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BIOSPECIFIC RECOVERY AND PURIFICATION
OF BETA AMYLASE FROM FERMENTATION BROTHS

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ELANKOVAN PONNAMPALAM

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Kris A. Berglund
Major professor

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BIOSPECIFIC RECOVERY AND PURIFICATION OF β-AMYLASE FROM FERMENTATION BROTHS

By

Elankovan Ponnampalam

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Abstract

Biospecific Recovery and Purification of β-amylase

from Fermentation Broths

By

Elankovan Ponnampalam

A thermostable extracellular enzyme, β-amylase is produced from Clostridium thermosulfurogenes with different substrates (carbon sources). Neither the size of the fermenter nor the substrates influenced the total fermentation time or the final β-amylase production. β-amylase produced with maltrin yields results in activity recovery than β-amylase produced with maltose with 100,000 molecular weight cut off (mwco) ultrafiltration membrane. Use of a larger mwco cut off UF membrane will reduce the process time and cost significantly.

Cell free concentrated β-amylase was further purified by ammonium sulfate and ethanol precipitation. The effects of substrate, temperature, and precipitant concentration on the β-amylase recovery process were investigated and compared. β-amylase was found to form a soluble complex with starch dextrins in the fermentation broth, which could be precipitated more easily by ethanol than ammonium sulfate. Light scattering studies revealed that the particle size of the precipitated complex was significantly larger for ethanol as compared to ammonium sulfate precipitation. Therefore, recovery of β-amylase was directly related to the particle size.

To confirm these results β-amylase was further purified by gel filtration and the molecular weight and the amino acid sequence were determined. A single band or single peak was obtained with sodium dodecyl sulfate polyacrylamide gel electrophoresis or HPLC gel filtration respectively, for β-amylase produced with maltose. Two different molecular weight fractions were obtained for maltrin fermented β-amylase by gel filtration. These were confirmed by obtaining two different single peaks with HPLC gel filtration. SDS-PAGE showed only a single band for both of these fractions in denatured form. These results further support the complexation of β-amylase subunits with starch dextrins. The complexation is the apparent cause of the higher effective molecular weight β-amylase. Amino acid sequence studies did not show any difference in the β-amylase produced with different substrates.

In a different study a combination of ethanol and ammonium sulfate precipitation was used to produce a highly purified β-amylase. This is very simple technique to purify proteins holds promise for a wide variety of proteins.

In summary, concentration and purification of β-amylase produced with different substrates was compared. The substrate forms a biospecific complex or helps to form a tetramer with β-amylase produced by fermenting starch dextrins (maltrin). This complex has an apparent larger molecular weight which could be easily purified by ultrafiltration and precipitation. This biospecific complexation technique could be used to purify similar types of protein.

DEDICATION

This work is dedicated to my loving wife Sugendrini Ponnampalam who has been a great source of inspiration and encouragement through my academic career.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Processing of biological materials and processing using biological agents such as cells, enzymes, or antibodies are the central domain of biochemical engineering. Success in biochemical engineering requires integrated knowledge of governing biological properties and principles and of chemical engineering methodology and strategy.

The most important step in the biochemical process industry is the downstream processing, which commands ninety percent of the overall process cost. This aspect usually demands converting a stream very dilute in final product to a stream of pure product. Downstream processing involves a series of purification steps, which each step bringing the product closer to purity. Simple and fewer purification steps yield high recovery with low cost. The main purpose of this work is to optimize the recovery process of β-amylase in large scale.

The amylase family of enzymes is a diverse group of starch degrading enzymes ubiquitous in the microbial, plant and animal kingdoms. They consist of three main

groups having exo-splitting, endo-splitting and debranching activities. β-amylase, which hydrolyses the α-1,4-glucan bonds in amylosaccharide chains from the non reducing ends and generates maltose, has great applications in food and beverage industries. Growth in biotechnology has placed high value on extreme thermostability and thermoactivity of β-amylase for the use in bioprocessing of starch (Bergmeyer, 1965 and Forgarty and Kelly, 1980). This project is focused on optimizing the production and purification of an extremely thermostable and thermoactive β-amylase produced from Clostridium thermosulfurogenes (Hyun and Zeikus, 1985). Producing β-amylase in large scale with a low price substrate and the subsequent optimum recovery is very important to realization of commercial application of this enzyme.

In this study the β-amylase was produced by fermenting different carbon sources (substrates), and its recovery was optimized by ultrafiltration, salt and solvent precipitation, and gel filtration. Simple scale-up studies were also conducted by proportionally increasing the raw materials with a constant stirring rate in the fermentor. Initial purification studies showed that the fermentation carbon source (substrate) strongly influenced the types of purification steps. For example, β-amylase produced with starch dextrins resulted in higher recovery during concentration with a 100,000 molecular weight cut off (MWCO) ultrafiltration (UF) membrane while maltose produced β-amylase showed less recovery with the same membrane. Therefore, initial concentration of β-amylase by UF with different size MWCO membranes for different substrates was extensively studied and compared. The recovery of enzymetic activity was also optimized with respect to the temperature, concentration of the precipitant, and fermentation carbon source during precipitation.

Precipitation studies were conducted in continuously stirred batch reactors and mean particle size and turbidity (absorbance) were obtained for β-amylase precipitates with ethanol and ammonium sulfate. A comparison showed that β-amylase produced with maltrin resulted in a larger mean particle size, (i.e. larger molecular weight complex) with ethanol. To further understand this complex formation, β-amylase with different carbon sources was purified by gel filtration and the effective molecular weight was estimated by sodium dodecyl sulfate slab gel electrophoresis and HPLC protein 300 SW pak gel filtration column. Fermentation and the three major purification steps used in this study are shown in figure 1.1. This study showed that purification steps are highly integrated with fermentation and the fermentation carbon source dictates the type of purification steps needed to recover β-amylase.

To complete the study, β-amylase was precipitated with the combination of ethanol and ammonium sulfate. β-amylase recovery was optimized with different concentrations of ethanol and ammonium sulfate in continuously stirred batch reactors. The combination studies were noteworthy because of their high purification fold with considerable activity recovery. Using small amounts of salt will decrease the salt disposal problem greatly. The combination of organic solvent and salt technique could be used for other purification studies.

BIOSPECIFIC RECOVERY AND PURIFICATION OF β-AMYLASE FROM FERMENTATION BROTHS

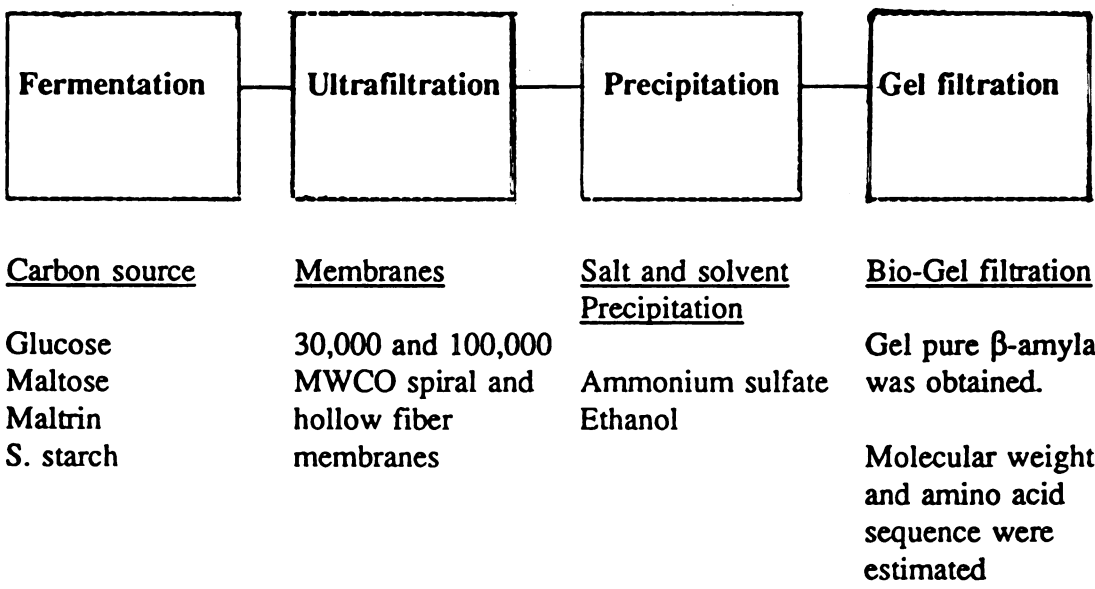

Figure 1.1 Flow diagram for recovery and purification of β-amylase from fermentation broths

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CHAPTER 2

LITERATURE SURVEY

2.1 Background:

In fermentation broths or cell culture supernatants, the product constitutes only a very small fraction of the total fermentation broth; therefore, the recovery requires extensive purification procedures. These procedures are called downstream purification and represents a large part of the product cost. In some cases, the down-stream separation cost is the major manufacturing cost of the final product.

A series of purification steps is used in a protein product recovery, the choicing which depends on the original contaminants and impurities, the stability of the biological product, and the required purity level of the end product. Each step removes some of the impurities and brings the product closer to the purity level. The specification of product purity level is a trade off between the product yield and purification cost. (Yield refers to that part of the active material initially present that is still present in the final product. Purity refers to the ratio of active substance to total material.) The final product purity can be increased through additional purification steps, but the yield will usually decrease. The improvement in one can occur only at the expense of the other (Gareil and Rosset, 1982). The optimum downstream process is the purification strategy that satisfies the quality requirement while minimizing the product cost.

A study was conducted to analyze the methods of purification used in successive steps in the purification schemes by Bonnerjea et al (1986). They analyzed one hundred papers published during 1984 in eight different journals with the following results:

1. More than half (57 percent) of the purification schemes used a precipitation step; more than three quarters of these used ammonium sulfate as a precipitant.
2. Ion-exchange chromatography and affinity chromatography were the most common purification procedures. Gel filtration was also included as a purification step.
3. On the average, four purification steps were necessary to purify the proteins to homogeneity with an overall yield of 28% and a purification factor of 6380.

In general, purification steps are performed in the following order, precipitation, ion-exchange chromatography, affinity separation, and gel filtration. Precipitation, which has the capability of handling larger quantities of materials than adsorption and chromatography, is used as a first step in purification. Since materials for affinity separation are expensive, it is better to use ion-exchange materials after the precipitation to reduce protein loads and to remove remaining fouling substances. Gel filtration has the least capacity for loaded proteins, but it serves as an important factor in removing the self aggregates of otherwise purified proteins. On the whole, precipitation methods have the highest average yield (81 percent) while affinity methods have the lowest average yield (61 percent). All the other purification techniques have average yields of 65-75 percent. Yields tend to be higher in processes with few overall steps.

2.1.1 SOURCES AND ISOLATION OF ENZYMES

Enzymes are obtainable from plant tissues, animal tissues and microbial organisms. Considerable quantities of enzymes are produced on a commercial scale from plant and animal sources, but for both technical and economic reasons microbial enzymes have become increasingly important. Large quantities of plant materials are required for production of small amounts of enzymes, and the types of enzymes recovered are also limited. Animal enzymes are by-products of the meat industry. Limited supply and other uses of meat narrow down the enzyme yielding tissues. On the other hand, the productive capacity for the microbial enzymes which are not subject to any other production or supply limitation, may be expanded without any limit to meet demand. Furthermore, the number of enzymes available from microorganisms are almost unlimited. Therefore, development of production methods for microbial enzymes has not only assured potentially unlimited supplies, but also made available enzyme systems which cannot be readily obtained from plant and animal sources.

Enzymes of similar types from different sources differ widely in their quantities and catalytic activity, although they catalyze the same reaction. A single enzyme rarely constitutes more than one percent of the source material, and much of the enzyme may be lost during purification. Enzyme are produced by living cells to bring about specific biochemical reactions involved in the metabolic and the digestic processes of the cells. Depending on the enzyme and its purpose, the enzyme may be secreted from the cell or retained within the cell. The important commercial enzymes are extracellular enzymes which are produced within the cells but excreted into the

medium. (The enzyme purified in the current project is such an extracellular enzyme secreted from Clostridium thermosulfurogenes). The majority of metabolic enzymes are found only intracellularly as bound or free within the cell. The isolation of extracellular enzymes is in general simpler than that of intracellular enzymes. The desired enzyme within the cell not only has properties much like many other extracellular enzymes but also contain a number of contaminants.

In many microorganisms the intracellular enzymes are protected by extremely tough cell walls. Nevertheless, in the last few years due to developments in genetics and gene cloning, several intracellular enzymes have begun to be produced industrially. Although the overall isolation processes for intracellular and extracellular enzymes are rather different, some of the operations are similar.

2.1.2 STARCH - HYDROLYSIS INDUSTRY

Starch, a glucose polymer, is naturally produced and one of the most widely available polysaccharides. It can be converted to glucose by either acid or enzyme hydrolysis. The advantage of starch hydrolysis over direct sugar production is that the initial materials used, such as wheat, corn, cassava, and potatoes are cheap and available in large quantities. Amylases and amyloglucosidases are used to hydrolyze starch in food and commercial industries, with the development of thermostable and thermoactive microbial amylases creating a new era in the food industry. These microbial enzymes are produced in large quantities and used in most industrial applications with a minimal purification. In 1979 alone, 300 tons of amylase was produced and marketed for about $8 million dollars (Aunstrup et al, 1979).

One of the main uses of amylases is in starch saccharification for the production of sweeteners such as glucose, fructose and maltose syrups. Amylases are used to hydrolyze starch initially into short-chain polymers (dextrins), then disaccharide (maltose) and finally glucose. Since glucose is not as sweet as its isomer fructose, glucose is converted into fructose using the enzyme glucose isomerase. The most important enzymes in the starch-saccharification process are α-amylases, β-amylases, glucoamylases, glucose isomerases, pullulanases, and iso-amylases (Crueger and Crueger, 1982). This study is focused on the purification of a thermostable and thermoactive enzyme β-amylase which could potiontially enter this class of industrial enzymes.

2.1.3 β-amylase

β-amylase [(1,4)-α-D-glucan maltohydrolase, EC 3.2.1.2] (1980)) is an exoacting enzyme which hydrolyses the α-1,4-glucosidic linkages from the non-reducing end of polysaccharides (amylose, amylopectin, glycogen, and dextrins) and produces both β-maltose and β-limited dextrins (Fogarty and Kelly (1979 and 1980) and Robyt and Whelan (1968)). In contrast to the action of α-amylase, only the penultimate bond from the non-reducing end group of the substrate molecule is selected for cleavage by β-amylase. Thus, one molecule of maltose after the other is detached from the substrate until the enzyme encounters a branching point. Accordingly, amylose is completely converted into maltose and amylopectin is converted into 60% of maltose and 40% of a higher molecular weight limit dextrin when the reaction is carried out properly (Bernfeld, 1955).

β-amylase is produced from the tissue of higher plants such as sweet potatoes (Balls et al, 1946), soybean (Fukumoto and Tsujisaka, 1954), wheat (Meyer et al, 1953), barley (Nummi et al, 1965), and sorghum (Okon and Uwaifo, 1984). It is also produced by microorganisms such as Bacillus megaterium (Higashihara and Okada, 1974), Bacillus polymyxa (Fogarty and Griffin, 1975), and Bacillus cereus var. mycoides (Takasaki, 1976a). These β-amylases have been classified according to their thermostability, pH optimum, and starch liquefying and/or saccharogenic effect (Crueger and Crueger, 1982).

The extreme thermostability and thermoactivity of amylases used in the bioprocessing of starch provides great potential. However, the above mentioned microbial β-amylases are not active or thermostable enough to substitute for the indigenous plant enzymes. It has been suggested that thermoanaerobes have application potential for the production of active and thermostable amylases from starch (Zeikus, 1979).

Only a few enzymes with higher activity and thermostability have been isolated and characterized from thermoanaerobes. They are endoglucanase of Clostridium thermocellum (Ng and Zeikus, 1981), alcohol dehydrogenase of Thermoanaerobium brockii and C. thermohydrosulfuricum (Lamed and Zeikus, 1981), and the polygalacturonase hydrolyze of C. thermosulfurogenes (Schink and Zeikus, 1983). These thermoanaerobic bacteria can produce faster metabolic rates and more thermostable enzymes than mesophilic micro-organisms (Zeikus, 1979). A thermoactive and thermostable β-amylase is also produced from C. thermosulfurogenes at the Michigan Biotechnological Institute. During the study of the β-amylase

synthesis in C. thermosulfurogenes a hyper productive mutant that produces eight fold more enzyme than the wild type was obtained (Hyun and Zeikus, 1985). The β-amylase isolated from C. thermosulfurogenes is concentrated and purified in this project.

2.2 PROTEIN PURIFICATION

Often protein purification processes consist of several separation steps. Generally, purification proceeds from one purification step to the next, until the desired protein is purified. It is economical to perform the largest step with the greatest degree of separation between the product and impurities first and the most expensive purification step last. It is also important to characterize the starting material in detail: source of the material, major contaminants, the presence of solid bodies, and the physical characteristics of the product. Defining physical properties such as thermal stability, isoelectric point, molecular weight, hydrophobicity, density and specific binding properties of the product are helpful in exploring all the options available for separation. All the separation procedures used in this project are discussed in detail later.

2.2.1 PURIFICATION BY DIFFERENTIAL SOLUBILITY

The most commonly used methods for purifying enzymes depend on separating active enzymes from other proteins and soluble substances by precipitation of one or the other. Precipitation is an operation in which a reagent is added to protein solution which causes the formation of insoluble particles of proteins.

In most cases, the intention is to recover the desired protein in either an unchanged molecular form or one which is readily returned to that native form. There are some exceptions such as in the food industry in which alteration of the enzyme structure is advantageous. There are some other industrially important ways to form insoluble proteins in solution. Heating may be employed either to coagulate the protein or to bring about drying.

The most important single characteristic of a protein is the arrangement of the backbone structure. This depends on the amino acid composition. Other charged groups, such as phosphate esters in lipids or coenzyme prosthetic groups, also have an effect.

The solubility of a protein is largely determined by its polypeptide structure. For example, proteins with relatively small non-peptide groups such as lipo-, nucleo-, and glycoproteins often exhibit distinctive solubility properties. Thus glycoproteins are very soluble in aqueous solution due to the hydration of the carbohydrate moiety. However, lipoproteins are relatively insoluble in aqueous solution due to their lipid component's hydrophobic nature.

The structure of proteins is an important factor in appreciating the function of precipitant. The polypeptide chain of water soluble proteins are folded in such a way that the majority of polar hydrophilic amino acid side-chain groups will lie on the exterior and the hydrophobic moieties are buried. However, this division is not so well defined that changes in the exterior environment brought about by the precipitant do not affect both types of groups as well as the backbone. The overall effect on the

protein results from the sum of individual effects which will often be opposed by one another. Thus, the resulting protein conformation may either increase, decrease or leave the solubility unaltered depending upon the moieties contained with in the protein. In addition, protein solubility largely depends on its structure and species present. Solubility may also be affected by changing the solvent composition.

Solubility effects may be explained by the Debye Huckel theory. Qualitatively, the protein in the solid phase may be pictured as held together by the columbic forces between opposite charges on adjacent molecules.

$$F = \frac{e\,e'}{Dr^2} \qquad (2.1)$$

where: F - columbic force
e,e' - opposing charges
r - distance
D - dielectric constant of the solvent

For example, the precipitating action of organic solvents is explained in which, by lowering the dielectric constant of the solvent, increases the attractive forces between the molecule.

Ions also affect protein solubility. In small amounts they act to shield the protein molecules from each other by coming between the opposing charges. They can increase the protein solubility ("salting in"). However, in high concentrations, salts decrease protein solubility. This behavior, called "salting out", is common to all nonelectrolyte solubles and appears to be due to "dehydration" of the protein molecule.

2.2.1.1 SALTING-OUT

As stated earlier, the precipitation of proteins by high concentration of neutral salts is called "salting out". This is one of the oldest and most widely used methods of recovering and/or fractionating proteins. The most frequently used salts are ammonium and sodium sulfate and potassium and sodium phosphates. Ammonium sulfate is inexpensive and highly soluble which permits the salting-out of practically any protein. Ammonium sulfate has minimal harmful effects on enzyme activity. Being the salt of a strong acid and weak base it does tend to become acid by hydrolysis and the release of ammonia at higher pHs is inconvenient on an industrial scale. Ammonium sulfate is corrosive material and is difficult to handle and dispose. Residues of it remaining in food products can be tasted at low level, and it is toxic with respect to clinical use so that it must removed. In this study ammonium sulfate is used and compared with organic solvent precipitation.

The solubility (s) of a protein depends on ionic strength (I) according to the equation

$$\log S = B - KI \qquad (2.2.)$$

where B and K are constants. The constant B varies markedly with the protein but is essentially independent of the salt: B is strongly dependent on pH and temperature, usually passing through a minimum at the isoelectric point. K is independent of pH and temperature but varies with the protein concentration and the salt used (Charm and Matteo, 1971). Most proteins are more soluble in concentrated salt solution at low temperatures and in dilute salt concentrations at higher temperatures. At a specified

pH, temperature and total protein concentration, the concentration of salt required to precipitate the enzyme varried with the concentration of enzyme.

According to a theoretical treatment by Melander and Horvath (1977) salting-out of proteins may be described as a balance between a salting-in process due to electrostatic effects of the salt and a salting-out process due to hydrophobic effects. Experimental procedures of salting-out are not complicated. There are three ways to add the salt; (1) add the solid salt; (2) add a saturated solution of the salt; and (3) dialyze the salt through cellulose membrane. All these procedures are more effective if carried out slowly with constant stirring so as to avoid localized zones of higher concentration. Sometimes it is necessary to permit the material to stand several hours or days for complete precipitation. The supernatant is usually removed by filtration or centrifugation.

The use and problems which can be encountered during protein precipitation by the salting-out method have been discussed by Charm and Matteo (1971), Melling and Phillips (1975), Wang et al. (1979), and Swimmer (1981).

