STUDIES ON SEED STORAGE PROTEIN METABOLISM IN COMMON BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH)

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ABSTRACT
OF THE THESIS
SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENT OF THE DEGREE OF
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IN
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NORTH EASTERN HILL UNIVERSITY
SHILLONG - 793022
INDIA
1996

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Seed storage proteins, intended as a source of nitrogen for germinating seedlings, constitute an important source of dietary proteins for human consumption. Although cereal grains and legume seeds are a major source of dietary proteins , the storage proteins in both are generally deficient in essential amino acids such as lysine , tryptophan and methionine . Over the years many attempts have been made to improve the amino acid composition in important crops through conventional breeding programmes. Molecular approaches towards improving the nutritional quality of seed proteins, however, provide alternative strategies to conventional breeding programmes . An important approach towards improving the amino acid composition of seed storage proteins could be to express the gene for a heterologous protein with a balanced amino acid composition . The generality of the approach by which foreign proteins rich in desired amino acids may be introduced is however constrained by scanty information in respect of suitable heterologous proteins . While a number of seed proteins rich in sulphur containing amino acids are available not many lysine rich seed storage proteins have been identified so far .

The North Eastern region of India is extremely rich in floristic wealth and is home to a large variety of traditional crops that could form an essential component of human diet in times to come. Common buckwheat (*Fagopyrum esculentum* Moench.) is one such plant that has been classified by IBPGR as an important but underutilized crop. The plant is a dicot psedocereal with a high protein content (18%) of its grains. Further unlike cereals, where the main storage proteins are the prolamins, the main storage protein in buckwheat seeds belongs to the globulin family . In order to use the available germpalsm for improvement programs , the characterization of seed storage proteins from the plant would be a prerequisite. The present investigation was undertaken to

1. determine the content and quality of storage protein in buckwheat seeds.

2. determine the extent of relationship between germination and mobilization of storage proteins in seeds of common buckwheat during early stages of germination.

3. Isolate and characterize the main seed storage protein of common buckwheat and to determine its homology with seed storage proteins of legumes and cereals.

In order to assess the protein quality and quantity of buckwheat seeds, the seeds of common buckwheat (*Fagopyrum esculentum* Moench), obtained from the Western Himalayas and the North Eastern Regional station of NBPGR, were analyzed for the content of total proteins. On dry weight basis, the seeds had a protein content of about 16 percent out of which more than 50 percent was present in the globulin fraction. Albumins, prolamins, glutelins and the residual proteins constituted 15.8, 7.83, 9.27 and 14.43 percent of the total protein content of the grains respectively.

The main storage protein in seeds of common buckwheat is localized within protein bodies present in the endosperm tissues of the seed. The protein bodies of common buckwheat are spherical in shape and range in diameter from 5-8 μm. The protein bodies are composed of an amorphous protein matrix with a single globoid inclusion. The protein matrix is enclosed by a single membrane. Electron microscopic examination of the isolated protein bodies of buckwheat seeds also revealed the presence of a membrane like boundary layer surrounding the globoid inclusion. The protein body of buckwheat seeds has been thus classified as of "complex" type.The protein bodies of buckwheat seeds had about 70 percent protein content; phytin and carbohydrates respectively constituted 25 and 2.7 percent of the protein body dry weight. An analysis of the pattern of accumulation of the main storage protein in buckwheat seeds revealed that the protein started to accumulate only after early mature stage of seed development.

When extracts from seeds at various stages of development were analyzed by double diffusion test with antibodies raised against the 280 kD protein from mature buckwheat seeds, no cross reactivity was observed with extracts from seeds harvested upto early mature stage of development. A precipitin line with extracts from seeds harvested at mid mature stage of development clearly indicated the presence of the protein at this stage.

Studies were carried out to determine the relationship between the levels of proteolytic activity and the mobilization of reserve proteins during early stages of buckwheat seed germination. Results of our investigations revealed a rapid uptake of water and mobilization of storage proteins in buckwheat seeds during early stages of germination. There was no marked change in the electrophoretic mobility of the main storage protein during the initial 24 hours of germination. Beyond 24 hours, the electrophoretic mobility of the protein increased with progressing seed germination. correspondingly there was no marked difference in the SDS-PAGE profile of the main storage protein extracted from ungerminated seeds and those allowed to imbibe water for 24 hours. After 24 hours a gradual decrease in the ammount of some high molecular weight subunits of the protein and increase in the levels of some low molecular weight proteins was observed with progressing germination. When tested by the Western blot assay, strong anti 280 kD activity could be detected in proteins extracted from seeds harvested at various stages of germination upto 96 hours. The immunoblot also confirmed the changes taking place in the electrophoretic mobility of the protein with progressing germination. These results indicate that during early stages of seed germination, the main seed storage protein in common buckwheat undergoes a partial cleavage leading to the release of some low molecular weight subunits. Results of the western blot assay obtained in the present investigation clearly establish that the 280 kD globulin of buckwheat seeds retained its antigenic identity even upto 96 hours of imbibition. Till this time the process of radical and plumule emergence is nearly complete.

A low level of proteolytic activity could be observed in the endosperm of buckwheat seeds upto 18 hours of germination. Increases in the activity of the enzyme occurred only after 18 hours of imbibition. In the embryo, however, the activity of the enzyme started to increase after after 6 hours of imbibition only. Corresponding with changes in the activity of proteases a marked increase in the tissue level of free amino acids was observed in the endosperm and embryo tissues with progressing germination after 14 hours. However, the content of soluble protein in these tissues showed a consistent increase throughout. Considered together with the changes taking place in the main storage protein and the levels of proteolytic activity in the seeds during germination, these results indicate that the 280 kD globulin may not be the source of amino acids that are utilized by the growing embryo during the initial stages of germination. Contributions towards this pool as a result of proteolytic cleavage of the main storage protein into small peptides and amino acids presumably start much later. The significant decrease in the level of free amino acids in the endosperm during the initial 2 hours of imbibition and the increase in the level of soluble protein in the embryo during the same period indicates that a pre-existing pool of free amino acids in the endosperm tissues could be the source of amino acids to be utilized for the synthesis of soluble proteins during the initial stages of germination.

The main seed storage protein of buckwheat was purified by $(NH_4)_2SO_4$ fractionation, dialysis and chromatography on Sepharose 6B column. The protein showed a molecular mass of 280 kD. Electrophoresis on a 7.5% polyacrylamide gel resolved the protein into a single band. Under denaturing conditions the protein resolved into three groups of subunits designated as α, ß and Γ with molecular weight ranging between (55-60 kD, α); (32- 44 kD, ß) and (16-29 kD , Γ). The 280 kD protein revealed an oligomeric structure typical to the 11-12S groups of legumin type globulins . When tested against antibodies raised against the 280 kD globulin from mature seeds of common buckwheat, the protein extract from ungerminated buckwheat seeds as well as the 280 kD globulin showed single precipetin lines indicating the antigenic homogeneity of the protein. The protein showed antigenic homology with 11-12S seed storage proteins from soya bean , mung bean and pea; the protein showed strongest homology with glycinin from soya bean seeds. No cross reactivity was observed for storage proteins extracted from broad bean , wheat , rice and grain amaranth. Based on the gross subunit composition and its antigenicity with seed storage proteins of other crops, as observed in the present investigation, it can be assumed that the 280 kD globulin of buckwheat seeds belongs to the legumin subfamily of proteins.

In order to determine which of the subunits of the 280 kD globulin had the highest lysine level, the protein was subjected to gel filtration on Sephadex G-120 and Sephadex G-50 after reduction and alkalization. Fractions of each peak were pooled together and lypholysed. Analysis of the partial amino acid composition of the separated proteins revealed that a protein which had separated under fractions 42-48 on Sephadex G-50 column had the highest (6.93 %) lysine content. Besides the protein was also rich in some sulphur amino acids.

On SDS-PAGE the purified subunit resolved into a single band corresponding to 26 kD molecular mass. The amino acid composition of the subunit revealed high levels of essential amino acids such as lysine, threonine, leucine and some sulphur containing amino acids. Compared to other seed storage proteins, the 26 kD subunit also showed a high content of glutamine and arginine . The role of high level of arginine is consistent with the role of crystalloid seed storage proteins as a nitrogen source during seed germination . The amino acid composition of the protein also closely matches the values of essential amino acids recommended by the World Health Organization for a nutritionally balanced protein.

The sequence of 17 N-terminal amino acid residues of the protein was established by automated sequencing. The sequence reads as:

GLY-ILE-ASP-GLU-ASN-VAL-CYS-THR-MET-LYS-LEU-ARG-GLU-ASN-ILE-LYS-SER

Alignment of the sequence with the corresponding regions of proteins from seeds of some economically important crops revealed considerable similarity in the sequences. The protein showed 73.3% sequence homology with the G_1, G_2, G_3 and G_4 precursors of soya bean glycinin. The second highest homology (66.7%) was shown with pea legumin. The least homology of 46.7% was observed with the ß subunit of 11S globulin from cucumber seeds. Out of the 17 residues compared, 6 were highly conserved and 5 residues matched closely in that they were amino acids with similar functional groups, thereby representing conservative replacements. Amongst the conserved bonds was the cysteine-threonine linkage at residue numbers 7, 8 and the asparagine-isoleucine linkage between residue numbers 14, 15. One of the significant aspects revealed by the alignment was the presence of lysine and serine at position numbers 16 and 17 respectively in the 26 kD globulin subunit of buckwheat seeds. None of the other sequence matched had these residues at position numbers 16 and 17.

The physical characterization of the 26 kD globulin subunit of buckwheat by circular dichrosim and fluorescence spectroscopy revealed that the protein had a predominantly α-helical structure. The protein had a 33% α-helix which under-phobicity .

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I **Manoj Kumar Rout**, hereby declare that the subject matter of thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anbody else, and that the thesis has not been submitted by me for any research degree in any other University/Institutes.

This is being submitted to the North-Eastern Hill University for the award of the degree of **Doctor of Philosophy in Botany**.

Forwarded

R.R. Mishra
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(M.K.ROUT)

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(Manoj Kumar Rout)

DEDICATION

Dedicated to my parents

ABBREVIATIONS

A	Absorbance
ATZ	Anitinotnoiozolinone
CFA	Complete Frerund's Adjuvant
CD	Circular dichroism
DAF	Days after flowering
DNP	Dinitropyrridine
DAB	Diamino benzoic acid
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
KD	Kilo dalton
2-ME	2 - Mercaptaethanol
OPD	Orthophenyl diamine
PAGE	Polyacrylamide gel electrophoresis
PMSE	Phenyl methyl suphonyl fluoride
PTH	Phynyl thio hydantoin
PVP	Polyvinyl pyrvolidone
PVDF	Poly vinylidene difluoride membrane
PR-HPLC	Reverse phase High Pressure Liquid Chromatography
SDS	Sodium dodecyl sulphate
TCA	Tri chloroacetic acid
TEMED	Tetramethyleithylenediamine
TFA	Trifluoro acetic acid

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CHAPTER I

INTRODUCTION:

Plant genetic resources are one of the important gifts of nature to mankind. They represent the sum total of diversity accumulated through years of cultivation under domestication and natural selection. Many of these genetic resources are presently, the important source of high nutritive value foods for human nutrition. Man has utilized only 20 crop plants as a major food source since the agricultural practices came into existence about 10000 years ago. Considering the ever increasing needs of food materials, it is not only necessary to use the available rich diversity and wide genetic base to improve the existing conventional cultivars but also to look for unconventional, lesser known food crops common buckwheat, chenopodium and grain amaranth. In this context common buckwheat (*Fagopyrum esculentum* Moench) assumes tremendous importance because of the high protein content of its seeds.

Seed proteins have attracted the attention of biochemists and other research workers mainly on account of their importance in human nutrition. The storage proteins of angiosperm seeds have been classified according to their solubilities into albumins, globulins, prolamins and glutelins. While the albumins are soluble in water and dilute aqueous buffers, the globulins are soluble in salt solutions but are insoluble in water. Glutelins are soluble in dilute acids or alkali solution and the prolamins are soluble in aqueous alcohol (Osborne, 1924). In legumes, the major storage proteins in seed are of the globulin type while in cereals the prolamin type of proteins predominate (Higgins, 1984). The storage proteins in both are, however, generally deficient in essential amino acids such as lysine, tryptophan and methionine (Hoffman, *et al.*, 1988).

While many an attempts have been made, over the years, to improve the amino acid composition in important crops through conventional breeding programmes (Coulter & Bewley, 1990); molecular approaches towards improving the nutritional quality of seed proteins provide alternative strategies to conventional breeding programmes. An important approach towards improving the amino acid composition of seed storage proteins could be to express the gene for a heterologous protein rich in the content of essential amino acids. The generality of the approach is, however, constrained by the small number of suitable heterologous proteins. While a number of seed proteins rich in sulphur amino acids are known, not many lysine rich seed storage proteins have been identified so far.

For improvement of seed protein quality, information about the type of storage proteins present in the seeds, their rate and pattern of synthesis as well as factors that regulate their synthesis is a prerequisite. The major seed storage proteins of most legumes belong to the globulin class. Proteins of this class consist of legumin and vicilin type proteins and have sedimentation values of 11S and 7S respectively (Derbyshire *et al.*, 1976). The legumin group has molecular weight in the range of 300-400 kD and contains about 30% amide amino acids and low levels of methionine and cysteine. On the other hand vicilin group of proteins is composed of many classes of subunits ranging in molecular weight from 140 to 200 kD. Since the biological role of seeds is much the same throughout the plants kingdom, it would appear likely that equivalent homologously related proteins could exist in a wide range of plants. These proteins can be identified on the basis of their size, subunit composition, serological cross reactivity and sequence homologies.

Even though seed storage proteins constitute an important component of dietary proteins for human nutrition, their primary role in seeds would be to provide free amino acids to the growing axis during early stages of seed germination. The physiological and biochemical processes underlying seed germination and early seedling growth are, thus, important to the establishment of a plant in its environment and ultimately determine its yield potential. One crucially important aspect is the mobilization of storage materials during the early stages of germination in plants. The degradation of storage proteins during seed germination has also been studied for a long time, the starting points being the establishments of protein degradation and the detection of proteolytic activity in seeds. Studies on the degradation of seed storage proteins during seed germination have provided information about histochemical and molecular characteristics of the process. Until recently, enzymatic aspects of storage protein degradation remained unclear since almost all studies dealt with proteolytic enzymes in seeds irrespective of function (Shutov and Vaintraub, 1987).

Even though much work has been done on soya bean (Hill and Briendenback, 1974), pea (Millard *et al.*, 1978; Higgins *et al.*, 1986; Hoffman *et al.*, 1988), *Lupinus albus* (Melo *et al.*, 1994), *Oryza sativa* (Takaiwa *et al.*, 1987; Krishnan and Pueppke 1993), Oat (Shotwell *et al.*, 1990), *Cumcumis sativus* (Kim and Smith, 1994) and *Phaseolus vulgaris* (Goossens *et al.*, 1994), not much information is available on seed storage proteins of common buckwheat (*Fagopyrum esculentum* Moench). An understanding of the individual protein fractions and the proteases involved in the degradation of these fractions is essential for a better utilization of the seed proteins as food for human nutrition.

Common buckwheat (*Fagopyrum esculentum* Moench) a pseudo cereal of extreme economic importance because of its short growth span, high nutritive value of its grains and capacity to grow on poor soils. A survey of the literature reveals that certain characteristics possessed by this crop give it an advantage over the non conventional crops like grain amaranth and chenopodium. The importance of the plant lies in the high protein content of its grains, short growth span and hardiness of the plants; besides the foliage is used as a green vegetable and is an important commercial source of the glucoside Rutin which is used as a medicine. However, because of its indeterminate growth habit, the crop has not been cultivated extensively and comes under the category of underutilized crops as classified by National Bureau of Plant Genetic Resource (NBPGR). Although some studies have been made on protease activity not many attempts has been made on characterization of the seed storage proteins in this crop.

The present investigation was therefore undertaken to

a) determine the extent of relationship between seed germination and the mobilization of storage proteins in common buckwheat.

b) isolate and characterize the main seed storage protein in common buckwheat and to determine its relationship with seed storage proteins of other crop plants.

CHAPTER II

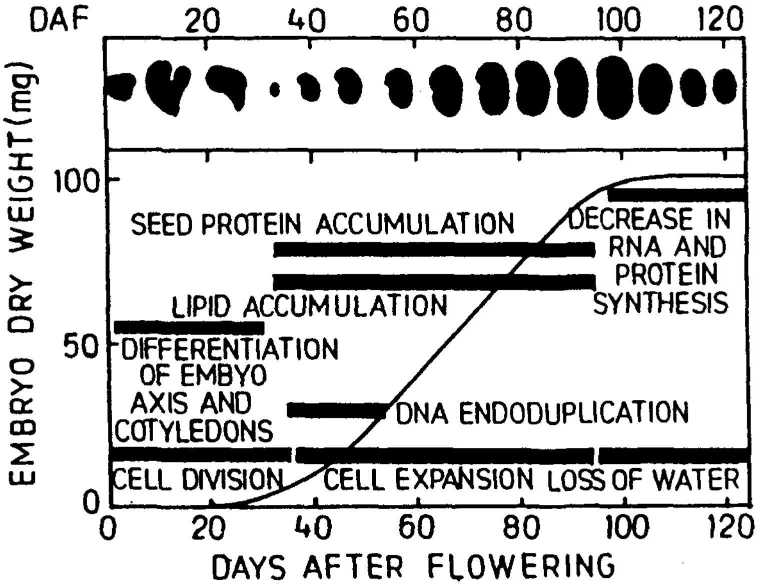
REVIEW OF LITERATURE

Seed storage proteins, intended as a source of nitrogen for germinating seedlings, constitute an important source of dietary proteins for human consumption. Seed storage proteins have attracted the attention of biochemists, nutritionists and other research workers mainly on account of their importance in human nutrition. The proteins of seeds can be subdivided into two categories: 1. the storage proteins, which account for the major part of the protein and 2. the house keeping proteins which are essential for the maintenance of normal cell metabolism. Seed storage proteins constitute an important source of dietary proteins for human consumption. Although cereal grains and legume seeds are a major source of dietary proteins, the storage proteins in both are generally deficient in essential amino acids. Over the years many an attempts have been made to improve the balance of essential amino acids in important crop plants through conventional breeding programme. Molecular approaches towards these goals, however, provide alternative to conventional breeding programmes.

The storage proteins of angiosperm seeds have been classified according to their solubilities as albumins, globulins, prolamins, and glutelins. The albumins are soluble in water and dilute aqueous buffer at neutral pH, globulins are soluble in salt solutions, prolamins in aqueous alcohol and glutelins in dilute acid or alkaline solution (Osborne, 1924). The most prevalent seed storage proteins of the dicotyledonous plants (legumes) are the globulins. In cereals, however, the principal storage proteins are the quite distinct prolamins (Higgins, 1984).

The storage proteins accumulate within the endosperm of seeds in spherical vesicles called protein bodies. Protein bodies were first demonstrated by Hartrig (1856) who named these organelles as `aleurone grains'. The protein bodies are small, more or less spherical organells varying in size from one plant to another and from one tissue to another. Depending on the plant species, the size of protein bodies ranges from 1-10 μm in diameter. While the protein bodies are bound by a membrane which has been reported to be single in most of the cases, some authors have described a double membrane sorrounding the protein body. The presence of a double membrane has been interpretted as inindicating plastid origin for protein bodies (Pernollet, 1978).

fig.2.1: Major physiological events during soyabean embryogenesis (Source:Goldberg *et al.*) 1989).


DAF
20
40
60
80
100
120
EMBRYO DRY WEIGHT (mg)
100
50
0
SEED PROTEIN ACCUMULATION
LIPID ACCUMULATION
DECREASE IN RNA AND PROTEIN SYNTHESIS
DIFFERENTIATION OF EMBYO AXIS AND COTYLEDONS
DNA ENDODUPLICATION
CELL DIVISION
CELL EXPANSION
LOSS OF WATER
0
20
40
60
80
100
120
DAYS AFTER FLOWERING

On the basis of their morphology, Tully and Beevers (1976) and Pernollet (1978), have described two types of protein bodies: 1.those consisting of an amorphous protein without any inclusions (peanut, soya bean, and corn), and 2. those consisting of the amorphous protein matrix with either globoid or a crystalloid inclusions (Cannabis, Cucurbit, Yucca, Cotton). Protein bodies without any inclusion have been described as "simple" while those with inclusion are described as "complex". While globoid is a phytic acid storage inclusion, the crystalloid is proteinaceous in nature. The crystalloids are composed of storage protein of the globulin type, where as the matrix contains non storage albumin proteins (Tully and Beevers, 1975).

Even though the biochemical composition of protein bodies varies greatly, the protein bodies are mainly composed of storage proteins (70-80%), salts of phytic acid (10%) carbohydrate (5-10%), hydrolytic enzymes, cations and ribonucleic acids (Pernollet, 1978).

Mills and Wettstein (1988) have shown the presence of three different components *viz.* a homogeneous component, a fibrillar or granular component and an electron- dense sphere in the protein bodies of the barley seeds. They have suggested that the fibrillar component could be characteristic of developing protein bodies and may reflect a progressive dehydration and condensation of storage proteins to yield the predominantly homogeneous structure. The structure of the barley protein bodies has been reported to resemble that of the protein bodies found in maize (Khoo and Wolf 1970; Larkins and Hurkman 1978) and wheat (Kim *et al.*, 1988).

The ontogeny of protein bodies, is still controversial. Protein bodies are assumed to develop in the lumen of endoplasmic reticulum with golgi apparatus playing an important role in their ontogeny (Larkins and Hurkman 1978, Tayor *et al.*, 1985, Gayler *et al.*, 1989; Torret *et al.*,1990, Rasmussen *et al.* ,1990, Hay *et al.*, 1991 and Robinson *et al.*, 1995). Two distinct path ways have been described for packing of the synthesized proteins into protein bodies. The prolamins type of proteins are deposited within the lumen of the ER; the ER-derived vesicles subsequently form the protein bodies in the cytoplasm (Khoo and Wolf, 1970; Larkins and Hurkman, 1978; Taylor *et al.*, 1985; Krishnan *et al.*, 1986; Lending *et al.*, 1988). On the other hand the glutelin type of proteins are suggested to be packaged into the vacuole as protein bodies via the Golgi complex (Krishnan *et al.*, 1988 and Kim *et al.*, 1988). The role of the Golgi apparatus in protein transport is well documented in developing seeds of dicotyledenous plants (Chrispeels, 1985). The general features of the pathway traversed as the legume globulins move to storage vacuoles has been described by Chrispeels *et al.* (1982, 1984, 1985). They have suggested that the proglobulin trimers move through Golgi complex prior to their arrival at protein storage vacuoles. Upon entry into the vacuole the proglobulins are cleaved to yield the acidic and basic polypeptides typical of a mature 11S globulin; the ASN-GLY bond being the site of cleavage for the formation of acidic and basic subunits of 11S globulin.

Following their appearance in vacuoles, the nature 11S subunits are isolated from the seed as hexamers. Thus, the transition of 11S globulins from trimers to hexamers is associated with cleavage of each subunit in the trimers into acidic and basic polypeptides (Barton *et al.*, 1982).

The strict conservation of the ASN-GLY bond between the acidic and basic polypeptide of globulins in a wide variety of legumes and non legumes indicates the functional significance of this bond. The first indication in this regard was demonstration by Dickinson *et al.* (1989). They showed that proglycinin trimers were incapable of assimilating into hexamers during reassembly of monomers and trimers that contained mature subunits. These authors were also demonstrated that limited digestion of proglycinin trimers by papain could restore this blockade.

To identify factors required for proglobulin folding and assembly, Gatenby (1992) added various purified components to a wheat germ assembly assay system. Addition of chaperonin 60 (cpn60) from *E. Coli*, and congnate Hsp 70 from pea, failed to stimulate oligomer formation. Supplementation of the wheat germ system with massive amount of BiP from pea seed ER also failed to stimulate trimer formation effectively. The observation lead Gatenby (1992) to suggest that if cpn60, BiP and Hsp70, were involved in post translational processing of the proglobulins, they probably exert their effect in combination with other factors. The author has pointed out about the purification of a TCP1-like chaperone which was similar to that purified from rabbit reticulyte and was analogous to cpn60 found in bacteria (Yaffe *et al.*,1992). Yaffe *et al.* (1992) have also isolated the TCP1-like chaperone from higher plants and implicated it in the folding of biologically active phytochrome. Thus, while there is no direct evidence that could establish that chaperones are involved in the assembly of 11S proglycinin trimers observations of Gatenby (1992) and Yaffe *et al.* (1992) could provide a starting point for additional investigations.

Historically the seed storage proteins of agriculturally important leguminous crop plants have been the most extensively studied. The most abundant seed proteins have been sub-divided into two groups based on their sedimentation coefficients; the 11S globulins (legumin-type) and the 7S globulins (Vicilin-type) (Danielsson, 1949). However, in Soya bean , seed storage proteins belonging to three sedimenting classes *viz.* 2.2S, 7.5S and 11.8S have been reported (Hill and Breidenbach, 1974). The subunit composition of 11-15S globulin seed storage proteins has been determined by combinations of SDS gel-electrophoresis, multidimensional gel electrophoresis and peptide mapping procedures. It has been shown that the 11-12S globulins existed as oligomeric molecules composed of six subunits. Each subunit consisted of a heavy α chain and a light β chain; both the chains were processed from a common precursor and remained bound to each other by disulphide bridges (Derbyshire *et al.*, 1976; Dalgalarrondo *et al.*, 1986; Melo *et al.*,1994).

Rodin and Rask (1990) have described the subunit composition of the 12S globulin (cruciferin) of *Brassica napus*. The protein has been shown to consist of six subunit pairs. Each of the six pairs is synthesized as a precursor containing one α and one ß chain. However, the relationship between mature chains and their precursors of cruciferin synthesized *in vitro* from rape seed mRNA has showed the existence of at least three major precursor forms (Rodin and Rask, 1990). On the other hand Sjodahl *et al*., (1991) have shown the existence of at least three different precursors (named P_1, P_2 and, P_3) giving rise to four different mature subunits. The relationship between mature chains and their precursors established by N-terminal sequence analysis of free as well as disulphide -linked ß chains has revealed that the cruciferin precursors are cleaved at a site homologous to the ones of 11-12S storage proteins. Using 2D electrophoresis they observed that the free cruciferin chains arose from disulphide interchange reactions during intracellular transport of the proteins. On the basis of their observations on amino acid sequence homologies of cruciferin with seed storage proteins from other plants, Sjodahl *et al*., (1991) have suggested that all Brassicaceae species might have three different cruciferin genes or gene subfamilies. This is unlike *Vicia faba* or *Glycine max* where only two types of globulin genes have been reported.

However, based on a comparison of the nucleotide and/or amino acid sequences of 13S globulin storage proteins from flowering plants (angiosperms) by dot matrix analysis and visualization of gross protein domains by hydropathy analyses, Borroto and Dure (1987), Hager *et al.* (1992)and Leal and Mishra (1993) have suggested that all the globulin storage proteins of flowering plants have emanated from genes that existed at the beginning of angiosperm evolution.

The degradation of storage proteins and mobilization of the released amino acids from storage tissues to the growing axis is an integral part of the process of seed germination. During grain development the basic storage reserves (carbohydrates, lipids and proteins) are laid down in all parts' of the grain or seed. After germination, hydrolysis of these reserves is seen first in the cotyledon and then in the endosperms (Jacobsen *et al.*, 1979). Investigation of the mechanism of storage protein hydrolysis has revealed that it proceeds in stages (Hara *et al.*, 1976; Pernollet, 1989; Shutov and Vaintraub, 1987; Dunaevsky and Belozersky, 1989). In legume seeds, limited proteolysis of the storage protein starts after 48-72 hours of imbibition (Bewely and Black, 1985; Shutov and Vaintraub, 1987). Hydrolysis of the storage protein in mung bean cotyledons has been shown to be catalyzed by a protease that is synthesized *de novo* between 3-6 days of germination (Baumgartner and Chrispeels, 1977). In pumpkin seeds, however, Hara *et al.*, (1976) and Hara and Matsubara (1980) have shown that limited proteolysis of t main storage proteins in pumpkin cotyledons starts almost simultaneously with the onset of water imbibition . The hydrolysis has been reported to be carried out by a metalloproteinase which is present in the dry seeds. The second stage of hydrolysis is performed by enzymes appearing in the growing seedlings. Specific mechanisms for the protection of the storage proteins from premature hydrolysis by the metalloproteinase present in dry seeds have also been reported to exists in plants. Such a mechanism has been reported to involve the formation of an enzyme-inhibitor complex. Belozersky *et al.*,(1982) have observed that the proteinase inhibitor found in dry buckwheat seeds could serve as such an inhibitor since it was capable of preventing proteolysis of the main storage protein *in vitro*.

The mobilization of storage proteins during seed germination is dependent on the action of a series of endo and exopeptidases working in concert within the protein bodies and cytosol of the storage parenchyma cells (Bewley and Black, 1985; Bewley and Greenwood, 1991; Shutov and Vaintraub, 1987). Baumgartner and Chrispeels (1977) and Shutov and Vaintraub (1987) have demonstrated that the initial cleavage of the major storage proteins of legume seeds is catalyzed by the endopetidic action of a specific cysteine proteinase(s) resident in the protein bodies . The initial cleavage has been observed to be essential for the subseque hydrolysis of the storage protein and peptide fragments by less specific endo - and exopeptidases resident in the protein bodies and cytoplasm (Wilson 1986).

An important set of questions is how proteolytic enzymes carry out the proteolysis? What is the sequence of their action, and how is this proteolysis regulated?

Two distinct classes of endopeptidases, one with an acidic pH optimum (characteristics of carboxylpeptidase

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activity) the other with a neutral/alkaline pH (typical of metallo endopeptidase activity) have been reported (Bond and Bowles, 1983).

Chrispeels and Baumgartner (1976) have described the results of studies on *in vitro* as well as *in vivo* digestion of seed globulins during seed germination. They have reported that a 50 kDa vicilin was degraded to 20 - 30 kDa peptides by a sulfhydryl- endopeptidase during the early stages of germination in *V. radiata*. An acid sulfhydryl protease capable of hydrolyzing pea proteins was also detected in cotyledons of pea (Basha and Beevers, 1975) and corn endosperm (Harvey and Oaks, 1974) 5 days after germination. In the well characterized system of castor bean, the proteinases responsible for mobilization of storage reserves have been shown to be synthesized 2 to 3 days after seed germination (Alpi and Beevers 1981; Chrispeels *et al.*, 1976; Nishimura and Beevers, 1979). Baumgartaner and Chrispeels (1977) and Shutov and Vaintraub (1987) have described a cooperative involvement of cysteine proteinases and carboxypeptidase in the digestion of storage proteins in germinating seeds of mung bean and Vetch respectively. For mung bean seedlings, such a function has been confirmed by a study of the intracellular localization of the enzymes which showed that the storage protein as well as the cystein proteinase and carboxypeptidase were located within the protein bodies. Shutov and Vaintraub (1987) have furhter suggested that such a cooperativity of cysteine proteinases and carboxypeptidases could indicate the presence of some common mechanism with respect to hydrolysis of storage proteins in the seeds of higher plants. The proteases that make up the enzymatic machinery of storage protei degradation must meet the following criteria.

