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Purification and characterization of fungal alpha-amylase

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PURIFICATION AND CHARACTERIZATION OF FUNGAL ALPHA-AMYLASE

by

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I. INTRODUCTION

Many highly important industrial processes are dependent upon imperfectly characterized, naturally occuring, enzyme systems. Among these the casual observer will note the amylaceous industries whose output of sizes, adhesives, and syrups, as well as industrial solvents make them a keystone of industry as a whole. Further development within these fields is greatly hampered by a lack of knowledge of the materials employed. It is probable that if the characteristics of the individual components of the enzyme systems used could be determined a more uniform and predictable end product of their utilization would result, with obvious benefits to the industry.

Ethyl alcohol is by far the most important organic industrial solvent and its production from a variety of saccharine and starchy materials has long been the subject of study. The saccharification required by starch before fermentation by yeast has been accomplished by a number of methods. Those of importance to the present industry involve the use of the enzyme systems of various malted grains and of fungi. Reports from this laboratory, and many others, have definitely established that the use of fungal saccharifying agents leads to higher alcohol yields than does the use of malt.

In an effort to determine the reason for this increased efficiency a program was set up in this laboratory to study separately the various carbohydrases of the fungi. In this connection Underkofler and Roy (74) have recently reported the isolation and crystallization of alpha-

amylase and limit dextrinase from submerged cultures of Aspergillus oryzae.

The present study was initiated to prepare, by the method already developed here, sufficient quantities of crystalline alpha-amylase for its characterization and determination of its role in the saccharification process. The commercial enzyme concentrate obtained by the previous investigators from Enzymes Incorporated, Eagle Grove, Iowa, was no longer available at the start of this investigation, thus necessitating a change in starting material. A second fungal enzyme concentrate available commercially is takadiastase and to solutions of this product the method of purification of Underkofler and Roy (74) was applied. However, the method did not yield crystalline enzyme from this source and attention was centered on the development of a scheme for the purification of alpha-amylase from takadiastase.

II. HISTORICAL

The characteristics of the amylolytic systems have been studied most extensively in the malted grains and discoveries in this field have led to investigations of other sources of these agents. As a consequence, this historical review is subdivided into sections dealing with first, the malt amylases, second, the fungal amylases, and lastly the isolation and characteristics of the fungal amylases.

A. Malt Amylases

Brown and Heron (12) give credit to Kirchhoff for conducting the first scientific investigations into the nature of the amylolytic systems of grains. In 1815 Kirchhoff demonstrated that albuminous material extracted from wheat was capable of converting starch to a crystallizable sugar. He noted that the activity of this albumin fraction was higher if the grain was first allowed to germinate. Several years earlier he had prepared the same crystalline sugar by the action of dilute sulfuric acid upon starch and Vögel had shown the presence of a gum-like material among the reaction products. The sugar received the name maltose from Dubrunfaut (18) years later and the gum was named dextrine by Biot and Persoz (10) because of its strong dextrorotatory power.

In an outstanding study by Payen and Persoz (60) of the action of the extract of malted grains upon starch it was stated that the action was due to a specific transforming agent which they termed diastase.

In addition to gravimetric measurements of the amount of sugar formed by diastatic action they utilized the change in iodine coloration of the starch paste as a measure of enzyme activity. By means of alcohol precipitation they were able to partially purify and concentrate the active agent from the malt extract.

Musculus (49, 51) showed by use of optical activity, reducing power, and alcohol precipitation of the products that the action of malt amylase led to the simultaneous production of maltose and dextrin from starch. This opposed the previously credited theory that starch was initially hydrated to dextrins which in turn were the source of the maltose. Schwarzer (63) showed that heat treatment of malt extract greatly affected its mode of action upon starch. At temperatures above 60°C the ability of malt extract to produce sugars fell off rapidly and above 75°C action was entirely halted. Further he observed that at temperatures above 60°C, where inactivation was rapid, a cloudiness due to coagulated protein formed, indicating that the saccharifying agent was associated with protein. Musculus and Gruber (50) presented further evidence that the breakdown of starch by malt extract resulted in the immediate production of dextrins and maltose, followed by the hydrolysis of the dextrins to achroödextrins and additional sugar.

Maercher (39) studying both the iodine reaction of the dextrins and the amount of reducing substances formed from starch by malt extract partially inactivated by heat concluded that two active agents were present in the original extract. Bourquelot (11) reiterated this belief on the basis of successive incomplete inactivations of malt

extract with high acid concentrations and temperatures in excess of 60°C.

Wijsman (80) first clearly showed the presence of these two agents and studied them in detail. His method was to place a measured quantity of malt extract in the center of a starch-gelatin plate and incubate for 12 to 60 hours at 20°C. The plate, when flooded with iodine, turned purple where no enzyme action had taken place, but in the center of the plate a colorless spot appeared and around it a mauve ring. He found that if part of the substance from the colorless area was transferred to a second plate the same phenomenon was repeated. On the other hand, if a portion of the outer ring was transplanted, only the mauve coloring was reproduced as a spot. Further he was able to show that the mauve ring was accompanied by a high production of sugar while the colorless inner area, though the starch had been decomposed to the achroödextrin stage, contained only a small concentration of sugar. From these data he concluded that indeed two enzymes were present and that one was largely dextrinogenic in nature while the other was saccharogenic. Using the above diffusion technique he proved that the saccharogenic enzyme was capable of more rapid diffusion through gelatin than the dextrinogenic and was more susceptible to heat inactivation. The dextrinogenic enzyme was inactivated by acids and dialysis.

This fine study unfortunately was neglected until 1922 when Ohlsson (53) restudied these same properties and devised a working method for the differential inactivation of the two amylases present. Ohlsson found that the dextrinogenic enzyme was highly unstable in acid solution and that at pH 3.3 and 0°C was completely inactivated in 15 minutes.

Under the same conditions the activity of the saccharogenic enzyme decreased only 20 to 30 per cent. Further it was shown that the saccharogenic enzyme was totally destroyed by a temperature of 70°C for 15 minutes at pH 6-7 leaving 75 per cent of the dextrinogenic activity. These preparations allowed the first study of the individual enzymes of malt extract and from this time on great numbers of papers have appeared dealing with these individual enzymes.

At about this same time Kuhn (34, 35) studied the amylases of the pancreas, takadiastase, and malt extract with particular attention to the mutarotation of the products of the reaction with starch. The action of the pancreatic amylase and takadiastase led to substances which mutarotated downwards but the products of malt extract hydrolysis showed a shift in optical activity in the opposite direction. In all cases the isolated sugar was maltose. This pointed to the initial production of alpha-maltose by the first two amylases followed by mutarotation to the lower optical activity of the equilibrium mixture of alpha- and beta-maltose. On the other hand the malt extract apparently gave rise to beta-maltose with subsequent upward shift in rotation due to establishment of equilibrium with the alpha-form.

Kuhn suggested that the enzymes be classified as alpha- and beta-amylases on the basis of the optical configuration of the sugar which they produced from starch. On this basis malt extract was termed a beta-amylase while pancreatic amylase and takadiastase were designated as alpha-amylases.

Application of Kuhn's classification to the enzymes of malt extract separated by Ohlsson's method showed that the dextrinogenic enzyme was

of the alpha type while the saccharogenic enzyme was a beta-amylase (54).

With these fundamental findings as a background numerous workers have attempted the purification of the alpha- and beta-amylases from a number of different sources and in recent years notable success has been achieved. In this regard may be mentioned the crystallization of beta-amylase from sweet potatoes by Balls, Thompson, and Walden (9), of alpha-amylase from malt extract by Schwimmer and Balls (65), and the outstanding achievements of Meyer and his co-workers in crystallizing the alpha-amylases of human saliva (40, 47) and pancreas (45), hog pancreas (42, 43, 44) and Bacillus subtilis (41), as well as the beta-amylase of barley malt (46). The methods used in these successful investigations are given in the appendix to this thesis.

B. Fungal Amylases

Amylolytic enzymes from molds and bacteria have long been an important factor in the industry of the Orient but have aroused interest in the Occident only in the last seventy-five years. Chief among the Oriental uses of these agents was in the production of koji and the alcoholic beverage sake, or rice beer. In each case the preparation is the result of the action of a mixed culture of several molds, yeasts, and bacteria upon a starchy substrate.

Korschelt (29-33) in a series of articles first described the Japanese process for preparing koji and sake. In this process a mold identified by Korschelt as Eurotium oryzae was grown on moist rice and allowed to sporulate and dry. The product, koji, was then used as

a saccharifying agent on more rice in conjunction with a mixture of molds and yeasts to produce the alcoholic beverage. The amylolytic agents could be easily extracted from koji with warm water, and below 50°C brought about rapid clarification of starch paste in a manner similar to malt extract. Temperatures in excess of 60°C caused extensive inactivation.

The organism isolated by Korschelt as the principal fungus in koji has been reclassified by later investigators as Aspergillus oryzae.

Atkinson (7) studied the change in soluble solids and specific rotation of starch solutions treated with aqueous extracts of koji. He found that the initially rapid hydrolytic reaction was followed by a slow but continuing hydrolysis. The optimum temperature for the rapid reaction appeared to be 45°C, while at 60°C the reaction was not complete, and at 70°C nearly total inactivation of the enzyme system took place. In contrast to malt extract, the amylase of Aspergillus oryzae rapidly converted maltose to glucose. The presence of sodium chloride caused a definite inactivation of the koji extract.

Further study revealed the presence of fungi belonging to the genera Mucor and Rhizopus as well as Aspergillus in koji. Gayon and Dubourg (23) tested several strains of Mucor and Aspergillus and drew the conclusion that Aspergillus oryzae possessed the most active saccharifying system of the molds studied.

Calmette (15) turned his attention to another fungal preparation called Chinese yeast cake. The principal organism present was Amylomyces rouxii, later classified as Mucor rouxii, which was not

only capable of saccharifying the starchy grains but also of converting the sugars to alcohol. This organism presented the interesting possibility of achieving starch conversion and fermentation by a single pure culture and was studied with this in mind. The process, though workable, proved too slow for distillery needs.

Calmette and Boidin, according to Owen (59), abandoning the single inoculum method, used a mold as the saccharifying agent in place of malt in the usual distillery operation. The organism originally used was Mucor rouxii but this was later replaced by other Mucors and finally by Rhizopus delemar, all isolated from Chinese yeast cake. These latter organisms had the advantage of higher alcohol tolerance, low acid production, greater resistance to contaminants, and shortened the time required to bring about saccharification. The modified process in which the mold and yeast are added simultaneously to the mash, known as the Amylo process, is still in use in Europe. Detailed descriptions of the Amylo process are those of Galle (21) and Owen (59).

The use of fungal amylases in the alcohol industry did not gain favor in this country although Takamine (68) patented a process for the cultivation of Aspergillus oryzae on moist wheat bran for use as an amylolytic agent and urged its acceptance. The moldy bran was termed "taka-koji" and the active agent obtained by water extraction of the bran and precipitation with 70 per cent ethanol was called "takadiastase."

In 1912 Ortved (57) actually made a plant scale run using taka-koji in place of malt as the converting agent in a corn-rye fermentation. His results indicated that the yields of alcohol were higher than when malt saccharification was used and that existing equipment and methods

were adequate for handling the newer agent. He strongly recommended the adoption of the fungal saccharifying agent in both industrial and potable alcohol production because of the economies introduced by its use and the more efficient utilization of the starch. Collens (16) used takadiastase for conversion of cassava starch for alcohol production and obtained a 7.8 per cent increase in alcohol yield over mashes converted by malt. In spite of these encouraging reports, the fungal amylases were not utilized by the alcohol distilleries.

Interest was again aroused in the fungal replacement of malt with the sudden expansion of starch fermentation required by World War II. Malt production could not be expanded to meet the demand and both price and quality of the material available was subject to wide variation. Underkofler, Fulmer, and Schoene (72) surveyed some 17 strains of Aspergillus, Mucor, and Rhizopus, grown in a rotating drum on wheat bran, as saccharifying agents for corn mashes. The results showed Aspergillus oryzae to be the most promising organism. Further work by Underkofler and co-workers (24, 76) resulted in the confirmation of these findings and the large scale production of mold bran, a dry preparation of Aspergillus oryzae grown on wheat bran. Application of mold bran saccharification to corn mashes on a commercial basis was begun in 1945 at the Farm Crops Processing Corporation, Omaha, Nebraska and reported by Underkofler, Severson, Goering, and Christensen (75, 76). Replacing a portion or all of the malt with mold bran in a commercial fermentation resulted in a significant increase in alcohol yield

and showed that on a weight basis 4 per cent mold bran was superior to 10 per cent malt in saccharification.

In the commercial method of production of mold bran devised by Underkofler, et al. (75) the mold was grown on shallow layers of moist wheat bran in trays at carefully controlled temperatures and humidities. Since the handling of the trays was time consuming and laborious, interest developed in the production of fungal amylases by submerged culture techniques. Erb and Hildebrandt (19) first successfully used submerged cultures of Rhizopus delemar and Rhizopus boulard for the saccharification of granular wheat flour mashes. The volume of mold inoculum required was 6 to 12 per cent of the total mash volume. Alcohol yields equalled those obtained by use of mold bran saccharification and were markedly superior to mashes using malt as the sole amylolytic agent.

Van Lanen and Le Mense (77) at the Northern Regional Research Laboratory tested 350 strains of fungi grown in submerged culture for alpha-amylase activity and in actual fermentation tests on corn mashes. Only seven of these strains showed commercial possibilities. Adams, Balankura, Andreasen, and Stark (1) using the best of the above strains, Aspergillus niger NRRL 337, and only a 0.5 per cent mycelial transfer obtained a 6 per cent increase in alcohol production over standard malting procedure.

