THE SPECTRUM OF LIPID PARTICLES IN *PHASEOLUS VULGARIS* SEEDS

by

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Abstract

The spectrum of lipid particles in *Phaseolus vulgaris* seeds

Lipid particles with similar morphologies have been reported in cells and circulatory systems of many organisms. The best studied of these in plants are oil bodies found in the cytosol of cells from oil-bearing seeds. Oil bodies are spherical consisting of a triacylglycerol core surrounded by a monolayer of phospholipid which is coated with a protein shell composed of oleosins. Oleosin is the only protein reported to be associated with oil bodies at any point in their genesis.

In the present study, distinguishable populations of lipid particles were isolated from developing and germinating seeds of wax bean (*Phaseolus vulgaris*). These particles have been termed low-density particles (LDPs), intermediate-density particles (IDPs) and high-density particles (HDPs) to reflect their different densities. LDPs and IDPs are both enriched in triacylglycerol indicating that they are oil bodies, and they also have three major proteins none of which is oleosin. One of the three major proteins identified in these particles is a ~ 52 kDa protein, which, despite a protease protected fragment, is too large for an oleosin and does not appear in developing seeds, as do oleosins. A second major protein, 22.9 kDa in size, associated with LDPs and IDPs was identified, by amino terminal sequencing and enzyme activity, as an α-amylase inhibitor originating from protein bodies and artifactually associated with the lipid particles. The third major protein associated with LDPs and IDPs is 17.7 kDa in size and was shown to be too hydrophilic to be an oleosin. In addition, a protein of the same molecular weight was also detectable in protein bodies

suggesting that the 17.7 kDa protein may also artifactually adhere to lipid particles during tissue homogenization of oil bodies. Thus LDPs, from *Phaseolus vulgaris* seeds, appear to be a unique class of oil bodies that do not contain oleosin. IDPs closely resemble LDPs and appear to be formed from LDPs by the shear forces generated during tissue homogenization.

The lipid composition of HDPs indicates that they are similar to lipid particles previously identified in carnation petals, called lipid-protein particles, which are thought to be involved in membrane turnover. HDPs are enriched in phospholipid metabolites including free fatty acids, diacylglycerol and steryl and wax esters and appear to have a different structure than that proposed for oil bodies.

The presence of phospholipid in LDPs and HDPs indicates that they both originate from membranes. This is also believed to be true for oil bodies and lipid particles from organisms other than plants. The mechanism employed by plants to accumulate triacylglycerol into domains within the membrane bilayer, prior to formation of an oil body, has not been described. The only proteins associated with oil bodies from developing seeds are oleosins, and it seems unlikely that oleosins are involved in this process, as naked triacylglycerol droplets have been described in different plants. Moreover, LDPs isolated from wax bean seeds do not appear to contain oleosins. A search of data bases using proteins involved in the generation of lipid particles in animals and bacteria resulted in the identification of several plant proteins with sequence similarities to these animal and bacterial proteins. Many of these plant proteins have been deduced from open reading frames identified by the *Arabidopsis* genome project and do not yet have assigned functions. This strategy

appears to be promising in the search for proteins involved in the ontogeny of lipid particles in plants.

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Table of Contents

Abstract iv

Acknowledgements vii

List of Tables xiv

List of Figures xv

Introduction 1

1 Triacylglycerol 1

2 Animal Lipid Particles 2

2.1 Mammalian Lipid Particles 2

2.1.1 Storage Particles 2

2.1.2 Transport Lipids 5

2.1.3 Milk Globule Lipids 8

2.2 Insect Lipid Particles 9

3 Lipid Particles in Microorganisms 11

4 Plant Lipid Particles 13

4.1 Seed Oil Bodies 13

4.1.1 Ontogeny of Oil Bodies 14

4.1.2 Involvement of Specialized Membrane Domains in Oil Body Formation 15

4.1.3 Formation of Localized Membrane Domains 16

4.1.4 Targeting of Oleosins 18

4.2 Anther Specific Lipid Particles 22

4.3 Plastid Lipid Particles 23

4.4 Nonstorage Cytosolic Lipid Particles 24

4.5 Fates of Oil Bodies and Other Lipid Particles 26

4.6 Lipid Particles Present in the Cytosol of *Phaseolus vulgaris* Cotyledons 31

Materials and Methods 33

1 Chemicals 33

2 Plant Material 33

2.1 Growth Conditions 33

2.1.1 Developing Seeds 33

2.1.2 Germinating Seeds 33

3 Tissue Fractionation 34

3.1 Isolation of Smooth Microsomal Membranes, Cytosol and Ultrafiltered High-Density Lipid Particles 34

3.2 Isolation of Microsomal Membranes and Low-Density, Intermediate-Density and High-Density Lipid Particles by Flotation 38

3.3 Homogenization Studies 44

3.4 Isolation of Protein Bodies 45

3.5 Isolation of Oil Bodies 46

3.6 Washing of Isolated Lipid Particles 47

3.7 Proteinase K Treatment 47

4 Analytical Procedures 48

4.1 Measurement of α-Amylase Inhibitor Activity 48

4.2 Cytochrome c Reductase Activity 48

4.2.1 Effects of Heat, Acid and Protease Treatment on Cytochrome c Reductase Activity 49

4.2.2 Effects of Sephadex G25 NAP Column Chromotography on Cytochrome c Reductase Activity 50

4.3 Effects of Abscisic Acid on the Induction of Protein Synthesis 50

4.4 Lipid Analysis 51

4.4.1 Lipid Extraction 51

4.4.2 Thin Layer Chromatography 51

4.4.3 Fatty Acid Analysis 52

4.5 Polyacrylamide Gel Electrophoresis 53

4.5 1 Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis 53

4.5.2 Isoelectric Focusing and Two-Dimensional Fractionation of Proteins 54

4.6 Delipidation of Protein Samples 56

4.7 Western Blotting 56

4.8 Protein and Chlorophyll Measurements 57

4.9 Light Scattering Measurements 58

4.10 Electrospray Mass Spectrometry 58

4.11 Protein Sequencing and Amino Acid Analysis 59

5 Nucleic Acid Isolation and Manipulation 60

5.1 DNA and RNA quantification 60

5.2 Agarose Gel Electrophoresis 61

5.3 Genomic DNA Isolation 62

5.4 Primer and Probe Design 64

5.5 Polymerase Chain Reaction Conditions 64

6 cDNA Library Construction 64

6.1 mRNA Isolation 64

6.2 cDNA Synthesis and Packaging 68

6.3 End-Labeling Probes for cDNA Library Screening 69

6.4 Screening 71

7 Data Bank Searches 73

8 Statistical Analyses 73

Results 75

1 Cotyledons Development 76

2 Membrane-Derived Cytochrome c Reductase Activity in the Cytosol of Senescing Cotyledons 76

2.1 Distribution of Cytochrome c-Reducing Activity in Fractionated Cytosol 91

3 Characterization of Low-, Low', Intermediate- and High-Density Lipid Particles from Cytosol of Bean Cotyledons 91

3.1 Protein Composition of Low-, Low', Intermediate- and High-Density Lipid Particles 97

3.1.1 Western Blot Analysis 104

3.1.2 Washing and Protease Treatment of the Lipid Particles 111

3.1.3 Analysis of Oleosin Antibodies 134

3.1.4 Sequencing and Identification of LDP Proteins 141

3.1.5 Amino Acid Composition of the 17.7 kDa Polypeptide and ~ 48 kDa Protected Fragment of Low-Density Particles 152

3.1.6 Isoelectric Focusing and Two-Dimensional PAGE 156

3.2 Lipid Analysis of High-, Intermediate- and Low-Density Particles 159

3.3 Effects of Homogenization on the Protein and Lipid Composition of Intermediate- and Low-Density Particles 178

4 PCR Amplification of the Oleosin Nucleotide Sequence 186

5 Developing Cotyledon cDNA Library Screening for the Oleosin Sequence 192

Discussion 195

References 231

Appendix 1 292

Appendix 2 301

List of Tables

Table 1 Abbreviations and brief descriptions of the subcellular fractions isolated from bean cotyledons 38

Table 2 Amino terminal sequence of 22.8 kDa protein from LDP isolated from 1 day old bean cotyledons compared with similar proteins using the BLAST program 144

Table 3 Amino acid composition of the 17.7 kDa protein and the protected fragment of 52 kDa protein isolated from LDPs of 1-day old bean cotyledons 153

List of Figures

Figure 1. Flowcharts describing the procedures for isolating cytosol, ultrafiltered HDPs, particle-free cytosol and smooth microsomal membranes from the homogenate of bean cotyledons after filtering through four layers of cheesecloth. 36

Figure 2. Flowchart describing the procedure for isolating LDPs, LDP's and HDPs from the homogenate of bean cotyledons after filtering through four layers of cheese cloth. 40

Figure 3. Flowchart describing the procedure for isolating microsomal membranes, IDPs and the sucrose-containing layer from the homogenate of bean cotyledons after filtering through four layers of cheese cloth. 42

Figure 4. DNA sequence from 24 kDa oleosin isoform B from soybean 66

Figure 5. Changes in fresh weight of bean cotyledons at specified stages of development and germination. 78

Figure 6. Changes in the NADPH-cytochrome c reductase specific and total activities and protein of subcellular fractions from bean cotyledons. 80

Figure 7. Effects of heat denaturation on the cytochrome c-reducing activity of cytosol and smooth microsomal membranes and effects of protein precipitation and protease digestion on the cytochrome c-reducing activity of cytosol from bean cotyledons. 83

Figure 8. Elution profile of NADPH-dependent reduction of cytochrome c and of protein during Sephadex G-25 chromatography of smooth microsomal membranes from 2 day-old bean cotyledons. 86

Figure 9. Elution profile of NADPH-dependent reduction of cytochrome c and of protein during Sephadex G-25 chromatography of cytosol from 7 day-old bean cotyledons. 88

Figure 10. pH profile for NADPH-dependent and NADPH-independent cytochrome c reducing activity by heat-denatured cytosol from 7 day-old bean cotyledons. 90

Figure 11. Comparison of the proportion of NADPH-dependent cytochrome c reducing activity from cytosol isolated from 7 day-old bean cotyledons fractionated into lipid particles and the remainder of the cytosol by two methods of fractionation: flotation and ultrafiltration. 93

Figure 12. Changes in the protein content of subcellular fractions isolated from bean cotyledons during development and germination. 96

Figure 13. Changes in the sum of the protein from intermediate-density particles and low-density particles isolated from bean cotyledons during development and germination. 99

Figure 14. SDS-PAGE of lipid particles from developing cotyledons and 1 day-old cotyledons of bean seeds on a 15% acrylamide gel. 101

Figure 15. SDS-PAGE of subcellular fractions isolated from cotyledons of 1 day-old bean seeds and oil bodies from canola and soybeans. 103

Figure 16. Mass spectrometric results indicating the molecular weights of the detected proteins in lipid particles isolated from cotyledons of 1 day-old beans. 106

Figure 17. Western blot analysis of fractions isolated from 1 day-old bean seeds probed with the 19 kDa canola oleosin antibody. 108

Figure 18. Western blot analysis of fractions isolated from 1 day-old bean seeds and probed with the 22 kDa canola oleosin antibody. 110

Figure 19. Western blot analysis of fractions isolated from 1 day-old bean seeds probed with HSP70 monoclonal antibody. 113

Figure 20. SDS-PAGE of washed canola oil bodies separated on 12 % acrylamide gels. Each lane contains 1 μg protein. 115

Figure 21. SDS-PAGE of washed intermediate-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. 117

Figure 22. SDS-PAGE of washed low-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. 119

Figure 23. Western blot analyses of washed intermediate-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. 121

Figure 24. Western blot analyses of washed low-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. 123

Figure 25. SDS-PAGE of canola oil bodies subject to proteolytic digestion for varying amounts of time. 126

Figure 26. SDS-PAGE of lipid particles isolated from 1 day-old bean seeds, subjected to proteolytic digestion for varying amounts of time and stained with Coomassie brilliant blue. 128

Figure 27. SDS-PAGE of lipid particles isolated from 1 day-old bean seeds, subjected to proteolytic digestion for varying amounts of time and stained with silver. 131

Figure 28. Western blot analyses of intermediate-density particles subjected to proteolytic degradation for varying periods of time. 133

Figure 29. Western blot analyses of low-density particles subjected to proteolytic degradation for varying periods of time. 136

Figure 30. Antigen competition analysis of 19 kDa canola oleosin antiserum by Western blot analyses of lipid particles from cotyledons of 1 day-old bean seeds and oil bodies from canola. 138

Figure 31. Antigen competition analysis of 22 kDa canola oleosin antiserum by Western blot analyses of lipid particles from cotyledons of 1 day-old bean seeds and oil bodies from canola. 140

Figure 32. Western blot analyses of subcellular fractions from 1 day-old bean seeds probed with preimmune sera. 143

Figure 33. Effects of denaturation and protease digestion on the ability of the low-density particles from cotyledons of developing and 1 day-old bean seeds to inhibit porcine α-amylase activity. 147

Figure 34. Comparative SDS-PAGE of protein bodies and lipid particles isolated from 1 day-old bean seeds. 149

Figure 35. Western blot analyses of protein bodies and lipid particles isolated from one day-old bean seeds. 152

Figure 36 Comparison of the proportion of nonpolar, polar and charged side groups on amino acids in the 17.7 kDa protein of low-density particles, the 52 kDa protein fragment of low density particles, *Arabidopsis* oleosin, canola oleosin, soybean oleosin and the average occurrence of these amino acids in proteins. 155

Figure 37. Isoelectric focussing of oil bodies from canola, and lipid particles and the sucrose-containing layer from 1 day-old bean seeds. Proteins from each sample were lipid-extracted before the proteins were fractionated. 158

Figure 38. Two dimensional SDS-PAGE of lipid particles from cotyledons of 1 day-old bean seeds. 161

Figure 39. Levels of protein and triacylglycerol (TAG) in cotyledons from developing and germinating bean seeds. 163

Figure 40. Changes in the lipid composition of lipid particles and microsomal membranes isolated from cotyledons of developing and germinating bean seeds. 165

Figure 41. Changes in the abundance of lipid particles in cotyledon tissue of developing and germinating bean seeds. 169

Figure 42. Light scattering measurements of lipid particles isolated from cotyledon tissue of developing and germinating bean seeds. 171

Figure 43. Changes in the levels of triacylglycerol (TAG), phospholipid (PL) and the other lipids (diacylglycerol, free fatty acids and steryl and wax esters) of lipid particles from developing and germinating bean seeds. 174

Figure 44. Changes in the phospholipid fatty acid composition of lipid particles and microsomal membranes isolated from cotyledon tissue of developing and germinating bean seeds. 177

Figure 45. Changes in the triacylglycerol fatty acid composition of lipid particles and microsomal membranes isolated from cotyledon tissue of developing and germinating bean seeds. 180

Figure 46. Changes in the lipid composition of lipid particles isolated from cotyledons of 2 day-old bean seeds isolated using different homogenization techniques. 183

Figure 47. Changes in the proportions of low-density particle lipid and intermediate-density particle lipid as a function of homogenization technique. 185

Figure 48. SDS-PAGE of lipid particles isolated from cotyledons of 2 day-old bean seeds using different homogenization techniques. 188

Figure 49. Agarose gel fractionation of PCR-amplified products from genomic DNA of wax bean and soybean. 191

Figure 50. Illustration of the most probable interactions of primer 2 with itself. 194

Introduction

1 Triacylglycerol

Biological energy reserves are most commonly found in three forms: carbohydrate, protein and lipid. Of these reserves, lipid is the most efficient on a weight basis (Brindley 1985, Miquel and Browse 1995). Although fatty acids are the primary energy-forming portions of storage lipids, they are toxic in free form due to their detergent effects on membranes (Thomas 1984). In most eukaryotic cells, free fatty acids are esterified to a glycerol backbone to form triacylglycerol, which is not as reactive as free fatty acids. Triacylglycerol has the added advantage of being insoluble in aqueous solutions, thereby not affecting the osmotic potential of the solution in which it exists. This, however, leads to difficulties in transport and in rapid mobilization of the lipid. In order to overcome these difficulties, triacylglycerol is usually packaged into lipid- and protein-containing particles (Couble *et al.* 1979, Body 1988, Holdsworth and Ratledge 1988, Murphy 1990, Huang 1992, Murphy 1993, Herman 1995, Davis and Vance 1996, Schneider 1996, Thompson *et al.* 1998). These triacylglycerol-containing particles have remarkably similar morphologies. The triacylglycerol is localized in the central portion of the particle and is surrounded by a monolayer of phospholipids. The phospholipids are oriented such that the hydrophobic acyl chains project into the interior of the particle where they interact with the triacylglycerol, and the polar headgroups are on the surface (Yatsu and Jacks 1972, Murphy 1990, Huang 1992, Davis and Vance 1996, Schneider 1996).

Lipid- and protein-containing particles have been reported in a diverse variety of organisms. Most organisms have intracellular lipid storage particles in which

triacylglycerol, in eukaryotic cells, or polyhydroxyalkanoic acid (PHA) in prokaryotic cells, is stored until energy is required. In addition, several organisms also have lipid-containing transport particles responsible for moving lipids between organs.

2 Animal Lipid Particles

2.1 Mammalian Lipid Particles

Mammalian transport and storage lipid particles are probably the best studied of all the lipid particles described for various organisms. This is due to the involvement of these particles in obesity and various diseases.

2.1.1 Storage Lipids

Mammals store their excess lipid in the form of triacylglycerol in adipose tissue made up primarily of adipocytes, although there is evidence that cytosolic lipid particles exist in almost all animal cells (Spector *et al.* 1981, Wheater *et al.* 1987). There are two types of adipose tissue, white and brown. White adipose tissue is a storage site for excess energy in the form of lipid derived from food, and it is metabolized under starvation conditions. Brown adipose tissue is found only in some animals. It is formed during fetal development, and this stored lipid is metabolized during extreme cold conditions and during hibernation (Bernlohr and Simpson 1996).

In white adipose tissue, fatty acids bound to albumin are believed to be taken up into adipocytes by fatty acid-binding proteins (Abumrad *et al.* 1993, Schaffer and Lodish 1994). These fatty acids, once inside the cell, are bound to small, soluble lipid-binding proteins, which are thought to be responsible for moving fatty acids throughout the cell (LaLonde *et al.* 1994, Bernlohr 1995). In addition, adipocytes can

readily take up glucose, which is rapidly converted to glucose-6-phosphate and goes through the glycolytic pathway leading to *de novo* synthesis of fatty acid. The fatty acids from both external and internal sources are condensed with coenzyme A and subsequently esterified to glycerol to generate triacylglycerol, and ultimately triacylglycerol droplets. This occurs in, or on, the endoplasmic reticulum membrane of the adipocyte (Senior and Isselbacher 1962, Brindley and Hubscher 1965, Vance 1996) by a process that is not understood, although the presence of a monolayer of phospholipids on the surface of the triacylglycerol droplets suggests that it may occur between the bilayers of the endoplasmic reticulum. Early during the formation of triacylglycerol droplets, a protein named adipose differentiation-related protein (ADRP), also known as adipophilin, associates with the particles (Jiang and Serrero 1992, Brasaemle *et al.* 1997, Heid *et al.* 1998). Despite the fact that ADRP appears to be associated with triacylglycerol particles early in their genesis while the particles are very small, it does not appear to be associated with endoplasmic reticulum and is synthesized on free polysomes with no recognized signal sequence (Brasaemle *et al.* 1997). This protein is particularly interesting as it appears to be widely expressed in animal tissue, suggesting that it may be associated with the intracellular lipid particles observed in most animal cells (Brasaemle *et al.* 1997, Heid *et al.* 1998). Eventually, the triacylglycerol droplets of the adipocyte coalesce, nearly filling the cell and pushing the organelles into a narrow rim around the perimeter (Behrlohr and Simpson, 1996).

The release of fatty acids from triacylglycerol in adipocytes is tightly regulated, but can be initiated by several different signals. Hormone-sensitive lipase is

the target of many of these signals. Upon phosphorylation, this lipase becomes active, releasing fatty acids from the glycerol backbone (Yeaman 1990). It is thought that this lipase binds to a protein associated with the perimeter of the large triacylglycerol droplets called perilipin (Greenburg *et al.* 1993, Blanchette-Mackie *et al.* 1995). Perilipin appears to replace ADRP in adipocytes as the triacylglycerol droplets grow larger, although the mechanism for this is not yet known. Perilipin is expressed solely in adipocytes and steroidogenic cells; the latter store cholesteryl esters in the form of droplets to be used as a source of cholesterol for steroid hormone biosynthesis (Greenberg *et al.* 1991, Blanchette-Mackie *et al.* 1995, Severtnick *et al.* 1995). Perilipin, like hormone-sensitive lipase, also appears to be regulated by a signal-sensitive phosphorylation site (Greenberg *et al.* 1991, Londos *et al.* 1996), and this phosphorylation seems to be required for lipolysis. Indeed, insulin, which blocks phosphorylation of perilipin, also inhibits the catabolism of storage lipids in adipocytes. It is thought that the nonphosphorylated form of perilipin acts as a barrier to hormone-sensitive lipase (Clifford *et al.* 1997).

Brown adipose tissue differs from white adipose tissue in several ways. The darker colour is due to the large number of mitochondria packed into the cells as well as the extensive blood supply to the tissue (Himms-Hagen 1989, Bernlohr and Simpson 1996). Triacylglycerol synthesis is much the same as in white adipose tissue except that the triacylglycerol droplets remain relatively small for reasons that are not clear. Lipolysis appears to be initiated by the sympathetic nervous system primarily in response to cold (Himms-Hagen 1989). The fatty acids released undergo β-oxidation in the mitochondria, but the presence of a mitochondrial uncoupling protein in brown

adipose tissue results in the generation of heat rather than the formation of ATP (Himms-Hagen 1989, Lafontan and Berlan 1993).

2.1.2 Transport Lipids

In addition to storage lipid particles, mammals have a myriad of lipid transport-particles, termed lipoproteins. Most of these have been identified on the basis of their densities. Chylomicrons and very low density lipoproteins (VLDL) are the two triacylglycerol transport particles. In animal systems, the formation of these particles requires the presence of two proteins: apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTP) (Gordon *et al.* 1995, Davis and Vance, 1996, Wetterau *et al.* 1997, Ginsberg 1998, White *et al.* 1998). ApoB is an extremely large protein (>500 kDa) present in two forms, apoB48 and apoB100. ApoB100, the intact form of the protein, is found in hepatic cells where triacylglycerol is synthesized, and is involved in the formation of VLDLs (Kane 1983, Chan 1992). ApoB48 is a truncated form (48%) of apoB100 which is located solely in intestinal cells in humans, where it is involved in the formation of chylomicrons used primarily for the transfer of dietary triacylglycerols and sterols (Young 1990, Innerarity *et al.* 1996). In other mammals, apoB48 is also found in hepatic cells where VLDLs are produced (Young 1990, Innerarity *et al.* 1996). Both of these forms of apoB are thought to act as a scaffold or structural protein bound to the surface of the lipoprotein (Kane 1983, Chan 1992, Boren *et al.* 1994, Hamilton *et al.* 1995). They require the presence of lipid to be cotranslationally inserted through the endoplasmic reticulum into the lumen (Davis *et al.* 1990, Chuck and Lingappa 1992).

The second protein required to form chylomicrons and VLDLs is MTP, a lipid transfer protein, which is located in the lumen of the endoplasmic reticulum in cells of the liver and intestine (Wetterau and Zilversmit 1984, Wetterau and Zilversmit 1985, Wetterau and Zilversmit 1986, Wetterau *et al.* 1991a, Wetterau *et al.* 1997, Jamil *et al.* 1998). This protein exists as a heterodimer with protein disulphide isomerase as one subunit (Wetterau *et al.* 1990, Wetterau *et al.* 1991b). *In vitro* assays have shown that MTP can shuttle triacylglycerol and cholesterol esters between membranes, and it has a putative role in loading apoB with lipid within the cells (Gordon *et al.* 1994, Haris *et al.* 1996, Shailendra and Grundy 1996, Jamil *et al.* 1998). Humans lacking functional MTP suffer from abetalipoproteinemia, a disease characterized by extremely low serum levels of lipid and apoB (Wetterau *et al.* 1992, Gregg and Wetterau 1994, White *et al.* 1998). MTP is thought to interact directly with apoB (Shailendra and Grundy 1996, Wu *et al.* 1996, Bakillah *et al.* 1998, Hussain *et al.* 1998) and recently, specific regions of apoB have been shown to bind to MTP, confirming the involvement of both of these proteins in the generation of apoB-containing particles (Bakillah *et al.* 1998, Hussain *et al.* 1998).

In addition to these lipoproteins, there are intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Both IDLs and LDLs are partially metabolized and modified VLDLs (Fielding and Fielding 1996, Ginsberg 1998, White *et al.* 1998). HDLs are structurally and functionally different from other lipoproteins: they are involved in reverse lipid transport, they do not contain apoB and the major lipids are cholesterol and cholesteryl esters (Fieldings and Fieldings 1996, Schneider 1996, Ginsberg 1998). There are several proteins

involved in the formation and metabolism of HDLs and, unlike apoB, all of these proteins are transferable between particles and have been found in lipid-poor forms in extracellular fluid (Segrest *et al.*, 1992). Several types of HDLs exist. These are believed to be formed on a structural protein called apolipoproteinA1 (apoA1). This protein is secreted from the cell into the extracellular spaces in a lipid-poor form in loose association with apoB-containing particles. This apoB complex releases apoA1 upon secretion from the cell. ApoA1 quickly associates with sphingomyelin and phosphatidylcholine. It is thought to have a unique structure that binds cholesterol from the cell surfaces; sphingomyelin also has a high affinity for cholesterol, making this particle effective in scavenging free cholesterol from plasma membranes (Asztalos and Roheim 1995, Fielding and Fielding 1995, Fielding and Fielding 1996, Ginsberg 1998). As the HDPs move through the circulatory system, several other proteins bind to them. One of these is lecithin-cholesterol acyl transferase (LCAT). LCAT catalyzes the removal of an acyl group from phosphatidylcholine and transfers it to free cholesterol, and the resulting cholesteryl ester forms a central core in these particles (Jonas 1987, Jonas 1998). In addition, a phospholipid-transfer protein moves phospholipid from the partially metabolized VLDLs to the HDLs (Day *et al.* 1994, Lagrost *et al.* 1998), and the cholesteryl ester transfer protein moves cholesteryl esters from HDL to VLDLs in exchange for triacylglycerol (Tollefson et al 1988, Jauhiainen *et al.* 1993, Tall 1993). This greatly increases the relative amounts of cholesteryl esters in LDLs, the metabolite of VLDLs.

The fates of all the lipoproteins appear to be determined by both lipases and specific receptors. All of the lipoproteins are modified as they circulate through the

body. As chylomicrons and VLDLs move through the circulatory system, a variety of apolipoproteins bind to them. These proteins are responsible for the lipoprotein binding to both lipolytic enzymes and to specific receptors on the surfaces of cells that eventually internalize the catabolized particles (Schneider 1996, Davis and Vance 1996, Ginsberg 1998, White *et al.* 1998). Lipoprotein lipase is responsible for hydrolyzing triacylglycerol of both chylomicrons and VLDLs for use primarily in muscle tissue and adipose tissue (Chappell *et al.* 1994, van Tilbeurgh *et al.* 1994). The majority of triacylglycerol in chylomicrons is metabolized before this particle is removed from the circulatory system by association with hepatic lipoprotein receptors, leading to receptor-mediated endocytosis (Herz and Willnow 1995). Although there are receptors specific for uptake of VLDLs, a large proportion of VLDLs stay in circulation and form IDLs, then LDLs, which exchange lipids with HDLs until they are also finally endocytosed as LDLs (Schneider 1989, Ginsberg 1998, White *et al.* 1998). The fate of HDLs is not as clear. It appears that the lipids and proteins of this particle are metabolized in different locations, and it may be possible that lipid-poor HDLs can be reused as a lipid shuttle (Schneider 1996, Ginsberg 1998).

2.1.3 Milk Globule Lipids

Another highly specialized triacylglycerol-containing lipid transport particle in mammals is formed during the synthesis of milk. In lactating mammary glands, small triacylglycerol-containing particles, surrounded by phospholipid and protein, appear to be synthesized on or within the bilayers of the endoplasmic reticulum membranes (Dylewski *et al.* 1984, Deeney *et al.* 1985, Zaczek and Keenan, 1990, Keenan *et al.* 1992). Within the cytosol, these droplets greatly increase in size; fusion is one

mechanism for this (Valivullah *et al.* 1988). Proteins associated with these droplets are ADRP (Heid *et al.* 1998) and fatty acid synthase, which appears to be associated with the droplets in a low density fatty acid synthase complex (Keon *et al.* 1994). In addition, GTP binding proteins, xanthine oxidase and butyrophilin have also been reported to be associated with these triacylglycerol droplets (Keon *et al.* 1994), although a more recent report suggests that the association of butyrophilin with the droplets may be artifactual and demonstrates that butyrophilin is a plasma membrane-bound protein (Banghart *et al.* 1998). The presence of GTP-binding proteins with the triacylglycerol droplets is interesting, as they are known to be involved in the targeting of vesicles (Reithmeier 1996).

Xanthine oxidase, a redox enzyme, is normally found in both membrane bound and soluble forms (Ishii *et al.* 1995). It, along with butyrophilin, is involved in the secretion of lipid droplets out of the cell (Frank *et al.* 1981, Jack and Mather, 1990, Zaczek and Keenan, 1990, Ishii *et al.* 1995, Banghart *et al.* 1998). It is thought that the cytoplasmic tail of membrane-bound butyrophilin interacts with both xanthine oxidase and the cytosolic lipid droplets, and that this complex is subsequently secreted by exocytosis (Jack and Mather, 1990, Banghart 1998).

2.2 Insect Lipid Particles

Insect transport lipid particles have been extensively studied as they are metabolized to provide the energy required for flight (Shapiro *et al.* 1984, Blacklock and Ryan 1994, Ryan 1996). Like mammals, insects store triacylglycerol in specialized tissue known as fat bodies, which are very similar to white adipose tissue, except that fat body tissue is only two cell layers thick and is surrounded by

hemolymph (van der Horst 1990). When energy for flight is required, adipokinetic hormone is released and binds to cell surface receptors. This results in lipolysis of triacylglycerol and the release of diacylglycerol which is loaded onto preexisting, reusable lipid shuttles called lipophorins found in the hemolymph (Chino 1985, van Heusden *et al.* 1987, van der Horst 1990, Ryan 1996, Arrese and Wells 1997). The loading of lipophorin requires a protein and lipid complex called the lipid transport particle (LTP) (Ryan *et al.* 1986, Ryan *et al.* 1988, van Heusden and Law 1989, Ryan *et al.* 1990a, Singh and Ryan 1991). Interestingly, LTP can also exchange lipid in both mammalian HDLs and LDLs and will catalyze this exchange by a carrier-mediated mechanism (Ryan *et al.* 1990b, Blacklock *et al.* 1992), although it does not seem to catalyze a net transfer of lipids between various lipid particles (Tsuchida *et al.* 1997).

Lipophorin, like HDLs, can exist in both a lipid-poor and lipid-rich form. In its lipid-rich form, it is spherical with a diacylglycerol filled core surrounded by proteins and phospholipids. Two proteins appear to be necessary as structural proteins: apolipophorin I (apoLpI) and apolipophorin II (apoLpII) (Shapiro *et al.* 1984, Blacklock and Ryan 1994, Ryan 1996, Sundermeyer *et al.* 1996). These appear to be different modifications of the same protein and bear resemblance to apoB in sequence, structure and size (Shapiro *et al.* 1984, Kawooya *et al.* 1989, van der Horst *et al.* 1993, Weers *et al.* 1993). As the lipophorins increase in size, a third protein, apolipophorin III (apoLpIII), binds to the particle (Cole *et al.* 1987). This is a soluble protein that undergoes a significant conformational change when it binds to lipophorins (Soulages and Bendavid 1998, Weers *et al.* 1998). ApoLpIII seems to

bind to exposed diacylglycerol on the surface of the lipophorins as they expand, and prevents aggregation of these particles (Demel *et al.* 1992, Wang *et al.* 1995, Soulages *et al.* 1996). Similar to the binding of many mammalian lipoproteins to lipid particles, the binding of apoLpIII to lipophorins is thought to be with the surface of the particle, and this binding is reversible (Wang *et al.* 1997a, Sahoo *et al.* 1998). In fact, the sequence of the N-terminal domain of mammalian apolipoprotein E is similar to apoLpIII (Fisher *et al.* 1997, Wang *et al.* 1997). Lipophorin ultimately moves the diacylglycerol to the muscle tissue where it undergoes extracellular lipolysis (Chino 1985, van Heusden *et al.* 1987, Ryan 1996). It is thought that the free fatty acids remain attached to the lipophorin until they are taken up into the muscle cells in order to avoid having toxic levels of free fatty acids in the hemolymph (Soulages *et al.* 1996).

3 Lipid Particles in Microorganisms

Lipid particles with similar morphologies to mammalian and insect lipid particles have also been observed in bacteria, yeast and fungi. Most bacterial lipid particles contain polyhydroxyalkanoic acids (PHA) and are called PHA granules (Anderson and Dawes 1990, Steinbuchel *et al.* 1995), although a few bacteria have lipid particles containing triacylglycerol or wax esters (Alvarez *et al.* 1997). Proteins associated with many of these particles include PHA synthase, involved in the synthesis of PHAs, PHA depolymerase, involved in the catabolism of PHAs, and phasins, thought to coat and stabilize the granules (Steinbuchel *et al.* 1995). Two phasins have been identified, one 14 kDa in size (Pieper-Furst *et al.* 1994) and the other 24 kDa in size (Wieczorek *et al.* 1995). In addition, an antibody against the 24

kDa phasin has been shown to cross-react with proteins of about the same molecular weight on PHA granules of many different bacteria (Wieczorek *et al.* 1996). Their attachment to the granules is similar to that for apoB in that the protein is not immersed in the hydrophobic interior of the granule, but is attached to the surface by two short hydrophobic amino acid sequences (Pieper-Furst *et al.* 1994, Pieper-Furst *et al.* 1995, Wieczorek *et al.* 1995).

Most yeast and fungal lipid particles contain a core of triacylglycerol. Some yeast lipid particles also contain steryl esters (Clausen *et al.* 1974) and some fungi lipid particles contain free sterols in addition to triacylglycerol (Mills and Cantino 1977). These particles appear to be involved in energy storage as their breakdown, in the event of carbon starvation, is extremely rapid (Holdsworth *et al.* 1988). The proteins associated with yeast particles have not been extensively studied, but none of them appear to be immersed in the interior of the particle or to protect the exterior phospholipids from degradation by phospholipase A_2 (Leber *et al.* 1994). One lipid particle-associated protein was identified through a yeast mutant defective in sterol uptake, which suggests that these lipid particles may also be involved in sterol transport (Keestler *et al.* 1992). It is also interesting to note that antibodies against this protein cross-react with animal apolipoprotein A2 (apoA2), although when the sequences of these two proteins were compared no regions of sequence similarity were found (Keestler *et al.* 1992). The function of apoA2 is not yet understood, but it is associated with HDLs (Davis and Vance 1996, Ginsberg 1998).

4 Plant Lipid Particles

4.1 Seed Oil Bodies

In plants, storage triacylglycerol is particularly abundant in the cytosol of oil-bearing seeds where it is packaged in small (~0.5-2.0 μm, average 1.0 μm, in diameter), spherical particles, surrounded by a monolayer of phospholipid, and known as oil bodies (Murphy 1990, Huang 1992, Murphy 1993, Herman 1995, Huang 1996, Napier *et al.* 1996). Recently, washing oil bodies in 9 M urea was shown to remove a considerable amount of phospholipid, leaving only phosphatidylcholine (Tzen *et al.* 1997). It is not clear whether this loss was due to destabilization of the isolated oil body or whether phospholipid does not actually completely surround the oil body. The phospholipids forming the monolayer surrounding the oil bodies are coated with oleosins. These are very abundant seed proteins which have characteristic structures. They are small (15-26 kDa), with a highly conserved central hydrophobic domain that is immersed in the hydrophobic, triacylglycerol core of the oil body. The amino and carboxyl termini of these proteins vary somewhat in length and are not well conserved. They are thought to be associated with the phospholipid head groups on the outside surface of the oil body and thus exposed to the surrounding cytosol (Murphy 1990, Huang 1992, Murphy 1993, Herman 1995, Huang 1996). Given the structure of oleosins, they may well compensate for an incomplete phospholipid monolayer such as is the case for apoLpIII on the surface of lipophorin in insects (Demel *et al.* 1992, Wang *et al.* 1995, Soulages *et al.* 1996). The function of oleosins, in fact, appears to be the same as that for apoLpIII of lipophorin in that they prevent the aggregation of oil bodies. Interestingly, in oil seeds of plants this propensity for

association of lipid particles appears to be greatest during rehydration of the mature seed (Leprince *et al.* 1998).

4.1.1 Ontogeny of Oil Bodies

Oil bodies are formed during the final stages of seed development. Their ontogeny is hypothesized to be related to the accumulation of triacylglycerol between the monolayers of the endoplasmic reticulum membrane where the enzymes required for the biosynthesis of triacylglycerol are localized (Huang 1992, Murphy 1993, Settlage *et al.* 1995). It has been proposed that physical forces resulting from the accumulation of triacylglycerol within the membrane bilayer force the bilayers apart and finally cause the release of triacylglycerol-filled particles from the surface of the endoplasmic reticulum into the cytosol, giving rise to oil bodies (Frey-Wysslin *et al.* 1963, Murphy 1990, Huang 1992, Murphy 1993, Huang 1996, Napier *et al.* 1996). This theory is similar to that proposed for the formation of lipid particles in adipose tissue (Senior and Isselbacher 1962, Brindley and Hubscher 1965, Vance 1996).

Oil bodies have never been reported in the lumen of the endoplasmic reticulum, indicating that the release of these particles is vectorial. In contrast, chylomicrons and VLDLs, animal lipid-protein particles involved in triacylglycerol transport which are morphologically and chemically similar to oil bodies, are found in the lumen of the endoplasmic reticulum before secretion from the cell (Davis and Vance 1996). Thus, in both cases these triacylglycerol-rich particles are released from the endoplasmic reticulum in a single direction. Chylomicrons and VLDLs require apoB and MTP, but no plant proteins have yet been described that are involved in the release of oil bodies.

Given the complexity of protein involvement in the formation of triacylglycerol-rich particles in animals, it seems highly probable that the formation and release of oil bodies in plants also involves proteins other than oleosins, which appear to function primarily in promoting the stability of the oil body. It is interesting to note that the maximum amount of triacylglycerol that can exist in a membrane is approximately 3 mol % (Miller and Small 1983), yet the concentration of triacylglycerol is 60 mol % in VLDLs and even higher in chylomicrons and plant oil bodies (Davis and Vance 1996, Murphy 1990, Huang 1993). There is, therefore, clearly a need to concentrate triacylglycerol during the formation of these particles, and in mammalian systems MTP is thought to be responsible for this. In plants, there has been no protein identified or proposed to function in this capacity thus far.

4.1.2 Involvement of Specialized Membrane Domains in Oil Body Formation

Triacylglycerol appears to be synthesized through either of the intermediates phosphatidic acid or phosphatidylcholine in oil-bearing seeds (Frentzen 1993, Murphy 1994, Miquel and Browse 1995, Ohlrogge and Browse 1995). There is no doubt that this pathway is localized in microsomal membranes, but it is not clear that it is localized exclusively on the endoplasmic reticulum. For example, oil body generation has been detected in granular areas of the cytosol by electron microscopy (Ichihara 1982), suggesting that endoplasmic reticulum may not be the sole site of storage lipid synthesis. In addition, a low density membrane fraction capable of synthesizing triacylglycerol has been isolated from *Lunaria annua* and canola (Lacey and Hill 1995), but this could correspond to a specialized region of the endoplasmic reticulum.

Some microscopic evidence also supports the assertion that oil bodies are formed by the endoplasmic reticulum membranes. For example, oil bodies in soybeans are evident in close proximity to tubular regions of endoplasmic reticulum (Herman 1987), and oleosins appear to collect in specific areas of endoplasmic reticulum (Sarmiento *et al.* 1997). In addition, diacylglycerol acyltransferase, the only enzyme known to be unique to triacylglycerol synthesis (Murphy 1993, Miquel and Browse 1995), has been localized to regions of endoplasmic reticulum apparently involved in oil body formation (Settlage *et al.* 1995). Based on this evidence, it has been proposed that there are specialized domains of this organelle involved in oil body biogenesis (Sarmiento *et al.* 1997, Napier *et al.* 1996).

There is evidence to suggest that, despite the classical model depicting a liquid crystalline lipid bilayer (Singer and Nicolson 1972), the endoplasmic reticulum and other membranes are made up of discrete lateral domains with distinctive lipid and protein compositions and functional specializations (Glaser 1993, Welti and Glaser 1994, Edidin 1997). A compelling example of domain formation is the lateral segregation of distinct membrane lipids synthesized on the endoplasmic reticulum membranes and destined for other organelles (Welti and Glaser 1994). Plant endoplasmic reticulum appears to be made up of many domains (Staehelin 1997). In fact, lipid synthesis in the endoplasmic reticulum of canola has been shown to occur in specific regions of the organelle during seed development (Lacey and Hills 1996).

4.1.3 Formation of Localized Membrane Domains

There are many possible interactions between membrane constituents that could result in domain formation. Lipid-lipid interactions and interactions of lipid

with proteins, both integral and peripheral proteins, may all be involved in this process (Glaser 1993, Welti and Glaser 1994, Edidin 1997). Domain formation has been demonstrated directly, in the case of plasma membrane, by examining the formation of clathrin-coated endocytotic vesicles using a fluorescent lipid analog which alters its colour upon concentration. This lipid analog is concentrated in the clathrin-coated vesicles immediately after they begin to bud from the membrane (Chen *et al.* 1997).

Integral membrane proteins interacting with lipid can also cause a lateral change in the lipid composition surrounding the protein. It has been demonstrated, for example, that some proteins collect an annulus of phospholipid which can be quite extensive (Selinsky 1992). Although most proteins only transiently associate with this boundary lipid (Oldfield *et al.* 1978, Seelig *et al.* 1982), some have a very high affinity for phospholipids with particular head groups (Marsh 1983, Knowles *et al.* 1981, Senak and Mendelsohn 1993, Rodgers and Glaser 1991). When these proteins are coupled to the cytoskeleton of the cell, these domains can be relatively long-lived (Kusumi and Yashushi 1996). In addition, domain formation may occur when particular proteins require a thicker bilayer, thus creating domains of lipids with longer acyl chains or higher cholesterol concentrations or taking advantage of existing domains of these lipids (Mouritsen and Bloom 1993, Lehtonen *et al.* 1996). Cholesterol itself appears to be partitioned within the plasma membrane into cholesterol-rich and -poor domains. It has been suggested that HDLs load cholesterol from these cholesterol-rich regions (Rothblat *et al.* 1992).

There are also bilayer differences in lipid composition within membranes, in which the two leaflets of a membrane bilayer can be composed of different lipids (op

den Kamp 1979, Devaux 1992), and these differences can affect the shape of a membrane or vesicle profoundly (Safran *et al.* 1991, Farge and Devaux 1992, Berndl *et al.* 1990). Indeed, this may in part account for the vectorial nature of oil body release from the endoplasmic reticulum. It seems possible, for example, that the physical forces generated by the accumulation of triacylglycerol, suggested to be responsible for the release of oil bodies from the endoplasmic reticulum, may be stronger on a single side of the bilayer. In support of this, phosphatidylcholine appears to be the most abundant phospholipid in the monolayer of oil bodies (Tzen *et al.* 1992).

4.1.4 Targeting of Oleosins

Oleosins, the major oil body proteins, are believed to be targeted to the endoplasmic reticulum by a mechanism that does not involve the traditional signal sequence (Lee *et al.* 1991, Batchelder *et al*, 1994, van Rooijen and Moloney 1995, Qu *et al.* 1986, Qu and Huang 1990, Hills *et al.* 1993, Loer and Herman 1993, Sarmiento *et al.* 1997), although the translation of synthetic oleosin transcripts from sunflower was found to be suppressed by the addition of the signal recognition particle in an *in vitro* translation system (Thoyts *et al.* 1995). This mechanism appears to be universal as a canola oleosin is correctly targeted in transgenic tobacco (Batchelder *et al.* 1994), and maize and soybean oleosins are correctly targeted in transgenic canola (Lee *et al.* 1991, Sarmiento *et al.* 1997). A more intriguing observation is that maize oleosin is also targeted to yeast lipid bodies (Ting *et al.* 1997). It is thought that oleosins may contain internal targeting information encoded in their highly conserved central domain, but deletion analysis has thus far failed to show a single region required for

insertion into the endoplasmic reticulum (van Rooijen and Moloney 1995, Thoyts *et al.* 1995). Deletion of the central hydrophobic region did, however, result in very low expression of oleosins in oil bodies (van Rooijen and Moloney 1995, Abell *et al.* 1997).

The notion that oleosins are targeted to the endoplasmic reticulum is supported by indirect evidence indicating that these proteins associate with oil bodies during their formation within the endoplasmic reticulum (Hills *et al.* 1993, Loer and Herman 1993, Tzen *et al.* 1993, Radetzky and Langheinrich 1994, Holbrook *et al.* 1991, van Rooijen and Moloney 1995). Recently, the conformation that oleosin assumes in this membrane has been examined (Abell *et al.* 1997). It appears that oleosin is incorporated into the membrane with the nonhydrophobic amino and carboxyl termini exposed to the cytosol and the central hydrophobic domain folded within the bilayer. Interestingly, the most conserved region of oleosin, consisting of three proline residues over a twelve amino acid region (proline knot) (Lee *et al.* 1994, Huang 1996), does not appear to be necessary for membrane targeting, but is required for oil body targeting (Abell *et al.* 1997). It may be that oleosin, like clathrin on the plasma membrane, can sequester lipids, specifically triacylglycerol, to a specific region of the endoplasmic reticulum. The unusual structure of oleosin within membranes and the highly conserved proline knot sequence found in the central region of the protein embedded in the bilayer suggest that it may have a role that goes beyond simply stabilizing the structure of the oil body.

The structure that oleosin is thought to assume within the bilayer of endoplasmic reticulum membranes may be unique for proteins associated with this

organelle, although cytochrome b_5, also found in the endoplasmic reticulum, could have a similar structure. Cytochrome b_5 is synthesized in the cytosol and is believed to spontaneously insert its hydrophobic domain into the membrane. It is not known whether this protein has a single transmembrane domain or forms a loop through the internal hydrophobic layer of the bilayer (Reithmeier 1996), which would be similar to the conformation proposed for membrane-associated oleosin.

Although it seems intuitive that the synthesis of triacylglycerol and oleosins should be regulated by a common signal, this may not be the case as the evidence remains contradictory (Murphy *et al.* 1989, Cummins *et al.* 1993, Tzen *et al.* 1993, Napier *et al.* 1996, Mazhar *et al.* 1998) More specifically, the expression of oleosin genes and the quantity of synthesized triacylglycerol do not appear to be correlated in high and low oil-producing maize seeds (Ting *et al.* 1996). In fact, a change in the oleosin to triacylglycerol ratio during seed development appears to affect the size and shape of the oil body (Ting *et al.* 1996). In addition, when triacylglycerol precursors are supplied, safflower microsomes can produce naked oil droplets *in vitro* (Stobart *et al.* 1986). This has also been observed very early in the development of oil bodies in plants (Ting *et al.* 1996). It has been proposed that triacylglycerol may be initially released from the endoplasmic reticulum in the form of oleosin-naked droplets, and as oleosins begin to be synthesized later in seed development the droplets thus formed become progressively more coated with these proteins. The naked and partially coated oil droplets are then thought to fuse, reducing the surface area to volume ratio until oleosin completely coats the particle (Sarmiento *et al.* 1997). This proposal does not,

however, account for the excess phospholipid that would accumulate on the oil body if each of the naked triacylglycerol droplets carried a full monolayer of phospholipid.

It appears that the synthesis of at least some oleosins is regulated by abscisic acid (ABA). Canola and maize oleosin genes have putative ABA-responsive elements in their promoter regions and are induced by treatment with ABA (Taylor *et al.* 1990, Qu *et al.* 1990, Holbrook *et al.* 1991, Lee and Huang, 1991, Holbrook *et al.* 1992, van Rooijen *et al.* 1992, Keddie *et al.* 1994, Zou *et al.* 1995). Anise oleosin is also induced by ABA when this growth regulator is applied to cells in culture (Radetzky and Langheinrich 1994). An oleosin gene is also induced in citrus cell culture exposed to salt stress (Naot *et al.* 1995). Interestingly, the synthesis of some very long-chain fatty acids, which are found primarily in triacylglycerol in some species, is also induced by ABA (Holbrook *et al.* 1992, Zou *et al.* 1995). ABA has been implicated in many plant processes, including desiccation tolerance and dormancy (Koorneef 1984, Kermode and Bewley 1987, Chandler and Robertson 1994). Given the location and putative function of oleosin, it is not surprising that it is affected by this growth regulator.

Another growth regulator which appears to induce the synthesis of oleosin is jasmonic acid. This induction has been demonstrated in microspore-derived embryos from canola (Holbrook *et al.* 1991, van Rooijen *et al.* 1992, Wilen *et al.* 1991). This growth regulator has also been implicated in other seed-specific processes such as inhibition of germination and expression of storage protein (Staswick 1990, Wilen *et al.* 1991).

The presence of two or more oleosins, of similar molecular weights, in a given plant species may have contributed to some of the confusion about the regulation of

oleosin and triacylglycerol synthesis. It has been speculated that these isoforms of oleosin form dimers or oligomers on the oil body (Tzen *et al.* 1990, Lee *et al.* 1995) and these isoforms are expressed at slightly different times during oil body formation (Lee *et al.* 1995, Peng and Tzen 1998). The function of having more than one isoform of oleosin on the oil body is not clear, but it has been suggested that it may increase stability of the oil body or enhance lipase binding (Huang 1996).

