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Purification and Properties of a Mannitol-Binding Protein from Pseudomonas Aeruginosa

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PURIFICATION AND PROPERTIES OF A MANNITOL-BINDING PROTEIN FROM *PSEUDOMONAS AERUGINOSA*

by

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PURIFICATION AND PROPERTIES OF A MANNITOL-BINDING PROTEIN FROM PSEUDOMONAS AERUGINOSA

Gregory V. Plano, M.S.

Western Michigan University, 1985

Mannitol-binding protein from Pseudomonas aeruginosa strain PAO has been purified 36-fold from magnesium chloride cold-shock supernatants. Sephadex G-100 gel filtration and carboxymethyl Sephadex ion exchange chromatography were utilized in the purification scheme. The position of the mannitol-binding protein band was identified on sodium dodecyl sulfate-polyacrylamide gels. The mannitol-binding protein has an apparent molecular weight of 45,000 as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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CHAPTER I

INTRODUCTION

Bacterial Transport

Bacteria have evolved several distinct mechanisms for the transport of solutes across cellular membranes. These mechanisms have been defined as facilitated diffusion, group translocation and active transport (Wilson & Smith, 1978). The most simple of these transport processes, facilitated diffusion, involves the translocation of solute molecules down a concentration gradient from one side of the cytoplasmic membrane to the other (Rose, 1976). The translocation step is mediated by a membrane transport protein which exhibits stereospecific specificity for its substrate (Dills, Apperson, Schmidt & Saier, 1980). Since the translocation step is not coupled to metabolic energy, the solute is not concentrated within the cell (Silhavy, Ferenci & Boos, 1978). This type of transport differs from passive diffusion in that a specific membrane transport protein acts to accelerate the rate of movement across the membrane; however, the final equilibrium obtained is not altered.

Active transport and group translocation resemble facilitated diffusion in that these mechanisms also require a membrane bound transport protein to catalyze the translocation step (Silhavy et al., 1978). They differ from facilitated diffusion in the one respect that the translocation step is coupled to metabolic energy, with the result

that a solute can be concentrated intracellularly (Rose, 1976). The mechanisms of coupling cellular metabolic energy to active transport and group translocation systems are quite diverse, providing another useful criterion for categorizing the different transport processes. Biochemical and genetic methods as well as the mode of action are other distinguishing characteristics which have been used in the classification of bacterial transport processes (Boos, 1974).

In bacteria, three distinct classes of concentrative transport systems have been defined. These are: (a) the group translocation systems, (b) the membrane bound transport systems, and (c) the binding protein transport systems (Wilson, 1978). Together, these transport systems enable bacteria to efficiently acquire and maintain high concentrations of solute within the cell.

In group translocation, the solute is chemically modified during transmembrane transport (Erni, Trachsel, Postma & Rosenbusch, 1982). The bacterial phosphotransferase system is a well-characterized group translocation transport system by which different sugars are transported and concomitantly phosphorylated (Saier, 1977). Sugar uptake by this system requires a number of soluble and membrane bound proteins. These proteins are responsible for the sequential transfer of phosphoryl groups from phosphoenolpyruvate to the transported sugars. The overall reaction requires magnesium ions, and the products formed are pyruvate and the translocated sugar phosphate (Dills, Apperson, Schmidt & Saier, 1980). Two soluble proteins, enzyme I and HPr, are nonspecific in regard to sugars. These proteins initiate phosphoryl group transfer from phosphoenolpyruvate

and subsequently pass the phosphoryl group to one of several specific enzymes II. These enzymes II are integral membrane proteins which recognize and translocate the sugars into the cell (Saier, 1977). In certain bacteria, a fourth component, the sugar specific soluble enzyme III, is required for glucose uptake (Erni, Trachsel, Postma & Rosenbusch, 1982). Since the translocated solute is chemically modified as it enters the cell, group translocation systems are not considered true active transport.

The true bacterial active transport systems are also coupled to metabolic energy allowing intracellular solute accumulation against a concentration gradient. However, in contrast to the group translocation systems, the translocated solute molecule found intracellularly is the same chemical species as removed from the extracellular medium. In bacteria, two types of active transport systems are known to operate. These are the membrane bound transport systems and the binding protein transport systems (Wilson & Smith, 1978).

The membrane bound transport systems utilize the proton motive force generated by cellular metabolism to transport ions and other molecules across the cytoplasmic membrane. Bacteria possess several independent mechanisms for generating proton electrochemical gradients. These include the membrane bound ATPase, the bacterial respiratory chain, and light sensitive electron transfer in photosynthetic bacteria (Dills, Apperson, Schmidt & Saier, 1980). The coupling of this energy source to the transport of a solute occurs so that an endergonic process (the accumulation of a nutrient) can be coupled to an exergonic process (protons moving down a concentration

gradient) (Dills et al., 1980). The translocation step involves a membrane bound protein specific for each transported substrate. The proton motive force generated by the cell can be used directly by a proton symport mechanism or indirectly by symport or antiport with some other ion (Wilson, 1978). The term symport refers to a protein catalyzing the transport of two substrates in the same direction. Antiport systems catalyze the translocation of two substrates in opposite directions (Silhavy, Ferenci & Boos, 1978). It has been demonstrated that the effect of energy coupling on the membrane bound lactose transport of Escherichia coli is to increase the K_m of substrate exit to above the K_m of entry. These kinetic changes are the result of coupling the lactose transport system to the proton motive force of the cell through proton symport (Ferenci, Boos, Schwartz & Szmelcman, 1977).

The bacterial binding protein transport systems are a group of specific high affinity active transport systems found exclusively in the gram negative bacteria (Wilson & Smith, 1978). Numerous binding proteins specific for a wide variety of sugars, amino acids, vitamins and ions have been reported (Harold, 1972). Generally, binding proteins refer to a group of soluble proteins consisting of a single polypeptide chain with molecular weights in the range of 22,000 to 45,000 (Silhavy, Ferenci & Boos, 1978). These proteins have no known enzymatic activity; however, they possess highly stereospecific active sites that reversibly bind substrates with K_D values in the range of 10^{-8} to 10^{-5} M (Silhavy et al., 1978). Binding proteins are located in the periplasmic space, a region between the outer

membrane and the cytoplasmic membrane in gram negative bacteria (Wilson, 1978). These proteins can be released from this region by osmotic shock treatment (Heppel, 1969; Heppel, Rosen, Friedberg, Berger & Weiner, 1972) or by spheroplast preparation (Neu & Heppel, 1965). In osmotic shock treatment, gram negative bacteria are first syspended in a hypertonic sucrose solution containing Ethylenediaminetetraacetate (EDTA). Next the cells are suddenly shifted to a cold solution of very low osmotic strength containing magnesium chloride (Heppel, 1969). Spheroplast formation can be facilitated by treatment of susceptible cells with EDTA and lysozyme (Neu & Heppel, 1965). These procedures selectively disrupt the outer membrane while leaving the cytoplasmic membrane intact. After treatment by either of these procedures, the released periplasmic proteins can be detected in the surrounding buffered medium. Modifications of the osmotic shock procedure have been developed for certain other gram negative bacteria, such as Pseudomonas aeruginosa (Cheng, Ingram & Costerton, 1970), which are not amenable to the above procedures. It is not clear whether the binding proteins exist free in the periplasmic space, or whether they are lightly attached to the outer surface of the cytoplasmic membrane (Rosen & Heppel, 1973). In any case, the binding proteins are not firmly attached to membrane, as is the case for the proteins associated with the membrane bound transport systems.

