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Further Studies on Purification of a Mannitol Binding Protein: Use of pH Gradients for Elution from Cation Exchange Resin

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FURTHER STUDIES ON PURIFICATION OF A MANNITOL BINDING PROTEIN: USE OF pH GRADIENTS FOR ELUTION FROM CATION EXCHANGE RESIN

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FURTHER STUDIES ON PURIFICATION OF A MANNITOL BINDING PROTEIN: USE OF pH GRADIENTS FOR ELUTION FROM CATION EXCHANGE RESIN

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Studies on the purification of a mannitol binding protein (MBP) from Pseudomonas aeruginosa PAO were performed. Utilizing the alkaline isoelectric point (pI 8.3) of MBP, pH gradients were used to elute MBP from carboxymethylcellulose cation exchange resins. Purification of MBP was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Only one protein band was seen on SDS-PAGE from MBP fractions eluted at pH 7.8 and 8.2. Although pH gradients appear to give good purification from cation exchange resins, these procedures lead to loss of MBP activity.

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I would like to dedicate this thesis to my parents and sister whose continuing support has been greatly appreciated.

I would also like to dedicate this thesis in the loving memory of my grandmother and also my great-aunt who both instilled in me the love of learning.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF TABLES v

LIST OF FIGURES vi

INTRODUCTION 1

Bacterial Transport Systems 1

Binding Proteins 4

Mannitol Binding Protein in Pseudomonas aeruginosa 5

MATERIALS AND METHODS 8

Maintenance of Bacterial Strains 8

Growth Conditions of Bacterial Strains 8

Starter Culture 8

Harvest Cultures 9

Harvesting and Cold-Shock Extraction 9

Harvesting 9

Cold-Shock Extraction 10

Buffers 10

Concentration of Samples 11

Protein Determination 11

Mannitol Binding Protein Assay 12

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 12

Pouring the Gel 12

Electrophoresis Procedure 14

Chemicals 14

Table of Contents--Continued

RESULTS 15

Dialysis of MBP Against CPA, pH 8.3 15

Sodium Chloride Elution of MBP 18

pH Gradient Elution of MBP 19

pH Gradient 6.0 to 9.0 19

pH Gradient 7.5 to 9.5 24

pH Gradient 7.5 to 8.5 25

SID-Polyacrylamide Gel Electrophoresis 28

DISCUSSION 36

BIBLIOGRAPHY 40

LIST OF TABLES

1. Effect of Dialysis Against CPA, pH 8.3 on MBP Activity . 16

2. Effect of Dialysis Against 1 M Magnesium Chloride and Against CPA, pH 8.3 on MBP Activity 17

3. Lack of Reversible Binding Activity of MBP 18

4. Loss of Binding Activity in MBP 25

5. Summary of Purification Scheme 38

LIST OF FIGURES

1. Sodium Chloride Elution of Crude Periplasmic Extract (FI) on a Sephadex CMC-50 Cation Exchange Column (2.5 by 23 cm). 20

2. pH Gradient (6.0 to 9.0) Elution of Partially Purified MBP (FIIB) on a Sephadex CMC-50 Column (2.5 by 23 cm) . . . 22

3. pH Gradient (7.5 to 9.5) Elution of Partially Purified MBP (FIIIB) on a Sephadex CMC-50 Column (2.5 by 23 cm) . . 26

4. pH Gradient (7.5 to 8.5) Elution on a FiiiB Fraction of MBP on a Sephadex CMC-50 Column (2.5 by 20 cm) 29

5. SDS-PAGE on Crude Periplasmic Extract (FI) and Partially Purified Fractions 31

6. SDS-PAGE on Crude Periplasmic Extracts and Partially Purified Fractions of MBP 34

INTRODUCTION

Bacterial Transport Systems

Bacteria take up nutrients from the environment by five general mechanisms of transport: passive diffusion, facilitated diffusion, membrane-bound transport systems, group translocation, and binding protein mediated systems (Hengge & Boos, 1983; Kaback, 1974). Passive diffusion is a non-energy requiring process. Because of the hydrophobic nature of the cytoplasmic membrane, only water and lipid-soluble substrates are able to freely pass across the membrane. This passage is a result of the random movement of substrate molecules and does not involve specific interaction with any molecular sybstances in the membrane. The random movement of solute molecules is dependent on external and internal concentrations of the solute. Therefore, the solute molecules will enter the bacterial cell only when their concentration is higher outside of the cell than inside (Dawes & Sutherland, 1976; Kaback, 1974).

Facilitated diffusion is also a non-energy requiring process. In this transport system, the solute molecule is transported down a concentration gradient from one side of the membrane to the other (Saier, 1971). The solute molecule reversibly binds to a specific carrier protein molecule located in the membrane. The solute-carrier complex travels across the cytoplasmic membrane of the bacterial cell effectively binding and releasing solute molecules (Kaback, 1974).

The remaining transport systems all require cellular energy.

These three systems may all be thought of as three distinct classes of active transport, although one of them (group translocation) does not fit the classical definition of active transport (Hengge & Boos, 1983; Kaback, 1974; Wilson, 1978).

