ISOLATION AND PRELIMINARY CHARACTERIZATION OF PROTEOLYTIC ENZYMES FROM MALTED WHEAT FLOUR

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ABSTRACT

A proteolytic enzyme from the flour of one variety of malted Canadian hard red spring wheat was isolated and partially characterized. A crude enzyme preparation was obtained by extracting the flour with 10% saturated ammonium sulfate solution followed by dialysis against 15% glycerol solution. This preparation was further fractionated by ammonium sulfate precipitation, membrane ultrafiltration, gel filtration and CM-cellulose chromatography. Approximately 60% of the proteolytic activity of the crude preparation was concentrated by precipitation with 40-80% saturated ammonium sulfate. The proteolytic activity in this fraction appeared to be associated with high molecular weight substances. This fraction was resolved into three different proteolytically active components by CM-cellulose chromatography.

A highly selective method was developed for the purification of proteolytic enzyme(s) in the crude extract. This method is based on the affinity of the enzymes for hemoglobin on a hemoglobin-Sepharose chromatographic column. The specific activity of the purified enzyme obtained by this procedure was twice that of the fraction obtained by precipitation with 40-80% saturated ammonium sulfate. By disc electrophoresis on poly-

acrylamide gel, the purified enzyme contained three major and one minor protein bands. It appeared to be homogeneous by Sephadex G-150 column chromatography and by ultrafiltration. The molecular weight of the purified enzyme, determined by gel filtration, was 34,000. The sedimentation coefficient ($S_{20,w}$) for the purified enzyme was 3.8. The activity of the purified enzyme was inactivated by sulfhydryl blocking agents, activated by reducing agents but was not affected by soybean trypsin inhibitor. Accordingly malted wheat flour protease appears to be a sulfhydryl protease, and is probably a papain-type enzyme.

The optimum pH of the purified enzyme was 3.8 on hemoglobin substrate and 7.5 on casein substrate. The proteolytic activity was linearly dependent of the enzyme concentration for both the 40-80% saturated ammonium sulfate precipitate and the purified enzyme preparation.

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INTRODUCTION

Wheat proteases through their action on flour proteins, are considered extremely important in the functional properties of flour. Nevertheless there is little precise fundamental information on the nature and mode of action of these enzymes in dough during the preparation of bakery products. The lack of fundamental information on their structure and function arises primarily from difficulties encountered in their isolation and purification. In general, the difficulties can be attributed to the very low activity of flour from sound wheat and to the tendency of the enzymes to associate with other proteins present in the flour.

The present study was undertaken with two main objectives: (1) to develop a simple isolation and purification procedure for the preparation of pure wheat protease(s), (2) to characterize the purified enzymes in terms of their fundamental physicochemical and functional properties.

Preliminary attempts to concentrate and purify the proteolytic enzymes present in flour from sound wheat were not very successful because of the extremely low activity of these flours. Accordingly, it was decided that a more fruitful, initial approach would be to use flour from malted wheat which is considerably higher in proteolytic activity. Results of experiments on the extraction, purification, and characterization of the proteolytic enzymes in flour from malted wheat will be the subject of this thesis.

LITERATURE REVIEW

I. Proteolytic Enzymes

Introduction

In recent years proteolytic enzymes have been extensively studied. This is partly because of the commercial availability of many of these enzymes from a variety of sources and partly because of their great utility as tools for studies of protein structure. The most important type of reaction catalyzed by these enzymes is the hydrolysis of the peptide bond as shown in the following equation:

$$\cdots NHCHRCO{-}NHCHR'CO\cdots + H_2O \xrightarrow{\text{protease}} \cdot\cdot NHCHRCOO^- + {}^+NH_3CHR'CO\cdot\cdot$$

Most of the peptide bond cleaving enzymes can also catalyze the reverse reaction, i.e. peptide bond synthesis. In certain cases they can also catalyze transpeptidation reactions (1). In addition, many proteolytic enzymes can hydrolyze certain ester bonds (1).

Classification of Peptide Bond Cleaving Enzymes

It was considered at one time that the specificity of proteolytic enzymes was dependent on the chain length of the substrate. Accordingly, these enzymes were classified into two broad groups, proteinases and peptidases. However, studies of the specificity of these enzymes on a large number of synthetic peptides have indicated that this distinction

was invalid, since proteinases are in fact capable of hydrolyzing small peptides of suitable structure.

On the basis of studies with synthetic substrates, Bergmann (2) suggested in 1942, that the specificity of proteases depends not on the length of the substrate but on the nature of the amino acid side chains and the presence or absence of ionic groups near the peptide bond that is hydrolyzed. Proteases were then divided into exopeptidases and endopeptidases.

Exopeptidases comprise enzymes that hydrolyze peptide bonds adjacent to terminal alpha-amino or alpha-carboxyl groups. This group of enzymes includes aminopeptidases, carboxyl-peptidases and dipeptidases. On the other hand, endopeptidases attack centrally located peptide bonds as well as terminal bonds. This group includes pepsin, trypsin, chymotrypsin, papain, ficin, cathepsin, and subtilism. The classification of proteolytic enzymes has been extensively reviewed by Bergmann (2), Smith (3), and Green and Neurath (4).

Proteases can also be classified into three different categories on the basis of the chemical nature of their active sites (5). Sulfhydryl proteases, comprise those enzymes whose activity depends on the presence of one or more free sulfhydryl groups. This group includes papain, ficin and similar plant proteases. Metallo proteases, are those enzymes whose activity depends on bound metal ions. This group includes carboxypeptidase A, leucine aminopeptidase, and certain bacterial proteinases. Serine proteases include the

enzymes whose activity is inhibited by di-isopropyl fluorophosphate (DFP) and similar organo-phosphorous compounds but not inhibited by sulfhydryl reagents or by metal poisons. Examples of this group are trypsin, chymotrypsin, subtilisin and thrombin. Thus far, most of the highly purified and well characterized proteases appear to fall into one of these three categories except pepsin which was classified as an 'acid protease' (6) because of the low pH at which it shows its optimum activity.

Occurrence and Properties of Plant Proteases

The most extensively studied plant proteases are those of papaya, fig, pineapple and cereal grains. The distribution and properties of some of these proteases are shown in Table 1.

Isolation, Purification, and Characterization of Plant Proteases

Isolation and Purification Procedures. Various extraction procedures depending on the type of material used as the enzyme source have been used for the preparation of plant proteases. For example, papain and ficin were originally prepared from the dried plant tissue powder, while bromelin was prepared from the acetone powder of squeezed pineapple juice. Water and dilute salt solution have been used most commonly for the initial extraction of the enzyme. Various conventional protein purification procedures were then used for the preparation of highly purified, and in some cases crystalline plant proteases.

Table 1. Occurrence and Properties of Plant Proteases

Enzyme	Source	Optimum pH	Activation	IUB-EC Nomenclature	Reference
Papain	Papaya (latex)	7-7.5[a]	CN, H_2S cysteine	3.4.4.10	(7,8)
Chymopapain	Papaya (latex)	7[a]	"	3.4.4.11	(9,10)
Ficin	Fig (latex)	7[a]	"	3.4.4.12	(11,12)
Bromelin	Pineapple (fruits or leaves)	6-7[a]	"	3.4.4.24	(13,14)
Proteases of cereal grain	Wheat, sprouted wheat, barley, malt	5[a] 3.5-3.8[b]	---	------	(15,16, 17,18)

(a) Casein as substrate

(b) Hemoglobin as substrate

Table 2 summarizes the methods used in the purification of five well-characterized plant proteases.

In the purification of proteases, their specific activity does not appear to increase as rapidly as for many other enzymes whose purification can give a hundred or thousand fold increase in activity. For example, the specific activity of crystalline papain is only 4.4 times that of its crude extract. In addition, proteases present a specific difficulty that is not encountered with other enzymes. With these enzymes, it is usually necessary to take special precautions to prevent autolysis during their purification. In the case of papain, cysteine must be used for its activation for maximum activity (8). Quite often metal ions poison enzyme activity; this can usually be obviated by dialysis against the chelating agent ethylene-diaminetetra-acetic acid (EDTA) (8).

Physicochemical Properties of Some Plant Proteases. Pertinent physical properties of four plant proteases that have been studied extensively are given in Table 3.

The molecular weights of all the plant proteases that have been so characterized are in the neighborhood of 20,000 to 40,000. In this property, plant proteases are similar to pepsin, trypsin, chymotrypsin, and some bacterial proteases.

Chemical Properties of Plant Proteases. The chemical properties of a number of plant proteases have been extensively investigated. The amino acid sequence of papain has been partially worked out (22). This enzyme is a single polypeptide chain, with 3 disulfide cross linkages, one -SH group, and

Table 2. Purification Procedures for Five Plant Proteases(19)

Enzyme	Purification Procedure					Degree of Purification
	$(NH_4)_2SO_4$ Precipitation	NaCl Precipitation	Anion Exchange Chromatography	Cation Exchange Chromatography	Gel-Filtration	
Papain(8)	X	X				4.4[a]
Chymopapain (10)	X	X		X		0.58[a]
Ficin(20)	X	X				2.0[a]
Stem-Bromelin(21)	X		X	X	X	1.4[a] 1.1[b]
Fruit-Bromelin(14)			X			1.7[a]

(a) Casein used as substrate (b) N-Benzoyl-L-Arginine Ethyl Ester used as substrate

Table 3. Physical Properties of Four Plant Proteases (19)

Enzyme	Sedimentation constant ($S_{w,20}$)	Diffusion constant (D)	Isoelectric point	Absorbancy at 280mμ	Molecular weight	Rotatory dispersion coefficient (λ_c)
		$cm^2\ sec^{-1}$	pH	$A^{1\%}_{1cm}$		mμ
Papain	2.42	10.23×10^{-7}	8.75	25	21,000	231
Chymopapain	2.71	--	10.0	18.7	27,000	258
Ficin	2.61	--	9.0	22.4	26,000	261
Stem-Bromelin	2.73	7.77×10^{-7}	9.55	19.0	33,200	241

an isoleucine residue at its N-terminal. Chymopapain has glutamine as its N-terminal amino acid residue. Stem and fruit bromelin have valine and alanine as their N-terminal amino acid residues respectively. In these enzymes the free -SH group is located at the active site and can be blocked by heavy metals ($HgCl_2$ or organic compounds containing mercury), iodoacetate or N-ethylmaleimide with a concomitant loss of activity.

Enzymatic Properties of Some Plant Proteases. Pertinent properties of plant proteases that have been investigated can be summarized as follows:

1. They are all sulfhydryl enzymes, and their activity can be activated by reducing agents such as cysteine or potassium cyanide, and inhibited reversibly by heavy metals or their derivatives.

2. DFP, an efficient inhibitor of trypsin, chymotrypsin and other serine proteases, has no effect on the activity of papain, ficin, and stem bromelin.

3. Plant proteases can also hydrolyze benzoyl-L-arginine-p-nitro-anilide (BAPA) and other synthetic substrates. In general, the reaction rates with synthetic substrates are relatively slow.

4. The pH-activity curves of plant proteases show optima near neutral pH, however these enzymes are stable over a relatively wide pH range.

II. Proteolytic Enzymes of Wheat and Its Products

Discovery of Proteolytic Activity in Wheat and Its Products

The existence of an enzyme or enzymes in wheat and wheat flours, capable of hydrolyzing proteins, was first reported by Balland (23) in 1884. Evidence in support of this observation was subsequently published by a number of investigators. The work by Ford and Guthrie (24) showed that aqueous extracts of wheat reduced the viscosity of gelatin. Baker and Hulton (25). observed that a similar extract of flour produced tryptophan from egg albumin. Swanson and Tague (26) detected the increase in soluble amino nitrogen in flour-water slurries by the Sorensen formol titration method. The work of Sharp and Elmer (27) showed that flour extracts were able to liquefy gelatin. This work also suggested that proteolysis or autolysis of flour was important in relation to the functional characteristics of certain flours.

Preparation and Properties of Wheat Proteases

In 1928, Cairns and Bailey (28) separated proteolytic enzymes from flour in the form of a safranine complex and showed that this preparation was capable of digesting casein. Few years later, Jorgensen (29,30) and Balls and Hale (31,32), reported independently that proteolytic enzymes of flour exist in a latent form and become active only when treated with reducing agents such as glutathione or cysteine.

Flour proteases were studied extensively by Balls and Hale (32), and Hale (33). In this work, a partially purified protease was prepared from wheat bran, patent flour, and whole

wheat by ammonium sulfate fractionation. The properties of this partially purified proteinase showed that it was similar to papain. It became inactive on standing in air and was readily reactivated by the addition of cysteine. The active enzyme digested casein, clotted milk, and lowered the viscosity of gelatin. It was inactivated by several oxidizing bread improvers namely, persulfate, bromate, and metavanadate. Further confirmation of papain-like character of wheat proteinase was based on its inactivation with iodoacetic acid. Hale and co-workers (33) suggested that their enzyme preparations might also contain peptidase activity.

Recent studies of wheat proteinases by McDonald and Chen(34) indicated that there are at least two different proteinases in sound wheat flour. One enzyme, which showed optimum activity at pH 3.8 on hemoglobin, was easily extracted from flour with water at slightly alkaline pH or with sodium acetate buffer solution of pH 3.8. The other proteinase was detected but could not be extracted by a number of different solvents. The pH for optimum activity for this unextractable enzyme was 4.4.

Various pH optima of flour proteinases have been reported: 4.4 with flour protein as substrate (34); 3.5 and 3.8 with hemoglobin (34,35); 5.0 with casein (36); and 5.0 with edestin(37).

Recently, Hanford (38) reported the presence of two types of proteolytic enzymes in wheat flours. One caused gluten softening, and the other produced low molecular weight substances that were soluble in trichloroacetic acid. They were named alpha- and beta-proteinases analogous to alpha- and

beta-amylases respectively. It was suggested that the alpha-enzyme hydrolyzes polypeptide chains into relatively large polypeptide units that are precipitated by trichloroacetic acid. This enzyme was assayed by the rate of softening of a gluten ball washed from the flour. On the other hand, it was postulated that beta-proteinase yields small peptide fragments and amino acids hydrolyzed from the end of the polypeptide chain which can be detected by the standard assay procedures used to measure the release of soluble nitrogen compounds.

In 1969, Kaminski and Bushuk (39), using a modified starch-gel electrophoretic technique showed that wheat proteases are extremely heterogeneous and that considerable activity is associated with the 70% ethanol soluble fraction of wheat proteins.

Distribution of Proteolytic Activity in the Wheat Kernel

The distribution of proteolytic activity in the wheat kernel was studied by Pett (37) and by Engel and Heins (40). These workers reported that protease activity was highest in the hull (bran) and the germ, and the lowest in the endosperm. The aleurone cells, directly under the bran, were also high in activity.

The distribution of proteolytic activity in mill fractions has also been investigated. Howe and Glick (41) reported that the activity of milled fractions, listed in increasing order was: patent flour, clear flour, lower grade flour, germ,

whole wheat flour, shorts, and bran. On the other hand, Balls and Hale (32) reported that the activity increased in the order: whole wheat, bran, and germ.

Proteolytic activity of air classified fractions of flours milled from French wheat was studied by Audidier and Seince(42). They showed that the medium and high protein fractions were higher in activity than the original patent flour, while the low protein fraction had the lowest activity. This study also showed that the high protein fraction had a lower activity per gram of protein than the patent flour or the two fractions of lower protein content.

Development of Proteolytic Activity during Germination and Malting

The proteolytic activity of malted wheat flours has been investigated extensively by Mounfield (43,44,16). In these studies, edestin was used as the substrate for the activity assay and the extent of proteolysis was followed by determination of the increase in free amino or carboxyl groups by the Sorensen formol titration. Mounfield found that sprouted wheat contained an enzyme capable of hydrolyzing edestin with an optimum activity at pH 4.1 at 40°C. He also demonstrated the presence of a dipeptidase capable of hydrolyzing leucylglycine and glycylglycine which had optimum activity at pH 7.5. Both enzymes lost activity simultaneously on standing in aqueous solution at room temperature. Mounfield found that the proteinase activity of wheat increased approximately six fold in four days, and ten fold in seven days of germination. The activity

after four days of germination decreased with increasing storage time of the sound grain.

Comparative studies of the activities of malted and unmalted wheat and wheat flour were carried out by Balls and Hale (32), Hildebrand (45), and Hildebrand and Burkert(46). The data of these workers were obtained by different assay methods, it is therefore not possible to make a direct quantitative comparison of their results. However these studies give some indication of the relative activity of malted wheat and its flour compared with that of unmalted wheat and flour. Balls and Hale (32), measured the increase in amino groups by titration in alcoholic solution and found that the protease activity of wheat malt was seven times that of patent flour, and from two to three times that of ground whole wheat. Hildebrand (45), on the other hand, measured protease activity by the rate of gelation procedure of Landis and Frey (47), and found that malt flour was from three to twenty times more active than patent flour but it had lower activity than a second clear flour produced from unmalted wheat. The values obtained by the Ayre-Anderson method (48) indicated that, in general, malt flours are from 2.5 to 5 times as active as patent flour from the same wheat (49).

The effects of malting procedure, wheat storage conditions, growth environment, variety, and class of wheat on the proteolytic activity of the wheat have been investigated by Fleming et al (50). The optimum malting conditions that were established are: steep moisture level-42%; germination

temperature-60°F; and kilning temperature below 110°F. Higher kilning temperatures caused a loss of enzyme activity. Proteolytic activity of harvested wheat did not reach the maximum level until after 2 months of storage. Storage at low temperatures (40°F) produced higher activity. On the basis of the increase in activity during malting, wheats of different classes were rated in the following order: soft white > soft red winter > durum > hard red spring. High moistures during the ripening stage of kernel development increased the activity of the germ, bran and endosperm of Japanese wheats(42).

