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THE EXPRESSION OF SEED STORAGE PROTEINS IN OAT, OTHER CEREALS AND IN LEGUMES

LAURIAN S. ROBERT

A thesis
presented to the University of Ottawa
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in
Biology

The University of Ottawa requires the signature of all persons using or photocopying this thesis. Please sign below, and give address and date.

DEDICATION

I wish to dedicate this thesis to my parents,
Irène and Eddy, and to my wife Michèle,
whose unfailing love and encouragement have
contributed immeasurably to the realization of
this project.

Je dédie cette thèse à mes parents, Irène et Eddy,
ainsi qu'à ma femme Michèle, puisque ce travail
représente le fruit de l'affection et de l'appui
inconditionnels qu'ils ont su me témoigner.

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ABSTRACT

The seed storage proteins of oat (Avena sativa L.) were characterized in detail. As shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) under reducing conditions, the oat globulins consist primarily of two major subunit groups, the α-subunits (30-40 kDa) and the β-subunits (20-25 kDa). These subunits were found to be associated via disulfide linkage in a 1α:1β ratio to form reducible molecules of 52 to 70 kDa. The reducible molecules assemble to form the oat 12S globulin holoprotein. Using isoelectric focusing (IEF) and two-dimensional (2-D) analysis (IEF followed by SDS PAGE), the α-subunits were demonstrated to be more heterogeneous and to possess relatively more acidic isoelectric points (pIs) than the β-subunits. These properties resemble closely those of legumin-like globulins of dicotyledonous plants.

Intervarietal differences were observed in the polypeptide composition of the globulin subunits in nine oat cultivars varying significantly in seed protein content (i.e. Hinoat, 19.1%; Dal, 18.1%; Sentinel 17.9%; Elgin, 17.0%; Harmon, 15.6%; OA-269, 15.6%; OA-424-1, 14.8%; OT-213, 14.5% and Donald, 11.9%). The differences in the total protein content of these cultivars did not appear to correlate with the presence, absence or quantity of specific globulin polypeptides.

Oat globulins are synthesized differentially during seed development as shown by the sequential accumulation of the individual components of

both the α- and β-subunit groups. The lower molecular weight components of both subunit groups are synthesized first, starting at one week post-anthesis. Differences in the total protein content of the oat cultivars Donald, Elgin and Hinoat were found not to result from developmental variations in the onset of the accumulation of the globulin fraction or specific globulin polypeptides.

The oat prolamins (avenins) obtained from the nine cultivars had molecular weights of 22 to 40 kDa, with minor components of 15 kDa and 16 kDa. Like other cereal prolamins, the avenins possess numerous charge variants upon IEF and 2-D analysis. They display a greater level of intervarietal polymorphism than the globulins and are thus better suited for cultivar identification. Cultivar variations in the avenin electrophoretic patterns did not appear to correlate with differences in total seed protein content. Major avenin accumulation during seed development does not occur until two weeks post-anthesis, that is, one week later than the onset of globulin synthesis. Avenin components varied in rate and in order of appearance within the first four weeks following anthesis.

The globulin fraction was found to constitute 65-75% of the total oat seed protein content, whereas the avenin fraction was shown to contribute as much as 20%. Thus, contrary to the results of earlier studies and unlike other cereals, the glutelin fraction is of relatively minor quantitative importance in oat. Solubilization studies along with electrophoretic and Western blot analyses have established (1) that oat glutelins contribute less than 5% of the total seed protein content and (2) that the high proportions previously allocated to the oat glutelin fraction resulted from the incomplete extractions of the prolamins and especially the globulins, which consequently became part of the residual glutelin fraction. The oat glutelin fraction may involve structural or metabolic proteins rather than storage proteins.

The observed similarity of the 12S globulin from oat (a monocotyledonous plant) and legumin-like globulins of dicotyledonous plants prompted a search for legume-like globulins in other cereals. The globulin fractions of wheat, rye and corn were shown by Western blot analyses (using antibodies raised against the purified oat 12S globulin holoprotein or its α-subunits) to contain polypeptides immunologically homologous with the α- and β-subunits of oat and pea globulins. The ∿40 kDa and ∿20 kDa subunits of wheat, rye and corn, like those of oat and pea, associate via disulfide bridging to form reducible ∿60 kDa polypeptides. In contrast to oat, the relative proportion of the wheat legumin-like proteins within the total globulin fraction was shown to be very small.

The rice glutelin fraction was also found to share common features with the oat 12S globulins: (1) rice glutelins are composed primarily of subunits of 19-22 kDa and 30-36 kDa, (2) these subunits are joined via disulfide linkage to form reducible 50-62 kDa molecules, (3) the 30-36 kDa subunits have relatively more acidic pIs than the 19-22 kDa subunits and are also more heterogenous, and (4) the rice glutelin subunits are

immunologically homologous with those of oat 12S globulin, as shown by Western blot analysis. The rice glutelin subunits were also detected within the rice globulin fraction. Hence, rice glutelin seems to be a legumin-like protein which exhibits an extreme version of the partial salt-solubility of oat 12S globulins.

The occurence of legumin-like proteins in cereals raises the possibility that globulins homologous to the other two classes of legume globulins, the 2-3S and 7-8S globulins, are also present in cereals. The wheat globulin fraction was separated by sucrose density gradient fractionation into principally two peaks of 3S and 7S. A minor shoulder of 10-11S was also apparent. The wheat (3S + 7S) fraction consists of polypeptides ranging from 10 to 70 kDa and shows features common to the oat (3S + 7S) globulins and pea vicilins upon 2-D analysis. The major polypeptides of the wheat (3S + 7S) fraction were found to be glycosylated. Western blot analysis using antibodies raised against the purified (3S + 7S) globulins of oat demonstrated the existence of antigenically related polypeptides within the pea vicilin fraction, as well as within the globulin fractions of wheat, rye, barley, rice and corn. Common regions of homology included polypeptides found at 15, 20 to 25, 40, 50 and 70 kDa.

RESUME

Les protéines du grain d'avoine font ici l'objet d'une caractérisation détaillée. Ainsi que le démontre l'électrophorèse en gel de polyacrylamide en présence de dodecyl-sulfate de sodium (SDS PAGE), les globulines d'avoine à l'état réduit sont essentiellement constituées de deux principaux groupes de sous-unités, soit les polypeptides α (30-40 kDa) et les polypeptides β (20-25 kDa). Il appert que ces sous-unités sont jointes par ponts disulfures dans la proportion 1α: 1β pour former des unités polypeptidiques réductibles. Ces unités s'associent à leur tour pour former, à l'état natif, la globuline 12S de l'avoine. En analysant les résultats de l'électrofocalisation et de l'électrophorèse bidimensionnelle, les sous-unités α se sont révélées plus hétérogènes et leur point isoélectrique (pI), relativement plus acide, par rapport aux sous-unités . Or, ces propriétés s'apparentent étroitement à celles des légumines de dicotylédones.

Des variations ont été observées au niveau de la composition des sous-unités des globulines de neuf cultivars d'avoine à teneur protéique considérablement différente (c.-à-d. Hinoat, 19,1%; Dal, 18,1%; Sentinel, 17,9%; Elgin, 17,0%; Harmon, 15,6%; OA-424-1, 14,8%; OT-213, 14,5% et Donald, 11,9%). Les différences au niveau du contenu protéique de ces cultivars ne semblent aucunement attribuables à la présence, à l'absence, ni à la quantité de certains polypeptides des globulines.

Comme en témoigne l'accumulation séquentielle des différents constituants de chacune des sous-unités α et β, la synthèse des globulines de l'avoine s'opère de façon différentielle pendant la maturation du grain. Les constituants de poids moléculaire inférieur sont synthétisés d'abord, environ une semaine après la fertilisation. Les différences dans la teneur protéique globale des cultivars d'avoine Donald, Elgin et Hinoat ne varient pas en fonction du moment où débute la phase d'accumulation des globulines, ni de la nature des polypeptides accumulés.

Le poids moléculaire des prolamines d'avoine (avénines) obtenues des neuf cultivars varie entre 22 et 40 kDa, à l'exception de certains constituants secondaires de 15 et 16 kDa. Tout comme les prolamines d'autres céréales, les avénines font preuve d'un polymorphisme accentué lorsqu'analysées par electrofocalisation ou par électrophorèse bidimensionnelle. Elles accusent un niveau supérieur de polymorphisme intervariétal par rapport aux globulines et, par conséquent, se prêtent mieux à l'identification des cultivars. Les variations remarquées chez les différents cultivars en ce qui concerne les spectres electrophorétiques des avénines ne semblent pas correspondrent aux différences dans la teneur globale des protéines du grain. Aucune accumulation d'importance en avénines ne se produit dans le développement du grain avant la deuxième semaine suivant la fertilisation, soit une semaine après le début de la synthèse des globulines. Au cours des quatre premières semaines d'accumulation, le taux et l'ordre d'apparition des constituants des avénines varient.

Des études de solubilisation, de pair avec l'analyse par électrophorèse et par la méthode dite "Western blot" ont démontrées (1) que les glutélines d'avoine comptent pour moins de 5% du contenu protéique global du grain et (2) que les pourcentages élevés antérieurement attribués à cette fraction sont imputables à l'extraction incomplète des avénines, et plus particulièrement des globulines, qui se retrouvaient ainsi avec les glutélines résiduelles. Par conséquent, contrairement au résultat d'études antérieures, et à l'opposé des autres céréales, les glutélines de l'avoine ne constituent qu'une faible proportion. La fraction glutéline de l'avoine pourrait alors tenir davantage des protéines de structure plutôt que des protéines de réserves. La fraction globuline est donc évaluée à 65-75% de la quantité protéique totale du grain, tandis que la fraction avénine représente jusqu'à 20%.

Les ressemblances observées entre la globuline 12S de l'avoine (monocotylédones) et les globulines de type légumine des dicotylédones a suscité une recherche plus approfondie des légumines chez d'autres céréales. L'analyse "Western blot" (au moyen d'anticorps engendrés par la globuline 12S de l'avoine à l'état natif ou ses sous-unités α, purifiées) a révélé que les globulines du blé, du seigle et du mais renferment des polypeptides homologues, sur le plan de l'immunologie, aux sous-unités α et β des globulines de l'avoine et du pois. Les sous-unités de ∿40 kDa et de ∿20 kDa du blé, du seigle et du mais, à l'instar de l'avoine et du pois, sont jointes par ponts disulfures pour former des polypeptides réductibles d'environ 60 kDa. Contrairement à l'avoine, la proportion relative des protéines de type légumine du blé s'est avérée infime par rapport à la fraction globuline totale.

Les glutélines du riz partagent également certains points communs avec les globulines 12S de l'avoine, à savoir: (1) les glutélines du riz se composent principalement de sous-unités de 19-22 kDa et de 30-36 kDa, (2) ces sous-unités sont jointes par ponts disulfures pour former des unités polypeptidiques réductibles de 50-62 kDa, (3) les sous-unités de 30-36 kDa ont un pI relativement plus acide que celles de 19-22 kDa, et sont également plus hétérogènes, et enfin (4) les sous-unités de glutélines du riz sont immunologiquement homologues à celles des globulines 12S de l'avoine, ce qui a été démontré à la lumière d'analyses "Western blot". En outre, les sous-unités des glutélines du riz ont également été détectées dans la fraction globuline du riz. Par conséquent, ces glutélines pourraient être des protéines de type légumine dont la solubilité partielle en solution saline serait encore inférieure à celle des globulines 12S de l'avoine.

L'occurence de protéines de type légumine chez les céréales suscite l'hypothèse qu'on puisse trouver, chez ces dernières, des globulines 2-3S et 7-8S homologues à celles présentes dans les légumineuses. La fraction globuline du blé a été séparée par fractionnement en gradient de densité de sucrose et donne essentiellement lieu à deux sommets de 3S et 7S; on peut également observer un autre point d'importance secondaire à 10-11S. La fraction du blé (3S + 7S) est composée de polypeptides variant entre 10 et 70 kDa et montre, par analyse bidimensionnelle, des caractéristiques communes aux globulines d'avoine (3S + 7S) et aux vicilines du pois. Les principaux constituants de la fraction globuline (3S + 7S) du blé sont des glycoprotéines. L'analyse "Western blot" effectuée au moyen d'anticorps engendrés par les globulines (3S + 7S) de l'avoine purifiées, démontre que les vicilines du pois, ainsi que les globulines du blé, de l'orge, du seigle, du riz et du maïs renferment des polypeptides immunologiquement apparentés. Ces plantes contiennent des polypeptides homologues de 15, 20 à 25, 50 et 70 kDa.

SCIENTIFIC PUBLICATIONS ARISING FROM THIS WORK

The original contributions of this thesis have been the subject of several manuscripts which have been published or submitted for publication:

Robert, L.S., G.J. Matlashewski, K. Adeli, C. Nozzolillo, and I. Altosaar. 1983. Electrophoretic and developmental characterization of oat (*Avena sativa* L.) globulins in cultivars of different protein content. Cereal Chem. 60 : 231-234.

Robert, L.S., C. Nozzolillo, and I. Altosaar. 1983. Molecular weight and charge heterogeneity of prolamins (avenins) from nine oat (*Avena sativa* L.) cultivars of different protein content and developing seeds. Cereal Chem. 60 : 438-442.

Robert, L.S., A. Cudjoe, C.Nozzolillo, and I. Altosaar. 1983. Total solubilization of groat proteins in high protein oat (*Avena sativa* L. cv. Hinoat): evidence that glutelins are a minor component. Can. Inst. Food Sci. Technol. J. 16 : 196-200.

Robert, L.S., C. Nozzolillo, and I. Altosaar. 1985. Homology among legumin-like polypeptides from cereals and pea. Biochem. J. 226 : 847-852.

Robert, L.S., C. Nozzolillo, and I. Altosaar. 1985. Characterization of oat (Avena sativa L.) residual proteins. Cereal Chem. in press.

Robert, L.S., C. Nozzolillo, and I. Altosaar. 1985. Homology between rice glutelin and oat 12S globulin. Biochim. Biophys. Acta. in press.

Robert, L.S., K. Adeli, and I. Altosaar. 1985. Homology among 3S and 7S globulins from cereals and pea. Plant Physiol. accepted.

Robert, L.S., C. Nozzolillo, and I. Altosaar. 1985. Developmental accumulation of seed globulins in oat (Avena sativa L.) cultivars varying in protein content. submitted to Plant Physiol.

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LIST OF ABBREVIATIONS

Tris	tris(hydroxymethyl)aminomethane
r.t.	room temperature
ME	2-mercaptoethanol
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
BIS	N,N'-methylene bisacrylamide
BSA	bovine serum albumin
IEF	isoelectric focusing
2-D	two-dimensional
IgG	immunoglobulin G
PBS	phosphate-buffered saline
NRS	normal rabbit serum
HAc	acetic acid
kDa	kilodalton
dH_2O	distilled water
pI	isoelectric point

Chapter I

INTRODUCTION

Sexual reproduction in flowering plants entails seed formation. To a flowering plant, a seed is both an end and a beginning. As the primary agent of reproduction, the seed is relied upon for both dispersal and survival. In order to fulfill these responsibilities, the seed must bear the essentials for the protection of the miniature embryonic plant it harbours in a state of suspended animation. It must also possess the necessary reserves of nutrients and the metabolic apparatus required for the early development of the seedling.

In addition to the newly emerging seedling, humanity has also developed a strong dependence on seed reserves for nutrition. This is particularly true of seeds obtained from cereal crops. In 1981 alone, world production of cereals was estimated at 1663 million tons (Lasztity, 1984). Although cereal grains store primarily starch, the reserve protein they contain is also of paramount importance to human nutrition. In fact, cereal crops not only provide approximately 60% of the calories (Righetti and Bosisio, 1981), but they also contribute 50% of the proteins consumed by humans (Pimentel et al, 1975; Belitz, 1978). In developing countries, cereals can supply on the average 80% of the dietary protein (Jansen, 1972). This dependence becomes particularly significant given the serious deficiency of cereal proteins in the essential amino acids lysine, threonine and tryptophan (Austin, 1979; Eggum and Beames, 1983).

Obviously, millions of years of selection pressures have ensured that seed reserve proteins met the specific requirements of the seedling, not man, to provide it with the best chances of survival. In view of this, scientists would like to manipulate the seed biosynthetic processes to produce proteins better suited to human requirements, but still allowing proper seedling development. However, to do so, they must first understand fully the fundamental biological mechanisms implicated directly or indirectly in seed storage protein biosynthesis. This represents an enormous undertaking to which this thesis is but a humble contribution.

1.1 DEFINITION OF A SEED STORAGE PROTEIN

Vegetable proteins have long been divided into four major fractions based on their solubility characteristics (Osborne, 1924). The albumins were defined as being "soluble in pure water and coagulable by heat", globulins were "proteins insoluble in water but soluble in saline solutions", prolamins corresponded to "the group of proteins soluble in relatively strong alcohol", while the glutelins were "those proteins which are not dissolved by neutral aqueous solutions, by saline solutions or by alcohol". The fact that this classification scheme has been used extensively and is still being employed today is a good indication of its utility and convenience. Unfortunately, it bears two important weaknesses. Firstly, because the seed is a very complex organ chemically and morphologically, and because storage proteins are usually present in an insoluble form inside the anhydrous seed environment, solubilization is not always complete and varies among different proteins and plant species. Secondly, this classification scheme reveals very little of the biochemical characteristics of the proteins involved and cannot account for the function, homogeneity or distinction of the constituents comprising the different fractions. Hence, solubility alone is not sufficient in defining a seed storage protein and additional characterization is required. Seed storage proteins are generally considered to:

(1) constitute at least 5% of the total seed protein content

(2) have no metabolic function

(3) be stored in an organelle (i.e. protein body)

(4) have a peculiar amino acid composition with a generally high amide content

(5) be composed of a limited number of related polypeptides

(6) increase preferentially with increases in nitrogen nutrition

(7) be degraded upon germination

(8) provide amino acids or nitrogen to the growing seedling

(Derbyshire et al, 1976; Miflin and Shewry, 1979a; Pernollet and Mossé, 1984).

A seed protein which satisfies the above criteria is thus, by definition, a storage protein. Knowledge of its solubility, size, charge, conjugated groups, secondary, tertiary and quaternary structure, and primary amino acid sequence should enable its distinction from other storage proteins.

1.2 CEREAL SEED STORAGE PROTEINS

The cereal grain or caryopsis is a simple dry fruit with the testa of its single seed fused to the ovary wall (pericarp). Since the cereal storage proteins of interest are found primarily in the endosperm of the seed and in order to facilitate comparisons with storage proteins of dicotyledonous seeds, the cereal grain storage proteins will be referred to as cereal seed storage proteins throughout this thesis. The genetic, biochemical and functional properties of the seed storage proteins from the major cereals have been the subject of numerous reviews (eg. Payne et al, 1982; Wrigley, 1982; wheat), (Simmonds and Campbell, 1976; rye), (Shewry and Miflin, 1982; barley), (Juliano, 1972; rice) and (Soave and Salamini, 1982; Paulis, 1982; corn.)

The fact that cereals constitute such an important portion of our daily diet, in comparison to other plant or animal foodstuffs, can be partly attributed to the special properties of the storage proteins they contain. Different cereals possess different relative proportions of the Osborne protein fractions, with the prolamins and/or the glutelins typically representing the major fractions (Table 1.1). Consequently, cereal proteins vary in their nutritional and functional values. Such variations in the proportion and composition of the nutritionally and functionally important cereal seed protein fractions necessarily govern the suitability of a cereal in providing certain food products for human consumption. For example, the superiority of wheat in providing various bread, pastry and pasta products depends largely on the quantities and

TABLE 1.1

Proportions of the different seed Osborne fractions in cereals
(% of total protein)

	Wheat	Rye	Barley	Oat	Rice	Corn
Albumins	4	4	3	0	3	5
Globulins	4	15	18	30	8	6
Prolamins	48	45	39	16	8	50
Glutelins	40	35	40	54	81	39

Mossé (1968)

particular characteristics of its prolamins and glutelins. The biosynthetic regulatory processes responsible for these phenotypic variations need to be understood.

1.3 WHY STUDY OAT?

There are essentially three stages in the development of seed storage tissue: (1) cell division - characterized by a high mitotic activity leading to the final cell number but to little increase in seed size, (2) cell expansion - involving the biosynthesis and accumulation in special organelles of proteins, starch and/or lipids, and (3) cell dehydration - where loss of water by the seed is accompanied by a decrease of metabolic activities (Muntz, 1978). In cereals, the greater portion of the storage proteins deposited during the cell expansion phase are found in the endosperm, a tissue resulting from the fusion of three nuclei and including the starchy endosperm and the proteinaceous aleurone layer. Hence, at a particular stage of seed development and within a relatively short period of time, there is a unique tissue-specific expression of a limited number of genes which results in the abundant synthesis of storage proteins. The deployment of such a concerted effort within a confined time period invokes the participation of a complex and precise genetic regulation of seed storage protein biosynthesis.

Storage proteins generally account for 80-90% of the total protein found in a mature seed. Therefore, variations in these proteins largely govern differences occurring in both the nutritive profile and total quantity of seed protein. Any information obtained on the mechanisms regulating seed storage protein biosynthesis will necessarily provide valuable clues to the appropriate and efficient man-inspired control of seed protein content and quality. Presently, one of the best approaches to gaining insight into the expression of a specific gene involves the detailed study of its product. The structure and activity of a gene can be inferred for example, from the size, quantity and primary amino acid sequence of the protein it codes for, as well as from the onset, rate and termination of protein synthesis. However, when dealing with complex genetic regulatory systems such as the one directing seed storage protein biosynthesis, this type of approach often represents a rather slow and tedious process producing only small scattered pieces of information. Fortunately, this process can be alleviated by the study of mutants or variants of the normal biological phenomenon under investigation. Scientists have repeatedly employed this strategy to penetrate intricate biological systems and decipher the important underlying mechanisms. If one hopes to learn more about the regulation of seed storage protein biosynthesis in cereals, one should specially consider a cereal such as oat which synthesizes seed proteins deviating from the normally observed cereal storage protein composition.

The oat seed contains proteins of nutritional value superior to those of other major cereals, having the best biological value and protein efficiency ratio (Howe et al, 1965; Youngs et al, 1973; Maruyama et al, 1975; Frey, 1977). The amino acid compositions of the proteins found in the major cereal grains are listed in Table 1.2. Unlike wheat, rye, barley

TABLE 1.2

Amino acid composition of cereal seed proteins
(g/100g protein)

Amino Acid	Oat[1]	Rice[2]	Wheat[3]	Rye[3]	Barley[4]	Corn[5]
Lys	4.2	3.4-4.3	2.1	3.5	3.5	2.6
His	2.2	2.5	2.3	2.1	2.2	2.8
Arg	6.9	7.4-8.8	3.7	4.5	4.8	3.8
Asx	8.9	9.5-10.4	3.4	6.8	5.3	26.9
Glx	23.9	18.3-21.1	40.5	30.5	23.4	26.9
Ser	4.2	5.7-6.9	4.1	4.1	4.4	4.8
Thr	3.3	3.8-4.1	2.5	3.3	3.6	3.6
Cys	1.6	1.4-1.8	2.5	2.6	2.2	1.1
Met	2.5	1.8-3.5	1.7	2.1	1.8	1.8
Gly	4.9	4.5-4.8	3.6	3.8	4.0	3.4
Ala	5.0	5.7-6.2	2.9	4.1	4.1	7.9
Val	5.3	6.2-7.3	4.3	5.2	5.0	5.3
Ile	3.9	4.8-5.3	3.9	4.2	3.5	3.7
Leu	7.4	7.8-9.6	7.1	6.6	6.9	13.6
Phe	5.3	5.6-6.3	6.1	5.2	5.3	5.1
Pro	4.7	4.7-5.0	12.5	15.3	11.1	8.3
Tyr	3.1	5.0-5.9	2.5	2.2	3.6	4.4
Trp	n.d.	1.0-1.4	n.d.	n.d.	1.4	0.7

[1] Robbins et al, (1971)
[2] Bressani et al, (1971)
[3] Bushuk (1974)
[4] Martens and Knudsen (1980)
[5] Baudet et al, (1966)

and corn, in which the lysine-deficient prolamins account for as much as 40-50% of the total seed protein, oat prolamins constitute only 16% of the seed protein (Table 1.1). In addition, the genus Avena displays a particularly wide variability in protein content. Estimates of the grain protein content in oat generally range from 12.4 - 25.4% (Clamot, 1979; Robbins et al, 1971), but values as high as 35% have been reported for A. sterilis (Frey et al, 1975). Furthermore, the nutritional quality of the oat proteins remains quite stable with increases in total seed protein content (Robbins et al, 1971; Maruyama et al, 1975; Peterson, 1976; Eppendorfer, 1977; Frey, 1977; Clamot, 1979). This does not hold true for other cereals (Doekes and Wennekes, 1982; Kirkman et al, 1982; Fullington et al, 1983). Obviously, the oat seed synthesizes proteins which differ in many respects from those of other cereals and just as importantly, these differences all result in a nutritionally advantageous cereal (i.e. a cereal with a high content of stable good quality protein). The occurrence of these differences provides a unique opportunity to gain insight into the regulatory mechanisms of cereal storage protein biosynthesis, yet research pertaining specifically to oat storage proteins is rather limited in comparison to the other major cereal crops.

1.4 OAT SEED PROTEIN RESEARCH

Much of the earlier work done on oat seed proteins dealt primarily with the Osborne fractionation and/or amino acid analysis of these proteins (Osborne, 1924; Csonka, 1941; Ewart, 1968; Robbins et al, 1971; Wu et al, 1972; Draper, 1973). Although there was general consensus regarding the low content of albumins and prolamins in oat, there were, and still remain, important discrepancies in the proportions allocated to the globulin and glutelin fractions. Values ranging from 5-80% of the total seed protein have been assigned to either the globulin or the glutelin fractions (Table 1.3). Such disagreement can hardly be attributed to inherent differences within the oat cultivars studied, but more likely results from variations in the extraction procedures employed by the various researchers (Kim et al, 1979a). Nevertheless, this situation renders the evaluation of these data very difficult and also points to a definite requirement for a better distinction of the various oat protein fractions.

More recently, Peterson (1976) studied the relationship between the levels of the individual oat protein fractions and total seed protein content. He reported that in different cultivars increases in total protein concentration were principally related to increased levels of the globulin fraction, which he considered the major protein fraction of the oat seed. In another study, Peterson and Smith (1976) examined the changes in total nitrogen, protein fractions and amino acids of six oat cultivars during seed development. They reported that total nitrogen concentration increased during seed development in all cultivars. They also found that the proportions of prolamins and globulins increased during maturation (2% to 11% and 28% to 55% respectively), while that of the albumins and glutelins decreased. These changes were believed to be responsible for the variations in amino acid composition observed during seed maturation. Since the individual oat protein fractions were defined

TABLE 1.3

Estimated proportions of the oat seed protein fractions (%)

Reference	Albumins	Globulins	Prolamins	Glutelins
Osborne (1924)	----	11	9	68
Csonka (1941)	-29-		16	45
Brohult and Sandegren (1954)	1	80	10-15	5
Ewart (1968)	14	16	6	67
Wu et al, (1972)	14	44	6	36
Draper (1973)	2	5	6	80
Peterson and Smith (1976)	10-11	54-56	9-13	23
Weiser et al, (1980)	20	12	14	54
Kim et al, (1979a)	8	13	14	66

chiefly on the basis of solubility, these studies inevitably become susceptible to the shortcomings associated with this approach, more particularly with respect to the composition and distinctness of these fractions.

A significant contribution to the characterization of oat globulins was later published by Peterson (1978). He found oat globulin to have a sedimentation value of 12.1S and a molecular weight of 322 kDa, as determined by analytical centrifugation. These properties are similar to those of the legume 11-12S globulins (Derbyshire et al, 1976). Upon SDS PAGE, the oat globulin was shown to consist primarily of polypeptides of 31.7 kDa and 21.7 kDa present in equimolar amounts. These polypeptides are now referred to as the α- and β-globulin subunits respectively (Brinegar and Peterson, 1982a). A model was proposed in which 6 α and 6 β subunits would associate to form the 322 kDa native oat globulin. Finally, the amino acid analysis of the individual subunits revealed the α-subunit to contain more glutamic acid/glutamine and glycine but less basic amino acids and aspartic acid/asparagine than the β-subunit. Support for the premise that globulins actually form the most abundant protein fraction in oat was provided by <u>in vitro</u> translation experiments (Luthe and Peterson, 1977). They found that the major <u>in vitro</u> products obtained mainly from membrane-bound polysomes were globulin-like proteins and suggested that the individual oat globulin subunits might possibly arise from the post-translational cleavage of a longer polypeptide.

The only other oat protein fraction which has received significant investigation is the prolamin or avenin fraction. The work of Kim et al, (1978, 1979a, 1979b) and Kim and Mossé (1979) is particularly notable. They showed avenin to constitute 10.6% of the total seed protein and to include at least eight different bands upon starch gel electrophoresis. They also demonstrated the close similarity in molecular weight, amino acid composition and N-terminal amino acid (Thr) of two avenin components (Kim et al, 1978). The inheritance of the avenin electrophoretic constituents was examined and four types of inheritance patterns were observed in the progeny: (1) identical to one parent, (2) partially recombined pattern of the parents, (3) all the bands of both parents and (4) a new band, absent in the parents. A significant degree of variability in the avenin electrophoretic patterns was also observed between and within species (Kim et al, 1979b). This variability was used to study the relationship among 17 different di-, tetra-, and hexaploid Avena species. A total of 17 distinct avenin bands was found, some being genome specific (Kim and Mossé, 1979). Finally, using polyacrylamide gel electrophoresis in 3M urea or SDS, the different oat protein fractions and their constituent polypeptides were shown to be hydrolyzed differentially during germination (Kim et al, 1979a). These authors, who considered the glutelins to be the major oat protein fraction (66%), pointed out the occurrence of possibly common bands in the globulin and glutelin electrophoretograms.

1.5 OBJECTIVE OF THE THESIS

A study of the expression of the different oat seed storage proteins within different cultivars or species requires firstly that these proteins be clearly defined and classified on the basis of several properties. The fact that in oat it is still uncertain whether the glutelin fraction (or the globulin fraction) is a major seed reserve fraction or whether the high proportions attributed to it are really extraction artefacts hinders the progress of a such a study and demands clarification. Experiments were initially aimed at the further characterization of the different types of oat seed proteins and the study of their relative contributions to the important varietal differences observed in total seed protein content. This work constitutes the first part of this thesis and is presented and discussed in Chapter III entitled: CHARACTERIZATION OF OAT SEED PROTEINS.

The second part of this thesis dealt primarily with the considerable differences observed in the storage protein composition of oat and that of other cereal grains. It is still not known whether these variations result principally from (1) the expression of different genes, or (2) the differential expression of common genes. The globulin fraction was chosen for comparative studies undertaken to gain insight into the relative importance of these two genetic mechanisms in determining the variations found in the storage protein composition of the major cereals and of legumes. The results of these experiments are presented and discussed in Chapter IV entitled: EXPRESSION OF HOMOLOGOUS GLOBULINS WITHIN CEREAL AND LEGUME SPECIES.

Chapter II

MATERIALS AND METHODS

2.1 PLANT MATERIAL

Seeds of nine oat (Avena sativa L.) cultivars: the high-nitrogen Hinoat and Dal, the commonly cultivated Sentinel, Elgin and Harmon, high-yielding OA-424-1, low-yielding OT-213 and two low-nitrogen cultivars, OA-269 and Donald; along with seeds of wheat (Triticum aestivum L. cv. Fredrick), barley (Hordeum vulgare L. cv. Perth), rye (Secale cereale L. cv. Puma) and corn (Zea mays L. cv. F64-11-6-1) were generously provided by Dr. V. Burrows, Ottawa Research Station, Agriculture Canada. Rice (Oryza sativa L. cv. M-101) seeds were kindly supplied by Dr. R. Ory, Southern Regional Research Center, USDA, New Orleans, U.S.A. Pea (Pisum sativum L. cv. Little Marvel) seeds were kindly furnished by Dr. K. Joy, Department of Biology, Carleton University, Ottawa. All seeds were obtained mature and dry and were kept at -20°C until use.

The oat cultivars Donald, Elgin and Hinoat were used for a developmental study. Each cultivar was grown in standardized four-row plots (3m long, 0.3m apart) at the Central Experimental Farm in Ottawa. The land had been summer-fallowed the previous year, and 75 kg/ha of seeds were sown. Nitrogen was applied at a rate of 33.6 kg/ha in a 10:10:10 (NPK) fertilizer mixture. The different growth stages were collected in the field at regular intervals from the time of anthesis until seed maturity. Harvested seed samples were stored at -20°C until use.

2.2 DEHULLING AND MILLING

The cereal seeds studied were first manually dehulled. Dry mature seeds were then milled to a flour in an electric coffee grinder. Immature seeds with a high moisture content were first frozen in liquid nitrogen and then finely ground with a mortar and pestle.

2.3 PROTEIN EXTRACTIONS

2.3.1 Salt-soluble proteins (albumins and globulins)

Salt-soluble proteins were extracted from seed flour according to a modification of the method used for oat by Peterson (1978). Flour samples were stirred magnetically for 2 hr at room temperature (r.t.) in 1.0 *M* NaCl, 0.05 *M* Tris adjusted to pH 8.5 with HCl. This saline solution was shown to be the most efficient in extracting oat globulins (Peterson, 1978). The flour to solvent ratio was 3.0 g/100 ml. The resultant slurry was centrifuged (15,000 x g; 30 min; r.t.) and the supernatant was dialyzed for 48 hr against several changes of distilled H_2O (dH_2O) at 4 °C to precipitate the globulins. The dialysate was centrifuged (22,000 x g; 45 min; 4°C) and both the supernatant (albumins) and pellet (globulins) were lyophilized. In some cases, the meal pellet was kept for further extractions.

2.3.2 Alcohol-soluble proteins (prolamins)

Prolamins were obtained either directly from the flour or from the meal pellet following the extraction of the salt-soluble proteins (section 2.3.1). Prolamins were extracted as above (i.e. 3.0 g/100 ml; 2 hr; r.t.) with 52% (v/v) ethanol (Kim et al, 1978) or with 55% (v/v) isopropanol, 2.0% (v/v) 2-mercaptoethanol (ME) at 60 °C (Shewry et al, 1978a). The slurry was then centrifuged (15,000 x g; 30 min; r.t.) and the pellet kept if needed for glutelin extractions. An equal volume of 0.7 M NaCl was added to the supernatant to precipitate the prolamins (this step also maintains any salt-soluble proteins in solution). The prolamins were precipitated overnight at 4°C, recovered by centrifugation (22,000 x g; 45 min; 4°C), dialyzed against dH_2O and lyophilized.

2.3.3 Residual proteins ("glutelins")

The meal pellet left after the extraction of the salt-soluble proteins (albumins and globulins) and the prolamins was air-dried. The remaining proteins (residual proteins) were solubilized by smooth homogenization of 0.4 g aliquots of residual flour in a glass tissue homogenizer for 20 min with 1.0% SDS or 7.0 M urea (4.0 ml and 5.0 ml respectively when using oat flour and 5.5 ml of either solvent when using rice flour). These extractions were performed either in the presence or absence of 1.0% ME and were followed by centrifugation (22,000 x g; 45 min; r.t.), the supernatant being kept for analysis.

2.3.4 Total seed proteins

Various solvents were tested in an attempt to extract in one step all the proteins from the oat flour. These included: acetic acid, pH 2.5; acetic acid, pH 5.0; NaOH, pH 7.3; NaOH, pH 9.2; NaOH, pH 12.1 (the pH was adjusted by the addition of 0.1 N acetic acid or 0.1 N NaOH to the slurries); 0.1 N NaOH, 0.5% SDS, 0.5% borax (Køie and Nielsen, 1977); 0.5% SDS, 0.6% ME (Juliano and Boulter, 1976) and 7 M urea, 1.0% ME. All extractions with the exception of those with SDS and urea consisted of stirring 3.0 g of flour in 200 ml of solvent for 3 hr at r.t. This was followed by centrifugation (15,000 x g, 30 min, r.t.). Each supernatant was then separately dialyzed against running water (24 hr, 10°C) prior to lyophilization. To avoid SDS or urea precipitation upon cold dialysis, extractions involving these compounds were done by homogenizing aliquots of 0.1 g of flour for 20 min with 2.0 ml of solvent in a tissue homogenizer. Each slurry was centrifuged as above and the supernatant kept for protein analysis. To determine protein extraction efficiency, the nitrogen contents of all the extracted pellets were compared to that of the intact oat flour by Kjeldahl analysis (section 2.4).

2.4 NITROGEN DETERMINATION

Nitrogen determinations were performed in duplicates (at least) on intact flour or residue samples dried overnight at 105°C using the standard micro-Kjeldahl method (AOAC, 1980). The conversion factor of 6.25 was used to calculate the protein percentage (Peterson, 1976).

