PREPARATION OF AMYLASE ACTIVE CONCENTRATES FROM MOLD BRAN

by

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INTRODUCTION

The utilization of enzymes to effect easily certain chemical reactions which normally require relatively severe conditions is being adapted on an increaeing scale in commercial processing. The eelectivity of these biocatylists finds application in many industries. The amylases, thoee enzymes which have the facility of breaking down starch into simpler subetances including sugars, are employed extensively. The textile industries utilize these enzymes in the preparation of sizings and likewise for desizing fabrics before dyeing. The syrup manufacturing industry benefits from the ability of the amylases to produce sugars from cereal starch. A direct sequel to this process is the production of industrial alcohol from these fermentable sugars. The selective action of the amylases is employed in the syrup and beverage industries to clarify the products by removal of insoluble carbohydrates.

The amylases are to be found in many places throughout the animal and plant kingdome. Their principal animal sources are the salivary and pancreatic secretions. In the plant kingdom amylases are found in malted grains, and as a product of the growth of certain microorganisms. The outstanding commercial sources of the amylases are pancreatin, malt, and certain fungal and bacterial enzymic preparatione.

As pointed out by Underkofler (1942) the use of fungal amylases in the preparation of various dietary articles has been a common practice in oriental countries for many years. The

most important species of mold associated with these products is Aspergillus oryzae, which is the predominant organism in the Japanese koji used for the preparation of sake (rice beer), soy sauce, and other fermented products. Scientifically, Aspergillus oryzae attracted the attention of occidental investigators as far back as 1875. Atkinson (1881) discussed the function of the organism in sake brewing.

A Japanese, Jokichi Takamine, was chiefly responsible for initiating the utilization of the amylase of Aspergillus oryzae in occidental countries. The chief industrial use at present is in the textile and paper industries. In a review of his own work, Takamine (1914) traced the history of his efforts, beginning in 1891, to produce an amylolytic material which would take the place of malt. He found that wheat bran was satisfactory as the substrate for culturing Aspergillus oryzae.

Although Aspergillus oryzae has become the dominant species of fungi used commercially for the production of amylases, the potentialities of many other species have been investigated (Dehn, 1923; Wei and Chin, 1934; Shih, 1937; Hao, Fulmer and Underkofler, 1943). Each species seems to have its own peculiar abilities in the production of enzyme systems, both as to kind and stability of the enzymes. It is interesting to note that though Aspergillus niger strains usually lag far behind those of Aspergillus oryzae in capacity to produce amylases, the amylases that are produced are more stable over a wide range of hydrogen-ion concentrations (Funke, 1922).

The preparation of the crude fungal enzyme, mold bran, has

been discussed quite completely by Underkofler, Severson, Goering and Christensen (1946). The wheat bran may be sterilized with steam, acidified to the desired hydrogen-ion concentration, pH 3.5 to 4.5, and inoculated with the spores of the fungus. It is then allowed to incubate with adequate aeration either in a rotating drum or in thin layers on porous trays. The mold is allowed to grow for 36 to 48 hours and the product is then dried at room temperature. For enzymic conversions, this material, mold bran, may be used as such or an extract of the bran may be employed. Water soluble concentrates of greater purity and uniformity frequently are the preferred materials for industrial applications. These may be prepared by precipitating the enzyme from a water extract of the bran.

The commercial preparation of amylases is closely linked with the milling industry. The use of an amylase supplement in the baking of bread is a standard commercial practice. The only supplements that may be used in the United States, as dictated by the Federal Food and Drug Laws, are malted wheat or malted barley flour. The technique of sizing and desizing textiles, the production of syrups and industrial alcohol, and the production of mold bran all utilize grain or grain products. The use of wheat bran as a substrate for controlled mold growth and enzyme production provides a valuable additional outlet for this mill by-product.

The purpose of the present investigation has been to determine the optimum conditions for producing stable, water soluble, industrially acceptable enzyme concentrates from

commercial mold bran. The ability of certain organic substances to precipitate the enzyme from a water extract is well established. But the influence of certain environmental factors upon enzyme activity and upon the physical characteristics of precipitates is not well known. Additional information should help not only in the commercial processing of mold bran but also should provide valuable information relative to the fundamental properties of the mold amylases.

Many approaches have been made to the problem of purification of the amylases. The problem has been mainly one of protein precipitation. The majority of the work done in this field has been for the development of a method of purification to obtain a relatively pure concentrate for further studies of the properties of the enzyme. However, Takamine (1894) was successful in the preparation of uniform marketable precipitate. His product is sold under the trade name of "Taka-diastase", understood to be an alcohol precipitated concentrate from Aspergillus oryzae grown on wheat bran.

One of the first references to a purification method for amylases is the report of Payen and Persoz (1833) that extracts of malt were precipitated with ethanol to concentrate the enzyme-active principle. A great deal of work has since been done on the use of ethanol as a precipitating agent. Newton and Naylor (1939) used 65 percent ethanol to precipitate the amylase from soy bean extracts. The work of Sherman and Schlesinger (1912) shows the feasibility of ethanol as a precipitating agent for the concentration of pancreatic amylases. The differential

solubility in ethanol of the alpha and beta components of malt was used by van Klinkenberg (1931), Giri (1933), and Kneen, Sandstedt, and Hollenbeck (1943), to obtain pure or relatively pure alpha and beta amylase.

Ammonium sulfate precipitation followed by resolution and precipitation with ethanol has been used as a means of amylase purification by several workers (Osborne, 1895; Osborne and Campbell, 1896; Sherman, Caldwell, and Doebbeling, 1934; Shukla, 1942).

The importance of acetone as a precipitating agent can be inferred from its incorporation in the purification methods of Sherman and Schlesinger (1913), Winkler and Köck (1929) and Tilden, Adams, and Hudson (1942).

Purification by adsorption has been employed by using alumina gel (Sherman, Caldwell, and Adams, 1930), tannins (Weidenhagen, 1933) and lignins (Wallerstein, Alba, and Hale, 1945). The same procedures have been employed for the purification of mold amylases. Ethanol (Takamine, 1894; Sherman and Tanberg, 1916; Harada, 1931; Tokyoka, 1937; Miyamoto, 1942), ammonium sulfate (Caldwell, Doebbeling, Chester, and Volz, 1945) and the lignins mentioned above, all have been used with this type of amylase. The work of Akabori and Kashimoto (1938) and Hayasi (1941) suggests the use of methanol as a precipitating agent for mold amylases. Purification of fungal amylases by adsorption was studied quite extensively by Nishimura (1926) and Kitano (1936).

As an aid to precipitation of mold amylases the addition of barium chloride to the enzyme infusion prior to precipitation

with ethanol is suggested by Moriga (1918) and Taketomi and Takeda (1935).

A detrimental effect of ethanol on the activity of the precipitate was reported by Münter (1911), Sherman and Schlesinger (1915), Sherman, Garard, and LaMar (1920) and Blish, Sandstedt, and Mecham (1937).

The starch-degradation system of molds consists of amylases and glucosidases either individually or in combination. The amylases have been classified as the alpha and beta types. The glucosidases include those splitting 1-4 and 1-6 alpha glucosidic linkages.

Alpha amylase is responsible for the fission of inter-glucosidic linkages deep within the starch molecule breaking it into less complex, indefinite carbohydrates called dextrins. These dextrins give no color with iodine and the progress of starch hydrolysis by this enzyme may be followed by the color changes produced with an iodine solution. Beta amylase effects hydrolysis of the starch molecule by the disruption of alternate glucosidic linkages, beginning at the end of a chain to produce maltose and a complex limit dextrin. The products of this limit hydrolysis, 60 percent maltose and 40 percent complex limit dextrin, give a similar color with iodine to that characteristic of the starch iodine complex. Therefore, this hydrolysis must be followed by some other means such as the production of reducing groups or fermentable sugars.

The 1-4 and 1-6 alpha glucosidases are responsible for the hydrolysis of the maltose and other di- and tri-saccharides

produced by the action of alpha and beta amylase on starch.

The enzyme system produced by Aspergillus oryzae has been described by several investigators (Wohlgemuth, 1912; Sano, 1922; Tamiya, 1942) as containing not only amylases but a great number of other enzymes. The present study is concerned with the amylase activity primarily and the maltase activity secondarily of the mold enzyme-complex. No attempt has been made to determine the content of other enzymes in either the original mold bran or the concentrates produced from it. The alpha amylase is the predominant and most important component of the mold amylase system (Kneen and Sandstedt, 1946), and was the only one considered in the research being reported. The existence of maltase in the mold enzyme-complex is well supported by several investigators (Atkinson, 1881; Kitano, 1935; Miwa and Miwa, 1940), and an attempt has been made in this research to show that its precipitation from a mold bran extract requires some other set of conditions than those optimum for amylase precipitation.

MATERIALS AND METHODS

In the investigation of precipitation procedures, four organic compounds have been compared under various conditions as precipitating agents. Methanol (99 percent), ethanol (95 percent), isopropanol (98 percent) and acetone (99 percent) were studied. The purity of the compounds was taken from the manufacturers' labels. The salts, acids, and bases used were of C.P. grade when available.

The starch substrate used for determining activity was made from "Merck Soluble Starch, According to Lintner, special for diastatic power determinations." The dextrin used for making the color standard was a "Merck Reagent Dextrin".

A quantity of commercial mold bran, "Eaglezyme", was obtained from the Mold Bran Company of Eagle Grove, Iowa, and this was used throughout the experiments as the crude product from which the enzyme was extracted for precipitation. This bran was produced by the growth of a strain of Aspergillus oryzae on wheat bran. Mold brans from other sources were used for comparison of enzyme systems. A mold bran produced by the growth of another strain of Aspergillus oryzae was obtained from the Bacteriology Department of the University of Nebraska. Other commercial mold brans were obtained through Dr. Eric Kneen from Wallerstein Company, Jeffreys Laboratories, and from Jacques Wolf and Company. Commercial concentrates from Rohm and Haas (RHozyme S), Wallerstein Company, and Schwarz Laboratories (Polidase S) also were used in some of the comparisons.

The principal amylase in fungal enzyme systems being of the alpha type, a modified Wohlgemuth (1908) method was used to follow precipitation recoveries. This method is based on the time required to obtain the red-brown color with iodine described by Sandstedt, Kneen and Blish (1939), when a known amount of extract is allowed to digest a given amount of starch substrate. The substrate was a 20 ml aliquot of one percent boiled starch buffered with sodium citrate-hydrochloric acid buffer to a pH value of 5.0. The time (dT in minutes, required by an appropriate

aliquot of the liquid culture to convert the substrate at 30°C. to a point where the "red brown" color is given with iodine, was determined.

The aliquots of the extracts or resolutions used for activity determinations varied from two ml to 10 ml, depending on activity of the precipitate. Since the total reaction volume was a constant 30 ml, it was necessary to adjust the volume when less than a 10 ml enzyme aliquot was employed; this was done with 0.2 percent calcium chloride solution (Hollenbeck and Blish, 1941). The accuracy of this procedure is about five percent so only differences in results of this order are significant.

The method outlined by Kneen and Beckord (1946) was used for comparison of the saccharification action of the various enzyme systems. This method measures the comparative amount of fermentable sugars produced. A starch substrate is digested by the enzyme, the sugars produced fermented by yeast, and this fermentation followed manometrically on the "pressuremeter" of Sandstedt and Blish (1934).

