Immunological and biochemical characterization of the major seed oil-body membrane proteins

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IMMUNOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE MAJOR SEED OIL-BODY MEMBRANE PROTEINS

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DEDICATION

This work is dedicated to the other five members of the Au's Family

ABBREVIATIONS

APS	ammonium persulphate
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Bis	N,N',-methylene bisacrylamide
BSA	bovine serum albumin
CNBr	cyanogen bromide
Da	dalton
EDTA	ethylenediaminetetra-acetate
EM	electron microscopy/microscopic
ELISA	enzyme-linked immunosorbent assay
Homo	total seed homogenate(s)
IgG	immunoglobulin G
Lys	Lys-C, endoprotease from *Lysobacter* enzymogenes
m.w.	molecular weight
NCS	N-chlorosuccinimide
n.d.	non-detectable
n.t.	not tested
OB	oil-body(ies)
PAGE	polyacrylamide gel-electrophoresis
PBS	phosphate buffer saline
PBSB	PBS + BSA
PBST	PBS + Tween20
PBSTB	PBST + BSA
SDS	sodium dodecyl sulphate (Lauryl sulphate sodium)
TEMED	N,N,N',N'-tetramethylethylemediamine

ABSTRACT

The major seed oil-body membrane proteins have been purified from a range of plant species by preparative SDS/PAGE. The purified proteins were used to elicit antibodies in rabbits and mice. Antisera were obtained from the following major oil-body membrane proteins: a) From mice (M), anti-*Brassica napus* 19kDa serum (Mnap19), anti-*Brassica napus* native oil-body proteins serum (Mnap native),anti-mustard 20kDa serum (Mmus20), anti-radish 20kDa serum (Mrad20), anti-*Crambe* 20kDa serum (Mcra20), anti-sunflower 20kDa serum (Msun20), anti-sunflower 19kDa serum (Msun19kDa), anti-safflower 20kDa serum (Msaf20) and anti-soybean 24kDa serum (Msoy24). b) From rabbit (R), anti-*Brassica napus* 19kDa (Rnap 19), anti-mustard 20kDa serum (Rmus20), anti-sunflower 20kDa serum (Rsun20) and anti-soybean 24kDa (Rsoy24). The cross-reactivity of each of these antibodies with oil-body proteins from species other than those to which they were raised was investigated by immunoblotting and ELISA. Considerable cross-reactivity was found, mostly within the Cruciferae, Compositae, and Leguminosae families. Cross-reactivity was also found between plant families and even between genera. There was also extensive cross-reaction between certain monocotyledonous species of Graminae (*Zea mays* and *Triticum durum*) with dicotyledonous species of the Cruciferae, Compositae and Leguminosae. The oil-body specific nature of the antibodies and their cross-reactivities were confirmed by immunogold labelling studies.

The total amino acid compositions of two of the purified oil-body membrane proteins, i.e. the *Brassica napus* 19kDa and radish 20kDa proteins, were

determined. Many similarities were observed in the amino acid compositions of these two proteins with those of the only other published plant species, i.e. the maize 15.5kDa and soybean 24kDa oil-body proteins. Similarities were also observed with the amino acid composition of the animal apolipoprotein B100 and bovin milk fat globule membrane proteins. These similarities included a moderately hydrophobic character and high levels of Glu and Leu. The immunological cross-reactivities and compositional similarities of these plant oil-body proteins imply that they may belong to a family of membrane proteins which share both structural and functional attributes. These plant proteins may also be related to animal lipoproteins which share the common function of enclosing oil-bodies, whether in blood serum, milk, adipose cells, egg-yolk or oilseeds.

CONTENT

1. INTRODUCTION

1.1 Lipid Reserves

Lipids are characterized by their hydrophobic nature. Fatty acids are the fundamental units of the acyllipids. Both prokaryotes and eukaryotes are able to synthesize fatty acids of considerable diversity. Triacylglycerols (esters of glycerol and three fatty acids) are the most common components found inside plant oil bodies, which are regarded as a highly reduced high energy reserviors especially in oilseeds (eg. rapeseed, mustard seed, soya, sunflower etc) and non-oil starchy seeds (eg. wheat (Morrison et al.,1975) and barley (Jones, 1969)) as well. Triacylglycerols provide an energy source during seed germination. Homologous cell inclusions have also been identified in animals (Angel, 1970; Mackenzie, 1980; and Yatsu, 1971). The fatty acid compositions in seeds are genotypically determined, unique and different from other vegetative cell constituents (Gurr, 1980). Moreover, different oilseed species have different sites of oil-body accumulation. Rapeseeds (Norton et al.,1975) concentrate their oil-bodies in cotyledons, castor beans store them in the endosperm, maize in the scutellum, while it is the pericarps of avocado which are rich in oil-bodies. (Sorokin, 1967).

1.2 Plant Lipid-Storage Vesicles

1.2.1 Nonemclature of Oil-bodies

Spherosomes (Frey-Wyssling et al., 1963), lipid-protein particles (Yatsu et al, 1963), reverve oil droplet (Sorokin, 1967), lipid-containing vesicles (Mollenhauer et al., 1971a) and oleosomes (Yatsu et al., 1971) are different types of nomenclature adopted by different authors describing the same oil-

rich inclusions in oilseeds (Stymme et al., 1987). Some authors believed that spherosomes are different from oil-bodies in terms of their differences in composition, function (Gurr, 1980) and tissue specifiity (Yatsu et al., 1971). Yatsu et al renamed them " oleosomes ", but this was objected to by Ichihara (1982) as he found that the site of triacylglycerol synthesis of safflower oil-bodies was not the oleosome. Wanner et al., (1981) and Huang (1987) regarded the two organelles as equivalent.

1.2.2 Structure of Oil-bodies

The presence of membrane around newly formed oil deposits in seeds is also controversial. It was reported that no membrane was found in new oil deposits of mustard seeds (Rest et al., 1972), in Crambe seed (Smith, 1974) nor in safflower seed (Ichihara, 1982) , instead, only a non-proteinaceous limiting boundary. Frey-Wyssling et al. (1963) claimed that tripartite membranes (4-6 nm thickness) were present in rapeseeds and mustard seeds. However, more evidence suggested the existence of an unusual half-unit membrane (2-3.5 nm thickness) around oil deposits of oilseeds and starchy seeds eg. mustard seeds (Bergfeld, 1987), cottonseeds (Yatsu, 1971), soya (Adams et al., 1983; Bair et al., 1980), peanut (Jacks et al., 1967; Yatsu et al., 1972) safflower and linseed (Slack et al., 1980), rapeseeds (Murphy and Cummins, 1988), maize (Huang et al., 1985; Vance et al.,1988) and bean cotyledons (Allen et al., 1971). These findings were based on electron microscopic observation and staining or through calculating the protein and phospholipid compositions. Wanner et al., (1981) were able to devise a model in which they tried to give a full picture of the ontogeny of oil-bodies. They suggested that the newly

synthesied triacylglycerols were sequestered between two phospholipid membrane monolayers in the endoplasmic reticulum (ER) and pinched off creating nascent, half-unit membraned or tripartite membraned oil-bodies. However, this model conflicts with the findings of Bergfeld et al. (1978) who observed that a proteinaceous membrane was only added onto nascent oil-bodies at the terminal seed maturation stage. Recently, similar findings had been reported also in rapeseed (Murphy, 1988). In developing rapeseed, oil-body membrane protein can only be detected well after the deposition of oil droplets. That is, oil droplets and their membrane protein are more likely under different spatial control and synthesized at different seed developmental stages.

In this report, oil-body is the term used to describe the oil droplets inside seeds. Such oil-bodies consist of a half-unit membrane with intergral proteins inserted in a monolayer of phospholipid. The hydrophobic terminals of both membrane proteins and phospholipids face inwards and interact with the triacylglycerol deposits in the core of oil-bodies (Jacks et al., 1967; Slack et al., 1980; Qu et al., 1986).

1.3 Animal Fat Reserves

1.3.1 Classification of Lipoproteins

The study of the mechanism of fat storage in animals is more well developed than that of plants. Lipoproteins and chylomicrons are regarded as homologous structures in animals to oil-bodies in seeds (Angel et al., 1971; Mackenzie et al.,1966; Yatsu et al.,1971). Chylomicron (in blood serum), very low density

lipoprotein (VLDL,in serum), low density lipoprotein (LDL,in serum and milk), high density lipoprotein (HDL, in serum) and ß-lipovitellin (in egg yolk) are the five classes of lipoprotein (Conn and Stumpf, 1976). They all have particular protein to lipid ratios. Every protein links with three types of fatty acids of cholesterol, triacylglcerol and phospholipid and is delimited by a half-unit membrane (Patton, 1975). The ER is directly involved in the synthesis of lipoprotein. The precursors of milk type liproteins were found to be naked when they were still inside the epithial cell of mammalian gland. Well-defined half-unit proteinaceous membranes could only be observed after their secretion in the lumen and in the milk fat globulin (Stein et al., 1967). This suggests that plant seed oil-body and animal lipoprotein are similar in physical appearance, membrane structure and in the of their ontogeny.

1.3.2 Apolipoproteins of Lipoproteins

Apolipoproteins are the major protein group that is found on the membranes of lipoproteins (Breslow, 1985). They are important in maintaining the structure and in the synthesis of lipoprotein. The apolipoproteins, apoAI, apoAII, apoAIV, apoB, apoCI, apoCII, apoCIII and apoE have been identified and some of them have also been characterized biochemically and molecular biologically (Breslow, 1985). Some of them act as receptors having regulatory effects eg. apoA and apoB. ApoAIII is involved in the biosynthesis of triacylglycerols while apoCII acts as a cofactor for the activation of lipoprotein lipase. Among them, apoB is the most abundant and has been under the most intensive study. Its amino acid composition (Kane, 1980) and partial amino acid sequence (LeBoenf, 1984) have been well documented.

1.4 Plant Major Oil-body Membrane Proteins

1.4.1 Ontogeny of Oil-body Membrane Proteins

In contrast to animal lipoproteins, plant oil-body membrane proteins are less well studied and their function is as yet unknown but it is believed by some that they may play a similar role to that of apolipoproteins in animals. These plant oil-body membrane proteins are hydrophohic with distinct polypeptides in different plant seeds (Qu et al., 1986). This group of proteins is synthesized during seed development after the deposition of oil droplets (Murphy, 1988) but not after germination (Huang, 1987). They are tissue specific and their expression is subjected to hormonal and spatial control (Vance, 1987). However, the site of protein synthesis is still debatable. Frey-Wyssling et al.(1987) and Wanner et al. (1981) believed that the proteins were synthesized directly in the ER and pinched off as entire oil-bodies. Qu et al. (1986) suggested that it was the ER associated polysomes were involved in the biosynthesis and supported the theory of ER synthesis. Herman (1987), however, identified that only free ribosomes responsible for the biosynthesis and believed the membranes proteins had similar ontogeny to that of lipases in germinating seed (Huang, 1987).

Until now, only a very few oil-body membrane protein species have been purified and characterized. Examples of some well documented ones are: i) the maize 15.5kDa, L_3 (Fernandez et al., 1987; Huang et al., 1986; Qu et al., 1986; Vance et al., 1987), ii) the soya 24 kDa, mP24 (Herman, 1987), and iii) rapeseed 19kDa (Murphy and Cummins, 1988; Murphy, 1988). Since the function of these

membrane proteins is unknown and they have no known enzymatic activity, immunoassay was the most useful technique with which to characterise them.

1.4.2 Similarities and Differences among Plant Species

In studies of the protein composition of oil-bodies, Slack et al. (1980) observed that linseed and safflower had similar membrane protein patterns when analysed on SDS/PAGE gels. They also found that one of the polypeptides (15kDa) exhibited similar behaviour towards *S.aureus* V8 protease digestion. Thence, they believed these similarities were significant. However, Qu et al. (1986) ignored the existence of similarity in oil-membrane proteins between non-related species.

1.5.0 Evidence of Similarities in other Plant Membrane Proteins

In fact, plant plasma membrane proteins from tomato (Grimes et al., 1987) and tobacco (Norman et al., 1986) have already been demonstrated to be able to cross-react with those that from non-related species. Both polyclonal and monoclonal antibody studies on plasma membrane proteins suggested that similar antigenic determinant(s) commonly exist throughout Angiosperms. Monoclonal antibodies that were raised against tobacco plasma membrane proteins were found to cross-react with similar proteins in other species (Norman et al., 1986). Similarly, polyclonal antibodies that were raised against tomato cell membrane proteins also could cross-react with corn root and soybean root plasma membrane proteins having the more or less similar molecular weights (Grimes

et al., 1987). Thus, the plasma membrane proteins are considered to have same function in dicotyledons (dicots) and monocotyledon (monocots).

1.6.0 Techniques used for Investigation in this Research

In this project, the major oil-body membrane proteins from a wide range of plant species have been purified. They are include species of the *Cruciferae* family such as rapeseed 19kDa (nap19), mustard seed 20kDa (mus20), radish 20kDa (rad20) and crambe 20kDa (cra20), species of the *Compositae* family such as sunflower 20 and 19 kDa (sun20 and sun19) and safflower 20kDa (saf20), and also species of the *Leguminosae* family, such as soya 24kDa (soy24). With the aid of antibodies to the respective purified membrane proteins, immunoblotting, immunocytochemistry and enzyme-linked immunsorbent assays (ELISA) were the major techniques used to characterise the polypeptides. The proteins were found to be oil-body membrane specific. Two of the proteins, nap19 and rad20 were further investigated to determine their total amino acid composition.

Although the oil-body membrane proteins are tissue specific, developmentally regulated (Vance et al., 1988) and different from other vegetative plasma membrane protein, we believed that, if this protein group serves a common function in seeds, the oil-body membrane proteins should have a certain degree of structural similarity among themselves. The extent of cross-reactivity between oil-body membrane proteins of different species should resesmble that of plasma membrane proteins. We tried to prove our hypothesis by producing a

wide spectrum of antibody types that were raised against oil-body membrane proteins from different oilseed species. Intensive cross-reactivity tests by immunoblotting and ELISA, plus immunocytochemical studies were carried out. Intra-genus, intra-family and inter-family cross-reactions of oil-body membrane proteins have been detected in this study. The data suggest that the oil-body membrane proteins among monocots and dicots have some structural similarities. Such structural similarities may also indicate functional similarities.

Two proteins (nap19 and rad20[1]) were also be investigated biochemically to reveal their amino-acid compositions. Their compositions were compared with two other plant oil-body membrane proteins (L_3 of maize and mP24 of soya) and animal apolipoproteins on milk fat globule and apoB that had already been documented in the literature. As all of these lipoproteins are believed to have similar functions, they should also have some structural similarities as is discussed in this report.

[1] : see Glossary on p.110 for explanation of these terms.

2.0.0 MATERIALS AND METHODS

2.1.0 Materials

All reagents are Analar grade. Chemicals are supplied by Sigma Chemical Co. unless otherwise stated.

2.2.0 Methods

2.2.1 Oil body Purification

Seeds were germinated in the dark (27°C for 48 h), then homgenised in a grinding buffer (40mM Tris, 5mM EDTA, 15mM β-mercaptoethanol, 0.3M D-sorbitol and 1% Polyvinyl-polypyrrolidone,PVP). Aliquots (100µl) of the homogenate were retained for Western blot analysis. The bulk of the homogenate was filtered through 3 layers of cheesecloth, centrifuged (70,000×g; 10 min). Oil pads were removed and resuspended in grinding buffer without PVP (1:4 oil bodies:buffer v/v) and overlayed with 4 times volume of half strength grinding buffer and centrifuged (150,000×g; 15 min). The oil bodies were removed and the purification process was repeated twice. The final pure oil bodies were resuspended in grinding buffer.

Oil-body Delipidation

The oil was removed by adding diethyl ether, 10 times volume, to oil body suspensions, vortexing for 1 min with maximum speed, centrifuged (4,000×g; 1 min) and the supernatant was discarded. The delipidation procedure was repeated twice and the residual ether was removed by a stream of nitrogen gas.

2.2.2 Gel Electrophoresis

The majority of analytical gel electrophoresis was done using Laemmli's method (1970) with a 1.5mm thick discontinuous SDS-system (SDS/PAGE), using Bio-Rad gel tank and accessary units. The resolving gel contained 15% acrylamide, 0.88% Bis gel, 0.475M Tris-HCl at pH 8.7, 0.1% SDS, 0.0825% TEMED and 0.03325% APS. The stacking gel contained 5% acrylamide, 0.14% Bis, 0.125M Tris-HCl at pH 6.9, 0.1% SDS, 0.25% TEMED and 0.05% APS. The gel was run in Tris-glycine buffer system containing 0.1% SDS at pH 8.4. The non-dissociating gel system used the same recipe except that SDS was replaced by water. Tris-borate electrophoresis buffer system (Neville, 1971)was used for purifying proteins for amino acid composition determination. Schägger's gel system (Schägger et al.; 1987) was employed for revealing low-molecular weight bands after digestion. The protein bands were revealed by staining with 10% coomassie blue for 1 h followed by shaking in destaining solution (40 % methanol, 10 % acetic acid in distilled water) for about 4 h or until the background became clear. Gels were allowed to shrink by storing in 50% methanol before taking photographs or drying in a vacuume gel drier.

Protein Purification

The total membrane proteins of the delipidated oil bodies were separated and purified by SDS-PAGE in 3mm thickness preparative gels based on the method of Laemmli (1970). The most heavily stained band was cut out and eluted by diffusion via homogenisation in protein extraction buffer containing 10mM Tris, 1 mM EDTA, 0.1% SDS at pH 7 and centrifuged at

3,000×g for 5 min. The supernatant was collected and the proteins were concentrated by ultrafiltration.

2.2.3 Anti-serum Production

Mice and New Zealand White rabbits were used for raising antibodies. An amount of purified protein (100μg for mice, 300μg for rabbits) was mixed with an equal amount of Complete Freund's Adjuvant. 1 ml of the emulsion was used for rabbit injection, 0.5 ml was intra-muscularly injected into left and right thighs and 0.25 ml injected subcutaneously at four sites on the back of the rabbit. Boostings were performed using incomplete Freund's adjuvant in an otherwise identical protocol. The first boosters were administered after six weeks and the first bleed taken 14 days later via the marginal ear vein. An ELISA was used to check the titre of the antisera.

Mice were immunised via the peritoneal cavity (100μl / mouse) using the same immunogens. Mice were bled via the tail vein.

2.2.4 Antibody Purification and Titre Determination

Recovery of serum from rabbit blood samples

Pre-immune and immune blood samples were collected into sterile boiling tubes and incubated at 37°C in water bath for two hours and stored in ice overnight for contraction of blood clot. Sera were poured off into a sterile centrifuge tube and centrifuged (70,000×g; 10 min). Sera were filter sterilized through a 0.22μm filter. The filtrates were collected

into universal bottles. A few grains of sodium azide were added as an antimicrobial agent.

Anti-serum recovery from mouse blood samples

About 100μl of mouse blood sample was collected, incubated for an hour at 37°C and left in ice overnight. The sample was centrifuged in a haematocrit centrifuge (7,000×g; 1 min) to pellet the cellular debris. Serum was transferred to a clean eppendorf tube. The pellet was resuspended in about 200μl of 1M borate-buffered saline at pH 8 and left for 1 h at 4°C in ice. The resuspended pellet was spun for 1 min in haematocrit centrifuge again. The washed serum was transferred to a clean eppendorf tube.

IgG purification

To the antiserum, an equal volume of sodium sulphate solution (32% w/v) was added dropwise over 10 to 20 min at RT. The mixtures were left standing for 5 min, centrifuged (15,000×g; 20 min). The supernatants were discarded while the pellets were resuspended in PBS to re-form the original volume. The precipitation procedure was repeated once. The final IgG-PBS solutions were dialysed against 1L PBS at 4°C for 2 d with 2 changes. The IgG concentration was calculated from U.V. absorption at 280nm using the following formula (Kang, A.S., personal communication):

$$\frac{\text{Absorbance at 280nm}}{1.34} = \text{mg / ml IgG}$$

2.2.5 Immunoblotting

Protein bands from SDS/PAGE gels were Western blotted by electrical transfer to a nitrocellulose membrane using current at 0.02mA cm^{-2} gel. A duplicate was made for Amindo Black staining of the protein. The unstained membrane was pre-blocked with 3% milk in Tris-NaCl buffer (10mM Tris, 150mM NaCl, pH 7.4) for ½ h. It was incubated in anti-serum (1/500 dilution) containing buffer for 2 h. After two washings with 0.5% Triton X-100 Tris-saline buffer and one washing in Tris-saline buffer, it was incubated with 0.05% 2° antibody for 1 h. The membrane was washed as before, incubated with catalase stain (25mg Diaminbenzidine, 50µl H_2O_2 in 100ml buffer) until colour developed, and then washed extensively with water.

2.2.6 Enzyme-Link Immunosorbent Assay (ELISA)

2.2.6.1 Direct ELISA

ELISA was used to check the titres, and for cross-reactions of possible related proteins with the antisera. Purified proteins were serially diluted to a range of 1 ng to 1µg ml^{-1} in coating buffer (0.3M Tris and 0.2M NaCl buffer at pH 9.5). Homogenates were diluted in a range of 10^{-2} to 10^{-8} in coating buffer. Aliquots (200µl) of the diluted samples were used to coat 96 well plates overnight at 4°C. The plates were then washed five times with PBST and once with dH_2O. Post-coating was achieved by incubating with 1%BSA-PBS (300µl / well) for 1 h at RT. After washing as before, aliquots (200µl) of 1° Ab in PBST-0.1% BSA were added and incubated for 2 h at RT. Washing was repeated,

2° antibody-enzyme conjugate added (200µl / well; 10^{-3} dilution in 0.1% PBSTB) and incubated for 2 h at RT. After washing, the enzyme substrate (1ml ABTS in every 24ml of citrate peroxide buffer at pH 4) was added (200µl / well) and the absorbance were read after 30-60 min using a plate reader (Titertek Multiskan MCC) at 415nm.

2.2.6.2 Immunometric Assay

Rabbit anti-*Brasicca napus* 19kDa (nap19) Ab was diluted to about 1µg/ml with Tris-NaCl buffer and used to coat 96 well plates (200µl / well) overnight at 4°C. The plates were washed and post-coated as in ELISA. The protein antigens were serially diluted in PBSTB and added (200µl / well) at RT for 2 h. Washing was followed by addition of mouse anti-nap19 (diluted in PBSTB; 200µl / well) and incubated at RT for 2 h. Then, incubated with secondary antibodies as in ELISA. After washing, the enzyme substrate was added (200µl / well) and the absorbance were read after 30-60 min using a plate reader.

2.2.7 Electron Microscopy

Small pieces of dry seed tissue were cut and fixed in standard osmium fixative containing 2.5% glutaraldehyde, 1.5% paraformaldehyde and 0.05M cacodylate buffer overnight at 4 °C after degassing in 2 cycles for 5 min. Fixed tissues were washed with 0.05M cacodylate buffer, pH 7 and followed by dehydration in 12.5%, 25%, 50%, 75%, 100% and absolute ethanol for 1 h each with 2 changes of ethanol. The tissues were stored in 50% Spurr's resin in absolute ethanol overnight at RT and then with Spurr's resin changed 3 to 4 times morning and night for 3 successive days. Finally, they were evenly distributed on moulds and allowed to

polymerize overnight at 60-70°C with a cover slide. The blocks were trimmed, sectioned, rested on 200 meshes and viewed under a transmissional electronmicroscopy (Philips EM 400).

2.2.8 Immunocytochemistry

Small pieces of tissue were fixed in standard immunocytochemical fixative containing 3% paraformaldehyde, 1.25% glutaraldehyde and 0.05% phosphate buffer at pH 7, overnight at 4°C after degassing. Hence, they were washed once in 0.05M phosphate buffer for 30 min, followed by a dehydration process the same as for electronmicroscopy and stored in 50% L-R White resin in absolute alcohol overnight at RT on a rotator. L-R White resin was changed 3 to 4 times morning and night for 3 successive days. Finally, specimens were placed on moulds with fresh L-R White and allowed to polymerize ovenight at 60°C. Gold-silver sections were prepared by microtome sectioning and placed on a fomvar-coated Ni 200 meshes grids. The specimens were processed as follows. They were firstly blocked with 10µl 5% dried skimmed milk powder in PBST for 15 min and blocked again with a 1/10 dilution of the relevant pre-immune serum for 15 min. We used mouse pre-immunse serum to block samples labelled with rabbit antiserum and vice versa. The specimens were washed with 3 drops of PBST and then incubated with a dilution of anti-serum between 1/10 to 1/500 in PBST for 1 h, follwed by washing with 10 drops of PBST. The sections were reblocked in the pre-immune serum before labelling with 2° antibodies, 10µl of 1/20 dilution goat-anti-rabbit (GAR 20) or goat-anti-mouse (GAM 20), antibodies conjugated to 20nm diameter gold partiles, were used as appropriate. After 15 min of incubation, they were washed with 10 drops of PBST and distilled

water. Uracil actetate as used to stain for 15 min and the specimens were washed extensively with distilled water. Structures recognised by the anti-bodies would be revealed as 20nm diameter black dots under transmission electron microscopy.

2.2.9 Protein Hydrolysis

Proteolysis

The digestability of membrane proteins was studied using protease *S. aureus* V.8, trypsin, N-Chlorosuccinimide/urea (NCS) (Lischwe et al., 1982) and endoprotease lys-C at different incubation time and concentrations. 20μg of PAGE purified proteins were denatured by boiling for 2 min, then cooled down pior to the addition of enzymes. The samples were incubated at 37°C for different lengths of time. Reactions were stopped by boiling samples for 5 min. in the presence of an equal volume of running sample buffer. The digestion pattern were reviewed first in Schägger's gel system (Schägger et al., 1987) and then by Laemmli PAGE.

Cyanogen Bromide Cleavage of Proteins

About 300μg of proteins was mixed with 70% formic acid. An equal weight of CNBr, dissolved in acetonitrile, was added to the samples, purged with nitrogen and stored in dark for 24 h with occassional shaking. Then, 15 times volume of distilled water was added, vortexed and frozen.

Mapping of Antigenic Determinants

After the optimum digestion conditions had been determined, the proteolytic patterns of various proteins were compared on Coomassie blue stained PAGE gels. Then, a similar gel was run for Western Blot and incubated with the appropriate antibodies to look for positively labelled peptide fragments. The antigenicity of same digestion products were checked by ELISA to detect the integrity of the epitopes.

Antigenicity of Intact Oil-bodies

Intact oil bodies of tested species were subjected to similar digestion conditions to purified proteins but without the prior denaturing process. The digestion pattern were checked on PAGE gel, Western blot and ELISA.

2.2.10 Amino Acid Composition Analysis

Protein Purification

PAGE purified nap19 and rad20 proteins bands were cut and extracted by shaking in 25ml of 0.1M NH_4HCO_3 (Schmidt, 1982) for 3 d at 4°C and then freeze dried the samples.

Reduction / Carboxylmethylation

About 300μg of each protein sample, 30μl of 6M granidin, 0.6M Tris-Cl at pH 8.6 was added (we use 300μg protein). Reduction was carrying out in a nitrogen-reducing environment for 3 h with the addition of 30μl β-mercaptoethanol. 0.1ml solution of a mixture of 0.268g iodoacetate plus 1 ml of 0.1M NaOH was then added for carbozylmethylation for 30 min

at RT in dark. The samples were dialysed with 5L 5mM NH_4HCO_3 in darkness for 24 h with 4 changes of buffer.

Hydrolysis and Derivatization Reactions

The subsequent reactions were carried out in reaction tubes which had been cleaned with 6N HCl, rinsed with Milli-Q water, 100% ethanol and dried under vacuum. All chemicals were transferred with a microliter pipette. About 10μg of samples were pipetted into reaction tubes and dried within a reaction vial in the oven of PICO.TAG Work Station before hydrolysing with 200μl of 6NCl 1% phenol which was added into reaction vial for sets of 24 h, 48 h and 72 h hydrolyses at 105°C. Hydrolysed samples were redried by adding 10μl of redrying solution (2:2:1 by volume of ethanol:water:triethylamine) and vacuum dried in the Work Station. 100μl of freshly prepared derivatization reagent (7:1:1:1 of ethanol: triethylamine: phenylisothiocyanate ; PITC) was then added to each of the reaction tubes for derivatizing for 20 min at RT before dry down. Standard samples were prepared in the same way.

Amino Acid Composition Determination

Hydrolysed, derivatized and dried samples were dissolved in sample diluent (5mM Na_2HPO_4 titrated to pH 7.4 with 10% phosphoric acid and added acetonitrile to be 5% of the final mixture). 5μl of the samples were transfered to sampling vials and run on a Waters Intelligent Sample Processor WISP 7108. Output was connected to IBM PC using Maxima 820 software. Detailed procedures followed PICO.TAG Work Station Operator's Manual (1984) and WISP Operator's Manual.

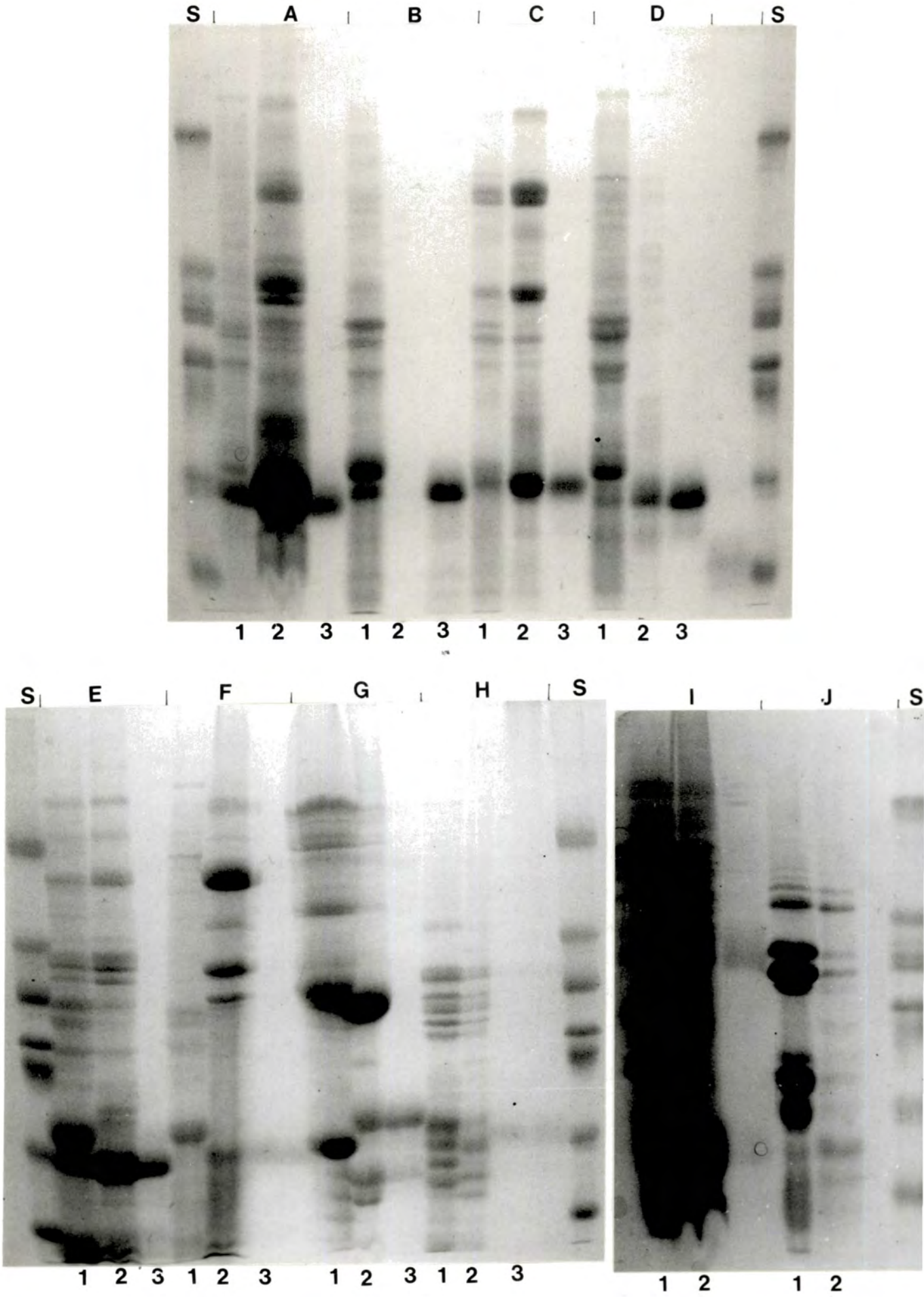
3.0.0 RESULTS

3.1.0 Protein Patterns of Seeds on SDS/PAGE Gels

Protein gels showing total homogenate, delipidated oil-bodies and the SDS/PAGE purified protein of those oilseeds species under study are shown in Fig.1. Species from same genus, eg Brassica, have similar patterns of distribution of the major membrane proteins on oil bodies (Fig.23). Those major bands were believed to be the antheutic oil-body membrane proteins while the minor bands might be oil-membrane proteins or impurities (Qu etal. 1986). All Brassica have major bands about 19 - 20 kDa and so do oil-body membranes of *Compositae*. Maize and tobacco also have one of their major oil-body membrane protein near 19 kDa (Fig. 1). In some cases, eg. rapeseed, the nap19 protein predominated also in thetotal homogenate. In contrast, eg. soy24 could only be detected in oil-body extracts. The SDS/PAGE purified proteins always represented a single band.

3.2.0 Titres of Various Anti-sera

The titres of antisera were determined by ELISA with comparison to normal pre-immune sera. The results are shown in Fig. 2 to 15. They all showed sigmoid curves when absorbance was plotted against serial dilution. The dilution corresponding to 1 unit of absorbance was assigned as the titre of each antiserum. The titres are shown in table 1.