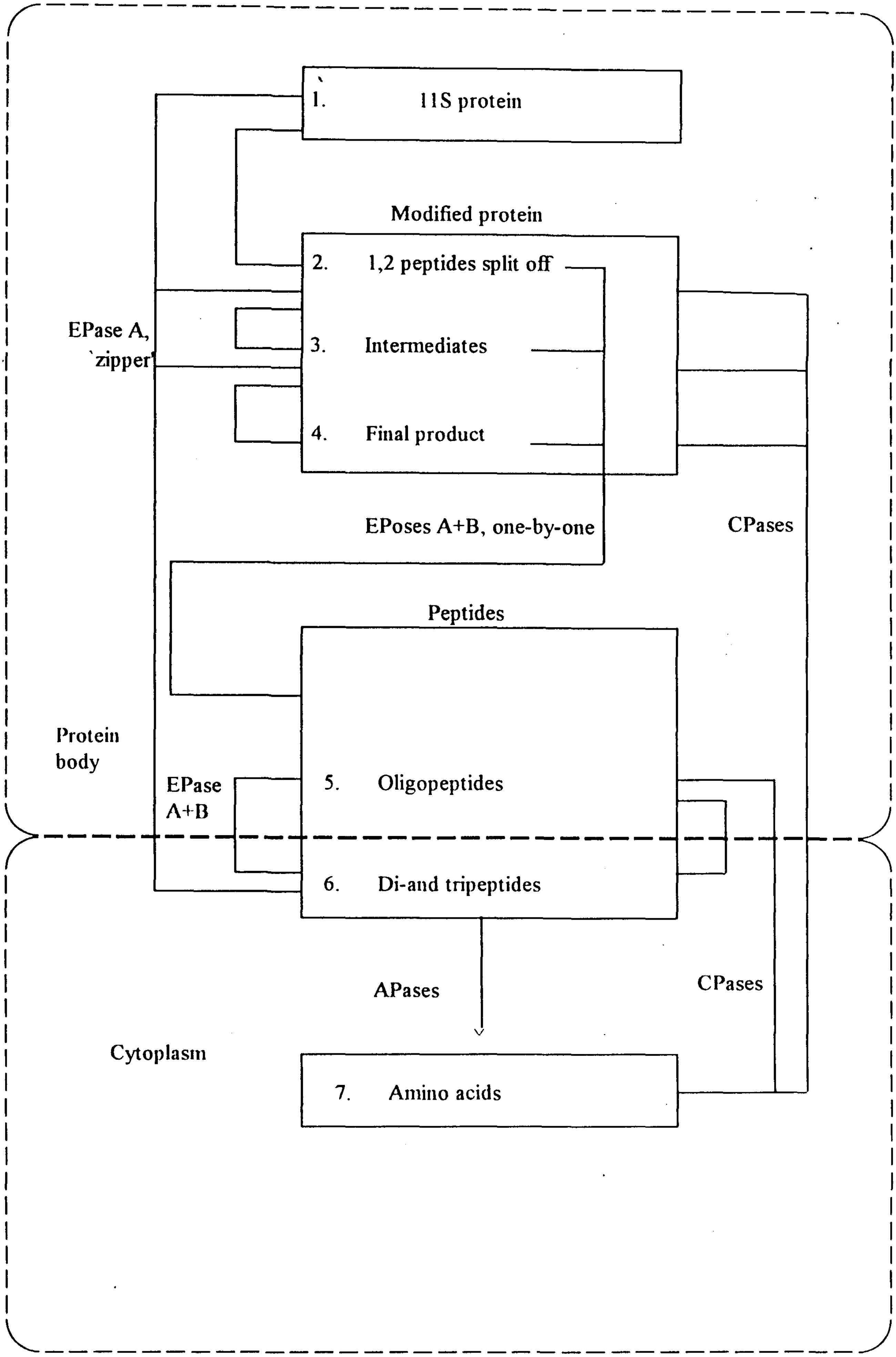
1. Ability to hydrolyze the storage protein of resting seeds or any of the products of their degradation during germination.

2. Lack of temporal or spatial hindrance to the action of protease on its expected natural substrate.

3. Ability to be active in the milieu of the corresponding cell compartment (pH, redox potential, absence or low level of inhibitory activity).

Vaintraub (1975) has described the role of the legumin like 11S proteins in regulating protein mobilization through the `zipper ' process during seed germination. The degradation of 11S protein from ungerminated seeds has been suggested to be initiated by proteinase` A ', the enzyme splitting off one or two short peptides from the storage protein. Further changes of the 11S protein are brought about by the continued `zipper' proteolysis *viz.* the formation of high molecular weight intermediates and subsequently that of a residue with a molecular mass which would be 10-15% lower than the molecular mass of the initial 11S protein. The peptide products of degradation are mainly the result of the one- by - one proteolysis. Both Zipper and one - by - one proteolysis may lead to the formation of di-and tri- peptides but the major products of proteolysis evidently comprise a wide range of intermediate oligopeptides. The hydrolysis of oligopeptides to free amino acids may follow two extreme

Fig. 22 : The pathways of 11S protein degradation in germinating seeds. EPases = endopeptidases (proteinases); CPases = carboxypeptidases; Apases = neutral aminopeptidases. After Shutov and Vaintraub 1986.


1. 11S protein
Modified protein
2. 1,2 peptides split off
3. Intermediates
4. Final product
EPase A, `zipper'
EPoses A+B, one-by-one
CPases
Peptides
Protein body
EPase A+B
5. Oligopeptides
6. Di-and tripeptides
APases
CPases
Cytoplasm
7. Amino acids

pathways: (a) the splitting off of amino acids from large peptides by carboxypeptidases until tri- and possibly di-peptides remain, (b) the break down of large peptides by proteinases` A' and` B' mainly to di- and tri- peptides which are transported from the protein bodies to the cytosol and further hydrolyzed by aminopeptideses. The release of free amino acids is largely due to the action of carboxypeptidases already present in ungerminated seeds.

The following factors regulate 11S protein mobilization in germinating seeds.

1. The resistance of the 11S protein to the action of proteases located in the protein bodies of resting seeds which prevents premature hydrolysis of the storage proteins.
2. Rate of *de novo* synthesis of proteinase` A 'and proteinase` B ' activity as well synthesis of some carboxypeptidases.
3. The rate of hydrolysis of 11S protein by proteinase `A'. leading to the susceptibility of the protein to the action of proteinase` B' and carboxypeptidases.
4. Changes in the ratio of the activity of proteinases and carboxypeptidases which controls the relative contribution of carboxypeptidases and aminopeptidases (dipeptidases) to the final stages of protein degradation.

Although cereal grains and legume seeds are a major source of dietary proteins, the storage proteins in both are generally deficient in essential amino acids. Over the years, many attempts have been made to improve the balance of essential amino acids in important crop plants through conventional breeding programmes. Molecular approaches towards these goals, however, provide alternatives to conventional breeding programmes.

As such first step towards production of transgenic plants with improved and balanced amino acid composition especially with respect to essential amino acids and those which can not be synthesized by human tissue, *viz.* lysine, the purification and characterization of the specific seed storage protein rich in essential amino acids followed by cloning a full length cDNA for the specific storage protein are pre-requisite. Besides other crops *viz.* rape seed (Coupe *et al.*, 1993); field bean (Heim and Baumlein, 1994); wheat (Gantier *et al.*, 1994); rice (Krishnan and Pueppke, 1993), such proteins have also been isolated and characterized in some lesser known crops *viz.* grain amaranth (Raina and Datta, 1992) and chenopodium (Dey and Mandal, 1993). (Utsumi *et al.*, 1993) have demonstrated the accumulation of both normal and modified glycinins accumulated in the leaves and stems of soya bean plants. There were no marked differences in the level of accumulation of the expressed proteins between leaves and seeds. The proteins expressed in each tissue were processed into the mature form and those expressed in the seeds assembled into hexamers. These results have supported the possibility that novel soya bean plants can produce modified glycinins having better nutritional and functional properties. In 1987, Susan Altenbach and her colleagues proposed that one way to increase the methionine content of legume seed proteins and hence their nutritional quality was to introduce the sulphur rich 2S albumen gene from Brazil nuts for which they had isolated a cDNA clone. Saalbach *et al.* (1995) placed the Brazil nut gene under the control of the CMV 35S promoter as well as the seed specific legumin B4 promotor. After inserting the constructs into binary vectors, they transfered the gene into *V. narbonensis* using *Agrobacterium* mediated gene transfer. Their results indicated that under the control of legumin LeB4 promoter sufficient 2S albumin was produced to significantly increas the nutritional quality of legume seeds.

Rao *et al.*, (1994) have approached a rate- limiting strategy to increase the lysine content of L - hardothionin, the barley seed storage protein, by mutating appropriate residues to lysine. The 3D structure of the protein has been determined by homology modeling with Crambin. Based on this model they have identified surface residues amenable to substitution with lysine. Their approach has permitted the creation of a modified L - hordothionin protein that has a lysine content of 27 percent.

Attempts have also been made to use *in vitro* mutagenesis to increase the number of methionine codons in genes that encode subunits of legume globulins. The first such report was by Hoffman *et al.* (1988), who inserted a high methionine containing DNA fragment from zein into the 7S phaseolin gene from *phaseolus vulgaris*. These workers demonstrated the production of translatable mRNA, but there was no accumulation of the mutant protein in storage vacuoles. They concluded that translation products from the transgene were rapidly degraded following synthesis. Saalbach *et al.* (1988) carried out similar experiments with a legB gene from *Vicia faba*. Four methionine codons were inserted towards the 3' -end of the legumin coding region. In this case also, there was no accumulation of the mutant protein in the seed tissues.

Considering the ever increasing needs of food materials, it is not only necessary to use the available rich diversity and wide genetic base to improve the existing conventional cultivars but also to look for unconventional, lesser known food crops common buckwheat, chenopodium and grain amaranth. In this context common buckwheat (*Fagopyrum esculentum* Moench) assumes tremendous importance because o the high protein content of its seeds.

Even though much work has been done on soya bean (Hill and Briendenback, 1974), pea (Millard *et al.*, 1978; Higgins *et al.*, 1986; Hoffman *et al.*, 1988), *Lupinus albus* (Melo *et al.*, 1994), *Oryza sativa* (Takaiwa *et al.*, 1987; Krishnan and Pueppke 1993), Oat (Shotwell *et al.*, 1990), *Cumcumis sativus* (Kim and Smith, 1994) and *Phaseolus vulgaris* (Goossens *et al.*, 1994), not much information is available on seed storage proteins of common buckwheat (*Fagopyrum esculentum* Moench). An understanding of the individual protein fractions and the proteases involved in the degradation o these fractions is essential for a better utilization of the seed proteins as food for human nutrition.

Common buckwheat (*Fagopyrum esculentum* Moench) a pseudo cereal of extreme economic importance because of its short fig.2.3: The geographicl distribution of buckwheat (<u>Fagopurum esculentam</u> Monech.)

Regions of Buckwheat Cultivation in India.

1. Leh
2. Pahal gaon
3. Srinagar
4. Udhampur
5. Chamba
6. Kangra
7. Lahaul & Spiti
8. Kinnaur
9. Mandi
10. Kulu
11. Shima
12. Uttarkashi
13. Chamoli
14. Pauri
15. Almora
16. Pithoragarh
17. Darjeeling
18. Siliguri
19. Assam
20. Meghalaya
21. Arunachal Pradesh
22. Nagaland
23. Manipur

growth span, high nutritive value of its grains and capacity to grow on poor soils. A survey of the literature reveals that certain characteristics possessed by this crop give it an advantage over the non conventional crops like grain amaranth and chenopodium. The importance of the plant lies in the high protein content of its grains, short growth span and hardiness of the plants; besides the foliage is used as a green vegetable and is an important commercial source of the glucoside Rutin which is used as a medicine. However, because of its indeterminate growth habit, the crop has not been cultivated extensively and comes under the category of underutilized crops as classified by National Bureau of Plant Genetic Resource (NBPGR). Although some studies have been made on protease activity not many attempts has been made on characterization of the seed storage proteins in this crop.

The present investigation was therefore undertaken to

a) determine the extent of relationship between seed germination and the mobilization of storage proteins in common buckwheat.

b) isolate and characterize the main seed storage protein in common buckwheat and to determine its relationship with seed storage proteins of other crop plants.

fig.2.4: Qualities and uses of common buckwheat (*Fagopyrum esculentam* Moench).

QUALITIES AND USES OF COMMON BUCKWHEAT

1. Buckwheat proteins have better amino acid composition with high level of lysine which is generally deficient in proteins in most of cereals and millets. The nutritive value of proteins is comparable to that of Casein. Therefore the grains, unlike other cereals, can serve as a source of good quality protein even without supplementing with pulses or lysine.

2. Common buckwheat is a rich source of honey. One acre of land of common buckwheat can produce about 60 kg of honey

3. Buckwheat is a rich source of rutin, a glucoside. The leaves and flowers are richer in rutin which varies from 1.0 to 6.0 percent. The stems have little while seeds have no rutin.

4. The grain is used as an article of food in different countries. In the hills of India, Bhutan and Nepal, the flour is the staple food.

5. The plant is also used as a food for livestock

ESSENTIAL AMINO ACIDS OF BUCKWHEAT GRAINS AS COMPARED TO OTHER CEREALS

Food Grain	Lysine	Methionine	Tryptophan	Leucine
Buckwheat	5.9	3.7	1.4	5.8
Amaranth	5.0	4.4	1.4	4.7
Wheat	2.6	3.5	1.2	6.3
Rice	3.8	3.0	1.0	8.2
Maize	1.9	3.2	0.6	13.0
FAO/WHO Recomendation	5.5	3.5	1.0	7.0

*

Source: Proceedings of 2nd Amarant conference, 1979 (USA)

(As percentage of protein)

CHAPTER III

MATERIALS:

Plant material: Buckwheat (*Fagopyrum esculentum* Moench) seeds were collected from western Himalayas (Almora, Nainital, Kosi) and National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Shillong. The grains were multiplied in experimental field of the University.

CHEMICALS:

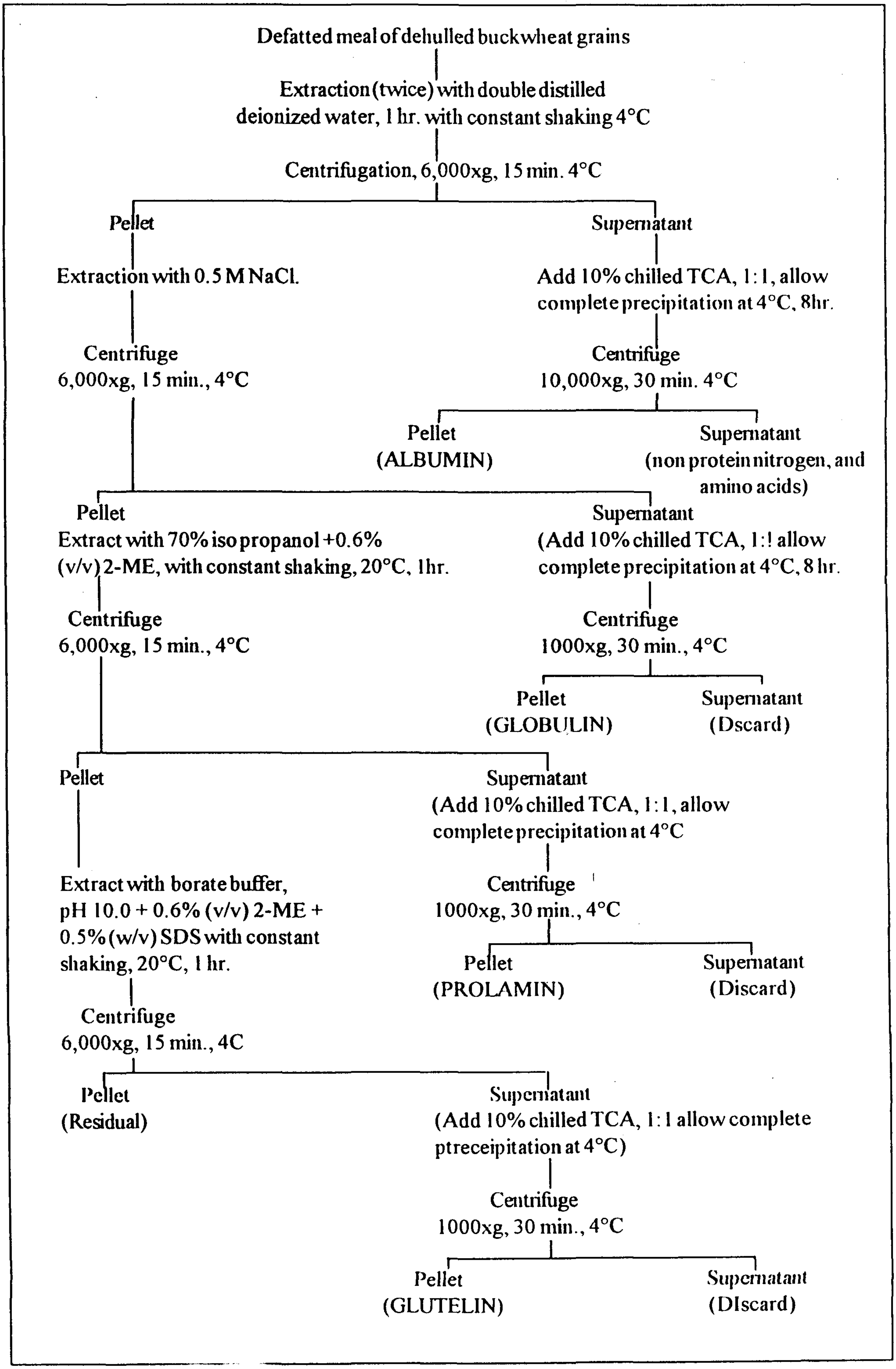
Sepharose 6B, Sephadex G.100-120, Sephadex G- 50, Blue dextran, Sodium azide, EDTA, PVP, Acrylamide, N-N methyl bisacrylamide, Ammonium persulphate, Commassie brilliant blue R-250, Commassie brilliant blue G-250, Ponceau S, Thymol, SDS, 2- ME, DDT, TEMED, Agarose, Protein molecular weight markers: bovine serum albumin (67 kD), egg albumin (45 kD), glyceraldehyde 3P dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), soya bean trypsin inhibitor (20 kD), lactalbumin (14 kD), ß-amylase (200 kD), papain (21kD), myoglobin (17.5 kD), gelonin (30 kD), catalase (240 kD), invertase (270 kD), Urea, Nitrocellulose (Sigma), PVDF membrane (millipore), Freunds Complete adjuvant, CFA (Sigma), ELISA Plate, O- phenyldiamine, Secondary antibody: goat anti -rabbit-HRP conjugate, Diamino benzoic acid (DAB), Casein, Ninhydrin, TCA. Amino acid Kit. Osmium tetraoxide, Gluterldehyte, Copper stubbs, Formavar coated copper mesh, Uranyl acetate, Lead Citrate, Phosphotungstic acid; H_2O_2, ANSA, D-Glucose, Methyl celloslove, Phosphoric acid, Whatman No. 3 filter paper, Dialysis tube (Spectrum), Trypsin, Dinitropyrridine (Fluka), Pepstatin (Fluka), PMSF. Glycine, Tris-base (molecular biology reagent). Ethyl acetate, Folin Cicalteau, Acetic acid (HPLC grade). Methanol (HPLC grade), All sequencing chemicals from Millipore.

PROTOCOLS:

Healthy seeds from the collection were washed with water and rinsed with distilled water. The hull portion of each of the seeds was removed and the groat used for the estimation of protein fractions and amino acid content. The groat was powdered in a ball mill and defatted with petrolem ether extraction. The defatted meal was used for protein fractionation and amino acid estimation.

Fractionation of total proteins:

The proteins were fractionated into albumins, globulins, prolamins and glutelins according to the protocol fig.3.1: Flow sheet for the fractionation of total storage proteins from seeds of common buckwheat (*fagpyrm esculentam* Moench) into albumins,globulins, prolamins and glutelins.


Defatted meal of dehulled buckwheat grains
Extraction (twice) with double distilled deionized water, 1 hr. with constant shaking 4°C
Centrifugation, 6,000xg, 15 min. 4°C
Pellet
Supernatant
Extraction with 0.5 M NaCl.
Add 10% chilled TCA, 1:1, allow complete precipitation at 4°C, 8hr.
Centrifuge 6,000xg, 15 min., 4°C
Centrifuge 10,000xg, 30 min. 4°C
Pellet (ALBUMIN)
Supernatant (non protein nitrogen, and amino acids)
Pellet
Extract with 70% iso propanol +0.6% (v/v) 2-ME, with constant shaking, 20°C, 1hr.
Supernatant (Add 10% chilled TCA, 1:! allow complete precipitation at 4°C, 8 hr.
Centrifuge 6,000xg, 15 min., 4°C
Centrifuge 1000xg, 30 min., 4°C
Pellet (GLOBULIN)
Supernatant (Dscard)
Pellet
Supernatant (Add 10% chilled TCA, 1:1, allow complete precipitation at 4°C
Extract with borate buffer, pH 10.0 + 0.6% (v/v) 2-ME + 0.5% (w/v) SDS with constant shaking, 20°C, 1 hr.
Centrifuge 1000xg, 30 min., 4°C
Pellet (PROLAMIN)
Supernatant (Discard)
Centrifuge 6,000xg, 15 min., 4C
Pellet (Residual)
Supernatant (Add 10% chilled TCA, 1:1 allow complete ptreceipitation at 4°C)
Centrifuge 1000xg, 30 min., 4°C
Pellet (GLUTELIN)
Supernatant (DIscard)

described by Landry- Moureaux (1970). The flow sheet for the fractionation is given in Fig. 3.1

Amino acid estimation:

Estimation of lysine: The lysine content of buckwheat grains was determined spectrophotometrically following the method of Tsai *et al.* (1972).

A known amount of the seed material was defatted in petroleum ether and air dried. 100 mg of sample was taken in a glass vial and 5 ml of 0.4% papain solution in 0.0[illegible] phosphate buffer (pH 7.4) was added to it. The vial was capped and incubated overnight at 65°C. A blank sample containing papain only was also processed similarly. The solution was vigorously shaken for one hour before and after taking out from the incubator. The hydrolyzed samples were allowed to adjust to room temperature and centrifuged at 3000xg for 5 minutes. To 1 ml of the supernatant, 0.5ml of 0.6M Carbonate buffer (pH 9.0) and 0.5 ml of copper phosphate suspension* was added. The solution was mixed thoroughly and centrifuged to precipitate excess copper phosphate. To 1 ml of aliquot from the supernatant 0.1 ml of 3% 2-chloro-3,5- dinitropyridine (DNP) prepared in methanol was added in a stoppered glass vial. The mixture was mixed by inversion and allowed to stand for 2 hours at room temperature with shaking at every 30 minutes. The reaction mixture was acidified by adding 5 ml of 1.2N HCl and vortexed. 5 ml of ethyl acetate was added to the solution and mixed thoroughly by inverting the tubes at least 10 times. The upper phase was extracted using a syringe adapted with a polyethylene tube. Absorbance of the aqueous phase containing E-DN-Pyr lysine was read at 390 nm against a sample free blank. The lysine content of the samples were calculated by using a standard curve prepared with (L- lysine in carbonate buffer).

*The copper phosphate solution was prepared separately as solution A and B as described below:

Solution `A' :2.8% $CaCl_2$

Solution `B':6.8% $Na_3PO_4.12H_2O$

Solution `A' was poured into`B' with swirling and centrifuged for 5 minutes at 3000xg. The precipitate was washed four times with 0.05M borate buffer (pH 9.0) followed by centrifugation. After the last washing the pellet was suspended in 8 ml of borate buffer.

Estimation of tryptophan: The content of lysine in the grains was estimated according to Spies and Chambers (1949).

The defatted protein extracted from the grains was hydrolyzed as described in the previous section. To 1 ml of the hydrolysate 4 ml of ferric chloride-sulphuric acid reagent (prepared as 1:1 ratio of 0.027% ferric chloride in glacial acetic acid with 30N sulphuric acid) was added. The sample was incubated at 65^{oC} for 15 minutes. Absorbance of the hydrolysate was determined at 545nm in a Hitachi UV-220 spectrophotometer against a reagent blank. The concentration of tryptophan in the sample was calculated from a standard curve prepared with DL-tryptophan.

Estimation of Methionine: The methionine content of the defatted grains was estimated following the method of Mc-Carthy and Paille (1959).

A known quantity of defatted buckwheat sample was hydrolyzed with 25 ml of 2N HCl and autoclaved at 15 lbs for 1hour. A pinch of activated charcoal was added to the hydrolysate. The solution was filtered through Whatman No. 1 filter paper and then neutralized with 10 N NaOH to bring pH to 6.5. To 50 ml of the neutralized hydrolysate 6 ml of 10% NaOH and 0.3 ml of 10% sodium nitroprusside were added in succession. After 10 minutes, 4 ml of concentrated orthophosphoric acid was added and the solution allowed to stand for further 10 minutes. Absorbance of the complex was measured at 520nm in a Hitachi UV 220 spectrophotometer with D-L methionine as the standard.

For the determination of changes in the content of proteins, amino acids and the activity of protease during germination of buckwheat grains, healthy seeds from the collection were surface sterilized for 15 minutes with 0.025% Sodium hypochlorite. The sterilized seeds were rinsed thoroughly with sterile deionized water. The seeds were kept for germination in petri plates on a moist Whatman No. 1 filter paper under continuous white fluorescent light at 25±2°C and 65% R.H. Samples were harvested in triplicate at 2, 6, 14, 18, 24, 36, 48 and 72 hours of imbibition. Following the harvest the seed coat was removed and the seeds were separated into the embryo and endosperm portions. Dry matter content of the embryo and the endosperm was determined by drying a known fresh weight of the tissue in a forced draught oven at 110°C for 72 hours. The dried sampled was cooled and weighed again. Moisture content of the tissue was calculated as the difference between fresh and dry weights. A suitable fresh weight of the harvested embryo and endosperm tissues was separately fixed in hot 80% ethanol for 24 hours. After 24 hours the tissue was macerated in a pestle and mortar and separated into alcohol soluble and alcohol insoluble fractions by centrifugation.

The alcohol soluble fraction was made to volume and used for the estimation of total α-amino nitrogen. An aliquot of the alcohol soluble fraction was also transferred to preweighed plate and allowed to dry under a constant stream of sterile air. The petri plate was weighed again; the weight of alcohol soluble solids was calculated as the difference between the initial and final weights. The alcohol insoluble residue was dried in a forced draught oven at 70°C for 72 hours over P_2O_5. The dried material was weighed to determine the content of total alcohol insoluble solids.

The level of free α-amino nitrogen in the endosperm as well as the embryo was estimated from the alcohol soluble fraction following the method of Rosen (1957). A suitable aliquot of the alcohol soluble fraction, from which alcohol had been completely removed by heating over a water bath, was made to 1 ml by distilled water. 0.5 ml of 0.002M acetate-cyanide buffer (pH 5.4) and 0.5 ml of 3% ninhydrin were added to the solution in succession. The mixture was heated in a boiling water bath for 15 minutes followed by addition of 4 ml of isopropyl alcohol - water diluent (1:1). After cooling absorbance of the coloured complex was recorded on a Hitachi

UV-220 spectrophotometer at 550 nm with glycine as the standard.

Extraction and estimation of soluble protein:

The level of soluble protein was estimated separately in the embryo and the endosperm tissues of germinating seeds following the dye-binding method (Bradford, 1976)

Grains harvested at different stages of germination were dissected to detach the embryo from the endosperm tissues. A suitable fresh weight of each embryo and the endosperm was separately homogenized in a chilled tissue homogenizer with chilled 50 mM Tris-Cl buffer (pH 8.0) containing 0.1M NaCl, 5% polyvinyl pyrrolidone (PVP) and 2 mM phenyl methysulphonyl fluoride (PMSF) to yield a 50% (w/v) homogenate. The homogenate was centrifuged at 10,000xg for 15 minutes at 4^{o}C and the supernatant collected for estimation of soluble protein.

A suitable aliquot of the supernatant, representing a known ammount of fresh weight, was diluted to 1.0 ml. 4 ml of Bradford's reagent were added to it; the solution was vortexed for 1 minute and allowed to stand at room temperature for 30 minutes for the complete development of colour. Absorbance of the coloured complex was measured at 595 nm using Bovine Serum Albumin (BSA) as the standard.

Assay of Protease activity:

A suitable fresh weight of the embryo and endosperm tissues, obtained from seeds harvested at different stages of germination, was homogenized separately in a cold glass tissue homogenizer with chilled 50 mM Tris-Cl buffer (pH 8.0) containing 0.1M NaCl, 5% polyvinyl pyrrolidone (PVP) and 2 mM phenyl methysulphonyl fluoride (PMSF) to yield a 50% (w/v) homogenate. The homogenate was centrifuged at 10,000xg for 15 minutes at 4^{o}C .

Protease activity was estimated from the supernatant traction according to the method described by Sarath et.al (1985) with BSA as the substrate. The assay mixture consisted of 1ml of 5% BSA, 0.5ml of 0.05M Tris-HCl buffer pH 6.5, 0.3ml of H_2O. The reaction was initiated by the addition of 0.2ml of the extract. Incubation of the assay mixture was carried out at 30^{o}C for 1hour in a water bath Shaker. The reaction was terminated by addition of chilled 50% TCA to the assay mixture in the ratio of 1:1. The mixture was centrifuged at 1000 x g for 15 minutes. The TCA soluble fraction obtained after centrifugation was brought to neutral pH with NaOH. Total free amino acids from the TCA soluble fraction were estimated by ninhydrin reaction according to Rosen (1957). Protease activity has been expressed as μ mol amino acids released per 100mg fresh weight of the tissue as well as per mg soluble protein present in the tissue.

Measurement of respiratory activity:

Changes in the respiratory activity in germinating seeds was determined in mitochondria isolated from the whole seeds, harvested at different stages of germination.

Isolation of mitochondria: A suitable fresh weight of the seeds harvested at different stages of germination homogtissue homogenizer with chilled 50 mM Tris-Cl buffer (pH 8.0) containing 0.1M NaCl, 5% polyvinyl pyrrolidone (PVP) and 2 mM phenyl methysulphonyl fluoride (PMSF) to yield a 50% (w/v) homogenate. The homogenate was centrifuged at 10,000xg for 15 minutes at 4°C .

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Measurement of respiratory activity:

Changes in the respiratory activity in germinating seeds was determined in mitochondria isolated from the whole seeds, harvested at different stages of germination.

Isolation of mitochondria: A suitable fresh weight of the seeds harvested at different stages of germination homogenized in a chilled pestle and mortar with 0.05M phosphate buffer (pH 7.3) containing 0.5M sucrose. The homogenate was filtered through two layers of musclin, and the filtrate was centrifuged at 1000xg for 5 minutes at $4^{o}C$. The supernatant was recentrifuged at 18,000xg for 25 minutes at $4^{o}C$. The pellet thus obtained was resuspended in the sucrose phosphate extraction medium and centrifuged again at 20,000xg for 25 minutes at $4^{o}C$. The pellet was suspended in 40 ml of suspending solution consisting of 0.02M phosphate buffer (pH 7.3), 0.4M sucrose, 0.005M $MgCl_2$ and 0.02M glucose.

Respiratory activity was estimated in a suitable aliquot of the isolated mitochondrial fraction by the Clarke type oxygen electrode (Rank Botham, England). The activity has been expressed as μmol O_2 consumed per seed per minute. The respiration rate has also been expressed on seed fresh weight as well as seed dry weight basis.

Estimation of free and total phosphorus:

A known fresh weight of the seed tissues, harvested at different stages of germination was extracted with chilled 10% trichloro acetic acid (TCA) in a chilled glass pestle and mortar. The homogenate was centrifuged at 10000xg for 15 minutes at $4^{o}C$. The pellet was designated as the TCA insoluble fraction and supernatant as TCA soluble fraction . The TCA insoluble as well as the TCA soluble fractions were digested with H_2SO_4 and H_2O_2 in a digestion rack until the digests turned colourless. The digests were made to volume with distilled water. Free phosphorus was estimated in suitable aliquots drawn from each TCA soluble as well as the TCA insoluble fractions following the method of Fiske and Subbarow (1925).

A suitable aliquot of the digest, representing a known ammount of fresh weight was made to 0.4 ml with distilled water. 0.4 ml of molybdate reagent and 0.2 ml of amino napthol sulphonic acid (ANSA) reagent were added to the solution. The solution was made to 5 ml with distilled water, vortexed and allowed to remain at room temperature for minutes. After 10 minutes absorbance of the coloured solution was measured at 640 nm against a reagent blank.

For the assessment of the effect of the growing axis on the mobilization of storage protein and amino acids as also proteolytic activity in the germinating buckwheat seeds, the seeds were dissected under aseptic conditions to remove the embryo before plating them for germination. The deembryonated seeds and the dissected embryos were separately kept for germination in petri plates on a moist Whatman No. 1 filter paper under continuous white fluorescent light at 25+^{o}C and 65% R.H. Samples were harvested in triplicate at 2, 6, 1 18, 24, 36, 48 and 72 hours.

The estimation of free α-amino nitrogen, soluble protein and the assay of protease activity from the tissues was carried out as described above.

Isolation and Electron Microscopy of protein bodies:

Protein bodies were extracted from dehulled buckwheat grains by the aqueous extraction protocol according to Pernollet (1978). The protein body suspension (20 µg/ml) was negatively stained with 2% phosphotungstic acid prepared in

0.1M cocodylate buffer (pH 7.3) containing 20 μg/ml bacitracin. The protein body to staining solution was in 1:1 ratio. The solution was loaded on 400-500 μm mesh copper grids and visualized under a Joel 100B electron microscope.

The protein bodies were also examined in ultrathin sections prepared according to Ludevid *et al.* (1985). Intact endosperm from the seeds was fixed in 3% glutaraldehyde in 0.1M cocodylate buffer (pH 7.2). The sections were post fixed in 1% OSO_4 in 0.1M cocodylate buffer for 2hours at $4^{o}C$. After post fixation the tissue was washed in 0.1M cacodylate buffer for 30 minutes with two changes. The tissue was left overnight in the buffer after washing. The tissue was cut into 1mm blocks and dehydrated gradually with 20%, 30%, 40%, 50%, 70%, 90% and 100% concentrations of acetone (analar) for 10 to 15 minutes in each step with 3 changes.

After dehydration the tissues were cleared with toluene in a flame cupboard with two changes of 5- 10 minutes each. Ultrathin sections of the fixed and dehydrated endosperm tissue were cut in LKB - Ultramicrotome at a scale of 60 μm. The sections were stained for 10 minutes in 5% uranyl acetate followed by 2 minutes in 0.4% Reynold's Lead citrate. Sections were viewed and photographed in the Joel 1000 Transmission electron microscope.

