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From Fermentation Broth By Two-Phase Partition

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RECOVERY OF *Clostridium thermosulfurogenes* β – AMYLASE FROM FERMENTATION BROTH BY TWO – PHASE PARTITION

By

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ABSTRACT

RECOVERY OF Clostridium thermosulfurogenes β – AMYLASE FROM FERMENTATION BROTH BY TWO – PHASE PARTITION

By

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Enzymes produced in commodity amounts, like amylases, require simple and low cost recovery and purification for commercial competitiveness. A procedure for recovering a very thermoactive and thermostable Clostridium thermosulfurogenes β-amylase from fermentation broth by partition to a hydroxypropyl methylcellulose phase has been developed. First, the enzyme is partitioned to a gel-like polymer phase with ammonium sulfate.This β-amylase containing polymer phase is separated by centrifugation. The resulting pellet can be redissolved and the polymer recovered by a second salt precipitation. The process is not dependent on polymer-enzyme solution pH, but it is affected by temperature, polymer nominal molecular weight and loading, polymer-enzyme solution dielectric constant, fermentation carbon source, and dilution and purity of the enzyme preparation. The partition mechanism seems to be adsorption of β-amylase to the insolubilized polymer in the presence of ammonium sulfate.

This work is dedicated to

my wife, Chia Miao

my mother, Dona Neide

my country, Brazil

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INTRODUCTION

Maltose containing syrups are experiencing increased demand due to their low hygroscopicity, low viscosity in solution, resistance to crystallization, low sweetness, reduced browning capacity, and good heat stability. These syrups are used as moisture conditioners, crystallization inhibiters, stabilizers, or bulking agents in brewing, baking, soft drink, canning, and confectionery industries. In Japan , the Hayashibara Company alone produces over 20,000 tons of crystalline maltose per year (Saha and Zeikus, 1987).

There are three types of maltose containing syrups: high maltose syrup, extremely high maltose syrup, and high conversion syrup. Their production is governed by the use of an appropriate enzymatic system in the saccharification step for each specific syrup. In high maltose syrup production the enzyme used is either β-amylase or fungal α-amylase. In order to produce extremely high maltose concentrations, a debraching enzyme such as isoamylase or pullulanase is used. For high conversion syrup, glucoamylase is substituted for the debraching enzyme.

Low temperature starch saccharification has drawbacks like starch retrogradation, high solution viscosity, and low hydrolysis rates. Slow rates may increase reversion product formation and microbial growth. Most of the β-amylases used industrially are of plant origin, such as barley, soybean, wheat, and sweet potato. They are expensive and thermally

unstable. On the other hand, most microbial β-amylases are not thermostable and thermoactive enough to substitute for the plant enzymes. Therefore, it is desirable to have a β-amylase which can operate at high temperatures, like the extracellular β-amylase from Clostridium thermosulfurogenes reported by Hyun and Zeikus (1985a). This enzyme presents an excellent opportunity for development of new processes for maltose production at higher temperatures than the ones currently used.

In addition to enzyme characteristics, downstream processing plays a key role in the development of commercial enzymes, since the recovery process often represents the major manufacturing cost. An optimum balance between yield and purification must be found by considering product specification and production costs, since high yield and purification are rarely achieved in a large scale process. Two examples are precipitation and affinity processes. Precipitation techniques, for example by ammonium sulfate or ethanol, are the most common recovery processes in protein recovery due to their operational simplicity and high yields obtained. However, purifications achieved by these processes are not high and sometimes very poor. Affinity purification processes, like affinity precipitation, chromatography, or ultrafiltration, often result in very large purification with reasonable recovery. The high costs of process development and ligand production make affinity separation processes applicable only to very expensive products, unlike the amylases currently under consideration.

Recovery process development for amylases must search for processes whose optimum cost, yield, and purification fall between those for precipitation and affinity methods. Partition in an aqueous two-phase system is a promising candidate for such processes. The simplicity and

mild conditions of this method produce enzymes at relatively low cost and high purity. Precipitation by non-ionic polymers is another suitable process. Even though there is clear distinction between precipitation and aqueous two-phase partition, when non-ionic polymers are used in both the distinction is not so obvious. In fact, some authors believe that, for some systems, they are the same basic phenomenon (Albertsson, 1986 and Fried and Chun, 1971).

The objective of this present work was to develop the basic features of a process for recovery of the extracellular C. thermosulfurogenes β-amylase from its fermentation broth by partition in an aqueous two-phase system. A polymer/salt system was chosen rather than a polymer/polymer system in order to produce simpler process which required removal of only one polymer for recycling and further β-amylase purification. The non-ionic polymers selected for this study were methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC). These compounds form a gel phase when salted-out by ammonium sulfate that can be pelleted by centrifugation. This characteristic could be exploited as a way to separate the enzyme containing phase and/or to carry out polymer recovery from the purified product for recycling.

MC and HPMC showed no harmful effects in β-amylase. These polymers have food-grade status, ready availability and modest cost. Their carbohydrate back-bone resembles the amylose structure opening the possibility for specific interaction between the polymers and the β-amylase.

The basic variables of the partition process (pH, polymer loading, temperature, fermentation carbon source, protein concentration and broth purity) were studied regarding yield and purification. Polymer recovery was also evaluated.

LITERATURE REVIEW

Extracellular β-amylase from Clostridium thermosulfurogenes

β-amylase* from C. thermosulfurogenes was first isolated by Hyun and Zeikus (1985a). The enzyme is produced in high yield as a primary metabolite during growth on starch, maltose, or glucose and it is excreted extracellularly. It was purified 2.4-fold by sequential precipitation with ethanol, ammonium sulfate, and acetone. Some physicochemical and kinetics properties of the β-amylase were studied.

Shen and co-workers (1988) purified this β-amylase 811-fold to homogeneity by ultrafiltration, precipitation by ethanol, and DEAE-Sepharose CL-6B and Sephacryl S 200 chromatographies. The physicochemical characteristics and catalytic and general molecular properties of this purified β-amylase were studied. The enzyme was found to be a tetramer of 210,000 daltons composed of a subunit of 51,000 daltons. The tetramer molecular weight is similar to the sweet potato β-amylase (200,000 daltons), but it is glycosylated. Moreover, immunological studies indicated that these two enzymes do not crossreact. The β-amylase from C. thermosulfurogenes is rich in acidic and hydrophobic amino acids but low in arginine and histidine. The sequence of the 20 first N-terminal amino

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* EC 3.2.1.2, α-1, 4-D-Glucan maltohydrolase, saccharogenic amylase is an exo-acting carbohydrase which cleaves alternative α-1, 4 glucosidic linkages of starch from the non-reducing end producing β-maltose.

acids showed only 45% homology when compared with the same sequence of β-amylase from Bacillus polymyxa. The specific activity of purified β-amylase was high (4215 U/mg) when compared with activities of β-amylase from other microorganisms (400-560 U/mg). A relatively high turnover number (400,000 minutes^{-1}) and a low Km value (1.68 mg/mL) may explain this high specific activity. Unlike the behavior of the β-amylase preparation reported by Hyun and Zeikus (1985a), this more pure β-amylase was inhibited by Schardinger dextrins (α- and β-cyclodextrin). The enzyme isoelectric point is 5.1 and its optimum activity pH is 5.5. However, at least 90% of the optimum activity is present in the pH range of 5.0 to 6.0. The enzyme thermoactivity and thermostability are supported by its optimum activity temperature, 75 °C, and its stability at 70 °C, respectively. Moreover, the enzyme is still stable at 80 °C if either Ca^{2+} (5 mM) or substrate (1%) is provided. Shen and co-workers (1988) concluded that the β-amylase purified from C. thermosulfurogenes is novel when compared to other described β-amylase because of the high specific activity, extreme thermostability, and glycosylation.

Saha and co-workers (1988) developed a purification process for this β-amylase based on the ability of the enzyme fermented with maltose to bind raw starch. First, β-amylase previously purified by ultrafiltration and ethanol precipitation was put in contact with raw starch to which it bound. The β-amylase containing starch was separated by centrifugation. Soluble starch was added to the pellet and the enzyme was desorbed from the raw starch. The soluble starch-β-amylase solution was passed through an octyl-Sepharose column pre-equilibrated with acetate buffer containing 30% ammonium sulfate. β-amylase was adsorbed to the matrix, but the soluble starch passed through. After washing the column with buffer, the enzyme

was eluted with the same buffer with no ammonium sulfate. The eluted active fraction was dialysed and showed a single protein band when analyzed by SDS-polyacrylamide gel electrophoresis. The purified β-amylase had a specific activity of 4188 U/mg of protein which is very close to the β-amylase purified by Shen and co-workers cited above.

Elankovan (1988) investigated the recovery of the C. thermosulfurogenes β-amylase from fermentation broth. He studied two operations (ultrafiltration and precipitation) and the effect of two types of fermentation carbon sources (maltose and starch/maltodextrin) on these separations. Both β-amylase fermented with maltose and with starch were efficiently concentrated by ultrafiltration with a 30,000 molecular weight cut-off membrane with 88 and 85% activity recovery, respectively. However, only the latter was efficiently concentrated in a 100,000 molecular weight cut-off membrane. In this case, activity recoveries were 93% for β-amylase fermented with starch and 51% for β-amylase fermented with maltose. β-amylase fermented with starch was assumed to have a effective molecular weight higher than the β-amylase fermented with maltose. This assumption was confirmed by HPLC gel filtration. It was postulated that β-amylase subunits form a biospecific complex with soluble starch or non-stable clusters among themselves which are stabilized by the presence of soluble starch.

Precipitation studies were carried out with two different precipitants and their combinations (ammonium sulfate and ethanol) at different temperatures (in the range of 4 to 50 °C). The optimum temperature and salt concentration for precipitation by ammonium sulfate was determined to be 20 °C at 50% saturation. Precipitation of β-amylase fermented with starch showed a poor activity recovery, from 57 to 84%. β-amylase

fermented with maltose was precipitated more efficiently with activity recovery of 98%. In both cases, purifications were low, 7-fold maximum. Precipitation by ethanol was efficient only for β-amylase fermented with starch due to the formation of a biospecific complex between the β-amylase and starch. The higher activity recovery for this case was correlated to the larger mean particle size obtained after precipitation for the enzyme produced from starch than for the enzyme produced from maltose. The optimum temperature and ethanol concentration were 20 °C and 20%, respectively, and purification was around 6.2-fold at maximum. The use of 3% ammonium sulfate in conjunction with the β-amylase precipitation by ethanol resulted in an an increase in purification to 31-fold at the same activity recovery. This purification is significantly higher than is usually the case for precipitation separations (Bonnerjea et al., 1986).

New processes for maltose syrup production have been developed with this unique β-amylase. Saha and Zeikus (1988a) studied the synergistic action between the β-amylase and two other amylases (isoamylase and α-amylase). They reported that isoamylase stimulated the starch hydrolysis of β-amylase 3.6-fold with approximately 97% of the low molecular weight products made up by products with degree of polymerization equal to two. On other hand, α-amylase had its activity stimulated by the presence of β-amylase. These same authors (Saha and Zeikus, 1988b) developed processes for high maltose, extremely high maltose, and high conversion syrups production from liquefied and raw starch taking advantage of the thermactivity and thermostability of this β-amylase. Environmentally compatible highly thermostable enzymes were used together with the β-amylase allowing starch conversion at higher temperatures than used in the past.

Miranda and Nikolov (1987) succeeded in immobilizing C. thermosulfurogenes β-amylase on porous silica activated with glutaraldehyde. The activity of the β-amylase containing silica was around 140 units per gram, but the half-life time was low, estimated to be 14 days at 60 °C.

Methylcellulose and Hydroxypropyl Methylcellulose

Methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC) belong to a family of cellulose ethers with applications ranging from components of construction products to pharmaceuticals. The basic molecular structure of these non-ionic polymers are shown on Figure 1. MC can be considered one of the five most important commercial gums and its produced worldwide under different brand names. It was first prepared by Suida in 1905 and its commercialization in the United States started in 1938. The production of the MC in this country in 1978 was 25,000 tons which corresponded to a value of more than 40 million dollars (Greminger, 1978; Greminger and Krumel*, 1980; and Whistler, 1978).

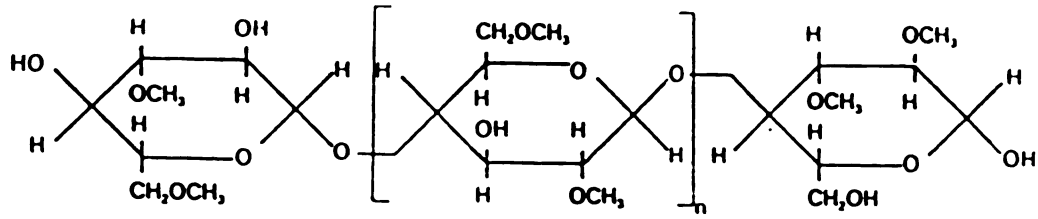
MC is prepared by reacting a cellulosic raw material, like cotton linters or wood pulp, with aqueous sodium hydroxide and methyl chloride. The main reactions that take place are:

$$R_{cell}OH + NaOH \longrightarrow R_{cell}OH\text{–}NaOH \text{ (complex)}$$

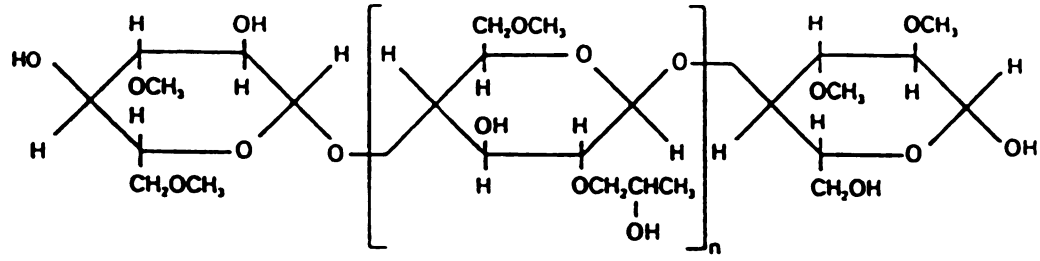
$$R_{cell}OH\text{–}NaOH \rightleftharpoons R_{cell}ONa + H_2O$$

$$R_{cell}ONa + CH_3Cl \longrightarrow R_{cell}OCH_3 + NaCl$$

* This is an excellent review on MC and HPMC. Most of the information presented in the literature review was taken from it.



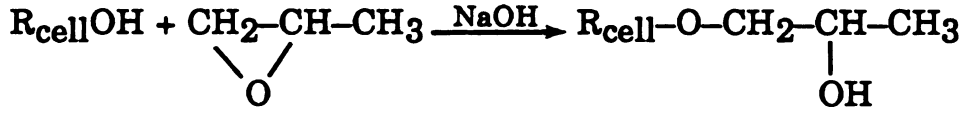
Methylcellulose



Hydroxypropyl Methylcellulose

Figure 1. Simplified chemical structure of methylcellulose and hydroxypropyl methylcellulose [from Handbook on Methocel Cellulose Ethers Products].

where R_{cell} represents the cellulose radical. If propylene oxide is added to the reaction mixture, HPMC is produced according to the following reaction



The reaction mixture composition (sodium hydroxide:cellulose, methyl chloride:cellulose, and propylene oxide:cellulose weight ratios and sodium hydroxide concentration) controls the level of substitution. The importance of this control is that differences in substitution markedly affect the properties of the cellulose derivatives like solubility, solutions viscosity, and biodegradation. The measurement of the substitution is given by the concept of degree of substitution (DS, the average number of hydroxyl groups substituted on the anydroglucose unit of cellulose) which is three at maximum. Molar substitution (MS) is the term used when side-chain formation is possible and its value can exceed three.

There are two difficulties in the production of MC or HPMC. First, there is the crystalline nature of cellulose which requires a precise pre-treatment of the raw material with sodium hydroxide. Secondly, the reactions are exothermic and cellulose pulps do not exhibit good heat transfer properties. Therefore, reactor design for these transformations must have special attention to heat dissipation.

