THE PURIFICATION OF PROTEINASE FROM MALTED WHEAT FLOUR

by

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INTRODUCTION

Proteolytic enzymes from various sources are employed industrially for many purposes. Included among those enzyme preparations of chief commercial importance are diastatic malt preparations, papain, pancreatin, and concentrates produced by culturing Aspergillus oryzae and Bacillus subtilis. Industries utilizing processes involving these enzymes include the textile, leather, photographic, and food industries.

Most proteolytic preparations represent crude products containing not only extraneous material but additional enzyme systems as well. Purification of these products would probably enhance their value in several ways. From an economic standpoint, purified preparations would permit better control of standardization and would also eliminate excessive bulk in the products. The elimination of undesirable factors in the preparations may, however, be the chief argument for removal of extraneous material and/or other enzyme systems. From an academic standpoint the availability of pure proteinases would permit elucidation of their fundamental properties.

The methods and conditions required for the purification of any specific enzyme cannot be simply defined. Since enzymes are essentially protein in nature, the problem is mainly one of protein precipitation, and only reagents and conditions known to have little or no injurious effect on proteins are of value. Several general methods and guiding principles that have been used with success in the past may be useful in developing methods applicable

to the purification of a particular enzyme. The general methods listed by Sumner and Somers (19) include fractional precipitation, adsorption, crystallization, ultra-centrifugation, and electrophoresis.

Fractional precipitation is almost invariably employed in enzyme purification procedures and may utilize such organic solvents as alcohol, acetone, and dioxane or may involve "salting out" with ammonium sulfate, magnesium sulfate, or other inorganic salts.

Adsorption may be accomplished by either a "batch" process in which the adsorbent is suspended in the enzyme solution or by passing the enzyme solution through a Tswett column. If the enzyme itself is adsorbed, it is removed from the adsorption complex by the use of a suitable eluant. Northrup et al. (14, p. 252) state that adsorption procedures have been useful in removing inert material or coloring matter from enzyme solutions, whereas adsorption of the active material usually results in the loss of substantial quantities of the enzyme. Schwimmer and Balls (18), however, employed adsorption on raw wheat starch from a 40 percent alcoholic solution as one of the major steps in the preparation of crystalline alpha-amylase from germinated barley. The utility of this method may depend upon the enzyme involved.

Crystallization procedures require a concentrated and partially purified solution of the enzyme. A supersaturated condition must exist before crystallization can take place. The solution should not be allowed to become to highly supersaturated, however, since this condition favors precipitation of the amorphous form of the enzyme (Northrup et al. 14, p. 252). These workers state that the

most favorable conditions for crystallization include a concentrated solution and very slight super-saturation. Supersaturation may be accomplished by cooling, by the slow addition of a precipitating agent, or by dialysis to remove salts.

In considering these general methods of enzyme purification, it should be recognized that any one method is usually insufficient. Successive use of several methods may be required before the desired state of purity is attained. In addition to the methods discussed, other techniques may be useful.

Extracts of original enzyme sources or of crude concentrates usually include other enzymes besides the one desired. Miller and Johnson (12) successfully applied differential inactivation techniques in preferentially inactivating either proteinase or alpha-amylase in extracts of malted wheat flour, malted barley, and a commercial fungal concentrate. In some cases such differential inactivation techniques would be useful in freeing the enzyme solution of undesirable activity either before or after other purification procedures were employed.

The existence of proteinases in cereal grains and their products has been known for many years but their importance in the baking industry was recognized only recently. Early data by Cairns and Bailey (3) presented evidence indicating the existence of small amounts of proteinases in wheat flour. Lüers and Malsch (8) described a papain-like gelatin-splitting proteinase in aqueous green malt infusions. Hopkins (5) and Hopkins and Kelley (6) reported a malted barley proteinase which was able to split

a variety of substrates including ovalbumin and edestin. An enzyme which had a disaggregating effect on gluten but which produced no appreciable increase in amino nitrogen was reported by Blagoveschenske and Soessiedov (1) to be present in a diastatic preparation from barley malt. A similar enzyme in wheat gluten has been described by Olcott, Sapirstein, and Blish (15). A proteinase from wheat bran has been partially purified by Balls and Hale (2) who used a fractional precipitation procedure. The properties of their preparation suggested that it was of the papain type of proteinase.

The effects of proteinases in dough have been regarded as deleterious in the past. Early data by Read and Haas (16), however, indicated that small amounts of proteinases produces beneficial effects on bucky doughs, and Conn, Johnson, and Miller (4) have shown that some proteinase activity is essential for the production of top quality bread. Recent work by Miller and Johnson (11) showed that the detrimental effects of excessive malt supplementation, previously attributed to high levels of alpha-amylase, could be more accurately ascribed to excessive amounts of proteinase accompanying the amylase.

It has become increasingly apparent that the proteinases present in diastatic supplements such as the germinated cereals or certain fungal preparations play an important role in baking. An adequate evaluation of the role of these enzymes has been difficult because of the relative impurity of the preparations used. Purification of such proteinases would not only provide a more suitable

preparation for evaluation in baking but should also permit more fundamental studies of proteinase action. The object of this study has been to purify the proteinase system of malted wheat flour preparatory to investigations of its properties and functions as a baking adjunct.

MATERIALS AND METHODS

Materials

The enzyme active material used throughout the experiments was a commercially prepared malted wheat flour high in proteolytic activity. Enzyme solutions for precipitation studies and other experiments were prepared by water extraction of this source under standardized conditions.

In the precipitation procedures studied, C. P. grade ammonium and magnesium sulfates were used. For adsorption experiments 95 percent ethanol and a commercial wheat starch were utilized. Acids and bases used for adjustment of hydrogen-ion concentration and salts used in buffer solutions were also of C.P. grade.

For proteolytic activity determinations the substrate was a commercially available dried hemoglobin preparation (Bacto-hemoglobin). The starch substrate used in alpha-amylase determinations was prepared from "Merck Soluble Starch, according to Lintner, special for diastatic power determinations". "Merck Reagent Dextrin" was used in preparing the color standard.

Methods

Proteinase Activity Determination. The basic method of proteinase activity determination was the modified Ayre-Anderson method as standardized by Miller (10). This procedure involves a five-hour digestion of a hemoglobin substrate at pH 4.7 and 40° C. A "blank" employing 15 minutes' digestion time is run for each enzyme concentration used. Following the digestion period, the undigested protein is precipitated with trichloroacetic acid and removed by filtering. Nonprotein nitrogen in the filtrate is determined by a standard Kjeldahl-Gunning procedure (9). The increase in nonprotein nitrogen, determined by difference from the two digestions, is a measure of proteolytic activity. For a comparison of the activity of unknown enzyme solutions reference may be made to a standard enzyme concentration curve. By raising the increase in nonprotein nitrogen to the 3/2 power, however, the data are transformed to a linear relationship so that a direct comparison of values so obtained may be made (13).

The analysis of filtrates by the Kjeldahl procedure was laborious and time consuming. Therefore, a spectrophotometric method of analysis was developed to replace the Kjeldahl procedure. The highly significant correlation ($r = 0.99$, 19 d.f.) between the two methods of analysis indicates that the spectrophotometric method may be substituted for the Kjeldahl technique with very little chance of obtaining different results. The straight line relationship for the data transformed to the 3/2 power also applied to this method of analysis. A detailed description of the experimental

work leading to this modification appears in the Appendix.