2.5 AMINO ACID ANALYSIS

Duplicate lyophilized samples of cereal flour (0.5 mg) were hydrolyzed at 110°C with 0.5 ml of 6.0 N HCl, 0.1% ME under vacuum for each of three time periods of 12, 36 and 72 hr. The amino acids were determined using a Durrum D500 amino acid analyzer.

2.6 PROTEIN ALKYLATION

Certain protein samples were alkylated in order to limit disulfide-disulfide interactions and to improve resolution upon electrophoresis. Alkylation was carried out according to the method of Shewry et al (1978a). Lyophilized protein samples (10 mg/ml) were stirred overnight at 4°C in 8.0 M urea, 0.01 M KCl and 1.0% ME in 0.133 M Tris/nitrate buffer at pH 7.5. The reduced samples were then alkylated for 2 hr at 20°C by adding 1.5% (v/v) 4-vinylpyridine. The reaction was stopped by the addition of glacial acetic acid to pH 3.0. The solution was dialyzed overnight against dH_2O and the alkylated proteins lyophilized.

2.7 ELECTROPHORESIS

2.7.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

The SDS PAGE procedure was a modified version of the Laemmli (1970) method. The resolving gel contained either 10.0%, 12.0% or 14.0% acrylamide, 0.20% N,N'-methylene bisacrylamide (BIS) and 0.1% SDS in 0.375 M Tris-HCl buffer, pH 8.8. The stacking gel was composed of 3.8% acrylamide, 0.63% BIS and 1.0% SDS in 0.125 M Tris-HCl buffer, pH 6.8. In some experiments 4.0 M urea was also included in the gels. The running buffer was a solution containing 0.025 M Tris, 0.2 M glycine and 0.1% SDS. The sample buffer consisted of 8.0 M urea, 1.0% SDS, 0.0% or 1.0% ME and 0.65 M Tris, pH 6.8. Lyophilized protein samples were dissolved in the sample buffer (10 mg/ml) and protein samples already in SDS or urea solution were mixed with an equal volume of sample buffer. The samples were heated in boiling water for 2-3 min prior to electrophoresis. Gels were typically run overnight at 50-60V constant voltage on a vertical slab electrophoresis apparatus (BioRad or Hoefer). They were stained for 3-6 hr with 0.2% Coomassie Brilliant Blue R-250 in acetic acid:methanol:water (10:25:65, v/v/v). Molecular weight standards (Pharmacia) used were: phosphorylase b (94 kDa), BSA (67 kDa), BSA (55 kDa in the absence of ME, Matta et al, 1981a), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

2.7.2 Isoelectric focusing (IEF)

The IEF gels (23.0 x 11.5 x 0.1 cm) consisted of 5.0% acrylamide, 0.2% BIS, 6.0 M deionized urea and either 5.0% pH 3-10 ampholytes (Pharmalyte, Pharmacia) or 2.0% pH 3.5-10 ampholytes (Ampholine, LKB). The latter ampholytes were used preferentially when performing two-dimensional analysis (see section 2.7.3 B). The sample buffer was composed of 10 mM glycine, 6.0 M urea, 1.0% ME, 1.0% Triton X-100 (pH 8.0 adjusted with Tris). Lyophilized protein samples were dissolved in the sample buffer (10 mg/ml) and samples already in urea solution were applied directly on the gel. The gels were prefocused at 9 watts constant power for 150 volthours prior to sample application and then focused at 12 watts for 2500 volthours. They were fixed in 10% trichloroacetic acid for 1 hr and washed overnight in running tap water. The gels were stained and destained as outlined in section 2.7.1 using 0.05% Coomassie Brilliant Blue R-250. Acetylated Cytochrome C standards (pI:4.1, 4.9, 6.4, 8.3, 9.7, 10.6; USB Ltd.) and a surface pH electrode were used to verify the completeness and linearity of the pH gradient.

2.7.3 Two-Dimensional (2-D) Analysis

A. SDS PAGE (-ME) and SDS PAGE (+ME)

Protein samples were separated in the first dimension by SDS PAGE in the absence of ME (section 2.7.1). The lane of interest was cut out without prior staining or fixing and equilibrated in reducing buffer (5.0%

ME, 2.3% SDS and 0.0625 M Tris-HCl, pH 6.8) for 30 min. The lane was then placed on top of a second gel (the lane was held within a layer of 1.5% agarose) and the reduced polypeptides resolved by SDS PAGE.

A similar 2-D analysis was also performed on selected polypeptide bands. The first dimension gel (-ME) was stained for 30 min with 0.1% Coomassie Brilliant Blue R-250 in acetic acid:methanol:water (10:50:40, v/v/v) and destained for 1 hr with acetic acid:methanol:water (10:25:65, v/v/v) in order to locate the bands of interest. These were cut out, placed in a microfuge tube and crushed in liquid nitrogen. SDS PAGE sample buffer (+ME) was added and the tubes strongly agitated. Gel debris was removed by centrifugation. The supernatants containing the eluted and reduced polypeptides were then applied into separate wells of a second gel and resolved by SDS PAGE.

B. IEF and SDS PAGE

This type of 2-D analysis is based on the method developed by O'Farrell (1975). Protein samples were first separated according to their pI by IEF as described in section 2.7.2. Individual lanes were cut out (without prior fixing or staining) and equilibrated for 30 min in 2.3% SDS and 0.0625 M Tris-HCl, pH 6.8. Each lane was then placed on top of a second gel and resolved according to molecular weight by SDS PAGE. The IEF lane was held in place within an additional layer of stacking gel. Following electrophoresis the 2-D gel was stained for 2 hr with

0.2% Coomassie Brilliant Blue R-250 in acetic acid:methanol:water (10:40:50, v/v/v). In some cases, silver staining was employed to promote the detection of minor polypeptides. The procedure followed was that outlined in Bulletin 1089, BioRad Silver Stain Kit.

All electrophoretic experiments reported in this thesis were, at the very least, duplicated. There was no significant gel to gel variation in the electrophoretic patterns. This is in agreement with the inherent stability of the seed storage proteins and is also indicative of intravarietal homogeneity. Equal amounts of sample were loaded on the gels unless stated otherwise in the accompanying figure legends.

2.8 SUCROSE DENSITY GRADIENT CENTRIFUGATION

Globulin samples were dissolved in 1.0 M NaCl, 50 mM Tris-HCl, pH 8.0 and fractionated at 27,000 x g for 24 hr on 5-20% continuous sucrose gradients made in the same buffer (Burgess et al, 1983). The gradients were monitored at 280nm and collected in 1.5 ml fractions. The appropriate fractions were pooled and dialyzed overnight against dH_2O. The precipitated protein was recovered by centrifugation and lyophilized.

2.9 CONCANAVALIN A SEPHAROSE CHROMATOGRAPHY

The globulin sample from wheat was incubated with Con A Sepharose (Sigma) for 30 min and the suspension poured into a column. Non-bound material was removed with 0.5 M NaCl, 0.05 M Tris-HCl, pH 7.4 and the bound proteins were eluted with 0.2 M α-methyl-D-glucoside (Adeli and Altosaar, 1984). Proteins were precipitated from the bound and non-bound fractions with trichloroacetic acid and analyzed by SDS PAGE.

2.10 ANTIBODY PREPARATION

Four different antibody preparations were used in this work. All of them were obtained from New Zealand White rabbits and employed oat globulins as antigens. The first preparation was raised against the total oat globulin fraction and was kindly provided by Dr. G. Matlashewski, Department of Biochemistry, University of Ottawa, Ottawa, Canada. The second preparation was raised against the purified acidic (α) subunits of oat 12S globulin obtained by preparative SDS PAGE (Matlashewski, 1983). This antibody preparation was generously donated by Dr. B. J. Miflin, Biochemistry Department, Rothamsted Experimental Station, Harpenden, England. The third and fourth antibody preparations were raised separately against the 12S holoprotein or the 3S and 7S holoproteins of oat globulin, which had been purified by isoelectric point precipitation and three cycles of sucrose density gradient centrifugation (Burgess et al, 1983; Adeli and Altosaar, 1984). These last antibody preparations were kindly supplied by Mr. K. Adeli, Department of Biochemistry, University of Ottawa, Ottawa, Canada. Separate IgGs were purified by affinity chromatography on protein A Sepharose as described by Matlashewski et al, (1982).

2.11 IMMUNODIFFUSION ANALYSIS

Double diffusion immunoprecipitation was carried out according to Ouchterlony (1968). Diffusion plates contained 1.5% agarose in 0.5 M NaCl, 20 mM NaH_2PO_4, pH 7.4. Globulin samples were dissolved, without prior freeze-drying in 1.0 M NaCl, 1.0% Triton X-100, 0.05 M Tris, pH 8.0 and placed in the antigen wells. The centre well contained the appropriate IgG preparation. Diffusion was allowed to proceed overnight at room temperature. Precipitin lines were photographed against a dark background.

2.12 WESTERN BLOT ANALYSIS

Labelling of IgG with ^{125}I was performed according to a modification of the procedure utilized by Greenwood et al, (1963). One mCi of $Na^{125}I$ (low pH, high concentration, NEN) was added to 50 µg of IgG in PBS (0.14 M NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$7H 0, 1.5 mM KH_2PO_4, pH 7.4). Ten µl of 18 mM chloramine T in PBS was added and the solution incubated on ice for 10 min. Following the addition of 10 µl of 52 mM sodium metabisulfite in PBS, the solution was applied to a 10 ml Sephadex G-25 column. The first peak fractions (containing the labelled IgG) were pooled, dialyzed against PBS and diluted with 30 ml of 5.0% BSA, 3.0% NRS in PBS. The specific activity was approximately 3 Ci/µg of IgG.

Western blotting was carried out similarly to the method of Towbin et al, (1979). Immediately following SDS PAGE, proteins were transferred electrophoretically onto nitrocellulose sheets (0.45 m pore size, Schleicher & Schuell, BA85) using a Hoefer transfer unit. The transfer buffer consisted of 25 mM Tris and 192 mM glycine in 20% methanol. Transfer at 135 mA had to be performed for at least 24 hr for efficient protein transfer. The gels were stained and destained to verify completeness of transfer and the nitrocellulose sheets were incubated for 1 hr at 40°C in 50 ml of 3.0% BSA and 3.0% NRS in PBS. The addition of NRS to the latter solution reduced background radioactivity significantly. After washing in PBS (2 x 15 min) the sheets were incubated for 6 hr in the hybridization mixture containing the appropriate ^{125}I- labelled IgG. The nitrocellulose sheets were then washed in PBS (6 x 10 min), air dried and exposed overnight at -70°C to Kodak X-Omat AT film in the presence of an intensifying screen.

Several control experiments were performed to verify the specificity of the antibody preparations utilized. No cross-reactivity was detected upon immunodiffusion analysis when the different antibody preparations were allowed to cross-react with the other oat seed protein fractions; including the 3S and 7S globulins in the case of the 12S globulin holoprotein antibody and vice versa (results not shown; Matlashewski et al, 1982; Adeli and Altosaar, 1984). Accordingly, each antibody preparation cross-reacted specifically with the antigen it had been raised against (see Chapters III and IV). The pre-immune serum did not cross-react with any of the oat protein fractions (results not shown). Upon Western blot analysis none of the antibody preparations reacted non-specifically to the nitrocellulose (for eg. see Figs. 4.2 and 4.19).

Molecular weight did not affect cross-reactivity since the antibodies did not bind to the molecular weight markers (results not shown). The quantity of protein on the nitrocellulose did not affect cross-reactivity since it was possible to observe both minor polypeptides cross-reacting strongly and major polypeptides not cross-reacting at all (for eg. compare Figs. 4.19 and 4.20). Finally, it was possible to observe a direct relationship between the cross-reactivity pattern and the taxonomic relationship of the species involved, a phenomenon which does not favour non-specific antibody binding (for eg. see Fig. 4.19).

Characterization and comparison of seed storage proteins in this thesis will be based on the assumption that the polypeptide composition of these proteins was not affected significantly by the environmental conditions to which the plants were exposed (for review see - Ladizinski and Hymowitz, 1979).

Chapter III

CHARACTERIZATION OF OAT SEED PROTEINS

3.1 RESULTS

3.1.1 Characterization of oat globulins

The groat (dehulled seed) protein concentrations of the nine oat (Avena sativa L.) cultivars used in this study were estimated by Kjeldahl analysis and are listed in Table 3.1. The nine cultivars ranged from 11.9 ± 0.1% to 19.1 ± 0.3% in protein concentration. The SDS PAGE patterns obtained for the globulin fractions from the nine different cultivars are shown in Figure 3.1. All nine globulin extracts exhibit a banding pattern similar to that previously described for the cultivar Froker by Peterson (1978). Two major subunit groups are consistently present. The α-subunits ranging from 30-40 kDa are comprised of one to three major bands and up to three minor ones, whereas the β-subunits found between 20-25 kDa include up to four bands of which at least one is predominant. Considerable differences in the position and intensity of protein bands were especially evident among the α-subunits of the various cultivars. Minor bands can also be seen at approximately 12-17 kDa and 50-70 kDa in all the cultivars.

TABLE 3.1

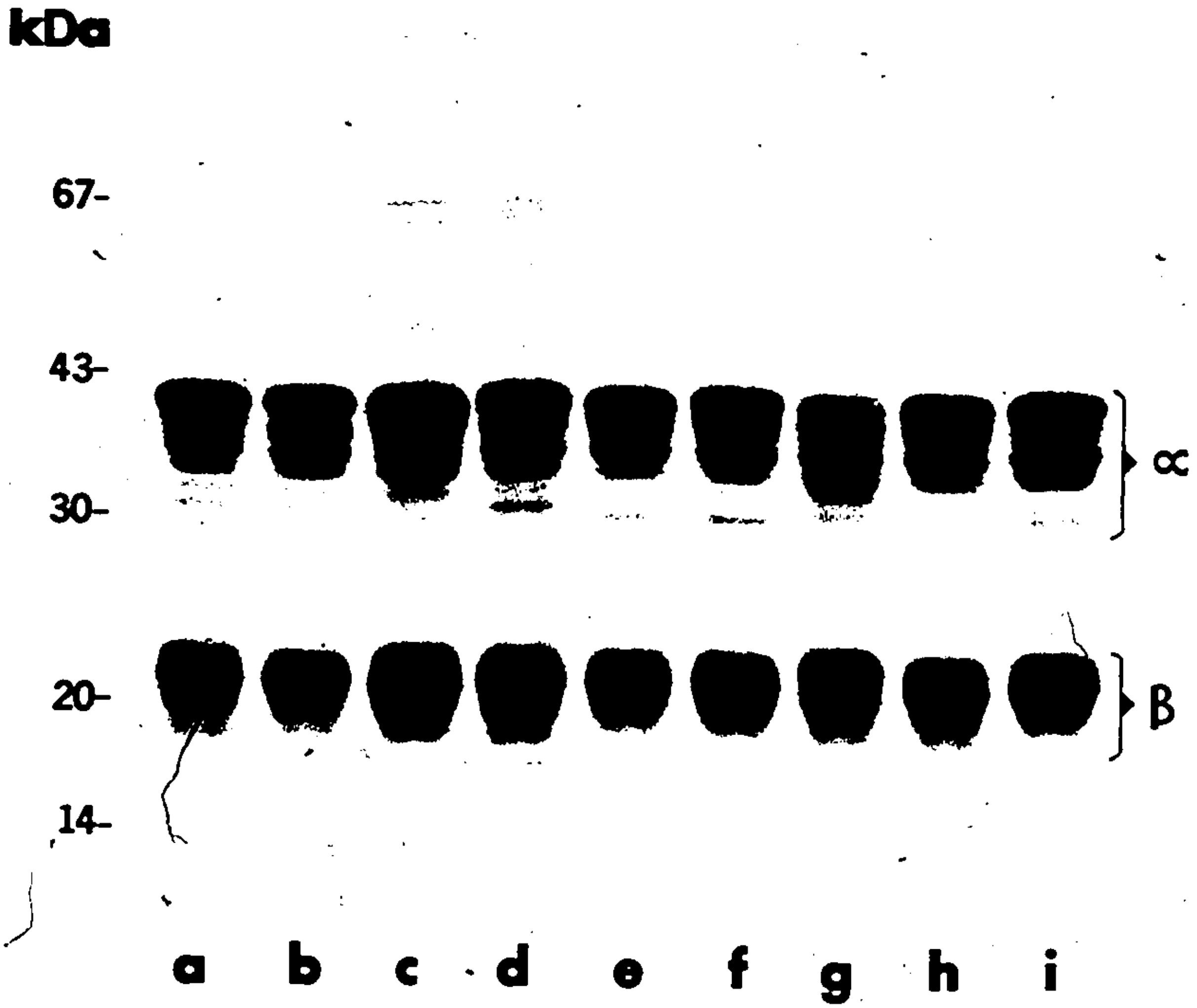
Groat protein concentration[1] of nine oat (*Avena sativa* L.) cultivars

Cultivar	Total Protein (g/100g)	Total Protein (mg/seed)
Hinoat	19.1 ± 0.3[2]	5.6 ± 0.1
Dal	18.1 ± 0.7	4.8 ± 0.2
Sentinel	17.9 ± 0.8	5.5 ± 0.2
Elgin	17.0 ± 0.5	5.9 ± 0.1
Harmon	15.6 ± 0.4	4.4 ± 0.1
OA-269	15.6 ± 0.4	4.6 ± 0.1
OA-424-1	14.8 ± 0.2	6.0 ± 0.1
OT-213	14.5 ± 1.0	4.1 ± 0.3
Donald	11.9 ± 0.1	3.7 ± 0.0

[1] Each nitrogen determination is a mean of at least six Kjeldahl values (N X 6.25).

[2] Sample standard deviation.

Figure 3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) in the presence of 2-mercaptoethanol of the total globulin fraction extracted from the oat (Avena sativa L.) cultivars: (a) Hinoat, (b) Dal, (c) Sentinel, (d) Elgin, (e) Harmon, (f) OA-269, (g) OA-424-1, (h) OT-213 and (i) Donald. The α-subunits (30-40 kDa) and β-subunits (20-25 kDa) are bracketed. The molecular mass of the standards is indicated in the left margin and expressed in kilodaltons (kDa).


kDa
67-
43-
30-
20-
14-
α
β
a
b
c
d
e
f
g
h
i

The globulin electrophoretograms were altered dramatically when resolved in the absence of a reducing agent (Fig. 3.2). The α- and β-subunit groups are absent and all the cultivars display only one major group of proteins ranging from 52-70 kDa . There are generally two or three major bands within this group, with variations occurring among cultivars. Minor protein bands are also present mainly in the 12-17 kDa and 35-40 kDa regions.

The oat globulin fraction was resolved by 2-D SDS PAGE analysis: the sample was first electrophoresed in the absence of a reducing agent as in Fig. 3.2 and the lane was cut out, equilibrated in reducing buffer and resolved in a second SDS PAGE gel (as described in section 2.7.3 A). The results show conclusively that the majority of the 52-70 kDa globulins break down to the α- and β-subunit groups under reducing conditions (Fig. 3.3). Proteins of 12-17 kDa and some proteins in the 52-70 kDa region are not involved in disulfide associations. Higher molecular weight proteins (>100 kDa) which become apparent upon greater sample loading under non-reducing conditions also break down to the α - and β-subunits. These proteins likely result from associations of the 52-70 kDa reducible proteins.

The reduced globulin fractions from the nine cultivars were separated by IEF in a pH 3.5 to 10 gradient (Fig. 3.4). A larger number of protein bands is evident upon IEF in comparison to SDS PAGE (Fig. 3.1). The bands cover practically the entire pH range but appear divided into a basic and a relatively more acidic group. However, in the absence of a

Figure 3.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) in the absence of 2-mercaptoethanol of the total globulin fraction extracted from the oat (Avena sativa L.) cultivars: (a) Hinoat, (b) Dal, (c) Sentinel, (d) Elgin, (e) Harmon, (f) OA-269, (g) OA-424-1, (h) OT-213 and (i) Donald.

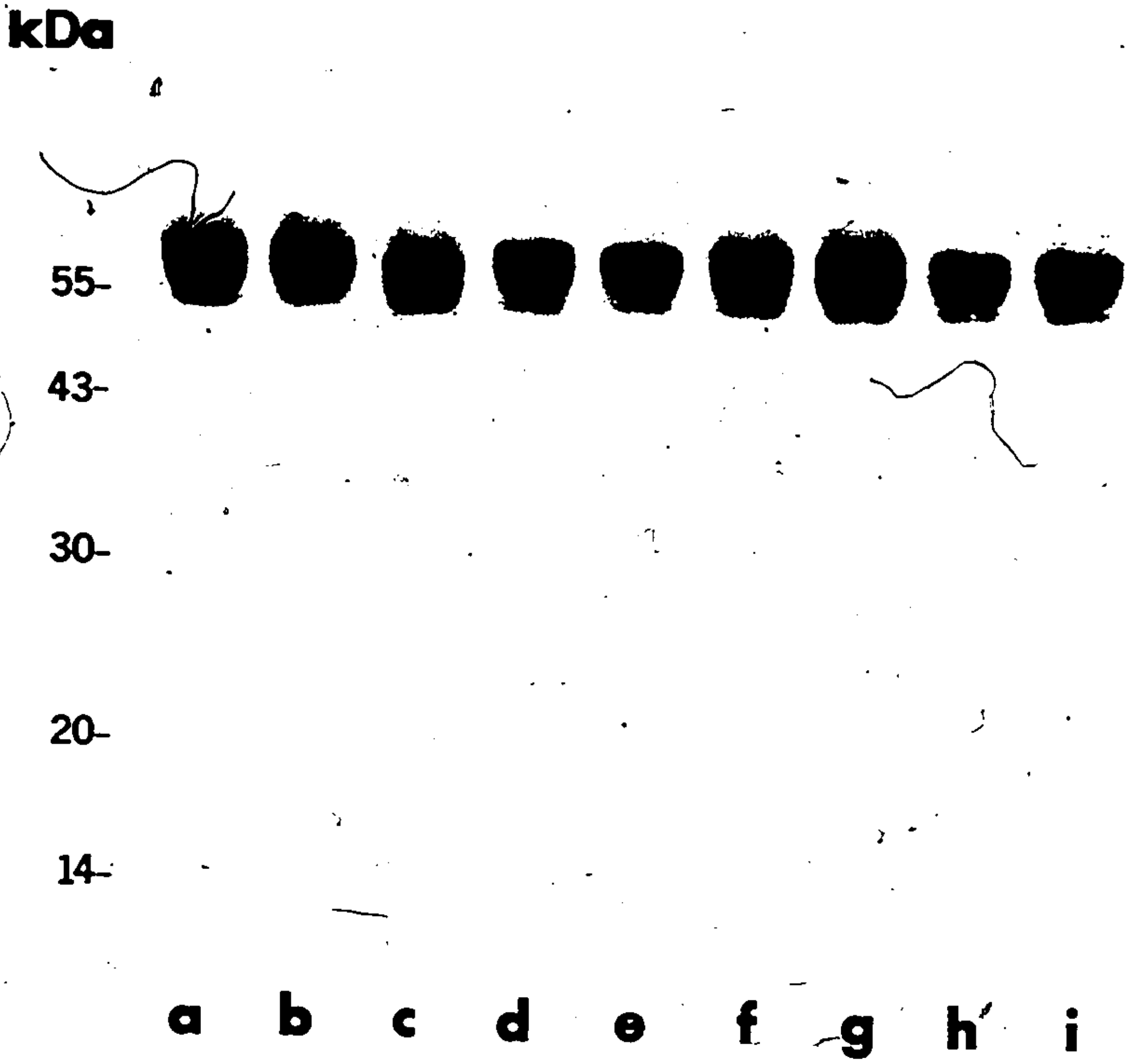

kDa
55-
43-
30-
20-
14-
a
b
c
d
e
f
g
h
i

Figure 3.3. Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.3A) of the total globulin fraction extracted from the oat cultivar Hinoat (first dimension resolved in the absence of 2-mercaptoethanol and the second dimension in its presence). To facilitate the interpretation of the results, a lane equivalent to the unreduced globulin lane which was electrophoresed in the second dimension was stained, similarly placed on top of the second gel and included in the photograph. Arrows indicate the direction of electrophoresis of the first and second dimensions.

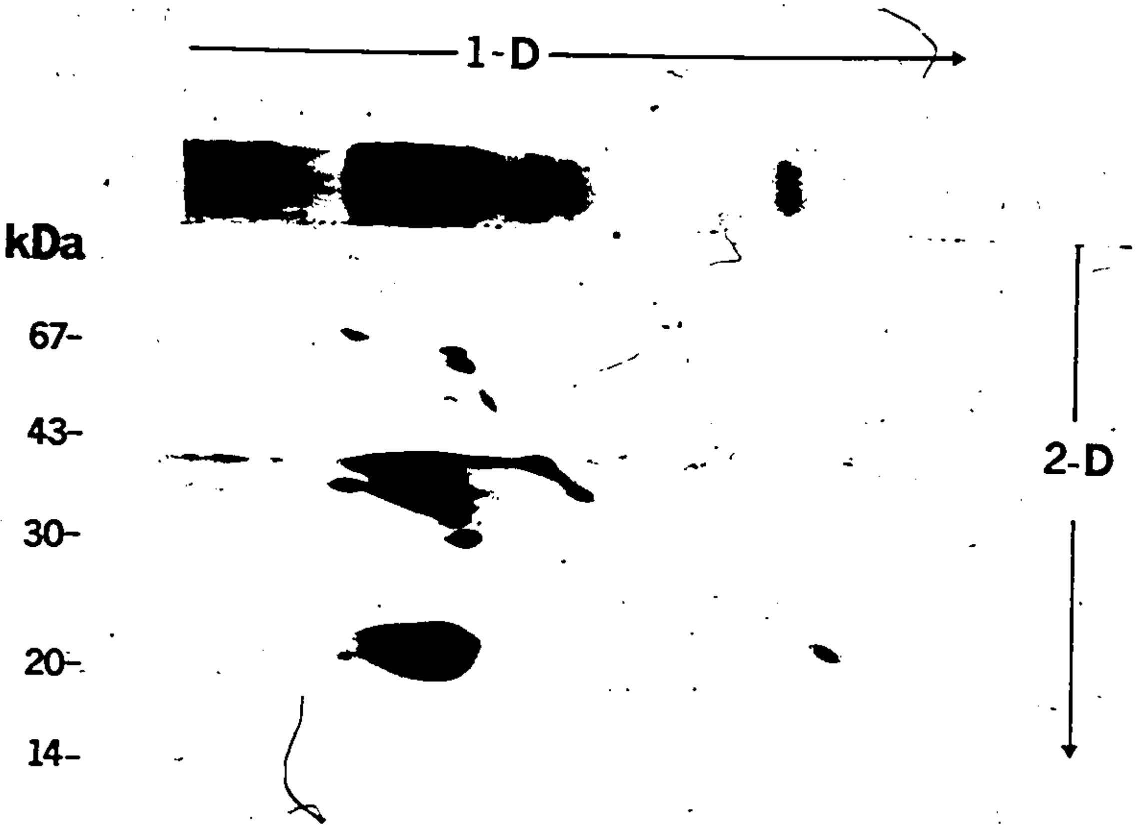
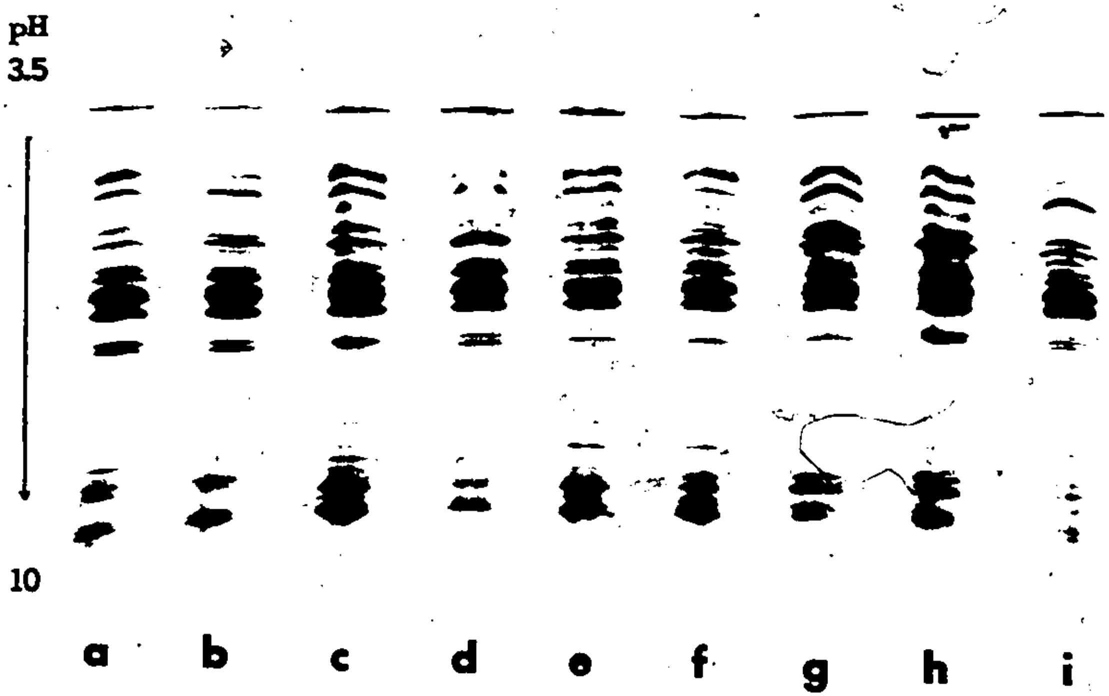

1-D
kDa
67-
43-
30-
20-
14-
2-D

Figure 3.4. Isoelectric focusing (section 2.7.2; pH 3.5-10) in the presence of 2-mercaptoethanol of the total globulin fraction extracted from the oat (Avena sativa L.) cultivars: (a) Hinoat, (b) Dal, (c) Sentinel, (d) Elgin, (e) Harmon, (f) OA-269, (g) OA-424-1, (h) OT-213 and (i) Donald.


pH
3.5
10
a
b
c
d
e
f
g
h
i

reducing agent, the globulin IEF patterns differ (Fig. 3.5). A decrease in the number of polypeptides with the more acidic and basic pIs is observed, with most protein bands occurring near the neutral region.

The large number of globulin IEF bands necessitated a 2-D analysis to reveal the charge distribution of the separate subunit groups. The reduced Hinoat globulins were first separated by IEF and then by SDS PAGE (Fig. 3.6). The α-subunits are found to be relatively acidic whereas the constituents of the β-subunit group are shown to be more basic. In spite of the large difference in protein content, the Hinoat globulin 2-D pattern is very similar to that of Donald, the cultivar with the lowest protein content (Fig. 3.7). In both cases, the subunit groups exhibit a greater level of polypeptide heterogeneity than was apparent upon SDS PAGE alone. This is especially true of the α-subunits which comprise numerous proteins spots. In a similar 2-D analysis of the unreduced Hinoat globulins (Fig. 3.8), the disulfide-linked dimers also displayed an increased amount of heterogeneity in comparison to their SDS-PAGE pattern.

Globulins were extracted from Donald (11.9% protein), Elgin (17.0%) and Hinoat (19.1%) seeds harvested at regular intervals during development following anthesis (Fig. 3.9). These were analyzed by SDS PAGE under reducing conditions (Fig. 3.10, A, B and C respectively). In all three cultivars, the seeds from the first three weeks post-anthesis (harvests 1 to 4) display globulin patterns which differ from the mature globulin profile (harvest 7). In the earlier stages of development, the

Figure 3.5. Isoelectric focusing (section 2.7.2, pH 3.5-10) in the absence of 2-mercaptoethanol of the total globulin fraction extracted from the oat (*Avena sativa* L.) cultivars: (a) Hinoat, (b) Dal, (c) Sentinel, (d) Elgin, (e) Harmon, (f) OA-269, (g) OA-424-1, (h) OT-213 and (i) Donald.

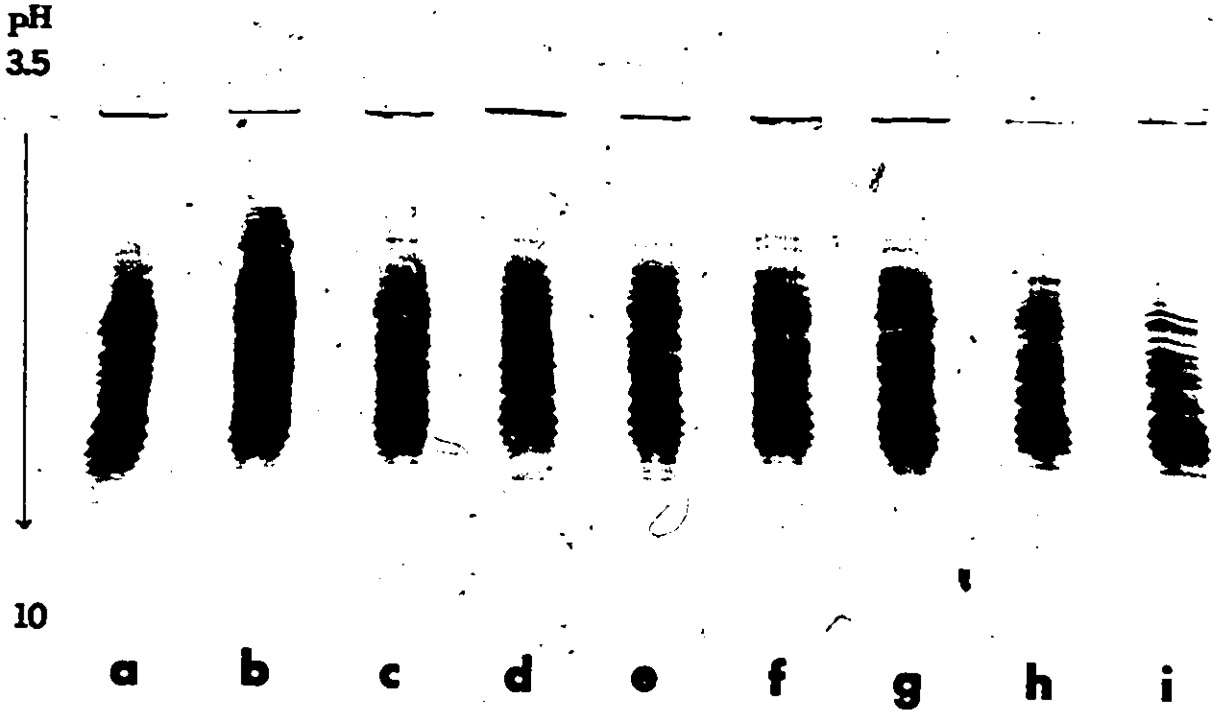

pH
3.5
10
a
b
c
d
e
f
g
h
i

Figure 3.6. Two-dimensional analysis (section 2.7.3B) in the presence of 2-mercaptoethanol of the total globulin fraction extracted from the oat cultivar Hinoat. Globulins were separated in the first dimension by isoelectric focusing (IEF; pH 3.5 (+) to 10 (-)) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). A standard SDS PAGE lane of reduced Hinoat globulins is included for comparison.