2.2.1.2 ORGANIC SOLVENT PRECIPITATION

The formation of precipitate by the addition of a weak polar solvent to an aqueous solution of a protein is called organic solvent precipitation. Addition of an organic solvent will lower the dielectric constant of the protein medium from its isoelectric point. The protein solubility decreases due to the increase in effectiveness of intra and intermolecular attractions. At low ionic strengths the addition of increasing amounts of organic solvents continuously depresses the solubility of the

protein at constant pH and ionic strength. In this way very soluble proteins may be made sufficiently insoluble to precipitate.

The solubility of a particular protein in an organic solvent also varies with temperature, total protein concentration, pH, ionic strength of the medium and the presence of multivalent cations (Kaufman, 1971). The solubility of most proteins in organic solvent-water mixture decreases as the temperature is decreased. The solubility of protein in an organic solvent-water mixture is minimized at its isoelectric points. Addition of salt to an organic solvent increases the solubility of the protein. This frequently makes it possible to adjust the solubility of a protein to a convenient value at a variety of hydrogen ion concentrations by balancing the solvent action of salt with the precipitating action of the organic solvent. This balance is different at constant ethanol and salt concentration for each pH and temperature, and for each protein component (Cohn et al. 1946). The protein may usually be precipitated at one half or one third the concentration of organic solvent if a suitable multivalent cation (Zn^{2+}, Mn^{2+} etc.) is added before the organic solvent is added. This allows organic solvent precipitation at lower solvent concentrations reducing protein damage.

Various organic solvents can be used in protein purification with methanol, ethanol, isopropyl alcohol, ether, and acetone the most commonly used. Ethanol has been the most common and widely used solvent due to its acceptability in the food and pharmaceutical industry. However, methanol and acetone have also proved valuable and might seem inherently better than ethanol. Methanol has less denaturing action on plasma protein than ethanol, and acetone is used for making dried protein preparations (acetone powder).

Organic solvents' relatively high volatility and bactericidal effect are also advantageous for easy recovery and media sterility. The main disadvantages of using organic solvents are flammability, denaturing effect, and government tax on ethanol. On the whole, organic solvents can provide an additional variable in the precipitation of protein.

2.2.1.3 ISOELECTRIC PRECIPITATION

The isoelectric precipitation of proteins depends on two factors: (1) All proteins exhibit minimum solubility, in solutions of constant ionic strength, at their isoelectric points; and (2) At low or zero concentrations of electrolyte, some proteins are sufficiently insoluble to form a precipitate. The isoelectric point of a protein solution can be reached by adjusting (with acids or bases) the pH to the point where the protein has no net charge.

Isoelectric precipitation is enhanced for proteins of low hydration constant or high surface hydrophobicity (Bell et al. 1983). Considering the Cohn equation (2.1), the constant B for a particular protein is pH dependent, reaching a minimum at or near the isoelectric point of the protein. Changes of solubility well in excess of an order of magnitude per pH unit in this region are common. Two proteins having the same isoelectric point but different solubility can be separated this way. The less soluble protein is removed at an electrolyte concentration just low enough to precipitate most of it, then the electrolyte concentration is lowered by dialysis or by dilution to precipitate the other protein. Another major advantage of isoelectric precipitation is the low cost of mineral acids and their acceptance in protein food products. The

major disadvantage of isoelectric precipitation is the potential denaturation of proteins at pH extremes.

2.2.1.4 PRECIPITATION BY METAL IONS

The combined use of multivalent cations with organic solvents has been discussed earlier in this chapter. Polyvalent metal ions are very effective in precipitating proteins (Morris and Morris, 1976). They are classified into three major groups according to the site to which they selectively bind. Ions such as Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} bind strongly to carboxylic acids and to nitrogenous compounds such as amines and heterocyclics. The second group of ions Ca^{2+}, Ba^{2+}, Mg^{2+}, and Pb^{2+} only binds to carboxylic acids, but not significantly to nitrogenous ligands. The final group, including Ag^{+}, Hg^{+} and Pb^{2+}, binds strongly to sulphydryl groups. Metal ions have greater precipitating power at dilute solutions and can be removed from solutions by ion exchange or chelating.

2.2.1.5 THERMAL PRECIPITATION

Since the constant B in the Cohn equation (Eqn. 2.1) is temperature dependent, solubility change may be brought by varying the temperature at constant ionic strength. Generally, protein solubility increases with temperature elevation. If a desired enzyme is thermostable at high temperature, much of the inert protein material may be denatured, precipitated, and removed without destruction of the desired enzyme by carefully heating the solution. This is very inexpensive and often the first step in many thermostable enzyme purifications. Sometimes it is advantageous to add a substance (salt, cofactor, substrates, and their analogs) which is known to protect or

stabilize the enzyme activity. The effectiveness of this method is attested to by its widespread use in purification of enzymes, including alpha amylase, adenylate kinase (Swimmer, 1981) and several of low volume high cost clinical diagnostic enzymes (Momsen and Brockman, 1977).

2.2.1.6 POLYELECTROLYTES AND PROTEIN PRECIPITATION

The recovery of proteins through insoluble complexes with polyelectrolytes seems to be a very attractive and practical approach. Compared to other methods used for large scale protein separation, polyelectrolytes are effective in low concentrations and do not pose problems with waste disposal or reagent recovery. Many laboratory and industrial works have been reported concerning general solubility behavior in the polyelectrolyte precipitation of proteins. When a protein solution is destabilized by the addition of polyelectrolyte, the protein and polymer combine by diffusion to form primary particles, then fluid driven particle-particle collisions lead to the formation and growth of aggregation.

Polyacrylic acid (PAA) is a widely used polyelectrolyte in protein precipitation. Polyacrylic acid is a linear polymer of weakly acidic carboxylic monomers. Sternberg et al. (1974 and 1976) has found that polyacrylic acid is well-suited to the large scale recovery of industrial enzymes without denaturation.

Purification and recovery of industrial enzymes by using polyacrylic acid is accomplished by using the procedure outlined below:

1. Polyacrylic acid is added to a crude enzyme solution in which pH is adjusted to 3.0-5.8. A precipitate forms between some of the proteins and the polyacrylic acid. Depending on chemical reactivity and conditions of the enzyme, the desired enzyme may end up in precipitate or stay in solution. If the desired enzyme precipitates, it will form a protein-polyacrylate complex which can be separated by filtration or centrifugation.
2. At pH > 6.0 conditions metallic ions (Ca^{2+}, Mg^{2+}) react with protein polyacrylate and form calcium or magnesium polyacrylate salts. In the meantime the enzyme is solubilized and released from precipitate to solution. Generally, the recovery of enzyme is more than 80%.
3. Recovery of polyacrylic acid from calcium or magnesium polyacrylate can be achieved by mixing with 2N H_2SO_4. H_2SO_4 will regenerate water soluble polyacrylic acid. In some cases the regeneration of polyacrylic acid is up to 90%.

The purification is based on the selectivity of the reaction of proteins with polyacrylic acid. Formation of an insoluble protein-polyacrylate complex is dependent on the affinity of particular proteins for polyacrylic acid, the molecular weight of polyacrylic acid, and the ionic strength and pH of the solution. Another advantage with polyacrylic acid at pH <6.0 is that mono- and polysaccharides, amino acids, oligopeptides, lipids, nucleotides, nucleic acids and inorganic salts do not form precipitates with polyacrylic acid (Sternberg, 1976).

2.2.1.7 NON-IONIC POLYMER PRECIPITATION AND TWO-PHASE PARTITIONING

Relatively few reports have appeared in the literature on the precipitation of proteins by nonionic high molecular weight polymers (Janssen and Ruelius (1968), Fried and Chun (1971), Foster et al. (1973), and Albertsson (1986)). The mechanism of precipitation is not fully understood, but the phenomenon is closely related to the formation of liquid-liquid two phase systems from mixtures of aqueous polymers. The most commonly used two-phase systems are higher molecular weight polymers dextran and polyethylene glycol (PEG) (Kula (1979), Hustedt et al. (1978) and Kroner (1982)). However, other polymers including methylcellulose, polyvinyl alcohol, and ficol (Albertson, 1986) have been used to form aqueous two-phase systems. Each of the above applications is based on the powerful influence of the polymer on the interaction of the protein with its aqueous environment. The partition is based on the principle that aqueous solutions of a mixture of two neutral polymers will form two phases, and each protein in this solution will have a unique distribution coefficient.

The partition of a compound in aqueous two-phase systems is mainly influenced by molecular weight, temperature, pH, ionic strength and the types of ions included in the system but it does not depend on the concentration of the desired product (Kroner et al. (1982) and, Mattiasson and Kaul, 1986). Unfortunately, the conditions for favorable partition have to be found empirically for the enzyme of interest. The main advantage of using the two-phase dextran-PEG system in protein purifications is its non-toxicity and resulting suitability for use in pharmaceuticals and safe consumption; but the high cost limits its application.

2.2.2 CHROMATOGRAPHIC METHODS

Chromatographic enzyme purification methods are the most effective of all the separation methods. The mechanism of the separation will depend in different cases on adsorption, ion exchange, specific affinity to immobilized ligands, and molecular sieving effects. The basic principles of chromatographic methods and the use of available ion exchange, gel chromatography and affinity chromatography media have been described by Himmelhock (1971), Cuatrecases and Anfiusen (1971), Cooper (1977), Kremmer and Boross (1979), and from the manufactures of the media (Pharmacia Fine Chemicals, Bio-Rad Laboratories, LKB Produckter Ltd., Whatman Biochemicals Ltd., Amicon Corporation and others).

2.2.2.1 NON-SPECIFIC ADSORPTION CHROMATOGRAPHY

Purification is performed by using substances that adsorb the proteins by Van der Waals forces and stearic interactions. These forces are most important for substances with few polar residues. Only a few non-specific absorbents are important in protein isolation. Calcium phosphate gel, particularly in its crystalline form hydroxyapatite (HA), has been widely used on a laboratory scale.

Although the mechanism by which HA separates proteins is not fully understood, it has been suggested that calcium and phosphate ions on the surface of HA crystals participate in the interactions with charged groups of protein amino acids under defined conditions of pH, temperature, type of ions, and ionic strength. Acidic and neutral proteins bind to calcium sites on HA, and the elution of these proteins is

achieved with low concentration of phosphate buffers at about pH 6.8. Basic proteins, however bind to phosphate groups on HA crystals with adsorption and elution being strongly affected by the presence of NaCl, KCl and $CaCl_2$. In enzyme purification procedures, HA is generally used as a final clean-up methods following other purification steps. Other non-specific absorbents that have been used are gamma-alumina gel and diatomaceous earth. As examples, alkaline phosphatase from bacteria was purified by using HA (Yeh and Trela, 1976) and diatomaceous earth (celite) is used in the purification of two beta-lactamases from B. census (Wang et al. 1979).

2.2.2.2 ION EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography is the most preferable and most widely used methods for industrial-scale protein purification because it can be easily scaled up by converting from a column technique to a batch process. It can be defined as the separation of one species from another, applied to the mobile phase, by the differential binding and release of these solutes to the fixed charges of the ion exchanger.

Ion-exchange chromatography of enzymes usually employs derivatives of cellulose, agarose, dextrans or resins. The cationic exchangers used for enzyme purification bear negatively charged groups attached to the matrix material, the pK values of these groups are in the range of 2-7. The anion exchangers most commonly used are diethylamino-ethyl (DEAE) cellulose, triethylamino-ethyl cellulose, and ECTEOLA cellulose; the basic substituents of the celluloses have pK values in the range of 7-10.

The separation of proteins by ion exchange chromatography is obtained by reversible adsorption. Most of the experiments are performed in two stages. The first stage is sample application and adsorption. Unbound substances can be washed from the exchanger. In the second stage, enzymes of interest are eluted from the column and collected in separate fractions. The fractionation is achieved since various proteins have different affinities for the ion-exchanger due to the differences in their charge. However, the net charge, charge density and molecular size of the protein as well as pH and ionic strength of the solution are all parameters which affect the separation of enzymes by ion exchange chromatography. Proteins are not bound to any type of ion-exchanger at its isoelectric point (IEP). Protein will bind to the exchangers when electrostatic interactions between protein and exchangers are strong. At this point, the charge on proteins is high and opposite to that of the exchanger. Normally, a cation exchanger should be used if the protein of interest is most stable below its IEP and an anion exchanger should be used if the protein is most stable above its IEP. The pH for binding should be at least one pH unity above or below the IEP's of proteins in order to give a high ionic charge on both the exchanger and the proteins.

In ion exchange chromatography one can choose whether to bind the enzymes of interest or to adsorb impurities and allow the substance of interest to pass through the column. Generally, it is more useful to adsorb the substance of interest, since this allows a greater degree of purification. A general reference for experimental details of ion exchange chromatography is the paper by Himmelhoch (1971).

2.2.2.3 GEL FILTRATION

This separation technique partitions two aqueous phases of identical composition on the basis of molecular size in a column or slab. One phase is freely flowing, the other extrapped by a gel maze in a beaded form. The sample is applied on the surface of the column of appropriate porous beads of the hydrated gel and solvent is percolated through the column. Large molecules which cannot diffuse into the porous structure of the beads, are excluded and pass through in the void volume of the column. Small molecules elute only when a volume equal to the total water content has passed through the column. Intermediate size molecules elute somewhere between the larger and smaller molecules according to their K_{ar} value. These intermediate size molecules do not become permanently trapped in the column if no other factors intervene. Separation occurs according to size, and the molecular weight can be estimated if calibrating proteins are run as references. Generally K_{ar} (defined as $V_{elution}/V_{void}$) is plotted against log M_r (molecular weight) giving a selectivity curve for the particular gel and column.

A wide variety of beaded gels is available: cross-linked dextrin (sephadex), polyacrylamide gel (sephacryl, Bio-Gel P), agarose (sepharose, Bio-Gel A) and cross-linked a gamse (sepharose CL). Together, they cover intermediate-size molecules (M_r) ranging from 500 to 10^6. In all of these gels the void volume is approximately 1/3 of the column volume and is determined by measuring the elution volume of an excluded substance like Blue Dextrin 200.

Much effort has been exerted to determine the molecular weight of globular proteins and their behavior in Sephadex or Agar-gel columns (Andrews, 1964). Agar

gels can be used to separate proteins over very wide molecular weight ranges. Over small ranges, much better separation can be obtained with gel filtration media composed of cross-linked dextrins or polyacrylamide. Therefore, a more critical appraisal of the relationship between the molecular weight of proteins and their gel-filtration behavior is possible with these media than with agar gels. The gel filtration chromatography technique is presented by Kremmer and Boross (1979), Delaney (1980) has reviewed industrial GFC of proteins. With respect to large scale use, GFC has been applied to the removal of lipase from microbial rennet (Somkuti, 1974).

2.2.2.4 AFFINITY CHROMATOGRAPHY

Affinity chromatography occupies a unique place in separation technology which relies not on the general physicochemical properties of a molecule, but rather on the presence of very specific biological and/or chemical functions in the molecule to be separated. In practice, this separation involves the following steps: 1) a solution with desired compound is passed through a column containing a highly specific ligand immobilized on a solid support; 2) as the fluid passes through the column, the desired component binds selectively, and reversibly to the ligand with most impurities passing unhindered; 3) any residual impurities are removed by flushing the column with an appropriate buffer solution; and 4) the compound now purified but still bound, is then recovered by passing through the column a solution that disrupts the ligand binding interaction by changing ionic strength or pH, for instance.

The solid support on which the ligand is immobilized should posses the following properties:

1. The matrix must form a loose porous network which permits unimpaired movement of large macromolecules.
2. The gel particles should be uniform, spherical, and rigid with good flow properties.
3. The matrix should not interact with proteins in general in order to avoid non-specific adsorption.
4. The inner support must have abundant supply of chemical groups which can be activated or functionalized to allow the covalent attachment of a variety of ligands.
5. The gel must be mechanically and chemically stable to the conditions of coupling, adsorption, and elution.

The most widely used solid supports are hydrophilic cellulose derivatives, polystyrene gels, cross-linked dextrans, beaded agarose, glass beads and polyacrylamide gels and ligands are antibodies, antigens, enzyme inhibitors, isolated receptors, and more recently, closed receptors (Bailow et al., 1987). The preparation of a number of chemical derivatives of agarose and polyacrylamide gel bead have been described by Cautrecases and Anfiusen (1971).

Dye-ligand chromatography, hydrophobic interaction chromatography, and immunoaffinity chromatography are similar to affinity chromatography. All these techniques have been used widely for fractionation and purification of proteins and are discussed in turn.

a. Dye-ligand chromatography; Textile dyes are used as general ligands and these dye-ligand media contain a mono- or dichloro substituted triazinyl group, and are sold as Cibacron dyes or Procion dyes. These dyes can be readily immobilized on agarose or other gels to form general purpose affinity matrices. Furthermore, these dyes are cheap, readily soluble, stable, and readily available. Since these are readily available and cheap, this method has been widely used in the industrial and large scale protein purification.

b. Hydrophobic interaction chromatography; A type of affinity chromatography in which the affinity arises through the hydrophobic interactions of the apolar ligands attached to the chromatographic carrier (usually agarose) and the hydrophobic regions of the proteins to be absorbed. The extent of these hydrophobic interactions depends on factors such as the length of the immobilized hydrophobic chains, ionic strength of the buffer, nature of the salts in the buffer, pH and temperature of the buffer, presence of polarity-reducing agents, and presence of a detergent (Rosengren et al. 1975 and Jennissen and Heilmeyer, 1975). For further references, reviews by Miles laboratories (1979) and by Pharmica Fine Chemicals (1982) are available. This technique has also been used for the large scale purification of glucocerebroside β-glucosidase from human placenta (Pharmica, 1982).

c. Immuno affinity chromatography; This chromatography exploits the unique specificity, high affinity, and reversibility of the antibody-antigen interactions. In this technique, the desired antigen is adsorbed to the antibody which has already been immobilized by covalent linkage to an insoluble matrix,

and then recovered by washing with an agent that disrupts the immune complex. Conversely, the antigen may be immobilized and used to purify its respective antibody. A great many applications of the technique have been described along with a number of review articles (Robbins and Schneerson, 1974, Eveleigh and Levy 1977, Fuchs and Sela, 1978, Krisfiansen 1978, Hudson and Hay 1980, and Eveleigh 1982).

In early work only polyclonal antibodies were used to purify antigens, but with the advent of the hybridoma technique, monoclonal antibodies have become available. Monoclonal antibodies have three main advantages over polyclonal antibodies.

1. Highly purified antigen is not required for immunization.
2. Monoclonal antibodies are directed against a single epitope.
3. Monoclonal antibodies can be produced in unlimited quantities.

Since monoclonal antibodies can be produced in large quantities this technique is also attractive for the purification of antigens on an industrial scale.

d. Affinity precipitation; Affinity precipitation uses the solubility of a ligand protein complex for separation. Two options are available, one in which the complex is insoluble upon formation; and the other in which the solution buffer must be changed to cause it to precipitate (Flygare et al., 1983).

2.3 ULTRAFILTRATION

Ultrafiltration (UF) is a pressure driven membrane separation process for dissolved and suspended materials according to the molecular weight and size. Substances

smaller than the pore size of the membrane will pass through with the solvent while larger particles, are retained. In general, UF membranes can separate species ranging from molecular weight 550 to 1,000,000 daltons and the pore size of the membrane ranges from 10 to 100 angstroms. UF is a very simple procedure and requires no phase change, no chemical addition, and less energy, time and cost. Recent advances in membrane technology and new developments in biotechnology have made UF the most useful method in bridging the gap between laboratory scale and industrial scale set ups (Underkofler (1976) and Blatt (1971)). In addition, this method has been used to replace and/or supplement many conventional separation techniques, such as centrifugation, dialysis, distillation, salt precipitation, lyophilization, evaporation, and chromatography. Presently, the need for the extensive isolation and concentration of recombinant DNA products and monoclonal antibodies show that the UF concentration process is an essential separation process for the pharmaceutical, chemical and food processing industries.

There are three basic configurations of UF membranes available. They are hollow-fiber, plate and frame and spiral cartridges. Hollow fiber membranes, which are supplied in self-contained cartridge housings, are easy to clean and allow good product recovery, but they are somewhat limited in pressure capability. On the other hand, plate and frame membranes are capable for high pressure operation, but can have cleaning and product recovery problems.

Traditionally cellulose membranes are the most common for membrane separation (Reid and Breton, 1959). These membranes exhibit remarkable selectivity between salts and water, but suffer chemical and biological attack and have low resistance to

temperature. Invention of synthetic polymer membranes which are physically and chemically more rugged and durable, replaced these cellulose membranes. Most commonly used synthetic membranes are aromatic amides, polycarbonates, polyacrylonitrile-polyvinyl chloride copolymer and polysulfone. Most recently, composite membranes also been used in membrane separation.

The basic problem in membrane separation is the interaction between the solutes and the membrane itself. This can decrease the flux significantly. It can be overcome either by back flushing with cleaning solution or using a membrane that has no affinity for the solutes or the membrane surfaces (Le and Howell, 1983). The application of UF can be categorized into three groups: concentration, diafiltration, or purification. In industry, UF also been used in the following ways:

1. To concentrate and diafiltrate of final product generally after gel filtration, thus substantially reducing volume prior to lyophilization.
2. To concentrate the product prior to salt precipitation, thereby reducing the amount of salt required. The smaller volumes are easier to separate after precipitation and salt disposal expenses are reduced.
3. To remove such low molecular weight contaminants as peptides, amino acids, sugars, and salts by initial diafiltration.
4. To remove alcohol by following solvent precipitation and redilution of the precipitate.

In this project UF is used to remove the cells, concentrate fermentation broth, and concentrate the product prior to another purification step.

