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DEDICATION

I wish to dedicate this thesis to my parents Marie and William for their love, trust and support throughout all my endeavors.

ABSTRACT

Oat globulin has been characterized on the basis of its protein chemistry, immunological relationships and biosynthesis. Results demonstrate widespread similarities between this storage protein derived from monocotyledons and storage proteins from dicotyledons.

Oat globulin subunits were shown to interact through disulfide linkages. Subunits were purified by preparative gel-electrophoresis and their amino acid profiles are reported. The α subunit (Mr, 20 000) was shown to have basic isoelectric points and the β subunit (Mr 40 000) contained neutral and acidic isoelectric points.

Antibodies were raised against oat globulin and isolated using protein A-Sepharose and globulin-Sepharose. These antibodies showed cross-reactivity against rye and pea salt soluble proteins.

Polysomes were isolated from developing oat seeds and used to direct in vitro translation in a wheat germ extract. Globulin was isolated from the translated products using anti-globulin IgG and protein A-Sepharose. Results suggest that oat globulin is synthesized as a 60 000 Mr precursor similar to legume globulins. Two dimensional analysis of translation products indicated that no α subunit was synthesized as such in vitro. Intact mRNA was isolated and translated in vitro.

High molecular DNA was isolated from groats and blotted onto nitrocellulose. A legumin clone was used to probe oat DNA. Results indicate that oat DNA and legumin DNA contain complementary sequences.

On the basis of these findings one may predict that oat and legume globulin arise from similar genes or gene families.

Oat lipase was also investigated. An assay using glycerol tri[1 - ^{14}C] oleate was developed which could be used to assay lipase from flour suspensions and aqueous extracts. Lipase was membrane bound, had a pH optimum of 7.5, a Km of 2-3 mM and appeared to act on di- and mono-glycerides. Lipase was purified 250 fold by extracting microsomes with 0.2% Triton X-100 followed by hydrophobic chromatography and gel filtration on Sephacryl S-200.

STUDIES ON OAT, AVENA SATIVA L.:

Part A. Partial Characterization of Oat Lipase

Part B. Structure and Biosynthesis of Globulin

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ABBREVIATIONS

2' + 3' AMP	2' + 3' adenosine monophosphate (mixed isomers)
ATP	adenosine triphosphate
cDNA	complementary DNA
CNBr	cyanogen bromide
DG	diglycerides
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GTP	guanosine triphosphate
FFA	free fatty acid
Hepes	2-(N-2-hydroxyethylpiperazine-N) ethane sulphonic acid
IEF	isoelectric focusing
IgG	immunoglobulin
K	one thousand
Mr	molecular weight
O.D.	optical density
Oligo-dT	oligo thymidylic acid
PBS	phosphate buffered saline
PMSF	phenylmethane sulphonyl fluoride
Poly A^+ RNA	polyadenylated RNA
Poly A^- RNA	RNA fraction lacking polyadenylic acid
RNAase	ribonuclease
S	Svedberg unit
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

TLC	thin layer chromatography
TCA	trichloracetic acid
TEMED	N,N,N',N-tetramethyl-ethylenediamine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol.
TG	Triglycerides

ACKNOWLEDGEMENTS

I wish to especially thank my supervisor Dr. I. Altosaar for his advice, patience and endless support throughout my graduate studies. Without his efforts this thesis would not have been possible.

I am grateful to Dr. B.J. Miflin for his many useful suggestions and allowing me the opportunity to work in his laboratory. I especially wish to acknowledge the kind help I received from Dr. P.R. Shewry, Dr. M. Kreis and Janice Forde during my stay at Rothamsted.

I would like to thank Dr. V. Mézl and Dr. B. Irving for their many useful discussions.

I wish to gratefully acknowledge the help and advice I received from Dr. S. Fabijanski. I am indebted to Dr. Croy for providing me with the legumin clone.

I wish to thank Dr A. Urquhart and Dr Sahasrabudhe for their help and suggestions.

I am indebted to L. Robert for providing me with various protein samples and K. Garson for help in isolating the various polysome preparations.

I thankfully acknowledge the financial support provided by Agriculture Canada.

Part A. Partial Characterization of Oat Lipase

A) INTRODUCTION

1) The Role of Lipase in Cereals

During the germination of cereals various physiological changes take place. One of these changes is the mobilization of nutrient reserves such as lipids, carbohydrates and storage protein. To breakdown these reserves various enzymes are required which include amylases, proteases and lipases. The aleurone layer (outer portion of the seed) acts as a source of these enzymes. The release of enzymes from the aleurone layer is largely under the control of the embryo. The embryo releases the hormone gibberellin which causes the release or synthesis of aleurone enzymes (Tomos and Laidman 1979).

Triglycerides may serve as the major source of metabolic energy during the early stages of germination (Tavener and Laidman 1972). An increase in lipase activity of aleurone tissue coincides with the stimulation of fatty acid oxidation (Tomos and Laidman 1979). Thus, lipase plays an important role during the germination of cereals.

Oats contain about 5 to 9% oil (Brown and Craddock 1972). Oats are unique among cereals in that 80% of the groat lipid is in the endosperm. About 50% of the total lipids in oats are made up of triglycerides and about 30% are phospholipids and glycolipids (Sahasrabudhe 1979). The predominant free fatty acids contained in triglycerides are oleic acid (45%) and linoleic acid (35%) (Sahasrabudhe 1979). In view of these observations

the predominant substrate for lipase in oat is probably triglycerides of which oleic acid and linoleic acid are the most likely hydrolysis products.

2) Plant Lipases

Several lipases have been described in plants of which castor bean lipase has been studied in detail (Muto and Beevers 1974, Ory et al. 1960, Ory et al. 1968). Castor bean contains two lipases, one has an acid pH optimum of 4.3 which hydrolyses predominantly triglycerides of chain length between C_4 to C_8. Unsaturated fatty acids of C_{18} are also hydrolyzed but not as rapidly as the short chain fatty acids. This lipase is most active after 2 days germination. The second castor bean lipase has an alkaline pH optimum (pH 9.0). The alkaline lipase is most active after 3 to 5 days germination. There is very little lipase activity in the ungerminated seed. The alkaline lipase hydrolyses only monoglycerides. The acid lipase is contained within fat-containing organelles (spherosomes). The alkaline lipase is associated with the glyoxysome membranes. Thus it appears that fatty acids are released in the spherosomes and are then transferred to glyoxysomes where β-oxidation occurs in plants. The exact function of the monoglyceride lipase of the glyoxysomal membrane is not understood.

Tavener and Laidman (1972) have studied lipase in germinating wheat. Wheat lipase activity appeared only after germination, similar to castor bean. It was revealed that glutamine was responsible for the induction of lipase activity. The lipase

activity originated in the aleurone cells. Inhibition of both RNA and protein synthesis prevented lipase induction suggesting that lipase is synthesized upon germination and is not stored in the mature grain.

Rosnitschek and Theimer (1980) have studied the properties of lipase from rapeseed. A microsomal preparation was used as the lipase source in this study. Lipase was assayed using a titrimetric procedure and sunflower oil as a substrate. The initial lipolysis was linear for at least 5 min and proceeded thereafter for another 10 min. The Km of rapeseed lipase was 6.5 mM. Using radioactive substrate (glycerol triolein [carboxyl C^{14}]) mixed with sunflower oil they monitored the production of free fatty acids, diglycerides and monoglycerides. The various products were separated by thin layer chromatography and the distribution of radioactivity in each was determined. In this manner it was determined that lipolytic membranes of rapeseed were capable of splitting triglycerides into glycerol and free fatty acids. 1,3 -diglycerides were utilized more efficiently than 1,2 -diglycerides. This lipase could not hydrolyse water-soluble triacetin or the phospholipid lecithin. Rapeseed lipase showed a preference for triglycerides that contained a high portion of unsaturated fatty acids such as oleic acid and linoleic acid. The activity of the microsomal lipase increased five fold in the presence of 0.1 M NaCl. The presence of 55 mM $CaCl_2$ also increased lipase activity. Low concentrations of digitonin, SDS, Triton X-100 and Tween 80 caused an inhibition of lipolysis.

Lipase activity was investigated in the storage tissue of several oil seeds during germination by Huang and Moreau (1978). Lipase activity was investigated from castor bean, peanut, sunflower, cucumber, cotton, corn and tomato. It was determined that the two-lipase system observed in castor bean was not present in the other oil seeds. The six other oil seeds studied contained no acid lipase and the alkaline lipase predominated. Furthermore lipase was not contained in the lipid bodies in the dry seed thus indicating that lipid hydrolysis occurred in other cellular compartments. It was determined that pea lipase was present in the glyoxysomes and mitochondrial membrane fractions. The authors suggested that each kind of oil seed contains its own unique mechanism of lipase action.

The study of rice bran lipase is interesting because this is the only seed lipase which has been purified (Funatsu et al. 1971). Lipase was extracted from defatted rice bran in 10 mM $CaCl_2$ pH 6.0 followed by centrifugation at 5 000 r.p.m., to obtain a crude extract. The extract was brought to 60% saturation with ammonium sulfate and the resulting precipitate was resuspended and dialysed. Insoluble material was removed resulting in a clear supernatant which was chromatographed on DEAE cellulose. In this manner 80% of the total enzyme activity was recovered in the clear supernatant and 40% of the total activity recovered from the DEAE column. The active peak from the DEAE column was chromatographed on Sephadex G-75 from which 20% of the total activity could be recovered. Lipase was further purified by two chromatography steps through carboxymethyl

Sephadex. The enzyme was purified 4700 fold of which the recovery from total was about 4%. The enzyme was electrophoretically homogeneous and had a basic isoelectric point of 8.56. It had a molecular weight of about 40 000. .The kinetics of the enzyme reaction and the substrate specficities were not investigated in their study. The results obtained on rice bran lipiase suggested that this enzyme was not membrane bound. Detergents were not required to solubilize the lipase prior to the various column chromatography techniques.

3) Oat Lipase

Oat lipase has been studies in the most detail by Martin and Peers (1953). The release of oleic acid and burtyric acid from triolein and tributyrin was studied in their investigation. They observed that the outer aleurone layers contained most of the lipase activity. It was reported that oat lipase was 2.5 times more active when using triolein as a substrate than if tributyrin was used.

Groats were ground in distilled water followed by centrifugation at 3 000 r.p.m. The result was an opalescent aqueous solution which contained lipase activity. Adjusting the pH of this solution to 5.5 and 4.8 caused precipitation to occur however 80% of the total lipase activity was lost by this procedure. Ethanol added to 75% (v/v) did not cause a precipi tate of this aqueous extract. The aqueous extract was made up to 80% saturation with ammonium sulfate. However, both the precipitate and supernatant were inactive even after dialysis against H_2O. Lipase could be adsorbed onto a calcium phosphate

gel; however it was not possible to elute the enzyme from the gel, although it remained active on this gel for 10 days at 4°C. Freezing and thawing the aqueous extract caused a precipitate to form which contained most of the total lipase activity. This precipitate was, however, insoluble in a wide variety of solvents, hence further purification of the oat lipase was not achieved. This precipitate represented a 2 000 fold purification on a dry weight basis.

Various solvents were used to extract lipids from groats which included petroleum ether, benzene and ether, acetone, butanol, methanol and chloroform. No loss in lipase activity was observed after extracting groats with petroleum ether or benzene and ether. The other solvents caused a partial or total loss in lipase activity (Martin and Peers 1953).

Paper electrophoresis of the crude aqueous solution demonstrated the presence of 3 protein components, two of which remained in solution after freeze-thawing.

The pH optimum of lipase obtained after freeze-thawing was 7.4. The optimum temperature for lipase activity was 37°C. The Km of the partially purified enzyme was 6.2 mM when using tributyrin as a substrate. It was also reported that this lipase could hydrolyse only tributyrin and not di- or monobutyrin (Martin and Peers 1953).

Oat lipase was then re-investigated by Berner and Hammond (1970). They investigated the specificity of lipases on lard and cocoa butter. Oat lipase was prepared by grinding oat in buffer or water followed by centrifugation to obtain an aqueous

extract. They confirmed Martin and Peers (1953) observation that oat lipase had a pH optimum of 7.4 and was most active against long chain unsaturated fatty acids similar to what Martin and Peers (1953) observed. Purification of lipase was not attempted.

Frey and Hammond (1975) investigaged the utilization of oil from oat seeds. One of the factors involved in the use of oat seed oil was the potent lipase which caused deterioration of the oil quality. Thus they investigated lipase activity from 352 oat varieties. To screen out cultivars for lipase activity seeds were pressed into tributyrin agar. After 24 hrs at room temperature the size of the clear zone was judged visually on a scale from 0-4. Zero activity represented no visible clear zone. By such a method it was shown that 58 samples had no lipase and 12 samples had large clear zones. Lipase was also measured colorimetrically in dough mixtures containing various levels of water. Additions of water to 25-50% of the seed weight increased lipase activity against soybean oil, while 75-100% water caused inhibition of lipase activity. Purification was not atempted.

4. RESEARCH OBJECTIVE

The objective of this investigation was to purify lipase from oats. A method of assaying oat lipase using radioactivity labelled substrate was developed. This method was used during the purification studies and to partially characterize oat lipase.

The purpose of purifying lipase was so that studies may be initiated regarding protein biosynthesis in oats. The strategy was to prepare anti-serum against purified lipase. Using immunoprecipitation lipase could then be identified from *in vitro* translation products directed by mRNA. In this manner it could be determined when the lipase gene is being expressed. The possibility that lipase is synthesized as a precursor may have also been investigated in this manner.

MATERIALS AND METHODS

B) Materials

Cereal grains were obtained from the Ottawa Research Station, Agriculture Canada. In most of the experiments on oat (Avena sativa L.), the cultivar Hinoat was used. Elgin, Harmon, and Sentinel were used where indicated. In one experiment, wheat (Neepawa), barley (Perth), and rye (Gazelle) were used. Grain was stored at -20°C until required. Gum arabic, olive oil, sodium dodecyl sulfate (SDS), sodium deoxycholate, sucrose, triolein, and Triton X-100 were obtained from Sigma Chemical Co. Glycerol tri[1-^{14}C]oleate (46.1 milli-Curie/mmol) was obtained from the Radiochemical Center, Amersham, IL, and diluted with cold triolein or olive oil in benzene before use. Silica gel G was obtained from Merck. All other chemicals were reagent grade.

Methods

1. Radioisotope Lipase Assay

Dry, ungerminated oats were manually dehulled and ground for 30 sec in a Phillips domestic electric coffee mill. After Soxhlet lipid extraction in petroleum ether (40-60°C) for 2 hr, the defatted flour was air-dried and reground in a mortar and pestle to disperse the powder. The standard assay mixture for oat flour suspensions contained 0.05 M Tris-HCl buffer, pH 7.5, 1% Triton X-100, 0.2% benzene, and 10 mM

glycerol tri[1-^{14}C]oleate (0.05 micro-Curie/mmol) in a final volume of 10 ml in a 50 ml Erlenmeyer flask. The assay mixtures were agitated vigorously and then placed in a shaking water bath (37°C, similar to Martin and Peers, 1953). After equilibration for 15 min, the reaction was started by adding 1 g samples of the defatted oat flour. Aliquots (1 ml) were transferred at intervals of 0 to 30 min into 15 ml glass centrifuge tubes containing 2 ml of chloroform/methanol (1:1). This mixture terminated the reaction and extracted the lipids. The tubes were then centrifuged at 3,000 x g for 5 min, the lipid-containing, chloroform layer transferred to small test tubes, and the chloroform evaporated to dryness under N_2. The lipid was then redissolved in small volumes of chloroform, and aliquots were spotted on silica gel G thin-layer plates (0.25 mm, prepared in the laboratory). The lipids were fractionated with petroleum ether/diethyl ether/acetic acid (85:15:1, v/v). Iodine vapor was used to locate the separated lipid components. Figure 1 demonstrates a TLC plate showing the migration of free fatty acids and triglycerides after various hydrolysis times. The free fatty acid, triglyceride, and other lipid fractions were then scraped into separate scintillation vials and radioactivity determined by liquid scintillation counting in 5 ml of Aquasol (New England Nuclear), (Kritchevski and Malhotra 1970). Variations in the procedure (eg. changes in the reaction mixture, temp. and pH) are given for individual experiments.

FIGURE 1

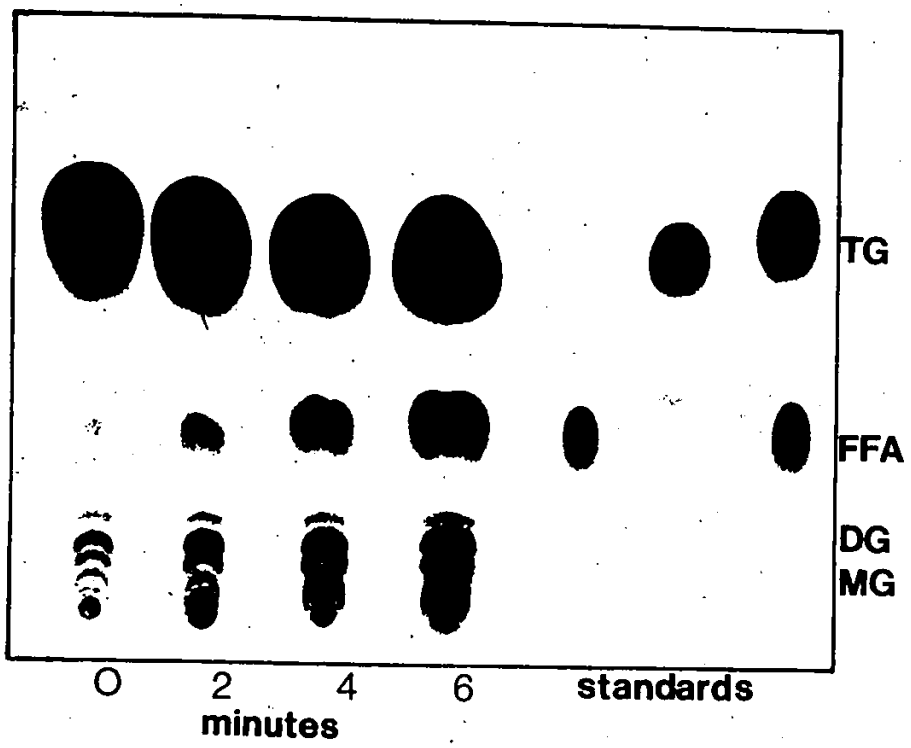


FIGURE I

Thin layer chromatography of lipids after various enzyme reaction times. Lipids were fractionated in the presence of petroleum ether/diethyl ether/acetic acid (85:15:1, v/v). Iodine vapour was used to locate lipid components. Triglycerides and free fatty acids were located by comparison with standards.

TG - Triglycerides
DG - Diglycerides
MG - Monoglycerides
FFA - Free Fatty Acids

Liberated fatty acid, labeled in the carbonyl group, was determined by calculating the ratio of radioactivity recovered in the free fatty acid fraction to that in the total lipid recovered. This ratio, multiplied by the number of moles of triglyceride supplied times 3 (moles of fatty acid per mole of triglyceride), gives the moles of fatty acid released for each time interval. Rates of triglyceride hydrolysis were calculated from the linear part of the time course. One unit of activity was defined as 1 μmole of fatty acid released per minute under the condition described for this assay. A sample calculation is as follows. Given the free fatty acid spot contains 115 DPM at a given time and the total lipid radioactivity for that time interval is 2175 DPM, the ratio of free fatty acid to the total is 0.053. At zero time the total radioactivity observed for free fatty acids was negligible. This ratio, .053, is multiplied by 10 mM (substrate concentration) and by 3 (3 moles FFA per mole of triglyceride). Thus the final concentration of free fatty acid is .053 x 10 x 3 = 1.59 mM or 1.59×10^{-2} mMole FFA present in the 10 ml assay volume, containing 1.0g flour. This calculation is carried out for each time interval.

Lipase was assayed in a similar manner in aqueous solutions. Extracts containing lipase were added into assay mixtures to yield a final substrate concentration of 10 mM triolein under the above described conditions.

2. Cytochrome c Reductase Assay

The reaction mixture contained 20 mM KH_2PO_4/K_2HPO_4, pH 7.2, 0.2 mM NADH, 0.02 mM oxidized cytochrome c. To this was added 0.2 ml of solution to be assayed, resulting in a final volume of 3.5 ml. The absorption at 550 nm was monitored and production of reduced cytochrome c was calculated using the formula A_{550} = 21.1 $mM^{-1}cm^{-1}$X path length X [concentration of reduced cytochrome c].

3. SDS-PAGE and Protein Determinations

Performed as described in Part B Chapter 2, Methods Section 2,4.

4. Many of the other techniques such as column chromatography are described during the Results and Discussion section.

C) RESULTS AND DISCUSSIONS

1. Lipase Activity in Oat Flour Extracts

The time course for triglyceride hydrolysis was linear for up to 6-10 min under the assay conditions described in the method section (Figure 2). The initial velocity was about 6.5 mMole X 10^{-3} FFA released/min. Velocities were based on the hydrolysis during the first 5 min. A linear relationship was obtained between initial velocity and defatted oat flour for the range investigated (Figure 3). For subsequent experiments 1.0 g of defatted flour was used. The effect of substrate concentration is shown in Figure 4. The intercept of -1/Km on the Lineweaver-Burk plot was -1/0.32. The apparent Michaelis constant was thus estimated to be about 3 mM. This was lower than the value reported by Martin and Peers (1953) who obtained a Km of about 6.5 mM. Martin and Peers (1953) were however using a manometric assay in the presence of tributyrin. Tributyrin was not used as a substrate in my assays because triolein is the major triglyceride in the mature seed (Sahasrabudhe 1979). The pH optimum for the 3 oat varieties tested was about 7.5 which is similar to the value of 7.4 reported by Martin and Peers (1953) (Figure 5). Hinoat appeared to have the highest lipase activity of the varieties tested here. Frey and Harmon (1975) reported a 20- fold variation in lipase activity among 352 varieties. They assayed lipase by inserting a seed in agar containing tributyrin. The lipase activity was proportional to the clearing zone. In my study it appeared that Hinoat contained about twice as much activity as Harmon.

FIGURE 2

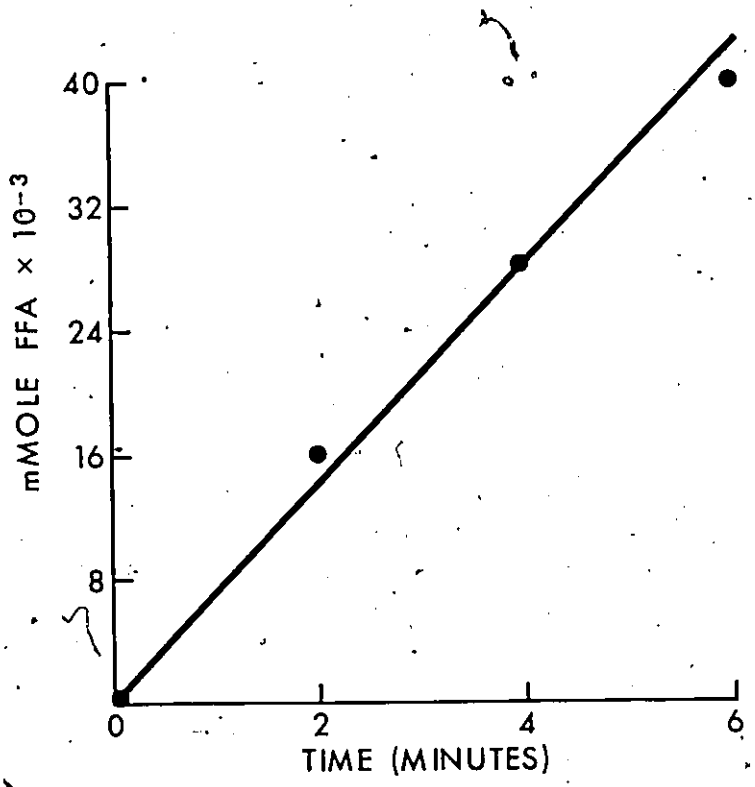


FIGURE 2

The release of free fatty acids in the presence of 1.0 gram defatted oat flour after various reaction times. Reactions were carried out according to Methods section 1. Each point is taken from an average of three assays. The standard deviations ranged from 1 to 2 mM FFA X 10^{-3}.

FIGURE 3

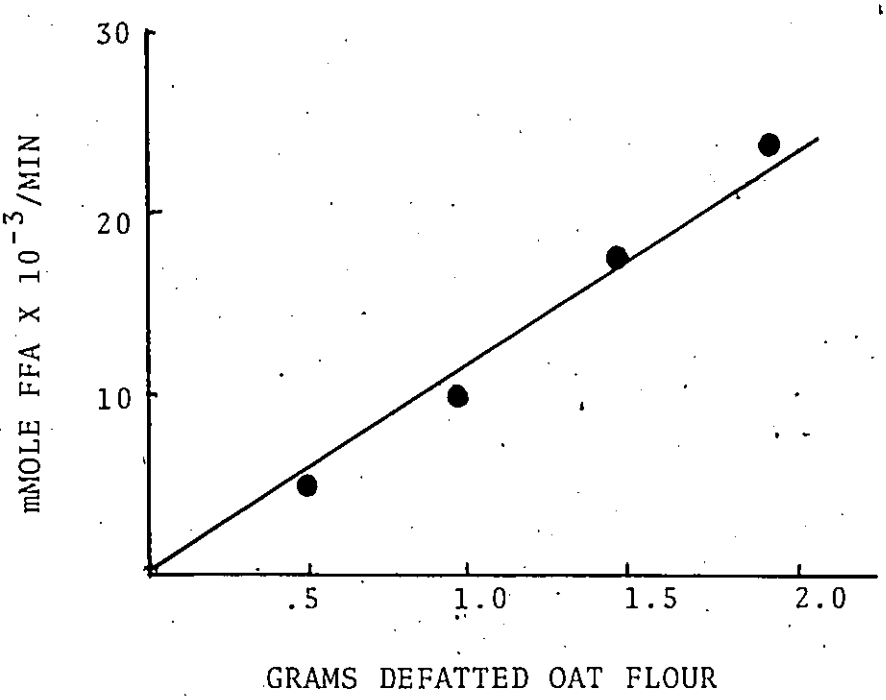
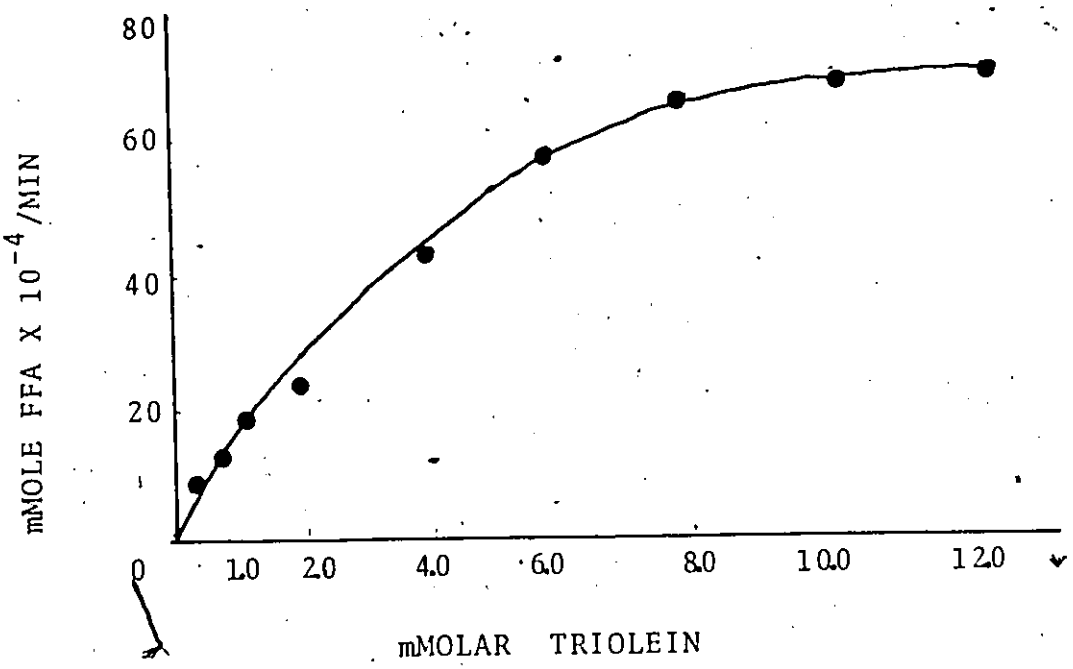


FIGURE 3

The release of free fatty acids in the presence of various amounts of defatted oat flour. Reactions were carried out according to Methods section 1. Each point is taken from an average of three assays. The standard deviations ranged from 2 to 4 mM FFA X 10^{-3}.

FIGURE 4


80
60
40
20
0
mMOLE FFA X 10^{-4}/MIN
1.0
2.0
4.0
6.0
8.0
10.0
12.0
mMOLAR TRIOLEIN

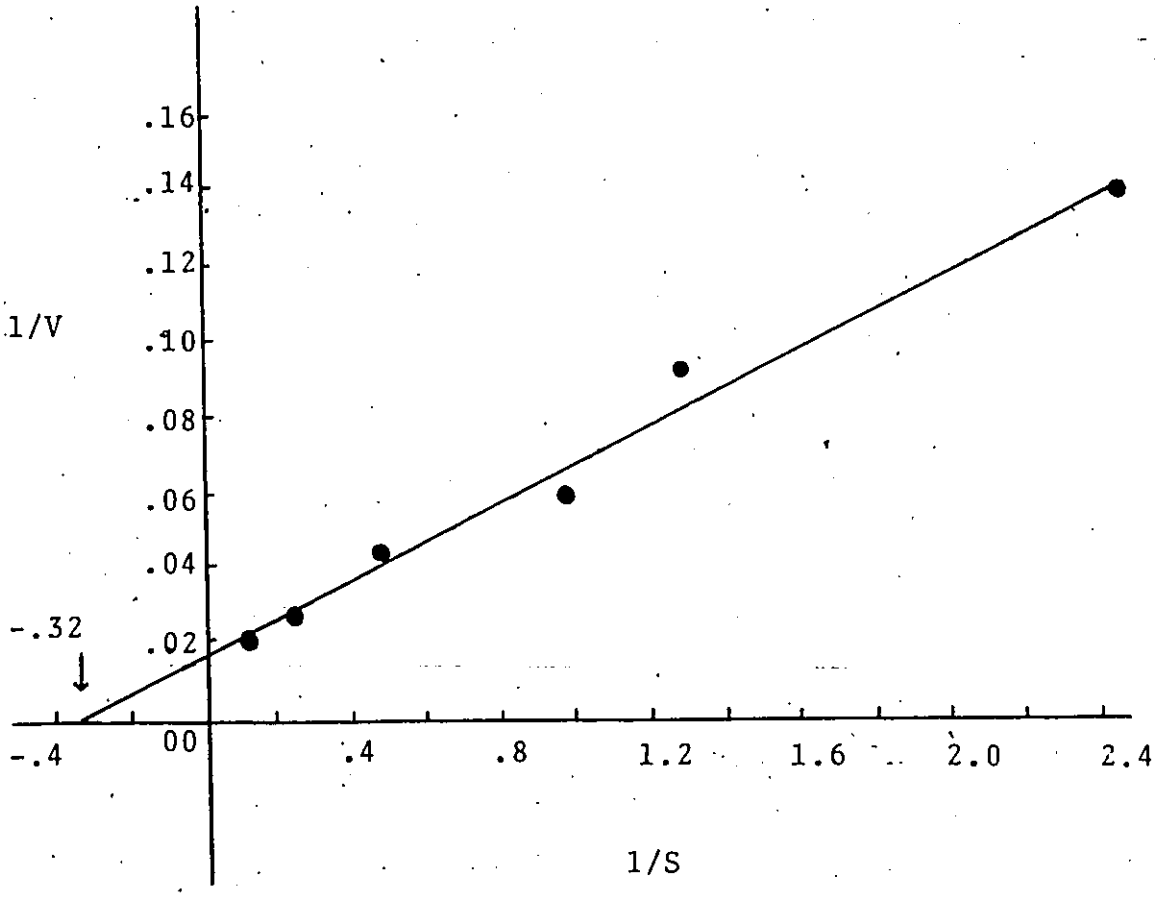

.16
.14
.12
.10
.08
.06
.04
.02
1/V
-.32
-.4
00
.4
.8
1.2
1.6
2.0
2.4
1/S

FIGURE 4

The release of free fatty acids in the presence of various amounts of triolein. One gram of defatted oat flour was used per assay. Assays were carried out as described in Methods section 1. A Lineweaver - Burk plot was obtained from plotting the inverse of the velocity versus the inverse of the substrate concentration. Standard deviations ranged from 5 to 10 mMole FFA X 10^{-4}.

FIGURE 5

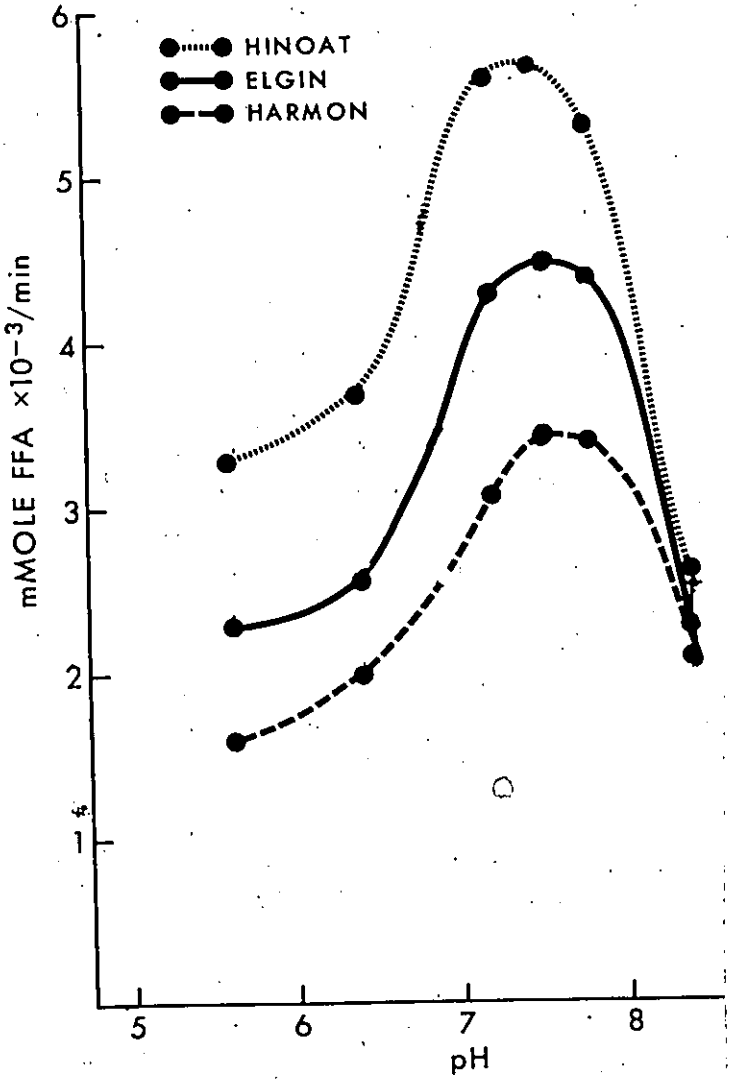


FIGURE 5

The effect of pH on lipase activity from oat flour suspensions using the three varieties indicated. Assays were carried out in the presence of 1.0 gram defatted oat flour and 10 mM triolein as described in Methods section 1.

Lipase activity required the presence of Triton X-100 (Table 1). Controls lacking detergent were almost inactive, presumably because triolein is insoluble in aqueous media. In samples containing 0.5-2.0% (v/v) Triton X-100, lipase activity was significantly higher. Triton X-100 at 1% (v/v) was routinely used because higher concentrations resulted in difficulties in separating the lipid fractions by thin layer chromatography.

Low levels of benzene (0.2 and 0.3%) increased lipase activity above the control with no benzene (Table II). Higher levels (0.5 and 1.0%) significantly decreased the activity. Hexane at 0.2% did not have the same effect as benzene. Higher levels of hexane were not tried.

Lipase is believed to act on the substrate at a water-lipid interface (Desnuelle 1961). Triton X-100 and benzene may facilitate arrangement of the long chain fatty acids at this interface. In rapeseed, however Triton X-100 inhibited lipase activity (Rosnitschek and Theimer 1980).

Several studies on plant lipases have used gum arabic to stabilize water-lipid emulsions (Dundas et al 1978, Muto and Beevers 1974, Theimer and Rosnitschek 1978). In contrast, this study showed no effect of gum arabic on oat lipase activity (Table III). The effect of gum arabic in the absence of Triton X-100 or benzene was not tried.

The methods used most commonly for assaying plant lipases are titrimetric (Tavener and Laidman 1972, Theimer and Rosnitschek 1978), colorimetric determination of copper soaps

TABLE I

Effect of Triton X-100 on Lipase Activity in Oat Flour Suspensions [a]

Triton X-100[b]	Lipase Activity (umole/min/g flour)
0	0.25 ± 0.06 [c]
0.5	4.40 ± 0.69
1.0	5.56 ± 0.40
2.0	6.19 ± 0.57

a) Reaction mixtures contained 0.05 M Tris/HCl, pH 7.5, 0.2% benzene and 10 mM triolein. Values are means of duplicate experiments.

b) Percent Triton X-100 (v/v) in 10 ml of reaction mixture.

c) Standard deviation values.

TABLE II

Effect of Benzene and Hexane on Lipase Activity[a]

Solvent[b] (% v/v)	Lipase Activity umole/min/g flour
Hexane	
none	1.44 ± 0.82[c]
0.2	1.17 ± 0.55
Benzene	
none	1.40 ± 0.80
0.2	4.76 ± 0.97
0.3	3.30 ± 0.02
0.5	0.50 ± 0.02
1.0	0.21 ± 0.06

[a]Reaction mixtures contained 0.05 M Tris/HCl, pH 7.5, 1.0% v/v Triton X-100 and 10 mM triolein. Values are means of duplicate experiments.

[b]Solvent as percentage (v/v) in the 10-ml reaction mixture.

[c]Standard deviation values.

TABLE III

Effect of Gum Arabic on Lipase Activity [a]

Gum Arabic [b] (%)	Lipase Activity (μ mole/min/g flour)
0	6.05 ± 1.82 [c]
1	7.02 ± 2.18
2	6.89 ± 2.45
4	6.24 ± 1.94
6	5.05 ± 0.19
8	4.26 ± 0.64

a) Reaction mixtures contained 0.05 M Tris/HCl, pH 7.5, 1% Triton X-100, 0.2% benzene, 10 mM triolein. Values are means of duplicate experiments.

b) Percent (w/v) gum arabic in 10 ml reaction mixture.

c) Standard deviation values.

(Huang and Moreau 1978) and fluorimetric methods (Mutor and Beevers (1974). Radioactive substrates have been used with castor bean (Borgstrom and Ory 1970) and rapeseed (Rosnitschek and Theimer 1980). My study on oat lipase also makes use of a radioactive substrate (triolein). This was a suitable substrate since this is the major storage triglyceride in oats (Sahasrabudhe 1979). Martin and Peers (1953) used tributyrin and triolein in their assays and determined that the ratio of release of oleic acid/butyric acid was about 2.5. Tributyrin however is not found in mature oat grain (Sahasrabudhe 1979). Nevertheless, the Km and pH optimum reported by Martin and Peers (1953) using tributyrin are similar to those values observed here using triolein. Martin and Peers (1953) did not perform kinetic studies using triolein as a substrate.

2. Lipase Activity in Flour Extracts from other Cereals

The same assay was performed on wheat, rye and barley and compared to oat. The flour suspensions from ungerminated grain had little activity as compared to oat (Table IV). Widhe and Onselius (1948) reported similarly that wheat and rye had no lipase activity in the ungerminated grain. They also found no increase in lipase activity in oats or rye after two days of germination. In comparison the results presented here showed increases in both oats and rye after two days germination. Little activity was observed in wheat or barley either before or after germination. Tavener and Laidman (1972) on the other hand, demonstrated an increase in lipase activity in wheat after

TABLE IV

Lipase Activity in Germinated and Ungerminated Cereals

cereal	Lipase Activity [a] μ mole FFA/min/g flour ungerminated	germinated [b]
Oat (Hinoat)	8.43 ± 1.01	11.90 ± 1.05 [c]
Wheat (Neepawa)	0.65 ± 0.04	0.80 ± 0.07
Barley (Perth)	1.09 ± 0.16	0.60 ± 0.19
Rye (Gazelle)	0.93 ± 0.05	3.20 ± 0.08

a) Lipase activity was determined from hydrolysis of glycerol tri [1- ^{14}C] oleate in 0.05 M Tris/HCl, pH 7.5, 1% (v/v) Triton X-100, 0.2% benzene, 10 mM triolein at 37°C. Values are means of duplicate experiments.

b) Soaked overnight in water, germinated on damp filter paper at 25°C for two days.

c) Standard deviation values.

two days of germination. It should be noted however that the optimum conditions for the assay of oat, wheat, rye and barley lipases may vary for each cereal. The activities reported in Table IV are under the specific assay conditions described here and have been optimized only for oat.

Hüang and Moreau (1978) have similarly reported that peanut, sunflower, cucumber, corn and tomato have little or no lipase activity in the mature grain. The reason why oat contains an active lipase in mature grain is unknown. Frey and Hammond (1975) reported that this leads to oil deterioration during grain storage. Dr. Urquhart in our laboratory has observed lipase activity throughout seed development. Martin and Peers (1953) similarly reported the presence of lipase during seed development. Lipase may be synthesized as an inactive precursor in oats. This precursor may become activated upon storage or during enzyme assays. The possibility also exists that there is more than one enzyme which is synthesized at various times during development or germination. This has been observed in castor bean (Muto and Beevers 1974).

