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The Concentration and Purification of α-Amylase Using A Methylcellulose-Salt, Two-Phase Partitioning Process

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THE CONCENTRATION AND PURIFICATION OF α-AMYLASE USING A METHYLCELLULOSE-SALT, TWO-PHASE PARTITIONING PROCESS

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ABSTRACT

THE CONCENTRATION AND PURIFICATION OF α-AMYLASE USING A METHYLCELLULOSE-SALT, TWO PHASE PARTITIONING PROCESS

By

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The downstream purification of α-amylase from E. coli by aqueous two-phase partitioning with methylcellulose (MC) and ammonium sulfate was studied. The partitioning of α-amylase into MC was optimized in a reversible purification scheme.

A portion of α-amylase has been genetically fused to alkaline phosphatase. Purification of this fusion protein via aqueous two-phase partitioning was investigated and some enhancement over regular precipitation was found. Comparing the partitioning, precipitation, and hydrophobicity of amylase to similar experiments using β-amylase and pullanase, indicated that the interaction of α-amylase with MC is not due to hydrophobicity alone. This interaction seems to be unique to starch binding enzymes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Introduction

α-Amylase, an enzyme that hydrolyzes starch, is utilized in many industrial applications. Providing amylase at practical prices for industrial applications requires that production and refining costs remain low. The refining cost alone is often half of the total production costs. To remain economical, the recovery process must achieve the desired purity of active enzyme at a high final yield without using costly equipment, or additives, or labor intensive techniques, and it must not result in waste disposal problems.

Large scale enzyme production processes usually require recovery techniques significantly different from the standard fractionation and chromatographic techniques that predominate the research literature[1]. Aqueous two-phase systems, particularly PEG/salt systems, have been employed in multistage processes for the purification of proteins[2]. The specific partitioning of an enzyme in aqueous two-phase systems results mostly from its interaction with the surroundings through hydrophobic, hydrogen, and ion bonding[2,3]. These interactions can be controlled to enhance partitioning purification by manipulation of the choice of polymer, its molecular weight, the concentration of the phase system components, the type and concentration of salts added, the solution pH, and the temperature during partitioning[2].

The specificity of partitioning in aqueous two phase systems can be increased by tailoring the polymer phase with ligands that are

hydrophobic or electrically charged, making the ligand "biospecific"[4]. The structure and composition of the protein can indicate the choice of ligand by its effect on proteins of similar composition[5].

A distinctive characteristic of α-amylase from E. coli, designated ATCC 29609, is its strong hydrophobic character[6]. Therefore, a hydrophobic polymer was chosen to form an aqueous two phase partitioning system with salt. Instead of synthesizing a hydrophobic polymer as the literature suggests[7], methylcellulose, an existing non-ionic polysacharide with an unbranched hyrophilic backbone interspersed along its length with small hydrophobic groups[8], was utilizized. Methylcellulose was used with dextran in 1960 to perform aqueous two-phase extraction of several proteins[9]. It has many advantages including well characterized properties[8], low cost at around $2.70 a pound[10], and FDA approval[8] as a polymeric surfactant for use as a food additive. It also comes in several different molecular weights and as methoxy or hydroxylpropoxyl and methoxyl substituted cellulose derivatives[8]. In aqueous solutions, methylcellulose exists as highly hydrated colloids which can be gelled or salted out of solution upon addition of certain limits of solutes or electrolytes[8]. In addition methylcellulose has an inverse solubility, i.e., it gels as temperature is increased.

The gene of the α-amylase used in the present study was cloned into E. coli from B. stearothermophilus[11]. α-Amylase from E. coli has the valuable and distinctive characteristics of thermostability and extracellular excretion, features which enhance its purification[11]. Extracellular excretion of enzymes by E. coli is rare[12,13], and therefore the structural regions which confer the α-amylase its excretion properties were investigated[14] to determine if fusions

containing these regions could produce extracellular release of other proteins. The enzyme alkaline phosphatase is naturally secreted into the periplasmic space, but not excreted extracellularly[14]. Alkaline phosphatase has been genetically fused with portions of α-amylase[14], and the fusion product was conferred extracellular release[14].

The objectives of this research are to:

1. Optimize the partitioning of thermophilic α-amylase in the methylcellulose/salt aqueous two-phase system with respect to the type, concentration and molecular weight of cellulose derivative, the type and concentration of salts added, the solution pH, and the temperature during extraction.
2. Determine if the partitioning of α-amylase can be reversed upon decreasing the salt concentration, enabling the methylcellulose to be recovered from the purified enzyme.
3. Demonstrate a recovery technique for α-amylase from crude broth using a methylcellulose/salt partitioning process.
4. Determine the nature of the interaction between the methylcellulose and α-amylase.
5. Determine if aqueous two phase partitioning with methylcellulose/salt can be applied to a phosphatase/amylase fusion protein.

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CHAPTER 2

LITERATURE SURVEY

2.1 Background

Enzymes used for large scale commercial applications such as food processing, household cleaning aids, and effluent treatment have to be produced and refined at low costs. Even so, industrial use of enzymes has been of increasing importance in recent years due to developments in microbial genetics, fermentation, and enzyme recovery and purification techniques. These developments were instrumental in making the production of these enzymes economically feasible.

In particular, the ability to recover and purify an enzyme economically often determines the overall feasibility of an industrial process. As well as a achieving a high final yield, the purification process must not reduce the biological activity of the enzyme. High yield and biological activity are difficult to obtain because enzymes are fragile molecules produced in very dilute solutions that can contain soluble portions of residual substrates, metabolic pathway intermediates, and cellular debris. Therefore, to recover the enzyme economically at the desired concentration and purity, the separation process must be efficient and reliable.

2.1.1 Starch-Hydrolysis Industry

Starch is a naturally produced and widely available glucose polymer. Amylases are enzymes used industrially to hydrolyze starch for such applications as brewing, baking, alcohol, milling, paper, textiles, feed, detergent, and sweeteners[1]. Bacterial amylases are commercially

produced in large quantities and generally used for industrial applications after only minimal refinement. In 1979, 300 tons of amylases were produced[2] and marketed for about 8 million dollars[3]. Studies on purification and crystallization of amylase from bacteria are numerous[4] due to its commercial importance.

The commercial production of sweeteners, such as glucose, fructose, and maltose from starch is one of the main uses of amylases. Amylases are used to hydrolyze starch into smaller saccharides including glucose. Because glucose is not as sweet as its isomer fructose, it is converted to fructose with the enzyme glucose isomerase. The enzymatic hydrolysis of starch, called starch-saccharification, includes α-amylases, β-amylases, glucoamylases, glucose isomerases, pullanases, and isoamylases[1]. The different actions of each of these enzymes is shown in Figure 2.1. The focus of the present study is on α-amylase.

2.1.2 α-Amylase

α-Amylase specifically hydrolyzes α-1,4-glucosidic bonds in polyglucans such as starch, glycogen, and dextrins while leaving the α-1,6 glucosidic bonds (i.e. branch points) unhydrolyzed[5]. The hydrolysis products are reducing groups such as maltose, short limit dextrins usually containing α-1,6-glucosidic branch points, and small amounts of glucose[6].

α-Amylases differ from β-amylases by attacking the interior of polyglucan chains (i.e. endoases) as opposed to attacking from a chain end (i.e. exoases)[5]. The products of both α- and β- amylases are maltose.

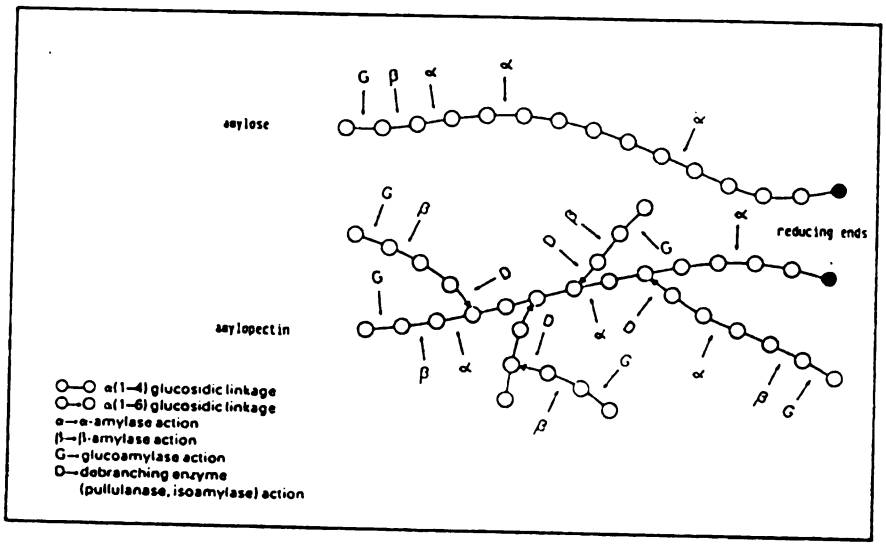

Figure 2.1. The action of enzymes involved in starch-saccharification as they act on the two components of starch[1].

Bacteria and fungi produce several different α-amylases, sometimes even within the same organism. These different α-amylases are classified according to their pH optimum, temperature range, stability, and starch liquefying and/or saccharogenic effect[1]. Free sugars are produced only by saccharogenic α-amylases. Starch liquefying α-amylase breaks down the starch into maltose and short limit dextrins.

2.1.3 Thermostability

Many α-amylases from bacteria of the genus Bacillus are thermostable, greatly facilitating the enzymes' industrial utilization. For example, operating at higher temperatures reduces contamination by foreign organisms during production. In the starch industry a thermostable α-amylase allows liquefaction of starch at temperatures above the gelatinising temperature of starch granules[5].

In addition, the number of purification steps required to isolate a thermostabile enzyme downstream from a fermentor are reduced by its thermostability. Just heating the broth for a short period of time to a

temperature slightly below the limit of the enzyme's stability precipitates by denaturation many contaminants in solution while leaving virtually all the thermostable enzyme.

2.1.4 Nature of the Thermostabile α-Amylase from B. stearothermophilus

As thermostability in α-amylase is a desired characteristic for commercial applications, discovery of such an enzyme in Bacillus stearothermophilus was of considerable importance. Subsequently, the α-amylase gene from Bacillus stearothermophilus was cloned into E. coli (designated as EC 147). The genetically modified E. coli releases thermostable α-amylase extracellularly and will grow on starch as the sole carbon source[7]. It was advantagous to clone the thermostable α-amylase gene into E. coli because the molecular biology of Escherichia coli is well known.

The α-amylase from both the parent B. stearothermophilus and EC 147 were found to have optimum temperature and pH of 70°C and 5.1, respectively. The amylase from both organisms were found to be stable with little loss of activity at 90°C over a 1 h period if in the presence of 5 mM $CaCl_2$[7].

The EC 147 did not grow efficiently on M9 minimal agar with glycerol[7]. However, a mutation of it was found that did grow well on M9-glycerol, -starch, or -soluble starch with significant release of α-amylase[7]. This mutation, designated EC 148, did not differ from the EC 147 in release of α-amylase or growth in L broth[8]. The EC 148 organism was used to produce the α-amylase in the present study.

Information on structure and biocomposition of thermostable α-amylase from B. stearothermophilus is highly variable, with significant differences reported for thermostability, molecular weights (15 to 90

kilodaltons), and other properties[9-11]. SDS-polyacrylamide electrophoresis indicated a molecular weight of 57 kilodaltons[7] for the α-amylase from ATCC 29609 which was used in this work. In addition, this enzyme appeared to be very hydrophobic.

2.2 Factors Affecting Protein Solubility, Fractional Precipitation, and Partitioning

The term precipitation will be used to describe an operation in which a reagent is added to a protein solution causing the formation of insoluble particles of protein. The intent of precipitation is to recover a protein in either an unchanged molecular form or one which is readily returned to that form.

The present research is concerned with proteins whose solubility properties are determined largely by their polypeptide structure. For example, proteins with relatively small non-peptide groups such as lipo-, nucleo-, and glycoproteins have solubility properties often distinctive to these groups. Thus, glycoproteins are very soluble in aqueous solution due to hydration of their carbohydrate moiety. However, lipoproteins are relatively insoluble in aqueous solution due the hydrophobic nature of their lipid components.

The choice of precipitating reagents can be suggested by the known effect of the reagent on the solubility of proteins of similar structure. 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 be on the exterior[12]. However, this division is not so well defined that changes in the exterior environment brought about by the precipitating reagent will not affect both hydrophobic and hydrophilic groups. The overall effect of the precipitating reagent on

the protein results from the sum of the individual effects which will often be opposed to one another. Thus, the resulting protein conformation may either increase, decrease or not change the solubility of the protein depending upon the moieties contained with the protein.

Because the solubility of a protein is largly determined by its primary structure, proteins may be genetically engineered to facilitate their purification. Recombinant DNA technology has made it possible to produce foreign gene products in bacteria[7]. This technique also makes it possible to direct the proteins to be secreted extracellularly using secretion vectors[13-15] and to simplify the protein's purification by another genetic modification[16]. One method for enhancing purification is to genetically fuse an additional peptide to the protein which would enable a simple, cheap, and efficient purification. The only requirements for the fusion are retention of biological activity and ready removal after purification[16]. Finally, if the same peptide is fused to other recombinant proteins, the same purification method may be applicable.

2.2.1 Salting out

Salting out involves precipitation of proteins from solution using high concentrations of neutral salts[12]. As the salt is added and the solution's ionic strength increases the protein's solubility decreases until it is driven out of solution. This change in solubility is described by the interaction between two opposing effects. Increasing the ionic strenth of solution increases the electrostatic effects which increases the protein's solubility[17]. Opposing this is the hydrophobic effect, which decreases the proteins solubility as the ionic strength increases[17].

Changing a protein's solubility by salting out is therefore a function of choice of salt and concentration. The Hofmeister series[17] rates the relative effectiveness of anions on salting out, where:

citrate > phosphate > sulfate >
acetate or chloride > nitrate > thiocyanate.

Salting out, however, may denature the protein and affect its function. The amount of protein denaturation caused by the salt is inversely related to its position in the Hofmeister series[17], e.g. nitrate would be more damaging than phosphate.

Cations have less effect than anions on the precipitation. Monovalent cations are preferred, with order of effectiveness being[12]:

$NH4^+ > K^+ > Na^+$

The salt of choice must also have a pH range within the limits of the enzymes stability. Finally, it must be inexpensive and either recoverable or have managable disposal problems.

The preferred salts used for isolating α-amylase by salting out are sodium chloride[18-20] and ammonium sulfate[4,6,18].

2.2.2 Organic solvent precipitation

Organic solvent precipitation involves the precipitation of protein from solution upon the addition of a weakly polar solvent. Solubility reduction by a polar solvent is a result of a shift in the solvent's dielectric constant away from the protein's isoelectric point, producing an increase in the effectiveness of intra- and intermolecular attraction[17]. The solubility of metal-protein complexes such as the calcium-α-amylase[21,22] is very dependent on the dielectric constant of

the medium[17]. Protein damage is reduced because of the lower solvent concentrations.

Precipitation by organic solvents can be combined with variation in pH, temperature, and ionic stength to provide very careful control of the protein fractionation. Additional advantagous properties of organic solvents include their relatively high volatility, which facilitates their recovery, and their bactericidal effect, which keeps the media sterile[17].

Some disadvantages of solvent extraction exist. There is a tendency for irreversibly denaturation of the protein, but this problem can be minimized by operating at low temperatures. Other disadvantages include flammability and government control of ethanol.

Solvent extraction of α-amylase has been frequently reported in conjunction with other extraction steps. The solvents of choice in previous methods include acetone[4,6,20,23-27], ethanol[20,23-25], ether[20,23-25] and NH_4OH[20,26,27].

2.2.3 Isoelectric precipitation

A protein's solubility is substantially reduced at its isoelectric point[12], i.e., the pH where the protein has no net charge[17]. In low ionic strength solutions, the isoelectric point can be reached by addition of either acids or bases to the solution.

Isoelectric precipitation is enhanced for proteins with high surface hydrophobicities[17]. A well defined isoelectric point enhances separation specificity. Additional advantages are low cost and acceptance for use in food products. Phosphoric, hydrochloric, and sulfuric acids are all available at low cost and are allowed food additives.

The overriding disadvantage of isoelectric precipitation is the potential for irreversible denaturation at pH extremes.

The preferred reagents for isoelectrically precipitating α-amylase are ammonia[24], and acetic acid[24,25].

2.2.4 Precipitation by metal ions

Polyvalent metal ions are even more effective at precipitating proteins than the salt ions mentioned above. Polyvalent ions have been classified into three groups according to the site they selectively bind[17]. Carboxylic acids and nitrogenous compounds such as amines and heterocyclics are bound strongly by ions such as Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+}. A different set of ions, including Ca^{2+}, Ba^{2+}, Mg^{2+} and Pb^{2+} bind carboxylic acids specifically but not nitrogenous groups. The third group, including Ag^{+}, Hg^{2+} and Pb^{2+} strongly binds sulfhydryl groups.

Metal ions have the advantage of precipitating proteins at low concentrations; they may be removed from solution by ion exchange.

α-Amylase has been precipitated[4] using Zn^{2+}.

2.2.5 Thermal precipitation

By increasing the temperature of a crude solution of proteins, fractionation can occur by selectively denaturing and hence precipitating proteins[12]. The selective denaturation of protein is not applicable to all protein systems, but it is of particular usefulness for the isolation of thermostable enzymes (see section 2.1.3). Thermal precipitation is inexpensive because it does not require the addition or removal of precipitating reagents.

The use of thermal precipitation to isolate α-amylase has been reported[26-28].

2.2.6 Polyelectrolyte precipitation

The recovery of proteins through reversible precipitation with polyelectrolytes, has been adapted for the purification and recovery of several industrial enzymes[29]. Polyelectrolytes such as polyacrylic acids[29], carboxymethylcellulose[30], anionic hydrocolloids[31], and heteropolyacids[32] have been used for precipitation of impurities, precipitation of the desired enzyme, and fractional precipitation of more than one enzyme.

Upon addition of a polyelectrolyte to a crude enzyme solution at pH 3.0-5.8, a precipitate forms between some of the protein and the polyelectrolyte[33]. The selectivity of the precipitation is such that at pH < 6.0 mono-, oligo- and polysaccharides, amino acids, oligopeptides, lipids, uncleotides, nucleic acids and inorganic salts do not form precipitates with polyacrylic acids[29]. The desired enzyme may end up in the precipitate or stay in solution depending upon the conditions of reaction and its chemical reactivity. In either instance a fractionation of the enzyme has begun. The polyelectrolye-enzyme complex can be isolated by centrifugation or ultrafiltration, and the enzyme unbound and solubilized at pH > 6.0 upon addition of a divalent metallic cation such as Ca^{2+} or Mg^{2+}. Thus the enzyme is isolated and the polyelectrolyte recovered as an insoluble salt of the polyacrylic acid is formed[33].

Polyelectrolytes have the advantage of low cost, recoverability and an overall effectiveness at low concentrations[29]. Their limitations for protein purification are due to denaturation within the pH range of precipitation[33].

The isolation of α-amylase using polyelectrolytes has been reported

using polyacrylic acid[29,33].

