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Studies on some Enzymes from Germinating oil and Cereal Seeds

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STUDIES ON SOME ENZYMES FROM GERMINATING OIL AND CEREAL SEEDS

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BY

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ABSTRACT

Degradation of three varieties of Brassica and wheat seed storage substances at different periods of germination have been studied to have a comparative data on their chemical composition. Free sugar contents of the three varieties of Brassica and wheat seeds differ slightly. Wheat seeds contain a little higher amount of free sugar than that of Brassica seeds. Reducing sugar contents also vary in Brassica and wheat seeds. During germination, the degradation of free sugar and reducing sugar in Brassica seeds is faster than in wheat seeds. Wheat seeds contain a larger amount of starch than that of Brassica seeds. Among the three varieties of wheat seeds, Akbar variety has the highest percentage of starch. The starch contents of both seeds decrease gradually during germination. Total protein and water-soluble protein content of the three varieties of Brassica seeds is slightly higher than that of wheat seeds. During germination, protein depletion starts after initial imbibition, and is completed in between 96 to 120 hours. Brassica seeds contain a significant amounts of lipid. Among Brassica seeds, napus variety contains the highest amount of lipid, followed by campestries and juncia varieties. Lipid degradation starts after 24 hours of germination and is completed within 72 to 96 hours. The results suggest that degradation of seed reserve nutrients accelerate the development of seedling growth during germination.

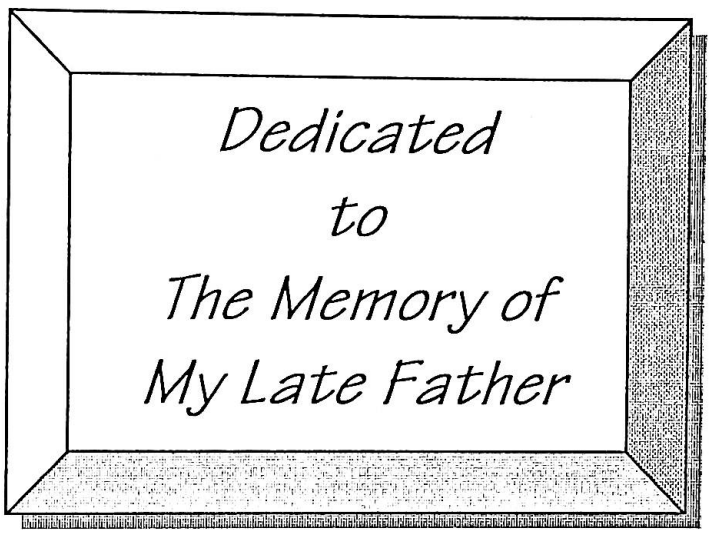
Lipid degrading enzyme lipase was identified in germinating *Brassica napus* L. The enzyme was purified to homogeneity by solvent extraction, followed by Sephadex G-75, DEAE and CM-cellulose ion exchange chromatography. The enzyme was purified to 68 fold with the final specific activity of 39-mmol min^{-1} mg^{-1} at 37^0C using olive oil as a substrate. The SDS-PAGE electrophoresis showed a molecular mass of 34 kD, while a 34.5 kD mass were obtained by gel filtration chromatography for this lipase. The optimum pH and temperature for the purified lipase for hydrolysis of oleic acid were found to be 7.0 and 35^0C respectively. In presence of Ca^{2+} and Ba^{2+}, the lipase activity was enhanced to 125 and 115% respectively. Fe^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} inhibited this lipase but Al^{3+}, Mn^{2+} showed no influence on lipolytic activity. K_m value of the purified lipase was determined to be 0.23 mM.

In germinating wheat (*Triticum aestivum* L) seeds after 42 hours, the abundant amylolytic activity was found to be due to β-amylase (α-1-4-glucan maltohydrolase). The enzyme was purified to homogeneity by ammonium sulphate precipitation followed by gel filtration on Sephadex G-75, and DEAE-cellulose chromatography. The enzyme was found to be more active against starch (pea) and amylopectin than soluble starch (used as substrate). The β-amylase showed maximum activity at pH 6.0 and at 45^{0}C. The enzyme was stable at a pH range of 4.0 – 8.0 and at 30 - 60° C for 15 min. The molecular weight of the enzyme was estimated to be 88 kD by Sephadex G-75 column chromatography and 89 kD by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). The Km value for β-amylase with soluble starch as substrate was found to be 1.47 mg/ml. The enzyme was completely inactivated by Cu^{2+}, Hg^{2+}, Pb^{2+}, Urea and Ag^{+} at 0.5mM concentration and its activity was increased by the addition of Fe^{3+}, Mn^{2+} and EDTA. The study indicates the importance of β- amylase as a starch-degrading enzyme.

Enzyme with proteolytic activity was also found in the germinating wheat seeds (*Triticum aestivum* L.,). The enzyme showed activity after 40 hours of dark germination and reached its maximum value after 72 hours and then declined sharply during rest of the germination period. After ion exchange chromatography, three proteins (pro-I, pro-II and pro-III) showed proteolytic activity. Properties like optimum pH and optimum temperature for the proteins, pro-I, pro-II and pro-III were found to be 7.1, 6.9, 6.8 and 45° C, 43° C, 42° C, respectively. Although Mg^{2+} and Mn^{2+} showed no effect on enzyme activities, EDTA, urea, Ca^{2+} showed an activating effect. Hg^{2+} and Fe^{2+} showed strong inhibitory action on the extracted enzyme. Molecular weights of pro-I, pro-II and pro-III were obtained as 108, 104 and 99kD respectively & the Km values were found to be 0.027, 0.032 and 0.037 mM respectively. The enzymes pro-I, pro-II and pro-III showed absorption maximum at 281 nm, 275 nm and 273 nm and minimum at 245 nm, 243 nm and 242 nm respectively. Maximum seed storage proteins (globulin) were found to be the best sources of substrate for these enzymes.

The functional properties of gluten treated with protease from germinating wheat seeds at pH 8.0 and at 25° C were also investigated. The treatment caused

deamidation of the gluten with a very limited proteolysis. Functional properties of the gluten such as solubility, emulsifying and foaming ability were found to be greatly improved by enzymatic deamidation. The solubility of the treated gluten was remarkably high in the pH range of 6 to 8, in which native gluten is usually insoluble. The foaming and emulsifying ability as well as the stability of the protein were also observed to increase in this treatment.


Dedicated
to
The Memory of
My Late Father

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Lastly, but not least, I owe a life long debt to my parents and relatives for their keen interest in my study.

The Author

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled **"Studies on some enzymes from germinating oil and cereal seeds"** prepared for submission to the University of Rajshahi, Bangladesh, for the Degree of Doctor of Philosophy in Biochemistry, are the original research works of mine and have not been previously submitted for the award of any Degree or Diploma anywhere.

(Niranjan Kumar Sana)
Signature of the candidate

CERTIFICATE

This is to certify that the thesis entitled **"Studies on some enzymes from germinating oil and cereal seeds"** has been prepared by Niranjan Kumar Sana under our supervision for submission to the Department of Biochemistry and Molecular Biology, University of Rajshahi for the Degree of Doctor of Philosophy in Biochemistry. It is also certified that the materials included in this thesis are the original work of the researcher.

We have gone through the draft of the thesis and found it acceptable for submission.

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DEAE-cellulose	:	Diethylaminoethyl cellulose
CM-cellulose	:	Carboxymethyl cellulose
Tris	:	Tris (hydroxymethyl) amino methane
UV	:	Ultraviolet
SDS	:	Sodium dodecyl sulphate
BSA	:	Bovine serum albumin
PBS	:	Phosphate buffer saline
PAGE	:	Polyacrylamide gel electrophoresis
rpm	:	rotation per minute
Pro-I	:	Protease-I
Pro-II	:	Protease-II
Pro-III	:	Protease-III
kD	:	kilo Dalton
EDTA	:	Ethylenediaminetetraacetic acid
O.D.	:	Optical density
Bis	:	N,N′-Methylene-bis-acrylamide
TEMED	:	N,N,N′,N′-Tetramethyl ethyl diamine.

CHAPTER-1

GENERAL INTRODUCTION

1.1 SEEDS: Seeds are the food for men, animals and birds. They are wealth, they are beauties, and they are symbol of beginning. Botanically seed is a ripened ovule-containing embryo, which is developed after fertilization of the egg cell by the male gamete in the embryo sac of the ovule in ovary of a flower. In agriculture, the seed is any part or organ of a plant which has the capability to regenerate a new plant and can be shown or distributed without much difficulty. It serves as the connecting link between two generations of a plant species and is responsible for maintaining the intrinsic qualities of that species through generations. Like a fortress the seed stores during its development special types of food and nourishment to be used for subsequent development of embryo into seedling during the process of germination. The nature and proportion of the inbuilt reserves of food and energy and the mechanism to overcome the hazards during its quescent period are different in different species.

Seed is used directly as food and for extraction of oil, malt and medicines and for propagation of a new generation of the species. It is a natural wealth, which sustains the life of animals and plants. Crop plants produce these useful seeds. Some seeds are not used as food or in any other form except for propagation of progeny plants. Weeds produce such seeds. Some seeds are, however, sources of trouble and health hazard for men and animals. The plants producing such unwanted and undesirable seeds are regarded as obnoxious weed. The dispersal of seeds carried out through various simple but innovative means help in the distribution and establishment of the plant species to balance ecosystem (1).

One of the unique features in seed is the production of food reserves concomitant to seed development and maturity. In cereals, like rice, wheat, maize, etc. the dominant food reserve is carbohydrate derivatives, which are metabolized in endosperm tissue and finally stored as starch. The stored food is predominantly lipid in the endosperm of castor. In exalbuminous seeds like pea, mungbean, soybean the reserve food is prepondarantly protein, metabolized in cotyledons of embryo, which serve as storage tissue. The constituents of these storage products are also specific so that a taxa can be characterized by the presence of a particular storage substance like inulin in

sunflower, phaseolin in most of grain legumes or erusic acid (fatty acid) in seed oil of Brassica sp. The stored carbohydrates are usually starch, amylose, amylopectin and hemicelluloses. Stored lipids are triglycerides of a number of fatty acids and stored proteins are complex polypeptides characteristic of different plant groups. In addition, there are auxins and various vitamins and various mineral salts.

1.1.1 CLASSIFICATION OF SEEDS: According to cotyledon, seeds are mainly classified into two groups: (a) Monocotyledons and (b) Dicotyledons.

(a) *MONOCOTYLEDONS:* Monocotyledonous seed is a single-seeded fruit in which the pericarp (ovary wall) is tightly fused with the seed. Such seed is called a caryopsis and is common in grasses or cereal. Embryo and endosperm are present, however, the true seed coat or testa is often absent. The endosperm is composed of an outer layer of cells, the aleurone layer, and the interior starchy endosperm. The cells of the aleurone layer contain proteins and fats, but little or no starch, whereas the cells of the starchy endosperm are filled with starch grains and protein bodies embedded in a proteinaceous matrix. The embryo is present just adjacent to the endosperm and consists of a scutellum or cotyledon and embryonic axis. The cotyledon, or scutellum, comprises a relatively large part of the embryo and lies in direct contact with the endosperm. A mature seed consists of the embryo, variable amounts of endosperm and the seed coat, or testa, derived from the integument's of the ovule.

(b) *DICOTYLEDONS:* A short synonym of dicotyledonous plant, the term refers to plants which have two seed leaves in the seed. Dicot stems always have definite wood and bark layers, and the leaf veins are branched. In dicotyledonous seed (soybean) the endosperm is absent while in monocotyledonous seed (corn) the endosperm is present at maturity. The seed coat is with a hilum or seed scar and a micropyle at one end. At the other end of the hilum, raphe is present. Each seed consists of two fleshy, large cotyledons. The hypocotyle is located below the cotyledons, and a short axis epicotyle, terminating in the shoot apex, is above the cotyledons. On the basis of preponderance of the type of stored substances the seeds can be generally classified into the following groups:

i) Cereals: which are rich in carbohydrate content, e.g. wheat, rice, maize, barley, oats, sorghum. All cereals are in a class of monocotyledon.

ii) Pulses: which are rich in proteins, e.g. cowpea, peas, beans, lentil.

iii) Oilseed: which are rich in lipids and fats, e.g. ground nut, mustard, rape, sesame, sunflower, soybean. All oil seeds are in a group of dicotyledon.

iv) Spices: which are rich in aromatic acids or alkaloids, e.g. cumin, cardamom, corriander, nutmag.

v) Beverages and medicines: which are rich in various secondary metabolites, e.g. cocoa, catharanthus, dioscoria.

vi) Fiber: fibers from seeds, e.g. cotton.

Table 1.1: Chemical composition of seed (% by dry weight)(2).

Crop	Moisture	Protein	Fat	Fibre	Carbohydrate	Minerals
Wheat	10.5	13.2	1.9	2.6	69.9	1.9
Rice	12.2	9.1	2.0	1.1	74.5	1.1
Maize	13.0	8.9	4.0	2.0	70.8	1.3
Oats	9.8	12.0	4.6	11.0	58.6	4.0
Sorghum	10.4	10.8	2.8	2.3	71.7	2.0
Cowpea	11.0	23.4	1.3	3.9	56.8	3.6
Pea	9.3	23.4	1.2	6.1	57.0	3.0
Beans	10.0	22.9	1.4	4.2	57.3	4.2
Soybean	10.1	37.9	18.0	5.0	24.5	4.6
Groundnut	5.4	30.4	47.7	2.5	11.7	2.3
Mustard	4.1	23.0	38.8	5.0	23.6	5.5
Rape	9.5	20.4	43.6	6.6	15.7	4.2
Sesame	8.0	22.3	42.6	10.3	10.9	5.6
Sunflower	6.4	16.8	25.9	29.0	18.8	3.1
Cotton	7.3	23.1	22.9	16.9	26.3	3.5
Safflower	6.9	16.3	29.8	26.6	17.5	2.9

Among monocot seeds, we use in this study wheat (*Triticum aestivum* L.), a cereal seeds, which is the second most important crop in our country. We also studied dicot seeds like rapeseed and mustard (*Brassica napus* L.), which are also the major rabi oilseed crops in Bangladesh.

1.2 WHEAT: Wheat (*Triticum aestivum* L.) is world's major crop sources of calories and protein (3). It is grown over a wide range of environments around the world; in fact it has the broadest adaptation of all the cereal crop species. More land is devoted worldwide to the production of wheat than to any other commercial crop. Wheat is the number one food grain consumed directly by humans, and its production leads all crops including rice, maize and potatoes. It is eaten in various forms by more than one thousand million human beings in the world. In Bangladesh it is second important staple food crop, rice being the first. In areas where wheat is the staple cereal food, it is eaten in the form of 'chapatis'. In areas where rice is the staple cereal food wheat is eaten in the form of 'puris' or in the form of 'upma' (cooked from suji or rawa). In addition to this wheat is also consumed in various other preparations such as 'dalia', 'halwa', 'sweet meals' etc. In most of the urban areas in the country the use of backed leavened bread, flakes, cakes, biscuits etc., is increasing at a fast rate. Besides staple food for human beings, wheat straw is a good source of feed for a large population of cattle in our country.

Wheat compares well with other important cereals in its nutritive value. It contains more protein than other cereals. Wheat has a relatively high content of niacin and thiamin. Wheat proteins are of special significance. Besides, their significance in nutrition, they are principally concerned in providing the characteristic substance 'gluten' which is very essential for bakers. In bakery gluten provides the structural framework for the familiar spongy, cellular texture of bread and other baked products. Flours of other cereals lacking gluten are, therefore, not good for bread making.

1.2.1 CLASSIFICATION OF WHEAT: Wheat belongs to the grass family Gramineae and the genus Triticum. It is an annual plant i.e., it grows and dies in the course of a year. It is grown in winter in Bangladesh. A number of species of wheat have been recognized and they are classified into three groups on the basis of chromosomal number (4).

Table-1.2: Classification of wheat:

Einkorn series(diploid) (2n-14)	Emmer series (tetraploid) (In-28)	Valgure series(hexaploid) (2n-42)
T. aegiloploids	T. dicoccoides	T. spelta
T. monecoccum	T. timopheevi	T. macha
T. thaudar	T. persicum	T. aestivum or vulgare
	T. polonicum	T. vulgave
	T. turgidum	T. compactum
	T. orientale	T. sphaerococcum
	T. durum	T. valvilovi
	T. dicoccum	

1.2.2 STORAGE SUBSTANCES IN WHEAT SEEDS: The composition of wheat grain varies greatly with the variety and climate and to a smaller extent with soil, fertilization and time of harvesting (5). The crude cereals contain 11% protein, 70% carbohydrate, 0.5-8% fat, 11% water and 2% minerals. Oatmeal is the richest in protein and fat, rice is the poorest. The main proteins of cereals are glutelins and gliadins. Small amount of albumins and globulin are generally present. The fat contains considerable amounts of olein to make them liquid at ordinary temperature (6).

1.2.3 PROTEIN STORAGE IN WHEAT SEEDS: Proteins in the wheat grain can be divided into four groups. The water-soluble albumins, the salt soluble globulins, the enzymatic and structural proteins distributed throughout the cytoplasm of the grain. Among the globulin alone, there are at least six major functions (7). The soluble proteins constitute most of the protein present during the first two weeks after anthesis, and may increase in amount for further two weeks, to 0.5-1.0 mg protein per grain (8-9). However, they soon exceed in amount by the prolamins (gliadins) and glutenins (10), stored in protein bodies in the endosperm, which appear 10-16 days after anthesis (11 -12). Storage of gliadins may begin later than that of glutenins. It is the characteristics of the glutens that are so important in bread making. The glutens and prolamins (gliadins) have amino acid compositions quite different from those of cytoplasmic proteins, being very high in glutamic acid and proline and relatively low

in several essential amino acids. Thus as protein storage proceeds the percentage of the essential amino acids falls progressively, e.g. from 8% lysine, 12 days after anthesis to 2.5% at maturity (9). Proteins of the aleurone layer are much higher in the basic amino acids such as arginine. High temperature seems to reduce starch storage more than that of protein (13), as does drought stress. Reduced light intensity lowers the grain nitrogen content to about the same extent as grain weight with the result that percentage of nitrogen is little affected (14). Late application of nitrogen fertilizers can increase grain protein substantially. For example, Abrol et. al., (15) found that application of 100 kg nitrogen per hectare increase the yield of protein by 250 kg per hectare, almost entirely through increase in the storage of protein, both prolamins and glutelins.

1.2.4 IMPORTANCE OF WHEAT: Wheat is the most widely used of all cereals, owing to high nutritive value combined with the dough forming properties of the gluten. While superior to rice and maize, especially in protein and mineral content, it is about equal in food value to barley, oats and rye, but is unique in possessing the coherent gluten makes it pre-eminently suitable for bread making.

The carbohydrate of wheat is almost completely starch in the form of grains covered by a thin membrane of cellulose. Small amounts of sugar are also often present in it. The abundant mineral elements are calcium and phosphate. The phosphate is partly in the form of phytic acid (Inositol hexaphosphate) which interferes the absorption of calcium. Wheat and rye contain the enzyme phytase which hydrolyses phytic acid and reduce the anti-calcifying effect (6).

Wheat contains many enzymes such as amylolytic, proteolytic and oxidizing. It contains vitamins B_1, E, B_2, niacin and carotene, which can act as a precursor of vitamin A, especially the first two are present in wheat, chiefly in the germ and bran. Wheat germ is by far the richest known source of vitamin E.

1.3 RAPE SEED AND MUSTARD: In Bangladesh, farmers, researchers, extension workers and policy members have neglected oil crops equally. Research institutes never had ample resources at their disposal to follow a systematic research and development program that would bring the oil seed crops high on their priorities. The

scope of reversing the situation exists, as is clear from the advances being made in both the developed and developing countries. In most oil seed crops, break through in yield have been made through breeding of HYV's (high yield variety), by following improved management practices and by developing appropriate post-harvest and post-management packages.

Brassica napus is a number of the botanical family cruciferae, which contains 220 genera and 1900 species. The genus Brassica is the most important agricultural plant above the oilseed rape. *Brassica napus* is not known as a wild plant and is derived from a cross between *Brassica oleracea* and *Brassica rapa* (formally known as *Brassica compestris*) (16). Still Schubart (1825) first term's turnip rape as *Brassica napus* and an oilseed rape as *Brassica oleracea*. The common name for all these similar species in the world statistics is "Rapeseed" at present (17). Vegetable oils, meals and seeds are the most important groups of agricultural commodities, in terms of value in the world trade. Rapeseed and mustard play an important role in international market. The importance of these crops is further enhanced by their wide adaptation. They are cultivated from the farming areas of Canada, Europe and Africa to the subtropics.

The oil obtained from the different types show slight variation in percentage of oil. The oil content varies from 37 to 49 percent. The seed and oil are used as condiment in the preparation of pickles and for flavoring curries and vegetables. The oil is utilized for human consumption throughout northern India in cooking and frying purposes. It is also used in the preparation of hair oils and medicines. It is used in soap making, in mixtures with mineral oils for lubrication. Rapeseed oil is used in the manufacture of greases. The oil cake is used as cattle feed and manure. Green stems and leaves are a good source of green fodder for cattle. The leaves of young plants are used as green vegetables as they contain enough sulphur and minerals. In the tanning industry, mustard oil is used for softening leather.

1.3.1 VARIETIES AND CHEMICAL COMPOSITION: Of the two Brassica rape species grown for oil in the world, Swede rape (*Brassica napus*) is virtually the only one grown in the EEC. The other, turnip rape (*Brassica campestris*) is not favoured

because of generally lower yields but, as it matures earlier, it is grown in areas subjected to early frost in Canada and constitute about half of the Swedish rape seed acreage (18). Chemical composition of Brassica seed contain protein-25.7%, oil-41.5%, sucrose-4.1%, oligosaccharide-2.0%, starch-0.4%, total fiber-20.4%, ash-5%, calcium-0.46%, others-0.44% (19).

1.3.2 IMPORTANCE OF MUSTARD SEEDS: The species Brassica (*Brassica napus*) play an important role in the industrial market. The oils and meals from these seeds have different characteristics. The seeds of Brassica contain 35-50% of fatty oil and about 20% of protein matter. Their free essential fatty acid content is usually very low, but they contain small proportion of glucosides, which on hydrolysis yield volatile mustard oils. The mustard oils are characterized by a high proportion of erucic acids, $C_{22}H_{42}O_2$ (45-50% of total fatty acids), oleic acid (20-30%) and linoleic acid (15-25%). The saturated acids, palmitic, stearic and lignoceric are present in very small proportions. Besides these it also contains trace amount of vitamin-C. The Bassica seeds are also rich in calcium, phosphorus, iron and magnesium (20). The oil is used for cooking and as salad oils. It is also used for oil-baths and it is believed to strengthen the skin to keep it cool and healthy. The oil cake (meal) is used as poultry and animal feeds. Besides these mustard seeds oil have many medicinal and industrial importance.

1.4 BIOCHEMICAL CHANGES DURING GERMINATION: Germination is indicated by sequential reactive changes taking place in the cells of seeds. Oxidative and hydrolytic function are triggered through activation of synthesis and mobilization of enzymes concerned. Gibberallic acid has been found to play a vital role in activation and initiation of these enzyme functions. This is followed by enzymatic degradation of stored reserves. The pattern or sequence of events controlling the hydrolysis of these reserves may vary from species to species (21).

Phytic acid is the main phosphate reserve in many seeds. Phytase hydrolyses the phytin to release phosphates which activate the protoplasm. Starch as amylose or amylopectin is degraded generally to glucose, α- and β–maltose, and dextrin by α–amylase and β–amylase. Starch breakdown during germination in cereal seeds is

caused by the synergistic action of hydrolytic enzymes. It is generally accepted that phosphorylase is not involved in this process. α-Amylase plays a major role during the degradation of native starch granules. Even though other amylolytic enzymes participate in the process of starch breakdown, the contribution of α-amylase is prerequisite for the initiation of this process. β-Amylase (1,4 α-glucan maltohydrolase) catalyzes the liberation of α-maltose from the non-reducing ends of starch-related α-1,4 glucans. In cereals, β-amylase helps mobilize the starch in germinating grains. In barley, the enzyme is already present in the dry seeds, where it accumulates during the process of grain development and is mainly bound to the starch granules. In rice, β-amylase is synthesized de novo during seed germination is almost absent in dry seeds. Lipase degrades the lipids to glycerol and fatty acids. The fatty acids are rapidly degraded and converted to carbohydrate; while glycerol is utilized directly as a substrate for respiration or translocated to cotyledones and embryonic axis. It has been known for many years that the early stages of germination of certain seeds with high fat content (e.g. castor been, sunflower, pumkin mustard) are accompanied by a decrease in fat and a simultaneous increase in sucrose. During the first several days of germination, the fat reserves are used as respiratory substrates. But instead of being oxidized to carbon dioxide and water, fat is converted in large measure to sucrose. Fat conversion to sucrose reaches its maximum rate on the fifth day of germination for seedlings maintained at 30°C. Once synthesized, sucrose is transported out of the endosperm and into the developing shoot-root system, where it is respired to carbon dioxide and water. The conversion of fat reserves to sucrose in endosperm tissue begins with the hydrolysis of fats to long-chain fatty acids (22). Then these fatty acids are oxidized via the β-oxidation pathway, which produces two-carbon units of acetyl-CoA capable of entering the tricarboxilic acid or the glyoxylase bypass, where fats are converted to sugars via reverse glycolysis. Proteases are responsible for storage protein hydrolysis, seeds evidently contain proteolytic enzymes performing other functions. Degradation of protein reserves by protease liberates amino acids. The amino acid pool thus formed is translocated to embryo axis for synthesis of proteins. The cells of bean cotyledones are filled with starch and proteins, those of soybean with oil and proteins. The germs (embryo, including scutellum or the single cotyledone) of wheat and maize contains

much oil and is rich in protein, but the endosperm is largely starchy. The stored food in date plam and carrot seeds is hemicellulose. Organic and inorganic phosphorus present during germination are also important for the transfer of energy for growth. Whether a seed is albuminous or exalbuminous, the pattern of mobilization of simple metabolites derived through degradation of food reserves are quite different in different species of angiospermous seeds. The differences indicate the possible genetic control of the amphibolic physiological functions. Reserve food mobilization during germination of cereal seed is a regulated function initiated by the residual hormone reserved in the seeds. The pattern of mobilization, therefore, is a sequential pathway of utilization of stored carbohydrates and proteins. In case of non-cereal seeds, however, the pattern of mobilization of food stored in endosperm or cotyledon may be different. Experimental results obtained from detached cotyledon or endosperm of non-cereal seeds have the drawback of incorporating some injury to storage tissues, unlike that of cereals. This may give rise to deviations from the interpretation derived from whole seed. It is evident that availability of oxygen is the most important factor in initiating food mobilization during germination. However, the picture will be clear to some extent by examining specific cases of the pattern of food mobilization in respect of protein rich cereal seeds and lipid rich oil yielding seeds.

1.5 ENZYMES: Enzymes are almost invisible protein molecules, which are available in the food we eat and are produced by our bodies. Scientists believe there are thousands of different enzymes, many of which have yet to be discovered. It controls catalytically the vast majority of chemical reactions in living system. In the past several decades an enormous number of such enzymatic reaction have been discovered. Industrial enzymes have been isolated and purified from bacteria, yeast, plants and animals. Modern enzyme chemistry is considered to have started with the crystallization of the enzyme urease in 1926 by Summer (23), who showed this material to be a crystalline protein. A series of quantitative experiments by Northrop and co-workers (24) and by Summer and co-workers (25) conclusively proved that enzymes were indeed proteins. A list of the amino acid composition of approximately

fifty enzymes (26) indicates that the gross composition of enzymes is not different than that of proteins.

Chemical reactions in biological systems rarely occur in the absence of a catalyst. These catalysts are specific proteins called enzymes. The striking characteristics of all enzymes are their catalytic power and specificity. Furthermore, the activity of many enzymes is regulated. In addition, some enzymes are intimately involved in the transformation of different forms of energy.

Enzymes accelerate reactions by factors of at least a million. Indeed, most reactions in biological systems do not occur at perceptible rates in the absence of enzymes. Even a reaction as simple as the hydration of carbon dioxide is catalyzed by an enzyme. Otherwise, the transfer of CO_2 from the tissues into the blood and then to the alveolar air would be incomplete. Each enzyme molecule can hydrate 10^5 molecules of CO_2 in one second. This catalyzed reaction is 10^7 times faster than the uncatalyzed reaction.

Enzymes are highly specific both in the reaction catalyzed and in their choice of reactants, which are called substrates. An enzyme usually catalyzes a single chemical reaction or a set of closely related reactions. The degree of specificity for substrate is usually high and sometimes virtually absolute.

All enzymes are proteins, metalloproteins, or conjugated proteins. The activity of pure protein enzymes is attributed to the reactive groups of the amino acids they contain. Many enzymes are proteins with traces of certain metals (Zn, Co, Ca) which stay with the protein even in crystalline form; these are metalloproteins. The complex enzymes are conjugated proteins, which contain an organic nonprotein and non-amino acid part, somewhat loosely attached to the protein part.

1.5.1 BREADTH OF ENZYMOLOGY: The area of enzymology is of special interest to both the biological and physical scientists. Enzymes are of universal occurrence in biological materials and life itself depends on a complex network of chemical reactions brought about by specific enzymes. Any alteration in the normal enzyme pattern of an organism may have far-reaching consequences. Enzymes, as catalysts, are of great interest to the physical chemist and investigation of the mechanisms of action of enzymes is a very important area of enzymology.

The area of enzymology has continued to grow rapidly for more than 60 years because of its importance to many field of sciences, especially biochemistry, physical chemistry, microbiology, genetics, botany, zoology, food science, nutrition, pharmacology, toxicology, pathology, physiology, medicine and chemical engineering. Enzymology has important practical applications to activities as diverse as brewing and industrial fermentation's, pest control and chemical warfare, dry cleaning, sizing and detergents, analytical determinations, and recombinant DNA technology (27).

1.5.2 USES OF ENZYMES: Enzymes have many uses in addition to their natural functions in the body. Manufacturers use enzymes in making a wide variety of products. For example, some detergents contain enzymes that break down protein matter, such as perspiration, that causes stains. Enzymes are also used in the manufacture of antibiotics, beer, bread, cheese, coffee, meat tenderizers, vinegar, vitamins, and many other products.

Physicians use medicines containing enzymes to help clean wounds, dissolve blood clots, relieve certain forms of leukemia and check allergic reactions to penicillin. Doctors also diagnose a number of diseases by measuring the amount of various enzymes in blood and other body fluids. Such diseases include anaemia, cancer, leukemia, heart and liver ailments (28).

1.6 LIPASES: Lipases are lipolytic enzymes which catalyze the hydrolysis of fats as well as esters of fatty acids to fatty acids and alcohols (29,30). According to the IUB (International Union of Biochemistry) lipase catalyze the following hydrolytic reaction:

Fat ⟶ Mono and diglycerides ⟶ Glycerol + Fatty acids.

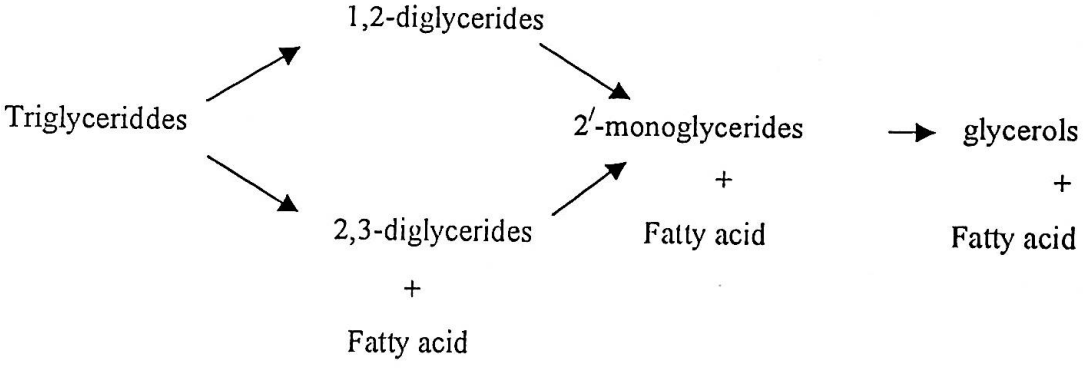
Fatty acid esters ⟶ Alcohol + Fatty acids.

Lipases have received increased attention after they were shown to be active even in nearly anhydrous immiscible organic solvents (31) and can be used for trans-

esterificaton (32), synthesis of esters (33) and peptides (34) and resolution of racemic mixture into optically active alcohols or acids (35).

Lipases constitute an important group of enzymes since they are associated with fat metabolism as well as fat degradation. They are widely distributed in nature, being present in numerous tissues and fluids of both animal and plant origin.

1.6.1 LIPASE ACTIVITY: The theory behind lipase action and assay is that it releases FFAs from the substrate and therefore, the lipase activity can be measured by determining, directly or indirectly, the disappearance of the substrate or the production of end product fatty acids. Lipases hydrolyze triglycerides and the products are formed in the following sequences:



Thus, the rate of lipase reaction can be measured by determining either the rate of disappearance of substrate or the rate of production of fatty acids. Automatic or manual titration of the system gives a direct measure of the lipase activity per unit time (36). This assay technique was used extensively for the measurement of lipase activity. As an alternative to the above method, the rate of acid production may be measured manometrically by determining the rate of liberation of carbon dioxide from a bicarbonate buffer (37). Several colorimetric methods have been described that employed a special substrate designated to give a coloured end product after hydrolysis. β–naphthyllaurate has been recommended and used as substrate for serum lipase. β–naphthol liberated is coupled with tetrazotized o-di-anisidine to give a coloured compound (38).

1.6.2 IMPORTANCE OF LIPASES: Lipase are physiologically important since they hydrolyze fats and oils giving rise to free fatty acids and partial glycosides which are essential for metabolic processes such as fatty acid transport, oxidation and resynthesis of glycosides and phospholipids. Additionally, these enzymes are of considerable economic significance in the food industries. If not appropriately controlled, lipase can hydrolyze lipids and produce undesirable rancid flavour in milk products, meat, fish and other food products containing fat. On the other hand, they are essential for the production of desirable and characteristic flavours in certain foods. In fact, lipases have been used in specific applications for the modification of lipids. Certain fatty acids released from milk fat are crucial to the development of cheese flavours (39).

An exciting development is the new focus on lipases that can be used in the mainstream of oleochemical processing. Three key areas with potential for improvement by enzymology are fat splitting for fatty acid production, lipid synthesis via reversal of hydrolysis (40) and lipid modification by ester interchange or inter-esterification (41). There is also currently great interest in biotechnological applications of lipases. One considerable advantage of lipase-mediated reactions over conventional chemical methods resides in the inherent specificity of the enzymes i.e., positional-, fatty acids- and stereospecificities (42).

1.7 AMYLASES: Enzymes responsible for the breakdown of starch are widely distributed in nature. Among these are the amylases, which act on starch, glycogen, and derived polysaccharides to hydrolyze the α-1,4-glycosidic linkages. The amylases may be divided into three groups: the α-amylases, which split the bonds in the interior of the substrate (endoamylases); the β-amylases, which hydrolyze units from the nonreducing end of the substrate (exoamylases); and the glucoamylases, which split off glucose units from the nonreducing terminal of the substrate molecules.

β-Amylase, also called saccharogenic amylase, is present in most higher plants; it is absent in mammals, and its existence in microorganisms is doubtful. It hydrolyzes the α-1,4-glycosidic linkages in starch and glycogen with an inversion of configuration about the C (1) position of the glucose from α to β. This configurational change is the reason why this enzyme is termed β, and it does not signify that the enzyme recognizes β-glycosidic linkages. Furthermore, β-amylase is unable to cleave the α-

1,6-glycosidic bonds in amylopectin and is unable to bypass such linkages; thus the degradation of amylopectin by the enzyme is incomplete. The amylopectin branches are, however, trimmed down by the enzyme, producing, generally, 50-60% maltose. The remaining portion is called a limit dextrin.