The first class of active transport is the membrane-bound transport system. In these systems, substrate molecules are transported against a concentration gradient with no change in the transported substrate molecules (Wilson, 1978). All the proteins required by these systems are firmly bound to the cytoplasmic membrane. The membrane-bound transport systems make use of the proton motive force across the membrane to drive active transport of the substrate molecules. The proton motive force is used either directly by a proton symport mechanism or indirectly by a symport or antiport of another ion (Wilson, 1978). The sodium ion symport is the most commonly used indirect mechanism.

About 40% of all the transport systems found in Escherichia coli belong to the membrane-bound transport system with the lactose transport system being the best understood (Hengge & Boos, 1983; Wilson, 1978). Important features of this system are: (a) an ion-substrate symport is involved in the coupling of substrate molecule translocation to the proton electrochemical gradient; (b) a symmetry of entry and exit of substrate; and (c) only one protein, possibly a dimer of identical subunits, is directly involved in the actual substrate transporting mechanism.

Group translocation is the second class of active transport systems, although, because it involves chemical modification of the

substrate, it does not fit the classical definition of active transport. Group translocation couples chemical modification of the substrate with transport across the membrane (Harold, 1972; Hengge & Boos, 1983; Kaback, 1974; Saier, 1977; Wilson, 1978). The most extensively studied group translocation process is the phosphotransferase system (PTS) first described in Escherichia coli. The PTS system consists of three soluble, cytoplasmic components (HPr, enzyme I, factor III) and various sugar-specific, membrane-bound components (enzyme II's). Each enzyme II is specific for a particular sugar (i.e., glucose, mannose, fructose, etc.). Transport of the sugar molecule is coupled to PTS-catalyzed phosphorylation. Thus, the solute molecule is taken up from the medium in its sugar form and enters the cytoplasmic space in its sugar phosphate form. It is the energy from the phosphorylation of the solute which is used by the bacterial cell to accumulate PTS substrate molecules.

Binding-protein mediated systems are the third class of active transport and thus far have been observed only in the periplasmic space of Gram-negative bacteria (Hengge & Boos, 1983). Adenosine triphosphate (ATP), or a related high energy metabolite, is required for transport of the substrate by the binding-protein mediated systems (Berger & Heppel, 1974; Wilson, 1978). These systems will be discussed more fully in the next section, but briefly, their important features are: (a) no substrate modification during translocation; (b) establishment of a concentration gradient ratio in excess of 10^4 (inside:outside); (c) unidirectional; and (d) expenditure of energy is directly coupled to transport. In addition to acting as the major

recognition sites of multicomponent transport systems, some binding proteins also act as specific chemoreceptor components of the tactic response (Hengge & Boos, 1983).

Binding Proteins

Gram-negative bacteria are bound by two distinct membranes. These are the cytoplasmic, or inner, membrane and the outer membrane (Brass, Higgins, Foley, Rugman, Birmingham, & Garland, 1986; Costerton, Ingram, & Cheng, 1974; Meadow, 1975). Between these two membranes is a region identified as the periplasmic space (Brass et al., 1986; Meadow, 1975). The volume of the periplasmic space has been estimated to be from 1 to 7% of the total volume of the cell (Brass et al., 1986).

Periplasmic proteins have been defined as those proteins which can be selectively released by mild osmotic shock and without contamination of cytoplasmic proteins (Ames & Higgins, 1983; Ames, Prody, & Kustu, 1984). Approximately 10-15% of the total bacterial cell protein has been reported to be present in the periplasmic space (Ames & Higgins, 1984). Brass et al. (1986) have identified three major classes of periplasmic proteins based on their function. One class plays a protective role, modifying such toxic compounds as heavy metals and antibiotics. Another class is comprised of hydrolytic enzymes which alter impermeable solutes to a form which can be transported into the cell. The third class of periplasmic proteins is the substrate-binding proteins which are the major recognition sites of multi-component transport systems and which are the main object of this discussion and study.

Substrate-binding proteins are water-soluble with a high affinity (K_D 0.1-1.0 μM) for their specific substrates (Brass et al., 1986; Hengge & Boos, 1983). Substrates for binding protein mediated transport include sugars, amino acids, peptides, vitamins, carboxylic acids, and inorganic ions (Brass et al., 1986; Hengge & Boos, 1983; Wilson, 1978). Each substrate has its own specific binding protein. Binding proteins range in molecular weight from 20,000 to 40,000 and have at least two separately functional binding sites (Boos, 1974). One site binds the specific substrate to be transported across the cytoplasmic membrane and the other site interacts with the membrane-bound component(s) of the transport system (Hengge & Boos, 1983). Binding protein mediated transport systems have been most extensively studied in Escherichia coli and Salmonella typhimurium; a wide variety of other Gram-negative bacteria have also been reported to contain these binding protein mediated transport systems (Boos, 1974).