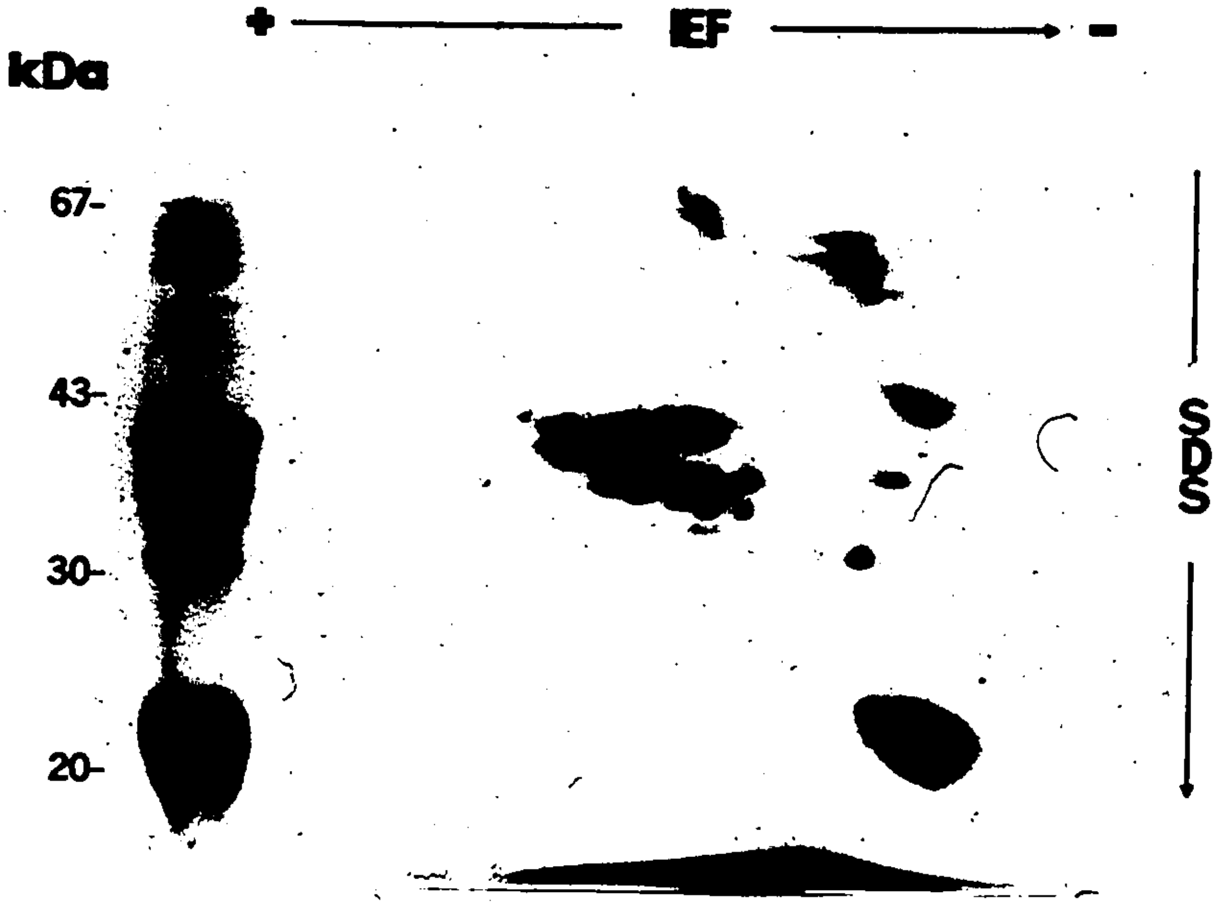

+
IEF
-
kDa
67-
43-
30-
20-
SDS

Figure 3.7. Two-dimensional analysis (section 2.7.3B) in the presence of 2-mercaptoethanol of the total globulin fraction extracted from the oat cultivar Donald. Globulins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of reduced Donald globulins is included for comparison.

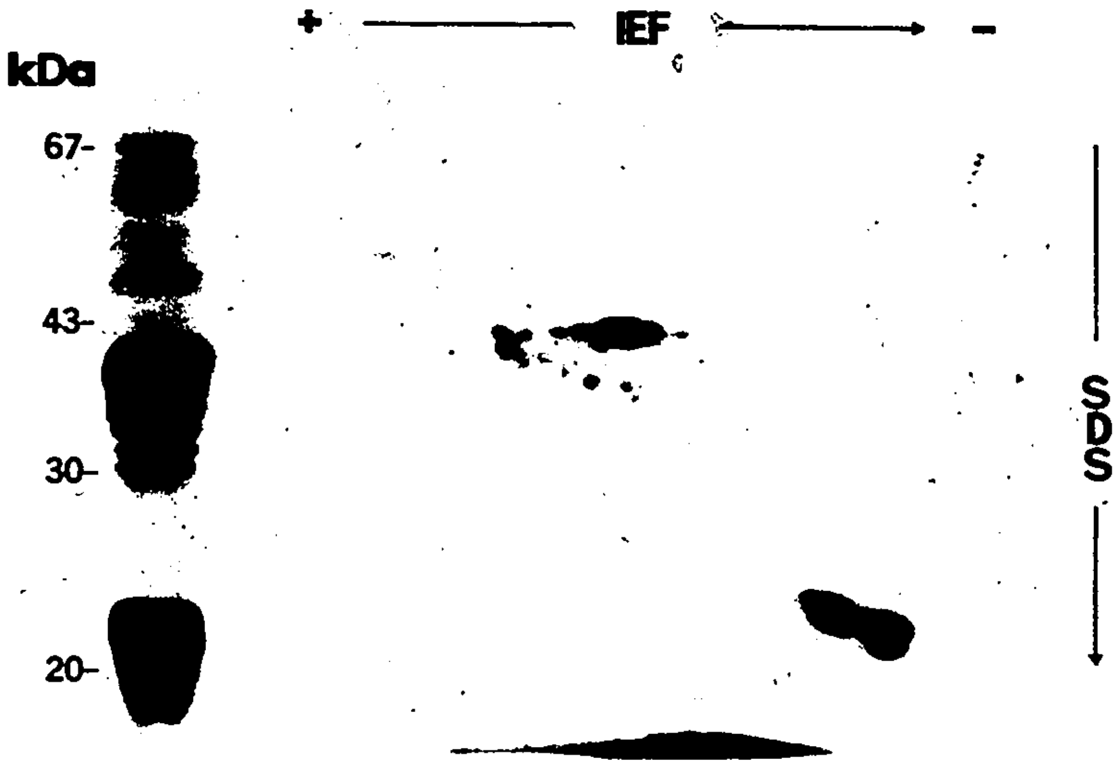

+
IEF
−
kDa
67-
43-
30-
20-
SDS

Figure 3.8. Two-dimensional analysis (section 2.7.3B) in the absence of 2-mercaptoethanol of the total globulin fraction extracted from the cultivar Hinoat. Globulins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of unreduced Hinoat globulins is included for comparison.

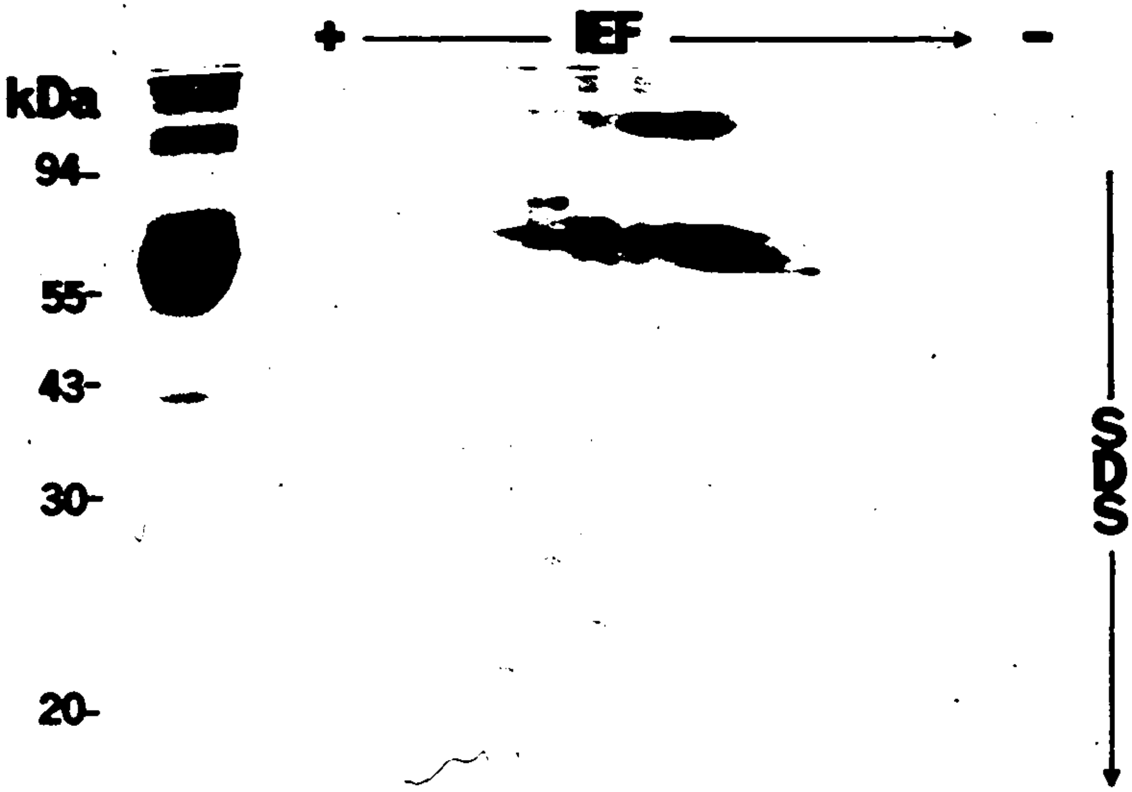

+
IEF
−
kDa
94
55
43
30
20
SDS

Figure 3.9. Donald, Elgin and Hinoat seeds harvested at seven consecutive intervals following anthesis. Number (1) corresponds to 7 days, (2) 12 days, (3) 16 days, (4) 21 days, (5) 28 days, (6) 35 days and (7) 42 days post-anthesis.

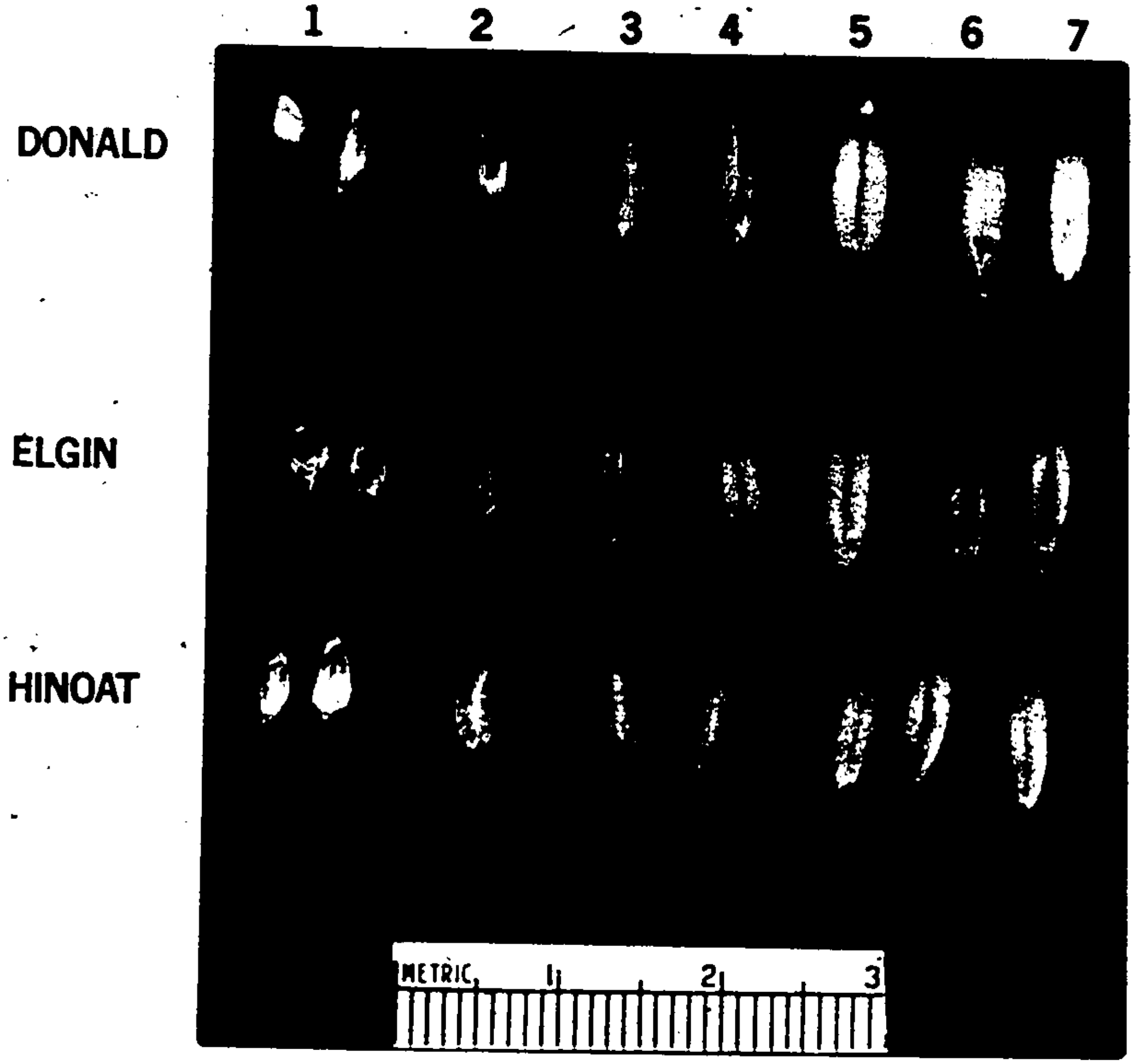
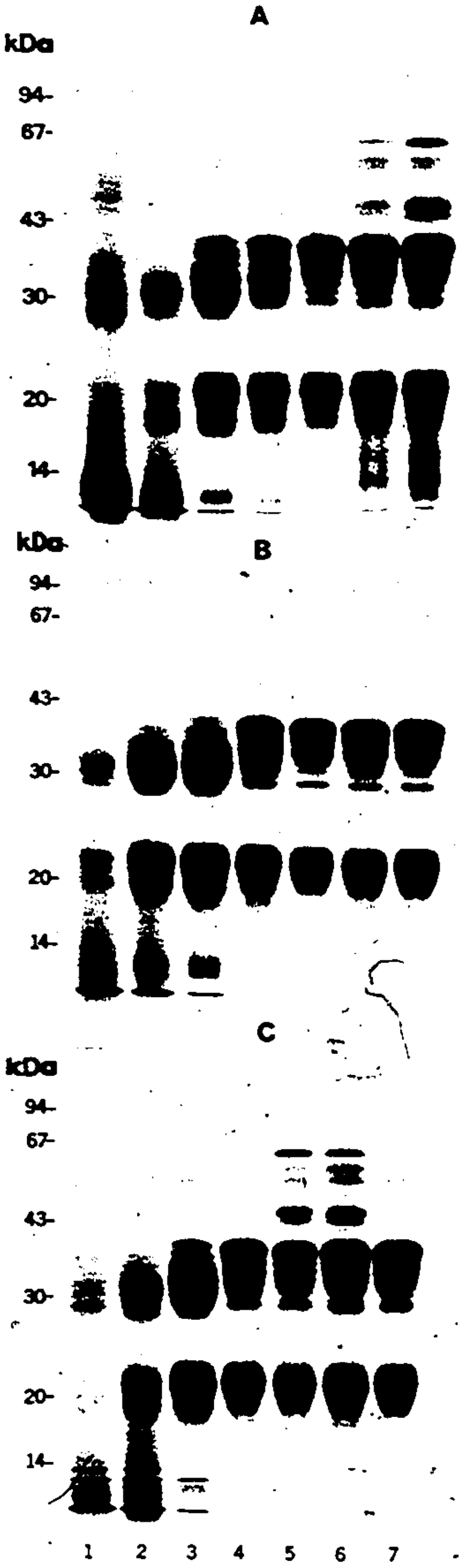

1
2
3
4
5
6
7
DONALD
ELGIN
HINOAT
METRIC
1
2
3

Figure 3.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) in the presence of 2-mercaptoethanol of the total globulin fraction extracted from (A) Donald, (B) Elgin and (C) Hinoat seeds collected at seven different times during development. The numbers 1 to 7 correspond to increasing stages of seed maturity as shown in Fig. 3.9. Differences occur during seed development in the proportion of non-protein material extracted by a saline solution. Therefore different amounts of sample were applied for the various stages: stage (1) 500 μg, (2) 400 μg, (3) 300 μg, (4) to (7) 200 μg.


A
kDa
94-
67-
43-
30-
20-
14-
kDa-
B
94-
67-
43-
30-
20-
14-
C
kDa
94-
67-
43-
30-
20-
14-
1
2
3
4
5
6
7

lower molecular weight components of both the α-subunit group (approx. 28 to 34 kDa) and the β-subunit group (approx. 17 to 22 kDa) predominate. However, their relative proportions gradually diminished until the mature globulin profile, in which the higher molecular weight components of the subunit groups predominate, was apparent at approximately the fourth week post anthesis (harvest 5). This phenomenon was consistent with the three cultivars examined.

To verify whether the lower molecular weight subunit group components of the earlier developmental stages were also disulfide-linked globulins, the globulin extracts from developing Donald, Elgin and Hinoat seeds were subjected to SDS PAGE in the absence of a reducing agent (Fig. 3.11, A, B and C respectively). Globulin patterns of developmental stages 2 to 7 display a predominance of the reducible molecules (52 to 70 kDa) in all three cultivars. However, in stages 2 to 4 the lower molecular weight reducible molecules are present to a relatively greater extent. These would presumably consist of the lower molecular weight α- and β-subunits described above, joined by disulfide linkage. As the seed matured, biosynthesis of the relatively higher molecular weight reducible molecules prevailed and the mature pattern can be observed in the last three harvests. Very little globulin is evident at the first stage which corresponds approximately to the first week post-anthesis. Developmental variations in polypeptide proportion and composition of globulins extracted from Donald seeds are also seen upon IEF (Fig. 3.12). However, in comparison to SDS PAGE, differences between the earlier and later stages of seed development are not as evident.

Figure 3.11. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) in the absence of 2-mercaptoethanol of the total globulin fraction extracted from (A) Donald, (B) Elgin and (C) Hinoat seeds collected at seven different times during development. The numbers 1 to 7 correspond to increasing stages of seed maturity as shown in Fig. 3.9. Differences occur during seed development in the proportion of non-protein material extracted by a saline solution. Therefore different amounts of sample were applied for the various stages: stage (1) 500 μg, (2) 400 μg, (3) 200 μg, (4) 120 μg, (5) to (7) 90 μg.

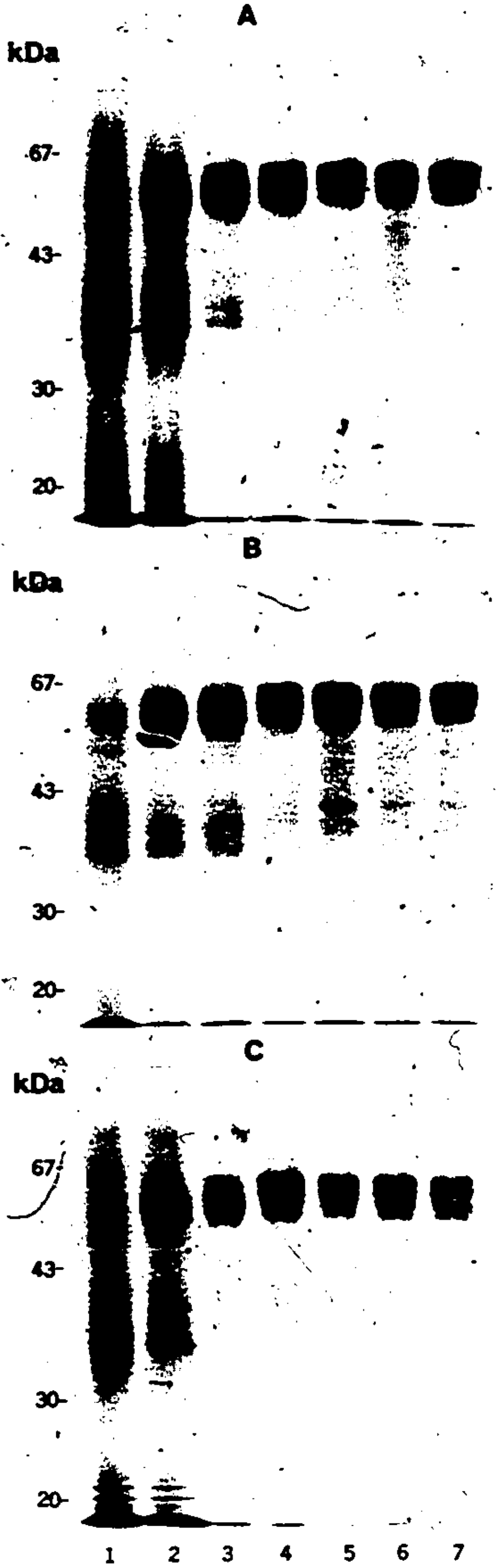
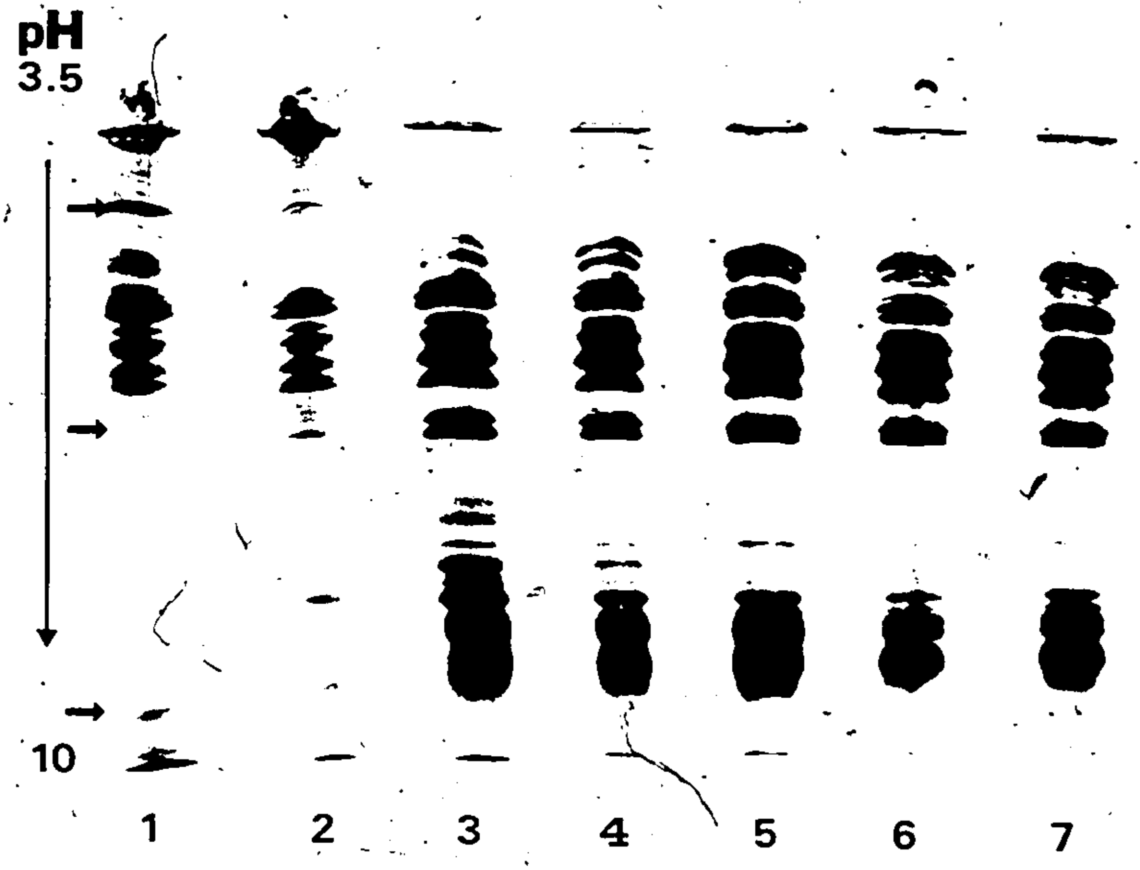

A
kDa
67-
43-
30-
20-
B
kDa
67-
43-
30-
20-
C
kDa
67-
43-
30-
20-
1
2
3
4
5
6
7

Figure 3.12. Isoelectric focusing (section 2.7.2; pH 3.5-10) in the presence of 2-mercaptoethanol of the total globulin fraction extracted from developing Donald seeds harvested at seven different times during development. Numbers 1 to 7 correspond to increasing stages of seed maturity as shown in Fig. 3.9. Differences occur during seed development in the proportion of non-protein material extracted by a saline solution. Therefore different amounts of sample were applied for the various stages: stage (1) 400 µg, (2) 300 µg, (3) 250 µg, (4) to (7) 190 µg.


pH
3.5
10
1
2
3
4
5
6
7

3.1.2 Characterization of oat prolamins (avenins)

A study similar to that described above for the globulins was also undertaken for the oat prolamin (avenin) fraction. Figure 3.13 represents the SDS PAGE patterns of reduced avenins extracted with 52% (v/v) ethanol from the nine oat cultivars. This concentration of ethanol was shown to extract the most avenin (Kim et al, 1978). The major avenin bands have molecular weights between 22 and 40 kDa. Two minor bands of approximately 15 and 16 kDa are also consistently present. Although the same avenin constituents were observed in the absence of a reducing agent, they displayed a decrease in their relative molecular weights of approximately 1 - 2 kDa (data not shown). For comparison, avenins were also extracted from the nine cultivars using 55% (v/v) isopropanol and 2.0% ME, a solvent commonly used to extract barley prolamins (Shewry et al, 1978a). In all cases the resulting avenin patterns (Fig. 3.14) differ to varying degrees from that shown in Figure 3.13. Regardless of the solvent utilized, each cultivar exhibited a distinct avenin pattern upon SDS PAGE with the notable exceptions of cultivars OT-213 (h) and Donald (i). Interestingly, these two cultivars are not closely related (V. Burrows, personal communication). There seemed to be fewer avenin components in the cultivars with higher protein content (eg. Hinoat, lane a, vs Donald, lane i, Figs. 3.13 and 3.14).

The charge distribution of the various avenin fractions was also examined using IEF (Fig. 3.15). The avenins display a considerable level of charge microheterogeneity as evidenced by the greater number of bands

Figure 3.13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) in the presence of 2-mercaptoethanol of the oat prolamin (avenin) fraction extracted with 52% (v/v) ethanol from the cultivars: (a) Hinoat, (b) Dal, (c) Sentinel, (d) Elgin, (e) Harmon, (f) OA-269, (g) OA-424-1, (h) OT-213 and (i) Donald.

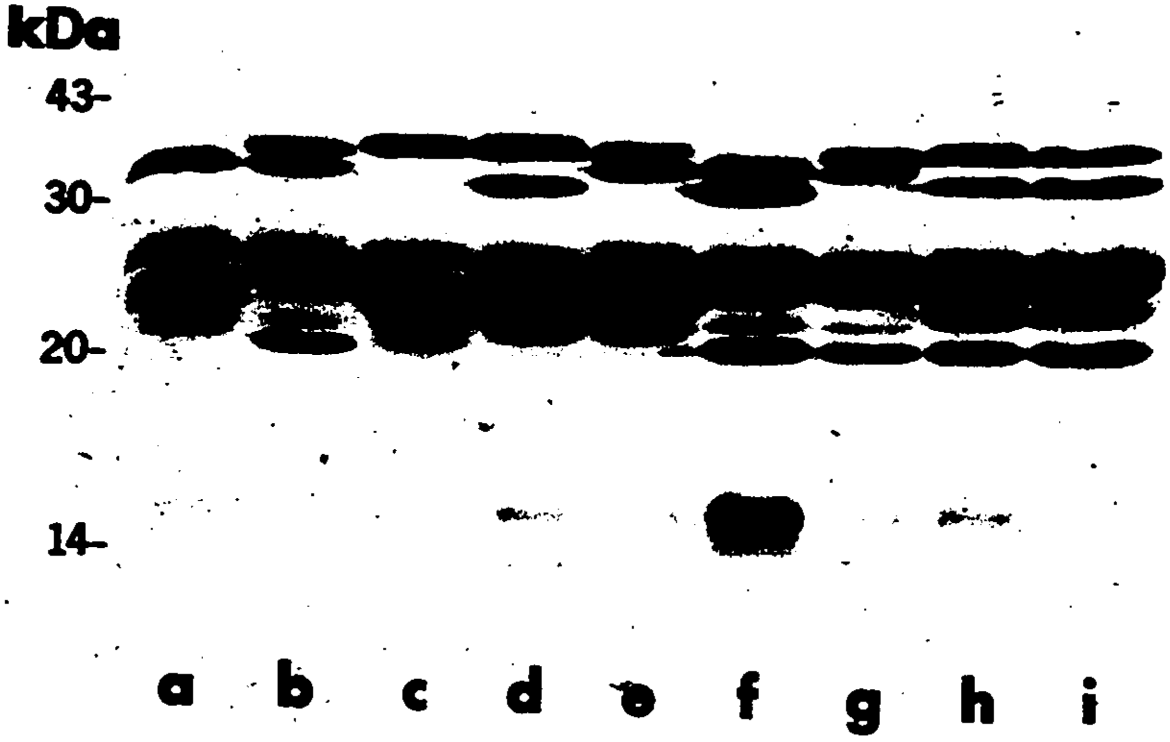

kDa
43-
30-
20-
14-
a
b
c
d
e
f
g
h
i

Figure 3.14. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) of the avenin fraction extracted with 55% (v/v) isopropanol and 2% 2-mercaptoethanol at 60 °C from the cultivars: (a) Hinoat, (b) Dal, (c) Sentinel, (d) Elgin, (e) Harmon, (f) OA-269; (g) OA-424-1, (h) OT-213, (i) Donald.

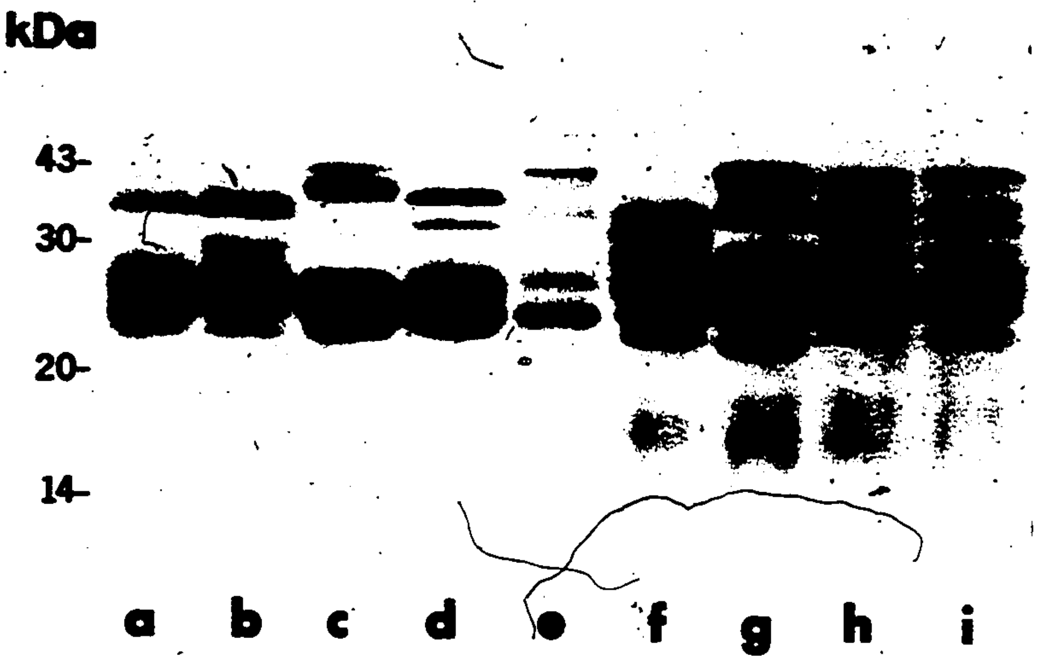
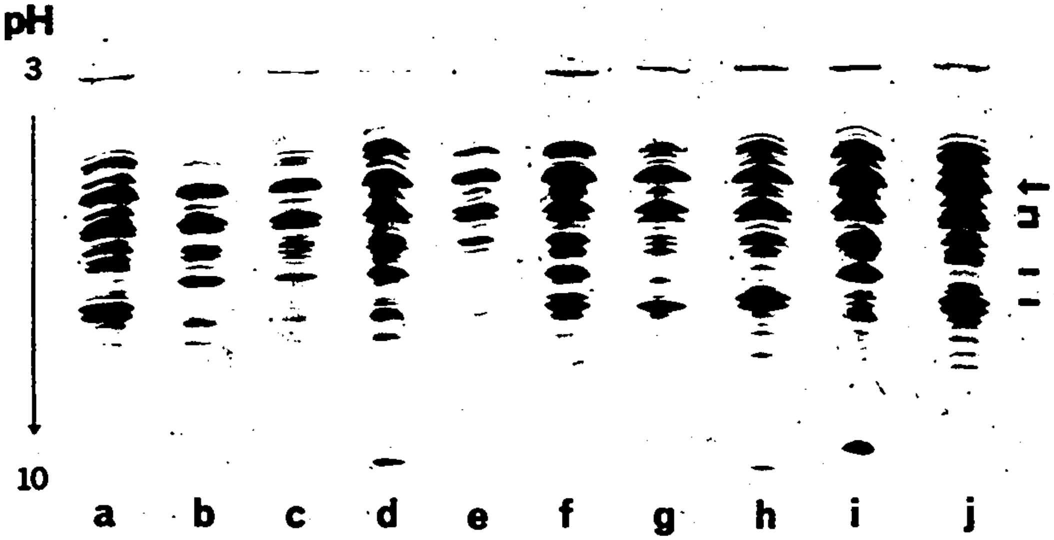

kDa
43-
30-
20-
14-
a
b
c
d
e
f
g
h
i

Figure 3.15. Isoelectric focusing (section 2.7.2; pH 3-10) in the presence of 2-mercaptoethanol of the avenin fraction extracted from the oat cultivars: (a) Hinoat, (b) Dal, (c) Sentinel, (d) Elgin, (e) Harmon, (f) OA-269, (g) OA-424-1, (h) OT-213, (i) Donald and (j) Hinoat. The small arrow and bracket point to common major bands; the bars indicate significant variations among cultivars.



pH
3
10
a
b
c
d
e
f
g
h
i
j

observed with IEF in comparison to SDS PAGE. The majority of the avenin bands lie approximately between pH 4.5 and 8.0 in all the cultivars. Both important similarities (Fig. 3.15, arrow and bracket) and variations (Fig. 3.15, bars) are apparent among the nine avenin patterns. The avenin patterns of the cultivars OT-213 (h) and Donald (i) are clearly different with IEF in contrast to SDS PAGE.

Cultivars Elgin (Fig. 3.15, lane d) and OA-424-1 (Fig. 3.15, lane g) possess two of the most different avenin patterns of the cultivars studied. Two-dimensional analysis of their avenins (Figs. 3.16 and 3.17 respectively) show that both cultivars have a similar protein spot (A) of molecular weight 28 kDa corresponding to the major IEF band pointed out by the arrow in Fig. 3.15. Similarly, the (B) region in Figs. 3.16 and 3.17 displays common spots of molecular weights between 35 to 43 kDa which include the major IEF doublet bracketed in Fig. 3.15. In cultivar OA-424-1, the more basic component of the IEF doublet also gives rise to a major 28 kDa protein spot (C).

Avenins were extracted from Elgin seeds harvested at six weekly intervals following anthesis and analyzed by SDS PAGE (Fig. 3.18) and IEF (Fig. 3.19). In both cases, avenins were not visible at one week post-anthesis (data not shown) and are barely detectable after two weeks. Differences in polypeptide composition were particularly evident between the third and fourth weeks (see arrows, Figs. 3.18 and 3.19). The avenin pattern typical of mature seeds (stage 6) is observed thereafter.

Figure 3.16. Two-dimensional analysis (section 2.7.3B) of the avenin fraction extracted (with 55% (v/v) isopropanol and 2% 2-mercaptoethanol, 60 °C) from the cultivar Elgin. Avenins were first separated by IEF (pH 3 - 10) followed by SDS PAGE. Standard IEF and SDS PAGE lanes of Elgin avenins are included for comparison. A and B refer to polypeptide spots discussed in section 3.1.2.

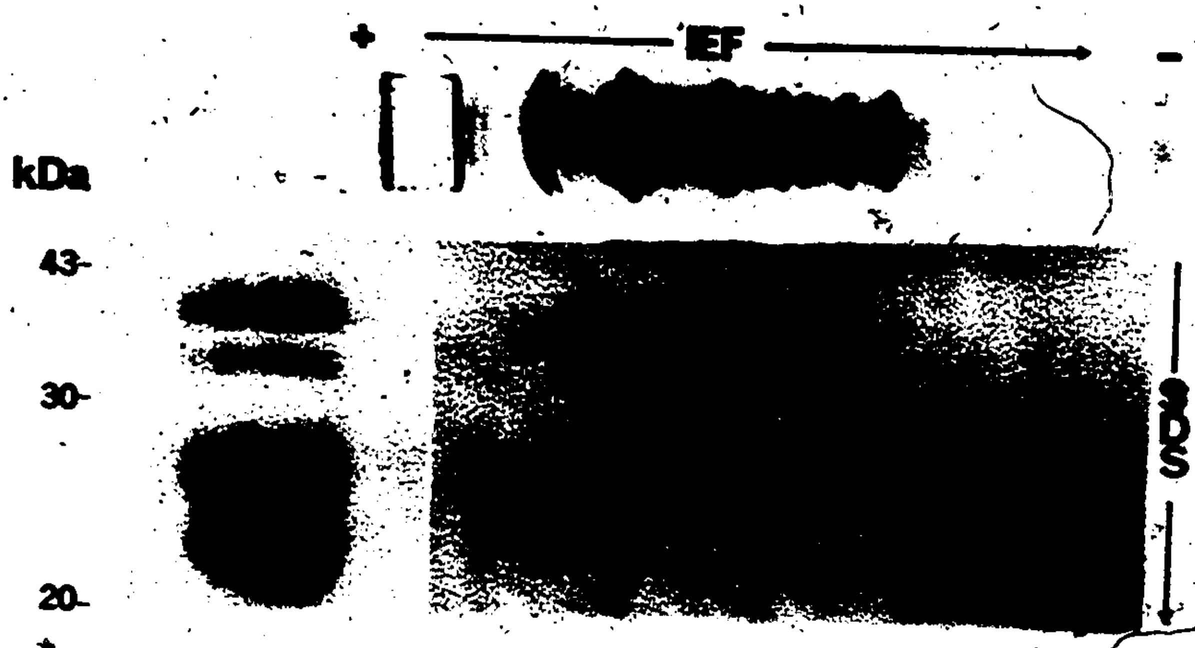

+
IEF
–
kDa
43-
30-
20-
SDS

Figure 3.17. Two-dimensional analysis (section 2.7.3B) of the avenin fraction extracted (with 55% (v/v) isopropanol and 2% 2-mercaptoethanol, 60 °C) from the cultivar OA-424-1. Avenins were first separated by IEF (pH 3 - 10) followed by SDS PAGE. Standard IEF and SDS PAGE lanes of OA-424-1 avenins are included for comparison. A, B and C refer to polypeptide spots discussed in section 3.1.2.

