FUNCTIONAL CHARACTERIZATION OF PURIFIED WHEAT (*Triticum aestivum* L.) ALPHA AMYLASE INHIBITOR(S) FOR RICE WEEVIL (*Sitophilus oryzae*) MANAGEMENT

THESIS

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WITH LOVE

TO

MA-PAPA

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Certificate

This is to certify that the thesis entitled "Functional characterization of purified wheat (*Triticum aestivum* L.) alpha amylase inhibitor(s) for rice weevil (*Sitophilus oryzae*) management" submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** with major in **Biochemistry** and minor in **Molecular Biology & Biotechnology and Microbiology** of the college of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by **Mr. Ashutosh Dubey, Id. No. 22081,** under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation and source of literature have been duly acknowledged.

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Certificate

We, the undersigned, members of the Advisory Committee of, **Mr. Ashutosh Dubey, Id. No. 22081,** a candidate for the degree of **Doctor of Philosophy** with major in **Biochemistry** and minors in **Molecular Biology & Biotechnology and Microbiology,** agree that the thesis entitled **"FUNCTIONAL CHARACTERIZATION OF PURIFIED WHEAT (*Triticum aestivum* L.) ALPHA AMYLASE INHIBITOR(S) FOR RICE WEEVIL (*Sitophilus oryzae*) MANAGEMENT"** may be submitted in partial fulfillment of the requirements for the degree.

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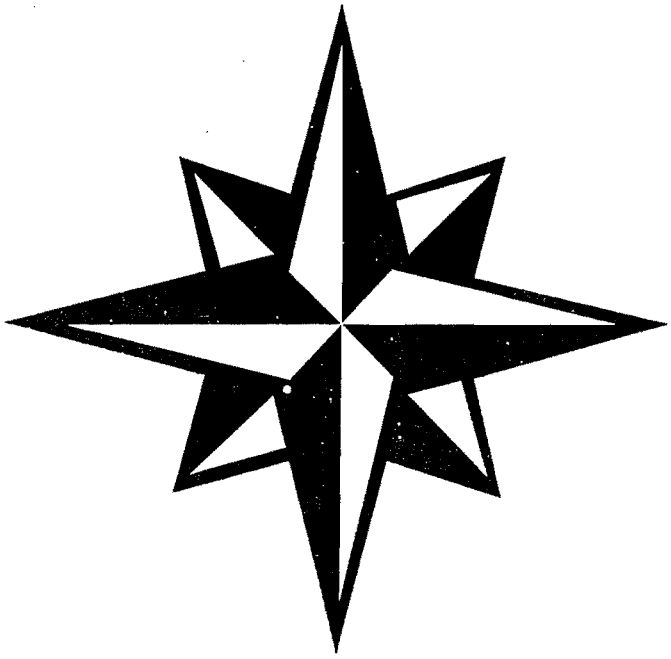
Chapter 1



INTRODUCTION

Chapter 1 INTRODUCTION

Plants evolved first and then arrived the herbivores. Therefore it is more likely that under the selection pressure of herbivores, plants evolved defense mechanisms. Some of these mechanisms were overcome by the herbivorous insects because of fast rate of adaptability amongst them. So, the insecticides had reigned the arena of pest control ever since the advent of DDT and BHC around forties. **Barfoat and Connett (1989)** estimated that just three crops- cotton, rice and maize, account for approximately half the total world expenditure on insecticides. However, the wisdom of using chemical insecticides on massive scale is now frequently being questioned. Apart from environmental and health risks associated with repeated exposures to chemical pesticides, it is becoming increasingly difficult to control insects due to the problem of insecticidal resistance and resurgence. Therefore, during the last two decades the emphasis has been shifted to explore biological means of pest suppression.

Host plant resistance is considered to be an ecologically sound approach of managing insect populations. In general plants defend themselves either by structural characteristics that act as physical barrier and inhibit the pathogen from gaining entrance and spreading through the plant or by means of biochemical reactions taking place in the cells/tissue to produce chemical substances that inhibit pathogen. Biochemical defense include release of inhibitor into the

environment, lack of essential factor, presence of several phenolics and tannins in high concentration in cells of young fruits or leaves. Some hydrolytic enzymes like glucanases, chitinases are also produced which help in the cell-wall breakdown of the pathogens. Some plant defences are induced by pathogen e.g. production of phytotoxins, inactivation of pathogen enzymes by inhibitor present in plants. Inhibitors of various enzymes are present in seeds of various plants e.g. protease, chitinase and amylase inhibitors. These inhibitors belong to the seed storage proteins, which involve lectins, protease inhibitor as well as protein with allergic properties, which lower the nutritional quality of food. But the development of insect resistant varieties through trial and error method generally takes long time. Therefore, resistant varieties develop at a very slow pace because of which insecticides have always dominated the pest control scenario. But due to the advances in molecular biology, the biotechnological and genetic engineering approaches can be exploited for host plant resistance. Several breakthroughs have been witnessed in the field of host plant resistance **(Fischhoff *et al.*, 1987; Nienhus *et al.*, 1987; Smith, 1989; Khush, 1977, 1989; Khush and Brar, 1991)**. Biotechnological approaches can help in developing transgenic plants carrying the genes of resistance from unrelated sources like insect pathogenic bacteria and viruses and plants containing inhibitors of proteinases/ amylases.

Proteinase inhibitor and α-amylase inhibitor genes have provided novel systems for investigations of the fundamental process that underlie the environmental and developmental regulation of natural defense systems in plants. These genes have considerable potential for the improvement of the plant defensive system either (a) through traditional breeding methods to select lines with enhanced expression, or (b) through direct transfer of genes(s) encoding inhibitor(s) of specific digestive enzymes of plant pests and pathogens through novel biotechnological techniques. Traditional breeding of major food crops for enhanced inhibitor contents has not been vigorously persued, but crop plants are now being engineered with a few amylase inhibitor and /or proteinase inhibitor genes, either individually or in combination with other defensive genes, for analysis of their effectiveness in enhancing defenses against insect pests. Amylase inhibitors are isolated from many cereals. It is used to inhibit enzyme(s) present in gut of insects thus, play an important role in plant defence.

α-amylase inhibitor was first reported in wheat. Alpha-amylase has been extensively studied in various cereals like wheat, maize and barley **(Weselake *et al.*, 1985; Maeda, 1986 and Mundy *et al.*, 1984)** and reported that these seeds contain a proteinaceous inhibitor

of α-amylase (MW-20,000) which inhibits the activity of α-amylase at its pI. Hard winter wheat is also found to contain amylase inhibitor of rice weevil *(Sitophilus oryzae)* amylase, which is the major insect pest of stored wheat throughout the world and considered the prime target insect. Beside the rice weevil, other lepidopteron insects are also reported to harm the production.

Mostly α-amylase occurs in two isozymic forms Amy 1 and Amy 2, which differ in their sensitivity to the various components of the inhibitor complex **(Baker, 1987)**. These inhibitors are dimeric. Genetic modification of these inhibitors may provide best means for improving overall nutritious value of protein in cereals; and increased resistance to insect attack.

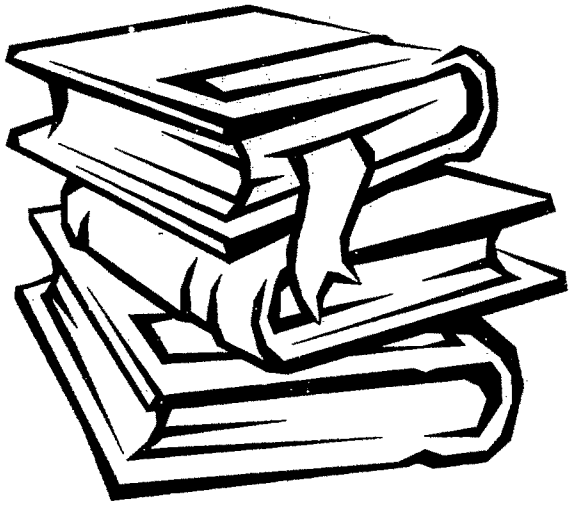
Inhibitors of α-amylases are used to modify the crop plants. cDNA encoding α-amylase inhibitors in various plants has been isolated, sequenced, cloned and biochemically characterized. **(Lullien *et al.*, 1991 and Titarenko and Chrispeels, 2000)**. Chemically synthesized gene for α-amylase inhibitor from wheat kernel has been expressed in *E. coli* **(Okuda *et al.*, 1997)**. Genes for three α-amylase inhibitors have been expressed in tobacco but the main emphasis has been on transferring the gene from the common bean (*Phaseolus vulgaris*) α-amylase inhibitor (αAI-Pv) to the other legumes **(Schuler *et al.*, 1998)**. The bifunctional inhibitors i.e. α-amylase/ trypsin or α-

amylase/ substilin, from different cereals as rye, maize are transferred to develop the resistance against the insects.

In the view of the current status of knowledge about α-amylase inhibitors from the wheat, the present investigation was undertaken with following objectives:

1. Screening of wheat varieties for α-amylase inhibitor activity.
2. Isolation and purification of amylase enzyme from target storage pest, i.e. rice weevil *(S. oryzae)* and germinating wheat seeds.
3. Quantitative estimation of inhibitor activity against different amylases.
4. Isolation and purification of the α-amylase inhibitor factor(s) from wheat.
5. Kinetic characterization of partially purified inhibitor from wheat variety UP 2425.
6. Subunit characterization of the purified α-amylase inhibitor from wheat variety UP 2425.
7. Feeding trails on target insect *S. oryzae.*

Chapter 2



REVIEW OF LITERATURE

Chapter 2 REVIEW OF LITERATURE

For the several millennia, man has selected crops for ease of cultivation and harvest, greater yield, appropriate quality and resistance to pests and diseases. Scientific breeding, involving deliberate hybridization followed by selection of the segregating progeny for particular traits of interest has been carried out for very long.

In 1960s, Green Revolution led to greatly increased yields of wheat and rice and brought the value and potential for improved crop cultivars to the public attention, heralding the possibility of sustainable food production. Unfortunately, these expectations were short lived because the large areas of high yielding but genetically identical cultivars proved to be susceptible to insect pests. In the absence of plant resistance, insecticides were required in larger amounts while new cultivars could be developed that were resistant to these pests.

Host plant resistance may be defined as the collective heritable characteristics by which a plant species, race, clone or individual may reduce the possibility of successful utilization of that plant as a host by an insect species, race, biotype or individual **(Beck, 1965)**. Reiterated in terms appropriate to crop production, host plant

resistance represents the inherent ability of crop plants to restrict, retard or overcome pest infestation **(Kumar, 1984)**.

2.2 CROP IMPROVEMENT BY TRADITIONAL BREEDING METHODS

The technology of plant breeding has developed from the science of genetics. But as crop improvement by breeding depends on recognition of particular traits needed for achieving high and stable yield, pest and disease resistance and quality, success in breeding also depends on an understanding of plant physiology, pathology and biochemistry. Conventional plant breeding is concerned mainly with the directed assortment of the allelic variants to produce a combination of genotype.

Over the period, there has been major changes in agricultural practices, not only much greater use of fertilizer and selective herbicides and improved insecticides and fungicides, but now the agricultural environment has also reached a near ideal level and varieties are produced which optimally exploit this environment, so that a new yield plateau is reached. Taking this concept, now the task is to maintain and improve resistance to pests/diseases, by genetically varying the composition of the crop plants and grain to increase production and pest control.

2.3 GENETICALLY MODIFIED CROPS

Over the last 50 years, insect pests have been controlled mainly by the application of insecticides. Much research effort has been

devoted to the development of new insecticides and methods of using them. In contrast, the control of pests by exploiting genetic resistance has received less attention. But now, due to excessive use of synthetic chemicals, environmental causes and saturation of these chemicals due to non-degradability of these chemicals, scientists have been looking for an alternative of these chemicals.

During the last few decades there has been a dramatic increase in our understanding of the chemistry and biology of the nucleic acids. It is now possible to isolate individual genes and determine their structures, to modify the structure in a desired way and for some organisms to achieve a particular change in the function of the organism, with the gene active either in its "natural" background or in a different organism. These techniques can be applied to crop plants, greatly to aid the production of genetically improved varieties

2.3.1 Development of Insect Pest Resistant Crops

Defence against pests and pathogens is a necessary property of plants allowing them to survive in nature. Physical features as epidermal hairs and dense fibrous tissues, as well as chemicals and also the patterns and timing of plant growth can all contribute to resistance against pests. Plants have defences that tend to be organized to maximize protection for a minimum metabolic cost **(Richardson, 1991)**. These defences render them virtually immune to all but a very few of the herbivorous species that encounter them. In

addition, the individual plant can be more economical in allocating resources to defence when it possesses more than one kind of resistance.

For several decades, researchers have been studying the biosynthesis and regulation in plants of specific chemicals associated with defense against pests and pathogens. For years, many of these chemicals, first considered to be "secondary plant compounds" with unknown functions are now considered to be defense chemicals that are acting either alone or in concort to contribute to the resistances of plants against insects on pathogens **(Janzen *et al.*, 1977, 1986; Mckey, 1979; Rhodes, 1979 and Rosenthal and Janzen, 1979)**. These defense chemicals are found as either constitutive components in various plant tissues **(Mckey, 1979; Rhodes, 1979)** or are synthesized in response to pests or pathogens attack **Baldwin, 1989; Carrol and Hoffman, 1980; Darvill and Albersheim, 1984; Ebel, 1986; Green and Ryan, 1972; Hildebrand *et al.*, 1986; McNaughton and Tarrants, 1983; Rhodes, 1979; Rossiter *et al.*, 1988; Valentine *et al.*, 1983** and **Wagner and Evans, 1985)**. The inducible chemicals, which can also occur constitutively, include such complex substances as antibiotics, alkaloids and terpenes as well as such as enzymes, enzyme inhibitors and lectins. Induced defense responses to pests and pathogens are activated by signals released during the early stages of infection or insect attacks. A natural

defensive role can be ascribed to many secondary metabolites, most of which are formed by biosynthetic pathways involving several enzymatic steps. Manipulation of these pathways by molecular biological techniques may be possible if the relevant gene can be isolated and transferred to a single plasmid which is then inserted into the genome of the desired host. Single genes that code for inducible defense proteins have same practical advantages over genes that code for complex pathways. By transferring single defense gene from one plant species genus to another and expressing them either with their own pathogen inducible or insect inducible promoters or with constitutive promoters, genetically modified plants can be readily obtained to test for possible roles of the gene products in defending plants against specific pathogen or pests **(Ryan, 1990)**.

Today, insect resistance transgenes, whether of plant, bacterial or other origin can be introduced into plants to increase the level of insect resistance, a technology that has dramatically extended the scope of resistance genes available to plant breeders. The first reports of transgenic insect resistant plants were only published in 1987 **(Hilder *et al.*, 1987)**, but technological development has been swift since then. Approximately 40 different genes conferring insect resistance have been incorporated into crops and the first insect resistant crops have been commercialized in several countries. This technology is seen as an additional tool for the control of crop pests

and could offer certain advantages over conventional insecticides, such as more effective targeting of insects protected within plants, greater resistance to weather conditions, fast biodegradability, reduced operator, exposure to toxins and financial savings **(Gatehouse *et al.*, 1992)**. The use of transgenic plants may lead to reduction in the use of broad spectrum insecticides, thereby extending the useful life of these compounds and reducing the ecological damage they cause.

2.3.2 Insect Resistance Genes

The insect resistance genes transferred into plants to date mainly target the insect digestive system. Most have been derived from a single species of bacterium or a range of higher plants, although some insect resistance genes from animals and other microorganisms have also been introduced. However, the search for new genes is ongoing and aims to expand the range of insects affected to combat the development of resistance in the target insects by identifying genes with different modes of action and to improve potency.

2.3.3 Resistance Genes From Microorganisms

The most prominent gene for insect resistance, from microorganism, is from *Bacillus thuringiensis* (Bt). Bt is an ubiquitous spore forming soil bacterium that produces insecticidal protein crystals, also called Bt toxin, endotoxins or crystal proteins, within its cells during the sporulation process. Spores and protein crystals of

several strains of this bacterium have been used as microbial insecticides since the 1950s and, owing to their selectivity, now have an established role in some integrated pest management system. Separate strains of Bt produce a variety of crystal toxins with distinct host ranges. At least ten genes encoding different Bt. toxins have been engineered into plants: cry1Aa, cry1Ab, cry1Ac, cry1Ba, cry1Ca, cry1H, cry2Aa, cry3A, cry6A and cry9C **(Gill *et al.*, 1992)**. Most cry proteins, even within the cry1A subfamily, have a distinctive insecticidal spectrum. The cry 1A and cry 1C genes code for the Cry 1A and Cry1C proteins, respectively, which are specific to larvae of lepidopteran pests such as the codling moth *(Cydia pomonella)*, the European corn borer *(Ostrinia nubilalis)* or heliathine boll worms. In contrast, the Cry 3A protein is toxic to coleopteran pests, including the Colorado potato beetle *(Leptinotarsa decemlineata)*. Bt toxins have been transferred and expressed in at least 26 different plant species. However, the level of resistance they confer will, in most cases depend on whether native bacterial or truncated codon optimized genes have been used **(Peferom, 1997)**.

Other microorganism-derived resistance genes include the isopentenyl transferase gene (*ipt*) from *Agrobacterium tumefaciens*, which codes for a key enzyme in the cytokinin biosynthetic pathway. Expression of *ipt* in tobacco and tomato by a wound inducible promoter has resulted in a decrease in leaf consumption by the

tobacco hornworm *(Manduca sexta)* and reduced survival of the peach potato aphid (*Myzus persicae*). However, the expression of *ipt* had negative effects on plant development, such as an underdeveloped root system and a reduced total chlorophyll content **(Smigock, 1997)**.

A cholesterol-oxidase gene from streptomycete fungus has also been engineered into tobacco. Cholesterol is necessary for the integrity and function of almost all cellular membranes and cholesterol oxidase is highly toxic to larvae of the boll weevil *(Anthonomus grandis)* and retards the growth of the tobacco budworm *(Heliothis virescens)*. Both species are major cotton pests and this protein damages the membrane of the insect midgut epithelium **(Purcell *et al.*, 1993)**.

2.3.4 Resistance Genes from Higher Plants

A defense role in plants can be ascribed to many secondary metabolites, most of which are formed by biosynthetic pathways involving several enzymatic steps. Manipulation of these pathways may be possible if the relevant gene(s) can be isolated and transferred to the desired host. There are two major groups of plant derived genes used to confer insect resistance on crops: 1) Lectins 2) Inhibitors of digestive enzymes. These genes have been transferred into crop plants without major alteration and expression has been at a similar level of codon optimized Bt toxins.

2.3.4.1 Lectins

Lectins are carbohydrate-binding proteins, some of which are toxic to insects. Invertebrate herbivores, lectins cause a number of effects including agglutination of blood cells and stimulation of mitosis. Various lectins have shown some toxic activity against species of the insect orders Homoptera, Coleoptera, Lepidoptera and Diptera. The mode of action of lectins against insect remains unclear, but it has been shown that at least some bind to midgut epithelial cells **(Gatehouse and Hilder, 1994)**.

Recent interest has mainly concentrated on the lectins from snow drop (GNA), because it has shown activity against aphids **(Down *et al.*, 1996)** and the rice brown plant hopper *(Nilaparvata lugens)* **(Powell *et al.*, 1995)**. GNA has been expressed in 9 different crops, including potato, oilseed rape and tomato. Laboratory tests with modified potatoes showed that GNA did not increase the mortality or development time of glasshouse potato aphid *(Aulocanthum solam)* but considerably reduced fecundity, an effect that led to a much slower population build up of this aphid in a glasshouse trial **(Down *et al.*, 1996). Gatehouse *et al.*, (1996)** concluded that the effect of GNA is antifeedant rather than insecticidal.

Other lectins i.e. from pea and snow drop *(Galanthus nivalis),* winged bean (*P. vulgaris*), soybean and wheat germ have demonstrated

insecticidal activity but had also shown significant toxicity to mammals **(Gatehouse and Hilder, 1994)**.

2.3.4.2 Inhibitors of Digestive Enzymes

These are small stable proteins that bind tightly to digestive enzymes and hinder digestion **(Mikola, 1983)**. Interest in the enzyme inhibitors from plants began in the 1940's when a heat labile protein from soybeans, isolated and purified which inhibited trypsin. **Kneen and Sandstedt (1943)** found an inhibitor of α-amylase in the grains of various cereals. The best-known groups of enzyme inhibitors from seeds are those which affect the activities of serine proteinases (EC 3.4.21._) such as trypsin, chymotrypsin and substilin. However, numerous examples are also known of inhibitors of sulphydryl (EC. 3.4.22._), Acidic (EC. 3.4.23._) and metallo proteinases (EC 3.4.12._). The range of inhibitor specificity exhibited by these proteins is highly variable. A number of bifunctional plant proteins have been isolated which are effective inhibitors of enzymes belonging to completely different classes. Seeds of ragi (Indian finger millet, *Eleusive caracanai)* contain a 13 kDa protein which inhibits not only mammalian trypsin but also the α-amylase from a number of different sources. On the other hand some inhibitors are capable of inhibiting only one or two closely related enzymes.

During the last decade the amino acid sequences of many of the enzyme inhibitors from plants have been sequenced. The various

amino acid sequences could be assigned to families on the basis of sequence homology, location of disulphide bridge pairing and the position of the reactive (peptide bond) site. Initially 4 families were prepared, but as more sequences became known it became clear that additional families could be distinguished. The following Table 1 shows a current listing of the 10 most likely families of the plant enzyme inhibitors.

Most of the enzyme inhibitors from seeds are simple protein with little or not detectable attached carbohydrate. A few, however, have a considerable carbohydrate content. Amylase inhibitors from cranberry beans contain 14% carbohydrate and the α-amylase inhibitors from various *Phaseolus* seeds have 8-15% carbohydrate **(Lajalo and Filho, 1985; Frels and Rupnow, 1985)**. The α-amylase inhibitors from wheat have much lower level (0.9%) of carbohydrate but this carbohydrate is thought to have a major role in the mechanisms of inhibitor **(Buonocore, *et al.*, 1981)**.

Several features of the enzyme inhibitors found in seeds indicate for their role as safe storage forms of protein which are immune to digestion until required during germination. Certainly a majority of these proteins are particularly resistant to denaturation by heat, extremes of pH and many proteolytic enzymes **(Pattabiraman, 1986; Silano, 1987)**. High content of cysteine and cystine in many plant inhibitors shows them as sulphur stores. The mRNA for the Bowman-

Table 1: Enzyme Inhibitor Families (Richardson 1991)

Family	Monomer		Enzyme Inhibited	Distribution
	Mr.	½ Cys.		
1. Bowman-Birk	8-9 (14)	14(18)	Trypsin[a] Chymotrypsin Elastase	Leguminosae Gramineae
2. Kunitz	21-22	4	Trypsin[b] Chymotrypsin Substilisin Kallikrein Amylase	Leguminosae Gramineae Araceae Alismataceae
3. Potato I	8-9	0-2	Chymotrypsin Trypsin Substilisin	Solanaceae Gramineae Leguminosae Polygonaceae Cucurbitaceae *Hirudo medicinalis*[c]
4. Potato II	6(12)	8	Trypsin Chymotrypsin	Solanaceae
5. Cucurbit	3	6	Trypsin Hageman factor	Cucurbitaceae
6. Cereal superfamily	12-13	10	Amylase[b] Trypsin Hageman factor	Gramineae (CM, 25 storage proteins in Cruciferae, Euphorbiaceae, Lecythidaceae, Leguminosae)
7. Ragi AI2/ barley, rice, PAPI	12-13	7-8	Amylase[b] Protease	Graminae
8.Thaumatin-PR like	22-23	16	Amylase[b] Trypsin	Gramineae Solanaceae
9. Carboxypeptidase	4	6	Carboxypeptidase	Solanaceae
10. Cystatin like	12	0	Cysteineproteinases	Gramineae animals

a: Double headed
b:Bifunctional (α-amylase/protease)
c:Leech (animal source)

Birk and Kunitz inhibitors, which are highly expressed at the mid maturation stage of seed development **(Vodkin, 1981)**, encode pre protein with N-terminal signal sequences. Such sequences would be expected if the inhibitors were to be transported and sequestered into membrane bound storage bodies. Many of these proteins are to be found alongside other storage protein in protein bodies, but they are also present in cell walls, intercellular spaces and in the cytosol **(Kapur *et al.*, 1989)**.

The enzyme inhibitors in seeds might control endogenous enzymes such as proteinases and α-amylases, thus preventing premature hydrolysis of reserve materials. Several inhibitors of the endogenous α-amylase enzymes are known, which are responsible for starch metabolism during germination. Inhibitors of this type has been isolated from wheat **(Mundy *et al.*, 1984; Weselake *et al.*, 1985; Maeda, 1986;** and **Abdul Hussain and Paulsen, 1989)**, barley, maize and rye. Few inhibitors are known that inhibit the endogenous trypsin like proteinases of seeds **(Ryan and Walker-Simmons, 1981**and **Mikola, 1983)**

The most important biological role of enzyme inhibitors, in seeds, is defense against pests. Many of the protein inhibitors found in seeds are effective *in vitro* against some of the digestive enzymes found in the guts of insects. This has been shown to be the case for the proteinase and α-amylase inhibitors. Along with the defence

against insect pests, these are also effective against microorganisms. The extracellular proteinases of plant pathogenic fungi and bacteria may play role in the invasion process of infection **(Sikes and Maxcy, 1979)**. Since many of the seed protein inhibitors are active *in vitro* against such microbially produced proteinases **(Boisen *et al.*, 1981; Boisen and Djurtoft, 1982; Mundy *et al.*, 1983, 1984** and **Richardson, 1991)**, it may be summarized that the inhibitors may act in the defence of the seeds against the microorganisms.

2.3.4.2.1 Proteinase Inhibitor

Plant proteinase inhibitor are polypeptide or proteins that occur naturally in wide range of plants and are a part of the plants natural defence system against herbivory. Proteinases in insects include serine-, cysteine-, aspartic- and metallo-proteinases that catalyze the release of amino acids from dietry proteins and so provide the nutrients crucial for normal growth and development. Different proteinases predominate in different insects, for example, serine like proteinases are dominant in lepidopteran larvae **(Schuler *et al.*, 1998)**, whereas coleopteran species have a wider range of dominant gut proteinases. Members of the serine and cysteine proteinase inhibitor families have been more relevant to the area of plant defence then metallo-and aspartyl proteinases inhibitors, since only a few of these latter two families of inhibitors have been found in plants.

Serine and cysteine proteinase inhibitors have been reported to inhibit the growth and development of a range of insects, mainly lepidopteran and coleopteran species, respectively **(Orr *et al.*, 1994; Broadway and Duffey, 1986)**. Gut digestive enzymes are not the only targets affected by proteinase inhibitors, but water balance, moulting and enzyme regulation of insects are also affected **(Schuler *et al.*, 1998)**.

At least 14 different plant proteinase inhibitor genes as listed below have been introduced into crop plant **(Schuler *et al.*, 1998)** (Table 2).

Most effort has concentrated on serine proteinase inhibitors from the plant families; Fabaceae and Poaceae, which are targeted mainly against lepidopteran pests. The most active inhibitor identified is the cowpea trypsin inhibitor (CpTI) which has been transferred into at least 10 other plant speices. CpTI affects a wide range of lepidopteran and coleopteran species **(Gatehouse and Hilder, 1994)**. CpTI transformed tobacco, caused significant larval mortality of cotton bollworm *(Helicoverpa zea)* larvae, but the protection provided by CpTI was less pronounced and consistent that of tobacco containing a truncated Bt. toxin gene **(Hoffmann *et al.*, 1992)**. **MacIntosh *et al.* (1990)** reported that genetically improved tobacco plants expressing a protease inhibitor fused to truncated *B. thuringenesis* insect control protein increased levels of activity. The presence of extremely low

Table 2: Genes of plant origin transferred to crop plants (Schuler *et al.*, 1998).

Proteinase Inhibitor	Target Insects	Transformed Plants
C-11 (Soybean serine proteinase inhibitor)	Coleoptera, Lepidoptera	Oilseed rape, poplar, potato, tobacco.
CMe (Barley trypsin inhibitor)	Lepidoptera	Tobacco
CMTI (Squash trp inhibitor)	Coleoptera, Lepidoptera.	Tobacco
CpTI (Cowpea trp inhibitor)	Coleoptera, Lepidoptera.	Apple, lettuce, oilseed rape, potato, rice, strawberry, sunflower, sweet potato, tobacco , tomato
14K-CI (bifunctional cereal inhibitor of Ser. proteinase and α-amylase		Tobacco
MTI-2 (Mustard Ser. proteinase inhibitor)	Lepidoptera	Arabidopsis, tobacco.
OC-1 (Rice Cys. proteinase inhibitor)	Coleoptera, Homoptera	Oilseed rape, poplar, tobacco
PI-IV (Soybean Ser. proteinase inhibitor)	Lepidoptera	Potato, tobacco
Pot PI-I (Potato proteinase inhibitor 1)	Lepidoptera, Orthoptera	Petunia, tobacco
Pot PI-II (Potato proteinase inhibitor II)	Lepidoptera, Orthoptera	Birch, lettuce, rice, tobacco
Proteinase inhibitor (unspecified)	Lepidoptera	Oilseed rape.
Soybean Kunitz trp. Inhibitor (Kti_3, SKTI)	Lepidoptera	Potato, tobacco
Tomato proteinase inhibitor I	Lepidoptera	Alfalfa, nightshade, tobacco, tomato
Tomato proteinase inhibitor II	Lepidoptera	Tomato, tobacco.

levels (4 μM) of proteinase inhibitors enhanced the insecticidal activity of *B.thuringenesis* var kurstaki by 2-20 folds.

Gatehouse *et al.* (1979) investigated the levels of trypsin inhibitors in the cultivar TVu 2027, of the cowpea *(Vigna unguiculata)* which had a high level of field resistance to the attack of the larvae of the bruchid beetle *(Callosobruchus maculatus)* and they concluded that resistance in this cultivar was due to the elevated levels of trypsin inhibitors, relative to those found in susceptible varieties. **Reese (1983)** proposed that the antinutritional effects of proteinase inhibitors were due to a reduced digestion of dietary protein produced by a direct inhibition of the host proteinases. Feeding trials in which purified inhibitors were added artificial meals have shown that seed proteinase inhibitors do significantly reduce the growth and development of various insect pests **(Gatehouse and Baulter, 1983; Broadway and Duffey, 1986)**. However in both cases supplementation of the artificial diet with the sulphur amino acids; methionine or cysteine largely prevented the adverse effects. Mode of action of the inhibitor is to cause pernicious hyper production of trypsin which in turn leads to depletion of the sulphur containing amino acids, thus being detrimental to the insects.

2.3.4.2.2 Amylase Inhibitors

Alpha amylases inhibitors are the second type of enzyme inhibitors of plant origin, which have been used to modify crop plants.

Proteins that inhibit α-amylases are found throughout the plant kingdom **(Garcia *et al.*, 1987)**. Many of the abundant proteins in cereal seeds are inhibitors of either α-amylases or proteinases or inhibitor of both **(Shivraj and Pattabhiraman, 1981; Boisen *et al.*, 1981; Mundy *et al*, 1983** and **Odani *et al.*, 1983)**. The amylase inhibitory activities of these proteins are usually detected against α-amylases from animals **(Buonocore *et al.*, 1977 and Jaffe *et al.*, 1973)** including a broad spectrum of insects **(Silano *et al.*, 1975; Yetter *et al.*, 1979; Powers and Gilbertson, 1982 and Gatehouse *et al.*, 1986)** or microorganisms **(Jaffe *et al.*, 1973 and Mundy *et al.*, 1983)** but rarely against amylases from plants **(Jaffe *et al.*, 1973 and Gatehouse *et al.*, 1986)**. A considerable variability is found in the specificities of the inhibitors towards α-amylase from different species of animals and microorganisms. Thus like proteinase inhibitors, α-amylase inhibitors are considered to be part of the protective chemicals of plant origin, against pathogens and pests **(Silano *et al.*, 1975 and Yetter *et al.*, 1979)**.

In cereals many of these inhibitor proteins are members of a large superfamily of storage proteins **(Keris *et al.*, 1985)** whose members are all thought to have arisen from the same 30-amino acid ancestor **(Keris *et al.*, 1985)**. Among α-amylase inhibitors are found homologies with member of 4 of the proteinase inhibitor families found in plants i.e. the Kunitz, Barley, Bawman-Birk and the

Ragi/Maize bifunctional inhibitor families. (Table1). These homologues are apparently not due to fusion of different proteinase and amylase inhibitor genes amino acid sequence identities are found that throughout the proteins. A few proteinase inhibitors also possess reactive sites that inhibit α-amylases and are therefore bifunctional inhibitors. These inhibitor proteins possess two types of independent reactive sites; one type specific for proteinases and the other for α-amylases **(Compose and Richardson, 1983; Odani *et al.*, 1983 and Nesterenke *et al.1987*)**, that are located on separate regions of the proteins. Thus the inhibitors can tightly bind and interactivate proteinase and α-amylase simultaneously **(Mundy *et al.*, 1983 and Mosolov and Shulgin, 1986)**. Substrate inhibitor interaction in the kinetics of alpha amylase inhibition by Ragi alpha-amylae/trypsin inhibitor (RATI) studies showed that RATI has a completely new motif of trypsin inhibitory site, which is not present in any known trypsin inhibitor structures. In RATI the alpha amylase inhibitory site resides at the N-terminal region and these two sites are independent of each other, these of the inhibitor forms a ternary (1:1:1) complex with trypsin and alpha-amylase **(Alam, 2001)**.

The primary sequence of an α-amylase inhibitors isolated from ragi **(Shivraj and Pattabhiraman, 1981** and **Sevendsen *et al.*, 1986)** is homologous with the Barley Trypsin Inhibitor family **(Compose and Richardson, 1983)**. The bifunctional inhibitors possess several

possess several disulphide bridges throughout the molecules, indicating that the bifunctional inhibitor is not a fusion protein. **Poerio *et al.* (1991)** assigned the 5 disulphide bridges in an α-amylase inhibitor from wheat kernel by fast atom bombardment mass spectrometery and Edman degradation. The ragi bifunctional inhibition contains two reactive sites for trypsin inhibitor, and one for α-amylase inhibition **(Compose and Richardson, 1983)** whereas only one trypsin reactive sites is present in the barley trypsin inhibitor. α-Amylase inhibitors have been characterized in the albumin and globulin fractions of barley and wheat endosperms **(Garcia *et al.*, 1987)** that are homologous with Barley Trypsin inhibitor family but that have no known proteinase inhibitor activity.