Chemical analysis in Protein bodies:

For the determination of partial chemical composition of the protein bodies, the Protein bodies were suspended in 0.1M Tris-Cl buffer (pH 8.) and sonicated to disrupt their membrane. The protein content of the suspension was determined by the dye binding method (Bradford, 1976).

The carbohydrate content of the suspension was determined by the anthrone-H_2SO_4 method of (Mc Cready *et al.*, 1950). To a suitable aliquot of the protein suspension 4 ml of 0.1 % anthrone prepared in conc. sulfuric acid was added. The solution was kept in a boiling water bath for 15 minutes for the development of colour. The solution was allowed to cool to room temperature. Absorbance of the solution was measured at 700 nm against a reagent blank.

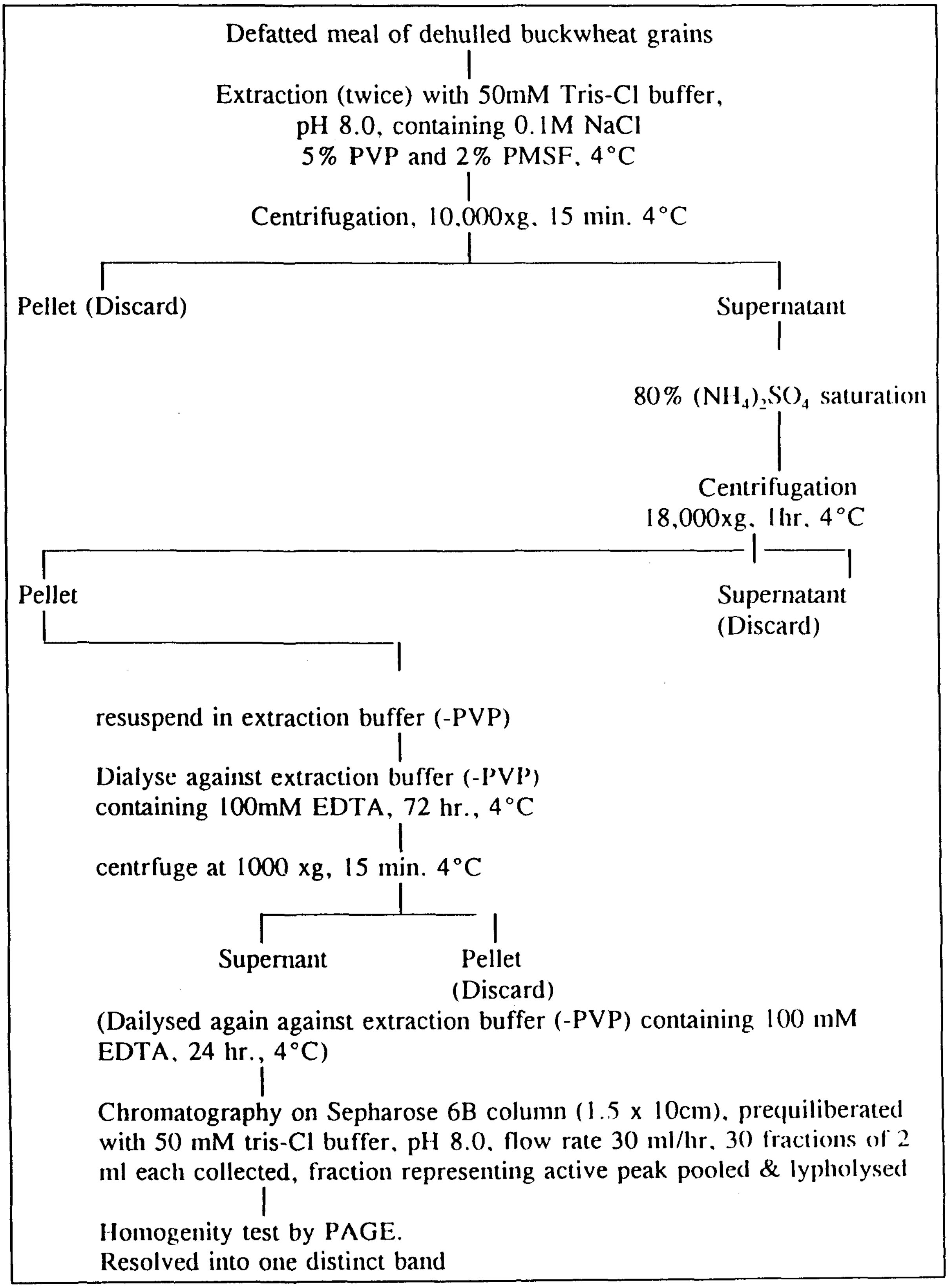
The phosphorus content of the protein bodies was estimated in suitable aliquots of the protein suspension according to the protocol of Fiske and Subbarrow (1925).

Purification of Buckwheat Main Seed Storage Protein:

Homogenization: The dehulled buckwheat grains were homogenized in a pre-chilled glass homogenizer with chilled 50 mM Tris- Cl buffer (pH 8.0) containing 0.1 M NaCl, 5% PVP a, 2mM PMSF to obtain a 50%(w/v) homogenate. The homogenate was centrifuged at 10,000xg for 15 minutes at 4^{o}C and the clear supernatant collected in pre-chilled glass beaker.

$(NH_4)_2SO_4$ Fractionation: The clear supernatant was brought to 80% ammonium sulphate saturation level by a gradual addition of AR grade ammonium sulphate to the supernatant. The solution was maintained under continuous stirring over a magnetic stirrer at 4^{o}C 6 hours. The solution was centrifuged at 18,000xg for 1hour at 4^{o}C and the resulting pellet resuspended in 2ml of extraction buffer.

Dialysis: The protein suspension obtained after ammoni sulphate precipitation was transferred to a dialysis sac and fig.3.2:Flow sheet for the isolation of 280 KD globulin from mature seeds of commo buckwheat (*Fagopyram esculentum* Monech)



Defatted meal of dehulled buckwheat grains

Extraction (twice) with 50mM Tris-Cl buffer,
pH 8.0, containing 0.1M NaCl
5% PVP and 2% PMSF, 4°C

Centrifugation, 10,000xg, 15 min. 4°C

Pellet (Discard)

Supernatant

80% $(NH_4)_2SO_4$ saturation

Centrifugation
18,000xg, 1hr, 4°C

Pellet

Supernatant
(Discard)

resuspend in extraction buffer (-PVP)

Dialyse against extraction buffer (-PVP)
containing 100mM EDTA, 72 hr., 4°C

centrfuge at 1000 xg, 15 min. 4°C

Supernant

Pellet
(Discard)

(Dailysed again against extraction buffer (-PVP) containing 100 mM EDTA, 24 hr., 4°C)

Chromatography on Sepharose 6B column (1.5 x 10cm), prequiliberated with 50 mM tris-Cl buffer, pH 8.0, flow rate 30 ml/hr, 30 fractions of 2 ml each collected, fraction representing active peak pooled & lypholysed

Homogenity test by PAGE.
Resolved into one distinct band

subjected to extensive dialysis for 72 hours at 4^{o}C against 50mM Tris-Cl (pH 8.0) containing 100mM EDTA. The dialysate was centrifuged at 18000xg for 15 mints at 4^{o}C and the clear supernatant collected in a pre-chilled glass vial.

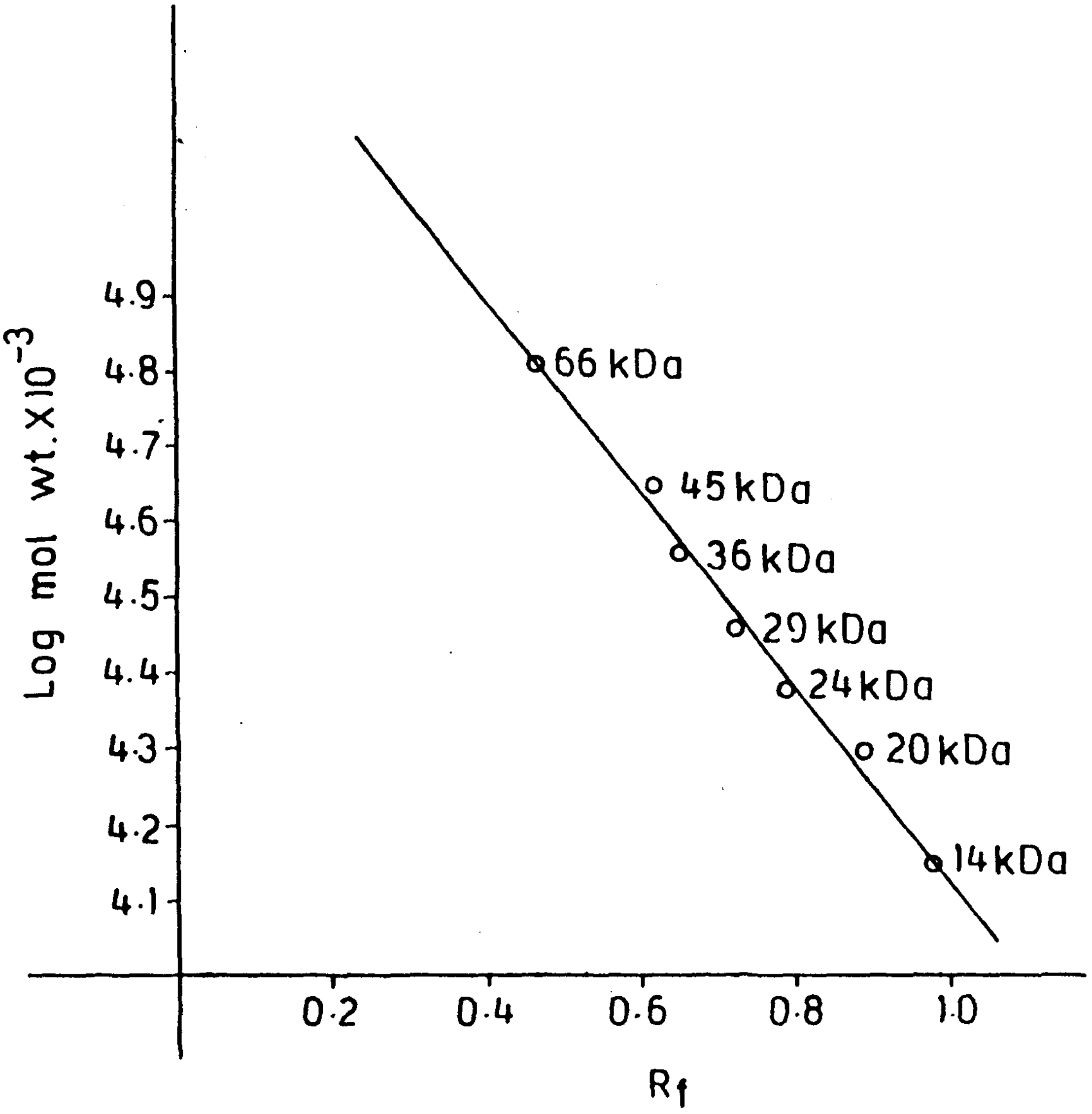
Chromatography: The clear supernatant was chromatographed on a pre-calibrated Sepharose-6B column size (1.5 cmx25 cm) having void volume of 12 ml . The column was eluted with 50mM Tris-Cl buffer (pH8.0) with a flow rate 30 ml/hour. 30 fractions of 2 ml each were collected. The absorbance of each fraction was recorded at 280nm in a Hitachi UV-220 spectrophotometer. The fractions showing absorbance at 280 nm were categorized into peaks and the fractions corresponding to each peak were pooled lypholyzed. The protein content of each fraction was determined by the dye binding assay (Bradford, 1979). The flow chart of the purification protocol of buckwheat main seed storage protein is given as Fig. 3.2.

Electrophoretic Methods:

Native Polyacrylamide Gel Electrophoresis: The electrophoresis of Buckwheat seed storage protein was carried out at room temperature on a 7.5% Polyacrylamide gel according to Davis (1964).

The gel was stained by for 2 hours in 0.2% commassie brilliant blue R-250 prepared in methanol, water and glacial acetic acid (4:5:1 v/v). The gel was destained using (methanol: acetic acid:water (5:1:4 v/v). Protein bands were visualized under a transilluminator.

SDS polyacrylamide gel electrophoresis: SDS-PAGE of the isolated protein was carried out on a 10% polyacrylamide slab

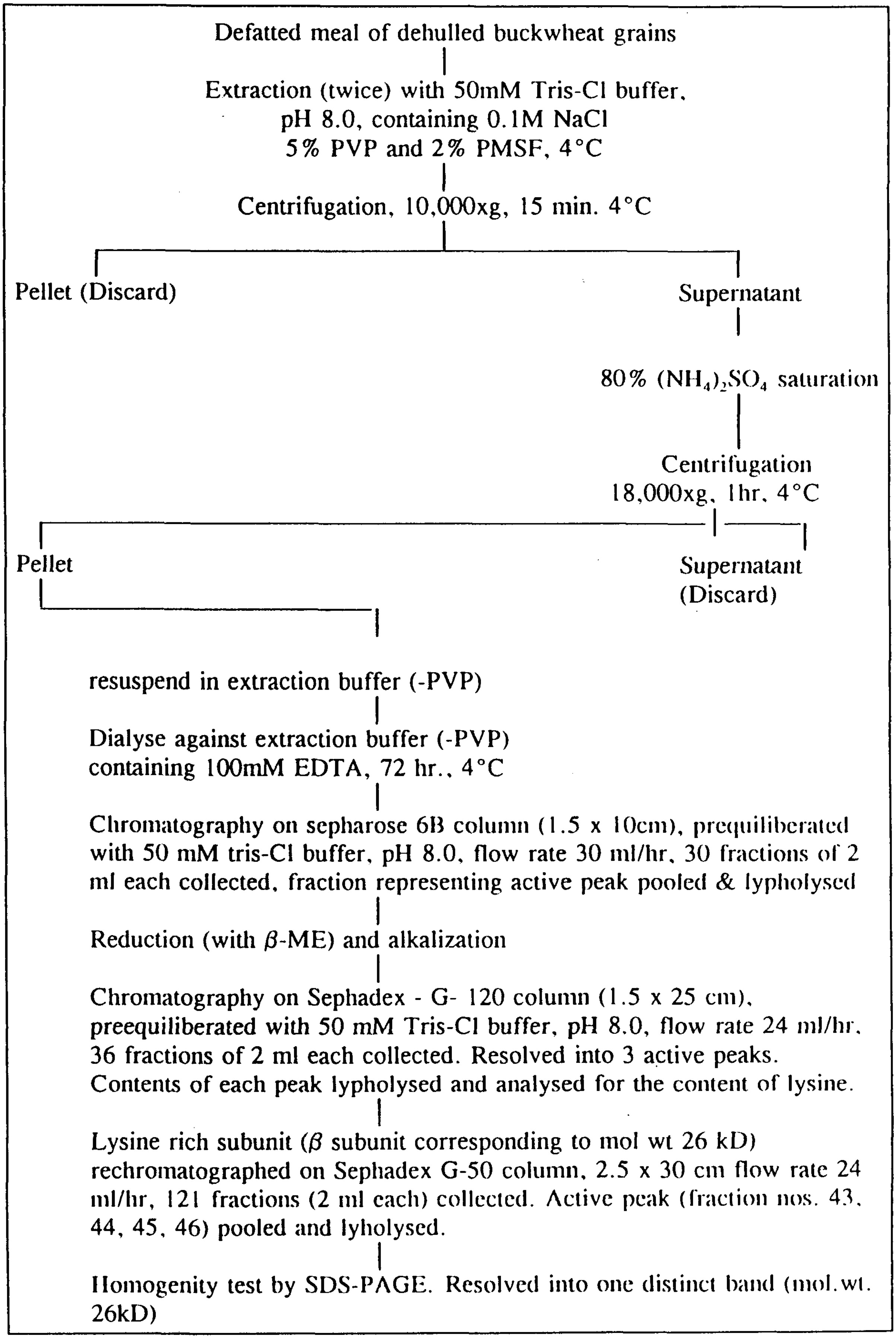

Log mol wt. X10^{-3}
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
0.2
0.4
0.6
0.8
1.0
R$_f$
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa

gel according to the method of Laemmli (1970). A suitable aliquot of the sample was mixed with sample buffer containing 1M Tris-Cl buffer (pH 7.3), 10% Glycerol, 5%(v/v) 2-ME and 2%(w/v) Sodium dodesyl Sulphate. The sample was heated for 3-5 minutes in a boiling water bath and loaded into the well of the gel. Standard markers were also similarly denatured and subjected to electrophoresis.

After electrophoresis the gels were stained using staining solution as mentioned in the previous section. The electrophoretic mobility of the marker proteins and the seed storage protein were calculated as the ratio of the distance traveled by the protein to the distance traveled by the tracking dye.

The molecular mass of the separated proteins was determined by comparing their electrophoretic mobility with that of the standard proteins and derived from a plot of electrophoretic mobility against log molecular weight of the marker proteins Fig. 3.3.

The purified High molecular weight (HMW) 13S globulin was reduced and alkalized by treatment with 5% 2-ME and 6M Urea as per Hager *et al.* (1993). The sample was chromotographed again on a pre- calibrated Sephadex G-120 column (1.5cmx25cm). The column was eluted with 50mM Tris-Cl buffer (pH8.0) with a flow rate 24 ml/hour.36 fractions of 2 ml each were collected; the absorbance of each fraction was determined in a spectrophotometer at 280 nm. The fractions corresponding to active peak were pooled together. Molecular weight of protein in each fraction was determined by referfig.3.3: Fow sheet for the isolaltion of the lysine rich subunit of the 280 KD globulin from mature seed of common buckwheat (*Fagopyrum esculentum* Moench).



Defatted meal of dehulled buckwheat grains

Extraction (twice) with 50mM Tris-Cl buffer,
pH 8.0, containing 0.1M NaCl
5% PVP and 2% PMSF, 4°C

Centrifugation, 10,000xg, 15 min. 4°C

Pellet (Discard)

Supernatant

80% $(NH_4)_2SO_4$ saturation

Centrifugation
18,000xg, 1hr, 4°C

Pellet

Supernatant
(Discard)

resuspend in extraction buffer (-PVP)

Dialyse against extraction buffer (-PVP)
containing 100mM EDTA, 72 hr., 4°C

Chromatography on sepharose 6B column (1.5 x 10cm), prequiliberated with 50 mM tris-Cl buffer, pH 8.0, flow rate 30 ml/hr, 30 fractions of 2 ml each collected, fraction representing active peak pooled & lypholysed

Reduction (with β-ME) and alkalization

Chromatography on Sephadex - G- 120 column (1.5 x 25 cm), preequiliberated with 50 mM Tris-Cl buffer, pH 8.0, flow rate 24 ml/hr, 36 fractions of 2 ml each collected. Resolved into 3 active peaks. Contents of each peak lypholysed and analysed for the content of lysine.

Lysine rich subunit (β subunit corresponding to mol wt 26 kD) rechromatographed on Sephadex G-50 column, 2.5 x 30 cm flow rate 24 ml/hr, 121 fractions (2 ml each) collected. Active peak (fraction nos. 43, 44, 45, 46) pooled and lyholysed.

Homogenity test by SDS-PAGE. Resolved into one distinct band (mol.wt. 26kD) ence to the standard graph prepared using standard markers.

The active fraction was rechromatographed on pre calibrated Sephadex G-50 column (2.5 cm x 30 cm). The column was eluted with 50mM Tris-Cl buffer (pH 8.0) at a flow rate of 24ml/hour. 121 fractions of 2ml each were collected. Fraction numbers. 43, 44, 45, 46 showing highest protein level and representing one peak were pooled together and lypholyzed. The molecular weight of the protein eluted in the peak was determined by reference the elution profile of standard markers. The flow chart for the purification of the lysine rich subunit of the 280 kD globulin isolated from buckwheat grains is given as Fig. 3.4.

The molecular weight of the proteins eluted from Sepharose 6B, Sephadex G120 and Sephadex G50 columns was calculated using the following equations:

.1s1 $$K_{av} = \frac{Ve - Vo}{V_t - V_0}$$

V_e = elution volume of the active material

V_o = elution volume of blue dextran

V_t = total volume of gel bed - r^2h

Where r = radius of the column

h = height of the column

K_{av} for standards was calculated and plotted against molecular weight on semi-logarithmic paper to prepare the standard curve. The molecular weight of the active fraction was calculated from standard graph.

Antibody preparation and immunochemical investigations:

Antibodies to the purified 280 kD buckwheat globulin were raised in Soviet male rabbit. The purified protein was dissolved in PBS to a concentration of 400 µg/ml. The solution was mixed thoroughly with an equal volume of Freund's complete adjuvant to make water -in-oil emulsion. The emulsion was injected intradermally at several sites into a 4 months old male Soviet Chinchilla rabbit weighing 1.5 Kg. 4 injections were administered at 15 days interval and the animal was bled 7 days after the booster doze to collect the antiserum.

The **agar gel double immunodiffusion test** (Ouchterlony, 1958) was carried out to test the antiserum. A 1% solution of agar prepared in phosphate buffer saline (PBS) was poured into a petridish and cooled to form a gel. Wells were punched into the agar gel for loading the antiserum and the test samples. 20 µl of the antiserum was placed in the central well and the protein (5-30 µg) loaded into the surrounding wells. The plates were incubated in a humid chamber for 24hours at 37°C. After 24 hours the plates were checked visually for the appearance of precipitin lines because of antibody - antigen interaction.

Enzyme linked immunoabsorbant assay (ELISA) was performed essentially according to the procedure of Clark (1981). The antigen was adsorbed to polystyrene plate by pipetting 100 µl (10 µg/well) aliquots of the antigen solution (prepared in 10 mM PBS (pH 7.4)) into each well of an

ELISA microtitre plate. The plate was allowed to stand at 37^{o}C for 2h. After 2 hours of incubation, the antigen solution was removed and the unoccupied sites were blocked by filling the wells completely with 0.2% BSA (prepared in PBS containing 0.02% sodium azide blocking buffer). The plate was incubated for 60 minutes at 37^{o}C . This step ensures binding of BSA to the unoccupied sites and eliminates the nonspecific adsorption of the antibody to the ELISA plates in the subsequent steps. Prior to addition of the antiserum, the wells were washed three times with PBS containing 0.5% Tween-20 and three times with PBS. The plates were then incubated wi suitably diluted antiserum for 2hours at 37^{o}C. After the incubation, the plates were washed with PBS as described earlier. Following this 100 μl of Goat anti-rabbit HRP conjugate was pipetted into each well and the plate again incubated at 25^{o}C for 2hours. After the incubation, the unbound double-antibody was removed and the plate was washed as described above. Color of the complex was developed with o-phenyldiamine and the reaction stopped by adding 100μl of 2N HCl per well. The absorbance of each well as determined in an ELISA reader (Bio-Tek auto reader, Model EL 311) with a 495 nm filter.

Western blot:

Western blot of the purified protein with antibodies raised against the protein was carried out according to Towbin *et al.* (1979). The total protein extract obtained after homogenizing the grains with Tris-Cl buffer was subjected to polyacrylamide electrophoresis following the method of Laemmli (1970). Immediately after gel electrophoresis, the unstained gel was rinsed in the transfer buffer for 15 minutes. Semidry electroblotting of the proteins to nitrocellulose (0.45 µm) membrane was carried out for 3 hours using a current of 1mA/cm^2 of filter paper. The proteins were thus transferred to the membrane at 90 mA for 3 hours at room temperature using the Novo blot (Pharamacia) apparatus. Immediately after the transfer, the blot was left overnight 4^{oC} in rinse buffer, prepared by mixing 10 ml of 20 X PBS, 0.1ml of Tween-20 and 190 ml of distilled water. The blot was washed 10 times for 10 minutes each with gentle rocking in the rinse buffer and incubated for 3 hours with gentle rocking with antibody diluting buffer (prepared by mixing 5 ml of 20 X PBS, 0.5 ml of Tween-20 and 94.5 ml of distilled water) containing the primary antibody (1:500 dilution). The blot was again rinsed 6 times for 10 minutes each with rinse buffer and incubated in secondary antibody (Goat-anti Rabbit IgG-HRP conjugate, 1: 2000 dilution) for 1 hour with rocking. The blot was rinsed 6 times for 10 minutes each and develop in dark by using diaminobenzoic acid.

Spectroscopy:

Fluorescence spectrophotometry: The 13S globulin isolated from buckwheat grains was subjected to fluorescence spectroscopy using Schimadzu RF 540 spectrofluorimeter equipped with a Xenon lamp, thermoregulated sample chamber, automatic polarizers and data recorder. Quartz cuvettes (3ml) with 1 cm path length were used for the measurements. It was ensured that the buffers did not interfere with the fluorescence measurement. The absorbance at the wavelength of excitation was less than 0.05 to avoid inner filter effects.

Circular dichroic spectrophotometry: Circular dichroic (CD) measurement of the purified protein were recorded in a Jasco 1-500A spectropolarimeter having JASCO DP-501A data processor attachment with cells of 1 mm and 2 mm. The instrument was calibrated with d-10 (Campho sulfonic acid (Cassium and Yang, 1969). The instrument was constantly purged with pure nitrogen silts were programmed to yield 10A^{o} band width at each wavelength. All the CD spectra were recorded in 0.1M Potassium phosphate buffer, using the same buffer as the blank. The spectra were recorded at 25 ± 1^{o}C at a time constant of 8 and chart speed was set to 2cm/minute. A known concentration of the protein was used for recording visible 500-350 nm CD spectra and far - UV(200-250 nm) CD spectra. Visible CD spectra was recorded at a Y-axis scale of 1 millidegree/ and X-axis scale of 10 nm/cm. Far-UV-CD spectra was recorded at Y-axis scale of 1 millidegrees/cm and X-axis scale of 10 nm/cm.

Specific elipticity and molecular elipticity of the protein was calculated by the Chou and Fasman (1969) equation as descried below:

Elipticity (deg)

$\phi = \pm H \times S$

S: CD scale (m^{o}/cm= x 10^{-3} deg / cm)

H: reading (cm)

Specific elipticity $[\psi_\lambda]$

$$[\psi_\lambda] = \frac{\theta_\lambda}{C \times 1}$$

C: concentration (g/ml)
1: cell length (dm)

Molecular ellipticity $[\theta_\lambda]$ (deg.Cm^2. deci mol^{-1}

$$[\phi] = [\psi_\lambda] \times M/100$$
$$= \frac{\phi}{C \times 1} \times 100$$

M: Molecular weight
C: Molar concentration (mol/l)
1: cell length (cm)

The protein CD spectra were plotted as molar elipticity $[\theta]$, molar or mean residue elipticity $[\theta_{mrw}]$ assuming a molec lar weight of 280 or a mean residue weight (mrw)of 115 and using the following formula (Greenfield and Fasman, 1969).

$[\theta]$ molar = x (Mr)/(10 x1xC)

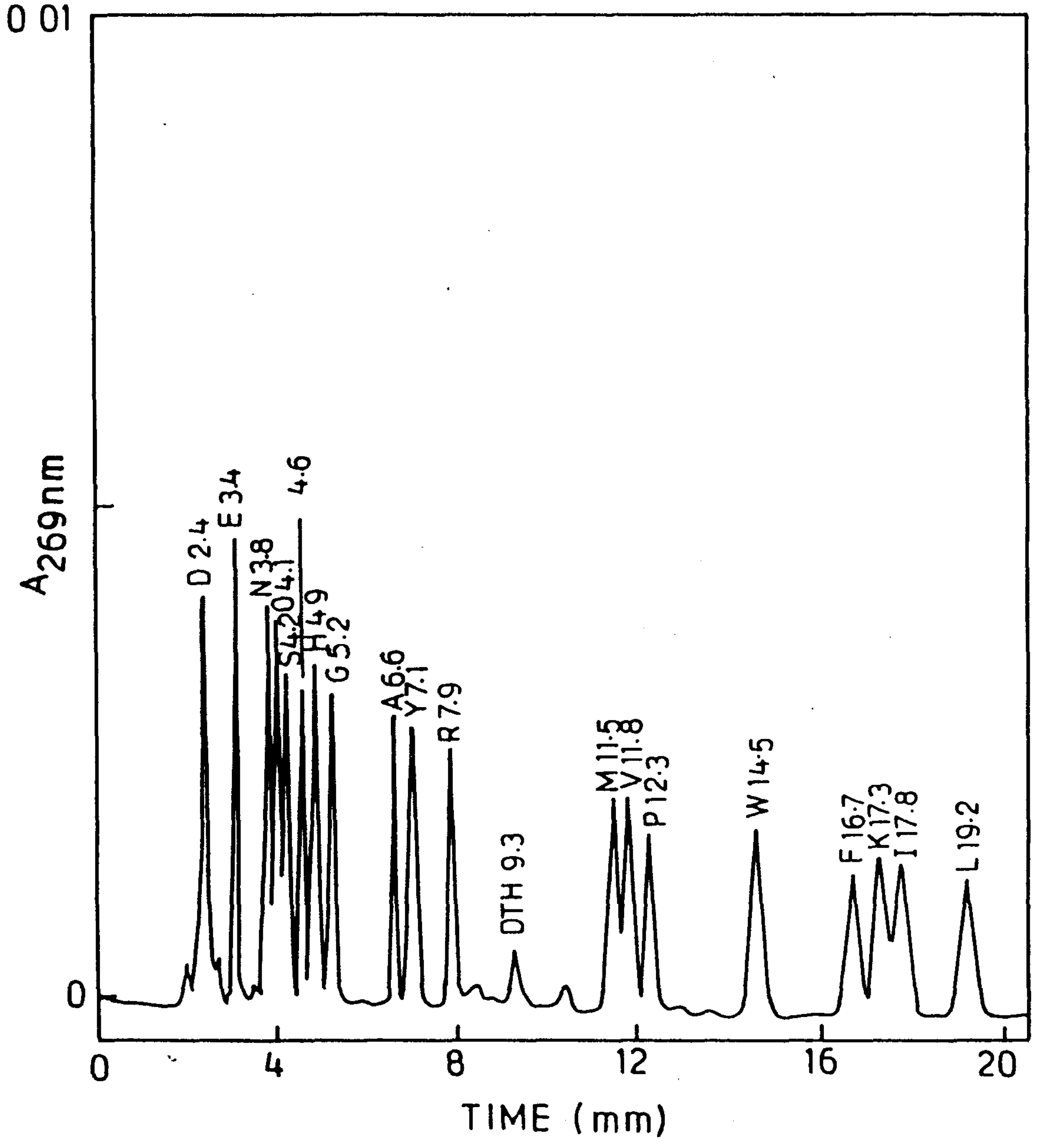
$[\theta]$ mrw = x (mrw)x(10x1xc)

where, $[\theta]$ is the observed elipticity in degrees, 1 is the optical path length in cm and C is the concentration of the protein in g/ml . The cells used for CD measurements had path lengths of 2 mm far visible CD and 1mm for far UV CD spectra.

Determination of amino acid composition and the N-termin amino acid sequence:

For the determination of amino acid composition, the purified 26 kD protein was subjected to acid hydrolysis at 110°C for 12 hours under vacuum. The amino acid composition of the hydrolysate was determined in an automated amino acid analyser according to the protocol described by Raina and Datta (1992).

For the determination of the N-terminal amino acid sequence of the 26 kD lysine rich protein subunit, the sample, was analyzed by SDS- PAGE on 10% gels according to Lamelli (1970). The protein was subsequently transferred to a PVDF membrane using the Nova according to the protocol of Matsudara (1987). Before transfer the PVDF (Polyvinyl difluoride) membrane was cut to the required size (slightly larger than the gel) and soaked in methanol (HPLC grade for 1-2 minutes) before use. After wetting the membrane in methanol, it was soaked for 10 minutes in 25 mM Tris-Glycine buffer (pH 8.3) containing 15% methanol (Matsudaria, 1987; Speicher, 1989). Transfer of the protein to the membrane was carried out in a semidry type Novoblot apparatus (Pharmacia) as described earlier. Detection of the protein on PVDF membrane was carried out by staining the membrane for 15 minutes with 0.1% commassie blue R-250 prepared in 50% methanol. The membrane was destained with 50% methanol. Care was taken not to use acetic acid during staining or de staining the membrane. The destained membrane was extensively washed Mili-Q deionized water (5 changes), air dried and stored at - 20°C. The segment having the protein was excised, washed with Mili-Q deionized water and then used directly for sequencing by automated Edman gas phase sequenator coupled to PTH - amino acid analyzer as described by Matsudara (1987). The protein transferred to PVDF membrane was directly loaded to the


0 01
A269nm
0
D 2.4
E 3.4
N 3.8
Q 4.1
S 4.2
4.6
H 4.9
G 5.2
A 6.6
Y 7.1
R 7.9
DTH 9.3
M 11.5
V 11.8
P 12.3
W 14.5
F 16.7
K 17.3
I 17.8
L 19.2
0
4
8
12
16
20
TIME (mm)

cartridge. Coupling was carried out by moistening the glass fiber disc with phenyl isothiocyanate, in the presence of trimethyl amine and ethyl acetate. The cleavage reaction was carried out with gaseous TFA to form anitinotniozolinone (ATZ) derivative. Both the coupling and cleavage reactions were carried out in a temperature - controlled reaction chamber. The free ATZ-amino acid extracted to the conversion flask by n-butyl chloride. The ATZ- amino acid was converted to the more stable PTH - amino acid by reaction with 25% TFA. The PTH-amino acid was dissolved in acetonitrile and injected into the HPLC. The residual amino acid was separated on RP-HPLC. The PTH- amino acid was identified and quantified. From the data on quantification recovery of the protein was calculated. The results were displayed and recorded. A standard PTH- amino acid profile is shown in Fig. 3.5.

