



A study of the amylase of *Aspergillus Oryzae*
by Victor C Bruski

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree
of Master of Science in Chemistry
Montana State University
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Abstract:

A study has been made of the amylase obtained from a submerged culture of *Aspergillus oryzae*. grown on a synthetic media of starch and inorganic salts. It was discovered that the amylase produced can be absorbed and eluted from the mold mycelium by pH adjustments. An initial concentration of 20-40 fold of the amylase from the filtrate is readily obtainable. The advantages claimed are: elimination of salts, carbohydrates, proteins, and other extraneous materials; also, the method is simple, rapid, and inexpensive. Elutes from the mycelium absorptions were subjected to repeated ammonium sulfate fractionations followed by a bentonite treatment. The preparations thus obtained gave exceedingly high amylase activities with very little evidence of maltase, limit dex-trinase, and proteolytic activity.

A STUDY OF THE AMYLASE
OF ³⁰
ASPERGILLUS ORYZAE

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VICTOR C. BRUSKI

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Submitted to the Graduate Faculty
in
partial fulfillment of the requirements
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I. ABSTRACT

A study has been made of the amylase obtained from a submerged culture of Aspergillus oryzae, grown on a synthetic media of starch and inorganic salts. It was discovered that the amylase produced can be absorbed and eluted from the mold mycelium by pH adjustments. An initial concentration of 20-40 fold of the amylase from the filtrate is readily obtainable. The advantages claimed are: elimination of salts, carbohydrates, proteins, and other extraneous materials; also, the method is simple, rapid, and inexpensive. Elutes from the mycelium absorptions were subjected to repeated ammonium sulfate fractionations followed by a bentonite treatment. The preparations thus obtained gave exceedingly high amylase activities with very little evidence of maltase, limit dextrinase, and proteolytic activity.

II. INTRODUCTION

Because of the demand for amylolytic enzymes in the brewing, distilling, milling, baking, textile, paper, and other industries, the amylases are one of the most important classes of enzymes used on a commercial basis.

There are two classes of amylases: alpha-amylase (the liquifying enzyme), and beta-amylase (the saccharifying enzyme). The substrate in both cases is starch. Amylases (alpha or beta) act very slowly on raw starch (20) but cooked starches, however, are very readily hydrolyzed.

Starch is believed to consist of two fractions known as amylose and amylopectin. Cereal starches are mixtures of 75 to 80 per cent amylopectin and 20 to 25 per cent amylose. The general opinion is that amylose is the straight-chain component of starch, containing glucose units bound by alpha-1, 4-glucosidic linkages. The straight chains contain 100 to 700 glucose residues (9) (10). According to Meyer (9) (10), amylopectin is a large branched-chain molecule, resembling a tree, containing 15 to 18 glucose molecules between branches. Most of these glucose units are linked by alpha-1, 4-glucosidic bonds having branches formed by alpha-1, 6-glucosidic linkages. Amylopectin consists of 500 to 2,000 or more glucose units.

The final products of hydrolysis of starch by alpha-amylase, give no color with iodine-potassium iodide solution. This is due to the rapid rupture of starch chains into low molecular weight dextrans (20). Alpha-amylase splits the amylopectin of starches into dextrans of 6 or 7 glucose units (20), whereas, the result of beta-amylase action on

amylopectin is the production of high-molecular glucosans in which alpha-1, 6-glucosidic bonds accumulate.

The final hydrolysis products of beta-amylase on starch, give a blue color with iodine-potassium iodide solution. Beta-amylase rapidly converts over 99 per cent of the amylose fraction of starch to maltose (10). Amylopectin, however, is only partially hydrolyzed (54 per cent) by beta-amylase (20). Beta-amylase appears to start at the non-reducing end of the chain, breaking off maltose units as it works toward the center; however, as soon as it reaches a branch, its action ceases. This results in obtaining a relatively large nucleus of branched structure from amylopectin. Since this nucleus has more than the minimum number of glucose units required to give the iodine test, beta-amylase will always give a purple colored end product with iodine.

Amylases are found in saliva, pancreatic juice, liver, other animal organs, seeds of cereals, molds, and bacteria. The important commercial sources are molds, malted cereals, and beef and hog pancreas.