3. Solubilization and Partial Purification of Oat Lipase

Martin and Peers (1953) reported that because of the insolubility of lipase obtained from their most pure preparation further purification was not possible. Berner and Hammond (1970) and Frey and Hammond (1975) studied lipase using aqueous extracts

or crude homogenates. Purification was not attempted.

The final strategy I adopted in attempting to purify lipase was arrived at after pursuing several unsuccessful approaches. The majority of the latter included various chromatography techniques using a lipase-containing aqueous extract. The difficulties encountered arose mostly because lipase was not behaving as a soluble protein. I then resorted to isolating membrane (a microsomal fraction) which contained lipase activity. Muto and Beevers (1974) and Rosnitschek and Theimer (1980) observed in castor bean and rapeseed that lipase was contained in microsomes. A fraction enriched in microsomal bound proteins could be obtained, and this fraction was then treated with various amounts of Triton X-100 in order to liberate lipase from the membrane fraction. Having achieved this, hydrophobic chromatography and gel filtration steps were employed to try to purify lipase further from the microsomal extract. However additional problems were encountered with stability and recovery once lipase had been liberated from membranes.

The following protocol is the one which has been the most successful. It is reproducible but unfortunately has not been improved upon. Mature seeds (30g) were soaked overnight in distilled water then ground to a homogeneous slurry with a mortar and pestle at 4°C in 100 ml of 0.05N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$, 15% (w/v) sucrose. The slurry was filtered

through cheesecloth and the filtrate was centrifuged at 5000 g for 15 min. The pellet was discarded and the opaque supernatant (aqueous extract) re-centrifuged in the Ti60 rotor at 100 000 g for 3 hours to separate the cell soluble fraction from the microsomal particles. The resulting supernatant was about 70 ml and the microsomal pellets were taken up in 40 ml of the above buffer. Lipase activity was determined for the microsomal suspension and the cell soluble fraction. Figure 6 shows a time course for the two reactions. Taking into account the difference in final volumes for each fraction, the microsomal suspension still demonstrated about 2 times more total activity than the cell soluble fraction (Table V, p.55). To verify that the pellet obtained after ultracentrifugation contained microsomes a cytochrome c reductase assay was performed on the resuspended pellet as described in the methods section 2. Figure 7 demonstrates that cytochrome c reductase is within this suspension. Theimer and Rosnitschek (1978) have likewise used cytochrome c reductase as a microsome marker for rapeseed. However, cytochrome c reductase should have also been assayed in the cell soluble fraction. It may be possible that the cell soluble fraction still contained microsomal particles. Thus, further experiments involving higher speeds of ultracentrifugation could increase the yield of lipase in the microsome pellet.

FIGURE 6

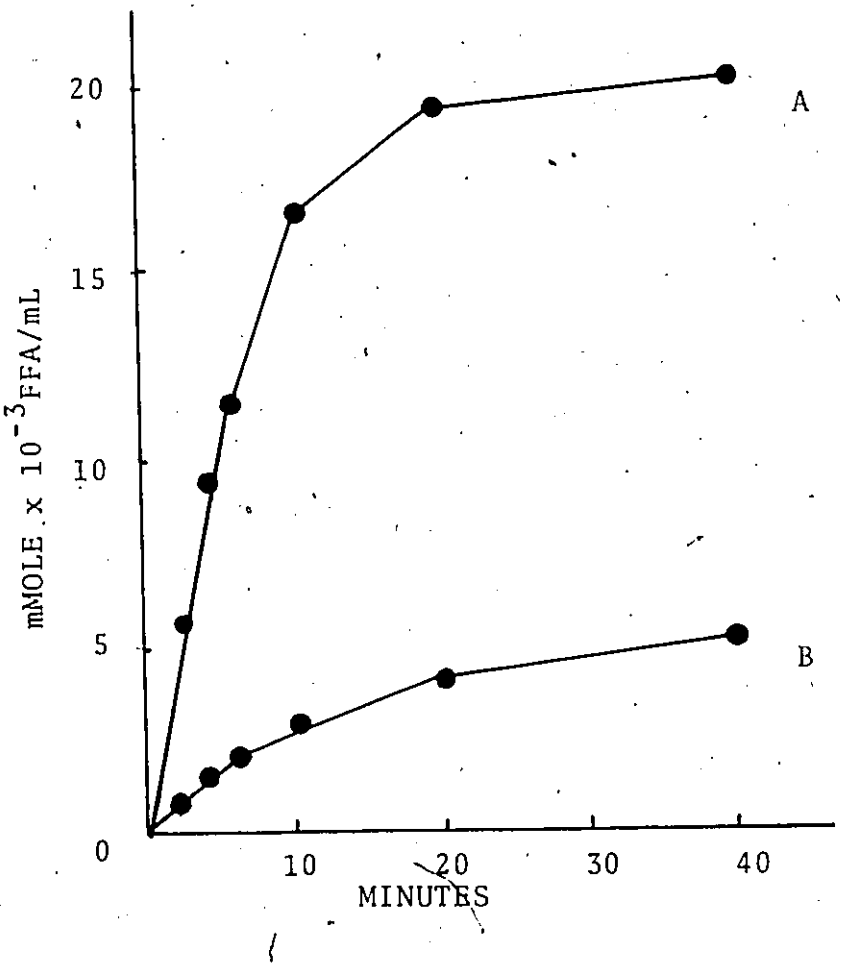


FIGURE 6

Lipase assays performed on microsomal suspensions and the cell soluble fraction. Samples (1.0 ml) were added to 1.0 ml of reaction mixture resulting in a mixture containing 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$, 1.0% (v/v) Triton X-100 and 10 mM triolein. Curve A was obtained from assaying the microsomal suspension and curve B was obtained from assaying the cell soluble fraction.

FIGURE 7

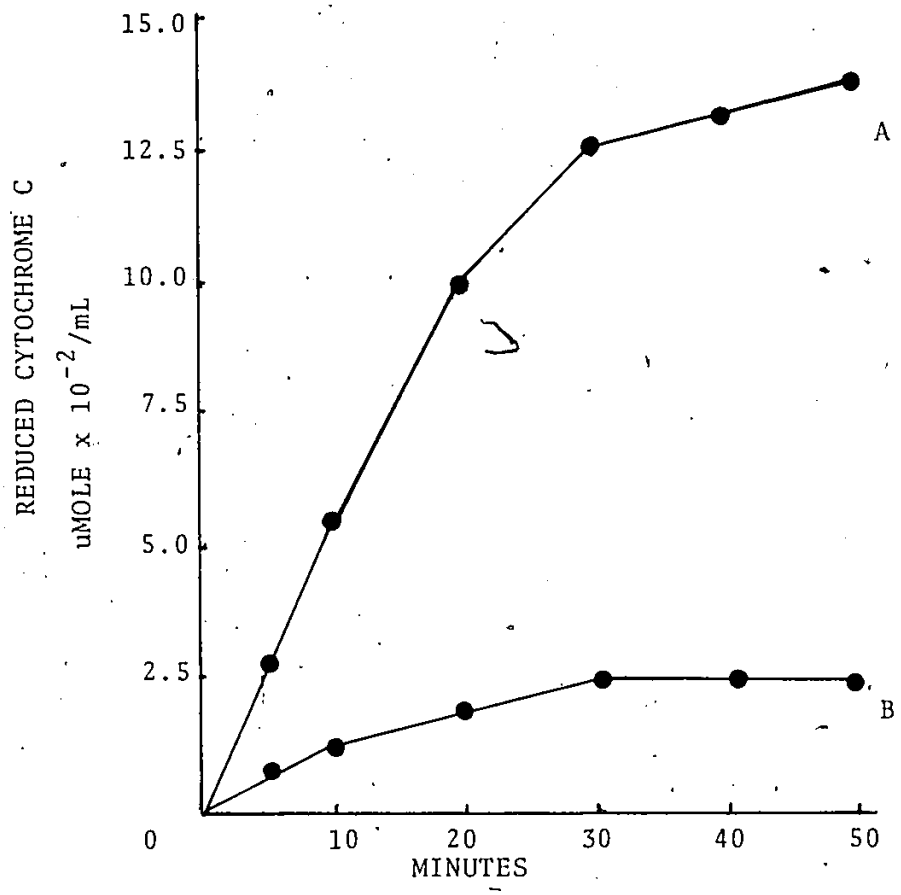

15.0
12.5
10.0
7.5
5.0
2.5
0
10
20
30
40
50
MINUTES
REDUCED CYTOCHROME C
uMOLE x 10^{-2}/mL
A
B

FIGURE 7

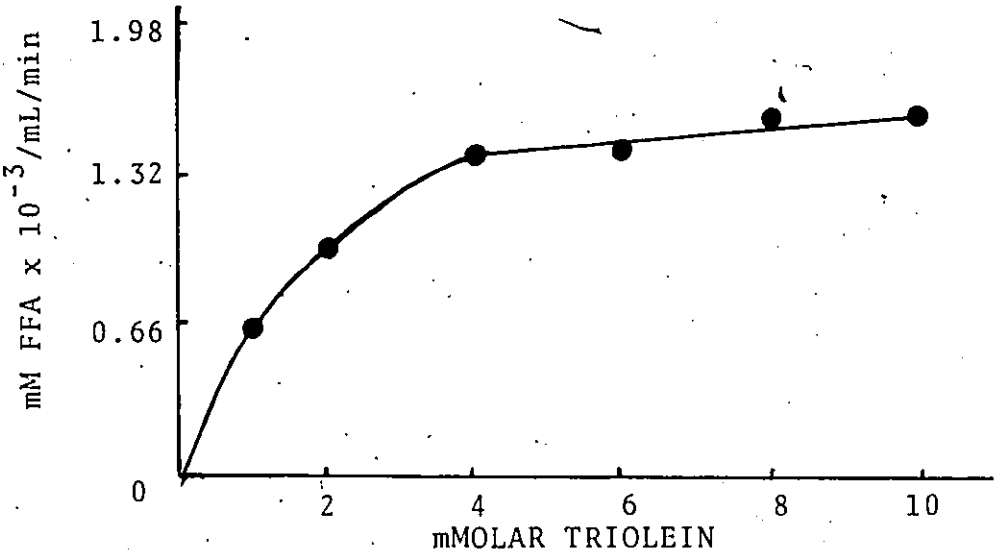
Cytochrome C reductase assay performed on the microsomal suspension. The assay described in Methods section 2 contained 20 mM KH_2PO_4/K_2HPO_4, pH 7.2, 0.2 mM NADH, 0.02 mM oxidized cytochrome C and 0.2 ml of microsomal suspension. The final volume was 3.5 ml. Curve A was obtained in the presence of microsomal suspension and curve B was obtained for the blank.

These results suggest that lipase is membrane bound in mature seeds or it becomes non-specifically attached to organelle membranes during the microsomal preparation. Lipase has been observed to be membrane bound in rapeseed (Rosnitschek and Theimer 1980). Martin and Peers (1953) also presented results which suggested that lipase may be membrane bound in oats. They observed that grinding oats in distilled H_2O followed by centrifugation at 3000 rpm resulted in an opalescent solution. They reported that this solution could be clarified by repeated filtration through filter paper. The resulting clear filtered extract contained very little lipase activity while the activity was associated with the material causing the opalescence. This opalescent material may have been rich in microsomal particles.

The effect of substrate concentration on lipase activity in the microsomal suspension was investigated (Figure 8). The Km value was approximately 2.1 as determined by the Lineweaver-Burk plot. This is slightly less than the value obtained when assaying the flour suspension. Such a small difference may not be significant, however this may also be due to a variety of reasons. Possibly this is because the substrate is more accessible to the enzyme in microsomal suspensions than in flour suspensions, or the substrate may bind non-specifically to other material in the flour. The pH optimum of the microsomal bound lipase (Fig. 9) shows a sharp peak with an optimum of 7.5 similar to the value reported for flour suspensions (Figure 5).

In order to determine if this lipase could act upon diglycerides and monoglycerides, a distribution was followed of the radioactivity in triolein and in the products of the

FIGURE 8


1.98
1.32
0.66
0
mM FFA x 10^{-3}/mL/min
2
4
6
8
10
mMOLAR TRIOLEIN

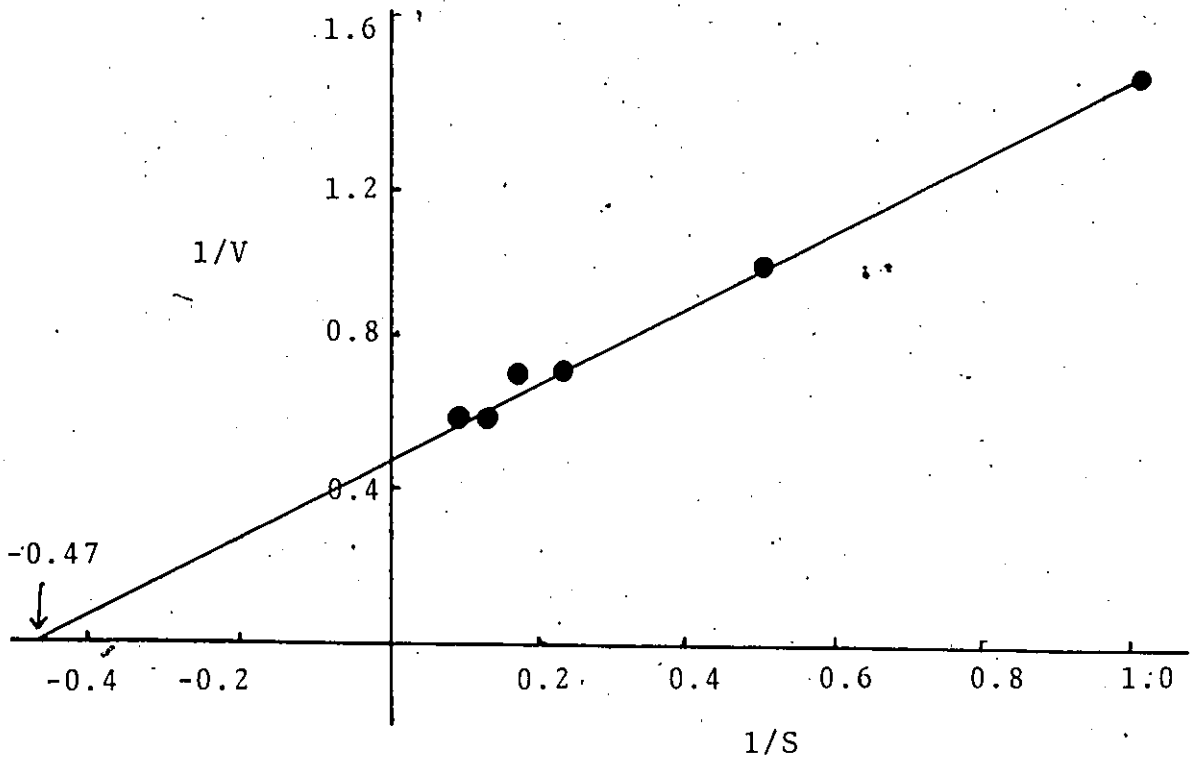

1.6
1.2
0.8
0.4
1/V
-0.47
-0.4
-0.2
0.2
0.4
0.6
0.8
1.0
1/S

FIGURE 8

The effect of triolein concentration on the release of free fatty acid when assaying the microsomal suspension. A Lineweaver - Burk plot was obtained from plotting the inverse of the velocity versus the inverse of the substrate concentration. Microsomal suspension samples (1.0 ml) were added to 1.0 ml of reaction mixture resulting in a mixture containing 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$, 1.0% (v/v) Triton X-100 and varying amounts of triolein.

FIGURE 9

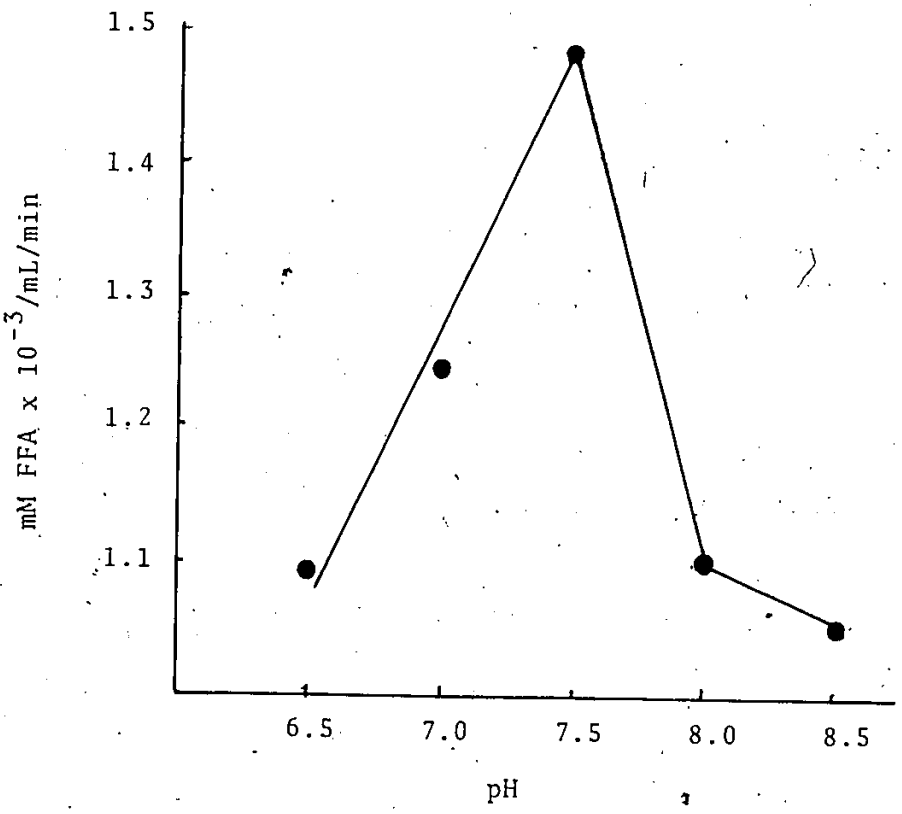


FIGURE 9

The effect of pH on lipase activity from the microsomal suspension. Microsomal suspension samples (1.0 ml) were added to 1.0 ml of reaction mixture resulting in a mixture containing 0.05 N Tris/HCl at various pHs, 2.0 mM $CaCl_2$, 1.0% Triton X-100 and 10 mM triolein.

enzyme reaction. The same method has been used to investigate lipase specificity in rapeseed (Rosnitschek and Theimer 1980). Results are presented in Figure 10. After 6 minutes of hydrolysis the diglyceride and monoglyceride fraction contained about 38% of the total radioactivity. After 10 minutes of hydrolysis this value dropped to about 30%. This suggests that the microsomal-bound lipase is capable of hydrolysing diglycerides and possibly monoglycerides. More studies however must be undertaken using either pure diglycerides or pure monoglycerides as the enzyme substrate to verify the enzymes' specificities.

Microsomal preparations (5.0 ml) were incubated with varying amounts of Triton X-100 in an attempt to release lipase from the membrane. The incubations in the presence of increasing amounts of Triton X-100 were carried out at 4°C with gentle shaking for 2 hrs.

The samples were re-centrifuged at 100 000 xg for 3 hrs and the pellets taken up in 5.0 ml of 0.05 N Tris/HCl, pH 7.5, 2 mM $CaCl_2$. The pellet suspensions and resulting supernatants were assayed for lipase activity (Figure 11). Of the Triton X-100 levels used the most effective was 0.2% (v/v). In the absence of detergent (0% Triton X-100) no enzyme activity could be detected in the supernatant. Concentrations of Triton X-100 in excess of 0.5% (v/v) resulted in a drop of enzymatic activity both in the supernatants and pellets. Thus it appears that lipase becomes inactive by prolonged exposure to high concentrations of Triton X-100. This may be a reason why enzymatic activity drops after about 10 min during a routine

FIGURE 10

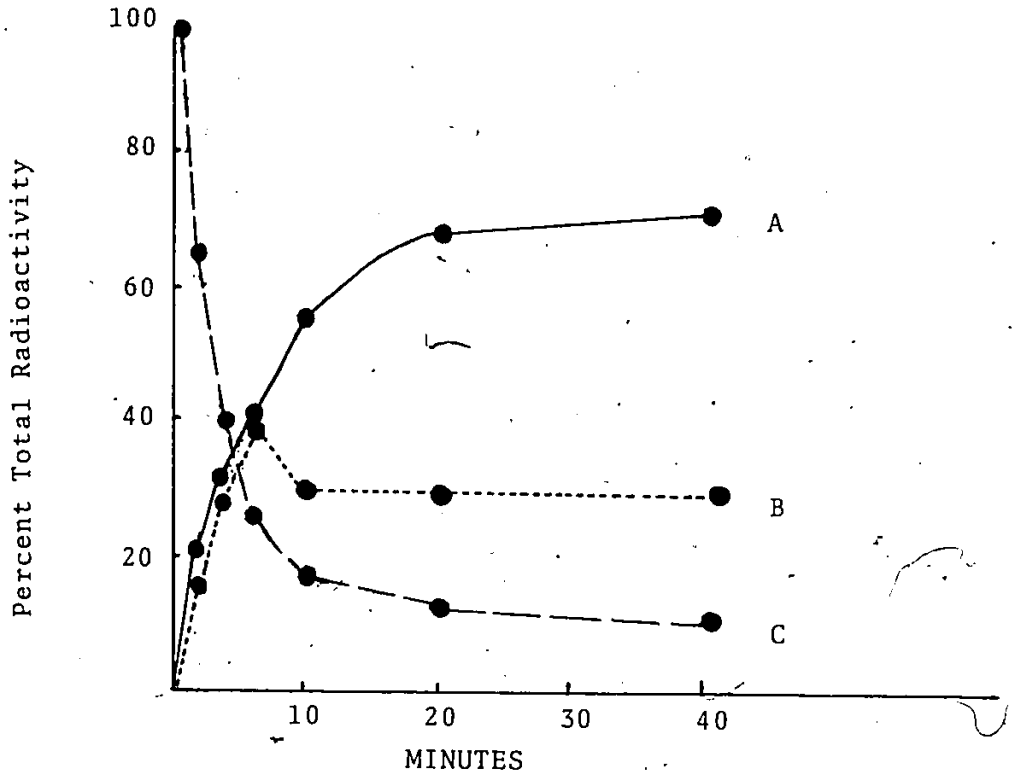

Percent Total Radioactivity
100
80
60
40
20
10
20
30
40
MINUTES
A
B
C

FIGURE 10

The effect of lipase on di- and monoglycerides. A microsomal suspension (1.0 ml) was added to 1.0 ml of reaction mixture resulting in a mixture containing 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$, 1.0% Triton X-100 and 10 mM triolein. Lipid components were fractionated by TLC then identified, isolated and counted. The di- and monoglycerides were (shown in Figure 1) were pooled together as one fraction.

A) Radioactivity contained in the free fatty acid fraction.

B) Radioactivity contained in the diglyceride fraction.

C) Radioactivity contained in the triglyceride fraction.

FIGURE 1[1]

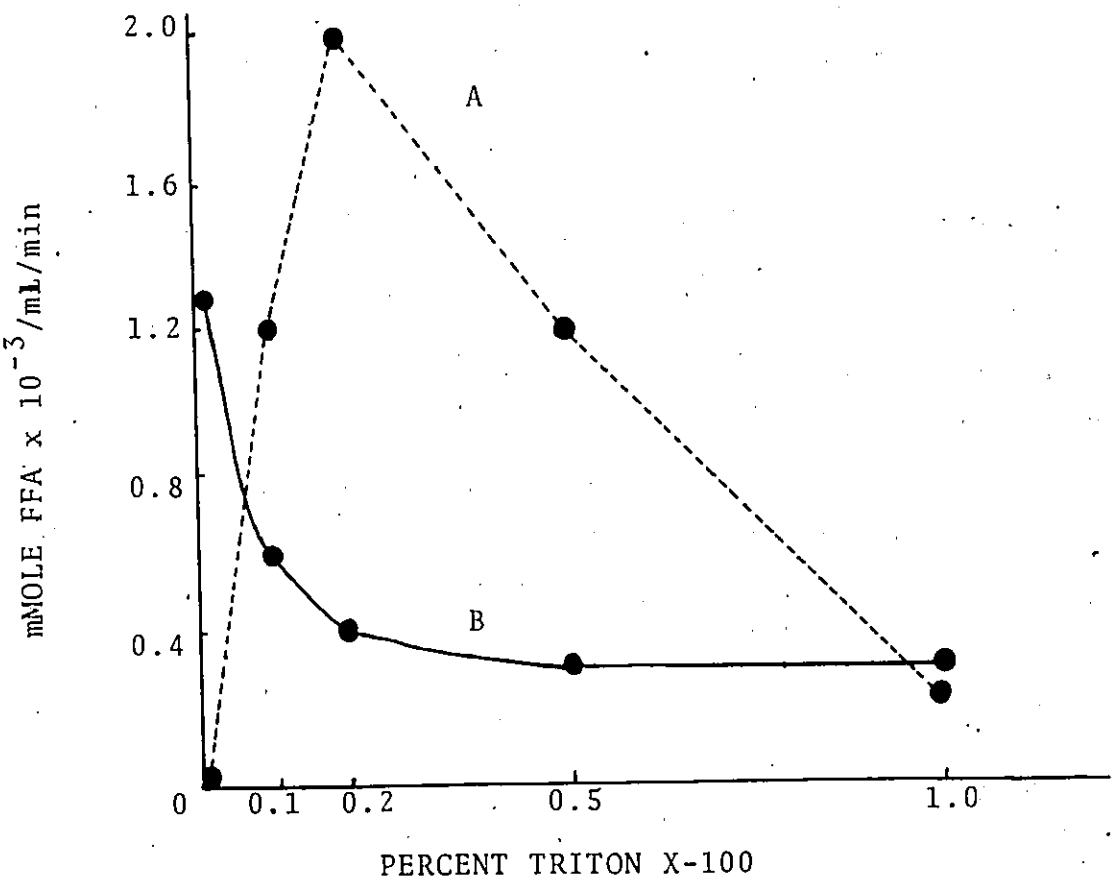

2.0
1.6
1.2
0.8
0.4
0
0.1
0.2
0.5
1.0
A
B
mMOLE FFA x 10^{-3}/ml/min
PERCENT TRITON X-100

FIGURE 11

The effect of varying amounts of Triton X-100 in solubilizing lipase from microsomes. Microsomal suspensions containing varying amounts of Triton X-100 were incubated at 4°C for 2 hrs followed by ultracentrifugation. Pellets were taken up in 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$. Assays were carried out as described in Methods section 1. Curve A was obtained when assaying the supernatants and curve B was obtained when assaying the resuspended pellets.

assay which uses 1% (v/v) Triton X-100. The effect of other detergents has not been investigated. This decrease in activity may also be due to the decrease in the amount of substrate.

Attempts were made to further purify lipase from the microsomal extract. Affinity chromatography of lipase on an hydrophobic matrix was investigated. Alkylated agaroses were used with alkyl chain lengths varying from 0 to 10 carbon atoms. The bed volume of each column which had been pre-equilibrated in 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$ was 1.0 ml. Soluble microsomal extract (0.5 ml) obtained from the 0.2% (v/v) Triton X-100 extract was applied to each column, then the columns were washed with an additional 2.0 ml of the above buffer. Virtually no enzyme was bound to the unsubstituted agarose column (Figure 12), while about 30% and 70% of the enzyme activity remained bound to the ethyl and butyl agarose columns, respectively. Almost all the enzyme activity remained on the hexyl, octyl and decyl agarose columns. Thus, as would be expected for a membrane bound enzyme, lipase demonstrated a greater affinity for the more hydrophobic columns.

The above procedure was scaled up using a 40 ml bed volume of butyl agarose. Microsomal extract (20 ml) was applied to this column and once the optical density at 280 nm returned to baseline value, protein was eluted in the presence of 0.05 N Tris/HCl, pH 7.5, 2mM $CaCl_2$ and 0.5 M NaCl (Figure 13). Judging from the optical density and enzyme activity profiles, the protein eluted with 0.5 M NaCl, had the higher specific activity. About 60% of the activity applied onto the column could be

FIGURE 12

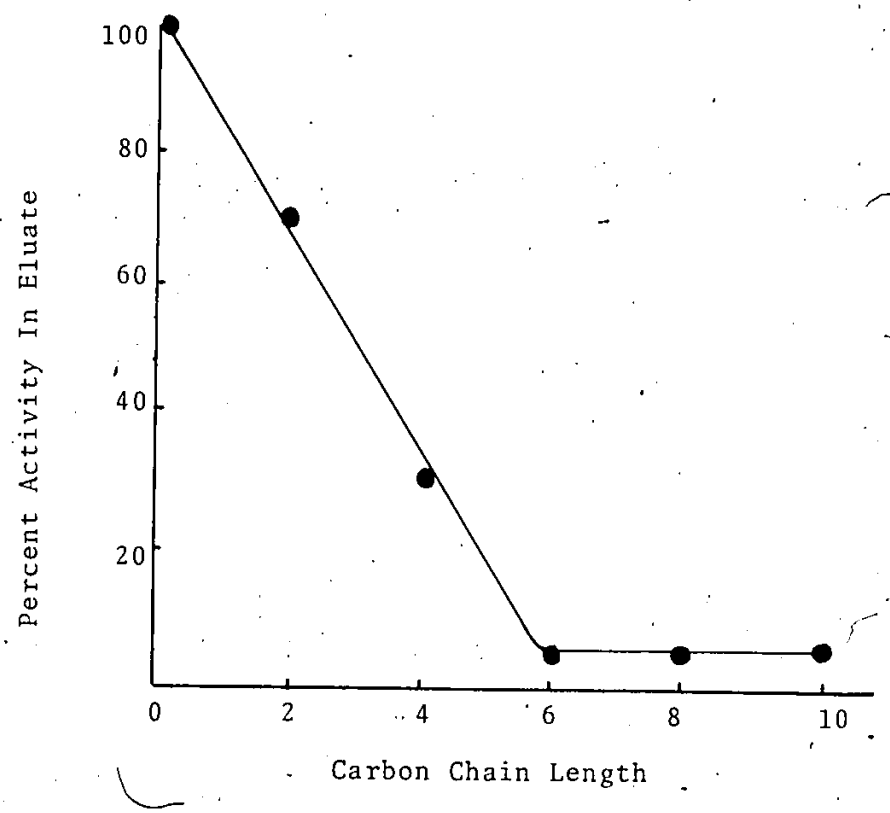

100
80
60
40
20
Percent Activity In Eluate
0
2
4
6
8
10
Carbon Chain Length

FIGURE 12

The affinity for lipase on various alkyl chain agarose columns. Lipase was solubilized from microsomes in the presence of 0.2% Triton X-100. Soluble extract (0.5 ml) was applied to each column which had a 1.0 ml bed volume. After the extract had entered the columns, each column was washed with 2.0 ml of 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$. The resulting eluates were assayed as described in Methods section 1. One hundred percent activity in eluate indicated that no lipase remained bound to the column.

FIGURE 13

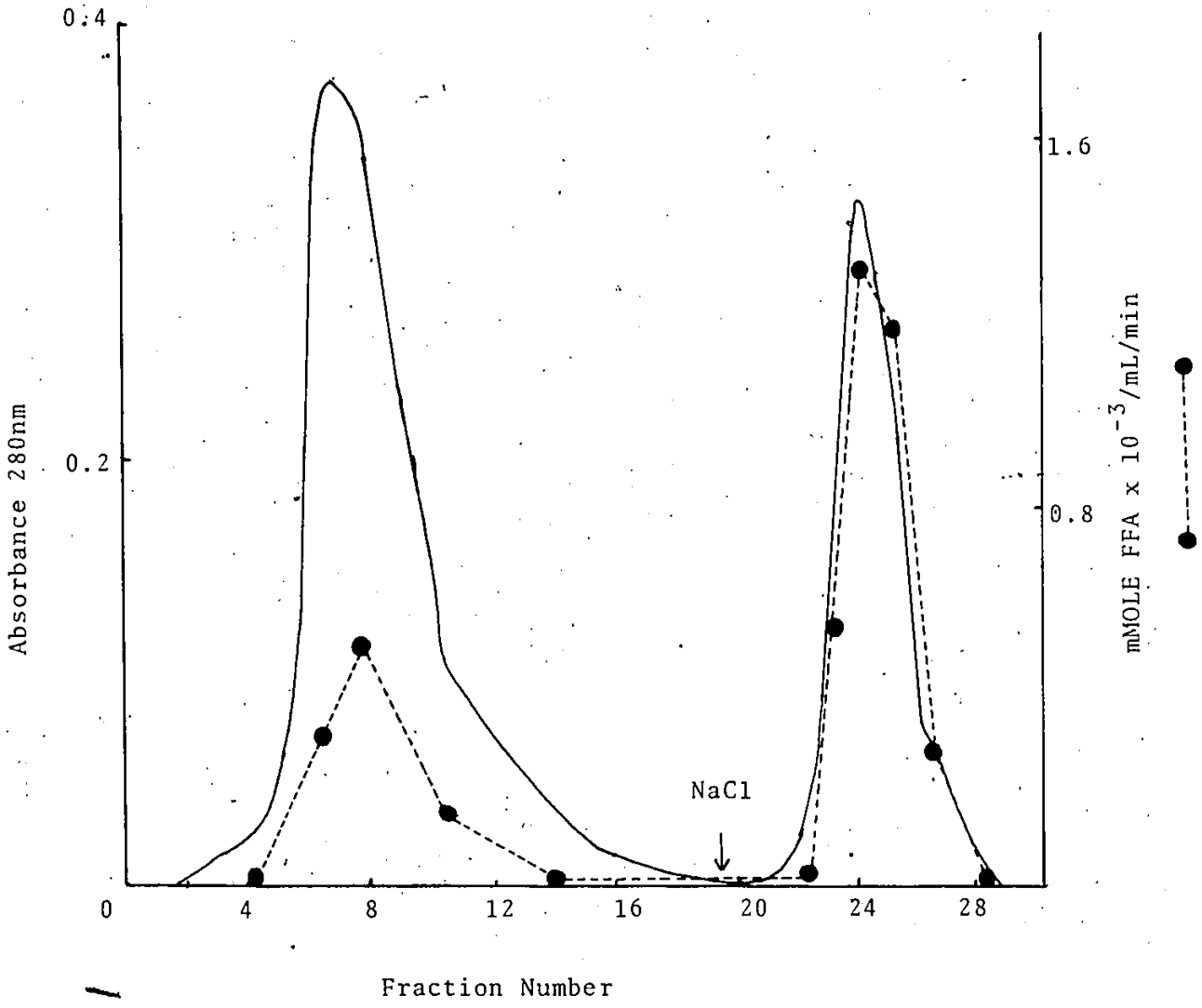

0.4
0.2
0
4
8
12
16
20
24
28
1.6
0.8
Absorbance 280nm
mMOLE FFA x 10^{-3}/mL/min
NaCl
Fraction Number

FIGURE 13

Hydrophobic chromatography of lipase on butyl agarose. Microsomal extract (20 ml) was applied to the column (8 X 2.6 cm) which contained a bed volume of 40.0 ml and the absorbance at 280 nm was monitored. The column was pre-equilibrated with 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$. Fractions contained 5.0 ml each and the flow rate was about 60 mls/hr. The above buffer containing 0.5 N NaCl was applied after fraction number 19. Fractions were assayed as described in Methods section 1.

recovered in this second peak (Table V page 55). Similar optical density profiles were obtained with hexyl-agarose. However no lipase could be recovered by increasing the salt concentration up to 1.5 M NaCl. Triton X-100 was also ineffective in eluting the bound lipase. However the lipase remained active while bound to the column (results not shown).

Lipase, eluted in the presence of 0.5 M NaCl from the butyl agarose column, was applied to a Sephacryl S-200 column which had been pre-equilibrated with 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$, 0.2% Triton X-100, 30% v/v glycerol (Figure 14). In the absence of glycerol, no enzyme activity could be recovered from the column. One peak of lipase activity was recovered from this column which appeared as a shoulder of the main 280 nm absorbing peak.

A summary of the isolation steps is given in Table V. Lipase could be purified about 250 fold using this procedure which resulted in about 7% recovery. Yield dropped substantially during the isolation of microsomes. This may be an indication that there is more than one lipase, one of which is not membrane bound. Indeed there was lipase activity in the cell soluble fraction which if added to the microsome bound enzyme fraction would account for about 80% of the total activity in the aqueous extract. Studies have not been performed to isolate the cell soluble enzyme. It was also observed that each step must be carried out promptly; not allowing any enzyme preparation to stand overnight.

FIGURE 14

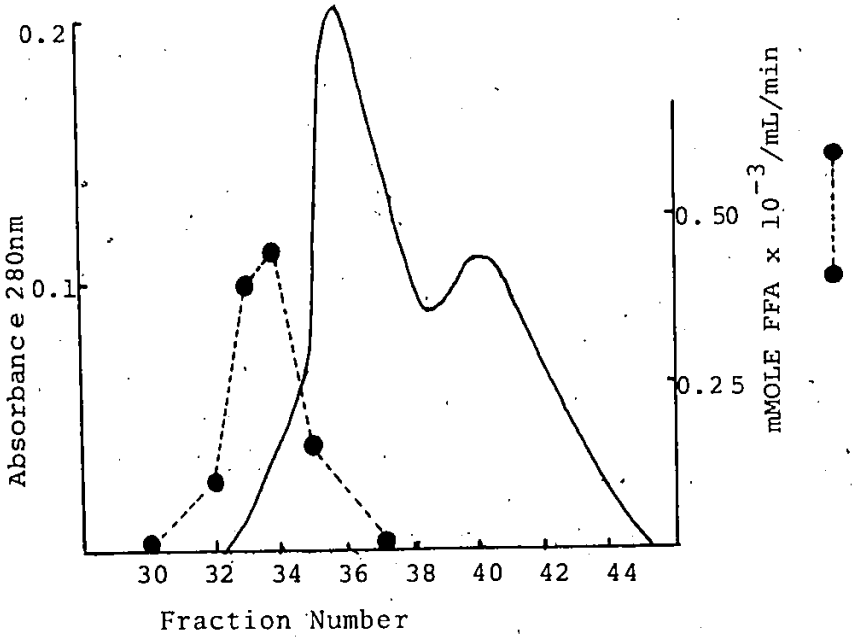

0.2
0.1
Absorbance 280nm
0.50
0.25
mMOLE FFA x 10^{-3}/mL/min
30
32
34
36
38
40
42
44
Fraction Number

FIGURE 14

Gel filtration on Sephacryl S-200 of the lipase eluted from butyl agarose. The column (2.6 X 100 cm) had been pre-equilibrated in 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$, 0.2% Triton X-100, and 30% (v/v) glycerol. The absorbance at 280 nM was monitored continuously. The bed volume was 250 ml and the sample volume was 15 ml. The flow rate was about 20 ml/hr and each fraction contained about 5.0 ml. Fractions were assayed as described in Methods section 1.

TABLE V

PURIFICATION OF LIPASE FROM OAT FLOUR[A]

PURIFICATION STEP	TOTAL VOLUME	ACTIVITY[B] UNITS/ml	TOTAL ACTIVITY UNITS.	PROTEIN[C] mg/ml	SPEC. ACT: UNITS/mg	YIELD	PURIFICATION
Crude Homogenate	100	1.5	150	45	0.033	100	1
Aqueous Extract	70	1.4	98	7.5	0.187	65	5.7
Microsomal Suspension	40	1.2	48	2.0	0.60	32	18
Cell Soluble Fraction	70	0.3	21	5	0.060	14	1.8
Triton X-100 Microsomal Extract	40	1.4	56	0.54	2.59	37	78
Butyl Agarose	20	1.6	32	0.27	5.93	21	178
Sephacryl S-200	20	0.5	10	0.06	8.33	7	251

[A]Assays were carried out in 0.05 N Tris/HCl, pH 7.5, 2 mM $CaCl_2$, 0.2% v/v benzene and 10 mM triolein

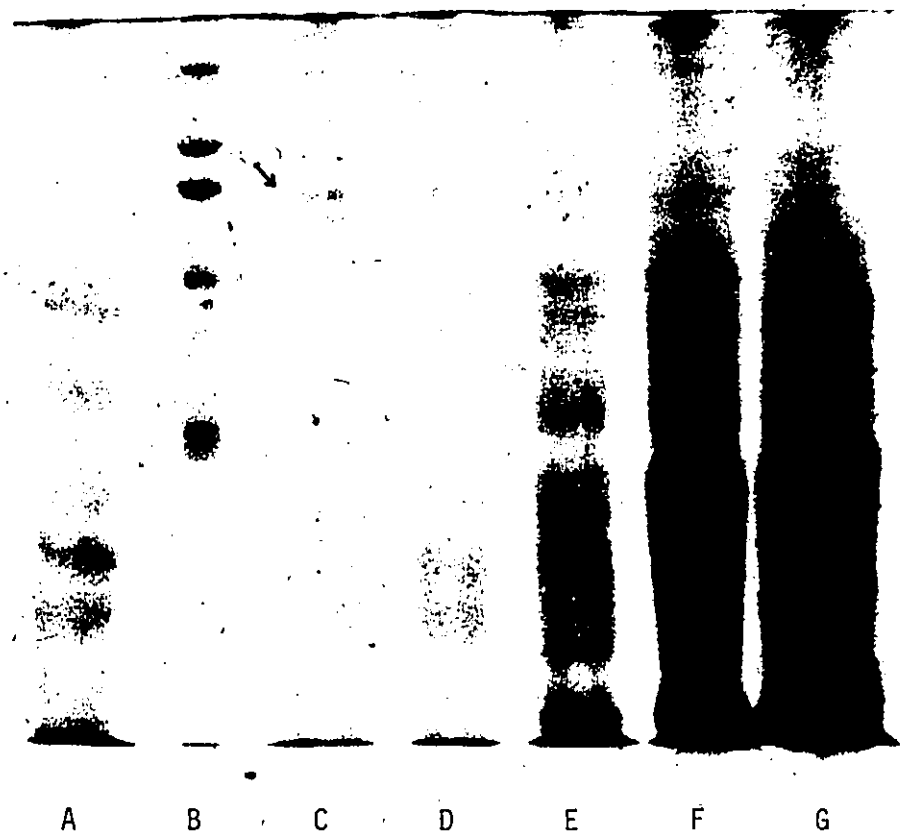
[B]one unit equals the hydrolysis of 1 μ Mole FFA per minute in the above assay.