2.4 GEL ELECTROPHORESIS

Electrophoresis, an enzyme purification technique based on the size and charge of the molecule, provides for high resolution of purification and is capable of purifying very minute quantities. This method enjoyed some popularity for enzyme separation for quite a few years but it is now largely used as a tool for separation of isozymes and other enzymes related diagnostic purposes like molecular weight determination. Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing are the techniques frequently used for laboratory scale enzyme purification and characterization. Polyacrylamide is the most common support matrix for the electrophoretic separation of proteins subunits (Southern, 1979 and Saeed and Boyde, 1980). However, other support media, such as Bio-Gel P-300 (Saeed and Boyde, 1980), can also be used. Only 10-200 micrograms are typically separated by analytical PAGE. The fractionation of larger quantities (up to 250 mg) by preoperative PAGE requires special equipment (Southern, 1979). Recently, an industrial scale continuous electrophoretic separator has been developed by the biochemistry group at Harwell Division of the U.K. Atomic Energy Authority (Didcot, England). This unit can draw off as many as 29 separate fractions in potential applications as isolation and purification of high value pharmaceutical enzymes and other products.

PAGE has become a common and important tool for the molecular biologist to determine the size of macromolecules, DNA, RNA, and protein. The procedure and the analysis are simple. Several methods have been developed for the estimation of the proteins using ionic detergents and solvents. Lambin et al. (1976) demonstrated

that electrophoresis in linear polyacrylamide gradient gels, combined with sodium dodecyl sulfate (SDS) treatment, can be successfully used to estimate the molecular weight (M_r) of proteins. Using only one electrophoretic step, this technique provides accurate measurement for proteins or glycoproteins with M_r's between 10^4 and 10^6. In the case of glycoproteins, this estimate is not modified by the carbohydrate content (Lambin and Fine, 1978). In this project the molecular weight of the β-amylase is determined by PAGE-SDS method and compared. For further details many review articles are available by Shaw (1969), Ornstein (1964), Davis (1964), Shuster (1971) and Smith (1967).

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CHAPTER 3

FERMENTATION AND CONCENTRATION OF A NOVEL THERMOSTABLE EXTRACELLULAR β-AMYLASE FROM CLOSTRIDIUM THERMOSULFUROGENES

3.1 ABSTRACT

An thermostable extracellular enzyme, β-amylase was produced from Clostridium thermosulfurogenes with different carbon sources. Different sizes of fermenters were used and the fermentation time was optimized. Neither the size of the fermenter nor the fermentation carbon sources influenced the final total β-amylase activity or the fermentation time for the production of β-amylase from C.thermosulfurogenes.

Concentration by ultrafiltration showed higher β-amylase activity recovery and purification due to the removal of peptides and small proteins with permeate. β-amylase produced by fermenting maltrin yields higher β-amylase activity recovery with 100,000 mwco UF membrane. On the other hand, β-amylase produced by fermenting maltose yields very poor recovery with the same membrane. It concluded that the residual maltrin forms a larger molecular weight complex with the β-amylase resulting in an increase in apparent molecular weight.

3.2 INTRODUCTION

Amylolytic enzymes are essential for a number of industrial processes, mainly in saccharification of starch and malting in beer production. The main amylolytic

enzymes used in the industry are α-amylase, β-amylase, glucoamylase, and pullulanase. A premium is placed on thermostability and thermoactivity of the above enzymes used in bioprocessing of starch. However, most of the microbial amylases used in industry are not thermostable and not active at higher temperature. It has been suggested that thermoanaerobes have the potential for the production of active and thermostable enzymes (Zeikus, 1979). The enzyme produced and partially purified in this project is an extracellular thermostable β-amylase (MBI β-amylase) developed from the thermoanaerobe C. thermosulfurogenes at the Michigan Biotechnology Institute (MBI).

3.2.1 β-AMYLASE

β-amylase (EC 3.2.1.2, α-1,4-D-Glucan maltohydrolase) is an exo-acting carbohydrase which cleaves α-1,4 glucosidic linkages from the non-reducing end of a starch molecule and generates maltose as a major final product. Since β-amylase cannot react or bypass the branching points of the carbohydrate chain, the hydrolysis stops near the branching points, thus yielding maltose and β-amylase limit dextrin (β-limit dextrin). β-amylases are well studied and characterized in higher plants and have also been reported in micro-organisms (Robyt and Whelan, 1968, Higashihara et al., 1974, Shinke et al., 1975, Takasaki, 1976, and Thomas et al., 1980). However, most of the β-amylases are not thermostable and not active at higher temperatures. The MBI β-amylase of the current study, is active at or above 75°C (Hyun and Zeikus, 1985). This enzyme has a wide pH stability between 3.5 to 7.0 with an optimum pH at 5.0. The general mechanism for regulation of β-amylase synthesis,

and its ability to bind starch and to produce maltose syrups have been studied and reported by Hyun and Zeikus (1985) and Saha et al., (1987). In a previous study, β-amylase produced from fermentation of maltose was purified and characterized at MBI (Shen et al., 1988). The purpose of the present work was to study the effect of different carbon sources on the β-amylase final activity during fermentation and initial concentration by ultrafiltration (UF) membranes. β-amylase was produced in different sizes of batch fermenters using different substances (maltose, maltrin, and glucose) to analyze a simple scale-up procedure.

3.2.2 ULTRAFILTRATION

Ultrafiltration is a pressure driven membrane separation technique based on molecular weight and size. Almost every bioseparation process uses UF to remove the cells, to clarify the whole broth, and to concentrate the bio-products from invariably dilute and complex aqueous mixtures. This is a low cost, short processing time unit operation which results in high product recovery. UF was used to remove the cells and to concentrate the fermentation product in the present study. Spiral and hollow fiber membranes with different pore sizes were used to concentrate β-amylase produced by fermentation of different carbon sources (substrates).

3.3 MATERIAL AND METHODS

3.3.1 ORGANISM AND FERMENTATION

The catabolic repression resistant mutant H-12-1 (Hyun and Zeikus, 1985b)

derived from C.thermosulfurogenes wild strain 4B (ATCC 33743) was used. The culture was grown at 60°C in 26 ml anaerobic pressure tubes (Bello Orlans, Inc. Vineland, N.J.) that contained 10 ml of TYE (trypticase and yeast-extract) medium supplemented with 0.5% soluble starch and N_2-CO_2 (95:5) gas head space. β-amylase was produced in TYE or corn steep liquor medium with different carbon sources. The components of the lab-medium are listed in Table 3.1. For simple scale-up, two different Bruan fermenters (14 and 120 liters) and a 300 liter Lab-Line fermenter were used to produce β-amylase. All the components were proportionally increased according to the fermentation volume. Initially, the seed culture was expanded to 500 ml in a round bottom flask and then was transferred anaerobically to the large fermenters. Fermenters were stirred at a constant rate (400 rpm.) and N_2-CO_2 (95:5) gas was purged for a few hours to remove the dissolved oxygen. β-amylase was produced anaerobically at 60°C with a slightly higher pressure than the atmosphere. An anti-foaming agent was added to avoid excessive foaming caused by high production of gases during fermentation. The turbidity of the culture was measured hourly and the fermentation was stopped after the exponential growth period of the enzyme. The culture was allowed to cool to ~ 15°C before concentration.

3.3.2 ENZYME ACTIVITY AND PROTEIN ASSAYS

Enzyme activity was assayed by incubation of the reaction mixture (1 ml) which consisted of 2% soluble starch, acetate buffer (50 mM, pH 6.0 with 5mM $CaCl_2$), and proportionally diluted enzymes at 60°C, for 30 minutes. The reducing sugars released by β-amylase action on starch were estimated by the DNS (dinitrosalicylic acid)

TABLE 3.1

COMPOSITION OF TRYPTICASE AND YEAST-EXTRACT MEDIA USED FOR THE PRODUCTION OF β-AMYLASE

Components	Per liter basis
Yeast	3 g
Trypticase	10 g
Salts	3.6 g
Carbon source	10 g
Vitamins	10 g
Trace elements	10 ml

method (Miller, 1969). One unit of enzyme activity is defined as a micromole of maltose released per unit time under the conditions of the assay. Total protein concentration was estimated using the Lowry et al., (1957) assay method with bovine serum albumin as a standard.

3.3.3 ULTRAFILTRATION

Concentration studies were conducted by UF before and after removal of cells by centrifugation (12,000 rpm, 30 min. at 4°C). These experiments showed that there is not a significant difference in recovery if centrifugation precedes UF. Therefore β-amylase was concentrated and cells removed by UF and centrifugation, respectivelly.

β-amylase produced with different carbon sources was initially concentrated by UF with 30,000 and 100,000 MWCO diaflo hollow fiber membranes (Amicon) and a 30,000 MWCO spiral membrane (Amicon). Recirculation rate was varied for different size membranes but was constant for each configuration. Membranes were back flushed at a different time intervals to avoid fouling. β-amylase activity and total protein concentration were assayed before and after each filtration study.

3.4 RESULTS AND DISCUSSION

3.4.1 FERMENTATION

β-amylase was produced more than twenty times in all three fermenters.

Depending on the activity of the inoculated culture, the fermentation time varied from 8 to 12 hours. In a few instances, when β-amylase activity or the turbidity of the media did not increase after inoculation, media were reinoculated with a fresh seed culture and successfully harvested. A typical plot for the change in turbidity with time for β-amylase produced with maltose and maltrin is shown in Figure 3.1. Final β-amylase activity was assayed in each fermentation, and it was not influenced by the size of the fermenter. β-amylase activities with different carbon sources are shown below.

Carbon Source	A_{660}	β-amylase Activity (activity unit)
1% Dextrins	2.6	30 to 40
1% Maltose	2.3	30 to 44
1% Glucose	0.8	20 to 35

These results also showed that there were no mass transfer problems during scale-up. The maximum activity obtained was 45 U/ml in a 120 liter fermenter with 1% maltose.

Fermentation results show that a growing seed culture is essential for the production of β-amylase. This would exclude the possibility of β-amylase produced from its substrate (β-amylase could be produced from plant tissues also) and confirmed that the β-amylase is produced only from C. thermosulfurogenes. The fermentation time neither depended on the size of the fermenter nor the carbon source

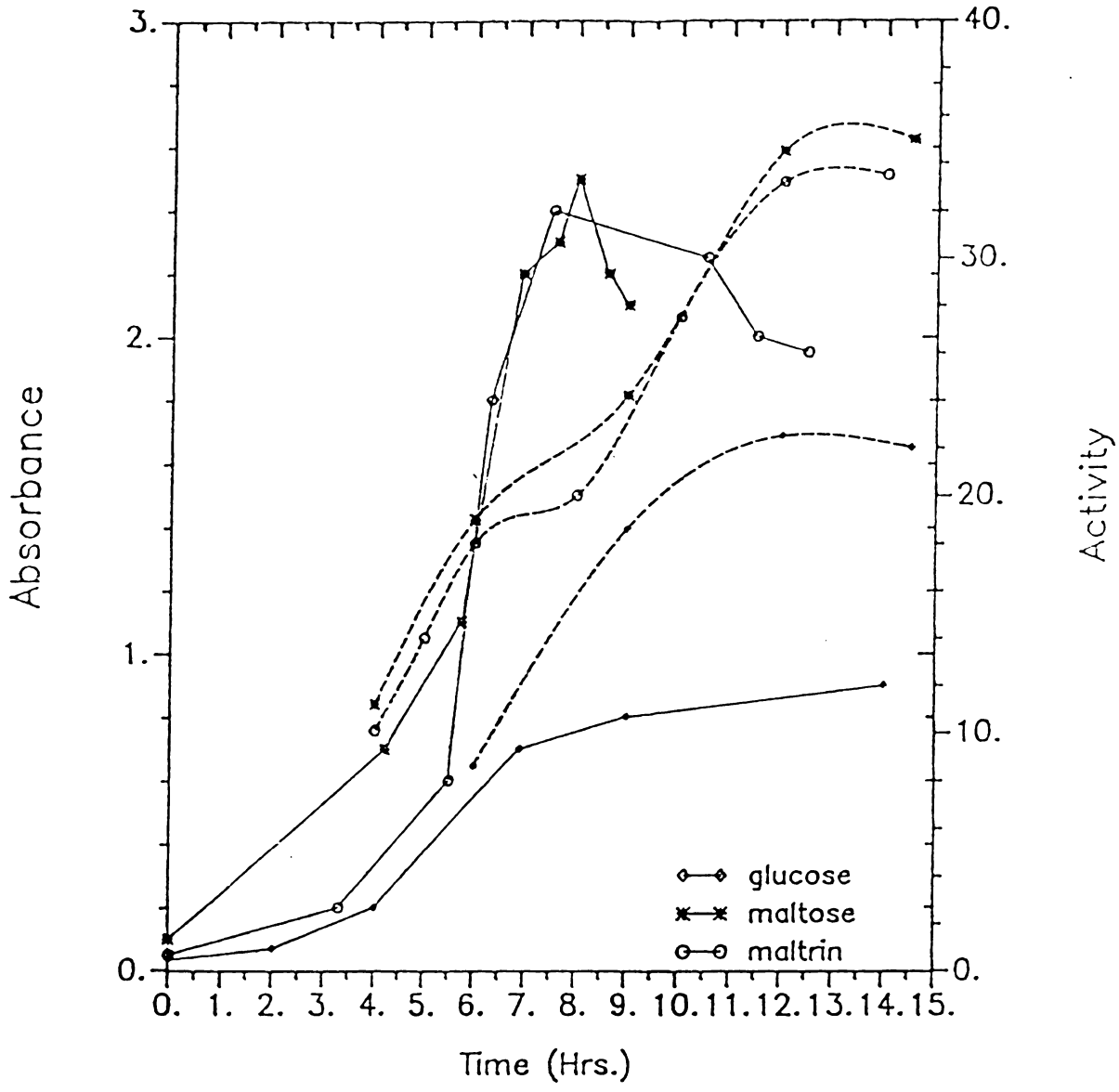

Figure 3.1. β-amylase activity and turbidity (absorbance) history during prodoction of β-amylase with three different carbon sources glucose, maltose and maltrin. ------ absorbance, ... β-amylase activity

(substrate) of the media, but it was only influenced by the activeness of the seed culture. Seeding a fermenter with culture at its exponential growth would reduce the fermentation time a great deal. Although the final turbidity was low in glucose fermented β-amylase, the activity was not influenced by the size of the fermenter or the fermentation carbon source. The turbidity increase was due to production of more cells without increasing the β-amylase activity.

3.4.2 ULTRAFILTRATION

Since there was no influence in β-amylase recovery for removal of cells before concentration, β-amylase produced by fermenting different carbon sources was concentrated with cells by UF. Two series of experiments were then conducted:

1. β-amylase produced by fermenting four different carbon sources was concentrated with a 30,000 MWCO spiral membrane and an average permeation rate of 500 ml/min through the membrane was maintained in each run. In all runs more than 85% of β-amylase activity recovery with ten fold purification was achieved (Table 3.2). There were no significant fouling problems or drop in permeation rate due to different carbon sources. Significant increases in specific activity were due to the removal of small peptides and proteins with the permeate. Fermentation carbon sources showed no influence in the recovery or in the purification fold of β-amylase with 30,000 MWCO membrane.
2. β-amylase produced with dextrins and maltose was concentrated with 30,000 and 100,000 MWCO hollow fiber membranes. Results are shown in Table 3.3. The highest purification and recovery for β-amylase with dextrins was

TABLE 3.2

INITIAL CONCENTRATION OF β-AMYLASE FERMENTATION BROTH WITH CELLS (WITH FOUR DIFFERENT CARBON SOURCES) BY A 30,000 MWCO SPIRAL MEMBRANE.

β-amylase	Carbon source	Volume (ml)	Specific activity (U/mg)	Recovery (%)
original	maltose	94,000	10.0	100
concentrated		6,000	141.1	88
original	soluble	11,000	6.0	100
concentrated	starch	1,200	62.8	89
original	maltrin-40	10,000	8.5	100
concentrated		840	88.3	88
original	maltrin-100	8,500	6.6	100
concentrated		740	75.5	85

TABLE 3.3

CONCENTRATION OF β-AMYLASE BY 30,000 AND 100,000 MWCO DIAFLO HOLLOW FIBER MEMBRANES AS A FUNCTION OF CARBON SOURCE

β-amylase	Carbon source	Membrane cut off (MWCO)	Specific activity (U/mg)	Recovery (%)
original	maltrin	100,000	56.0*	100
concentrated			248.2	93
original	maltrin	30,000	57.3*	100
concentrated			149.5	85
original	maltose	100,000	10.5	100
concentrated			50.0	51
original	maltose	30,000	10.0	100
concentrated			141.0	88

* higher specific activity β-amylase was obtained from recycle cells experiments by Dr. A. Nipkow at MBI.

obtained by the 100,000 MWCO UF membrane but the same membrane resulted in very poor recovery and purification for β-amylase produced by fermenting maltose. This suggests that particles formed in dextrins fermented β-amylase are larger or form a more stable aggregate than in those formed in maltose. The starch dextrins may form a biospecific complex with β-amylase or help to form stable β-amylase aggregates. Using a larger MWCO UF membrane, reduces the purification cost and time for dextrin fermented β-amylase. The molecular weights of the β-amylases produced with maltose and dextrin were estimated by gel filtration chromatography (results are shown chapter 4). β-amylase produced from dextrins showed a portion of β-amylase activity at higher molecular weight, and the remainder is at a lower molecular weight similar to the maltose derived case. The effective higher molecular weight may be due to the aggregation of β-amylase subunits or binding of β-amylase to dextrin thereby forming a complex.

3.5 CONCLUSIONS

1. Production time of β-amylase in a batch fermenter requires eight to twelve hours, and could be reduced by inoculating with a seed culture at its exponential growth stage.
2. Final β-amylase activity during fermentation does not depend on the size of the fermenter or the fermentation carbon source (substrate) for the range of fermenter sizes studied.
3. Concentration of β-amylase with 30,000 MWCO UF membrane gives 85%

activity recovery with ten fold purification of β-amylase from all four carbon sources considered.

4. A 100,000 MWCO UF membrane recovers approximately 90% activity of β-amylase produced by maltrin fermentation and only 50% activity recovery of β-amylase produce by maltose fermentation. This effect must be due to complexation between β-amylase and maltrin resulting in a larger effective molecular weight.

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CHAPTER 4

EFFECT OF FERMENTATION CARBON SOURCE ON THE PRECIPITATION OF β-AMYLASE FROM CLOSTRIDIUM THERMOSULFUROGENES

4.1. Abstract

β-amylase from Clostridium thermosulfurogenes was recovered from cell free broth by ammonium sulfate and ethanol precipitation. The effects of the fermentation carbon source, temperature, and precipitant concentration on the β-amylase recovery process were studied and compared. Fifty percent saturated ammonium sulfate or twenty percent ethanol was the optimum concentrations for the enzyme recovery and purification. The β-amylase was found to form a soluble complex with starch dextrins in the fermentation broth. Light scattering studies revealed that the particle size of the precipitated complex was significantly larger for ethanol as compared to ammonium sulfate precipitation. Therefore, recovery of β-amylase activity was directly related to the particle size obtained. Smaller particles were obtained in the absence of starch; however, the ammonium sulfate resulted in larger particles as compared to the ethanol precipitation. The complexation of the β-amylase with the starch and subsequent precipitation of the complex can be interpreted as a bio-specific recovery process. The temperature effect on the precipitation process was also related to the carbon source

used in the fermentation. Gel filtration gave high β-amylase specific activity, and a single band or single peak was obtained with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or HPLC gel filtration for β-amylase produced with maltose. Two different molecular weight fractions were obtained for maltrin fermented β-amylase, and they were confirmed as two different single peaks in HPLC gel filtration. SDS-PAGE showed only a single band for both of these fractions in denatured form. These results further support the complexation of β-amylase subunits with starch dextrins, and the complex may be the reason for the higher effective molecular weight β-amylase observed.

4.2. Introduction

Protein precipitation with the subsequent recovery of the precipitate is one of the most important unit operations in downstream processing. More than half of all purification schemes use a precipitation step somewhere in the process (Steven Oh, et al., 1986). The potential applicability of precipitation is being expanded with the incorporation of novel precipitants and processes. The major role of protein precipitation as a separation process is in its ability to handle large quantities of dilute streams. This attribute allows precipitation to be used as an initial recovery process with the resulting product being further purified by more expensive techniques as the end use dictates.

Although precipitation is used for both product recovery and purification, it is most effective in achieving the former. Precipitation is followed by a solid-liquid separation whose efficiency is dependent on the characteristics of the precipitate (Devenux et al., 1986).

4.2.2 Protein precipitation kinetics studies

The understanding of protein precipitation requires the study of the formation of a stable solid phase, the subsequent aggregation, and efficient separation of the precipitate. In a destabilized protein solution a primary particle is formed by nucleation and it subsequently aggregates due to the diffusive motion of the particles. The final size of the particles is dependent on agglomeration and disruption. The primary particles continue to grow to a size where fluid dynamics become important and fluid driven particle-particle collisions lead to the formation and growth of final aggregates. These growth processes are termed perikinetic and orthokinetic aggregation according to zero and uniform shear fields, respectively (Bell et al., 1983). However, in practice most precipitations are conducted in turbulent conditions, so other mechanisms and rate relations should be considered. Grabenbauer and Glatz (1981), in a study of isoelectric precipitation of soy proteins, have identified that the protein concentration, agitation, and ionic strength are all significant factors in shaping the particle size distribution. They found that protein concentration influences the number of aggregates more than it does their growth or break-up rates (Grabenbauer and Glatz, 1981). Agitation affects the growth rates of aggregates, but affects aggregates breakup much more. In other studies, protein precipitation is described as a rapid formation of submicron particles followed by aggregation via collision between particles. Collision efficiencies are high when one of the particles is small, but are quite low otherwise (Petenate and Glatz, 1983).

On the other hand, particle size plays a major role in the recovery of precipitates. Because of similar densities of the protein precipitate and the aqueous phase, the

particle size of the precipitate is crucial to the effectiveness of particle removal (Petenate and Glatz, 1983). In fact, there is evidence that small particle size may be responsible for poor yields associated with some precipitation and recovery processes. Since centrifugal recovery is related to the square of the particle size, the improvement in the aggregate strength could result in a significant improvement in separation efficiency (Bell and Dunnill, 1982).