Complications arise in the digestion of amyloses derived from common starches. As pointed out above, a complete conversion of this polymer requires perfect linearity of amylose. It appears, however, that this may not be the case, since, generally, only 70-90% conversion may be achieved. Its incomplete degradation may also be caused by the modification of amylose (e.g., oxidation) during preparation of starch and glycogen. When traces of α-amylase are present, the degradation of amylose proceeds to completion.

The cleavage of glycosidic bonds in the α-1,4-glycans take place in a stepwise (exo) fashion starting at a nonreducing end of the chains. Since the enzyme hydrolyzes alternate glycosidic bonds, the product of this reaction is maltose when the enzyme acts on a straight-chain molecule with an even number of glucose residues; when it acts on a chain consisting of an uneven number of residues, some glucose and maltotriose are also found among the end products. The breakdown of maltotriose, resulting in maltose and glucose, proceeds at a much slower rate than the initial β-amylolysis, and it requires the presence of a high concentration of the enzyme. β-Amylase action on the branched macromolecules, e.g., amylopectin, is incomplete, since it is arrested in the vicinity of 1,6-glycosidic linkages. Typical amylopectins yield 50-60% maltose, and the more highly branched glycogen 40-50% maltose (43). The undigested residue is a high molecular weight polymer (β-amylase limit dextrin) containing all the branch linkages of the original polymer.

1.7.1 ACTIVITY OF β-AMYLASE: The exoglycosidase β-Amylase successively removes glucosyl-α-1,4-glucosyl (maltosyl) units from the nonreducing end of the polymer to yield the disaccharide maltose with an OH group in β-configuration at C-1 (44). The amylase activity was measured by estimating the release of maltose.

$$\text{Starch} + H_2O \xrightarrow{\beta\text{-Amylase}} \text{Maltose} + \text{Polymer}_{n-2}$$

Amylases (45-47) comprise the most important group of carbohydrates. They are produced in far larger quantities than any other enzymes and make up the group of amylase enzyme, which hydrolyze starch. Although all amylases hydrolyze the D-glycosidic linkage, they are different in many respects. Most of them hydrolyze only linkages between C-1 and C-4 of two monosaccharides units; others, in addition, hydrolyze bonds between C-1 and C-6.

1.7.2 IMPORTANCE OF AMYLASE: Amylases are among the most important enzymes in present-day biotechnology. The amylase family of enzymes is of great significance due to its wide area of potential application. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorder (48). Amylases find potential application in a number of industrial processes such as in the food, fermentation, textiles and paper industries. Microbial amylases have successfully replaced the chemical hydrolysis of starch in starch-processing industries. They would be potentially useful in the pharmaceutical and fine-chemical industries if enzymes with suitable properties could be prepared (49). With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medicinal and analytical chemistries. Recently Witczak (50), presented a review on the biological relevance of thio-sugars as potential new therapeutics, which are gaining substantial attention. The new developments, especially in the synthetic and medicinal chemistry of thio-sugars are critically important for carbohydrate drug design. Enzyme-controlled mechanisms, involving enzymes such as amylase and lipase, have contributed to the understanding of the biological processes. There are several processes in the medicinal and clinical areas that involve the application of amylases (51-61). Sutton et al. (51), assessed 13 analytes, including amylase, for serum evaluation. Amylases were used for sugar syrup analysis and also used as an enzyme thermistor for the biochemical analysis of cyclo-dextrins (58). They have been used as chiral selector in chromatography (60). β-Amylase is uesful in structural studies of starch and glycogen. Marshal and Whelan (62), report on the removal of any contaminating β–glucosidase.

1.8 PROTEASES: The International Union of Biochemistry and Molecular Biology (1984) has recommended to use the term peptidase for the subset of peptide bond hydrolases. The widely used term protease is synonymous with peptidase. Peptidases comprise two groups of enzymes: the endopeptidases and the exopeptidases, which cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C-terminus respectively. The term proteinase is also used as a synonym word for endopeptidase and four mechanistic classes of proteinases are recognized by the IUBMB as detailed below:

i) The serine proteinases
ii) The cysteine proteinases
iii) The aspartic proteinases
iv) The metallo proteinases

This classification by catalytic types has been suggested to be extended by a classification by families based on the evolutionary relationships of proteases (63). In addition to these four mechanistic classes, there is a section of the Enzyme nomenclature, which is allocated for proteases of unidentified catalytic mechanism. This indicates that the catalytic mechanism has not been identified but the possibility remains that novel types of proteases do exist.

1.8.1 ACTIVITY OF PROTEASE: Proteases hydrolyze peptide bonds in polypeptides into amino acids. The proteolytic activity was measured by detecting the release of amino acid, tyrosine by protease. Proteases hydrolyze proteins and the product are formed in the following way:

$$\text{Proteins} + H_2O \xrightarrow{\text{Protease}} \text{Amino acid} + \text{Polypeptides}$$

Many methods have been developed for the quantitative measurement of proteolytic activity. Protein-based substrates are often used for this purpose because of their sensitivity to the majority of proteases. These substrates are especially useful when activity of endoproteases with unknown specificity is to be determined such as in the case of newly discovered and isolated proteases of microbial origin. Both soluble and insoluble protein-based substrates can be used in the assays. Proteins are usually labelled with an appropriate marker, such as a dye (64), fluorescent label (65) or an appropriate radioactive label (66). The simplest assays use chromolytic substrates with spectrophotometric detection.

1.8.2 IMPORTANCE OF PROTEASE: The proteases are usefull in various ways and their applications are increasing at a fantastic rate. Proteases are used in the degumming of silk goods, in the manufacture of liquid glue, in the preparation of cosmetics, in the preparation of detergents, in the meat tenderization, in the preparation of cheese, in medicine preparation and in agriculture as growth promoters (67-69). The major sources of these proteases are microorganisms (70) but proteases of plant origin have not been extensively examined. So far studies have been conducted on proteolytic enzymes occuring in plant seeds and beans including wheat (71), barley (72), sorghum (73), corn (74), potato tubers (75), mung beans (76), soybean (77), sunflower seeds (78), rice seeds (79), Moringa oleifera seeds (80) and lotus seeds (81). Ichishima (82) have reported that most of the plant proteases are neutral or alkaline and there are few acid proteases with a pH optimum at 2-3 are widely distributed in the plant seeds and play some important physiological roles in the metabolism of seed proteins.

1.9 REVIEW OF THE LITERATURE:

All over the world much works were performed on the chemical composition, nutritive value and industrial uses of Brassica and wheat seeds. Lipase, β-amylase and protease were isolated, purified and characterized by several workers. Some reports are summarized below.

Geddes (83) reported the following data on the composition of the Canadian wild mustard seeds: Nitrogen (7.2%), fixed oil (25.4%) and ash (4.38%). Rollet and Raquet (84) determined the composition of French rapeseed oil by fractional crystallography of the fatty acid followed by fractional distillation of the methyl esters. Jean, et al., (85) reported the amino acids composition of the protein found in the oil cake of rape which is used as fertilizer in French. They found that 20 amino acids are commonly present in protein and reported a total of 28 amino acids. Degradation of nutrient compounds namely sugar, protein and starch in germinating Brassica seeds are in good agreement with earlier studies by Gad *et al* (86). The results of oil degradation in germinating Brassica seeds are quite similar with previous studies by Miled Ben *et al.* (87).

Lipases are versatile enzymes that catalyze the hydrolysis of ester linkages, primarily in neutral lipids such as triglycerides. They hydrolyze the acyl chains either at primary (88) or secondary positions (*Candida antarctica* lipase, *Geotrichum condidum* lipase, etc.). Storage substances, particularly the storage protein, lipid and their regular degradation play an important role in the germinating seeds, determined the first steps of the seedling development. Lipases play a key role in the degradation of lipid of germinating oil seeds. Germinating oil seeds have been investigated by several workers (89).

During recent years considerable attention has been devoted to lipase of botanical origin and microorganism (90). Several lipases of animal origin, especially pancreas lipase, have been purified and their chemical and enzymatic properties were elucidated in detail (91,92).

The lipase activity in the pancreas was first demonstrated in 1846 and a gastric lipase was first reported in 1858 (93). The presence of lipase in plant seeds was demonstrated by Green in 1890 (94).

In plants, the regulation, in some cases the location and the exact physiological roles of lipase are not very clear. Oil seeds (*Brassica napus*) lipases that are the best described have been shown to be localized in oil bodies (95,96) or glyoxyzomes (97,98). They are known to play an essential role in the mobilization of seed-storage lipids to support germination and postgerminative embryonal growth. Lipases are generally considered to be absent in most dry seeds and are probably synthesized de novo after the germination; a triacylglycerol lipase has been purified from ungerminating dry seeds of *Vernonia galamensis* (99).

Iwai et al. (100) have purified two lipases from *Penicillium cyclopium* having molecular weights of 27,000 and 36,000. Iwai and Tsujisaka (101) also reported the presence of three lipases in *Rhizopus delemer*. Funatsu et al. (102) have purified two lipases from *Oryza sativa* having molecular weights of 32,000 and 40,000.

Most of the lipases are active up to 45^0C and the activity declined sharply to 65% at 65^0C and then gradually decreased thereafter. The pancreatic lipases and the reported

plant triacylglycerol lipases lose activity at high temperatures, but some microbial lipases have been reported to be resistant to temperature up to 50^0C (103). The rice bran lipases retained 65% activity at temperature 60^0C. Thermally stable lipase was isolated from *Oryza sativa,* shows maximum activity at temperature 80^0C and stable up to 100^0C.

Pancreatic lipase (104) and rice bran lipases were stimulated in the presence of calcium. The primary role of Ca^{++}seems to be to remove the released fatty acid as its calcium salt. Other divalent cation like Mg^{++}, Zn^{++}, Cu^{++} and Cd^{++} inhibited enzyme activity about 70% in *Oryza sativa* thermal stable lipases.

β-amylase enzymes have been purified and characterized from the different types of plant sources and a few of microbial origin. Such as-

Yamasaki (105), isolated β-amylase from germinating millet seeds by a procedure that include ammonium sulfate fractionation, chromatography on DEAE-cellufine and CM-cellufine and reported that the molecular weight of the enzyme was estimated to be 58 kD with a single sub-unit. Amylose, amylopectin and soluble starch were the most suitable substrates for the enzyme.

Ekon et al. (106), purified β-amylase from Sorghum bicolor (L.) by ammonium sulfate fractionation followed by gel filtration on Sephadex G-200 and reported that the molecular weights of 20 kD, 40 kD and 80kD based on the selectivity curve for the Sephadex column determined with standard proteins. The optimum pH and Km value of the enzyme were 5.0 – 5.5 and 1.272 g of starch/L, respectively.

Pauline et al. (107), isolated β-amylase from pea epicotyle by anion exchange and gel filtration chromatography and showed that the molecular weight of the enzyme was approximately 55 – 57kD, a pH optimum of 6.0 and a Km of 1.67 mg/ml (soluble starch). The enzyme was strongly inhibited by heavy metals, p-chloromercuriphenylsulfonic acid and N-ethylmaleimide but much less strongly by iodoacetamide and iodoacetic acid.

Rashad et al. (108), isolated β-amylase from radish-root by following DEAE-cellulose, hydroxyapatite and Sephadex G-200 chromatography and reported that the

enzyme was active against starch, glycogen and α-dextrin but it failed to hydrolyze sucrose, maltose and lactose. Its molecular weight was 58.88kD, as estimated by gel filtration on Sephadex G-200, Km value was 2.85% for soluble starch at optimum pH 5.0 and 45°C. The enzyme was completely inactivated by Cu^{+2}, Fe^{+2}, Ag^{+}, Hg^{+2}, but only moderately inhibited by p-chloromercuribenzoate and strongly activated by EDTA, Zn^{+2}, K^{+}, Ca^{+2} and Co^{+2}.

Chang et al. (109), extracted β-amylase from sweet potatoes by ammonium sulfate fractionation and Sephacryl S-400 HR gel filtration and observed that the molecular masses of the β-amylases were 209kD for Tainung No. 57 and 239kD for Chailai variety, an optimum pH of 5.0 and an optimum temperature of 50°C. Heavy-metal ions, Cu^{+2}, Ag^{+} and Hg^{+2}, and chemical modification agents, PMSF, p-hydroxymercuribenzoic acid and N-bromosuccinimide significantly inhibited the enzyme activities.

Nielsen et al. (110), purified β-amylase from leaves of potato by following ammonium sulfate precipitation, anion exchange chromatography, affinity chromatography and gel filtration and found that the molecular weight of the enzyme was 111kD, an optimum pH of 6.5 and an optimum temperature was 40 °C. The Km value was 0.73mM and the activity was inhibited by cyclodextrins.

Arai et al. (111), studied β-amylase from tubers of ichoimo by ion-exchange chromatography and hydrophobic chromatography and reported that the molecular weight of the enzyme was 60kD, optimum pH of 6.0, optimum temperature of 55°C and a stable pH range of 4.3 to 8.5.

Dicko et al. (112), purified β-amylase from the bulbs of G. klattianus by ammonium sulfate precipitation and gel filtration and observed that the enzyme was a heterodimer protein of 60 and 12kD sub-units, optimum pH of 5.5 and optimum temperature was 55°C.

Yamasaki et al. (113), isolated β-amylase from Ginseng by column chromatography on DEAE-Sephadex A-50, Sephadex G-100 and hydroxyapatite and reported that the molecular weight of the enzyme was 63kD, optimum pH of 5.0 and optimum temperature of 50°C.

Subbramaiah and Sharma (114), purified β-amylase from Sinapis alba cotyledons by affinity chromatography on a starch column and found that the enzyme was a monomer of 58kD. The Km value of the enzyme was 0.24% and activity of the enzyme was inhibited by heavy metal ions (Cu^{2+}, Pb^{2+}, Ag^{+}).

Shen et al. (115), purified β-amylase from Clostridium thermosulphurogenes and reported that the enzyme was a tetramer of 210kD composed of a single type sub-unit, an optimum pH of 5.5 and an optimum temperature of 70°C. The enzyme activity increased with amylose, amylopectin, glycogen and limit dextrins but inhibited by α- and β- cyclodextrins. Km value of the enzyme was 1.68 mg/ml.

Protease enzymes have been purified and characterized from the different types of plant sources and some of microbial origin. Such as, Dutta et al. (116), purified protease from Indigenous medicinal plants, Shinamo and Fukushima, (117), isolated protease from lotus seeds and Hashinage et al. (118), purified protease from fruit juice.

Usha and Singh, (119), purified proteases from germinating winged-bean seeds by following gel filtration on a Sephacryl S-200, CM-cellulose column, DEAE-cellulose column and gel filtration on a Bio-Gel P-60 column and reported that the enzyme was a monomer with a molecular mass of 35kD and a pH optimum of 6.0 and the enzyme was highly activated with divalent ions of Mg^{2+} and Ca^{2+} and completely inhibited by benzamidine, pepstatin, WbCI and SbTI.

Guerra and Nicolas, (120), isolated two proteases, one peptidehydrolase and one aminopeptidase, from the cotyledons of germinating lentils and reported that Peptidehydrolase had an apparent molecular weight of 89kD and an isoelectric point of 4.7. Peptidehydrolase activity was not affected by metal chelators but it was affected by N-bromosuccinimide, phenylmethylsulfonyl fluoride and N-ethylmaleimide, suggesting the presence of tryptophan and serine residues together with free--SH groups in its active site. Peptidehydrolase activity was maximally active from pH 6.0 to 9.0 being practically zero below pH 5.0. It was stable at temperatures up to 40 degrees C, and complete inactivation was obtained at or over 70 degrees C. Aminopeptidase had an apparent molecular weight of 83kD and an isoelectric point of

4.5. Its activity was affected by N-bromosuccinimide, suggesting the presence of tryptophan residues in its active site. The aminopeptidase presents its maximal activity at pH 5.5. It was stable at temperatures up to 40 degrees C, and complete inactivation was detected at over 70 degrees C.

Basu et al. (121), isolated protease from germinated seeds of rice bean, Vigna umbellata (Thunb), by ammonium sulphate precipitation, gel filtration, ion-exchange chromatography and by high performance liquid chromatography (HPLC). The purity of the enzyme was checked by polyacrylamide gel electrophoresis (PAGE) and reported that the molecular weight of the enzyme was about 16kD and an optimum pH of 8.4. The enzyme activity was studied on natural substrates like casein, haemoglobin and vicilin, a rice bean storage protein. The activity of the enzyme was completely inhibited by phenylmethylsulfonyl fluoride, but not by iodoacetamide and $HgCl_2$, suggesting it to be a serine protease. Loss of activity in presence of EDTA was reversed by addition of Ca^{2+}.

Umar Dahot, (122), studied protease activities from ten plant seeds and showed that the optimum pH of the enzyme was 2.5 – 5.5 and an optimum temperature was 30°C and it was stable up to 40°C. The enzyme activity was markedly increased in presence of Ca^{2+}, cysteine and mercaptoethanol but strongly inhibited with EDTA, iodoacetic acid, o-phenanthroline, Hg^{2+} and PMSF.

Chung and Goldberg, (123), purified protease from Escherichia coli by conventional procedures with casein as the substrate and reported that its molecular weight was about 140kD, a broad pH optimum of 6.5 – 8.0 and Km value of 1.4 μM for casein. The enzyme activity was not inhibited by chelating agents, divalent cations but strongly inhibited by N-tosyl-L-phenylalanine chloromethyl ketone.

Thangam and Rajkumar, (124), isolated alkaline protease from Alcaligenes faecalis by DEAE-cellulose and Sephadex G-100 chromatography and observed that the molecular weight of the enzyme was 67kD, optimum pH of 9.0 and optimum temperature of 55°C. The enzyme had a Km of 1.66 mg/ml and a Vmax of 526 units /min per mg of protein and its activity was inhibited by PMSF, suggesting the presence of serine residues at the active site.

Besides these, many other researchers have done their research on protease enzyme from various sources. Such as:

Adinarayana et al. (125), have purified thermostable serine alkaline protease from Bacillus Subtilis PE-11; Beilison et al. (126), from soybean; Csoma and Polgar, (127), from germinating bean cotyledons; Bulmaga and Shutov, (128), from germinating vetch seeds; Forward et al. (129), from Douglas-fir seeds; Mukhopadhyay, (130), from winged bean.

1.10 AIM OF THE PRESENT STUDY: Brassica is the major rabi oil seed crops and wheat is the second most important cereal crops in Bangladesh. Brassica seeds are not only rich in oils and fats but also contain an appreciable amount of proteins, carbohydrates, vitamins and minerals. Oils of these seeds are mostly used for edible purposes but a reasonable amount is used for industrial purposes as well. The cakes are usually used as cattle feed or as fertilizer in our country. Wheat is the number one grain consumed directly by humans. Its nutritional value is very high. It is eaten in various forms by more than one thousand million human beings in the world. Storage substances, particularly the storage carbohydrate and protein in wheat and their regular degradation play an important role in germination of seeds, determining the first steps of the seedling development.

Both types of seeds contain a high amount of storage substances and some enzymes. Few of them are synthesized de novo in the seeds during germination. Lipases are generally considered to be absent in most dry seeds and are probably synthesized de novo after the germination. They are known to play an essential role in the mobilization of seed-storage lipids to support germination and post-germinative embryonal growth. Lipases are extensively used in the dairy for the hydrolysis of milk fat. Current applications include the flavour enhancement of cheeses, the acceleration of cheese ripening, the manufacturing of cheese like products, and the lipolysis of butterfat and cream.

These seeds also contain amylases and proteases. β-amylase is an enzyme that releases successive maltose units from the non-reducing end of a polysaccharide chain by hydrolysis of α-1,4-glucan linkages. It may play a role in the mobilization of starch during germination or the sprouting of tubers (131). It has potential application in

many fields, such as clinical, medicinal and analytical chemistries, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries. Protease enzymes are involved in the breakdown of proteins. It may involve in the seed storage-protein mobilization process (132). It is used in the pharmaceutical industry, in medicine as well as in the food processing industry (e.g., in the preparation of vaccines and for the treatment of hard skin). It is also used in the tanning of leather, in the paper and adhesive industries.

Many workers have been isolated and characterized these enzymes from different sources but the reports from germinating oil seeds (Brassica) and cereal seeds (wheat) are relatively very few. Moreover, the physiological roles of these enzymes in plant during germination of seeds are unclear. To understand these, a systematic study is required.

Keeping all these in mind, in this study we made an attempt to detect the roles of lipase from germinating Brassica seeds, and amylase and protease from germinating wheat seeds. The study comprises the following:

1. Estimation of storage substances of Brassica and wheat seeds during, before and after germination.
2. Purification and characterization of the enzymes lipase, amylase and protease from the germinating seeds of Brassica and wheat.
3. To study the effect of physico-chemical agents on the stabilities of the enzymes.
4. To improve the functional properties of gluten by protease from germinating wheat seeds.

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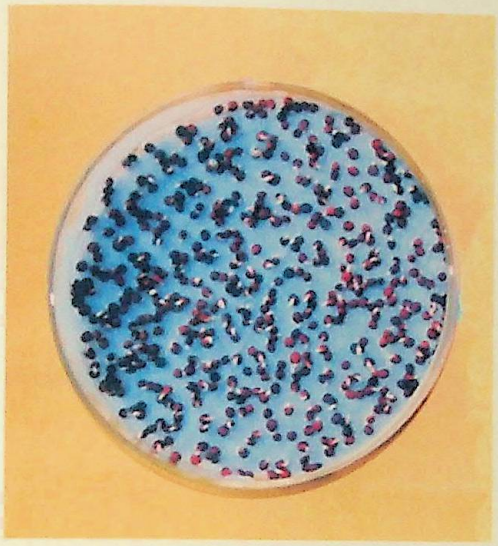
CHAPTER-2

MOBILIZATION OF NUTRIENTS IN BRASSICA AND WHEAT SEEDS DURING GERMINATION

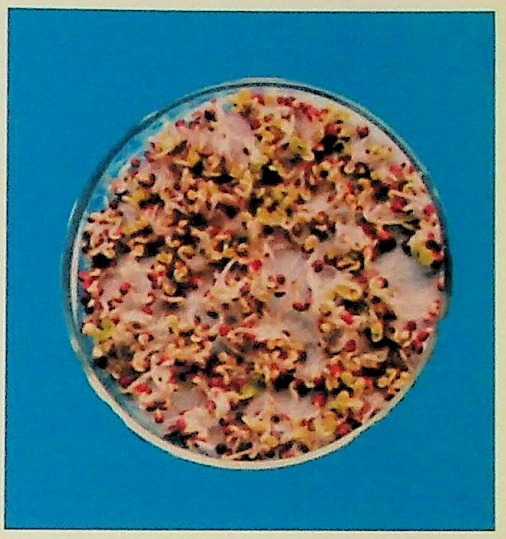
2.1 INTRODUCTION: In plant seeds the storage nutrient substances are protein, fat and carbohydrate. During germination these storage nutrients used up for seedling growth (1). Seeds are classified into two distinct types according to the main compounds stored: those that accumulate mostly lipids and proteins and those that accumulate mostly carbohydrates and proteins (2). These reserve substances are degraded during germination and early plantlet growth and translocated to the growing parts. In plants a large proportion of carbon reserves as triacylglycerols (TAG), such as rapeseed (B. napus) and Arabidopsis, the activation of the (-oxidation and glyoxylate cycle during germination ensures conversion of fatty acid to carbohydrates necessary for the growth of the seedling before establishment of photosynthesis (3,4). This is the first step of TAGs conversion to sugars required for growth of the germinating embryo (5). The germination of lipid-rich seeds such as rapeseed has been involved among other processes, the rapid mobilization of storage triacylglycerols (TAGs) in the cotyledons of seedlings. Such hydrolysis of TAGs is catalyzed by highly active lipases. Enzymes involved in the process of lipid mobilization, such as malate synthase and isocitrate lyase, are detectable towards the late stages of embryo development (6). Starch is the major component of most of the word's crop yield and the degradation of starch is essential in the germination of these plants (7). Starch degradation in the cereal grain requires the concerted action of several enzymes (8) including limit dextrinase, β-amylase, α-glucosidase (9), and α-amylase. Plants accumulate and store proteins in protein storage vacuole during seed development. Upon seed germination, storage proteins are degraded by hydrolytic enzymes to provide nutrients for embryo/seedling growth (10). Some metabolic changes in reserve compounds can be detected histologically in germinating seeds with the use of appropriate and specific histochemical procedures. During soybean germination, protein content remained constant inside the phloem elements whereas it decreased in other tissues thus suggesting that transportation of this storage material occurred (11). In Dalbergia miscolobium histochemical data suggested a similar pattern of events for protein and lipids during the germination and initial phases of seedling growth (12). There have been very few reports on the degradation of seed storage substances particularly on oil and cereal seeds (13,14). Therefore, the aim of the present work was to study the degradation of seed storage substances of Brassica (Brassica napus L.) and wheat (Triticum aestivum L.) seeds during germination.

2.2 MATERIALS AND METHODS: Three varieties of Brassica seeds (Brassica napus L., Brassica juncea L. and Brassica campestris L.) and three varieties of wheat seeds were collected from Bangladesh Agriculture Research Institute (BARI), Pabna, Dinajpur, Rajshahi and Jessore. Some seeds were also collected from local market for preliminary work. Successful seed storage starts with collection. If seeds are not mature and healthy, when stored, their germination rate will be poor, their seedlings will have poor vigor, and their storage life will be short. Nothing can be done after collection to improve seeds that were of poor quality to begin with. Collectors should strive to collect mature seeds from healthy plants. For avoiding these problems, the seeds were collected from different places and the seeds, which were immature, broken and damaged by insects, were cut out. The appropriate seeds were washed in water, dried in the sunlight and kept in a polyethylene bag and stored in an airtight desiccator with absorbent in a polyethylene bag for experimental purpose. Shephadex G-75, DEAE-cellulose and SDS were purchased from Sigma Chemical Co. Ltd., USA. CM-cellulose, Marker proteins and Petroleum ether (40-60° C) were the products of British Drug House (BDH), Poole, England. All other chemicals used in this study were commercially available and of high purity.

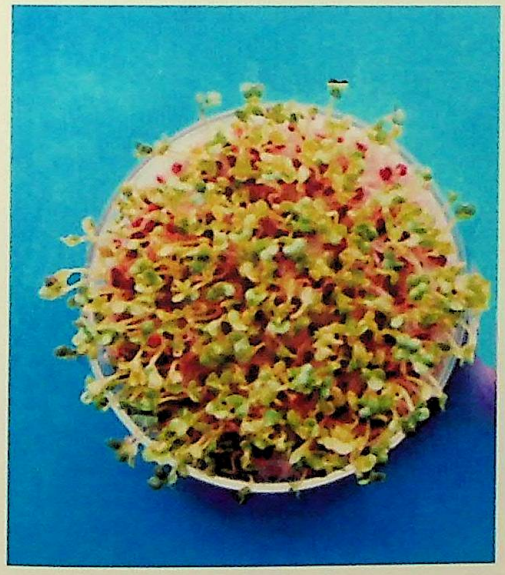
2.2.1. GERMINATION OF SEEDS: Germination does not take place unless the seed has been transported to a favorable environment by one of the agencies of seed dispersal. The primary conditions of a favorable environment are adequate water and oxygen and suitable temperature. Different species of plants germinate best in different temperatures; as a rule, extremely cold or extremely warm temperatures do not favor germination. Some seeds also require adequate exposure to light before germination. For this reason, good and mature seeds of Brassica and wheat were soaked in distilled water within a glass beaker with potassium permanganate for six hours. Potassium permanganate was used for avoiding the growth of microorganism on seed surfaces during germination. In order to germinate, the seeds were taken out from water and scattered in 20-cm Petri dishes on two sheets of Whitman No. 1 filter paper moistened with little amount of distilled water. The dishes were then placed in the light room at 25° C for 120 hours including soaking time. The germinating seeds at different hours (0, 24, 32, 48, 96 and 120 h) were collected and stored separately in the deep freeze for further experimental purposes. Various germinating stages (24, 48, 72 and 96h) of Brassica and wheat seeds are shown in the following figures as sample.



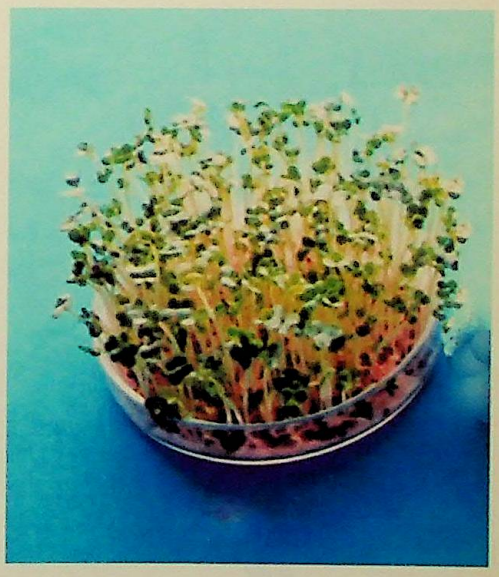
24h



48h



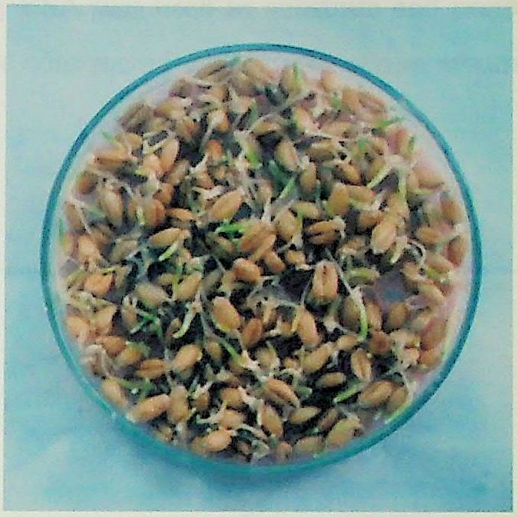
72 h



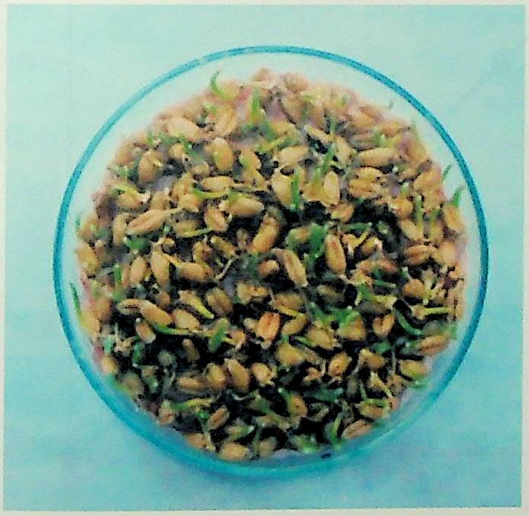
96 h

Figure-2.1: Photographic representation of Brassica seeds at different periods of germination.

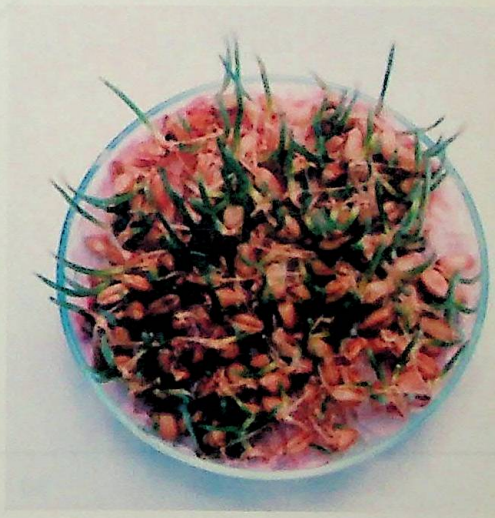
24h



48h



72 h



96 h

Figure-2.2: Photographic representation of wheat seeds at different periods of germination.

2.3 BIOCHEMICAL ANALYSIS: Total protein content of Brassica and wheat seeds was determined by the method of Micro-Kjeldahl (15) and the water-soluble protein content by the method of Lowry et al. (16). BSA was used as substrate and protein content was calculated from a standard curve constructed with bovine serum albumin (figure-2.3).

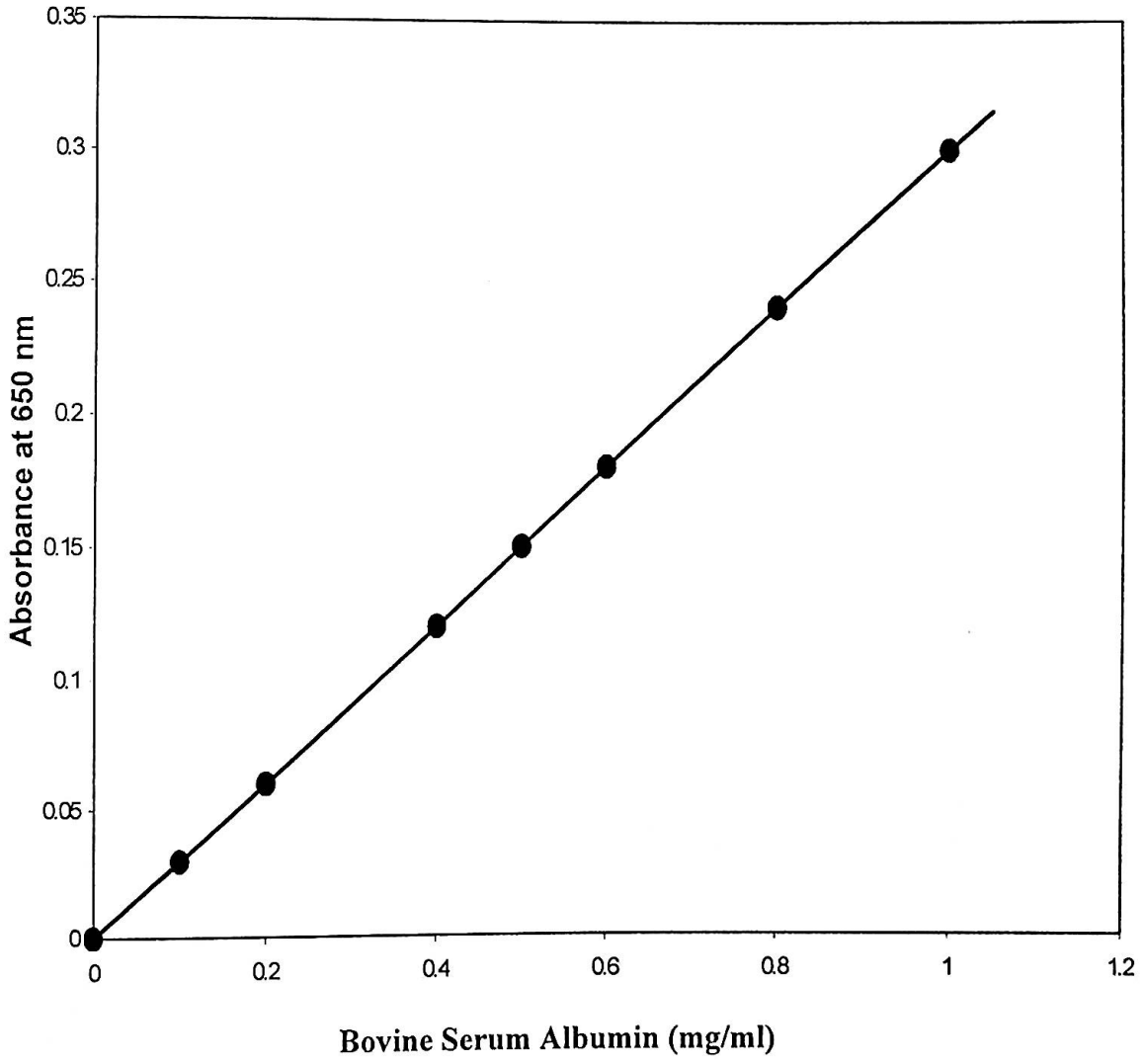


Figure-2.3: Standard curve for estimation of water-soluble protein by Lowry method.