MC and HPMC are insoluble in water at high temperatures (80 - 90 °C). However, they do not dissolve by just adding polymer powder preparations to cold water. Under these conditions, the particles gelatinize on the surface and agglomerate among themselves prohibiting the

hydration and dissolution of the entire particle. In order to achieve complete polymer dissolution, the powder must be dispersed in hot water (80-90 °C) by agitation to wet the particles. Cold water or ice is added as the suspension is cooled under mixing. The particles dissolve and a marked increase in solution viscosity takes place.

Aqueous solutions of MC and HPMC behave as pseudoplastic nonthixotropic fluids at room temperature. Solutions with more than 5% of polymer may show some thixotropy due to weak chain-to-chain interaction. Dilute solutions are very nearly Newtonian fluids. All the rheological properties are strongly affected by polymer molecular weight and molecular weight distribution .

MC and HPMC can function as polymeric surfactants since they reduce surface tension and interfacial tensions of aqueous systems. Thereby, they can act as emulsifiers for two-phase systems or as foaming agents. They are effective even at very low concentrations, like 0.001%, due to their polymeric structure.

One of the most remarkable characteristics of these polymers is their thermal gelation properties (Sarkar, 1979). In contrast to the majority of other polymers, MC and HPMC gel when the temperature is raised. This gelation is usually preceded by precipitation. Figures 2 to 5 illustrate these phenomena*, which are probably due to a decrease in hydration of the

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* Some variables used to quantify gelation and precipitation are "Incipient Gelation Temperature" (IGT) and "Incipient Precipitation Temperature" (IPT), and " Cloud Point" (CP), respectively. IGT is defined as the temperature at which a sharp increase in viscosity takes place when polymer solution temperature is increased. IPT and CP are defined as the temperatures at which light transmission through polymer solution reaches 97.5 and 50%, respectively, when such solutions are heated.

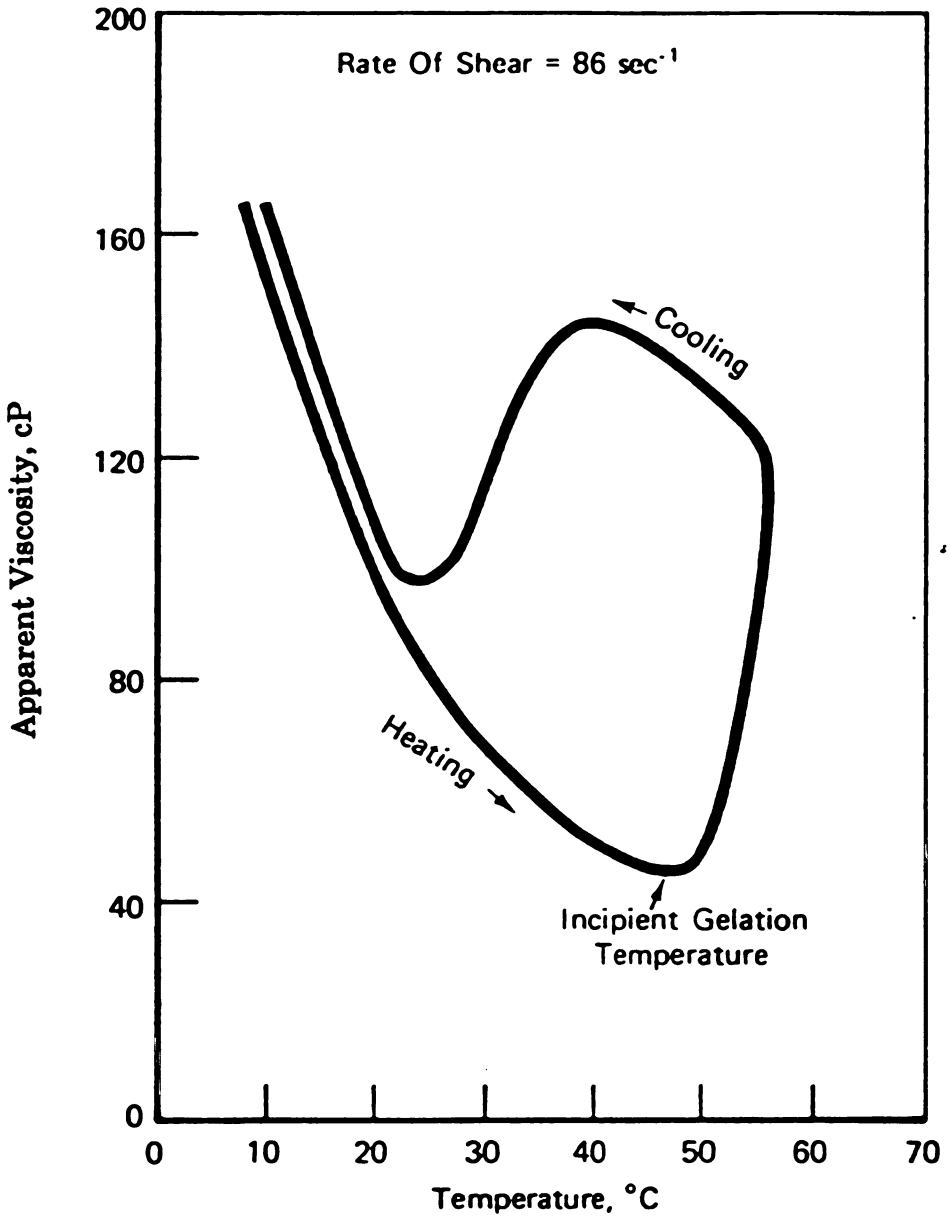


Figure 2. Gelation of 2% aqueous solution of Methocel A100 on heating at 0.25 °C/min [from Sarkar, 1979].

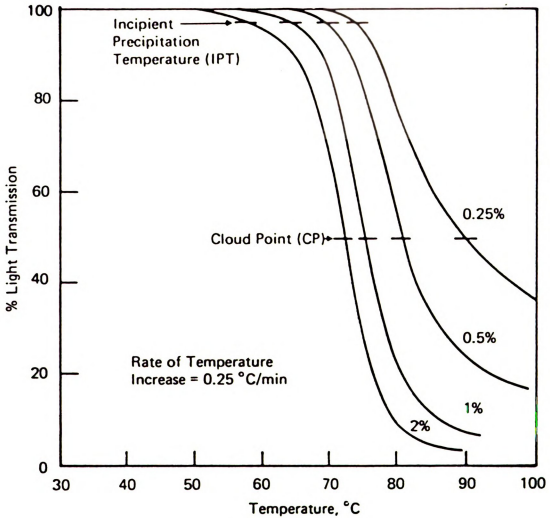


Figure 3. An ilustration of the change of the 545 nm light transmission for an aqueous solution of hydroxypropyl methylcellulose sample having degree of substitution and molar substitution (see footnote for definitions) of 1.45 and 0.11, respectively, as function of temperature at different concentrations [from Sarkar, 1979].

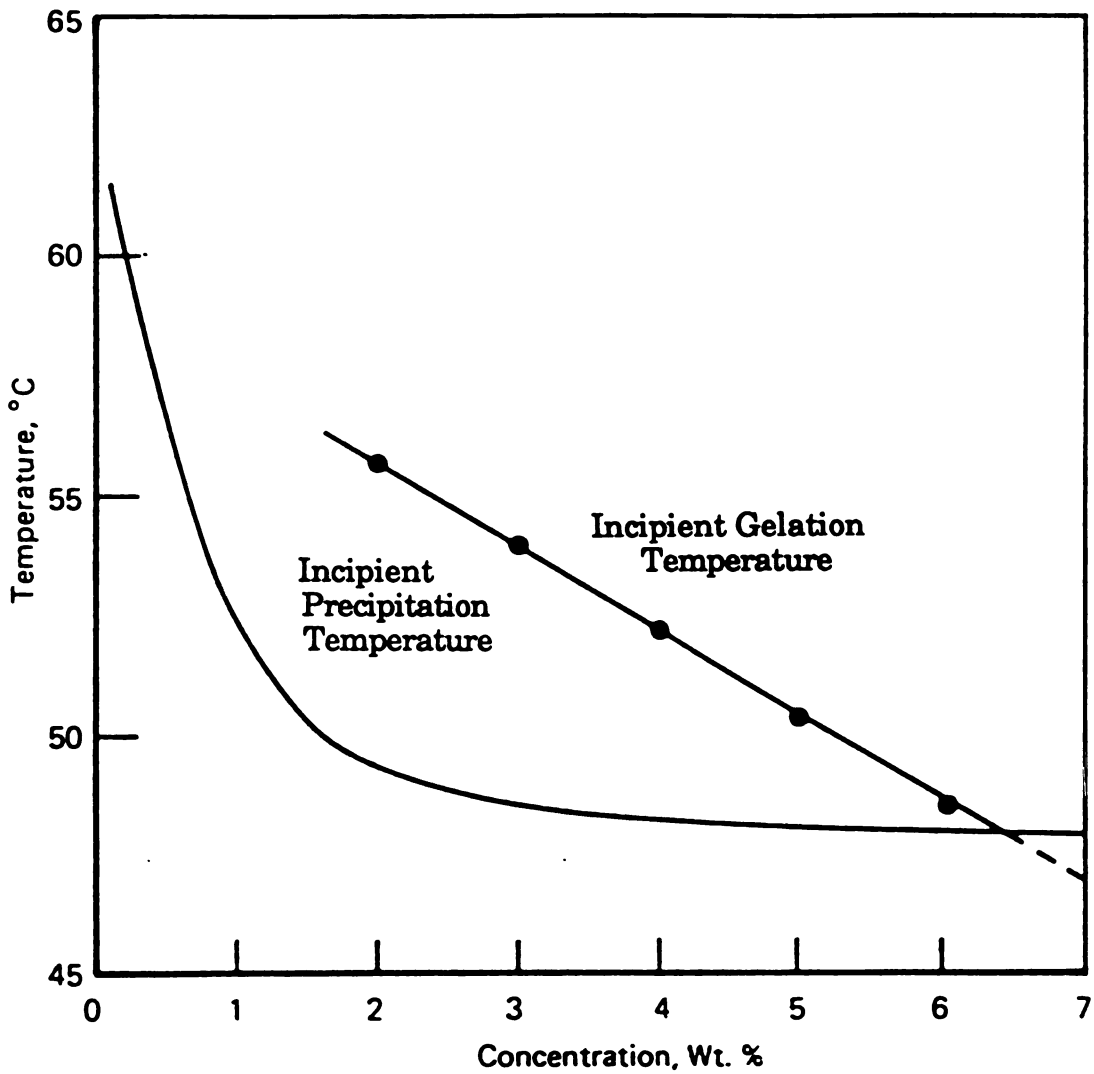


Figure 4. Temperature of sol-gel transformation for aqueous solutions of Methocel F50 as function of concentration [from Sarkar,1979].

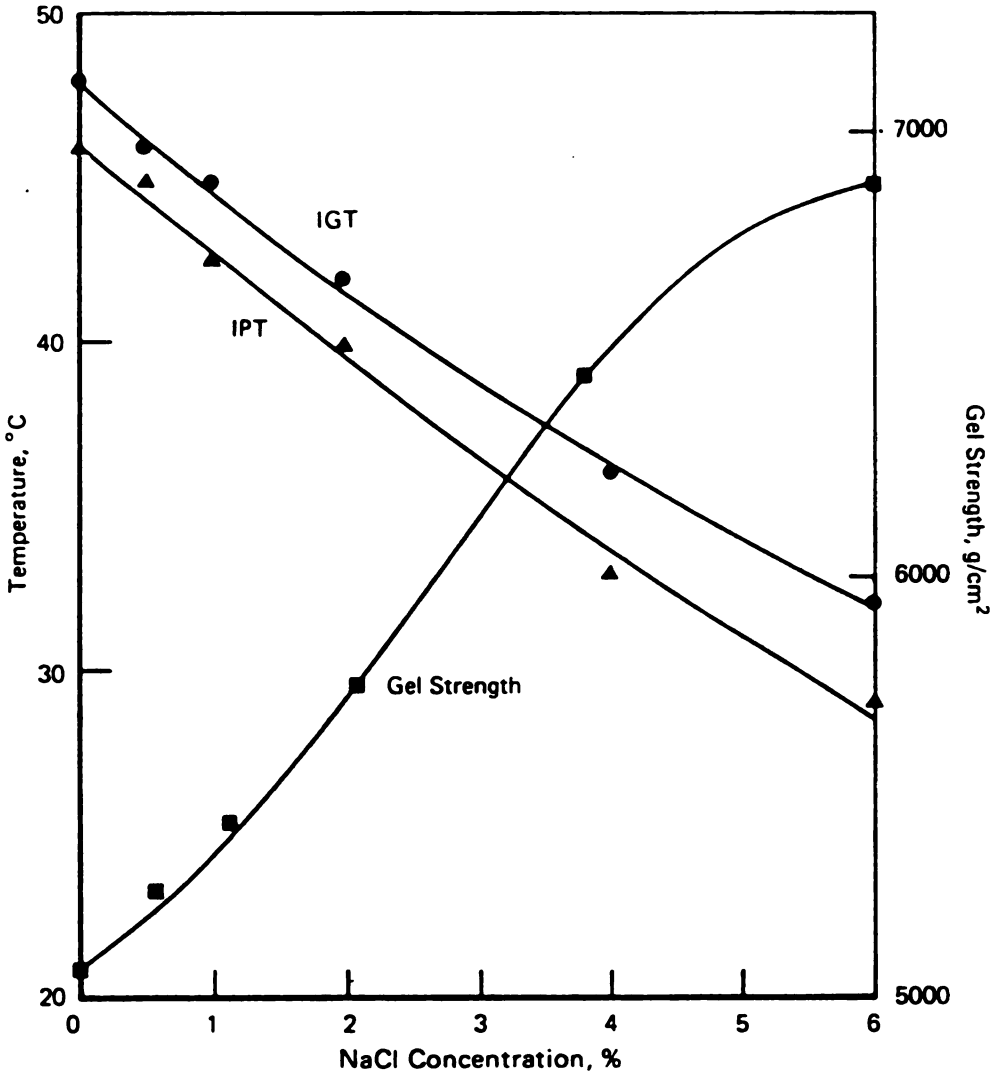


Figure 5. Incipient gelation temperature (IGT), incipient precipitation temperature (IPT), and gel strength of 2% methylcellulose (Methocel A15C) solution as function of NaCl concentration (see footnote for definitions) [from Sarkar, 1979].

polymer molecules and an increase in hydrophobic interaction among themselves when polymer solutions are heated.

The IGT and IPT of commercial MC and HPMC, which have wide molecular weight distributions, are not very dependent on their average molecular weights. This is due to the presence of high molecular weight fractions, which precipitate out first and strongly influence the IGT and IPT values. As a consequence, commercial polymers exhibit similar IGT and IPT.

The degree of hydroxypropyl and methoxyl substitutions are important in determining the gelation properties of these cellulose ethers. Methoxyl groups are responsible for gelation. However, the presence of hydroxypropyl groups in the polymers alters the gelation characteristics significantly, like increasing precipitation temperature and decreasing gel strength (Sarkar, 1979).

Additives can strongly alter the gelation properties of MC solutions. A decrease in IGT usually takes place when electrolytes, dextrose, and glycerol, are added to MC solutions. However, some components, (e.g., ethanol, and propylene glycol), have a opposite effect, increasing the IGT due to their large solvent power. Figure 5 shows the effect of salt on the gelation and precipitation of MC. The salting out phenomena is not explained simply on the basic of competition between the polymer and the electrolyte for the water molecules, since the relative efficiency of the insolubilization by different salts is function of the polymer (Sarkar, 1979). Albertsson (1986) used this property to separated MC from a dextran/MC solution by salting out it with ammonium sulfate.

Both polymers, MC and HPMC, are very stable in the pH range of 3 to 11, are very resistant to microorganisms, and have food grade status. The

Food and Drug Administration lists MC as Generally Recognized as Safe (GRAS). HPMC compounds whose methoxyl and hydroxypropyl substitutions ranges from 19 to 30% and 4 to 12%, respectively, meet the requirements of Food Additive Regulations 182.1480 and 172.874 as miscellaneous and/or general purpose food additives for nonstandardized foods.