Several distinct lines of evidence have implicated the involvement of periplasmic binding proteins in transport processes. In all binding protein transport systems studied it has been found that the release of binding proteins by osmotic shock or spheroplast formation

is accompanied by a concurrent reduction in transport activity (Boos, 1974). This reduction is not due to general membrane damage since the membrane bound and group translocation transport systems remain unimpaired. The binding specificity of purified binding proteins generally reflects the specificity of the corresponding transport system (Boos, 1974). Mutations in binding proteins drastically diminish the ability of the transport system to accumulate solute and revertents require both binding and transport activities simultaneously. It has been shown that binding protein synthesis and transport activity are coregulated in several binding protein transport systems. More recently, the reconstitution of binding protein dependent transport has been achieved using a glutamine-binding protein point mutant as a source of transport vesicles (Hunt & Hong, 1983). These reconstitution experiments included well designed controls and were generally free of the ambiguities encountered in earlier attempts to restore transport by the addition of purified binding protein.

Although there is a wealth of evidence implicating binding proteins as essential components of binding protein transport systems, very little is known about their actual function in the transport mechanism. Kinetic studies of binding protein transport systems suggest that the binding protein is the rate limiting step in the translocation process. However, for all systems studied so far, the product of at least one gene in addition to the binding protein is required for functional transport (Wilson, 1978). These other gene products are believed to be integral membrane bound protein(s) which

interact with the binding protein and catalyze the translocation step. Direct bio-chemical evidence supporting the existence of these membrane bound components has proved difficult to obtain. Genetic studies, in contrast, have provided good evidence supporting the proposed existence of these additional components. Genetic analysis of the galactose (Wilson, 1978), maltose (Kellerman & Szmelcman, 1974), and glutamine (Hunt & Hong, 1983) systems of Escherichia coli as well as the histidine transport system of Salmonella typhimurium (Ames & Spudich, 1976) have confirmed the presence of these additional components. In the histidine transport system of Salmonella typhimurium, the histidine-binding protein (the J protein) possesses two independent active sites necessary for its function in transport; i.e., the site of substrate binding and a second site called the interaction site. The interaction site is believed to interact with the second (membrane) component of the histidine transport system, the P protein (Ames & Spudich, 1976). The P protein has recently been identified by two-dimensional polyacrylamide gel electrophoresis. In this same study, it was determined that the P protein is a membrane bound protein (Ames & Nikaido, 1978). Similar studies have been conducted with the glutamine-binding protein transport system of Escherichia coli. The glutamine-binding protein has also been reported to possess separate sites for substrate binding and for interaction with the membrane bound components of the transport system. Chemical modification of the sole tryptophan and histidine residues in the binding protein allowed for the selective alteration of the interaction site without affecting the glutamine binding properties of the protein.

The chemically modified binding proteins, possessing normal substrate binding activity, were unable to restore glutamine transport in vesicles (Hunt & Hong, 1981). As indicated above, reconstitution of the binding protein dependent active transport of glutamine in Escherichia coli vesicles has been successfully demonstrated (Hunt & Hong, 1981).

Energy coupling to the binding protein transport systems is less clearly understood than that of the membrane bound and group translocation transport systems. However, the following observations indicate an obligatory requirement of phosphate bond energy (ATP) or a closely related compound, for these transport systems: (a) ATPase negative mutants cannot use oxidative energy supplied by D-lactate to drive binding protein transport processes. The membrane bound transport systems, however, are active with these electron donors. (b) Uncouplers of oxidative phosphorylation, such as dinitrophenol, strongly inhibit membrane bound transport activity while binding protein transport systems are relatively resistant to these uncouplers when supplied with an ATP generating energy source. (c) ATPase negative mutants supplied with an ATP generating energy source also show binding protein mediated active transport. (d) Arsenic destroys binding protein transport activity; however, it has little effect on membrane bound transport systems (Berger & Heppel, 1974). Recently, evidence supporting acetyl phosphate as the immediate energy source for binding protein transport systems has been presented. A significant reduction in transport activity was observed when cellular levels of acetyl phosphate were lowered (Hong,

Hunt, Masters & Lieberman, 1979). However, under the experimental conditions employed, ATP synthesis was repressed in order to limit acetyl phosphate production. Thus, it is not possible to rule out ATP, or other closely related high energy compounds, on the basis of this report.

In addition to their function in active transport, certain periplasmic binding proteins also serve as receptor proteins for chemotaxis. Although not all chemotactic receptors are binding proteins, a number of sugar binding proteins appear to be necessary for chemotaxis toward their respective substrates. Even though binding proteins seem to serve as the initial recognition components in both transport and chemotaxis, other independent components for both systems are necessary for each specific activity (Oxender & Quay, 1976). Apparently, independent receptor sites on the cell membrane exist for both transport and chemotaxis.

Mannitol Transport and Catabolism in *Pseudomonas aeruginosa*

Escherichia coli and *Salmonella typhimurium* have been shown to accumulate mannitol by means of a phosphoenolpyruvate: hexose phosphotransferase system (Saier, 1977). However, no phosphenolpyryvate phosphotransferase system for mannitol or mannitol-1-phosphate dehydrogenase has been detected in *Pseudomonas aeruginosa* (Phibbs & Eagon, 1970). Instead, growth on mannitol as a sole carbon and energy source results in the induction of a nicotinamide adenine dinucleotide-linked mannitol dehydrogenase, an adenosine 5'-triphosphate-dependent fructokinase, and a shock sensitive mannitol specific active

transport system (Phibbs & Eagon; Phibbs, McCowen, Feary & Blevins, 1978). Recently, Pseudominas aerruginosa strain PAO has been shown to form a mannitol-binding protein, which is specifically induced in the presence of mannitol (molecular weight 37,000), had an isoelectric point (pI) of 8.3 and an apparent dissociation constant K_D of 2.3 μM for mannitol (Eisenberg, & Phibbs, 1982). In the Thesis I will describe the partial purification and characterization of mannitol-binding protein.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Pseudomonas aeruginosa strain PAO was cultured in a basal salts medium containing 50 mM Potassium phosphate (pH 7.0), 15 mM ammonium sulfate, 0.80 mM magnesium chloride and 2 μM ferrous sulfate. In addition the medium contained 30 mM lactate and 10 mM mannitol as energy sources and to induce mannitol binding protein. Two 25 ml cultures were inoculated from slants and incubated at 37°C on a reciprocating shaker. These cultures were used to inoculate 7 one liter volumes of the same medium contained in 2.8 liter Fernbach flasks. The cultures were incubated on a New Brunswick gyratory shaker at 37°C until mid logarithmic growth, usually 8 to 10 hours. Growth was followed by measuring turbidity with a Klett-Summerson photoelectric colorimeter (no. 66 filter). Cultures with optical densities between 140 and 180 Klett units were harvested by centrifugation at 10,000 rpm at 10°C rotor temperature for 10 minutes. Harvested cells were washed twice with 50 mM potassium phosphate buffer (pH 7.0) and were immediately subjected to magnesium chloride cold shock extraction.

Magnesium Chloride Cold-Shock Extraction

Extraction of periplasmic proteins was performed by the method

of Hoshino and Kageyama (1980) developed for use in making vesicles from Pseudomonas aeruginosa. Cells harvested at midexponential phase were suspended in 50 mM tris (hydroxymethyl) aminomethanehydrochloride (pH 7.4) containing 0.2 M magnesium chloride at a concentration of 0.2 g (wet weight) of cells per ml. This suspension was incubated for 10 minutes at 30°C with gentle agitation, then rapidly chilled in an ice bath for 15 minutes. The cold-shock procedure was repeated twice more. The cell suspension was centrifuged at 15,000 x g for 10 minutes to remove cells, followed by centrifugation at 27,000 x g for 20 minutes. The resulting supernatant was collected, dialyzed against CPA buffer (50 mM dibasic sodium phosphate, 50 mM citric acid, 0.02 percent sodium azide, pH 6.0) and stored at minus 70°C until used.