The idea that the enzyme inhibitors might be part of a general defence mechanisms against tissue damage, environmental stress, invasion by pathogens and/or consumption by predators has been given considerable support by several observations. The maize bifunctional trypsin/α-amylase inhibitor **(Richardson *et al.*, 1987)** has striking sequence homology with virus induced pathogenesis related (PR) proteins and other stress induced protein **(Singh *et al.*, 1987, 1989)**. An α-amylase inhibitor from *Eaix* is also an endochitinase in common with several of the known PR proteins **(Legrand *et al.*, 1987 and Josten and deWit, 1989)**, and an α-amylase inhibitor from beans is now known to be like certain lectins

(Moreno and Chrispeels, 1989) which are believed to be toxic to animal predators **(Osborn *et al.*, 1988)**. Another bifunctional α-amylase/ proteinase inhibitor family isolated from maize and sequenced **(Richardson *et al.*, 1987)** is a potent inhibitor of insect α-amylase and bovine trypsin but it exhibits no homology with ragi bifunctional inhibitor. However it exhibits homology with ragi α-amylase inhibitor **(Compose and Richardson, 1984** and **Svensson *et al.*, 1986)** and with the Kunitz Soybean Trypsin Inhibitor family (SBTI) **(Hejgaard *et al.*, 1983)**. A new alpha-amylase inhibitor from rye (Secale cereale), purified and characterized, which have no activity against mammalian or insect trypsin but active against porcine pancreatic amylase and human salivary amylase. This inhibitor has high homology with the bifunctional alpha-amylase/ trypsin inhibitors from barley and wheat **(Iulek *et al.*, 2000)**.

A large family of α-amylase inhibitors that exist in various multimeric forms **(Garcia *et al.*, 1987)**, exhibits homology with the Barley Trypsin Inhibitor family. These α-amylase inhibitors exhibit different specificities toward amylases from animals, including insects and microorganisms **(Silano *et al.*, 1975)**. This family of protein has also been reported to possess homology with another trypsin inhibitor family (Kazal) that is found in the pancreases and various other tissues and fluids of higher plants **(Odani *et al.*, 1983)**.

Gatehouse *et al.*, 1986 found that α-amylase inhibitors, purified from wheat **(Shainkin and Birk, 1970; Silano *et al.*, 1973 Kashlan and Richardson, 1981 and Maeda 1986)** were potent inhibitors of amylases found in the midguts of the larvae from two species of insects *Callosobruchus maculatus* and *Tribolium confusum*, which are storage pests of legumes. Wheat α-amylase inhibitors are also potent inhibitors of the α-amylases present in several stored grain insects including *Tenebrio, Tribolium, Sitophilus* and *Oryzaephilus* **(Silano *et al.*, 1975)**. Amylase from rice weevil *(S. oryzae)* were also inhibited by wheat amylase inhibitors **(Silano *et al.*, 1975 ;Yetter *et al.*, 1979 and Baker, 1987)**.

2.4 WHEAT PROTEIN INHIBITORS OF ALPHA AMYLASE

2.4.1 Distribution

Proteinuons α-amylase inhibitors have been found only in kernel. **Kneen and Sandstedt (1946)** first described these α-amylase inhibitor from wheat. **Kneen and Sandstedt (1943)** reported that most of the inhibitor is located in the endosperm. This observation has been confirmed by microscopic studies **(Sandstedt and Beckard, 1946)** and by the assay of inhibitor contents in different milling fractions **(Buonocore *et al.*, 1977)**. The linear relationship observed between inhibitor and starch contents of different wheat milling fractions indicates that the inhibitors are closely associated with starch and are probably endospermic in nature.

The inhibitor production starts about 8 days after fertilization and rapidly increases with maturation upto a maximum reached at the full maturity. The inhibitor content rapidly decreases after germination and no detectable amount of inhibitors was found in roots and coleoptiles from germinated seeds **(Pace *et al.*, 1977).**

2.4.2 Albumin Inhibitors

Kneen and Sandstedt (1943, 1946) established the protein nature of the α-amylase inhibitor from wheat flour. The inhibitor extracted from flour with water and precipitated by ammonium sulphate, was readily inactivated upon treatment with pepsin and ficin. It is highly non dialyzeable and highly sensitive to the action of a number of oxidizing and reducing agents. It is now well established that the inhibitor precipitation used in this work contained a mixture of protein inhibitors.

Shainkin and Birk (1970) isolated two protein inhibitors (coded as AmI_1 and AmI_2) from an aqueous extract of wheat kernel by ammonium sulphate fractionation and ion exchange column chromatography. AmI_1, and AMI_2 were electrophoretically homogenous and differed in their specificity towards α-amylases from different origin.

By subjecting a water extract from wheat flour to DEAE-Sephadex column chromatography, **Saunders and Lang (1973)** isolated two amylase inhibitors (coded as I and II) which showed

identical molecular weight and inhibitory activity but different electrophoretic mobility.

An inhibitor of 0.19 gel electrophoretic mobility was isolated from a typical albumin preparation from wheat flour by means of DEAE-chromotography and preparative electrophoresis **(Silano *et al.*, 1973; Sodini *et al.*, 1970 and Petrucci *et al.*, 1976). O'Donnel and McGeenay (1976)** also isolated an α-amylase inhibitor very likely identical to 0.19 from the water extract of whole wheat flour by means of alcohol fractionation, ion-exchange chromatography and gel-filtration.

AmI_2, InhI and 0.19 showed similar inhibitor pattern (Table 3), and almost identical amino acid composition (Table 4) and Mr close to 25,000 but they are different proteins. **Saunders and Lang (1973)** showed by means of direct comparison that AmI_2, InhI and 0.19 have different electrophoretic mobilities. Moreover the isoelectric point of AmI_2 was much higher than that of 0.19. Beside, these findings others also indicated the existence of several related forms of wheat proteins capable of inhibiting mammalian, insect and other α-amylases **(Petrucci *et al.*, 1974 and Bedetti *et al.*, 1974** and **O'Donnel and McGreeney, 1976).**

2.4.3 Gliadin Inhibitors

The α-amylase inhibitor of gliadin nature from wheat flour has been described by **Strumeyer and Fischer, (1973)** and **Bu nocore *et***

Table:3 Specificity of protein inhibitors from wheat towards α-amylases from different sources.

Source of α-amylase	Inhibitor of Kneen *et al.*,	AmI_1	AmI_2	Inh I	Inh II	0.19	0.28
Mammalian (human saliva)	+	-	+	+	Nd	+	-
Avian (chicken pancreas)	Nd	Nd	Nd	+	+	+	-
Insect (yellow mealworm)	+	+	+	Nd	Nd	+	+
Plant (wheat kernel)	-	-	-	Nd	Nd	-	-
Fungal *(A. oryzae)*	-	-	-	Nd	Nd	-	-
Bacterial (*B. subtilis*)	-	-	-	Nd	Nd	-	-

Nd = Not detected

Table : 4 **Amino acid composition (Mol. %) of several amylase inhibitors purified from wheat**

Amino acid	AmI_1	0.28	0.19	AmI_2	InhI
Lys	5.2	5.0	2.6	2.5	2.8
His	Trace	Trace	1.6	1.4	1.4
Arg.	6.5	6.4	5.6	6.6	5.4
Asp.	7.8	7.5	5.9	6.1	6.4
Thr	1.9	2.7	2.9	2.7	2.8
Ser	7.2	7.3	6.3	5.4	6.7
Glu	8.5	11.4	12.8	10.7	11.6
Pro	9.0	7.6	7.3	8.2	7.7
Gly	9.1	7.8	8.4	8.4	10.4
Ala	9.1	7.8	13.4	13.4	14.9
½ Cys	5.7	8.2	6.1	6.9	3.3
Val.	12.2	10.8	7.4	7.2	8.4
Met	2.7	2.6	2.0	2.3	1.7
Ile	2.2	1.6	2.4	2.3	2.5
Leu	8.0	8.2	8.3	8.2	8.4
Tyr.	3.6	3.3	3.3	4.2	3.6
Phe	Trace	Trace	1.6	1.8	1.6

***al.* (1977). Buanocore *et al.* (1977)** extracted from commercial wheat gliadin, a protein inhibitor active towards several amylases including pig pancreatic amylase. Molecular seive chromatography gave a Mr of 55,000 for the inhibitor. Disc elctophoresis of the purified inhibitor showed, two major bands corresponding in electrophoretic mobility to the α-glidins. Amino acid analysis of the inhibitor showed on extremely high content of glutamine (30%) and proline, but a very low lysine (1%). However, **Pace *et al.*, (1977)** were not able to detect any inhibitor activity toward human salivary, *T. malitar* and pig pancreatic α-amylases. Hence, further investigation is needed to establish whether amylase inhibitors of a gliadin nature are present in wheat flour. It should be pointed out that the possibility of extracting amylase inhibitors from commercial preparations of wheat gliadin or even of wheat gliadin is not a proof of their gliadin in nature. These preparation, in fact, may even contain 10% or more wheat albumin. Similarly it cannot be considered a proof of gliadin nature that amylase inhibitors can be extracted from wheat flour or other wheat products with solvents commonly considered typical for wheat gliadin such as 70% ethanol, dilute acidic solutions and others. These solvents, in fact, extract wheat albumins as well. Moreover, since albumin amylase inhibitors display their action at concentration as low as 0.01-0.3μg/ml, the possible presence of active albumin

impurities, hardly detectable with the usual homogeneity tests, should be taken into account when isolating gliadin amylase inhibitors.

2.3.5 Inhibition specificity of wheat amylase inhibitors

Silano *et al.* (1975) tested the inhibitory activity of the albumin fractions of wheat, with Mr 60,000, 24,000 and 12,000 towards 58 animal amylases and number of α-amylases from cereal species or from immature and germinated wheat kerneles. On the basis of the effectiveness with which the 3 albumin fractions inhibit their activities, the amylase preparations tested were divided into 3 groups ; (1) susceptible (2) partially susceptible and (3) resistant. Susceptible amylases inhibited by any of the 3 albumin fractions, were found mainly in insects that attack wheat and in marine species. Partially susceptible amylases, inhibited by only one of two of the 3 albumin fractions, were present in a few avian and mammalian species including man. Resistant amylases were largely distributed in cereal, avian and mammalian species as well as in insect species that do not usually attack wheat grain or wheat flour products. At no stage of development was wheat α-amylase inhibited. The albumin fractions with Mr 12,000 was the most effective in inhibiting insect amylases. The albumin fraction with MW 24,000 was the most effective in inhibiting amylases from marine, avian and mammalian species. **Iulek *et al.* (2000)** purified and biochemical characterized a new alpha amylase inhibitor from rye (*Secale cereale)*, with high homology with

the bifunctional alpha amylase/ trypsin inhibitors from barley and what and did not showed activity against mammalian or insect trypsin although activity against porcine pancreatic amylase and human salivary amylase was absorbed.

Although valid interspecies comparison of amylases are difficult because amylase activity and action pattern are easily affected by experimental conditions, it appears that only certain amylases have groupings which can combine with the amylase protein inhibitors from wheat kernel. This suggests that inhibitors do not act by competing with starch for the active site of the enzyme; in this case we would expect all the amylases to be affected.

2.3.6 Features essential for activity of wheat amylase inhibitor

Although the effect of a number of chemical and physical treatments on albumin inhibitors has been studied, little is known on the inhibitor structural features essential for their activity. Inactivation of the inhibitor of **Kneen *et al.*, (1946)** by a number of oxidizing and reducing agents has been reported **(Buonocore *et al.*, 1977).** Both AmI_1 and AmI_2 were inactivated **(Shainkin and Birk, 1970)** by digestion with pepsin and pronase, but were not affected by carboxypeptidase A and B or by 0.02 M HCl. AmI_1 lost its activity when subjected to digestion with trypsin and chymotrypsin whereas AmI_2 was poorly affected by these proteases. Both inhibitors were inactivated upon reduction with mercaptoethanol, and did not lose

activity after treatment in 6.4 M urea. Esterification with methanol-HCl or carboxymethylation at different pH values caused a stronger impairment of the inhibitory activity of AmI_2 towards human salivary amylase than towards *T. molitor* amylase.

Cyanogen bromide treatment, at controlled concentrations, removed all the activity of AmI_2 towards the salivary amylase, but had little effect on the activity of AmI_2 or AmI, towards *T. molitor* amylase.

As shown by circular dichroism measurements in the for UV, the 0.19 inhibitor is a protein with about 50% of ordered structure **(Petrucci *et al.*, 1976)**. Significant and largely reversible changes have been observed in the aromatic CD spectrum of 0.19 at alkaline pH values or in the presence of sodium dodecyl sulphate. These changes, which were associated with only a partial loss of inhibitory activity, indicate that ionizable tyrosine groups contribute significantly to the ellipticity bands of 0.19 in the near UV. Incubation for 1 hr. at room temperature of a 12 μM. solution of 0.19 at alkaline pH values (upto 11.7) or at acidic pH values (down to 3.0) did not affect the inhibitory activity of 0.19 towards both human salivary and *T. molitor* α-amylases. The 0.19 inhibitory activity was resistant to trypsin and thermal treatments, but it was completely destroyed by incubation with pepsin or by reduction of disulphide bonds. Incubation of the 0.19 inhibitor in 1% SDS and then removing the dissociating agent caused only a minor loss of activity.

Shainkin and Birk (1970) suggested that the polypeptide chain of AmI_2 consisted of AmI_1 plus an additional peptide segment, **Silano *et al.* (1973)**, proposed that AmI_2 like 0.19 contained two subunits, one of which was AmI_2 or another member of 0.28 family.

3.3.4 Role of wheat amylase inhibitor in the wheat kernel

The heterogeneity pattern of albumin α-amylase inhibitors from wheat kernel is typical of storage protein whose biological role essentially is that of being broken down to peptides and amino acids upon germination of the seed to provide a source of nitrogen for the proteins being synthesized by the developing embryo **(Varner 1965 and Kasarda *et al.* 1971)**.

Albumin inhibitors are located in the endosperm and appear to be closely associated with starch **(Kneen and Sandstedt 1946, 1943; Sandstedt and Beckred 1946; Saunders, 1975** and **Pace *et al.* 1977)**. According to **Barlow *et al.* (1973)** and **Simmonds *et al.* (1973)** albumins strongly adhere to starch granules in the endosperm and make a significant contribution to endosperm hardness playing the role of cementing substance between starch granules and storage proteins (mainly gliadins). Although wheat albumins surrounding starch granules in the seed have not been tested for α-amylase inhibitory activity, they exhibited electrophoretic mobilities and differential staining with aniline blue black **(Minetti *et al.* 1973)**, identical to albumin amylase inhibitors. Moreover some purified

inhibitors showed a tendency to associate with polysaccharides **(Shainkin and Birk, 1970** and **O'Donnel and McGeeney, 1976).**

Although amylase inhibitors are inactive towards wheat α-amylase *in vitro,* these albumin amylase inhibitors affect, during maturation of the kernel, starch digestion by preventing the access of amylase to starch granules. The area surrounding starch granules, that is filled of albumins and other water-soluble substances, is capable of rapid swelling on hydration **(Barlow *et al.*, 1973).** So during germination wheat albumins serve as hydrocals in promoting water absorption. Thus upon germination, hydrated albumins get detached from starch granules which become available to α-amylase. In the meantime, hydrated albumins are broken down to proteases, behaving as an easily digestible protein pool useful for the earliest development of the embryo to plant. **Kanzaki *et al.* (1993)** studied the localization of α-amylase inhibitor in germinating seed and found histochemically that the inhibitor was present between the scutellar parenchyma cells of the embryo at 0 and 24 hrs and disappeared later in germinating seeds, while on the other hand in the dry seeds the inhibitor was present in the aleurone cells and starchy endosperm, while during imbibition the inhibitor in the starchy endosperm disappeared from the area to which α-amylase was secreted. It was concluded by **Kanzaki *et al.* (1993)** that the aleurone cells were the only place where α-amylase could come into contact with inhibitor.

However, due to early secretion of α-amylase from the aleurone cells after imbibition and rapid disappearance of the inhibitor, it was suggested that the inhibitor does not work as an effective inhibitor of endogenous α-amylase synthesized following germination rather they act as stored proteins.

2.4 GENES ENCODING α-AMYLASE INHIBITORS IN WHEAT

The location of genes controlling the synthesis of 7 wheat albumin fractions including 0.19 and, presumably, other components of the 0.19 family has been investigated by **Cubadda (1973)**. Albumin fractions were extracted from the kernel of a number of nulli-tetrasomic lines of Chinese spring with 0.13 M magnesium sulphate, precipitated with ammonium sulphate, between 0.8 and 1.2 M and analyzed by polyacrylamide gel electrophoresis. Only one albumin fraction is coded by chromosome 3A, two are coded by chromosome 3B and the remaining 4 fractions including 0.19 by chromosome 3D.

Genes coding for 5 albumin components, extracted with 70% ethanol from flour and separated by combined electrofocusing and electrophoresis studied were studied by **Aragancillo *et al.* (1975)**. Coding gene for one of these components (named component 5 and probably corresponding to 0.19) was located in the chromosome arm 3DB and coding genes for the other 4 components were in 3BS. In conclusion, it seems likely that albumins are mainly controlled by homologous chromosome groups 3, whereas genes coding for gliadins

are located in homologous chromosome group 1 and 6. **Masojc *et al.* (1993)** studied the polymorphism and chromosomal location of endogenous alpha amylase inhibitor genes in common wheat, using isoelectric focusing followed by monoclonal antibody based immunoblotting and three alpha-amylase inhibitor loci (Isa-1) were identified in common wheat and located on the long arms of chromosomes 2A, 2B and 2D. A high resolution isoelectric focusing separation demonstrated two active and one null allele at the Isa-A1, two alleles at the Isa-B1, one allele at the Isa-D1, four alleles at the isa-S1, and one allele at the Isa-G1 locus. The most frequent electrophoretic pattern of common wheat cultivars consisted of two isoforms, encoded respectively by the Isa-B1b, Isa-D1a alleles and the Isa-A1 null allele. All the durum wheat had only one inhibitor form controlled by allele Isa-B1b, which was accompanied by the null allele at the Isa-A1 locus **(Lullien *et al.*, 1991)**.

2.5 THE INSECT PESTS OF STORED GRAIN

Stored grains are subject to attack by a group of insects that have adopted themselves to a diet of dried vegetable material. Stored grain pests live in granaries, silos and warehouses where they are favoured by shelter, temperatures somewhat higher than outdoors, and by great concentrations of food material. Whether or not it is the result of leading such an easy life and the lack of need for flight, a number of the pests of stored grains have completely lost the power to

fly, although small number of insects are able to fly in. The granary weevil, *Sitophilus granarius* (L.), in fact has lost all but a vestige of its wings, although the closely related rice weevil, *Sitophilus oryzae* (L.), is still a powerful flier.

Owing to the small size of many of the insects that attack stored grain and the ease with which they can conceal themselves in grain, many of them have been carried by commerce to all parts of the world and have became truly cosmopolitan in distribution. Conditions in all parts of the world, however, are not equally favourable for the development of all of these insects, so that in some regions, where some species flourish, others are barely able to exist. **(Cotton, 1960)** (Table 5).

Among these insects the *S. oryzae* L. is most dominating insect pest and undoubtedly the most destructive to stored grain. It is a small reddish brown beetle, about 1/8 of an inch long, with head prolonged into a long slender snout at the end of which is a pair of stout mandibles or jaws. It is further characterized by being marked on the back with four light reddish or yellowish spots. It is world wide in distribution being found wherever grain is stored. It prefers a temperate or subtropical climate **(Cotton, 1960)**.

2.5.1 Stored Grain Pest: *Sitophilus oryzae*

Sitophilous oryzae (L.), commonly known as rice weevil is a main stored grain pest of *Curculionidae* family which attacks the ripe seeds,

Table 5: Insect Pests of Stored Grain (Khare, 1972).

Sl. No.	Common Name	Scientific Name	Family
1.	Rice weevil	*Sitophilus oryzae* Linnaeus	Curculionidne
2.	Lesser grain borer	*Rhizopertha dominica* Fabricius	Bostrychidae
3.	Kharpa beetle	*Trogoderma ganarium* Everts	Dermestidae
4.	Rust flour beetle, (Red)	*Tribolium castaneum* Hebrst	Tenebrionidae/ cdeoptera
5.	Saw toothed grain beetle	*Oryzaephilus surinamensis* Linnaeus	Salvinidae/ cucujiidae
6.	Angoumois grain moth	*Sitotroga cereeablla* alivier	Gelechiidae
7.	Indian meal moth	*Plodia interpunctella* Hubener	Phycitidae
8.	Almond moth	*Cadra cautella* Walker	Phycitidae
9.	Corn sap beetle	*Carpophilus pilosellus* Motsch	Nitidulidae
10.	Pulse beetle	*Callosobruchus chinensis* Linnaeus	Bruchidae
11.	--	*Evorinea* sp. near indicus Arrow	Dermestidae
12.	Black fungus beetle	*Alphitobius laeuigatus* (Fabricius)	Tenebrionidae
13.	--	*Mesomorphus villiger* Blanchard	Tenebrionidae
14.	Saw toothed grain beetle (variety)	*Oryzaephilus mercator* (Fauvel)	Salvinidae/cucujiidae
15.	Grain beetle	*Cryptolesters* sp.	Cucujidae
16.	--	*Uropoda* sp.	Uropodidae
17.	--	*Xylocoris flavipes* (Reuter)	Anthocoridae
18.	Psocides	*Liposcelis* sp.	Liposcelidae
19.	Rice moth	*Corcyra cephalonica* stainton	Pyralidae
20.	--	*Choetospila elegans* Westwood	Pteromalidae

storage. It is a very serious major pest of rice, wheat and other cereals in the warmer parts of the world. The developing larva lives and feeds inside the grain, hollowing it out, in the process. The entire grain is usually destroyed by the time the adult emerges.

2.5.1a Life History of *Sitophilus oryzae* (L)

Eggs of *S. oryzae* are white and oval. The female lays the eggs inside the grain by chewing a minute hole in which each egg is deposited, followed by the sealing of the hole with a secretion. These eggs hatch into tiny grubs which stay and feed inside the grain and are responsible for most of the damage. Mature larvae are plimp, legless and white about 4 mm long. Pupation takes place inside the grain. The adult beetle emerges by bitting a circular hole through outer layer of the grain. They are small brown weevils, virtually indistinguishable from each others, about 3.5-4.0 mm long with rostrum and thorax large and conspicuous. The elytra are uniformly dark brown. Each female is capable of laying 300-400 eggs and the adults live for upto 5 months and are capable fliers. The life cycle is about 5 weeks at 30°C and 70% RH, optimum conditions for development are 27-31°C and more than 60% RH; below 17°C development ceases **(Hill, 1993)**

2.5.1b. Distribution of Sitophilus Genus

Two species, *S. oryzae* and *S. zeamais* are virtually cosmopolitan throughout the warmer parts of the world, extending as

far north as Japan and Southern Europe. They have been reported from Asia, Europe, Africa, Australia, USA, Central and South America. In Europe they are replaced by the temperate Palaearatic species *S. granaries*, which is distinguished by the punctate sculpturing on the prathorax and elytra, and by the fact that it is wingless and hence can not fly.

2.5.2 Physiological Adaptations to Food Components In Insect Pests

Sitophilous oryzae has a higher capacity for population increase than *S. zeamais* or *S. granaries* when reared in wheat **(Birch, 1953; Evans, 1977a, b)** and *S. oryzae* is more efficient than *S. granaries* in utilizing food **(Baker, 1974)**. A number of behavioral and physiological responses probably account for these differences among such closely related species. One difference in enzymology related to food conversion in these weevils is the alpha amylase level. Larvae of *S. oryzae* contain the highest midgut amylase activities and amylase/ proteinase ratio of ten tested species of stored product insects **(Baker, 1986)**, and the alpha amylase levels in adult *S. oryzae* were about three-fold and eight-fold greater than those of *S. granaries* and *S. zeamais* respectively **(Baker and Woo, 1985)**. Since starch makes up about 55% of the wheat kernel and is the major nutrient in endosperm **(D'Appalonia *et al.*, 1971)**, the high levels of amylase in *S. oryzae* may be partly responsible for its competitive superiority.

Wheat also contains alpha-amylase inhibitors **(Kneen and Sandstedt, 1943)** which inhibit amylases from *Tenebrio molitor* **(Applebaum, 1964; Silano *et al.*, 1973)**, *Tribolium castaneum* **(Applebaum and Kanijn, 1965)** and *Sitophilus* spp. **(Yetter *et al.*, 1979; Baker, 1983)**. High levels of amylase in *S. oryzae* may act as a detoxifying mechanism by actually flooding ingested inhibitor with enzyme to allow normal digestive mechanisms to proceed.

2.5.2a Physiological Changes Induced by fluctuating moisture levels in *S. oryzae*

Arbian (1979) studied the effect on water flux of feeding on wheat with different moisture contents in adult *S. oryze.* At 22.5% RH, loss by transpiration exceeds total gain by metabolism, diet intake and sorption and feeding rate is significantly reduced. At 65 and 85% RH, water gained from ingested food and metabolism exceeds that lost via transpiration. Sorption at these humidities provides additional water. At 99% RH, food consumption is also markedly reduced, but water intake by passive sorption more than compensates for loss of ingested water. Metabolic rate is independent of relative humidity, and metabolic water production is insignificant to total water exchange at any humidity. **Arbian (1979)** concluded that *S. oryzae* makes a behavioral adjustment to significantly reduce food consumption at low and high humidities, and suggested that water balance and potential for water gain may be a motivation factor for feeding.

2.5.2b Population changes Induced by fluctuating moisture levels in *S. oryzae*

Successful compensatory responses to changes in food moisture content and relative humidity are reflected in maintenance or increase in population size. Population parameters including developmental time, immature mortality, net reproductive rate, adult survival and rm were determined for *S. oryzae* reared at 15, 18 and 21°C on wheat with 14 or 11.2% moisture content **(Evans, 1982)**. In almost every case, parameters measured in wheat with 14% moisture content are more favorable for population increase than those obtained at 11.2% moisture, regardless of temperature.

2.5.3 Effects of Insect Feeding on Food Grain Quality

Infestation by stored product insects results in loss of both, food quality and quantity throughout the food distribution system. Stored grain with heavy insect population has decreased protein content **(Girish *et al.*, 1975)**, decreased levels of essential amino acids **(Ranjan *et al.*, 1975)** and lower palatability **(Khare *et al.*, 1974)**. Hot spots induced by insect heating of stored grain **(Howe, 1962)** are after sites of dramatic buildup of insects, mites and microflora **(Sinha and Wallace, 1966)**. These population increases result in decreased grain quality through an increase in fat acidity values and decreased seed viability and germination **(Sinha, 1983)**. Decrease in seed germination is caused by direct feeding injuries on the germ tissue and by the substantial heat produced by large insect population **(Howe, 1973)**.

Quality loss of finished foods is usually due to displeasing presence of insects, insect fragments, excreta and secretions.

2.6 AMYLASE/ PROTEINASE INHIBITORS FOR INSECT CONTROL

Pittendrigh *et al.* (1997) studied the effects of amylase and proteinase inhibitors, lectins and Bt delta-endotoxin, on the development of the *Sitophilus oryzae* using an artificial seed bioassay and concluded that weevils reared in artificial seeds compared to those reared in whole maize developed faster, had similar within seed developmental mortalities, were lighter in weight upon emergence and laid the same number of eggs. The artificial diet containing E-64, a cysteine proteinase inhibitor, decreased the number of emerged adults per seed and delayed within seed development time, suggesting that *S. oryzae* utilizes a cysteine proteinase to digest its dietary proteins. While on the other hand, weevil fed inhibitors of trypsin and chymotrypsin, Bowman-Birk and Kunitz inhibitors, Bt endotoxin and lectin, developed almost normally.

Bean and wheat α-amylase inhibitors have effect on the α-amylase activity and growth of the stored product insects. The bean inhibitor was effective *in vitro* against α-amylase from *Tribolium castaneum, T. confusum, Tenebrio molitor* and *Sitophilus oryzae.* Adults of *S. oryzae* fed a diet containing 1% wheat inhibitor exhibited more mortality than those fed the control diet. When the wheat amylase inhibitor was combined with cystein proteinase inhibitor and fed to

larvae of *T. castaneum,* a reduction in the growth rate and increase in the time required per adult eclosion was observed **(Pueyo *et al.*, 1995)**.

2.6.1 Wheat Amylase Inhibitor Activity Against *S. oryzae*

Dietary modulation of α-amylase activity in *S. oryzae* and *S. zeamais*, indicated that levels of these predominant enzymes were highest in adults feeding on hulled maize and lowest in those fed on wheat **(Baker, 1988)**.

Although extracts prepared from barley had an inhibitory effect on 2 isoamylases from *S. oryzae*, levels of the 2.2 fold and 6.1 fold, respectively, more concentrated in wheat. Ingestion of these amylase inhibitors may account for the lower activity of amylase in adults of both species, *S. oryzae* and *S. zeamais,* feeding on wheat. Amylase levels across all strains feeding on a given diet were about twice as high in *S. oryzae* as in *S. zeamais.* Significant differences in activity levels were also found between strains in both species **(Baker, 1988)**. Since alpha-amylase is a predominant digestive hydrolase in these species, the degree to which cereal diet affect amylase levels may indicate their suitability as potential hosts.

A positive correlation of α-amylase inhibitor content in wheats with the development parameters of *S. oryzae* was observed, in the form of average number of days to adult emergence **(Baker *et al.*, 1991)**. Based on a population model for *S. oryzae* developing on wheat

a 25°C and 75% RH, the slight delay in mean development time (0.7 days) on cultivars with relatively higher Amylase Inhibitor Units (ATU)/ gms results in a 20.9% reduction in total number of weevils after 180 days.

Amylase isoenzymes from adults of *S. oryzae* were purified by HPLC, and designated RW-1 and RW-2, having similar pH optima (pH 4.5-5.0), molecular weight of about 56 kDa with similar amino acid profiles containing high concentration of aspartic acid and low levels of cystine and methionine and with identical action patterns for hydrolysis of amylase, but the kinetic constants. (Km and Vmax) of RW-1 and RW-2 for hydrolysis of soluble starch were significantly different **(Baker, 1987)**. α-Amylase inhibitor content of millet fractions of hard red winter wheat was determined by assay against these isoenzymes, RW-1 and RW-2, from *S. oryzae* **(Baker and Lum, 1989)**. Electrophoretic analysis indicated no major qualitative difference in inhibitor composition among flour and bran fraction, but flour extract was more inhibitory against RW-1 and RW-2, than bran extracts. Isoenzyme RW-2 was about 3-fold more sensitive than RW-1 to extracts from each fraction. Bran is generally not ingested by larvae or adults of *Sitophilus* spp. thus ingestion of inhibitor from wheat occurs primarily through consumption of endosperm components **(Baker and Lum, 1989)**.

occurs primarily through consumption of endosperm components **(Baker and Lum, 1989).**

Baker (1988, 1989) isolated and purified α-amylase inhibitor protein from the albumin fraction of wheat flour and designated inhibitor 0.31 and 0.12 respectively because of their mobility on gel. Inhibitor 0.31 combined with an isoamylase (RW-1) from *S. oryzae* at pH 4 -5, the protein complex thus formed was stable during electrophoresis and intermediate in mobility between that of free enzyme and free inhibitor. Inhibitor 0.31 complexed with amylase RW-1 an apparent 2:1 molar ratio and this inhibitor- enzyme compared was not dissociated even in the presence of substrate, starch. The binding mechanism(s) of this major amylase inhibitor from wheat to the weevil amylase is unknown. While on the other hand inhibitor 0.12 was not as effective as inhibitor 0.31 in complexing and inactivating amylases from this granivorous insect.

The plant inhibitors of insect digestive enzymes act as growth inhibitor of insects and possibly as plant defence proteins and open the way to the use of the genes of these inhibitors for genetically improving the resistance of cereals of storage pests **(Pueyo *et al.*, 1995).**

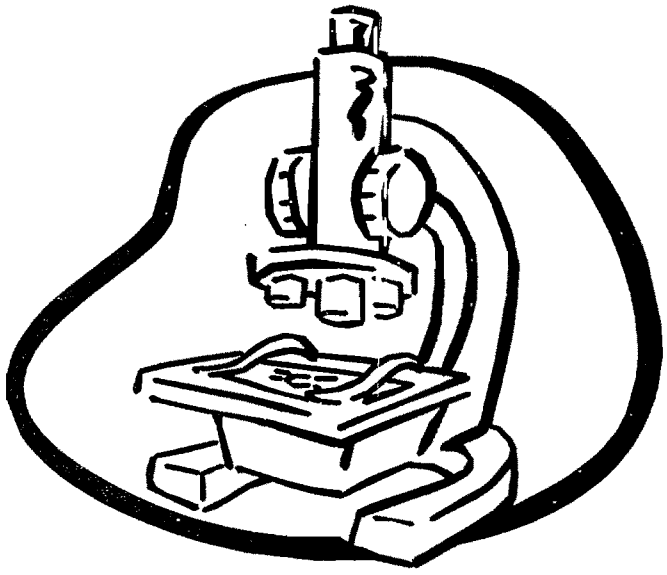
3.7 INSECT RESISTANT TRANSGENIC CONTAINING CEREAL α-AMYLASE / PROTEINASE INHIBITOR

Plant proteinase inhibitors of hydrolases from heterologous systems have been implied in plant defence and have been extensively

toxins and inhibitors that are active against heterologous systems. More than 20 different members from a single multigene family of α-amylase/ trypsin inhibitors, specifically expressed in endosperm, have been characterized. Their apparent molecular weight are in the 12,000-16,000 range. **Lullien *et al.* (1991)** isolated a cDNA clone, which codes for a subunit of the tertromeric heterologous alpha-amylase inhibitor. This cDNA contained a open reading from of 429 nucleotides encoding a putative preparation of 143 residues with a molecular weight of 13,275 or 13,432 Da depending on the position of signal peptide cleavage site. The α-amylase inhibitors can be classified, according to their degree of aggregation into monomeric. (as the trypsin inhibitors) dimeric and tetrameric forms During kernal development, their synthesis precedes that of the storage protein and they are rapidly degraded upon germination. Genes encoding these inhibitors are scattered on the Triticase chromosomes: at least 5 out of the 7 homologous groups carry genes encoding different family members. Structure-functional relationships have been pursued through site-directed-mutagenesis of the wheat monomeric α-amylase inhibitor. In this molecule both the carboxy and amino terminal ends that are proximal in the 3D-structure due to the position of disulphide bridges, have been shown to be important for activity against the *Tenebrio molitor* amylase. NMR structural studies in the finger- millet bifunctional inhibitors have shown that the reactive site for trypsin is located in a loop opposite to the reactive site for α-amylase (in the C- and N-terminal ends of the molecule), strongly suggesting that the

activity of these inhibitors can be improved by domain swapping among different members of the family. More recently transgenic tobacco and wheat plants have been obtained, expressing the gene encoding barley trypsin inhibitor BTI-Cme, and these where shown to be more resistant to lepidopterous *Agrotis ipsilon* and *Sittroga cerealella* pests, respectively, than non transformed controls. **Gatehouse *et al.* (1996)** transformed the potato plants (*Solanum* tubesum) cv. Desiree with genes encoding the protein bean chitinase (BCH), show drop lectin (GNA) and wheat alpha-amylase inhibitor (WHI), which as enhanced resistance to the peach potato aphid, M*yzus persicae*. **Titarenko and Chrispeels (2000)** cloned and characterized two α-amylase isozymes from western corn root warm larvae which were substantially inhibited by amylase inhibitors from wheat and common beans. Preliminary data with other transgenically expressed members of the family confirmed the potential of these inhibitors for increasing insect resistance in genetically modified (GM) plants. This methodology is aimed at complementing an integrated pest management system, by amplifying the natural variability outside the species barrier, in a more environmental friendly agriculture less dependent on chemical pesticides.