Fig, 1 Total Homogenates(Homo), oil-bodies(OB) and SDS/PAGE purified proteins on SDS/PAGE gel,
A, *Brassica napus* B, Raphanus sativus C, *Sinapis alba* D, *B,campestris* E, R,sativus
F, *B,oleracea* G, *Glycine max* H, *Carthamus tinctorius* I, *Zea mays* J, *N,tobacum Wiscosin 38*
1, Homogenate 2, oil-body 3, SDS/PAGE purified protein

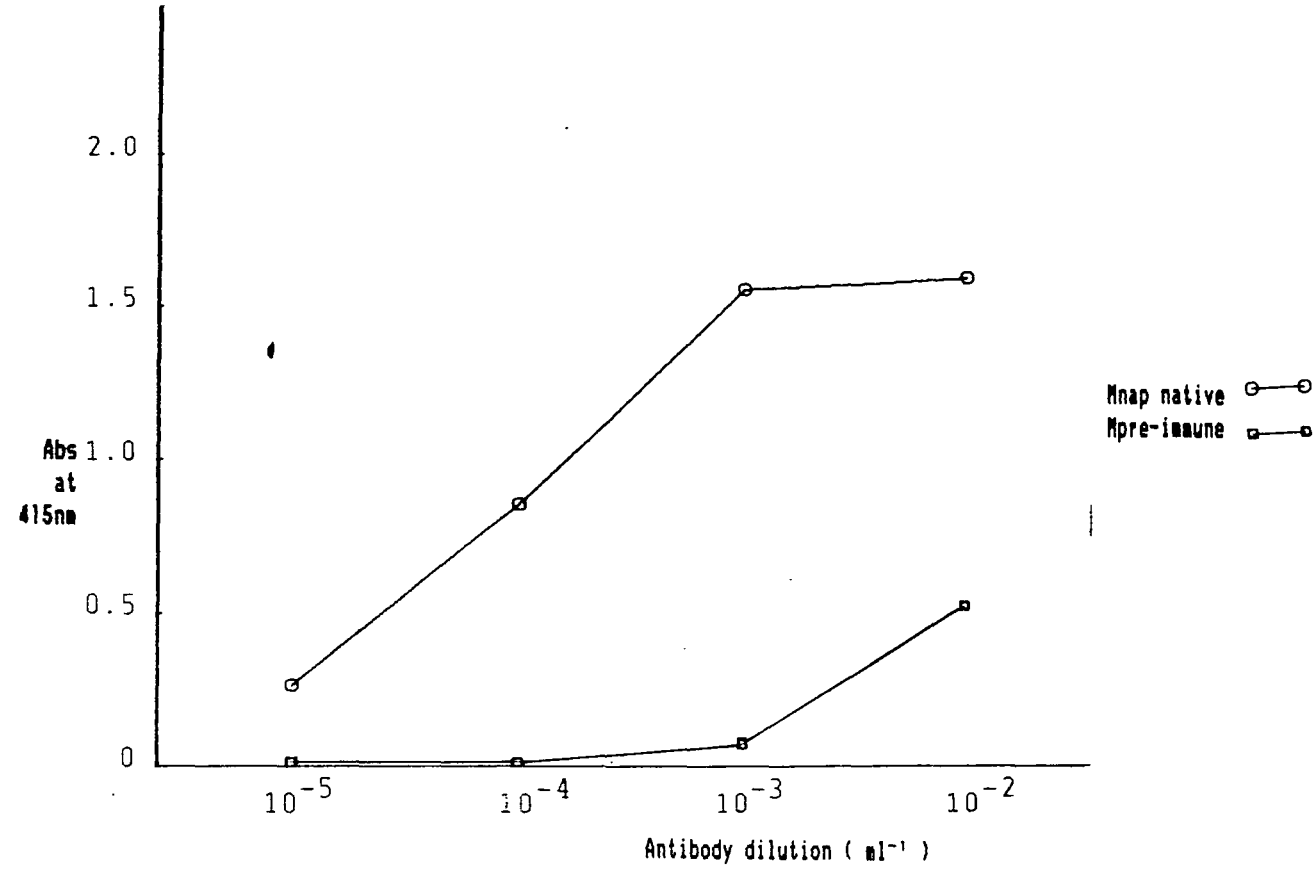


Fig.2 Mnap native Titration determination

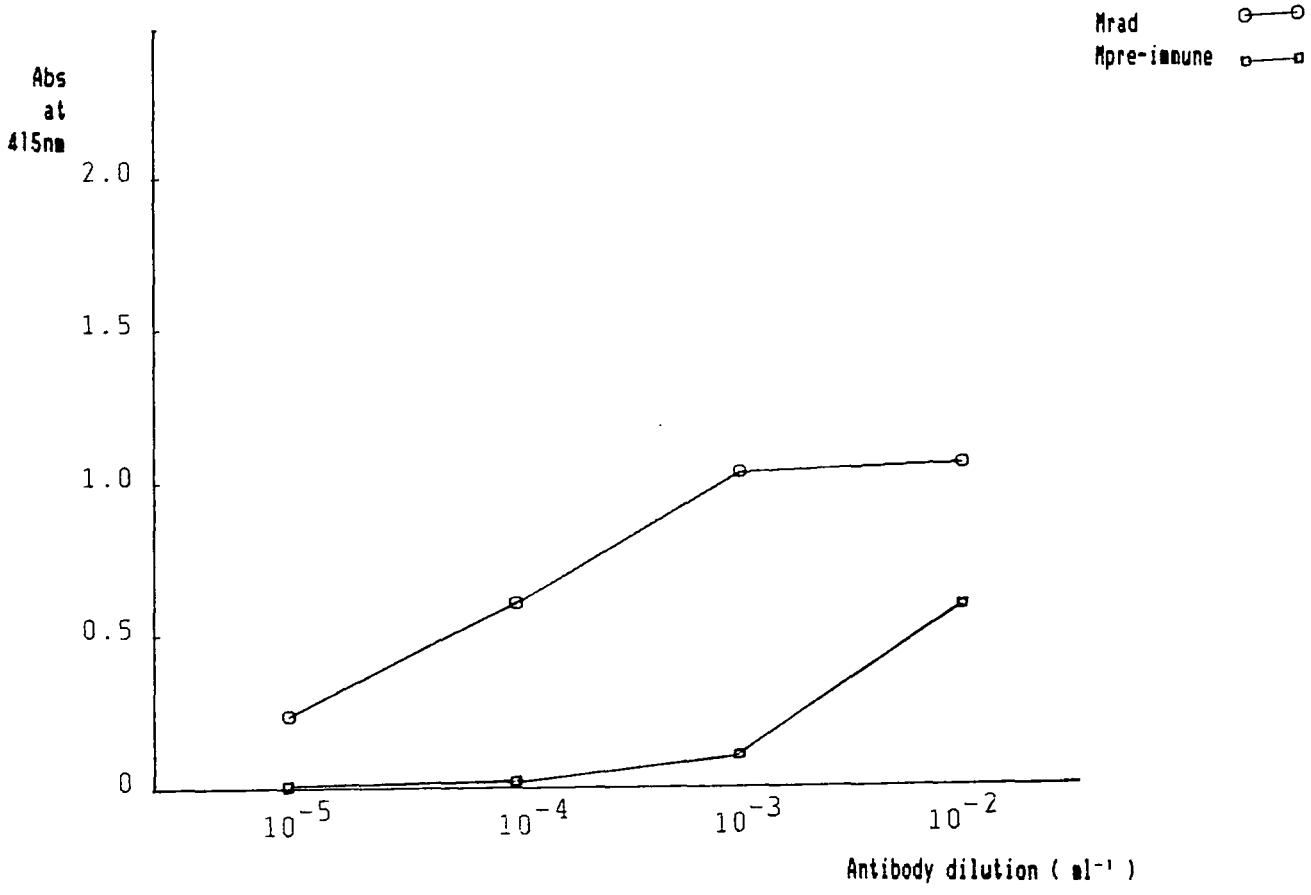
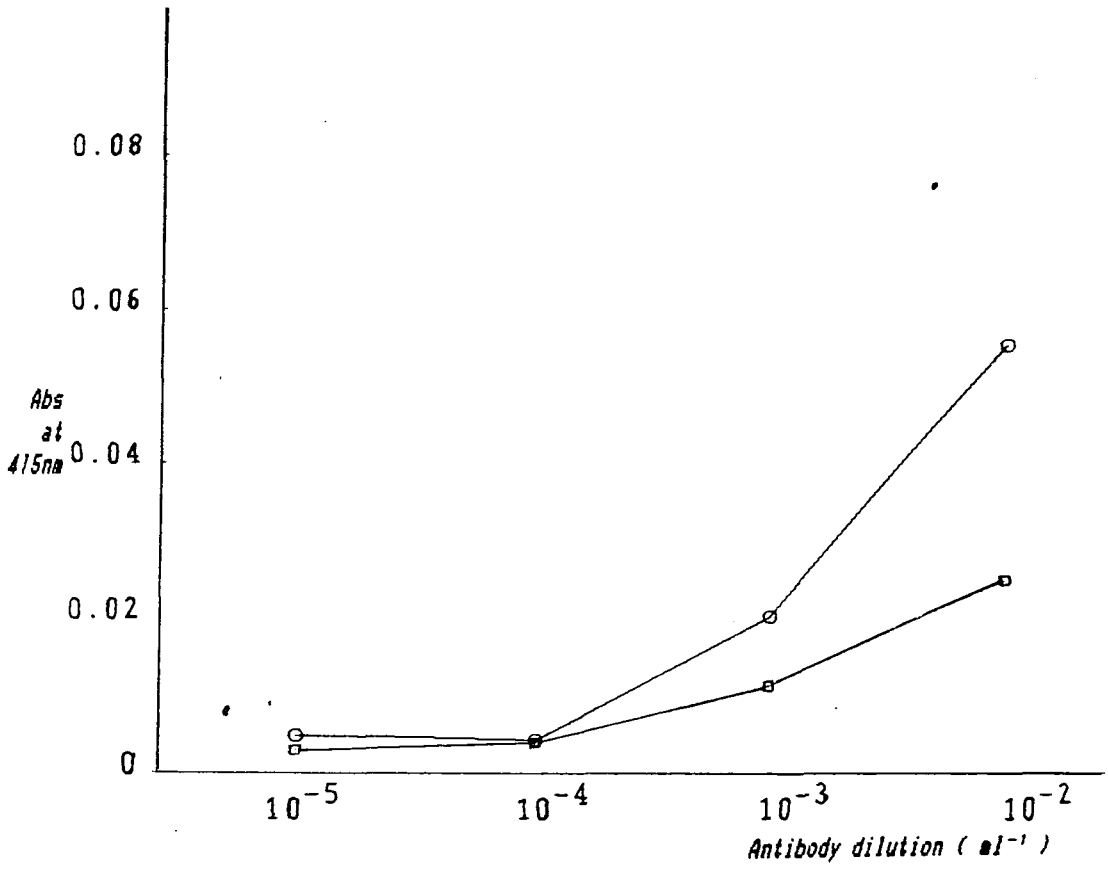
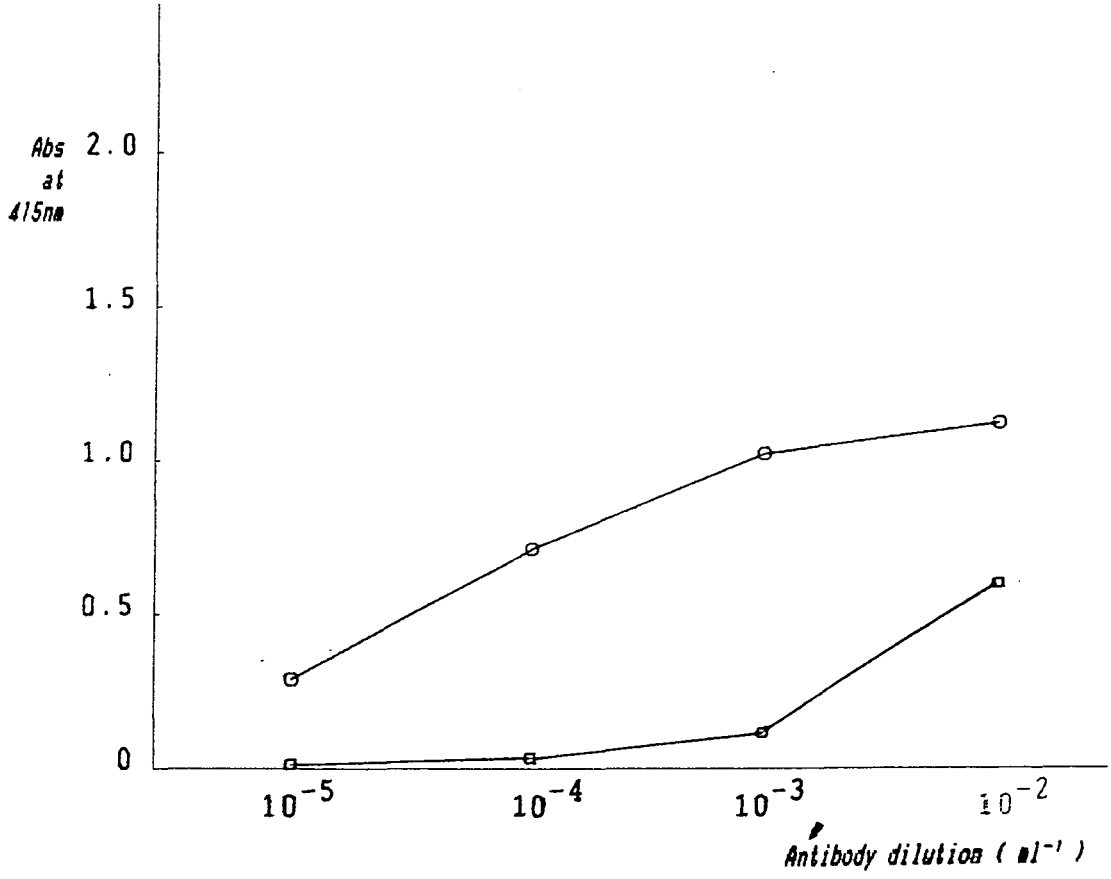


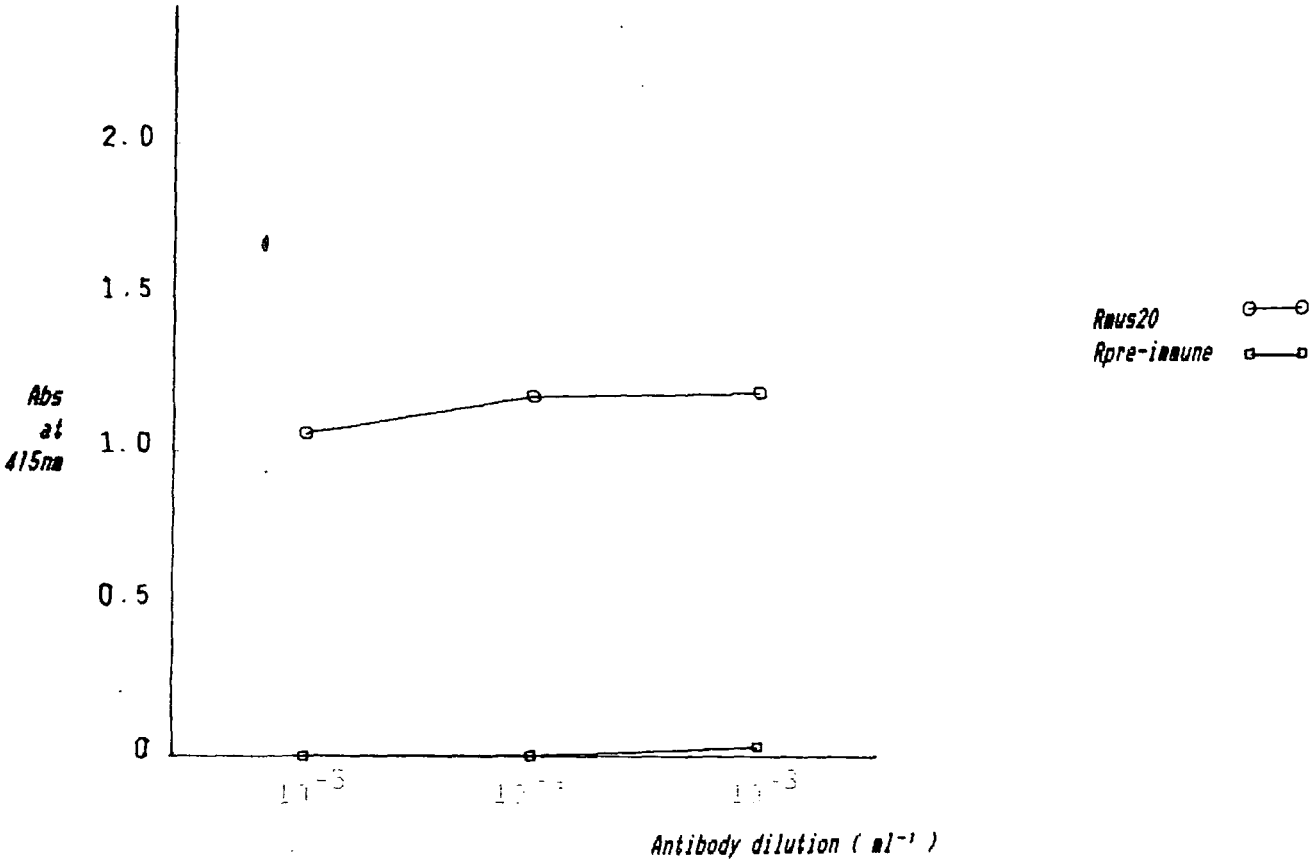
Fig.3 Mrad20 Titration determination



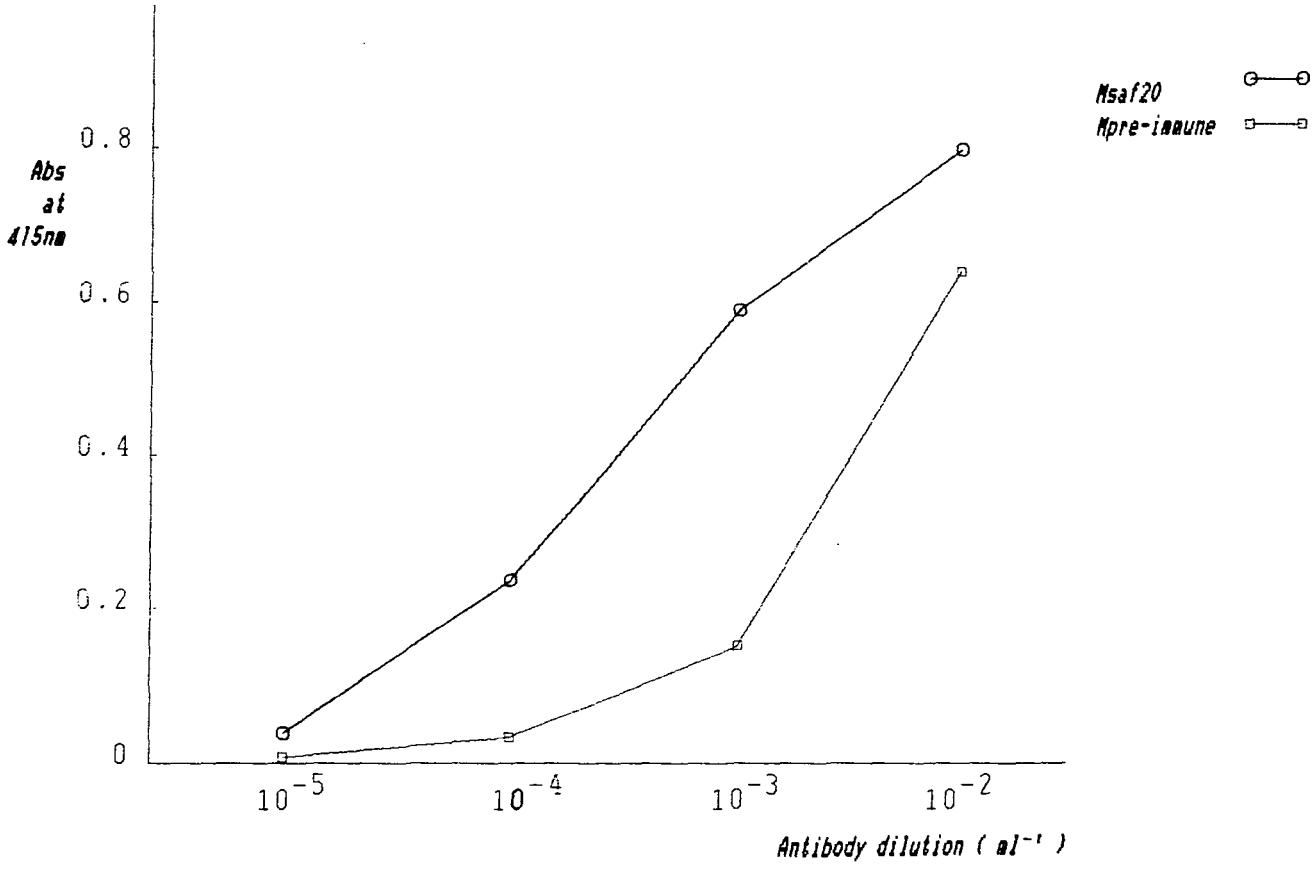
Fig,4 Mcra20 Titration determination



Fig,5 Mmus20 Titration determination



Fig,6 Rmus20 BI Titration determination



Fig,7 Msaf20 Titration determination

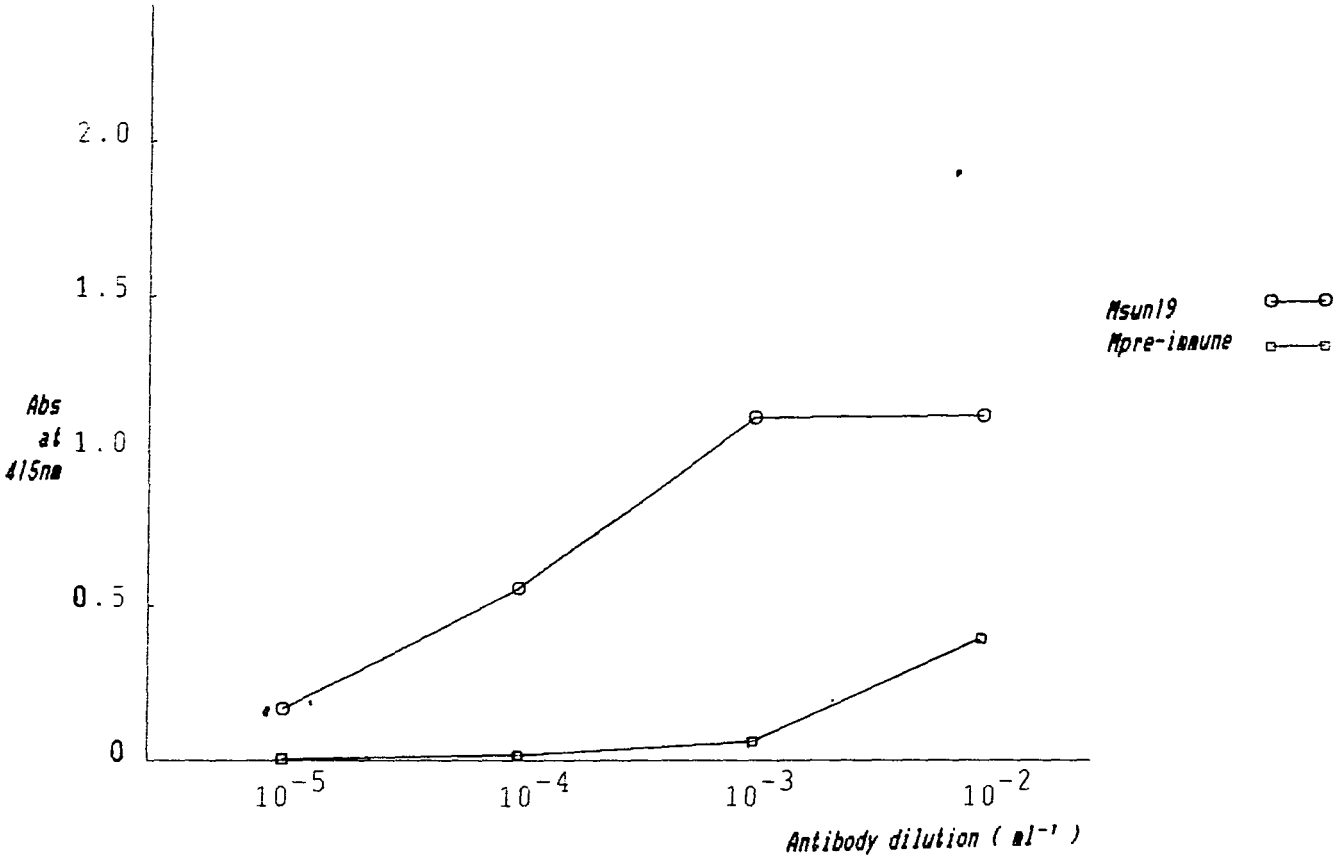


Fig.8 Msun19 Titration determination

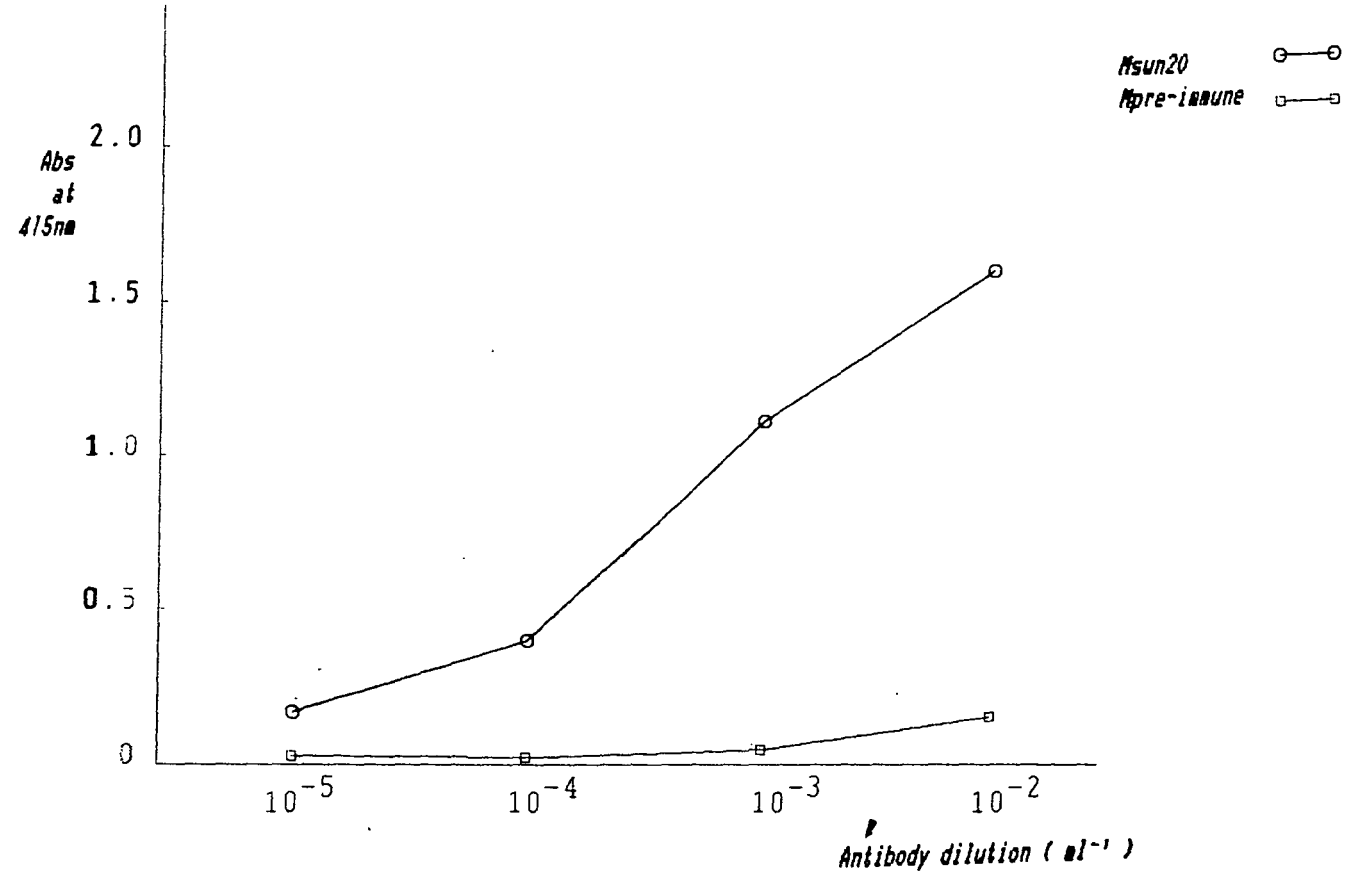
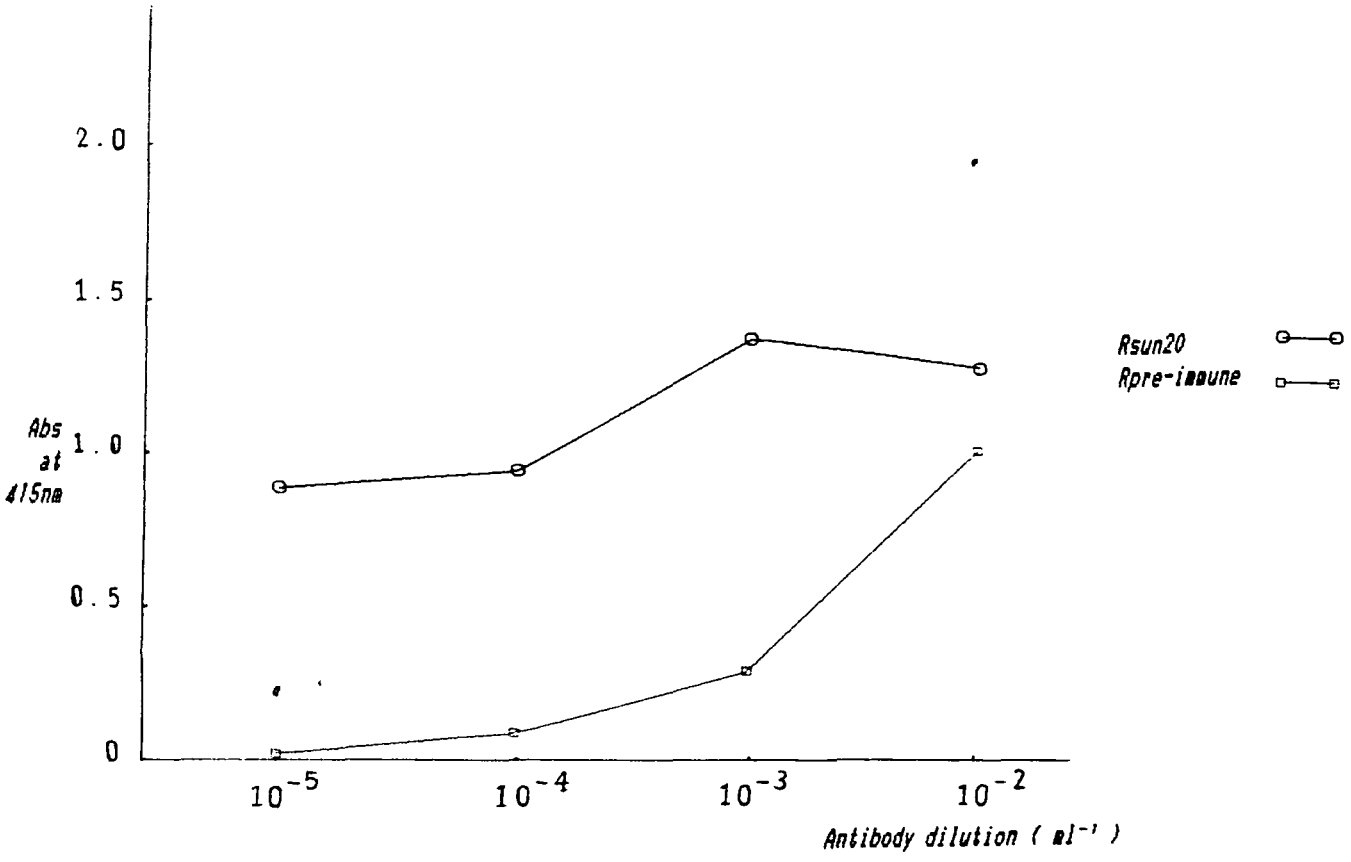
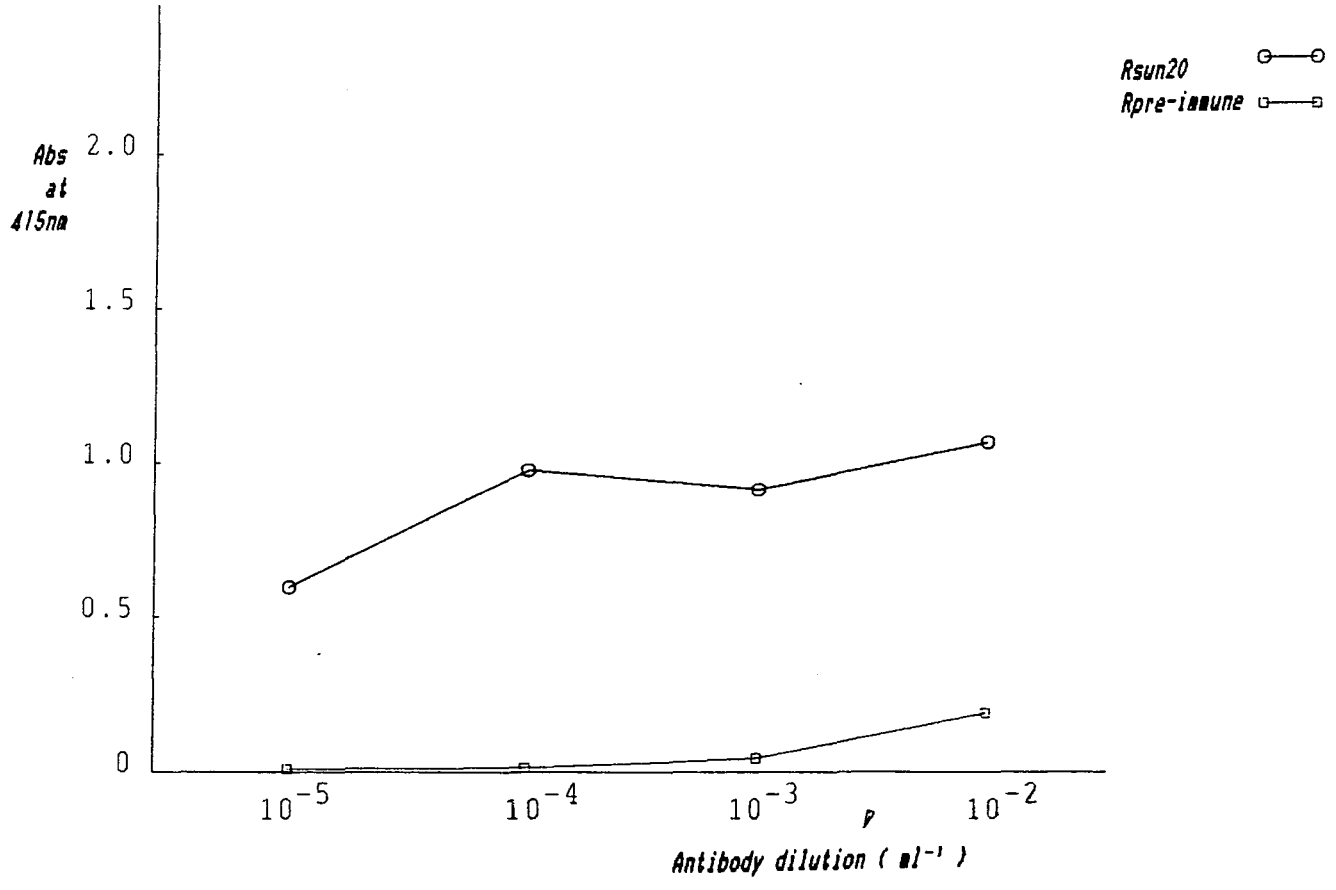