Binding Protein Assay

Mannitol-binding activity was determined by a nitrocellulose membrane filtration assay. Protein solutions, up to 200 μg total protein were mixed with 0.5 ml 2 μM D-(1-^{14}C)-mannitol (50 mCi/mmole) in CPA buffer (pH 6.0) and taken up in CPA buffer to 1 ml final volume. The solution was immediately filtered on a premoistened 24 mm filter (Millipore type HA, pore size 0.45 μm) and washed with 2 ml of CPA buffer. The filter was removed while still under vacuum, transferred to a liquid scintillation vial containing 10 ml of Aquasol (New England Nuclear L.S.C. cocktail), and counted in a Searle Isocap/300 spectrophotometer. One unit of MBP activity is defined as one picomole D-(1-^{14}C)-mannitol bound per filter.

Specific activity is expressed as units per mg protein.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to the methods of Weber and Osborn (1969). Samples solubilized in a solution of 1% SDS, 1% β-mercaptoethanol, a drop of glycerol and 5 μl of bromophenol blue were layered onto 10% polyacyrlamide gel columns (0.5 by 7.5 cm). Electrophoresis was performed in a Buchler Polyanalyst instrument at a constant current of 8 ma per gel. Gels were stained with Coomassie brilliant blue R250. The gels were removed from the staining solution, rinsed with distilled water, and placed in destaining solution (75 ml of acetic acid, 50 ml of methanol, and 875 ml of distilled water). Bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), Trypsinogen (24,000), Trypsin inhibitor (20,100) and α-Lactalbumin (14,200) were used as molecular weight standards. Relative mobility was plotted against the known molecular weights on semi-logarithmic paper. Estimations of molecular weight of unknown proteins were made from calibration curves using the above mentioned molecular weight standards.

Protein Determinations

Protein concentrations were determined by the method of Lowry (1951) using bovine serum albumin as a standard or by the methods of

Kalb and Bernlohr (1977).

Chemicals

D-(1-^{14}C)-mannitol (50 mCi/mmole) was purchased from New England Nuclear. All other compounds used were of analytical or reagent grade.

Preparation of Antisera

Antisera against both the MBP induced ion exchange fraction and the MBP noninduced ion exchange fraction were prepared separately in two adult white rabbits (details for the preparation of these fractions in Results section). Approximately 0.5 mg of each sample was emulsified in Freund's complete adjuvant and injected intradermally at multiple sites with a 26-gauge needle. After about three weeks, three booster injections containing 0.25 mg of antigen in Freund's incomplete adjuvant were injected intramuscularly at approximately 10 day intervals. Rabbits were bled from the central ear artery after each injection and precipitating antibody was checked qualitatively by double diffusion gel precipitation. After the third booster injection the MBP noninduced sensitized rabbit was bled via cardiac puncture. Approximately 10 ml of serum was recovered after removal of red blood cells.

CHAPTER III

RESULTS

Mannitol-Binding Protein Purification

Shock fluid was prepared from 14 liters of a culture of Pseudomonas aeruginosa strain PAO. This crude periplasmic extract (FI) containing 354 mg of protein in 100 ml was used for purification. The FI fraction was stored at minus 70°C until further purification steps were performed.

The crude periplasmic extract (FI) was subjected to Sephadex carboxymethyl-cellulose 50 (CM-C50) cation exchange column chromatography. The CM-C50 gel was prepared by equilibration with CPA buffer (pH 6.0). The swollen and equilibrated gel was degassed and poured at room temperature (ca. 23°C). The column (2.5 by 26 cm) was then moved to the refrigeration unit (4°C) and several bed volumes of cold degassed CPA buffer were passed through to ensure equilibration. After sample application, the column was washed with CPA buffer at a flow rate of 40 ml per hour followed by elution with 400 ml of a sodium chloride gradient (50 to 150 mM). The flow rate was maintained by using an LKB 2132 Microperpex peristaltic pump. Ultraviolet absorbance at 280 nm was followed using a Gilson model 111 LC detector in conjunction with a Fisher Recordall series 5000 chart recorder. The effluent was collected in 5 ml fractions using a Gilson FC-100 Microfractionator. A profile of the chromatography is shown in Figure 1.

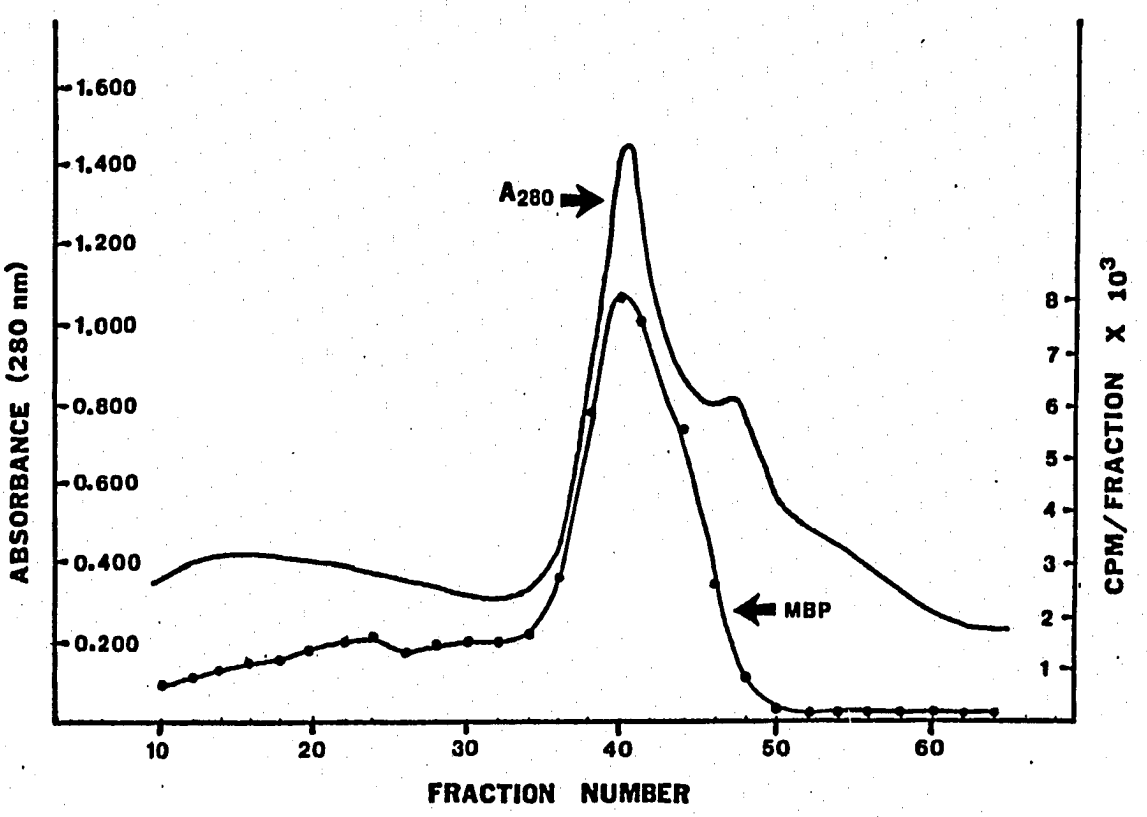

Figure 1. Elution profile of the crude periplasmic extract (FI) on a Sephadex CM-C50 column (2.5 x 26 cm). The sample solution (100 ml) was loaded onto the column, which had been equilibrated with CPA buffer (pH 6.0). After the column had been washed with 200 ml of the same buffer, mannitol-binding protein was eluted with a linear gradient of 50 to 150 mM NaCl in CPA buffer. The fraction volume and flow rate were 5 ml and 40 ml per hour, respectively. Symbols: (——) absorbance at 280 nm; (—•—) mannitol-binding activity.