Le Mense, Corman, Van Lanen, and Langlykke (36) reported that only those organisms of the Aspergillus group of 35 varied fungi tested were capable of high amylase elaboration. Le Mense, Sohns, Corman, Blom,

Van Lanen, and Langlykke (37) have shown that submerged cultures of Aspergillus niger NRRL 337 and NRRL 330 can replace malt saccharification in commercial corn fermentation with an estimated saving of 2.4 to 3.6 cents per gallon of 190 proof alcohol produced.

C. Isolation of Fungal Amylases

Takamine (69) had early used precipitation of the aqueous extract of taka-koji with 70 per cent alcohol as a means of concentrating the amylolytic agents of Aspergillus oryzae. The precipitate if further dehydrated with absolute alcohol and dried could be preserved over long periods of time. Sherman and Tanberg (66) found that the amylase of Aspergillus oryzae could be precipitated by ammonium sulfate and ethanol-diethyl ether mixtures and that the increase in amylolytic activity was accompanied by an increase in protein nitrogen. A thirty-fold concentration of the enzyme was achieved by precipitation of a takadiastase solution with 0.75 saturated ammonium sulfate, dissolving the precipitate in 50 ml. of water, dialysis against 800 ml. of distilled water, and the dialysed solution fractionally precipitated with ethanol in the presence of salt. The most active fraction was obtained between 65 and 70 per cent ethanol.

Sherman, Thomas, and Baldwin (67) determined the optimum pH for the action of takadiastase to be 4.8 as compared to 4.4-4.5 for malt amylase and approximately 7.0 for pancreatic amylase.

Ohlsson and Swaetichin (55) following Ohlsson's success in separating the alpha- and beta-amylases of malt extract (53) briefly studied

takadiastase. They found no evidence for the presence of a beta-amylase. The enzyme was capable of rapidly hydrolysing starch to achroödextrins, but produced reducing sugars slowly, and thus was a typical dextrinogenic amylase. The enzyme was inactivated rapidly below pH 2.0 or above pH 11.5 but regained nearly full power in one hour in neutral solution. The importance of this regeneration to oral therapy was pointed out. The reversibility of inactivation by acid has been confirmed by Akabori, Hayasi and Kasimoto (2), Rau and Sreenivasan (61), and others.

Harada (25) prepared fungal amylase solutions by the method of Takamine (69) and demonstrated that the amylase became progressively more unstable as the temperature increased above 45°C. He further noted that while the pH optimum for amyloclastic action was 5.2 below 50°C at higher temperatures the optimum increased. The stabilizing effect of calcium ion and to a lesser extent phosphate ion on takadiastase was pointed out by Nakamura (52).

In an extensive investigation of "koji-diastase" prepared from pure cultures of Aspergillus oryzae grown on steamed rice, Ito (28) reported that activity was not destroyed by temperatures of 100°C to 130°C for a short time, that the active material was dialysable and thus not a protein. He proposed that the enzyme was a complex of a polypeptide, polysaccharide, and 5 to 12 per cent minerals (largely magnesium and phosphate) and attempted to combine constituents of these types to recreate the enzyme. Mixtures of monosaccharides, glycogen, dextrin, peptone, amino acids, magnesium monohydrogen phosphate, and other salts were tried and were actually found to have some hydrolysing

action on starch at pH 4.8-5.0. No further report of this work has appeared.

Oshima and Church (58) conducted a survey of some 34 strains of the Aspergillus flavus-oryzae group of organisms and clearly showed the species variation which occurred in the production of amylase and protease. Of a large number of media tested wheat bran proved most encouraging for the production of an active amylase. These workers were the first to show the vast differences in enzyme production by different strains of organisms identical morphologically.

Caldwell and Doebbeling (14) found that the amyloclastic activity of takadiastase as measured by a modified Wohlgemuth (81) starch-iodine method was very dependent upon ionic strength and suggested the use of a 1 per cent starch substrate buffered at pH 5.0 with 0.01 M acetate and 0.05 M sodium chloride. The rate of production of sugars was found to be nearly independent of the ionic strength.

Akabori and co-workers (3, 4, 6) were able to separate takadiastase into two fractions by dialysis through shark swim-bladder membrane in 50 per cent methanol. The active solutions gave no precipitate with trichloroacetic acid, were not inactivated by trypsin or erepsin, showed a strong Molisch reaction, and had molecular weights, by the diffusion method, of 1240 to 1370. These workers concluded that the amylase was not a protein, but an acidic polysaccharide (5).

Hollenbeck and Blish (27) measured the starch dextrinizing and liquifying powers of several amylases and concluded that since the activities were decreased in a parallel manner by partial inactivation

of the amylases by acid or heat that the two observed properties were manifestations of the same enzyme action.

Tokunago (71) purified the amylase of Aspergillus oryzae by fractional precipitation with ammonium sulfate at 0.50 to 0.66 saturation and by precipitation with saturated magnesium sulfate or sodium chloride followed by dialysis. The resulting solution brought about typical antibody response in rabbits. The immune serum and amylase solution gave a precipitin reaction which removed all enzyme activity from solution. Immune serum developed against boiled amylase solution still brought about a precipitin reaction with fresh amylase but the activity was not removed from solution. Pepsin was capable of destroying the enzyme but neither trypsin or papain had an effect. The enzyme was thought to belong to the albumin class of proteins.

Caldwell, Chester, Doebbeling, and Volz (13) in an extensive study of takadiastase found that extraction of the material with dilute sodium chloride solutions, 50 per cent ethanol, acetate buffers, and papain at 5°C offered no advantages over the use of distilled water for removal of the active agent. Precipitation of the amylase with sodium sulfate, magnesium sulfate, and basic lead acetate were not as satisfactory as ammonium sulfate. Working in the cold, 5°C, the amylase of takadiastase was precipitated at 0.66 saturation with solid ammonium sulfate. The precipitate was transferred to a dialysis bag with a minimum of distilled water and the salt removed, followed by pervaporation. Three to five repetitions of the procedure achieved maximum concentration. The product was free of maltase activity. It was found that pH control made no

appreciable difference in the effectiveness of the precipitation. Adsorption on alumina and silica gel proved ineffective in purifying the enzyme. Evidence that the dextrinizing and saccharifying roles of takadiastase are carried out by the same enzyme was presented by comparing the ratios of dextrinogenic to saccharogenic activity for the enzyme partially inactivated by heat (60°C to 70°C) and by acid (pH 3) and alkali (pH 9). In all cases the ratio of the activities remained the same.

Wallerstein, Alba, and Hale (79) reported that the amylase of Aspergillus oryzae can be completely precipitated by the addition of lignin at pH 3.6-4.5 and resuspended in active form at a pH of 6.5 and higher.

Volz and Caldwell (78) investigated the course of the hydrolysis of starch by highly purified takadiastase. Measuring rate as a function of the production of reducing sugars (saccharogenic activity) they found that the initially rapid reaction decreased markedly when about 20 per cent of the theoretically complete hydrolysis was reached. That this slowing down of the reaction was not due to enzyme inactivation was readily shown by the rapid breakdown of additional fresh starch substrate. The evidence, therefore, points to a lower rate of reaction with the hydrolysis products of starch than with starch itself.

In an oral paper Lipps, Roy, Andreasen, Vernon, and Kolachov (38) described a procedure capable of isolating alpha-amylase from submerged cultures of Aspergillus niger, NRRL 337. The procedure adopted was as follows:

> One liter of freshly prepared, chilled mold filtrate at pH 4.1-4.2 was treated with 2 per cent bentonite and allowed to adsorb for one hour with mild, continuous stirring. The mixture was centrifuged and the bentonite residue, containing extraneous protein, was discarded. The supernatant liquid was treated with 0.75 saturated ammonium sulfate for 30-40 minutes, centrifuged, and the supernatant liquid discarded. The precipitate was dissolved in 20 ml. of 0.137 M acetic acid-sodium acetate buffer of pH 4.6. The resulting solution was treated with 0.75 saturated ammonium sulfate for 30-40 minutes, centrifuged, and the precipitate dissolved in 20 ml. of 0.2 M potassium phosphate of pH 4.6. This solution contained alpha-amylase and limit dextrinase. Maltase was destroyed by the treatment.
>
> The potassium phosphate solution, containing alpha-amylase and limit dextrinase, was treated with 2 per cent bentonite for one hour with moderate continuous stirring and then centrifuged. The supernatant liquid contained pure alpha-amylase, while the limit dextrinase was adsorbed on the bentonite.

The alpha-amylase solution obtained by this procedure was twenty times more active than the original mold filtrate. Further it was shown that the limit dextrinase could be eluted from the bentonite with phosphate buffer at pH 7.0 to give pure limit dextrinase solutions uncontaminated by alpha-amylase.

Gates and Kneen (22) made an excellent and detailed study of organic precipitating agents for the amylase of Aspergillus oryzae. The starting material, mold bran prepared by the Mold Bran Company of Eagle Grove, Iowa, was extracted with ten parts of water at 30°C for one hour. The resulting infusion was strained through cloth and centrifuged. The precipitating characteristics of methanol, ethanol, isopropyl alcohol, and acetone were determined with regard to temperature, pH, electrolytes present, and inactivation of the enzyme by contact with the precipitating agent. Methanol was found to cause extensive inactivation even at 10°C,

and required an 80 per cent concentration to be effective. Acetone, while precipitating the enzyme completely at 60 per cent concentration with little or no inactivation at 25°C, caused some discoloration and the precipitate was gummy and difficultly soluble in water. Ethanol and isopropyl alcohol, on the other hand, precipitated the enzyme completely at 70 and 60 per cent concentrations respectively, yielding precipitates of good color and water solubility. Hydrogen ion concentrations had a marked effect upon both the extent of precipitation and character of the precipitate caused by miscible organic solvents. Conditions on either the alkaline or acid side of the optimum for the given solvent gave a relatively stable colloid which would not precipitate, while at the optimal pH, 6.0-6.5 for ethanol and 5.5-6.0 for isopropyl alcohol and acetone, the precipitates flocculated rapidly and in the presence of the proper electrolyte were easily handled and readily redissolved in water.

Determination of the per cent recovery of activity in precipitates obtained as described above in the presence of a number of common electrolytes indicated that magnesium and phosphate ions were advantageous in stabilizing the enzyme and enhanced the desirable characteristics of the precipitate. Further it was shown that other multivalent cations had a detrimental effect upon the enzyme. Surprisingly this list included calcium and barium ions which had shown stabilizing action on other alpha-amylases.

The conclusion reached by Gates and Kneen concerning optimal conditions for concentration of the amylase was precipitation with isopropyl

alcohol at 60 per cent concentration, pH 5.5-6.0, in the presence of 0.25 per cent magnesium sulfate and 0.72 per cent disodium phosphate at room temperature. Harada (25) confirmed the findings of Gates and Kneen with regard to the action of phosphates in promoting better precipitate characteristics using ethanol, but stated that calcium chloride or magnesium chloride in the solution causes lower diastatic recovery.

Dirks and Miller (17) investigating means of removal of proteolytic enzymes from mold bran concentrates reported that a pH of 10 and high concentrations of sodium chloride (4.3 M) inactivate up to 90 per cent of the proteolytic enzymes while decreasing the amylase activity by only 10 to 15 per cent.

The paper chromatopile has been used successfully for the partial separation of amylase and adenosine deaminase from commercial taka-diastase by Mitchell, Gordon and Haskins (48) who suggested that sufficiently large quantities for isolation might be realized by this method.

Terui, Okada, and Fujiwara (70) have succeeded in partially purifying the amylase of Aspergillus oryzae from saturated sodium sulfate solutions by adsorption upon hydrocellulose and an agar sol, followed by elution with water. Granulous starch and native cellulose were reported ineffective.

Underkofler and Roy (74) have recently reported a scheme for the crystallization of alpha-amylase and limit dextrinase from concentrates of submerged cultures of Aspergillus oryzae provided by Enzymes Incorporated, Eagle Grove, Iowa. The procedure utilizes ammonium sulfate

precipitation and bentonite adsorption at controlled acidities to achieve purification and is given in detail below.

Fifty liters of concentrated mold culture filtrate was 0.75 saturated with ammonium sulfate at ice box temperatures and allowed to stand overnight. The precipitate obtained by decanting the clear supernatant liquid was dissolved in 5 liters of water, adjusted to pH 4.2 with 2 N acetic acid, and stirred for one hour with 50 g. of bentonite. Following centrifugation the bentonite was discarded and the centrifugate 0.75 saturated with solid ammonium sulfate and stored overnight in the refrigerator. The precipitate was removed by centrifugation, dissolved in 500 ml. of distilled water and the solution again 0.75 saturated with ammonium sulfate, placed in the refrigerator overnight, and centrifuged. The precipitate was dissolved in 50 ml. of 0.2 M phosphate buffer at pH 4.6 and stirred for one hour with 2 g. of bentonite, centrifuged, and the bentonite treatment repeated. The clear solution obtained upon centrifugation was 0.75 saturated with solid ammonium sulfate and chilled overnight. The precipitate obtained was largely crystalline and on solution in the minimum amount of water followed by the addition of an equal volume of saturated ammonium sulfate and chilling in the refrigerator a completely crystalline material having high alpha-amylase and only a trace of limit dextrinase activity was achieved. Crystallization took place from a solution approximately 728 times as concentrated as the original extract. The crystalline enzyme had an activity of 4,800 Sandstedt, Kneen, and Blish (62) units per milligram dry weight.

Subsequent to the completion of the experimental work reported in this thesis, Fischer and de Montmollin (20) published a report of the crystallization of alpha-amylase from takadiastase. Crystallization was accomplished by fractionation of a suspension of commercial takadiastase with ammonium sulfate, then with acetone, followed by sodium chloride treatment and refractionation with ammonium sulfate.