Fig.9 Msun20 Titration determination



Fig, 10 Rsun20 B1 Titration determination



Fig, 11 Rsun20 B2 Titration determination

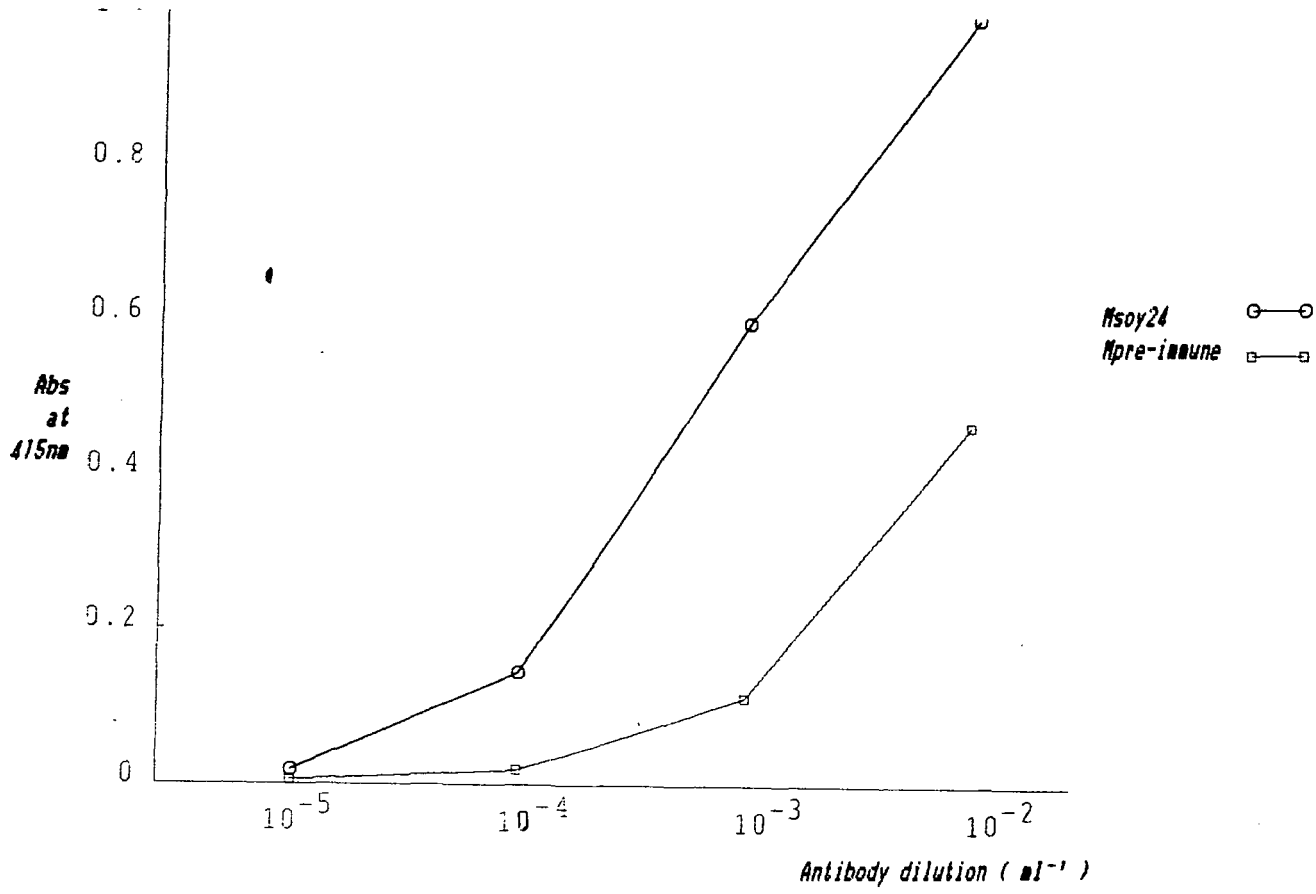


Fig. 12 Msoy24 Titration determination

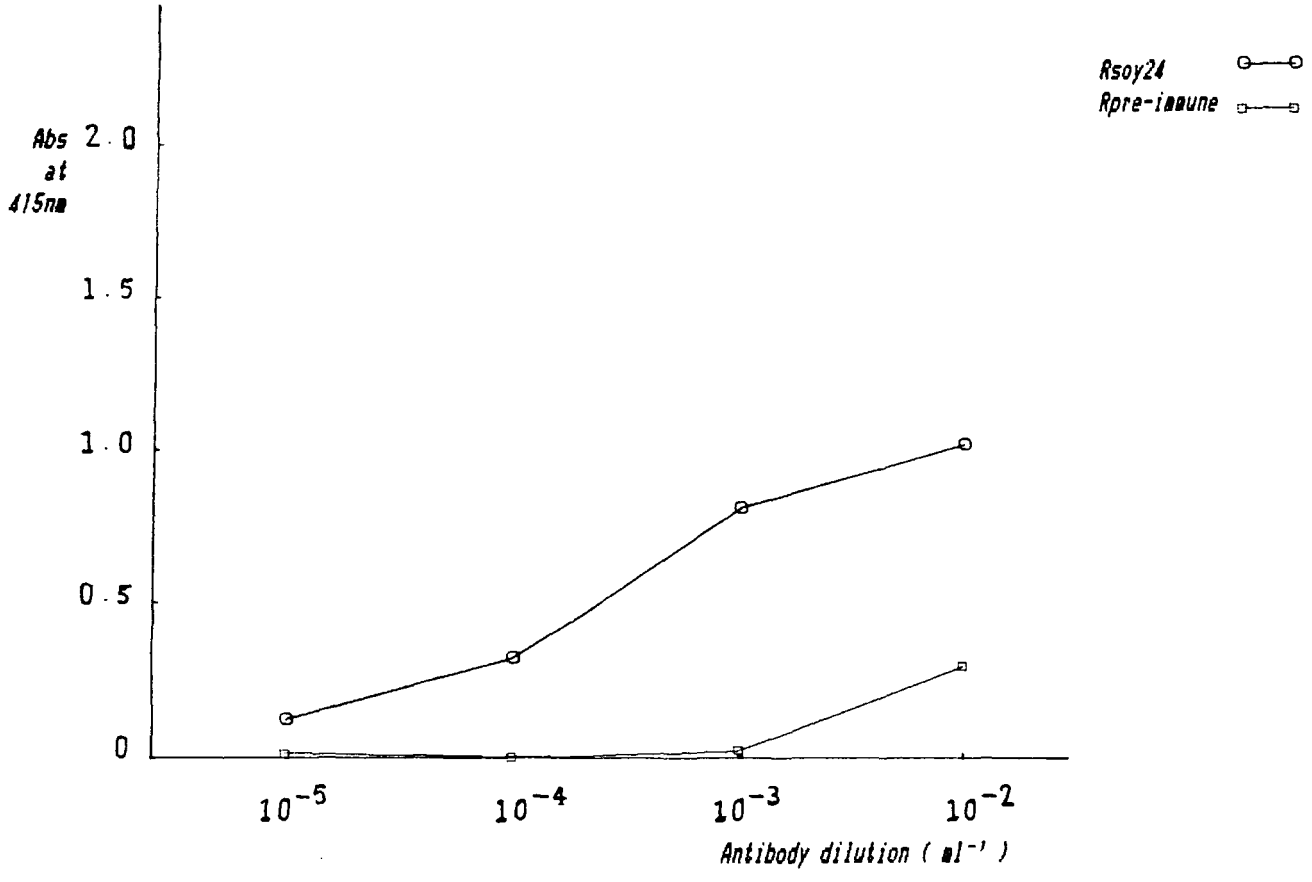
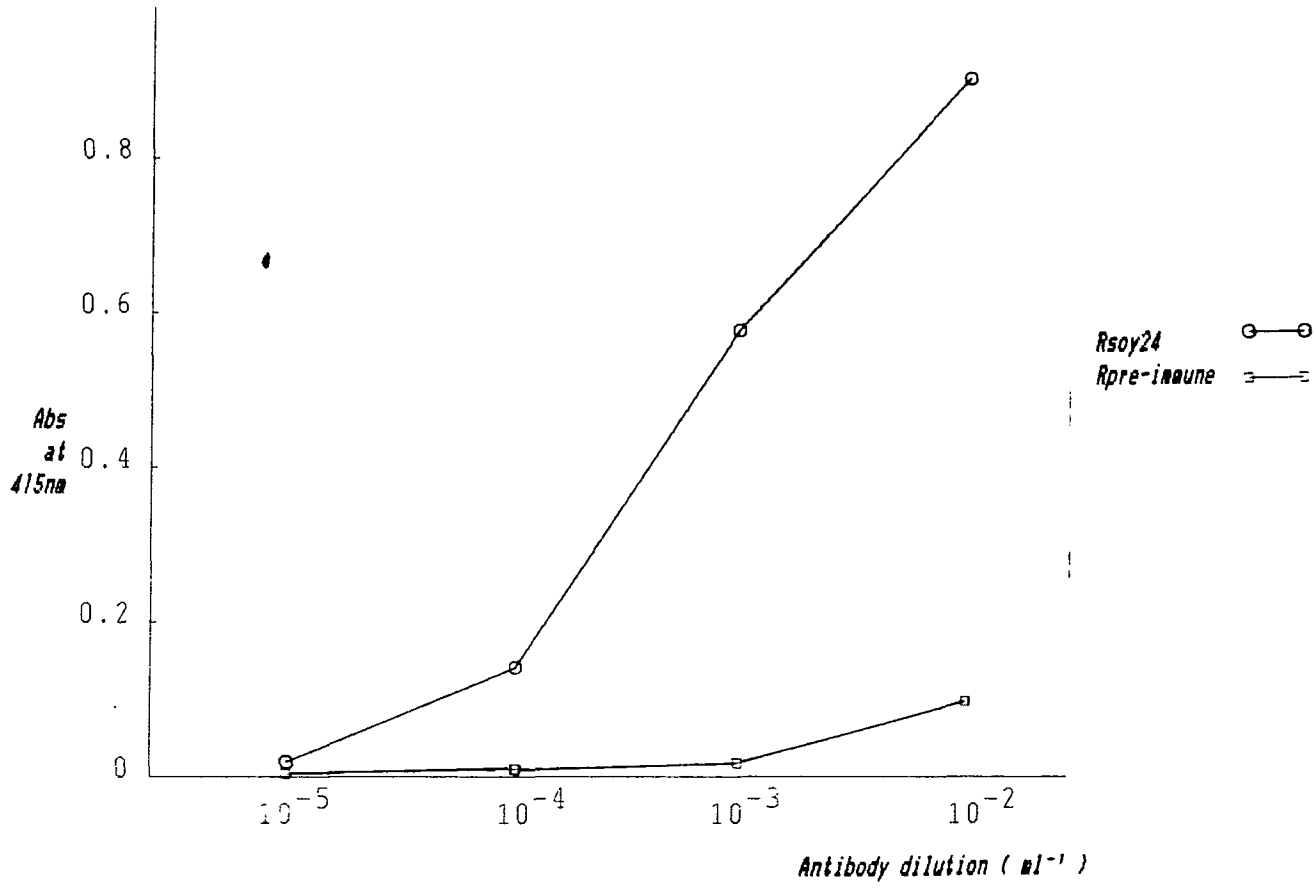
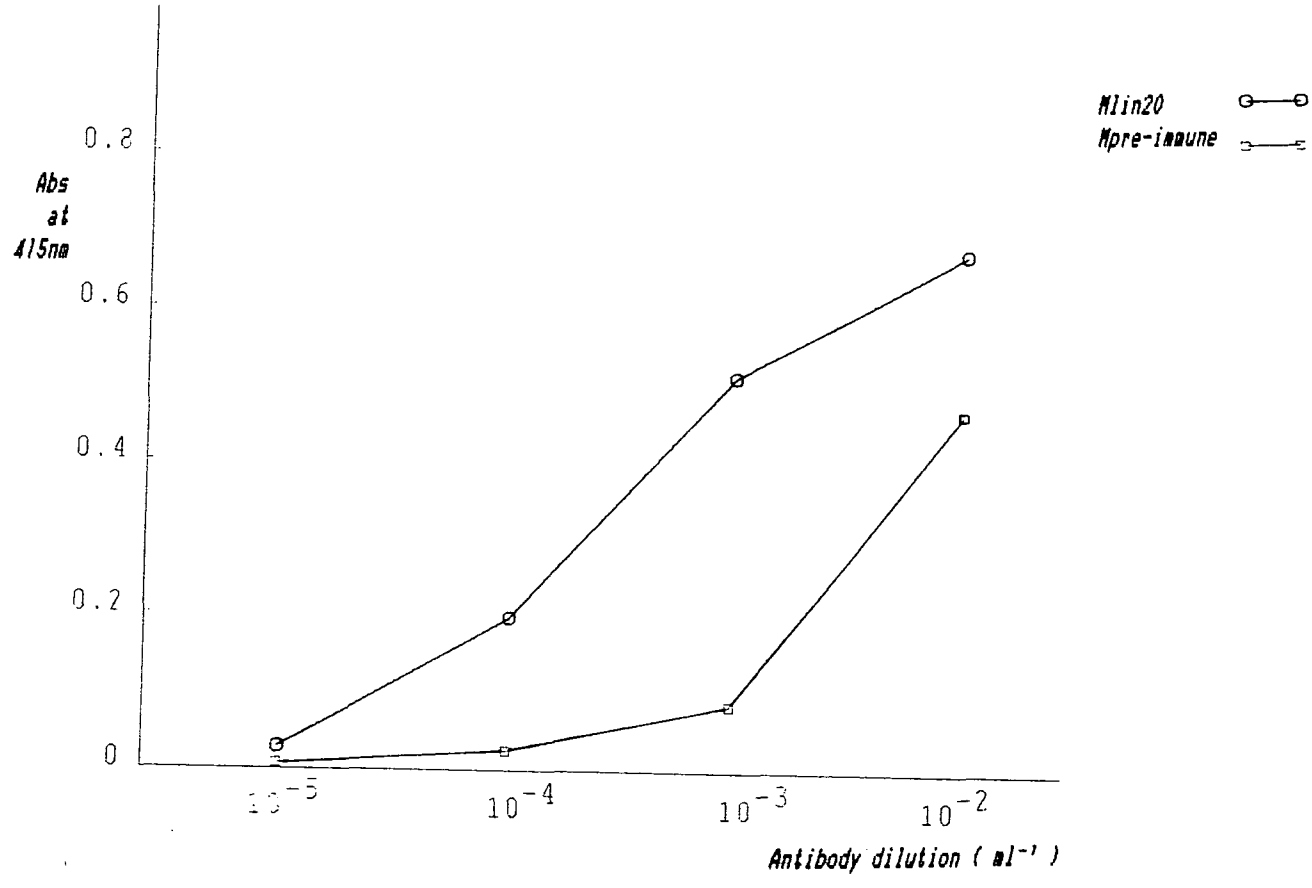


Fig. 13 Rsoy24 BI Titration determination



Fig, 14 Rsoy24 B2 Titration determination



Fig, 15 Mlin20 Titration determination

Table 1. Titres of antisera

Antisera	Titre of first bleed (B1)	Titre of second bleed (B2)
Mnap native	2×10^{-4} (Fig.2)	
Mnap denatured		-
Mrad20	1×10^{-3} (Fig.3)	
Mcra20	$>1\times10^{-2}$ (Fig.4)	
Mmus20	1×10^{-3} (Fig.5)	
Rmus20	1×10^{-6} (Fig.6)	
Msaf19	$>1\times10^{-2}$ (Fig.7)	
Msun19	9×10^{-4} (Fig.8)	
Msun20	9×10^{-4} (Fig.9)	
Rsun20	2×10^{-4} (Fig.10)	7×10^{-4} (Fig.11)
Msoy24	1×10^{-2} (Fig.12)	
Rsoy24	1×10^{-6} (Fig.13)	1×10^{-2} (Fig.14)
Mlin20	$>1\times10^{-2}$ (Fig.15)	

'-': non-detectable

The lower the figure, the more dilute was the antiserum concentration required to produce the appropriate colour response and the higher the amount of IgG in the antiserum.

3.3.0 Cross-reactions

3.3.1 Information from Immunoblotting

The specificity of the existing IgG in an antiserum towards its original antigen could be reviewed using immunoblots. Antisera that recognised a single band from a total seed homogenate indicated they were more specific.

Immunoblots of *Brassiceae* specific antisera are shown in Fig. 17 to 22. The specificity of our antisera varied, Mnap19 was the most specific. The specificity increased in the subsequent bleeds and also increased towards other related species. A summary of the bands that were labelled in immunoblot by various antisera to homogenates of different species is shown in Table 2.

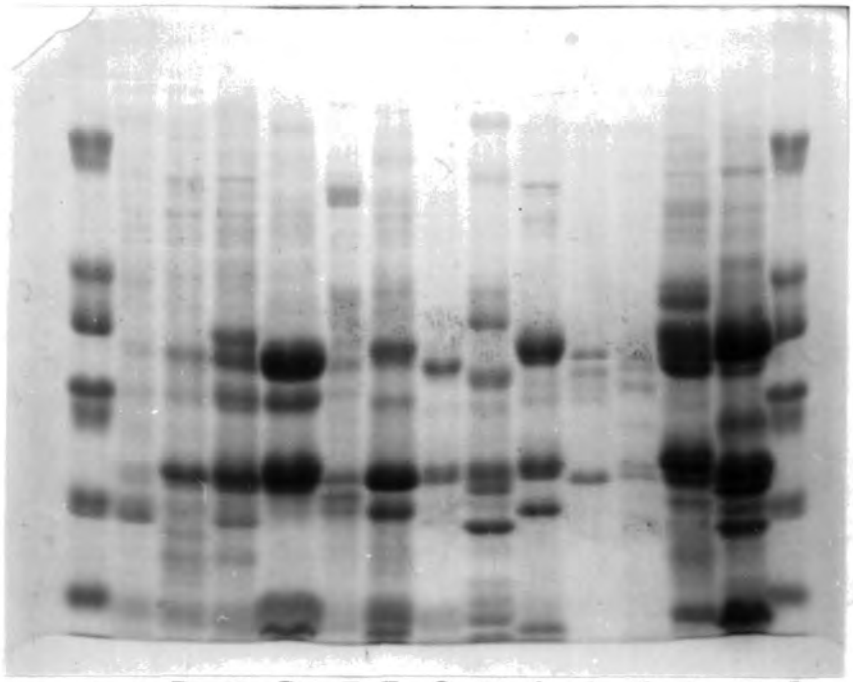
The cross-reactivities between antibodies and different seed homogenates were first tested by immunoblotting. As indicated in Table 2, the antisera Mnap19, Mnap nat, Mrad20, Mmus20, Mcra19 and Rmus20 could cross-react with proteins in all the 15 crucifer tested of which 6 were *Brassiceae*. This left 10 non-crucifer species including seeds from the Leguminosae, Compositae and Graminae families unlabelled. Blots are shown in Fig. 17 to 22. The labelled bands lay between 18kDa to 22kDa. The labelled bands are further indicated to be specifically associated with oil-bodies as shown in Fig.24.

Table 2 Molecular weight of labelled bands on immunoblots of total seed homognates probed with various, antibodies

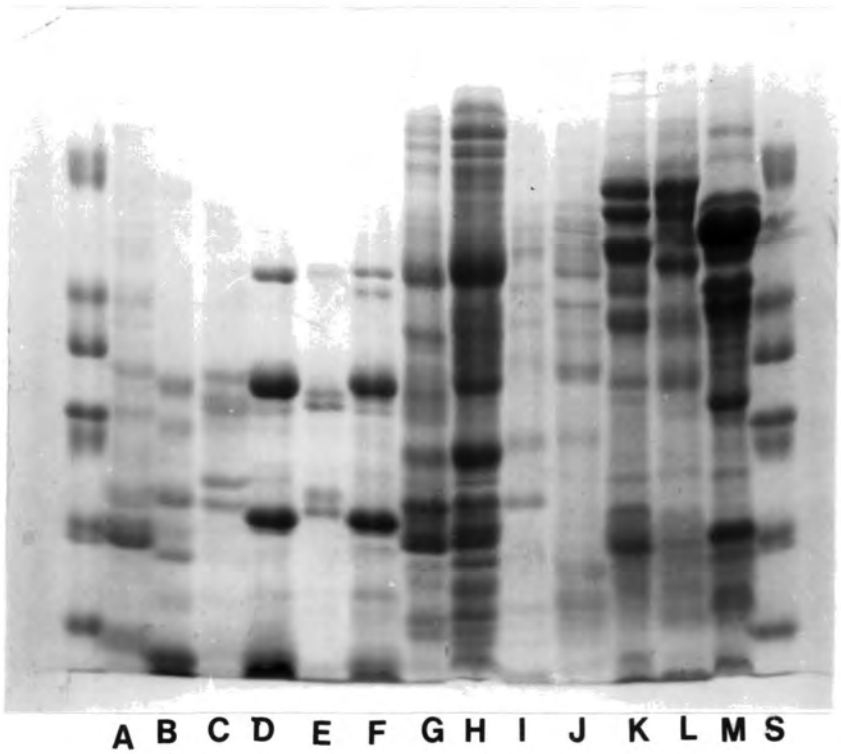
Family	Specie	Antibodies: Cruciferae Mnap	Mrad	Mcra	Mmus	Rmus	Compositae Msun20	Rsun20	Msun19	Msaf	Leguminosae Msoy	Rsoy
	B, napus				20				19	(19)		
Cruciferae		19	19	19	19	19	*	*	*	*	*	*
Brassiceae	*B, oleracea*	19	19	*	19	19	*	*	*	*	*	*
		16	16		16							
	S, alba	20	20	*	20	20	*	*	*	*	*	-
	B, alboglabra	19	19	19	19	19	*	*	*	*	*	*
	B, campestris	19	19	*	19	19	*	*	*	*	*	*
			17		17							
					16							
	R, sativum	20	20	20	20	20	*	*	*	*	*	*
			17									
other	*C, maritima*	19	*	19	*	19	*	*	*	*	*	*
non-	*T, arvense*	21	21	*	*	*	*	*	*	*	*	*
Brassiceae		19	19		19	19						
species	*A, alpina*	19	*	*	*	19	*	*	*	*	*	*
	A, thaliana	19	*	*	*	19	*	*	*	*	*	*
	B, vulgaris	19	*	*	*	19	*	*	*	*	*	*
	C, officinalis	21	21	*	21	21	*	*	*	*	*	*
		19	19									
	C, cheiri	19	19	*	19	19	*	*	*	*	*	*
	I, tinctoria	19	*	*	*	19	*	*	*	*	*	*
	M, bicornis	19	19	*	19	19	*	*	*	*	*	*
			17		17							
	Helianthus annuus	-	*	*	*	*	32	*	32	*	-	-
Compositae							20	20				
							19	19	19			
								17	17			
	C, tinctorius	-	*	*	*	*	-	-	20	*	*	24
	A, absinthium	-	*	*	*	*	*	*	20	*	*	*
Leguminosae	*Glycine max*	-	*	*	*	-	-	*	*	*	24	24
	P, sativum	-	*	*	*	-	*	*	*	*	-	24
	P, sativum JI827	-	*	*	*	-	*	*	*	*	-	24
	L, albus	-	*	*	*	*	*	*	*	*	-	24
	L, arborseus	-	*	*	*	*	*	*	*	*	-	24
	L, nanus	-	*	*	*	-	*	*	*	*	-	24
Resedaceae	*Reseda lutea*	-	*	*	*	-	*	*	*	*	*	-

'-'; no cross-reaction; '*'; not tested,
see Glossary on p,109 for full systematic names and common names,

i .



i i .



Fig, 16 Total Homogenates on SDS/PAGE gel, See key of Fig,17 i, and ii,

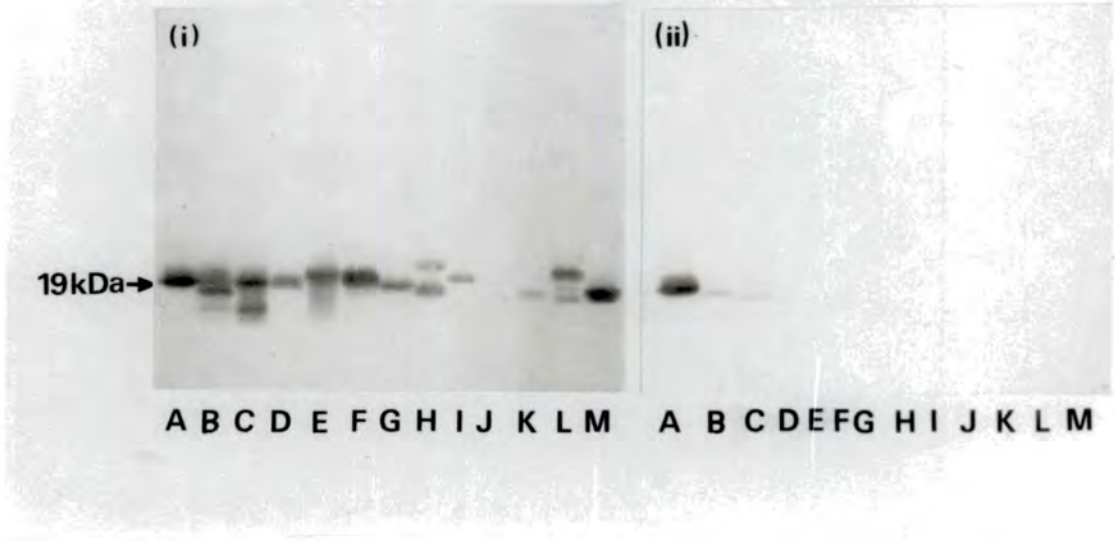
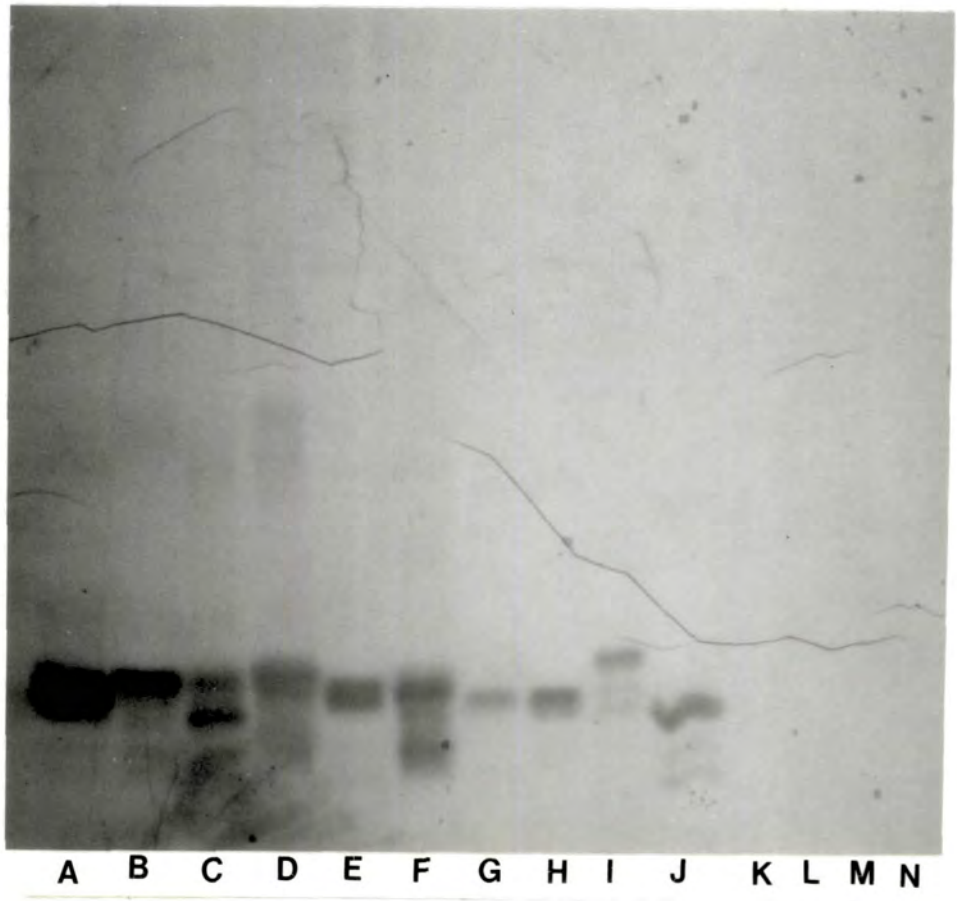


Fig. 17 Immunoblotting on Total Homogenates of Fig.16 using Mnap19

Key for Fig.16 and Fig.17;

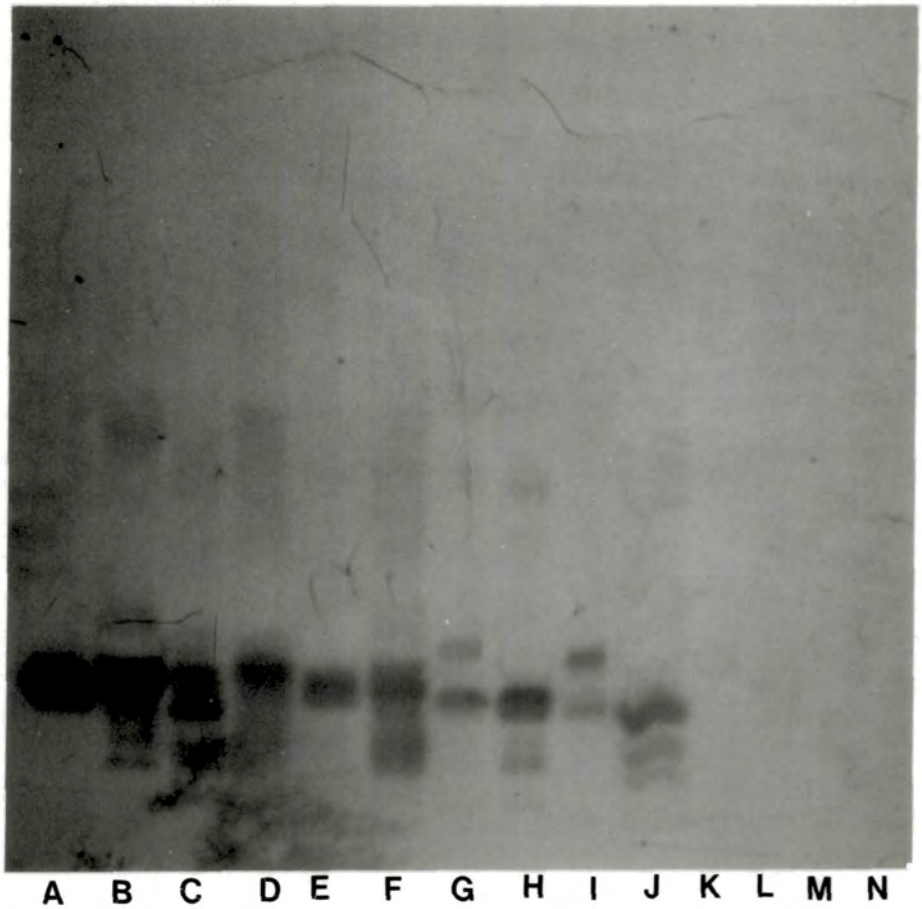
i, A, *Brassica napus* B, *Brassica oleracea* C, *Brassica campestris* D, *Brassica alboglabra*
E, *Sinapis alba* F, *Raphanus sativus* G, *Crambe maritima*
H, *Thlaspi arvense* I, *Arabis alpina* J, *Arabidopsis thaliana* K, *Berberis vulgaris*
L, *Cochlearia officinalis* M, *Cheiranthus cheiri*

ii, A, *Brassica napus* B, *Isatis tinctoria* C, *Matthiola bicornis* D, *Reseda lutea*
E, *Reseda odorata* F, *Reseda odorata* G, *Pisum sativum* H, *Pisum sativum JI827* I, *Zea mays*
J, *Triticum durum* K, *Lupinus nanus* L, *Lupinus arboreus* M, *Lupinus albus*
S, standard markers; 66kDa, 45kDa, 36kDa, 29kDa, 24kDa, 20,1kDa and 14,2kDa respectively.

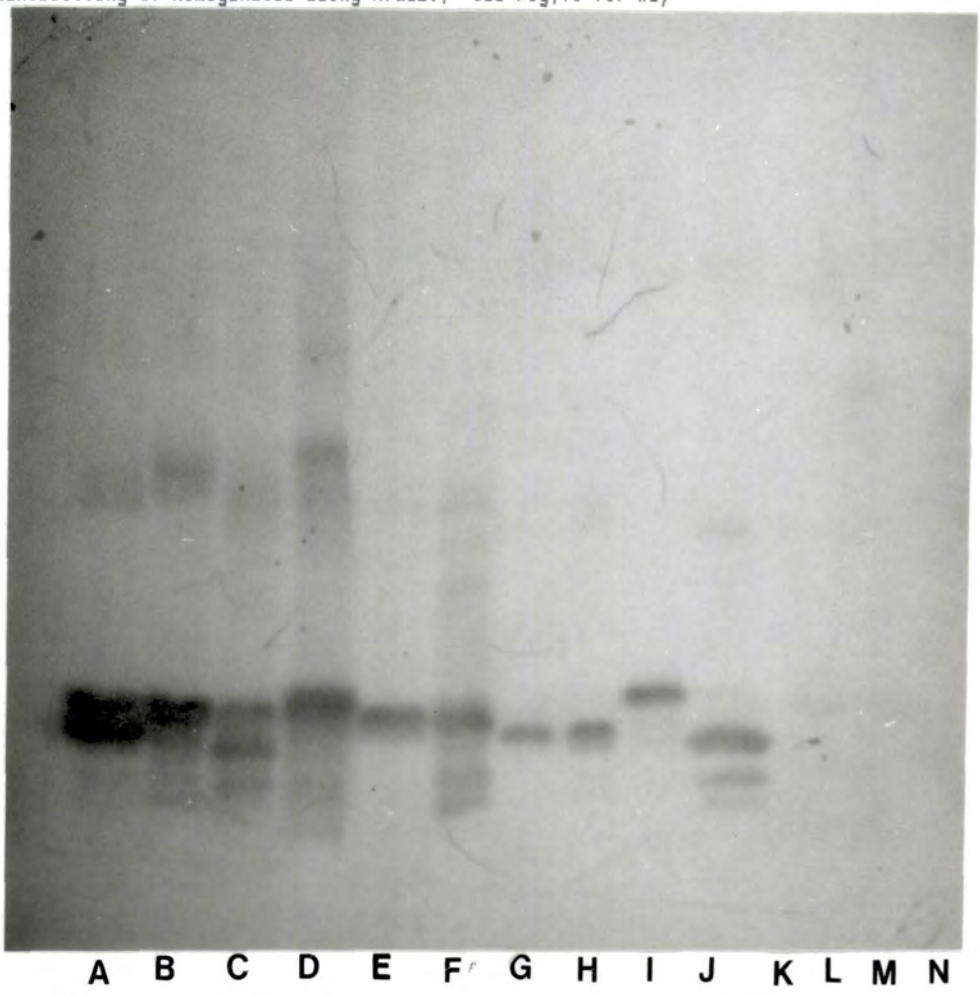


Fig, 18 Immunoblotting of Homogenates using Mnap native

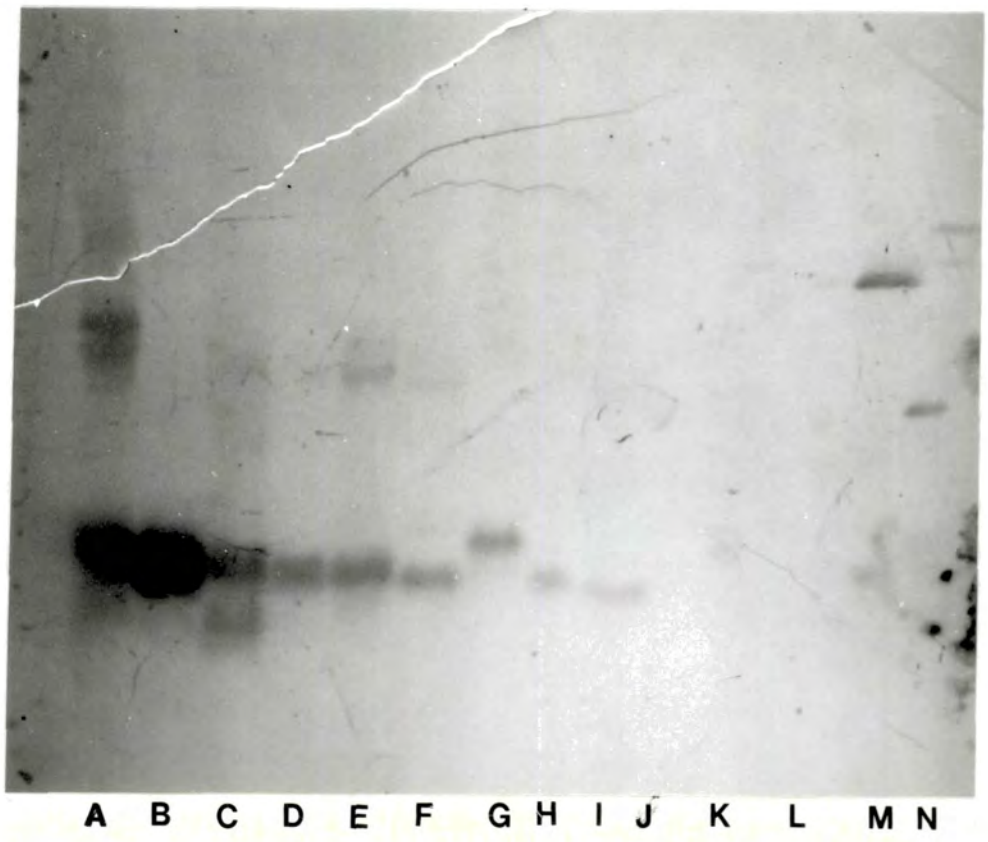
Key for Fig, 18 - 20 ;
A, *Brassica napus* B, *Raphanus sativus* C, *Brassica oleracea* D, *Sinapis alba*
E, *Brassica alboglabra* F, *Brassica campestris* G, *Thlaspi arvense*
H, *Cheiranthus cheiri* I, *Cochlearia officinalis* J, *Matthiola bicornis* K, *Reseda lutea*
L, *Lupinus nanus* M, *Pisum sativum JI827* N, *Zea mays* S, standard markers



Fig, 19 Immunoblotting of Homogenates using Mrad20, See Fig,18 for key

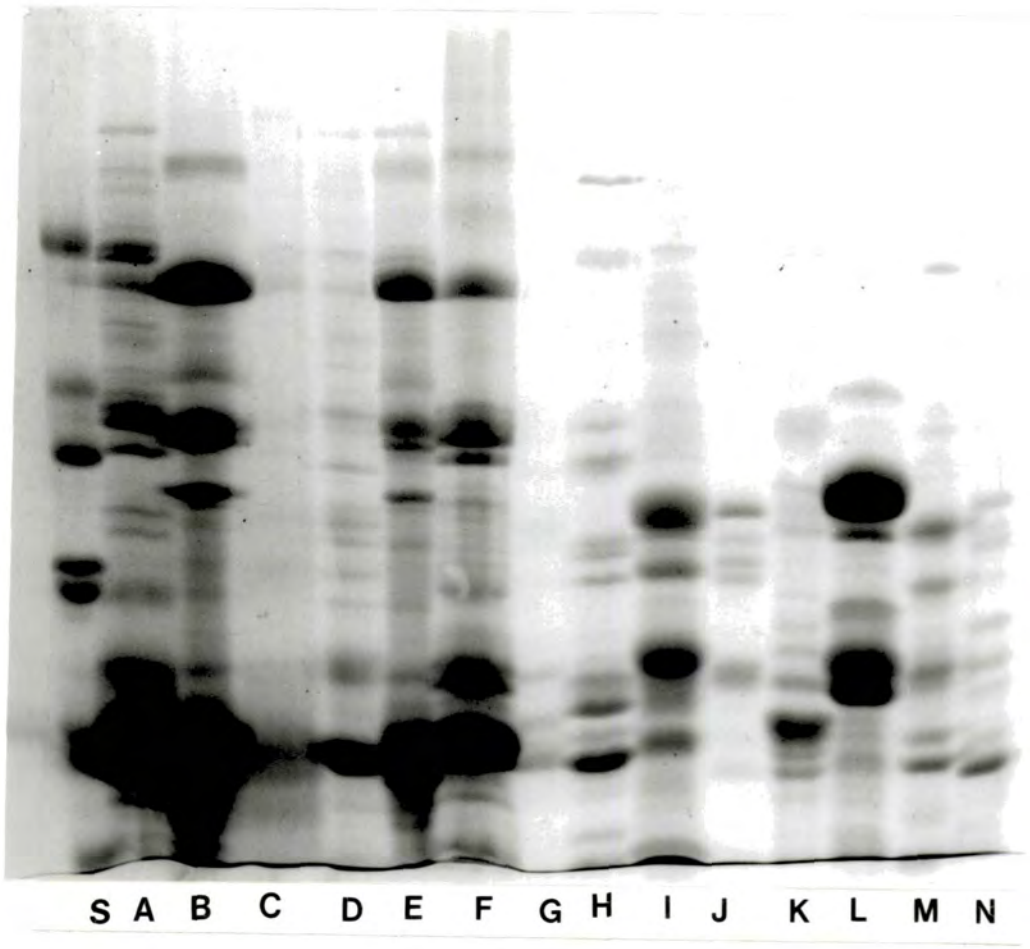


Fig, 20 Immunoblotting of Homogenates using Mmus, See Fig,18 for key

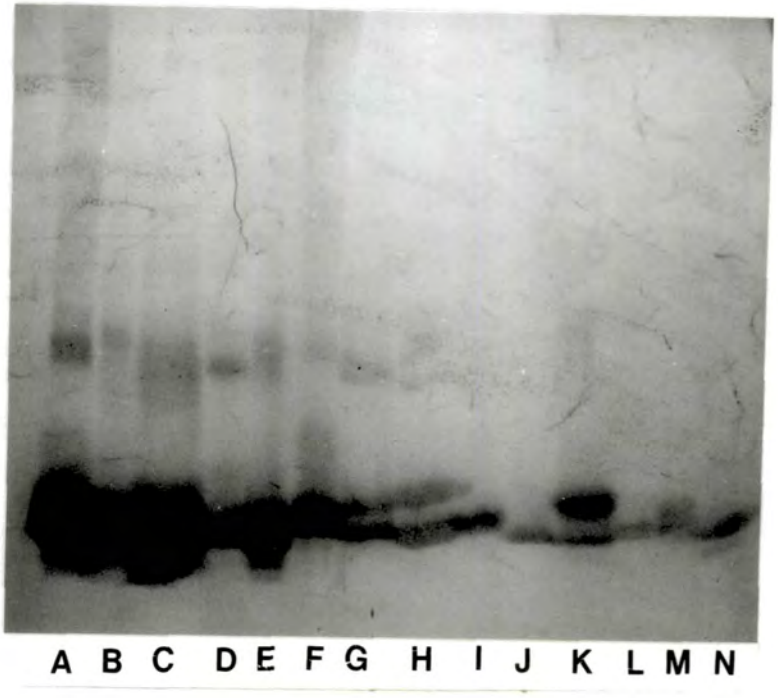


Fig, 21 Immunoblotting of Homogenates using Rmus, A, *Sinapis alba* B, nap19 C, *Brassica campestris* D, *B,alboglabra* E, *Crambe maritima* F, *Thlaspi arvense* G, *Cochlearia officinalis* H, *Cheiranthus cheiri* I, *Matthiola bicornis* J, *Reseda lutea* K, *Pisum sativum JI827* L, *Lupinus nanus* M, *Zea mays* N, *Triticum durum* S, standard markers