The amylase which is the subject of this study is produced by the mold Aspergillus oryzae, and is of the alpha type. Aspergillus oryzae has been cultivated in the Orient for centuries for the production of enzymes, particularly starch hydrolyzing enzymes. The amylase produced has been used as a partially purified product in this country since 1898 (19). However, surprisingly little is known of its action and properties. This lack of quantitative information is probably due to the fact that many investigations have dealt with rather crude alcohol precipitates of the enzyme extracts from the mold. These contained high concentrations

of other enzymes, and similar protein materials. Tauber (20) listed 23 different enzymes which have been reported for this organism.

III. STATEMENT OF PROBLEM

Preliminary investigation had indicated that the amylase produced by Aspergillus oryzae on a media made up of inorganic salts and starch had somewhat different properties than commercial preparations of this enzyme. There were even indications that it might not be protein in nature due to the fact that a negative biuret test was obtained on some samples (8). Furthermore, tyrosine appeared to be absent while surface cultures of the same organism grown on organic media always gave a positive tyrosine test. In order to study this problem and determine if a more highly purified product would show the same properties the following problems were to be investigated:

1. Extraction and purification of amylase from Aspergillus oryzae grown on synthetic media of starch and inorganic salts.
2. Work out a feasible scheme for the purification of the amylase.
3. Obtain highest possible activity by some type of fractionation from other proteins and carbohydrates and, if possible, obtain the enzyme in crystalline form.
4. Determine physical and chemical properties of highly purified enzyme. This includes analysis for nitrogen and determining its activity.
5. Compare with properties of amylases from other sources.

IV. LITERATURE SURVEY

In 1898, Takamine (19) introduced into this country under the name Taka-diastrase a partially purified powder prepared by alcohol precipitation of the aqueous extract of "Koji", which is made by cultivating the mold Aspergillus oryzae on steamed wheat bran. Since then, the commercial purification has been improved, and the amylase has found many applications in industry.

Sherman and Tanberg (18) obtained more concentrated preparations of this amylase in 1916. They precipitated the enzyme from aqueous solutions of the commercial product (Taka-diastrase) by use of ammonium sulfate, dissolved in precipitate in water, dialyzed the solution free from sulfate, and then fractionally precipitated it with alcohol in the presence of added sodium chloride. The most active products obtained in this manner had 30 times the activity of the starting material.

In 1926, Nishimura (14) reported still greater purification of the amylase by adsorption on alumina gel, and elution with phosphate solutions. The adsorption was carried out in a weak acid solution, and the adsorbed material eluted with a weak acid-phosphate solution at a pH of 8.0. By repeating this process several times, he obtained a product with approximately 45 times greater activity than the starting material.

Chester obtained more concentrated preparations of this amylase in 1933 (4). Her work consisted of following the adsorption procedure of Nishimura (14) along with fractionation with ammonium sulfate and alcohol. Her best preparation had an activity of approximately 53 times that of the original material.

Because of the different conditions used in measuring the enzyme activities of the purified products in the references cited above, it is difficult to make an accurate comparison of the purified products with this work. In the light of the studies that have been made, the best one can do is make an approximation of the comparative activities.

Caldwell, Chester, Doebbeling and Volz, in 1945 (3) prepared an exceedingly potent amylolytic precipitate which gave no evidence of maltase activity. Their method of purification consisted of fractional precipitation of the aqueous extract of a commercial Taka-diaxase concentrate with ammonium sulfate, suspension of the precipitate in a minimum of distilled water, dialysis to remove sulfate, and concentration of the dialyzed solutions. This process of fractionation and dialysis was repeated on the most active solutions until no further increases occurred in activities expressed on the basis of total solids in the dialyzed solutions. The number of fractionations necessary was three to five. Their most active preparation had an activity of 16,000 alpha units per gram. The amylase activity is defined on page 12.

Crystalline alpha-amylases have been obtained from malt, pancreas, saliva, and bacteria. Since their action and many of their properties are similar, a short review of the methods used for obtaining the crystalline material seems appropriate at this time.

Malt alpha-amylase was crystallized by Sigmund Schwimmer and A. K. Balls (17). The procedure consists essentially of heating concentrated malt extract to destroy the beta-amylase activity, precipitating the remaining protein with ammonium sulfate, adsorbing the enzyme from an

alcoholic solution on wheat starch granules, and crystallizing the eluted enzyme with ammonium sulfate. The conditions for the adsorption were adjustment of pH to 5.9-6.0 with 0.1 N ammonium hydroxide in the presence of calcium ions at a temperature of 30°C.