The changes in the proteolytic activity of wheat during maturation and sprouting were investigated by Safanova (51). This author reported that the enzymes from endosperm and embryo were distinctly characteristic and had different pH optima. It was presumed that embryo proteinases do not penetrate into the endosperm when the grain germinates. The existence of more than one protease in germinated wheat was observed also by Prentice et al (52). These workers partially purified and characterized two peptide hydrolases, A and B, from germinated wheat. Comparison of the thermal stabilities, pH optima, stability at various pH values, kinetic constants, and effects of metal ions for these hydrolases showed that germinated wheat contained a neutral peptide hydrolase with properties similar to those of barley hydrolase B, one acidic peptide hydrolase similar to barley hydrolase A, and a third hydrolase that was more resistant to low pH and thermal denaturation.

The activation or synthesis of proteolytic enzymes during

germination of wheat has not been studied in detail. However, an extensive study of the biosynthesis of proteases in germinating barley has been carried out. Jacobsen and Varner(53) in 1967 observed that production of proteases by isolated aleurone layers of barley is increased markedly by gibberellic acid. The enzymes seem to originate in the aleurone layer and are subsequently released from the aleurone cells. On the basis of this evidence these workers proposed that proteases, like amylases, are synthesized de novo in response to the hormone. Whether the development of proteases during germination follows the same mechanism in wheat as in barley remains to be investigated.

Methods for the Determination of Proteolytic Activity

Numerous methods for the determination of proteolytic activity in wheat flour have appeared in the literature. In general, these methods depend on either a physical or a chemical analysis of the changes brought about during proteolysis. In 1928 Cairns and Bailey (28) made a comparative study of eight chemical procedures for measuring progressive proteolysis in flour suspensions. The eight procedures are:

1. Ritthausen method (54) as modified by Blish (55).
2. Scherning method (56,57) as modified by Olsen & Bailey(58).
3. Sorensen formol titration method (59).
4. Van Slyke amino nitrogen method (60).
5. Trichloroacetic acid method (28).
6. Tungstic acid method as developed by Rumsey (61) in 1922.
7. Water soluble protein method (28).

8. Foreman' amino acid titration method (62).

These workers concluded that methods 5 and 7 appeared to be of limited value. Method 2 was somewhat cumbersome and the results of replicate determinations were more variable than with methods 1 and 6. Methods 1 and 6, although somewhat laborious, gave acceptable results. Method 8, although rapid, apparently was less acceptable than the Sorensen formol titration (method 3), which appeared to be the best of all the methods examined. Method 4 was also quite useful but somewhat more laborious than method 3.

Hildebrand (45), compared the rate of gelation (47) and viscometric methods (63) with the Sorensen formol titration procedure, and concluded that the first two methods gave results that were appreciably different from those obtained by the third method. This was attributed to the fact that dipeptidase and/or polypeptidase activity had an appreciable effect on the results obtained by the formol titration method, whereas with the rate of gelation and viscometric procedures the results depended much more on the proteinase activity. Hildebrand (64) also compared the rate of gelation (47) and the Ayre-Anderson methods (48) for the determination of proteolytic activity using twelve experimentally produced samples of malted wheat flour. Both methods gave similar results and had approximately the same precision and ability to distinguish different samples. The Ayre-Anderson technique was preferred because of its greater simplicity and convenience.

Several methods for the determination of proteolytic activity in dough were compared by Bowlby et al (65).

Results from this study indicated that the Ayre-Anderson hemoglobin digestion method with either Kjeldahl or spectrophotometric determination of the soluble nitrogen, the bromosulfthalein method (66), and the formol titration procedure gave similar results to those obtained with the Farinograph. Results obtained by methods based on gluten digestion and milk clotting did not agree with those obtained by the other methods.

The Ayre-Anderson method was considered the best method for the measurement of proteolytic activity in flour. On the basis of a detailed and critical study by Miller (35), the use of this method for flours has been investigated in collaborative study sponsored by the American Association of Cereal Chemists (67). Recently it has been adopted as a standard method of the Association of Official Agricultural Chemists (35,68). The Kjeldahl procedure used for determining non-protein nitrogen in the original Ayre-Anderson method has been generally replaced by the spectrophotometry (69) or by the Lowry procedure (70).

III. The Role of Proteolytic Enzymes in Breadmaking

Effects of Reducing and Oxidizing Agents on Proteolytic Activity in Dough

There was considerable controversy over the actual role of flour proteases in dough properties following the publication of Jorgensen's hypothesis (29,30) that sulfhydryl compounds activated these enzymes which in turn, by hydrolyzing the

gluten proteins, caused a softening and ultimately liquefaction of the dough. Oxidizing agents, on the other hand were thought to inhibit the activity of proteases in dough, and thereby produce a stiffer dough. Independently, at about the same time, Balls and Hale (71,31) came to the same conclusion as Jorgensen. They observed that the proteolytic activity of wheat products was increased by the addition of sulfhydryl compounds such as cysteine and glutathione, and decreased by treatment with oxidizing agents. In the same year, Balls and Hale (32) published another paper on the effect of reducing agents on flour proteins and concluded that reducing agents act in two distinct ways: (1) by activating proteinases present in doughs, (2) by direct chemical action on gluten proteins.

First results that contradicted Jorgensen's hypothesis came from the work by Ford and Maiden (72), who observed distinct differences in the actions of glutathione and papain preparations on doughs. Glutathione caused an immediate softening of dough, whereas the softening effect of papain increased gradually with time. Evidence in support to this finding was later provided by Sandstedt and Fortmann (73). They reported that the action of glutathione could be reversed by oxidation, whereas the effect produced by papain was more permanent and could not be reversed by addition of oxidizing agents.

The discrepancies between the proteinase hypothesis of Jorgensen and the observed action of reducing and oxidizing agents in doughs was reviewed by Hildebrand (49). This author summarized the arguments into the following points:

1. Proteinases are present in a very low concentration in patent flour.

2. Flour proteinases are of papain type, and therefore can be activated by reducing agents and inactivated by the oxidizing agents.

3. Despite the analogy between the effect of oxidizing agents on proteinases and their action on dough, it does not necessarily follow that the primary function of oxidizing agents that are used as flour improvers is to inhibit the activity of these enzymes in flour.

More recently, it has been confirmed that reducing agents cause no or only a small increase on the rate of proteolysis in dough (34,74,75). Oxidizing agents such as potassium bromate, which improve the breadmaking quality of most flours, had no effect on the activity of flour proteases (34,75,76). Potassium iodate under some conditions produced a partial inhibition. More recent studies with reducing, oxidizing, and sulfhydryl blocking agents indicate that not all of the wheat proteases are activated by reducing agents and inhibited by oxidizing agents. Sulfhydryl blocking agents such as p-chloromercuribenzoate, N-ethylmaleimide, and iodoacetamide inhibited 4-43% of the proteolytic activity in patent flours (34) and 20-30% of the proteolytic activity in whole wheat (34,77).

The Action of Proteolytic Enzymes on Flour Proteins

Studies of the action of proteolytic enzymes on flour proteins are significant for two reasons: (1) they can provide

useful information on the role of these enzymes in the functional properties of flour in breadmaking, (2) they provide information on the structure of flour proteins. An early study of the effects of flour proteases on leucosin (albumin), gliadin, and glutenin by Blagoveshenski and Yurgenson (78) showed that proteinases extracted from wheat flour exhibited different pH optima on these substrates. The pH optima obtained were 3.7 to 4.7 with leucosin; 3.7 with gliadin; and 4.9 to 5.0 with glutenin. Moreover, they also observed that the action of flour proteases on its proteins was primarily a viscosity reducing process and produced only small increases in amino nitrogen.

Later, Harris (79,80) studied the effect of proteolytic enzymes on the solubility of wheat gluten in sodium salicylate solutions. He observed that proteases added to doughs affected the relative distribution of protein fractions obtained from their glutens. Different proteolytic enzymes affected the distribution in a different way. Papain had a disruptive action on the gluten complex. Flour proteases, activated by glutathione, appeared to coagulate rather than disperse the gluten complex. Pancreatin had a similar effect. Pepsin did not have any definite effects under the conditions used.

Studies of enzymatic hydrolysis of wheat gluten were carried out independently by Oka et al (81), Finlayson(82), and McCalla et al (83,84,85). In 1966 Verma and McCalla (84) reported that sedimentation analysis of gluten hydrolyzates suggested that papain produced large quantities of low molecular weight products whereas pepsin produced somewhat larger fragments.

Oka et al (81) found that pepsin hydrolysis of glutenin caused a rapid cleavage of a few peptide bonds to produce relatively large polypeptides (molecular weights >10,000) followed by further cleavage to produce small peptide fragments. Yang and McCalla (85) compared the modes of action of pepsin, papain, and trypsin on wheat gluten. Their results indicated that trypsin was considerably less effective than either papain or pepsin for hydrolyzing wheat gluten. Papain produced non-protein nitrogen most rapidly during the early stages of hydrolysis. However the rate of release of terminal amino nitrogen was similar for papain and pepsin during the early stage of hydrolysis. The polypeptides produced by papain were of larger average size than those produced by pepsin during the early stage of hydrolysis; the order of relative size was reversed after 48 hours of hydrolysis. Only pepsin released appreciable amounts of amino acids. Using gliadin as substrate, Finlayson (82) obtained only small amounts of amino acids after consecutive hydrolysis by pepsin and trypsin.

Effects of Proteolytic Enzymes on Rheological Properties of Dough and on Bread Quality

Early work on the role of proteases in the baking process was reviewed by Hildebrand (49) in 1946. It appeared at that time that the proteinases in flour milled from sound wheat, or those added in the malted wheat flour supplement, had no significance in the preparation of baked products. Few years later, this question was re-investigated by Johnson

and Miller (86). The supplements used in this study were prepared from malted wheat flour and fungal extracts, and contained no alpha-amylase activity. A commercial sponge-dough procedure was used in the baking. The observed effects of increasing concentrations of the two enzymes were different. This difference depended to some extent on the flour used in the formula. Hard red spring wheat flour exhibited a marked positive response in loaf volume whereas winter wheat flour did not show a positive response. High concentrations of the fungal proteinase were definitely detrimental to loaf volume, grain, and texture of the bread. With fungal extracts that had alpha-amylase activity, the decrease in dough consistency depended very strongly on the ratio of amylase to proteinase activity.

The usefulness of certain proteases in breadmaking has been demonstrated by Johnson et al (17). These workers showed that addition of protease to the sponge in the sponge and dough procedure reduced the mixing requirements at the dough stage. Protease-treated doughs were more extensible and could be sheeted thinly and molded with greater ease. These effects were particularly beneficial with certain flours that normally produced bucky doughs.

Malted wheat and barley flour, and fungal preparations are the main sources of protease supplements used in the baking industry. The fungal enzyme preparation is considered superior to malt flour for the following reasons (87):

1. The ratio of amylolytic to proteolytic activity can be conveniently varied to suit the requirements of the flour.

2. Flours with excessively strong gluten proteins can be mellowed by increased levels of fungal supplements with low amylolytic activity.

3. Baking schedules can be more readily maintained because fungal proteinases reduce mixing time and optimum fermentation time.

4. The lower inactivation temperature of fungal alpha-amylase precludes the appearance of gumminess in bread crumb arising from excessive starch degradation during the initial part of the baking.

Fungal proteases are normally added to the sponge rather than the dough in the sponge and dough baking procedure. The main reasons for this are to obviate the inhibiting effect of the salt which is included in the dough but not in sponge and to give the enzyme sufficient time to act on the flour proteins during the long fermentation period. Fungal proteases are normally omitted when active dry yeast is used for fermentation since the yeast itself releases proteolytic enzymes that mellow the gluten proteins of the flour in the sponge. According to Silberstein (88), the use of protease supplements produced faster proofing due to better gas retention, and loaves with better symmetry and improved grain and texture characteristics.

EXPERIMENTAL

The experimental work of this investigation will be presented in four sections. Each section is written in the form of a scientific paper which can be submitted for publication with only minor editorial changes. This format was adopted in an attempt to decrease the time lapse between the preparation of the thesis and the preparation of the scientific papers resulting from the investigation.

The first section deals with the extraction and isolation of proteolytic enzyme(s) from malted wheat flour. Some pertinent properties of a crude enzyme preparation are discussed in this section.

The second section deals with the fractionation and further purification of the crude enzyme extract. The techniques used in this study include ammonium sulfate precipitation, membrane ultrafiltration, and gel filtration and CM-cellulose ion-exchange chromatography.

The third section describes a rapid method of purification using affinity chromatography. The highly purified enzyme preparation obtained by this technique was characterizied in terms of physicochemical properties and this information is included in the fourth section of the experimental part.

I. Preparation and Properties of a Crude Enzyme Extract from Malted Wheat Flour

Abstract

A crude proteinaceous fraction showing high proteolytic activity on hemoglobin substrate was obtained by extracting the flour from one variety of malted hard red spring wheat for 1 hour at 3°C with 10% saturated ammonium sulfate solution followed by dialysis of the extract against 15% glycerol solution. The pH of optimum activity of this preparation with hemoglobin substrate was 3.8. The activity was fully stable to heat treatment for 15 min. at 40°C, decreased to 70% at 50°C, and was completely inactivated at 80°C. Activity decreased with increasing urea concentration in the range from 0 to 9 M. Specific activity of the crude extract from malted wheat flour was about twice that of analogous extract from unmalted flour. Disc electrophoretic patterns of the two extracts were essentially the same.

Introduction

In recent years, numerous attempts have been made to isolate and purify the proteolytic enzyme(s) from wheat flour (89,90). So far, these attempts were unsuccessful. The main difficulties seem to arise from the relatively

low proteolytic activity of sound wheat flour, and from the lack of effective procedures for separating the small amount of the enzyme protein from the relatively large proportion of other proteins. To obviate the first difficulty, flour from malted wheat was used in this study as the initial source of the enzyme(s).

The first paper of this series will describe a procedure by which a relatively active crude enzyme preparation was obtained from malted wheat flour. Some pertinent properties of this enzyme preparation were studied and will be discussed.

Materials

Manitou, a variety of Canadian hard red spring wheat, was malted by a standard malting procedure (91). The malted wheat was milled into flour on a Buhler experimental mill to 69.5% extraction. The protein and ash contents of the flour were 14.7 and 0.40% (14% m.b.), respectively. Flour from the same variety of unmalted wheat was milled by the same procedure; its ash content was 0.48%.

Methods

Extraction Procedures. To determine the optimum extraction conditions, three solvents, three extraction times, and two temperatures were examined. The solvents used were; water, 5% sodium chloride solution, and 10%

saturated ammonium sulfate solution. Extraction times were 15, 30, 60, and 120 min. Extraction temperatures were 3° and 25°C. All solvents contained 0.125 g. L-cysteine hydrochloride to maintain the proteolytic activity, and 0.1 g. disodium ethylenediaminetetraacetate (EDTA) to eliminate possible adverse effects of metal ions.

The ratio of solvent to flour used in each extraction was 4 to 1. Each slurry was stirred in a Waring Blendor at medium speed through-out the extraction time. The slurry was then centrifuged for 30 min. at 3,000xg. and the supernatant was dialysed against two 1-liter portions of distilled water over two days in the cold room (3°C) to remove salt ions Ammonium sulfate solution extracts were dialysed against distilled water and also against 15% glycerol solution. Protein contents of these extracts were determined by the Lowry method (92) using gamma-globulin as the standard.

Determination and Expression of Proteolytic Activity. Proteolytic activity of the crude extracts was determined by a modified Ayre-Anderson procedure(93) using hemoglobin as the substrate and the Lowry method (92) to estimate the tyrosine produced. In the standard assay, one ml. of enzyme solution of appropriate dilution was incubated with 5 ml. of 1% hemoglobin solution for two hours at pH 3.8 and 37°C. The reaction was stopped by the addition of 5 ml. of 5% trichloroacetic acid (TCA) solution. The resulting

mixture was centrifuged for 10 min. at 12,000xg. and the tyrosine content of 1 ml. aliquot of supernatant was determined. Blank determinations, in which the enzyme solution was added after the addition of TCA, were made for all experiments.

Proteolytic activity was expressed in terms of the tyrosine released from hemoglobin. The activity, in protease units (P.U.), was defined as the amount of tyrosine released in μmoles per minute under the assay condition, raised to 3/2 power as suggested by Miller and Johnson (93). Specific activity was expressed in P.U. per mg. of protein.

Disc Electrophoresis. Disc electrophoresis on 7.5% polyacrylamide gels was carried out at pH 4.0 as described by Davis (94). The electrophoresis was run at 2 mA per tube during the first half an hour, and 4 mA per tube during the final two hours. The gels were stained with 0.5% Aniline Blue Black in 7% acetic acid solution over night and destained electrophoretically with 7% acetic acid solution as the electrolyte.

Results and Discussion

Effects of Extraction Solvent, Time and Temperature. Results for the three solvents for 60 min. extraction time at 3°C are given in Table 1. Each value is the average of duplicate extractions. The extracts were dialyzed against distilled water. Variation of extraction time from 15 to

120 min. or increase of extraction temperature to 25°C did not affect the specific activity significantly, accordingly these data are not included in Table 1.