Chapter 3



MATERIALS AND METHODS

Chapter 3 MATERIALS AND METHODS

3.1 EXPERIMENTAL MATERIALS

The present study on the isolation, purification and characterization of the factors from wheat that bind specifically to and inhibit insect amylase was conducted in the Department of Biochemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar.

The study was performed with 15 varieties of wheat which were obtained from Department of Genetics and Plant Breeding, College of Basic Sciences and Humanities, G.B.P.U.A.&T., Pantnagar.

The chemicals, glassware and instruments with browse are listed in Appendices 1, 2 and 3, respectively. Triple glass distilled water was used for preparing reagents and enzyme assays.

3.2 EXPERIMENTAL METHODS

3.2.1 Isolation and Screening of α-Amylase inhibitor

3.2.1a Isolation of α-Amylase inhibitor(s)

Aqueous Extraction : Wheat seeds were ground to powder using Grinding Mill flouted at department of PHPCFE, College of Technology, G.B.P.U.A.&T., Pantnagar. This seed powder was suspended in distilled water (1:3 w/v), stirred for 1 hr and filtered. The residue was washed with water (1:1 w/v) and again stirred for 30 min and filtered. Both filtrates were pooled and one part was heated at 70°C for 30 min

on water bath (to destroy endogenous amylase enzyme) and the other part was used as such.

3.2.1 Quantitative screening of wheat varieties for α-Amylase inhibitor

Activity on starch-agar plates:

Soluble starch (1%) and agar (0.6%) were dissolved in water and boiled up to clear solution, and this hot solution was poured into the petriplates to have a thickness of 5 mm and was allowed to cool and solidify. Wells of 1 cm diameter (4 wells in each plates) were put at equal distance in solidified gel with a precaution to have a thin layer or gel at the bottom of the wells.

Amylase inhibitory activity on starch-agar gel:

To screen the amylase inhibitory activity, different amounts (30, 40 and 50 µl) of water extract (heat treated and untreated) were taken in the 3 wells. In fourth well only water was added, which served as control. The starch-agar plates were then incubated at 30°C for 2 hrs for the diffusion of water extract(s) in the starch-agar. Then 50 µl of porcine pancreatic amylase enzyme (20 times diluted) was added in each well, including the well, in which no water extract was added.

After the addition of amylase enzyme these plates were again incubated at 30°C for 12 hrs. After incubation, acidic KI solution was poured in the plates and left for 30 min at room temperature for development of reduced starch zone.

3.2.2 Quantitation of α-Amylase Inhibitor Activity

α-Amylase inhibitor activity was checked with amylase from germinating wheat seeds, *S. oryzae* and porcine pancreas.

3.2.2a Amylase enzyme isolation from different sources

(i) **Germinating Wheat Amylase:** The wheat seeds were surface sterilized with $HgCl_2$ solution and were put on wet paper towels for 7 days at 30°C. These 7 days germinated wheat seedlings were finely ground in pestle-mortar with 20mM Tris HCl+1mM $CaCl_2$ (pH 7.5) solution in cold conditions. Suspension was filtered with Whatman no.1 filter paper and the filtrate was centrifuged at 5000 rpm for 15 min at 4°C and the supernatant was stored at 4°C to be used as α-Amylase.

(ii) **Amylase from *Sitophilus oryzae*:** Pure culture of *S. oryzae*, a storage pest of wheat, was taken from the Department of Entomology, College of Agriculture, Pantnagar and the insects were reared on wheat seeds in the lab at 30°C, 75% relative humidity (R.H.) and 13.5% moisture containing wheat seeds.

Approximately 1 gm insects were separated from seeds with the help of sieve and then kept at –20°C for 30 min. These frozen insects were homogenized with ice cold autoclaved distilled water (1:20 w/v) in a pestle – mortar in cold conditions. The suspension was centrifuged at 10,000 rpm for 25 min at 4°C. The clear supernatant was used for the test of amylase activity and amylase inhibitor assay.

The crude insect α-amylase preparation was subjected to 95% ammonium sulphate precipitation in cold conditions for 12 hrs and then centrifuged at 10,000 rpm for 20 min. The precipitate was dissolved in 0.03 M acetate buffer containing 0.44 M NaCl and 0.001 M $CaCl_2$, pH 5.4 and dialyzed against the same buffer. The dialyzed enzyme preparation was further purified by passing through sephadex G-25 column, using the same 0.03 M acetate buffer as elution buffer. The eluted enzyme was lyophilized, stored at 4°C and was used as partially purified amylase enzyme preparation as well as for amylase inhibitory activity assay.

(iii) **Porcine pancreatic amylase:** The porcine pancreatic amylase was obtained commercially and it was diluted 20 times before use.

3.2.2b Amylase Assay:

The amylase inhibitor activity of wheat seed extracts was estimated by measuring the degree of inhibition of α-amylase activity in different amylase preparations i.e. germinating wheat amylase, insect amylase and porcine pancreatic amylase. The α-amylase enzyme activity was assayed in the absence and presence of the extracted inhibitors **(Bernfeld, 1955)**.

To assay α-amylase 1 mL of 1% starch solution was incubated for 30 min at 30°C with 1 ml of the enzyme preparation(s) and this reaction was stopped by the addition of 1 ml of dinitrosalicylic acid

(DNS) reagent. The tubes containing the mixture were heated for 5 min in boiling water bath and then cooled in running tap water. After cooling of the tubes, 1 ml of 40% potassium sodium tartarate solution was added in each tube and then final volume was made up to 10 ml with distilled water. The optical density of the solution was observed at 510 nm. A calibration curve was prepared with maltose, to convert the colorimetric reading into μmoles of maltose. Beside this standard curve for protein estimation were also prepared **(Lowry *et al.*, 1951)** and **Bradford (1974)** method to estimate the protein in the samples.

Enzyme activity was expressed as International units (IU) and one IU was defined as the formation of 1 μ moles of maltose formed per unit time of reaction (per min).

A Standard curve of Maltose :

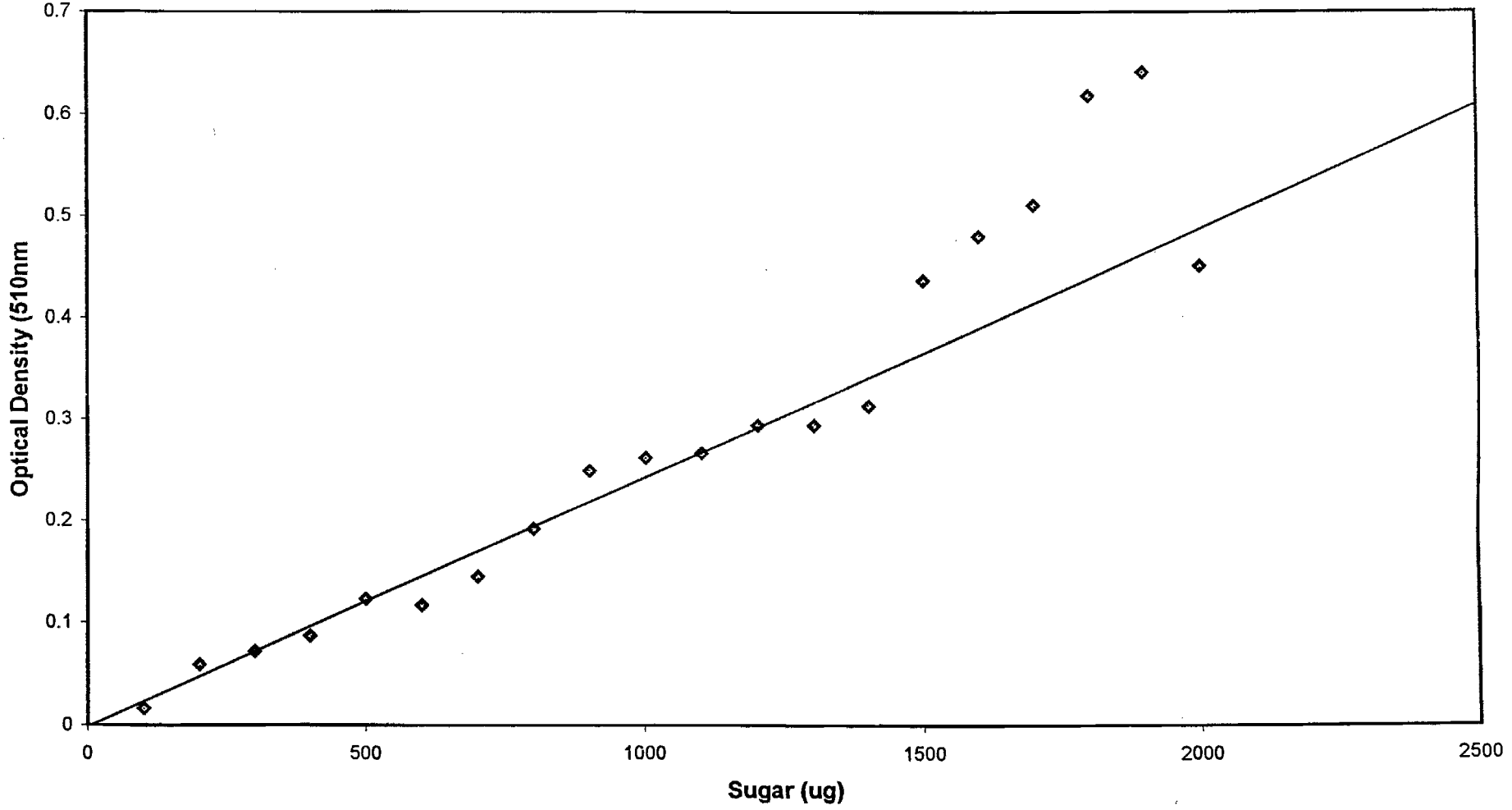
Principle: Maltose reduces 3, 5-dinitrosalicylic to 3 amino-5-Nitrosalicyltic acid and resulting in change of yellow colour to red. Intensity of red colour is read at 510 nm.

Chemicals

(1) DNS reagent: 1 gm of dinitrosalycylic acid, 200 mg crystalline phenol and 50 mg sodium sulphate were dissolved in 100 ml of 1% NaOH. Since reagent deteriorates due to sodium sulphite, if long storage is required, it may be added at the time of use.

(2) 40% Potassium Sodium Tartarate (Rochelle salt) solution.

FIG.3.1 STANDARD CURVE OF MALTOSE (DNS METHOD)



Procedure

(i) Pipette out 0.2 to 2 ml of standard solution (1 mg/ml maltose solution) in test tubes and equalize the volume to 2 ml with water in all the tubes and one blank tube without sugar was also taken.

(ii) Add 1 ml of DNS reagent

(iii) Heat the contents in a boiling water bath for 5 min.

(iv) Add 1 ml of Rochelle salt solution in warm solution.

(v) Cool in running tap water and read the intensity of dark red colour at 510 nm.

The standard curve for maltose is shown in Fig. 3.1

B. Standard Curve for Proteins

(1) Method of Lowry *et al.* (1951)

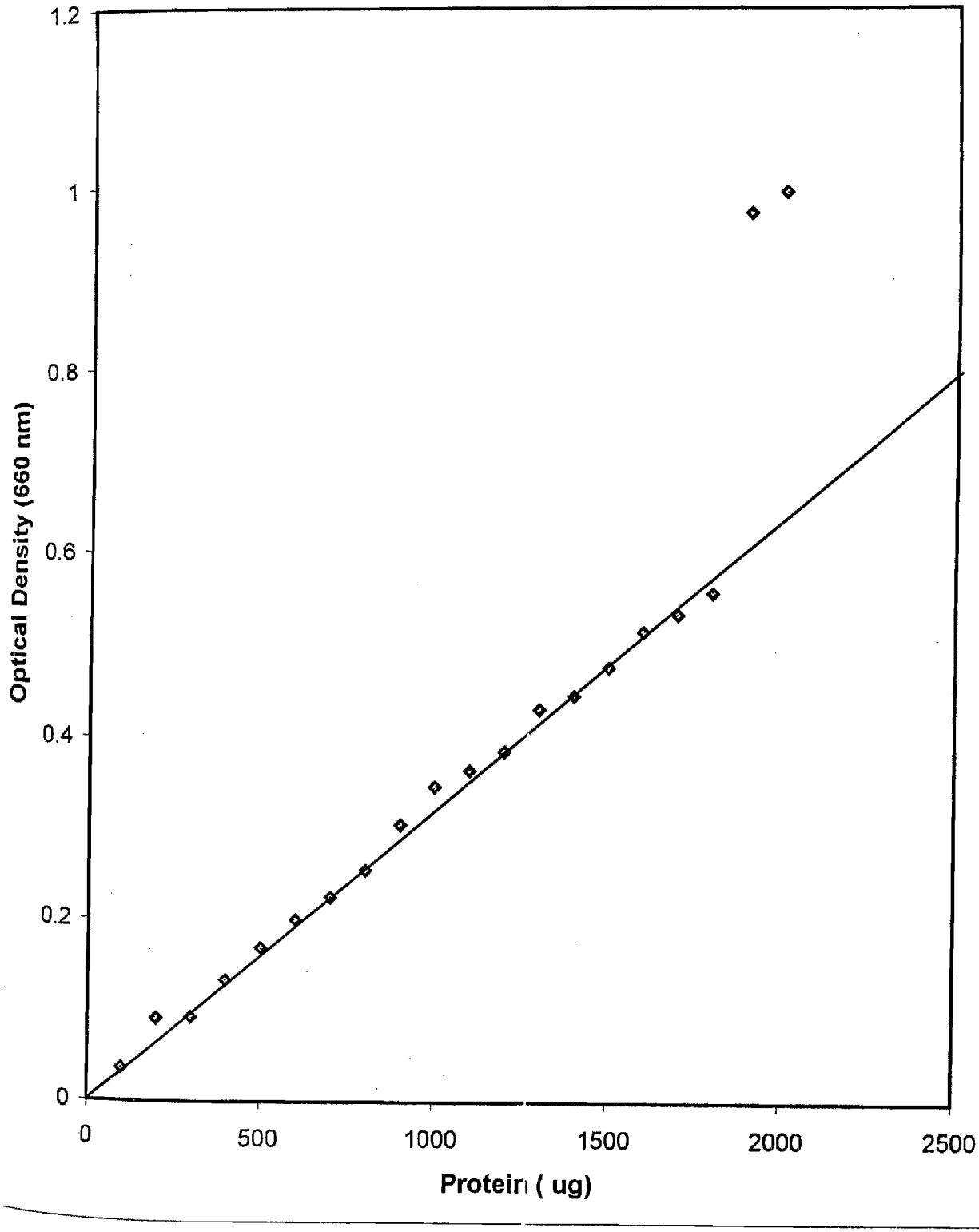
Principle

The blue colour developed by the reaction of the phosphomolybdic phosphotungstic components in the Folin-Cicalteau reagent by amino acids : Tyrosine and Tryptophan present in the protein plus the colour developed by biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry's method.

Chemicals

(i) Reagent A : 2% Sodium carbonate in 0.1 N NaOH.

FIG.3.2 STANDARD CURVE OF PROTEIN (LOWREY METHOD)



(ii) Reagent B : 0.5% $CuSO_4.5H_2O$ in 1% Potassium sodium tartarate.

(iii) Reagent C (Alkaline Cu solution) : Mix 50 ml of A and 1 ml of B prior to use.

(iv) Folin reagent : Diluted 2 times.

(v) Stock protein solution : 1 mg/ml BSA solution.

(vi) Working protein solution : Stock solution diluted 5 times.

Procedure

(i) Take working solution from 0.1 ml to 1 ml in test tubes and equalize the each tubes content to 1 ml with water.

(ii) One blank tube, without protein solution was also taken, which contains only water.

(iii) Add 5 ml of reagent C in each tube, mix well and allow to stand for 10 min. at room temperature.

(iv) Now add 0.5 ml of Folin reagent, mix well and incubate at room temperature in dark for 30 min.

(v) Blue colour is developed and the O.D. was taken at 660 nm.

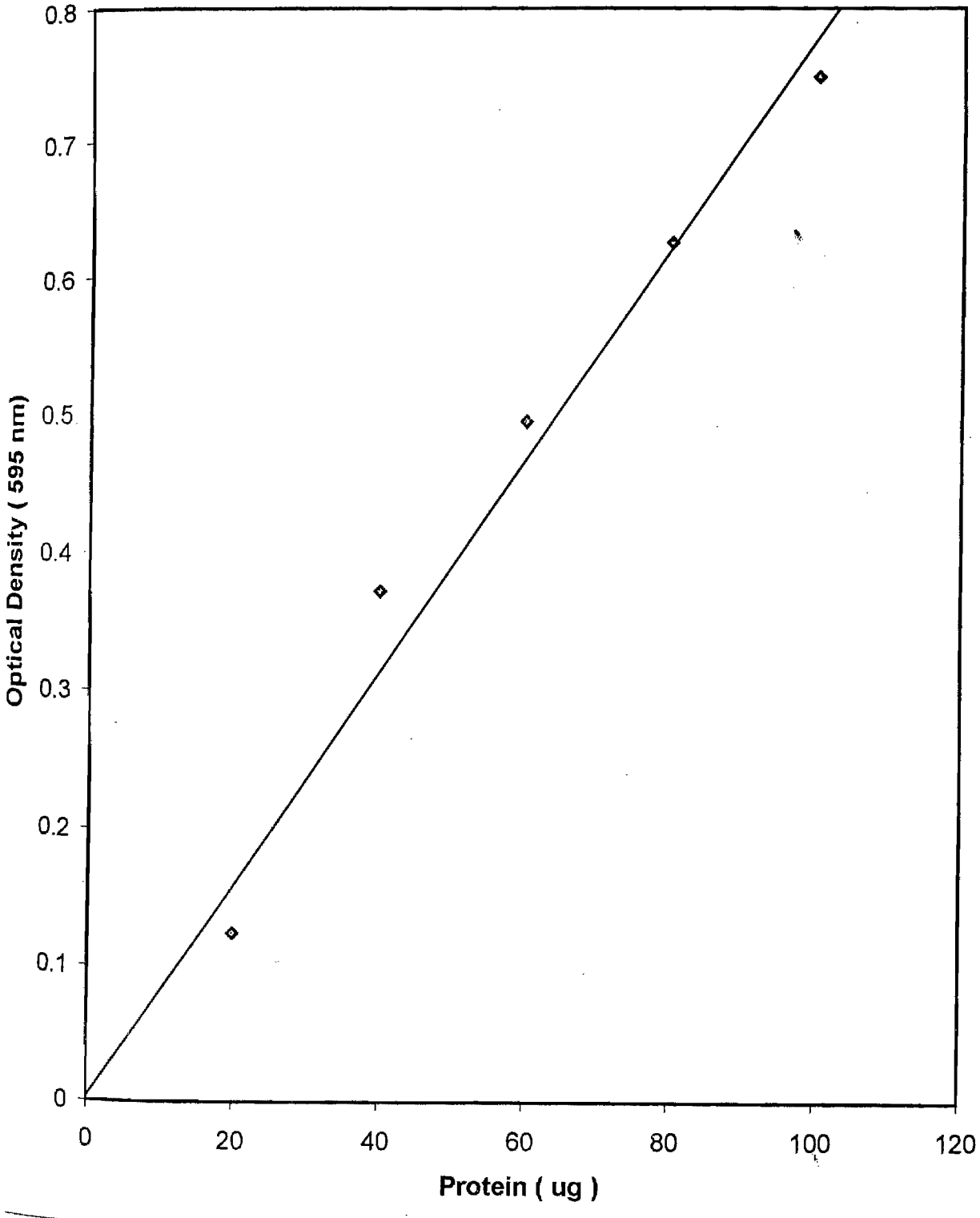
The standard curve for protein by Lowry *et al.* method is shown in Fig.3.2

(2) Method of Bradford (1974): (Fig. 3.3)

Chemicals

(i) 100 mg of commassive brilliant blue G-250 dissolved in 50 ml ethanol, 100 ml of phosphoric acid (85% w/v) added and then

FIG.3.3 STANDARD CURVE OF PROTEIN (BRADFORD METHOD)



final volume made one litre with distilled water, kept in dark bottle.

(ii) Stock protein solution : 1 mg/ml BSA

Procedure:

(i) Dilute the stock protein solution to make 3-20 µg in different tubes.

(ii) Make the final volume 0.3 ml with distilled water.

(iii) Add 3 ml of dye, blue colour developed and observe O.D. at 595 mm.

3.2.2C Amylase Inhibitor Assay:

α-Amylase inhibitory activity was measured by assaying α-amylase activity in presence of wheat seed extract prepared discussed earlier (section 3.2.1a). 500 µl of wheat seed extract was mixed with 1 ml of 1% starch and incubated at 30°C for 30 min, then 1 ml of amylase preparation was added and again incubated at 30°C for 30 min. Further steps were same as for amylase enzyme assay (section 3.2.2b).

The α-amylase inhibitor activity was expressed as per cent amylase activity in presence of inhibitor, which was calculated by using following formulae:

Enzyme activity = µmol of sugar produced per min per unit of enzyme

$$= \frac{\mu \text{ mol of product} \times \text{enzyme dilution factor}}{\text{Incubation time}}$$

$$\% \text{ inhibition} = \frac{\text{Amylase activity in presence of inhibitor}}{\text{Amylase activity in absence of inhibitor}} \times 100$$

Amylase assay was carried out in presence of inhibitory protein and inhibition of amylase was calculated as reduction in micromoles of maltose produced per unit time per mg of inhibitory protein (i.e. specific inhibitory activity, Sp. I.A.) and reduction in micromoles of maltose produced per unit time per gm seed (i.e. inhibitory activity per gm seed, I.A.).

3.2.3 Purification of α-amylase Inhibitor protein(s)

The α-amylase inhibitory protein(s) were isolated (as described earlier) and further purified from wheat variety UP 2425, which exhibited maximum inhibitory activity against the amylase insect *S. oryzae* and porcine pancreatic amylases. Purification of inhibitory protein from UP2425 was performed by heat treatment of crude aqueous extract to inactivate endogenous amylase, salting out by ammonium sulphate precipitation followed by dialysis, gel filtration chromatography on sephacryl S200 and ion exchange chromatography by DEAE-sephadex A50.

3.2.3a Heat treatment for inactivation of Endogenous amylase

Initially the aqueous seed extract was subjected to heat treatment at 70°C to inactivate any endogenous amylase activity. But to optimize temperature for heat treatment, crude water extract was subjected to heat treatment for 30 minutes at different temperatures (i.e. 60°C, 65°C, 70°C, 75°C, 80°C and 85°C). After and then

centrifuge at 8000 rpm for 15 minutes, the clear heat treated supernatant was assayed for amylase activity at 30°C. The heating temperature at which seed extract had minimum amylase activity in comparison to unheated aqueous extract, was the optimum temperature for inactivation of endogenous amylase and then the amylase activity of these heated fractions were assayed at 30°C.

3.2.3b Ammonium Sulphate Precipitation

For the isolation of the protein fraction from the heat treated aqueous extract, it was subjected to salting out with ammonium sulphate precipitation. Initially 30% ammonium sulphate saturation was achieved by adding of solid ammonium sulphate to 100 ml of heat treated aqueous extract (Ammonium sulphate was dissolved gently in cold conditions). The solution was kept at 4°C for 12 hrs and then it was centrifuged at 5000 rpm for 30 min at 4°C. The inhibitor activity against the insect amylase was checked in the supernatant and precipitate.

Subsequently the supernatant was further subjected to ammonium sulphate precipitation with gradual increase of 10% saturation, (30-40, 30-50, 30-60, 30-70, 30-80, 30-90 and 30-100%) and the inhibitors activity was checked in each ammonium sulphate precipitate fraction after through dialysis to remove excess ammonium sulphate. The ammonium sulphate precipitate fraction, showing the maximum inhibitory activity, was washed with 2M, NaBr and this

fraction was stored at 4°C and used as partially purified inhibitor protein(s).

3.2.3c Dialysis

Dialysis of the partially purified inhibitor was done to remove the excess ammonium sulphate and for this the dialysis tubes of length 10 to 20 cm were cut and soaked for 6 to 8 hours in distilled water. Then kept in 2% $NaHCO_3$ solution for 60°C for 15 minutes followed by a washing with warm water 2-3 times. Again the tubings were soaked in 1 mM EDTA solution at 60°C for 15 minutes followed by washing repeatedly with lukewarm water.

The ammonium sulphate fraction was carefully transferred into dialysis tube, which was tightly tied with thread at one end and after pouring the ammonium sulphate fraction the other end was also tied tightly. These tubes were dialyzed overnight against the distilled water in which the ammonium sulphate precipitate was dissolved, in the cold conditions. After the dialysis, any cloudiness was removed by centrifugation at 6000 rpm for 10 min at 4°C. Then these inhibitory protein(s) fractions were lyophilized and stored at 4°C.

Since inhibition of insect amylase by the wheat inhibitor was dependent on several enzyme inhibitor preincubation conditions, the detailed kinetic study of the partially purified inhibitor was carried out, to study the effect of temp, pH time of preincubation, stability of enzyme inhibitor complex and type of inhibition.

3.2.3 Kinetic characterization of partially purified α-amylase inhibitor protein

3.2.3.1 Heat Stability of α-amylase inhibitor protein

The water extract of the wheat variety UP 2425 was heated at different temperatures (i.e. 50°C, 60°C. 70°C, 80°C and 90°C) for different time periods (i.e. 0, 15 30, 45, 60, 75, 90, 105 and 120 min), centrifuged and then the inhibitory activity of these heat treated fractions, on insect amylase was measured (section 3.2.2c). Beside the inhibitory activity of the aqueous extract the endogenous amylase activity was also checked in the fractions heated at different temperatures for various time intervals.

3.2.3.2 Effect of pH

3.2.3.2a pH stability of insect enzyme

Insect amylase after 95% ammonium sulphate fractionation was dissolved in different buffers i.e. citrate buffer (pH 3.3), acetate buffer (pH 5.3) and phosphate buffer (pH 7.2). The amylase activity of insect amylase at these different buffers were checked.

3.2.3.2b pH stability of α-amylase inhibitor protein

The water extract of the wheat variety UP 2425, was heated at 65°C for 30 min and then subjected to 30-90% ammonium sulphate precipitation, the precipitates were dissolved in buffers of different pH values i.e., citrate buffer (pH 3.3), acetate buffer (pH 5.6) and phosphate buffer (pH 7.2) and then α-amylase inhibitory activities in these fractions were measured. The α-amylase activity in the same

fraction was also measured to check for any endogenous amylase activity.

3.2.3.3 Effect of time of incubation

To optimize the time of incubation, the heat treated aqueous extract was 30-90% ammonium sulphate precipitated, dissolved in citrate buffer (pH 3.3) and after dialysis, it was incubated with insect amylase at 30°C for various time intervals (i.e. 0, 15, 30, 45 min, 1, 2, 3, 12 and 24 hrs.) and the inhibition of amylase, which was also stored at 30°C, activity was monitored at each time interval.

3.2.3.4 Stability of Insect enzyme and enzyme inhibitor complex

Stability of the insect enzyme and the enzyme-inhibitor complex at 30°C was also checked by incubating the only enzyme and enzyme-inhibitor complex (1:0.1) at 30°C and checking the amylase activity of insect amylase and the inhibitory activity in enzyme inhibitor complex at different time intervals (at 15, 30, 45 min, 1, 3, 6 hr and 24 hrs).

3.2.3.5 Type of Inhibition

To determine the type of inhibition the different amount of inhibitor (0, 0.1, 0.2, 0.3 and 0.4 ml), in presence of varying substrate concentration (0.1 ml, 0.118 ml, 0.143 ml, 0.182 ml, 0.25 ml, 0.4 ml and 0.91 ml of 1% starch solution) was subjected to inhibit the insect amylase at 30°C.

3.2.4 Chromatographic Purification of Partially Purified Inhibitor Protein(s) of UP 2425.

3.2.4.1 Gel filtration chromatography

Beaded gel used for gel filtration chromatography are composed of cross linked macro polymer which constitute large or small pores in the beads depending upon the extent of cross linkage. These beads take up large quantities of water forming swollen gel which would have at least two kinds of water medium: (1) freely flowing inter beads water, and (ii) stationary water inside the beads which is bound to it and flows very slowly, if at all.

An aqueous medium containing molecules of different sizes, is forced through gel beads having a certain pore size, those molecules which are larger then the pores and therefore can not enter the beads, move along with medium flowing through the interbead space, while other molecules entering the gel beads get retarded. The relative retardation of molecules entering the gel beads had been shown to correspond to the average time. Thus molecules come out of the gel bed in order to descending molecular weight.

For the separation of molecule of interest Sephacryl S-200 that had a cut off size limit of these beads ranges from 5 to 200 kDa therefore, only the molecules ranging between 5-200 kDa were retarded by the gel particles. Besides this, sephadex G-75 was also used to check the homogeneity of amylase inhibitor. The exclusion limits of Sephadex G-75 are similar to that of Sephacryl S-200.

Requirements

A Sephacryl S-200

100 mM Ammonium Acetate Buffer (pH 6.8)

Glass columns with sintered glass filter (34×2 cm)

B Standard proteins for column calibration

Bovine Serum Allumin (67 kDa)

Haemoglobin (60 kDa)

Trypsin Inhibitor (20 kDa)

Lysozyme (14.3 kDa)

Packing of the column

Suspended Sephacryl S-200 solution was shaken gently and poured into the column and allowed to settle until about half of the column was packed by slurry. The outlet was opened for average flow rate and more slurry was added up to the appropriate height. After packing the matrix, it was introduced with 100mM Ammonium acetate (pH 6.8) buffer to equilibrate the packed column. The washing of the column with ammonium acetate buffer was done until the eluted buffer pH reached up to 6.8.

Sample Application and Elution

The maximum quantity of protein sample which can be loaded/ applied on the column is, 10% of the total bead volume of the column and 0.5 mg/ml to 1mg/ ml protein quantity can be applied on the column for proper resolution of proteins.

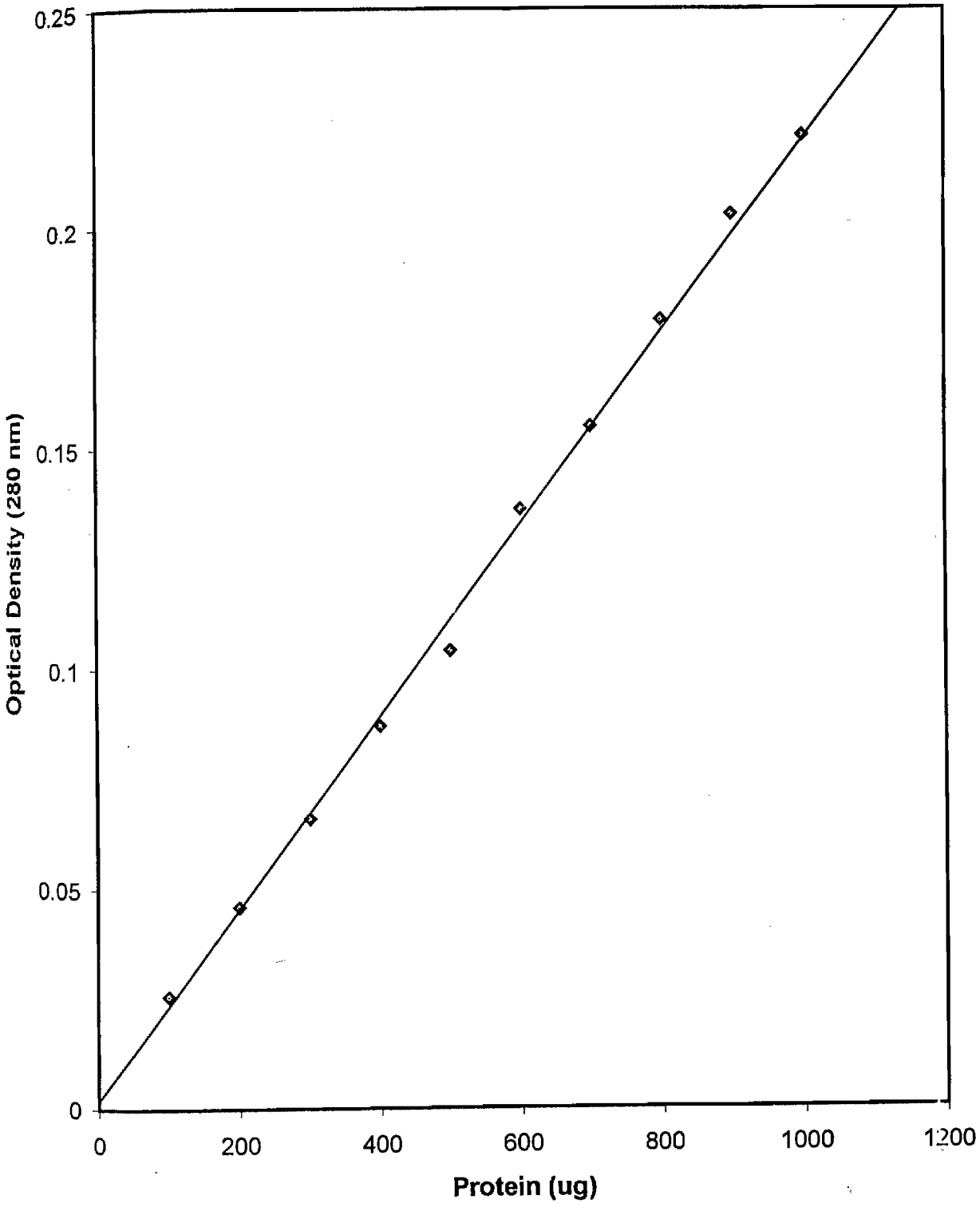
To load the sample onto the column, the buffer present of the top in column was allowed to flow down and stopped slightly above the level of the slurry and then the outlet was closed. The concentrated sample was gently poured over the slurry bed with the help of pipette, with out disturbing the slurry. The outlet was opened till the entire sample had entered the slurry and immediately the elution buffer was allowed to run. Fraction of 5 ml volume were collected by maintaining 1 ml per min. flow rate. Protein content in the fractions were measured by recording absorbance at 280 nm (Fig 3.4) as well as by dye binding method (Fig 3.3).