III. MATERIALS AND METHODS

The commercial enzyme takadiastase was obtained from Takamine Laboratory, Inc., Clifton, New Jersey in undiluted form. The enzyme is precipitated with alcohol from aqueous extracts of cultures of Aspergillus oryzae grown on moist wheat bran and dried under vacuum to a pale cream-colored powder for handling. The usual commercial material is diluted with starch or dextrin to a standard potency. The enzyme used here had not been so diluted.

Alpha-amylase activity was determined by a modification of the method of Sandstedt, Kneen, and Blish (62). Since the enzyme system of Aspergillus oryzae has no beta-amylase activity this enzyme was omitted in preparing the starch substrate. The unit of activity thus obtained was 11.6 times that of the original Sandstedt, Kneen, and Blish unit and is designated by U throughout this paper.

The color standard used for the red-brown endpoint was that recommended by Olson, Evans, and Dickson (56) which is made up of inorganic salts and has the advantage of complete stability.

The method of Back, Stark, and Scalf (8) for the determination of limit dextrinase was followed with the exception that the limit dextrin used was purchased from Wahl-Henius Laboratories as limit-dextrin prepared according to Kneen.

The method of Underkofler, Guymon, Rayman, and Fulmer (73) was followed without modification for the quantitative determination of reducing sugars.

The determination of protein nitrogen was carried out by two methods. Samples which had been exhaustively dialysed were analysed by means of the micro-kjeldahl technique using a solid copper sulfate-potassium sulfate catalyst. Samples containing ammonium sulfate could be more rapidly handled by determination of the optical density at 281 millimicrons in the Beckmann DU spectrophotometer using a hydrogen arc source and one centimeter quartz cuvettes. The optical density values were then referred to a standard curve established with Kjeldahl-analysed samples. The readings were not affected by ammonium sulfate, but precautions against traces of acetone, which absorbs in this region, must be observed.

The apparatus used for electrophoretic analysis of the various alpha-amylase solutions was a Klett Electrophoresis Apparatus made by the Klett Manufacturing Company, New York. The optical system of this particular instrument had a magnification of 0.341. The standard cell used had an approximate volume of 11 ml. and a cross section area of 0.725 square centimeters.

All solutions to be analysed were dialysed in the cold against several changes of distilled water for at least 24 hours and then against potassium phosphate buffer, pH 7.5, ionic strength 0.20, for at least 48 hours to assure establishment of equilibrium. Solutions were adjusted, when necessary, to approximately 0.5 per cent protein by dilution with the buffer against which they had been dialysed.

The electrophoretic mobility of components of the system was calculated by means of the standard formula:

$$\mu = \frac{q \times d}{L \times M_c \times M_e \times t \times i}$$

where: μ = electrophoretic mobility

q = cross section of the cell

d = distance the boundary has moved

L = specific conductance of the sample

M_c = magnification of the instrument

M_e = magnification of the enlarger if measurement is made from an enlarged photograph

t = time of electrophoresis in seconds

i = current in amperes.

The units are in $cm^2\ sec^{-1}\ volt^{-1}$.

IV. EXPERIMENTAL RESULTS

In common with most enzyme purification schemes, the methods which have proved most useful in the purification of the alpha-amylases have involved precipitation with salts, and organic solvents and adsorption on a variety of solids. The method employed successfully by Underkofler and Roy (74) for the crystallization of fungal alpha-amylase was dependent upon ammonium sulfate precipitation with several adsorptions of impurities by bentonite interspersed and was used directly on concentrated culture filtrates.

A. Ammonium Sulfate - Bentonite Purification Scheme

The method of Underkofler and Roy (74) was applied to a solution of takadiastase with the hope of achieving crystallization of the alpha-amylase. The concentration of the starting solution was adjusted to duplicate the alpha-amylase content of the culture filtrates for which the method was devised. The steps of the process will not be discussed in detail, as they were given in the historical portion of this thesis, but the scheme is outlined in the following flow diagram and analytical data are given in Table 1.

5 g. takadiastase in 8.6 l. of distilled water, cooled in ice box, 0.75 saturated with solid ammonium sulfate. Kept overnight in refrigerator and decanted.

A. Precipitate — Supernatant

Dissolved in 860 ml. distilled water, pH adjusted to 4.2 with 2N acetic acid, stirred with 8.5 g. bentonite for one hour and centrifuged. — Discarded

Precipitate — B. Supernatant

Discarded

Solid ammonium sulfate added to 0.75 saturation. Chilled overnight and centrifuged.

C. Precipitate — Supernatant

Dissolved in 85 ml. distilled water, solid ammonium sulfate added to 0.75 saturation, chilled overnight and centrifuged. — Discarded

D. Precipitate — Supernatant

Dissolved in 50 ml. of 0.2 N phosphate buffer at pH 4.6, stirred two hours with 2 g. bentonite and centrifuged. — Discarded

Precipitate — E. Supernatant

Discarded

Two g. bentonite added, stirred one hour and centrifuged.

Precipitate — F. Supernatant

Discarded

Solid ammonium sulfate added to 0.75 saturation, chilled overnight and centrifuged.

G. Precipitate — Supernatant

Dissolved in minimum volume of distilled water to make 13.6 ml. — Discarded

Table 1

Enzyme Activities Obtained During Ammonium Sulfate-Bentonite Purification

Fraction	Alpha-amylase		
	U/ml.	Total U	% original
Original solution	1.8	15,800	100
B. Supernatant	8.6	7,420	47
C. Precipitate	59.9	5,030	32
F. Supernatant	86.2	3,660	23
G. Precipitate	134	2,700	17

It will be noted that the procedure resulted in marked concentration of the active alpha-amylase but was accompanied by high losses. The major loss occurred with the initial ammonium sulfate precipitation and is due in part to precipitate which remained suspended and was lost by decantation. The loss of alpha-amylase on bentonite adsorption was generally observed and apparently indicates a greater affinity of the amylase of takadiastase for bentonite than was evidenced by alpha-amylase of concentrated submerged culture filtrates.

The precipitate designated as G. was amorphous, white, and flocculent. It was dissolved in a minimum amount of distilled water to give a volume of 13.6 ml. To 10 ml. of this solution 10 ml. of saturated ammonium sulfate was slowly added with stirring and a small quantity of white precipitate appeared. One half of this solution was placed in the refrigerator and the other half in a 30°C incubator. Each solution was examined microscopically for evidence of crystallization, but none was found within three days, although a considerable quantity of amorphous precipitate had formed.

The solution which had been held in the refrigerator was saturated with ammonium sulfate, centrifuged after one hour, and the precipitate redissolved in a minimum amount of distilled water. Saturated ammonium sulfate was added to this solution until a very faint cloud appeared, toluene was layered on the surface and the flask placed in the 30°C incubator. The precipitate which formed during the following week gave no evidence of crystallinity.

Attempts to crystallize other precipitates obtained in the manner given above did not meet with success and attention was turned to the use of organic solvents as precipitants in the hope that if ammonium sulfate were eliminated from the solution crystallization might take place, yielding a salt free product.

To this end, several precipitates obtained by ammonium sulfate-bentonite precipitation procedures, as previously described, were combined and dissolved in a minimum amount of distilled water. The solution was 0.75 saturated with solid ammonium sulfate, centrifuged, and the

precipitate dissolved in a minimum of distilled water. This solution was dialysed against distilled water at room temperature with daily changes of water until no test for sulfate was given by the dialysate. Pervaporation before a small fan brought the volume to 49 ml. at which point it was slightly viscous and had a faint odor of putrefaction. The alpha-amylase content was 10,190 U.

To 10 ml. of this solution 75 ml. of absolute ethanol was added to give a concentration of 91 per cent by volume. A cloud formed but no precipitate could be obtained by centrifugation. The solution was chilled, the pH adjusted to 5.0 with 2 N acetic acid, and a flocculent white precipitate formed immediately. After centrifuging and decanting the supernatant, the precipitate was redissolved in 11 ml. of distilled water made to pH 7.8 with 1 N ammonium hydroxide. Upon standing two days in the refrigerator a few micro-crystals similar in appearance to the bipyramidal alpha-amylase crystals of Underkofler and Roy were observed in this solution along with considerable amorphous material. Partial evaporation of the solution yielded additional amorphous precipitate but no increase in crystalline substance. The amounts were too small to permit isolation.

To a second 10 ml. portion of the enzyme solution 92 ml. of isopropyl alcohol was added to give 91 per cent concentration by volume. The cloudy solution which formed was cooled in the refrigerator, the pH adjusted from 7.3 to 5.0 with 2 N acetic acid and a flocculent precipitate appeared. The precipitate obtained on centrifugation was suspended in 15 ml. of water. The pH of the suspension was 3.7 and upon adjustment to 5.5 with 1 N ammonium hydroxide the solution cleared

considerably leaving only a faint cloud. No crystalline precipitate was formed from this solution upon extended storage in the refrigerator.

To a third 10 ml. portion of the enzyme solution 97 ml. of acetone was added giving a concentration of 92 per cent by volume. No precipitate formed until the pH was lowered from 7.7 to 5.0 with 2 N acetic acid. The solution was centrifuged, the supernatant decanted, and the precipitate suspended in 19 ml. of distilled water. Solution was accomplished by raising the pH to 7.9 with 1 N ammonium hydroxide and when solution was complete the pH was lowered to 5.3 with 2 N acetic acid. At the final pH a cloud again formed and the solution was stored in the refrigerator. The precipitate which formed in 48 hours was largely amorphous, but numerous crystals of the form indicated by the work of Underkofler and Roy (74) were present. Again the quantities were too small to be handled for isolation.

A repetition of the last given procedure failed to yield anything but an amorphous precipitate and a very viscous solution. The system would, however, appear to be worth further study.

In an effort to evaluate the effect of the various stages of the ammonium sulfate-bentonite procedure, previously outlined, electrophoretic patterns were made of the active solution at each step of the purification. A 20 g. sample of takadiastase dissolved in one liter of distilled water was treated as shown in the following flow diagram:

20 g. takadiastase dissolved in 1 l. distilled water, ammonium sulfate added to 0.75 saturation. Held overnight and centrifuged.

A. Precipitate — Supernatant

Dissolved in distilled water to make 256 ml., ammonium sulfate added to 0.75 saturation, held overnight and centrifuged. — Discarded

B. Precipitate — Supernatant

Dissolved in distilled water to make 108 ml., adjusted to pH 4.2 with 2 N acetic acid, stirred one hour with 4 g. bentonite and centrifuged. — Discarded

Precipitate — C. Supernatant

Discarded

Ammonium sulfate added to 0.75 saturation, held overnight and centrifuged.

D. Precipitate — Supernatant

Dissolved in distilled water to make 60 ml., ammonium sulfate added to 0.75 saturation, held overnight and centrifuged. — Discarded

E. Precipitate — Supernatant

Dissolved in distilled water to make 41 ml., ammonium sulfate added to 0.75 saturation, held overnight and centrifuged. — Discarded

F. Precipitate — Supernatant

Dissolved in 0.2 N phosphate buffer at pH 4.6 to make 24 ml., stirred for one hour with 0.8 g. bentonite and centrifuged. — Discarded

Precipitate	G. Supernatant
Discarded	Ammonium sulfate added to 0.75 saturation, held overnight and centrifuged.
H. Precipitate	Supernatant
Dissolved in distilled water to make 6 ml.	Discarded

The analytical data for each step of the procedure are given in Table 2. It should be pointed out that while the final precipitate represented only 3.3 per cent of the original activity that 41.4 per cent of the alpha-amylase was removed in samples for electrophoresis studies and that a correspondingly higher yield could be expected if these had not been removed. On the other hand it is clearly evident that unaccountable losses of 47.2 per cent did occur. The largest single loss of activity was the result of the first bentonite adsorption at pH 4.2 (step C) and accounts for over half of the total loss.

The solution of the final precipitate, though representing only a small percentage of the total amylase in the original solution, was 5.5 times as concentrated in alpha-amylase as the starting solution. The specific activity, i.e., the activity per milligram of protein nitrogen, of the precipitate, H, is 146 U/mg. N., while the original takadiastase has a specific activity of 41.9 U/mg. N.

The electrophoretic patterns for each of the fractions obtained are given in Figures 1 and 2. These patterns indicate that the initial ammonium sulfate precipitation and the first bentonite adsorption at

Table 2

Distribution of Alpha-amylase in Ammonium Sulfate-Bentonite Purification

Fraction	Precipitate				Supernatant		Lost, % total
	Used in next precipitation, U	% total	Sample analysed, U	% total	U	% total	
Original solution	53,010	95.3	2790	5.0	--	--	+ 0.3
A.	46,200	82.8	5000	9.0	3150	5.7	+ 2.2
B.	39.350	70.5	3650	6.5	548	1.0	4.8
C.	23,470	42.1	2130	3.8	--	--	24.6
D.	15,000	26.9	3000	5.4	398	0.7	9.1
E.	7,440	13.3	2400	4.3	327	0.6	8.7
F.	4,430	7.9	1840	3.3	88	0.1	2.0
G.	2,270	4.1	2270	4.1	--	--	+ 0.3
H.	1,860	3.3	--	--	0	--	0.8
Totals		3.3		41.4		8.1	47.2

Figure 1. Electrophoretic Patterns of Alpha-amylase Concentrates Obtained by the Ammonium Sulfate-Bentonite Procedure.

All patterns obtained in phosphate buffer, pH 7.5, ionic strength 0.20.

(1) Original solution approximately 0.5 per cent protein, time 5 hours at 50 milliamperes and 120 volts.

(2) Step A. Solution approximately 0.35 per cent protein, time 4 hours 30 minutes at 40 milliamperes and 140 volts.

(3) Step C. Solution approximately 0.5 per cent protein, time 4 hours 50 minutes at 40 milliamperes and 150 volts.

(4) Step D. Solution approximately 0.75 per cent protein, time 2 hours 55 minutes at 40 milliamperes and 150 volts.