Table 1. Specific Activity of Extracts Obtained with Various Solvents

Solvent	Protein Extracted	Specific Activity
	mg.	(P.U./mg.) X 10^3
Water	90.5	0.22
5% NaCl	69.9	0.95
10% Sat'd $(NH_4)_2SO_4$	--	1.06

The data show that extracts obtained with either sodium chloride or ammonium sulfate have higher specific activities than the water extract. This is probably due to the partial fractionation which results from the presence of salt. Ammonium sulfate solution extract gave the highest specific activity and therefore was adopted as the extract for subsequent fractionations and purifications.

Effect of Dialysing Medium. Dialysis is commonly used to remove salt ions from enzyme preparations. Usually this process results in a partial loss of enzyme activity especially when the dialysing medium is distilled water. Glycerol solution, which has a low dielectric constant. was used by Balls & Hale in the preparation of wheat proteinase (15)

with some success. It was therefore of interest to compare the activities of the enzyme preparations obtained with these two dialysing media.

In this experiment, dialysis was carried out at 3°C against two changes of solvent over 2 days. Extracts obtained with all the extraction times used were dialyzed in this manner. However, only the specific activities of the 60 min. extracts are shown in Table 2. Results for other extraction times were parallel and are therefore not shown.

It appears that 15% glycerol solution has a slight beneficial effect for maintaining activity of the proteolytic enzymes during dialysis. Dialysis against this medium was therefore adopted for all the subsequent preparations.

Table 2. Effect of Dialysing Medium on Specific Activity

Dialysing Medium	Specific Activity
	(P.U./mg.) X 10^3
Distilled water	1.06
15% glycerol solution	1.14

Effect of pH. The pH range covered in the present study was from 2.4 to 8.0 using the following buffer solutions:

pH	Buffer Composition
2.4 to 3.4	0.05M glycine-hydrochloric acid
3.0 to 6.0	0.20M sodium acetate-acetic acid
6.0 to 8.0	0.05M potassium di-hydrogen phosphate-sodium hydroxide

Figure 1 gives the relative activity versus pH curve for the extract obtained with 10% saturated ammonium sulfate solution. The optimum activity was at pH 3.8. This agrees with 3.5 and 3.8 obtained by Miller (35), and McDonald and Chen (34) respectively. In these studies hemoglobin was used as the substrate for the activity assay. Considerably higher pH optima (from 5.0 to 6.0) were obtained with gelatin and gluten substrates (16). With the crude enzyme preparations the pH optimum seems to depend on the substrate used for the activity assay.

Effect of Temperature. Thermal stability of the activity of the crude extract was investigated to determine if this enzyme showed any unusual heat denaturation behavior. Figure 2 shows the activity of the crude extract after incubation for 15 min. at various temperatures. Most of the activity was retained up to about 40°C. Above this temperature, activity was lost very quickly; full inactivation, at the conditions used, was obtained at 80°C. These results indicate that thermal lability of wheat proteases in a crude extract is similar to that of other plant enzymes.

Figure 1. Effect of pH on Proteolytic Activity of Crude Extract of Malted Wheat Flour

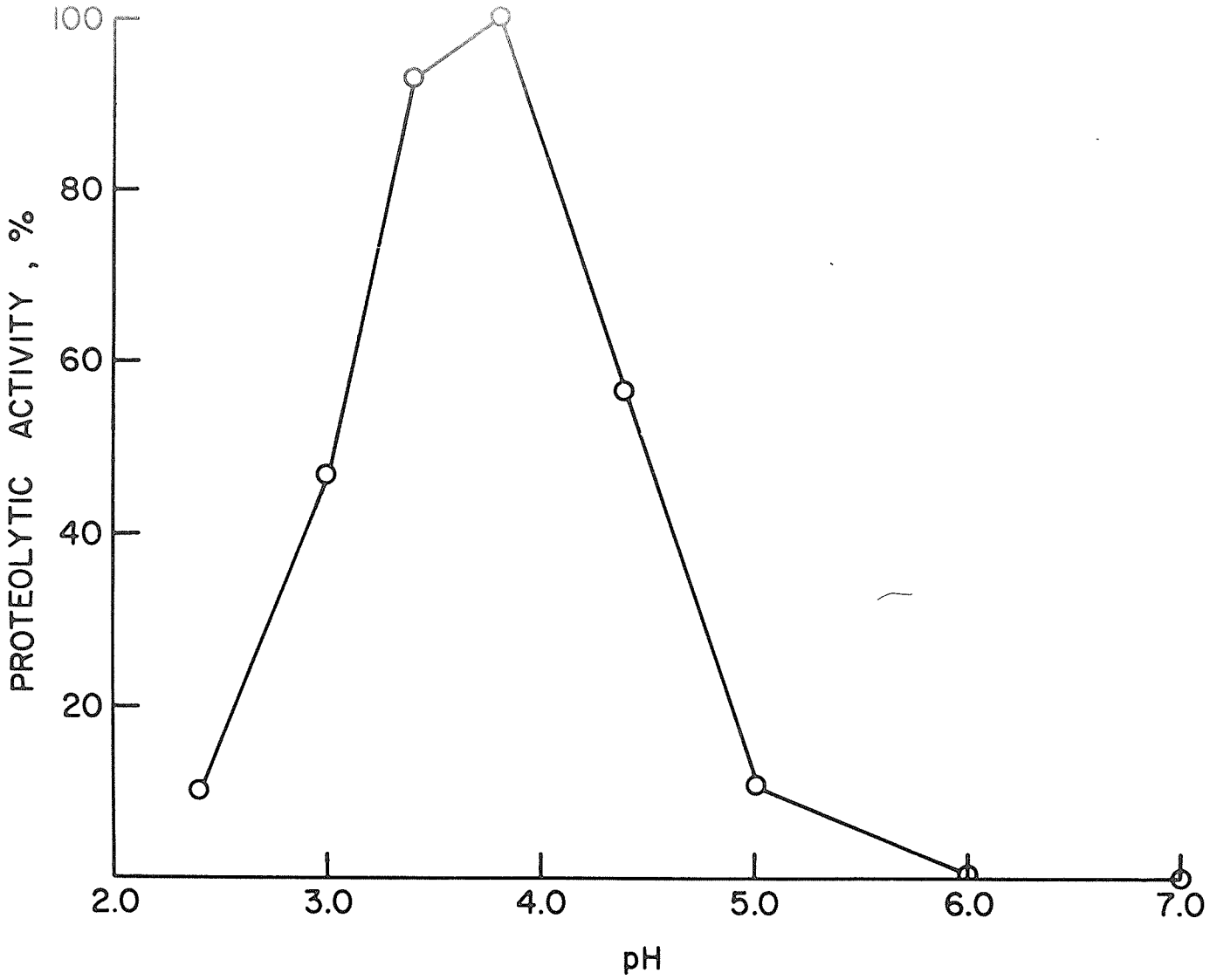
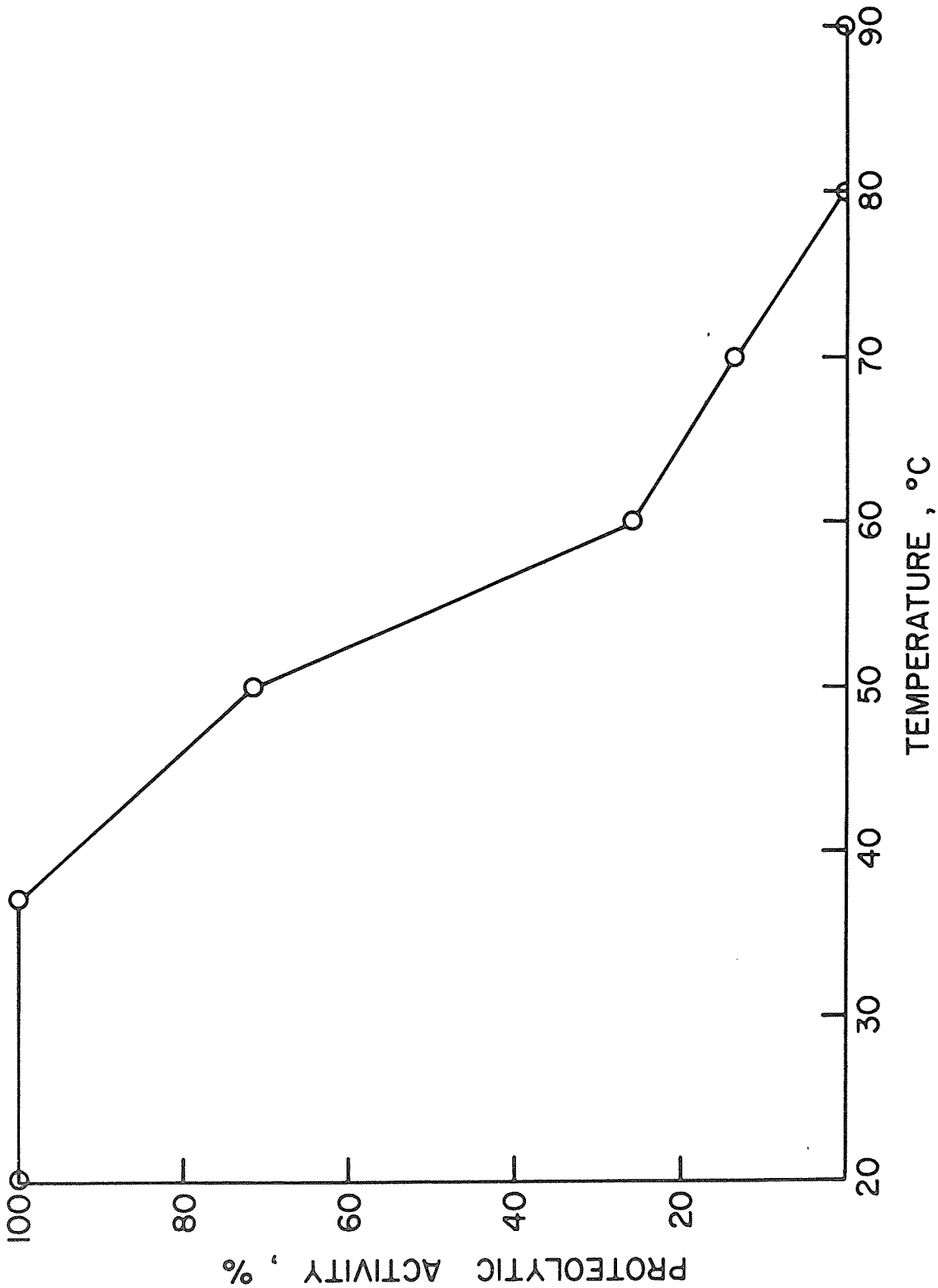

100
80
60
40
20
PROTEOLYTIC ACTIVITY , %
2.0
3.0
4.0
5.0
6.0
7.0
pH

Figure 2. Effect of Incubation Temperature on Proteolytic Activity of Crude Extract of Malted Wheat Flour

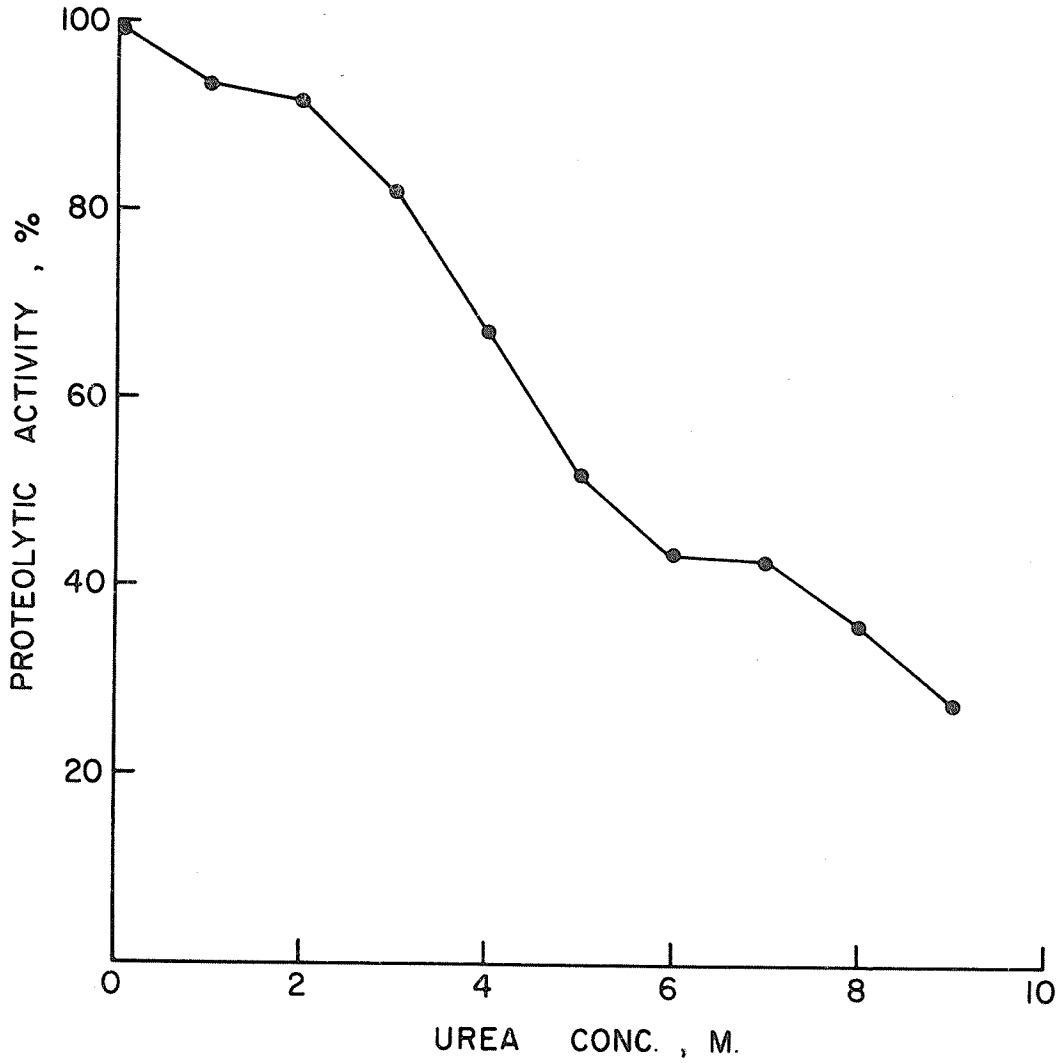

PROTEOLYTIC ACTIVITY , %
100
80
60
40
20
20
30
40
50
60
70
80
90
TEMPERATURE , °C

Effect of Urea. Urea is a strong dissociating agent commomly used for dissociation of protein aggregates. It has been suggested that proteolytic enzymes of wheat flour seem to be associated with other protein components (89). The effect of urea on the activity of crude extract was therefore examined as a preliminary to possible use of this reagent in the purification procedure. For these experiments 9.0 ml. of urea solution, from zero to 9M, were added to 1 ml. of crude extract containing 2.8 mg of protein and thoroughly mixed. One ml. of this solution was used for the enzyme activity determination.

Figure 3 shows the plot of proteolytic activity versus urea concentration. The activity decreased gradually with increasing urea concentration. There is suggestion of a multi-step inactivation mechanism. Such a mechanism would be consistent with the suggestion that the proteolytic activity of wheat flour comprises a number of different enzymes (39).

Comparison of Proteolytic Activities of Crude Extracts from Normal and Malted Wheat Flour. For technological reasons it was of interest to compare the activities of crude extracts obtained with 10% saturated ammonium sulfate solution from normal and malted wheat flours. Published results show a wide variation in the increase in activity during malting. Activities of malted wheat flour ranged from 2 to 20 times the activity of normal flour (31,45).

Figure 3. Effect of Urea on the Activity of the Crude Extract from Malted Wheat Flour


100
80
60
40
20
PROTEOLYTIC ACTIVITY , %
0
2
4
6
8
10
UREA CONC. , M.

Actual activity seems to depend on wheat variety, malting conditions, and the method used to measure the activity.

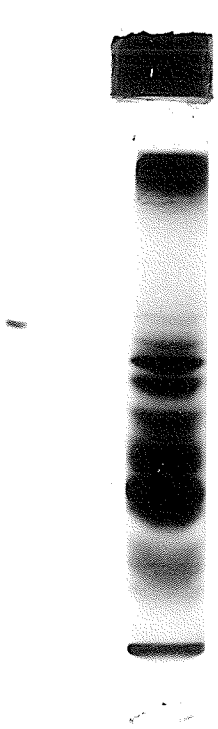
Protein contents and specific activities of crude extracts obtained in the present study are shown in Table 3. The activity of the extract obtained from malted wheat flour is approximately 2 times that of the extract from normal flour. The protein content per 100 g. of flour of the extract from malted wheat flour is only slightly higher than that of the extract from normal flour.

The electrophoretic patterns of these two extracts are shown in Figure 4. Both extracts showed essentially the same electrophoretic patterns with protein staining. On the basis of these experiments, it appears that the proteases of flour from malted wheat are qualitatively the same as those of sound wheat flour. More detailed studies are required to confirm this preliminary observation.

Table 3. Proteolytic Activity of Crude Extracts of Normal and Malted Wheat Flours

Flour	Protein Extracted	Specific Activity	Total Activity
	g./100g. flour	(P.U./mg.) X 10^3	P.U.X 10^3/100g. flour
Normal	0.98	0.59	578.2
Malted	1.09	1.14	1,242.6

Figure 4. Disc Electrophoretic Patterns of Crude Extracts of Normal and Malted Wheat Flour



NORMAL FLOUR

MALTED FLOUR

II. Fractionation and Purification of the Crude Enzyme Extract from Malted Wheat Flour

Abstract

The crude extract from malted wheat flour was further fractionated by precipitation with ammonium sulfate. Most of the proteolytic activity was concentrated in the fraction precipitated between 40-80% saturation. This fractionation gave a 1.6 fold increase in specific activity over that of initial extract. Membrane ultrafiltration and chromatography on Sephadex G-75 and G-100 showed that the molecular weight of the proteases in the partially purified extract was higher than 50,000. Ion exchange chromatography on CM-cellulose showed that the active fraction that precipitated between 40 and 80% saturation of ammonium sulfate comprised at least three proteolytically active components.