[C]Protein determinations were performed as described in Part B methods section 4.

Gel electrophoresis was performed on aliquots of each purification step (Figure 15). The fractions containing the highest specific activity (fractions 33,34,35) obtained from the Sephacryl S-200 column were pooled and are shown in lane C. This lane contains several protein bands including a distinct band at about Mr 90,000 (migrated to a similar position as did phosphorylase b which has a Mr of 92000). Lane A contains proteins recovered from the major 280 nm absorbing peak (fractions 36 and 37) which demonstrated no detectable lipase activity. Lane A and C contain similar proteins, however lane C contains the 90,000 Mr protein which is not present in lane A. Since lane C contains protein with lipase activity and lane A does not, this 90,000 Mr protein is a candidate for lipase. However, in order to verify that this is lipase, further purification must be achieved.

Lipase has successfully been purified from rice bran (Funatsu et al 1970) who reported a Mr of about 40,000. Since oat and rice are both cereals it might be expected that they should have similar Mr lipases. However the lipase enzyme isolated from rice bran was an aqueous soluble protein. During their purification it was reported that 80% of the enzyme activity could be recovered in an aqueous extract and no detergents were required to remove it from membrane particles. They were able to proceed to column chromatography using the aqueous extract. I observed that gel filtration on a Sephacryl S-200 column using an aqueous extract (obtained after centrifuging a crude homogenate at 5000 g) resulted in lipase eluting

FIGURE 15


A
B
C
D
E
F
G

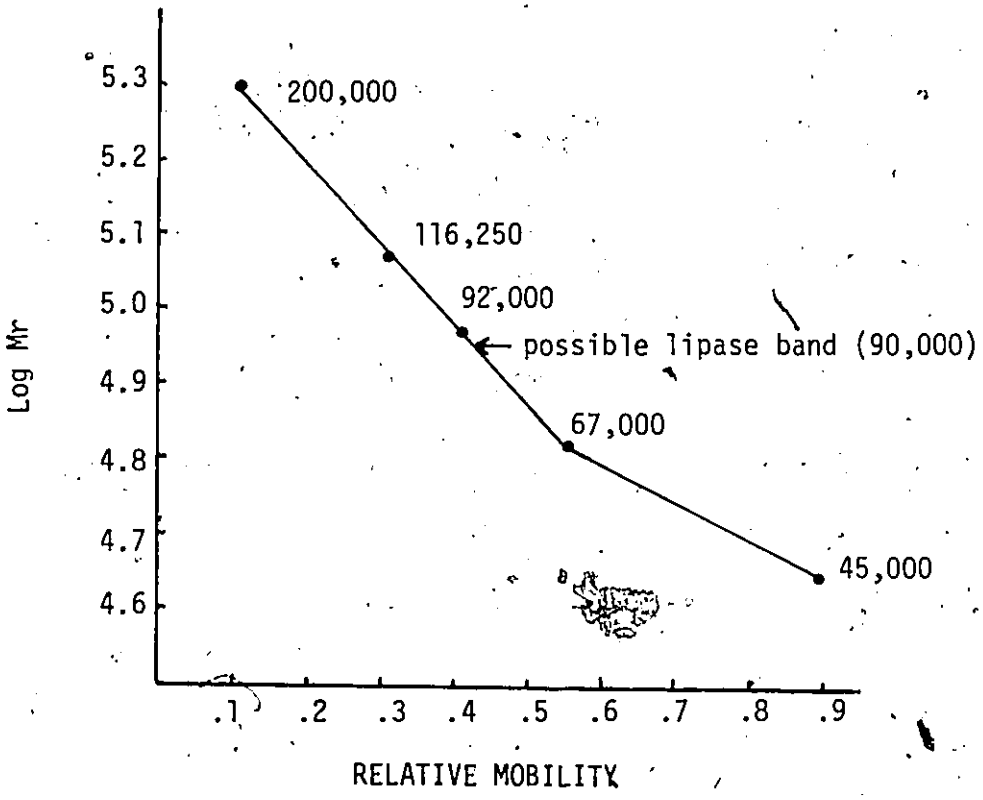

Log Mr
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
200,000
116,250
92,000
← possible lipase band (90,000)
67,000
45,000
.1
.2
.3
.4
.5
.6
.7
.8
.9
RELATIVE MOBILITY

FIGURE 15

Gel electrophoresis (SDS-PAGE) performed on various lipase containing fractions. Electrophoresis was performed similarly to that described in Part A, Methods section 2. The gel contained 9.0% acrylamide. Fractions containing lipase activity were dialysed against H_2O then freezedried prior to electrophoresis. The various lanes contained the following fractions. Protein amounts in each lane are not known.

lane A: tubes 36 and 37 from the Sephacryl S-200 column.

lane B: Mr standards containing myosin 200 000, phosphorylase b 92 000, BSA 67 000, ovalbumin 45 000, β-galactosidase 116 250.

lane C: tubes 33, 34, 35 from the Sephacryl S-200 column.

lane D: tubes 24 and 25 from the butyl agarose column.

lane E: 0.2% Triton X-100 extraction of microsomes.

lane F: microsomal suspension proteins.

lane G: aqueous extract proteins.

only in the void volume (Figure 16). This indicated that lipase was membrane bound. No enzyme activity was detected in the soluble fractions.

Further purification of oat lipase will be successful only if a means of further stabilizing the enzyme is found. Possibly higher concentrations of glycerol would be useful however, this makes column chromatography increasingly difficult. The presence of BSA or substrate in lipase containing solutions has not been tried and may be helpful. The use of other non polar detergents such as Tween 80, Nonidet P-40 should be investigated.

Another approach may be to include detergent in the aqueous extract. Possibly affinity chromatography or gel filtration following this treatment would result in a greater enzyme recovery than I obtained by isolating microsomes. Furthermore, the yield of lipase may be increased if a protease inhibitor was included in all the buffers.

FIGURE 16

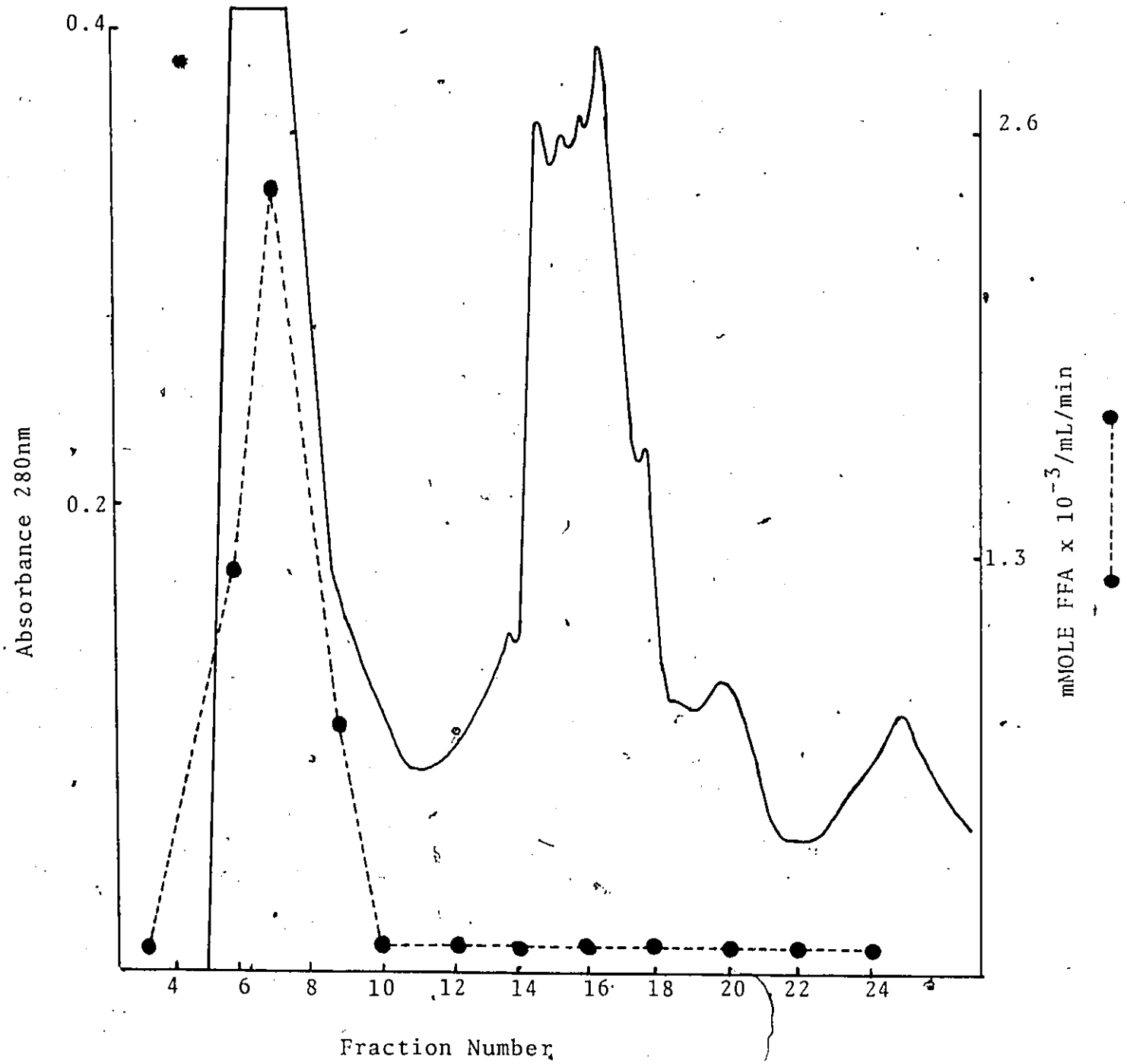

0.4
0.2
Absorbance 280nm
2.6
1.3
mMOLE FFA x 10^{-3}/mL/min
4
6
8
10
12
14
16
18
20
22
24
Fraction Number

FIGURE 16

Gel filtration of an aqueous extract (supernatant following centrifugation at 5000 g of the crude homogenate) on Sephacryl S-200 (1 X 45 cm). The absorbance at 280 nm was monitored continuously. The column had a bed volume of 30 mls and was pre-equilibrated in 0.05 N Tris/HCl, pH 7.5, 2.0 mM $CaCl_2$. The sample volume was 2.0 ml and the fractions contained about 2.0 mls. Lipase assays were performed as described in Methods section 1.

CONCLUSION AND FUTURE WORK

Preliminary experiments have been conducted to study oat lipase. Lipase activity (triglyceride hydrolysis) was assayed in oat flour suspensions and soluble extracts by measuring the increase in [1-^{14}C] oleic acid. Triton X-100 (0.5 - 2.0%) and benzene (0.2%) enhanced lipase activity. The pH optimum of lipase in flour suspensions and microsomal suspensions was 7.5. The observed Km for lipase was 3 mM in flour suspensions and 2.1 in microsomal suspensions. Using these assay conditions it was determined that oat had a greater lipase activity than wheat, rye and barley.

Preliminary evidence was presented which suggested the lipase, contained in microsomal suspensions, may be capable of hydrolysing di- and monoglycerides. Lipase could be released from microsomes in the presence of 0.2% Triton X-100. Lipase was partially purified from the microsomal extract using hydrophobic chromatography on butyl agarose and gel filtration on Sephacryl S-200. Gel electrophoresis suggested that lipase may have a subunit Mr of about 90 000.

Work is being continued in our laboratory to try and determine the Mr of lipase. Presently Triton X-100 acrylamide gels are being run of our most pure lipase preparation (Figure 15 lane C). Such gels are reported to separate proteins on the basis of Mr. In this manner it may be possible to verify if the 90 000 Mr protein contains lipase activity. If this is successful it will be the final purification step and the

procedure described here will be scaled up. Once a pure lipase has been obtained it will be used to conduct further kinetic studies. Structural analysis will be initiated to possibly determine which amino acids are involved in the active site of this enzyme. Research at this level may some day provide clues as to how this enzyme may be inhibited which may hopefully result in a longer shelf life for oats.

Further studies using purified lipase will include preparing antibodies against this preparation. Antibodies may then be used to determine if lipase can be sythesized as a precursor molecule. Furthermore in vitro translation experiments may be used to determine when the lipase is being transcribed during development. To do so, polysomes must be isolated during various developmental stages, translated in vitro followed by immunoprecipitation. Such a study might provide information as to the best time to harvest oats. For instance lipase may be synthesized at a period after the protein and lipids have been laid down. It thus may be possible to harvest seeds before lipase is sythesized and after the proteins and lipids are present.

However, because of the difficulties encountered in obtaining a pure lipase preparation, the next experiments were performed on oat globulins which are abundant and easy to obtain. The biosynthetic study of oat globulin polypeptides would provide a model system to investigate protein synthesis in oats. This

is also an intersting theoretical problem since globulin is normally a low abundance protein in cereals. Furthermore, in pursuing the study of globulin biosynthesis various methodologies would be developed which could be used for the similar future study on lipase.

Part B. The Structure and Biosythesis of Globulin

CHAPTER 1

INTRODUCTION

1. Oat as a Model System

One of the objectives of modern agriculture is to improve crops with respect to protein quality (content of essential amino acids) and quantity. Much work has centered in this area because of the important nutritional implications.

Oat is a cereal crop which combines the benefits of a high protein content with an excellent amino acid profile and is capable of thriving over many areas of the world (Pomeranz, 1973). Oat varieties grown in North America have an average protein content of 17% (Youngs 1972, Robbins et al., 1971, Pomeranz et al., 1973) while most other cereals contain about 10% protein. Oat proteins contain the highest amount of lysine compared to other cereals and are probably second with respect to methionine and threonine (Youngs et al, 1973).

Aside from these favourable qualities, oat contains several poor characteristics. Oat is a poor yielding crop compared to other cereals such as wheat, barley, and corn (Shukla, 1975). Programs have been initiated however, to breed a high yielding oat variety (Youngs et al, 1973). Another unfavorable characteristic in oats is its lipase activity. This enzyme is active during grain storage (Hutchinson and Martin, 1952) which gives rise to fatty acid rancidity resulting in off flavours (Dundas et al, 1978). For this reason oat has been used predominantly for animal feed

and seldom for human consumption. Recently, studies have been undertaken to characterize oat lipase so that its high activity may be better understood (Matlashewski, et al, 1982).

Apart from these important practical aspects, developing oat seeds provide an excellent system with which to study gene expression. During seed development, gene expression takes place predominantly within the endosperm tissue. This results in the synthesis of a major protein fraction (Tumer, et al, 1981) known as storage protein. During recent studies on protein synthesis in developing seeds it has been established that polypeptides of similar physical and antigenic characteristics to authentic storage protein can be synthesized in vitro (Larkins and Dalby, 1975; Burr and Burr, 1976; Hall, et al, 1978; Beachy et al, 1978; Matthews and Miflin, 1981). Characterizing these in vitro synthesized products has been essential in understanding their synthesis.

The characterization of oat storage protein and the study of its synthesis must be further investigated. Such an investigation may yield information regarding why oat proteins have superior qualities to other cereal storage protein. For instance, the synthesis of oat storage protein may be regulated in a different manner than other cereal storage protein.

2. Storage Proteins

Oats like other cereals and legumes, store seed nitrogen in the form of protein. Storage proteins are deposited within

cellular organelles called protein bodies (Quail, 1979). Protein bodies are generally spherical and have diameters from 0.1 to 25 μm (Pernollet, 1978). Storage proteins are synthesized during seed development which begins after anthesis (flowering). During this period through to seed maturation nitrogen continues to be deposited in the form of storage proteins. During the period between two and five weeks after anthesis it has been demonstrated in several seeds that the increase in seed dry weight is paralleled by the accumulation of storage protein (Shewry et al, 1979; Bain et al, 1966).. Within this period the endosperm which is in a soft milky state is the site of protein synthesis. Upon reaching maturation the endosperm dehydrates and protein synthesis stops. In cereals this complete process occupies a period of between six and eight weeks. Upon germination of the seed, these storage proteins are broken down and the nitrogen is utilized for plant development.

In the mature seed, storage proteins are located predominantly in the sub-aleurone and outer endosperm layers. The middle of the endosperm contains mostly starch (Weber and Neumann, 1980).

3. Protein Body Contents

Storage protein were first classified by Osborn (1895) into four classes according to their solubilities. They are:

globulins - salt soluble,

albumins - aqueous soluble,

prolamins - alcohol soluble,

glutelins - weak acid or weak alkali soluble.

This type of classification has come under much criticism because of its crude nature. Never-the-less, a better system of classification has not been forthcoming.

Albumins constitute a minor portion of the storage protein. Their predominant role is to provide the seed with enzymatic activity (Weber and Neumann, 1980). Prolamins and glutelins are the major storage proteins in most cereals (monocytoledons) such as wheat, rye, barley and rice (Weber and Neumann, 1980). Prolamins have the general characteristics of being rich in proline (10 - 20%) and glutamine (15 - 30%) and low in lysine content (Miflin and Shewry, 1979). Glutelins generally contain more lysine and less proline and glutamine when compared to prolamins.

In legumes (dicotyledons) globulins constitute the major storage protein fraction (Matta et al, 1981). Globulins generally contain about 5% lysine and proline with about 10 - 20% glutamine (Miflin and Shewry, 1979; Pernollet et al, 1982).

Among the cereals, oat is unique. The major class of storage protein is globulin which constitutes about 50% of the total seed protein (Peterson, 1976). Thus, this renders oat similar to legumes with respect to its storage protein characteristics. This does seem interesting since oat is classified as a monocotyledon while legumes are dicotyledons.

1. Formation of Protein Bodies

Several hypotheses have been introduced to explain the formation of protein bodies in seeds. One theory suggests that storage protein in legumes are synthesized on the endoplasmic reticulum and subsequently enter into the lumen. Once in the lumen the storage protein enters into dictyosomes (Golgi body), then are deposited into vacuoles which are known as protein bodies (Boutler, 1979; Dieckert and Dieckert, 1976). This is analogous to the situation in animal cells. For example, in pancreatic cells, proteins enter into the endoplasmic reticulum, then are deposited into the golgi bodies and finally enter storage granules prior to being excreted (Bhagavan, 1978).

Observations using electron microscopy of developing endosperm show it to be highly vacuolated. When cells start synthesizing protein these vacuoles begin filling at the inner surface (Briarty et al, 1969). Pronase digestion of these deposits suggests they are protein (Neumann and Weber, 1978).

It has also been suggested that the dictyosomes may not play an active role and that vesicles break away from the endoplasmic reticulum and are transported directly to the vacuoles (Boulter, 1981).

In maize, electron microscopy of endosperm cells has revealed membranes surrounding the protein bodies. These membranes are continuous with the endoplasmic reticulum membrane (Larkins and Hurkman, 1978). Both protein body and

endoplasmic reticulum membranes contain ribosomes and cytochrome C reductase activity. Furthermore, polyribosomes isolated from protein bodies and endoplasmic reticulum both were capable of synthesizing storage protein in vitro. These results indicated that in maize, storage proteins are formed as deposits in the endoplasmic reticulum, then bud off to form protein bodies. This would seem feasible in light of the fact that storage proteins are generally insoluble. Thus, as they accumulate in the endoplasmic reticulum they would be expected to precipitate causing aggregation which could lead to the budding process.

Burr and Burr (1976) however, have demonstrated that polyribosomes isolated from protein bodies in maize synthesize only storage protein in vitro and have thus postulated that protein bodies are independent protein synthesizing organelles.

5. Protein Body Degradation During Germination

Storage protein degradation during seed germination corresponds to changes in protein body ultra-structure. Protein bodies in legumes have been shown to undergo internal degradation which proceeds by a flocculation of its contents (Briarty et al, 1970; Kirk and Pyliotis, 1976). It has been suggested that flocculent appearance of protein bodies indicates endogenous proteolytic activity within the organelle (Ashton, 1976). Alternately, protein bodies may undergo peripheral degradation as seen in bean seedlings (Chrispeels et al, 1976). In this case, degradation of storage proteins

starts after de novo synthesis of proteases (Baumgartner and Chrispeels, 1977, Baumgartner et al, 1978). These proteolytic enzymes presumably originate from outside the organelle (Ashton, 1976).

6. Storage Protein: Future Prospects and the Role of Oat Globulin

Recombinant DNA techniques may soon allow the transfer and expression of specific genes among plants. The evolution of such technology will overcome many barriers inherent in conventional breeding techniques. For instance, it may become favorable to have oat globulin in a high yielding crop such as wheat or barley. However, a more complete understanding of eukaryotic molecular genetics is necessary before this is accomplished.

Storage proteins have been cloned into prokaryotic organisms (Forde et al, 1981; Barton et al, 1982; Croy et al, 1982). This will provide an understanding of the organization and structure of these genes which may provide an understanding of their regulation. This level of research should be extended towards oat globulin because:

A) its high level of expression is unique among cereals, and

B) preliminary results indicate it may have more in common with storage protein from legumes (dicotyledons) than from cereals (monocotyledons).

The work presented in this thesis has focused on comparing authentic globulin with in vitro synthesized globulin so that its biosynthesis may be better understood. During the course

of this research, much of the ground work has been provided which will allow a similar study to be performed on oat lipase.

CHAPTER 2

GLOBULIN SUBUNIT CHARACTERIZATION

A) INTRODUCTION

1. Globulin Structure in Legumes

The accepted model for the structure of legumin, the major storage protein of pea (a legume) is as follows. Each molecule contains six subunit pairs. Each subunit pair has a molecular weight (Mr) of 60 000. Thus, the complete legumin complex has a Mr of about 350 000 and a sedimentation coefficient of 12S. A subunit pair contains polypeptides of approximate Mr 40 000 and 20 000. Each of these proteins are in fact a heterogeneous group of polypeptides with similar Mr. The 40 000 Mr group are considered acidic proteins and the 20 000 Mr group are considered basic (Matta et al, 1981). The terms acidic and basic for the legumin large and small subunits must be considered as relative since many of the so called acidic proteins are in the neutral range. The smaller basic subunits however all have higher isoelectric points than the larger acidic subunits. There is considerably less heterogeneity in the basic subunits than in the acidic subunits. The 40 000 and 20 000 Mr subunits are held together by one or more disulfide bonds (Derbyshire et al, 1976). Upon reduction in the presence of 2-mercaptoethanol the disulfide bonds are dissociated and the subunits can be

separated by sodium dodecylsulfate (SDS) gel-electrophoresis. If reducing agent is not present during SDS gel-electrophoresis the disulfide bonds are not broken and legumin migrates as the dimer with a Mr of about 60 000 (Matta et al, 1981).

In soybean, also a legume, the major storage proteins fall in the globulin class. This class contains a group of proteins called glycinins which are analogous to legumin in pea. The glycinin complex has a sedimentation coefficient of 12.3S, a Mr of 300 - 350 000 and is made up of six subunit pairs (Badley et al, 1975). Six different polypeptides with acidic isoelectric points and Mr in the region of 40 000 and five with basic isoelectric points of 20 000 Mr have been identified. Each of these polypeptides have similar but unique primary sequences (Moreira et al, 1979). It has been established using SDS gel-electrophoresis under reducing and non-reducing conditions that the acidic and basic subunits are linked together through disulfide bonds to form a subunit pair (Staswick et al, 1981).

2. Globulin Structure in Cereals

Aside from oat globulin very little is known about cereal globulins, probably due to their low concentrations. According to Peterson (1978) the definition of oat globulins is those proteins which are extracted from seeds with 1.0 M NaCl, Tris/HCl, pH 8.5, and are precipitated upon dialysis against H_2O. These precipitated globulins contain two major subunits (α and β)

(major globulins) present in equimolar amounts and one to four minor components (minor globulins). Peterson and Smith (1976) have shown that an increase in total oat protein is paralleled with an increase in the globulin fraction. Globulin increases linearly from day 4 to 16 after anthesis and constitutes up to 50% of the total protein by the twelfth day (Peterson, 1976). Oat globulin has a sedimentation coefficient of 12.1S and a holoprotein molecular weight of 32 000 (Peterson, 1978). Oat globulin was separated into two major subunits by SDS-electrophoresis under reducing conditions. The Mr were 21 700 (α-subunit) and 31 700 (β-subunit). Heavier Mr components (Mr 56 000) were always present in smaller quantities. It was speculated that the heavier peptides could be dimers or precursors of the α and β subunits (Peterson 1978). In the absence of SDS globulin migrates as a single diffuse band during electrophoresis (Peterson 1973).

Peterson (1978) isolated the two major globulin subunits by excising them from SDS polyacrylamide gels. The amino acid composition of these subunits was reported and will be presented in a subsequent section.

3. Research Objective

Peterson's results suggest homology between oat and legume globulin. The objective of this chapter is to continue studying this aspect of oat globulin using the work of Peterson (1978 , 1973) as a foundation.

This chapter focuses on some physical characteristics which have been reported for legume storage globulin but not oat storage globulin. They are:

1) Mr and pI heterogeneity within the α and β subunits.
2) The effect of the presence and absence of reducing agent on the α and β subunits during SDS-PAGE.
3) The pI characteristics of the α and β subunit.

MATERIALS AND METHODS

B) MATERIALS:

1. Tissue:

Oat (*Avena sativa* L.) was used throughout this investigation. The cultivar Hinoat was grown at the Ottawa Research Station, Agriculture Canada. Samples were harvested at maturity (8 weeks after anthesis) and stored at -20°C.

2. Chemicals:

Sigma: bromophenol blue, Coomassie Brilliant Blue R, dithiothreitol (DTT), 2-mercaptoethanol, Tris (2-amino-2-hydroxymethylpropane-1,3-diol).

Bio-Rad Laboratories: acrylamide, bis (N,N'-methylene-bis-acrylamide), ammonium persulfate, Temed (N,N,N'N'-tetramethyl-ethylenediamine), Bio-Rad Protein assay.

Pharmacia: Pharmalyte 3-10.

All other chemicals were analytical grade.

C) METHODS:

1. Isolation of Globulin:

The procedure for isolating globulin was based on that of Luthe and Peterson, 1977. Twenty grams of mature seed were ground to a fine powder in a coffee mill then defatted with 100 ml of cold acetone. The flour was allowed to dry at room temperature followed by regrinding in a coffee mill. Protein was extracted by stirring the defatted flour in 500 ml of extraction buffer (0.05 M Tris/HCl, pH 8.0, 1.0 M NaCl, 1.0% (v/v) 2-mercaptoethanol) for 2 hrs at room temperature. The suspension was centrifuged at 17 000 x g for 20 min and the supernatant retained. The supernatant was brought to 60% saturation with $(NH_4)_2SO_4$ at 4°C and the suspension centrifuged at 17 000 x g for 10 min. In this manner, the protein was precipitated and pelleted. The pellet was redissolved in 100 ml of extraction buffer and allowed to stir overnight at 25°C. Undissolved particles such as precipitated prolamins, glutelins and albumins which may have been present in trace amounts in the salt soluble extract were pelleted at 27 000 x g for 30 min. The supernatant was dialyzed against 0.1% (v/v) 2-mercaptoethanol for 1 hr followed by dialysis against distilled H_2O for an additional 2 hrs. The precipitate was pelleted at 20 000 x g for 20 min and the globulin pellet taken up in a small qunatity of distilled H_2O and lyophilized.

2. Laemmli Gel-Electrophoresis:

The method was a modification of that described by Laemmli, 1970. The separating gel (18 x 14 x 0.15 cm) contained between 12 - 18% (w/v) acrylamide, 0.26% (w/v) bisacrylamide, 0.10% (w/v) sodium dodecylsulphate (SDS), 1.0 mM DTT, 0.375 M Tris/HCl pH 8.8, 4.0 M urea, 0.05% (v/v) Temed, and 0.005% (w/v) ammonium persulphate. The stacking gel contained 5.0% (w/v) acrylamide, 0.625% (w/v) bisacrylamide, 125 mM Tris/HCl pH 6.8, 0.1% (w/v) SDS, 4.0 M urea, 0.10% (v/v) Temed and 0.005% (w/v) ammonium persulphate. The sample buffer contained 4.0 M urea, 1.0% (w/v) SDS, 1.0% (v/v) 2-mercaptoethanol, 65 mM Tris/HCl pH 6.8 and 0.001% bromophernol blue. Samples were dissolved in this buffer and heated for 20 min at 60 C prior to electrophoresis. For non-reducing conditions 2-mercapto-ethanol was omitted from the sample buffer and DTT omitted from the resolving gel. Electrode buffer contained 25 mM Tris/HCl, pH 8.3, 200 mM glycine, and 0.1% (w/v) SDS. Electrophoresis was performed under constant voltage (60v) for about 16 hrs. After electrophoresis the gel was fixed and stained in 10% (w/v) trichloroacetic acid (TCA), 40% (v/v) methanol and 0.20% (w/v) Coomassie Brilliant Blue R for 4 hrs. Gels were destained in 30% (v/v) methanol, 10% (v/v) acetic acid.

3. Preparative Gel Electrophoresis:

Electrophoresis was performed on a 170 ml cylinder containing 12% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 0.125 M Tris-borate pH 8.9, 0.05% (v/v) Temed, 0.1% SDS, 0.04% (w/v) ammonium persulphate. Electrode buffer contained 0.125 M Tris-borate, pH 8.9, 0.1% (w/v) SDS. Samples were prepared by dissolving 150 mg of freeze dried globulin in 1.0% (w/v) SDS, 10% (v/v) 2-mercaptoethanol and stirred at 60 C for 30 min followed by dialysis against 0.1% (w/v) SDS, 5.0% (v/v) 2-mercaptoethanol. The sample was placed on the gel cylinder and electrophoresis was conducted under constant current (50 ma) for about 25 hrs. Protein was allowed to run off the bottom of the gel into a dialysis bag. The contents of the dialysis bag were pumped out every 10 minutes and collected in 3.0 ml fractions.

4. Protein Determinations:

Protein concentrations were determined by the Coomassie Blue dye-binding method described by Bradford, (1976). Bio-Rad Protein Assay was used for these determinations. Bovine gamma globulin was used as a standard. Dye reagent (Bio-Rad) was diluted five fold and filtered through Whatman No. 1 paper. The diluted dye was added to the protein samples, allowed to stand for 10 min, and the absorption read at 595 nm.

5. Analysis of Preparative Gel Electrophoresis Fractions:

The mini slab apparatus (GE-2/4) by Pharmacia was used as follows. The separating gel (10 x 10 x 0.2 cm) contained 17.5% (w/v) acrylamide, 0.18% (w/v) bisacrylamide, 0.10% (w/v) sodium dodecylsulphate (SDS), 0.125 M Tris/HCl, pH 8.9, 0.05% (v/v) Temed and 0.005% (w/v) ammonium persulphate. The stacking gel contained 3.0% (w/v) acrylamide, 0.28% (w/v) bisacrylamide, 50 mM Tris/HCl, pH 6.8, 1.0% SDS, 0.05% (v/v) Temed and 0.001% (w/v) ammonium persulphate. Sample buffer contained 0.5M Tris/HCl, pH 6.8, 8.0 M urea, 1.0% (w/v) SDS and 10% (v/v) 2-mercaptoethanol. Electrode buffer contained 0.125 M Tris-borate, pH 8.9, 0.1% (w/v) SDS. Samples were dissolved in this buffer then heated for 20 min at 60 C prior to electrophoresis. Electrophoresis was performed under constant current (40 ma) for 4-6 hrs. Gels were stained and destained as previously described.

6. Isoelectric Focusing (IEF):

Gels contained 5.0% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 6.0 M urea, 5.0% (v/v) Pharmalyte pH 3-10, 0.03% (w/v) ammonium persulfate, 0.05% (v/v) Temed. The anode and cathode buffers contained 0.05 M H_2SO_4 and 1.0 M NaOH respectively. The slab was prefocused at 10 watts for 30 min. Samples were dissolved in 0.01 M glycine pH 8.0, 6.0 M urea. 1.0% (v/v) Triton X-100. Focusing was performed at 15 watts for 2.5 hrs followed by fixing, staining and destaining as previously described. The pH gradients were found to be linear. They were measured by soaking gel slices in H_2O followed by testing the pH with a pH electrode.

7. Two Dimensional Electrophoresis (IEF and SDS-PAGE):

Tracks were removed from an unfixed focusing gel with a razor blade, placed in 65 mM Tris/HCl pH 6.8, 1.0% (w/v) SDS and heated to 60 C for 10 min. The strip was set on an SDS electrophoresis resolving gel. Stacking gel solution was poured on top of the isoelectric focusing strip and allowed to polymerize. Electrophoresis was carried out as described in section 2.

D. RESULTS AND DISCUSSION

1. Molecular Weight Determination and Heterogeneity of Globulin Subunits:

The globulin fraction was obtained from mature seeds on the basis of its salt solubility (Osborne 1895) essentially by the method of Peterson (1978). The major subunit components described by Peterson (1978) were always present in each preparation. However, the presence and absence of minor bands during SDS-PAGE varied among preparations, possibly due to the crude nature of the Osborne fractionation technique. This thesis focuses on the major components (α and β subunits) which were isolated by preparative gel electrophoresis (section 3).

About 400 mg of the globulin fraction was obtained from 20 gm of mature seeds. Globulin was subjected to SDS-PAGE as described in methods section 2. Globulin was applied to track B of Figure 1. Molecular weight standards (phosphorylase B 92 000, bovine serum albumin 67 000, ovalbumin 45 000, carbonic anhydrase 30 000, trypsin inhibitor 20 000) were treated in a similar manner to globulin and applied to track A.

The banding pattern in track B demonstrates two major groups of polypeptides. The α subunit contains at least 3 polypeptides from Mr 21,000 to 23,000. The β subunit contains at least 4 polypeptides

FIGURE 1

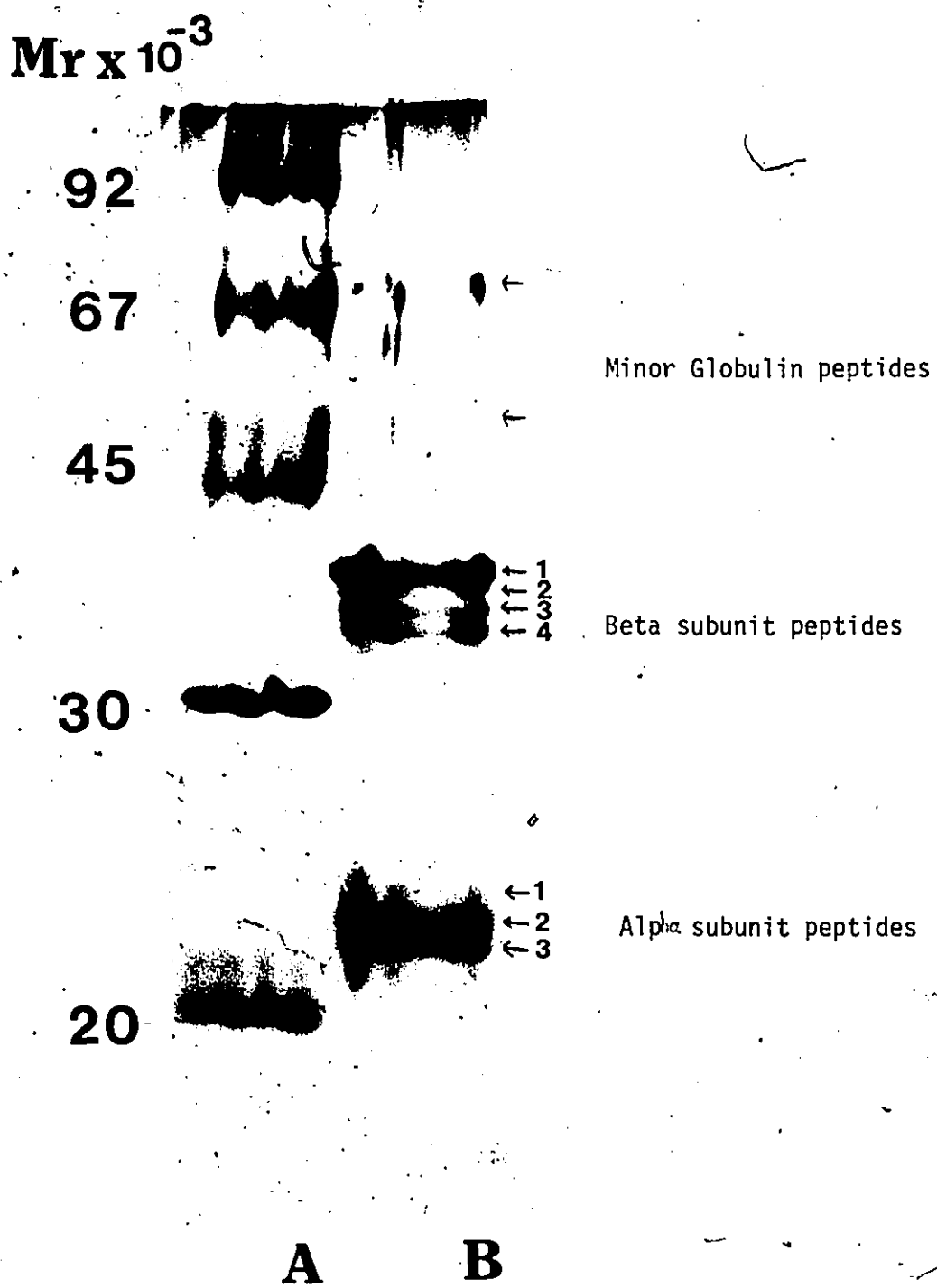

Mr x 10-3
92
67
45
30
20
Minor Globulin peptides
1
2
3
4
Beta subunit peptides
1
2
3
Alpha subunit peptides
A
B

Figure 1. SDS-PAGE of the globulin fraction (Methods section 2).

Track A) Molecular weight standards.

Track B) Globulin isolated according to methods section 1.

from Mr 35,000 to 41,000 (Figure 2). Thus, each subunit exhibits a certain amount of Mr heterogeneity. Such heterogeneity may be due in part to proteolysis occurring in the mature seed or during the isolation procedure. In addition, there are small amounts of polypeptides in the Mr region from 50 000 to about 69,000 (Figure 2). A minor globulin band is also observed at Mr 30,000 which will be discussed further in the subsequent chapter.

This is similar to Peterson's (1978) previous results. Peterson (1978) however reported no Mr heterogeneity within the α and β subunits. Such heterogeneity has also since been observed by Adeli (M.Sc. Thesis, University of Ottawa 1982). The Mr of the β subunit reported here is slightly higher than that reported by Peterson (1978). A possible explanation for this discrepancy is the different Weber and Osborn (1969) gel system, gel analysis or the differences in cultivars used. Peterson's (1978) previous study uses the cultivar Froker and a gel scanner following electrophoresis while this study uses Hinoat and direct measurement of stained proteins. Further differences may have arisen from comparing different molecular weight marker proteins.