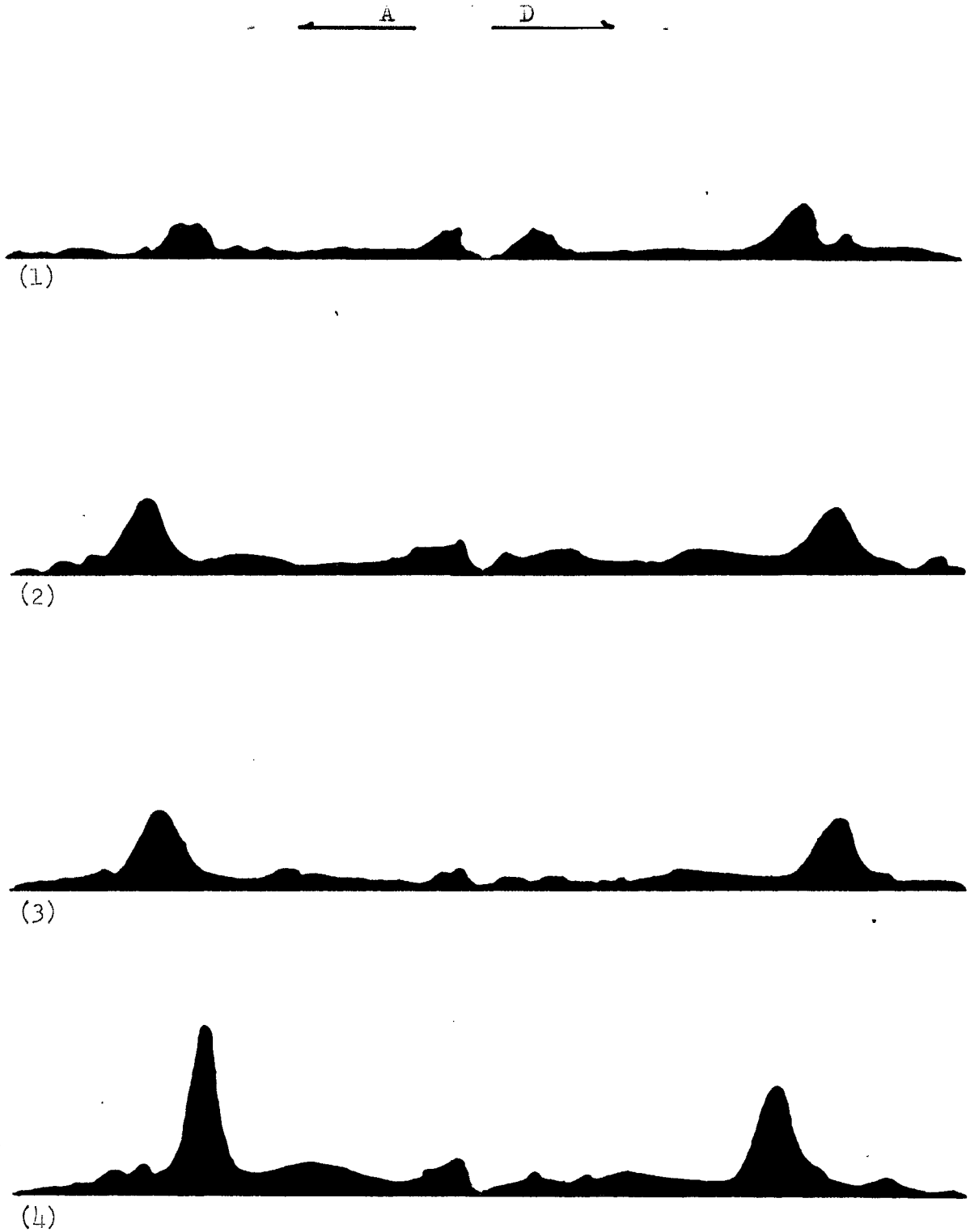

A
D
(1)
(2)
(3)
(4)

Figure 2. Electrophoretic Patterns of Alpha-amylase Concentrates Obtained by the Ammonium Sulfate-Bentonite Procedure.

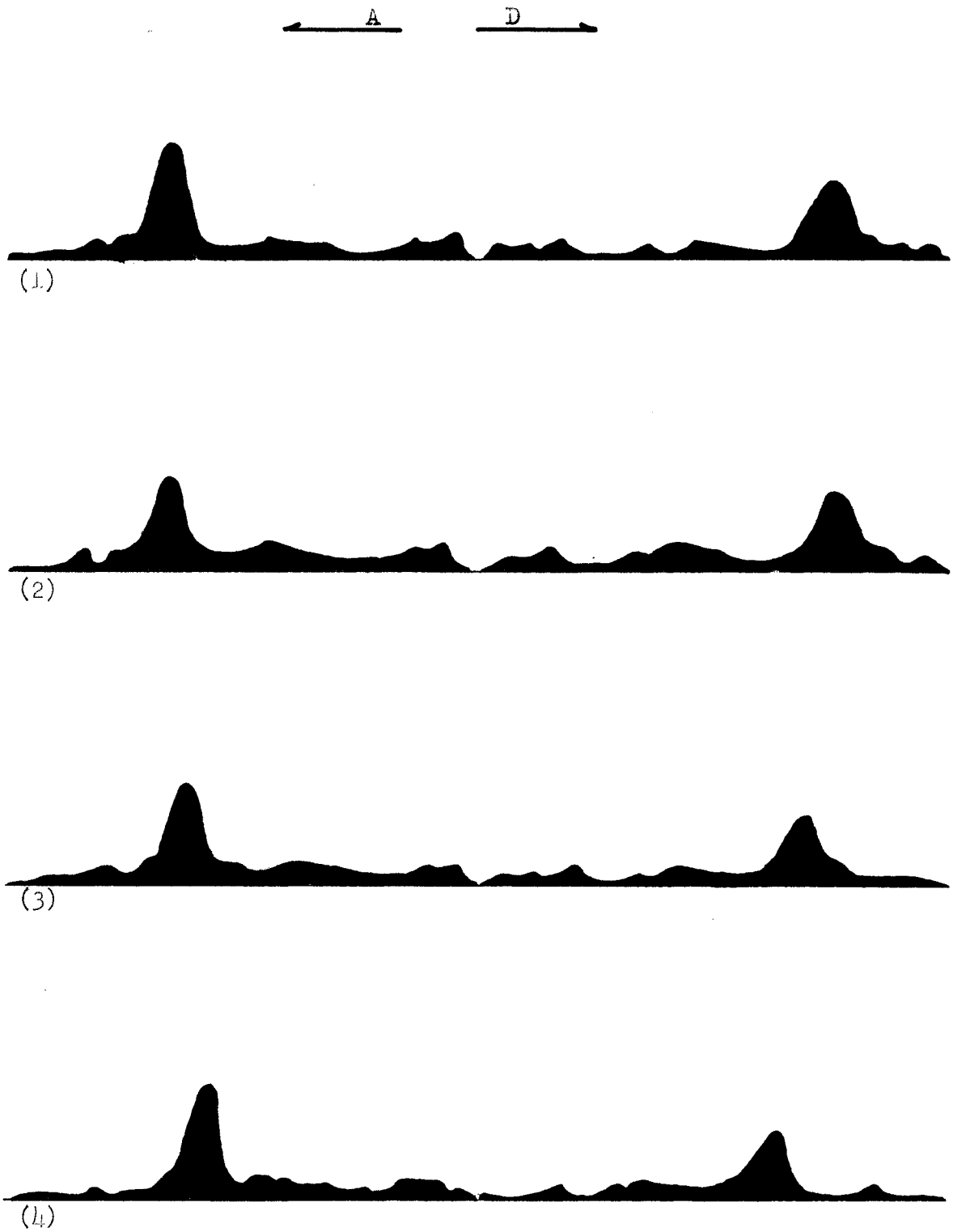
All patterns obtained in phosphate buffer, pH 7.5, ionic strength 0.20.

(1) Step E. Solution approximately 0.7 per cent protein, time 3 hours 40 minutes at 40 milliamperes and 145 volts.

(2) Step F. Solution approximately 0.5 per cent protein, time 3 hours 15 minutes at 36 milliamperes and 145 volts.

(3) Step G. Solution approximately 0.5 per cent protein, time 3 hours 25 minutes at 35 milliamperes and 140 volts.

(4) Step H. Solution approximately 0.15 per cent protein, time 3 hours 8 minutes at 35 milliamperes and 140 volts.


A
D
(1)
(2)
(3)
(4)

pH 4.2 bring about a considerable purification of one of the components of the system. Repeated precipitations with ammonium sulfate at 0.75 saturation, thereafter, appear to accomplish very little in the way of eliminating additional components of the system. On the other hand, the second bentonite adsorption at pH 4.6 did bring about a decrease in the amounts of the smaller peaks, especially the several slow moving components crowded together near the initial boundry. The final ammonium sulfate precipitation had little effect upon the electrophoretic picture.

Thus ammonium sulfate appears to accomplish its maximum purification with a single use, while bentonite serves to decrease the number of components in the system both early and late in the procedure.

No proof can be presented here that the major component shown in these patterns is due to alpha-amylase. However, it was true of all the studies made that as the alpha-amylase was concentrated this peak became more prominent.

B. Fractional Precipitation with Ammonium Sulfate

Several workers (13, 44, 71, 74) have reported the use of different concentrations of ammonium sulfate for precipitation of the alpha-amylases from various sources. In order to determine the optimum concentration to be used for the precipitation of the alpha-amylase component of takadiastase a fractional precipitation was carried out. The procedure was as follows: One gram of takadiastase was dissolved in 500 ml. of one half saturated calcium sulfate solution and sufficient

solid ammonium sulfate was slowly added with stirring to give the desired degree of saturation. A sample was then removed for analysis and sufficient solid ammonium sulfate added to increase the concentration of precipitant by a predetermined amount. Account was taken of the change in volume due to the addition of salt and removal of the sample in calculating the amount of salt to be added. These steps were repeated until the solution was saturated with ammonium sulfate. After each addition of ammonium sulfate the 20 ml. sample was removed, centrifuged, and both centrifugate and precipitate analysed for alpha-amylase. Analytical results given as percentage of the total activity found in the precipitate and supernatant are given in Table 3.

Table 3

Fractional Precipitation of Alpha-amylase of Takadiastase with Ammonium Sulfate

Ammonium sulfate, % saturation	Alpha-amylase, % found in	
	Supernatant	Precipitate
0 - 50	100	none
60	41	59
65	35	65
70	12	88
75	14	86
80	16	84
90	13	87
100	10	90

It is evident from the data of Table 3 that the maximum precipitation is achieved near 0.70 saturation with ammonium sulfate. Thus the use of the 0.75 saturated solution recommended by previous investigators (74) is more than adequate for precipitation of alpha-amylase from the highly concentrated takadiastase used here.

Application of the 0.70 saturated salt level to the previously described ammonium sulfate-bentonite purification scheme gave results comparable in all regards to those already reported.

C. Adsorbents for Limit Dextrinase and Alpha-amylase

The work of Lipps, Roy, Andreasen, Vernon, and Kolachov (38) with the amylase of Aspergillus niger NRRL 337 and of Underkofler and Roy (74) with concentrates of submerged cultures of Aspergillus oryzae made use of the selective adsorption of impurities from alpha-amylase solutions by bentonite at controlled hydrogen ion concentrations. In each case the addition of two to four per cent bentonite to a solution of the partially purified enzyme resulted in only very slight adsorption of alpha-amylase but complete removal of the maltase and limit dextrinase activities, with the limit dextrinase proving to be the more difficult to remove.

1. Bentonite as a selective adsorbent for limit dextrinase

In an effort to determine the ability of bentonite to achieve the same success with takadiastase solutions, the activity of alpha-amylase and limit dextrinase were followed through a series of steps suggested by the procedure of Underkofler and Roy (74). A 0.1 per cent solution

of takadiastase in 0.025 M calcium chloride was adjusted to pH 4.2 with 2 N acetic acid and stirred with 2 per cent bentonite for one hour. The solution was centrifuged, the bentonite discarded, and a sample of the supernatant analysed (A). Solid ammonium sulfate was then added to 0.75 saturation, the precipitate allowed to settle for 2 hours, and the solution centrifuged. The precipitate was taken up in 20 ml. of sodium acetate-acetic acid buffer at pH 4.6 and reprecipitated by 0.75 saturation with ammonium sulfate. The precipitate obtained on centrifugation was dissolved in potassium phosphate buffer at pH 4.6 and diluted to exactly 25 ml. This solution was analysed for both enzymes (B).

A second 0.1 per cent takadiastase solution in 0.025 M calcium chloride was treated in an identical manner. The last precipitate, obtained as above, was dissolved in only 20 ml. of phosphate buffer at pH 4.6, stirred with 2 per cent bentonite for one hour, followed by centrifugation, and precipitation with ammonium sulfate at 0.75 saturation. The precipitate was redissolved in potassium phosphate buffer at pH 4.6 and diluted to exactly 25 ml. for analysis (C).

The procedure, given above, is summarized in the following flow diagram, and the analytical data are given in Table 4.

0.1 per cent solution of takadiastase in 0.025 M $CaCl_2$ at pH 4.2 stirred with 2 per cent bentonite for 1 hour and centrifuged.

Precipitate — Discarded

A. Supernatant

Solid ammonium sulfate added to 0.75 saturation, held 2 hours and centrifuged.

Precipitate

Dissolved in 20 ml. of sodium acetate-acetic acid buffer at pH 4.6, reprecipitated with ammonium sulfate at 0.75 saturation and centrifuged.

Supernatant — Discarded

Precipitate

B. Dissolved in 25 ml. of potassium phosphate buffer at pH 4.6.

Supernatant — Discarded

- - - - - - - - - -

Second sample identical except dissolved in only 20 ml. of phosphate buffer. Stirred with 2 per cent bentonite for 1 hour and centrifuged.