Introduction

A variety of different techniques used in enzymology were applied to the purification of proteases extracted from malted wheat flour with 10% saturated ammonium sulfate solution. The most successful procedure was the fractional precipitation with ammonium sulfate followed by further fractionation of the active fraction by ion exchange chromatography on CM-cellulose. This paper describes these

purification procedures and gives some information on the homogeneity of the enzyme preparation at various stages of purification.

Materials

Ammonium sulfate (granular) was of reagent grade. Ultrafiltration cell (model 52) and Diaflo membranes (XM-100, XM-50, UM-1 and UM-2) were obtained from Amicon Corporation, Sephadex G-100 and G-75 from Pharmacia Canada Ltd., and carboxymethyl cellulose (CM-32, microgranular) from Whatman Laboratories.

Methods

Fractional Precipitation with Ammonium Sulfate. The starting solution (100 ml.) was the extract from malted wheat flour obtained with 10% saturated ammonium sulfate as described in the previous section. The amount of ammonium sulfate required to produce a specific fraction of saturation was determined from the nomogram of Dixon (95). The salt was added slowly to the extract with gentle stirring. Stirring was continued for 10 min. after all the salt was added. The precipitate that formed at a particular saturation was separated by centrifugation at 10,000xg for 20 min. Additional salt was then added to the supernatant to produce the next level of saturation. In this manner the precipitates produced by 20, 40, 60, 80 and 100%

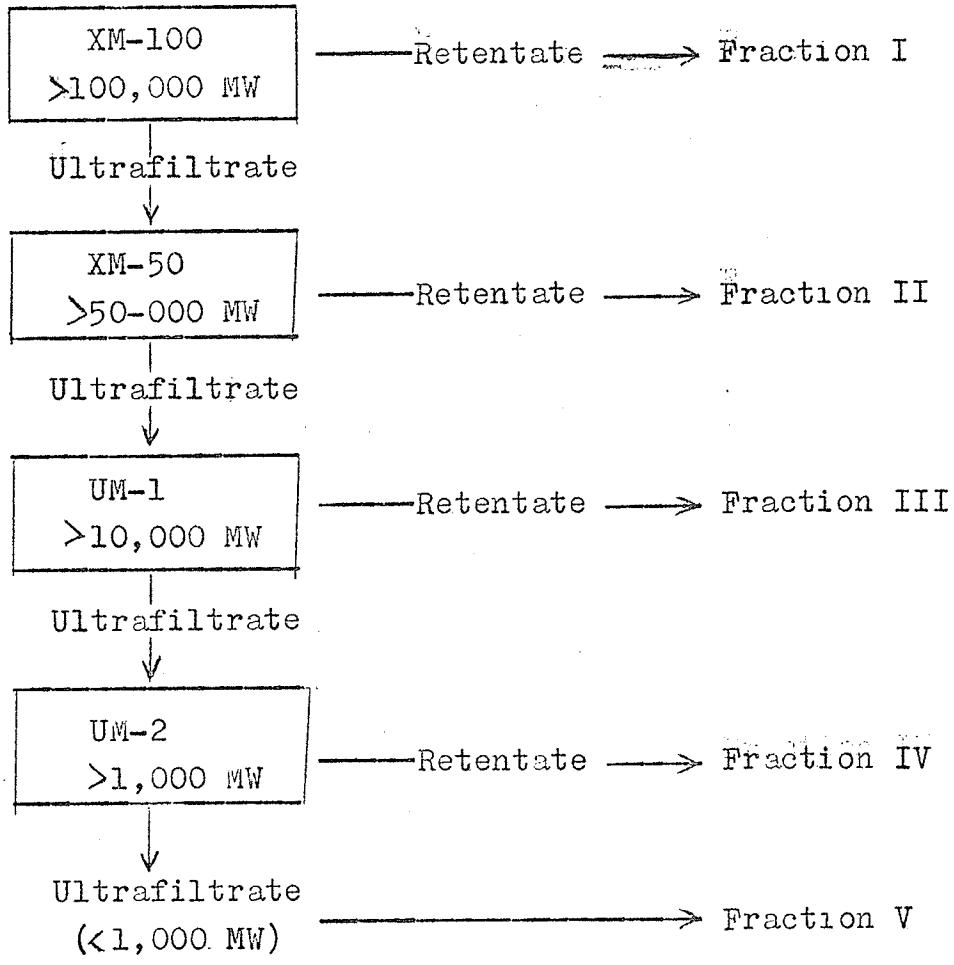
saturated ammonium sulfate were prepared. The fractionation was carried out at 0°C, pH 5.5.

Each precipitate was dissolved in about 20 ml. of distilled water and dialysed against 50 volumes of 15% glycerol solution for 48 hours at 2-3°C with two changes of dialysing solution. The glycerol solution used in the dialysis contained 125 mg of L-cysteine and 100 mg of EDTA per liter. A small amount of insoluble material formed during dialysis was removed by centrifugation for 20 min. at 10,000xg. The resulting supernatant was assayed for enzyme activity and protein content. The starting enzyme preparation and the four fractions obtained by ammonium sulfate fractionation were examined by disc electrophoresis.

Fractionation by Membrane Ultrafiltration. The fraction that precipitated between 40-80% saturation of ammonium sulfate was collected and lyophilized. One gram of the lyophilized material was dissolved in 100 ml. of 0.05M sodium acetate buffer (pH 5.5) containing 12.5mg L-cysteine and 10 mg EDTA. The solution was dialysed against two 2-l. volumes of the same acetate buffer for 48 hours. The insoluble residue that precipitated during dialysis was removed by centrifugation.

Fifty ml. of the supernatant was used for ultrafiltration. Fractionation by membrane ultrafiltration was carried out as shown in Fig. 1. The supernatant (50 ml.) was placed into the Amicon ultrafiltration cell fitted with a XM-100

Figure 1. Fractionation by Membrane Ultrafiltration Enzyme Solution



membrane, and filtered with nitrogen pressure of 15 p.s.i. at 2-3°C. After 80% of the solution had passed through the membrane, 40 ml. of sodium acetate buffer (pH 5.5) was added to the solution in the cell. Ultrafiltration was continued until the volume was reduced to about 10 ml. The solution remaining on the XM-100 membrane (fraction I) was withdrawn from the cell with a syringe. The filtrates were combined and filtered on a XM-50 membrane with 30 p.s.i. of nitrogen pressure. The final 10 ml. (fraction II) was withdrawn from the cell. Two further fractions (fractions III and IV) were prepared similarly using membranes UM-1 and UM-2 and 50 p.s.i. of nitrogen pressure. The filtrate that passed through membrane UM-2 will be referred to as fraction V. The protein contents and proteolytic activities of these five fractions as well as that of the original enzyme solution were determined. The five fractions were also examined by disc electrophoresis.

Molecular Sieve Chromatography. Standard procedures were used to prepare the Sephadex Columns (96). Twenty g. of dry Sephadex G-100 powder was added slowly to 500 ml. distilled water with continuous stirring. The fines were removed by decantation. The Sephadex was allowed to swell for 48 hours at room temperature. During this time the gel was stirred and decanted intermittently. Before the gel was poured into the column, it was equilibrated with 0.05M

sodium acetate buffer (pH 5.5). The swollen gel was mixed with enough eluant to permit the trapped air bubbles to rise to the surface after the column was filled. The column was equilibrated by allowing 2 to 3 column volumes of eluant to pass through the Sephadex. A constant flow rate was maintained by using a mariotte bottle to provide a constant pressure head. Five ml. of 0.2% Blue Dextran 2000 was passed through the column to check the homogeneity of the packing and to determine the exclusion volume before the start of each experiment. Sephadex G-75 column was prepared and packed in a similar manner. The chromatographic separation was carried out in a cold room (2-3°C).

Preparation of Carboxymethyl Cellulose Columns. Fifty g. of CM-cellulose 32 was weighed out, mixed with 750 ml. of 0.5N sodium hydroxide solution, and allowed to equilibrate for 30 min. This slurry was filtered and the resin was washed with water until the pH of the filtrate reached a value of 8.0. The resin was then stirred into 750 ml. of 0.5N hydrochloric acid and left for 30 min. The acid treatment was repeated once and this was followed by washing with water until the filtrate pH was near neutral. The washed resin was equilibrated with 20 volumes of starting buffer, and allowed to settle for 15 to 30 min. The supernatant liquor containing the fines was decanted. This equilibration step was repeated at least 6 times.

After the final equilibration, the pH of the filtrate was checked to ensure it was the same as that of the starting buffer solution. The prepared ion exchange resin was mixed with a relatively large volume of starting buffer. This mixture was degassed by slight suction and poured into the column to the desired height.

After each experiment, the column was washed with 0.1N sodium hydroxide solution to remove any residual proteins. Finally, it was re-equilibrated with the starting buffer. The size of the column used was 2.0 X 40 cm., and the starting buffer was 0.05M sodium acetate solution (pH 5.5) containing 125 mg. cysteine and 100 mg. of EDTA per liter of buffer solution. A linear salt gradient (from zero to 0.5M NaCl) used in the elution was produced by an ISCO Dialagrad gradient apparatus.

Disc Electrophoresis on Polyacrylamide Gels. Disc electrophoresis was carried out on 7.5% polyacrylamide gels at pH 4.0 as described in the previous section.

Determination of Protein Content. Protein content of all solutions was determined by the Lowry method (92) using gamma-globulin as the standard. The protein concentration in the effluent from chromatographic columns was measured as optical density at 280 mμ with a Zeiss PMQII Spectrophotometer.

Proteolytic Activity Assay. Proteolytic activity was assayed as described in the previous section.

Results and Discussion

Fractional Precipitation with Ammonium Sulfate. Table 1 gives the protein contents, proteolytic activities, and the degree of purification of the original extract and the five fractions obtained by precipitation with ammonium sulfate. Most of the activity was in the fractions precipitated by 40-60% and 60-80% saturation of ammonium sulfate. The specific activities of these two fraction were 1.7 and 1.5 times that of the original extract. Total recovery of protein in this fractionation was about 84%. The fraction that precipitated between 40 and 80% saturation contained 38% of the protein and 60% of the proteolytic activity. Total activity recovered in the five fractions was 66%. Apparently some inactivation occurred during the fractionation.

Disc electrophoresis was used to examine the homogeneity of the fractions obtained by ammonium sulfate precipitation (Fig. 2). For discussion purposes, the bands can be divided into four groups A, B, C, and D of similar mobility. All the major bands of the original extract were also present in the two active fractions but some of the bands were considerably more concentrated in these fractions. Groups B, C, and D appear to be concentrated in the 40-60% saturated ammonium sulfate, while C and D were concentrated in the 60-80% ammonium sulfate saturation. The results of Fig. 2 showed that the two enzymatically active fractions

Table 1. Fractionation of Proteolytic Activity with Ammonium Sulfate

$(NH_4)_2SO_4$ % Saturation	Protein Content	Specific Activity	Degree of Purification
	mg	(PU/mg) X 10^3	
Extract (10%)	242	1.14	1.0
Precipitates			
10 - 20%	30	0.20	0.2
20 - 40%	59	0.23	0.2
40 - 60%	40	1.95	1.7
60 - 80%	49	1.71	1.5
80 - 100%	22	0.007	0.0

Figure 2. Disc Electrophoretic Patterns of Fractions Obtained by Ammonium Sulfate Fractionation

(0) - 10% saturated ammonium sulfate extract

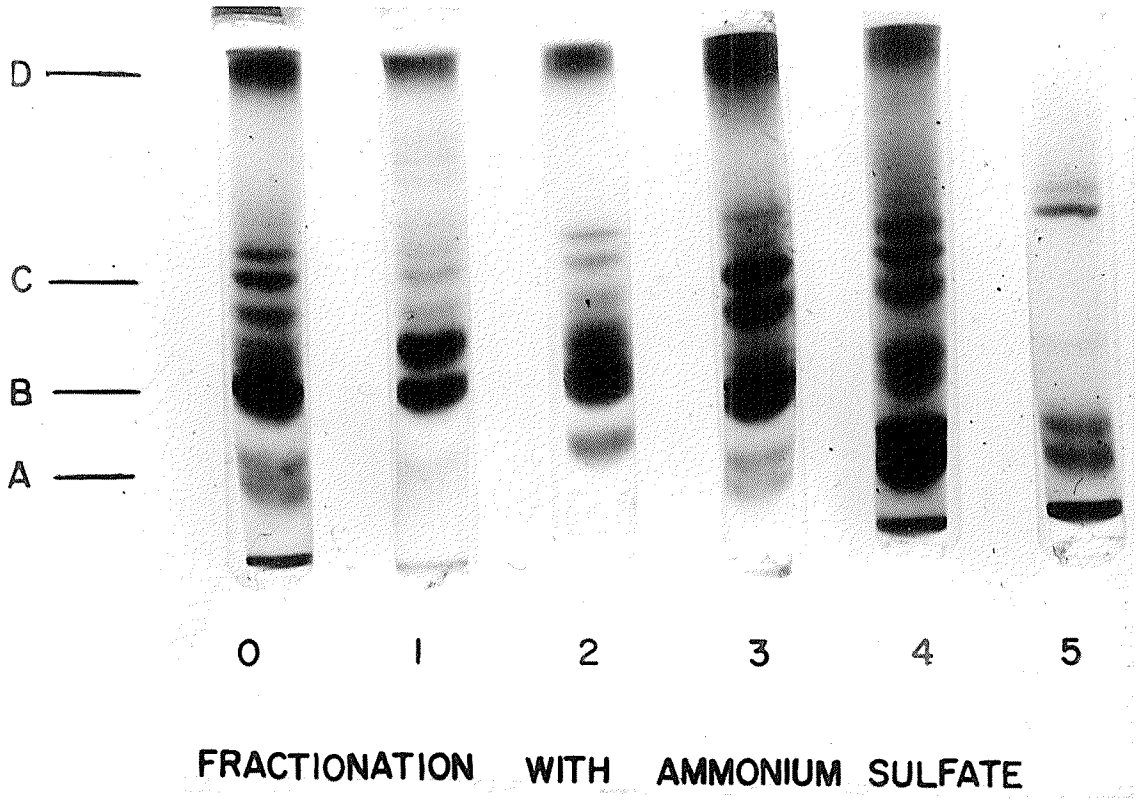
(1) - 10% to 20% saturated $(NH_4)_2SO_4$ ppt.

(2) - 20 to 40% "

(3) - 40 to 60% "

(4) - 60 to 80% "

(5) - 80 to 100% "


D
C
B
A
0
1
2
3
4
5
FRACTIONATION WITH AMMONIUM SULFATE

were still extremely heterogeneous. Accordingly, no attempts were made to characterize these enzyme preparations any further.

Fractionation by Membrane Ultrafiltration. Membrane ultrafiltration has proven to be a time saving device for a rough fractionation of protein components in a mixture(97). With some proteins, considerable purification has been obtained by this fractionation technique. In the present study, this technique, employing membranes of gradually finer porosity, was used in an attempt to purify further the enzymes in the fraction obtained by precipitation between 40 and 80% saturation of ammonium sulfate. Results of this fractionation are summarized in Table 2.

Most of the proteolytic activity was found in fractions I and II. These fractions comprise species of molecular weight larger than 50,000. The specific activities of fractions I and II, were 1.3 and 1.2 times that of the starting solution (fraction obtained with 40-80% saturation of ammonium sulfate) and contained 35 and 62% of the total activity respectively. Although the degree of purification of the fractions obtained by ultrafiltration was low, the recoveries of protein and proteolytic activity were almost 100%.

The homogeneity of the fractions obtained by membrane ultrafiltration was examined by disc electrophoresis (Fig. 3).

Table 2. Fractionation of Proteolytic Activity with Membrane Ultrafiltration

Fraction	Range of Mol. Wt.*	Protein Content	Specific Activity	Degree of Purification
		mg	(PU/mg) X 10^3	
Original solution	--	40.75	1.78	1.0
I	100,000	12.20	2.32	1.3
II	50,000-100,000	19.60	2.26	1.2
III	10,000-50,000	6.05	0.15	0.1
IV	1,000-10,000	1.15	0.00	0.0
V	1,000	--	--	--

* Molecular weights based on membrane specifications.