Fig, 22 Immunoblotting of Seed Fractions using Mcra20, A, nap19 B, *Brassica napus* oil-body(OB) C, *B,alboglabra* Homogenate (Homo) D, *Crambe maritima* Homo E, *C,maritima* OB F, *C,cheiri* Homo G, *Z,mays* Homo S, standard markers



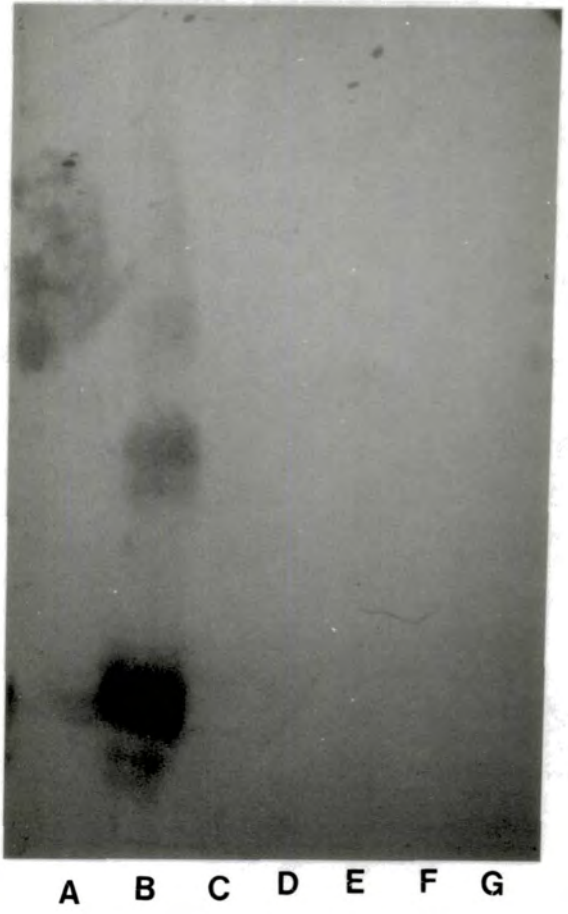
Fig, 23 Oil-body SDS/PAGE gel of Crucifers



Fig, 24 Immunoblotting of Oil-bodies using Mnap19

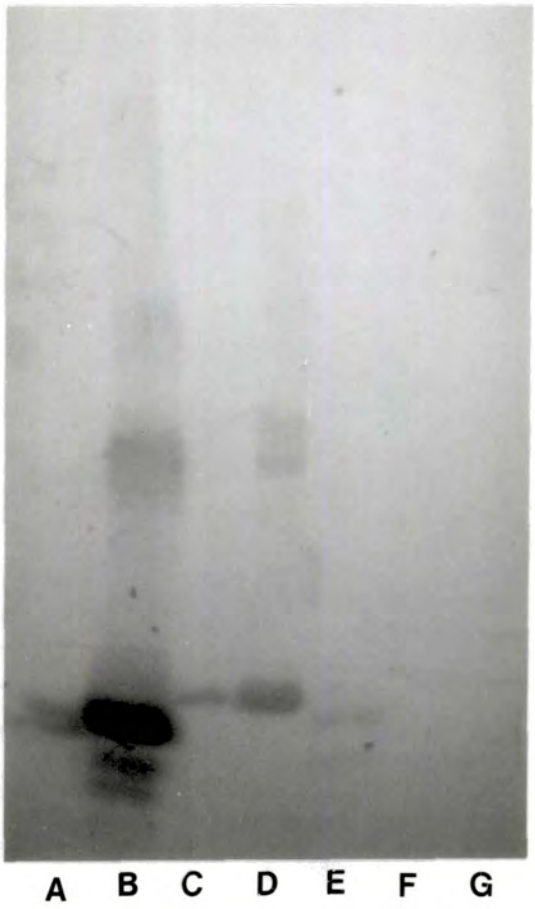
Key for Fig, 23 - 24;

A, *Brassica napus* B, *B,oleracea* C, *B,campestris* D, *B,alboglabra* E, *Sinapis alba*
F, *Raphanus sativus* G, *Crambe maritima* H, *Thlaspi arvense* I, *Arabis alpina*
J, *Arabodipsis thaliana* K, *Cochlearia officinalis* L, *Cheiranthus cheiri* M, *Isatis tinctoria*
N, *Matthiola bicornis* S, standard markers

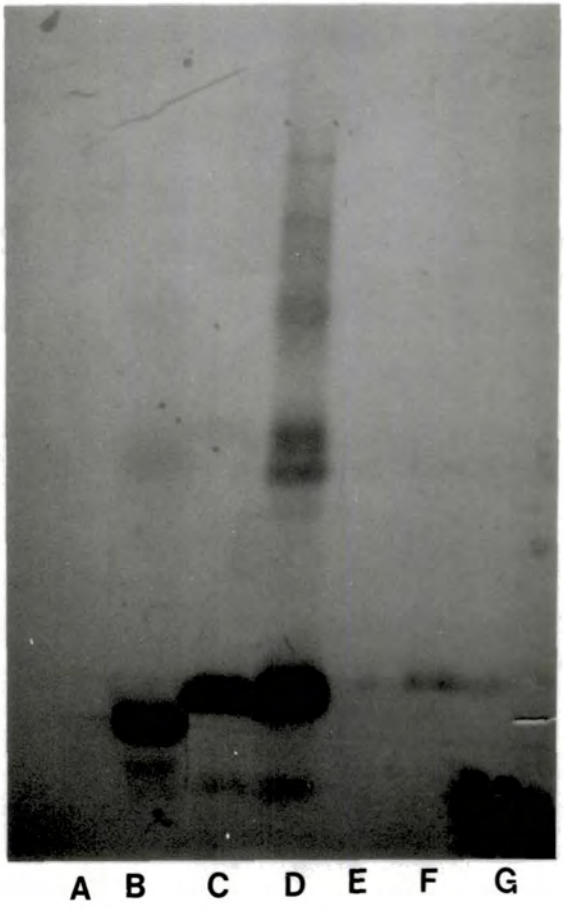


Fig, 25 Immunoblotting of Seed Fractions using Rsun20 B2, Key for Fig,25-28; A, *Helianthus annuus* Homogenate(Homo) B, *H,annuus* oil-body(OB) C, *Carthamus tinctorius* Homo D, *C,tinctorius* OB E, *Artemisia absinthium* Homo F, *Brassica napus* Homo G, *Zea mays* Homo

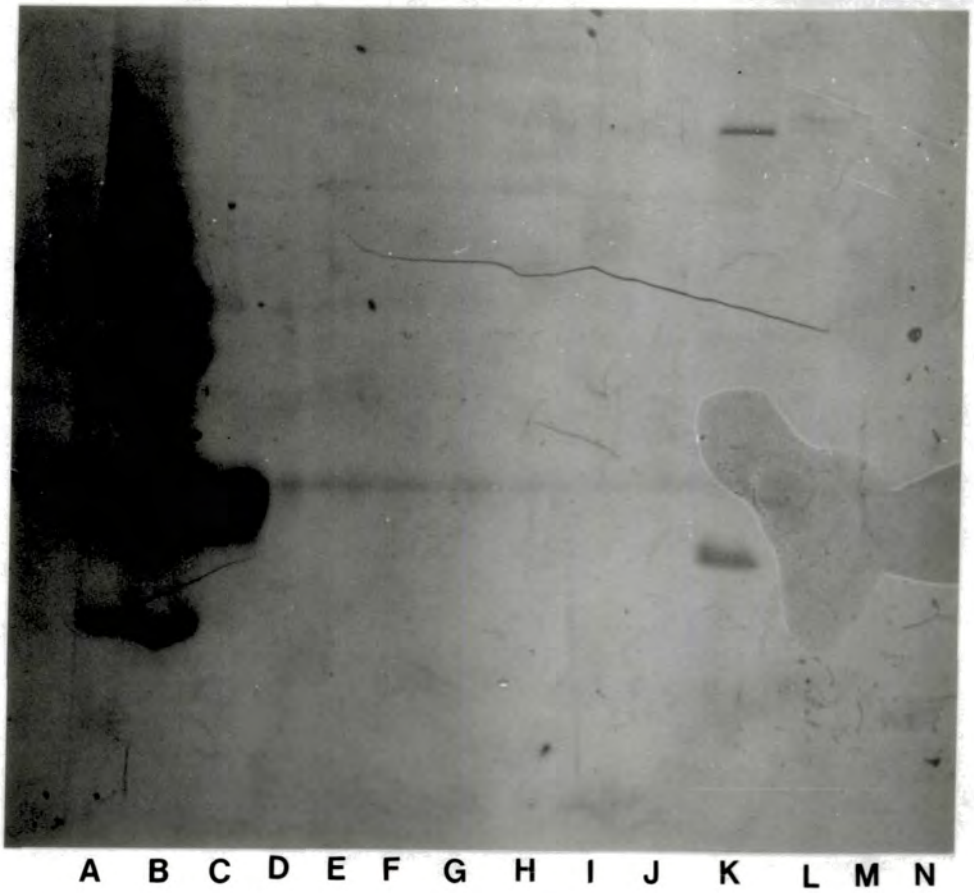
Fig, 26 Immunoblotting of Seed Fractions using Msun20, See Fig,25 for key



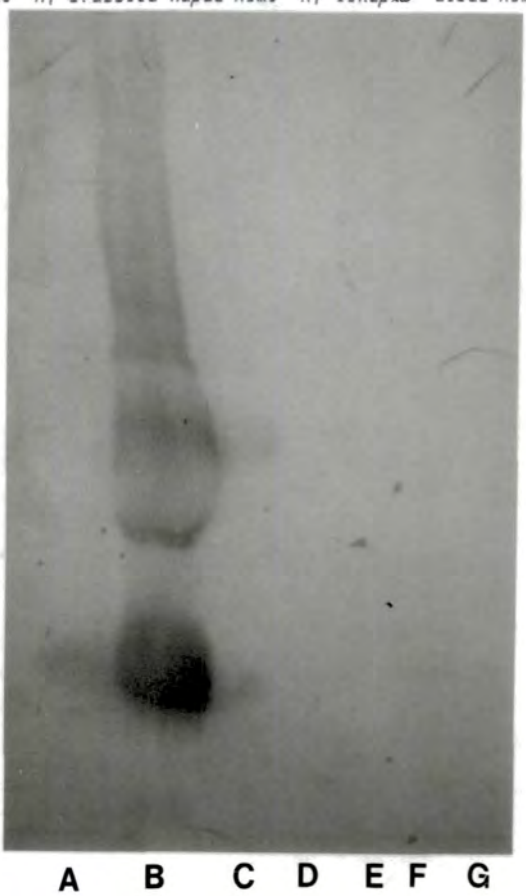
Fig, 27 Immunoblotting of Seed Fractions using Msun19



Fig, 28 Immunoblotting of Seed Fractions using Msaf20

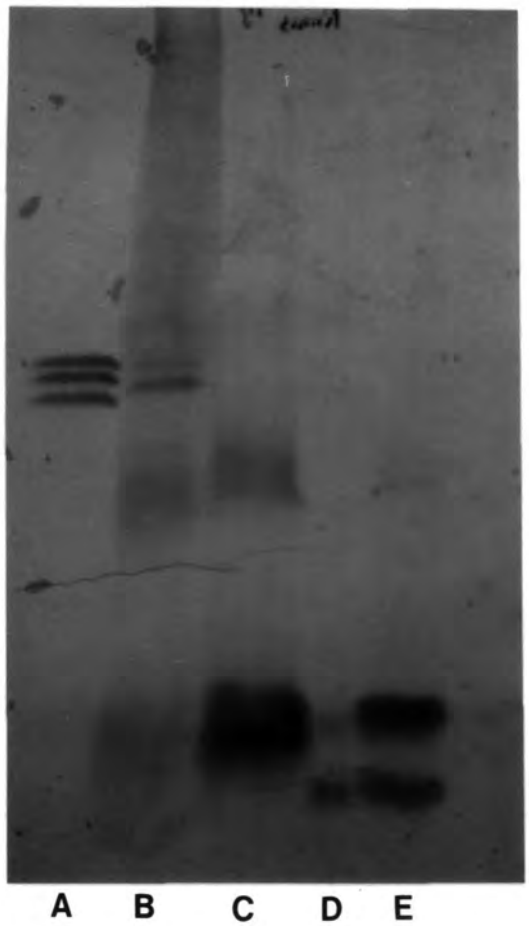


Fig, 29 Immunoblotting of Seed Fractions, using Rsoy B1, A, soyHomo B, soyOB C, soy24
D, *Lupinus albus* Homogenate(Homo) E, *L,arboreus* Homo F, *L,nanus* Homo G, *Pisum sativum* Homo
H, *P,sativum JI827* Homo I, *Zea mays Homo J, Reseda lutea* Homo K, *Carthamus tinctorius* Homo
L, *Helianthus annuus* Homo M, *Brassica napus* Homo N, *Sinapis albus* Homo



Fig,30 Immunoblotting of Seed Fractions using Msoy24, A, soyHomo B, soyOB C, *Pisum sativum JI827*
Homogenate(Homo) D, *Lupinus albus* Homo E, linseed Homo F, linseed Oil-body G, *Brassica napus* Homo

Fig, 31 Immunoblotting of oil-bodies of A, *Sinapis alba* B, *Zea mays* C, *Triticum durum* using Rmus20



Fig, 32 Immunoblotting of Seed Fractions using Rmus20, Key for Fig,32-35; A, *Zea mays* Homogenate(Homo)
B, *Z,mays* Oil-body(OB) C, *Z,mays* 19kDa D, *Nicotiana tobacum Wiscosin 38* Homo
E, *N,tobacum Wiscosin 38* OB

Fig, 33 Immunoblotting of Seed Fractions using Mnap19, See Fig,32 for key

Fig, 34 Immunoblotting of Seed Fractions using Mrad20, See Fig,32 for key

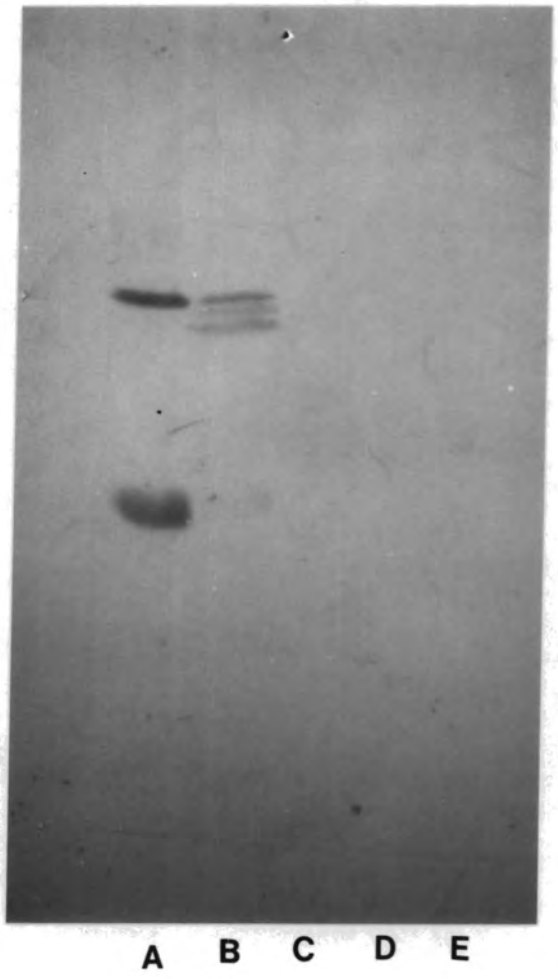


Fig. 35 Immunoblotting of Seed Fractions using Rsun20. See Fig.32 for key

The antisera, Msun19, Msun20, Rsun20, Msaf19 showed positive immunoblots with homogenates of seeds from members of Compositae family including sunflower (both sun19 and sun20 were labelled at the same time), safflower (19kDa) and *Artimisia absinthium* (19kDa). Immunoblots are shown in Fig. 25 to 28.

The antiserum Rsoy B1 showed positive results on legumes at about 24kDa. The immunblottings are shown in Fig. 29 to 30. However, in the blots of Msoy24 Fig. 30 and the second bleed of Rsoy24 B2, only soybean homogenate proteins were recognised, but,not those of other species.

Some non-specific inter-family cross-reactivity was observed in immunoblottings with the antisera Rmus20 (Fig.19,29,31,32), Mnap19 (Fig.33), Mrad20 (Fig.34), Rsun20 (Fig. 26), Msaf (Fig. 28), and Rsoy24 (Fig.29). All of them except Rsoy24 were able to cross-react with monocots while Rsoy24 B1 also cross-reacted with safflower. Results are shown in Table 3 .

Table 3 Inter-family cross-reactivity

Ab	Molecular weight of labelled bands (kDa) *Z.mays*	*T.durum*	*N.tobaccum Wiscosin 38*	safflower
Rmus20	45, 43, 40, 19	46, 42, 34	19, 14	-
Rsun20	47, 45, 36	-	-	-
Msaf20	19			
Mnap19	19	-	19, 14	-
Mrad20	50,	-	-	*
Rnap19	-	-	-	-
Mnapnat	-	-	-	*
Mmus20	-	-	-	*
Rsoy24	-	-	*	24

key: underlined m.w. indicate also present in oil-body preparation
'-': no cross-reaction; '*': not tested.

The cross-reactivity was not entirely oil-body membrane protein specific but the majority of antibodies-labelled were from oil-body proteins. The labelled band from tobacco seed homogenates were particularly specific to oil-body membrane proteins.

3.3.2 Information from ELISA

Five *Brassiceae* species and one legume were selected for cross-reactivity tests with Mnap19, Rnap19 and Rmus20 by ELISA. All brassica gave positive results and showed sigmoid curve patterns (Fig. 36, 37, 40). Immunoassays that used Rnap19 and Mnap19 in a sandwich format also gave sigmoid curve patterns but the responses were delayed and lowered (Fig. 38 to 39). The cross-reactivity graph of Rsoy24 is shown in Fig. 49. The degree of cross-reactivity was expressed as a percentage by comparing the amount of antibodies required so as to obtain equal reaction to the native antigens at 50% cross-reaction. The results are shown in table 4.

Table 4 Percentage of Cross-reactions:

	Antibodies					
	Mnap	Rnap	Rmus	Rsoy	Rnap + 0.1μg/μl	Mnap 1μg/μl
Proteins-Ag						
nap19	100%	100%	112.0%		100%	100%
cam19	103	62	42.9		<.0001	↑
cam16	103	50	75.7		67.5	|
ole19	33	29	147.4		52.1	|
mus20	92.5	82	100		↑	<.0001
rad20	148	64	80		<.0001	|
soy24	←-------	<.0001	-------→		↓	↓
soyHomo				100%		
safHomo				56.7		
safOB				63		

The general trends in decending order of cross-reactivity were as follows:

Mnap	rad20> cam19> cam16> nap19> mus20> ole19
Rnap	nap19> mus20> rad20> cam20> cam16> ole19
Rmus	ole19> nap19> mus20> rad20> cam16> cam20
Rnap0.1-Mnap	nap19> cam16> ole19
Rnap1.0-Mnap	nap19
Rsoy	soyHomo> SafOB> SafHomo

The ability of antibodies to cross-react with other proteins was also indicated by immunocytochemical EM as shown in Fig. 42 to Fig. 43. Thin sections of rapeseed, radish and mustard seeds were specifically labelled on the oil-body

membranes by an antibody to the rapeseed 19kDa oil-body protein. For maize, Fig. 43 A, proteins other than those of the oil-body mebrane were also labelled although to a lesser extent. Tobacco seed, Fig. 43 B, had a lot of labels and some of them were believed to be non-specific.

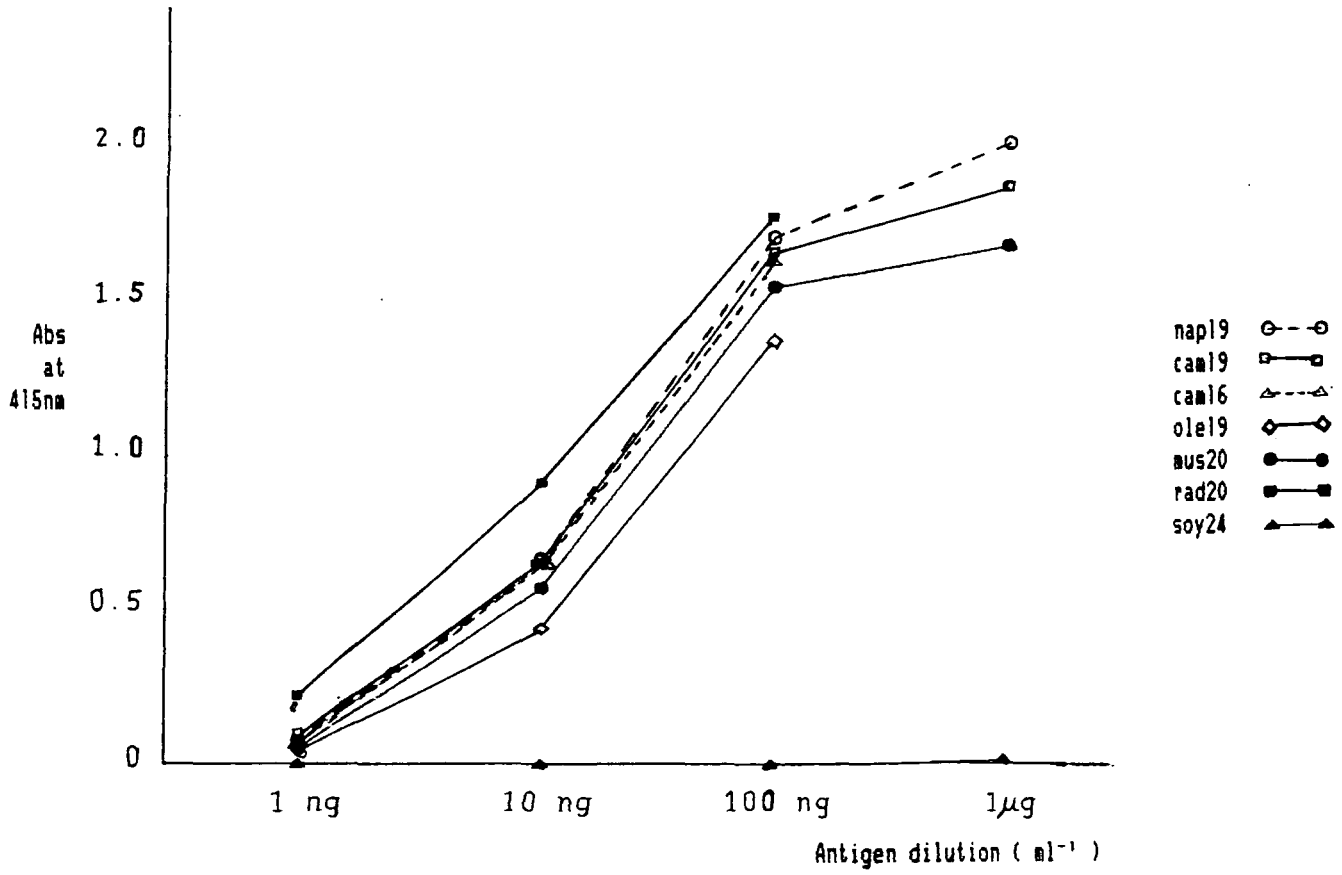


Fig. 36 Mnapl9 ELISA Cross-reaction

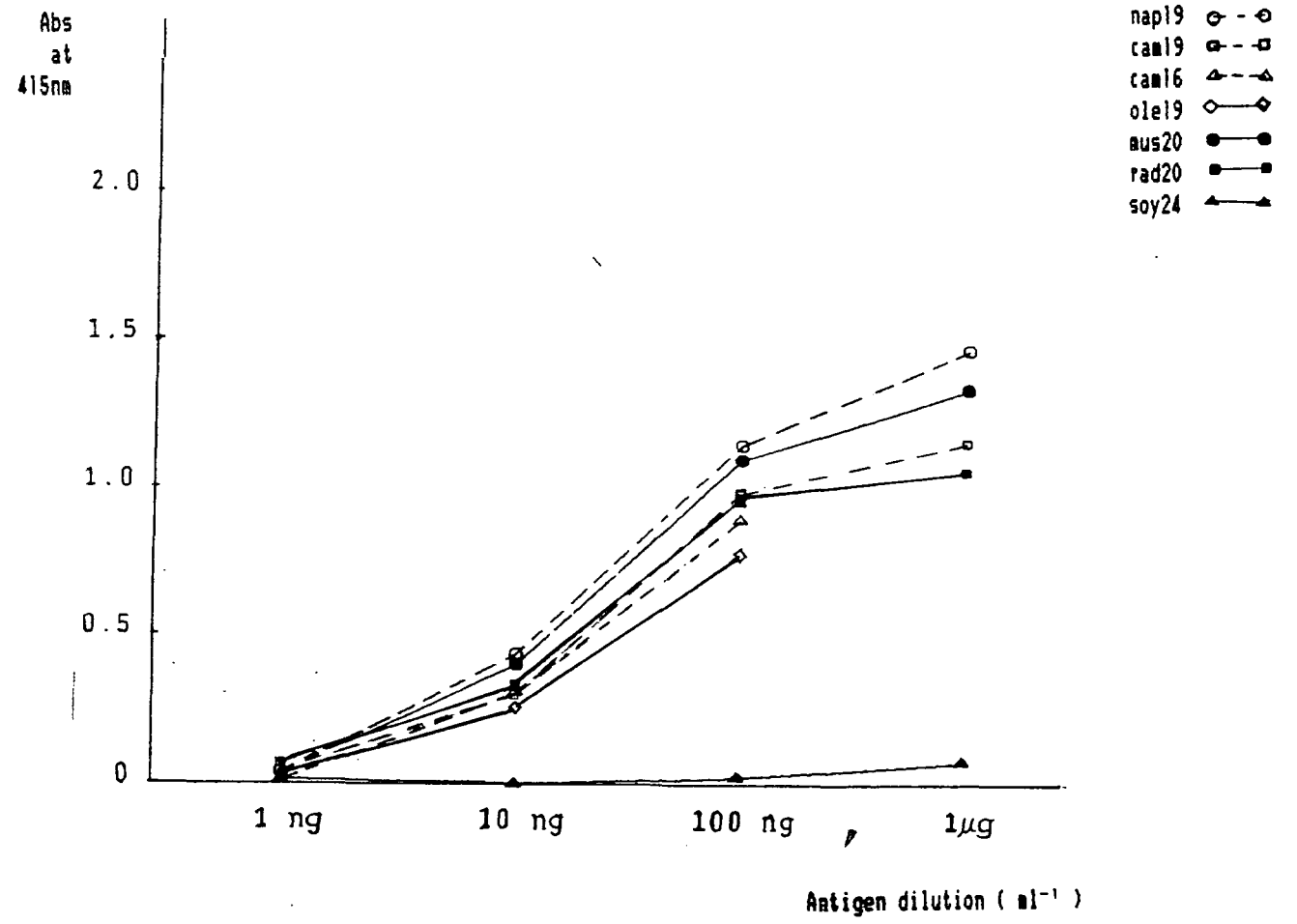


Fig. 37 Rnapl9 ELISA Cross-reaction

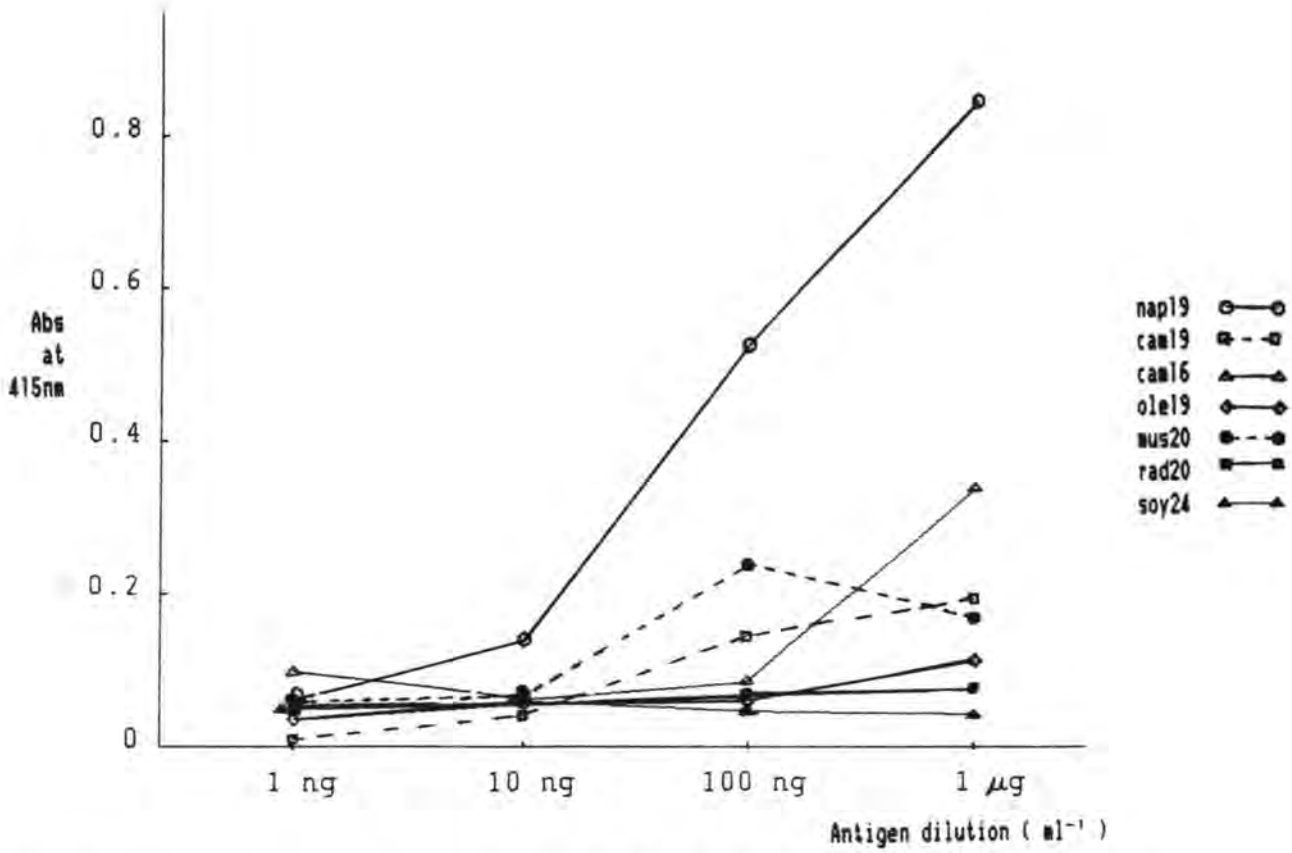


Fig. 38 Cross-reaction of Immunometric assay using Rnapl9 (1μg ml^{-1}) and Mnapl9 (1/10000 dilution)

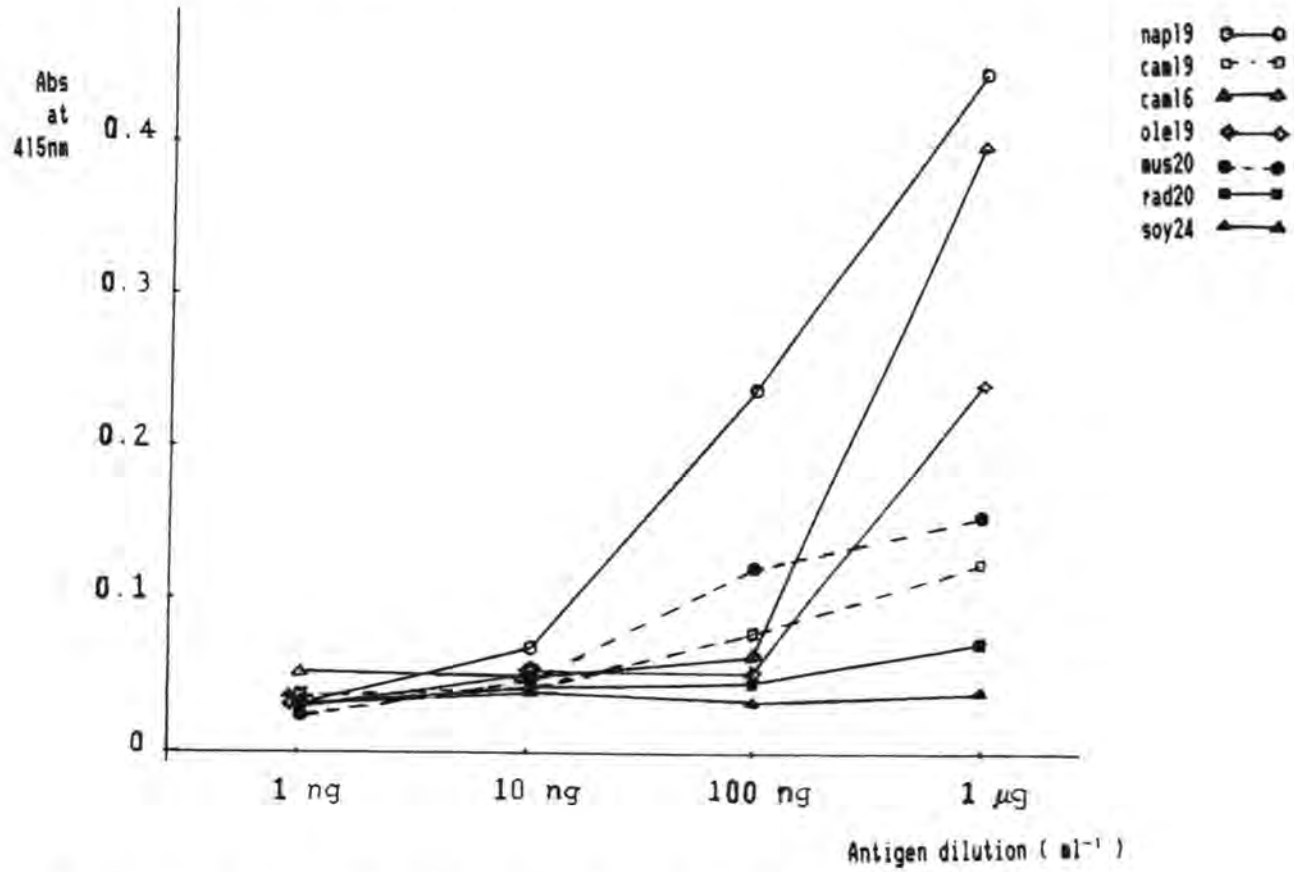


Fig. 39 Cross-reaction of Immunometric assay using Rnapl9 (0.1μg ml^{-1}) and Mnapl9 (1/10000 dilution)