4.2 Anther Specific Lipid Particles

There are reports that oleosins, identified by homology with the hydrophobic region of seed oleosins, are also present in anther tissue (Roberts *et al.* 1993, Roberts *et al.* 1994, Ruiter *et al.* 1997). Pollen and tapetum cells have both been shown to contain cytosolic lipid particles (Hess and Hess 1994, Hess 1995). In addition, the pollen coat contains a layer enriched in lipid called the tryphine; oleosins, which end up in the tryphine of pollen, are thought to be released with lipid particles from tapetal cells during their lysis (Ross and Murphy 1996, Murphy and Ross 1998). It has been proposed that these oleosins may serve a role in the rehydration of pollen immediately after fertilization (Ross and Murphy 1996).

Most of the anther oleosins differ from seed oleosins in that they have large carboxyl terminal extensions with a repetitive motif, the function of which remains unknown (Roberts *et al.* 1995). Oleosins associated with lipid bodies in the tapetum cells also have this repetitive motif (Ross and Murphy 1996, Wang *et al.* 1997b, Murphy and Ross 1998, Ferreira *et al.* 1997, Wu *et al.* 1997) and interestingly, they appear to be targeted to endoplasmic reticulum and assume the same conformation in this membrane as the seed oleosins (Murphy and Ross 1998). They also appear to be

post-translationally modified by proteolysis and possibly other processes (Ross and Murphy, 1996, Murphy and Ross 1998, Wang *et al.* 1997b).

4.3 Plastid Lipid Particles

Most plastids also contain lipid particles, and the best studied of these are the plastoglobuli. These particles are similar in structure to other lipid bodies and vary in size and number depending on the species examined (Sarafis 1998). They are almost always abundant in senescing chloroplasts and are thought to be formed during the dismantling of the thylakoid membranes that accompanies senescence. However, plastoglobuli are also evident in the chloroplasts of young healthy leaves (Sarafis 1998). It has been suggested that the plastoglobuli of young leaf tissue may serve as a source of reserve components for thylakoid formation (Pozueta-Romero *et al.* 1997, Sarafis 1998). Plastoglobuli have been reported to contain proteins, polar lipids, pigments and neutral lipids, most commonly triacylglycerol (Hansmann and Sitte 1982, Steinmuller and Tevini 1985, Tevini and Steinmuller 1985, Young *et al.* 1991, Sarafis 1998), which suggests that the endoplasmic reticulum is not, in fact, the only organelle to contain the enzymes that synthesize triacylglycerol. Interestingly, reports on the chemical composition of plastoglobuli are often contradictory, and it has been suggested that there may be more than one population of these particles (Simpson and Lee 1976, Pozueta-Romero *et al.* 1997). In fact, lipid-protein particles isolated from young *Phaseolus vulgaris* leaves have been reported to contain photosynthetic protein catabolites (Ghosh *et al.* 1994) and the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Smith *et al.* 1997), and it has been proposed that these particles may be involved in thylakoid membrane turnover in healthy leaves.

Recently, an abundant protein termed plastoglobuli associated protein (PAP) has been reported (Pozueta-Romero *et al.* 1997). This protein binds to the boundary of plastoglobuli in chromoplasts of capsicum fruit and is present in the plastids of other organs of capsicum and other plants. It has been proposed that this protein may have a stabilizing function similar to oleosin by virtue of its location on the boundary of a lipid particle (Pozueta-Romero *et al.* 1997).

4.4 Nonstorage Cytosolic Lipid Particles

As with animal cells, there are reports of lipid-containing particles in the cytosol of nonstorage cells of plants (Frey-Wyssling *et al.* 1963, Walek-Szernecka 1965, Sorokin and Sorokin 1968, Trelease 1969, Yatsu *et al.* 1971, Yatsu and Jacks 1972), and some efforts have been made to distinguish these particles from oil bodies in seeds (Yatsu *et al.* 1971). More recently, these lipid particles have been proposed to be involved in the turnover of membranes during normal growth of plants (Yao *et al.* 1991a, Yao *et al.* 1991b, Hudak *et al.* 1995). This proposal is based on the following observations. First, healthy membranes in young cells contain enzymes capable of forming lipid metabolites, during lipid turnover, which will disrupt the integrity of an intact bilayer if allowed to accumulate in the membrane (Brown *et al.* 1991). Second, these lipid metabolites have been found to be abundant in the cytosolic lipid-protein particles (Yao *et al.* 1991a, Yao *et al.* 1991b, Hudak *et al.* 1995, McKegney *et al.* 1995, Hudak and Thompson 1996). Third, during senescence, these lipid metabolites accumulate in the membranes disrupting the structure of the membrane, and at the same time the abundance of these cytosolic lipid particles decreases (Yao *et al.* 1991b, Hudak *et al.* 1995, McKegney *et al.* 1995).

Lipid particles thought to be involved in membrane turnover were first described in *Phaseolus vulgaris* cotyledons (Yao *et al.* 1991a) and later in carnation petals (Hudak and Thompson 1996). Initially, they were thought to be vesicles based on their appearance in freeze-fracture electron microscopy, and were termed deteriosomes (Yao *et al.* 1991a, Yao and Thompson 1993, Yao *et al.* 1991b), but this interpretation was later revised when it was ascertained that these particles contain lipids similar to those in oil bodies (Hudak and Thompson 1996) and they are presently thought to be lipid particles, with a similar structure to oil bodies (Thompson *et al.* 1997, Thompson *et al.* 1998). These lipid particles contain phospholipid and triacylglycerol, and they are enriched in free fatty acids, steryl and/or wax esters, diacylglycerol and peroxidized lipids when compared to microsomal membranes (McKegney *et al.* 1995, Hudak and Thompson 1996, Yao *et al.* 1991b, Hudak *et al.* 1995). Two techniques have been used to isolate these particles: ultrafiltration and flotation centrifugation. It is not clear if these two techniques isolate the same population of particles, but particles obtained by either technique appear to have similar characteristics. For example, the same lipid metabolites are present in both ultrafiltered and floated lipid particles from carnation petals (Hudak and Thompson 1996). It has also been observed that lipid particles from both carnation petals and *Phaseolus vulgaris* cotyledons contain proteins. Protease activity has been described in *Phaseolus vulgaris* cotyledon lipid particles (Yao and Thompson 1993), and catabolites of H^+-ATPase have been found in lipid particles from carnation petals (Hudak *et al.* 1997). The latter observation has prompted speculation that these particles may be involved in removing protein catabolites from membranes in addition

to lipid metabolites (Hudak *et al.* 1997). In fact, there have been several reports of activities of membrane-associated enzymes in the cytosol from *Phaseolus vulgaris* cotyledon cells. These enzymes include 5'-nucleotidase, glucose-6-phosphatase (Lai *et al.* 1971), ATPase (Lai and Thompson 1972) and cytochrome c reductases (Thompson 1974). These observations indicate that the soluble forms of these enzymes are removed from the membranes in such a way as to retain their activities, possibly through the release of lipid particles from the membrane bilayer.

4.5 Fates of Oil Bodies and Other Lipid Particles

The documented functions of oleosins are somewhat speculative. It seems certain that they prevent the coalescence of oil bodies during the desiccation of seeds during dormancy, thereby maintaining the large surface area to volume ratio required for rapid metabolism of triacylglycerol during germination (van Staden *et al.* 1975, Slack *et al.* 1980, Tzen and Huang 1992, Tzen *et al.* 1992, Murphy 1993). The major evidence supporting this contention is the finding that when the exposed regions of oleosin are removed proteolytically from the surface of oil bodies, they coalesce into much larger particles (Tzen and Huang 1992) or aggregate when the pH is lowered (Tzen *et al.* 1997). Furthermore, oleogenic fruits, which have very large oil globules that are not metabolized, and certain desiccation-sensitive seed oil bodies lack the oleosin coat (Ross *et al.* 1993, Murphy 1993, Lee *et al.* 1994, Murphy *et al.* 1995).

It has also been proposed that oleosins may serve as platforms for the attachment of triacylglycerol lipases to the oil body (Huang *et al.* 1987, Wang and Huang 1987, Huang 1992, Huang 1996). Further to this notion, it is conceivable that oleosin acts as a lipase activator analogous to the activators documented for animal

lipases (Benatsson-Olivecrona and Olivecrona 1994). For example, one of the oleosins of canola contains a domain that has 54% homology with a 24 amino acid region of apolipoprotein C2 found associated with animal LDPs. This region of apolipoprotein C2 serves as a lipase binding site and activator (Murphy 1990). Lipases have been shown to be firmly attached to oil bodies in germinating seeds of maize, canola, castor bean and sunflower (Lin *et al.* 1983, Lin and Huang 1983, Lin and Huang 1984, Ory *et al.* 1962, Ory 1969, Bahri *et al.* 1995), but there are also reports of both membrane-bound and soluble lipases in germinating seeds (Theimer and Rosnitschek 1978, Maeshima and Beevers 1985, Hills and Murphy 1988). There is a considerable lack of consistency in the observations made on the characteristics of lipases involved in the breakdown of triacylglycerol in oil bodies. It has been suggested that this reflects the method of lipase binding to oil bodies rather than the lipases themselves (Hoppe and Theimer 1997). It is also of interest to note that most of the lipases described are not present in the oil bodies of developing seeds, suggesting that they are synthesized and become associated with oil bodies only during germination (Huang 1992).

There are reports that proteins other than oleosins are associated with oil bodies, but these reports are difficult to accept without immunocytochemical evidence as it is clear that contaminating proteins can associate tightly with oil bodies (Herman 1987, Herman *et al.* 1990, Kalinski *et al.* 1990, Kalinski *et al.* 1992, Thoyts *et al.* 1996). Recently, three unidentified proteins have been proposed to associate with oil bodies in sesame seeds (Chen *et al.* 1998). It has been shown by immunofluorescence

microscopy that these proteins are found in the same region as oil bodies, but their precise location has not yet been adequately pinpointed (Chen *et al.* 1998).

Another protein, which is proposed to be associated with oil bodies, is a specific isozyme of lipoxygenase. It is thought to be synthesized and targeted to the surface of oil bodies very early during the germination of cucumber seeds (Feussner and Kindl 1992, Feussner *et al.* 1996) coincident with the onset of triacylglycerol mobilization. The location of this lipoxygenase has been confirmed immunochemically in cucumber (Feussner *et al.* 1996) and biochemically in cucumber, soybean and barley (Feussner and Kindl 1992, Feussner *et al.* 1995, Holtman *et al.* 1997). It has been suggested that this lipoxygenase is responsible for oxygenating the linoleate esterified to triacylglycerol, thereby initiating the mobilization of oil body triacylglycerol (Feussner *et al.* 1997a, Grechkin 1998). Indeed, oxygenated products of linoleic acid have been found to increase with time in cucumber cotyledons, following the onset of germination, in parallel to increased expression of this lipoxygenase (Feussner *et al.* 1997b). Peroxidized lipids have also been described for cytosolic lipid particles from *Phaseolus vulgaris* cotyledons, and these lipids were proposed to be products of membrane lipoxygenase (Yao *et al.* 1993). Given the proposed role for lipoxygenase in oil bodies, it is possible that these peroxidized lipids were formed in the lipid particles rather than membranes and that these lipid particles are subject to the same cascade of steps hypothesized to be involved in the catabolism of oil bodies.

Oleosins have been shown to minimize the size of oil bodies (Fasman and Gilbert, 1990), thereby maintaining a large surface area to volume ratio, which

facilitates the metabolism of triacylglycerol during seed germination and cotyledon senescence. In oil seeds, glyoxysomes, which are specialized microbodies capable of metabolizing fatty acids, rapidly appear following imbibition and disappear coincident with the decrease in oil bodies and depletion of triacylglycerol. This is thought to reflect the conversion of other microbodies into glyoxysomes rather than glyoxysome biogenesis (Huang *et al.* 1983, Sautter 1992). Glyoxysomes are considered to be a type of peroxisome containing the glyoxylate pathway as well as the peroxisomal enzymes, catalase and flavin oxidases. Both glyoxysomes and peroxisomes are capable of fatty acid β-oxidation. However, peroxisomes only shorten long-chain fatty acids to medium-chain fatty acids, whereas glyoxysomes are capable of metabolizing fatty acids completely to acetyl CoA (Schulz 1985). Although the ontogeny of microbodies is not yet understood, the transformation of peroxisomes to glyoxysomes has been documented for several plant seedlings (Sautter 1992). Furthermore, there is evidence indicating that peroxisomes become glyoxysomes in senescing leaf, petal and photosynthetic cotyledon tissue (De Bellis and Nishimura 1991, De Bellis *et al.* 1990, Landolt and Matile 1990, Pistelli *et al.* 1991, Nishimura *et al.* 1993, Gut and Matile 1988, Kudielka 1981). The transformation is hypothesized to be involved in metabolizing lipids derived from the breakdown of organelles during cellular senescence (Gut and Matile 1988, De Bellis and Nishimura 1991).

In oil seeds, glyoxysomes are thought to metabolize fatty acids resulting from the action of lipase on the triacylglycerol of oil bodies (Cooper and Beever 1969, Hutton and Stumpf 1969). It has been demonstrated that there is close physical contact between oil bodies and glyoxysomes, which may facilitate this transfer

(Frederick *et al.* 1968, Mollenhauer and Toten 1970, Vigil 1969, Vigil 1970, Wanner *et al.* 1982). The fatty acids are metabolized by β-oxidation to acetyl CoA, which enters the glyoxylate cycle and is converted to succinate. Succinate is then transferred to the mitochondrion where it is converted to malate or oxaloacetate through the TCA cycle. Malate supports gluconeogenesis in the cytosol producing glucose, which can be translocated (Bewley and Black 1994). It is interesting to note that although the peroxisome contains all the enzymes for β-oxidation, it does not convert the products of this cycle to malate or succinate. Rather, the peroxisome may be involved in metabolizing lipid removed from the membrane in the normal course of turnover in order to provide anabolic building blocks. In fact, this may be the fate of the cytosolic lipid particles observed in plant tissues. However, there is evidence that the abundance of cytosolic lipid particles decreases during senescence both of bean cotyledons (Yao *et al.* 1991b, Yao *et al.* 1993, McKegney *et al.* 1995) and carnation petals (Hudak *et al.* 1995), which may reflect the onset of glyoxysomal β-oxidation.

The fates of lipid-depleted oil bodies or cytosolic lipid particles have not been well studied. It is generally thought that as the triacylglycerol stores of oil bodies are depleted, the oleosin coat is also proteolytically degraded, for the triacylglycerol and oleosins of oil bodies decline in parallel after germination (Murphy and Cummins 1989, Murphy *et al.* 1989). It has been further proposed, based on electron microscopic evidence for maize seeds, that the collapsed phospholipid monolayer of the triacylglycerol-depleted oil body becomes associated with the tonoplast, in order to allow expansion of the vacuole (Wang and Huang 1987). In mustard, however, these phospholipid monolayers appear to become associated with the glyoxysome (Bergfeld

et al. 1978). As yet, any conclusion drawn from these results would be highly speculative and need further support.

4.6 Lipid Particles Present in the Cytosol of *Phaseolus vulgaris* Cotyledons

In the present study, four distinguishable classes of lipid particles were identified in developing and germinating, wax bean seeds (*Phaseolus vulgaris*). All developing legume seeds store carbohydrate, protein and lipid, but the predominate reserves tend to be protein stored in protein bodies, and carbohydrate in the form of starch (Mayer and Poljakoff-Mayber 1982, Bewley and Black 1994). The particles from wax bean were classified as low-density, low-density', intermediate-density, and high-density lipid particles (LDPs, LDP's, IDPs and HDPs, respectively) on the basis of differences in density established by flotation centrifugation, and were found to have distinctive lipid compositions that accounted for the differences in density. LDPs and IDPs resemble oil bodies of oil-bearing seeds in that they contain predominantly triacylglycerol and have proved to be abundant in developing seeds and to decline in abundance after the onset of germination coincident with mobilization of energy reserves. However, LDPs and IDPs are also distinguishable from the well characterized oil bodies of oil-bearing seeds in that they do not contain detectable levels of oleosin. They appear, therefore, to be a distinctive and previously uncharacterized type of seed oil body. HDPs proved to be clearly distinguishable from LDPs and IDPs on the basis of their lipid composition, particularly in that they contain only low levels of triacylglycerol. These particles are thought to be similar to previously described cytosolic lipid particles found in wax bean cotyledons which are believed to be involved in membrane turnover. Finally, several proteins involved in

the formation of lipid particles in other organisms were compared with plant proteins in various data banks in an effort to gain some further insight into the ontogeny of plant lipid particles, and preliminary results from these searches are described.

Methods and Materials

1 Chemicals

Chemicals were purchased from Sigma (Mississauga, Ontario) and Boehringer Mannheim (Laval, Quebec) unless otherwise noted. Solvents were purchased from BDH (Toronto, Ontario). Radiochemicals were purchased from ICN (Mississauga, Ontario).

2 Plant Material

2.1 Growth Conditions

2.1.1 Developing Seeds

Seeds of *Phaseolus vulgaris* (var. Kinghorn) were obtained from the Ontario Seed Company (Waterloo, Ontario, Canada). In order to obtain developing seeds, plants were grown in a soil mix (promix BX), four seedlings to a pot, in ten inch pots supported by wire mesh. The plants were grown under a 16 hour photoperiod, with 24°C days and 20°C nights, and fertilized with 20-20-20 twice weekly. Developing seeds were harvested from pods just beginning to dry, and at this stage contained 17.5 $\mu g \pm 2.1$ chlorophyll$\cdot g^{-1}$ fresh weight.

2.1.2 Germinating Seeds

Seeds of *Phaseolus vulgaris* were also germinated in vermiculite at 27°C in a dark growth chamber to promote etiolation. For this purpose, the seeds were planted in trays containing dry vermiculite, and the vermiculite was saturated with water. Germinating seeds at different stages were harvested at specified periods after planting

with the exception of those classified as being harvested 1 day after planting. These were simply soaked in water for 24 h in the dark with vigorous aeration.

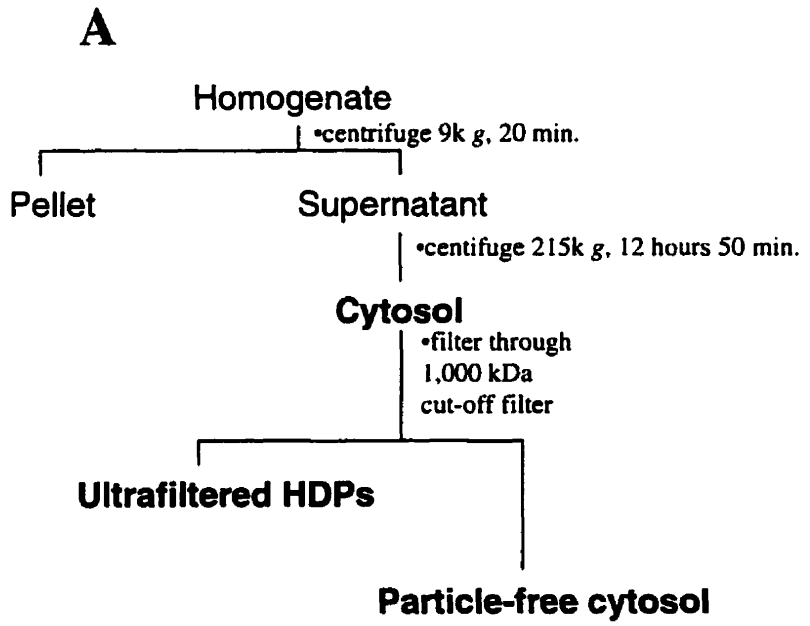
3 Tissue Fractionation

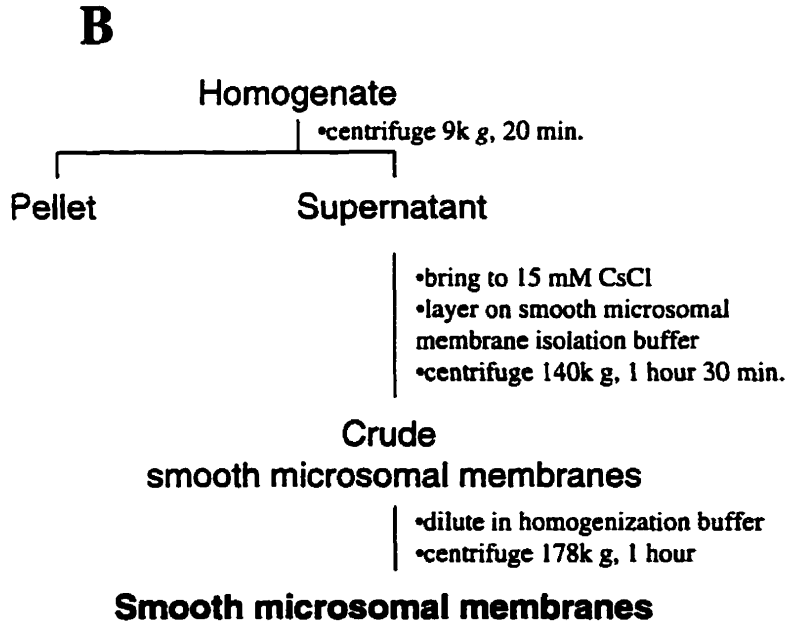
Developing and germinating bean seeds were homogenized with a Polytron homogenizer for 50 seconds in homogenizing buffer (3ml•g^{-1} fresh weight) containing 50 mM sodium phosphate buffer, pH 7.5, 2mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 8.5 % sucrose. Phenylmethanesulfonylfluoride (PMSF) (1 mM) and benzamidine hydrochloride (1mM), each dissolved in dimethylsulphoxide (DMSO), were added immediately before homogenization to minimize proteolytic degradation. All steps were performed on ice or at 4°C.

3.1 Isolation of Smooth Microsomal Membranes, Cytosol and Ultrafiltered High Density Lipid Particles

Smooth microsomal membranes and cytosol were isolated as described in Yao *et al.* (1991a). The homogenate was filtered through four layers of cheesecloth and centrifuged at 9,000 *g* for 20 minutes (Fig. 1). To collect smooth microsomal membranes (Fig. 1B), the resulting supernatant was made 15 mM with respect to CsCl, layered on top of a sucrose cushion (homogenizing buffer containing 1.3 M sucrose and 15 mM CsCl) and centrifuged in a Beckman SW 28 rotor at 140,000 *g* for 90 minutes. The layer formed at the interface was collected and diluted approximately four times in homogenizing buffer, mixed thoroughly and centrifuged again at 178,000 *g* for 60 minutes. The resulting pellet, which consisted of smooth microsomal membranes, was resuspended in homogenizing buffer.

Figure 1. Flowcharts describing the procedures for isolating cytosol, ultrafiltered HDPs, particle-free cytosol and smooth microsomal membranes from the homogenate of bean cotyledons after filtering through four layers of cheesecloth. A. Protocol describing the isolation of cytosol, ultrafiltered HDPs and particle-free cytosol. B. Protocol describing the isolation of smooth microsomal membranes. Subcellular fractions used to measure cytochrome c-reducing activity are shown in bold letters.


A
Homogenate
•centrifuge 9k g, 20 min.
Pellet
Supernatant
•centifuge 215k g, 12 hours 50 min.
Cytosol
•filter through
1,000 kDa
cut-off filter
Ultrafiltered HDPs
Particle-free cytosol


B
Homogenate
•centrifuge 9k g, 20 min.
Pellet
Supernatant
•bring to 15 mM CsCl
•layer on smooth microsomal
membrane isolation buffer
•centrifuge 140k g, 1 hour 30 min.
Crude
smooth microsomal membranes
•dilute in homogenization buffer
•centrifuge 178k g, 1 hour
Smooth microsomal membranes

Cytosol was collected by centrifuging the post-9,000 *g* supernatant for 12 hours and 50 minutes at 215,000 *g* and collecting the supernatant beneath the floating fat pad (Fig. 1A).

To obtain ultrafiltered high-density lipid particles and particle-free cytosol, 25 ml of cytosol was placed in a 1000 kDa filter (Pharmacia, Baie d'Urfe, Quebec) and concentrated to a volume of approximately 5 ml (Fig. 1A). The filtrate was collected as particle-free cytosol. Homogenizing buffer (10 ml) was added to the retentate (ultrafiltered particles), and the diluted rententate was reduced to a volume of 5 ml by further filtration. This was repeated twice to reduce cytosolic contamination of the ultrafiltered high-density lipid particles. This is a modification of the procedure described by Yao *et al.* (1991a).

3.2 Isolation of Microsomal Membranes and Low-Density, Intermediate-Density and High-Density Lipid Particles by Flotation

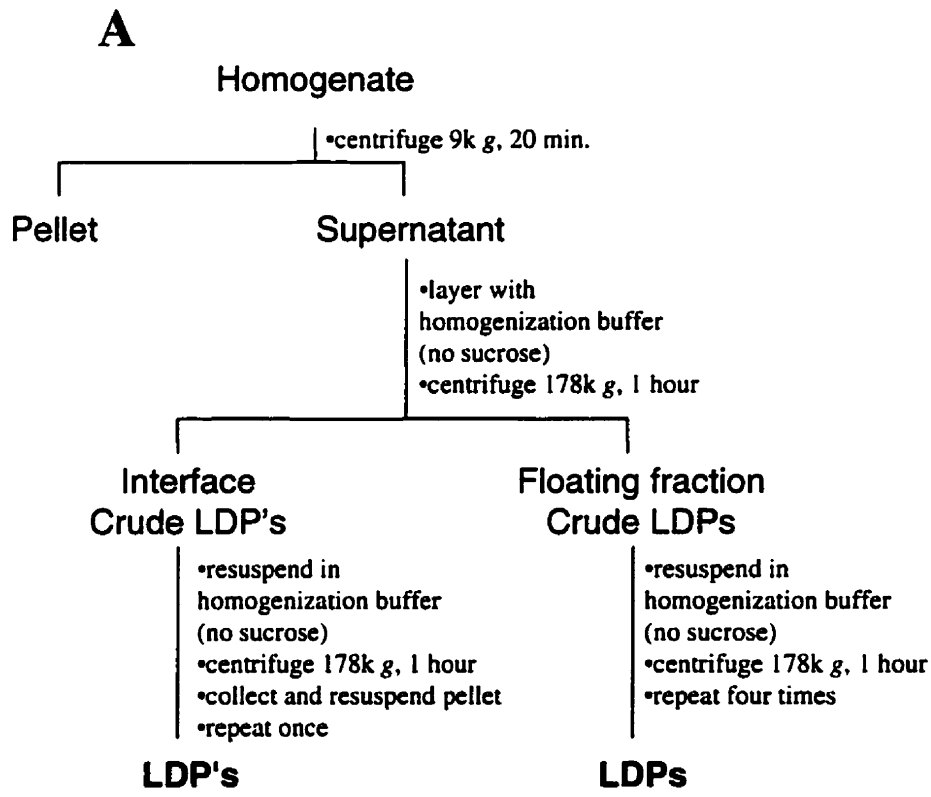
To obtain microsomal membranes, low-density lipid particles (LDPs), slightly higher-density particle (LDP's), intermediate-density particles (IDPs) and high-density particles (HDPs) (Table 1), the homogenate was filtered through four layers of cheesecloth and centrifuged at 9,000 *g* for 20 minutes at 2°C (Figs. 2 and 3). The supernatant was collected and centrifuged again at 178,000 *g* for one hour. The floating white pad, the remaining supernatant and the pellet were collected and processed separately (Figs. 2 and 3). Each step was performed at 2°C or on ice.

HDPs were isolated from membrane-free cytosol. For this purpose, the initial post-178,000 *g* supernatant was centrifuged at 215,000 *g* for 12 hours in order to sediment any residual membrane. Sucrose (20%) was then added to the resulting

Table 1. Abbreviations and brief descriptions of the subcellular fractions isolated from bean cotyledons

Abbreviations	Description
Cyt	Cytosol isolated after a prolonged centrifugation
SM	Smooth microsomal membranes
LDP	Low density lipid particle
LDP'	Slightly higher density particles than the low density lipid particle
IDP	Intermediate density lipid particle
HDP	High density lipid particle
SCL	Sucrose-containing layer isolated from directly beneath the IDP fraction
TM	Total microsomal membranes

Figure 2. Flowchart describing the procedure for isolating LDPs, LDP's and HDPs from the homogenate of bean cotyledons after filtering through four layers of cheese cloth. A. Protocol describing the isolation of LDPs and LDP's. B. Protocol describing the isolation of HDPs. Subcellular fractions used for subsequent analysis are shown in bold letters.


A
Homogenate
•centrifuge 9k g, 20 min.
Pellet
Supernatant
•layer with
homogenization buffer
(no sucrose)
•centrifuge 178k g, 1 hour
Interface
Crude LDP's
Floating fraction
Crude LDPs
•resuspend in
homogenization buffer
(no sucrose)
•centrifuge 178k g, 1 hour
•collect and resuspend pellet
•repeat once
•resuspend in
homogenization buffer
(no sucrose)
•centrifuge 178k g, 1 hour
•repeat four times
LDP's
LDPs

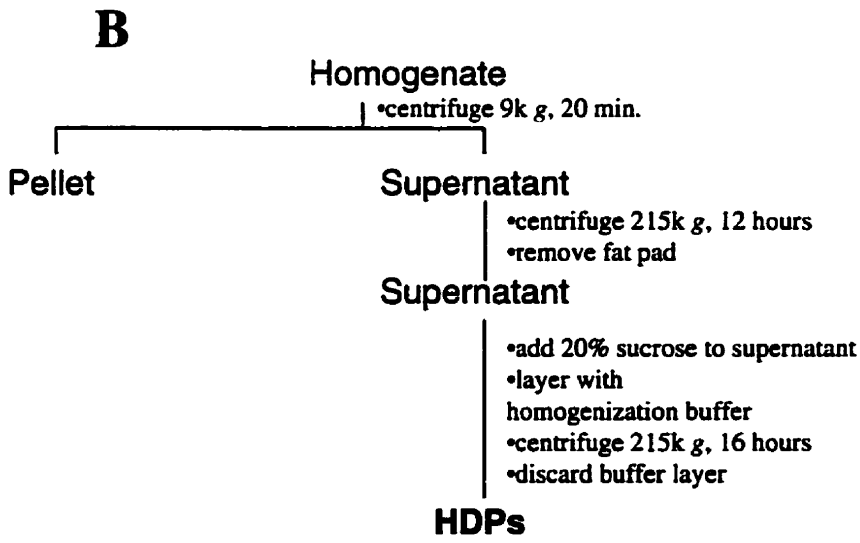
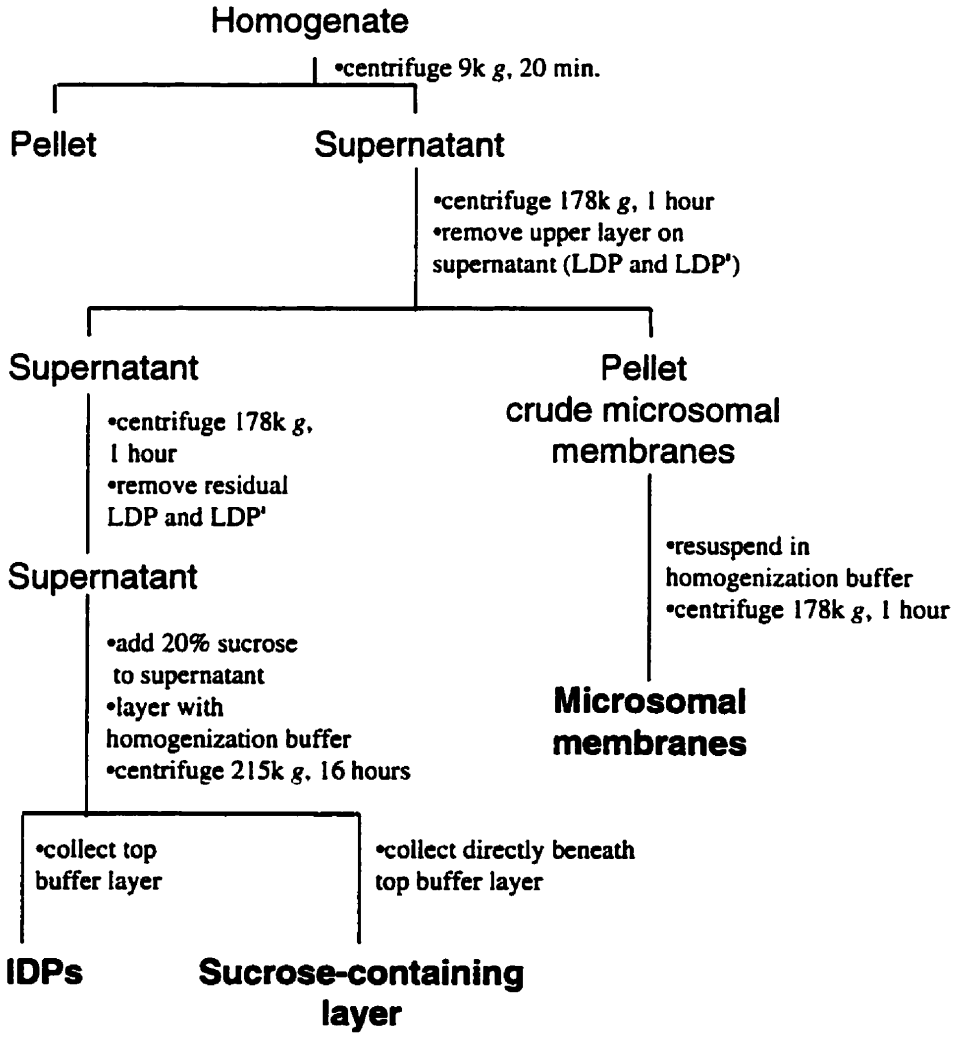

B
Homogenate
•centrifuge 9k g, 20 min.
Pellet
Supernatant
•centrifuge 215k g, 12 hours
•remove fat pad
Supernatant
•add 20% sucrose to supernatant
•layer with
homogenization buffer
•centrifuge 215k g, 16 hours
•discard buffer layer
HDPs

Figure 3. Flowchart describing the procedure for isolating microsomal membranes, IDPs and the sucrose-containing layer from the homogenate of bean cotyledons after filtering through four layers of cheese cloth. This protocol describes the isolation of microsomal membranes, IDPs and the sucrose-containing layer. Subcellular fractions used for subsequent analysis are shown in bold letters


Homogenate
•centrifuge 9k g, 20 min.
Pellet
Supernatant
•centrifuge 178k g, 1 hour
•remove upper layer on
supernatant (LDP and LDP')
Supernatant
Pellet
crude microsomal
membranes
•centrifuge 178k g,
1 hour
•remove residual
LDP and LDP'
•resuspend in
homogenization buffer
•centrifuge 178k g, 1 hour
Supernatant
Microsomal
membranes
•add 20% sucrose
to supernatant
•layer with
homogenization buffer
•centrifuge 215k g, 16 hours
•collect top
buffer layer
•collect directly beneath
top buffer layer
IDPs
Sucrose-containing
layer

HDPs were isolated from membrane-free cytosol. For this purpose, the initial post-178,000 *g* supernatant was centrifuged at 215,000 *g* for 12 hours in order to sediment any residual membrane. Sucrose (20%) was then added to the resulting supernatant, and 22 ml of this sucrose-containing solution was overlaid with 3 ml of homogenization buffer and centrifuged at 215,000 *g* for 16 hours in order to float the IDPs into the buffer layer. The upper buffer layer was removed, and the sucrose-containing supernatant was retained as the HDP preparation.

The floating white pad, containing the LDPs and the LDP's, was diluted by 2 volumes of homogenizing buffer. Each 25 ml centrifuge tube contained 20 ml of diluted LDPs and LDP's, which was layered with 5 ml homogenizing buffer without sucrose and centrifuged again at 178,000 *g* for 1 hour. The floating upper layer (LDPs) and the interface (LDP's) were collected and processed separately (Fig. 2A). The floating upper layer was resuspended in homogenizing buffer without sucrose and floated to the top of the centrifuge tube again by centrifuging at 178,000 *g* for 1 hour. This step was repeated three times to ensure minimal cytosolic contamination. The final preparation of LDPs was resuspended in homogenizing buffer without sucrose. The interface (LDP's) was also resuspended in homogenizing buffer without sucrose and pelleted by centrifuging at 178,000 *g* for 1 hour (Fig. 2A). The small white pellet was collected and resuspended in homogenizing buffer without sucrose.

IDPs were isolated from the supernatant (Fig. 3). For this purpose, the post-178,000 *g* supernatant was centrifuged again at 178,000 *g* for 1 hour, and the residual white floating pad was removed. Sucrose (20 %, w/v) was added to the resulting

supernatant, and 22 ml of this solution was overlaid with 3 ml of homogenization buffer, in each tube, and centrifuged at 215,000 *g* for 16 hours in order to float the IDPs into the buffer layer. The buffer layer containing the IDPs was retained. A portion of the sucrose-containing layer (SCL) below the IDPs was also collected to check for contamination of the IDPs with SCL.

The pellet, which consisted of microsomal membranes, was washed by resuspension in homogenizing buffer and centrifuging again at 178,000 *g* for 1 hour in order to remove residual cytosolic contamination (Fig. 3). This final pellet was resuspended in homogenizing buffer and retained as microsomal membranes.

3.3 Homogenization Studies

Two methods of homogenization, in addition to the standard procedure of homogenizing the tissue (section 3), were tested to examine possible homogenization effects on the particles isolated. In one of these methods, LDPs and IDPs were isolated under nonaqueous conditions, as described by Yatsu and Jacks (1968), by homogenizing cotyledon tissue in pure glycerol (1 g•3 ml^{-1}) at 4°C with a Sorval Omnimixer for six 10-second bursts. The glycerol homogenate was then layered with homogenization buffer containing sucrose and centrifuged at 178,000 *g* for 4 hours. The upper buffer layer was then used for isolation of LDPs and IDPs as described in section 3.2.

In the second method, LDPs and IDPs were isolated from a powder obtained by freezing the cotyledon tissue in liquid nitrogen and grinding in a Waring blender. This powder was suspended in homogenization buffer containing sucrose (1 g•3 ml^{-1}),

filtered through four layers of cheese cloth and used for isolation of LDPs and IDPs as described in section 3.2.

3.4 Isolation of Protein Bodies

Protein bodies were isolated from bean cotyledons one day after germination by two methods due to the difficulty of isolating these organelles in aqueous media without rupturing their membranes. In one of the methods, the 1-day-old seedlings were homogenized with a Sorval Omnimixer for ~50 seconds in glycerol ($3ml \cdot g^{-1}$ fresh weight) according to Yatsu and Jacks (1968). The homogenate was filtered through four layers of cheesecloth and centrifuged at 1100 *g* for 5 minutes. The pellet was discarded, and the supernatant was centrifuged at 41,000 *g* for 20 minutes. This pellet was retained and resuspended in 50 mM sodium phosphate buffer, pH 7.5 containing 2 mM EDTA, 5 mM EGTA and 8.5% sucrose. All steps were performed at 2°C or on ice.

For the alternate method, the cotyledons were cubed into ~$2mm^2$ pieces and homogenized, with a mortar and pestle, in 100 mM MES (morpholinoethanesulfonate) (pH 5.5) with 0.6 M mannitol and 1mM EDTA (2 cotyledons$\cdot ml^{-1}$) as described by Mader and Chrispeels (1984). The homogenate was filtered through four layers of cheesecloth and centrifuged at 100 *g* for 1 minute. The supernatant was then layered on a solution of 5% Ficoll (w/v) in the MES homogenizing buffer. This was centrifuged at 100 *g* for 10 minutes, and the pellet (protein bodies) was resuspended in MES homogenizing buffer and centrifuged at 100 *g* for 10 minutes. This last step was

repeated, and the purified protein bodies were resuspended in MES homogenizing buffer. All steps were performed at 2°C or on ice.

3.5 Isolation of Oil Bodies

Oil bodies were isolated from canola seeds (*Brassica napus* var. scimitar or *Brassica napus* var. topaz) as described by van Rooijen and Moloney (1995). Dry canola seeds were homogenized (10 $ml \cdot g^{-1}$ fresh weight) using a Polytron homogenizer for 50 seconds in ice cold 0.15 M Tricine-KOH buffer (pH 7.5) containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA and 0.6 M sucrose. The homogenate was filtered through four layers of cheesecloth and centrifuged at 5000 *g* for 10 minutes. The fat pad (oil bodies) was removed from the top of the centrifuge tube and resuspended in the Tricine homogenizing buffer. This was layered with the same buffer, except it contained only 0.1 M sucrose, and centrifuged at 18,000 *g* for 20 minutes. This step was repeated three times to minimize cytosolic contamination. The purified oil body layer was resuspended in Tricine homogenizing buffer containing 0.1 M sucrose. All steps were performed on ice or at 2°C.

Oil bodies were also isolated from soybean seeds (*Glycine max* var.victory) according to the method of Loer and Herman (1993). Soybean seeds were soaked in water for 24 hours and homogenized ($3ml \cdot g^{-1}$ fresh weight) for 50 seconds in ice cold 100 mM Tris-HCl buffer (pH 8.6) containing 3 mM $MgCl_2$ using a Polytron homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 53,000 *g* for 20 minutes in a SW 28 rotor. The floating fat pad (oil bodies) was resuspended in the same buffer with the addition of 0.5 M NaCl and

centrifuged at 53,000 *g* for 20 minutes. The oil body pad was recovered, resuspended in 100 mM Na_2CO_3, allowed to incubate on ice for 30 minutes and centrifuged at 53,000 *g* for 20 minutes. The oil body pad was recovered, resuspended in 2 mM Hepes buffer (pH 7.5) containing 100 mM KCl and 3 mM $MgCl_2$ and centrifuged at 53,000 *g* for 20 minutes. This final step was repeated twice, and the oil body pad was resuspended in Hepes buffer containing 100 mM KCl, 3 mM $MgCl_2$ and 2 mM DTT. All steps were performed at 2°C or on ice.

3.6 Washing of Isolated Lipid Particles

Purified LDPs, IDPs and canola oil bodies were incubated in 100 mM Na_2CO_3 for 30 minutes on ice (Fujiki *et al.* 1982) or in 1% (v/v) Triton X-100 or 1% (w/v) sodium deoxycholate for 1 hour on ice (Bergfeld *et al.* 1978) in an attempt to remove proteins which might be artifactually associated with the particles. Following these incubations, the particles were reisolated in accordance with the standard procedures described in sections 3.2 and 3.5 and also figures 2 and 3.

In another experiment, LDPs and soybean oil bodies were isolated in the presence of 4.5 M urea (Millichip *et al.* 1996) in an effort to remove contaminating proteins. In this case, the particles were isolated as described in section 2.3 and figure 1 except that 4.5 M urea was added to the homogenization buffer.

3.7 Proteinase K Treatment

Isolated lipid particles were treated with Proteinase K in order to determine whether proteins associated with the particles were anchored in the interior of the particle. For this purpose, suspensions of isolated lipid particles, containing ~0.1

$mg \cdot ml^{-1}$ protein, were incubated in 10 $\mu g \cdot ml^{-1}$ Proteinase K at 37°C with gentle agitation. The reaction was stopped at specified time points by adding PMSF (dissolved in DMSO) to a final concentration of 2 mM and placing the samples on ice (Hills *et al.*, 1993).

4 Analytical Procedures

4.1 Measurement of α-Amylase Inhibitor Activity

The ability of LDP preparations to inhibit α-amylase was measured using the assay for α-amylase inhibitors originally described by Bernfeld (1955) and modified by Pueyo *et al.* (1993). Porcine α-amylase was dissolved in 15 mM succinate (pH 5.6) containing 20 mM $CaCl_2$, 0.5 M NaCl and 2 $mg \cdot ml^{-1}$ BSA at a concentration of 1.53 $units \cdot ml^{-1}$. This enzyme preparation (50 µl) was added to 950 µl of a 1% solution of soluble potato starch (w/v) dissolved in 40 mM potassium phosphate (pH 6.9) containing 50 mM NaCl. This reaction mixture was incubated for 5 minutes at 20°C and stopped with 1 ml of degassed 1% dinitrosalicylic acid (w/v) dissolved in 0.4 M NaOH containing 30% potassium sodium tartrate. The reaction mixture was then boiled for 10 minutes and diluted with 8 ml of water. The absorbance of this solution was read at 530 nm against a blank prepared in the same way but omitting the enzyme. The absorbance was compared to a standard curve for maltose. Inhibition of ∀-amylase was determined by incubating the enzyme with specified amounts of LDP suspension for 60 minutes at 37°C before measuring α-amylase activity.

4.2 Cytochrome c Reductase Activity

Cytochrome c-reducing activity of isolated subcellular fractions was assayed according to Pohl and Wiermann (1981) with slight variations. The basic reaction mixture consisted of 50 mM $NaPO_4$ buffer (pH 7.5), 1 mM KCN, 30 µM rotenone (dissolved in 5 µl ethanol), 70 µM cytochrome c and 0.4 mM NADPH in a total volume of 1 ml. In TCA-precipitated and Proteinase K-treated samples, the KCN and rotenone were omitted from the assay mixture. The reaction was started by adding 0.5 ml of isolated subcellular fraction containing 200 $\mu g \cdot ml^{-1}$ protein. Cytochrome c reduction was recorded at room temperature as the change in absorbance at 550 nm. In some cases, 0.4 mM NADH was added instead of NADPH.

The pH profile of NADPH-dependent cytochrome c-reducing activity was measured using the following buffers in the assay mixture: MES (pH 6.0, 6.5, 7.0), sodium phosphate (pH 6.5, 7.0, 7.5, 8.0), TRIZMA (pH 7.5, 8.0, 8.5, 9.0), CHES (pH 8.5, 9.0, 9.5, 10.0) and CAPS (pH 9.5, 10.0, 10.5). Each buffer overlapped the next by two pH points to assess the possibility of buffer effects on the activity.

4.2.1 Effects of Heat, Acid and Protease Treatment on Cytochrome c Reductase Activity

To determine whether the cytochrome c-reducing activity of smooth microsomal membranes and cytosol was enzymatic, the isolated fractions were boiled for 20 minutes and centrifuged for 5 minutes in a clinical centrifuge to pellet aggregated proteins. The resultant supernatant was then used for enzyme assays. Cytosolic protein was also precipitated by adding trichloroacetic acid (TCA) to a final

concentration of 10% followed by incubation for 20 minutes on ice. This mixture was centrifuged for 5 minutes in a clinical centrifuge, and the supernatant was then boiled for 20 minutes and centrifuged again for 5 minutes in a clinical centrifuge. The final supernatant was adjusted to pH 7.5 by addition of concentrated KOH before being assayed for cytochrome c reductase. The volume of KOH was recorded and more sample added to the assay mixture accordingly.

In other experiments, the effects of degrading cytosolic protein by proteolysis were determined. Proteinase K was added to the cytosol at a concentration of 1 $mg \cdot ml^{-1}$, and the sample was incubated at 4°C overnight, boiled for 20 minutes and centrifuged for 5 minutes in a clinical centrifuge. The resultant supernatant was assayed for cytochrome c reductase activity.

4.2.2 Effects of Sephadex G25 NAP Column Chromatography on Cytochrome c Reductase Activity

In order to determine whether the cytochrome c-reducing activity was proteinaceous, smooth microsomal membranes and cytosol were fractionated on NAP 25 columns (Pharmacia, Baie d'Urfe, Quebec). This column is specifically designed for desalting and allows separation of molecules 5 kDa and larger from lower molecular weight components. For this purpose, cytosol and smooth microsomal membranes were diluted to approximately 1.5 $mg \cdot ml^{-1}$ and 4 $mg \cdot ml^{-1}$, respectively, and loaded onto the columns. The fractions were eluted with homogenizing buffer, and 2 ml fractions were collected and assayed for cytochrome c reductase activity and protein.

4.3 Effects of Abscisic Acid on the Induction of Protein Synthesis

Abscisic acid (ABA) was used to induce the synthesis of putative oleosins in wax bean seeds as described by Zou *et al.* (1995). To this end, ABA dissolved in 70% ethanol was added, at a final concentration of 10 μM, to the water in which the bean seeds were allowed to imbibe for 24 hours. The seeds were then fractionated as described in section 3.2.

4.4 Lipid Analysis

4.4.1 Lipid Extraction

For lipid analysis, total lipids were extracted from 3 ml of HDP, IDP and LDP suspensions, from 3 ml SCL and from 1 ml of microsomal membrane suspensions according to Bligh and Dyer (1959). Prior to extraction, the following internal standards were added: diheptadecanoyl phosphatidylcholine, diarachidinglycerol, heptadecanoic acid and arachidic acid oleoyl ester. Extraction was accomplished by adding one volume of sample to three volumes of a 1: 2 chloroform: methanol mixture, vortexing and allowing the mixture to stand at room temperature for 10 minutes. One volume of chloroform and 0.8 volumes of NaCl (0.73% w/v) were then added to the samples, and they were vortexed thoroughly and left covered, overnight, at room temperature. The following day, the samples were centrifuged in a table top centrifuge for five minutes, and the lower phase was removed, dried under nitrogen, resuspended in 25 μl of 6: 1 chloroform: methanol and either further processed or stored, sealed under nitrogen, at -20°C.

4.4.2 Thin Layer Chromatography

Lipid extracts were fractionated by thin layer chromatography. To achieve this, the solvated lipids were streaked with a Hamilton syringe onto 250 μm thick Sil G-25 plates (Rose Scientific, Edmonton, Alberta) which had been conditioned at 110°C for 30 min, and the plates were developed in petroleum ether: diethyl ether: acetic acid (70: 30: 1, v/v/v). Separated lipids were visualized with iodine vapour and identified using the following standards: phosphatidylcholine (mixed acyl chains), dilinolein glycerol (mixed isomers), linolenic acid, trilinolein glycerol and cholesteryl linoleate. Individual lipids were extracted from the silica gel by scraping the silica off the plate into 2.5 ml of 2: 1: 0.8 methanol: chloroform: water and vortexing thoroughly. The samples were centrifuged for 5 minutes in a tabletop centrifuge and the solvent removed from the pelleted silica. This process was repeated once to extract any residual lipid from the silica, and the solvent removed was pooled with the original solvent. Chloroform (1.5ml) and water (2 ml) were added to the lipid extract, and the mixture was vortexed and allowed to phase-separate. The chloroform phase containing the extracted lipid was collected and dried under nitrogen (McKegney *et al.*, 1995).