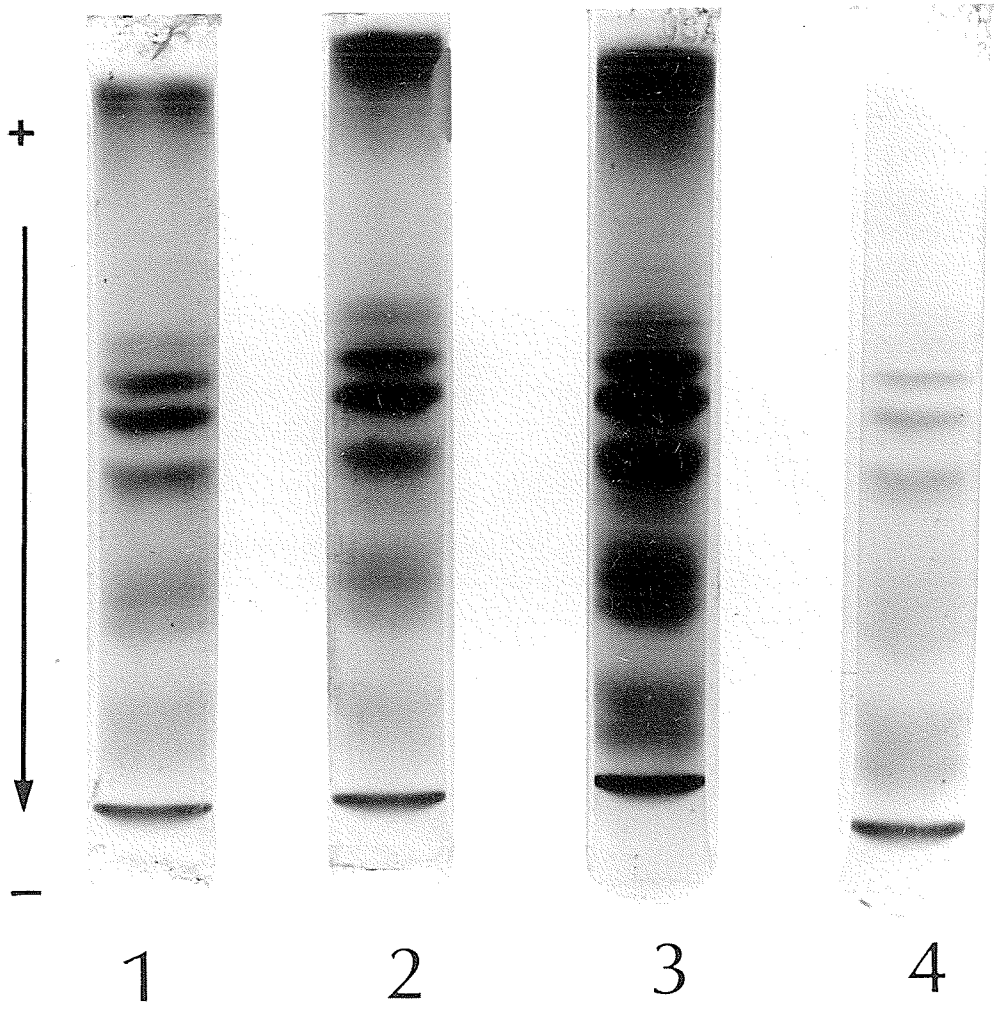
Figure 3. Disc Electrophoretic Patterns of Fractions Obtained by Membrane Ultrafiltration

(1) 40 - 80% sat'd $(NH_4)_2SO_4$ ppt.

(2) Fraction I

(3) Fraction II

(4) Fraction III


+
−
1
2
3
4

Fractions IV and V did not show any protein bands and thus are not included in Fig. 3.

In comparison to the original solution, fraction I (Tube 2) showed a high concentration of relatively slow moving protein components of groups C and D (see Fig. 2) and very small amounts of the faster moving protein components of groups A and B (see Fig. 2). It is presumed that these minor bands represent low molecular weight proteins which were not removed by ultrafiltration. Fraction II (Tube 3), which had the highest protein content, contained relatively high concentrations of proteins of groups B, C, and D. Fraction III (Tube 4), which had very little proteolytic activity, contained mainly components of the fast moving group A. Most of the proteolytic activity seems to be associated with the slow moving protein components of groups B, C, and D. These groups also represent the species of high molecular weight.

In summary, membrane ultrafiltration produces some fractionation but only a small degree of purification. The resolution was very low and there was considerable overlap between consecutive fractions. Disc electrophoresis showed that fractions obtained by this technique are highly heterogeneous. To produce relatively homogeneous fractions by fractionation on the basis of molecular weight, the technique must have a higher resolution than that obtainable by membrane ultrafiltration. Accordingly gel filtration on Sephadex columns was adopted for further purification.

Fractionation by Gel Filtration on Sephadex. The proteolytically active fraction that precipitated between 40-80% saturated ammonium sulfate was fractionated by gel-filtration on Sephadex G-75 (Fig. 4). By this procedure, the proteins in the starting solution were separated into five protein peaks. Most of the proteolytic activity appeared in the first two peaks. The second peak (fractions 16 to 24) had the highest activity. Peaks I and II were both eluted in the void volume of the column. Accordingly, the molecular weights of the proteins represented by these peaks must be higher than 50,000. These results agree with the approximate molecular weights obtained by ultrafiltration. Fractionation on Sephadex G-75 produced some fractionation but the active peaks were still quite heterogeneous.

The next step in the use of gel filtration was to fractionate fraction II obtained by membrane ultrafiltration. Sephadex G-100 was used for this fractionation. The curves representing protein content and proteolytic activity by this fractionation are shown in Fig. 5. The shape of this curve is similar to that of Fig. 4, except that the separation on Sephadex G-100 in the region of high molecular weight seems to be slightly better than on G-75. However, the protein fractions that contained the proteolytic

FIGURE 4. Separation of Proteolytic Enzymes from 40-80% Saturated Ammonium Sulfate Precipitate on Sephadex G-75.

Eluting Buffer- 0.05M sodium acetate buffer, pH 5.5 containing 125mg of L-cysteine and 100 mg of EDTA per liter of solution.

Bed- 2.5 X 40 cm

Flow rate - 12 ml/hr.

Sample - 6ml of 40-80% saturated ammonium sulfate ppt.

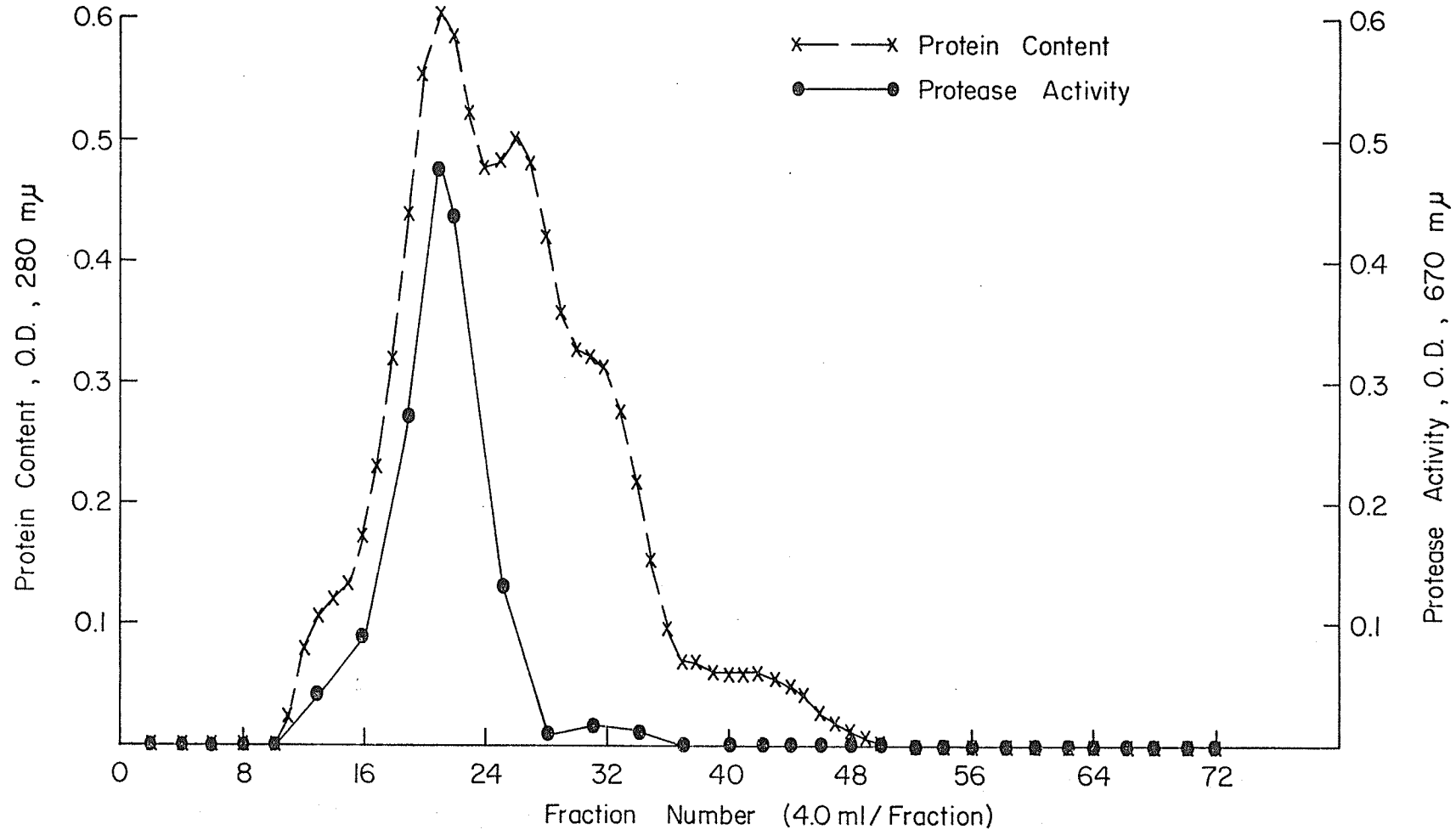

Protein Content
Protease Activity
Protein Content , O.D. , 280 mμ
Protease Activity , O.D. , 670 mμ
0.6
0.5
0.4
0.3
0.2
0.1
0
8
16
24
32
40
48
56
64
72
Fraction Number (4.0 ml / Fraction)

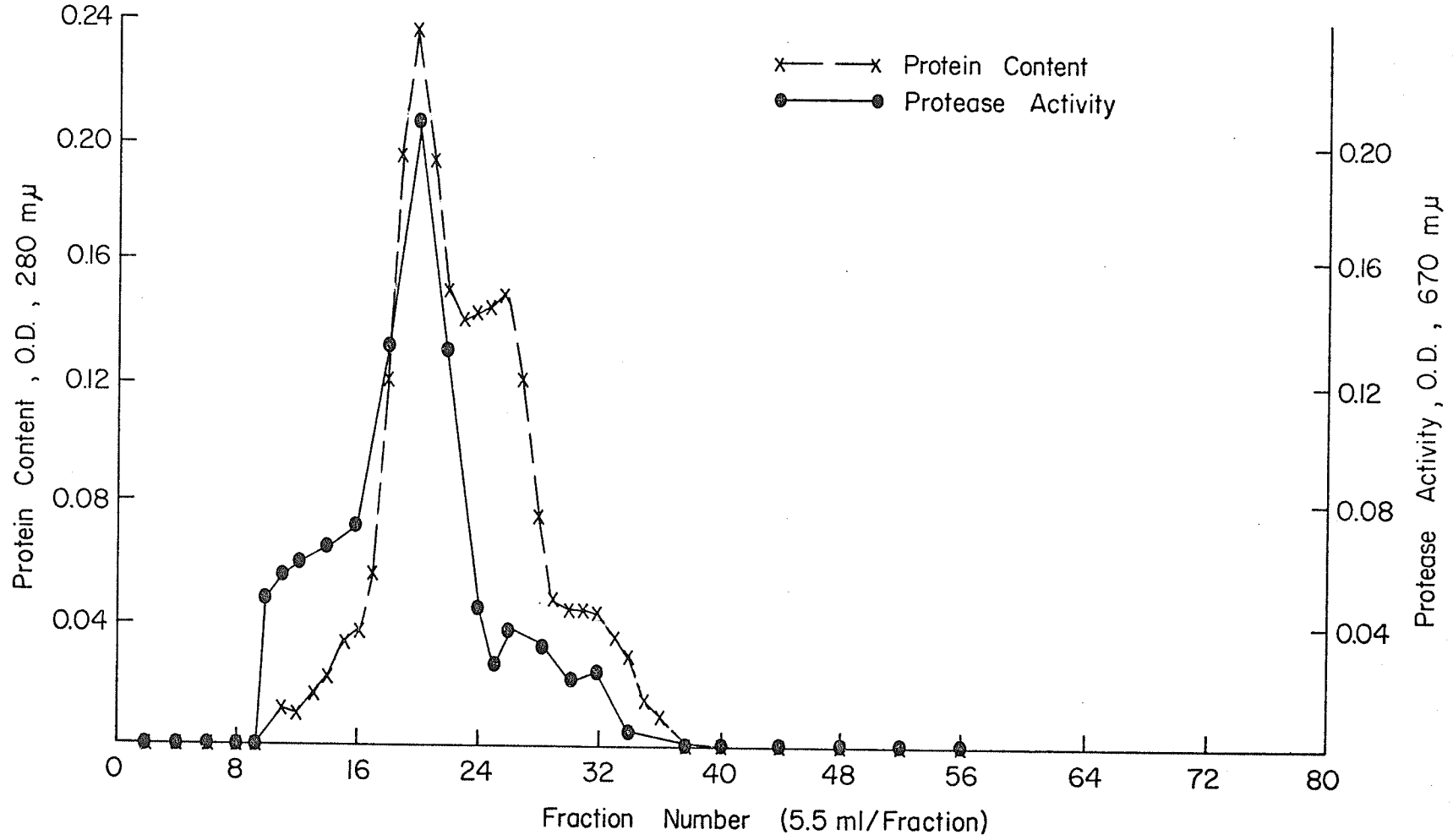
FIGURE 5. Separation of Proteolytic Enzymes of Fraction II Obtained from Membrane Ultrafiltration on Sephadex G-100.

Eluting Buffer- 0.05M sodium acetate buffer, pH 5.5 containing 125 mg of L-cysteine and 100 mg of EDTA per liter of solution.

Bed- 2.5 X 40 cm

Flow Rate- 5.5 ml/hr.

Sample- 5 ml of fraction II obtained from membrane ultrafiltration.


Protein Content
Protease Activity
Protein Content , O.D. , 280 mμ
Protease Activity , O.D. , 670 mμ
0.24
0.20
0.16
0.12
0.08
0.04
0
8
16
24
32
40
48
56
64
72
80
Fraction Number (5.5 ml/Fraction)

activity obtained with Sephadex G-100 were also highly heterogeneous when examined by disc electrophoresis.

Purification by Ion Exchange Chromatography on Carboxymethyl Cellulose. The active fraction obtained by 40-80% saturation of ammonium sulfate, was fractionated by ion exchange chromatography on CM-cellulose (Fig. 6). The column was first eluted with 0.05M sodium acetate buffer of pH 5.5. This was followed by elution with a sodium chloride gradient increasing linearly from zero to 0.5M. Elution was continued with the final salt concentration until no more protein was detected in the effluent. This fractionation produced two major peaks (A and B) and one minor peak (C) which had proteolytic activity. Peak A was the break-through peak and was not adsorbed on the CM-cellulose. Peak B was adsorbed and eluted with about 0.3M salt concentration. Peak C which followed immediately after peak B contained very little proteolytic activity.

Table 3 shows the specific activity and the degree of purification of the three peaks obtained by ion exchange chromatography on CM-cellulose. Peak A which had the highest protein content had a specific activity of 1.53 times that of the original enzyme solution. The specific activities of peaks B and C were 1.9 and 0.25 times that of the original enzyme solution respectively.

Figure 6. Chromatography of Proteolytic Enzymes on CM-Cellulose. The solid line represents the protein concentration measured at 280 mu whereas the dashed line represents the proteolytic activity measured at 670 mu. The column was first eluted with 0.05M sodium acetate buffer of pH 5.5 and then with a sodium chloride gradient from 0 to 0.5M and with the final salt concentration until no further protein was eluted. The salt concentration is represented by the dotted line. Flow rate was 15 ml/hr.

PROTEIN CONTENT (——), O.D. at 280mμ
PROTEOLYTIC ACTIVITY (- - - -), O.D. at 670mμ
NaCl CONCENTRATION (M)
FRACTION NUMBER (4ml.)
A
B
C

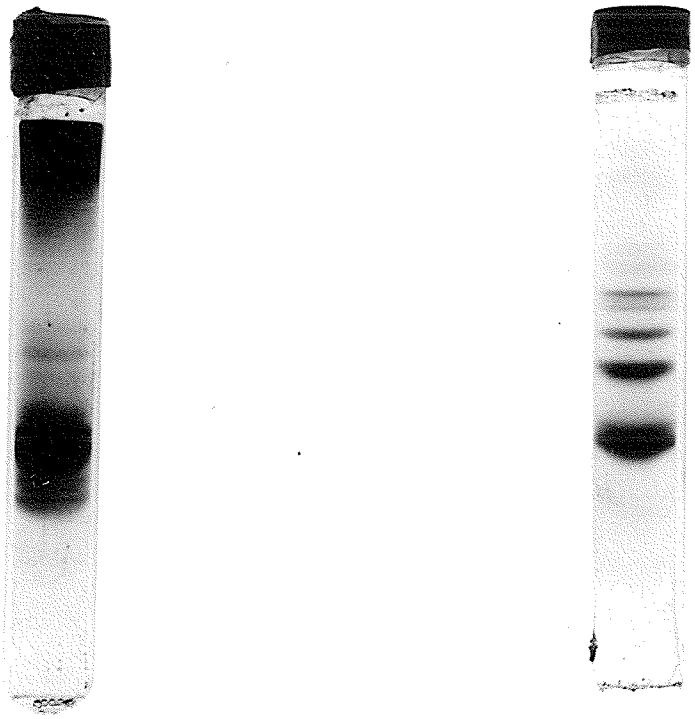
Table 3. Purification of Proteolytic Enzymes by CM-Cellulose Column Chromatography

Fraction	Specific Activity	Protein Content	Degree of Purification
	(P.U./mg.) X 10^3	mg	
Original Solution	1.78	--	1.0
Fraction A	2.76	24.7	1.53
Fraction B	3.39	12.4	1.90
Fraction C	0.445	22.4	0.25

The tubes that contained the proteins represented by A and B were pooled separately, concentrated by ultrafiltration, and examined by disc electrophoresis. Figure 7 shows the patterns of Fractions A and B obtained from CM-cellulose chromatography. Fraction A comprised two major bands and a number of minor bands. One of the major bands has moderate mobility whereas the other is a slow moving band which appeared near the origin.