Alpha-amylase Activity Determination. Alpha-amylase appears to be the major complicating enzyme system present in many proteolytic preparations (Johnson and Miller, 7). Assay for activity of this enzyme was carried out using a modified Wolgemuth procedure as described by Sandstedt, Kneen, and Blish (17). This method is based on the time required to obtain a standard red-brown color with iodine when the enzyme is allowed to digest a given amount of boiled starch substrate. The time required to reach the standard red-brown color is inversely proportional to the alpha-amylase activity.

Adsorption Procedure. Studies of adsorption of alpha-amylase from malted wheat flour extracts utilized a batch process in which the adsorbent (wheat starch) was stirred into the extract which contained 40 percent ethanol (by volume). The hydrogen-ion concentration was adjusted as rapidly as possible and adsorption continued for 30 minutes at 0° C. At the end of this period, the adsorbent was removed by centrifugation and filtration. Proteinase and alpha-amylase activities were determined on the filtrates.

Protein Nitrogen Analysis of Enzyme Solutions. Since enzymes are essentially protein in chemical composition, enzyme activity per unit weight of protein nitrogen is a measure of enzyme purity. The protein nitrogen content of enzyme preparations was determined in most cases by a standard Kjeldahl-Gunning procedure (9). Nitrogen analysis of dilute purified enzyme solutions, however, was performed by a micro-Kjeldahl technique. Enzyme solutions of

precipitates obtained by ammonium sulfate precipitation were dialyzed prior to nitrogen analysis.

Precipitation and Fractionation Procedures. Initial experiments on the precipitation of proteinase with inorganic salts were carried out using a saturated solution of the salt. This procedure, however, gave volumes of solution too large for convenient separation of the precipitate. Therefore, a standardized general procedure was adopted using the solid salts. The desired amount of salt was slowly added to portions of the extract with stirring and the stirring continued until solution of the salt was complete. This required approximately 30 minutes for the largest amounts of salt. After adjusting the hydrogen-ion concentration of each solution to the desired level, the solutions were allowed to stand for a definite time at constant temperature. At the end of the standing period, the solutions were filtered using Sargent #500 filter paper, usually with a small amount of filter-aid (Hyflo-supercel). Gentle suction was applied to remove as much of the mother liquor as possible without excessive drying of the filter-cake. The precipitates were held at 5° C. until proteinase activity was determined.

In fractionation experiments, the salt concentration was adjusted to an arbitrary initial level and the solution allowed to stand for a definite time at the desired pH and temperature. The precipitate formed was then removed and the filtrate treated with additional salt under similar conditions. This process was repeated until several successive precipitates had been obtained.

The proteolytic activity of precipitates was determined on solutions prepared by dispersing the filter-cake (when filter-aid had been used) or filter paper plus precipitate (when filter-aid had not been used) in a volume of water equal to the volume of extract from which the precipitate was obtained. These suspensions were stirred 5 to 10 minutes, adjusted to a stable hydrogen-ion concentration (pH 5.7), centrifuged, and filtered. Proteinase determinations were run on the filtrates.

EXPERIMENTAL RESULTS AND DISCUSSION

Extraction of Proteinase

Recent work by Miller and Johnson (12) indicated that extraction of malted wheat flour in water at 30° C. for one hour with continuous stirring gave enzyme solutions of convenient proteinase activity when a 2 to 5 ratio of flour to water was used. This procedure was followed in preparing extracts for experiments designed to study methods of removal of alpha-amylase from malted wheat flour extracts. Later experiments on extraction procedures indicated that extraction periods longer than 30 minutes had but little effect on extraction of proteinase but produced extracts containing increasing amounts of inactive protein. Extracts for precipitation experiments were prepared, therefore, by extracting the source for only 30 minutes at 30° C. in order to keep the inactive protein concentration as low as practicable. A slightly higher ratio of malted wheat flour to water (1 to 2) was used to compensate for the small loss of proteinase resulting from the shorter extraction time.

Following the extraction period, the insoluble material was removed by centrifuging and filtering through cotton.

Purification of Proteinase by Removal or Inactivation of Alpha-amylase

Most proteolytic preparations are contaminated not only with enzymatically inactive material but, also, with other active enzyme systems. The amylases are probably the chief enzymes, other than proteinase, present in malted wheat flour. The removal or inactivation of the amylase systems, especially the alpha-amylase, would preclude complications in proteinase studies which would otherwise be associated with this contaminant. Miller and Johnson (12) reported that alpha-amylase from malted wheat flour was unstable at low pH. The work of Schwimmer and Balls (18) suggested the possibility of removal of alpha-amylase by adsorption. Unpublished work by Underkofler and Roy indicated that low concentrations of mercuric chloride might be effective in preferentially inactivating the alpha-amylase.[1]

The Effects of Hydrogen-ion Concentration, Ethonal, and Adsorption on Starch on the Alpha-amylase and Proteinase Activity of Malted Wheat Flour Extracts. In order to separate the individual effects of hydrogen-ion concentration, ethanol, and adsorption, three similar experiments were performed. In the first, portions of an aqueous extract were allowed to stand at 26° C. for one hour

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[1] L. A. Underkofler and D. K. Roy, "Crystallization of Fungal alpha-amylase and Limit Dextrinase." This paper was presented at the May (1946) Meeting of the A.A.C.C.

at various hydrogen-ion concentrations. The pH was readjusted to a stable value and analyses made on the various portions. In the second experiment, portions of the extract were subjected to various hydrogen-ion concentrations at 0° C. for one hour in the presence of 40 percent ethanol. The third experiment involved a study of the effect of hydrogen-ion concentration on the adsorption of alpha-amylase on wheat starch in 40 percent ethanol solution. Adsorption was continued for 30 minutes at 0° C. with periodic stirring.

The results of this series of experiments are presented graphically in Figs. 1, 2, and 3. In aqueous solution the alpha-amylase was rapidly inactivated at hydrogen-ion concentrations greater than pH 5.0, and at pH 3.5 only about one and one-half percent of the original alpha-amylase activity remained. The retention of proteinase activity, however, decreased only slightly with increasing hydrogen-ion concentration, and at pH 3.5 about 80 percent of the proteinase activity was retained (Fig. 1). In the presence of 40 percent ethanol the effect of increasing hydrogen-ion concentration on alpha-amylase and proteinase activity was essentially the same as in aqueous solution, but there was an overall decrease of about 40 percent in the retention of proteinase activity under these conditions (Fig. 2). This effect may be attributed to the deleterious effect of ethanol on the proteinase.

When the alcoholic extract was treated with wheat starch, there was very little change in proteinase activity whereas there was a great decrease in alpha-amylase activity, even at stable pH values (Fig. 3). At pH 5.0, for example, the retention of alpha-amylase activity in alcoholic solution was about 88 percent while

the retention at this pH after adsorption was only 9 percent. At a hydrogen-ion concentration corresponding to pH 4.0 the loss of alpha-amylase activity by inactivation or adsorption was about 99 percent.

In general the data from these experiments show that, while the proteinase of malted wheat flour was inactivated to some extent by increasing hydrogen-ion concentration (about 20 percent loss at pH 3.5), the biggest factor affecting retention of proteinase activity was the presence of ethanol. On the other hand, alpha-amylase activity was only slightly decreased by ethanol in the concentration used but this enzyme was very susceptible to inactivation at high hydrogen-ion concentrations and was readily adsorbed on raw wheat starch.

The Effect of Low Concentrations of Mercuric Chloride on Alpha-amylase and Proteinase in Malted Wheat Flour Extracts. An experiment to determine the effect of mercuric chloride was performed by adding various amounts of this agent to portions of the extract. Alpha-amylase and proteinase activities were determined immediately. The results of this experiment are shown in Table 1.