The variation of the MC and HPMC properties over a wide range give these polymers an extensive spectrum of uses. They are used commercially as compounding and formulating ingredients in adhesives, agricultural chemicals, chemicals specialities, construction industry products, cosmetics, food products, latex painting, paint removers, paper products, pharmaceuticals, printing inks, resins, elastomers, textiles, and tobacco sheet. In addition, MC and HPMC are commercially used as processing aids in ceramics, leather, and polyvinyl chloride industries. MC has also been used as phase forming agent in partition in aqueous two-phase systems applied to separate biomolecules and cell particles (Albertsson, 1986 and Kula et al., 1982).

Protein Precipitation by Non-Ionic Polymers

Non-ionic polymers have been successfully used as protein precipitants based on their ability to stabilize proteins and room temperature use. As they are soluble in water, they can be added to proteins as aqueous solutions in concentrations generally less than twenty percent (Bell et al., 1983).

Cornwell and Kruger (1961) discussed the use of sulfated polysaccharides, including some non-ionic, for the isolation and characterization of plasma proteins by precipitation. However, Polson and

co-workers (1964) were the first to publish a report of protein precipitation with non-ionic polymers. In their pioneer work, they studied the precipitation of plasma proteins with five different non-ionic polymers : dextran, polyethylene glycol, nonylphenolethoxylate, polyvinyl alcohol, and polyvinyl pyrrolidone. After preliminary experiments, polyethylene glycol was the only one submitted to further studies because the latter three produced significant denaturation of the protein precipitates and dextran solutions had very high viscosity.

Martin and Landon (1975) included dextran in the antibody precipitation technique to improve the separation of antibody-bound and free antigen fraction in radioimmunoassays. They found dextran markedly increased the rate of immunoprecipitation of the antibody-bound antigen fraction. However, it was not clear whether dextran coprecipitated with the antibody or a partition to a second phase occurred.

Polyethylene glycol has been the only non-ionic polymer extensively studied as protein precipitant (Bell et al., 1983; Ingham, 1984; Patel, 1985 and Scopes, 1987). Examples of proteins precipitated by polyethylene glycol are plasma proteins (including immunoglobulins), alcohol oxidase, fumarase, alcohol dehydrogenase, carboxyhaemaglobin, and invertase. Precipitation conditions vary in polyethylene glycol concentration from a few percent to 70% w/v and in ionic strength from 0.1 M to 2.5 M. Precipitation pH varies from acid to alkaline (from 3,0 to 9.0). At very high values of ionic strength, a two-phase liquid system is formed instead of a precipitate. Protein purification varies from crystalline forms to precipitates with no significant purification. Protein mixtures of low protein concentration can be fractionated by polyethylene glycol precipitation, but good yield results only when working with highly

concentrated protein mixtures (Janssen and Ruelius, 1968; Fried and Chun, 1971; Juckes, 1971; Foster et al., 1973; Ingham, 1978 and Hao et al., 1980).

The amount of polyethylene glycol needed for a specific precipitation a is function of pH and ionic strength. pH affects not only polymer-protein interaction, but also protein-protein interaction by changing the net charge on the molecules. Similar effects are provided by certain ions, the sulfate anion in particular. These salt effects correlate roughly with the Hofmeister series and their effective concentrations are far below the concentrations known to work as salting-out agents (Ingham,1978). Miekka and Ingham (1978) attributed the ammonium sulfate enhancement in the precipitation of chymotrypsin and chymotrypsinogen by polyethylene glycol to the ability of these proteins to undergo self-association in the presence of this salt.

Haire and co-workers (1984) described hemoglobin precipitation by polyethylene glycol as a two step phenomenon. First, an amorphous solid phase is formed by the protein solution upon polyethylene glycol addition. This phase is very hydrated with a water mass fraction from 0.5 to 0.7. After 17% polyethylene glycol concentration is reached, the amorphous solid phase becomes metastable and a new phase of crystalline hemoglobin appears.

Protein precipitation with non-ionic polymers is said to be closely related to the formation of liquid-liquid two-phase systems from mixtures of aqueous polymer solutions. However, its mechanism is not fully understood. Some authors explain it as either coprecipitation or partition between polymer/water phases. Others believe in the exclusion theory by which the polymer excludes the protein molecule from part of the solution

thereby reducing the amount of water available for solvation and bringing the protein solution to its solubility limit (Fried and Chun, 1971; Atha and Ingham, 1981 and Bell et al., 1983).

The exclusion volume theory has been supported by experimental data that show insensitivity of some precipitation characteristics to changes in solution conditions (pH, temperature or salts) and the observation that polymer-protein interaction depends primarily on the size and shape of polymer and protein molecules. Moreover, there is an apparent absence of specific interactions between water soluble polymers with proteins or with groups that commonly occur on the surface of proteins molecules. Thermodynamic studies of the polymer-protein interaction based on the exclusion volume theory by computer simulation agreed very well with experimental data (Knoll and Hermans, 1983 and Baskir et al, 1987).

The removal of non-ionic polymers from a protein precipitate is sometimes a necessity. The polymer may be considered an undesirable contaminant due to toxicity, adverse effects in further processing, viscous product solutions, and interference with analytical procedures. Moreover, if the polymer is present at high concentrations in the precipitate, its recycling is of major interest in large scale processing. Polymers have a linear non-compact configuration that give them a large exclusion radius, making them difficult to remove from globular proteins of the same or even larger molecular weight using conventional molecular sieve methods. Since they are nonvolatile, they can not be removed by lyophilization, a common operation in the pharmaceutical industries. Thus polymer removal is difficult and results in increased processing costs (Ingham and Busby, 1980 and Smith, 1985).

However, there are some methods available for polymer removal. Polyethylene glycol can be separated from other purified proteins by ultrafiltration when the differences in molecular weight are large enough. Other methods are ion exchange or affinity methods since polyethylene glycol molecules have no charge or a ligand can be chosen with no specificity towards the polymer. Precipitation of either polyethylene glycol by salts or of proteins by any suitable method can be carried out. Formation of a two-phase system by adding salts, like ammonium sulfate or phosphates, under appropriate conditions can be exploited for the separation of polyethylene glycol from the proteins, which tend to partition into the salt-rich phase (Ingham and Busby, 1980).

Protein Partition in Aqueous Two-Phase Systems

Liquid two-phase partition, also called liquid-liquid extraction or solvent extraction, is a very well developed technique. Such processes are based on the distribution* of components of a mixture into phases of a multiphase system, usually two-phase systems, due to differences in affinity of these components to each phase. Initially a tool of analytical chemistry, solvent extraction has emerged into a major unit operation at the industrial scale. However, common applications of this technique to biochemical processes have been very limited (basically separation of proteins and nucleic acids in phenol/water two-phase system) because proteins were precipitated or denaturated in the organic phases or

* Quantification of distribution is given by the partition coefficient K defined as the ratio between top and bottom phase concentration of the partitioned substance .

concentrated only in the aqueous phases (Albertsson, 1958; Kroner et al., 1978).

Some of these difficulties have been overcome by using two-phase systems containing water, salts, and organic solvents soluble in water, like ethanol and glycol ethers. However, partition using these systems require careful temperature control to avoid changes in phase composition which affect separation.

Albertsson (1958) introduced aqueous two-phase systems containing high polymers and opened a new era for two-phase partition in biotechnology. The high water contents of both phases (from 80 to 99 percent) make them an excellent environment for cell organelles or biologically active proteins that distribute differently between the two phases (Albertsson, 1962 and Kula et al., 1982). These two-phase polymer systems are still sensitive to temperature changes, but they can be made far from the plait point so temperature changes lead to little concentration variation and consequently little change in partitioning.

When solutions of two different polymers are combined, phase separation occurs with each polymer concentrating preferentially between the phases. The phase formation is governed by interactions between molecules of the two polymers. The interaction energy among polymers is proportional to polymer molecule size since it is the sum of interactions of repetitive groups throughout the molecule. Thus, this interaction energy per mol of polymer is very high, usually much higher than the entropy per mol due to mixing, which is based on the number of molecules involved in the mixing process. This additive phenomenon allows second phase formation,even though the polymers are very similar to each other or are present in very low concentration. This theory is in good agreement with

experimental data that shows that the higher the molecule weight of the polymer, the lower its concentration necessary for phase separation (Albertsson, 1986).

In some cases salts of low molecular weight compounds are part of the system. Addition of salts to some polymer solutions may cause phase separation with one phase polymer-rich and the other salt-rich, as in the polyethylene glycol/ammonium sulfate system. Depending on the polymers and conditions used, a gel or solid-like phase may be formed. Examples are dextran/potassium phosphate, methylcellulose/ammonium sulfate, and low molecular weight polyethylene glycol/dextran systems (Albertsson, 1986 and Kroner et al., 1982). The presence of salts can reduce the water available for polymer hydration, as in protein precipitation by non-ionic polymers. Such a reduction in the water available increases polymer-polymer interaction and results in polymer segregation in a gel phase. At the same time, ions like HPO_4^{2-}, SO_4^{2-} or Cl^- can form complexes with polymer chains leading to the formation of gel structure.

Another interesting and useful feature of salts in aqueous two-phase systems is the unequal partition of small ions. This creates a small electrical potential φ across the interface given by

$$\varphi = R\,T / [\,(\,Z^+ + Z^-\,)\,F\,]\,\ln(\,K_- / K_+\,)$$

where R is the gas constant, T the absolute temperature, F the Faraday constant, Z^+ and Z^- the net charge of the cations and anions, respectively and K_+ and K_- the partition coefficient of the cations and anions, respectively. Charged proteins will have their partition altered by the

interfacial electrical potential φ according to the following expression

$$\ln K_p = \ln K^o_p + [\, Z_p F / (RT)\,]\, \varphi$$

where Z_p is the net charge of the protein, K_p the protein partition coefficient, K^o_p the partition coefficient of the protein at condition of zero interfacial potential or at its isoeletric point where Z_p becomes zero. Therefore, pH has an important role in such partition because it can determine, not only the protein net charge, but also the interfacial potential by establishing multivalent ions dissociation (Albertsson, 1986 and Kula et al., 1982).

Among 63 different aqueous polymer two-phase system listed by Albertsson (1986), 20 were composed of two non-ionic polymers, and 38 have one of the polymer components non-ionic. Polyethylene glycol and dextran appear as components in 11 and 9 systems, respectively. Other polymers were polypropylene glycol, polyvinyl alcohol, polyvinylpyrrolidone, methylcellulose, ethylhydroxyethylcellulose, hydroxypropyldextran, methoxypolyethylene glycol, sodium dextran sulfate, sodium carboxymethyldextran, sodium carboxymethylcellulose, ficoll, and DEAE dextran-HCl. Despite the number of polymers available for aqueous two-phase partition, large scale processes have been developed using only polyethylene glycol/dextran or polyethylene glycol/salts (Kula et al., 1982).

Partition of macromolecules and cell particles is a very complex phenomenon. The mechanism by which molecules (or particles) are preferentially distributed between the different phases is largely unknown. Hydrogen bonds, charge interaction, van der Waals forces, hydrophobic interactions, and steric effects are expected to take part in the interaction

between the partitioned substance and the components of each phase. Therefore, partition depends on the size and chemical properties of both the phase forming polymers and partitioned molecules (or particles).

This complex situation makes it difficult to predict behavior of a partition system or to interpret precisely experimental results. On the other hand, the versatility of the approach is great.

Like in precipitation, ultrafiltration, and chromatography, chemical modification of separating agents can greatly increase specific interactions resulting in specificity of the separation. Hydrophobicity of one of the phases can be increased by attaching hydrophobic groups to polymers, like fatty acid esterified groups to polyethylene glycol. Separation of serum albumin in a system of such modified polyethylene glycol/dextran was increased by approximately 56-fold (Shanbhag and Axelsson, 1975). Similar improvement in separation can be achieved when the attached group is an affinity ligand. Those ligands can be very specific to a certain product or have affinity to to a variety of compounds. The formers are ligands such as enzyme substrates and inhibiters; the latters are such as triazine dyes. These specific partitions are called hydrophobic and affinity partition, respectively (Kula, 1982).

Partition can be used as a research tool to detect and quantify interactions of protein-protein, protein-small ligand, nucleic acid-small ligand, protein-nucleic acid, and protein-lipid since these interactions change the partition of the individual substances. However, the main application of partition in biotechnology is on large scale concentration, purification of enzymes, and extractive bioconversion.

Concentration of a desired product (biomolecules, viruses and cell particles) is achieved when the system used is such that the product

partitions to a phase that has a small volume compared to the original volume. Examples of virus concentration by this method are bacteriophage T2, influenza, parotitis, New Castle disease, vaccinia, poliovirus, and tobacco mosaic virus (Albertsson, 1962; Albertsson and Frick, 1960 and Albertsson, 1960).

A great variety of proteins have been purified by partition in laboratory or pilot plant scale. Some examples are interferon, amylases (e.g., pullulanase and glucose isomerase) and dehydrogenase (e.g., glucose dehydrogenase, leucine dehydrogenase and formate dehydrogenase). Overall purification and yield were as high as 33-fold and 98 percent, respectively, for a maximum of 4 partition steps (Kula et al., 1982).

Albertsson (1986) lists the following advantages for large scale enzyme purification by partition:

1. Scale-up can easily and reliably be predicted from small laboratory experiments.
2. Rapid mass transfer and equilibrium is reached by relatively little input of energy in the form of mechanical mixing.
3. It can be developed as as continuous process.
4. The polymers stabilize the enzymes.
5. Separation can be made selective and rapid.
6. Because of the rapid separation it may be carried out at room temperature instead of in the cold, thus at lower investment costs.
7. It is more economical than other purification methods.

Extractive bioconversion is accomplished in a two-phase system where an enzyme and its substrate are contained in one phase while the reaction products segregate to the other phase. The renewing of the phase

containing the products by repetitive partitions or a continuous extraction may increase conversions and favor kinetics by avoiding product inhibition and reverse product formation, respectively (Albertsson, 1986).

Removal of the polymers from the concentrated or purified products is subject to the same problems and solutions discussed for removal of polymers from products concentrated or purified under precipitation by non-ionic polymers. In the case of products that are large particles such as cells or cell fragments, centrifugation of the phase followed by repeated washing can produce pellets of polymer-free products (Albertsson, 1986).

MATERIALS AND METHODS

C. thermosulfurogenes Fermentation for β-amylase Production

The catabolic repression resistant mutant H-12-1 (Hyun and Zeikus, 1985b) derived from C. thermosulfurogenes wild type strain 4B (ATCC 33743) was used. The culture was grown at 60 °C in 26 ml anaerobic pressure tubes, Bellco Glass Inc., NJ, USA, containing 10 mL of TYE (trypticase and yeast-extract) medium (Hyun and Zeikus, 1985b) supplemented with 0.5% soluble starch and a N_2-CO_2 (95 : 5) gas headspace. The culture was expanded twice, to 500 mL in a round bottom flask and to 16 L in a 18 L carboy. All the transfers were made under anaerobic conditions and the medium used was TYE with 0.5% of maltose or partially hydrolysed starch (maltodextrin) added as the fermentation carbon source (substrate). A last transfer for final growth, still under anaerobic conditions, was made to a 250 L fermentor containing 180 L of the same medium used previously.The fermentor content was stirred at constant rate (400 rpm), N_2-CO_2 was purged for a few hours to remove any dissolved oxygen present and temperature was kept at 60 °C. Antifoam emulsion FG-10, Dow Corning Corp., MI, USA, was added to suppress excessive foaming due to high production of gases during fermentation. Growth was followed by hourly turbidity measurements and the fermentation was stopped after detecting that the stationary phase was

achieved. The culture was cooled down to approximately 15 °C before cell removal and concentration.

Cell Removal and Broth Concentration

Cell removal and concentration was done under aerobic conditions. In the cases where maltose was used as the substrate, the cells were removed by ultrafiltration of the culture broth in an Amicon Hollow Fiber Ultrafiltration Cartridge, 100,000 MWCO membrane, model H26MP01-43. A batch of 20 L of this broth free of cells (called maltose broth) was then concentrated to 800 mL using an Amicon Spiral Ultrafiltration Cartridge, 30,000 MWCO membrane, model S10Y30. Three aliquots of 500 mL were taken during the concentration. These aliquots were denoted by their concentration ratio: ratio between the initial volume (20 L) and the volume of the concentrated at the time sampling was performed. For the maltodextrin case, the cell removal was done by centrifugation at 8,000 rpm for 10 minutes and the concentration of the resulting broth (called starch broth) was performed the same as for the maltose broth case.