The fractions were assayed for mannitol-binding activity using the membrane filter assay (0.1 ml assay sample). Fractions containing mannitol-binding activity (35-36) were pooled and dialyzed overnight against CPA buffer. This CM-C50 fraction was designated FII and contained 38.5 mg of protein with a specific activity of 910 units per mg protein. This represents a 7.7-fold purification over the FI fraction with an 84% recovery of MBP activity (Table 1).

The FII fraction was rechromatographed on a CM-C50 column (2.5 by 26 cm) equilibrated with CPA buffer (pH 7.0). The column was washed with CPA buffer at a flow rate of 30 ml per hour, followed by elution with 400 ml of a sodium chloride gradient (0 to 100 mM). The fractions with significant mannitol-binding activity were collected, concentrated using Millipore immersible CX-10 ultrafiltration units, and dialyzed against CPA buffer (pH 6.0). This fraction was designated FIII and contained 21.3 mg of protein with a specific activity of 1670 units per mg protein. A 14-fold purification was attained by these two chromatography steps (Table 1).

The FIII fraction was subjected to Sephadex G-75 gel filtration chromatography. The column (2.0 by 100 cm) was equilibrated with CPA buffer (pH 6.0) at a flow rate of 15 ml per hour. The sample volume and fraction volume were 4.0 ml (4.3 mg per ml) and 1.5 ml, respectively. The elution profile is presented in Figure 2. Fractions 28 to 44 were collected, concentrated by ultrafiltration (Amicon model M-3 ultrafiltration cell), and dialyzed against CPA buffer overnight.

Table 1

Purification of Mannitol-Binding Protein

Fraction	Total Activity (units)	Volume (ml)	Total Protein (mg)	Specific Activity (units/mg)	Protein (g/ml)	Yield (%)	Purification (-fold)
Crude Periplasmic Extract*	41,772	100	354.0	118	3,540	100.0	1.0
Sephadex CM-C50 Column (FII)	35,035	134	38.5	910	287.2	83.9	7.7
Sephadex CM-C50 Column (FIII)	35,570	106	21.3	1670	208.1	85.2	14.2
Sephadex G-75 Column (FIV)	19,152	24.8	11.4	1680	457.8	45.8	14.2
Sephadex CM-C50 Column (FV)	11,900	30.0	2.8	4250	92.5	28.5	36.0

*Extracted from 115 grams cell wet weight.

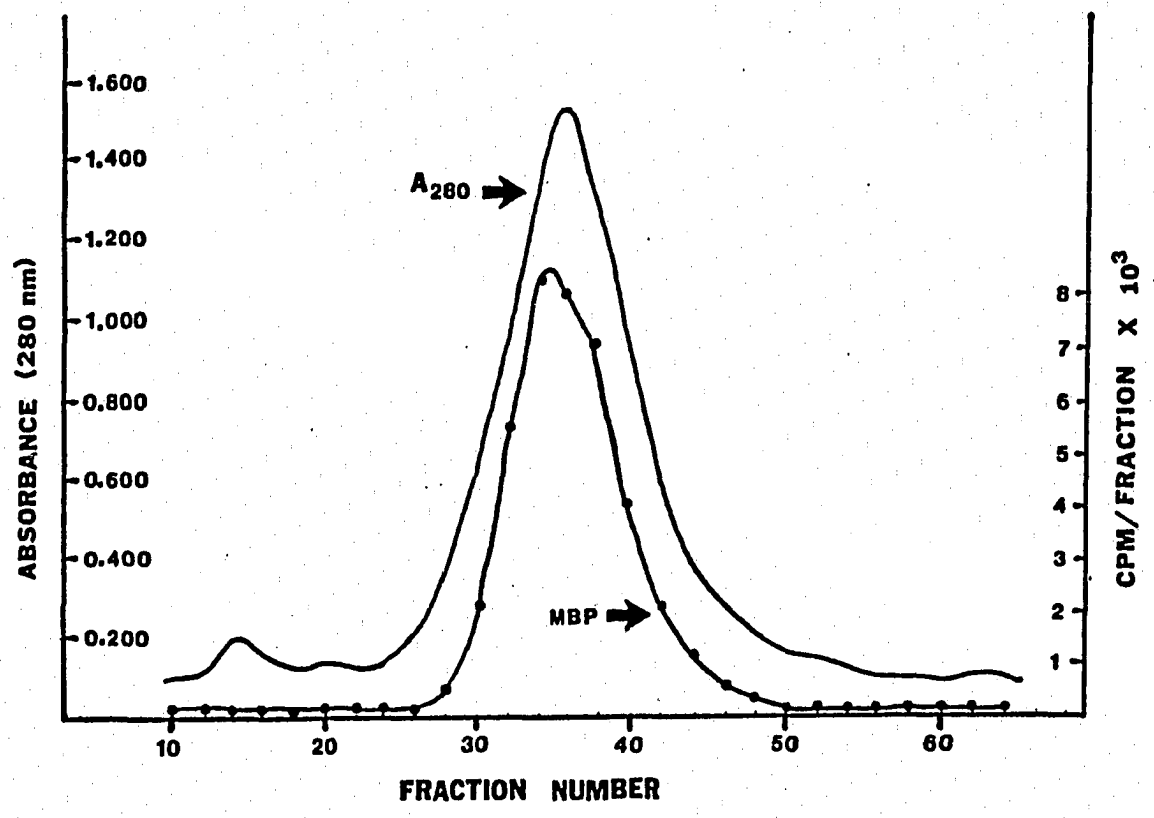

Figure 2. Elution profile of the FII fraction on a Sephadex G-75 column (2.0 x 100 cm). The sample volume and fraction volume were 6.0 ml and 3.5 ml, respectively. Flow rate 15 ml per hour. Symbols: (——) absorbance at 280 nm; (—●—) mannitol binding activity.

This was the G-75 fraction (FIV) and contained 11.4 mg of protein with a specific activity of 1680 units per mg protein.

The concentrated G-75 fraction (FIV) was loaded on a third CM-C50 column (1.5 by 23 cm) which had been equilibrated with CPA buffer (pH 5.5). After the column was washed with buffer, materials adsorbed to the gel were eluted with 200 ml of a sodium chloride gradient (0 to 120 mM). Elution profile is shown in Figure 3. Fractions 25 through 46 were collected, concentrated and dialyzed against CPA buffer (pH 6.0). This preparation was designated FV and had a specific activity of 4250 units per mg protein. The third CM-C50 fraction (FV) was purified 36-fold over the crude periplasmic extract with a recovery of 28.5% (Table 1). The purified sample (FV) showed one major band and several minor bands when analyzed by SDS-polyacrylamide gel electrophoresis.