Precipitate — Discarded

Supernatant

Solid ammonium sulfate added to 0.75 saturation, held for 30 minutes and centrifuged.

Precipitate

C. Dissolved in exactly 25 ml. of phosphate buffer at pH 4.6.

Supernatant — Discarded

Table 4

Enzyme Recovery from Bentonite Adsorptions and Ammonium Sulfate Precipitation

Solution	Alpha-amylase			Limit dextrinase		
	U/ml.	total	% remaining	units/ml.	total	% remaining
Original	3.0	3000	100	2.5	2500	100
A	2.4	2400	80	2.3	2300	92
B	20	500	16.7	10.0	250	10
C	21.4	536	17.9	10.7	268	10.7

The data of Table 4 indicate a very large loss in activity of both enzyme systems by the ammonium sulfate precipitations between solutions A and B. This loss is due in part to inefficient centrifugation of the large volumes of solution. The significance of these data, however, lie in the tendency of the two enzyme activities to parallel one another through these steps which are able to separate them in other types of preparations. It would appear that under these conditions bentonite holds little promise for selective adsorption.

The effect of pH on the bentonite treatment was determined in two experiments. In the first, a series of 0.1 per cent takadiastase solutions in 0.2 M acetate buffer at pH 4.6 were adjusted to pH values from 4.0 to 6.0 with glacial acetic acid or 6 N sodium hydroxide, as required. The solutions were then stirred with 2 per cent bentonite for

1 hour at the end of which time they were centrifuged and the alpha-amylase and limit dextrinase assayed. The results given in Table 5 indicate a very high loss of both enzyme activities at pH 4.0 while at pH 4.5 and 5.0 the limit dextrinase suffers correspondingly more than the alpha-amylase, but not sufficiently so to warrant use as a separation scheme.

Table 5

The Effect of pH Upon the Adsorption of Alpha-amylase and Limit Dextrinase by 2 per cent Bentonite Treatment

pH of solution	Alpha-amylase		Limit Dextrinase	
	U/ml.	% remaining	units/ml.	% remaining
Original solution	2.4	100	4.0	100
4.0	< 0.12	< 5	1.0	25
4.5	2.0	83	2.7	68
5.0	2.0	83	2.4	60
5.5	1.7	71	1.8	45
6.0	1.2	50	2.2	55

A second experiment using smaller increments in the lower range of pH was made in the hope that the nearly complete loss of activity of both enzymes, as noted previously, would not proceed in a parallel manner between pH 4.1 and 4.5. A 1 per cent solution of takadiastase and 4 per cent bentonite were used as more indicative of the concentrations

encountered in actual separation procedures. The data of Table 6 indicates that the adsorptions do not occur in parallel but, in fact, favor the removal of alpha-amylase from limit dextrinase solution at these low pH levels.

Table 6

The Effect of pH Upon the Adsorption of Alpha-amylase and Limit Dextrinase by 4 per cent Bentonite

pH of solution	Alpha-amylase		Limit Dextrinase	
	U/ml.	% remaining	units/ml.	% remaining
Original solution	27.4	100	30.6	100
4.10	4.45	16	19.5	63.6
4.20	10.4	38	21.9	71.5
4.30	18.7	68	21.6	70.5
4.50	24.6	90	25.0	81.6

2. The effect of successive bentonite adsorptions at pH 4.2

The pH 4.2 was chosen as being intermediate between the point of very high enzyme loss, pH 4.0, and the point of little effect, pH 4.5. It was hoped that successive adsorptions by small amounts of bentonite might be able to accomplish a separation of alpha-amylase and limit dextrinase not realized by a single use of a large amount of adsorbent.

To a 1 per cent solution of takadiastase in acetate buffer at pH 4.2, 2 per cent of bentonite was added and the slurry stirred for

30 minutes. The bentonite was removed by centrifugation and a sample of the supernatant adjusted to pH 4.6 to minimize the inactivation by low pH levels on alpha-amylase. To the supernatant 2 per cent bentonite was again added and stirred for 30 minutes. Four additions of bentonite were made in this way and finally all samples were analysed for alpha-amylase and limit dextrinase.

The analytical results in Table 7 indicate that while limit dextrinase is removed more rapidly than alpha-amylase the differential is not sufficient for good separation. There is the possibility, however, that if the limit dextrinase were already at a low level it might be removed more effectively than at this high activity.

Table 7

The Effect of Successive Bentonite Adsorptions at pH 4.2 upon Alpha-amylase and Limit Dextrinase

No. of bentonite treatments	Alpha-amylase		Limit Dextrinase	
	U/ml.	% remaining	units/ml.	% remaining
0	26.0	100	27.4	100
1	21.8	83.9	22.2	81
2	20.0	77.0	16.6	60.5
3	17.8	68.5	11.2	40.8
4	16.6	63.8	10.8	39.4

3. Adsorption on starch

One of the final steps used by Schwimmer and Balls (65) in the purification of malt alpha-amylase was adsorption of the enzyme on wheat starch from cold 40 per cent ethanol solution followed by elution with water. These workers surveyed a number of starches and found that they differed widely in ability to adsorb malt alpha-amylase (64).

In the hope that the alpha-amylase of takadiastase could be selectively adsorbed by starch, thereby separating it from limit dextrinase, the technique was tried. The test method of Schwimmer and Balls (64) was applied as follows to the starches available in the laboratory: to 50 ml. of a cold solution of takadiastase 50 ml. of cold 80 per cent ethanol, containing 0.5 per cent calcium chloride, was slowly added with stirring. The solution was held in an ice bath and 10 g. of starch added, stirred for 5 minutes, the suspension centrifuged, and the supernatant analysed for residual alpha-amylase.

No difference in the alpha-amylase adsorbing power of corn, potato, rice, or arrowroot starch could be discovered if the original takadiastase solution was of high activity, i.e. alpha-amylase of 50 U/ml. On the other hand, if the takadiastase was more dilute, i.e. 5 U/ml., rice starch proved to be the most effective adsorber.

The ability of rice starch to selectively adsorb alpha-amylase in the presence of limit dextrinase was determined by the method given above. The starch after centrifugation was eluted with one 50 ml. portion of distilled water and the eluate analysed for both enzymes. More complete recovery of the enzymes could, no doubt, be achieved by elution of the starch by several small volumes of water; however, as a method of

comparison the single elution was deemed adequate. The data of Table 8 clearly indicate that the eluate is relatively more concentrated in alpha-amylase than the original solution, but that no clean-cut separation was accomplished by the method.

Preliminary experiments served to show that adsorptions at higher temperatures were more inefficient and accompanied by higher inactivation than under the conditions used here.

Table 8

Adsorption of Alpha-amylase and Limit Dextrinase from Takadiastase Solutions by Rice Starch

Amount of starch, grams	Recovered in eluate			
	Alpha-amylase		Limit dextrinase	
	U total	% recovery	units total	% recovery
0	260	100	290	100
5	85	33	62	21
10	100	38	33	11

D. Fractional Precipitation with Organic Solvents

The solvents used were chosen because of the encouraging results obtained in the crystallization attempts cited earlier in this section.

1. Fractional precipitation of takadiastase with ethanol

One gram of takadiastase was dissolved in 100 ml. of distilled water; a 5 ml. sample was removed and saved for analysis. To the remaining 95 ml. of solution 110 ml. of absolute ethanol was added slowly with stirring to give an alcohol content of 55.6 per cent. After standing for 5 minutes the solution was centrifuged for 15 minutes, the supernatant decanted and the precipitate immediately taken up in 35 ml. of water and allowed to stand. A 5 ml. sample of the alcoholic supernatant was removed and to the remaining solution was added 50 ml. of absolute ethanol to bring the concentration to 65.6 per cent. The solution was centrifuged for 20 minutes and the precipitate dissolved in 14 ml. of distilled water. The procedure was continued in this manner to give precipitates at 73, 80.7, 85.5 and 90.5 per cent ethanol.

Analytical data are presented in Table 9.

These data show that while between 65.6 and 90.5 per cent ethanol, 62.4 per cent of the alpha-amylase can be precipitated, there is no point of rapid break which would suggest that effective separation of closely similar proteins might occur in good yield.

Table 9

Fractional Precipitation of Alpha-amylase from Takadiastase with Ethanol

% ethanol	Alpha-amylase in the precipitate	
	U	% total
0	2500	100
55.6	47	1.9
65.6	54	2.2
73.0	557	23.3
80.7	327	13.1
85.5	387	15.5
90.5	263	10.5

2. Fractional precipitation of takadiastase with acetone

To 10 ml. portions of a 1 per cent takadiastase solution various amounts of acetone were added slowly with stirring to give the desired concentrations. After five minutes the solutions were centrifuged for 10 minutes and the precipitates taken up in distilled water. No precipitation was achieved at less than 50 per cent acetone by volume. The results of alpha-amylase determinations on both supernatant and precipitate are given in Table 10.

Table 10

Fractional Precipitation of Alpha-amylase from Takadiastase Solutions with Acetone

% acetone (by vol.)	Alpha-amylase			
	Centrifugate		Precipitate	
	U	% total	U	% total
0	310	100	- -	- -
40	310	100	- -	- -
50	252	81.3	10	3.2
60	< 1	- -	220	70.5
70	< 1	- -	230	73.7
80	< 1	- -	235	75.3
90	< 1	- -	184	59.0

The great increase in precipitability of alpha-amylase between 50 and 60 per cent acetone concentration indicated by these data suggest that a procedure utilizing this property might prove of value where others fail. The precipitate obtained at 50 per cent acetone concentration was about one-half the volume of the active precipitate from 60 per cent acetone, indicating a considerable removal of inactive solids.

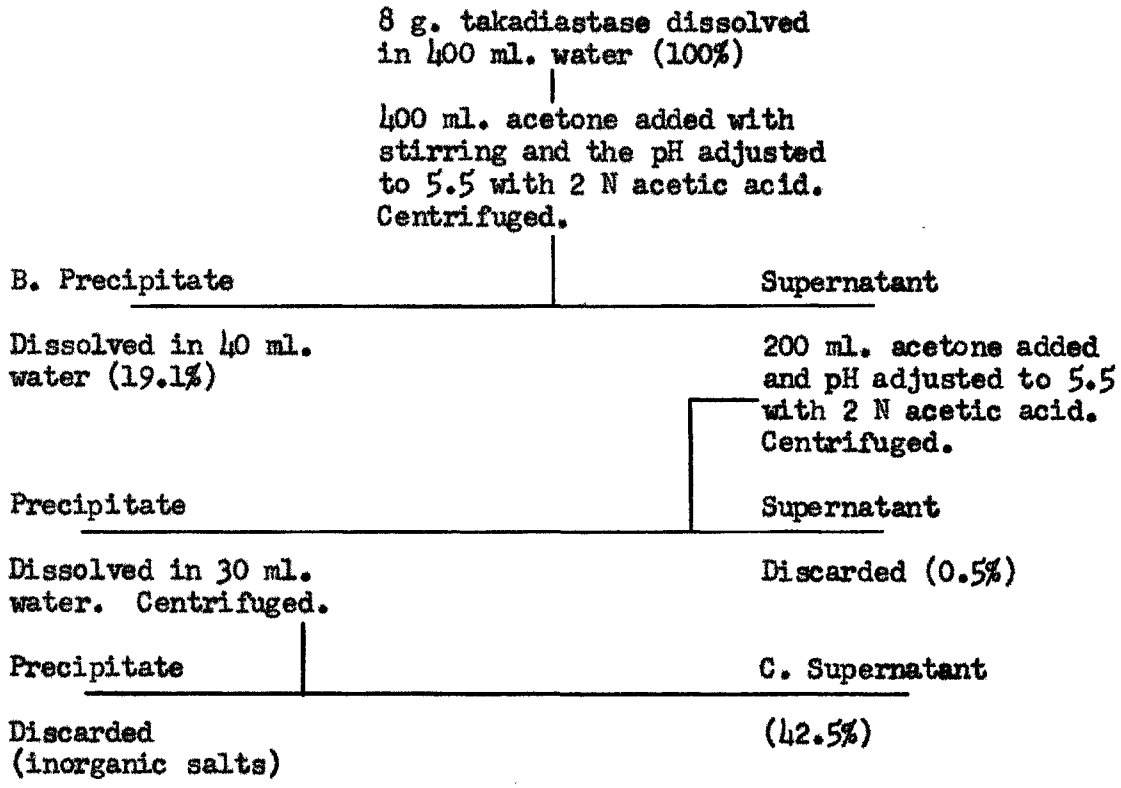
The results of the fractional precipitation with acetone were encouraging as was the previously shown case when small amounts of crystals which may have been alpha-amylase were obtained from a solution

of an acetone precipitate. In view of these results efforts were concentrated upon the use of acetone in isolation procedures.

3. Electrophoresis of solutions from acetone precipitation

At this point a series of runs were made involving fractionation of the alpha-amylase of takadiastase between acetone concentrations of 50 and 60 per cent to obtain samples of sufficient size for electrophoretic analysis at each stage. The procedure followed in each case was the same. Eight grams of takadiastase was dissolved in 400 ml. of distilled water and 400 ml. of acetone was added slowly with constant mechanical stirring. The pH of the resulting cloudy solution was reduced from approximately 7.3 to 5.5 with 2 N acetic acid and the solution centrifuged for five minutes. The supernatant was decanted, the precipitate allowed to drain for one minute in the inverted centrifuge cup, then 40 ml. of distilled water was added, mixed and the solution allowed to stand. To the supernatant, decanted above, an additional 200 ml. of acetone was added slowly with mechanical stirring, the pH readjusted to 5.5, and the solution again centrifuged. To the precipitate, drained as above, approximately 30 ml. of distilled water was added and thoroughly mixed for several minutes. The resulting cloudy, brown solution on centrifugation gave a clear yellow-brown solution and a precipitate made up almost entirely of inorganic salts.