Free sugar content was determined colorimetrically by the anthrone method (17). Glucose was used as standard substrate and sugar content was calculated from a standard curve constructed with glucose (figure-2.4).

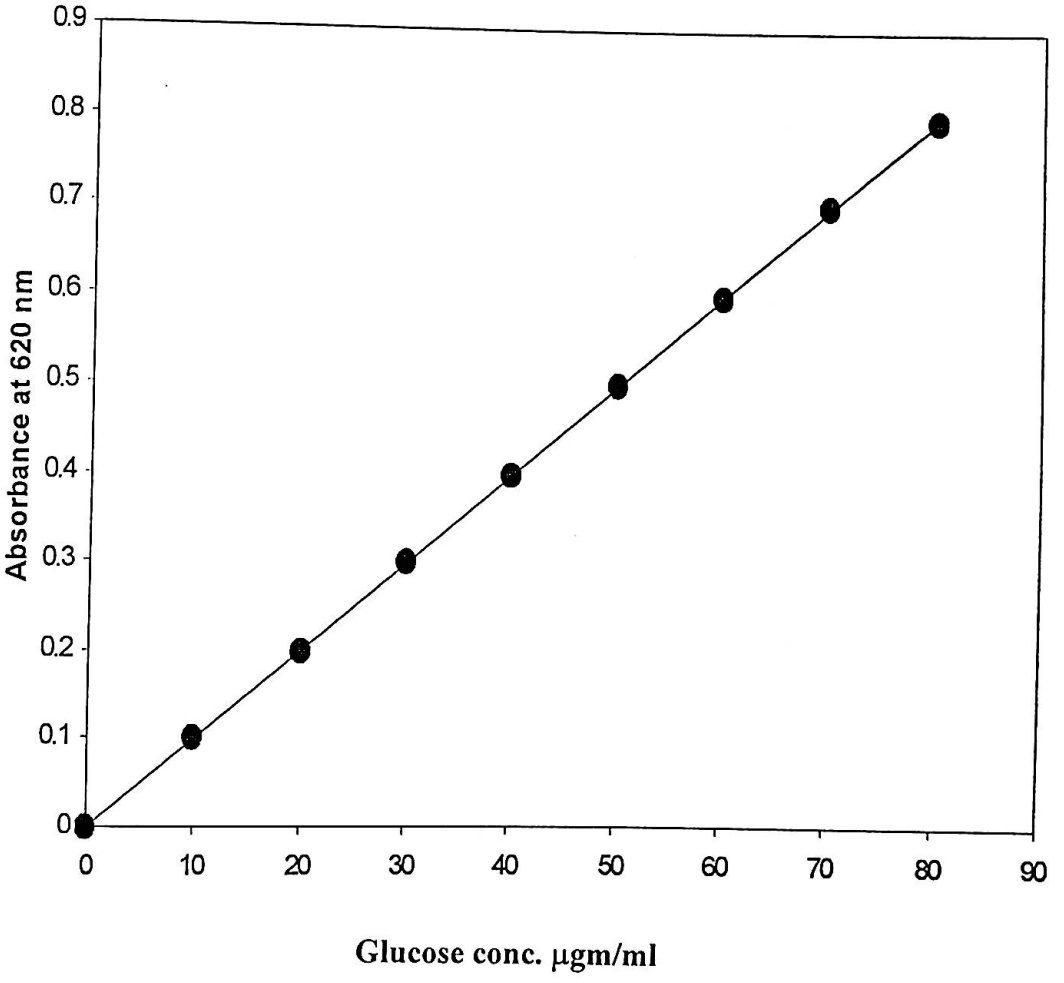


Figure-2.4: Standard curve of glucose for estimation of free sugar and starch.

Reducing sugar content was estimated by DNS (Dinitrosalicylic acid) method (18). Extraction of sugar from Brassica and wheat seeds was done following the method of Loomis and Shull (19) and the amount of reducing sugar was calculated from a standard curve constructed with glucose (figure-2.4).

The starch content of Brassica and wheat seeds was determined by the anthrone method (17,20) and the amounts of starch were also calculated from a standard curve constructed with glucose (figure-2.4).

The oil was extracted from the Brassica and wheat seeds of previously cleaned, dried and stored seeds by the solvent extraction process. For this purpose, the seeds were crushed in a glass mortar and the oil was extracted with petroleum ether (40^0-60^0C): acetone (1:1 v/v) in a Soxhlet apparatus as reported in the literature (21). The amount of oil was calculated using electrical balance (mettler H18). Each analysis was performed in triplicates and the averages were taken.

2.4 RESULTS

Table-2.1: Amounts of free sugar in the three varieties of Brassica and wheat seeds at different periods of germination.

Seeds	Varieties	Free Sugar (gm %) at different hour (h)					
		(0,h)	24h	48h	72h	96h	120h
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Brassica	B. Napus	4.20 ± .02	2.92 ± .05	2.58 ± .07	1.12 ± .04	0.99 ± .03	0.95 ± .03
	B. Campestries	4.15 ± .02	2.87 ± .07	2.55 ± .05	1.11 ± .03	0.95 ± .06	0.93 ± .06
	B. Juncia	4.27 ± .03	2.94 ± .04	2.58 ± .05	1.18 ± .02	1.05 ± .05	0.99 ± .09
Wheat	Akbar	4.88 ± .07	4.79 ± .07	4.72 ± .04	4.66 ± .04	4.03 ± .04	3.82 ± .04
	Kanchan	4.87 ± .07	4.78 ± .03	4.75 ± .05	4.67 ± .07	4.22 ± .05	3.96 ± .06
	Agrani	4.76 ± .06	4.65 ± .05	4.63 ± .04	4.59 ± .04	4.20 ± .07	3.83 ± .05

Table-2.2: Amounts of reducing sugar in the three varieties of Brassica and wheat seeds at different periods of germination.

Seeds	Varieties	Reducing Sugar (gm%) at different hour (h)					
		0, h	24h	48h	72h	96h	120h
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Brassica	B. Napus	.093± .06	.070± .06	.042± .06	.037± .07	.022± .06	.012± .04
	B. Campestries	.091± .04	.070± .04	.042± .05	.037.± .05	.022± .04	.012± .07
	B. Juncia	.089± .07	.069± .04	.042± .07	.037± .06	.022± .05	.012± .05
Wheat	Akbar	.078± .05	.068± .05	.057± .06	.054± .05	.049 ± .07	.042± .06
	Kanchan	.067± .07	.056± .07	.047± .06	.044± .07	.039± .05	.033± .06
	Agrani	.064± .04	.054± .07	.042± .07	.041± .05	.037± .06	.032± .05

Table-2.3: Amounts of starch in the three varieties of Brassica and wheat seeds at different periods of germination.

Seeds	Varieties	Starch (gm%) at different hour (h)					
		0, h	24h	48h	72h	96h	120h
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Brassica	B. Napus	4.67± .06	4.32± .06	2.57± .06	1.79± .07	1.58± .07	1.44± .05
	B. Campestries	4.57± .06	4.30± .05	2.52± .06	1.75± .06	1.55± .06	1.42± .08
	B. Juncia	4.70± .05	4.40± .05	2.67± .06	1.85± .07	1.65± .05	1.52± .06
Wheat	Akbar	69.65± .05	58.49± .08	38.29± .07	28.25± .05	18.36± .05	10.26± .05
	Kanchan	68.25± .05	58.15± .05	38.12± .06	28.14± .05	18.32± .06	10.28± .07
	Agrani	67.35± .06	56.39± .09	38.11± .05	27.34± .06	17.28± .06	09.38± .07

Table-2.4: Amounts of total protein in the three varieties of Brassica and wheat seeds at different periods of germination.

Seeds	Varieties	Protein (gm%) at different hour (h)					
		0, h	24h	48h	72h	96h	120h
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Brassica	B. Napus	25.37± .06	22.62± .07	18.87± .06	9.98± .07	7.9± .05	4.5± .05
	B. Campestries	25.27± .06	21.62± .05	17.82± .06	8.95± .05	6.8± .07	3.4± .05
	B. Juncia	25.17± .06	21.32± .07	17.52± .04	8.55± .05	6.3± .03	3.1± .02
Wheat	Akbar	14.24± .03	9.88± .03	8.32± .03	5.87± .04	3.90± 2	1.5± .02
	Kanchan	13.57± .03	8.92± .03	7.85± .02	5.12± .03	3.11± .03	1.6± .02
	Agrani	12.35± .04	7.95± .05	6.98± .05	4.82± .04	3.88± .04	1.5± .03

Table-2.5: Amounts of water-soluble protein in the three varieties of Brassica and wheat seeds at different periods of germination.

Seeds	Varieties	Protein (gm%) at different hour (h)					
		0, h	24h	48h	72h	96h	120h
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Brassica	B. Napus	18.37± .06	15.62± .06	11.87± .02	08.98± .05	5.91± .04	3.2± .03
	B. Campestries	17.85± .05	14.60± .04	10.85± .04	07.96± .02	4.90± .03	2.4± .04
	B. Juncia	17.80± .03	14.55± .02	10.82± .02	07.91± .03	4.85± .05	2.3± .02
Wheat	Akbar	6.50± .03	6.42± .03	5.35± .05	3.57± .02	1.98± .03	1.1± .03
	Kanchan	6.48± .04	6.52± .03	5.27± .04	3.20± .03	1.99± .04	1.2± .04
	Agrani	6.14± .04	6.02± .04	5.29± .05	3.85± .04	1.62± .04	1.0± .02

Table-2.6: Amounts of oil in the three varieties of Brassica and wheat seeds at different periods of germination.

Seeds	Varieties	Oil (gm%) at different hour (h)					
		0,h	24h	48h	72h	96h	120h
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Brassica	B. Napus	43.6± .04	38.33± .03	15.43± .05	5.79± .05	3.34± .03	2.92± .04
	B. Campestries	42.4± .05	38.30± .02	15.40± .04	5.78± .04	3.32± .03	2.90± .05
	B. Juncia	41.3± .02	38.28± .04	15.40± .03	5.76± .02	3.30± .02	2.89± .05
Wheat	Akbar	1.92± .04	1.61± .03	1.52± .05	1.43± .04	1.15± .04	1.08± .03
	Kanchan	1.73± .04	1.54± .03	1.35± .05	1.29± .04	1.20± .04	1.10± .03
	Agrani	1.65± .05	1.46± .02	1.40± .05	1.33± .04	1.27± .02	1.13± .04

2.5 DISCUSSIONS

Free sugars, particularly glucose, are important energy fuels in the nervous systems, muscles and many other tissues. Combined with proteins as glycoproteins, the sugars play a role in secretion and external recognition properties of cell membranes. The free sugar content in three varieties of Brassica and wheat seeds is shown in table-2.1. From the tables, it is found that wheat seeds contain a larger amount of free sugar than that of Brassica seeds. In Brassica seeds, Juncia variety contains a little higher amount of free sugar (4.27%) than that of Napus (4.20%) and Campestries (4.15%) varieties. While in wheat seeds, Akbar variety contains higher amount of free sugar (4.88%) than that of Kanchan (4.87%) and Agrani (4.76%) varieties. During germination, the degradation of free sugar in Brassica seeds is rapid while the degradation in wheat seeds is comparatively slow.

Reducing sugar content in three varieties of Brassica and wheat seeds is represented in table-2.2. From the tables, it is observed that reducing sugar content in the Brassica seeds is higher than that of wheat seeds. Both in Brassica and wheat seeds, there is a little variation of reducing sugar content in the three varieties. Degradation of reducing sugar in different periods of germination in the Brassica and wheat seeds are significant. In Brassica seeds reducing sugar is converted from .093% to .012%, whereas in wheat seeds the conversion is from .078% to .032%. The result indicates that degradation of reducing sugar is very faster in Brassica seeds than that of wheat seeds.

One of the nutritional reservoirs in plants, which is used as a fuel, is starch. Starch is the most important polysaccharides in the storage form of carbohydrate in plant. Most plant cells have the ability to form starch, but it is especially abundant in tubers such as potatoes and in seeds. It is found that, wheat seeds contain a larger amount of starch (69.65% - 67.35%) than that of Brassica seeds (4.70% - 4.57%)(table-2.3). In wheat seeds, Akbar variety contain the highest amount of starch (69.65%), followed by Kanchan (68.25%) and Agrani (67.35%). Similarly, in Brassica seeds, Juncia variety contains the highest amount of starch (4.70%), followed by Napus (4.67%) and Campestries (4.57%). The starch content of both the seeds decrease gradually during germination. In light, germination probably first degrades starch for embryo growth and latter other enzymes utilize seed's storage substance, for the energy supply, required for seedling growth.

Total protein and water-soluble protein content of the different varieties of Brassica and wheat seeds are presented in table-2.4 and 2.5 respectively. From the tables, it is found that the Brassica seeds contain a significant amount of protein (total 25.37% - 25.17% and water-soluble 18.37% - 17.80%). In wheat seeds, Akbar variety contain the highest amount of protein (total 14.24%, water-soluble 6.50%), while Agrani contain the lowest amount of protein (total 12.35%, water-soluble 6.14%). The present results clearly demonstrate that the percentage of both types of proteins present in different varieties of Brassica and wheat seeds decrease gradually up to 48 hours and then sharply decline up to 120 hours of germination. This indicates that after 48 hours of germination, the proteolytic enzymes may vigorously involve for hydrolysis on seed storage proteins.

The oil content of different varieties of Brassica and wheat seeds is shown in table-2.6. The oil content in Brassica seeds is much higher than that of wheat seeds as usual. In Brassica seeds, Napus variety contains the highest amount of oil (43.6%), followed by Campestries (42.4%) and Juncia (41.3%) varieties. While in wheat seeds, Akbar variety contains slightly higher amount of lipid than that of other two varieties. The oil content found to degrade quickly up to 48 hours and then gradually decline up to 120 hours of germination. The decrease in oil content in the germinating seeds probably caused by the involvement of a lypolytic enzyme, which is responsible for hydrolysis of triacylglycerol, that ultimately generate sugars for the growth of germinating embryo. This finding is in good agreement with those reported by Miled Ben. *et al.* (5).

The degradation of nutrient components namely sugar, protein, starch and lipid were found to be much different and the results are in good agreement with earlier results reported by Gad *et al.* (22). The seed storage substances gradually decrease with the increase of germination time. The decrease in different types of nutrient content in the germinating seeds probably caused by the involvement of the hydrolytic enzymes, which hydrolyses seed storage nutrient, a process that generates amino acids and sugars for the development of embryo and seedling growth.

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CHAPTER-3

PURIFICATION AND CHARACTERIZATION OF LIPASE FROM GERMINATING OIL SEEDS (BRASSICA NAPUS L)

3.1 INTRODUCTION: The storage nutrient substances in plant seeds are used up for seedling growth during germination (1). During mobilization of storage lipids in seeds in postgerminative growth, the triacylglycerol is converted to sugars and other metabolites for the growth of the embryonic axis (2,3). The lipase (lipid converting enzyme) activity is absent in the ungerminated seeds and increases in seedling growth. Lipases are active in lipid-storing tissues in seeds during germination. Corn lipase is most active on triacylglycerols of linoleic acid and oleic acid, which are the major fatty acid constituents of corn oil (4). Rapeseed and mustard seed (whose storage triacyiglycerols contain substantial amounts of erucic acid) lipases are more active on triacylglycerols of erucic acid than of stearic acid or behenic acid (5). The palm kernel lipase is more active on tricaprin than trilaurin or tripalmitin (6). Lipases are versatile enzyme that catalyze the hydrolysis of ester linkage, primarily in neutral lipids such as triglycerides and are widely distributed in various animals, plants and microorganisms (7-13). Some lipases are shown to exist in multiple forms in animal tissues such as rat liver (14), rat adipose tissue (15), porcine pancrease (16) and human adipose tissue (17), and in some species of mold (18,19). With respect to the plant lipase, the existence of two lipases, acid and alkaline lipase, has been pointed out in castor bean endosperm (20). They hydrolyze the acyl chains either at primary or secondary positions (21,22). Mammals, bacteria and plants produce lipases in large amounts. Plant lipases hydrolyze triglycerols at much lower rate (usually <0.5 μmol min^{-1} mg^{-1}) as compared with animal or microbial lipases. The plant lipase is located in the endosperm of the seeds and is bound to the oleosin/phospholipid coat, which surrounds the storage oil bodies (23,24). Oil seeds (*Brassica napus* L) lipases have been shown to be localized in oil bodies (25,5) or glyoxysomes (20,26). They are known to play an essential role in the mobilization of seeds storage lipids to support germination and post-germinative embryonal growth. In the last decade, lipases often have been perceived by research scientists as one of the most important class of industrial enzymes (27). For instant, lipases have been used extensively in the dairy industry, in the oleo chemical industry and to produce structured triglycerols (28,16). So far lipases have been purified from several different sources by using several different methodologies (29,30). The characteristics of the rape seed enzyme are quite different from those reported earlier (31-33). The present study describes the purification and characterization of lipases from germinating oil seeds as sources, specially *Brassica napus* seeds for the first time.

3.2 MATERIALS AND METHODS: Discussed in chapter 2.2.

3.2.1 GERMINATION OF BRASSICA SEEDS: Discussed in chapter 2.2.1.

3.2.2 PREPARATION OF DRY POWDER: The germinated seeds were crushed in a mortar and pestle and then homogenized with twice the volume of ice-cold acetone. The suspension was filtered through cheese cloth and quickly washed with successive portions of acetone, acetone-ether (1:1, v/v) and ether. The mass was then air dried with gentle stirring. The powder, thus obtained called the dry powder was stored in a refrigerator for further experiment.

3.2.3 PREPARATION OF CRUDE EXTRACT: The stored dry powder (250 gm) was suspended in 1 liter ice-cold water. After occasional gentle stirring for 3 hours at 4^0C the suspension was filtered through cheese cloth. The filtrate was collected and centrifuged in a refrigerated centrifuge at 6000 rpm for 20 minutes at 4^0C. The supernatant was used as "crude extract".

3.2.4 AMMONIUM SULPHATE FRACTIONATION: The "crude extract" was saturated to 80% by addition of solid ammonium sulphate under constant and gentle stirring at 4^0C. The resulting precipitate was collected by centrifugation, dissolved in minimum volume of pre-cold distilled water and dialyzed against distilled water for 24 hours at 4^0C. The dialyzed solution was then centrifuged in a refrigerated centrifuge at 8000 rpm for 5 minutes to remove the insoluble materials. The clear supernatant thus obtained was designated as "crude enzyme solution".

3.2.5 GEL FILTRATION: Gel filtration was carried out on Sephadex G-75 column. The "crude enzyme solution" after dialysis against 50 mM phosphate buffer, pH 7.2, was loaded onto the Sephadex G-75 column previously equilibrated with the same buffer and the protein was eluted with the buffer at 4^0C, at a flow rate of 1.0 ml per min. The fractions were collected for analysis of protein concentration and enzyme activity using 280 nm absorbance.

3.2.6 DEAE-CELLULOSE COLUMN CHROMATOGRAPHY: The enzymatically active protein fractions after gel filtration were collected and dialyzed against 10 mM

Tris-HCl buffer, pH 8.4 for 24 hours and then concentrated to 1/4th of its volume by freeze dryer and the sample was loaded on the DEAE-cellulose column (32x1.0 cm, flow rate 25ml/hour) previously equilibrated with 10 mM Tris-HCl buffer, pH 8.4, at 4°C. The proteins were eluted from the column with the same buffer containing (100-500 mM) NaCl gradient. Using absorbance at 280 nm, protein concentration and enzyme activities were determined and the active fractions were collected.

3.2.7 CM-CELLULOSE COLUMN CHROMATOGRAPHY: The active fraction from DEAE-cellulose column chromatography was dialyzed against distilled water for 12 hours and against 10 mM phosphate buffer, pH 6.5 for 12 hours at 4°C. The dialyzed sample was loaded on the CM-cellulose column at 4°C. The proteins were eluted from the column with the sample buffer containing (100-500 mM) NaCl gradient.

3.2.8 ENZYME ASSAYS (METHOD A): Lipase activity was assayed essentially as described by Sugihara *et. al.* (34). Olive oil was used as substrate. The lipase activity was measured by estimating the release of fatty acids. One unit of lipase activity is defined as the amount that liberates one μ mole of fatty acid under the specific condition. Specific activity of lipase was expressed as the enzyme unit per mg of protein.

METHOD B: The lipase activities were quantified by the hydrolysis of triolein as described by Macedo and Pastore (35). One unit (U) of lipase activity was defined as 1 mmol of oleic acid released per minute at 37° C. All the experiments were carried out in triplicate and the average were reported.

3.2.9 MOLECULAR WEIGHT DETERMINATION OF LIPASE (a): The molecular weight of *Brassica napus* lipase was determined by gel filtration on a shephadex G-75 column (90x0.9 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.2, following the procedure as described by Andrews (36). Trypsin inhibitor (12.028 kD), carbonic anhydrase (29 kD), α-amylase (58 kD), bovine serum albumin (68 kD) and β–galactosidase (116 kD) were used as marker proteins (37).

(b) *ELECTROPHORESIS*: SDS-PAGE was performed according to the method of Laemmli on a Bio-rad mini electrophoresis system (38). The standard proteins were lysozyme (14 kD), β-lactoglobulin (18.4 kD), trypsinogen (24 kD), pepsin (36 kD), albumin (egg white 45 kD), albumin (BSA, 67 kD). PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A.

3.2.10 EFFECT OF pH ON ENZYME ACTIVITY: For the purpose, the enzyme solutions (0.5-0.6%) were dialyzed against 50 mM buffer of different pH's (pH 2.0-3.0. AcONa-HCl; pH 4.0-5.0, AcONa-CH_3COOH; pH 5.5-8.0, NaH_2PO_4-Na_2HPO_4; pH 8.5-9.0, $Na_2B_4O_7$-HCl; pH 9.5, $Na_2B_4O_7$-$NaCO_3$.) for 24 hours with frequent change of buffers. After necessary adjustment of pH values by addition of 0.2 N HCl or 0.2 N NaOH, the enzyme activities were assayed using olive oil as substrate at room temperature (25-28^{0}C).

3.2.11 OPTIMUM TEMPERATURE FOR THE ENZYME ACTIVITY: In order to determine optimum temperature, the enzyme solutions (0.5%) in 50 mM sodium acetate-HCl buffer, pH 7.0, were incubated at various temperature (10-70°C) for 30 minutes in a temperature controlled water bath and the activities remaining were assayed.

3.2.12 SUBSTRATE SPECIFICITY: Substrate specificity of *Brassica napus* lipase was measured using substrate as castor oil, coconut oil, corn oil, linseed oil, olive oil, peanut oil, rapeseed oil and soybean oil. In order to determine substrate specificity, 4.5 ml of 50 mM Phosphate buffer pH 7.0 and 0.5 ml $CaCl_2$, was taken in different conical flask and 1 ml of substrate of different type was taken in the flask. Then 1 ml of enzyme solution were added in each flask and incubated at 35^{0}C for 30 minutes. Then the amount of fatty acids released during the incubation was estimated by titrating the mixture with 50 mM KOH.

3.2.13 TREATMENT WITH UREA, EDTA AND VARIOUS SALTS: Lipase solutions (0.3mg/ml) were dialyzed separately against deionized water at 4^{0}C for 48 hours before incubation. The dialyzed lipase solution (200 µl) were added to urea, EDTA and various salts solutions of different concentrations and incubated for 30 minutes at 35^{0}C pH 7.0, using olive oil as substrate (1 ml/tube). The activities were assayed at room temperature.

3.2.14 MEASUREMENT OF Km VALUE OF LIPASE: Michaelis constant (Km) was determined by Lineweaver-Burk double reciprocal plot. The initial velocity (Vi) is equal to the amount of product formed per unit time. The initial velocity (Vi) is determined quantitatively by measuring the amount of one of the products at various time intervals (39).

3.2.15 PROTEIN ASSAY: Protein concentration of each fraction was determined by UV-visible spectrophotometer at 280 nm. The amount of protein was estimated by the published method of Lowry et al., (40) using BSA as standard substrate.

3.3 RESULTS

3.3.1 TIME COURSE STUDY OF LIPASE: The lipase from the three varieties of germinating oilseeds (*Brassica napus, Brassica campestries* and *Brassica juncia*) in water treatment showed their maximum activity after 40 hours of germination (in crude and in partial purified form) and then declined rapidly (figure-3.1). The lypolytic activity of Brassica napus variety was higher than that of other two varieties. So for the extraction of lipase we used 40 hours germinated Brassica napus extract.

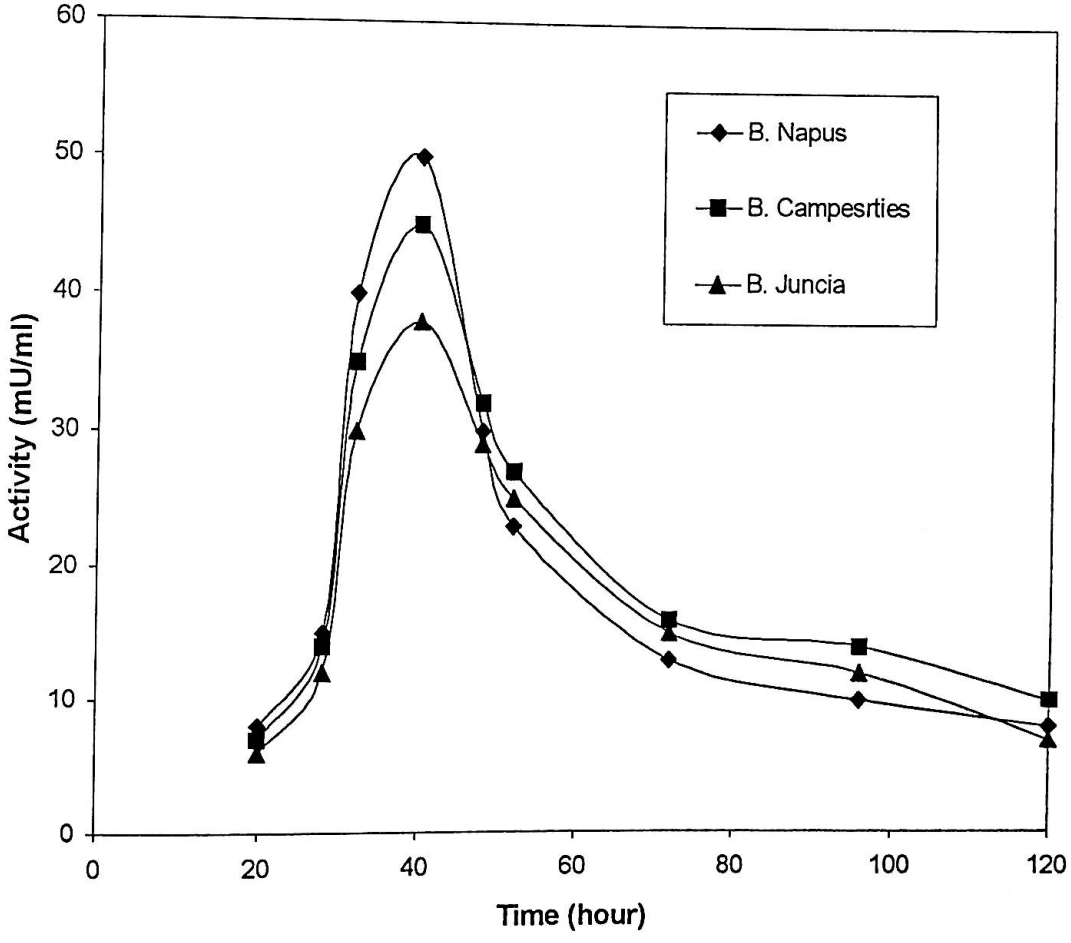


Figure-3.1: Time course study of crude lipase extract from three varieties of Brassica seeds.

3.3.2 GEL FILTRATION: The crude enzyme solution from *Brassica napus's* seeds, after dialysis against 50 mM phosphate buffer (pH 7.2), was applied to Sephadex G-75 column at 4^0C previously equilibrated with the same buffer and was eluted with the same buffer. As shown in the figure-3.2, the components of the crude enzyme solution was separated as two major peaks (F-1 and F-2 fraction) and a minor peak (F-3 fraction). Only F-2 (tube nos. 32-37) fraction showed the enzyme activity and the area as indicated by solid line was pooled, concentrated by freeze dryer and stored for further purification by ion exchange chromatography.

3.3.3 DEAE- CELLULOSE CHROMATOGRAPHY: The freeze dried stored active enzyme were applied to a DEAE-cellulose column at 4^0C, equilibrated with 10 mM Tris-HCl buffer at pH 8.4 and eluted by a linear gradient of sodium chloride solution (from 0.0 to 0.5 M) in the same buffer. As shown in the figure-3.3, the components of F-2 fraction were separated into three major peaks (F-2a fraction, F-2b fraction and F-2c fraction) and a minor peak, F-2d fraction. Of these fractions F-2a was eluted by buffer only while F-2b, F-2c and F-2d were eluted by the buffer containing 0.1M, 0.2M and 0.3M NaCl respectively. Only F-2a fraction showed lipase activity while other fractions showed no lipase activity. The homogeneity of the lipase existing fraction (F-2a) was checked by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE). The fraction (F-2a), containing lipase activity was not pure as it gave two bands on the gel. The fraction F-2a was collected separately dialyzed against 10 mM phosphate buffer, pH 6.5 and then concentrated for further purification on CM-cellulose column chromatography.

3.3.4 CM-CELLULOSE CHROMATOGRAPHY: The concentrated fraction of F-2a (tube nos.10-18) was applied to the CM-cellulose column, which was previously equilibrated with the phosphate buffer pH 6.5, at 4^0C and eluted by a linear gradient of sodium chloride (0.1 to 0.5 M) in the same buffer. As shown in the figure-3.4, F-2a fraction obtained from DEAE-cellulose column was separated into a major peak F-2′a and a minor peak F-2″a. These two fractions, F-2′a and F-2″a were eluted by buffer containing 0.2M and 0.3M NaCl respectively. Of these two fractions only F-2′a fraction showed lipase activity. Fraction F-2″a was not used for further study, as that fraction did not show lipase activity.

The purity of the fraction F-2'a was checked by polyacrylamide gel electrophoresis. This fraction gave single band on polyacrylamide gel electrophoresis (figure-3.5) indicating that the enzyme was in pure form.

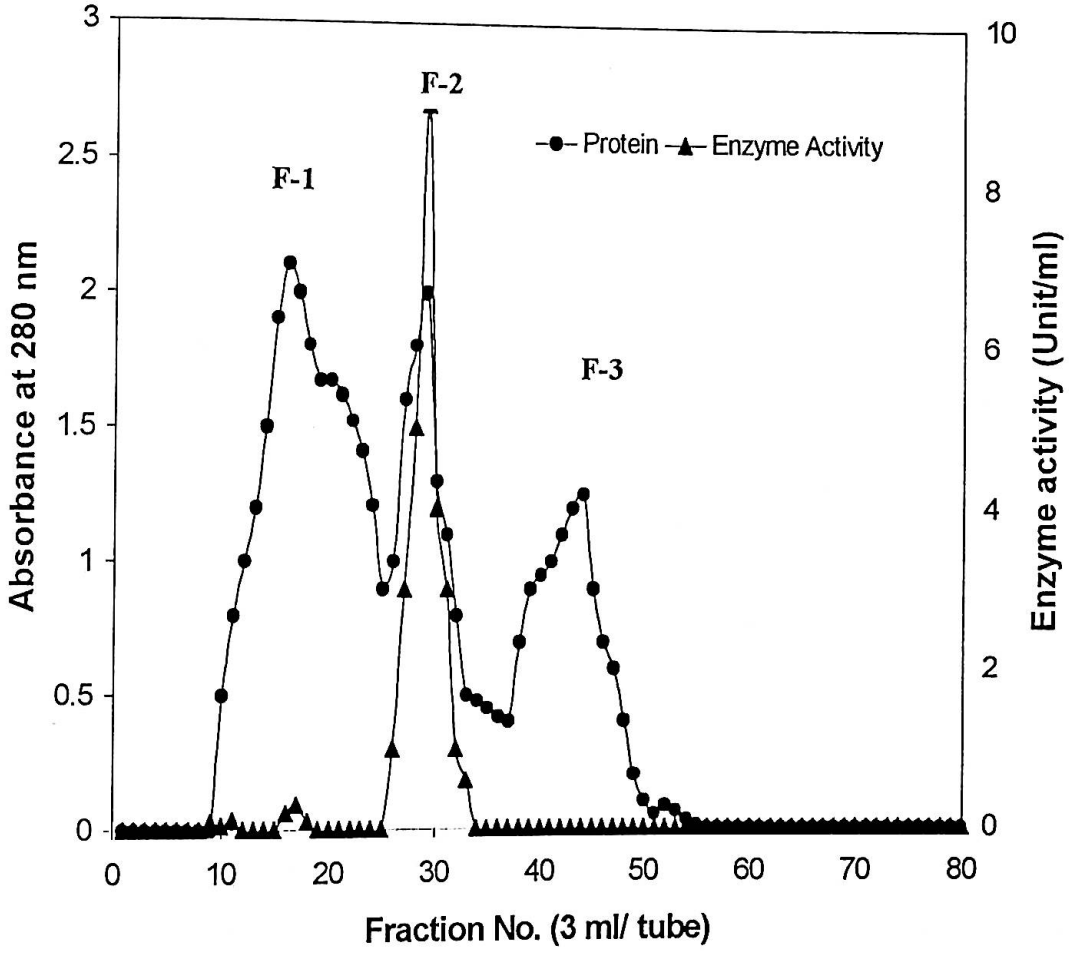


Figure-3.2: Gel filtration profile of 85% ammonium sulphate saturated crude extract on Sephadex G-75 column (407.33 mg for 2.0 × 50.0 cm column) pre-equilibrated with 50 mM phosphate buffer, pH 7.2, at 4^0C and developed with the same buffer. Flow rate: 25ml/hour.