Table 1. Effect of mercuric chloride on proteinase and alpha-amylase.

Concentration of $HgCl_2$ (percent)	Percent retention of activity	
	Proteinase	Alpha-amylase
0.05	100.0	99.2
0.10	100.0	85.5
0.50	83.2	9.5

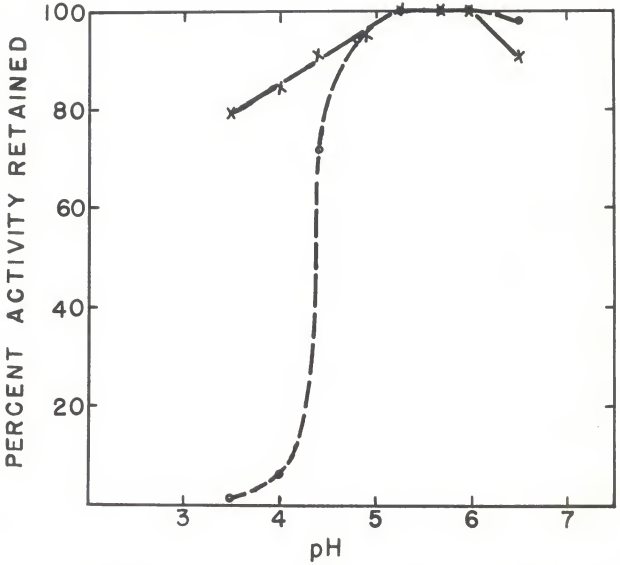

Figure 1. The influence of hydrogen-ion concentration on alpha-amylase and proteinase retention in aqueous solution. Treatment for one hour at 26° C. Solid lines represent proteinase and dashed lines represent alpha-amylase.

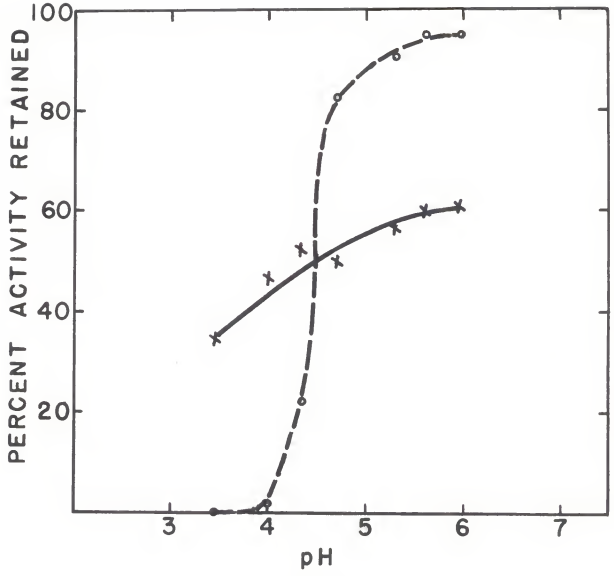

Figure 2. The influence of hydrogen-ion concentration on alpha-amylase and proteinase retention in the presence of 40 percent ethanol. Treatment for one hour at 0° C. Solid lines represent proteinase and dashed lines represent alpha-amylase.

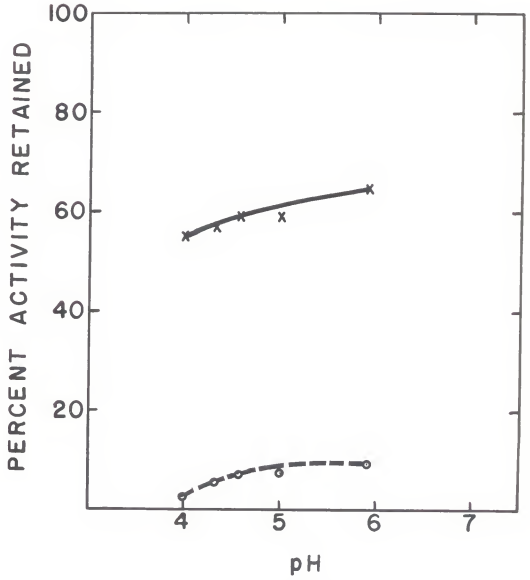

Figure 3. The influence of hydrogen-ion concentration on alpha-amylase and proteinase retention in the presence of 40 percent ethanol and 20 percent raw wheat starch. Treatment for 30 minutes at 0° C. Solid lines represent proteinase and dashed lines represent alpha-amylase.

Concentrations of mercuric chloride up to 0.10 percent had no effect upon proteinase and relatively little effect upon alpha-amylase. At 0.50 percent mercuric chloride, however, only 9.5 percent of the alpha-amylase activity remained while proteinase retention was 83.2 percent. These results indicate that this agent may be useful in differentially inactivating the alpha-amylase of malted wheat flour without extensive treatment.

While all three procedures for removal of undesirable alpha-amylase were effective, the inactivation of this enzyme by high hydrogen-ion concentration appears to be the best. Although the adsorption procedure was very effective, the presence of ethanol in the final solution together with the deleterious effect of this agent on the proteinase makes this method undesirable. The mercuric chloride treatment was somewhat less effective than the pH treatment and also had the disadvantage that the treatment time could not be controlled.

Precipitation of Proteinase

For proteinase precipitation studies the general procedures outlined in the Introduction, together with the numerous enzyme purification techniques described by Northrup et al. (14), served as guides in designing experiments. The detrimental effect of ethanol on proteinase, noted in previous experiments, suggested that other organic solvents might have similar effects. Therefore, precipitation by inorganic salts seemed the most logical approach to the problem.

Precipitation with Magnesium Sulfate. In a preliminary experiment various amounts of a saturated solution of $MgSO_4 \cdot 7H_2O$ were added to 25 ml portions of malted wheat flour extract. No attempt was made to control the hydrogen-ion concentration during the precipitation. The precipitates were recovered by centrifugation and were redissolved in water. Proteinase activity determinations on these solutions showed slight activity in the precipitates recovered above 0.45 saturation, but the activity was very low compared with the original extract. Analysis of the supernatant solutions showed that the greater part of the proteinase system remained unprecipitated.

The effect of hydrogen-ion concentration on recovery of proteinase. It seemed possible that the low recovery of proteinase in the first experiment may have been due to a hydrogen-ion concentration unfavorable for precipitation. This was investigated in a series of experiments in which precipitation was carried out at various hydrogen-ion concentrations and at two temperatures. In the first of this series a saturated solution of the salt was used but the volumes of solutions were so unwieldy to handle that solid salt was used for the remainder of the experiments. The results of these experiments are shown in Tables 2, 3, and 4.

It is apparent from the data that in all cases the recovery of proteinase increased with increasing hydrogen-ion concentration. The best recovery obtained was 71.3 percent at pH 3.0 and 30° C. (Table 3) but in no case was an optimum pH apparent. Since the proteinase of malted wheat flour is relatively unstable at hydrogen-ion concentrations greater than that represented by pH 3.5,

Table 2. The effect of hydrogen-ion concentration on proteinase precipitation using saturated $MgSO_4 \cdot 7H_2O$ solution at 30° C. Final salt concentration was 0.80 at 30° C. Solutions stood 1 1/2 hours at 30° C.

pH	Percent recovery of proteinase
4.5	36.4
5.0	26.5
5.5	22.0
6.0	19.6
6.8	19.6

Table 3. The effect of hydrogen-ion concentration on proteinase precipitation using solid $MgSO_4 \cdot 7H_2O$. Final salt concentration was 0.77 saturation[1] at 30° C. Solutions stood two hours at 30° C.

pH	Percent recovery of proteinase
3.0	71.3
3.6	60.4
4.0	50.3
5.0	38.4
6.0	19.6
7.1	30.6

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th ed.