2.2.7 Non-ionic polymer partitioning

Aqueous two-phase partitioning results from the incompatability between aqueous solutions of two polymers or between one polymer and an appropriate salt if above certain reagent concentrations[34,35]. The phases formed have characteristics ranging from precipitate to liquid droplet, or to something in between. The most common two-phase system employed are with polyethylene glycol (PEG) and dextran. However, other polymers including methylcellulose[36,37], polyvinyl alcohol[35], and ficol[35] have been used to form aqueous two-phase systems.

Aqueous two phase partitioning is a well described purification method particularly suited to the separation of cell debris and enrichment of a desired protein[34,38,39] found in low concentrations. These systems provide very mild conditions for partitioning due to their high water content, approximately 75-95 percent for both phases[40-42]. A gentle partitioning is also due to the very low surface tension between the two phases, about 0.1 dyne/cm[43], allowing the creation of an emulsion even upon only light mixing. However, water-organic solvent two-phase extraction systems are harsh on biomolecules, partly due to surface tension around 400 times greater than for aqueous systems[42].

The partitioning of biomolecules between two-phases is governed mainly by their surface properties[39,42] and the composition of the phase system[39,42]. The phase system is characterized by the partition coefficient, K_{part}, which is defined as the ratio of the biomolecule's concentration in the top and bottom phases. The partitioning results from the biomolecule's interactions with the surrounding phases, which are mainly through hydrophobic, hydrogen and ionic bonding[34,42]. The

many different interactions provides several methods of controlling the partitioning by manipulation of:

1. kind of polymers,
2. molecular weight of the polymer,
3. concentration of the phase systems components,
4. type and concentration of salts added,
5. solution pH, and
6. temperature during extraction[34].

Over a wide range, partitioning does not depend on the concentration of the desired product[39,41]. In addition, it has been reported[34] that it is the K_{part} value that has the largest influence on the economics of an extraction.

The advantages for using PEG, and dextran or salt to form aqueous two phase systems are the density and viscosity differences between the two phases[40]. In addition, dextran as well as PEG are nontoxic substances and have been thoroughly tested for pharmaceutical and food purposes.

However, most of the aqueous two phase systems have the disadvantage of high costs because of highly purified phase components, particularly dextran[44]. Even so, two-phase systems are economical for large scale isolation of certain proteins; e.g., utilizing PEG/salt[34] and PEG/crude dextran[34,44].

The purification of α-amylase by non-ionic partitioning has been reported previously using PEG/crude dextran[44] and PEG/dextran[44].

2.3 Biospecific and Affinity Separations

2.3.1 Biospecific and Affinity Chromatography

Biospecific and affinity chromatography techniques utilize

immobilized ligand adsorbents to form reversible complexes with the complementary biomolecules to be isolated[45-47]. Affinity chromatography techniques have absolute enzyme specificity by covalently attaching ligands that function with the enzyme as either substrates, inhibitors, cofactors, products or antibodies[45-48]. Biospecific chomatography techniques have selectivity based upon ligands that are group specific[40], such as electrically charged groups[49-51] or groups hydrophobic in nature[51-58]; these ligands are group specific and are capable of binding a number of similar biomolecules.

Techniques have been reported that use chromatography of α-amylase by ion exchange[26,27,59], hydrophobic[60,61], and affinity[18,19,23,26-28,62-64] adsorbents.

2.3.2 Affinity precipitation

Affinity precipitation utilizes specific interactions between the enzyme and its substrates, inhibitors, cofactors, products or antibodies to selectively form complexes that can be isolated by precipitation. Two methods of precipitation are available, one wherein the complex is insoluble upon formation and the other wherein the solution buffer must be changed to cause the complex to precipitate[65].

α-Amylase has been purified by affinity precipitation by utilizing its interactions with glycogen[20,23,24] and dextrin[25].

2.3.3 Biospecific and affinity partitioning

Aqueous two-phase partitioning has been developed by using polymers that have been tailored to increase their specificity. This procedure is sometimes referred to as biospecific and affinity partitioning[39,42]. Biospecific partitioning of proteins has been most effective by covalently combining polymers with ligands that are electrically charged

or hydrophobic in nature[40]. These polymer-ligands are biospecific adsorbents, i.e., capable of binding a number of similar biomolecules.

Biospecific partitioning differs from affinity partitioning only in the degree of polymer-enzyme binding specificity[42,47]. Affinity partioning like affinity chromatography has absolute enzyme specificity by covalently attaching substrates, products, inhibitors, cofactors or antibodies specific to the enzyme to one of the polymer phases[38,39,42,47]. Both biospecific and affinity partitioning take advantage of principles of affinity chromatography to chemically modify a ligand so that it partitions exclusively, or nearly exclusively, to the phase opposite of the impurities. A favorable effect on the distribution of the protein will be obtained after it has been specifically bound by the ligand-polymer[34,38-40,42,47].

A multistage process for the isolation of proteins by affinity partitioning and the subsequent recycle of modified liquid polymer is a somewhat more complex process than conventional affinity chromatography using solid matrices. However, there are many advantages of multistage affinty partitioning processes in large scale enzyme purification. Extraction techniques scale up much better than chromatograpic techniques[41]. Aqueous phase systems have considerably higher binding capacities per unit volume than chromatography columns due to their higher ligand density and availability[66]. In addition, approach to equilibrium binding is faster in solution[38]. Finally, chromatography is intrinsically a batch operation, while partitioning can be performed in multistage operations[34].

The literature on hydrophobic and affinity chromatography includes techniques using the covalent attachment of ligands to carbohydrate

supports[45,46,48,67-80]. Because the carbohydrate support is composed of crosslinked carbohydrate polymers, the techniques used to attach ligands for affinity chromatography can be used to design specific affinity and biospecific partitioning reagents. However, there are reasonably priced and FDA approved modified polymers available, though as yet not developed for this use, for biospecific partioning of enzymes. The polymer of choice for the present research is one of these existing polymers.

Methylcellulose is a non-ionic polymer that was used with dextran over 28 years ago to perform aqueous two-phase partitioning of proteins[37] and virus particles[36]. Methylcellulose has many advantages in that its properties have been well characterized[81,82], it is inexpensive at about 2.70/lb[83], and it has been approved many times by the Food and Drug Administration as a polymeric surfactant for use as a food additive[81]. Methylcellulose is available as a methoxyl substituted or as a methoxyl/-hydroxypropoxyl substituted cellulose derivative; it is available in several molecular weights ranging from 15 to 250 kilodaltons. It also has unique solubility characteristics[81,82], allowing it to be gelled or salted out of solution. Also, methylcellulose exhibits an inverse solubility, gelling and precipitating at high temperatures more readilly than at low ones. This is a good property to exploit with α-amylase thermostability.

The diversity of physical and chemical properties and FDA approval provide impetus to use methylcellulose in aqueous two phase partitioning of α-amylase.

2.4 Membrane Separations

2.4.1 Ultrafiltration

Ultrafiltration and microfiltration are membrane separation processes that separate dissolved substances according to molecular weight and size based upon their ability to pass through membrane pores while under an applied pressure. Pore sizes of the membranes ranges from 10 to 200 angstroms. Dissolved substances and solvents whose size and molecular weight are below the membrane cutoff will pass through the membrane while large molecules are retained. Molecules that pass through the membrane are called the permeate; while those which are retained by the membrane are called the concentrate.

Ultrafiltration is a relatively new but already popular concentration and separation process. It still has drawbacks that include low operating pressures[84] and fouling problems[85]. However, improvements in its performance and new applications such as affinity ultrafiltration[86-89] are occuring rapidly.

2.4.2 Affinity ultrafiltration

Affinity ultrafiltrafiltration is a new purification method that combines affinity binding with ultrafiltration separations[86-89]. Affinity ultrafiltration is initiated by contacting a crude enzyme extract with a solution of macromolecular ligand specific for the desired enzyme (see section on biospecific partitioning). Upon contact, the enzyme binds reversibly to the high-molecular-weight ligand, this complex is retained on one side of a membrane while other material is washed out through the membrane pores. Following isolation of the ligand-enzyme macromolecule complex, the enzyme is liberated from the

ligand by addition of a dissociative media. The enzyme is isolated and the ligand-macromolecule recovered by making a second pass through the ultrafiltration membrane.

2.5 Summary

There are many publications describing protein separation, including major reviews covering developments in separation and purification of biomolecules[12,17,89]. Techniques such as aqueous two-phase extraction with non-ionic polymers[34,38-44], polyelectrolytes[29,30,32,33], and biospecific[40,42,47] and affinity[34,38,39,42,47] polymers have been detailed. There are also numerous papers on the chromatography of enzymes by hydrophobic[51-58], ion exchange[49-51], and affinity[45-48] adsorbants. Ultrafiltration[84,85], and affinity ultrafiltration[86-89] provide alternatives for protein isolation.

There are many reports dealing with the purification of α-amylases isolated from animals[6,18,20,24,29,63] plants[23,26-28,62], fungi[29], and bacteria[4,19,25,33,59-61,64,91,92]. α-Amylase has been isolated using fractionation techniques such as salting out[4,6,18,19,20], solvent precipitation[4,6,23-27,61], isoelectric precipitation[24], metal ion precipitation[4], thermal precipitation[26-28], polyelectrolyte precipitation[29,33], and non-ionic polymer precipitation[19,44]. Affinity precipitation[20,23-25] has been used as well. Techniques involving chromatography of α-amylase by ion exchange[26,27,59], hydrophobic[60,61] and affinity[18,19,23,26-28,62-64] adorbents have been developed to a similar extent.

With the number of purification techniques available, selection of the "best" technique for the commercial production of protein becomes a function of economics, and requirements for final purity and yield.

Techniques such as aqueous two-phase biospecific or affinity partitioning can be economical, with high yields at high puritities[39]. A technique such as this was used in the present study of α-amylase (chapter 3). Recombinant-DNA technology has been tried to enhance purification techniques by modifying the protein to facilitate its isolation. The effectiveness of some protein modifications are evaluated in chapters 3 and 4.

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CHAPTER 3

CONCENTRATION AND PURIFICATION OF α-AMYLASE USING A METHYLCELLULOSE-SALT, TWO-PHASE PARTITIONING PROCESS

3.1 Abstract

The downstream purification of thermostable α-amylase was studied utilizing a technique involving aqueous two-phase partitioning with methylcellulose and ammonium sulfate. The partitioning was optimized with respect to the type of cellulose derivative, its molecular weight and concentration, the type and concentration of salts added, the solution pH, and the temperature during extraction. The partitioning was shown to be reversible upon lowering the salt concentration, allowing the recovery of methylcellulose from the enzyme. A process was demonstrated for recovering α-amylase from crude broth using methylcellulose/salt partitioning.

3.2 Introduction

Molecular biology and recombinant-DNA technology have combined to provide control over the kinds and composition of proteins biosynthesized by microorganisms such as E. coli. Commercial applications require development of efficient methodologies to concentrate and purify extracellular secreted enzymes from culture broths. Production of low priced industrial enzymes on a large scale, however, often requires significantly different recovery processes than the standard fractionation and chromatographic techniques that have predominated in the research literature[1]. Aqueous two-phase systems, e.g. PEG/salt systems, have seen some use in multistage processes for the

purification of proteins, but require PEG recycling to remain economical[2]. This technique has been fairly successful due to the gentleness of partitioning[1,3,4], the large density and viscosity differences between the two phases[3], the ability to control the partitioning specificity[2,5], and the acceptance of PEG by the Food and Drug Administration.

The specific partitioning results from the various interactions of the biomolecules with the surrounding phases mainly through hydrophobic, hydrogen, and ionic bonding[2,4]. These interactions provide options for controlling the partitioning by manipulation of[2]

1. kind of polymers,
2. molecular weight of the polymer,
3. concentration of the phase system components,
4. type and concentration of salts added,
5. solution pH, and
6. temperature during extraction.

To increase the specificity of partitioning in aqueous two phase systems, the polymer phase can be tailored with ligands that are hydrophobic or electrically charged[3]. The ligand-tailored polymer phase is then group specific, or in this case biospecific, because it is capable of binding a number of similar biomolecules. Selecting a biospecific polymer to partition the desired enzyme therefore depends upon the chemistry of the enzyme. The choice of ligand may be indicated by the ligands effect on the solubility of proteins with similar structure[6]. For example, proteins with relatively small non-peptide groups such as lipo-, nucleo-, and glycoproteins have solubility properties often distinctive to these groups.

When the purification of the enzyme α-amylase from E. coli

(designated ATCC 29609) was investigated, it was found that the enzyme was strongly hydrophobic[7]. Thus, a hydrophobic polymer/salt, two-phase aqueous partitioning system was selected.

An existing hydrophobic polymer could be utilized or one could be synthesized[5], utilizing a PEG or carbohydrate backbone. Hydrophobic ligands can be attached to a carbohydrate polymer backbone, as descibed by techniques used for hydrophobic and affinity chromatography[8-24], because the carbohydrate support matrix is composed of crosslinked carbohydrate polymers, and similarly for PEG backbones. However, there are reasonably priced and FDA approved hydrophobic polymers available, though not as yet developed for this use, that fill the requirements for a two-phase partitioning process that could be biospecific.

Methylcellulose is one such non-ionic polymer that was used with dextran over 28 years ago to perform aqueous two-phase extractions of proteins[25]. In addition, it has many advantages including well-characterized properties[26,27], low cost at $2.70 per lb[28], and FDA approval[26] as a polymeric surfactant for use as a food additive. Also, because methylcellulose comes in several molecular weights and as methoxy (MC) or hydroxylpropoxyl/ methoxy (HPMC) substituted cellulose derivatives, the partitioning can be optimized for molecular weight and somewhat for ligand substitution.

The nature of interaction that methylcellulose brings to the partitioning is indicated by its structure (Figure 3.1). MC and HPMC are neutral, non-ionic polysaccharides whose unbranched hydrophilic backbones, are interspersed with small hydrophobic groups along their lengths[26]. They have an inverse solubility, gelling at increasing temperatures, which could be used advantageously with thermostable α-

amylase. They are not susceptible to chemical gelation or precipitation with di- or trivalent metals, with borates, or by interaction with other polymers to form complexes or coacervates[26]. However, in aqueous solution, where they exist as highly hydrated colloids, MC and HPMC can be gelled or salted out of solution when the concentration of added solutes or electrolytes exceeds certain limits[26].

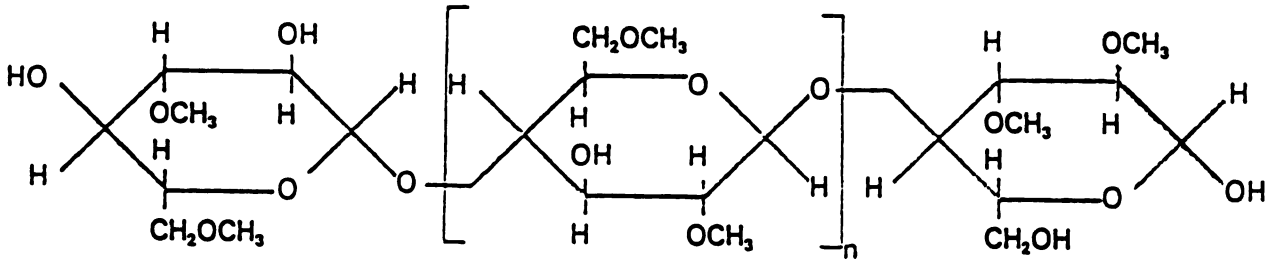

Figure 3.1. The structure of methoxy substituted cellulose (MC)

α-Amylase excreted extracellularly by E. coli is thermostable (with little loss of activity at 90°C for 1 hr)[29]. Taking advantage of the characteristics of α-amylase and methylcellulose described above, a process for concentration and purification of the enzyme downstream from the fermentor has been studied. The process involves removal of insolubles by centrifugation, concentration by ultrafiltration, soluble contaminant removal by thermal precipitation, and aqueous two-phase partitioning for further amylase purification.

3.3 Experimental

Materials

The methylcellulose and hydroxypropyl methylcellulose were donated

by Dow Chemical and have brand name METHOCEL type A4M, K4M, K15M, and K100M designating the cellulose derivative (A for 26-33 % methoxy substitution and K for 19-24 % methoxy/7-12 % hydroxypropoxyl substitution) and the approximate molecular weight (4, 15 and 100 corresponding to 85, 15, and 250 kilodaltons, respectively). The α-amylase was obtained as described below. All other chemicals were reagent grade.

Amylase Preparation

α-Amylase purified to homogeniety was used in the six partition optimization experiments. The α-amylase was prepared and purified from transformant EC 147 periplasmic extracts[30], grown overnight in cultures shaken at 37°C in L broth[31], as described by P. Oriel[29]. Purification of the amylase extract involved fractionation by heat treatment at 80°C for 45 min in the presence of 15 mM $CaCl_2$, and salting out with ammonium sulfate, retaining the 0 to 55% saturation fraction. After redissolving the precipitate in buffer (0.05 M Tris, pH 8.0, containing 0.025 M $CaCl_2$), further impurities are removed by DEAE Sepharose chromatography, which passes the α-amylase. Purification to homogeneity is obtained by gel permeation chromatography on Sephadex G-200, and validated by SDS-PAGE electrophoresis. The pure α-amylase was stored frozen in distilled water, and prior to experiments diluted in 50 mM acetate buffer, pH 6.0, containing 5 mM CaCl2.

After the α-amylase partitioning was optimized, further experiments were carried out to evaluate the α-amylase purification by methylcellulose/salt partitioning from a broth. The α-amylase was produced as just described. However, prior to separation of the extracellular fraction from the cells by centrifugation, the volume was

reduced with a 10,000 moleculer weight cut off, cross flow ultrafiltration unit. Following α-amylase extraction from the periplasm by osmotic shock with sucrose[30], both the periplasmic extract and the supernatant were further fractionated by heat treatment as described above. Following the heat fractionation step, the further purification of α-amylase from the periplasmic extract and the extracellular supernatant was studied for comparison purposes (sections 3.4.2 & 3.4.3).

Methylcellulose Preparation

The methylcellulose powder was dispersed and wetted in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ solution by slow addition of the powder to the hot buffer, around 80-90°C. The wetted methylcellulose was then poured into an equal volume of cold buffer, around 4°C, and mixed. Methylcellulose solutions thus formed[26] were stored at 4°C between experiments for several months with no apparent changes.

Partitioning

The term partitioning is used here to indicate the localization of protein upon formation of two phases. The exact physical nature of the two phases is somewhat unclear. With ammonium sulfate-methylcellulose partitioning, the polymer phase can appear as liquid droplets under a microscope while resembling a precipitate in other characteristics.

Experiments involving homogeneously pure α-amylase were performed on a small scale using 1.5 ml Eppendorf tubes. However, experiments evaluating purification and the nature of partitioning were performed on a scale of several ml.