β-amylase Preparations

Two β-amylase preparations were used for the first set of experiments at small scale. They will be denoted pre-purified and purified enzymes (or preparations) to take into account that one has a higher specific activity than the other due their different processing as described below.

β-amylase contained in 1 L of maltose broth was precipitated by adding 500 mL of saturated solution of ammonium sulfate at room temperature. After slow mixing for 1 hour the suspension was left

overnight at 4 °C. The precipitate formed was separated by centrifugation at 8,000 rpm for 30 minutes, suspended in 200 mL of 50 mM acetate buffer pH=6.0 with 5 mM $CaCl_2$, and dialysed overnight at 4 °C against this same buffer. The dialysed enzyme solution was frozen in aliquots of 10 mL. This is denoted as pre-purified β-amylase. Prior to use, the enzyme solution was thawed, centrifuged for 10 minutes in a clinical centrifuge, and filtered through Gelman Acrodisc 0.45 μm filters.

Chilled ethanol was added dropwise to pre-purified β-amylase while stirring to give a final alcohol concentration of 20% v/v. The precipitate formed was suspended and dialysed against the acetate buffer above. This is denoted as purified β-amylase. SDS-polyacrylamide gel electrophoresis of this preparation showed only one protein band.

Polymers

The MC and HPMC used were the commercial products Methocel* donated by Dow Chemical Company, Midland, USA. MC was type A4M with nominal molecular weight of 85,000 daltons and methoxyl degree of substitution of 1.6-1.9. Three types of HPMC were used: K15M, K4M and K100 M Premium with nominal molecular weight of 15,000, 85,000 and 250,000 daltons, respectively. These HPMC's have methoxyl degree of substitution and hydroxypropyl molar substitutions in the range of 1.1 to 1.4 and 0.1 to 0.3, respectively. The solutions were prepared according to the manufacturer's instructions : wetting and dispersing the particles in hot acetate buffer (temperature in the range of 80 to 90 °C), then adding cold buffer to dissolve the wetted particles. Stock solutions of the various

* Trademark of the Dow Chemical Company, USA.

polymers were prepared (1%) and kept at 4 °C. Dilutions to lower concentrations were made by weight.

Assays

A reaction mixture containing 800 µL of 2.5% w/v boiled starch and 200 µL of enzyme solution was used for the routine enzyme assay for β-amylase activity. Both solutions were prepared in 50 mM acetate buffer pH=6.0 with 5 mM $CaCl_2$ and the assay temperature was 60 °C. Reducing sugars released by enzymatic hydrolysis of starch were determined by the dinitrosalicylic acid method (Bernfeld, 1955). One unit of β-amylase was defined as the number of µmol of reducing sugars as maltose produced per minute under the above conditions.

Total carbohydrates content was determined by slight modification of the phenol-sulfuric method of Dubois et al. (1956). Aliquots of 200 µL were added to 200 µL of 5% phenol solution in a cuvette. After mixing, 1 mL of 95.5% sulfuric acid was added and after 5 minutes the content of the cuvettes were again thoroughly mixed. The absorbance at 470 nm was measured after 15 minutes. MC A4M was used as the standard. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. SDS-polyacrylamide slab gel electrophoresis was performed as described by Laemmli (1970). Molecular weight standards were bovine serum albumin (66,000 daltons), egg albumin (45,000 daltons), glyceraldehyde 3 - phosphate dehydrogenase (36,000 daltons), carbonic anhydrase (29,000 daltons), trypsinogen (24,000 daltons), and α-lactoalbumin (14,200 daltons).

The effects of ammonium sulfate or HPMC present in samples on activity, total carbohydrates, and protein assays were investigated. The only

significant interference for the practical concentration ranges observed was ammonium sulfate on the activity assay for concentrations higher than 12% saturation. Therefore, all samples containing ammonium sulfate were diluted at least to 10% prior to the activity assay.

Partition Experiments

Quantitative experiments were done in two ways. First, screening experiments were performed to study the use of MC and HPMC in the partition of β-amylase. These were small scale experiments carried out in 1.5 mL Eppendorf tubes and the enzyme preparation used was pre-purified or purified β-amylase. Subsequently, experiments were performed with broth in a batch fashion.

In both the small scale and batch experiments, the parameter used for evaluation regarding activity partition was "percentage partitioned". This is defined as the percentage of the activity missing from the supernatant compared with the initial value determined in a control experiment. This is an indirect measurement of how much activity was partitioned to the pellet. "Percentage precipitated" was defined in a similar way to account for the polymer or β-amylase salted-out by ammonium sulfate.

Percentage of saturation was used as the concentration unit for ammonium sulfate assuming that all solutions were at 21°C. The solubility for ammonium sulfate at this temperature is 757 g/L.

Small Scale Experiments

Small scale experiments were conducted in 1.5 mL Eppendorf tubes using 500 μL of enzyme preparation and 500 μL of HPMC or MC solution at

twofold the final desired concentration. Polymer solutions and enzyme dilutions were prepared with the acetate buffer cited before, unless specified otherwise. Very viscous solutions of these polymers were measured by weight. After homogenization in a vortex for 10 seconds, the appropriate volume of saturated ammonium sulfate solution was added and the content of the tubes was mixed again in a vortex for another 10 seconds. The reaction mixture was kept at 21 °C for approximately 10 minutes and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was separated from the pellet and filtered through Millipore Millex HV 0.45 μm filters. The filtrate was used for enzyme activity and total carbohydrates assays.

Batch Experiments

Batch experiments were done with cell-free maltose and starch broths, with different levels of concentration fixed by ultrafiltration (concentration ratios from 1 to 17). HPMC and ammonium sulfate concentration were 0.200% w/v and 30% saturation, respectively. Broth pH was in the range of 5.1 to 5.2 and after addition of HPMC very little change was observed.The batch experiments were carried out in a 50 mL beaker at room temperature, unless specified otherwise. Agitation was provided by a magnetic stirrer. No significant difference in activity recovery was noticed when mixing was provided by paddles indicating the stir bars did not denature the protein. Broth and HPMC aliquots, 20 and 5 mL, respectively, were homogenized for 5 minutes. 10.7 mL of ammonium sulfate saturated solution was then added dropwise and mixing was continued for more 10 minutes. The pellet and supernatant were separated by centrifugation at 8,000 rpm for 20 minutes and the pellet was dissolved in 100 mL of buffer. This solution was used to study HPMC recovery and for electrophoresis

after concentration in 10,000 MWCO disc membrane using an Amicon Ultrafiltration Cell, model 8010. The same solution was also used for enzyme activity, total carbohydrates, and protein assays. The volume of the supernatant was measured and the supernatant was used for activity assay. One aliquot of the supernatant was dialysed and used for electrophoresis after concentration using the same disc membrane and ultrafiltration cell as above.

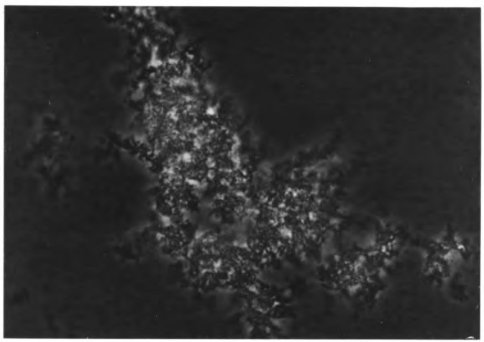
RESULTS

Preliminary Experiments

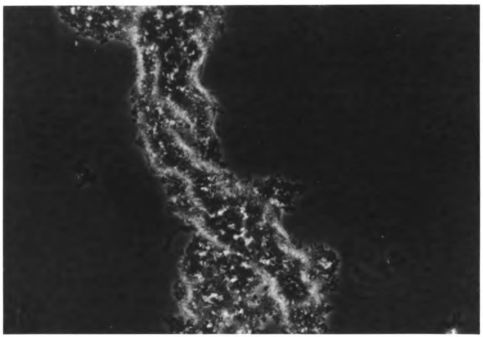
The continuous addition of ammonium sulfate solution to enzyme-MC or enzyme-HPMC solutions, under mixing, produced a cloudy suspension. As the ammonium sulfate concentration achieved a certain level, the particles agglomerated among themselves in a filamentous form. Observing this suspension with a microscope, one can see particles and an arrangement of them in linear form which twist around each other forming a helix-like structure (Figures 6). Droplets, probably gelified polymer, were detected inside clusters of the filaments (Figure 7). Therefore, the insoluble phase seems composed of two and not just one phase. A decrease in solution viscosity was noticeable during the insolubilization, since the polymer was been removed from the solution. Centrifugation of this mixture produces a gel-like compact pellet. This pellet gradually lost its white color while dissolving slowly in water. It became a transparent gel before complete dissolution.

Experiments with β-amylase Preparations

Figure 8 shows the ability of HPMC to promote partitioning of β-amylase to the polymer phase. β-amylase activity recovery of 98% was achieved for ammonium sulfate concentration of 30% saturation.



A



B

Figure 6 - Particle agglomerate (A) and helix-like conformation (B) (200X).

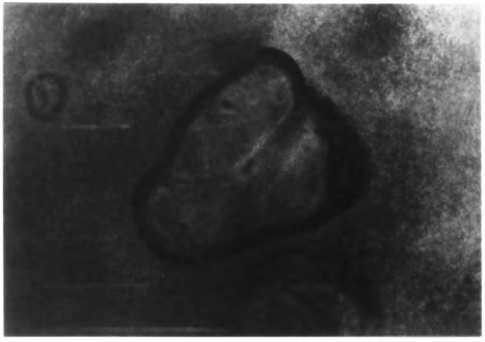


Figure 7 - Droplets inside cluster of filaments (400X).

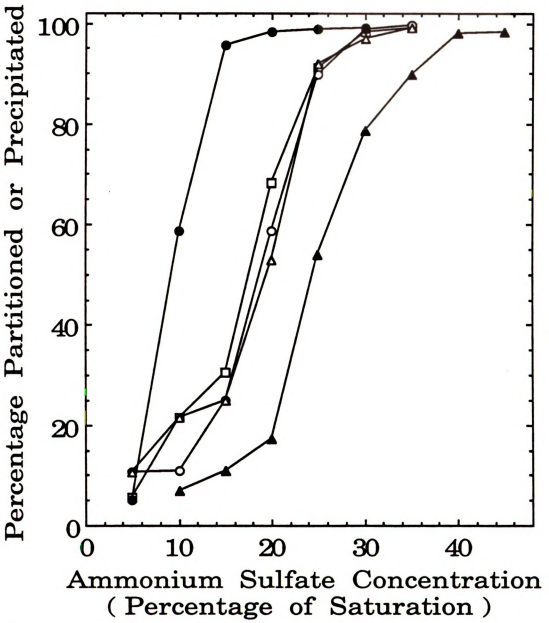


Figure 8 - Effect of ammonium sulfate concentration on β-amylase partition at different HPMC concentrations (□ , 0.500% w/v ; O , 0.200% w/v ; Δ , 0.005% w/v) and on precipitation of β-amylase (▲) and HPMC (● , 0.200% w/v). Enzyme used : different pre-purified β-amylase preparations. Conditions of experiments : enzyme activity, from 33.0 to 80.0 U/mL; protein concentration from 0.7 to 2.4 mg/mL.

Approximately 10% less ammonium sulfate is necessary for partition as compared to precipitation. However, this occurs at a ammonium sulfate concentration approximately 14% higher than the one necessary to precipitate HPMC from a 0.200% w/v solution with no enzyme present.

Comparison of the two polymers, MC and HPMC, as partitioning agents showed that they are equally effective in partitioning β-amylase at 30% ammonium sulfate saturation (Figure 9). However, HPMC is more effective at lower ammonium sulfate concentrations.

β-amylase partition takes place at a ammonium sulfate concentration intermediate to the concentrations necessary to precipitate the enzyme and to segregate HPMC from its solution. There are two possibles explanations for this. First, it may be that polymer and β-amylase interact with each other and form a complex that is insoluble at a specific ammonium sulfate concentration. Second, the enzyme may partition to the polymer phase during its formation, since low ammonium sulfate saturation, for example 15%, is enough to separate more than 95% of the HPMC from its solution.

To check this second possibility, a pellet of MC was prepared in a Eppendorf tube from 1 mL of 0.400% w/v MC solution by adding ammonium sulfate solution up to 30% saturation. The pellet was washed twice with 1 mL of 30% ammonium sulfate saturated solution and 1.43 mL of pre-purified β-amylase with 30% ammonium sulfate saturation was added to it. The tube was shaken for 30 seconds, let stand at room temperature for 10 minutes, and after centrifugation the supernatant was filtrated and analyzed for activity. Only 9% of the initial activity was found on this supernatant. If only β-amylase precipitation by ammonium sulfate

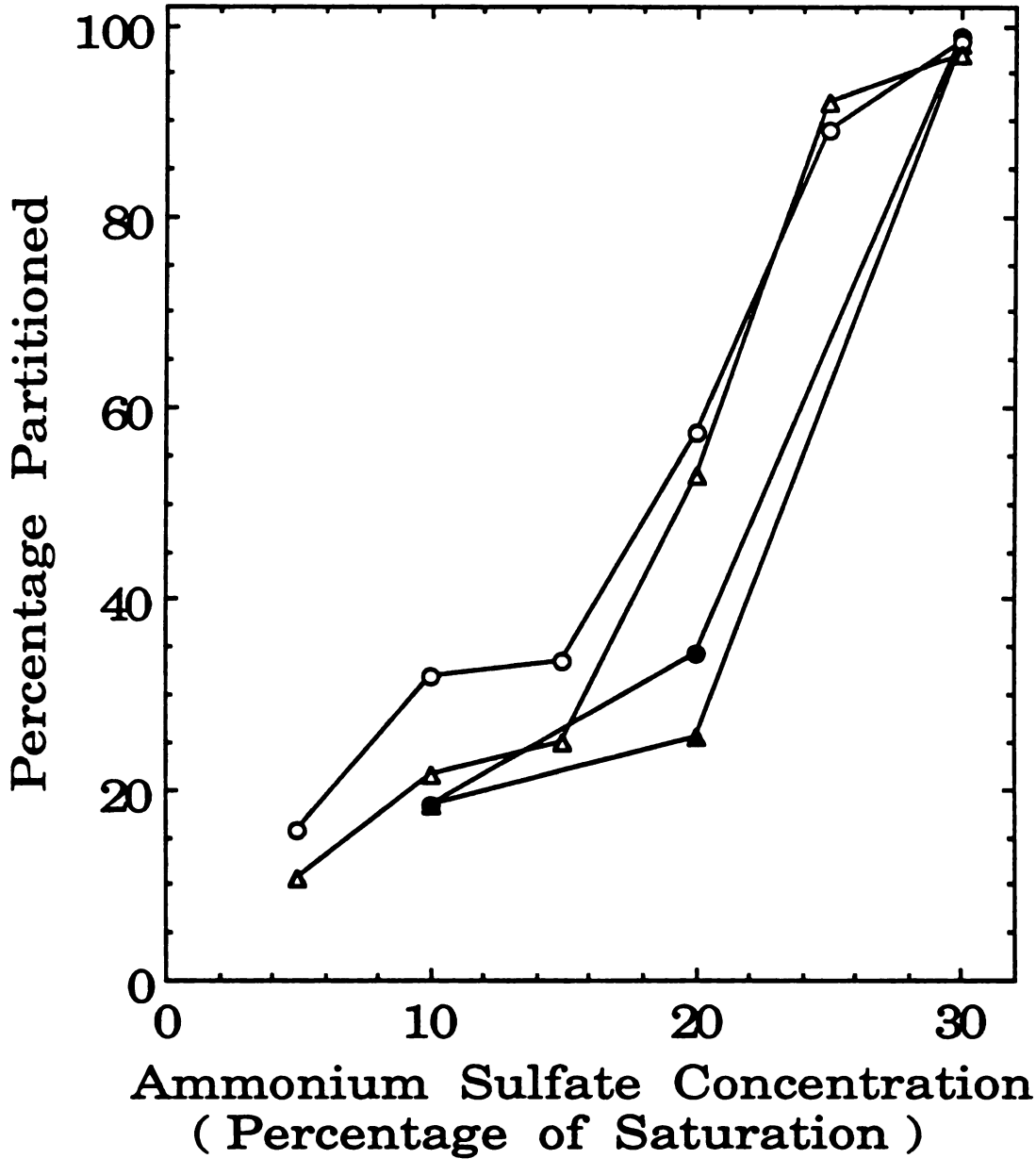


Figure 9 - Comparison between the two types of polymers, MC (● , 0.200% w/v ; ▲ , 0.005% w/v) and HPMC (O , 0.200% w/v ; Δ , 0.005% w/v) in partitioning β-amylase as function of ammonium sulfate concentration. Enzyme used : pre-purified β-amylase. Conditions of experiments : enzyme activity, 41.1 U/mL; protein concentration, 0.7 mg/mL.

had occurred, the supernatant would have around 21% of the initial activity. This indicates that β-amylase partitions to the polymer phase rather than coprecipitates with it.