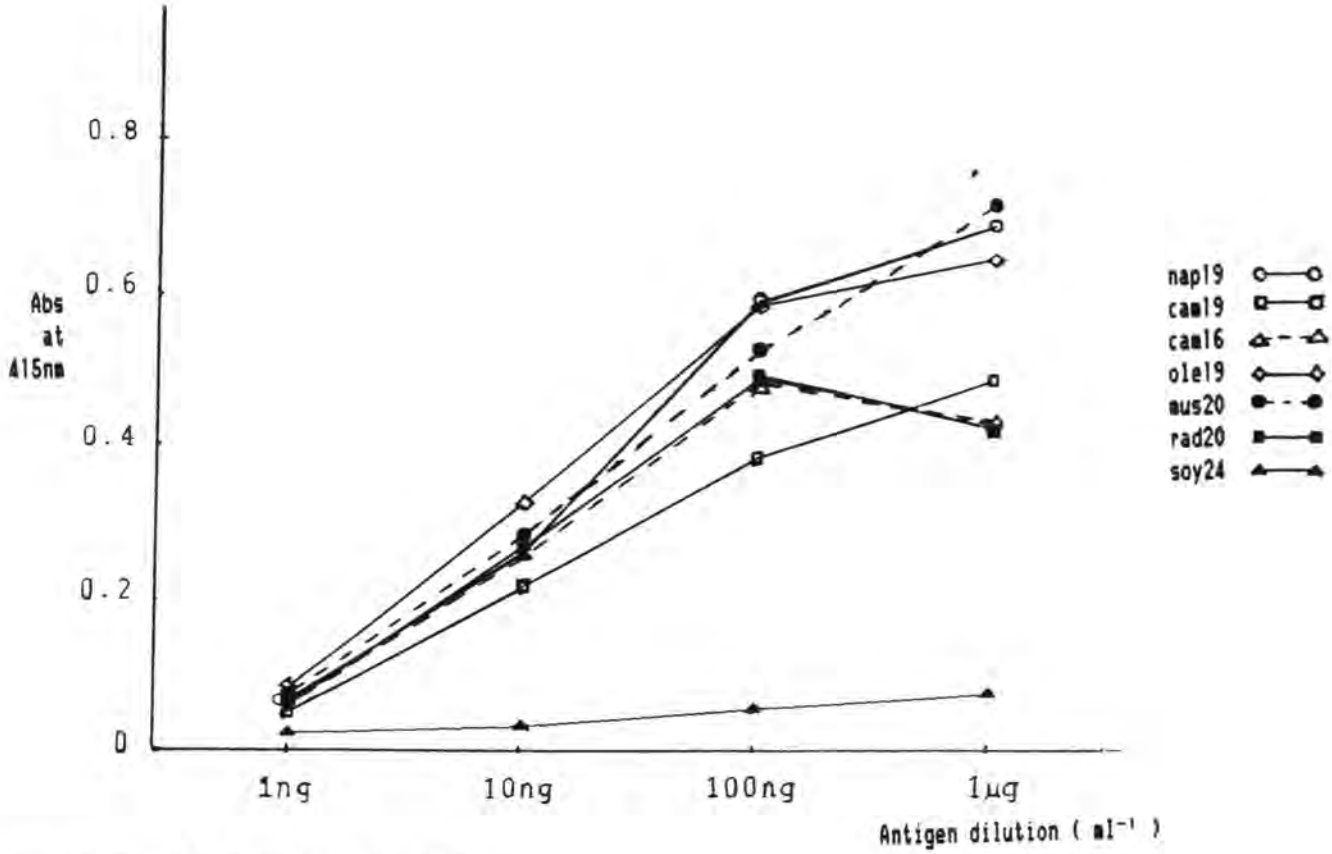


Fig. 40 Raus20 B1 ELISA Cross-reaction

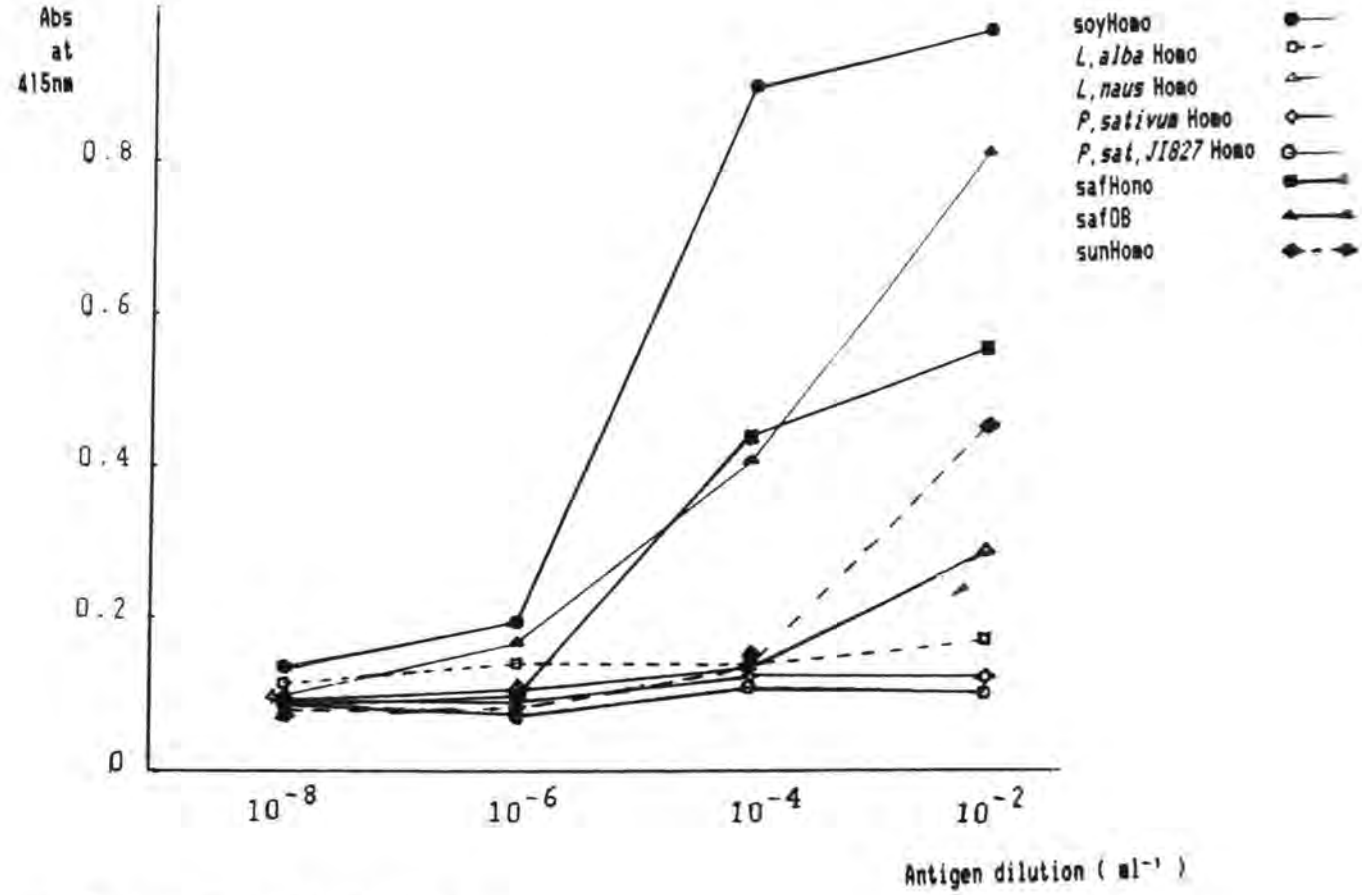


Fig. 41 Rsoy B1 ELISA Cross-reaction

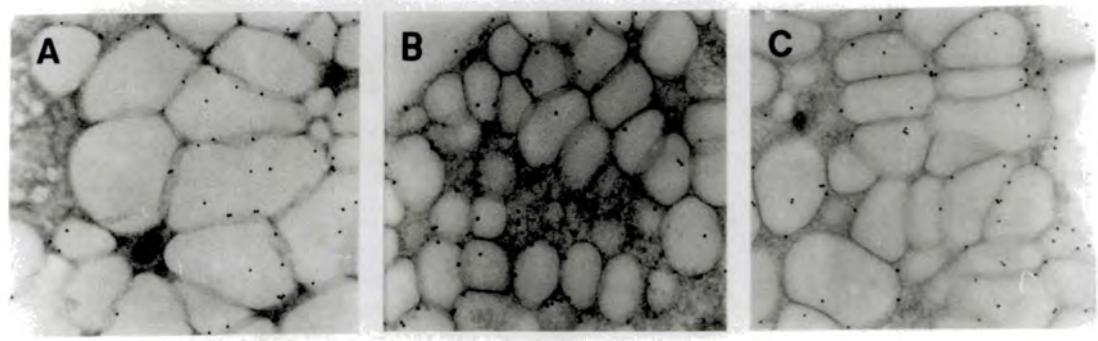


Fig. 42 Immunogold labelling of ultra thin sections of seeds using 1/100 dilution Mnap19;
A, rapeseed B, mustard C, radish
[Thanks Ian Cummins for preparing and immunogold labelling the above sections]

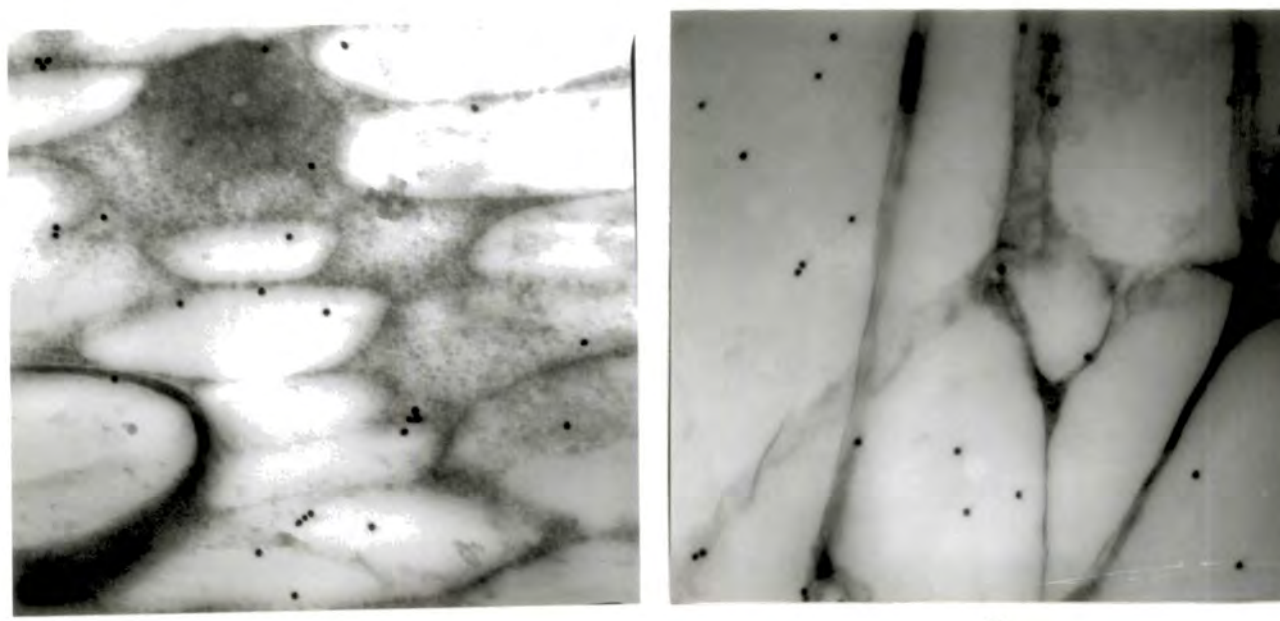


Fig. 43 Immunogold labelling of ultra thin sections of seeds using 1/200 dilution Rmus20;
A, maize (55,000x) B, tobacco (55,000x)

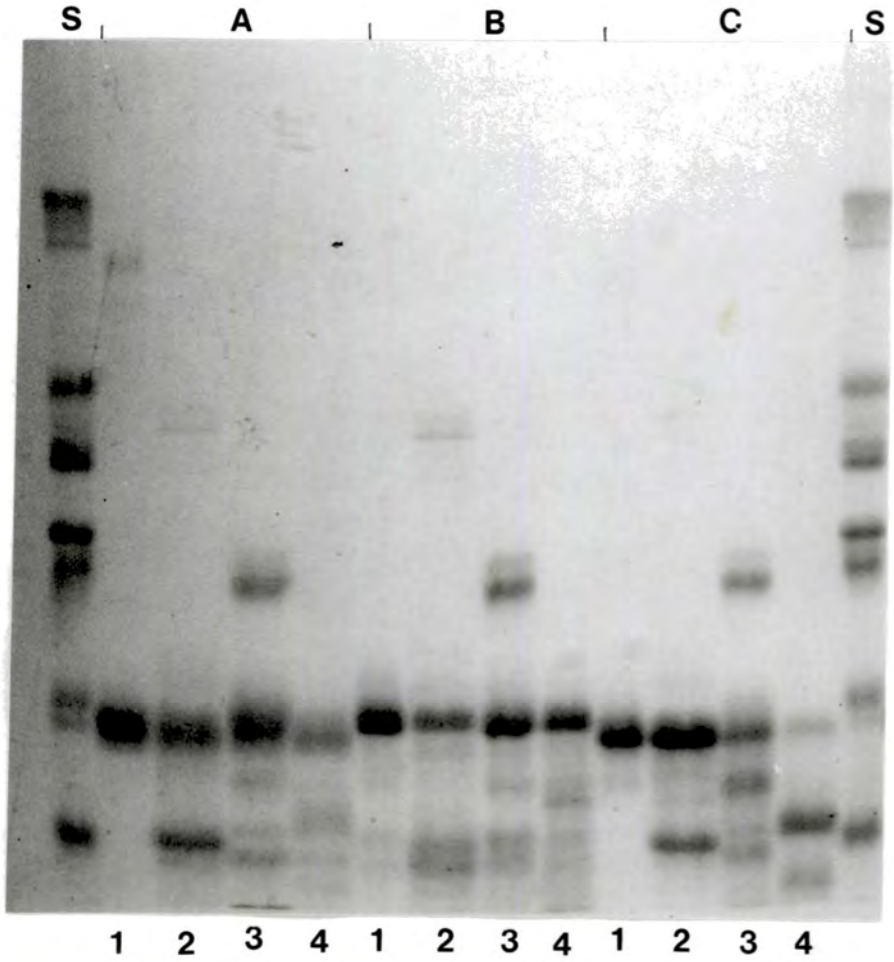
3.4.0 Peptide Mapping

The proteolytic maps of protein being digested with V8, trypsin and lys-C are shown in Fig 44 to 45. The peptide mapping of antibody to the Western blot of such maps are shown in Fig. 46. Major bands that could be observed after proteolysis and that remained immunogenic are summarized in Table 5.

Table 5 Summary of the major peptide fragments and immunogenic peptide fragments following proteolytic of purified oil-body proteins

Enzyme	Proteins	major peptides (kDa)	immunogenic peptides (kDa)
V8	nap19	16, 14	16, 14
	ole19	16, 14	16, 14
	cam19	16, 14	16, 14
	cam16	14	-
	rad20	17, 14	17
	mus20	16, 14	16, 14
Lys-C	nap19	15, 13	15
	ole19	15, 13	15
	cam19	15, 13	15
	cam16	13	-
	rad20	16, 14	16
	mus20	16, 14	16, 14
Trypsin	nap19		15
	ole19		15
	cam19		17
	cam16		-
	rad20		16
	mus20		15

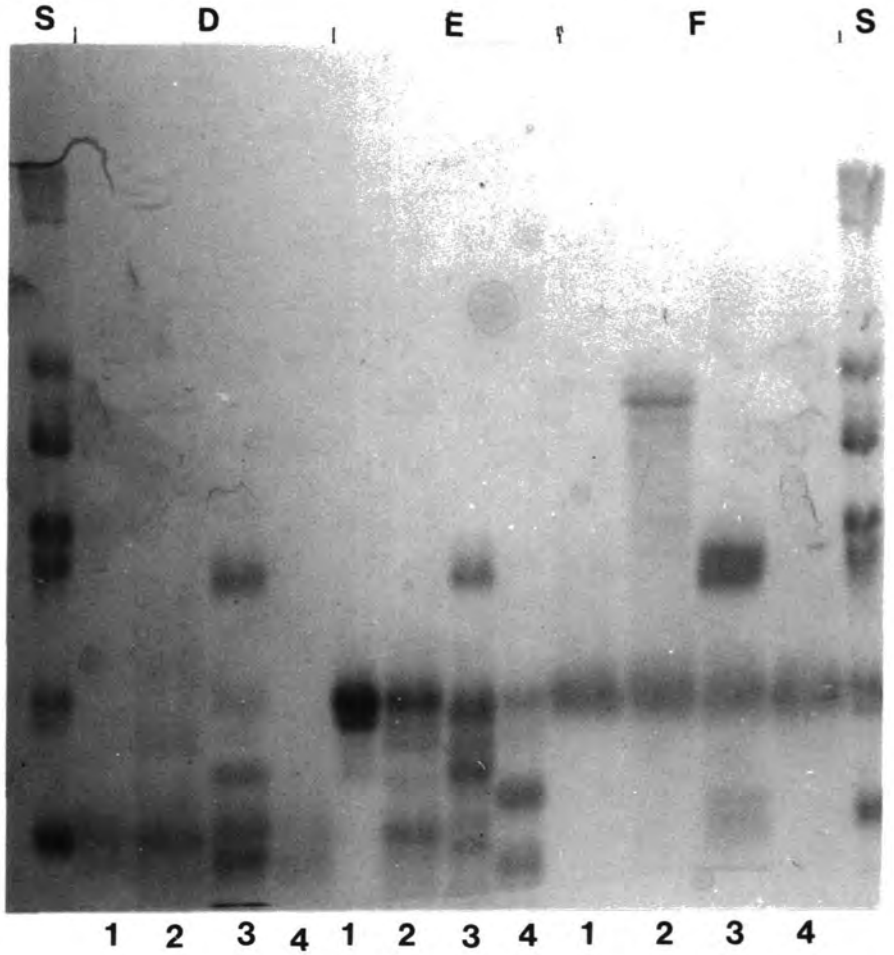
'-': no detectable peptide
Remark: The above molecular weights are determined based on several gels which may not included in the figures.



Fig, 44a, Proteolytic Maps of Denatured Proteins on SDS/PAGE of nap19, rad20 and cam19 using *S,aureus V8* protease (V8), Trypsin (Try) and endoprotease *Lysobacter* Lys-C (Lys);

A	B	C
1, nap19 control	1, rad20 control	1, cam19 control
2, nap19 + V8	2, rad20 + V8	2, cam19 + V8
3, nap19 + Try	3, rad20 + Try	3, cam19 + Try
4, nap19 + Lys	4, rad20 + Lys	4, cam19 + Lys

S, standard markers



Fig, 44b, Proteolytic Maps of Denatured Proteins on SDS/PAGE of caml6, mus20 and olel9 using *S,aureus V8* protease (V8), Trypsin (Try) and endoprotease *Lysobacter* Lys-C (Lys);

D	E	F
1, caml6 control	1, mus20 control	1, olel9 control
2, caml6 + V8	2, mus20 + V8	2, olel9 + V8
3, caml6 + Try	3, mus20 + Try	3, olel9 + Try
4, caml6 + Lys	4, mus20 + Lys	4, olel9 + Lys

S, standard markers

Remark; olel9 remains inert towards proteolysis, Digested olel9 is shown in Fig,45,

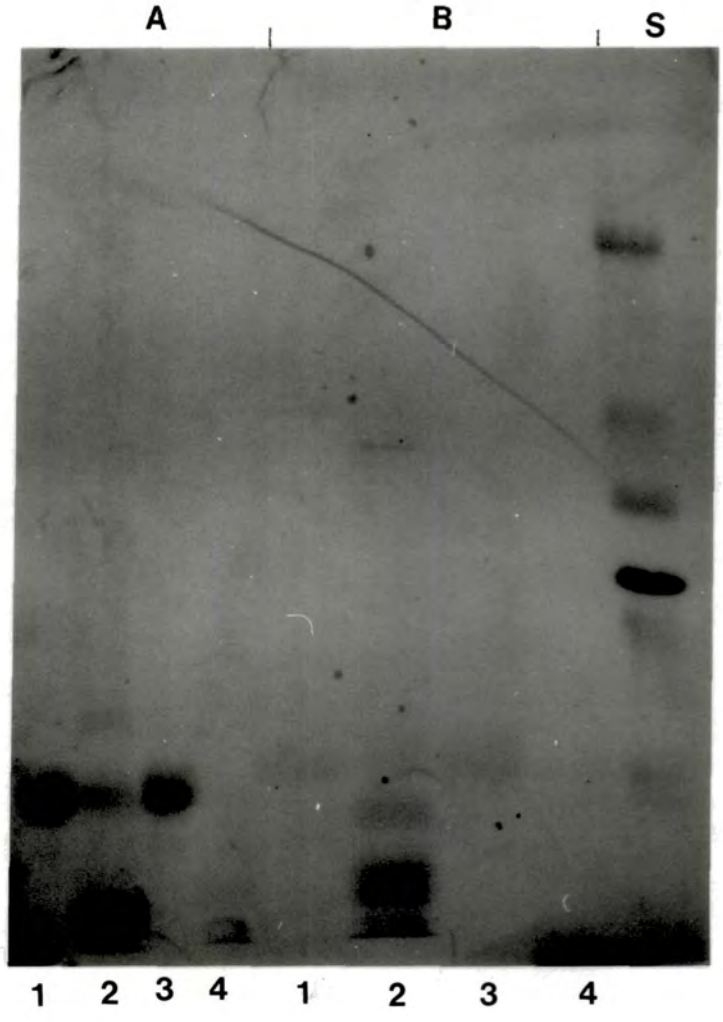


Fig. 45 Amido Black Staining of Proteolytic Map of Denatured Proteins of caml9 and olel9 on Nitrocellose Membrane

A 1, caml9 control
2, caml9 + V8
3, caml9 + Try
4, caml9 + Lys

B 1, olel9 control
2, olel9 + V8
3, olel9 + Try
4, olel9 + Lys

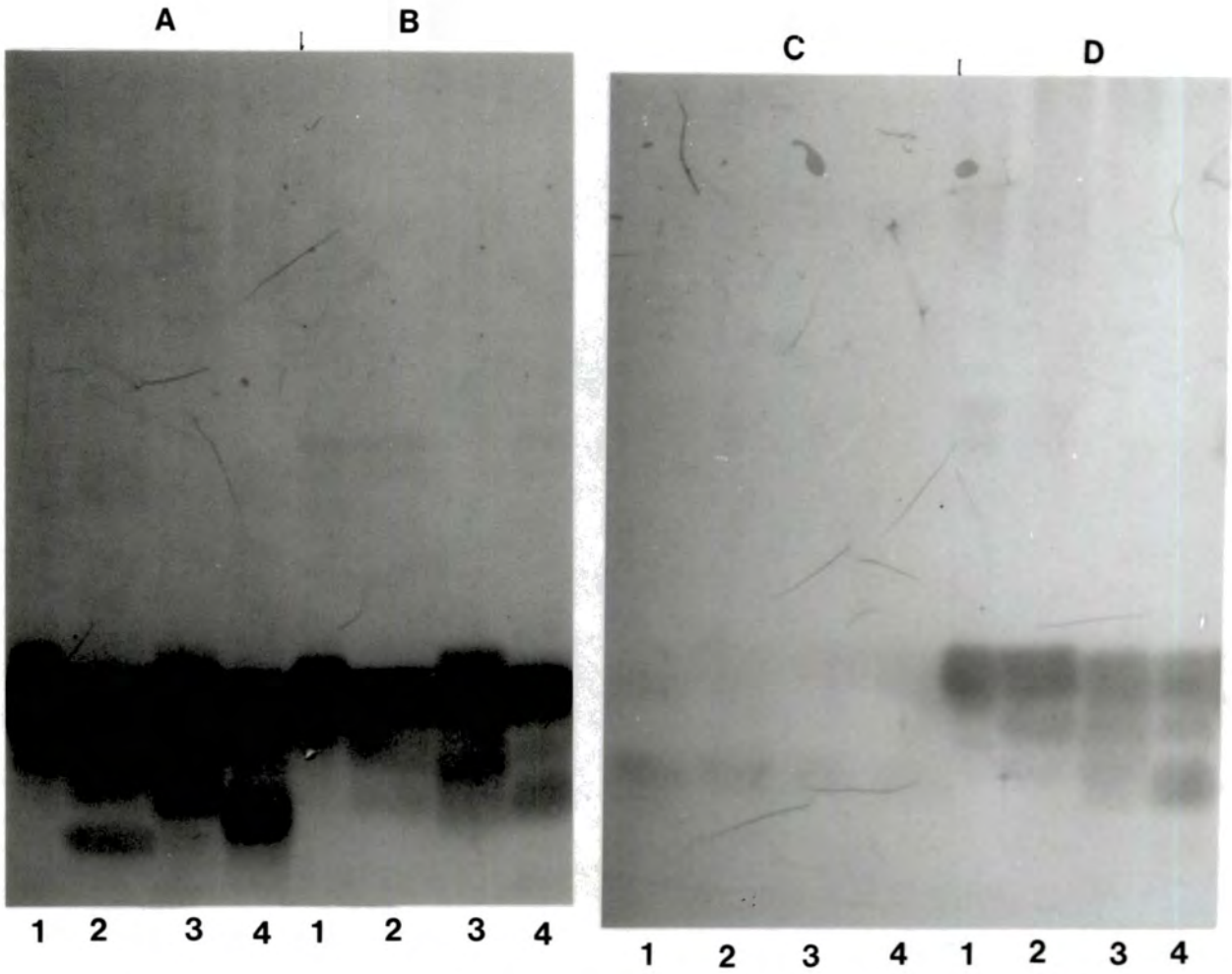


Fig. 46a Immunoblotting of Proteolytic Maps of Denatured Proteins after running on SDS/PAGE of nap19, rad20, cam16 and mus20

A	B	C	D
1, nap19 control	1, rad20 control	1, cam16 control	1, mus20 control
2, nap19 + V8	2, rad20 + V8	2, cam16 + V8	2, mus20 + V8
3, nap19 + Try	3, rad20 + Try	3, cam16 + Try	3, mus20 + Try
4, nap19 + Lys	4, rad20 + Lys	4, cam16 + Lys	4, mus20 + Lys

Fig. 46b. Immunoblotting of Proteolytic Map of Denatured Proteins of caml9 and ole

E 1, caml9 control
2, caml9 + V8
3, caml9 + Try
4, caml9 + Lys

F 1, olel9 control
2, olel9 + V8
3, olel9 + Try
4, olel9 + Lys

Digestion of oil-bodies under non-denaturing condition was carried out. The peptide map shown in Fig. 47 was PAGE run under non-dissociating conditions (without SDS in buffer) while Fig. 48 was the same product that ran in dissociating SDS/PAGE gels. Immunonblotting on SDS/PAGE wasalso performed andis shown in Fig. 49. The molecular weight of immunolabelled peptide bands is shown in Table 6. The trypsin digested products gave no labelling at all.

Table 6 Immunogenic peptide of digested oil-bodies

Enzyme	Species	molecular weight of Immunogenic peptides (kDa)
V8	nap	16
	ole	16
	cam	19-14 (faint)
	rad	16
	mus	-
Lys-C	nap	19-15
	ole	16, 15
	cam	15
	rad	16
	mus	16
Trypsin	nap	-
	ole	-
	cam	-
	rad	-
	mus	-

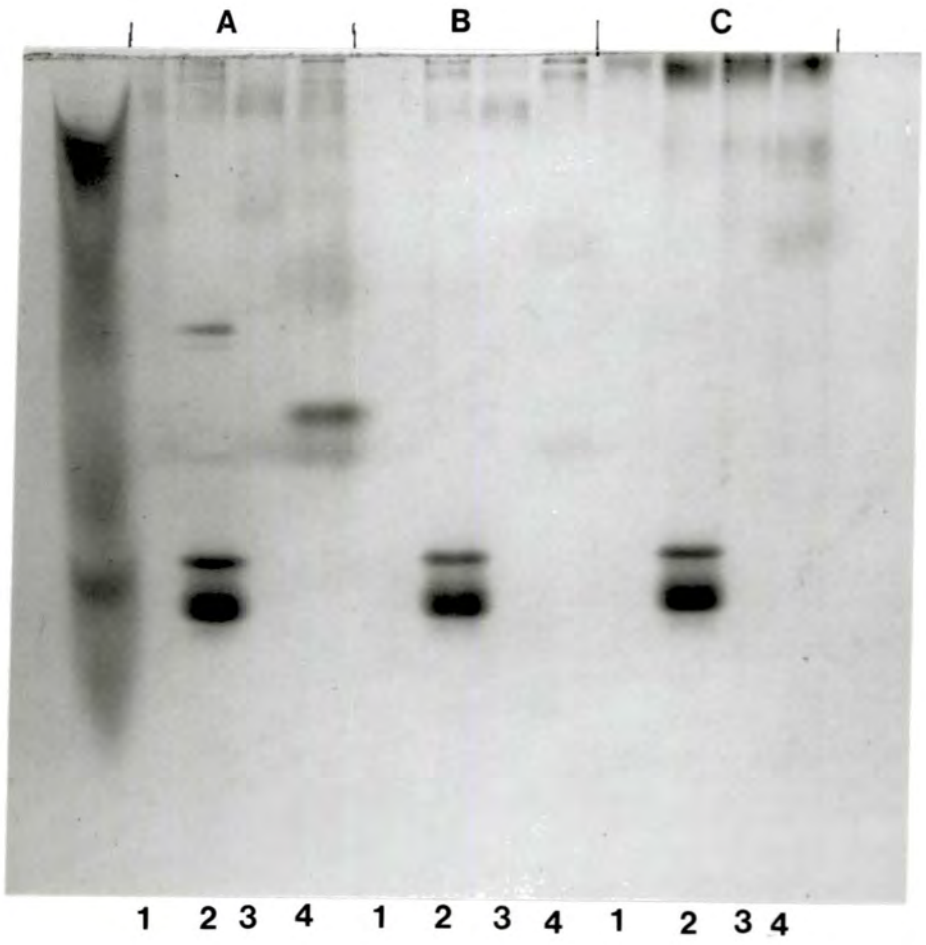
'-': no detectable immunolabelled peptide.

The changes in antigenicity of proteolytic products in native and denaturing digestion conditions were checked by ELISA. The proteolytic products were serially diluted and the dilution/response curves are presented as line-graphs in Fig. 50, 52, 54, 56 and 58. Dilution at 10^{-3}, which gave reasonably sensitive response in ELISA, was used to draw bar charts (Fig. 51, 53, 55 and

57) revealing the drops in antigenicity. The percentage of fall in antigenicity was calculated with reference to a control (undigested proteins), data are shown in Table 7. Generally, trypsin digestion of both native and denatured proteins led to a greater reduction in antigenicity compared to the other proteolytic enzymes.

Table 7 The percentage decrease in antigenicity of proteins

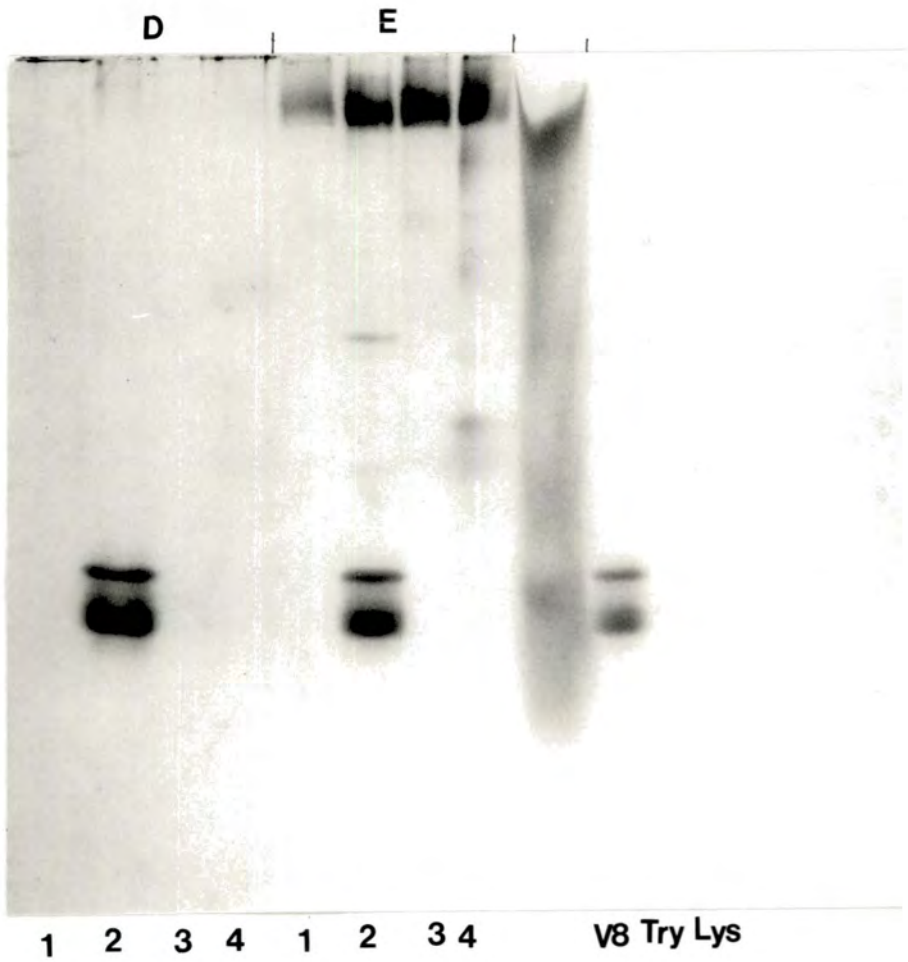
species	enzyme	% drop in OB	% drop in denatured protein	
nap	V8	59.1	nap19	56.5
	Try	79.4		69.4
	Lys	52.8		50.4
ole	V8	80.5		28.2
	Try	90.3		65.4
	Lys	69.3		29.6
cam	V8	89.7	cam19: 40.5	cam16: 59.8
	Try	95.8	60.4	70.9
	Lys	56.2	54.2	72.7
rad	V8	56.0	rad20	49.6
	Try	95.0.		50.8
	lys	54.4		40.1
mus	V8	85.1	mus20	33.0
	Try	95.0		50.8
	Lys	54.4		40.1



Fig, 47a Digestion Map of Non-denatured Oil-body Proteins og napOB, radOB and camOB on Non-dissociating Gel,

Key for Fig,47 - 49;

A1, napOB control	B1, radOB control	C1, camOB control	D1, musOB control	E1, oleOB control
2, napOB + V8	2, radOB + V8	2, camOB + V8	2, musOB + V8	2, oleOB + V8
3, napOB + Try	3, radOB + Try	3, camOB + Try	3, musOB + Try	3, oleOB + Try
4, napOB + Lys	4, radOB + Lys	4, camOB + Lys	4, musOB + Lys	4, oleOB + Lys



Fig, 47b Digestion Map of Non-denatured Oil-body Protein of MusOB and OleOB on Non-dissociating Gel, See Fig 47a for key,

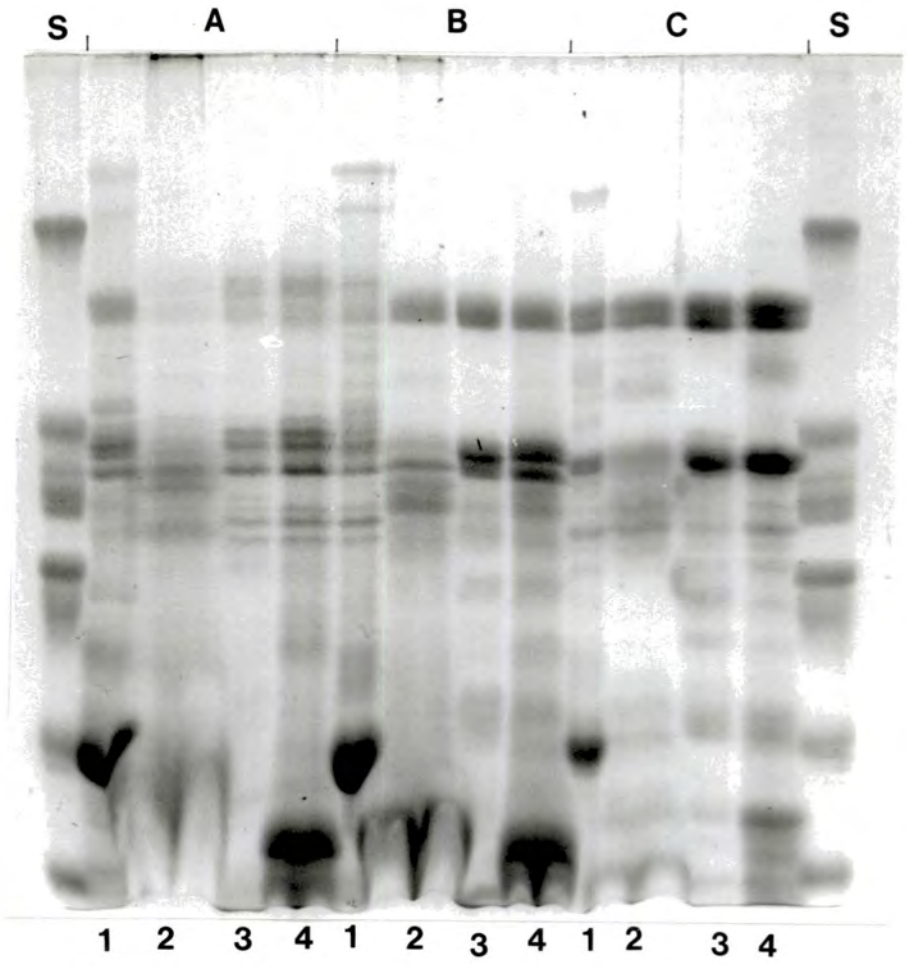
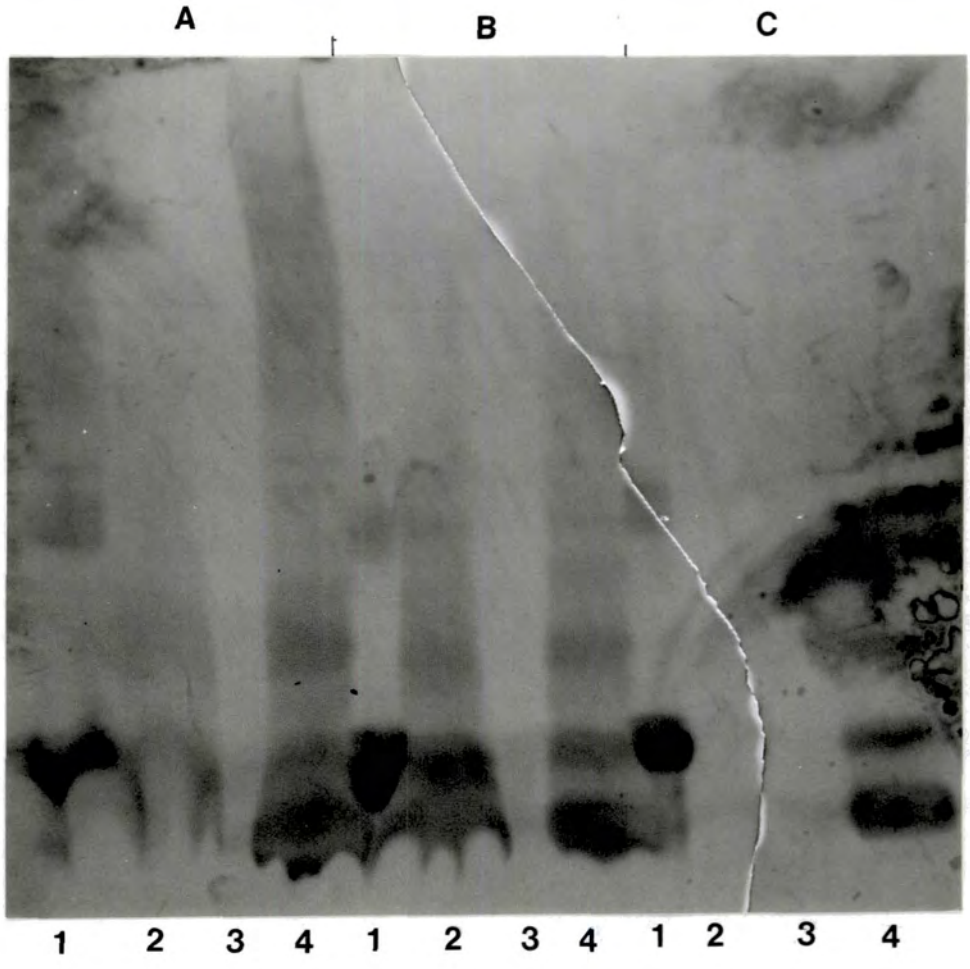


Fig. 48 Digestion Maps of Oil-body Proteins digested under Non-denaturing conditions and run on SDS/PAGE Gels. See Fig. 47 for key.