Identification of the MBP Band on SDS-Polyacrylamide Gels

Shock fluid was prepared separately from two 7 liter cultures of Pseudomonas aeruginosa strain PAO. The first 7 liter culture was grown in a basal salts medium containing 30 mM lactate and 10 mM mannitol as energy sources and to induce mannitol-binding protein. The second 7 liter culture was grown in a basal salts medium containing 30 mM lactate as the sole carbon and energy source. No mannitol was present to induce mannitol specific enzymes or a mannitol transport system. Approximately 60 ml of crude periplasmic extract was obtained from each 7 liter culture. After dialysis against CPA

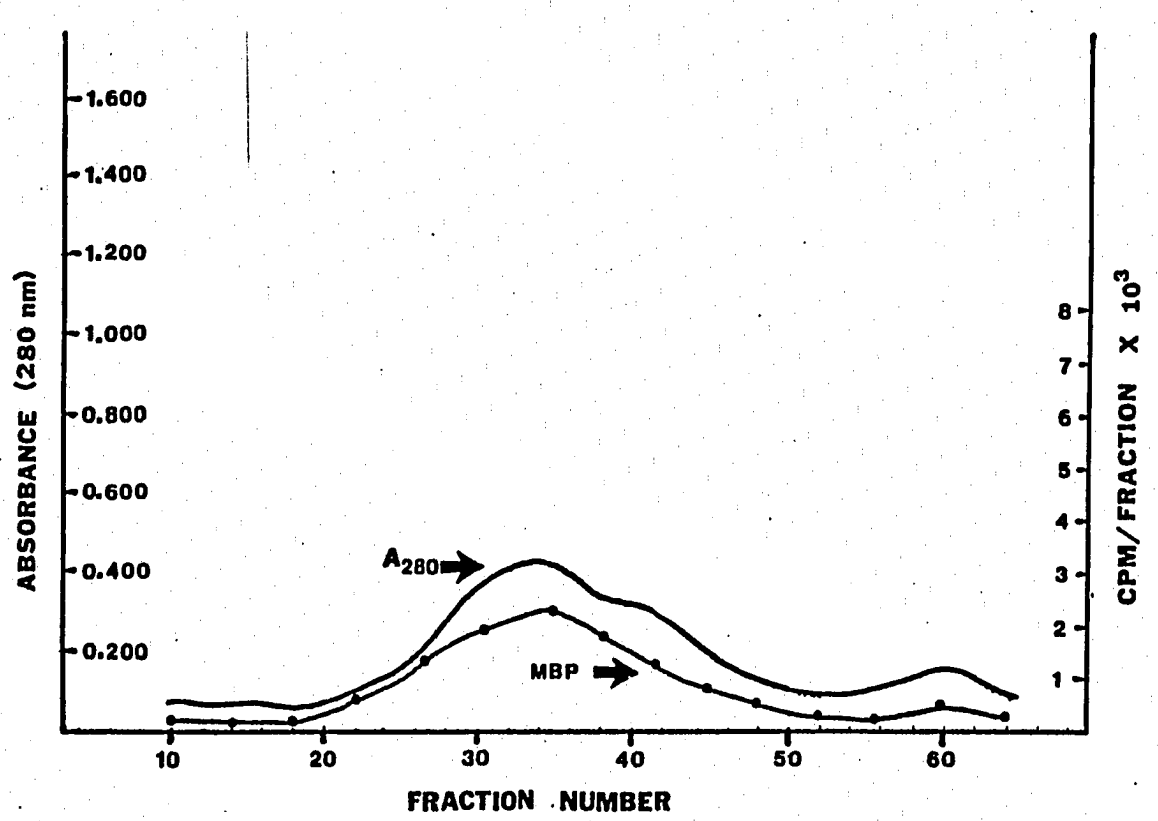

Figure 3. Elution profile of the G-75 fraction (FV) on a Sephadex CM-C50 column (1.5 x 23 cm). The sample solution (5 ml) was loaded onto the column, which had been equilibrated with CPA buffer (pH 5.5). After the column had been washed with 150 ml of the same buffer, the mannitol-binding protein was eluted with a linear gradient of 0 to 120 mM NaCl in CPA buffer. The fraction volume and flow rate were 3 ml and 30 ml per hour, respectively. Symbols: (——) absorbance at 280 nm; (—•—) mannitol-binding protein activity.

buffer, each extract was subject to Sephadex CM-C50 cation exchange column chromatography. Following sample application each column was washed with CPA buffer and eluted with 120 mM sodium chloride in CPA buffer. The major peak from each chromatography was combined and dialyzed against CPA buffer. The lactate and mannitol grown fraction (MBP induced) contained 31.2 mg of protein with a specific activity of 465 units per mg protein. The fraction prepared from those cells grown on lactate as the sole carbon and energy source (MBP not induced) contained 13.7 mg of protein with no measurable mannitol specific binding activity (Figure 4). The samples were dialyzed against distilled water and lyophilized overnight in preparation for SDS-polyacrylamide gel electrophoresis. For each sample, 25 to 125 μg of protein was mixed with 5 μl of tracking dye, one drop of glycerol, 5 μl of β-mercaptoethanol and 50 μl of SDS dialysis buffer. The protein solutions were applied to 10% poly acrylamide gels. Electrophoresis was performed at a constant current of 8 ma per gel. The gels were stained with Coomassie Brilliant Blue R250. Protein bands were detected by scanning the stained gels in a Gilford 2000 spectrophotometer at 500 nm. Gels were scanned at 0.5 cm per minute with a scanning slit aperture of 0.10 x 2.36 mm. The resulting SDS-polyacrylamide gel band patterns were compared to facilitate the identification of any mannitol specific protein bands. Comparisons between the MBP induced fraction and the MBP noninduced fraction revealed two separate protein bands seemingly present only in the MBP induced fraction. One band corresponding to a molecular weight of 45,000 and another corresponding to a molecular weight of 30,000.

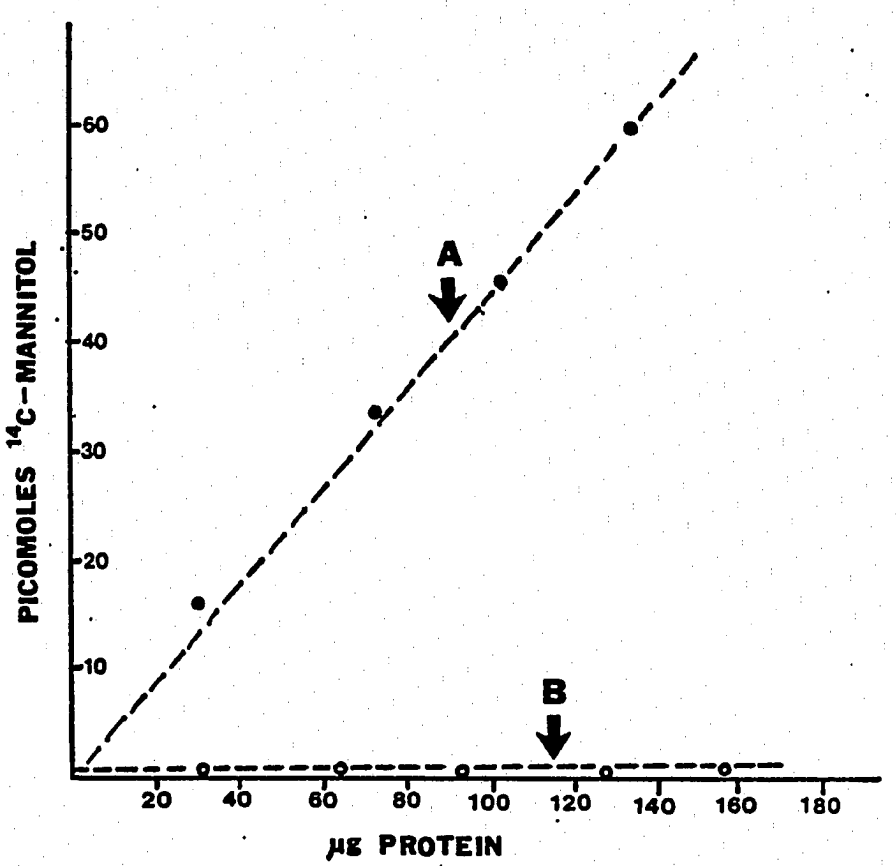

Figure 4. Binding assay of the lactate and mannitol grown ion exchange fraction (A) and the lactate grown ion exchange fraction (B). Specific activity of (A) was 465 units per mg protein. The lactate grown fraction (B) had no measurable mannitol binding activity.

Positive identification of the MBP band was not possible at this point.