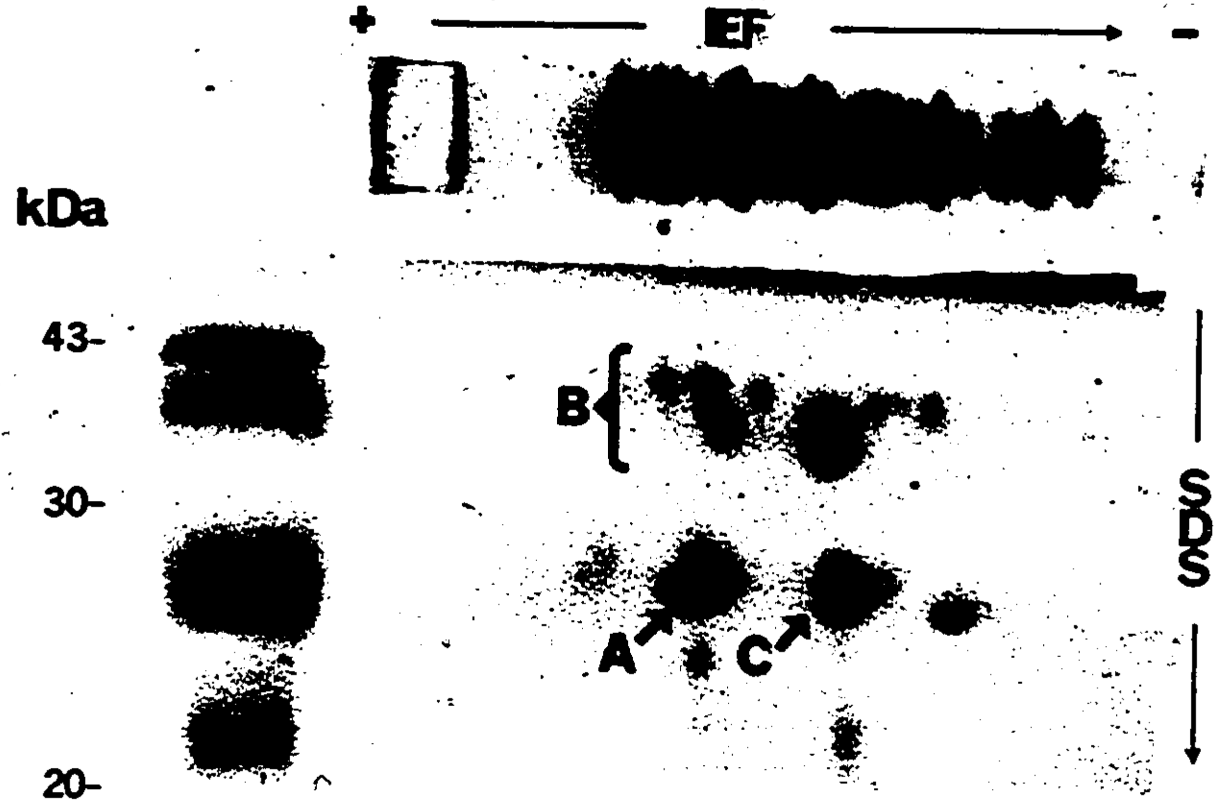

+
EF
−
kDa
43-
30-
20-
B
A
C
SDS

Figure 3.18. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) of the avenin fraction extracted (with 55% (v/v) isopropanol and 2% 2-mercaptoethanol, 60°C) from developing Elgin seeds harvested at weekly intervals following anthesis. Numbers 2 to 6 correspond to weeks post-anthesis. Arrows point to differences in polypeptide composition which are apparent between the third and fourth weeks. Differences occur during seed development in the proportion of non-protein material extracted by an alcohol solution. Therefore different amounts of sample were applied for the various stages: stage (2) 500 µg, (3) 400 µg, (4) to (6) 200 µg.

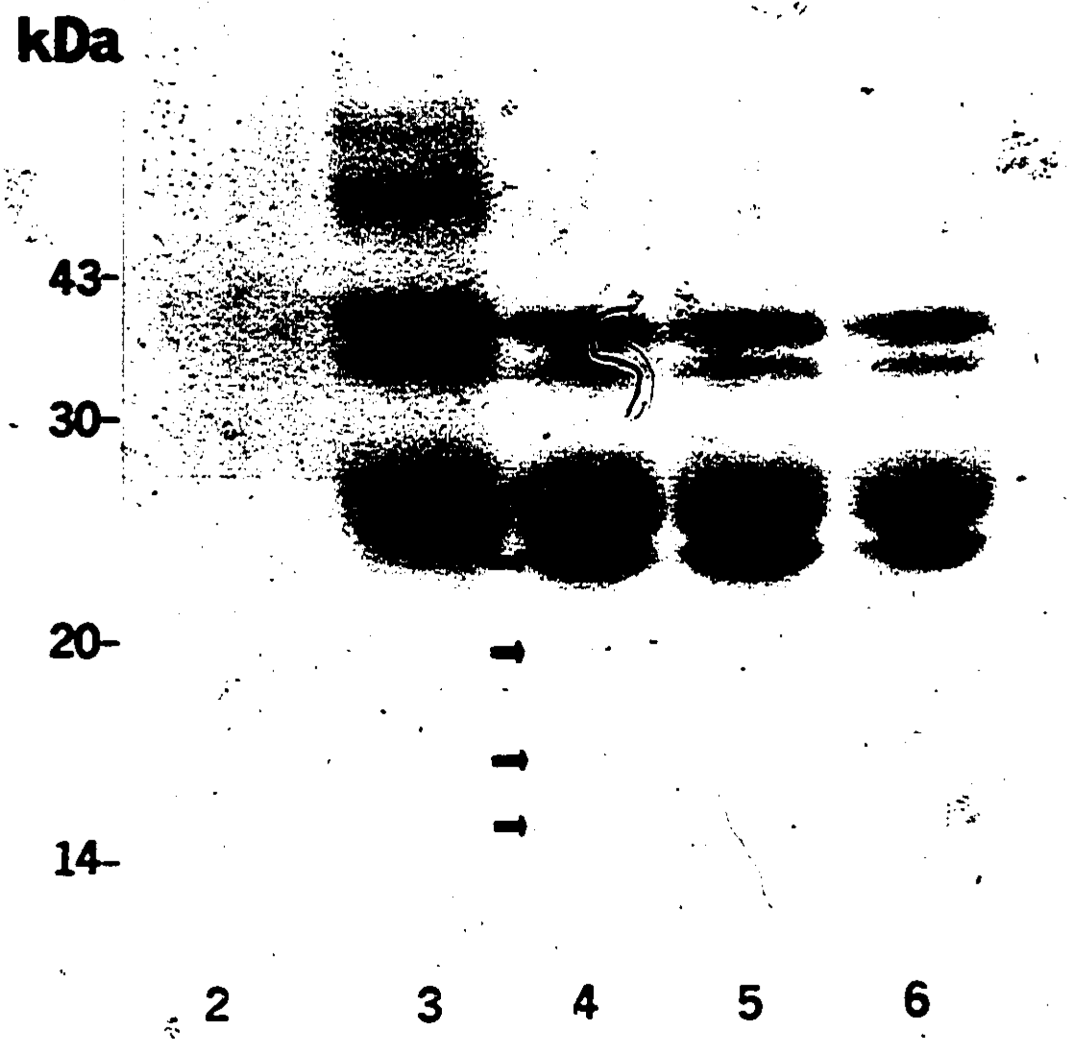
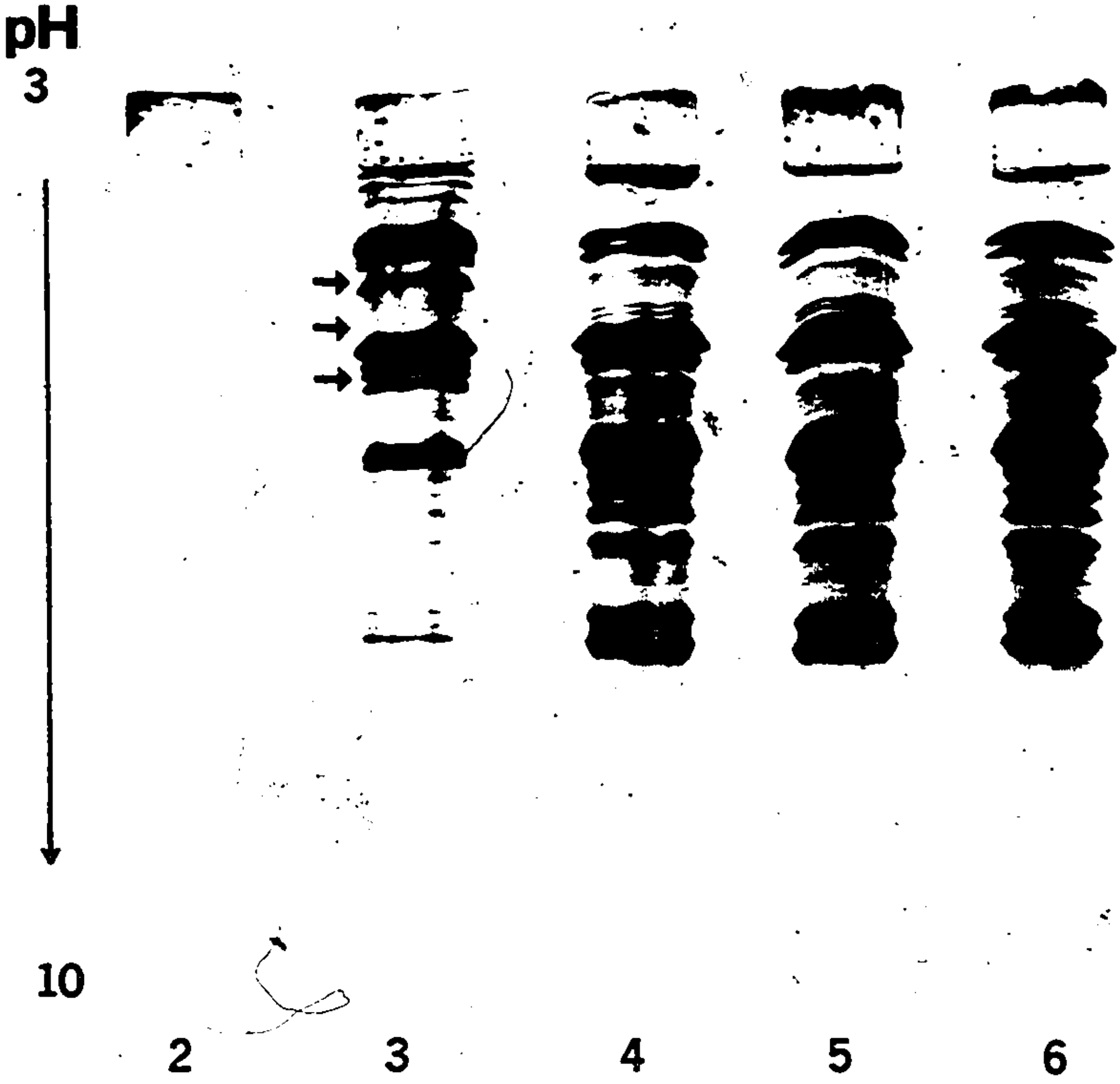

kDa
43
30
20
14
2
3
4
5
6

Figure 3.19. Isoelectric focusing (section 2.7.2; pH 3-10) in the presence of 2-mercaptoethanol of the avenin fraction extracted (with 55% (v/v) isopropanol and 2% mercaptoethanol, 60°C) from developing Elgin seeds harvested at weekly intervals following anthesis. Numbers 2 to 6 correspond to weeks post-anthesis. Arrows point to significant variations occurring in avenin polypeptide patterns between weeks 3 and 4. Differences occur during seed development in the proportion of non-protein material which is extracted by an alcohol solution. Therefore different amounts of sample were applied for the various stages: stage (2) 500 μg, (3) 400 μg, (4) to (6) 200 μg.


pH
3
10
2
3
4
5
6

3.1.3 Characterization of oat glutelins

To establish the importance of the glutelin fraction relative to the other protein fractions occurring in the oat seed, it is necessary to extract completely all the protein fractions involved. Groat proteins were extracted with eight different solvents typically employed for cereal glutelin solubilization. Since these solvents are believed to extract the least soluble seed proteins, they should represent the best candidates for a complete solubilization of the oat seed protein.

The various solvents utilized and the percent protein extracted from oat flour are listed in Table 3.2. The preponderance of NaOH- and HAc-containing solutions results from their favoured use in oat glutelin solubilization (Ewart, 1968; Draper, 1973; Peterson and Smith, 1976; Wu et al, 1977; Kim et al, 1979a). The different solvents extracted from 2% to 98% of the total oat seed proteins. As the pH of the NaOH and HAc solutions became extreme, more protein was removed from the flour, the basic solutions being much more effective. The percent protein extracted by the urea (+ME) solution is not reported in Table 3.2 because of variability resulting from starch gelatinization and residual urea contamination. Solutions containing SDS were quite efficient in extracting oat seed proteins and the combination of SDS and ME provided the maximum solubilization with approximately 98% of the proteins being removed from the flour. Similar SDS (+ME) solutions have been found to extract practically all the seed proteins in other cereals, for example, rice (Juliano and Boulter, 1976), barley (Landry et al, 1972) and wheat (Fullington et al, 1980).

TABLE 3.2

Percent protein extracted from oat flour (cv. Hinoat) by various solvents[1]

Solvent	pH	% Protein extracted
(a) Acetic acid	2.5	9 ± 1[2]
(b) Acetic acid	5.0	1.9 ± 0.2
(c) NaOH	7.3	9 ± 1
(d) NaOH	9.2	41 ± 6
(e) NaOH	12.1	86 ± 4
(f) 0.1 _M_ NaOH, 0.5% SDS, 0.5% borax	11.0	78 ± 5
(g) 7 _M_ urea, 1.0% ME	7.8	n.d.
(h) 0.5% SDS, 0.6% ME	7.6	98 ± 2

[1] Data are averages of duplicate Kjeldahl analyses (N X 6.25) of dried samples from triplicate extractions.

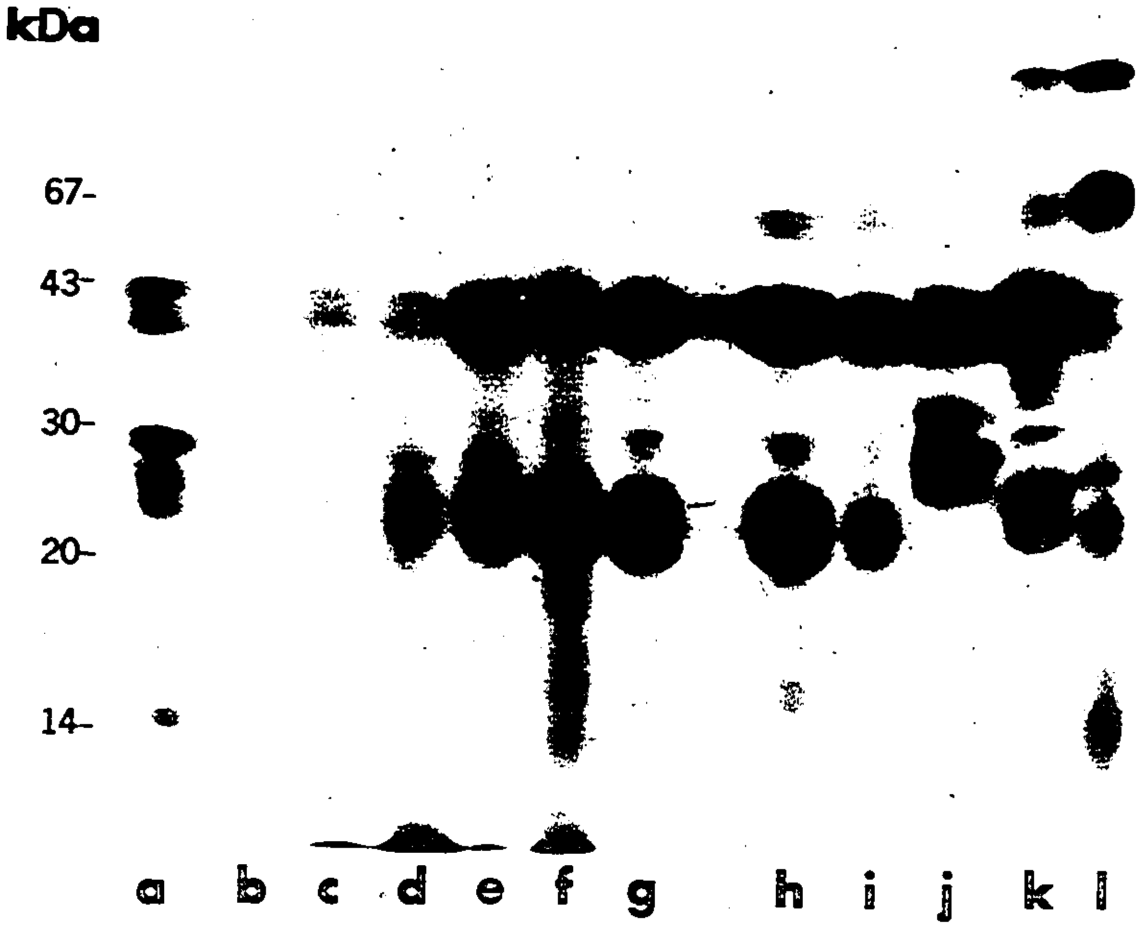
[2] Sample standard deviation.

The proteins obtained from the various extracts were analysed by SDS PAGE (Fig. 3.20) and the polypeptide patterns were compared to those of the typical Osborne fractions (Fig. 3.20, lanes i-l). Except for the pH 5.0 HAc extraction (lane b) which removed only 2% of the flour proteins, all solvents normally used for glutelin extraction (lanes a-i) show the capacity to solubilize proteins which co-migrate with the oat globulin polypeptide groups of approximately 20-25 kDa and 30-40 kDa (lane k). In addition, some solutions also extract polypeptides co-migrating with the prolamins and albumins. The latter Osborne fractions are shown in lanes (j) and (l) respectively.

Protein bands obtained with NaOH-containing solvents (Fig. 3.20, lanes c-f) did not resolve clearly, this may indicate some degree of degradation (Wilson et al, 1981). Nevertheless, with increasing pH, a concomitant increase in the capacity to extract globulins occurred. The pH 2.5 HAc extract (lane a) displays bands which co-migrate with the globulins and prolamins. The latter two fractions (lanes j and k respectively) seem to share common bands of 40-43 kDa. However, oat prolamins stain differently from globulins (also shown in wheat, Minetti et al, 1973), so that the prolamin bands in this region can be distinguished from the globulins, although this is not obvious from a black and white photograph.

There is a high degree of similarity between the electrophoretic patterns of the urea (+ME) (lane g) and SDS (+ME) (lane h) extracts. Since the SDS (+ME) solution extracted nearly all of the flour protein (98%),

Figure 3.20. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) of Hinoat proteins soluble in : (a) Acetic acid, pH 2.5; (b) Acetic acid, pH 5.0; (c) NaOH, pH 7.3; (d) NaOH, pH 9.2; (e) NaOH, pH 12.1; (f) 0.4% NaOH, 0.5% SDS, 0.05% borax; (g) 7.0 M urea, 1.0% 2-mercaptoethanol; (h) 0.5% SDS, 0.6% 2-mercaptoethanol and Hinoat Osborne fractions: (i) glutelins; (j) prolamins; (k) globulins and (l) albumins. Samples (a) to (f) and (k) were reduced and alkylated prior to analysis.


kDa
67-
43-
30-
20-
14-
a
b
c
d
e
f
g
h
i
j
k
l

this stronly indicates that the urea (+ME) extraction was also quite extensive. As would be expected, all the proteins bands found in the different Osborne fractions are detectable within these two patterns.

Since the SDS (+ME) solution was found to be the most efficient solvent in extracting oat seed proteins, it was chosen to solubilize the glutelins in the final step of the Osborne sequential fractionation scheme. Following the extractions of the salt- and alcohol-soluble proteins, the SDS (+ME) solution removed all the proteins remaining in the residue (Table 3.3). Therefore, the resulting electrophoretogram should best describe the oat glutelin composition. Surprisingly, this protein pattern (Fig. 3.20, lane i) greatly resembled that of the total protein extract (lane h). This suggests that the albumin, globulin and prolamin extraction conditions were not optimal since these proteins were still found in the last fraction. Moreover, the "glutelin" electrophoretogram (lane i) displayed only one polypeptide (∿50 kDa) which could be distinguished from those of the other fractions. The possibility that some glutelin bands co-migrate with other proteins cannot, however, be excluded.

These results seem to indicate that the "glutelin" fraction does not represent a distinct polypeptide group, but rather consists primarily of proteins co-migrating upon SDS PAGE with prolamins and especially globulins. For this reason, proteins found in this so-called glutelin fraction should more appropriately be referred to as residual proteins (i.e. proteins remaining in the residue following the extraction of the

TABLE 3.3

Percent protein extracted from oat flour (cv. Hinoat) in an Osborne sequential extraction[1]

Solvent	pH	% Protein extracted	% Protein remaining in residue
1M NaCl, 0.05M Tris	8.5	51 ± 3[2]	49 ± 3
52% Ethanol (v/v)	---	12 ± 2	37 ± 5
0.5% SDS, 0.6% ME	7.6	37 ± 5	0

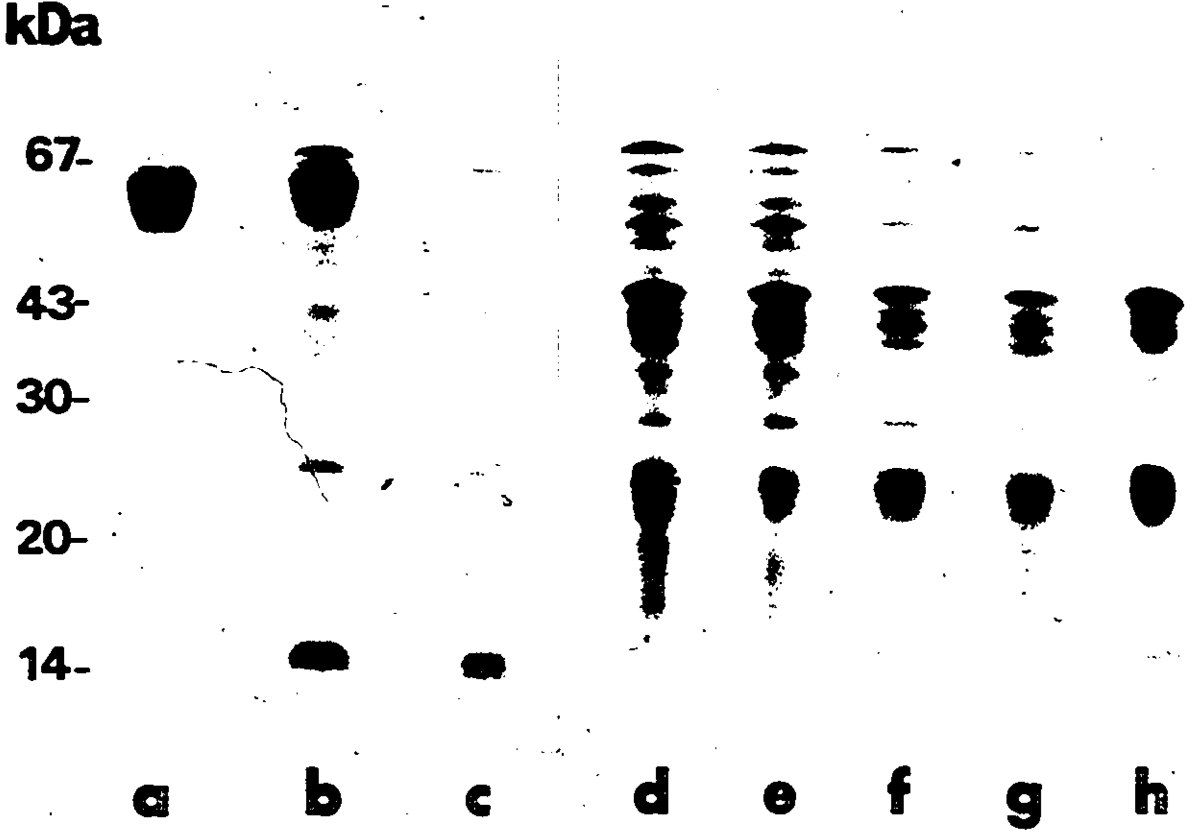
[1] Data are averages of duplicate Kjeldahl analyses (N X 6.25) of dried samples from triplicate extractions.

[2] Sample standard deviation.

albumins, globulins and prolamins). Nonetheless, SDS PAGE is limited in resolving capacity and the residual proteins co-migrating with the prolamins and globulins could possibly conceal (or even be) different proteins, especially in view of the large number of proteins that can be found in a seed. The composition of the oat residual protein fraction was thus examined in greater detail.

To perform IEF for the 2-D analysis of the oat residual proteins, an extracting solution without SDS (which alters the native charge distributrion) had to be found that would also provide the extensive solubilization of these proteins obtained with this detergent. Since the protein patterns of the urea (+ME) and SDS (+ME) extracts appeared very similar (Fig. 3.20, lanes g and h) and urea can be utilized in IEF, further comparisons were made between these two extracts. The SDS PAGE patterns of the 7.0 M urea and 1.0% SDS extracts of the oat residual proteins are shown in Fig. 3.21. If globulins make up a significant fraction of the residual proteins, a major group of polypeptides should comigrate with standard unreduced oat globulins (lane a, 52-70 kDa) in the absence of ME(-ME). This is apparently the case with the SDS (-ME) extract (lane b), but not with the urea (-ME) extract (lane c). If the residual proteins found at molecular weights 52-70 kDa in the SDS extract include globulins, the latter should dissociate into the individual globulin α- (30-40 kDa) and β-(20-25 kDa) subunit groups upon addition of ME. Indeed, a predominance of polypeptides co-migrating with the standard globulin α- and β-subunit groups (lane h) is observed for the SDS (+ME) extract (lane f). There is a concomitant decrease in the amount of

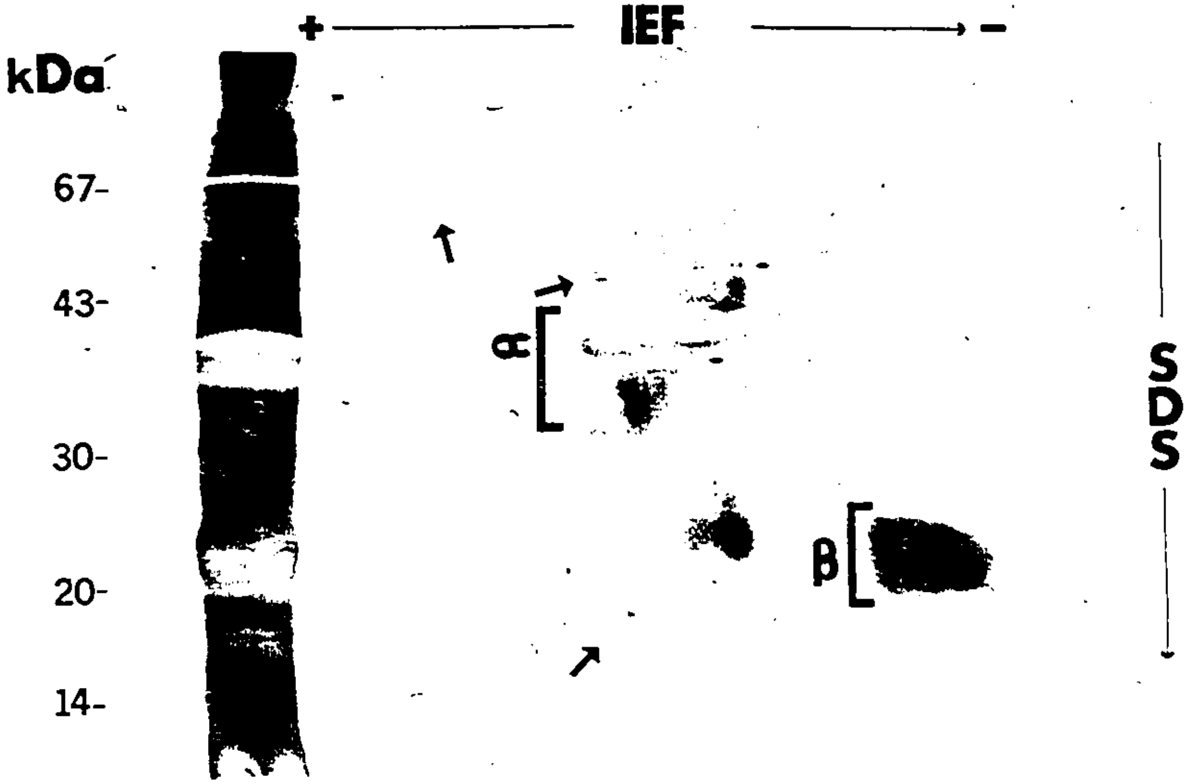
Figure 3.21. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) of: (a) standard Hinoat globulin in the absence of 2-mercaptoethanol (-ME); Hinoat residual proteins extracted with: (b) 1.0% SDS (-ME), (c) 7.0 M urea (-ME) and resolved (-ME); Hinoat residual proteins extracted with: (d) 7.0 M urea (-ME), (e) 7.0 M urea (+ME), (f) 1.0% SDS (-ME), (g) 1.0% SDS (+ME) and resolved (+ME), and (h) standard Hinoat globulin (+ME).


kDa
67-
43-
30-
20-
14-
a
b
c
d
e
f
g
h

the higher molecular weight proteins. The urea extract (lane d) similarly displays proteins co-migrating with the reduced globulin subunits despite the low amount of 52-70 kDa proteins in the unreduced extract (lane c). This difference seems to be due to a higher level of protein aggregation in the urea extract limiting globulin entry into the gel. In the presence of ME, the SDS PAGE patterns of the urea (lane d) and SDS (lane f) extracts are essentially identical. This is true whether the reducing agent is added to the final sample as above or is originally present in the extracting solution (lanes e and g respectively). These results are in agreement with those obtained in the preceding electrophoretogram (Fig. 3.20, lanes g and h)). The two extracts were therefore assumed to be equivalent and the urea (+ME) extract was used for IEF and subsequent 2-D analysis.

If the residual proteins with molecular weight equivalent to the globulin α- and β-subunit groups are really globulins, they should also exhibit the respectively acidic and basic charge distribution typical of these subunits. The 2-D analysis might also reveal different proteins co-migrating with the globulins or prolamins which were not evident upon one-dimensional SDS PAGE. Figure 3.22 represents a 2-D analysis of the urea-extracted proteins remaining in the residue following the extraction of the albumins, globulins and prolamins. The great majority of the polypeptides of molecular weights equivalent to those of the globulin subunit groups also displayed their characteristic charge distributions (Fig. 3.22, brackets). Major protein spots found between the globulin α- and β-subunit groups consisted primarily of prolamins (data not

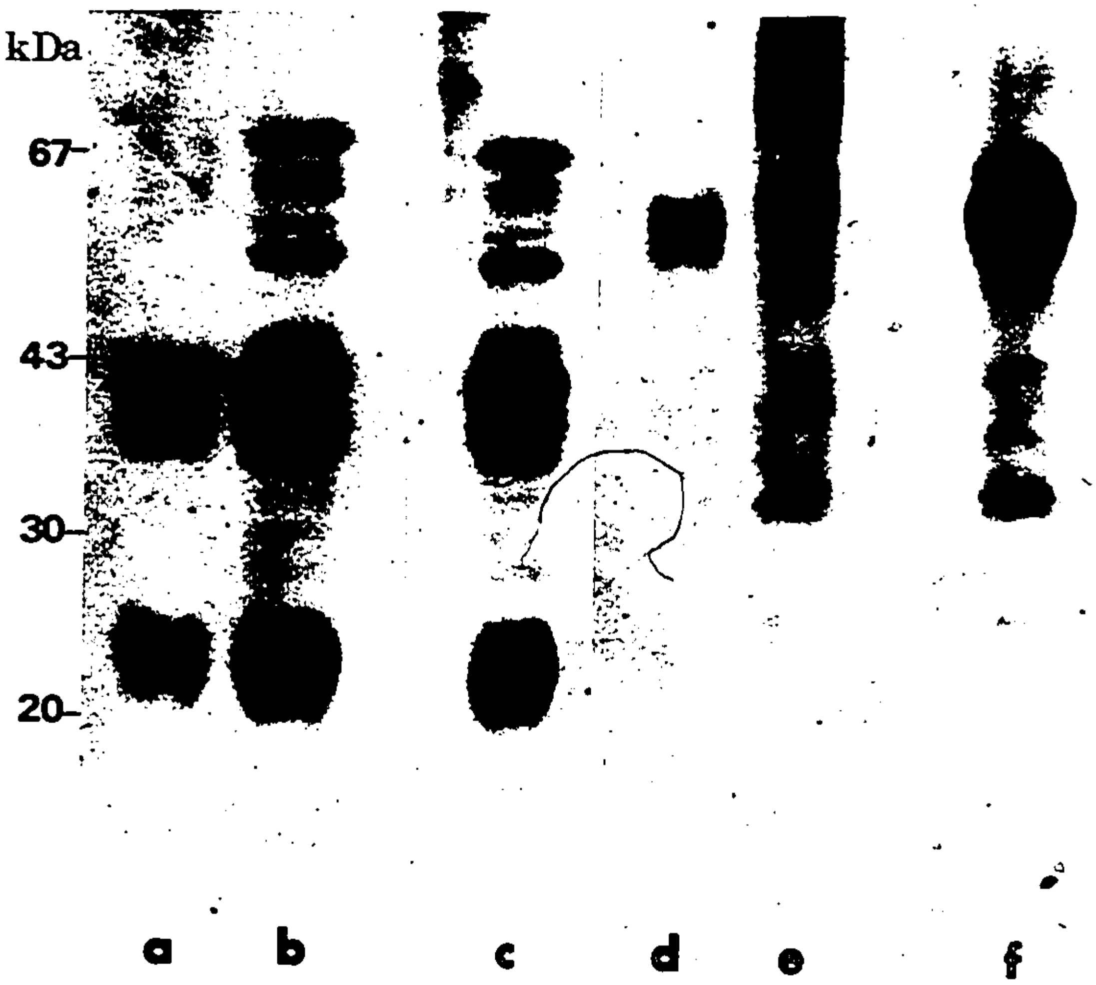
Figure 3.22. Two-dimensional analysis (section 2.7.3B) in the presence of 2-mercaptoethanol of Hinoat residual proteins. Proteins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of Hinoat residual proteins is included for comparison. Brackets denote the α- and β- globulin subunits and arrows point to polypeptides which are probably glutelins. This gel was silver stained.


+
IEF
−
kDa
67-
43-
30-
20-
14-
α
β
SDS

shown). There were, however, small clusters of protein spots which did not comigrate with the two-dimensional pattern of either the globulins or the prolamins (Fig. 3.22, arrows). By process of elimination, these proteins are likely true candidates for the glutelin fraction (albumins, being soluble in both aqueous and saline solutions, are not likely to be still present in significant amounts in the residue).