The recovery of β-amylase from C. thermosulfurogenes produced at the Michigan Biotechnology Institute was studied using salting-out and organic solvent precipitation in conjunction with a biospecific interaction between β-amylase and starch dextrin molecules. The effects of fermentation carbon source, temperature, and protein concentration on precipitation of β-amylase recovery were studied. Crude β-amylase was further purified by a Bio-Gel gel filtration column and the different size fractions were collected. Higher β-amylase activity peak fractions were pooled and concentrated by UF (PM10, Plate membrane, 10,000 MWCO). The effective molecular weight and the purity of the enzyme were analyzed by SDS-polyacrylamide gel electrophoresis and HPLC gel filtration. Finally, the amino acid sequence of the β-amylase was determined and compared for each fermentation carbon source.

4.3 Materials and Methods

4.3.1 Production of β-amylase.

The thermostable, solvent resistant extracellular enzyme crude β-amylase was produced using trypticase and yeast extract (TYE) or corn steep liquor medium as the nitrogen source, and maltose, glucose, or maltodextrin (maltrin-10) as the carbon source. Two different Braun fermenters (14 and 100 liters) and a 300 liter

Lab-Line-Bioengineering fermenter were used to produced β-amylase from Clostridium thermosulfurogenes. The fermentation broth was initially concentrated by ultrafiltration using a 30,000 MWCO spiral membrane (Amicon Spiral Ultrafiltration Cartridge model S10Y30). Subsequently, the cells were removed by centrifugation. The fermentation broth was purified about six fold during the above procedures. β-amylase specific activity of approximately 50 U/mg was used in all precipitation experiments.

4.3.2 Enzyme activity and protein assays

Enzyme activity was determined by incubation of the enzyme solution with 2% soluble starch at 60°C, pH 6.0 for 30 minutes. The reducing sugars released by β-amylase action on starch were estimated by the DNS (dinitrosalicylic acid) method. One unit of enzyme activity is defined as a micromole of maltose released per unit time under the conditions of the assay. Total protein concentration was estimated using the Lowry et al. (1951) assay method with bovine serum albumin as a standard.

4.3.3 Gel filtration

A Bio-Gel column (1.5 cm x 150 cm) equilibrated with 50mM acetate buffer (pH 6.0) containing 50mM $CaCl_2$ was used for gel filtration. Since, this was a small column, only 2-3 ml of concentrated β-amylase were purified at a time. The elution rate was 0.1 -0.2 ml/min. Fractions of β-amylase fermented with different carbon sources were collected and assayed for specific activity, total protein content, and total

carbohydrate content. The higher activity fractions were pooled, concentrated by UF (PM10, plate membrane, 10,000 mwco), and dialyzed against the same buffer. These concentrated peak fractions were used as purified enzymes for SDS-polyacrylamide gel electrophoresis and HPLC gel filtration.

4.3.4 Molecular weight determination by HPLC gel filtration

Purified enzyme fractions were chromatographed on a Protein Pak 300SW column (Waters Associates) equilibrated with 50mM acetate buffer, pH 6.0 with 5mM $CaCl_2$. A 100uM of enzyme sample was loaded and eluted with the same buffer at a rate of 1.0 ml/min. The protein peaks were detected by a UV detector and recorded by a Waters 700 recorder. The retention time for each β-amylase peak was obtained. Molecular weights and retention times were calibrated for different molecular weight protein standards. Effective molecular weights were estimated for β-amylase produced with maltose and maltrin. In order to compare the differences in β-amylase produced with maltose or maltrin, the amino acid sequence of electrophoretically pure β-amylase produced with maltose and maltrin were determined at the Department of Biochemistry, Michigan State University.

4.3.5 Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed for all gel filtered and concentrated enzymes as described by Laemmli (1970). Bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), trypsinogen (24,000), carbonic anhydrous (20,000) and

α-lactalbumin (14,200) were used as standards. Gels were then stained with coomassie brilliant blue G-280 and compared.

4.3.6 Stirred batch precipitation studies

Three different kinds of β-amylase protein mixtures were used in batch experiments.

1. β-amylase produced with maltose or glucose as the fermentation carbon source.
2. β-amylase produced with starch as the fermentation carbon source.
3. Synthetic β-amylase mixture: β-amylase precipitated by fifty percent ammonium sulfate was redissolved in acetate buffer (50mM), pH 6.0, containing 5mM calcium chloride and dialyzed against the same buffer for 24 hours. The above β-amylase was added to pretreated corn steep liquor and acetate buffer (50mM), pH 6.0, containing 5mM calcium chloride to match the protein concentration and β-amylase activity of the original fermentation broth.

The results of preliminary batch precipitation studies indicated no significant difference in protein precipitation when TYE or corn steep liquor was used as fermentation medium nitrogen source or when maltose or glucose was used as fermentation carbon source. The optimum precipitant concentrations were 50% saturated ammonium sulfate or 20% (v/v) ethanol for stirred batch precipitations of β-amylase. These concentrations were used in all subsequent stirred batch precipitation studies.

Concentrated β-amylase fermentation broth and the synthetic β-amylase mixtures were used for stirred batch precipitation studies. Saturated ammonium sulfate or ethanol was added dropwise to 75ml of β-amylase solution with gentle stirring (using a rubber coated stirring rod) in a 250 ml beaker. The final concentration of the mixture was 50% saturated ammonium sulfate or 20% (v/v) ethanol. Each batch was stirred for two hours at a stirring rate of 80 rpm. The precipitate was collected by centrifugation (12000 rpm: 30 min: 4°C), redissolved in 20 ml acetate buffer (50mM), pH 6.0, containing 5mM calcium chloride, and dialyzed against the same buffer for 24 hours. Protein assay and activity tests were performed before and after the precipitation.

4.3.7 Protein precipitation kinetics studies

A Coulter Electronics Model N4 sub-micron particle analyzer was used to measure the particle size distributions of the protein precipitate. An auto-sampling time of 200 seconds was used in all experiments. The auto-correlation functions were generated and transformed into particle size distribution through standard estimation techniques, yielding the mean size and standard deviation. β-amylase with the precipitant were mixed in a continuous stirred batch reactor and 2ml samples were analyzed at different time intervals.

A Perkin-Elmer Lambda 3A spectrophotometer was used to measure the turbidity at 660 nm (i.e, absorbance) of the protein precipitate with time. The kinetics of initial particle formation were monitored immediately after mixing using 50% saturated ammonium sulfate or 20% ethanol to β-amylase broth produced with maltose or

maltrin. 50% saturated ammonium sulfate or 20% ethanol solution was used as blank for the above experiments. In addition, 1% soluble starch was precipitated with 50% ammonium sulfate or 20% ethanol and initial kinetics studies were also performed and compared.

4.4 Results

4.4.1 Batch precipitation studies

Preliminary batch precipitation results for different concentrations of ammonium sulfate and ethanol are reported in tables 4.1 and 4.2, respectively. The temperature and the carbon source effect on total protein and β-amylase activity recovery with 50% saturated ammonium sulfate are shown in Figures 4.1 and 4.2, respectively. Total protein and activity recoveries were generally better for β-amylase with maltose than than with maltrin as the carbon source. However, at 50°C β-amylase recovery dropped for β-amylase produced with maltose.

The effects of temperature on total protein and β-amylase activity recovery for ethanol precipitate are shown in Figures 4.3 and 4.4, respectively. The maximum specific activity recovery occurred at room temperature. Further increases in temperature resulted in precipitation of other proteins without additional precipitation of β-amylase. The activity recovery was enhanced by the strong binding of β-amylase to starch dextrins and the resulting low solubility of the complex in aqueous ethanol.

The addition of maltrin-10 to the maltose containing broth resulted in a 3 fold increase in activity recovery with ethanol precipitation and is shown in Table 4.3.

TABLE 4.1

Purification of β-amylase by different concentration of ammonium sulfate precipitation (carbon source: maltose).

steps	Specific activity (U/mg of protein)	Purification ratio	Activity recovery (%)
Pretreated broth	26.0	1	100
50% ammonium sulfate ppt.	174.0	7	98
50-65% ammonium sulfate ppt.	4.0	-	1.4

Experiments were conducted at room temperature for two hours stirring.
Purification ratio = (final specific activity/ initial specific activity)

TABLE 4.2

Purification of β-amylase by different concentration of ethanol precipitation (Carbon source: maltrin)

Steps	Specific activity (U/mg of protein)	Purification ratio	Activity recovery (%)
Pretreated broth	57.5	1	100
20% ethanol precipitation	358.7	6.2	72
30% ethanol precipitation	241.6	4.2	87
40% ethanol	120.9	2.1	74

Experiments were conducted at room temperature for two hours stirring.
Purification ratio = (final specific activity / initial specific activity).

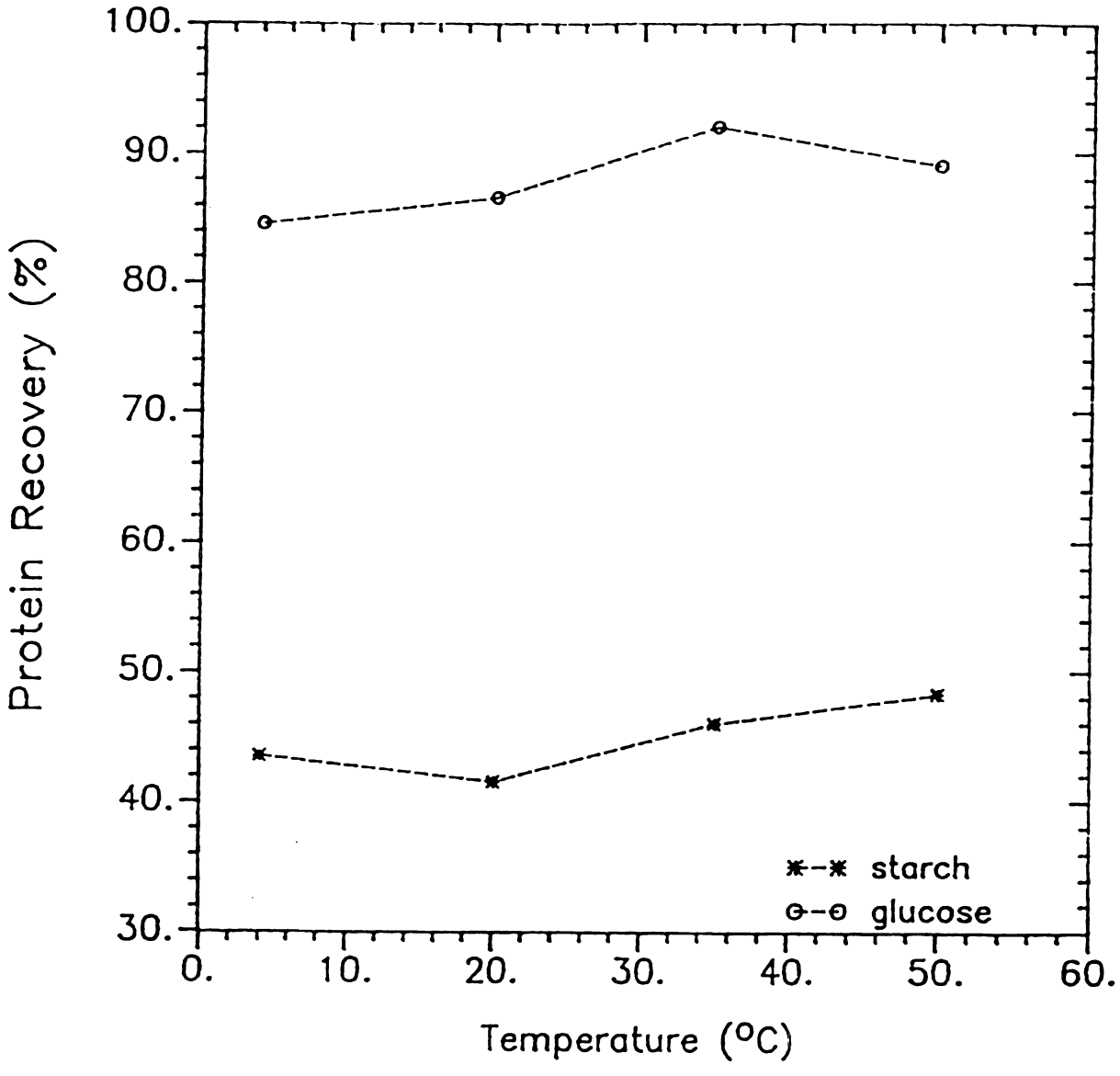

Figure 4.1. Effects of temperature and carbon source on total protein recovery of β-amylase precipitation with 50% saturated ammonium sulfate

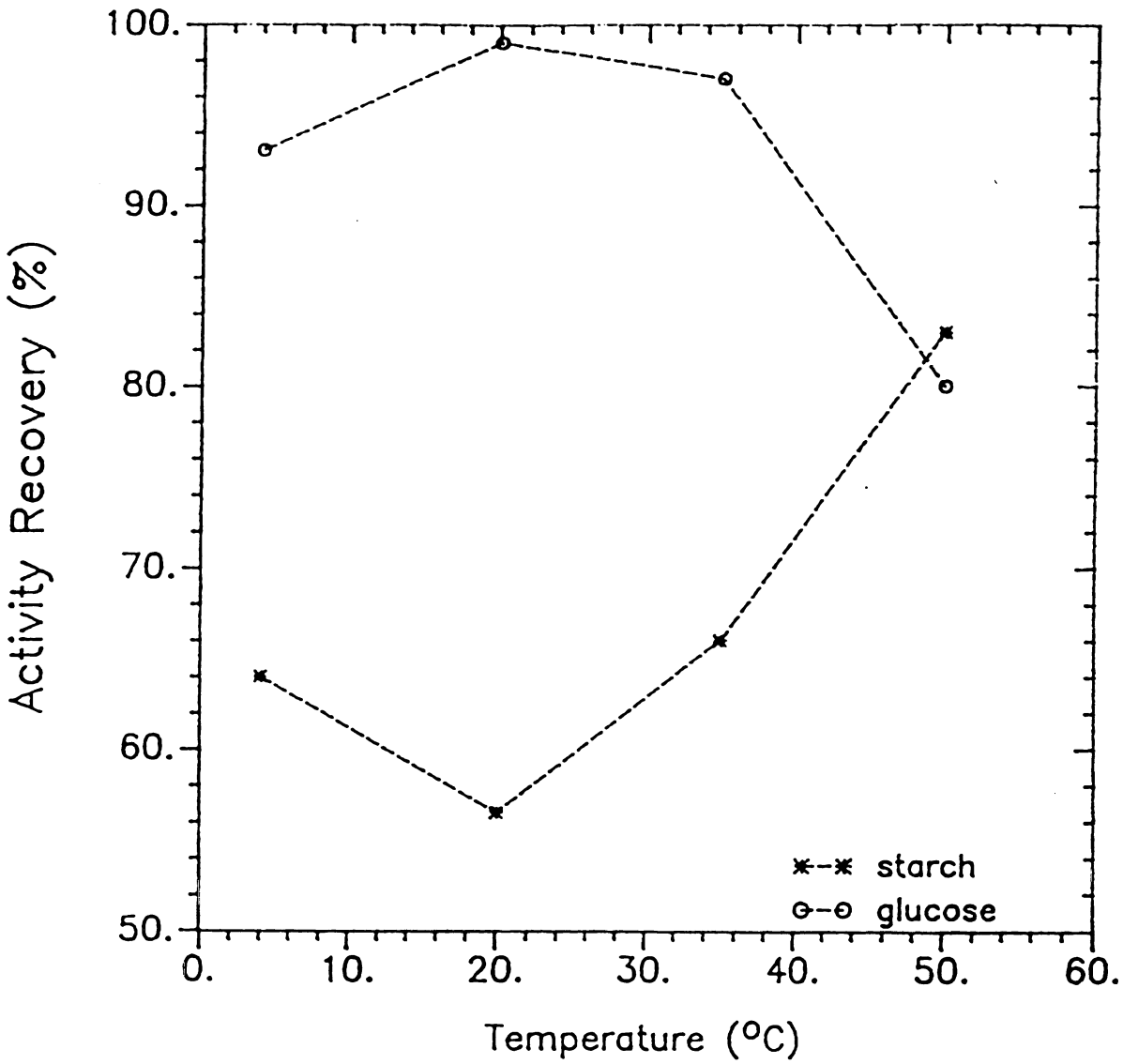

Figure 4.2. Effects of temperature and carbon source on β-amylase activity recovery during β-amylase precipitation with 50% saturated ammonium sulfate

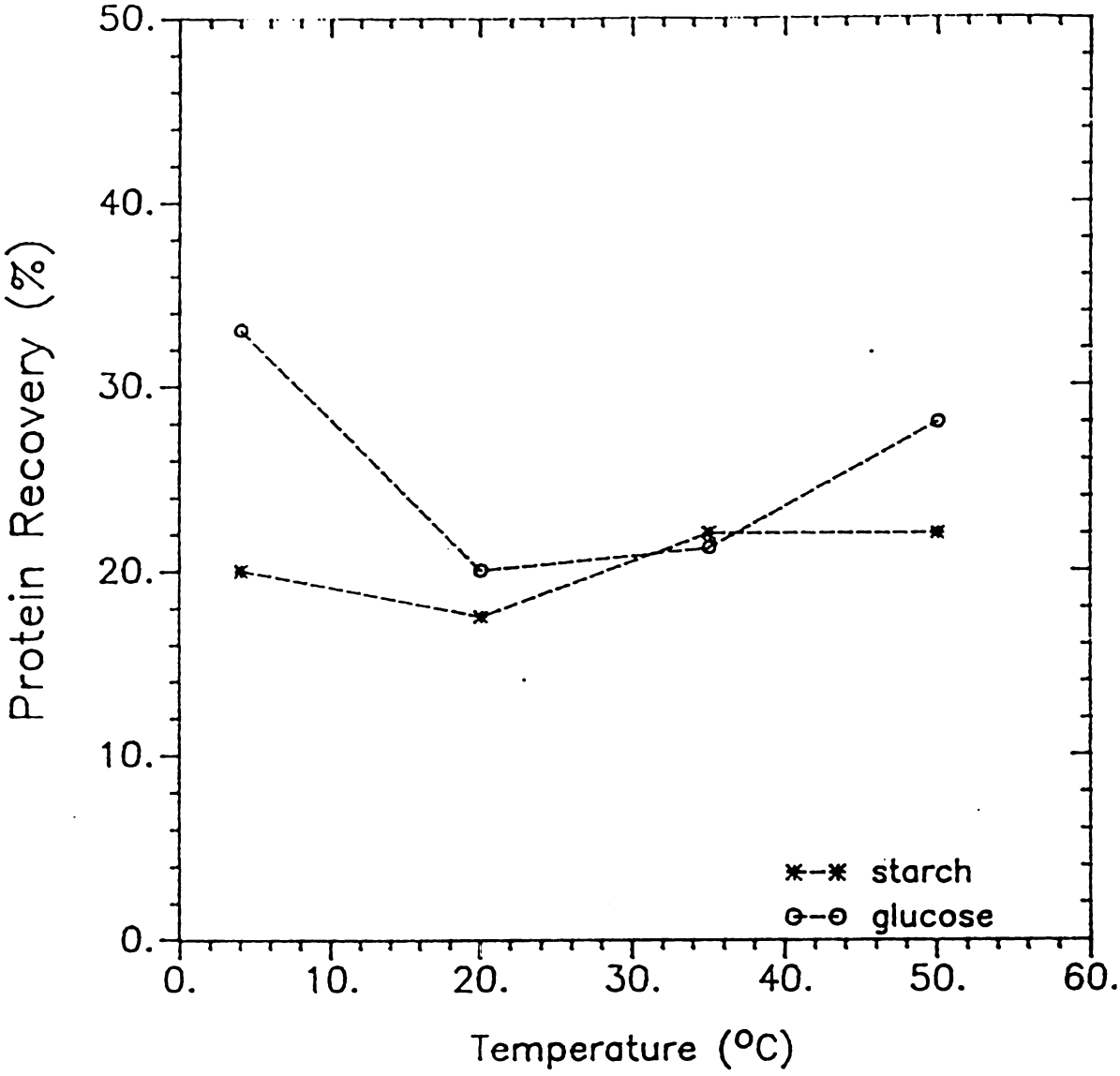

Figure 4.3. **Effects of temperature and carbon source on total protein recovery of β-amylase precipitation with 20% (v/v) ethanol**

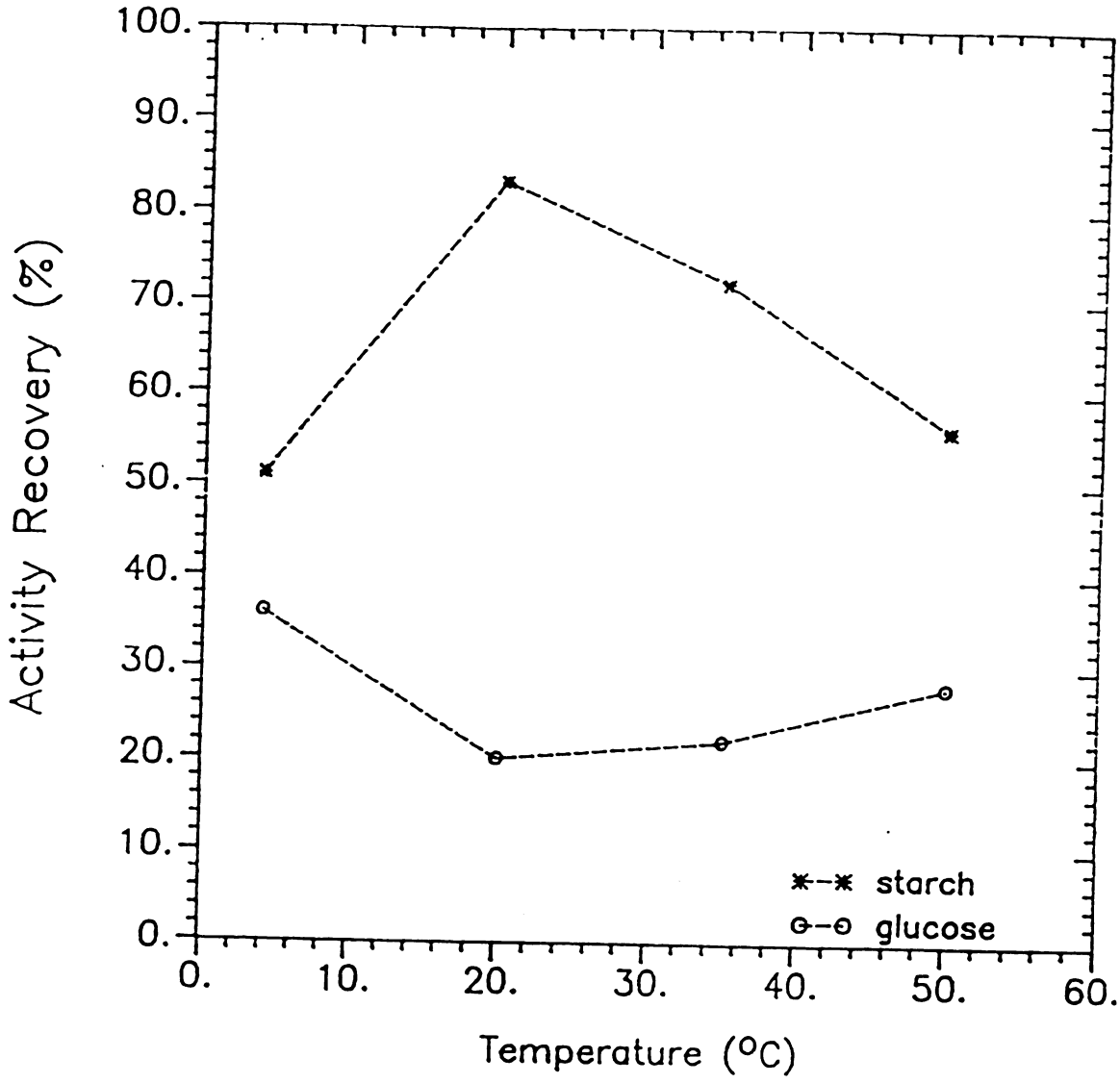

Figure 4.4. Effects of temperature and carbon source on β-amylase activity recovery during β-amylase precipitation with 20% (v/v) ethanol

TABLE 4.3

Effect of starch dextrin on β-amylase recovery with 20% ethanol. β-amylase was initially concentrated by ultrafiltration.