Table 4. The effect of hydrogen-ion concentration on proteinase precipitation using solid $MgSO_4 \cdot 7H_2O$. Final salt concentration was 0.80 saturation at 40° C.[1] Solutions stood 2 hours at 40° C.

pH	Percent recovery of proteinase
3.8	64.5
4.1	64.0
4.3	59.3
4.6	46.5
5.8	33.6

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

a range between pH 3.5 and pH 4.0 was selected for use in further precipitation studies.

It will be noted that the recovery of proteinase was generally slightly higher at 40° C. than at 30° C. The explanation for this may lie in the fact that at any given degree of saturation there is a greater amount of salt present in solution at 40° than at 30°.

The effect of magnesium sulfate concentration on proteinase recovery. The influence of salt concentration on precipitation of proteinase and protein was studied at two temperatures (Tables 5 and 6). Although the results at the two temperatures are not strictly comparable, it seems apparent that up to about 0.2 to 0.3 saturation, very little of the proteinase was precipitated while about 18 percent of the total protein was removed. Above this level increasing amounts of protein and proteinase were recovered. Precipitation at 40° C. gave more complete recovery of the proteinase than at 30° and also appeared to effect a slight purification at 0.80 saturation.

Fractionation of malted wheat flour extract with magnesium sulfate. On the basis of the results obtained in the study of the effects of salt concentration on enzyme and total protein recovery, it seemed that a partial purification of the proteinase might be effected by successive fractionation of the extract. An experiment was performed using an initial fractionation at 0.26 saturation and 30° C. After removal of the precipitate obtained at this level, portions of the filtrate were subjected to a second precipitation under several different conditions. The treatment given the various portions and the results of each treatment appear in Table 7.

Table 5. The effect of magnesium sulfate concentration on proteinase precipitation at pH 3.8 and 30° C. Solutions stood 2 hours at 30° C.

Saturation[1] with $MgSO_4 \cdot 7H_2O$	Percent of total protein nitrogen recovered	Percent recovery of Proteinase	Activity per mg N compared to control
0.09	5.7	1.3	0.23 X
0.17	10.7	1.6	0.15 X
0.26	17.8	1.9	0.10 X
0.43	28.6	8.9	0.31 X
0.60	40.4	26.8	0.67 X
0.77	41.3	44.5	1.08 X
Control	100.0	100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

Table 6. The effect of magnesium sulfate concentration on proteinase precipitation at pH 3.7 and 40° C. Solutions stood 2 hours at 40° C.

Saturation[1] with $MgSO_4 \cdot 7H_2O$	Percent of total protein nitrogen recovered	Percent recovery of proteinase	Activity per mg N compared to control
0.20	17.9	6.0	0.33 X
0.40	32.2	20.4	0.62 X
0.50	41.5	40.2	0.95 X
0.60	--	55.4	--
0.80	33.8	59.6	1.74 X
Control	100.0	100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

Table 7. Fractional precipitation of proteinase from malted wheat flour extracts using two precipitation temperatures and three contact times. Initial precipitation was at 0.26 saturation[1], pH 3.8 to 4.1, and a temperature of 30° C.

Treatment[1]	Percent of total Protein N recovered	Percent recovery of proteinase	Activity per mg N compared to control
Control	100.0	100.0	1.00 X
Initial cut at 0.26 saturation	27.2	2.9	0.11 X
Filtrate from initial cut	62.5	73.5	1.17 X
2nd cut, 0.77 saturation, 30° C., 2 hours	17.4	51.5	2.96 X
2nd cut, 0.77 saturation, 30° C., 5 hours	17.7	47.0	2.65 X
2nd cut, 0.77 saturation, 30° C., 20 hours	18.0	49.5	2.80 X
2nd cut, 0.76 saturation, 40° C., 2 hours	18.4	59.7	3.25 X
2nd cut, 0.76 saturation, 40°C., 5 hours	18.0	61.7	3.44 X
2nd cut, 0.76 saturation, 40° C., 20 hours	18.0	59.2	3.29 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook, 6th Ed.

Some purification was obtained by each of the treatments. Recovery of the proteinase and purification of the enzyme was better at 40° than at 30°. Precipitation time appeared to have little effect on enzyme recovery. The most active precipitates were recovered at 0.76 saturation and 40° C. These contained about 60 percent of the original proteinase activity and were approximately three times as active as the control when compared on an equivalent protein nitrogen basis.

The results of these studies using magnesium sulfate as a precipitating agent showed that up to 70 percent of the proteinase could be recovered and that some purification was possible by fractionation with this salt. Of the conditions investigated, those found to be most favorable for precipitation of proteinase included high hydrogen-ion concentration, (pH 3.0 to 4.0), high salt concentration (0.8 saturation), and a temperature of 40° C. Since, however, the total recovery was lower than desired and precipitation of appreciable amounts of the proteinase was apparent at relatively low salt levels (0.3 to 0.4 saturation), the use of this agent was abandoned in favor of ammonium sulfate which gave more promising results.

Precipitation Using Ammonium Sulfate. The studies involving the use of ammonium sulfate as the precipitating agent followed, in general, the same pattern as those employing magnesium sulfate. The solid salt was used in all cases, however, and a precipitating temperature of 30° C. was maintained throughout.

The influence of hydrogen-ion and salt concentration on proteinase precipitation. In the first experiment with ammonium sulfate, an arbitrary high level of salt (0.83 saturation) was added to separate portions of the extract and the hydrogen-ion concentration of the different portions adjusted to various levels. The data from this experiment appear in Table 8. With the exception of the highest hydrogen-ion concentration (pH 2.9) there was very little difference in the recovery of proteinase at the hydrogen-ion concentrations used. The best recovery obtained was 90.6 percent at pH 6.8.

A similar experiment using constant pH (6.8 to 7.0) and varied amounts of ammonium sulfate was performed. The data recorded in Table 9 show that below 0.32 saturation very little of the proteinase was precipitated while above this level the recovery of proteinase increased markedly with increasing salt concentration. These data suggest the merits of fractional precipitation of the proteinase with this salt.

Fractional precipitation of proteinase. On the basis of the preceding experiments an extensive series of fractionation studies was carried out. Since the precipitation of proteinase by ammonium sulfate appeared to occur above 0.32 saturation, a single fractionation was employed in the initial experiments, the first precipitation being at 0.32 saturation while the final precipitation was at 0.83 saturation. Table 10 shows the results of this treatment. At 0.32 saturation only about 4 percent of the proteinase was precipitated while nearly 30 percent of the total protein was removed. The fraction between 0.32 and 0.83 saturation, however, contained

Table 8. The effect of hydrogen-ion concentration on proteinase precipitation using solid ammonium sulfate. Concentration of salt was 0.83 saturation.[1] Solutions stood 2 hours at 30° C.

pH	Percent recovery of proteinase
2.9	73.6
4.0	87.3
5.0	87.8
6.0	84.5
6.4	87.8
6.8	90.6

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

Table 9. The effect of salt concentration on proteinase precipitation using solid ammonium sulfate. Solutions stood 2 hours at pH 6.8 to 7.0 and 30° C.

Saturation[1] with ammonium sulfate	Percent recovery of proteinase
0.16	1.8
0.32	4.6
0.48	22.3
0.64	67.8
0.83	86.1

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

80 percent of the original proteinase but only 11 percent of the protein. Thus, a considerable purification was effected as shown by the comparison of activity with the control on an equivalent protein nitrogen basis. This fraction was purified about 7.6 times by the single fractionation treatment.