Although very slight, the possibility still exists that the residual proteins co-migrating with the globulins are glutelins with similar molecular weights and charge distributions. Convergent evolution brought about by functional constraints for the biosynthesis, assembly, packaging and hydrolysis of these proteins might be a possible cause of such a similarity. To investigate this possibility, the residual proteins from oat were challenged with ^{125}I-labelled anti-oat 12S globulin IgG. Results presented in Fig. 3.23 show that the residual proteins co-migrating with globulins evidently possess antigenic determinants recognized by the antibody. The antibody cross-reacted selectively to the reduced standard globulin subunits (lane a) and also cross-reacted with polypeptides of identical molecular weights in both the urea (+ME) (lane b) and SDS (+ME) (lane c) extracts. These polypeptides can, therefore, be considered as being globulins. The antibody also recognized polypeptides in the 60 kDa molecular weight region within these extracts. The individual oat globulin subunits are known to arise from the post translational cleavage of precursors of approximately 60 kDa (Matlashewski et al, 1982; Brinegar and Peterson, 1982b; Walburg and Larkins, 1983; Adeli and Altosaar, 1983; Rossi and Luthe, 1983; Adeli et al, 1984). The polypeptides recognized

Figure 3.23. Western blot analysis (section 2.12) of oat protein extracts using ^{125}I- labelled anti-12S oat globulin IgG: (a) standard Hinoat globulin in the presence of 2-mercaptoethanol (+ME); Hinoat residual proteins extracted with: (b) 7.0 M urea (+ME), (c) 1.0% SDS (+ME), (d) standard Hinoat globulin (-ME); Hinoat residual proteins extracted with: (e) 7.0 M urea (-ME) and (f) 1.0% SDS (-ME). Approximately 10μg of standard oat globulin and 100μg of residual proteins were applied to the gel.


kDa
67
43
30
20
a
b
c
d
e
f

by the antibody in this region may correspond to uncleaved globulin precursors having a reduced salt-solubility.

With the SDS extract, in the absence of ME, the radioactive signal shifted from the subunit groups to the 52-70 kDa region (lane f). This supports the hypothesis that the latter proteins are the result of the disulfide association of the globulin subunits (as in lane d). With the urea (-ME) extract, a smear of radioactivity was detected in the high molecular weight region (lane e) again suggesting poor resolution due to protein aggregation. These results can be compared with the equivalent Coomassie Blue-stained lanes of Fig. 3.21.

3.2 DISCUSSION

3.2.1 Characterization of oat glutelins

Problems have resulted in cereal seed protein classification mainly because of an excessive reliance on a method based solely upon the solubility properties of these proteins. For precision and completeness, a classification method must also encompass all other relevant biochemical, biological and chemical information and preferably deal with pure gene products. Although both a necessary and a great step forward, the method of classification of vegetable proteins devised by Osborne possesses important limitations which he was the first to recognize:

> "A chemical classification should be based on definite properties of individual substances, but such treatment of the proteins is at present manifestly impossible. It is, however, desirable to have some scheme by means of which the proteins can be brought together in an orderly fashion. All attempts, thus far made, to classify them have been based chiefly on their solubility under different conditions. This method of classification has proved in many ways unsatisfactory and inadequate, but seems, for the present, to be the best available."
> Osborne (1924)

As a result of recent advances in biochemical technology, it is now possible to obtain additional information on the "definite properties" of individual proteins and protein fractions which can supplement solubility characteristics and provide a more precise categorization of seed proteins. Hence, based on solubility, electrophoretic and immunological findings, it was possible to determine that the globulins, and not the glutelins, constitute the major reserve protein fraction of the oat seed (see Figs. 3.20 to 3.23). Prolamins and especially globulins were found to be still present in the oat residual protein fraction. This was taken as an indication that the extraction procedures typically employed were not optimal and did not remove these proteins completely from the oat flour. The glutelin fraction is always the last to be extracted in an Osborne fractionation scheme and since this extraction generally involves strong protein solubilizing solvents, it becomes obvious that these solvents could also extract albumins, globulins and prolamins remaining from the preceding extractions. The less efficient the extractions of the oat albumins, globulins and prolamins, the greater the proportion assigned to the glutelin fraction. The results obtained are in agreement with the proposal of Shewry et al, (1978b) which attributes the high glutelin proportion in barley, wheat and corn to the incompleteness of the preceding extractions.

Although some polypeptides were unique to the glutelin fraction (see Fig. 3.22), it was difficult to ascribe a qualitatively (or quantitatively) major polypeptide assemblage to this fraction. In contrast, this criterion was easily fulfilled even for the smaller Osborne fractions in oat, the albumins and the prolamins. Therefore, the proportion of the total protein content allocable to the glutelin fraction in cultivated oat must be very small.

Miflin and Shewry (1979a) proposed that glutelins serve a metabolic and/or structural role rather than a storage function in wheat, corn and barley. This now seems to apply to oat as well. The low proportion of glutelins observed in oat favours a limited storage role for this fraction. This also implies that the only storage protein fractions in oat are the prolamins and globulins. The albumins, generally considered to play a metabolic role in cereal seeds, comprise approximately 10% of the oat total seed protein (Appendix I; Peterson, 1976). The oat prolamins contribute 10-15% (Table 3.3; Kim et al, 1978), although their presence in the residual protein fraction revealed these values to be slight underestimates. These two fractions thus account for about 20-30% of the oat seed protein. Assuming the amount of glutelins to be at most 5%, the globulin proportion in oat may be as high as 65-75%, a unique feature among major cereals. Consequently, the possibility also arises that data associated with oat glutelins in the past may have pertained largely to the globulins. Kim et al., (1979a) have noted some similarity between the electrophoretic patterns of oat globulins and glutelins.

The above findings illustrate the complications inherent in extracting proteins tightly packaged into discrete protein bodies within the dehydrated environment of mature grains. The physical, biochemical and physiological parameters which may affect the solubility of these proteins are many (temperature, particle size, solvent, pH, membrane- binding, protein aggregation, phenolic interactions, etc.). Thus, for example, a seed globulin may be defined as a protein soluble in saline solution, but a protein not solubilized by such a solution may still be a product of the globulin gene family. While acknowledging the utility of the Osborne classification scheme in simplifying the inherent complexity of vegetable proteins, its limitations must also be kept in mind. Highly discriminatory data from electrophoretic and immunological analyses should be used, in combination with other criteria available, to define individual gene products and assign each protein to its specific fraction. Advances in molecular genetics may eventually supersede these techniques and provide direct means of identifying individual oat storage protein molecules.

Unlike other major cereals the oat seed contains unusually high amounts of globulins and very low levels of prolamins and glutelins. This serves to explain several properties peculiar to the oat grain and its flour. For example, oat proteins have been shown to be of superior nutritional value to proteins from other major cereals (Howe et al, 1965; Youngs et al, 1973; Maruyama et al, 1975). This can now be attributed to both a higher level of globulins and the lower level of prolamins in oat. The oat globulin fraction possesses a better balanced nutritional profile than the avenin fraction (Draper, 1973; Wieser et al, 1980). In addition, unlike other cereals, the nutritional quality of oat proteins is known to remain practically unchanged with increases in total seed protein content (section 1.3). In oat, it is the globulins rather than the prolamins which increase preferentially with increasing total protein content (Peterson, 1976). On the other hand, the low quantities of prolamins and glutelins in oat may account for its poor baking quality in comparison to wheat. In order to achieve a better understanding of the oat seed storage protein system, the oat storage proteins (namely the globulins and prolamins) were characterized to a greater extent.

3.2.2 Characterization of oat globulins

The globulin α- and β-subunits from nine oat cultivars ranging widely in total seed protein content were found to be much more heterogeneous than previously reported (Peterson, 1978). These subunits were shown to be associated to form molecules of 52-70 kDa under non-reducing conditions (see Figs. 3.1, 3.2 and 3.3). Six of these reducible molecules would presumably assemble to form the oat globulin holoprotein possibly ranging from 312-420 kDa and corresponding to the 12.1S protein described by Peterson (1978).

Interestingly, the type of disulfide-associated globulin subunits observed in oat has not been reported among other cereals but is quite common among dicotyledonous plants. Indeed, disulfide-linked subunits having molecular weights surprisingly similar to those of the oat α- and β-subunits have been shown to occur within the globulin fractions of Pisum sativum L. (legumin, Thomson et al, 1978; Croy et al, 1979; Matta et al, 1981b), Vicia faba L. (legumin, Matta et al, 1981a), Glycine max L. (glycinin, Moreira et al, 1979; Staswick et al, 1981), Arachis hypogea L. (arachin, Singh and Dieckert, 1973), Helianthus annuus L. (helianthin, Murray and Vairinhos, 1982), Cicer arietinum L. (Vairinhos and Murray, 1982), Lupinus albus (Duranti and Cerletti, 1983) and Cucurbita species (O'Kennedy et al, 1979; Ohmiya et al, 1980). In addition, legumin-like proteins, like the oat globulin, associate into holoproteins with sedimentation values of 11-12S and molecular weights of 300-400 kDa (Derbyshire et al, 1976).

Although the nine oat cultivars displayed the typical α- and β-globulin subunit pattern, considerable differences were noted in the polypeptide composition of their subunit groups. This was especially true of the α-subunits. Genotypic variability was also demonstrated within the subunits of legumins (Casey, 1979; Thomson et al, 1979; Utsumi and Mori, 1980; Matta and Gatehouse, 1982). Generally, variability is also more pronounced within the acidic (α) subunits of legumin (Casey, 1982). The presence or absence of particular protein bands as well as their differing relative intensities may be indicative of variations in the genetic regulation of the biosynthesis of oat globulins. However, there was no evident correlation of these variations with differences in total protein content of the cultivars. For example, oat cultivars with a higher protein content did not exhibit corresponding increases in the relative amount or number of specific constituent globulin polypeptides. Similarly, the greater resolution of IEF did not uncover any existing correlation between the globulin pattern and the protein content of the cultivars.

Two-dimensional analysis demonstrated that the α-subunits were not only more heterogeneous than the β-subunits but also displayed significantly more acidic pIs. The unreduced globulin molecules were also found to involve several charge variants (see Figs. 3.6, 3.7 and 3.8). Interestingly, the respective acidic and basic nature of the oat α- and β-globulin subunits, along with their extensive charge microheterogeneity, correspond to features peculiar to the α- and β-subunits forming legumin-like proteins (Derbyshire et al, 1976; Moreira et al, 1979;

Gatehouse et al, 1980; Matta et al, 1981a,b). Furthermore, as was shown in legumes (Croy et al, 1980a; Spencer and Higgins, 1980; Turner et al, 1981), the oat globulin subunits were also found to arise from the post-translational cleavage of precursor polypeptides of approximately 60 kDa (Matlashewski et al, 1982; Brinegar and Peterson, 1982b; Walburg and Larkins, 1983; Adeli and Altosaar, 1983; Rossi and Luthe, 1983; Adeli et al, 1984). Therefore, results point to either the conservation of globulin genes in oat which would have evolved from the same ancestral gene as the legumin-like globulins of dicotyledonous plants, or a convergent evolution of these proteins.

The properties of oat globulins that I have reported are in close agreement with the findings of recently published reports (Brinegar and Peterson, 1982a; Walburg and Larkins, 1983; Burgess et al, 1983). Homology with legumin-like proteins was further supported by the close similarity of the N-terminal sequences of the β-subunits from oat globulin, Pisum sativum L. and Vicia faba L. legumin and Glycine max L. glycinin (Walburg and Larkins 1983).

Even though compositional differences within the globulin fraction did not appear to correlate with total seed protein content, it is still possible that variations occur in the rate and sequence of the biosynthesis of these proteins during the seed development of high and low protein cultivars. Welch et al, (1980) showed that the nitrogen percentage of different oat cultivars varied significantly at all stages of seed development and that differences in total seed protein content were established early in grain development and persisted until maturity. Similar results were reported by Peterson and Smith (1976), who also found a marked increase in globulin synthesis within the first 16 days following anthesis.

In this study, three cultivars displayed essentially the same sequential pattern of globulin accumulation despite important differences in their total protein content and the differential biosynthesis of their globulin subunits (see Figs. 3.10 and 3.11). Other electrophoretic studies dealing specifically with the developmental accumulation of globulin polypeptides in cereals seem to be lacking. However, such is not the case with the legumes where globulin polypeptides have been demonstrated to accumulate differentially during development (Millerd et al, 1978; Thomson et al, 1979; Spencer et al, 1980; Mutschler et al, 1980; Meinke et al, 1981). Like oat 12S globulin, pea legumin was also shown to exhibit a developmental shift in the preferred synthesis of the low- to the high-molecular weight components of its α- and β-globulin subunits (Thomson et al, 1979). The delayed synthesis reported for the higher molecular weight components of the glycinin α-subunits (Meinke et al, 1981) and pea legumin β-subunits (Spencer et al, 1980) may implicate similar regulatory mechanisms. The reasons for this sequential gene expression are not yet known, but this phenomenon points to the existence of a discriminatory biosynthetic process which can regulate the differential accumulation of specific members of a same storage protein family.

The time of onset of globulin synthesis was not related to the final protein content of the three oat cultivars studied. Hence, the high protein content of the cultivar Hinoat cannot be attributed to a precocious synthesis of its globulins relative to the low-protein cultivar Donald. Likewise, the shift to the preferential synthesis of the higher molecular weight globulin subunits did not occur earlier in the cultivar Hinoat. Results obtained by Peterson and Smith (1976) showed that protein synthesis did not end prematurely in a low-protein cultivar in comparison to a high-protein cultivar. The variations observed in total seed protein content in oat thus ensue from different rates of globulin synthesis. Cataldo et al, (1975) suggested that the main factor affecting the protein content of a high-protein (Dal) and a low-protein (Orbit) oat cultivar involved a decreased deposition of non-structural carbohydrates in the groat of the cultivar Dal and not necessarily a difference in nitrogen metabolism. It is difficult to assess from their results whether the cultivar Dal contains more or less protein per seed than the cultivar Orbit. However, a difference in carbohydrate metabolism cannot solely account for the important spread in protein content between the cultivars Hinoat and Donald, since the cultivar Hinoat contains a significantly larger amount of protein per seed than the cultivar Donald (Table 3.1), in spite of a smaller average seed weight (data not shown). The higher seed protein content of the cultivar Hinoat is probably related to both a higher rate of globulin synthesis and a greater capacity to accomodate it. More work is required to determine whether this is due to a more efficient transcription and/or translation, a greater number of genes, a higher level of gene expression or a greater mRNA stability.

3.2.3 Characterization of oat prolamins

The alcohol-soluble prolamins of barley (hordein), corn (zein), rye (secalin), sorghum (kafirin) and wheat (gliadin) constitute major seed storage protein fractions (Mossé, 1968). However, prolamins of oat (avenin) represent only a minor fraction relative to the total protein content. In view of this, the role of avenins as storage proteins may be questioned, especially given the unusually high concentration of reserve globulins in oat. Avenins may simply correspond to structural or metabolic proteins soluble in aqueous alcohol. However, the avenin fraction displays notable similarities to other cereal prolamins. For example, avenins (1) contain unusually large amounts of glutamate and proline while being especially low in lysine (Draper, 1973; Weiser et al, 1980; Kim et al, 1978), (2) exhibit a significant degree of polymorphism (Kim and Mossé, 1979), (3) are present in protein bodies (Pernollet et al, 1982) and (4) are hydrolyzed upon germination (Kim et al, 1979a). Hence, avenins are probably reserve proteins which have evolved to become (or to remain) of limited quantitative importance in the oat seed. This low avenin concentration is particularly interesting in view of the prominent role played by prolamins in determining the nutritive and functional value of other cereal crops.

The avenins obtained from the nine cultivars examined in this study displayed a wider molecular weight range than the estimate of 20-34 kDa mentioned by Kim et al, (1979b). This probably results from varietal differences. Two minor bands appearing consistently in the nine different avenin SDS PAGE patterns may belong to a class of prolamins similar to the low molecular weight A-hordeins of barley (Shewry et al, 1978a). The higher molecular weight of the avenin components observed under reducing conditions may be attributed to the breakage of intramolecular disulfide bonds, causing the polypeptides to unfold and thus retarding their migration. Such an increase in the apparent molecular weight of avenin in the presence of a reducing agent has been reported previously (Bietz, 1982). The prolamins from cultivated oat appear devoid of major polypeptides of molecular weights greater than 40 kDa unlike the equivalent fractions from wheat (Kasarda et al, 1976), barley (Shewry et al, 1984a) and rye (Shewry et al, 1983a). This, along with the low levels of glutelins, likely contributes significantly to the poor baking quality of oat flour. The genes coding for these proteins may be repressed or absent in cultivated oat.

The avenins displayed both significant intervarietal polymorphism and increased microheterogeneity when separated according to their pI by IEF (see Figs. 3.13 to 3.17). Genotypic differences among the cultivars examined were more evident with this fraction than with the globulin fraction. The electrophoretic analysis of avenins would thus be better suited to the needs of cultivar identification. The considerable level of charge heterogeneity shown by the avenins is typical of cereal prolamins and has been reported in wheat (Kasarda et al, 1976), barley (Shewry et al, 1978a), rye (Shewry et al, 1983a) and corn (Righetti et al, 1977). Although the quantity of prolamins is much lower in oat, they do not demonstrate a reduced degree of polymorphism when compared to that observed in cereals where these proteins occur in greater proportions. Thus, extensive heterogeneity does not appear to be either a consequence or a requirement for a major storage protein role in cereals.

The absence or presence and different relative intensities of avenin bands may point to a differentiated mode of biosynthetic regulation of these polypeptides, but it seems uncorrelated with the total protein content of the seed. Furthermore, Frey (1951) reported no significant quantitative changes in the proportions of the alcohol-soluble nitrogen fraction in oat cultivars with varied total protein content. Therefore, the contribution of the prolamin fraction to differences in total seed protein content among oat cultivars appears rather limited.

The different avenin constituents of the cultivar Elgin were shown to accumulate differentially during seed development. Analogous results have been reported for wheat (Mecham et al, 1981) and barley (Rahman et al, 1982) prolamins. It is interesting to note that avenin biosynthesis begins at least one week after that of the globulins. This may contribute in part to their relatively low concentration in oat.

3.2.4 Oat storage protein heterogeneity

The extensive level of heterogeneity displayed by the oat globulins and prolamins is a reflection of storage protein structure and evolution and deserves further attention. There are three principal sources of storage protein charge microheterogeneity and these include: (1) in vitro artefacts caused by extracting conditions, protein-ampholyte interactions, protein aggregation, deamidation and carbamylation; (2) in vivo modifications such as post-translational cleavage, amidation, deamidation, glycosylation, phosphorylation, etc. and (3) genetic polymorphism resulting from multiple allelism and multiple genes. There is presently a considerable amount of evidence favouring a genetic origin of most of the storage protein heterogeneity observed as opposed to in vitro and in vivo modifications. In vitro artefacts produced during IEF are generally considered to play a minor role in seed storage protein heterogeneity (Soave and Salamini, 1982). This premise is strongly supported by the work of Righetti et al, (1977) who dismissed extraction procedures, deamidation, aggregation, carbamylation and ampholyte interactions as possible sources of zein heterogeneity. In addition, an artefactual or random origin of the heterogeneity of the oat globulins and prolamins appears unlikely in view of the reproducibility of the IEF patterns obtained and the consistent similarities found among them in spite of the large number of bands present.

The extent to which microheterogeneity is caused by in vivo post-translational modifications is unknown. Glycosylation seems improbable since oat 12S globulin contains essentially no carbohydrate (Peterson, 1978; Adeli and Altosaar, 1984) and glycosylation of cereal prolamins with the exception of zein (Burr, 1979), has not been reported (Miflin and Shewry, 1981). Several lines of evidence corroborate the genetic basis of seed storage protein microheterogeneity: (1) differences in primary amino acid sequence (Bietz et al, 1979; Moreira et al, 1979;

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Vitale et al, 1980; Shewry et al, 1981; Faulks et al, 1981; Ohmiya et al, 1980), (2) mRNA microheterogeneity (Park et al, 1980; Marks and Larkins, 1982), (3) microheterogeneity of in vitro products (Viotti et al, 1978; Matthews and Miflin, 1980), (4) multiple gene copies (Viotti et al, 1979; Croy et al, 1982; Schuler et al, 1982 a, b; Kreis et al, 1983), (5) inheritability of components and response to gene dosage (Righetti et al, 1977; Shewry et al, 1980a) and (6) persistent polymorphism in homozygous populations (Miflin and Shewry, 1979b; Shewry et al, 1983a).

For example, even though zein consists only of 3 to 5 major polypeptides upon SDS PAGE and 8 to 15 components with IEF, the number of zein genes is estimated at 110 to 130 genes per haploid genome (i.e. 300 to 350 genes per triploid endosperm cell) involving 15 multigene families (Viotti et al, 1979). It is not known how many of these genes are active. A similar estimate is believed to be reasonable for the number of genes coding for hordeins in barley (Shewry and Miflin, 1982). This implies that the current electrophoretic techniques probably underestimate the heterogeneity of seed storage proteins, probably because these cannot separate polypeptides which differ only in minor deletions, additions or substitutions of their numerous uncharged residues.

Hence, seed storage proteins are believed to be encoded by complex multigenic families derived from the duplication and mutational divergence of single ancestral genes (Kasarda et al, 1976; Thomson and Doll, 1979). This appears also to be the most suitable interpretation of the observed microheterogeneity of the globulins and prolamins found in oat; especially if one takes into consideration the facts that the large number of nucleotides composing a gene can allow a theoretically infinite number of alleles and that oat is an allohexaploid (i.e. 9 haploid genomes of seven chromosomes per endosperm cell) thus permitting countless possible combinations.

The purpose of simple multigene families is to increase an organism's capacity to synthesize a product (Brown, 1981). In view of the abundance of reserve proteins which must be synthesized within the relatively short period of seed development, this seems a likely explanation for the presence and expression of multigenic families in seeds. Although this may apply quite well to the oat globulins, one might rightfully question the need for so many avenin genes. Oat might have evolved from an ancestor which possessed multigenic families coding for the prolamins and the globulins, the multigene family coding for the latter being favoured through the course of evolution. On the other hand, the sort of molecular promiscuity observed, is apparently permitted because of the weaker function-related constraints imposed on the structure of storage proteins relative to that of other seed proteins such as enzymes (Robbelen, 1979). The low selective pressures imposed on the avenin genes might have permitted the formation of a multigene family coding only for a minor storage protein fraction.

Obviously, the fact that an infinite number of storage proteins does not occur within a seed, points to the existence of limits to the evolutionary divergence of these proteins. The constraints would involve specific requirements for the recognition, processing, assembly, packaging and degradation of the seed storage proteins (Derbyshire et al, 1976; Thomson and Doll, 1979). Indeed, N-terminal amino acid sequence data have revealed significant homology among the prolamins within species, such as Triticum aestivum (Bietz et al, 1977; Shewry et al, 1984b), Hordeum vulgare (Shewry et al, 1980b), Secale cereale (Shewry et al, 1983b), Zea mays (Bietz et al, 1979) and Avena sativa (Bietz, 1982), as well as within genera in the tribe Triticeae (i.e. wheat, barley, rye; Autran et al, 1979; Shewry et al, 1980c, Kasarda et al, 1983) and in the subfamily Panicoideae (i.e. Zea Mays, Tripsacum dactyloides, Sorghum bicolor, Pennisetum americanum; Bietz, 1982). Although amino acid sequencing of globulins has not been done to the same extent, homology among the constituents of both the acidic and basic subunits of glycinin has been reported (Moreira et al, 1979). Likewise, shared sequences have been demonstrated among the legumin-like basic subunits of Glycine max and Vicia faba (Gilroy et al, 1979) and those of Pisum sativum and Avena sativa (Walburg and Larkins, 1983). In all cases, the degree of homology is directly proportional to the taxonomic relatedness of the species compared.

To reconcile both the existing laxity and stringency of the genetic controls implied, the observed seed storage protein heterogeneity might be accounted for by mutational changes being allowed only in particular portions of the molecules. Such "localized mutagenesis" may account for the greater heterogeneity of the acidic subunits of oat 12S globulin in comparison to the basic subunits. For example, regions of the molecules involved in specific interactions such as subunit association, membrane transport and proteolytic cleavage would be expected to be conserved. This would explain why it is possible to form molecular hybrids involving legumin-like subunits from different legume species (Utsumi et al, 1980). For this reason also, the N- and C-terminal regions are generally atypical of the amino acid composition of the whole storage protein (Shewry et al, 1984a).

Recently, the isolation and sequencing of cDNA clones coding specifically for prolamins of corn (Geraghty et al, 1981; Pedersen et al, 1980), wheat (Bartels and Thompson, 1983) and barley (Miflin et al, 1984) have provided invaluable insight into the structure and evolution of these proteins. The considerable proportion of these genes encoded for by tandem repeats was one of the most outstanding features uncovered. This finding has led Miflin et al, (1984) to propose that, in its simplest form, a prolamin polypeptide would consist of three domains; an N-terminal domain, a C-terminal domain and an internal domain containing sequence repeats that are variants of one or more consensus sequences. The prolamin genes would presumably have arisen by cycles of internal sequence duplication followed by cycles of gene duplication bringing about complex loci. The polymorphism observed would result from insertions, deletions, point mutations and unequal crossing over. For example, partial sequencing of a C-hordein has revealed repeated octapeptides having the sequence Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln. The importance of this repeat is evidenced by the fact that 80% of the amino acids of the

C-hordeins are glutamine, proline and phenylalanine in the same proportions (i.e. 4:3:1) as the observed repeat (Miflin et al, 1984). Whether the oat avenins fit in the above proposal will have to await sequencing. Interestingly, glutamine, proline and phenylalanine are also the most abundant amino acids of avenin and account for about 55% of the total amino acid content in the ratio 3:1:1 (Draper, 1973; Kim et al, 1978; Weiser et al, 1980). In an attempt to explain these unusual proportions, one might speculate on the importance of a sequence repeat within the avenins which would contain these three amino acids in the same ratio.

The sequences of cDNA clones coding for pea legumin have also been determined recently (Croy et al, 1982; Lycett et al, 1984). Three 54 base pair direct repeats were found within the legumin sequence and all three were located in the region coding for the acidic subunit of legumin (Lycett et al, 1984). The low number of repeats observed in comparison to prolamin genes may indicate that the globulin genes have arisen via a different evolutionary process and might also indicate the stronger structural constraints imposed on the globulins, since they have to fulfill the specific requirements of holoprotein formation. Such stringent structural controls would also explain the similarity exhibited by the N-terminal sequences of the oat 12S globulin and pea legumin basic subunits (Walburg and Larkins, 1983). In addition, the presence of all three repeats within the acidic subunit coding region might partially account for its consistently greater heterogeneity with respect to the basic subunit. Whether oat 12S globulin displays similar characteristics will have to await sequencing of its constituent polypeptides.

Many aspects of oat storage protein genetics require further investigation. It would be interesting to know more about the number, complexity, chromosomal arrangements and regulatory mechanisms of the loci coding for the oat globulins and prolamins. Naturally, knowledge of the sequence of these proteins would also be very helpful in determining for example, the presence and frequency of repeats, the position of proteolytic cleavage sites and the extent of homology with related proteins. One of the most important questions raised by the findings discussed in this chapter involves the regulation of globulin biosynthesis in cereals. Are there legume-like proteins in cereals other than oat? Oat is the only monocot which has been demonstrated to possess firstly, globulins as a major seed storage protein fraction and secondly, globulins which show obvious structural similarities to the major globulin fractions of legumes, the legumins and 3S and 7S globulins.

CHAPTER IV

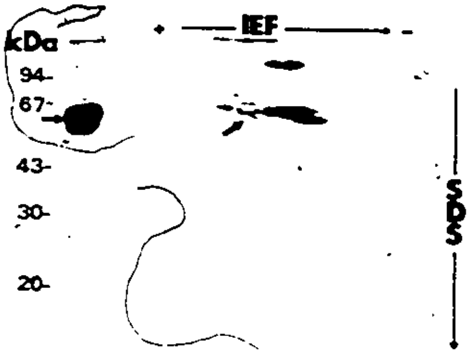
EXPRESSION OF HOMOLOGOUS GLOBULINS WITHIN CEREAL AND LEGUME SPECIES

4.1 RESULTS

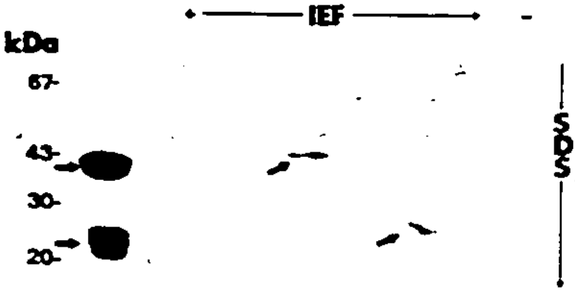
4.1.1 Expression of legumin-like proteins in cereals and pea

Significant differences among the total globulin fractions from oat, pea and wheat become apparent upon comparison of their two-dimensional (IEF and SDS PAGE) electrophoretic patterns (Fig. 4.1). As previously mentioned, the oat globulins consist primarily of legumin-like proteins (section 3.1.1). The oat globulin reducible molecules (∿60 kDa) predominate in the absence of a reducing agent (Fig. 4.1 O-, arrows) and dissociate to their constituent α- and β-subunits in the presence of a reducing agent (Fig. 4.1 O+, arrows). Pea legumin is observed as both the unreduced molecules (Fig. 4.1 P-, arrows) and the reduced α- and β-subunits (Fig. 4.1 P+, arrows) despite the presence of a significant amount of other polypeptides, the vicilins. A minor group of proteins is also found in the 60 kDa region of the wheat globulins under non-reducing conditions (Fig. 4.1 W-, arrows), which is absent under reducing conditions (Fig. 4.1 W+). Dissociation products of the wheat ∿60 kDa group of polypeptides, are not immediately evident and may have co-migrated with other wheat globulins.

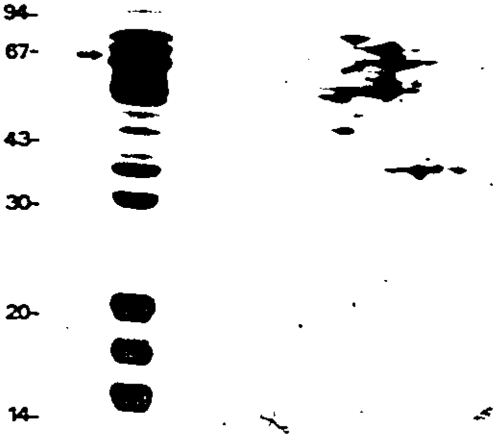
Figure 4.1. Two-dimensional analysis (section 2.7.3B) of the unreduced total globulin fraction from (O-) oat, (P-) pea and (W-) wheat (arrows point to the ~60 kDa reducible dimers) and the reduced total globulin fraction from (O+) oat, (P+) pea and (W+) wheat (arrows point to the subunits). Proteins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of the globulins is included with each gel for comparison.


kDa
IEF
94-
67-
43-
30-
20-
SDS

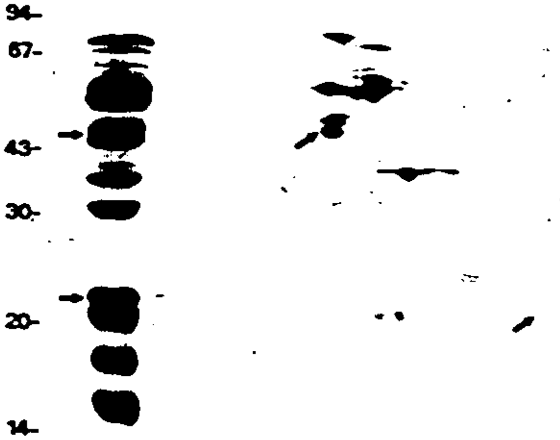
O^-


IEF
kDa
67-
43-
30-
20-
SDS

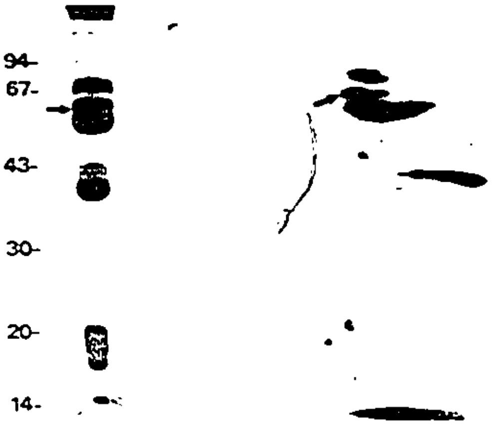
O^+


94-
67-
43-
30-
20-
14-

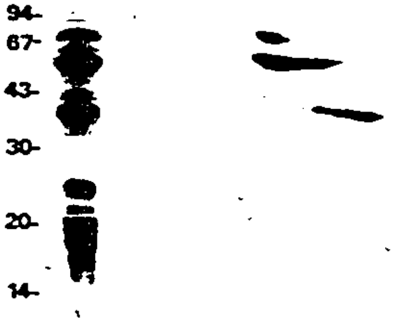
P^-


94-
67-
43-
30-
20-
14-

P^+


94-
67-
43-
30-
20-
14-

W^-

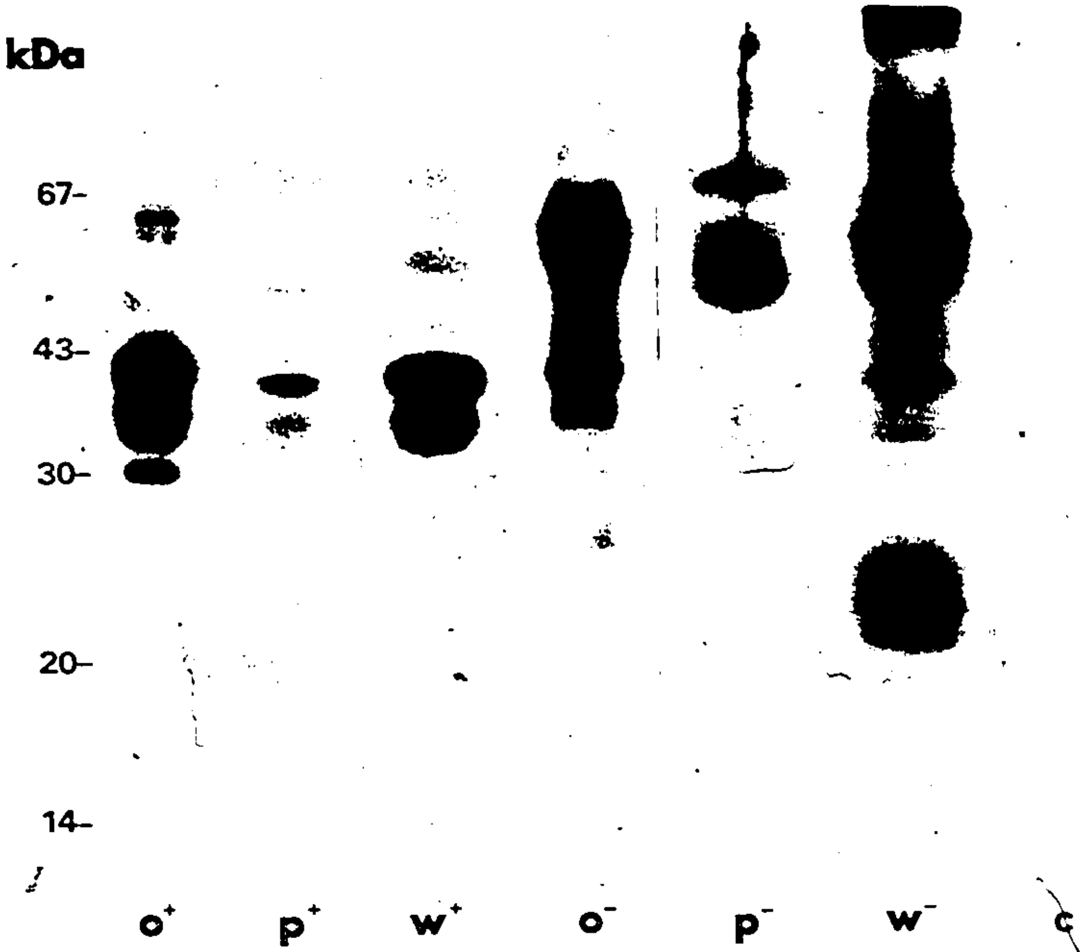

94-
67-
43-
30-
20-
14-

W^+

The SDS PAGE patterns of oat, pea and wheat globulins challenged with the ^{125}I-labelled oat globulin α-subunit antibody show that various polypeptides of all three plants are selectively recognized (Fig. 4.2). As expected, the antibody cross-reacts strongly to the ∿40 kDa oat globulin α-subunits under reducing conditions (Fig. 4.2, lane o+). The antibody also cross-reacts with polypeptides of the pea α-subunit (Fig. 4.2 p+). The wheat globulin fraction also contains polypeptides of ∿40 kDa which are selectively recognized by the oat globulin α-subunit antibody and these show a strong cross-reactivity (Fig. 4.2 w+). These ∿40 kDa polypeptides of oat, pea and wheat globulins are therefore immunologically homologous.