4.4.3 Fatty Acid Analysis

In order to identify and quantify individual fatty acids, the separated lipids were transmethylated as described by Morrison and Smith (1964) by adding 1ml boron trifluoride-methanol, sealing the vial under nitrogen and heating in a water bath at 90°C for 1 hour. After allowing the samples to cool, the resultant fatty acid methyl

esters were extracted by adding 1ml hexane and 0.5 ml water, vortexing and allowing the phases to separate. The hexane phase, containing the fatty acid methyl esters, was removed, and 1ml hexane was added to the lower phase to remove any residual methylated fatty acids. The hexane extracts were pooled and dried under nitrogen. These samples were dissolved in a small amount of hexane and sealed under nitrogen.

Fatty acid methyl esters were quantified using a Hewlett-Packard model 5890 series II gas chromatograph equipped with a flame ionization detector and a 15 m x 0.25 mm i.d. fused silica capillary column, 0.20 μm film (SP-230, Supelco, Bellefonte, Pennsylvania) and optimized to separate a standard mixture of 37 fatty acid methyl esters. The injector temperature was set at 250°C and the detector temperature at 275°C. The split ratio was 89 using helium as a carrier gas at 2.9 ml•minute^{-1} with a head pressure of 50 PSI and a split flow rate of 63 ml•minute^{-1}. The initial oven temperature was 130°C, which was maintained for 1.0 minute and then ramped by 10°C•minute^{-1} to 180°C. This temperature was maintained for 3.0 minutes and was followed by a second ramp of 10°C•minute^{-1} until 250°C was reached. The oven was maintained at this final temperature for 4.0 minutes.

4.5 Polyacrylamide Gel Electrophoresis

4.5.1 Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

Proteins were fractionated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) in Mini protean Dual Slab Cells (BioRad, Mississauga, Ontario). Separating gels were prepared with 1.5 M Tris-HCl (pH 8.8) or 0.5 M Tris-HCl (pH 6.8), acrylamide/bis (30%T, 2.7%C, w/v), 10%

SDS (w/v), fresh 10% ammonium persulfate (w/v), 10% bromophenol blue (w/v) and TEMED. The final concentrations of ingredients in the separating gels were: 0.75 M Tris-HCl, 0.1% SDS (w/v), 0.1% ammonium persulfate (w/v), 0.001% TEMED (v/v) and acrylamide/bis as specified. Once poured, the gels were covered with 1-butanol and allowed to polymerize for at least one hour. Stacking gels contained 4% acrylamide/bis (w/v), 0.125 M Tris-HCl (pH 6.8), 0.1% SDS (w/v), 0.1% ammonium persulfate (w/v), 0.001% TEMED (v/v) and 0.005% bromophenol blue (w/v). The stacking gel was added to the exposed surface of the separating gel after the 1-butanol had been completely removed and the surface of the gel rinsed thoroughly with deionized, distilled water (ddH_2O). The stacking gel was also allowed to polymerize for at least one hour. Gradient gels (10-20% acrylamide) were purchased from BioRad (Mississauga, Ontario).

Protein samples to be fractionated were dissolved in an equal volume of 2 x SDS-sample buffer consisting of 0.125 M Tris-HCl (pH 6.8), 20% glycerol (v/v), 10% SDS (w/v), 10% β-mercaptoethanol (v/v) and 0.01% bromophenol blue (w/v). Delipidated samples were dissolved directly in 2 x SDS-sample buffer and diluted with an equal volume of ddH_2O. These mixtures were incubated at 100°C for 5 minutes before being loaded into rinsed wells of the gel.

Fractionation was carried out at 25 mA in a buffer consisting of 25 mM Tris, 19.2 mM glycine and 0.2% SDS (w/v) (pH 8.3). The gels were stained with Coomassie brilliant blue R250 (Fairbanks *et al.* 1971) or silver (Wray *et al.* 1991)

4.5.2 Isoelectric Focusing and Two-Dimensional Fractionation of Proteins

Denaturing isoelectric focusing (IEF) gels were prepared in Mini protean Dual Slab Cells (BioRad, Mississauga, Ontario) according to Robertson *et al.* (1987). A slab gel, 0 .75 mm thick, was made from the following components: ddH_2O, acrylamide/bis (30% T and 2.7% C w/v), 50% glycerol (v/v), ampholytes, pH 3-10 (BioRad, Mississauga, Ontario), urea, 10% Triton X-100 (v/v), 10% ammonium persulfate (w/v) and TEMED. Specifically, a 6% acrylamide gel was prepared with these solutions by mixing 2.7 ml water, 2 ml acrylamide/bis, 2.4 ml glycerol, 0.6 ml ampholytes and 6 *g* urea. This solution was degassed, and 40 µl Triton X-100, 25 µl ammonium persulfate and 20 µl TEMED were added. This final solution was poured immediately into the slab gel apparatus and allowed to polymerize for one hour. In some experiments, a higher percentage acrylamide gel was placed below the isoelectric focusing gel. The solution for this gel was prepared exactly as for the IEF gel except that the final acrylamide concentration was 12%. Five ml of this solution was placed in the bottom of the slab gel apparatus, covered with 1-butanol and allowed to polymerize for one hour before filling the rest of the apparatus with the 6% acrylamide gel solution. Samples to be fractionated by IEF were dissolved in an equal volume of lysis buffer containing: 8 M urea, 2% ampholytes (v/v), 2% Triton X-100 (v/v) and 1% β-mercaptoethanol (v/v). Delipidated samples were resuspended directly in IEF-lysis buffer and diluted with an equal volume of ddH_2O. The samples were incubated for 15 minutes at room temperature. The gels were placed in the anode and cathode buffers (25 mM H_3PO_4 and 50 mM NaOH, respectively), and the wells were

rinsed with ddH_2O, then filled with anode buffer and finally filled with 40 µl IEF-lysis buffer diluted four times. The samples were then added to the wells, and the electrodes were connected in reverse, consistent with the electrode solutions. The gels were run for 30 minutes at 150 V followed by 120 minutes to 180 minutes at 200 V and stained using Coomassie brilliant blue R250 (Fairbanks *et al.* 1971). Some gels were rinsed three times for 3 minutes in ddH_2O, and the non-sample lanes were cut into 6 mm slices which were eluted into ddH_2O for pH measurements.

For two dimensional fractionation, IEF gels were incubated in SDS-PAGE sample buffer for 30 minutes at room temperature with gentle agitation and placed at -20°C for 15 minutes. Strips corresponding to the lanes were then cut from the IEF gel and immediately placed horizontally along the top of a 15% SDS polyacrylamide gel. The proteins from the IEF strip were run into the gel and fractionated in the second dimension by SDS-PAGE.

4.6 Delipidation of Protein Samples

In some experiments, protein samples were delipidated prior to being fractionated by PAGE. Lipids were extracted, based on the method described by Bligh and Dyer (1959), by adding 3 volumes of a 1: 2 chloroform: methanol mixture to one volume of sample, vortexing and allowing the mixture to stand at room temperature for 10 minutes. One volume of chloroform and 0.8 volumes of NaCl (0.73% w/v) were then added, and the samples were vortexed and left overnight at 4°C. The lower chloroform phase was removed, and 3 volumes of chloroform were added to the upper phase. The mixture was vortexed, allowed to stand for 15 minutes

and centrifuged in a clinical centrifuge for 5 minutes. The lower chloroform phase was again removed. This procedure was repeated once, and then the upper methanol-water phase was removed leaving the interface (the aggregated proteins) in a small volume of chloroform and methanol-water. The interface suspension of aggregated protein was dried under nitrogen, and the samples were resuspended in SDS-sample buffer or IEF-lysis buffer in preparation for electrophoresis.

4.7 Western Blotting

Western blotting of SDS-PAGE fractionated proteins was performed according to Ghosh *et al.* (1989). The proteins were electrophoretically transferred to a 0.45 μm nitrocellulose membrane (BioRad, Mississauga, Ontario) at 4°C and 50 V for one hour. The transfer buffer consisted of 25 mM Tris-NaOH (pH 8.8), 160 mM glycine and 20% methanol (v/v). The nitrocellulose membrane was then blocked by agitation in 1% BSA (w/v) in buffer B (25 mM Tris-HCl, pH 7.5, 140 mM NaCl) for one hour and agitated overnight with primary antibody diluted in buffer B containing 1% BSA.

All polyclonal antibodies directed against oleosins (gifts from M.M. Moloney, Department of Biological Sciences, University of Calgary, Calgary, Alberta) were diluted 1: 10,000. Monoclonal antibodies directed against HSP70 (Sigma, Mississauga) and the phosphoamino acids (Sigma, Mississauga) were diluted 1: 5,000.

Following treatment with the primary antibody, the nitrocellulose membrane was washed twice for 10 minutes in buffer B containing 1% BSA, and twice more for 10 minutes in buffer B. The washed membrane was then incubated in anti-rabbit goat (Boehringer Mannheim, Germany) or anti-mouse goat (Rockland, Gilbersville,

Pennsylvania) secondary antibody conjugated to alkaline phosphatase and diluted in buffer B (1: 2,500) for one hour. Following this, the membrane was washed for 10 minutes in buffer B, for ten minutes in 1% Triton X-100 in buffer B, and twice again for ten minutes in buffer B. Bound antibody was visualized by developing the membrane in 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) containing 66 µl nitroblue tetrazolium (50 $mg \cdot ml^{-1}$ in 70% dimethylformamide, v/v) and 33 µl of 3-bromo-4-chloro-indolyl phosphate (50 $mg \cdot ml^{-1}$ in dimethylformamide). Colour development was stopped by rinsing the membrane three times in ddH_2O and allowing it to dry.

4.8 Protein and Chlorophyll Measurements

Proteins were quantified by the methods of Ghosh *et al.* (1988) or Bradford (1976) using BSA as a standard. Chlorophyll levels were determined by measuring absorbances at 647 nm and 664 nm after diluting the samples at least 50 times in DMSO as described by Pora *et al.* (1989).

4.9 Light Scattering Measurements

Light scattering measurements were performed by R. Hallet (University of Guelph, Guelph, Ontario) using a 50 mW Nd:YAG laser (Model 532-50, Coherent, Santa Clara, CA. USA) at a wavelength of 532.0 nm focused into a four-sided clear optical cuvette (Helma, Concord, MA. USA) at room temperature. The scattered light was collimated by two 400 µm diameter pinholes placed 35 cm apart and detected with a photomultiplier (Model 9863, EMI Electronics Ltd., Hayes, England). Photon counting and pulse shaping were performed using a quantum photometer (Model

1096, Langly Ford, Amherst, MA. USA). The procedures to recover size distributions are detailed in Hallet *et al.* (1988).

4.10 Electrospray Mass Spectrometry

Electrospray mass spectrometric analysis was performed by J. Chen, L. Taylor and G. Lajoie (University of Waterloo, Waterloo, Ontario) on a Micromass Quattro II triple-stage quadrupole mass spectrometer (Micromass, UK) equipped with an electrospray ionization source in flow-injection mode. The solvent mixture was 50: 50 acetonitrile: water with 1% formic acid at a flow rate of 40µl•min^{-1}. The sample was injected through a Rheodyne 7125 valve and transported to the ionization source by 40 cm of PEEK tubing (0.125 mm i.d.), which connects the injection valve to the electrospray tubing (75 µm i.d.). Nitrogen, the nebulization gas and drying gas, generates a stable spray at a flow rate of about 20 l•min^{-1}, and the flow rate is 300 l•min^{-1} for drying gas.

The electrospray ionization source was operated in positive ionization mode and was calibrated with myoglobin (MW=16,951.6, 10 pmol•µl^{-1}). Accurate mass measurement mode was used with a sampling frequency of 16 point-per-dalton.

The capillary voltage was set at 3.5 kV. The voltages of the HV lens and cone were 0.5 kV and 25 V, respectively. The values of resolution at high and low molecular weight were all adjusted to 15 allowing one dalton mass resolution. The electrospray source temperature was maintained at 80°C.

The mass spectra were recorded under multi-channel A (MCA) mode, in which the scans were added together and the total signal intensity was displayed. Ten to

twenty scans were accumulated for one spectrum acquisition. A Digital Celebris TM computer was used for system control, parameter adjustment, data acquisition and spectrum display with Masslynx TM version 2.0 (Micromass, UK). The raw data were deconvoluted using the Transform or Maximum Entropy programs provided by Micromass as part of the system operation and data acquisition software.

4.11 Protein Sequencing and Amino Acid Analysis

Selected proteins of LDPs were isolated for amino-terminal sequencing and amino acid analysis. For this purpose, LDPs were isolated as described in section 3.2 except that 2 mM β-mercaptoethanol was added to the isolation buffers. The purified LDPs were delipidated as described in section 3.6 and resuspended in 2 x SDS-sample buffer, diluted with an equal volume of ddH_2O and incubated at 90°C for 5 minutes. The solubilized proteins were fractionated by SDS-PAGE on 10% acrylamide gels as described in section 4.5.1 except that the gels were allowed to polymerize at 4°C for at least 48 hours. After SDS-PAGE, the gels were rinsed in 400 ml of polyvinyldifluoride (PVDF) membrane transfer buffer (100 mM sodium borate with 20% methanol, v/v, pH 9.2) three times for 5 minutes to remove any residual glycine remaining from the SDS-PAGE running buffer.

The fractionated proteins were electrophoretically transferred to PVDF membranes (BioRad, Mississauga, Ontario) at 50 V. To keep the gel cool, the apparatus was kept at 4°C and surrounded by ice. The membranes were immersed in methanol and incubated in PVDF transfer buffer for at least 15 minutes prior to the transfer. The transfer was stopped after 2 hours, and the membrane was washed 3

times in ddH_2O for 5 minutes and stained for 30 minutes in 0.1% Coomassie brilliant blue R250 (w/v) dissolved in 50% methanol (v/v). The membrane was destained in several changes of 50% methanol and dried. The bands of interest were cut out and stored in nitrogen at 20°C until they were sent to Commonwealth Biotech Inc., Richmond, Virginia, for N-terminal sequencing or to The Biotechnology Service Centre, Toronto, Ontario for N-terminal sequencing and amino acid analysis.

5 Nucleic Acid Isolation and Manipulation

All solutions and labware used for isolation or manipulation of RNA or DNA were purchased sterile or treated according to Sambrook *et al.* (1989) to avoid contamination by foreign nucleic acids or degrading enzymes.

5.1 DNA and RNA quantification

DNA and RNA were quantified and assessed for purity by measuring their optical densities at 260 nm and 280 nm according to Sambrook *et al.* (1989). The ratios of OD_{260}/OD_{280} nm were routinely ~1.8 and 2.0 for DNA and RNA, respectively, indicating little contamination from protein or phenol (Sambrook *et al.* 1989).

DNA was also quantified by using the ethidium bromide plate assay. Agarose (0.8% w/v) was prepared in TAE buffer (40 mM Tris-acetate, pH 8.0 and 1 mM EDTA), cooled, and 10 µl ethidium bromide stock (10 $mg \cdot ml^{-1}$) was added. This was poured into a petri plate and allowed to harden. DNA was spotted onto the plate, and the fluorescence of the samples was viewed under UV light and compared to known concentrations of DNA in the range of 10-200 $\mu g \cdot ml^{-1}$ (Sambrook *et al.* 1989).

5.2 Agarose Gel Electrophoresis

Agarose gels for separating RNA were prepared according to instructions given in the ZAP Express cDNA Gigapack Gold Cloning Kit (Stratagene). Agarose (1% w/v) was dissolved in 1 x MOPS buffer (20 mM 3-[*N*-morpholino] propanesulfonic acid, 5 mM sodium acetate and 1 mM EDTA, pH 7.0), cooled and brought to 2% formaldehyde (v/v) before being poured into an acrylic tray, sealed on each end with masking tape, and allowed to solidify. MOPS buffer was added as the running buffer. The RNA to be fractionated was dried in a vacuum, resuspended in 10 µl freshly prepared formaldehyde gel loading buffer (720 µl formamide, 160 µl 10 X MOPS, 260 µl 37% formaldehyde (v/v), 100 µl ddH_2O, 100 µl ethidium bromide (10 $mg \cdot ml^{-1}$), 80 µl sterile glycerol and 80 µl saturated bromophenol blue in ddH2O) and boiled for 2 minutes before being loaded into the gel. The gel was run at 150 V in a horizontal system for submerged gel electrophoresis, (Model H5-Life technologies Ltd., Gaithersburg, MD) and photographed under a UV transilluminator.

Agarose gels for separating DNA were prepared according to Sambrook *et al.* (1989). Agarose (2% w/v) was dissolved in TAE buffer and allowed to cool to 70°C, and 5 µl ethidium bromide (10 $mg \cdot ml^{-1}$) was added. This was poured into an acrylic tray, sealed on each end with masking tape, allowed to solidify and covered in TAE buffer. DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in sterile ddH_2O) was added to each sample (1 volume loading buffer to 5 volumes sample), and the gel was run at 5 V/cm in a horizontal system for submerged gel electrophoresis (Model H5-Life technologies Ltd., Gaithersburg, MD)

until the bromophenol blue had migrated about two thirds of the way down the gel. The gels were photographed under a UV transilluminator.

5.3 Genomic DNA Isolation

Genomic DNA was extracted from 2.5 *g* fresh leaves of wax beans or soybeans essentially as described by Ausubel *et al.* (1987). The leaf tissue was ground to a powder in liquid nitrogen, and then 1.25 ml of 0.1 M Tris-HCl (pH 8.0), 0.5 ml of 0.25 M EDTA and 2.5 ml of 0.1 M SDS (w/v) were added and mixed with the powder. This was followed by the addition of 10 ml phenol: chloroform: isoamyl alcohol (25: 24: 1), further mixing and centrifugation at 4340 *g* for 5 minutes. The upper aqueous layer was transferred to a clean tube, and the remaining lower layer was extracted once more with 2.5 ml of 0.1 M Tris-HCl (pH 8.0) and 0.5 ml of 10% SDS (w/v), and centrifuged at 4340 *g* for 5 minutes. The upper layer was pooled with the previously collected aqueous layer. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added to the pooled aqueous layers, and the suspension was mixed, centrifuged at 4340 *g* for 5 minutes and the aqueous layer collected. This was repeated, and the aqueous layer was then ethanol-precipitated by adding 0.1 volume of 3.3 M sodium acetate (pH 5.5) and 2.2 volumes of 95% ice cold ethanol (v/v) and precipitation was allowed to occur overnight at -20°C. The mixture was then centrifuged at 4340 *g* for 15 minutes, and the pellet, containing the DNA, was gently resuspended in 9.2 ml buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 5 mM EDTA). For CsCl purification of the DNA, 9.2 *g* CsCl was dissolved in the isolated DNA solution. Ethidium bromide (500 µl of 10mg•ml^{-1} stock solution) was added to a

heat-sealable ultracentrifuge tube (Beckmann, Mississauga, Ontario), followed by the CsCl-containing DNA sample. The tubes were topped with paraffin oil, sealed and centrifuged at 250,000 *g* for 20 hours at 15°C. The DNA band was visualized under UV light, and the tube was punctured at the top and about 1 mm below the band with syringes. Drops were collected until fluorescent material was no longer visible.

Ethidium bromide was removed from the purified DNA by adding an equal volume of isopropanol saturated with a solution of CsCl-saturated ddH_2O to the tube, allowing the phases to separate and removing the upper layer. This was repeated until the upper layer was no longer pink. One volume of ddH_2O was added, and the sample was then ethanol-precipitated as described above. The resulting pellet was dried under vacuum and gently resuspended in 400 µl TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and ethanol-precipitated once more. Without disturbing the resulting pellet, 500 µl of 70% ice-cold ethanol was added. This was centrifuged at 15,800 *g* for 5 minutes, the ethanol removed and the pellet dried under vacuum. The DNA was dissolved by shaking overnight at 4°C in 100 µl TE buffer and stored at -20°C.

5.4 Primer and Probe Design

Primers and probes for a putative oleosin in *Phaseolus vulgaris* were designed based on the soybean oleosin sequence (Fig. 4). Regions of homology were determined by comparing the soybean oleosin gene with oleosin genes from other plants.

Figure 4. DNA sequence from 24 kDa oleosin isoform B from soybean (Rowly, D.L. and Herman, E.M. 1994, unpublished, submitted to Entrez). The start codon is underlined and a box surrounds the EM1-type ABA box found in the promoter region. Each primer or probe is shown in a different colour and underlined. The direction of each of the primers is illustrated by the arrow heads shown at the ends of the lines. The location of primer 1 is shown in blue; the location of primer 2 is shown in pink, the location of primer 3 is shown in green and the location of probe a is shown in brown.

GACTATACATAGAGAATTAAACTTAATGTTATATTTAAGGAAAAGGACGAA
ACTTAAAACTTAAATACAATTGTATGATTAATTTTAGTATTGTCTTTAATG
AGAATTAAAGTTTTATTCACTAATTTATGATTATTTCATTTACTAATTTAT
GTAATGTGATTTCAATAAGTGAGGTAAACTCCGATTGATTGAAGATACCAC
CAACACCAACACCACCACCACCTGCGAAACTGTACGTATCTCAATTGTCCT
TAATAAAAATGTAAATAGTACATTATTCTCCTTGCCTGTCATTATTTATGT
GCCCCCAGCTTTAATTTTTCTGATGTACTTAACCCAGGGCAAACTGAAACA
TGTTCCTCATGCAAAGCCCCAACTCATCATGCATCATGTACGTGTCATCAT
CCAGCAACTCCACTTTTGCTATATAACTCCTCCCCCATCACACTCCCCATC
TCTCTAACACACACATACCCCCAACTAACAATAATTCCTTCACTTGCAGAA
CTTAGTTCTCTGTTGCATCATCATCATCTTCATTAGTGTTAGCCCTAACTT
CACCTTAACCATGACCACAGTGCCACCACACAGTGTCCAAGTGCACACAAC
AACACACCGCTACGAAGCCGGCGTCGTCCCGCCGGCTCGTTTTGAGGCGCC
GCGTTACGAAGCCGGCATCAAGGCGCCCTCTTCCATTTACCATTCCGAGAG
AGGTCCGACGACCTCTCAGGTTCTCGCAGTTGTCGCCGGCCTTCCGGTCGG
CGGCATCCTCCTGCTCCTGGCAGGACTGACCCTGGCTGGAACTCTCACCGG
GCTGGTGGTGGCAACACCGCTCTTCATCATCTTCAGCCCGGTGCTGATTCC
GGCCACGGTCGCAATTGGCCTGGCCGTGGCCGGGTTCCTGACGTCGGGAGT
CTTTGGGCTCACGGCGCTTTCGTCCTTCTCCTGGATCCTGAACTACATCCG
GGAGACCCAGCCGGCGTCGGAGAATCTGGCCGCTGCGGCGAAGCACCATCT
GGCGGAGGCGGCGGAGTACGTGGGGCAGAAGACCAAAGAAGTAGGGCAGAA
AACCAAGGAAGTTGGGCAGGATATTCAGAGCAAGGCACAAGATACAAGGGA
AGCAGCTGCAAGAGATGCAAGAGATGCAAGGGAAGCAGCTGCAAGAGATGC
AAGGGATGCAAAGGTGGAGGCGAGAGATGTAAAGAGAACAACAGTGACAGC
AACAACCGCAACCGCATGAACGTGATGAGTATTAATGTGTTGTTATGAACT
TATGATGTTGGTTTATGTGGGGAAATAAATGATGTATGTACCTCTTCTTGC
CTATGTAGTAGGTTTGGGTGTTTTGTTGTCTAGCTTTGCTTATTTAGTAAT
TAGTAGAAGGGATGTTCGTTCGTGTCTCATAAAAAGGGGTACTACCACTCT
GGCAATGTGATTTGTATTTGATGAATTCAATGTGAAATGTGTTTTGCGTTG
GTGTATGTAATTTCTCGTCCAATGTGCTTTTAGAACAACGTACGGTGAAAT
TTAAGCTGTGCATTGTTGATAATTCTTCGTTCAAAATCAAATTGCTAGCAA
ATTTTAACTAACATGAAATTTTGTCAACAGTCAGCACTCATTAACCAAGTT
CCAAACTAAGATCGCCATAACATCAACCAATAGCATTTTAGCATAAGGAAG
GCTTGACATGCAAAATATCTTAATATATAGATAAGCATGTGAAGTAGGAAT
TAACTTGTTAAGATTTGTTTCGCTTTTTCTTCAATGAAACAGTTCTTATAT
GTATAAGAAAAAAAATAAATGAGTTTTCCTATAATTTTAAATTAACTTATA
CACAGGCTAGCCTTTAAAAAAAAAACCTATACACAGGCTAAGTTTTAGAAG
TTTTCTAGAAATTATGACAATAAGCTT

5.5 Polymerase Chain Reaction Conditions

Polymerase chain reaction (PCR) was performed in a final volume of 50 µl. An amplification reaction mixture was freshly prepared in sterile ddH_2O with the following components: PCR buffer (20 mM Tris-HCl, pH 8.3, 3.0 mM $MgCl_2$ and 100 mM KCl), 0.4 mM each of dNTP and 80 units•ml^{-1} Taq DNA polymerase. One hundred pmol of each specified primer and 100 ng of soybean or wax bean $CsCl_2$-purified DNA were added to a sterile, thin walled PCR tube, and this was brought to 25 µl with ddH_2O. The primer and DNA mixture was brought to 94°C and held for 5 minutes before adding 25 µl of the amplification mix and proceeding with the cycle.

The reaction proceeded through 40 cycles consisting of 94°C for 45 seconds, 45°C for 30 seconds and 72°C for 90 seconds. After cycling, the temperature was held at 72°C for 10 minutes and then left at 4°C until the PCR-generated fragments were examined by agarose gel electrophoresis as described in section 3.13.

6 cDNA Library Construction

6.1 mRNA Isolation

Total RNA was isolated by Y. Hong (Skye Pharmatech Inc., Mississauga, Ontario) from cotyledons of developing seeds of *Phaseolus vulgaris* by the method of Chomezynski and Sacchi (1987) as modified by Puissant and Houdebine (1990). For isolation of RNA, 15 *g* of seed tissue was frozen in liquid nitrogen and homogenized on ice in 150 ml of RNA isolation buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5 % sarkosyl (w/v) and 0.1 M β-mercaptoethanol in DEPC water) with a Polytron homogenizer. This homogenate was then mixed with 10 ml of

2 M sodium acetate (pH 4.0) and 100 ml phenol (saturated with DEPC water), after which 20 ml of chloroform were added. The resulting suspension was centrifuged at 10,000 *g* for 10 minutes, and the upper phase was collected, mixed with 100 ml of isopropanol and incubated at -20°C for 3 hours. Following this, the mixture was centrifuged at 3,000 *g* for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 20 ml of 4 M LiCl and centrifuged at 3,000 *g* for 10 minutes. The resulting pellet was resuspended in 2 ml TE buffer containing 0.5% SDS and 2 ml chloroform. This suspension was centrifuged at 3,000 *g* for 10 minutes, and the upper phase was collected, brought to 0.2 M with sodium acetate (pH 5.0), mixed with 2 ml isopropanol and incubated at -20°C overnight. The mixture was then centrifuged at 3,000 *g* for 10 minutes at 4°C, and the resultant pellet of total RNA was collected and resuspended in DEPC water. A small portion of total RNA was fractionated on an agarose gel to confirm that the RNA was not degraded during the isolation procedure.

The PolyATtract mRNA Isolation Systems Kit (Promega, Madison, Wisconsin) was used to isolate mRNA from the total RNA according to the supplier's instructions. Briefly, 5 mg RNA was diluted to a final volume of 2.43 ml in DEPC water and incubated at 65°C for 10 minutes. After incubation, 10 µl of the biotinylated oligo(dT) probe (50pmol•µl^{-1}) and 60 µl of 20 x SSC (175.3 g•l^{-1} NaCl and 88.2 g•l^{-1} sodium citrate) were added, and the tube was allowed to cool to room temperature. The RNA-oligo(dT) mixture was then added to streptavidin-paramagnetic particles (SA-PMP), which had previously been washed and

resuspended in 0.5 ml of 0.5 x SSC. The SA-PMP and RNA-oligo(dT) mixture was incubated at room temperature for 10 minutes before being placed on the magnetic stand, and the supernatant was removed. The particles were washed four times with 0.5 x SSC by gently agitating the tube and placing it back on the magnetic stand to remove the supernatant. The first supernatant was retained and the integrity of the RNA confirmed by agarose gel electrophoresis.

The mRNA was eluted by gently resuspending the SA-PMP particles containing the mRNA in 1.0 ml of DEPC water. The mRNA was separated from the particles by placing the tube on the magnetic stand and removing the mRNA-containing supernatant to a clean tube.

6.2 cDNA Synthesis and Packaging

A cDNA library was prepared with 5.2 µg mRNA using the ZAP Express cDNA Synthesis Kit (Stratagene, La Jolla, California) according to the supplier's instructions. Briefly, the first strand synthesis was primed with an oligo(dT)-linker primer which contained a *Xho* I site. This strand was synthesized using Moloney murine leukemia virus reverse transcriptase and 5-methyl dCTP to prevent digestion of the cDNA strand when later treated with *Xho* I. The second strand was synthesized using nick translation by treating the cDNA-RNA hybrid with *Eschericia coli* RnaseH to create RNA fragments used as primers for *E. coli* DNA polymerase I . This reaction is supplied with excess dCTP to prevent incorporation of 5-methyl dCTP into the restriction site in the primer used for first strand synthesis. Uneven termini of the

cDNA were blunted with *Pyrococcus furiosus* DNA polymerase and ligated to an *Eco*R I adapter with a blunt end.

A *Xho* I digest followed by size fractionation on a Sephadex S-500 spin column resulted in cDNA with a *Xho* I sticky end on one side and an *Eco*R I sticky end on the other allowing for directional insertion into the predigested ZAP Express vector arms. The lambda insertion vector (Uni-ZAP XR vector) library was packaged in a packaging extract provided in the kit and plated on the host cell line *E. coli* XL1-Blue MRF'. This cell line is McrA$^-$ and McrB$^-$, and the lack of these restriction systems prevents digestion of hemimethylated DNA. After the library has passed through this *E. coli* strain once, it is no longer hemimethylated and can be grown on XL1-Blue strain.

The XL1-Blue strains of bacteria contain a mutation in the lacZ gene which is complemented by ZAP Express vector without an insert. Therefore, successful recombination was determined by the colour of the plaque when bacteria were plated on media containing isopropyl-1-thio-∃-S-galactopyranolside and 5-bromo-4-chloro-3 indoyl-∃-D-galactopyranoside (IPTG-X-gal). Clear plaques contained the inserted DNA. The titre of the constructed library was 2.5 x 10^5 pfu/ml above background. The library was amplified immediately, and the titre of the amplified library was 1.55 x 10^9 pfu/ml.

6.3 End-Labeling Probes for cDNA Library Screening

The probes used to screen the library included primer 1, primer 2 and probe a (Fig 4). Each of these was end-labeled with [(^{32}P] ATP using DNA 5'-End Labeling

Kit (Boehringer Mannheim, Laval, Quebec) according to supplier's instructions. The probes to be end-labeled were purified by ethanol precipitation by adding 0.1 volumes of 8 M lithium chloride and 2.2 volumes of ice-cold ethanol to 30 pmol DNA in 100 µl sterile ddH2O. This was incubated at -70°C for 30 minutes and centrifuged at 15,850 *g* for 15 minutes at 4°C. The pellet was marked and the supernatant removed. The pellet was then washed with 70% ice-cold ethanol (v/v), centrifuged at 15,850 *g* for 15 minutes, the ethanol removed and the pellet suspended in 8 µl dephosphorylation buffer (50 mM Tris-HCl, pH 8.2) and 0.5 units alkaline phosphatase. This was allowed to incubate for 30 minutes at 37°C at which point the reaction was stopped by adding 20 µl of 50 mM EGTA and incubating for 30 minutes at 65°C. The mixture was allowed to cool to room temperature, and 100 µl TE-saturated phenol was added, thoroughly mixed and the suspension centrifuged for 1 minute. The aqueous layer was retained, and 41 µl of 12.5 mM Tris-HCl (pH 8.2) with 0.125 mM EDTA was added to the phenol to dissolve any remaining polynucleotides. This was mixed, centrifuged for 1 minute and the aqueous layer pooled with the previously retained aqueous layer. The phenolization step was repeated on the pooled aqueous layers with 150 µl TE-saturated phenol after which 500 µl chloroform was added and the mixture was centrifuged for 1 minute. The chloroform layer was discarded, and this step was repeated on the aqueous layer. After the final chloroform extraction, the DNA was purified by ethanol precipitation as described above. The pellet was resuspended in 10 µl of 50 mM Tris-HCl, pH 8.2, containing 0.5 mM EDTA and 2 µl of phosphorylation buffer (500 mM Tris-HCl, pH

8.2, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM dithiothreitol and 1 mM spermidine), and 20 pmol [(^{32}P]ATP was added. This was brought to 20 µl with sterile ddH_2O and incubated for 30 minutes at 37°C at which point the reaction was stopped by cooling on ice. The DNA was purified by ethanol precipitation, resuspended in 100 µl TE buffer and the level of radioactivity determined by adding a 5 µl aliquot to 3 ml of scintillation fluid (EcoLite, ICN, Mississauga, Ontario) and counting in a Beckman LS 1701 liquid scintilation counter.

6.4 Screening

Screening of the cDNA library was performed according to the supplier's instructions (Stratagene, La Jolla, California). Briefly, fresh host cells were prepared by streaking on plates containing liquid broth (10 $g \cdot l^{-1}$ NaCl, 10 $g \cdot l^{-1}$ tryptone and 5 $g \cdot l^{-1}$ yeast extract) with 0.2% agar and 12 $\mu g \cdot ml^{-1}$ tetracycline and incubated overnight at 37°C. These plates were stored at 4°C and used within a week. A single colony was isolated from the plate and used to inoculate NZY broth (5 $g \cdot l^{-1}$ NaCl, 2 $g \cdot l^{-1}$ $MgSO_4 \cdot H_2O$, 6 $g \cdot l^{-1}$ yeast extract, 10 $g \cdot l^{-1}$ NZ amine) containing 10 mM $MgSO_4$ and 0.2% maltose (w/v). The bacteria were incubated with shaking at 30°C overnight, pelleted by centrifuging at 500 *g* for 10 minutes and resuspended in sufficient 10 mM $MgSO_4$ such that the OD_{600} was 0.5. The library was diluted to 1000 $pfu \cdot ml^{-1}$ and, for each plate poured, 50 µl of the library was added to 600 µl of the host cells. These were incubated together for 15 minutes at 37°C and added to 6.5 ml of NZY top agar (NZY broth with 0.7% agar, w/v) which had been prepared and allowed to cool to ~48°C. The infected host cells in NZY top agar were spread on 150 mm plates of

NZY agar (NZY broth, pH 7.5 with 1.5% agar, w/v) which had been prepared at least five days in advance. The top agar was allowed to solidify and then incubated overnight at 37°C. These plates were cooled at 4°C for at least 2 hours and then transferred onto Biotrans nylon membranes (ICN, Mississauga, Ontario) for 2 minutes using pins to mark the orientation of the membrane on the plate. The DNA was denatured by submerging the nitrocellulose in 1.5 M NaCl and 0.5 M NaOH for 2 minutes followed by neutralization in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) for 5 minutes. The membranes were then rinsed for 0.5 minutes in 2x SSC buffer with 0.2 M Tris-HCl (pH 7.5), allowed to dry and the DNA crosslinked to the membrane using the GS Gene Linker UV chamber (BioRad, Laval, Quebec). The plates were stored at 4°C.

Membranes were wetted in prehybridization solution, containing hybridization solution (6 x SSC, pH 7.0, 20 mM NaH_2PO_4, 0.4 % SDS w/v and 500 $\mu g \cdot ml^{-1}$ denatured and sonicated salmon sperm DNA) containing 5 x Denhardt's reagent (1 $g \cdot l^{-1}$ Ficoll, 1 $g \cdot l^{-1}$ polyvinylpyrrolidone and 1 $g \cdot l^{-1}$ BSA), rolled in mesh and placed, two to a bottle, into a hybridization incubator (Model 2720, VWR, Mississauga, Ontario). These were incubated with prehybridization solution at 42°C for at least 2 hours before the prehybridization solution was removed and replaced with hybridization solution and the radiolabelled probe. The reaction mixture was incubated overnight at 5°C-10°C below the calculated melting temperature of the probe as described by Sambrook *et al.* (1989). The hybridization solution and the probe were removed, and the membrane was washed at room temperature with 6 x SSC buffer and 0.1% SDS

(w/v) until background counts diminished. The membranes were blotted briefly on Whatmann paper and wrapped between layers of plastic wrap and exposed to photographic film (Kodak X-OMAT) in a cassette with intensifying screens overnight at -70°C. The film was developed and lined up with the original plate to identify labeled plaques. These plaques were removed with an inverted 200 µl pipet tip and resuspended in 1 ml SM buffer (200 mM Tris-HCl, pH 7.5, 5.8 $g \cdot l^{-1}$ NaCl, 2.0 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, and 5.0 ml 2% gelatin, w/v) and 20 µl chloroform in preparation for secondary screening.

Secondary and tertiary screenings were performed as described above. The positive plaques isolated from the tertiary screening were stored in 500 µl SM buffer with 20 µl chloroform at 4°C.

7 Data Bank Searches

Data bank comparisons and sequence analyses were performed by using various programs found on the internet. The most commonly used programs are listed below.

Isoelectric points and molecular weights were determined from amino acid sequences by pI/Mw tool (Bjellqvist *et al.* 1993, Bjellqvist *et al.* 1994) from ExPASy Molecular Biology Server, Swiss Institute of Bioinformatics, Geneva Switzerland. Previously identified amino acid and nucleic acid sequences were retrieved using Entrez search system from the National Center for Biotechnology Information, Bethesda, Maryland, USA and ENZYME Enzyme nomenclature data base (Bairoch 1996) from ExPASY Molecular Biology Server, Swiss Institute of Bioinformatics, Geneva Switzerland.

Both nucleic acid and protein sequences were compared to previously identified sequences using BLAST (Altschul *et al.* 1990, Gish and States 1993) from the National Center for Biotechnology Information and BEAUTY (Worley *et al.* 1995) from the Human Genome Center, Baylor College of Medicine, Houston Texas. To compare one sequence to another, LALIGN (Pearson and Lipman 1988, Huang and Miller 1991) from the Genestream network server, IGH, Montpellier France was used. To examine protein sequences for possible post-translational modification sites, PPSearch was used with the Prosite database (Fuchs 1991, Bairoch 1993, Fuchs 1994) from the Human Genome Center, Baylor College of Medicine, Houston, Texas. To compare amino acid compositions with those of other proteins, Propsearch (Hobahm *et al.* 1994) on the European Bioinformatics Institute, Cambridge, UK server was used. TMpred (Hofmann and Stoffel 1993) for predicting transmembrane domains in proteins and Hydrophilicity/hydrophobicity plot (provided by Bioinformatics Unit, Weizmann Institute of Science, Israel) for generating Kyte-Doolittle hydropathy plots were both found on the BCM Search Launderer server, Human Genome Center, Baylor College of Medicine, Houston, Texas.

8 Statistical Analysis

Two-tailed t-tests were used to determine statistically significant differences between means at $\alpha = 0.05$. Formulae for both paired and independent t-tests were described by Sokal and Rohlf (1981).

Results

1 Cotyledon Development

During seed development, lipid and protein reserves are stored in the cotyledons. After the seeds germinate and these reserves are metabolized, the cotyledons senesce. When seeds of *Phaseolus vulgaris* are germinated under conditions of etiolation, the cotyledons rapidly imbibe water for the first six hours, and then germinate and senesce over a period of eight to nine days. This is accompanied by a decrease in the size of the cotyledons reflecting a decline in fresh weight (Fig. 5). By the eighth and ninth day after germination, many of the cotyledons dehisce from the seedlings.

2 Membrane-Derived Cytochrome c Reductase Activity in the Cytosol of Senescing Cotyledons

Microsomal membranes from cotyledons of bean (*Phaseolus vulgaris*) and other higher plant tissues have been shown to reduce cytochrome c in an NADPH-dependent fashion (Hackett 1964, Thompson 1974, Pohl and Wiermann 1981, Lesot *et al.* 1990). This has been attributed to cytochrome P450 reductase activity (Sottocasa *et al.* 1967). In one report, this activity was also found in the cytosol of senescing bean cotyledons (Thompson 1974). The activities of other membrane-associated enzymes have also been observed in the cytosol (Lai *et al.* 1971, Lai and Thompson 1972), suggesting the possibility that active forms of some proteins, possibly their catabolites, may be released from the membranes into the cytosol. To examine this possibility further, microsomal and cytosolic levels of NADPH-dependent cytochrome c reductase were compared during cotyledon senescence. The data in Figure 6 illustrate the specific activity of NADPH-dependent cytochrome c reductase in smooth

Figure 5. Changes in fresh weight of bean cotyledons at specified stages of development and germination. Ages of cotyledons are expressed as days after planting except for developing seeds, which were harvested just as the seed pods begin to dry. Standard errors of the means are given for $n > 3$.

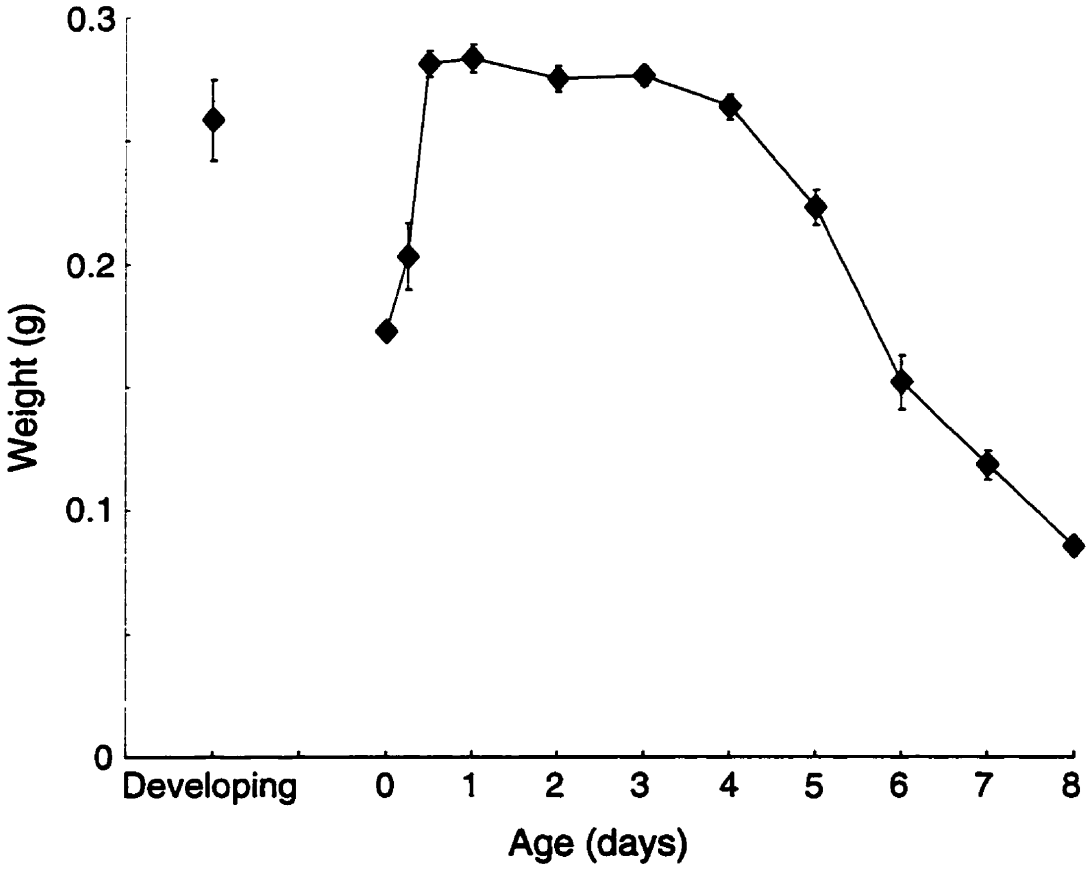
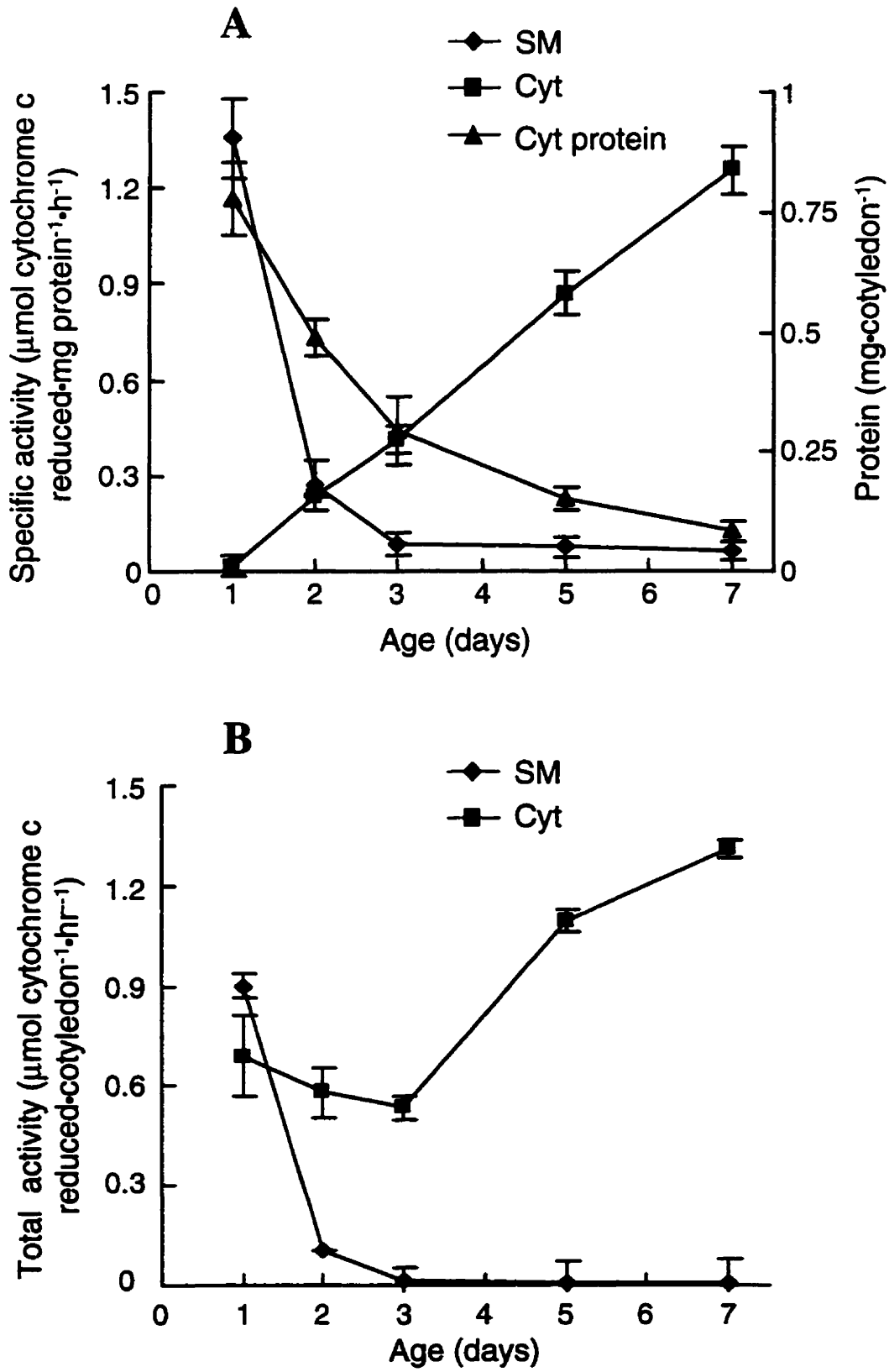

0.3
0.2
0.1
0
Weight (g)
Developing
0
1
2
3
4
5
6
7
8
Age (days)

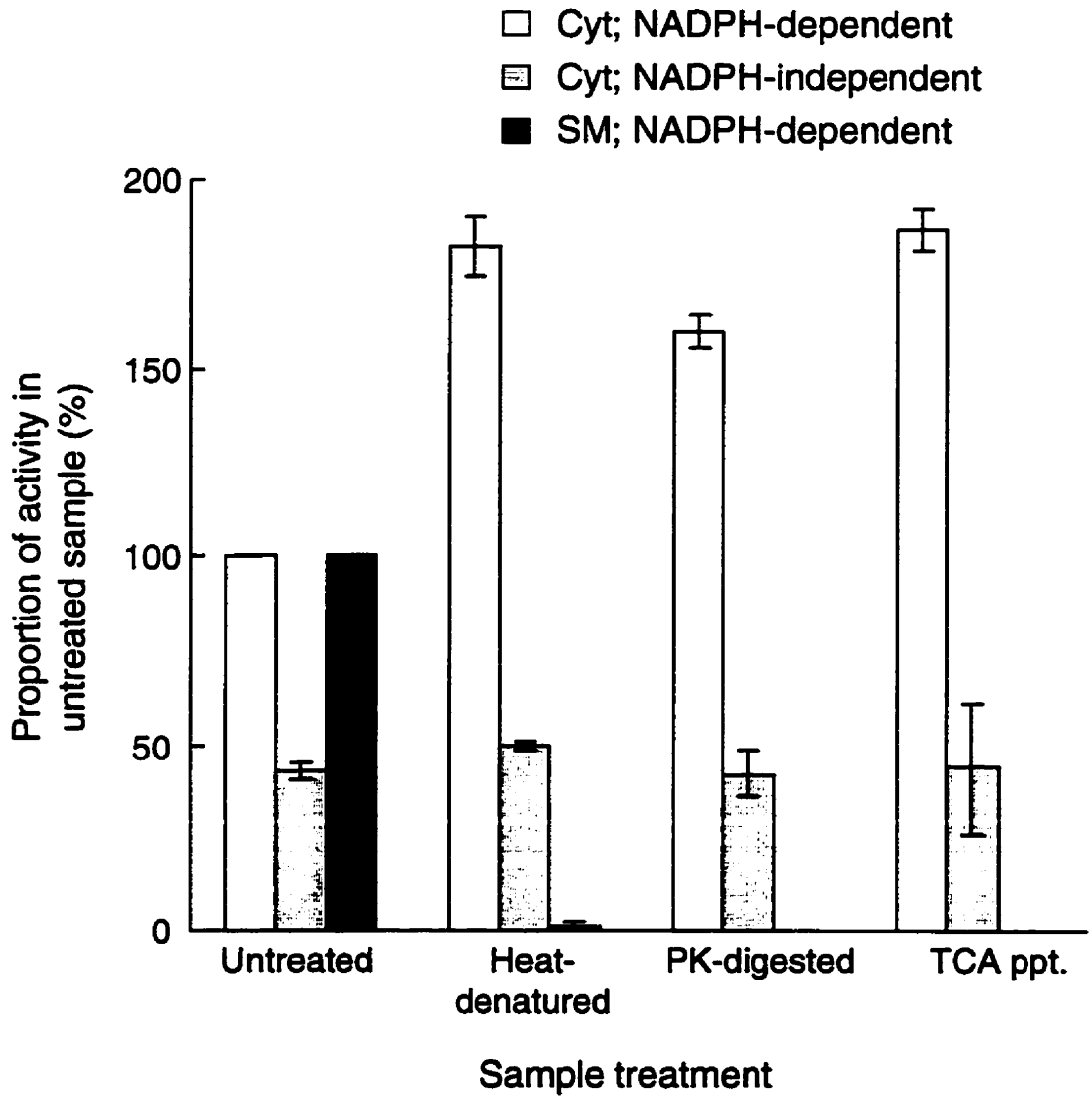
Figure 6. Changes in the NADPH-cytochrome c reductase specific and total activities and protein of subcellular fractions from bean cotyledons. **A**. Specific activities of smooth microsomal membranes (SM) and cytosol (Cyt), as well as cytosolic protein (Cyt protein). **B**. Total activities of smooth microsomal membranes (SM) and cytosol (Cyt). Ages of cotyledons are expressed as days after planting. Standard errors of the means are indicated for n = 3 - 5.