Equal volumes of methylcellulose and α-amylase solutions, prepared

as described above, were added in various concentrations and mixed vortically at room temperature. After approximately 30 minutes with occasional mixing, saturated solutions of ammonium sulfate were added to the samples, then immediately mixed to produce the desired salt concentration. The addition of ammonium sulfate to the α-amylase and dilute (0.5-0.0001%) methylcellulose solution creates a precipitate-like polymer phase and a salt phase. After waiting an additional 30 minutes, while mixing ocasionally, the precipitate was pelleted by centrifuging the Eppendorf tubes at 13,000 rpm for 20 minutes. The resulting supernatant and/or pellet were then tested, after appropriate dilutions, for total reducing activity, total carbohydyrate, and/or total protein. A measure of total protein was not always possible because effort to obtain highly pure α-amylase and the dilutions used in most of the experiments reduced the protein levels to less than that assayable by the Lowry et al. procedure[33].

Assays

α-Amylase activity was estimated by a modification of the dinitrosalicylate assay by Bernfeld[32]. The assay requires photometric measurement at 640 nm of the optical density arising from the reducing power of a solution of soluble starch in the presence of α-amylase[32]. One unit of activity is defined as the amount of enzyme that liberates starch hydrolysis products at a rate equivalent to the reducing capacity of one umol of maltose per minute at 60°C.

The method of Lowry et al[32] was used to estimate total protein; bovine serum albumin was the protein standard. The contribution of the methylcellulose to the total protein measurement was negligible.

The phenol sulfuric method by Dubois et al[34] was used to estimate

total carbohydrate; the appropriate methylcellulose calibration curve was the carbohydrate standard. The contribution of the pure α-amylase to the total carbohydrate measurement was insignificant.

3.4 Results and Discussion

3.4.1 Optimization of the Partitioning Process

Partitioning can be optimized by the choice of polymer, its molecular weight, its concentration, the type and concentration of salt added, the solution pH, and the temperature during extraction[2]. The effects of all of these variables on the partitioning of homogeneously pure α-amylase were evaluated in the present study.

The partitioning is presented in terms of the amount of starch reducing activity remaining in the aqueous salt phase. An experiment was carried out to verify that the activity missing from the salt phase is actually in the methylcellulose phase. The results showed 93% of the total activity remained in the polymer phase, 1% remained in the salt phase, and the 6% unaccounted was possibly due to experimental error. The two phase system included 0.02% w/v MC, 30% saturation ammonium sulfate and 90 units/ml α-amylase.

Choice of polymer and the effect of its molecular weight

The choice of polymer and the effect of its molecular weight on partitioning α-amylase was evaluated for solutions of 0.0005% w/v polymer A4M, K4M, K15M, and K100M containing 30% saturated ammonium sulfate, and 90 units/ml α-amylase. The percent activity remaining in the salt phase after partitioning was 13.8, 12.9, 6.0, and 5.4% for A4M, K4M, K15M and K100M polymer types, respectively. The HPMC, of molecular weights 15 and 250 kilodaltons, enables better partitioning than MC or HPMC of molecular weight 85 kilodaltons, with MC and HPMC of similar

molecular weights partitioning similarly. In addition, when choosing between HPMC of molecular weights 15 or 250 kilodaltons, the lower molecular weight was used for lower solution viscosity.

Effect of methylcellulose concentration

The effect of methylcellulose concentration on partitioning α-amylase was evaluated for solutions of varied methylcellulose concentrations, each containing 30% saturated ammonium sulfate, and 90 units/ml α-amylase. The results were evaluated as a percentage of activity remaining in the salt phase as a function of the enzyme loading (Figure 3.2). In another experiment, the concentration of enzyme was varied while the methylcellulose concentration and ammonium sulfate saturation were fixed at 0.005% and 30%, respectively. A similar loading profile was produced (Figure 3.2). The results indicate that a loading of between 1 and 10 g A4M per g amylase is required to partition the majority of α-amylase.

Effect of different salts

Ammonium sulfate and sodium chloride were evaluated to determine their ability to partition α-amylase as a function of their concentration. The concentrations of K4M and α-amylase were 0.05% w/v and 90 units/ml, respectively. The starch reducing activity of the salt phase was measured after partitioning with ammonium sulfate or sodium chloride at 0, 15, 20, 25, and 30% of saturation. The starch reducing activity was measured as the percentage of activity remaining in the salt phase after partitioning as a function of salt concentration (Figure 3.3). At less than 10% of saturation, ammonium sulfate had little or no effect on partitioning α-amylase, but at 30% saturation, nearly all of the amylase was separated from solution. At similar

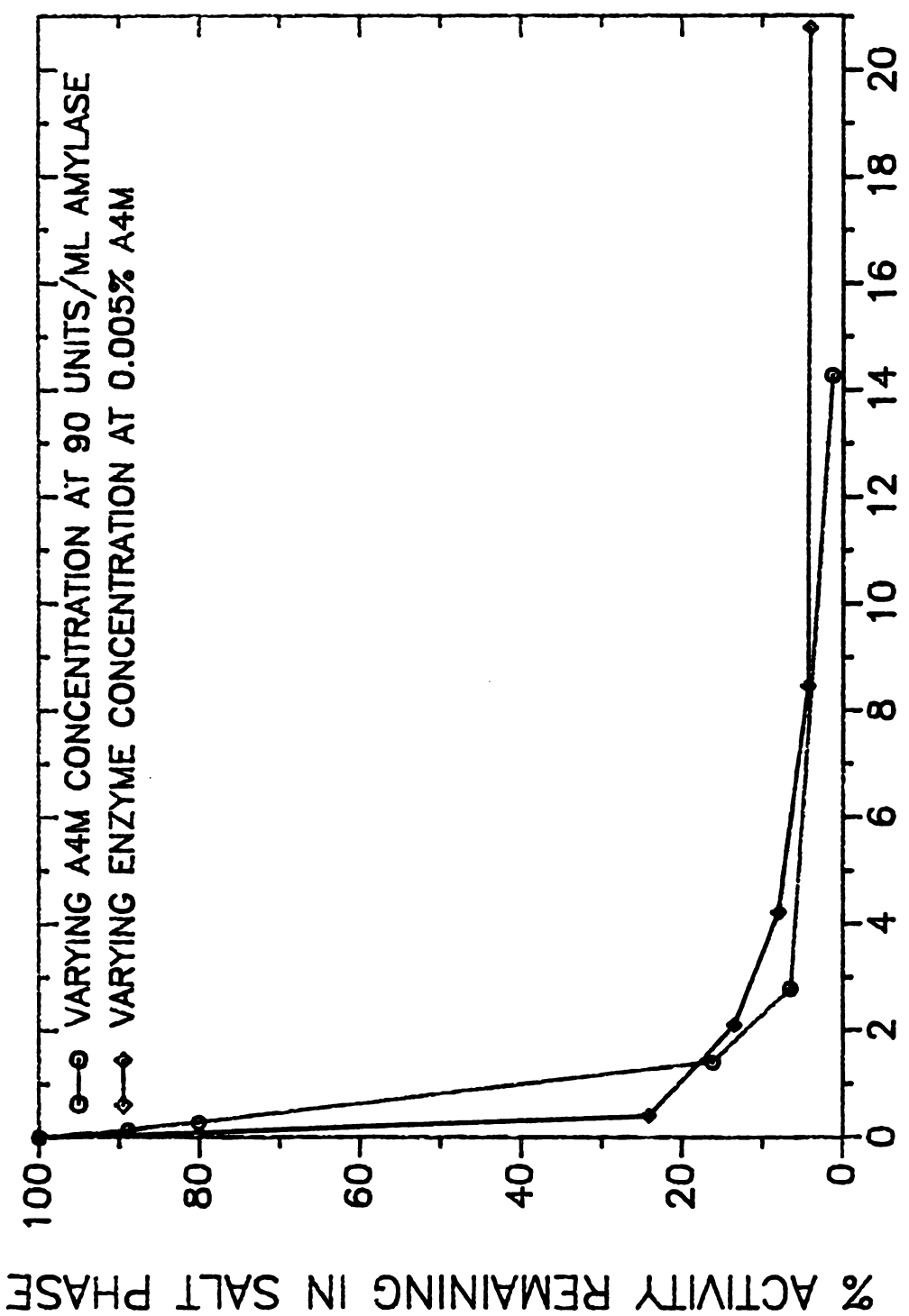

FIGURE 3.2: The percentage of activity remaining in the salt phase as a function of the enzyme loading at 30% of ammonium sulfate saturation.

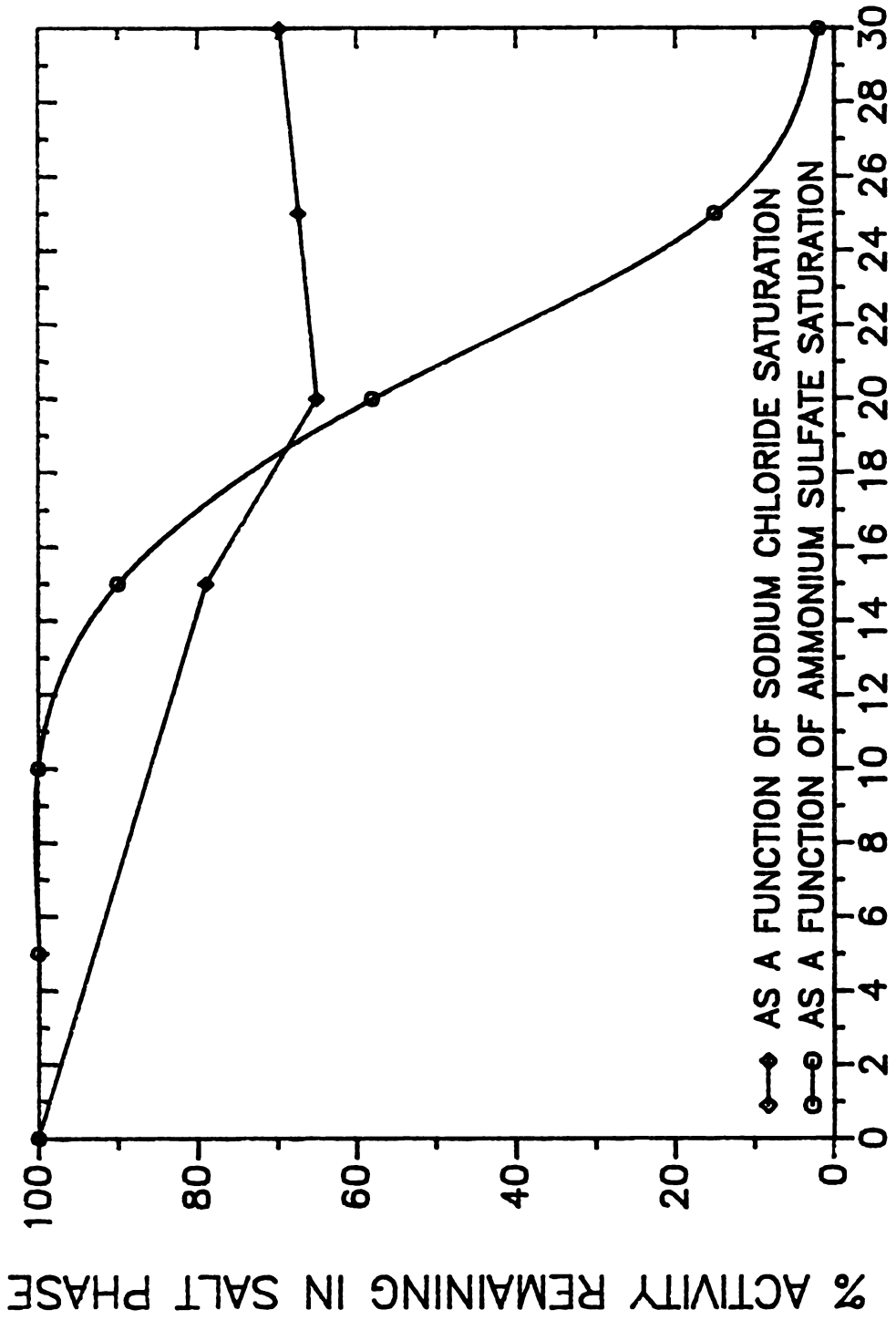

FIGURE 3.3: The percentage of activity remaining in the salt phase as a function of the ammonium sulfate or sodium chloride saturations.

saturations, sodium chloride partitioned less α-amylase than ammonium sulfate did.

Effect of pH

The effect of pH on partitioning α-amylase was investigated in solutions containing 0.05% w/v K4M, and 90 units/ml α-amylase. Each solution was prepared with buffers of pH 4.0, 5.0, 6.0 or 7.0 prior to partitioning with ammonium sulfate to 30% of saturation. After partitioning, the starch reducing activity was measured and reported at each pH as a percentage of that remaining in an identical, unpartitioned solution (Figure 3.4). In addition, the pH of each solution was measured following partitioning with ammonium sulfate to get a true measure of the solution pH. The results showed little difference in partitioning at the different pH's tested with maximum partitioning between 5 and 6.

Effect of temperature

The effect of temperature on partitioning was investigated in solutions containing 0.05% K15M, 90 units/ml α-amylase, and ammonium sulfate at 30% of saturation. The solutions were held at constant temperature from 30 minutes prior to partitioning until 30 minutes after partitioning, when the activity of the salt phase was sampled. The temperatures investigated were 1, 23, 46, and 60°C. The percentage of α-amylase activity remaining in the salt phase at each temperature (Appendix) are within 1% of each other, leading to the conclusion that temperature does not play an important role in partitioning α-amylase into methylcellulose.

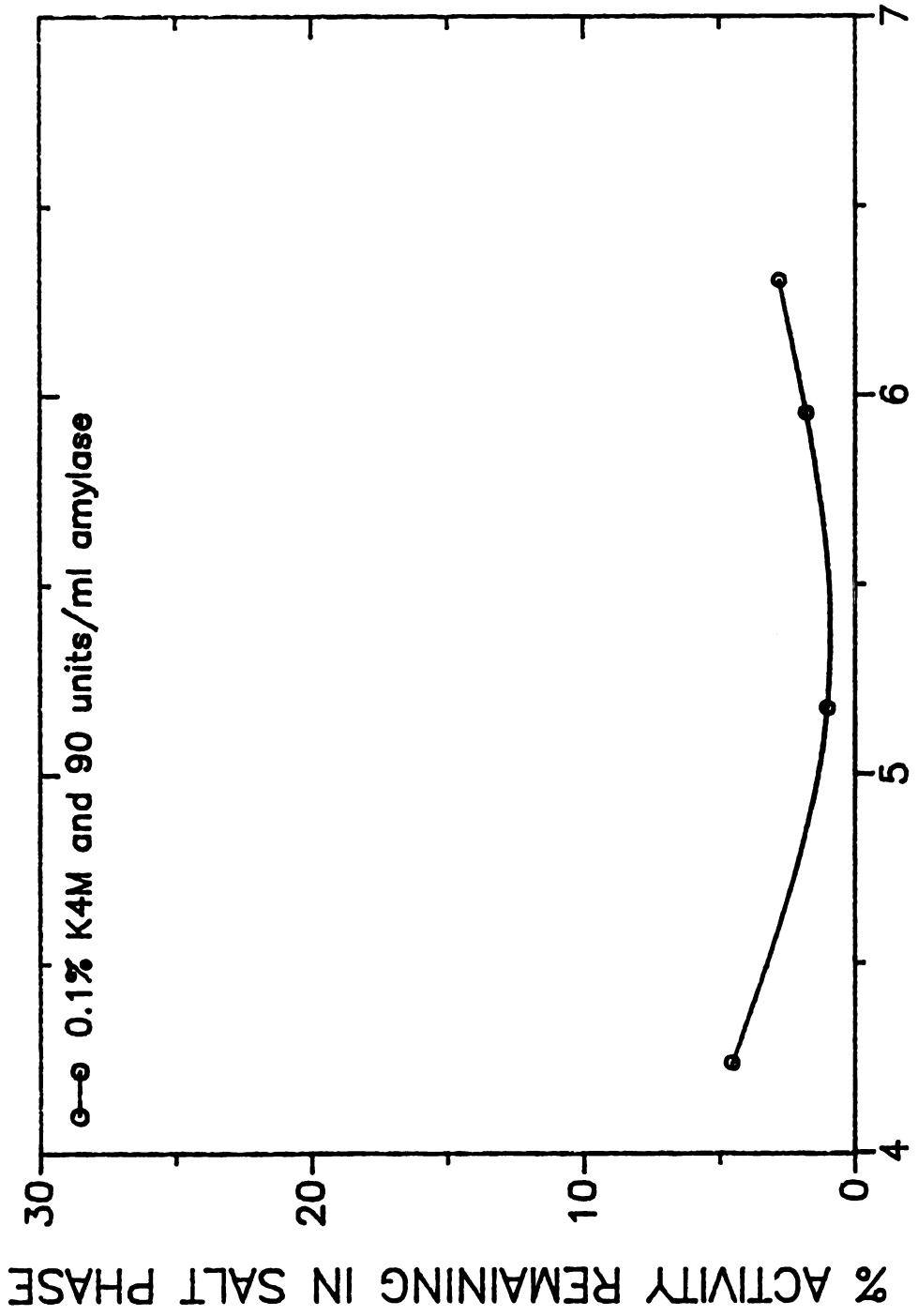

FIGURE 3.4: The percentage of activity remaining in the salt phase as a function of pH at 30% of ammonium sulfate saturation.

3.4.2 Purification

The purification of α-amylase from contaminating proteins is demonstrated by indicating the percent total protein as well as the percent amylase activity remaining in the salt phase after partitioning. The two incompletely purified α-amylase solutions, described in the experimental section, were used to test for purification by partitioning as a function of (i) methylcellulose concentration and (ii) ammonium sulfate saturation. In addition, the purifications obtained by methylcellulose partitioning as a function of ammonium sulfate saturation were used for comparison against ammonium sulfate precipitation.

The experiments on ammonium sulfate precipitation and two-phase partitioning were done with 3 ml solutions, to which 100% saturated ammonium sulfate was added. The concentration of methylcellulose, ammonium sulfate saturation, enzyme activity, and total protein of these solutions are given in Table 3.1. The total protein was measured after membrane dialysis, using 12 to 14 kilodalton molecular weight cutoff bags, against 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$

The results shown in Figures 3.5a or 3.5b indicate the percentage of activity or total protein remaining in the salt phase after partitioning of, extracellular or periplasmic extract α-amylase, respectively. The results shown in Figures 6a and 6b indicate the percentage of activity or total protein remaining in the salt phase after precipitation of extracellular or periplasmic extract α-amylase, respectively. The above results are shown with respect to ammonium sulfate saturation. However, the results shown in Figure 3.7 are with

Table 3.1. The a-amylase activity and total protein of solutions prepared for ammonium sulfate precipitation and methylcellulose/salt partitioning (partitioning as a function of (i) ammonium sulfate saturation and as a function of (ii) methylcellulose concentration).

Solution	[K15M] (% w/v)	Salt (% Sat.)	Activity (units/ml)	Tot. Prot. (ug/ml)
Extracellular				
Precipitation	0.00	var.[a]	120	830
Partitioning (i)	0.05	var.[b]	52	440
Partitioning (ii)	var.[c]	30	28	230
Periplasmic Extract				
Precipitation	0.00	var.[a]	1850	300
Partitioning (i)	0.05	var.[b]	950	96
Partitioning (ii)	var.[c]	30	102	10

[a]Ammonium sulfate saturations varied from 0 to 35%.

[b]Ammonium sulfate saturations varied from 0 to 70%.