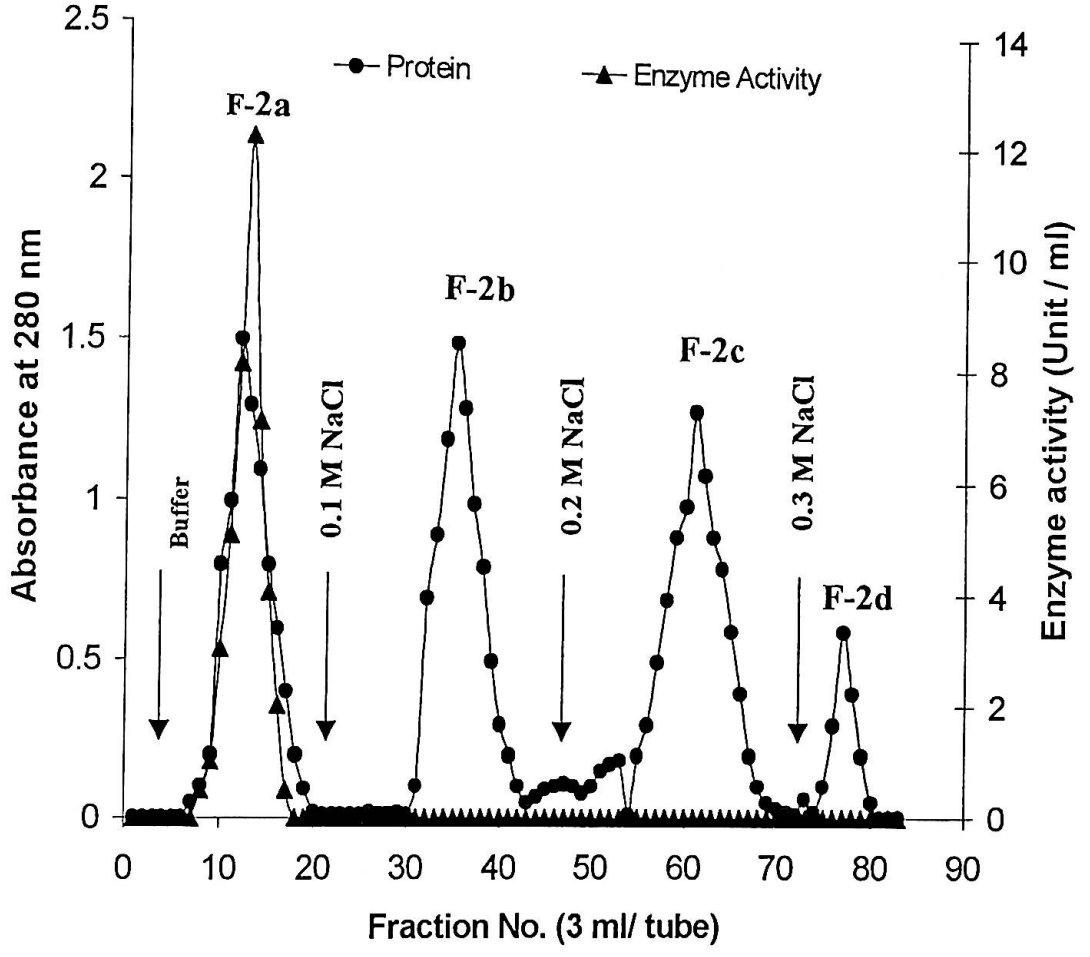


Figure-3.3: Ion exchange chromatography of F-2 fraction (91.95 mg) on DEAE-cellulose column (1.5×30 cm) prewashed with 10 mM Tris-HCl buffer, pH 8.4, at 4^0C and eluted by NaCl gradient (100-500 mM) in the same buffer. Flow rate: 25 ml/hour.

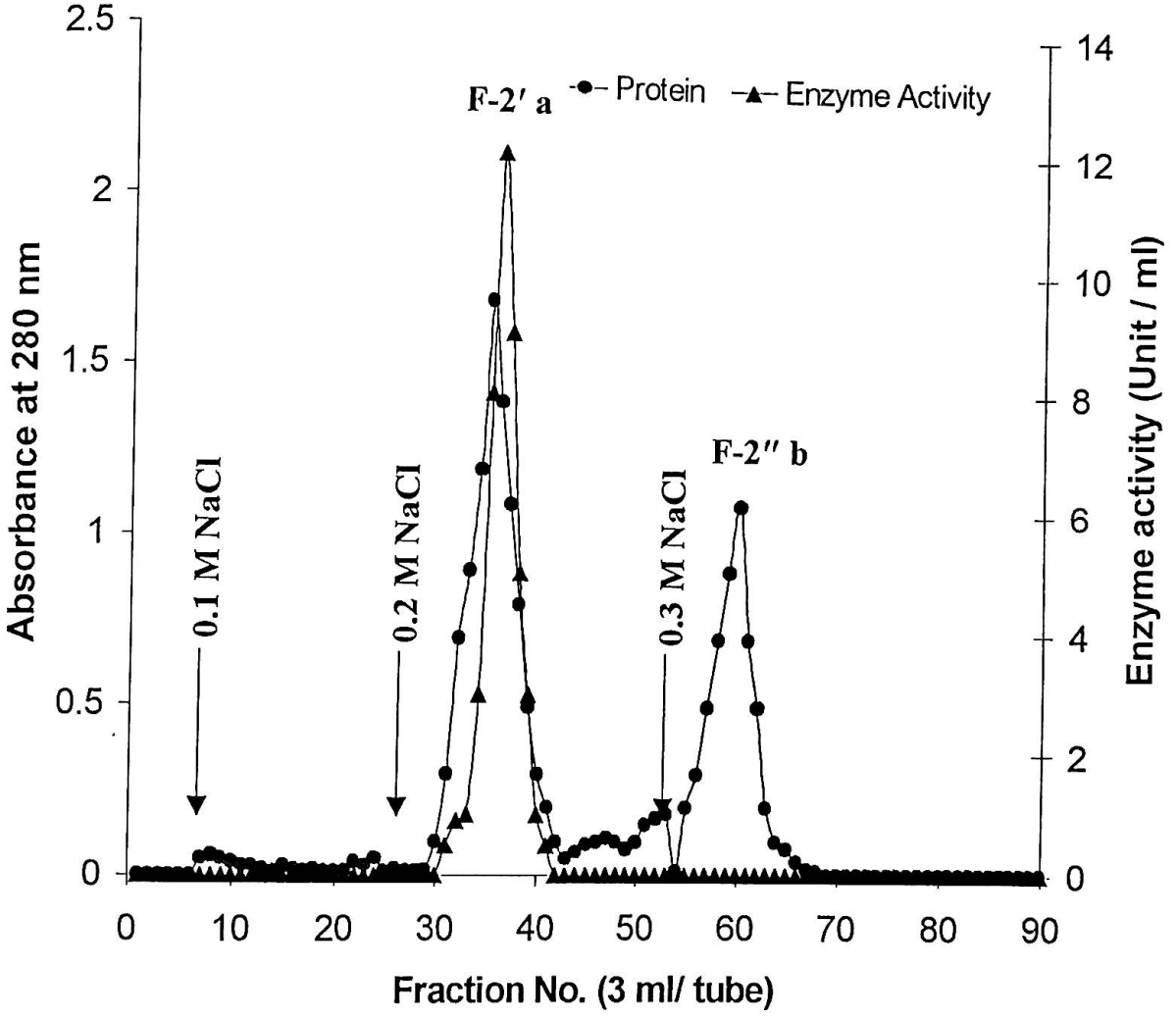


Figure-3.4: **CM-cellulose chromatography of F-2a fraction (18.29 mg) to a column (1.5 ×25 cm) pre-washed with 10mM sodium phosphate buffer, pH 6.5 at 4^0C and eluted by the same buffer containing 0.1M to 0.3M NaCl concentration. Flow rate: 25ml/hour.**

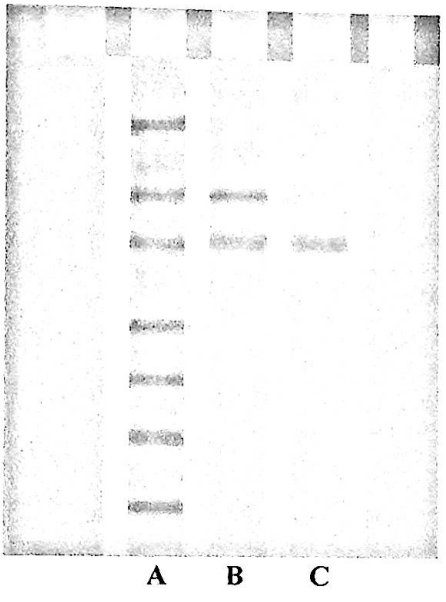


Figure-3.5: Photographic representation of SDS-polyacrylamide slab gel electrophoresis of the various fractions obtained during the purification of lipase.

A: Crude enzyme solution

B: After DEAE-cellulose column

C: After CM-cellulose column

A brief scheme of the overall purification steps of lipase from *Brassica napus's* seeds:

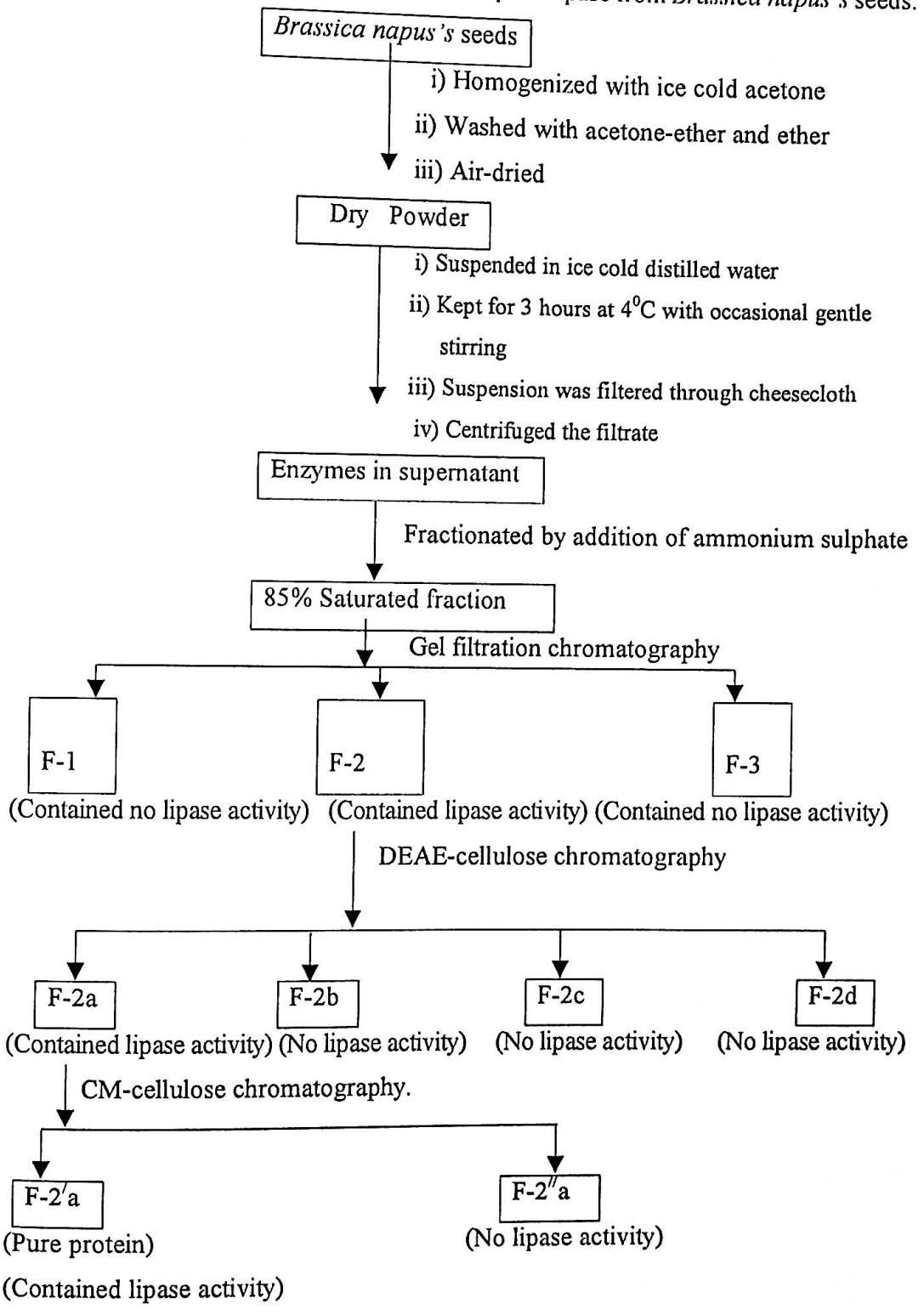


Figure-3.6: Schematic representation of the purification steps of lipase.

The purification results of lipase from germinating brassica seeds are summarize in Table (3.1). The specific activities of extracted enzyme were increased at each purification steps and the purification fold was achieved from crude extract nearly 67.59. The specific activity of the final preparation was 366.54 U mg^{-1} with 24.60% overall yield of enzyme where olive oil was used as a substrate.

Table-3.1: Purification summary of lipase from germinating Brassica seeds.

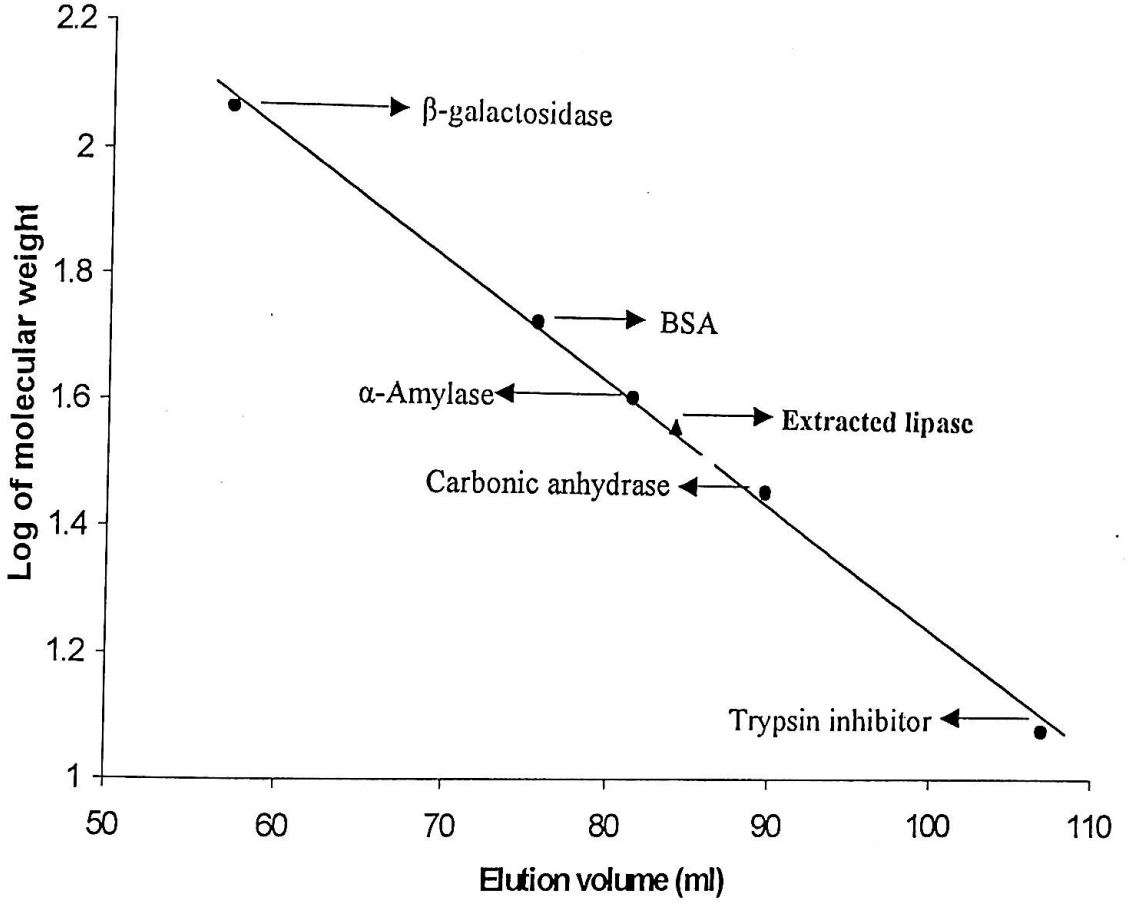
Purification steps	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification Fold.
Crude extract	1597.00	15942	9.98	100.00	1.00
Salting out and dialysis	407.33	11324	27.80	71.03	1.98
Gel filtration	91.95	6749	73.39	42.33	9.90
DEAE-cellulose	18.29	5742	313.94	36.01	34.50
CM-cellulose	10.7	3922	366.54	24.60	67.59

The molecular weight of the enzyme was determined by gel filtration technique on Sephadex G-150 using β-galactosidase (116kD), Bovin serum albumin (66kD), α-amylase (58kD), Carbonic anhydrase (29kD) and trypsin inhibitor (12kD)) as standard proteins. The molecular weight was calculated from the standard curve of reference proteins, which was constructed by plotting log of molecular weight against elution volume on gel filtration (figure-3.7) and estimated to be 34.5 kD for purified lipase.

The molecular weight of the enzyme was also determined by SDS-polyacrylamide slab gel electrophoresis method using lysozyme (14kD), β-lactoglobulin (18.4kD), trypsinogen (24kD), pepsin (36kD), albumin (egg white, 45kD), albumin (BSA, 67kD) as standard proteins under reducing and non-reducing conditions. The molecular weight was found to be unchanged under both the conditions indicating that the enzyme contained only one sub-unit. The standard curve was constructed by plotting log of molecular weight against relative mobility of the reference proteins (figure-3.8) and the molecular weight of the enzyme were calculated from the plot as 34 kD.

3.3.5 OPTIMUM pH: The enzymatic activity of lipase was assayed at different pH values varying from 2.0 to 10.0 are shown in figure (3.9). As demonstrated in figure-3.9, the activity of lipase enzyme was greatly influenced by pH changes. The enzyme showed the maximum activity at the physiological pH 7.0 and the activity of lipase decreased abruptly with the pH changes. The activity of lipase was stable at pH values between 5.5-9.0.

3.3.6 OPTIMUM TEMPERATURE: The effect of temperature on the Brassica seed's lipase was examined in the range of 10 - 70^{0}C. The activities of the purified enzymes increased sharply at 37^{0}C and then began to decrease gradually with the rise in temperature (figure-3.10). Very little activities were found below 20^{0}C and at or above 50^{0}C.



Figure–3.7: Standard plot for molecular weight versus elution volume for different proteins in gel permeation chromatography.

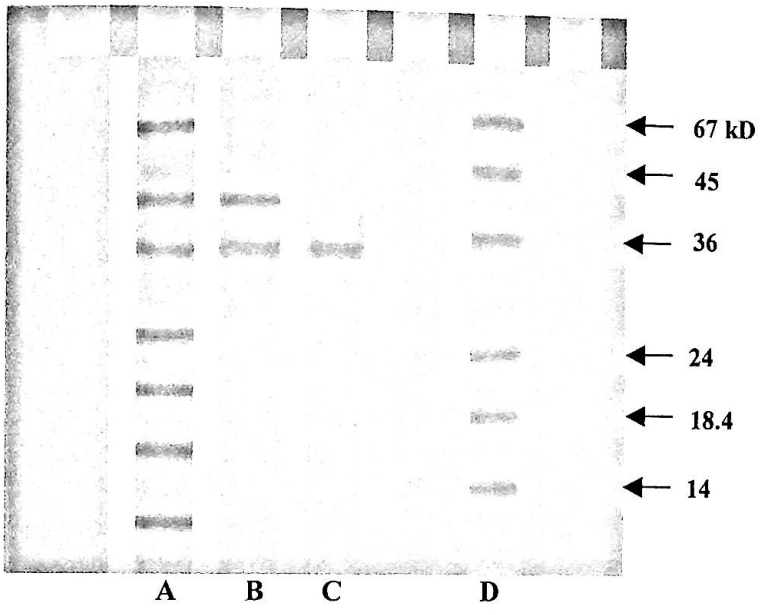


Figure-3.8: Photographic representation of SDS-polyacrylamide slab gel electrophoresis of the various fractions obtained during the purification of lipase for determination of molecular weight.

A: Crude enzyme solution

B: After DEAE-cellulose column

C: After CM-cellulose column

D: Marker protein solution containing lysozyme (14 kD), β-lactoglobulin (18.4 kD), trypsinogen (24 kD), pepsin (36 kD), albumin (egg white 45 kD) and albumin (BSA, 67 kD)

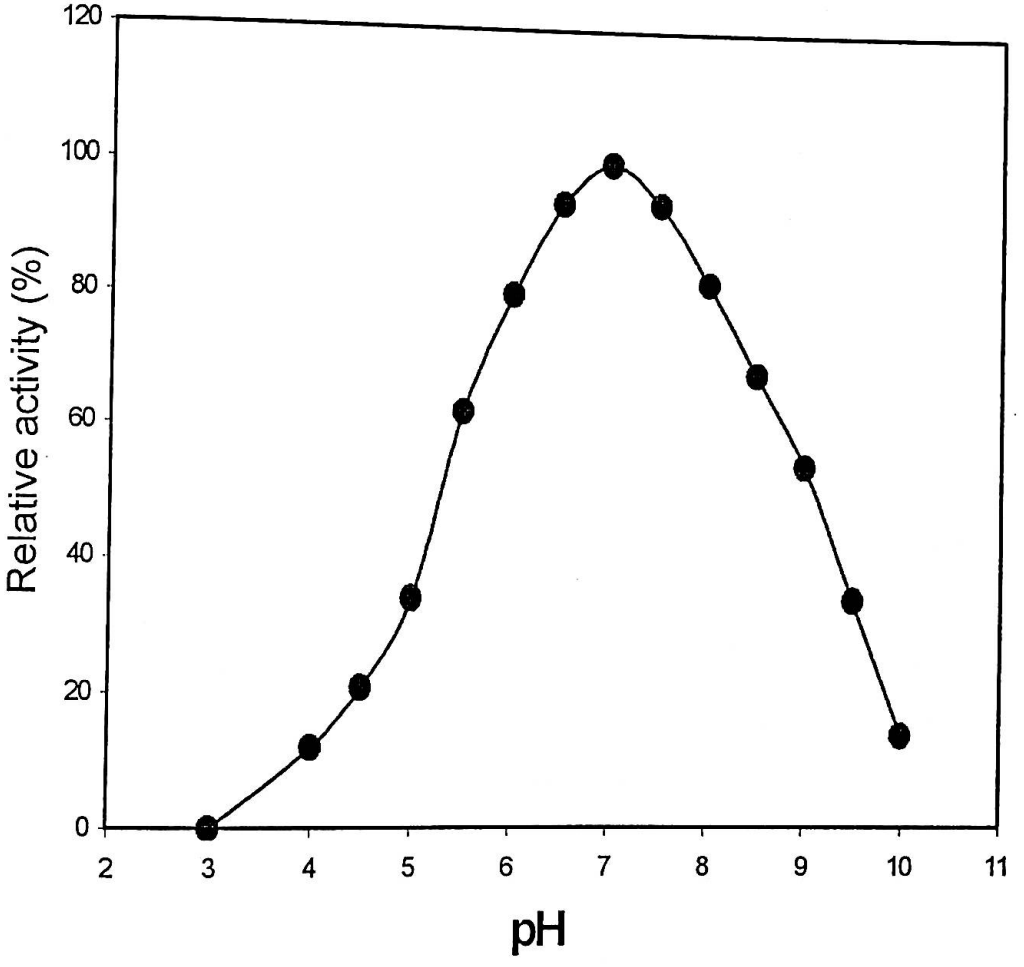
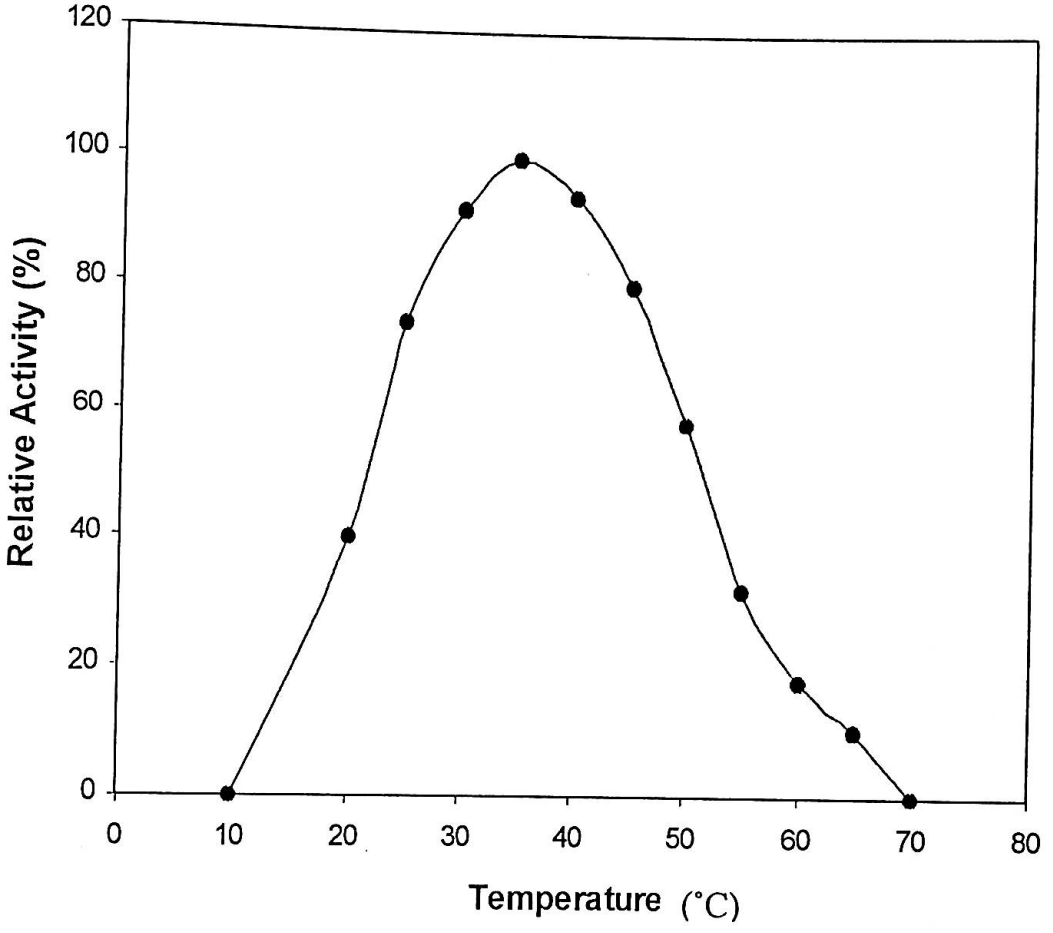


Figure-3.9: Plot of enzyme activities versus pH at 35° C.



Figure–3.10: Effect of temperature on the activity of Brassica seed's lipase.

3.3.7 SUBSTRATE SPECIFICITY OF LIPASE: Activity of the lipase of Brassica seeds was measured using the different triglycerides as substrate and the result was presented in table-3.2. The lipase is found to be capable of hydrolyzing the vegetable oils such as castor oil, coconut oil, corn oil, cottonseed oil, linseed oil, olive oil, peanut oil, rapeseed oil and soybean oil. The specific activities of the lipase were found to be varied from 60 to 186 mU/mg/min. on different oils. Except coconut oil, all the vegetable oils exhibited very similar specific activity on lipase treatment.

Table-3.2: Substrate specificity of Brassica seed's lipase on different oils.

Substrate	Specific activity, mU/mg/min.
Coconut oil	60
Castor oil	165
Corn oil	178
Cottonseed oil	134
Linseed oil	153
Olive oil	170
Peanut oil	186
Rape seed oil	164
Soybean oil	142

3.3.8 EFFECT OF UREA, EDTA AND SOME OTHER SALTS: Table-3.3, shows the relative activity of the lipase of Brassica seeds after treatment with different concentrations of urea and EDTA. It was found that, the activity of lipase decreases gradually with the increase of urea and EDTA concentrations. The lipase lost their activity completely with 7M urea. The effect of some metallic salts was also tested at the specified concentrations of the reagents and the results was presented in table-3.3. The activity of lipase was enhanced at a very low concentration of calcium while the activity decreases gradually with the increase in concentration of calcium. From the table, it was observed that the activity of lipase began to decrease after 0.005M concentration of $CaCl_2$.The other metallic salt solutions $BaCl_2$, $MgCl_2$ and $MnCl_2$ enhance the activity of lipase rather slowly while solutions of $CuSO_4$, $ZnSO_4$, $HgCl_2$, $FeSO_4$ and $ZnCl_2$ reduce the activity of lipase remarkably.

Table-3.3: Effect of Urea, EDTA and some other salts on the activity of lipase purified from *Brassica napus* seeds.

Chemicals	Concentration (Molarity)	Relative activity (%)
Urea	0.0	100.00
	1.0	82.50
	3.0	52.30
	5.0	21.78
	7.0	0.00
EDTA	0.000	100.00
	0.001	81.32
	0.003	69.57
	0.005	54.20
$CaCl_2$	0.000	100.00
	0.001	115.00
	0.003	125.00
	0.005	96.50
	0.010	91.35
$BaCl_2$	0.001	107.78
	0.003	111.45
	0.005	115.00
$MgCl_2$	0.001	103.30
	0.003	108.00
	0.005	97.21
$MnCl_2$	0.001	96.00
	0.003	94.00
	0.005	92.00
$AlCl_3$	0.001	95.43
	0.003	91.89
	0.005	87.90
$CuCl_2$	0.001	87.98
	0.003	83.37
	0.005	77.56
$ZnCl_2$	0.001	55.76
	0.003	46.55
	0.005	39.78
$HgCl_2$	0.001	54.87
	0.003	47.55
	0.005	33.51
$CuSO_4$	0.001	31.77
	0.003	23.49
	0.005	12.16
$FeSO_4$	0.001	64.54
	0.003	51.29
	0.005	37.93

3.3.9 Km VALUE OF LIPASE: The activity of purified lipase was analyzed using different concentrations of olive oil as substrate. The extrapolated Km value was estimated to be 0.23 mM for purified lipase from *Brassica napus* seeds (Figure-3.11.) [S] represents the substrate concentration and Vi is the initial velocity for the reaction.

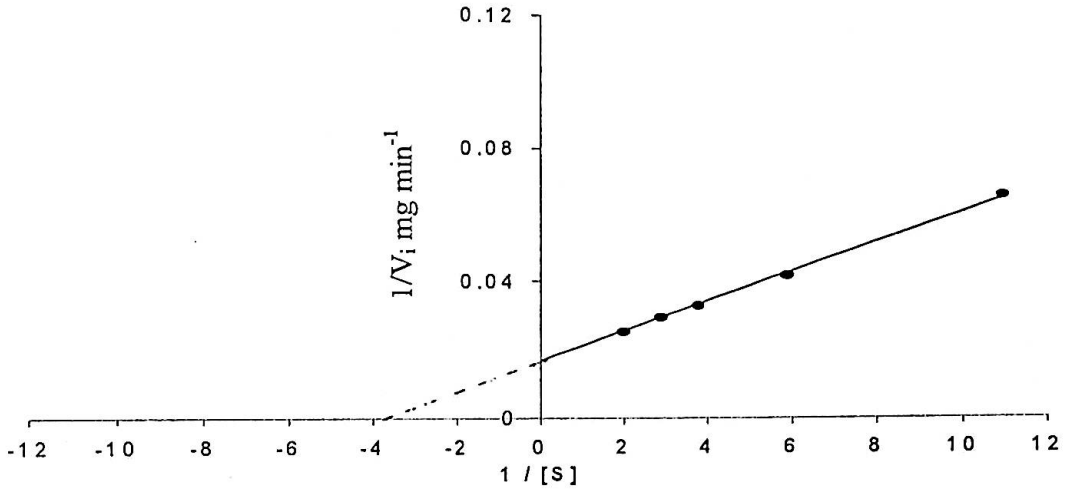


Figure-3.11: Lineweaver-Burk double reciprocal plots for the determination of Km value of the *Brassica napus* seed lipase.

3.4 DISCUSSIONS

The enzyme lipase has shown significant lipolytic activities. It rapidly hydrolyzed olive oil releasing free fatty acids (FFAs). The lipases are glycoprotein (41) in nature as they gave pinkish red colour in presence of phenol-sulphuric acid. Lipases from different sources such as from rice bran (41), from germinating sunflower seedling (42), from corn and other seeds (43), from Jatropha curcas L. seeds (44), from *Aspergillus niger* (45), from *Geotrichum candidum* (34) have been purified and characterized earlier. The lipase purified in the present study from *Brassica napus* seeds is quite different from the others on the basis of some characteristics. Although over 75% of the lipase activity was destroyed during the purification procedure, the lipase from Brassica seeds was purified with an increase in purification fold to 36.72. The loss in enzymatic activity during purification steps might be due to denaturation of the enzyme during longer purification procedure or for some other unknown reasons.

The molecular weight of lipase from germinated *Brassica napus* seeds estimated in our investigation is in reasonable agreement with the molecular weights estimated for lipase from other sources. Hiol et al., (46) have purified a lipase from Rhizopus oryzae strain isolated from palm fruit has a molecular mass of 32kD by SDS-polyacrylamide gel electrophoresis and gel filtration; while Downey and Andrews (15) employing gel filtration techniques, determined the molecular weight of a triacetin-hydrolysing wheat germ enzyme to be 51kD. Aizono *et al.*(47) and Fujiki *et al.* (48) have been isolated two lipases (Lipase-I and Lipase-II) from *Oryza sativa* whose molecular weight are 40kD and 32kD respectively and Kohno et al. (49) have been purified two other lipases (Lipase I and Lipase II) from Rhizopus niveus having molecular mass of 34kD and 30kD respectively. The molecular weight of the enzyme was found to be unchanged in the presence or absence of β-mercaptoethanol indicating that the lipase contained no subunit.

The optimum pH calculated for the lipase activity of Brassica seeds was 7.0 (figure-3.9). From the result it might be concluded that the lipase isolated from *Brassica napus's* seeds belong to the category of neutral lipase similar to lipase in peanut as reported by Sanders and Pattee (50) but unlike lipases in castor bean (51) and in rape and mustard seedling (5) in which optimum pH stay at acidic range. Aizono *et al.* (47) and Fujiki *et al.* (48) isolated two lipases from *Oryza sativa* and reported its optimum pH 7.5.

The purified enzyme showed maximum activities at 37^0C. Lipases from other sources were found to be highly active in the temperature range 30^0C to 40^0C. The optimum temperature for lipase from wheat germ was reported to be 37^0C (52) while those of three lipases from *Rh. Delemer* were from 30^0C to 35^0C (18). Aizono *et al.* (47) and Fujiki *et al.*(48) isolated two thermal stable lipases from *Oryza sativa which* showed 16% and 34% activity at 37^0C and 90^0C, respectively.

Although the enzyme catalyzed the hydrolysis of many vegetable oils but the extent of hydrolysis varied from each oil according to the constituent fatty acids. Olive oil, soybean oil, corn oil, peanut oil and cottonseed oil, containing mainly unsaturated fatty acids of eighteen carbon atoms, were hydrolyzed more rapidly while coconut oil,

containing saturated fatty acids of twelve carbon atoms, undergo hydrolysis to a smaller extent. Linseed oil, which has almost similar constituent fatty acid as olive oil or soybean oil, was also found to be highly hydrolyzable.

Ca^{2+} is reported to be required for activity of many enzymes ($Sr^{2+}$and $Mg^{2+}$are less effective activators). The activities of all the lipases studied here are found to be increased in presence of calcium ion at low concentration (1 mM or less) but inhibited at higher concentrations. This is consistent with the results reported earlier (53). Rice bran lipase (54) and Brassica seed's lipase were found to be stimulated in the presence of calcium ions at certain concentrations. A few lipases/phospholipases required Ca^{2+} for their activity reported by Channon and Leslie, (55) and Scott et al. (56). The primary role of Ca^{2+} seems to be to remove the released fatty acid as its calcium salt. The activating effect of calcium on a lipase derived from *Humicola lanuginosa* was explained by the removal of free fatty acids from the interface (57). In a calcium free system, the lipase can not adsorb at the water-fat interface, and consequently no lipolytic activity occurs.

In the presence of EDTA and Urea, the activity of the enzyme was decreased significantly. The decrease in activity may be due to the removal of metal ions located on or near the active site which seems to be essential to require fatty acids or salt and probably for the denaturing properties of the urea respectively.

Metal ions Ba^{2+} and Mg^{2+} slightly stimulate the lipase at 1 mM or 2 mM and 3 mM concentration whereas Mn^{2+} and Al^{3+} produce no effect on the activity. Activity of Brassica seeds lipase inhibited by heavy metals such as Cu^{2+}, Zn^{2+}, Hg^{2+} and Fe^{2+}. Similar results have been reported for Wheat germ lipase (58), lipase from Rhizopus japonicus lipase (59) and lipase from Aspergillus terreus (60). Salts of Fe were found to inhibit lipase from *Aspergillus niger* (61) and lipase from fungi of the genus *Geotrichum* (62). One possible explanation for the results of the above investigation is that some metal ions bind to the lipase, and alter enzyme activity by stabilization or destabilization of the enzyme's conformation. However, we have not yet demonstrated this experimentally due to limitations of our laboratory facilities.