Pancreatic alpha-amylase was crystallized by Meyer, Fisher, and Bernfeld (11) (12). Their method consisted of the dialysis of extracts of hog pancreas, fractional precipitation with ammonium sulfate (pH 6.9), removal of denatured protein by shaking with chloroform in the presence of ammonium hydroxide, and then crystallized from water at 3°C.

Salivary alpha-amylase was crystallized by Meyer, Fisher, Bernfeld, and Staub (13). Human saliva was centrifuged, and the precipitate discarded. Then two fractional precipitations with acetone, and two fractional precipitations with ammonium sulfate were carried out. In the next step, the sulfate ions were replaced by acetate by using a basic ion exchange resin. An additional precipitation was made with acetone. The precipitate was taken up in water and cooled. Crystals formed in 48 hours at a pH of 7.0.

When this project was started, no report had been made of crystallization of fungal alpha-amylase. However, in February of this year, Underkofler and Roy at Iowa State College, published an outlined procedure for obtaining crystals of the alpha-amylase from submerged cultures of Aspergillus oryzae (21). In brief, the method consists of six ammonium sulfate precipitations, with three bentonite adsorptions to remove limit dextrinase. Crystallization was completed in one-half saturated ammonium sulfate at a refrigerated temperature.

The authors of the above procedure obtained only 10 mg of crystals. This amount was insufficient to allow physical properties to be determined. As a result only a picture was made of the crystals and the amylolytic activity determined. It was learned through correspondence that the authors of this procedure had great difficulty in repeating the above process.

V. ANALYTICAL PROCEDURES

Throughout the course of this investigation the following analytical methods were used.

Alpha-amylase was determined by a modified method of Sandstedt, Keen,^N and Blish (16), the activity being expressed as "alpha units." An "alpha unit" represents the number of grams of Lintners soluble starch dextrinized in one hour, at 30°C, by one milliliter of enzyme solution, unless otherwise indicated. The modification is the elimination of the preliminary treatment of the starch with beta-amylase since beta-amylase is not produced by this particular mold. One alpha unit, as described here, represents 8.8 Sandstedt, Keen, and Blish "alpha units."

Limit dextrinase was determined by the method of Back, Stark, and Scalf (2). The unit of measurement is expressed as per cent hydrolysis of limit dextrin to fermentable sugar in one hour, at 30°C, by one milliliter enzyme solution unless indicated otherwise.

Maltase activity was determined by the method of Gorman and Langlykke (5). The activity is expressed as per cent hydrolysis of maltose to glucose in two hours at 30°C.

Proteolytic activity was run by the method of Northrop (15). A change of one per cent per minute in specific viscosity of a standard gelatin solution is defined as one unit of proteolytic activity.

The Kjeldahl nitrogen determination used is an adaptation of the official method of the A. O. A. C. (1), used by the Chemistry Research Department of the Agricultural Experiment Station at Montana State College. The catalytic mixture contains 1,000 grams of potassium

sulfate, and 50 grams of mercuric oxide. A mixed indicator (22) was used in the titration.

All pH determinations were made with a Beckman pH meter, which employs the glass electrode.

VI. METHODS AND RESULTS

In determining the type of fermentation media to be used, a series of synthetic preparations made up of inorganic salts and cornstarch were tried. This was done by varying the salt concentration in the media, but keeping the basic constituents the same. As a result of this preliminary experiment, it was concluded that the medium preparation that would serve the needs of this investigation most fully was of the following nature:

<u>Ingredients</u>	<u>% by Weight</u>
Cornstarch	1.5
KNO_3	0.4
CaCO_3	0.1
KH_2PO_4	0.05
MgSO_4	0.025
KCl	0.0235
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5.0 P.P.M.
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	5.0 P.P.M.
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	5.0 P.P.M.

