THE INACTIVATION AND REMOVAL OF PROTEOLYTIC ENZYMES FROM AMYLOLYTIC SUPPLEMENTS

by

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B. S., Kansas State College
of Agriculture and Applied Science, 1948

A THESIS

submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

Department of Milling Industry

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1948

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INTRODUCTION

The role of enzymes in baking technology has been reviewed recently in monograph form (2). Even before the existence of enzymes was recognized, malt supplements were used in baking solely on the basis of the desirable results obtained. Ensuing investigations resulted in the concept that this beneficial action was due to added amylolytic activity. Unfortunately, many amylase preparations suitable for flour supplementation are accompanied by considerable quantities of proteolytic enzymes. It is generally believed that these enzymes attack the flour proteins and thus impair the quality of the final product. The importance of amylolytic action and proteolytic activity control in brewing and distilling processes, as well as in the breadmaking industry, makes it desirable to separate the proteolytic enzymes from the amylolytic preparations. It was the purpose of this investigation to study the efficacy of various adsorbents and associated treatments in accomplishing this separation.

The influence of added amylase on the quality of bread was recognized as early as 1885 by Jago and Jago (23) who had a clear concept of the value of amylolytic supplements. The first mention of a direct relationship of "diastatic" activity and loaf volume was made by Wood (59). In 1890, Brown and Morris (9) and, later, Baker and Hulton (4) observed that the beneficial effect was due to a "starch-

liquefying" enzyme developed during germination of the cereal grain. It was not until 1930, however, that Ohlsson (42) successfully differentiated two "diastases" and named them alpha-amylase and beta-amylase, the starch-liquefying enzyme and the starch-saccharifying enzyme, respectively. Normally milled flour from sound wheat is deficient in alpha-amylase, but contains ample beta-amylase. Accordingly, Kneen and Sandstedt (33) concluded that the logical evaluation of a malt supplement should be a measure of its alpha-amylase activity.

Nearly all workers emphasize the value of malt supplements in preventing "yeast starvation" and maintaining gas production in doughs. Even in cases where gas production is not a factor, alpha-amylase supplementation is observed to improve bread quality. The reasons underlying the use of such supplements were summarized by Freeman and Ford (12). These included increased gas production, improvement in crust color, increased moistness of crumb and keeping quality, and additional flavor. To these Kneen and Sandstedt (33) have added the apparent increase in gas retaining ability of the dough.

Several sources of alpha-amylase have been studied in relation to flour supplementation. The practicability of using malted wheat or barley for mill control of amylase activity was demonstrated in extensive investigations by Sherwood and Bailey (54). Certain strains of the mold, Aspergillus oryzae, have been found to produce alpha-amylase suitable for flour

supplementation. The organism is customarily cultured on wheat bran. Data obtained by Read and Haas (46), Johnson and Miller (24) and others indicated the successful application of fungal amylases in baking. Green (15), in comparative studies, also found pancreatic amylase to be satisfactory but impractical. Certain bacterial amylases may also be applicable although their properties as reported by Kneen and Beckord (32) are quite different from the mold and cereal amylases. It appears, then, that the commercially feasible amylase sources for flour supplementation are cereal and mold amylases. Both are available commercially in concentrated form.

Alpha-amylase supplementation may be beneficial or harmful depending on the amount or type used. Detrimental effects have frequently been observed and bakers are reluctant to add more than a very small percentage in their doughs. Hildebrand and Burkert (21) presented two conflicting theories on the cause of this detrimental action. The older theory proposed by Ford and Guthrie (11) and substantiated by Baker and Hulton (4) and others postulated that the effects were due to high proteolytic activity. A later theory advanced by Kozmin (34) contended that the production of sticky doughs and moist crumb was due to the liquefaction and dextrinization of starch resulting from excessive alpha-amylase activity. This theory was further amplified by Read and Haas (46), Sandstedt, Jolitz, and Blish (51), Geddes, Hildebrand, and Anderson (13) and others. No satisfactory solution to the problem, however, has been proposed. Recent studies by Johnson and Miller (24)

and Miller and Johnson (38) appear to support the proteolysis theory. These authors reported that high levels of alpha-amylase may be used in straight doughs without detrimental effects. However, when malt preparations were added to sponge doughs in sufficient quantities to materially improve diastatic activity, the proteolytic enzymes present effected a degradation of protein resulting in inferior bread. Salt, which is present in the straight dough, but not in sponge, was shown to inactivate a portion of the proteolytic enzymes.

Hard wheat flours normally contain negligible amounts of proteolytic enzymes; therefore, it is desirable to consider the addition to doughs of other products known to be rich in these enzymes. Olson and Bailey (43) found that the proteases of sound normal yeast are negligible in their effect on gluten properties. Proteases do not diffuse through living yeast cell walls to any appreciable degree. Other ingredients in dough do not have proteolytic activity. It would appear that a method for removing proteolytic enzymes from amylolytic supplements would mitigate the detrimental effects of high levels of malt supplementation.

Several general procedures have been used for purification and concentration of enzymes. These include dialysis; precipitation, either single or repeated; and adsorption of the enzyme on selected adsorbents followed by elution. Little information is available concerning separation of proteolytic enzymes from amylases by dialysis, but some work has been done with precipitation methods.

Marston (36) called attention to the fact that certain

water soluble compounds containing an azine nucleus, such as safranine, would completely precipitate proteolytic enzymes from acqueous solutions. No effect on the amylases was noted. Tissue and Bailey (55) and Read and Haas (46) found, however, that safranine did not give a strictly quantitative separation of the proteases from malt preparations and, in addition, experienced difficulty in removing the excess dye. Other precipitation methods also have been tried with little success.

The use of adsorbents has produced the most promising results. The process of adsorption has long been used in the isolation and purification of chemical compounds which are similar in structure and reaction. European chemists used adsorbents extensively for purification and concentration of enzymes. Willstatter and Waldschmidt-Leitz (57) and Willstatter, Waldschmidt-Leitz and Hesse (58) found that the lipase, amylase, and trypsin of pancreatic extracts could be separated by adsorption on alumina and kaolin. Previously, Rakuzin and Flier (45) reported that diastase was exhaustively adsorbed by aluminum hydroxide gel but not by kaolin.

The problem of selective adsorption is complex and involves the interaction of many factors. Sherman, Caldwell, and Adams (53) and Hemmi and Inami (19,20) reported pancreatic amylase to be readily adsorbed by kaolin and by alumina gel, while Hemmi and Inami noted that the amylase of takadiastase was only weakly adsorbed by kaolin. Neither of these investigations resulted in complete separation of proteolytic from amylolytic enzymes. Work on yeast extracts by Grassman

and Haag (14) indicated the use of aluminum hydroxide and kaolin for separating the yeast proteases, trypsin, and erepsin. Young and Hartman (61) investigated a series of adsorbents in a study involving the adsorption of lipase, amylase, and trypsin from pancreatic extract. These authors reported several adsorbents to be specific for the adsorption of trypsin, of lipase, and of amylase. Techniques were developed by which any two of the enzymes could be removed from the third.