In comparing the maltase activity of the various systems a method was devised in which the selective fermentation of glucose in the presence of maltose by "Maca Yeast" was utilized. Additional information on this method will be given later under experimental data. These last two methods were used only in the characterization of the concentrates obtained and in the comparison of various enzyme systems.

In order that a greater number of precipitation factors might be studied it was deemed desirable to use a simple and

rapid method of following precipitation recovery. Ten ml of a one to ten extract of the mold bran were placed in a 100 ml centrifuge tube and adjusted to the desired set of conditions for precipitation. To this was added a calculated amount of precipitating agent. The precipitate formed was centrifuged out and the supernatant liquid discarded. To the residue was added 40 ml of distilled water. The dextrinizing activity of an aliquot of this solution was compared with a similar quantity of the original extract and from this was calculated the percent recovery of activity.

The addition to the precipitate of a volume of distilled water equal to four times the original volume of the extract minimized errors due to dilution by water remaining in the tube, and those due to traces of precipitating agents or salts from the precipitation. Normally a one ml aliquot of the dissolved precipitate was used for determination of activity. This one ml of enzyme solution was in turn diluted thirty times in the reaction flask. The procedure kept contamination errors to a minimum.

EXPERIMENTAL

After standardizing a method of extraction, the problem of preparation of an enzyme concentrate became a process of determining, step by step, the effect of precipitant concentration, temperature, hydrogen-ion concentration, and the kind and concentration of salts present during precipitation on the activity and physical characteristics of the precipitates. The goal one

might hope to attain in such a problem would be to find a set of conditions under which the enzyme could be recovered 100 percent, as a crystalline, water-soluble precipitate which would be many times as active on a weight basis as the original mold bran.

Extraction of Amylase

The studies on the technique of extraction showed that the amount of enzyme extracted is directly proportional to the ratio of extraction, i.e., the concentration of enzyme in the extract is doubled when the water-bran extraction ratio is doubled. But the amount of liquid extract recovered varies inversely with the water-bran ratio of extraction. It was concluded that an extraction ratio of 1:10 was most adapted to the research. Dilute calcium chloride solutions have been suggested as appropriate extractants for the alpha type of amylase, (Hollenbeck and Blish, 1941). With the particular mold bran used the activity of the extract seemed to be independent of the presence of calcium chloride in the extraction medium.

Three methods of extraction procedure were investigated. Method one was the standard method of Sandstedt, Kneen, and Blish (1939). The extraction mixture was allowed to stand for one hour at 30°C. with agitation at 15 minute intervals. Method two made use of a mechanical laboratory stirrer, the extraction mixture being in a beaker in a 30° bath. Method three employed a Ward's Liqui-mixer (similar to a Waring Blender). A greater concentration of enzyme was obtained from a three minute extraction

with the Liqui-mixer than with a one hour extraction by the standard method. Fifteen minute extraction with method two was equal to a one-hour standard extraction.

The effect of temperature upon the extraction procedure was not studied. A temperature of 30°C. was the most convenient temperature to use and since satisfactory enzyme infusions were obtained at this temperature, it was used as the standard extraction temperature throughout the work.

The severe agitation of the Liqui-mixer suspends a quantity of material which cannot be removed even by centrifugation. This appeared to be the only disadvantage of such a method of extraction. However, the suspended material did not interfere in preliminary studies and the time saved facilitated the laboratory work.

Difficulty was encountered in the extraction process in removing the bran particles and other foreign matter from the prepared extract. The mixture could not be filtered by suction or by gravity. The volume of the mixture did not lend itself well to centrifuging in 100 ml tubes. The procedure finally used was to strain the mixture through coarse cloth followed by centrifuging to remove the remaining small particles.

Precipitation of the Amylase

Influence of Concentration of Precipitant. The influence of concentration of various organic compounds was studied by adding a calculated amount of the precipitant to 10 ml of the

enzyme extract such as to bring the resulting mixture to a certain percentage by volume of the organic compound. A comparison of the precipitating potential of methanol, ethanol, isopropanol and acetone was made by precipitation with 60 and 70 percent precipitant at approximately 35°C. Acetone and isopropanol are superior to the other two as estimated from the activity recovered (Table 1).

It is of interest to note that the desirability of the precipitate as judged by its appearance, varied inversely with the activity recovered at the 70 percent level. Methanol gave a very white, flocculent precipitate that settled rapidly to leave a clear, supernatant liquid. The other precipitating agents gave increasingly cloudy supernatant liquids in the order mentioned. The apparent stickiness of the precipitate followed the same order as the activity recovered.

In an attempt to determine the optimum concentration of methanol for the complete precipitation of the amylase it was found impossible to obtain complete recovery of the enzyme at 20°C. (Table 2, Fig. 1). There was a continued increase in the recovery obtained with increase in concentration to 75 percent methanol. Further increases in concentration gave no further increase in amylase recovery.

Ethanol gave a similar curve but with more complete recovery of enzyme (Table 2, Fig. 1).

Studies with acetone as a precipitation agent showed it to be similar to isopropanol and superior to the other two alcohols (Table 2, Fig. 1). However, this precipitant tended to produce

Table 1. Effect of concentration of certain organic compounds on the activity recovered in amylase precipitation.

Precipitant	Percent recovery Concentration of precipitant 70%	 60%
Methanol	0	0
Ethanol	94	0
Isopropanol	98	41
Acetone	100	39

Table 2. Effect of concentration of precipitant on recovery of amylase activity in precipitates (20-25°C.).

Concentration of precipitant (by volume)	Percent recovery			
	Methanol	Ethanol	Isopropanol	Acetone
40	--	1.5	--	3
45	--	--	13	32
50	--	3	33	76
55	--	4	90	90
60	--	32	100	93
65	11	63	100	100
70	35	94	100	100
75	75	95	--	--
80	79	--	--	--

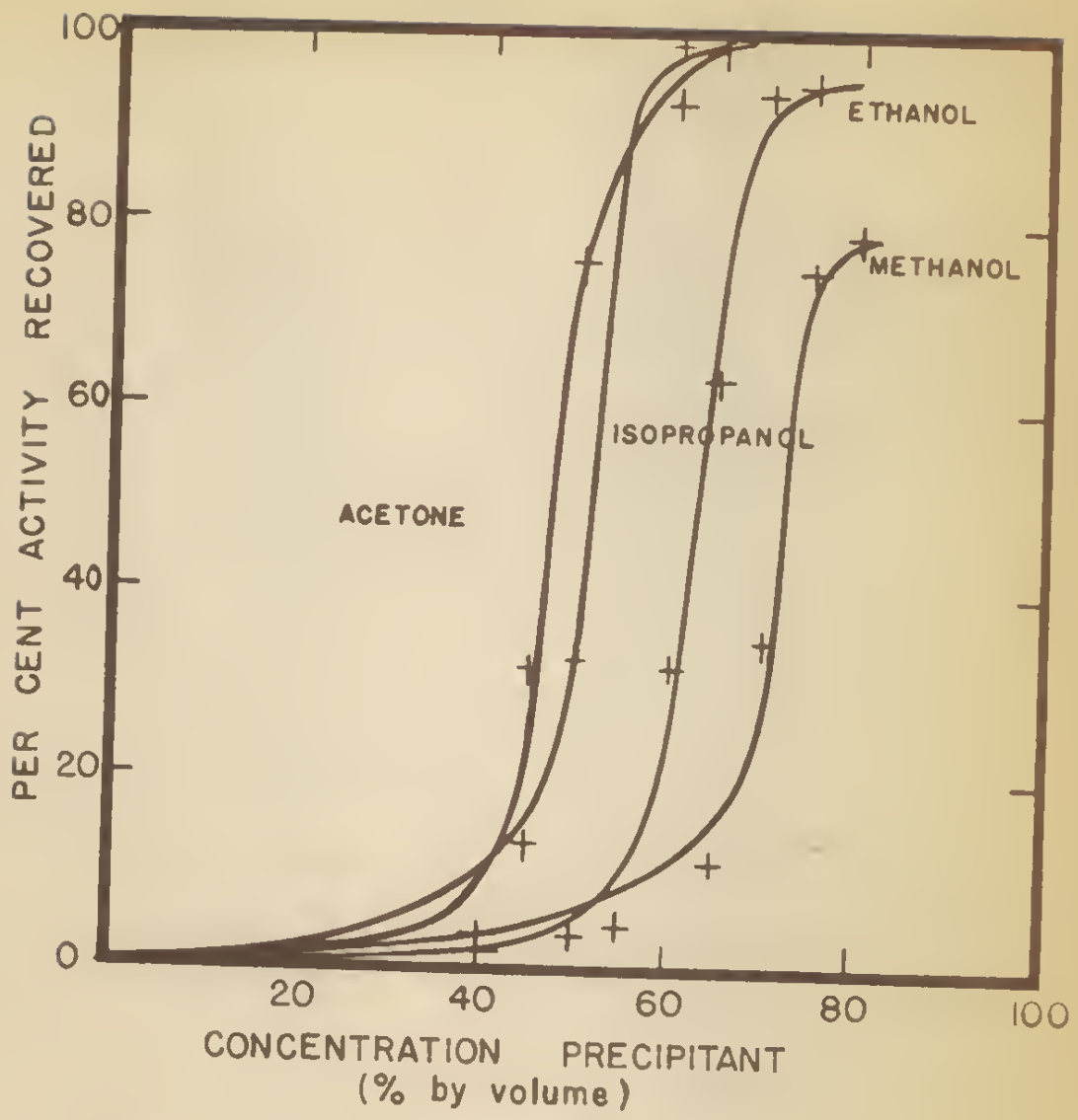

Figure 1. Effect of precipitant concentration on recovery of amylase activity from mold bran extract.

stickiness and discoloration in the precipitates. The precipitates produced also are more difficultly soluble than those of the alcohols.

The concentration studies with isopropanol indicated that this compound was superior to the other alcohols studied (Table 2, Fig. 1). Isopropanol seemed to be the most promising precipitating agent since an abrupt rise in the activity of the precipitate occurred between 50 and 55 percent alcohol.

Studies with isopropanol showed that at low concentrations, 10 to 20 percent, very small amounts of enzyme were precipitated. This suggested a method for purification. The possibility exists of precipitating out foreign matter by an initial purification precipitation with a small quantity of alcohol. Initial precipitations with 25 percent isopropanol resulted in no appreciable loss of enzyme (Table 3). The second precipitate obtained by making the concentrations of alcohol up to 60 percent was taken up in the original volume of water and again precipitated with 60 percent alcohol. The process produced a loss of 30 to 40 percent of the enzyme.

To determine the effect of variations in the concentration of the alcohol in the third precipitation, the second precipitate was taken up with one-third the original volume of 0.2 or 0.4 percent calcium chloride solution and again precipitated with various concentrations of isopropanol. Approximately 80 percent recovery was obtained with 60 percent isopropanol on the third precipitation (Table 4). With higher concentrations of alcohol there was a decrease in activity. This approach to the problem

Table 3. Effect of various concentrations of isopropanol in the initial purifying precipitation on the activity of the second and third precipitates.

Concentration initial precipitation (percent)	Percent recovery	
	Second precipitation	Third precipitation
0	96	70
14	89	69
25	89	63
33	86	63
39	83	62
45	80	62

Table 4. Effect of isopropanol and calcium chloride concentration in the third precipitation on the activity of the precipitate produced.