This scheme in outline is given in the following flow diagram. The percentage values indicate the amounts of the original alpha-amylase activity found at those points. Each figure is an average of at least three runs.



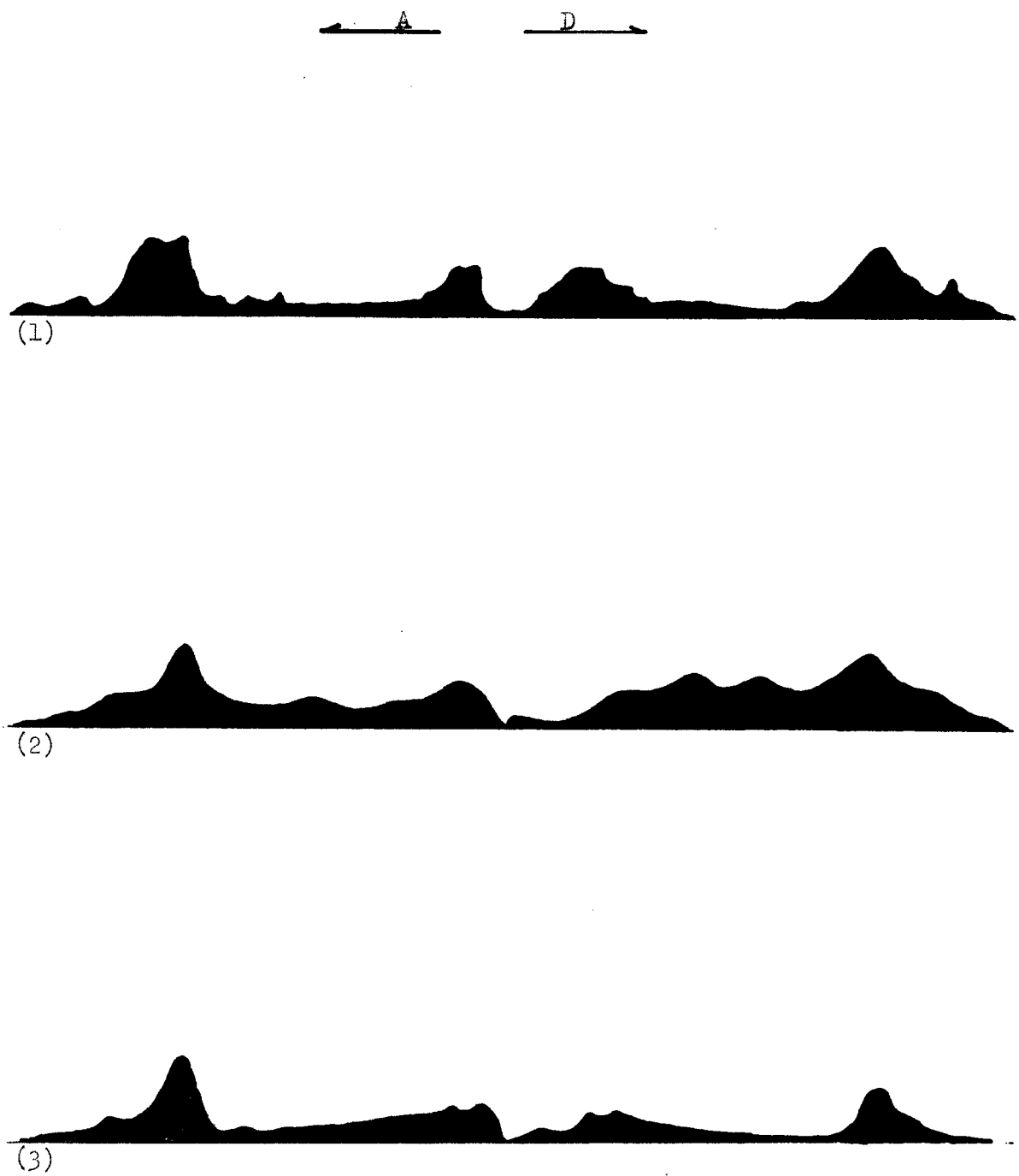
Electrophoretic patterns of solutions of the precipitates obtained at 50 and 60 per cent concentrations of acetone and the original takadiastase were made. These patterns, shown in Figure 3, indicate a definite concentration of one component of the system in the precipitate obtained from acetone at 60 per cent concentration. In the precipitates formed in 50 per cent acetone solutions at least four components of the system are in relatively high proportion compared with the largest fraction. Acetone at a concentration of 60 per cent acetone on the other hand, appears to precipitate this same main fraction but with much smaller amounts of impurities. The chief contaminants appear to be two or more bodies which have little or no mobility under these conditions, i.e., pH 7.5, ionic strength 0.20, phosphate buffer.

Figure 3. Electrophoretic Patterns of Alpha-amylase Concentrates Obtained by Acetone Precipitation.

(1) Original solution: Solution approximately 0.5 per cent protein, time 5 hours at 50 milliamperes and 120 volts.

(2) Precipitate formed at 50 per cent acetone: Solution approximately 1.0 per cent protein, time 3 hours 25 minutes at 35 milliamperes and 138 volts.

(3) Precipitate formed at 60 per cent acetone: Solution approximately 0.65 per cent protein, time 4 hours 19 minutes at 35 milliamperes and 140 volts.


A
D
(1)
(2)
(3)

E. Acetone-Bentonite-Ammonium Sulfate Purification Scheme

If the solution of the precipitate obtained by fractionation with acetone to 60 per cent as previously described, is three-fourths saturated with ammonium sulfate the precipitate obtained may be dissolved in small volumes of water to give exceptionally high activities per unit volume. In several runs these activities ranged from 760 to 1260 U/ml. This higher figure represents a 22.5 fold increase over the starting solution. In addition the specific activity of the alpha-amylase increased from 42 U/mg N. to 171 U/mg N., a four-fold concentration.

The electrophoretic diagrams show that this ammonium sulfate precipitate has an increased concentration of a relatively fast moving component over the fractions of very low mobility. This is clearly shown by comparison of its pattern, Figure 4 (2), with that of the solution from which it is derived, Figure 4 (1).

On the other hand, if the 60 per cent acetone precipitate in acetate buffer pH 4.2 is treated with 4 per cent bentonite for one hour only a slight relative decrease in concentration of the slowly moving components is observed, but the small component B is eliminated entirely, as shown in Figure 4 (3).

If, then, these steps are combined, first, the acetone fractionation, followed by treatment of the solution of the 60 per cent acetone precipitate with 4 per cent bentonite at pH 4.2 for one hour, and finally precipitation with three-fourths saturated ammonium sulfate the main component is concentrated with only very small amounts of impurities

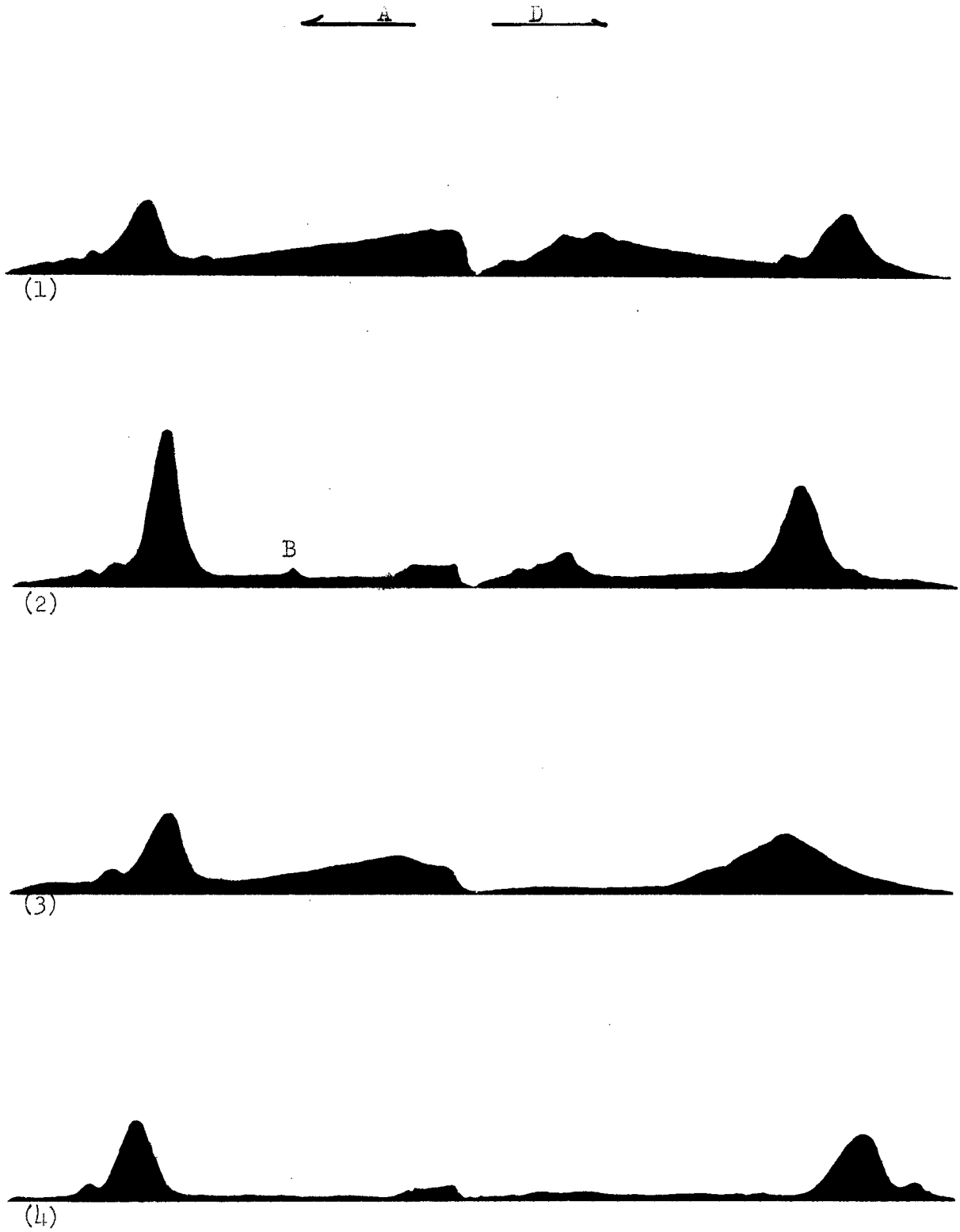
Figure 4. Electrophoretic Patterns of Alpha-amylase Concentrates Obtained by Combining Acetone and Ammonium Sulfate Precipitation with Bentonite Adsorption.

(1) Solution from precipitate obtained from 60 per cent acetone. Solution approximately 0.65 per cent protein, time 4 hours 19 minutes at 35 milliamperes and 140 volts.

(2) Precipitated once with acetone (60 per cent), once with ammonium sulfate (0.75 saturation). Solution approximately 0.65 per cent protein, time 4 hours 13 minutes at 35 milliamperes and 138 volts.

(3) Precipitated once with acetone (60 per cent), treated once with 4 per cent bentonite. Solution approximately 0.7 per cent protein, time 3 hours 55 minutes at 35 milliamperes and 138 volts.

(4) Precipitated once with acetone (60 per cent), treated with 4 per cent bentonite followed by precipitation with ammonium sulfate. Solution approximately 0.45 per cent protein, time 4 hours 19 minutes at 35 milliamperes and 135 volts.


A
D
(1)
B
(2)
(3)
(4)

contaminating it. Such a scheme is shown in the flow diagram on the next page and the accompanying analytical data are in Table 11. In the run shown here the final yield was less than half that expected owing to the collapse of one of the centrifuge tubes used for collecting the final precipitate.

The electrophoretic pattern of this final precipitate is shown in Figure 4 (4). There is still a remnant of the very slow moving material as well as a small amount of a component which moves more rapidly than the main fraction.

Calculation of concentration from this diagram indicates that the main component constitutes 72 per cent of the material present and has a mobility of 6.59×10^{-5} om^2 sec^{-1} $volt^{-1}$ at pH 7.5 in phosphate buffer of ionic strength 0.20.

The precipitate evinced an activity of 480 U/ml., an 8.6 fold concentration, and a specific activity of 152 U/mg. N as compared to 41.9 U/mg. N in the original takadiastase solution.

In other experiments, solutions of the final precipitate showed activities as high as 1260 U/ml. and specific activities up to 170 U/mg. N.