The reaction or pH of the adsorbent suspension also affects the specificity and efficiency of the adsorption process. Lüers and Malsch (35) and Sabalitschka and Weidlich (50) found that malt amylase was best adsorbed at pH values approximating 4.5 while a pH optimum above 5.0 was observed for the adsorption of malt proteases. Protease was reported by Hopkins (22) to be adsorbed from 40 per cent acetone by alumina and less completely by kaolin at a pH of 4.7. Kitano (25,26,27,28,29, 30,31) reported investigations on the use of a number of adsorbents in the separation of maltase from the amylase in taka-diastase. All adsorbents were found to be more efficient as the pH was increased and became more or less selective with changes in pH. Selectivity of any particular adsorbent for amylase or maltase was not absolute but was influenced by temperature, pH, and enzyme concentration. Yamagisi (60) obtained similar results with adsorption of rice amylase on aluminum hydroxide gel. The optimum pH range for adsorption was found to be from 4.0 to 4.5.

In 1938, Waldschmidt-Leitz and Ziegler (56) obtained a patent on the use of bauxite, a natural aluminum ore, for selectively adsorbing proteases from enzyme mixtures obtained from malted grains, molds, and bacteria. This process succeeded only in increasing the ratio of amylolytic to proteolytic enzymes. A pH value between 5.5 and 7.5 was recommended. Miller and Johnson (38) have investigated bentonite and kaolin and found these agents also to be effective in differentially removing proteolytic enzymes although the separation was not complete. The purpose of the present work was to investigate various adsorbents and treatments in an effort to improve the inactivation or removal of proteolytic from amylolytic enzymes.

MATERIALS AND METHODS

Materials

The chief source of amylase investigated was a mold bran obtained through the courtesy of Dr. K. J. Goering, President, The Mold Bran Company, Eagle Grove, Iowa. This preparation was a culture of the mold, Aspergillus oryzae, grown on wheat bran. Additional fungal sources included "E-Z-1" from The Mold Bran Company; a mold bran product of the Wallerstein Laboratories, New York; and Rohzyme-S, Rohm and Haas Company, Philadelphia. A bacterial bran of the Jeffreys Laboratories, Salem, Virginia, was used as well as a Wallerstein bacterial amylase preparation. Also examined were a commercial malted wheat flour, The Kansas

Milling Company, Wichita, Kansas; a commercial malted barley, Hales and Hunter Company, Chicago; and Polidase-S, a vegetable amylase preparation of the Schwartz Laboratories, Inc., New York. The papain used was a product of the Merck Chemical Co., Rahway, New Jersey.

Samples of adsorbents were obtained from the following commercial sources:

Activated Alumina
The Aluminum Ore Co., East St. Louis, Illinois

Amberlite IR4B
An acid adsorbent or anion exchange resin. The Resincus Products and Chemical Co., Washington Square, Philadelphia

Amberlite IR100
A synthetic resinous cation exchanger in the sodium form. The Resinous Products and Chemical Company

De-Acidite
An amine resin anion exchanger. The Permutit Co., 330 West 42nd St., New York

Decalso fines
A synthetic sodium alumino-silicate in the sodium form. The Permutit Company

Duolite A-2
A resinous anion exchanger. The Chemical Process Co., 58 Sutter Street, San Francisco

Duolite C-3
A resinous cation exchanger in the hydrogen from. The Chemical Process Company

Egg Albumin
Merck & co., New York

Florex XXX
Florida Fuller's Earth consisting largely of SiO_2 and Al_2O_3. The Floridin Co., Warren, Pennsylvania

Florisil
An analytical adsorbent. The Floridin Company

Folin Decalso
A synthetic sodium alumino-silicate with exchangeable

hydrogen ions. The Permutit Company, 330 West 42nd Street, New York

Isco Adsorbol A-420
An activated clay. Innis, Spieden & Co., 117 Liberty Street, New York

Isco Adsorbol N-100
Unactivated or natural clay. Innis, Spieden & Company

Lloyd's Reagent
A form of hydrated aluminum silicate. The Hartman-Leddon Co., Philadelphia

Norit A
A decolorizing carbon. The Pfanstiehl Chemical Co., Waukegan, Illinois

Permutite
A material in the laboratory whose exact source is unknown, but which is evidently very similar to the Decalso of the Permutit Company, 330 West 42nd Street, New York

Pumice
A finely powdered product in the laboratory, the source of which is unknown

Santocel C
A processed sodium silicate. The Monsanto Chemical Co., Merrimac Division, Boston

Santocel CX
A processed sodium silicate of different density and particle size. The Monsanto Chemical Company

Turkish Emery
Arthur H. Thomas Co., Philadelphia

Zeo-Dur
A processed glauconite or greensand in the sodium form. The Permutit Company, 330 West 42nd Street, New York

Zeo-Karb H
An organic cation exchanger of the sulfonated coal type. The Permutit Company, 330 West 42nd Street, New York

Zeolite
A resinous adsorbent. Zeolite Chemical Co., 140 Cedar Street, New York

Zeo-Rex H
A resinous cation exchanger treated to operate in the hydrogen cycle. The Permutit Company, 330 West 42nd Street, New York

Other adsorbents were prepared in the laboratory as follows:

Activated Bauxite
Crude bauxite ore was obtained from the Department of chemistry, Kansas State College. It was finely ground and activated at 200° C. for 24 hours.

Hydrous aluminum oxide gel
Prepared by adding 250 ml of 3 M NH_4OH to 250 ml 0.5 M $Al(SO_4)_3$. The precipate was washed, dried at 130°C., and activated for 24 hours at 200°C.

Hydrous aluminum oxide gel on asbestos support
Prepared in the same manner as above but in the presence of 10 g asbestos fiber.

Hydrous aluminum oxide gel in alcohol
Prepared in the same manner as above with the alcohol being added at the time of adsorption.

Magnesium silicate
Made by diluting sodium silicate to a specific gravity of 1.06 with distilled water and adding a molar solution of magnesium chloride to the silicate as long as precipitation occurred. The precipitate was washed, dried at 130°C., and activated 24 hours at 200°C.

Preparation of Extracts

A solution of amylolytic and proteolytic enzymes was prepared by extracting the source with 0.2 per cent calcium chloride solution at room temperature. The mixture was stirred every 15 minutes for one hour after which it was filtered and centrifuged. The resulting clear solution was stored under toluene in a refrigerator until used. Under these conditions, Sandstedt, Kneen, and Blish (52) demonstrated that as much as 8 per cent of the alpha-amylase activity is lost in 48 hours. Consequently, fresh enzyme extracts were prepared daily to minimize interactions in the solution. A ratio of 1 g of preparation to 10 ml of 0.2 per cent calcium chloride solution pro-

vided an extract of convenient enzyme activity.