Hydrophobic interaction is known to be the mechanism responsible for protein binding to some polysaccharide columns, such as Sepharose 4B and cellulose, just as in hydrophobic chromatography (Arakawa, 1986). To check if this is the case of the process under investigation, partition experiments were performed with a protein well known as hydrophobic, bovine albumin. The experiment conditions were 30% ammonium sulfate saturation and 0.200% w/v HPMC (loading of 10 milligrams of polymer to one milligram of protein). No more than 30% of the bovine albumin was partitioned, dismissing hydrophobic interaction as the major mechanism on β-amylase partition.

The influence of ammonium sulfate concentration on the β-amylase separation to the polymer pellet was checked by a "reverse experiment". β-amylase-containing pellets were prepared by adding 429 μL of saturated solution of ammonium sulfate to 500 μL of 1% w/v MC solution,mixing, and then adding 500 μL of enzyme solution. After 15 minutes under gentle mixing, the supernatant was separated by centrifugation. Ammonium sulfate solutions at different concentrations, called "equilibrium solutions", were added to the pellets. The Eppendorf tubes containing the mixtures were shaken for 30 seconds and allowed to stand at room temperature overnight. The supernatant was separated by centrifugation and analyzed for activity and total carbohydrates. The results in Figure 10 show that polymer dissolution was negligible. The percentage of activity in solution was a function of the ammonium sulfate concentration and it was lower

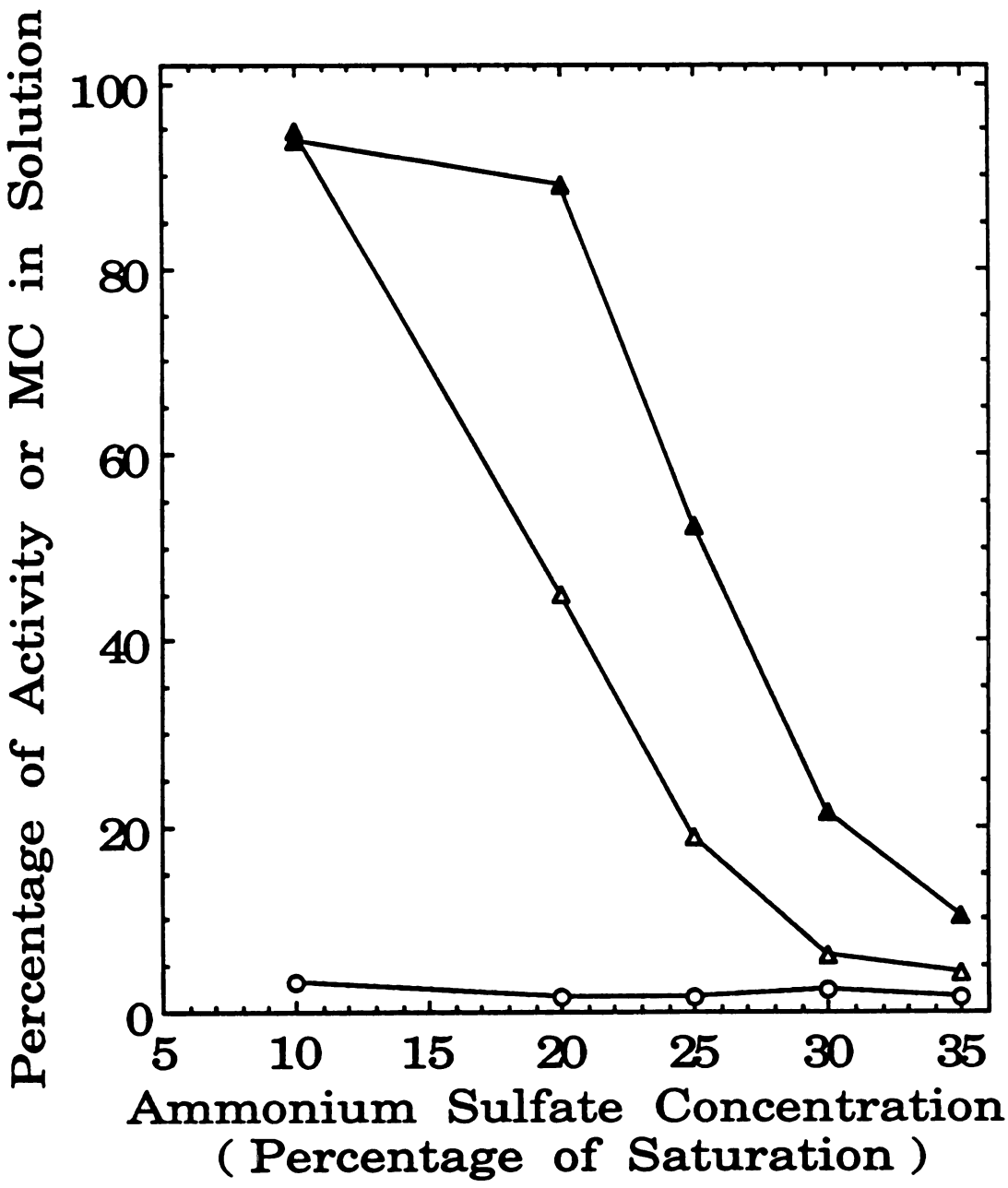


Figure 10 - β-amylase and MC dissolution from a β-amylase containing pellet (Δ and O , respectively) and expected β-amylase dissolution from a pellet of β-amylase precipitated with 30% ammonium sulfate saturation (▲) as function of ammonium sulfate concentration of the "equilibrium solutions". β-amylase containing pellets were obtained by adding saturated ammonium sulfate solution to a MC solution, mixing, and then adding the enzyme solution.

than the percentage expected from dissolution of just β-amylase precipitated by ammonium sulfate.

HPMC loading (mass of polymer per mass of protein) is shown in Figure 11. A minimum of 0.8 is required for maximum partition. At the final protein concentration used, 1.2 mg/mL, this loading correspond to a HPMC concentration of 0.100% w/v.

Table 1 contains the results of experiments made with HPMC of different nominal molecular weights. The HPMC type K4M, with nominal molecular weight of 85,000 daltons, produced the highest partition among the three polymers studied.

The effect of the enzyme dilution was investigated at three different levels of contaminant proteins concentration (different specific activities). Two preparations of pre-purified β-amylase (specific activities of 17.5 U/mg and 38.9 U/mg) and a purified β-amylase (specific activity of 608.8 U/mg) were diluted with acetate buffer to different activities. These dilutions were partitioned at 0.200% w/v HPMC and 25% ammonium sulfate saturation. Results of these experiments were plotted based on the concentration of pure β-amylase present in each dilution, assuming a specific activity of 4215 U/mg (Figure 12). The lower the concentration of pure β-amylase, the lower the partition. However, this behavior was much stronger as the β-amylase preparation was more impure. Enzyme preparations with specific activity of 608.8 U/mg is not affected significantly by dilution in the range concentration studied (from 11.18 to 66.72 micrograms of pure β-amylase per milliliter).

A better quantification of the influence of protein impurities on the partition was made with enzyme solutions at different specific activities but at the same concentration as pure β-amylase (19 μg/mL). Volumes of pre-

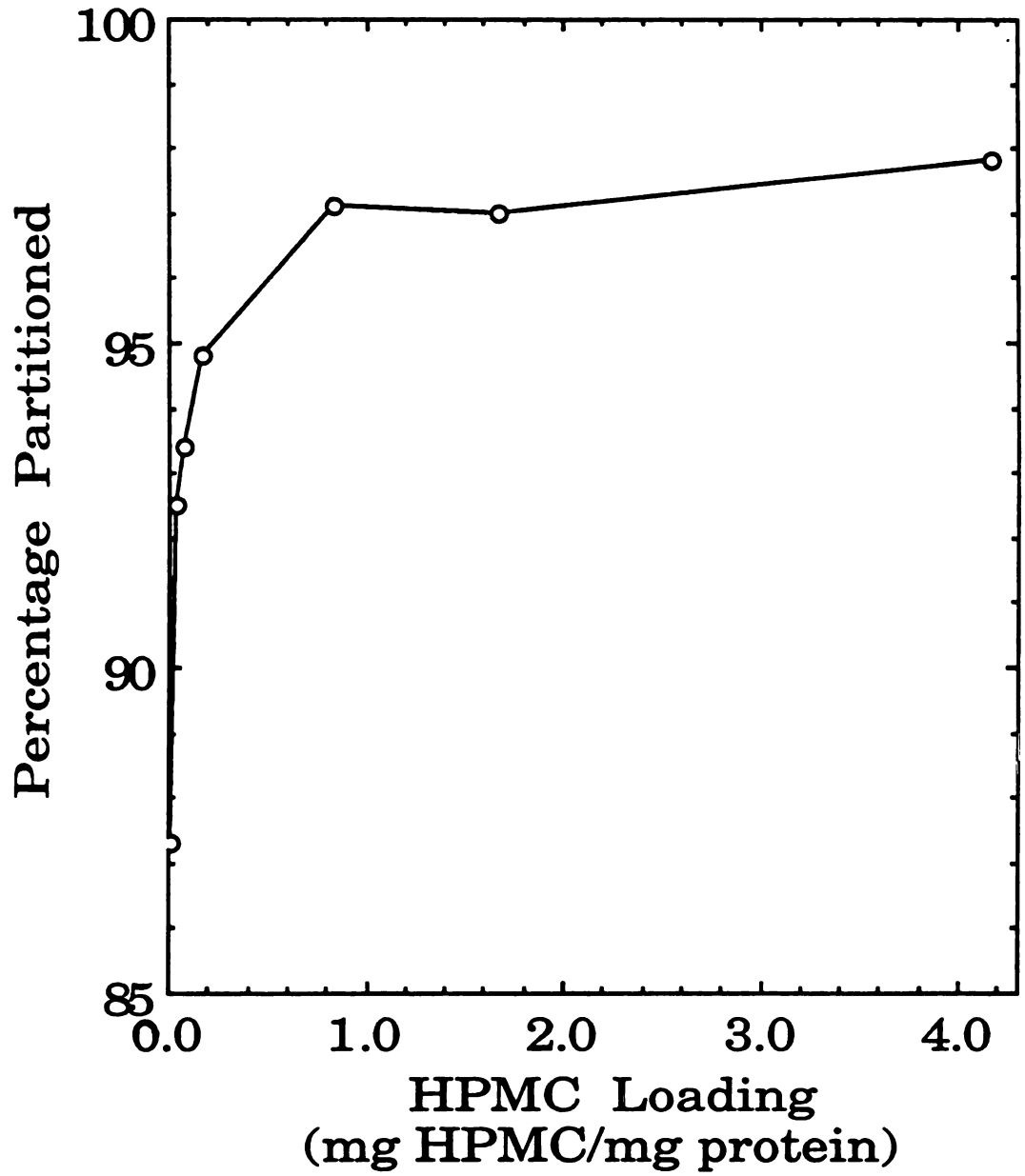


Figure 11 - β-amylase partition as function of the HPMC loading. Enzyme used : pre-purified β-amylase. Conditions of experiments : enzyme activity, 36.7 U/mL and protein concentration, 1.2 mg/mL ; ammonium sulfate concentration, 30% saturation.

Table 1. Effect of the nominal molecular weight of HPMC on β-amylase partition from pre-purified preparation.

HPMC	Nominal molecular weight	Percentage partitioned
K15M	15,000	65.5
K4M	85,000	84.6
K100M Premium	250,000	74.6

Conditions of experiments : HPMC concentration, 0.200% w/v ; HPMC loading, 0.7 ; ammonium sulfate concentration, 25% saturation.

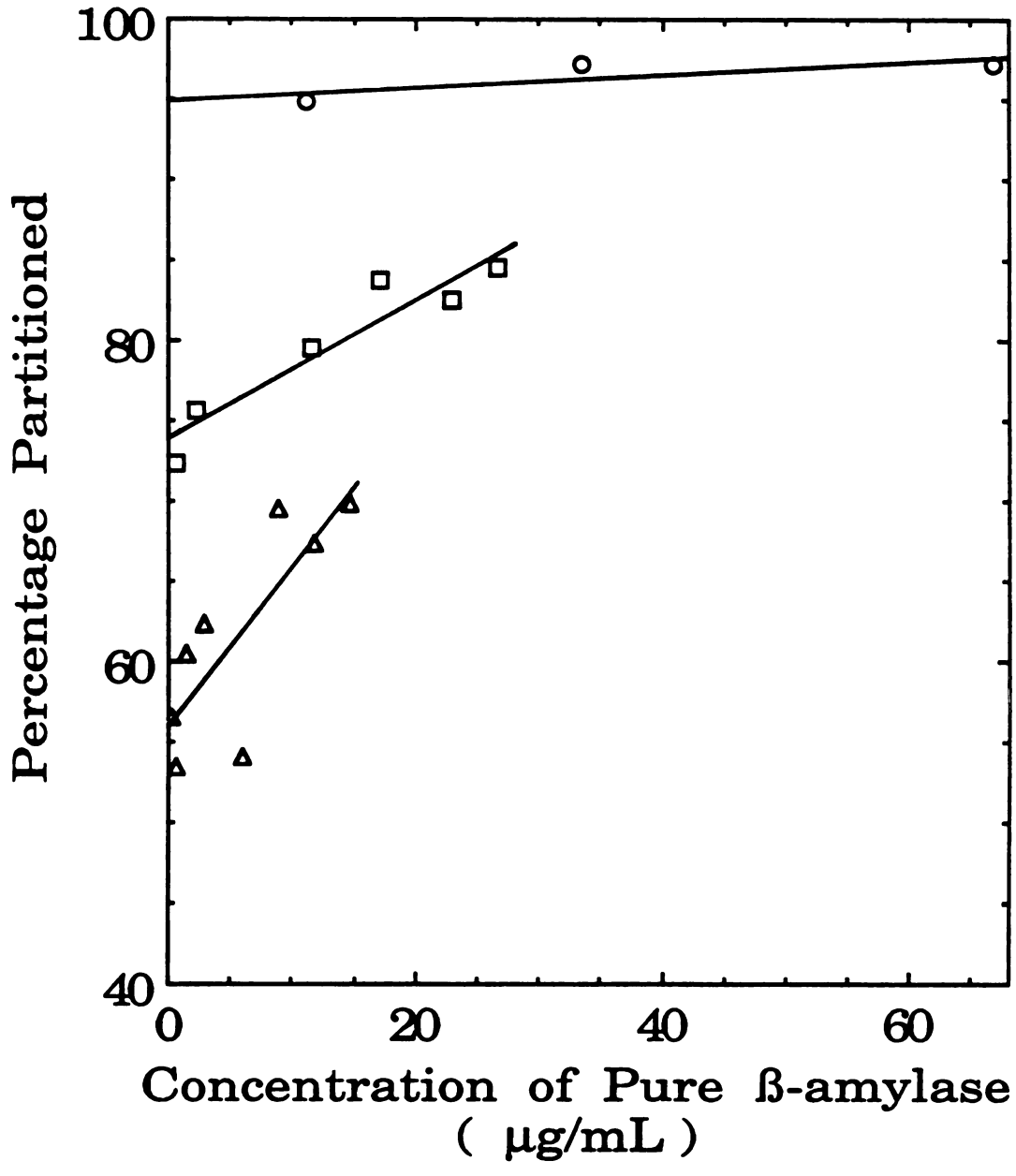


Figure 12 - β-amylase partition as function of concentration of pure β-amylase. Enzyme used : pre-purified β-amylase (Δ , 17.5 U/mg ;□, 38.9 U/mg) and purified β-amylase (O , 608.8 U/mg). Conditions of experiments: HPMC concentration, 0.200% w/v ; ammonium sulfate concentration, 25% saturation. The lines shown are linear regression lines.

purified and purified β-amylase (specific activities of 17.5 and 386.7 U/mg, respectively) were combined, diluted, and the resulting solutions were partitioned at 0.200% w/v HPMC with 25% of ammonium sulfate saturation. Results are plotted on Figure 13. The influence of contaminant proteins, negligible for specific activity higher than 150 U/mg, becomes increasingly important as the purity of the enzyme preparation decrease from this point. At specific activity of 17.5 U/mg, 24% decrease in partition is observed as compared to the value at 150 U/mg.