A comparison of the results obtained here with those obtained by ammonium sulfate fractionation and membrane ultrafiltration, suggests that the two major protein bands of Fraction A represent most of the proteolytic activity of the original extract. On the other hand Fraction B contained only one major band with a moderate mobility.

Figure 7. Disc Electrophoretic Patterns of Fractions A and B Obtained from CM-Cellulose Column Chromatography



A B

In addition, this fraction showed three to four minor, slower-moving bands. Since both fractions (A and B) contain more than one protein band, it is difficult to identify which of the protein bands contain proteolytic activity. These fractions are still relatively heterogeneous. Further purification will be required before attempting to characterize the enzyme(s) in these fractions.

III. Purification by Affinity Chromatography on Hemoglobin-Sepharose Column (102)

Abstract

A rapid chromatographic procedure for the isolation and purification of proteases from malted wheat flour is described. The separation was achieved by passing a relatively crude preparation through a hemoglobin-Sepharose column. Unadsorbed proteins were eluted with the starting buffer. The adsorbed proteases were then eluted with 0.1N acetic acid. Recoveries of proteins and proteolytic activity were over 90%. A two-fold increase in specific activity was achieved by this purification technique. Disc electrophoretic analyses showed that all of the non-proteolytic components were separated from the proteolytically active proteins. The active peak comprised three major and one minor proteins of similar mobility.

Introduction

Difficulties encountered in the isolation and purification of wheat proteases have prevented the fundamental characterization of these technologically important enzymes. The main difficulty appears to be due to the very low proteolytic activity of wheat (42) and the tendency of its proteases to aggregate with other proteins (89, 98). Attempts to purify the proteolytic enzymes in malted wheat

flour with a number of standard procedures described in the first two section were not very successful.

Recently Cuatrecasas et al.(99) have purified several enzymes by first adsorbing them on Sepharose containing covalently bound substrate analogues. This section will describe an analogous chromatographic procedure which was used successfully in the purification of wheat proteases. The insoluble support material was also Sepharose but it contained covalently linked hemoglobin. With this support material proteins other than proteases passed directly through the column, whereas the proteolytic enzymes which have an affinity to form a complex with hemoglobin were adsorbed and subsequently eluted by changing the pH of the eluting buffer solution.

Materials

Sepharose 4B was obtained from Pharmacia, cyanogen bromide (MP 50-52°C) from Matheson Coleman & Bell Company, and hemoglobin (lyophilized, salt free) from Mann Research Laboratory Inc. Other chemicals used were of reagent grade and the water was distilled and demineralized.

Methods

Preparation of Enzyme Solutions. The starting enzyme solution was obtained by extracting malted wheat flour with four volumes of 10% saturated ammonium sulfate solution.

The crude extract was immediately fractionated by precipitation with ammonium sulfate. The fraction that precipitated between 40-80% saturation of ammonium sulfate was collected and lyophilized. One gram of the lyophilized material was dissolved in 100 ml. of the acetate buffer of pH 5.5. The solution was dialysed against two 2-l. volumes of the same buffer for 48 hours at 2-3°C. The insoluble residue which precipitated during dialysis was removed by centrifugation. Portions of the supernatant were concentrated by ultrafiltration before application to the column.

Protein Content Determination. Protein content of all solutions was determined by the Lowry method (92) using gamma-globulin as standard. The protein in the effluents from chromatographic columns was measured as optical density at 280 mu with Zeiss PMQII spectrophotometer.

Proteolytic Activity Determinations. Proteolytic activity was measured by the Ayre-Anderson method as modified by McDonald and Chen (70). The procedure was described in detail in the previous section.

Preparation of Hemoglobin-Sepharose Column. The procedure described by Cuatrecasas et al.(99) was used to activate the Sepharose with cyanogen bromide. The Sepharose suspension (125 ml) was mixed with an equal volume of water, and 12.5 g. of cyanogen bromide, dissolved in 125 ml of water, was then added to the Sepharose. The pH was adjusted immediately to 11 by titration with 4N sodium hydroxide. After ten

minutes, the activated Sepharose was washed with 20 volumes of cold 0.1N sodium bicarbonate solution of pH 9.0, and 50 ml of 4% hemoglobin solution was added quickly to the Sepharose. This mixture was stirred gently for 24 hours and was washed extensively with water. Finally it was poured into the column and equilibrated with the starting buffer solution used to dissolve the crude, lyophilized enzyme preparation. The column was prepared and run in a cold room (2-3°C).

Disc Electrophoresis. Disc electrophoresis on 7.5% polyacrylamide gel was carried out at pH 4.0 as described in the previous section.

Results and Discussion

When the crude enzyme solution was chromatographed on the unsubstituted Sepharose column (Fig. 1) all the protein and the enzyme activity was eluted as a single peak with 0.05M acetate buffer of pH 5.5. No additional protein or enzyme activity was eluted when the eluent was changed to 0.1N acetic acid of pH 3.1. The recoveries of protein and enzyme activity were close to 100%. These results indicate clearly that the enzyme proteins did not have substantial affinity for the unsubstituted Sepharose column.

Results obtained with the hemoglobin-Sepharose column are shown in Fig. 2. Two protein peaks were obtained in

Figure 1. Protein content and protease activity curves for fractions from crude extract separated on an unsubstituted Sepharose column (1.5 X 12 cm) using 0.05M acetate buffer of pH 5.5 as the starting eluant. Volume of extract used was 3 ml and elution flow rate was 50 ml/hr.

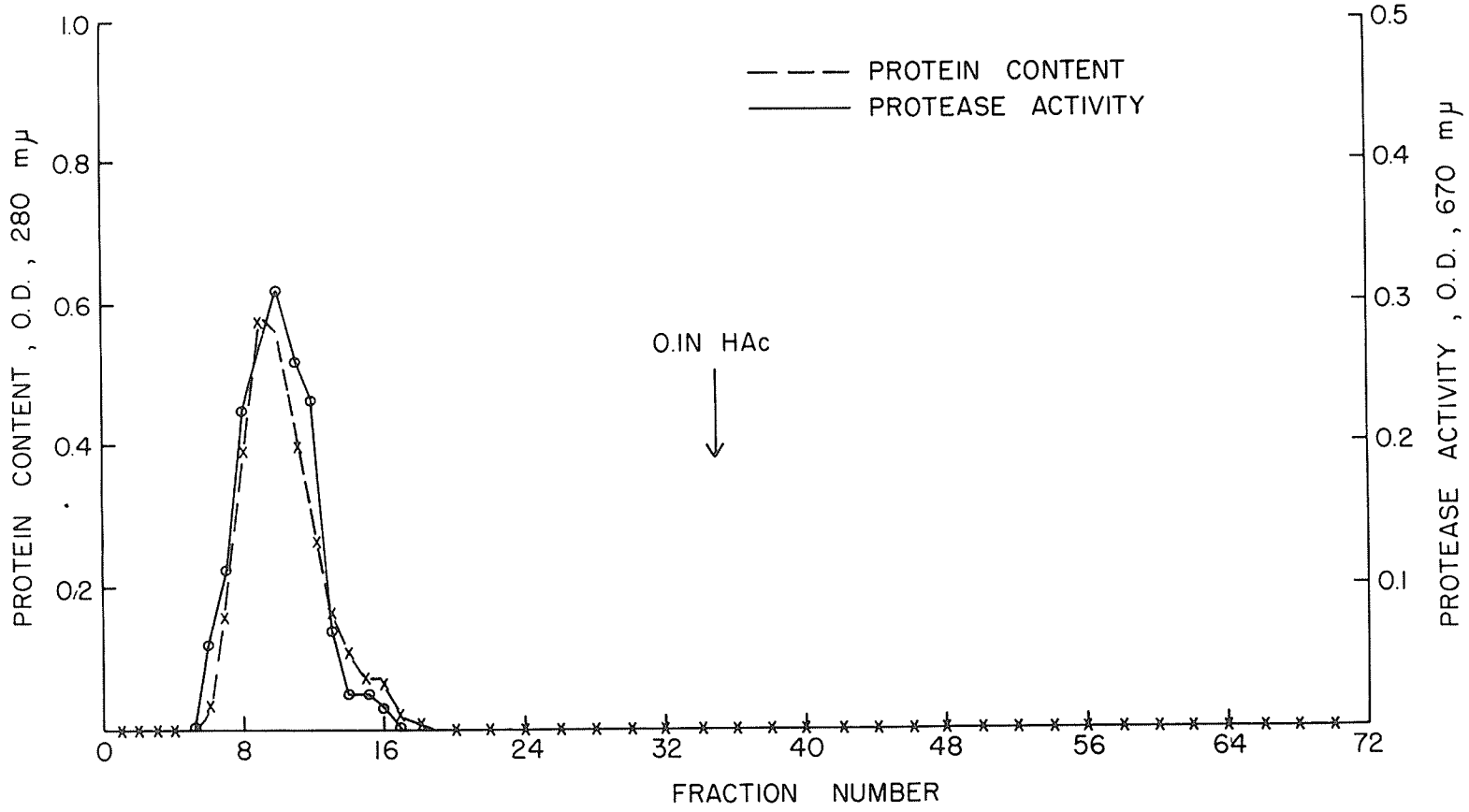
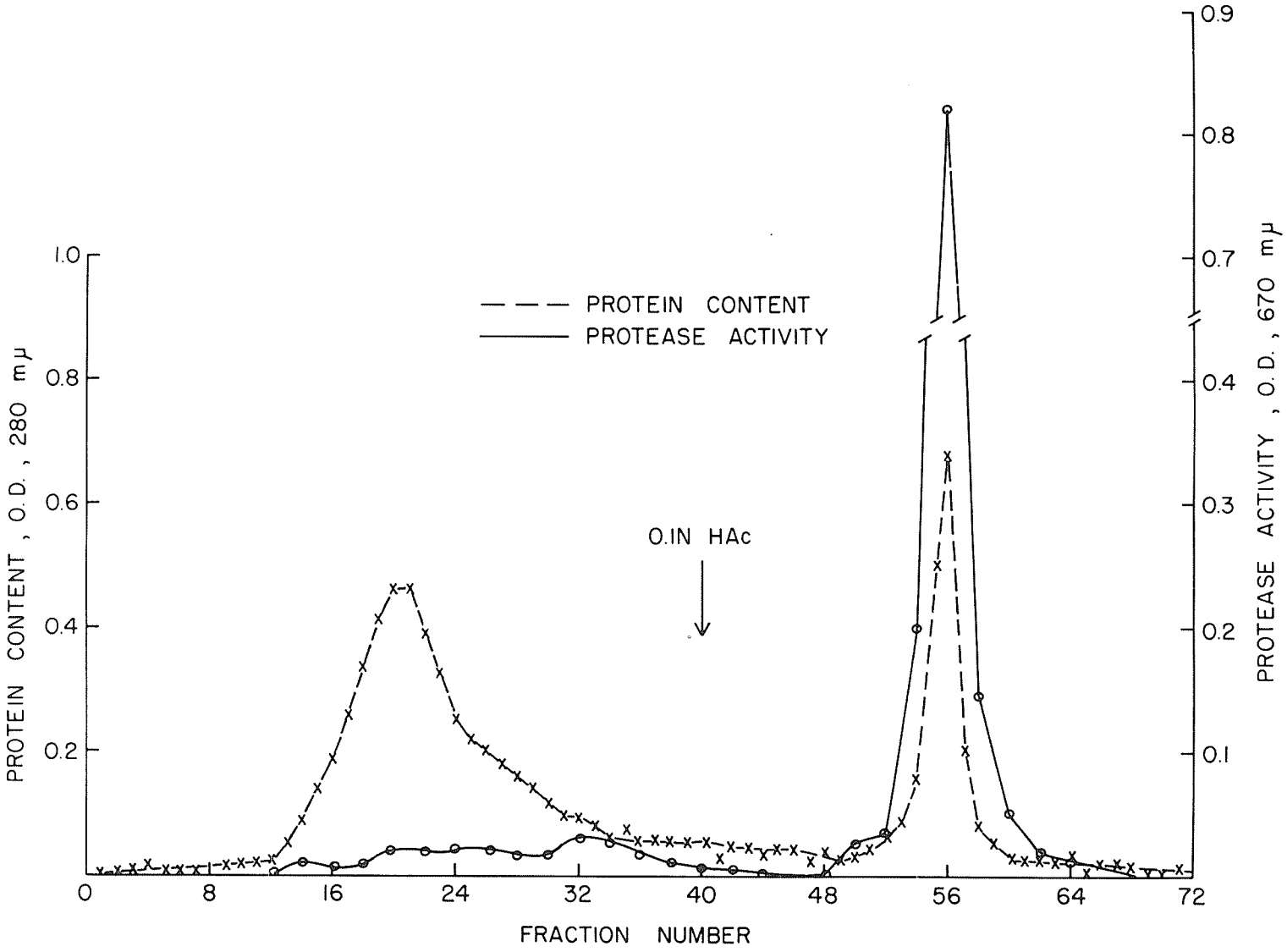

PROTEIN CONTENT
PROTEASE ACTIVITY
0.1N HAc
PROTEIN CONTENT , O.D. , 280 mμ
PROTEASE ACTIVITY , O.D. , 670 mμ
FRACTION NUMBER
1.0
0.8
0.6
0.4
0.2
0.5
0.4
0.3
0.2
0.1
0
8
16
24
32
40
48
56
64
72

Figure 2. Protein content and protease activity curves for fractions from crude extract separated on a hemoglobin-Sepharose column (1.5 X 18 cm) using 0.05M acetate buffer of pH 5.5 as the starting eluant. Volume of extract used was 5 ml and elution flow rate was 50 ml/hr.


PROTEIN CONTENT
PROTEASE ACTIVITY
0.1N HAc
PROTEIN CONTENT , O.D. , 280 mμ
PROTEASE ACTIVITY , O.D. , 670 mμ
FRACTION NUMBER
0
8
16
24
32
40
48
56
64
72
0.2
0.4
0.6
0.8
1.0
0.1
0.2
0.3
0.4
0.7
0.8
0.9

the chromatogram. The first peak, which was eluted with starting buffer solution (pH 5.5), contained about 55% of the total protein applied to the column, but only a small portion (about 7%) of the proteolytic activity. This activity was associated with the tailing portion of this peak and was probably due to the removal of some of the adsorbed enzyme from the support material by the relatively high flow rate of elution. The majority of the proteolytic activity (93%) had a strong affinity for the bound hemoglobin, and remained on the column. The adsorbed enzyme activity could not be eluted from the column by acetate buffer (pH 5.5) or acetate buffer containing 0.5M NaCl. With 0.1N acetic acid of pH 3.1 as eluant, the adsorbed enzyme protein emerged sharply and was collected in few tubes. This enzyme peak contained about 45% of the total proteins. The specific activity of this enzyme protein after the fractions had been pooled was two times that of the original enzyme preparation that was applied to the column.

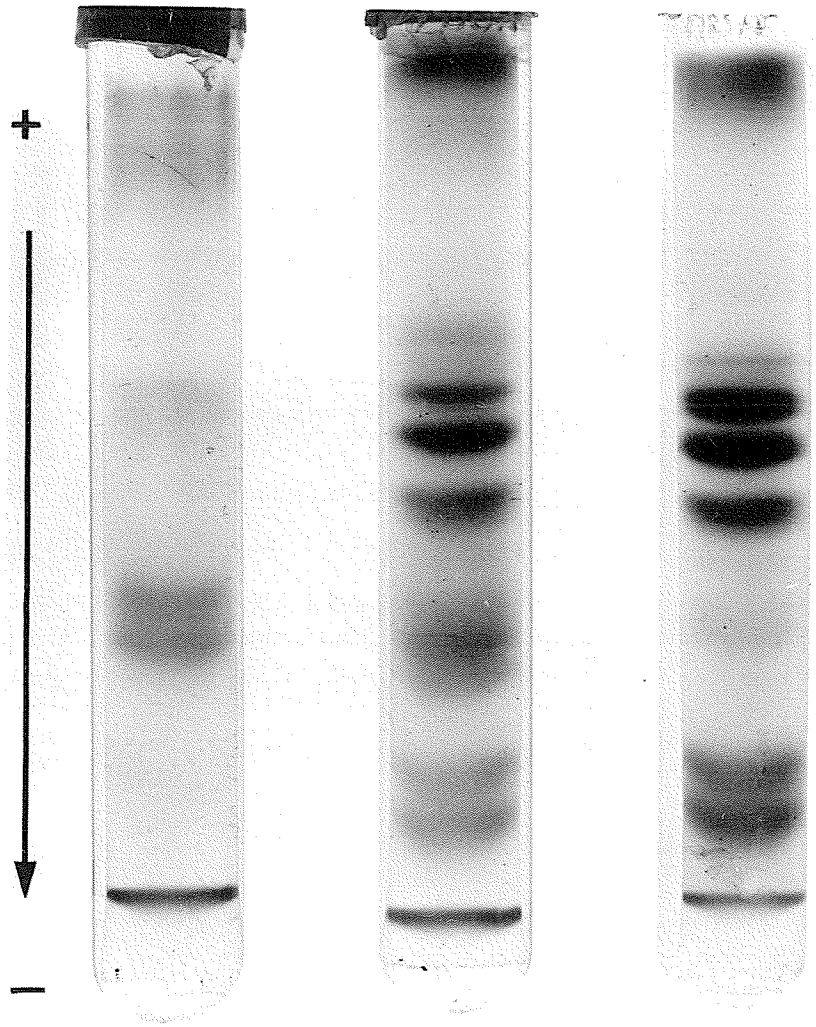
At pH 4.0 (acetate buffer) the enzyme did not have a strong affinity for the hemoglobin on the column and therefore most of the protein and proteolytic activity was eluted with the starting buffer. At pH 8.0 (Tris-HCl buffer) most of the enzyme activity was lost. Accordingly, 0.05M acetate buffer of pH 5.5 was adopted as the starting buffer.