Determination of Proteolytic Activity

Proteolytic activity was determined by a modified Ayre-Anderson procedure as standardized by Miller (37). Briefly, this method involves the hydrolysis of a hemoglobin substrate by the enzyme extract in a mixture buffered at pH 4.7. After the hydrolysis period, undigested protein is precipitated with trichloroacetic acid, filtered, and aliquots of the filtrate analyzed for total nitrogen. Titration values indicate the extent of proteolytic activity in the sample.

The detailed procedure consisted of adding 25 ml of 0.1 M sodium acetate-0.1 M acetic acid buffer (pH 4.7) to 125 ml Erlenmeyer flasks containing 0.625 g (moisture free) of Bacto-hemoglobin and approximately 2 g of powdered pumice. One ml of the appropriate dilution of the enzyme extract was added, the flasks tightly stoppered and placed in an automatic shaking device fitted in a constant temperature water bath held at 40.0 ±0.1°C. After a five-hour digestion period, a 4 ml portion of trichloroacetic acid (45 per cent by weight) was added to each flask and shaken for an additional five minutes. The reaction mixture was then filtered and duplicate 5 ml aliquots of the filtrate pipetted directly into Kjeldahl flasks for analysis of soluble nitrogen.

The standard Kjeldahl-Gunning-Arnold procedure (3) was used; however, a digestion time of 20 minutes was ample. Each

still was checked for leaks and duplicability by determining the nitrogen in aliquots of an ammonium oxalate solution. The water used for dilution after digestion was added in such a way as to wash down all of the trichloroacetic acid which condensed on the neck of the flask during digestion. Concentrated alkali was also added so that any acid remaining in the neck of the flask would be neutralized.

Unneutralized acid was back-titrated with 0.0714 N sodium hydroxide. Blank determinations were carried out in exactly the same way except that the enzymatic digestion period was terminated after 15 minutes. The proteolytic activity of the enzyme preparation was expressed in equivelant ml of 0.0714 N sodium hydroxide derived from duplicate titration values and their appropriate blanks.

Determination of Alpha-Amylase Activity

Alpha-amylase activity was determined by the starch dextrinization procedure described by Sandstedt, Kneen, and Blish (52). Values for alpha-amylase activity are expressed as the time in minutes required to produce the standard red-brown end point with iodine.

Several reagent solutions were required. A stock iodine solution consisting of 5.5 g of iodine crystals and 11 g of potassium iodide was diluted to 250 ml with water and stored in the dark in a glass-stoppered bottle. The iodine solution (A) for the standard was prepared by diluting 15 ml of the

stock iodine and 8 g potassium iodide to 200 ml with water. The dilute iodine solution (B) was composed of 2 ml of the stock solution and 20 g potassium iodide in 500 ml of water solution. A standard dextrin solution was prepared by transferring quantitatively a suspension of 0.528 g (moisture-free) of "Merck's Reagent Dextrin" into boiling water. This preparation was cooled, made up to 1000 ml. saturated with toluene and stored in a refrigerator. The buffer solution consisted of 73.5 g sodium citrate (0.25 M) and 21 ml concentrated hydrochloric acid (0.25 M) made up to 1000 ml volume with water.

The buffered limit-dextrin substrate was prepared by suspending 5 g (moisture-free) of "Merck Soluble Starch, according to Lintner, Special for Diastatic Power Determination" in 25 ml of water and pouring this suspension slowly into approximately 250 ml of boiling water. Boiling with stirring was continued for approximately one minute after which the beaker, covered with a watch glass to prevent a starch film from forming on the surface, was cooled in cold running water. Fifty ml of buffer solution and 125 mg of special beta-amylase powder, prepared by the Wallerstein Laboratories were suspended in a few ml of water and transferred quantitatively to the starch solution. The mixture was then brought to a volume of 500 ml, saturated with toluene, and stored at 30°C. for not less than 24 nor more than 72 hours.

The color standard was prepared by pipetting 5 ml of iodine solution (A) and 1 ml of the dextrin solution into a comparison tube. Test tubes measuring 0.5 by 4 inches were satisfactory.

A fresh standard was made up for each series of determinations.

For each amylase determination, 1 ml of the appropriate dilution of the enzyme extract was introduced into a 50 ml Erlenmeyer flask and diluted to 10 ml with 0.2 per cent calcium chloride. After sufficient time had elapsed for the flask and contents to reach 30°C. in a constant temperature water bath, a 20 ml portion of the buffered limit-dextrin was blown into the flask from a fast-flowing pipette and the exact time recorded. At appropriate time intervals 1 ml portions of the hydrolyzing mixture were withdrawn and introduced into 5 ml of the dilute iodine solution (B) in a comparison tube. The contents were thoroughly mixed and compared for color with the standard. Color comparisons were conveniently made through openings in a black surface before a 100 watt daylight bulb screened by thin paper. The exact time for the hydrolyzing mixture to produce a color in the iodine solution matching that of the standard was recorded. It was found that in most cases this time could be estimated to the nearest 0.25 minutes. The time interval required to reach the standard color is inversely proportional to the alpha-amylase activity.

Adsorption Procedure

Batch methods were employed in all adsorption experiments. A 25 ml portion of the enzyme extract diluted 1:10 was added to 2 g of the adsorbent in a small beaker and stirred periodically during a 30 minute period. The pH of the suspension

was measured immediately after mixing and pH adjustments were made at this time unless otherwise noted. After standing, the suspension was filtered with suction through Whatman No. 5 filter paper. Alpha-amylolytic and proteolytic activity were determined on appropriately diluted aliquots of the filtrate. All dilutions were made with 0.2 per cent calcium chloride solution.

EXPERIMENTAL

Enzyme Concentration-Activity Relationships

Sandstedt, Kneen and Blish (52) have established the linear relationship between alpha-amylase concentration and the rate of dextrinization, but no such simple relationship was evident in the determination of proteolytic activity. It was therefore necessary to determine the relation existing between proteolytic enzyme concentration and the titration values obtained from the activity determination. Accordingly, the proteolytic activity in several dilutions of the mold bran extract was determined. The activity expressed in ml of 0.0714 N sodium hydroxide (blank minus sample titrations) was plotted as shown in Plate I. Curve A demonstrates that the enzyme-substrate reaction is not zero order and titration values must consequently be referred to a previously determined curve to get an accurate estimate of proteolytic activity. However, if extracts are diluted so that titration values do not exceed 3 ml, the values fall on the

essentially linear portion of the curve plotted in Curve B. Errors in assuming a straight line relationship between activity and concentration in this region would be quite small. In the course of this investigation, these limits were adhered to and a linear relation assumed.

Examination of Adsorbents

The preliminary work on available adsorbents was designed to eliminate those showing little or no faculty for selective adsorption of the proteolytic enzymes. Each adsorbent was run in duplicate and average values for the percentage loss of both proteolytic and amylolytic enzyme activity are tabulated in Table 1. The pH value of the adsorbent suspension is also recorded. Duplicate results are listed individually when they are substantially different.