In order to verify the effect of β-amylase-HPMC solution pH on the partition, pre-purified enzyme (7.1 mg/mL) and 1% w/v HPMC solutions, both in 50 mM acetate buffer pH=6.0 with 5 mM $CaCl_2$, were diluted 3- and 2.5-fold, respectively, with buffers at pH's of 3.3, 4.0, 5.0, 6.0, 7.5, 8.5, and 10.0. Buffers with pH lower than 7.0 were 50 mM acetate with 5 mM $CaCl_2$ and those with pH higher than 7.0 were 50 mM Tris with 5 mM $CaCl_2$. The combination of 500 μL of enzyme solution diluted with each one of the above buffers and 500 μL of enzyme solution diluted with the same buffer provided final concentration of polymer of 0.200% w/v and pH's of 3.6, 4.2, 5.7, 6.0, 7.4, 8.5, and 9.3. The results of partition of these preparations showed that the effect of pH is negligible (Figure 14). Differences in partition for mixtures with pH's above and below 7.0 is probably due to the use of different buffers for each one of these ranges.

Introduction of ethanol to the system prior to ammonium sulfate addition was deleterious to the β-amylase partition (Table 2).

Experiments with Fermentation Broths

Batch partition experiments with 0.200% w/v HPMC and 30% ammonium sulfate saturation were carried out with both types of broth at

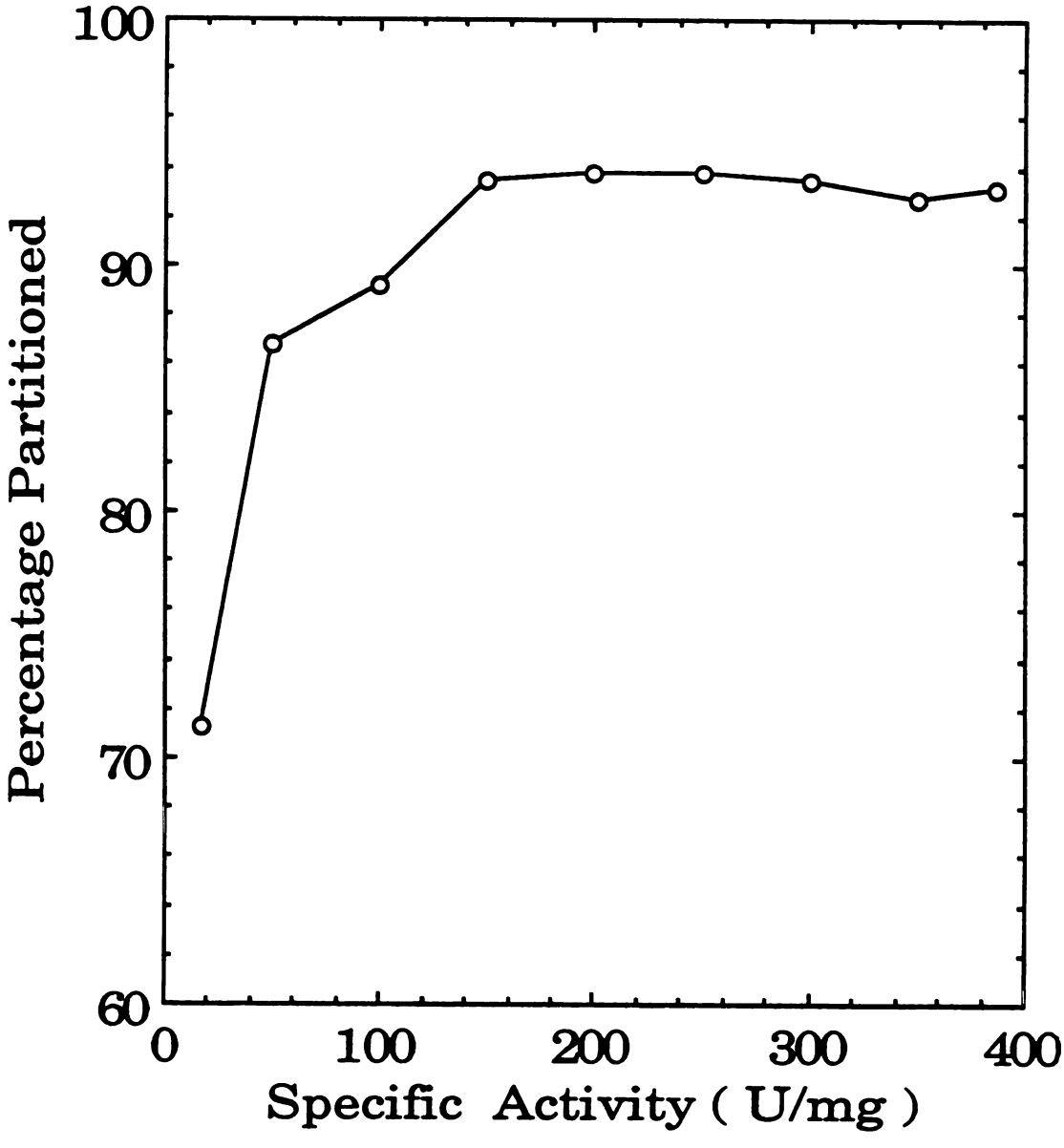


Figure 13 - β-amylase partition as function of enzyme preparation purity. β-amylase preparations at different specific activities were obtained by mixing appropriate volumes of pre-purified β-amylase (17.5 U/mg, 7.1 mg/mL) and purified β-amylase (608.8 U/mg, 1.1 mg/mL). Conditions of experiments : enzyme activity, 40.0 U/mL ; HPMC concentration, 0.200% w/v ; ammonium sulfate, 25% saturation.

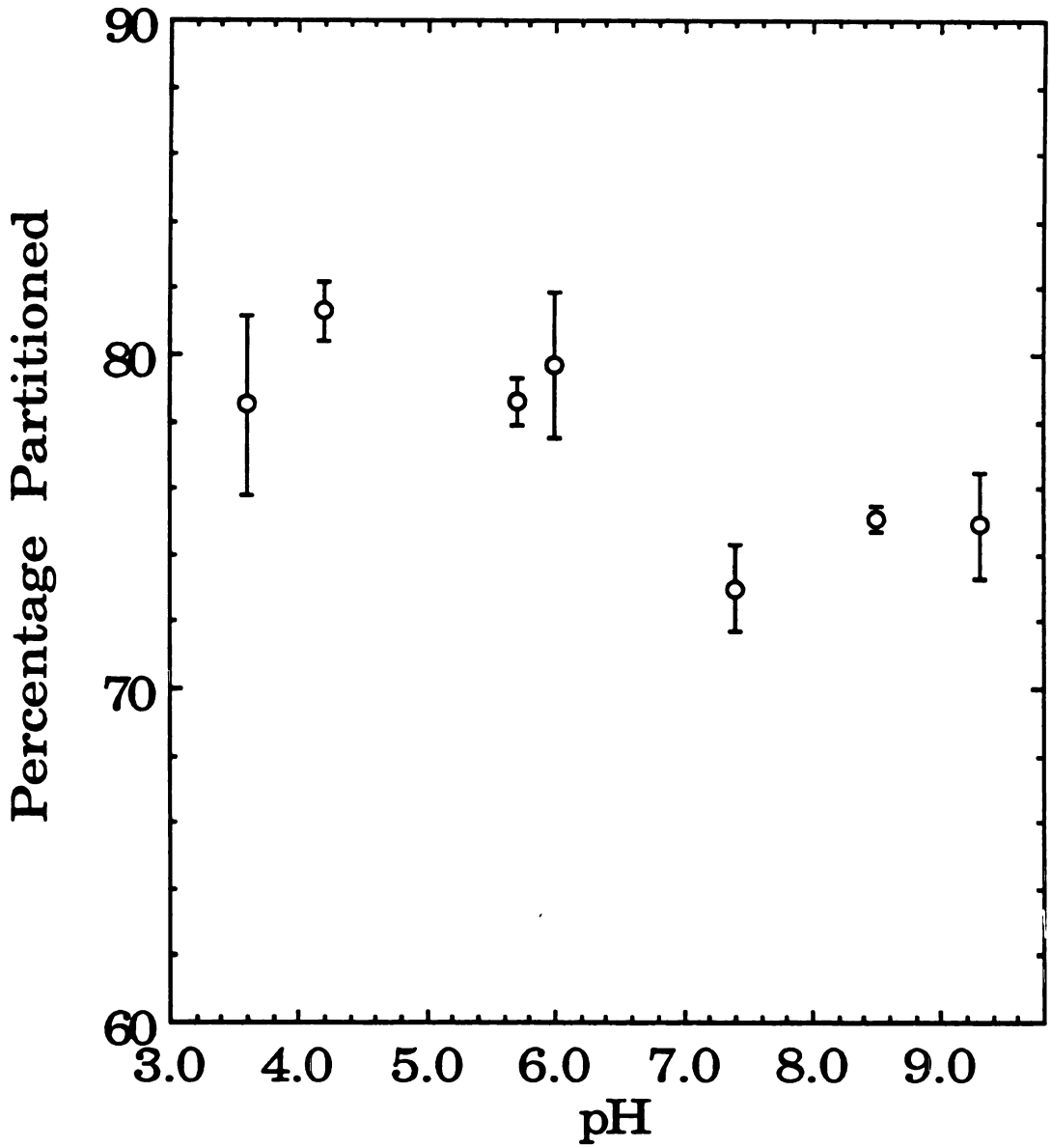


Figure 14 - β-amylase partition as function of HPMC-β-amylase solution pH. Buffers for pH lower or higher than 7.0 were acetate or Tris, respectively, both at 50 mM with 5 mM $CaCl_2$. Enzyme used : pre-purified β-amylase. Conditions of experiments : enzyme activity, 38.3 U/mL ; protein concentration, 2.4 mg/mL ; HPMC concentration, 0.200% w/v ; ammonium sulfate concentration, 25% saturation.

Table 2 - Effect of ethanol on β-amylase partition from pre-purified preparation.

Ethanol concentration*	Percentage partitioned
0	84.6
5	67.9
10	36.4

*in v/v before addition of ammonium sulfate

Conditions of experiments : HPMC concentration, 0.200% w/v ; HPMC loading, 0.7 ; ammonium sulfate, 25% saturation.

various levels of concentration (Figure 15). HPMC loading, measured for the maltose broth case, was in the range of 0.78 to 0.83. Basically, no β-amylase denaturation took place. The behavior of maltose broth was the same as in the small scale experiments, with more than 95% of the enzyme recovered in the pellets even though broth specific activities were low, in the range of 2.0 to 11.5 U/mg. However, β-amylase from starch broth was not efficiently partitioned, with only 30% of the activity found on the pellets at most.

Partition of the β-amylase from maltose broth achieved different purification according to the concentration given by ultrafiltration (Figure 16). The purification for the maltose broth concentrated 17-fold in volume was confirmed by SDS-polyacrylamide gel electrophoresis (Figure 17).

Temperature increases were investigated to improve the partition of β-amylase from starch broth. Since HPMC precipitates at relatively high temperatures, solutions of this polymer were added dropwise to the broth to which ammonium sulfate had already been added in sufficient amount to give a final concentration of 30% saturation. Starch broth, ammonium sulfate, and HPMC solutions were equilibrated at the temperature of the experiment prior to addition of one to the other. The centrifugation was done using a pre-warmed rotor. Even though high levels of partition were not achieved, a reasonable amount of β-amylase activity was partitioned at 75 °C (approximately 25%) while partition was almost negligible at 21 °C (Figure 18). The maximum enzyme denaturation was around 10% at 75 °C.

Separation and recovery of the HPMC from the β-amylase containing pellet by a second precipitation with ammonium sulfate was studied at two different temperatures, 21 and 56 °C, in small scale. The pellet used for this experiments was produced by partitioning β-amylase from 8-fold

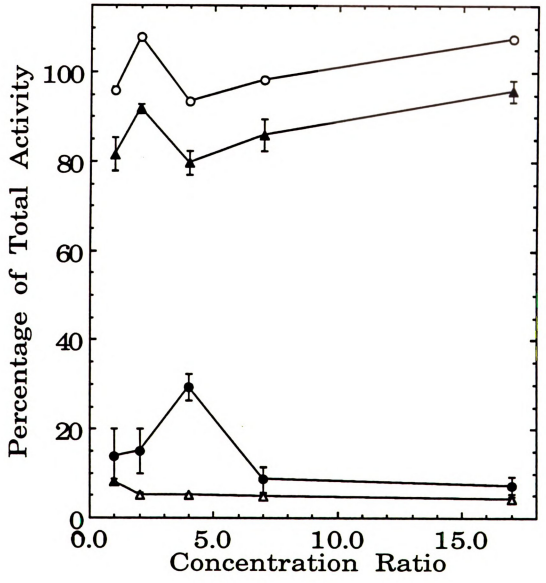


Figure 15 - Activity distribution between precipitate (O ,●) and supernatant (Δ , ▲) on β-amylase partition as function of concentration for maltose broth (open symbols) and starch broth (close symbols). Conditions of experiments: enzyme activities from 5.0 to 29.2 U/mL and from 13.1 to 127.5 U/mL for maltose and starch broth, respectively ; protein concentration from 2.4 to 2.6 mg/mL for maltose broth ; HPMC concentration, 0.200% w/v ; ammonium sulfate concentration, 30% saturation.

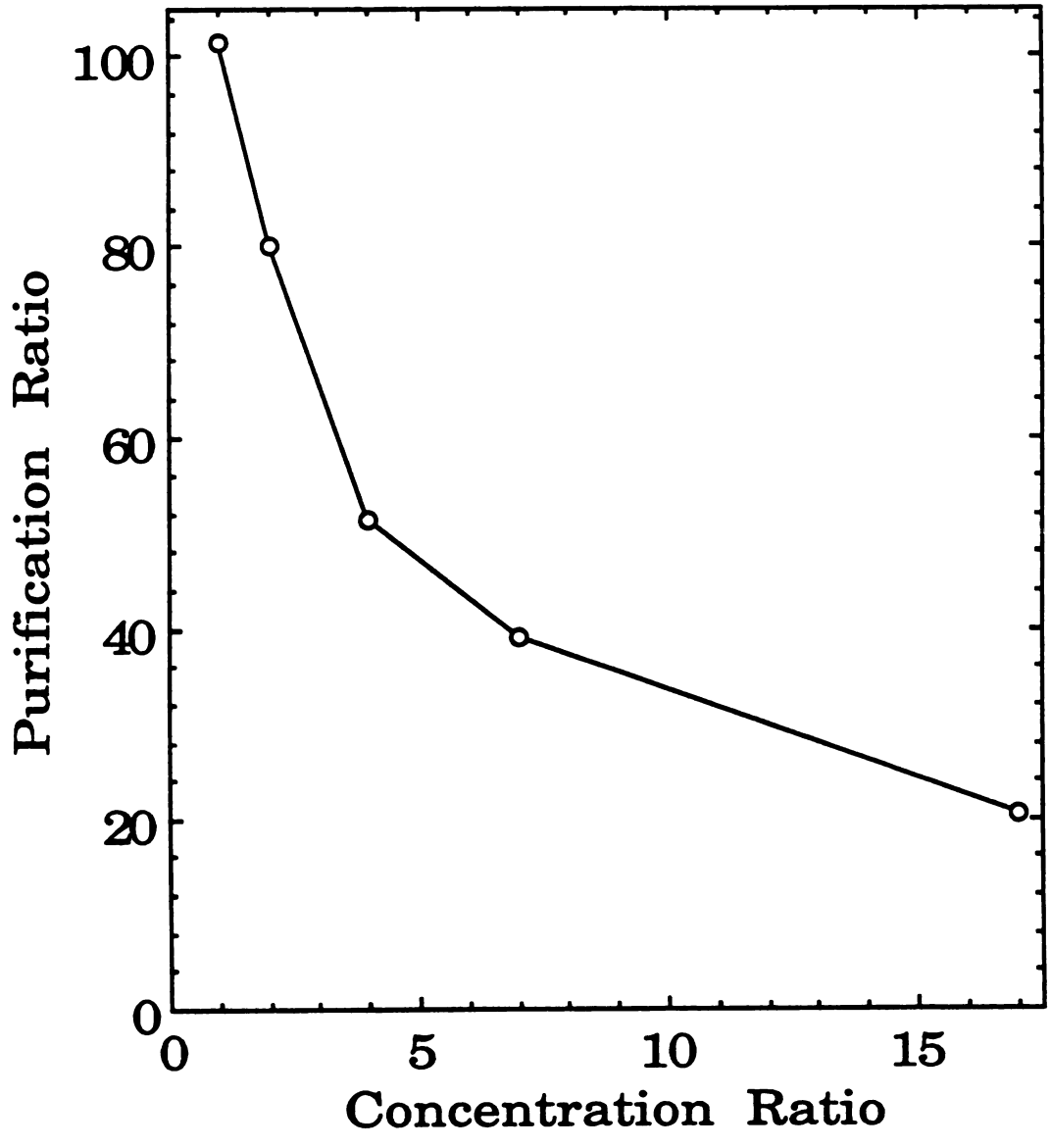


Figure 16 - β-amylase purification achieved by partition from maltose broth with 0.200% w/v and 30% ammonium sulfate saturation as function of broth concentration.