Although the single fractionation described produced considerable purification of the proteinase, a greater degree of purification was desirable. Therefore, a more extensive fractionation was attempted with results which are shown in Table 11. Although there is considerable variation in the recoveries shown for the same fraction in different trials, this may be attributed to experimental error associated with the use of relatively small volumes of solution. The greatest precipitation of proteinase occured above 0.48 saturation with the best recovery in the fraction between 0.48 and 0.64 saturation. This fraction contained about 38 percent of the starting proteinase and was 11 times as active as the control on an equivalent nitrogen basis. The fraction obtained between 0.64 and 0.83 saturation, while having only 15 percent of the starting activity, was only slightly less active than the 0.48 to 0.64 fraction.

Since the fractions about 0.48 saturation were the most active and contained most of the proteinase, a single fractionation at this level was attempted. It was found, however, that if a single initial precipitation was performed at 0.48 saturation, considerable amounts of proteinase were precipitated in the first fraction with a corresponding loss of activity in the fraction from 0.48 to 0.83

Table 10. Fractionation of malted wheat flour extract with ammonium sulfate. Precipitation performed at pH 6.9 and 30° C. for one hour.

Saturation[1] level	Trial	Percent recovery of proteinase	Percent of total nitrogen recovered	Activity per mg N compared to control
0.00 to 0.32 saturation	1	3.9	--	--
	2	3.4	32.9	0.10 X
	3	3.5	31.2	0.11 X
	4	3.8	29.6	0.13 X
Average		3.7	31.2	0.11 X
0.32 to 0.83 saturation	1	77.4	12.4	6.24 X
	2	83.3	10.9	7.62 X
	3	80.5	10.6	7.61 X
	4	79.2	9.9	8.00 X
Average		80.1	10.9	7.62 X
Control		100.0	100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

Table 11. Fractionation of malted wheat flour extract with ammonium sulfate. Precipitation performed at pH 7.0 and 30° C. for one hour.

Saturation[1] level	Trial	Percent recovery of proteinase	Percent of total nitrogen recovered	Activity per mg N compared to control
0.00 to 0.32 saturation	1	2.8	--	--
	2	6.6	18.2	0.36 X
	3	2.5	24.6	0.10 X
Average		4.0	21.4	0.23 X
0.32 to 0.48 saturation	1	14.0	5.5	2.62 X
	2	12.7	5.2	2.45 X
	3	6.4	2.6	2.50 X
Average		11.0	4.4	2.52 X
0.48 to 0.64 saturation	1	31.8	2.6	12.04 X
	2	41.5	3.5	11.81 X
	3	40.4	4.4	9.26 X
Average		37.9	3.5	11.04 X
0.64 to 0.83 saturation	1	18.1	1.5	11.83 X
	2	10.4	1.3	8.11 X
	3	17.7	2.3	7.64 X
Average		15.4	1.7	9.19 X
Control		100.0	100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

saturation. If, however, a stepwise precipitation was carried out up to 0.48 saturation followed by a single precipitation from 0.48 to 0.83 saturation, a much smaller amount of proteinase was precipitated in the fractions below 0.48 saturation with a corresponding increase in the recovery in the 0.48 to 0.83 fraction. The data for this experiment are presented in Table 12. The explanation for these results appears to lie in the physical properties of the precipitates. In this and previous experiments it was noted that precipitates produced by high initial salt concentration (0.48 to 0.83 saturation) were flocculent and either floated in or on top of the solution. If, however, a stepwise fractionation was performed, the precipitates in all fractions settled. It would appear that in the single fractionation the proteinase was occluded in the flocculent mass of inactive protein precipitated in the initial precipitation.

In most of the preceding experiments precipitation and purification of the proteinase was the only object and no evaluation of alpha-amylase activity in the precipitates was attempted. In one experiment, however, the alpha-amylase activity of the different fractions was determined. It is apparent from the data in Table 13 that appreciable amounts of alpha-amylase were included in the fractions. Therefore, an attempt was made to inactivate this enzyme by a preliminary treatment of the extract at low pH prior to fractionation. A one-hour treatment of the extract at pH 3.5 before precipitation reduced the recovery of proteinase somewhat but almost completely inactivated the alpha-amylase, especially in the fractions containing the most proteinase (Table 13).

Table 12. Comparison of a single initial precipitation to a stepwise fractionation with ammonium sulfate. Precipitation performed at pH 6.9 and 30° C. for one hour.

Saturation[1] level	Percent recovery of proteinase	Percent of total nitrogen recovered	Activity per mg N compared to control
0.00 to 0.48 saturation	29.7	36.8	0.81 X
0.48 to 0.83 saturation	46.1	6.4	7.38 X
0.00 to 0.32 saturation	3.4	32.0	0.11 X
0.32 to 0.48 saturation	11.9	6.4	1.90 X
0.48 to 0.83 saturation	61.2	6.5	9.45 X
Control	100.0	100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

Table 13. The effect of preliminary low pH treatment on proteinase and alpha-amylase recovery in fractions from malted wheat flour. Fractionation performed at pH 7.0 and 30° C. for one hour.

Treatment	Saturation[1] level	Percent recovery of proteinase	Percent recovery of alpha-amylase	Proteinase Activity per mg nitrogen compared to control
No preliminary pH treatment	0.00 to 0.32	2.5	39.0	--
	0.32 to 0.48	6.4	27.1	--
	0.48 to 0.64	40.4	12.2	9.26 X
	0.64 to 0.83	17.7	0.8	--
One hour preliminary treatment at pH 3.5 to 3.6	0.00 to 0.32	4.6	1.3	--
	0.32 to 0.48	7.0	0.8	--
	0.48 to 0.64	26.2	0.1	10.54 X
	0.64 to 0.83	8.2	0.0	--
Control		100.0	100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

In the most active fraction (0.64 to 0.83 saturation), proteinase recovery from the treated extract was lower than from the untreated extract but the degree of purification was about the same in both cases due to the lower protein content of the fraction from the treated extract. From these results it would appear that hydrogen-ion treatment would be desirable in order to free the proteinase of alpha-amylase activity. The low recovery of proteinase observed in this experiment is undesirable, however.

In an effort to further increase the purity of the proteinase by fractionation, re-fractionation of solutions of precipitates was investigated. Re-fractionation of a highly active fraction was first attempted employing a stepwise fractionation of 600 ml. of the extract at pH 7.0 and 30° C. The fraction obtained between 0.48 and 0.83 saturation was redissolved in 150 ml. of distilled water and re-fractionated. The second fractionation was carried out at pH 4.0 in an attempt to inactivate the alpha-amylase during the precipitation procedure. The results presented in Table 14 show that the initial precipitate between 0.48 and 0.83 saturation contained 34.1 percent of the starting proteinase activity and was about 8.6 times as active as the control. Re-fractionation of this precipitate gave two highly active fractions. The one from 0.32 to 0.48 saturation contained 10 percent of the starting proteinase activity while the one from 0.48 to 0.83 saturation contained about 18 percent of the original activity. Both of these fractions were about 12 to 13 times as active as the control extract when compared on a nitrogen basis. The alpha-amylase activity was nearly completely eliminated by the treatment used.

Table 14. Re-fractionation of proteinase from a fraction obtained between 0.48 and 0.83 saturation[1] with ammonium sulfate. First fractionation for one hour at 30° C. and pH 7.0. Re-fractionation at pH 4.0.