From these data, it was possible to divide the adsorbents into three general groups with respect to selective adsorption of proteolytic enzymes. Several of the adsorbents, when in contact with the enzyme solution, produced a reaction which inactivated the enzymes present. Those making up this first group and their respective pH values in suspension were as follows:

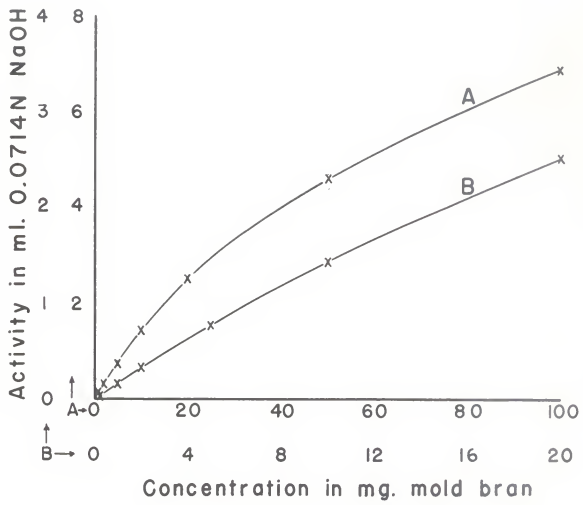
Santocel C	3.3	Isco Adsorbol A-420	3.4
Santocel CX	3.5	Duolite A-2	3.6
Zeo-Karb H	1.7	Duolite C-3	2.1
Zeo-Rex H	1.3		

Those adsorbents which adsorbed both proteolytic and amylolytic enzymes and exhibited little selectivity in their adsorption characteristics comprised a second group. This group included Florex XXX and Norit A which adsorbed both enzymes strongly, Zeo-Dur and Egg Albumin with weak adsorptive characteristics,

EXPLANATION OF PLATE I

Curves showing the effect of concentration on measured proteolytic activity of mold bran extracts. Curve B represents the first portion of Curve A plotted on a different scale.

PLATE I


Activity in ml. 0.0714N NaOH
4
8
3
6
2
4
1
2
0
A
B
A→0
20
40
60
80
100
B→ 0
4
8
12
16
20
Concentration in mg. mold bran

and aluminum hydroxide gel which moderately adsorbed both enzymes. Egg Albumin was difficult to handle and, due to its gel-like properties, a large proportion of the enzyme suspension was retained. A marked variability in results was noted when using aluminum hydroxide gel prepared in the laboratory. This may be expected since the properties of aluminum hydroxide vary markedly with changes in preparation (Hawk, Oser, and Summerson, 18).

A third group consisting of the remaining adsorbents exhibited definite possibilities for selective adsorption of proteolytic enzymes. These were subjected to further study by varying the hydrogen ion concentration of the adsorbent suspension. This pH adjustment was accomplished by carefully adding dilute acid (HCl) or alkali (NaOH) to the suspension immediately after mixing the enzyme extract and adsorbent. Dilution was kept constant by adding appropriate amounts of 0.2 percent calcium chloride solution. After a 30-minute contact period, the suspension was filtered and the enzyme activities determined in aliquots of the filtrate.

During the contact period, there was a tendency in all cases for the pH value to change slowly toward the natural pH of the adsorbent suspension. The degree of change was as much as 2.8 pH units, but varied with the adsorbent used. Apparently, this change is also an adsorption phenomena, the adsorbent removing either anions or cations from the solution. The first or most extreme pH reading was taken to be the critical value.

The results of this investigation are presented in Plates II,III, and IV. Percentage losses of proteolytic and amylolytic activity are plotted against pH levels for each adsorbent.

Table 1. The effect of various adsorbents on the adsorption of proteolytic and amylolytic enzymes.

Adsorbent	pH	Percent loss in activity: amylolytic	proteolytic
Amberlite IR 4B	10.8	61.1	85.6
Amberlite IR 100	10.2	30.0	88.2
Florex XXX	6.5	92.7	97.6
Florisil	9.2	66.7	100.0
Santocel C	3.3	83.7	81.1
Santocel CX	3.5	85.0	80.4
Folin decalso	6.3	7.2	33.8
Decalso fines	7.2	7.2	61.8
Zeo-Dur	6.1	10.4	13.5
Zeo-Karb H	1.7	100.0	94.8
Zeo-Rex H	1.3	100.0	93.6
De-Acidite	7.6	29.0	51.8
Activated alumina	7.5	53.4	77.2
Magnesium silicate	10.2	22.8	78.9
Turkish Emery	5.5	3.5	17.0
Pumice (Pwd.)	5.8	13.0	44.4
Egg Albumin	6.2	10.0	20.5
Isco adsorbol A-420	3.4	100.0	100.0
Isco adsorbol N-100	6.4	29.0	84.7
$Al(OH)_3$	4.4	15.2	24.8
$Al(OH)_3$	6.2	9.6	0.0
$Al(OH)_3$	6.5	44.0	31.1
$Al(OH)_3$on asbestos	6.5	42.8	52.8
$Al(OH)_3$on asbestos	4.3	70.1	57.1
$Al(OH)_3$in alcohol	4.3	19.4	22.8
Activated Bauxite	6.2	22.2	40.5
Zeolite	4.7	17.6	51.8
Norit A	7.2	100.0	97.1
Permutite	6.9	6.6	31.5
Duolite A-2	3.6	100.0	53.5
Duolite C-3	2.1	100.0	96.5
Lloyd's reagent	6.7	12.1	78.0

EXPLANATION OF PLATE II

The relationship of the adsorption of amylolytic and proteolytic enzymes in mold bran extracts to changes in hydrogen-ion concentration for different adsorbents. Amylolytic activity is represented by "o" and proteolytic activity by "x".

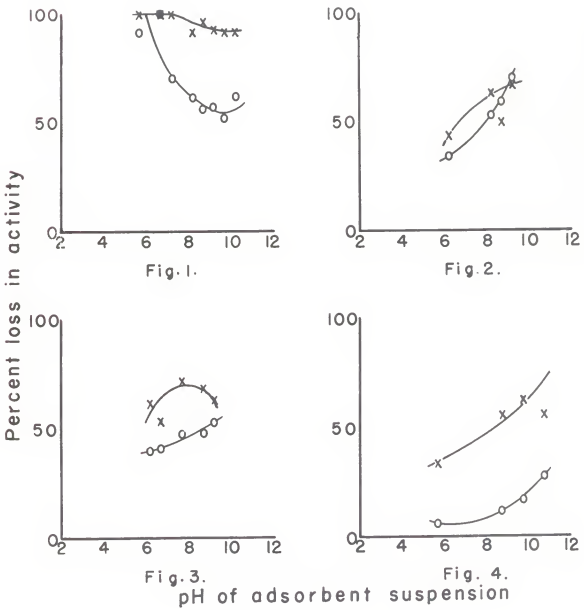
Fig. 1. Florisil

Fig. 2. Activated Bauxite

Fig. 3. Activated Alumina

Fig. 4. Pumice

PLATE II


Percent loss in activity
100
50
0
2
4
6
8
10
12
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
pH of adsorbent suspension

EXPLANATION OF PLATE III

The relationship of the adsorption of amylolytic and proteolytic enzymes in mold bran extracts to changes in hydrogen-ion concentration for different adsorbents. Amylolytic activity is represented by "o" and proteolytic activity by "x".