A
SM
Cyt
Cyt protein
Specific activity (µmol cytochrome c reduced•mg protein⁻¹•h⁻¹)
Protein (mg•cotyledon⁻¹)
Age (days)
B
SM
Cyt
Total activity (µmol cytochrome c reduced•cotyledon⁻¹•hr⁻¹)
Age (days)

microsomal membranes from bean cotyledon tissue undergoing a rapid decrease with increasing age of the tissue. Between days 1 and 3 after planting, this activity decreased by 15-fold, and then remained essentially constant through day 7 by which time the cotyledons were extensively senescent, containing only 42% of their fully hydrated fresh weight (Figs. 5 and 6 A). Bean cotyledon cytosol, on the other hand, exhibited a significant increase in specific cytochrome c-reducing activity as senescence proceeded (Fig. 6 A). However, the pattern of increase in specific activity seen in the cytosol did not mirror the decrease observed in microsomal membranes. The membrane-associated specific activity was close to background level by the third day of germination, whereas the cytosolic specific activity increased in a linear fashion throughout all ages of the tissue examined (Fig. 6 A). The increase in cytosolic specific activity can be largely accounted for by a decrease in cytosolic protein with advancing age (Figs. 6 A and B). Microsomal and cytosolic cytochrome c-reducing activities per cotyledon also show no correspondence during senescence. Microsomal activity per cotyledon declined with age, whereas cytosolic activity per cotyledon showed an approximately 2-fold increase 5 and 7 days after planting (Fig. 6 B).

In order to examine the nature of the cytochrome c-reducing activity, several methods were used to eliminate the native protein present in the sample. The effects of these treatments on cytochrome c-reducing activity are illustrated in Figure 7. The activity found in smooth microsomal membranes was reduced to background level by heat-denaturation. The activity of the cytosol, however, showed a statistically significant increase after heat denaturation ($\alpha = 0.05$, Appendix 1, Table A.1.4). Furthermore, neither high concentrations of Proteinase K nor TCA precipitation

Figure 7. Effects of heat denaturation on the cytochrome c-reducing activity of cytosol and smooth microsomal membranes and effects of protein precipitation and protease digestion on the cytochrome c-reducing activity of cytosol from bean cotyledons. Protein precipitation was achieved by treatment with TCA and digestion by treatment with Proteinase K. Smooth microsomal membranes (SM) were isolated from 1 day-old cotyledons and cytosol (Cyt) from 7 day-old cotyledons. Values for cytosolic heat-denatured, Proteinase K-treated, TCA precipitated and NADPH-independent cytochrome c-reductase are expressed as a percentage of the untreated NADPH-dependent cytosolic cytochrome c-reductase. Values for heat-denatured smooth microsomal membranes are expressed as a percentage of cytochrome c reduction in the untreated membranes. Standard errors of the means are indicated for $n = 3$.


Cyt; NADPH-dependent
Cyt; NADPH-independent
SM; NADPH-dependent
Proportion of activity in untreated sample (%)
200
150
100
50
0
Untreated
Heat-denatured
PK-digested
TCA ppt.
Sample treatment

followed by heat-treatment, significantly altered the level of cytochrome c-reducing activity in the cytosol when compared to the corresponding heat-treated sample (Fig. 7).

The association of cytochrome c-reducing activity with protein was examined further by Sephadex G25 chromatography. When smooth microsomal membranes from cotyledons harvested 2 days after germination were fractionated on a NAP column, designed for removing small molecular weight compounds from protein or nucleic acid samples, the protein and the cytochrome c-reducing activity coeluted in the void volume (Fig. 8). This reducing activity was absent when the membranes were heat-denatured before fractionation (Fig. 8).

When the cytosol of cotyledons harvested 7 days after germination was fractionated on the same NAP column, protein and cytochrome c-reducing activity did not coelute, suggesting that cytosolic cytochrome c-reducing activity is nonenzymatic. Protein eluted in the void volume whereas cytochrome c-reducing activity eluted in the delayed volume (Fig. 9 A). Moreover, the capability of fractions in the delayed volume to reduce cytochrome c appeared to increase after heat-denaturing the sample (Fig. 9 A). In the absence of NADPH, the fractions in the delayed volume continued to reduce cytochrome c, although with considerably lower efficiency (Fig. 9B).

The pH optimum of the nonenzymatic activity was determined in the presence and absence of NADPH (Fig. 10). The activity in the presence of NADPH was optimal between pH 8.5 and 9.5, whereas the NADPH-independent activity showed a pH optimum between pH 9.0 and 10.5.

Figure 8. Elution profile of NADPH-dependent reduction of cytochrome c and of protein during Sephadex G-25 chromatography of smooth microsomal membranes from 2 day-old bean cotyledons. NADPH-dependent cytochrome c-reductase activity of untreated membranes, NADPH-dependent cytochrome c-reductase activity of heat-denatured membranes and protein are measured. Data are from one of two separate experiments showing similar results.

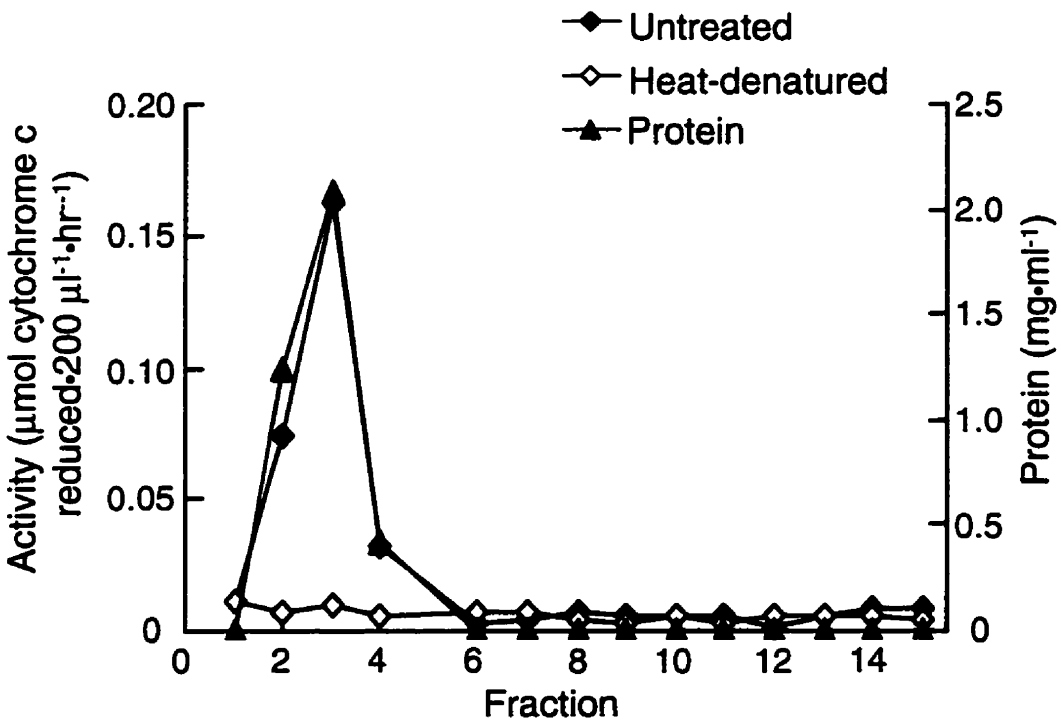
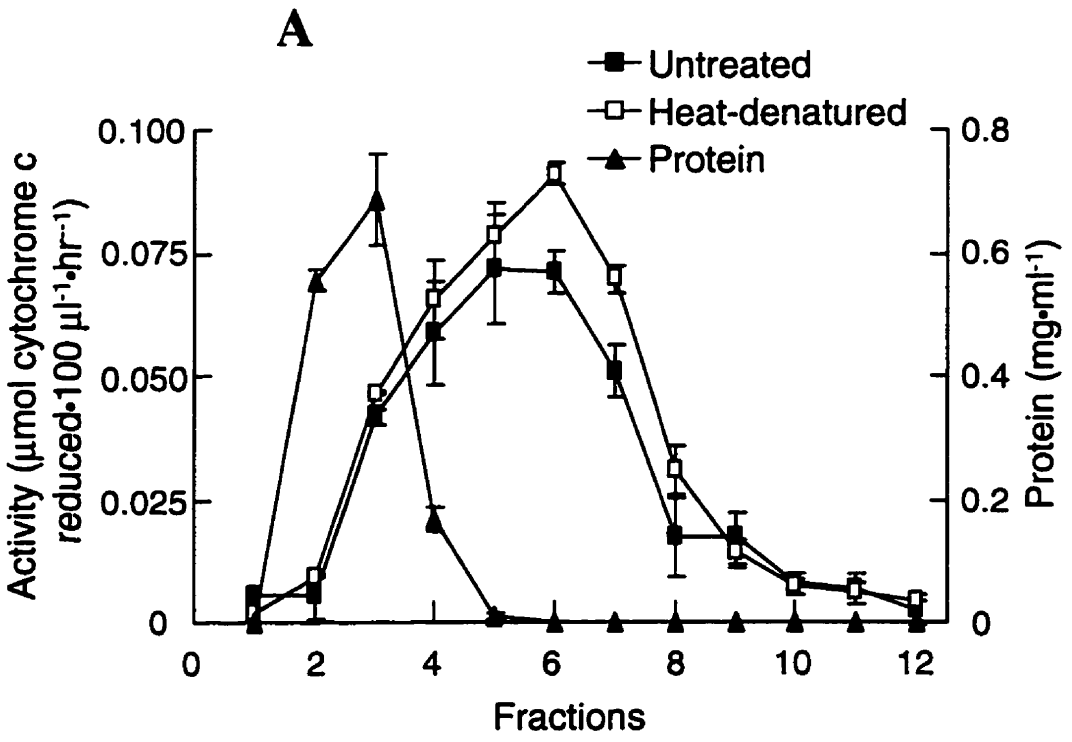

Untreated
Heat-denatured
Protein
Activity (µmol cytochrome c reduced·200 µl⁻¹·hr⁻¹)
Protein (mg·ml⁻¹)
0.20
0.15
0.10
0.05
0
2.5
2.0
1.5
1.0
0.5
0
0
2
4
6
8
10
12
14
Fraction

Figure 9. Elution profile of NADPH-dependent reduction of cytochrome c and of protein during Sephadex G-25 chromatography of cytosol from 7 day-old bean cotyledons. **A**. NADPH-dependent reduction of cytochrome c. **B**. NADPH-independent reduction of cytochrome c. Cytochrome c reducing activity by untreated cytosol, cytochrome c reducing activity by heat-denatured cytosol and protein are measured. Standard errors of the means are indicated for n = 3.


A
Untreated
Heat-denatured
Protein
Activity (µmol cytochrome c reduced•100 µl⁻¹•hr⁻¹)
Protein (mg•ml⁻¹)
0.100
0.075
0.050
0.025
0
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12
Fractions

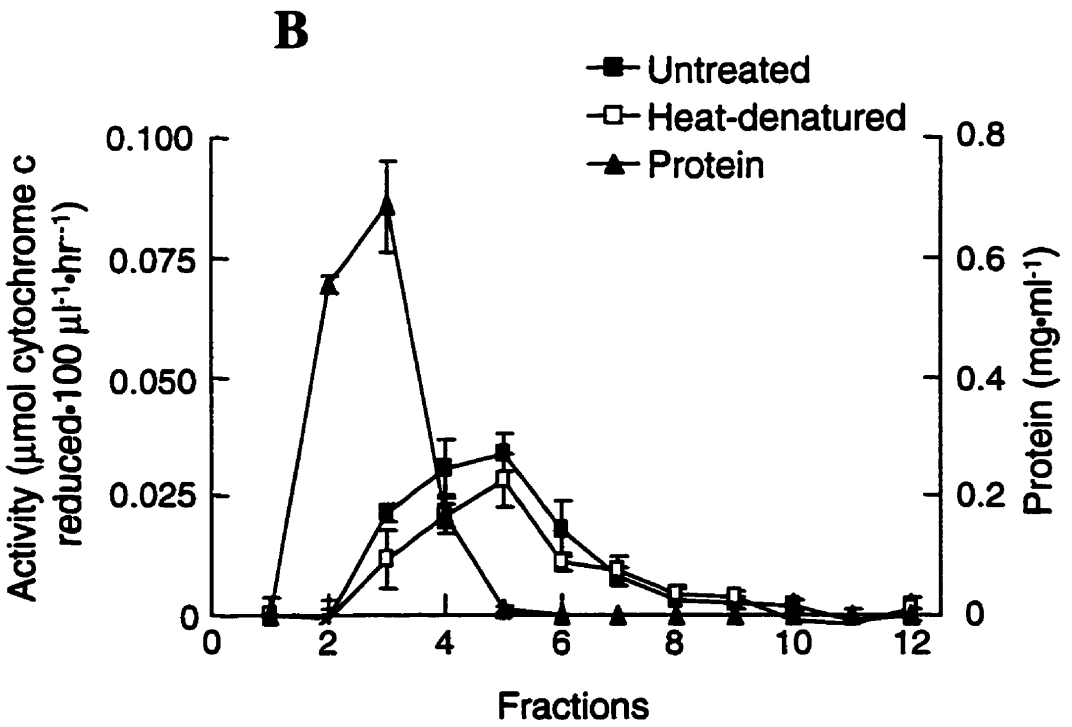
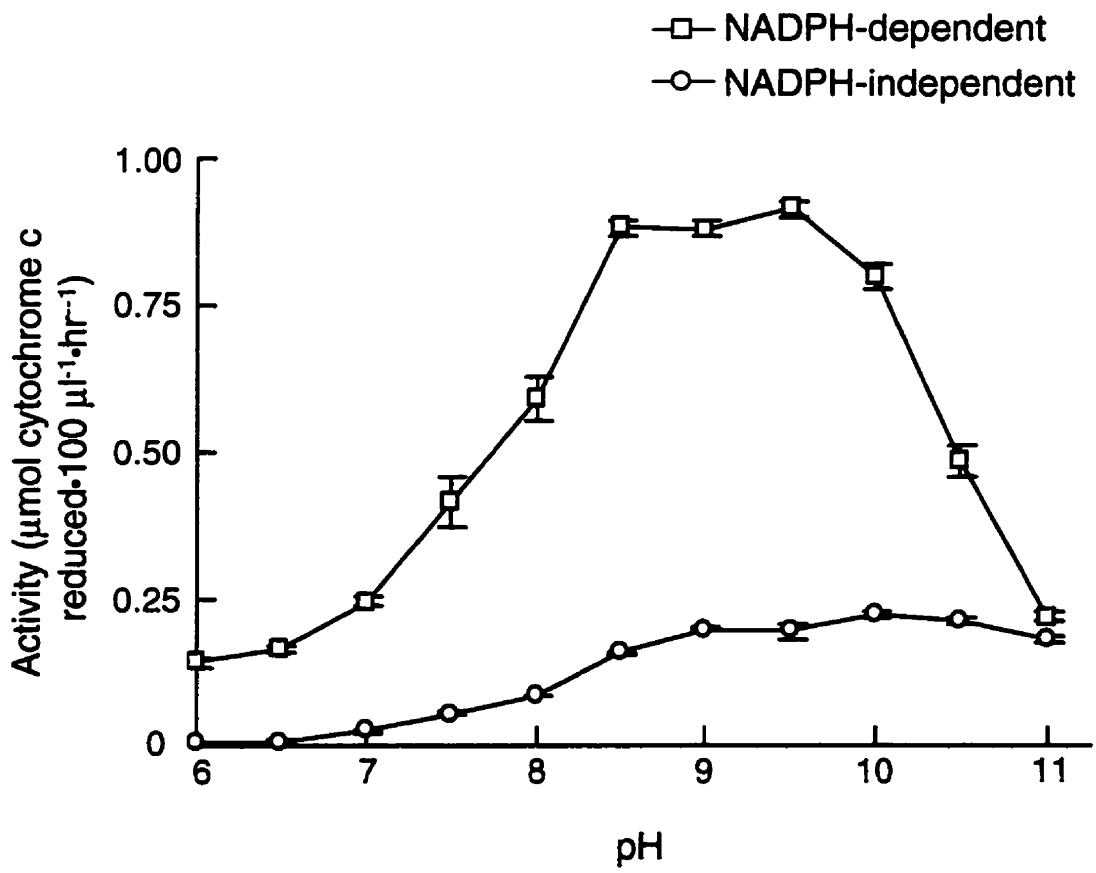

B
Untreated
Heat-denatured
Protein
Activity (µmol cytochrome c reduced•100 µl⁻¹•hr⁻¹)
Protein (mg•ml⁻¹)
0.100
0.075
0.050
0.025
0
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12
Fractions

Figure 10. pH profile for NADPH-dependent and NADPH-independent cytochrome c reducing activity by heat-denatured cytosol from 7 day-old bean cotyledons. Standard errors of means are indicated for n = 3.


NADPH-dependent
NADPH-independent
Activity (µmol cytochrome c reduced•100 µl⁻¹•hr⁻¹)
1.00
0.75
0.50
0.25
0
6
7
8
9
10
11
pH

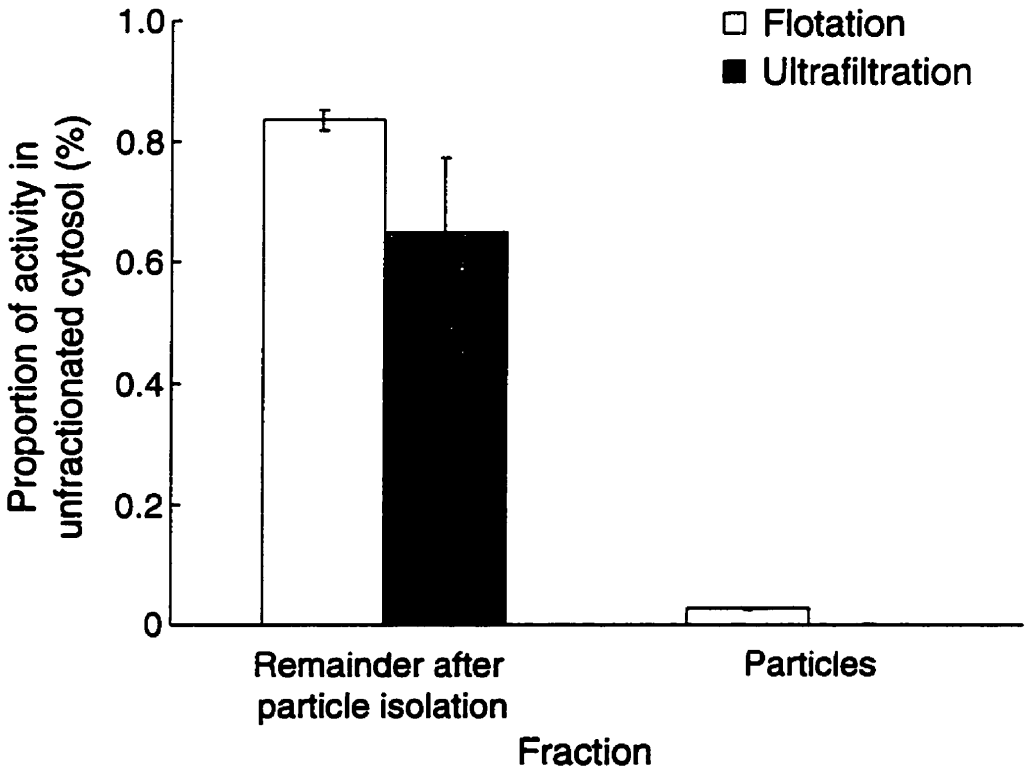
2.1 Distribution of Cytochrome c-Reducing Activity in Fractionated Cytosol

The hypothesis that soluble cytochrome c-reducing activity in the cytosol of germinating bean cotyledons is attributable to the release of the native protein or an active portion of the protein from the endoplasmic reticulum, appears to be unlikely in light of the fact that the soluble cytochrome c-reducing activity can not be attributed to a protein. Furthermore, as illustrated in figure 11, the soluble cytochrome c reductase is not associated with lipid particles that were previously identified as lipid-protein particles and believed to be formed from membranes (Thompson *et al.* 1998). The lipid particles examined were isolated by two different methods: ultracentrifugation and flotation. Trace amounts of cytochrome c-reducing activity were detected in floated particles; however, this activity was undetectable in lipid particles isolated by ultrafiltration (Fig. 11). These results indicate that there is no cytochrome c-reducing activity in these particles. Nonetheless, in an effort to confirm the contention that these particles are derived from membranes other properties were characterized. The method of isolation by flotation centrifugation was chosen, as the particles obtained in this fashion could be isolated more rapidly and consistently.

3 Characterization of Low-, Low'-, Intermediate- and High-Density Lipid Particles from Cytosol of Bean Cotyledons

Four distinguishable populations of lipid particles were isolated from cotyledon cytosol (Figs. 2 and 3, Table 1). These were present in both maturing and germinating bean seeds and were termed low-density lipid particles (LDPs), low-density lipid particle's (LDP's), intermediate-density lipid particles (IDPs) and high-density lipid

Figure 11. Comparison of the proportion of NADPH-dependent cytochrome c reducing activity from cytosol isolated from 7 day-old bean cotyledons fractionated into lipid particles and the remainder of the cytosol by two methods of fractionation: flotation and ultrafiltration. Both types of lipid particles and the remainder of the cytosol are expressed as a percentage of the activity of unfractionated cytosol. Standard errors of the means are shown for n = 3.

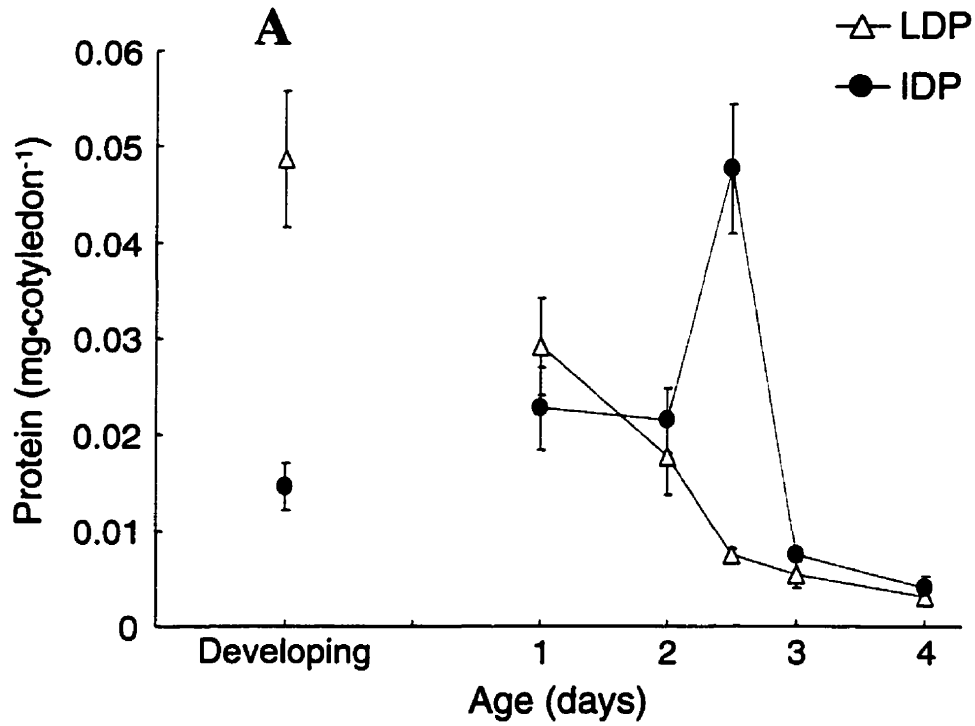

1.0
0.8
0.6
0.4
0.2
0
Proportion of activity in unfractionated cytosol (%)
Flotation
Ultrafiltration
Remainder after particle isolation
Particles
Fraction

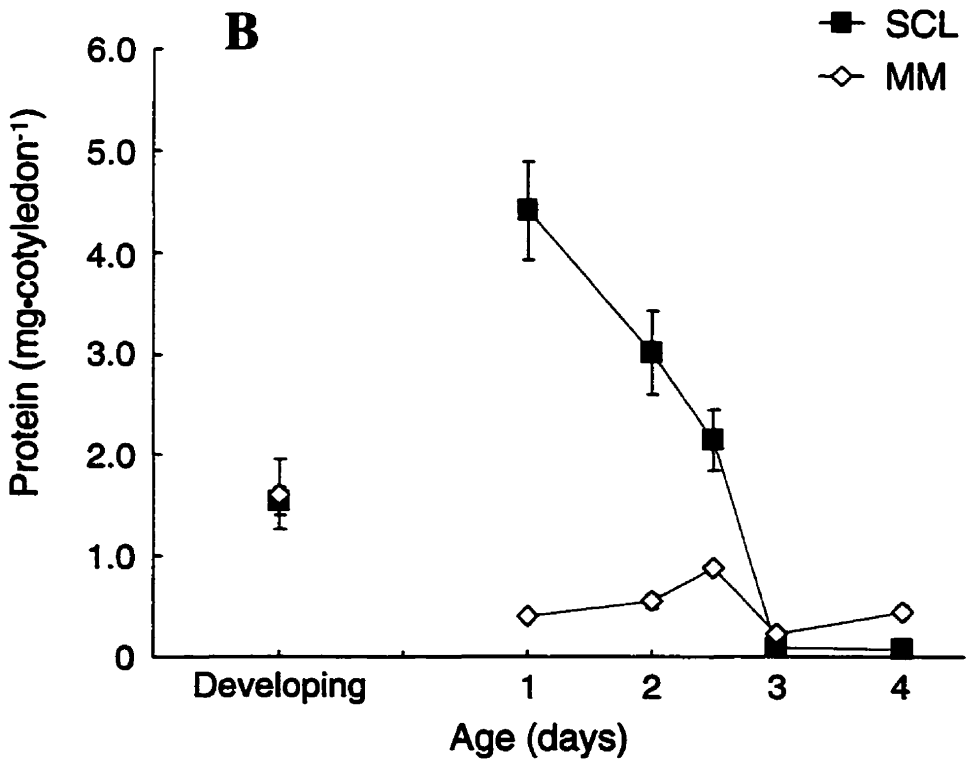
particles (HDPs), reflecting density differences that allow them to be separated by flotation and differentiation centrifugation (Figs. 2 and 3, Table 1). LDP and IDP protein levels both decline with advancing senescence of the cotyledons, but in distinct temporal patterns (Fig. 12 A). Changes in the levels of LDP and IDP protein during germination may reflect changes in the abundance of these particles. In this context, it is of interest to note that the developing seed level of LDP protein is approximately 3-fold higher than the corresponding level of IDP protein (Fig. 12 A). After germination, LDP protein declines progressively, whereas the IDP protein levels increase significantly between days 2 and 2.5 before decreasing to very low levels (Fig. 12 A, $\alpha = 0.05$, Appendix 1, Table A.1.8).

This unusual pattern of IDP protein content suggests the possibility that IDPs are contaminated with protein from another source. To examine this possibility the sucrose-containing fraction (SCL), located directly below the IDP fraction during the isolation procedure (Fig. 3, Table 1), was also collected and examined for protein (Fig. 12 B). Although the concentration of protein is about 100-fold higher in the SCL, the protein levels in the SCL decline progressively during germination until, at day 3, there is relatively little protein remaining (Fig. 12 B). This pattern is similar to that of LDPs except that the protein content of the SCL in developing cotyledons shows a proportionately lower level of protein than LDPs at the same stage of development (Figs. 12 A and B).

Interestingly, when the protein contents of LDPs and IDPs are summed they decrease from 1 day onward although there are no statistically significant differences

Figure 12. Changes in the protein content of subcellular fractions isolated from bean cotyledons during development and germination. **A.** Low-density particles (LDP) and intermediate-density particles (IDP). **B.** The sucrose-containing layer (SCL) and microsomal membranes (MM). Each graph is shown on a different scale. Standard errors of the means are shown for $n > 3$.


A
LDP
IDP
Protein (mg-cotyledon-1)
0.06
0.05
0.04
0.03
0.02
0.01
0
Developing
1
2
3
4
Age (days)


B
SCL
MM
Protein (mg-cotyledon-1)
6.0
5.0
4.0
3.0
2.0
1.0
0
Developing
1
2
3
4
Age (days)

in the protein levels, when they are compared in sequence, until 3 days after planting (Fig. 13, $\alpha = 0.05$, Appendix 1, Table A.1.8).

3.1 Protein Composition of Low-, Low'- and Intermediate-Density Lipid Particles

An examination of the protein composition of IDPs and LDPs from cotyledons of seeds harvested after germination revealed three major bands with molecular weights of ~ 17, 23 and 52 kDa (Fig. 14, lanes 3 and 4). For developing seeds, the data are comparable with the exception that the ~ 52 kDa protein is not present (Fig. 14, lanes 1 and 2).

It is also possible to isolate an additional population of lipid particles from cotyledons of seeds harvested 1 day after planting. These particles, termed low-density lipid particle's (LDP's), appear to have a density between LDPs and IDPs (Figs. 2 A and 3) and exhibit the same protein profile as LDPs and IDPs from the same tissue (15 B, lanes 2, 3 and 4). LDPs, LDP's and IDPs all have protein compositions distinguishable from microsomal membranes and the SCL isolated from bean cotyledons of the same age (Fig. 15 A, B and C) and they all have low molecular weight proteins within the range given for oleosin from oil bodies (Fig 15 A and B, Murphy 1990, Huang 1992, Murphy 1993, Herman 1995, Huang 1996, Napier *et al.* 1996).

The precise molecular weights of the major proteins in LDPs, LDP's and IDPs were determined by mass spectrometry. The smallest protein appears to have a molecular weight of 17,689 $\pm$ 2.17 daltons, and the ~ 23 kDa protein appears to have a

Figure 13. Changes in the sum of the protein from intermediate-density particles and low-density particles isolated from bean cotyledons during development and germination. Standard errors of the means are shown for $n \geq 3$.

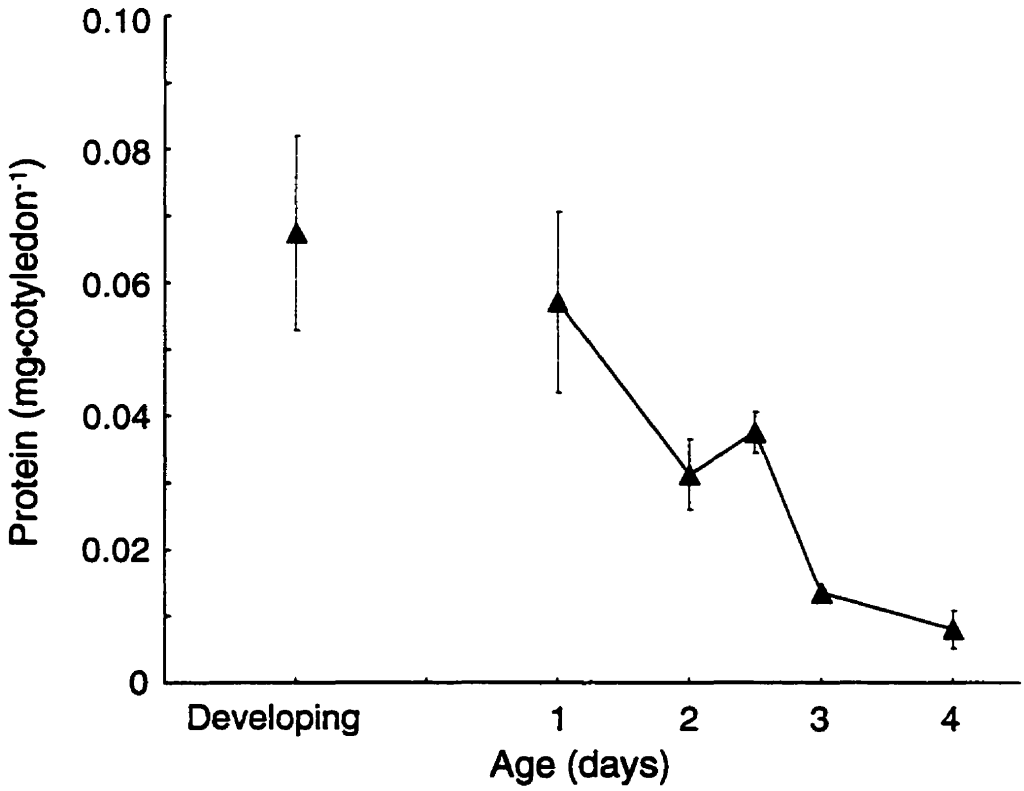

0.10
0.08
0.06
0.04
0.02
0
Protein (mg•cotyledon-1)
Developing
1
2
3
4
Age (days)

Figure 14. SDS-PAGE of lipid particles from developing cotyledons and 1 day-old cotyledons of bean seeds on a 15% acrylamide gel. Lane 1, low-density particles from cotyledons of developing bean seeds; lane 2, intermediate-density particles from cotyledons of developing bean seeds; lane 3, low-density particles from cotyledons of 1 day-old bean seeds; lane 2, intermediate-density particles from cotyledons of 1 day-old bean seeds. Each lane contains 0.25 µg protein and the gel is stained with silver. Approximate molecular weights in kilodaltons are indicated.

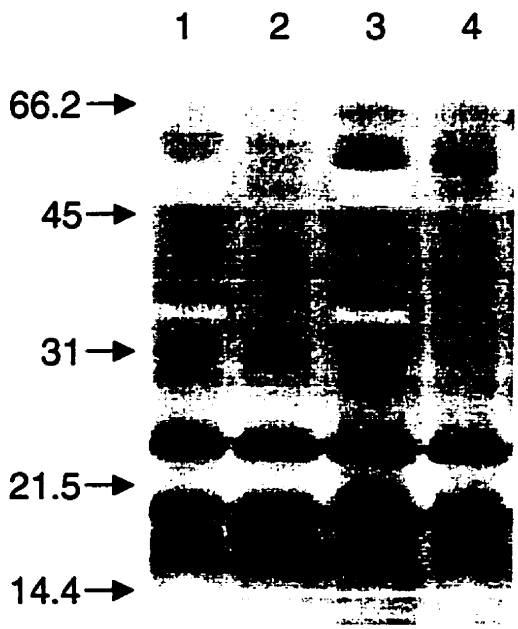
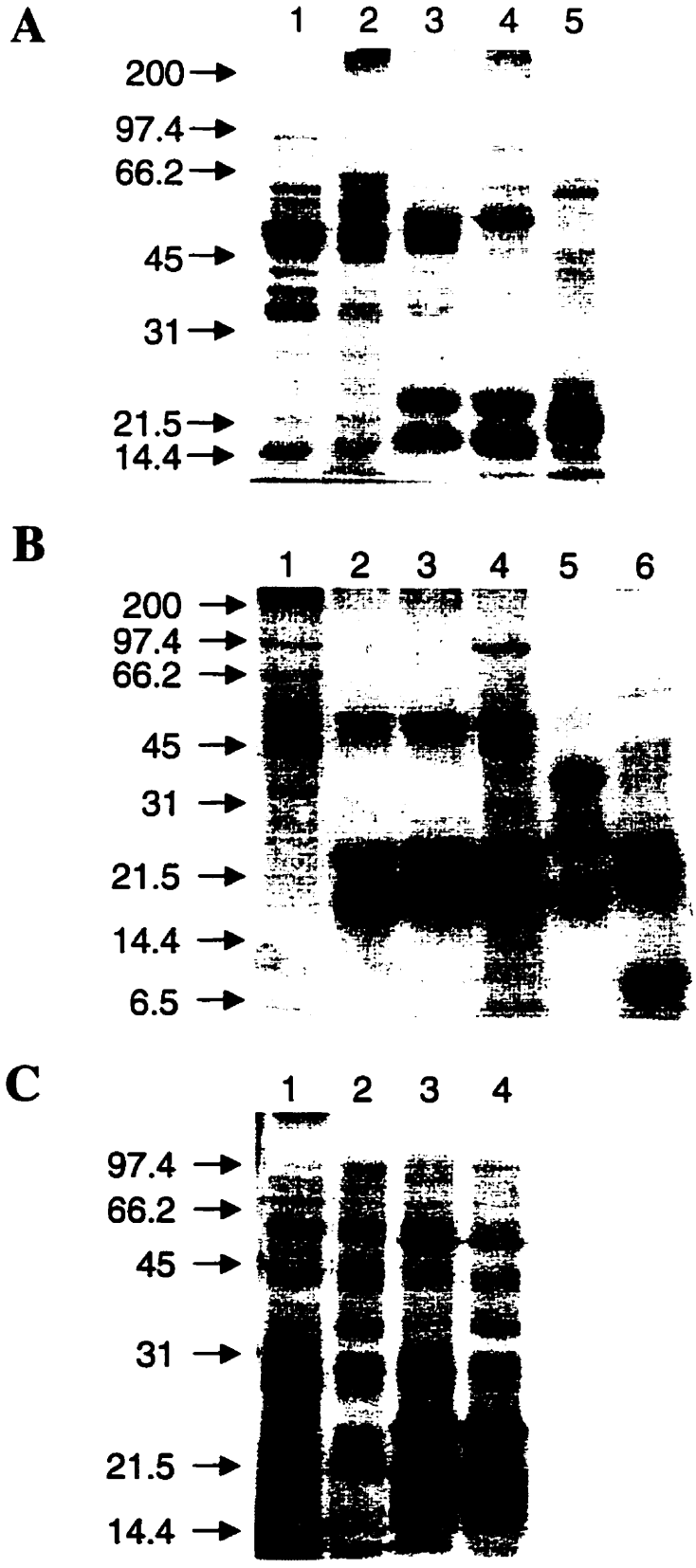

1
2
3
4
66.2
45
31
21.5
14.4

Figure 15. SDS-PAGE of subcellular fractions isolated from cotyledons of 1 day-old bean seeds and oil bodies from canola and soybeans. **A**. A 12% acrylamide gel stained with Coomassie brilliant blue. Lane1, microsomal membranes; lane 2, the sucrose-containing layer; lane 3, low-density particles; lane 4, intermediate-density particles; lane 5, canola oil bodies. **B**. A 15% acrylamide gel stained with Coomassie brilliant blue. Lane 1, microsomal membranes; lane 2, intermediate-density particles; lane 3, low-density particles; lane 4, low-density particles'; lane 5, soybean oil bodies; lane 6, canola oil bodies. **C**. A 13% acrylamide gel stained with silver. Lane 1, microsomal membranes; lane 2, the sucrose-containing layer; lane 3, intermediate-density particles; lane 4, low-density particles. The lanes in individual gels are loaded with equal protein. Approximate molecular weights in kilodaltons are indicated.


A
1 2 3 4 5
200
97.4
66.2
45
31
21.5
14.4
B
1 2 3 4 5 6
200
97.4
66.2
45
31
21.5
14.4
6.5
C
1 2 3 4
97.4
66.2
45
31
21.5
14.4

molecular weight of 22,858 ± 2.11 daltons (Fig. 16). The ~ 52 kDa protein was not resolvable by mass spectrometry. Of particular interest was the finding that the 22.9 kDa protein consisted of two populations differing by a range of 79 - 81 daltons. This size difference could be attributable to a phosphate group (80.99 daltons) or a sulfate group (82.07 daltons). The possibility of phosphorylation was explored using monoclonal phosphoamino acid antibodies, but these did not bind to the 22.9 kDa protein (data not shown). This could be due to the absence of a phosphate on the protein, but could also be due to an inability of the antibody to access the phosphoamino acid due to steric hindrance. Alkaline phosphatase treatment of the protein followed by mass spectrometry resulted in the complete disappearance of the protein. This may reflect activation, through dephosphorylation, of proteases and the consequent degradation of the protein of interest.

3.1.1 Western Blot Analysis

The possibility the LDPs, LDP's and IDPs isolated from bean seeds during germination contain a protein immunologically similar to oil body oleosins was examined by Western blotting. For this purpose, blots were probed with a polyclonal antibody directed against a canola 19 kDa oleosin protein. This antibody cross-reacted with the 22.9 kDa protein found in LDPs, LDP's and the IDPs, but did not bind with any proteins in the SCL or microsomal membranes (Fig. 17). When Western blots of the same fractions were probed with an antibody directed against a 22 kDa oleosin from canola, there was no reaction with the 22.9 kDa protein, but this antibody did react with the ~ 52 kDa protein from the LDPs, LDP's and IDPs (Fig. 18). The lack of

Figure 16. Mass spectrometric results indicating the molecular weights of the detected proteins in lipid particles isolated from cotyledons of 1 day-old beans. **A**. Detected proteins from the low-density particle fraction. **B**. Detected proteins from the low-density particle' fraction. **C**. Detected proteins from the intermediate-density particle fraction. All fractions were lipid extracted and the protein isolated before mass spectroscopic analysis. Molecular weights are indicated for each major peak.

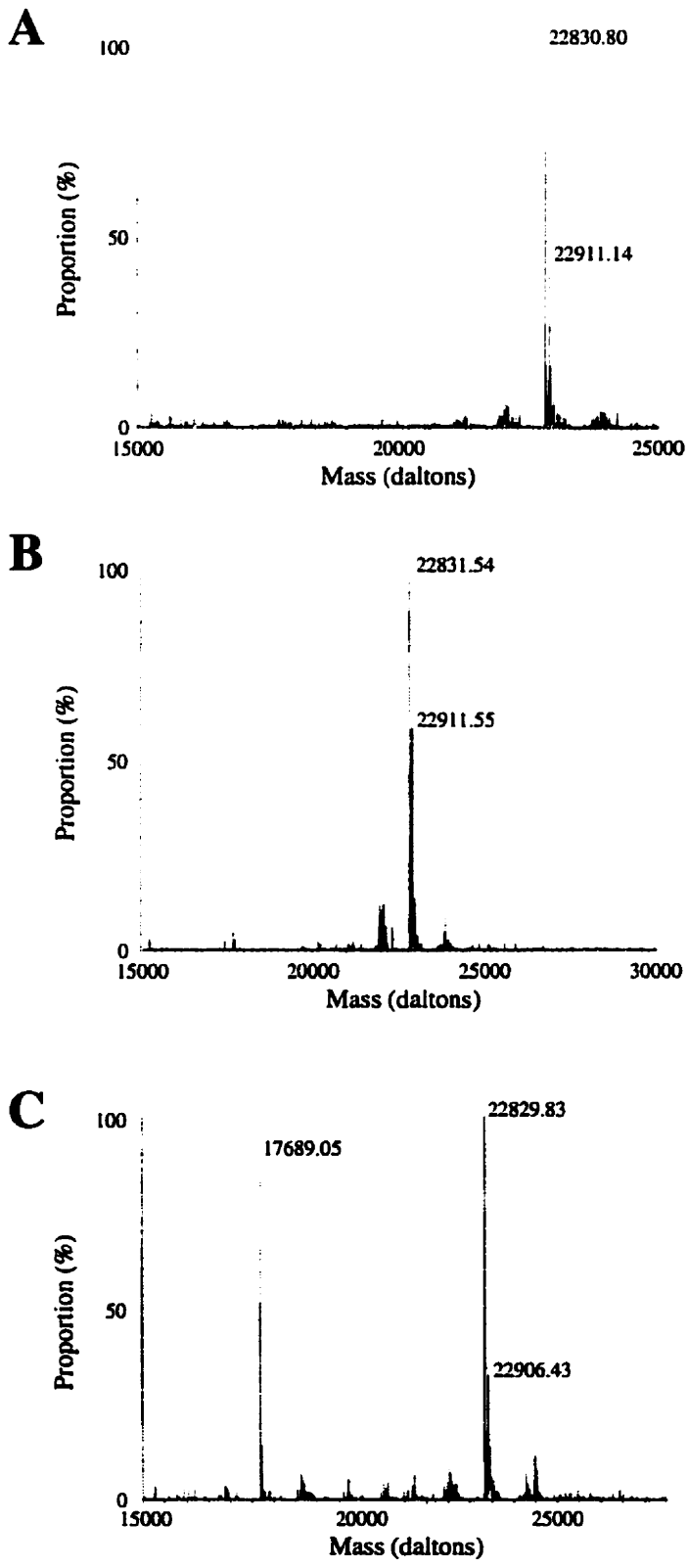

A
100
22830.80
Proportion (%)
50
22911.14
0
15000
20000
25000
Mass (daltons)
B
100
22831.54
Proportion (%)
22911.55
50
0
15000
20000
25000
30000
Mass (daltons)
C
100
22829.83
17689.05
Proportion (%)
50
22906.43
0
15000
20000
25000
Mass (daltons)

Figure 17. Western blot analysis of fractions isolated from 1 day-old beans probed with the 19 kDa canola oleosin antibody. Each lane was loaded with 3 μg protein and the proteins were separated by SDS-PAGE on a 12% acrylamide gel. Each lane was loaded with 3 μg protein. Lane 1, microsomal membranes; lane 2, the sucrose-containing layer; lane 3, low-density particles; lane 4; low-density particles'; lane 5, intermediate-density particles; lane 6, soybean oil bodies; lane 7, canola oil bodies. Approximate molecular weights in kilodaltons are indicated.

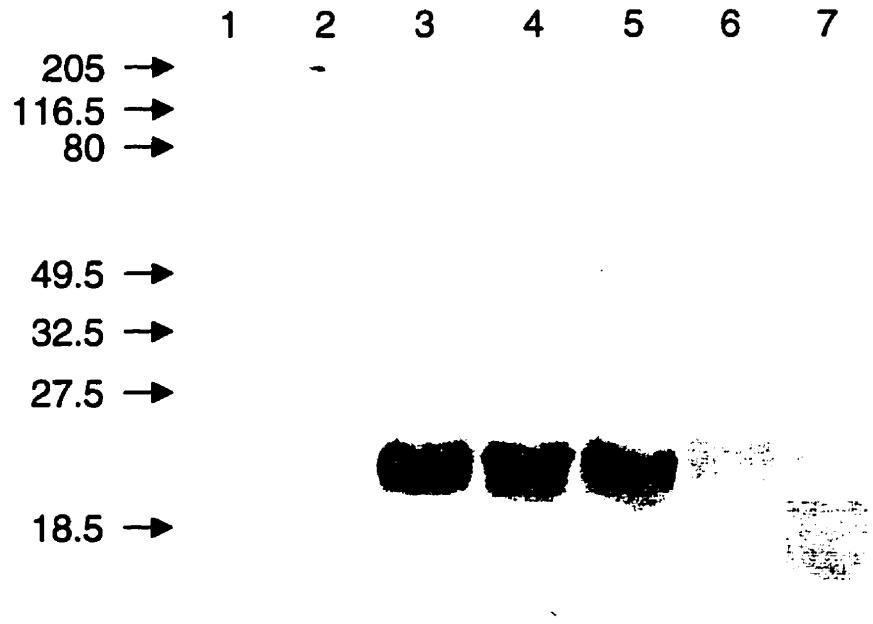
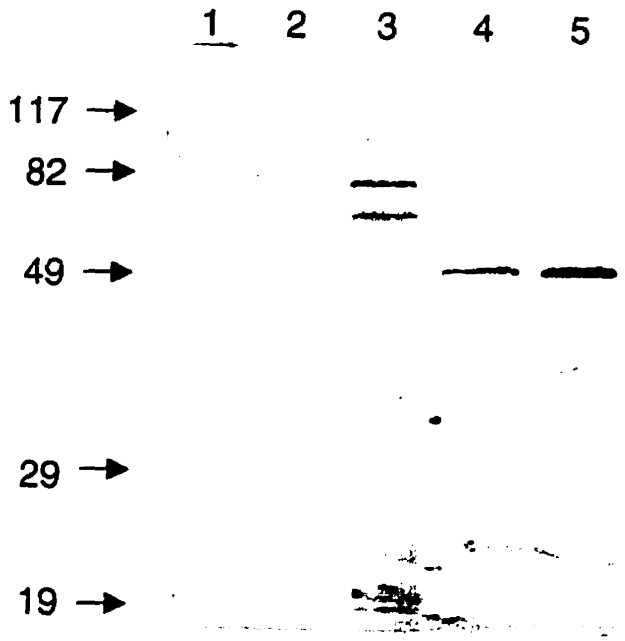

1
2
3
4
5
6
7
205
116.5
80
49.5
32.5
27.5
18.5

Figure 18. Western blot analysis of fractions isolated from 1 day-old bean seeds and probed with the 22 kDa canola oleosin antibody. Each lane was loaded with 1 μg protein and the proteins were separated by SDS-PAGE on a 12% acrylamide gel. Lane 1, the sucrose-containing layer; lane 2, microsomal membranes; lane 3, canola oil bodies; lane 4, intermediate-density particles; lane 5, low-density particles. Approximate molecular weights in kilodaltons are indicated.


1
2
3
4
5
117
82
49
29
19

recognition of the 22.9 kDa protein was unexpected, as this antibody has been shown to react with oleosins from many species of plants (M.M. Moloney, personal communication).