Treatment	Saturation[1] level	percent recovery of proteinase	Percent recovery of alpha-amylase	Proteinase Activity per mg nitrogen compared to control
1st fractionation	0.48 to 0.83	34.1	1.38	8.58 X
2nd fractionation	0.00 to 0.32	2.1	0.01	--
	0.32 to 0.48	10.0	0.02	13.10 X
	0.48 to 0.83	18.3	0.05	12.00 X
Control		100.0	100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

Another re-fractionation experiment was performed using a slightly different procedure. In this experiment an initial precipitation at 0.83 saturation (pH 7.0, 30° C.) gave a precipitate containing 85 percent of the proteinase and having about 2.7 times the activity of the control on an equivalent nitrogen basis. When a solution of this precipitate was re-fractionated at pH 4.0, the fraction obtained between 0.32 and 0.83 saturation retained nearly 60 percent of the starting proteolytic activity and was approximately 16 times as active as the original extract. The results obtained in this experiment (Table 15) represented the highest degree of purification yet obtained together with a high recovery of proteinase.

Batch Fractionation Followed by Lyophilization of Purified Proteinase. From the information gained from previous experiments on the influence of various factors on recovery and purification of proteinase, the best set of conditions was selected and fractionation carried out on a large volume of extract (2 liters). An initial precipitation at 0.85 saturation was performed at pH 5.4 and 30° C. for one hour. This precipitate was then redissolved in one liter of distilled water and re-fractionated at pH 4.0. The fraction obtained from 0.32 to 0.85 saturation contained 59 percent of the starting proteinase and was about 19 times as active on an equivalent nitrogen basis.

In order to obtain a dry preparation, free of salts, a solution of this purified fraction was dialyzed against distilled water at 5° C. for 24 hours and then lyophilized. The dry product thus

Table 15. Re-fractionation of proteinase from fraction obtained between 0.00 and 0.83 saturation[1] with ammonium sulfate. First fractionation for one hour at 30° C. and pH 7.0. Re-fractionation at pH 4.0.

Treatment	Saturation[1] level	Percent recovery of proteinase	Activity per mg N compared to control
1st fractionation	0.00 to 0.83	85.5	2.72 X
2nd fractionation	0.00 to 0.32	19.4	1.04 X
	0.32 to 0.83	58.9	15.85 X
Control		100.0	1.00 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

obtained was readily soluble in water. It retained about 58 percent of the original proteinase and was approximately 15 times as active on an equivalent protein nitrogen basis. It contained less than 0.05 percent of the original alpha-amylase activity. These results are listed in Table 16. The activity of the final lyophilized proteinase was compared to the original source in several ways. When compared to the starting extract, the purified proteinase was 15 times as active on an equivalent nitrogen basis. It was 32 times as active as the original malted wheat flour when compared on an equivalent nitrogen basis and about 115 times as active when compared on a weight basis. The ratio of proteinase to alpha-amylase activity in the purified preparation was about 1500 times the ratio found in malted wheat flour extract.

In the light of information obtained from the numerous studies on purification of the proteinase in malted wheat flour by ammonium sulfate precipitation, the conditions found most favorable for purification of this enzyme system suggest the following purification procedure:

Add sufficient solid ammonium sulfate to a 1 : 2 extract of malted wheat flour to give 0.85 saturation. Allow to stand one hour at pH 4.0 and 30° C. Filter with a small amount of filter-aid using gentle suction to remove as much mother liquor as possible without excessive drying of the precipitate. Redissolve the filter-cake in a volume of water equal to one-half the volume of extract used. To this solution add solid salt to give 0.32 saturation and allow to stand one hour at pH 4.0. Remove the

Table 16. Batch fractionation of malted wheat flour extract.

Treatment	Saturation[1] level	Percent recovery of proteinase	Activity per mg N compared to control
1. Initial prep't'n using 2000 ml. extract. One hour, pH 5.4, 30° C.	0.00 to 0.85	88.5	--
2. P'p't from #1 redissolved in 1000 ml H_2O and refractionated at pH 4.0, 30° C, for 1 hour.	0.00 to 0.32	discarded	--
	0.32 to 0.85	59.4	19.75 X
	0.32 to 0.85 (lyophilized)	58.0	14.75 X

[1]Saturation calculated on the basis of salt solubility listed in Lange's Handbook of Chemistry, 6th Ed.

precipitate after one hour's standing and discard. To the filtrate add salt to give 0.85 saturation and, after one hour's standing at pH 4.0 and 30° C., remove the active precipitate as in preceding steps. This precipitate contains about 60 percent of the starting proteinase activity and is about 15 times as active on an equivalent nitrogen basis. The proteinase active filter-cake may be preserved by freezing it or a solution of it at -10° C. a highly active dry preparation may be prepared by dialyzing a solution of the active precipitate against distilled water for 24 hours at 5° C. and lyophilizing the dialyzed solution.

In addition to affording a means of preparing a purified preparation of malted wheat flour proteinase suitable for evaluation as a baking adjunct, the procedure outlined may serve as the basis for further studies which might well lead to the separation of the proteinase in crystalline form.

Properties of Purified Proteinase

The method of proteinase activity determination employed was not suitable in itself for comparison of the properties of the proteinases in the purified fractions with those of the original proteinase. In order to make such a comparison the purified proteinase and the control extracts were subjected to various treatments and the effects of these treatments on the proteolytic activity compared.

The Stability of Proteinases on Standing at 5° C. at Various Hydrogen-ion Concentrations. For a comparison of the stability of

a purified fraction to that of the control, the fraction obtained between 0.48 and 0.83 saturation after stepwise fractionation was used. Portions of the solution of this fraction and of the control were allowed to stand at several hydrogen-ion concentrations in the refrigerator at 5° C. The proteinase activity at the end of 48 hours and 192 hours standing was determined (Table 17).

Table 17. The stability of proteinase in malted wheat flour extract and purified fraction on standing at 5° C. and at various hydrogen-ion concentrations.

pH	Percent of initial activity retained[1]					
	Malted wheat flour extract: Hours			Purified proteinase solution: Hours		
	1	48	192	1	48	192
3.0	--	85.3	77.6	--	62.7	62.7
4.0	--	100.0	97.5	--	98.8	89.2
5.0	--	100.0	97.5	--	100.0	92.8
6.0	--	100.0	95.5	--	100.0	85.6
7.0	--	56.4	45.5	--	15.7	6.9
Control	100.0	--	--	100.0	--	--

[1]Retention of activity based on initial activity of each solution before treatment.

The data in this table show that both the purified proteinase and that in the original extract were relatively stable from pH 4.0 to pH 6.0. Both became unstable below pH 4.0 and above pH 6.0 with the greater loss of activity occuring above pH 6.0. The greater loss of activity by the purified fraction at unstable hydrogen-ion concentrations is probably due to the absence of protective

material in the solution. The similarity between the stability of the purified proteinase and the original may be considered presumptive evidence of the identity of the two proteinases.

The Effects of Redox Reagents on Original and Purified Malted Wheat Flour Proteinases. Using the same enzyme solutions employed in the preceding experiment, the influence of reducing agents on proteinase activity was studied. The data in Table 18 indicate that the activities of both the control extract and the purified proteinase were increased by treatment with cysteine.

Table 18. The influence of reducing agents on purified and unpurified malted wheat flour proteinase.

Treatment	Percent of initial activity retained[1]	
	Malted wheat flour extract	Purified proteinase solution
No treatment	100.0	100.0
5.0 mg cysteine added to digestion mixture	105.7	116.8
4 days standing at 30° C.	80.2	89.2
10 mg cysteine added to digestion mixture after enzyme solution stood 4 days at 30° C.	87.2	103.6
H_2S bubbled through enzyme solution for 30 minutes after 4 days standing at 30° C.	88.5	113.2

[1]Retention of activity based on initial activity of each solution before treatment.