Fig. 5. Amberlite IR4B

Fig. 6. Amberlite IR100

Fig. 7. Zeolite

Fig. 8. Decalso Fines

PLATE III

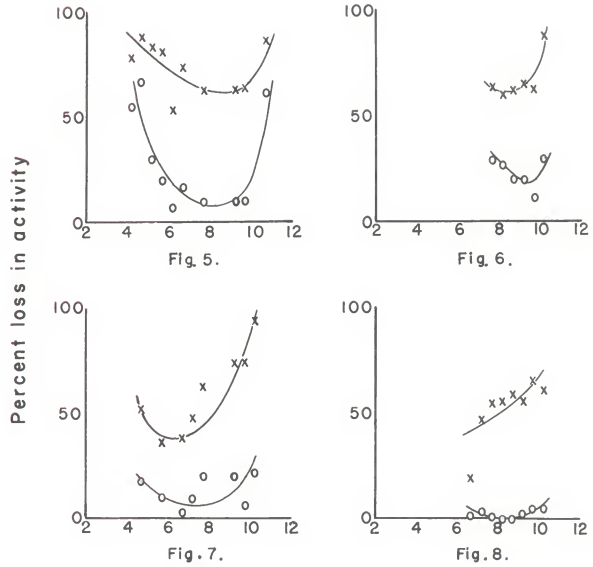


Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

EXPLANATION OF PLATE IV

The relationship of the adsorption of amylolytic and proteolytic enzymes in mold bran extracts to changes in hydrogen-ion concentration for different adsorbents. Amylolytic activity is represented by "o" and proteolytic activity by "x".

Fig. 9. Isco Adsorbol M100

Fig. 10. Lloyd's Reagent

Fig. 11. Folin Decalso

Fig. 12. Magnesium Silicate

PLATE IV

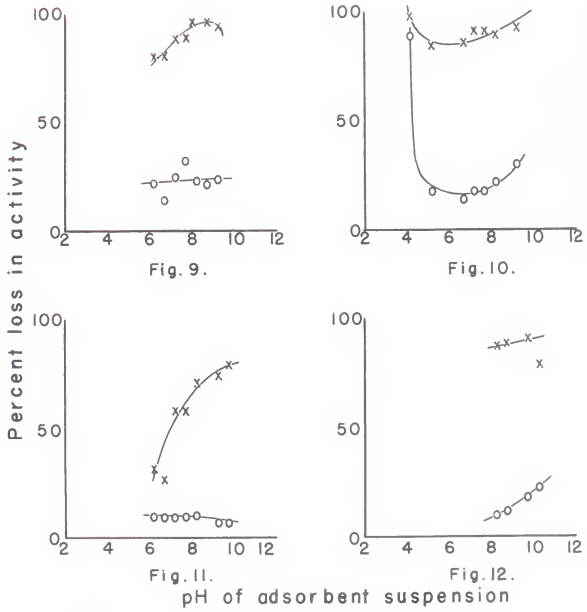


Fig. 9.

Fig. 10.

Fig. 11.

Fig. 12.

The points represent average values of several determinations within a pH range of 0.5 unit. The degree of adsorptive selectivity is thus represented by the distance between the two curves at any particular pH value.

Examination of the curves revealed that an optimum selectivity existed near a pH of 10.0 in nearly all cases. Except for this tendency toward a high optimum pH, there was a wide variation in the effect of pH changes. Florisil was unusual in that it removed the proteolytic enzymes almost completely over the entire pH range studied. Both Decalso products exhibited very little adsorption of amylases. Folin Decalso, however, had the highest selective adsorption of proteolytic enzymes.

From these data it was possible to choose several adsorbents with distinctly superior selective properties. These were Folin Decalso, Decalso Fines, Lloyd's Reagent, Isco Adsorbol N100, and magnesium silicate. It was interesting to note that all of these adsorbents were silicates or were high in silicate content. The Amberlites, IR4B and IR100, exhibited the most promising characteristics of the resinous adsorbents.

Effect of Hydrogen-ion Concentration

The tendency toward a uniform selective adsorption optimum at pH 10.0 stimulated further investigation of pH effects. It was found that raising the pH value of the extract to 10.0 caused a 50 per cent loss of proteolytic activity with no significant

decrease in amylolytic activity. An adsorbent must therefore show selective adsorption for proteolytic enzymes at least 50 per cent above that for amylolytic enzymes to be of value. Since few of the adsorbents studied exhibited a selectivity significantly greater than this, a study of the nature of the differential inactivation of proteolytic enzymes by pH was undertaken.

Plate V illustrates the effect of pH on proteolytic and amylolytic activity. Values above pH 10.0 resulted in marked inactivation of amylolytic as well as proteolytic activity. A pH of 10.0 appeared to be optimum for selective inactivation of proteolytic enzymes. The data in Table 2 revealed that this inactivation was irreversible. When the pH was reduced to 5.5 with hydrochloric acid after adjusting to 10.1 with sodium hydroxide there was a further inactivation of proteolytic enzymes as well as a slight decrease in amylolytic activity. Lowering the pH value with acetic acid did not alter the proteolytic activity appreciably. Barium hydroxide had the same effect as sodium hydroxide. Data on the effect of prolonged standing at various pH levels and temperatures is given in Table 3. No significant differences were noted and it was concluded that the inactivation was practically instantaneous.

It would be desirable commercially to adjust the pH of the extraction mixture and thus eliminate extra steps in the process. The data of Table 4 indicated that favorable results were obtained by this method.

The problem of eliminating the proteolytic activity re-

EXPLANATION OF PLATE V

The effect of hydrogen-ion concentration on the activity of amylolytic and proteolytic enzymes in mold bran extracts. Amylolytic activity is represented by "o" and proteolytic activity by "x".

PLATE V

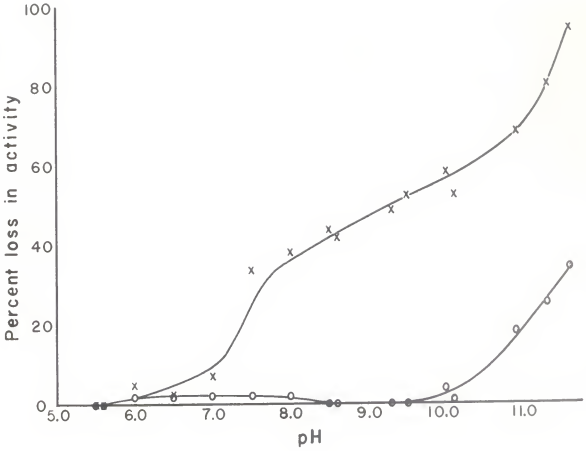

100
80
60
40
20
0
Percent loss in activity
5.0
6.0
7.0
8.0
9.0
10.0
11.0
pH

Table 2. The effect of hydrogen-ion concentration on amylolytic and proteolytic activity of mold bran extracts.