Of particular interest is the finding that HSP70 appears to be associated with LDPs and IDPs. This was established by Western blotting using a monoclonal HSP70 antibody (Fig. 19). HSP70 was also discernible in microsomal membranes and the cytosol (Fig. 19). In addition, canola oil bodies also cross-reacted with this antibody showing a reactive band corresponding to the molecular weight of HSP70 (Fig. 19).

3.1.2 Washing and Protease Treatment of Lipid Particles

When canola oil bodies, LDPs and IDPs were incubated in sodium deoxycholate, Triton X-100 and sodium carbonate and reisolated, there was no change in their protein profiles when examined by SDS- PAGE stained with either Coomassie brilliant blue or silver (Figs. 20, 21 and 22). This was confirmed by Western blotting of LDPs and IDPs. The proteins recognized by the antibodies against the 19 kDa and 22 kDa canola oleosins were unaffected by the washes of both IDPs and LDPs (Figs. 23 A and B, and 24 A and B). In some blots probed with the 19 kDa oleosin antibody, an artifact appears in the sample lanes as well as the marker and buffer lanes (Figs. 23 A, 24 A, and 30 A) and is not related to the sample.

In contrast to the proteins recognized by the canola oleosin antibodies, HSP70 was partially removed by treatment with Triton X-100 or sodium deoxycholate in IDPs, but unaffected by treatment with sodium carbonate (Fig 23 C, lanes 2 and 3, Fig. 24 C, lanes 2 and 3).

Figure 19. Western blot analysis of fractions isolated from 1 day-old bean seeds probed with HSP70 monoclonal antibody. Each lane was loaded with 3 µg protein and the protein was separated by SDS-PAGE on a 12% acrylamide gel. Lane 1, low-density particles; lane 2, intermediate-density particles; lane 3, the sucrose-containing layer; lane 4; microsomal membranes; lane 5, canola oil bodies. Approximate molecular weights in kilodaltons are indicated.

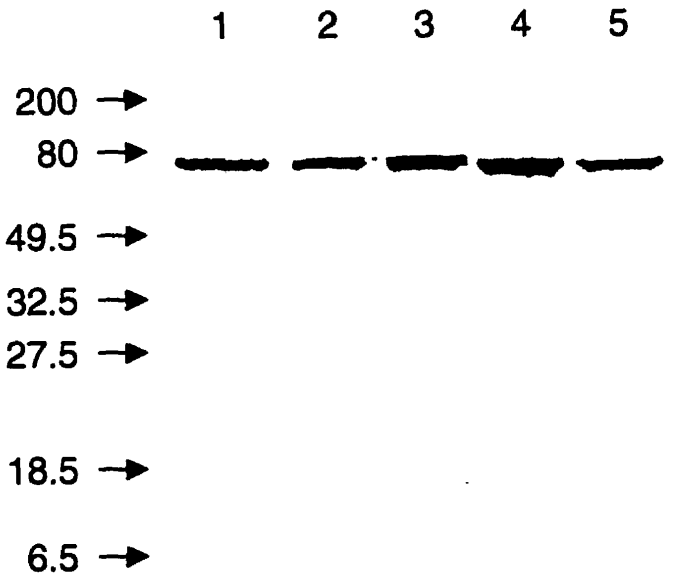

1
2
3
4
5
200
80
49.5
32.5
27.5
18.5
6.5

Figure 20. SDS-PAGE of washed canola oil bodies separated on 12 % acrylamide gels. Each lane contains 1 μg protein. Lane 1, untreated oil bodies; lane 2, oil bodies washed in water; lane 3, oil bodies washed in Triton X-100; lane 4, oil bodies washed in sodium deoxycholate; lane 5, oil bodies washed in sodium carbonate. **A.** Gel is stained with Coomassie brilliant blue. **B.** Gel is stained with silver. Approximate molecular weights in kilodaltons are indicated.

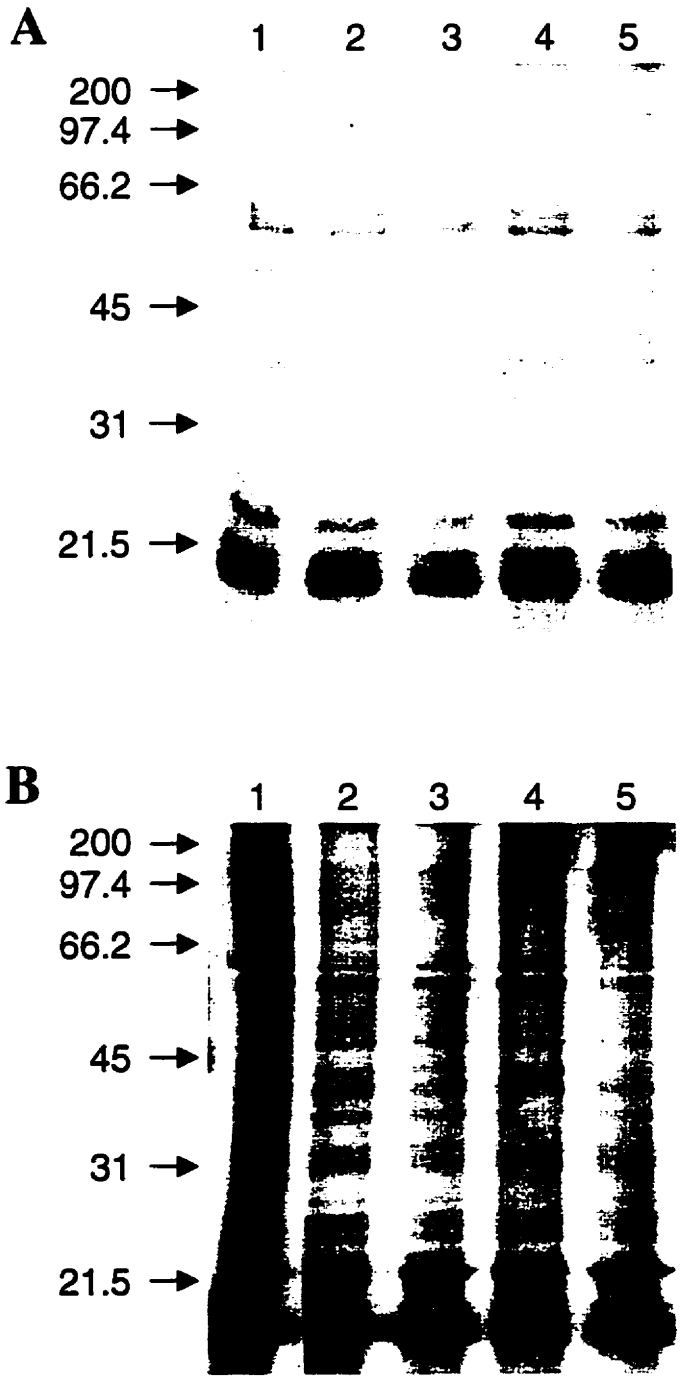

A
1
2
3
4
5
200
97.4
66.2
45
31
21.5
B
1
2
3
4
5
200
97.4
66.2
45
31
21.5

Figure 21. SDS-PAGE of washed intermediate-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. Each lane contains 1 μg protein. Lane 1, intermediate-density particles washed in water; lane 2, intermediate-density particles washed in sodium deoxycholate; lane 3, intermediate-density particles washed in Triton X-100; lane 4, intermediate-density particles washed in sodium carbonate. **A.** Gel stained with Coomassie brilliant blue. **B.** Gel stained with silver. Approximate molecular weights in kilodaltons are indicated.

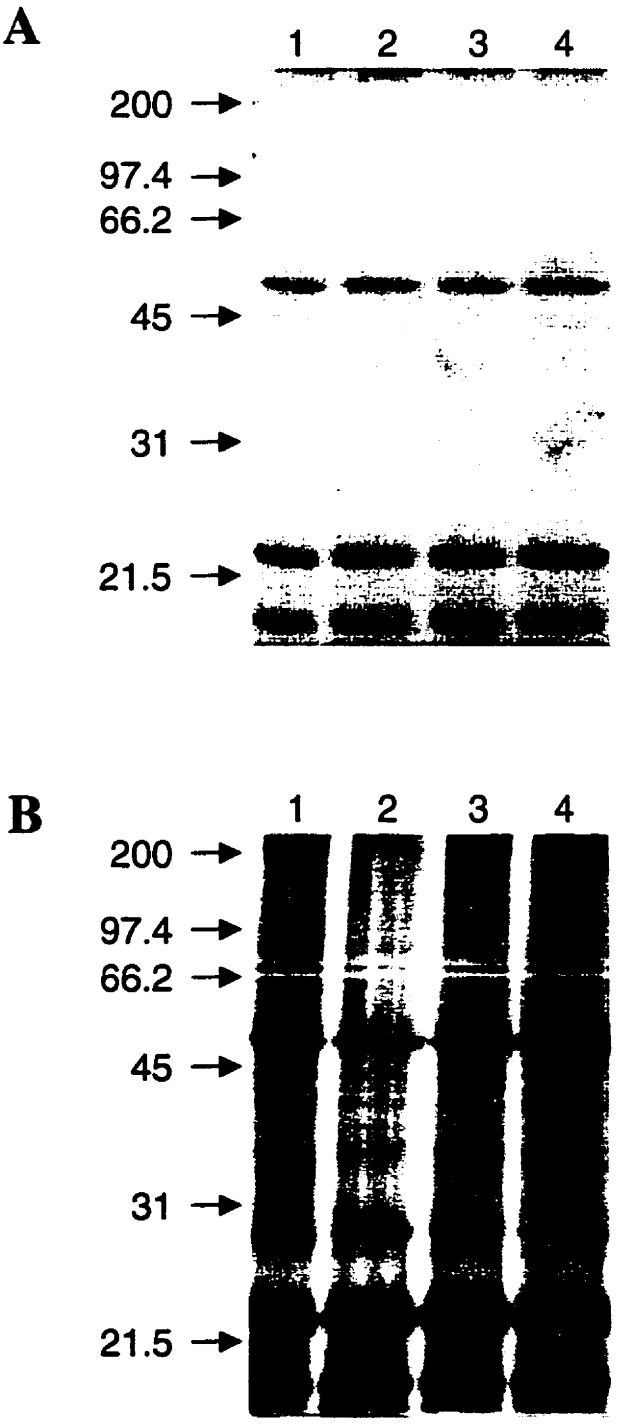
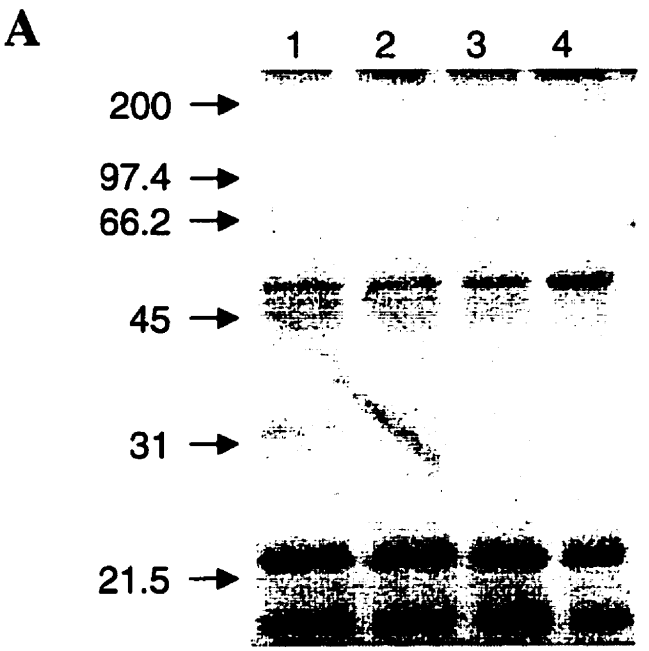

A
1
2
3
4
200
97.4
66.2
45
31
21.5
B
1
2
3
4
200
97.4
66.2
45
31
21.5

Figure 22. SDS-PAGE of washed low-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. Each lane contains 1 μg protein. Lane 1, low-density particles washed in water; lane 2, low-density particles washed in sodium deoxycholate; lane 3, low-density particles washed in Triton X-100; lane 4, low-density particles washed in sodium carbonate. **A**. Gel stained with Coomassie brilliant blue. **B**. Gel stained with silver. Approximate molecular weights in kilodaltons are indicated.


A
1
2
3
4
200
97.4
66.2
45
31
21.5

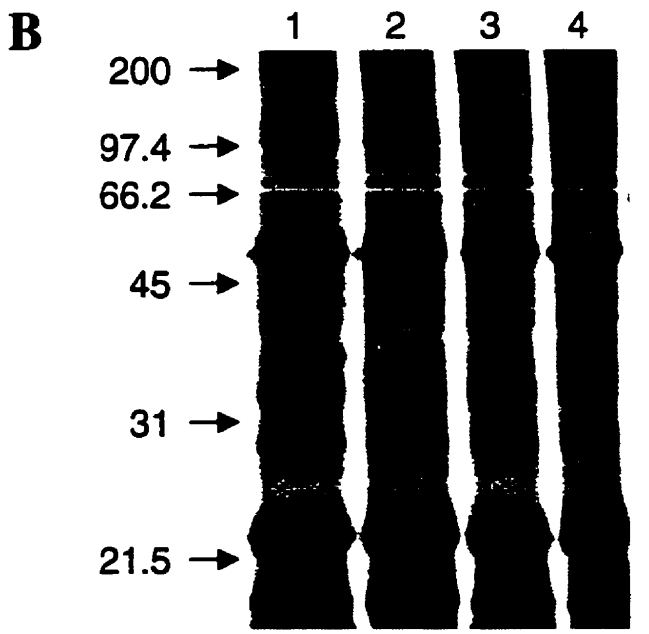

B
1
2
3
4
200
97.4
66.2
45
31
21.5

Figure 23. Western blot analyses of washed intermediate-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. Each lane contains 2 μg protein. Lane 1, intermediate-density particles washed in sodium carbonate; lane 2, intermediate-density particles washed in Triton X-100; lane 3, intermediate-density particles washed in sodium deoxycholate; lane 4, intermediate-density particles washed in water. **A**. Blot probed with 19 kDa canola oleosin antibody. **B**. Blot probed with 22 kDa canola oleosin antibody. **C**. Blot probed with HSP70 antibody. After primary antibody application all blots were treated with secondary antibody and stained for alkaline phosphatase activity. Approximate molecular weights in kilodaltons are indicated.

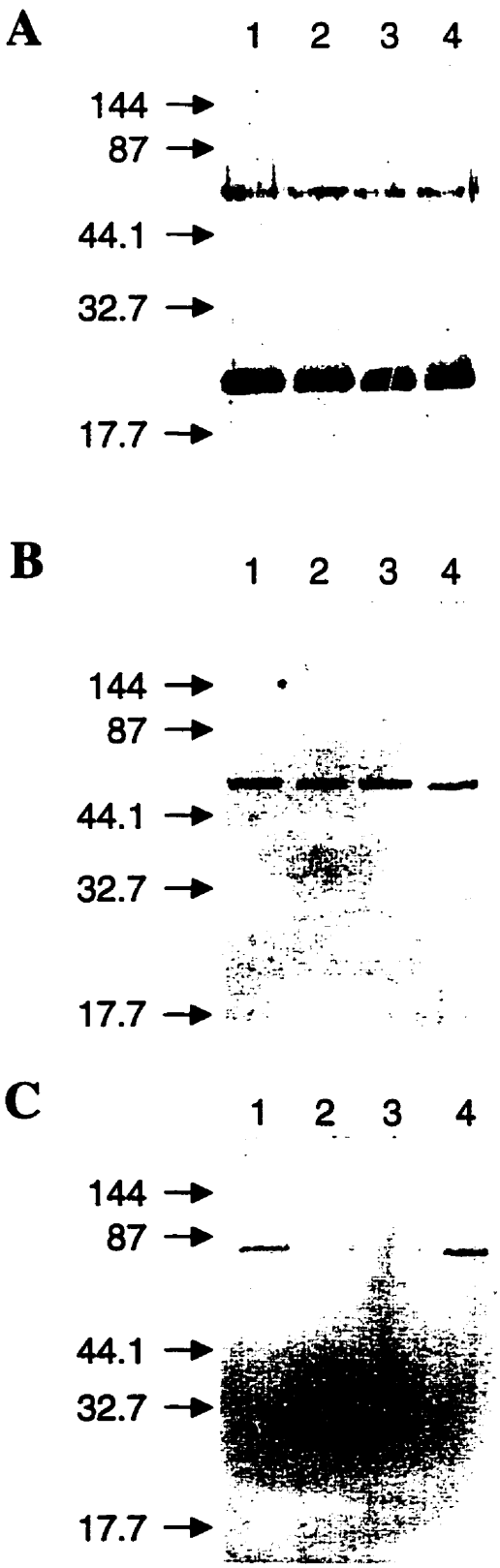
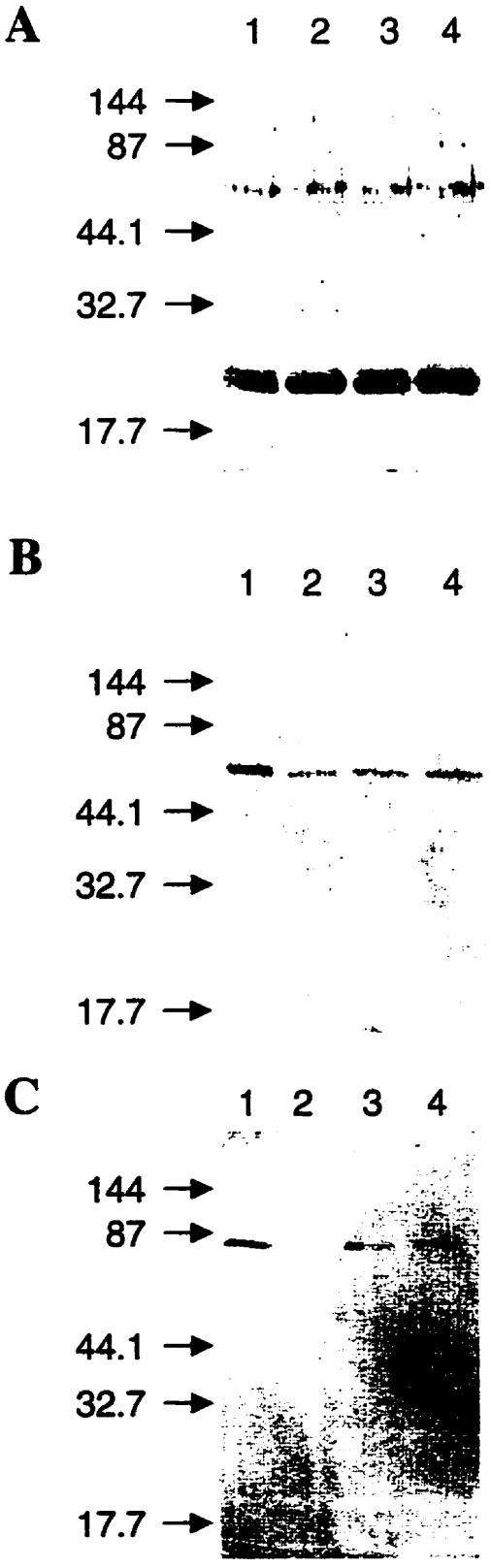

A
1 2 3 4
144
87
44.1
32.7
17.7
B
1 2 3 4
144
87
44.1
32.7
17.7
C
1 2 3 4
144
87
44.1
32.7
17.7

Figure 24. Western blot analyses of washed low-density particles isolated from 1 day-old beans separated on 12 % acrylamide gels. Each lane contains 2 µg protein. Lane 1, low-density particles washed in sodium carbonate; lane 2, low-density particles washed in Triton X-100; lane 3, low-density particles washed in sodium deoxycholate; lane 4, low-density particles washed in water. **A.** Blot probed with 19 kDa canola oleosin antibody. **B.** Blot probed with 22 kDa canola oleosin antibody. **C.** Blot probed with HSP70 antibody. After primary antibody application all blots were treated with secondary antibody and stained for alkaline phosphatase activity. Approximate molecular weights in kilodaltons are indicated.


A
1 2 3 4
144
87
44.1
32.7
17.7
B
1 2 3 4
144
87
44.1
32.7
17.7
C
1 2 3 4
144
87
44.1
32.7
17.7

When canola oil bodies were treated with Proteinase K, there was a rapid degradation of the oleosins visualized by SDS- PAGE and silver staining (Fig. 25). After one minute of digestion, there was a faint diffuse band at a slightly lower molecular weight than the oleosins apparent at the start of the reaction, and five minutes into this reaction there is no trace of this lower molecular weight protein (Fig. 25, lanes 1 and 2). During further protease digestion, the protein profile remains unchanged (Fig. 25, lanes 3 - 6). A broad, diffuse, low molecular weight band or group of bands at approximately 6 - 10 kDa appears at all time points during the digestion (Fig. 25). This is approximately the size of the hydrophobic portion of oleosin, which is known to be protected from proteolytic degradation (Tzen and Huang 1992). It is interesting to note that the 6 - 10 kDa fragments do not appear to increase in intensity to the same degree that the 19 kDa band decreases, and its presence at the beginning of the experiment suggests a certain amount of proteolytic degradation may be occurring during the isolation procedure (Fig. 25).

IDPs and LDPs were treated with Proteinase K in the same manner as canola oil bodies, and the effects were visualized by SDS-PAGE and staining with Coomassie blue (Fig. 26). For both types of particles, the 22.9 kDa protein was not discernible after one minute of Proteinase K digestion, and the ~ 52 kDa protein appeared to be somewhat degraded as several new bands were apparent directly below the original band (Figs. 26 A, lane 3 and 26 B, lane 3). The 17.7 kDa protein band proved to be fainter after 1 minute of Proteinase K treatment, but was still clearly visible. After five minutes of digestion, the 17.7 kDa protein was almost undetectable and the ~ 52 kDa protein continued to be degraded as shown by an accumulation of ~ 48 kDa band

Figure 25. SDS-PAGE of canola oil bodies subject to proteolytic digestion for varying amounts of time. Samples are separated on a 15% acrylamide gel and stained with Coomassie brilliant blue. Each lane is loaded with equal protein. Lane 1, 0 minutes digestion; lane 2, 1 minute digestion; lane 3, 5 minutes digestion; lane 4, 10 minutes digestion; lane 5, 60 minutes digestion; lane 6, 120 minutes digestion; lane 7, 120 minutes of incubation under the same conditions with no protease. Approximate molecular weights are indicated in kilodaltons.

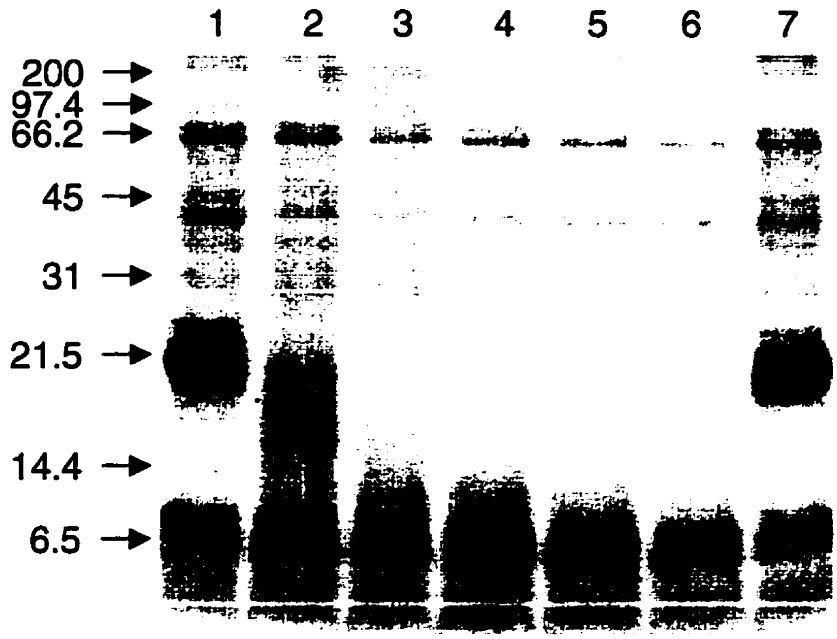
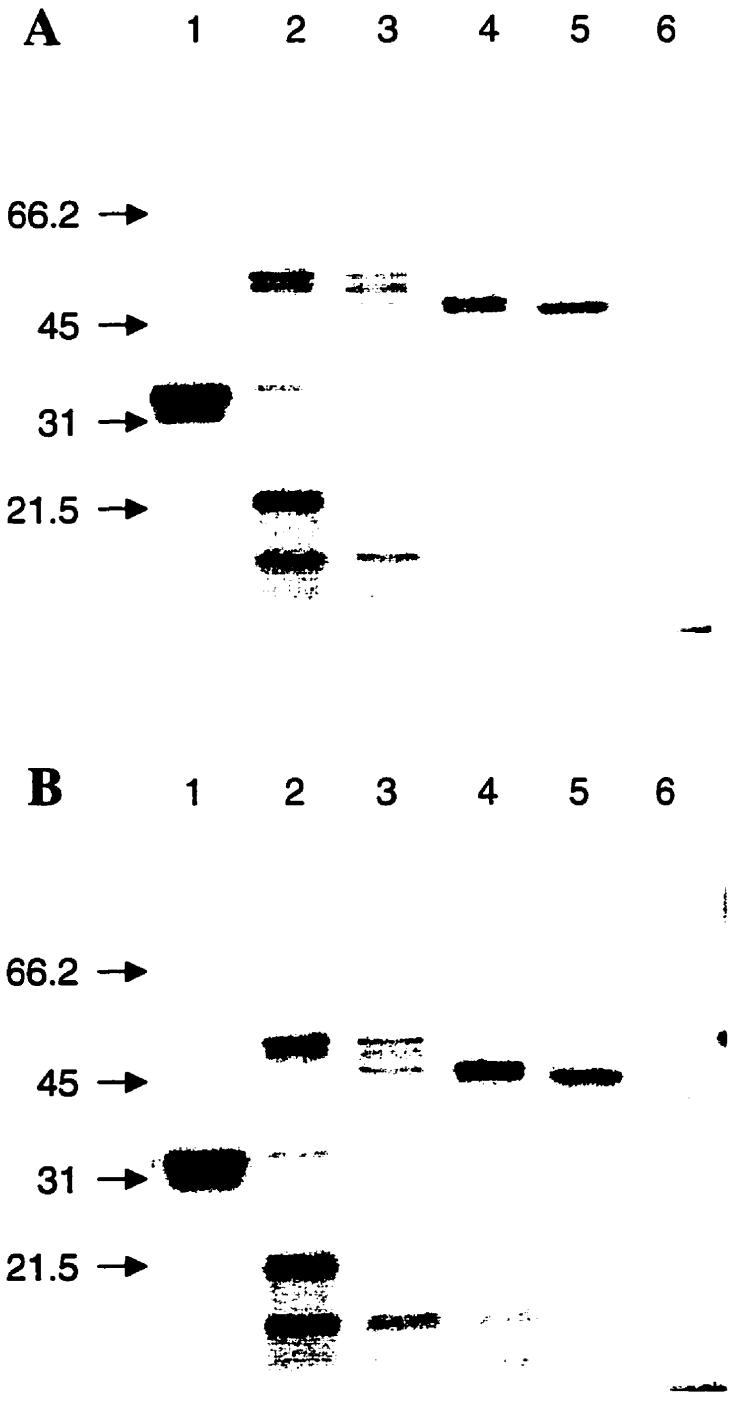

1
2
3
4
5
6
7
200
97.4
66.2
45
31
21.5
14.4
6.5

Figure 26. SDS-PAGE of lipid particles isolated from 1 day-old bean seeds, subjected to proteolytic digestion for varying amounts of time and stained with Coomassie brilliant blue. Each lane is loaded with equal protein and the protein is separated on a 13% acrylamide gel. Lane 1, Proteinase K; lane 1, 0 minutes digestion; lane 3, 1 minute digestion; lane 4, 5 minutes digestion; lane 5, 40 minutes digestion; lane 5, 5 minutes digestion with 1% SDS added. **A**. Digestion of intermediate-density particles. **B**. Digestion of low-density particles. Approximate molecular weights are indicated in kilodaltons.

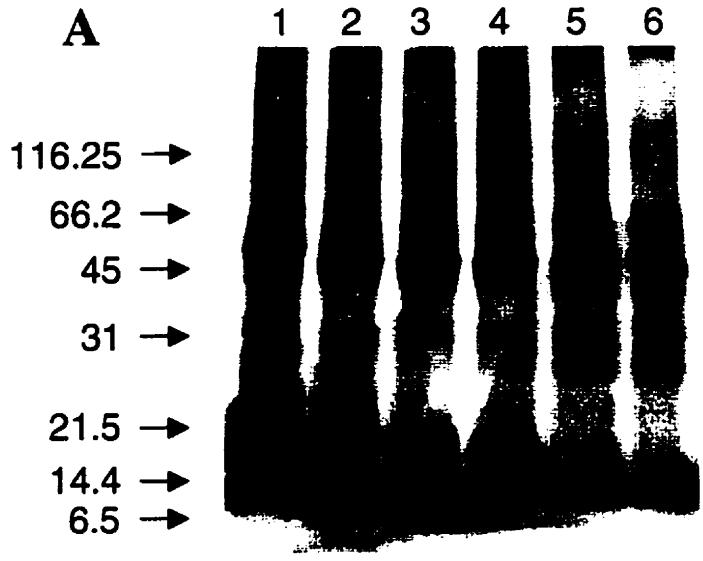

A
1
2
3
4
5
6
66.2
45
31
21.5
B
1
2
3
4
5
6
66.2
45
31
21.5

(Figs. 26 A, lane 4 and 26 B lane 4). After 60 minutes of Proteinase K digestion, a proteolytic fragment, ~ 48 kDa in size, was the only distinct band visible on the gel (Figs. 26 A, lane 5 and 26 B, lane 5). This is presumably a fragment from the ~ 52 kDa protein that is resistant to proteolysis by Proteinase K. Even when the Proteinase K reaction was run for 3 hours, the ~ 48 kDa polypeptide remained intact (data not shown). When the particles were treated with 1% SDS before protease digestion, there were no bands visible on the gel (Fig. 26 A, lane 6) showing that solubilization of the lipid in the sample resulted in complete degradation of the proteins.

Proteinase K-digested IDPs and LDPs were also fractionated on a 10 - 20% gradient gel in order to examine the smaller polypeptides more effectively. These smaller fragments were visualized by overstaining with silver (Fig. 27). It is clear that there are several proteins not visible by staining with Coomassie that do appear when stained with silver, but the results of the digestion are similar to those obtained on a 13% acrylamide gel (Figs. 26 and 27). The exceptions are that one low molecular weight fragment (~ 10 kDa) is apparent and remains stable in the IDP fraction, and three low molecular weight fragments in the LDP fraction (~ 14.4, 12 and 10 kDa) appeared within 1 minute of digestion and remained clearly distinct even after 60 or 120 minutes of Proteinase K treatment (Figs. 27 A, lane 6 and 27 B, lane 5).

In further experiments, these protease digests were probed with selected antibodies. In Western blots of IDP digests probed with the 19 kDa canola oleosin antibody, the reactive epitopes of the 22.9 kDa protein showed rapid degradation. Indeed, this protein is only faintly detectable after 1 minute of digestion (Fig. 28 A, lanes 1 and 2). Corresponding blots of LDP digests indicated that the 22.9 kDa protein

Figure 27. SDS-PAGE of lipid particles isolated from 1 day-old bean seeds, subjected to proteolytic digestion for varying amounts of time and stained with silver. Each lane is loaded with equal protein and the proteins are separated on 10-20% acrylamide gradient gels. Lane 1, 0 minutes digestion; lane 2, 1 minute digestion; lane 3, 5 minutes digestion; lane 4, 10 minutes digestion; lane 5, 60 minutes digestion; lane 6, 120 minutes digestion. **A**. Digestion of intermediate-density particles. **B**. Digestion of low-density particles. Approximate molecular weights are indicated in kilodaltons.


A
1
2
3
4
5
6
116.25
66.2
45
31
21.5
14.4
6.5

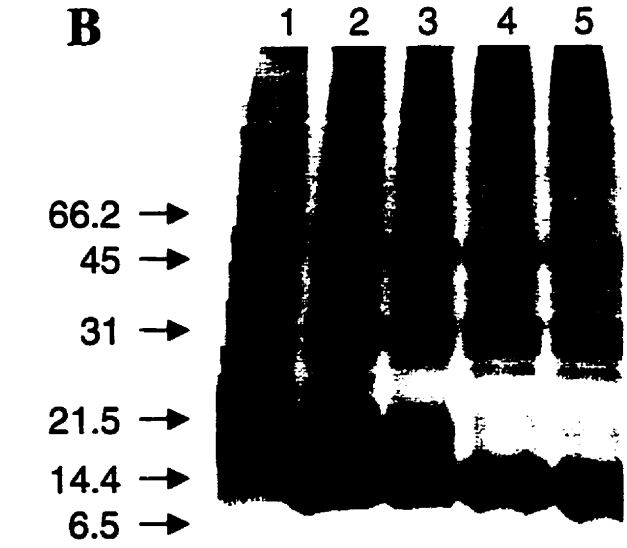
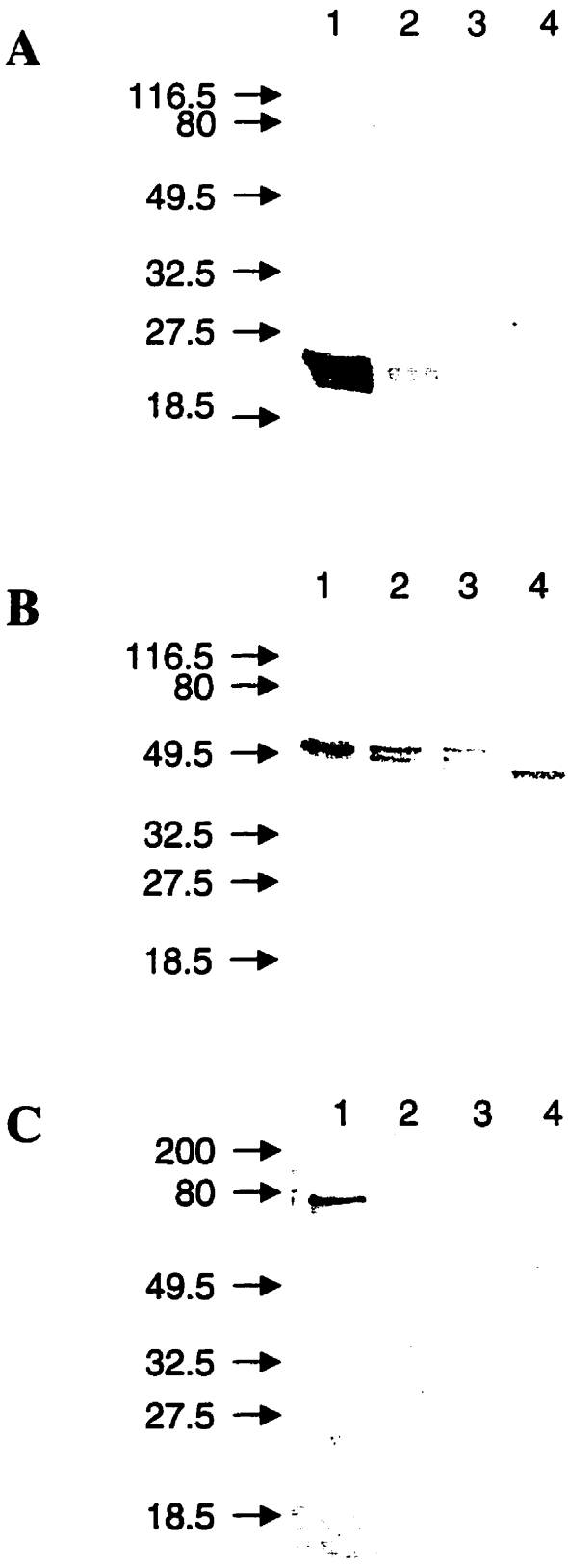

B
1
2
3
4
5
66.2
45
31
21.5
14.4
6.5

Figure 28. Western blot analyses of intermediate-density particles subjected to proteolytic degradation for varying periods of time. Intermediate-density particles were isolated from cotyledons of 1 day-old bean seeds. Equal protein is loaded and separated on 12% acrylamide gels. Lane 1, 0 minutes incubation; lane 2, 1 minute incubation; lane 3, 5 minutes incubation; lane 4, 60 minutes incubation. **A.** Blot probed with 19 kDa canola oleosin antibody. **B.** Blot probed with 22 kDa canola oleosin antibody. **C.** Blot probed with HSP70 antibody. After primary antibody application all blots were treated with secondary antibody and stained for alkaline phosphatase activity. Approximate molecular weights in kilodaltons are indicated.


A
1 2 3 4
116.5
80
49.5
32.5
27.5
18.5
B
1 2 3 4
116.5
80
49.5
32.5
27.5
18.5
C
1 2 3 4
200
80
49.5
32.5
27.5
18.5

is undetectable after one minute of digestion (Fig. 29 A, lanes 1 and 2). In contrast, when LDP and IDP digests were probed with the 22 kDa canola oleosin antibody, which cross reacts with the ~ 52 kDa protein from the particles, evidence for protected fragments of this protein was again obtained for both types of particles (Figs. 28 B and 29 B). Finally, the Western blots of LDP and IDP digests were also probed with the HSP70 antibody. This protein was not detectable in either LDP or IDP fractions after 1 minute of incubation with the protease, and no proteolytic fragments were apparent (Figs. 28 C and 29 C).

3.1.3 Analysis of Oleosin Antibodies

In order to determine if the cross-reaction of oleosin antibodies with the proteins of IDPs and LDPs is specific to regions of proteins in common with canola oleosins, competition experiments were performed. In one of these experiments, the 19 kDa canola oleosin antiserum was incubated with canola oil bodies prior to Western blotting. Despite the rather weak control reaction with the canola oleosin antibodies (Fig. 30 A lane 3), the IDPs and LDPs showed little change in antiserum-binding capacity (Figs. 30 A, lanes 1 and 2 and 30 B, lanes 1 and 2) when compared to the canola oleosins (Figs. 30 A, lane 3 and 30B, lane 3), suggesting that the antibodies reacting with the proteins of IDPs and LDPs are a nonoleosin-specific component of the antiserum.

The same experiment was performed using the antibodies generated against the 22 kDa oleosin of canola (Fig. 31). Once again, despite a reduced reaction of oleosin antibody-depleted antiserum with canola oleosin (Figs. 31A, lane 3 and 31B, lane 3),

Figure 29. Western blot analyses of low-density particles subjected to proteolytic degradation for varying periods of time. Low-density particles were isolated from cotyledons of 1 day-old bean seeds. Equal protein is loaded and separated on 12% acrylamide gels. Lane 1, 0 minutes incubation; lane 2, 1 minute incubation; lane 3, 5 minutes incubation; lane 4, 60 minutes incubation. **A**. Blot probed with 19 kDa canola oleosin antibody. **B**. Blot probed with 22 kDa canola oleosin antibody. **C**. Blot probed with HSP70 antibody. After primary antibody application all blots were treated with secondary antibody and stained for alkaline phosphatase activity. Approximate molecular weights in kilodaltons are indicated.

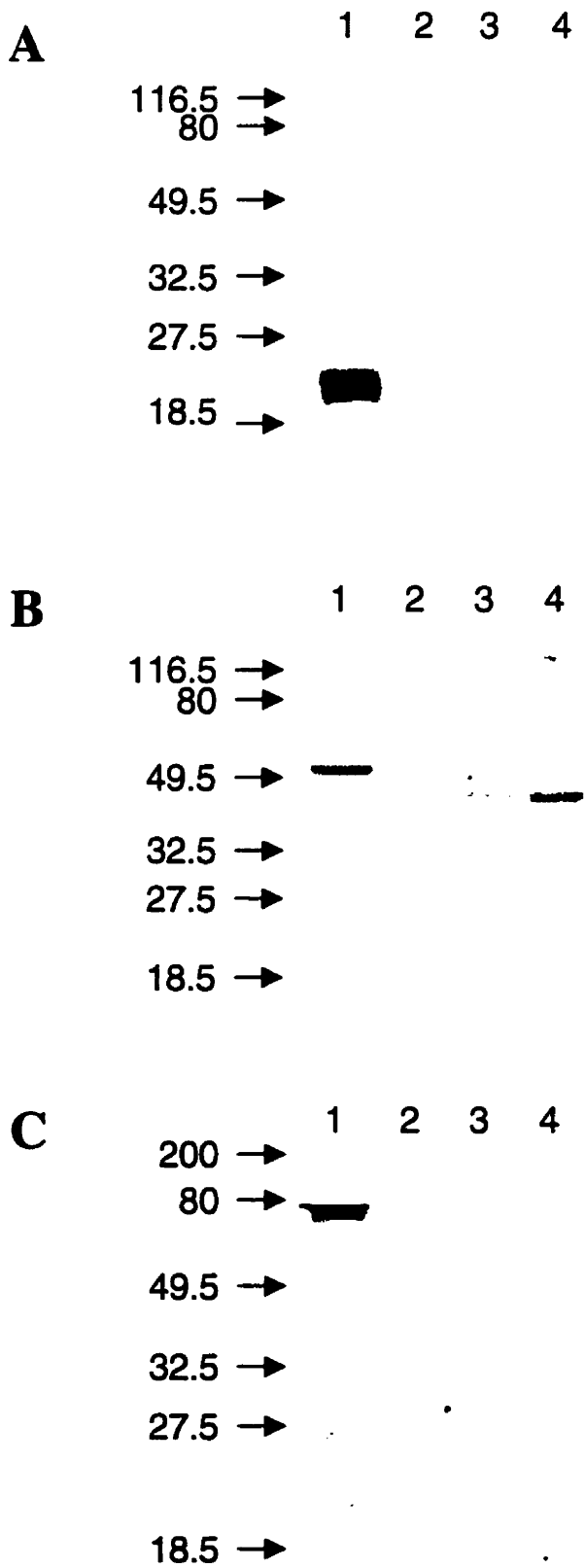
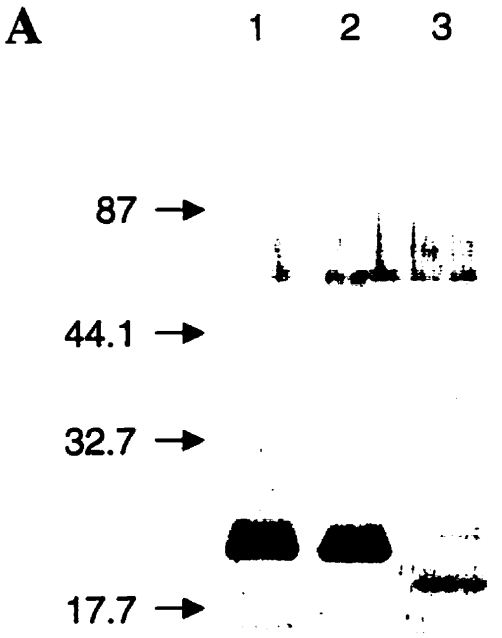
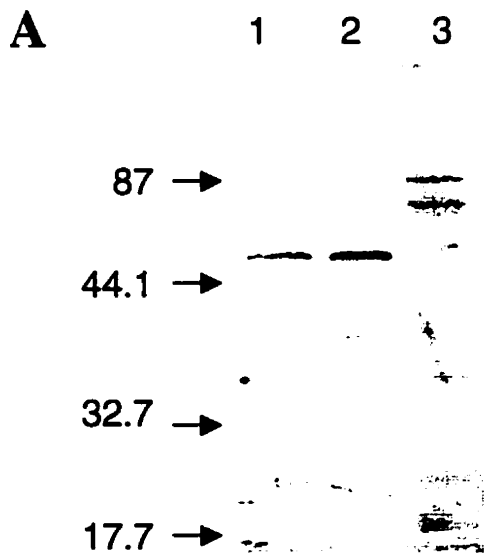

A
1 2 3 4
116.5
80
49.5
32.5
27.5
18.5
B
1 2 3 4
116.5
80
49.5
32.5
27.5
18.5
C
1 2 3 4
200
80
49.5
32.5
27.5
18.5

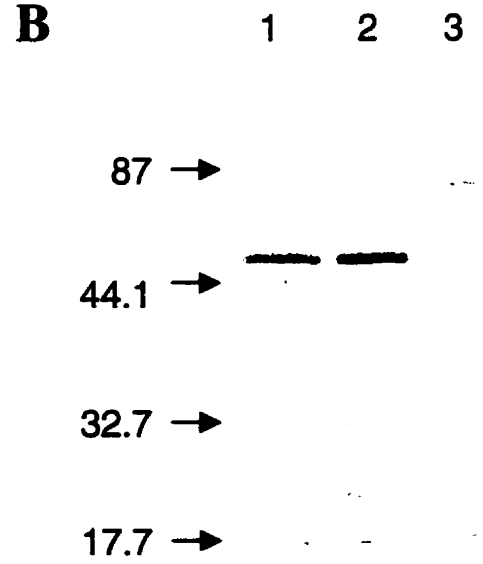
Figure 30. Antigen competition analysis of 19 kDa canola oleosin antiserum by Western blot analyses of lipid particles from cotyledons of 1 day-old bean seeds and oil bodies from canola. Each lane is loaded with 1 µg protein and separated on 12% acrylamide gels. Lane 1, low-density particles; lane 2, intermediate-density particles; lane 3, canola oil bodies. **A**. Blot probed with untreated 19 kDa canola oleosin antibody followed by secondary antibody treatment and stained for alkaline phosphatase activity. **B**. Blot probed with 19 kDa canola oleosin antibody incubated for 5 minutes with 1 ml canola oil bodies followed by secondary antibody treatment and stained for alkaline phosphatase activity. Approximate molecular weights in kilodaltons are indicated.


A
1
2
3
87
44.1
32.7
17.7


B
1
2
3
87
44.1
32.7
17.7

Figure 31. Antigen competition analysis of 22 kDa canola oleosin antiserum by Western blot analyses of lipid particles from cotyledons of 1 day-old bean seeds and oil bodies from canola. Each lane is loaded with 1 μg protein and separated on 12% acrylamide gels. Lane 1, low-density particles; lane 2, intermediate-density particles; lane 3, canola oil bodies. **A**. Blot probed with untreated 22 kDa canola oleosin antibody followed by secondary antibody treatment and stained for alkaline phosphatase activity. **B**. Blot probed with 22 kDa canola oleosin antibody incubated for 5 minutes with 1 ml canola oil bodies followed by secondary antibody treatment and stained for alkaline phosphatase activity. Approximate molecular weights in kilodaltons are indicated.


A
1
2
3
87
44.1
32.7
17.7


B
1
2
3
87
44.1
32.7
17.7

the cross-reaction with the 52 kDa protein of IDPs and LDPs was unaffected by oil body treatment of the antiserum (Figs. 31 A, lanes 1 and 2, and 31 B, lanes 1 and 2). This suggests that, again, the antibodies in the serum that cross-react with this protein are not those that cross-reacted with the canola oleosin.

The oleosin antibodies were provided by M.M. Moloney (University of Calgary, Calgary, Alberta), and no preimmune serum was available to examine the array of antibodies naturally present in the rabbit serum before the antigen was introduced. In order to examine the possibility that these proteins are common antigens in rabbit serum, preimmune sera from three different rabbits were used to blot subcellular fractions from bean cotyledons (Fig. 32). It is apparent that all of the preimmune sera examined cross-reacted with proteins from bean cotyledons, although it is not clear if any of the LDP and IDP proteins corresponded to those identified by the canola oleosin antisera.

3.1.4 Sequencing and Identification of LDP Proteins

Each of the three major proteins of LDPs was prepared for amino-terminal sequencing in an effort to identify them by comparison with corresponding sequence data for other proteins in the data banks. Unfortunately, the ~ 52 kDa protein (as well as the protected proteolytic fragment of this protein) and the 17.7 kDa protein were N-terminally blocked. However, using BLAST, the N-terminal amino acids of the 22.9 kDa protein showed a strong homology with a protein identified as an α-amylase inhibitor (Table 2). These proteins serve as defense proteins by impairing the ability of eukaryotic organisms to digest starch (Powers and Whitaker 1977).

Figure 32. Western blot analyses of subcellular fractions from 1 day-old bean seeds probed with preimmune sera. Each lane is loaded with 1 μg protein and separated on 15% acrylamide gels. Lane 1, microsomal membranes; lane 2, the sucrose-containing layer; lane 3, intermediate-density particles; lane 4, low-density particles. **A.**, **B.** and **C.** each represent blots probed with preimmune serum from different rabbits followed by secondary antibody treatment and stained for alkaline phosphatase activity. Approximate molecular weight in kilodaltons are indicated.

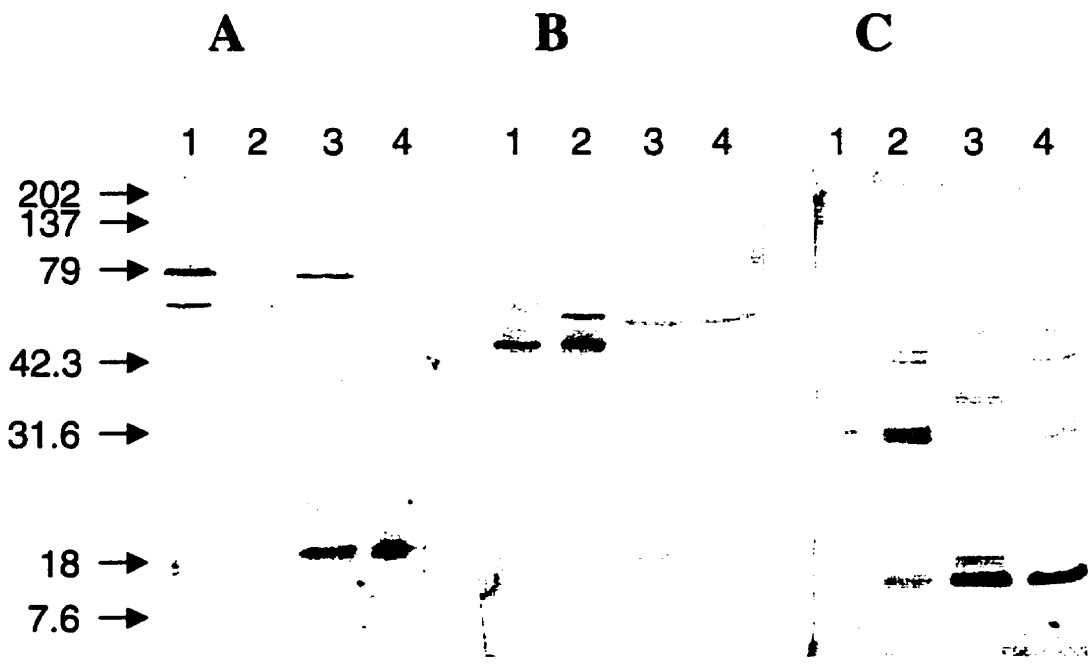

A
B
C
1 2 3 4
1 2 3 4
1 2 3 4
202
137
79
42.3
31.6
18
7.6

Table 2. Amino terminal sequence of 22.8 kDa protein from LDP isolated from 1 day old bean cotyledons compared with similar proteins using the BLAST program (Altschul *et al.* 1990, Gish and States 1993).