Inhibitory activity in each fraction was also measured (as described in section 3.2.2c). The fractions showing higher specific inhibitory activity were pooled and lyophilized, which were further used for the ion-exchange chromatography.

Calibration of the Column

Calibration of the column was done different by proteins of known molecular weight, e.g., BSA, , Hb ,Lysozyme and Trypsin Inhibitor. Proteins were dissolved in the elution buffers (1 mg/ml of each) and these solutions were loaded on the column. One inhibitor fractions were collected, protein content in each fraction was measured by taking absorbance at 280 nm and extrapolating absorbance on the standard curve. A calibration curve of log mal cut of standard proteins vs. V_e/V_o was drawn (Fig 3.5), where V_e is the

FIG 3.4 PROTEIN STANDARD CURVE
(At 280 nm)



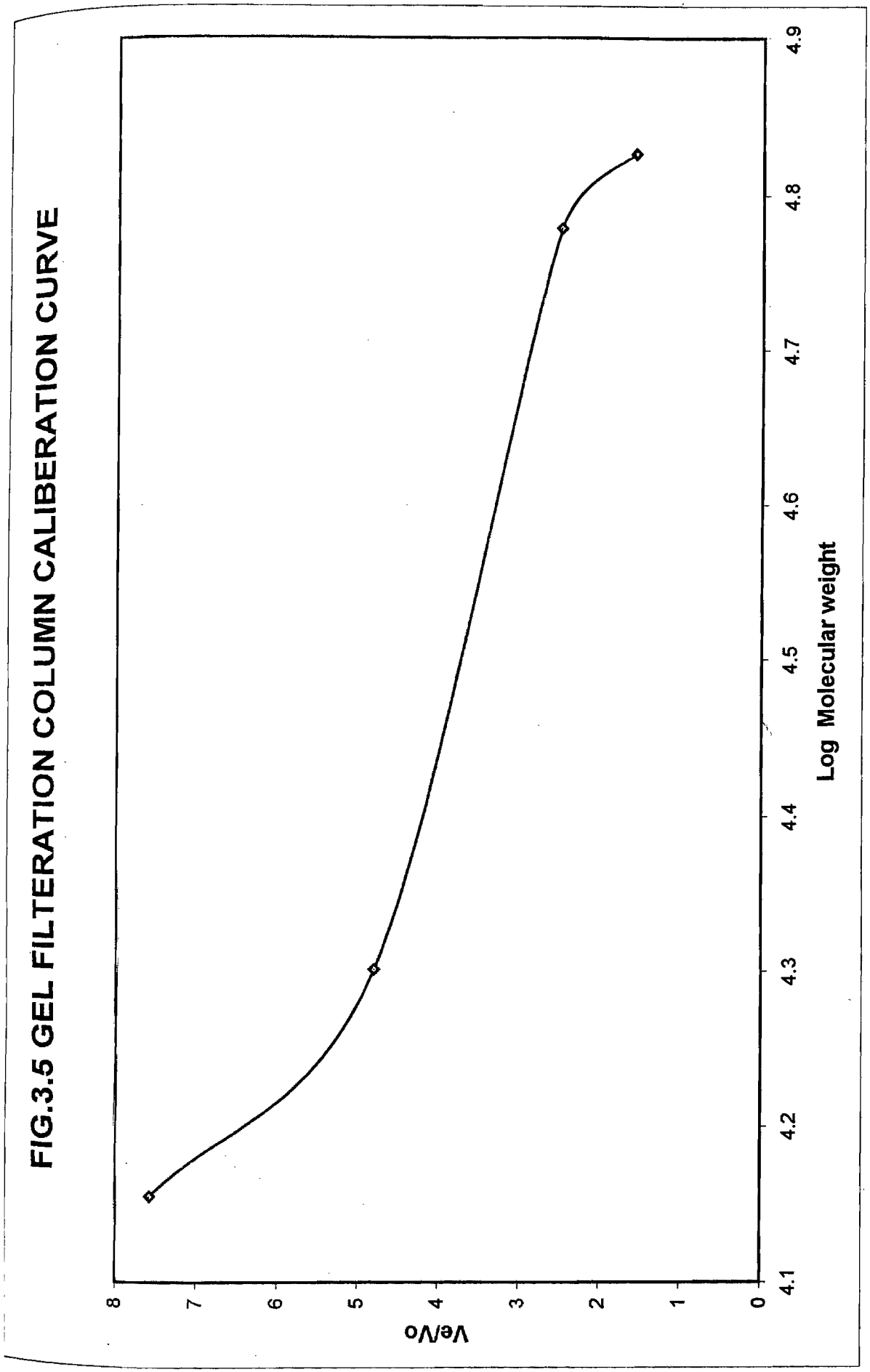


FIG.3.5 GEL FILTERATION COLUMN CALIBERATION CURVE
Ve/Vo
0
1
2
3
4
5
6
7
8
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
Log Molecular weight

elution volume of the specific protein and V_o is the void volume of the column.

3.2.4.2 Ion-Exchange Chromatography

On an ion-exchange column proteins are separated on the basis of net charge. Both cation or anion exchange resins may be used separately, depending net charge on proteins. Negatively charged proteins are separated on an anion exchanger such as DEAE-Sephadex; which itself bears a positive charge. Most proteins being a net negative charge at pH 8.0, hence they bind to the anion exchange resin. They are released gradually by decreasing the strength of binding by either increasing the ion concentration or reducing the pH of elution buffer. Thus, proteins having high negative charges are eluted later than the proteins with less negative charges.

Requirement

- DEAE- Sephadex A-50
- 0.2 M NaOH
- 0.2 N HCl
- 20 mM Tris Cl (pH 7.5)
- 60 mM NaCl
- 150 mM NaCl
- Column with sintered glass filter
- Plastic tubing with stopper
- Gradient maker

3.2.4.2a Activation of DEAE Sephadex A-50

DEAE Sephadex A-50 (1gm) was allowed to swell in 250 ml of distilled water for 4-5 hrs. Water was decanted and an equal volume of 0.2 M NaOH was poured to it and kept for 30 minutes with intermittent stirring. The basic solution was removed and the slurry was washed thoroughly to make the pH neutral. Again the sediment was treated with an equal volume of 0.2 N HCl for 30 minutes and then washed with distilled water till the pH became neutral. Now the slurry was washed with buffer, 20 mM Tris Cl (pH 7.5) and used for column packing after degassing of the slurry.

3.2.4.2b Packing of the column

Thick slurry was poured along the sides of the wall of the column, so that no air bubbles were trapped, till the slurry rose to the tap. The slurry was allowed to stand for sometime under gravity till the required length of the column was packed. A plastic tubing with a stopper was attached at the outlet of the column and the outlet was opened to give an average flow rate. The column was washed repeatedly until the pH of the eluted buffer becomes 7.5. The buffer used was degassed before use to avoid air bubbles in the column.

3.2.4.2c Samples application and elution

Out of the two aspirator bottles filled with 20 mM Tris Cl buffer containing 60 mM NaCl and the other was filled with 20 mM Tris Cl buffer containing 150 mM NaCl. An outlet from the bottle containing

60mM NaCl was connected to the column. The aspirators were placed at a higher level to that of column to have a flow of buffer by siphoning.

The concentrated protein fraction, collected from the Sephacryl S-200 column, was loaded at the top of the slurry and the outlet was opened to allow the sample to enter the column. A flow rate of 1 ml/10 min was maintained by adjusting the flow of elution buffer between the two aspirator bottles and at a speed similar to the outlet of the column and 2 ml each fractions were collected.

The amount of protein was monitored by recording absorbance at 280 nm and inhibitor activity was estimated in each fraction by DNS method (section 3.4.2). The fractions with inhibitory activity were pooled and lyophilized.

3.2.5 Subunit Characterization

3.2.5.1 Poly Acrylamide Gel Electrophoresis

Principle

Gel electrophoresis is among the most powerful and conveniently used method for macromolecules separation. Charged particles more under the electrical field and so do the proteins. Proteins are separated on the polyacrylamide gel on the basis of both molecular size and the net charge.

The native PAGE and SDS-PAGE were performed to determine the molecular weight and subunit composition of amylase inhibitor protein by the following method of **Laemmli (1970)**.

In native PAGE, separation of protein in their native state is effected by charge as well as molecular size. Hence, it gives information about the different type of proteins present in sample. While in SDS-PAGE, SDS, an anionic detergent subjects a protein to disintegrate into subunits and imparts a net negative charge on them and polypeptides are separated on the basis of molecular size. SDS-PAGE thus gives the idea about the subunits of the proteins.

Both native-PAGE and SDS-PAGE were performed by the following method of **Laemmli (1970)**.

Reagents

1. Acrylamide
2. N.N.-methylene bisacrylamide
3. Ammonium per sulphate (APS)
4. TEMED
5. Sodium dodecyl sulphate (SDS)
6. β-Mercaptoethanol
7. Glycerol
8. Coomassie Brilliant Blue R 250
9. Glacial acetic acid
10. Methanol

11. Tris buffer,

12. Sodium chloride

Native PAGE

Staking gel and separation gel (7.5% and 10%) were prepared from the stock solution by mixing them in the following proportions:

Stock Solutions	Separating Gel		Stacking Gel	
	7.5% (ml)	10% (ml)	7.5% (ml)	10% (ml)
1 M Tris buffer, (pH 8.8) separating gel buffer	2.5	2.5	-	-
1 M Tris buffer, (pH 6.8) stacking gel buffer	-	-	2.5	2.5
Acrylbis solution (30% Acrylamide + 0.8% bis)	5.0025	6.67	0.9379	1.25
Distilled water	17.79	9.83	5.801	5.50
TEMED	0.010	0.010	0.0075	0.0075
APS (1.5%)	1.0	1.0	0.75	0.75
TOTAL	**20 ml**	**20 ml**	**10 ml**	**10 ml**

Before gel preparation, glass plates were assembled to make leak proof gel cassette. APS was added to the separating gel in the last and was immediately poured into the glass cassette with 20 ml glass syringe. The volume of the filled gel solution was kept 2.5 cm below the top and was immediately layered with water to avoid air entrapment. After the gel had set, water was drained out by tilting the glass cassette.

The comb was then inserted between the glass plates leaving a 10 mm. distance between comb and the separating gel. APS was

added to the stacking gel solution and immediately the solution was poured over the separating gel using 5 ml syringe. The comb was removed after the gel had properly set and the wells were cleaned. Now the side and base clamps were removed and the cassette was clamped into the electrophoresis assembly, with care that no air bubble was trapped. The upper and lower troughs were filled with electrode buffer (Tris glycine buffer, pH 8.3) and the electric terminals were connected to the power pack. Protein solutions containing glycerol (15%) and bromophenol dye (5µl for each well) were loaded (30-50µl having 30-35 µg protein per well).

The gel was electrophoresed at a constant current of 10 mA and voltage of 90V. The gel was run till the bromophenol blue dye reached just 0.5 cm above the bottom of the gel. The power supply was switched off and the glass plates were removed. The gel was carefully removed from the glass plate with the help of spatula. The position of the last well of the gel was marked by cutting the bottom of the last well and the gel was put into staining solution.

The gel was transferred for staining in a tray containing about 200 ml of staining solution and stained overnight. The staining solution was then drained off and the gel was then floated into the destaining solution (10% acetic acid or 3% NaCl solution). Destaining solution was changed three to four times. Then gel was stored after drying in gel dryer at 80°C for 2 hrs.

SDS-PAGE

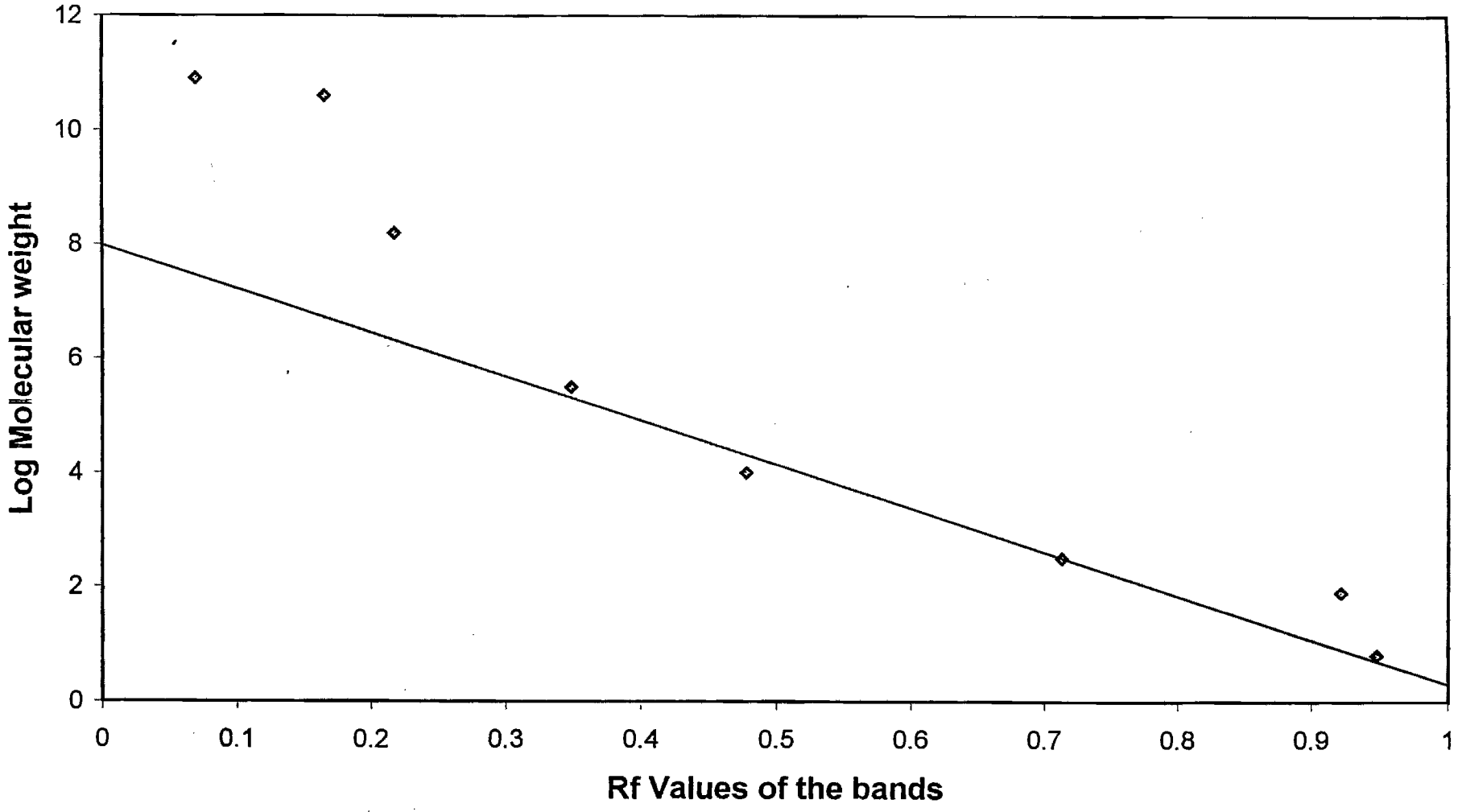
In SDS-PAGE, SDS an anionic detergent disrupts the non-covalent interactions in the native proteins which are then electrophoresed on a polyacrylamide gel. Thus, the subunit profile of the protein can be elucidated. SDS-PAGE gel was prepared using following composition.

Stock Solutions	Stacking Gel		Separating Gel	
	7.5% (ml)	10% (ml)	7.5% (ml)	10% (ml)
Stacking gel buffer (pH 6.8)	2.5	2.5	-	-
Separating gel buffer (pH 8.8)	-	-	5.00	5.00
Distilled water	6.1	5.89	7.95	9.28
Acrylbis solution (37.5% Acryl+1% Bis)	0.6	0.8	5.33	3.99
TEMED	0.015	0.015	0.015	0.015
APS (1.5%)	0.7	0.7	1.5	1.5
SDS	0.1	0.1	0.2	0.2

The inhibitor protein samples (20-30 µl) containing 30 to 35 µg protein and molecular weight marker protein were mixed with 20-30 µl of the sample buffer (pH 6.8) containing β-mercaptoethanol. It was heated for 5 min. at 65°C, then centrifuged for 10 minutes at 3000 rpm and rest of the procedure followed was same as that of the native-PAGE (Fig 3.6).

In SDS-PAGE, different molecular weight markers were also loaded along with the protein samples. After destaining, the bands of the protein samples were compared with the bands of the molecular

FIG.3.6 STANDARD CURVE OF PROTEIN MARKER BANDS



81

weight markers and the relative mobility of the different samples were calculated by using the formula:

$$\text{Rm value} = \frac{\text{Distance travelled by the protein band}}{\text{Total distance travelled by dye front}}$$

3.2.6 Feeding Trials

S. oryzae were reared on the wheat samples and after one life cycle, newly emerged adults of F 1 generation were taken for the feeding trials. For the feeding trials three sets of partially purified inhibitory protein coated wheat seed were used i.e. 20 µg/ seed, 50 µg/seed and 100 µg/seed along with control, which was not coated with inhibitor. These treated and control wheat samples were taken in trplicate.

For each set of test and control 25 seeds were taken in vials and 5 adults of *S. oryzae* (F1 generation) were released in them and then these vials were kept at 28-30^{0}C$\pm$1^{0}C with 65%$\pm$ 5% RH. Growth rate of the insects, protein content of the seeds and germination efficacy of these treated and untreated seeds were checked, to determine the effect of partially purified protein on the insect activity and growth.

Chapter 4

RESULTS

Chapter 4 RESULTS

Alpha amylase is an important enzyme for carbohydrate digestion in the diet of animals. Proteinaceous inhibitors of enzyme α-amylase are widespread in plants, notably certain cereals, such as wheat, maize, millets and also in legumes like pigeonpea. Since α-amylase plays an important role in starch breakdown in human beings as well as in the insects, the presence of such inhibitors in foodstuffs may be responsible for impaired starch digestion in insects as well as to protect the crops from insect pests. Alpha amylase inhibitor genes have provided novel system for investigation of the fundamental of developmental biology and natural defense systems in plants. Induction of such defensive genes into plants by using gene technologies may increase their insect resistance potential. It will also help to reduce the amount of conventional insecticides(s) up to some extent, which in turn will help in the efforts for safer environment.

In the present investigation wheat varieties were screened for their amylase inhibitory activity against insect (*S. oryzae*) amylase, germinating wheat seed amylase and porcine pancreatic amylase, isolation of inhibitory protein(s), their purification, kinetic and subunit characterization and insect feeding trials.

4.1 SCREENING OF DIFFERENT WHEAT VARITIS FOR α-AMYLASE INHIBITOR ACTIVITY

4.1.1 Qualitative Test

Seeds of 10 varieties of wheat were screened for their amylase inhibitor activity. Aqueous extract of finally grounded wheat verities were prepared and porcine pancreatic amylase inhibitory activity of heat untreated and heat treated (at 70°C for 20 min) supernatants were checked on the starch-agar plate (Table 4.1). Heat untreated supernatant did not show any inhibitor activity while the heat-treated supernatant showed the inhibitory activity in all the varieties. On the starch agar plate, in the control well there was only amylase and in the other test wells the amylase and heat treated water extract was present. In the control well, the starch was hydrolyzed due to the activity of the enzyme and a clear zone was farmed when acidic KI was poured in the plate, while in the test wells due to the presence of inhibitor proteins, the amylase activity was inhibited as the inhibitor concentration was increased and less starch was hydrolyzed giving rise to smaller zones of hydrolysis. (Photoplate 4.1 showed the inhibitory activity of heat treated aqueous extract from UP2425 where well 4 was control and 1, 2 & 3 were tests with increasing concentration of inhibitor). Although slight differences in the zone of

Table 4.1: Detection and selection of wheat varieties base on inhibitory activity.

Controls	Development of zones on Starch agar plates	
	Heat Treated extract	**Heat untreated**
Only Enzyme 50 µl	-	+
Only Inhibitor 50 µl	-	-
Only D.W. 50 µl	-	-
Tests		
50 µl (E) + 50 µl (I)		
Sonalika	+	-
UP 115	+	-
UP 262	+	-
UP 319	+	-
UP 1109	+	-
UP 2003	+	-
UP 2113	+	-
UP 2338	+	-
UP 2382	+	-
UP 2425	+	-

PHOTO PLATE 4.1: **INHIBITION OF PORCINE PANCREATIC AMYLASE BY THE HEAT TREATED AQUEOUS INHIBITORY PREPARATION OF UP 2425 ON STARCH AGAR PLATE.**

C: Control (only 50 µl enzyme)

1: 50 µl enzyme + 20µl inhibitor

2: 50 µl enzyme + 30µl inhibitor

3: 50 µl enzyme + 40µl inhibitor

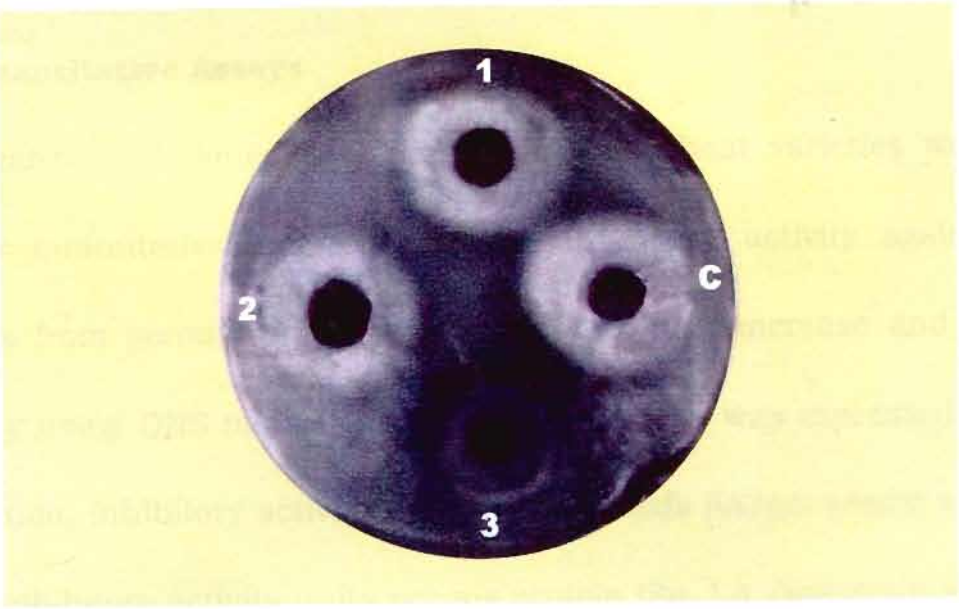


Photo Plate: 4.1 : Inhibition of porcine pancreatic amylase by the heat treated aqueous inhibitor preperation of UP-2425 on starch-agar plate.

starch hydrolysis were absorbed, which were too less apparent to distinguish varieties for higher or lower amylase inhibitor activity. Thus quantitative assays for amylase inhibitory activities were performed against wheat amylase, porcine pancreatic amylase and *Sitophilus oryzae* amylase.

4.1.2 Quantitative Assays

Heat-treated aqueous extracts from ten wheat varieties were used for quantitative assay of amylase inhibitory activity against amylases from germinating wheat seeds, porcine pancrease and *S. oryzae* by using DNS method The inhibitory activity was expressed in % inhibition, inhibitory activity units per gm seeds (IA/gm seeds) and specific inhibitory activity units per mg protein (Sp. I.A./mg inhibitory protein).

4.1.2.1 Germinating wheat amylase inhibition by wheat amylase inhibitory protein(s)

Germinating wheat seed amylase was subjected to inhibition by heat treated amylase inhibitors from different wheat varieties (Table 4.2). It showed that there was no amylase inhibitory activity against wheat amylase in any of the varieties, since in all the wheat varieties the production of maltose was increased, even after the addition of heat treated inhibitor along with wheat amylase (Table 4.2).

Table 4.2: **Alpha amylase activity in germinating wheat seed amylase in absence and presence of amylase inhibitory protein(s) extracted from different wheat varieties.**

Wheat varieties	**Amylase Activity** (μg maltose produced / 30 min by wheat amylase)	**Residual Amylase Activity** (μg maltose produced/ 30 min in the presence of inhibitor
Sonalika	110.0	212.5
UP 115	55.0	227.0
UP 262	50.0	63.0
UP 319	95.0	100.0
UP 1109	120.0	223.0
UP 2003	100.0	177.0
UP 2113	107.0	125.0
UP 2338	73.0	167.0
UP 2382	125.0	213.0
UP 2425	70.0	77.0

* values are the mean of triplicate readings.

4.1.2.2 Porcine pancreatic amylase inhibition by wheat Amylase inhibitor protein(s)

When commercially available porcine pancreatic amylase (20 times diluted) was subjected to heat treated aqueous inhibitor preparation, the porcine pancreatic amylase was inhibited by amylase inhibitor(s) from all the wheat varieties (Table 4.3). The percentage inhibition ranged between 24 -77% (Table 4.3 and Fig 4.1). The specific inhibitory activity of the wheat amylase inhibitor against the porcine pancreatic amylase ranged between 2.53 to 24.43 while the units per gm of seed ranged between 0.294 to 4.284 (Table 4.3). The maximum % inhibition-77% was shown by UP1109 while Sonalika had minimum percentage inhibitory activity 24% against porcine pancreatic amylase (Fig. 4.1). The units/ gm seed and specific inhibitory activity had similar results as the maximum in both units were found in the UP2113 specific inhibitory activity /mg protein was 24.43 while inhibitory activity units/gm seed was 4.284 in UP 2113. Both the minimum specific inhibitory activity /mg protein and inhibitory units/gm seed were minimum in Sonalika (Table 4.3 and Fig. 4.2).

4.1.2.3 Insect Amylase Inhibition by Wheat Amylase inhibitory protein(s)

Amylase inhibitors from all wheat varieties were able to inhibit the activity of amylase isolated from *S. oryzae* (Table 4.4). Percent inhibition of insect amylase by wheat amylase inhibitor ranged

Table 4.3: Inhibition of porcine pancreatic amylase activity by alpha amylase inhibitor protein(s) extracted from seeds of different wheat varieties.

Wheat Varieties	% of Inhibition*	Inhibitory Activity units (/gm seed)**	Specific Inhibitory Activity (per mg protein) ***
Sonalika	24.0	0.294	2.53
UP 115	38.0	1.598	11.22
UP 262	57.0	0.542	7.36
UP 319	65.0	1.000	10.24
UP 1109	77.0	1.394	21.56
UP 2003	26.0	1.458	21.92
UP 2113	32.0	4.284	24.43
UP 2338	52.0	0.656	4.57
UP 2382	37.0	1.413	16.64
UP 2425	33.0	1.37	17.22

* sem = $\pm$ 0.20 cd at 1% = 0.83 cd at 5% = 0.61 cv = 5.37

** sem = $\pm$ 0.06 cd at 1% = 0.24 cd at 5% = 0.18 cv = 15.40

*** sem = $\pm$ 0.92 cd at 1% = 3.76 cd at 5% = 2.74 cv = 11.60

FIG.4.1 PERCENT PORCINE PANCREATIC AMYLASE INHIBITION BY ALPHA AMYLASE INHIBITOR FROM DIFFERENT WHEAT VARITIES

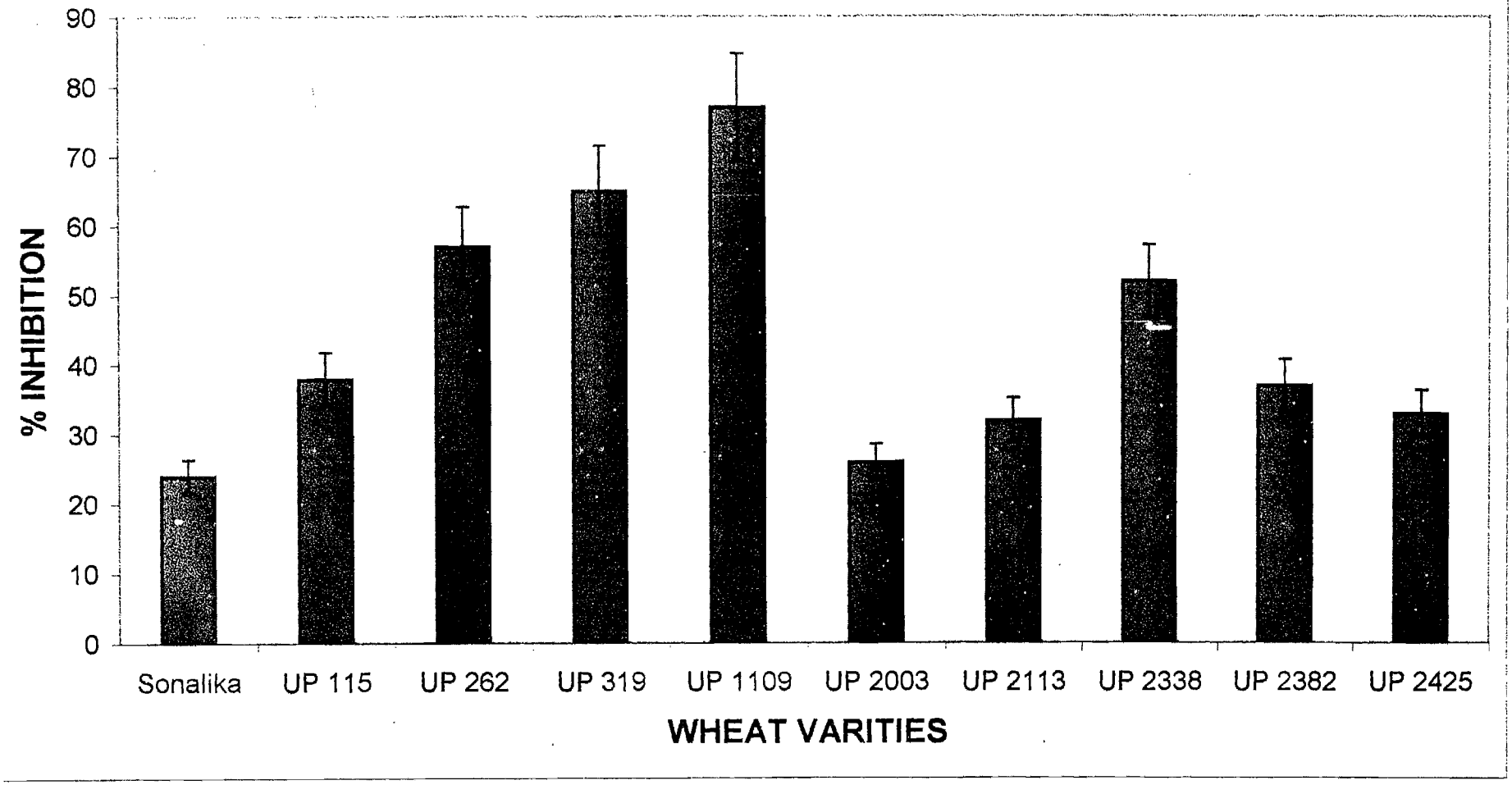
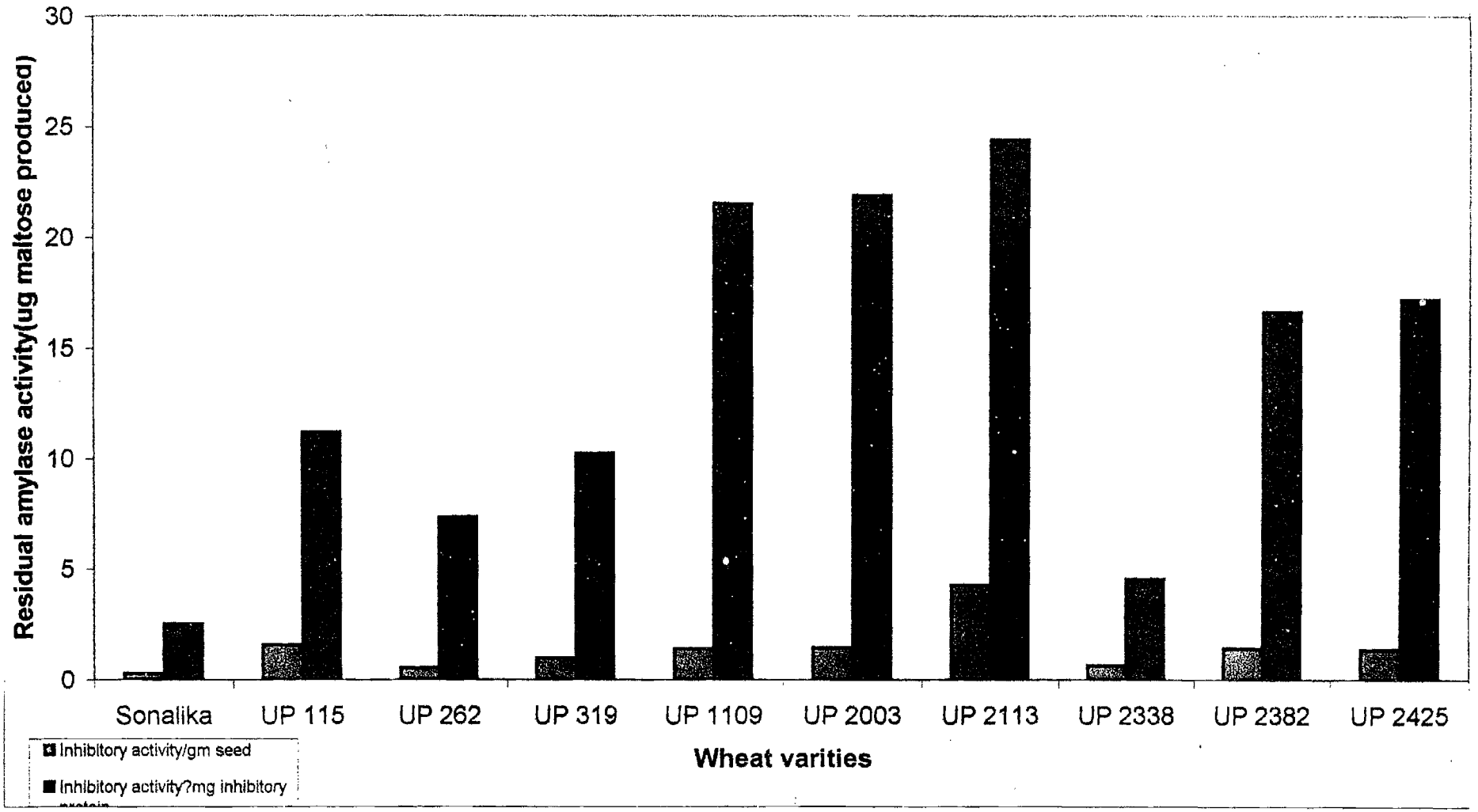


FIG.4.2 PORCINE PANCREATIC AMYLASE INHIBITORY ACTIVITY/gm SEED AND SP.INHIBITORY ACTIVITY/mg PROTEIN



between 25 to 60%, inhibitory activity/gm seed ranged 0.278 to 0.754 and specific inhibitory activity ranged 0.585 to 4.762. Maximum % inhibition of insect amylase was shown by UP 2425 along with UP1109 (60%) and the minimum by UP115 and UP319 (25%) (Fig. 4.3, Table 4.4). Maximum specific inhibitory activity and. inhibitory activity / gm seed was also shown by UP2425 while the minimum specific inhibitory activity and min. inhibitory activity / gm seed was shown by UP115 (Table 4.4, Fig. 4.4). The specific inhibitory activity/mg protein in UP 2425 was 4.762 and 0.585 in UP 115 and the inhibitory activity /g seed in UP 2425 was 0.754 and in UP 115 it was 0.278.