[c]K15M concentration varied from 0 to 0.01% w/w.

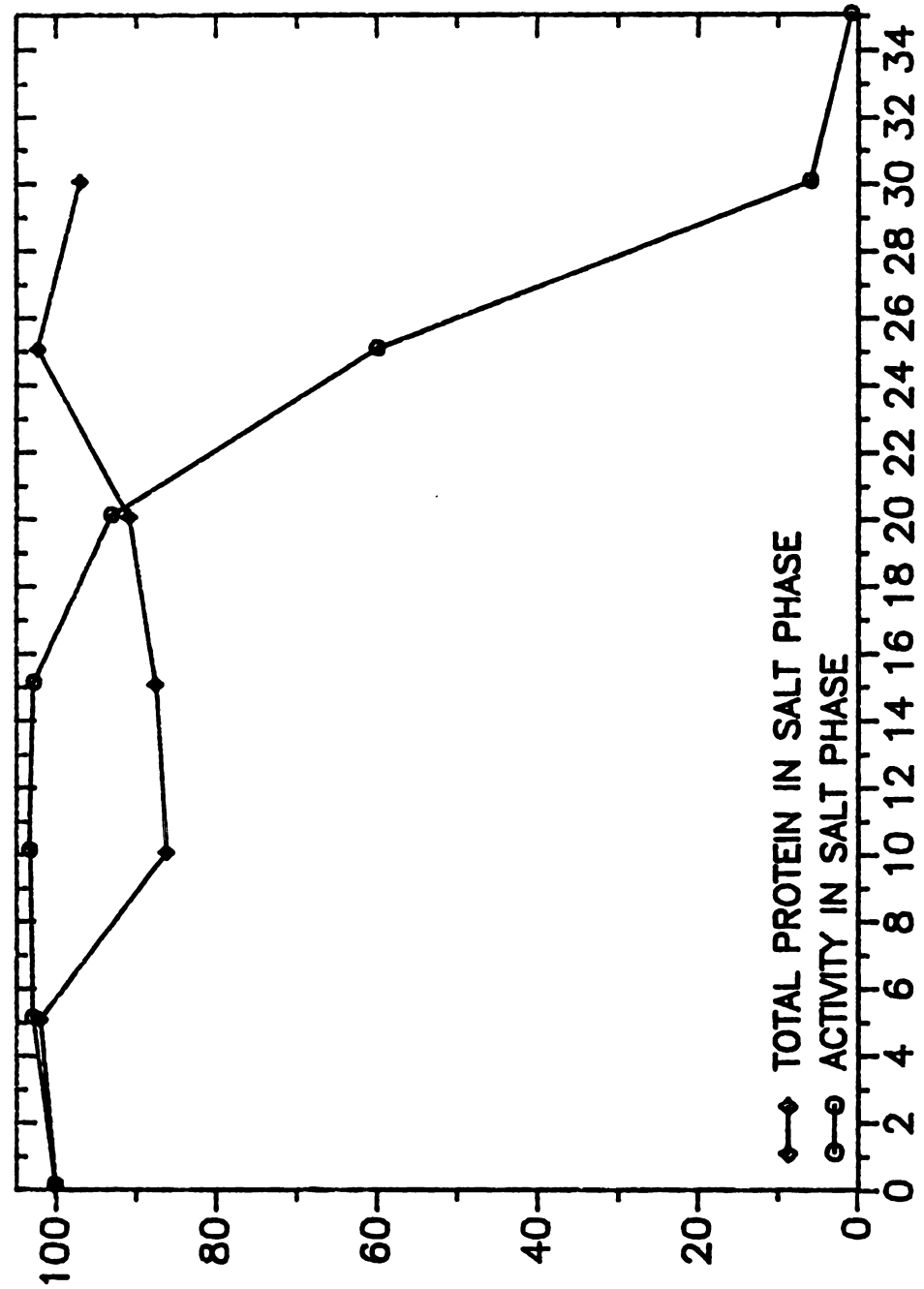

FIGURE 3.5a: The percentage of activity or protein remaining in the salt phase after partitioning of extracellular amylase.

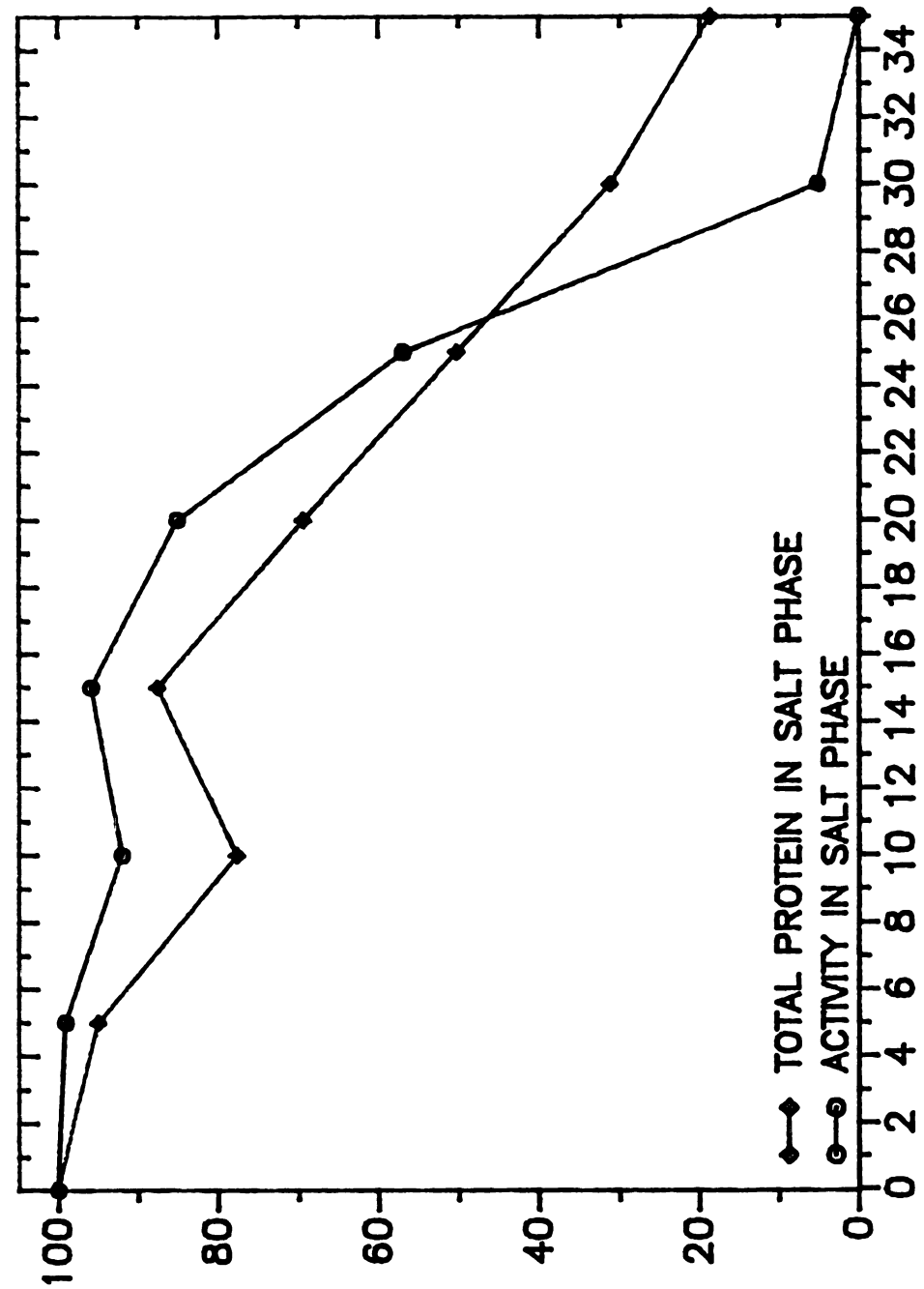

FIGURE 3.5b: The percentage of activity or protein remaining in the salt phase after partitioning of periplasmic extract amylase.

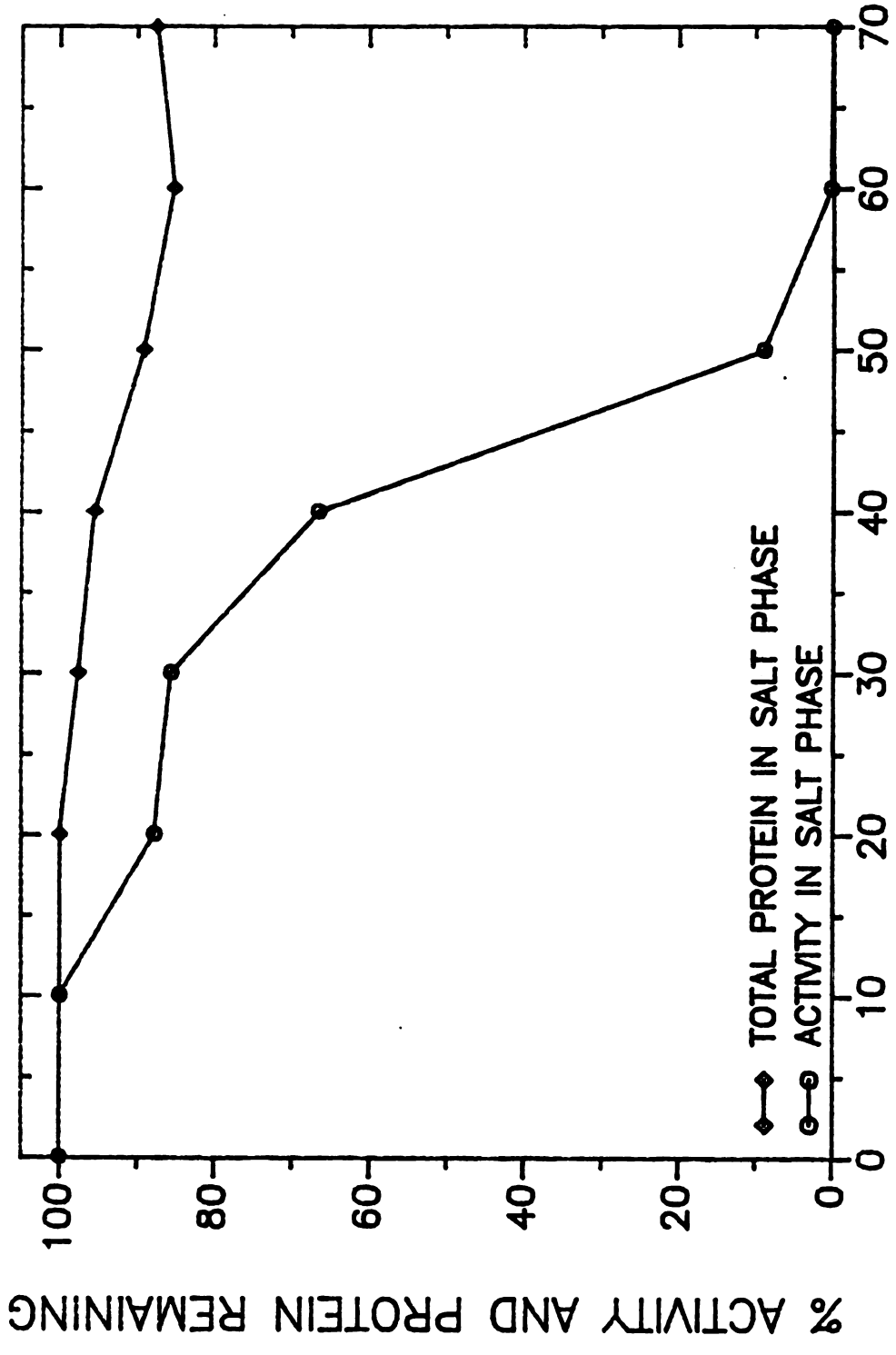

FIGURE 3.6a: The percentage of activity or protein remaining after precipitation of extracellular amylase.

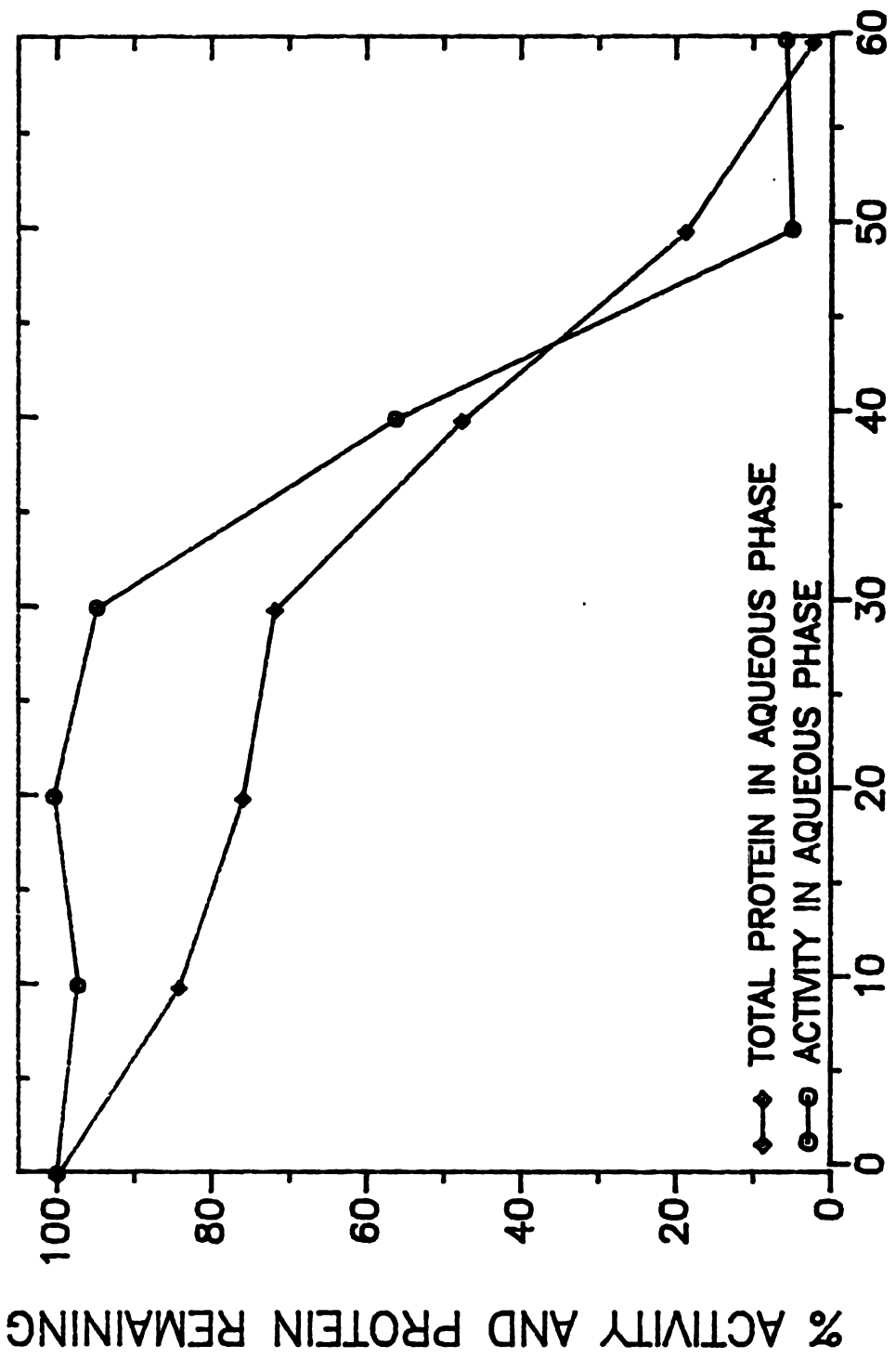

FIGURE 3.6b: The percentage of activity or protein remaining after precipitation of periplasmic extract amylase.

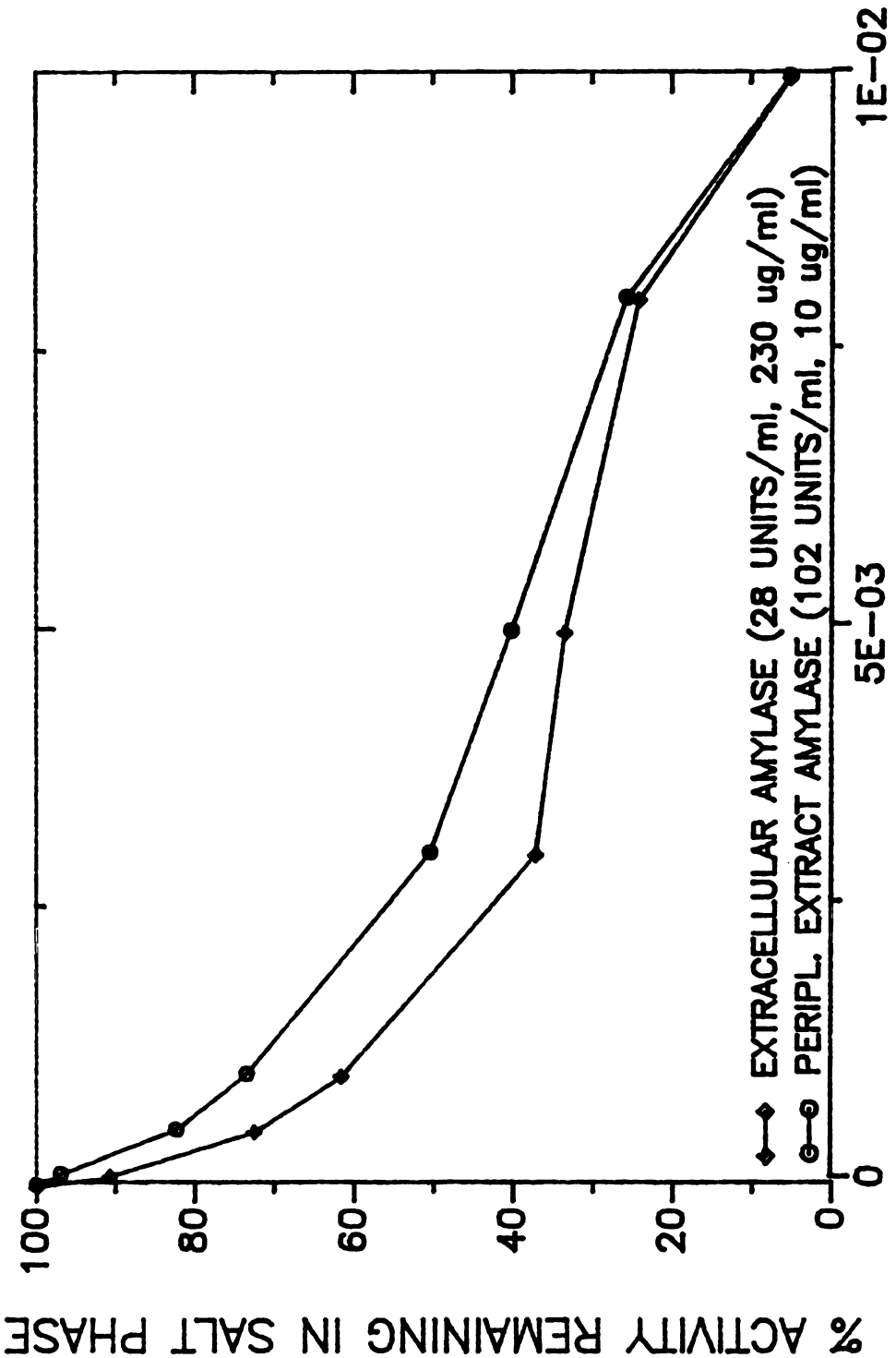

FIGURE 3.7: The percentage of activity remaining in the salt phase after partitioning with 30% of sat. salt, as a function of K15M concentration.

respect to the methylcellulose concentration, and are indicated as the percentage of activity remaining in the salt phase after partitioning extracellular and periplasmic extract α-amylase with 30% of saturation ammonium sulfate.