The Km value of the purified lipase was found to be 0.23 mM using olive oil as substrate. Estressangles and Desnuelle (63) observed the Km value of 10 mM for porcine pancreatic lipase using tripropionin as substrate. Sonnet and Baillargeon (64) determined kinetic constants of lipase from *Rhizomucor miehei* (0.03 mM) and *Candida cylindracea* (0.21 mM) using p-nitrophenyl octanoate and p-nitrophenyl-2-methyloctanoate as substrates respectively. Shastry and Raghavendra, (53) determined Km value for 44 kD rice bran lipase was 7.4 mM using triolein as substrate.

3.5 REFERENCES

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CHAPTER-4

PURIFICATION AND CHARACTERIZATION OF β-AMYLASE FROM GERMINATING WHEAT SEEDS (Triticum aestivum L.).

4.1 INTRODUCTION: Amylolytic enzymes are widely distributed in plant tissues e.g. in storage tissues such as seeds and tubers, and in vegetative organs such as leaves. There exits two types of amylases in some species of plants, α-(α-1-4-glucan glucohydrolase) and β-(α-1-4-glucan maltohydrolase) amylases (1,2). It has long been known that the mature ungerminated kernels of cereal (barley and rye) possess activities of β-amylase. When the seed germinate, this activity increases considerably (3,4). β-amylase is considered as one of the enzymes which degrade starch, may play a role in the mobilization of starch during germination or the sprouting of tubers (1,5,6). Extensive studies have been done on the isolation of β-amylase enzyme from seeds (2,7-10); tubers (11-16) and leaves (17-19). The saccharifying activity of cereal seed β-amylases is also exploited in bread making and in the use of malt as an additive in other foodstuffs and even as a 'digestive' (20). Cereal β-amylases also has applications in the production of maltose and maltose-rich syrups, sweeteners and vaccines (21). There are a few reviews dealing extensively with cereal β-amylases (22,23) and a few researches is concerned exclusively with these enzymes (24). Studies on the purification of β-amylases from cereals are relatively few. Barley and millet β-amylases have been isolated and characterized by several workers (25-27). The literature on wheat *beta*-amylase is particularly scanty. The accumulation of *beta*-amylase in the seeds of cereals and the activity increases during germination is interesting from both physiological and biotechnological standpoint. We present here the isolation and characterization of β-amylase from germinating wheat seeds.

4.2 MATERIALS AND METHODS: Three varieties of wheat seeds (Akbar, Agrani and Kanchan) were collected from Bangladesh Agricultural Research Institute (BARI), Irshardi, Pabna. The seeds were cleaned, dried in the sunlight, sealed in a polythene bag and stored in a desiccator for future experiment. Starch, SDS-PAGE-chemicals and Sephadex G-75 were purchased from Sigma Chemicals Co. Ltd. USA. Standard proteins, DEAE-cellulose were purchased from Pharmacia Fine Chemicals Co. Ltd. Sweden. All other chemicals used for this research purpose were commercially available and were of high purity.

4.2.1 GERMINATION OF WHEAT SEEDS: Discussed in chapter 2.2.1.

4.2.2 MEASUREMENT OF AMYLASE ACTIVITY (METHOD A): Amylase activity was assayed following the method described by Jayaraman (28). One percent of starch solution (soluble) was used as substrate (1 gm in 100 ml of 0.1M phosphate buffer, pH 6.7). The amylase activity was measured by estimating the release of maltose. The amount of maltose released was calculated from the standard curve (figure-4.1) prepared with maltose. One unit of amylase activity was defined as the amount required for liberating 1 mg of maltose in 15 minutes at 37 ° C.

METHOD B: The standard reaction mixture contained 100 mM phosphate buffer pH 6.0, 1.0% (w/v) amylose (soluble starch) and an appropriate amount of enzyme solution in a final volume of 0.3 ml, and was incubated for 1 hour at 40° C. The reaction was stopped by boiling for 10 min, followed by measuring the increase of the reducing power by Somogyi (29) and Nelson methods (30). One unit of enzyme activity was defined as the amount of enzyme which would catalyze the formation of 1 μM maltose under the standard assay conditions and the specific activity was expressed as units per mg of protein.

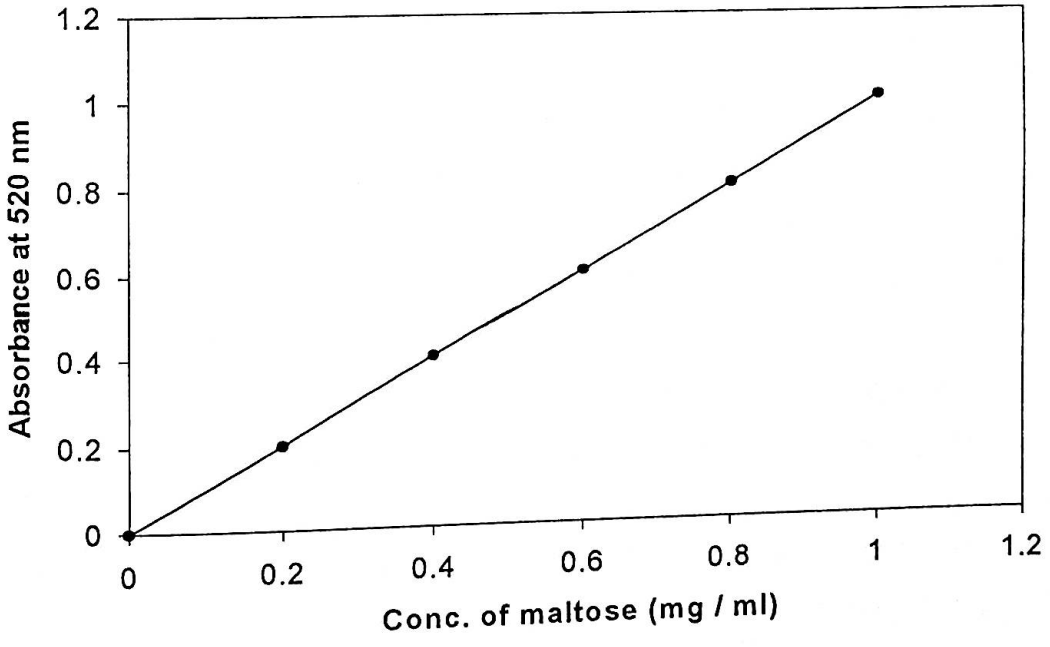

Figure-4.1: Standard curve of maltose for the determination of amylase activity.

4.2.3 PREPARATION OF CRUDE ENZYME EXTRACT: About 30 grams of germinated wheat seeds were taken in a small pot of homogenizer and homogenized well with cold 0.1 M phosphate buffer, pH 7. The extracts were filtered by few layer of cheese cloth and further clarified by centrifugation at 6000 rpm, for 15 minutes at 4°C. The supernatants was collected and precipitated by $(NH_4)_2SO_4$ at different concentration and collected the precipitate at 100% with high amylase activity as previously described (31). The precipitate was redissolved in small amount of pre-cooled distilled water and dialyzed (first in distilled H_2O, for 24 hours and then in 50 mM phosphate buffer, for overnight, pH 7.8, 4°C). After centrifugation, the clear supernatant obtained was used as crude enzyme extract.

4.2.4 GEL FILTRATION: The crude extract after dialysis with 50 mM phosphate buffer, pH 7.8, 4° C was loaded onto a Sephadex G-75 gel column (2.5 x 120 cm) which was equilibrated with the same buffer. The column was eluted with 50 mM phosphate buffer, pH 7.8, at a flow rate of 1.0 ml min^{-1}. Absorbance of each fraction at 280 nm, amylase activities and protein concentration were measured. The active fractions were collected.

4.2.5 DEAE-CELLULOSE COLUMN CHROMATOGRAPHY: The enzymatically active protein fractions after gel filtration were collected and dialyzed against 50 mM phosphate buffer, pH 7.8 for overnight and then concentrated to its $1/4^{th}$ volume by freeze dryer and applied to a DEAE-cellulose column (32 x 1.0 cm, flow rate 25 ml h^{-1}) previously equilibrated with 50 mM phosphate buffer, pH 7.8 and eluted with the same buffer containing a linear gradient of NaCl (100-500 mM). Absorbance at 280 nm, protein concentration and amylase activities were determined. The active fractions were collected.

4.2.6 MOLECULAR WEIGHT DETERMINATION OF β-AMYLASE: SDS-PAGE was performed according to the method of Laemmli on a Bio-rad mini electrophoresis system (32). The standard proteins were lysozyme (14.3 kD), β-lactoglobulin (18.4 kD), carbonic anhydrase (29 kD), ovalbumin (43 kD), bovine serum albumin (68 kD) and phosphorylase-b (97.4 kD). PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A.

All other methods used in purification and characterization of β-amylase from germinating wheat seeds were same as those described in Chapter-3.

4.3 RESULTS

4.3.1 TIME COURSE STUDY: The amylolytic activity of β–amylase from the three varieties of germinating wheat seeds (Agrani, Kanchan & Akbar) showed their maximum activity at 42 hours of germination. After that the activity declined rapidly (figure-4.2). The activity of Agrani variety of amylase was found higher than that of the other two varieties. So, in further studies, we used the extracts of Agrani variety of wheat seeds germinated at 42 hours.

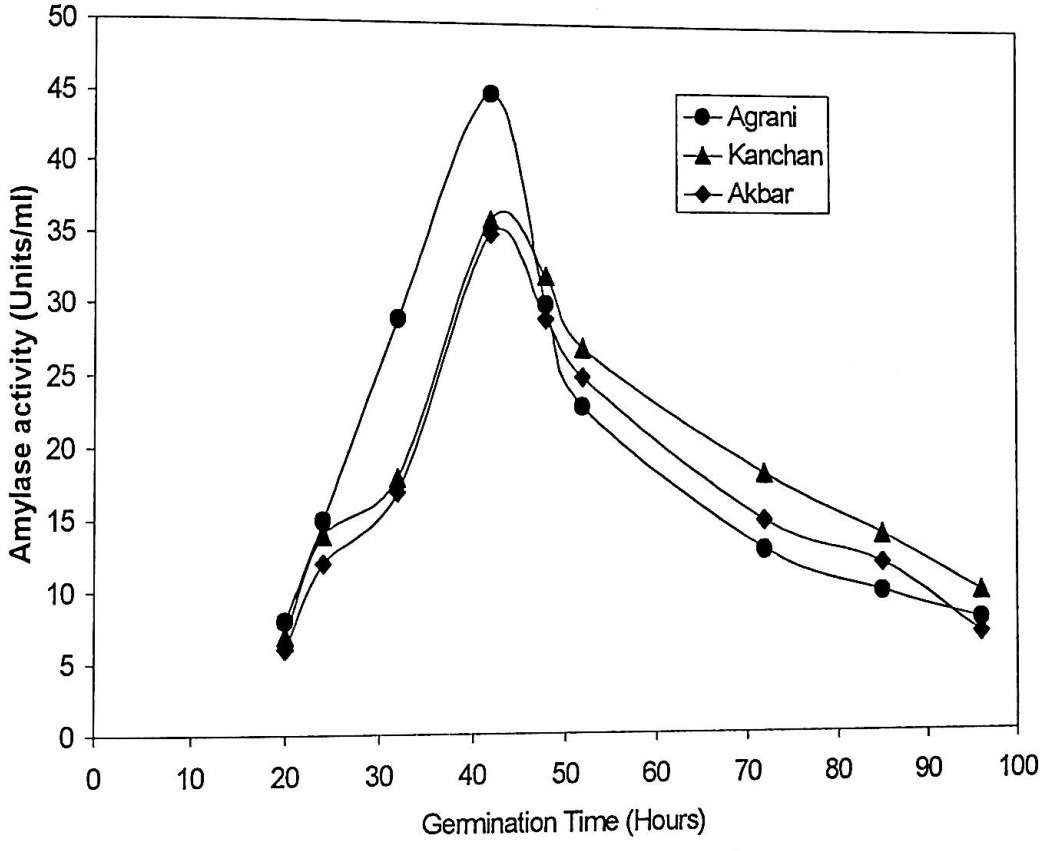

Figure-4.2: Time course of the three varieties of wheat seeds amylase activities during germination.

4.3.2 GEL FILTRATION: The crude enzyme solution from Agrani variety of germinating wheat seeds showed two main peaks F-1 (tube nos. 19-26) and F-2 (tube nos. 56-64) (figure-4.3) on sephadex G-75 column. Only the F-1 fraction showed the amylolytic activity while the F-2 fraction showed no activity. The F-1 fraction was pooled, concentrated by freeze dryer and further purified by DEAE-cellulose column chromatography.

4.3.3 DEAE-CELLULOSE COLUMN CHROMATOGRAPHY: The enzyme active fraction F-1 obtained after Gel filtration was dialyzed against distilled water for 12 hours and then against 50 mM phosphate buffer, pH 7.8 for 24 hours and was applied to a DEAE-cellulose column at 4° C previously equilibrated with the same buffer and eluted by a linear gradient of sodium chloride (0.1 – 0.5 M) in the same buffer.

The fractions F-1 (tube nos. 19-26) were separated into three peaks: F-1a (tube nos. 8-18), F-1b (tube nos. 43-50) and F-1c (tube nos. 64-71) (figure-4.4). Fraction F-1a eluted with the buffer showed no amylolytic activity. The other two fractions F-1b & F-1c eluted with salt gradient showed amylolytic activity. The minor peak (F-1c) was not collected for further study because of low amylolytic activity. Only major peak (F-1b) having high amylolytic activity was collected separately, dialysed against same buffer for over night at 4° C and concentrated by freeze dryer and then rechromatographed on DEAE-cellulose under identical conditions. The fraction was eluted only as a sharp single peak (Figure not shown) and its purity was judged by polyacrylamide slab gel electrophoresis. This fraction contained pure amylase as it gave single band on polyacrylamide gel electrophoresis (figure-4.5).

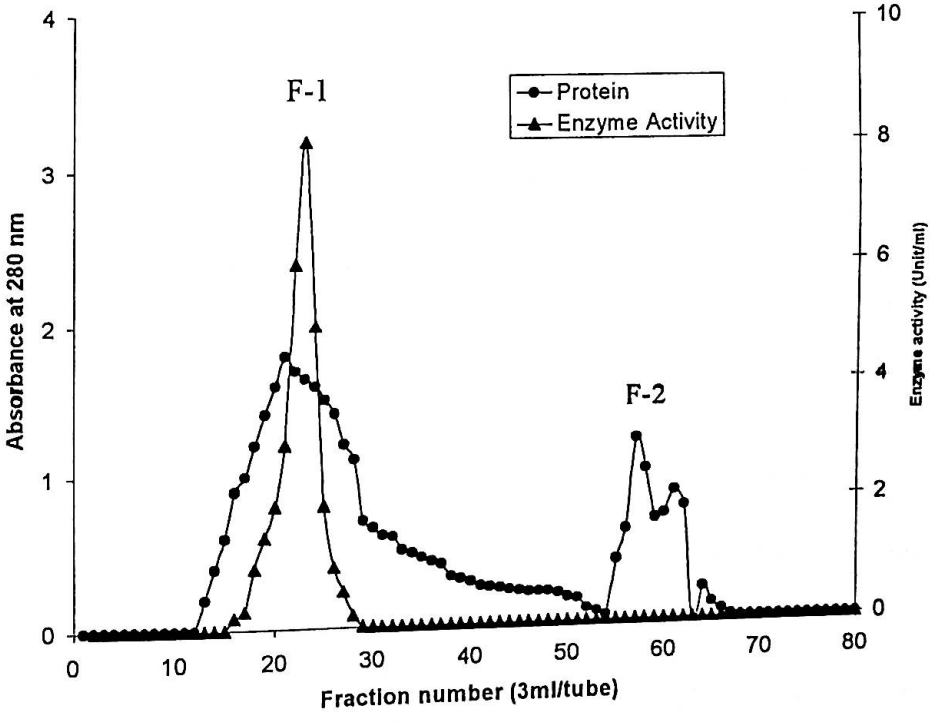

Figure-4.3: Gel filtration pattern of crude extract of Agrani variety on Sephadex G-75 column chromatography.

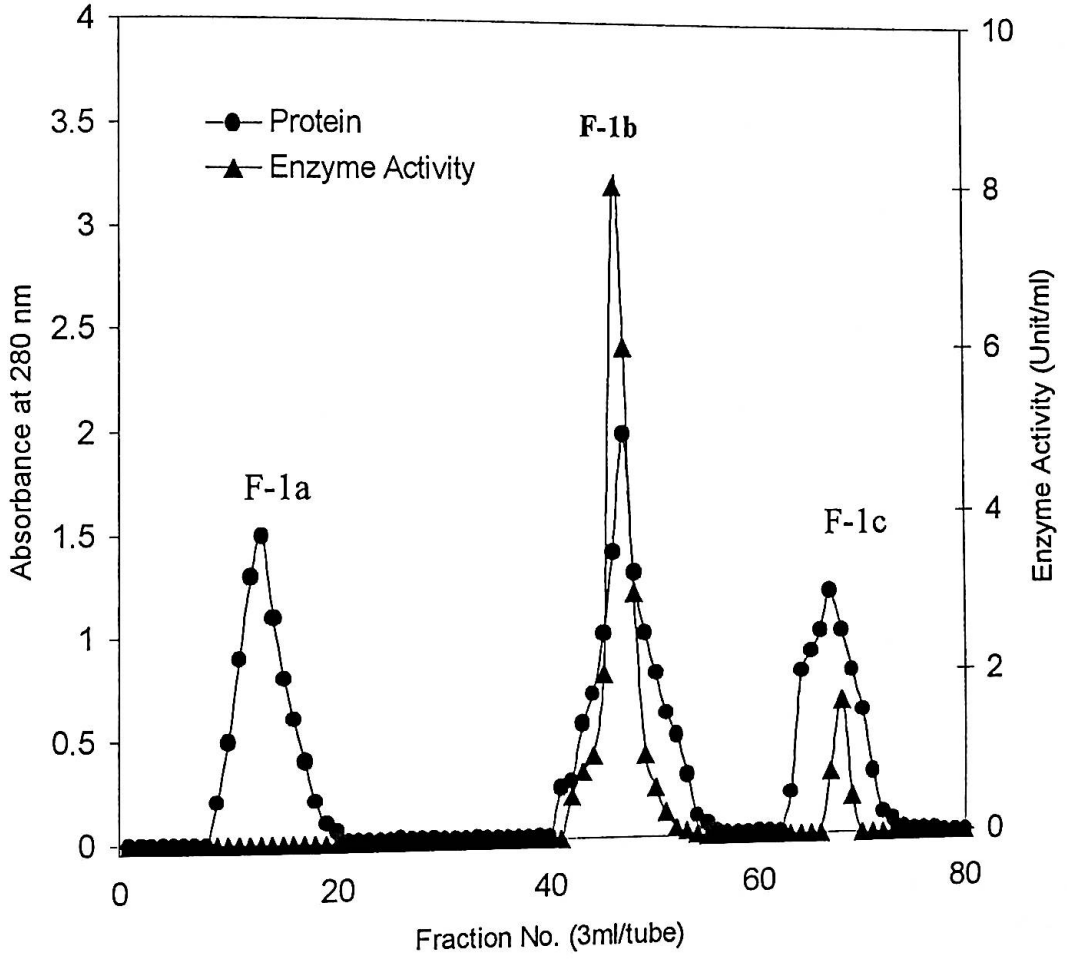

Figure-4.4: DEAE-cellulose column chromatography of wheat amylase from F-1 fraction obtained from gel filtration.

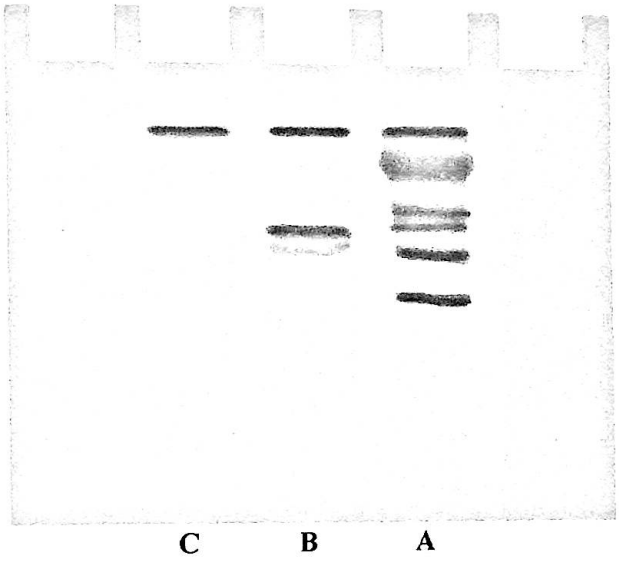

Figure-4.5: Photographic representation of SDS-polyacrylamide slab gel electrophoresis of the various fractions obtained during the purification of *β*-amylase.

A: Crude enzyme solution

B: After gel filtration column

C: After DEAE-cellulose column

A BRIEF SCHEME OF THE OVERALL PURIFICATION STEPS:

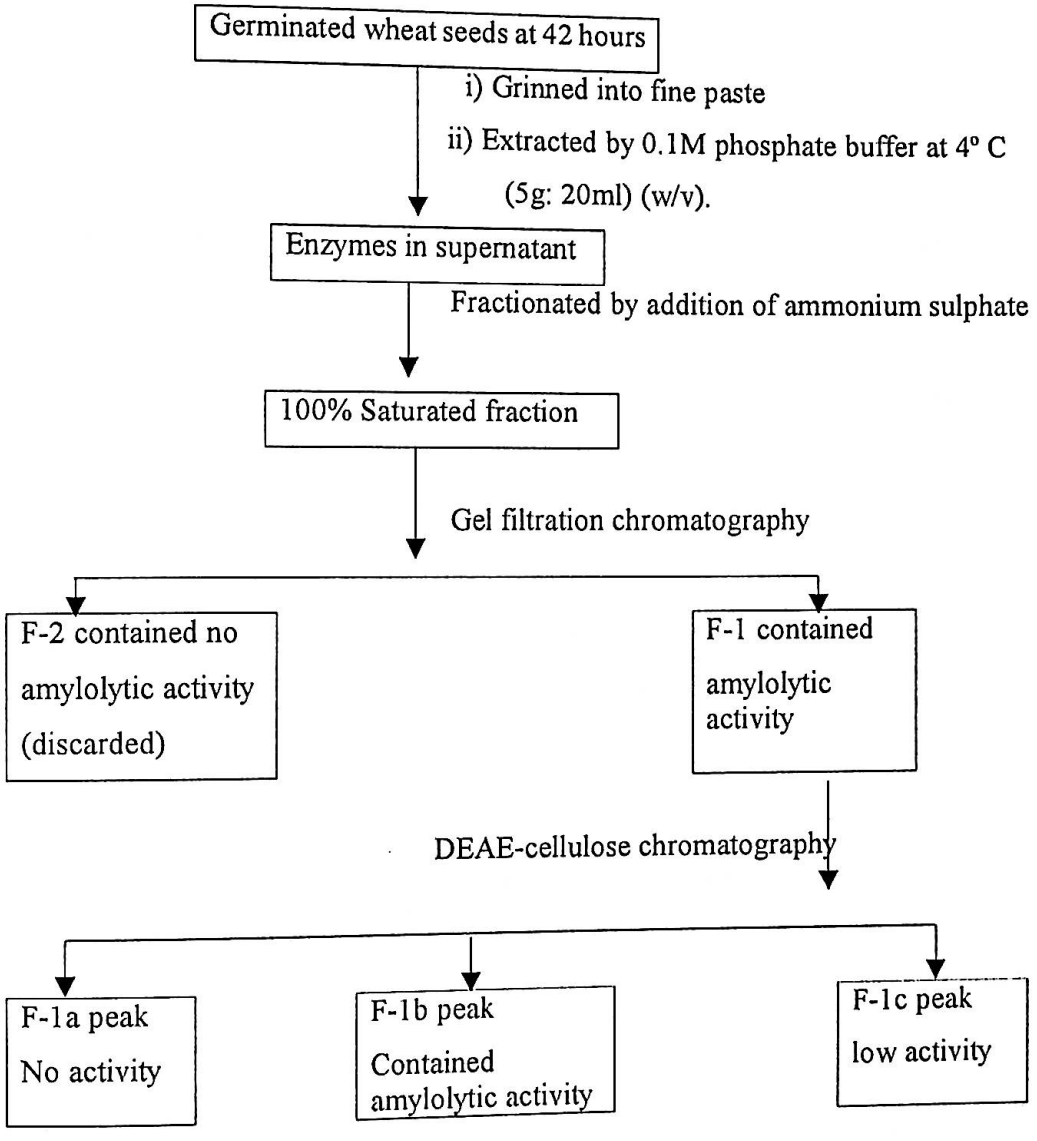

Figure-4.6: Schematic representation of the purification steps of β-amylase from germinated wheat seeds.

The purification results of amylase from germinating wheat seeds are summarized in table-4.1. The specific activities of extracted enzyme increases at each step and the purification fold was achieved from crude extract nearly 65.59. The specific activity of the final preparation was 135.77-unit mg^{-1} with 37.07 % overall yield of enzyme where soluble starch used as a substrate.

Table-4.1: Purification summary of germinating wheat seeds β–amylase (Agrani variety)

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (folds)
Crude extract	924	1915.00	2.07	100	1.00
Salting out and dialysis	690	1504.20	2.18	78.50	1.05
Gel filtration	24.35	1230.89	50.55	64.27	24.42
DEAE-cellulose	5.23	710.07	135.77	37.07	65.59

The purified amylase enzyme gave 100% hydrolytic activity when treated with the substrate in absence of $HgCl_2$, but no hydrolytic activity was found if the substrate solution was pre-mixed with $2x10^{-3}$M $HgCl_2$, an inhibitor of β-amylase. Further the hydrolytic activity of the purified enzyme was found to be unchanged in the presence of $2x10^{-3}$M EDTA (α-amylase inhibitor)(33). These findings clearly indicated that the purified amylase was of β-type.

4.3.4 DETERMINATION OF OPTIMUM pH AND pH STABILITY: The activity of β–amylase at various pH values from 3.0 to 10.0 were shown in figure-4.7. The activity of the enzyme was greatly influenced by pH changes. The pH optimum of the purified enzyme was found in a range of 5.5 – 6.5, with a maximum at pH 6.0. The enzyme was found to be stable between pH 4.0 – 8.0 and completely inactive below and above the range.

4.3.5 OPTIMAL TEMPERATURE AND THERMOSTABILITY: The activity of the purified β–amylase was measured at various temperatures (25 – 80 ° C) at optimum pH 6.0. The activity was found to increase with increasing temperature to 40 - 50° C, with an optimum temperature of 45° C (figure-4.8) at which the activity was

maximum. The effect of temperature on the stability of the enzyme was studied by keeping the enzyme at various temperatures (25 - 80° C) for 30 min and the remaining activities were measured. The enzyme was stable below 65° C and its activity significantly decreased at and above 80° C.

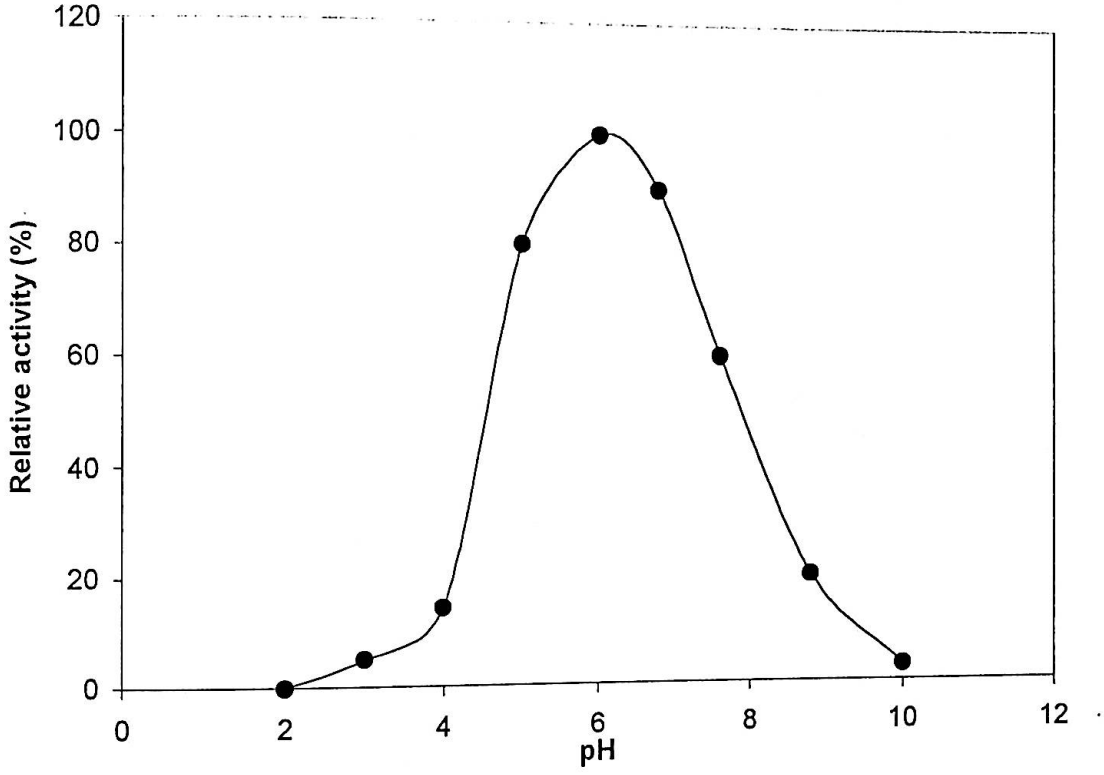

Figure-4.7: Effect of pH on the amylase activity from germinating wheat seeds.

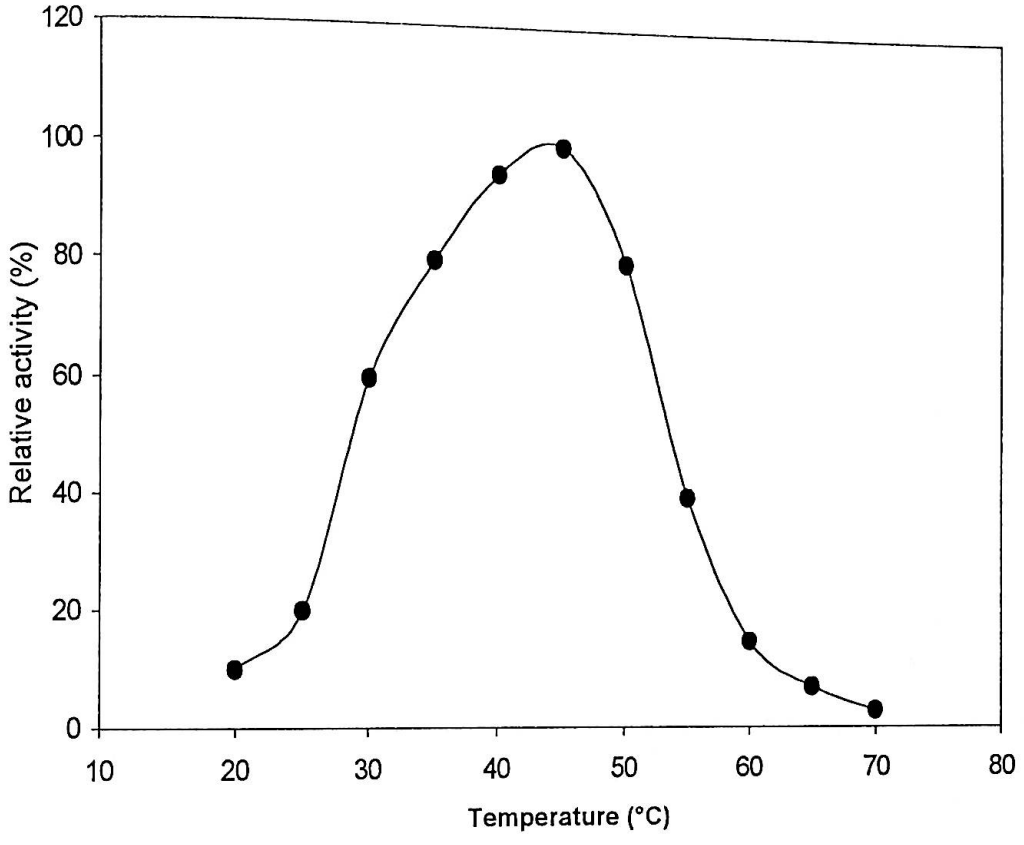

Figure-4.8: Effect of temperature on amylase activity from germinating wheat seeds.

4.3.6 MOLECULAR WEIGHT DETERMINATION: The molecular weight of the purified enzyme was determined by comparing their elution volume on Shephadex G-75, with those of the marker proteins, Tripsin inhibitor (12.028 kD), Carbonic anhydrase (29 kD), α-amylase (58 kD), Serum albumin (66 kD) and β-galactosidase (116 kD) under identical experimental conditions. A logarithmic plot of molecular weight against relative elution volume (figure-4.9) gave a linear relationship, and the molecular weight of the enzyme was found to be 88 kD. In order to elucidate the sub-unit structure of the enzyme, SDS-polyacrylamide gel electrophoresis was performed. As shown in figure-4.10, the enzyme gave a single band of the same mobility with or without SDS and β-mercaptoethanol treatment. The result indicated that the enzyme has no subunit structure. A logarithmic plot of the molecular weight against relative mobility indicated that the molecular weight of the enzyme was 88-89 kD.

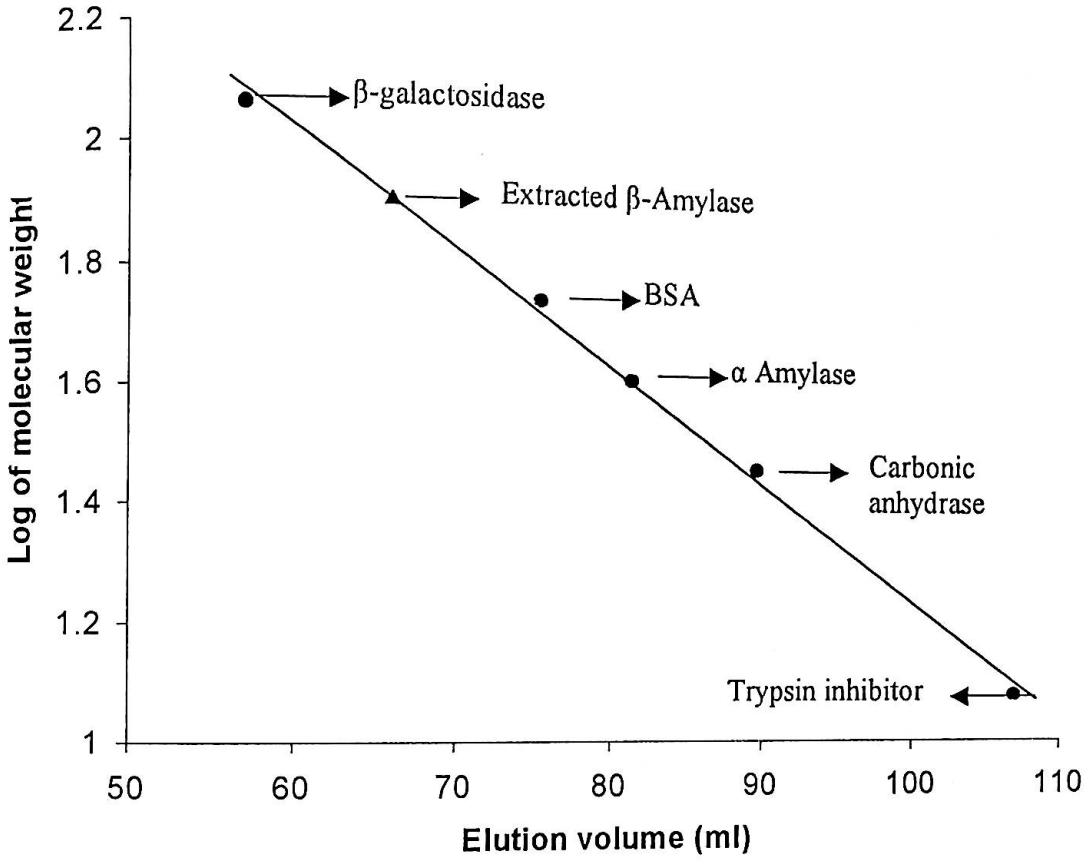

Figure-4.9: Standard curve for the determination of molecular weight of β-amylase by gel filtration methods.