	Starch dextrin conc. (%)	Total activity recovery (%)
Concentrated β-amylase	0.0	15
Concentrated β-amylase	0.5	50

Experiments were conducted at room temperature for two hours stirring.

4.4.2 Fundamental kinetic studies

The change in mean particle size with time after mixing for two different precipitants and fermentation carbon sources is shown in Figure 4.5. Light scattering studies showed that the particle size of the precipitated complex was significantly larger when ethanol was used as precipitating agent in presence of starch β-limit dextrins. Mean particle size of the precipated complex increased with time up to two hours, and decreased afterwards for ethanol when starch β-limit dextrins were present in the fermentation broth. Particles were initially formed and maintained a constant mean size with time for the other three conditions. Ammonium sulfate precipitate had a larger mean particle size than ethanol in the absence of starch dextrins. Pure soluble starch dextrins (1%) was precipitated with 50% ammonium sulfate or 20% ethanol to compare precipitate growth (if any) in the absence of β-amylase. When soluble starch was precipitated with ethanol, particles were formed quickly and increased in size rapidly with time but no precipitate was obtained with ammonium sulfate. Mean particle size reached the instrument's measuring limit within few minutes for ethanol. This indicates that the β-limit dextrins are precipitated by ethanol.

Change in relative absorbance (turbidity) with time is shown in Figure 4.6. Figure 4.6A shows that particles are larger for ethanol precipitation when starch is present and increase with time slowly to a maximum size. A decrease in mean particle size occurred in few hours after mixing. A sharp increase in absorbance was observed (Fig. 4.6B) immediately after mixing for starch dextrins fermented β-amylase with ammonium sulfate with similar results for the other treatments.

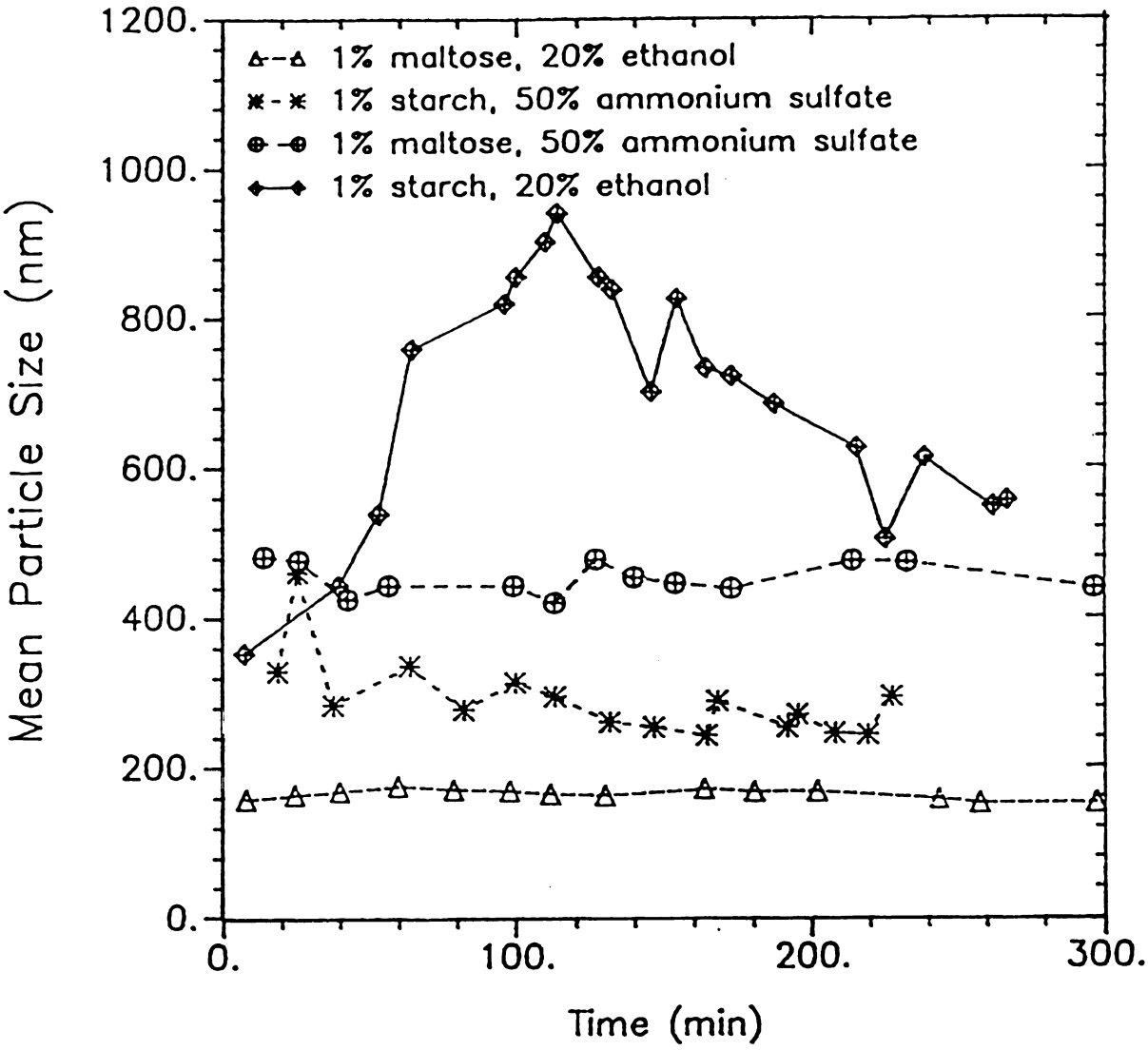

Figure 4.5. Change in mean particle size with time for β-amylase precipitate with 50% saturated ammonium sulfate and 20% (v/v) ethanol β-amylase was produced by fermentating maltose or maltodextrin and was initially concentrated by ultrafiltration

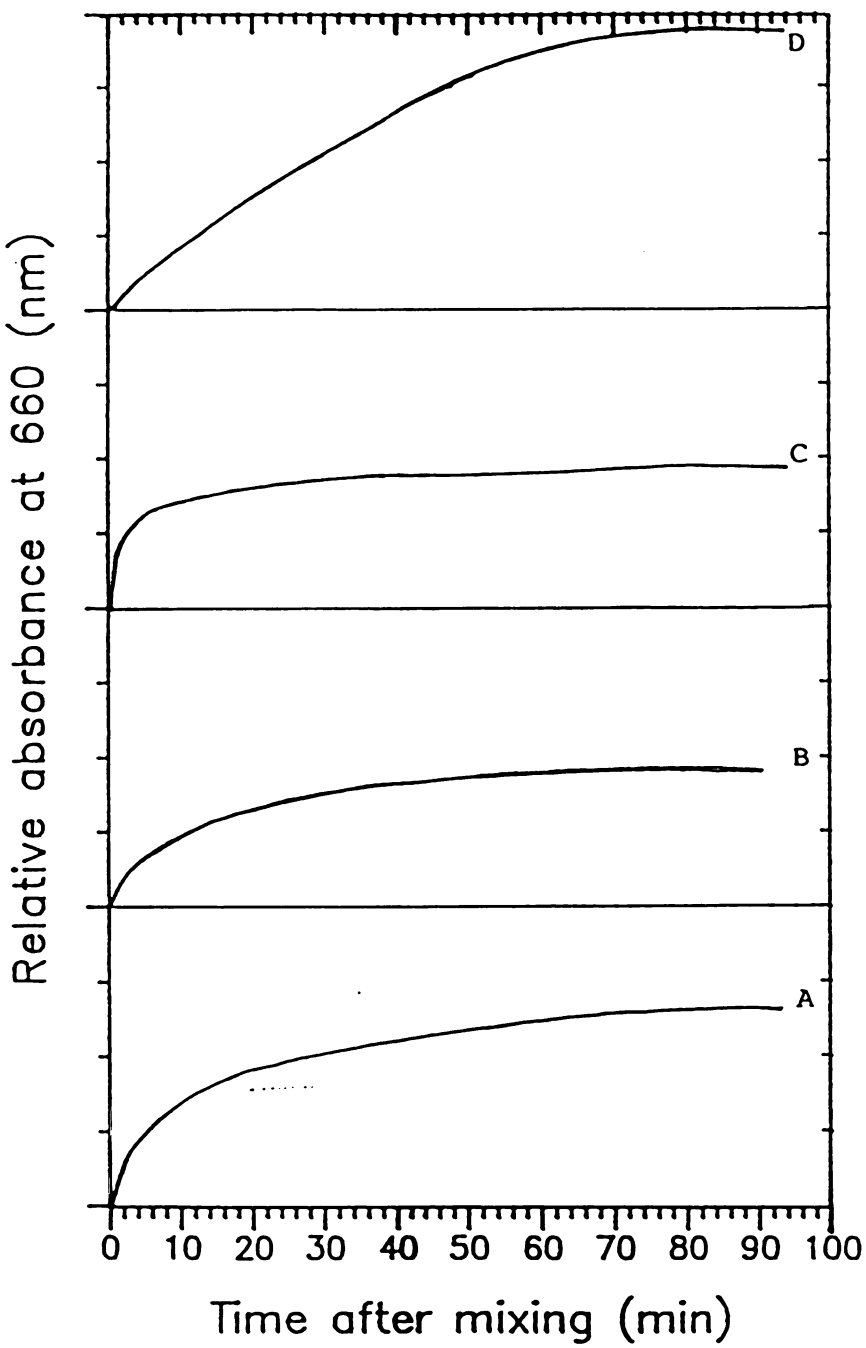

Figure 4.6. **Change in relative absorbance (turbidity) with time for β-amylase precipitate with 50% saturated ammonium sulfate and 20%(v/v) ethanol. β-amylase was produced by fermentating maltose or starch dextrins and was initially concentrated by ultrafiltration**

A-- Maltose-ammonium sulfate, B-- Maltose-ethanol
C-- Maltodextrin-ammonium sulfate D-- Maltodextrin--ethanol

Photomicroscopic pictures were taken at two different time intervals for β-amylase precipitates with ethanol. The pictures are shown in figure 4.7A and B. The breakage of protein clusters due to longer stirring is obvious in figure 4.7B.

4.4.3 Molecular weight estimation

The protein contaminants were removed by gel filtration on a Bio-Gel column. Total protein content and β-amylase activity profiles for β-amylase produced with maltose is shown in Figure 4.8. Profiles for β-amylase produced with maltrin are shown in Figure 4.9. Only one β-amylase activity fraction was obtained for β-amylase produced with maltose but two different activity fractions were obtained for β-amylase produced with maltrin. The final yield and purification for β-amylase are shown in Table 4.4.

SDS-polyacrylamide gel electrophoresis in denatured form showed a single band for β-amylase produced with both carbon sources (Figures 4.10 and 4.11). Likewise, the HPLC gel filtration column gave single peak for higher β-amylase activity fractions and are shown in Figures 4.12 to 4.14. The gel permeation purified enzyme was considered to be homogeneous by detection of a single peak on SDS-PAGE and a single peak on HPLC gel filtration column. Although the first and second maximal activity fractions from β-amylase produced with maltrin showed single peaks for HPLC gel filtration, their different retention times show that they have different effective molecular weight or size. An estimation of total carbohydrate content didn't reveal any correlation among the fractions. Carbohydrate contents are similar and distributed among all fractions. The amino acid sequences for β-amylase produced

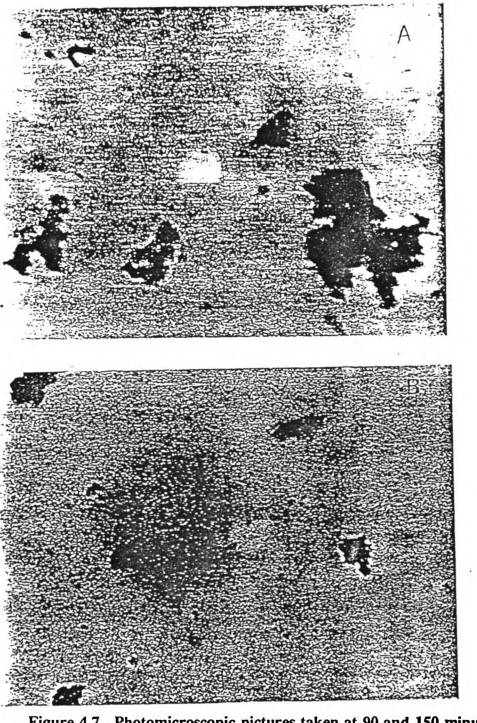

Figure 4.7. **Photomicroscopic pictures taken at 90 and 150 minutes after the addition of ethanol to β-amylase fermentation broth during precipitation studies**

TABLE 4.4

Major steps for purification of β-amylase from C. thermosulfurogenes

Steps	Total protein (mg/ml)	Total activity (U)	Purification fold	yield (%)
Original Broth	6534	69428	1.0	100
Ultrafiltration	730	55266	7.6	80
Precipitation	28	52166	179.0	75
Gel filtration	4	17765	435.0	26

Fermentation Carbon Source : 1% maltose

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Diaflo Hollow Fiber (Amicon)

Final β-amylase activity: 4562 U/mg

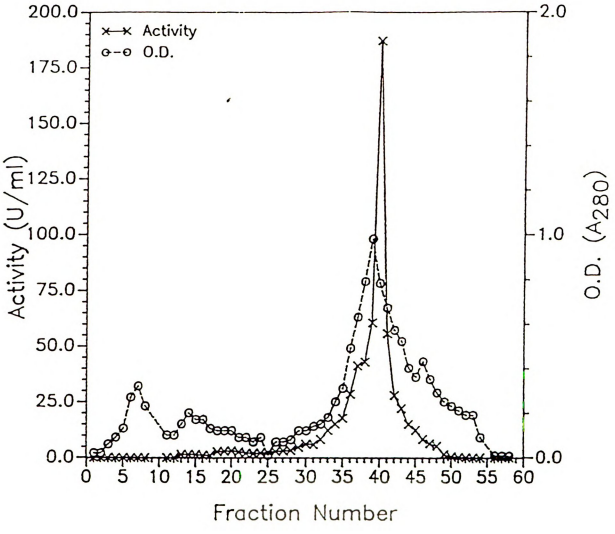

Figure 4.8. **Activity** and total protein distribution for β-amylase through a Bio-Gel column. Carbon source was maltose and β-amylase was initially concentrated by ultrafiltration and ammonium sulfate precipitation

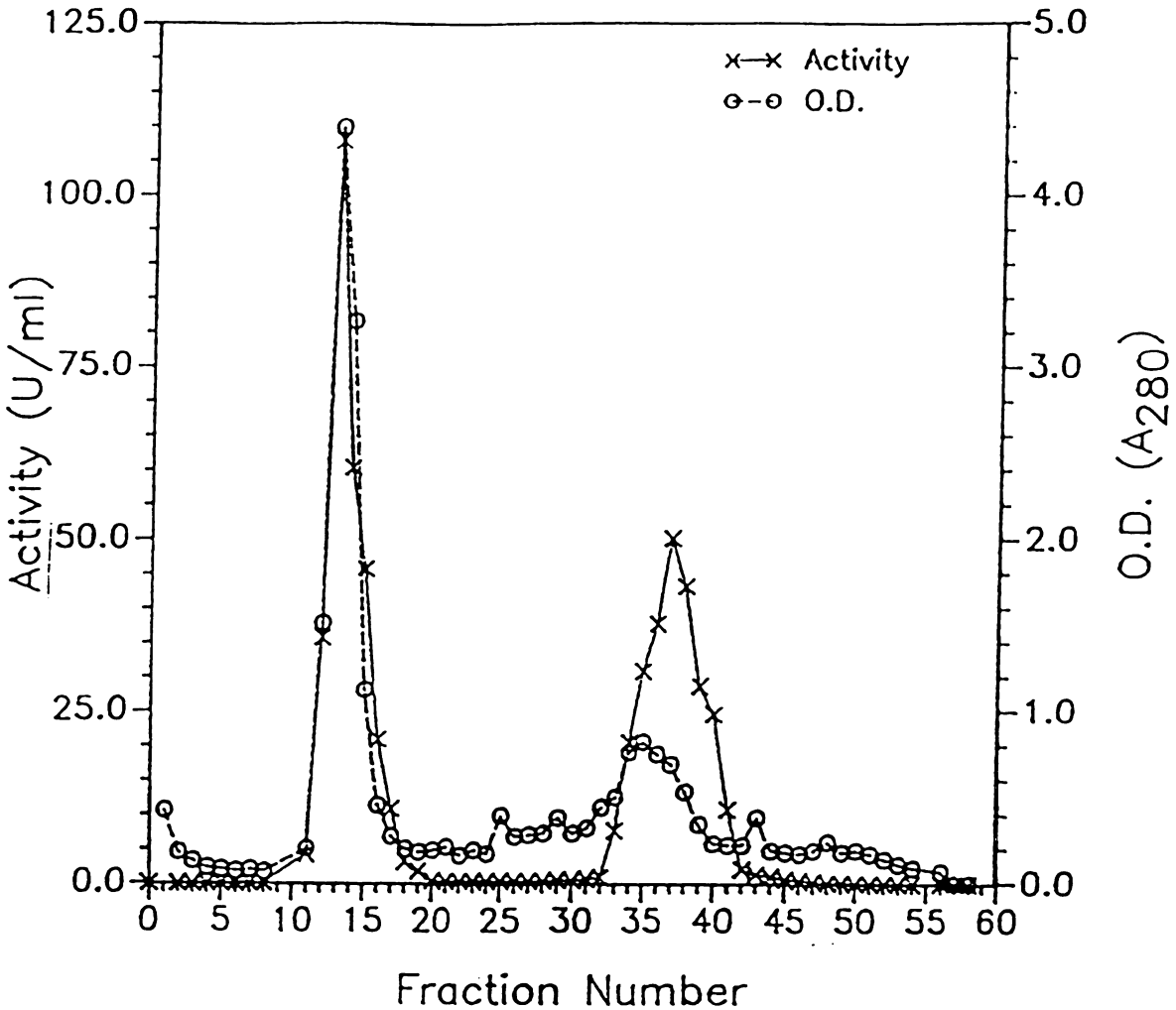

Figure 4.9. Activity and total protein distribution for β-amylase through a Bio-Gel column. Carbon source was maltodextrin and β-amylase was initially concentrated by ultrafiltration and ethanol precipitation

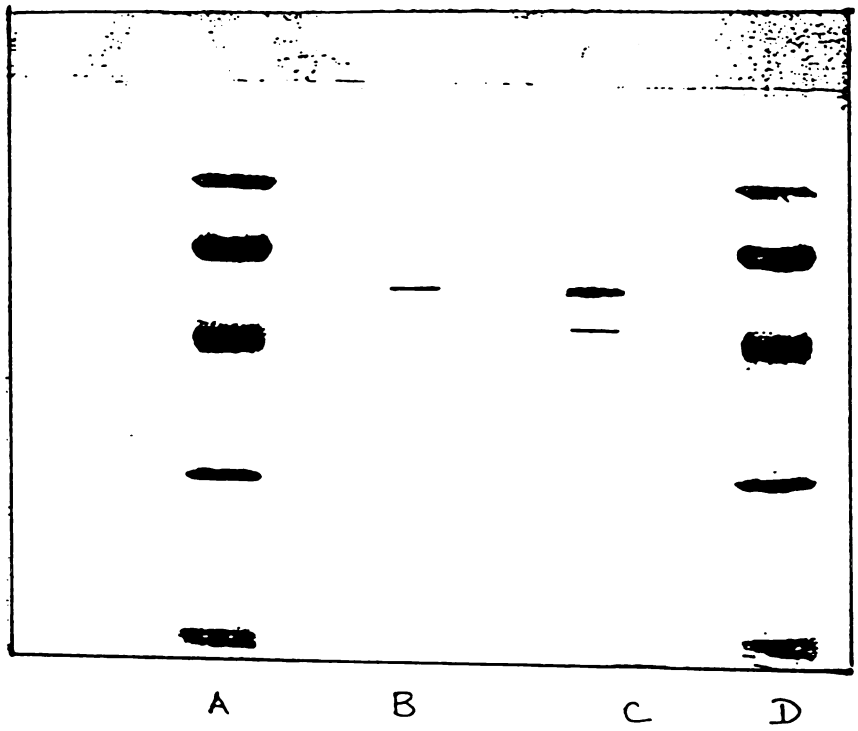

FIGURE 4.10 SDS-polyacrylamide gel electrophoresis of purified β-amylase. Carbon source was maltose. The β-amylase was electrophoresed on 10% acrylamide gel and stained with Coomassie Brilliant blue R-250. B- bio-gel filtration or C- combination of 3% ammonium sulfate and 20% ethanol; A- and D- standard marker proteins. The standards were: bovine serum albumin (66,000); egg albumin (45,000); glyceraldehyde 3-phosphate dehydrogenase (36,000); trypsinogen (24,000); carbonic anhydrous (20,000); and α-lactalbumin (14,000).