The medium was sterilized in 10 liter quantities in pyrex bottles equipped with air-dispersing canvas bags. The medium was sterilized twice on successive days to insure highest possible sterility. Each bottle was inoculated with 600 ml of 48 hour pure culture of Aspergillus oryzae. This inoculum was cultivated in 300 ml quantities of the same medium as listed above, except for the addition of 0.24 per cent by weight bentonite. The bentonite was added to keep the mold mycelium growing in small particles. These seed cultures were inoculated from a slant culture of the mold grown

on the same media except for the addition of 2.5 per cent agar, and then shaken for 48 hours to insure growth under aerobic conditions. The medium, after inoculation, was incubated at a temperature of 32-34°C with continuous aeration. The air was supplied by compressed air from the air line which was passed through a sterile cotton filter, and then through sterile distilled water. Approximately one-third volume of air per volume of media per minute was used. After 72-96 hours of fermentation, the alpha amylase activity reached a value of 1.0 to 4.0 units per ml with an average of approximately 1.5 units per ml.

The mold filtrate consists of 0.8 per cent solid matter, of which 40 per cent is salts. The other components are carbohydrates (which are products of enzyme action on starch), proteins (which diffuse from mold cells), and enzymes (Tauber lists 23) (20).

A number of procedures were tried for the concentration and purification of the alpha-amylase from the mold filtrate. These involved:

1. Concentration by vacuum distillation and fractional precipitation with alcohol.
2. Concentration by vacuum distillation and fractional precipitation with ammonium sulfate.
3. Concentration by vacuum distillation and adsorption of alpha-amylase on starch and pure amylopectin.
4. The Underkofler method.
5. Adsorption of the alpha-amylase from mold filtrate on the mold mycelium, elution and fractional precipitation with ammonium sulfate.

Method number five was the most successful. The first four methods attempted will be described in brief, and method five described in detail.

1. Concentration by Vacuum Distillation

and Fractional Precipitation with Isopropyl Alcohol.

The vacuum distillation was carried out at temperatures below 35°C, the concentration being 8-10 fold. The loss in activity in this step was from 0-40 per cent depending on the time and contamination in the medium. After a concentration of 8-10 fold, the medium was filtered through a mat of celite, placed in dialyzing bags made of cellulose acetate, and dialyzed at refrigerated temperatures until free of salts. This salt-free solution was then precipitated with alcohol. The best conditions for this precipitation were found to be the use of two parts alcohol to one part of enzyme solution at 0°C and pH 7.0. Twice precipitated material, upon drying, gave a maximum alpha activity of 6,800 units per gram. In order to check the amount of fractionation from other components, just before precipitating, a small amount of the dialyzed enzyme solution was taken to dryness in a dialyzing bag by hanging in a stream of warm air. The activity of this material was 7,500 alpha units per gram. This indicated that part of the enzyme activity was destroyed by the alcohol, and very little fractionation had taken place. Therefore, this approach to the purification was abandoned.

2. Concentration by Vacuum Distillation

and Fractional Precipitation With Ammonium Sulfate

This procedure was carried out exactly the same as above except ammonium sulfate fractionation was used instead of alcohol. The enzyme

solution which was precipitated four times with three-fourths saturated ammonium sulfate, dialyzed and taken to dryness, yielded an activity of 8,000 alpha units per gram.

3. Concentration By Vacuum Distillation

and Adsorption of Enzyme on Starch and Amylopectin

Concentration, filtering, and dialysis was carried out as in the above two procedures. Since one of the intermediate steps in the purification of malt amylase (17) was to adsorb the enzyme on starch in the presence of 40 per cent alcohol, this method was tried. The best results that could be obtained in this manner were 20 per cent adsorption of the enzyme on cornstarch and 40 per cent adsorption on amylopectin. Because of the large losses, this procedure was discarded.

4. The Underkofler Method

This method was carried out as outlined by Underkofler (21). No crystals could be obtained by this method. On removal of the ammonium sulfate by dialysis and taking to dryness by hanging in a stream of warm air, this material yielded an alpha activity of 10,000 units per gram. The quantity of ammonium sulfate needed for this purification scheme was too great to warrant further attempts.