The specific activities of the extracellular α-amylase before either purification step is around 0.13 units/ug. However, after partitioning or precipitating with 30% or 50% saturated ammonium sulfate, respectively, the specific activity is approximately 3.8 or 1.1 units/ug, respectively (Figures 3.5a and 3.6a). Similarly, the specific activities of the periplasmic α-amylase before either purification step is around 9 units/ug. After partitioning or precipitating with 30 or 50% saturated ammonium sulfate, respectively, the specific activity is approximately 13.6 or 7.2, respectively (Figures 3.5b and 3.6b). The specific activity of α-amylase after the purification step was determined by assaying only the salt phase; therefore, the concentrations in the polymer or precipitate phases had to be extrapolated.

The results suggest that the purification (i.e. the ratio of specific activities) of extracellular α-amylase was nearly 30 fold by partitioning, and nearly 10 fold by precipitation. These approximate ratios indicate that partitioning extracellular α-amylase into a methylcellulose phase with 30% saturated ammonium sulfate is about three times more effective in purification than precipitating the α-amylase with 50% saturated ammonium sulfate. The periplasmic extract α-amylase had a specific activity 50 times larger than the extracellular amylase prior to the partitioning and precipitation experiments. Possibly for this reason, neither the partitioning nor the precipitation step

resulted in any substantial degree of additional purification.

3.4.3 Nature of the Partitioning Process

Equilibrium

The equilibrium of α-amylase between the salt and methylcellulose phases was investigated as a function of ammonium sulfate concentration in solutions containing 0.05% w/v A4M and 1200 units/ml α-amylase. Two experiments were carried out, one to test the equilibrium of α-amylase partitioned into a polymer phase with 30% sat. ammonium sulfate and its subsequent equilibration when the salt phase is replaced with solutions of 0, 10, 20 and 30% sat. ammonium sulfate. The second experiment tests the equilibrium of α-amylase, in solutions of 0, 10, 20 and 30% sat. ammonium sulfate, when a polymer phase that contains zero amylase is added. The polymer phase is formed by addition of 30% sat. ammonium sulfate after the subsequent salt phase had been removed. Equilibrium was assumed after 24 hours.

The percentage of initial activity remaining in the salt phase after attaining equilibrium, as a function of the equilibrium salt concentration, indicates that α-amylase is partitioned into the polymer phase with the addition of salt (Figure 3.8). In addition, the α-amylase resolubilizes out of the polymer phase with a reduction in salt concentration. The partitioning equilibrium of α-amylase in the methylcellulose/salt system is therefore only a function of ammonium sulfate concentration.

Hydrophobicity

The interaction between α-amylase and methylcellulose may be hydrophobic. It is already known that methylcellulose is slightly hydrophobic due to the degree and kind of substitution of its hydroxyl

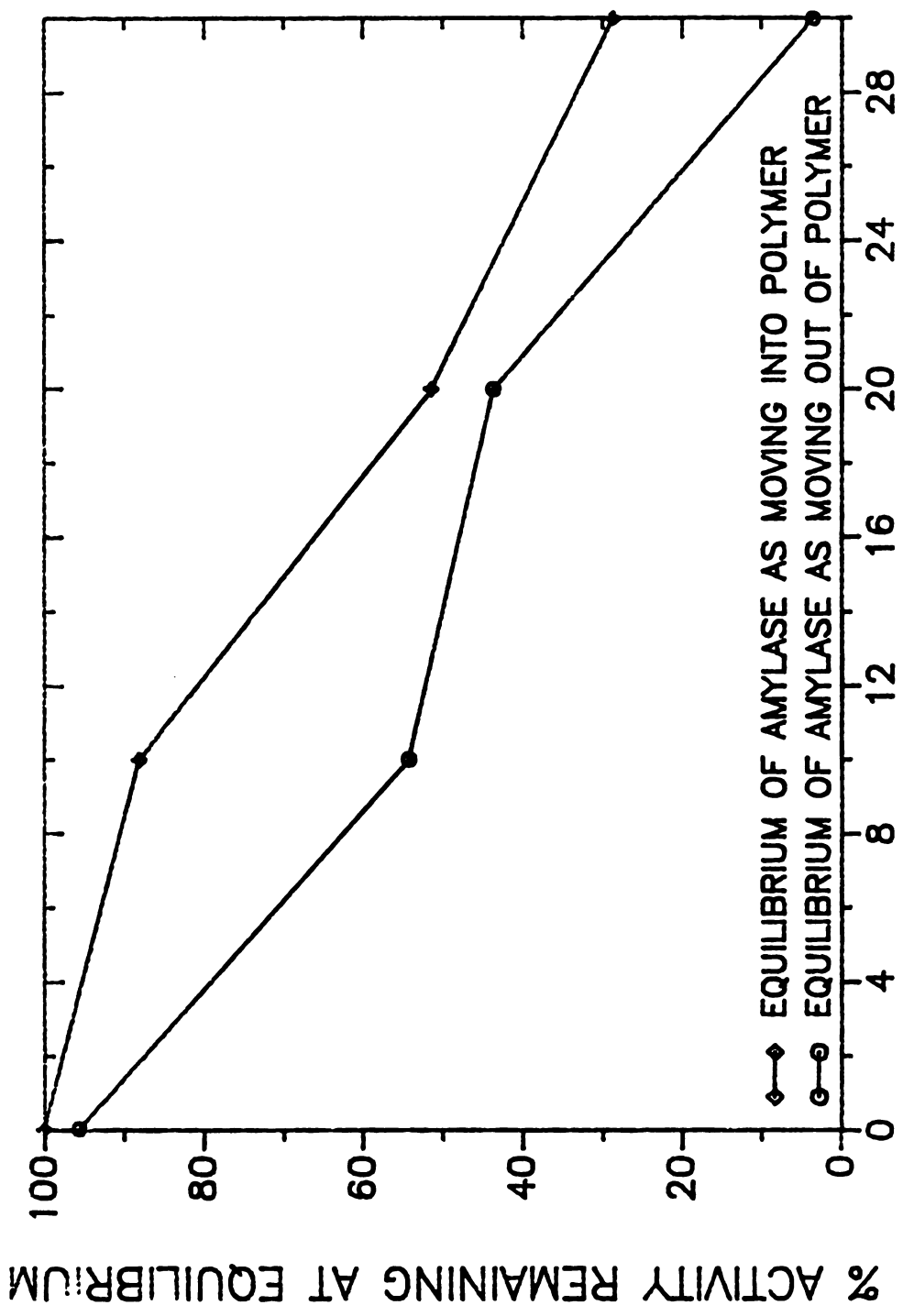

FIGURE 3.8: The percentage of activity remaining after partitioning has attained equilibrium as a function of ammonium sulfate saturation.

groups[26,27]. To test the degree of hydrophobic interaction corresponding to α-amylase, its adsorption, as a function of ammonium sulfate concentration, to two known hydrophobic gels, octyl and phenyl sepharose Cl 4B, was tested. Batch type adsorption experiments were performed separately after the sepharose gels were equilibrated with 50 mM Acetate, pH 6.0, containing 5 mM $CaCl_2$ and the appropriate ammonium sulfate concentration. The adsorption of extracellular α-amylase out of the aqueous phase was determined by measuring the dissappearance of starch reducing activity from that phase. The total protein remaining in the aqueous phase was measured. After removal of the ammonium sulfate, the amount of α-amylase desorbing into the aqueous phase from the sepharose was measured also. The adsorption/desorption results using octyl and phenyl sepharose-Cl 4B are shown in Figures 3.9a and 3.9b, respectively.

The results indicate that α-amylase is very strongly adsorbed to hydrophobic gels, with more affinity towards phenyl than octyl sepharose. The experiment demonstrated that α-amylase is a hydrophobic enzyme that can be strongly adsorbed to a very hydrophobic polymer. A strong affinity makes it difficult to recover the enzyme. However, these data do not provide insight to accurately draw conclusions about the nature of interaction between the α-amylase and methylcellulose in the precense of ammonium sulfate. Any explaination for the selectivity of α-amylase partitioning into methylcellulose requires a comparison of the interaction of several enzymes with methylcellulose and also a hydrophobic polymer, such as octyl sepharose (Chapter 4).

3.4.4 Methylcellulose Recovery

After purification, methylcellulose can be recovered and α-amylase

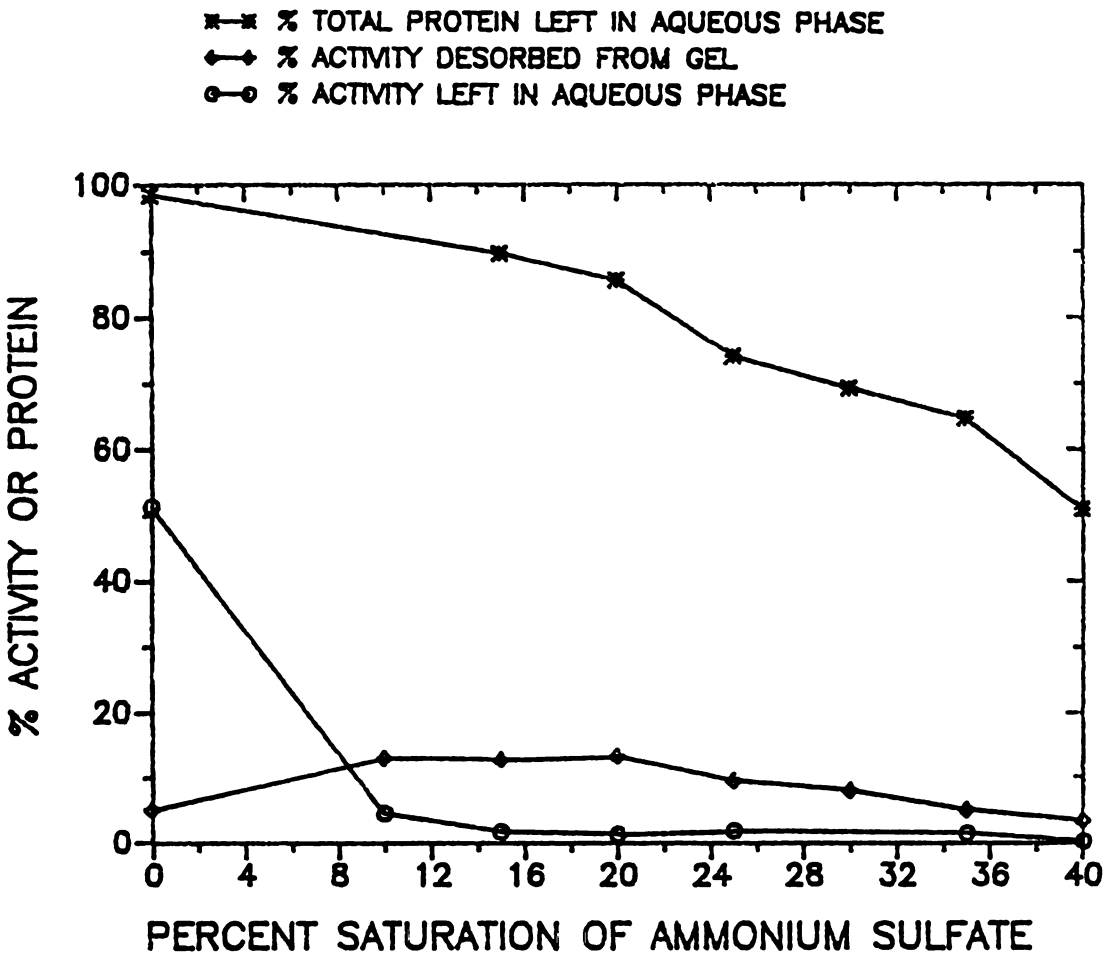

FIGURE 3.9a: The adsorbtion/desorbtion of amylase and adsorbtion of protein using octyl sepharose–Cl 4B.

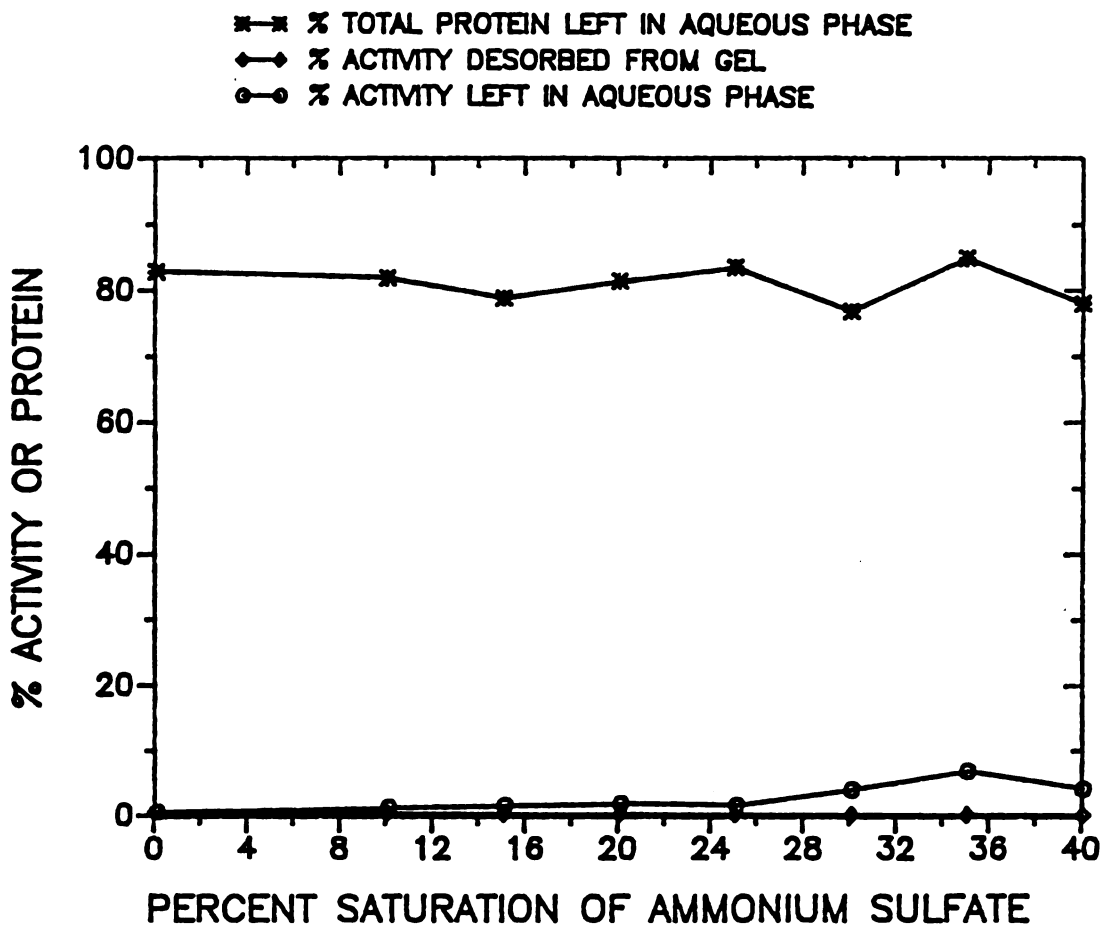

FIGURE 3.9b: The adsorbtion/desorbtion of amylase and adsorbtion of protein using phenyl sepharose–Cl 4B.

subsequently freed from most of the polymer. The recovery and liberation of the polymer and enzyme, respectively, is due to the reversibility of partitioning and the unique partioning profiles of methylcellulose and α-amylase upon addition of ammonium sulfate (described below).

The findings (Figure 3.10) suggests that extracellular α-amylase would require a purification step occuring at 30-35% sat. of ammonium sulfate, as over 94% of the enzyme was partitioned into the polymer phase there, while approximately 97% of the contaminating proteins were left in the salt phase. The contaminants could then be removed by disposing of the salt phase. In addition, the α-amylase can be reversibly resolubilized from the methylcellulose, as Figure 3.10 indicates, at an ammonium sulfate saturation of 15% and after equilibrium between amylase and methylcellulose has been reached. Under these conditions none of the α-amylase is partitioned into the methylcellulose phase while over 80% of the methylcellulose is partitioned out of the salt phase. After disposing of the methylcellulose precipitated at 15% salt saturation, the majority of the remaining methylcellulose can be precipitated by increasing the ammonium sulfate saturation to 20%. However, removing methylcellulose at an ammonium sulfate saturation of 20% results in a loss of α-amylase.

3.5 Conclusions

Recombinant DNA technology made it possible to produce a robust, thermostable α-amylase in E. coli, and to direct its excretion extracellularly[29]. The excretion of α-amylase extracellularly simplifies its downstream refining steps because it contains fewer contaminating proteins. An aqueous two phase partition step was studied to determine its potential for purifying α-amylase.

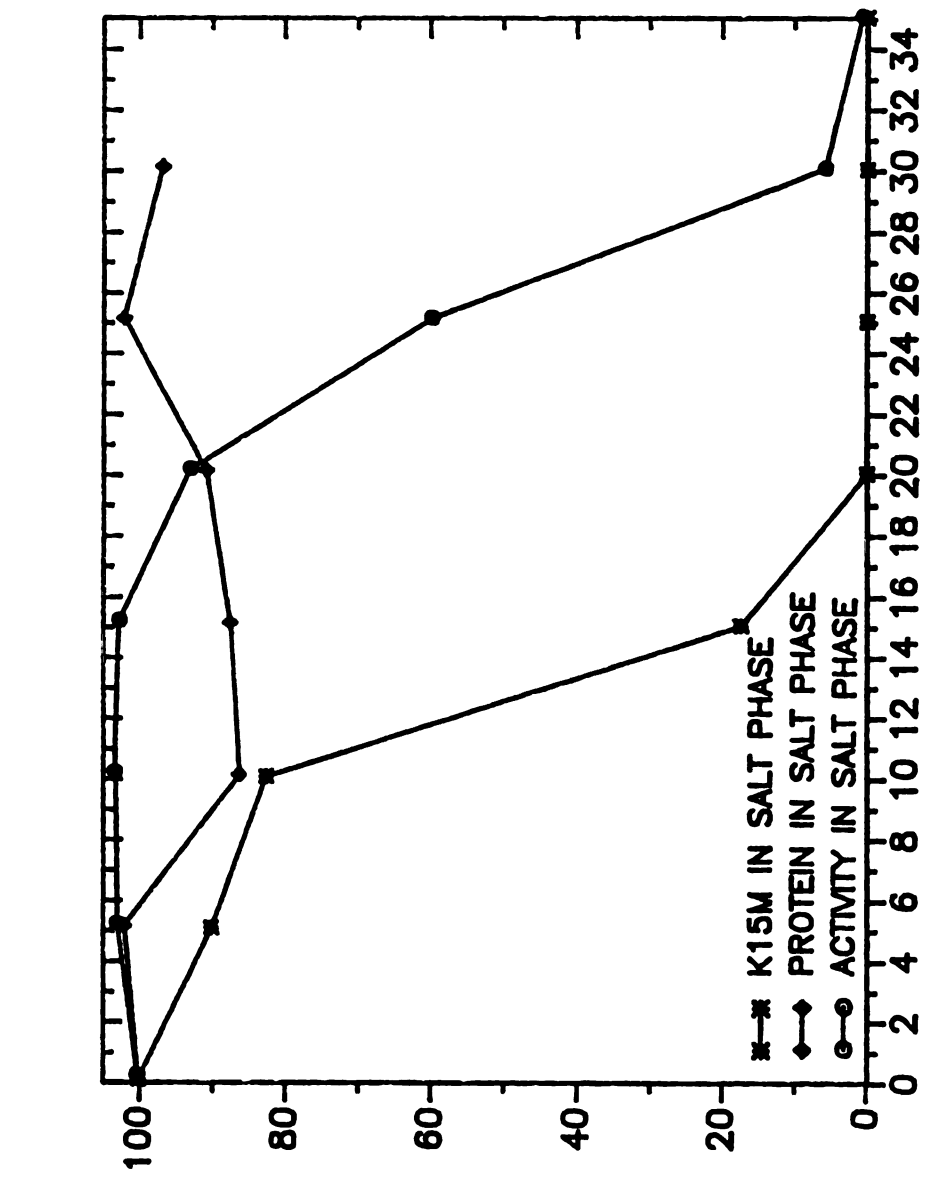

FIGURE 3.10: The percentage of activity, total protein or K15M remaining in the salt phase after partitioning of extracellular amylase.