Since heat-treated aqueous extracts from seeds of UP2425 exhibited maximum specific inhibitory activity (4.762 units/mg) as well as inhibitory units per gm seed (0.754 units/g) against *S. oryzae* amylase, further studies were conducted with alpha amylase inhibitor from UP 2425 (Table 4.4).

4.2 PURIFICATION OF α-AMYLASE INHIBITOR FROM UP 2425

The proteinaceous inhibitors, obtained after the aqueous extraction from seed powder of variety UP 2425, was purified by following purification steps:

- Heat treatment to inactivate endogenous amylase enzyme
- Ammonium sulphate precipitation
- Dialysis

Table 4.4: **Inhibition of insect *(S. oryzae)* amylase activity by α-amylase inhibitor protein(s) extracted from different wheat varieties.**

Wheat Varieties	% of Inhibition*	Inhibitory Activity units (/gm seed)**	Specific Inhibitory Activity (per mg protein)
Sonalika	50	0.406	1.141
UP 115	25	0.278	0.585
UP 262	45	0.696	4.363
UP 319	25	0.394	2.268
UP 1109	60	0.630	2.952
UP 2003	45	0.424	1.899
UP 2113	40	0.566	1.995
UP 2338	45	0.686	1.624
UP 2382	55	0.694	1.995
UP 2425	60	0.754	4.762

* sem = ± 0.243 cd at 1% = 0.99 cd at 5% = 0.72 cv = 7.39

** sem = ± 0.069 cd at 1% = 0.28 cd at 5% = 0.21 cv = 43.05

*** sem = ± 0.36 cd at 1% = 1.46 cd at 5% = 1.06 cv = 31.54

FIG.4.3 PERCENT INHIBITION OF INSECT AMYLASE BY ALPHA AMYLASE INHIBITOR(S) FROM DIFFERENT WHEAT VARITIES

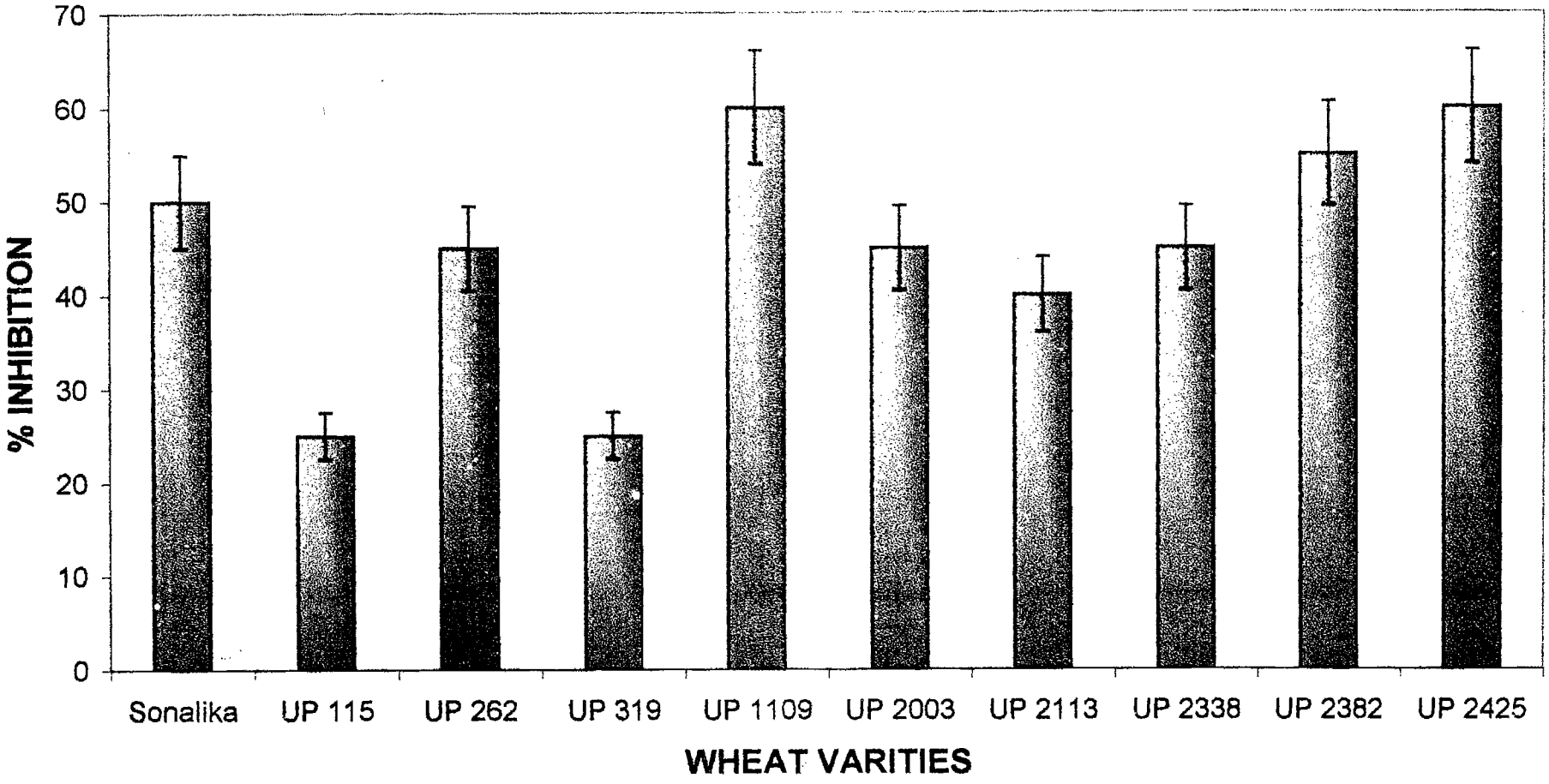
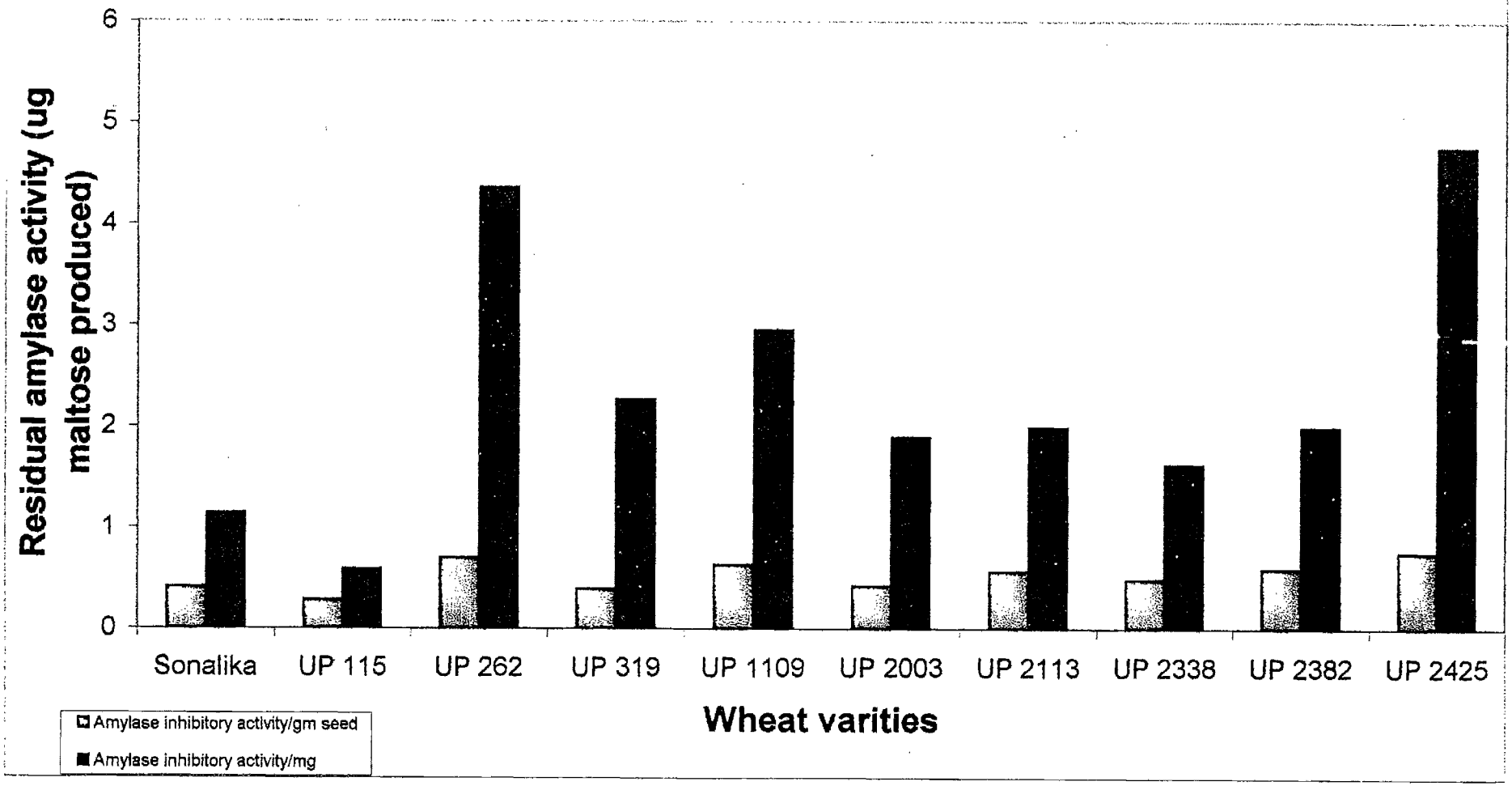


FIG.4.4 INSECT AMYLASE INHIBITORY ACTIVITY/gm SEED AND SP.INHIBITORY ACTIVITY



- Gel filtration chromatography
    - On sephacryl S-200
    - On sephadex G 25
- Ion exchange chromatography on DEAE-Sephadex A50

4.2.1 Heat Inactivation of Endogenous Amylase

Since wheat grains contained high amount of α- and β-endogenous amylase, it is necessary to inactivate the endogenous amylase prior to any purification of the proteinaceous amylase inhibitor. The crude extract was therefore subjected to heating at different temperature from 60⁰ to 85⁰C for 30 min. and alpha amylase activity was measured in the clear supernatant fractions of these variably heated fractions, as these aqueous extracts after heat treatments were used as amylase preparation and produced maltose was measured by DNS method (Table 4.5, Fig. 4.5).

Table 4.5: Inactivation of aqueous endogenous amylase in inhibitor preparations by heat treatment.

Heating Temperature (⁰C)	**Amylase activity** (μg maltose produced /30 min)	**% Inhibition of endogenous amylase**
Control (no heating)	215	-
60	200	7.00
65	32.5	85.00
70	207.5	2.50
75	240	*
80	277	*
85	433	*

* no inhibition observed due to hydrolysis of starch present in aqueous extract.

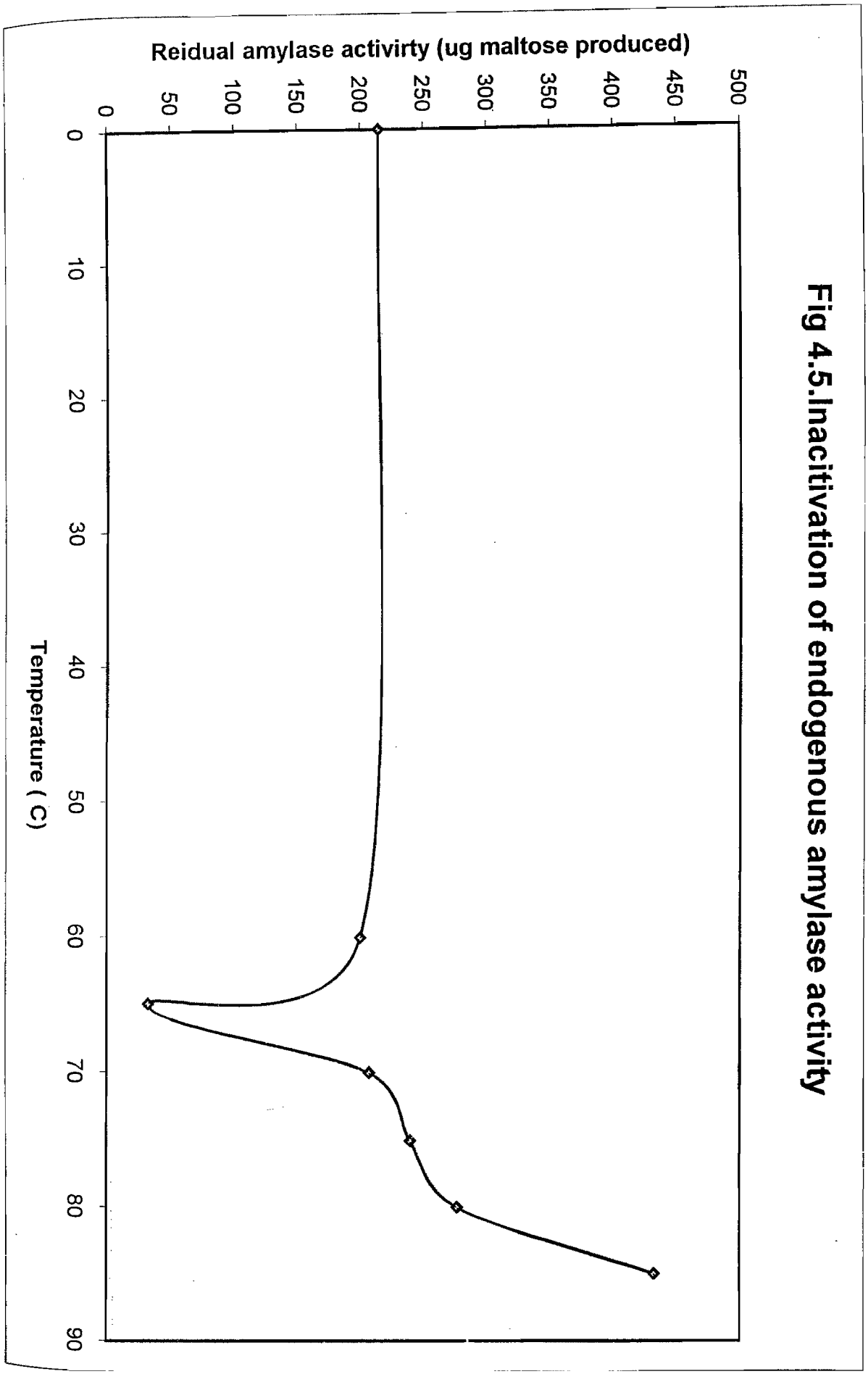


Fig 4.5.Inacitivation of endogenous amylase activity

4.2.2 Purification After Ammonium Sulphate Precipitation

After heat treatment the clear supernatant was subjected to ammonium sulphate precipitation for salting out of the inhibitor proteins. Initially it was 0-30% ammonium sulphate saturation, however, inhibitory activity was only in the supernatant and precipitate after dialysis did not show any inhibitory activity against insect amylase. A gradual increase in ammonium sulphate concentration at intervals of 10% saturation (i.e. 30-40, 30-50, 30-60, 30-70, 30-80, 30-90 and 30-100) beyond 30% saturation was done. As the ammonium sulphate saturation increased the inhibitor activity in the precipitate, after dialysis, it was further also increased (Table 4.6). The maximum inhibitory protein fractions were precipitated in 30-90% saturation, as after 30-90% ammonium sulphate saturation negligible inhibitor activity was found in supernatant and major inhibitory activity was confined to precipitate. In 30-100% ammonium sulphate precipitation, ppt again did not show any increase in % inhibition of insect amylase (Table 4.6). Thus the 30-90% ammonium sulphate saturation was found optimum for salting out the amylase inhibitor proteins fractions in heat treated aqueous extracts.

The precipitate containing bulk of the inhibitory protein was washed with 2 M NaBr to remove excess carbohydrates moieties present in the precipitate and then this precipitate was dissolved in distilled water and dialyzed.

Table 4.6: **Ammonium sulphate fractions used for amylase inhibitor purification.**

%Ammonium sulphate ppt. Fractions (after dialysis)	% Inhibition of insect amylase
0-30	0.0
30-40	1.57
30-50	3.21
30-60	20.21
30-70	33.4
30-80	57.00
30-90	97.31
30-100	97.26

4.2.3 Dialysis

Amylase inhibitor protein(s) after the salting out with 30-90% ammonium sulphate saturation and 2 M NaBr washing were subjected to dialysis against the distilled water in cold conditions. The inactive precipitate which was formed during the dialysis was separated by centrifugation and discarded. The active supernatant was designated "dialysate's supernatant" and used for the kinetic characterization and further purification by gel filtration and ion-exchange chromatography.

Deployment amylase inhibitors for insect control required a detailed analysis of the amylase inhibitor-insect amylase interaction. Since inhibition of insect amylase by the wheat inhibitor(s) was dependent on several enzyme – inhibitor preincubation conditions, therefore, the detailed kinetic characterization of inhibitor protein should be carried out at least after partial purification for the studies of effect of temperature, pH, time of preincubation for inhibition, stability of enzyme-inhibitor complex and type of the inhibition.

4.3 KINETIC PROFILE OF PARTIALLY PURIFIED AMYLASE INHIBITOR

4.3.1 Effect of temperature

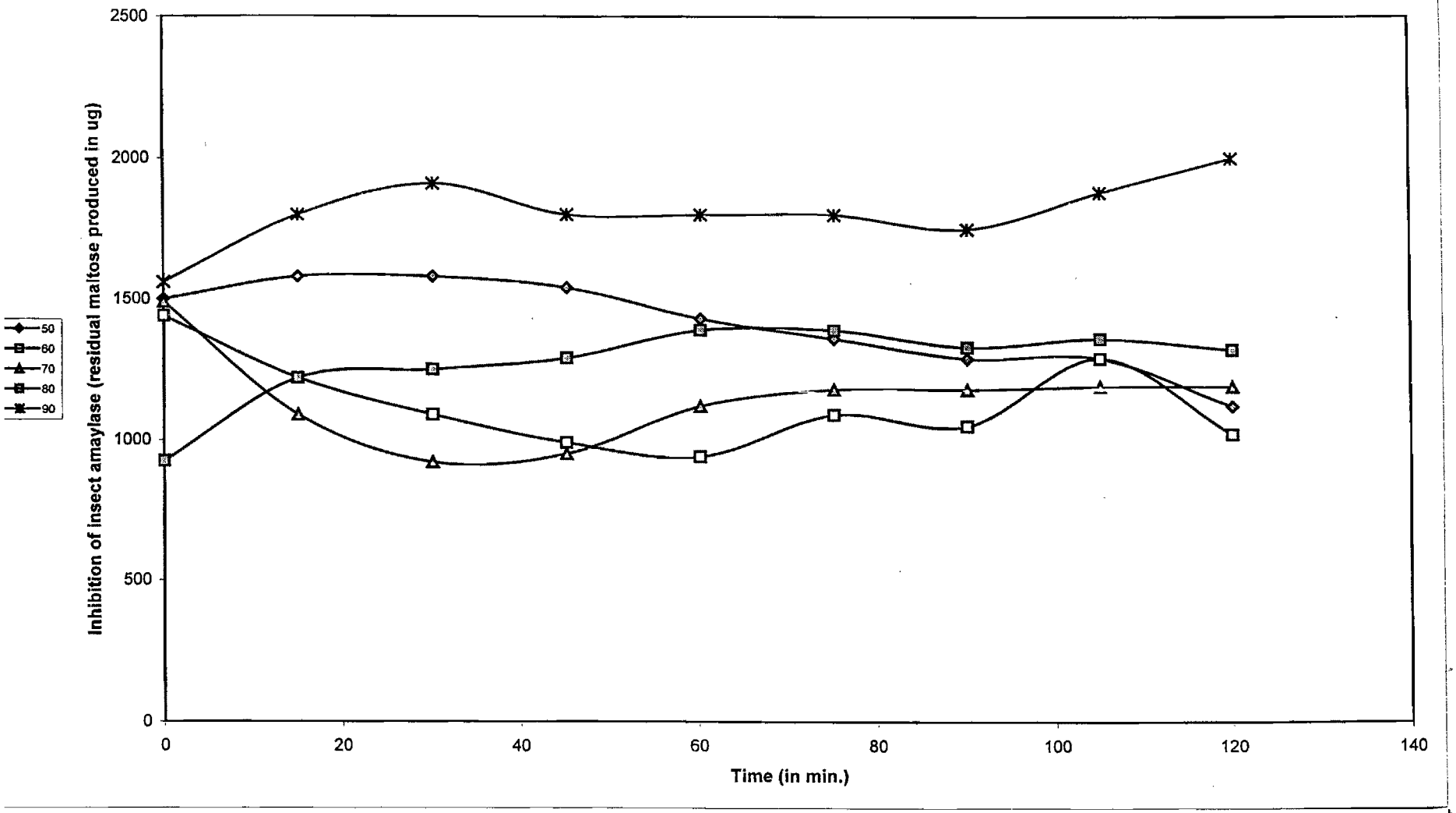
4.3.1a Inhibitory activity in aqueous extracts fraction heated at different temperatures

Aqueous extracts was subjected to heat treatment at different temperatures for various time intervals and these different fractions

were tested for their amylase inhibitory activity against insect amylase. When 50°C treated aqueous extract used to inhibit insect amylase, initially upto 45 min. heating, no inhibitory activity was observed instead their was increase in the reducing sugars which might be due to the endogenous amylase activity. After 60 min of heating at 50°C the aqueous extract exhibited the insect amylase inhibitory activity and there was an increase in the inhibitory activity after heating upto 2 hrs at 50°C (Fig.4.6). Heating at 60°C gave the inhibitory activity within the 15 min. of heating and their was gradual increase in the inhibitory activity upto 60 min of heating, but after the 60 min. of heating at 60°C there is increase in the reducing sugars in the reaction mixture. When aqueous extract was heated at 70°C, the inhibition of insect amylase was observed after 15 min of heating, and the aqueous extract exhibited inhibitory activity upto 30 min heating at 70°C. After 45 min of heating again there was increase in reducing sugars in the reaction mixture (Fig. 4.6). Heating at the 80°C and 90°C the aqueous extract did not show any inhibitory activity even after only 15 min of heating instead there was always increase in the reducing sugars when fractions heated at 80°C and 90°C were used in the reaction. Heating at 90°C gave more reducing sugars in the reaction then heating at 80°C (Fig. 4.6).

Hence, it was found that the heating between 60-70°C for 15-30 min gave the maximum inhibitory activity.

FIG.4.6 INHIBITOR ACTIVITY IN AQUES EXTRACT AFTER HEATING AT DIFFERENT TEMPERATURE FOR DIFFERENT TIME PERIODS



4.3.1b Effect of temperature on endogenous amylase

Since heating at 60-70°C gave the maximum inhibitory activity, so aqueous extract was heated at 60°C, 65°C and 70°C for various time intervals i.e. 15 min, 30 min and 45 min. In these heat treated fractions the endogenous amylase activity and insect amylase inhibitory activity were checked.

For the endogenous amylase activity, heating at 60°C, 65°C and 70°C upto 30 min gave the decrease in amylase activity (Fig. 4.7). Heating at 65°C for 30 min gave the maximum inactivation of endogenous amylase activity. Heating at 60°C and 70°C upto 30 min also inactivated the endogenous amylase which was lesser in degree in comparison to 65°C heating for 30 min (Fig. 4.7). 70°C heating for 30 min also gave the sufficient inactivation of endogenous amylase. So the insect amylase inhibition activity by the fractions heated at 65°C and 70°C for 15 min and 30 min were also checked.

The aqueous extract fractions had shown insect amylase inhibition at after heating at 65°C and 70°C. Heating for 30 min had shown more insect amylase inhibition then 15 min. of heating at both the temperatures (Fig. 4.8). The inhibitor activity was slightly higher in the fraction heated at 70°C, then the fraction heated at 65°C.

Since, heating at 65°C for 30 min gave the minimum endogenous amylase activity and high insect amylase inhibitory

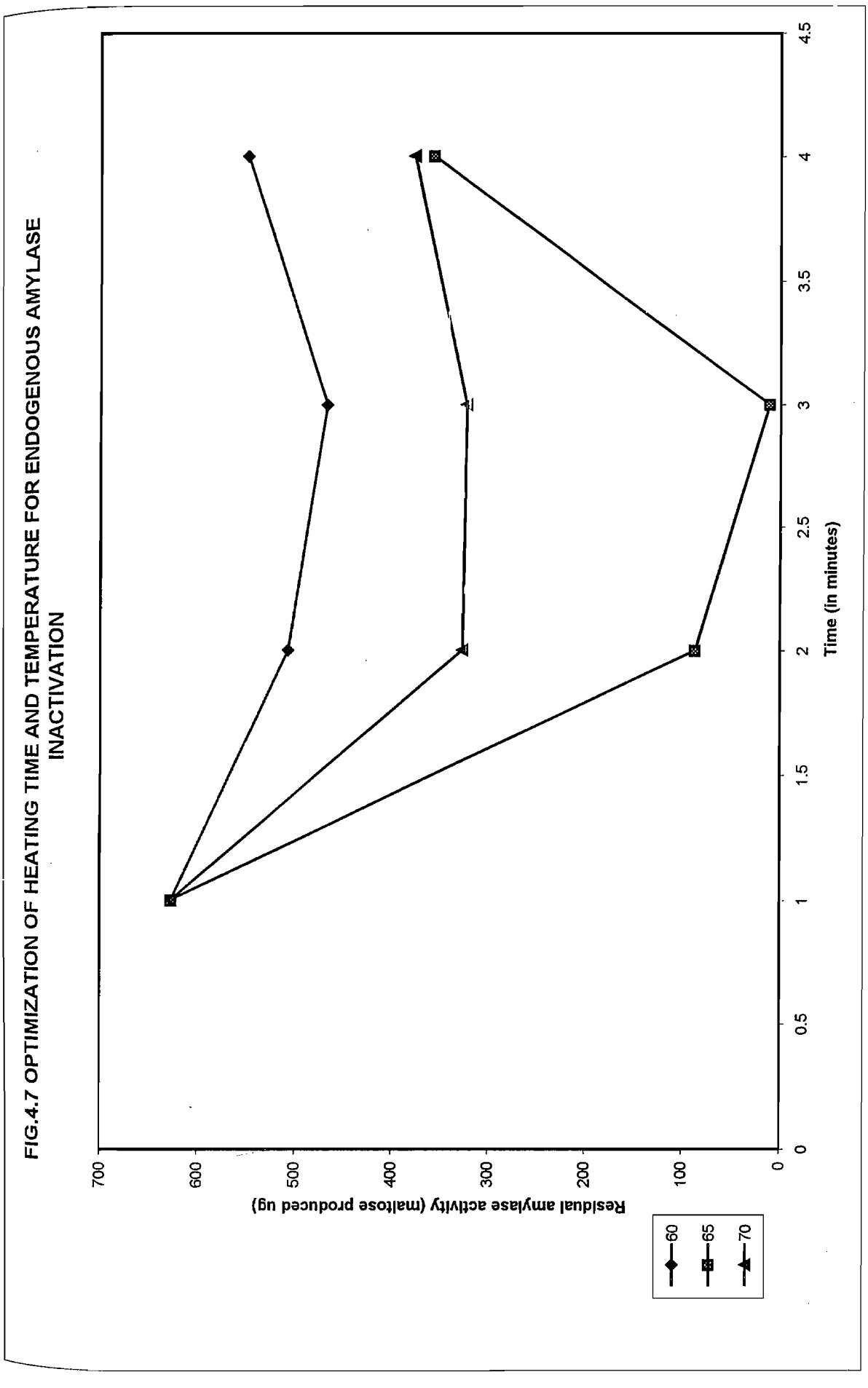
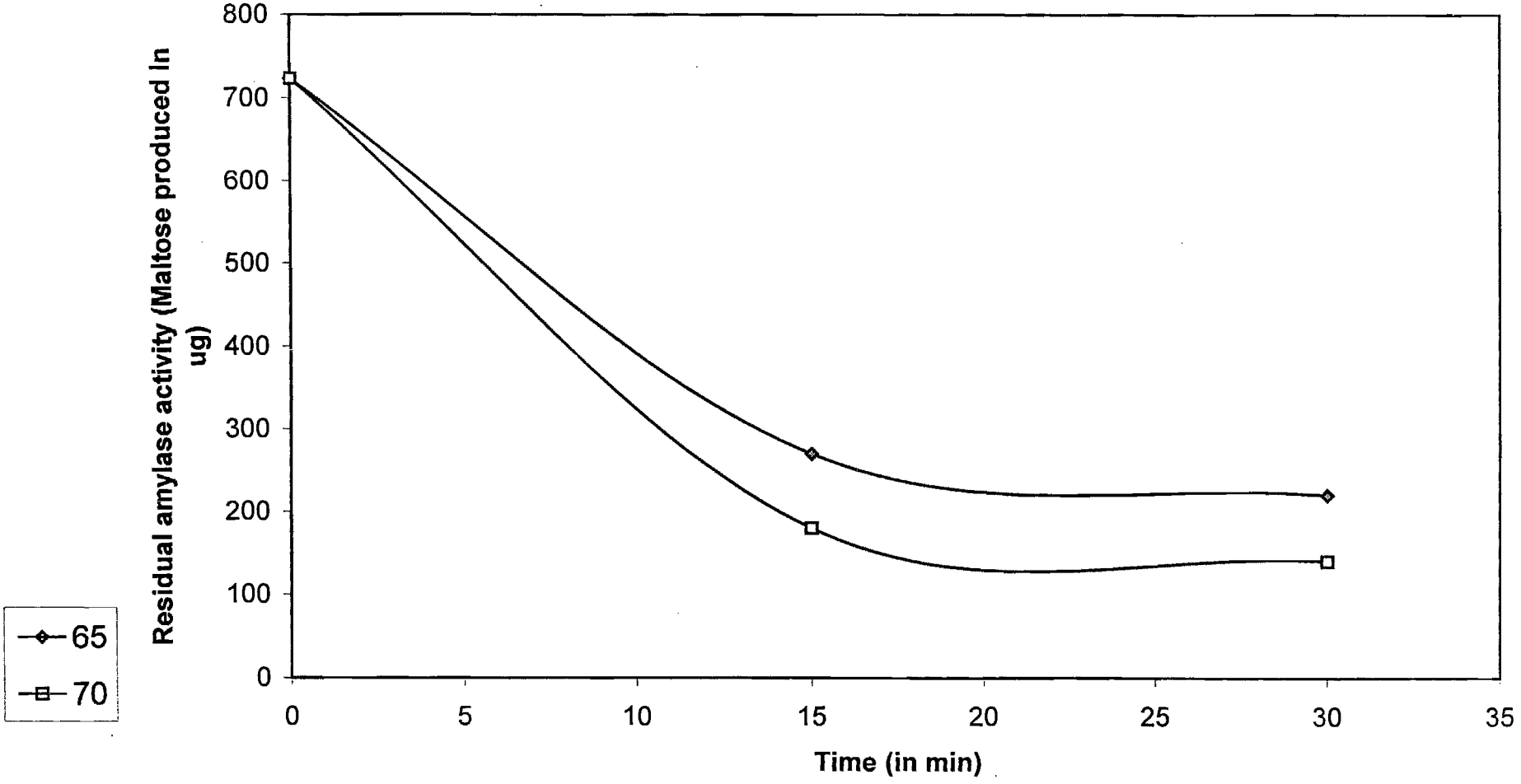


FIG.4.7 OPTIMIZATION OF HEATING TIME AND TEMPERATURE FOR ENDOGENOUS AMYLASE INACTIVATION

FIG.4.8 INSECT AMYLASE INHIBITORY ACTIVITY IN HEAT TREATED AQUEOUS EXTRACTS



activity this temperature and time duration was considered optimum for wheat amylase inhibitor activation.