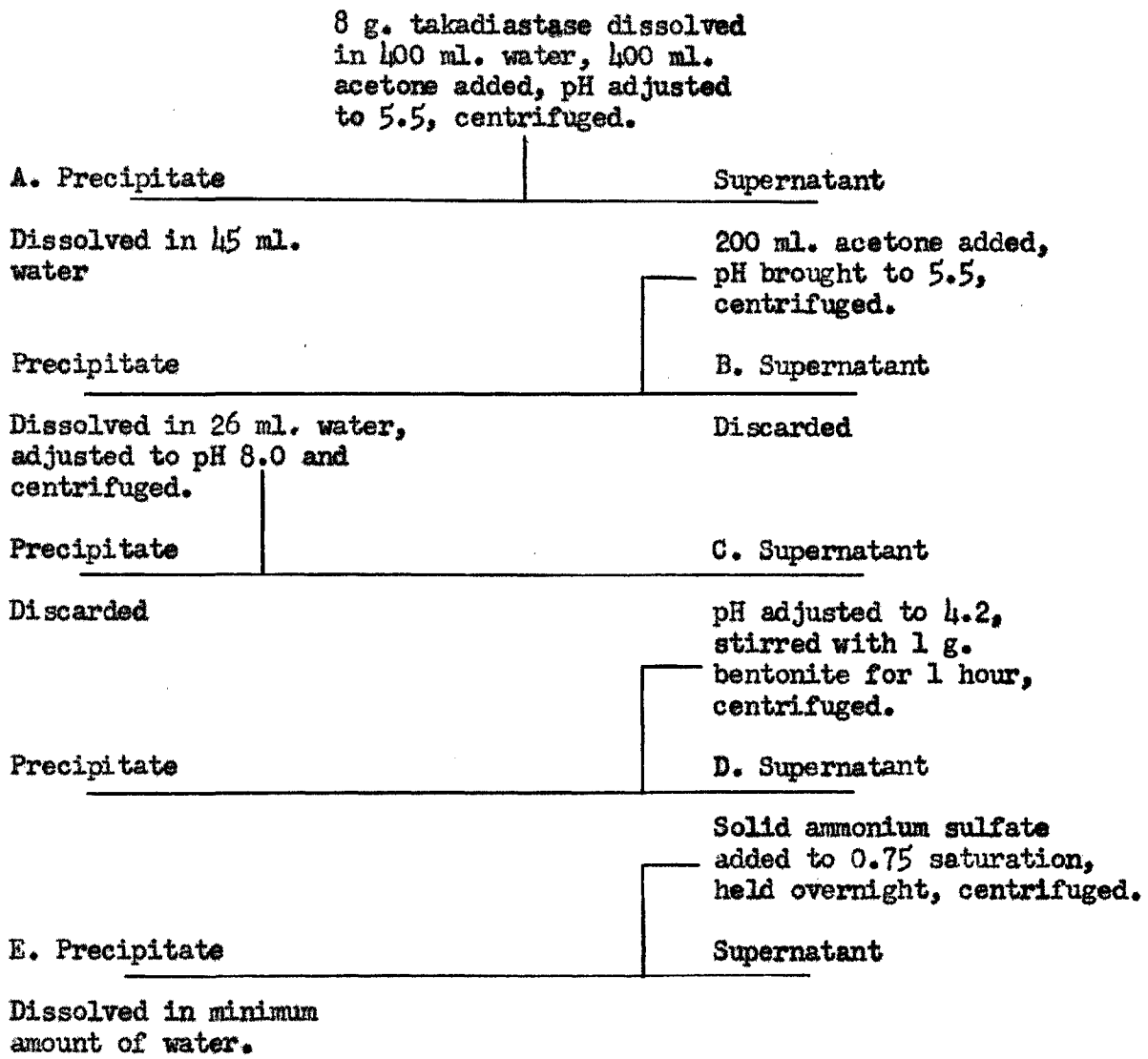

8 g. takadiastase dissolved in 400 ml. water, 400 ml. acetone added, pH adjusted to 5.5, centrifuged.
A. Precipitate
Supernatant
Dissolved in 45 ml. water
200 ml. acetone added, pH brought to 5.5, centrifuged.
Precipitate
B. Supernatant
Dissolved in 26 ml. water, adjusted to pH 8.0 and centrifuged.
Discarded
Precipitate
C. Supernatant
Discarded
pH adjusted to 4.2, stirred with 1 g. bentonite for 1 hour, centrifuged.
Precipitate
D. Supernatant
Solid ammonium sulfate added to 0.75 saturation, held overnight, centrifuged.
E. Precipitate
Supernatant
Dissolved in minimum amount of water.

Table 11

Alpha-amylase Activities Recovered in the Acetone-Bentonite-Ammonium Sulfate Purification of Takadiastase

Step	Alpha-amylase		
	U/ml.	U total	% recovery
Original solution	56	22,400	100
A	94	4,420	19.7
B	0.076	72	0.3
C	331	9,600	42.9
D	343	7,140*	31.9
E	480	2,060	9.2

* 3260 U of alpha-amylase removed from this solution for electrophoretic study before proceeding to Step E.

F. Filter Paper Chromatography

The application of filter paper chromotography as a test for purity of an enzyme preparation would be highly desirable since it is rapid, gives a lasting record if photographed, and requires little in the way of equipment. Preliminary experiments with this in mind were carried out with some of the amylase solutions prepared and with crude takadiastase.

The chromatogram was made on sheets of No. 1 Whatman filter paper cut to 19 cm. x 20 cm. The samples, four in number, were placed

equidistant from each other along a line one inch above the bottom of the paper. The paper was formed into a cylinder 20 cm. tall by bringing the edges together and loosely joining them with metal staples. The paper cylinder was then placed in a wide mouth chemical reagent bottle which contained a layer about one centimeter deep of developing solvent and the bottle capped. The chromatogram was developed by allowing the solvent to pass upwards to the top of the paper which required six to eight hours. When the solvent reached the top of the paper the cylinder was removed and air dried.

Samples were placed on the paper in duplicate so that by cutting the paper in half vertically two identical chromatograms were obtained. One of these was sprayed with 0.1 per cent ninhydrin in butanol and heated for five minutes in an oven at 100°C to produce prominent purple areas where protein materials were present. The other half of the chromatogram was sprayed with 1 per cent starch solution and incubated at 30°C for 30 minutes in a moist chamber, then dried in air and sprayed with dilute iodine solution. The location of alpha-amylase was indicated by a colorless area on a deep blue-purple background.

In these experiments it made no discernable difference whether the original sample spot was wet or allowed to dry before the paper was placed in the developing solution.

A number of solvents were surveyed and it was found that water and aqueous buffers carried the amylase and most of the other protein material to the top edge of the paper, thereby giving no separation. Various concentrations of acetone in steps of 5 per cent by volume from

30 to 80 per cent were tested as developing solvents. The mobility of the enzyme on the paper decreased with increasing acetone concentration. When 65 per cent acetone solutions were used as developing solvents the enzyme of crude takadiastase did not move from the original spot but a minimum of six inactive, ninhydrin positive materials were apparent with Rf values of 0.32, 0.42, 0.53, 0.65, 0.71, and 0.77. No attempt was made to identify any of these constituents. At lower acetone concentrations the amylase became more mobile and tended to overrun the inactive materials giving a more confused picture. At the lower end of the acetone concentration scale the reverse influence was evident. The enzyme moved rapidly to the upper portion of the picture leaving inactive protein material behind, but this was observed as a diffuse streak on the paper and gave no definite spot. Ethyl alcohol and isopropyl alcohol gave similar results and appeared to offer no advantages over acetone in this regard.

It is important to note that solutions of highly purified alpha-amylase obtained by either the ammonium sulfate-bentonite method or by acetone precipitation showed no ninhydrin positive material other than the enzyme in chromatograms developed at 65 per cent and 35 per cent acetone.

The author regrets that time permitted no further exploration of this field. There are a wealth of solvents and conditions to be investigated which might indeed make this technique a valuable tool in the further purification of the amylase of Aspergillus oryzae. As was suggested by Mitchell, Gordon and Haskins (48) and further pointed up by this

preliminary work it may well be possible to isolate workable quantities of alpha-amylase by the chromatopile technique, or with a filter paper pulp column. The approach is certainly worth further investigation.

V. DISCUSSION

The original objective of this research, namely, the preparation of large quantities of crystalline alpha-amylase, was not achieved. The inability of the ammonium sulfate-bentonite purification scheme devised by Underkofler and Roy (74) to yield crystalline product from takadiastase is not so surprising if we realize how radically the starting material was changed. The organism used by Enzymes Incorporated, Eagle Grove, Iowa, for their submerged culture work was originally obtained from Rohm and Haas and is maintained in the culture collection of the Biophysical Chemistry section of the Chemistry Department as Aspergillus oryzae No. 38. Takadiastase, produced by Takamine Laboratory, is reported to be elaborated by a strain of Aspergillus oryzae, but no further information is available. There is every reason to believe that the organism used by the Takamine Laboratory is different from that used by Enzymes Incorporated.

The work of Oshima and Church (58), Van Lanen and Le Mense (77) and Le Mense, Corman, Van Lanen, and Langlykke (36) have clearly shown the vast differences which occur in the ability of various substrains of microorganisms to elaborate amylases. It is quite probably that these amylases produced in such varying amounts are indeed different in their physical and chemical makeup and would require different purification methods.

In addition, it is impossible to overlook the great difference in the conditions under which the organisms were grown. The culture filtrates previously used were produced by submerged culture, on wholly

synthetic media, with vigorous forced aeration. Thus the mycelium was violently agitated, constantly surrounded by nutrients, under precise temperature control, and sporulation did not occur. On the other hand, the organism used by Takamine Laboratory was grown on shallow trays of moist bran as a surface culture. Temperatures of such cultures fluctuate widely and sporulation takes place before the enzymes are harvested. Furthermore the enzymes after being extracted from the mold bran with warm water are precipitated with ethyl alcohol and dried for ease of handling. Each of these cultural and processing differences could contribute to variations in the properties of the alpha-amylases from the two sources.

Nevertheless, it should be noted that the ammonium sulfate-bentonite treatment did achieve a 3.4 increase in specific activity of the amylase of takadiastase even though crystals were not obtained.

Toward the end of this investigation efforts were made to grow Aspergillus oryzae No. 38 in submerged culture in existing pilot plant equipment. The preliminary experiments were encouraging and it is suggested that filtrates of such cultures may well give results comparable to those of the Enzymes Inc. filtrates and should be investigated further.

The effect of bentonite upon the alpha-amylase and limit dextrinase of takadiastase was significantly different from its effect on the enzymes from culture filtrates. Underkofler and Roy (74) found that at pH 4.2 and pH 4.6 alpha-amylase was adsorbed very poorly while at pH 4.6 limit dextrinase was removed from solution efficiently. However, the

experiments made here indicate that limit dextrinase and alpha-amylase of takadiastase are adsorbed under very similar conditions. The degree of adsorption of the two enzymes is not the same, but it is of the same magnitude and thus not suited to batchwise separation, as attempted.

The difference in adsorption shown by the two enzymes, while not sufficiently great to make batchwise separations possible might yield to column chromatography. In the column continued development might allow the activities to separate to a sufficient degree to permit recovery of essentially pure enzymes in successive eluates.

The differential adsorption of the two enzymes, alpha-amylase and limit dextrinase, by rice starch was more pronounced than that shown by bentonite. However, recoveries were very poor and the selectivity of adsorption not sufficiently great to make batchwise adsorptions a practical means of separation. The use of a column with this type of adsorbent might well prove a fruitful field for further investigation for the same reasons as cited above.

In connection with earlier work with crystalline alpha-amylase obtained from ammonium sulfate solutions, Underkofler and Roy (74) have stated that the ammonium sulfate appeared to have a detrimental effect upon the enzyme activity. It was in the hope of eliminating this difficulty that attention was first turned to the use of organic solvents as precipitants. Final crystallization from such solvents or from water solutions derived from them would yield enzyme uncontaminated with inorganic salts and excess precipitant could readily be removed by evaporation at low temperatures and pressures. A solvent of high

volatility is desirable for such uses, hence the widespread application of acetone. The attainment of crystals of the desired form from water solutions of acetone precipitates early in the investigation focussed attention upon this solvent. Further, acetone showed a much steeper solubility gradient curve than any other solvent tried.

Along with the advantages offered by organic solvents as precipitants must be considered their prime disadvantage of inactivation. Typically only 40 to 60 per cent of the total activity of the original amylase solution was recovered in the precipitate obtained at a concentration of 60 per cent acetone by volume, while from 20 to 40 per cent was lost by inactivation and the remainder of the activity was retained by other precipitates and supernatants.

The final scheme proposed for the purification of the alpha-amylase of takadiastase utilizes the best features of the various procedures tried and attains a marked purification. As shown by electrophoresis, the initial fractionation of the enzyme between 50 per cent and 60 per cent acetone by volume eliminates a number of components of the system, most of them being retained by the precipitate from 50 per cent acetone. In addition, bentonite was shown to remove one of the remaining components completely. A final precipitation with ammonium sulfate heightens the concentration of the substance making up the major electrophoretic peak at the expense of the few remaining components. The scheme provides exceedingly active concentrates having from 10 - 20 times the activity of the original solutions and specific activities up to four times as high as the original takadiastase.

The final precipitation of the enzyme with ammonium sulfate, above, of course means that if crystallization could be induced in the precipitate that the product would be grossly contaminated. It is suggested that the precipitate might be dialysed to free it from inorganic salts followed by a second acetone fractionation between 50 per cent and 60 per cent concentrations. The final precipitate dissolved in a minimum amount of water and adjusted to pH 5.5 might indeed be induced to crystallize in pure form.

The work with paper chromatography, while of a preliminary nature, suggests several interesting possibilities. The ability to vary the mobility of the amylase on paper with different solvents offers a qualitative test which may prove valuable as a means of characterization of the enzyme. Further, since on paper the enzyme may clearly be separated from other ninhydrin positive materials the use of filter paper pulp in column form is suggested as a medium for selective adsorption of the amylase.

If the paper chromatograms could be adapted to the qualitative and/or quantitative determination of fungal maltase and limit dextrinase an aid of inestimable value to the investigator would be available. Use of paper chromatography could permit relatively rapid determination with the added advantage of a permanent record.

VI. SUMMARY AND CONCLUSIONS

1. The alpha-amylase of takadiastase can be markedly concentrated by repeated precipitation with 0.75 saturated ammonium sulfate and adsorption of precipitates by bentonite. The highest specific activity obtained was 145 U/mg. N.

2. Ammonium sulfate at 0.70 saturation was shown to be sufficient to precipitate the alpha-amylase of takadiastase.

3. Adsorption of alpha-amylase and limit dextrinase by rice starch and bentonite proceed in a roughly equivalent manner. These adsorbents, therefore, do not lend themselves to batchwise separations of these two enzymes.

4. The precipitation of alpha-amylase by acetone is the method of choice because of the high solubility gradient occuring in solutions containing 50 to 60 per cent of the organic solvent.

5. The purification of alpha-amylase from takadiastase by precipitation from 60 per cent acetone solution followed by bentonite adsorption at pH 4.2 and final precipitation with ammonium sulfate at 0.75 saturation yields a product which when dissolved in a minimum quantity of water has an activity of 1260 U/ml. and a specific activity of 170 U/mg. N.