Concentration of alcohol in the third precipitation	Percent recovery	
	0.2% $CaCl_2$	0.4% $CaCl_2$
60	78	78
70	80	74
80	67	74

of purification did not seem promising and was discontinued.

It will be noted that dilute calcium chloride extracts were used to obtain the data of Tables 3 and 4. The use of calcium chloride solution as an extraction medium was suggested by the work of Kneen, Sandstedt, and Hollenbeck (1943) which points out the beneficial effect of this salt in stabilizing malt amylase at high temperatures. Later studies in the present investigation showed certain ions, including calcium, to have a pronounced effect on the activity recovered. Accordingly, the practice of using calcium chloride solution extractions was discontinued.

Influence of Temperature. The temperature of precipitation has considerable effect on the amount of recovery obtained when methanol is used as the precipitating agent (Table 5). It was found that by reducing the temperature of precipitation to 0°C., it was possible to obtain fair recovery. However, small increases in temperature reduced appreciably the amount of activity recovered in the precipitate.

In the ethanol-temperature relation studies difficulty was encountered in controlling the temperature. When ethanol or any of the investigated organic compounds is mixed with water, heat is liberated. This necessitated that the alcohol be added very slowly and with constant agitation to control the temperature. Later information showed this factor to be unimportant. The precipitating agent could be added rapidly and the tube and contents allowed to return to temperature equilibrium before centrifuging without any additional loss of activity.

The data obtained and recorded in Table 6 are the averages

Table 5. Effect of temperature on the recovery obtained when methanol is used as the precipitating agent.

Precipitation temperature (°C.)	Percent recovery	
	75% methanol	80% methanol
0	90	92
10	82	88
20	75	78

Table 6. Effect of precipitant concentration and precipitation temperature on the activity recovered in the precipitate.

Concentration precipitant (% by volume)	Percent recovery at indicated temperature (°C.)		
	10	20	30
Ethanol			
60	57	32	17
65	84	63	55
70	93	94	92
Isopropanol			
50	7	4	3
55	74	38	19
60	96	96	93
Acetone			
50	26	12	5
55	86	76	62
60	96	96	93

of from one to six determinations in each temperature-concentration combination. These data indicate that if a precipitation concentration of ethanol of 70 percent or higher is used the influence of temparature is not critical in recovery of activity in the precipitate. When the concentration was reduced to 65 percent, precipitation at low temperatures yielded a good recovery but variations in temperature had a profound effect on the activity of the precipitate formed (Fig. 2).

The investigation into the effect of temperature on precipitation with acetone and isopropanol gave results similar to those obtained with ethanol (Table 6, Fig. 2). The information indicates that a certain optimum concentration the influence of temperature up to 30°C. is unimportant. But when the precipitation concentration is reduced below this optimum the influence of temperature is most pronounced in determining the activity recovered in the precipitate. Isopropanol seems to be more sensitive to changes in concentration and temperature than acetone.

Influence of Standing in Contact with Precipitant. To study the effects of other factors on the recovery of enzymes from methanol precipitation a determination of the recovery obtained with 75 percent methanol at 20°C. was used as the standard precipitation method. To ascertain the effect of standing in contact with methanol, precipitates were allowed to remain in the precipitant for various periods of time before centrifuging. The activity of the precipitate was then determined (Table 7). Methanol apparently has a detrimental effect on the activity of the precipitate from a mold bran extract.

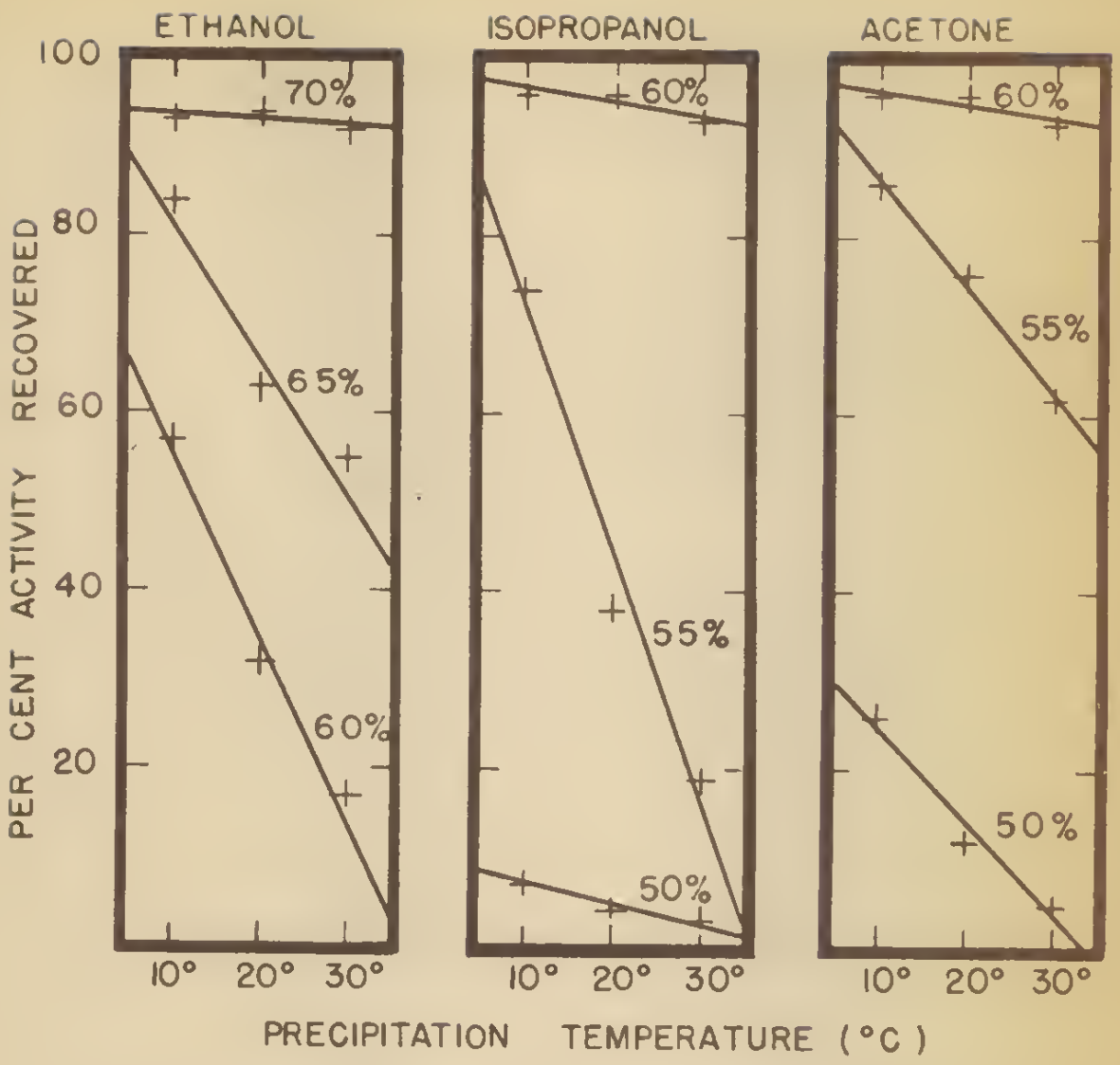

Figure 2. Effect of temperature and precipitant concentration on recovery of amylase activity from mold bran extract.

Table 7. Effect of contact with methanol before centrifuging on the activity of the precipitate.

Period allowed to stand (hrs.)	Percent recovery
1/6	69
1	63
2	54
3	45

The same procedure was followed in the study of ethanol. The precipitations were carried out at 30°C. and allowed to stand at the same temperature. The data of Table 8 indicate that over a period of one hour the loss may be assumed to be negligible. There was appreciable loss on longer standing with 25 hours producing almost 50 percent loss.

Isopropanol and acetone also were studied to determine the stability of the enzyme in the presence of each of these precipitants (Table 9). Acetone precipitates were stable in distilled water but not in calcium chloride solution. Isopropanol precipitates were stable in both water and calcium chloride solution. An additional study with acetone showed that the prscipitate, when it was made from a distilled water extract of mold bran, was stable for at least three hours.

It will be noted that the data given in Table 9 for ethanol precipitation is with 65 percent ethanol. This is in the critical range of prscipitant concentration effect and therefore variations in contact effect may be exaggerated. However, the trend shown in loss of activity on standing is valid.

The loss of enzyme activity during the period necessary for drying the precipitate was next considered. The precipitates were centrifuged out, the supernatant liquid poured off and the precipitate allowed to dry in the bottom of the centrifuge tube. One series was allowed to dry for two hours in front of a fan before the activity was determined. Another was allowed to dry for 20 hours with no forced air circulation. The third series was centrifuged and the activity of the precipitate determined

Table 8. Effect of standing in contact with ethanol on the activity of the precipitate (70% ethanol).

Time allowed to stand (hrs.)	Percent recovery
1	94
3	88
7	83
25	55

Table 9. Effect of time and precipitating agent on the activity of the amylase in the precipitate from distilled water and .2% $CaCl_2$ extract of mold bran, (precipitant - 65% by volume).

Time allowed to stand (min.)	Percent recovery		
	Ethanol	Isopropanol	Acetone
	Distilled water extract		
0	73	100	100
30	67	100	100
60	63	93	93
	0.2% Calcium chloride extract		
0	86	86	96
30	75	89	75
60	65	89	65

immediately. The data shown in Table 10 indicate that there is more danger of loss from the drying of methanol and ethanol precipitates than those from the other two precipitants.

Influence of Hydrogen-ion Concentration. The difficulty of obtaining complete recovery with methanol and its apparent detrimental effect on the activity of the precipitate discouraged any additional work with this compound and only ethanol, isopropanol and acetone were investigated further.

To determine the effect of hydrogen-ion concentration on 70 percent ethanol precipitation a series of determinations were made on various samples, the pH values of which had been adjusted by adding hydrochloric acid or sodium hydroxide. The results, given in Table 11, show a tolerance over the range of pH 5.7 to 6.9 through which there is only a slight variation in recovery (Fig. 3). Above and below these values there was a decrease in activity.

With an increase in pH there was an increase in the density of the precipitate. Precipitates formed at pH 2.5 and 3.5 remained in suspension forming cloudy solutions. They could be clarified only by centrifuging. Precipitates formed at hydrogen-ion concentrations near neutral flocced down very readily.

The studies on precipitation with isopropanol at various hydrogen-ion concentrations were carried out with 60 percent isopropanol and by adjusting the pH with approximately 0.1 N hydrochloric acid of sodium hydroxide. Precipitations were made both with distilled water and 0.2 percent calcium chloride solutions.

Table 10. Effect of drying on the activity recovered from precipitation with 75% methanol, 70% ethanol, and 60% isopropanol and acetone (30°C.).

Time allowed to dry (hrs.)	Percent recovery			
	75% Methanol	70% Ethanol	60% Isopropanol	60% Acetone
0	70	100	88	100
2	69	100	85	100
20	48	83	91	94

Table 11. Effect of hydrogen-ion concentration on the activity of the precipitate formed from mold bran extracts.