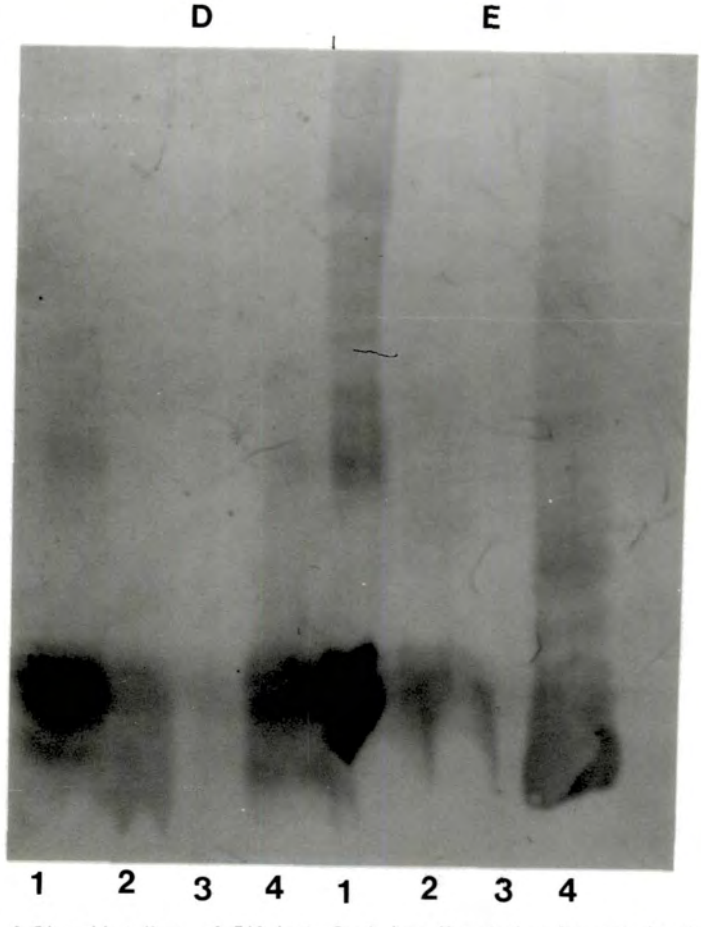
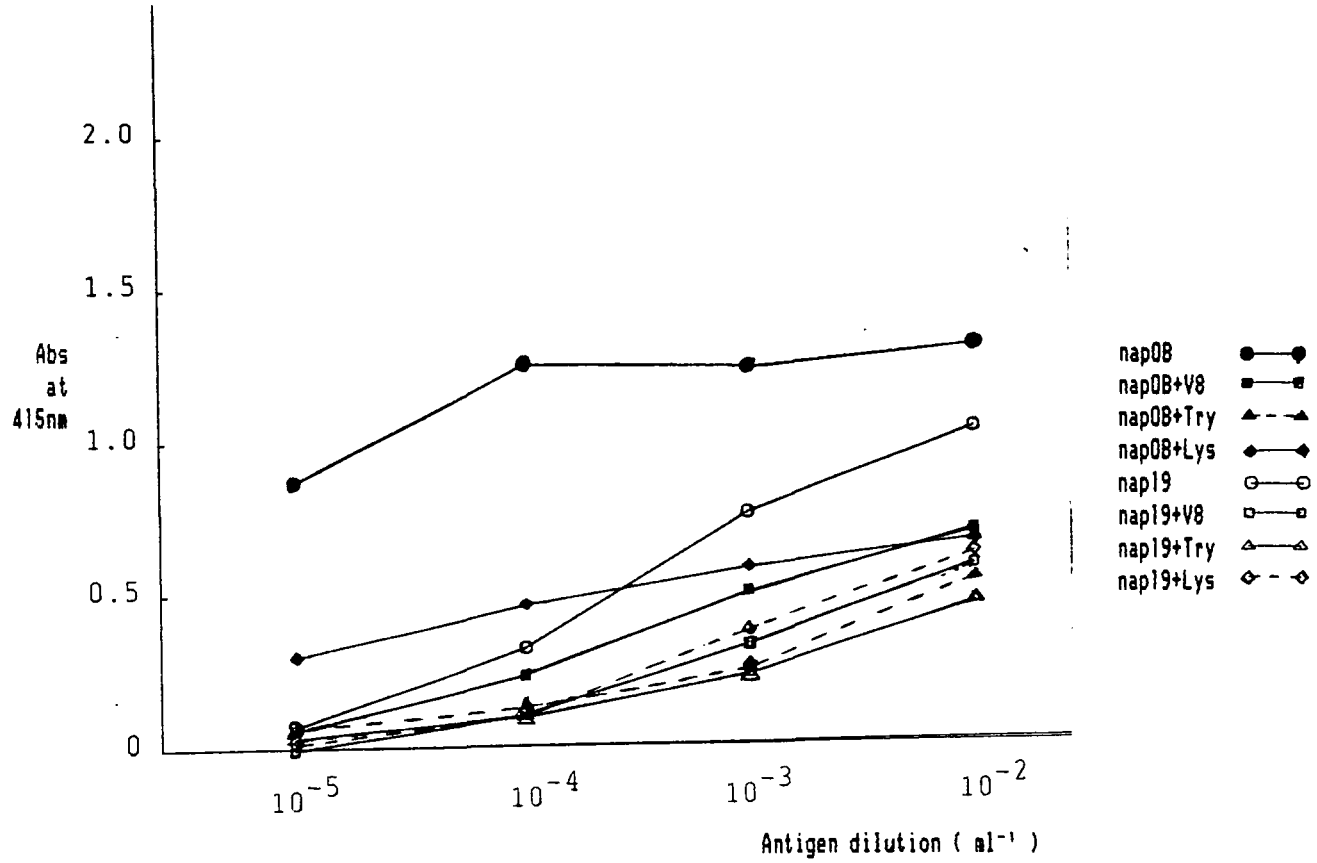
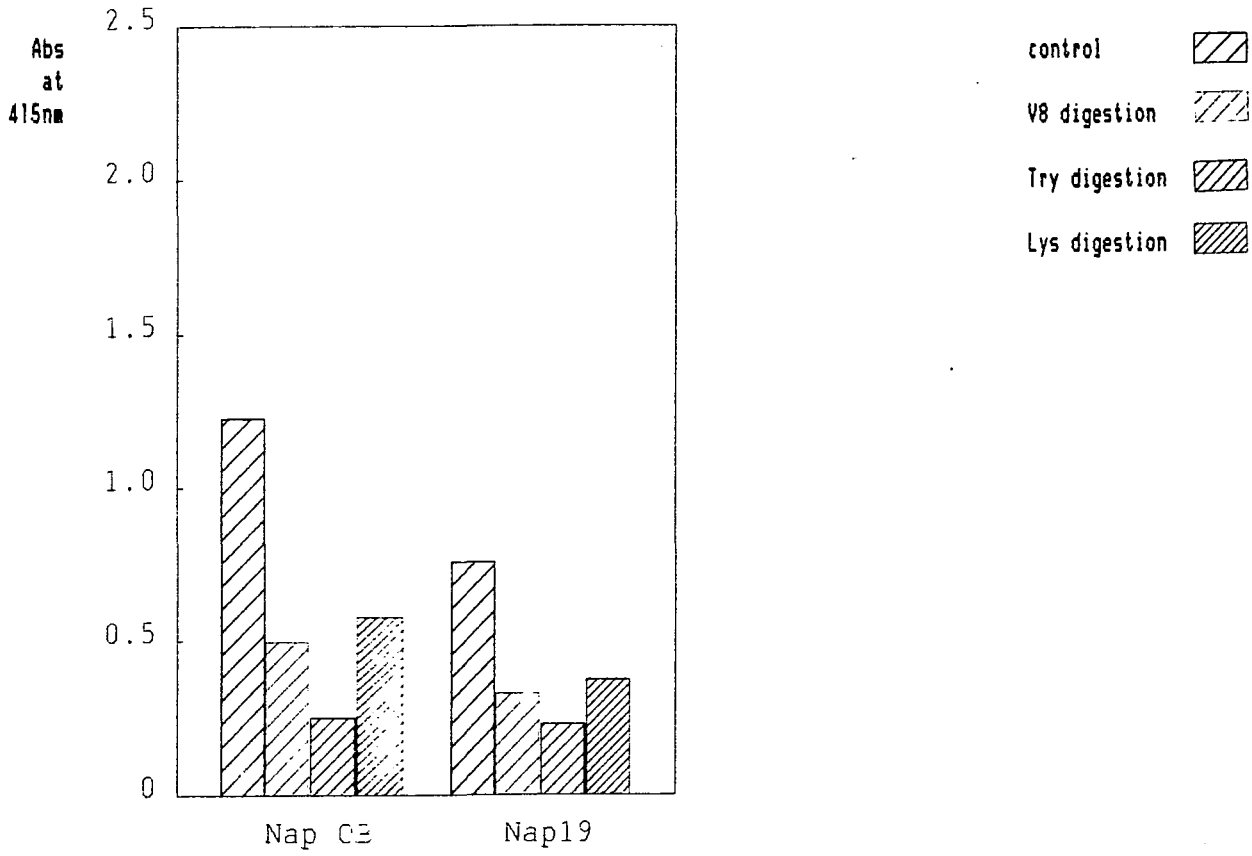


Fig. 49 Immunoblotting of Digestion Maps of Oil-body Proteins digested under non-denaturing conditions after running on SDS/PAGE Gels. See Fig.47 for key.



Fig, 50 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis by ELISA



Fig, 51 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis at 10^{-3} dilution by ELISA

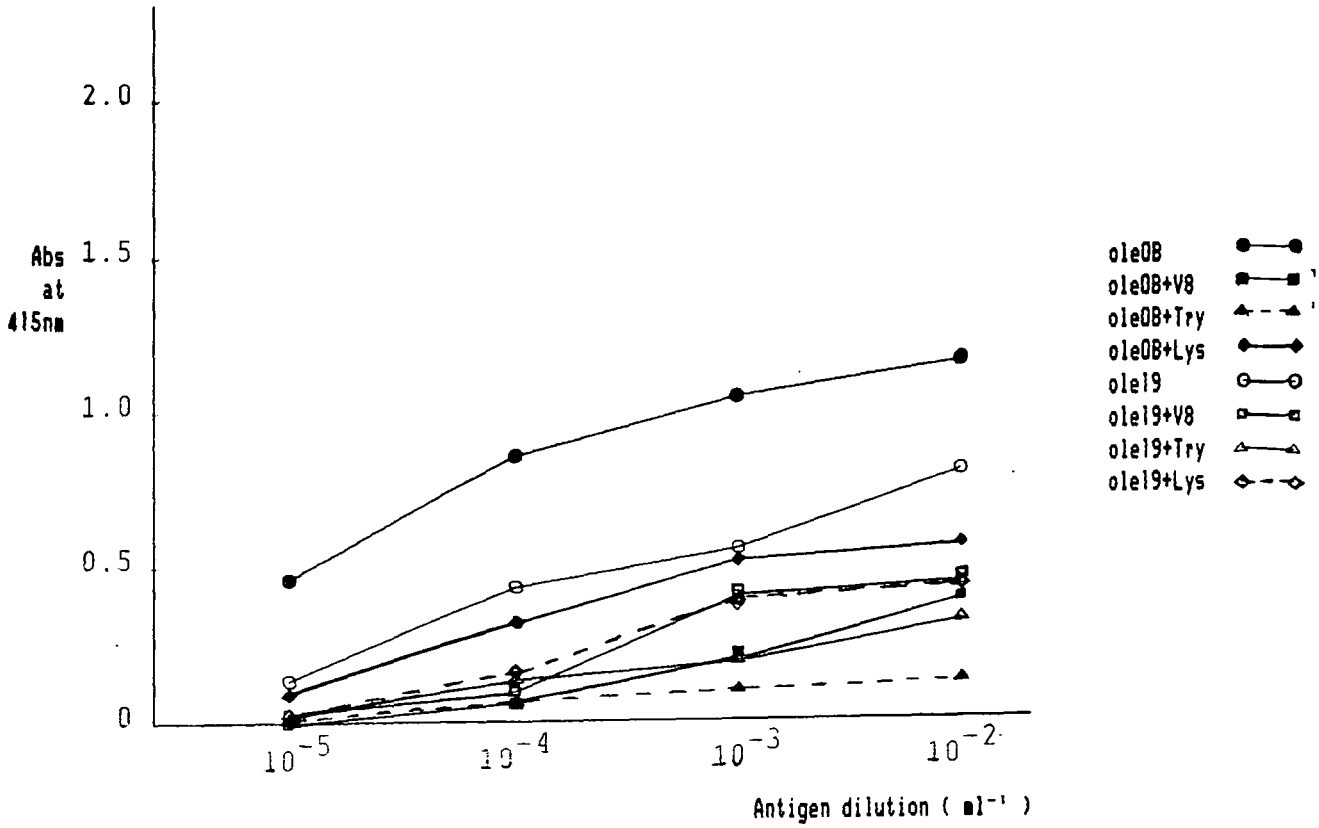


Fig. 52 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis by ELISA

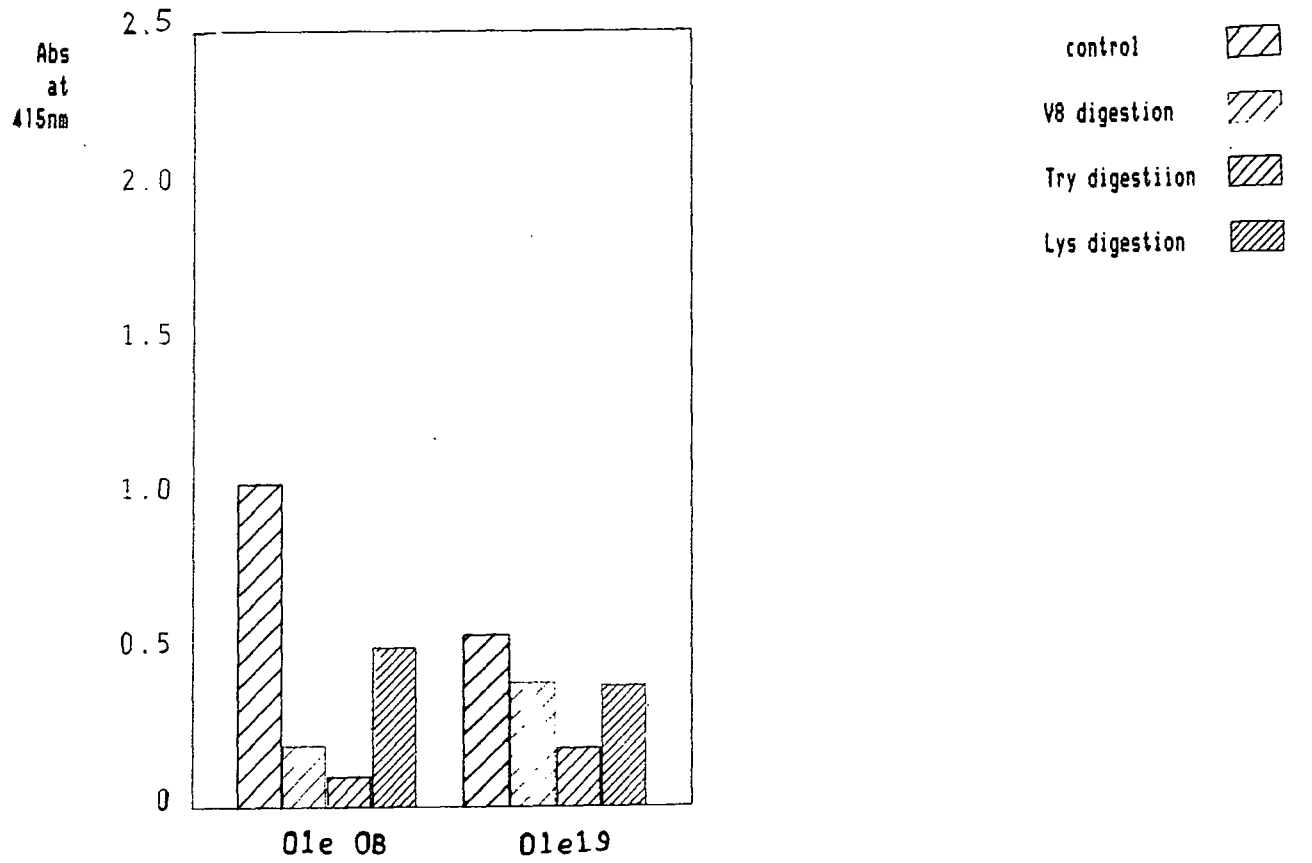


Fig. 53 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis at 10^{-3} dilution by ELISA

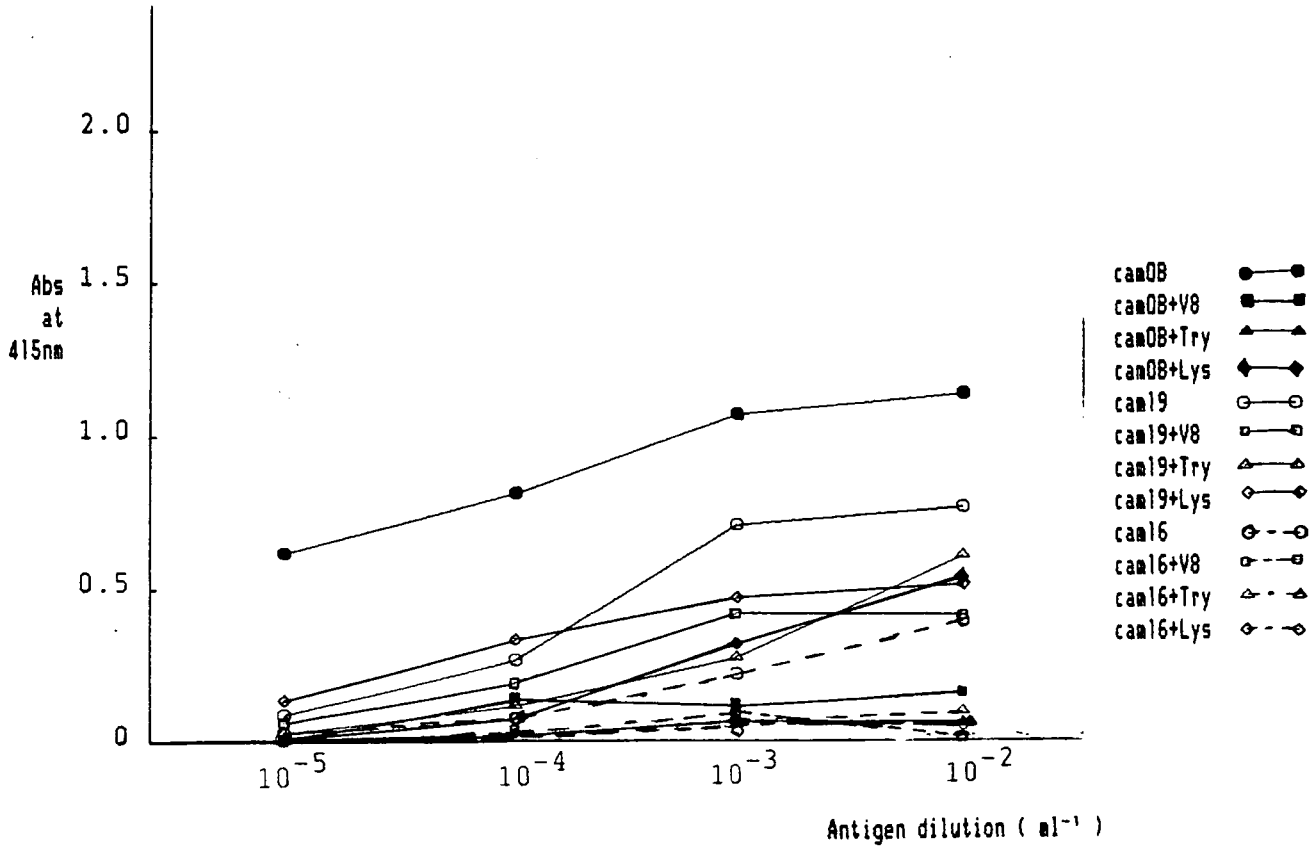


Fig. 54 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis by ELISA

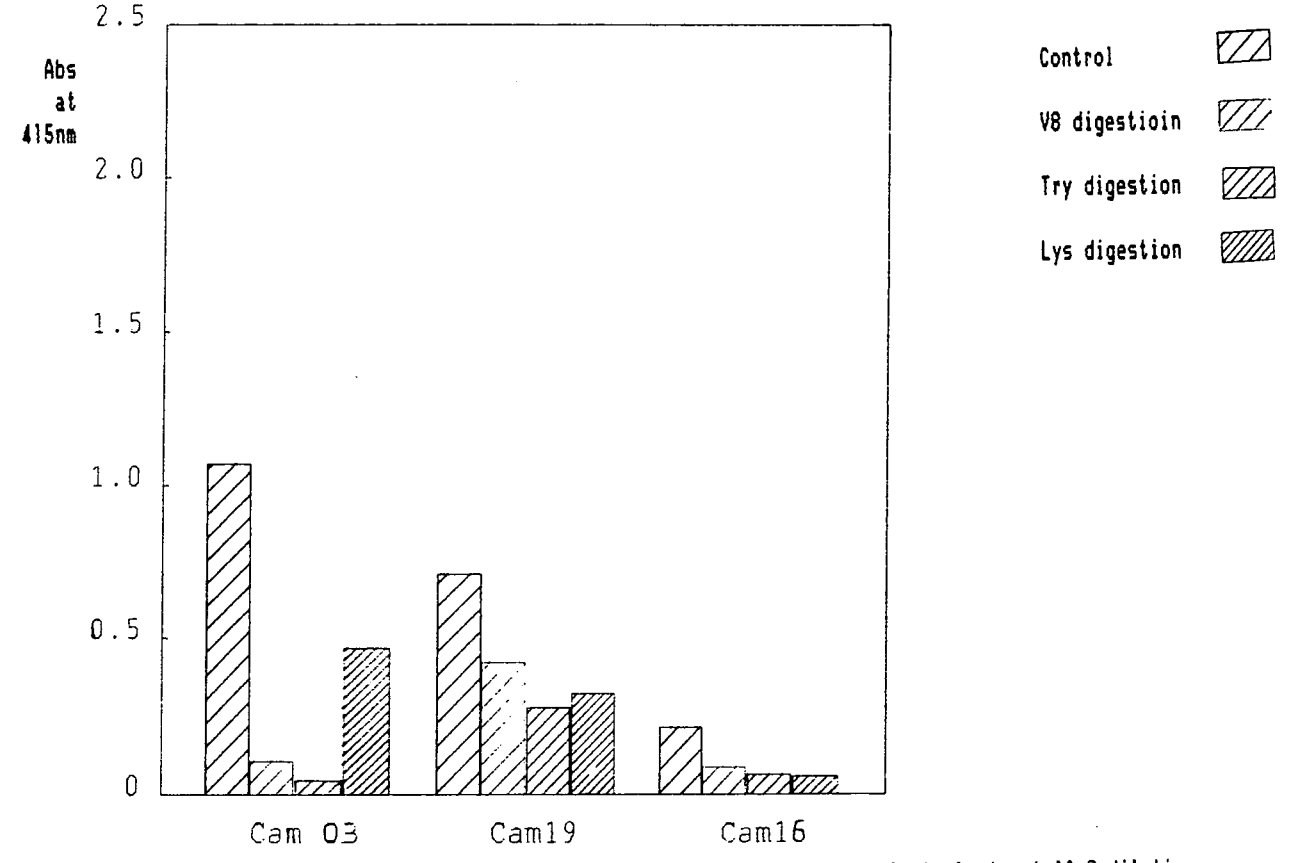


Fig. 55 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis at 10^{-3} dilution by ELISA

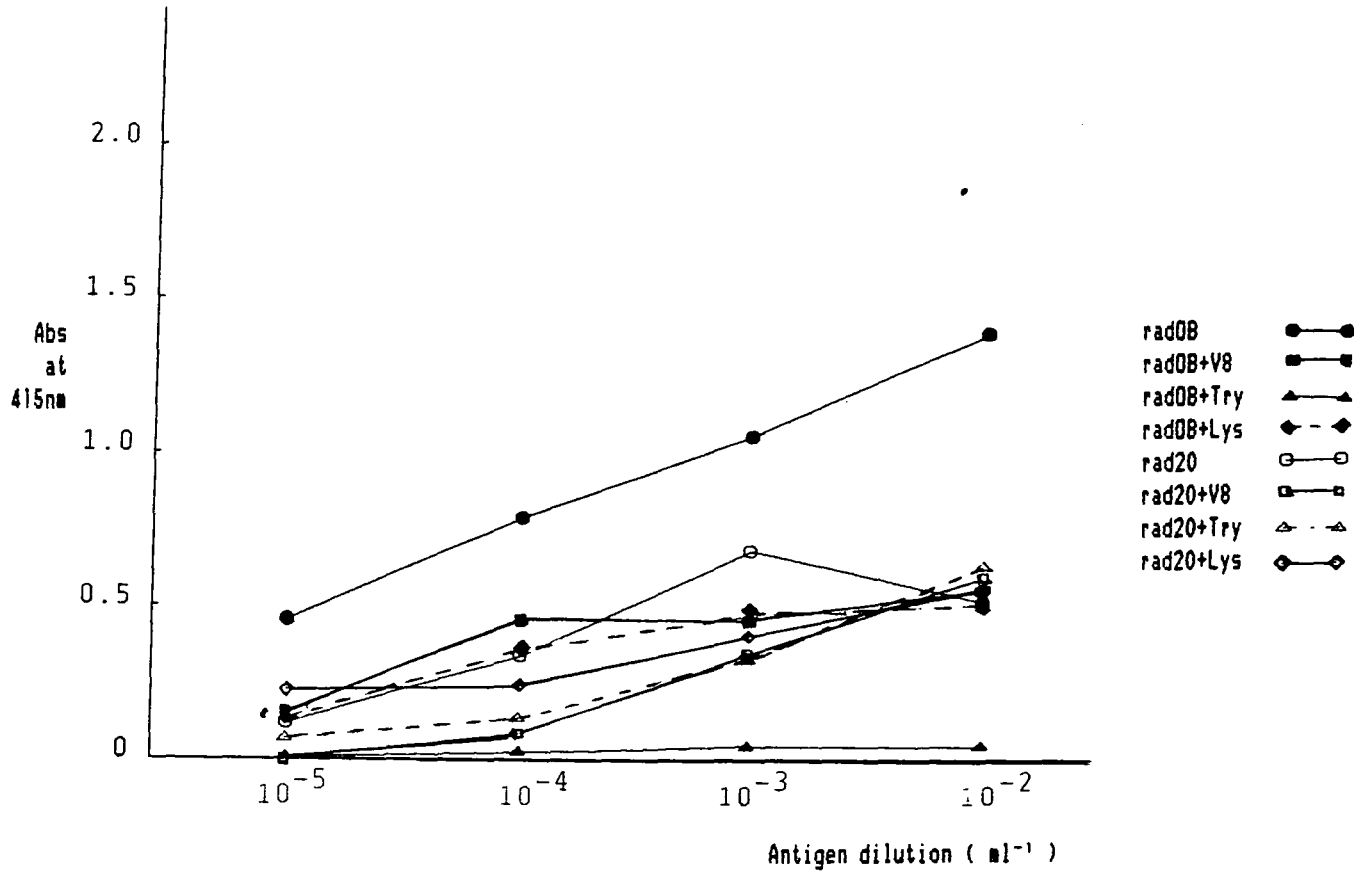


Fig. 56 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis by ELISA

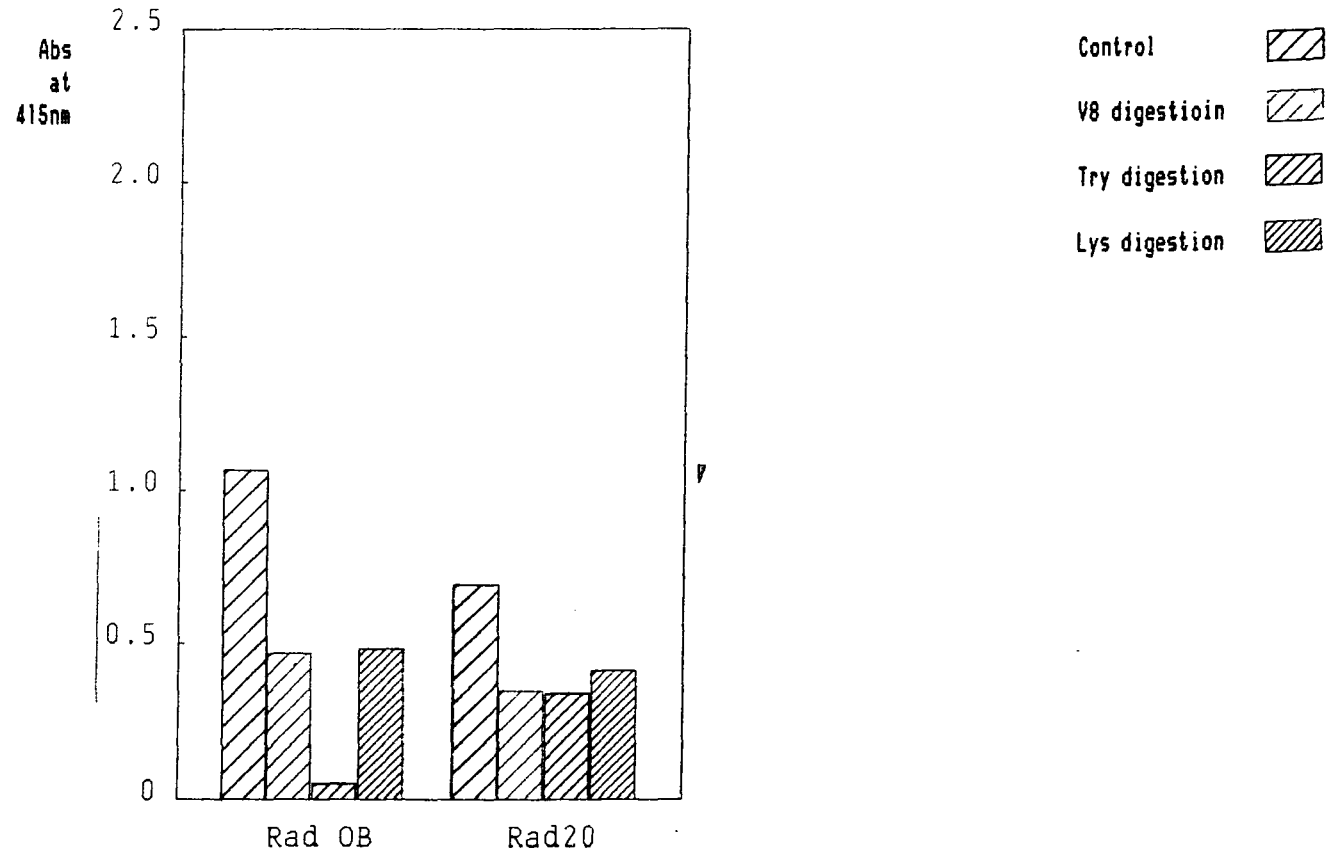
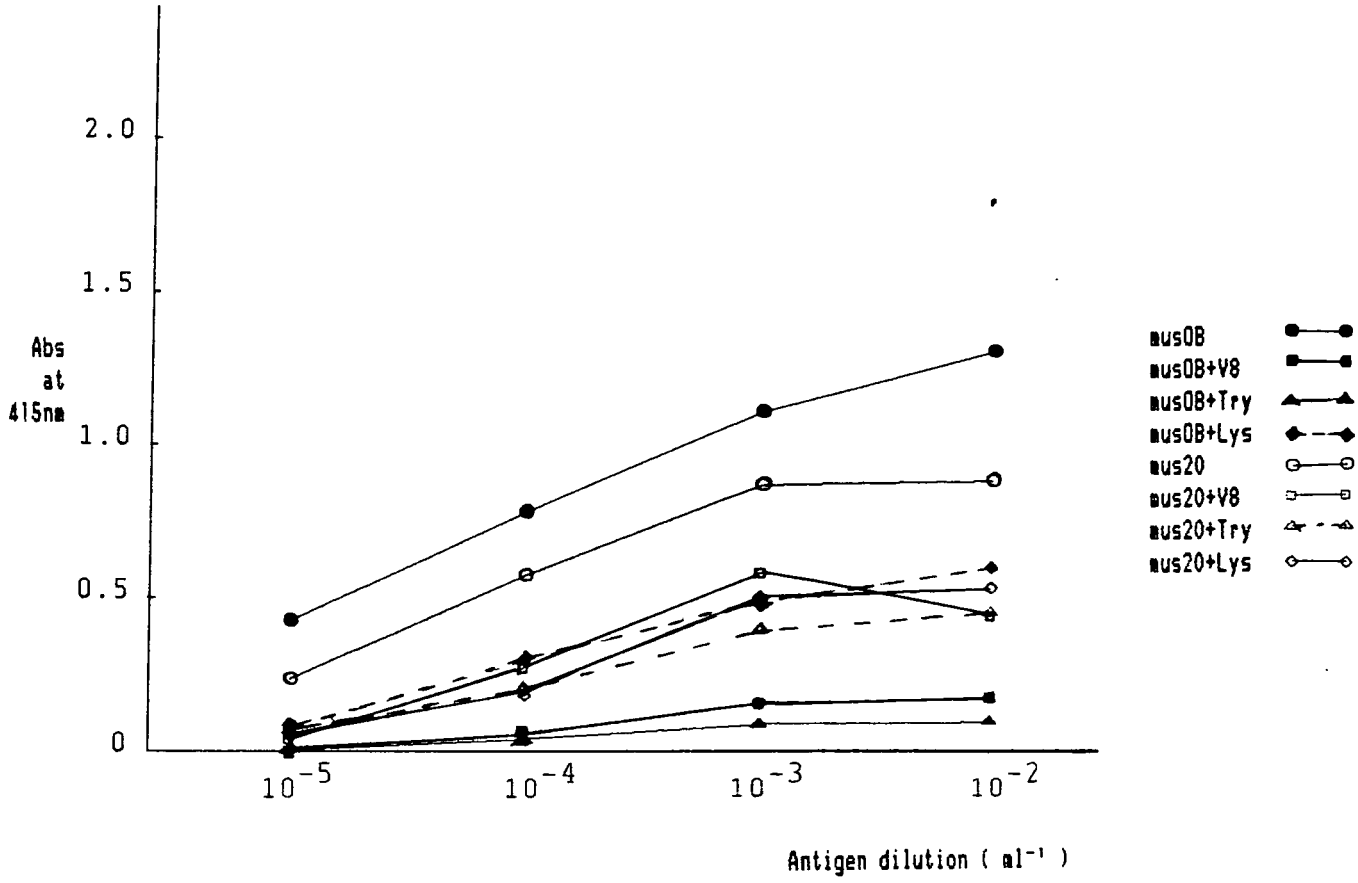
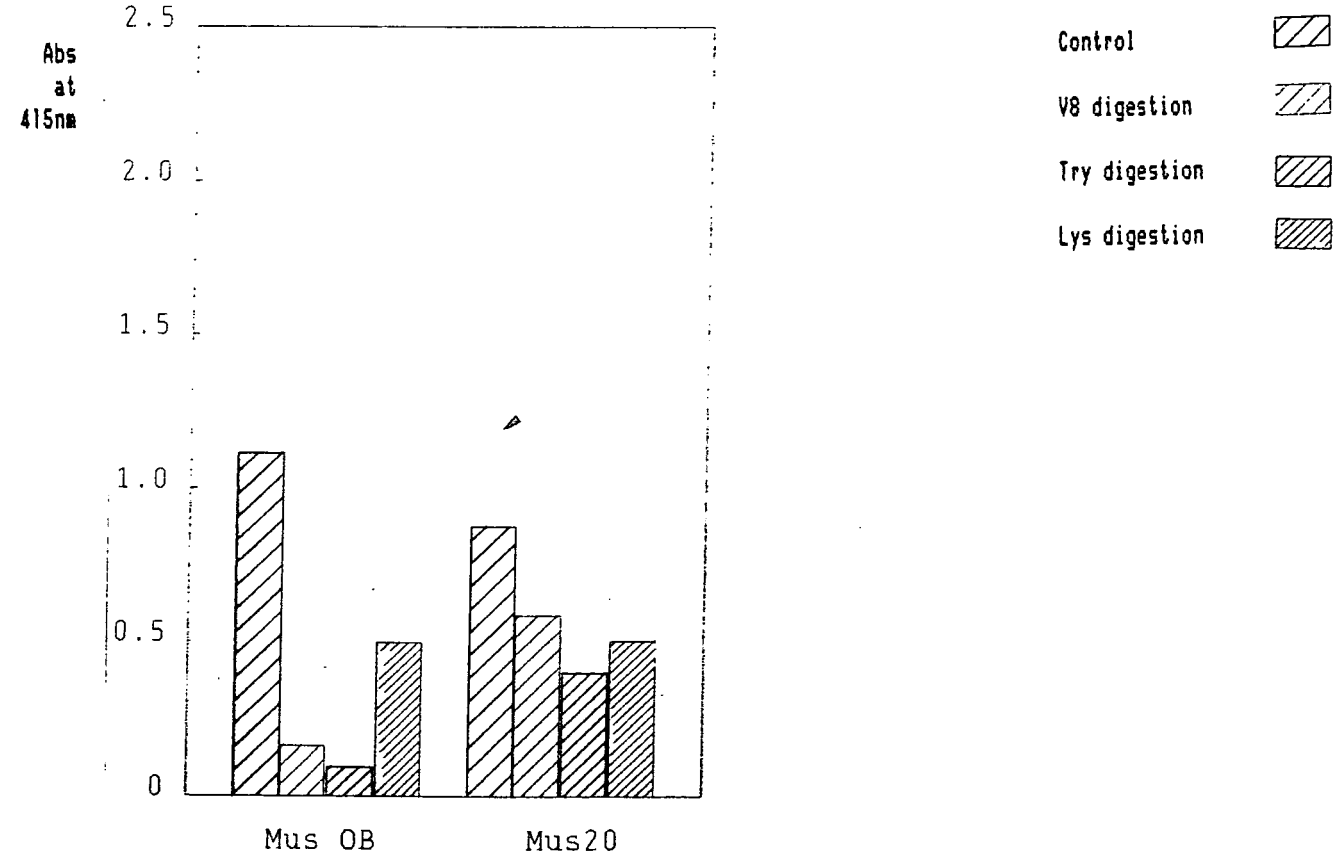


Fig. 57 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis at 10^{-3} dilution by ELISA



Fig, 58 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis by ELISA



Fig, 59 Antigenicity of Non-denatured and Denatured protein before and after Proteolysis at 10^{-3} dilution by ELISA

3.5.0 Amino Acid Composition of Nap19 and Rad20

The total amino acid compositions of nap19 and rad20 were determined. The result is showed in Table 8. The amount of each amino acid is arranged in decending order as follows (compositions are expressed in residues mol^{-1}):

nap19: Gly 17.1, Leu 17.1, Ala 15.5, Glu 14.7, Pro 14.3, Thr 12.7, Asp 12.2, Val 11.6, Ser 11.3, Ile 9.5, Arg 8.9, Tyr 6.9, Lys 5.4, His 4.5, Phe 4.3 Cys 0, Met 0

rad20: Gly 17.3, Glu 16.6, Pro 13.9, Leu 13.7, Val 13.5, Asp 12.3, Ala 12.3, Thr 11.0, Ser 10.5, Arg 8.9, Ile 8.4, Tyr 6.7, Phe 5.6, Lys 5.3, His 3.7 Cys 0. Met 0

Both proteins had quite similar amino acid compositions and were particularly rich in Gly, Glu, Leu and Pro when the most abundant five amino acid were considered. Also, they both contained no Cys nor Met.