Gene Bank Data Base Search:

The FASTA programme (Pearson and Lipman, 1988) was used for a search of sequences in data bases available with EMBL (European Molecular Biology Laboratory, Heidelberg, Germany) wing the first 17 N-terminal amino acid residues of purified BWG 26 as a query sequence. A total of 19214 residues in 41 sequences were compared by using protein matrix. The alignment of the sequence on the basis of homology to amino acid sequence of the 26kD protein isolated from buckwheat grains was carried out.

CHAPTER IV

EXPERIMENTAL:

For the determination of the extent of relationship between the level of proteolytic activity and the mobilization of reserve proteins during early stages of germination in seeds of common buckwheat (*Fagopyrum esculentum* Moench), healthy and mature seeds of common buckwheat procurred from the regional station of NBPGR were washed under running tap water and sterilized with 0.025% sodium hypochlorite for 15 minutes. The seeds were subsequently washed thoroughly with sterile MilliQ deionized water and kept for germination in petriplates on moist Whatman no. 1 filter paper. Samples were harvested in triplicate a 2, 6, 14, 18, 24, 36, 48 ad 72 hours of imbibiytion. Following the harvest the seed coat was removed and the seeds discected to separate the embryo a endosperm tissues. Portions of the harveted samples were used for the determination of moisture and the dry matt content of the endosperm and embryo tissues. The rest of the harvested samples were used for the estimation of free α-amino nitrogen, soluble protein, and proteolytic activity in the endosperm and the embryo tissues. The tissue level of TCA insoluble as well as TCA soluble phosphorus was measured separately in the embryo, endosperm and the seed coat of seeds harvested at various stages of germination. Respiratory activity has been measured in mitochondria isolated from intact seeds of common buckwheat harvested at various stages of germination.

All the samples were taken in triplicate and the experiments repeated at least twice to ensure reproducibility of the results.

RESULTS:

Immediately upon exposure to water the seeds showed a rapid uptake of water from the ambient environment. Within two hours of incubation, there was a more that 3 fold increase in the moisture content of endosperm in the grains of common buckwheat. After 2 hours, the rate of increase in the moisture content slowed down till it attained a plateau at 24 hours of incubation. The pattern of changes in the moisture content in the embryo of germinating seeds was, howeve different than that observed for the endosperm. In the embryo the uptake of moisture showed a lag upto 14 hours of incubation with no marked change in the level of moisture. After 14 hours the moisture content of the embryo started to increase gradually with progressing time. Between 14 and 72 hours, there was a more than 3 fold increase in the moisture content of the embryo tissues (table 4.1)

Corresponding with changes in the moisture content, the dry matter content of endosperm in buckwheat seeds showed a rapid decline with germination. The endosperm tissues showed a more than two fold decrease in the dry matter content during the initial 6 hours of incubation. Between 6 and 72 hours, however, the rate of decrease in the total dry matter content of the endosperm slowed down till there was no significant change in the dry matter content of the endosperm after 24 hours of incubation (table 4.2, Fig. 4.1). In the embryo there was a more than two fold increase in the content of total dry matter during the first 14 hours of incubation; the magnitude of increase was, however, more marked during the initial 2 hours of incubation. Beyond 14 hours the dry matter content in the embryo showed a marginal decline with progressing germination (table 4.2, Fig. 4.1).

Irrespective of the reference parameter there was no marked change in the respiratory activity in mitochondria isolated from germinating seeds during the initial two hou of imbibition. After two hours of imbibition, respiratory activity in the mitochondria showed a gradual increase with progressing germination. While there was a more than five fold increase in the rate of respiration in germinating seeds between 6 and 72 hours of incubation, the magnitude of increase in respiratory activity was more marked between 14 and 48 hours (table 4.3; Figs. 4.2, 4.3).

Irrespective of the reference parameter, the content of

FIG4.1 Changes in the dry matter content of endosperm and embryo expressed as mg total dry weight/endosperm and mg total dry weight/embryo respectively in seeds of common buckwheat during germination Vertical lines represent LSD at P 0.05.

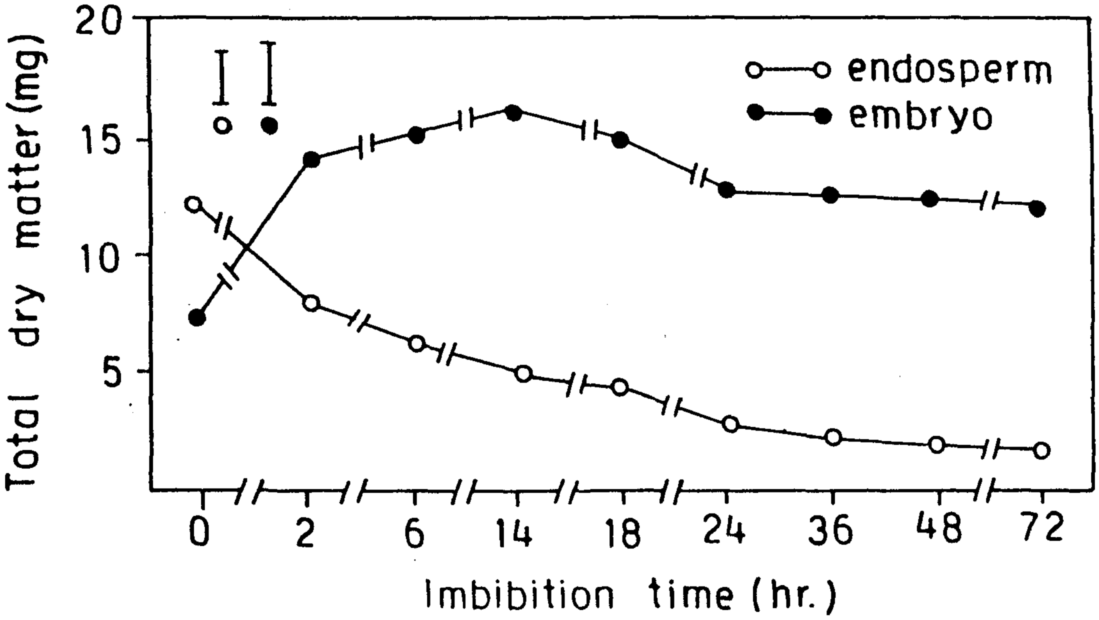

Total dry matter (mg)
20
15
10
5
endosperm
embryo
0
2
6
14
18
24
36
48
72
Imbibition time (hr.)

Table 4.1: Changes in the moisture content of the endosperm and embryo in seeds of common buckwheat (*Fagopyrum esculentum* Moench) during germination upto 72 hours.

Hours after imbibition	Percent moisture endosperm	embryo
0	18.2	20.0
2	57.1	25.0
6	70.0	25.0
14	75.0	22.3
18	85.7	40.0
24	93.3	58.3
36	93.7	64.3
48	94.1	68.7
72	94.4	76.5

Table 4.2: Changes in the total dry matter content of the endosperm and embryo in seeds of common buckwheat (*Fagopyrum esculentum* Moench) during germination upto 72 hours.

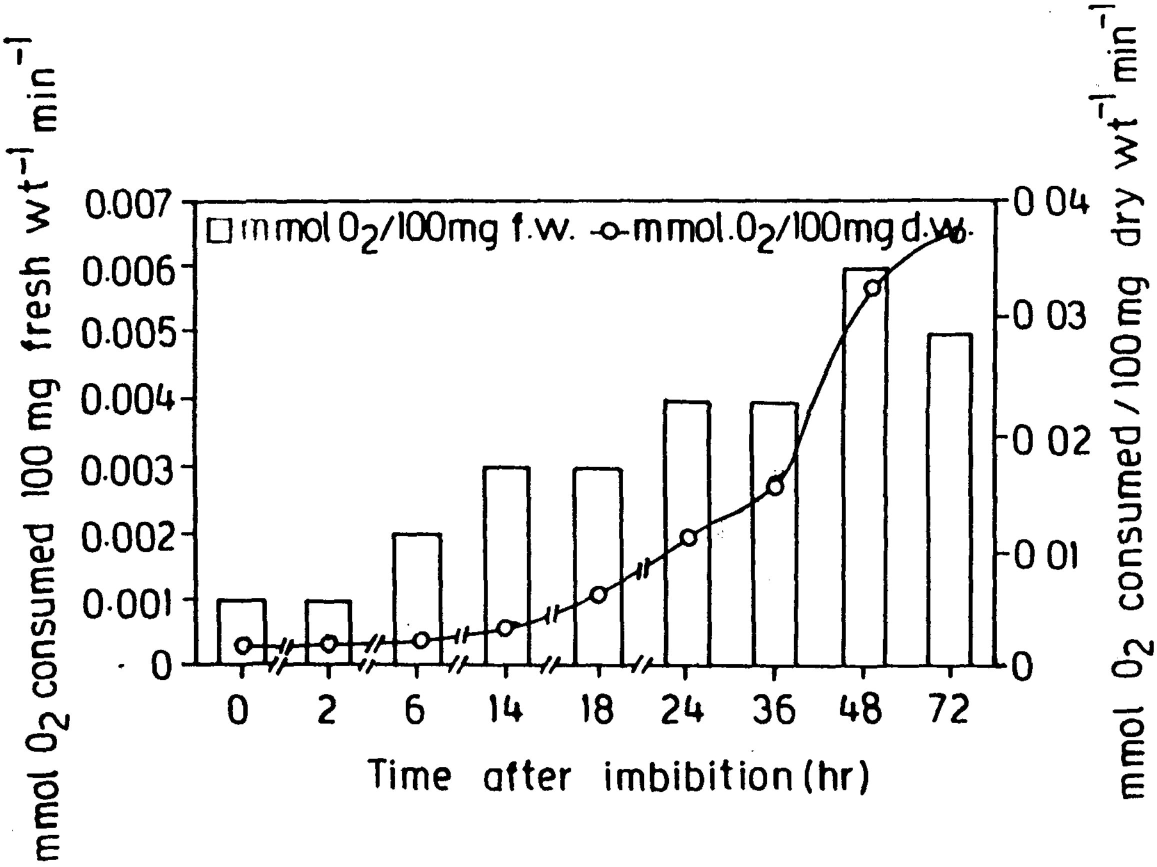
Hours after imbibition	Total dry matter (mg) endosperm	embryo
0	12.27	7.0
2	7.5	14.0
6	6.0	15.0
14	5.0	17.5
18	5.0	15.0
24	2.5	12.5
36	2.5	12.5
48	2.5	12.5
72	2.4	10.0

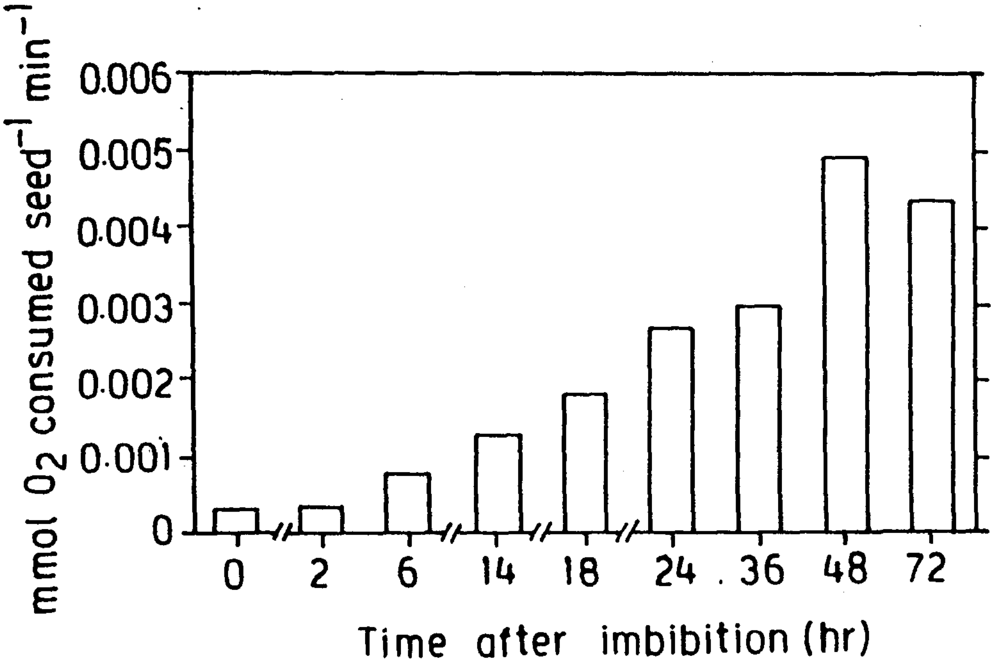
Table 4.3 Changes in respiratory activity in mitochondria isolated from seeds of common buckwheat (*Fagopyrum esculentum* Moench) at various stages of germination upto 72 hours.

Hours after imbibition	m mole O_2 consumed 100mg fresh wt.$^{-1}$ minute^{-1}	m mole O_2 consumed 100mg dry wt.$^{-1}$ minute^{-1}	m mole O_2 consumed seed^{-1} minute^{-1}
0	0.001	0.001	3.24×10^{-4}
2	0.001	0.002	3.75×10^{-4}
6	0.002	0.002	8.0×10^{-4}
14	0.003	0.003	1.27×10^{-3}
18	0.003	0.006	1.8×10^{-3}
24	0.004	0.011	2.7×10^{-3}
36	0.004	0.015	3.0×10^{-3}
48	0.006	0.033	4.95×10^{-3}
72	0.005	0.037	4.37×10^{-3}

fig 4.2: Respiratory activity in asolated mitocondria of germination seeds of common buckwheat (*Fagopyrum esculetum* Moench) as expressed m mole O_2 consumed per 100 mg fresh weight per minute and m mole O_2 consumed per 100 mg dry weight per minute respec tively.

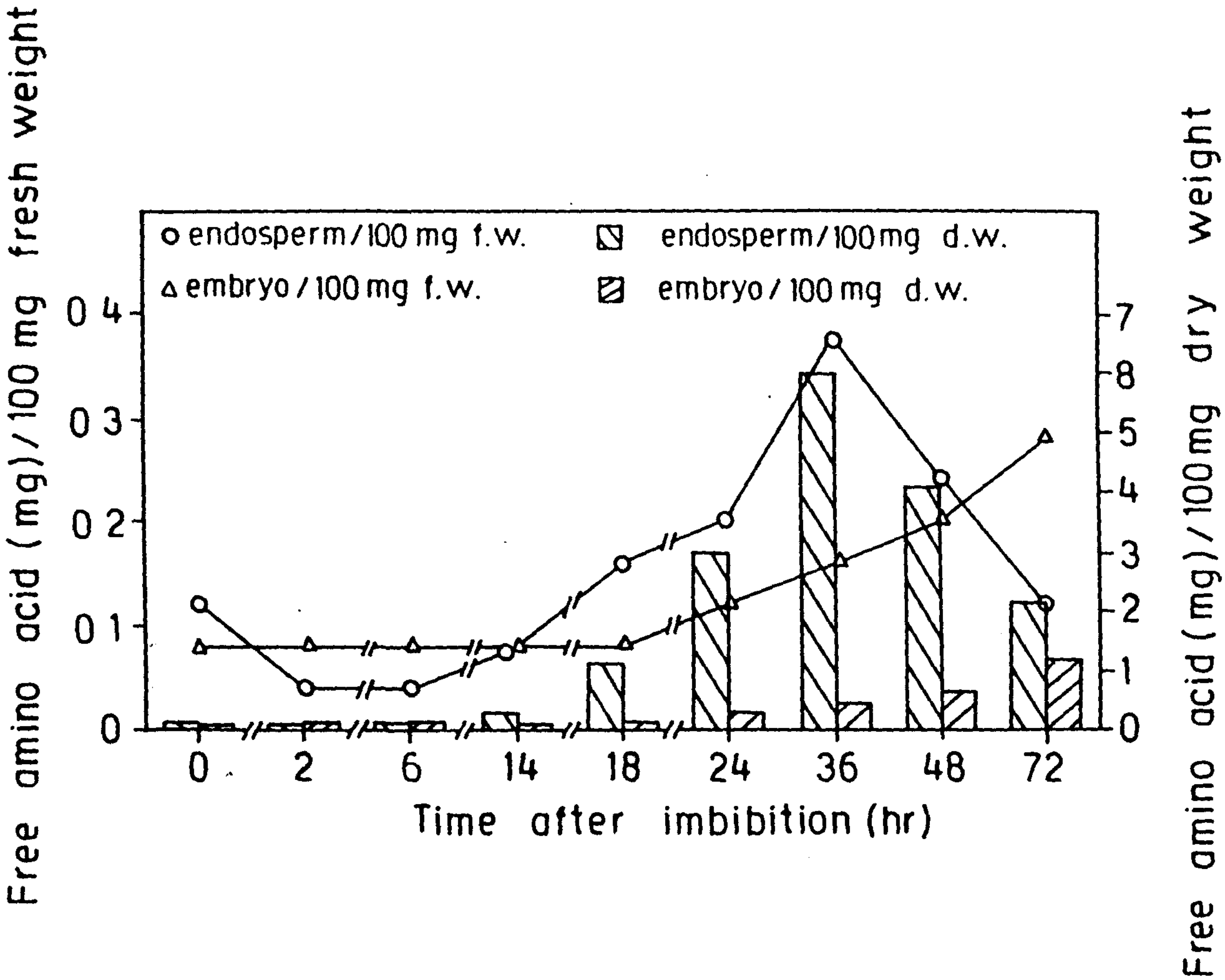
fig 4.3 : Respiratory activity in isolated mitocondria of germinating seeds of common buckwheat (Fagopyrum esculemtum Moench)expressed as m mole O_2 consumed per seedling per minute.


□ mmol O₂/100mg f.w. -o- mmol.O₂/100mg d.w.
mmol O₂ consumed 100 mg fresh wt⁻¹ min⁻¹
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
0.04
0.03
0.02
0.01
0
mmol O₂ consumed/100 mg dry wt⁻¹ min⁻¹
0
2
6
14
18
24
36
48
72
Time after imbibition (hr)


mmol O₂ consumed seed⁻¹ min⁻¹
0.006
0.005
0.004
0.003
0.002
0.001
0
0
2
6
14
18
24
36
48
72
Time after imbibition (hr)

free amino acids in the endosperm of germinating seeds showed a marked decline during the initial 2 hours of imbibition. Thus, between 0 and 2 hours a nearly five fold decrease in the level of free amino acids could be observed in the endosperm of germinating buckwheat seeds. The content of fr amino acids in the endosperm started to increase thereafter till 48 hours of incubation. During this period, there was a more than 10 fold increase in the content of free amino acids in the endosperm tissues. A sharp decline in the content of free amino acids was, however, observed between 48 and 72 hours (table 4.4; Figs. 4.4, 4.5a). When expressed as percent of fresh weight, there was no marked change in the level of free amino acids in the embryo tissues during seed germinaion upto 18 hours. Between 18 and 72 hours, the content of free amino acids showed a gradual increase with progressing seed germination; during the period a nearly three fold increas in the level of free amino acids was observed in the embryo tissues. However, when expressed either as percent of dry weight or as mg free amino acids per embryo, a four fold increase in the content of free amino acids was observed in the embryo during the initial 2 hours of incubation. There was no significant change in the level of free amino acids in the embryo with progressing seed germination between 2 and 18 hours after which it increased gradually with progressing germination upto 72 hours (table 4.5; Figs. 4.4, 4.5a).

Expressed as percent of fresh weight the level of soluble protein in the endosperm showed a gradual decrease with progressing seed germination. When expressed as percent of fig 4.4 Changes in the lavel of fre amino acids in endosperm and emboyo expressed as 100mg fresh weght and 100mg dry weight of seeds of common buckwheat during germination


○ endosperm/100 mg f.w.
△ embryo/100 mg f.w.
▧ endosperm/100mg d.w.
▨ embryo/100 mg d.w.
Free amino acid (mg)/100 mg fresh weight
0 4
0 3
0 2
0 1
0
Free amino acid (mg)/100mg dry weight
7
8
5
4
3
2
1
0
0
2
6
14
18
24
36
48
72
Time after imbibition (hr)

grain dry weight, there was marked change in the level of soluble protein upto 14 hours. Beyond 14 hours, the conte of soluble protein showed a sharp increase with progressing seed germination. However, when changes in the level of soluble protein where expressed on per endosperm basis, a progressive increase in the level of soluble protein was observed upto 36 hours of germination. During this period an almost four fold increase in the level of soluble protein was observed in the endosperm. Beyond 36 hours, the content of soluble protein in the endosperm decreased somewhat upto 72 hours (table 4.4; Figs. 4.6a; 4.7a). Expressed either as percent of grain fresh weight or grain dry weight, the level of soluble protein in the embryo showed a gradual increa upto 36 hours of germination after which it decreased marginally upto 72 hours. However, when expressed as mg soluble protein per embryo the content of soluble protein showed a more than six fold increase with progressing seed germination upto 72 hours. The increase in the level of soluble protein was, however, more marked during the initial 2 hours of incubation; during this period there was a nearly two fold increase in the content of soluble protein in the tissue (table 4.5; Figs. 4.6b, 4.7b).

Irrespective of the reference parameter, the ratio of free amino acids to soluble protein in both endosperm as well as the embryo tissues followed a trend similar to that observed for free amino acids (tables 4.4, 4.5; Fig. 4.5b). There was no marked change in the electrophoretic mobility of the 280 kD globulin of buckwheat grains during the initial 24

Table 4.4: Changes in the content of free amino nitrogen, soluble protein and the ratio of free amino nitrogen to soluble protein in the endosperm tissues of seeds of common buckwheat (*Fagopyrum esculentum* Moench) during germination upto 72 hours.

Hours after imbibition	free amino nitrogen	total soluble protein	amino nitrogen / soluble protein
mg per 100 mg fresh weight			
0	0.12	1.229	0.0976
2	0.04	1.629	0.0245
6	0.04	1.858	0.021
14	0.08	1.987	0.040
18	0.16	2.087	0.076
24	0.20	1.945	0.102
36	0.376	1.945	0.193
48	0.240	0.973	0.246
72	0.12	0.902	0.133
mg per 100 mg dry weight			
0	0.146	1.542	0.0976
2	0.093	3.801	0.0245
6	0.133	6.193	0.021
14	0.320	7.948	0.040
18	1.120	14.614	0.076
24	3.000	29.204	0.102
36	6.016	31.120	0.193
48	4.080	16.547	0.246
72	2.162	16.252	0.133
mg per endosperm			
0	0.032	0.337	0.0976
2	0.007	0.285	0.0245
6	0.008	0.371	0.021
14	0.016	0.397	0.040
18	0.056	0.730	0.076
24	0.075	0.729	0.102
36	0.150	0.778	0.193
48	0.102	0.413	0.246
72	0.054	0.405	0.133

fig 4.5 a. Changes in the level of free amino acids in endosperm Cmg free aminoacids/endosperm) and embryo C mg free amino acids / embryo) of seeds of common buckwheat during germination vertical line represents LSD at Po. .05.

b. Changes in the ratio fo free amino acids to soluble protien in endosperm and embryo of seeds of common buckwheat during germination. Vertical lines represent LSD at P O. .05.

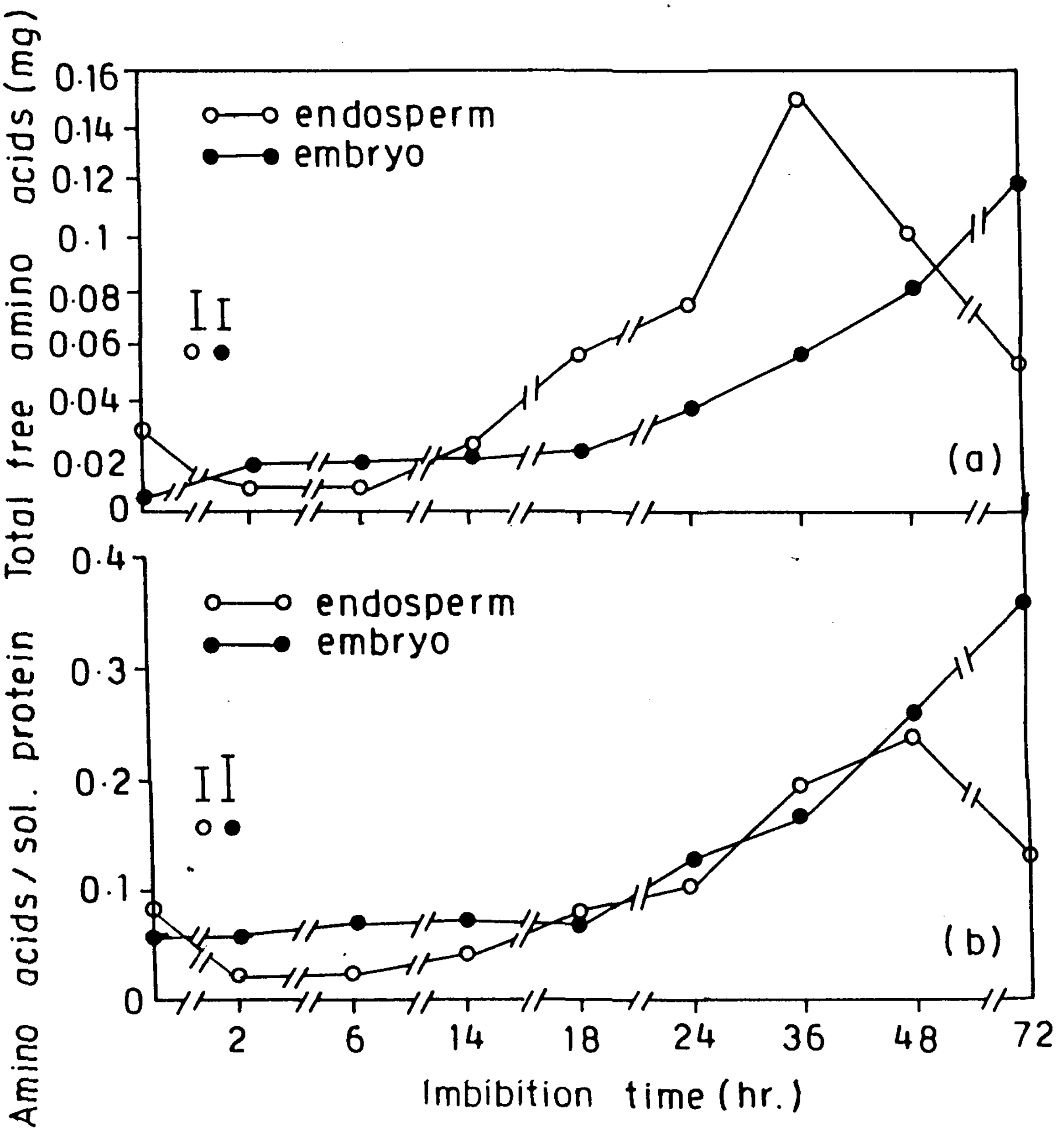

Total free amino acids (mg)
0·16
0·14
0·12
0·1
0·08
0·06
0·04
0·02
0
endosperm
embryo
(a)
Amino acids / sol. protein
0·4
0·3
0·2
0·1
0
endosperm
embryo
(b)
2
6
14
18
24
36
48
72
Imbibition time (hr.)

Table 4.5: Changes in the content of free amino nitrogen, soluble protein and the ratio of free amino nitrogen to soluble protein in the embryo tissues of seeds of common buckwheat (*Fagopyrum esculentum* Moench) during germination upto 72 hours.

Hours after imbibition	free amino nitrogen	total soluble protein	amino nitrogen / soluble protein
	mg per 100 mg fresh weight		
0	0.08	1.287	0.062
2	0.08	1.287	0.062
6	0.08	1.116	0.071
14	0.08	1.116	0.071
18	0.08	1.116	0.071
24	0.12	0.945	0.126
36	0.16	0.945	0.169
48	0.20	0.773	0.258
72	0.28	0.773	0.362
	mg per 100 mg dry weight		
0	0.100	1.608	0.062
2	0.106	1.716	0.062
6	0.106	1.488	0.071
14	0.102	1.434	0.071
18	0.133	1.860	0.071
24	0.288	2.268	0.126
36	0.448	2.646	0.169
48	0.640	2.473	0.258
72	1.190	3.255	0.362
	mg per embryo		
0	0.004	0.064	0.062
2	0.0159	0.257	0.062
6	0.0159	0.223	0.071
14	0.0178	0.251	0.071
18	0.0199	0.279	0.071
24	0.036	0.283	0.126
36	0.056	0.330	0.169
48	0.080	0.309	0.258
72	0.119	0.328	0.362

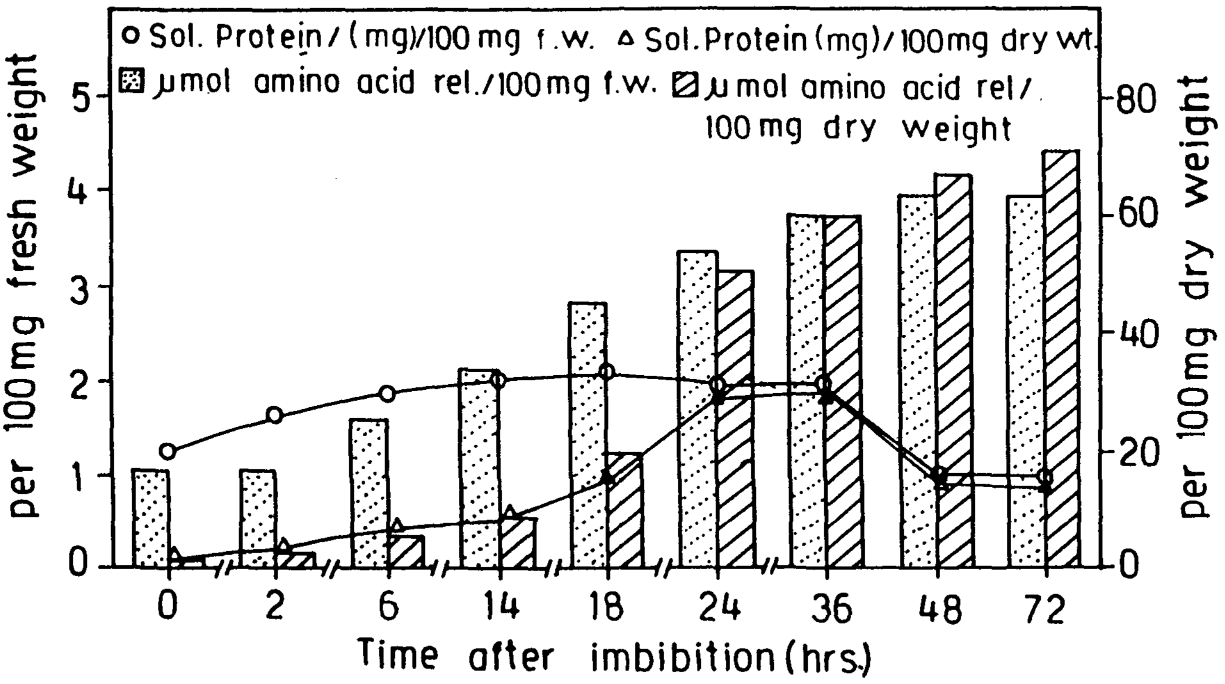
hours of germination. After 24 hours, the electrophoretic mobility of the protein increased with progressing seed germination. The magnitude of increase in the electrophoretic mobility was, however, more marked after 48 hours of incubation (Fig. 4.8a). When subjected to electrphoresis under denaturing conditions, there was no marked difference between the electrophoretic profile of 13S globulin extracted from dormant grains and those allowed to imbibe water for 24 hours. After 24, hours there was a gradual decrease in the width and intensity of bands representing protein subunits 58, 48 and 37 kD molecular weight. During the same period, a protein corresponding to 31 kD molecular weight could be detected as a band on the electrophotogram (Fig. 4.8b).

When analyzed by agar double diffusion test, protein extracted from grains harvested at 24, 48 and 72 hours of imbibition showed positive cross reactivity with antibodies raised against the 280 kD globulin from mature buckwheat grains. However, no precipitin line was observed for proteins extracted from seeds harvested at 96 hours (Fig. 4.9a). However, when tested by the Western immunoblot assay, anti 280 kD activity was detected in proteins extracted from seeds harvested at various stages of germination upto 96 hours. The immunoblot confirmed the changes in the electrohoretic mobility of the protein (Fig. 4.9b)

Irrespective of the reference parameter there was no marked change in the activity of protease, leading to the release of free amino acids, in the endosperm during the initial 18 hours of germination. After 18 hours the activity

FIG. 4.6 a: Changes in the lavel of soluble protien (0) and protease activity ( ) in endosperm expressed as mg protien / 100 mg frwt, and µ mole of amino acid rel./100mg f.w. in endosperm tissue respectively. [ ] represent mg soluble protien/100 mg dry [ ] and [ ] represent protease actively expressed as [ ] mole of amino acid [ ]/100 mg dry wt. in germinating seeds of buckwheat.