The aqueous two-phase partioning step utilized a unique system consisting of methylcellulose and ammonium sulfate. Only one polymer phase was necessary for selective partitioning, an improvement over other aqueous two-phase partitioning techniques described using PEG and dextran[2,35]. Methylcellulose, designated MC or HPMC, were found to be equally effective especially at a molecular weight of 15 kilodaltons. Nearly complete partioning of α-amylase occurs at ammonium sulfate saturations of around 30-35% and methylcellulose concentrations of at least 1 g methylcellulose per g α-amylase. There is no pH optimum for partitioning, however, optimum pH for α-amylase activity is between 5.0 and 6.0. Similarly, there is no optimum temperature for partioning, however, ambient would be the most economical.

Preliminary studies indicated that a single aqueous two-phase partitioning step at an ammonium sulfate saturation of 30%, purified (according to specific activity) a fairly crude extracellular solution of α-amylase by 30 fold; 94% of the enzyme was partitioned out of the salt phase. Preliminary studies also indicated that approximately 80% of the methylcellulose can be separated from the α-amylase and nearly all of the enzyme recovered by resolubilizing the α-amylase at an ammonium sulfate saturation of 15%.

A scaled up purification process for the isolation of α-amylase was tested with approximately one liter of fermentation broth. The process consisted of insolubles removal by centrifugation, concentration by ultrafiltration, soluble contaminant removal by thermal precipitation, and aqueous two-phase partitioning steps for purification and methylcellulose recovery (Figure 3.11). The results are indicated in Table 3.2, and demonstrate a very good purification. However, the

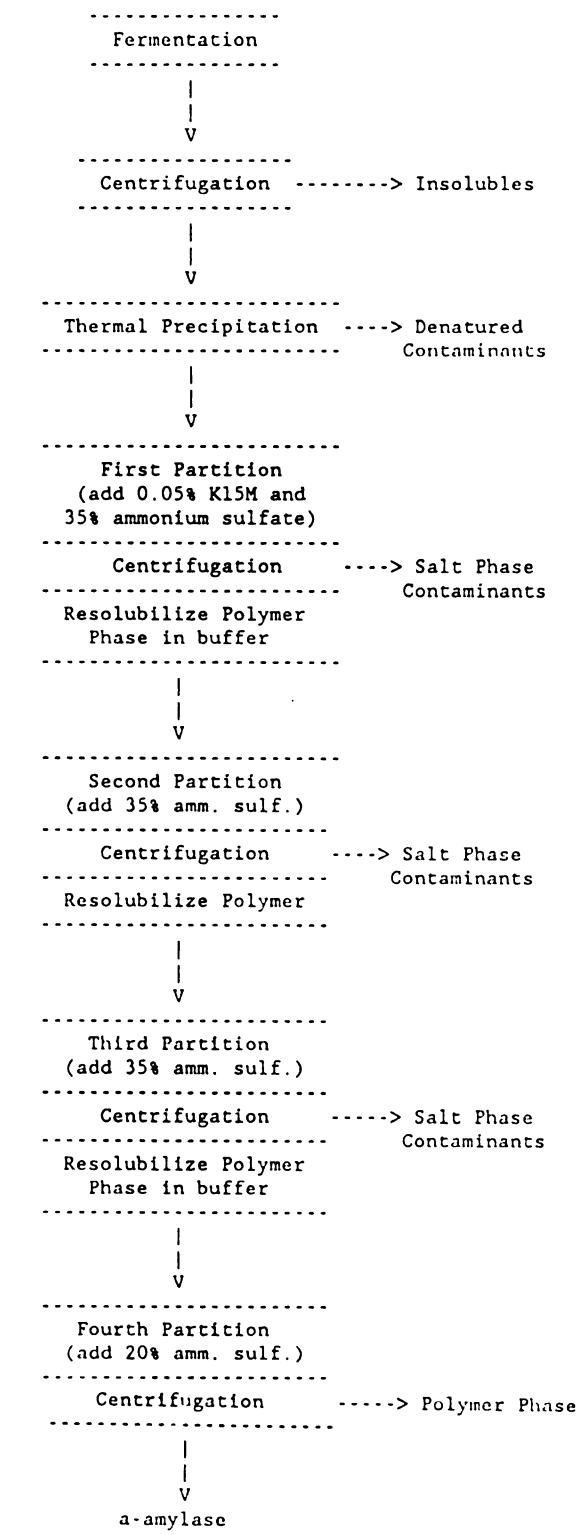

Figure 3.11. The downstream purification of a-amylase.

Table 3.2: The downstream refining of α-amylase.

Process	Volume (ml)	Activ. (units)	Protein (ug)	Carbohyd. (ug)	Purific.	Recovery
Centrifugation	970	3112	2,648,000	52.0		
Ultrafiltration	200	3034	1,380,000	35.2	1.9	97.5
Heat Precipit.	200	2318	1,255,000	30.6	2.1	74.5
First Partition	400	1960	18,000	19.2	147	63.0
Second Partition	200	1596	2,000	16.2	1320	51.3
Third Partition	100	1084	300	11.7	8800	34.8
MC Precipitation	50	510	?	0.7	?	16.4

process did not excell at recovery of α-amylase, around 16%. If small quantities of methylcellulose could be tolerated, the recovery of amylase could be increased to around 70%, with approximately 20 ug protein/ml remaining, by limiting the process to removal of only the insolubles followed by two partioning steps. Each partitioning step results in a loss of 10-15% of the α-amylase.

The concentration and purification success of α-amylase upon aqueous two-phase partitioning with methylcellulose and ammonium sulfate provides what appears to be a successful purification scheme for α-amylase. Moreover, methylcellulose has a low cost and a good standing with the FDA who have often approved its use in the food industry[26].

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CHAPTER 4

EFFECT OF A GENETICALLY ATTACHED SECRETION SEQUENCE ON PARTITIONING ALKALINE PHOSPHATASE FROM E. COLI

4.1 ABSTRACT

Alkaline phosphatase was previously fused genetically to a sequence of α-amylase. Purification of the fusion protein via aqueous two-phase partitioning (a technique successful for the purification of α-amylase) was investigated and some enhancement over regular precipitation was found. Investigation of the hydrophobicity, precipitation, and partitioning of α-amylase, alkaline phosphatase, and the amylase/phosphatase fusion protein indicate that partitioning of α-amylase into methylcellulose is not governed by hydrophobic interactions. Further partitioning experiments with other starch binding enzymes indicates a specific α-amylase/ methylcellulose interaction possibly similar to interaction between amylase and starch.

4.2 INTRODUCTION

Extracellular excretion of an enzyme simplifies its subsequent collection and purification by eliminating traditional problems of recovery from a heterogeneous mixture of periplasmic proteins or cellular debris. There are very few enzymes known to be naturally excreted by E. coli[1,2]. This lack of extracellular enzymes in gram-negative bacteria is thought to be due to transport limitations imposed by the lack of a general outer membrane secretion mechanism[2]. However, foreign gene products are known to be secreted, at least if they carry bacterial signal peptides whose function is similar for many

organisms[2,3]. These signal peptides function in transporting the protein into the periplasmic space[3] in E. coli and are removed during transport. The excretion mechanisms for transporting proteins from the periplasmic space to the culture medium are unknown[3]; however, the structure of the protein is known to play an important role[4]. Techniques exist for coupling protein synthesis to protein excretion[2].

Recently, Oriel and Alexander[5] discovered a peptide sequence in α-amylase transformed from Bacillus stearothermophilus into E. coli that enabled approximately 28% of this enzyme to be excreted extracellularly. The enzyme is excreted throughout the growth phase; however, the excretion is not accompanied by general periplasmic rupturing[5]. The structural regions which confer α-amylase its excretion properties were investigated to determine if fusions containing these regions could confer extracellular release to other proteins that are not normally excreted[3]. One such enzyme of commercial interest is alkaline phosphatase from E. coli, which is naturally secreted across the inner membrane to the periplasmic space, but not secreted extracellularly[3,4]. With this in mind, they took N-terminal regions containing approximately 10 and 65 percent of the amylase gene and fused them in frame to an alkaline phosphatase gene in which the upstream control and leader sequence had been removed[4]. They found alkaline phosphatase activity in both gene fusions produced at the predicted molecular weights.

Both fusion products were produced under culture conditions similar to those that generated the 25 percent extracellular excretion of α-amylase[4]. Oriel and Alexander found that the amount of fusion protein excreted under these conditions to be 17% and 7% of the larger and smaller alkaline phosphatase fusions, respectively[4]. These results

indicated that extracellular release of normally periplasmic proteins can be conferred by fusion to sequences of extracellular proteins, with longer sequences of the amylase being more effective[4]. The successful excretion is thought to be a result of the α-amylase sequence conferring desirable conformational changes on the alkaline phosphatase domain[4].

With a similar enhancement of purification in mind, recovery studies of the α-amylase and alkaline phosphatase gene fusion were conducted to determine if the fusion protein had separation properties which could be exploited commercially. This is of importance because a simple recovery and partial purification process has been demonstrated for α-amylase (Chapter 3) and a similar technique could be applicable to the fusion protein if the fused portion of amylase confers enough structural similarity.

In addition, experiments were designed to determine the nature of the interaction between α-amylase and methylcellulose. The adsorption to octyl sepharose, precipitation, and partitioning into methylcellulose of α-amylase, alkaline phosphatase, and the phosphatase/amylase fusion protein were determined upon addition of ammonium sulfate. These results were used to indicate if the α-amylase portion of the fusion protein conferred any hydrophobicity to the fusion protein. They also can give an indication of the nature of the interaction between α-amylase and methylcellulose in the precense of ammonium sulfate.

4.3 Experimental

Materials

The methylcellulose and hydroxypropyl methylcellulose were donated by Dow Chemical and have brand names A4M, K4M, K15M, and K100M designating the type of cellulose derivative (A for 26-33 percent

methoxy substitution and K for 19-24 percent methoxy and 7-12 percent hydroxypropoxyl substitution) and the approximate molecular weight (4, 15, and 100 corresponding to 85, 15, and 250 kilodaltons, respectively). The octyl sepharose-Cl 4B was obtained from Sigma. Alkaline phosphatase from E. coli was obtained from Sigma (type III-N). The alkaline phosphatase/α-amylase fusion protein and the α-amylase were obtained as described below. All other chemicals were of reagent grade.

Amylase/phosphatase fusion preparation

The fusion of a 10% portion of α-amylase from B. stearothermophilus to alkaline phosphatase from E. coli was performed by Dr. P. Alexander[4], and designated EC 243. EC 243 grown on agar plates (LB medium[6]) was obtained from Dr. P. Oriel, Department of Microbiology and Public Health, Michigan State University, E. Lansing, Michigan. EC 243 cells were transferred to 5 ml LB medium and grown in a 37°C shaker incubator, overnight. The innoculum was transferred to 1 l of LB medium contained in a 2800 ml large bottom Erlenmeyer flask and grown in a 37°C shaker incubator, overnight. The cells were pelleted and both the supernatant and cell pellet collected. The cell pellet was resuspended in approximately 50 ml of 50 mM tris-Cl buffer, pH 7.5. The method of Nossal and Heppel[7] was used to osmotically shock the cells and recover the periplasmic fusion phosphatase. The extracellular and periplasmic extract alkaline phophatase solutions were made up to 1000 ml and 100 ml, respectively, to give final concentrations of 50 mM tris-Cl and 0.01 M $MgSO_4$. These solutions were heated to 75°C for 15 minutes and stored at 4°C overnight to precipitate contaminants by denaturation as described by Schwartz and Lipmann[8]. The precipitates were pelleted and discarded. The extracellular phosphatase solution was further

concentrated by ultrafiltration (10,000 molecular weight cut off) from 1000 ml to 15 ml. After refrigeration overnight, the concentrated extracellular solution was cloudy with precipitate. The precipitate was removed by centrifugation.

Amylase preparation

The α-amylase was prepared from EC 147 cultures, provided by Dr. P. Oriel (Michigan State University, E. Lansing, MI 48824), by the same method described for the phosphatase/amylase fusion preparation, with slight modifications. The modifications consist of: using 50 mM acetate buffer, pH 6.0, including 5 mM Ca^{++}, instead of tris buffer; and, heat treating at 80°C for 45 min in the presence of 15 mM Ca^{++} instead of at 75°C for 15 min.

Methylcellulose preparation

The methylcellulose is prepared in 50 mM tris-Cl buffer, pH 7.5, or in 50 mM acetate buffer, pH 6.0, including 5 mM Ca^{++}, depending on whether partitioning alkaline phosphatase and the phosphatase/amylase fusion protein or α-amylase, respectfully. The methylcellulose powder is dispersed and wetted in the appropriate buffer solution by the slow addition of the powder to the hot buffer, around 80-90°C. The wetted methylcellulose is then poured into an equal volume of cold buffer, around 4°C, and well mixed. Methylcellulose solutions thus formed[9] were stored at 4°C between experiments for several months with no apparent qualitative changes.

Partitioning

The term partitioning is used here to indicate the selective localization of protein into either of the two phases formed. The exact physical nature of the two phases is somewhat unclear. With ammonium

sulfate-methylcellulose partitioning, the polymer phase can appear as liquid droplets under a microscope while resembling a precipitate in other characteristics.

Experiments involving homogeneously pure alkaline phosphatase were performed on a small scale using 1.5 ml Eppendorf tubes. However, experiments involving α-amylase or the phosphatase/amylase fusion protein were performed on a scale of several ml.

Equal volumes of methylcellulose and enzyme solution, prepared as described above, were added in various concentrations and mixed vortically at room temperature. After approximately 30 minutes with occasional mixing, saturated solutions of ammonium sulfate were added to the samples, then immediately mixed to produce the desired salt concentration. The addition of ammonium sulfate to the enzyme and dilute (0.5-0.0001%) methylcellulose solution created a precipitate like polymer phase and a salt phase. After an additional 30 minutes, while mixing occasionally, the precipitate was pelleted by centrifuging the Eppendorf tubes at 13000 rpm for 20 minutes. The resulting supernatant and/or pellet were then tested, after appropriate dilutions, for total enzymatic activity and when possible total protein. A measure of total protein was not possible on every sample because pure alkaline phosphatase along with the dilutions used in most of the experiments reduced the protein levels to less than that assayable by the Lowry et al. procedure[10].

Assays

Alkaline phosphatase was assayed as described by Torrianni[11]. Equal 1 ml volumes of substrate, 4.0 mM p-nitrophenol phosphate (pNPP) in 1 M Tris-Cl (pH 8.0), and enzyme solution were added and allowed to

react at 37°C for 10 minutes. The reaction was stopped by the addition of 0.2 ml 20% K_2HPO_4. Adsorbance was measured at 410 nm in solutions diluted to give adsorbance changes of less than 0.1/minute. The molar extinction coefficient of p-NP at 410 nm is 16.2 E3, and one unit of phosphatase was defined as 1 nmole p-nitrophenol/min/ml.

Total protein was measured using the method of Lowry et. al[10] using bovine serum albumin as the protein standard. The contribution of the methylcellulose to the total protein measurement was negligible.

4.4 Results and Discussion

4.4.1 Comparison of partitioning of alkaline phosphatase, amylase, and the phosphatase/amylase fusion protein

It has previously been shown[4] that the alkaline phosphatase/α-amylase fusion protein was conferred the α-amylase property of extracellular excretion. If the fusion protein was also conferred isolation properties similar to those of α-amylase, it could be isolated using a previously described and effective technique involving aqueous two-phase partitioning (Chapter 3). The experiments described in this section were designed to test the partitioning of the phosphatase/fusion protein and determine if either fusion portions, i.e. the phosphatase or the amylase, conferred observable selectivity to the fusion proteins partitioning. The concentration of methylcellulose, ammonium sulfate saturation, initial enzyme activity, and total protein used in these experiments are listed in Table 4.1.

Separate solutions of alkaline phosphatase, α-amylase, and phosphatase/amylase fusion protein were used to test for selectivity of partitioning as a function of both ammonium sulfate saturation (Figure 4.1) and methylcellulose concentration (Figure 4.2). The results show

Table 4.1. The initial enzyme activity and total protein of solutions prepared for ammonium sulfate precipitation and methylcellulose/salt partitioning (partitioning as a function of (i) ammonium sulfate saturation and as a function of (ii) methylcellulose concentration).

Solution	[K15M] (% w/v)	Salt (% Sat.)	Activity (units/ml)	Tot. Prot. (ug/ml)
a-Amylase				
Precipitation	0.00	var.[a]	122	820
Partitioning (i)	0.05	var.[b]	52	440
Partitioning (ii)	var.[c]	30	56	231
Alkaline Phosphatase				
Precipitation	0.00	var.[a]	7.06E-5	2.9
Partitioning (i)	0.05	var.[a]	7.06E-5	2.9
Partitioning (ii)	var.[c]	30	1.54E-5	2.5
Fusion Protein				
Precipitation	0.00	var.[a]	4.65E-4	55
Partitioning (i)	0.05	var.[a]	4.61E-4	192
Partitioning (ii)	var.[c]	30	3.13E-4	117

[a]Ammonium sulfate saturations varied from 0 to 70%.

[b]Ammonium sulfate saturations varied from 0 to 40%.

[b]K15M concentration varied from 0 to 0.03% w/w.