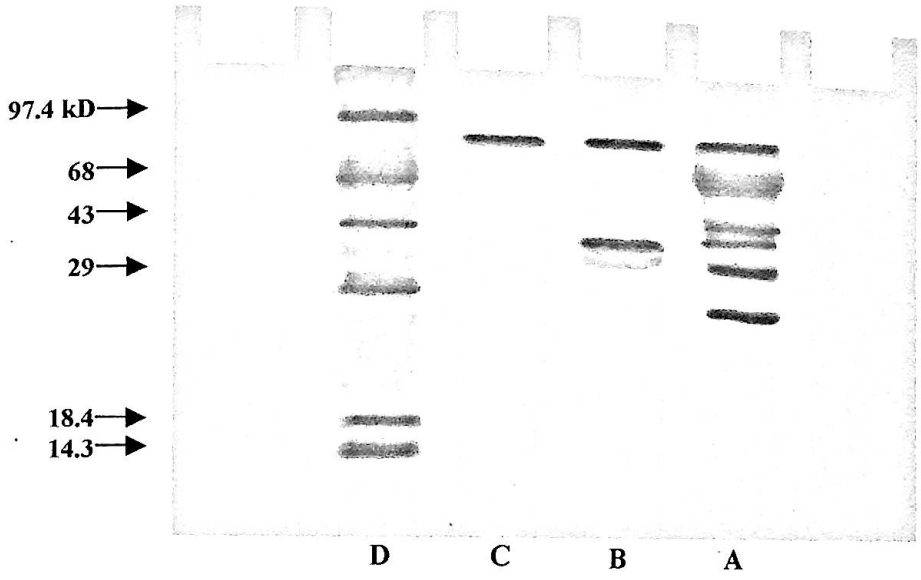

Figure-4.10: Photographic representation of SDS-polyacrylamide slab gel electrophoresis of the various fractions obtained during the purification of β-amylase for determination of molecular weight.

A: Crude enzyme solution

B: After gel filtration column

C: After DEAE-cellulose column

D: Marker protein solution containing lysozyme (14.3 kD), β-lactoglobulin (18.4 kD), carbonic anhydrase (29 kD), ovalbumin (43 kD), bovine serum, albumin (68 kD) and phosphorylase-b (97.4 kD).

4.3.7 MICHAELIS CONSTANT (Km): The affinity of the enzyme for substrate was investigated. Soluble starch was used as the substrate. The Km value of β–amylase was estimated from Lineweaver-Burk plots using various concentrations of starch (figure-4.11). The Km value for the hydrolysis of starch was found to be 1.45 mg/ml. Ultra-violet absorbance of the pure enzyme in aqueous solution was maximum at 262 – 266 nm and minimum at 240 – 244 nm. No significance absorbance was detected above 320 nm, indicating the absence of chromophore (34) (figure-4.12).

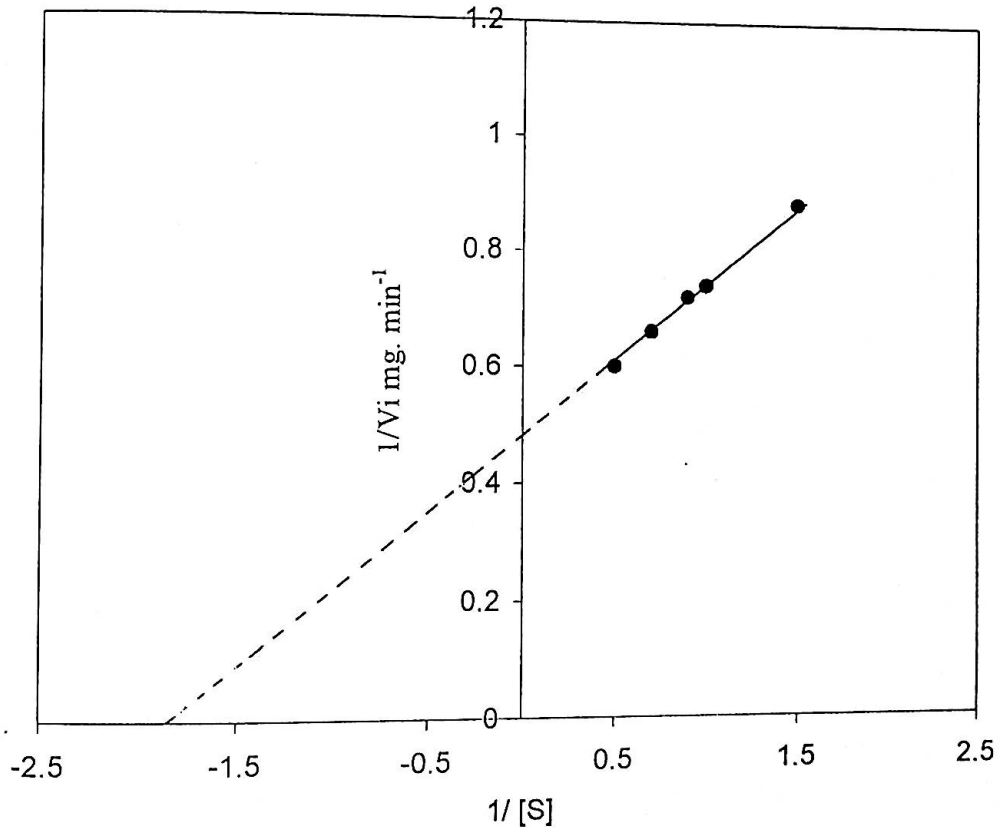

Figure-4.11: Linewaever-Burk double reciprocal plots for the determination of Km value of β-amylase.

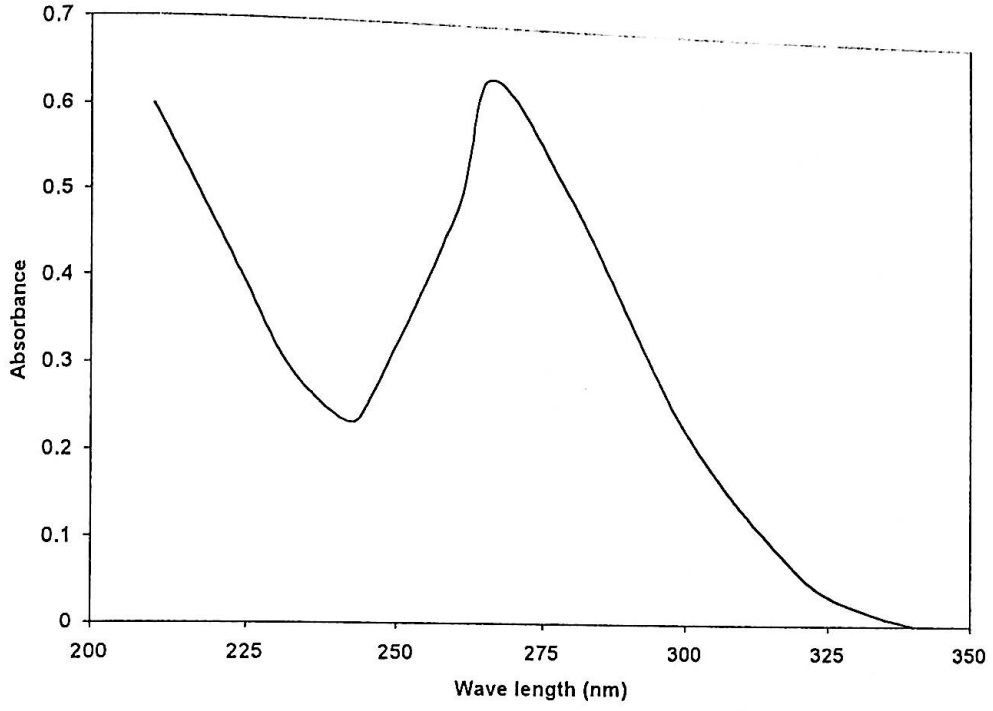

Figure-4.12: Ultraviolet absorption spectrum of β-amylase from germinating seeds.

4.3.8 SUBSTRATE SPECIFICITY: As given in table-4.2, the β–amylase gave about 100% activity when soluble starch and amylopectin were used but gave more than 200% activity when starch grains from pea was used. It was also shown that when amylose and starch grains from potato were used, low activity was found. On the other hand, no hydrolytic activity was observed when the enzyme was incubated with maltose and maltotriose.

Table-4.2: Substrate specificity of the purified β–amylase from germinating wheat seeds.

Substrate	**Relative activity (%)**
Soluble starch	100
Amylopectin	108
Amylose	60
Maltose	0
Maltotriose	0
Starch (pea)	214
Starch (potato)	56

4.3.9 ACTIVATORS & INHIBITORS: The effects of various metallic salts and chemical reagents on the activity of β–amylase were examined. Enzyme was added to the substrate solution pre-incubated with a reagent at 45° C for 5 min, and residual activity (%) was determined.

As shown in the table-4.3, the presence of Fe^{3+}, Mn^{2+}, Mg^{2+} and EDTA increased the activity of the enzyme while the presence of Cu^{+2}, Hg^{+2}, Pb^{+2} and Urea reduced the activity of the enzyme remarkably.

Table-4.3: Effects of Metal ions and Chemical Reagents on β–amylase activity from germinating wheat seeds.

Metal or Chemical Reagent	% Relative activity
Control (soluble starch)	100
$CuCl_2$	4.0
$HgCl_2$	2.6
$FeCl_3$	104
NaCl	94
$CaCl_2$	93
MgC_2	91
$ZnCl_2$	80
$MnCl_2$	105
$BaCl_2$	79
$CoCl_2$	71
$NiCl_2$	63
$PbCl_2$	5.0
$SnCl_2$	20
AgCl	7.5
EDTA	110
Urea	2.5

4.4 DISCUSSIONS

The enzyme amylase has high amylolytic activity. It rapidly hydrolyzes poly- and oligoglucans from the nonreducing ends of the chains releasing successive maltose units. Although the enzyme was purified with an increase of purification fold of about 65 but the yield was found to be about 37% only. This decrease in yield may be due to denaturation of enzyme during the lengthy purification procedures or for some other unknown reasons.

The molecular weight of β–amylase from germinating wheat seeds determined by Shephadex G-75 gel filtration column, was 88kD. This is in good agreement with the molecular weight (89kD) determined by SDS-PAGE (figure-4.10). Hence, wheat

seeds ,β–amylase is a monomer. The molecular weight of wheat seeds β–amylase is similar to that of β–amylases from bulbs of Klattianus (35), Sorghum bicolor moench (11) and Bacillus megaterium B(6), (36). Larger multimeric β–amylases have been reported from vicia faba leaves (107kD) (37), leaves of potato (111kD) (19), potato tubers (122kD) (38), C. thermosulphurogenes (210kD) (39) and β–amylase produced by Xanthophyllomyces dendrorhous (240kD) (40).

β–amylase from germinating wheat seeds has a pH optimum for starch hydrolysis 6.0

(figure-4.7) which is higher than that reported by Fan (38) for potato tuber β–amylase (pH 5.1-5.5), Chen-Tien et al., (16) for sweet potatoes β–amylase. However, the optimum pH of amylase activity in extracts of sprouted potato tubers reported by Ross and Davies (41) as 6.0. Hydrolysis of soluble starch by β–amylase isolated from pea epicotyl and leaves of Arabidopsis had optima at pH 6.0 and at 6.0-6.5 respectively (42,43), Cereal β–amylases have pH optima at 5.0-6.0 reported by Yamamoto (44), with which our result is in good agreement.

The optimum temperature for β–amylase activity from germinating wheat seeds was 45° C. Shen et al. (39) reported that the temperature optimum was as 75° C for Clostridium thermosulphurogenes β–amylase, Dicko et al. (35) reported as 55° C for bulbs of G. Klattianus β–amylase and Rashap et al. (45) reported as 50° C for Bacillus polymyxa N3 β–amylase, which are higher than our result but our result is very close to those reported by Lizotte et al. (43), Serafimova et al. (46) as 42° C, and Rashad et al. (15) as 45° C.

The Km value of β–amylase using starch as substrate was calculated from Lineweaver-Burk plot and found to be 1.45 mg/ml (figure-4.11). This result is close to those reported by Diaz et al. (40), Ekong and Anthony (11), Pauline et al. (47) & Shen et al. (39), and is lower than that reported by Kotha and Rameswar (48), Katsuhiro et al. (49) & Rashad et al. (15).

Some disaccharides and polysaccharides were tested as substrate for β–amylase activity shown in table-(4.2). From the results it is clear that the rate of hydrolysis is highest in case of starch (pea) followed by amylopectin and soluble starch (potato). This result is very similar in substrate specificity for pea epicotyl β-amylase as reported earlier (47).

The effects of metal ions and chemical reagents on the wheat seeds β–amylase enzyme activity were studied (table-4.3). The purified enzyme was inactivated in presence of Cu^{2+}, Hg^{2+}, Pb^{2+}, Ag^{+} and Urea, and was strongly activated in presence of Fe^{3+}, Mn^{2+} and EDTA. These results are in agreement with those reported by Okamato and Akazawa, (2) from rice seed β–amylase, Rashad et al. (15) from radish root and Tsao et al. (50) from small abalone.

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CHAPTER-5

PURIFICATION AND CHARACTERIZATION OF PROTEASE (S) FROM GERMINATING WHEAT (Triticum aestivum, L) SEEDS.

5.1 INTRODUCTION: Seeds are the main source of protein in plant. Wheat is the most widely used of all cereals, owing to high nutritive value combined with the dough forming properties of gluten. The protein stored in seeds is used chiefly in the formation of protoplasm in new cells when seeds germinate (1). Protein content rather seeds size may be the main factor influencing seedling development (2). The reserve proteins are stored in two separate sites of the cereal grain: the aleurone grains (bodies) of the aleurone layer, and the protein bodies (sometimes disrupted) of the endosperm. Protein of the aleurone layer is rich in the basic amino acids. Proteolysis within the cells of the aleurone grain proteins appear to be important for the provision of amino acids from which hydrolytic enzymes are synthesized. High and increased activity during seed germination may also indicate a high probability of the participation of the protease in the degradation process. Proteolytic enzymes play critical roles in homeostasis, developmental processes and in responses to environmental stimuli. Proteolysis involves not only an important recycling system for amino acids, but also the final step of a complex cascade of regulatory events (3). These enzymes are classified by group according to the amino acids that are essential for active-site integrity. A survey of plant proteinases, their mechanisms of action and their functions has been published recently (4). The most well characterized proteinases are those which are associated with seed germination, and are employed to mobilize stored reserves to provide amino acids and amides for embyrogenesis and /or early seedling development (5). Seed reserve proteins were used for studying proteolytic enzymes in Vicia faba (6), Phaseolus vulgaris (7,8), Lupinus angustifolius (9), Cucurbita moschata (10), Phaseolus mungo (11), and Cucurbita sp. (12). In addition azo-storage proteins were used to determine proteolytic activities in Pisum sativum (13), black-eyed pea (14), and Phaseolus vulgaris (15). Some researchers investigate about the degrading enzymes, which act on storage substance during germination (16-17). The modification of the 11S and 7S proteins in germinating seeds is caused mainly by limited proteolysis (18). The wide diversity and specificity of proteases is used to great advantage in developing effective therapeutic agents. Proteases of the subtilisin group are used in the treatment of burns and wounds. Oral administration of proteases produces an anti-inflammatory response in burn patients and speeds up the process of healing (19). These enzymes are also used for tenderisation of meat and the production of dough for pizzas and batters for waffles

and wafers in the food processing industry. Bangladesh is an agriculture dependent country. Agricultural commodities are used in food industries for the production of various kinds of food products. But presently, many good quality proteins are lost in food industries through wastage due to their less solubility and other poor functional properties.

Till now there is no detailed study about the degrading enzymes from germinating wheat seeds. This research describes the purification and characterization of the proteolytic enzyme from germinating Akbar, Agrani and Kanchan varieties of wheat seeds. Further more, the proteolytic enzyme from Akbar variety only was purified and characterized.

5.2 MATERIALS AND METHODS: As described in chapter 4.2.

5.2.1 PREPARATION OF CRUDE ENZYME EXTRACT: The germinating wheat seeds were crushed into powder and paste by using a homogenizer with 5 mM phosphate buffer, pH 7.0. The temperature was maintained at 4° C by putting ice in the outer chamber of the homogenizer. The suspension was then filtered through few layers of cheese cloth in the cold room. The filtrate was collected and clarified further by centrifugation in a refrigerated centrifuge at 6000 rpm for 15 minutes at 4° C. The clear supernatant was collected and saturated to 100% saturation by adding solid ammonium sulfate with gentle stirring. The precipitate was collected by centrifugation at 7000 rpm for 10 minutes at 4° C. Then the precipitate was dissolved in minimum volume of pre-cooled distilled water, and dialyzed against water for 12 hours and then against 5 mM phosphate buffer, pH 7.0 for overnight at 4° C. It was again centrifuged at 7000 rpm for 6 minutes to remove any insoluble material present in the solution and the clear supernatant was used as crude enzyme extract.

5.2.2 GEL FILTRATION: The crude extract after dialysis with 5 mM phosphate buffer (pH 7.0) was loaded onto the Sephadex G-75 gel column which was equilibrated with same buffer. The column was washed with 5 mM phosphate buffer, pH 7.0, containing 1M sodium chloride. The fractions were collected on a automatic fraction collector and monitored for activity of protease and protein concentration at 280 nm. The fractions containing enzyme activity were collected and further purified by DEAE-cellulose chromatography.

5.2.3 DEAE-CELLULOSE CHROMATOGRAPHY: The enzymatically active protein fractions after gel filtration were collected and dialyzed against 10 mM Tris-HCl buffer (pH 8.4) overnight at 4° C and then concentrated to its 1/4th volume by freeze-dryer and loaded onto DEAE-cellulose column. The column was washed with 10 mM Tris-HCl buffer (pH 8.4) and then elution was done with sodium chloride gradient (0-0.5M) containing the same buffer. The fractions were collected on an automatic fraction collector and monitored for enzyme activity and protein concentration at 280 nm.

5.2.4 MEASUREMENT OF PROTEOLYTIC ACTIVITY: The proteolytic activity was measured following the method of Kunitz (20). The milk protein casein was used as a substrate. The activity is determined by detecting the release of amino acid, tyrosine by protease. The amount of tyrosine released was calculated from the standard curve (figure-5.1) constructed with tyrosine. The activity was expressed as the amount of μ mole of tyrosine produced per minute at 45° C. The specific activity was expressed as the number of enzyme unit per mg of protein per unit time. The crude enzyme from Akbar variety shows higher proteolytic activity than that of Agrani and Kanchan variety. Only seeds of Akbar variety were used for further purification and characterization of the enzyme.

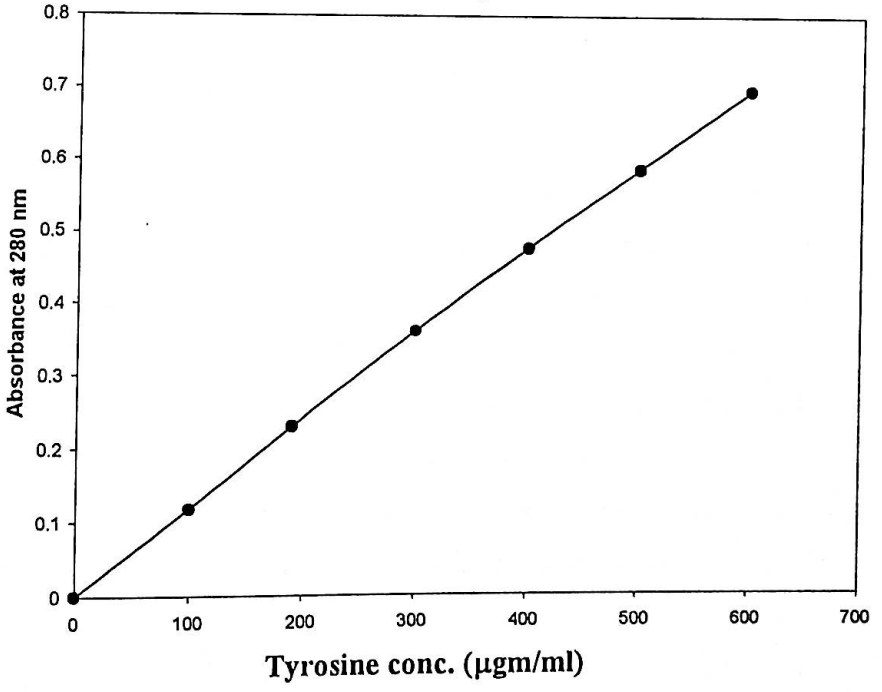


Figure-5.1: Standard curve for the determination of protease activity.

5.2.5 MOLECULAR WEIGHT DETERMINATION: SDS-PAGE was performed according to the method of Laemmli on a Bio-rad mini electrophoresis system (22). The standard proteins were β-lactoglobulin (18.4 kD), carbonic anhydrase (29 kD), ovalbumin (43 kD), bovine serum albumin (68 kD), phosphorylse-b (97.4 kD) and β-galactosidase (116kD). PAGE was performed with 7% gels and the electrophoresis was run at 2000 V and 50 A.

5.2.6 EFFECT OF VARIOUS INCUBATION PERIOD:

In order to ascertain the effect of incubation period on the enzyme activity, the enzyme solutions were kept at 45° C, pH 7.2 for 0.5, 1.0, 2.0, 3.0 and 4.0 hours respectively, and the residual caseinolytic activity was measured by the method of Kunitz (20).

All other methods used in purification and characterization of protease from germinating wheat seeds were the same as those described in Chapter-3.

5.3 RESULTS

5.3.1 TIME COURSE STUDY: In the germinating wheat seeds (Akbar, Agrani& Kanchan), the proteolytic activity appears in the crude extract after 40 hours (figure-5.2) and the activity increases dramatically during further germination. Time course studies of the three varieties of germinating wheat seeds were performed after salt precipitation with ammonium sulfate following dialysis firstly with distilled water and finally with 5mM phosphate buffer, pH 7.0. The protease from three varieties of germinating wheat seeds showed their maximum activity after 72 hours of germination and then declined rapidly. Yang and Davies (23) reported a sharp drop of protease activity in the midgut of the Aedes mosquito during its development. The result indicates that the activity of protease's is higher at the early stage of larval development. It has been demonstrated that secretion of the proteolytic enzymes in the midgut actually stops when maximum activity is reached (24). The activity of Akbar variety of protease was higher than that of Kanchan and Agrani varieties. So, in further studies, we used the extracts of Akbar variety of wheat seeds germinated at 72 hours.

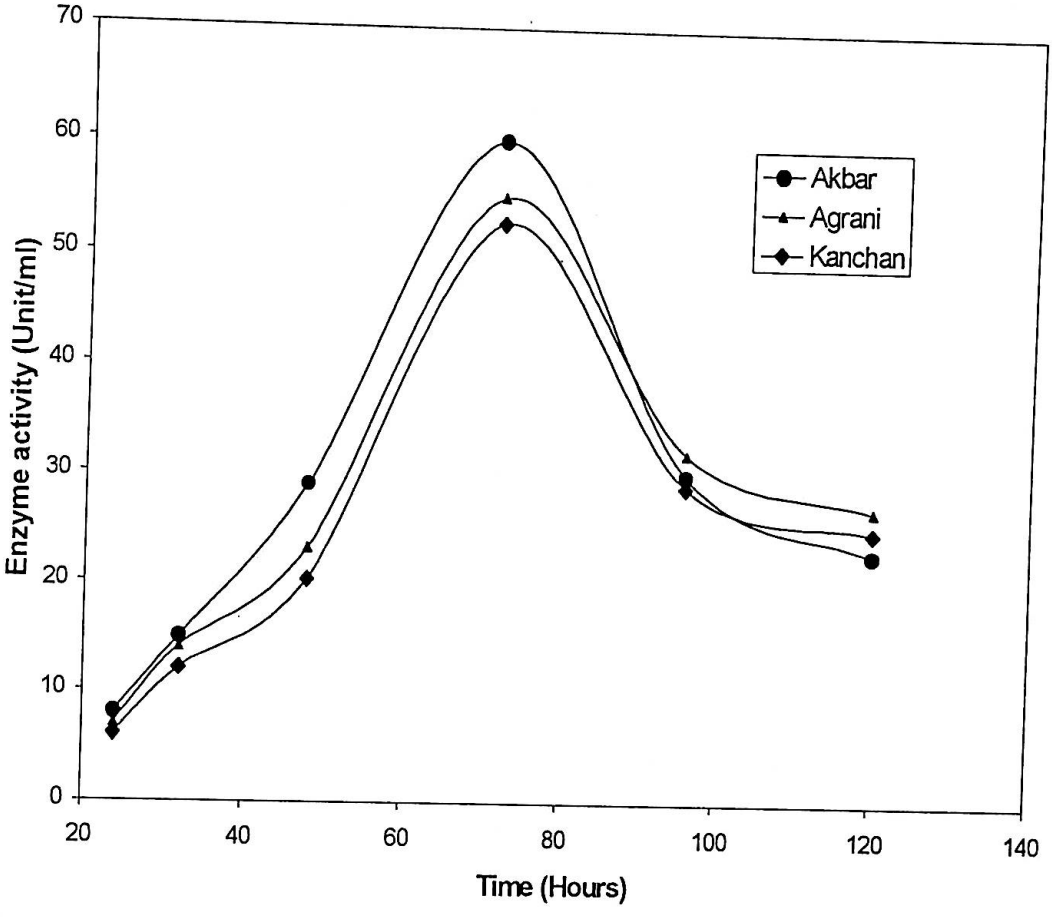


Figure-5.2: Time course of the three varieties of wheat seeds protease activities during germination (Crude extract).

5.3.2 GEL FILTRATION: The crude enzyme solution from Akbar variety of germinating wheat seeds, after dialysis against distilled water and then against 5mM phosphate buffer, pH 7.0 was applied to Sephadex G-75 column at 4° C previously equilibrated with and eluted with the same buffer. The enzyme was eluted as two main peaks F-1 (tube nos. 24-36) and F-2 (tube nos. 52-60) (figure-5.3). It was found that only F-1 fraction contained the proteolytic activity while the F-2 peak showed no activity. The F-1 fraction containing the proteolytic activity was pooled, concentrated by freeze dryer and stored for further purification on DEAE-cellulose column chromatography.

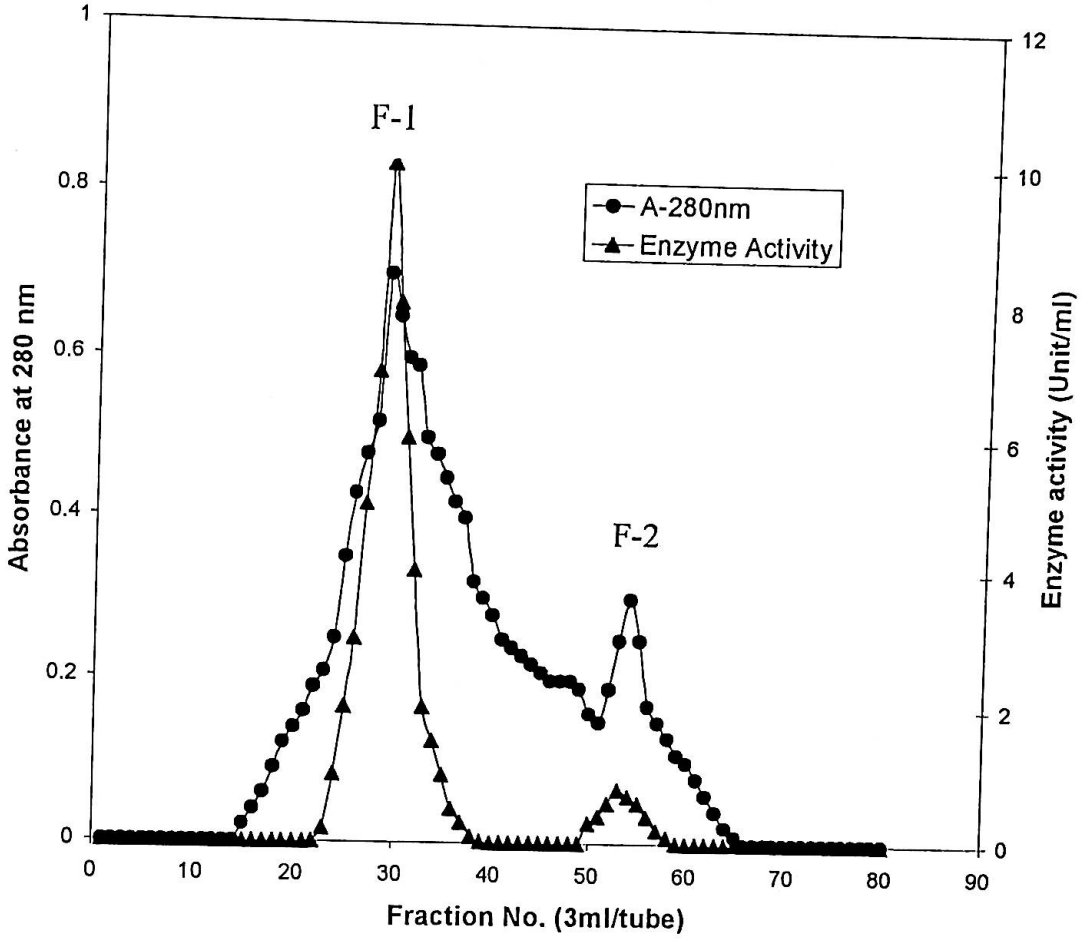


Figure-5.3: Gel filtration pattern of ammonium sulpfate saturated crude extract of Akbar variety on Sephadex G-75 column.

5.3.3 DEAE-CELLULOSE CHROMATOGRAPHY: The enzyme active fraction F-1 obtained after Gel filtration was dialyzed against distilled water for 12 hours and then against 10 mM Tris-HCl bufer, pH 8.4 for 24 hours was applied to a DEAE-cellulose column at 4° C, which was previously equilibrated with the same buffer and eluted by a linear gradient of sodium chloride from 0.0 to 0.5 M in the same buffer.

As shown in figure-(5.4), the F-1 fraction were separated into four peaks: F-1a (tube nos. 5-32), F-Ib (tube nos. 33-54), F-Ic (tube nos. 55-66) and F-1d (tube nos. 67-81). The enzymatic activity of all these fractions were investigated and it was found that the fractions F-1a, F-1b and F-1c showed proteolytic activity while the fraction F-1d possessed no proteolytic activity. The fractions having proteolytic activity were

collected separately, dialyzed against 10 mM Tris-HCl buffer (pH 8.4) overnight and then concentrated. All these fractions having proteolytic activity are pure protease enzyme as they gave single band on polyacrylamide slab gel electrophoresis (figure-5.5). The pure protease containing fractions F-1a, F-1b and F-1c were named as protease-I (pro-I), protease-II (pro-II) and protease-III (pro-III) respectively.

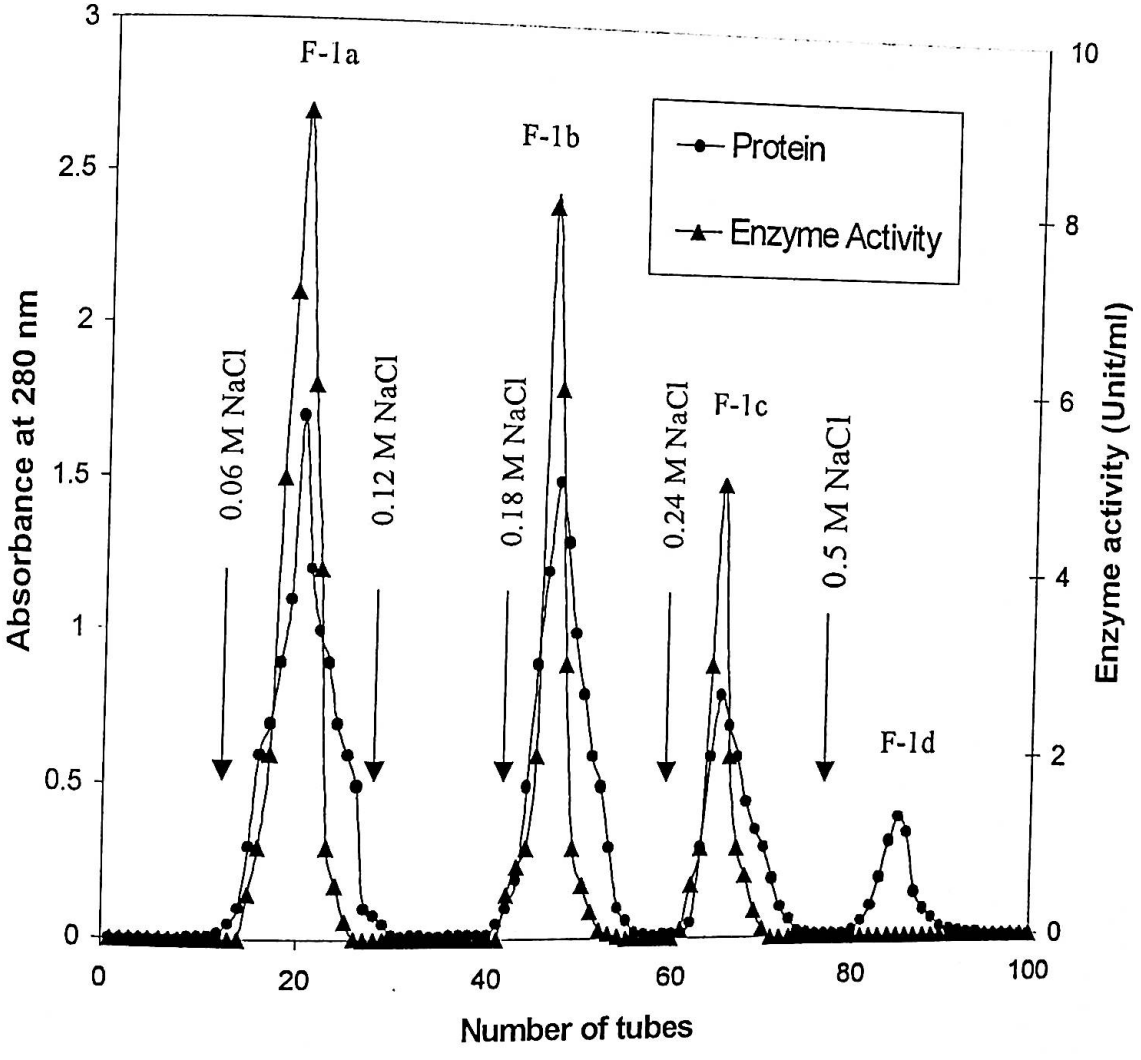


Figure-5.4: DEAE-cellulose column chromatography of protease active fraction from Gel filtration.