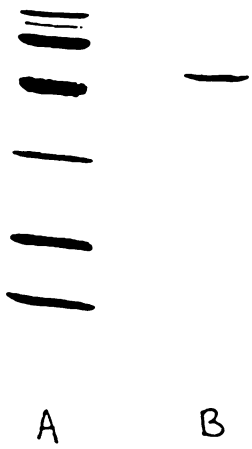

FIGURE 4.11 SDS-polyacrylamide gel electrophoresis of purified β-amylase. Carbon source was maltodextrin. The β-amylase was electrophoresed on 10% acrylamide gel and stained with Coomassie Brilliant blue R-250. B- bio-gel filtration, A- standard marker proteins. The standards were: bovine serum albumin (66,000); egg albumin (45,000); glyceraldehyde 3-phosphate dehydrogenase (36,000); trypsinogen (24,000); carbonic anhydrous (20,000); and α-lactalbumin (14,000).

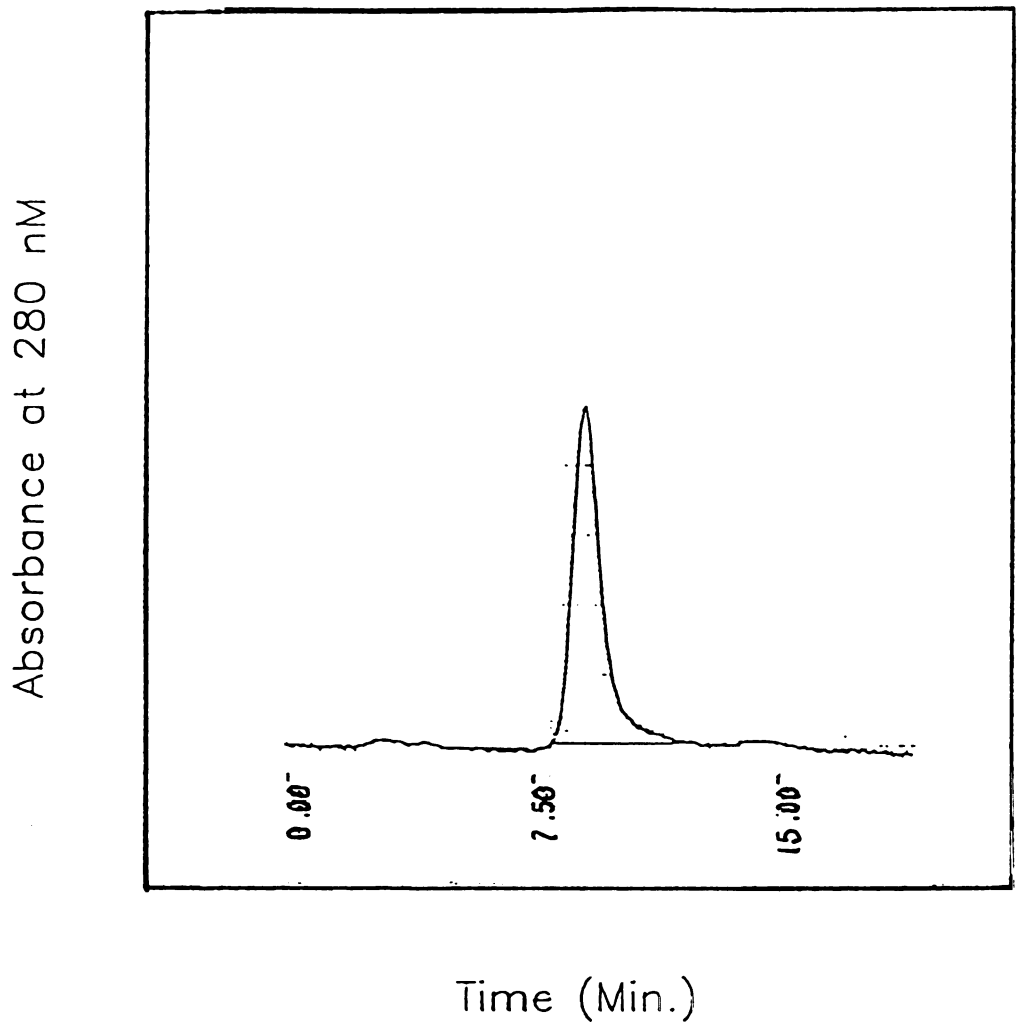

FIGURE 4.12 Separation of gel pure β-amylase (previously separated by Bio-Gel column chromatography. The dialyzed β-amylase was applied to a column (1.5cm*150cm) equilibrated with 50mM acetate buffer pH 6.0 with $CaCl_2$ and eluted with same buffer. 5ml fraction were collected and β-amylase specific activity and total protein content were assayed.) by a HPLC protein pak 300sw gel filtration column. Carbon source was maltose.

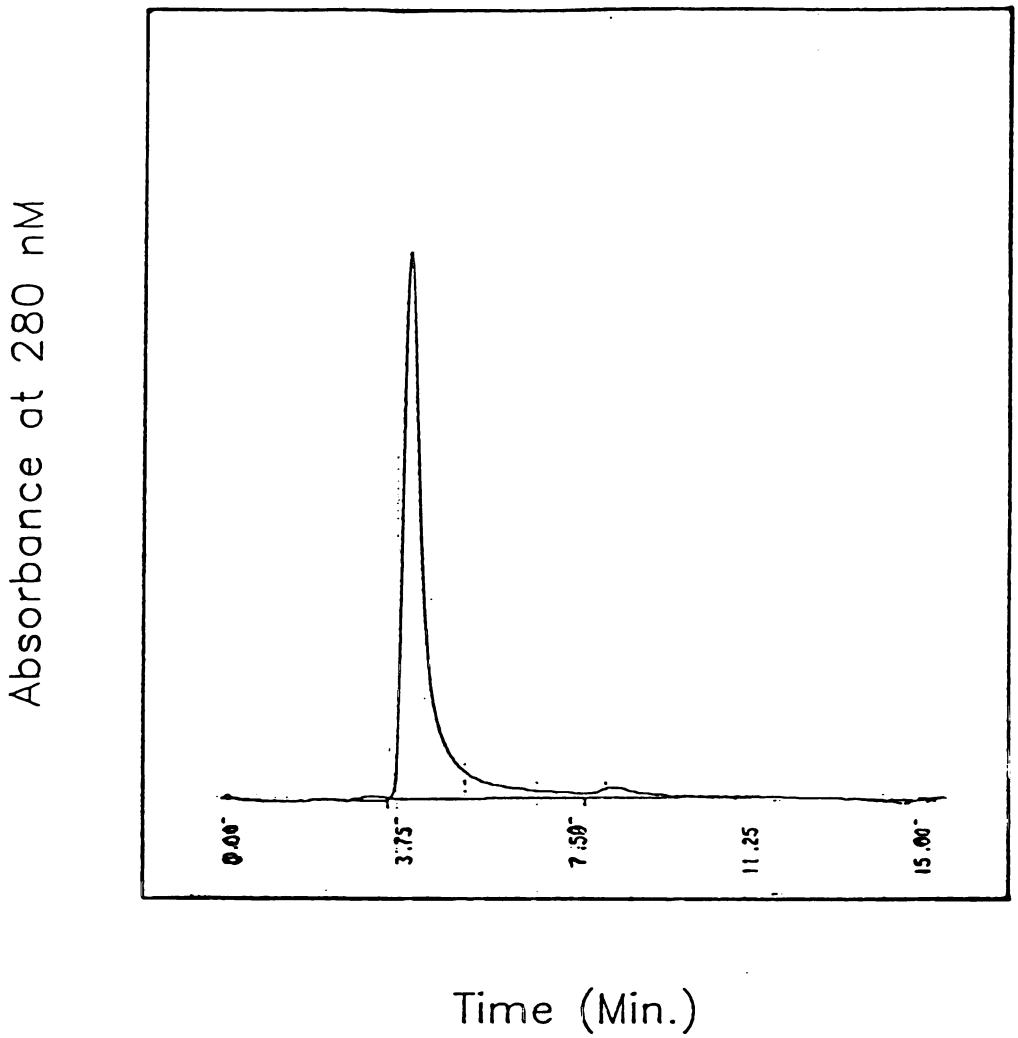

FIGURE 4.13 Separation of gel pure β-amylase (previously separated by Bio-Gel column chromatography. The dialyzed β-amylase was applied to a column (1.5cm*150cm) equilibrated with 50mM acetate buffer pH 6.0 with $CaCl_2$ and eluted with same buffer. 5ml fraction were collected and β-amylase specific activity and total protein content were assayed.) by a HPLC protein pak 300sw gel filtration column. Carbon source was maltodextrin. First peak fraction by gel filtration.

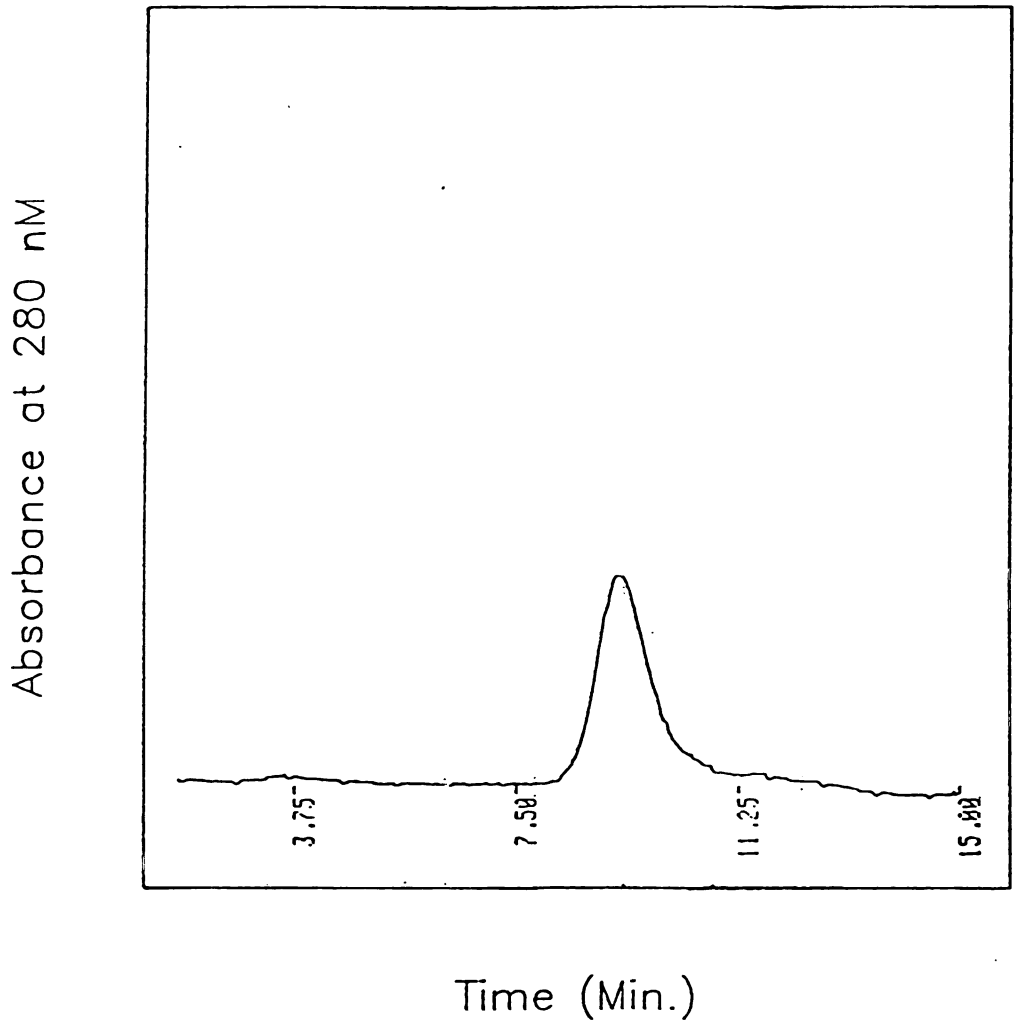

FIGURE 4.14 Separation of gel pure β-amylase (previously separated by Bio-Gel column chromatography. The dialyzed β-amylase was applied to a column (1.5cm*150cm) equilibrated with 50mM acetate buffer pH 6.0 with $CaCl_2$ and eluted with same buffer. 5ml fraction were collected and β-amylase specific activity and total protein content were assayed.) by a HPLC protein pak 300sw gel filtration column. Carbon source was maltodextrin. Second peak fraction by gel filtration.

from C.thermosulfurogenes with maltose and maltrin and from B.polymyxa are shown bellow.

	1			5			10			15				
I	Ser-	Ile-	Ala-	Pro-	Asn-	Phe-	Lys-	Val-	Phe-	Val-	Met-	Gly-	Pro-	Leu-	Glu-
II		-Ile-	Ala-		-Asn-	Phe-	Lys-	Val-	Phe-		-Met-	Gly-	Pro-	Leu-	Glu-
III	Ala-	Val-	Ala-	Asp-	Asp-	Phe-	Gln-	Ala-	Ser-	Val-	Met-	Gly-	Pro-	Leu-	Ala-

I **β-amylase produced with maltose**

II **β-amylase produced with maltrin**

III **β-amylase from B.polymyxa**

These results show that β-amylase produced from C.thermosulfurogenes is different from that produced from B.polymyxa thereby showing the sensitivity of the sequence. The use of different carbon sources didn't reveal any significant difference in sequence.

4.5 Discussion

In the preliminary experiments, the β-amylase activity and the assay results for 40% saturated ammonium sulfate precipitation showed that a considerable amount of β-amylase activity was left in the supernatant. The 50% saturated ammonium sulfate precipitation gave the best recovery and purification (Table 4.1). The supernatant of

the 50% precipitation was reprecipitated with 65% of the ammonium sulfate resulting in a precipitate with a very low activity. For ethanol precipitation 30% (V/V) ethanol showed higher recovery than for 20%, but the purification ratio was low due to the precipitation of unwanted proteins (Table 4.2). Therefore, it was concluded that 50% ammonium sulfate or 20% (v/v) ethanol were the optimum conditions for recovery and purification ratio. It was also found that there was no significant difference in protein recovery when the fermentation medium was TYE or corn steep liquor. Furthermore, no difference precipitate formation or activity recovery was found between β-amylase produced with maltose or glucose; however, the results were different from TYE or corn steep liquor. Therefore, maltose and maltrin-10 were used for the protein recovery, and activity experiments, and in fundamental kinetic studies.

Initially, fermentation broth was concentrated by spiral membrane ultrafiltration. The choice of UF with a high molecular cut off spiral cartridge resulted in relatively high fluxes. At the same time a rather large portion of small peptide proteins was removed with the filtrate. Therefore, protein specific activity was increased more than six fold during UF and centrifugation.

The effect of temperature and fermentation carbon source on total protein and activity recovery could be related to the kinetics of nucleation/agglomeration and solubilities of proteins in the broth. This effect could be explained very clearly by first explaining the particle formation and agglomeration behavior given in Figures 4.5 and 4.6. The change in mean particle size and relative absorbance (turbidity) follow similar behavior with time for corresponding precipitation curves (Figures 4.5 and 4.6). Since the light scattering instrument requires 200 seconds of sampling time it was

impossible to obtain initial particle formation (nucleation) kinetics, but these were studied by turbidity measurements (Fig. 4.6). Similar turbidity curves were obtained by Chan et al. (1986) in soy protein precipitation with ammonium sulfate and ethanol. Particle size is highly dependent on protein concentration and the concentration of precipitants. The primary particles aggregated by further collision, the final size being controlled by a balance between growth and break up, which are both shear controlled. Break up is dependent on concentration (Virkar et al., 1982 and Twineham et al., 1984), on the shear history of the particles (Bell and Dunnill, 1982), and on the degree of binding between the basic particles. In ethanol, starch dextrins cause particles to aggregate with mixing for about two hours and break up with further mixing. This phenomenon is further supported by the photomicroscopic pictures taken at 90 and 150 minutes after the ethanol addition to β-amylase solution. Figure 4.7A shows the agglomeration of the protein precipitates and 4.7B shows the breaking up of the aggregates at longer stirring time. This is why longer times of stirring resulted in less protein activity recovery for ethanol precipitation. Several mechanisms have been proposed to describe the break up of the precipitate particles (Petante and Glatz, 1982), although a satisfactory theoretical prediction is not available.

The mean particle size of the ammonium sulfate precipitate from β-amylase produced with maltose was greater than the size of the precipitate from β-amylase produced with maltrin and resulted in better protein and activity recovery for maltose fermented protein (Figures 4.1 and 4.2). An increase in solubility of the precipitate at 50°C for ammonium sulfate may have resulted in less protein recovery in Fig. 4.1. The salting-out process was inhibited by the presence of starch dextrins and β-amylase

was readily recovered at ambient temperature with 50% saturated ammonium sulfate from β-amylase produced with maltose.

The ethanol precipitated β-amylase produced with maltose had a smaller mean particle size (Figure 4.5) and relative absorbance (Figure 4.6) as compared to β-amylase produced with maltrin. A 1% soluble starch ethanol precipitate tended to aggregate very fast and reached 10mm in a few minutes. Comparing these three levels of particle size or relative absorbance (turbidity) showed that the formation of the soluble complex with starch dextrins in the fermentation broth inhibited the starch precipitation and/or enhanced the protein activity recovery. The activity recovery was enhanced by the strong binding of β-amylase to starch dextrins and the resulting low solubility of the complex in aqueous ethanol. The large difference in activity recovery for starch fermentation (Figure 4.4) and no significant difference in total protein recovery (Figure 4.3) support the notion that enzyme-starch dextrins adsorption is a biospecific adsorption. Microbial β-amylase is known to be adsorbed on starch (Hoshino et al., 1975). The addition of soluble starch to the glucose containing broth resulted in a 3 fold increase in activity recovery with ethanol as a precipitant (Table 4.3). This result confirmed the previous observations and indicated that ethanol precipitation of β-amylase in the presence of starch dextrins is a biospecific precipitation.

The major protein contaminants were separated by gel filtration. Pure β-amylase was obtained for β-amylase produced with maltose and two pure fractions were obtained from β-amylase produced with maltrin. β-amylase produced with maltose and the second fraction of β-amylase produced with maltrin show the same molecular

weights, which is about 60,000 daltons determined by HPLC gel filtration column and about 50,000 daltons in denatured form determined by SDS-PAGE. The first fraction obtained in β-amylase produced with maltrin shows about 228,000 daltons determined by HPLC gel filtration; but, the same molecular weight was obtained in the denatured form by SDS-PAGE. The difference in effective molecular weights could be explained by three different hypotheses:

1. β-amylase subunits (each 60,000 daltons) form nonstable clusters among themselves and the clusters are stabilized by the presence of dextrins, but they are separated by sodium dodecyl sulfate.
2. The dextrins present in the fermentation broth form a biospecific complex with different amounts of β-amylase subunits resulting in higher effective molecular weight complex. They are also breakable in denatured form. In a different study, 85% of β-amylase activity was adsorbed by raw starch from crude broth for β-amylase produced with maltose but very little was adsorbed for β-amylase produced with maltrin. This experiment supports that the starch dextrins binding sites are already bound with maltrin in β-amylase produced with maltrin. This phenomenon is called substrate-enzyme complexation.
3. β-amylase produced with maltrin is entirely different from that produced by maltose. Both are produced only by the micro-organism C.thermosulfurogenes. The amino acid sequencing study showed that there is no difference between both carbon sources produced β-amylase. To narrow down these hypotheses further enzymological studies and binding studies are required, and they are beyond the scope of this project.

In summary, the above results show that the β-amylase downstream purification is dependent on the carbon source (substrate) used in fermentation. Maltose or glucose fermented β-amylase results in smaller mean particle size and can be precipitated by 50% saturated ammonium sulfate. On the other hand, β-amylase produced with starch dextrins shows a larger mean particle size and is easily precipitated by 20% ethanol. Only three precipitation steps were needed to purify β-amylase to electrophoretically homogeneity. The specific activity of β-amylase purified from C.thermosulfurogenes was high (~4,000 U/mg) when compared to the enzymes from Thermoactinomyces (408 U/mg) by Obi and Odibo(1984) or from sweet potato (560 U/mg) (Bernfeld, 1955).