Ethanol		Isopropanol		Acetone	
pH	% recovery	pH	% recovery	pH	% recovery
3.5	00	3.4	39	3.5	38
--	--	4.0	78	4.4	88
5.4	92	4.7	93	4.8	90
5.7	95	6.1	95	5.7	99
6.9	95	7.0	97	7.0	96
7.4	89	7.3	90	7.4	92
8.1	80	8.9	82	8.1	85
--	--	--	--	9.8	72

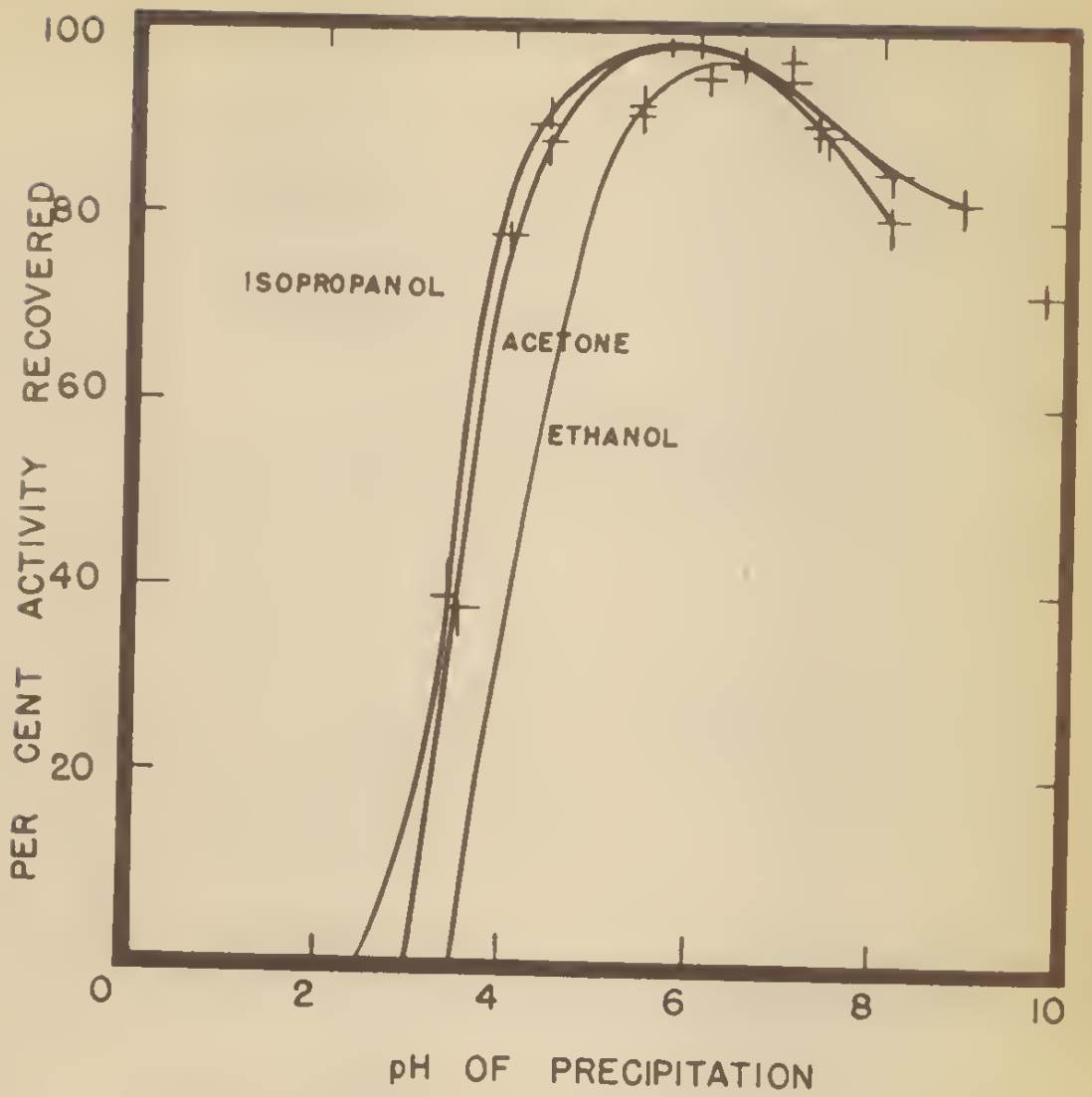

Figure 3. Effect of Hydrogen-ion concentration on recovery of amylase activity from mold bran extract.

As shown in Table 11 for a water extract, the optimum was in the range of pH 6.1 to 7.0. This likewise was found optimal for calcium chloride extracts. The density of the precipitate produced in the calcium chloride extract, as indicated by the rate of settling, varied with the pH; low hydrogen-ion concentrations gave rapid settling and high hydrogen-ion concentrations produced only milky solutions with very slow rates of settling. The precipitate produced at pH 6.5 seemed to have optimum physical characteristics. Conversely, the precipitates formed with the distilled water extracts showed very little variation in density. They all settled slowly.

The effect of hydrogen-ion concentrstion on precipitation and recovery with acetone likewise was considered. The pH was adjusted with 4.0 N hydrochloric acid or sodium hydroxide before precipitation with 60 percent acetone. The data from this experiment are included in Table 11 and Fig. 3 and show that over the pH range from 5.7 to 7.0 there was very little loss in activity, with maximum recovery at about pH 6.0.

Influence of Quality and Quantity of Ions Present. There are two kinds of salt addition which might be made in an enzyme precipitation study. Certain techniques depend solely on the addition of salt to precipitate from solution the enzyme active principle. Other methods incorporate the addition of a small amount of some salt as a precipitation aid to help carry down the active principle when an organic compound is used as the precipitating agent.

The precipitation studies with ammonium sulfate as the sole

precipitating agent were very brief. One series of precipitations was effected, using concentrations from 10 to 60 grams of salt per 100 ml of extract in 10 percent increments. This series displayed a maximum recovery in the neighborhood of 50 grams of salt per 100 ml of extract. A second series was run using concentration variations from 40 to 55 grams per 100 ml of extract in three percent increments. This series showed a leveling off at the 46 gram level. Approximately 93 percent recovery was obtained with concentrations of 46 through 55 grams of salt per 100 ml of extract.

Difficulty was encountered in this investigation in separating the precipitate from the liquid. The high concentration of salt produced a liquid system with a density greater than that of the precipitate. Consequently the precipitate rose to the top and it was impossible to obtain clear separation by centrifugation. With the first series of determinations, separation was by decantation and, undoubtedly, there was an appreciable loss. The second series was separated by gravity filtration. Though the ability to remove the active precipitate by gravity filtration is desirable, the whole procedure did not lend itself well to additional studies. Accordingly, the major investigations of salt effects were directed towards their use as precipitation aids.

There were very noticeable effects of certain salts, especially calcium chloride, on the physical properties of the precipitate obtained when the organic compounds, ethanol, isopropanol and acetone, were used as precipitants. As has been indicated

previously, calcium chloride apparently aids in the flocculation and rate of settling of the precipitate from mold bran extracts. The precipitate formed from a distilled water extract settled quite readily and good recovery was obtained but it was not filterable nor did it dry satisfactorily when centrifuged out.

The study of the effect of salts on precipitation was undertaken to find an aid in improving the physical characteristics of the precipitate, i.e., rate of flocculation and settling, filterability, and ease of powdering, without causing any loss of activity. Before precipitation with 70 percent ethanol, calculated amounts of various salts were added to 10 ml aliquots of extract to make the concentration 0.1 N based on the replaced hydrogen ions in the molecule.

Most salts except those containing calcium and barium, as shown in Table 12, have very little effect on enzyme activity recovered from ethanol precipitation. However, certain salts have noticeable effects on the degree of flocculation and rate of settling. Calcium and barium ions produced a large amount of white floc which settled out leaving a very clear supernatant liquid. The phosphates tended to form gummy appearing precipitates. All the other salts produced rapid but incomplete settling, leaving a cloudy supernatant liquid. The precipitate formed in the presence of calcium and barium ions seemed to have preferable physical characteristics but the activity loss is undesirable.

In the isopropanol precipitation studies on the effect of

Table 12. Effect of various salts on the activity of the precipitate produced. (Salt concentrations: ethanol - .10 N; isopropanol - .04 N; acetone - .10 N).

Salt	Percent recovery		
	70% ethanol	60% isopropanol	60% acetone
NaCl	97	89	100
KCl	97	89	--
$BaCl_2$	83	87	82
$CaCl_2$	73	84	83
K_2SO_4	--	--	95
$(NH_4)_2SO_4$	--	96	--
Na_2SO_4	98	--	93
KH_2PO_4	--	91	--
K_2HPO_4	--	95	--
$Na_2HPO_4 \cdot 12\ H_2O$	93	--	93
$NaH_2PO_4 \cdot H_2O$	93	--	--
$MgSO_2 \cdot 7\ H_2O$	93	--	93
$MgCl_2 \cdot 6\ H_2O$	--	--	95
$Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24\ H_2O$	--	0	--
$FeNH_4(SO_4)_2 \cdot 12\ H_2O$	--	0	--

ions several common salts were chosen to include representatives of mono-, di-, and tri-valent cations and anions. Precipitations were made in extracts modified only with regard to the salts dissolved. Also precipitations were made with extracts to which the salts had been added and then the hydrogen-ion concentration adjusted to pH 7.0 for a study of the cations and to pH 5.0 for the study of the anions. It was assumed from the information on pH optima that the isoelectric point of the protein being studied was between pH 5.0 and 7.0. Assuming that proteins act as amphoteric compounds and therefore acquire a negative charge on the alkaline side of the isoelectric point and a positive charge on the acidic side of the isoelectric point, the adjustment made in the pH should give a better comparison of the various anions and cations as precipitating aids. However these changes of hydrogen-ion concentrations yielded no important information except that as the pH was adjusted to a value nearer 6.5 to 7.0 the activity of the precipitate was increased. This would seem to support the previous contention that the optimum pH for precipitation is between 6.0 and 7.0.

The data of Table 12 show that with an increase in the valence of the cation the activity of the precipitate decreases. However, the di- and tri-valent cations produced a larger volume of precipitate than the mono-valent cations. The ferric and aluminum salts produced a voluminous precipitate which had no activity. Calcium and barium produced a lesser amount of precipitate. The mono-valent cations produced the least precipitate but with the greatest activity. Again the separation obtained

in the presence of the di-valent cations seemed to be preferable and more complete than that obtained with the mono-valent cations.

In the study of the anions the grouping by valence was not so distinct. An anion series based on the activity of the precipitates produced from extracts to which had been added these ions, may be arranged as follows: phosphate, acetate, sulfate, chloride, nitrate, oxalate, citrate, tartrate, and sulfite. A variation of about 20 percent recovery of activity existed between the first and last of this series. There is no marked difference in the density of the prscipitate from the different anion solutions, as was noticed in the precipitation studies with the cations.

Data relative to acetone precipitation in the presence of various salts likewise are given in Table 12. Sufficient salt was added to aliquots of the enzyme extract to make the salt concentration .10 N. The results support evidence gained from studies with other precipitants that calcium and barium ions have a detrimental effect on the recoverable activity.

Besides the detrimental effect of calcium and barium ions on the activity of the precipitate it was found that the sulfate salts had a very pronounced effect on the physical characteristics of the acetone-produced precipitates from mold bran extracts. With magnesium sulfate it was found that in a concentration of .10 N the precipitate appeared as a semi-liquid, sticky mass after centrifugation. If an excess of disodium phosphate was added to the aliquot containing magnesium sulfate the precipitate appeared as a white floc that settled rather rapidly leaving a

clear, supernatant liquid. In the tubes containing solutions of calcium and barium ions the precipitates had much the same appearance as those which contained the magnesium sulfate and disodium phosphate.