In order to clarify the location and molecular weight of MBP on SDS-polyacrylamide gels a modified cold-shock procedure (Wolff, 1984) was used to decrease the number and concentration of contaminating proteins in both the MBP induced and noninduced fractions. The modified cold-shock procedure was identical to the magnesium chloride cold-shock method employed originally, except that the extraction buffer contained no magnesium chloride. The deletion of magnesium chloride from the extraction buffer was reported to dramatically reduce the total protein released; however, the MBP specific activity of that protein released was found to be greater than that of the magnesium chloride shock fluids (Wolff, 1984). Using the modified cold-shock procedure a 7 liter culture of Pseudomonas aeruginosa grown on 30 mM lactate and 10 mM mannitol yielded 30.5 ml (5.3 mg per ml) of shock fluid with a specific activity of 134 units per mg protein. The specific activity of this shock fluid was approximately 20 units per mg higher than that of the magnesium chloride shock fluids. Approximately 162 mg of total protein was obtained by using the modified shock procedure compared to 180 mg of total protein obtained by using the magnesium chloride shock procedure. A second 7 liter culture containing 30 mM lactate as the sole source of carbon and energy yielded 157 mg of total protein with no measurable mannitol specific binding activity. The cold-shock fluid from each fraction was subjected to Sephadex CM-C50 cation exchange column chromatography. Bound proteins were eluted with 200 ml of a sodium chloride gradient (50 to 150 mM). The lactate grown ion exchange

fraction (MBP not induced) contained 1.5 mg of protein. The lactate and mannitol grown ion exchange fraction (MBP induced) contained 7.5 mg of protein with a specific activity of 1150 units per mg protein.

Lyophilized samples from each of the two fractions were run on SDS-polyacrylamide gels. The gels were scanned and compared to identify any mannitol specific protein bands (Figure 5). Band #12 with an approximate molecular weight equal to 45,000 was found exclusively in the MBP induced fraction. All other protein bands present in the MBP induced fraction were found to correlate with a parallel protein band of similar mobility in the MBP noninduced fraction. Thus, protein band #12 was tentatively identified as MBP..

Figure 6 shows SDS-polyacrylamide gel scans of MBP preparations at different levels of purification. In order to confirm band #12 as being MBP a plot of specific activity vs fraction total area of detected bands was made for each band remaining in the FV fraction (specific activity 4250 units per mg protein). The fraction total area of detected bands was determined by the following method. The area under a single gel scan peak was found by first drawing tangent lines through the sides of the peak and then equating the peak area to the area of the triangle thus constructed, area = 1/2 (base width) X (peak height). The addition of all peak areas for each preparation studied was equal to the total area of that particular MBP preparation. Thus, the fraction total area for each peak in a sample is the area of that particular peak divided by the total area under all peaks in the sample. Figure 7 shows the results of this plot. Correlation coefficients were calculated for each line plotted using the equation:

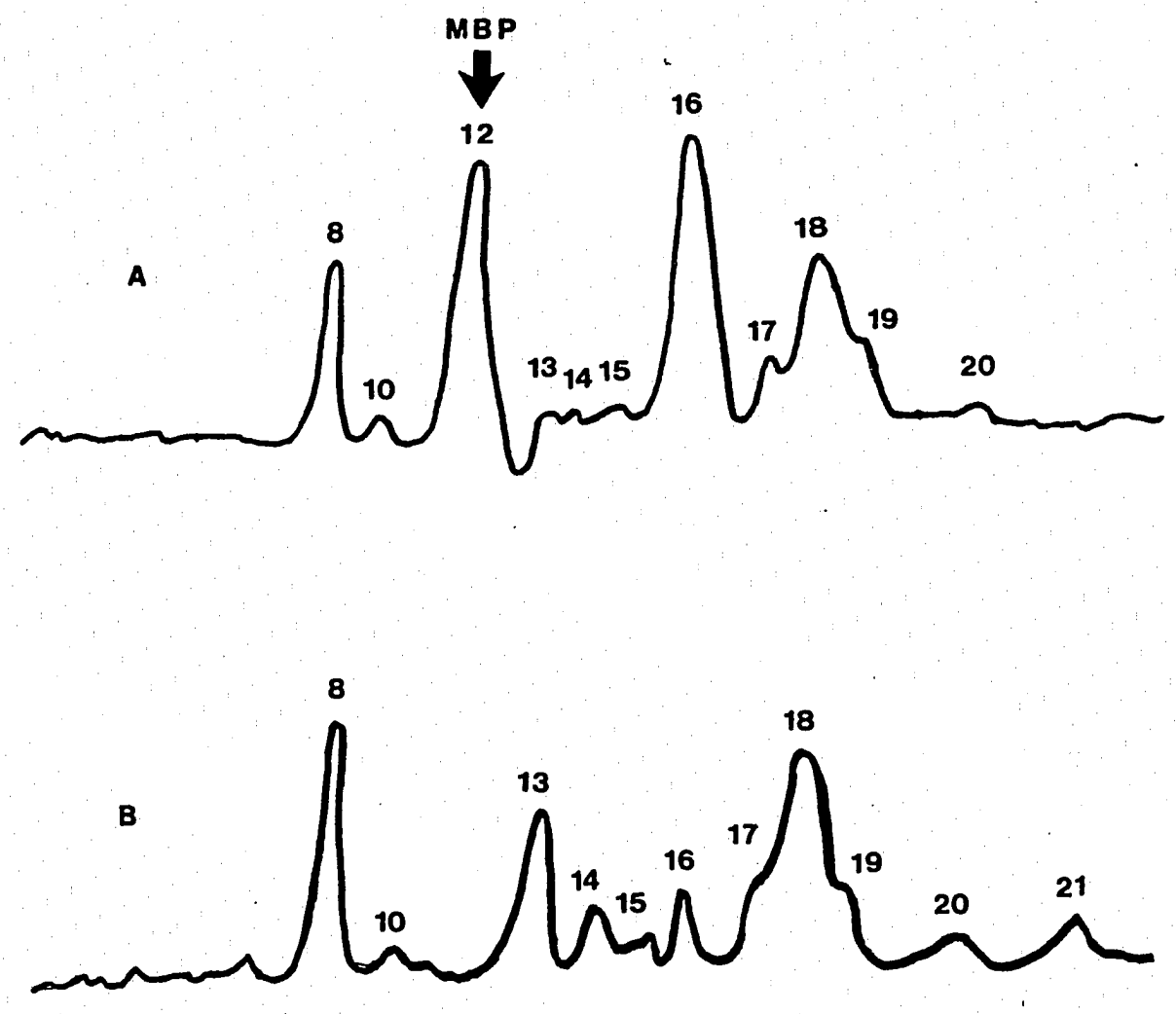

Figure 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the lactate and mannitol grown fraction (A) and the lactate grown fraction (B). Band #12 was identified as the mannitol-binding protein (MBP). MBP was found exclusively in the lactate and mannitol grown fraction (A).