The homogeneity of the proteolytically active fraction

was examined by disc electrophoresis (Fig. 3). The active peak still contains three or four distinguishable protein bands of slightly different mobilities. However, a clear cut separation was achieved as can be seen from a comparison of the pattern for the active peak (Tube 1) and the pattern for the original enzyme solution used in the purification (Tube 2). The electrophoretic pattern for the protein peak eluted from hemoglobin-Sepharose column with the acetate buffer of pH 5.5 (Tube 3) has all the bands of the crude extract except the bands of the active peak.

The disc electrophoretic pattern of the active peak does not contain any extra proteins not present in the crude enzyme preparation. Thus there is no indication of contamination with hemoglobin fragments that might result from the possible proteolysis during chromatography. Whether the protein bands in the active peak are isoenzymes or other contaminants remains to be clarified.

Figure 3. Disc electrophoretic patterns for (1) the proteolytically active fraction, (2) the crude enzyme extract, and (3) the unadsorbed nonactive protein fraction obtained by fractionation on hemoglobin-Sepharose column.


+
−
1
2
3

IV. Characterization of the Proteolytic Enzyme Purified by Hemoglobin-Sepharose Affinity Chromatography

Abstract

The proteolytic enzyme preparation obtained by hemoglobin-Sepharose affininty chromatography was partially characterized. Gel filtration and ultracentrifugal measurements showed that the preparation comprised one component. The molecular weight of this component determined by chromatography on Sephadex G-150 was 34,000. The activity of this enzyme was inhibited by -SH blocking agents, and activited by reducing agents. It was not affected by soybean trypsin inhibitor. pH of its optimum activity was 3.8 with hemoglobin substrate and 7.5 with casein. The enzyme did not show BAPA-ase activity. With 1% hemoglobin as substrate, the relationship between enzyme concentration and proteolytic activity was essentially linear in the range of concentration examined. The purified enzyme from malted wheat flour appears to be a papain-type enzyme.

Introduction

The previous section described a chromatographic procedure by which a relatively homogeneous, proteolytically active enzyme preparation was obtained from the 10% saturated ammonium sulfate extract of malted wheat flour. The present section deals with a preliminary characterization of this preparation.

Homogeneity of the preparation was examined by gel filtration and ultracentrifugation. At the same time, these techniques provided a good estimate of the molecular weight of the enzyme. The effects of -SH blocking agents, reducing agents, and a natural protease inhibitor from soybeans were investigated to obtain information on the nature of the active site of this enzyme. Substrate specificity of the enzyme was studied using hemoglobin, casein, and benzoyl-DL-arginine-p-nitro-anilide. The effect of enzyme concentration on the activity with hemoglobin substrate was examined.

Materials

The enzyme was prepared by concentrating the proteolytically active fraction obtained by hemoglobin-Sepharose affinity chromatography (see previous section) by ultrafiltration using a UM-2 membrane.

p-Chloromercuribenzoic acid, sodium salt (PCMB) and 2-mercaptoethanol (ME) were obtained from Aldrich Chemical Co. Inc.; N-ethylmaleimide (NEM) from Sigma Chemical Co.; iodoacetic acid, potassium salt (IAA) from Eastman Kodak Company; mercuric chloride ($HgCl_2$) from Fisher Scientific Co.; and glutathione, reduced form (GSH) from Schwarz Bio-Research Inc. Hemoglobin, casein (Hammersten) and benzoyl-DL-arginine-p-nitro-anilide (BAPA) were obtained from Mann Research Laboratories. Blue dextran 2000 and Sephadex G-150 were from Pharmacia Canada Ltd. Soybean trypsin inhibitor(STI),

gamma-globulin, (bovine, fraction II, B grade), bovine serum albumin, (A grade), myglobin (equine heart, 2X cryst.) and cytochrome c(equine heart, salt free, A grade) were purchased from Calbiochem. Trypsin(2X cryst.) and pepsin (3X cryst.) were obtained from Nutrition Biochemical Co.

Methods

Gel Filtration. Sephadex G-150 was used as the support material. The Sephadex was equilibrated for 72 hours in 0.05M acetate buffer of pH 5.5 before it was poured into the column (2.5 X 40 cm). Five ml of the enzyme solution was applied to the column and elution flow rate was 7.0 ml/hr. Fractions (3.5 ml) were collected, and protein content and proteolytic activity of each fraction were measured. Elution volumes of the enzyme peak and six reference proteins of known molecular weights were determined for the same column in the usual manner (100). The reference proteins used were γ-globulin, bovine serum albumin, pepsin, trypsin, myoglobin and cytochrome c. The void volume of this column was determined using a 0.02% solution of blue dextran 2,000. All operations were carried out in a cold room (3°C).

Ultracentrifugation. The sedimentation pattern was obtained with a Spinco model E analytical ultracentrifuge at 60,000 rpm using a AN-H-181 rotor and 12 mm single sector cell at 20°C.

Activation and Inhibition Studies. In these experiments 0.01M solutions were used for all the substances except the

soybean trypsin inhibitor which was 1%. One ml. of each solution was added to 4 ml. aliquots of the enzyme solution and incubated at room temperature for 30 min. One ml. of each solution was assayed for proteolytic activity with hemoglobin substrate as described in previous sections.

Substrate Specificity Studies. Substrate specificity of malted wheat flour protease was examined using hemoglobin, casein and BAPA. With hemoglobin, the activity was measured in the pH range from 3.0 to 8.5 using the buffers solution given below. Casein could only be used at alkaline pH because of its low solubility in acidic conditions.

Buffer composition	pH range
Tris-HCl (0.05M)	7.5 - 8.5
KH_2PO_4 - NaOH (0.05M)	6.0 - 8.0
CH_3COOH - CH_3COONa (0.2M)	3.0 - 6.0

With the two natural substrates, 5 ml. of substrate solution was incubated with 1 ml. of the enzyme preparation for 2 hours at 37°C. Proteolytic activity was measured as described in the first experimental section.

BAPA-ase activity was determined by the procedure described by Erlanger et al (101). Trypsin was used as a standard in this assay.

Effect of Enzyme Concentration. Both the purified enzyme preparation and the 40-80% saturated ammonium sulfate precipitate were examined. One ml of each preparation, appropriately diluted with acetate buffer pH 3.8, was assayed

for proteolytic activity with hemoglobin as substrate.

Effect of Time of Incubation. The effect of incubation time on the action of malted wheat protease on hemoglobin at 37°C was examined using 1% substrate concentration and incubation times of 30, 60, 120 and 180 min. As in the normal assay, the reactions were stopped after the appropriate time by addition of TCA solution.

Results and Discussion

Gel Filtration and Ultracentrifugation Results. Gel filtration of the purified protease preparation on Sephadex G-150 gave one relatively symmetrical protein peak (Fig. 1). This single peak contained all of the proteolytic activity of the solution applied to the column. The Sephadex chromatographic column used in gel filtration was calibrated using six proteins of known molecular weight (Fig. 2). The approximate molecular weight of the malted wheat protease obtained from its effluent volume and the calibration curve is 34,000. This value is almost the same as the molecular weight of pepsin (33,400) but is somewhat lower than the value obtained for the crude enzyme preparation (see section II above). It is quite possible that in the crude preparation, the enzyme might be associated with higher molecular weight non-active proteins (39, 98) which are separated from the enzyme by affinity chromatography on the hemoglobin-Sepharose column. In molecular weight, malted wheat protease

Figure 1. Chromatography of the purified wheat protease fraction on Sephadex G-150. Sample size: 7 ml. Column size: 2.5 X 40 cm. Eluting buffer: 0.05M acetate buffer, pH 5.5. Elution flow rate: 7.0 ml/hr. Fractions of 3.5 ml were collected. Arrows show effluent volumes of reference materials used to calibrate the column: (1) Blue dextran 2000, (2) gamma-globulin, (3) bovine serum albumin, (4) pepsin, (5) trypsin, (6) myoglobin, (7) cytochrome c.

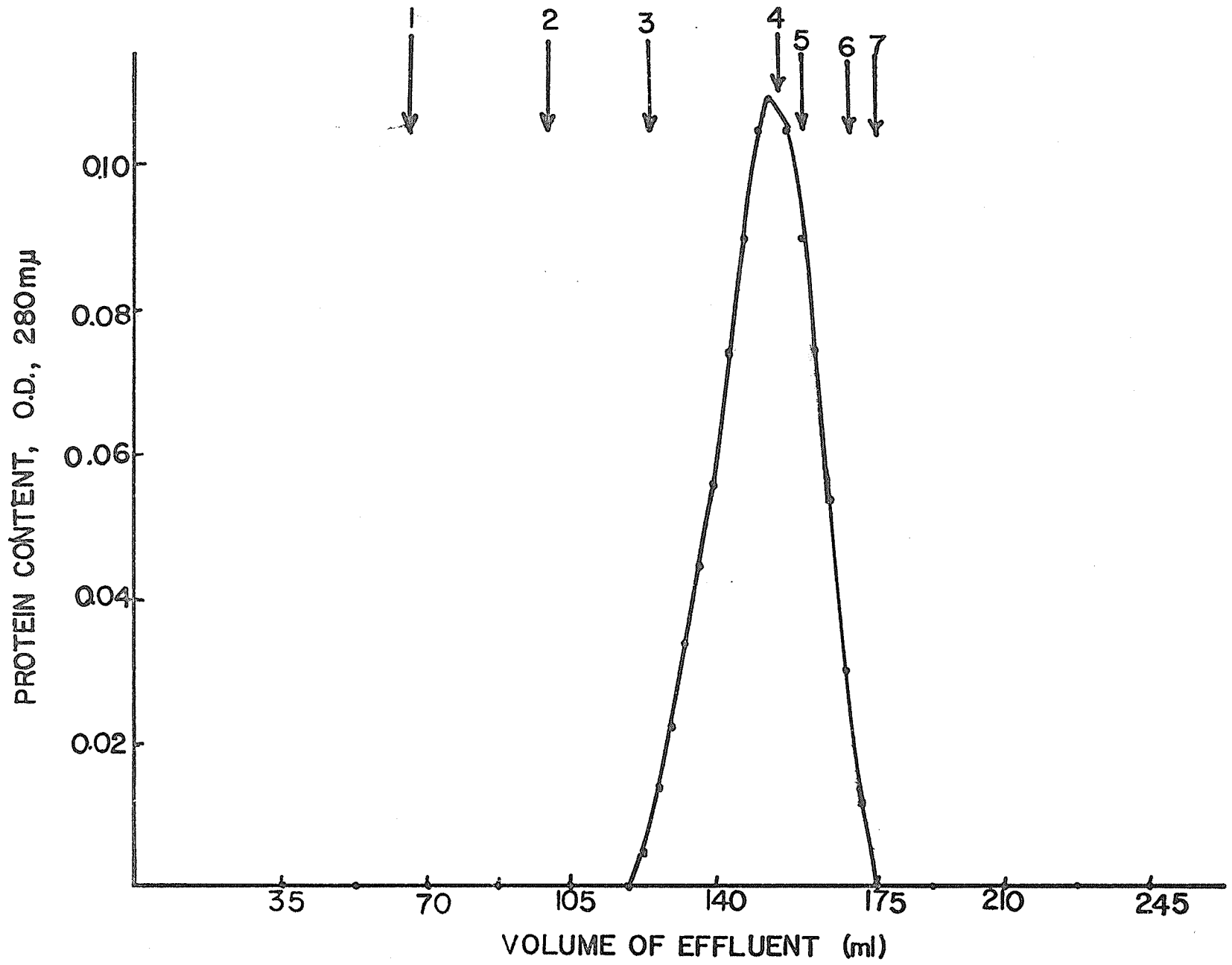
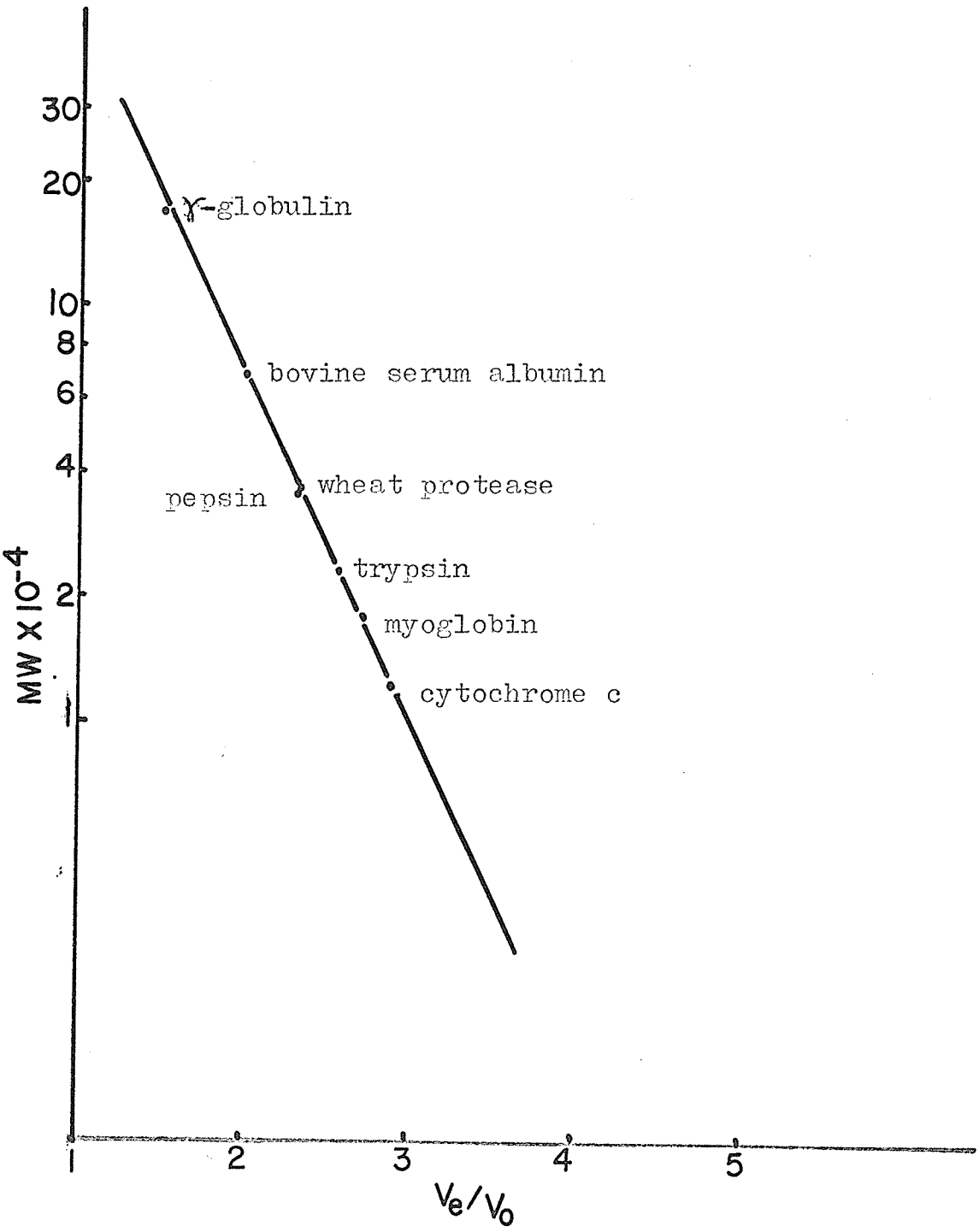

1
2
3
4
5
6
7
0.10
0.08
0.06
0.04
0.02
PROTEIN CONTENT, O.D., 280mμ
35
70
105
140
175
210
245
VOLUME OF EFFLUENT (ml)

Figure 2. Molecular weight calibration curve for Sephadex G-150 Column.


30
20
10
8
6
4
2
1
MW X 10⁻⁴
γ-globulin
bovine serum albumin
pepsin
wheat protease
trypsin
myoglobin
cytochrome c
1
2
3
4
5
Ve/Vo

is similar to other plant proteases which have molecular weights in the range from 20,000 to 40,000 (19).

The sedimentation pattern of the purified enzyme (Fig. 3) showed that the preparation was relatively homogeneous. The sedimentation coefficient ($S_{20,w}$) for this protein peak calculated from the sedimentation data was approximately 3.8 which is slightly larger than 3.2 for pepsin under identical conditions.

Disc electrophoresis results discussed in the previous section showed that the purified enzyme preparation comprised three major and one minor protein bands of similar mobility. The gel filtration and ultracentrifugation results suggest that these components, which could be isoenzymes, have similar molecular weights.

Activation and Inhibition Studies. There appears to be some controversy regarding the importance of sulfhydryl groups in active wheat protease(s) (15,75). It seems that wheat might contain two types of proteolytic enzymes: 1) those that are activated by reducing agents, and 2) those that do not require reducing agents. It was therefore of interest to examine if the purified enzyme from malted wheat flour required sulfhydryl groups for its activity. Three groups of substances were used in the experiments: the sulfhydryl blocking agents PCMB, $HgCl_2$, IAA and NEM; reducing agents GSH and ME; and natural soybean trypsin inhibitor (STI). The results of these experiments are summarized in Table 1.