Since the α-subunits of oat and pea are components of ∿60 kDa reducible molecules, the absence of a reducing agent should cause a shift of the radioactive signal from the ∿40 kDa α-subunits to the disulfide-linked molecules. A similar phenomenon should be observed with the wheat globulins, if the cross-reacting ∿40 kDa polypeptides are involved in the formation of the ∿60 kDa reducible polypeptides observed in Figure 4.1 (W-). Indeed, under non-reducing conditions a relative increase in the radioactive signal found in the 60 kDa region (accompanied by a decrease in the 40 kDa region) was observed not only within the oat and pea globulin patterns, but with that of wheat also (Fig. 4.2 o-, p-, w-). The radioactivity found in the 40 kDa region under non-reducing conditions, might have resulted from disulfide bonds broken during sample boiling prior to electrophoresis. The antibody also bound to additional polypeptides found between 50-70 kDa, as well as polypeptides smaller

Figure 4.2. Western blot analysis (section 2.12) autorodiagraph: ^{125}I-labelled antibodies raised against the purified oat globulin α-subunits cross-reacted with the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of the reduced (o+) oat, (p+) pea, (w+) wheat and unreduced (o-) oat, (p-) pea and (w-) wheat total globulin fractions transferred onto nitrocellulose. Lane (c) contains no protein. Since differences occur in the proportion of legumin-like proteins within the total globulin extracts and also in species interrelationship, different quantities of protein were applied to the gel for the various species: o+ (10 μg), p+ (120 μg), w+ (250 μg), o- (7.5 μg), p- (100 μg) and w- (250 μg). The cross-reactivity patterns can be compared to the equivalent Coomassie Blue-stained lanes presented in Fig. 4.20.

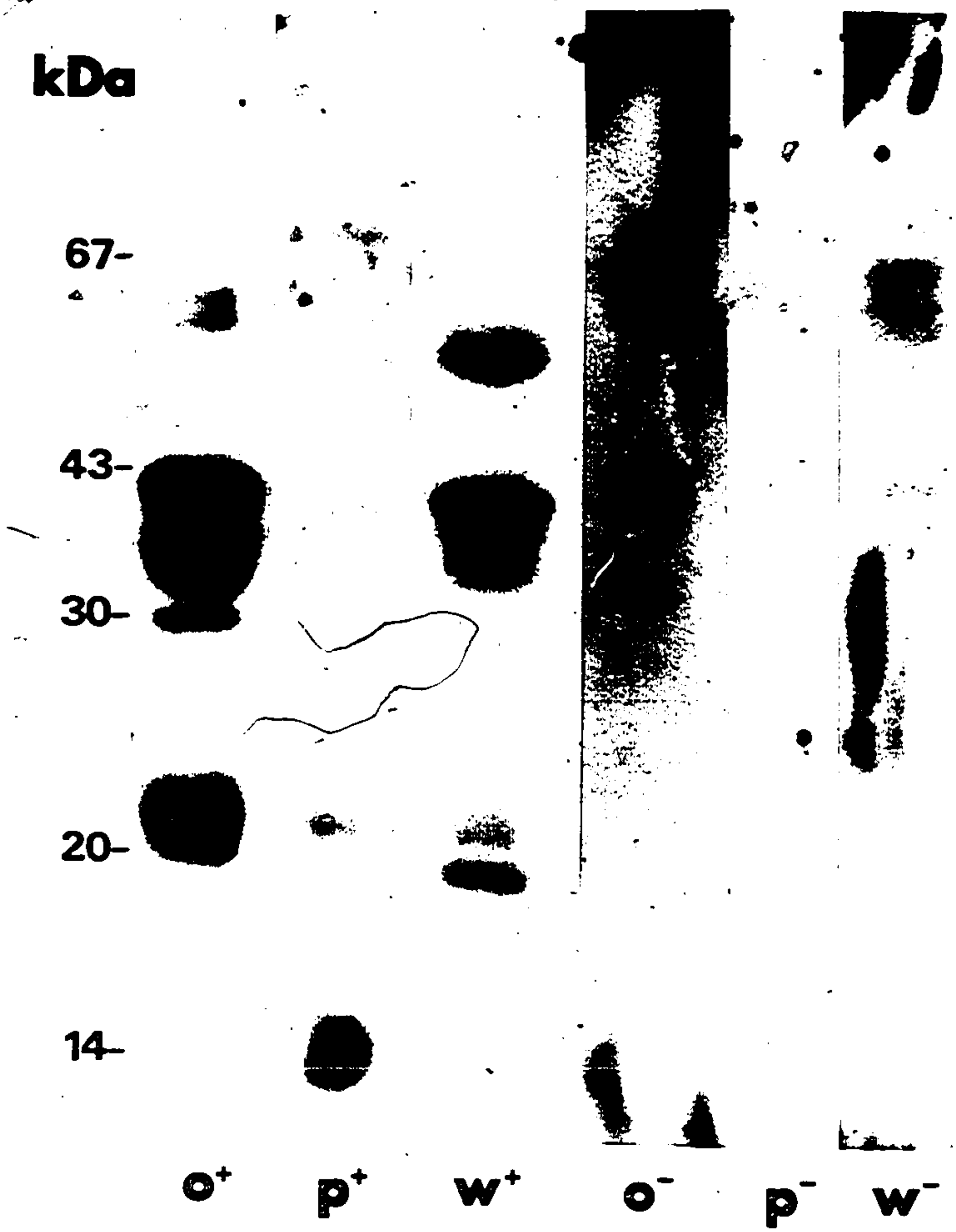

kDa
67–
43–
30–
20–
14–
o⁺
p⁺
w⁺
o⁻
p⁻
w⁻
c

than 40 kDa in the oat, pea and wheat globulin fractions. The positions of these bands were apparently not affected by the presence or absence of reducing agent (Fig. 4.2).

Western blot analysis shows, that along with their α-subunits, the β-subunits of both oat and pea globulins are detected by the labelled oat 12S globulin holoprotein antibody under reducing conditions (Fig. 4.3 o+, p+, respectively). Similarly, wheat globulin polypeptides of ~40 kDa and ~20 kDa are also recognized (Fig. 4.3 w+). If the ~20 kDa wheat polypeptides are the necessary counterparts which associate with the ~40 kDa subunits to form the reducible ~60 kDa polypeptides observed in Figs. 4.1 (W-) and 4.2 (w-), the absence of a reducing agent should cause a shift of the radioactive signal from these polypeptides to the region of the 60 kDa polypeptides. The same phenomenon should take place with the homologous oat and pea globulins. The cross-reaction patterns of the oat, pea and wheat globulins observed under non-reducing conditions display a relatively stronger radioactive signal in the 60 kDa region and much weaker signals in the 20 kDa and 40 kDa regions (Fig. 4.3 o-, p-, w-), in comparison to the patterns obtained under reducing conditions (Fig. 4.3 o+, p+, w+).

Some of the polypeptides (other than the ~40 kDa subunits) which had cross-reacted with the oat globulin α-subunit antibody (Fig. 4.2) were also recognized by the 12S gobulin holoprotein antibody (Fig. 4.3). This was particularly the case with the polypeptides in the 50-70 kDa region.

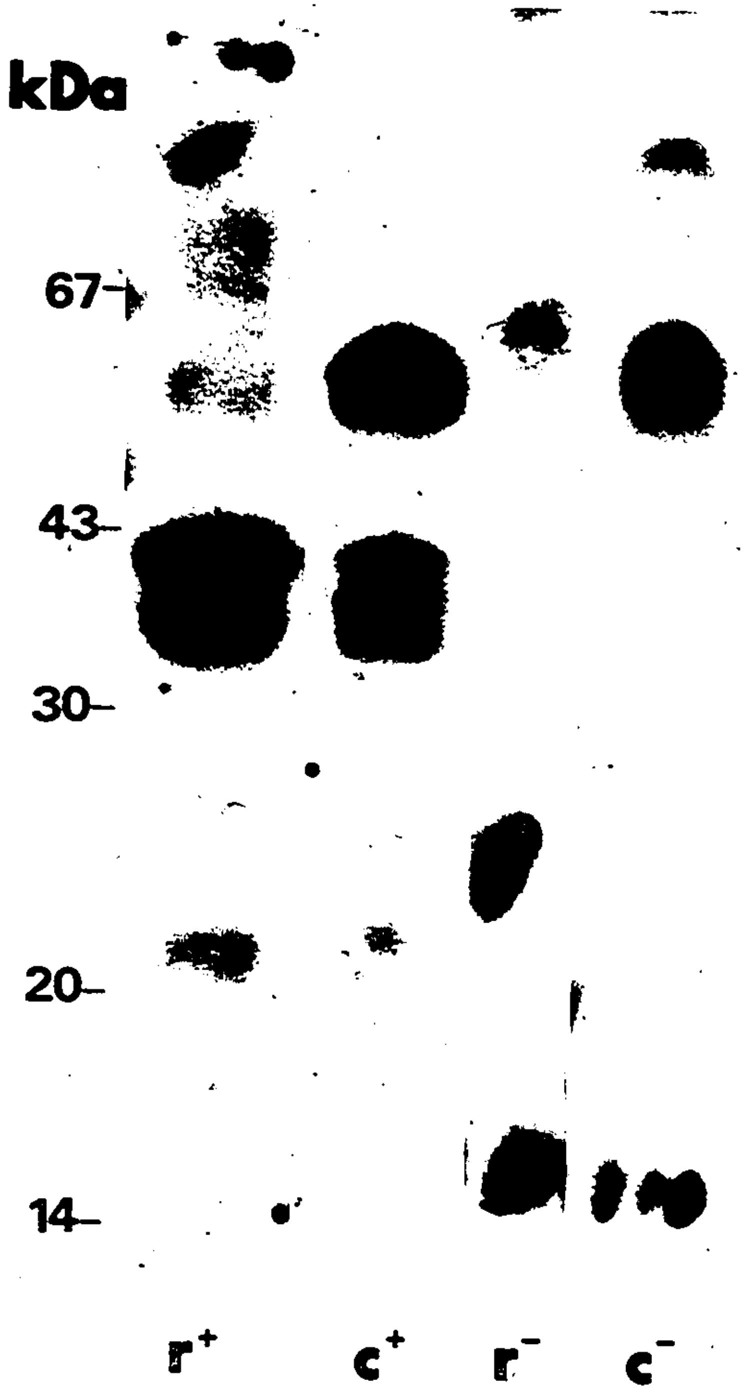
Figure 4.3. Western blot analysis (section 2.12) autoradiograph: ^{125}I-labelled antibodies raised against the purified oat 12S globulin holoprotein cross-reacted with the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of the reduced (o+) oat, (p+) pea, (w+) wheat and unreduced (o-) oat, (p-) pea and (w-) wheat total globulin fractions transferred onto nitrocellulose. Since differences occur in the proportion of legumin-like proteins within the total globulin extracts and also in species interrelationship, different quantities of protein were added to the gel for the various species: o+ (10 µg), p+ (250 µg), w+ (300 µg), o- (7.5 µg), p- (250 µg) and w- (300 µg). The cross-reactivity patterns can be compared to the equivalent Coomassie Blue-stained lanes presented in Fig. 4.20.


kDa
67-
43-
30-
20-
14-
o+
p+
w+
o-
p-
w-

Variations in the intensity of the radioactive signals are evident among the different Western blot analyses. For example, the 12S holoprotein antibody cross-reacted weakly to the ∼60 kDa disulfide-linked molecules (Fig. 4.3 o-, w-, p-) in comparison to the α-subunits antibody (Fig. 4.2 o-, w-, p-). This is especially true of the ∼60 kDa reducible molecules from pea (Fig. 4.3 p-). Since background staining was reduced to a minimum in these experiments, these variations are believed to arise from differences in the availability of the antigenic sites among the three forms of the protein (i.e. subunits, disulfide-linked molecules, holoprotein) and in the affinity of the antibodies to proteins from the different plants studied.

A Western blot analysis was also performed on the globulin fractions from rye and corn using the oat 12S globulin holoprotein antibody. Under reducing conditions, the labelled antibody binds primarily to rye globulin polypeptides of ∼20 kDa and ∼40 kDa (Fig. 4.4 r+). In the absence of a reducing agent, however, a radioactive signal is observed only in the 60 kDa regions (Fig. 4.4 r-). Similar results were obtained with the corn globulins, except for the additional presence of strongly cross-reacting polypeptides in the 60 kDa region observed under reducing conditions (Fig. 4.4 c+) and of a high molecular weight band (>100 kDa) seen under non-reducing conditions (Fig. 4.4 c-). This high molecular weight band may correspond to a similar band sometimes observed with oat globulins (see Fig. 3.3) and rice glutelin (see Fig. 4.8 below). Western blot analysis of barley globulins repeatedly gave very weak cross-reactions (results not shown).

Figure 4.4. Western blot analysis (section 2.12) autoradiograph: ^{125}I-labelled antibodies raised against the purified oat 12S globulin holoprotein cross-reacted with the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of the reduced (r+) rye, (c+) corn and unreduced (r-) rye and (c-) corn total globulin fractions transferred onto nitrocellulose. The quantities of protein applied to the gel were approximately: r+ (250 µg), c+ (350 µg), r- (400 µg) and c- (400 µg). The cross-reactivity patterns can be compared to the equivalent Coomassie Blue-stained lanes presented in Fig. 4.20.


kDa
67
43
30
20
14
r^+
c^+
r^-
c^-

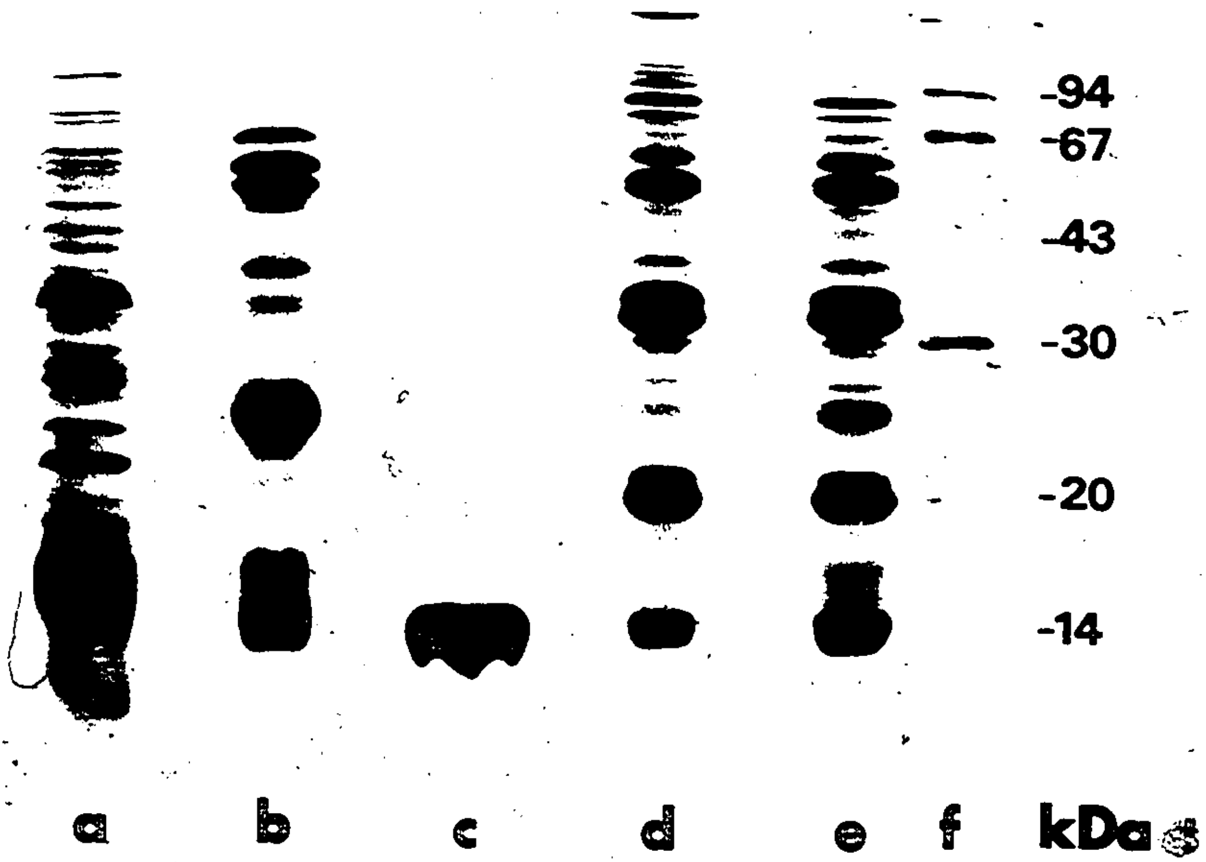
4.1.2 Expression of legumin-like proteins in rice

Rice is treated separately mainly because its glutelin fraction, rather than its globulin fraction, seems to bear the most resemblance to legumin-like globulins. Rice glutelins have been poorly characterized because of their low solubility. Therefore, an attempt was first made to characterize these proteins in a fashion similar to that used for the oat residual proteins (section 3.1.3). Western blot analysis was then performed to assess possible homology.

The different Osborne protein fractions of the rice seed were extracted as done with oat (section 2.3) and resolved by SDS PAGE under reducing conditions (Fig. 4.5). Each fraction exhibits a distinct electrophoretic pattern. The albumins (lane a) consist of an heterogeneous group of polypeptides with a major band of 15 kDa (lane a). The major globulins (lane b) were found at 13-16, 24 and 50-60 kDa. The prolamin fraction (lane c) displays a strong band of 13 kDa as observed by Tanaka et al, (1980). The glutelin fraction (lane d) contains numerous polypeptides including major subunits of 19-22 and 30-36 kDa. As seen in the total protein extract (lane e), the glutelin subunits represent the most abundant rice seed proteins.

The efficient extraction of rice glutelin generally involves the use of SDS and ME (Yamagata et al, 1982). Since SDS alters native polypeptide charge distributions and ME breaks disulfide bonds, it becomes difficult firstly, to detect the presence of intra- and

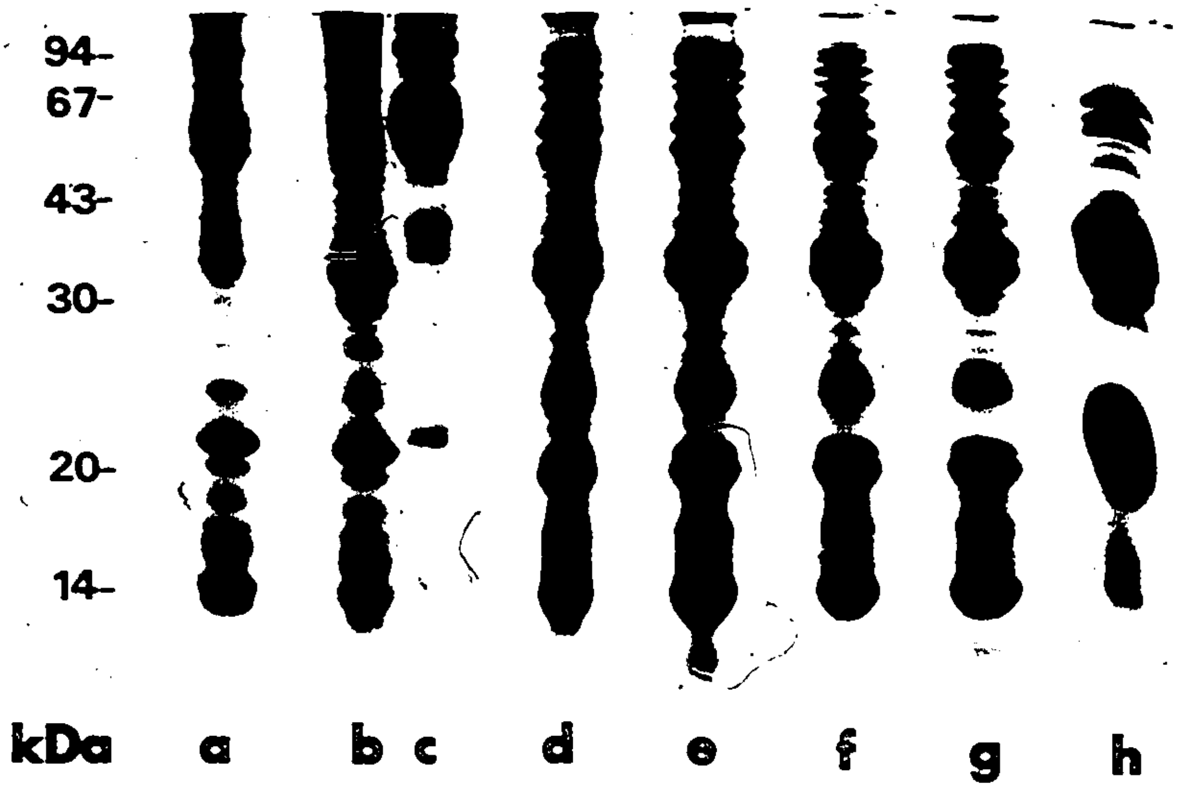
Figure 4.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) in the presence of 2-mercaptoethanol of rice seed Osborne fractions: (a) albumins, (b) globulins, (c) prolamins, (d) glutelins, and (e) total protein extract and (f) molecular weight standards.


-94
-67
-43
-30
-20
-14
a
b
c
d
e
f
kDa

interchain disulfide associations or secondly, to perform an IEF analysis. To circumvent this problem, extractions of rice proteins were performed in the absence of ME and also with solutions containing urea rather than SDS, as done with the oat residual proteins (section 3.1.3). The electrophoretic patterns obtained for the various SDS and urea extracts are shown in Fig. 4.6. The pattern observed for the unreduced SDS total protein extract (SDS (-ME); lane a) shows a prominent group of polypeptides of 50-62 kDa and reduced levels of the glutelin subunits of 19-22 and 30-36 kDa in comparison to the pattern obtained under reducing conditions (Fig. 4.5, lane e). This behaviour is typical of oat globulins in the absence (Fig. 4.6, lane c) and presence (lane h) of a reducing agent.

The best recovery of the rice proteins obtained with a urea solution was achieved at a concentration of 7.0 M urea (data not shown). The electrophoretic pattern of the proteins from the 7.0 M urea (-ME) extract (Fig. 4.6, lane b) resembles closely that of the SDS (-ME) extract (lane a), except that the proteins found at 50-62 kDa are not as evident and a smear in the high molecular weight region (>50 kDa) is typically observed. With the addition of ME to the urea extract (lane d) and the SDS extract (lane f), the glutelin subunits (19-22 and 30-36 kDa) become prominent with a concomitant decrease in the amount of smearing (urea) or in polypeptides of 50-62 kDa (SDS). The resulting patterns are equivalent to each other and to those obtained for proteins directly extracted from seeds under reducing conditions (urea (+ME), lane e; SDS (+ME), lane g).

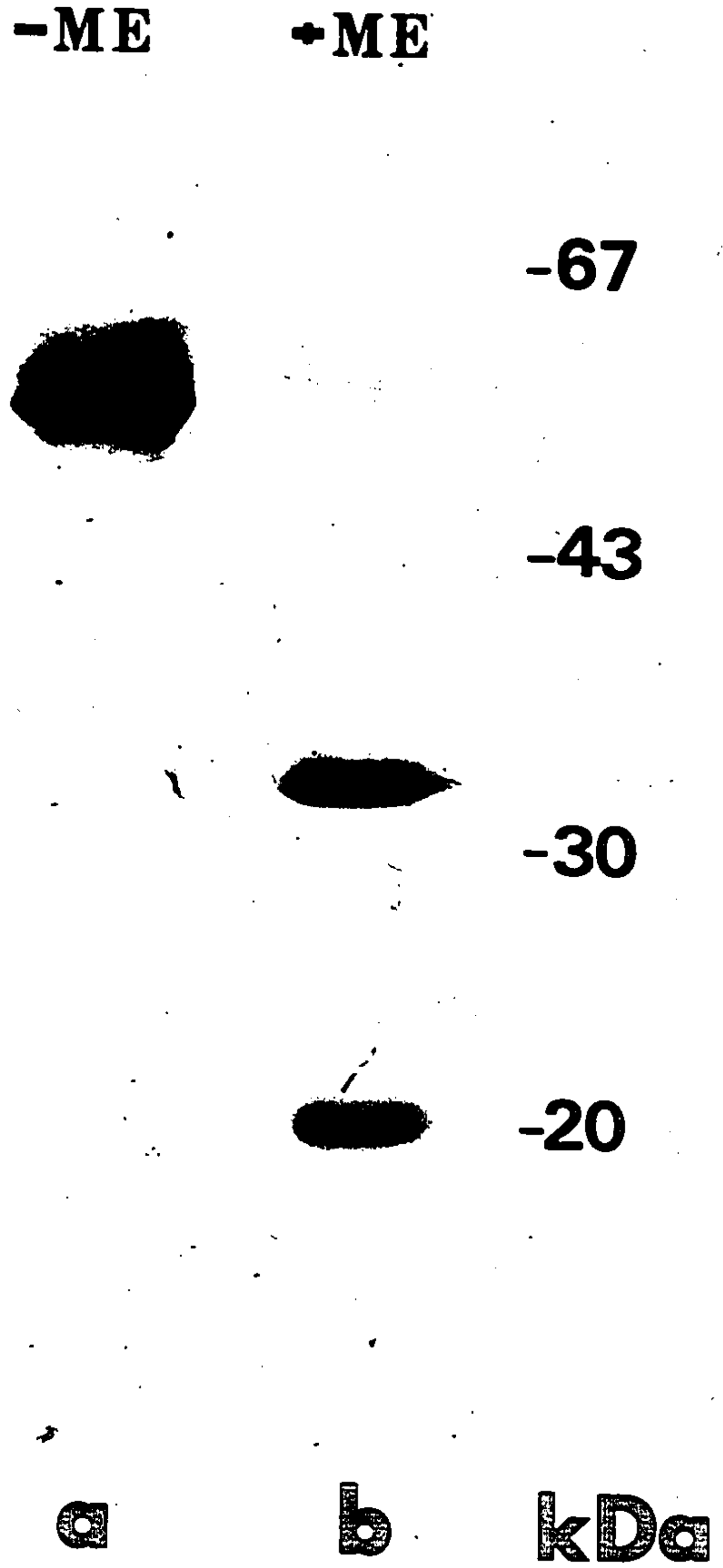
Figure 4.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) of different rice total protein extracts compared to oat total globulins: (a) rice proteins extracted with 1.0% sodium dodecyl sulfate (SDS) in the absence of 2-mercaptoethanol (-ME), (b) rice proteins extracted with 7.0 M urea (-ME), (c) oat total globulin (-ME), (d) rice proteins extracted with 7.0 M urea (-ME) resolved (+ME), (e) rice proteins extracted with 7.0 M urea (+ME), (f) rice proteins extracted with 1.0% SDS (-ME) resolved (+ME), (g) rice proteins extracted withe 1.0% SDS (+ME) and (h) oat total globulin (+ME).


94-
67-
43-
30-
20-
14-
kDa
a
b
c
d
e
f
g
h

To verify whether the larger amount of proteins of 50-62 kDa observed under non-reducing conditions resulted from the association of the glutelin subunits, this region of the gel was eluted and electrophoresed in the presence and absence of ME (Fig. 4.7). In the absence of ME, only the 50-62 kDa polypeptides can be seen (lane a), whereas in the presence of ME, one subunit of 20 kDa and at least three subunits of 30-36 kDa become apparent (lane b). Not all bands of 50-62 kDa dissociate under reducing conditions (lane b). These results were substantiated by a 2-D analysis of a SDS (-ME) total protein extract electrophoresed without ME in the first dimension and with ME in the second SDS PAGE gel (Fig. 4.8). The 50-62 kDa polypeptides of the first dimension (exemplified by the top horizontal lane) dissociated to the subunits observed in Figure 4.7. High molecular weight proteins (>100 kDa) were also affected by reducing conditions and these probably represent associations of the disulfide-linked 50-62 kDa polypeptides (since the former also broke down to the 19-22 and 30-36 kDa subunits in the second dimension). A major globulin band increased in apparent molecular weight from 22 kDa to 26 kDa under reducing conditions probably due to unfolding upon breaking of intrachain disulfide bonds.

The rice seed Osborne protein fractions were characterized by IEF (Fig. 4.9). Each fraction exhibits a distinct charge distribution. The albumin fraction (lane a) contains few basic polypeptides, whereas the globulins (lane b) cover practically the entire pH range. The five prolamin bands observed upon IEF (lane c) contrast with the single major band evident upon SDS PAGE (Fig. 4.5, lane c). Charge heterogeneity also

Figure 4.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) of the unreduced rice glutelins of 50-62 kDa extracted with 1.0% SDS: (a) in the absence of 2-mercaptoethanol and (b) in the presence of 2-mercaptoethanol.



-ME
+ME
-67
-43
-30
-20
a
b
kDa

Figure 4.8. Two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.3A) of the rice 1.0% SDS (-ME) total protein extract (first dimension resolved in the absence of 2-mercaptoethanol and the second dimension in its presence). A lane equivalent to the unreduced total rice protein lane which was electrophoresed in the second dimension was stained, similarly placed on top of the second gel and included in the photograph. Arrows indicate the direction of electrophoresis in the first and second dimensions.

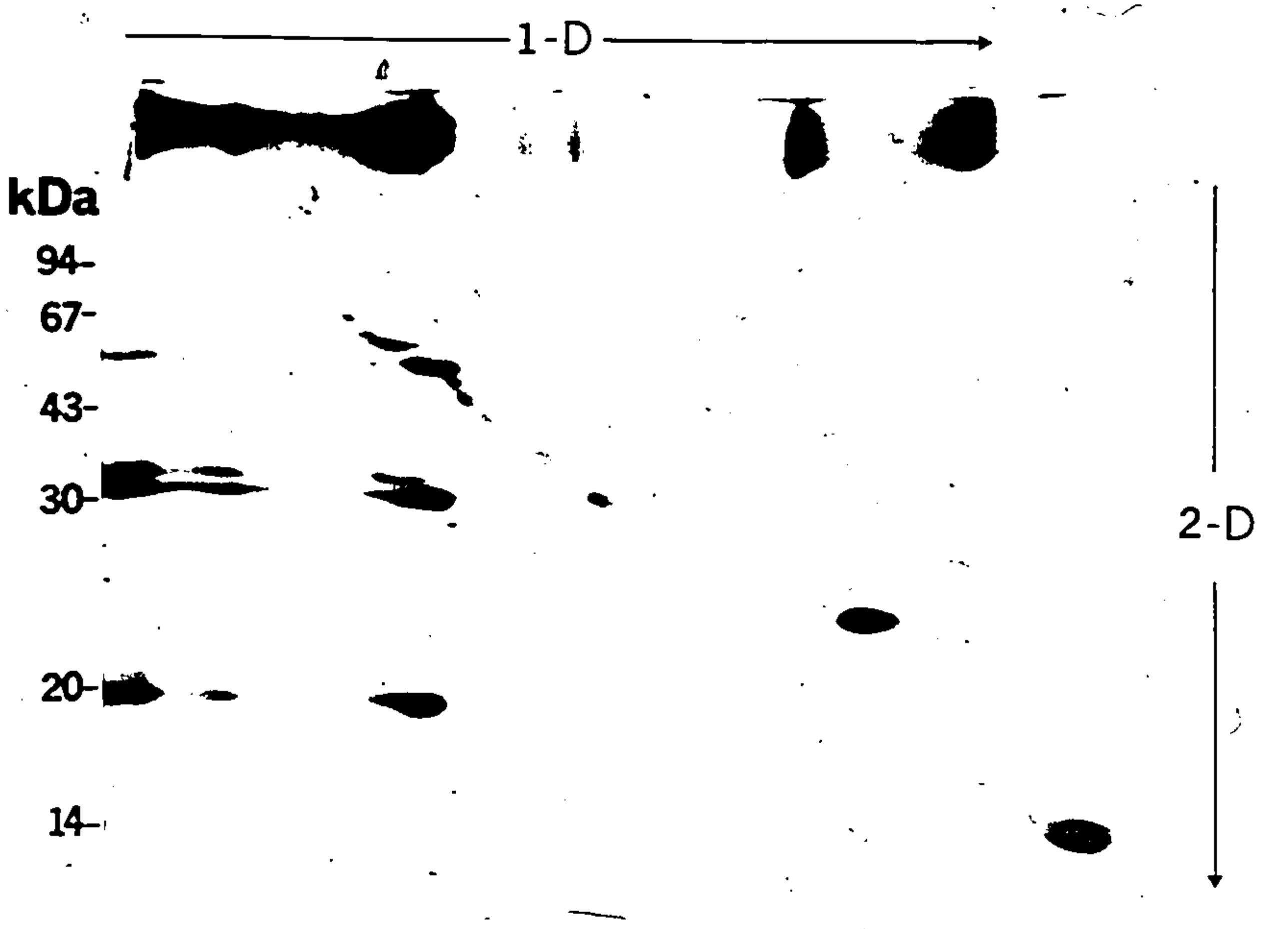
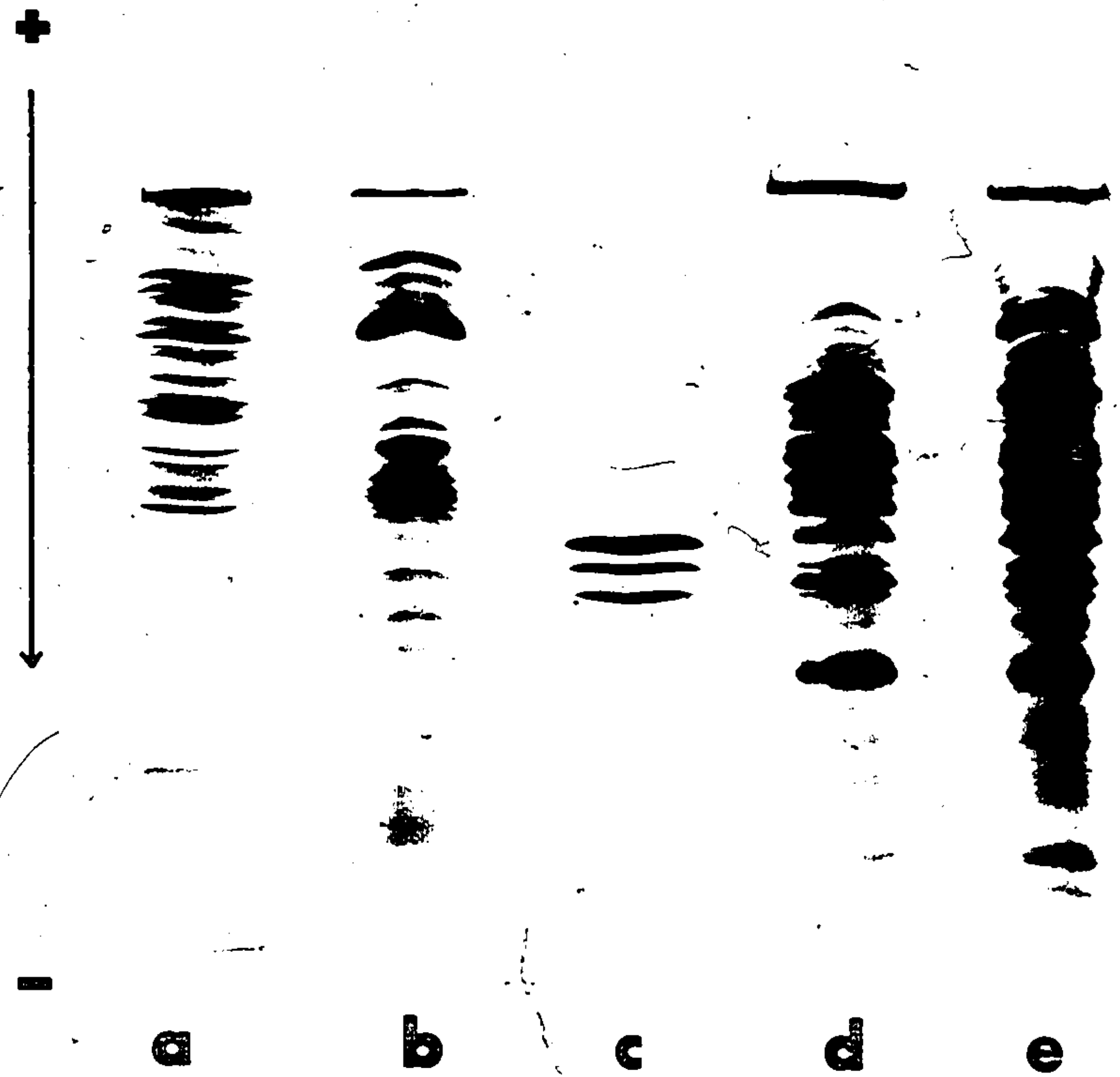

1-D
2-D
kDa
94-
67-
43-
30-
20-
14-

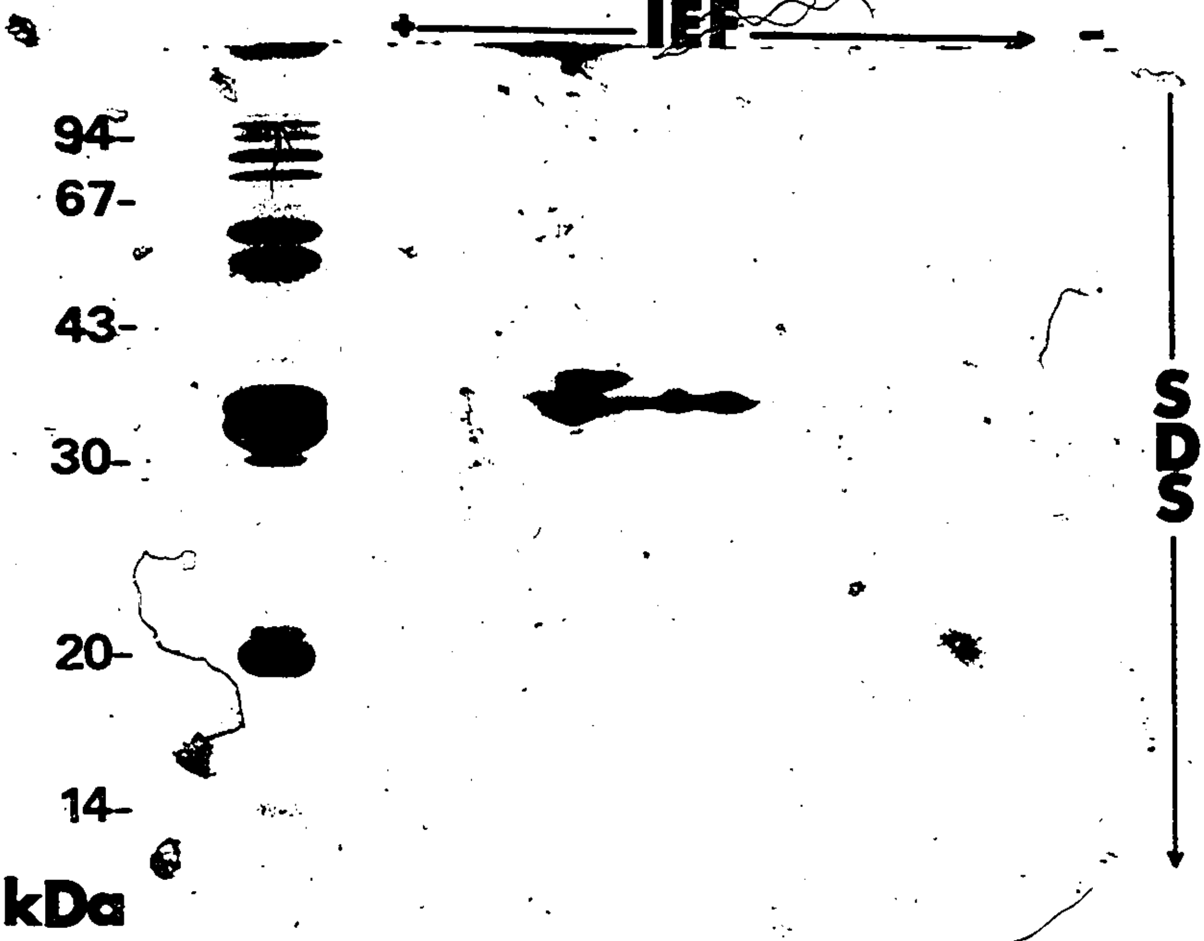
Figure 4.9. Isoelectric focusing (section 2.7.2; pH 3.5-10) in the presence of 2-mercaptoethanol of the rice seed protein Osborne fractions: (a) albumins, (b) globulins, (c) prolamins, (d) glutelins and (e) total proteins extracted with 1.0% SDS.