4.3.2 Effect of pH

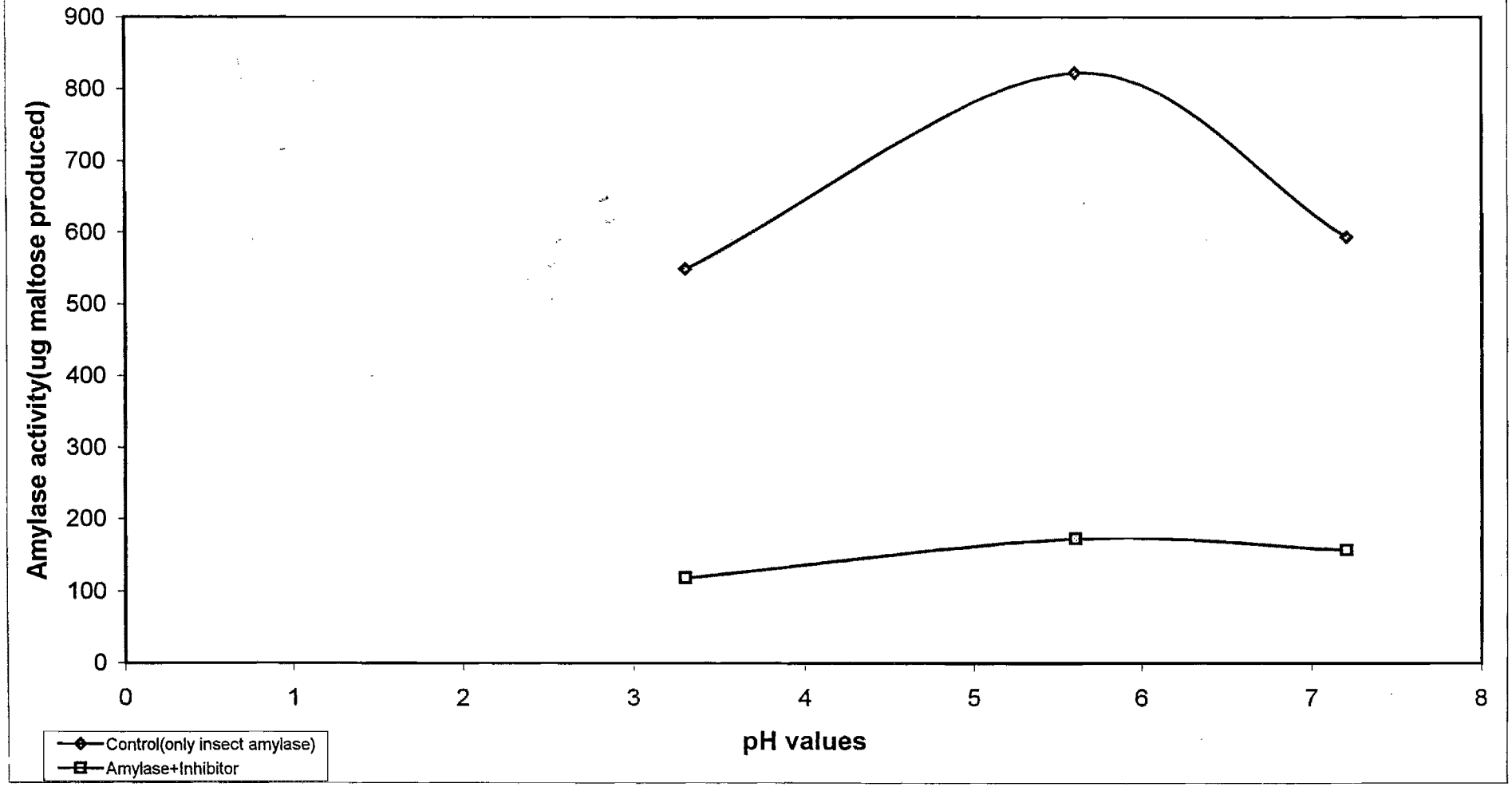
4.3.2a Effect of pH on insect *S. oryzae* amylase

For the isolation of insect amylase 0.5 M acetate buffer pH 5.4 was used because it has been earlier reported that the insect amylases were optimally active between the range of 5-6 pH **(Appelubaum *et al.*, 1961).** So in the present study activity of insect *S. oryzae* amylase was checked at different pH i.e. 3.3, 5.4 and 7.2. Activity of insect amylase at these pH were also the control to check the inhibitory activity of wheat amylase inhibitor at these pH, against the insect amylase. It was found that insect amylase was active at all three pH values, but the pH 5.4 (acetate buffer) showed highest activity among all pH values and it was also evident that with increase or decrease in pH lead to reduction in the insect amylase activity (Fig. 4.9). So the optimum pH for insect *S. oryzae* amylase was 5.4.

4.3.2b Effect of pH on wheat amylase inhibitory activity

Effect on pH on partially purified inhibitory protein(s) were also checked against the insect amylase. Inhibitor was active at all the three pH values i.e. 3.3 (Citerate buffer), 5.4 (acetate buffer) and 7.2 (Phosphat buffer). However, the maximum inhibition of insect amylase was observed at pH 5.4 (Fig. 4.9) in comparison to insect amylase activity. Since inhibition at all pH values leads to production of

FIG.4.9 EFFECT OF pH ON INSECT AMYLASE ACTIVITY AND WHAET AMYLASE INHIBITOR ACTIVITY



almost same amount of reducing sugar so it clearly demonstrated that inhibitor protein(s) were active at a broad range of pH values (Fig. 4.9).

4.3.2c Effect of pH on endogenous amylase activity

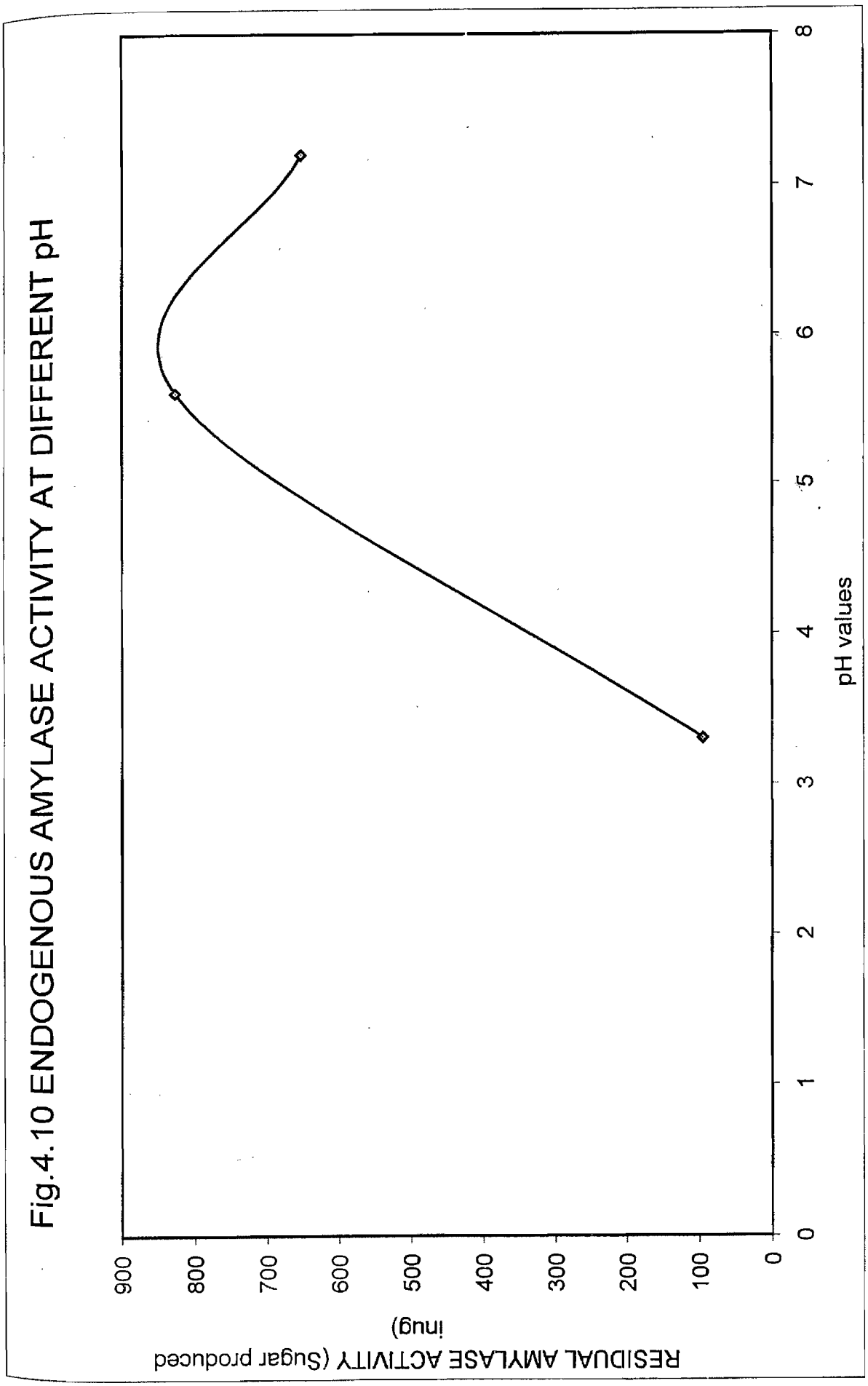
Effect of pH on endogenous amylase which was present in aqueous extract, was also studied. Heating treatment did not fully inactivate the endogenous amylase activity. (Table 4.5). The endogenous amylase was active at pH 5.4 but the maximum activity was at pH 5.4. But at the pH 3.3 endogenous amylase activity was almost negligible (Fig. 4.10).

The wheat amylase inhibitor and insect amylase both were active at all the three pH (i.e. 3.3, 5.4 and 7.2) but the maximum inhibitory activity against amylase was observed at pH 5.4 (Fig 4.9). But at pH 3.3 the inhibitory activity of wheat amylase inhibitor against insect amylase was also high and the endogenous amylase was almost inactive (Fig. 4.9 & 4.10). Hence the wheat amylase inhibitor protein(s) were stored at pH 3.3 and insect amylase was stored at pH 5.4.

4.3.3 Effect of time of incubation

When the insect amylase and the partially purified inhibitor was preincubated at 30°C, the inhibition was observed within the 15 min of incubation but the maximum degree of inhibition was observed after 30 min of incubation (Fig. 4.11). Although inhibition of amylase by the wheat amylase inhibitor has shown gradual increase upto 24

Fig.4.10 ENDOGENOUS AMYLASE ACTIVITY AT DIFFERENT pH



hrs of incubation but the increase in degree of inhibition was very less, so the maximum degree of inhibition was attained within 30 min (Fig 4.11). After 30 min. the inhibition degree was very slow.

4.3.4 Effect of time on insect amylase – amylase inhibitor complex

When insect amylase and insect amylase-inhibitor complex were incubated at 30°C for 24 hrs, the insect amylase was active even after 24 hrs and the amylase inhibitor complex was also active after 24 hrs of incubation at 30°C. Initially up to 15 min no inhibition was observed but within 30 min the insect amylase was inhibited. The degree of inhibition in enzyme- inhibitor complex was almost similar after 24 hrs. in comparison to the insect amylase activity (Fig. 4.12).

4.3.5 Type of inhibition

With the increasing concentration of substrate and the inhibitor protein the V_{max} remain unchanged and the Km values were increased in Linevever –Burk plot between reciprocal curve of reaction velocity and substrate concentration (Fig.4.13), hence showed the competitive inhibition where substrate and the inhibitor were competiting for the same site or very closely situated reaction site on enzyme.

4.4 CHROMATOGRAPHIC PURIFICATION OF INHIBITOR PROTEIN(S) FROM UP2425

4.4.1 Gel Filtration Chromatography on Sephacryl S-200

Gel filtration by Sephacryl S-200 has exclusion limit of 5 to 250 kDa. Hence, it offers an advantage to screen out small protein

Fig.4.11 EFFECT OF INCUBATION TIME ON INHIBITORY ACTIVITY OF WHEAT AMYLASE INHIBITORY PROTEIN ON INSECT AMYLASE

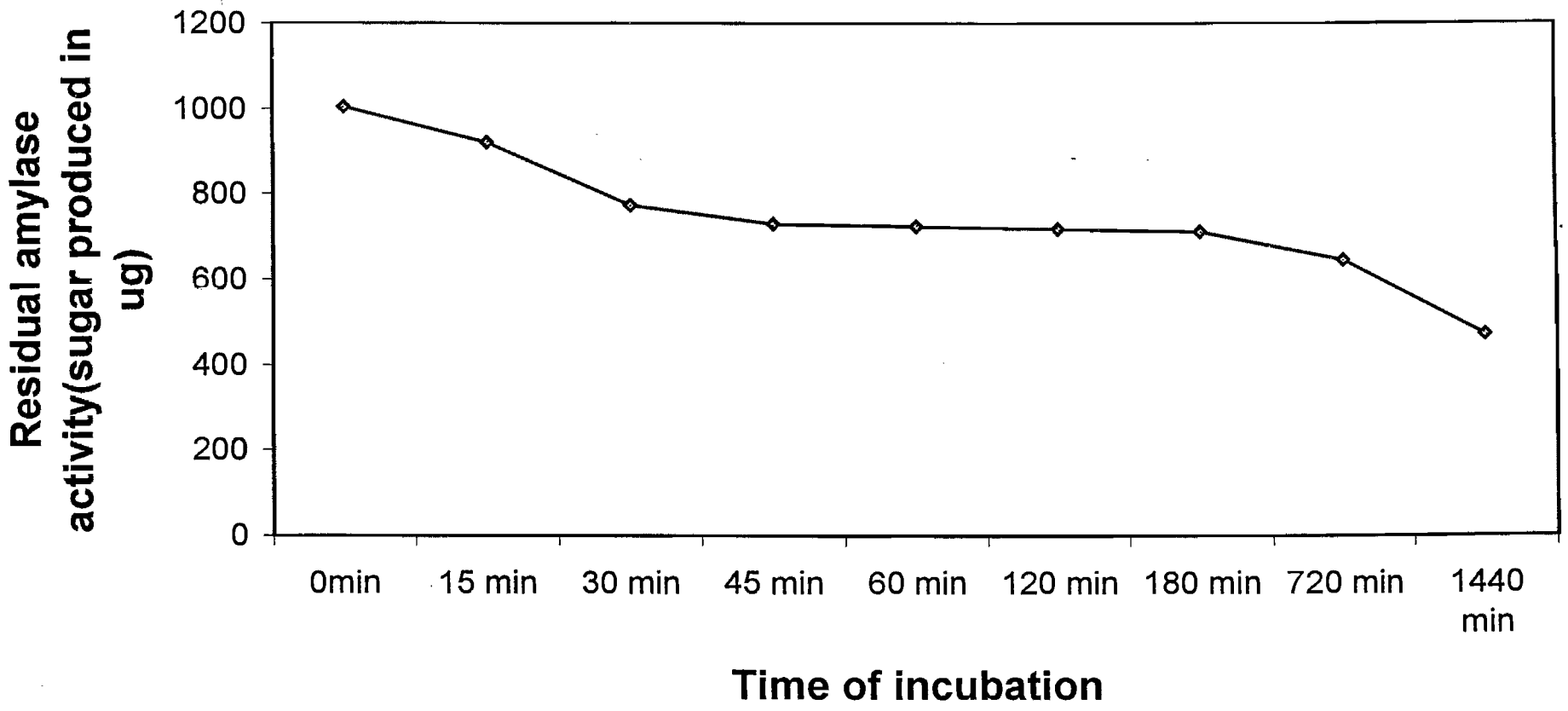
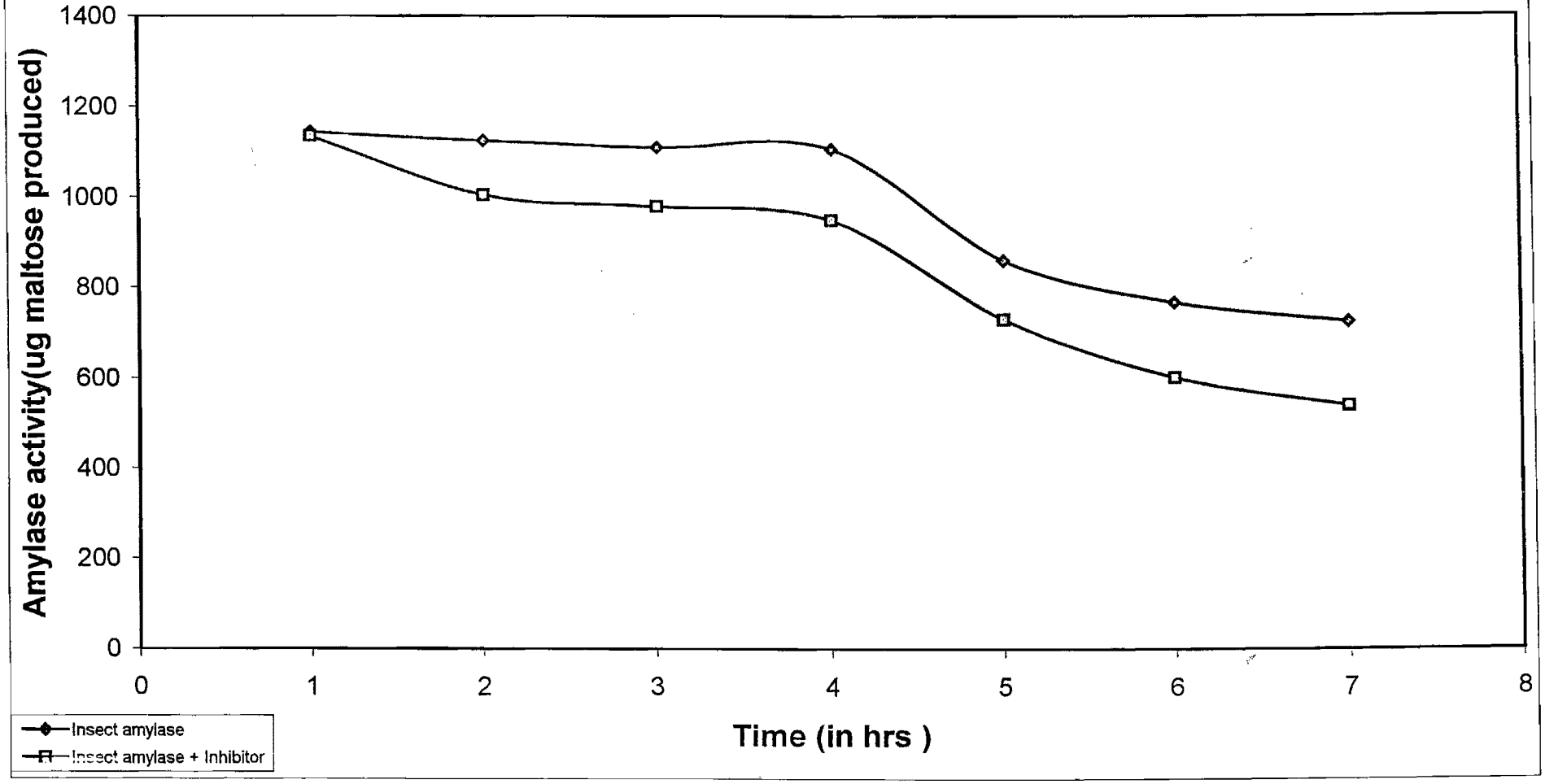


FIG.4.12 EFFECT OF INCUBATION TIME ON INSECT AMYLASE AND AMYLASE+INHIBITOR COMPLEX



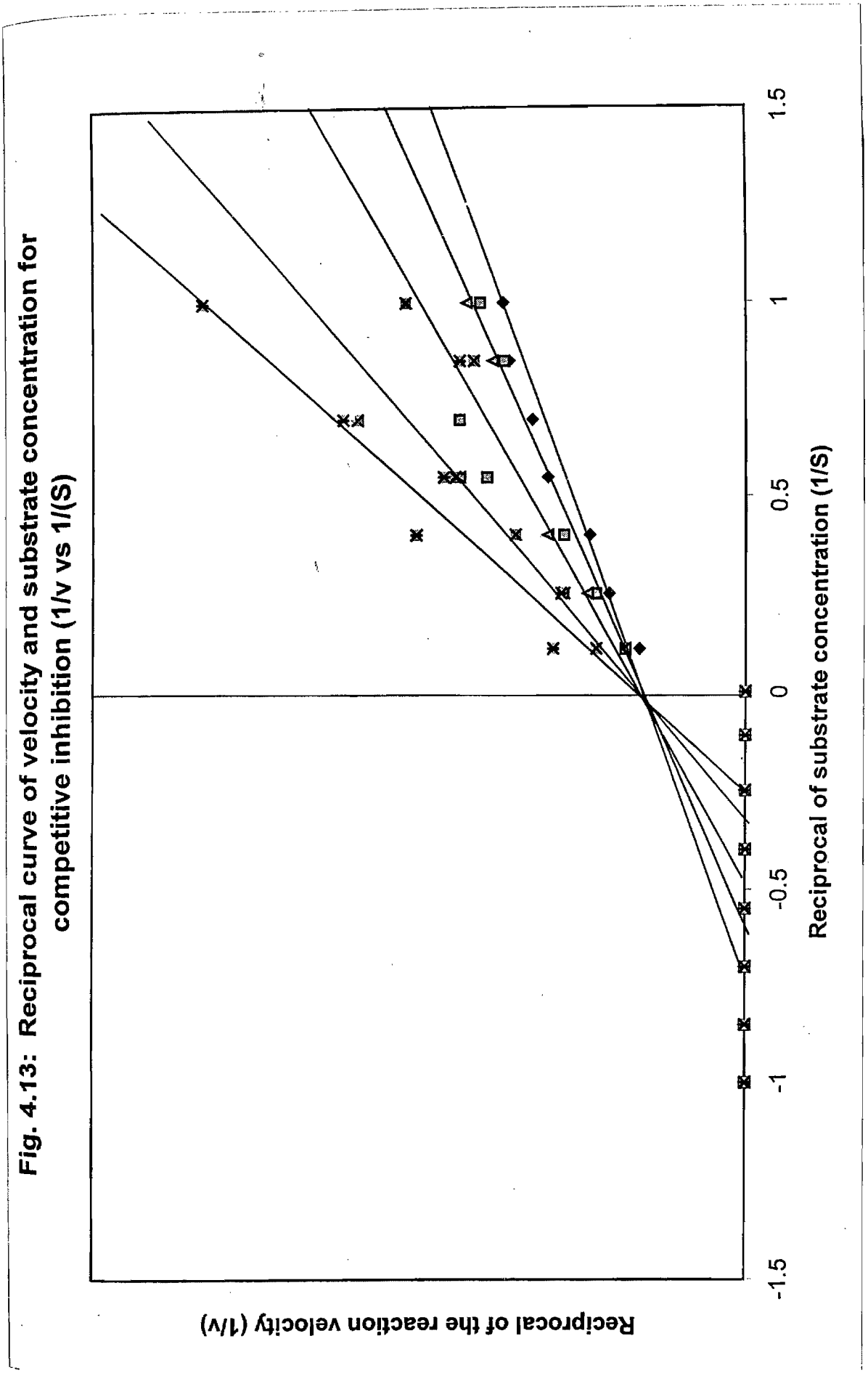


Fig. 4.13: Reciprocal curve of velocity and substrate concentration for competitive inhibition (1/v vs 1/(S)

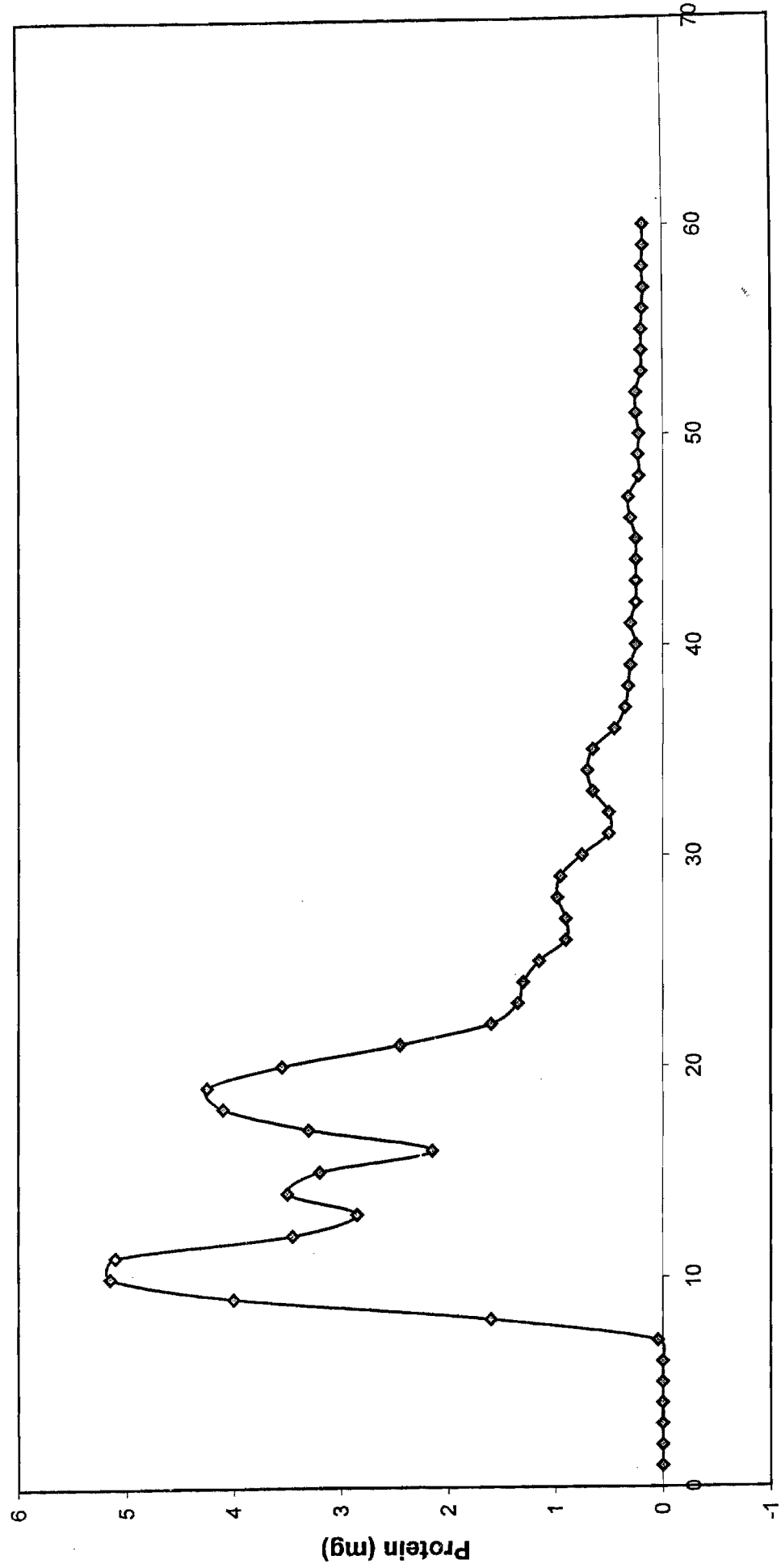
fractions having biological activities. Protein obtained after ammonium sulfate fractionation was first concentrated by lyophilization and then subjected to purification by gel filtration on Sephacryl S-200 column. 50 fractions of 5 ml each were collected. Protein content by Bradford method and inhibitory activity in each fraction were determined (Fig. 4.14 and 4.15). With the protein content of each fraction and the inhibitory activity against the insect amylase, the specific inhibitory activity of each fractior was calculated (Fig. 4.16) and found in fractions 21-39. Only one broad peak from fraction 26-32 was pooled and lyophilized. The molecular weight of this peak ranges between 43-44 kDa approximately (Fig. 3.5 of Calibration curve of Sephacryl S-200 column).

The pooled, lyophilized protein fraction had protein content 0.1951 mg/ml, inhibitory activity 0.7602 units/ml, specific inhibitory activity 5.009 unit/mg protein. After gel filtration the fold purification was 6.46 and the recovery was 2.56% of total protein (Table 4.7).

4.4.2 Gel Filtration Chromatography on Sephadex G-25

Purified and concentrated inhibitor protein was passed through Sephadex G-25 and eluted with distilled water, to remove the ammonium acetate molecule which were used for the elution from Sephacryl S-200 column, because during subsequent concentration of pooled peak area the concentration of ammonium acetate could also increased.

FIG.4.14 PROTEIN CONTENT OF SEPHACRYL S-200 COLUMN FRACTIONS (UP 2425)



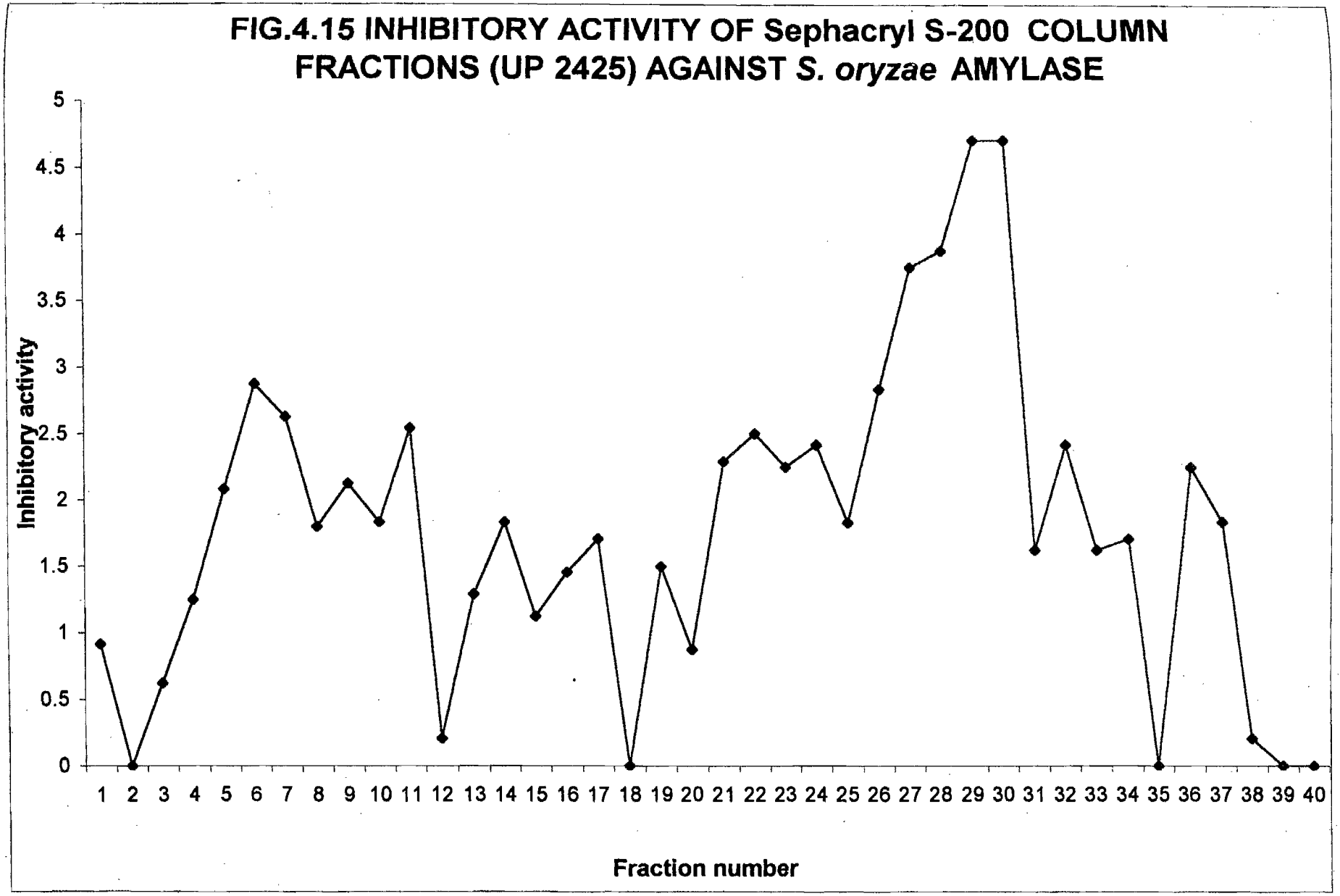


FIG.4.15 INHIBITORY ACTIVITY OF Sephacryl S-200 COLUMN FRACTIONS (UP 2425) AGAINST S. oryzae AMYLASE
Inhibitory activity
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Fraction number

FIG.4.16 SPECIFIC INHIBITORY ACTIVITY OF SEPHACRYL S-200 FRACTIONS (UP 2425) AGAINST *S. oryzae* AMYLASE

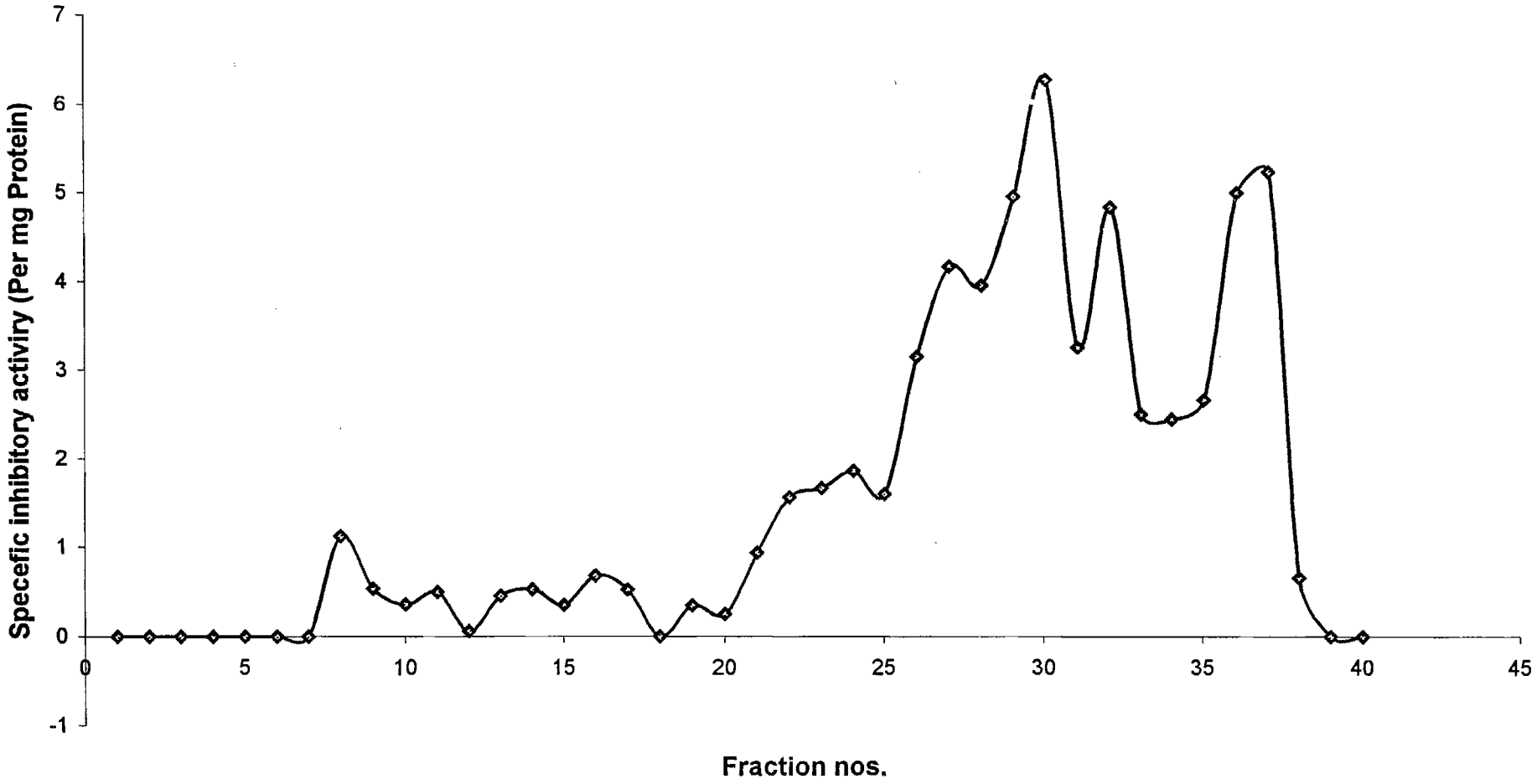


Table 4.7: Purification chart of inhibitory protein(s) from UP 2425.