4.6 Conclusions

1. The mechanism of the precipitation of β-amylase with ammonium sulfate and ethanol depends on the fermentation carbon source.
2. Fifty percent saturated ammonium sulfate or twenty volume percent ethanol is the optimum concentration for the recovery and purification of C.thermosulfurogenes derived β-amylase for salting out or organic solvent precipitation, respectively.
3. Room temperature is the optimum temperature for β-amylase recovery and purification with both agents.
4. Ammonium sulfate precipitation results in higher activity yield from fermentation broths with maltose as a carbon source.
5. Starch dextrins present in the fermentation broth form a biospecific

complex with β-amylase which could be easily precipitated with ethanol.

6. Ethanol precipitation of β-amylase gives higher recoveries with starch dextrin containing broths and is a biospecific recovery process.
7. Three purification steps are enough to purify the β-amylase to homogeneity with β-amylase specific activity about 4,000 U/mg.
8. β-amylase produced with starch dextrins has higher effective molecular weight (228,000 daltons) clusters formed by β-amylase subunits.
9. About 50,000 daltons molecular weight was obtained in denatured form with maltose and maltrin determined by sodium dodcyl sulfate polyacrylamide slab gel electrophoresis.

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CHAPTER 5

IMPROVED RECOVERY AND HIGH PURIFICATION OF β-AMYLASE FROM CLOSTRIDIUM THERMOSULFUROGENES BY COMBINATION OF ORGANIC SOLVENT AND SALT PRECIPITATION

5.1 ABSTRACT

An thermostable, extracellular, solvent resistant β-amylase from C thermosulfurogenes was purified to 205 fold with 72 percent of total activity recovery by the combination of ethanol and ammonium sulfate precipitation in a batch reactor. The purification fold and the total recovery were highly dependent on the ethanol and ammonium sulfate concentration. The protein precipitates were stable and tend to aggregate with time. This alternative technique is a simple, low cost, short time process compared to other traditional purification methods.

5.2 INTRODUCTION

Major development in genetic engineering and biotechnology has successfully produced a wide spectrum of protein products for food and health industries. These new proteins are produced either by large-scale bacterial fermentation or by mammalian cell culture. The large scale fermentation of these products has increased

the need for an efficient downstream protein concentration and purification scheme. In spite of the increasing need for large-scale purification, there is very little information available on separation techniques which are more efficient to replace the traditional methods.

Precipitation of protein by a variety of reagents is an important stage in their recovery and fractionation (Bell et. al., 1983) and more than half of purification schemes use a precipitation step, mostly with ammonium sulfate (Bonnerjea, 1986). Ammonium sulfate is one of the best known neutral salts used for precipitation by salting-out and ethanol is the most widely used agent for bringing about precipitation by reduction of the dielectric constant of the medium. Ammonium sulfate presents costly disposal problems in large scale applications, but organic solvents may be used on a large scale since they can be recycled efficiently. On the other hand the tendency for alcohol-water mixtures to denature protein is also well established (Schubert and Finn, 1981).

There are advantages to the combination of salt and solvent precipitation. In organic solvent precipitation, salt itself is not the precipitating agent. The addition of small amounts of solvent and salt have opposite effects on the solubility of protein. The neutral salts, in general, increase the solubility of proteins in the presence of organic solvents. The balance between the precipitating action of organic solvents and the interaction with salts permits attainment of a variety of conditions under which the protein to be separated may be brought to any desired solubility (Cohn et. al., 1946). This balance is different at constant ethanol and salt concentration for each pH, temperature, and each protein components. This paper will focus on the

combination of two traditional protein precipitation techniques, organic solvent and salt precipitation to obtain highly purified β-amylase from C. thermosulfurogenes.

β-amylase is an exo-splitting enzyme which attacks amylose, amylopectin, and glycogen from the nonreducing terminal, resulting in the formation of maltose in the β-configuration. β-amylase is specific for α-1,4 linkages and is unable to bypass α-1,6 branch points. It is a commercially important enzyme useful for food and beverage industries. β-amylase is widely distributed in plants and also has recently been found in the microbial world. Hyun and Zeikus(1985) have developed and characterized a novel thermostable extracellular β-amylase from Clostridium thermosulfurogenes, that is stable and optimally active at 75°C (Hyun and Zeikus, 1985). Some microbial β-amylases are reported to adsorb onto raw starch (Hoshino et. al., 1975) and digest it (Ueda and Marchal, 1980).

The specific binding of an enzyme to its substrate has been extensively studied in several cases to constitute a useful purification step (Wilchek et. al., 1984). The raw starch adsorption, elution and digestion behavior of the β-amylase from C. thermosulfurogenes were studied and reported by Saha et. al. (1987). The present study will introduce an alternative method to purify β-amylase by combination of ethanol and ammonium sulfate precipitation.

5.3 MATERIAL AND METHODS

Cultivation of organism and concentration of β-amylase:

The catabolic repression resistant mutant H-12-1 derived from C. thermosulfurogenes wild strain 4B (ATCC 33743) was used (Hyun and Zeikus, 1985).

The culture was grown anaerobically in a 300 liter fermenter containing 250 liters TYE medium with 1.0% maltose as carbon source (substrate) at 60°C for 10 hours. The culture broth containing cells was initially concentrated 8 times by a 30,000 MWCO spiral ultrafiltration membrane (model DCIO, Amicon) and the cells were separated by centrifugation (10,000 rpm, 30 min). Precipitation studies were conducted in a stirred batch reactor. First, ethanol was added dropwise to the concentrated β-amylase and then ammonium sulfate was added while stirring. Each experiment was stirred for two hours at room temperature. The precipitate was collected by centrifugation (18,000 rpm, 30 min), dissolved in acetate buffer (50 mm, pH 6.0 with 5 mm $CaCl_2$), and dialyzed against the same buffer. β-amylase activity and total protein were estimated before and after each precipitation study. The purity of the β-amylase was analyzed with a high pressure liquid chromatography (HPLC) protein pak 300 SW gel filtration column and sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (Laemmli, 1970). The purity of the enzyme was compared with Bio-Gel filtered pure β-amylase. The change in mean particle size with time was measured with a Coulter N4 submicron particle analyzer. Two different particle kinetic studies, 1) 20% (V/V) ethanol with 5% ammonium sulfate, 2) 30% ammonium sulfate with no ethanol, were conducted and compared.

Enzyme assays:

β-amylase activity was assayed using a 5ml reaction mixture consisting of 2% boiled soluble starch solution, acetate buffer (50 mm, pH 6.0 with 5 mm $CaCl_2$) and appropriately diluted enzyme solution. After incubation for 30 minutes at 60°C, the reducing sugar released was measured by the dinitrosalicylic acid method (Miller,

1969). One unit of β-amylase activity is defined as the amount of enzyme that releases 1 mM mole of reducing sugar as maltose per minute under the above conditions. Total protein was estimated by the method of Lowry et. al., with bovine serum albumin as standard (Lowry et. al., 1951).

5.4 RESULTS

Table 5.1 summarizes β-amylase specific activity, total activity recovery, and one step purification fold for different concentrations of ethanol and ammonium sulfate. 20% ethanol with 3% ammonium sulfate precipitation gave about seventy-eight percent β-amylase activity recovery with 31 times purification in a single purification step. Purity of β-amylase for the above combination was analyzed by gel filtration and the results are shown in figure 5.1. β-amylase purified by gel filtration is shown in figure 5.2. Comparing these two figures only a small amount of impurity is present in the combination of precipitation. For the same experiment SDS-PAGE also shows a small amount of impurity (figure 5.3). The effect of agglomeration/aggregation after addition of a combination of precipitants and precipitation only by ammonium sulfate, are compared in figures 5.4. Particles were stable and tended to grow or aggregate with time for the combination of precipitation (figure 5.4) but particles were formed immediately after mixing and maintained their size throughout for ammonium sulfate precipitated β-amylase. Specific activity and recovery results for each purification step involved in β-amylase partial purification is given Table 5.2.

TABLE 5.1

Precipitation of β-amylase with combinations of ethanol and ammonium sulfate. The activity recovery and purification fold for different concentration of ethanol and ammonium sulfate precipitate.

Ethanol conc. (%)	Ammonium Sulfate (%)	Specific Activity (U/mg)	Purification ratio	Activity Recovery (%)
0	30	2.37	8.3	93
20	0	125	4.4	23
10	5	298	10.4	11
10	10	322	11.3	22
10	20	785	27.5	88
20	3	889	31.0	78
20	5	867	29.7	59
20	10	661	23.2	40
20	20	442	15.4	60
30	10	25.3	--	5
30	20	14.2	--	3

β-amylase with cells was initially concentrated 8 times by 30,000 MWCO spiral UF membranes and the cells were separated by centrifugation (10,000 rpm, 30 min) prier to precipitation.

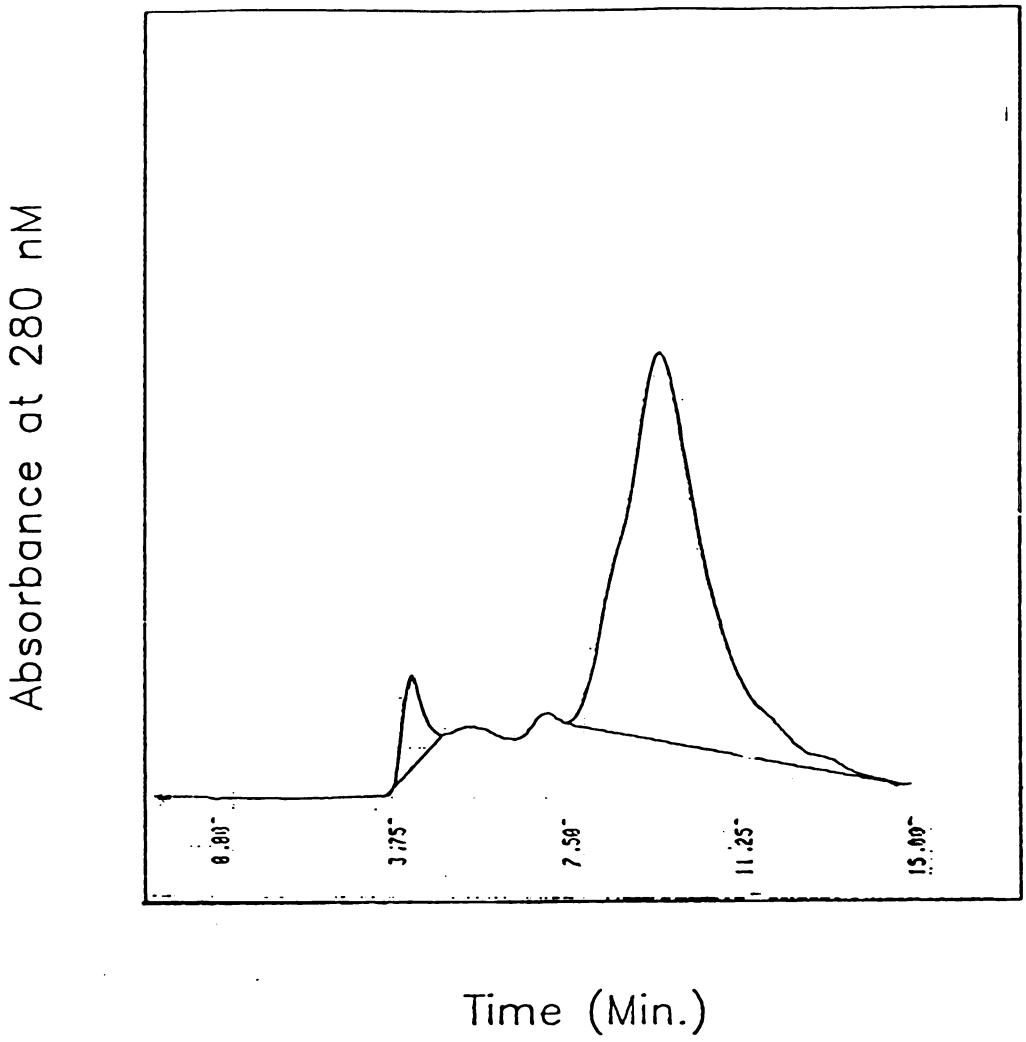

FIGURE 5.1 **Separation of β-amylase (previously precipitated with 3% ammonium sulfate and 20% of ethanol) by a HPLC protein pak 300sw gel filtration column. Area under the smaller peak shows the amount of impurity left with β-amylase.**

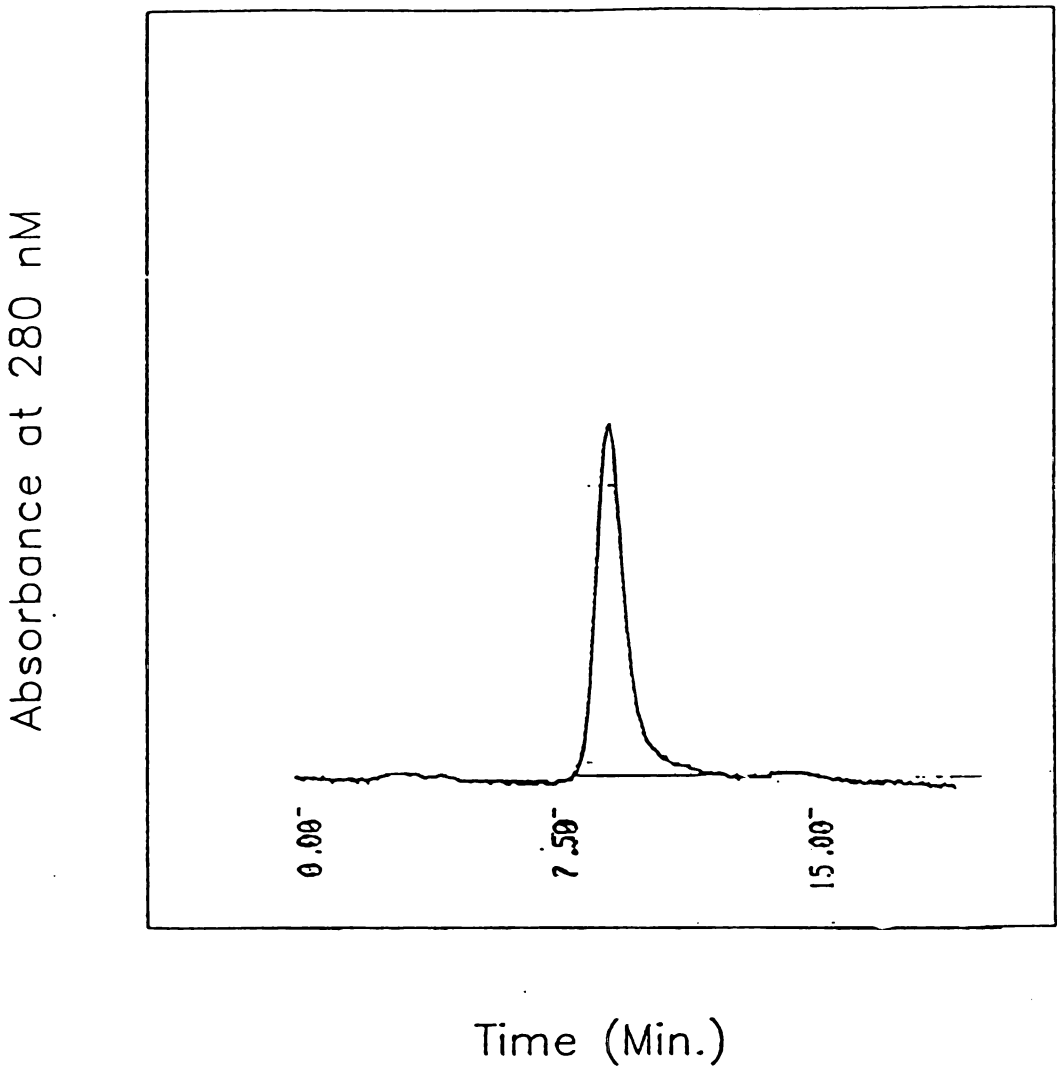

FIGURE 5.2 Separation of gel pure β-amylase (previously separated Bio-Gel column chromatography of β-amylase. The dialyzed enzyme was applied to a column (1.5cm*150cm) equilibrated with 50mM acetate buffer pH 6.0 with $CaCl_2$ and eluted with same buffer. 5ml fraction were collected and β-amylase specific activity and total protein content were assayed.) by a HPLC protein pak 300sw gel filtration column.

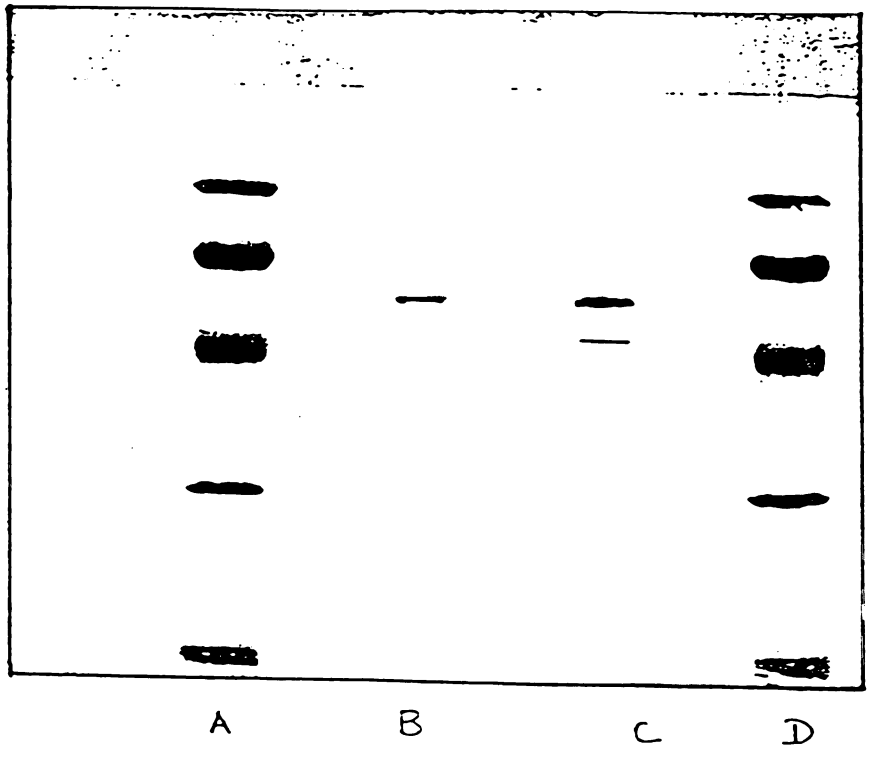

FIGURE 5.3 SDS-polyacrylamide gel electrophoresis of purified β-amylase. The β-amylase was electrophoresed on 10% acrylamide gel and stained with Coomassie Brilliant blue R-250. B- bio-gel filtration or C- combination of 3% ammonium sulfate and 20% ethanol; A- and D- standard marker proteins. The standards were: bovine serum albumin (66,000); egg albumin (45,000); glyceraldehyde 3-phosphate dehydrogenase (36,000); trypsinogen (24,000); carbonic anhydrous (20,000); and α-lactalbumin (14,000).

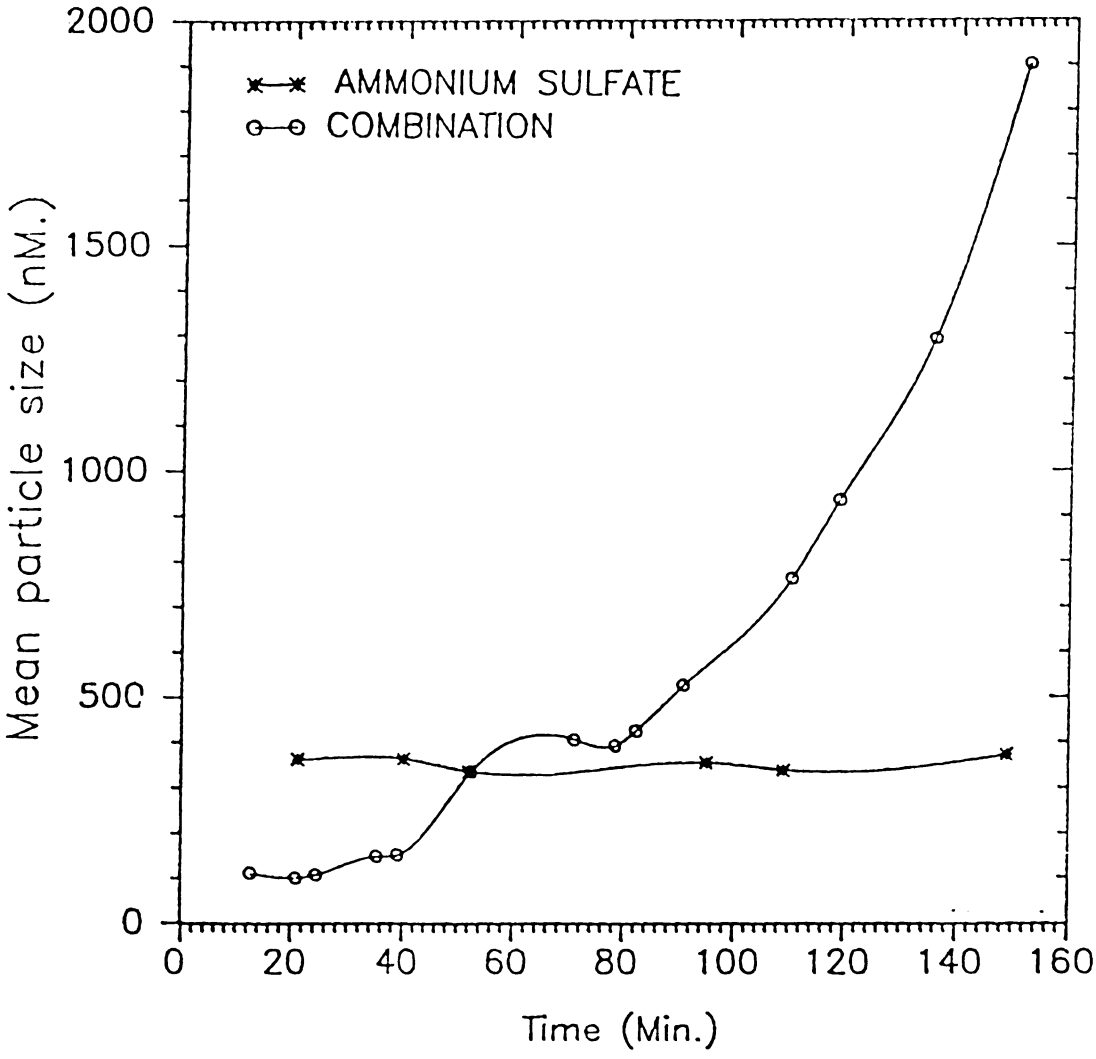

FIGURE 5.4 Change in mean particle size with time for β-amylase precipitated by a combination of 3% ammonium sulfate and 20% ethanol and by 50% ammonium sulfate alone. A Coulter Electronics Model N4 sub-micron particle analyzer with 200 seconds auto sampling time was used in all the experiments.