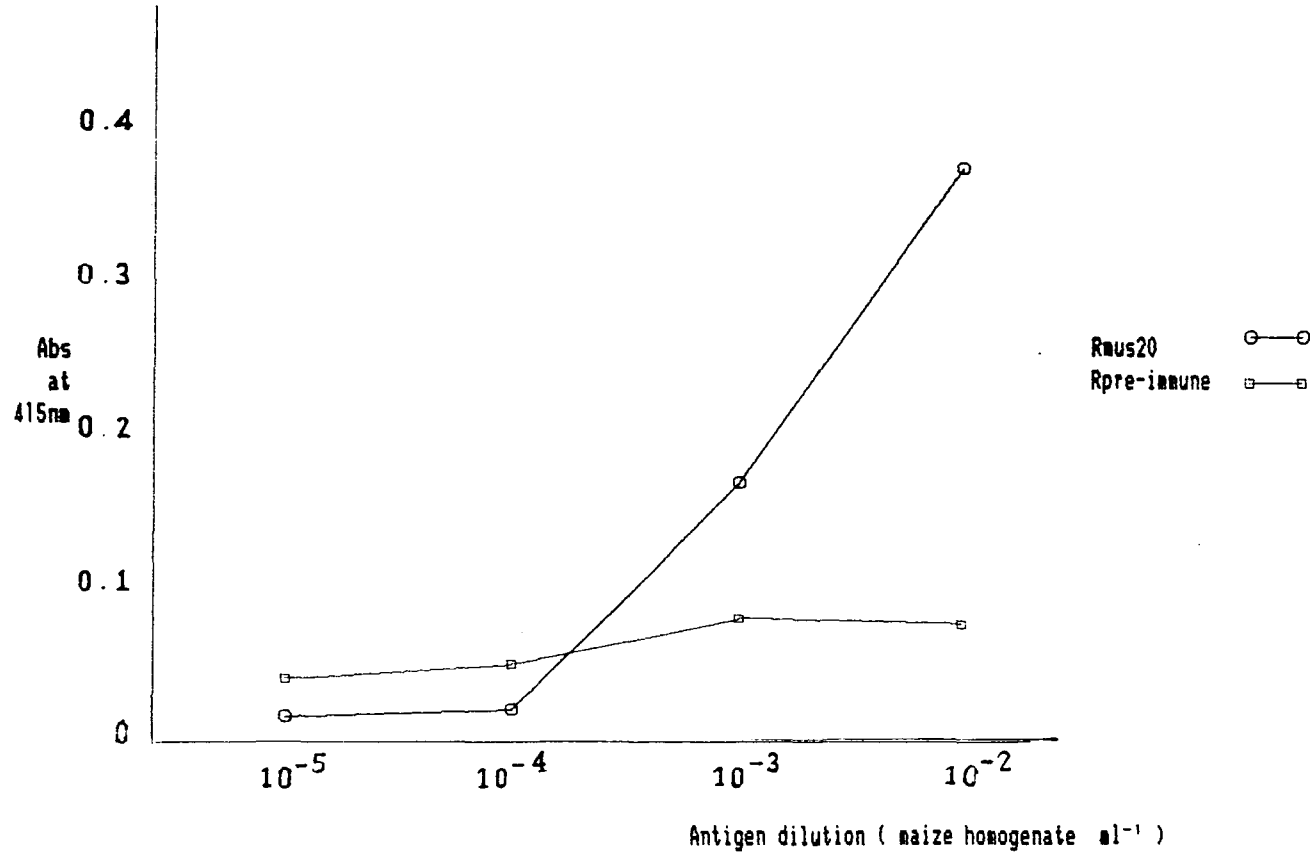


Fig. 60 Rmus20 and Rpre-immune Cross-reaction with Maize Homogenate by ELISA

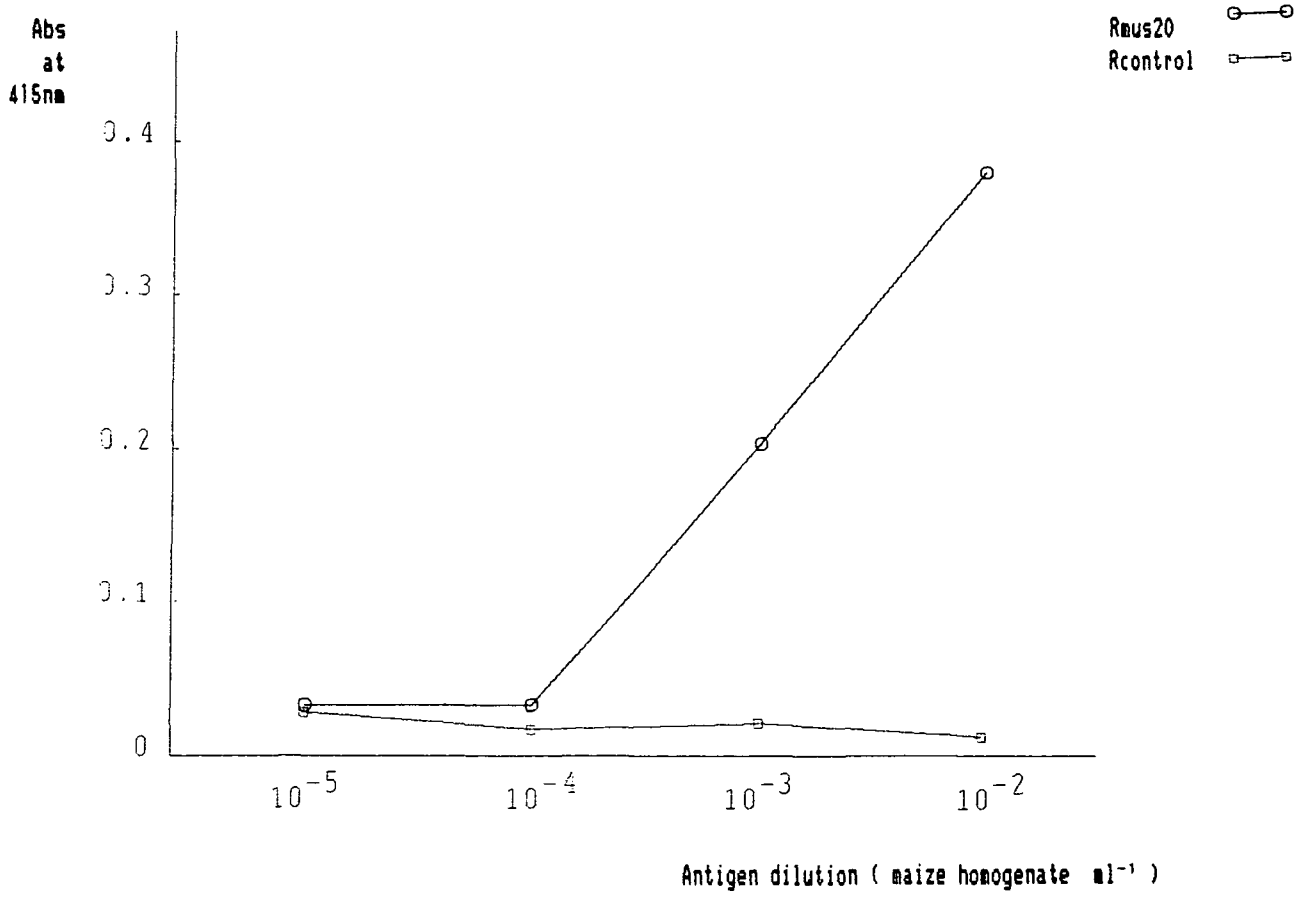


Fig. 61 Rmus20 and Rcontrol Cross-reaction with Maize Homogenate by ELISA

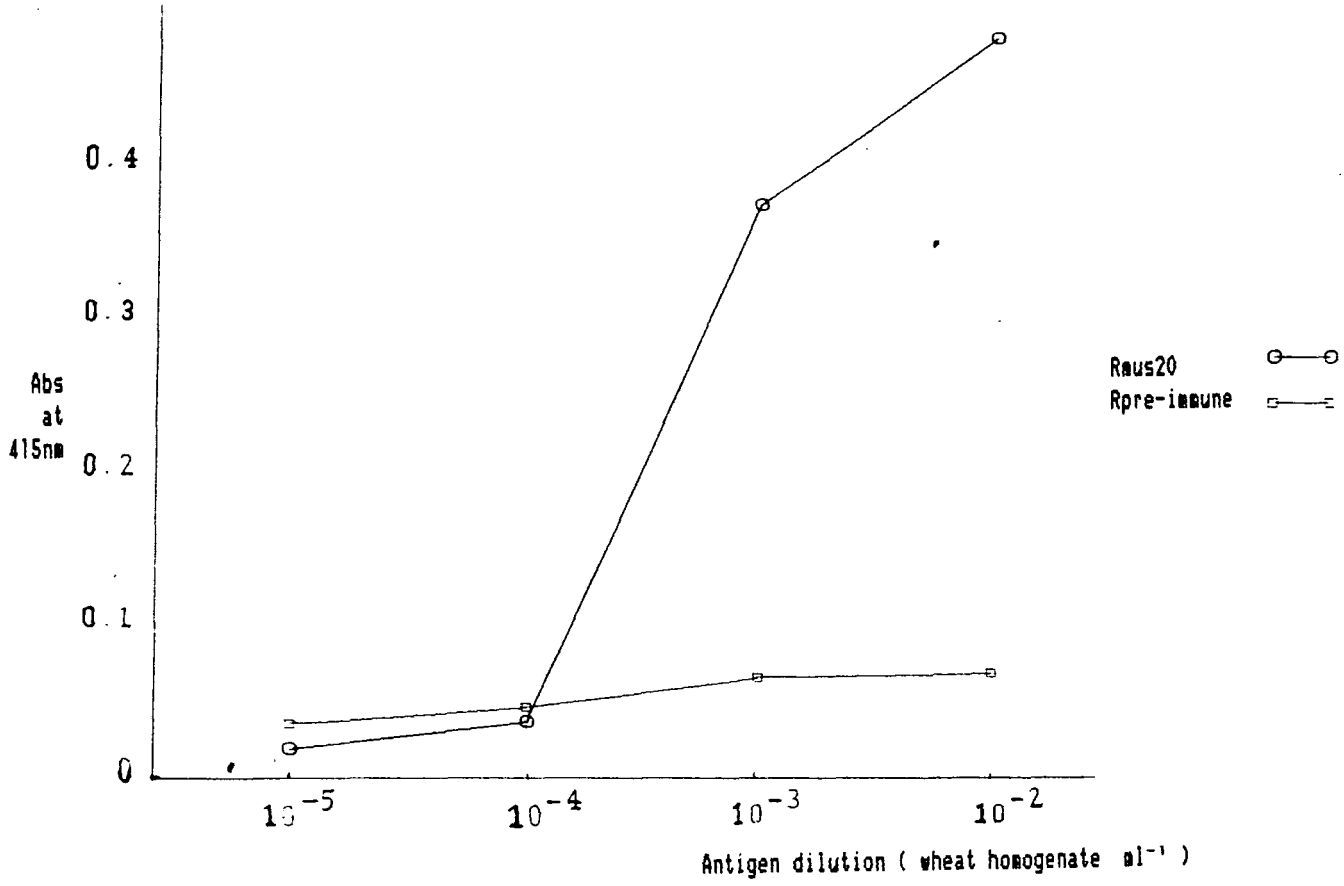


Fig. 62 Rmus20 and Rpre-immune Cross-reaction with Wheat Homogenate by ELISA

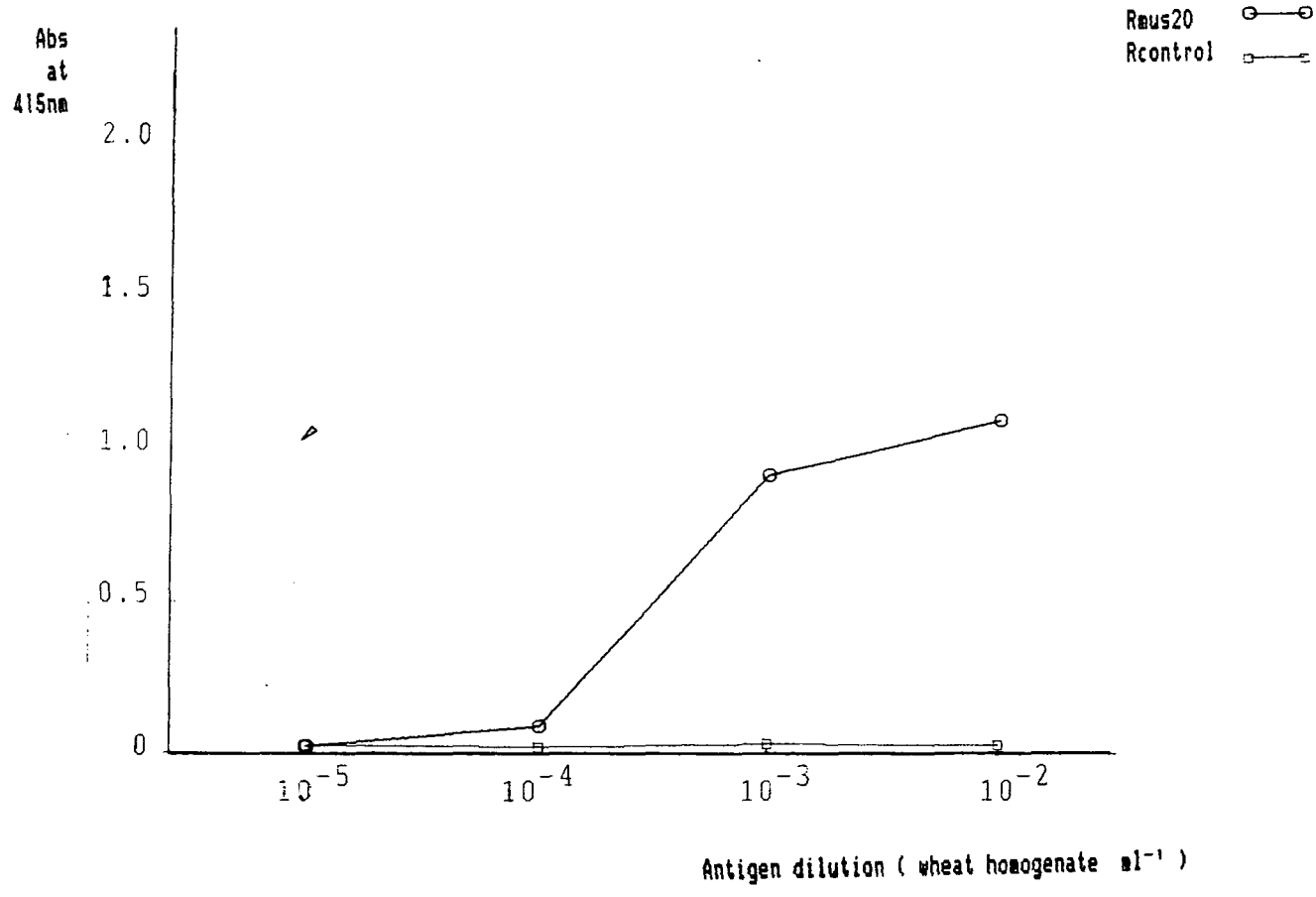


Fig. 63 Rmus20 and Rcontrol Cross-reaction with Wheat Homogenate by ELISA

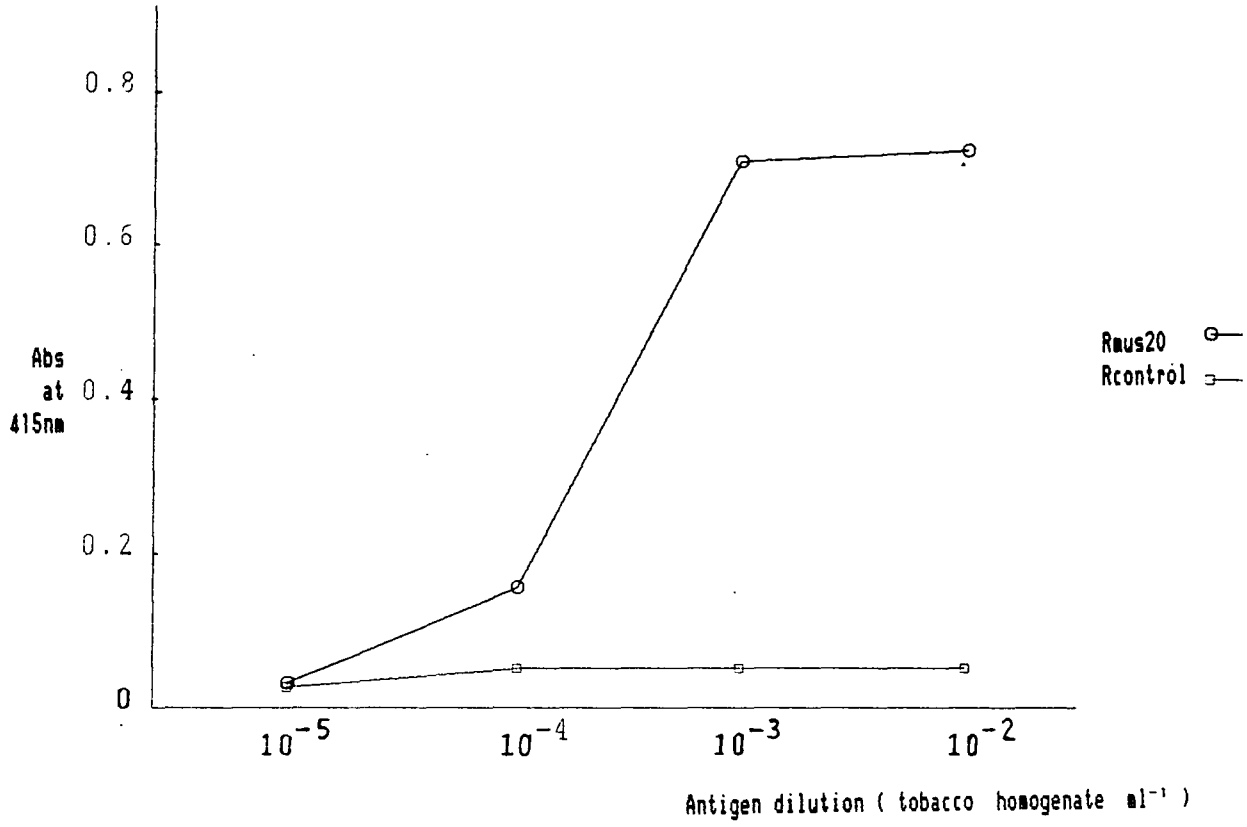


Fig. 64 Raus20 and Rcontrol Cross-reaction with Tobacco Homogenate by ELISA

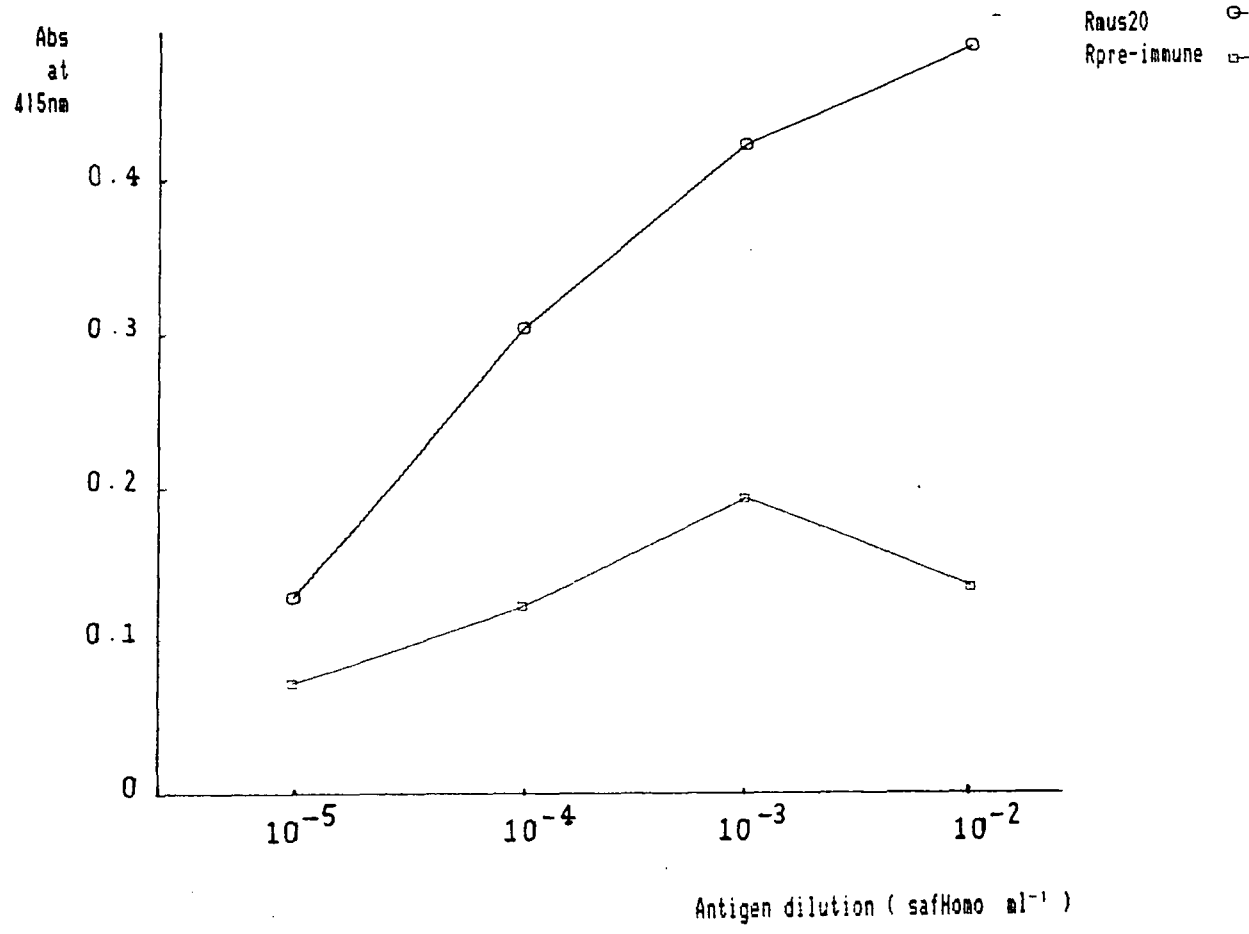


Fig. 65 Raus20 and Rpre-immune Cross-reaction with Safflower Homogenate by ELISA

4. DISCUSSION

4.1 Techniques

4.1.1 Protein Purification

The purity of PAGE puried proteins would be increased if the oil body preparation were reasonable pure and completely delipidated so that fewer charged particles, other than the membrane proteins, interfered with the migration of the proteins. It was found that when non-delipidated oil-bodies were used in SDS-PAGE, especially in high concentration, the bands below 20kDa would not separate and no sharp bands could be distinguished. Moreover, a loading of 2 ml to 4 ml sample per gel was more perferable as a distinct and intense band at 19kDa (in the case of *B.napus*) was revealed after a brief staining process (2 mins if fresh stain were used). Destaining could then be omitted. The shorter the time in stain and destain, the higher the recovery of proteins since they are not then firmly fixed into the gel material. It was also benificial if the proteins were concentrated down to 1 mg / ml (about 1 ml recovery) so as to reduce the volume used in sequencing experiments. The SDS/PAGE purifed proteins were revealed as single bands in analytical gels and thus they are considered to be pure. However, since the lowest detection limit of Coomassie blue is 0.2-0.5 μg of any protein in a sharp band (Hames, 1981), any contaminant below this amount would not be detected and could interfere subsequent experiments. This was especially troublesome in the course of antibody raising and led to the isolation of multi-antibody populations.

4.1.2 Antibody raising and their Immuno-assays

The titres of harvested antisera were checked by ELISA. Different antigens in different animals gave different titres since the immune response of the animals varied. Comparatively, in our trials, rabbits give higher titres than mice. Msoy, Mcra, Msaf and Mlin gave very poor responses in ELISAs. However, when immunoblotting was performed, only Mlin showed negative results to all the oilseed homogenates which were tested - even the linseed homogenate. While in Mnap native (the boosting of mouse for Mnap19) gave no response on ELISA and immunoblotting at all. Msun20, which had moderate ELISA response, in constrast, was insensitive to low protein concentrations i.e. only the oil-body lane which was highly concentrated in sun20 was positively labelled. Meanwhile, the other two low titre antibodies Mcra20 and Msoy24 responsed well in immunoblotting. This suggested that low concentration but extremely high affinity antibodies were present. Furthermore, interference might affect the sensitivity of the ELISA. It was found that the presence of ionic detergents (eg. SDS) reduced the sensitivity of ELISA through altering the antigen binding to the plates (McCabe et.al., 1988). Since these proteins were obtained from batches of low concentration, thus the effective concentration of SDS might be in excess (i.e. more than the need for solubilising the membrane protein) and the excess SDS micelles could interfere with the ELISA. Thus, it would be better if all protein preparations were prepared at more or less similar concentration, i.e. by adjusting the amount of starting materials as different seeds have different oil contents.

Moreover, the antibodies that we raised were found to be specifically recognised by only a few antigenic determinants as suggest in the immunometric assays. This result will be discussed in section 4.2.1.2. When the antibodies were checked by immunblotting, usually, more than one band was labelled (Fig.18 - 30). There are several factors which may lead to this phenomonon. Firstly, the presence of low concentration but highly immunogenic impurities eg. other cell wall or membrane debris in the SDS/PAGE purifed proteins which generated a common population of antibodies. Secondly, it may due to the property of the antibodies (recognised only a few antigenic determinants). It was believed that the higher the specificity of antibodies (i.e. the fewer antigenic determinant recognition domains), the higher the chance of the antibodies recognising carbohydrate determinants rather than proteinaceous determinants. This effect has previously caused serious problems with immunoblottings using monoclonal antibodies (Grime et al., 1987). In addition, plant plasma membrane proteins are always heavily glycosylated (Gamborg et al., 1981). Due to the comparitively high specificity of our polyclonal antibodies, their antigenic recognition sites against oil-body membrane proteins might be a mixture of proteinaceous and carbohydrate orientated domains. Such low specificity carbohydrate recognition domains might recognise the common glycosylated side groups of different polypeptide on oil-body membrane proteins and result in multi-labelled bands in immunoblottings. However, we have no evidence as yet that the oil-body membrane proteins are glycosylated and, given their intracellular location and small size, it is not thought likely that they are glycosylated.

Thirdly, common peptide structures may really exist within same species and thus antibodies recognised them at the same time. However, such degree of similarity should be very low and their recognition of would depend on the batch of antiserum. Thus, not all of the antisera would recognise oil-body proteins equally as we found.

4.1.3 Proteolysis

It was found to be difficult to proteolytically digest proteins using NCS and CNBr. NCS lowered the pH of the loaded samples to an extent that interfered with the separation of proteins in SDS/PAGE gels, even affecting the migration of neighbouring lanes. The failure of CNBr in protein cleavage was disappointing in the first place. However, after total amino acid composition analysis, it was not suprising when methionine was found to be absent in at least two of the representive protein species, nap19 and rad20, that we tested.

The five selected protein species were found to be digestable by protease V8, trypsin and endoprotease Lys-C. V8 cleaves at the carboxyl side of aspartate and glutamate. Trypsin hydrolyses at the carboxyl end of the basic amino acids lysine and arginine, while lys-C cleaves at the carboxyl end of lysine. Trypsin was less informative in protein gels, since at the concentration required for proteolysis, the trypsin peptides themselves were visible on the gels and some of them overlapped with the test proteins their digested peptides. However, it became more informative in immunoblotting since trypsin showed no reaction with the oilseed antibodies.

It was also found that the digestion maps were reproducible, although there were difficulties in assigning the molecular weight of peptide fragments since their migration on SDS/PAGE gels varied slightly each time which is a common phenomonon with hydrophobic polypeptides. As long as digestion occurred, any variation in time or concentration of enzyme gave raise to different intensities of proteolytic products but otherwise the same peptide pattern, which is a characteristic using SDS for limited proteolysis (Cleveland, 1977). There was a tendency of slightly increasing the amount of lower molecular weight products during prolonged incubations. The disproportion in concentration of some peptides was suggested to be due to the unequal sensitivity of peptide bonds towards proteolysis (LeBoeuf, 1984).

4.1.4 Amino Acid Composition Analysis

During the course of amino acid compositional analysis, the presence of SDS and Tris salt in SDS/PAGE purified sample created inconsistancies in some sets of data as they interfer with HPLC by increasing the background noise. Nevertheless, the molecular ratios of amino acids were worked out based on subjectively selected sets data with good resolution peaks for each amino acid.

4.2 Intra-family Similarities

4.2.1 Cruciferae Family

4.2.1.1 Oil-body Pattern

As shown in Fig.1 and Fig.23, the patterns of oil-bodies revealed in SDS/PAGE agreed with Qu et al. (1986) that they could serve as fingerprints to indentify different taxa. Species from the same genus although still retaining a unqiue

polypeptide pattern nevetheless had certain degree of similarity in the distribution of major oil-membrane proteins. All *Brassica*s had 19-20 kDa as a major oil-body band. Although other non-*Brassica* members of the *Cruciferae* also had similar protein bands, they might not be the most predominant one.

4.2.1.2 Cross-reactivities

4.2.1.2.1 Immunoblottings

From our results in immunoblottings and ELISA, we are able to show that proteins that from oil-bodies having non-identical oil-body patterns in SDS/PAGE can behave equally in turn of antigenicity.

All the 15 *Cruciferae* used in this study including *Brassica* and non-*Brassica* species can be recognized by anti-napus 19kDa antibodies (Rnap and Mnap). Immunoblots on purified oil-bodies from the crucifers reveal patterns of labelling which coincide with these of the homogenates. This suggests that the members of the family share some common protein domains which are located on oil body membrane proteins. Such proteins with slightly different molecular weights may have similar antigenic determinants as evidenced by the binding of antibody. Hence these proteins have both physiological and immunological similarities.

The *Cruciferae* family of oil-body associated proteins exhibited a similar pattern of cross-reativity with Rmus to that observed for Mnap and Rnap. Mnap native, Mmus, Mrad and Mcra, all of which cross-reacted with all the crucifer homogenates that were tested, and all of which gave virtually identical

patterns in immunoblotting. However, they all had a similar problem in recognising other oil-body proteins bands than the 19kDa proteins, although the 19kDa were always the most heavily labelled.

4.2.1.2.2 Direct ELISA

In the direct ELISA, which involves the immobilisation of antigen to the plastic wells followed by subsequent immunodetection, the 6 selected *Brassicae* species (nap19, cam19, cam16, ole19, rad20, mus20) exhibited sigmoid curve responses over a range of antigen levels. These *Brassicae* species should have a common ancestor. Soy24 gave a base-line response which indicated Mnap19 and Rnap19 could not cross-react with soy24 i.e. *Brassicae* and legumes are very distantly related.

When comparing results of direct ELISA using Mnap and Rnap, Rnap seemed to be more selective and antigen-specific since none of the other proteins exceeded the degree of binding exhibited by nap19. In contrast, the ELISA with Mnap showed that the antibodies appeared to have a higher affinity for the other proteins (i.e. rad19, cam19 and cam16) than for nap19 itself. Nevertheless, the antibody Mnap exhibited an enhanced specificity for nap19 at high antigen concentration. This may be due to lower affinity but comparatively higher specificity and higher titre of a subpopulation of Mnap antibodies specific for nap19. The observation of heteroclictic antibodies (i.e. antibodies that bind to more avidly to proteins other than the immunising protein immunogen) may be a reflection of the ability of the immune response of the animal to confer cross-protection against minor structural variants of the antigen. This

possibility was further confirmed by the labelling pattern on immunoblotting as Mnap only specifically labeled nap19 while Rnap recognized oil membrane proteins other than the 19kDa polypeptide.

4.2.1.2.3 Immunometric Assay

Immunometric assay using two different antibodies in a sandwich format is a more sensitive assay than that of direct ELISA (Kuffner et al.,1988). In order to develop an adequate immunometric assay, the antibodies used must be able to detect at least two distinct antigenic determinants. This is possible for nap19 protein. When the two antibodies Rnap and Mnap are used in a sandwich format (i.e. immunometric assay), a new trend of cross-reactivity was observed, although sigmoid kinetics were still observed. The cross-reaction with the related proteins was lower since both antibodies may only be able to bind to similar antigenic determinants. The assay as performed is a non-competitive assay since antigen binding to Rnap is in the presence of excess antibody. This may introduce bias in the assay as reflected by the antibody specificity, i.e. the preferential binding of a particular antigenic determinant, which would leave exposed fewer antigenic domains available for binding to the second antibody Mnap. Using a higher level of Rnap to prepare the immobilised phase, with the exception of nap19, all the other related proteins did not give significant cross-reactions. When Rnap was diluted to 0.1μg ml^{-1} in the immobilised phase, more species including cam16 and ole19 showed 50% cross-reactivities. This may be due to the fact that specific antibodies which are of lower avidity are present in high concentration. Thus, as they are diluted out,

the whole system becomes relatively less specific due to the presence of a high-avidity, low-titre, broad specificity antibodies.

In the two-sites assay, the cross reactivity of rad20 is significiantly diminished and is equivalent to the level of binding exhibited by soy24, which exhibited very low binding in all three asays. However, in the direct ELISA, rad20 protein gave a strong reaction to both the individual antisera Rnap and Mnap. It appears that the antibodies we raised recognize only a few and possibly a single key antigenic determinant on the rad20 protein. The inability of rad20 to bind both the antisera would imply that Rnap binding inhibits Mnap binding. This postulate is supported by evidence in the two-sites assay and also the later results on peptide mapping.

4.2.1.2.4 Immunocytochemistry

As shown in Fig.42, the three *Brassica* species are all labelled by Mnap19 which was in turn labelled with gold-conjugated anti-mouse IgG. This further indicated the ability of cross-reaction. Moreover, all the labels were evenly distributed on oil-body membranes. Non-specific labels on other structures were rarely found. Thus, antigens were common in these species and they were restricted to oil-body membrane but not other structures in seeds.