Purification Step	Volume (ml.)	Protein (mg/ml)	Total Protein (mg)	Inhibitory Activity (units/ ml)	Total Inhibitory Activity (Units)	Sp. Inhibitory activity (Units/mg/protein)	% Yield	Fold Purification
Aqueous Extract	100	3.5	350	-	-	-	-	-
Heat Treated Aqueous Extract (65^0C, 30 min)	98	3.22	315.56	2.495	244.51	0.775	90.16	1
Ammonium Sulfate fraction (30-90%) after dialysis	10	26.732	267.32	24.95	249.5	0.933	84.78	1.20
Gel Filteration (Sephacryl S-200)	35	0.1951	6.83	0.9774	34.21	5.009	2.56	6.46
Ion-exchange (DEAE-Sephadex A-50)	10	0.00309	0.0309	0.2945	2.945	95.31	0.34	122.92

4.4.3 Ion exchange Chromatography on DEAE Sephadex A-50

The lyophilized protein obtained after gel filtration were applied on DEAE Sephadex A-50 (cut off limit 200 kDa) column. Seventy fractions of 2 ml each were collected using a linear NaCl gradient (60 mM-150 mM) in 100 mM ammonium acetate (pH 6.8) buffer. Absorbance at 280 nm for protein content (Fig.4.17) and specific inhibitory activity for each fraction were monitored (Fig. 4.18). There were several minor peaks and one major peak was observed for the specific inhibitory activity at fraction 37-41 (Fig. 4.18). Hence these fraction were pooled and lyophilized as well as assessed for protein content (22 µg/ml), inhibitory activity (0.2945 units/ml), specific inhibitory activity (95.31 units/ mg protein). After ion exchange chromatography the fold purification was 122.95 but percent recovery was very low (0.34% of total protein) (Table 4.7).

4.5 Subunit Characterization of Purified Amylase Inhibitor by Native and SDS-PAGE.

Most biological polymers are electrically charged hence shows differential mobility in an electrical field. A useful way to characterize macromolecule in by their rate of movement in the electric field. This property can be used to determined proteins molecular weight. Both native PAGE and SDS-PAGE were performed to characterize the purified inhibitory protein. In native PAGE the separation of proteins in their natural state affected by charge as well as molecular size. Hence it gives information about the different types of proteins present

FIG.4.17 PROTEIN CONTENT OF DEAE-SEPHADEX A 50 COLOUMN FRACTIONS OF PURIFIED INHIBITORY PROTEIN (UP 2425)

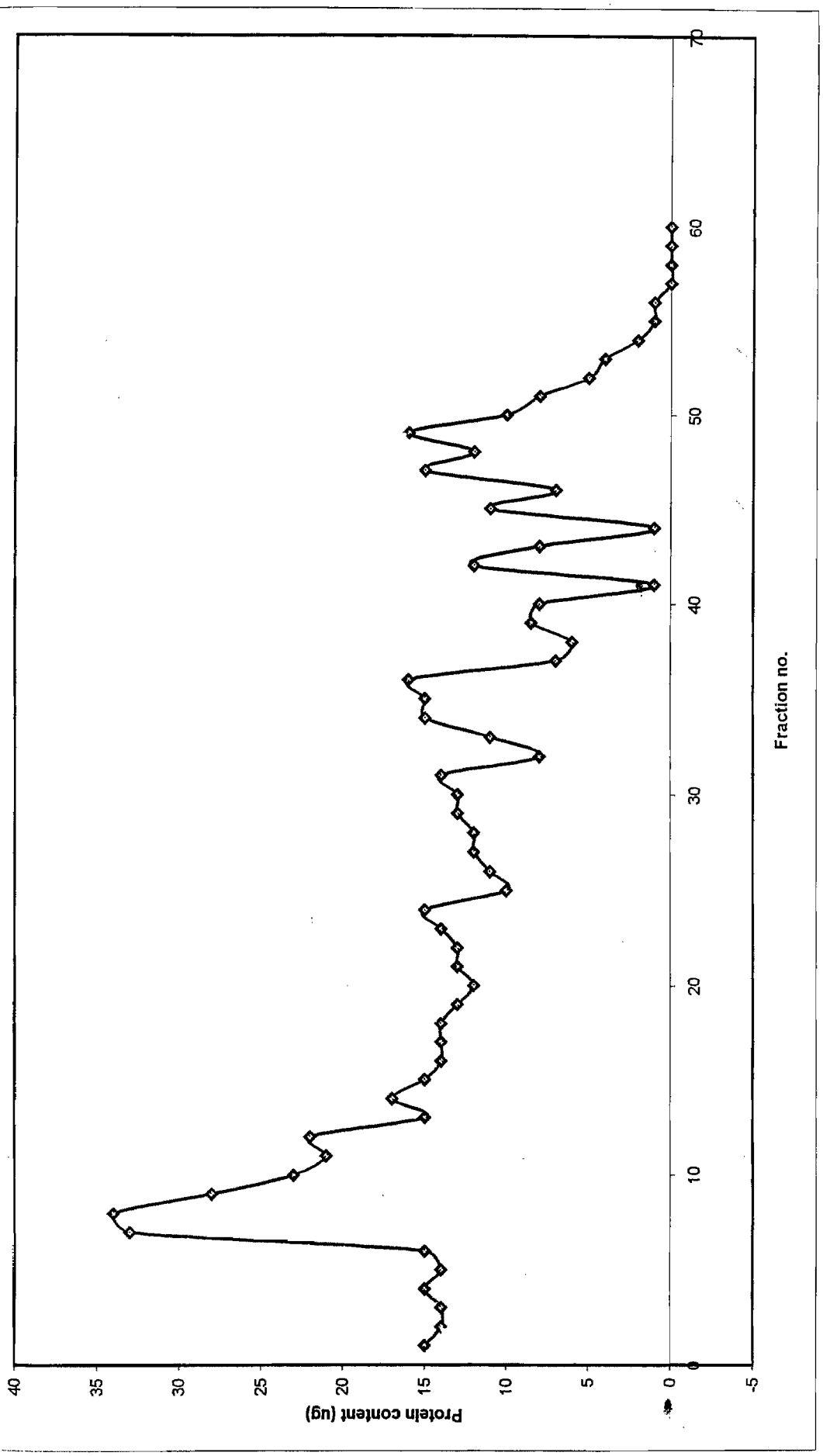
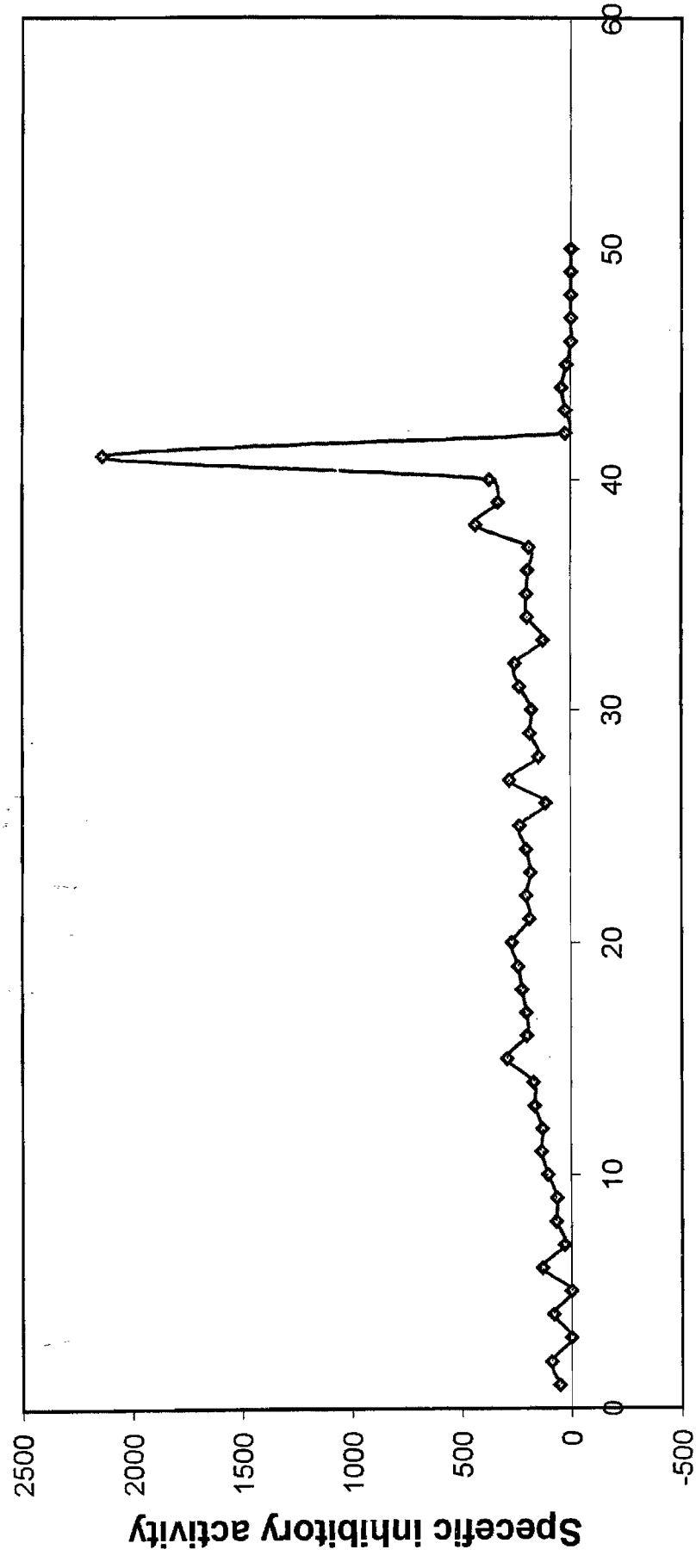


Fig.4.18 SPECEFIC INHIBITORY ACTIVITY OF (DEAE-Sephadex A 50 coloumn) FRACTIONS OF PURIFIED UP 2425



in the sample. While SDS interacts with protein in such a mannor that it imparts a net negative charge. Moreover, SDS also dissociates protein-protein and protein-lipid interaction, SDS-PAGE gives an idea of a polypeptide profiling of a protein sample.

Native PAGE was done at 10% gel of water extract of all varieties (Photo plate 2). Partially purified (Photo plate 3) and purified inhibitory (Photo plate 4) protein form the wheat variety UP2425 was also electrophoresed at 10% Native gel. One thick major band was observed in purified inhibitor and one faint band. Thick band was supposed to be for amylase inhibitor protein and faint band due to some impurity.

SDS-PAGE was also performed at 10% gel after denaturation of crude inhibitory protein (Photo plate 5) and purified inhibitor protein (Photo plate 6). Purified inhibitor also showed only major thick band with Rm value (corresponding molecular weight ~ 44.0 kDa) was observed (Photo plate 6). Rf value of marker band was 0.327 and purified protein had of value 0.349.

Therefore, the subunit characterization of purified inhibitor protein, exhibited the monomeric nature of inhibitor protein with an molecular weight of ~ 44 kDa in their natural conformation.

4.6 FEEDING TRIALS

Target insects, *S. oryzae* is F_1 generation was fed on the wheat seeds of UP2425, coated with 20 µg, 50 µg, and 100 µg partially purified inhibitor protein along with a control in a triplicates. Damage caused by these insects was measured in terms of insect growth,

PHOTO PLATE 4.2: NATIVE-PAGE OF CRUDE INHIBITORY PROTEIN OF DIFFERENT WHEAT VARIETIES

Lane 1: Sonalika

Lane 2: UP 115

Lane 3: UP 262

Lane 4 &5: NONE

Lane 6: UP 319

Lane 7: UP 1109

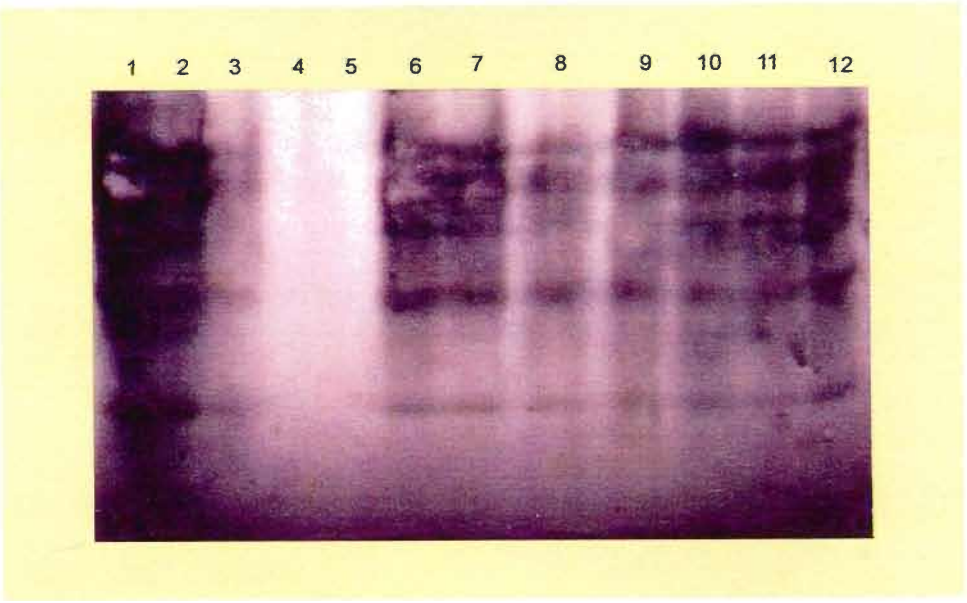
Lane 8: UP 2003

Lane 9: UP 2113

Lane 10: UP 2338

Lane 11: UP 2382

Lane 12: UP 2425



Phot Plate : 4.2 : Native PAGE of crude inhibitory proteins of different wheat varieties.

PHOTO PLATE 4.3: NATIVE PAGE OF PARTIALLY PURIFIED INHIBITORY PROTEIN OF UP 2425 WHEAT VARIETY.

PHOTO PLATE 4.4: NATIVE PAGE OF PURIFIED INHIBITORY PROTEIN OF UP 2425 WHEAT VARIETY.

Lane 1,2 & 3: After gel filtration chromatography

Lane 4& 5 : After ion-exchange chromatography

Lane 6 : none

Lane 7 : Partially purified inhibitory protein

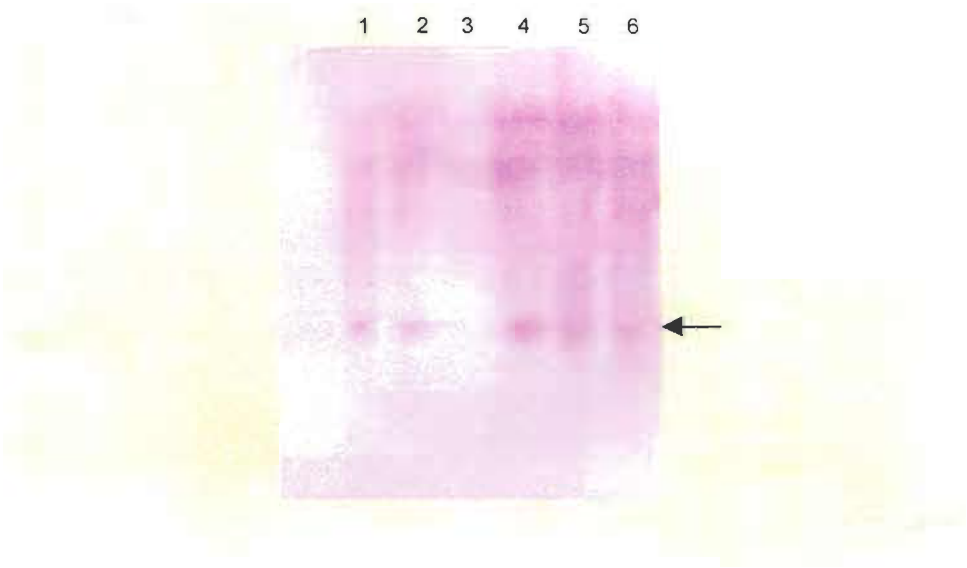


Photo Plate: 4.3 : Native PAGE of parially purified inhibitory proteins of UP-2425 wheat variety

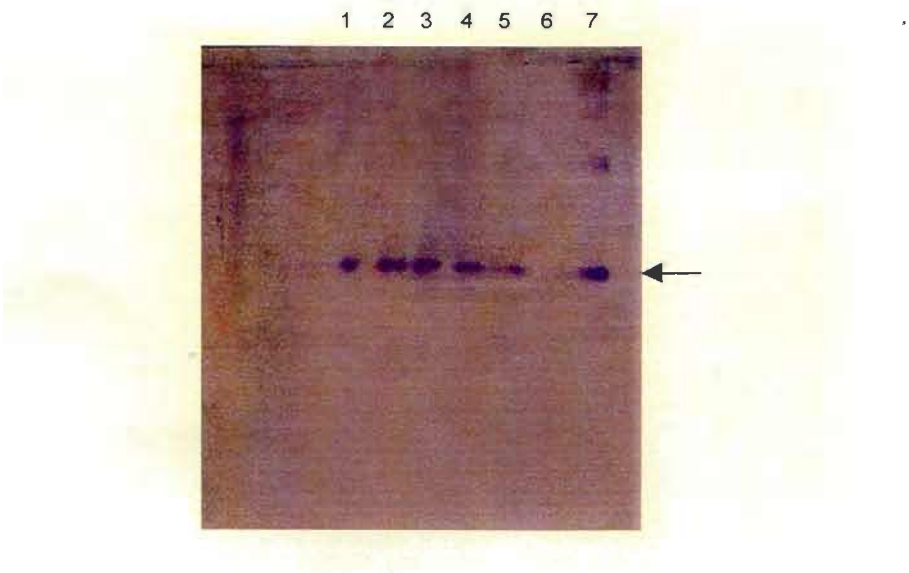


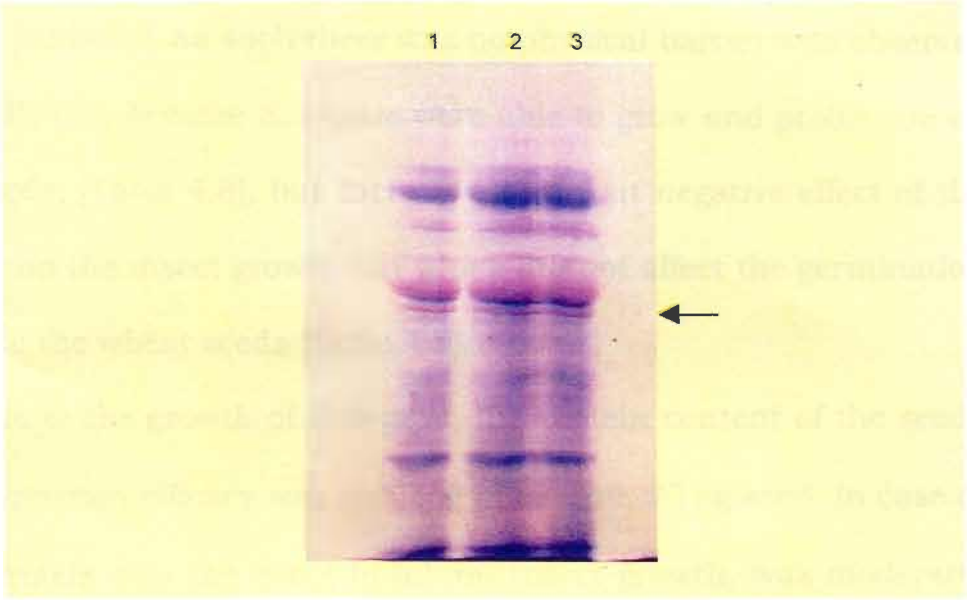
Photo Plate :4.4 : Native PAGE of purified inhibitory proteins of UP-2425 wheat variety.

PHOTOPLATE 4.5: SDS-PAGE OF PARTIALLY PURIFIED INHIBITORY PROTEINS OF UP 2425 WHEAT VARIETY

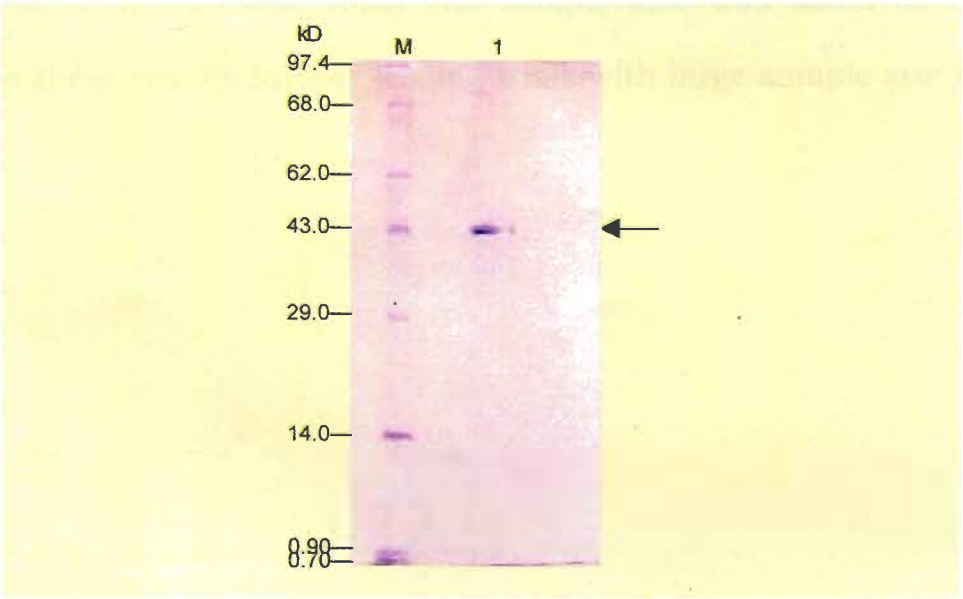
PHOTOPLATE 4.6: SDS-PAGE OF PURIFIED INHIBITORY PROTEIN OF UP 2425 WHEAT VARIETY

Lane M : Protein marker

Lane 1 : Purified inhibitory protein



Phot Plate : 4.5 : SDS-PAGE of parially purified inhibitory proteins of UP-2425 wheat variety.



Phot Plate : 4.6 : SDS-PAGE of purified inhibitory proteins of UP-2425 wheat variety.

protein content and the germination efficacy of seeds treated with amylase inhibitor. As such there was no physical barrier was observed in the UP2425, became *S. oryzae* were able to grow and proliferate on these seeds, (Table 4.8), but there is a clear cut negative effect of the inhibitor on the insect growth but which did not affect the germination efficacy of the wheat seeds (Table 4.8).

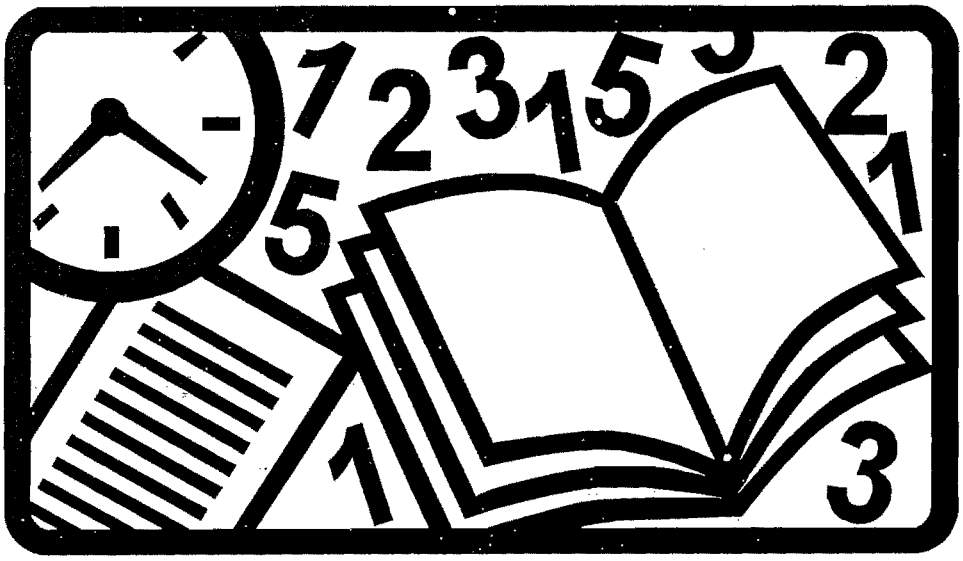
Due to the growth of *S. oryzae*, the protein content of the seeds and germination efficacy was reduced in control. 20 μg seed. In case of coated protein was the effect to inhibit insect growth, was moderate, however, 50 μg/seed and 100 μg/seed was very much effective as there is no insect growth, and insect could not feed on them. (Table 4.8). Furthermore, there was no decrease in germination efficacy.

Since in the present study the sample size was small so to confirmed these results further feeding trials with large sample size is required.

Table 4.8: Effect of purified inhibitor protein on insect growth using the protein coated seed.

Treatment on wheat seeds	Initial			After 60 Days			
	Insects (F_1 generation)	Protein Content (µg/ seed)	Germination (% efficacy)	Insect		Protein Content (µg/ seed)	Germination (% efficacy)
				Live	Dead		
Control	5	301.67	100	12	8	154.67	0
A. 20 µg Protein per seed	5	318.33	100	2	6	304.67	80
B. 50 µg protein per seed	5	355.67	100	-	5	354.33	100
C. 100 µg protein per seed	5	405.67	100	-	5	406.67	100

Chapter 5



DISCUSSION

Chapter 5 DISCUSSION

There is continuing need to increase food production, particularly in the developing countries in Asia, Africa and Latin America. This increase has to come from increased yields of major crops grown on existing cultivable lands. One practical means of achieving greater yields is to minimize the pest associated losses, which are estimated at 14% of the total agricultural production. The losses are far more significant in food crops, e.g. 52% in wheat, 83% in rice, 59% in maize, 74% in potato, 58% in soybean and 84% in cotton **(Sharma *et al.*, 2000)**. In addition to direct losses caused by insects, there are additional costs in the form of pesticides applied for pest control, which not only affect the non-target organisms, but also leaves harmful residues in the food, and results in environmental pollution. Also, despite integrated pest management strategies combining the use of chemicals, resistant germplasm and the modification of planting, harvesting and handling practices, yield losses due to insects have actually increased slightly for most crops over the last two decades **(Duck and Evalva, 1997)**.

With the advent of genetic transformation and recombinant DNA techniques, it has become possible to clone and introduce insect resistant genes into the plant genome that confer resistance to insects. The technology allows the extension of the "gene pool" available to a particular crop species, and thus engineered inherent

resistance to pests based on resistance genes from other plant species, or on resistance genes from species in other kingdom or even on entirely novel resistance genes, becomes possible. Pesticide usage can be decreased, with concomitant economic benefits and benefits to the environment.

Thus, genes encoding endogenous plant defensive proteins are most useful for enhancing the resistance of crops to insect pests. Interfering with digestion, and thus affecting the nutritional status of the insect, is a strategy widely employed by plants to defend themselves against pests. A major inhibition of digestion is the presence of protein-aqueous inhibitors of digestive enzymes (both amylases and proteinases) in the plant tissues. These proteins interact with digestive enzymes, bind tightly to the active site and prevent access of the normal substrates **(Garcia-Olmedo *et al.*, 1987)**. The inhibition of digestive enzymes not only has a direct effect on the insect's nutritional status, but is also thought to lead to secondary effects where oversynthesis of digestive enzymes occurs as a feedback mechanism in an attempt to utilize ingested food. If the insect cannot overcome the inhibition of digestion, death by starvation occurs. Inhibitors of digestive enzymes are normally accumulated in storage tissues both in seeds and vegetative storage tissues. Direct evidence for a defensive role of protein inhibitors of digestive enzymes is shown by the induced synthesis of serine proteinase inhibitors that occur

when many plant species are wounded **(Ryan, 1984)**, such induction can be caused by insect feeding, or mimicked by mechanical damage. Other evidence suggests that insect feeding may lead to more rapid accumulation of inhibitors than simple wounding, providing further evidence of their defensive role against insect predators **(Korth and Dixon, 1997)**.

Inhibitors of α-amylase from both higher animals and insects are widespread in plants, and are accumulated in similar tissues as proteinase inhibitors and are reported as bifunctional protein **(Odani *et al.*, 1983 and Nesterenke *et al.*, 1987)**. The amylase inhibitors have significant insecticidal activity towards phytophagous coleopterans, particularly pests of stored seeds, as α-amylase inhibitors purified from wheat and *P. vulgaris* have been shown to be insecticidal to coleopteran species when tested in artificial diet **(Gatehouse *et al.*, 1986** and **Ishimoto and Kitamura, 1988)**.

The function of α-amylase inhibitors is to inhibit α-amylase. Alpha-amylase inhibitor from wheat inhibits a wide range of amylases i.e. insect amylases and mammalian pancreatic α-amylase. Alpha-amylase catalyses the cleavage of α (1-4) glucosidic bonds found in starch and glycogen. The α-amylase inhibitor is found in the kernel of the wheat plant. There are two classes of α-amylase inhibitors, endogenous and exogenous. Endogenous alpha amylase inhibitors inhibit wheat alpha amylase (AI) and take part in germination, while

the exogenous AI inhibits alpha amylase of insects/ animals, and is under consideration for present study.

Pueyo *et al.* (1995) reported that adults of *S. oryzae* when fed on a diet containing wheat inhibitor exhibited more mortality than those fed on the control diet. Wheat amylase inhibitor along with cystein proteinase inhibitor in the diet had reduction in the growth rate of the insects. Plants have responded to insect attacks by depositing often copious amounts of amylase inhibitors in their seeds **(Feng *et al.*, 1996; LeBerre *et al.*, 1997 and Giri and Kachole, 1998)**. Bruchid beetles, common stored product pests, feed on the seeds of a limited number of leguminous plants and are especially susceptible to the action of amylase inhibitor **(Reis *et al.*, 1997)**. When expressed at high levels in transgenic peas, α-amylase inhibitor (AI was capable of completely blocking weevil larval development on seeds and neutralized the effects of weevil damage on overall seed yield **(Schroeder *et al.*, 1995)**. Hence, the α-amylase inhibitor proteins and gene(s) encoding them from wheat have the potential for use in the genetic transformation against the coleopteraon insect i.e. *Sitophilus oryzae* storage pest. Although several amylase inhibitor gene constructs have been introduced into different transgenic crops **(Schuler *et al.*, 1998)**, deployment of amylase inhibitors for insect control requires a detailed analysis of the particular amylase inhibitor-insect amylase interaction. Hence the present study was undertaken

to study the insect, *S. oryzae* amylase and wheat amylase inhibitor interaction. The amylase inhibitor protein from the wheat having maximum potent inhibitor against insect amylase was purified and characterized for its kinetic and subunit composition, for the future study of the gene(s) encoding the inhibitor protein for the genetic transformation.

Seeds of 10 varieties of wheat (*Triticum aestivum* L.) were screened for α-amylase inhibitors, in the heat treated water extracts of the seeds. Wheat seed extracts were prepared in water because amylase inhibitors are albumins **(Pace *et al.*, 1975** and **Silano *et al.*, 1975).** Although these inhibitors are also soluble in NaCl **(Limas *et al.*, 1990)** the salt extracted inhibitors show lesser degree of inhibition of insect amylase in comparison to water extract inhibitor (data not reported). Screening was done on starch agar plate by cutting wells and putting in wells a mixture of porcine pancreatic α-amylase and proteinaceous inhibitors. After incubation, iodine solution was poured in the plate to look for intensity of blue colour of the starch and the developed uncoloured zones surrounding the wells due to the activity of porcine pancreatic amylase with and without the wheat amylase inhibitor (Plate 4.1). On the starch-agar plate, the zone of hydrolysis varied depending on the concentration of the inhibitors. As the inhibitor concentration was increased, the zone of hydrolysis decreased, because of the porcine pancreatic amylase activity was

interrupted or inhibited due to presence of proteinaceous inhibitor. Only the heat-treated inhibitor exhibited inhibition of porcine pancreatic amylase (Table 4.1) perhaps due to the fact that heat treatment inactivated endogenous amylase activity in the inhibitor preparation.

Although on the starch-agar plates, slight differences in the starch hydrolysis zones were observed, but these were too less apparent to distinguish varieties for higher or lower amylase inhibitor activity. Thus α-amylase inhibitor activity was quantitatively estimated in the heat-treated water extracts of all 10 varieties against the amylases from different sources i.e. germinating wheat seeds amylase, porcine pancreatic amylase and target insect *S. oryzae* amylase. There was no inhibition of germinating wheat amylase by any wheat varieties (Table 4.2). There are several other reports which state that there is no inhibition of α-amylase by wheat amylase inhibitor preparations **(Silano *et al.*, 1975; Bunocore *et al.*, 1977 and Pace *et al.*, 1973)**. The porcine pancreatic amylase was inhibited by heat-treated water extracts of all 10 varieties (Table 4.3). Percent inhibition of porcine pancreatic amylase varied from 24 to 77 percent (Fig. 41.), inhibitory activity units/gm seed varied from 0.294 to 4.284 (Table 4.3, Fig. 4.2) and specific inhibitory activity/mg protein varied from 2.53 to 24.43 (Table 4.3, Fig. 4.2). Percent inhibition shown by different varieties did not water to I.A. units/gm seed and sp. I.A./mg

protein, however, the latter parameter showed similar trend. Activity against porcine pancreatic amylase expressed as inhibitory activity/gm seeds and specific inhibitory activity/mg protein was maximum in UP 2113, i.e. 4.284 and 24.43 respectively, while the minimum activity was in Sonalika (Table 4.3, Fig 4.2). Heat treated extracts of all 10 varieties also inhibited the amylase extracted from *Sitophilus oryzae* (a common stored grain pest of wheat and rice) (Table 4.4). Percent inhibition ranged between 25 to 60 percent (Table 4.4, Fig 4.3), inhibitory activity/gm seed varied from 0.278 to 0.754, and the specific inhibitory activity/ mg protein varied from 0.585-4.762 (Table 4.4, Fig. 4.4). Inhibitory activity/gm seed and specific inhibitory activity were maximum for UP2425, i.e. 0.754 and 4.762 respectively, while in UP 115 these were minimum i.e. 0.278 and 0.585 respectively (Table 4.4, Fig. 4.4).

Quantitative assay for inhibitory activity revealed that these proteinaceous inhibitor preparations were effective against the insect/ animal amylase but not against endogenous amylase. Hence, it may be concluded that these protein inhibitors provide a measure of insect resistance to wheat kernel, and do not regulate the endogenous amylase activity. The pattern of inhibition of amylases from different sources by wheat seed is similar to earlier reports **(Silano *et al.*, 1975 and Bunocare *et al.*, 1977). Silano *et al.* (1975)** have reported that the amylases susceptible to wheat amylase inhibitors were found

mainly in insects those attack wheat and stored wheat products, partially susceptible amylases were found in mammals, while the resistant amylases were largely distributed in cereals as well as in insect species that do not attack wheat grain or wheat product.

The inhibitor preparations from wheat variety UP 2425 had the maximum inhibitory activity/gm seed and specific inhibitory activity/mg protein, against the *S. oryzae* amylase, so this variety was used for further isolation, purification and characterization of the wheat amylase inhibitor protein(s).

Heating of the aqueous inhibitor preparation was a pre-requisite, as in this preparation the endogenous α- and β-amylases were also present in high concentration. As it had been experimented earlier that the endogenous amylases were not inhibited by the wheat amylase inhibitor (Table 4.2), so the aqueous extract was subjected to heat treatment to inactivate the endogenous amylases.