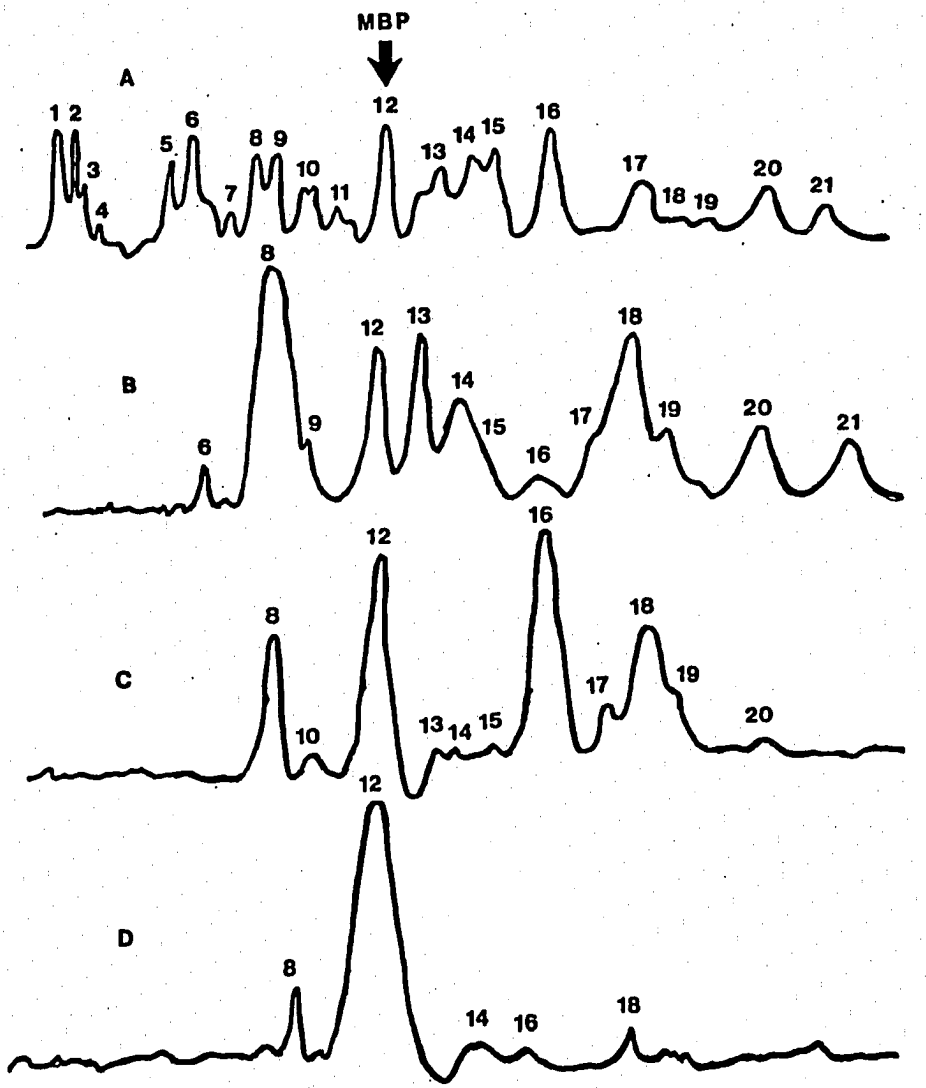

Figure 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of partially purified mannitol-binding protein preparations at different levels of purification. (A) crude periplasmic extract (specific activity 92 units per mg protein); (B) Sephadex CM-C50 fraction (specific activity 465 units per mg protein); (C) Sephadex CM-C50 fraction (specific activity 1150 units per mg protein); (D) FV fraction (specific activity 4250 units per mg protein).

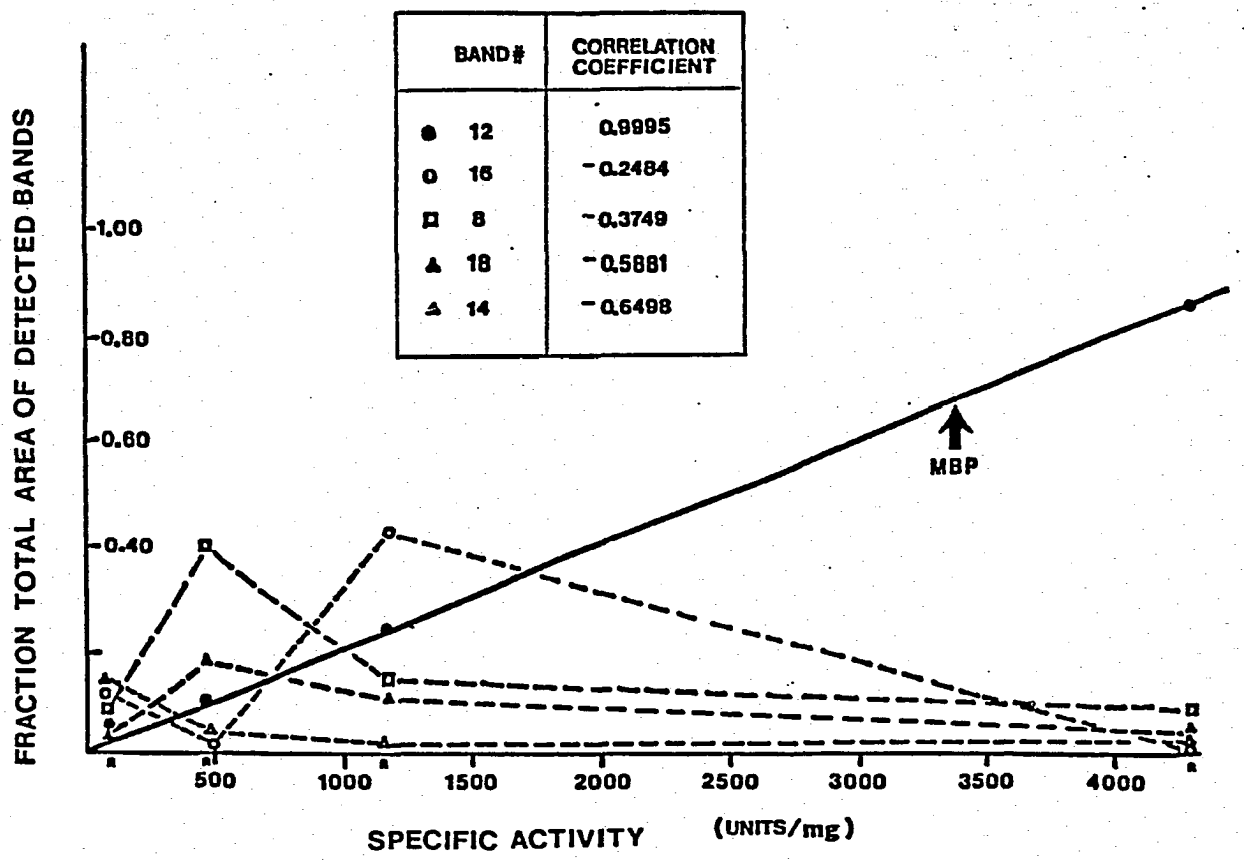

Figure 7. Plot of the fraction total area of detected bands vs specific activity for all bands detected in the FV fraction. Symbols: (—●—) Band #12 (MBP); (-o-) Band #16; (-□-) Band #8; (-▲-) Band #18; (-△-) Band #14.

$$r = \frac{xy\Sigma - \frac{\Sigma x \, \Sigma y}{n}}{\sqrt{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right]}}$$

Band #12, with a correlation coefficient of 0.9995, clearly, is the only band which varies directly with MBP specific activity.

Molecular Weight of MBP

The molecular weight of MBP was estimated by SDS-polyacrylamide gel electrophoresis. On 10% polyacrylamide gels MBP migrated with a mobility of 0.388 with respect to bromophenol blue. By comparisons with appropriate standards in two separate experiments, a molecular weight of 45,000 was determined (Figure 8).

Stability of MBP

Crude shock fluid or partially purified binding protein preparations were stable at minus 70°C for several months without loss of binding activity. Similar to other binding proteins, MBP had appreciable thermal stability ($t_{\frac{1}{2}}$ at 60°C in CPA buffer, 4 minutes). However, at 100°C all binding activity was lost within one minute.