+
-
a
b
c
d
e

appeared to contribute to the large number of glutelin IEF bands (lane d) and a 2-D analysis was performed to reveal the charge distribution of the major glutelin subunits (Fig. 4.10). The 30-36 kDa subunits shows even more heterogeneity than was first observed with SDS PAGE (Fig. 4.7) and consists of several polypeptides . The 19-22 kDa subunit does not display the same level of heterogeneity, but there seems to be two charge variants. The smaller subunits possess more basic pIs than their larger counterparts.

Urea and SDS extracts of rice proteins were electrophoresed, transferred onto nitrocellulose and allowed to cross-react with ^{125}I-labelled anti-oat 12S globulin IgG. Under reducing conditions, the 30-36 kDa subunits and two of the 19-22 kDa subunits cross-react strongly in both the urea and SDS extracts (Fig. 4.11, lanes b and c respectively). Bands of 50 and 63 kDa and a minor low molecular weight band are also recognized by the oat 12S globulin antibody. The antibody also binds to polypeptides in the rice globulin fraction (lane d) which co-migrate with the glutelin subunits. The glutelins subunits were not detected in the albumin or prolamin fractions (data not shown). With the SDS (-ME) glutelin extract, the 19-22 kDa and 30-36 kDa subunits are not detected, but the antibody binds strongly to proteins in the 50-65 kDa region (Fig. 4.12, lane b) supporting the disulfide association of these subunits. A high molecular weight band (>100 kDa) also cross-reacts with the oat antibody under non-reducing conditions. This band probably corresponds to the high molecular weight proteins which break down into the individual glutelin subunits under reducing conditions (Fig. 4.8). The

Figure 4.10. Two-dimensional analysis (section 2.7.3A) of the 7.0 M urea (+ME) rice glutelin extract. Glutelins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of rice glutelins extracted with 7.0 M urea (+ME) is included for comparison.



+
IEF
-
94-
67-
43-
30-
20-
14-
kDa
SDS

Figure 4.11. Western blot analysis (section 2.12) autoradiograph: ^{125}I- labelled antibodies raised against the purified oat 12S globulin holoprotein cross-reacted to the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of: (a) reduced oat globulins, (b) 1.0% SDS (+ME) rice glutelin extract, (c) 7.0 M urea (+ME) rice glutelin extract and (d) rice globulins (+ME) transferred onto nitrocellulose. The quantities of protein applied to the gel were approximately: (a) 10 μg, (b) 200 μg, (c) 200 μg and (d) 300 μg.

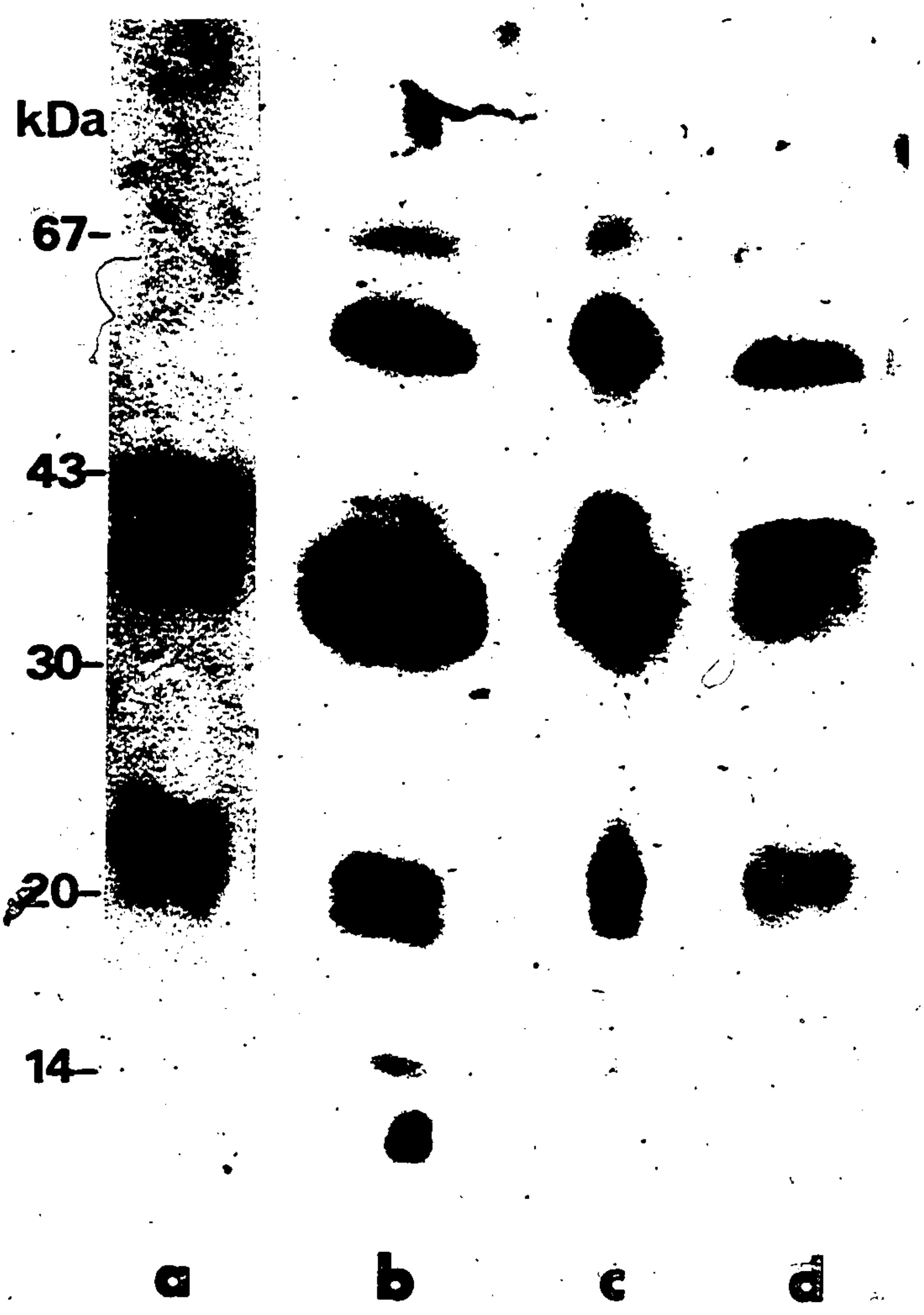
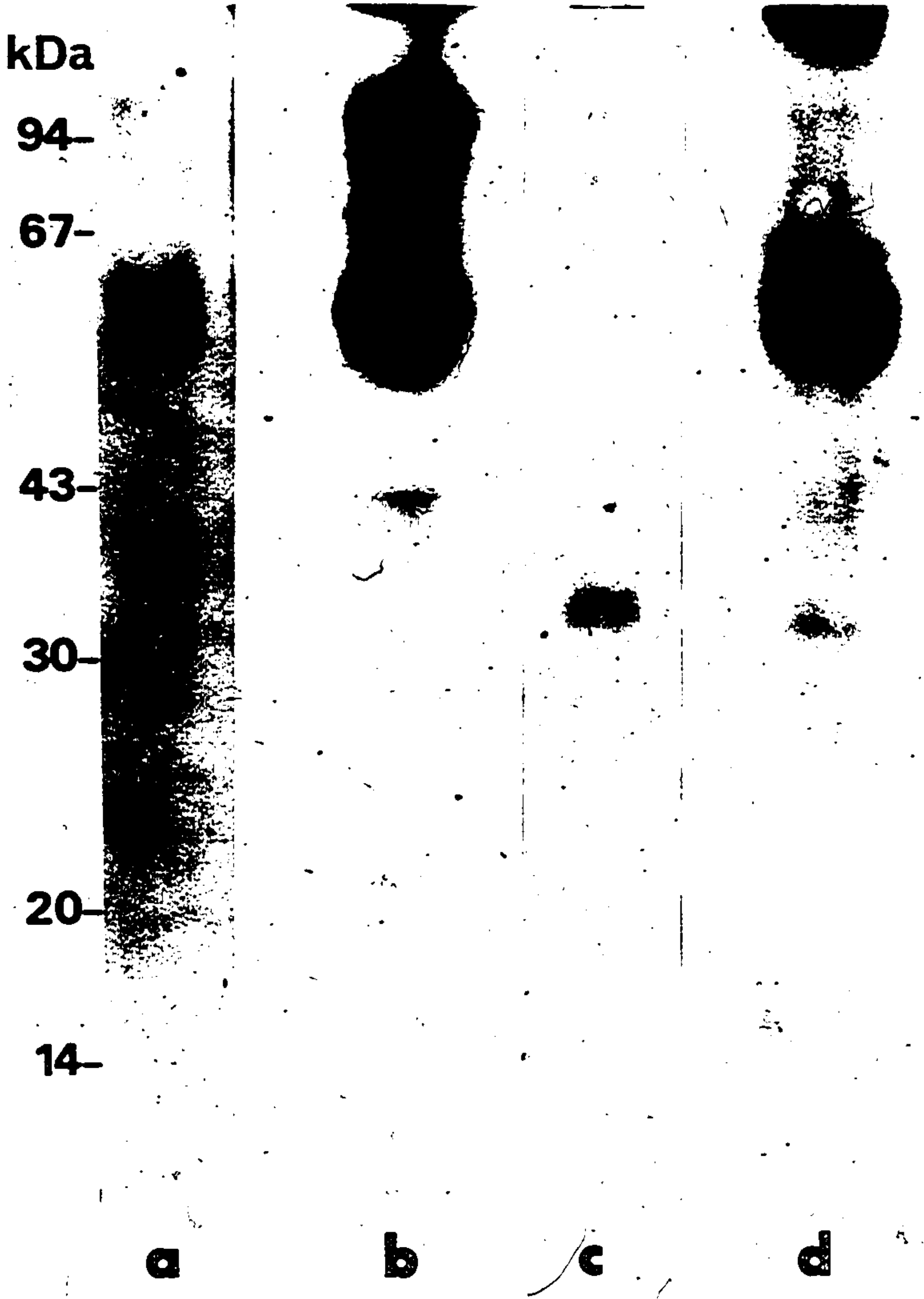

kDa
67–
43–
30–
20–
14–
a
b
c
d

Figure 4.12. Western blot analysis (section 2.12) autoradiograph: ^{125}I - labelled antibodies raised against the purified oat 12S globulin holoprotein cross-reacted to the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of: (a) unreduced oat globulins, (b) 1.0% SDS (-ME) rice glutelin extract, (c) 7.0 M urea (-ME) rice glutelin extract and (d) rice globulins (-ME) transferred onto nitrocellulose.


kDa
94-
67-
43-
30-
20-
14-
a
b
c
d

urea (-ME) extract showed practically no radioactivity except for a faint band of 35 kDa (Fig. 4.12, lane c), reiterating the requirement for ME to disaggregate rice glutelin and permit its entry into the gel. Under non-reducing conditions, the rice globulin extract does not display the polypeptides which co-migrated with the rice glutelin subunits anymore, but shows rather a strong radioactive signal in the 50-65 kDa region (Fig. 4.12, lane d).

4.1.3 Expression of 3S and 7S globulins in cereals and pea

The wheat globulins were fractionated by sucrose density gradient centrifugation and the resulting pattern was compared to that of the purified oat 3S and 7S globulins (Fig 4.13). These oat proteins have been characterized in detail elsewhere (Burgess et al., 1983; Adeli and Altosaar, 1984). The sedimentation coefficients of the two major peaks observed for wheat globulins very closely match the 3S and 7S peaks of the purified oat proteins. Only a small shoulder was apparent at 10-11S with wheat globulins (Fig. 4.13).

The proteins from the wheat 3S and 7S peaks (fractions 5 to 16, Fig. 4.13) were pooled and resolved by two-dimensional analysis (Fig. 4.14). The wheat (3S + 7S) preparation consists of numerous polypeptides with the major ones found at approximately 70, 55, 36-43, 24, 17-20 and ~10 kDa. The relative molecular weights and charge distributions of the major (3S + 7S) wheat polypeptides show features common to the (3S + 7S) globulins found in oat (Fig. 4.15). However, different relative

Figure 4.13. Absorbance at 280 nm of sucrose density gradient fractions (section 2.8) of wheat total globulins and oat purified (3S + 7S) globulins: wheat (▲——▲), oat (■——■). Purified pea 7S vicilin and 11S legumin holoproteins were used as reference standards.

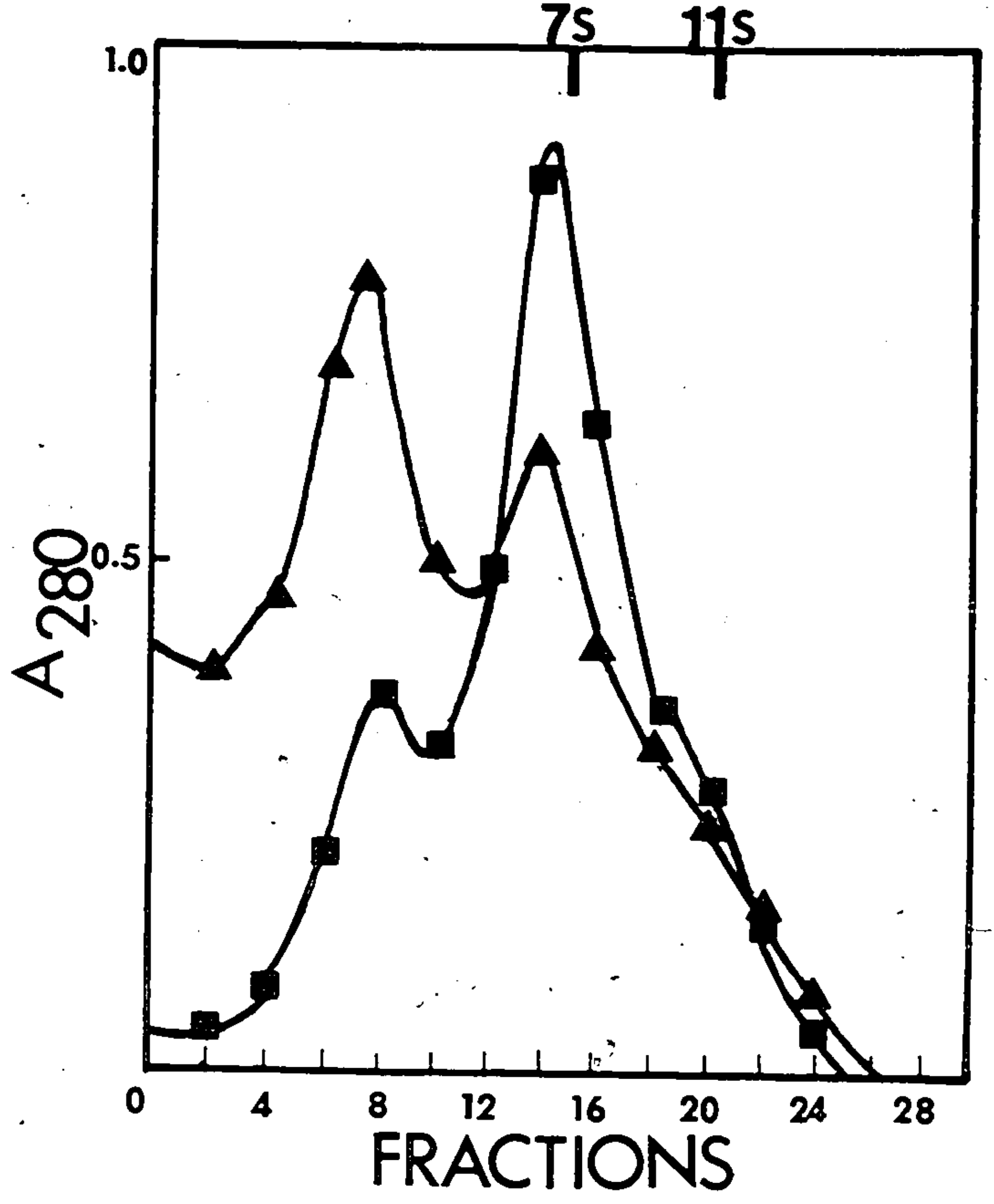
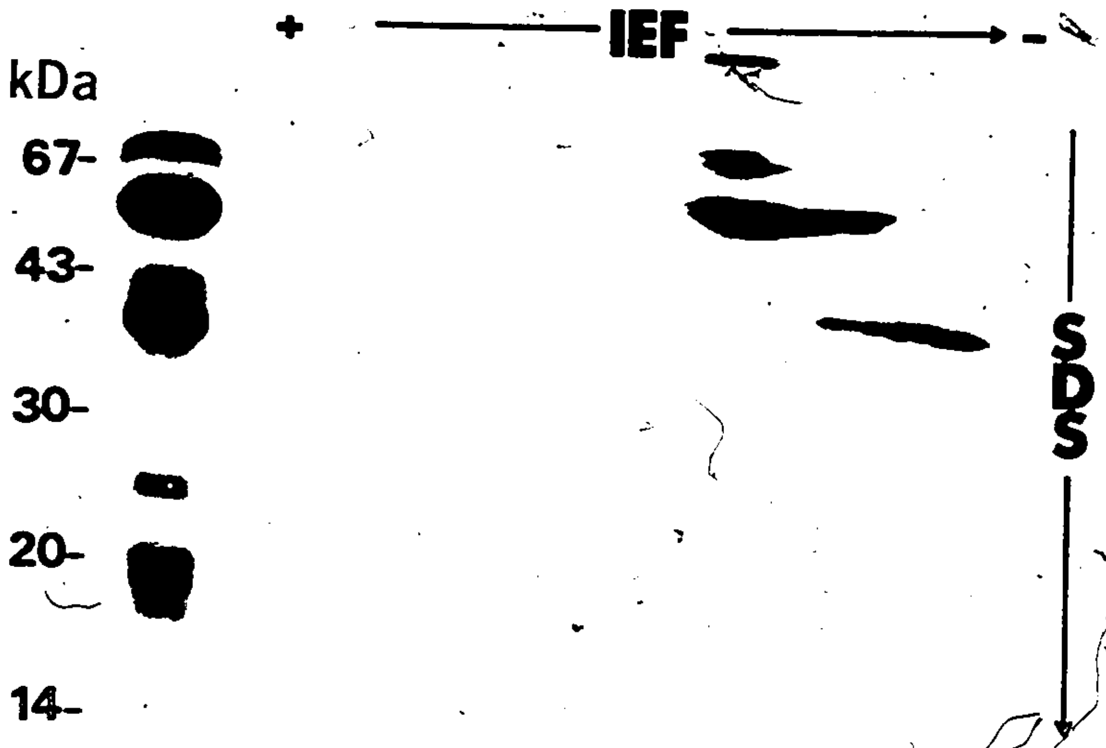
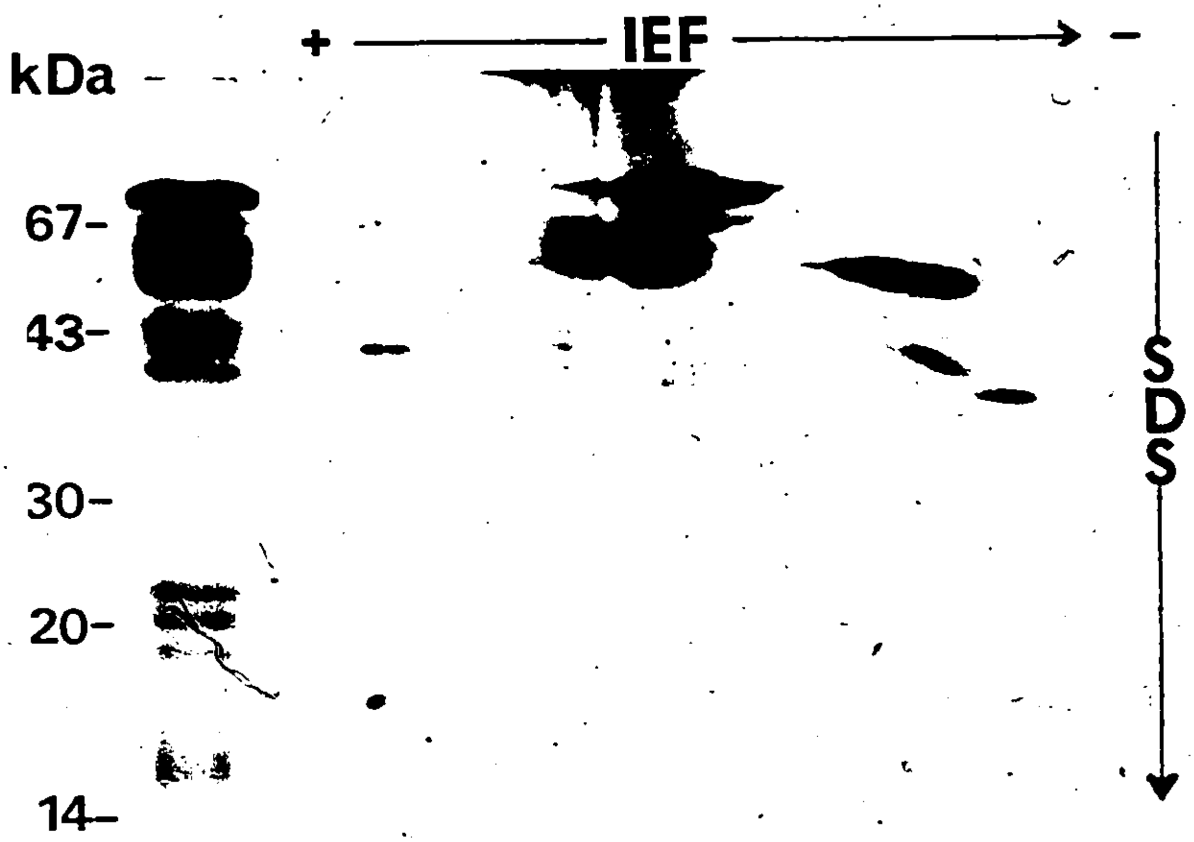

7S
11S
1.0
0.5
A280
0
4
8
12
16
20
24
28
FRACTIONS

Figure 4.14. Two-dimensional analysis (section 2.7.3B) in the presence of 2-mercaptoethanol of wheat (3S + 7S) globulins. Globulins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of the wheat (3S + 7S) globulins is included for comparison.



+
IEF
-
kDa
67-
43-
30-
20-
14-
SDS

Figure 4.15. Two-dimensional analysis (section 2.7.3B) in the presence of 2-mercaptoethanol of oat (3S + 7S) globulins. Globulins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of the oat (3S + 7S) globulins is included for comparison.


kDa
+
IEF
–
67–
43–
30–
20–
14–
SDS

proportions of the major subunits were evident between the two cereals. For example, scanning the stained SDS PAGE lanes showed the proportion of 70 kDa bands in the oat fraction to be approximately three times that of the equivalent bands in wheat, whereas the reverse proportions were observed for the bands at ∿ 40 kDa (data not shown). The purified pea vicilins are composed of an array of polypeptides uniformly distributed between 14 kDa and 100 kDa (Fig. 4.16). Unlike wheat and oat, no pea proteins are found in the basic region of the gel.

To test for the presence of glycoproteins within the wheat globulins, the total globulin fraction was subjected to affinity chromatography. Bound and non-bound fractions were analyzed by SDS PAGE and compared to the original extract and the purified wheat (3S + 7S) globulin fraction (Fig. 4.17). Many wheat globulins did not bind to the Con A Sepharose column (Fig. 4.17, lane d). However, a large proportion did bind (lane c), including the major polypeptides of 70 kDa, 55 kDa and 36-43 kDa found in the wheat (3S + 7S) fraction (lane b).

Antibodies raised against the purified oat (3S + 7S) globulins were labelled with ^{125}I and allowed to cross-react with the oat and wheat (3S + 7S) fractions and pea vicilins previously electrophoresed and transferred onto nitrocellulose (Fig. 4.18). The labelled oat (3S + 7S) antibody recognizes the members of the oat fraction (Fig. 4.18, lane a). It also binds to the 100, 70, 50, 40, 20, 18 and 15 kDa polypeptides of the pea vicilins. Common antigenic determinants are also evident within all the major polypeptide groups of the wheat (3S + 7S) fraction (lane c),

Figure 4.16. Two-dimensional analysis (section 2.7.3B) in the presence of 2-mercaptoethanol of pea vicilins. Vicilins were first separated by IEF (pH 3.5 - 10) followed by SDS PAGE. A standard SDS PAGE lane of pea vicilins is included for comparison.

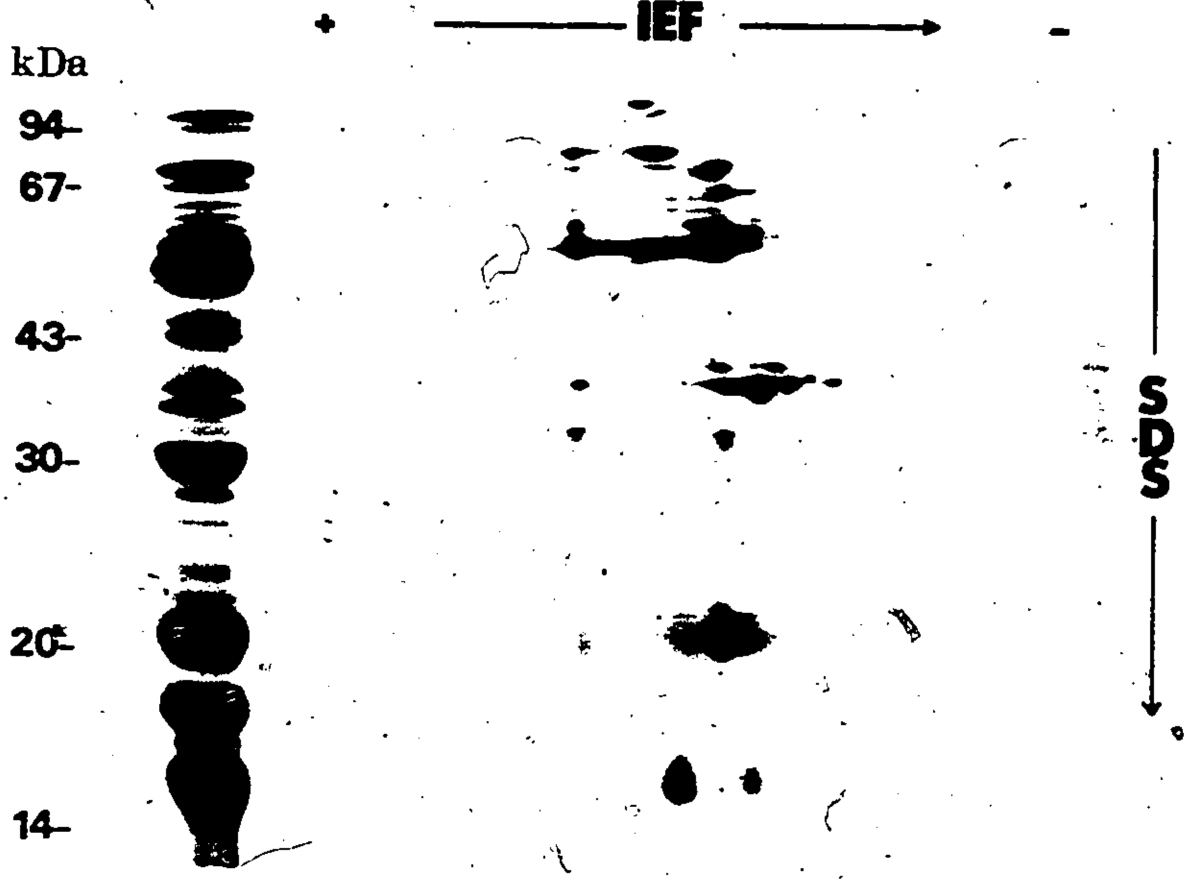
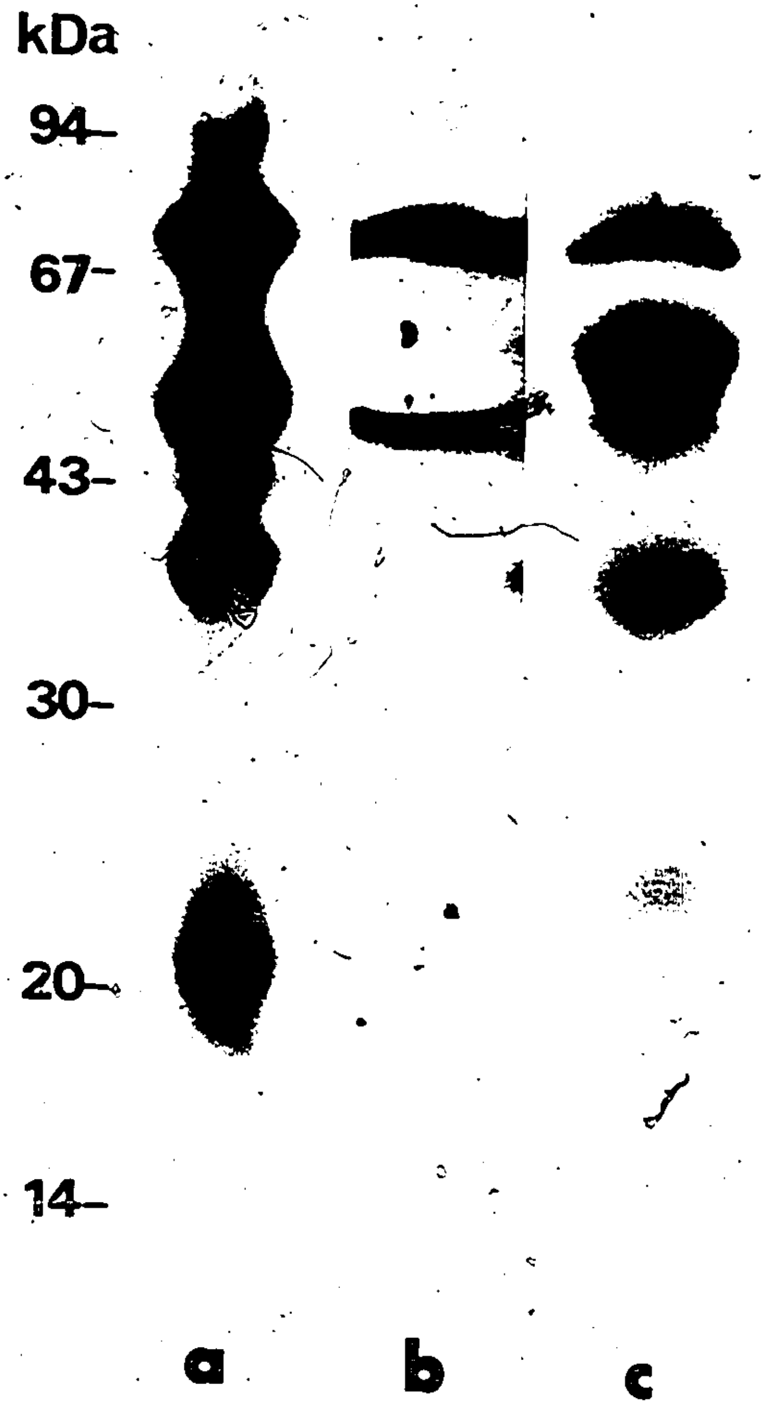

+
IEF
–
kDa
94-
67-
43-
30-
20-
14-
SDS

Figure 4.17. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) of (a) total wheat globulins, (b) wheat (3S + 7S) globulins and the Con A Sepharose chromatography fractions of (c) wheat globulins which bound to the column and (d) wheat globulins which did not bind to the column.