5. Concentration and Purification by Adsorption

of Alpha-amylase From Mold Filtrate on Mold Mycelium,

Elution and Fractional Precipitation With Ammonium Sulfate

It was discovered in the course of this investigation that by proper adjustment of temperature, pH, salt concentration, and selection of mycelium of the proper age, the alpha-amylase produced by Aspergillus

crystals was completely adsorbed on one-fifth of the mycelium present in the growth medium. By elution from this mycelium, a concentration of 20-40 fold was obtained and at the same time the enzyme was separated from the contaminating carbohydrates, salts, and over 80 per cent of the protein present. A second adsorption allowed an additional 2-3 fold concentration.

This procedure eliminated the usual methods of concentration under reduced pressure and precipitation by salts, alcohol, or other organic solvents thus allowed for an easy and inexpensive method of concentration.

In this procedure, just as in the others, the culture was grown under the usual conditions of forced aeration on a starch and nitrogen containing medium. When the optimum amylolytic activity was obtained, the mycelium was removed from the fermentation liquor by filtration, after first adjusting the pH to 8.5 with dilute sodium hydroxide. This was necessary to allow complete separation of mycelium and enzyme. Approximately one-fifth of the mycelium was washed and then resuspended back into the mold filtrate. The pH of the medium was adjusted to 5.0 with glacial acetic acid, the temperature adjusted to 37°C, and the entire mixture gently agitated for one hour. At the end of this time the mycelium was then washed with water with the pH adjusted to 5.0. The mycelium was resuspended in a minimum of water (.05-.025 of the original volume of fermentation) to which had been added 1.0 per cent sodium chloride, and 25 per cent by volume borate buffer prepared in the following manner: (50 ml 0.2 M KCl; 50 ml 0.2 m H_3BO_3 ; 8.5 ml 0.2 M NaOH; mixed and

diluted to 200 ml). The pH of elution liquid was then adjusted to 8.5 with sodium hydroxide with careful stirring. After one-half hour, the mycelium was removed from the enzyme solution by filtration, and a concentration of 20-40 fold was obtained.

This process was repeated, thus allowed a further concentration of 2-3 fold. However, one of two things must be done to the elution liquid from above: 1. dilution until the total salt concentration is below one-half per cent, or 2. dialysis to remove the salts. If a more concentrated liquor is desired, the material from the second adsorption can be concentrated by vacuum distillation over 6 fold more with little or no loss in activity. Since at this stage the total volume handled is approximately one hundredth of the original, the length of time required for evaporation is much less, allowing less time for contamination and loss of activity. With this third step, concentrations of 400-600 times were readily obtainable.

After preliminary experiments indicated that this method might be feasible, the optimum pH, and temperature for the adsorption were determined as indicated in Tables I and II. The same source of media and mycelium was used for all adsorption analyses.

It can readily be seen from observing the two tables, that the best conditions of adsorption are pH of 5.0 at a temperature of 37°C.

There was no problem of eluting the enzyme from the mycelium. As was mentioned previously, the pH of the elution liquid was adjusted to 8.5, which allowed the enzyme to separate from the mycelium. Elutions of 98-100 per cent can be obtained every time in the presence of 1.0 per

cent sodium chloride. The buffer was found necessary to avoid local concentration of sodium hydroxide on the pH adjustment, since the activity is destroyed at once in an alkaline condition.

After this preliminary work had indicated adsorption and elution of the enzyme by this process to be feasible, the next step was to determine the limits of concentration, the amount of fractionation from other proteins and enzymes and finally, if this concentrated enzyme solution obtained by adsorption and elution procedures could be further concentrated, by vacuum distillation. The latter would be useful for preparing concentrates of the enzymes for commercial purposes. The data obtained in behalf of these questions is set forth in Table III.

From the results listed on Table III, it can be observed that a concentration of 29 times that of the original was obtained on the first adsorption and elution with an elimination of approximately 84 per cent of the compounds which contained Kjeldahl nitrogen. This adsorption also eliminated 92 per cent of limit dextrinase. On the second adsorption and elution, a concentration of 73 times that of the original was obtained, however, the amount of limit dextrinase and those compounds which contained Kjeldahl nitrogen and that were fractionated were very small. It was less than 3.0 per cent for each. In order to test the suitability of further concentrating, the concentrate (E, Table III) by vacuum distillation the following experiment was run: 90 ml of the 110 unit material was concentrated by vacuum distillation to 14 ml (a little more than 6 times). The concentrate was analyzed and found to have an activity of 700 units per ml. Recovery was 100 per cent within the limits of experimental

error on the measuring of volumes of these concentrated solutions. There is no question but at these concentrates could be concentrated even more if necessary. Qualitative tests also indicate the removal of the original salts and most of the carbohydrate by this adsorption procedure.