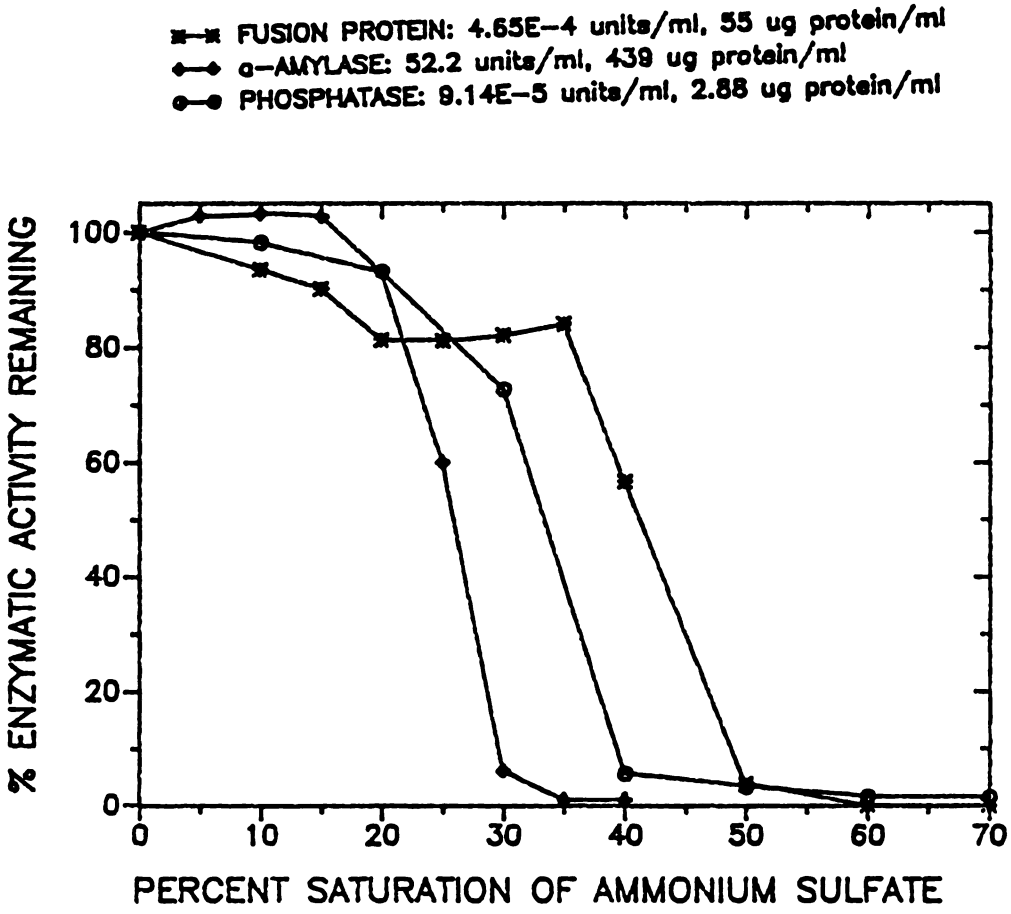

FIGURE 4.1: The percentage of phosphatase, amylase or fusion protein activity remaining after partitioning upon addition of ammonium sulfate.

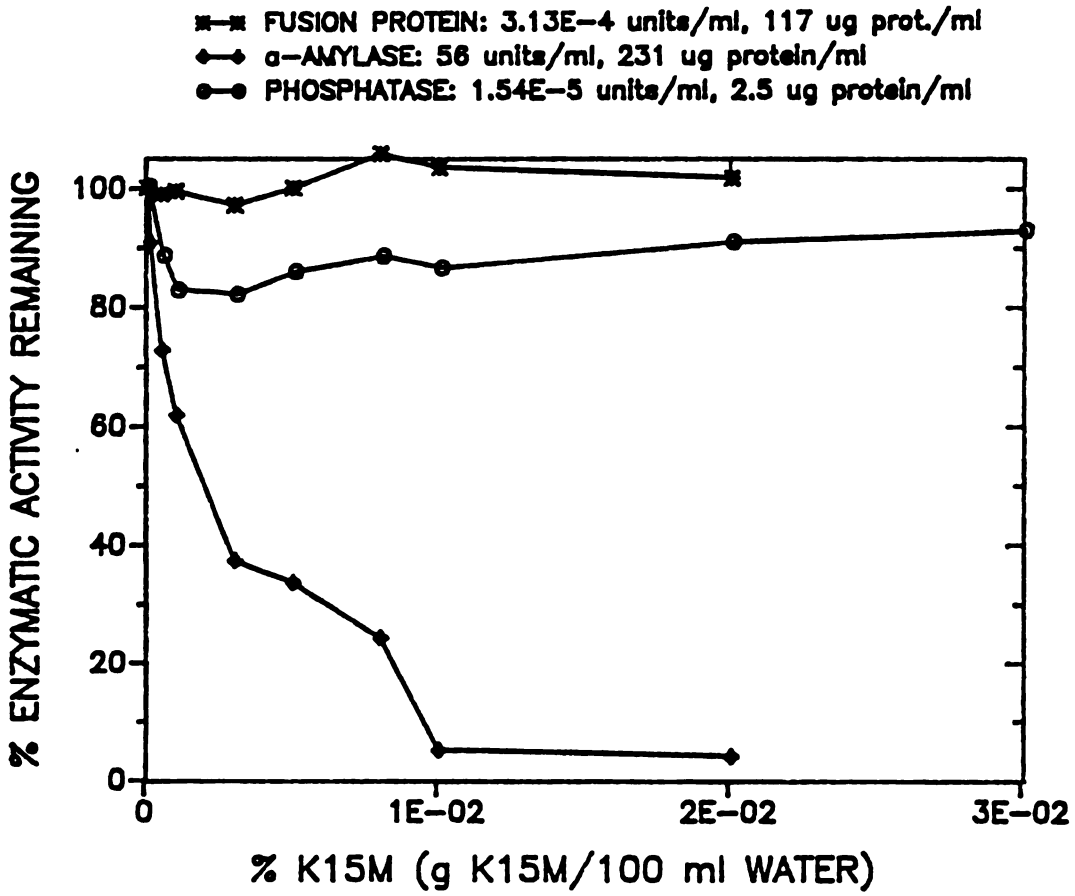

FIGURE 4.2: The percentage of phosphatase, amylase or fusion protein activity remaining in the salt phase with respect to % K15M present.

the percentage of each of the three enzymatic activities remaining in the salt phase after partitioning with respect to either ammonium sulfate (Figure 4.1) or methylcellulose added (Figure 4.2).

In addition, the effect of ammonium sulfate saturation on precipitation of the three proteins was tested to ensure that precipitation and partitioning were not confused. The results show the percentage of enzymatic activity remaining in solution after either partitioning or precipitation of solutions of alkaline phosphatase (Figure 4.3a), α-amylase (Figure 4.3b), or phosphatase/amylase fusion protein (Figure 4.3c) with respect to ammonium sulfate added.

A comparison of partitioning and precipitation of each enzyme, upon addition of ammonium sulfate, indicates that the exclusion of α-amylase (Figure 4.3b) from the salt phase is greatly enhanced in the presence of methylcellulose. Whereas, the exlusion of alkaline phosphatase (Figure 4.3a) or the fusion protein (4.3c) from the salt phase is not as affected by the presence of methylcellulose. However, the fusion protein partitions before it precipitates, similar to α-amylase (Figures 4.3b and 4.3c); and, the alkaline phosphatase precipitates slightly before it partitions (Figures 4.3a and 4.3c). In addition, comparing each enzymes partitioning (Figure 4.1) indicates that α-amylase is the first to be partitioned out of the salt phase as ammonium sulfate is added, followed by alkaline phosphatase and lastly the phosphatase/amylase fusion protein. Similarly, at 30% of saturation ammonium sulfate more α-amylase, by percentage, is partitioned out of solution, with increasing methylcellulose concentration, than alkaline phosphatase or the fusion protein (Figure 4.2). Again, the percentage of phosphatase partitioning out of the salt phase is greater than that

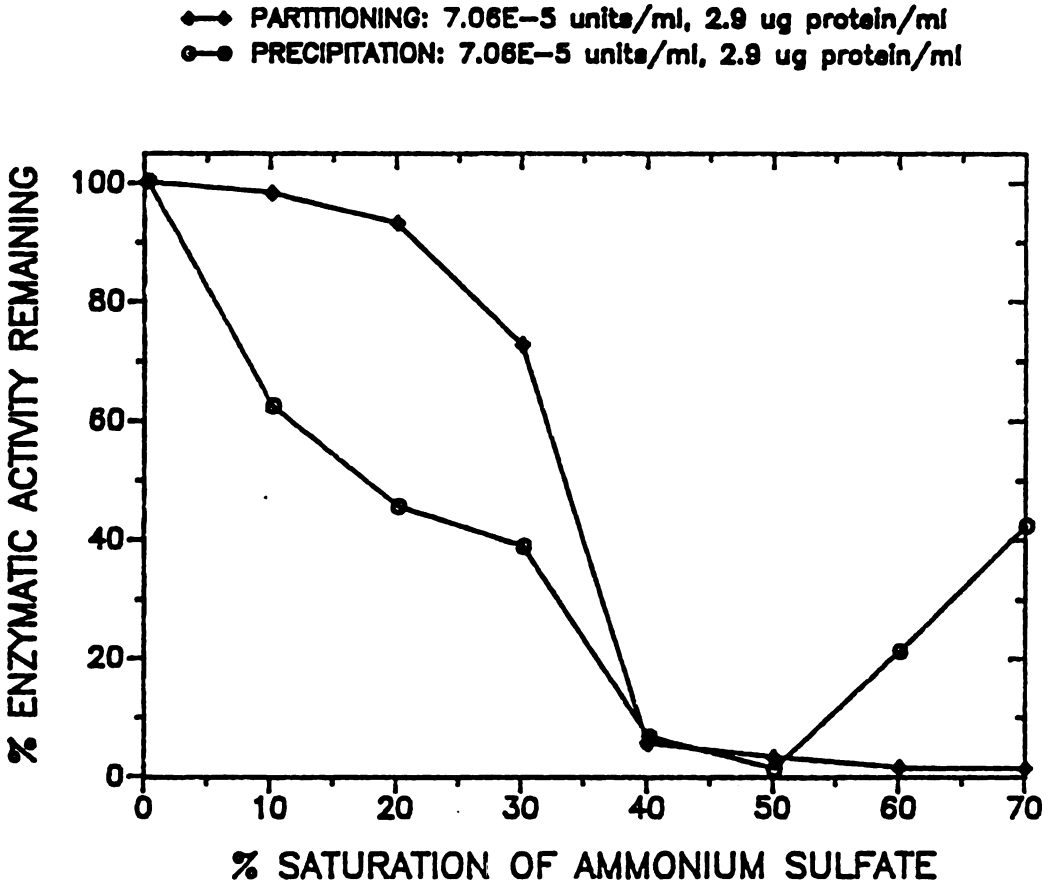

FIGURE 4.3a: The % of phosphatase activity remaining in the salt phase, as a function of salt added, for either partitioning or precipitation.

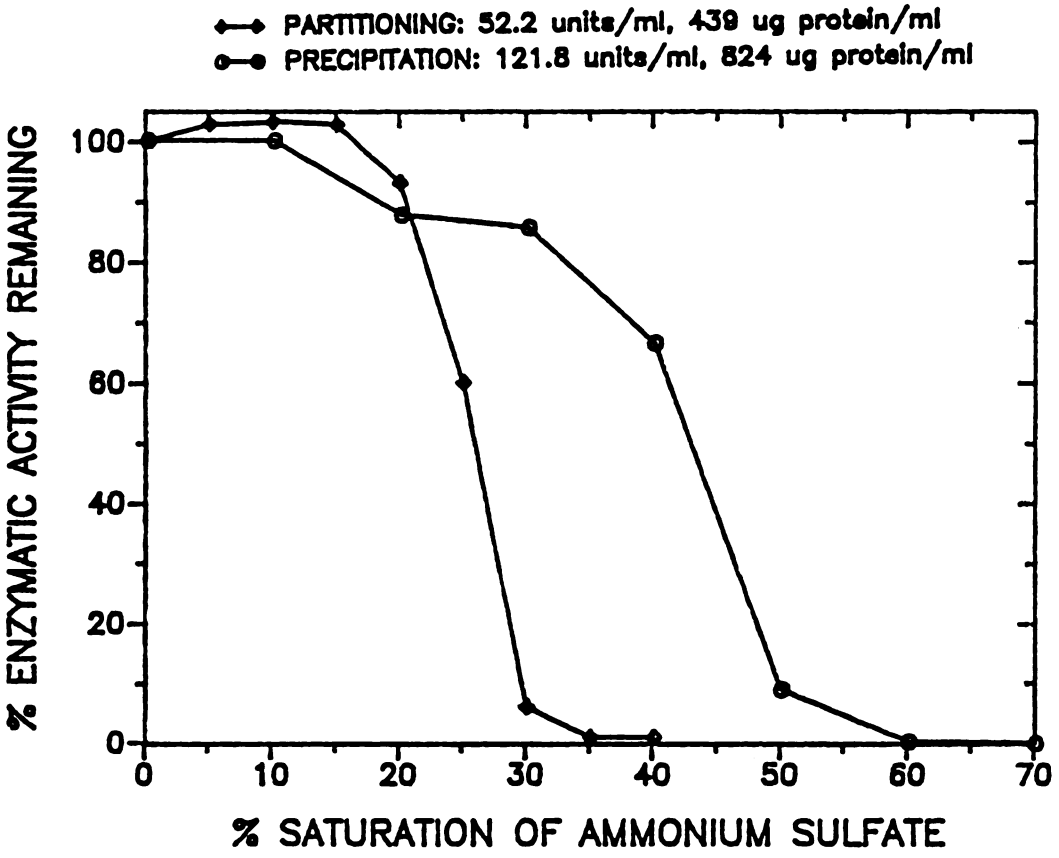

FIGURE 4.3b: The percentage of a-amylase activity remaining in the salt phase, as a function of salt added, for either partitioning or precipitation.

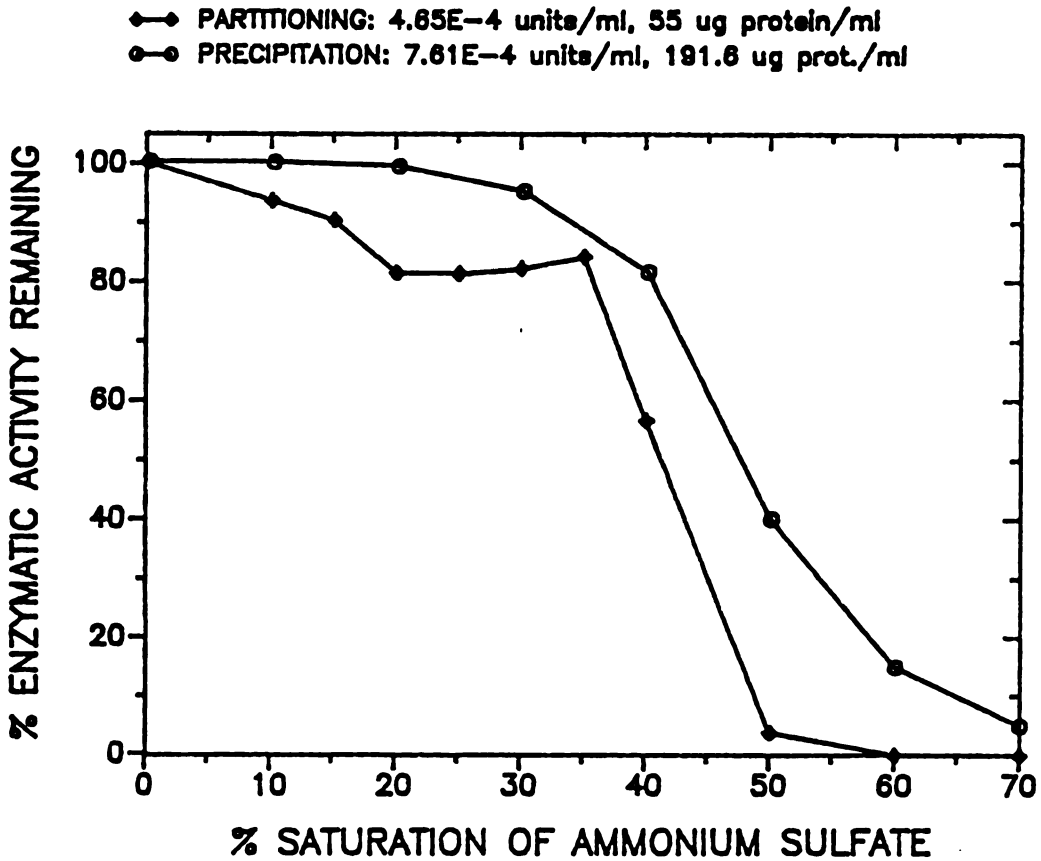

FIGURE 4.3c: The percentage of phosphatase activity, due to the fusion protein, remaining in the salt phase with respect to salt added.

for the fusion protein (Figure 4.2).

These results indicate that the α-amylase portion (approximately 10% of the α-amylase) of the fusion protein conferred little of the partitioning selectivity unique to α-amylase. These results seem to indicate that the amylase portion of the fusion protein works against partitioning into methylcellulose (Figure 4.2) and also against precipitation (Figure 4.4). The results indicate that the partitioning specificity of α-amylase into methylcellulose is probably not due to the simple hydrophobic interactions assumed. It should be mentioned that the quantitative comparisons of the results should be viewed with caution due to the different sources and purities of the enzymes.

4.4.2 Comparison of hydrophobicity of alkaline phosphatase, amylase, and the phosphatase/amylase fusion protein

Prior to the experiments just described, the nature of interaction between α-amylase and methylcellulose was assumed to be hydrophobic in nature (Chapter 3). Comparing the partitioning into methylcellulose of alkaline phosphatase, α-amylase and the phosphatase/amylase fusion protein upon addition of ammonium sulfate, however, indicated that the selectivity for amylase partitioning was much more complex than the simple hydrophobic interaction previously assumed.