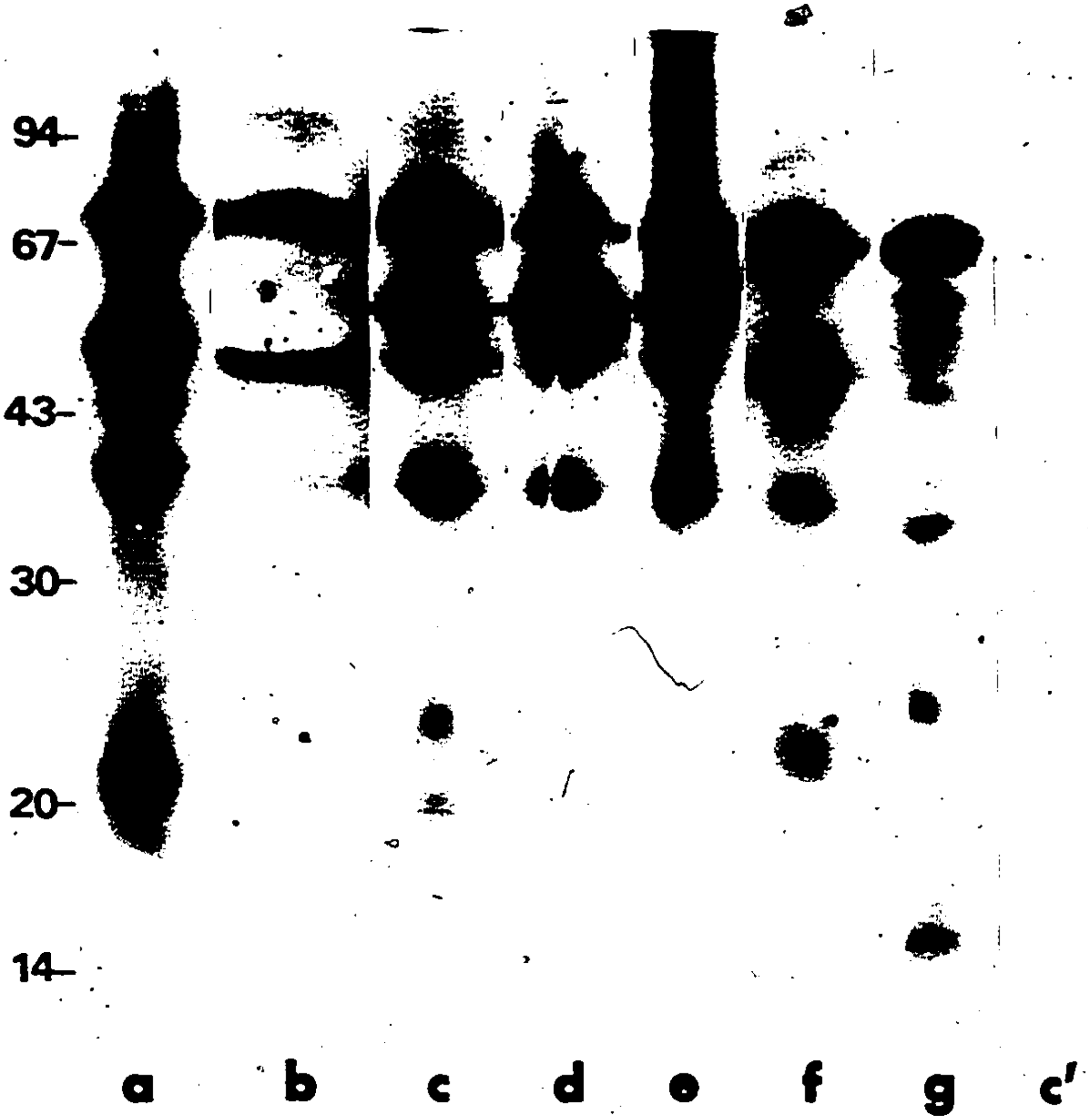

kDa
94-
67-
43-
30-
20-
14-
a
b
c
d

Figure 4.18. Western blot analysis (section 2.12) autoradiograph: ^{125}I- labelled antibodies raised against the purified (3S + 7S) oat globulins cross-reacted with the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of: (a) oat (3S + 7S) globulins, (b) pea vicilins and (c) wheat (3S + 7S) globulins transferred onto nitrocellulose. The quantities of protein applied to the gel were approximately: (a) 7.5 µg, (b), 350 µg, and (c) 200 µg.


kDa
94
67
43
30
20
14
a
b
c

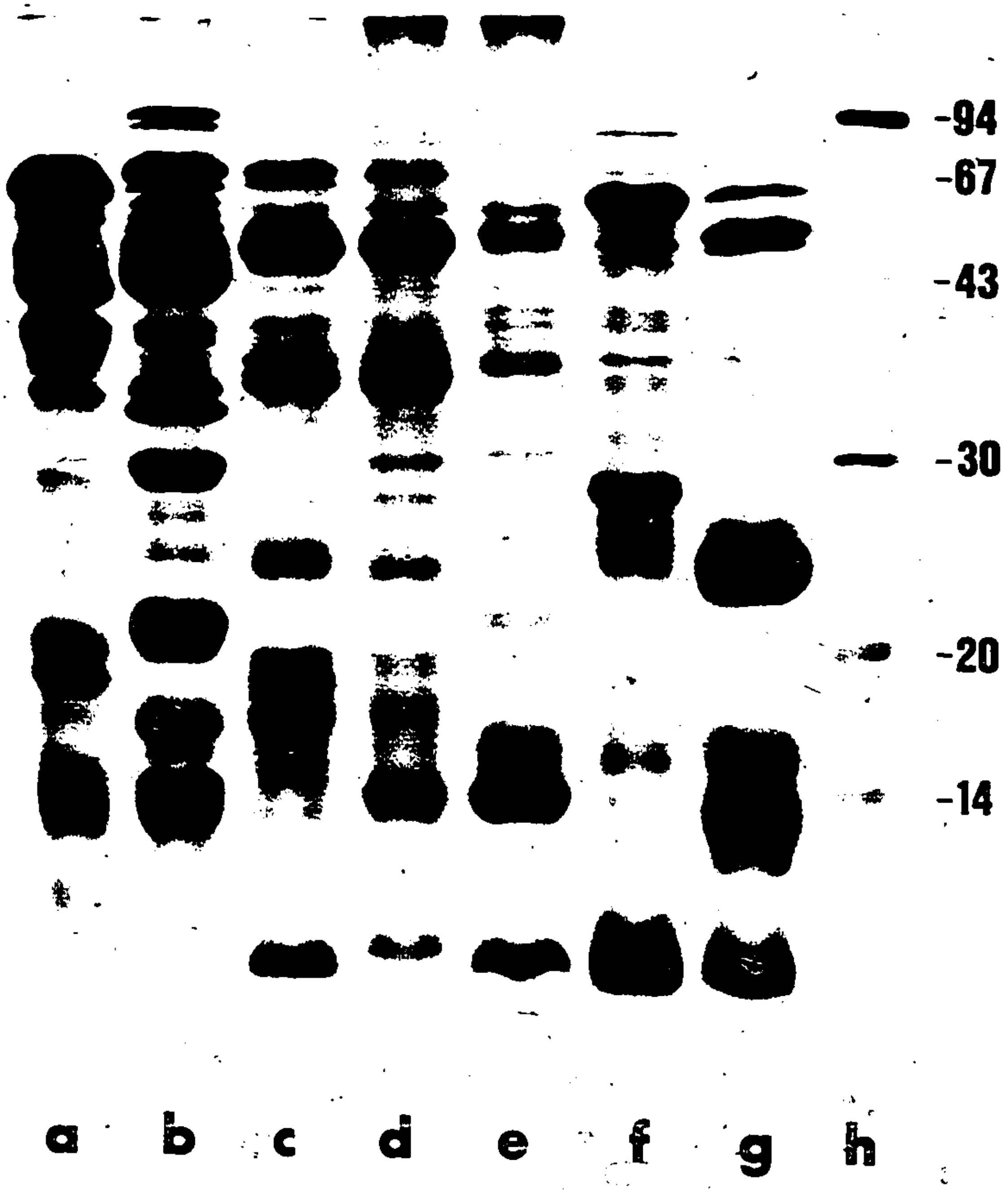
the affinity being stronger than that observed with pea. The occurrence of these antigenically similar polypeptides among oat, wheat and pea raised the possibility of this homology extending to other major cereals. When similarly challenged with the labelled anti-oat (3S + 7S) globulin IgG, the globulins obtained from rye, barley, corn and rice also exhibit cross-reactivity (Fig. 4.19). The cross-reactivity patterns can be compared to the equivalent Coomassie Blue-stained lanes (Fig. 4.20). The molecular weight patterns of the recognized polypeptides within the various cereal globulin fractions are very similar. Globulin bands co-migrating at approximately 40 kDa, 50 kDa and 70 kDa cross-react with the antibody in all the plants examined. Variations are observed primarily in the cross-reacting polypeptides found between 50 and 70 kDa and those of molecular weight less than 30 kDa. The degree of similarity is directly proportional to the taxonomic relatedness of the species. For example, the closely related members of the tribe Triticeae (wheat, rye and barley; lanes c, d and e respectively) show a typical pattern of cross-reactivity, with a major band of ∿55 kDa being recognized (see arrows, Fig. 4.19). The taxonomic relationship of the plants studied are shown in Appendix II.

Figure 4.19. Western blot analysis (section 2.12) autoradiograph: ^{125}I- labelled antibodies raised against the purified (3S + 7S) oat globulins cross-reacted with the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of: (a) oat (3S + 7S) globulins, (b) pea vicilins, (c) wheat globulins, (d) rye globulins, (e) barley globulins, (f) corn globulins and (g) rice globulins transferred onto nitrocellulose. Lane (c) contains no protein. Arrows point to a common 55 kDa band of the Triticeae members (wheat, rye and barley). Since differences occur in the proportions of the 3S and 7S globulins within the total globulin extract and also in species interrelationship, different quantities were applied to the gel for the various species: (a) 7.5 µg, (b) 350 µg, (c) 200 µg, (d) 250 µg, (e) (350 µg, (f) 350 µg, and (g) 200 µg. The cross-reactivity patterns obtained can be compared to the equivalent Coomassie Blue-stained lanes presented in Fig. 4.20.



7b

Figure 4.20. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (section 2.7.1) in the presence of 2-mercaptoethanol of: (a) oat (3S + 7S) globulins, (b) pea vicilins, (c) wheat globulins, (d) rye globulins, (e) barley globulins, (f) corn globulins, (g) rice globulins and (h) molecular weight markers. The quantity of protein applied to the gel was between 200 to 250 μg.


-94
-67
-43
-30
-20
-14
a
b
c
d
e
f
g
h

4.2 Discussion

4.2.1 Expression of legumin-like proteins in cereals and pea

The close similarity of oat 12S globulin to pea legumin points to either the conservation of legumin-like globulins in these plants from the time of divergence of monocotyledonous and dicotyledonous plants, millions of years ago, or to a convergent evolution of these proteins brought about by common structural requirements. It also raises the possibility of the occurrence of these proteins in other cereals. However, few data are available to indicate the presence of legumin-like globulins among cereals other than oat. Cereal globulins have generally been considered as cytoplasmic or metabolic proteins and their relatively low proportions and practical importance have diverted possible research efforts aimed at identifying legumin-like constituents. Nonetheless, a 11-12S or δ-globulin component has been identified in barley (Danielsson, 1949) and in wheat (Pence and Elder, 1953) which may involve proteins similar to legumin. Globulins resembling those of legumes have also been reported to occur in corn (Khavkin et al, 1977).

Pea (Pisum sativum L.) was chosen to represent the legumes in this comparative study because of the impressive amount of research which has been devoted to its globulin fractions (for review see Casey, 1982). Pea globulins have typically been separated into two major fractions, legumin and vicilin (Osborne, 1924). Pea legumin characteristically displays a sedimentation value of approximately 12S and is composed of subunit pairs (∿60 kDa molecules) of disulfide-bridged acidic (∿40 kDa) and basic (∿20 kDa) subunits (Croy et al, 1979; Krishna et al, 1979). The individual legumin subunits are believed to arise from the post-translational cleavage of precursor polypeptides of approximately 60 kDa (Croy et al, 1980a).

The globulin fractions of wheat, rye and corn all possess disulfide-linked polypeptides of similar molecular weight which are immunologically related to the subunits of oat and pea legumin-like proteins (see Figs. 4.1 to 4.4). Western blot analyses using the oat 12S globulin subunit or holoprotein antibody were also performed with barley globulins, but these repeatedly yielded poor results. This is believed to arise from the significant amount of phenolics which are co-extracted with barley globulins, although attempts to remove these phenolic compounds did not improve upon the Western blot results. However, barley globulins did cross-react (as well as wheat, rye and pea globulins) upon immunodiffusion analysis using antibodies raised against the total oat globulin fraction (Appendix III). This result suggests a likeness in the native oligomeric structure of these globulins to that of oat. In addition to the immunological homology, the amino acid composition of barley globulins resembles closely that of wheat and rye globulins (Appendix IV). This is to be expected in view of the close taxonomic relationship of these three cereals (Appendix II). Finally, the presence of a 12S globulin in barley (Danielsson, 1949) also supports the occurrence of legumin-like proteins in this cereal.

It is interesting to note that although oat, pea and wheat all possess legumin-like proteins, the relative proportions of these proteins within the respective total globulin fractions vary considerably. These variations point to important differences in the biosynthetic regulation of these proteins. In contrast to oat, wheat not only displays a low percentage of seed globulins (4% of total protein, Table 1.1), but it also synthesizes a relatively small amount of legumin-like polypeptides within this fraction. Hence, assuming the latter proteins are not being prematurely hydrolyzed in wheat, the legumin-like globulins in these two cereals are being expressed at extremely different levels. Further work is required to investigate the relative importance of gene copy number, transcriptional and translational controls in this differentiation.

At such a low level, the legumin-like proteins of wheat can hardly be expected to play a significant storage protein role. One might reasonably question their function in wheat altogether. A possible explanation for the occurrence of these proteins in wheat might involve its evolution from an ancestor in which legumin-like globulins were relatively more important. These proteins would have presumably become insignificant as a storage protein fraction, possibly being replaced by the gliadins. Cereal globulins are known to be metabolically more expensive to produce than prolamins or glutelins (Mitra et al, 1979). Hence, switching to these latter fractions might have conferred some survival value to the "low-globulin" cereals. On the other hand, wheat globulins may be performing a specialized role in the wheat seed, for example, as the immediate source of reserve protein utilized by the embryo upon germination. Further research is required in this area.

Globulin polypeptides not affected by the presence or absence of reducing conditions consistently cross-reacted with both the α-subunit and 12S globulin holoprotein antibodies in all the plants examined. This was particularly true of polypeptides found in the 50-70 kDa region. These polypeptides might represent a group of uncleaved globulin molecules. In oat and pea, precursors of the individual α- and β-subunits fall within this molecular weight range (Matlashewski et al, 1982; Brinegar and Peterson, 1982b; Walburg and Larkins, 1983; Adeli and Altosaar, 1983; Rossi and Luthe, 1983; Adeli et al, 1984; Croy et al, 1980a). The factors responsible for the presence of these uncleaved polypeptides might vary from mutations in the globulin gene regions coding for the cleavage sites, to the inability of the seed to process all the precursor molecules available. Whatever the reason, these would be recognized by both antibody preparations. Further study is required to establish the relationship existing between the typical legumin-like constituents in these plants and the other antigenically related polypeptides.

4.2.2 Expression of legumin-like proteins in rice

Like oat globulins, rice glutelins have been reported to consist of an heterogeneous collection of polypeptides with the major components being of approximately 22-23 kDa and 37-39 kDa. These major polypeptides are also believed to be subunits arising from the post-translational cleavage of a putative precursor of 57 kDa (Yamagata et al, 1982; Luthe, 1983). Hence, the possibility arises that rice glutelins are in fact globulins exhibiting an extreme version of the partial salt-solubility shown by the oat globulins (see section 3.2.1). In other words, instead of only a portion of the globulins ending up in the glutelin fraction as in oat, in rice most of the globulins, if not all, remain insoluble and are classified as glutelins.

The rice Osborne fractions each displayed a distinct electrophoretic pattern. Especially notable was the low molecular weight and the reduced level of heterogeneity of the rice prolamin fraction. This is atypical of prolamins from other cereals. The major rice glutelin subunits of 19-22 kDa and 30-36 kDa corresponded to the most abundant rice seed proteins. The molecular weight values observed for these subunits are somewhat lower than the molecular weight estimates previously reported for the rice cultivar Koshihika (Yamagata et al, 1982). This variation may be due to differences in the rice cultivars studied. The major rice glutelin subunits were shown to be associated by disulfide linkage to form reducible polypeptides of 50-62 kDa (see Figs. 4.7 and 4.8). The molecular weights of the reducible polypeptides are best accounted for by the association of one small to one larger rice glutelin subunit.

Similar to legumin-like proteins, the smaller rice glutelin subunit group of 19-22 kDa was shown to be basic, while the larger subunit group of 30-36 kDa displayed greater microheterogeneity and relatively more acidic pIs. A multigene family may be coding for the numerous rice glutelin components. In view of these obvious similarities to legumin-like proteins, the major rice glutelin subunits should be referred to as the α- and β-subunit groups respectively, as is done with legumin-like subunits in other plants.

The α- and β-subunits of rice glutelin possess antigenic sites recognized by the oat 12S globulin antibody. The rice globulin fraction displayed an essentially identical pattern of cross-reactivity to that observed with the glutelin fraction (see Figs. 4.11 and 4.12). This is considered a strong indication that rice glutelin is in fact a globulin-like protein which is only barely solubilized by a saline solution. Both globulins and glutelins are found in the same rice protein bodies (Tanaka et al, 1980). The factors affecting the solubility of a seed storage protein are numerous (section 3.2.1). Nonetheless, it is interesting to note that both rice glutelin and the residual oat 12S globulin exhibit a strong aggregation phenomenon in urea, which is probably the result of tight packaging within discrete organelles in the dehydrated seed environment and/or disulfide interchange. This may account for part of the salt-insolubility of these proteins. However, adding ME to the salt-containing extraction solution produces an increased, but still incomplete, solubilization of rice glutelin (results not shown). In spite of all their similarities, rice glutelin is much less salt-soluble than oat globulin and both are less salt-soluble than legume globulins. This difference may point to important variations in their primary sequences or to varying interactions with constituents of the different seeds (eg. membranes, phenolics, lipids...).

The oat 12S globulin antibody also cross-reacted with rice glutelin and globulin polypeptides of approximately 55 and 65 kDa which were not affected by the presence or absence of a reducing agent. As was suggested for pea and the other cereals examined, these polypeptides may represent uncleaved precursor molecules, although rice glutelin precursor molecules have been reported to be salt-soluble (Yamagata et al, 1982). However, the latter may be only partially salt-soluble.

Recently, Zhao et al, (1983) demonstrated that two major rice glutelin polypeptides of 22 kDa and 36 kDa were associated by disulfide linkage. They also reported significant homology between the partial N-terminal amino acid sequence of the 22 kDa glutelin subunit and that of the β-subunit of pea legumin. These results are in agreement with the premise that rice glutelin is in fact a legumin-like protein.

It is also possible to compare directly the N-terminal amino acid sequences of the rice glutelin β-subunit (Zhao et al, 1983) to that of the oat globulin β-subunit (Walburg and Larkins, 1983) and estimate the degree of evolutionary conservation found in these two proteins. Using the first 15 amino acids from the published sequences, the corresponding nucleic acids were derived, providing for maximum homology. When these two presumed sequences were compared, only 6 of the 45 bases differ between the two cereals. This represents 87% homology between the two hypothetical nucleic acid sequences. The properties common to rice glutelin and oat 12S globulin, including previously published findings, are summarized in Table 4.1.

TABLE 4.1

Properties Common to Rice Glutelin and Oat 12S Globulin

Property	Rice	Oat
constitute 70-80% of total seed protein	(Juliano, 1972)	(This thesis; Colyer and Luthe, 1984)
composed of ~20kDa and ~40kDa subunits	(This thesis; Yamagata et al, 1982; Luthe, 1983; Zhao et al, 1983)	(This thesis; Brinegar and Peterson, 1982a; Walburg and Larkins, 1983; Burgess et al, 1983)
disulfide-linked subunits (one large + one small)	(This thesis; Zhao et al, 1983)	
larger subunits are acidic and smaller subunits are basic	(This thesis)	
microheterogeneity, enhanced among the larger subunits	(This thesis)	
post-translationally cleaved precursors (~60 kDa)	(Yamagata et al, 1982; Luthe, 1983)	(Matlashewski et al, 1982; Brinegar and Peterson 1982a; Walburg and Larkins, 1983)
found in protein bodies	(Tanaka et al, 1980)	(Pernollet et al, 1982; Adeli et al, 1984)
partially salt-soluble	(This thesis)	(This thesis)
synthesized before prolamins during seed development	(Yamagata et al, 1982)	(This thesis)
differential biosynthesis of subunit members during development	(Yamagata et al, 1982)	(This thesis)

low carbohydrate content	(Juliano and Boulter, 1976)	(Peterson, 1978; Adeli and Altosaar, 1984)
less methionine in larger than smaller subunit	(Yamagata et al, 1982)	(Brinegar and Peterson, 1982a, Burgess et al, 1983)
need for ME to dissociate in urea extract	(This thesis)	(This thesis)
common antigenic determinants	(This thesis)	(This thesis)
homologous N-terminal amino acid sequences	(Zhao et al, 1983)	(Walburg and Larkins, 1983)

4.2.3 Expression of 3S and 7S globulins in cereals and pea

Legumin-like proteins have been shown to be expressed in cereals. It follows that, similarly to legumes, homologous 2-3S and 7-8S proteins may also be present. In fact, such proteins have been purified as a minor component of the oat globulin fraction and shown to possess sedimentation values of approximately 3S and 7S, subunits with molecular weights from 12-75 kDa and glycosylated components (Burgess et al, 1983; Adeli and Altosaar, 1984).

The ultracentrifugation results obtained for the wheat globulins are in agreement with the sedimentation constants previously reported for wheat α-(2.5S) and γ-(8.2S) globulins respectively (Danielsson, 1949). A minor shoulder was also evident at 10-11S which probably corresponds to the wheat δ-globulins observed by Pence and Elder (1953). The polypeptides present in this 10-11S shoulder may be related to the minor wheat globulins found to be antigenically homologous to 11-12S legumin-like globulins. The predominance of the 3S(α) and 7S(γ) globulin fractions relative to the 10-11S(δ) globulins in wheat is reversed in oat, where the 11S globulins largely predominate (Burgess et al, 1983; Adeli and Altosaar, 1984). Barley, rye, corn and rice, like wheat, all possess mainly α- and γ-globulins, the δ-globulins being absent or found as a minor component (Danielsson, 1949; Morita and Yoshida, 1968). The occurrence of these broad variations in the relative levels of the 3S, 7S and 10-12S cereal globulin fractions may implicate varying gene copy number or differential expression of the genes involved. Such variability in the content of legumin and vicilin has been observed among different genotypes of the Pisum sativum L. species alone (Thomson et al, 1979). There is a possibility that legumin-like proteins of wheat, rye and corn display the partial salt-solubility observed in rice and oat, thus explaining the low levels observed within the globulin fractions of the former cereals. However, there is no evidence available to support the presence of legumin-like proteins within the other seed protein fractions of wheat, rye or corn.

Like pea, both oat and wheat globulins displayed a considerable level of charge microheterogeneity. They contained, however, a larger number of relatively more basic polypeptides (Figs. 4.14, 4.15) than was observed within the pea vicilins (Fig 4.16). Gatehouse et al, (1981) also reported that pea vicilins were generally situated between a pI range of 4.5 to 7. Although the molecular weight distribution of the oat and wheat (3S + 7S) fractions and pea vicilins shared common features, variations in the relative quantities of the major subunits were evident. This variability among genera can be expected since different relative proportions of vicilin subunits have been observed within the single genus Phaseolus (Derbyshire et al, 1976) and the single species Pisum sativum L. (Thomson et al, 1980).

Vicilin preparations in legumes have often been shown to contain carbohydrates (Derbyshire et al, 1976). Glycoproteins have also been found in the oat (3S + 7S) protein fraction (Adeli and Altosaar, 1984). The molecular weight distribution of the wheat (3S + 7S) glycoproteins was very similar to that observed with oat and represented all the major subunit groups. These results also significantly resembled those obtained for pea vicilins (Badenoch-Jones et al, 1981), the differences arising from the presence of glycoproteins at 36-43 kDa and their absence at ~14 kDa in the cereals.

Polypeptides displaying antigenic determinants common to pea vicilins were detected within the oat and wheat (3S + 7S) fractions. In view of such strong conservatism among these taxonomically distant dicots and monocots, one might expect to find homologous proteins within other cereals. Indeed, this study showed that polypeptides antigenically related to the oat (3S + 7S) globulins do occur in the barley, rye, corn and rice globulin fractions (Fig. 4.19). This is in agreement with the presence of (2-3S) and (~8S) globulins in all these cereals (Danielsson, 1949; Morita and Yoshida, 1968).

The molecular weight variability observed in the cross-reacting polypeptides among the different genera can be expected in view of the variant subunit forms which have been observed intraspecifically, for example, with pea (Thomson et al, 1978; Matta and Gatehouse, 1982) and oat globulins (section 3.1.1). Such variability might also be expected to contribute to heterogeneity at the holoprotein level as shown to exist in pea (Thomson et al, 1980) and soybean (Hill and Breidenback, 1974; Thanh et al, 1975).

The 70 kDa and 50 kDa polypeptides in pea are known to cross-react immunologically (Croy et al, 1980b), but the exact interrelationship of these high molecular weight vicilins awaits sequencing for elucidation. However, pea vicilins with molecular weights less than 50 kDa are believed to arise from the post-translational proteolytic cleavage of precursor 50 kDa polypeptides (Gatehouse et al, 1982; Spencer et al, 1983). The possibility exists that cereal vicilin-like globulins of $<$ 50 kDa arise in a similar fashion. Small differences occurring among the genera in these low molecular weight subunits ($<$50 kDa) might be attributed to evolutionary divergence in the sites of proteolytic cleavage. If this is the case, the sequences of the subunits of $<$ 50 kDa and of the 50 kDa group should be related. Further work is required to test this possibility.

This preliminary study has revealed that the major protein fractions of wheat globulin share similar sedimentation values, molecular weights, glycosylation patterns and antigenic determinants with the equivalent fractions of oat and pea globulins. Other cereals including barley, rye, rice and corn were also found to contain globulins homologous with the (3S + 7S) fraction of oat. With the exception of oat, these proteins largely predominate within the globulin fractions of cereals. Further characterization of the individual components of the cereal 2-3S and $\sim$ 8S globulins is required to clearly identify the vicilin-like components and evaluate the degree of homology which exists between these proteins and the major reserve vicilins of legumes.

It would thus appear that the globulin fractions of cereals do not consist primarily of metabolic proteins as was generally believed. Accordingly, these proteins should occur within protein bodies rather than the cytoplasm, but this remains to be shown. Considering the low levels of globulins in wheat (4%), barley (18%), rye (15%), rice (8%) and corn (6%) (Table 1.1), their role as seed storage proteins in these cereals must be rather limited. They may perform a specialized function within the cereal seed which would require only small amounts of these proteins or they may simply be "vestigial" proteins which have lost their importance (or never gained any) throughout evolution.

CHAPTER V

CONCLUSION

The basis of an organized man-inspired control of seed protein quality and quantity involves firstly an integral comprehension of the biosynthetic processes implicated. The primary objective of this thesis was to provide a meaningful contribution to the vast amount of knowledge required to achieve such a goal. In view of the significant differences in seed protein composition which distinguish oat from the other cereals, oat was chosen as the "variant" whose study would allow a better insight into the differential genetic mechanisms responsible for the observed variations.

The detailed characterization and delineation of the oat seed proteins was deemed a necessary first step to this study. Solubility studies combined with electrophoretic and Western blot analyses established that, in comparison to other cereals, oat contains unusually high levels of seed globulins and much reduced amounts of prolamins and especially glutelins. These findings served firstly, in clarifying the controversies surrounding the composition of the major storage protein fraction in oat and secondly, in emphasizing the limitations associated with a classification system relying primarily on the solubility characteristics of seed proteins. There are, therefore, essentially two reserve protein fractions in oat, the globulins and the prolamins (avenins). The uncommon proportions of these proteins in oat account for the particular nutritional and functional properties of the oat proteins and flour.

The study of the polypeptide composition of globulins and avenins obtained from oat cultivars varying markedly in total seed protein content revealed a significant degree of intervarietal polymorphism. The observed heterogeneity is best accounted for by the existence of separate multigenic families coding for these two protein fractions. There was no evident correlation between the total protein content of a cultivar and the presence, absence or quantity of a particular polypeptide. Hence, differences in the seed protein content of oat cultivars do not result from the increased or decreased production of specific storage polypeptides already present or from the expression of additional genes. A developmental study revealed the sequential accumulation of globulin polypeptides in oat cutlivars differing in protein content, but differences in the onset of synthesis of the globulin fraction or individual polypeptides were not responsible for final variations in total protein content. Avenins are also accumulated sequentially during seed development, however their contribution to differences in total seed protein content is considered minimal. Therefore, intervarietal differences in oat seed protein content are likely the result of significant variations in the net rate of globulin synthesis. More research is necessary to uncover the genetic mechanisms responsible for these biosynthetic variations.

Detailed characterization of oat 12S globulins also demonstrated the close structural similarity of these proteins to legumin-like globulins of dicotyledonous plants. Such extensive homology between distantly related plants might have resulted from the conservation of legumin-like genes from the time of divergence of monocots and dicots or from the convergent evolution of these genes. Other cereals were investigated for the presence of legume-like globulins. The data presented indicate that, in addition to oat, wheat, rye, corn and rice (and probably barley also) all contain legumin-like proteins. Rice differs in that it possesses large amounts of these proteins in an essentially salt-insoluble form. In addition, the same cereals were all found to harbour proteins immunologically homologous with the (3S + 7S) globulins of oat. Many of these homologous cereal globulins also shared antigenic determinants with pea vicilin components of equivalent molecular weight. These findings support the presence of legume-like genes in cereals other than oat.

Therefore, as far as seed globulins are concerned, the different levels observed among the major cereals examined appear to result principally from the differential expression of homologous genes rather than the expression of unrelated genes. The similarities shared by the avenins and other cereal prolamins, as well as the extent of heterogeneity they display in spite of their low proportions in oat may also signify that interspecific differences in cereal prolamin content also result, at least in part, from a similar phenomenon. Whether glutelin genes homologous to those of other cereals also exist in oat remains to be shown.

APPENDIX I

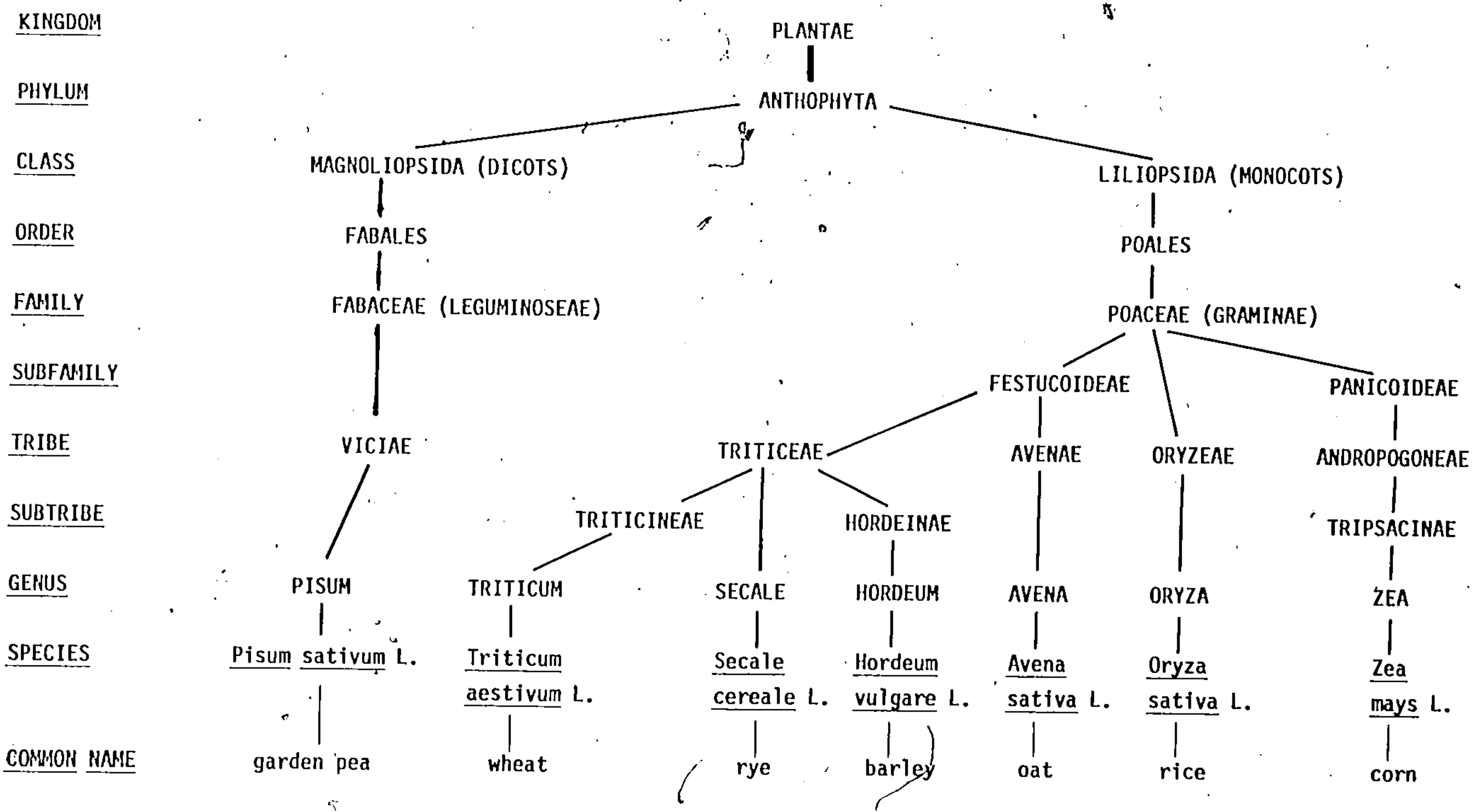
Quantities of albumins and globulins extracted by a saline solution from nine oat cultivars[1]

Cultivar	Total Protein (g/100g)	Globulins (% of total protein)	Albumins (% of total protein)
Hinoat	19.1 ± 0.3[2]	39	9
Dal	18.1 ± 0.7	36	11
Sentinel	17.9 ± 0.8	34	11
Elgin	17.0 ± 0.5	37	9
Harmon	15.6 ± 0.4	38	12
OA-269	15.6 ± 0.4	36	11
OA-424-1	14.8 ± 0.2	42	11
OT-213	14.5 ± 1.0	38	12
OA-366	11.9 ± 0.1	35	11

[1] Each nitrogen determination is a mean of at least six Kjeldahl values (N X 6.25).

[2] Sample standard deviation.

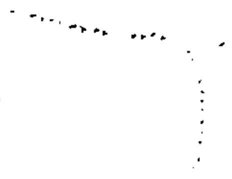
APPENDIX II

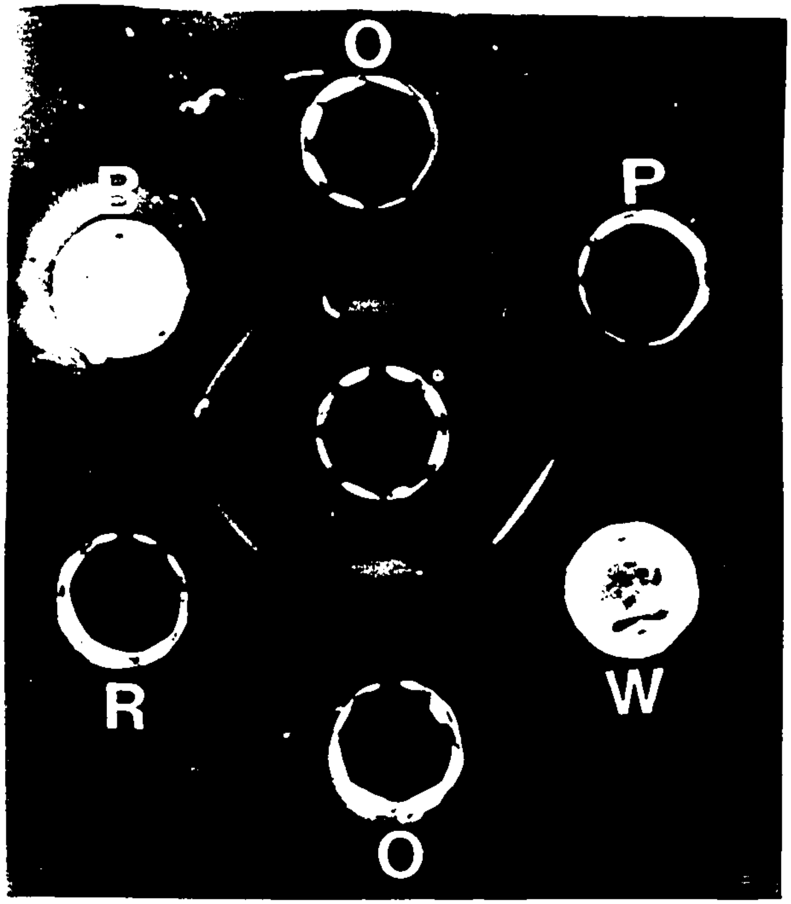


(Jones and Luchsinger, 1979)

APPENDIX III

Double immunodiffusion analysis (section 2.11) using antibodies raised against the total oat globulin fraction. The center well contained the antibody, whereas the surrounding antigen wells contained oat globulins (O), pea globulins (P), wheat globulins (W), rye globulins (R) and barley globulins (B). The continuity of the immunoprecipitin line indicates that the antibody cannot distinguish the different globulins.




O
B
P
R
W
O

APPENDIX IV

AMINO ACID COMPOSITION OF GLOBULINS [1]

AMINO ACID	OAT	BARLEY	RYE	WHEAT	PEA
Asx	10.2	8.7	8.4	8.4	12.4
[2]Thr	4.6	5.1	4.9	4.4	4.6
[2]Ser	7.6	7.5	7.6	7.6	8.0
Glx	21.6	12.7	15.3	15.2	16.4
Pro	5.6	5.9	5.7	5.8	5.2
Gly	8.5	11.7	10.7	11.0	7.4
Ala	6.8	9.3	9.1	8.3	7.0
Val	6.6	7.5	7.2	7.0	6.5
Met	0.9	1.0	1.2	0.9	0.7
Ile	5.1	3.8	4.3	3.8	4.9
Leu	8.4	8.2	8.2	7.6	9.5
Tyr	3.7	3.0	2.8	2.8	3.4
Phe	5.0	4.1	4.0	3.8	4.8
His	2.3	2.7	3.0	3.2	2.3
Lys	3.5	5.6	5.8	5.6	7.5
Arg	6.9	9.3	8.8	10.5	6.7

[1] Reported as mole percent.

[2] Extrapolated from duplicate 12, 36 and 72 hr hydrolysis values.

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