A number of additional runs of adsorption and elution of the enzyme have been made. The results of some of these are found in Table IV. Each of the adsorptions was carried out in 10 liter quantities. The mycelium and medium were grown in separate bottles at different times.

The adsorption process is very selective, since Aspergillus oryzae mycelium fails to absorb the amylase from malt extract and Aspergillus niger.

The most successful purification procedure is outlined on Table V. This resulted in a final dry concentrate which had high amylase activity and only traces of maltase, limit dextrinase, and proteolytic activity. The purification involved concentrating the enzyme by mycelium adsorption and then using three-fourths saturated ammonium sulfate fractionation, along with bentonite adsorption as was used by Underkofler (21). The initial concentration (fraction labeled A and B) was carried out in ten different steps, spread over a period of five weeks. The elutes were stored at refrigerated temperatures until the desired quantity needed to continue further purification had been obtained. Crystallization was attempted four different times with fraction labeled H, twice at refrigerated temperatures and twice at room temperatures. Crystallization at room temperature was attempted because malt alpha-amylase was crystallized in that manner. Ammonium sulfate was added in each case over a period of

24 hours until one-half saturation was reached. The pH was adjusted to 5.5-6.0.

After crystals of the amylase failed to appear, the enzyme solution was dialyzed free of ammonium sulfate, and then taken to dryness in a dialyzing bag. The enzyme solution, before drying, had a total activity of 58,000 alpha units. This yielded 3.4 grams of dry enzyme preparation, therefore, the enzyme preparation had an activity of 17,350 units per gram before drying. The dry material had an activity of 14,300 units per gram, a loss of 3,050 units on drying. Kjeldahl nitrogen analysis of the dry concentrate indicated 12.15 per cent nitrogen.

TABLE I

OPTIMUM TEMPERATURE FOR ADSORPTION
(5 g wet mycelium per 100 ml of media)
Assumed opt. pH5 from preliminary results

Temperature °C	% of Total Amylase Adsorbed
25	77
30	86
35	97
40	96

TABLE II

OPTIMUM pH FOR ADSORPTION
(5 g wet mycelium per 100 ml of media)
Temperature of adsorption 37°C

pH	% of Total Amylase Adsorbed
4.50	66
4.75	66
5.00	93
5.50	72
6.00	70

TABLE III

INITIAL PURIFICATION SCHEME

FRACTION	AMYLASE UNITS/ML	% AMYLASE OF ORIG.	CONC. BY ACTIVITY	KJELDAHL NITROGEN MG/ML	% KJELDAHL NITROGEN OF ORIG.	LIMIT DEXTRINASE HYDROL/ML	% LIMIT DEXTRINASE OF ORIG.
(A) Mold filtrate as grown (20 liters)	1.50	100	----	0.1475	100	2.01	100
(B) Filtrate from A after mycelium adsorption (20 liters)	0.22	----	----	0.1380	----	1.90	----
(C) Elution liquid from mycelium adsorp- tion (550 ml)	43.0	78.5	29.0	0.855	15.9	5.67	7.75
(D) Filtrate from C after second mycelium adsorption (550 ml)	0.32	----	----	0.255	----	4.50	----
(E) Elution liquid from second mycelium adsorption D (200 ml)	110.0	73.5	73.0	1.80	12.2	12.5	6.2
(F) Vacuum distilled conc. 6.4 more times; no measurable loss in activity	700.0	73.5	465.0	----	----	----	----

TABLE IV

ADDITIONAL ADSORPTION DATA

Performed on Separate Grown Bottles
(10 liter quantities)

<u>Run No.</u>	<u>% Recovery</u>	<u>Concentration</u> (times by activity)
1.	75	15
2.	80	16
3.	60	19
4.	75	37
5.	74	32
6	8	—
7.	41	15.5
8.	27	14
9.	0	—
10.	46	22.3
11.	52	26
12.	62	31
13.	90	25
14.	78	34
15.	85	35