2. Involvement of Disulfide Linkages in the Native Globulin Structure:

Globulin was separated by electrophoresis in both the presence and absence of 2-mercaptoethanol as shown in Figure 3. Track A contains globulin run under reducing conditions and track B contains globulin resolved under non-reducing conditions. Track A and B demonstrate widely differing banding patterns. Under non-reducing conditions a major group of polypeptides

FIGURE 2

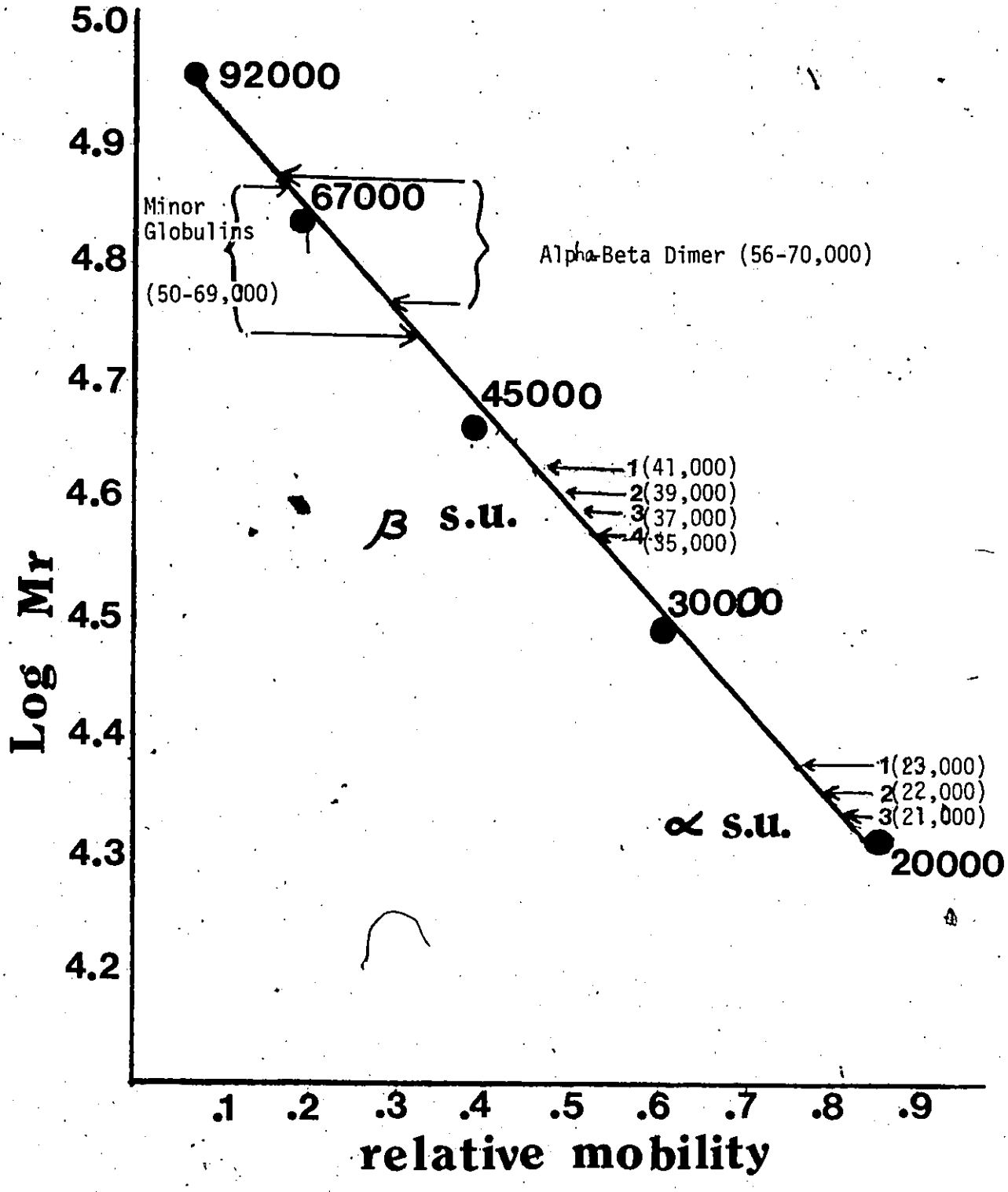

5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
Log Mr
92000
67000
45000
30000
20000
Minor Globulins
(50-69,000)
Alpha-Beta Dimer (56-70,000)
1(41,000)
2(39,000)
3(37,000)
4(35,000)
β s.u.
1(23,000)
2(22,000)
3(21,000)
α s.u.
.1
.2
.3
.4
.5
.6
.7
.8
.9
relative mobility

Figure 2. Graph of log Mr vs relative mobility.
Gel electrophoresis was performed according to method section 2.
Mr weight standards were
Phosphorylase B, 92 000
bovine serum albumin, 67 000
ovalbumin, 45 000
carbonic anhydrase, 30 000
trypsin inhibitor, 20 000
α S.U., β S.U., minor globulins and the α - β dimer are indicated.

FIGURE 3

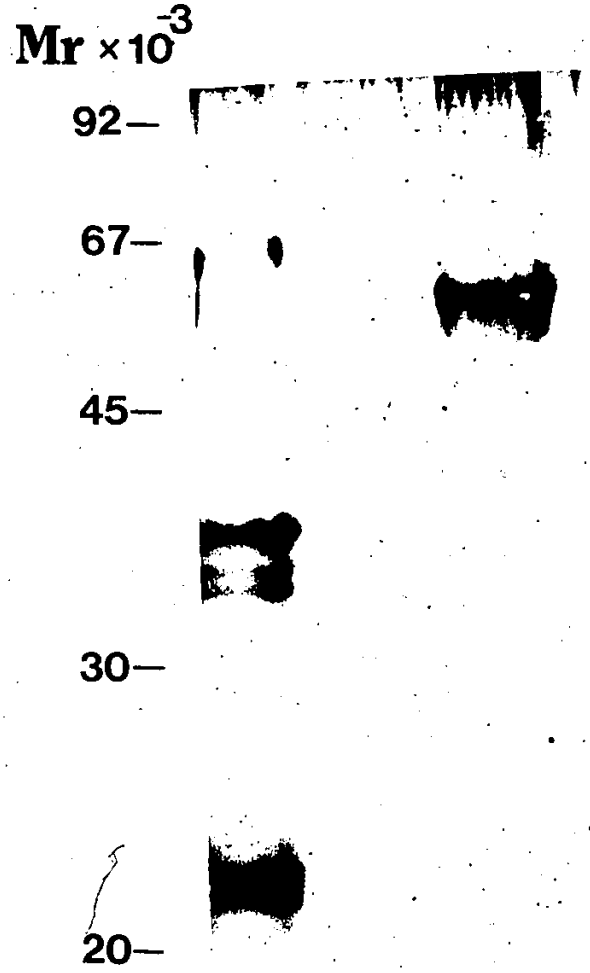


Figure 3. SDS-PAGE of oat globulin in the presence and absence of reducing agent (method section 2). Mr's are as indicated.

Track A) Globulin (20 - 30 ug) exposed to 2-mercaptoethanol and 4.0 M urea prior to electrophoresis.

Track B) Globulin (20 - 30 ug) exposed to 4.0 M urea prior to electrophoresis.

have Mr of 56-70000 (Figure 2). This is approximately the sum of the two subunits. This suggests that the α and β subunits contain disulfide bonds which gives rise to the larger molecular weight products shown in track B. Also observed in track B are small amounts of protein corresponding to the reduced α and β subunits shown in track A. It is presumed that these are globulin subunits which have been dissociated from the dimer during the isolation procedure since mercaptoethanol was used in the extraction buffer (Methods section 1).

This experiment suggests the involvement of cystine cross-links to form a dimer between the α and β subunits. Such cross-linking may be between the two subunits or between two half cystines within the same or both subunits. Such disulfide linkages are probably important for proper association into the holoprotein described by Peterson, (1978).

3. Purification of Globulin Subunits:

The purification of the individual subunits was performed by preparative gel-electrophoresis as described in methods section 3. This was a modification of the method described by Peterson (1978) which separated globulin subunits by SDS-PAGE followed by excising the subunit-containing gel portions. Protein was then eluted from the excised portions of the gel.

The procedure used in this study allows the protein to continue migrating out of the gel into a dialysis bag. The contents of the dialysis bag were collected every 10 min. Samples were run under constant current for about 25 hrs. The

elution profile is shown in Figure 4. Samples containing equal volumes (20 ul) from each fraction were collected and subjected to electrophoresis as described in methods section 5 (Figure 5).

In addition to the α and β subunits the early fractions contain polypeptides in the 14 000 Mr region. These polypeptides were not apparent in the globulin preparation shown in Figure 1. This globulin preparation was allowed to dialyse overnight rather than for 2 hrs. The longer dialysis time may have resulted in the lower Mr proteins precipitating or breakdown of the subunits. Furthermore, the α and β subunits themselves are not resolved as well in Figure 5 as in Figure 1. This is probably due to the different electrophoresis conditions used (Method section 5) for analysing eluted fractions which is considerably less expensive and more rapid than the method described in Method section 2, which however does not offer the same high level of resolution.

The fractions containing the individual subunits were pooled, dialyzed and lyophylized. In this manner the β subunit could be obtained with little contamination (Figure 6). However, the α subunit still contained a considerable amount of the lower Mr contaminating protein. Altering the current (i.e. length of run) did not affect the purity of the subunits. The α subunit containing fractions were pooled and re-subjected to preparative gel-electrophoresis. The fractions were collected and analysed for purity in a manner similar to that already described. Figure 6 is an electrophoresis banding pattern of the final isolated subunits. Electrophoresis was performed according to methods section 2. About 20 ug of each sample was applied. Again the heterogeneity within the subunits is observed.

FIGURE 4

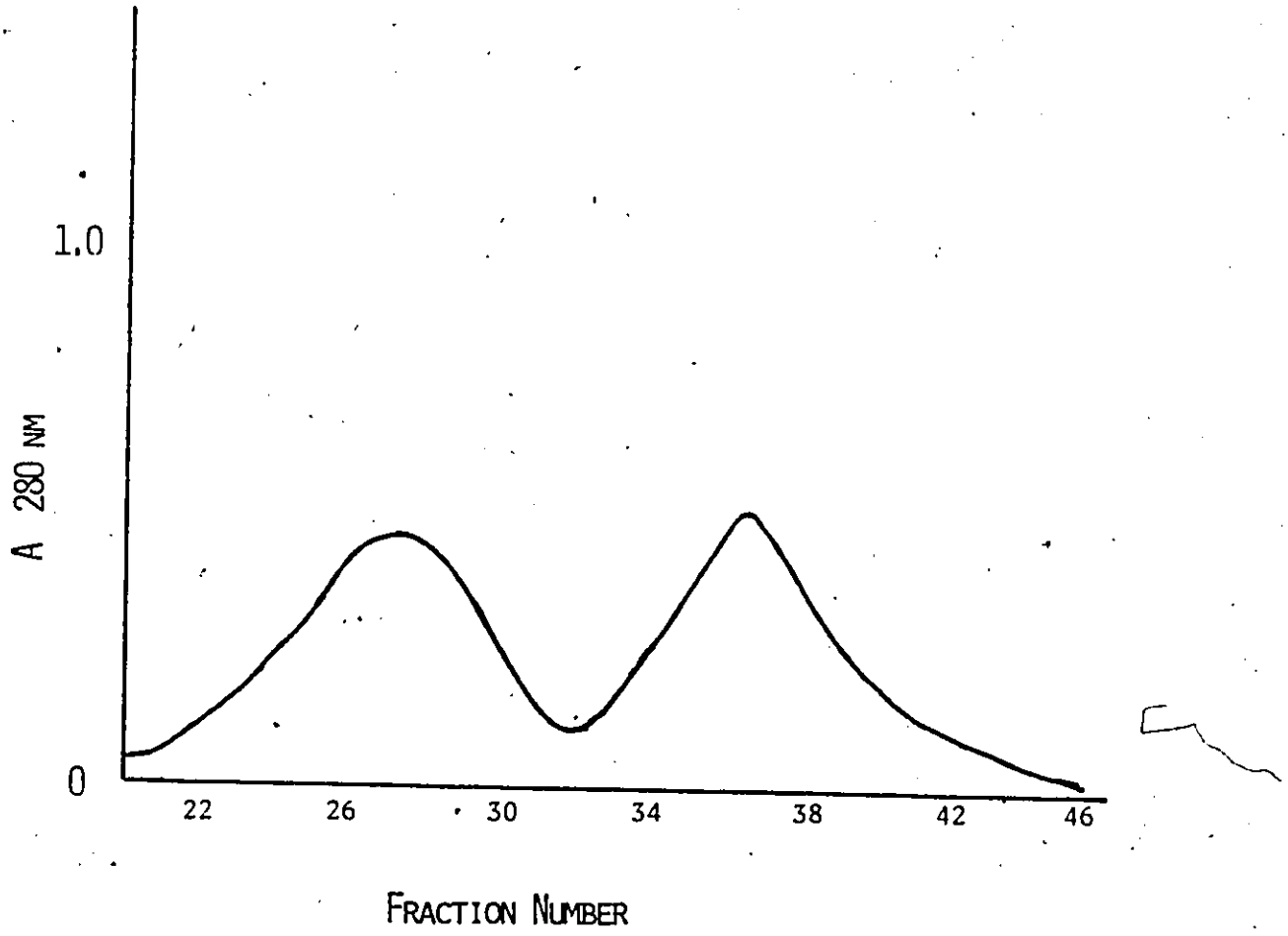


Figure 4. Preparative electrophoresis of oat globulin. Electrophoresis was performed under constant current (50 ma) for about 25 hours. The elution was monitored continuously at 280 nm. Fractions containing 3.0 ml were collected. (methods section 3).

FIGURE 5

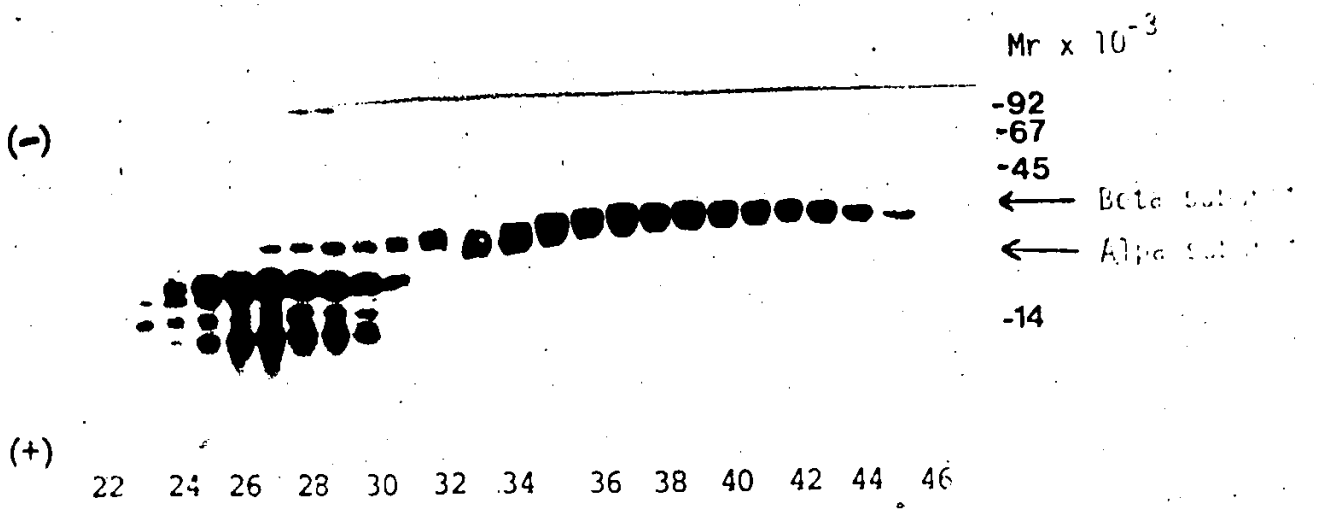


Figure 5. Analysis of subunit fractions from Figure 4 using SDS-PAGE (method section 5). 20 µl Volumes from the fractions shown in Figure 4 were applied to each well. Molecular weight markers used were phosphorylase b (92,000), BSA (67,000) ovalbumin (45,000) and lysozyme (14,000). The expected areas for the α and β subunits are indicated.

FIGURE 6

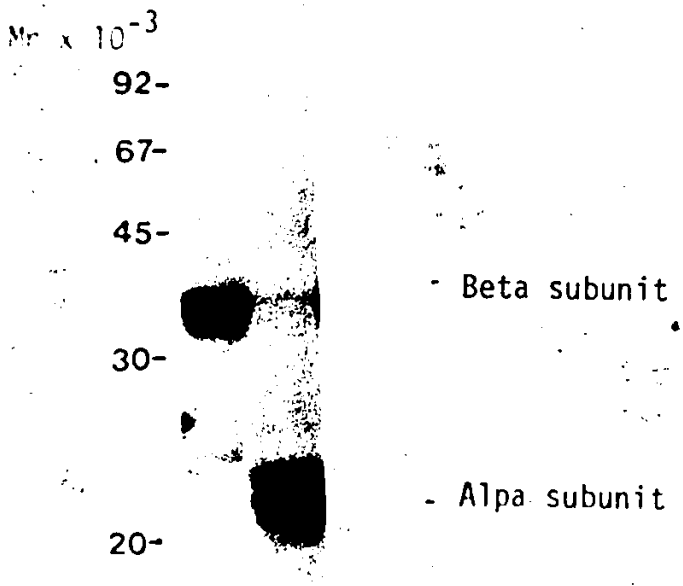


Figure 6. Electrophoretic pattern of isolated α and β subunits (method section 2). About 20 ug of each sample was applied to each track.

Track A) Beta subunit.

Track B) Alpha subunit.

However, the β subunit does not appear to be resolved as well in Figure 6 as in Figure 1. The minor bands (Figure 1, band 2, 3, β S.U.) appear to be compressed in Figure 6. This may be due to slight differences in running conditions from gel to gel. Each subunit demonstrates only small amounts of contaminating protein as judged by this electrophoresis gel.

It is difficult to determine if the isolation procedure described here is an improvement over that described by Peterson (1978). His study did not show a gel of the purified subunits to which a comparison may be made.

4. Isoelectric Focusing of the Subunits:

Isoelectric focusing was performed on the purified subunits to determine their respective pI ranges. The procedure is described in methods section 6. The results are shown in Figure 7. The α subunit migrates towards the basic range between pH 10 and 8.5 while the β subunit is situated in the neutral and acidic region between pH 7.5 and 4.

Furthermore, considerable heterogeneity is demonstrated within each subunit. The acidic β subunit demonstrates more heterogeneity than the basic α subunit. This may however be due to the narrow range of pI values within the α subunit making it more difficult to resolve. Matta et al, (1981) however, similarly reports more heterogeneity in the acidic subunit of legumin. The basic subunit may be more conserved than the acidic subunit. This heterogeneity may be due in part to deamidation of glutamine and asparagine resulting in a shift toward the acidic region. Heterogeneity arising from artifacts in the isoelectric focusing procedure are unlikely since protein standards focused under the same conditions demonstrated no such multiple banding patterns. Brinegar and Peterson (1982) have also demonstrated such heterogeneity.

FIGURE 7

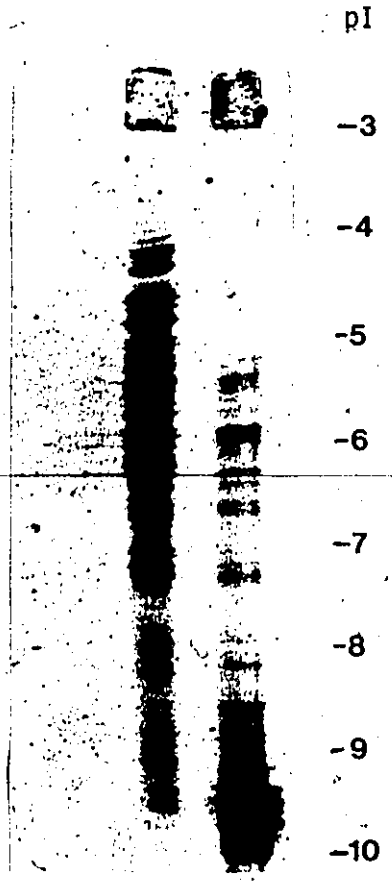


Figure 7. Isoelectric focusing pattern observed for isolated α and β subunits. A pH range of 3 to 10 was used (Methods section 6) and was generally found to be linear by this procedure

Track A) Beta subunit.

Track B) Alpha subunit.

High conservation in proteins generally indicates highly specialized functions. For example, calf thymus and pea histone 4 differ in sequence by only 2 out of 102 residues (Delange 1980). It is believed that such conservation is required because the function of this protein is dependant on its entire structure, thus small changes in sequence would result in a non-functional protein. Similarly, the structure of the basic subunit of globulin may be more critical for globulin's biological activities than the structure of the acidic subunit. These biological activities would include its biosynthetic pathway, transport within the cell, packaging in the cell and its degradation.

5. Two Dimensional Electrophoresis (IEF and SDS-PAGE):

The isoelectric focusing results obtained by using the purified subunits were repeated by two dimensional electrophoresis of total globulin. Isoelectric focusing of total globulin in the first dimension was followed by SDS gel-electrophoresis in the second dimension. Results are shown in Figure 8. The neutral and acidic group of polypeptides (observed between pH 4.5 and 7) co-migrated with the β subunit and the basic group of polypeptides (observed between pH 10 and 8.5) with the α subunit. This indicates that the acidic polypeptides correspond to the β subunit and the basic polypeptides are derived from the α subunit.

FIGURE 8

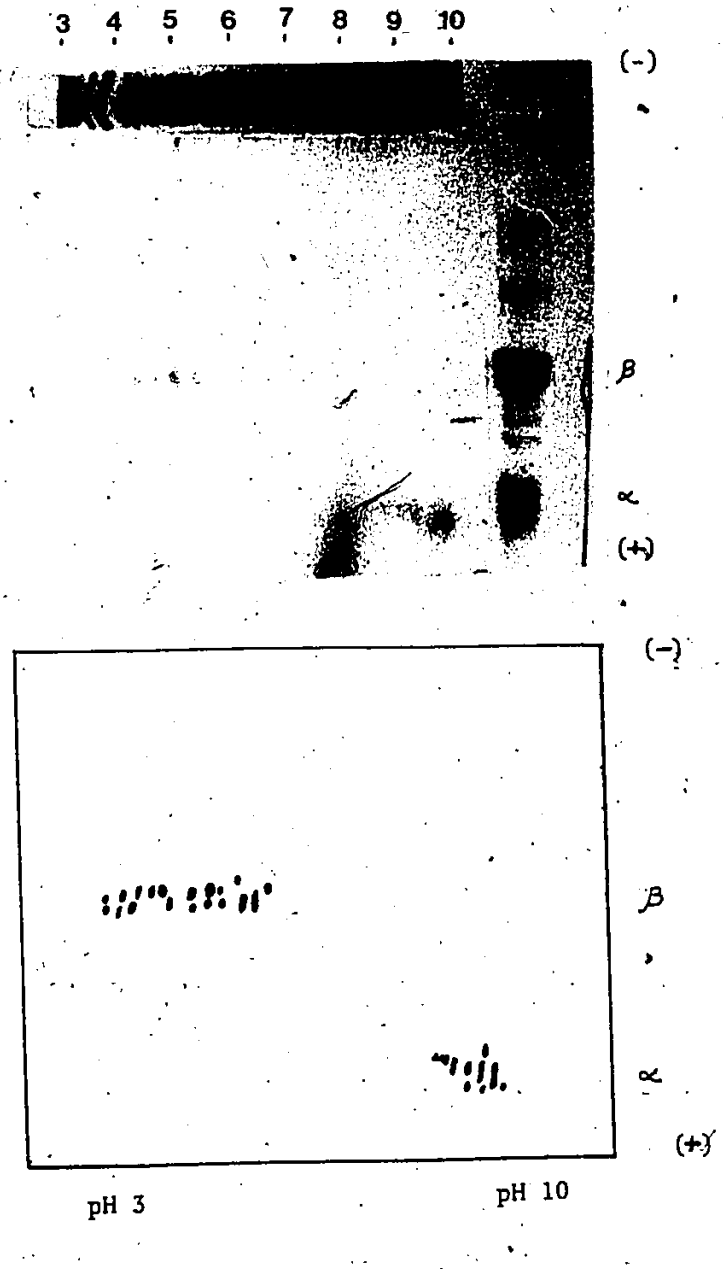


Figure 8. Two dimensional electrophoresis (IEF and SDS-PAGE) performed on total globulin (Methods section 10).

A) First dimension: Total globulin subjected to isoelectric focusing.

Second dimension: SDS-PAGE performed on the isoelectric focusing gel from the first dimension.

B) Schematic taken from the original gel.

The isoelectric values for the subunits are similar but not identical to those observed in Figure 7. It has been noted in our laboratory that these values do vary slightly for all isoelectric focusing experiments, even when the same protein preparation is used. Similar isoelectric characteristics have been demonstrated in legume globulin (Matta et al, 1981; Moreira et al, 1979).

6. Amino Acid Composition of Oat Globulin Subunits:

The amino acid compositions of the isolated globulin subunits were determined by the staff at the John Innes Institute (Norwich,U.K.) and are presented in Table 1. These values are compared to those obtained by Peterson (1978), soybean globulin (glycinin) subunits and pea globulin (legumin). No notable differences are observed between Peterson's values and those reported here. The only exception is the availability of sulfur containing amino acid values. There is a striking resemblance in the amino acid compositions of oat and legume globulin, especially between oat and soybean basic subunits.

As could be predicted by the isoelectric points of the subunits the basic (α) subunit contains more lysine, histidine and arginine than does the acidic (β) subunit.

TABLE I

Amino Acid Composition of Oat Globulin Subunits

(MOLE PERCENT)

	Oat Globulin				Soybean Globulin[3] (Glycinin)		Pea Globulin[3] (Legumin)
	Acidic		Basic		Acidic	Basic	Total
	(β)[1]	(β)[2]	(α)[1]	(α)[2]			
ASP/ASN	8.3	9.1	12.4	12.8	12.6	12.9	12.0
THR	3.3	3.3	4.4	4.0	3.4	4.7	3.5
SER	6.6	7.9	7.0	6.3	6.0	6.9	6.8
GLU/GLN	23.7	21.4	14.9	15.3	25.0	14.4	20.1
PRO	5.1	4.5	5.2	5.2	7.0	5.8	5.4
GLY	9.9	12.1	8.3	7.0	7.7	6.8	7.5
ALA	6.1	5.8	7.5	7.2	3.8	6.3	6.0
CYS	0.8	N/A	1.0	N/A	-	1.6	5.1
VAL	5.8	5.6	6.1	6.2	3.9	6.8	0.6
MET	0	N/A	0.2	N/A	0.5	1.1	0.5
ILE	4.0	3.9	5.3	5.3	4.0	4.8	4.0
LEU	7.7	7.7	7.5	7.9	5.7	9.2	7.6
TYR	3.0	3.0	3.1	3.6	2.2	2.8	1.7
PHE	5.5	5.7	4.4	4.9	3.4	5.4	3.6
HIS	2.1	1.6	3.0	2.2	2.6	1.5	5.2
LYS	2.5	2.1	3.7	3.3	6.3	3.1	2.6
ARG	5.5	5.9	6.0	7.2	6.4	5.4	8.6

1. Amino acid composition of the globulin subunits purified according to methods section 1, 3, 5.
2. Peterson, 1978.
3. Derbyshire et al, (1976).
4. N/A values were not reported.

Oat and legume globulins contain more aspartic acid/asparagine and glutamic acid/glutamine than any other amino acid. It is assumed that in both cases these amino acids are predominately in the amide form, since the function of storage protein is to store nitrogen (Weber and Neumann 1980). Large amounts of glutamine and asparagine should not have had a large effect on the pI characteristics since these are neutral amino acids. Both oat subunits contained about 1.0 mole % cysteine which is consistent with the disulfide stabilized globulin dimer proposed in this chapter. Methionine was not observed in the acidic subunit and only 0.2 mole % was reported in the small subunit. These values may be underestimates because of oxidation of methionine during acid hydrolysis. This is probably so since Dr Shewry (Rothamsted, U.K.) has informed me (personal communication) that the acidic subunit is susceptible to CNBr cleavage. Furthermore, Luthe and Peterson (1977) estimated that methionine residues are distributed between the two polypeptides.

E) CONCLUSION:

Experiments were performed to further understand the physical characteristics of oat globulin. Results are presented which demonstrate:

A) Mr and pI heterogeneity with the α and β subunit groups.

B) The α and β subunits exist as dimers stabilized by disulfide bonds.

C) The α subunit has basic pI values and the β subunit has acidic pI values.

Such characteristics have thus far not been described for oat α and β subunits. These observations substantiate those reported by Peterson (1973, 1978) in showing further homologies between oat and legume globulin.

These similarities suggest these proteins have evolved such that they are present both in monocotyledons and dicotyledons. Sequence analysis or immunological studies would be useful in determining further homologies between these proteins.

In vitro studies have revealed that the globulin storage proteins of soybean (Tumer et al, 1981), and pea (Croy et al, 1980) are synthesized as precursor polypeptides of Mr 60 000. Contained in this precursor are both the acidic and basic peptides and an NH_2-terminal signal peptide. It was speculated that disulfide bonding occurs before the post-translational cleavage into the two subunits. In vitro synthesis studies in cereals would be useful in establishing whether a precursor

molecule is also present during globulin biosynthesis in monocotyledons.

F) ADDENDUM:

After the completion of the experiments presented in this chapter, similar work by Peterson was brought to my attention (Arch. Biochem. Biophys. 219: 71-79, 1982). His results demonstrate the α subunit is 22-24 000 Mr, and the β subunit is 33-37 500 Mr which is again somewhat different to my values. Each group was shown to contain considerable Mr and pI heterogeneity and they were associated through disulfide linkages which is similar to what is reported in this chapter.

The β subunit contained components with isoelectric points varying from 5.9 to 7.5 and the α subunit contained values from 8.5 to 9.2. These values are not identical to the ones I obtained but they are consistent in showing the α subunit as basic and the β subunit as neutral - acidic. The reason for differences in pI values is probably due to the technique not being completely reproducible for each experiment. Increased methionine values of 0.4 and 1.5 mole % for the β and α subunits respectively are reported. These values are most likely more accurate than mine since the presence of methionine has also been detected by CNBr cleavage in the β subunit (Dr Shewry, personal communication).

CHAPTER 3

IMMUNOCHEMICAL INVESTIGATIONS

A) Introduction

1. Antibodies as an Analytical Reagent

Antibodies may be used as analytical reagents for the identification of specific proteins. Antibodies have binding sites which are specific for the structural features (tertiary structure of the protein) of the antigen. Thus, if two proteins from different sources undergo similar antigen-antibody interactions they contain to some degree structural homology.

Antibody specificity may be used to reflect phylogenetic relationships. Homologous proteins from closely related species will be more reactive with a particular antibody than proteins from distant species. This difference in cross-reactivity is due to differences in tertiary structure which presumably arise from altered primary sequences. Such differences may arise during evolution through converging or diverging processes.

Antibodies have two antigen binding sites. When these sites are occupied a three-dimensional lattice of alternating antibody, and antigen molecule is formed. This will result in the formation of a precipitin which is visually apparent on an Ouchterlony (1968) plate. For a more detailed description of antibody binding reactions refer to Barrett (1978).

More recent techniques are available for the detection of antigens and antibodies such as radioimmune assays or Western

blots. However for the purpose of this study double immunodiffusion was sufficient for detecting the presence of globulin related proteins. Double immunodiffusion has been the method used for detecting storage proteins from moth larvae (Miller and Silhacek 1982) and even the expression of cloned genes in B. subtilis (Palva 1982).

As with most proteins the production of antibodies to plant proteins depends on the immunogenicity of the latter. If the carbohydrate content of the protein is high and the Mr is lower than 20 000 the immune response can be weak (Baudner, 1977). Oat globulin having a Mr of 60 000 under non-reducing conditions and little or no carbohydrate (Peterson, 1978) should in theory be immunogenic. Baudner (1977) has reported that plant maturity and isolation procedures may alter the antigenic properties of the protein being investigated. For taxonomic studies, mature seeds which represent well defined and physiologically stable states constitute the material most often used in these studies. As with oat globulin, raising antiserum to a storage protein class usually entails producing antibodies to several protein components. This will present no problem if the antiserum reacts only with a particular class of protein. If however, the antiserum cross-reacts with other protein fractions then it cannot be used to specifically identify the particular protein of interest.

Antiserum has successfully been raised against legume globulin which did not cross-react with other protein fractions

(Evans et al, 1979, Tumer et al, 1981). An advantage with oat globulin is that contamination by small amounts of prolamin (the second most abundant protein in oats) (Peterson, 1976) should present no problems with respect to antiserum specificity. Cereal prolamins have been shown to be very poor antigens; from 0.5 mg to 40 mg of barley prolamin has been injected into rabbits resulting in the production of very little detectable anti-prolamin antibody activity (Miflin, personal communication). The physical properties of prolamins (alcohol soluble) may render them insoluble once they are injected into rabbits.

2. Antibodies used in the Study of Seed Proteins

Immunological techniques have been extended to the study of seed proteins. For instance, Catsimpoolas et al. (1971) have looked at the effect of thermal denaturation on the main storage protein in soybean. The protein (glycinin) retained its immunological activity after heating to 65 C. A rapid loss in antigenicity was observed between 70 C and 90 C. Electrophoretic analysis of the heat denatured glycinin showed a beakdown in the native structure accompanied by the appearance of subunits. As judged by immunodiffusion these subunits did not cross-react with glycinin antiserum. The results suggested that loss in antigenicity was associated with changes in the glycinin quaternary structure and possibly alteration of the tertiary structure of the individual subunits.

The properties of phaseolin (the major storage protein in bean) during germination have been studied using antibody

cross-reactivity. Phaseolin undergoes modification with respect to charge during germination causing a shift towards the anode during electrophoresis. Double diffusion immunoprecipitation revealed that this modification did not alter the antigenic specificity of phaseolin (Daussant, 1975). It was suggested that such charge modifications resulted from deamidation of asparagine and glutamine. Such a modification would not alter the antigenic specificity of the protein.

Daussant and Renard (1972) studied alpha-amylases in developing and germinating wheat seeds using double immunodiffusion. In this manner it was determined that the alpha-amylase synthesized during germination was structurally different from the alpha-amylase present in developing seeds.

A fourth example involves aqueous soluble protein in wheat (Piazzi and Cantogalli, 1969). Gel-filtration chromatography of bread wheat and macaroni wheat albumins showed the former to contain a unique protein fraction. Antiserum was prepared against this fraction. Immunodiffusion precipitation revealed positive reactions against extracts from 16 bread-wheat cultivars and negative reactions against extracts from 16 macaroni-wheat cultivars. In this manner, immunological investigation demonstrated differences in the albumin fraction from various sources.

Thus, immunological techniques have been successfully applied to a wide variety of seed protein studies. Such techniques have been applied in this chapter to demonstrate homologies between pea and oat globulin.

3. Research Objective

The objective of this study was firstly to raise antibodies against the total oat globulin fraction. Having achieved this, anti-oat globulin antibodies were used to determine if they recognize any pea globulins as an antigen. Anti-oat globulin antibodies were also used to determine if cross-reactivity occurs among other cereal globulins.

If such cross-reactivity does exist it will strengthen the evidence from the protein chemistry studies (Chapter 2) that cereal and legume globulin are evolutionarily similar proteins.

Another purpose for the production of these antibodies was to use them to identify *in vitro* synthesized globulin. It must be emphasized that antibodies were raised against the total globulin fraction in order that all the *in vitro* synthesized globulin could be immunoprecipitated. In this manner the number of globulins synthesized *in vitro* might be estimated.

MATERIALS AND METHODS

B) Materials

Rabbits weighing about four pounds (New Zealand White) were obtained from Charles River Canada Incorp. and maintained at the Animal Care Service Facility at the University of Ottawa.
Complete Freunds adjuvant was obtained from Calbiochem-Behring Corp.
Agarose was obtained from Sigma Chemical Co.
Protein A-Sepharose and CNBr Activated-Sepharose were obtained from Pharmacia Fine Chemicals.
All other materials were reagent grade.

C) Methods

1. Production of Antiserum

Unreduced globulin (0.5 mg as shown in Figure 3,B Chapter 2) was dissolved in 0.50 ml of 20 mM Tris/HCl, pH 7.5 containing 1.0 M NaCl. This buffer had been autoclaved prior to addition of globulin. To this solution was added 0.50 ml complete Freunds adjuvant and the mixture emulsified by blending in a Polytron. The resulting emulsion was injected intra-muscularly (0.50 ml into each hind leg). Prior to the first injection, a sample of blood was collected as a control. Injections were performed at seven day intervals for a total of four weeks. After the fourth and subsequent weeks, 5 ml of blood was collected from an ear vein. Prior to collection, the ear was swabbed with xylene to cause the vein to dilate.

Xylene was washed off with ethanol and water.

After 10 weeks, rabbits were sacrificed. Blood (about 50 mls) was obtained from the ear as described above followed by an injection of Innovar-Vet (0.22 ml/kilogram body weight). Once the rabbits were anesthetized a heart puncture was performed yielding about 60 ml of blood. Blood was allowed to clot at 37 C for 30 min followed by centrifugation at 6 000 x g to separate serum from red blood cells. Serum was stored in 5.0 ml aliquots at -20 C.

2. Assay for Anti-globulin Antibodies

Serum or purified antibodies (IgG) were routinely assayed for anti-globulin activity according to the double diffusion immunoprecipitation method described by Ouchterlony (1968). Diffusion plates contained 1.5% (w/v) agarose in phosphate buffered saline, 20 mM Na_2HPO_4, pH 7.4, 0.14 M NaCl (PBS) which had been autoclaved. Antigen wells contained 10 µl of 5.0 mg/ml globulin in 0.05 M Tris/HCl pH 7.5 in 1.0 M NaCl. Antibody wells contained 10 µl of serum. Diffusion was allowed to proceed overnight. If a visible precipitin could be observed, the reaction was positive.

Titre was determined by performing a serial dilution of the serum in PBS followed by double diffusion immunoprecipitation.

3. Isolation of Total IgG from Serum

One gram of protein A-Sepharose was washed with 200 ml of 20 mM Na_2HPO_4, pH 7.4, 0.14 M NaCl. The slurry was poured

into a column (5 cm x 1.2 cm) resulting in a bed volume of about 3.5 ml. Serum (4 ml) was applied onto the column at room temperature and washed with the above buffer until 280 nm absorbing material no longer eluted. Bound protein was released from the column with 1.0 M acetic acid in the above buffer (pH 3.0). Protein was collected at 0°C and the pH immediately re-adjusted to 7.4 with 1.0 M NaOH. The column containing 0.02% sodium azide could be stored for several weeks at 4°C.

4. Isolation of Anti-globulin IgG from Total IgG

Globulin-Sepharose was prepared based on the procedure of Tan-Wilson et al, (1976). Globulin (20 mg) was placed in 10 ml of 0.05 M Tris/HCl, pH 7.5, 1.0 M NaCl then dialyzed against 0.1 M $NaHCO_3$, pH 9.0, 1.0 M NaCl. The globulin containing solution was centrifuged and the supernatant mixed with 3.5 ml (about 1.0 gram dry weight) of CNBr activated Sepharose which had been prewashed with 200 ml of 1.0 mM HCl. The slurry was gently mixed at room temperature on an end over end shaker for 2 hrs. The slurry was subsequently washed with 25 ml of the above buffer followed by incubation with 1.0 M ethanolamine for 2 hrs. The slurry was poured into a column (1.2 cm x 5 cm) followed by washing with 25 ml of 0.1 M sodium acetate pH 4.0, 1.0 M NaCl, then 25 ml of 0.02 M sodium tetraborate pH 8.0, 1.0 M NaCl. The column was then re-equilibrated with PBS.

Protein recovered from the protein A-Sepharose column was assayed for anti-globulin activity then applied onto the globulin-Sepharose column. The column was washed with PBS until 280 nm absorbing material no longer eluted. Bound protein was released from the globulin-Sepharose with 0.2 M glycine pH 2.8 and collected at 0 C. The eluant was immediately neutralized to pH 7.4 with 0.2 M Na_2HPO_4, 0.14 M NaCl. The eluted samples were tested for anti-globulin activity and stored in aliquots at -20 C.

5. Isolation of Pea Salt Soluble Proteins

Ten grams of mature pea seeds were homogenized in a coffee blender. The powder was added into 50 ml of buffer containing 0.05 M Tris/HCl, pH 8.0, 1.0 M NaCl and 1.0% (v/v) Triton X-100. The slurry was allowed to stir for 2 hours at -25 C then centrifuged at 20 000 xg for 15 min. The supernatant was filtered through Whatman No. 1 paper then dialysed at 4 C overnight against distilled water. The precipitate was collected by centrifugation followed by lyophilization and stored at -20 C.

6. Analysis of the Anti-globulin IgG - Globulin Complex

Ten grams of mature oat were homogenized in a coffee blender. The powder was added into 50 ml of buffer containing 0.05 M Tris/HCl, pH 8.0, 1.0 M NaCl and 1.0% (v/v) Triton X-100. The slurry was allowed to stir for 2 hrs at 20°C then centrifuged at 20 000 x g for 15 min. The supernatant was filtered through Whatman No. 1 filter paper. A portion (100 µl of the resulting supernatant was then added to an equal volume of anti-globulin IgG in PBS buffer. This mixture was allowed to stand overnight at room temperature. The resulting

precipitate was centrifuged, and the pellet washed with PBS and re-suspended in electrophoresis sample buffer. Electrophoresis was carried out as previously described (Chapter 1, Methods section 2).

D) RESULTS AND DISCUSSION

1. Production of Anti-globulin Antibodies

Antiserum was raised as described in methods section 1. Assays for anti-globulin antibodies were performed using the double diffusion immunoprecipitation method described in methods section 2.

Serum collected six weeks after the first injection was assayed and the results shown in Figure 1. Titre was estimated by performing a serial dilution of the serum in PBS. Serum could be diluted 64 fold and still produce a precipitin line. No precipitin lines were obtained when pre-immune serum was assayed.

The effect of globulin concentration in the antigen wells was investigated. Globulin (5.0 mg) was placed in 1.0 ml of buffer (0.05 M Tris/HCl, pH 7.5) containing either 0.10 M NaCl (low salt) or 1.0 M NaCl (high salt). Antigen wells contained 10 µl of either the low salt suspension (Figure 2, A) or the high salt suspension (Figure 2,B). Antibody wells contained 10 µl of serum. The precipitin lines in Figure 2,B are larger and have migrated further towards the antibody wells than those in Figure 2,A. This indicates that more antigen is available for immunoprecipitation. This is consistent with the solubility characteristics of globulin. More globulin is available in high salt thus the precipitation lines are larger.