When the untreated enzyme solutions were allowed to stand four days at room temperature, the control lost about 20 percent of its activity while the purified solution lost only about 10 percent. Some bacterial growth was observed in the control in spite of the presence of toluene. This may account for its greater loss of activity on standing. The control extract regained only about one-half of its lost activity when treated with reducing agents whereas reducing agents more than restored the lost activity in the purified proteinase solution.

In another experiment the effects of both an oxidizing agent and a reducing agent were studied. The lyophilized proteinase prepared in an earlier experiment was used together with a malted wheat flour extract. The effects of these agents are shown in Table 19.

Table 19. The effects of oxidizing and reducing agents on purified and unpurified malted wheat flour proteinase.

Treatment	Percent of initial activity retained[1]	
	Malted wheat flour extract	Lyophilized proteinase
No treatment	100.0	100.0
0.06 mg KIO_3 added to digestion mixture	95.5	100.0
6.00 mg KIO_3 added to digestion mixture	90.4	93.4
6.00 mg cysteine added to digestion mixture	103.7	109.2

[1]Retention of activity based on initial activity of each solution before treatment.

These data show that oxidizing agents produced a slightly smaller decrease in the activity of the purified proteinase than of the original proteinase while the activating effect of reducing agents was greater in the case of the purified enzyme. Both the original proteinase and the purified preparation were affected in the same manner by these redox reagents. The smaller effect of oxidizing agents and the greater effect of reducing agents on the activity of the purified enzyme suggests that some oxidation of the proteinase may occur during purification.

While the comparisons of the purified proteinase and the original were inadequate to establish the identity of the two proteinases, the evidence obtained from these experiments indicates that they are essentially the same enzyme systems.

SUMMARY

The purification of malted wheat flour proteinase has been investigated. The influence of the principal factors related to precipitation of the proteinase system by each of two inorganic salts has been considered. In addition the effects of several treatments designed to free the proteinase of undesirable alpha-amylase activity have been studied. From these studies the following information has been obtained:

1. Alpha-amylase activity in malted wheat flour may be most effectively inactivated by low pH treatment of the extract. The effect of this treatment on the proteinase is comparatively small. Removal of alpha-amylase activity may also be effected by adsorption on raw wheat starch in 40 percent ethanol solution or by treatment with 0.50 percent mercuric chloride, but each of these methods has disadvantages not present in the pH treatment.

2. Recovery of proteinase by magnesium sulfate precipitation increases with increasing hydrogen-ion concentration up to pH 3.0, the highest concentration used.

3. Precipitation of proteinase using magnesium sulfate occurs chiefly above 0.2 to 0.3 saturation with increasing amounts of proteinase being recovered at higher salt concentrations up to about 0.8 saturation.

4. A single fractionation of malted wheat flour extract at 0.26 and 0.76 saturation (40° C.) yields a fraction which is about three and one-fourth times as active as the original extract compared on an equivalent nitrogen basis.

5. Recovery of proteinase at 40° C. is better than at 30° C. for a given degree of saturation with magnesium sulfate.

6. Using ammonium sulfate for precipitation of proteinase the hydrogen-ion concentration has little influence on recovery between the limits of pH 4.0 to pH 7.0.

7. Precipitation of proteinase by ammonium sulfate occurs chiefly above 0.32 saturation with increasing amounts of proteinase being recovered at higher salt concentrations up to about 0.85 saturation.

8. Multiple fractionation of malted wheat flour extract with ammonium sulfate at pH 7.0 and 30° C. permits considerable purification of the proteinase. The most active precipitates are obtained above 0.48 saturation.

9. Precipitates obtained by fractionation with ammonium sulfate at pH 7.0 contain appreciable amounts of alpha-amylase. Treatment of the extract at high hydrogen-ion concentration (pH 3.5) before fractionation effectively inactivates the alpha-amylase, but also reduces recovery of proteinase. Fractionation at pH 4.0 with no prior treatment, however, has little effect on proteinase recovery but almost completely inactivates the alpha-amylase.

10. Re-fractionation of a solution of the precipitate obtained from zero to 0.83 saturation with ammonium sulfate produces an active precipitate which contains about 60 percent of the starting proteolytic activity and which is about 16 times as active as the control extract when compared on an equivalent nitrogen basis.

11. Batch fractionation of malted wheat flour extract followed by lyophilization produces an active dry product which contains approximately 60 percent of the starting proteinase. This dry product is about 15 times as active as the malted wheat flour extract when compared on an equivalent nitrogen basis and is about 115 times as active as the starting malted wheat flour when compared on a weight basis. The ratio of proteinase to alpha-amylase in this preparation is about 1500 times the ratio found in malted wheat flour extract.

12. As judged on the basis of stability and by the effects of oxidizing and reducing agents, the purified proteinase appears to be essentially the same enzyme system as found in the original malted wheat flour.

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APPENDIX

A Spectophotometric Modification of the Ayre-Anderson Method for Estimation of Proteinase Activity

The modified Ayre-Anderson procedure as standardized by Miller (10) has been found useful in estimating the proteolytic activity of enzyme preparations. This procedure, which is described under 'Materials and Methods', employs a standardized Kjeldahl procedure for determination of the nitrogen content of filtrates after precipitation by trichloroacetic acid. The Kjeldahl procedure is one of the greatest sources of error in the method. It is also very laborious and time consuming. It seemed desirable, therefore, to investigate the possibility of finding a substitute for the Kjeldahl procedure in this method of proteinase determination.

Anson has described a rapid colorimetric method for proteinase assay which utilizes denatured hemoglobin as the substrate.[1] The hemoglobin is digested for 10 minutes under standard conditions. At the end of the digestion period the undigested hemoglobin is precipitated with trichloroacetic acid and the amount of unprecipitated protein split-products, which is a measure of proteinase present, is estimated with a phenol color reagent which gives a blue color with tyrosin and tryptophane. The values obtained are standardized against a standard tyrosine solution. A modification of Anson's method has been described by Northrup, Kunitz, and Herriot (14, p. 305) for estimating pepsin activity. The procedure

[1] M. L. Anson, "The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin." Jour. Gen. Physiol. 22:79-89, 1938.

for obtaining the filtrate containing the protein split-products is identical with Anson's. The concentration of these products is then estimated by extinction coefficient determinations using light at 280 millimicrons. Pepsin activity is determined by reference to a standard curve.

The successful application of a spectrophotometric technique for analysis of filtrates obtained in a method using essentially the same substrate and precipitation procedures as the modified Ayre-Anderson method indicated that this technique might be applied to filtrate analysis in the latter method. Therefore, experiments were undertaken to investigate this problem. A series of optical density curves was established using filtrates obtained from digestion at three different enzyme concentrations. The filtrates were diluted 1 to 10 with distilled water and the optical density read at various wave-lengths using a 1 to 10 dilution of the buffer and trichloroacetic acid solvent for the blank. A Beckman spectrophotometer was employed for the optical density measurement. The curves presented in Fig. 4 show that the maximum optical density for these filtrates occurs near 275 to 280 millimicrons. For spectrophotometric analysis of filtrates 280 millimicrons was adopted.