TABLE V

MOST SUCCESSFUL PURIFICATION SCHEME

FRACTION	AMYLASE UNITS/ML	% AMYLASE OF ORIG.	CONC. BY ACTIVITY
(A) Mold Filtrate (100 liters)	1.44	100	---
(B) Elution Liquid (2.5 liters)	41.5	72	29
(C) First Ammonium Sulfate Precipitate Dissolved in Distilled Water to Volume of (950 ml)	92.5	61	64
(D) Second Ammonium Sulfate Precipitate Dissolved in Distilled Water to Volume of (180 ml)	458	57.2	318
(E) Third Ammonium Sulfate Precipitate Dissolved in Distilled Water to Volume of (50 ml)	1,560	54	1,080
(F) After Bentonite Adsorption to Remove Limit Dextrinase (50 ml)	1,380	48	---
(G) Fourth Ammonium Sulfate Precipitate Dissolved in Distilled Water to Volume (50 ml)	1,350	47	---
(H) Fifth Ammonium Sulfate Precipitate Dissolved in Distilled Water to Volume (50 ml)	1,160	40.2	---
(I) Fraction (H) Dialyzed Free of Ammonium Sulfate, Taken to Dryness in Dialyzing Bag (3.4 grams)	17,350 Units/gram, wet bases 14,300 Units/gram, dry bases		

ANALYSIS OF FRACTION (H)

Proteolytic Activity	Trace of Original
Maltase Activity	0.1% of Original
Limit Dextrinase Activity	0.05% of Original

VII. DISCUSSION

The emphasis of the investigation on the present problem was confined to experimental work on methods of concentration and purification of the amylase obtained from a submerged culture of Aspergillus oryzae.

One of the most important factors in this investigation that deserves attention and still is unsolved, is the large variation in the amount of amylase that the mold mycelium adsorbed, (0-90%, Table IV). For a time this adsorption appeared very uniform and averaged 73 per cent, then for no apparent reason poor results were obtained. The media, rate of air flow, and growth conditions were in each case as uniform as possible. This brings up the question, "Why do these variations occur?" As the investigation progressed several possibilities presented themselves:

1. Salt concentration

At the beginning of this project, a salt mixture was prepared which contained 50% KH_2PO_4 ; 25% MgSO_4 ; 23.5% KCl ; 0.5% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The medium used was made up of 0.1 per cent by weight of this salt mixture. Since this mixture was not ball milled, it seems very likely that the elements present in trace amounts ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) may not have been distributed homogeneously. Foster (6) states that these elements are probably tied up with some enzyme system of the mold. Too much or too little of these elements may have interfered with the production of the characteristic property of the mold mycelium that takes part in the adsorption. A protein analysis was performed on several batches of mycelium. It was found that the good adsorbing mycelium had much lower protein content than the mycelium

that did not adsorb. Foster (6) said that the protein content of the mycelium is inversely proportional to the amount of zinc present. Therefore, a series was run by varying the zinc concentrations, keeping all the other salt concentrations constant. The results obtained at this time indicate that this might be the limiting factor but checks must be run on this ion. The possibility of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ have not been checked.

2. Bentonite

Another possibility for explaining the variation in the adsorption is the bentonite concentration of the seed cultures used to inoculate the media. Although the bentonite was always added in constant amounts by weight, it was not of uniform particle size. It was noticed that different amounts of bentonite had a decided effect on the shape of the mycelium particles. Large amounts made the mycelium take on a stringy appearance, while smaller amounts made the mycelium take on the shape of tiny balls with tentacles coming out of the center. The amount of surface available for adsorption may be tied up with this factor. It appears as though an intermediate form between strings and balls gives the best adsorption. Further work must be done on this to establish the influence of bentonite.

3. pH

It was observed by keeping a record of the pH of the growing media that when the pH turned alkaline in a short period of time (24-28 hours), the mycelium adsorbed the amylase on almost every occasion, however, if the media stayed on the acid side from 96-120 hours, no adsorption took place. This indicates that there must be some reaction taking

place in the growing mycelium which decides the course early in the growth. Artificially controlled pH did not prove successful.

4. Mutation

The possibility of mutation cannot be overlooked. Foster (6) has stated that the elements of zinc and copper induce mutations to fungi under certain conditions. However, this possibility for producing mycelium with properties with different characteristics is unlikely. Cultures preserved for twelve months under paraffin oil did not give any different results than those repeatedly transferred at regular intervals.