FIG 4.6b:Changes in the level of soluble protein (o) and protein and protease activity [ ] in embryo expressed as mg protein/100 mg frwt and µ mole of amino acid [ ]/100 mg f.WL respectively. [ ] represents mg soluble protein/100 mg dry ut. and [ ] represent protease activity expressed as µ mole of amino acid rel/1-- mg dry ut. in germinating seeds of buckwheat.


○ Sol. Protein / (mg)/100 mg f.w.
△ Sol. Protein (mg)/100mg dry wt.
▨ µmol amino acid rel./100mg f.w.
▨ µmol amino acid rel/ 100mg dry weight
per 100mg fresh weight
0
1
2
3
4
5
per 100mg dry weight
0
20
40
60
80
0
2
6
14
18
24
36
48
72
Time after imbibition (hrs.)

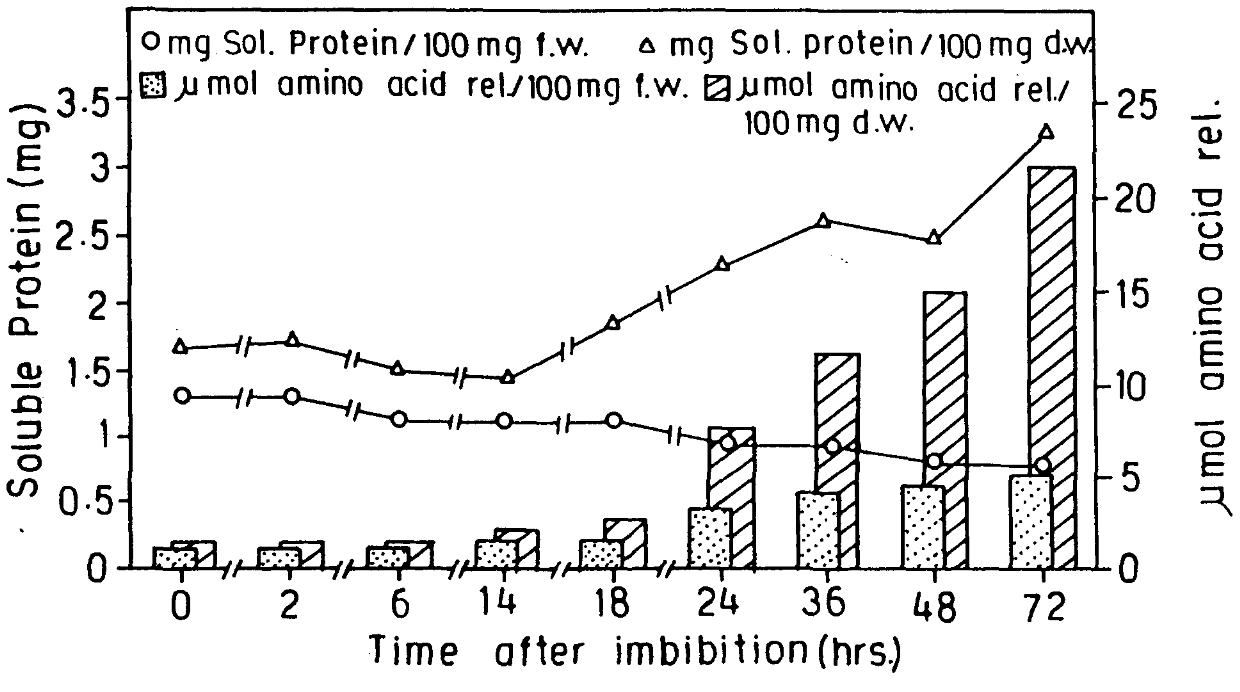
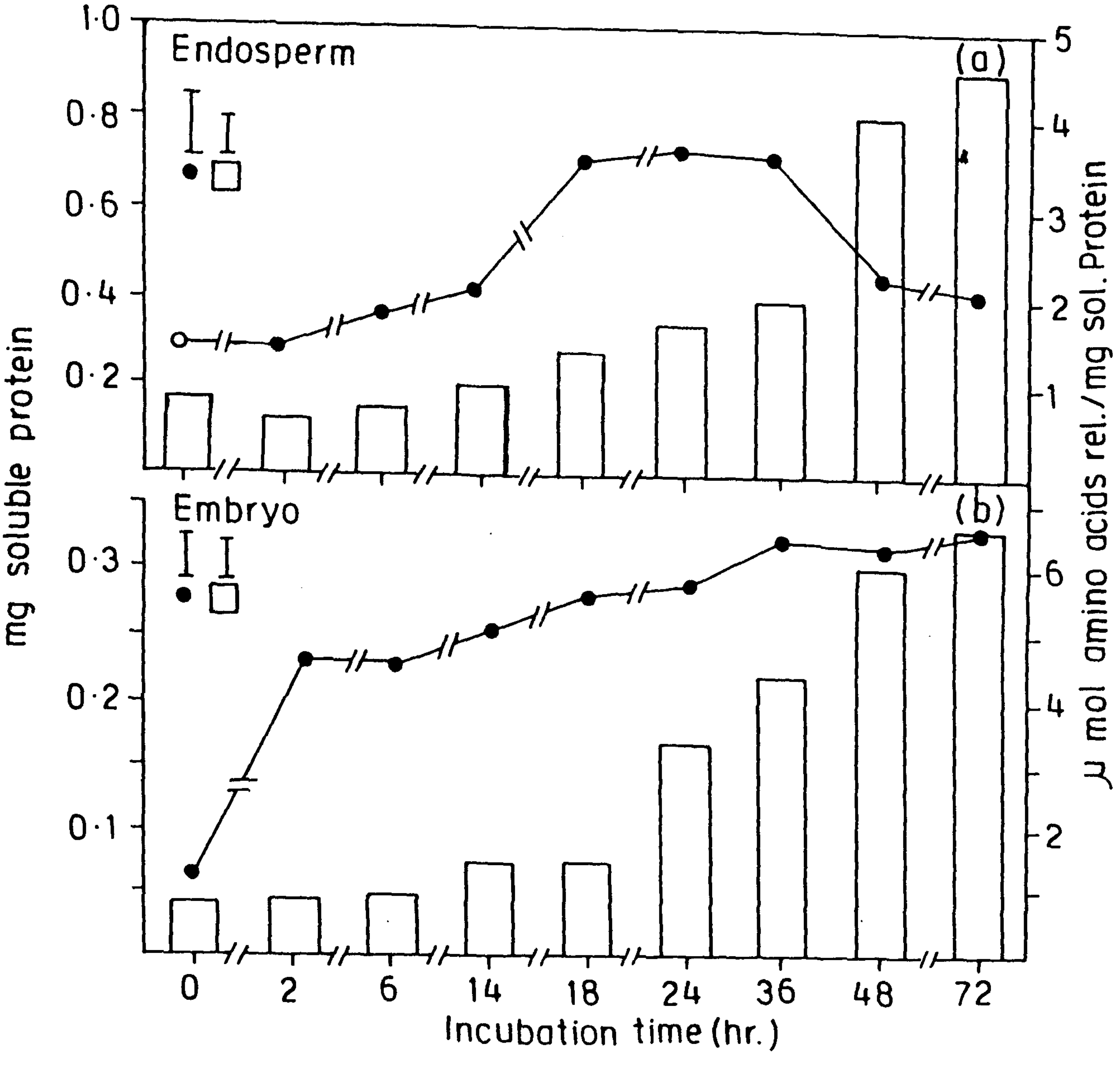

○ mg Sol. Protein/100mg f.w.
△ mg Sol. protein/100mg d.w.
▨ µ mol amino acid rel./100mg f.w.
▨ µmol amino acid rel./ 100mg d.w.
Soluble Protein (mg)
0
0.5
1
1.5
2
2.5
3
3.5
µmol amino acid rel.
0
5
10
15
20
25
0
2
6
14
18
24
36
48
72
Time after imbibition (hrs.)

FIG 4.7 (a) Changes inthe level of soluble protien (.●) expressed as mg soluble protien / endosperm and proteolytic activity (□) expressed as μ mole amino acid released / mg soluble protien/hr in the endosperm of seeds of common buckwheat during germination verticle lines represent LSD at PO .05.



1·0
0·8
0·6
0·4
0·2
Endosperm
(a)
5
4
3
2
1
Embryo
(b)
0·3
0·2
0·1
6
4
2
mg soluble protein
μ mol amino acids rel./mg sol. Protein
0
2
6
14
18
24
36
48
72
Incubation time (hr.)

Table 4.6 Changes in proteolytic activity in the endosperm tissues of seeds of common buckwheat (*Fagopyrum esculentum* Moench) during germination upto 72 hours.

Hours after imbibition	µ mol amino acids released 100 mg fresh weight^{-1}	100 mg dry weight^{-1}	mg soluble protein^{-1}
0	1.065	1.301	0.866
2	1.065	2.485	0.653
6	1.598	5.326	0.860
14	2.098	8.392	1.055
18	2.820	19.747	1.351
24	3.364	50.510	1.729
36	3.730	59.680	1.917
48	3.930	66.836	4.035
72	3.930	70.828	4.358

Table 4.7: Changes in proteolytic activity in the embryo tissues of seeds of common buckwheat (*Fagopyrum esculentum* Moench) during germination upto 72 hours.

Hours after imbibition	µ mol amino acids released 100 mg fresh weight^{-1}	100 mg dry weight^{-1}	mg soluble protein^{-1}
0	1.620	1.820	1.258
2	1.620	1.820	1.258
6	1.620	1.820	1.451
14	1.809	1.915	1.620
18	1.809	2.340	1.620
24	2.989	7.820	3.160
36	3.890	12.860	4.110
48	4.980	15.129	6.110
72	5.029	22.360	6.500

FIG 4.8a:Electro phoretic analysis of the purified 12S globulin at 0, 48, 72 and 96 hours samples of the total globulin previously isolated on the sepharose-6B column from buckwheat seeds. Lane 1 to 4, on 7% PAGE.

FIG 4.8b:Changes in the SDS-PAGE (10%) profile of 13S globulin from seeds of common buckwheat molecular weight markers [] Lane 2, 3, 4, 5, 6, 7, and 8 : seeds imbibed foro, 24, 48, 72 and 96 hours respectively with and without 2-ME..pa

Fig. 4.8

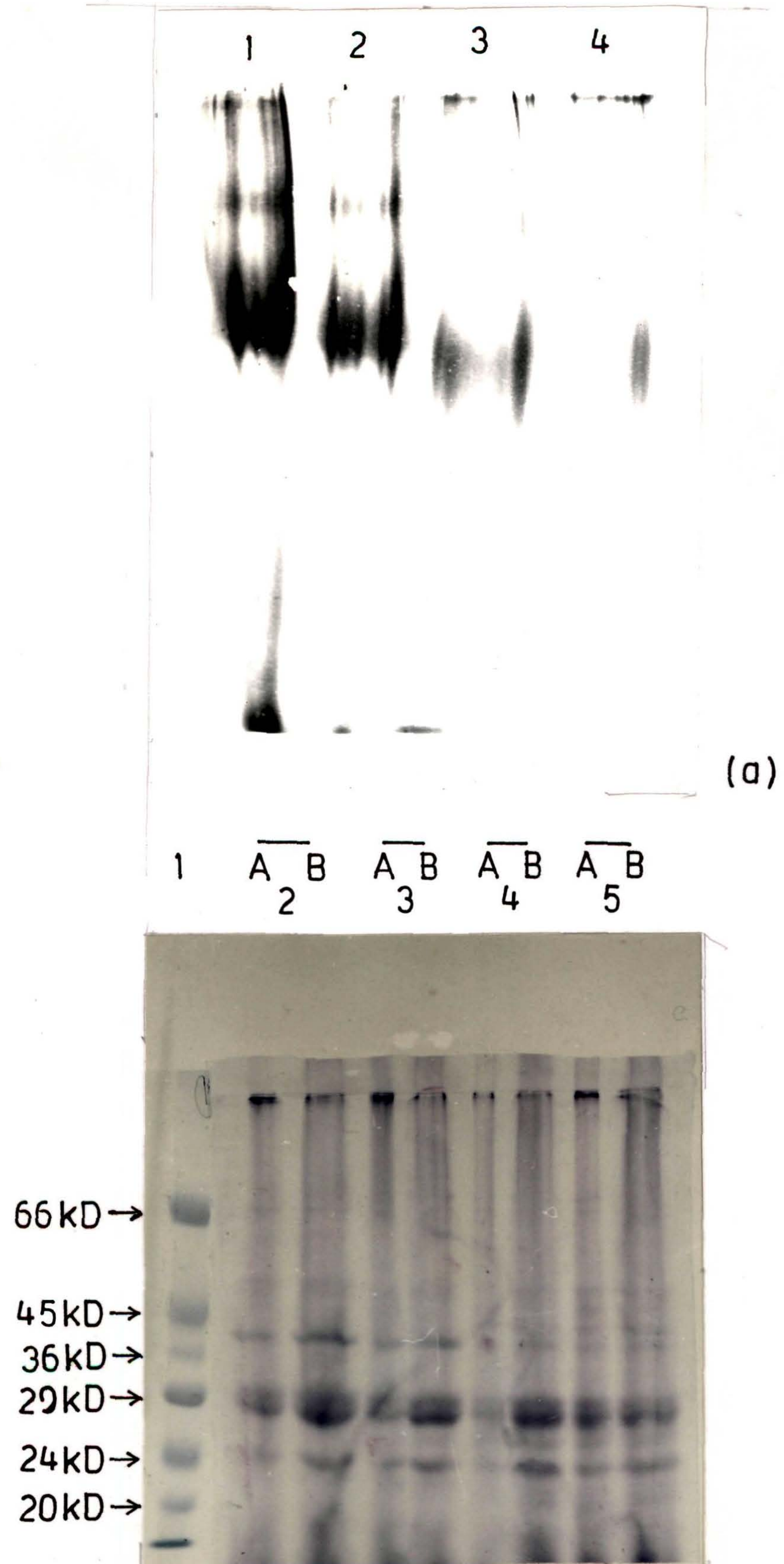
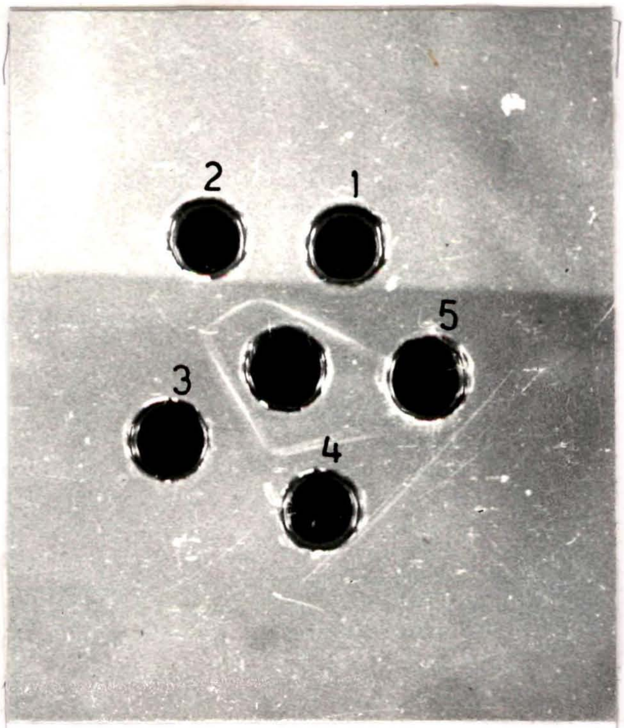


FIG 4.9a : Double immune diffusion in 0.8% agar gel of extracts from dormant buckwheat seeds (a), seeds imbibed for 24 hours (b) and seeds germinated for 45 hours (c), 72 hours (d) and 96 hours (e). Polyclonal antibodies to 135 globulin from dormant seeds were used. The antibodies were placed in the central well.

FIG 4.9b:Western blot of total protien extracts of seeds of common buckwheat with antiserum directed against the 280· [ ] globulin Lane 1 : dry seeds, lanes 2, 3, 4 and 5 seeds imbibed for 24, 48, 72 and 96 hours respectively.

Fig. 4.9

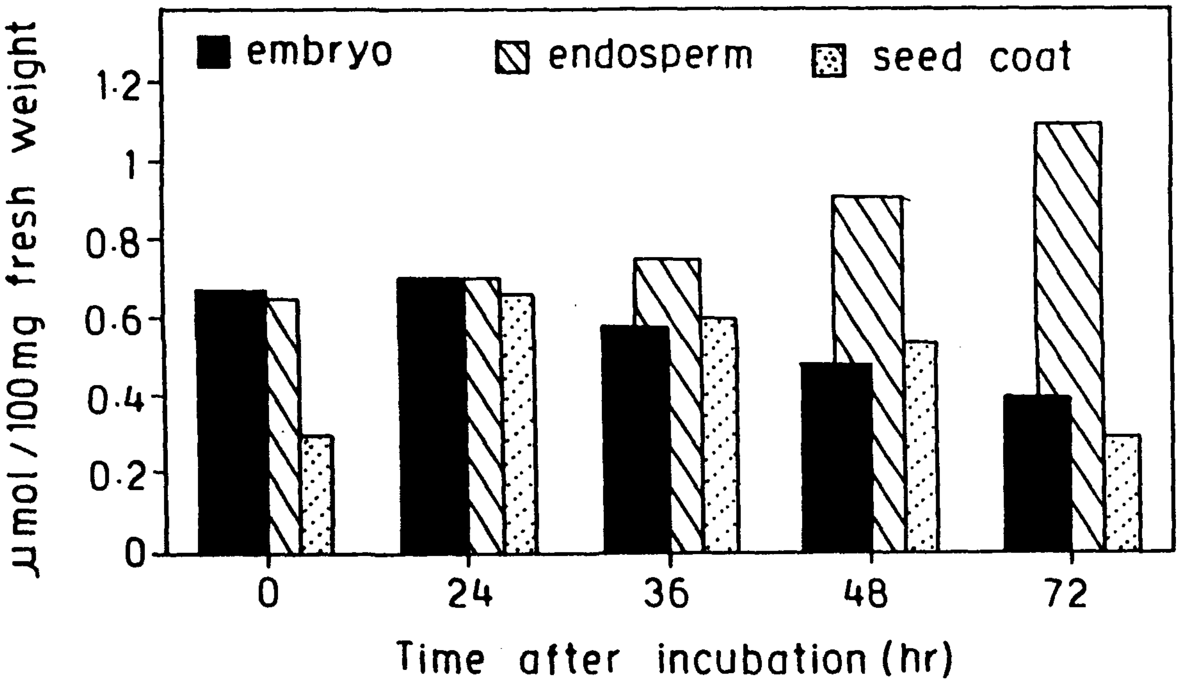


(a)

(b)

FIG 4.10: Changes in total TCA soluble phosphorus in embryo ■ endosperm ▨ and seed coat ▩ expressed as μ mole [ψ]/100 mg fresh weight, at 0.24, 36, 48 and 72 hours of imbibition.

FIG 4.11 :Changes in total TCA insoluble phosphorus in embryo ■ endosperm ▨ and seed coat ▩ expressed as μ mole if/100 mg fresh weight ixt 0,24,36,48 and 72 hours of imbibition.


embryo
endosperm
seed coat
µmol / 100mg fresh weight
0
0·2
0·4
0·6
0·8
1
1·2
0
24
36
48
72
Time after incubation (hr)

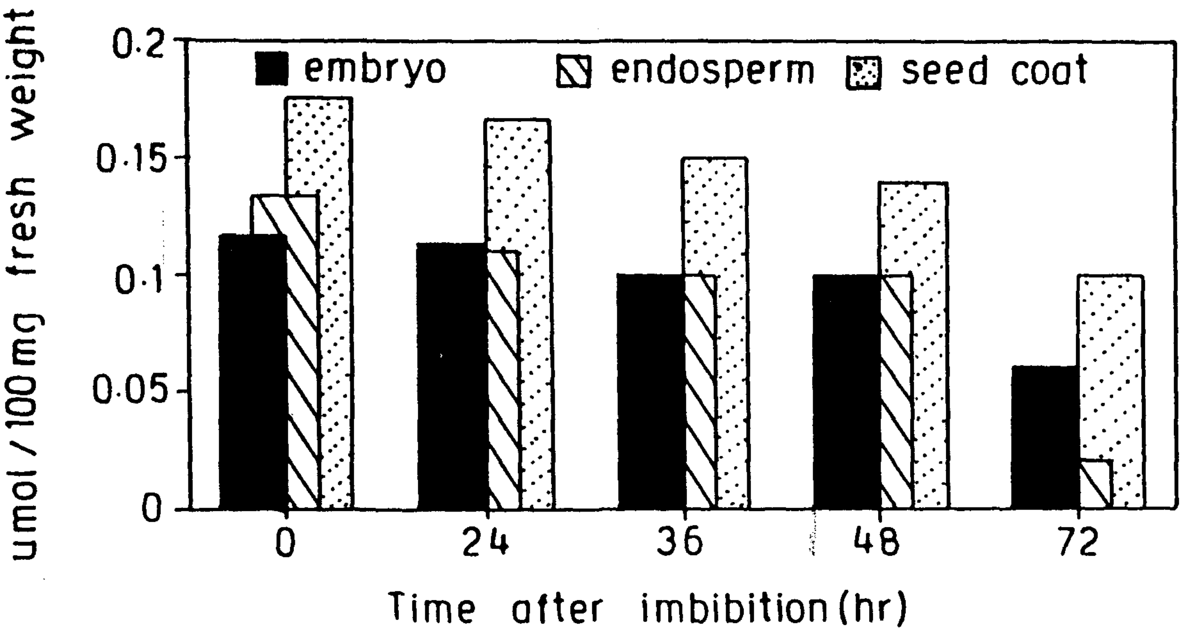

embryo
endosperm
seed coat
µmol / 100mg fresh weight
0
0·05
0·1
0·15
0·2
0
24
36
48
72
Time after imbibition (hr)

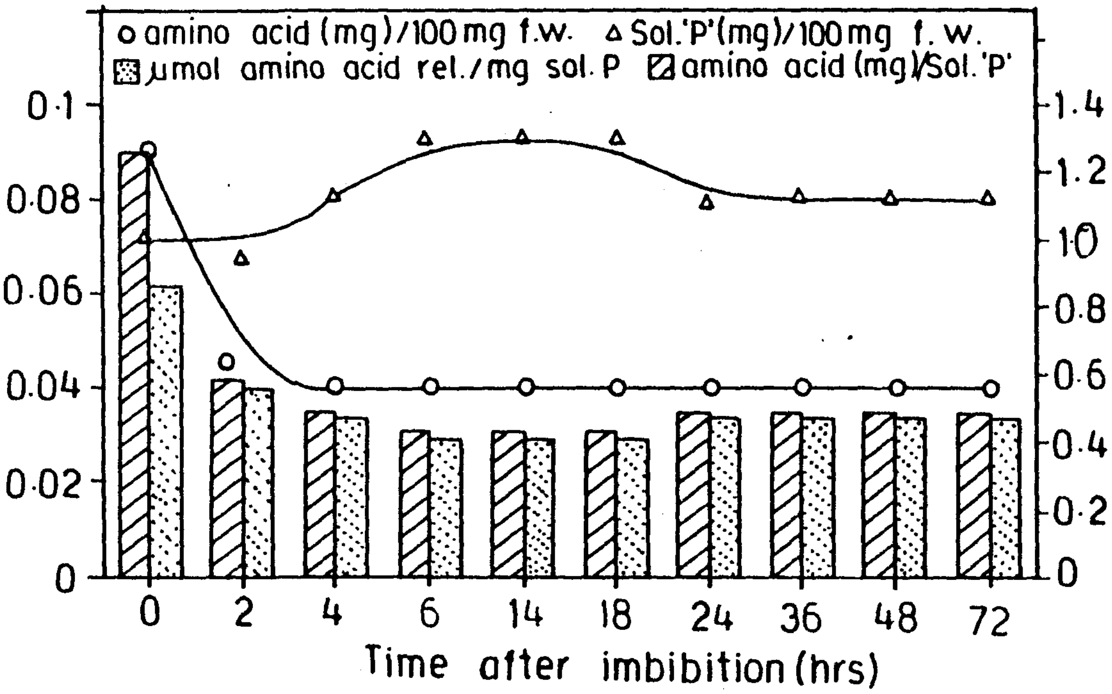
Table 4.8 :Changes in the content of TCA soluble phosphorus in the endosperm, embryo and seed coat of seeds of common buckwheat (Fagopyrum esculentum Moench) during germination upto 72 hours.

Hours after imbibition	mol iP per 100 mg fresh weight		
	endosperm	embryo	seed coat
0	0.65	0.67	0.3
24	0.70	0.68	0.66
36	0.75	0.58	0.60
48	0.91	0.48	0.54
72	1.20	0.40	0.30

Table 4.9:Changes in the content of TCA insoluble phosphorus in the endosperm, embryo and seed coat of seeds of common buckwheat (Fagopyrum esculentum Moench) during germination upto 72 hours.

Hours after imbibition	mol iP per 100 mg fresh weight		
	endosperm	embryo	seed coat
0	0.11	0.11	0.16
24	0.10	0.11	0.153
36	0.09	0.10	0.14
48	0.09	0.10	0.13
72	0.02	0.06	0.11

Fig 4.12



○ amino acid (mg)/100 mg f.w.
△ Sol.'P'(mg)/100 mg f.w.
▩ µmol amino acid rel./mg sol. P
▨ amino acid (mg)/Sol.'P'
0.1
0.08
0.06
0.04
0.02
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
14
18
24
36
48
72
Time after imbibition (hrs)

of the enzyme increased gradually with progressing germination upto 72 hours. During this period a nearly four fold increase in the activity of the enzyme could be observed (table 4.6; Figs. 4.6a, 4.7a). In the embryo, however, .the activity of protease remained stationary during the initial six hours of imbibition after which it showed a progressive increase with seed germination upto 72 hours. During th period there was a nearly 7 fold increase in the activity of the enzyme in the embryo tissues of germinating buckwheat grains (table 4.7; Figs. 4.6b, 4.7b).

Marked changes were observed in the content of various phosphorus fractions in buckwheat seeds guring germination. The content of acid soluble free phosphorus showed significant increase in the endosperm tissues of germinating seeds during the enitre period of germination upto 72 hours. In the embryo, however, the content of free phosphorus showed a a marked decline during the same period. In the seed coat too, the level of acid soluble free phosphorus showed mark decline between 24 and 72 hours of imbibition (table 4.8; Fig. 4.10). In contrast to changes taking place in the level of free phosphorus, the level of TCA insoluble phosphorus showed marked decline in embryo, endosperm and the seed coat tissues of germinating buckwheat seeds. The magnitude of decrease was, however, more marked in the endosperm tissues where it registered a more than five fold decrease during seed germination upto 72 hours (table 4.9; Fig. 4.11).

DISCUSSION:

The physiological and biochemical processes underlying seed germination and early seedling growth are important to the establishment of a plant in its environment. The degradation of storage proteins during seed germination has been studied for a long time, the starting points being the detection of changes in proteolytic activity in seeds. Since then studies on protein degradation have provided a lot of information on the temporal, histochemical, physiological and molecular characteristics of the process, which would eventually lead to the release of free amino acids for transport to the growing axis.

Two important events associated with seed germination are the uptake of water and the initiation of hydrolysis of the storage reserves, the products of which are used by the growing embryo for sustaining its growth. Immediately upon exposure to water the seeds of common buckwheat (*Fagopyrum esculentum* Moench) showed a rapid uptake of water from the ambient environment without any lag phase. Thus, within two hours of incubation, there was a more that 3 fold increase in the moisture content of endosperm. The pattern of changes in the moisture content in the embryo was, however, different from that observed for the endosperm. In the embryo the uptake of moisture showed a lag upto 14 hours, after which the level of moisture started to increase gradually with progressing time. Between 14 and 72 hours, there was a more than 3 fold increase in the moisture content of the embryo. A large number of reports have appeared in literature concerning the uptake of water by seeds during their early stages of germination. Bewely and Black (1978, 1985) have reported a triphasic pattern of uptake of water by seeds of *Allium cepa*, *Daucus carota*, *Impatiens* and *Apium* during germination. While the Ist and 2nd phase of uptake has been ascribed to matric forces the third phase has been reported to be associated with the emergence of radicle. In the present study the uptake of water by the endosperm followed a typical behaviour. While there was a clear distinction between the Ist and 2nd phase with the phase I lasting for 6 hours, phase III could not be distinctly identified from phase II. In the embryo a clear distinction could be made into phase I II and III. Phase I lasted for initial 2 hours and was followed by phase II which lasted between 2 and 14 hours. After 14 hours of incubation there was a sharp increase in the moisture content of the embryo indicating the onset of phase III.

Corresponding with changes in the moisture content, the dry matter content of endosperm in buckwheat seeds showed a rapid decline with germination. In the embryo there was a more than two fold increase in the content of total dry matter during the first 14 hours after which it decreased marginally upto 72 hours of incubation. Our observations on the changes in the dry matter content of the endosperm and the embryo indicate a remobilization of stored reserves from endosperm to the embryo during seed germination. The decrease in the total dry matter content of the embryo during later stages of germination could be ascribed to increased respiratory activity in the tissues during later stages of germination.

Nawa and Asahi (1971) have demonstrated a rapid increase in the mitochondrial respiration in Alaska pea seeds during the first 6 hours of imbibition. In the present study there was no marked change in the respiratory activity during the initial two hours of imbibition after which the rate of respiration showed a progressive increase with seed germination. While a number of studies have revealed a rapid increase in the respiratory activity with incubation time in germinating seeds (Levari, 1960; Kolloffel, 1967; Nawa and Asahi, 1971), our studies reveal an initial lag in mitochondrial respiration during germination of buckwheat seeds. Such a lag could be ascribed to the fact that this period coincides with phase I of water uptake, a period during which the metabolic activity is quite low. Such a lag phase in the respiratory activity has also been described for germination seeds of *Pisum sativum, Phaseolus vulgaris, Zea mays* and *Glycine max* (Bewley and Black, 1983). Bewley and Black (1983) have suggested that the lag phase could perhaps represent a time of temporary anaerobiosis due to restricted oxygen supply by the surrounding structures or the time taken for the development of a secondary respiratory system (i.e. mitochondria with efficiently coupled oxidative phosphorylation); mitochondria present in dry seeds have been suggested to be deficient in cristae.

The degradation of storage proteins and mobilization of the released amino acids from storage tissues to the growing axis is an integral part of the process of seed germination. Our investigations on changes in the pool of free amino acids in the endosperm as well as the embryo of germinating buckwheat seeds reveal a marked decline in the content of free amino acids during the initial 2 hours of imbibition. The initial decline was subsequently followed by a consistent increase in the level of free amino acids with progressing seed germination. However, a four fold increase in the content of free amino acids could be observed in the embryo during the initial 2 hours of imbibition after which the content of free amino acids showed a progressive increase with progressing seed germination. The increase in the content of free amino acids could be a result of degradation of reserve proteins in the storage tissues of the seed leading to the release of free amino acids for sustaining the growth of embryonic axis. An analysis of the changes in the level of free amino acids, soluble protein and the ratio of free amino acids to soluble protein, during the first 6 hours of imbibition by buckwheat seeds, as observed in the present investigation, indicates a utilization of the pre existing free amino acids in the endosperm for the synthesis of soluble proteins in the embryo. While Nawa and Asahi (1973) have shown that cotyledons of germinating pea seeds were capable of incorporating ^{3}H-leucine within 6 hours of the start of imbibition. Similar observations had been reported earlier by Maherchandani and Naylor (1972) for aleurone tissues of *Avena fatua* seeds. However, in this case the tissues were shown to be capable of incorporating amino acids within 10 minutes of imbibition. They concluded that the tissues had an innate capacity for protein synthesis and that all the requirements for this synthesis were present in the dry mature seed tissues. Changes in the level of free amino acids and solub- protein in the embryo of germinating buckwheat seeds observed in the present investigation indicate the existence of such a capacity in embryos of buckwheat seeds also.