	Amino terminal sequence																														
22.9 kDa LDP protein	[GAVS]	X	[EV]	G	L	[FD]	I	V	D	A	F	V	Q	P	N	L	I	L	Q	G	D	A	K	V	E	D	N	G	[FL]	L
α-amylase inhibitor-5		T		T	S				D	A	F	N	K	T	N	L	I	L	Q	G	D	A	T	V	S	S	N	G	N	L
α-amylase inhibitor-4		T		T	S				D	A	F	N	K	T	N	L	I	L	Q	G	D	A	T	V	S	S	N	G	N	L
Lectin precursor		T		T	S				D	A	F	N	K	T	N	L	I	L	Q	G	D	A	T	V	S	S	N	G	N	L
α-amylase inhibitor		T		T	S		N		D		F	N	K	T	N	L	I	L	Q	G	D	A	I	V	S	S	N	G	N	L
α-amylase inhibitor		T		T	S		N		D	A	F	N	K	T	N	L	I	L	Q	G	D	A	T	V	T	S	K	G	Y	L
Lectin-2 precursor		N					N		D	T	F	N	E	T	N	L	I	L	Q	G	D	A	T	V	S	S	K	G	Q	L
Lectin-3 precursor		N					N		D	T	F	N	E	T	N	L	I	L	Q	G	D	A	T	V	S	S	K	G	Q	L
α-amylase inhibitor		N			S		N		T	T	F	N	E	T	N	L	I	L	Q	G	D	A	T	V	S	S	N	G	N	L

Identical amino acids

Conserved amino acids

To confirm the identity of this sequenced protein, the ability of LDPs to serve as an α-amylase inhibitor was examined. It is clear that LDPs from both developing and germinating cotyledon tissue inhibit porcine α-amylase (Fig. 33, $\alpha = 0.05$, Appendix 1, Table A.1.9). This activity was abolished by heat denaturation as well as by five minutes of degradation with Proteinase K (Fig. 33). Attempts to confirm that the corresponding protein of IDPs is also an α-amylase inhibitor were confounded by the fact that the IDP preparation contained considerable innate amylase activity, presumably a contaminant from the cytosol (data not shown).

α-Amylase inhibitors are thought to be localized in protein bodies of *Phaseolus vulgaris* seeds (Santino *et al.* 1992). These organelles are easily destroyed during homogenization and proteins contained therein have been shown to contaminate preparations of oil bodies (Kalinsky *et al.* 1992, Thoyts *et al.* 1996). In order to examine the possibility of protein body contamination of LDPs and IDPs, protein bodies were isolated by two different methods, and the protein profiles were compared with those of isolated lipid particles (Fig. 34). Although the two methods of protein body isolation resulted in different protein profiles as resolved by SDS- PAGE and silver staining, there are several polypeptides at molecular weights in common to both protein body preparations and to LDPs and IDPs (Fig. 34, lanes 3 and 4). Of these common polypeptides, one corresponds in molecular weight to the 22.9 kDa protein (α-amylase inhibitor) of LDPs and IDPs, and another corresponds to the 17.7 kDa protein of IDPs and the LDPs (Fig. 34).

The 19 and 22 kDa canola oleosin antibodies were utilized to confirm the identity of these proteins in protein bodies by Western blotting. Blots of fractionated

Figure 33. Effects of denaturation and protease digestion on the ability of the low-density particles from cotyledons of developing and 1 day-old bean seeds to inhibit porcine α-amylase activity. Values are expressed as $mg \cdot ml^{-1}$ maltose released from starch in 10 minutes in the presence and absence of 1 μg low density particle (LDP) protein. Standard errors of the means are indicated for n = 3.

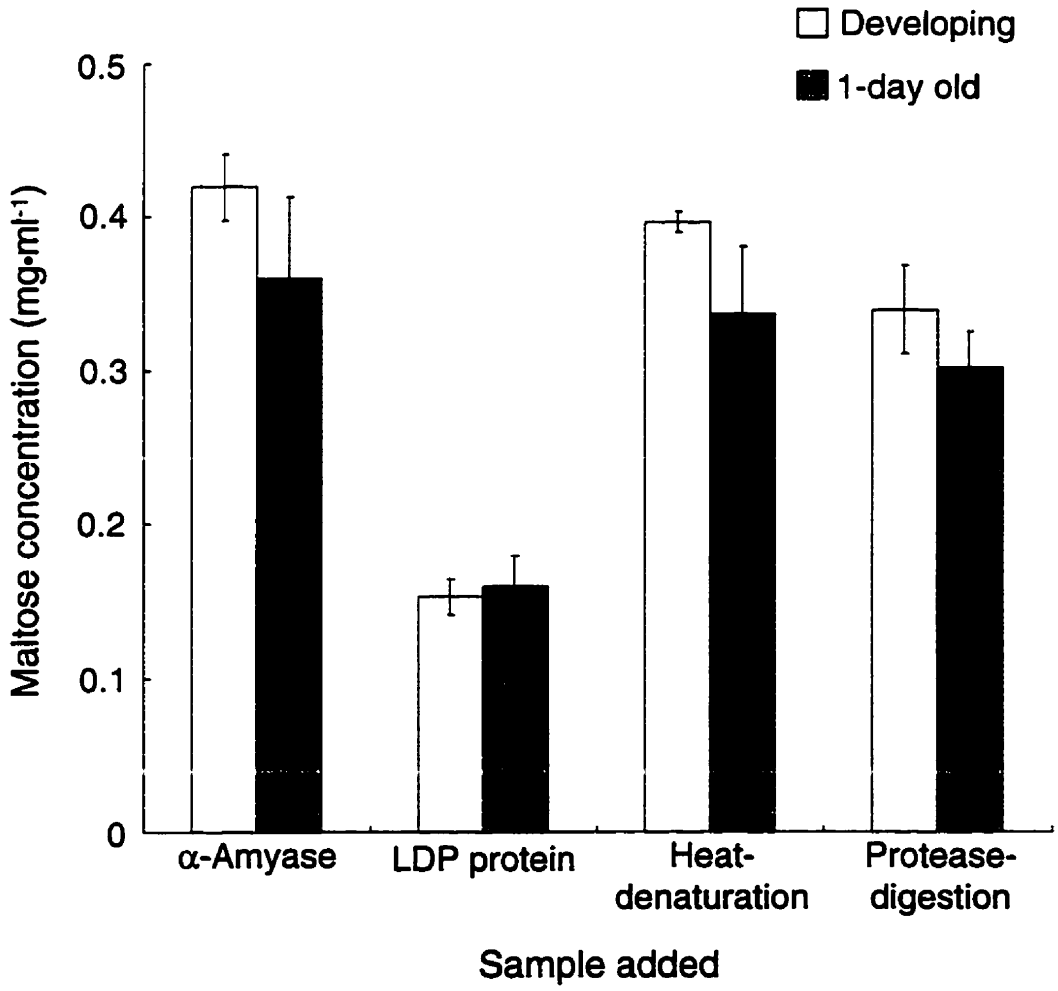
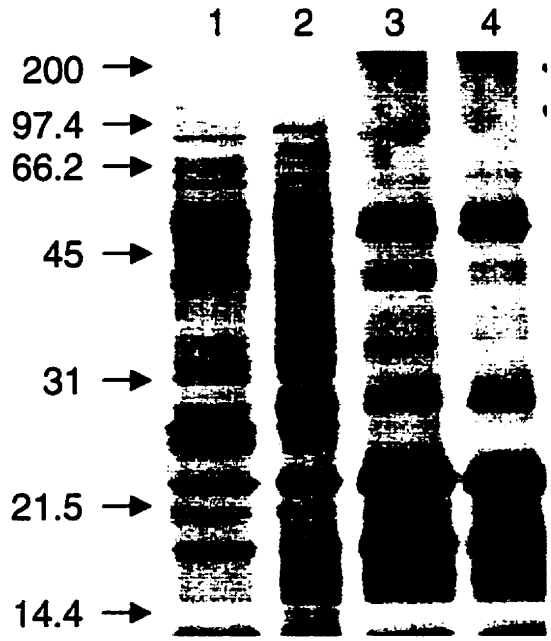

Developing
1-day old
0.5
0.4
0.3
0.2
0.1
0
Maltose concentration (mg·ml-1)
α-Amyase
LDP protein
Heat-denaturation
Protease-digestion
Sample added

Figure 34. Comparative SDS-PAGE of protein bodies and lipid particles isolated from 1 day-old bean seeds. One μg protein was loaded in each lane, separated on a 15% acrylamide gel and stained with silver. Lane 1, protein bodies isolated by the method described by Mäder and Chrispeels (1984); lane 2, protein bodies isolated by the method of Yatsu and Jacks (1968); lane 3, low-density particles; lane 4, intermediate-density particles. Approximate molecular weights in kilodaltons are indicated.

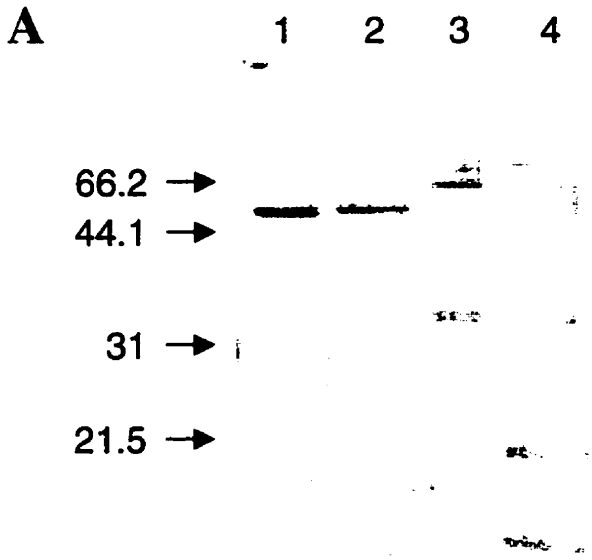

1
2
3
4
200
97.4
66.2
45
31
21.5
14.4

IDPs and LDPs probed with the 22 kDa canola oleosin antibody both featured a band corresponding to the ~ 52 kDa protein, which typically reacts with this antibody (Fig. 35 A, lanes 1 and 2). Corresponding Western blots of the two protein body preparations did not show the presence of this protein, suggesting that the ~ 52 kDa protein is not a contaminant from protein bodies (Fig. 35 A, lanes 3 and 4). On the other hand, when the blot was probed with the 19 kDa canola oleosin antibody, there was clear evidence in both lipid particles, and protein bodies, for a protein of approximately the same molecular weight which cross reacts with this antibody, supporting the contention that at least the 22.9 kDa α-amylase inhibitor is a contaminant from protein bodies.

3.1.5 Amino Acid Composition of the 17.7 kDa Polypeptide and ~ 48 kDa Protected Fragment of Low-Density Lipid Particles

The 17.7 kDa polypeptide from LDPs and the ~ 48 kDa protected fragment derived from the ~ 52 kDa protein of LDPs were analyzed for their amino acid compositions (Table 3). When examined for homology to proteins registered in the data banks using Propsearch (Hobahm *et al.* 1994), no significant similarities were detected. However, the ~ 48 kDa protected fragment contains a lower proportion of charged amino acids than is found in several oleosins from different seeds and in proteins on average (Fig. 36). The 17.7 kDa protein, however, proved to have a higher proportion of charged amino acids and a lower proportion of nonpolar amino acids than any of the oleosins examined, and in fact is quite similar in terms of its

Figure 35. Western blot analyses of protein bodies and lipid particles isolated from 1 day-old beans. Two µg protein were loaded in each lane and separated on 13% acrylamide gels. Lane 1, intermediate-density particles; lane 2, low-density particles; lane 3, protein bodies isolated by the method of Yatsu and Jacks (1968); lane 4, protein bodies isolated by the method of Mäder and Chrispeels (1984). **A.** Blot probed with 19 kDa canola oleosin antibody. **B.** Blot probed with 22 kDa canola oleosin antibody. After primary antibody application all blots were treated with secondary antibody and stained for alkaline phosphatase activity. Approximate molecular weights in kilodaltons are indicated.


A
1
2
3
4
66.2
44.1
31
21.5

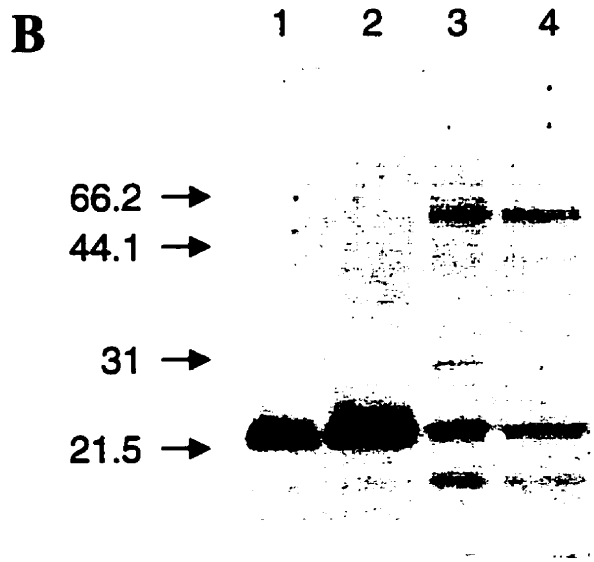
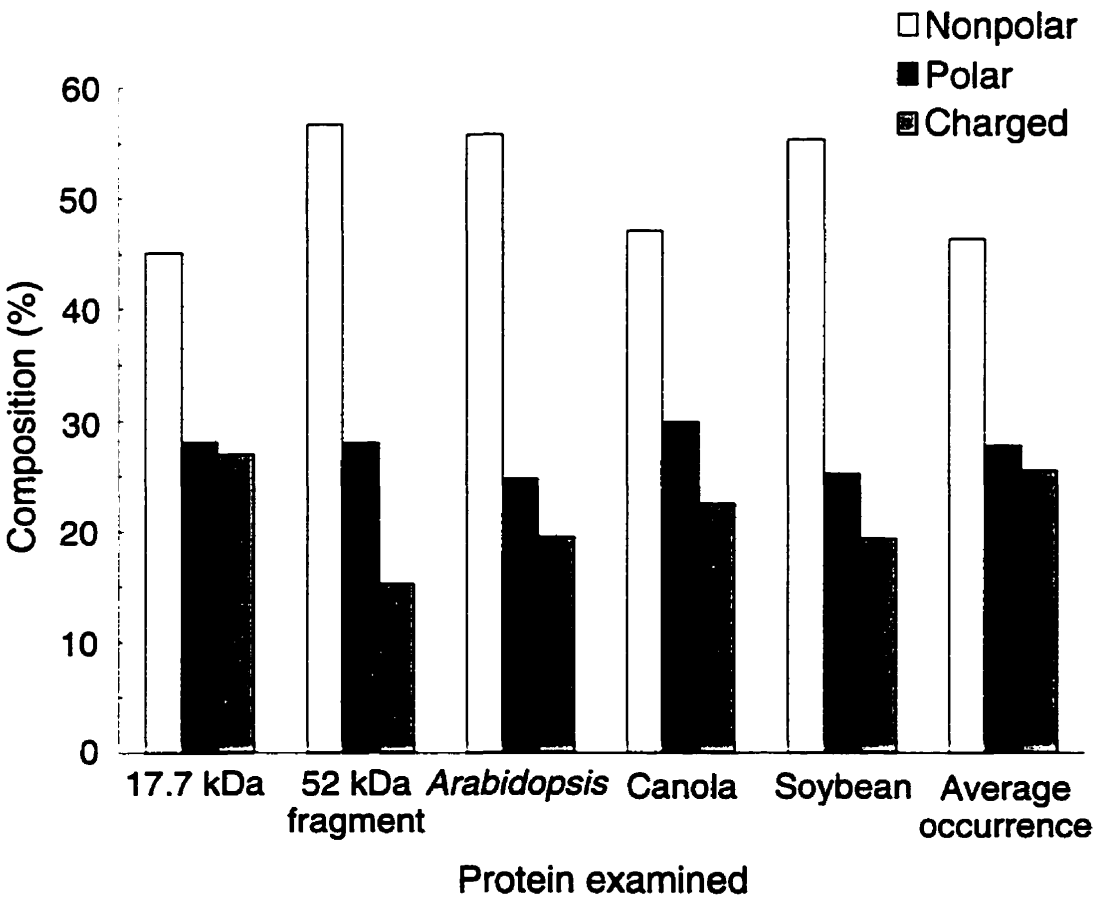

B
1
2
3
4
66.2
44.1
31
21.5

Table 3. Amino acid composition of the 17.7 kDa protein and the protected fragment of 52 kDa protein isolated from LDPs of 1-day old bean cotyledons. Amino acids are expressed as a percentage of total amino acids. Data bank analysis using Propsearch (Hobahm *et al.* 1994) identified no proteins of similar amino acid composition.

Amino acid	**Percent composition of each amino acid** %	
	17.7 kDa protein	52 kDa protein fragment
Arg	7.11	3.36
Glx	12.43	17.88
Ser	9.85	8.04
His	2.24	0
Gly	17.01	16.25
Thr	6.35	4.22
Ala	7.81	6.90
Pro	9.16	4.88
Tyr	0.2	2.72
Val	1.42	9.10
Met	10.52	1.57
Ile	0.42	5.09
Leu	6.56	9.45
Phe	0.14	3.43
Lys	0	0
Asx	9.64	6.04
Cys	0.15	1.06

Figure 36 Comparison of the proportion of nonpolar, polar and charged side groups on amino acids in the 17.7 kDa protein of low-density particles, the 52 kDa protein fragment of low-density particles, *Arabidopsis* oleosin (Kirk, V., Kolle, K. and Baumlein, H. 1995, unpublished), canola oleosin (Lee and Huang 1991), soybean oleosin (Rowly, D.L. and Herman, E.M. 1994, unpublished) and the average occurrence of these amino acids in proteins (Voet and Voet 1990). The sequences were obtained from Entrez.


Nonpolar
Polar
Charged
60
50
40
30
20
10
0
Composition (%)
17.7 kDa
52 kDa fragment
Arabidopsis
Canola
Soybean
Average occurrence
Protein examined

complement of nonpolar, polar and charged amino acids to the average for proteins in general (Fig. 36, Voet and Voet 1990)

3.1.6 Isoelectric Focussing and Two-Dimensional PAGE

In a further attempt to determine whether IDPs and LDPs contain oleosin, their protein compositions were examined by isoelectric focussing. This strategy was based on the fact that oleosins are reported to be basic proteins (Huang 1992). However, when canola oil bodies (oleosins pIs: 9.18 - 9.69) and pure cytochrome c (pI: 10.6) were used as positive controls, only cytochrome c ran as a basic protein during IEF-PAGE, and no basic proteins were detectable for IDPs and LDPs (data not shown). Two possible reasons for the poor resolution of basic proteins in the samples are: the presence of high relative quantities of lipids in the oil bodies and the lipid particles, and a pI high enough that the proteins would run off the bottom of the gel. To eliminate alterations in the mobility of the proteins by lipid in the particles, separation of the lipids from the proteins was undertaken. SDS-PAGE of untreated and lipid-extracted IDPs, LDPs and canola oil bodies revealed that the major proteins from the lipid particle and canola oil bodies were retained following lipid extraction (data not shown). These lipid-free protein preparations were also fractionated by IEF-PAGE containing 6 % acrylamide (Fig. 37). To avoid the possibility that highly basic proteins might run off the bottom of the gel, the lower portion of the gel was prepared with 12% acrylamide, and the gel was run for 120 minutes (Fig. 37 A) and 180 minutes (Fig. 37 B). Despite removing the lipid from the samples and the 12% acrylamide base, the only basic protein visible was cytochrome c (Fig. 37, lane 1).

Figure 37. Isoelectric focussing of oil bodies from canola, and lipid particles and the sucrose-containing layer from 1 day-old bean seeds. Proteins from each sample were lipid-extracted before the proteins were fractionated. The proteins are focussed on a 6% acrylamide gel with a base of 12% acrylamide (border shown by the arrow) and stained with Coomassie brilliant blue. Lane 1, cytochrome c; lane 2, canola oil bodies; lane 3, low-density particles; lane 4, intermediate-density particles; lane 5, the sucrose-containing layer. **A**. Gel is run for 120 minutes. **B**. Gel is run for 180 minutes. Positions of the electrodes above and below the gels are shown on the right, and the charges are indicated.


A
1
2
3
4
5
+
-
B
1
2
3
4
5
+
-

After 120 minutes the cytochrome c had not reached the 12% acrylamide portion of the gel, and no other proteins had run as far as the cytochrome c (Fig. 37 A). After 180 minutes, the cytochrome c had penetrated into the 12% acrylamide gel, whereas none of the proteins from the other samples had made much movement toward the negative electrode (Fig. 37 B). When the LDPs and IDPs were run in the second dimension, the ~ 52 kDa protein and the 17.7 kDa protein were visible as individual spots, but the 22.9 kDa band resolved as two proteins (Fig. 38). This was particularly clear for the 2-dimensional analysis of the IDPs (Fig. 38 B).

3.2 Lipid Analysis of High-, Intermediate- and Low-Density Lipid Particles

Developing seeds of *Phaseolus vulgaris* store both protein and triacylglycerol, and both are metabolized after germination. Indeed, when the seeds are germinated under conditions of etiolation, levels of protein and triacylglycerol in the cotyledons decrease by ~ 90% within 3 days after imbibition (Fig. 39), which precedes the first statistically significant decrease in the weight of the cotyledons (Fig. 5, $\alpha = 0.05$, Appendix 1, Table A.1.1) and also the first morphological changes apparent in the cotyledons. There is a slight lag in mobilization of protein when compared with the lipid stores at 2.5 days after germination.

Of particular interest is the finding that triacylglycerol is present in all three types of lipid particles, LDPs, IDPs and HDPs, in both developing and germinating seeds, but in different relative proportions (Figs. 40 A - C). These differences in relative triacylglycerol concentration may account for the different densities of LDPs, IDPs and HDPs (Figs. 2 and 3). Moreover, the lipid compositions of LDPs, IDPs and

Figure 38. Two dimensional SDS-PAGE of lipid particles from cotyledons of 1 day-old bean seeds. Individual lanes from the IEF gels were placed on 15% acrylamide gels for SDS-PAGE in the second dimension and stained with Coomassie brilliant blue. **A.** Low-density particles. **B.** Intermediate-density particles. Approximate molecular weights in kilodaltons are indicated.

A



B



Figure 39. Levels of protein and triacylglycerol (TAG) in cotyledons from developing and germinating bean seeds. Ages of cotyledons are expressed in days after planting except for developing seeds, which were harvested just as the seed pods began to dry. Standard errors of means are indicated for $n \geq 3$.

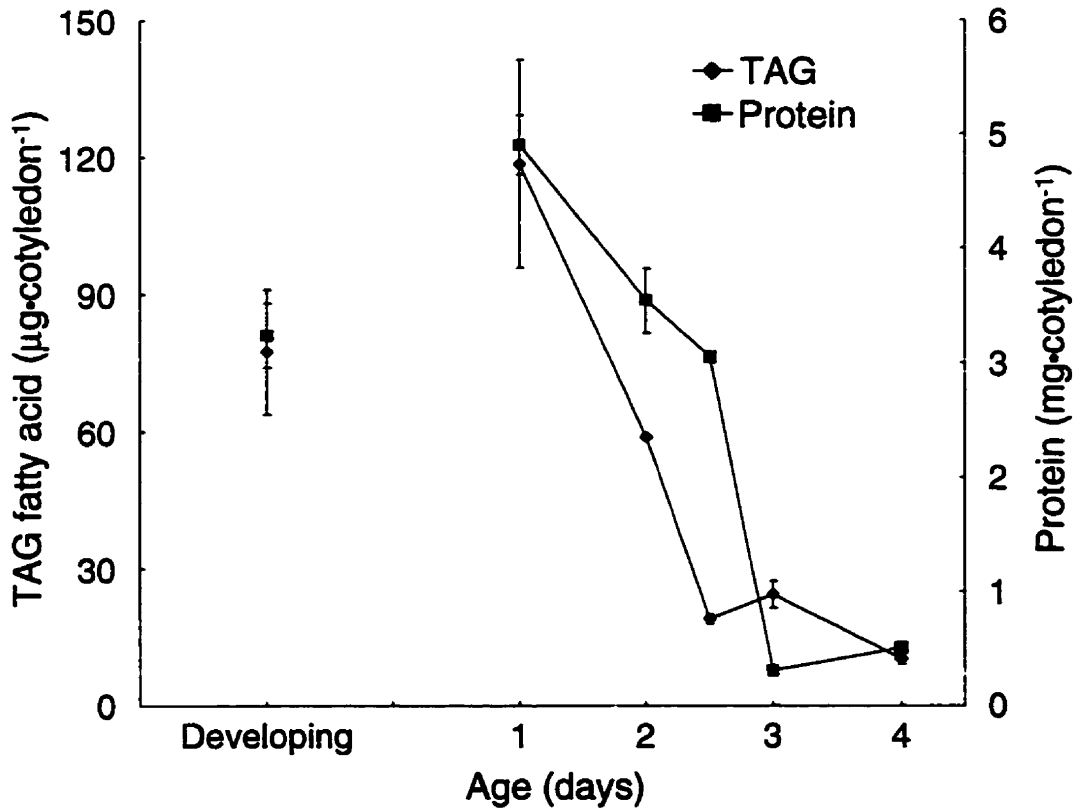
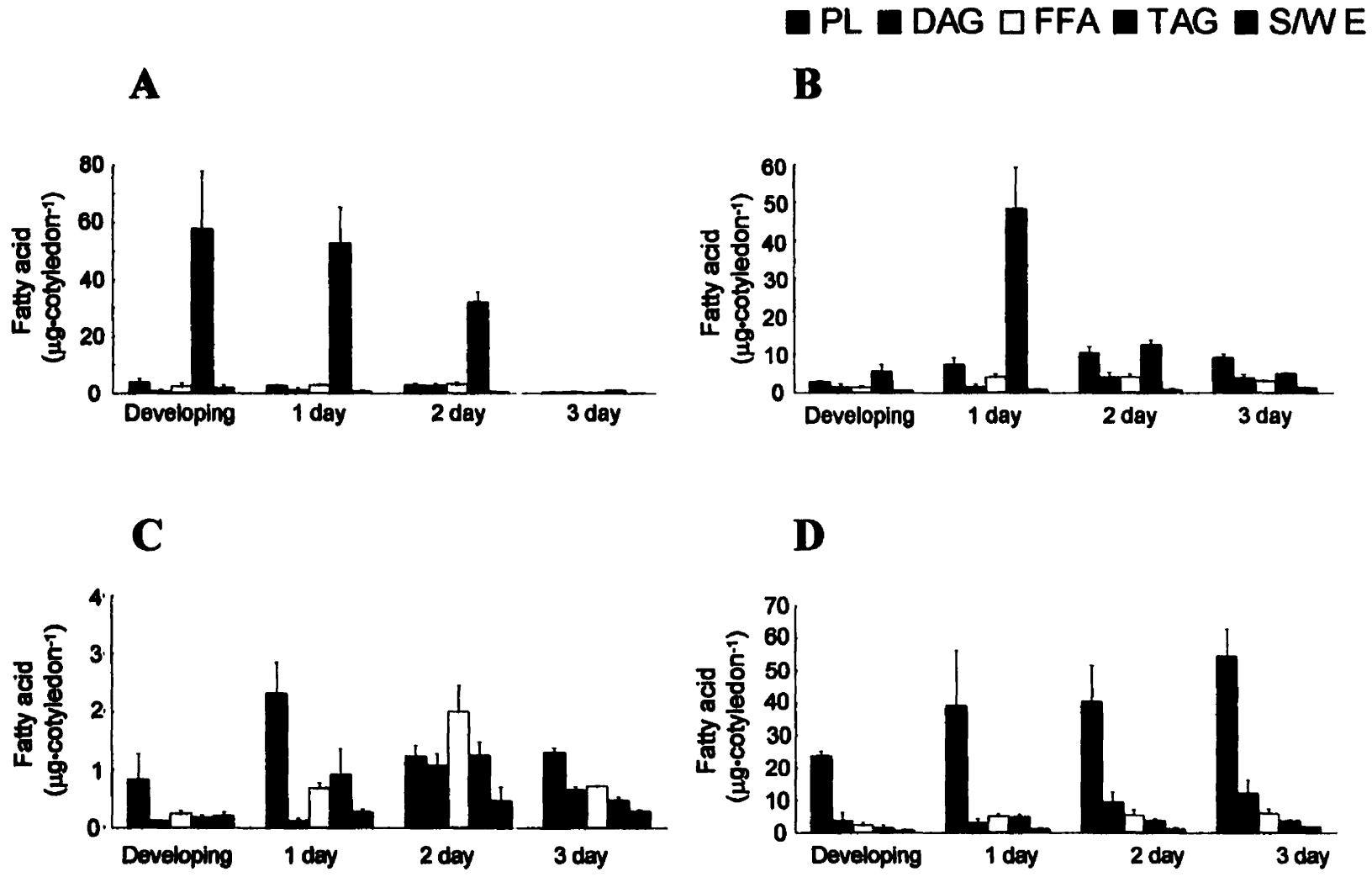

150
120
90
60
30
0
TAG fatty acid (µg·cotyledon⁻¹)
6
5
4
3
2
1
0
Protein (mg·cotyledon⁻¹)
TAG
Protein
Developing
1
2
3
4
Age (days)

Figure 40. Changes in the lipid composition of lipid particles and microsomal membranes isolated from cotyledons of developing and germinating bean seeds. Phospholipid (PL), diacylglycerol (DAG), free fatty acids (FFA), triacylglycerol, (TAG) and steryl and wax esters (S/WE) have been quantified in terms of fatty acid levels. **A**. Low-density particles. **B**. Intermediate-density particles. **C**. High-density particles. **D**. Microsomal membranes. Ages of cotyledons are expressed in days after planting except for developing seeds, which were harvested just as the seed pods began to dry. Each graph is shown on a different scale. Standard errors of means are indicated for n = 3 - 4.


PL DAG FFA TAG S/W E
A
B
C
D
Fatty acid (µg-cotyledon-1)
Developing
1 day
2 day
3 day

HDPs are not only distinguishable from each other, but are also clearly distinct from the lipid composition of microsomal membranes (Figs. 40 A - D).

The most abundant lipid in the LDPs is triacylglycerol, which accounts for 80 to 90% of the total fatty acid content of the particle in both developing and young germinating seeds (Fig. 40 A). The LDPs also contain some phospholipid (equivalent to 5 to 8% of the total fatty acid), and smaller amounts of diacylglycerol, free fatty acids and steryl and wax esters. The relative proportions of these lipids in the particles remain essentially constant during seed development and within 2 days after germination (Fig. 40 A).

IDPs resemble LDPs, particularly at day 1, in that they are enriched in triacylglycerol (Fig. 40 B), but they are also distinguishable from LDPs based on their lipid composition. Specifically, they contain proportionately more phospholipid, diacylglycerol and free fatty acids than LDPs (Figs. 40 A and B). The lower proportion of triacylglycerol relative to other lipids and protein (Fig. 12 A) in the IDPs presumably accounts for their higher density, compared with the LDPs. In addition, the lipid composition is much more variable during seed development and germination for IDPs than for LDPs (Figs. 40 A and B). For example, although triacylglycerol and phospholipid are the dominant lipids of IDPs at all stages of development, the ratios are quite different. However, the relatively large amount of phospholipid found in the IDPs may be due to contamination from the SCL directly below the floating IDPs (Fig. 3), which contains a considerable amount of phospholipid (data not shown).

HDPs resemble the other particles in that they also contain phospholipid, diacylglycerol, free fatty acids, triacylglycerol, and steryl and wax esters, but they are

chemically distinguishable from LDPs and IDPs (Fig. 40 C). For example, triacylglycerol accounts for only 10 - 20 % of the total lipid in HDPs compared with 80 - 90% of the total lipid for LDPs (Figs. 40 A and C), and this presumably accounts, at least in part, for their differences in density. In addition, HDPs have proportionately higher amounts of the other lipids examined than LDPs and IDPs (Fig. 40 C). It is also apparent that the lipid composition of HDPs changes as the seeds mature and germinate (Fig. 40 C).

The different classes of lipid particles are not equally abundant in maturing and germinating seeds. In maturing seeds harvested just as the pods begin to dry, LDPs expressed in terms of fatty acid equivalents are ~ 3.5-fold more abundant than IDPs, and ~ 35-fold more abundant than HDPs (Fig. 41). This abundance of LDPs is maintained during the early stages of germination before quickly declining by 95% between 2 and 3 days after planting (Fig. 41). This decrease in LDP fatty acid equivalents is not simply due to a decrease in particle size inasmuch as the decrease in LDP diameter between days 2 and 3 of germination is less than 2-fold (Fig. 42). In contrast, IDP levels increase by ~ 3.5-fold between the stage of seed development sampled (i.e. just as the pods begin to dry) and 1 day after planting, reaching an abundance comparable to that for LDPs (Fig. 41). Thereafter, the level of IDPs decreased as the cotyledons aged but more slowly than for LDPs, such that between 2 and 4 days after planting IDPs were 2- to 8-fold more abundant than LDPs (Fig. 41). There was little change in IDP diameter after germination, indicating that these changes in IDP fatty acid equivalents are not attributable to changes in particle size

Figure 41. Changes in the abundance of lipid particles in cotyledon tissue of developing and germinating bean seeds. Lipids from low-density particles (LDP), intermediate-density particles (IDP), high density particles (HDP) and microsomal membranes (MM) have been quantified in terms of total fatty acid levels. Ages of cotyledons are expressed in days after planting except for developing seeds, which were harvested just as the seed pods began to dry. Standard errors of means are indicated for n = 3 - 4.

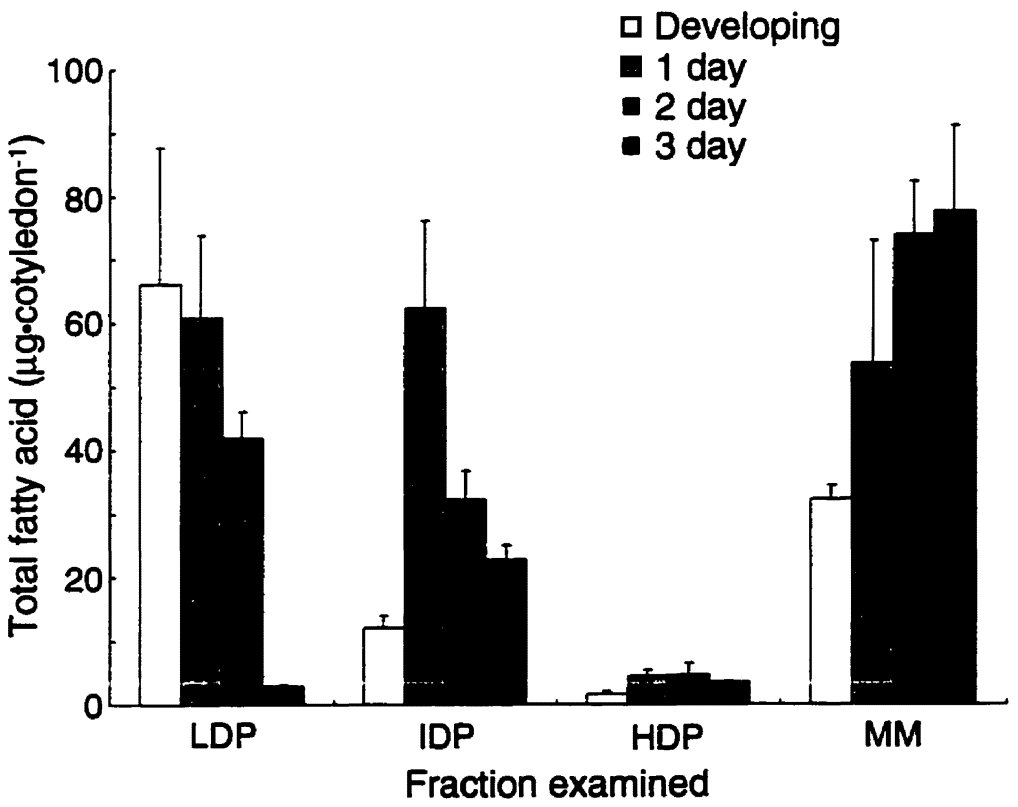
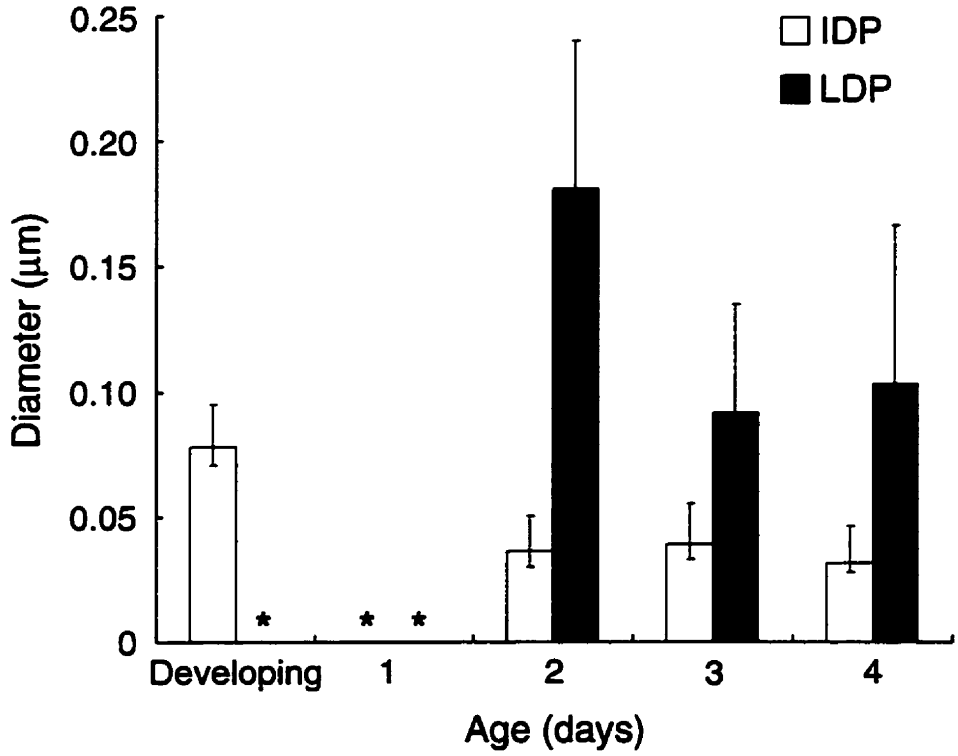

Developing
1 day
2 day
3 day
100
80
60
40
20
0
Total fatty acid (µg•cotyledon-1)
LDP
IDP
HDP
MM
Fraction examined

Figure 42. Light scattering measurements of lipid particles isolated from cotyledon tissue of developing and germinating bean seeds. Clear bars, intermediate-density particles (IDPs); gray bars, low-density particles (LDPs). Ages of cotyledons are expressed in days after planting except for developing seeds, which are harvested just as the seed pods begin to dry. Each bar represents the mode for the size distribution. Stars indicate that numeric data were not obtainable for these ages due to polydispersity of the samples. Interquartile ranges for the distributions are indicated.

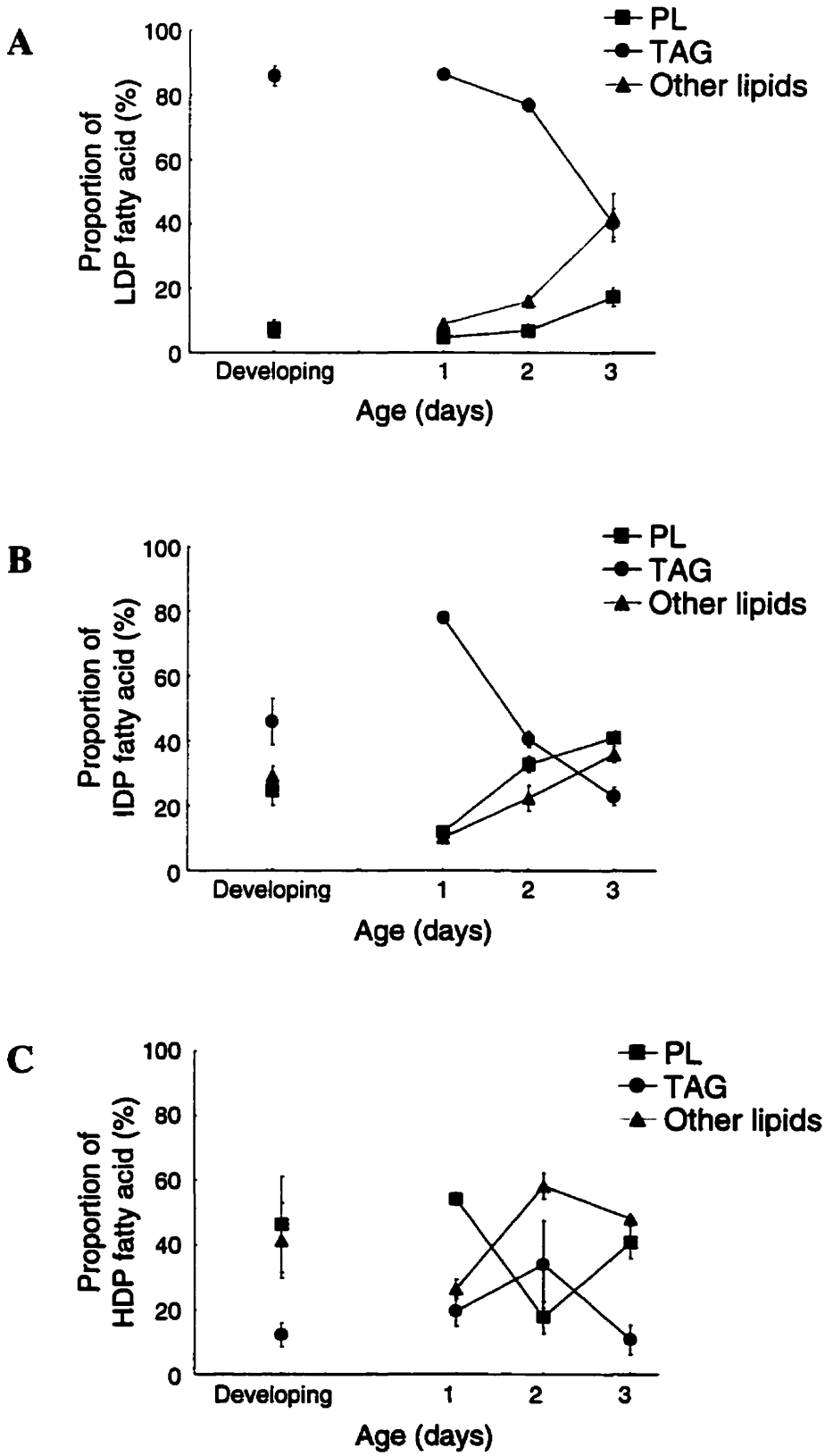

0.25
0.20
0.15
0.10
0.05
0
Diameter (µm)
IDP
LDP
*
* *
Developing
1
2
3
4
Age (days)

(Fig. 42). IDPs are also much more abundant (5- to 10-fold) than HDPs in germinating seeds (Fig. 41).

The sizes of LDPs and IDPs from cotyledons of maturing and germinating seeds were examined by laser light scattering. LDPs had size distributions with modes ranging from about 91 to 181 nm in diameter after germination, whereas the IDP size distributions had modes ranging from about 32 to 78 nm in diameter (Fig. 42), with the largest of these particles evident in the developing seeds. This difference in size is consistent with the higher proportion of triacylglycerol in the LDPs. The possibility that these fractions are made up of vesicles is unlikely given the higher density of phospholipids, which would pellet after the protracted centrifugation used for their isolation (Figs. 2 and 3), as well as the presence of a high proportion of other lipids which would not support a normal bilayer structure (Figs. 40 A and B).

The relative proportions of triacylglycerol, phospholipid and the other lipids (diacylglycerol, free fatty acids and steryl and wax esters) change in LDPs, IDPs and HDPs with development of the cotyledons. LDPs undergo only small changes in their relative triacylglycerol, phospholipid and the other lipids content until 3 days after germination when the proportions of phospholipid and the other lipids increase and triacylglycerol decreases significantly (Fig. 43 A). This suggests that the triacylglycerol in these particles is being preferentially metabolized between 2 and 3 days after planting. This preferential metabolism of triacylglycerol also occurs in IDPs (Fig. 43 B). There are relative decreases in phospholipid and the other lipids, and increases in triacylglycerol when comparing IDPs of developing cotyledons to those 1 day after planting (Fig. 43 B). Thereafter, there is a relative decrease in triacylglycerol

Figure 43. Changes in the levels of triacylglycerol (TAG), phospholipid (PL) and the other lipids (diacylglycerol, free fatty acids and steryl and wax esters) of lipid particles from developing and germinating bean seeds. Triacylglycerol fatty acid, phospholipid fatty acid and other lipid fatty acids are expressed as a percentage of total fatty acid. **A**. Low-density particles. **B**. Intermediate-density particles. **C**. High-density particles. Ages of cotyledons are expressed in days after planting except for developing seeds, which were harvested just as the seed pods began to dry. Standard errors of means are indicated for n = 3 - 4.

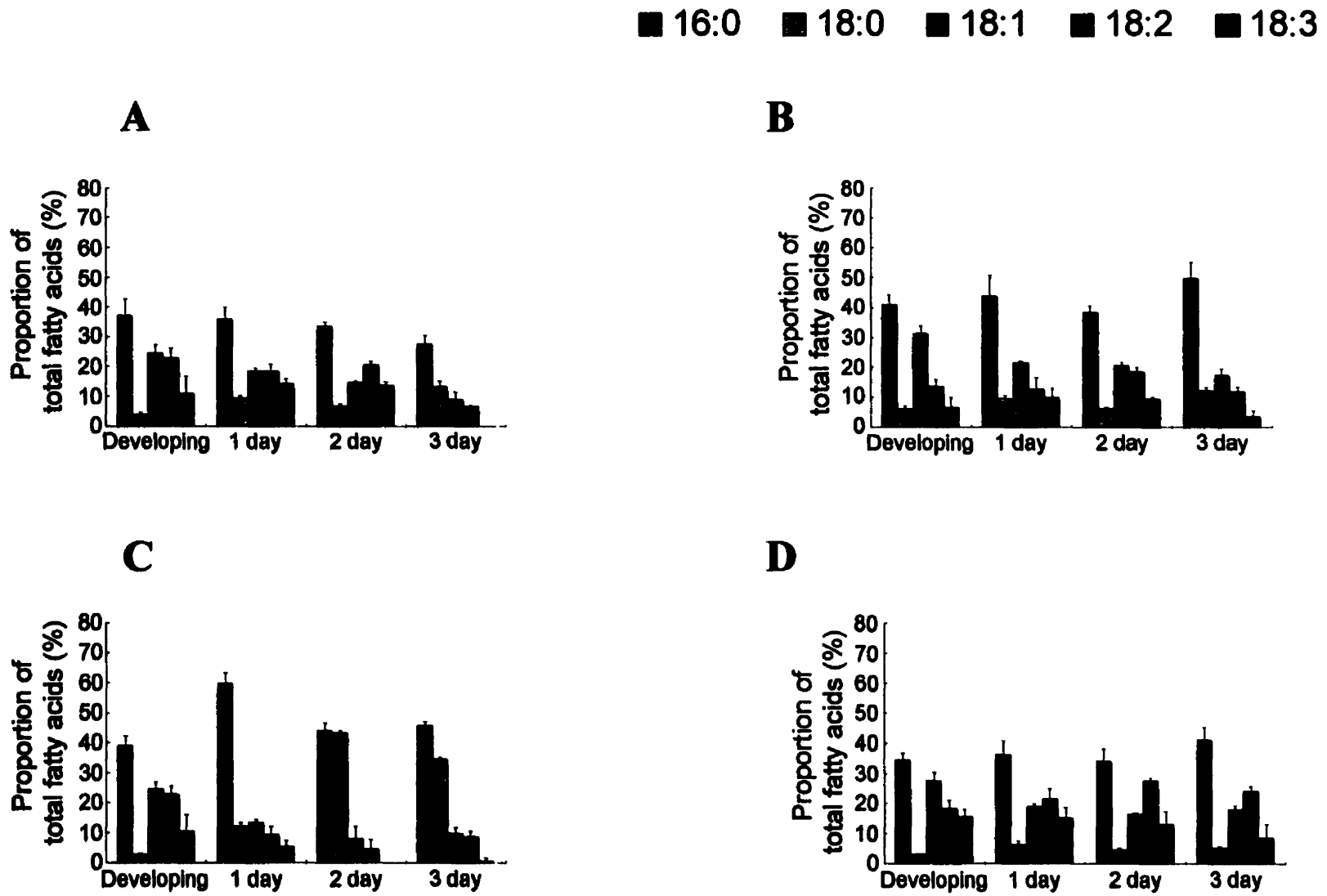

A
PL
TAG
Other lipids
Proportion of LDP fatty acid (%)
100
80
60
40
20
0
Developing
1
2
3
Age (days)
B
PL
TAG
Other lipids
Proportion of IDP fatty acid (%)
100
80
60
40
20
0
Developing
1
2
3
Age (days)
C
PL
TAG
Other lipids
Proportion of HDP fatty acid (%)
100
80
60
40
20
0
Developing
1
2
3
Age (days)

and increase in the phospholipid and the other lipids (Fig. 43 B). In contrast, HDPs show a larger or nearly equal proportion of phospholipid at every stage when compared to levels of triacylglycerol (Fig. 43 C). In LDPs and IDPs the changes in triacylglycerol and other lipids are approximately inversely proportioned (Figs. 43 A and B). For HDPs, on the other hand, this is not the case (Fig. 43 C).