Treatment	pH	Percent loss in activity	
		amylolytic	proteolytic
None	5.4	0.0	0.0
NaOH	10.1	1.9	51.7
NaOH + HCl	5.5	12.7	61.4
NaOH + HAc	4.6	5.8	53.3
$Ba(OH)_2$	10.0	5.9	45.5

Table 3. The effect of standing at various hydrogen-ion concentrations and temperatures on amylolytic and proteolytic activity of mold bran extracts.

Time : hours :	Temperature : °C.	pH : beginning	pH : ending	Percent loss in activity : amylolytic	Percent loss in activity : proteolytic
0.0	30	5.4	5.4	0.0	0.0
0.0	30	10.1	10.1	1.9	51.7
0.5	30	10.2	10.0	3.6	52.4
0.5	6	10.2	10.1	0.0	51.4
1.0	30	10.2	9.8	3.6	56.2
1.0	6	10.2	9.9	0.0	48.1
3.0	30	5.4	4.9	0.0	-6.7
3.0	30	10.2	9.5	3.6	52.4
3.0	6	5.4	5.6	0.0	-1.0
3.0	6	10.2	9.7	0.0	43.8
36.0	30	5.4	---	2.1	0.0
36.0	6	5.4	---	2.1	13.8

Table 4. Effects of hydrogen-ion concentration in the extraction slurry on amylolytic and proteolytic activity of mold bran extracts.

pH adjustment	pH	Percent loss in activity	
		amylolytic	proteolytic
In extract	10.1	1.9	51.7
In slurry (10.1)	7.6	---	52.7
Reversed with HCl	5.6	---	59.1

maining after treatment at high pH was subjected to further study. Accordingly, the pH adjusted extract was treated by several of the more efficient adsorbents. The results are tabulated in Table 5. It was possible to remove all proteolytic activity with Lloyd's Reagent at the expense of a significant loss in amylolytic activity. Folin Decalso and Amberlite IR4B provided better differentiation, but did not completely eliminate proteolytic activity.

Effect of Sodium Chloride

The use of adsorbents for further removal of proteolytic activity would necessitate extra handling and expense in a commercial process. A chemical method would be more simple and less costly. Conclusive evidence was presented by Miller and Johnson (33) showing that proteolytic activity was inhibited or inactivated by sodium chloride in flour doughs. The effect of salt on the proteolytic activity in extracts of mold bran was therefore thought worthy of investigation.

Salt was first added to the digestion mixture in the same proportion as that found in normal flour doughs. The loss of activity as shown in Table 6 represented 66.4 per cent of the proteolytic enzymes and 14.8 per cent of the amylolytic enzymes. Since the nature of this loss of activity could not be studied conveniently in the digestion mixture, a series of activity determinations was made with different concentrations of salt in the diluted extract. These results are also record-

ed in Table 6. Equal salt concentrations in the digestion mixture and in the extract are shown to give similar results. A slight loss of amylolytic activity with increasing salt concentration was observed, but this loss was not proportional to the decrease in proteolytic activity. A salt concentration of 250 mg per ml of extract was chosen for further comparison work. This represents about 70 per cent saturation of the enzyme extract.

The loss of proteolytic activity by salt treatment was shown by the data in Table 7 to be virtually instantaneous and irreversible. Neither prolonged standing at 30° and 6° C. nor dialysis against 0.2 per cent calcium chloride solution caused consistent significant changes in activity. Tests for chloride ions (Cereal Laboratory Methods, 1) after 36 hours' dialysis confirmed the absence of all added salt. Again with commercial aspects of the problem in mind, salt added to the extraction slurry achieved similar results.

Combined Effect of Hydrogen-ion Concentration and Sodium Chloride

The combined effects of hydrogen-ion concentration and sodium chloride were investigated next. The results are tabulated in Tables 8 and 9. A definite additive effect was observed, but the only significant effect of additional treatment was the effect of salt on the adsorption of amylases. Loss of amylolytic activity in adsorption was in all cases increased over similar

Table 5. Effects of treating extracts of mold bran at pH 10.0 with different adsorbents.

Adsorbent	pH	Percent loss in activity	
		amylolytic	proteolytic
None	10.1	1.9	51.7
Amberlite IR4B	9.0	16.6	82.9
Folin Decalso	8.4	17.8	82.0
Lloyd's Reagent	8.2	48.9	100.0

Table 6. Effects of salt (NaCl) on the amylolytic and proteolytic activity of mold bran extracts.

Treatment	Salt conc.	pH	Percent loss in activity: amylolytic	Percent loss in activity: proteolytic
In digest	860 mg	5.2	14.8	66.4
In digest	250 mg	5.2	2.0	26.2
In extract	30 mg/ml	5.1	0.0	3.3
In extract	100 mg/ml	5.0	5.8	13.3
In extract	250 mg/ml	4.8	5.8	25.2
In slurry	250 mg/ml	5.4	3.8	17.6

Table 7. Effect of dialysis and standing on the amylolytic and proteolytic activity of salt-treated mold bran extracts.

Treatment	Temp. °C.	Salt mg/ml	pH	Percent loss in activity amylolytic	proteolytic
None	30	0	5.6	0.0	0.0
Standing 36 hrs.	6	0	5.4	2.1	13.8
Standing 36 hrs.	30	0	5.4	2.1	0.0
Standing 36 hrs.	6	250	4.7	8.0	30.8
Standing 36 hrs.	30	250	4.7	6.0	25.2
Dialysis 36 hrs.	30	---	6.5	19.3	29.5

Table 8. Combined effects of salt and hydrogen-ion concentration on amylolytic and proteolytic activity of mold bran extracts.

Treatment	Salt: mg./ml.	pH	Percent loss in activity: amylolytic	proteolytic
None	0	5.4	0.0	0.0
NaOH	0	10.1	1.9	51.7
NaCl	250	4.8	5.8	25.2
NaCl + NaOH	250	10.1	6.5	86.2
NaCl + NaOH In slurry	250	6.9	10.7	95.1
Standing 36 hrs.(6°C)	250	10.0	8.0	84.6
Standing 36 hrs.(30°C)	250	10.0	10.0	81.9
Dialysis 36 hrs.(30°C)	---	----	15.4	79.2

Table 9. Effect of adsorbents on amylolytic and proteolytic activity of mold bran extracts treated with salt at pH 10.0.

Adsorbent	pH	Percent loss in activity	
		amylolytic	proteolytic
None	10.0	4.2	76.0
Lloyd's Reagent	8.7	61.7	100.0
Folin Decalso	8.0	28.1	80.0
Amberlite IR4B	9.0	14.8	78.4

losses in adsorption from extracts at pH 10.0 with no salt present. The data obtained from subsequent treatment by dialysis, standing at various temperatures, and the use of adsorbents are also included in Tables 8 and 9.