FIGURE 1

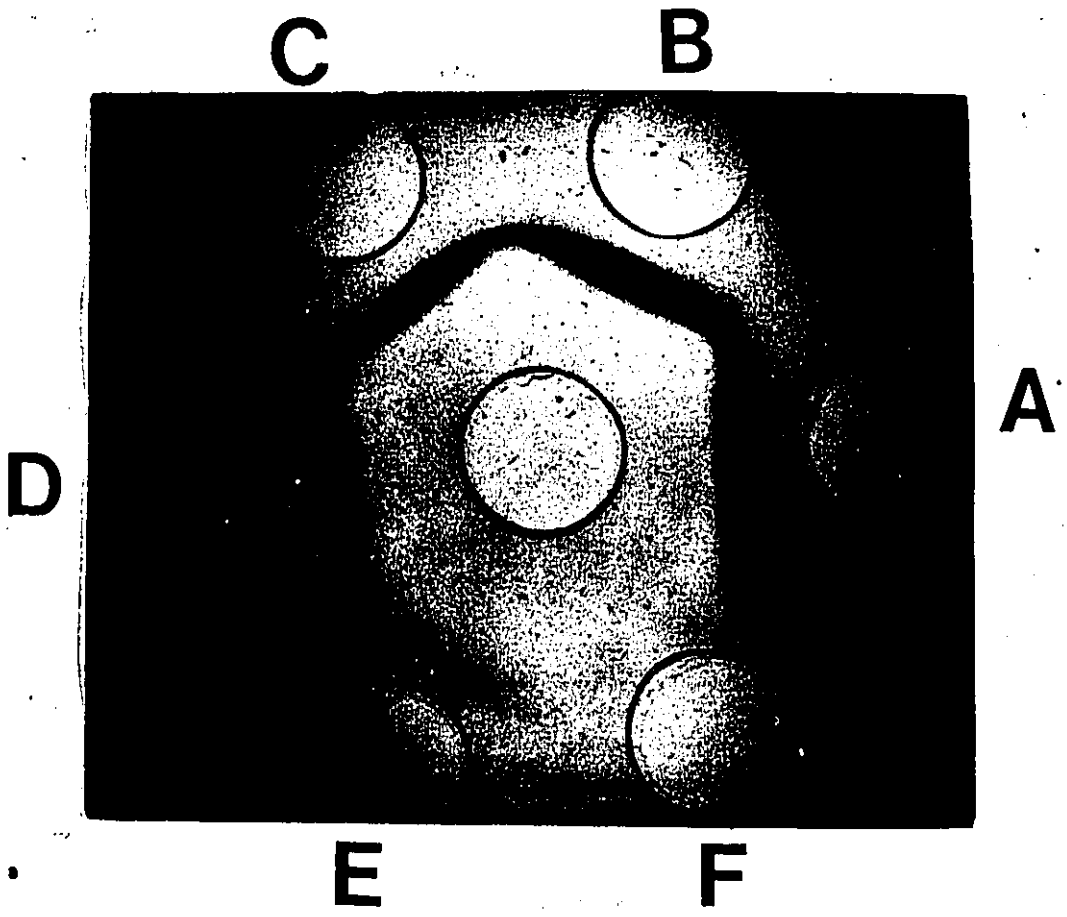


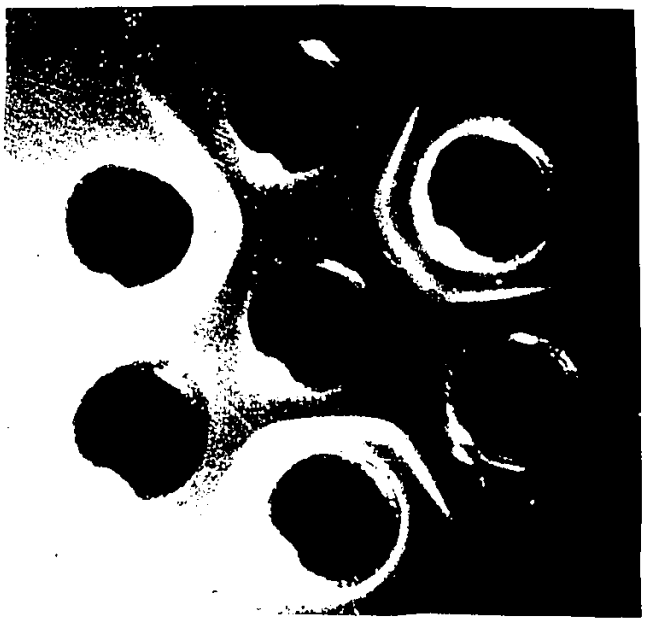
Figure 1. Assay for anti-globulin IgG using double immuno-precipitation. (methods section 2).

The center well contained 10 µl of a solution containing 5.0 mg/ml of globulin in 0.05 M Tris/HCl, pH 7.5, 1.0 M NaCl. Outside wells contained serial dilutions of serum in 20 mM Na_2HPO_4, pH 7.4, 0.14 M NaCl.

Dilutions: A X 4, B X 8, C X 16, D X 32, E X 64, F X 128. The plate was washed in 1.0 M NaCl for 48 hrs followed by staining and destaining according to Chapter 1, methods section 2.

FIGURE 2

A



B

Figure 2. Immunoprecipitin lines obtained when globulin is dissolved in different salt concentrations.

A) Immunoprecipitin lines obtained using globulin dissolved in 0.10 M NaCl

Wells A,B,C, contain 10 µl of a solution containing 0.75 mg/ml globulin in .05 N Tris-HCl, pH 7.5, 1.0 M NaCl. All other wells contained 10 µl of serum.

B) Immunoprecipitin lines obtained using globulin dissolved in 1.0 M NaCl.

Wells A,B,C, contain 10 µl of a solution containing 4.2 mg/ml of globulin in .05 N Tris-HCl, pH 7.5, 1.0 M NaCl. All other wells contained 10 µl of serum.

Proteins assays were performed (Chapter 1, method section 4) on the globulin solutions to determine the amount of soluble globulin present in each. The high salt solution contained 4.2 mg/ml (42 µg globulin/antigen well; Figure 2,B) and the low salt solution contained 0.75 mg/ml (7.5 µg globulin/antigen well; Figure 2,B). There is about 5 times more globulin soluble in 1.0 M NaCl than in 0.1 M NaCl. The precipitin lines observed in Figure 2B are larger than in Figure 2A which is consistent with their respective different amounts of antigen. This provides evidence suggesting that the serum contains antibodies which are specific for salt soluble proteins.

2. Purification of Anti-globulin IgG from Serum

The purification procedure is described in methods section 3 and 4. Serum was chromatographed through a protein A-Sepharose column giving rise to the elution profile shown in Figure 3. Bound protein was released by lowering the pH of the eluting buffer. The second peak was pooled, neutralized and tested for anti-globulin activity as described in methods section 2. A 32-fold dilution could be performed on this fraction and still produce a precipitin indicating the presence of anti-globulin IgG. The IgG fraction was applied onto a globulin-Sepharose column prepared as described in methods section 4. Again, bound protein was eluted in the presence of low pH. The elution profile is demonstrated in Figure 4. Protein from the second peak could be diluted 32-fold and still yield a precipitin similar to the IgG fraction obtained from

FIGURE 3

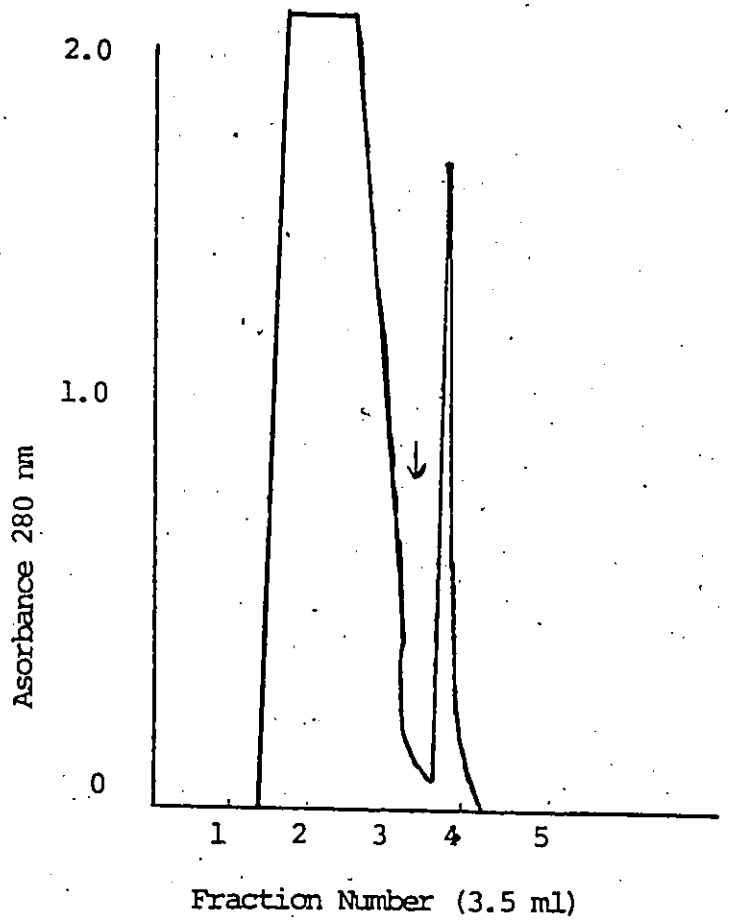


Figure 3. Affinity chromatography of rabbit serum on protein A-Sepharose.

Serum (4 ml) was applied to the column (5 x 1.2 cm) followed by PBS until 280 nm absorbing material no longer eluted.

The arrow indicates when PBS buffer containing 1.0 M acetic acid was applied to the column.

FIGURE 4

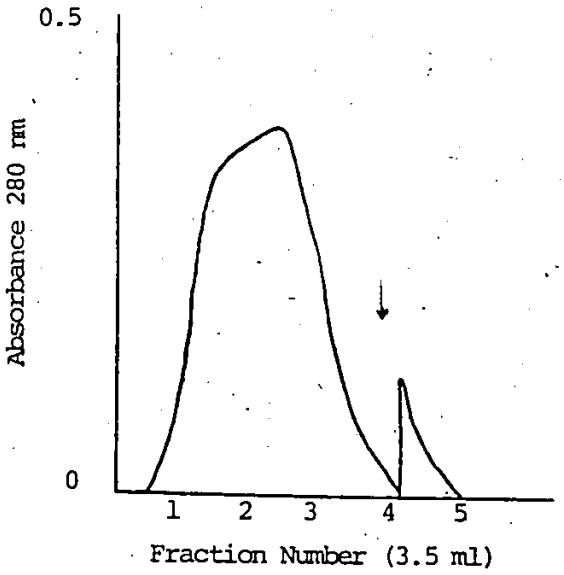


Figure 4. Affinity chromatography of IgG on globulin-Sepharose.

5.0 ml of PBS containing IgG (40 mg) was applied to the column (1.2 x 5.0 cm) followed by PBS buffer.

The arrow indicates when 0.2 M glycine, pH 2.8 was applied to the column.

the protein A-Sepharose column. Protein assays were performed on the antibody containing fractions and Table 1 summarizes the purification steps. Anti-globulin IgG could be purified 250-fold. The specific activity was calculated as the maximum serial dilution still yielding a precipitin, divided by the protein concentration. For example, the specific activity of anti-globulin eluted from globulin-Sepharose was 128. This value was arrived at by dividing 32 (maximum serial dilution still yielding a visible precipitation line) by 0.25 (total protein recovered/volume).

3. Cross Reactivity Among Other Oat Protein Fractions

The final anti-globulin IgG preparation eluted from the globulin-Sepharose column was tested for cross-reactivity against oat albumins, glutelins and prolamins prepared by Laurian Robert in our Laboratory. Anti-globulin IgG reacted only against globulin (Figure 5).

It should be mentioned that the prolamins and glutelins may not have been available for reaction since they are not salt soluble. Thus, these proteins should not have been in the original globulin preparation (also see page 78) in which case no antibodies would have been produced against them. No precipitin line would have occurred in this case.

The reason for the double precipitation is unknown. It is generally not observed in most double immunodiffusion tests. However this may be due to globulin being present in various forms such as the 360 000 Mr holoprotein (Peterson 1978), the

TABLE I

Purification of Anti-globulin IgG

	Protein concentration (mg/ml)	Specific Activity[1] (serial dilution/mg/ml	Purification
Serum (4 ml)[2]	125.0	0.51	1.0
IgG eluted from protein A-Sepharose with 1 M acetic acid (5 ml)	8.0	4.0	7.8
IgG eluted from globulin-Sepharose with 0.2 M glycine pH 2.8, (5 ml)	0.25	128.0	251.0

[1]Specific activity was determined by dividing the maximum serial dilution factor, where a positive double immunodiffusion test could be obtained, by the protein concentration.

[2]Serum was obtained by letting the blood clot at 37°C for 15 min followed by centrifugation at 6 000 x g for 15 min.

FIGURE 5

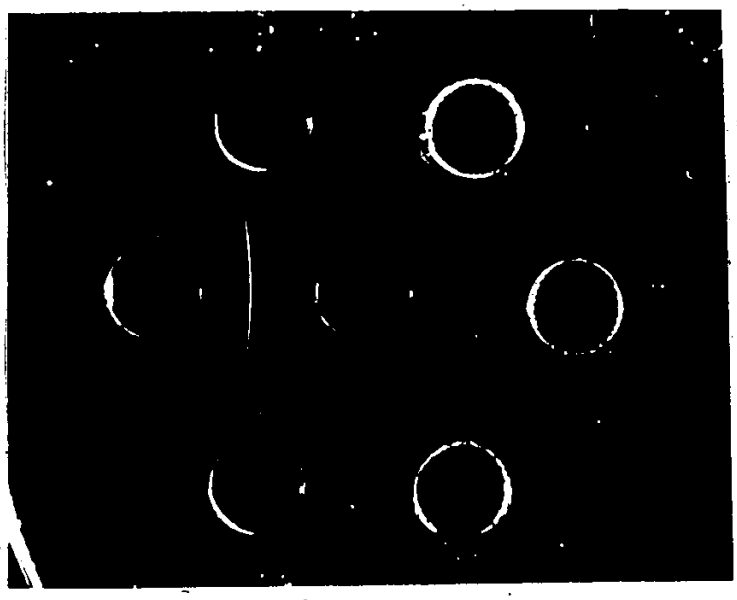


Figure 5. Immunodiffusion analysis using anti-globulin IgG against oat seed protein fractions.

Wells, A,B,C,D,E contained 10 µl aliquots from a 5.0 mg/ml dispersion containing globulin, albumin, prolamin, glutelin and a blank in .05 N Tris-HCl, pH 7.5, 1.0 N NaCl.

The centre well contains 10 µl of anti-globulin IgG.

dimer (Figure 3, Chapter 2) and the individual subunits. Furthermore since this is a polyclonal antibody preparation immunodominance of some of the polyclonals for one or more form of globulin may have given rise to this double line. For example, one antibody population may recognize the holoprotein but not the dimer and vice versa.

Oat salt soluble extract was mixed with the isolated IgG and allowed to incubate overnight as described in Methods section 6. The resulting precipitate was washed and subjected to SDS-PAGE under reducing conditions. Figure 6 demonstrates that the resulting precipitate contains predominantly the α and β subunits. These subunits probably have arisen from the holoprotein (see page 140 for further discussion). Minor bands are observed from Mr 30 to 35 000 and the β subunit shows major bands from Mr 35 to 41 000. These minor bands from 30 to 35 000 are slightly visible in Figure 1, Chapter 2 and are more pronounced in Figure 9 of this chapter especially the one of Mr 30 000. Since these are salt soluble and precipitate upon dialysis against H_2O they are presumed to be globulin, however their exact relationship with the β subunit is questionable. The band at Mr 30 000 is also observed in pea and rye globulins and will be discussed further in a following section.

This experiment does however suggest that the double diffusion immunoprecipitate observed in Figure 5 does arise from a globulin antibody complex. The higher Mr proteins from

FIGURE 6

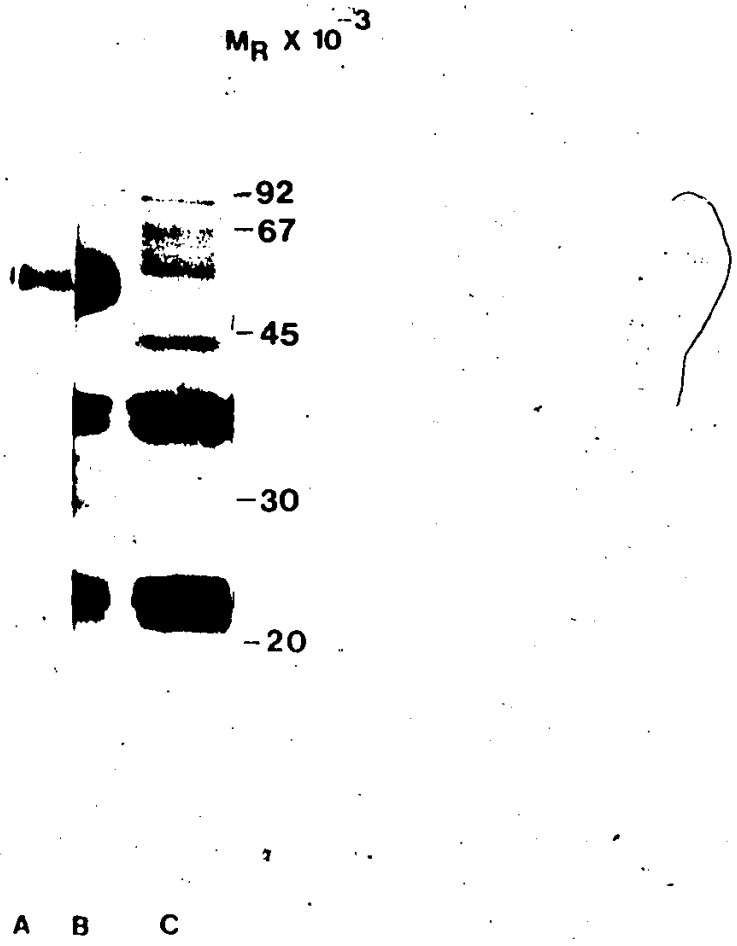


Figure 6. Analysis of the Immunoprecipitate by SDS-PAGE

Samples were prepared as described in Methods section 6.

Track A) Anti-globulin IgG prepared according to Methods section 3 and 4.

Track B) Proteins precipitated from the total salt soluble fraction using anti-globulin IgG.

Track C) Total protein soluble in 0.05 M Tris-HCl, pH 7.5 1.0 M NaCl, 1.0% (V/V) Triton X-100.

65 to 100 000 and 40 to 50 000 do not appear to be recognized by these antibodies.

Taken together, the antigen solubility and the cross-reactivity and SDS-PAGE experiments provide evidence that the isolated anti-globulin IgG is specific for the globulin fraction.

4. Antigenic Relationships between Cereal and Legume Globulin

Anti-globulin IgG was tested for cross-reactivity against rye globulin (prepared in a similar manner to oat globulin by Laurian Robert in our laboratory) and pea globulin (method section 5). Anti-globulin IgG prepared against oat globulin showed cross-reactivity against rye and pea salt soluble protein (Figure 7, 8). All the intercepts formed by the precipitation lines in Figure 7 and 8 merge to form single arcs. The precipitation lines do not cross or form spurs. This indicates that the antibody cannot distinguish one antigen from the others (Barrett 1978).

One would expect cross-reactivity to occur against rye globulin since it is a cereal (subclass Monocotyledoneae) similar to oat. What is more significant however is that cross-reactivity also occured against pea (subclass Dicotyledoneae) globulins. This was not unexpected in view of the results obtained in Chapter 2 which revealed that oat and legume globulins have similar subunit associations, pI values and amino acid compositions.

Both oat globulin and pea legumin are present in the mature seed as hexamers (Mr 360 000) of the dimers (Mr 60 000). This has yet to be established for rye globulin. The hexamer is probably a very compact molecule since it is the storage form. Oat globulin was not denatured priorto injection into the rabbits therefore the antibodies may be predominantly against the hexamer. It may be this hexamer structure which is cross-reacting from pea and possibly rye. It is possible that the hexamers are more homologous in size and structure than the dimers and subunits thus explaining why spurs were not observed.

FIGURE 7

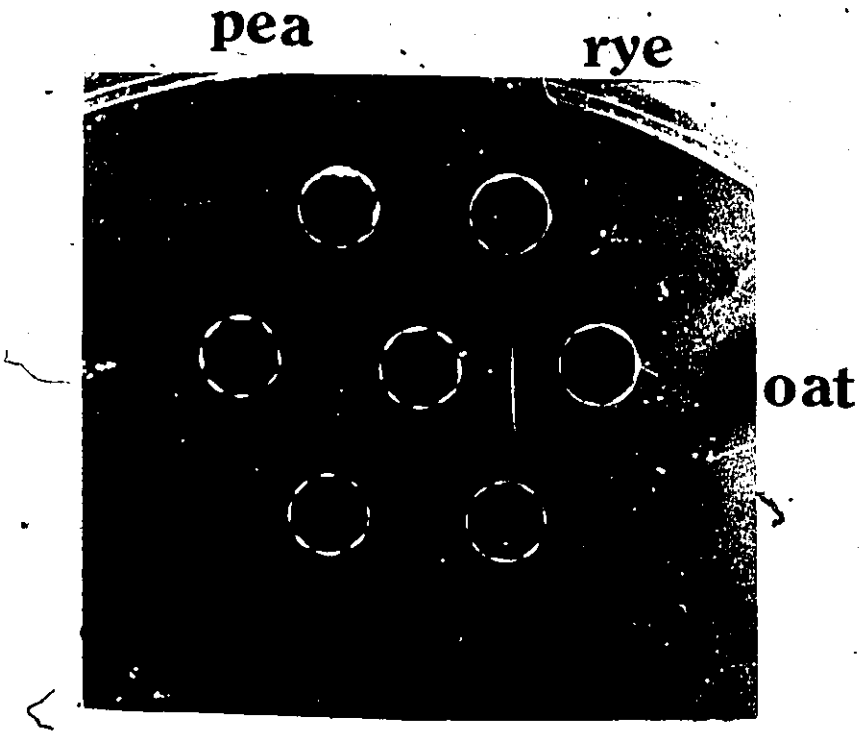


Figure 7 . Immunodiffusion plate showing cross-reactivity between anti-globulin IgG and salt soluble protein from rye and pea.

Oat, rye and pea wells contain 20 µg, 110 µg, and 150 µg of salt soluble protein respectively.
The middle well contains 10 µl of anti-globulin IgG prepared according to method section 3 and 4.

FIGURE 8

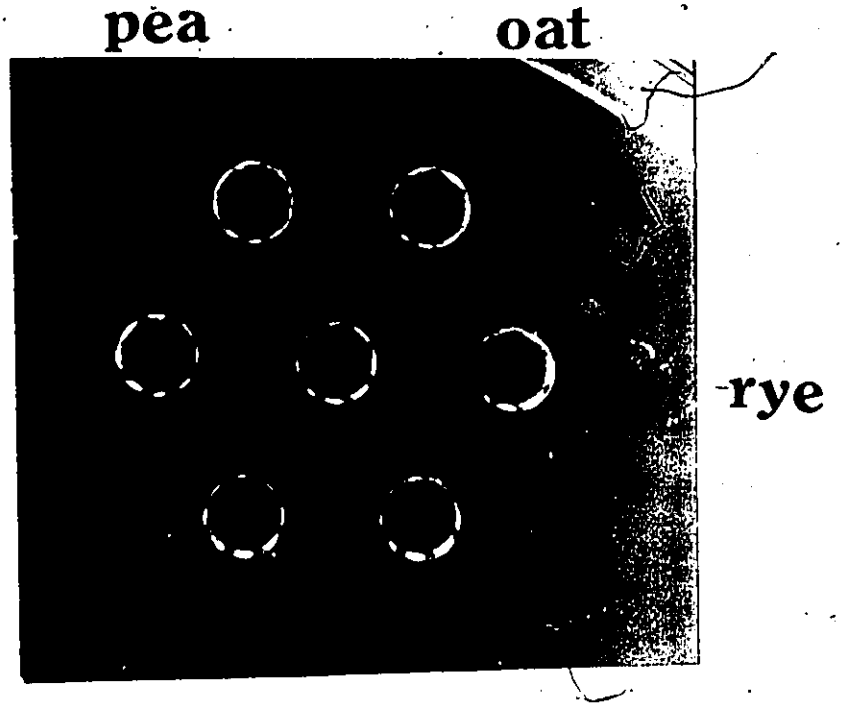


Figure 8. Immunodiffusion plate showing cross-reactivity between anti-globulin IgG and salt soluble protein from rye and pea.

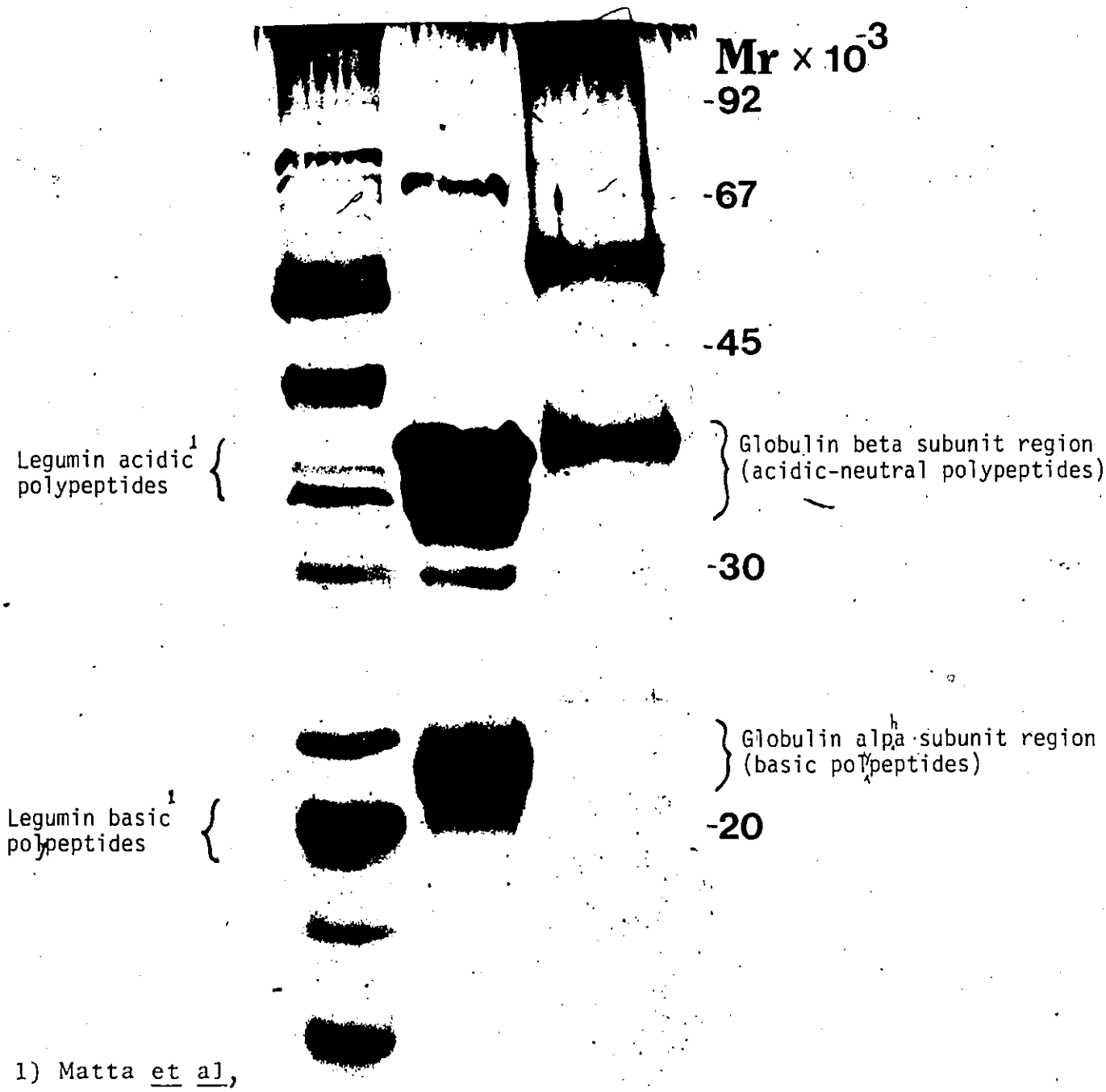
Oat, rye, and pea wells contain 20 µg, 90 µg and 130 µg of salt soluble protein respectively.
The middle well contains 10 µl of anti-globulin IgG prepared according to method section 3 and 4.

For a similar size precipitation to occur for pea or rye about 5 - 7 times more protein than oat globulin was required (page 141 and 143). This indicates that the required protein concentration was less for oat globulins than for pea and rye globulins. This was not unexpected since the anti-serum was raised against oat globulins thus should have a higher titre against oat globulins.

In an attempt to further understand this cross-reactivity the various globulin fractions were submitted to SDS-PAGE (Figure 9). Under reducing conditions pea legumin contains 2 major heterogeneous subunits (Matta et al. 1981). The smaller subunit group is basic and has a Mr of about 20,000 and the larger acidic group has a Mr of about 37,000. The rest of the pea salt soluble proteins are defined as vicilins (Matta et al., 1981). The legumin areas are also indicated in Figure 9 . These groups migrated to the same vicinity on the gel as did the oat basic α subunit and the acidic β subunit. It is possible that these are the antigenically related proteins. Rye also appears to share a major similar Mr protein to the acidic subunit of oat globulin and pea legumin.

The pattern obtained here for oat globulin is similar to the one in Figure 1, Chapter 2. The globulin band of Mr 30 000 is more apparent on this gel. This polypeptide is generally visible when more globulin is applied on the gel. This band is also shared by pea and rye globulins.
This protein is immunoprecipitated (Figure 6). L. Robert (personal communication) has observed that during the early stages of development, the beta subunit contains polypeptides from Mr 30-35,000. During the later stages the beta subunit contains predominantly polypeptides in the Mr region from 35 to 40,000.

FIGURE 9



1) Matta et al, 1981

Figure 9. SDS-PAGE pattern of the salt soluble protein fraction obtained from pea oat and rye (Chapter 2, method section 2). Pea legumin subunits[1] and oat globulin subunits are indicated. About 50 - 60 ug of each protein sample was applied on this gel.

1) Matta et al. 1981

E) CONCLUSIONS AND FUTURE EXPERIMENTS

This work shows that cereal and legume globulins share similar antigens. Thus, they contain to some extent proteins with analogous tertiary structure which presumably arise from similar primary sequences. Such observations provide additional evidence for the homology between cereal and legume globulins.

Oat and pea globulin polypeptides may have arisen from the same gene(s) or gene families. These gene(s) may have been altered during the past few million years giving rise to the different electrophoresis banding patterns seen in Figure 8. However, these alterations have not been extensive enough to completely disrupt antigenic sites shared by these globulins.

Alternatively, these globulins may have originated from completely different genes and evolved such that oat and pea now contain similar globulins.

In view of these findings experiments confirming gene homology should be initiated at both the protein and DNA level. A legumin (pea globulin) clone does exist in Boulter's (Croy et al. 1982) laboratory and has been made available to our group. Experiments using Southern methodology will try to confirm gene homology. Amino acid sequencing is being conducted on the purified globulin subunits. When this data is available, it will be compared to known sequences of legumin.

Larkins (personal communication) has shown that oat globulin and pea legumin basic subunits share about 50% sequence

homology in the first 18 NH_2 terminal amino acids. Such homology substantiates the immunocrossreactivity demonstrated in this chapter in suggesting that these globulins may have arisen from a common ancestral gene(s).

CHAPTER 4

GLOBULIN BIOSYNTHESIS

A) INTRODUCTION

1. Globulin Biosynthesis

Globulin biosynthesis has been extensively studied in legumes (Croy et al, 1980, 1982; Tumer et al, 1981, 1982; Barton et al, 1982; Chrispeels et al, 1982) presumably because it is the major storage protein and because of the nutritional significance of legume proteins in general. It was established by in vitro and in vivo methods that the acidic and basic subunits arise from a post-translational modification of a globulin precursor.

Luthe and Peterson (1977) have reported the in vitro translation of oat polysomes. Their results indicated that polysomes from developing groats synthesize the 21 000 and 31 000 Mr peptides of globulin. They also suggested that a precursor may be synthesized in vitro.

Oat globulin biosynthesis was re-investigated in this chapter to investigate the possibility of a precursor.

2. Messenger RNA and In Vitro Translation

The first extensively studied mRNA was derived from rabbit reticulocyte polysomes which code for globin. Zone sedimentation of polysomes treated with SDS showed the occurrence of a minor 9S peak as well as the ribosomal RNA

species. It was later established that this 9S RNA component was capable of inducing globin synthesis when introduced into frog oocytes (Lane et al, 1971). Since that time, the structure and function of mRNA has been studied in detail (Adesnik et al, 1972); Weissman et al, 1973; Bronson et al, 1973); Rosen et al, 1976; Bina et al, 1980).

Plant mRNA thus far has yielded few surprises. The presence of poly A^+ sequences has been demonstrated for several plant species (Van de Wall, 1973;Verma et al, 1974;Esnault, et al, 1975). Gray and Cashomore (1976) however have suggested that as much as 35% of mRNA in leaves is non-polyadenylated.

The assay used for poly A^+ was its ability to bind to poly U-Sepharose. These results may not have been accurate since some of the poly A^+ RNA may not have been retained on the column. Furthermore, poly A^+ tails may have been removed during the phenol-chloroform extraction performed on polysomes. Alternatively, the leaf tissue may have removed or shortened the poly A^+ sequences.

Plant mRNA has been shown to translate in vitro in a similar manner to animal mRNA (Tumer et al, 1982 ; Matthews and Miflin 1980; Larkins et al, 1978; Hall et al, 1978). Throughout the rest of this thesis I will refer to mRNA to describe its general properties and poly A^+ RNA to describe experimental details.

The isolation of seed mRNA however does deserve mention. The common phenol extraction procedure (Maniatis et al, 1982) of a tissue homogenate has limited success when dealing with developing seeds. In doing so, large amounts of starch accompany the RNA in the aqueous phase. Starch tends to precipitate in the presence of ethanol thus contaminating RNA, making subsequent purification steps difficult.

A better method developed by Larkins et al, (1976) is to isolate polysomes from a tissue homogenate by means of ultra-centrifugation through a sucrose cushion. These polysomes (free of starch) may be dissociated and poly A^+ RNA isolated in a manner similar to animal mRNA using oligo-dT cellulose.

A further advantage of this method is that polysomes may be used to direct in vitro translation. This yields valuable information with regards to which proteins are being synthesized in vivo at the time of polysome isolation.

Numerous eukaryotic cell-free protein synthesizing extracts (in vitro translation systems) have been shown to translate mRNA. In vitro translation systems have been reported using extracts from Krebes II ascites cells (Housman et al, 1971; Swan et al, 1972), mouse liver cells (Sampson et al, 1972), Hela cells (McDowell et al, 1972), reticulocytes (Lodish, 1971;Pelham and Jackson, 1976) and wheat germ (Roberts and Paterson 1973). Of these, the most commonly used cell free systems are derived from reticulocytes and wheat germ. Wheat germ extracts are easier and more rapidly prepared than reticulocyte lysates. Wheat germ cell free systems however, have been termed "early quitters" due to premature termination. When this occurs the efficency for synthesizing large polypeptides is decreased. Reticulocyte lysate cell free systems do not suffer such drawbacks however, they often contain high background levels of mRNA. This endogenous mRNA often prevails even after nuclease treatment.

3. Protein Transport

Blobel in 1975 postulated the role of the signal (leader) sequence. He used this sequence to explain how a cell

identifies proteins destined for secretion. This sequence extends from the NH_2-terminus by 15-30 amino acids.

The leader sequence is required for the vectorial transfer of proteins across the membrane of the endoplasmic reticulum (Blobel and Dobberstein 1975, Talmadge et al, 1980). As the sequence emerges from the large ribosomal subunit it directs the ribosomal complex towards a specific binding site located on the endoplasmic reticulum membrane. Upon forming this association the nascent protein is translocated across the membrane. During this event the signal sequence is proteolytically removed and the remainder of the synthesized protein is transfered across the membrane into the cisternal space.

Signal sequences have subsequently been shown to contain non polar middle regions flanked by hydrophylic residues (Dobberstein 1981). No homology among the primary sequences of these leader sequences has been established.

The mechanisms in which the signal sequence carries out its function has remained elusive. Recently, a cytoplasmic protein complex of Mr 250 000 referred to as the signal recognition protein (SRP) has been shown to block translation of nascent secretory proteins (Walter and Blobel 1981). A specific protein located on the endoplasmic reticulum referred to as the "docking protein" releases the block and allows translation to proceed (Meyer et al, 1982). Such a mechanism

insures that a secretory protein is not completely synthesized in the cytoplasm and subsequently released from the ribosome.

An alternate method of protein translocation has been reported in the case of ribulose bisphosphate carboxylase. This enzyme consists of two subunits; a large subunit synthesized in the chloroplast and a small subunit synthesized in the cytosol. The small subunit translocates into the chloroplast to produce the holo-enzyme. This translocation occurs only after it has been completely synthesized by free polysomes in the cytoplasm (Highfield and Ellis, 1978). This subunit contains a leader sequence which is removed during translocation. Thus, the translocation is post-translational and not co-translational.

Plant storage proteins have been shown to be synthesized on membrane bound polysomes (Chrispeels et al, 1982; Tumer et al, 1981; Matthews and Miflin, 1980; Croy et al, 1980). The isolation of such polysomes followed by in vitro translation should result in a product which contains no leader sequence. Alternatively, isolating poly A^+ RNA from these polysomes followed by in vitro translation should result in a product which still contains a leader sequence. Thus, SDS-PAGE followed by flourography should reveal that the poly A^+ RNA translation products are larger than the polysomal products. Comparing the size of oat globulin synthesized in vitro by membrane bound polysomes and poly A^+ RNA may reveal if a leader sequence is present.

B) CELL FREE SYNTHESIS OF SEED STORAGE PROTEIN

1. Initial Studies

Early work involving cell free synthesis of storage protein was carried out in French bean (*Phaseolus vulgaris*) by Sun *et al* (1975) and in maize (*Zea may L.*) by Larkins *et al*. (1976). Both these studies were performed using polysomes from developing seeds in the presence of wheat germ extracts. *In vitro* synthesis polypeptides containing radioactive amino acids were separated by gel-electrophoresis. Gel slices were isolated and the migration of radioactivity was compared to the migration of authentic storage protein.

2. In Vitro Synthesis of Zein

Larkins and Hurkman (1978) used fluorography rather than gel slices to obtain more accurate molecular weight determinations of the translation products. It was revealed that *in vitro* synthesized zein (maize storage protein) was larger than native zein by a Mr of 2000. This suggested that storage proteins are synthesized as precursors, similar to animal proteins made on endoplasmic reticulum (Blobel and Dobberstein, 1975).

To verify if endoplasmic reticulum was involved in processing zein, Larkins and Hurkman (1978) compared the *in vitro* synthesis of zein under two conditions. One *in vitro* translation reaction contained microsomes and polysomes

while the other contained only polysomes. In vitro products synthesized in the presence of microsomes had a smaller molecular weight than products made in their absence. The results of this study were however, not conclusive since in vitro synthesis was carried out using polysomes obtained from membranes. Such polysome preparations have probably already lost their leader sequences due to proteolytic activity in the endoplasmic reticulum (Blobel and Dobberstein, 1975).

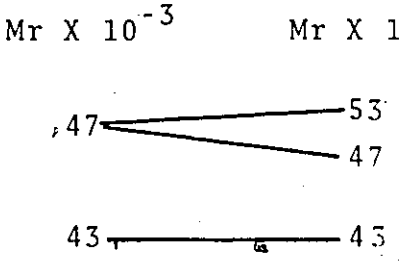
Larkins et al. (1979) continued studying the biosynthesis of maize storage protein by injecting its mRNA into Xenopus oocytes. The results demonstrated that Xenopus oocytes will translate plant mRNA. It was further demonstrated that the storage protein translation products were approximately 2 000 Mr smaller than the translation products from either wheat germ or reticulocyte cell free systems. Previous reports have shown oocytes efficiently translate a number of animal mRNA's and cleave signal peptides from secretory proteins (Kindas-Mugge et al, 1974; Zehavi-Willner and Lane, 1977). Thus, the results of Larkins' study demonstrated that the signal peptide of a plant protein is recognized by an animal system. These observations added additional support to the hypothesis that the oocyte enzyme(s) required to cleave signal sequences are not spetific (Rapoport et al. 1978).

3. In Vitro Synthesis of Phaseolin

Hall et al. (1978) isolated the mRNA for the storage protein (phaseolin) of french bean seeds. Authentic phaseolin has three polypeptide subunits of Mr 53 000, 47 000 and 43 000 (McLeester et al, 1973). In vitro synthesis of phaseolin showed polypeptides of about 47 000 and 43 000 Mr corresponding to two of the three authentic subunits. Immunoprecipitation of these products with monospecific antibodies demonstrated that these were in vitro synthesized phaseolin. However, no trace of the largest (53 000 Mr) subunit was observed among the in vitro products. Furthermore, these in vitro synthesized products were slightly smaller than native subunits.