LDPs and IDPs from developing and germinating seeds were further characterized in an effort to determine whether IDPs are derived from LDPs. Specifically, the fatty acid compositions of phospholipid and triacylglycerol, the major lipids in both LDPs and IDPs, were compared for both particles and with the fatty acid composition of the same lipids in corresponding HDPs and microsomal membranes. Palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and linolenate (18:3) were the major acyl constituents of the lipids found in all fractions examined.

The fatty acid composition of phospholipid is similar for LDPs, IDPs and microsomal membranes from cotyledon tissue of both developing and germinating seeds (Figs. 44 A, B and C), and different from the fatty acid composition for phospholipid in corresponding HDPs (Fig. 44 C). In particular, palmitic acid is the dominant phospholipid fatty acid in LDPs, IDPs and microsomal membranes, as well as in HDPs. The other fatty acids, especially stearic acid, are present in more variable proportions relative to palmitic acid in HDPs by comparison with LDPs, IDPs and microsomal membranes (Fig. 44). In addition, the phospholipid fatty acid composition of LDPs, HDPs and microsomal membranes remains fairly constant during the stages of development and germination examined (Figs. 44 A, B and C), whereas that for

Figure 44. Changes in the phospholipid fatty acid composition of lipid particles and microsomal membranes isolated from cotyledon tissue of developing and germinating bean seeds. Each fatty acid is expressed as a percentage of total phospholipid fatty acid. **A.** Low-density particles. **B.** Intermediate-density particles. **C.** High-density particles. **D.** Microsomal membranes. Ages of cotyledons are expressed in days after planting except for developing seeds, which were harvested just as the seed pods began to dry. Standard errors of means are indicated for n = 3 - 4.


16:0
18:0
18:1
18:2
18:3
A
B
C
D
Proportion of total fatty acids (%)
0
10
20
30
40
50
60
70
80
Developing
1 day
2 day
3 day

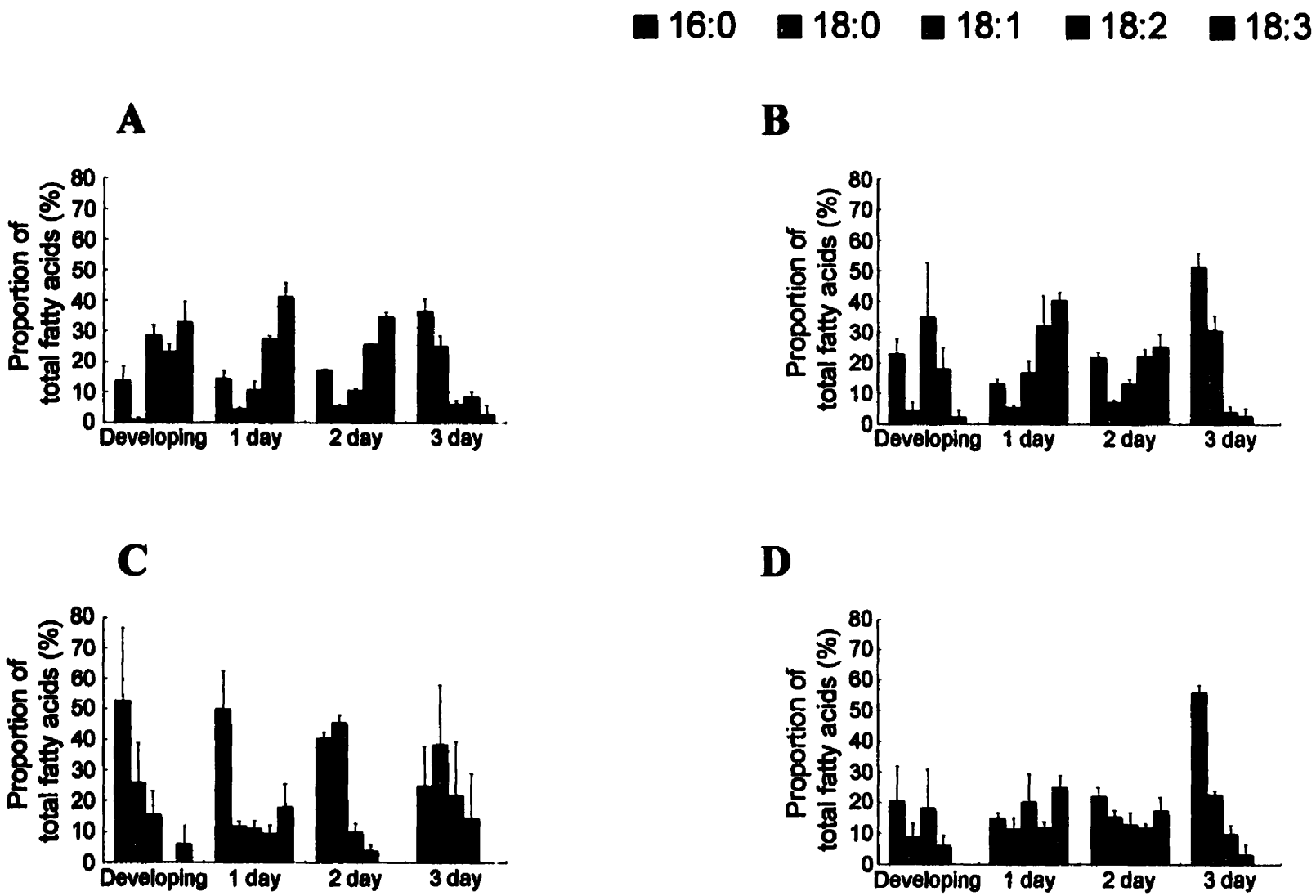
HDPs shows more variation over the same period (Fig. 44 C). The presence of phospholipid in the particles suggests that they originate from membranes.

The triacylglycerol fatty acid profiles for each fraction isolated are distinguishable (Fig. 45). This is particularly true of the LDPs and IDPs when compared to the HDPs and microsomal membranes, as there are relatively higher proportions of linoleic and linolenic acids in LDPs and IDPs at 1 and 2 days after planting (Fig. 45). In fact, the triacylglycerol of LDPs and IDPs is significantly more unsaturated than that of either HDPs or microsomal membranes. For example, the unsaturated to saturated fatty acid ratios for triacylglycerol in LDPs and IDPs from seeds 1 day after planting are 4.50 and 4.62, respectively, whereas corresponding values for triacylglycerol in HDPs and microsomal membranes are 0.76 and 2.48, respectively (Fig. 45). Thus, the triacylglycerol content of HDPs bears greater similarity to that of microsomal membranes than to that of either LDPs or IDPs. The fatty acid composition of triacylglycerol in LDPs and IDPs also changes during germination. In particular, there is a disproportionate depletion of linoleic and linolenic acids as germination progresses (Figs. 45 A and B).

3.3 Effects of Homogenization on the Protein and Lipid Compositions of Intermediate- and Low-Density Lipid Particles

The possibility of altering the structure or composition of IDPs and LDPs during the process of their isolation was examined by varying the method of tissue homogenization used in the procedure, and examining any changes in the protein and lipid compositions of the particles. Two methods were used: homogenization in pure

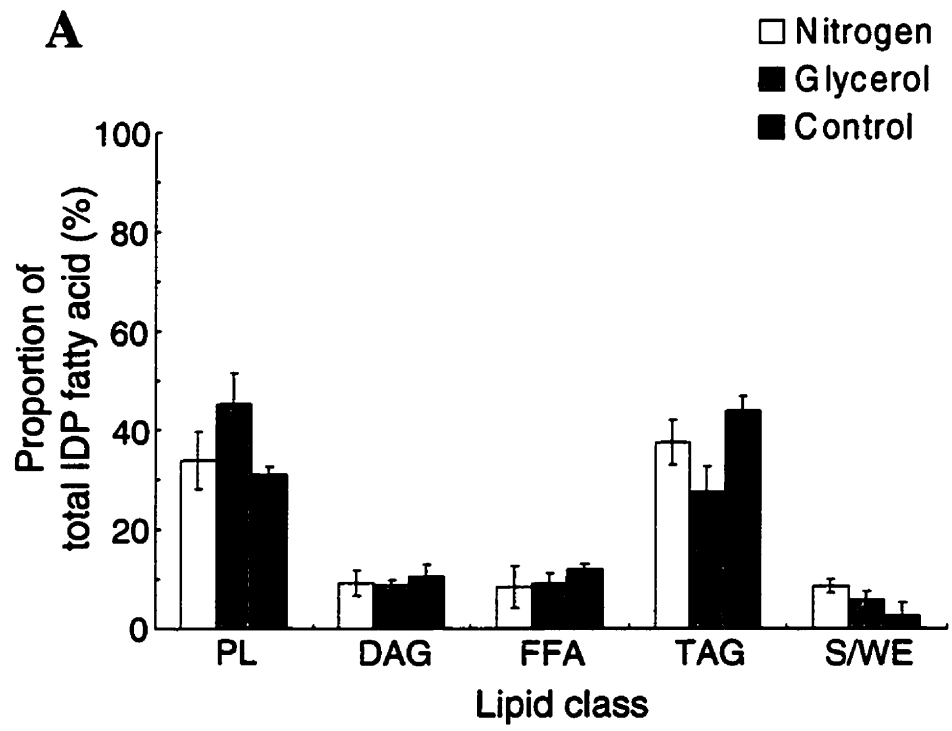
Figure 45. Changes in the triacylglycerol fatty acid composition of lipid particles and microsomal membranes isolated from cotyledon tissue of developing and germinating bean seeds. Each fatty acid is expressed as a percentage of total triacylglycerol fatty acid. **A**. Low-density particles. **B**. Intermediate-density particles. **C**. High-density particles. **D**. Microsomal membranes. Ages of cotyledons are expressed in days after planting except for developing seeds, which were harvested just as the seed pods began to dry. Standard errors of means are indicated for n = 3 - 4.


16:0
18:0
18:1
18:2
18:3
A
B
C
D
Proportion of total fatty acids (%)
0
10
20
30
40
50
60
70
80
Developing
1 day
2 day
3 day

glycerol, and freezing in liquid nitrogen followed by grinding to a powder. In both of these methods, the tissue was homogenized under nonaqueous conditions in an attempt to minimize hydrophobic associations in the homogenate. The effects of these different homogenization methods on the lipid composition of LDPs and IDPs were examined by TLC followed by GLC. Although the trends in the lipid classes appear to be similar for each of the different homogenization methods, there are statistically significant differences between many of the lipid classes (Fig 46, $\alpha = 0.05$, Appendix 1, Table A.1.17). The IDPs homogenized in glycerol have a higher proportion of phospholipid and lower proportion of triacylglycerol compared with the buffer control (Fig. 46 A), and the IDPs homogenized normally in buffer have a lower proportion of steryl and wax esters than those obtained by either of the alternate homogenization methods (Fig. 46 A). The LDPs homogenized in liquid nitrogen have a higher proportion of phospholipid than the LDPs isolated from homogenates obtained by the other two methods (Fig. 46 B). In addition, the LDPs homogenized in glycerol contain a lower proportion of free fatty acids and steryl and wax esters than either the LDPs isolated by homogenization in liquid nitrogen, or in normal buffer (Fig. 46 B).

There are also statistically significant differences in the relative proportions of IDP and LDP lipid (Fig. 47, $\alpha = 0.05$, Appendix 1, Table A.1.18). Homogenization in glycerol or in liquid nitrogen resulted in a strikingly reduced proportion of IDP lipid and a corresponding increase in the proportion of LDP lipid (Fig. 47). Furthermore, the differences in the proportions of IDP and LDP lipid for particles isolated from glycerol, and liquid nitrogen homogenates, were also statistically significant (Fig. 47, $\alpha = 0.05$, Appendix 1, Table A.1.18).

Figure 46. Changes in the lipid composition of lipid particles isolated from cotyledons of 2 day-old bean seeds isolated using different homogenization techniques. Phospholipid (PL), diacylglycerol (DAG), free fatty acids (FFA), triacylglycerol (TAG) and steryl and wax esters (S/W E) have been quantified in terms of fatty acid levels. Each lipid is expressed as a percentage of the total fatty acid content. Clear bars, lipid composition from cotyledons homogenized by grinding after freezing in liquid nitrogen; gray bars, cotyledons homogenized in glycerol; hatched bars, cotyledons homogenized by the standard method in homogenization buffer. **A.** Intermediate-density lipid particles. **B.** Low-density lipid particles. Standard errors of the means are indicated for n = 3 - 4.


A
Nitrogen
Glycerol
Control
Proportion of total IDP fatty acid (%)
100
80
60
40
20
0
PL
DAG
FFA
TAG
S/WE
Lipid class

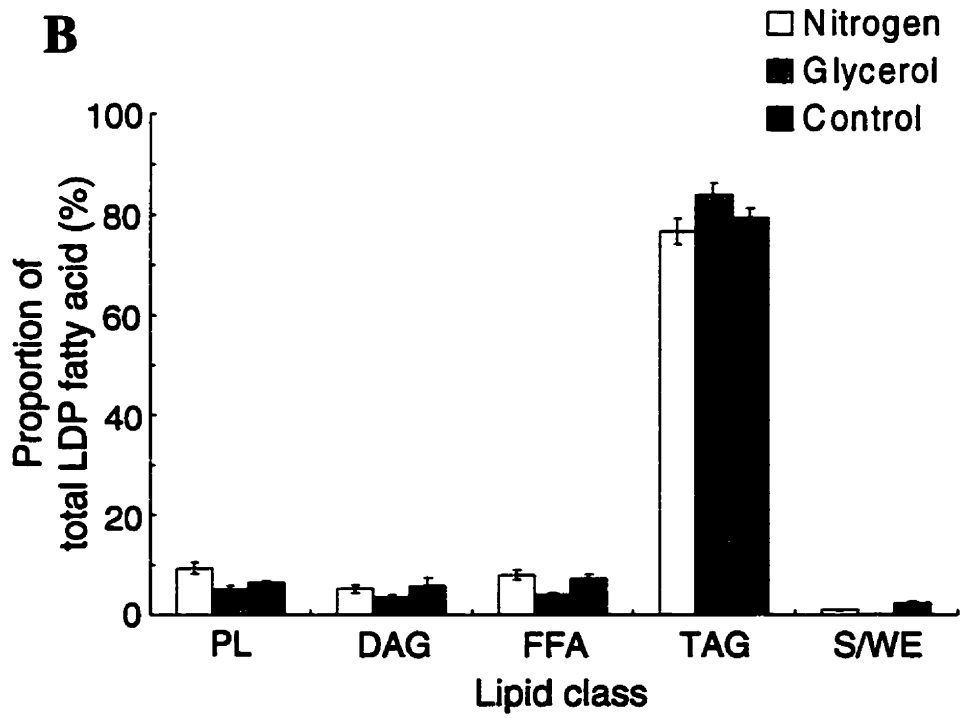
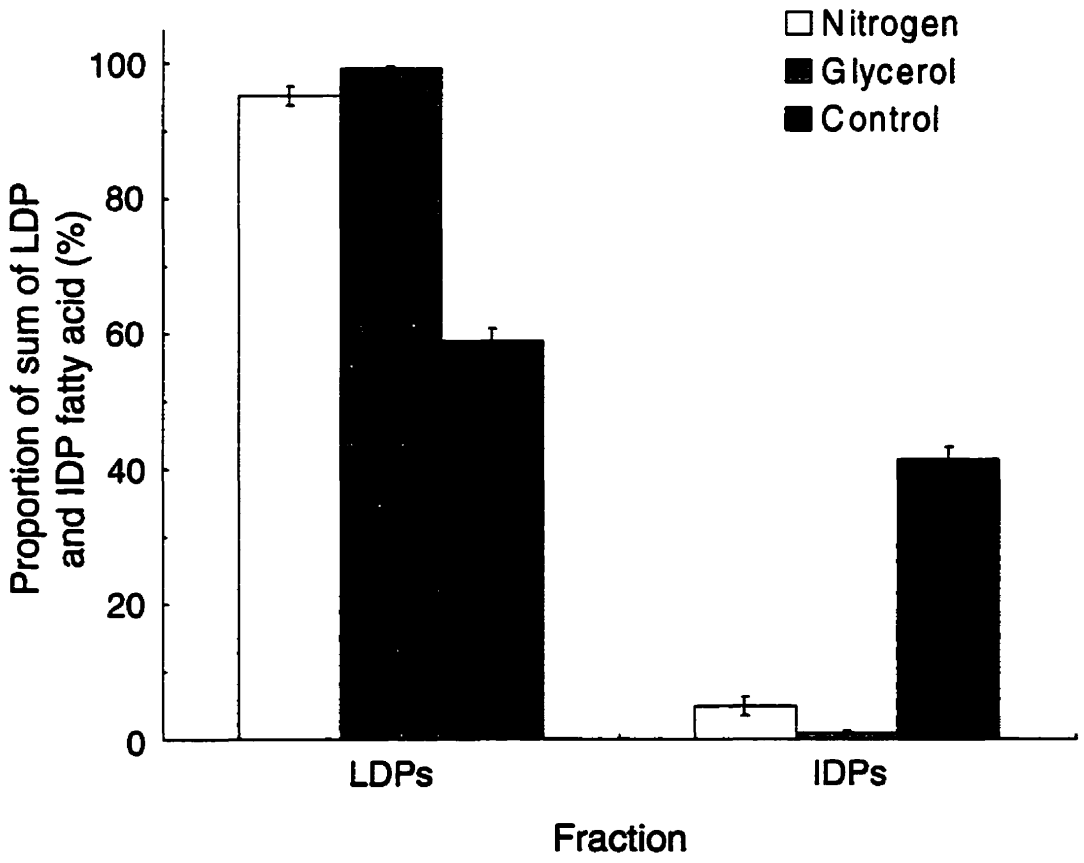

B
Nitrogen
Glycerol
Control
Proportion of total LDP fatty acid (%)
100
80
60
40
20
0
PL
DAG
FFA
TAG
S/WE
Lipid class

Figure 47. Changes in the proportions of low-density particle lipid and intermediate-density particle lipid as a function of homogenization technique. The lipid for each particle is represented as a percentage of the sum of lipid found in both particles. Clear bars, proportion of lipid in low-density particles (LDPs) and intermediate-density particles (IDPs) isolated from cotyledons homogenized by grinding after freezing in liquid nitrogen; gray bars, proportion of lipid in low-density and intermediate-density particles isolated from cotyledons homogenized in glycerol; solid dark bars, proportion of lipid in low-density and intermediate-density particles isolated from cotyledons homogenized by the standard method in homogenization buffer. Standard errors of the means are indicated for n = 3 - 4.

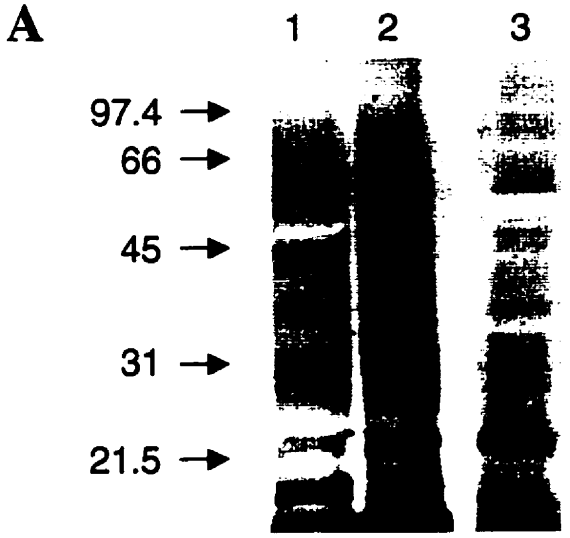

Nitrogen
Glycerol
Control
100
80
60
40
20
0
Proportion of sum of LDP and IDP fatty acid (%)
LDPs
IDPs
Fraction

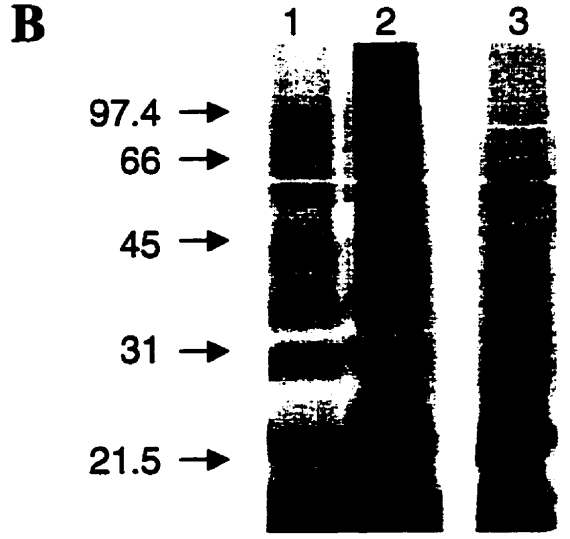
The effects of these different homogenization methods on the protein composition of LDPs and IDPs were also examined by SDS-PAGE (Fig. 48). Although there were minor differences in the protein profiles of the IDPs and the LDPs isolated from glycerol and liquid nitrogen homogenates by comparison with those isolated from tissue disrupted in normal homogenization buffer, the major proteins are present in every case (Figs. 48 A and B). Interestingly, irrespective of the homogenization method, the relative abundance of the 22.9 kDa α-amylase inhibitor in LDPs was unaffected (Fig. 48 B). This result is surprising as the α-amylase inhibitor is almost certainly a contaminant and suggests that this protein has a very high affinity for lipid particles. Control IDPs and LDPs (Figs. 48 A, lane 3 and 48 B, lane 3) were isolated from homogenates obtained by the standard method of tissue disruption in buffer with the exception that protease inhibitors were not added during homogenization in order to make the conditions more consistent with the other two methods of homogenization. Both IDPs and LDPs show a very faint band at 17.7 kDa with a more intense band directly below (Figs. 48 A, lane 3 and 48 B, lane 3) whereas in the presence of protease inhibitors the 17.7 kDa band is always the most dominant (Figs. 13, 14, 25, 26 and 33). This likely reflects degradation by endogenous proteases.

4 PCR Amplification of the Oleosin Nucleotide Sequence

There is considerable variation in the nucleic acid sequences of seed oleosins from species to species (Murphy 1990, Murphy 1993, Lee *et al.* 1994). The portions of these sequences encoding the central hydrophobic domain of the protein are, however, relatively homologous from species to species. This is particularly true of

Figure 48. SDS-PAGE of lipid particles isolated from cotyledons of 2 day-old bean seeds using different homogenization techniques. Each lane is loaded with 1 μg protein, separated on 12% acrylamide gels and stained with silver. Lane 1, cotyledons homogenized by grinding after freezing in liquid nitrogen; lane 2, cotyledons homogenized in glycerol; lane 3, cotyledons homogenized by the standard method in homogenization buffer. **A**. Intermediate-density particles. **B**. Low-density particles. Approximate molecular weights in kilodaltons are indicated.

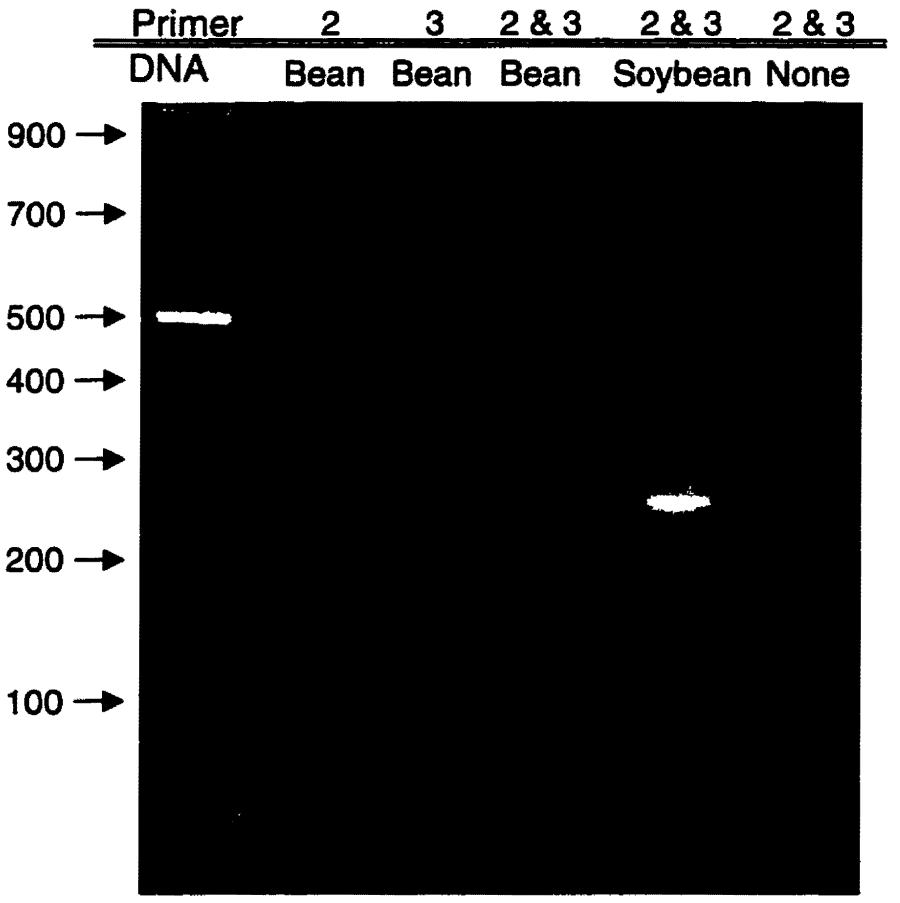

A
1
2
3
97.4
66
45
31
21.5


B
1
2
3
97.4
66
45
31
21.5

the mid region of this central domain which contains a 12-residue segment thought to form a proline-mediated loop structure (Murphy 1990, Murphy 1993, Lee *et al.* 1994). A primer was designed from this region of soybean oleosin sequence (Fig. 4, primer 2). A second primer (Fig. 4, primer 3) was designed corresponding to an amino acid sequence of the protein directly outside the hydrophobic central domain. Primer 2 is at least 94% homologous to sequences from maize, barley, *Bromus secalinus*, carrot, cotton and rice. Primer 3 is at least 80% homologous to sequences from cotton, carrot and sunflower. These primers were used to amplify isolated genomic DNA from *Phaseolus vulgaris* by PCR using soybean genomic DNA as a positive control. Genomic DNA was used instead of mRNA or cDNA as only canola oleosins have been found to contain introns in their sequences (Murphy 1993).

The PCR reaction products were fractionated on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light. The fragment amplified from soybean DNA appeared as a 242 nucleotide fragment as anticipated from the sequence information for soybean oleosin (Fig. 49 and Fig. 4). As well, there was a fragment of approximately 200 nucleotides in size that was amplified from the wax bean DNA (Fig. 49). No products were formed in the absence of DNA or in the presence of only one of the primers (Fig. 49). In addition, there was considerable amplification of the primers in reactions with and without the DNA template. In fact, some experiments showed considerably more amplification of the primers than of the DNA template (data not shown). These inconsistent results were thought to be due, in part, to the ability of the primers to bind each other. Moreover, from an examination of the

Figure 49. Agarose gel fractionation of PCR-amplified products from genomic DNA of wax bean and soybean. Primers used in the PCR reaction are indicated on the top line and type of DNA is indicated on the second line. Lane 1 contains size standards and the sizes are indicated in base pairs.


Primer
2
3
2 & 3
2 & 3
2 & 3
DNA
Bean
Bean
Bean
Soybean
None
900
700
500
400
300
200
100

sequence of primer 2 it is also apparent that this primer has a strong propensity to bind to itself, forming dimers (Fig 50 A) or a hairpin structure (50 B).

All attempts at sequencing the 200 bp fragment amplified from the wax bean DNA were unsuccessful. Introduction of restriction enzyme cut sites into one or both of the primers in order to subclone the amplified fragment into a suitable vector for sequencing resulted in complete elimination of the 200 bp PCR fragment generated from the wax bean DNA. A third primer, (Fig. 4, primer 1), was designed to replace primer 2 and circumvent the problem of self-association of primer 2. This sequence was at least 66% homologous to maize, *Arabidopsis* and rice. Once again, there was no consistent amplification of the wax bean DNA (data not shown).

5 Developing Cotyledon cDNA Library Screening for the Oleosin Sequence

A cDNA library was constructed from bean cotyledons in the final stage of development (just as the pods were beginning to dry). The synthesis of oleosins is reported to be at its maximum at this point in development in most plant species (Murphy 1993). All three primers used for PCR (Fig. 4) were end labeled and used as probes to screen the library with no positive results (data not shown). A separate degenerate probe was also designed from oleosin sequences of several different species (Fig. 4). This probe was used for further screening of the cDNA library and resulted in isolating three positive colonies. When the cDNAs from the isolated colonies were sequenced, they proved to be unrelated to oleosin, and one was tentatively identified by homology to a putative ribosomal protein from carrot (data not shown). In fact, the region complementary to the probe where hybridization would be expected to occur could not be detected in any of these sequences.

Figure 50. Illustration of the most probable interactions of primer 2 with itself. A. Ability for primer 2 to form dimers with itself. B. Ability of primer 2 to form a hair pin structure.

A



B



Discussion

Lipid particles from plants, animals, bacteria and yeast are similar in that they are all enriched in lipid, but they are also distinguishable on the basis of their lipid and protein compositions. Lipid particles appear to be derived from membranes, although in most cases their ontogenies have not been fully elucidated. Different strategies can be used to identify the origins of these particles. One way is by tracking the putative movement of proteins from the membranes to lipid particles, and another is to examine these particles for their particular characteristics.

In the present study, four distinct classes of lipid particles, LDPs, LDP's, IDPs and HDPs, have been identified in developing and germinating seeds of *Phaseolus vulgaris*. These particles are similar in that they contain the same lipid classes, viz., phospholipid, diacylglycerol, free fatty acids, triacylglycerol and steryl and wax esters. However, these particles are also distinguishable by their differing relative proportions of these lipids, triacylglycerol in particular.

Determining the protein composition of the lipid particles from developing and germinating seeds of *Phaseolus vulgaris* proved to be complicated by the possibility of non-specific association of proteins with the surfaces of the particles. Because the particles are localized in the cytosol, but appear to be derived from membranes, the hypothesis that they contain cytosolic forms of membrane-associated enzymes was examined. Specifically, non-mitochondrial NADPH- and NADH-dependent cytochrome c reductases are marker enzymes for microsomal membranes (Sottocasa *et al.* 1967, Donaldson *et al.* 1972, Hirai and Asahi 1975) and have been detected in the cytosol isolated from cotyledons of *Phaseolus vulgaris* (Thompson 1974). These

cytochrome c reductase assays measure cytochrome P450 and cytochrome b5 reductases, respectively, and it seemed highly unlikely that these membrane-associated proteins would retain conformations consistent with activity in the aqueous environment of the cytosol. Rather, it seemed probable that these enzymes are released from membranes in association with lipid and perhaps in the form of lipid particles.

There have been reports of plant microsomal cytochrome P450 reductases being solubilized by proteolytic activity (Fujita and Asahi 1985, Lesot *et al.* 1990). However, it is clear from the present study that the cytosolic cytochrome c-reducing activity in bean cotyledons is not attributable to proteolytic cleavage of the microsomal enzyme. The specific activity of the soluble cytochrome c reductant increased with advancing senescence of the cotyledons, and there was also an age-related decrease in the specific activity of the corresponding microsomal activity. However, these changes were not temporally reciprocal as one would expect if the cytosolic activity were a proteolytic cleavage product of the microsomal enzyme. Moreover, in keeping with the finding that cytosolic cytochrome c-reducing activity in the cotyledons is not attributable to protein and presumably not derived from membranes, practically none of the heat-stable activity in the cytosol co-isolated with lipid particles isolated by flotation, and only about 2% of this activity co-isolated with lipid particles isolated by ultrafiltration; this result can be reasonably attributed to cytosolic contamination.

Several lines of evidence support the contention that the cytosolic cytochrome c-reducing activity is independent of protein. Specifically, it is resistant to boiling,

TCA-precipitation and extensive Proteinase K digestion. Furthermore, unlike thermosensitive microsomal cytochrome c-reducing activity, the cytosolic thermostable reductant can be separated from protein using a desalting column, indicating that this reductant has a molecular weight of < 5kDa. Thermostable cytochrome c-reducing activity resolvable from protein by Sephadex G-25 chromatography has also been reported for coleoptiles and root tips from maize, root tips from oat and hypocotyls from red beet (Pohl and Wiermann 1981). The activity from these tissues increases toward a pH of 8.5, (higher pH was not examined), which is consistent with the broad pH optimum found for the cytosolic activity of the bean cotyledons.

To date, the molecule responsible for this cytochrome c-reducing activity in plants has not been identified. Flavin nucleotides and chlorogenic acid (Hackett 1964) have been suggested as possible candidates, as has ascorbic acid (Slinde *et al.* 1983). Quinones, abundant in plants, are also capable of reducing cytochrome c (Masters *et al.* 1967). Also, a thermostable cytochrome c reductant in insect larvae has been identified as 3-hydroxykynurenine (Goshima *et al.* 1987). However, these compounds can be ruled out as candidates for the bean cotyledon thermostable activity as it is stimulated by the cofactors NADPH or NADH. Pohl and Wierman (1981) reported that the nonenzymatic activity found in the tissues they examined had no significant dependence on a cofactor. This is consistent with the notion that flavin nucleotides, chlorogenic acid, quinones, ascorbic acid or 3-hydroxykynurenine could be responsible for this activity. In bean cotyledons, however, there is an approximately 2-fold increase in the nonenzymatic activity in the presence of a

cofactor. Both NADPH and NADH (data not shown) have the same effect. This suggests that the cytochrome c-reducing activity in bean cotyledons is not the same as that reported by Pohl and Wierman (1981). Further, it is apparent that the compound responsible for this activity is not a flavin nucleotide, chlorogenic acid, a quinone, ascorbic acid or 3-hydroxykynurenine, as none of these compounds have enhanced cytochrome c-reducing activity in the presence of NADPH or NADH. It can not be ruled out, however, that there is more than one compound responsible for the nonenzymatic activity found in bean cotyledons: one dependent and the other not dependent on a cofactor. This possibility is suggested in several ways. First, the NADPH-dependent and -independent activities have overlapping but not identical pH optima. Second, there is no increase in NADPH-independent activity after heat denaturation, whereas there is for NADPH-dependent activity, and third, the NADPH-dependent and -independent activities do not coelute exactly during fractionation on Sephadex G-25. However, the possibility that the NADPH-independent activity reflects the presence of an endogenous cofactor is not precluded.

Recognition of the existence of this heat-stable molecule is important for studies in which cytochrome c or other compounds are being used as electron acceptors for identification of a specific enzyme. In preliminary experiments, the thermostable reductant was observed in unwashed microsomal membranes, and may in fact be responsible for the residual reductase activity found after immunoinhibition of cytochrome P450 reductase which was attributed to microsomal diaphorases (Benveniste *et al.* 1989). The increase in NADPH-dependent cytochrome c-reducing

activity observed after heat denaturation of bean cotyledon cytosol is puzzling, but may simply reflect removal of compounds which interfere with the assay.

Goshima *et al.* (1987) speculated that 3-hydroxykynurenine in insect larvae plays a role in scavenging active oxygen species. Given that the bean cytochrome c-reducing activity is found in cotyledons from the onset of germination, this molecule may be also involved in protecting cellular structures of storage tissue from active oxygen species after germination and perhaps even during seed dormancy.

LDPs, LDP's, IDPs and HDPs were isolated from the cytosol by flotation centrifugation. That this was possible is consistent with the finding that these classes of particles have different densities reflecting differences in lipid and protein composition. However, in spite of this, two classes of particles, LDPs and IDPs, were both highly enriched in triacylglycerol (LDP's were not examined) and LDPs, LDP's and IDPs had very similar protein compositions as resolved by SDS-PAGE. The high levels of triacylglycerol, about 86% of total fatty acid for LDPs and about 78% for IDPs, in 1 day-old seeds, indicate that LDPs and IDPs are oil bodies and accordingly either have separate or common ontogenies. In the event of a common ontogeny, one would be derived, metabolically or artifactually during tissue homogenization, from the other, and it would seem likely that IDPs are derived from LDPs inasmuch as IDPs have lower triacylglycerol levels. Whether the ontogenies are separate or common, from the differences in densities, triacylglycerol content and the similarities in protein profiles, it seems likely that LDPs, LDP's and IDPs represent a range of particles with similar protein profiles, but differing proportions of triacylglycerol and hence different

densities. As LDPs and IDPs appear to represent the extremes of this range, in terms of density, these two particles were chosen for further study.

In an effort to distinguish between the possibilities that LDPs and IDPs originate from a common source or one from the other, LDPs and IDPs were characterized in detail. One facet of this characterization involved measuring changes in levels of LDP and IDP protein during seed development and germination. Changes in protein levels of the particles during seed development and germination can be interpreted as reflecting changes in particle abundance, and it is noteworthy in this context that LDPs appear to be rapidly catabolized beginning at the onset of germination. Indeed, LDP protein levels after one day of germination were 1.7-fold lower than those for developing seeds and decreased progressively during germination to very low levels by 3 days after planting. Developing seeds were routinely harvested from pods that were just beginning to dry. In spite of this, the developing seeds appeared to be at different stages of development in that their fresh weights were variable. However, there was no significant difference in the average fresh weight of mature seeds and that of fully hydrated germinating seeds ($\alpha = 0.05$, Appendix 1, Table A.1.1). The developing seeds also still contained residual levels of chlorophyll. The average level of chlorophyll in microsomal membrane preparations from different batches of seeds was 17.5 μg/cotyledon, and variation among membrane preparations from different seed batches was within 2 standard deviations of the mean.

In contrast to LDPs, IDP protein content increased after germination. The differences between days 1 and 2 after planting were not statistically significant, but the change by 2.5 days after planting was significant ($\alpha = 0.05$, Appendix 1, Table

A.1.6). The finding that LDP and IDP protein levels change reciprocally after germination suggests that LDPs are converted to IDPs. The sums of the protein contents of LDPs and IDPs isolated from the same homogenates decline after germination but do not decrease significantly again until 3 days after planting (α = 0.05, Appendix 1, Table A.1.7).

Fractionation of the proteins associated with IDPs and LDPs isolated from seeds during germination revealed the presence of three major proteins with apparent molecular weights of ~ 52 kDa, 23 kDa and 17 kDa when the gels were stained with Coomassie brilliant blue. The molecular weights of the smaller two proteins were confirmed by mass spectroscopy at 22.9 kDa and 17.7 kDa. Proteins of these molecular weights were not apparent in the corresponding microsomal membranes or the sucrose-containing layer (isolated from directly below the IDPs during their preparation) when the protein gels were stained with either Coomassie brilliant blue or silver. These observations indicate that these major IDP and LDP proteins are not cytosolic or membranous contaminants adhering to the particles. Some lower molecular weight bands were evident after silver staining when IDPs and LDPs were fractionated on higher percentage SDS-polyacrylamide gels. Moreover, the protein banding pattern for IDPs and LDPs from germinating seeds remained essentially unchanged up to 3 days after planting. The protein banding patterns of LDPs and IDPs from developing seeds were also closely similar to those for corresponding particles from germinating seeds, with one difference. That is, LDPs and IDPs from developing seeds do not contain the major ~ 52 kDa protein clearly visible in protein gels for IDPs and LDPs isolated from germinating seeds. These observations are

consistent with the possibility that this protein may be involved in mobilizing the contents of these particles during germination. Intriguingly, its apparent molecular weight (~ 52 kDa) is similar to the average molecular weight of 54 triacylglycerol lipases reported in the Enzyme data bank (51.056 kDa).

The LDPs and IDPs isolated from developing and germinating seeds resemble oil bodies from oil-bearing seeds in that they are enriched in triacylglycerol. In addition, the 17.7 and 22.9 kDa proteins of LDPs and IDPs are within the size range of oleosins, the major oil body protein for oil bodies from canola, soybean and other oil-bearing seeds (Murphy 1990, Huang 1992, Huang 1996, Napier *et al.* 1996, Galili *et al.* 1998). Wax bean (*Phaseolus vulgaris*) seeds, unlike most typical oil-bearing seeds, store large quantities of protein in the form of protein bodies for use by the germinating seedling as a source of energy (Vitale and Bollini 1995). However, thin sections of young *Phaseolus vulgaris* cotyledons and an examination of fractions isolated by density gradient centrifugation clearly show the presence of oil bodies, as well (Opik 1966, Allen *et al.* 1971, Mollenhauer and Totten 1971a, Mollenhauer and Totten 1971b, McKegney *et al.* 1995). These, like their counterparts from typical oil-bearing seeds, are likely to contain oleosin, and in an effort to determine whether one of the major proteins associated with IDPs and LDPs is oleosin, these proteins were further characterized.

Mass spectrometry of the 22.9 kDa protein revealed the presence of two proteins differing in molecular weight by 79-81 daltons. The precision of the mass spectrometric analysis allowed that this difference could be accounted for by either a phosphate group or a sulfate group. No other posttranslational modifications were

detected on these proteins. Two techniques were used in an attempt to identify whether a phosphate group was associated with this protein. Alkaline phosphatase was used to remove the putative phosphate group before the samples were examined by mass spectrometry, and this resulted in complete digestion of the proteins in the sample, suggesting that proteolytic enzymes activated by dephosphorylation were present. In addition, Western blots of the LDP and IDP proteins were probed with phosphoamino acid antibodies to examine the possibility that they were phosphorylated. However, this antibody did not cross-react with the 22.9 kDa protein. This finding does not, however, rule out the possibility that the protein is phosphorylated, for steric hindrance may have precluded effective cross reaction.

Western blot analysis using antibodies directed against a 19 kDa oleosin from canola (kindly provided by M.M. Maloney, University of Calgary) did show a positive reaction with the 22.9 kDa protein of LDPs and IDPs, suggesting that this protein might be oleosin. This antibody also cross-reacted with the 24 kDa oleosin of soybean oil bodies. However, a second antibody directed against a 22 kDa oleosin from canola (provided by M.M. Maloney), shown to bind with a broad number of oleosins from different plants, did not bind with the 22.9 kDa protein of LDPs and IDPs, but there was a clear reaction with the ~ 52 kDa protein from these particles. This protein is at least twice the size of any reported oleosin, and its absence in particles from developing seeds makes it an unlikely candidate for wax bean oil body oleosin. It is evident from the Western blots of fractionated LDP and IDP proteins probed with the 19 and 22 kDa canola oleosin sera that the 22.9 and ~ 52 kDa proteins are identical in both fractions.

Western blots probed with monoclonal HSP70 antibodies disclosed the presence of HSP70 in LDPs and IDPs from bean seeds as well as in canola oil bodies. The significance of this finding is unclear; it may reflect an association of HSP70 with the proteins of these particles related to targeting of the proteins, but it is more likely that HSP70 becomes associated with proteins of these fractions in response to some level of protein denaturation incurred during their isolation.

Contaminating proteins have been reported to associate with oil bodies during their isolation (Kalinski *et al.* 1992, Thoyts *et al.* 1996), and in recognition of this several washes were used in an attempt to rid IDPs and LDPs of any artifactually-associated proteins. For this purpose Triton X-100, a nonionic detergent, sodium deoxycholate, an ionic detergent and sodium carbonate, previously shown to eliminate trapped or nonspecifically-bound proteins from membranes, were used to wash LDPs and IDPs from wax bean as well as oil bodies from canola. No differences in the protein profiles of any of these particles could be detected by SDS-PAGE as a result of any of these washes, using either Coomassie brilliant blue or silver staining. Also, Western blot analysis of LDPs and IDPs using both canola oleosin antibodies showed no clear effect of any of the washes on the protein content of the particles. Interestingly, levels of HSP70 associated with the particles were reduced by all of the washes, most particularly the two detergent washes. This is not surprising given the demonstrated association of HSP70 with hydrophobic stretches of amino acids in unfolded proteins (Boston *et al.* 1996, Miernyk 1997, Netzer and Hartl 1998), and it suggests that the other proteins associated with the particles are not associated by hydrophobic interactions.

When Proteinase K was used to examine the topography of the proteins associated with LDPs and IDPs, the only clearly protected fragment was that derived from the ~ 52 kDa protein. Furthermore, in the presence of SDS, which would solubilize the particles, the ~ 52 kDa protein is completely degraded. This supports the notion that this protein is partially embedded in the lipid of the particles and is a native constituent of these particles. The 22.9 kDa protein and the 17.7 kDa protein were both degraded by Proteinase K; in fact after 1 minute, the 22.9 kDa protein appeared to be completely degraded in gels that were stained with Coomassie brilliant blue. The 17.7 kDa protein was degraded more slowly, but was not detectable in Coomassie-stained gels after 40 minutes of digestion with Proteinase K. When gels of Proteinase K-treated LDPs were stained with silver, a more sensitive stain, a different pattern of protected fragments was evident. The protected fragment of the ~ 52 kDa protein remained clear, but the 17.7 and 22.9 kDa proteins also appeared to have possible protected fragments.

It is difficult to ascertain the precise origin of the smaller fragments from these results; the low molecular weight peptides that begin appearing after 1 minute of digestion could originate from any of the lipid particle proteins. However, it seems reasonable to assume that the ~ 48 kDa protected fragment derives from the ~ 52 kDa protein inasmuch as it is larger than either of the other two major proteins with molecular weights of 22.9 kDa and 17.7 kDa.

The canola oleosins showed rapid disappearance in the presence of Proteinase K. After 1 minute of treatment there was little intact oleosin remaining, and after 5 minutes there was a broad band or collection of bands at 6 - 10 kDa, consistent with

the size of the portion of oleosin embedded in the oil bodies as judged by their sequences. This result confirms the reported arrangement of oleosin in oil bodies.

The Western blots of the protease digests of LDPs and IDPs confirmed the pattern of proteolytic degradation observed for the proteins of the lipid particles. When Western blots probed with the 19 kDa canola oleosin antiserum were examined, it became clear that the epitopes of the 22.9 kDa protein recognized by this antiserum are digested extremely rapidly and that there is no residual protected fragment identified by this antibody. The ~ 52 kDa protein, on the other hand, which has epitopes recognized by the 22 kDa canola oleosin antiserum, showed progressive digestion to the expected ~ 48 kDa protected fragment, confirming that this fragment is derived from the ~ 52 kDa protein and that this protein is likely embedded in the particles. Western blots of LDPs and IDPs probed with anti-HSP70 antibody showed that the epitope of this protein recognized by the antibody is completely digested by Proteinase K within 1 minute, suggesting that it is associated with the surface of the lipid particle.

Both antibodies against canola oleosins are polyclonal antibodies. Moreover, the finding that these antibodies cross-react with proteins associated with LDPs and IDPs that do not appear to be oleosins, raised the possibility that the rabbit polyclonal antisera contains antibodies apart from those generated against the two oleosins. Preimmune sera were not available to explore this possibility.

Accordingly, the prospect that the reactions of the 19 kDa and 22 kDa canola oleosin antisera with LDP and IDP proteins reflected spurious cross-reaction was investigated by incubating the antisera with canola oil bodies in order to deplete it of

the antibodies against the canola oleosins. This process did not affect binding of antibodies to LDPs and IDPs, but completely eliminated cross-reactivity of the antisera with the canola oleosins. The ineffectiveness of the treatment to block cross-reactivity with IDP and LDP proteins suggests that the antibodies against these proteins are not oleosin-induced, but are rather contaminating antibodies in the sera. Furthermore, a second batch of 19 kDa canola oleosin antiserum, also provided by the Calgary lab, did not recognize either the 22.9 kDa protein or the ~ 52 kDa protein of LDPs and IDPs. These observations further support the contention that the 22.9 kDa and ~ 52 kDa proteins of IDPs and LDPs are not oleosins. It is likely that proteins from plants are present in the diet of most rabbits, and it would seem plausible that rabbits develop antibodies against some of the proteins found in these plant organs. This notion is supported by the finding that when preimmune sera from three different rabbits were used to probe Western blots of different fractions from bean seeds, many proteins in these fractions were recognized.

Further support for the contention that the oleosin antisera were binding to proteins unrelated to oleosin was obtained by amino terminal sequencing. Although all of the three major proteins of LDPs and IDPs, the 17.7, 22.9 and ~ 52 kDa proteins, as well as the ~ 48 kDa fragment of the ~ 52 kDa protein, were prepared for amino terminal sequencing, only the 22.9 kDa protein was not blocked at the amino terminus. The amino terminus amino acid sequence of this protein shows high homology to the lectin-like group of proteins known to be abundant in seeds of *Phaseolus vulgaris* (De Mejia *et al.* 1990), and these proteins have no sequence similarity to oleosin. Although numerous members of the lectin-like group of proteins have been identified

and sequenced, the 22.9 kDa protein appears to be a unique member. The proteins with highest homology to the 22.9 kDa protein are the α-amylase inhibitors, and LDPs from both developing and germinating bean seeds showed considerable porcine α-amylase inhibitory activity. This activity was destroyed after denaturation or protease digestion of the sample.

It is of considerable interest that this protein appears to be post-translationally modified by a phosphate or a sulfate group when examined by mass spectrometry. No members of the lectin-like group of proteins have been reported to have either of these modifications, and despite the fact that the presence of a phosphate group could not be confirmed by probing Western blots with anti-phosphoamino acid antibodies nor by alkaline phosphate treatment, it is not out of the question that there is some control of this inhibitory activity by phosphorylation or dephosphorylation.

Proteins of the lectin–like group are usually located in the protein bodies of cotyledons in germinating seedlings (Santino *et al.* 1992). This organelle has been reported to contain proteins that adhere to the surface of oil bodies during homogenization of the tissue (Kalinski *et al.* 1992, Thoyts *et al.* 1996), and it has been shown to rupture easily during homogenization and even during sectioning of tissue for examination by electron microscopy (Kalinski *et al.* 1992). The protein bodies isolated from cotyledons of germinating *Phaseolus vulgaris* showed some variation in protein profile when examined by SDS-PAGE depending on the protocol used for isolation. In every case, however, a protein of the same apparent molecular weight as the 22.9 kDa protein found in LDPs and IDPs was present in the protein bodies. This was also the case for the 17.7 kDa protein. By contrast, the ~ 52 kDa protein did not

appear to have a counterpart in the protein bodies. This was confirmed by probing Western blots of protein body proteins with the 22 kDa canola oleosin antibody which recognizes the ~ 52 kDa protein in LDPs and IDPs. The 19 kDa canola oleosin antiserum, which recognizes the 22.9 kDa protein of LDPs and IDPs, did cross-react with the protein body protein of similar molecular weight, confirming that the α-amylase inhibitor from the LDPs and IDPs is very likely a protein body constituent. Thus, the 22.9 kDa protein of LDPs and IDPs, and possibly the 17.7 kDa protein as well, appear to be proteins from protein bodies, which become associated with the particle surfaces during homogenization and fractionation. The ~ 52 kDa protein of LDPs and IDPs, on the other hand, is not a protein body protein and appears to be a *bona fide* component of the particles.