Comparison of Enzyme Sources

The application of salt and pH treatment to enzyme extracts from various sources is summarized in Table 10. Significant differences in the response of fungal and bacterial enzymes were noted. A pH value of 10.0 had virtually no effect on bacterial proteolytic activity while salt inactivated significant amounts of bacterial amylolytic as well as bacterial proteolytic enzymes. All fungal sources exhibited the same relative effects as those observed in mold bran, and the enzymes from cereal sources, particularly those of malted barley, reacted similarly.

The comparative effects of similar treatment on papain were significant. There was no marked change in papain activity at pH 10.0. Salt concentrations comparable to those used with other sources actually increased the activity of this enzyme.

Table 10. The effect of hydrogen-ion concentration and salt on the amylolytic and proteolytic activity in extracts from different enzyme sources.

Source	Type	Percent loss in activity					
		pH 10.0		Salt /1		pH + Salt	
		amyl:	prot.:	amyl:	prot:	amyl:	prot
Goering Bran	Fungal	0.0	52.0	14.8	66.4	14.8	88.0
Wallerstein Bran	Fungal	4.8	76.5	9.1	54.1	16.7	88.8
"E-Z-1"	Fungal	0.0	75.0	10.5	48.3	10.5	86.7
Rohzyme S	Fungal	0.0	85.7	5.9	45.7	5.9	92.1
Wallerstein	Bacterial	0.0	1.1	10.5	40.4	10.5	40.4
Jeffrey's Bran	Bacterial	3.8	3.9	35.1	71.0	35.9	80.6
Polidase S	Vegetable	0.0	30.5	0.0	56.6	5.3	78.6
Malted Barley	Cereal	0.0	70.6	2.4	26.5	4.7	78,4
Malted Wheat	Cereal	0.0	30.4	6.7	15.8	12.5	42.9
Papain	-	---	6.8	---	31.0	---	-31.0

/1 860 mg salt in the digestion flask represents the same proportion as that usually mixed in bread doughs.

DISCUSSION

The properties of proteolytic enzymes in flour doughs and in brewing media have not been completely investigated. For many years, workers have avoided studies involving proteolytic activity because of the lack of suitable methods. The method used in this investigation employed hemoglobin as a substrate and was standardized recently by Miller (37). The assumption that the action of enzymes in baking or brewing will follow the same pattern as their action on an unrelated substrate, such as hemoglobin is not strictly true. However, until a more suitable substrate is prepared, the proteolytic activity of different preparations can be followed by this procedure.

Determinations of relative proteolytic activity by this method have proved readily reproducible and reliable. Miller and Johnson (38) used it successfully to predict the extent of proteolysis in flour doughs and they were able to follow the effect of adsorption in separating proteolytic and amylolytic enzymes. In this study, 39 different determinations of proteolytic activity in mold bran extracts resulted in a mean titration value of 1.200 ml of 0.0714 N sodium hydroxide. Twenty-seven duplicate determinations showed a mean difference of only 0.038 ml with a standard deviation of 0.040 ml. Thus it appears that the method for proteolytic activity determination is satisfactory and should stimulate further work on this enzyme system.

Proteolytic activity in mold bran extracts was shown by this method to follow a curvilinear relationship with enzyme concen-

tration (Plate I). A curve of similar shape was also obtained with different concentrations of malted wheat flour and it is to be expected that the activity of proteolytic enzymes from other sources would follow like curves. A unit of proteolytic activity is therefore suggested, but its development was beyound the scope of this work.

The removal of proteolytic enzymes from amylolytic supplements by adsorption was only partially successful. Investigation of numerous adsorbents of various types resulted in the selection of four superior ones; viz., Lloyd's Reagent, Isco-Adsorbol M100, Folin Decalso, and magnesium silicate. It is significant that these four and also other effective adsorbents were either silicates or were high in silicate content. This selection should not be construed to eliminate conclusively all remaining adsorbents from further study. Several ion-exchange resins, for example, were eliminated because their natural pH value in suspension was low enough to inactivate both amylolytic and proteolytic enzymes. Preliminary treatment with basic solutions could adjust the pH to almost any level desired with probable improvement in selective properties. It is quite possible that other adsorbents might have much better selective characteristics under different treatment but the conditions of this study indicate a definite trend toward the silicates as the material best suited for preferential adsorption of proteolytic enzymes.

Most of the adsorbents listed in Table 1 increased the ratio of amylolytic to proteolytic activity. Lloyd's Reagent

removed as much as 78 per cent of the proteolytic enzymes and only 12 per cent of the amylolytic enzymes at the natural pH level of the adsorbent suspension. Changes in hydrogen-ion concentration of the various adsorbent suspensions produced a wide variety of results (Plates II, III, and IV). The striking feature of this phase of the investigation was a definite tendency for all adsorbents to reach a maximum selectivity for proteolytic enzymes at pH 10.0. Lloyd's Reagent, for example, removed approximately 90 per cent of the proteolytic activity and about 20 per cent of the amylolytic activity at this level.

The data plotted in Plate V showed that the effect of high pH values was largely due to selective inactivation rather than to adsorption. A pH of 10.0 resulted in the inactivation of over 50 per cent of the proteolytic activity with no significant loss in amylolytic activity. Thus there appears to be no advantage in the use of adsorbents for removal of proteolytic enzymes unless a selective adsorption of at least 50 per cent over the amylolytic enzymes was obtained. Adjustment of pH with barium hydroxide achieved the same degree of inactivation as adjustment with sodium hydroxide. The proteolytic enzymes did not recover their activity either with prolonged standing or with subsequent lowering of the pH level. The inactivation, apparently due to hydroxyl ions, was concluded to be instantaneous and irreversible. Indeed, hydrochloric acid caused an additional loss of proteolytic activity. Since acetic acid resulted in no significant changes, it was assumed that the additional loss of proteolytic activity by hydrochloric acid was due to the chloride ion.

Sodium chloride likewise caused significant losses of proteolytic activity. A salt concentration of 250 mg per ml of mold bran extract reduced the activity 20 to 30 per cent. This inactivation was also shown to be irreversible and instantaneous (Table 7). Since concentrations of calcium chloride supplying an equivelant amount of chloride ion achieved similar results, the inactivating influence of the chloride ion was further substantiated.

The additive effect of hydroxyl and chloride ions on proteolytic activity was adequately illustrated by the data of Table 8. Treatment of mold bran extracts at pH 10.0 with 250 mg sodium chloride per ml resulted in over 80 per cent inactivation of proteolytic enzymes and less than 10 per cent loss of amylolytic enzymes. Significant losses of amylolytic activity prevented the use of higher concentrations of either hydroxyl or chloride ions. These data appear to indicate the presence of at least two types of proteolytic enzymes, one inactivated by hydroxyl ions and the other by chloride ions. Berger, Johnson and Peterson (8) reported that the proteolytic enzyme system of most common molds consisted of several types. They found at least one protease in addition to at least five peptidases. Otani (44) found a papain-type protease, a peptide decomposing enzyme and other proteolytic enzymes in cultures of Aspergillus oryzae. It is probable that hydroxyl ions and chloride ions inactivate certain specific groups of these enzymes.