As determined by western blot assay using antibodies raised against the 280 kD globulin from mature buckwheat grains, there was no marked change in the electrophoretic mobility of the main storage protein of buckwheat grains during the initial 24 hours of germination. After 24 hours, the electrophoretic mobility of the protein showed a significant increase with progressing seed germination. The magnitude of increase in the electrophoretic mobility was, however, mo marked after 48 hours of incubation. Under denaturing conditions too, no marked differences in the electrophoretic profile of the protein could be observed upto 24 hours of imbibition. After 24 hours, however, there was a gradual decrease in the width and intensity of bands representing some high molecular weight subunits of the main storage protein. These results indicate that during early stages of seed germination, the main seed storage protein in common buckwheat undergoes partial cleavage leading to the release of some low molecular weight subunits. These results are in agreement with the observations of Dunaevsky and Belozers (1989). They have observed that the protein retained its structural integrity upto 3 days following imbibition. Results of the western blot assay obtained in the present investigation, however, clearly establish that the 280 kD globulin of buckwheat seeds retained its antigenic identity even upto 96 hours of imbibition. Till this time the process of radicle and plumule emergence is nearly complete. It can therefore be assumed that the *in vivo* proteolysis of the 280 kD globulin during seeds germination in common buckwheat is not completed even upto 96 hours of germination and probably the basic elements responsible for the structural integrity of the protein are not affected during this period. Similar observations have been reported for ß-conglycinins of so bean (Bryant *et al.*, 1995).

During grain development the basic storage reserves (carbohydrates, lipids and proteins) are laid down in all parts of the grain or seed. After germination, hydrolysis of these reserves is seen first in the cotyledon and then in the endosperms (Jacobsen *et al.*, 1979). In the present study a low level of proteolytic activity could be detected even in ungerminated seeds of common buckwheat. There was no marked increase in proteolytic activity in the endosperm tissues during the initial 6 hours of imbibition. After 6 hours, however, activity of the enzyme showed a nearly four fold increase with progressing seed germination upto 72 hours. In the embryo, protease activity remained stationary during the initial two hours of imbibition. Between 2 and 72 hours, proteolytic activity in the embryo showed a progressive increase with seed germination, registering a nearly 7 fold increase during the period.

According to the available data there are two possible pathways for the degradation of storage proteins during seed germination. In legume seeds a limited proteolysis of the storage proteins has been reported to start a few days after the beginning of seedling growth (Bewely and Black, 1985; Shutov and Vaintraub, 1987). In contrast, Hara and Matsubara (1980) have shown that limited proteolysis of the main storage protein in germinating pumpkin seeds starts almost simultaneously with the onset of seedling growth. Buckwheat seeds too showed a slight increase in proteolytic activity immediately upon imbibition of water. However, marked increases in the activity of the enzyme occurred only after 18 hours of imbibition. These results are in agreement with the observations of Fujimiki *et al.* (1977) and Hay *et al.* (1991) who have also reported a low level of general protease activity in endosperm of germinating maize seeds during the initial 48 hours of imbibition followed by a sharp increase in the activity of the enzyme with progressing seed germination.

Considered together with changes taking place in the main seed storage protein and the levels of proteolytic activity in buckwheat seeds during the process of germination, our results indicate that the 280 kD globulin may not be the source of amino acids that are utilized by the growing embryo during early stages of germination. Contributions towards this pool as a result of proteolytic cleavage of the main storage protein presumably start much later. It is possible that the source of amino acids required for sustaining the growth of embryonal axis during the early stages of buckwheat seed germination may either lie in the aleurone tissues or in the pre-existing pool of free amino acids in the endosperm of the seed.

Shutov and Vaintraub (1987) have observed that the onset of degradation of reserve material in the seeds is not directly related to uptake of water and increase in the metabolic activity in the seeds during germination. They have suggested that the onset of degradation of stored materials is to a large extent determined by other processes taking place in the seed. They ahve implicated a positive role of embryonal axis in the degradation of storage proteins in germinating seeds. In the present investigation too, there was no marked increase in the level of soluble proteins and free amino acids in the endosperm of seeds from which t' embryonal axis had been removed. Endosperm tissues of such seeds showed a marked drop in the level of free amino acids during the initial two hours of imbibition after which the level of amino acids remained stationary (Fig. 4.12). These results indicate a marked suppression of protein degradation, mos probably due to the removal of the sink effect of the embryonal axis.

CHAPTER V

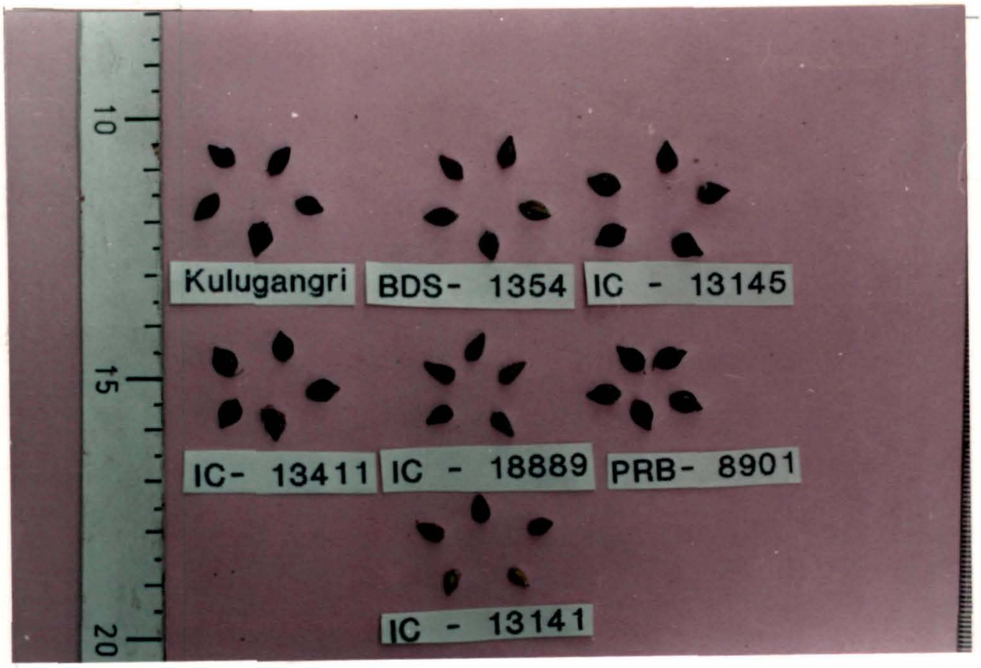
EXPERIMENTAL:

Grains of common buckwheat (*Fagopyrum esculentum* Moench) were procured from cultivators fields in the Western Himalayas and the North Eastern Regional Station of the National Bureau of Plant Genetic Resources, Shillong. Healthy seeds from the collection were screened out and used for the present study. The hull portion of the seeds was removed and the groat powdered in a ball mill. The powdered groat was defatted by extraction with petroleum ether and then used for protein fractionation into albumins, globulins, prolamins and glutelins. The freshly harvested seeds were also fixed in glutaraldehyde for electron microscopic examination of protein bodies after ultra microtomy. Protein bodies were also extracted from the freshly harvested seeds by the aqueous extraction protocol. The protein bodies were stained with

PLATE

A

B


10
15
20
Kulugangri
BDS- 1354
IC - 13145
IC- 13411
IC - 18889
PRB- 8901
IC - 13141

phosphotungstic acid and visualized under the electron microscope.

For the isolation and purification of the seed storage protein, 50% (w/v) extracts of the dehulled seeds was made in 50 mM Tris-Cl buffer (pH 8.0). The extract after centrifugation was used for protein purification using combinations of ammonium sulphate precipitation and gel filtration techniques. The purified protein was analyzed for its secondary structure and amino acid sequence using CD spectral analysis and automated amino acid analyzer respectively.

For the determination of antigenic homology of the protein with seed storage proteins from other crops, antibodies to the purified protein were raised in rabbit. The antiserum collected after the last booster doze was used for the determination of antigenicity of proteins using the Ouchterlony agar double diffusion test.

RESULTS:

Grains of common buckwheat (*Fagopyrum esculentum* Moench), obtained from the Western Himalayas and the North Eastern Regional Station of the National Bureau of Plant Genetic Resources were analyzed for grain protein quality and quantity. When expressed on dry weight basis, the grains had a more than 16 percent protein content. The grain storage proteins were fractionated into albumins, globulins, prolamins and glutelins. When expressed as percent of dry weight, the grains had 8.06 percent globulins, 2.42 percent albumins, 1.2 percent prolamins and 1.4 glutelins. Residual proteins constituted 2.2% of the total grain dry weight (table 5.1).

Thus, the globulin fraction constituted about more than 50 percent of the total protein content of the grains. Albumins, prolamins, glutelins and the residual proteins constituted 15.8, 7.83, 9.27 and 14.43 percent of the total protein content of grains, respectively (table 5.1).

The main storage protein of common buckwheat is present within protein bodies located in the endosperm tissues of the grains (Fig. 5.1). Protein bodies were isolated by the aqueous extraction protocol. The protein bodies were intact spherical in shape and ranged in diameter form 5-8 μm. The protein bodies were composed of an amorphous protein matrix with a single globoid inclusion. The amorphous proteinaceous matrix was in turn enclosed by a single membrane (Fig. 5.2). Electron microscopic examination of the isolated protein bodies of buckwheat grains revealed the presence of a membrane like boundary layer surrounding the globoid inclusion.

When expressed as percent of protein body dry weight, the protein bodies of buckwheat grains had a 70 percent protein content. Phytins and carbohydrate constituted 25 percent and 2.7 percent of the protein body on dry weight (table 5.2).

Among the large number of buffers used to extract total seed proteins of dicotyledonous plants, the buffer used in our study was always found to give reproducible results. The defatted meal of dehulled buckwheat grains was extracted with 50 mM Tris-Cl buffer (pH 8.0) containing 0.1 M NaCl, 5% polyvinyl pyrrolidone (PVP) and 2% phenyl methyl sulphonyl fluoride (PMSF). The extract was brought to 80 percent

Table 5.1: The content of albumins ,globuilns ,prolamins and glutelins and residual protein in grains of common buckwheat (*Fagopyrum esculentum* Moench.) expressed as percent of grain dry weight as well as percent of total grain protein.

Protein fraction	mg per 100mg grain dry weight	mg per 100 mg total grain protein
Albumins	2.42	15.80
Globulins	8.06	52.64
Prolamins	1.20	7.83
Glutelins	1.42	9.27
Residual	2.21	14.43

Table 5.2: Partial chemical composition of the protein bodies isolated from mature grains of common buckwheat (*Fagopyrum esculentum* Moench.) expressed as percent of protein body dry weight.

Constituent	mg per 100 mg protein body dry weight
Protein	70.00
Phytin	25.00
Carbohydrate	2.72

fig.5.1: Electronmicrograph of an ultrathin section of buckwheat endosperm from mature grains, stained with uranyl acetate and lead citrate showing the cellulr localization of protein bodies.

Fig. 5.1

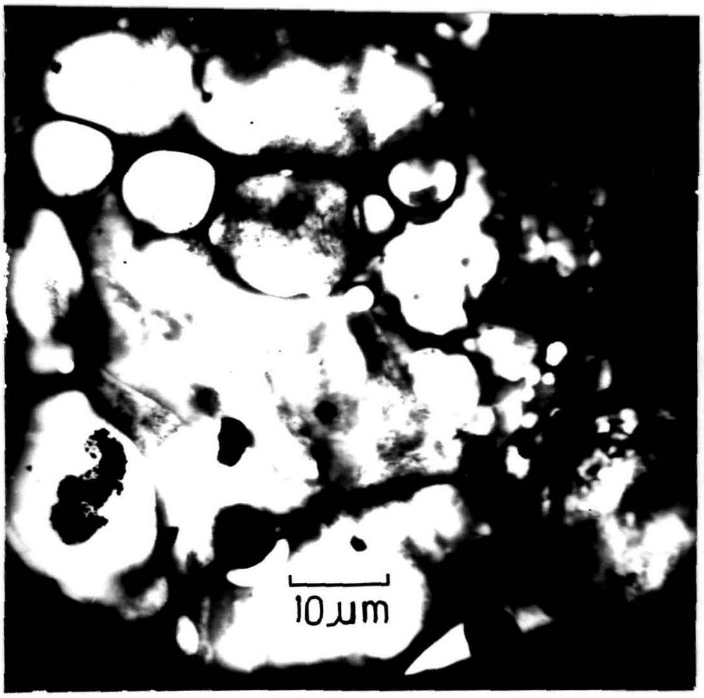
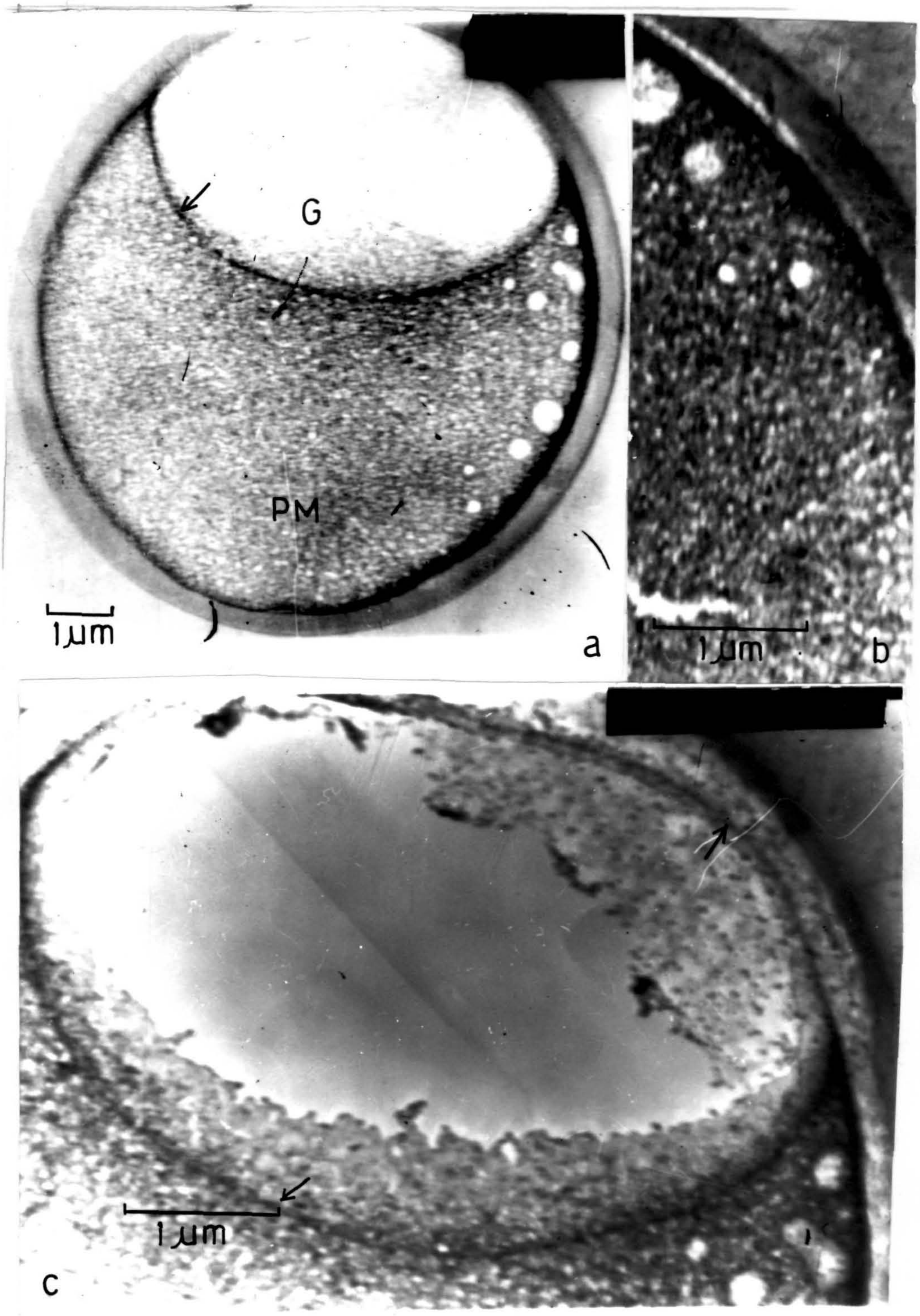


fig.5.2: (a) A single isolated protein body showing the protein matrix (PM) and the phytin globoid(G); (b) a magnified view of a protion of PM showing the electron dense amorphous protein matrix and the protein boundry membrane, (c) an enlarged view of the phytin globoid (G). Note the presence of a membrane like boundary (arrow) sorrounding the phytin globoid.

Fig. 5.2



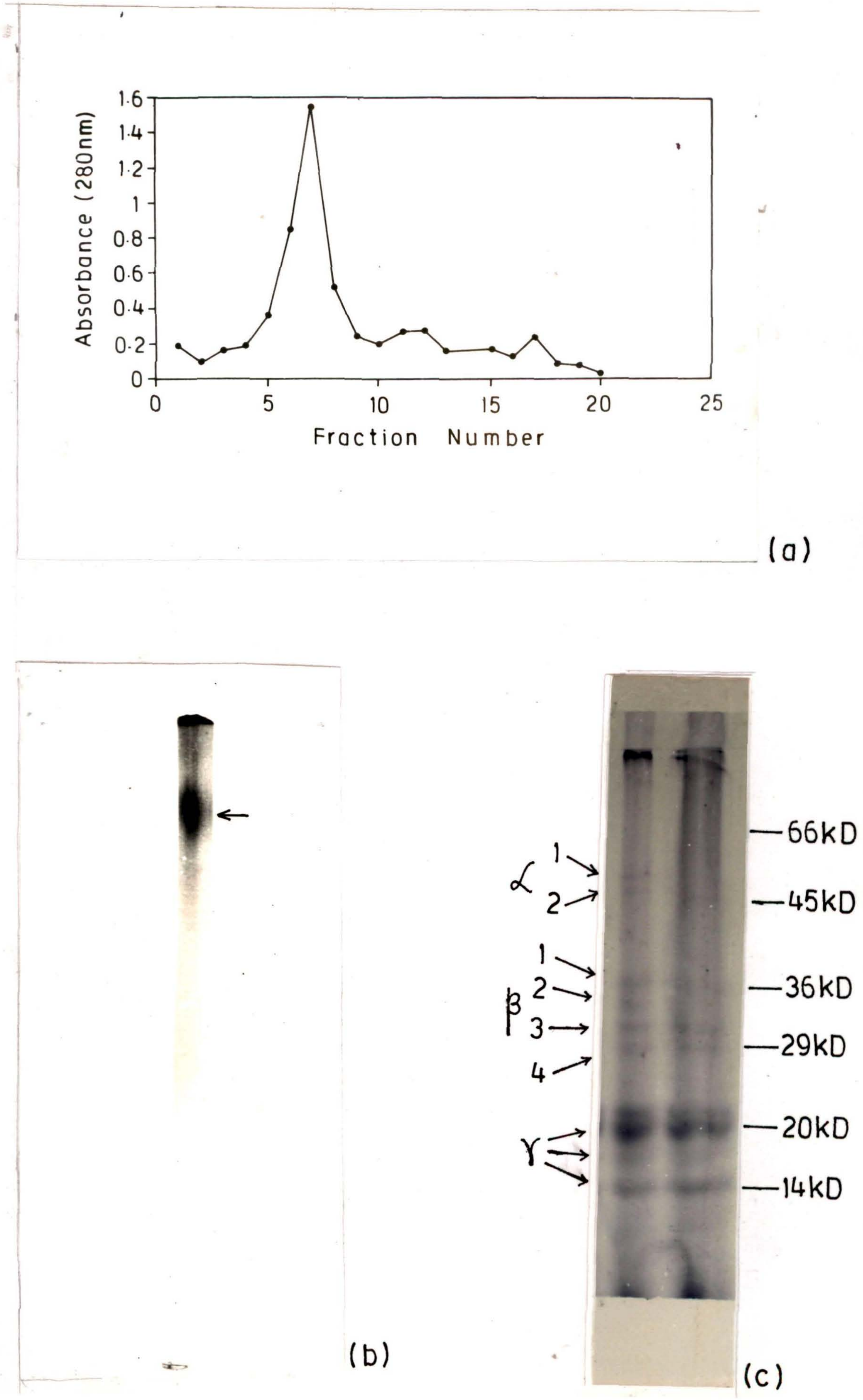
saturation level of ammonium sulfate and then eluted on a Sepharose 6B column with 50 mM Tris-Cl buffer (pH 8.0). The elution pattern of the ammonium sulphate fractionated and dialyzed extract on Sepharose 6B column is shown in Fig. 5.3a. The extract could be fractionated into two peaks. Of these the major peak corresponded to fractions numbers 6, 7, 8; the other peak corresponded to fraction numbers 15, 16 and 17. 13S globulin, the major storage protein in grains· of common buckwheat (*Fagopyrum esculentum* Moench) was eluted in a single peak corresponding to fraction numbers 6,7 and 8 and a native molecular mass of approximately 280 kD (Fig. 5.3a). The fractions were pooled together and lypholysed. When subjected to electrophoresis on a 7.5% acrylamide gel, the protein resolved into a single band (Fig. 5.3b). Analysis of the subunit composition of the 13S globulin under reducing and non-reducing conditions on SDS-PAGE revealed that the protein was composed of 3 main groups of subunits designated as α, ß and Γ. The polypeptides of α group ranged in molecular mass from 55-60 kD. The polypeptides of the ß .and the Γ groups had molecular weights ranging between 32-44 and 16-29 kD respectively (Fig. 5.3c). Polypeptides belonging to the α group could not be detected on the electrophoretogram when the protein was subjected to electrophoresis under reducing conditions. Correspondingly there was an increase in the intensity of bands representing polypeptides of the ß and Γ groups (Fig. 5.3c).

The 280 kD globulin isolated from buckwheat grains had a 5.7 percent lysine, 2.4 percent tryptophan, 1.8% methionine fig.5.3:(a) Elution profile of the ammonium sulphate fractionated crude protein from grains of common buckwheat from Spharo 6B column.

(b) Polyacrylamide gel electrophoresis of the pooled peak A fractions eluted from sepharose 6 B column.

(c) SDS-PAGE profile under non-reducing conditions (lane 1) and redcing conditons (lane 2) of the purified 13 s globulin from grais of common buckwheat.

Fig. 5.3


Absorbance (280nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Fraction Number
(a)
(b)
α
1
2
β
1
2
3
4
γ
66kD
45kD
36kD
29kD
20kD
14kD
(c)

and 5.8 percent leucine content (table 5.3).

Figure 5.4a shows the immunoprecipitation of the 280 kD globulin of buckwheat grains with antibodies raised against the protein in rabbit. A single precipitin line obtained against the antiserum indicated the antigenic homogeneity of the purified protein. In order to study the accumulation pattern of the 280 kD globulin in buckwheat grains during their development, grain development in buckwheat was classified into 5 stages *viz.* 1. milky (10 DAF), 2. milk mature (20 DAF), 3. early mature (30 DAF), 4. mature (40 DAF) and 5. late mature (45 DAF). From each collection total proteins were extracted and analyzed for anti 280 kD activity by agarose double immunodiffusion. No cross reactivity was detected in extracts from grains harvested at milky (10 DAF), milk mature (20 DAF) and early mature (30 DAF) stages of development. Extracts of grains harvested at mid maturation stage of development showed strong cross reactivity with antibodies raised against the 280 kD globulin from mature buckwheat grains. The sharp precipitin line indicated the presence of the protein in grains at this stage (Fig. 5.4b). Correspondingly developing buckwheat grains accumulated most of their dry matter between milky and early mature stages. During this period there was a nearly 4 fold increase in the grain dry weight (table 5.4; Fig. 5.5).

When tested for antigenic homology with seed storage proteins of other crops by agar immunodiffussion test, the 280 kD globulin from buckwheat grains showed antigenic homology with the legumin group of seeds storage proteins. Anti-

Table 5.3: Percentage of some essential amino acids in the 280 kD globulin isolated from mature grains of common buckwheat (*Fagopyrum esculentum* Moench) compared with the values of same amino acids for grain amaranth, chenopodium, cow milk casein and the WHO recommended values of amino acids for a nutirinationlly balanced protein

Amino acid	mole %				
	1	2	3	4	5
Lysine	5.9	5.0	4.9	6.7	5.5
Methionine	1.8	4.4	2.8	2.1	3.5
Tryptophan	2.4	1.4	ND	0.9	1.0
leucine	5.8	4.7	3.0	8.2	7.0

[1] 280 kD globulin from buckwheat grains ; [2] grain amaranth; [3] chenopodium ; [4] cow milk casein ; [5] WHO recommended values

source: 1: present study, 2: Raina & Datta (1992), 3: Dey and Mandal (1993), 4:cow milk casein (Gordon *et al*. (1949); 5: WHO recommended values.

Table5.4: Changes in the fresh and dry weight of seeds of common buckwheat (*Fagopyrum esculentum* Moench) during various stages of seed development.

Stage	fresh weight (gm) per 100 seeds	dry weight (gm) per 100 seeds
I (10 DAF)	2.38	0.714
II (20 DAF)	3.57	1.428
III (30 DAF)	4.545	2.181
IV (45 DAF)	6.25	3.125

fig 5.4a: Ouchterlony double diffusion test with undilulted ntiserum raised against purified 13s globulin of buckwheat grains showing a single precipitin line.

fig.5.4b:Ouchterlony doubel diffusion with undiluted antiserum raised aginst purified 13s buckwheat globulin in the central well and extracts from buckwheat grains at different stages of maturity in the sorrounding wells. 1:milky stage; 2: milk mature stage; 3:early mature stage; 4:mid mature stge; 5:late mature stage.

Fig. 5.4

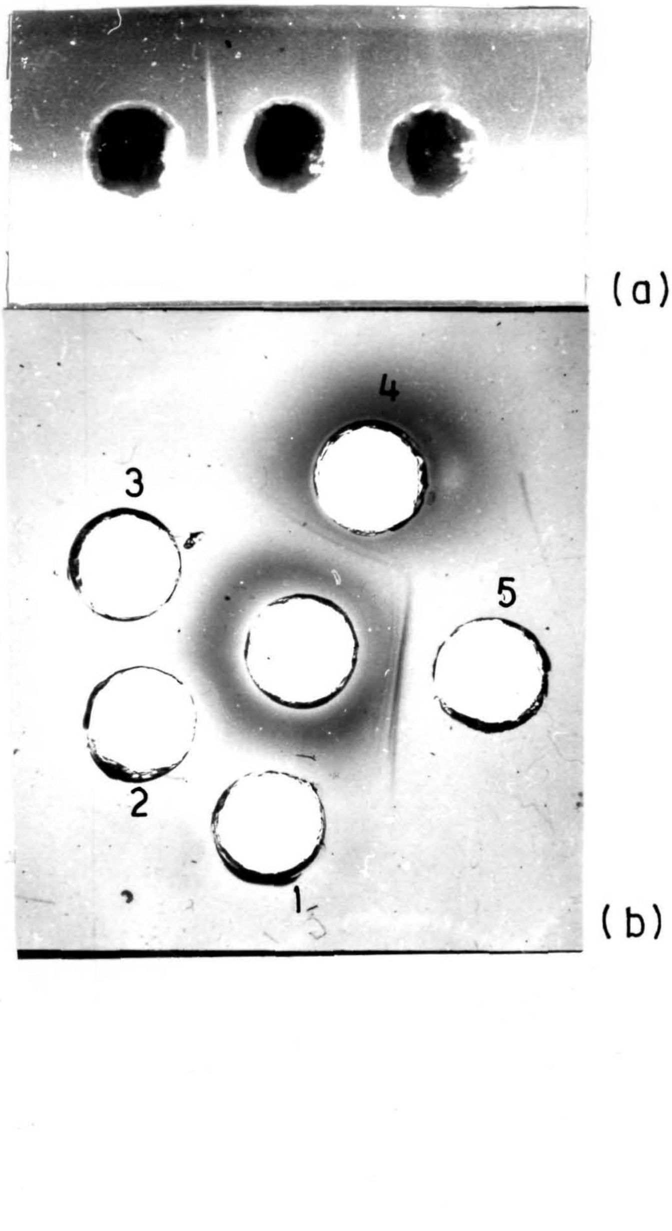
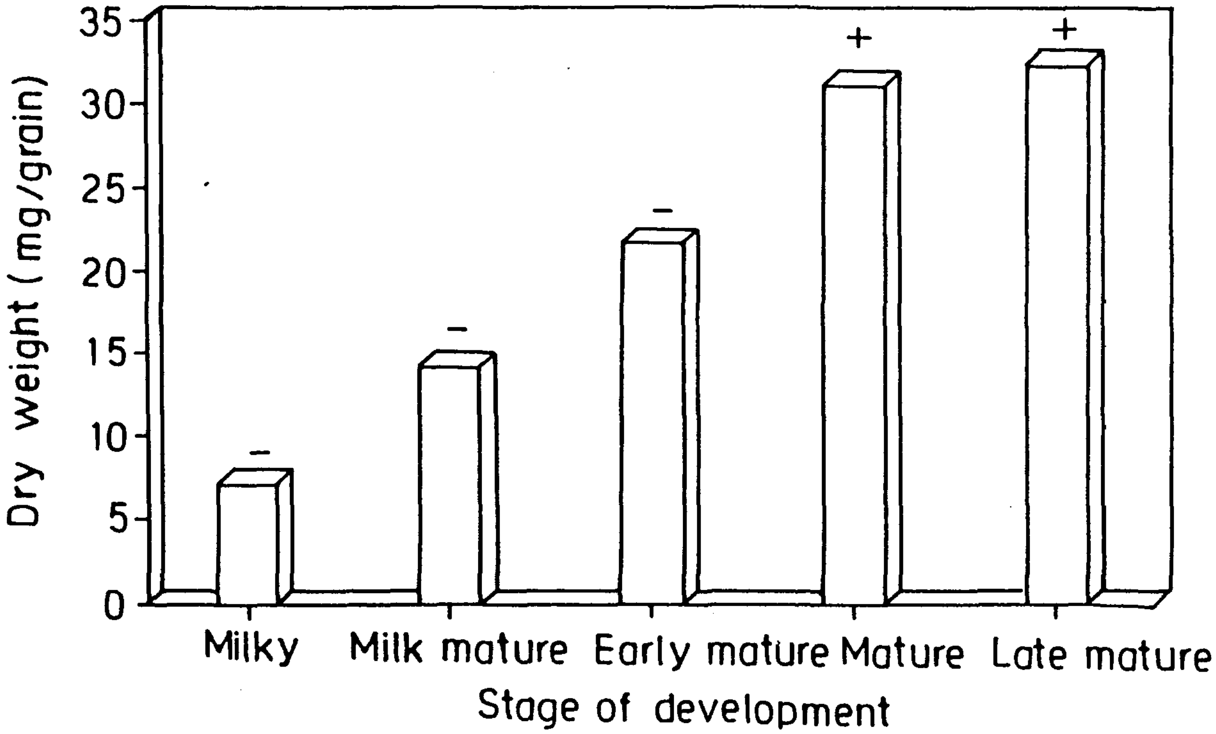


fig.5.5:Changes inthe total dry weight (mg) of seeds of common buckwheat (*Fagopyrum esculentum* Moench) during various stages of development.


Dry weight (mg/grain)
35
30
25
20
15
10
5
0
−
−
−
+
+
Milky
Milk mature
Early mature
Mature
Late mature
Stage of development

bodies raised against the 280 kD globulin from grains of common buckwheat cross reacted positively with the 11-12S proteins form soya bean, mung bean and pea. However no such cross reactivity could be detected for storage proteins fr broad bean, wheat, rice and grain amaranth (Fig. 5.6a,b).

A comparison of the globulin content in the protein bodies of buckwheat with some other crops and the extent of antigenic homology of the 280 kD buckwheat globulin with storage proteins of some important crops is presented in table 5.5. When compared with storage proteins from wheat, rice, soya bean, mung bean, pea, broad bean and grain amaranth, the 280 kD globulin from buckwheat seeds showed the highest antigenic homology with soya bean glycinin. The intensity of the precipitin line was relatively low for pea globulin. No serological homology could be detected with prolamins from wheat, glutelins from rice, globulins from broad bean and albumins from grain amaranth.

Fig. 5.7 depicts the fluorescence emission spectra of the partially purified 280 kD globulin from buckwheat grains. The maxima for excitation wavelengths of 275 and 295 nm centered at 317 and 325 nm respectively. The two excitation maxima are typical of a tryptophan containing protein.

In order to determine which of the subunits of the 280 kD protein had the highest lysine level, the protein was subjected to gel filtration on Sephadex G-120 after reduction and alkalization. The elution profile of the protein is shown in Fig. 5.8. The protein resolved into 3 peaks designated A, B and peak C. The contents of each peak were pooled to-fig.5.6:Ouchterlony double diffusion with undiluted antiserum raised against purified 13S globulin of grains of common buckwheat in the central well and crude protein extracts from grains/seeds of different crps in .the sorrounding wells. (a) 1, buckwheat; 2,wheat;3, rice; 4, kidney ben; 5, soyabean; (b) 1, buckwheat; 6, mung bean; 7, broad bean; 8, pea; 9, french bean and 10, grain amaranth.