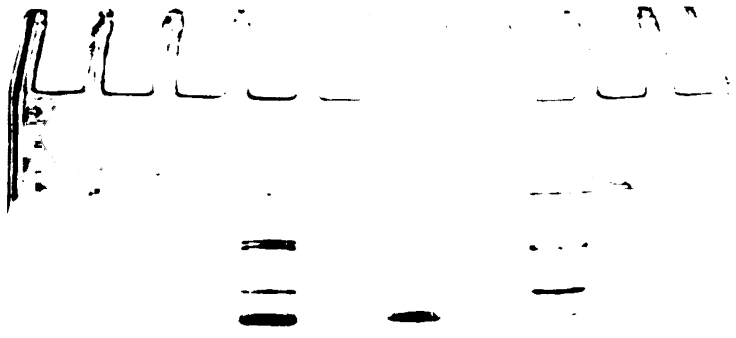


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Figure 17 - SDS-polyacrylamide gel electrophoresis of (1) maltose broth, (2) supernatant, and (3) dissolved β-amylase containing pellet obtained by partition from maltose broth (16-fold concentrated) with 0.200% w/v HPMC and 30% ammonium sulfate saturation. Concentrations were adjusted to have the same intensity for each band.

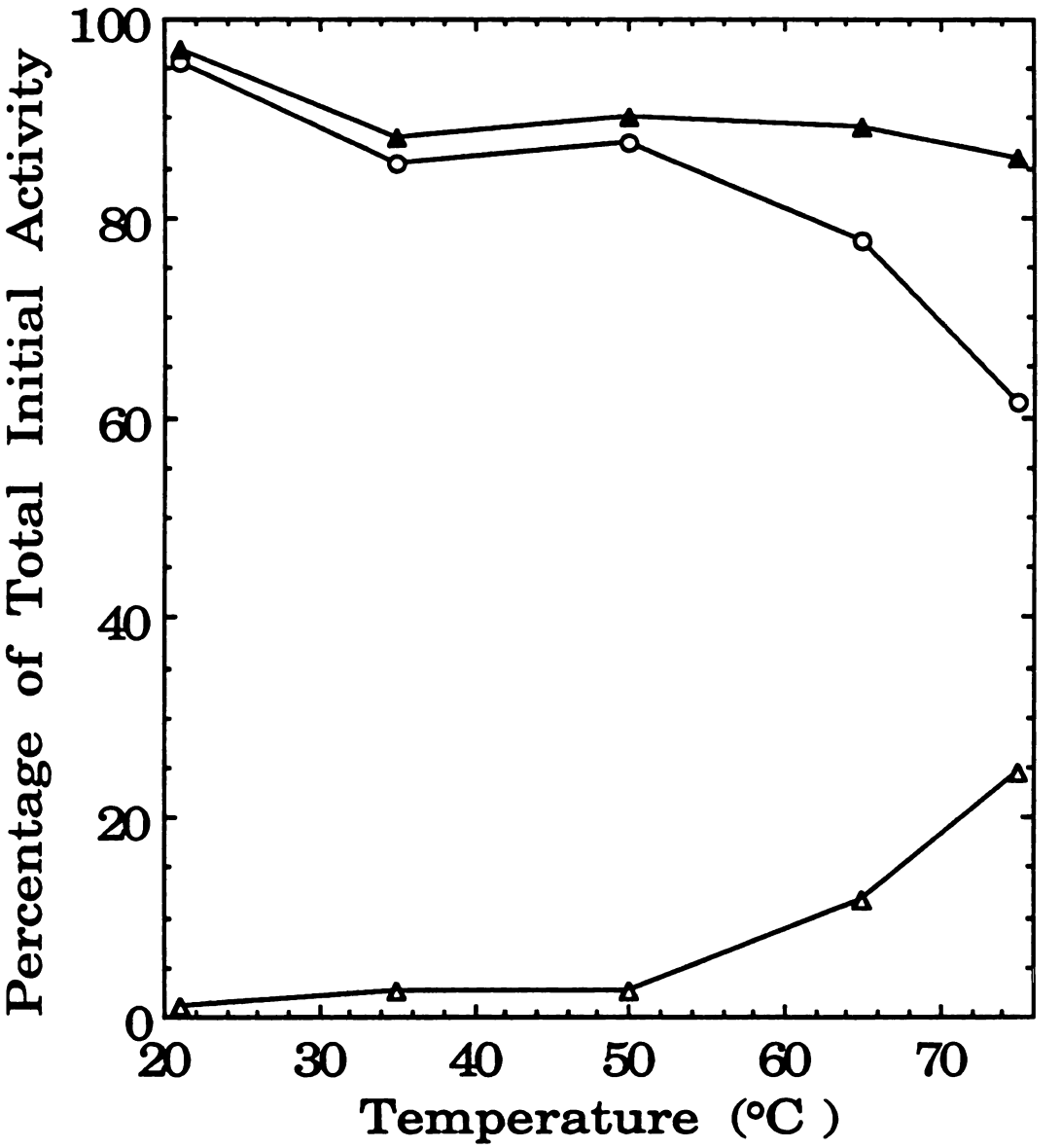

Figure 18 - Activity distribution and total recovery in β-amylase partition from starch broth (8-fold concentrated) as function of temperature (Δ , pellet ; O , supernatant ; ▲ , total recovery). Conditions of experiments : HPMC concentration, 0.200% w/v ; ammonium sulfate concentration, 30% saturation.

concentrated maltose broth. More than 90% of the polymer could be precipitated along with only 5% of activity partition at both temperatures (Figure 19). However, only 5% ammonium sulfate saturation was required for the same separation at 56 °C while at 21 °C the salt concentration required was 15% saturation. This is the result of the shift of the partition and precipitation curves to the left produced by the raising in the temperature. It is interesting to note that at 56 °C the partition and precipitation curves are more separated from each other than at 21 °C. A batch polymer separation was made and samples were analyzed to check for further β-amylase purification by SDS-polyacrylamide gel electrophoresis. No improvement in purification was observed (gel pictures not shown).

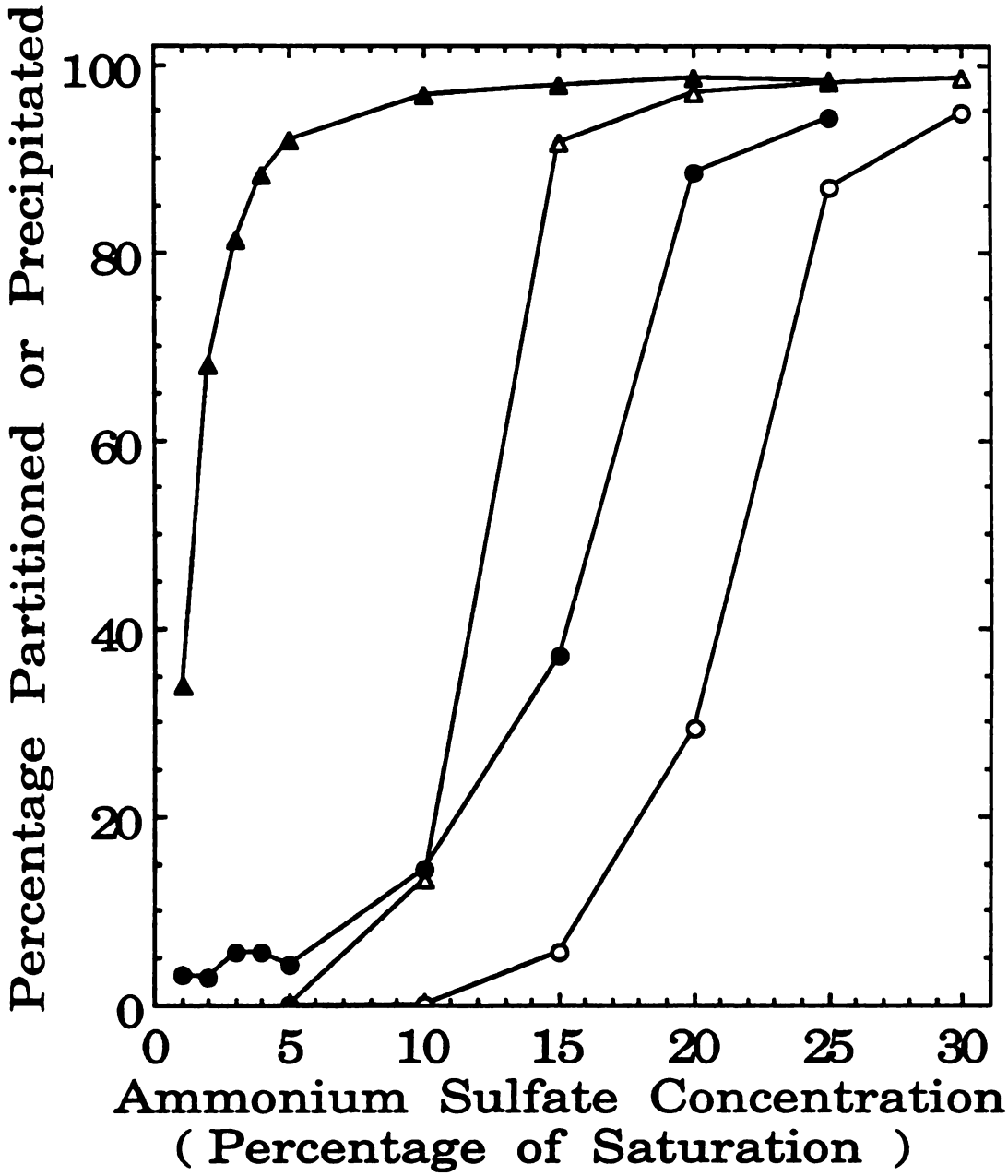

Figure 19 - Effect of ammonium sulfate concentration on β-amylase partition (O , ●) and HPMC precipitation (Δ , ▲) at 21 °C (open symbols) and at 56 °C (close symbols) from dissolved β-amylase containing pellet obtained by partition from maltose broth (16-fold concentrated) with 0.200% w/v HPMC and 30% ammonium sulfate saturation.

DISCUSSION

The small scale experiments showed that β-amylase partition to a HPMC second phase is a very fast, simple and efficient technique, even though its mechanism does not seem to be simple. The experiments suggest that the polymer is first insolubilized and then the β-amylase is adsorbed to it according to the ammonium sulfate concentration of the solution.

The slightly better behavior of HPMC for partition of β-amylase in comparison to MC may be explained by comparison of the methoxyl degree of substitution of HPMC (in the range of 1.1 to 1.4) and MC (in the range of 1.6 to 1.9), since it is known that degrees of substitution lower than 1.4 give products with low water solubility (Greminger and Krumel, 1980).

The minimum HPMC loading necessary for maximum partition, 0.8, is a relatively low loading if compared with some polyethylene glycol loadings that can be as high as 31.1 (Foster et al., 1973). This low loading contributes to low recovery cost and low viscosity of the HPMC containing solutions.

The nominal molecular weight of HPMC has a significant influence on the partition of β-amylase, contrary to what was expected since it is known that IGT and ITP for commercial HPMC have little dependence on this variable. Based on the results showed in Table 1, it seems that there is an optimum HPMC molecular weight for the β-amylase partition.

Dilution and purity were found to be important variables with respect to how much enzyme partitions to the HPMC phase while pH was not. A competition of the contaminant proteins for the polymer, β-amylase, or both could explain this behavior. Self-association of protein is known to increase protein precipitation by polyethylene glycol. If such protein interaction plays a significant role in the β-amylase partition, competition of contaminant proteins with the β-amylase may be the mechanism by which partition of preparations with low specific activity/high dilution results in low recovery. Partition insensitivity to enzyme-polymer solution pH may be due to the buffer effect of the relatively high ammonium sulfate content used on the preparation (around 1.7 M) when compared to acetate or Tris buffer concentrations (50 mM).

The approach of decreasing the dielectric constant of the solution prior partition by adding ethanol does not improve β-amylase separation. Again, the concentration of ammonium sulfate, a strong electrolyte, may be the explanation for poor recovery since it may overcome the ethanol effect. Moreover, ethanol is known to increase solubility of HPMC in water.

The batch experiments showed that partition with HPMC can be applied to recover and purify C. thermosulfurogenes β-amylase from the fermentation broth with very high yield (95% or more). Purification achieved (21- to 101-fold) was very high when compared with purification obtained by ammonium sulfate, ethanol, or ethanol/ammonium sulfate precipitations (7-, 6.2- and 31-fold, respectively).

The behavior of maltose and starch broth in β-amylase partition is similar to the behavior in precipitation by ammonium sulfate reported by Elankovan (1988). In each case, β-amylase fermented in maltose was efficiently separated (activity recovery higher than 90%), while β-amylase

fermented with starch was poorly separated (activity recovery of 57% or lower). These results suggest that interaction with starch makes β-amylase unable to interact and partition with HPMC.

The conclusion above provides an explanation as to why partition of β-amylase fermented in starch takes place only at high temperatures and is a function of the temperature increase (Figure 18). The reason may be in the kinetics of the starch hydrolysis by the enzyme, since increasing in the temperature increases the reaction rate. As the temperature gets closer to the β-amylase optimum temperature, 75 °C, it is plausible to expect that each enzyme molecule is more efficient in hydrolysing the starch molecule(s) bound to itself or to other β-amylase molecules. Eventually, the enzyme molecules would be free of the starch moiety and, then, able to interact with HPMC molecules. Another contribution to the success in partition at high temperature came from the HPMC solubility properties. HPMC is less soluble at higher temperature, (e.g., 75 °C), than at 21 °C, and β-amylase partition at higher temperatures is more efficient for the same ammonium sulfate concentration, as one can see on Figure 19.

In a companion work, two other amylases, a thermophilic α-amylase and a pullulanase, were also partitioned by the same HPMC system. These facts suggest that there is a specificity involved in amylase partition to HPMC and that this specific interaction may mimic amylases' binding to starch.

The low solubility of HPMC at higher temperatures is probably the reason why recovery of HPMC is more efficient at 56 °C. The shift in partition and precipitation curves caused by temperature increase (Figure 19) shows a very important feature of this technique. Recovery of more than 95% of β-amylase activity took only 20% saturation in ammonium sulfate.

This is one third less than the value at 21 °C and 40% the salt needed by precipitation with only ammonium sulfate. Therefore, the use of temperatures higher than 21 °C can substantially reduce the amount of ammonium sulfate needed, not only for polymer recovery, but for β-amylase partition, also.