In the investigation of the relation of quantity of salt to the activity of the precipitate a brief study was made of the effect of concentration of calcium chloride and sodium chloride in distilled water extracts on the activity of the precipitates from 70 percent ethanol. The results (Table 13, Fig. 4) indicate a very detrimental effect of the calcium ion when compared to the sodium ion.

Additional information was obtained on the effect of calcium chloride concentration with a comparison of the tolerance of the three precipitating agents, ethanol, isopropanol, and acetone to the presence of this salt. Aliquots of extract were adjusted to .10 and .20 N calcium chloride before being precipitated with 70 percent ethanol or 60 percent isopropanol or acetone. When the activity of the precipitates thus formed were determined (Table 14, Fig. 5) it became evident that isopropanol precipitation is more tolerant to the presence of excess calcium ion than either of the other precipitants.

To isolate the individual salt effects, aliquots of extract were dialyzed against various media and their behavior determined after dialysis. Four aliquots of extract were dialyzed respectively against distilled water, .043 N sodium chloride, .060 N calcium chloride and .060 N disodium phosphate solutions for 48 hours at 10°C. After dialysis the extracts were adjusted to

Table 13. Comparison of the effect of various concentrations of calcium chloride and sodium chloride on the activity of the precipitates formed with 70% ethanol.

Salt concentration (N)	Percent recovery	
	NaCl	$CaCl_2$
.02	100	91
.05	98	83
.10	96	72
.20	94	46
.50	91	22
1.00	71	6

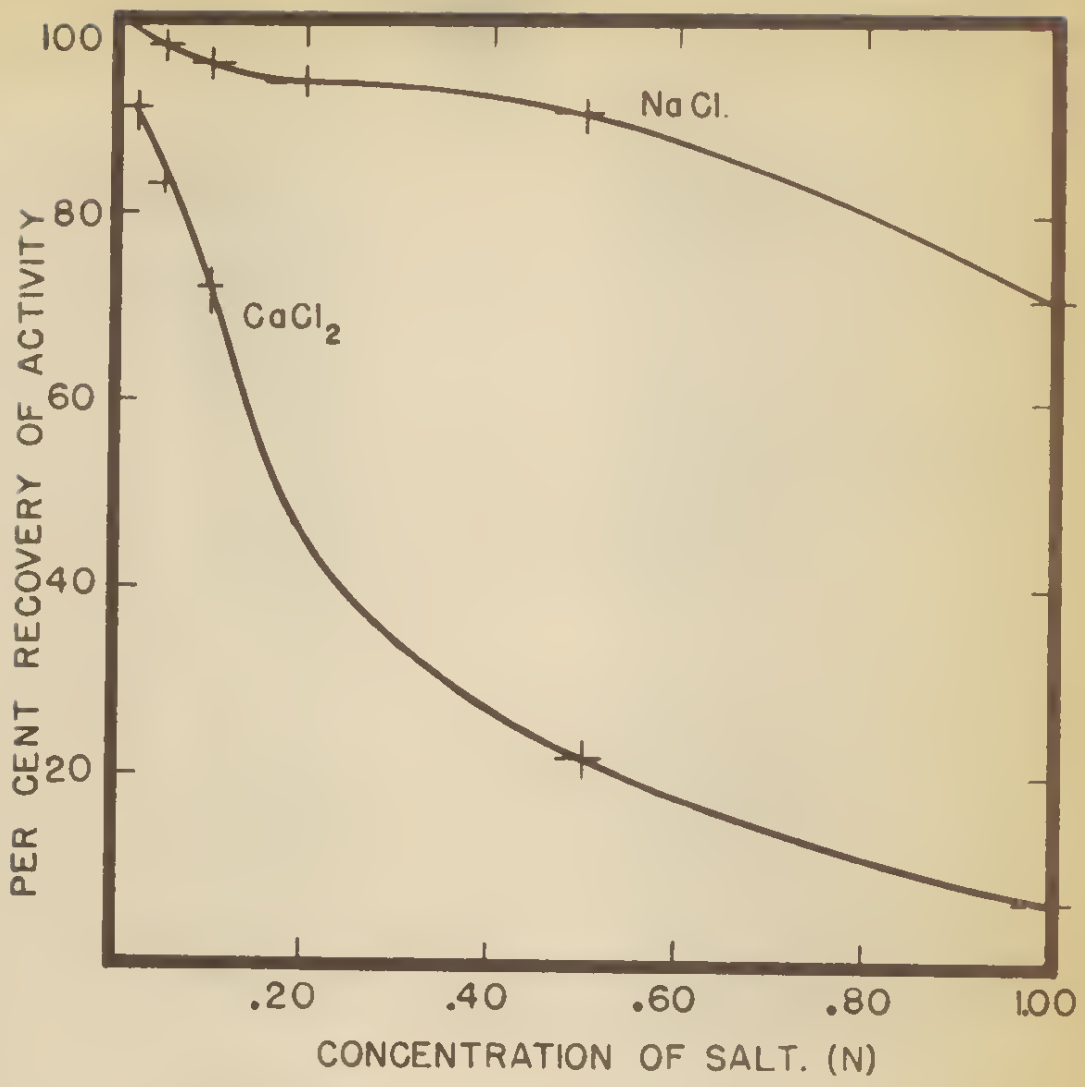

Figure 4. Effect of salt concentration contrasting NaCl and $CaCl_2$ on the recovery af amylase activity from mald bran extract.

Table 14. Effect of calcium ion concentration on the activity recovered from ethanol, isopropanol, and acetone precipitation.

Precipitant	Percent recovery	
	.10 M $CaCl_2$	.20 M $CaCl_2$
Acetone	68	47
Ethanol	74	50
Isopropanol	89	81

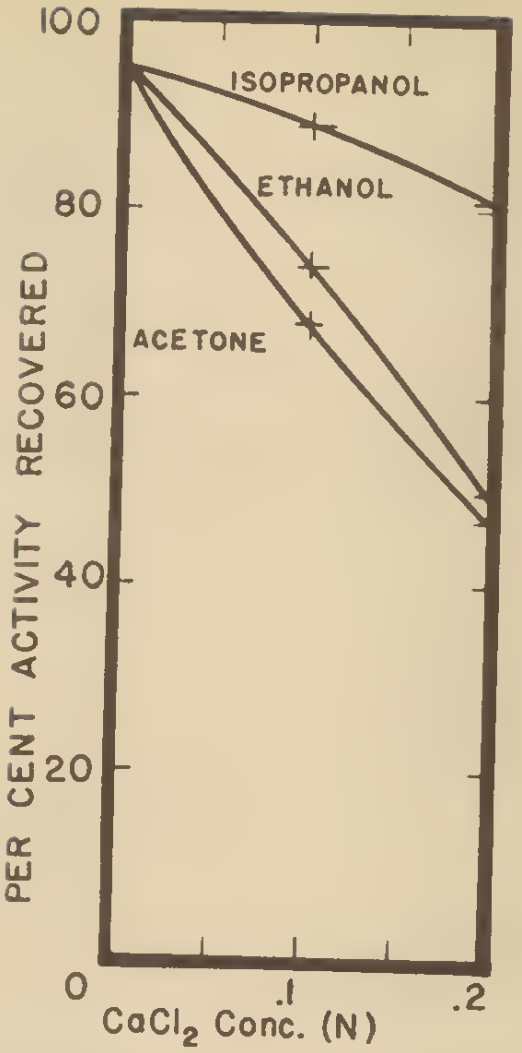

Figure 5. Effect of calcium concentration on recovery of amylase activity.

their original volumes and the activity of each was compared with that of an original extract. No loss of activity was observed in any of the solutions, or in a control allowed to stand at the same temperature without dialysis.

Aliquots of each of these dialyzed solutions were precipitated with 70 percent ethanol. For comparison aliquots of an undialyzed extract to which had been added equal amounts of salt were precipitated under the same conditions. The results in Table 15 demonstrate that the presence of some salt is imperative to recovery of activity in the precipitate. However, none of the salts used in the dialyzing solutions gave recoveries comparable to that obtained with the undialyzed extracts.

The extracts dialyzed against distilled water produced only milkiness when ethanol was added, and when these were centrifuged very little precipitate settled out. The other dialyzed solutions produced a small amount of precipitate with a cloudy supernatant liquid indicating incomplete separation of the precipitated phase. From the undialyzed extract to which had been added calcium chloride, a white, flocculent precipitate formed which settled out to leave a clear supernatant liquid.

The foregoing information indicates that there is some dialyzable substance extracted from the bran which is an aid in recovering an active precipitate. To determine the source of this precipitating aid, i.e., whether it is in the natural wheat bran or is something formed or added in the production of the mold bran, a sample of mold bran and a sample of ordinary wheat bran were extracted, filtered and precipitated under the same

Table 15. Effect of dialysis on activity recovered in the precipitate from 70% ethanol.

Dialysis medium	Salt concentration (N)	Percent recovery	
		Dialyzed solution	Undialyzed solution
Distilled water	--	21	100
NaCl	.043	89	100
$CaCl_2$	.060	89	83
Na_2HPO_4. 12 H_2O	.060	59	100

conditions. The wheat bran produced a precipitate with similar physical characteristics to those of the mold bran as determined simply from appearance. It was therefore assumed that the precipitating aid is something found naturally in wheat bran.

An analysis reported by Bailey (1944) indicates that the content of the four elements, phosphorus, magnesium, calcium and potassium, is high in wheat bran. These elements were considered singly and in combinations to determine their effect on the appearance of the precipitate.

A quantity of mold bran extract was dialyzed for 24 hours against distilled water. Aliquots of this extract were adjusted to various concentrations with various salts and precipitated with 70 percent ethanol. Any one of the salts studied aids in recovery of the enzyme but some appear to cause an increasing loss of enzyme with increases in concentration (Table 16).

Only the magnesium chloride-disodium phosphate combination produced a white, flocculent precipitate characteristic of undialyzed solutions. All other salts or combinations produced milky, slow settling precipitates. The potassium phosphate produced a liquid precipitate at a concentration of .50 N.

The role of magnesium and phosphate ions was investigated further to determine the optimum concentration of each. In the investigation of optimum concentration of magnesium sulfate and disodium phosphate as precipitation aids, no sharp optimum occurred. However, concentrations of .028 N and greater left increasing amounts of insoluble residue when the precipitates were redissolved. Magnesium sulfate was substituted for magnesium

Table 16. Effect of various salts on the activity of enzymes precipitated from dialyzed extracts.

Salt added	Percent recovery		
	.10 N salt	.50 N salt	
No salt added	less than 5	--	
NaCl	97	97	
KCl	93	93	
$MgCl_2 \cdot 6 H_2O$	90	75	
$CaCl_2$	52	less than 5	
K_2HPO_4	13	30	
	.012 N salt		
$CaCl_2$) $Na_2HPO_4 \cdot 12 H_2O$)	69		
	.023 N salt	.036 N salt	.092 N salt
$MgCl_2 \cdot 6 H_2O$) $Na_2HPO_4 \cdot 12 H_2O$)	94	98	96

chloride because of its greater ease of handling. There is no difference in the two salts as precipitating aids with ethanol precipitations. Variations in the concentration of magnesium sulfate and disodium phosphate from zero to .046 N have no effect on the activity of the precipitate.