TABLE 5.2

A comparison of partial purification of β-amylase from C.thermosulfurogenes by various methods

Step	specific Activity (U/mg)	Protein Conc. in Supernatant (mg/ml)	Recovery (%)	Purification Fold
β-amylase Cultured broth	4.33	2.93	100	1
Concentration by UF	28.6	3.25	92	6.60
Precipitation by Combination	889	0.41	72	205
Precipitation only by ammonium sulfate *	237	1.40	86	54.7
Precipitation only by ethanol	125	0.49	21	28.9

* 20% ethanol and 3% ammonium sulfate precipitate

5.5 DISCUSSION

The extracellular β-amylase produced by C thermosulfurogenes was easily purified to near homogeneity with 72 percent activity recovery and with 205 purification fold by the combination of ethanol and ammonium sulfate precipitation. About 30 fold purification with 78 percent recovery was obtained in a simple batch precipitation step. The purity of the β-amylase is shown in figures 5.1 and 5.2, and compared with the highly pure β-amylase obtained by gel filtration chromatography (figure 5.2). These results suggest that only a small amount of impurity is present in the β-amylase after precipitation. The combination effect of salt and solvent is not well characterized but it was suggested that the opposite effects on the solubility of proteins makes it possible to adjust the solubility of a protein to a convenient value by balancing the solvent action of salt with the precipitating action of the organic solvent. Different amounts of salt and solvent have produced different results in precipitation (Table 5.1). Mean particles were stabilized by the presence of ammonium sulfate and increased in size for longer stirring (figure 5.4). This result is different from the ammonium sulfate precipitation.

In summary, the combination of organic salt and solvent precipitation is a very efficient, simple, cheap, and short time purification process. Four times more purification fold can be obtained than precipitation by ammonium sulfate alone in the present case. In a different study, only 91 fold purification with 64 percent recovery was obtained for β-amylase partial purification by ethanol and followed by raw starch adsorption and soluble starch elution in a column (Saha and Zeikus, 1988). Comparing these results, the combination of ethanol and ammonium sulfate

precipitation is better for purification of β-amylase from low activity broth to higher activity. This is also a simple, fast, and low cost purification process. All these experiments were conducted at room temperature with different amounts of salts and solvents. The purification can be further optimized by conducting the precipitation at different temperature and pH.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

6.1 Conclusions

From fermentation and concentration studies (Ch. 3):

1. A simple scale-up in β-amylase production has no influence in mass transfer rate or final β-amylase activity.
2. Production time of β-amylase in a batch fermenter is eight to twelve hours and can be reduced by inoculating with a seed culture at its exponential growth stage.
3. Concentration of β-amylase with 30,000 mwco UF membrane yields more than 85% activity recovery with ten fold purification for all four carbon sources used in the β-amylase production.
4. β-amylase produced by maltrin fermentation yields about 90% activity recovery with 100,000 MWCO UF membrane while β-amylase produced by maltose fermentation yields only about 50% activity recovery with the same membrane. Using a bigger MWCO membrane reduce the process time and cost a great deal.

From precipitation and molecular weight estimation studies (Ch. 4):

1. The mechanism of the precipitation of β-amylase with ammonium sulfate and ethanol depend on the fermentation carbon source.
2. Fifty percent saturated ammonium sulfate or twenty volume percent ethanol is the optimum concentration for the recovery and purification of C.thermosulfurogenes derived β-amylase for salt and organic solvent precipitation, respectively.
3. Room temperature is the optimum temperature for β-amylase recovery and purification with both agents.
4. Ammonium sulfate precipitation results in higher activity yield from fermentation broths with maltose as a carbon source.
5. Starch dextrins present in the fermentation broth form a biospecific complex with β-amylase which can be easily precipitated with ethanol.
6. Ethanol precipitation of β-amylase gives higher recoveries with starch dextrin containing broths and is a biospecific recovery process.
7. Three purification steps are enough to purify the β-amylase to homogeneity with β-amylase specific activity of about 4,000 U/mg.
8. Maltrin fermented β-amylase has a higher effective molecular weight (228,000 daltons) clusters formed by β-amylase subunits.
9. About 50,000 daltons molecular weight was obtained in denatured form with maltose and maltrin determined by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis.

10. β-amylase purified by gel filtration shows higher specific activity compare to another highly purified β-amylases from Thermoactinomycel or from sweet potato.

From combination of precipitation studies (Ch. 5):

1. β-amylase could be easily purified to near homogeneity by combination of 20% ethanol and 3% ammonium sulfate batch precipitation.
2. Combination of organic solvent and salt precipitation is a very efficient, simple and low cost purification process, and has great potential in protein purification.

6.2 Proposals for future research

1. More ultrafiltration studies are necessary with pure β-amylase to further understand the β-amylase-substrate complex formation. This can be performed by adding known amount of dextrin into pure β-amylase produced with maltose and then concentrating with 100,000 MWCO UF membrane. The increase in recovery in the concentrate could be related to the complex formation.
2. More light scattering and turbidity studies are necessary to predict the agglomeration/break up mechanism in ethanol precipitated β-amylase (produced by maltrin fermentation) and in the combination of ethanol and ammonium sulfate precipitation.

3. Further studies are necessary to optimize temperature, pH, and initial β-amylase concentration for combination of salt and solvent precipitation. This will lead to a simple purification process of proteins.
4. The combination of precipitation techniques should be applied to other proteins.

TABLE A.1

Components used in fermentation to produce β-amylase

Components	Amount per Liter
NH_4Cl	1.0 gram
KH_2PO_4	0.3 gram
$Na_2HPO_4.7H_2O$	2.1 gram
$MgCl_2.6H_2O$	0.2 gram
Trypticase	10.0 gram
Yeast Extract	3.0 gram
Vitamin Solution	10.0 ml
Trace Mineral	10.0 ml
$FeSO_4$ (2.5%)	0.25 ul
Resazurin	1.0 ml
Maltose/ maltrin	10.0 gram

TABLE A.2 RAW DATA

ULTRAFILTRATION STUDIES #1

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	1520	13.0	4.15	100
Concentrate	220	80.0	4.07	90
Permeate	1300	3.0	4.32	--

Fermentation Carbon Source : 1% maltose

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Diaflo Hollow Fiber (Amicon)

Purification Fold: 7.0

TABLE A.3 RAW DATA

ULTRAFILTRATION STUDIES #2

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	110,000	45.0	4.34	100
Concentrate	3,000	1396.0	8.18	85
Permeate	107,000	8.1	----	--

Fermentation Carbon Source : 1% maltose

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Diaflo Hollow Fiber (Amicon)

Purification fold: 16.5

TABLE A.4 RAW DATA

ULTRAFILTRATION STUDIES #3

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	11,000	26.7	4.46	100
Concentrate	1,200	216.0	3.45	88
Permeate	9,800	3.0	4.12	--

Fermentation Carbon Source : 1% soluble starch

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Spiral (Amicon)

Purification Fold: 10.5

TABLE A.5 RAW DATA

ULTRAFILTRATION STUDIES #4

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	12,000	25.93	3.03	100
Concentrate	840	315.72	2.88	85
Permeate	11,160	---	----	--

Fermentation Carbon Source : 1% Maltrin-40

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Spiral (Amicon)

Purification Fold: 12.7

TABLE A.6 RAW DATA

ULTRAFILTRATION STUDIES #5

Concentration and purification of β-amylase by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	8,500	27.8	4.19	100
Concentrate	740	271.4	3.59	85
Permeate	7,760	---	----	--

Fermentation Carbon Source : 1% Maltrin-100

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Spiral (Amicon)

Purification Fold: 11.4

TABLE A.7 RAW DATA

ULTRAFILTRATION STUDIES #6

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	2,000	213.3	3.81	100
Concentrate	200	2080.0	8.39	90
Permeate	1,800	19.0	2.70	--

Fermentation Carbon Source : 1% Maltrin-100

Membrane Size: 100,000 MWCO Cartridge

Membrane Type: Diaflo Hollow Fiber (Amicon)

Purification Fold: 4.5

TABLE A.8 RAW DATA

ULTRAFILTRATION STUDIES #7

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	21,000	216.38	3.79	100
Concentrate	1,600	2496.0	16.70	86
Permeate	20,000	----	----	--

Fermentation Carbon Source : 1% Maltrin-100

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Spiral (Amicon)

Purification Fold: 2.6

TABLE A.9 RAW DATA

ULTRAFILTRATION STUDIES #8

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	1027	6.79	3.26	100
Concentrate	127	19.22	3.60	35
Permeate	900	3.97	2.70	--

Fermentation Carbon Source : 1% maltose

Membrane Size: 100,000 MWCO Cartridge

Membrane Type: Diaflo Hollow Fiber (Amicon)

Purification Fold: 2.7

TABLE A.10 RAW DATA

ULTRAFILTRATION STUDIES #9

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	2075	34.0	3.23	100
Concentrate	225	150.0	3.18	50
Permeate	1850	10.0	4.03	--

Fermentation Carbon Source : 1% maltose

Membrane Size: 100,000 MWCO Cartridge

Membrane Type: Diaflo Hollow Fiber (Amicon)

Purification Fold: 4.7

TABLE A.11 RAW DATA

ULTRAFILTRATION STUDIES #10

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Yield (%)
Original Broth	94,000	34.0	3.21	100
Concentrate	6,000	471.0	3.34	88
Permeate	88,000	3.0	----	--

Fermentation Carbon Source : 1% maltose

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Spiral (Amicon)

Purification Fold: 14.0

TABLE A.12 RAW DATA

ULTRAFILTRATION STUDIES #10

Concentration and purification of β-amylase with cells by ultrafiltration

	Volume (ml)	Activity (U/ml supernatant)	Protein conc. (mg/ml)	Yield (%)
Original Broth	2042	34.0	---	100
Concentrate	192	288.0	---	80
Permeate	1850	10.0	---	--

Fermentation Carbon Source : 1% maltose

Membrane Size: 30,000 MWCO Cartridge

Membrane Type: Diaflo Hollow Fiber (Amicon)

Purification Fold: 7.6

TABLE A.13 RAW DATA

PRECIPITATION STUDIES #1

Precipitation of β-amylase by 50% saturated ammonium sulfate or 20 % (v/v) ethanol

	Activity (U/ml supernatant)	Protein conc. (mg/ml)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	135.2	3.70	1.0	100
Concentrate (ammonium sulfate)	254.8	1.07	6.47	94
Concentrate (ethanol)	61.5	0.49	3.43	23

Fermentation Carbon Source : 1% maltose

Original volume: 50.0 ml

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.14 RAW DATA

PRECIPITATION STUDIES #2

Precipitation of β-amylase by 50% saturated ammonium sulfate or 20 % (v/v) ethanol

	Activity (U/ml supernatant)	Protein conc. (mg/ml)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	332.1	3.60	1.0	100
Concentrate (ammonium sulfate)	149.8	0.72	2.25	15
Concentrate (ethanol)	659.4	1.30	5.50	80

Fermentation Carbon Source : 1% maltrin

Original volume: 75.0 ml

Sttirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

Purification ratio = (final enzyme activity (U/mg)/ initial enzyme activity (U/mg)

TABLE A.15 RAW DATA

PRECIPITATION STUDIES #3

Precipitation of β-amylase by 50% saturated ammonium sulfate or 20 % (v/v) ethanol

	Total Protein (mg)	Total Activity (Units)	Purification Ratio	Yield (%)
Concentrated β-amylase	871.2	22275.0	1.0	100
Concentrate (ammonium sulfate)	125.7	21875.0	7.0	98
Concentrated β-amylase	594.0	15186.0	1.0	100
Concentrate (ethanol)	35.1	6636.0	7.2	45

Fermentation Carbon Source : 1% maltrin

Original volume: 50.0 ml for ammonium sulfate ppt.
75.0 ml for ethanol ppt.

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

Purification ratio = (final enzyme activity (U/mg)/ initial enzyme activity (U/mg)

TABLE A.16 RAW DATA

PRECIPITATION STUDIES #4

Precipitation of β-amylase by 20 % (v/v) ethanol at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	141.0	18802.0	1.0	100
Precipitate at 4°C	18.7	6908.0	2.75	37
Precipitate at 25°C	13.7	4021.0	2.25	21
Precipitate at 35°C	15.7	2626.0	1.24	14
Precipitate at 5°C	53.9	6578.0	0.90	34
Supernatant at 4°C	116.5	10149.0	----	--
Supernatant at 25°C	107.5	12628.0	----	--
Supernatant at 35°C	124.9	11162.0	----	--
Supernatant at 5°C	58.2	13260.0	----	--

Fermentation Carbon Source : 1% maltose
Original volume: 75.0 ml
Stirring time during precipitation: 120 min
Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.17 RAW DATA

PRECIPITATION STUDIES #5

Precipitation of β-amylase by 20 % (v/v) ethanol at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	205.0	29823.0	1.0	100
Precipitate at 4°C	21.5	9345.0	2.90	31
Precipitate at 25°C	28.4	5620.0	1.40	19
Precipitate at 35°C	24.8	7850.0	2.20	23
Precipitate at 50°C	49.8	6986.0	0.54	34
Supernatant at 4°C	165.9	21819.0	----	--
Supernatant at 25°C	167.3	22584.0	----	--
Supernatant at 35°C	158.9	23599.0	----	--
Supernatant at 50°C	99.4	19782.0	----	--

Fermentation Carbon Source : 1% maltose
Original volume: 75.0 ml
Stirring time during precipitation: 120 min
Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.18 RAW DATA

PRECIPITATION STUDIES #6

Precipitation of β-amylase by 20 % (v/v) ethanol at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	253.9	25387.0	1.0	100
Precipitate at 4°C	53.2	11948.0	2.2	47
Precipitate at 25°C	57.0	22762.0	4.0	90
Precipitate at 35°C	76.1	18180.0	2.4	72
Precipitate at 50°C	46.7	12850.0	2.7	57
Supernatant at 4°C	206.8	7665.0	----	--
Supernatant at 25°C	195.4	3523.0	----	--
Supernatant at 35°C	187.0	6390.0	----	--
Supernatant at 50°C	193.4	7247.0	----	--

Fermentation Carbon Source : 1% maltrin
Original volume: 75.0 ml
Stirring time during precipitation: 120 min
Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.19 RAW DATA

PRECIPITATION STUDIES #7

Precipitation with 50 % sat. ammonium sulfate at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	187.6	15204.0	1.0	100
Precipitate at 4°C	89.4	9395.0	1.3	62
Precipitate at 25°C	80.2	8147.0	1.3	54
Precipitate at 35°C	91.6	9883.0	1.3	65
Precipitate at 50°C	100.6	14040.0	1.7	92
Supernatant at 4°C	82.7	8997.0	----	--
Supernatant at 25°C	82.1	8323.0	----	--
Supernatant at 35°C	83.6	8273.0	----	--
Supernatant at 50°C	81.6	7140.0	----	--

Fermentation Carbon Source : 1% maltrin .
Original volume: 50.0 ml
Stirring time during precipitation: 120 min
Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.20 RAW DATA

PRECIPITATION STUDIES #8

Precipitation of β-amylase by 50 % sat. ammonium sulfate at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	187.6	15204.0	1.0	100
Precipitate at 4°C	74.1	10009.0	1.7	66
Precipitate at 25°C	75.3	9027.0	1.5	59
Precipitate at 35°C	80.1	10170.0	1.6	67
Precipitate at 50°C	78.2	11237.0	1.8	78
* Precipitate at 4°C	83.6	13068.0	1.9	86
* Precipitate at 25°C	86.6	10562.0	1.5	70

Fermentation Carbon Source : 1% maltrin

Original volume: 50.0 ml

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

* Precipitation was conducted in a baffled reactor

TABLE A.21 RAW DATA

PRECIPITATION STUDIES #9

Precipitation of β-amylase by 50 % sat. ammonium sulfate at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	155.6	7367.5	1.0	100
Precipitate at 25°C	56.1	5072.5	2.0	69
Precipitate at 35°C	43.3	7043.0	3.5	96
Precipitate at 50°C	42.0	7246.0	3.7	98

Fermentation Carbon Source : 1% maltrin

Original volume: 50.0 ml

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.22 RAW DATA

PRECIPITATION STUDIES #10

Precipitation of β-amylase by 20 % (v/V) ethanol at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	259.5	14897.0	1.0	100
Precipitate at 4°C	50.9	8647.3	3.0	58
Precipitate at 25°C	29.8	10617.9	6.2	72
Precipitate at 40°C	36.4	10080.5	4.8	68
Precipitate at 50°C	65.4	9788.7	2.6	66

Fermentation Carbon Source : 1% maltrin

Original volume: 75.0 ml

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.23 RAW DATA

PRECIPITATION STUDIES #11

Precipitation of β-amylase by 50 % saturated ammonium sulfate at different temperatures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	340.0	12721.0	1.0	100
Precipitate at 4°C	241.0	12561.0	1.4	98.7
Precipitate at 20°C	265.6	11465.9	1.3	99.0
Precipitate at 40°C	268.4	11465.5	1.2	90.0
Precipitate at 50°C	274.2	11232.7	1.1	88.0

Fermentation Carbon Source : 1% maltose

Original volume: 50.0 ml

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.24 RAW DATA

PRECIPITATION STUDIES #12

Precipitation of β-amylase by 50 % saturated ammonium sulfate at different temeratures.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	201.8	7345.0	1.0	100
Precipitate at 4°C	170.4	6878.0	1.1	93
Precipitate at 25°C	174.4	7291.0	1.2	99
Precipitate at 35°C	185.5	7156.0	1.1	97
Precipitate at 50°C	180.0	5856.0	1.0	79

Fermentation Carbon Source : 1% maltose

Original volume: 50.0 ml

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.25 RAW DATA

PRECIPITATION STUDIES #13

Precipitation of β-amylase by different concentration of ethanol at room temperature.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	331.3	31275.0	1.0	100
Precipitate with 10% ethanol	54.0	7884.0	1.8	25
Precipitate with 20% ethanol	57.1	8394.0	1.8	27
Precipitate with 30% ethanol	68.0	8798.0	1.6	28
Precipitate with 40% ethanol	105.0	8629.0	1.0	28
Precipitate with 50% ethanol	150.4	7069.0	0.6	23
Supernatant, 10%	285.5	20767.0	----	--
Supernatant, 20%	275.3	20232.0	----	--
Supernatant, 30%	267.1	23304.0	----	--
Supernatant, 40%	204.1	22464.0	----	--
Supernatant, 50%	106.1	25082.0	----	--

Fermentation Carbon Source : 1% maltose
Original volume: 75.0 ml
Stirring time during precipitation: 120 min
Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.26 RAW DATA
PRECIPITATION STUDIES #14

Precipitation of β-amyalse by different concentration of ethanol at room temperature.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	253.9	25387.0	1.0	100
Precipitate with 10% ethanol	29.4	7621.0	2.6	30
Precipitate with 20% ethanol	56.9	22762.0	4.0	90
Precipitate with 30% ethanol	98.3	11745.0	1.8	70
Precipitate with 40% ethanol	75.0	18245.0	2.5	72
Precipitate with 50% ethanol	70.1	12745.0	1.8	50
Supernatant, 10%	223.1	15292.0	----	--
Supernatant, 20%	195.4	3523.0	----	--
Supernatant, 30%	204.0	6576.0	----	--
Supernatant, 40%	207.0	9477.0	----	--
Supernatant, 50%	213.1	9040.0	----	--

Fermentation Carbon Source : 1% maltrin
Original volume: 75.0 ml
Stirring time during precipitation: 120 min
Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.27 RAW DATA

PRECIPITATION STUDIES #15

Precipitation of β-amylase by different concentration of ethanol at room temperature.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	259.5	14897.0	1.0	100
Precipitate with 20% ethanol	29.8	10618.0	6.2	72
Precipitate with 30% ethanol	53.8	13019.0	4.2	87
Precipitate with 40% ethanol	90.5	10952.0	4.2	74
Precipitate with 20% ethanol 4 hrs. stirring	34.5	13567.0	7.0	91
Precipitate with 30% ethanol 4 hrs. stirring	61.8	13548.0	3.6	50

Fermentation Carbon Source : 1% maltrin

Original volume: 75.0 ml

Stirring time during precipitation: 120 min

Centrifucation time: 30.0 min at 12,000 rpm

TABLE A.28 RAW DATA

PRECIPITATION STUDIES #16

Precipitation with different concentration of ethanol at room temperature.

	Total Protein (mg)	Total Activity (Units)	Purific-cation Ratio	Yield (%)
Concentrated β-amylase	220.9	13919.0	1.0	100
Precipitate with 10% ethanol	17.8	2562.0	1.5	19
Precipitate with 20% ethanol	26.4	11794.0	7.1	85
Precipitate with 30% ethanol	32.2	12410.0	5.4	89
Precipitate with 40% ethanol	36.5	11482.0	5.0	82
Precipitate with 50% ethanol	37.9	11219.0	4.7	80

Fermentation Carbon Source : 1% soluble starch

Original volume: 75.0 ml

Stirring time during precipitation: 120 min

Centriufcation time: 30.0 min at 12,000 rpm