Phaseolin accumulates in membrane-bound protein bodies, thus it seemed likely that they would be translated as precursor molecules of higher molecular weight than the native proteins. A possible explanation for this discrepancy may be a lack of post-translational modification such as glycosylation. Higgins and Spencer (1977) cited this as a possible reason for their failure to detect immunochemically any pea globulin among polysome-directed in vitro synthesized products. Hall et al. (1978) cited several possible reasons for the absence of the 53 000 Mr subunit. Lack of a poly A^+ sequence in its mRNA or lack of recognition of the initiation sequence by the wheat germ system were suggested. Brown et al. (1981) further studied this problem by injecting phaseolin mRNA into Xenopus

oocytes in the presence and absence of a glycosylation inhibitor (tunicamycin). In the presence of the inhibitor the 43 000 and 47 000 Mr products were synthesized. In the absence of glycosylation inhibitor the 43 000, 47 000 and 53 000 Mr phaseolin subunits were synthesized. It was concluded that Xenopus oocytes are capable of post-translational glycosylation of phaseolin in the manner shown below:



4. In Vitro Synthesis of Hordein

Matthews and Miflin (1980) studied the in vitro synthesis of the major storage proteins in barley (Hordeum vulgare). They are prolamins which are termed hordeins. In vitro synthesized products were judged to be prolamins on the basis of

A) solubility characteristics,

B) low levels of incorporation of lysine, and

C) electrophoretic mobility.

Hordeins derived from mRNA *in vitro* translation were larger than authentic and polysomal run off *in vitro* synthesized hordein. This was consistent with the Blobel hypothesis.

5. *In Vitro* Synthesis of Legumin

Higgins and Spencer (1977) using polysomes from developing pea were unable to demonstrate the *in vitro* synthesis of the legumin 40 000 and 20 000 Mr subunits. Evans *et al*. (1979) again using polysomes from developing pea could not demonstrate the *in vitro* synthesis of these subunits. Croy *et al*. (1980) however was successful in characterizing the legumin storage protein synthesized *in vitro* by using anti-legumin antibodies for selective immunoprecipitation. Analysis of the anti-legumin immunoprecipitated polypeptides on SDS gel-electrophoresis and fluorography revealed a single band of Mr 60 000. This single immunoprecipitated product could be recognized as a major polypeptide from the total translation products. The 60 000 Mr immunoprecipitated polypeptide was unaffected by the presence of mercaptoethanol whereas native legumin dissociates into two subunits (Matta *et al*. 1981). Croy *et al*. (1980) concluded from this study that legumin is synthesized as a 60 000 Mr precursor which is post-translationally modified into two subunits of Mr 20 000 and 40 000.

During the same study, *in vivo* labelling (at a period 13 days after anthesis) of legumin was performed to determine if the 60 000 Mr precursor is processed in the intact seed. The

intensity of the in vivo labelled 60 000 Mr polypeptide in the fluorograph relative to the 20 000 and 40 000 Mr subunits was greater after short term labelling (12 hours) than after long term labelling (6-10 days). This indicated that processing was occuring. These results could also have however been due to a number of factors involving the isolation of these subunits.

Croy et al. (1980) also compared the polysomal directed translation products from bean (Vicia faba) which also contains legumin as a major storage protein. Specific immunoprecipitation of a single polypeptide of Mr 60 000 was demonstrated. No proteins of Mr 40 000 or 20 000 were immunoprecipitated or were present as prominent bands among the total translation products. Thus, the possibility that the legumin precursor is unique in pea was eliminated by this observation. This was contrary to the report by Muntz (1978) which claimed that membrane-bound polysomes from beans directed the synthesis of the 20 000 and 40 000 Mr subunits. In this study however, antibodies were not used to characterize the translation products. Mobility in SDS gel-electrophoresis was the only criterion used to identify the products. This is analogous to Luthe and Peterson's (1977) report on oat globulin. They also claimed separate synthesis of subunits, but did not verify it using immunoprecipitation.

Chrispeels et al. (1982) studied the labelling kinetics of the smaller legumin polypeptides and the site of their

assembly into oligomers. Cotyledons were pulse labelled with ^{14}C amino acids for one hour and chased for various times. Legumin was first observed as the precursor (60 000 Mr) at zero hours chase and was completely processed after a period of three hours. Upon isolating both endoplasmic reticulum and protein bodies it was established that the precursor was contained in the former and processing of the precursor occured in the latter. Furthermore, it was determined that legumin is formed as an 8S product in the endoplasmic reticulum and assembled into the 12S product in the protein bodies. Bollini et al. (1982) has also determined that the endoplasmic reticulum plays a similar role during the synthesis of phaseolin in bean.

6. In Vitro Synthesis of Glycinin

Glycinin in soybean (Glycinine max L.) contains two major subunits analogous to legumin in pea (Moreira et al. 1979). Beachy et al, (1978) claimed to have demonstrated the in vitro synthesis of the individual 40 000 and 20 000 Mr subunits. In Beachy's study however, in vitro synthesized glycinin was not identified by immunoprecipitation.

Tumer et al. (1981) investigated the biosynthesis of glycinin by in vitro translation followed by immunoprecipitation. Glycinin with Mr 58 000 to 63 000 were identified in this manner. The precursors were also immunoprecipitated by antibodies raised against either of the purified 40 000 or 20 000 Mr subunits. The authors concluded that a glycinin precursor exists which contains the two smaller subunits linked

via a peptide bond which is apparently cleaved post-translationally. Furthermore, it was observed that *in vitro* synthesized glycinin precursors had slightly lower electrophoretic mobilities than authentic unreduced glycinin. The decreased mobility was attributed to an increase in molecular weight of about 3 000 which could reflect the presence of a leader sequence.

In the same study, mRNA was fractionated on linear sucrose gradients. It was established that 18S RNA was capable of synthesizing the 60 000 molecular weight glycinin precursor.

Tumer *et al.* (1982) attempted to investigate the processing of the glycinin precursor by injecting glycinin 18S mRNA into *Xenopus* oocytes. The immunoprecipitated products did not contain the individual subunits but rather the precursor. Thus, *Xenopus* oocytes do not contain the proper processing enzyme or, the enzyme may not have been present in the proper cellular compartment to cleave the glycinin precursor.

The glycinin polypeptides synthesized in oocytes were however 1000 to 2000 Mr smaller than those synthesized in the reticulocyte lysate system. This suggested the removal of a leader sequence from the precursor. This was confirmed by NH_2-terminal sequence analysis of glycinin synthesized in oocytes which further revealed the presence of the acidic subunit at the NH_2-terminal end of the precursor polypeptide. It was concluded from this study that glycinin precursors have

an NH_2-terminal leader sequence followed by the acidic then basic peptide components joined by a peptide bond.

7. In Vitro Synthesis of Oat Globulin

Luthe and Peterson (1977) reported the cell free synthesis of globulin by developing oat. Polysomes were isolated by centrifugation through a 0.25 M sucrose cushion. These polysomes were subjected to sucrose gradient ultracentrifugation. There were only small amounts of large polysomes, however a 9-mer could be resolved. These polysomes were translated in a wheat germ cell free translation system. The pH and Mg^{++} optimum were 6.8 and 4 mM respectively. The dependance on polysome concentration was linear for up to 40.0 O.D. units/ml. Incorporation of ^3H amino acids into TCA precipitable products was linear for up to 5 - 10 min and continued for up to 60 min. An 8 fold stimulation of translation was obtained using polysomes sedimented through 2 successive sucrose cushions. Polysomes sedimented once through the sucrose cushion stimulated translation 6 fold. The amount of initiation taking place was determined by T-2 toxin inhibition. About 30% of the amino acid incorporation was due to initiation.

In vitro synthesized products were co-electrophoresed on SDS-denaturing gels with authentic globulin. Gel slices were prepared, counted and the migration of radioactivity was compared to the migration of authentic globulin. Eighty-seven percent of the total radioactivity co-migrated with the globulin subunits. They also observed a small amount of radioactivity migrating

to a Mr of 56 000. Two possible explanations for this were presented. They speculated that it may have been a polypeptide unrelated to globulin or a precursor for the globulin subunits. They suggested that a precursor may be present since a proportionally greater amount of this polypeptide was present within the *in vitro* products than was present in authentic globulin. *In vitro* products and authentic globulin subunits were subjected to CNBr cleavage. Authentic globulin yielded six fragments thus indicating the presence of 4 methionines. *In vitro* products yielded several fragments and two of them co-migrated in SDS-PAGE with authentic globulin fragments. It should however be noted that *in vitro* synthesized globulin in the precursor or subunit form would have migrated essentially the same after CNBr cleavage. Furthermore the evidence for a precursor is difficult to accept since protein staining and protein labeling are not accurately comparable in terms of intensities.

It was determined that free polysomes contained a larger monosome peak and translated only one half as efficiently as did membrane bound polysomes. However, free and membrane bound polysomes directed *in vitro* synthesis of similar products. It was concluded that membrane bound and free polysomes synthesize the two globulin peptides.

The identification of *in vitro* synthesized products on the basis of Mr is no longer valid since it has led to misleading conclusions for legumin and glycinin biosynthesis

in the past (Muntz 1978, Beachy et al, 1978). Both these studies reported the separate synthesis of subunits, however it was later revealed that these proteins were synthesized as precursors (Tumer et al, 1981, Croy et al, 1980). There was little homology between the CNBr fragments of the in vitro products and authentic globulin (Luthe and Peterson 1977). Furthermore CNBr fragments of a precursor or the individual subunits would be very similar thus any conclusions would be difficult to arrive at. Thus, it appears that there is ample justification for a reinvestigation of the in vitro synthesis of globulin using more advanced techniques such as immunoprecipitation and two dimensional analysis.

Adeli (M.Sc. thesis, University of Ottawa 1982) has also isolated polysomes and poly A^+ RNA from developing oat seeds. Polysomes were used as a template for translation in a New England Nuclear rabbit reticulocyte translation kit. Incorporation of TCA precipitate counts over background was about 3 fold and the Mg^{++} optimum was reported to be 3 mM. Translation products were not analysed by SDS-PAGE and polysomes were not analysed by surcrose gradient ultracentrifugation. Poly A^+ RNA was isolated from polysomes and translated in a similar manner. Translation was about 5 fold over background and a Mg^{++} optimum between 1.4 and 2.4 was reported. Translation products were not analysed.

In conclusion, in vitro translation of polysomes and poly A^+ RNA should be continued so that the products may be characterized as described above. In this manner it may be possible to understand in more detail the biosynthesis of oat globulin.

C) RESEARCH OBJECTIVE

It is well documented that legume globulins are synthesized as precursors (Croy et al, 1980, 1982; Tumer et al, 1981, 1982; Barton et al, 1982; Chrispeels et al, 1982). In light of the structural and antigenic homologies between oat and legume globulins demonstrated in this thesis, oat globulin may also be synthesized as a precursor.

The purpose of this chapter was:

A) To provide evidence if a precursor is synthesized.

B) Isolate intact poly A^+ enriched RNA which is capable of directing the in vitro synthesis of high Mr polypeptides. Such poly A^+ RNA will be required to continue the study of oat globulin synthesis.

MATERIALS AND METHODS

D) MATERIALS

Oat (Avena sativa L.) was used throughout this investigation. Samples were harvested two to three weeks after anthesis. Wheat germ (Triticum aestivum L.) was obtained from Marriages Mills, Essex, England or Karla's Health Food Store, Ottawa.

Amino acids, adenosine triphosphate (ATP), 2' and 3' adenosine monophosphate (2' + 3' AMP), creatine phosphate, creatine phosphokinase, dithiothreitol (DTT), diethylpyrocarbonate, ethylenediaminetetraacetic acid (EDTA), guanosine triphosphate (GTP), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), ribonuclease A, were obtained from Sigma Chemical Co.

Sucrose (RNAase free), agarose, rabbit globin mRNA, E. coli RNA standards were obtained from Bethesda Research Laboratories.

L-[3,4,5-^{3}H]-leucine 100 Ci/mmole, L-[^{35}S]-methionine 1000 Ci/mmole and EN^{3}HANCE were obtained from New England Nuclear.

Fuji RX X-ray film was obtained from Fuji Ltd.

Phenylmethyl Sulfonylfluoride (PMSF) was obtained from Calbiochem-Behring Corp.

E) METHODS

1. Preparation of Starting Material

The starting material used for the isolation of oat polysomes was very critical. The different starting materials used were:

A) Whole seeds (hull and seed)

B) Dehulled seeds (caryopsis)

C) Seed endosperm

Developing oats were placed directly into liquid N_2 and used as such or dehulled in the presence of liquid N_2 in a Quaker Oats experimental dehuller. An air classifier attached to the dehuller was adjusted to remove hulls from seeds. Alternately, developing oats were pressed through a mangle and the endosperm tissue was collected in liquid N_2. All samples were stored at -80°C.

2. Isolation of Polysomes

Sterile glassware and buffers were mandatory throughout the procedure. All manipulations were carried out at 4°C or on ice. The method of Matthews and Miflin (1980) was used.

Starting material (100 g) was placed in liquid N_2 then ground to a fine powder in a coffee mill. The powder was added to 300 ml of grinding buffer (0.2 M Tris/HCl, pH 9.0, 120 mM KCl, 50 mM magnesium acetate, 0.15 M sucrose, 4 mM DTT, 2 mM 2' + 3' AMP) and homogenized in a Waring blender for 1 min. The 2' + 3' AMP is used as a ribonuclease inhibitor.

The suspension was centrifuged at 500 x g for 5 min to pellet the unbroken cells. The resulting supernatant was recentrifuged at 35 000 x g for 30 min. The pellet was re-extracted with a glass homogenizer in 100 ml of grinding buffer containing one percent Triton X-100. This suspension was centrifuged at 35 000 x g for 30 min and the clear supernatant retained and layered onto 4 ml of 54% (w/v) sucrose in grinding buffer. Polysomes were pelleted at 200 000 x g for 4 hours in a Beckman Ti60 rotor. The surfaces of the polysome pellets were washed with cold sterile distilled deionized water then resuspended in 20 mM Hepes, pH 7.6, 100 mM KCl, 4.0 mM magnesium acetate, 1.0 mM DTT and stored at -80°C.

3. Isolation of Total RNA from Polysomes

Sterile glassware and buffers were used throughout the isolation procedure. Phenol was redistilled by heating the crystals in a round bottom flask to 180°C. The vapour was air cooled and the condensate was stored at -20°C.

Polysomes were resuspended in 10 ml of phenol extraction buffer (0.2 M Tris/HCl, pH 9.0, 0.1 M NaCl, 10 mM EDTA, 0.5% (w/v) SDS). Ten milliliters of 1:1 phenol-chloroform was heated to 30°C then added to the polysome solution and gently shaken for 5 min. The suspension was centrifuged at 600 x g for 5 min at 22°C and the upper aqueous phase collected. The phenol phase was extracted with a further 10 ml of phenol extraction buffer and centrifuged as before. The aqueous

phases were pooled then re-extracted with phenol-chloroform in the same manner. The resulting aqueous phase was then extracted with an equal volume of chloroform. The aqueous phase was collected and made 0.2 M with ammonium acetate followed by addition of 2.5 volumes of absolute ethanol previously cooled to -20°C. The RNA was precipitated at -20°C overnight and pelleted by centrifugation at 12 000 x g for 30 min. The pellet was washed with 75% (v/v) ethanol precooled to -20°C then taken up in sterile, distilled deionized water and stored at -80°C.

All RNA concentrations were determined by using one optical density unit at 260 nm equal to 40 µg RNA/ml (Maniatis et al 1982).

4. Isolation of Poly A^+ Enriched RNA from Total RNA

Oligo-dT cellulose chromatography was performed similar to the method described by Bantle et al (1976). Oligo-dT cellulose (0.5 gram) was poured into a column (1.2 x 5 cm) and washed with 0.1 NaOH, then 10 ml of sterile distilled water followed by 50 ml of binding buffer (10 mM Tris/HCl, pH 7.5, 1.0 mM EDTA, 0.5 M NaCl, 0.1% (w/v) SDS). Total RNA was resuspended in binding buffer and heated to 60°C for 5 min, then cooled to room temperature . This sample was applied to the pre-equilibrated oligo-dT cellulose column. Binding buffer was washed through the column until 260 nm absorbing material no longer could be detected in the eluant. Eluting buffer (10 mM Tris/HCl, pH 7.5, 0.1% (w/v) SDS, 1.0 mM EDTA) was then applied to the column to release poly A^+

RNA. Fractions were made 0.2 M in ammonium acetate followed by addition of 2.5 vol of absolute ethanol previously cooled to -20°C. The poly A^+ RNA was allowed to precipitate overnight at -20°C, pelleted by centrifugation and redissolved in sterile distilled deionized water. The sample was re-precipitated in the same manner and washed with 75% (v/v) ethanol precooled to -20°C. Poly A^+ RNA was pelleted by centrifugation and allowed to dry slowly under a slight vacuum then redissolved in sterile distilled deionized water and stored at -80°C.

5. Preparation of Wheat Germ Cell-free Extract

Wheat germ extract was prepared similar to the method described by Roberts and Paterson (1973) but omitting the pre-incubation step. Sterile glassware and buffers were used throughout the procedure. All manipulations were carried out at 4°C. Wheat germ (8.0 grams) was ground in a chilled mortar and pestle in the presence of 2 volumes grinding buffer containing 20 mM Hepes, pH 7.6, 150 mM KCl, 1.0 mM MgAc, 2.0 mM $CaCl_2$, 6.0 mM 2-mercaptoethanol and a broken pasteur pipette. The slurry was centrifuged at 30 000 x g for 20 min and the aqueous layer carefully collected avoiding the pellet and the floating lipid layer. The aqueous solution was applied on a column containing Sephadex G-25 course (1 cm x 18 cm bed volume) which had been pre-equilibrated with 10 mM Hepes, pH 7.6, 100 mM KCl, 4.0 mM magnesium acetate, 1.0 mM DTT. As soon as the eluant appeared cloudy it was collected. About

the same volume was collected as was applied to the column. The eluant was then centrifuged at 30 000 x g for 20 min at 4°C and the supernatant dispensed through a pasteur pipette into liquid nitrogen to form frozen beads. Beads were stored at -80°C.

6. Preparation of Wheat Germ Cell Free Translation Mixtures

The following stock solutions were prepared using sterile distilled deionized H_2O and stored at -80°C unless otherwise stated:

A) 40 mM ATP, 320 mM creatine phosphate, 0.4 M Tris/HCl pH 7.6

B) 12.0 mM spermidine

C) 5.0 mM GTP

D) 200 mM DTT

E) Creatine phosphokinase, 5.0 mg/ml made up in 50% (v/v) glycerol and stored at -20°C

F) 5.0 mM of each amino acid in 5.0 mM DTT omitting the radioactively labelled amino acid

G) Salt solutions containing 0.6 M KCl and from 0.0 to 24 mM magnesium acetate.

For a 20 µl translation mixture the solutions were combined to yield final concentrations of 1.0 mM ATP, 8.0 mM creatine phosphate, 300 mM spermidine, 50 mM GTP, 2.0 mM DTT, 50 µg/ml creatine phosphokinase, 50 µM of each amino acid, 100 mM KCl, 4.0 µCi [^{3}H]leucine, or 1.0 µCi [^{35}S]methionine and varying

amounts of magnesium acetate. Contained in the translation mixture were 2.5 µl of wheat germ extract and varying amounts of polysomes or RNA. Assays were carried out at 28°C for 1 hr.

7. Determination of Incorporated Radioactivity into TCA Precipitable Products

Aliquots (2.0 µl) were removed from the translation mixture at appropriate times and spotted onto 2 cm x 1 cm strips of Whatman No. 1 filter paper. The strips were then placed in 10% (w/v) TCA at 0°C for 10 min to precipitate protein, boiled in 5% (w/v) TCA for 5 min to dissociate aminoacyl-tRNAs, then placed in 5% (w/v) TCA at 0°C for an additional 5 min. Strips were then washed in ethanol followed by diethylether and they were allowed to air dry. Strips were counted in non-aqueous scintillation fluid.

8. Separation of In Vitro Translated Products by Electrophoresis and Fluorography

Translation mixtures were suspended in equal volumes of electrophoresis sample buffer and electrophoresis was carried out as described in Chapter 1, methods section 2.

Fluorography was performed according to the method of Chamberlain (1979). A solution containing 250 ml of 1.0 M sodium salicylate was neutralized with NaOH. After the gel had been fixed and washed in distilled H_2O it was placed in the above solution for 30 min. The gel was then removed and dried under

vacuum at 65°C using a Bio Rad gel dryer. Alternately gels which had been stained and fixed were placed in En^{3}Hance for 1 hr then in water for 1 hr. Dried gels were exposed to Fuji X-Ray film for various times at -80°C.

9. Immunoprecipitation of Translation Products

The method used was based on that described by Roberts and Lord (1981). Cell free translation mixtures were dispersed in an equal volume of one percent Triton X-100, 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2.0 mM EDTA, 40 µg PMSF/ml. For a translation mixture containing 60 µl of the above buffer, 2.0 µl of preimmune serum was added and allowed to incubate at 20°C for 20 min, followed by the addition of 50 µl of a 1:1 slurry of protein A-Sepharose in above buffer. The solution was incubated for an additional 30 min with gentle shaking. Ten micrograms of anti-globulin IgG in PBS was added and incubated for 1 hr at 20°C followed by the addition of 75 µl of protein A-Sepharose and the mixture incubated for an additional 30 min with gentle shaking. The mixture was centrifuged for 30 sec and the beads washed 4 times with 1.0 ml of 2.0% (v/v) Triton X-100 in the above buffer, 3 times with 1.0 ml of 0.2% (v/v) Triton X-100, 500 mM NaCl in the above buffer and twice with 10 mM Tris/HCl, pH 7.5. SDS gel-electrophoresis sample buffer (40 µl of 0.65 M Tris/HCl, pH 6.8, 1.0% (w/v) SDS, 1.0% (v/v) 2-mercaptoethanol, 8.0 M urea) was added to the beads containing the globulin-(anti-globulin)-(protein A - Sepharose) complex.

The samples were heated to 60°C for 20 min and analysed by SDS gel-electrophoresis and fluorography.

10. Analysis of Polysomes by Sucrose Gradient Ultracentifugation

Polysomes (10 A_{260} units/ml) were suspended in 1.0 ml of 0.2 M Tris/HCl pH 9.0, 100 mM KCl, 50 mM magnesium acetate. Sucrose gradients (20-50% w/w) were prepared in the above buffer and formed using an ISCO gradient former. Samples were centrifuged at 25 000 rpm in a SW 28.1 rotor for 3 hrs at 4°C. Similarly, a sample was treated with bovine pancreatic RNAase A (20 µg/ml) for 15 min at 28°C prior to centrifugation in order to determine the position of the monosome peak. Gradients were analyzed with an ISCO gradient fractionator and monitored at 254 nm.

11. Analysis of RNA by Sucrose Gradient Ultracentrifugation

Sucrose gradients (5-20% w/w) were prepared in 10 mM Tris/HCl, pH 7.4, 0.1 M NaCl, 1.0 mM EDTA. About 10 A_{260} units of total RNA was suspended in 1.0 ml of H_2O. Samples were heated to 70°C for 5 min then placed on ice for 10 min. Samples were gently layered onto the gradient and centrifuged at 10°C and 25 000 rpm on a SW 28.1 rotor for 20 hrs. Gradients were fractionated and monitored at 254 nm. Fractions were adjusted to 0.2 M NaCl and 2.5 volumes of absolute ethanol were added. The RNA was allowed to precipitate overnight. Samples were centrifuged, washed, dried and stored at -80°C as previously described.

12. Analysis of RNA by Agarose Electrophoresis

Agarose (0.8% w/v) was dissolved by boiling in buffer (0.05 M boric acid, 0.005 M sodium borate, 0.01 M sodium sulfate and 0.001 M EDTA, pH 8.2). The solution was cooled to 60 C and adjusted to 10 mM with methylmercuric hydroxide then poured onto a glass plate (22 x 16 cm). This operation was carried out in a fumehood. Both buffer chambers contained the above buffer with no methylmercury. Samples were applied in a two fold concentration of the above buffer containing 10 mM methylmercury. Electrophoresis was carried out for 600-800 volt hours at room temperature using a vertical gel apparatus.

Bands were located by incubating the gel in 0.5 M ammonium acetate containing 1.0 µg/ml ethidium bromide for 30 min followed by examination with a transluminator at 310 nm.

13. Determination of Relative Amounts of Radioactivity in Gels

Following electrophoresis of translated products gel tracks (2 mm) were removed with a razor blade. Each slice was placed into a scintillation vial followed by 0.5 ml of 0.5 M ammonium hydroxide. The vials were incubated at 60°C overnight then 50 µl of acetic acid added to each. Scintillation fluid was added and the vials were then counted.

RESULTS AND DISCUSSION

F) IN VITRO SYNTHESIS OF OAT GLOBULIN

1. Isolation of Oat Polysomes

Polysomes were first isolated from developing seeds according to the method used for barley, wheat and rye (Matthews and Miflin, 1980) as described in methods section 2 . This entailed squeezing endosperm tissue into liquid N_2 using a mangle. However, because of the large ratio of fibre to endosperm in the oat panicle the procedure was very inefficient. Very little endosperm could be squeezed out and rolled away from the fibrous residue. Thus, this approach became an unattractive prospect for future studies.

The first alternative explored was to freeze whole seeds (spikelets) in liquid N_2 as in methods section 1. Polysomes were isolated from this material as described in methods section 2. The second alternative was to freeze whole seeds (spikelets) in liquid N_2 then remove the fibrous hulls in an automatic dehuller (methods section 1). The resulting dehulled seeds (caryopses) were subsequently used for polysome isolation. Yield of polysomes and preparation times are reported in Table 1.

Using dehulled seeds or endosperm tissue as a starting tissue yielded almost three times as much polysomes as did total seeds. However, dehulled seeds required much less preparation time than did endosperm tissue.

TABLE 1

POLYSOME ISOLATION

Starting Material	Yield[1] (O.D.260 units/ 100 gram)	Preparation Time[2] (Man hrs/100 g starting material)
Whole seeds (spikelets)	40	0
Dehulled seeds (caryopsis)	110	0.25
Endosperm tissue	110	19.0

[1] $O.D._{260}$ Units when polysomes are in a 1.0 ml volume.

[2] Time required to prepare material to be used for the start of polysome isolation.

Polysomes were subjected to sucrose gradient ultra-centrifugation. In this manner it was possible to determine which method yields intact polysomes by studying their respective profiles (Figure 1 A,B,C). The monosome peaks were determined by comparison against profiles of polysomes which had been nuclease treated prior to ultracentrifugation (Figure 1D). All profiles were obtained from the same centrifugation. Davies and Larkins (1974) have demonstrated that small amounts of RNAase caused a decrease in the number of interribosomal bonds and an increase in the monosome peak. The monosome peak produced by RNAase digestion appeared at 1.25 cm on the chart paper starting from the top of the gradient (Figure 1D). On Figures 1 A,B,C, the peak observed at 1.25 cm was thus designated as the monosome peak(indicated with an arrow).

Preparations containing increasing amounts of large polysomes are present in profiles using dehulled seeds or endo-sperm as the starting material (Figure 1, B and C). Furthermore, the large polysomes are the most abundant fractions as is evident from these profiles. Starting with dehulled seeds, polysomes contained the smallest monoribosome peak. Whole seeds yielded much fewer large polysomes and resemble the polysome profile shown in Luthe and Peterson's (1977) report. The reason for this degradation is not known. Possibly the outer hulls themselves or micro-organisms which they harbour are rich in ribonuclease activity. Hulls may also contain minerals and salts, destabilizing polysomes or causing them to run off.

FIGURE 1

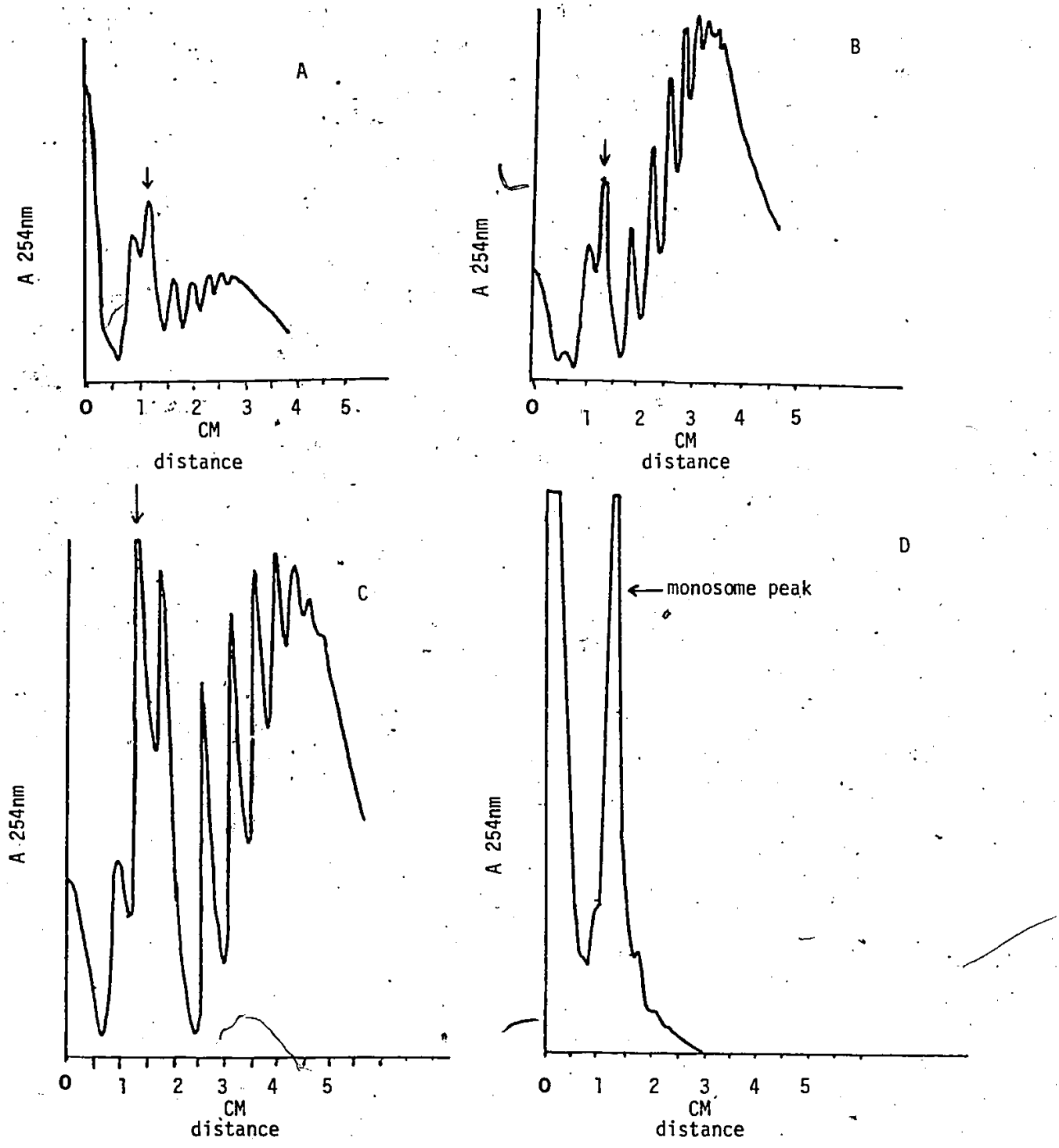

A
A 254nm
0 1 2 3 4 5
CM
distance
B
A 254nm
0 1 2 3 4 5
CM
distance
C
A 254nm
0 1 2 3 4 5
CM
distance
D
monosome peak
A 254nm
0 1 2 3 4 5
CM
distance

Figure 1. Size fractionation of polysomes by sucrose gradient ultracentrifugation (methods section 10)

Profiles represent polysomes isolated from three different starting materials.

A) Whole seeds

B) Dehulled seeds

C) Endosperm tissue

D) RNAase digested polysomes.

Polysomes were centrifuged at 25 000 rpm on 20-50% (w/v) sucrose gradients for 4 hrs in a Beckman SW 28.1 rotor. Profiles were monitored at 254 nm. Sample D was incubated with 20 ug of bovine pancreatic RNAase A for 15 min at 28° C prior to centrifugation. The distance of the monosome peak migration was obtained by direct measurement on the chart paper of the RNAase digested sample.

2. Wheat Germ Cell Free Translation

The translation characteristics reported here are derived from polysomes obtained from squeezed endosperm. The optimum concentration of magnesium in the system was a broad range from 1.4 to 2.3mM (Figure 2). For subsequent experiments 2.0 mM was used. The effect of polysome concentration (Figure 3) was linear for the range used (25 µg/20µl, 31.25 O.D. 260 units/ml). A time course is given in Figure 5. Luthe and Peterson (1977) reported a magnesium optimum of 4 mM and the reaction linear up to 4 OD 260/ml. Adeli (M. Sc. thesis University of Ottawa) reported a Mg^{++} optimum for polysome translation in a reticulocyte lysate to be 3 mM. Matthews and Miflin (1980) reported a Mg^{++} optimum for barley polysomes in wheat germ to be 4 mM similar to Luthe and Peterson (1977). The reason for the lower requirement in my wheat germ system is unknown. Differences may arise from the different wheat germ or polysome preparations. Possibly my system has been contaminated with Mg^{++} from a source which I was not aware of. Further differences may have been due to the different translation parameters used by Luthe and Peterson such as the reaction mixtures which contained different amounts of ATP, GTP, amino acids and no DTT, creatine phosphate, creatine phosphokinase or spermidine.

Oat polysomes caused a 40 fold increase of incorporation of ^{3}H -leucine into TCA insoluble products after 60 min over background (Figure 4). For convenience, all subsequent translations were carried out for 60 min. This stimulation of translation caused by oat polysomes is considerably higher

FIGURE 2

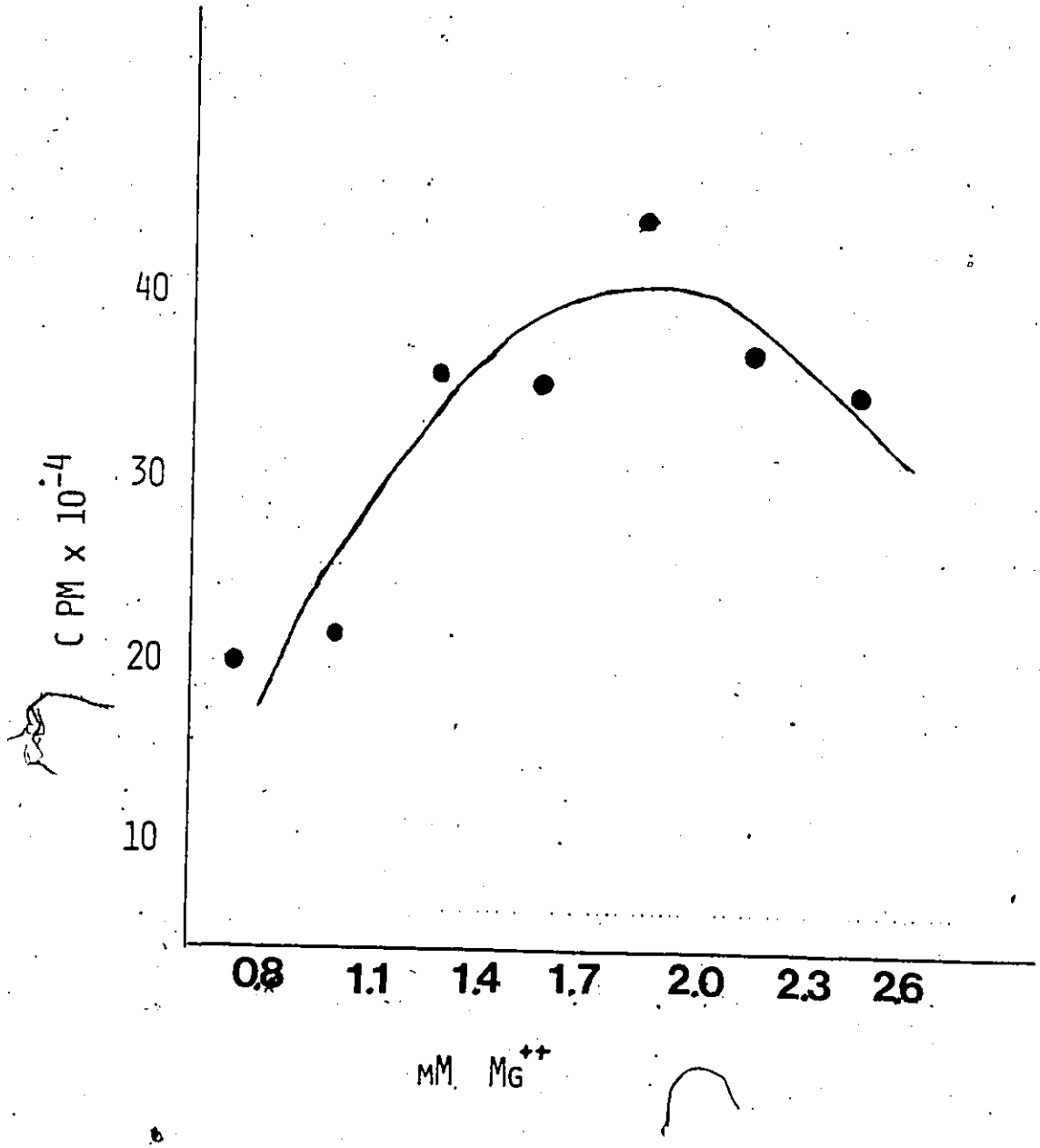


Figure 2. Magnesium concentration required for optimum translation. Translations were carried out as described in methods section 6 in the presence of 25 μg of polysomes derived from endosperm. (^{3}H)-leucine was used as the labelled amino acid.

FIGURE 3

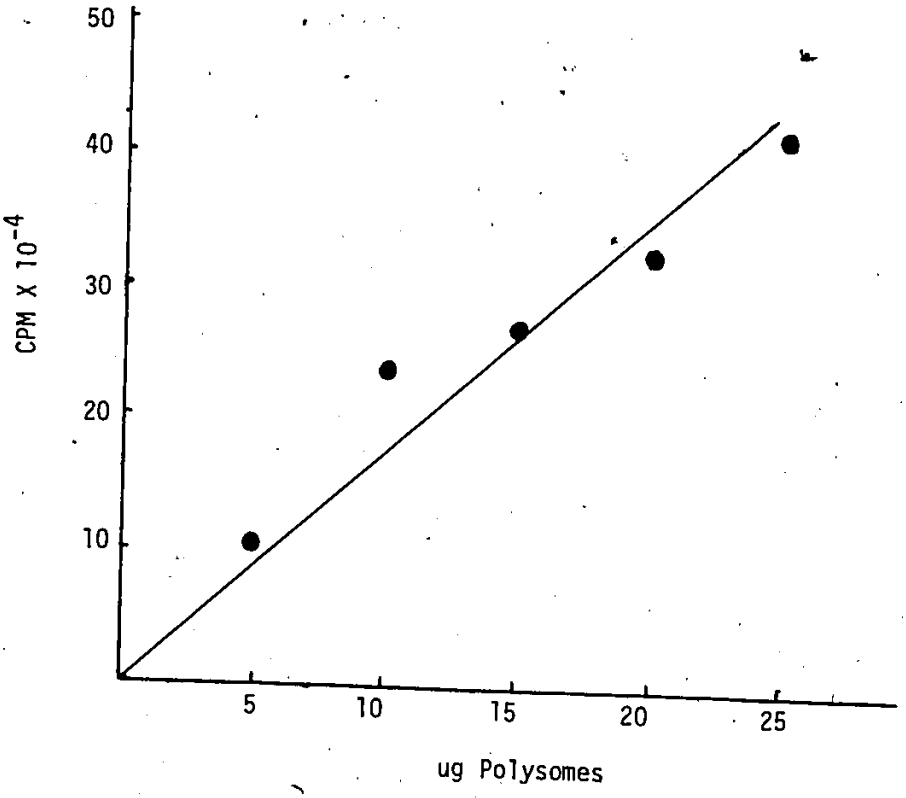


Figure 3. The effect of polysome concentration on the incorporation of [^{3}H]-leucine into TCA precipitable products. Translations were carried out as described in methods section 6 in the presence of polysomes derived from endosperm.

FIGURE 4.

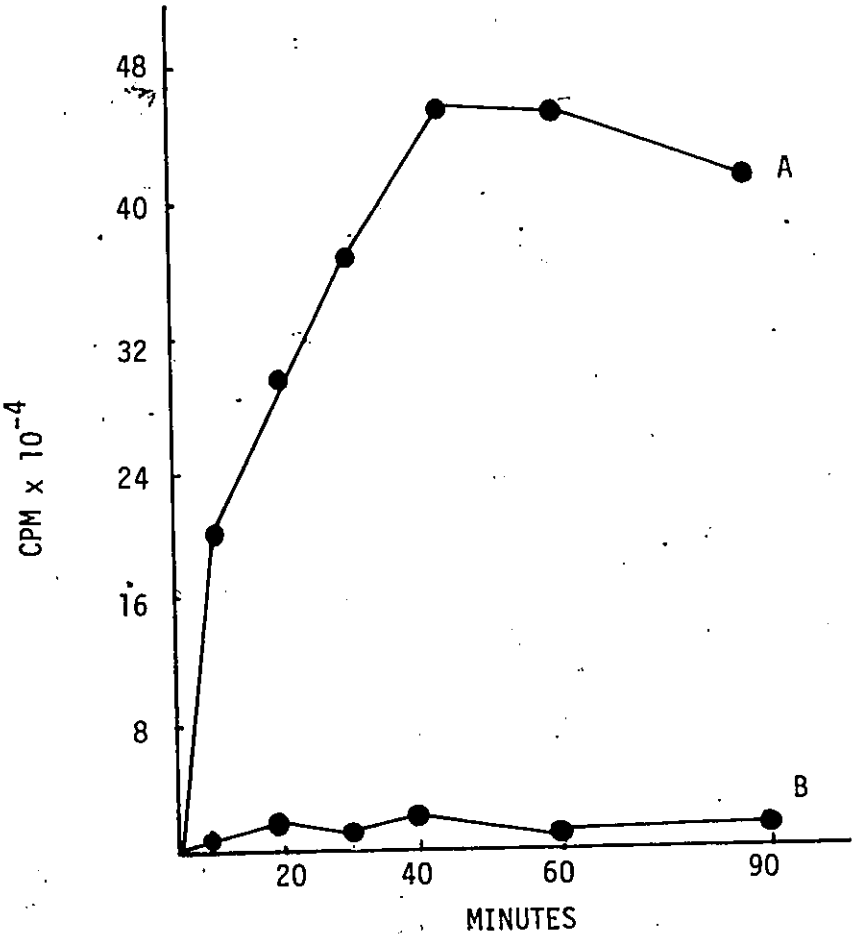


Figure 4. Kinetics of the incorporation of [^{3}H]-leucine into TCA precipitable products. Translation was carried out as described in methods section 6 in the presence of 25 µg of polysomes derived from endosperm. A demonstrates the curve in the presence of polysomes. B demonstrates the curve in the absence of polysomes.

than that reported by Luthe and Peterson (1977) or Adeli (1982) which were both under 10 fold. Rabbit globin mRNA (1.0 µg) translated under similar conditions resulted in a 12 fold increase over background. The level of incorporation caused by globin mRNA was lower than expected. This was probably due to improper magnesium ion concentration. The optimum magnesium concentration required for globin is 3.0 mM (Roberts and Paterson 1973). However, the system was not altered because the level of incorporation caused by polysomes was satisfactory for the purposes of this investigation.