4.2.1.3 Peptide Mapping after Proteolysis

All six protein species studied (nap19, rad20, cam16, cam19, ole19 and mus20) were digestable by V8, lys-C and trypsin but exhibited different susceptibilities to proteolytic digestion. V8 cleaves at the carboxyl side of

aspartate and glutamate. Trypsin hydrolyses at the carboxyl end of the basic amino acids lysine and arginine, while lys-C cleaves at the carboxyl end of lysine. Nap19 and rad20 were more readily digestable. A minimun of 3 h digestion gave rise to clearly defined peptide fragments. Cam19 and cam16 required 18 h digestion while mus20 and ole19 needed 24 h digestion. These results may suggest that the proteins are disimilar. However, such disimilarity may partly due to artifacts. For instance, ole19 was found to resist proteolytic action even over 24 h of enzymatic digestion. It was eventually found that the low pH of the ole19 sample retarded its digestability. As soon as the pH was readjusted to about pH 7 with Tris buffer, a good proteolytic pattern could be observed. The differences in pH among the samples was believed to be due to the prolonged staining and destaining processes when the bands were rather faint by the time they were purified. The acetic acid that bound with the protein might then exceed the buffering capacity of the extraction buffer, leading to a final pH below the operational limit of proteolytic enzymes.

4.2.1.3.1 Proteolytic Maps of Denatured Proteins

The amount of similarity between the five *Brassica* species can be further compared via their respective proteolytic digestion patterns. It is believed that the more similar the digestion pattern, the more closely related are the species. Closely related peptides should carry the same cleavage sites and have similar molecular weights before they can be viewed as the same in protein gels. Thus, peptides that lay at the same band position, most probably, have the similar amino acid compositions and highly homologous amino acid sequences.

When one considers the V8 digestion pattern, Nap19, cam19, ole19 and mus20 all had digested bands more resembling each other than rad20 and cam16. The four former proteins all had the major proteolytic fragments at 16 and 14kDa. In contrast, rad20 had bands at 17 and 14kDa and cam16 has one band at 14kDa. Under lys-C digestion, mus20 became distinguishable from other three as it was hydrolysed into two major bands of 16 and 13kDa, while Nap19, cam19 and ole19 again showed more or less similar patterns. Rad20 and cam16 remained distinct following lys-C treatment. All of these proteins gave fragments between 17 to 15kDa after trypsin digestion, although they might have overlapped with trypsin itself. Information from protein gels of peptide mapping suggests that nap19, cam19 and ole19 are more likely to resemble to each other than the remaining three (cam16, rad20 and mus20). Rad20 and mus20 also have distinguishable characters.

Presumably, if different proteins that can be hydrolysed by same enzyme, giving rise to peptide fragments of the same molecular weight and retaining same antigenicity, the original proteins should have very similar amino acid sequences. When the digested proteins were immunoblotted, it was found that the 16 and 14kDa fragments that observed following V8 hydrolysis of nap19, ole19, mus20 retained their antigenicity. Only the 16kDa of cam19 and the 17kDa of rad20 were labelled but there was no label at all in cam16. Most likely, the 16kDa peptides contain a highly conserved amino acid sequence in the major oil-body membrane proteins (i.e. either 19 or 20kDa) of all of the *Brassicae* species. The labelled 15kDa fragments of nap19, cam19 and ole19 or the 14kDa fragments of rad20 and mus20 should be virtually identical for the same

reasons. Furthurmore, following the trypsin hydrolysis, only cam16 gave no label, mus20 labelled at 17kDa fragment, otherwise, the remainder had their 16kDa fragments positively labelled. Therefore, it suggested that *B. napus* and *B. oleracea* are more closely related than *B. campestris*, radish and mustard.

Additionally, the mapping result also reduces the possibility of saying that cam16 was an auto-degradation product of cam19 since none of the peptide fragments from cam16 digestions showed antigenicity wheras those from cam19 did show antigenicity. To a certain extent, cam19 and cam16 share similar antigenic determinants but their overall structures are more dissimilar to each other than similar molecular weight major oil-body membrane proteins of other brassica species.

4.2.1.3.2 Proteolytic Maps of Non-denatured Oil-body Protiens

In addition to hydrolysing pure proteins under denaturing conditions, intact proteins associated with non-delipidated oil-bodies were also found to be susceptible to proteolysis under non-denaturing conditions. The digestion patterns on SDS/PAGE gels were, as expected, rather complicated since digested peptides from other proteins on the same oil-body can be revealed at the same time. Moreover, the gels did not run well enough to give distinct bands at molecular weights below 18kDa. As stated before, this might due to the presence of lipid (most probablily, the charged phospholipid). When comparing the information obtained from immunoblotting, a general pattern could be drawn. *B.napus* and *B.oleracea* had similar patterns on all the three digestion maps and they both unique in not having any labelled bands in trypsin

and lys-C treatments. In contrast, *B. campestris* showed similarity on trypsin and lys-C digestion maps. Mustard had a more distinct pattern as it showed no labelling in both V8 and trypsin maps.

When similar digestion products were loaded onto a non-denaturing gel, quite different patterns were observed. Oil-bodies as a whole cannot migrate through the low porosity resolving gel. Hence only multiply-cut peptides were small enough to enter the gel while very small peptides were lost from the gel. Since the migration of peptide in a non-denaturing gel was governed by both charge and molecular weight, no standard can be used. But as the pattern revealed was simpler than on a denaturing gel, it facilitated the comparison of the peptide mapping. From Fig.47 a,b, it was obvious that *B. napus* and *B. oleracea* had nearly identical maps, radish and *B. campestris* coincided with the former two in the lys-C map whereas mustard remained distinct. This confirmed that nap19 and ole19 were more closely related to one another, while mus20 was more distinct.

4.2.1.3.3 Reduction in Antigenicity after Hydrolysis

There were two general phenomona in the immunoblotting of native protein digestion. First of all, fewer peptide bands were labelled. Secondly, no label at all was found in the trypsin digested oil-bodies. The fewer labelled bands in digested oil-bodies compared to digested denatured proteins showed that the overall loss of antigenicity in native proteins digestion was greater than the denature ones. This hypothesis was proved by ELISA on native and denatured proteins.

In the ELISA work, it was found that following proteolytic cleavage, the total antigenicity of both native and the denatured proteins dropped. This indicated that some of the cleavage sites were adjacent to or within the antigenic determinants. Moreover, the digested native proteins exhibited a much larger fall in their antigenicity when compared with the denatured proteins. Native proteins always showed a drop in antigenicity of more than 50% and even up to 95%, while denatured proteins had drops between 30% to 70%. This difference may suggest that membrane bound native proteins are more susceptible to proteolysis than denatured proteins, i.e., their peptide bonds may be more accessable to the enzymes. This may be due to differences in their conformations. As intact oil body preserve all the 2° and 3° structure of polypeptides, they will fold up into a more stable and less sterically hindered format. However, polypeptides are linearized in the presence of SDS and shield the bonds by side groups. Thence, their digestion is limited compared to the native proteins.

The antigenicity drop also suggested that the intact proteins are more reactive to antibodies than denatured ones. Probably, some antigenic determinants are destroyed by SDS. That is, the antibodies we raised recognised 1°, 2° and 3° structures of their antigens and it is also believed that the denatured immunogens we used may have partially renatured inside the body of animal. Thus, the effective amount of antigenic determinants on denatured proteins is lower than that of native proteins. Both hidden antigenic determinants and lower initial antigenic sites contribute to the less severe drop in antigenicity following proteolysis of denatured proteins compared to native proteins.

Furthermore, in all the cases except cam16, trypsin-digestion of proteins led to the greastest drop in antigenicity. Trypsin digestion of nap19, cam19, ole19 and mus20, also V8 digestion of cam19 and mus20, all showed drops exceeding 80%. This agreed with immunoblot data since all these proteins were undetectably labelled on Western blots. This indicated that the drop of 80% in antigenicity was sufficient to destroy nearly all the antigenic sites and the remaining sites were so low in molecular weight that they ran out of the gel. It is also suggested that most of the antigenic determinants contained the amino acids lysine and arginine which were unlikely to be linked with proline, otherwise they may be indigestable. Furthermore, lysine and arginine seem to be located on non-related antigenic determinants and the appearance of lysine on an antigenic determinant is more frequent than arginine. This argument is reinforced by consideration of amino acid composition data. The use of trypsin (which cleaves at lysine and arginine) caused a drop in antigenicity nearly twice as much as that of the drop caused by lys-C (which cleaves only at lysine), whereas the amount of arginine is about three times that of lysine - at least in the case of nap19 and rad20.

The high drop in antigenicity of intact native oil-body proteins compared to denatured proteins, following proteolysis, also gives an insight into the location of the antigenic determinant sites. The majority of the antigenic determinants must be located on the outer surface of the membrane in order to be accessable to the enzymes. More antigenic determinants are hydrophilic than hydrophobic, i.e. the antibodies are raised more specifically and more preferably to hydrophilic amino acid sequences.

The differences in the trend of drop in antigenicity between cam16 and that of cam19 and camOB further supports the notion that cam16 is not the breakdown product of cam19. The drops in antigenicity of cam16 in trypsin and lys-C digestions were very close. It is therefore suggested that arginine is less likely to be present in any extra antigenic sites other than those co-existing with lysine. When comparing the native oil-body protein , camOB had the greatest drop in antigenicity after V8 and trypsin hydrolysis but not lys-C even though it is the highest in cam16. Additionally, although the summations of reduction in cam19 and cam16 were always greater than 100% in all cases, the drops in camOB were always less than 100%. It was particularly obvious in lys-C digestion of cam16 where the drop in antigenicity exceeded that of camOB. Thus, one can conclude that cam16 is a distinct protein on camOB that can be recognized by nap19 Ab but is present in lower concentration than cam19.

4.2.1.4 Amino Acid Composition of Nap19 and Rad20

There were only minor differences in amino acid composition between rad20 and nap19. Basically, they both were rich in Gly, Glu, Leu and Pro but contain no Met and Cys at all. They also have similar hydrophobic index (Capaldi et al, 1972); nap19 was 40.6%, rad20 was 42.5 %.

4.2.2 Compositae Family

4.2.2.1 Oil-body Pattern

Their patterns on SDS/PAGE gels also looked similar but distinct. Members of the Compositae family also had the major oil-body membrane proteins at 19 or 20 kDa.

4.2.2.2 Cross-reactivities in Compositae Family

Cross reactivity within members of the same family was also observed as in the case of Cruciferae family using corresponding antibodies. Rsun20, Msun20, Msun19, Msaf19 are antibodies raised against Compositae. Generally, sun19, saf20 and *Artemisia absinthium*19 kDa were equally labelled in immunoblottings. Among the two major proteins in sunflower, sun19 is more likely to share structural or even functional similarity with other species of the same family since Msun19 could cross react with other species but neither Rsun20 nor Msun20 could do so. Also, Msaf20 and Msun19 only recognise sun19 but not sun20. These antibodies could therefore cross-react with each others antigen belonging to Compositae family. Thus, sun20 and sun19 although were labelled equally with Msun20 and Rsun20, did not originate from the same protein. A more or less similar situation were observed in cam19 and cam16, from *B. campestris*, as they both were capable of cross-reaction with antisera that raised against other *Brassiceae* species. One might suspect that the observation of major proteins of slightly differing molecular weight with similar antigencities was caused by an artifact of partial self-degradation, especially in the denaturing gel system, i.e. the higher molecular weitght protein may be degraded to release a lower molecular one that retains the same antigenic determinants. This argumnt is unlikely to be true when Msun19, Msaf are take into account. They both recognise sun19 but not sun20, suggesting that sun19 and sun20 are probably not derved from same protein. Cam19 and cam16 were also concluded to be different after peptide mapping which had been discussed in previous section(4.2.1.3). Thus, these proteins might carry a few common antigenic determinants but structurally, they were diversed.

4.2.3 Leguminosae Family

4.2.3.1 Oil-body Pattern

Similar to the observation in other families and Qu et al. (1986), members of the Leguminosae family also had their distinct protein patterns of oil-bodies on SDS/PAGE gels. Nevertheless all legumes have their major oil-body proteins located at about 24kDa even in non-oilseed species such as pea.

4.2.3.2 Cross-reactivity

Rsoy cross reacts with 24kDa bands in all species of legume tested. However, since the titre of this antibody was low, and was even worse in the case of Msoy, the cross-reaction was very weak. This could have been due to the fact that animals in Durham were all fed with soya protein concentrated feeds. The animals were thus substaintially exposed to high a dosage of soya protein, which might have reduced their sensitivity towards the soy24 antigen.

4.3 Inter-Family Similarties

4.3.1 Appearance of Oil-bodies

The various seed species from which oil-bodies were purified, including species from Cruciferae, Compositae, Leguminosae, Graminae and Solanacea families all had a similar appearance of the thick oil-pads floating on top of grinding buffer after centrifugation. In all cases, the oil-bodies were 0.5 - 1.5μm in diameter and were surrounded by an electron-dense membrane. Such similar physical appearances among different species has also been observed by other authors(Slack et al., 1980; Gurr et al., 1974).

4.3.2 Cross-reactivities

Extensive inter-family cross-reactions were observed. Some brassica antibodies also recognise the Solanacea species, tobacco (*N. tobaccum Viscosin 38*) and even Graminae species of monocots such as *Z.mays, T.durum*. Many of the proteins were later found to be localised specially on oil-bodies. In the case of tobacco, only the 14kDa and 19kDa oil-body proteins were able to cross react with Mnap, Mrad and Rmus. Similar events were also observed in the case of Rsoy which cross reacts with safflower oil-body proteins. It was thought that this might be due to the animals being inadvertantly predisposed to these antigens from the soya protein-concentrated diet that they are fed in Durham. Pre-immune sera and control sera (i.e. sera from animals that had been raised in Durham but without any artifical antigen treatment) were tested in the ELISA for the presence of endogenous anti maize, wheat, tobacco and safflower antibodies (Fig. 60-65). The negative results show that our animals were not pre-exposed to soybean, maize, wheat and tobacco proteins. Recently, Msaf20 was found to able to cross-react with *B. napus* and maize homogenates, the bands at 19kDa and 18.5kDa respectivily, were labelled in immunoblottings (Fig. 28). Although more detailed work eg. ELISA and immunocytochemistry has not yet done, this gives addition evidence on the existence of inter-family cross-reactivity.

In fact Slack et al. (1980) had already raised the similarity in oil-body pattern of linseed and safflower. They were able to demonstrate the 15.5 kDa protein from these two unrelated species had identical V8 hydrolysed peptide map although Qu et al. (1986) opposed this similarity. Interestingly, soya and

linseed both belong to Rosidae, and they may therefore have some attributes in common, as was found by Slack et al. (1980).

Base on the argument of Slack et al. (1980), the protein gels of oil-bodies were compared among different taxa (Fig.1). Signs of similarities were observed between *Brassiceae* species, maize and tobacco as they all had an extremely intense band at 18-20kDa while the 24kDa was the second abundant protein on oil-bodies of soya and safflower. Thence, all the evidence suggests that species with different oil-bodies are not neccessary unrelated, which ruled out the idea of Qu et al. (1986).

Evidence of cross-reactivitites between unrelated taxa and even between monocots and dicots had already reported with regard to plant plasma membrane proteins (Grimes, et al., 1987; Norman et al., 1986). Grimes et al. using polyclonal antibodies against tomato cell membrane proteins demonstrate the existence of a similar group of proteins on plant plasma membrane from various species. Their polyclonal antibodies were able to cross react with plasma membrane proteins from the dicotyledonous species, soybean, and also the monocotyledonous species, corn. Some clones of monoclonal antibodies that were raised against *Nicotiana glutinosa* L. plasma membrane proteins were also found able to cross react with other solanaceous species and also with other families of dicots including legumes and even monocot species such as wheat (Norman et al., 1986). Since only a few clones out of the monoclonal antibodies showed cross-reaction, it further suggested that only a particular group of proteins on plant plasma membrane was common. A similar situation is believed to exist in

oil-body membrane proteins. The ability of cross-reaction between a wide range of different taxa suggested that a similar group of proteins was present in oil-body membranes. As most of the cross-reactions were one-way (i.e. for example, Rsoy24 cross-reacts with safflower but Msaf20 does not cross-react with soybean) and not all species of same kind showed cross-reactivities (i.e. Rmus and Mrad show cross-reactivities but not Mmus nor other *Brassica* antibodies), it suggested that only a few proteins were common. These major oil-body membrane proteins most likely belong to a family of proteins which is common throughout the different plants species. They have structural similarities and probably play the same role in encapsulating seed oil-bodies.

When the immunocytochemistry of three of these species was studied, it was found that all three were labelled with Rmus. Labels were concentrated mostly on oil body membranes but other protein bodies were occassionally labelled. This result coincides with immunoblots of maize and wheat since bands other than oil membrane protein were labelled. However, in the study of tobacco, due to the availability of stock, the variety that was used for immunocytochemistry was different from that for immunblotting. This might cause the difference in the result. Moreover, the excessive labels on tobacco might be due to non-specific binding which could be eliminated if more dilute antiserum was used in later trials. Anyway, such inter-family cross-reaction implies that most probably, monocots have some antigenic determinants similar to that of *Brassicae* oil body membrane proteins. If these determinants were present in small amount and were less concentrated, only a few subpopulations of antibodies would then capable of recognising them. Moreover, the random

mixture of subpopulations in polyclonal antibodies leads to variation in the antigenic recognition. As a result, only certain brassica antibodies that we raised recognized monocot proteins. A similar explanation can be applied to soy24 antibodies since the rabbit but not mouse antibody was found cross react with safflower.

4.3.3 Comparison of Amino Acid Composition of Nap19, Rad20, L_3 from Maize and mP24 from Soya

When comparing the amino acid compositions of nap19 and rad20 with that of other oil-body membrane proteins such as the maize 16.5kDa protein (Qu,et al, 1986) and the soya 24kDa protein (Herman, 1987) for which compositional data are available, the following table is drawn:

Amino acid	Residues (mol^{-1})			
	mP24 (Herman, 1987)	L_3 (Qu, 1986)	Nap19	Rad20
Asp	9	8.1	12.2	12.3
Thr	22	4.8	12.7	11.0
Ser	11	7.3	11.3	10.5
Glu	22	9.9	14.7	16.6
Pro	10	2.8	14.3	13.9
Gly	22	15.2	17.1	17.3
Ala	42	13.4	15.5	12.3
Cys	3	0	0	0
Val	20	5.6	11.6	13.5
Met	0	2.2	0	0
Leu	21	9.0	17.1	13.7
Ile	8	3.2	9.5	8.4
Tyr	4	3.5	6.9	6.7
Phe	6	3.7	4.3	5.6
HLys	1	n.d.	n.d.	n.d.
Lys	10	4.8	5.4	5.3
His	6	4.0	4.5	3.7
Arg	14	n.d.	8.9	8.9
Trp	n.d.	0	n.d.	n.d.
Total	231	97.5	165.97	159.7
Hydrophobic index	42.6%	41.5%	40.6%	42.5%

n.d. : non-detectable

From the above summary, these major oil-body membrane proteins are common in four out of the first five richest amino acids, they are Gly, Ala, Glu and Leu. They are all moderately hydrophobic proteins (Capalai, et. al., 1972). Indeed, their indices are very close to each other. It may give an insight that they

probably share a similar biochemical role in seeds during germination. As suggested by others, they may serve as a receptor site for common enzyme(s) such as lipase. The inter-family cross-reactivities that were observed in the previous experiments also suggest that these proteins share extensive structural similarities.

4.4 Comparasion of Plant Major Oil-body Membrane Proteins and Animal Apolipoproteins

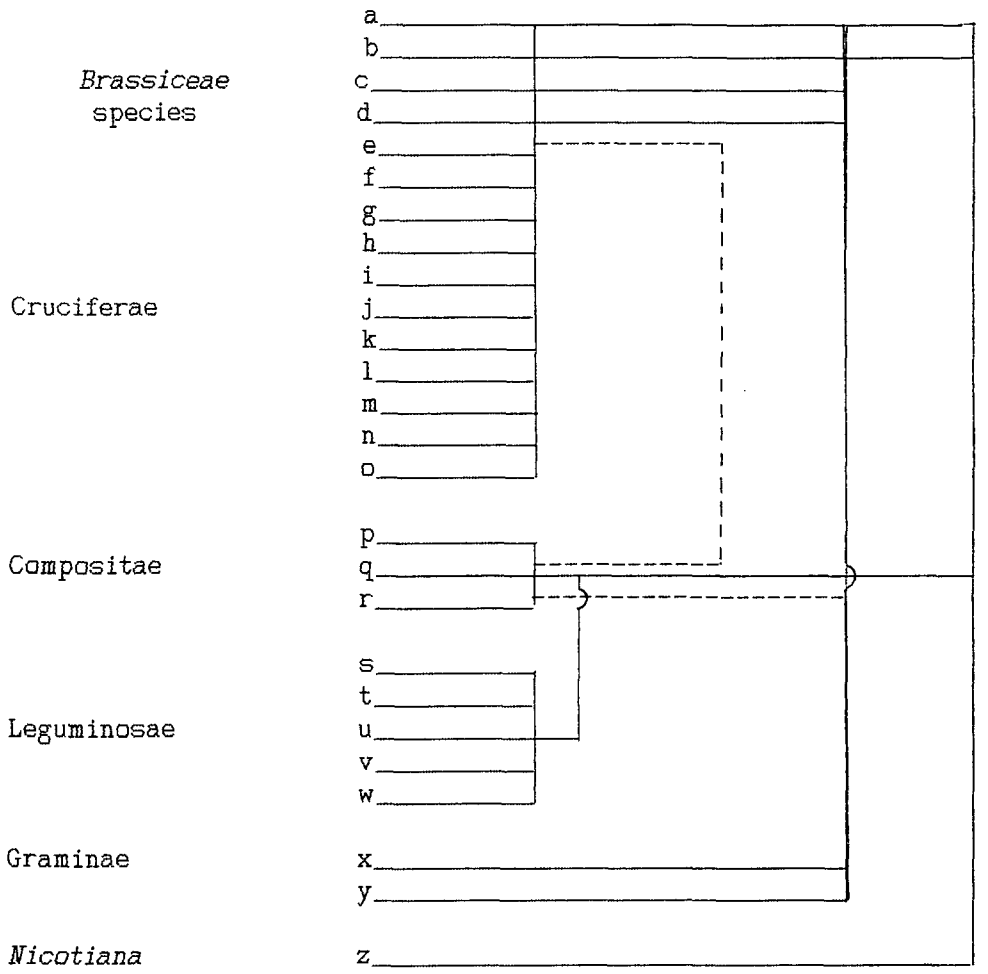
The physical appearance of animal lipoproteins was reported in the literture as a floating thick oil-pads (Angel et al.,1971) after centrifugation and their size and shape are also similar to that of plant oil-bodies. The apolipoprotein and major oil-body membrane proteins are also believed to have similar ontogenies (Herman, 1987, Murphy et al., 1988; Stein et al., 1967)

Their degree of similarity is investigated at biochemical level through comparing the amino acid composition of plant oil-body membrane proteins (as discussed in 4.3.3) with apolipoproteins of ApoB (Kane, 1980) and that of milk fat globule (Patton et al., 1975). They were all characterized by having high levels of Glu and Leu, and low in sulphur amino acids. They belong to group of moderately hydrophobic proteins with hydrophobic indices between 43 - 41 %. Such similarities between these animal lipoproteins may suggest some structural similarities and also implies similarities in function, both with each other and with the plant oil-body membrane proteins.

SUMMARY

From the above information, it is possible to draw the following conclusions. First of all, members of the same family eg. Cruciferae, Compositae or Leguminosae, show great similarity between their major oil-body membrane proteins as they readily cross-react with each other. However, these proteins of different speices within same family are not identical. Even though they have similar molecular weights, their amino acid composition and sequences are different. These differences contribute to their differential suceptibilities towards hydrolysis of peptide bonds, different proteolytic maps and differential antigenicity of these proteins. Thus, those proteins from different species having the same molecular weight and the same antigenicity should contain highly conserved amino acid domains. Among the *Brassiceae, rapeseed (Brassica napus)* is believed to be more closely related to savoy cabbage seed (*Brassica oleracea*), *Brassica campestris* comes next and then radish while mustard seed (Sinapis alba) is quite distinct from the remainder. Since radish seems to less related and mustard is the least related to other *Brassiceae* species, this may be one reason for their greater ability to cross-react to certain extent with monocotyledon proteins while the other *Brassiceae* cannot. Our results also support the idea proposed by Slack (1980) suggesting the existence of similarities between major oil-body membrane proteins from different taxanomic groups, and even further extend it to many different classes in plants including dicotyledonous and monocotyledonous oilseeds and starchy seeds .

Based on our findings, a flow diagrame on the ability of intra- and inter-families cross-reaction is summaried as below:



___ : evidence of cross-reactivity based on immunoblotting and ELISA
--- : evidence of cross-reactivity based on immunoblotting only
Each letter represents a separate plant species.
Joined letters represent separate cross-reactions.

Cross-reactions between Cruciferae and Compositae, and Cruciferae and Compositae were not found. Hopefully, when more intensive investigations are carried, the gaps can be filled in. In deed, some signs of cross-reaction

between Msaf20 and nap19 and maize 18.5kDa had been detected as shown in Fig.28 although more evidence will be required to confirm this cross-reactivity.

The animal lipid storage organelles (VLDL and chylomicrons) were observed to have similar physical appearance to seed oil-bodies (Angel, 1970). It was believed that the apolipoproteins found on animal lipoproteins should share some similarities with plant oil-body membrane proteins if they had similar functions. Based on the amino acid composition analysis data that is available from animal apolipoprotein-B (Kane, 1980), apolipoproteins of milk fat globule (Patton et al.,1975) and the four plant oil-body proteins listed in this report, it can be seen that all five proteins are rich in Glu and Leu. They all belong to a class of intermediate hydrophobic proteins and have very close hydrophobic indices. Animal apolipoproteins and plant oil-body membrane proteins need to be co-investigated in order to determine in more detail their degree of similarity. Immunological cross-reactivity tests on them may be a way to explore this.

GLOSSARY:

1. Oilseeds:

Systematic name	Common name	Abbreviation
Brassica napus	rapessed	nap
Brassica olearcea	savoy cabbage	ole
Brassica campestris	*turnip rape*	cam
Sinapis (Brassica) alba	*white mustard*	*mus*
Brassica alboglabra		
Raphanus sativus	radish	rad
Crambe maritima	seakale	cra
Thlaspi arvense		
Arabis alpina		
Arabidopsis thalina		
Berberis vulgaris		
Cochlearia officinalis		
Cheiranthus cheiri	wallflower	
Isatis tinctoria		
Matthiola bicornis		
Helianthus annuus	sunflower	sun
Carthamus tinctorius	safflower	saf
Artemisia absinthium		
Glycine max	soya/soy bean	soy
Pisum sativum	pea	

Systematic name	Common name	Abbreviation
Pisum sativum JI827	pea	
Lupinus albus	lupin	
Lupinus arboreus		
Lupinus nanus		
Reseda lutea		
Nicotiana tobaccum Wiscosin38	tobacco	
Zea mays	maize / corn	
Triticum durum	wheat	

2. Antibodies:

Animal in which antibody was raised	Full name of protein (antigen)	Abbreviation for protein	Abbreviation for antibody
mouse	rapeseed 19kDa	nap19	Mnap19
rabbit	rapeseed 19kDa	nap19	Rnap19
mouse	rapeseed total native oil-body proteins	nap native	Mnap native
mouse	mustard 20kDa	mus19	Mmus19
rabbit	mustard 20kDa	mus20	Rmus20
mouse	radish 20kDa	rad20	Mrad20
mouse	*Crambe* 20kDa	cra20	Mcra20
mouse	sunflower 20kDa	sun20	Msun20

Animal in which antibody was raised	Full name of protein (antigen)	Abbreviation for protein	Abbreviation for antibody
rabbit	sunflower 20kDa	sun20	Rsun20
mouse	sunflower 19kDa	sun19	Msun19
mouse	safflower 20kDa	saf20	Msaf20
mouse	soya 24kDa	soy24	Msoy24
rabbit	soya 24kDa	soy24	Rsoy24
mouse	linseed 20kDa	lin20	Mlin20

REFERENCES

Angel, A. (1970) Comparatmentation of lipid in adipose cells, I, Subcellur distribution, composition, and transport of newly synthesized lipid; liposomes. *Journal of Lipid Research 11:420- 432.*

Allen, C.F., Good, P., Moiienhauer, H.H., Totten, C. 1971. Studies on seeds IV Lipid composition of bean cotyledon vesicles. The Journal of Cell Biology 48:542-546.

Bergfeld, R., Hong, Y-N., Kühnl, T., and Schopfer, P. (1978) Formation of oleosomes (storage lipid bodies) during embryogenesis and their breakdown during seedling development in cotyledons of *S.alba L. Planta* 143:297-307.

Breslow, J.L. (1985) Human apolipoprotein molecular biology and genetic variation. *Annual Review Biochemistry* 54:699-727.

Capaldi, R.A., and Vanderkooi,G. (1972) The low polarity of many membrane proteins. *Processings of the Natural Academy of Science* 69:930-932.

Cleveland, Don W., Fisher, S.G., Kirschuner,M.W. and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *The Journal of Biological Chemistry* 252(3):1102-1106

Conn, E.E., and Stumpf, P.K. (1976) *Outline of Biochemistry.* 4th ed., Wiley Eastern Limited, New Delhi.

Frey-Wyssling, A., Grieshaber, E., and Mühlethaler, K. (1963) Origin of spherosomes in plant cells. *Journal of Ultrastructure Research* 8:506-16.

Fernandez, D.E., Wu, R., Huang, A.H.C., and Staehelin, L.A. (1987) Immunogold localization of the L3 portein of maize lipid bodies during germination and seedling growth. *Plant Physiology* 86:270-274.

Grimes, H.D., and Breidenbach, R.W. (1987) Plant plasma membrane proteins immunological characterization of a Major 75 kilodalton protein group. *Plant Physiology* 85:1048-1054.

Gurr, M.I. (1980) 'The biosynthesis of triacylglycerols'. In *The Biochemistry of Plant*. Ed. P.K.Stumpf. Vol.4 pp.205-248.

Gurr, M.I., Blades, J., Appleby, R.S., Smith, C.G., Robinson, M.P., and Nichols, B.W. (1974) Studies on seed oil triglycerles. Triglyceride biosynthesis and storage in whole seeds and oil bodies of *Crambe abyssinica*. *European Journal of Biochemistry* 43:281-290.

Hames, B.D. (1981) 'An introduction to polyacrylamide gel electrophoresis' In *Gel Electrophoresis of Proteins*. Eds. B.D.Hames and D. Rickwood. IRL Press, Oxford, pp.1-86.

Herman, E.M. (1987) Immunogold-localization and synthesis of an oil-body membrane protein in developing soybean seeds. *Planta* 172:336-345.

Hofsten, A.V. (1878) 'Cellular structure of rapeseed'. In *Proceedings of the 5th International Rapeseed Conference*. vol.2, pp.70-85.

Huang, A.H.C. (1987) 'Lipase'. In *The Biochemistry of Plants*. Eds. P.K. Stumpf and E.E.Conn. Vol.9, Academic Press, INC., London, p.91-119

Huang, A.H.C., Qu, R., Wang, S.M., Vance, V.B., Cao, Y.Z., and Lin, Y.H. (1986) Synthesis and degradation of lipid bodies in the scutella of maize. In *Metabolism, Structure, and Function of Plant Lipids*. Eds P.K. Stumpf, J.B. Mudd, and W.D. Nes. Plenum Press, New York, pp.239-246.

Ichihara, K. (1982) Formation of oleosomes in maturing safflower seeds. *Agricultural and Biological Chemistry*. 46(7):1767-1773.

Jacks, T.J., Yatsu, L.Y., Altschul, A.M. (1967) Isolation and characterization of peanut spherosomes. *Plant Physiology* 42:585-597.

Jones, L. (1969) The fine structure of barley aleuron cells. *Planta* 85:359-375.

Kane, J.P., Hardman, D.A, and Paulus, H.E. (1980). Heterogeneity of apolipoprotein b: isolation of a new species from human chylomicrons. *Processings of the Natural Academy of Sciences* 77(5):2464-2469.

Kuffner, T.A., McKinney, R.M., Wells, D.E., Reeves, M.W., Hunter, S.B., and Plikaytis, B.D. (1988) Two-site monoclonal antibody quantitative ELISA for toxic shock syndrome toxin-1. *Journal of Immunological Methods* 109:85-92.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

LeBoeuf, R.C., Miller, C., Shively, J.E., Schumaker, V.N., Balla, M.A., and Lusis, A.J. (1984) Human apolipoprotein B: partial amino acid sequence. *FEBS* 170(1):105-108.

Lischwe, M.A., and Ochs, S. (1982) A new method for partial peptide mapping using N-Chlorosuccinimide/Urea and peptide silver staining in sodium dodecyl sulfate-polyacrylamide gels. *Analytical Biochemistry* 127:453-457.

Mackenzie, C.G., Mackenzie, J.B., Reiss, O.K., and Philpott,D.E. (1966) Regulation of cell lipid metabolism and accumulation IV the isloation and composition of cytoplasmic lipid-rich particles. *Biochemistry* 5:1454-1461.

McCabe, J.P., Eletcher, S.M., and Jones, M.N. (1988) The effects of detergent on the enzyme-linked immunosorbent assay (ELISA) of blood group substances. *Journal of Immunological Methods* 108:129-135.

Mollenhauer, H.H., and Totten, C. (1971) Studies on Seeds II. Origin and degradation of lipid vesicles in pea and bean cotyledons. *The Journal of Cell Biology* 48:395-405.

Mollenhauer, H.H., and Totten, C. (1971) Studies on Seeds III. Isolation and structure of lipid-containing vesicles. *The Journal of Cell Biology* 48:533-541.

Morrison, I.N., Kuo, J., and O'Brien, T.P. (1975) Histochemistry and fine structure of deeloping wheat aleuron cell. *Planta* 123:105-116.

Murphy, D.J., and Cummins, I. (1988). 'Oil-body formation in developing rapeseed, *B.napus*'. In *Biotechnology for the Fat and Oils Industry*. Amerucab Oil Chemists' Society, Camparign, USA., in press.

Murphy, D.J., Cummins, I., and Kang, A.S. Synthesis of the major oil-body membrane protein in developing rapeseed embryos. Integration with storage lipid and storage protein synthesis and implication for the mechanism of oil-body formation. *Biochemical Journal,* in press.

Neville, D.M. (1971) Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophesis in a discontinous buffer system. *The Journal of Biological Chemistry* 246(20):6328-6334.

Norman, P.M., Wingate, V.P.M., Fitter, M.S., and Lamb C.J. (1986) Monoclonal Antibodies to Plant Plasma Membrane Antigens. *Planta* 167:452-459.

Norton, G., and Harris, J.F. (1975) Compositional changes in developing rape seed (*Brassica napus L.*). *Planta* 123:163-174.

Patton, S., and Keenan, T.W. (1975) The milk fat globule membrane. *Biochimica et Biophysica Acta* 415:273-309.

Qu, R., Wang, S.M., Lin, Y.H., Vance, V.B. and Huang A.H.C. (1986). Characteristics and biosynthesis of membarne proteins of lipid bodies in the scutella of maize (*Zea mays L.*). *Biochemical Journal* 235:57-65.

Rest, J.A., and Vaughan, J.G. (1972) The development of protein and oil bodies in the seed of *S.alba L. Planta* 105:245-262.

Roughan, P.G., and Slack,C.R. (1982) Cellular organization of glycerolipid metabolism. *Annual Review of Plant Physiology* 33:97-132

Schägger, H., and Jagow, G.V. (1987) Tricine-sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for the Seperation of Proteins in the Range from 1 to 100 kDa. *Analytical Biochemistry* 166:368-379.

Schmidt, M.F.G. (1981) 'Preparation of viral envelope components'. In *Techniques in Lipid and Membrane Biochemistry* Part 1. Eds. T.R.Hesketh, H.L.Kornberg, J.C.Metcaffe, D.H.Northcote, C.I.Pogson and K.F.Tipton. Elsevier Biomedical, Ireland, B419/1-15.

Slack, C.R., Bertand, W.S., Shaw, B.R., Holland, R., Browise, J., and Wright, H. (1980) Some studies on the composition and surface properties of oil bodies from seed cotyledons of safflower (*Carthamus tinctorius*) and linseeds (*Linum usitatissimun*). *Biochemical Journal* 190:551-561.

Smith, C. G. (1974) The ultrastructural development of spherosomes and oil bodies in the developing embryo of Crambe abyssinica. *Planta 119*:125-142.

Stein, O., and Stein., Y. (1967) Lipid synthesis intracellular transport, and secretion II Electron microscopic radioautographic study of the mouse lactating mammary gland. *The Journal of Cell Biology* 34:251-263.

Sorokin, H.P. (1967) The spherosomes and the reserve fat in plant cells. *Americian Journal of Botany* 54(8):1008-1016.

Stymne, S. and Stobart, A.K. (1987) Triacylglycerol biosynthesis. In *Biochemistry of Plants*. Eds. P.K. Stumpf, and E.E. Conn. Vol. 9 Academic Press INC., USA., pp.175-214.

Vance, V.B., and Huang, A.H.C. (1987) The major protein from lipid bodies of maize characterization and structure based on $_c$DNA Cloning. *The Journal of Biological Chemistry* 262(23):11275-11279.

Vance, V.B., and Huang, A.H.C. (1988) Expression of lipid body protein gene during maize seed development spatial, temporal, and hormonal regulation. *The Journal of Biological Chemistry*. 263(3):1476-1487.

Wanner, G., Formanek, H., and Theimer, R.R. (1981) The ontogeny of lipid bodies (spherosomes) in plant cells. *Planta* 151:109-23.

Wanner, G., and Theimer, R.R. (1978) Membranous appendices of spherosomes (oleosomes) possible role in fat utilization in germinating oil seeds. *Planta* 140:163-169.

Yatsu, L.Y., and Jacks, T.J. (1972) Spherosome membranes half unit-membranes. *Plant Physiology* 49:937-943.

Yatsu, L.Y., Jacks, T.J., and Hensarling, T.P. (1971) Isolation of spherosomes (oleosome) from onion, cabbage, and cottonseed tissues. *Plant Physiology* 48:675-682.