Adsorption after treatment with hydroxyl and chloride ions proved of little value. Lloyd's Reagent provided a solution

entirely free of proteolytic activity, but over half the amylolytic activity was lost in the process. It may have been possible to recover the amylolytic activity by appropriate elution methods, but that phase of the work was not investigated at this time.

Proteolytic and amylolytic enzymes from several fungal sources reacted similarly to treatment with sodium chloride and sodium hydroxide. The cereal and vegetable enzymes were also found to be inactivated in the same manner, although the proteolytic activity of malted wheat flour was not decreased to the same extent as in malted barley and fungal extracts. Mounfield (39,40) reported at least two types of proteolytic enzymes in extracts of sprouted wheat. A protease stable at a pH of 4.0 to 6.0 was reported destroyed by standing three days at pH 8.0. This enzyme may be the one specifically inactivated by hydroxyl ions. A dipeptidase which lost its activity at pH 4.0 could be the one affected by the chloride ion. For all fungal and cereal sources tested, there was a definite additive effect of pH and salt inactivation resulting in as much as 90 per cent loss of proteolytic activity with as little as 6 per cent loss of amylolytic activity. It would appear that the proteases and amylases of fungal sources are of the same type as those found in cereals.

Bacterial sources, however, provided proteolytic enzymes with different properties. Proteolytic activity was not decreased significantly at pH 10.0. Amylolytic activity decreased sharply with pH values above 10.0 in the same manner as the fun-

gal amylolytic activity. The same inactivation pH for bacterial amylases was reported by Di Carlo and Redfern (10). Treatment with sodium chloride, however, achieved a degree of proteolytic enzyme inactivation comparable to that of fungal sources, thus adding support to the assumption that hydroxyl and chloride ions inactivate two different types of proteolytic enzymes. With higher salt concentrations, it was possible to obtain an inactivation of bacterial proteolytic activation of bacterial proteolytic activity roughly the same as that obtained with fungal extracts treated with both salt and base. However, a relatively large amount of amylolytic activity was also lost.

The chief enzyme of the proteolytic system of wheat and flour has long been considered to be of the papain type (5,16). Balls and Hale (6,7) based this conclusion on the fact that both papain and wheat proteinase were inhibited by oxidizing agents such as potassium bromate and activated by reducing agents such as cysteine hydrochloride and glutathione. Concurrently, Mounfield (41) found the activity of both papain and a proteinase in sprouted wheat to be accelerated by cyanide. It was concluded that the proteolytic enzymes in sprouted wheat were also of the papain type.

Extracts of papain were treated with hydroxyl and chloride ions in the same manner as the fungal, cereal, and bacterial extracts. The results clearly indicated that the properties of proteolytic enzymes from fungal and cereal sources are not the same as those of papain. A pH level of 10.0 produced no sig-

nificant decrease in papain activity and the same chloride ion concentration as used previously actually accelerated papain activity. Read and Haas (47, 48, 49) presented data indicating that malt protease was not of the papain type, but the "papain-like" concept has persisted. Potassium bromate was found by these authors to inhibit, but not inactivate, the proteases of malt and taka-diastase. Bromelin and papain were adversely affected. Recently, Harris, Johnson and Jesperson (17) made comparisons between native flour proteinases and papain and found distinct differences in regard to temperature of activation and temperature of optimum activity. It must be concluded from these results that the proteolytic enzymes in malt and fungal extracts are not of the papain type as previously supposed.

The results of this investigation indicate a process for commercial production of a fungal amylolytic supplement relatively free from proteolytic activity. It was shown that extractions of mold bran at pH 10.0 in the presence of sodium chloride achieve the same degree of proteinase inactivation as similar treatment of filtered extracts. Thus a single extraction process would provide a solution of alpha-amylase low in proteolytic activity which could be concentrated and packaged for commercial distribution. The inactivation and removal of proteolytic enzymes from amylolytic supplements was not complete, but a significant increase in the ratio of amylolytic to proteolytic activity was successfully obtained. However, only a few of the many aspects of the problem have been cov-

ered. It is desireable, for instance, to obtain free, active proteolytic enzymes as well as amylases for application to research problems. The use of adsorption and elution techniques should be exploited to determine proper conditions for this separation. Extensive investigation of the amylolytic and proteolytic enzymes of cereals, molds, and bacteria will then be possible. It is hoped that the results of this study will stimulate further inquiry into the many problems associated with these and other enzyme systems.

SUMMARY

Several commercial adsorbents were studied with regard to their ability for selectively removing proteolytic enzymes from amylolytic supplements. In the course of the investigation it was observed that 50 to 60 per cent of the proteolytic activity in a mold bran extract was irreversibly inactivated at pH 10.0. Ensuing investigations of this effect and the effects of added sodium chloride resulted in the following conclusions:

1. The modified Ayre-Anderson procedure as standardized by Miller (37) appears to be a satisfactory and reliable method for determining proteolytic activity in various preparations.

2. The adsorbents exhibiting the best characteristics for selective adsorption of proteolytic enzymes from amylolytic extracts of mold bran were either silicates or were high in silicate content; viz., Lloyd's Reagent, Isco Adsorbol N100, Folin Decalso, and magnesium silicate.

3. Fifty to sixty per cent of the proteolytic activity in mold bran extracts was irreversibly inactivated at pH 10.0 which had little effect on the amylolytic activity. Lower hydrogen-ion concentrations inactivated significant amounts of the amylases.

4. Sodium chloride in concentrations of 250 mg per ml of mold bran extract irreversibly inactivated 20 to 30 per cent of the proteolytic enzymes. A small but significant loss of amylolytic activity was also observed.

5. The effects of hydrogen-ion concentration (pH 10.0) and

sodium chloride were additive thus indicating that mold bran extracts contain several specific proteolytic enzymes, some of which are inactivated by hydroxyl ions and others by chloride ions. Combined treatment resulted in a loss of over 80 percent of the proteolytic activity and a decrease of less than 10 percent in amylolytic activity.

6. Fungal, cereal and vegetable proteolytic enzymes responded similarly to treatment with hydroxyl and chloride ions. Bacterial proteolytic enzymes, however, were only slightly affected at pH 10.0.

7. Papain was not affected at pH 10.0 and sodium chloride actually accelerated its activity. This appears to refute the popular concept that the proteinases of molds and cereals are of the papain type.

8. Commercial production of an amylolytic supplement essentially free from proteolytic activity is suggested.

ACKNOWLEDGMENTS

Acknowledgment is made to Byron S. Miller, Assistant Chemist, United States Department of Agriculture, for invaluable aid in directing the investigation and checking the manuscript; to Mr. Donald C. Abbott for preparing the plates; and to Dr. J. A. Shellenberger, Head, Department of Milling Industry, and other members of the department without whose cooperation the investigation could not have been completed.

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