6. Electrophoresis showed that the product of the acetone-bentonite-ammonium sulfate purification consisted of a major component making up 72 per cent of the total material and two or more components in very small concentration.

7. Filter paper chromatography using aqueous-organic developing solvents proved capable of separating the alpha-amylase of takadiastase from other ninhydrin positive impurities present in the starting material. The enzyme purified by the acetone-bentonite-ammonium sulfate scheme gave no evidence of retaining these impurities.

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VIII. APPENDIX

The following procedures have resulted in the successful crystallization of amylases by various investigators during recent years.

A. Crystallization of Alpha-amylase from Saliva

The following procedure was developed by Meyer, Fischer, Staub, and Bernfeld (47). All steps were carried out at 0 to 4°C.

1. To 1500 ml. of centrifuged saliva, 1380 ml. of acetone (40 per cent) was added over a 40 minute period with stirring. The precipitate was rejected. To the solution (pH 6.3) was added 2120 ml. of acetone (70 per cent); after centrifugation the precipitate was dissolved in 350 ml. of water.

2. To the solution was added 2 g. of sodium acetate and 350 ml. of acetone (50 per cent) in 20 minutes. The precipitate was rejected. A volume of 470 ml. of acetone (70 per cent) was added and the precipitate obtained dissolved in 250 ml. of water.

3. The solution was adjusted to pH 8.0 with 0.1 N ammonium hydroxide and 209 ml. of saturated ammonium sulfate solution (pH 8.0) (0.455 saturation) was added, stirred for 30 minutes and centrifuged. The precipitate was dissolved in 80 ml. of water.

4. The solution at pH 8.0 was adjusted to 0.40 saturation with ammonium sulfate solution, stirred for 30 minutes and centrifuged. The precipitate was dissolved in 40 ml. of water.

5. The solution was adjusted to pH 7.0 with 0.1 N ammonium hydroxide

and 43 ml. of acetone (52 per cent) was added in 15 minutes. The precipitate was rejected and to the supernatant 1 g. of sodium acetate and 50 ml. of acetone (70 per cent) was added in 15 minutes. The precipitate was taken up in 15 ml. of water.

6. The solution was treated three times with 3 g. of Amberlite IR-4B which had been regenerated with sodium acetate. The final volume was 28 ml.

7. Sixty-five ml. of acetone (70 per cent) was added to the solution over a 10 minute period, the solution centrifuged and the precipitate redissolved in 2 ml. of 0.1 N ammonium hydroxide.

8. Cooling the solution in the refrigerator for 24 - 28 hours gave 60 to 80 per cent of the active material in crystalline form.

B. Purification and Crystallization of Human Pancreatic Amylase

Meyer, Fischer, Bernfeld, and Duckert (45) published the following procedure in 1948, the same year in which Meyer, et al., announced the crystallization of alpha-amylase from saliva.

1. Human pancreatic tissue was finely minced and defatted first with acetone then with ether and finally dried in vacuo. All operations were carried out at 0 to 2°C.

2. The powder was extracted with 15 times its weight of 0.5 M sodium acetate for 48 hours.

3. The solution was fractionally precipitated with acetone. The precipitate obtained between 46 and 69 per cent acetone was retained.

4. The precipitate was dissolved and again fractionated with acetone between the same two concentrations.

5. The precipitate was dissolved and reprecipitated at 42.5 per cent saturation with saturated ammonium sulfate solution at pH 8.0.

6. A solution of the enzyme was reprecipitated by ammonium sulfate at 0.25 saturation.

7. The dissolved precipitate was treated three times with Amberlite IR-4B ion exchange resin to replace sulfate with acetate.

8. The solution was shaken twice with a mixture of chloroform and amyl alcohol and the coagulated protein discarded.

9. The enzyme was precipitated with acetone up to 70 per cent concentration.

10. The precipitate was dissolved in the least possible volume of 0.01 N ammonium hydroxide and the solution adjusted to pH 6.4 - 6.6 with 1 N acetic acid. After standing one week 75 per cent of the enzyme deposited in crystalline form. Recrystallization was achieved by repeating the process.

C. Crystallization of Alpha-amylase from Hog Pancreas

The following method was reported by Meyer, Fischer and Bernfeld (44) in 1947.

1. Sixty grams of minced hog pancreas was shaken slowly for 48 hours with 900 ml. of 0.5 N sodium acetate at pH 7.2 in the presence of a few drops of decyl alcohol, to prevent foaming, and a small amount of

toluene. The suspension was centrifuged for 75 minutes at 3000 rpm. and the precipitate discarded.

2. The 800 ml. of crude extract was diluted with 800 ml. of water, chilled in ice and 1670 ml. of acetone (50.5 per cent) added slowly with stirring. The precipitate was discarded and an additional 1430 ml. (64 per cent) of acetone was added slowly.

3. The active precipitate was dissolved in 800 ml. water and 860 ml. (51 per cent) of acetone added. The precipitate was discarded and an additional 1140 ml. (69 per cent) of acetone added.

4. The precipitate was dissolved in 200 ml. of water and to it was added 88 ml. of saturated ammonium sulfate solution (0.325 saturation) and the solution was centrifuged 30 minutes.

5. The precipitate was dissolved in 100 ml. of water and saturated ammonium sulfate solution added to bring the concentration to 0.225 saturation. The solution was centrifuged, the precipitate dissolved in a minimum of water, 25 ml. of boiled enzyme solution (a portion of solution from step 3 boiled and filtered) added and the whole diluted to 50 ml.

6. The solution was adjusted to pH 7.9 with 0.1 N ammonium hydroxide and acetone at pH 8.3 added to 55 per cent concentration. The precipitate was rejected. Acetone was added to a final concentration of 65 per cent and the solution centrifuged. The precipitate was dissolved in a small volume of water, 20 ml. of boiled enzyme added and the volume made up to 50 ml.

7. The solution was shaken vigorously for 10 minutes with 10 ml.

chloroform and 2.5 ml. of amyl alcohol, centrifuged, and the solution decanted. This step was repeated 5 to 6 times.

8. The solution was shaken for 45 minutes with 2.5 g. Wolfatite. The liquid was decanted and the operation repeated.

9. Acetone was added slowly; at the appearance of cloudiness (50 - 55 per cent acetone) the solution was centrifuged. The precipitate was discarded and acetone was added to 70 per cent. Following centrifugation the precipitate was dissolved in water and lyophylized.

10. The dried product was dissolved in 20 times its weight of water, centrifuged and the solution at pH 7.0 adjusted to 50 per cent acetone. After 3 to 4 hours an abundant quantity of crystals formed. They were slightly soluble in water and saline, and were coagulated by heat.

D. Crystallization of Bacterial Alpha-amylase

The method given below was reported by Meyer, Field and Bernfeld (41) in 1947 and is without further amplification in the literature.

1. The starting material was "Biolase," a product of Kalle and Co. of Wiesbaden, Germany and is prepared from Bacillus subtilis or B. mesentericus.

2. The initial purification was made by means of four precipitations with salts and organic solvents at predetermined temperatures and hydrogen ion concentrations. (details not given).

3. The last precipitate obtained was dissolved in a minimum volume of water and chilled for 24 hours. The clear colorless crystals which

formed could be recrystallized by solution in dilute ammonium hydroxide (pH 7.8) followed by reduction of pH to 5.6 with acetic acid. Crystals formed readily in the cold.

E. Crystallization of Alpha-amylase of Aspergillus oryzae

The method given below was published by Fischer and de Montmollin (20) in October, 1951, after the completion of the work reported in this thesis.

1. An aqueous 1:6 suspension of "Clarase 900," a product of Takamine Laboratories, Clifton, N. J., was left for 12 hours in the cold and centrifuged.

2. The dark brown liquor was 0.8 saturated with solid ammonium sulfate and the precipitate obtained by filtration on Filter-Cel.

3. The precipitate was dissolved, the solution adjusted to pH 6.0 and fractionated by acetone, the 40-58 per cent acetone fraction being retained.

4. The acetone precipitate was dissolved in a small amount of water and this solution at pH 7.2 brought to 0.75 saturation with sodium chloride and then fractionated between 0.54 and 0.70 saturation with a saturated solution of ammonium sulfate.

5. The fractionation was repeated twice under approximately the same conditions. The last precipitate was then dissolved in the minimum quantity of water, and the slightly brown solution, adjusted to pH 7.2, left in the cold. Crystals appeared after two or three days, and, after

a week, at least 50 per cent of the activity was found in the crystalline material.

F. Isolation and Properties of Crystalline Alpha-amylase from Germinated Barley

The procedure below was developed by Schwimmer and Balls (65).

1. Two liters of commercial malt syrup was heated to 70°C for 15 minutes and then filtered on a Buchner funnel with 60 g. of Celite. The residue was washed with calcium sulfate solution until the volume was again 2 liters.

2. The filtrate was 0.43 to 0.50 saturated with ammonium sulfate and the pH adjusted to 5.6 to 6.0. After 2 hours at room temperature nearly all of the alpha-amylase had precipitated; it was filtered onto a fluted paper and washed with 5 or 6 times its volume of 0.33 saturated ammonium sulfate (pH 5.6 to 6.0) containing 2 g. calcium sulfate per liter.

3. The washed precipitate was taken up in 50 ml. of water, then an equal volume of cold 80 per cent ethanol containing 5 g. of calcium chloride per liter was added. The insoluble material was filtered out with a little Celite and washed with diluted alcoholic calcium solution (40 per cent alcohol) until the total volume of filtrate and washings was 200 ml. This was kept for 30 to 60 minutes at 5°C. and filtered again if a precipitate formed. It was poured onto a well packed column of starch and Celite and sucked through. The column was kept at room temperature. The column was next washed with 40 per cent alcohol until the outflow was colorless. Elution was made by pouring 50 ml. portions

of water saturated with calcium sulfate through the column at room temperature. Each portion of eluate was kept separate until assayed and only those containing much enzyme were combined and used.

4. The enzyme was precipitated from the combined eluates by 0.66 saturation with ammonium sulfate (pH 6.0). The precipitate was collected on a little Celite on a small Buchner funnel and washed thereon with about 20 ml. of half saturated ammonium sulfate (pH 6.0). It was then dissolved in as small a volume as convenient (15 to 20 ml.) of half saturated calcium sulfate. The pH of the solution was adjusted to 5.9 to 6.0 with 0.1 N ammonia, and saturated ammonium sulfate solution (pH 6.0) was added slowly up to 0.26 saturation. When placed at 30 to 33°C., crystals of the enzyme appeared in 1 to 3 hours. Later ammonium sulfate was added to 0.33 saturation, and after standing overnight in the incubator the suspension was centrifuged.

5. For recrystallization the sedimented crystals were taken up in 15 ml. of cold half saturated calcium sulfate solutions and crystallization carried out as above. Crystal formation was not observed below 25°C or below pH 5.6.

G. Beta-amylase from Malt

This procedure, reported by Meyer, Fischer, and Piguet (46) is the first instance of the application of these general procedures to an amylolytic agent other than alpha-amylase.

1. The starting material was "Diastafor" a commercial malt extract prepared by Wander of Berne, Switzerland. All steps were carried out

at 0 to 5°C.

2. One kg. of "Diastafor" was suspended in 2 l. of distilled water to give 2700 ml. The solution was adjusted to pH 3.6 with 250 ml. of 8 N acetic acid with constant agitation. The precipitate formed during 30 minutes of stirring was inactivated alpha-amylase.

3. The addition of 300 ml. of 4 N ammonium hydroxide raised the pH to 4.5 saturated ammonium sulfate was added to 0.60 saturation. The solution was centrifuged in the Sharples supercentrifuge at 50,000 rpm. The precipitate was taken up in distilled water, filtered through Filter-Cel and made up to 600 ml. at pH 5.0 and 0.1 N ammonium hydroxide.

4. To the solution 360 ml. of acetone (37 per cent) was added in 10 minutes, the precipitate discarded and an additional 590 ml. of acetone added (60 per cent). The precipitate was dissolved in 400 ml. of distilled water at pH 5.0.

5. To the solution was added 275 ml. of acetone (40 per cent) and the precipitate which formed in 20 minutes discarded. An additional 145 ml. of acetone (50 per cent) was added, the solution centrifuged and the precipitate taken up in 150 ml. of water.

6. Four ml. of 4 N sodium acetate solution (pH 7.8) was added, the solution diluted to 200 ml. and 0.1 N ammonium hydroxide added to bring the pH to 7.8. The precipitate obtained upon the addition of 217 ml. of acetone (51 per cent) was discarded and 50 ml. of acetone (56 per cent) added to the supernatant. The precipitate which formed was dissolved in 15 ml. of water.

7. The solution was 0.24 saturated with solid ammonium sulfate,

then concentrated with moderate stirring over silica gel at 3°C. for 24 hours. The precipitate was both crystalline and amorphous. Solution of the precipitate in a small quantity of water followed by repetition of the ammonium sulfate precipitation led to a wholly crystalline product.

H. Crystallization of Beta-amylase

Balls, Thompson, and Walden (9) published the following brief scheme for the preparation of beta-amylase from sweet potatoes.

1. The press juice of sweet potatoes was heated to 60°C., cooled immediately, lead acetate solution added and filtered.

2. The protein in the filtrate was precipitated with ammonium sulfate at 70 per cent of saturation and then dissolved and purified by dialysis.

3. The dialyzed solution was precipitated by successive acidification to pH 4.6, 4.0 and 3.2 and finally 25 per cent saturation with ammonium sulfate at pH 3.5 to 4.0 with removal of the precipitate after each step.

4. Repeated fractionation of the remaining solution between 25 and 50 per cent saturation with ammonium sulfate at pH 4.0 finally yielded crystals on cautious addition of ammonium sulfate in the cold.

5. Recrystallization was carried out in the same manner to yield tetragonal prisms.