3. Comparison of Translation Products Directed by the Different Polysome Preparations

Polysomes derived from squeezed endosperm or dehulled seeds were equally capable of directing the incorporation of TCA insoluble counts (Figure 4). Polysomes derived from whole seeds contained about half this activity. A comparison of the in vitro synthesized products is shown in Figure 5. Equal amounts of radioactivity were applied to each track. Gel-electrophoresis and fluorography were performed as described in methods section 8.

Translation products directed by polysomes isolated from dehulled seeds or squeezed endosperm both contained major products between Mr 50 - 60 000. Translation products directed by polysomes isolated from whole seeds were lacking in these products or any other high molecular weight products. This was not unexpected in light of the polysome profile (Figure 1, A) which showed relatively small amounts of intact polysomes.

FIGURE 5

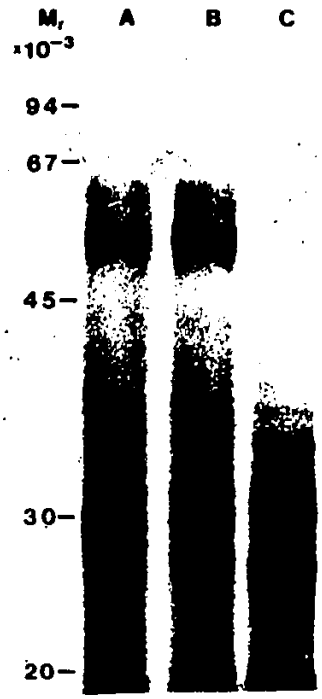


Figure 5. Fluorogram of translation products directed by the various polysome preparations in the presence of [^{35}S]-methionine.

Track A) Endosperm polysome directed translation products

Track B) Dehulled seed polysome directed translation products

Track C) Whole seed polysome directed translation products

Translation products were subjected to electrophoresis and fluorography as described in methods section 8. About 200,000 cpm of TCA insoluble material were placed in each lane.

4. Preparation Time

A major consideration in these experiments is the time factor. For instance, to obtain 80 gm of squeezed endosperm 15 hrs of preparation is required. To obtain the same amount of dehulled material about 15 min is sufficient using the automatic dehuller. Whole seeds require no preparation time. Thus, for future large scale preparation of RNA dehulled seed is the most attractive starting material.

5. Characterization of Polypeptides Translated in Vitro

Legume globulin is synthesized as a 60 000 Mr precursor (Croy et al, 1980, 1982; Tumer et al, 1981, 1982; Chrispeels et al, 1982). I also found that translations directed by polysomes isolated from dehulled oat seeds or endosperm contained a major product of about Mr 60 000 (Figure 5, A and B). To determine the relative amount of this major product gel slices were prepared and counted after polysome translation and SDS-PAGE (Method section 13). Flourography was not used because X-ray film can become saturated. Figure 6 demonstrates the migration of radioactivity and molecular weight markers. This demonstrates that the major translation product has a molecular weight of about 60 000. Depending on where the base-line is set for Figure 6 this major peak around Mr 60 000 occupies from 10 to 20% of the total radioactivity.

This is however contrary to Luthe and Peterson (1977) who reported a minor peak of radioactivity in this Mr region and 2 major peaks around 31 000 and 20 000. My studies have shown that the methods used for isolating polysomes are

FIGURE 6

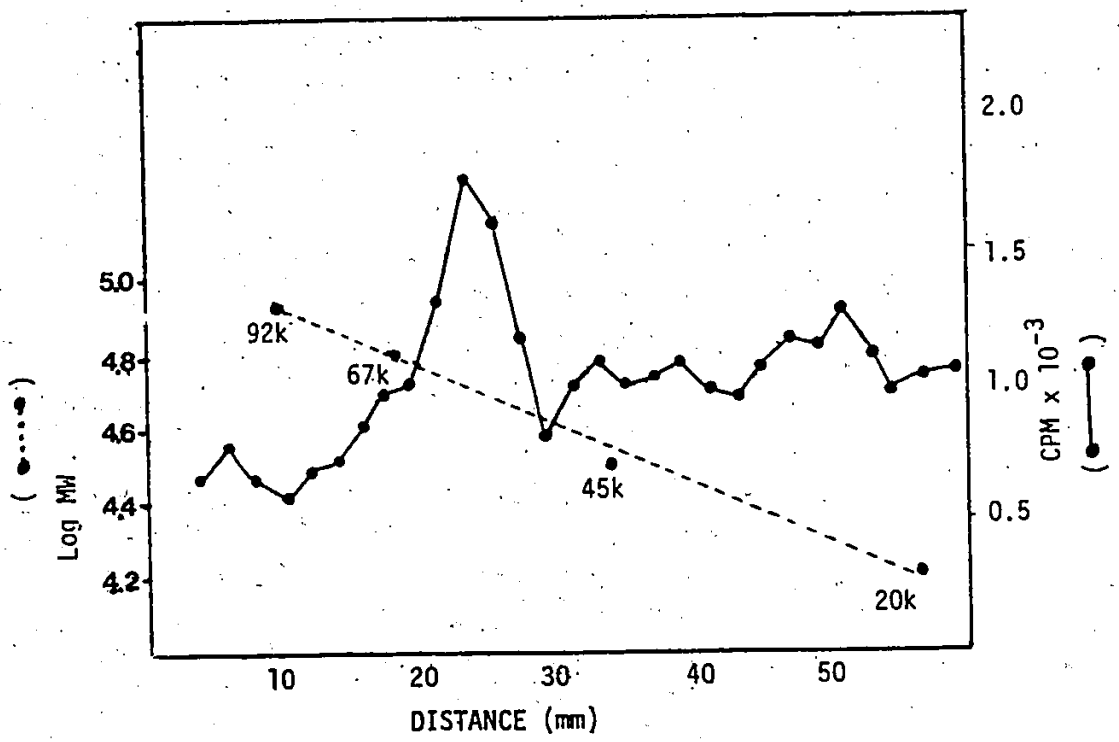

Log MW
5.0
4.8
4.6
4.4
4.2
92k
67k
45k
20k
2.0
1.5
1.0
0.5
CPM x 10^{-3}
10
20
30
40
50
DISTANCE (mm)

Figure 6. Distribution of radioactivity on the basis of molecular weight. Translation products were subjected to electrophoresis then the gel was sliced (2 mm sections) and counted (Methods section 13). Translation was carried out in the presence of [^{35}S]-methionine. About 100 000 cpm were applied onto the gel.

crucial. The polysome profile reported in 1977 showed similar characteristics to the profiles we obtained with polysomes from whole seeds (Figure 1, A). Such polysomes were probably not capable of translating into a 60 000 Mr polypeptide (Figure 5, C). Another possibility for Luthe and Peterson (1977) obtaining two peaks at Mr 31 000 and 20 000 may be the presence of a processing enzyme in their system which was not present in mine.

Immunoprecipitation studies were performed on the total translation products directed by endosperm polysomes in order to identify globulin. Anti-globulin IgG (Chapter 3 Table 1) was used to probe the translation products (methods section 9). The antigen antibody complex was recovered by using protein A-Sepharose which binds to the Fc of IgG. This complex was denatured and analysed by SDS gel-electrophoresis followed by fluorography (method section 8). Results are presented in Figure 7. Only about 2% of the total radioactivity was recovered in the immunoprecipitate. This value is only about 10 to 20% of the radioactivity present in the major peak observed in Figure 6. The low recovery is most likely due to the washing steps whereby protein A-Sepharose beads are lost. Losses in recovery may also be due to the antigen-antibody-protein A-Sepharose complex being unstable during the isolation and washing steps. Furthermore, the antibodies may not have bound all the *in vitro* synthesized globulin.

Only one *in vitro* synthesized polypeptide is selectively recognized by the anti-globulin IgG. This polypeptide occupies a region of the gel which is most heavily labeled (Figure 6) as would be expected for the major seed protein. This polypeptide has a

figure 7

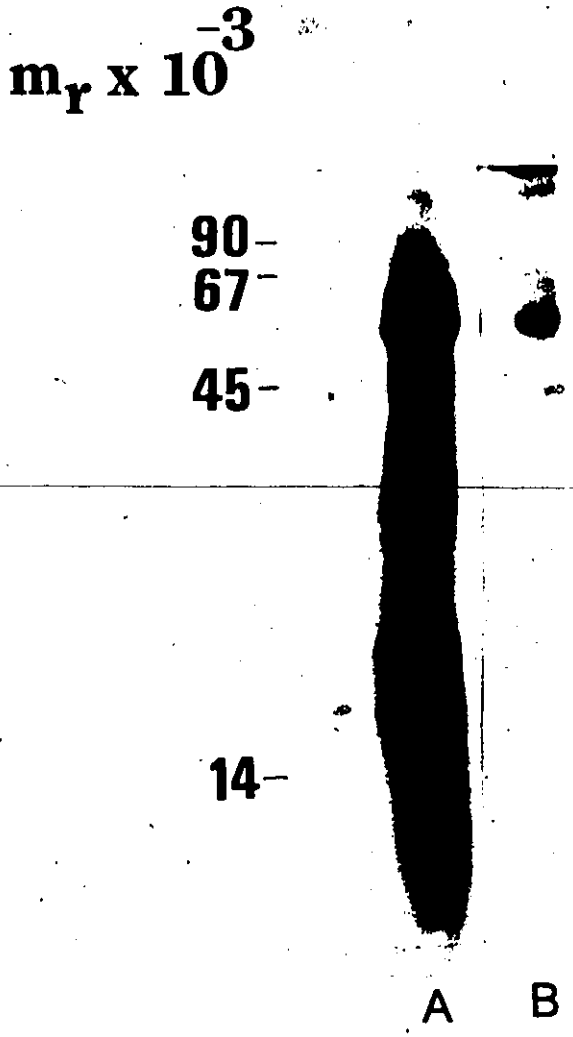


Figure 7. Fluorogram of total translation products and immunoprecipitated products.

Track A) Total polysomal translation products in the presence of [^{35}S]-methionine. About 100 000 cpm were applied on the gel.

Track B) Selective immunoprecipitation of globulin using anti-globulin IgG as described in methods section 9. About 2 000 cpm were recovered and applied on the gel. Electrophoresis and fluorography were carried out as described in methods section 8.

molecular weight of 58 000 - 60 000. The identity of this polypeptide as globulin on the basis of its antigenic properties is confirmed by this selective immunoprecipitation. A minor band located at the top of the fluorogram corresponds to a Mr of about 150 000. This is most probably background since it has not been observed in any subsequent immunoprecipitation analysis. This 58 000 - 60 000 molecular weight polypeptide is not linked by disulfide bonds (as is native globulin, Chapter 2 Figure 3) since this in vitro product is unaffected by treatment with mercaptoethanol and DTT which are in the SDS-PAGE buffers. This therefore suggests that this in vitro synthesized globulin is the precursor for the α and β subunits.

Such results are consistent with the biosynthesis of pea (Croy et al, 1980) and soybean globulins (Tumer et al, 1981). Furthermore, these results are compatible with the structural and antigenic similarities between oat and legume globulin demonstrated in Chapter 2 and 3.

However, these antibodies have not been shown to cross-react with the purified subunits. Therefore, the possibility exists that the α and β subunits are translated, as well as a 60 000 Mr globulin. Small amounts of radioactivity can be detected in the 20 000 Mr region of the immunoprecipitate track (Figure 7B). Two dimensional analysis should be performed on total translation products to determine if any radioactivity co-migrates with authentic α and β subunits. This will provide an indication whether or not the α and β subunits are being synthesized in vitro.

6. Isolation of Total RNA and Poly A^+ Enriched RNA from Polysomes

A phenol-chloroform extraction of polysomes was performed as described in methods section 3. In this manner, total polysomal RNA could be isolated. This total RNA preparation was subjected to oligo-dT cellulose chromatography (methods section 4) to obtain an enriched fraction of poly A^+ containing RNA. Yield of the RNA containing fractions is given in Table II.

These RNA fractions were introduced into the wheat germ translation system and translated under the conditions previously described (methods section 6). Incorporation of ^{35}S -methionine into TCA precipitable products was determined for various RNA fractions (also shown in Table II).

The level of incorporated isotope into TCA insoluble material directed by oat poly A^+ RNA was more efficient than for globin mRNA under these conditions. However, globin mRNA obtained from BRL generally always translated inefficiently regardless of the conditions used.

The poly A^+ RNA contains the highest specific activity with respect to level of incorporated isotope. The lower levels of incorporation caused by total RNA and poly A^- RNA are due to less mRNA in these preparations.

TABLE II

RNA ISOLATION FROM POLYSOMES

	Level of Incorporation[1] cpm/µg	Total Incorporation[1] (cpm/µg X µg)	Yield µg/100g endosperm
Rabbit globin mRNA	158 000		
Oat polysome (intact)	48 000	19×10^7	4 000
Total polysomal RNA[2]	23 200	5.5×10^7	2 400
Poly A^+ enriched RNA[4]	890 000	2.8×10^7	32
Poly A^- RNA	12 900	3.0×10^7	2 370[3]

[1]Level of incorporation of [^{35}S]-methionine into TCA precipitable counts when translated as described in methods section 6.

[2]Obtained by performing a phenol-chloroform extraction of the polysomes as described in methods section 3.

[3]Approximated by obtaining the difference between total RNA and poly A^+ enriched RNA.

[4]Poly A^+ enriched RNA was isolated according to methods section 3.

The total incorporation capabilities present in the poly A^- RNA and poly A^+ RNA samples were almost equal and together added up to the value obtained for total RNA. This suggests that there is an equal amount of mRNA in the poly A^- RNA and poly A^+ RNA fractions. This observation may be due to a variety of reasons. Possibly oat does contain a substantial amount of poly A^- mRNA similar to what Gray and Cashomore (1976) reported. Alternatively, poly A^+ tails may have been removed during the phenol-chloroform extraction or by the endosperm tissue. The poly A^+ tails may also have been too short to bind oligo-dT cellulose.

Adeli from this laboratory (M.Sc. thesis University of Ottawa 1982) has also isolated mRNA from oat polysomes in the same manner. He reported a 5 fold greater yield of polysomes and 5 times more poly A^+ RNA than what is reported here.

The reason for this discrepancy is unknown. In vitro translations were performed in a rabbit reticulocyte lysate kit purchased from New England Nuclear. He found that poly A^+ RNA translated 5 times more efficiently than poly A^- RNA or total RNA which probably indicated degradations of mRNA or that the translation kit was not performing properly. RNA preparations and translation products, however, were not analyzed on gradients and gels respectively, thus comparisons with this thesis cannot be made.

7. Comparison of Translation Products Directed by Polysomes Total RNA and Poly A^+ Enriched RNA

Translation products were subjected to electrophoresis followed by staining and fluorography (methods section 9). Results are shown in Figure 8. The larger globulin polypeptides are not as prominent in the poly A^+ or total RNA tracks as in the polysome track (assuming the region around Mr 60 000 is globulin as shown by immunoprecipitation, Figure 7). This may be to a variety of reasons. The larger mRNA's may be lost during the phenol-chloroform extraction or due to ribonuclease activity. There may be extensive secondary structure within the globulin mRNA resulting in decreased translation ability. Heating the RNA prior to translation however did not effect the final product size.

8. Analysis of Total Polysomal RNA by Sucrose Gradient Ultracentrifugation

Total polysomal RNA was isolated by extracting polysomes with phenol-chloroform as described in methods section 3. Ultracentrifugation on sucrose gradients (5%-20% w/v) was performed as described in methods section 11. A profile obtained for oat RNA is shown in Figure 9,B. This profile was compared to a profile obtained for E. coli 4S, 5S, 16S and 23S RNA shown in Figure 9, A. In this manner the location of oat ribosomal RNAs were determined to be

FIGURE-8

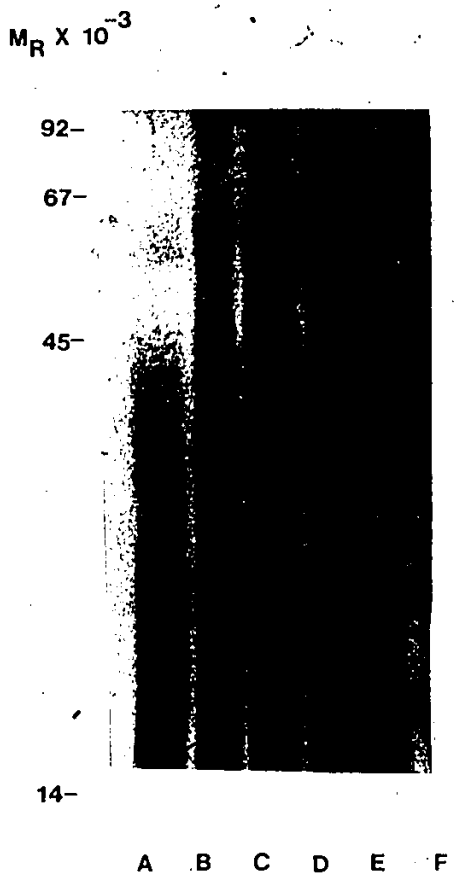


Figure 8. Fluorogram of various RNA directed translations. Gel-electrophoresis and fluorography was performed as described in methods section 8. About 150 000 cpm were placed in each lane except the poly A^- lane which had about 60 000 cpm. Translations were carried out as described in methods section 6 in the presence of [^{35}S]-methionine.

Track A: Poly A^- RNA directed translation.

Track B: Poly A^+ enriched RNA directed translation.

Track C: Total RNA (polysomal) directed translation.

Track D: Polysomal directed translation.

Track E: Globin mRNA directed translation.

Track F: Blank, no RNA added into the translation mixture.

FIGURE 9

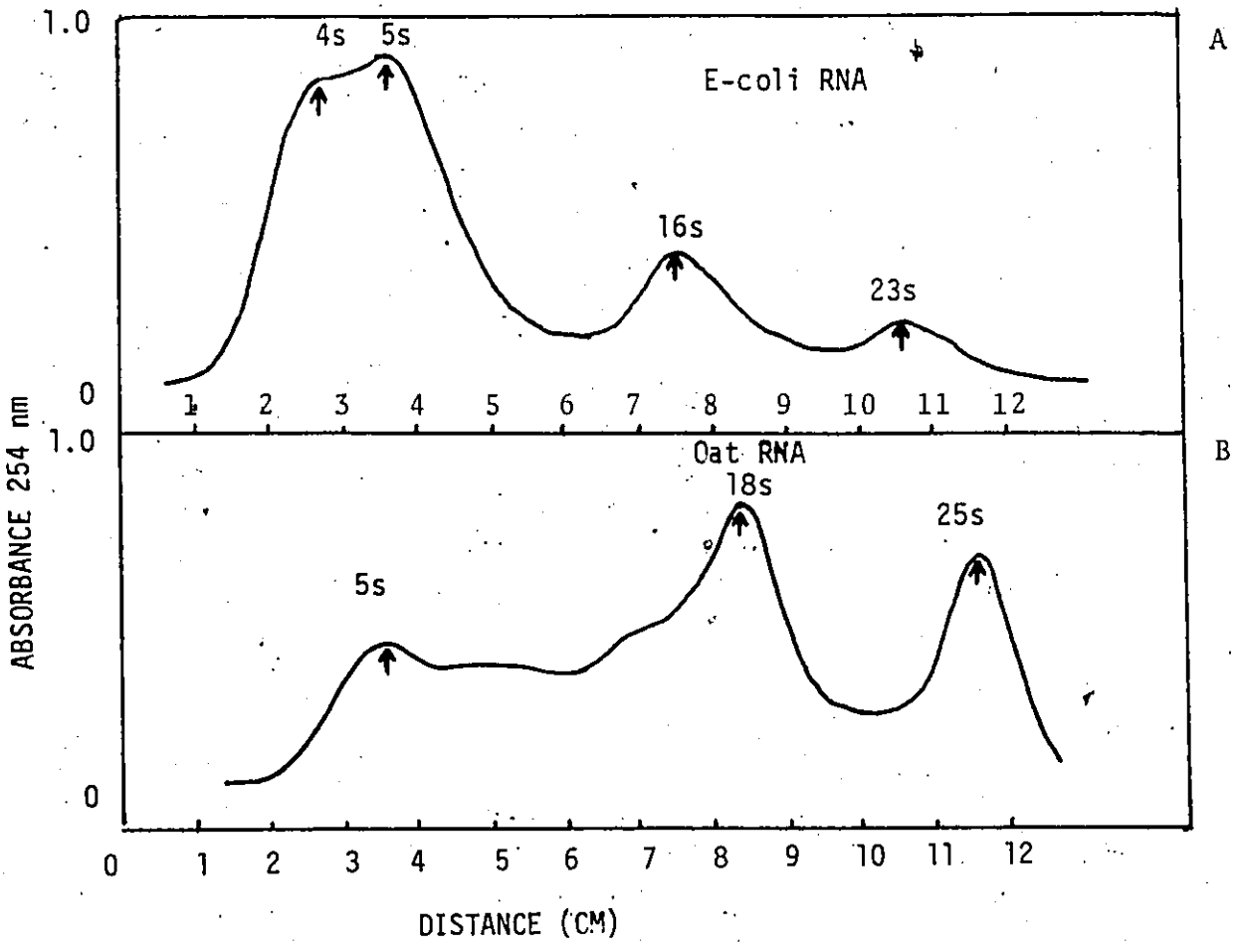

1.0
4s
5s
E-coli RNA
A
16s
23s
0
1
2
3
4
5
6
7
8
9
10
11
12
1.0
Oat RNA
18s
B
25s
5s
ABSORBANCE 254 nm
0
0
1
2
3
4
5
6
7
8
9
10
11
12
DISTANCE (CM)

Figure 9. Sucrose density gradient profiles of E. coli (A) and Oat RNA (B)

Gradients (5-20% w/w) were prepared as described in methods section 11. Samples were centrifuged in a SW 28.1 rotor at 25 000 rpm for 20 hrs then monitored at 254 nm. About 10 $O.D._{260}$ units were applied to each gradient. Distances of migration were compared by direct measurement on the chart paper.

approximately 5S, 18S and 25S (Figure 10) and is in agreement with Cifferi (1975) who reports similar sizes for plant ribosomal RNA. An interesting observation is that the 25S oat ribosomal RNA peak is smaller than the 18S peak. Normally, the 25S is larger than the 18S ribosomal RNA peak in eukaryotes. The E. coli RNA preparation was purchased from Miles Laboratories. When this E. coli RNA was run on agarose gels in our laboratory it was also observed that the 23S RNA was of weaker intensity than 4S and 5S RNA. Therefore the smaller peaks observed for oat and E. coli 25 and 23S RNA were probably not due to degradation during the sucrose gradient experiments. This may be an indication that there is degradation of the total RNA during its isolation from polysomes.

Further evidence for degradation may be observed in the smaller peaks between 5S and 18S. The RNA in these peaks appear to be in larger quantities than would be expected for mRNA. These peaks may contain some degraded RNA. This may account for the decreased ability of total RNA and poly A^+ RNA to translate into high Mr products of similar intensity to polysomal translation products (Figure 8, C,D).

FIGURE 10

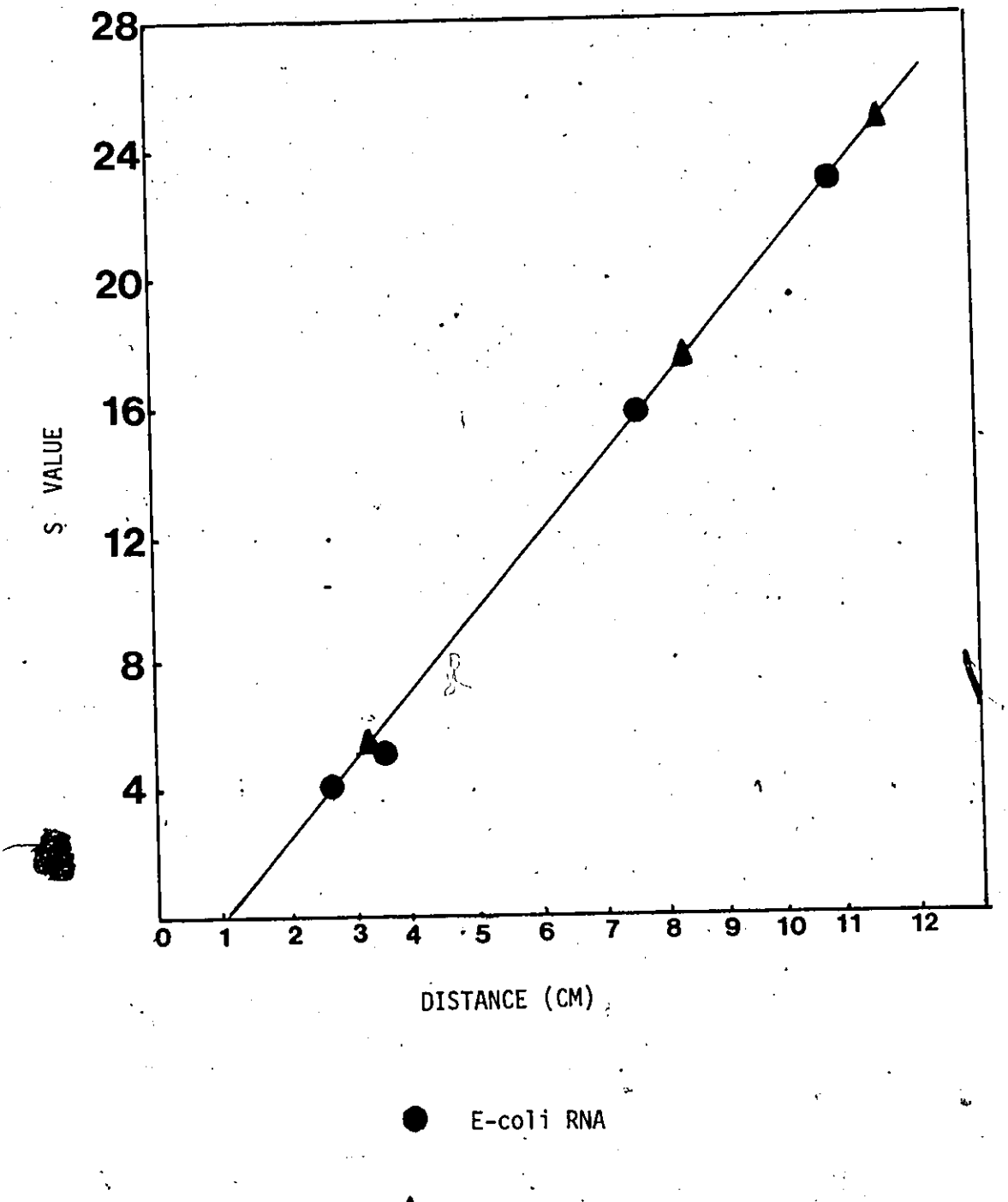

28
24
20
16
12
8
4
S VALUE
0 1 2 3 4 5 6 7 8 9 10 11 12
DISTANCE (CM)
E-coli RNA
Oat Ribosomal RNA

Figure 10. Plot of the distance traveled by E. coli RNA and oat ribosomal RNA versus their respective sedimentation coefficients.
The distance was obtained by direct measurement from the chart paper.
S values were given for E. coli RNA (●) and determined for oat ribosomal RNA (▲).

9. Isolation of 18S Containing Poly A^+ RNA

Further experiments were conducted in order to isolate an mRNA fraction rich in 18S RNA. Croy et al, (1982) and Tumer et al, (1981) have shown legume globulin mRNA to be between 18S and 19S. The same isolation procedures as described in methods section 3 and 4 were used with the following changes. Heparin (1.0 mg/ml) was used as a ribonuclease inhibitor in the phenol extraction buffer and mRNA was selected by three rounds of chromatography on oligo-dT cellulose to remove as much rRNA as possible.

Poly A^+ enriched RNA and poly A^- RNA were subjected to electrophoresis on methylmercury hydroxide agarose gels as previously described (methods section 12). Results are presented in Figure 11. Track B contains poly A^- RNA showing the 25S, 18S and 5S rRNA.

In this track the 25S band appears to contain more RNA than the 18S band. This is more consistent with the normal distribution for eukaryotic systems. Track A contains poly A^+ RNA and demonstrates an enrichment of the 18S region over the 25S and 5S regions. This 18S RNA may be globulin mRNA or it may be residual 18S rRNA or a mixture of both. 18S RNA contains about 2 000 nucleotides thus about 666 codons which could code for a protein up to Mr 70 000 (Tumer et al, 1981). However since not all mRNA sequences are involved in coding the 18S RNA is a good size for a 60 000 Mr protein.

FIGURE 11

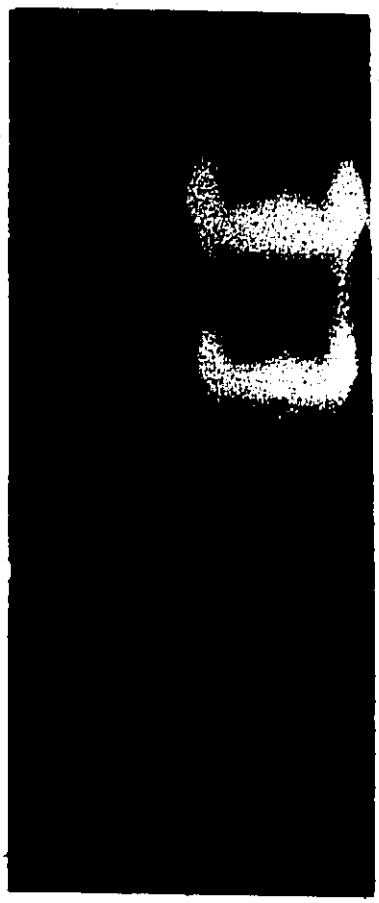


Figure 11. Agarose electrophoresis of poly A^- RNA and poly A^+ RNA following three rounds of chromatography on oligo-dT cellulose. Samples were subjected to electrophoresis as described in methods section 12.

Track A) Poly A^+ RNA. Containing about 1.0 ug RNA

Track B) Poly A^- RNA. Containing about 10 ug RNA

Also observed in the poly A^+ track is RNA in the 10S and 12S regions which appear in larger quantities than 18S RNA. The nature of these RNA fractions is unknown. They may also code for globulin subunits (i.e. 10S&12S RNA). To investigate this possibility two dimensional analysis of total translation products may confirm the presence or absence of such subunits (see p 218). Of course there is a strong possibility that the lower Mr RNA fractions may be degradation products. However if degradation was occuring one may have expected more RNA smaller than 5S in track A.

10. Characterization of mRNA Translation Products

The poly A^+ mRNA (Figure 11) and the polysomes it was obtained from were translated as previously described. Results are presented in Figure 12 A, B. Both preparations translated into similar products with similar intensities at the 60 000 Mr region. This is an indication that this poly A^+ RNA contains some intact mRNA large enough to code for a 60 000 Mr protein.

Immunoprecipitation was performed on the poly A^+ RNA directed translation products (Figure 12C). Anti-globulin IgG selectively precipitated a doublet, one band of about 59 000 Mr the other about 61 000. Immunoprecipitation of polysomal translation showed a single region at Mr 58 000 to 60 000 (Figure 7). Allowing for small errors in Mr determinations the immunoprecipitated products from polysomes and poly A^+ are essentially the same size. Indeed if the poly A^+

FIGURE 12

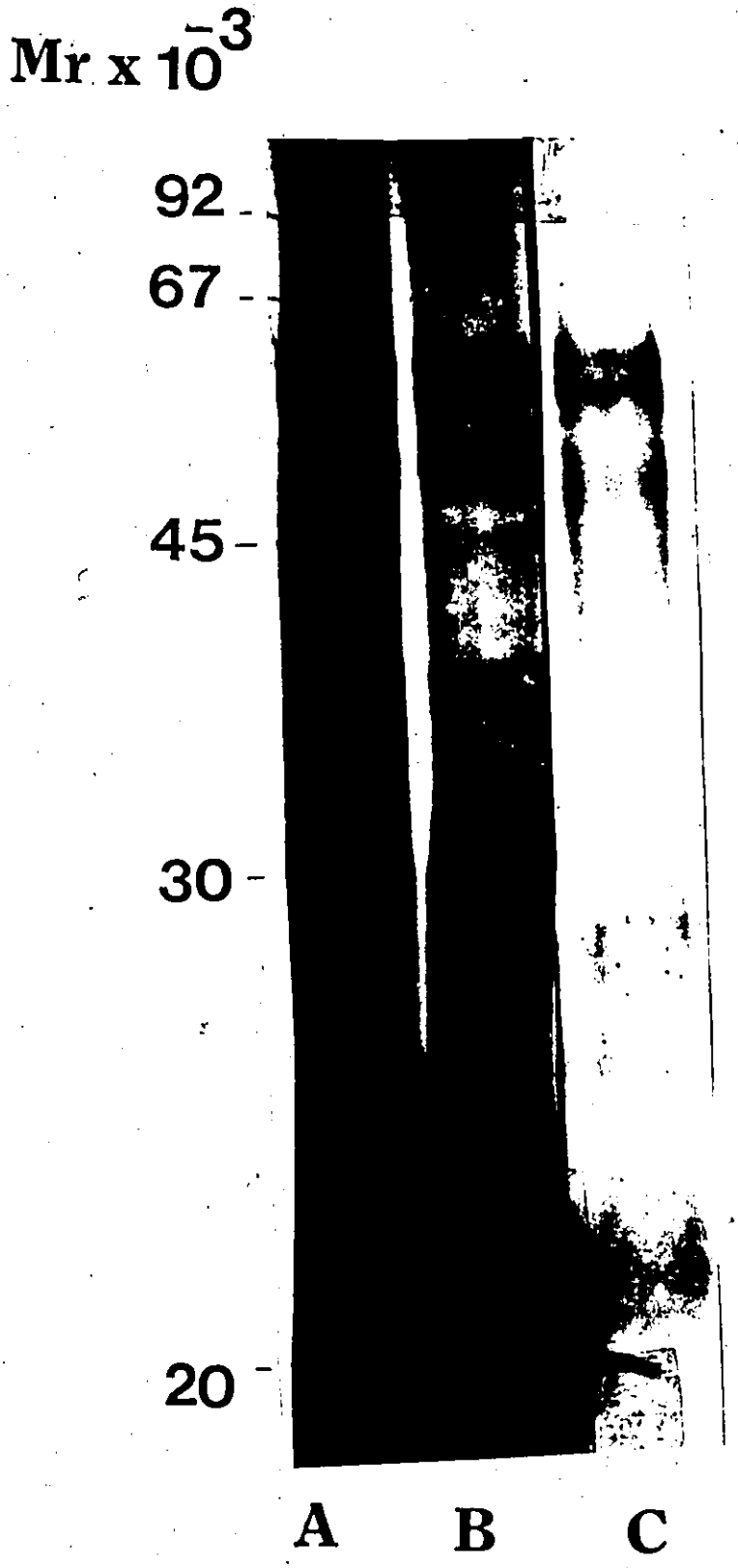


Figure 12. *In vitro* translation of poly A^+ RNA followed by immunoprecipitation (Method section 6,9).

Track A) Polysomal translation products. (200 000 cpm)

Track B) Poly A^+ translation products. (200 000 cpm)

Track C) Immunoprecipitation of poly A^+ translation products. (5 000 cpm).

products were to be 1 000 Mr larger this could reflect the presence of a leader sequence which has been removed from the membrane bound polysomes. Presumably the doublet observed for poly A^+ immunoprecipitates has been distinguished here due to an increase in resolution. It has been my experience the mRNA translation products appear "cleaner" on a fluorogram than do polysomal translation products as can be observed when comparing track A and B from Figure 12. This may be due to nascent chains present in polysomes. Furthermore, Luthe, Peterson and Larkins (personal communications) have all observed a similar doublet which is recognized by anti-globulin antibodies. The doublet may indicate that more than one mRNA species is responsible for the synthesis of globulin. Alternatively this may be due to 2 start codons or 2 stop codons on the same mRNA between which the wheat germ system cannot distinguish.

11. Two Dimensional Analysis of In Vitro Translated Products

The question regarding the possible presence of the individual globulin subunits within the total translation products was addressed. Two dimensional electrophoresis (IEF, SDS-PAGE), (Chapter 2, methods section 7) was performed with minor revisions on the total polysomal translation products. Translation assay (4.0 µl) was mixed with 40 µl sample buffer containing 10 M urea, 20 mM DTT and 10 mg/ml authentic globulin. Isoelectric focusing was performed followed by SDS-PAGE and flourography.

Assuming the α and β subunits are synthesized in vitro then they should have co-migrated with authentic α and β subunits. Figure 13 demonstrates the two dimensional gel of authentic globulin. The α subunit migrated to a Mr region of 20 - 23 000 with a pI range of 8-10. This is similar to what was reported in Chapter 2 which showed a Mr range of 21 - 23 000 and a pI range of 8.5-10. The β subunit (Figure 13) showed a Mr range of about 35 - 40 000 and a pI range of between 5 and 6.5. This is also similar to chapter 2 which reported a similar Mr range and a pI between 4.5 and 7. Therefore, globulin resolved in a similar manner in Figure 13 as it did in Chapter 2, Figure 8.

Figure 14 demonstrates the flourogram of in vitro synthesized products resolved on the same gel as the authentic globulin (Figure 13). A major product which has the doublet characteristic and a Mr of about 60 000 is observed. On the basis of its Mr and doublet characteristics I am interpreting this major product

FIGURE 13

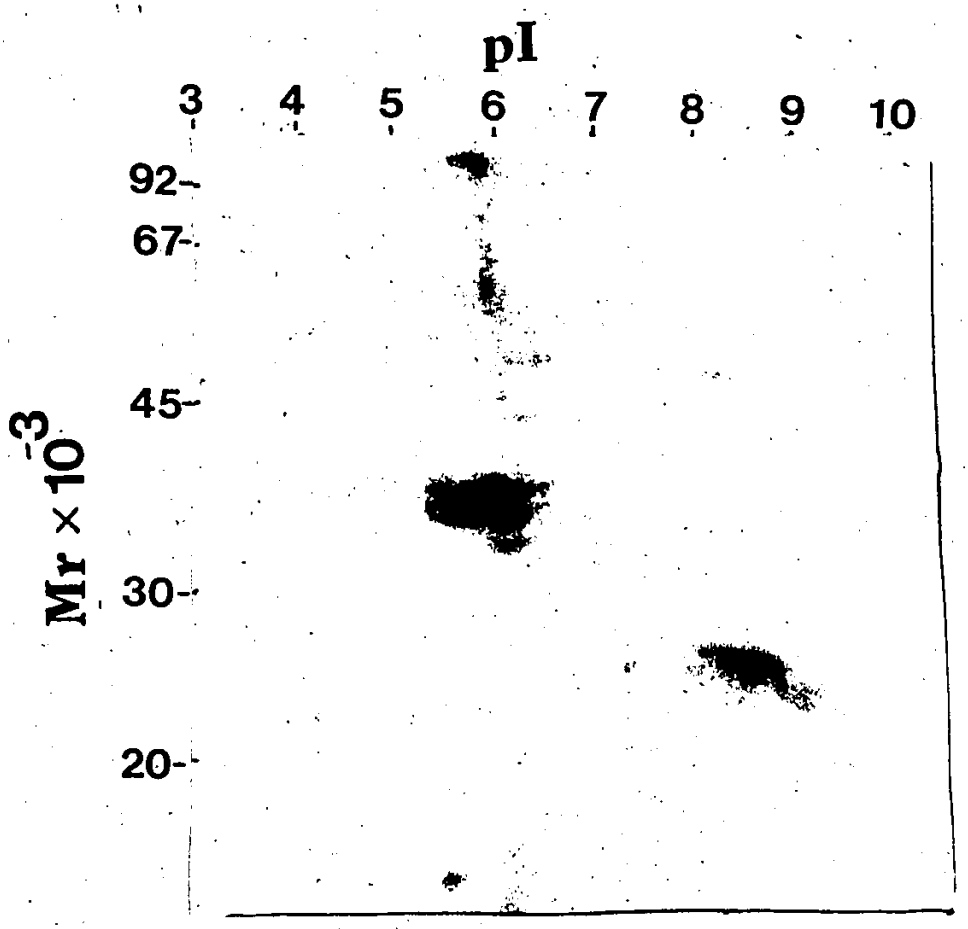


Figure 13. Two-dimensional stained gel of authentic globulin resolved by IEF between pH 3-10 followed by SDS-PAGE (Chapter 4, method section 6). This gel was run in the presence of *in vitro* translation products.