Figure 3. Sedimentation pattern of purified wheat protease. Photographs, from right to left, were taken at 0, 16, 32, 48, and 64 min. after full speed (60,000 rpm) had been attained.

0
16
32
48
64
min.

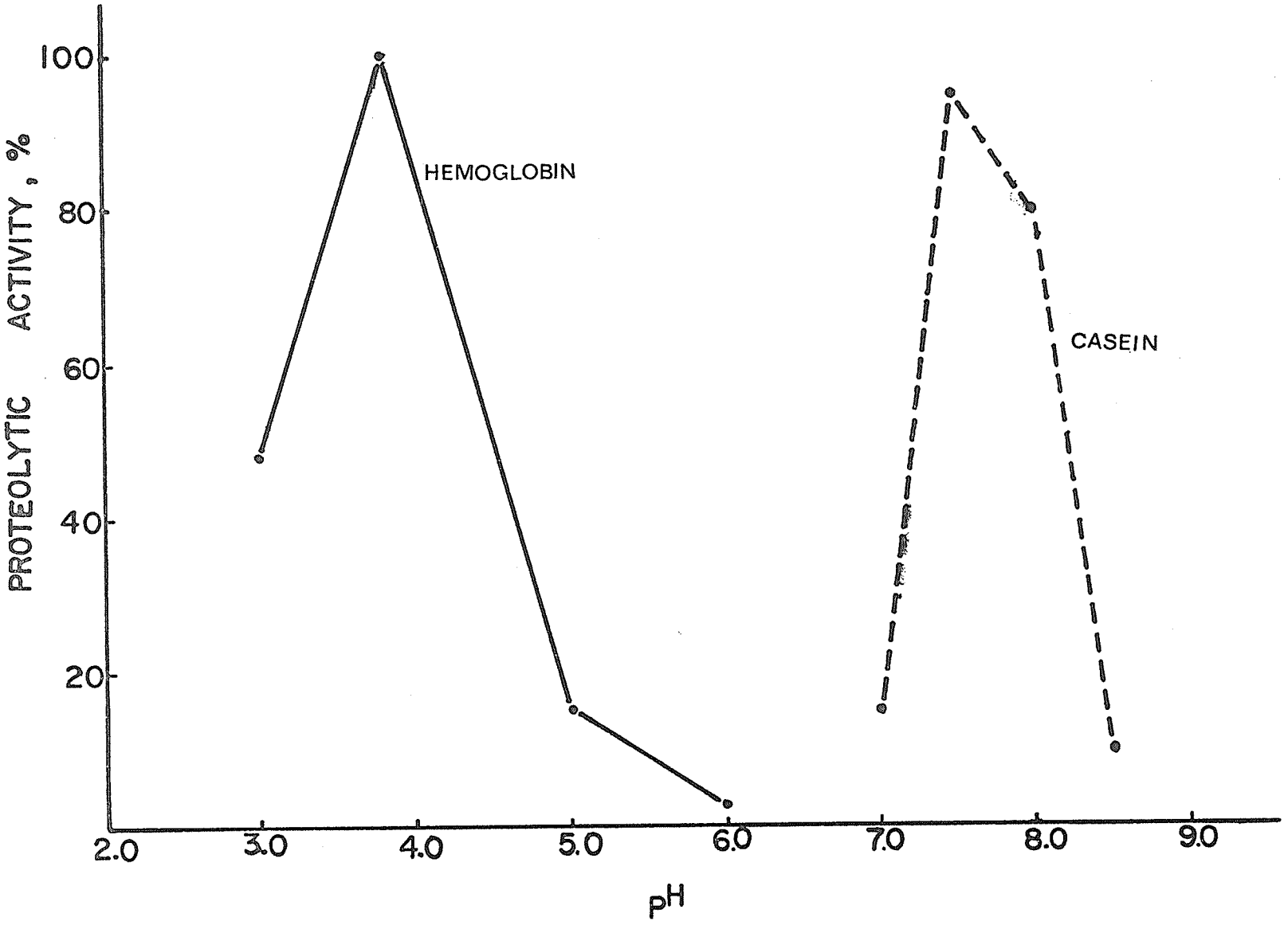
Table 1. Inhibition and Activation of Purified Malted Wheat Protease

Reagent added	Concentration	Activity	Effects
	mM	%	
none	--	100	--
PCMB	2	39	inhibition
NEM	2	92	inhibition
$HgCl_2$	2	72	inhibition
GSH	2	120	activation
IAA	2	75	inhibition
ME	2	114	activation
STI	0.2(%)	100	none

The results clearly indicate that sulfhydryl groups are essential for most of the activity of malted wheat protease. At the concentration level studied, PCMB was the most effective inhibitor, $HgCl_2$ and IAA produced partial inhibition, while NEM produced only a slight inhibition. Both GSH and ME produced approximately 15% activation over the control. Soybean trypsin inhibitor, a highly effective inhibitor of trypsin, did not show any inhibition on the activity of malted wheat protease.

Substrate Specificity Studies. Substrate specificity was examined using two natural and one synthetic substrates. The pH-activity curves for the hydrolysis of hemoglobin and casein are shown in Figure 4. The optimum pH on hemoglobin is 3.8, which is the same as that obtained for the crude

Figure 4. Effect of pH on the activity of purified malted wheat protease on hemoglobin and casein.


PROTEOLYTIC ACTIVITY, %
100
80
60
40
20
HEMOGLOBIN
CASEIN
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH

enzyme extract. The optimum pH on casein is 7.5, which is considerably higher than the pH optimum of 5.0 reported by Johnson et al (17) for malted wheat flour. A pH optimum for casein of 5 is somewhat surprising since the solubility of this substrate at this pH is extremely low.

BAPA is a good synthetic substrate for measuring trypsin activity. The use of BAPA in this study was to determine if the purified wheat protease preparation contained any trypsin like activity. Results of duplicate experiments showed that purified malted wheat protease does not hydrolyze BAPA.

Effect of Enzyme Concentration. Figure 5 shows the effect of enzyme comcentration on proteolytic activity on hemoglobin substrate for the purified enzyme and the partially purified preparation obtained by precipitation with 40-80% saturated ammonium sulfate. Both curves show that proteolytic activity is directly proportional to enzyme concentration in the range investigated. As expected the specific activity (slope) for the purified enzyme is considerably higher than that for the crude preparation.

Effect of Time of Incubation. The amount of tyrosine released after various incubation times are shown in Fig. 6. The relationship between the proteolytic activity and time of incubation is linear for the first 120 min. The rate of tyrosine released decreases slightly during incubation longer than 120 min. During the initial 120 min. the release of tyrosine from hemoglobin appears to follow zero order kinetics.

Figure 5. Effect of enzyme concentration on proteolytic activity. (Upper curve-purified wheat protease; lower curve-40-80% saturated ammonium sulfate precipitate).

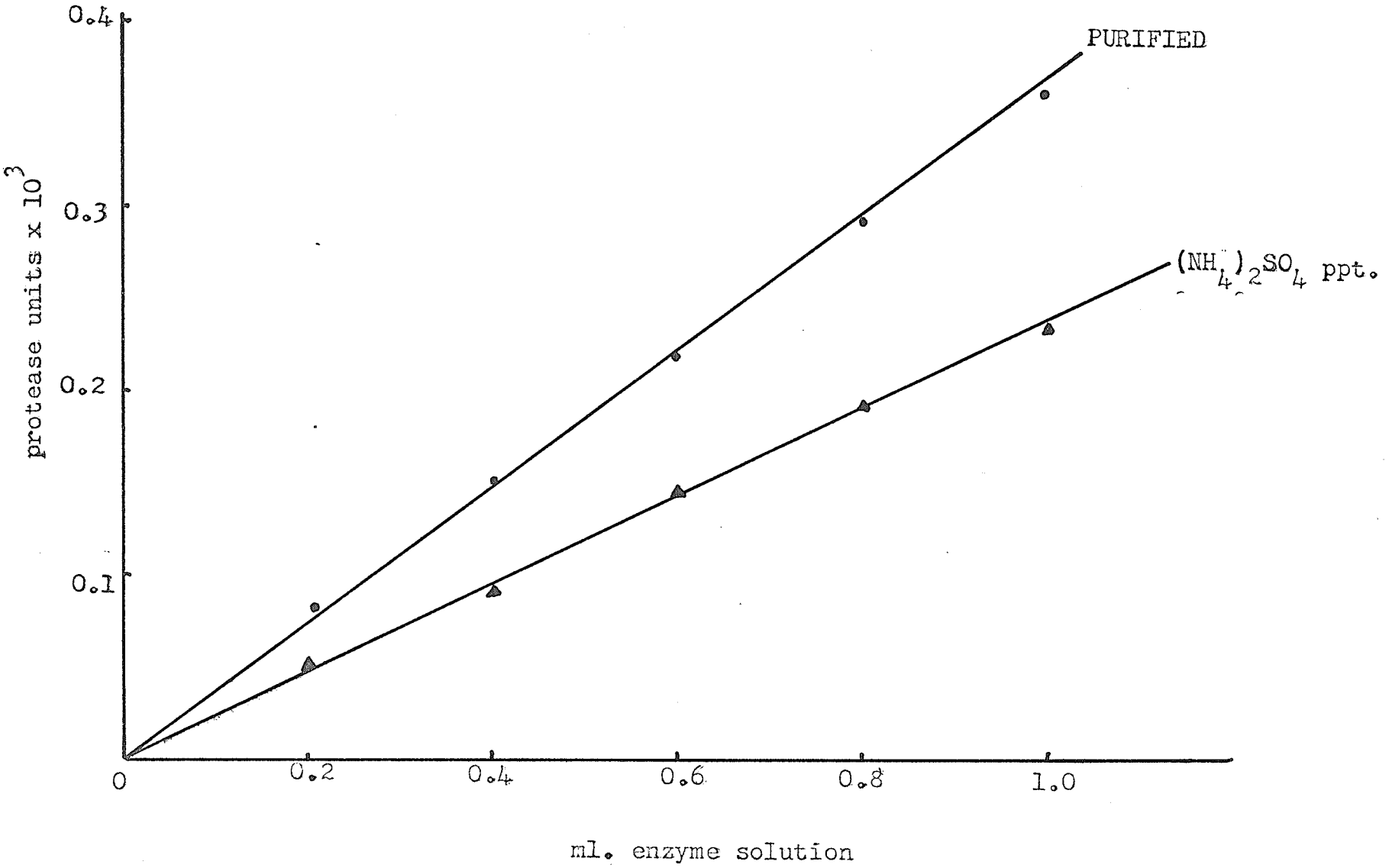
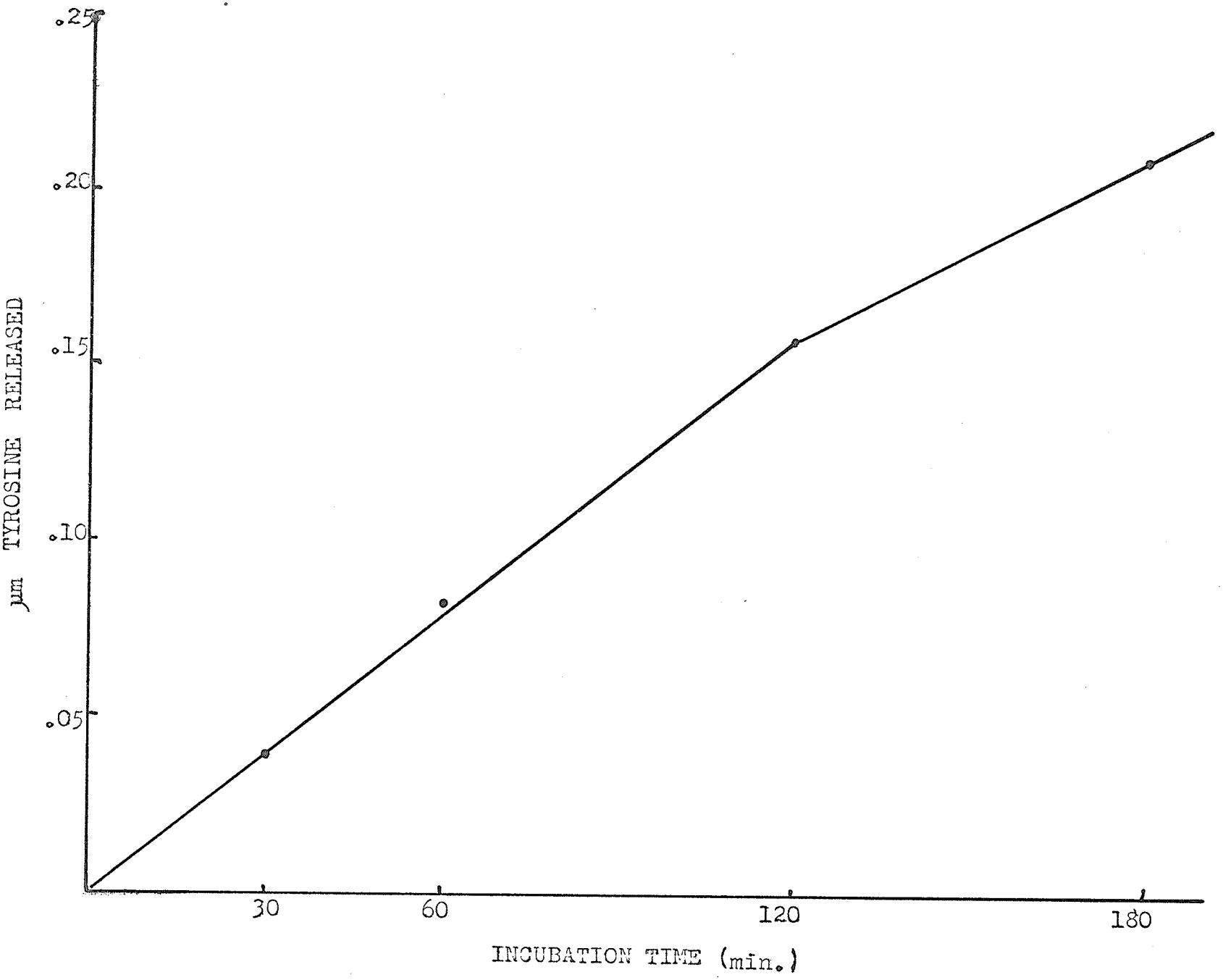

0.4
0.3
0.2
0.1
protease units x 10^3
PURIFIED
$(NH_4)_2SO_4$ ppt.
0
0.2
0.4
0.6
0.8
1.0
ml. enzyme solution

Figure 6. Effect of incubation time on the hydrolysis of hemoglobin.


.25
.20
.15
.10
.05
μm TYROSINE RELEASED
30
60
120
180
INCUBATION TIME (min.)

SUMMARY AND CONTRIBUTIONS TO KNOWLEDGE

1. Proteolytic enzymes of malted wheat flour were isolated, purified and partially characterized.
2. A highly active crude extract was obtained by extracting the flour with 10% saturated ammonium sulfate solution followed by dialysis against 15% glycerol solution.
3. The pH of optimum activity of the crude extract with hemoglobin substrate was 3.8.
4. The proteolytic activity of the crude extract was heat stable for 15 min. at 40°C: lost 30% of its activity at 50°C; and was completely inactivated at 80°C.
5. The activity of the crude extract decreased directly with increasing urea concentration in the range from 0 to 9M.
6. The specific activity of the crude extract from malted wheat flour was twice that of the analogous extract from unmalted flour.
7. Disc electrophoretic patterns of the two crude extracts from malted and normal wheat flours were qualitatively the same.
8. Two thirds of the proteolytic activity of the crude extract was concentrated in the fraction precipitated between 40-80% saturation of ammonium sulfate. This fractionation produced a 1.6 fold increase in spectific activity over that of the initial extract.

9. Membrane ultrafiltration and chromatography on Sephadex G-75 and G-100 indicated that the molecular weight of the proteolytically active fraction of the precipitate obtained by 40-80% saturated ammonium sulfate was above 50,000.
10. The proteolytically active fraction that precipitated between 40-80% saturation of ammonium sulfate showed three proteolytically active peaks after separation by ion exchange chromatography on CM-cellulose.
11. A rapid chromatographic procedure(102) based on the affinity of wheat protease with hemoglobin on a hemoglobin-Sepharose column was developed for purification of the crude extract obtained with 10% saturated ammonium sulfate solution.
12. A two fold increase in specific activity was achievied by the affinity chromatography purification.
13. Disc electrophoretic examination showed that the purified enzyme preparation contained three major and one minor protein components of similar mobility.
14. Gel filtration and ultracentrifugation results showed that the purified enzyme preparation contained one protein component.
15. The molecular weight of the purified wheat protease determined by chromatography on Sephadex G-150 was 34,000.
16. The activity of the purified enzyme was inhibited by PCMB, $HgCl_2$, IAA and NEM, and activated by GSH and ME.

The activity was not affected by soybean trypsin inhibitor at the conditions used.

17. The purified enzyme exhibited an optimum pH of 3.8 for hemoglobin substrate and 7.5 for casein substrate.

18. Purified malted wheat protease did not have BAPA-ase activity.

19. With 1% hemoglobin solution as substrate, the relationship between the activity and enzyme concentration was essentially linear in the range of concentrations investigated.

20. It is concluded that the purified enzyme obtained from malted wheat flour in this investigation is a sulfhydryl enzyme.

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