Enzyme concentration series employing malted wheat flour extract were conducted on three different days. The filtrates obtained after "zero" time and five hour digestions were analyzed by two methods, one being the usual Kjeldahl procedure and the other the spectrophotometric method using the Beckman

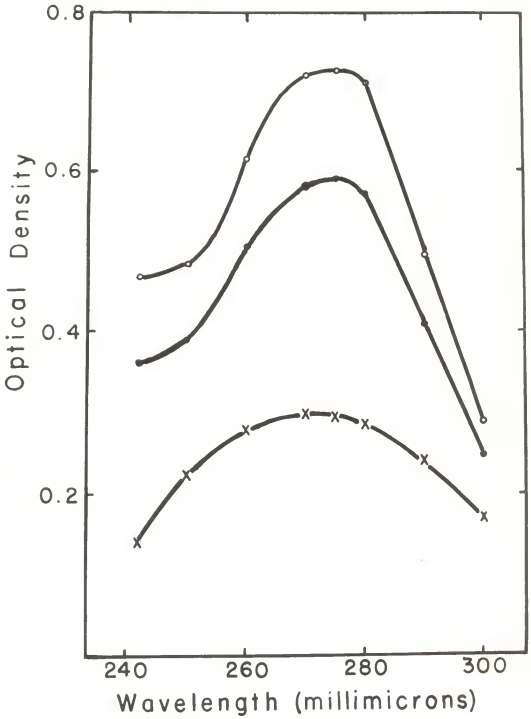

Figure 4. The relationship between optical density and wave length for filtrates containing different amounts of hemoglobin split-products not precipitated by trichloroacetic acid. Upper curve represents highest concentration of products.

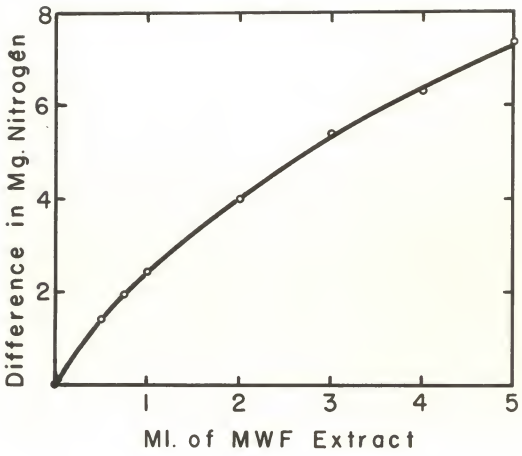

Figure 5. The relationship between proteinase concentration and activity as measured by Kjeldahl procedure. Proteinase activity measured as the difference in nonprotein nitrogen content of filtrates obtained after zero time and 5 hour digestions of hemoglobin.

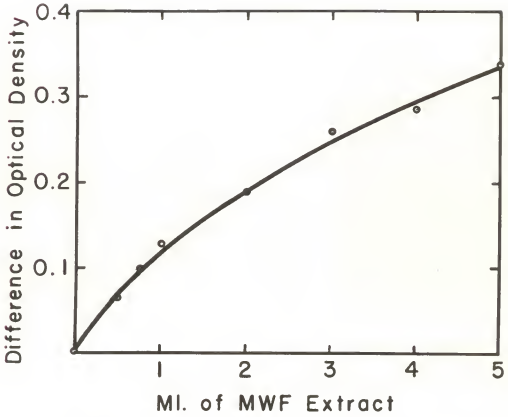

Figure 6. The relationship between proteinase concentration and activity as measured by spectrophotometric procedure. Proteinase activity measured as difference in optical density of filtrates obtained after zero time and 5 hour digestions of hemoglobin.

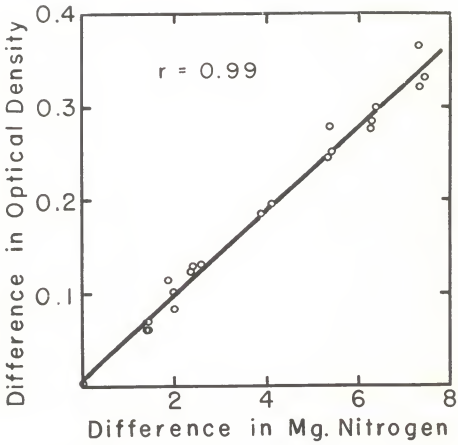

Figure 7. Scatter diagram for correlation of results of proteinase activity determinations obtained by Kjeldahl and spectrophotometric methods of filtrate analysis.

spectrophotometer. The results of the analysis by the two methods described are shown in Figs. 5, 6, and 7. The highly significant correlation ($r = 0.99$, 19 d.f.) between these two methods of analysis indicates that the spectrophotometric method may be substituted for the Kjeldahl technique with very little possibility of obtaining different results. On the basis of these results, proteinase determinations during the latter part of the experimental work presented in this thesis used this method of analysis.

While this spectrophotometric analysis has been successfully used with malted wheat flour proteinase on hemoglobin, it should be noted that the relationship between this and the Kjeldahl technique may not hold true for other proteinases or other substrates. Further work must be done before such applications may be made.

THE PURIFICATION OF PROTEINASE FROM MALTED WHEAT FLOUR

by

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Investigations of methods for freeing malted wheat flour extracts of alpha-amylase activity while retaining the proteolytic activity showed that inactivation of the alpha-amylase at high hydrogen-ion concentrations was more effective and desirable than adsorption on wheat starch in alcoholic solution or treatment with mercuric chloride. Subjecting the extract to pH 3.5 for one hour at 26° C. resulted in a loss of about 98 percent of the alpha-amylase activity while 80 percent of the proteinase activity was retained.

Precipitation of proteinase from malted wheat flour extract by magnesium sulfate increased with increasing hydrogen-ion concentration and increasing salt concentration up to about pH 3.0 and 0.8 saturation, the highest concentrations used. The recovery of proteinase was greater when precipitation was carried out at 40° C. than at 30° C. A single fractionation of the extract at 0.26 and 0.76 saturation (40° C.) yielded a fraction which was about three and one-fourth times as active as the original extract when compared on an equivalent nitrogen basis.

The precipitation of proteinase from malted wheat flour extract by ammonium sulfate was essentially independent of hydrogen-ion concentration over the range from pH 4.0 to 7.0. Precipitation of proteinase occurred chiefly above 0.32 saturation with ammonium sulfate. Increasing amounts were recovered at higher salt concentrations up to about 0.85 saturation, the highest concentration used. Multiple fractionation of the extract with this salt (pH 7.0 at 30° C.) effected considerable purification of the

proteinase with the most active precipitates being recovered above 0.48 saturation.

Precipitates obtained by ammonium sulfate fractionation at pH 7.0 retained appreciable amounts of alpha-amylase. Treatment of the extract at pH 3.5 prior to fractionation effectively eliminated alpha-amylase but also considerably reduced recovery of proteinase. Fractionation at pH 4.0 with no prior pH treatment, however, had little effect on proteinase recovery but almost completely inactivated alpha-amylase.

Re-fractionation of a solution of the precipitate obtained at 0 to 0.83 saturation with ammonium sulfate produced an active precipitate which contained about 60 percent of the starting proteolytic activity and which was approximately 16 times as active as the control extract compared on an equivalent nitrogen basis. Lyophilization of a solution of this active precipitate produced a dry product which was about 15 times as active as the control extract compared on an equivalent nitrogen basis and nearly 115 times as active as the starting malted wheat flour compared on a weight basis. The ratio of proteinase to alpha-amylase in this preparation was about 1500 times the ratio found in the original malted wheat flour extract.

As judged on the basis of stability and by the effects of oxidizing and reducing agents the purified proteinase appeared to be essentially the same enzyme system as found in the original malted wheat flour.

In addition to the work on proteinase purification, a spectrophotometric modification of the Ayre-Anderson method of proteinase determination was developed. This modification was found to be highly correlated with the standardized Ayre-Anderson procedure.