Fig. 5.6

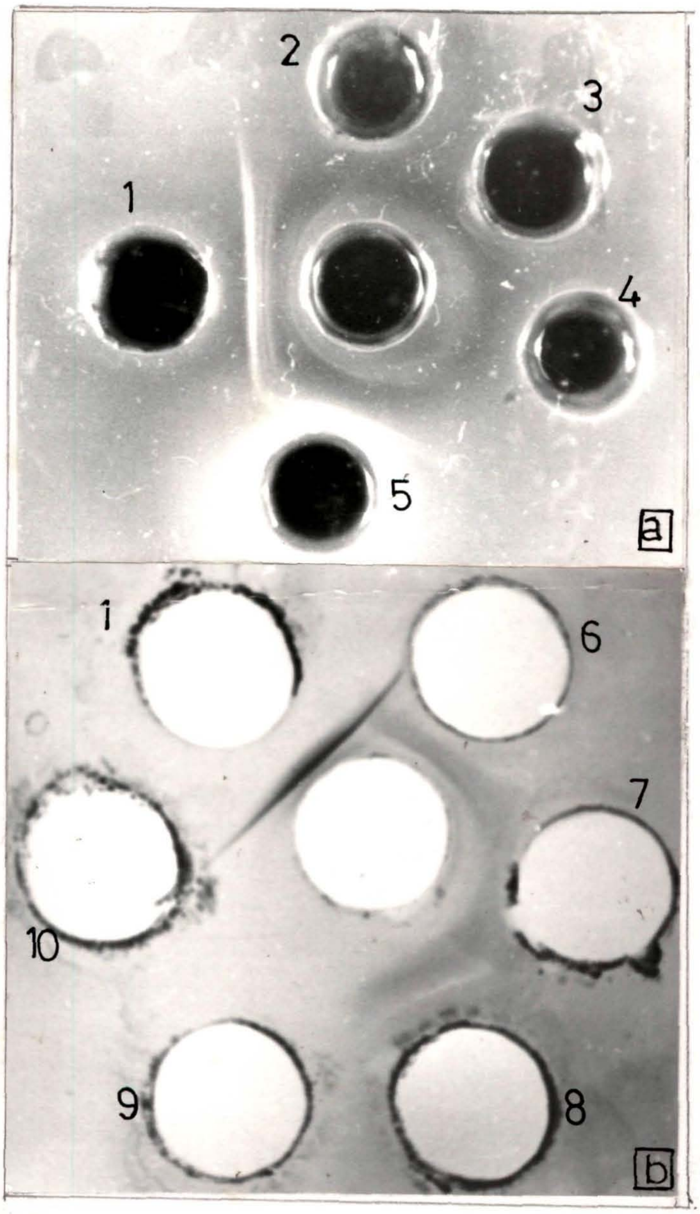
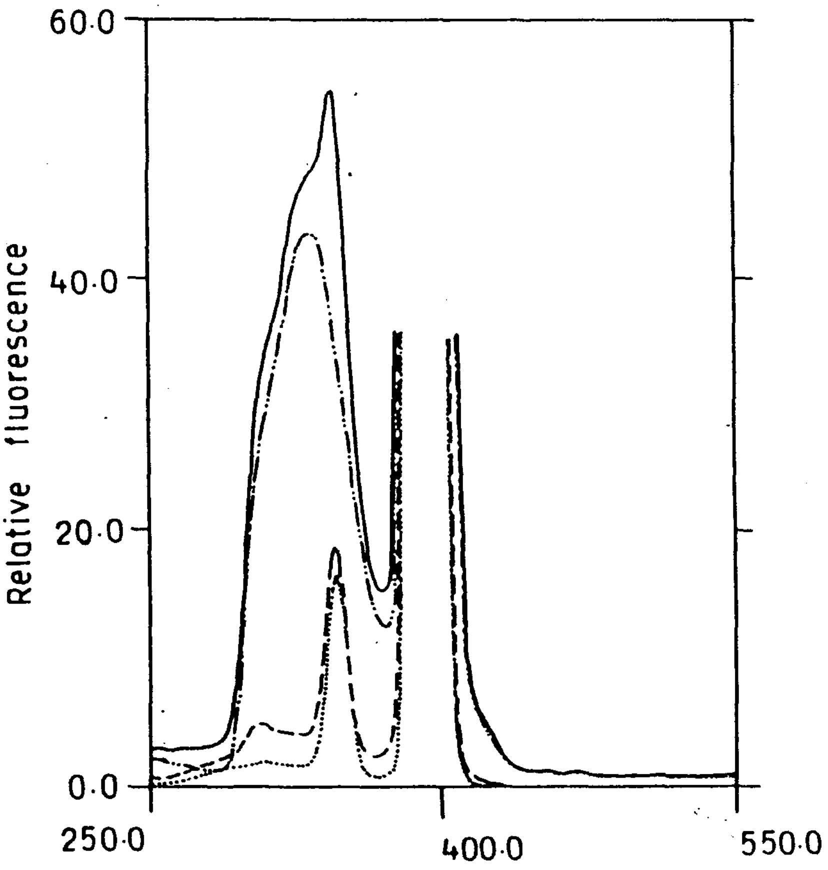
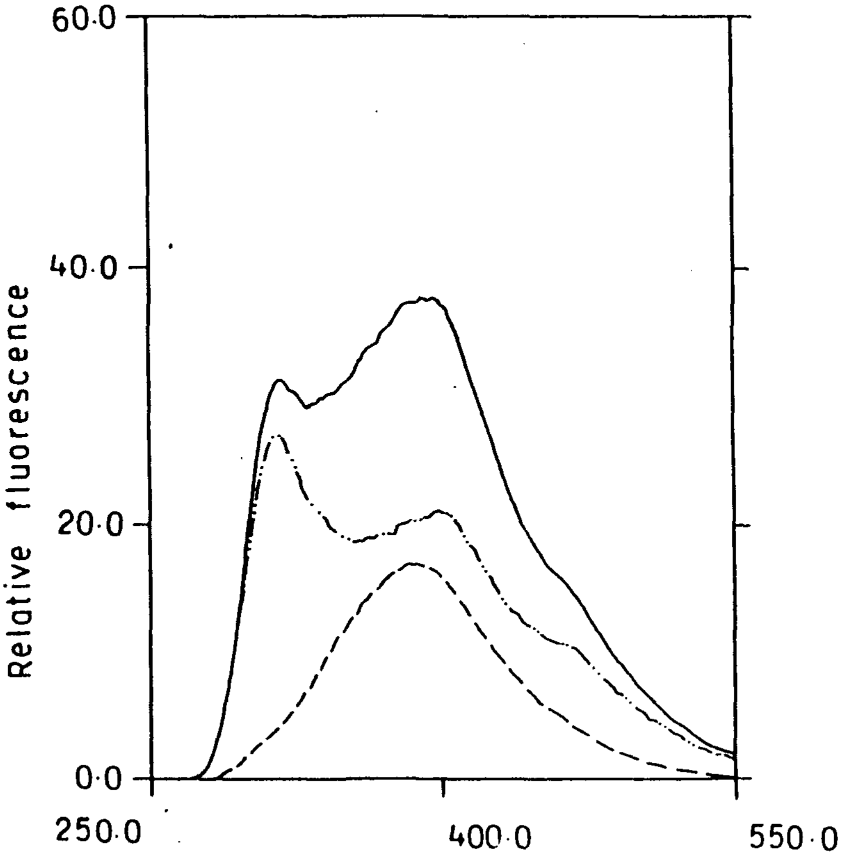


Table 5.5: Antigenic homology of the 280 kD globulin isolated from mature grains of common buckwheat (*Fagopyrum esculentum* Moench) with storage proteins from grains of some important crops .

Speccies	Major storage	Total protein as percent of protein body dry weight	Antigenic homology with 280 kD buckwheat globulin
Buckwheat	280 kD globulin	70	++++
Wheat	Prolamin	72	-
Rice	Glutelin	82	-
Soyabean	Glycinin (Legumin)	83	+++
Mung bean	Globulin (Legumin)		++
Pea	Globulin (Legumin)		+
Broad bean	Globuin (Vicilin)		-
Amaranthus	Albumin	(Cytosolic)	-

++++ > +++ > ++ > + Reletavie intensity of cross reactivity ; - No cross reactivity fig5·7a: Fluorescence emission spectra of the 280 KD globulin isolated from seeds of common bckwheat (*Fagopyrum esculentum*Moench)(_________)buffer,(--------------------) protein(a) buffered protein.


Relative fluorescence
60.0
40.0
20.0
0.0
250.0
400.0
550.0


60.0
40.0
20.0
0.0
Relative fluorescence
250.0
400.0
550.0

gether and lypholysed. Analysis of the proteins of each pe for the content of lysine revealed a lysine content of 1.8 percent in proteins of peak A, 6.4 percent in proteins of peak B and 1.2 percent in proteins present in peak C. Thus the protein having the highest lysine content was eluted in peak B corresponding to fraction number 11-15. The protein was again chromatographed on a pre calibrated Sephadex G-50 column. (2.5 x 30 cm). The column was eluted with 50 mM Tris-Cl buffer (pH 8.0) at a flow rate of 24 ml per hour. A total of 121 fractions of 2 ml each were collected. The protein resolved into 9 peaks.(Fig. 5.9). Fractions of each peak were pooled together, lypholysed and subjected to partial ami acid analysis. Analysis of the amino acid composition of the separated proteins revealed that the protein which had separated under fractions 42-48 had a 6.93 percent lysine , 2.73 percent threonine and 6.17 percent leucine. Besides the protein was also rich in sulphur amino acids. On SDS-PAGE the purified protein resolved into a single band corresponding to a molecular mass of 26 kD (Fig. 5.10).

The complete amino acid composition of the 26 kD subunit of the 280 kD globulin from buckwheat grains is presented in table 5.6. In addition to the high levels of some essential amino acids the protein also had a high content of glutamine and arginine. For comparison of the nutritional quality of this protein, the amino acid composition of cow milk casein, considered as an ideal protein and also *Brassica compestris* 2S protein, chenopodium & grain amaranth are also presented in the table along World Health Organization recomfig.5.8: Elution profile of the reduced and alkalized glolbulin isolated from seeds of common buckwheat(*Fagopyrum esculentum* Moench) on sephadex G-120 column.

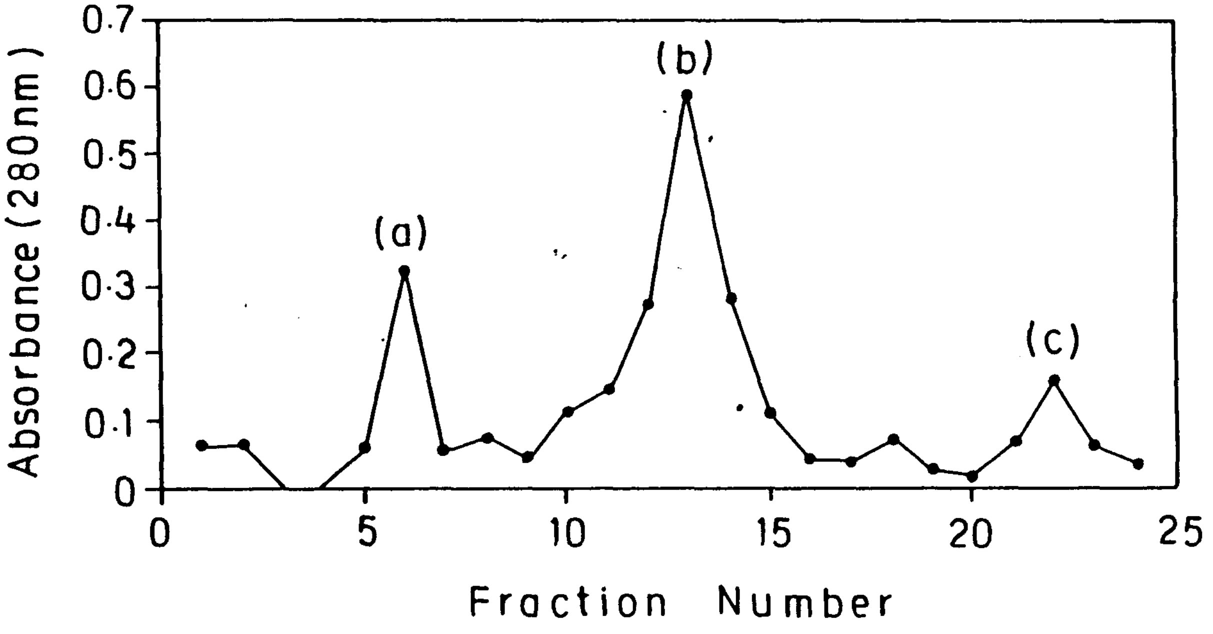

Absorbance (280nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(a)
(b)
(c)
0
5
10
15
20
25
Fraction Number

fig.5.9: Column chromatography profile of the proteins eluted in Peak `B' of sephadex G-120 column rechromatographed on sephadex G-50.

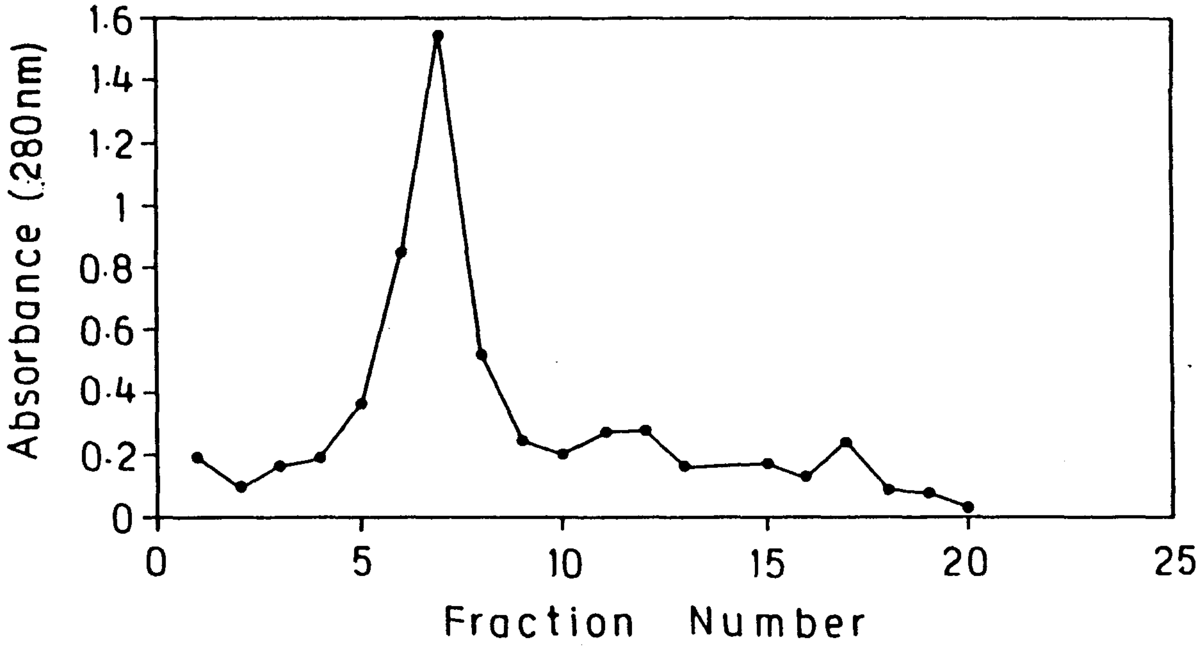

Absorbance (280nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Fraction Number

fig.5.10: SDS-PAGE of the 26 KD subunit of globulin of seed of common buckwheat (*Fagopyrum esculentum* Moench).

Fig. 5.10

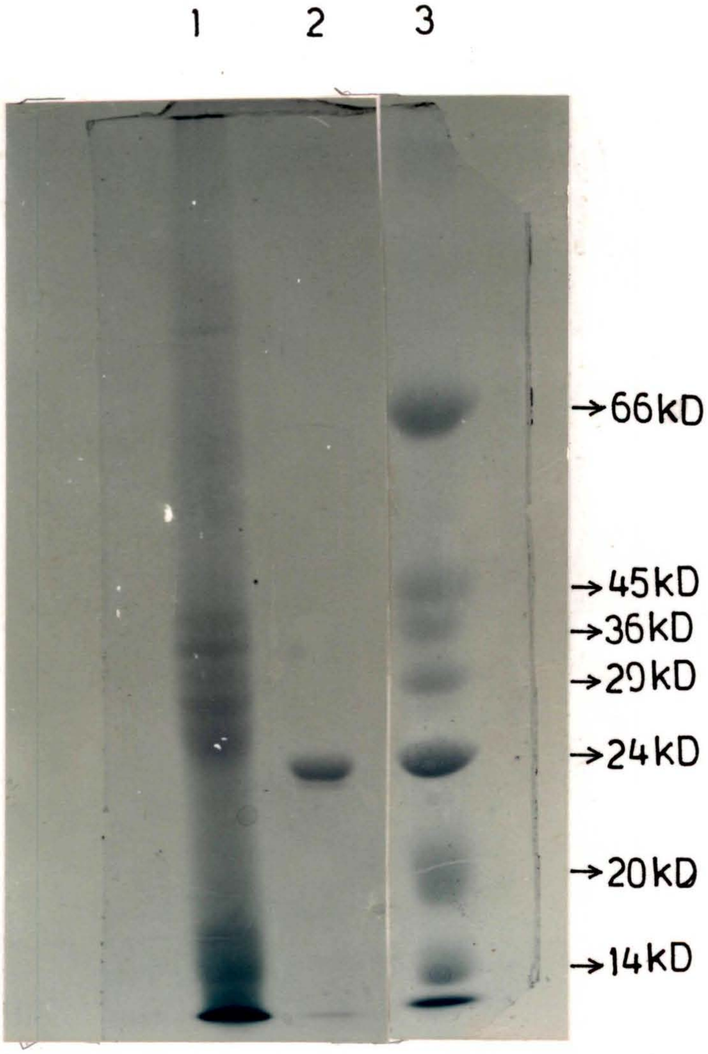


Table 5.6: Comparison of the amino acid composition of the purified 26 kD subunit of the 280 kD globulin from mature buckwheat grains with the 31 kD protein of grain amaranth, 15 kD protein from chenopodium seeds, 2S protein from Brassica seeds, casein from cow milk and the WHO recommended values of amino acids for a nutritionally balanced protein.

Amino acid	Crop					
	buckwheat	grain amaranth	Chenopodium	Brassica	cow milk	WHO values
	amino acid content (mole %)					
Asx	12.30	16.6	5.0	1.6	6.3	-
Glx	25.70	8.9	17.9	23.9	18.0	
Ser	5.11	6.6	5.6	4.9	7.4	
Thr	2.73	5.3	5.1	3.3	4.9	4.0
His	2.40	3.3	5.7	3.3	2.4	
Gly	10.40	12.0	5.8	6.6	4.3	
Ala	4.06	5.3	4.3	4.2	3.9	
Tyr	2.13	2.13	4.8	3.0	0.8	
Arg	5.93	5.3	7.5	4.2	3.9	
Met	1.57	1.6	2.8	3.3	2.1	3.5
Val	3.00	5.6	3.4	4.9	7.2	5.0
Pro	2.57	3.6	6.1	8.2	11.6	
Phe	1.93	5.6	5.4	1.6	3.6	
Lys	6.93	6.6	4.9	6.6	6.7	5.5
Ile	3.02	5.0	3.8	4.2	5.5	4.0
Leu	6.17	7.6	3.0	6.6	8.2	7.0
Trp	ND	ND	ND	0.8	0.9	1.0
CyS	1.03	0.7	5.8	6.6	0.2	

ND: Not determined
source: buckwheat, our study; grain amaranth, Raina and Datta (1992); chenopodium, (Dey and Mandal (1993); Brassica, Dasgupta and Mandal (1991); cow milk, Gordon *et al*. (1949).

Table 5·7 : Amino acid composition of the 26 kD subunit of the 280 kD globbulin isolated from mature grains of common buckwheat (*Fagopyrum esculentum* Moench)

Amino acid	residues (mole %)
Asx	12.30
Glx	25.70
Ser	5.11
Thr	2.73
His	2.40
Gly	10.40
Ala	4.06
Tyr	2.13
Arg	5.93
Met	1.57
Val	3.00
Pro	2.57
Phe	1.93
Lys	6.93
Ile	3.02
Leu	6.17
Trp	*
CyS	1.03

* Not determined

mend values for a protein with nutriotionally balanced amino acid composition. As is evident from the table , the purified 26 kD protein from buckwheat grains contains appreciable amounts of all essential amino acids like lysine, Leucine methionine and threonine. An analysis of the amino acid composition of the 26 kD subunit of buckwheat globulin reveals that content of some essential amino acids *viz.* methionine, lysine, leucine matches the requirements of a nutritionally balanced protein as per WHO requirements.

Figs. 5.11 shows the CD spectrum of the purified 26 kD protein from buckwheat grains taken at pH 7.0, 8.0 and 10.0. Two distinct negative peaks at 208 and 222 nm could be detected. Alkalization did not affect the overall conformation of the 26 kD protein. The protein depicted a predominantly α-helical structure which could undergo a transition to α -ß structure, with a decrease in hydrophobicity. When the pH of the ambient medium was 7.0, the protein showed a 33.0% α-helical structure.With increase in the hydroxyl ion concentration of the ambient medium, however, the α-helical content of the protein showed a consistent decrease. Thus, pH 8.0, the protein had a 13.79% α-helix which dropped to 12.8% at pH 10.0 (table 5.7). The α-helical content was calculated from the far-UV-CD spectral data according to the method of Greenfield and Fasman (1969) using the following formula :

$$\text{helix} = \frac{[\phi\ ]208-4000}{33000-4000} \times 100$$

fig.5.11: Circular dichroism (CD) spectra in the far and near-UV regions of 26 KD subunit of the 280 KD globulin from seed of common buckwheat (*Fagopyrum esculentum* Moench.) at pH 7.0(a); pH 8.0(b) and pH 10.0(c).

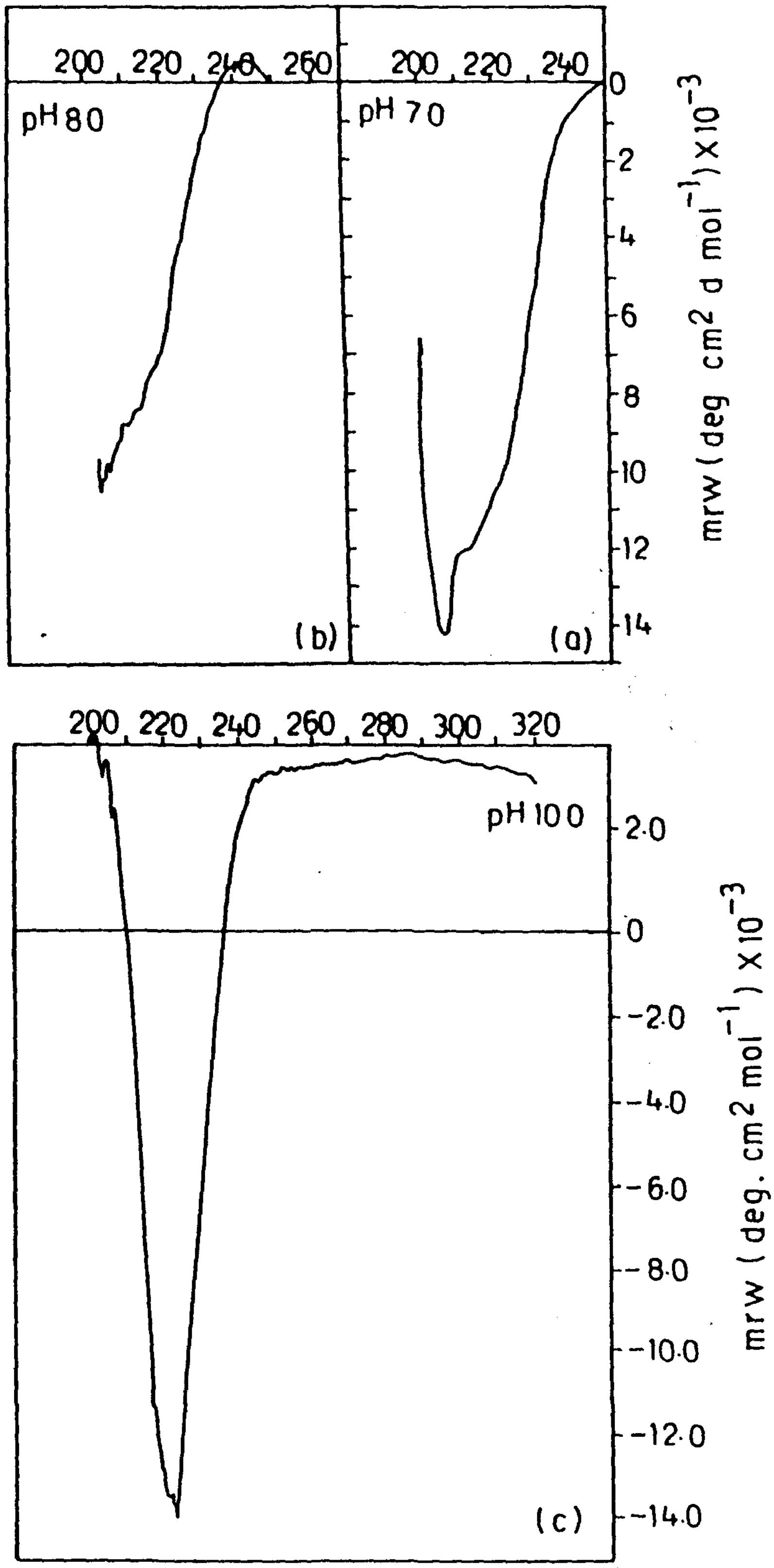

200
220
240
260
pH 8.0
(b)
200
220
240
pH 7.0
(a)
0
2
4
6
8
10
12
14
mrw (deg cm2 d mol-1) X 10-3
200
220
240
260
280
300
320
pH 10.0
2.0
0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
(c)
mrw (deg. cm2 mol-1) X 10-3

Table 5.8 :Elipticity $[\theta]$, specific elipticity $[\psi]_\lambda$, molecular elipticity $[\theta]_\lambda$ and the percent helical content of the purified 26 kD subunit of the 280 kD globulin isolated from buckwheat seeds, at varying H+ ion concentrations of 50 mM Tris-Cl buffer.

pH	Elipticity (mo/cm)	Specific elipticity (deg.cm2decimol-1)	Molecular elipticity (deg.cm2 mol-1x10-3)	Percent -helix
7.0	1.0	2.0 x 104	-14.0	33.00
8.0	1.0	2.0 x 103	-9.8	13.79
10.0	1.0	1.2 x 103	0.9	12.80

The elipticity, specific elipticity and molecular elipticity of the 26 kD protein at different ambient hydroxyl ion concentrations is shown in table 5.8. With change in the pH of the ambient medium there was a marked decrease in the electronegativity of the molecular elipticity of the protein. Thus with a change in pH from 7.0 to 10.0, the molecular elipticity of the protein changed from -14×10^{-3} deg. cm^2 mol^{-1} to 0.9×10^{-3} deg. cm^2 mol^{-1}. Changes were also observed in the specific elipticity of the protein which showed an increase from 2×10^4 deg. cm^2 deci mol^{-1} to 1.2×10^3 deg. cm^2 deci mol^{-1} with change in pH of the ambient medium from 7.0 to 10.0 (table 5.8).