The amino acid compositions of the 17.7 kDa protein and of the protected fragment from the ~ 52 kDa protein from LDPs and IDPs show no homology with those of any reported databank sequences when analyzed using the Prosite program. When the proportions of hydrophobic, polar and charged amino acids of the 17.7 kDa protein are compared with the corresponding groups of amino acids for *Arabidopsis*, canola and soybean oleosins and with the average occurrence of these amino acids in proteins generally, this protein appears to be relatively hydrophilic. This is consistent with the contention that the 17.7 kDa protein is a contaminant adhering to the surface of the lipid particles. That the protected fragment from the ~ 52 kDa protein has a low proportion of charged amino acids, by comparison with oleosins, is not surprising since the most hydrophilic portion of the protein would be expected to be degraded during protease treatment of the particles. On the other hand, less than 10% of this

protein appears to be lost during digestion with Proteinase K, and if these amino acids were all charged or polar it would still be a relatively hydrophobic protein as compared with the average occurrence of amino acids in proteins generally. The oleosin sequences examined all have a smaller than average proportion of charged amino acids, which is consistent with the subcellular localization and topography of these proteins. That is, oleosins characteristically possess a long hydrophobic midsection that extends into the hydrophobic interior of oil bodies (Murphy 1990, Huang 1992, Murphy 1993, Murphy 1994, Napier *et al.* 1996).

These various analyses of the major proteins associated with LDPs and IDPs collectively indicate that none of them correspond to oleosin. Nonetheless, several additional approaches were undertaken to identify an oleosin in the LDPs and IDPs from wax bean seeds. One such approach was based on the fact that oleosin genes from several plants, including soybean, appear to have an abscisic acid responsive element in their promoters (Huang 1992). Accordingly, germinating wax bean seeds were treated with abscisic acid for 24 hours. The relative amounts of the individual LDP or IDP proteins were unaffected by this treatment. This suggests that, if oleosin is present in wax bean seeds, its abundance is not regulated by an abscisic acid inducible promoter or, less likely, that each one of the major proteins present in the lipid particles are equally affected by this treatment.

Most oleosins are basic, and an examination of the sequences of 18 different oleosins revealed an average isoelectric point of 9.10 with a range of 6.91 – 9.99. Interestingly, when this property was examined by isoelectric focussing of these lipid particles, none of the proteins in solubilized LDPs, IDPs and canola oil bodies

migrated as basic proteins. Even when the isolated lipid particles were lipid-extracted prior to isoelectric focussing to exclude the alteration of protein migration by adhering lipid, once again all of the proteins ran as acidic proteins, including the canola oleosin. The possibility that highly basic proteins had run off the gel was eliminated by placing the isoelectric focussing gel over a gel with a higher percentage of acrylamide and carrying out the isoelectric focussing for varying lengths of time. Moreover, cytochrome c with a pI of 10.6 behaved as anticipated, and ran as a basic protein. Given the small size (12.8 kDa) and charge of cytochrome c, it seems highly unlikely that the larger and less basic oleosins could have been lost from the gel. The finding that canola oleosins, which are net basic proteins, do not migrate as basic proteins is puzzling, but may be due to the varying charges on the domains of the protein, although examination of the sequences making up the cytosolic domains of oleosins reveals that these domains have net positive charges. However, it has been observed that if oil bodies are placed in an acidic solution they aggregate (Tzen *et al.* 1992, Tzen *et al.* 1997) suggesting that the surfaces of oil bodies carry a neutral charge at low pH. In addition, entire oil bodies isolated from maize have a pI of between 5.7 and 6.6 (Tzen *et al.* 1993). It is thought that the structure of oil bodies is maintained in part by the interaction of exposed regions of oleosin and negative charges of the phospholipid. Interestingly, phosphatidylcholine, the major phospholipid found on the surface of oil bodies (Tzen *et al.* 1992), is not negatively charged. Thus, the behaviour of oil bodies and oleosins at acidic pH is difficult to explain.

The absence of proteins in LDPs and IDPs that migrate as basic proteins during isoelectric focussing was confirmed by two-dimensional analysis. The ~ 52 kDa and

17.7 kDa proteins were clearly discernible in the two-dimensional gel. At a molecular weight of ~ 23 kDa where the α-amylase inhibitor migrates, there appeared to be either two proteins of similar molecular weights or two forms of the same protein. This is consistent with the finding by mass spectrometry that this protein may be modified by a sulfate or a phosphate group. Moreover, amino terminal sequencing of the protein at this Rf value isolated by SDS-PAGE resulted in only one sequence, which supports the contention that the two spots on the two-dimensional gel reflect two forms of the same protein. The better resolution of the two forms of the protein in two-dimensional gels than in single dimension SDS-PAGE may reflect a change in the isoelectric point of the modified form of the protein attributable to the negatively charged phosphate or sulfate. However, the prospect that there are two separate proteins of similar molecular weight, one being amino terminally blocked, is not precluded.

The amino acid and nucleotide sequence similarity of the central hydrophobic region of oleosins among different species of plants (Murphy 1990, Huang 1992, Murphy 1993, Murphy 1994, Huang 1996, Napier 1996) suggests that the presence of this protein in wax bean seeds might be discernible by PCR using either DNA or RNA as a template. In fact, when PCR was performed using wax bean DNA as a template and primers designed from soybean oleosin, one of which was a highly conserved region within the central hydrophobic domain of oleosin, a product ~ 200 bp in size was obtained. These primers also generated a 242 bp PCR fragment using soybean DNA as a template, as expected. However, this PCR product was obtained only inconsistently, using either the wax bean or the soybean DNA templates, suggesting

that there were problems with the primers themselves. Examination of the sequence of the primer generated from the most homologous region of the oleosin gene showed that this primer has a strong proclivity to form dimers. A second alternative primer was designed but no PCR products were obtained using wax bean DNA as a template. Attempts to sequence the PCR fragment generated from the first set of primers directly, in order to confirm its identification, were unsuccessful, and two different attempts to introduce restriction enzyme sites into the primers in order to subclone the PCR product for sequencing were also unsuccessful. In addition, screening of a cDNA library from maturing seeds with four different oligonucleotide probes, corresponding to conserved regions of the oleosin genes, failed to uncover any oleosin-like proteins.

In summary, SDS-PAGE, western blotting, protease digestion, abscisic acid-induction, IEF analysis, amino acid composition, amino-terminal sequencing, PCR and cDNA library-screening were used in an attempt to identify oleosin in LDPs and IDPs. None of these strategies confirmed the presence of oleosin, and only PCR suggested the possibility that oleosin might be expressed in wax bean seeds, but the identification of this PCR fragment could not be obtained. In addition, examination of sequences from 12 oleosins from different plant species revealed that each would generate a fragment of 242 bp in size using the same PCR primers suggesting that perhaps the 200 bp fragment from the wax bean DNA template was not oleosin. Therefore, the possibility that there is no oleosin in wax bean seeds must be considered. This may not be unreasonable given the fact that oleosin is normally found in oil-bearing seeds. Such an interpretation, however, leads to the speculation

that lipid particles in wax beans might contain a hitherto undescribed surface protein that would function in a similar role to oleosin to prevent lipid particle coalescence.

There is compelling evidence that maximum oleosin synthesis occurs concomitant with, or slightly after maximum triacylglycerol synthesis in oil-bearing seeds (Herman 1987, Slocombe *et al.* 1992, Cummins *et al.* 1993, Tzen *et al.* 1993, Plant *et al.* 1994, Aalen 1995, Sarmiento *et al.* 1997), and it has been estimated that oleosins make up about 8 to 20% of total seed protein (Murphy 1993, Tzen *et al.* 1993). These oil-bearing seeds are characterized by having very high levels of triacylglycerol. In fact, electron micrographs show an abundance of oil bodies tightly juxtaposed within the cells in such seeds (van Staden *et al.* 1975, Bergfeld *et al.* 1978, Murphy *et al* 1989, Kalinski *et al.* 1992, Cummins *et al.* 1993, Murphy 1993, Ting *et al.* 1996, Tzen *et al.* 1997, Leprince *et al.* 1998, Tzen *et al.* 1998). By contrast, in electron micrographs of *Phaseolus vulgaris* cotyledon tissue this is not the case (Opik 1964, Mollenhauer and Totten 1971a, Mollenhauer and Totten 1971b, McKegney *et al.* 1995). Unlike oil-bearing seeds, *Phaseolus vulgaris* seeds store protein and starch as primary sources of energy for use by the developing embryo during germination and, although lipid-containing particles corresponding to oil bodies are clearly visible in electron micrographs of these seeds, they do not appear to be as tightly packed as they are in oil-bearing seeds (Opik 1964, Mollenhauer and Totten 1971a, Mollenhauer and Totten 1971b, McKegney *et al.* 1995). The proposed function of oleosin is to prevent oil bodies from coalescing during the rapid uptake of water preceding germination (Sarmiento *et al.* 1997), thereby keeping the surface to volume ratio high for rapid mobilization of the lipid. Since the oil bodies in *Phaseolus vulgaris*

cotyledons do not appear to be tightly packed, coalescence of lipid may not be a problem during imbibition of these seeds and, accordingly, oleosin may not be necessary.

The ubiquitous nature of oleosin in oil bodies of all oil-bearing species of plants examined to date, suggests it would be an anomaly to have one plant which does not contain oleosins. However, there are no known reports of oleosin being associated with oil bodies in seeds which do not store large quantities of oil, of which *Phaseolus vulgaris* is an example. It is conceivable that the absence of oleosin is a characteristic feature of oil bodies in such plants. It is appropriate to note in this context that lipid particles in oil-rich fruit tissue do not contain oleosin, and this is thought to be related to the fact that there is no requirement for oil bodies, in these systems, to retain a high surface to volume ratio as the oil is used to attract seed dispersers rather than for energy (Murphy 1993). Also, lipid particles in seeds that do not undergo desiccation, do not contain oleosin (Murphy *et al.* 1995).

It is clear that the protein profiles of LDPs and IDPs are very similar to each other. It was not possible to obtain preparations of HDPs that were free of contamination by cytosolic proteins, but the three major proteins of LDPs and IDPs were not detectable in polypeptide profiles of corresponding cytosol containing HDPs (sucrose-containing layer). In addition, the lipid complements of LDPs and IDPs are similar to each other and distinguishable from the lipid composition of HDPs. Not only do LDPs and IDPs both have relatively high levels of triacylglycerol, compared with HDPs, but the major lipids, triacylglycerol and phospholipid, are also more unsaturated in LDPs and IDPs than in HDPs. The unsaturated to saturated fatty acid

ratios for triacylglycerol in LDPs and IDPs are much higher than for HDPs. In fact, the lipid composition of LDPs, as well as their size, are similar to the corresponding attributes of oil bodies in the seeds of a range of oil-bearing species (Murphy 1990), supporting the contention that LDPs are oil bodies.

The striking similarity between LDPs and IDPs and the ability to isolate a third class of similar particles intermediate in density and presumably triacylglycerol content (LDP's), suggests that IDPs and LDP's may be derived as parts of a continuum of metabolites from LDPs as oil body triacylglycerol is mobilized. The finding that IDPs contain less triacylglycerol than LDPs, but are otherwise similar, is consistent with this possibility. However, several lines of evidence indicate that this is not the case. For example, LDPs and IDPs are both present in maturing seeds at a time when triacylglycerol is being synthesized rather than metabolized. Moreover, there is a large increase in the level of IDP lipid, during the latter stages of seed development or soon after planting, that is not matched temporally by the corresponding decline in the level of LDP lipid. In addition, the ~ 90% decrease in LDP lipid during the early stages of germination (between 2 and 3 days after planting) is not matched by a corresponding increase in IDP lipid; in fact, the change in IDP lipid is not significantly different at this stage ($\alpha = 0.05$, Appendix 1, Table A.1.13). Thus, although the possibility that IDPs are metabolic derivatives of LDPs is not precluded, this seems unlikely to be the case.

Isolation of organelles from the cells of tissues and organs is complicated by the need to deploy disruptive forces, in the form of tissue homogenization, to break open the cells and release their organelles into the isolation medium. This is

particularly true of plant cells which have cell walls surrounding the plasma membranes. The difficulties are manifold: organelles may be disrupted during tissue homogenization, and the possibility that contaminating proteins and/or lipids will spuriously associate with the surfaces of organelles once the structural integrity of cells has been destroyed must be considered. Oil bodies, for example, have been shown to pick up contaminating proteins and possibly also lipid during tissue fractionation (Kalinski *et al.* 1992, Thoyts *et al.* 1996) as have plastoglobuli, lipid particles located in the plastids of plant cells (Steinmuller and Tevini 1985). The composition and morphology of some animal lipoproteins have also been shown to be affected by isolation and manipulation (Kunitake and Kane 1982, Guyton *et al.* 1991).

In the present study, this potential difficulty was addressed by using two alternative techniques for disruption of cotyledon tissue apart from the standard method of homogenizing the tissue with a Polytron homogenizer in aqueous buffer. One of these involved freezing the tissue in liquid nitrogen followed by grinding to a powder, and the second entailed homogenizing the tissue in pure glycerol. Both of these techniques are nonaqueous and provide a very different environment for interactions of cellular contents after the membranes have been disrupted. Of interest was the finding that the protein profiles of both LDPs and IDPs visualized by SDS-PAGE are similar, no matter which of the three techniques of tissue disruption is used. The most notable difference in the protein profiles of these fractions is probably not due to the method of homogenization, but more likely to the presence or absence of protease inhibitors during the homogenization. Specifically, in the absence of protease inhibitors, for both LDPs and IDPs the ~ 52 kDa protein is no longer a strong band,

and the 17.7 kDa protein appears to be partially degraded. Aside from these changes, the different techniques of tissue disruption had no major effects on the protein profiles of LDPs and IDPs.

There was, however, a distinct effect of homogenization on the relative abundance of the different types of lipid particles. Specifically, when the particles are isolated from homogenates obtained by disrupting the seed tissue in aqueous buffer, LDPs account for ~ 60% of the total fatty acid complement of LDPs and IDPs combined. By contrast, when the particles are isolated from homogenates obtained by freezing in liquid nitrogen followed by grinding or homogenizing in glycerol, LDPs account for 95% and 99% of the total fatty acid complement, respectively. This strongly suggests that IDPs do not have a separate ontogeny nor are they metabolically derived from LDPs. Rather, they appear to be artifacts derived from LDPs during homogenization in aqueous medium. In fact, it is likely that homogenization in aqueous medium shears a continuum of particles from LDPs which differ in size, density and relative amounts of triacylglycerol. Given this evidence, it is not surprising that there are also small, but statistically significant differences in amounts of lipid classes between IDPs and the LDP ($\alpha = 0.05$, Appendix 1, Table A.1.17).

Oil bodies are believed to be formed *in situ* from the surface of the endoplasmic reticulum (Murphy 1990, Huang 1992, Murphy 1993, Huang 1996, Napier *et al.* 1996). There is also evidence that the size of oil bodies is determined by the availability of oleosin. More specifically, in the event that oleosin is a limiting factor, the size of oil bodies is increased due to an increased number of fusion events (Sarmiento *et al.* 1997). Oleosin synthesis increases during the latter stages of seed

maturation relative to triacylglycerol synthesis (Murphy and Cummins 1989, Cummins *et al.* 1993, Batchelder *et al.* 1994, Aalen 1995), and this could account for differences in oil body size and density. Alternatively, given the findings of the present study, it is also conceivable that oil bodies, which are isolated in essentially the same manner as LDPs, are likely to shear in the same manner thus generating a continuum of particles with varying sizes. On the other hand, if LDPs lack oleosin, as seems likely, they may be more prone to shearing during homogenization in aqueous buffer than oil bodies from oil-bearing seeds, which are known to be stabilized by the presence of oleosin coating their external surfaces.

The much lower abundance (10- to 30-fold) of HDPs in comparison with LDPs and IDPs in both developing and germinating seeds suggests that they are not storage organelles. This contention is further supported by the fact that HDPs contain low levels of triacylglycerol by comparison with LDPs and IDPs, and have proportionately higher levels of free fatty acids and steryl and wax ester than LDPs, IDP and microsomal membranes. In fact, lipid particles of a similar nature have been identified in carnation petals, which do not store triacylglycerol, and previously in wax bean cotyledons (Thompson *et al.* 1997, Thompson *et al.* 1998). These lipid particles, referred to as deteriosomes or lipid-protein particles, resemble HDPs in that they are enriched in free fatty acids and steryl and wax esters (Yao *et al.* 1991a, Yao *et al.* 1991b, McKegney *et al.* 1995, Hudak and Thompson 1996). Moreover, lipid-protein particles from carnation petals appear to contain catabolites of membrane proteins (Hudak *et al.* 1997). These observations have prompted the proposal that the formation of these particles may be a feature of membrane turnover, allowing removal

of lipid and protein metabolites from the membrane that would otherwise accumulate and destabilize the bilayer (Yao *et al.* 1991a, Thompson *et al.* 1998).

Although lipid-protein particles isolated from carnation petals and wax bean cotyledons are thought to have a structure similar to oil bodies, this is difficult to assert unequivocally. One method which has been used to examine the structure of oil bodies is based on calculating the amount of phospholipid required to form a monolayer surrounding a spherical core of triacylglycerol. This proportion can be roughly calculated given the diameter of the particles (Huang 1992, Appendix 2). With a diameter of 0.181 μm, LDPs isolated from 2 day old cotyledons would be expected to contain about 7.8% phospholipid fatty acid and 92.2% triacylglycerol fatty acid by weight, if they were constructed from only these two lipids. Experimentally, considering only phospholipid and triacylglycerol, the phospholipid fatty acid and triacylglycerol fatty acid proportions are 17.8% and 82.2%, respectively. These proportions do not take into account the other lipids present in the LDPs. Diacylglycerol, steryl esters and wax esters would all partition into the core of a particle. This has been shown for diacylglycerol in insect lipophorins (Ryan 1996), for steryl esters in high- and low-density lipoproteins in mammals (Jonas 1998) and for wax esters in lipid bodies of jojoba (Huang 1992). Free fatty acids have been suggested to reside immediately below the phospholipid head groups in particles with a monolayer of phospholipid (Miller and Small 1983). The fatty acids from diacylglycerol, free fatty acids and steryl and wax esters, make up about 16% of the total fatty acid in LDPs, and taking these additional lipids into account suggests that the structure of these particles is very likely to be similar to the structure proposed for

oil bodies. Even at 3 days after planting, when the lipid stores have clearly been depleted and the diameter of LDPs is about half the diameter of LDPs from 2 day-old bean seeds, these calculations support the contention that these particles are made up of a neutral lipid core surrounded by phospholipid.

Using this method to examine the theoretical structure of IDPs shows that the phospholipid fatty acid content of the particles is 25-30% higher than would be required to form a monolayer on the surface of particles except in IDPs isolated from 2 day-old seeds. This may reflect a structure different from the sphere proposed for oil bodies, but seems more likely to be due to contamination of the IDPs by phospholipid vesicles. In fact, fractions from bush bean and pea cotyledons of about the same density as IDPs have been reported to contain a mixture of oil body-like particles and structures described as composite lipid vesicles, which presumably are made up primarily of phospholipid (Mollenhauer and Totten 1971). HDPs also contain a higher proportion of phospholipid than would be required to form a monolayer on the surface of particles and hence may also be a mixture of vesicles, particles and possibly individual lipids.

Notwithstanding the high lipid content of LDPs, IDPs and HDPs, differences in their lipid compositions suggest that they may have different structures and physiological functions. Inasmuch as LDPs and IDPs contain high levels of triacylglycerol and decline in abundance during the early stages of germination, they are likely lipid storage particles. HDPs, on the other hand, are more abundant in germinating seeds than in maturing seeds, contain only small amounts of triacylglycerol and, based on their similarity to lipid and protein particles previously

isolated from petal and bean cotyledon tissues, may be involved in membrane turnover.

HDPs are unlikely to be homogenization-induced derivatives of LDPs inasmuch as the lipid complement and fatty acid composition of HDP phospholipid and triacylglycerol are quite different from those of LDPs or IDPs. Moreover, although the proteins of HDPs were not isolated and characterized, cytosol containing HDPs (sucrose-containing layer) showed no evidence of the three major proteins associated with LDPs and IDPs. If HDPs are created as a result of homogenization, it is much more likely that they are derived from cellular membranes. It is well documented, however, that neutral lipids are released from membranes in the form of particles, not only in plants as in the case of the triacylglycerol of oil bodies, for example, but also in animals, fungi, yeast and bacteria. Thus, although homogenization could explain the existence of these particles, or alter them in some manner, their distinct lipid composition (compared to microsomal membranes, from which they would presumably originate) makes such an interpretation problematic. If HDPs are formed from membranes, it must be at specific sites along the plane of a membrane, sites corresponding to domains within the bilayer in which there is an enrichment of free fatty acids and steryl and wax esters relative to bulk membrane lipid. Indeed, these localized domains are thought be released from the surface of the membrane *in situ*, as part of normal membrane turnover, forming lipid particles that are circumscribed by a monolayer of phospholipid (Thompson *et al.* 1998). It is nevertheless possible, that the release of membrane domains enriched in these lipids is

induced during tissue homogenization, reflecting an accelerated version of a natural process; this is a possibility that needs further exploration.

Particles similar to HDPs have been found in carnation petals and, with evidence that glyoxysomes are synthesized in senescing tissues including petal and cotyledon tissues (Kudielka 1981, Gut and Matile 1988, De Bellis *et al.* 1990, Landolt and Matile 1990, De Bellis and Nishimura 1991, Pistelli *et al.* 1991, Nishimura *et al.* 1993), it seems reasonable to suggest that the lipid particles in each of these systems may be a result of membrane lipid catabolism, followed by further metabolism of these lipids in glyoxysomes. It is interesting to note that in both petals and cotyledons the abundance of these lipid particles decreases as senescence progresses (Yao *et al.* 1991b, Hudak *et al.* 1995). The decrease in particle abundance has been suggested to reflect the impairment of particle blebbing from membranes with advancing senescence and a consequent accumulation of the lipid metabolites in the bilayer, leading to loss of membrane function (Thompson *et al.* 1998). The reappearance of glyoxysomes in senescing tissues suggests that there is a system in place for converting lipid into a form of energy which can be transported to other plant organs, and the presence of these organelles may explain the disappearance of these lipid particles. In the present study, the abundance of HDPs was not examined beyond 3 days after planting, well before the first significant decrease in fresh weight of the cotyledons or any morphological manifestation of senescence. Thus the possible involvement of glyoxysomes in the process of their metabolism was not determined.

The ontogeny of oil bodies has been described as resulting from an accumulation of triacylglycerol in the endoplasmic reticulum where the enzymes

required for triacylglycerol synthesis are located, and subsequent release of these particles after the accumulation of triacylglycerol reaches a certain level (Murphy 1990, Huang 1992 Murphy 1993, Murphy 1994, Huang 1996, Napier *et al.* 1996). Recent work on oleosins has demonstrated that these proteins are targeted to the endoplasmic reticulum (Loer and Herman 1993, van Rooijen and Moloney 1995, Abell *et al.* 1997) suggesting that oleosin and triacylglycerol accumulate together in these membranes. It seems unlikely that oleosin is responsible for the release of triacylglycerol, as naked triacylglycerol particles are also released from the membranes in oil-bearing seeds (Stobart *et al.* 1986, Sarmiento *et al.* 1997), and LDPs of *Phaseolus vulgaris* cotyledons also appear to lack a protein with the characteristics of oleosin.

The model proposed for the release of triacylglycerol from endoplasmic reticulum membrane is thought to be caused by physical forces resulting from an accumulation of triacylglycerol between the bilayers until the outer layer of the phospholipid is forced to bud and eventually break off from the membrane (Murphy 1990, Huang 1992 Murphy 1993, Murphy 1994, Huang 1996, Napier *et al.* 1996). This model presents some difficulty. It seems unlikely that triacylglycerol, once formed within the membrane, would remain stable in one location until it accumulated to the levels necessary for the physical release of a lipid particle from the membrane surface. Rather, it would be likely to diffuse rapidly throughout the fatty acyl chains of the phospholipid bilayer in accordance with a concentration gradient. Thus, the sequestering of triacylglycerol in a particular membrane location that allows for its accumulation is likely to be a proactive event. The presence of a protein, microsomal

triglyceride transfer protein (MTP), required for the formation of triacylglycerol-rich particles, has been documented in animal cells (Wetterau and Zilversmit 1985, Wetterau *et al.* 1997). Other proteins which may have similar functions in animals are the adipose-differentiation related protein (ADRP) in mammals, which is found associated with lipid particles early in their genesis (Brasaemle *et al.* 1997), and the lipid transfer particle (LTP) in insects, which is capable of transferring lipids between particles (Ryan *et al.* 1986, Ryan *et al.* 1988, van Heusden and Law 1989, Ryan *et al.* 1990a, Singh and Ryan 1991). Studies involving lipid particles from bacteria and yeast cells have not yet uncovered proteins of similar functions.

It seems probable that there are plant proteins involved in the process of oil body ontogeny. Sequence information is available for MTP as well as other proteins involved in the ontogeny and modification of lipid particles from different organisms, and these can be used to examine plant data bases for similar sequences. This type of search has become more fruitful for finding plant proteins with the continuing genome sequencing project and growing data base information on *Arabidopsis*. In searching these data bases in the present study, gapped BLAST similarity searches were used. The data are presented with a percentage of gaps, which is the number of gaps calculated as the percentage of the total amino acids examined in the sequences. An E value is also presented as a way of describing the probability of matching a sequence by chance. An E value of 1 means that the match would be expected to occur by chance once in the data base examined. Conserved amino acids are reported rather than identical amino acids to identify functionally similar proteins. The findings presented are strictly preliminary, as regions of similarity would be expected to occur

in many different proteins for various reasons. For example, proteins which fold in a particular way or those associated with lipid would be likely to have similar domains. No attempt was made to use only functional regions of animal and bacterial proteins as most of these have not been elucidated. However, the abundance of sequences showing some similarities is startling.

The sequences of the proteins involved in the animal lipoprotein system which were used in examining the data banks were: MTP, apolipoprotein B (apoB), apoA1, lethicin-cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP). Of these proteins, only apoA1 and MTP did not show plant proteins with sequence similarities.

ApoB is a protein of 4563 amino acids which functions as a scaffold for the biosynthesis of chylomicrons and very low density lipoproteins in mammals. In order to examine this protein for similar sequences it was divided into three sections. The first third of apoB (1530 amino acids) generated no sequences with striking similarities. The central third of this protein (1743 amino acids) showed homology with three *Arabidopsis* sequences (gi 3482922, E=0.00011, gi 2864624, E = 0.0032, gi 836950, E = .0059). The first two of these sequences are proteins with no assigned functions as yet. They are both hydrophilic proteins as judged by hydropathy plots, and the subcellular locations, as predicted by PSORT, are cytosolic and nuclear, respectively. The first protein shows 49% amino acid conservation with ApoB over a sequence of 249 amino acids with 4.5% gaps, whereas the second protein has 45% amino acid conservation over a sequence of 397 amino acids with 5.0% gaps. The third *Arabidopsis* protein to show similarity to the middle sequence of apoB has been

identified as CIP1, a protein found in cotyledons and hypocotyls of seedlings and thought to be associated with the cytoskeleton (Matsui *et al.* 1995). CIP1 has 45% conserved amino acids when compared to apoB over a 397 amino acid stretch with 7.2% gaps. The final 1530 amino acids of apoB show little similarity with plant proteins.

LCAT (440 amino acids) is involved in transferring fatty acyl groups from phosphatidylcholine to cholesterol in the HDPs of mammals. This protein shows sequence similarity with one *Arabidopsis* protein (gi 3250695, E = 0.017) showing 47% conserved amino acids over a 128 amino acid stretch and 2% gaps. This protein appears to have large regions of hydrophobic amino acids judging by hydropathy plots, but no clear subcellular location was predicted by PSORT. This protein also shows some similarity to poly-β-hydroxybutyrate synthase (PHB synthase), a component of polyhydroxyalkanoic acid granules in bacteria. Both LCAT and PHB synthase contain a domain responsible for binding fatty acids, and this may be the region detected in the *Arabidopsis* protein.

Adipose tissue also contains proteins associated with lipid particles. These are perilipin, which associates with lipid particles in the adipocytes after their formation, and adipose differentiation-related protein (ADRP), which associates with the lipid particles of adipose tissue very early in their formation as well as with other intracellular lipid particles in mammalian cells. Although sequence comparisons to perilipin show no strong similarities with any plant proteins, ADRP (425 amino acids) shows some homology with two plant proteins (genbank AF058919, E = .0047, swiss-prot P31568, E = .056). The first of these is a protein of unknown function with 50%

conserved amino acids in common with a 154 amino acid region of ADRP with 3% gaps. This sequence shows a mixture of hydrophobic and hydrophilic regions in hydrophobicity plots with no clear transmembrane regions as predicted by TMpred. Also no subcellular location was predicted by PSORT. The second protein is predicted from an open reading frame in chloroplast DNA from *Oenothera picensis* and reported to be found in other plant chloroplast DNA (Nimzyk *et al.* 1993). This protein has 56% conserved amino acids over a 234 amino acid region with 10% gaps when compared to ADRP. The chloroplast protein contains highly repetitive, tandemly arranged sequences, which may affect the results of the match. This protein shows a relatively hydrophobic amino terminus in a hydrophobicity plot and a transmembrane region is predicted for this area by TMpred.

The lipid transport system of insects involves four proteins or complexes, apolipophorin I (apoLpI), apoLpII, apoLpIII and the lipid transfer particle. No primary sequence data appeared to be available for the proteins of the lipid transfer particle, and no plant proteins showed sequence similarity with apoLpI or II. However, apoLpIII (189 amino acids) shows similarity with several *Arabidopsis* proteins (embl ATTS1928, E = 4.8 x 10^{-6}, embl ATTS4003, E = 0.00015, embl ATLEA76T1, E = .00021, swiss-prot JC6171, E = .00039). Most of these are homologues with the late embryogenesis abundant (LEA) group of proteins, which are expressed in seed tissue of plants and are thought to be involved in maturation and desiccation of seeds. These are hydrophilic proteins, but no strong subcellular locations were predicted by PSORT. The most similar of these LEA homologues showed a region of 75 amino acids with 57% conservation to apoLpIII and 8.7% gaps.

Bacterial PHA granules appear to be associated with proteins called phasins. Although there have been both 14 kDa and 24 kDa phasins identified, the 24 kDa phasin appears to be present in several species of bacteria. This protein (192 amino acids) shows similarity to an *Arabidopsis* protein of unknown function (genbank AF058919, E = 0.0078). The similarity was 50% over a 150 amino acid region of phasin with 4.5% gaps. This unknown protein shows some homology to the heavy chain of myosin and is predicted to be located in the endoplasmic reticulum membrane.

Many of the plant proteins with similarities to animal and bacterial lipid particle-associated proteins uncovered in data bank searches are of unknown function. This fact is worthy of notice and suggests a possibly valuable area of research in the study of the formation of lipid particles in plants.

Despite the fact that four distinguishable classes of lipid particles were identified in developing and germinating *Phaseolus vulgaris* seeds, only two of these appear to exist *in situ*, the LDPs and HDPs. LDP's and IDPs, with varying but low densities and the same protein profiles, are derivatives of LDPs. These appear to be populations of lipid particles created as a result of the homogenization step in the isolation of these particles. In addition, of the three major proteins associated with these particles, only one of them appears to be a native constituent. This ~ 52 kDa protein has a protease protected fragment as do oleosins in the oil bodies of oil-bearing seeds. The triacylglycerol-rich nature of LDPs, combined with their putative structure, suggest that these are also oil bodies, but the protein associated with these particles is too large to be considered an oleosin. The absence of oleosin and the rapid

disappearance of the lipid and protein of these particles indicates they are unique lipid storage structures.

The HDPs are structures of a different nature than LDPs altogether based on their lipid composition. The structure of these particles is unlikely to be the same as that for LDPs due to the high proportion of phospholipid. They are enriched in phospholipid catabolites (free fatty acids and diacylglycerol) relative to LDPs or membranes, and this characteristic indicates they are similar to lipid-protein particles previously found in carnation petals and wax bean cotyledons, which are involved in membrane turnover. It can not be ruled out, however, that these particles are also either modified or derived from membranes as a result of homogenization.

The presence of phospholipid in both these particles suggests that they originate from membranes, but the high proportion of neutral lipids and/or free fatty acids by comparison with membranes suggests an unusual degree of catabolic complexity. The model for the release of neutral lipids from plant endoplasmic reticulum membranes is incomplete, since possible mechanisms by which membranes concentrate these lipids into small regions remain to be elucidated. It seems possible that oleosin is but one in a class of proteins responsible for lipid particle integrity and/or formation in plants. Data bank searches for novel plant protein candidates that could play a role in these processes appear to be a promising strategy.

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Appendix 1

Statistical calculations

P values were calculated using a two-tailed test with $\alpha = 0.05$.

Table A.1.1 Changes in weights of bean cotyledons during seed development and germination (Fig. 5)[1]

Independent sample t-tests

Ages examined	P values
Dev. - 0.5 d	0.0105
Dev. – 0 d	0.3787
0 d – 0.25 d	0.3010
0.25 d – 0.5 d	6.40×10^{-4}
0.5 d – 1 d*	0.8509
1 d* – 2 d	0.3079
1 d* – 3 d	0.1476
1 d* – 4 d	0.0136
2 d – 3 d	0.8311
3 d – 4 d	0.1251
4 d – 5 d	7.03×10^{-5}
5 d – 6 d	7.14×10^{-4}
6 d – 7 d	0.0198
7 d – 8 d	0.0316

* highest value

Table A.1.2 Changes in specific activity of cytochrome c reductase with age (Fig. 6A)

Independent sample t-tests

Cytosol

Ages examined	P values
1 d – 2 d	0.0328
2 d – 3 d	0.1129
3 d – 4 d	0.0460
4 d – 5 d	0.1354

[1] Abbreviations: day (d), precipitated (ppt.), Proteinase K (PK), phospholipid (PL), diacylglycerol (DG), free fatty acids (FFA), triacylglycerol (TG), steryl and wax esters (S/WE), low-density particle (LDP), intermediate-density particle (IDP), high density particle (HDP), microsomal membrane (MM)

Smooth microsomal membranes

Ages examined	P values
1 d – 2 d	0.0213
2 d – 3 d	0.3440
3 d – 4 d	0.9223
4 d – 5 d	0.8913
1 d – 3 d	0.0229

Cytosolic protein

Ages examined	P values
1 d – 2 d	0.0275
2 d – 3 d	0.0790
3 d – 4 d	0.1356
4 d – 5 d	0.1031

Table A.1.3 Changes in total activity of cytochrome c reductase with age (Fig 6B)
Independent sample t-tests

Cytosol

Ages examined	P values
1 d – 2 d	0.3744
2 d – 3 d	0.7350
3 d – 4 d	0.0569
4 d – 5 d	0.3536

Smooth microsomal membranes

Ages examined	P values
1 d – 2 d	9.62×10^{-5}
2 d – 3 d	5.87×10^{-4}
3 d – 4 d	0.1146
4 d – 5 d	0.5226

Table A.1.4 Differences in sample treatment effects on cytochrome c reductase with age (Fig. 7)
Paired t-tests

Treatments	P values
Control – heat treated	0.0102
Control – TCA ppt.	0.0039
Control – PK treated	0.0055
Control: cofactor – no cofactor	0.0014

Table A.1.5 Optimal pH of cytochrome c reductase activity (Fig. 10)
Paired t-tests

With cofactor

pH	P values
8.5 – 9.5*	0.071
9.0 – 9.5*	0.2381
9.5* – 10.0	0.0142

*highest value

Without cofactor

pH	P values
8.5 – 10.0*	0.0113
9.0 – 10.0*	0.0704
9.5 – 10.0*	0.0759
10.0* – 10.5	0.4337
10.0* – 11.0	0.0333

* highest value

Table A.1.6 Fractionation of cytosol by ultrafiltration or flotation to examine partitioning of cytochrome c reductase activity (Fig. 11)
Independent sample t-tests

Fractions examined	P values
Particles	0.0004
Remainder of cytosol	0.3981

Table A.1.7 Changes in quantities of protein found in different subcellular fractions (Fig. 12)
Independent sample t-tests

Low-density particles (Fig. 12 A)

Ages examined	P values
Dev. – 1 d	0.0365
1 d – 2 d	0.1240
2 d – 2.5 d	0.0450
2.5 d – 3 d	0.1897
3 d – 4 d	0.2470

Intermediate-density particles (Fig. 12 A)

Ages examined	P values
Dev. – 1 d	0.1469
1 d – 2 d	0.8105
2 d – 2.5 d	0.0021
2.5 d – 3 d	2.70×10^{-4}
3 d – 4 d	0.0424

Sucrose-containing layer (Fig. 12 B)

Ages examined	P values
Dev. – 1 d	9.90×10^{-4}
1 d – 2 d	0.0504
2 d – 2.5 d	0.1800
2.5 d – 3 d	4.80×10^{-4}
3 d – 4 d	0.0016

Microsomal membrane (Fig. 12 B)

Ages examined	P values
Dev. – 1 d	0.0152
1 d – 2 d	0.0905
2 d – 2.5 d	0.0135
2.5 d – 3 d	5.20×10^{-5}
3 d – 4 d	0.0240

Table A.1.8 Changes in sums of low-density particle and intermediate-density particle protein with age (Fig. 13)
Independent sample t-tests

Ages examined	P values
Dev. – 1 d	0.6187
1 d – 2 d	0.1175
2 d – 2.5 d	0.3340
2.5 d – 3 d	0.0015
3 d – 4 d	0.1944

Comparing every second age

Ages examined	P values
Dev. – 2 d	0.0421
1 d – 2.5 d	0.2092
2 d – 3 d	0.0203
2.5 d – 4 d	0.0004

Table A.1.9 Changes in α-amylase inhibitor activity of low-density particles with different sample treatments (Fig. 33)
Paired t-tests

Treatments	Developing	1 day-old
	P values	
α-amylase – native sample	0.0013	0.0283
α-amylase – heat denatured sample	0.4140	0.5090
α-amylase – PK digested sample	0.1039	0.7867

Table A.1.10 Changes in α-amylase inhibitor activity of LDPs with age (Fig. 33)
Independent sample t-test

Ages examined	P value
Dev. – 1d	0.7802

Table A.1.11 Change in total protein and total triacylglycerol with seed development (Fig. 39)
Independent sample t-tests

Total protein

Ages examined	P values
Dev. – 1 d	0.0004
1 d – 2 d	0.0039
2 d – 2.5 d	0.1442
2.5 d – 3 d	2.42×10^{-7}
3 d – 4 d	0.0387

Total triacylglycerol

Ages examined	P values
Dev. – 1 d	0.2194
1 d – 2 d	0.1199
2 d – 2.5 d	4.93×10^{-6}
2.5 d – 3 d	0.2289
3 d – 4 d	0.0208

Table A.1.12 Changes in each lipid class in each fraction with age (Fig. 40)
Independent sample t-tests

Low-density particles (Fig. 40 A)

Ages examined	P values for each lipid class				
	PL	DG	FFA	TG	S/WE
Dev. – 1 d	0.4344	0.6771	0.0590	0.8453	0.3611
1 d – 2 d	0.7230	0.1865	0.5246	0.2494	0.1786
2 d – 3 d	0.0281	0.0403	0.0124	0.0028	0.0745

Intermediate-density particles (Fig. 40 B)

Ages examined	P values for each lipid class				
	PL	DG	FFA	TG	S/WE
Dev. – 1 d	0.1144	0.9786	0.0369	0.0599	0.5467
1 d – 2 d	0.2316	0.1519	0.9717	0.0816	0.9903
2 d – 3 d	0.5609	0.9641	0.2587	0.0072	0.1279

High-density particles (Fig. 40 C)

Ages examined	P values for each lipid class				
	PL	DG	FFA	TG	S/WE
Dev. – 1 d	0.097564	0.882149	0.017686	0.010853	0.391002
1 d – 2 d	0.194717	0.039185	0.098468	0.089879	0.493677
2 d – 3 d	0.528701	0.163554	0.104809	0.02986	0.473251

Microsomal membranes (Fig. 40 D)

Ages examined	P value for each lipid class				
	PL	DG	FFA	TG	S/WE
Dev. – 1 d	0.4606	0.8336	0.0722	0.0283	0.6714
1 d – 2 d	0.5507	0.0938	0.5265	0.2400	0.4661
2 d – 3 d	0.8727	0.7647	0.8472	0.6837	0.3732

Table A.1.13 Changes in total fatty acids found in each fraction with age (Fig. 41)
Independent sample t-tests

Ages examined	P values for each fraction			
	LDPs	IDPs	HDPs	MM
Dev. – 1 d	0.8459	0.0702	0.1411	0.3867
1 d – 2 d	0.3021	0.1759	0.2586	0.4106
2 d – 3 d	0.0028	0.1348	0.0568	0.8213

Table A.1.14 Changes in proportions of phospholipid, triacylglycerol and other lipids with age (Fig. 43)
Independent sample t-tests

Low-density particles (Fig. 43 A)

Ages examined	P values for each lipid		
	Phospholipid	Triacylglycerol	Other lipids
Dev. – 1 d	0.4347	0.8877	0.2746
1 d – 2 d	0.1007	0.0160	0.0172
2 d – 3 d	0.0751	0.0050	0.0747

Intermediate-density particles (Fig. 43 B)

Ages examined	P values for each lipid		
	Phospholipid	Triacylglycerol	Other lipids
Dev. – 1 d	0.1117	0.0463	0.0093
1 d – 2 d	0.0033	0.0001	0.0424
2 d – 3 d	0.0421	0.0084	0.0328

High-density particles (Fig. 43 C)

Ages examined	P values for each lipid		
	Phospholipid	Triacylglycerol	Other lipids
Dev. – 1 d	0.6549	0.2847	0.3361
1 d – 2 d	0.0080	0.8545	0.0029
2 d – 3 d	0.0477	0.1779	0.1271

Table A.1.15 Changes in the proportions of phospholipid fatty acid with age (Fig. 44)

Independent sample t-tests

Low-density particles (Fig. 44 A)

Ages examined	P values for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.8638	0.0073	0.1921	0.3195	0.6569
1 d – 2 d	0.6610	0.0891	0.0434	0.4589	0.8602
2 d – 3 d	0.1279	0.0367	3.91×10^{-5}	0.0009	0.0007

Intermediate-density particles (Fig. 44 B)

Ages examined	P values for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.7225	0.0979	0.4384	0.8779	0.5019
1 d – 2 d	0.5179	0.1291	0.4141	0.2852	0.8621
2 d – 3 d	0.1336	0.0359	0.3168	0.0226	0.0002

High-density particles (Fig. 44 C)

Ages examined	P values for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.0116	0.0318	0.0185	0.0229	0.4364
1 d – 2 d	0.0246	0.0211	0.3268	0.3170	0.1109
2 d – 3 d	0.5550	0.2125	0.7082	0.3239	0.4227

Microsomal membranes (Fig. 44 D)

Ages examined	P value for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.7299	0.0926	0.0555	0.4870	0.9683
1 d – 2 d	0.7456	0.1828	0.0918	0.2003	0.6744
2 d – 3 d	0.2970	0.2351	0.2290	0.1164	0.5105

Table A.1.16 Changes in the proportion of triacylglycerol fatty acid with age (Fig. 45)

Independent sample t-tests

Low-density particles (Fig. 45 A)

Ages examined	P values for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.8792	0.0080	0.0154	0.2068	0.3430
1 d – 2 d	0.1763	0.1392	0.6471	0.6777	0.1768
2 d – 3 d	0.0257	0.0164	0.1758	0.0022	0.4801

Intermediate-density particles (Fig. 45 B)

Ages examined	P values for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.1404	0.7512	0.3197	0.5207	0.0040
1 d – 2 d	0.0158	0.1165	0.3824	0.4878	0.0397
2 d – 3 d	0.1534	0.0025	0.1185	0.5213	0.7903

High-density particles (Fig. 45 C)

Ages examined	P values for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.9246	0.3941	0.6360	0.0806	0.2783
1 d – 2 d	0.5358	0.0015	0.8022	0.1985	0.1388
2 d – 3 d	0.3520	0.7558	0.5702	0.5454	-

Microsomal membranes (Fig. 45 D)

Ages examined	P value for each fatty acid				
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Dev. – 1 d	0.6104	0.6681	0.8999	0.2219	0.0221
1 d – 2 d	0.0519	0.3481	0.5773	0.8119	0.1962
2 d – 3 d	0.1903	0.0312	0.1368	0.1614	0.1064

Table A.1.17 Changes in proportions of individual lipid classes with altered homogenization (Fig. 46)

Independent sample t-tests

Intermediate-density particles (Fig 46 A)

Lipid type	P value for each homogenization techniques		
	Nitrogen-Control	Glycerol-Control	Glycerol-Nitrogen
PL	0.5606	0.0333	0.2593
DG	0.7250	0.6045	0.8881
FFA	0.3305	0.1950	0.8993
TG	0.2726	0.0259	0.2168
S/WE	0.0022	0.0553	0.2679

Low-density particles (Fig. 45 B)

Lipid type	P value for each homogenization technique		
	Nitrogen-Control	Glycerol-Control	Glycerol-Nitrogen
PL	0.0152	0.1102	0.0268
DG	0.7967	0.3546	0.1330
FFA	0.5871	0.0362	0.0180
TG	0.4198	0.1837	0.1120
S/WE	0.3501	0.0397	0.0053

Table A.1.18 Changes in the proportions of LDPs and IDPs collected using different methods of homogenization (Fig 47)
Paired t-tests

	P value for each homogenization technique		
	Nitrogen-Control	**Glycerol-Control**	**Nitrogen-Glycerol**
LDP-IDP	3.79×10^{-6}	1.04×10^{-5}	0.0483

Appendix 2

Calculations

These are formulae for calculating the amount of phospholipid and triacylglycerol that would fit into a particle of a given size with a triacylglycerol core and a phospholipid monolayer from Huang (1992).

To calculate the volume of triacylglycerol and phospholipid required:

$$\text{Volume of triacylglycerol} = {}^{4}/_{3}\,\pi\,(r-t)^3$$

$$\text{Volume of phospholipid} = {}^{4}/_{3}\,\pi\,r^3 - {}^{4}/_{3}\,\pi\,(r-t)^3$$

where r is the radius of the particle and t is the thickness of the phospholipid monolayer.
The thickness of the monolayer is estimated at 0.0025 μm (Huang 1992).

To calculate the mass of triacylglycerol and phospholipid in grams:

Triacylglycerol = volume of triacylglycerol x density of triacylgycerol

Phospholipid = volume of phospholipid x density of phospholipid

where the average density of triacylglycerol is 0.92×10^{-3} g•μm^{-3} and the average density of phospholipid is 1.03 x 10-3 g•μm^{-3} (Huang 1992)

To calculate the mass of fatty acid found in the triacylglycerol and phospholipid:

Triacylglycerol fatty acid = mass of triacylglycerol x 0.8973 g fatty acid•g triacylglycerol^{-1}

Phospholipid fatty acid = mass of triacylglycerol x 0.7726 g fatty acid•g phospholipid^{-1}

where the average proportion of fatty acids making up triacylglycerol is 0.8793 and the average proportion of fatty acids making up phospholipid is 0.7726.

For example, low density particles isolated from 2 day-old seeds have a diameter of 0.181 μm:

$$\begin{aligned}\text{Volume of triacylglycerol} &= {}^{4}/_{3}\,\pi\,(r-t)^3 \\ &= {}^{4}/_{3}\,\pi\,(0.0905\ \mu m - 0.0025\ \mu m)^3 \\ &= {}^{4}/_{3}\,\pi\,6.815 \times 10^{-4}\ \mu m^3 \\ &= 2.854 \times 10^{-3}\ \mu m^3\end{aligned}$$

Volume of phospholipid = $^4/_3 \pi r^3 - {}^4/_3 \pi (r-t)^3$
$= {}^4/_3 \pi\ 0.0905\ \mu m^3 - {}^4/_3 \pi\ (.0905\ \mu m - 0.0025 \mu m)^3$
$= 3.105 \times 10^{-3}\ \mu m^3 - 2.854 \times 10^{-3}\ \mu m^3$
$= 2.503 \times 10^{-4}\ \mu m^4$

Mass of triacylglycerol = $2.854 \times 10^{-3}\ \mu m^3 \times 0.92 \times 10^{-3}\ g \bullet \mu m^3$
$= 2.626 \times 10^{-6}\ g$

Mass of phospholipid = $2.503 \times 10^{-4}\ \mu m^3 \times 1.03 \times 10^{-3}\ g \bullet \mu m^3$
$= 2.578 \times 10^{-7}\ g$

Mass of triacylglycerol fatty acid = $2.626 \times 10^{-6}\ g \times 0.8973$
$= 2.356 \times 10^{-6}\ g$

Mass of phospholipid fatty acid = $2.578 \times 10^{-7}\ g \times 0.7726$
$= 1.9918 \times 10^{-7}\ g$

Calculating proportions of triacylglycerol and phospholipid fatty acids in a particle made up solely of these two lipids:

Percentage triacylglycerol = $2.356 \times 10^{-6}\ g / (2.356 \times 10^{-6}\ g + 1.9918 \times 10^{-7}\ g) \times 100\ \%$
$= 92.20\ \%$

Percentage phospholipid = $1.9918 \times 10^{-7}\ g / (2.356 \times 10^{-6}\ g + 1.9918 \times 10^{-7}\ g) \times 100\ \%$
$= 7.80\ \%$

Experimental values ± standard deviations are: 76.92 ± 4.07 % triacylglycerol, 6.95 ± 1.56 % phospholipid and 16.14 ± 3.01 % other lipid.