HPMC-β-amylase pellets were dissolved in beakers with magnetic stirring for two to three hours. Even when this dissolution took place at 21 °C, no loss in activity was detected. HPMC may stabilize the β-amylase , as it is the case of polyethylene glycol with some enzymes (Hao et al., 1980).

A process for recovery of C. thermosulfurogenes β-amylase from fermentation broth is illustrated by the general scheme in Figure 20. If the gain from purification and volume reduction due to ultrafiltration does not overcome the cost and drawbacks of introduction of this processing step, the operation should be omitted. The specific enzyme application will determine what further processing steps (e.g., desalinization, concentration, or precipitation) must follow partition in order to achieve specifications.

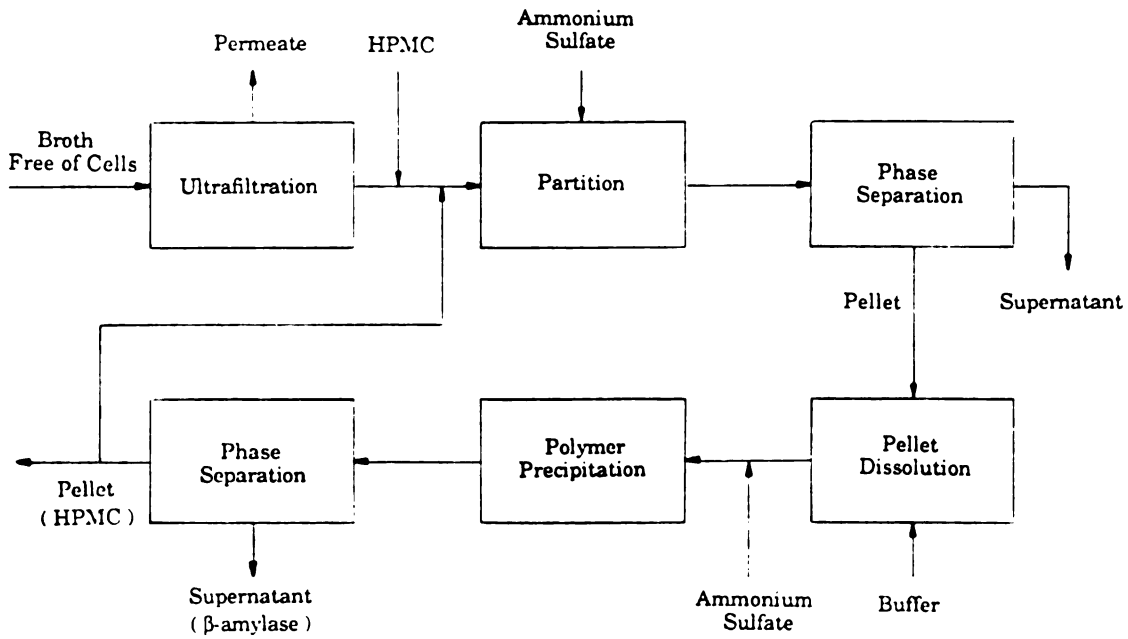

Figure 20. Flow diagram of the developed process for β-amylase recovery from fermentation broth by partition to a gel-like phase.

CONCLUSIONS

1. Addition of ammonium sulfate to a final concentration of 30% saturation to a solution of C. thermosulfurogenes β-amylase preparation and HPMC or MC produces a gel-like phase which contains the enzyme. The mechanism by which β-amylase is separated to the gel-like phase is partition to the polymer. First the polymer precipitates at approximately 15% saturation at 21 °C. The enzyme adsorbs to this polymer phase as the salt concentration is increased from this point.

2. A minimum loading of 0.8 milligram of HPMC per milligram of protein is necessary to assure maximum β-amylase partition from pre-purified preparations.

3. HPMC is a better partition agent for β-amylase than MC for ammonium sulfate concentrations lower than 30% saturation. The two polymers seem to be equivalent to each other for partition at 30% saturation.

4. β-amylase partition is a function of the nominal molecular weight of the HPMC in the range of 15,000 to 250,000 daltons.

5. Dilution and impurity of the β-amylase preparation affect its partition for pure β-amylase concentrations from 0.3 to 26.7 μg/mL and specific activities from 17.5 to 608.8 U/mg. The higher the dilution or impurity, the lower the partition.

6. β-amylase partition does not depend on the HPMC-enzyme solution pH in the range of 3.6 to 9.3.

7. β-amylase partition is decreased by the introduction of ethanol to the system prior to the addition of ammonium sulfate. 10% ethanol causes a reduction of 57% in partition.

8. β-amylase produced from maltose partitions from the fermentation broth to HPMC phase with high yield (activity recovery of 95% or more) and high purification (at least 20-fold). 30% saturation in ammonium sulfate and 0.200% w/v are required conditions for the separation.

9. β-amylase does not partition efficiently from starch broth with HPMC (maximum activity recovery of 25% at 75 °C) due to its binding to starch.

10. HPMC can be recovered from a dissolved β-amylase containing pellet solution by a second ammonium sulfate precipitation. Recovery of 90% of the polymer along with only 5% of the total activity previously partitioned can be obtained with 5% ammonium sulfate saturation at 56°C.

RECOMMENDATIONS

The following points are proposed topics for future research:

1. A better understanding of the partition mechanism is needed in order to optimize and access the general applicability of this technique. One approach is to apply this partition to other enzymes which have very well-known structure or properties (like hydrophobicity, affinity bindings). The general applicability and clues to the partition mechanism may be provided simultaneously by such experiments.

2. Optimization of partition and polymer recovery temperatures are needed, since it was shown that the amount of ammonium sulfate required for a specific partition yield decreases as the temperature increases.

3. Kinetics of HPMC phase formation and engineering aspects of pellet separation from solutions in partition and polymer recovery steps must be studied in order to optimize polymer separation and, consequently, activity yield and polymer recovery. These pellets have a density approaching the broth density indicating that large scale centrifugation may not be an efficient separation technique. Filtration, macro or micro (membrane separation) filtration, may be considered as centrifugation substitute.

4. Polymer recovery was shown to be feasible. However, it is necessary to determine if the recycled polymer has the same partition properties as the original one and how many times recycling would be possible.

5. Other salts (e.g., sodium chloride, magnesium chloride, and sodium carbonate) should be considered as substitute for ammonium sulfate. Besides processing cost, pellet physical characteristics, HPMC loading, and enzyme purification may be improved by using other salting-out agents.

APPENDIX

Appendix : Raw Data Used on the Preparation of Tables and Figures

Table 3 - Raw data of Figure 8

Ammonium sulfate concentration as percentage of saturation	Percentage of β-amylase partitioned at different HPMC concentration*(in w/v)			Percentage of precipitation*	
	0.500%	0.200%	0.005%	β-amylase	0.200% w/v HPMC
5	5.6	10.6	10.6	–	5.0
10	21.4	10.9	21.4	6.8	58.7
15	15.0	30.6	25.1	11.0	95.7
20	68.2	58.7	52.8	17.2	98.2
25	91.2	89.9	91.8	54.0	98.7
30	98.4	98.8	97.0	78.7	99.2
35	99.0	99.5	99.0	89.7	–
40	–	–	–	97.9	–
45	–	–	–	98.3	–

*Average of the results of two or three experiments.

Conditions of experiments: enzyme activity, from 33.0 to 80.0 U/mL; protein concentration,from 0.7 to 2.4 mg/mL.

Table 4 - Raw data of Figure 9

Ammonium sulfate concentration as percentage of saturation	Percentage partitioned			
	MC		HPMC	
	0.020% w/v	0.005% w/v	0.020% w/v	0.005% w/v
5	–	–	15.6	10.6
10	18.2	18.2	31.9	21.4
15	–	–	33.4	25.1
20	34.2	25.4	57.5	52.8
25	–	–	89.0	91.8
30	98.8	98.1	98.2	97.0

Conditions of experiments: enzyme activity, 41.1 U/mL; protein concentration, 0.7 mg/mL.

Table 5 - Raw data of Figure 10

Ammonium sulfate concentration as percentage of saturation	Percentage of activity or total carbohydrates dissolved from MC pellet				Expected activity to dissolve from pellet of β-amylase precipitated by ammonium sulfate* (in percentage)
	Act 1	Act 2	Average	TCH	
10	93.2	96.1	94.7	3.2	93.2
20	44.6	45.4	45.0	1.7	82.8
25	18.9	18.8	18.9	1.7	46.0
30	5.4	6.7	6.1	2.3	21.3
35	–	4.3	–	1.5	10.3

i- Act 1, Act 2 are values of percentage of activity dissolved from MC pellets of two experiments.

ii- TCH are the average of percentage of total carbohydrates dissolved from MC pellets of two experiments.

* Values obtained from data on Table 3.

Table 6 - Raw data of Figure 11

mg of HPMC / mg of protein	Percentage partitioned			
	Experiment 1	Experiment 2	Experiment 3	Average
0.008	86.3	83.1	93.5	87.6
0.042	94.4	98.1	83.3	91.9
0.083	93.4	91.3	95.5	93.4
0.167	87.1	92.6	108.9	96.2
0.833	101.5	92.7	97.1	97.1
1.667	101.4	88.2	105.8	98.5
4.167	86.9	103.2	108.7	99.6

Conditions of experiments: enzyme activity, 36.7 U/mL; protein concentration, 1.2 U/mL; ammonium sulfate concentration, 30% saturation.

Table 7 - Raw data of Table 1

HPMC	Nominal molecular weight	Percentage partitioned		
		Experiment 1	Experiment 2	Average
K15M	15,000	62.6	68.4	65.5
K4M	85,000	84.6	84.6	84.6
K100M Premium	250,000	77.5	71.6	74.6

Conditions of experiments: HPMC concentration, 0.200% w/v; HPMC loading, 0.7; ammonium sulfate concentration, 25% saturation.

Table 8 - Raw data of Figure 12

Concentration of pure β-amylase (μg/mL)	Percentage partitioned for different specific activities								
	17.5 U/mg			38.8 U/mg			608.8 U/mg		
	Exp 1	Exp 2	Average	Exp 1	Exp 2	Average	Exp 1	Exp 2	Average
14.77	–	–	69.8						
11.81	–	–	67.3						
8.86	–	–	69.6						
5.91	–	–	54.0						
2.95	–	–	62.3						
1.48	–	–	60.5						
0.59	–	–	53.5						
0.30	–	–	56.6						
26.74				84.6	84.6	84.6			
22.94				82.5	82.5	82.5			
17.22				77.4	90.3	83.9			
11.50				80.9	78.1	79.5			
2.30				77.5	73.7	75.6			
0.59				76.1	68.5	72.3			
66.72							112.5	81.9	97.2
33.55							102.3	92.1	97.2
11.18							–	–	94.9

Conditions of experiments: HPMC concentration, 0.200% w/v; ammonium sulfate concentration,25% saturation.

Table 9 - Raw data of Figure 13

Specific activity (U/mg)	Percentage partitioned (average of two experiments)
386.7	93.1
350.0	92.7
300.0	93.4
250.0	93.7
200.0	93.7
150.0	93.4
100.0	89.2
50.0	86.8
17.0	71.2

Conditions of experiments: enzyme activity, 40.0 U/mL; HPMC concentration, 0.200% w/v; ammonium sulfate concentration, 25% saturation.

Table 10 - Raw data of Figure 14

pH	Percentage partitioned			
	Experiment 1	Experiment 2	Average	|Experiment 1 - Experiment 2|
3.60	75.8	81.2	78.5	2.7
4.20	80.4	82.2	81.3	0.9
5.70	79.3	77.9	78.6	0.7
6.00	81.9	77.5	79.7	2.2
7.40	71.7	74.3	73.0	1.3
8.50	74.7	75.6	75.1	0.4
9.30	73.3	76.5	74.9	1.6

Conditions of experiments: enzyme activity, 38.3 U/mL; protein concentration, 2.4 mg/mL; HPMC concentration, 0.200% w/v; ammonium sulfate concentration, 25% saturation.

Table 11 - Raw data of Table 2

Ethanol concentration (Percentage in volume)	Percentage partitioned		
	Experiment 1	Experiment 2	Average
0	84.6	84.6	84.6
5	71.5	64.3	67.9
10	36.7	36.1	36.4

Conditions of experiments: HPMC concentration, 0.200% w/v; HPMC loading, 0.7; ammonium sulfate concentration, 25% saturation.

Table 12 - Raw data of Figure 15

Broth	Concentration ratio	Total activity distribution (in percentage)							
		Precipitate				Supernatant			
		Exp 1	Exp 2	Average	Δ	Exp 1	Exp 2	Average	Δ
Maltose broth	1	95.0	96.7	95.8	1.7	7.7	8.7	8.2	1.0
	2	107.5	108.2	107.8	0.7	4.7	5.7	5.2	1.0
	3	93.0	94.0	93.5	1.0	5.0	5.4	5.2	0.4
	7	98.3	97.9	98.1	0.4	5.4	4.4	4.9	1.0
	17	107.3	107.7	107.5	0.2	4.7	3.7	4.2	1.0
Starch broth	1	20.0	7.6	13.8	12.4	77.8	85.4	81.6	7.6
	2	9.8	20.2	15.0	10.4	90.8	92.8	91.8	2.0
	3	26.4	32.4	29.4	3.0	82.4	76.8	79.6	5.6
	7	5.7	11.5	8.6	2.9	82.3	89.5	85.9	7.2
	17	9.3	5.3	7.3	4.0	93.3	98.3	95.8	2.5

Conditions of experiments: enzyme activity, from 5.0 to 29.2 U/mL and from 13.1 to 127.5 U/mL for maltose and starch broth, respectively; protein concentration, from 2.4 to 2.6 mg/mL for maltose broth; HPMC concentration, 0.200% w/v; ammonium sulfate concentration, 25% saturation.

$\Delta = |\text{Exp 1} - \text{Exp 2}|$

Table 13 - Raw data of Figure 16

Concentration ratio	Purification ratio		
	Experiment 1	Experiment 2	Average
1	98.7	104.1	101.4
2	92.7	67.3	80.0
4	61.6	40.9	51.3
7	38.5	39.5	39.0
17	20.1	20.9	20.5

Table 14 - Raw data of Figure 18

Temperature (°C)	Initial activity distribution (in percentage)						Total activity recovered (Average)
	Precipitate			Supernatant			
	Exp 1	Exp 2	Average	Exp 1	Exp 2	Average	
21	0.8	1.3	1.1	98.4	92.9	95.7	96.8
35	3.2	2.2	2.7	84.3	86.2	85.3	88.0
50	3.4	1.9	2.7	84.0	90.8	87.4	90.1
65	11.7	11.4	11.6	74.0	81.0	77.5	89.1
75	25.1	23.8	24.5	58.7	64.3	61.5	86.0

Conditions of experiments: HPMC concentration, 0.200% w/v; ammonium sulfate concentration, 30% saturation.

Table 15 - Raw data of Figure 19

Ammonium sulfate concentration as percentage of saturation	Percentage partitioned						Percentage of HPMC precipitated					
	Temperature = 21°C			Temperature = 56°C			Temperature = 21°C			Temperature = 56 °C		
	Expl	Exp2	Average	Expl	Exp2	Average	Expl	Exp2	Average	Expl	Exp2	Average
1	–	–	–	3.2	–	–	–	–	–	39.3	28.1	33.7
2	–	–	–	2.0	3.5	2.8	–	–	–	67.9	67.8	67.9
3	–	–	–	5.2	6.1	5.7	–	–	–	80.7	81.5	81.1
4	–	–	–	6.6	4.8	5.7	–	–	–	87.2	89.0	88.1
5	0.0	0.0	0.0	2.6	5.8	4.2	0.0	0.0	0.0	91.6	92.2	91.9
10	0.0	0.0	0.0	9.1	9.8	14.5	13.2	13.1	13.2	96.8	96.4	96.6
15	1.0	10.0	5.5	36.0	38.1	37.1	91.6	91.5	91.6	97.4	97.9	97.7
20	29.0	29.4	29.2	86.7	89.8	88.3	97.4	96.3	96.9	98.4	98.5	98.5
25	85.0	88.3	86.7	93.5	95.2	94.4	97.8	97.9	97.9	97.8	98.9	98.4
30	94.8	94.9	94.9	–	–	–	98.5	98.5	98.5	–	–	–

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