The sequence of the first 17 N-terminal amino acid residues of the protein was established by automated sequencing. The sequence reads as : GLY-ILE-ASP-GLU-ASN-VAL-CYS-THR-MET-LYS-LEU-ARG-GLU-ASN-ILE-LYS-SER. The alignment of the N-terminal sequence with the corresponding regions of proteins from grains of some economically important crops is shown in Fig. 5.12. Analysis of the alignment reveals considerable similarity between the 26 kD protein isolated from buckwheat grains an storage proteins subunits of soya bean, pea, rice etc. The protein had 73.3 percent sequence homology with the G_1, G_2, G_3, and G_5 precursors of soya bean glycinin and 66.7 percent homology with G_4 subunit of soya bean glycinin. The pea legumin A and A_2 subunits had 66.7 percent with the purified lysine rich 26 kD subunit of the 280 kD globulin from buckwheat grains. The protein had the least (46.7%)

```
BIOCHEM.SEQ, 17 aa vs C:\EMBL\GLOBULIN 3 library
using protein matrix


     initn   initl
  2     0     0:
  4     0     0:
  6     0     0:
  8     0     0:
 10     0     0:
 12     5     5:===
 14     0     0:
 16     9     9:=====
 18     9     9:=====
 20     0     0:
 22     1     1:=
 24     2     2:=
 26     0     0:
 28     0     0:
 30     0     0:
 32     0     0:
 34     0     0:
 36     1     1:=
 38     0     0:
 40     6     6:===
 42     0     0:
 44     0     0:
 46     2     2:=
 48     1     1:=
 50     0     0:
 52     0     0:
 54     1     1:=
 56     1     1:=
 58     0     0:
 60     0     0:
 62     0     0:
 64     0     0:
 66     2     2:=
 68     1     1:=
 70     0     0:
 72     0     0:
 74     0     0:
 76     0     0:
 78     0     0:
 80     0     0:
 80     0     0:
 19214 residues in    41 sequences
 mean initn score:  24.0 (12.70)
 mean init1 score:  24.0 (12.70)
   15 scores better than 27 saved, ktup: 2, fact: 8  scan time:  0:00:
The best scores are:                                    initn init1   op
GLC2_SOYBN GLYCININ G2 PRECURSOR (CONTAINS: A2 AND B1A   67    67    6
GLC3_SOYBN GLYCININ G3 PRECURSOR (CONTAINS: A AND B CH   66    66    6
GLC1_SOYBN GLYCININ G1 PRECURSOR (CONTAINS: A1 AND BX    66    66    6
GLC5_SOYBN GLYCININ PRECURSOR (CONTAIN: A3 AND B4 CHAI   55    55    6
GLC4_SOYBN GLYCININ G4 PRECURSOR (CONTAINS: A5, A4 AND   53    53    6
GLU3_ORYSA GLUTELIN TYPE II PRECURSOR.                   48    48    5
GLU2_ORYSA GLUTELIN TYPE I PRECURSOR (CLONE PREE 103).   46    46    5
GLU1_ORYSA GLUTELIN TYPE I PRECURSOR (CLONE PREE 61).    46    46    5
```

```
11SB_CUCMA 11S GLOBULIN BETA SUBUNIT PRECURSOR.               40     40     5
GLU4_ORYSA GLUTELIN PRECURSOR.                                40     40     5
GLUB_ORYSA GLUTELIN TYPE-B 1 PRECURSOR.                       40     40     5
GLU5_ORYSA GLUTELIN PRECURSOR.                                39     39     4
LEG2_PEA   LEGUMIN A2 PRECURSOR.                              39     39     5
LEGA_PEA   LEGUMIN A PRECURSOR.                               39     39     5
GLUC_ORYSA GLUTELIN TYPE-B 2 PRECURSOR.                       36     36     4

GLC2_SOYBN GLYCININ G2 PRECURSOR (CONTAINS: A2 AND B1A    67     67     67
  73.3% identity in 15 aa overlap

                                               10
BIOCHE                                 GIDENVCTMKLRENIKS
                                       X:::..:::.::.:X
GLC2_S   QEEDDDDEEEQPQCVETDKGCQRQSKRSRNGIDETICTMRLRQNIGQNSSPDIYNPQAGS
                280       290       300       310       320       330

GLC2_S   ITTATSLDFPALWLLKLSAQYGSLRKNAMFVPHYTLNANSIIYALNGRALVQVVNCNGER
                340       350       360       370       380       390

GLC3_SOYBN GLYCININ G3 PRECURSOR (CONTAINS: A AND B CH    66     66     66
  73.3% identity in 15 aa overlap

                                               10
BIOCHE                                 GIDENVCTMKLRENIKS
                                       X:::..:::.::.:X
GLC3_S   PTEEQQQRPEEEEKPDCDEKDKHCQSQSRNGIDETICTMRLRHNIGQTSSPDIFNPQAGS
          270       280       290       300       310       320

GLC3_S   ITTATSLDFPALSWLKLSAQFGSLRKNAMFVPHYNLNANSIIYALNGRALVQVVNCNGER
          330       340       350       360       370       380

GLC1_SOYBN GLYCININ G1 PRECURSOR (CONTAINS: A1 AND BX     66     66     66
  73.3% identity in 15 aa overlap

                                               10
BIOCHE                                 GIDENVCTMKLRENIKS
                                       X:::..:::.::.:X
GLC1_S   EEEEDEKPQCKGKDKHCQRPRGSQSKSRRNGIDETICTMRLRHNIGQTSSPDIYNPQAGS
                290       300       310       320       330       340

GLC1_S   VTTATSLDFPALSWLRLSAEFGSLRKNAMFVPHYNLNANSIIYALNGRALIQVVNCNGER
                350       360       370       380       390       400

GLC5_SOYBN GLYCININ PRECURSOR (CONTAIN: A3 AND B4 CHAI     55     55     67
  73.3% identity in 15 aa overlap

                                               10
BIOCHE                                 GIDENVCTMKLRENIKS
                                       :..X:.::::::.:X
GLC5_S   EDQPRPDHPPQRPSRPEQQEPRGRGCQTRNGVEENICTMKLHENIARPSRADFYNPKAGR
            320       330       340       350       360       370

GLC5_S   ISTLNSLTLPALRQFGLSAQYVVLYRNGIYSPDWNLNANSVTMTRGKGRVRVVNCQGNAV
            380       390       400       410       420       430

GLC4_SOYBN GLYCININ G4 PRECURSOR (CONTAINS: A5, A4 AND    53     53     65
  66.7% identity in 15 aa overlap
```

```
                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :..X:.::.::.::X
GLC4_S RKSREWRSKKTQPRRPRQEEPRERGCETRNGVEENICTLKLHENIARPSRADFYNPKAGR
       350       360       370       380       390       400

GLC4_S ISTLNSLTLPALRQFQLSAQYVVLYKNGIYSPHWNLNANSVIYVTRGQGKVRVVNCQGNA
       410       420       430       440       450       460

GLU3_ORYSA GLUTELIN TYPE II PRECURSOR.                       48   48   56
  52.9% identity in 17 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :.X:. :::..:.:X..
GLU3_O QEQEQGQMQSREHYQEGGYQQSQYGSGCPNGLDETFCTMRVRQNIDNPNRADTYNPRAGR
        280       290       300       310       320       330

GLU3_O VTNLNSQNFPILNLVQMSAVKVNLYQNALLSPFWNINAHSIVYITQGRAQVQVVNNNGKT
        340       350       360       370       380       390

GLU2_ORYSA GLUTELIN TYPE I PRECURSOR (CLONE PREE 103).    46   46   54
  47.1% identity in 17 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :.X:. ::...:.:X..
GLU2_O QEQEQGQVQSRERYQEGQYQQSQYGSGCSNGLDETFCTLRVRQNIDNPNRADTYNPRAGR
        280       290       300       310       320       330

GLU2_O VTNLNTQNFPILSLVQMSAVKVNLYQNALLSPFWNINAHSVVYITQGRARVQVVNNNGKT
        340       350       360       370       380       390

GLU1_ORYSA GLUTELIN TYPE I PRECURSOR (CLONE PREE 61).     46   46   54
  47.1% identity in 17 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :.X:. ::...:.:X..
GLU1_O QEQEQGQVQSRERYQEGQYQQSQYGSGCSNGLDETFCTLRVRQNIDNPNRADTYNPRAGR
        280       290       300       310       320       330

GLU1_O VTNLNTQNFPILSLVQMSAVKVNLYQNALLSPFWNINAHSVVYITQGRARVQVVNNNGKT
        340       350       360       370       380       390

11SB_CUCMA 11S GLOBULIN BETA SUBUNIT PRECURSOR.             40   40   58
  46.7% identity in 15 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :..:..X:..:..:X
11SB_C DEDFEVLLPEKDEEERSRGRYIESESESENGLEETICTLRLKQNIGRSVRADVFNPRGGR
        270       280       290       300       310       320

11SB_C ISTANYHTLPILRQVRLSAERGVLYSNAMVAPHYTVNSHSVMYATRGNARVQVVDNFGQS
        330       340       350       360       370       380

GLU4_ORYSA GLUTELIN PRECURSOR.                                40   40   51
  52.9% identity in 17 aa overlap
```

```
                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :..X: ::.:.: :X..
GLU4_O LTQQQEQAQAQDQYQQVQYSERQQTSSRWNGLEENFCTIKVRVNIENPSRADSYNPRAGR
            280       290       300       310       320       330

GLU4_O ITSVNSQKFPILNLIQMSATRVNLYQNAILSPFWNVNAHSLVYMIQGRSRVQVVSNFGKT
            340       350       360       370       380       390

GLUB_ORYSA GLUTELIN TYPE-B 1 PRECURSOR.                  40   40   51
  52.9% identity in 17 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :..X: ::.:.: :X..
GLUB_O LTQQQEQAQAQDQYQQVQYSERQQTSSRWNGLEENFCTIKVRVNIENPSRADSYNPRAGR
            280       290       300       310       320       330

GLUB_O ITSVNSQKFPILNLIQMSATRVNLYQNAILSPFWNVNAHSLVYMIQGRSRVQVVSNFGKT
            340       350       360       370       380       390

GLU5_ORYSA GLUTELIN PRECURSOR.                           39   39   47
  58.8% identity in 17 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :.X:: ::.: : :X..
GLU5_O QQEQAQQQEQAQAQYQVQYSEEQQPSTRCNGLDENFCTIKARLNIENPSHADTYNPRAGR
           280       290       300       310       320       330

GLU5_O ITRLNSQKFPILNLVQLSATRVNLYQNAILSPFWNVNAHSLVYIVQGHARVQVVSNLGKT
           340       350       360       370       380       390

LEG2_PEA   LEGUMIN A2 PRECURSOR.                         39   39   53
  66.7% identity in 15 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :..:.X:: ::: :X
LEG2_P HQRRRGEEEEEDKKERRGSQKGKSRRQGDNGLEETVCTAKLRLNIGPSSSPDIYNPEAGR
         310       320       330       340       350       360

LEG2_P IKTVTSLDLPVLRWLKLSAEHGSLHKNAMFVPHYNLNANSIIYALKGRARLQVVNCNGNT
         370       380       390       400       410       420

LEGA_PEA   LEGUMIN A PRECURSOR.                          39   39   53
  66.7% identity in 15 aa overlap

                                             10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :..:.X:: ::: :X
LEGA_P HQRRRGEEEEEDKKERGGSQKGKSRRQGDNGLEETVCTAKLRLNIGPSSSPDIYNPEAGR
            310       320       330       340       350       360

LEGA_P IKTVTSLDLPVLRWLKLSAEHGSLHKNAMFVPHYNLNANSIIYALKGRARLQVVNCNGNT
            370       380       390       400       410       420
```

```
GLUC_ORYSA GLUTELIN TYPE-B 2 PRECURSOR.                        36    36    47
  52.9% identity in 17 aa overlap

                                              10
BIOCHE                               GIDENVCTMKLRENIKS
                                     :..X: ::.: : :X..
GLUC_O PTLTQQQEQAQAQYQEVQYSEQQQTSSRWNGLEENFCTIKARVNIENPSRADSYNPRAGR
       270       280       290       300       310       320

GLUC_O ISSVNSQKFPILNLIQMDATRVNLYQNAILSPFWNVNAHSLVYMIQGQSRVQVVSNFGKT
       330       340       350       360       370       380

Library scan:  0:00:02  total CPU time:  0:00:17
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fig.5.12: Homology of the N-terminal region of the 26 KD globulin subunit from seeds of common buckwheat (*Fagopyrum esculentum* Moench) with the corresponding regions of soyabean glycinin precursors (G_1,G_2,G_3, G_4, and G_5) rice glutelin, pealegumin and cucumber globulin. Conserved residues are boxed with solid lines and identical amino acids are in bold type. Figures in parenthesis represent % homology.

																	17	
BUCKWHEAT 26 kD GLBLN	G	I	D	E	N	V	C	T	M	K	L	R	E	N	I	K	S	
SOYBN GLYCININ G1	G	I	D	E	T	I	C	T	M	R	L	R	H	N	I	G	Q	(73.3)
SOYBN GLYCININ G2	G	I	D	E	T	I	C	T	M	R	L	R	Q	N	I	G	Q	(73.3)
SOYBN GLYCININ G3	G	I	D	E	T	I	C	T	M	R	L	R	H	N	I	G	Q	(73.3)
SOYBN GLYCININ G4	G	V	E	E	N	I	C	T	L	K	L	H	E	N	I	A	R	(66.7)
SOYBN GLYCININ G5	G	V	E	E	N	I	C	T	M	K	L	H	E	N	I	A	R	(73.3)
ORYZA GLUT TYPE I	G	L	D	E	T	F	C	T	L	R	V	R	Q	N	I	D	N	(47.1)
ORYZA GLUT TYPE I	G	L	D	E	T	F	C	T	L	R	V	R	Q	N	I	D	N	(47.1)
ORYZA GLUT TYPE II	G	L	D	E	T	F	C	T	M	R	V	R	Q	N	I	D	N	(52.9)
ORYZA GLUT 4	G	L	E	E	N	F	C	T	I	K	V	R	V	N	I	E	N	(52.9)
ORYZA GLUTN 5	G	L	D	E	N	F	C	T	I	K	A	R	L	N	I	E	N	(58.8)
ORYZA GLUTN B1	G	L	E	E	N	F	C	T	I	K	V	R	V	N	I	E	N	(52.9)
ORYZA GLUTN B2	G	L	E	E	N	F	C	T	I	K	A	R	V	N	I	E	N	(52.9)
PEA LEGUMIN A	G	L	E	E	T	V	C	T	A	K	L	R	L	N	I	G	P	(66.7)
PEA LEGUMIN A2	G	L	E	E	T	V	C	T	A	K	L	R	L	N	I	G	P	(66.7)
CUCUM 11S GLOB B SUBUNT	G	L	E	E	T	I	C	T	L	R	L	K	Q	N	I	G	R	(46.7)

sequence homology with the ß-subunit of 11S globulin from cucumber seeds. A statistical evaluation of these alignments revealed that the sequence homologies were highly significant. Out of the 17 residues compared 6 residues were highly conserved and 5 residues matched closely in that they are amino acids with similar functional groups there by representing conservative replacements. Amongst the conserved amino acids are glutamic acid, cysteine, threonine, asparagine and isoleucine at position numbers 4, 7, 8, 14 and 15 respectively. Amongst the conserved bonds was the cysteine-threonine linkage at residue numbers 7, 8 and the asparagine-isoleucine linkage at residue numbers 14, 15. One of the significant aspects revealed by the alignment was the presence of lysine and serine at position numbers 16 and 17 respectively in the purified 26 kD globulin purified fr buckwheat grains. None of the other sequences matched had these residues at position numbers 16 and 17.

DISCUSSION:

Seed storage proteins, intended as a source of nitrogen for germinating seedlings, constitute an important source of dietary proteins for human consumption. Although cereal grains and legume seeds are a major source of dietary proteins, the storage proteins in both are generally deficient in essential amino acids such as lysine, tryptophan and methionine (Higgins, 1984; Hoffman *et al.*, 1988; Shotwell *et al.*, 1989 and Krishnan *et al.*, 1993). Likewise zein, t storage protein of *Zea mays* has been reported to be deficient in tryptophan and lysine (Nelson, 1969). While many attempts have been made over the years to improve the amino acid composition of storage proteins of important crops through conventional breeding programmes, molecular approaches towards improving the nutritional quality of seed proteins provide alternative strategies to conventional breeding programmes. An important approach towards improving the amino acid composition of seed storage protein could be to express the gene for a heterologous protein with a balanced amino acid composition. The generality of the approach by which foreign proteins rich in desired amino acids may be introduced is, however, constrained by scanty information in respect of suitable heterologous proteins. While a number of seed protein rich in sulphur containing amino acids are available (Coulter & Benley, 1990), not many lysine rich seed storage proteins have been identified so far.

In common buckwheat more than 50% of the seed storage protein is the soluble globulin. Thus, the main storage protein of buckwheat seeds is of globulin type. Another characteristic feature of buckwheat seed proteins is the low content of prolamins. These results are in agreement with those of Sokolov and Semihov (1976). That the protein fractions are an important element in protein quality was shown by Kirilenko (1981) in his study of protein composition in nine buckwheat varieties derived by different techniques. The main fraction in all the varieties was the globulin. Globulins and albumins showed a positive correlation to the content of total proteins. In contrast the prolamin and glutelin fractions were smallest at higher levels of total proteins in the seeds. Storage proteins are not found at the early stage of seed formation. It is synthesized during the developmental stage of seed formation and accumulated within the seed. As observed by agar immunodiffusion test the main seed storage protein of buckwheat started to accumulate in the seeds 40 days after flowering. The protein could not be detected in the early stage of seed formation. During this period there was a significant increase in the content total dry matter in the seed. Our results on the temporal pattern of accumulation of the 280 kD globulin in buckwheat seeds are however, not in agreement with those of Sokolov and Semihov (1974) who have reported the highest rate of globulin accumulation in the seeds at milky stage. Our results suggest that the globulin accumulation starts from early maturation stage of seed development.

The main storage protein of common buckwheat is present within protein bodies located in the endosperm tissues of the grains. Protein bodies isolated by the aqueous extraction protocol were intact spherical in shape and ranged in diameter form 5-8 μm. Miege and Mascherpa (1976) and Pusztai *et al.* (1978, 1979) have laid emphasis on the medium to be used for the isolation of protein bodies. They have observed that the protein bodies were readily destroyed during homogenization of the seeds in an aqueous medium. They have suggested the use of organic non-aqueous medium and density gradient centrifugation for the isolation of protein bodies from seeds. In the present investigation intact protein bodies could be obtained by homogenization in Tris-buffer containing appropriate concentrations of salt and protease inhibitors followed by gel filtration. The protein bodies were composed of an amorphous protein matrix with a single globoid inclusion; the matrix was enclosed by a single membrane. Electron microscopic examination of the isolated protein bodies buckwheat grains revealed the presence of a membrane like boundary layer surrounding the globoid inclusion also. On the basis of their morphology, Tully and Beevers (1976), Sobolev *et al.* (1976) and Pernollet (1978), have divided protein bodies into two types: 1. those consisting of an amorphous protein without any inclusions (peanut, soya bean, and corn), and 2. those consisting of the amorphous protein matrix with either globoid or a crystalloid inclusions (Cannabis, Cucurbit, Yucca, Cotton). Protein bodies without any inclusion have been described as "simple" while those with inclusion are described as "complex". On the basis of this classific tion , the protein bodies in seeds of common buckwheat can described as of "complex" type. While the existence of a single membrane surrounding a protein body has been long established (Pernollet, 1978), the question regarding the presence of a similar membrane surrounding the globoid has not been fully resolved. Poux (1965) has observed that the globoids of *Cucumis sativus* were membrane bound. However, Lott *et al.* (1971) could not find any evidence for the existence of a membrane surrounding the globoid inclusion in *Cucurbita maxima*. The presence of a membrane like boundary, which did not have the ultrastructure of a typical biologic membrane, has been reported for protein bodies isolated from barley aleurone (Jacobsen *et al.*, 1971), castor bean endosperm (Tulley, 1976; Mettler and Beevers, 1979) and *Pinus sylvestris* seeds (Simola, 1974). Our results on the ultrastructure of protein bodies of buckwheat seeds clearly show the presence of a membrane-like boundary layer surrounding the globoid inclusion. The globulin content of protein bodies of buckwheat seeds closely matches the values reported for soya bean, broad bean, barley and rice (Pernollet, 1978).

The 280 kD globulin, the main storage protein of buckwheat seeds, is a large neutral oligomeric protein. t' is composed of three groups of polypeptides with molecular weight ranging between 55-60 kD (α - group), 33-44 kD (ß - group) and 16-29 kD (Γ- group). Under reducing conditions the α- group species of polypeptides, in 55 kD range, disappeared and gave two kinds of polypeptides with M_r corresponding to those of the initial ß and Γ protein species. Such behaviour under reducing conditions is common to the legumin group of proteins (Schwenke *et al.*, 1981; Dalgalavvondo *et al.*, 1986; Sjodahl *et al.*, 1991 and Rodin and Rask, 1990). These results supports the notion that native 280 kD globulin of buckwheat seeds is composed of subunits with large and small polypeptides linked by disulphide bonds and of similar or closely related polypeptides which are not covalently bonded. That the proteins of ß and Γ groups could be detected ev under reducing conditions indicates that contrary to the α group, the ß and Γ group proteins are not constituted by the association of disulfide bridge linked polypeptides. Staswick (1984) has reported that in maize globulins at least two polypeptides components are non-covalently associated. Dalgalarrando *et al* (1986) have suggested that such non covalent linkages could be the hydrophobic interactions.

Antibodies raised against the 280 kD globulin of buckwheat seeds reacted positively with the globulins extracted from soya bean, mung bean and pea. However, no such cross reactivity was observed for salt soluble proteins extracted from rice, wheat, broad bean and grain amaranth. These results clearly demonstrate the existence of immunological homology between seed globulins from soya bean, mung bean, pea and common buckwheat. However, no such homology could be detected with the globulin from seeds of broad bean. While the seed storage globulin from soya bean, pea and mung bean belong to the legumin family, the globulin from broad bean belongs to the vicilin family of proteins.

The partial amino acid composition of the oligomeric complex revealed the presence of a high proportion of lysine, methionine and tryptophan that are otherwise deficient in most legumes and cereals. The composition closely matches the WHO recommended values for a nutritionally balanced lysine rich protein.

When screened by SDS-PAGE, the 26 kD subunit of the 280 kD globulin protein resolved into a single band. The protein had 6.93 percent lysine , 2.73 percent threonine and 6.17 percent leucine. Besides the protein was also rich in sulphur amino acids. Compared to other storage proteins, the 26 kD subunit also has a high content of glutamine and arginine. The presence of high levels of arginine is consistent with the role of crystalloid seed storage proteins as a nitrogen source during seed germination (Shotwell and Larkins, 1989). As is evident from the data on amino acid composition, the 26 kD protein from buckwheat grains contains appreciable amounts of all essential amino acids like lysine, Leucine methionine and threonine. An analysis of the amino acid composition of the 26 kD subunit of buckwheat globulin reveals that content of some essential amino acids *viz*. methionine, lysine, leucine matches the values for essential amino acids recommended by World Health Organization making it more important nutritionally.

The sequence of the first 17 N-terminal amino acid residues of the protein was established by automated sequencing . The alignment of the N-terminal sequence with the corresponding regions of proteins from grains of some economically important crops reveals considerable similarity between the 26 kD protein isolated from buckwheat grains and storage proteins subunits of soya bean, pea, rice etc. The protein had 73.3 percent sequence homology with the G_1, G_2, G_3, and G_5 precursors of soya bean glycinin and 66.7 percent homology with G_4 subunit of soya bean glycinin. The pea legumin A and A_2 subunits had 66.7 percent with the purified lysine rich 26 kD subunit of the 280 kD globulin from buckwheat grains. The protein had the least (46.7%) sequence homology with the ß-subunit of 11S globulin from cucumber seeds. A statistical evaluation of these alignments revealed that the sequence homologies were highly significant. Out of the 17 residues compared 6 residues were highly conserved and

5 residues matched closely in that they are amino acids with similar functional groups there by representing conservative replacements. Amongst the conserved amino acids are glutamic acid, cysteine, threonine, asparagine and isoleucine at position numbers 4, 7, 8, 14 and 15 respectively. Amongst the conserved bonds was the cysteine-threonine linkage at residue numbers 7, 8 and the asparagine-isoleucine linkage at residue numbers 14, 15. One of the significant aspects revealed by the alignment was the presence of lysine and serine at position numbers 16 and 17 respectively in the purified 26 kD globulin purified from buckwheat grains. None of the other sequences matched had these residues at position numbers 16 and 17. Staswick *et al.* (1984) have provided evidence for the involvement of cysteine residue at position no. 7 in t formation of disulphide bridge linking the basic and acidic subunits of glycinin. The strict conservation of this residue in the buckwheat 26 kD protein as well as the precursors of soya bean glycinin (Nielsen *et al.*, 1989), pea legumin (lycett *et al.*, 1984) and the ß subunit of 11S globulin from cucumber (Hayashi *et al.*, 1988) indicates the importance of these interchain bonds in the stability of these proteins. Sequence conservation observed in reported groups of storage proteins has been interpreted as identifying regions of structural importance. On the basis of our observations on gross subunit composition, serological as well as sequen homologies with legumin group of proteins from seeds of some other crop plants, it is clear that the 280 kD globulin from seeds of common buckwheat is a legumin type of protein.

Globulins from a range of flowering plants have been shown to have a conserved N-terminal sequence GLY-LEU/ILE-GLU/ASP-GLU, the probability of the sequence at random being 1 in 10^6 sets of amino acids (Simon *et al.*, 1985). This sequence of amino acids is conserved in the 26 kD subunit of 280 kD buckwheat globulin also where it reads as GLY-ILE-ASP-GLU-ASN-VAL-CYS. Comparative analysis of the protien sequences, as well as the gene sequences, suggests that the 11s globulins of angiosperms have arisen from a common ancestor (Borroto and Dure, 1987; Mishra and Green, 1994)).

Legumin group of proteins are known to be synthesized as precursors of the form of α - β - CO_2H, which undergo a limited post - translational proteolysis during maturation. Lycett *et al.* (1984) and Nielsen (1984) have observed that the cleavage site for the post translational maturation of legumin precursors always occurred between the C-terminus ASN of the α - polypeptide and an N-terminal GLY of the β subunit. They have emphasized the conserved nature of ASN-GLY cleavage site. On the basis of these arguements, Borroto and Dure (1987) have proposed the existence of a conserved endoprotease recognition site in these proteins.

The amino acid composition of the protein clearly revealed the lysine rich nature of the protein. This signifies the importance of the protein in human nutrition. Common buckwheat being an unconventional food cultivar with the seed storage protein having a high content of essential amino acids, the 26 kD subunit of the 280 kD globulin from the plant could be used as a tool in future work related to compensating the amino acid deficiencies of the legumin group of seed storage proteins.

GENERAL SUMMARY
&
CONCLUSION

GENERAL SUMMARY AND CONCLUSIONS:

Seed storage proteins, intended as a source of nitrogen for germinating seedlings, constitute an important source of dietary proteins for human consumption. Although cereal grains and legume seeds are a major source of dietary proteins , the storage proteins in both are generally deficient in essential amino acids such as lysine , tryptophan and methionine . Over the years many attempts have been made to improve the amino acid composition in important crops through conventional breeding programmes. Molecular approaches towards improving the nutritional quality of seed proteins, however, provide alternative strategies to conventional breeding programmes . An important approach towards improving the amino acid composition of seed storage proteins could be to express the gene for a heterologous prote with a balanced amino acid composition . The generality of the approach by which foreign proteins rich in desired amino acids may be introduced is however constrained by scanty information in respect of suitable heterologous proteins . While a number of seed proteins rich in sulphur containing amino acids are available not many lysine rich seed storage proteins have been identified so far .

The North Eastern region of India is extremely rich. in floristic wealth and is home to a large variety of traditional crops that could form an essential component of human diet in times to come. Common buckwheat (*Fagopyrum esculent* Moench.) is one such plant that has been classified by IBPGR as an important but underutilized crop. The plant is a dicot psedocereal with a high protein content (18%) of its grains. Further unlike cereals, where the main storage proteins are the prolamins, the main storage protein in buckwheat seeds belongs to the globulin family . In order to use the available germpalsm for improvement programs , the characterization of seed storage proteins from the plant would be a prerequisite.

Even though seed storage proteins constitute an important component of dietary proteins for human nutritio their primary role in seeds would be to provide free amino acids to the growing axis during early stages of seed germination. The physiological and biochemical processes underlying seed germination and early seedling growth are, thus, important to the establishment of a plant in its environment and ultimately determine its yield potential. One crucially important aspect is the mobilization of storage materials during the early stages of germination in plants. The degradation of storage proteins during seed germination has also been studied for a long time, the starting points being the establishments of protein degradation and the detection of proteolytic activity in seeds. Studies on the degradation of seed storage proteins during seed germination have provided information about histochemical and molecular characteristics of the process. Until recently, enzymatic aspects of storage protein degradation remained unclear since almost all studies dealt with proteolytic enzymes in seeds irrespective of function (Shutov and Vaintraub, 1987).

Even though much work has been done on soya bean (Hill and Briendenback, 1974), pea (Millard *et al.*, 1978; Higgins *et al.*, 1986; Hoffman *et al.*, 1988), *Lupinus albus* (Melo *et al.*, 1994), *Oryza sativa* (Takaiwa *et al.*, 1987; Krishnan and Pueppke 1993), Oat (Shotwell *et al.*, 1990), *Cumcumis sativus* (Kim and Smith, 1994) and *Phaseolus vulgaris* (Goossens *et al.*, 1994), not much information is available on seed storage proteins of common buckwheat (*Fagopyrum esculentum* Moench). An understanding of the individual protein fractions and the proteases involved in the degradation of these fractions is essential for a better utilization of the seed proteins as food for human nutrition.

Studies were carried out to determine the relationship between the levels of proteolytic activity and the mobilization of reserve proteins during early stages of buckwheat seed germination. Results of our investigations revealed a rapid uptake of water and mobilization of storage proteins in buckwheat seeds during early stages of germination. The pattern of increase in the level of moisture in endosperm followed a typical hyperbolic behaviour without any lag phase. In the embryo, however the uptake of water showed lag upto 18 hours of incubation after which it followed a pattern similar to that shown by the endosperm. During the same period there was a six fold decrease in the dry matter content of the endosperm. The embryo, however, registered a two fold increase upto 14 hours after which it decreased marginally upto 72 hours of incubation.

There was no marked change in the electrophoretic mobility of the main storage protein during the initial 24 hours of germination. Beyond 24 hours, the electrophoretic mobility of the protein increased with progressing seed germination. correspondingly there was no marked differen in the SDS-PAGE profile of the main storage protein extracted from ungerminated seeds and those allowed to imbibe water for 24 hours. After 24 hours a gradual decrease in the ammount of some high molecular weight subunits of the protein and increase in the levels of some low molecular weight proteins was observed with progressing germination. When tested by the Western blot assay, strong anti 280 kD activity could be detected in proteins extracted from seeds harvested at various stages of germination upto 96 hours. The immunoblot also confirmed the changes taking place in the electrophoretic mobility of the protein with progressing germination. These results indicate that during early stages of seed germination, the main seed storage protein in common buckwheat undergoes a partial cleavage leading to the release of some low molecular weight subunits. Results of the western blot assay obtained in the present investigation clearly establish that the 280 kD globulin of buckwheat seeds retained its antigenic identity even upto 96 hours of imbibition. Till this time the process of radical and plumule emergence is nearly complete.

A low level of proteolytic activity could be observed in the endosperm of buckwheat seeds upto 18 hours of germination. Increases in the activity of the enzyme occurred only after 18 hours of imbibition. In the embryo, however, the activity of the enzyme started to increase after after 6 hours of imbibition only. Corresponding with changes in the activity of proteases a marked increase in the tissue level of free amino acids was observed in the endosperm and embryo tissues with progressing germination after 14 hours. However, the content of soluble protein in these tissues showed a consistent increase throughout. Considered together with the changes taking place in the main storage protein and the levels of proteolytic activity in the seeds during germination, these results indicate that the 280 kD globulin may not be the source of amino acids that are utilized by the growing embryo during the initial stages of germination. Contributions towards this pool as a result of proteolytic cleavage of the main storage protein into small peptides and amino acids presumably start much later. The significant decrease in the level of free amino acids in the endosperm during the initial 2 hours of imbibition and the increase in the level of soluble protein in the embryo during the same period indicates that a pre-existing pool of free amino acids in the endosperm tissues could be the source of amino acids to be utilized for the synthesis of soluble proteins during the initial stages of germination. A low level of proteolytic activity could be observed in the endosperm of buckwheat seeds upto 18 hours of germination. Increases in the activity of the enzyme occurred only after 18 hours of imbibition. In the embryo, however, the activity of the enzyme started to increase after after 6 hours of imbibition only. Corresponding with changes in the activity of proteases a marked increase in the tissue level of free amino acids was observed in the endosperm and embryo tissues with progressing germination after 14 hours. However, the content of soluble protein in these tissues showed a consistent increase throughout. Considered together with the changes taking place in the main storage protein and the levels of proteolytic activity in the seeds during germination, these results indicate that the 280 kD globulin may not be the source of amino acids that are utilized by the growing embryo during the initial stages of germination. Contributions towards this pool as a result of proteolytic cleavage of the main storage protein into small peptides and amino acids presumably start much later. The significant decrease in the level of free amino acids in the endosperm during the initial 2 hours of imbibition and the increase in the level of soluble protein in the embryo during the same period indicates that a pre-existing pool of free amino acids in the endosperm tissues could be the source of amino acids to be utilized for the synthesis of soluble proteins during the initial stages of germination.

In order to assess the protein quality and quantity of buckwheat seeds, the seeds of common buckwheat (*Fagopyrum esculentum* Moench), obtained from the Western Himalayas and the North Eastern Regional station of NBPGR, were analyzed for the content of total proteins. On dry weight basis, the seeds had a protein content of about 16 percent out of which more than 50 percent was present in the globulin fraction. Albumins, prolamins, glutelins and the residual proteins constituted 15.8, 7.83, 9.27 and 14.43 percent of the total protein content of the grains respectively.

The main storage protein in seeds of common buckwheat is localized within protein bodies present in the endosperm tissues of the seed. The protein bodies of common buckwheat are spherical in shape and range in diameter from 5-8 μm. The protein bodies are composed of an amorphous protein matrix with a single globoid inclusion. The protein matrix is enclosed by a single membrane. Electron microscopic examination of the isolated protein bodies of buckwheat seeds also revealed the presence of a membrane like boundary layer surrounding the globoid inclusion. The protein body of buckwheat seeds has been thus classified as of "complex" type.The protein bodies of buckwheat seeds had about 70 percent protein content; phytin and carbohydrates respectively constituted 25 and 2.7 percent of the protein body dry weight. An analysis of the pattern of accumulation of the main storage protein in buckwheat seeds revealed that the protein started to accumulate only after early mature stage of seed development. When extracts from seeds at various stages of development were analyzed by double diffusion test with antibodies raised against the 280 kD protein from mature buckwheat seeds, no cross reactivity was observed with extracts from seeds harvested upto early mature stage of development. A precipitin line with extracts from seeds harvested at mid mature stage of development clearly indicated the presence of the protein at this stage.

The main seed storage protein of buckwheat was purified by $(NH_4)_2SO_4$ fractionation, dialysis and chromatography on Sepharose 6B column. The protein showed a molecular mass of 280 kD. Electrophoresis on a 7.5% polyacrylamide gel resolved the protein into a single band. Under denaturing conditions the protein resolved into three groups of subunits designated as α , ß and Γ with molecular weight ranging between (55-60 kD, α); (32- 44 kD, ß) and (16-29 kD , Γ). The 280 kD protein revealed an oligomeric structure typical to the 11-12S groups of legumin type globulins . When tested against antibodies raised against the 280 kD globulin from mature seeds of common buckwheat, the protein extract from ungerminated buckwheat seeds as well as the 280 kD globulin showed single precipetin lines indicating the antigenic homogeneity of the protein. The protein showed antigenic homology with 11-12S seed storage proteins from soya bean , mung bean and pea; the protein showed strongest homology with glycinin from soya bean seeds. No cross reactivity was observed for storage proteins extracted from broad bean , wheat , rice and grain amaranth. Based on the gross subunit composition and its antigenicity with seed storage proteins of other crops, as observed in the present investigation, it can be assumed that the 280 kD globulin of buckwheat seeds belongs to the legumin subfamily of proteins.

In order to determine which of the subunits of the 280 kD globulin had the highest lysine level, the protein was subjected to gel filtration on Sephadex G-120 and Sephadex G-50 after reduction and alkalization. Fractions of each pe were pooled together and lypholysed. Analysis of the partial amino acid composition of the separated proteins revealed that a protein which had separated under fractions 42-48 on Sephadex G-50 column had the highest (6.93 %) lysine content. Besides the protein was also rich in some sulphur amino acids.

On SDS-PAGE the purified subunit resolved into a single band corresponding to 26 kD molecular mass. The amino acid composition of the subunit revealed high levels of essential amino acids such as lysine, threonine, leucine and some sulphur containing amino acids. Compared to other se storage proteins, the 26 kD subunit also showed a high content of glutamine and arginine . The role of high level of arginine is consistent with the role of crystalloid seed storage proteins as a nitrogen source during seed germination . The amino acid composition of the protein also closely matches the values of essential amino acids recommended by the World Health Organization for a nutritionally balanced protein.

The sequence of 17 N-terminal amino acid residues of the protein was established by automated sequencing. The sequence reads as:

GLY-ILE-ASP-GLU-ASN-VAL-CYS-THR-MET-LYS-LEU-ARG-GLU-ASN-ILE-LYS-SER

Alignment of the sequence with the corresponding regions of proteins from seeds of some economically important crops revealed considerable similarity in the sequences. The protein showed 73.3% sequence homology with the G_1, G_2, G_3 and G_4 precursors of soya bean glycinin. The second highest homology (66.7%) was shown with pea legumin. The least homology of 46.7% was observed with the ß subunit of 11S globulin from cucumber seeds. Out of the 17 residues compared, 6 were highly conserved and 5 residues matched closely in that they were amino acids with similar functional groups, thereby representing conservative replacements. Amongst the conserved bonds was the cysteine-threonine linkage at residue numbers 7, 8 and the asparagine-isoleucine linkage between residue numbers 14, 15. One of the significant aspects revealed by the alignment was the presence of lysine and serine at position numbers 16 and 17 respectively in the 26 kD globulin subunit of buckwheat seeds. None of the other sequence matched had these residues at position numbers 16 and 17.

The physical characterization of the 26 kD globulin subunit of buckwheat by circular dichrosim and fluorescence spectroscopy revealed that the protein had a predominantly α-helical structure. The protein had a 33% α-helix which undergoes a transition to α-ß structure with a decrease in hydrophobicity .

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