An experiment was designed to determine quantitatively the degree of hydrophobicity, if any, that was conferred to the fusion protein by its amylase fusion portion. The resulting experiment tested the hydrophobicity of the three enzymes by comparing their enzymatic activity left in the salt phase after adsorbtion to octyl sepharose, with respect to ammonium sulfate added (Figure 4.5). Adsorbtion to octyl sepharose can be used to test for hydrophobicity because it is

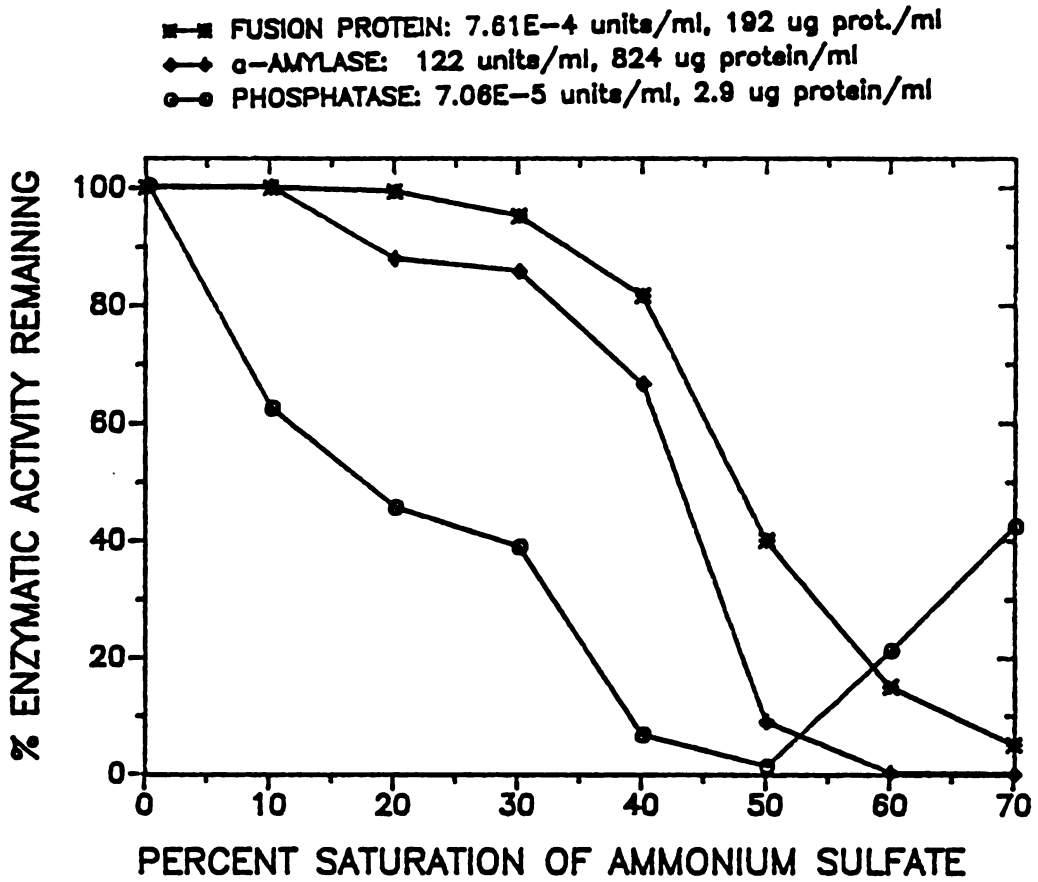

FIGURE 4.4: The percentage of phosphatase, amylase or fusion protein activity remaining after precipitation upon addition of ammonium sulfate.

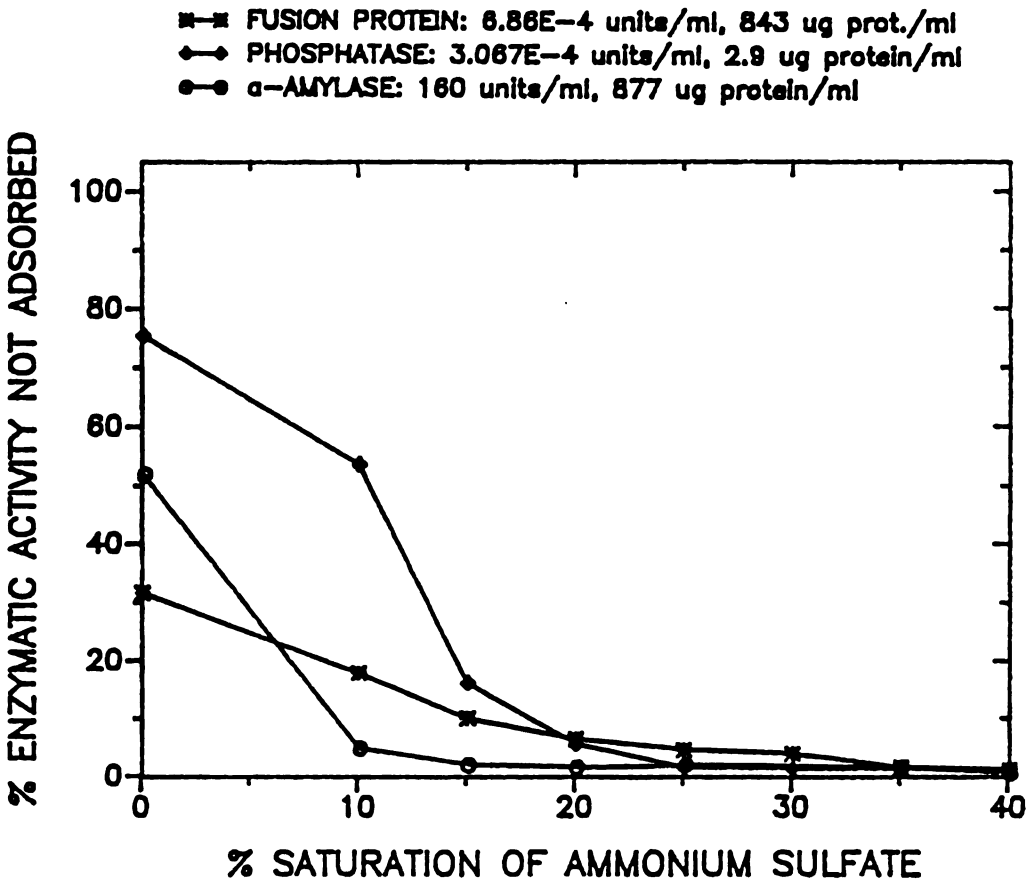

FIGURE 4.5: The adsorbtion of the fusion protein, amylase or phosphatase to octyl sepharose as a function of the salt added.

known to interact hydrophobically with proteins[12]. Also, the addition of ammonium sulfate increases the van der Waals attraction between proteins and nonpolar-hydrocarbon-coated adsorbents[13], i.e. the hydrophobic effect. Proteins with a relatively large proportion of nonpolar molecules exposed are attracted to hydrophobic adsorbents more than proteins with relatively few nonpolar molecules exposed[13].

Because the hydrophobic effect is increased with addition of ammonium sulfate, and the electrostatic effect decreased[14], the relative hydrophobicity of the enzymes is best indicated only after ammonium sulfate was added. Without this assumption, the results (Figure 4.5) would not give a clear indication if any hydrophobic interactive nature was conferred upon the fusion protein (Figure 4.5). At ammonium sulfate saturations of 10% and above, the results indicate that α-amylase has more hydrophobic interaction with octyl sepharose than the other two proteins, as assumed. The results also indicate that the fusion protein is more hydrophobic in its interaction than alkaline phosphatase. Thus, the fusion protein was conferred considerable hydrophobic nature from its α-amylase portion.

4.5 Summary

Recent developments in recombinant-DNA technologies have made it possible to direct the extracellular excretion of proteins produced in *E. coli*[2], thus enhancing the proteins subsequent purification by limiting contaminants. One such extracellular, recombinant-DNA transformed protein is an alkaline phosphatase fusion to a portion of α-amylase (10% of the amylase)[4]. Because a successful technique for purifying α-amylase had already been developed, based upon aqueous two phase partitioning into methylcellulose from a salt phase (Chapter 3), a

similar technique could be applicable to the fusion protein if the fused portion of amylase confered enough structural similarity.

The results indicate that the phosphatase/amylase fusion protein has been confered an increased hydrophobic nature but not a similar selectivity for partitioning into methylcellulose. Therefore, the conclusion can be made that it is not hydrophobic interactions that govern the partitioning of α-amylase into methylcellulose. The highly selective partitioning of α-amylase indicates that there must be a specific interaction between the methylcellulose and the enzyme. Somehow, possibly through an allosteric or active site, the α-amylase may recognize some portion of the methylcellulose structure, while existing in its precipitated state, as similar to its substrate. This assertion must be confirmed by subsequent experiments.

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CHAPTER 5

SUMMARY

5.1 Conclusions

The purpose of this research was to evaluate the concentration and purification of α-amylase from E. coli using a methylcellulose/salt, two-phase partitioning process. The following objectives were achieved in this study:

1. The partitioning of α-amylase in the methylcellulose/salt aqueous two-phase system was optimized with respect to the type, molecular weight, and concentration of cellulose derivative, the type and concentration of salts added, the solution pH, and the temperature during extraction.
2. The partitioning of α-amylase was reversed upon decreasing the salt concentration, and a large percentage of the methylcellulose recovered from the purified enzyme.
3. A process was demonstrated for recovering α-amylase from crude broth using methylcellulose/salt partitioning.
4. The nature of interaction between the methylcellulose and α-amylase was not hydrophobic in nature. The nature of interaction is most likely some specific interaction similar in nature to the interaction of starch and α-amylase. This conclusion is supported by work on partitioning of β-amylase with methylcellulose[2].
5. Aqueous two phase partitioning with methylcellulose/ salt is not an effective technique for the purification of a phosphatase/amylase fusion protein containing a portion of α-amylase.

5.2 Proposals For Future Research

It would be fruitful to focus future research on several aspects of the purification process:

1. Investigate partitioning with other polymers such as water soluble gums like curdlan or carboxymethyl amylose. In addition, specific polymers could be tailor-made[1].
2. Investigate the exact interaction between methylcellulose and α-amylase which this paper concluded could be similar in nature to the starch-amylase interaction. Spectroscopic techniques seem applicable.
3. Investigate the application of methylcellulose/salt partitioning with other starch binding enzymes. (This has already been attempted with β-amylase, resulting in similar partitioning to α-amylase.)
4. Investigate the purification of other enzymes that were designed specifically to enhance their separation.
5. Investigate the interaction of cellulose and α-amylase at different ammonium sulfate saturations.
6. Investigate further the function of temperature on partitioning, especially at low ammonium sulfate saturations.
7. Investigate the nature of the polymer phase, whether it is liquid, precipitate or a combination of the two. Light scattering techniques seem applicable.

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APPENDIX

ORIGINAL DATA

Effect of methylcellulose concentration, Figure 3.2:

The percentage of activity remaining in the salt phase as a function of the enzyme loading.

30% ammonium sulfate; 90 units/ml of initial amylase activity; 3.5 ug/ml of initial protein; T = 23°C

MC (w/v)	Final Activity (units/ml)	Loading (g A4M/g amylase)	% Activity
0.005	2.6	14.3	1.3
0.001	13.6	2.8	6.6
0.0005	33.6	1.4	16.2
0.0001	167.9	0.28	80.1
0.00005	184.3	0.14	88.9
0.0	207.4	0.0	100.0

30% ammonium sulfate; 0.005% (w/v) A4M initially; T = 23°C

Initial		Final		
Activity (units/ml)	Protein (ug/ml)	Activity (units/ml)	Loading (g A4M/g amylase)	% Activity
			0.0	100.0
2847	117.9	685.7	0.424	24.1
569	23.6	77.1	2.12	13.5
285	11.8	22.8	4.24	8.0
142	5.9	6.1	8.47	4.3
56.9	2.4	2.3	20.8	4.0

Effect of different salts, Figure 3.3:

The percentage of activity remaining in the salt phase as a function of the ammonium sulfate or sodium chloride saturations.

0.05% w/v K4M; T = 23°C

% NaCl	Final Activity (units/ml)	% Activity	% NS*	Final Activity (units/ml)	% Activity
0.0	129	100.0	0.0	26.0	100
15.0	102	78.9	15.0	22.9	88
20.0	84	65.0	20.0	13.6	52
25.0	87	67.3	25.0	3.6	14
30.0	90	69.7	30.0	0.6	2

* % of ammonium sulfate saturation

Effect of pH, Figure 3.4:

The percentage of activity remaining in the salt phase as a function of pH.

0.05% w/v K4M; T = 23°C

Final Activity			
0% NS	30% NS	Final pH	% Activity
53.7	2.4	4.23	4.5
102.7	1.0	5.17	1.0
88.3	1.6	5.95	1.8
112.2	3.2	6.76	2.8

Effect of temperature:

The percentage of activity remaining in the salt phase as a function of temperature.

0.05% w/v K4M

Final Activity			
0% NS	30% NS	Temperature	% Activity
113.1	1.62	1	1.4
118.7	1.46	23	1.2
120.6	1.94	46	1.6
101.3	2.80	60	2.7

Purification, Figures 3.5a, 3.5b, 3.6a, 3.6b and 3.7:

The percentage of activity or protein remaining in the salt phase after partitioning of extracellular amylase.

0.05% w/v K15M; 52 units/ml of amylase; 440 ug/ml of total protein

% NS	Final Activity (units/ml)	% Activity	Final Protein (ug/ml)	% Protein
0	52.2	100.0	438.8	100
5	53.7	102.9	447.5	102
10	53.9	103.3	378.1	86
15	53.7	102.8	425.1	97
20	48.6	93.1	399.0	91
25	31.3	60.0	449.1	102
30	3.2	6.1	425.8	97

The percentage of activity or protein remaining in the salt phase after partitioning of periplasmic amylase.

0.05% w/v K15M; 950 units/ml of amylase; 96 ug/ml of total protein

% NS	Final Activity (units/ml)	% Activity	Final Protein (ug/ml)	% Protein
0	946.8	100.0	96.0	100
5	935.1	99.1	91.2	95
10	870.3	92.0	74.5	77
15	906.2	95.8	84.1	86
20	801.2	85.1	66.6	69
25	536.2	56.9	48.2	50
30	48.8	5.2	29.9	31
35	1.2	0.1	17.9	19

The percentage of activity or protein remaining after precipitation of extracellular amylase.

0.00% w/v K15M; 120 units/ml of amylase; 830 ug/ml of total protein

% NS	Final Activity (units/ml)	% Activity	Final Protein (ug/ml)	% Protein
0	121.8	100.0	832.9	100
10	121.8	100.0	---	---
20	106.8	87.7	---	---
30	104.3	85.6	804.4	98
40	81.0	66.5	787.1	96
50	10.5	9.0	734.4	89
60	0.3	0.3	702.2	85
70	0.1	0.1	721.3	87

The percentage of activity or protein remaining after precipitation of periplasmic amylase.

0.00% w/v K15M; 1850 units/ml of amylase; 300 ug/ml of total protein

% NS	Final Activity (units/ml)	% Activity	Final Protein (ug/ml)	% Protein
0	1846	100.0	298.1	100
10	1797	97.3	250.9	84
20	1854	100.4	226.6	76
30	1750	94.8	214.2	72
40	1038	56.2	142.0	48
50	92.3	5.0	55.6	19
60	106.9	5.8	7.0	2

The percentage of activity remaining in the salt phase as a function of K15M concentration.

30% saturation of ammonium sulfate;
28 units/ml of extracellular amylase;
230 ug/ml of extracellular total protein;
102 units/ml of periplasmic amylase;
10 ug/ml of periplasmic total protein

% K15M	Periplasmic Amylase		Extracellular Amylase	
	Activity	% Activity	Activity	% Activity
0.0	102.4	100.0	28.0	100.0
0.0001	99.2	96.9	25.4	90.7
0.0005	84.3	82.3	20.3	72.5
0.001	75.2	73.4	17.2	61.6
0.003	51.6	50.4	10.4	37.1
0.005	41.1	40.1	9.4	33.4
0.008	26.2	25.6	6.8	24.1
0.01	5.1	5.0	1.4	5.0

Equilibrium, Figure 3.8:

The percentage of activity remaining after partitioning has attained equilibrium as a function of ammonium sulfate saturation.

0.05% w/v A4M

Equilibrium of amylase as moving out of pellet
(100% activity = 1193 units/ml)

% NS	Final Activity	% Activity
0	1141	95.6
10	646.6	54.2
20	520.1	43.6
30	42.4	3.6

Equilibrium of amylase as moving into polymer
(100% activity = 1425 units/ml)

% NS	Final Activity	% Activity
0	1425	100.0
10	1256	88.1
20	621.0	51.4
30	341.5	28.6

Hydrophobicity, Figures 3.9a and 3.9b:

The adsorbtion/desorbtion of amylase and adsorbtion of protein using octyl sepharose-Cl 4B.

0.033% w/v K15M
(100% activity = 160.5 units/ml; 100% protein = 877 ug/ml)

	Adsorbed		Desorbed			
% NS	Activity	% Activity	Activity	% Activity	Protein	% Protein
0	82.4	51.3	8.08	5.0	863.7	98.5
10	7.3	4.5	20.8	13.0	---	----
15	2.7	1.7	20.6	12.8	786.3	89.7
20	2.1	1.3	21.3	13.3	751.0	85.6
25	3.0	1.8	15.2	9.5	649.8	74.1
30	---	---	12.8	8.0	607.0	69.2
35	2.2	1.4	8.0	5.0	566.1	64.6
40	0.35	0.2	5.4	3.3	446.4	50.9

The adsorbtion/desorbtion of amylase and adsorbtion of protein using phenyl sepharose-Cl 4B.

0.033% w/v K15M
(100% activity = 160.5 units/ml; 100% protein =877 ug/ml)

	Adsorbed		Desorbed			
% NS	Activity	% Activity	Activity	% Activity	Protein	% Protein
0	0.17	0.21	0.235	0.29	725.6	82.7
10	0.81	1.01	0.134	0.17	717.2	81.8
15	1.08	1.35	0.088	0.11	690.6	78.7
20	1.35	1.68	0.090	0.11	712.3	81.2
25	1.17	1.46	0.080	0.10	731.3	83.4
30	3.14	3.92	0.082	0.10	673.0	76.7
35	5.49	6.84	0.098	0.12	743.5	84.8
40	3.32	4.14	0.124	0.16	683.6	77.9

Comparison of partitioning of alkaline phosphatase, amylase, and the phosphatase/amylase fusion protein, Figures 4.1, 4.2, 4.3a, 4.3b, 4.3c and 4.4:

The percentage of phosphatase, amylase, or fusion protein activity remaining after partitioning as a function of ammonium sulfate saturation.

See Table 4.1 for initial conditions.

	% Activity		
% NS	**Fusion Protein**	**Amylase**	**Phosphatase**
0	100.0	100.0	100.0
5	---	102.9	---
10	93.5	103.3	98.2
15	90.2	102.8	---
20	81.3	93.1	93.1
25	81.2	60.0	---
30	82.1	6.1	72.6
35	84.1	1.0	---
40	56.6	---	5.6
50	3.8	---	3.3
60	0	---	1.6
70	0	---	1.5

The percentage of phosphatase, amylase, or fusion protein activity remaining in the salt phase with respect to % K15M present.

See Table 4.1 for initial conditions.

	% Activity		
% K15M	**Fusion Protein**	**Amylase**	**Phosphatase**
0.00	100.0	100.0	100.0
0.0001	---	90.7	---
0.0005	98.9	72.5	88.3
0.001	99.4	61.6	82.5
0.003	97.1	37.1	81.8
0.005	100.0	33.4	85.7
0.008	105.7	24.1	88.3
0.01	103.4	5.0	86.4
0.02	101.7	---	90.9
0.03	---	---	92.9

The percentage of phosphatase, amylase, or fusion protein activity remaining in the salt phase after precipitation as a function of ammonium sulfate added.

See Table 4.1 for initial conditions.

	% Activity		
% NS	Fusion Protein	Amylase	Phosphatase
0	100.0	100.0	100.0
10	99.9	100.0	62.2
20	99.3	87.7	45.4
30	95.1	85.6	38.7
40	81.5	66.5	6.7
50	40.0	9.0	1.4
60	15.0	0.3	21.3
70	5.1	0.1	42.5