Studies were conducted to determine the effect of varying ratios of the magnesium ion and monohydrogen phosphate ion on the activity of the precipitate (Table 17). These experiments indicated that an excess of magnesium is detrimental to the activity of the precipitate. This fact supports evidence of loss caused by excess calcium and barium ions when these salts were studied as precipitation aids. When the element of time was introduced along with the factor of ionic ratio it was demonstrated that an excess of monohydrogen phosphate ion stabilized the precipitate (Table 17, Fig. 6). One series of precipitations was made and allowed to stand for three hours before being centrifuged, while another was centrifuged immediately after precipitation.

With a decrease in the $(HPO_4)/(Mg)$ ratio from three to one the rate of settling increases and the clarity of the supernatant liquid decreases. Ratios above three produce a cloudy supernatant liquid and rapid settling.

In a study of the effect of these salts on isopropanol precipitation, with concentrations from 250 percent excess magnesium ion to 250 percent excess monohydrogen phosphate ion complete recovery was obtained. However, it was noted that an excess of magnesium ion tended to cause a gumminess of the precipitate. Also it was noted that the flocculation obtained at 60 percent

Table 17. Effect of variable $(Mg)/(HPO_4)$ ratio and time on the activity of the precipitate produced with 70% ethanol by volume.

Concentration (N) $MgSO_4 \cdot 7H_2O$	Concentration (N) $Na_2HPO_4 \cdot 12\ H_2O$	$(Mg)/(HPO_4)$ ratio	Percent recovery: 10 min.	Percent recovery: 3 hrs.
.047	.019	2.6	90	62
.038	.019	2.0	--	72
.029	.019	1.5	90	78
.019	.019	1.0	--	87
		$(HPO_4)/(Mg)$ ratio		
.019	.029	1.5	98	92
.019	.038	2.0	--	92
.019	.056	3.0	98	92
.019	.073	4.0	--	96

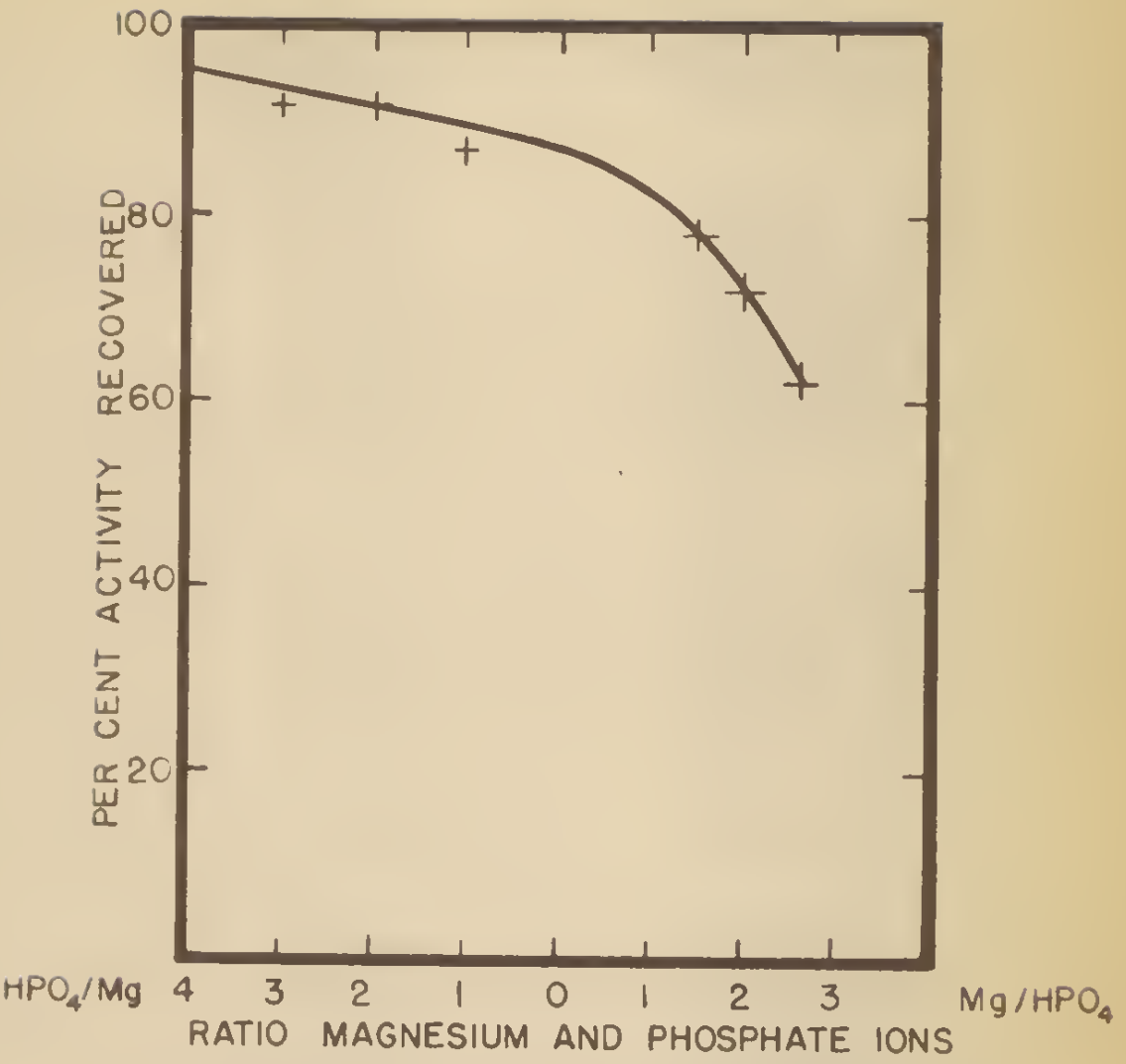

Figure 6. Effect of ionic ratio of magnesium and monohydrogen phosphate ions on the recovery of activity from mold bran extract.

isopropanol was preferable to that obtained at greater alcohol concentrations.

From the study of the effect of different ions on activity recovered in the precipitate from isopropanol it was concluded that mono-valent cation-phosphate salts would give the best recovery of activity. Consequently a study was made of the effect of mono- and di-potassium phosphate on the activity of the precipitate from 60 percent isopropanol. The activity of the precipitate remained constant up to .20 molar mono-potassium phosphate. Further increases gave appreciable loss. At .50 molar phosphate concentration there was a 24 percent loss. This may be a combination of the influence of hydrogen-ion concentration and precipitant concentration. It is interesting to note that in a similar study with calcium chloride there was a 50 percent loss at a salt concentration level of .20 N.

When an attempt was made to make a similar study of di-potassium phosphate, high concentrations of this salt produced coacervates, the activity of which could not be determined.

A study was made of the effect of a variable hydrogen-ion concentration on the activity of the precipitate at a phosphate concentration of .50 molar. The hydrogen-ion concentration was adjusted by using various ratios of mono- and di-potassium phosphates. An optimum, though not marked, occurred at pH 5.3 to 5.6. Lower hydrogen-ion concentrations produced liquid precipitates on centrifugation.

Phosphate concentration studies were made at pH 6.1 and 5.5. At pH 6.1 there is an optimum at .20 molar concentration

but the precipitates tended to liquify and were difficult to handle.

The precipitation at pH 5.5 showed an optimum recovery at .40 molar and all precipitates were of good handling characteristics except the .10 molar which was amorphous and gummy. The quantity of the precipitate increases with the concentration of salt present. The use of the potassium phosphates as precipitating aids was later considered impractical because of the high concentration of salt required.

The tendency of acetone to produce discoloration, gumminess and insolubility in precipitates removed this precipitant from those considered for additional investigation.

Batch Precipitation. From the information accumulated on the influence of separate factors on precipitation, it was believed that ethanol and isopropanol were the more promising precipitating agents. The choice set of conditions was selected for each factor and an effort was made to prepare a quantity of the enzyme concentrate with each of these precipitants.

Attempts to dry precipitates from ethanol precipitation were rewarded with isolates of high activity and good physical characteristics except for water solubility. By using a concentration of .25 percent magnesium sulfate and .72 percent disodium phosphate in the enzyme extract a white to buff precipitate was formed that had ten times the activity of the original mold bran. The precipitate dried rapidly and was readily powdered. The precipitate was separated by centrifugation, resuspended in 95 percent ethanol and filtered. Approximately 68 percent yield

was obtained.

In the batch precipitations with isopropanol similar results were obtained. In Table 18 are listed the data on four trials at isolating and drying concentrates from isopropanol precipitation.

In each case .25 percent magnesium sulfate and .50 percent disodium phosphate were added to the extract before precipitation with 60 percent isopropanol. The residue after centrifugation was dispersed in a volume of isopropanol one-fifth the original volume of the extract to which had been added the anhydrous sodium sulfate. All the precipitates dried as buff to white powders. The sodium sulfate materially aided the filtration of the suspension.

In the use of potassium phosphate as a precipitation aid for batch precipitation certain limitations soon were discovered. When using .20 molar phosphate a decrease in the isopropanol concentration below 60 percent produced no filterable precipitate. An increase in the alcohol concentration to 65 percent increases both quantity and activity of the precipitate. By decreasing the amount of salt from .50 to .13 molar and precipitating with 65 percent isopropanol the activity of the precipitate increases. With concentrations of .13 molar the precipitate tends towards gumminess and is presumably the workable lower limit of phosphate concentration.

The precipitates formed were separated principally by centrifugation. They were not sufficiently crystalline to be easily filtered either by gravity or suction. Either a super-centrifuge or large capacity centrifuge were found necessary to obtain

Table 18. Recovery of activity in precipitates with isopropanol.

Amount Na_2SO_4 (gms.)	Amount extract (ml)	Activity ppt. dT .005 gms.	Yield	
			Weight (gms.)	Percent
1.5	100	40.5	1.94	92
0.0	100	9.0	0.39	71
0.5	100	13.0	0.68	86
2.0	1000	10.5	6.45	74

satisfactory separation.

The use of the anhydrous sodium sulfate in the isopropanol precipitation studies arose from an attempt to dry the precipitates more rapidly. The procedure worked very well and made the concentrate a very easily dried powder.

Properties of Precipitates

It was deemed desirable to make a comparison of the two choice precipitates, from ethanol, magnesium sulfate and disodium phosphate and from isopropanol, magnesium sulfate and disodium phosphate, with the source enzyme material. In this comparison the dextrinizing activity of each was determined. Table 19 indicates that a more concentrated enzyme preparation was obtained with the isopropanol precipitation than with the ethanol. When it is considered that the isopropanol precipitate had been diluted about 30 percent with anhydrous sodium sulfate the concentration of the enzyme obtained is still greater.

In evaluating and comparing the saccharifying power of mold bran and concentrates prepared from it, extracts of these three sources were adjusted to the same dextrinizing activity and then equal amounts (dT - 20 min.) of the extracts were added to a two percent starch substrate and allowed to digest for one hour at 30°C. At the end of this hour 0.5 gram of compressed yeast was added to the fermentation cup and the fermentation was followed manometrically. Readings were taken at 1, 3, 5, 10, 21, and 22 hours. The data in Table 20 include, for comparison, information

Table 19. Comparison of the amylase activity of the original mold bran with concentrates prepared therefrom.