5. Increasing the amounts of mycelium

It was observed that when the proportion of mycelium was increased, the amount of enzyme adsorbed also increased. Calculations were not made to determine if the laws of adsorption hold true. The emphases of this part of the investigation was to concentrate the enzyme as much as possible, therefore, concentration of fifty times by volume was arbitrarily taken and all adsorptions carried out and calculated on that basis. There is no question but that this could be increased considerably if concentrations of fifty fold by volume were not desired. In general, an increase of 50 per cent in adsorption was obtained when the amount of mycelium used was tripled. However, this would mean that it could only be concentrated approximately 18 times and, therefore, these concentrations were not run.

It is believed that crystals of the enzyme could be obtained by the procedure outlined, provided that a large enough quantity of mold filtrate was available at one time. This would eliminate storing until

the desired quantity was available. Losses in activity due to contamination and age were considerable over long periods of time. Furthermore, the properties of the enzyme might change on extended storage.

VIII. SUMMARY

1. A method was presented for concentrating the amylase from a submerged culture by adsorbing on one-fifth the mycelium present, eluting and recovering in a concentrated form from 20-40 times the original activity in one step, thus eliminating the costly procedures usually encountered.
2. A method was presented whereby concentrated fungal amylolytic liquors can be further concentrated from 2-3 times (yielding a total concentration of 60-120 times that of the original) by a simple procedure of a second adsorption on the mycelium followed by elution.
3. A method was presented whereby the fungal concentrate (60-120 times original) can be further concentrated 6-10 times giving a final concentration of 360-1200 times the original culture liquor by a process of vacuum distillation.
4. A method was presented whereby concentrated fungal amylase can be further concentrated and purified by fractional precipitation with ammonium sulfate, along with a bentonite adsorption.

IX. LITERATURE CITED

- (1) A.O.A.C., p. 26, 2.24 sixty edition, (1945).
- (2) Back, T. M., Stark, W. H., and Shalf, R. E., Anal. Chem 20, 50-60 (1948).
- (3) Caldwell, Chester, Doebbeling, and Volz., J. Biol. Chem. 161, 361 (1945).
- (4) Chester R.M., Dissertation, Columbia University (1933).
- (5) Corman, J., and Langlykke, A.F., Cereal Chem. 25, 190-201 (1948).
- (6) Foster, J. W., Chemical Activities of Fungie., New York Academic Press, (1949).
- (7) Hassid, W.Z., and McCready, R.M., J. Am. Chem. Soc., 65, 1157 (1943).
- (8) LeMense, E.H., Enzymes Incorporated, Unpublished work (1949).
- (9) Meyer, K.H., Advances in Colloid Sci., 1, 143 (1942).
- (10) Meyer, K. H. Advances in Enzymol. 3, 109 (1943).
- (11) Meyer, K. H., Fisher, E.H., and Bernfeld, P., Helv. Chim. Acta, 30, 64-78 (1947).
- (12) Meyer, K.H., Fisher, E.H., and Bernfeld, P., Experientia 3, 106 (1947).
- (13) Meyer, K.H., Fisher, E.H. Bernfeld, P., and Staub, A., Experientia 3, 455 (1947).
- (14) Nishimura, A.S., Bull, Agric. Chem. Soc. Japan 2, 129 (1926).
- (15) Northrop, J. H., J. Gen. Physiol. 16, 41-58 (1932).
- (16) Sandstedt, Kneen, and Blish Cereal Chem. 16, 712-732 (1939).
- (17) Schwimmer, S., and Balls, A.K., J. Biol. Chem., 179, 1063-1074 (1949).

- (18) Sherman, H.C., and Tanberg, A.P., J. Am. Chem. Soc., 38, 1638 (1916).
- (19) Takamine, Jokichi,, J. Soc., Ind. 17, 118 (1898).
- (20) Tauber, Chemistry and Technology of Enzymes, New York, John Wiley and Sons, Inc. (1940).
- (21) Underkofler, L.A., and Roy, D.K, Cereal Chemistry, 28, 18-29 (1951).
- (22) Zuazaga, G., and Ma, T.S., Ind. Eng. Chem. Anal. Ed. 14:2, 281 (1942).

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