Heat treated extract from variety UP 2425 was subjected to further purification by salting out (30-90% ammonium sulphate saturation, gel chromatography on Sephacryl S-200 and DEAE-sephadex A-50 ion exchange chromatography.

For inactivation of endogenous amylase present in the aqueous extract, it was subjected to heat treatment at different temperature for 30 minutes. Heating at 65°C for 30 min was found to be optimum (Table 4.5). After heat treatment clear supernatant was subjected to

ammonium sulphate fractionation for salting out of the proteins. Initially upto 30% ammonium sulphate, only supernatant exhibited inhibitory activity. A gradual increase in ammonium sulphate concentration at intervals of 10% saturation beyond 30% saturation, revealed that at 90% saturation amylase inhibitory activity was confined to pellets only (Table 4.6). Hence, the heat-treated extract was first subjected to 30% ammonium sulphate saturation and then to 90% saturation to precipitate the all amylase inhibitor proteins. The pellets thus obtained were washed with 2 M NaBr to remove access carbohydrate moieties and dialyzed. The specific inhibitory activity after the ammonium sulphate precipitation was 0.933/mg protein, with 84.78% recovery and a 1.20 fold purification was achieved.

To understand the behaviour of the amylase inhibitor protein(s) kinetic characterization was done after the partial purification of the inhibitor proteins precipitated after 30-90% ammonium sulphate precipitation and dialysis. The kinetic parameters studied were the effect of temperature, pH, time of pre-incubation for inhibition, stability of enzyme-inhibitor complex and the type of the inhibition shown by the purified inhibitor.

Heating of aqueous extract was to inactivate endogenous amylase, but the heat stability of the amylase inhibitor proteins were also tested, by heating aqueous extract for different time intervals at various temperature (Fig. 4.6). It was evident that after heating at

50°C, inhibitory activity was observed after 45 minutes of heating, while at 60°C and 70°C, heating for 15 minutes was sufficient for inhibitory activity against insect amylase. But the aqueous extract heated at 80°C and 90°C did not show any inhibitory activity (Fig. 4.6). With these findings it can be concluded that heating at 60-70°C for 15-30 min is sufficient.

Endogenous amylase activity in the fractions heated at 60°C, 65°C and 70°C for 15 min, 30 min and 45 minutes were checked (Fig. 4.7). The minimum endogenous amylase activity was present at 65°C for 30 min. and 70°C heating for 30 min. also had second highest amylase inhibitory activity, but at all temperature concentration of reducing sugar was increased after 45 minutes of heating (Fig.4.7). This increase in reducing sugar in aqueous extract might be due to hydrolysis of starch present in aqueous extract.

The inhibitory activity against insect amylase due to these heat treated fractions was found maximum in the fraction heated at 70°C for 30°C. Fractions heated at 65°C for 30 min also had very high inhibitory activity (Fig. 4.8).

For the heat treatment, thus we selected 65°C heating for 30 min, become at this temperature, endogenous amylase activity was almost nil (Fig. 4.8, Table 4.5) and the inhibitory activity of insect amylase was also very high.

Optimum pH for insect amylase has been reported to range between pH 5-6 **(Applebaum *et al.*, 1961)**. Hence the inhibitor activity was checked at pH 3.3, 5.4 and 7.2 (Fig. 4.9). The data revealed that the insect amylase and wheat amylase inhibitors activity were highest at pH 5.4. At pH 3.3 and 7.2, insect amylase activity decreased relatively to a greater extent than the inhibitor activity. Further the wheat seed amylase activity decreased drastically at pH 3.3 (Fig. 4.10). Therefore, after salting out and subsequent purification steps the inhibitor proteins were suspended and stored at pH 3.3. **Wagenaor and Lugteuburg (1972)** have also reported that endogenous α- and β-amylase are inactivated at pH 3.3 and by heating at 70°C for 20 min.

Although various workers have suggested that the inhibitor and substrate are incubated for 30 min before adding amylase, effect of incubation time was studied to confirm it in the present investigation. An incubation for 30 min. was found to be optimum (Fig. 4.11). The insect amylase wheat amylase inhibitor complex was comparatively more stable than insect amylase alone (Fig. 4.2). The complex was active when stored at 30°C for 24 hrs. So it can be concluded that the maximum inhibition of insect amylase by the wheat amylase inhibitor is achieved at pH 3.3, after 30 minutes of pre-incubation at 30°C.

The wheat amylase inhibitor from seeds of variety UP 2425 was found to behave as a competitive inhibitor (Fig. 4.13). Our findings are in comformity to earlier reports **(Garcia-Olmedo *et al.*, 1987)**.

For further purification of wheat amylase inhibitor after 30-90% precipitation and dialysis, the protein fraction was subjected to gel filtration by Sephacryl S-200. Sephacryl S-200 has an exclusion limit of S-250 kDa, hence it offers an advantage to screen out a wide range of proteins. In wheat variety UP 2425, total 60 fractions of 5 ml each were collected and checked for the inhibitory activity against insect amylase and the protein content in each fraction (Fig.4.). Although the major protein fraction was present in fractions 8-22, the inhibitory activity was present in fractions 8-38, with maximum specific inhibitory activity in fractions 21-39 (120-195 ml of eluate). Hence, the ascending region i.e. fraction 26 to 32 (130-160 ml of the eluate) were taken for further purification. This peak area had 5.009 specific inhibitory activity/mg protein and it was 6.46 fold purified. The specific inhibitory activity in fraction 33-39 (165-195 ml) was also high, but this fraction was a shoulder to the peak present on the descending side of the main peak. Hence, it was not considered, while pooling the fractions. Fractions showing maximum specific inhibitory activity (fractions 26-32) had a very low protein content. This indicated that the inhibitor protein in very less amount was effective to inhibit the insect amylase. This finding also suggested that the

potent amylase inhibitor protein was present in wheat seed in very low concentration, in comparison to the total protein of the wheat seed, and this low concentration of the wheat amylase inhibitor was able to inhibit the amylase of storage pest insect, *Sitophilus oryzae.* On the comparison of these fractions containing inhibitory protein, with the column calibration curve (Fig. 3.9), it was found that the molecular weight of these fractions was approximately 43 kDa. So, the fraction no 26-32 were pooled, concentrated by lyophilization and used for ion-exchange chromatography on DEAE-Sephadex A-50. Before loading on the ion-exchange column, this concentrated fraction was passed through Sephadex G-25 column, using autoclaved distilled water as eluting buffer, because for the gel-filtration from sephacryl S-200, 100 mM Ammonium acetate buffer was used that would have further increased upon lyophilization. The elute from sephadex G-25 column was again pooled and lyophilized and then used for ion-exchange chromatography.

Ion-exchange chromatography relies on the interaction between oppositely charged particles. Many biological materials e.g. amino acids, proteins have ionizable group and they may carry a net charge, so the fractionation by this technique exploits differences in net charge between the biomolecules. From the ion-exchange chromatography DEAE-sephadex A-50 was used in the present study which was a positively charged gel type ion-exchanger; which has

combined separation properties of gel filtration with those of ion exchange. Due to their high degree of substitution of charged groups, which results from their porous structure, these gels have a high loading capacity, but were easily compressed thereby greatly reducing the flow. Total 60 fractions of 2 ml each were collected and tested for amylase inhibitory activity and protein content. Upon ion exchange all the fractions collected showed amylase inhibitory activity. However, maximum specific inhibitory activity against *S. oryzae* amylase was found in fractions 37-41 (74-82 ml). Hence, those fractions with high specific inhibitory activity against insect amylase (fraction 74-82 ml) were pooled.

Ion-exchange fractions 37-41 (74-82 ml) had very high specific inhibitory activity, 95.31 units per mg protein and this peak with highest specific inhibitory activity were pooled and lyophilized for further study. Fold purification after the ion-exchange chromatography was 122.92 fold with 0.34% yield (Table 4.7).

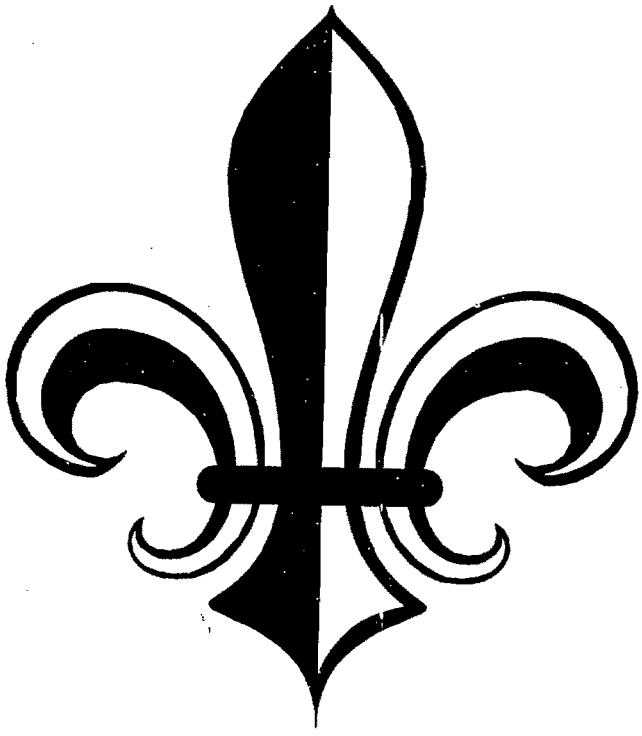
For subunit characterization, native and SDS-PAGE were performed and it was found that in both cases a single thick band was present (Photoplate 4.4 and 4.6). On SDS-PAGE, this thick band corresponded to the 43.6 kDa marker band. Gel filtration also revealed an about 43 kDa molecular weight for the inhibitor protein (Fig.3.9). **Le-Berre Anton *et al.* (1997)** above reported a monomeric inhibitor protein of 43 kDa from kidney beans. Although in the previous report the inhibitor proteins are reported monomeric dimeric

and tetrameric in nature. The monomeric subunit is reported 12-14 kDa **(Silano *et al.*, 1993)** but **Garcia-Maroto *et al.* (1991)** reported monomeric wheat protein which has the molecular weight as reported in our study.

Pueyo *et al.* (1995) reported the retarded growth of *S. oryzae* fed on a diet containing wheat inhibitory. Similarly when the *S. oryzae* were fed on seeds coated with wheat amylase inhibitor protein solution, they had retarded growth (Table 4.8). No damage was caused to seeds when coated with higher concentrations of inhibitor (50 µg or more protein per /seed) and the F1 generation of insects had 100% mortality and no further increase in number (Table 4.8). However at lower concentration (20 µg/ seed) showed some marginal insect infestation (Table 4.8). Seeds coated with higher amount of proteinaeceous inhibitor retained their germinability and total protein content but loss of germination and decrease in protein content of seeds were observed in uncoated seeds (Table 4.8). **Girish *et al.* (1975)** have reported that the stored grains pest population lead to the decreased protein content and germination efficacy.

Since, the inhibitory protein had the potent activity against the target storage pest, *Sitaphilus oryzae* amylase both as *in vivo* and *in vitro* and beside this, the low optimum pH for alpha-amylase inhibitory activity, inhibitor containing diets such as transgenic crops expressing high concentration of alpha-amylase inhibitor should be devoid of any harmful effect on human health and will be effective against the storage pests.

Chapter 6



SUMMARY AND CONCLUSION

Chapter 6 SUMMARY AND CONCLUSION

Since the wide scale mechanization of agriculture, and the revolution in plant breeding that has length high yielding crop varieties. Unfortunately, as has been all too clearly shown in both the developed and undeveloped worlds, the price for achieving maximum production can be too high, with irreversible depletion or destruction of the natural environment making certain agricultural practices unsustainable in the longer term. One of these practices is the indiscriminate use of pesticides to combat insect and other pests. Non specific pesticides are harmful to non-target organisms, which would normally act to keep the pest population in check. They are toxic to beneficial insects, that act as predators or parasites to the pest species and have a harmful effect on higher animals that also act as predators for crop pests. Many pesticides are also toxic to humans. Further, to clearly demonstrate that over reliance on pesticides in non sustainable, many insect pests have become resistant to pesticides.

In this context, the emergence of technologies that have allowed plants to be stably transformed with foreign genes has been timely, and the genetic engineering of crops for insect resistance has now been adopted. Considerable progress has been made over the past decade in genetic engineering of crop plants for providing resistance to insects. The ideal transgenic technology should be commercially feasible, environmentally benign (biodegradable), and easy to use in

diverse agro ecosystems. It should also harmless to the natural enemies of damaging insect, target the sites in insects in insects that have developed resistance to the conventional pesticides, flexible enough to allow ready deployment of alternative (in case of development of resistance in pest), and preferably produce acute rather than chronic effects on the target insect. Transgenic crops satisfy many of these requirements. Whereas the strategy of employing genes encoding Bt toxins to produce insect resistant transgenic plants has its origins in established practices with conventional insecticides, where an exogenous compound is used to protect the host plant, a number of other strategies for protecting crops from insect pests take as their starting point the endogenous resistance shown by plants to most insect predators. Most plants survive attack by most potential insect predators and as a result of selection pressure, have evolved many strategies of endogenous resistance. As well as physical defences and ecological strategies such as dispersal and growth habits, plants make extensive use of biochemical defences, based primarily on a rich and varied secondary metabolism **(Harborne, 1988)**, but also on the use of defensive proteins. Genes encoding endogenous plant defensive proteins were thus obvious candidates per enhancing the resistance of crops to insect pests.

Interfering with digestion, and thus affecting the nutritional status of the insect, is a strategy widely employed by plants to defend

themselves against pests. A major factor in inhibition of digestion is the presence of protein inhibitors of digestive enzymes, both amylases and proteinaceous, in the plant tissues. Cereals contain α-amylase inhibitors, which can actively inhibit the activity of amylase.

The present study was undertaken to screen wheat varieties for their α-amylase inhibitors activity and to purify the proteinaceous amylase inhibitor exhibiting maximum inhibition of target insect *S.oryzae* amylase, then kinetically and subunit characterization of this inhibitor and feeding trials of the purified inhibitor on the *S. oryzae*, the target insect.

(i) Out of the 10 wheat varieties, all the heated (65°C for 30 min) aqueous extracts tested positive for α-amylase inhibitors against the porcine pancreatic amylase qualitatively.

(ii) Wheat amylase inhibitors are not effective against the wheat amylase.

(iii) During the quantitative estimation of the porcine pancreatic amylase, it was found that percent inhibition of porcine pancreatic amylase ranges between 24-77% in all varieties. However, the specific inhibitory activity (units of α-amylase inhibited per mg of inhibitor proteins) ranged from 2.53-24.43, being highest in the UP 2113 and the same variety had maximum inhibitory activity/gm seed (4.284).

(iv) Insect *Sitophilus oryzae* amylase was also inhibited, upon quantitative estimation, percent inhibition was found to vary

from 25 to 60 percent in all varieties. However the specific inhibitory activity ranged from 0.585-4.762; being highest in UP 2425. The inhibitory activity per gm seed ranged from 0.278-0.754, and the maximum was in UP 2425.

Since the UP 2425, had max specific inhibitory activity against insect amylase, it was selected for further purification and characterization.

(v) The heat-treated seed extracts were treated with ammonium sulphate (30-90% saturation). The pellets obtained were dissolved and dialyzed against distilled water. The specific inhibitory activity after ammonium sulfate fractionation and dialysis was 0.933 units/mg protein resulting in 1.20 fold purification.

(vi) Aqueous extracts of wheat amylase inhibitor exhibited insect amylase inhibition after heating at 60 to 70°C for 15-30 min. Optimum temperature and time of heating to inactivate endogenous amylase was 65°C for 30 min. and at the same heating temperature and time the amylase inhibitor fraction also had high inhibitory activity against insect amylase.

(vii) Endogenous amylase was almost inactivated at pH 3.3, while the amylase inhibitor protein had the inhibitory activity against the insect *Sitophilus oryzae* amylase at the pH 3.3.

(viii) For the optimum inhibitory activity, amylase enzyme and wheat amylase inhibitor were pre-incubated 30 min at 30°C. The enzyme inhibitor complex was very stable, which has the activity upto 24 hrs.

(ix) The inhibitory protein shows the competitive inhibition, in the presence of varied substrate concentration.

(x) Protein obtained after ammonium sulphate fractionation were subjected to gel filtration on Sephacryl S-200. The fraction showing highest inhibitory activities were poled and concentrated. The specific activity was 5.009 units/mg protein and 6.46 fold purification was achieved. Percent yield was 2.56.

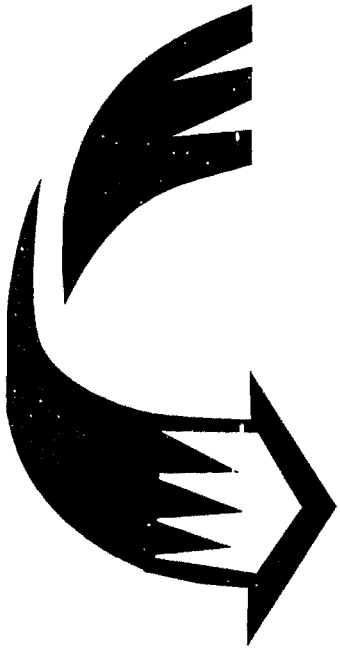
(xi) After gel filtration, the inhibitory protein was subjected to DEAE-sephadex. A-20 gel type ion exchange chromatography. Fractions showing highest inhibitory activity pooled and concentrated by lyophilization. The specific inhibitory activity was 95.31 /mg protein and 122.92 fold purification. But the yield percentage was very low, only 0.34%.

(xii) On native PAGE, the purified protein gave a single band on, while on the SDS-PAGE same single band of ~43 kDa was observed. The presence of single band on the SDS-PAGE conformed that this purified inhibitor protein was monomeric in nature.

(xiii) Insect feeding trials also showed that the inhibitor protein had negative effect on the insect proliferation.

Thus, the present study concludes that wheat is the one of the good source for amylase inhibitor, in which the UP 2425 variety is a potential sources having the highest specific inhibitory activity against target insect *S. oryzae* amylase. Although the fold purification was very high but the yield percentage was relatively very low. The electrophoretic pattern reveals that the purification was upto near homogenicity and the purified protein is monimeric in nature.

Owing to the low optimum pH found for alpha amylase inhibitor activity and their effectiveness against *S. oryzae* amylase, inhibitor gene containing transgenic plants expressing alpha amylase inhibitor should be devoid of any harmful effect on human health.



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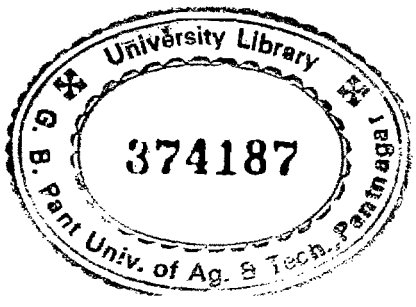

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APPENDIX

APPENDIX I

CHEMICALS/ REAGENTS

Name of Chemical	Name of Company
Acrylamide	Hi-Media
Agar	Hi-Media
Ammonium Per Sulphate	SRL
Ammonium Sulphate	Hi-Media
Bisacrylamide	Hi-Media
Bovine Serum Albumin	Spectrochem
Bromophenol Blue	SRL
Calcium Chloride	Qualigen
Coomassie Brilliant Blue R	Merck
Cupric Sulphate	Analytical Rasayan
DEAE-Sphadex A 50	Spectrochem
EDTA	Analytical Rasayan
Folin's ragent	SRL
Glacial Acetic Acid	SRL
Glycine	Hi-Media
Iodine Cystals	SRL
Maltose	Merck
Methoanol	Spectrochem
Potassium Iodide	SRL
Rochelles Salt	Merck
SDS	Qualigen
Sephacryl S 200	SRL
Silicone	Hi-Media
Sodium Azide	Spectrochem
Sodium Bicarbonate	Merck
Sodium Carbonate	Analytical Rasayan
Sodium Chloride	Merck
Sodium Hydroxide	Merck
Sodium Sulphate	Analytical Rasayan
β-mercaptoethanol	SRL
Starch	Hi-Media
TCA	SRL
TEMED	Spectrochem
Tris buffer	Spectrochem
Tris-Cl	Spectrochem

APPENDIX II

GLASSWARES AND INSTRUMENTS

Balance	Tech-Du
Deep Freezer	Blue Star
Digital Balance	Adair Dutt & Co.(P) Ltd., India
Electrical Balance	K-Roy, India
Electrophoresis apparatus	Biotech, Salem, India
Eppendorf Tubes, Centrifuge Tubes and Tips	Tarson, India
Glasswares	Borosil, India
Heating mantle	Sunvil, UK
Incubator	Tanco, India
Megnetic Stirrer	MAC
Micropipettes	Tarson, India
Oakridge Tubes	Tarson, India
Oven (Nucriwave)	BPL Sanyo, India
pH meter	Elico, India
Power pack	Biotech, Salem, India
Refrigerator	Zenith
Refrigerated Speed Centrifuge	Sarval RC-5C
UV-VIS Spectrophotometer	Elico, India
Water Purification System	Millipore, USA

APPENDIX III

PREPARATION OF STOCK SOLUTION

1. Acidic iodine solution

I_2	0.0125 g
KI	0.125 g
HCl	0.05 N

Dissolved in 180 ml. Make up the volume 250 ml and stored at $4^{o}C$.

2. Starch-agar gel

Starch	1g
Agar	0.7 g

Dissolved in 100 ml water. Used freshly prepared solution.

3. 1 M NaOH

NaOH crystals	40 g
Distilled water	90 ml

Make up the volume 100 ml. Stored at room temperature in plastic bottle.

4. 1M NaCl

NaCl	58.5 g
Distilled water	90 ml

Make the volume up to 100 ml. Stored at room temperature.

5. 20 mM Tris-Cl (pH 7.5)

Tris-Cl	3.152 g

Distilled water 90 ml

Made the volume to 100 ml. Stored at room temperature.

6. 5 mM Tris Cl (pH 8.9)

Tris-Cl 0.788 g

Distilled water 80 ml

Made the volume to 100 ml. Stored at room temperature.

BUFFERS AND STOCK SOLUTION FOR NATIVE-PAGE

1. 2X separating gel buffer (pH 8.9)

Tris base 22.4

Dissolved in 230 ml of distilled water. Concentrated HCl was added dropwise until pH had fallen to 8.9. The volume was made up to 250 ml and stored at 4°C.

2. Stock acrylamide solution for preparing separating gel (0.8% C)

Acrylamide 75 g

Bisacrylamide 0.6 g

Dissolved in 181 ml of distilled water. The volume was made up to 250 ml (30% acrylamide, 0.24% bisacrylamide. 0.8% C) and stored at 4°C.

3. 2X stacking gel buffer (pH 6.8)

Tris base 6.06 g

Dissolved in 190 ml water. Added concentrated HCl dropwise until pH fell to 7.2 and the 1N HCl was added till the pH fell to 6.8. Total volume was made up to 200 ml and stored at 4°C

4. Stock acrylamide for stacking gel

Acrylamide	15 g
Bisacrylamide	0.4 g

Dissolved in 36 ml distilled water. The volume was made upto 50 ml (30% acrylamide, 0.8% bisacrylamide, 2.7% C) and stored in dark bottle at 4°C.

5. 10X Electrode buffer (pH 8.3)

Tris base	30.3 g
Glycine	144.2 g

Dissolved in 885 ml water. PH was approximately 8.6. The volume was made upto 10 litre and stored at room temperature. For using one part of this solution was mixed with nine parts of distilled water. The pH was approximately 8.4.

BUFFER AND STOCK SOLUTION FOR SDS-PAGE

1. 2X separating gel buffer (pH 8.9)

Tris base	22.7 g
SDS	0.5 g

Dissolved in 240 ml of distilled water. Concentration was added dropwise until pH fell to 8.9. The volume was made upto 500 ml and stored at 4°C

2. 2X stacking gel buffer (pH 6.8)

Tris base	6.06 g
SDS	0.40 g

Dissolved in 180 ml of distilled water. Concentration HCl was added drop-wise until pH fell to 6.8. The volume was made upto 200 ml and stored at 4°C.

3. 10 X electrode buffer (pH 8.3)

Tris base	30.3 g
Glycine	144.2 g
SDS	10.0 g

Dissolved in 885 ml of distilled water. pH was approximately 8.4. The volume was made up to 1 liter and stored at room temperature.

10% APS

APS	0.1 g

Dissolved in 1ml distilled water and kept at 4°C for a maximum of 4 days

Staining Solution

Solution A

Coomassie brilliant blue R 0.25 g in 100 ml

Solution B

TCA	60 G
Methanol	180 ml
Glacial acetic acid	60 ml
Distilled water	600 ml

Added solution A to solution B and mixed by shaking. Volume was made to 1 liter, stored in dark bottle at room temperature.

Destaining Solution

NaCl	150 g

Dissolved in 5 liters of distilled water (3% NaCl)

2X sample buffer for SDS-PAGE (pH 6.8)

Tris base	1.51 g
SDS	8.0 g
Glycerol	30.0 ml
Bromophenol blue	2.0 mg

Dissolved in 84 ml distilled water (warmed and shaker to dissolve). Allowed to cool at room temperature. Added dropwise concentration HCl until the pH had fallen to 7.4. Then added 1 N HCl until pH fell to 7.1. Left for few minutes to allow the pH to go down to 6.8. Made

2X Sample buffer for native PAGE

Except for the addition of SDS, the composition was similar to that of sample buffer of SDS-PAGE.

50% Glycerol

Glycerol	50 g
Distilled water	90 ml

Made upto volume 100 ml. Stored at room temperature.

0.5M EDTA

EDTA	9.306 g

Dissolved in 50 ml water and stored at room temperature after autoclaving.

Dinitrosalicyclic Reagent (DNS)

DNS acid	10.0 g
Phenol	2.0 g
Na_2SO_3	0.5 g
Rochellis salt	200 g

Dissolved in 500 ml of 2% NaOH (%) solution and then diluted to 1 liter with distilled water. Stored at room temperature.

Lowry's Reagent A

$NaHCO_3$	2.0 g

Dissolved in 100 ml 0.1 N NaOH

Lowry's Reagent B

$CuSO_4$	1.0 g

Dissolved in 100 ml of Na-K tartarte

Lowry's Reagent C

Dissolved 1 ml of reagent B to 50 ml of reagent A, just prior to use.

APPENDIX IV

STATISTICAL ANALYSIS OF WHEAT AMYLASE INHIBITORY PROTEIN ACTIVITY

Table identification 1: Percentage inhibition of porcine pancreatic amylase.

a-n-o-v-a

source	d.f.	s.s.	m.s.	fvalue
repl	2.0	0.6647949E-01	0.3323974E-01	0.2653507
treat	9.0	45.49695	5.055216	40.35546 **
error	18.0	2.254810	0.1252672	
total	29.0	47.81824		

gm= 6.585797 sem = 0.2043422

cd at 1% = 0.8317165 cd at 5% = 0.6071094

Comparison at 5%

1---- 2 * 3 * 4 * 5 * 6 ns 7 * 8 * 9 * 10 *
2---- 3 * 4 * 5 * 6 * 7 ns 8 * 9 ns 10 ns
3---- 4 ns 5 * 6 * 7 * 8 ns 9 * 10 *
4---- 5 * 6 * 7 * 8 * 9 * 10 *
5---- 6 * 7 * 8 * 9 * 10 *
6---- 7 ns 8 * 9 * 10 *
7---- 8 * 9 ns 10 ns
8---- 9 * 10 *
9---- 10 ns

cv= 5.374157

Table identification 2: Porcine pancreatic amylase inhibitory activity per gm seeds.

a-n-o-v-a

source	d.f.	s.s.	m.s.	fvalue
repl	2.0	0.1273231E-01	0.6366157E-02	0.5893650
treat	9.0	4.068218	0.4520242	41.84742 **
error	18.0	0.1944310	0.1080172E-01	
total	29.0	4.275381		

gm= 0.6748667 sem = 0.6000479E-01

cd at 1% = 0.2442323 cd at 5% = 0.1782768

Comparison at 5%
1---- 2 * 3 ns 4 ns 5 * 6 * 7 * 8 ns 9 * 10 *
2---- 3 * 4 * 5 ns 6 ns 7 * 8 * 9 ns 10 ns
3---- 4 * 5 * 6 * 7 * 8 ns 9 * 10 *
4---- 5 * 6 * 7 * 8 ns 9 * 10 *
5---- 6 ns 7 * 8 * 9 ns 10 ns
6---- 7 * 8 * 9 ns 10 ns
7---- 8 * 9 * 10 *
8---- 9 * 10 *
9---- 10 ns

cv= 15.40028

Table identification 3 : Porcine pancreatic amylase inhibitory activity per mg wheat amylase inhibitory protein.

a-n-o-v-a

source	d.f.	s.s.	m.s.	fvalue
repl	2.0	3.117676	1.558838	0.6103671
treat	9.0	1595.107	177.2341	69.39651 **
error	18.0	45.97083	2.553935	
total	29.0	1644.196		

gm= 13.76867 sem = 0.9226655

cd at 1% = 3.755446 cd at 5% = 2.741278

Comparison at 5%
1---- 2 * 3 * 4 * 5 * 6 * 7 * 8 ns 9 * 10 *
2---- 3 * 4 ns 5 * 6 * 7 * 8 * 9 * 10 *
3---- 4 * 5 * 6 * 7 * 8 * 9 * 10 *
4---- 5 * 6 * 7 * 8 * 9 * 10 *
5---- 6 ns 7 * 8 * 9 * 10 *
6---- 7 ns 8 * 9 * 10 *
7---- 8 * 9 * 10 *
8---- 9 * 10 *
9---- 10 ns

cv= 11.60682

Table identification 4: Percentage inhibition of insect amylase

a-n-o-v-a

source	d.f.	s.s.	m.s.	fvalue
repl	2.0	0.1059326	0.5296631E-01	0.3002284
treat	9.0	39.96301	4.440335	25.16910 **
error	18.0	3.175561	0.1764201	
total	29.0	43.24451		

gm= 5.682592 sem = 0.2425009

cd at 1% = 0.9870306 cd at 5% = 0.7204806

Comparison at 5%

1---- 2 ns 3 ns 4 * 5 * 6 * 7 * 8 * 9 * 10 *
2---- 3 ns 4 * 5 * 6 * 7 * 8 * 9 * 10 *
3---- 4 * 5 * 6 * 7 * 8 * 9 * 10 *
4---- 5 * 6 * 7 * 8 * 9 * 10 *
5---- 6 ns 7 ns 8 * 9 * 10 ns
6---- 7 ns 8 ns 9 ns 10 ns
7---- 8 ns 9 ns 10 ns
8---- 9 ns 10 ns
9---- 10 ns

cv= 7.391414

Table identification 5 :Insect amylase inhibitory activity per gm seeds.

a-n-o-v-a

source	d.f.	s.s.	m.s.	fvalue
repl	2.0	0.5193901E-02	0.2596951E-02	0.1804163
treat	9.0	0.3471543	0.3857270E-01	2.679737 *
error	18.0	0.2590958	0.1439421E-01	
total	29.0	0.6114440		

gm= 0.2787000 sem = 0.6926811E-01

cd at 1% = 0.2819360 cd at 5% = 0.2057985

Comparison at 5%

1---- 2 ns 3 ns 4 ns 5 ns 6 ns 7 * 8 * 9 ns 10 *
2---- 3 ns 4 ns 5 ns 6 ns 7 * 8 * 9 * 10 *
3---- 4 ns 5 ns 6 ns 7 * 8 * 9 ns 10 ns
4---- 5 ns 6 ns 7 * 8 * 9 ns 10 ns
5---- 6 ns 7 ns 8 ns 9 ns 10 ns
6---- 7 * 8 ns 9 ns 10 ns
7---- 8 ns 9 ns 10 ns
8---- 9 ns 10 ns
9---- 10 ns

cv= 43.04840

Table identification 6: Insect amylase inhibitory activity per mg wheat amylase inhibitory protein.

a-n-o-v-a

source	d.f.	s.s.	m.s.	fvalue
repl	2.0	1.845990	0.9229950	2.404540
treat	9.0	33.50069	3.722299	9.697145 **
error	18.0	6.909392	0.3838551	
total	29.0	42.25607		

gm= 1.964133 sem = 0.3577034

cd at 1% = 1.455929 cd at 5% = 1.062752

Comparison at 5%

1---- 2 ns 3 * 4 ns 5 * 6 ns 7 ns 8 ns 9 ns 10 *
2---- 3 * 4 ns 5 * 6 * 7 ns 8 * 9 * 10 *
3---- 4 ns 5 ns 6 ns 7 ns 8 ns 9 ns 10 *
4---- 5 * 6 ns 7 ns 8 ns 9 ns 10 *
5---- 6 ns 7 * 8 ns 9 ns 10 *
6---- 7 ns 8 ns 9 ns 10 *
7---- 8 ns 9 ns 10 *
8---- 9 ns 10 *
9---- 10 *

cv= 31.54370

VITA

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ABSTRACT

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Thesis title: "Functional characterization of purified wheat (*Triticum aestivum* L.) alp amylase inhibitor(s) for rice weevil (*Sitophilus oryzae*) management".

Advisor: Dr. D.P. Mishra

Inhibitors of α-amylase from bath higher animals and insect are widespre in plants and have significant insecticidal activity. Hence, the α-amylase inhibitc proteins and gene(s) encoding them from wheat have the potential for use in t genetic transformation. Although several amylase inhibitory gene constructs ha been introduced into different transgenic crops, but for deployment of amyl inhibitors for insect control requires a detailed analysis.

The present study was undertaken to study the insect *S. oryzae* amylase a wheat amylase inhibitor interaction and their respective characterization. Wh varieties were screened for the amylase inhibitory protein and the wheat vari UP 2425 which had maximum inhibition of insect amylase was purified by heati at 65^{0}C for 30 min to inactivate endogenous amylase, salting out (30-9(ammonium sulphate saturation), dialysis, gel filtration by Sephacryl S-200 and i exchange chromatograph on DEAE-sephadex A-50.

The inhibitory protein was active even after heating at 70^{0}C for 30 min a at the pH 3.3. Insect amylase –inhibitory protein complex was active upto 24 hrs 30^{0}C and the inhibitory protein exhibited competitive inhibition with ins amylase. Subunit characterization on SDS-PAGE revealed that inhibitory prot was monomeric protein of approximately 43 kD. Feeding trials of insects w inhibitory protein coated seeds (50 μg/seed or more) also had totally inhibit insect growth. Since, the inhibitory protein had the potent activity against t target storage pest, *S oryzae* amylase as *in vivo* and *in vitro* and beside this, the l optimum pH for α-amylase inhibitory activity, inhibitor containing diets such transgenic crops expressing high concentration of α-amylase inhibitor should devoid of any harmful effect on human health and will be effective against stora pest.

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Advisor

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