Enzyme source	Activity	
	Weight (gms.)	dT (min.)
Mold bran	.050	13.0
Ethanol precipitate	.005	13.0
Isopropanol precipitate	.005	10.5

Table 20. Saccharifying power of various amylase sources.

Enzyme source	Wt. equal (mg) dT - 20 min.	Pressure - mm Mercury 1 hr.	3 hr.	5 hr.	10 hr.	21 hr.	22 hr.
		Fungal concentrates					
Wallerstein	1.3	17	81	147	251	327	336
Polidase-S (Schwarz)	1.0	15	80	138	270	323	329
RHozyme-S (Rohm & Haas)	0.76	17	67	144	276	331	338
Ethanol precipitate	3.5	14	59	123	272	339	341
Isopropanol precipitate	2.6	15	65	140	293	383	387
		Fungal brans					
Wallerstein	6.1	16	67	145	281	361	364
Jacques Wolf	19.7	13	71	153	290	363	366
Jeffreys	12.5	14	72	153	280	343	346
Eaglezyme	31.4	15	63	138	296	390	390

on other mold brans and fungal concentrates.

It will be noted that all of the enzyme sources listed in Table 20 gave approximately the same fermentation pattern. However, the Eaglezyme mold bran and the isopropanol precipitate produced from it gave greater total conversion than any other of the enzyme sources, as indicated by the reading at 22 hours. It should also be noted that the isopropanol precipitate is superior to the ethanol precipitate in total starch conversion.

To obtain information of the completeness of the carbohydrase enzyme system precipitated it was necessary to have a method of determining maltase activity. This entails a differentiation between glucose and maltose content of a given solution. The possibility of selective fermentation was investigated. In a comparison of different commercial yeasts, Maca Yeast exhibited the greatest selectivity. After a series of fermentations using various concentrations of yeast to ferment known quantities of glucose and studying the relation of various periods of fermentation it was found that by using the same complete nutrient solution of Kneen and Beckord (1946) and 1.5 grams of Maca Yeast per cup, the difference (dP) between the reading of one-fourth and one and one-fourth hours is proportional to the glucose concentration regardless of the maltose concentration, (Table 21).

This principle of selective fermentation may be used to evaluate maltase content by allowing an aliquot of enzyme extract to digest a given quantity of maltose under specified conditions and then after killing the enzyme, fermenting the digested solution with Maca Yeast. Two factors had to be determined before

Table 21. Selective fermentation of glucose in the presence of maltose with Maca Yeast.

Amount (mg) glucose	Theoretical dP	dP without maltose	dP with maltose	Amount (mg) maltose
600	310	317	301	0
480	248	252	255	120
360	186	180	178	240
120	61	59	62	480

this method could be used for assaying maltase content. The optimum temperature for digestion of the maltose would depend upon the thermostability of the enzyme. Some relation must be established between maltase content and the percent conversion of the maltose.

To determine the thermostability of the enzyme, 10 ml aliquots of a 1:10 extract of mold bran were allowed to digest 600 mg of maltose for two hours at 30°, 40°, 50°, 55°, 60°, and 70°C. From these data included in Table 22, it was concluded that the optimum temperature for maltose conversion was approximately 50°C.

The percent conversion of the maltose was calculated by fermenting three samples with the Baca Yeast: (1) 600 mg of glucose with the enzyme extract added; (2) 600 mg of maltose in which the enzyme had been killed by placing the fermentation cup in a boiling water bath immediately on the addition of the enzyme extract; and (3) 600 mg of maltose to which the aliquot of extract had been added and allowed to digest for two hours before killing the enzyme by placing the fermentation cup in a boiling water bath. One, (1), gave the fermentation of 100 percent conversion; another, (2), gave the fermentation of zero percent conversion and the third (3) gave the fermentation due to the conversion produced by the action of the enzyme.

When the relation between maltase content and percent conversion of maltose was studied it was found that the relation between the amount of enzyme extract used and the calculated percent conversion of maltose was approximately linear up to 60 percent conversion. Therefore the percent conversion of maltose

Table 22. Effect of temperature on maltase activity as measured by the conversion of fermentable glucose from maltose.

Temperature of digestion (°C.)	Percent conversion
30	13
40	23
50	44
55	40
60	31
70	2

may be used as a measure of the maltase content up to a 60 percent conversion figure.

This method of maltase determination with digestion at 50°C. for two hours was used to compare the maltase activity of the original mold bran with the maltase activity of the concentrates prepared with ethanol and isopropanol. Also included in the comparison is the maltase activity of certain other fungal brans.

The comparisons were made by adjusting extract concentrations from the different sources so that one ml of extract had a dextrinizing activity (dT) of 10 minutes. Then 10 ml of each extract was allowed to digest 600 mg of maltose for two hours. These data (Table 23) show isopropanol to be more efficient as a maltase-precipitating agent than ethanol. The information also indicates that the Eaglezyme type of mold bran has a higher content of maltase than any of the other commercial brans studied.

DISCUSSION

The precipitation of an amylase active concentrate from a mold bran extract is not a difficult procedure when one of the common organic compounds methanol, ethanol, isopropanol or acetone is used. The influence of the concentration of the precipitant is of fundamental importance. It was found in this study that isopropanol and acetone are of about equal efficiency in the precipitation of the enzyme, while ethanol and methanol require greater concentrations to produce complete precipitation. In other words, the more complex organic compounds are the most

Table 23. Maltase activity of several mold brans and the precipitates prepared from the Eaglezyme bran.

Enzyme source	Percent conversion 600 mg maltose
Wallerstein	25
Jeffreys	13
Eaglezyme	38
Isopropanol precipitate	27
Ethanol precipitate	13

efficient precipitating agents and the efficiency of the precipitant decreases with the decrease in complexity of the organic compound. It might be of interest to study, perhaps, some of the butyl alcohols as precipitating agents from a theoretical standpoint, but the fact that these alcohols are not completely miscible with water and the fact that they would be uneconomical to use on a commercial scale removed these compounds from the list of those investigated as precipitating agents.

In the study of the effect of temperature on precipitation it was found that temperature affected the precipitation potential of the various compounds in the same manner, i.e., by lowering the temperature of precipitation, the concentration of precipitant required to obtain satisfactory recovery was lowered. For example, in the case of methanol with which it was impossible to obtain a recovery anywhere near 100 percent at a temperature of 30°C., by reducing the precipitation temperature to 0°C., recovery in the neighborhood of 90 percent could be obtained with approximately 75 percent methanol.

Of prime importance when attempting to work out an economical process for concentration of enzymes is the effect of the precipitant on the enzyme being studied. In the studies conducted methanol was found to be quite destructive to the activity of a precipitate when the precipitate was allowed to stand for various periods of time in contact with the precipitant. This was also true in the case of ethanol precipitation only to a lesser degree. Precipitates that were allowed to stand in the presence of either acetone or isopropanol showed no loss of activity in

the time periods studied. This would indicate that the latter two precipitants would be superior in commercial processes, particularly those that would require long periods of contact with the precipitant.

From the information obtained in the study of hydrogen-ion extract, it was concluded that this factor is relatively unimportant so long as the pH of the extract prior to precipitation is between pH 5.5 and 7.0. This was true in the case of the three precipitants ethanol, isopropanol, and acetone. The pH relationships of methanol were not studied.

The most interesting influences of environmental factors were found in the studies on the effect of ions on precipitation with ethanol, isopropanol and acetone. The ability to produce a workable precipitate depends on the presence and precipitation of sufficient inorganic salt with the protein to avoid the gumminess of purer protein precipitates. As the dialysis studies demonstrated, without some salt present the enzyme is not precipitated from dilute solutions in a form that can be removed from suspension. But when a small amount of salt is present the enzyme is almost completely recovered. The presence of magnesium phosphate in the natural wheat bran and its extraction with the enzyme aids in the precipitation of the amylase from distilled water extracts. With the addition of more of the same salt a clean and satisfactory separation of the amylase active fraction is obtained. The presence of a high percentage of magnesium and phosphorus in the ash of the takadiastase prepared by the method of Takamine (1914) is reported by Harada (1931). Whether

this was added before precipitation of the enzyme or extracted from the bran was not mentioned.

The detrimental effect of an excess of certain bi-valent metallic ions during precipitation, especially in the ethanol and acetone precipitations, could find an advantageous application in the industrial concentration of amylases. This factor, if not appreciated, could be the source of much difficulty and unexplained loss of enzyme during precipitation.

By using the optimum set of conditions for each factor satisfactory concentrates of the enzyme were obtained which were white in color, though not completely water soluble, and were more than ten times as active as the original mold bran.

The precipitates obtained are almost as efficient as the original mold bran in furnishing an enzymatic starch degradation system. The isopropanol precipitate is superior to that produced with ethanol. This seems to be due to the presence of more maltase in this precipitate than is to be found in the ethanol precipitate.

From the information gained in this investigation the amylases may be classified as albumins since they are soluble in water and dilute salt solutions and coagulated by heat. The fact that cations seem to have a more pronounced influence on the precipitation of the enzyme than anions might indicate that though the protein is amphoteric it has a greater affinity for cations.

It is most difficult to postulate an explanation of the cause of loss of activity when the enzyme is precipitated in the presence of excess multivalent cations. Perhaps these ions attach

themselves to the enzyme prosthethic groups and thereby block the action of the enzymes. Whatever the cause might be, this would be an interesting point of attack for another problem.

Since the object in mind in undertaking this research was to produce a commercially acceptable amylase concentrate from mold bran, it is believed that a certain degree of success may have been attained, since the corporation supporting the research has been able to utilize the information gained for the production of an enzyme concentrate on a commercial scale. This concentrate is to be sold on the open market and will undoubtedly find application in the fields of commercial alcohol production and textile sizing and de-sizing. In order that such a production of an enzyme concentrate would be profitable, such a concentrate must necessarily possess a carbohydrase enzyme system comparable to that found in the original mold bran. Also, if the process is to be economically feasible, the advantages gained in the production and marketing of the concentrate must be such that they will off-set the additional expense of the concentrate production. Though the determination of these economic factors has not been included in this piece of research, it is believed that simply the fact that a commercial organization finds it possible to utilize the factors determined in the research in the preparation of a concentrate on a commercial scale is indicative of the practicability of the process.

SUMMARY

The possibility of preparing amylase active concentrates from mold bran has been investigated. The influence of the principal environmental factors has been considered in their relation to precipitation of the enzyme active principle with each of four organic compounds. From this study it has been demonstrated that:

1. Methanol, ethanol, isopropanol, or acetone under the proper set of conditions may be used to precipitate amylases from a mold bran extract.

2. At room temperature (25°C.) it is possible to get complete recovery of the amylase with 70 percent ethanol or 60 percent isopropanol or acetone. The maximum recovery of amylase activity obtainable with methanol at this temperature is 70 percent.

3. By lowering the precipitation temperature the concentration of precipitant required to obtain complete recovery is reduced. At 0°C. complete recovery of the amylase may be obtained with 75 percent methanol.

4. There is a loss of activity in the precipitate when it is allowed to stand for a period of time in contact with either methanol or ethanol. Contact with isopropanol or acetone exhibits no such loss even when allowed to stand for long periods of time.