FIGURE 14

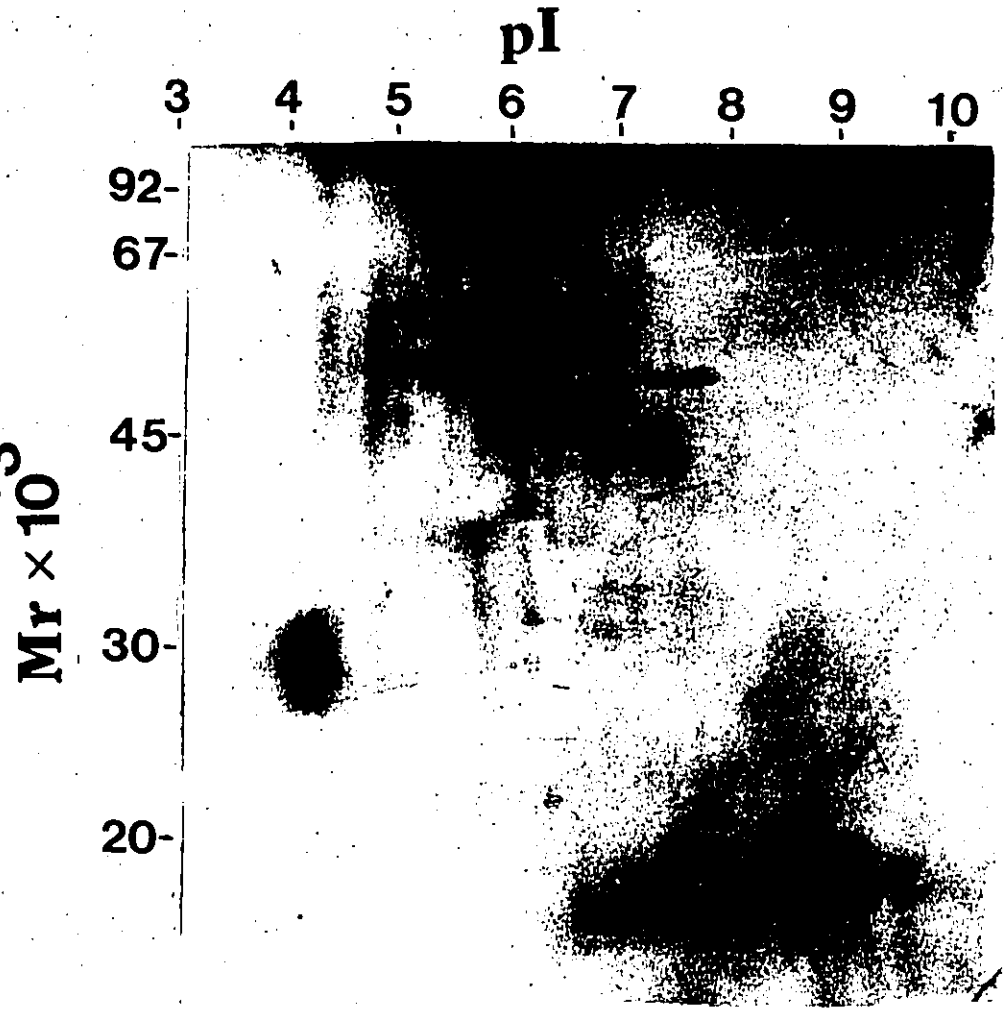


Figure 14. Two dimensional flourogram of *in vitro* translation products (Chapter 2, method section 7, Chapter 4 method section 6). The first dimension was resolved by IEF using a pH range from 3 to 10. The second dimension was resolved by SDS-PAGE.

to be the same product as observed in Figure 12C. This is believed to be the globulin precursor. This product has a pI range from 5.5 to 7.0. Figure 14 demonstrates few if any products in the Mr and pI regions of the individual subunits seen in Figure 13. This assay appears to be sensitive enough to observe the precursor, however it may not be sensitive enough to observe small levels of the individual subunits. This experiment therefore suggests that if the subunits are synthesized in vitro they are made in only small amounts as compared to the precursor.

As shown in both Figure 13 and 14 a minor spot is observed at about Mr 50 000 with a pI value of 7.5. This indicates that this protein was synthesized faithfully in vitro and co-migrated faithfully with its authentic counterpart. If the individual globulin subunits were synthesized in vitro they also would have been expected to co-migrate with their authentic counterparts.

This experiment suggests that no distinct α and β subunit mRNA populations exist in developing seed or are present in only small amounts. However, developing seeds contain an mRNA fraction capable of translating into a doublet of about Mr 60 000 which is recognized by anti-globulin IgG (Figure 12C) and has a pI range of 5.5-7.0. The hypothesis that this 60 000 Mr doublet is a globulin precursor is strengthened by the fact that if the α and β subunits are synthesized in vitro, this occurs only at a low level.

The major product observed in the 30 000 Mr region on the one dimensional gel (Figure 12 A,B) is also present as a diffuse region at the acidic portion of the flourogram (Figure 13). It is not observed in the stained gel (Figure 14). The nature of this product is unknown. It does not possess globulin characteristics or any other oat protein fraction characteristics on the basis of two dimensional analysis (L. Robert personal communication). Possibly it is labelled tRNA which would be acidic due to its phosphate groups and would not be present within the authentic globulin proteins.

G) CONCLUSIONS

In vitro translation followed by immunoprecipitation has revealed the presence of globulins in the Mr region of 60 000. These were major proteins among the total *in vitro* synthesized products. No subunits could be detected by two dimensional analysis of the *in vitro* synthesized products. These results meet the first objective of this chapter by providing evidence that oat globulin is synthesized *in vitro* as a precursor similar to what Peterson speculated in 1977. These results are consistent with Chapter 2 and 3.

Caution must however be exercised. It remains to be demonstrated that this 60 000 Mr globulin is processed *in vivo*. Pulse-chase experiments are presently underway in this laboratory to investigate this.

The presence of a major translation product of Mr 60 000 is somewhat different to Luthe and Peterson (1977) who reported it as a minor product. I suggest that the differences in techniques used has resulted in this discrepancy. The polysome profile obtained during 1977 indicated degradation. Such polysomes were probably not capable of synthesizing products of Mr 60 000 as was demonstrated in this chapter.

The second objective of this chapter was to isolate poly A^+ RNA from oats which is capable of translating into high Mr polypeptides. This has been achieved. Translation products of Mr 59-61 000 were obtained which could be immunoprecipitated with anti-globulin IgG. Furthermore, on the basis of translation

products there may be at least two mRNA species responsible for the synthesis of globulin precursors. Such observations are consistent with globulin having more than one gene similar to legume globulin (Croy et al, 1982, Fisher and Goldberg, 1982).

Walburg G. and Larkins B.A. at Purdue have obtained parallel results concerning the globulin precursor using polysomal directed in vitro translation followed by immunoprecipitation with antibodies prepared against the β- subunit. These results provide additional evidence for the presence of a globulin precursor and the absence of β subunit from in vitro translations. (Annual meeting, Americal Society of Plant Physiology, Urbana, 111., June 1982).

Peterson reports similar observations concerning the globulin precursor using poly A^+ enriched RNA directed in vitro translation followed by immunoprecipitation (Brinegar and Peterson, Plant Physiol. 70; 1767-1769 (1982)).

CHAPTER 5

DNA HOMOLOGY

A) INTRODUCTION

For legume and oat globulin to have arisen from similar genes or gene families they must contain to some degree homologous DNA sequences. Preliminary experiments have been conducted to determine if oat genomic DNA contains legumin-like DNA sequences. In order to pursue this study a legumin clone was made available from Dr. Boulter (Croy et al, 1982). The clone pRC 2117, containing a 790 bp insert in the Bam HI site of pBR 322, (corresponding to approximately one half of the pea legumin mRNA) was used in this study.

High molecular weight DNA was isolated from developing groats. Southern (1975) analysis was employed using [^{32}P] labelled pRC 2117 to probe oat genomic DNA. Results are presented which indicate that oat genomic DNA contains legumin-like sequences.

MATERIALS AND METHODS

B) Materials

Developing dehulled seeds as described in Chapter 4, materials section were used for DNA isolation. Agarose, restriction enzymes, λ DNA markers and cesium chloride were obtained from Bethesda Research Laboratories.
Nitrocellulose was obtained from Schleicher and Schuell.
Formamide was obtained from Eastman Kodak Co.
Ethidium bromide, Ficoll, polyvinylpyrrolidone, BSA dextran sulfate, Pipes, calf thymus DNA were obtained from Sigma Chemical Co.
Deoxycytidine 5'-triphosphate [α^{32}P], and the nick translation system was obtained from New England Nuclear.

C) Methods

1. Isolation of DNA

Developing groats (5.0 gm) were gently disrupted with a chilled mortar and pestle. 25 mls of 0.05M Tris/HCl, pH 7.6, 0.025M KCl, 0.005M $MgCl_2$, 0.35M sucrose and 400 µg/ml ethidium bromide was added followed by additional grinding for 1 min. The slurry was then homogenized with a polytron for 30 sec and filtered through cheesecloth. The filtrate was centrifuged at 3000 x g for 10 min at 4 C. The crude nuclear pellet was resuspended gently in 5 mls of 0.05M Tris/HCl, pH 8.0, 0.1M EDTA, 0.015M NaCl. The resulting nuclear suspension was added dropwise to 50 ml of 0.1M Tris/HCl,

pH 9.5, 0.3M EDTA preheated to 65°C followed by the dropwise addition of SDS to a final concentration of 1.0%. The solution was adjusted to 50°C and proteinase K was added to 500 µg/ml and incubated for 3 hrs. The solution was then gently extracted with phenol: $CHCl_3$ three times and the DNA was precipitated from the aqueous phase by the addition of 2 volumes of ethanol. DNA was resuspended in 10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1.0 mM EDTA then dialyzed against 10 mM Tris/HCl, pH 8.0, 1.0 mM EDTA.

2. Plasmid Isolation

Plasmid pRC 2117 in host E. coli 910 was isolated in the following manner following chloramphenicol amplification.

Cells were collected by centrifugation at 5000 r.p.m. for 10 min at 4°C. The cells were washed in 5.0 mls of 10 mM Tris/HCl, pH 7.5, 1.0 mM EDTA then resuspended in 5.0 ml of 25% (w/v) sucrose, 50 mM Tris/HCl, pH 8.5. Lysozyme (1.0 ml from a 10 mg/ml stock solution containing 0.25M Tris/HCl, pH 8.0) was added and stirred for 5 min followed by addition of 1.25 ml of 0.5M EDTA, pH 8.5. Cells were lysed at 37°C by the addition of 7 mls of 1.0% Nonidet P-40, 0.14% deoxycholate 70 mM EDTA 50 mM Tris/HCl, pH 8.5. The lysate was then centrifuged at 40 000 r.p.m. in a Ti 60 rotor for 40 min at 4°C to pellet most of the chromosomal DNA and cell debris. To the supernatant CsCl was added to a final density of 1.56 gm/ml then centrifuged at 10 000 r.p.m. for 15 min in a JA-20 rotor.

The cleared supernatant was recovered and ethidium bromide was added to a final concentration of 500 µg/ml. The density was re-adjusted to 1.57 gm/ml and centrifuged in a Ti 60 rotor at 44 000 r.p.m. for 40 hrs. The lower plasmid band was visualized using U.V. light and withdrawn by side puncture with a 18 ga. needle. Ethidium bromide was removed by butanol extraction and the plasmid was dialyzed against 10 mM Tris/HCl, pH 8.0, 1.0 mM EDTA. If needed, plasmid was concentrated by ethanol precipitation.

3. DNA Gel Electrophoresis

DNA was separated on 0.8% (w/v) agarose slabs poured and run in 40 mM Tris/HCl, pH 7.8, 5.0 mM Na acetate, 1.0 mM EDTA. Buffer was recirculated during electrophoresis. DNA was dissolved in sample buffer containing 10 mM Tris/HCl, pH 7.8, 20% glycerol, 0.25% bromophenol blue. Hind III λ DNA fragments were used as markers. Electrophoresis was for 300 to 600 volt hours, usually the bromophenol blue migrated 2/3 of the bed length. DNA was visualized by ethidium bromide and U.V. light.

4. Southern Transfer

The method was similar to Fabijanski and Pellegrini (1982). After electrophoresis DNA gels were soaked in 1.5M NaCl, 0.5M NaOH for 45 min to denature the DNA. Gels were neutralized by soaking in 1.0 M Tris/HCl, pH 8.0, 1.5M NaCl for 45 min. Transfer was essentially according to Southern (1975), but was in 20 X SSC (3.0 M NaCl, 0.3M Na citrate, pH 7.0).

After the transfer, the nitrocellulose was soaked in 10 X SSC for 5 min and allowed to dry at room temperature. Filters were baked for 2 hrs at 80°C under vacuum then soaked in 6 X SSC. Filters were then prehybridized at 42°C in 50 mls of 50% (v/v) formamide, 0.1M pipes, 0.8M NaCl, 5x Denhard's (50x = 1% Ficoll 1% polyvinylpyrrolidone, 1% BSA 500 mg/ml) sonicated, denatured calf thymus DNA, 0.1% N-lauryl sarcosine for at least 2 hrs. This solution was changed two times. The probe was hybridized to the filter in 50% formamide, 0.1M pipes, 0.8M NaCl, 5x Denhard's, 500 µg/ml calf thymus DNA, 0.1% N-lauryl sarcosine, 10% (w/v) dextran sulfate containing 2.5 X 10^7 cpm of [^{32}P] labelled probe. Usually a total volume of 50 mls was used. Hybridization was carried out at 42°C for about 15 hrs with gentle agitation. The filter was then rinsed 2 times for 5 min in 2x SSC, 0.1% (w/v) N-lauryl sarcosine, 0.1% (w/v) sodium acid pyrophosphate (NPP) then for 2 hrs in 1 x SSC containing 0.1% (w/v) N-lauryl sarcosine and 0.1% (w/v) sodium acid pyrophosphate (NPP) at 50°C changing the solution 4 times. The filter was air dried, wrapped in Saran wrap and exposed to XAR-5 X-ray film (Kodak) with an intensifying screen.

5. DNA Restrictions

Restriction enzymes were purchased from BRL and used in the core buffer provided. Enzyme stocks contained 10 units/µl. A unit of enzyme was defined as that amount which will digest

1.0 µg of DNA in one hour in a 50 µl volume. A two fold excess of enzyme was used (20 units per 10 µg DNA) and the reaction was allowed to proceed at 37°C for 5 hrs. Reactions were stopped by the addition of 10 mM Tris/HCl, pH 7.5, 50 mM EDTA, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue and heated to 65°C for 5 min.. Samples were cooled and loaded directly onto gels. Usually 10 µg of DNA were applied onto 0.8% (w/v) agarose gels as previously described.

6. Nick Translation

Nick translations were performed using the system supplied by New England Nuclear. Routinely 0.5 µg of DNA was nick translated in the presence of 50 µCi [α^{32}P] dCTP, as described in the manual. Reactions were allowed to proceed for 2 hrs at 12°C. The reaction was stopped by the addition of 10 mM Tris/HCl, pH 7.5, 50 mM EDTA, 20% glycerol, 0.2% bromophenol blue then loaded on a Sephadex G-50 column (bed volume 10 mls, 16 cm x 1 cm). The void volume was 5 mls and the column was pre-equilibrated with 10.0 mM Tris/HCl, pH 8.0, 10 mM NaCl, 0.1 mM EDTA. Fractions containing 1.0 ml were collected and monitored using Cerenokov radiation. The void volume fractions were pooled and an aliquot removed for determination of specific activity.

D) Results and Discussion

1. DNA Isolation and Restriction

Genomic DNA was isolated according to Methods section 1. Routinely 100 µg of DNA was obtained per gram of groat. This genomic DNA was digested with the indicated enzymes and the fragments separated by agarose electrophoresis as shown in Figure 1. Undigested DNA is greater than 30 kb in length and shows very little low Mr material. The visible DNA bands, due to repetitive genomic DNA sequences, varied with the restriction enzymes. The bright bands at the bottom of the gel are due to small DNA fragments resulting from the digestion with the various restriction enzymes.

This gel demonstrates that the DNA isolation procedure provides high Mr DNA which is susceptible to restriction enzyme digestion and can be used for Southern analysis.

FIGURE 1

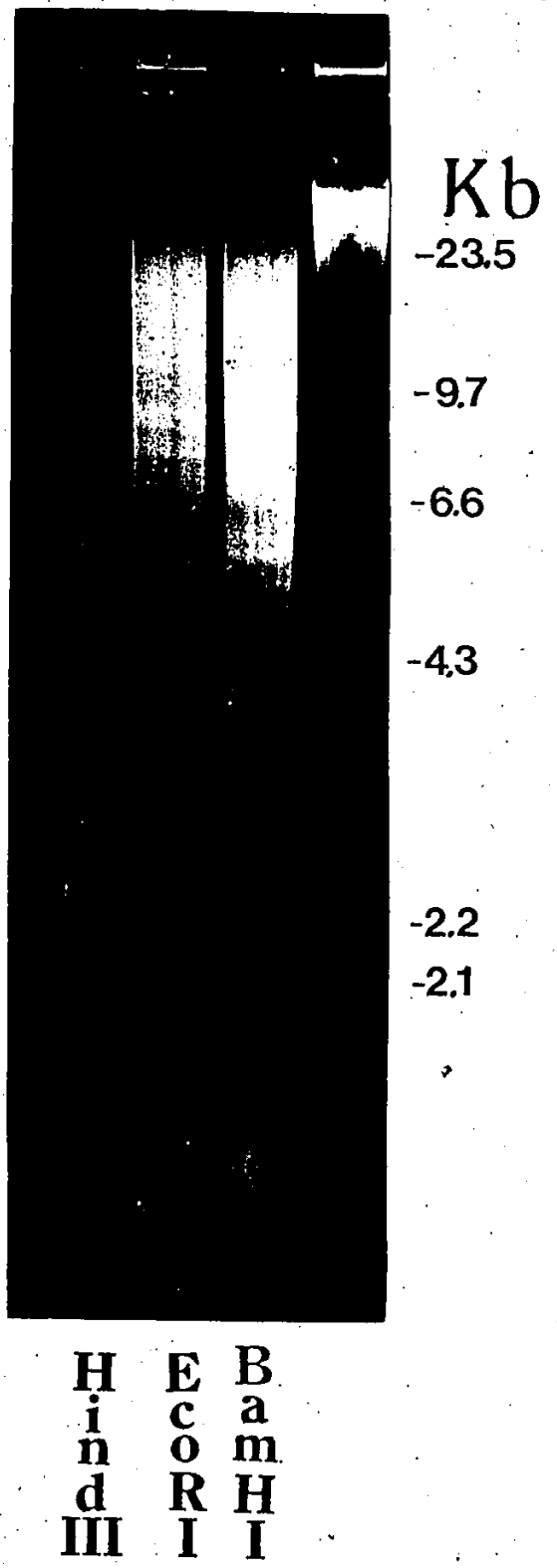


Figure 1. Analysis of oat genomic DNA by gel electrophoresis (Method section 3).

DNA was isolated according to method section 1 and restrictions were performed using Bam HI, Eco RI and Hind III (Method section 5).

2. Plasmid Isolation

Plasmid DNA from clone 2117 was isolated according to Method section 2. In order to verify that it contained an insert of 790 base pairs located in the Bam HI site, this enzyme was used to digest the plasmid according to method section 5. Figure 2 demonstrates a DNA gel of the digested plasmid. As predicted, the Bam HI digestion resulted in the release of the insert. The size of this insert was estimated to be about 900 base pairs when comparing its migration to the λ DNA Hind III fragments. Allowing for a small margin of error, this is consistent with the information provided by Croy which stated the size of the insert was 790 base pairs. Furthermore, there appears to be little of no bacterial chromosomal DNA contamination.

3. Nick Translation of Plasmid 2117

Nick translation was carried out as described in methods section 6. Reactions were carried out in the presence of 0.5 µg plasmid pRC (2117) and 50 µCi of [α^{32}P] dCTP (600 Ci/mM). Unincorporated nucleotides were removed by Sephadex chromatography and the fractions containing the labelled DNA pooled. An aliquot was used for determination of specific activity. The specific activity of probe was 5.0×10^7 cpm/µg assuming that all the DNA loaded on the column has been recovered in the void volume. Control pBR 322 was labelled to a specific activity of 1×10^8 cpm/µg.

FIGURE 2

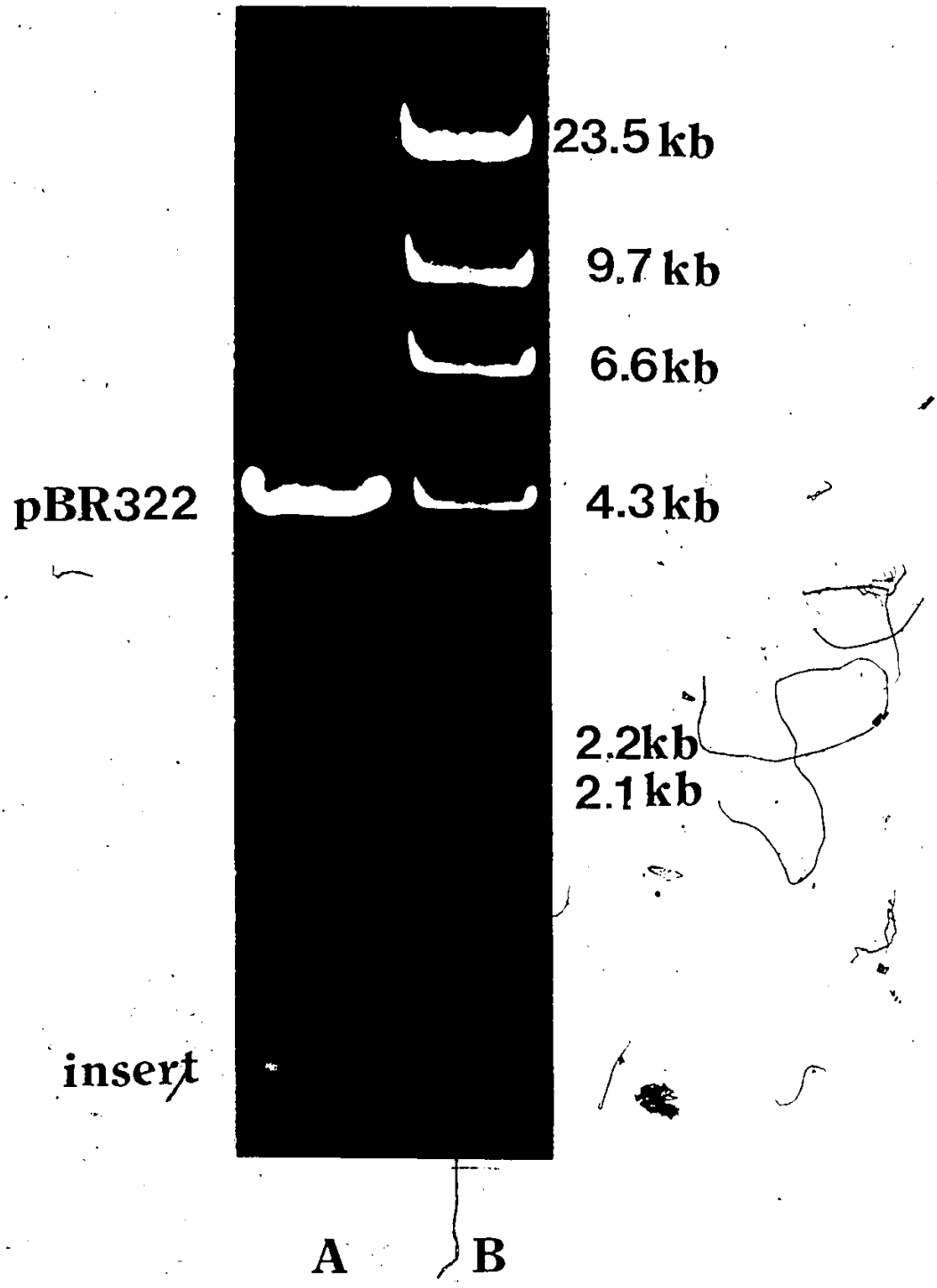


Figure 2. Gel electrophoresis of the legumin-containing plasmid pRC 2117.

The plasmid was isolated according to method section 2 and restricted with Bam HI according to method section 5.

λ Hind III restriction fragments containing 23.5, 9.7, 6.6, 4.3, 2.2 and 2.1 KB.

4. Southern Transfer Analysis

Southern transfer was carried out as described in methods section 4 and the blot was probed using nick translated pRC 2117. Oat genomic DNA was restricted with Hind III and Bam HI. As shown in Figure 3, pRC 2117 hybridizes to oat DNA. A control nick translated pBR 322 was also used to probe the same restriction fragments. No specific hybridization of pBR 322 to oat DNA was observed. However; one more control should have been performed. It is possible that legumin DNA hybridizes non specifically to unrelated DNA sequences. Thus the legumin probe should also have been used to probe DNA such as calf thymus DNA. If no hybridization occurred then legumin DNA does not hybridize non specifically to unrelated sequences.

Under the same hybridization conditions it was observed that 1×10^{-4} µg of pRC 2117 could be detected when treated in a similar manner to oat DNA. This would represent 0.001% of the amount of oat DNA applied to each track in Figure 3. This value should be in the range of a low gene copy number.

Bam Hl digested DNA showed a small number of fragments that hybridize to the legumin clone. Hind III digested DNA shows a strongly hybridizing fragment and a few fragments showing weaker hybridization. It is most likely that Bam HI cuts the sequence responsible for the strong hybridization in the Hind III digested DNA. It is possible that Bam HI cuts inside an intron located in the oat-like legumin sequence. Alternatively, Bam HI may cut a site present in oat legumin-like sequences which is not present in pea legumin sequences.

FIGURE 3

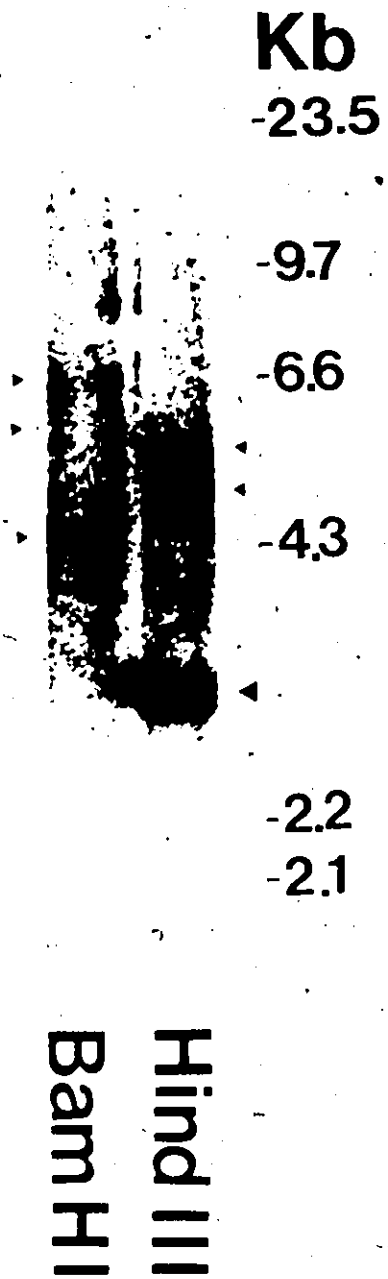


Figure 3. Southern blot analysis of oat genomic DNA using [^{32}P] labelled pRC 2117 as a probe.

DNA was isolated according to method section 1 and electrophoresis was carried out on 0.8% (w/v) agarose gels (method section 3). The blot was performed as described in method section 4. Hybridization was allowed to proceed for 15 hrs in the presence of pRC 2117 labelled to a specific activity of 5.0×10^7 cpm/µg. Blots were exposed for 6 days.

Track A) Blot of Bam HI digested DNA.

Track B) Blot of Hind III digested DNA.

▲ Strong Hybridization

▲ Weak Hybridization

Either of these possibilities may have led to the diminished hybridization observed in the Bam HI lane. The larger weaker hybridizing regions in the Hind III lane may also be due to partial digestion. This strong hybridization of the pea legumin clone to a small Hind III fragment and weaker hybridization to other Hind III and Bam HI fragments has also been demonstrated in this laboratory for wheat (S. Fabijanski). This may serve as an indication that more than one gene is responsible for the production of cereal globulin.

E) Conclusion and Future Work

On the basis of Southern analysis and under the assumption that legumin DNA binds specifically to oat DNA this investigation has demonstrated homology between legumin DNA and oat genomic DNA sequences. This provides further evidence that a legumin like gene(s) are contained within oat genomic DNA. These results further substantiate the main body of this thesis in demonstrating oat and legume globulin proteins may be derived from similar genes or gene families.

From these experiments however, it is not possible to predict the number of globulin genes present. More information may be gained by using a larger variety of restriction enzymes and oat globulin cDNA as the probe. The Southern blot analysis is preliminary. This result should therefore be substantiated using a globulin clone to screen pea genomic DNA to determine if it hybridizes to similar restriction fragments as does legumin cDNA. Finally, sequency data on the globulin clone must be compared to that of legumin clones.

CHAPTER 6

GENERAL DISCUSSION

A) Questions Arising

This investigation has provided an increased understanding of a cereal globulin. Results demonstrate widespread similarities (protein structural properties, antigenic properties, biosynthetic pathway) between oat and legume globulin. Oat was a good cereal to choose because its major storage protein is a globulin. However, it is premature to draw conclusions about other cereal globulins based on the results obtained for oat globulin. Antibodies raised against oat globulin do however cross-react with other cereal (rye) globulin. A more detailed study on other cereal globulins should be undertaken before generalizations can be made.

The similar properties shared by legume and oat globulins may be due to a converging evolutionary phenomenon. The present structures of globulin may have arisen independently in oat and legumes possibly due to selective pressures. Alternately, oat and legume globulins may have arisen from a common ancestral gene(s). Indeed, this gene(s) might have been conserved if globulin is to be properly packaged within the cell. If this is so, the biosynthesis and packaging of this storage protein is a sensitive and delicate system with which

evolution has not been able to tamper.

From an evolutionary point of view, rye salt soluble protein was tested for antigenic similarity because it is of the same subclass (Monocotyledon) as oat. However, nothing is known about the structure of rye globulins thus it is difficult to say which proteins are antigenically related.

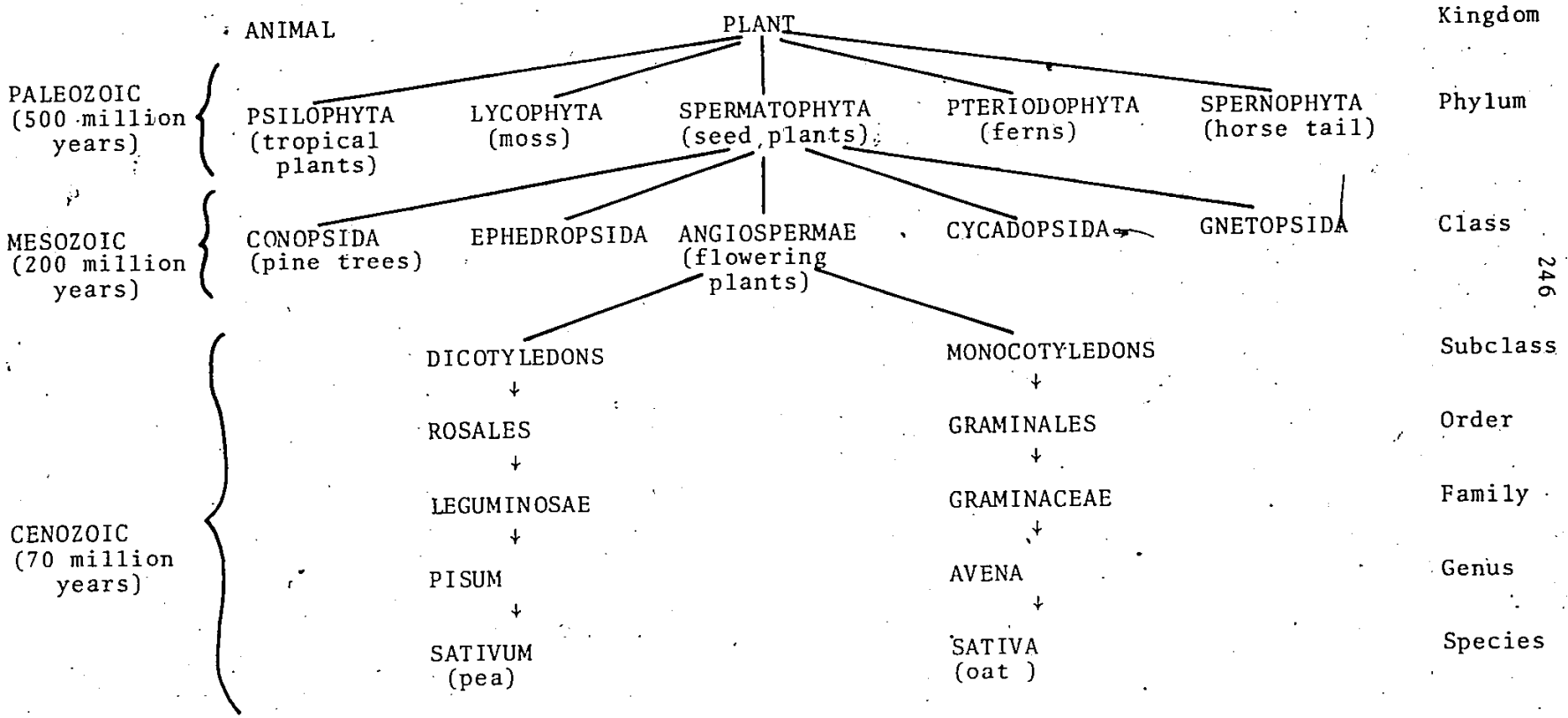
Alternatively, pea salt soluble protein was tested because it contains legumin which has similar physical characteristics to oat globulin. However, pea (Dicotyledon) is from a different subclass than oat (Monocotyledon).

My results suggest a common evolutionary trait contained in the salt soluble extracts from these seeds. On the basis of what is known about the physical characteristics of oat globulin and pea legumin these proteins are likely the evolutionary similar antigen.

Figure I represents the phylogenetic relationship between oats (Avena sativa L.) and pea (Pisum sativum L.). They both originate from the class angiospermae. Angiospermae evolved some 200 million years ago at a time when dinosaurs were at their zenith. According to my results, if globulin has been conserved then this gene must date back in evolution at least to this period. As dramatic or undramatic as this may seem it is indeed dwarfed when comparing globulin to leghemoglobin. Assuming leghemoglobin genes were conserved (also found in legumes; Verma et al, 1974) the globin genes may be followed back through evolution past the class and phylum taxa into the kingdom taxa which spans the paleozoic period some 500 million

FIGURE I[1]

PHYLOGENETIC RELATIONSHIP BETWEEN OAT AND PEA



[1]Compiled from Benson (1967).

years ago. It must have been during this era that the ancestral globin genes were adopted by both plants and animals.

Similarly, to establish how far back in evolution the globulin gene dates one might consider extracting and studying salt soluble proteins from Conopsida (pine seeds) and Lycophyta (mosses). If globulin is not present in this class or phylum possibly the gene is still present but not being expressed. Southern analysis using oat globulin cDNA to probe genomic DNA derived from these sources may reveal further interesting homologies.

To state that the globulin gene(s) (legume or oat) has been conserved is perhaps an over-simplication. The heterogeneity of the native subunits must be addressed. Such heterogeneity may be introduced at the level of the gene. For example, gene duplication is probably one of the most important mechanisms for generating newly related genes. Gene duplication has been implicated in numerous families of related proteins. Globin genes are a prime example. The human β-globin gene cluster contains five active globin genes together with two pseudogenes. Within this gene cluster are two closely related fetal genes which have arisen by a recent duplication within the cluster (Jeffreys et al. 1982).

Recently, Croy et al. (1982) has cloned the globulin (legumin) precursor from pea into pBR 322. During their study legumin cDNA was used to probe for homologous sequences in genomic DNA. Four genomic fragments hybridized to legumin cDNA. The intensity of these hybridizations, to genomic

fragments indicated a single gene copy per fragment. Thus, in pea there appears to be four legumin genes per haploid genome, assuming the probe hybridized to all legumin sequences. Such a genomic arrangement could give rise to heterogeneity observed in the legumin subunits. The situation may be analogous for oat globulin gene(s). Fisher and Goldberg (1982) prepared DNA blots from digested soybean leaf DNA. Blots were probed with a glycinin cDNA containing plasmid (containing a 700 base pair insert). The autoradiographic signals were similar to those expected for single copy sequences. It was concluded that approximately three genes are present in the glycinin gene family. This was somewhat similar to what Croy et al. (1982) reported. Thus it appears that legume globulins are expressed through a small gene family.

Probably the best example of a large storage protein gene family is that of the prolamins from maize. They are called zein which are contained in major groups of polypeptides with Mr 22 000, 15 000 and 19 000. cDNA clones have been synthesized from zein mRNA (Marks and Larkins, 1982). During their investigation they could distinguish different mRNAs for each of the Mr 22 000, 15 000 and 19 000 zeins. Comparison of Mr 22 000 and 19 000 gene nucleotide sequences revealed about 60% homology in the coding regions. They were able to distinguish at least five different families of zein sequences. There were two Mr 19 000 groups and two or three Mr 22 000 groups which contained short repetitive nucleotide

sequences common to both groups of clones. This suggested that they originated by a duplication and subsequent divergence of an ancestral gene. In this investigation on oat evidence for two mRNA populations for globulin precursors is presented. This may also have originated from a gene duplication.

More recently Larkins et al. (1983) using cDNA clones has predicted that the different sub-families of Mr 22 000 and 19 000 zeins are comprised of 40 to 60 of these genes in the genome and only 1-2 copies of the Mr 15 000 gene.

Thus it appears that this prolamin is made up of a much larger gene family that what has been reported for legume globulin gene families.

The size of the oat globulin gene family (if one exists) at present is unknown. This may be answered in our laboratory within the next few months. However, in view of the Mr and pI heterogeneity, immunoprecipitated translation products and Southern analysis presented in this thesis, I feel confident in predicting that there is more than one oat globulin gene.

Heterogeneity in the oat globulin polypeptides may also be due in part to differential processing of RNA transcripts. In this manner, RNA processing is used to increase the diversity of gene products. Such processing could further increase the protein heterogeneity caused by a gene family. Similar processing has been demonstrated for the calcitonin gene in rat (Amara et al. 1982).

Further heterogeneity within the globulin subunits may be introduced at the protein level such as glycosylation. Also post-translational proteolytic cleavage of the precursor may not be strictly controlled giving rise to final polypeptides of different size.

Finally, the heterogeneity among globulin subunits may have arisen from a combination of these above factors.

B) Future Work

S. Fabijanski is continuing to study the globulin gene(s) in our lab. Complimentary DNA produced against poly A+ mRNA was inserted into pBR 322. These hybrid plasmids have been used to transform E. coli HB 101. About 600 clones have been identified on the basis of antibiotic resistance and hybridization to [^{32}P] labelled cDNA. Clones have been classified according to whether they have been derived from high, medium and low abundance mRNAs. High abundant cDNA clones will be screened for legumin-like sequences with pRC 2117. Globulin clones must be verified using hybrid selected translation which is presently underway.

Globulin clones may be initially used to probe genomic DNA in an attempt to determine the number of globulin genes present. Northern analysis and dot hybridization will be useful in further understanding globulin mRNA with respect to size, abundance and expression.

Globulin clones could be used to probe endosperm genomic DNA at different developmental stages. This may provide information regarding possible genomic rearrangements during development. Clones could be used to probe genomic DNA from cultivars with various levels of protein and protein to yield ratios. In doing so, yield and protein levels may be correlated to certain genomic organizations. Furthermore, cultivars containing different ploidy levels may be studied with respect to genomic organizations.

These kinds of experiments may be very useful to plant breeders. Such approaches may result in a practical application of plant molecular biology in the near future.

In the more distant future techniques may soon become available which will allow genes to be transferred from one plant to another in the laboratory. In order to do so, several barriers must be overcome. Firstly, the regulation of such genes must be understood. What role do the flanking sequences play and how may they be used to regulate the expression of the structural gene? Possibly, there is a mediator which recognizes the flanking regions of the globulin gene which stimulates its expression. If such a mediator was isolated it may be used to increase globulin synthesis both in oat and other cereals. Secondly, a vector must become available which could carry a gene into other plant genomes. To do so plant mutants must also be developed in order to identify transformed plants. Ti plasmid has been extensively studied (Cocking et al. 1981) but thus far has not yielded dramatic results, especially in the monocotyledons. Thirdly and probably most important, protoplast regeneration techniques must be developed for cereals. Only a select few plants are presently capable of being regenerated from protoplasts (such as potatoe and tobacco).

In order to overcome these obstacles, intensive research must be conducted in several disciplines. One of these is

undoubtedly the understanding of the regulation and biosynthesis of cereal storage proteins. Ultimately, globulin genes may be manipulated so that they will become highly expressed in all cereals. If this is accomplished, cereals will have the nutritive qualities that legumes have.

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