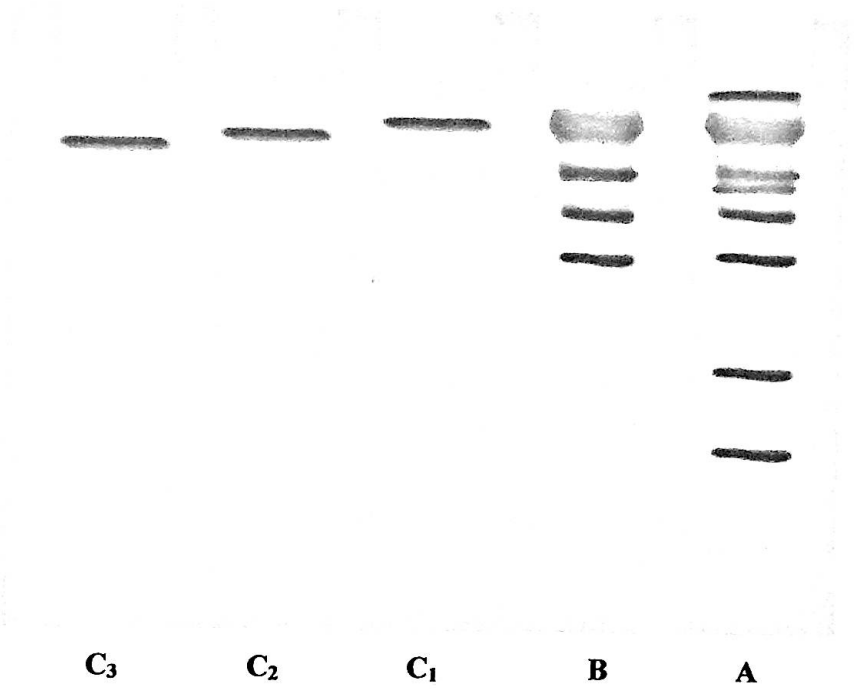


Figure-5.5: Photographic representation of SDS-polyacrylamide slab gel electrophoresis of the various fractions obtained during the purification of proteases.

A: Crude enzyme solution.

B: After gel filtration column.

C_1, C_2 and C_3: After DEAE-cellulose column.

A BRIEF SCHEME OF THE OVERALL PURIFICATION STEPS:

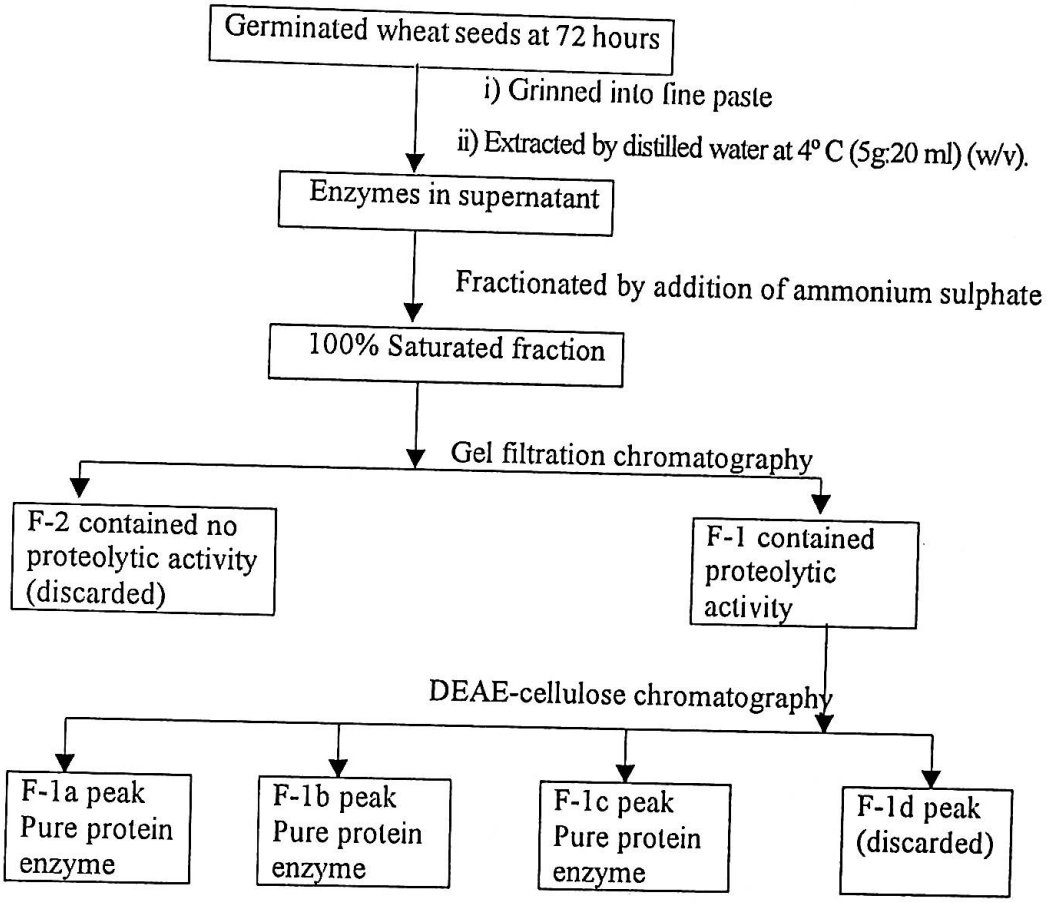


Figure-5.6: Schematic representation of the purification steps of protease.

Table-5.1, summarizes the purification of proteolytic enzymes from germinating wheat seeds (Akbar variety). The specific activities of the extracted enzyme increased at each purification step and the purification fold was nearly 30-40.

Table-5.1: Purification summary of germinating wheat seeds protease (Akbar) variety.

Steps of purification		Total protein (mg)	Total activity (units)	Specific activity (unit/mg)	Yield (%)	Purification folds
Crude extract		460	1015	2.21	100	1
Ammonium sulfate		370	890	2.41	87.68	1.09
After gel filtration		4.12	122.57	29.07	12.08	13.46
DEAE-cellulose fraction	pro-I	0.87	76.34	87.75	7.52	39.71
	pro-II	0.66	52.71	79.86	5.19	36.14
	pro-III	0.45	30	66.66	2.95	30.16

5.3.4 DETERMINATION OF OPTIMUM TEMPERATURE: The effects of temperature on the proteolytic activities were examined in the range of 10-100° C. The activities of the purified enzyme increases remarkably having the maximum activities at 45, 43 and 42° C for pro-1, pro-II and pro-III, respectively (figure-5.7). The activities began to decrease gradually with the rise of temperature at or above 60° C. Very little activities were found at or above 90° C and at or below 10° C.

5.3.5 DETERMINATION OF OPTIMUM pH: The activities of the protease's were greatly influenced by pH changes. The enzymes pro-1, pro-II and pro-III gave maximum activities in the ranges of pH 7.1, 6.9, and 6.8, respectively (figure-5.8). From the results it might be concluded that enzymes pro-II and pro-III isolated from germinating wheat seeds belong to the category of slightly acidic proteases while pro-I was slightly alkaline proteolytic enzyme in nature.

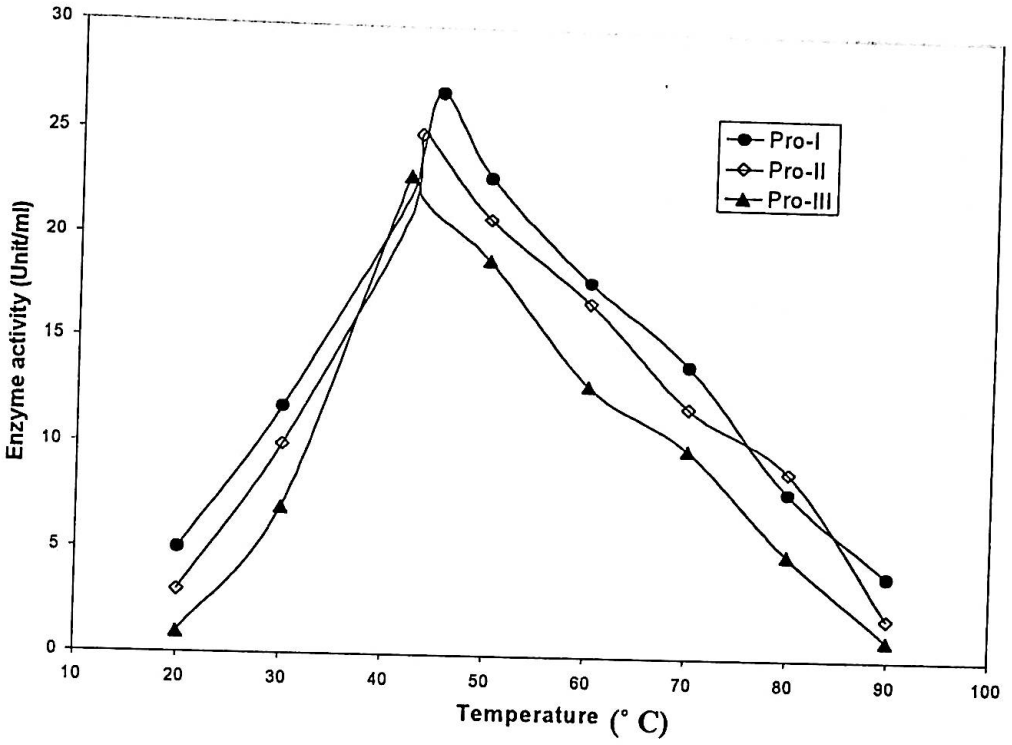


Figure-5.7: Effect of temperature on the protease activities from germinating wheat seeds.

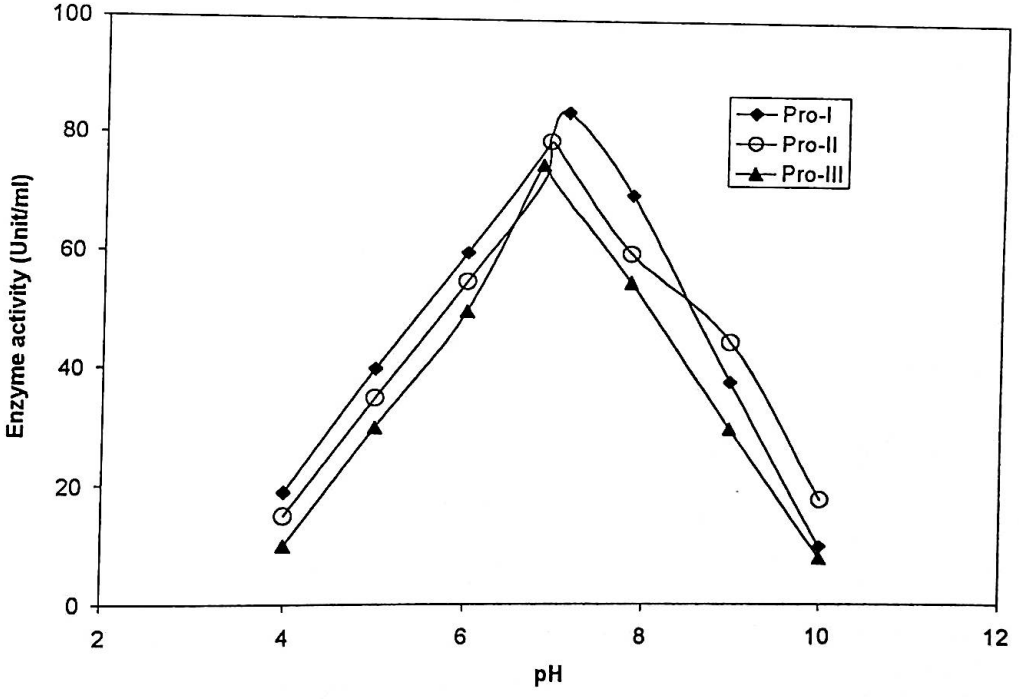


Figure-5.8: Effect of pH on the protease activities from germinating wheat seeds.

5.3.6 MOLECULAR WEIGHT DETERMINATION: The molecular weights of the enzyme were determined by comparing their elution volume on Sephadex G-75 with those of the marker proteins under same experimental conditions. The molecular weights were calculated from the standard curve of the reference proteins, constructed by plotting log of molecular weight against elution volume on gel fraction. The molecular weights were found to be 108 kD, 104 kD and 99 kD for pro-I, pro-II and pro-III respectively (figure-5.9). The molecular weights of proteases were also determined by SDS-polyacrylamide gel electrophoresis using another set of marker proteins under reducing and non-reducing conditions. The molecular weights of the enzymes were found to be same under both the conditions indicating that the enzymes have no sub-unit (figure-5.10) structure.

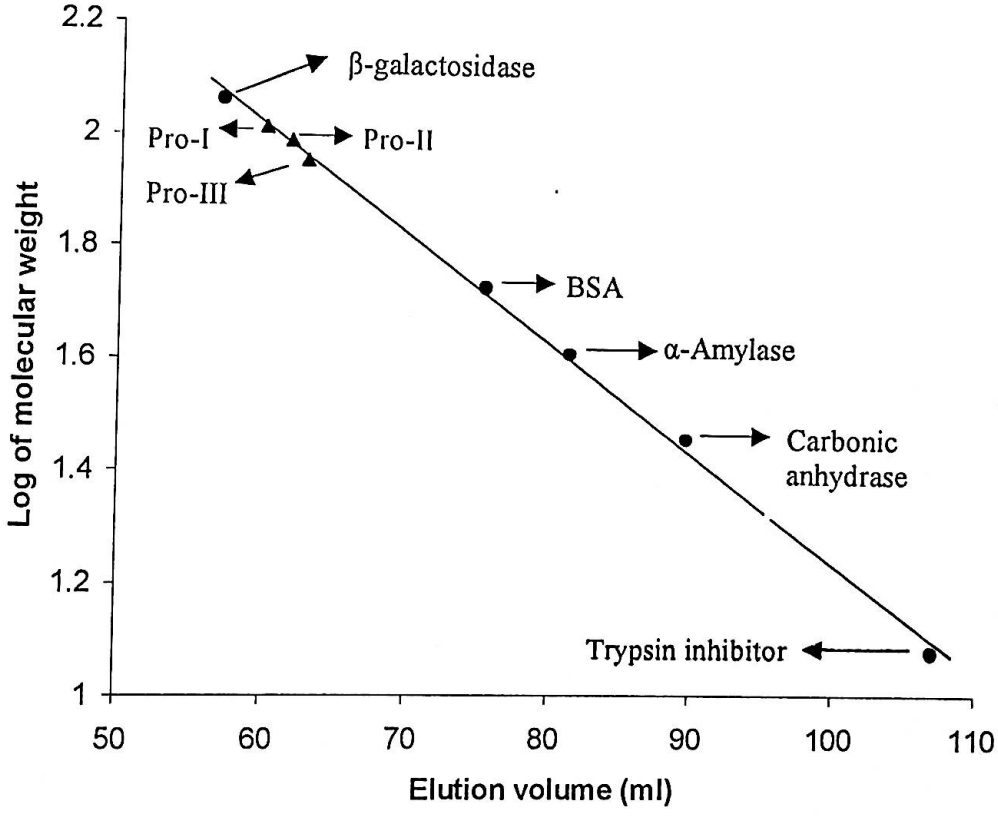


Figure-5.9: Standard curve for the determination of molecular weight of protease by gel filtration method.

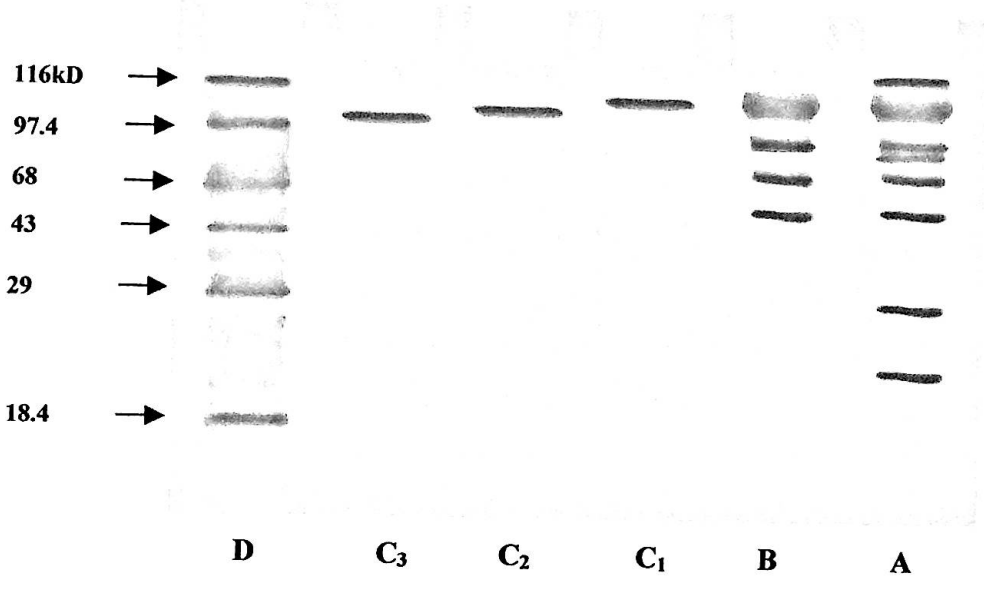


Figure-5.10: Photographic representation of SDS-polyacrylamide slab gel electrophoresis of the various fractions obtained during the purification of proteases for determination of molecular weight.

A: Crude enzyme solution.

B: After gel filtration column.

C_1, C_2 and C_3: After DEAE-cellulose column.

D: Marker protein solution containing β-lactoglobulin (18.4kD), carbonic anhydrase (29kD), ovalbumin (43kD), bovine serum albumin (68kD), phosphorylase-b (97.4kD) and β-galactosidase (116kD).

5.3.7 EFFECT OF EDTA, UREA AND VARIOUS METAL IONS ON THE ENZYME ACTIVITIES: EDTA, a metal chelating agent had an activating effect on the extracted enzyme from germinating wheat seeds (Table-5.2). The enzymes present in the germinating wheat seeds homogenate may thus be a characteristic cysteine type proteolytic enzyme, specifically for the enhancement of activity by EDTA. The activities of the proteases were found to be increased in the presence of urea. As shown in the table, the activities of Pro-I, Pro-II and Pro-III were gradually increased with the increase of urea concentrations up to 4M but with further rise of urea concentrations (6 M or above) the activities were found to be decreased gradually. The effect of calcium as metallic salt on the activities of enzymes is presented in the table-5.2. The activities of pro-I, pro-II and pro-III were gradually increased with the increase in concentration of calcium. The effect of other metal salts on germinating wheat seed proteases is also presented in the same table (table-5.2). From the table we have observed that Mg^{2+} and Mn^{2+} have no significant effect on the activities of proteases, while the presence of Hg^{2+} and Fe^{2+} completely inhibited all the three enzymes. The presence of Zn^{2+} and K^{+} increases the activities of the enzymes slightly.

Table-5.2: Effect of EDTA, urea and various metal ions on the activities of proteolytic enzymes.

Chemicals	Concentration (Molarity)	Relative activity % pro-I	Relative activity % pro-II	Relative activity % pro-III
EDTA	0.000	100	100	100
	0.001	99	98	96
	0.002	104	102	99
	0.005	109	108	106
	0.010	112	109	108
	0.020	116	113	110
Urea	1	104	102	101
	2	108	105	103
	4	112	108	104
	6	109	107	106
	8	105	104	103
$CaCl_2$	0.001	101	100	100
	0.002	104	102	102
	0.005	107	104	103
	0.010	109	108	107
	0.020	113	111	108
	0.050	116	114	110
	0.100	120	117	115
	0.500	127	122	119
$MgCl_2$	0.001	97	95	92
	0.002	90	90	87
	0.005	84	80	79
$MnCl_2$	0.001	100	100	94
	0.002	98	96	92
	0.005	91	86	85
KCl	0.001	100	100	100
	0.002	104	103	101
	0.005	106	104	102
$ZnCl_2$	0.001	100	100	100
	0.002	103	103	102
	0.005	105	103	102
$HgCl_2$	0.001	25	23	18
	0.005	00	00	00
$FeCl_2$	0.001	17	14	11
	0.005	00	00	00

5.3.8 DETERMINATION OF Km VALUE: The activities of purified enzyme were analyzed using different concentrations of casein as substrate. The Km values for pro-I, pro-II and pro-III were estimated to be 0.027, 0.032 and 0.032 mM respectively (figure-5.11, 5.12 and 5.13).

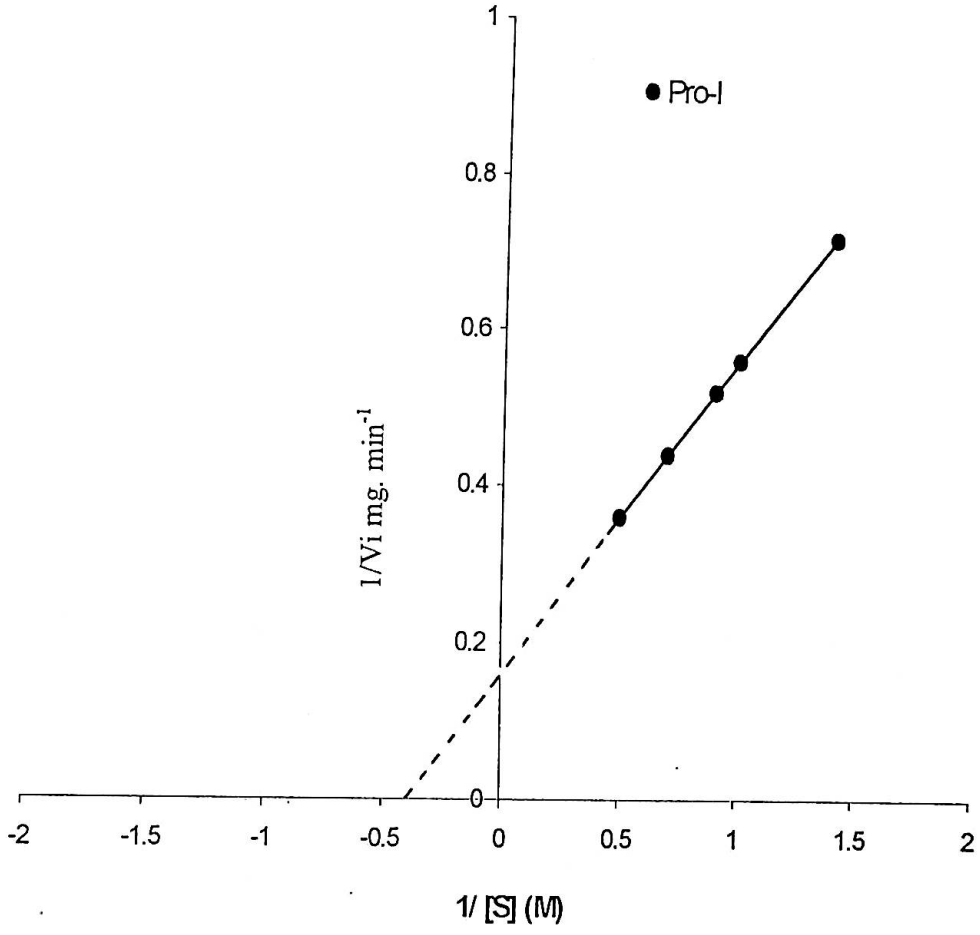


Figure-5.11: Lineweaver–Burk double reciprocal plots for the determination of Km value of protease-I.

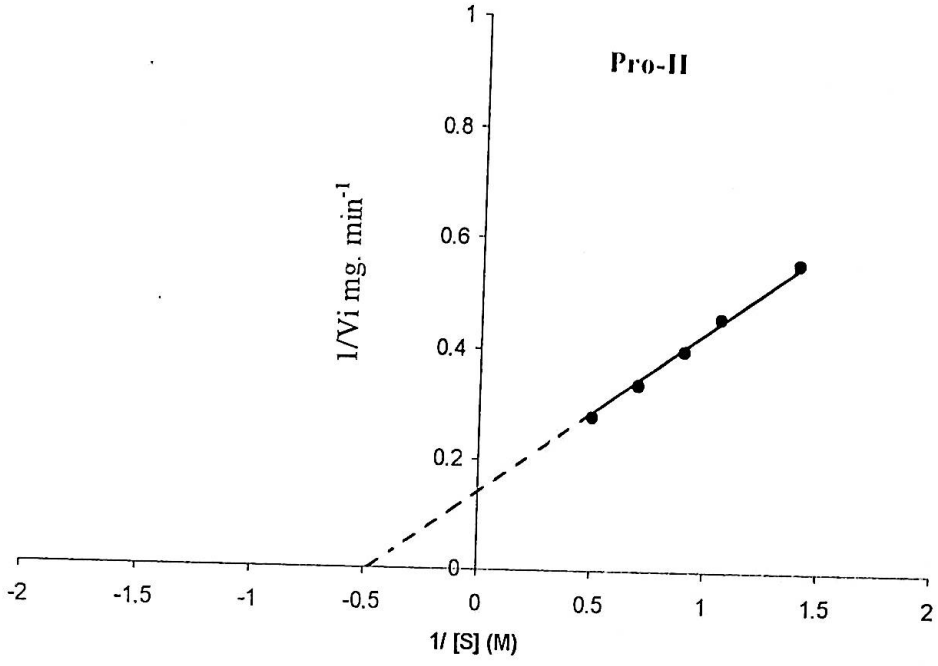


Figure-5.12: Lineweaver–Burk double reciprocal plots for the determination of Km value of protease-II.

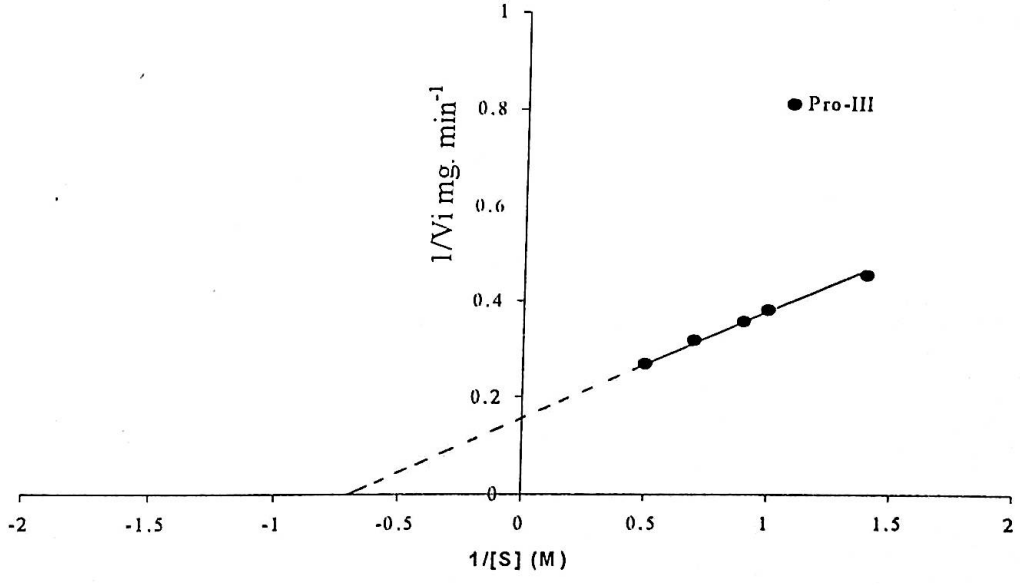


Figure-5.13: Lineweaver–Burk double reciprocal plots for the determination of Km value of protease-III enzyme

5.3.9 EFFECT OF INCUBATION PERIOD:

Pure protease activity at various incubation periods at assay temperature (45° C) is shown in (figure-5.14). It was observed that the enzyme activities gradually decreases with increase of time. Highest proteolytic activities were found when the homogenate assayed after heat treatment for 30 minutes. After 3 hours or 4 hours, the activities of Pro-I, Pro-II and Pro-III decreases to 50%, 45% and 43% respectively.

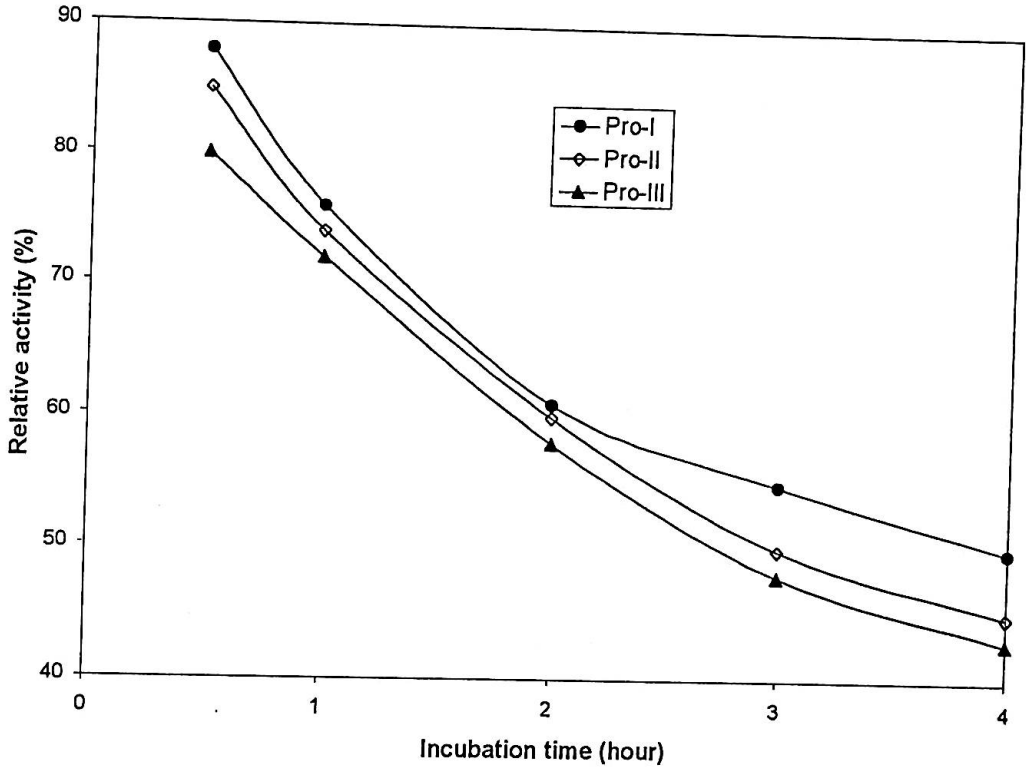
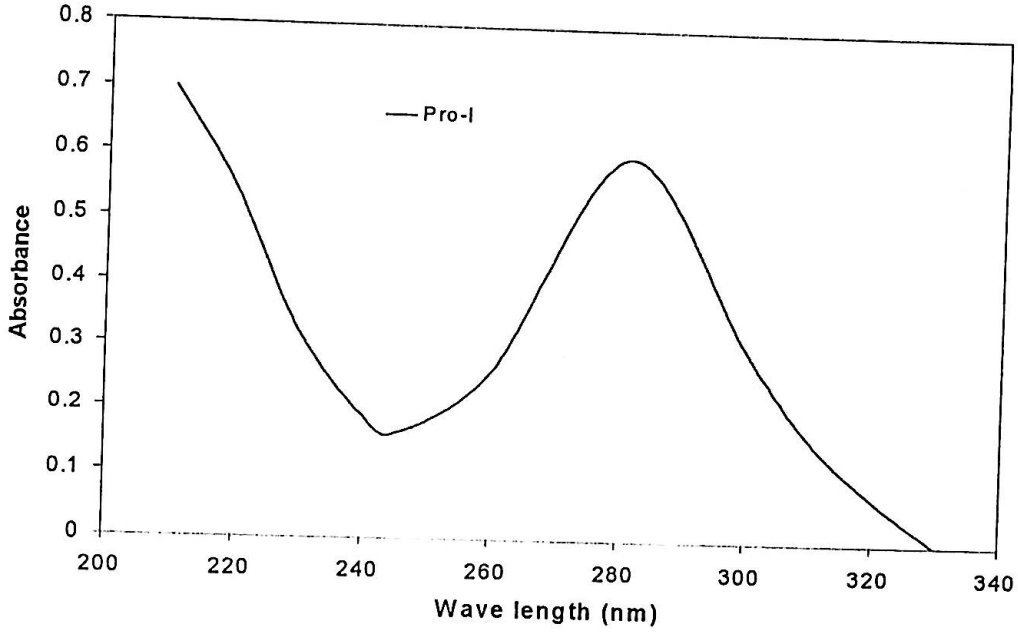
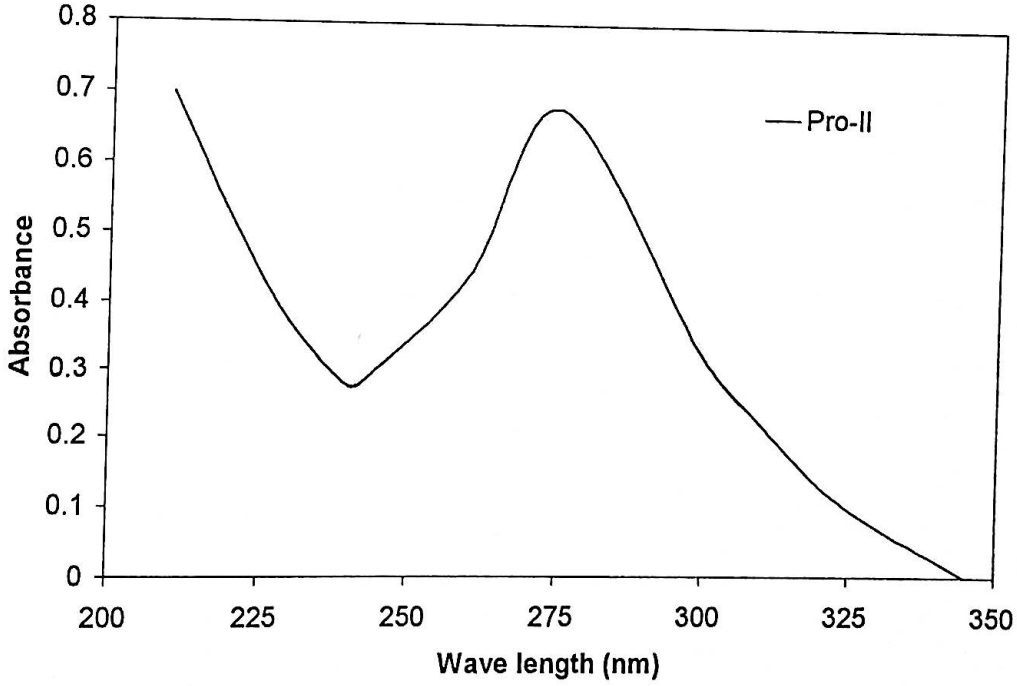


Figure-5.14: Effect of incubation time at optimal temperature on the protease activities of germinating wheat seeds.

5.3.10 ULTRAVIOLET ABSORPTION SPECTRUM:

The purified enzymes, Pro-I, Pro-II and Pro-III in aqueous solution gave absorption maxima at around 281, 275 and 273 nm; and minima at around 245, 243 and 242 nm respectively (figure-5.15).