5. When the precipitate from methanol or ethanol is allowed to dry by evaporation of the precipitant at room temperature and at atmospheric pressures, a loss of activity is incurred. No

such loss of activity is experienced in the drying of the precipitates from isopropanol or acetone.

6. Methanol produces the sharpest separation of the precipitated phase with ethanol, isopropanol, and acetone producing less sharp separation in that order. The separation of the precipitated phase may be aided by the addition of certain salts.

7. Acetone produces the least soluble precipitate of the four organic compounds studied.

8. The influence of hydrogen-ion concentration on the activity recovered in the precipitate is not critical so long as the pH of the extract prior to precipitation is between 5.7 and 7.0.

9. The presence of an excess of multi-valent cations in the extract is detrimental to the activity of the precipitate. Isopropanol precipitation is most tolerant to the presence of these ions.

10. By the addition of magnesium sulfate and disodium phosphate with a slight excess of the latter salt to the extract prior to precipitation, the precipitated phase separates and settles very well.

11. The concentrates produced by precipitation with ethanol and isopropanol are almost as efficient as starch degradation agents as the original mold bran. The precipitate produced with isopropanol is more efficient than that produced with ethanol due primarily to the greater concentration of maltase precipitated by the isopropanol.

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LITERATURE CITED

Akabori, Shiro and Katsuhiko Kashimoto.
Chemical nature of takadiastase. II. Molecular weight of takadiastase. Bul. Chem. Soc. Japan, 13: 291-8. 1938. Original not seen. Abstract in Chem. Abs. 32: 5012. 1938.

Atkinson, R. W.
On the diastase of koji. Proc. Royal Soc. London, 32: 299-322. 1881.

Bailey, C. H.
The constituents of wheat and wheat products. New York. Reinhold, 210 p. 1944.

Blish, M. J., R. M. Sandstedt and D, K. Mecham.
Temperature effects in the preparation of wheat amylase. Cereal Chem. 14: 328-30. 1937.

Caldwell, M. L., S. E. Doebbeling, R. M. Chester and G. W. Volz.
Further studies of the purification and properties of the amylase of Aspergillus oryzae. Jour. Biol. Chem., 161: 361-5. 1945.

Dehn, F. B.
Diastatic preparation. British patent 207,225. 1923. Original not seen. Abstract in Chem. Abs. 18: 1167. 1924.

Funke, G. L.
The influence of hydrogen-ion concentration upon the action of the amylase of Aspergillus niger. Proc. Acad. Sci. Amsterdam, 25: 6-8. 1922. Original not seen. Abstract in Chem. Abs. 16: 3321. 1922.

Giri, K. V.
Separation of the two components of amylase. Current Sci., 2: 128-9. 1933. Original not seen. Abstract in Chem. Abs. 28: 5090. 1934.

Hao, L. C., E. I. Fulmer and L. A. Underkofler.
Alcoholic fermentation of corn. Indus. Engin. Chem., 35: 814-8. 1943.

Harada, Taichi.
Preparation of Aspergillus oryzae enzymes. Indus. Engin. Chem. 23: 1424-7. 1931.

Hayasi, Sigeru.
Purification of takadiastase by precipitation methods. Jour. Chem. Soc. Japan 62: 1-4. 1941. Original not seen. Abstract in Chem. Abs. 37: 3779. 1943.

Hemmi, Fumio and Goro Inami.
Selective separation of protease and amylase from takadiastase and pancreatin. Jour. Agr. Chem. Soc. Japan, 5: 660-73. 1929. Original not seen. Abstract in Chem. Abs. 24: 1655. 1930.

Hollenbeck, C. M. and M. J. Blish.
Parallelism between starch dextrinizing and liquifying activities of amylase. Cereal Chem. 18: 754-71. 1941.

Kneen, Eric and L. D. Beckord.
Quantity and quality of amylase produced by various bacterial isolates. Arch. Biochem. 10: 41-54. 1946.

_____ and R. M. Sandstedt.
Enzymes and their role in wheat technology. New York. Interscience. 115 p. 1946.

_____ and C. M. Hollenbeck.
The differential stability of the malt amylases - separation of the alpha and beta components. Cereal Chem. 20: 399-423. 1943.

Kitano, Toshio.
Takadiastase. II. The constants of the reaction velocities of the amylolytic and maltatic actions. Jour. Soc. Chem. Ind. Japan, 38: 381-5. 1934. Original not seen. Abstract in Chem. Abs. 29: 8015. 1935.

_____.
Takadiastase. VI. The relation of the amylolytic and maltatic activities of takadiastase after purification. Jour. Soc. Chem. Ind. Japan, 38 Suppl. binding 449-50. 1935. Original not seen. Abstract in Chem. Abs. 29: 8015. 1935.

_____.
Taka-amylase. VII. The purification of the taka-amylase by adsorption. Jour. Soc. Chem. Ind. Japan, 39: Suppl. binding 22-24. 1936. Original not seen. Abstract in Chem. Abs. 30: 4182. 1936.

van Klinkenberg, G. A.
The separation and action of the two malt amylases; the relation of starch to glycogen. Proc. Acad. Sci. Amsterdam, 34: 893-905. 1931. Original not seen. Abstract in Chem. Abs. 26: 1383. 1932.

Miwa, Tomo and Ayako Miwa.
Alpha glucosidase. II. Enzymes of mold fungi. Jour. Chem. Soc. Japan, 61: 1172-5. 1940. Original not seen. Abstract in Chem. Abs. 36: 2577. 1942.

Miyamoto, Katshuhei.
Treatment of koji solution for the isolation of amyloclastase. II. Amyloclastase I, maltase and dextrinase. U. S. patent 2,282,492. 1942.

Moriga, M.
Pure diastase. Japanese patent, 32,116. 1918. Original not seen. Abstract in Chem. Abs. 12: 2232. 1918.

Münter, F.
Enzymes. Land w. Jahrb. 39, (Erg.) 3, 298-314. 1911. Original not seen. Abstract in Chem. Abs. 5: 1627. 1911.

Newton, J. M. and N. M. Naylor.
Soybean amylase. I. The concentration and characterization of soy bean amylase. Cereal Chem. 16: 71-8. 1939.

Nishimura, S.
Uber die Reinigung der Amylase aus Aspergillus oryzae. Bul. Agr. Chem. Soc. Japan, 2: 129-46. 1926. Original not seen. Abstract in Chem. Abs. 21: 2287. 1927.

Osborne, Thomas B.
The chemical nature of diastase. Jour. Amer. Chem. Soc., 17: 587-603. 1895.

_____ and G. F. Campbell.
The chemical nature of diastase. II. Jour. Amer. Chem. Soc., 18: 536-42. 1896.

Payen, A. and J. Persoz.
Memoire sur la Diastase, les principaux produits de les Raction, et leurs application aux arts industriels. Annales de Chem. et Phys., ser. 2, 53: 73-92. 1833.

Sandstedt, R. M. and M. J. Blish.
Yeast variability and its control in flour gassing power tests. Cereal Chem., 11: 368-73. 1934.

_____ Eric Kneen and M. J. Blish.
A standardized Wohlgemuth procedure for alpha-amylase activity. Cereal Chem., 16: 712-23. 1939.

Sano, Tokuichi.
The enzymes contained in takadiastase preparation. Japan Jour. Med. Sci., 2: 25-6. 1922. Original not seen. Abstract in Chem. Abs. 20: 1634. 1926.

Sherman, H. C., M. L. Caldwell and Mildred Adams.
Enzyme purification; further experiments with pancreatic amylase. Jour. Biol. Chem., 88: 295-304. 1930.

Sherman, H. C., M. L. Caldwell and S. E. Doebbeling.
Further studies upon the purification and properties of malt amylase. Jour. Biol. Chem., 104: 501-9. 1934.

_____, I. D. Garard and V. K. LaMar.
Further studies of the process of purifying pancreatic amylase. Jour. Amer. Chem. Soc., 42: 1900-7. 1920.

_____ and M. D. Schlesinger.
Studies on amylases: IV. A further investigation of the properties of pancreatic amylase. Jour. Amer. Chem. Soc., 34: 1105-11. 1912.

_____.
Studies on amylases: V. Experiments upon the purification of the amylase of malt. Jour. Amer. Chem. Soc., 35: 1617-23. 1913.

_____.
Studies on amylases: X. Comparison of certain properties of pancreatic and malt amylase preparations. Jour. Amer. Chem. Soc., 37: 1305-19. 1915.

Sherman, H. C. and A. P. Tanberg.
Experiments upon the amylase of Aspergillus oryzae. Jour. Amer. Chem. Soc., 38: 1638-45. 1916.

Shih, Y. K.
Comparative study of the amylase of Aspergillus species found at Wuchang, Hupeth Province (China). Lingnan Sci. Jour., 16: 71-6. 1937. Original not seen. Abstract in Chem. Abs. 31: 3091. 1937.

Shukla, J. P.
Enzymes. II. Purification of amylase from Kaseru (Scirpus grossus, Linn.). Jour. Indian Chem. Soc., 19: 121-4. 1942. Original not seen. Abstract in Chem. Abs. 37: 148. 1943.

Takamine, J.
Diastatic substance and method of making.
U. S. Patent 826, 699. 1894.

_____.
Enzymes of Aspergillus oryzae and the application of its amyloclastic enzymes to the fermentation industry. Jour. Indus. Engin. Chem., 6: 824-8. 1914.

Taketomi, Noboru and Sadae Takeda.
Purification of koji amylase by precipitation method. Bul. Waseda Applied Chem. Soc., 25: 11-15. 1935. Original not seen. Abstract in Chem. Abs. 29: 7352. 1935.

Tamiya, Hiroshi.
Advances in enzymology. New York. Interscience. 183 p. 1942.

Tilden, E. B., M. Adams and C. S. Hudson.
Purification of the amylase of Bacillus macerans. Jour. Amer. Chem. Soc., 64: 1432-3. 1942.

Tokyoka, Yuzo.
Koji amylase IX. Existence of beta-amylase. Jour. Agr. Chem. Soc. Japan, 13: 586-94. 1937. Original not seen. Abstract in Chem. Abs. 32: 2153. 1938.

Underkofler, L. A.
Microbial amylases - their application to alcoholic fermentation. The Brewers' Digest, Nov. 1942.

_____, G. M. Severson, K. G. Goering and L. M. Christensen.
Commercial production and use of mold bran. In press. 1946.

Wallerstein, G. S., R. T. Alba and M. G. Hale.
Precipitation and recovery of malt and mold amylase by alkali cook lignin. Arch. Biochem. 8: 275-84. 1945.

Wei, Nie-Shou and Kuei-Shih Chin.
The diastatic activity of Aspergillus. Science (China), 18: 1193-8. 1934. Original not seen. Abstract in Chem. Abs. 29: 2977. 1935.

Weidenhagen, I. R.
Uber die Reinigung Pflanzlichen Amylasen I. Z. Ver deut. Zucker-Ind., 83: 505-15. 1933. Original not seen. Abstract in Chem. Abs. 28: 3428. 1934.

Winkler, Ferdinand and Franz Köck.
The precipitation of diastase. Chem. Ztg. 53: 457. 1929. Original not seen. Abstract in Chem. Abs. 23: 4012. 1929.

Wohlgemuth, J.
Uber eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments. Biochem. Z., 9: 1-9. 1908.

_____.
Zur Kenntniss der takadiastase. Biochem. Z., 39: 324-8. 1912.