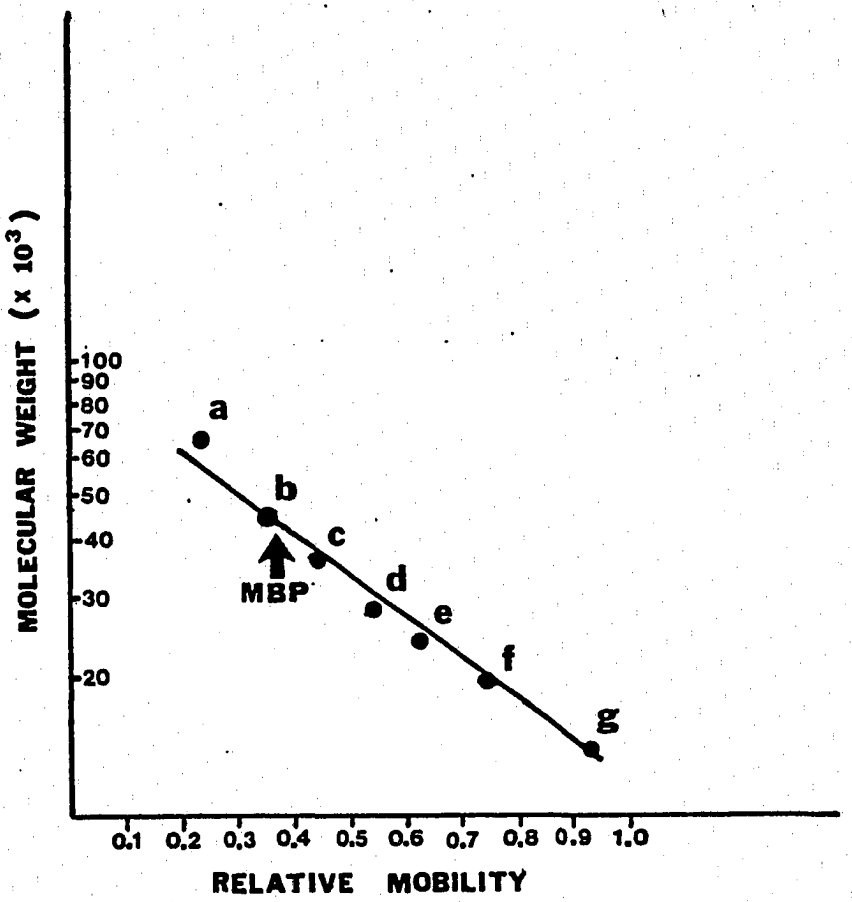

Figure 8. Molecular weight determination of the mannitol-binding protein. Relative mobility signifies the relative migration of a protein on SDS-polyacrylamide gel to that of bromophenol blue. Molecular weight standards: (a) bovine serum albumin; (b) egg albumin; (c) glyceraldehyde-3-phosphate dehydrogenase; (d) carbonic anhydrase; (e) trypsinogen; (f) trypsin inhibitor and (g) -Lactalbumin.

CHAPTER IV

DISCUSSION

A mannitol-binding protein was purified 36-fold from cold-shock supernatants extracted from 115 grams (wet weight) of logarithmic phase Pseudomonas aeruginosa. Cation exchange and gel filtration chromatography were employed in the purification scheme. Two steps utilized in the purification are worthy of comment. First, it is clear that the advantage of magnesium chloride cold-shock supernatants over disrupted cell suspensions cannot be overemphasized. The magnesium chloride cold-shock procedure enables the efficient and highly selective release of MBP and other periplasmic proteins without the release of cytoplasmic proteins (Wolff, 1984). Secondly, the initial CM-C50 cation exchange chromatography step removed approximately 90% of the total protein in the crude periplasmic extract (FI) with a recovery of over 80% of the MBP activity (Table 1). The overall effectiveness of this procedure is due largely to the alkaline pI (8.3) of MBP, which contrasts to the lower isoelectric points of the majority of proteins in the crude periplasmic extract. The alkaline pI of MBP gives the protein an overall net positive charge at the pH of the chromatographic buffer (pH 6.0), while the majority of other proteins retain a net negative or neutral charge. Together the above mentioned procedures provide an efficient and reproducible means of obtaining a partially purified sample suitable for use in identifying the MBP band on SDS-polyacrylamide gels.

The Sephadex G-75 gel filtration chromatography step was of little value in the overall purification scheme. SDS-polyacrylamide gel scans of the G-75 fraction (FIV) revealed the removal of one light protein band (mw~72,000) from the Sephadex CM-C50 (FIII) fraction. The large loss of MBP activity that resulted from this step was due largely to the loss of protein during the concentration of the FIII fraction. A millipore immersible CX-10 ultrafiltration unit was used to concentrate 106 ml of the FIII fraction down to a 4 ml fraction which was applied to the G-75 chromatography column.

MBP was identified on SDS-polyacrylamide gels by comparing partially purified samples from lactate grown cells (no measurable MBP activity) against a partially purified MBP preparation. A single protein band (band #12), corresponding to a molecular weight of 45,000 was found exclusively in those fractions with MBP activity (Figure 5). A plot of MBP specific activity vs fraction total area of detected bands indicated a direct linear relationship for band #12 (correlation coefficient = 0.9995). All other detectable bands in the FV fraction had negative correlation coefficients when plotted as above (Figure 7). On the basis of the prementioned evidence, band #12 was identified as MBP.

An estimation of the molecular weight of MBP was determined by SDS-polyacrlamide gel electrophoresis. The molecular weight of MBP was estimated by comparing its electrophoretic mobility with known protein markers. An approximately linear relationship is obtained if the logarithms of the molecular weights of standard polypeptide chains are plotted against their respective electrophoretic mobilities.

Determination of the molecular weight of MBP by SDS-polyacrylamide gel electrophoresis yielded a value of 45,000. In contrast, the molecular weight of MBP determined previously by calibrated G-100 gel filtration was found to be ~37,000 (Eisenberg & Phibbs, 1982). This discrepancy is unresolved, although differences in molecular weight as determined by gel filtration and SDS-electrophoresis for several binding proteins possessing alkaline pI's have been noted previously (Kuzuya, Bromwell & Guroff, 1971; Ahlem, Huisman, Neslund & Dahms, 1981). In each case the molecular weight as determined by calibrated gel filtration was lower than the molecular weight as determined by SDS-polyacrylamide gel electrophoresis and other methods. It seems possible that the binding protein adheres or interacts with the Sephadex to some extent. This would retard its progress through the column and lead to an incorrect and lower apparent molecular weight. In order to better resolve the discrepancy in the molecular weight estimates of MBP, alternative methods of molecular weight determination will be necessary.

A complete and accurate characterization of MBP cannot be accomplished until purification to homogeneity is achieved. Attempts to purify MBP have centered around chromatographic and electrophoretic techniques. These efforts have met with only limited success. Current attempts to purify MBP involve the use of immunological techniques. Antisera against the partially purified lactate grown extract and antisera against the partially purified MBP preparations have been prepared in two adult white rabbits. The high specificity of antibodies provides a unique approach to the purification problem.

Two separate approaches utilizing the prepared antisera to construct immunoaffinity columns are being considered. First, the IgG fraction from the antisera against the partially purified lactate grown extract could be immobilized on a solid matrix. After attachment of the antibodies to the column matrix a partially purified MBP fraction would be run through the column. SDS-polyacrylamide gels indicate MBP is the only mannitol specific protein found in the partially purified ion exchange fractions. Therefore, all contaminating proteins would be absorbed out by the immobilized antibodies. MBP would pass through the column and be collected in a purified form. Possible problems that may be encountered include antibody cross reactions with MBP and lack of sufficient antibody titer or selectivity to absorb out all contaminants.

In the second approach the protein (Ag) from the partially purified lactate grown extract would be attached to an affinity matrix. A suitable gel matrix should provide spacer groups to provide proper orientation of the bound antigen. The affinity column with coupled antigen would be used to selectively absorb out the majority of the IgG fraction from the antisera against partially purified MBP fractions. The elutent from the column would contain antibodies directed against MBP. These antibodies could then in turn be used to construct an immunomatrix which would specifically absorb out MBP from crude protein extracts. Antigen-antibody cross reactions would once again be a potential problem.

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