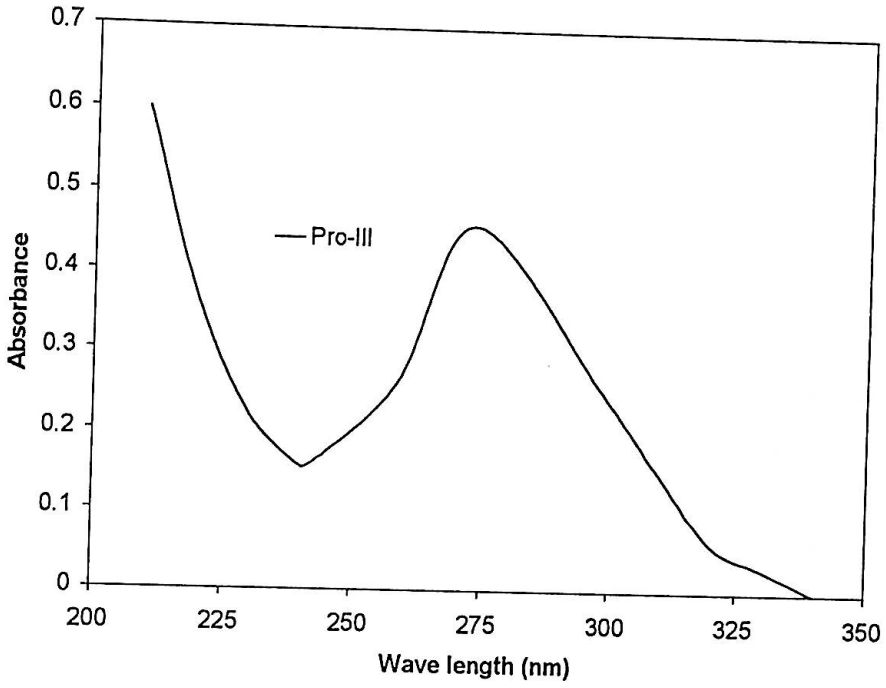


Figure-5.15: Ultraviolet absorption spectrum of proteases from germinating wheat seeds.

5.3.11 SUBSTRATE ACTIVITY:

Protease from germinating wheat seeds showed activity on several substrates. Casein solutions were used as a substrate. But seed storage proteins (globulin) were found to be the good sources of substrate for these enzymes than casein. As shown in the table-5.3, all three proteases showed about 49-52% activity with casein substrate. On the other hand the activities were within the range of 104-118% and 83-92% on wheat and bean respectively.

Table-5.3: Substrate activity.

Substrate	Relative activity %		
	Pro-I	Pro-II	Pro-III
Casein	52	50	49
Wheat (Akbar)	118	109	108
Wheat (Kanchan)	112	106	104
Bean (Ipsha-I)	92	87	84
Bean (Dolicos)	90	86	83

5.4 DISCUSSIONS

Many researchers have undertaken the task of purifying and characterizing a variety of proteases and peptidases in the recent past from various sources. Some of which occur only transiently in germinating seeds (8, 25-27). However, the main drawback in these studies is the lack of reliable assay methods for these enzymes, since seed extracts usually contain protease enzymes as well as other proteins that compete with the added substrate. Proteolytic enzymes pro-I, pro-II and pro-II purified from germinating wheat seed showed molecular weights of 108 kD, 104 kD and 99 kD respectively. Many laboratories purified protease enzyme with variation in molecular weight from plant sources. A protease enzyme with molecular weight of 100-112 kD was purified from the latex of Akanda (*Calotropis procerra*) while Shevchenko et al. (28), purified a thiol protease from skeletal muscle junctional sarcoplasmic reticulum with molecular weight of 94 kD, Ciborowski et al. (29), purified two cysteine ptoteinase from Porphyromonas gingivalis of molecular weight 120 kD and 150 kD, Raphael et al. (30), purified a cysteine protease from Plasmodium falciparum of molecular weight of 155 kD and Kageyama and Takahashi (31), purified a cysteine proteinase from silkworm eggs of molecular weight of 350 kD by gel filtration and 47 kD by electrophoresis on sodium dodecyl sulfate/ polyacrylamide gels, suggesting an octameric structure.

The enzymes pro-I, pro-II and pro-III showed maximum activities at the pH of 7.1, 6.9 and 6.8 and at temperatures of 45, 43 and 42 °C respectively. The optimum pH of protease around 6.3-7.5 were reported by Keay et al. (32) and Klaper et al. (33), 6.5-8.0 was reported by Banno et al. (34) and Chen et al. (35) was also reported a pH optimum in the neutral to alkaline range, which is nearly similar to pH value obtained from our investigated enzymes. The optimum temperature of protease at 45°C was reported by Mercado-Flores et al. (36) and around 50-55°C was also reported by Kundu et al. (37). These reports support our results.

Metallic ions like Mg^{2+} and Mn^{2+} had no effects on proteolytic activities while EDTA, urea and Ca^{2+} had an activator effect. Hg^{2+} and Fe^{2+} had strong inhibitory action on proteolytic activities. Ahmed et al. (38), reported that protease from the larval gut of *Spodoptera litura,* which were not inhibited by Mg^{2+} and Mn^{2+} while the presence of

Hg^{2+} and Fe^{2+} completely inhibited the three enzymes, Kumura et al. (39), reported that the protease from Pseudomonas fluorescens , were inhibited by Hg^{2+}, Cu^{2+} and Fe^{2+} , Cazzulo et al. (40), reported that the protease from Trypanosoma cruzi, was activated by urea, Darby et al. (41) and Abe et al. (42), also reported that the proteolytic activity is increased in the presence of EDTA and Ca^{2+}. These reports support our results. The increase of enzyme activity in the presence of urea and EDTA is difficult to explain at this stage. The enhancement of enzyme activity observed with different concentrations of urea and EDTA may probably be due to the unfolding effect on casein (substrate) thus making it a better substrate.

The Km values of the enzymes pro-I, pro-II and pro-III were found to be 0.027, 0.032 and 0.032 mM for casein as substrate respectively. The Km value of protease from Entamoeba invadens has been reported to be .022 mM reported by Scholze and Schulte (43), 0.055 mM from tomato juice reported by Islam (44) and .183 mM from Thermotoga neapolitana also reported by Dong and Zeikus (45).

Protease's, extracts from microorganism are used in the food industries to improve the food quality which are very expensive. Our laboratory reported the existence of proteolytic enzyme in the germinating wheat seeds. So, purified proteolytic enzyme from germinating wheat seeds might open new possibilities by changing the functional properties of food proteins. Research work on this field is going on in our laboratory for future use.

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CHAPTER-6

IMPROVEMENT OF FUNCTIONAL PROPERTIES OF GLUTEN BY PROTEASE FROM GERMINATING WHEAT SEEDS

6.1 INTRODUCTION: The insolubility of proteins limits their utilization in formulated food systems. Wheat gluten is a typical insoluble protein and an abundant byproduct of the food industry. The poor solubility of gluten has been mainly attributed to its amino acid composition, particularly to the presence of a large number of nonpolar amino acid residues such as proline and leucine. On the other hand, the amide groups in the side chains of glutamine and asparagine in gluten play an important role in stabilizing the protein structure and in promoting the association of gliadine and glutenine molecules through hydrogen bonding (1). Many researchers have developed methods to change the solubility and functional properties of gluten. Finley (2) suggested a mild acid treatment of wheat gluten to increase its solubility in fruit-based acidic beverages. Wu et al., (3) found a significant improvement in the functional properties of gluten by mild acid hydrolysis. It has been reported that deamidation to an extent as low as 2%-6% could enhance the functional properties of proteins (4,5). Shaha et al., (6) reported that deamidase from germinating wheat seeds improved the functional properties of gluten. Nevertheless, there is little information on the deamidation of proteins by protease. Here we try to study the effect of crude protease (from germinating wheat seeds) on gluten. This may be of value in the utilization of proteins by improving their functional properties.

6.2 MATERIALS AND METHODS: For the extraction of crude enzyme, we used the Akbar variety of wheat seeds (Triticum aestivum L.) germinated at 25° C in the dark for 72 hours. The germinating seeds were taken in a mortar and ground uniformly into fine powder. Five grams of seed-powder mixed uniformly with pre-cooled 50 ml of deionized water was kept overnight at 4° C with occasional gentle stirring. The extract was then centrifuged at 8000 rpm for 15 minutes and the proteins were salted-out from the supernatant by gradually adjusting to 100% saturation with ammonium sulfate. The precipitate was dissolved in a minimum volume of cooled deionized water and dialyzed against 5 mM phosphate buffer, pH 7.0. After centrifugation, the clear supernatant was applied to the Sephadex-G-75 column previously equilibrated with 5 mM phosphate buffer, pH 7.0. Two peaks appeared in the chromatogram. The first peak containing protein showed the protease activity and was pooled, concentrated, freeze-dried and used as a crude protease enzyme. Protease activity was measured according to Kunitz (7). The second peak containing eluent did not show protease activity and it was discarded.

The following experiments described in 6.2.1 to 6.2.7 were performed in Japan by the help of Prof. R.K. Shaha and Prof. A. Kato.

6.2.1 WHEAT GLUTEN PREPARATION: Gluten was prepared by washing flour dough with water until the washings were free from soluble protein. The gluten ball thus obtained was dialyzed against distilled water and then freeze-dried in a freeze–dryer (VD-80, Taitec Co., Japan).

6.2.2 CRUDE ENZYME TREATMENT OF GLUTEN SAMPLES: The freeze-dried sample (4.0 g) of gluten was suspended in 200 ml of 0.05 M Tris-HCl (pH 7.5) buffer containing 0.05% sodium azide. To it 100 mg of crude protease from germinating wheat seeds was added. The mixture was incubated at 35° C with shaking for 24 hours. After incubation, the reaction mixture was immediately heated for 5 min at 90° C to inactivate the protease. The enzymatically treated gluten was centrifuged (8000 rpm for 10 minutes) to remove any undigested protein, and then the supernatant was dialyzed (3000-4000, MW cut off) against distilled water for 24 hours at 4° C and freeze-dried. The yield of crude protease digests obtained after dialysis was in the range of 40%-50%. Control gluten was similarly treated without protease.

6.2.3 PREPARATION OF COMMERCIAL PROTEASE-DIGESTED GLUTEN: A freeze-dried sample (4.0 g) of gluten was suspended in 400 ml of 0.05 M Tris-HCL (pH 8.0) buffer containing 0.05% sodium azide and 40 mg of Pronase-E was added (10). The mixture was incubated at 37° C with shaking for 24 hours. After incubation, Pronase-E was inactivated by heating at 100° C for 3 minutes. The Pronase-E-treated gluten was centrifuzed (8000 rpm for 10 minutes) to remove any undigested protein, and the supernatant was then dialyzed against distilled water for 24 hours at 4° C and freeze-dried. The yield of protease digests obtained after dialysis was in the range of 60%-75%.

6.2.4 DETERMINATION OF AMMONIA: Direct determination of the ammonia released from the gluten during treatment with crude protease and Pronase-E was carried out using a Toyosoda amino acid analyzer (HLC-805). The enzyme reaction was performed in a Thunberg tube at 25° C in vacuum; a 0.5% gluten

pH 8.5) containing crude protease (1.5mg) or Pronase-E (0.5mg) was placed in the main tube and 2.0 ml of 0.05 M H_2SO_4 was placed in the side tube as an absorbent of the ammonia. After incubating without mixing, 1.0 ml of the H_2SO_4 solution was diluted with 9.0 ml of de-ammonized water, and 100 µl of the diluted solution was injected into the amino acid analyzer.

6.2.5 DETERMINATION OF THE DEGREE OF PROTEOLYSIS: After the enzymatic treatment, the treated gluten solution (3.0 ml) was mixed with equal volumes of 20% trichloroacetic acid (TCA). The mixture was filtered, and the absorbance of the filtrate was measured at 280 nm. The degree of proteolysis is expressed as the ratio of absorbance of the TCA soluble fraction in the treated gluten solution to that of the native gluten solution treated with 2.0 M HCl for 2 hours at 110° C. Freeze-dried samples (0.2%) of crude enzyme and protease digests was used for the determination of solubility at various pHs: pH 2-3, 0.05 M citrate buffer, pH 4-5, 0.05 M acetate buffer, pH 6-8, 0.05 M phosphate buffer, pH 9-11, 0.05 M carbonate buffer; and pH 12, 0.05 M NaOH adjusted with 0.05 M HCl. Samples were dissolved in the buffer and shaken with a vortex mixer (Scientific Industries, adjusted on digit 4 to work on touch) for 10 s, and the turbidity was measured at 500 nm. Values reported are averages of triplicate studies.

6.2.6 MEASUREMENT OF EMULSIFYING PROPERTIES: The emulsifying property of freeze-dried sample solutions was determined by the method of Pearce and Kinsella (8). To prepare the emulsion, 1.0 ml of corn oil and 3.0 ml of crude protease treated protein solution (2%) in 0.1 M phosphate buffer, pH 7.0, were shaken together and homogenized in an Ultra Turrax (Hansen & Co., West Germany) at 12000 g for 1 min at 20°C. A 50µl sample of emulsion was taken from the bottom of the container at different times and diluted with 5 ml of 0.1% sodium dodecyl sulfate solution. The absorbance of the dilute emulsion was then determined at 500 nm. The relative emulsifying activity was determined from the absorbance immediately measured after the emulsion formation. The emulsion stability was expressed as the time taken for the initial turbidity of the emulsion to halve (9-10). Values reported are averages of triplicates.

6.2.7 MEASUREMENT OF FOAMING PROPERTIES: The foaming properties of freeze-dried sample solution were determined according to the conductivity method of Kato et al. (11). Electric conductivity of foams was measured when air was introduced into 5 ml of 2% protease-treated protein solution in 0.02 M phosphate buffer, pH 7.0, in a glass filter (G-4) at a constant flow rate (1.5 cm^3/s). The foam stability is expressed as C_0 x dt/dc, where dc is the change in conductivity occurring during the time interval dt, and C_0 is the conductivity at zero time obtained by extrapolating the plot of linear conductivity Vs time. The foam stability is indicated as the time for the disappearance of the conductivity. Values reported are averages of triplicates.

6.3 RESULTS AND DISCUSSIONS

Table-6.1 shows the amount of ammonia released from the gluten during treatment with crude protease from germinating wheat seeds and commercial Pronase-E at pH 8.5 and at 25° C. As the incubation time increased the ammonia released reached 90 µg-105 µg at an incubation time of 2.5 hours. On the other hand, the ammonia released from the untreated gluten was low even at an incubation time of 3.5 hours. This indicates that the amide ammonia of gluten was released by treating with the extracted crude protease and with Pronase-E.

Table-6.1. Release of ammonia from treated gluten

	Incubation Time (h)						
	0	0.30	0.45	1.0	2.0	2.5	3.5
Untreated Gluten	3.5	4.2	6.0	9.0	11.5	12.0	14.1
Gluten treated with extracted crude protease	5.0	12.0	36.0	40.0	85.0	90.0	92.0
Gluten treated with standard Pronase-E.	6.0	11.5	38.5	42.8	90.0	105.0	112.0

Figures-6.1 and 6.2 show the time course of the deamidation and proteolysis respectively of gluten by extracted crude protease and Pronase-E at pH 8.5 and at 25°C. The degree of deamidation increased linearly up to 2.5 hours and increased slightly after that. After incubation for 2.5 hours, the deamidation of gluten was about 25%-28%. A small percentage of deamidation also occurred in the untre

under the experimental conditions. The changes in proteolysis of the extracted enzyme and Pronase-E treated gluten were similar to each other, reaching a maximum of 10% in 1 hour. Figure-6.3 shows the gel filtration patterns of gluten samples. As shown in figure-6.3A, the elution pattern of native gluten showed two major peaks corresponding to gluten and gliadin. Gluten treated with extracted crude protease as well as with Pronase-E gave a third peak in addition to these two peaks, this new peak being eluted after the gliadin fraction (figures-6.3B and 6.3C). The values of the free amino groups determined by using 2,4,6-trinitrobenzensulfonic acid (TNBS) did not show any changes compared with that of native gluten. Therefore, it is suggested that proteolysis of gluten by extracted crude enzyme and Pronase-E at pH 8.5 and at 25°C may have hardly occurred during the 2.5 hours enzymatic incubation. The functional properties of enzymatically (extracted as well as Pronase-E) treated gluten were then examined.

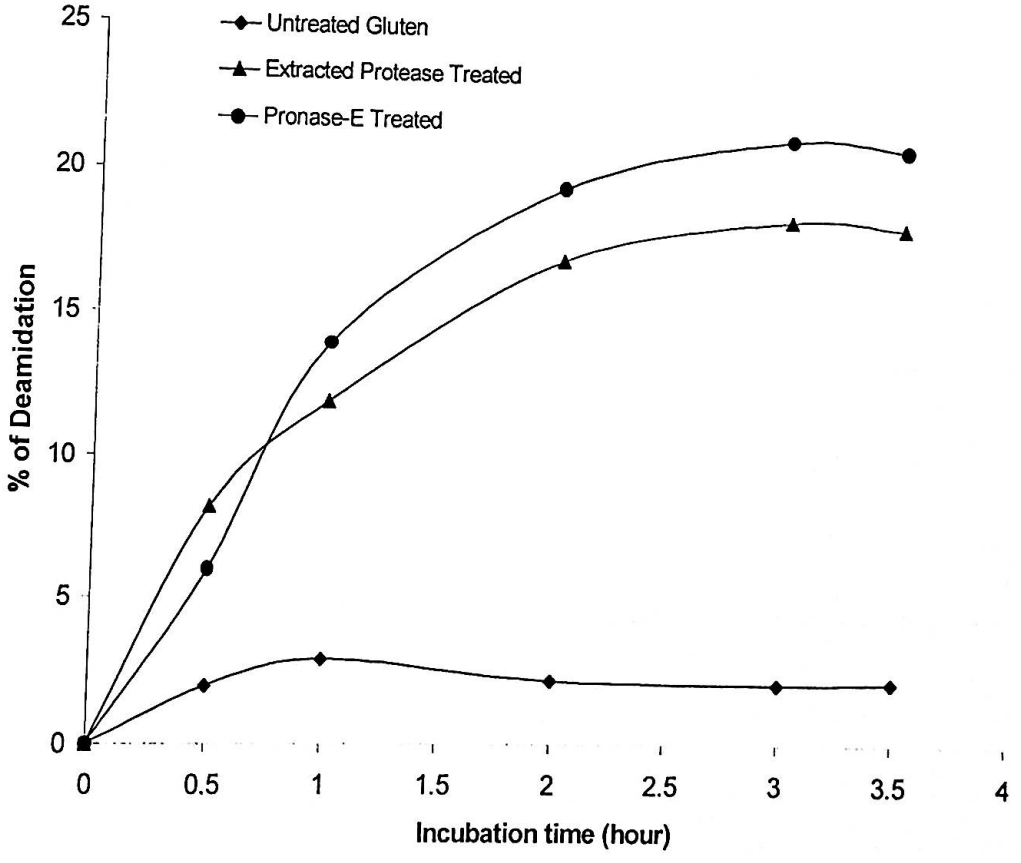

Figure-6.1: Time course for deamidation of gluten, treated with extracted protease and Pronase-E.

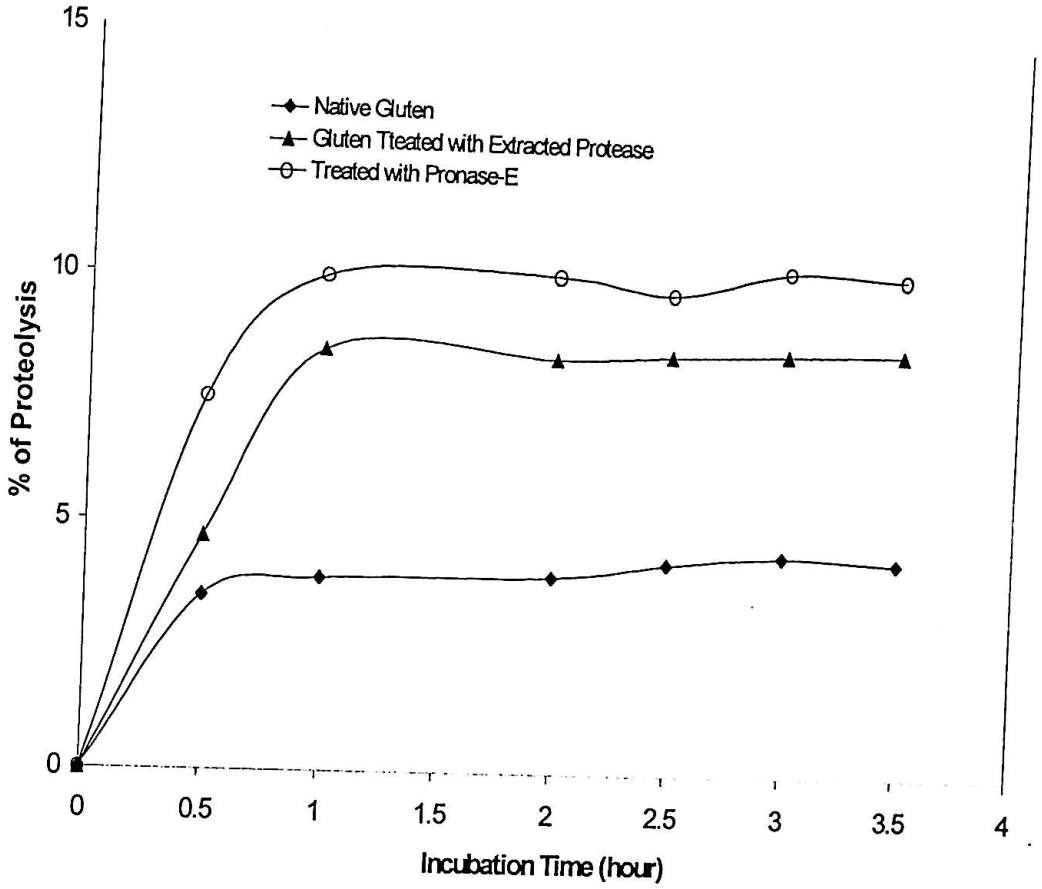

Figure-6.2: Time course for proteolysis of gluten, treated with extracted protease and Pronase-E

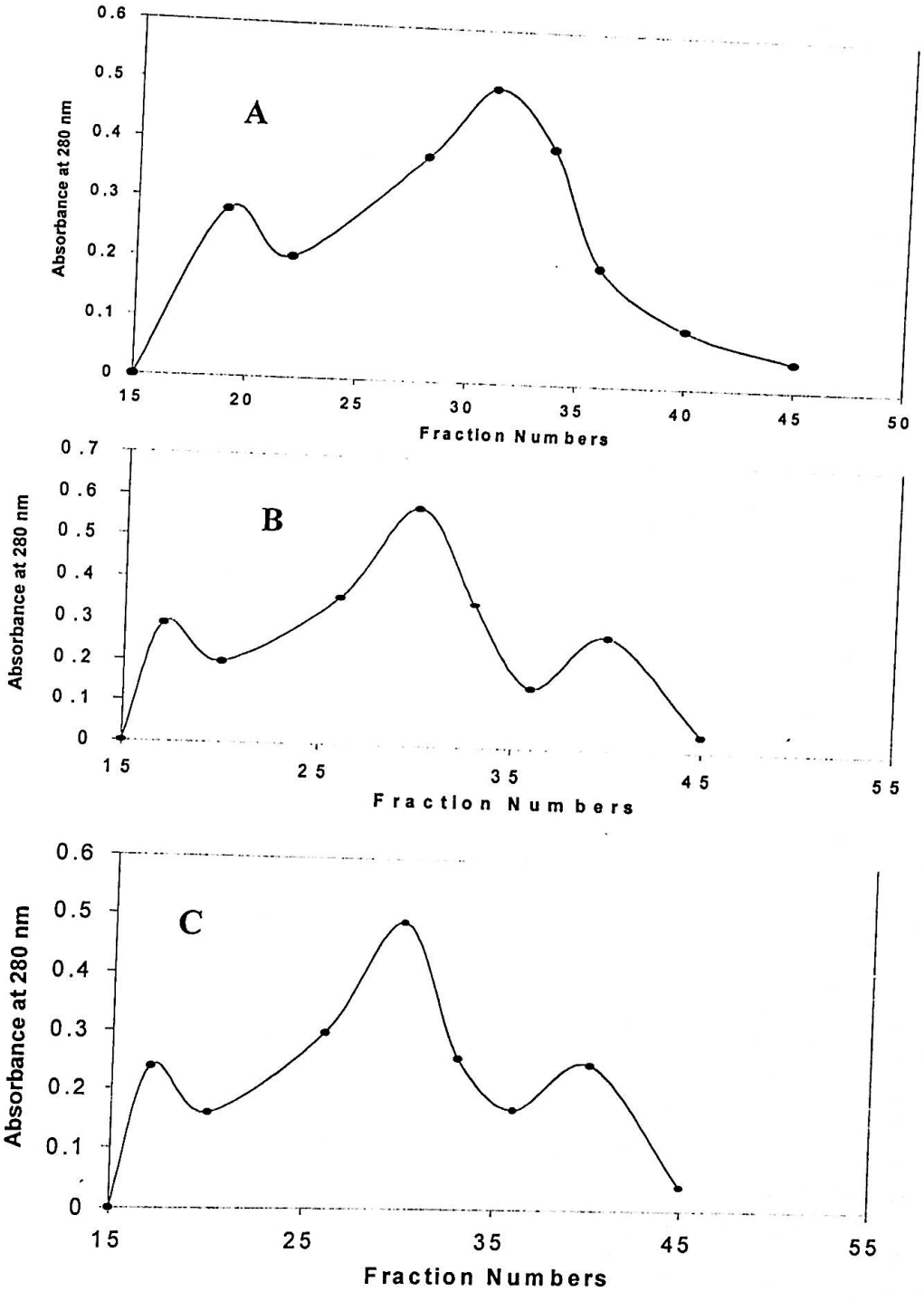

Figure-6.3: Gel filtration chromatogram of gluten treated with protease from germinating wheat, Pronase-E and native gluten in the presence of 2-mercaptoethanol. A: Native gluten, B: Treated with Pronase-E, and C: Treated with protease from germinating wheat seeds.

Figure-6.4 shows the solubility of the treated gluten. Enzymatically deamidated gluten was more soluble in a pH range above 6.0 when compared with native gluten. The solubility of the enzymatically treated gluten was shifted to the acidic pH range. This indicates that the free carboxyl groups in gluten may have been increased by deamidation of the glutaminyl and asparaginyl residues in gluten. Increased polar amino acid residues on the surface of treated proteins may contribute to increased solubility.

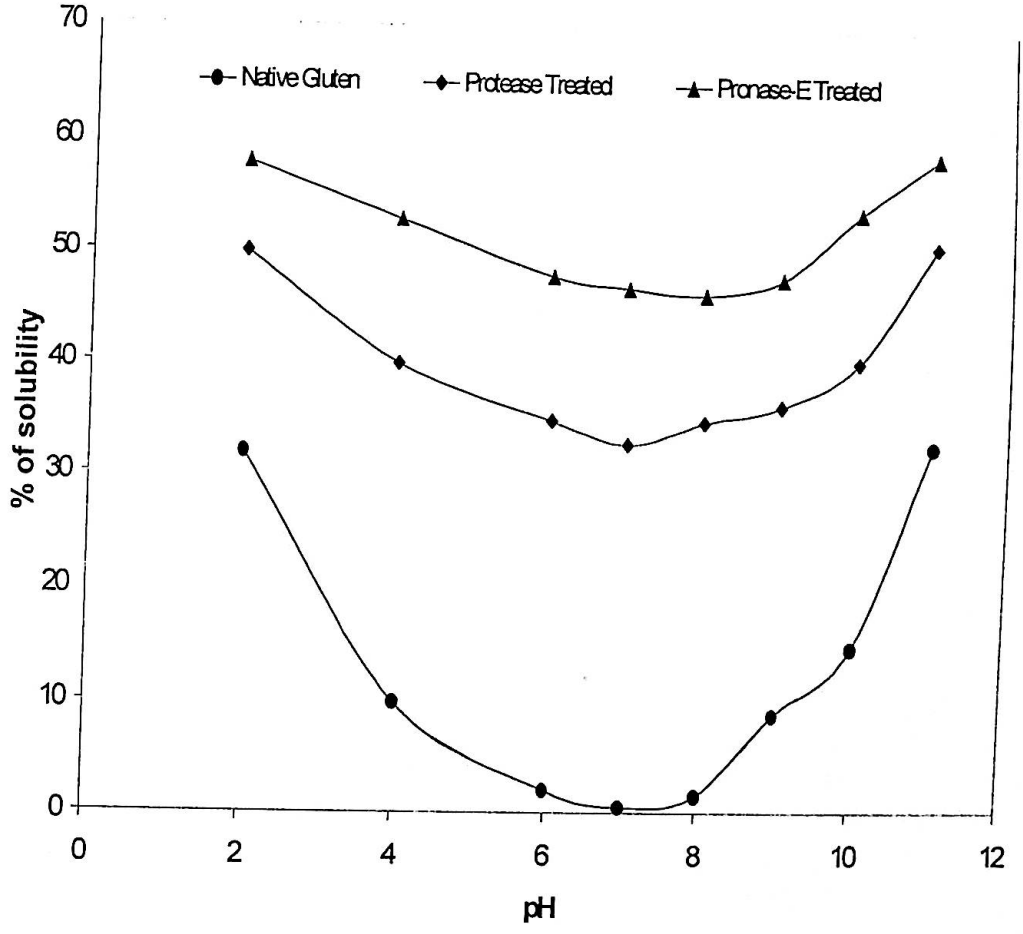

Figure-6.4: Effect of pH on the solubility of gluten treated with crude protease, Pronase-E and native gluten.

Figure-6.5 shows the emulsifying property of gluten. The emulsifying activities of gluten treated with the extracted crude enzyme as well as Pronase-E were higher than that of native gluten. In addition, the emulsions of the enzymatically treated glutens, were more stable than that of native gluten. The increase emulsifying property may probably due to induction of an amphiphilic nature by protease-catalyzed deamidation. Figure-6.6 shows the foaming property of gluten. The foaming properties of glutens treated with the extracted crude enzymes as well as Pronase-E were higher compared with that of native gluten. The improvement of foaming property was probably due to solubilization of the glutenin molecule by the enzymatic treatment.

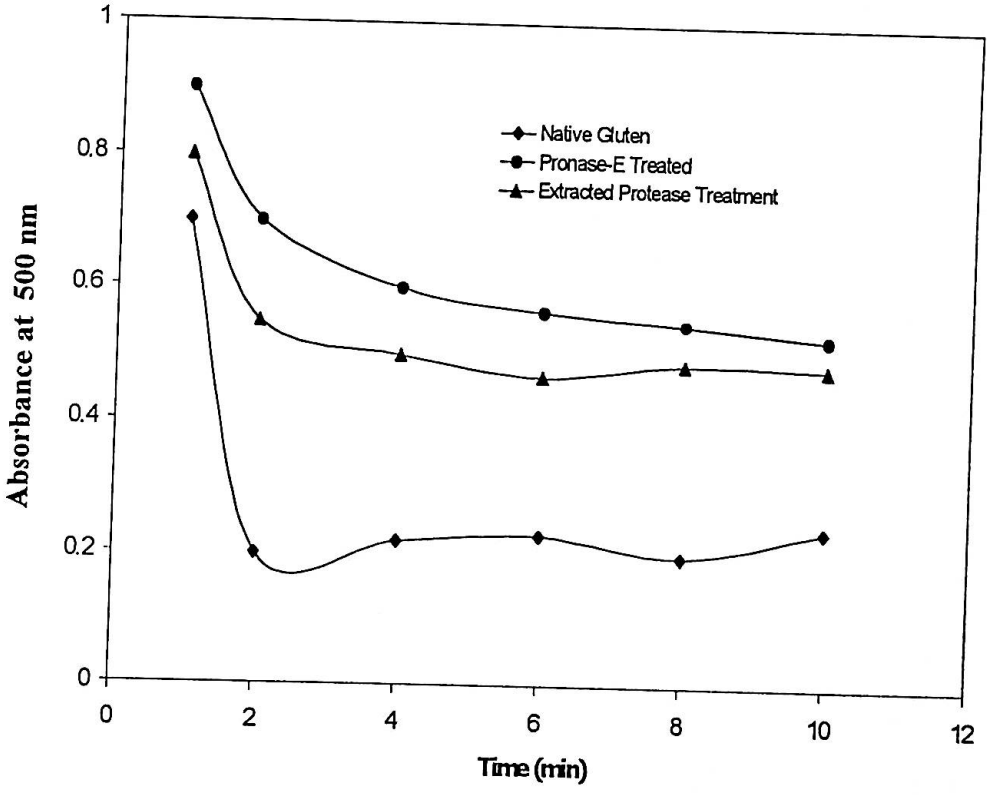

Figure-6.5: Emulsifying properties of gluten treated with extracted protease and Pronase-E

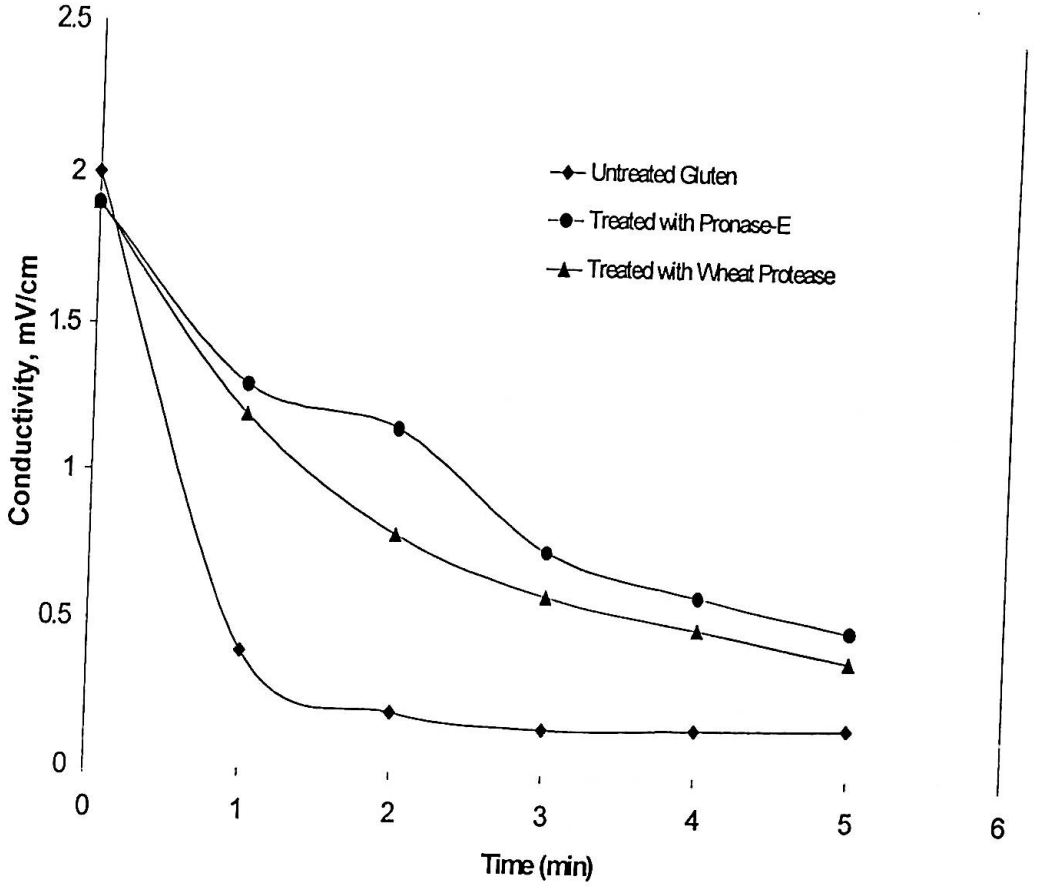

Figure-6.6: Foaming properties of gluten treated with protease from germinating wheat seeds, Pronase-E and of native gluten.

From this study it may be concluded that when gluten is treated with extracted crude protease from germinating wheat seeds and commercial Pronase-E at slightly alkaline media (pH 8.5) at 25°C, deamidated gluten is obtained without acid and heat-induced denaturation. The deamidated gluten improved functional properties appreciably like solubility, emulsifying and foaming ability. It is suggested therefore, that enzymatic deamidation of plant seed proteins containing a large amount of asparagine and glutamine could be a useful procedure for improving their functional properties without denaturation.

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