Mannitol Binding Protein in Pseudomonas aeruginosa

The mannitol transport system in Psuedomonas aeruginosa was first studied by Phibbs and Eagon in 1970. In 1982, a mannitol binding protein (MBP) was reported to have been partially purified and characterized by Eisenberg and Phibbs. Via preparative isoelectric focusing, an isoelectric point (pI) of 8.3 was reported. This alkaline pI is in contrast to the acidic pI's reported for the majority of periplasmic binding proteins isolated from bacteria. An approximate molecular weight of 37,000 was reported using Sephadex

G-100 exclusion chromatography. This reported molecular weight is consistent with molecular weights reported for other bacterial periplasmic binding proteins (Boos, 1974). MBP was found to have a dissociation constant (K_D) of 2.3 μM mannitol, again consistent with dissociation constant data reported for other bacterial periplasmic proteins (Brass et al., 1986; Hengge & Boos, 1983). The substrate specificity of partially purified MBP was examined in competitive binding studies. Neither glucose nor glycerol caused detectable inhibition of mannitol binding. Mannose, fructose, and sucrose caused 15 to 25% inhibition of mannitol binding when present in concentrations ten times higher than mannitol.

Wolff (1984) investigated the localization of MBP and confirmed that it is a periplasmic protein. Analysis of mutants deficient in mannitol uptake and MBP activity also resulted in strong evidence implicating MBP as a required component of the mannitol transport system in Pseudomonas aeruginose. Arsenate was reported to inhibit the uptake of mannitol almost as well as azide, indicating that the transport of mannitol requires ATP. This finding agrees well with other binding protein mediated transport systems and their requirements for ATP as an energy source (Berger & Heppel, 1974; Wilson, 1978). Wolff (1984) also isolated and characterized a catabolite repression resistant (crr) mutant for mannitol uptake. This was the first time that a crr mutant has been identified for Pseudomonas.

An attempt to purify MBP to homogeneity was undertaken by Plano in 1984. By using a series of sodium chloride gradient elutions on cation exchange columns and gel filtration chromatography, Plano was

able to purify MBP 36-fold. Analysis of the partially purified MBP by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with coomassive blue, revealed one major band and four lesser bands. Comparison of samples from a non-mannitol induced fraction and a mannitol-induced fraction by SDS-PAGE revealed that the major band only appeared in the mannitol-induced fraction while the lesser bands appeared in both fractions, indicating that the major band was MBP. Further investigation of the suspected MBP electrophoretic band resulted in a reported molecular weight of 45,000 for MBP.

The present study is an extension of the previous work by Wolff (1984) and Plano (1984) in efforts to purify MBP to homogeneity. This study will explore the possibility of resolving MBP from other alkaline pI proteins by using pH gradients for elution of MBP from cation exchange columns.

MATERIALS AND METHODS

Maintenance of Bacterial Strains

Pseudomonas aeruginosa strain PAO was maintained on nutrient agar slants. Stock slants were stored at room temperature and transferred once a month. Working slants were stormed at room temperature and transferred at least once a week.

Growth Conditions of Bacterial Strains

Starter Culture

In a 50-milliliter culture flask, 25 ml of basal salts medium (BSM) was added. BSM contained 50 mM dibasic potassium phosphate ($K_2HPO_4 \cdot 3H_2O$), 15 mM ammonium sulfate [$(NH_4)_2\ SO_4$], 0.8 mM magnesium chloride ($McCl_2 \cdot 6H_2O$), and 2 μM ferrous sulfate ($FeSO_4 \cdot 7H_2O$) (Hyleman & Phibbs, 1972). Carbon and energy sources added were lactate (30 mM) and mannitol (10 mM). Each was sterilized separately. When the induction of MBP synthesis was not desired, lactate (30 mM) was the only carbon and energy source added. The starter culture flask was incubated at 37°C for one hour in a New Brunswick rotatory shaker (150 rpm) to ensure equilibration of temperature and aeration. After equilibration, one ml of a bacterial suspension was added and the starter culture was incubated at 37°C for 10-12 hours in the rotatory shaker at 150 rpm.

Harvest Cultures

Five 2.80 liter Fernbach flasks, each containing 1,000 ml BSM (pH 7.0), 30 ml lactate (1 M), and 10 ml mannitol (1 M) were incubated for one hour at 37°C in the New Brunswick rotatory shaker (150 rpm) to ensure equilibration as described above. An appropriate volume of bacteria from the starter culture was added to each Fernbach flask to permit serial growth. Approximately 10 hours after inoculation, the first flask (with the heaviest inoculum) was harvested when a reading of 140-180 Klett units (#66 filter) was obtained. The other flasks were harvested at a Klett reading of 140 to 180 (and in order of the volume of inoculum). If the induction of MBP was not desired, the mannitol solution was not added to the flasks.

Harvesting and Cold-Shock Extraction

Harvesting

One liter of bacterial culture (the contents of one Fernbach flask) was transferred to six large centrifuge bottles when the turbidity of the culture reached a Klett reading of 140-180 Klett units (#66 filter). The bacteria were centrifuged at 10,000 rpm for 30 minutes. After the supernatant was decanted, another one liter of bacterial culture was added to the bottles and centrifuged. This was repeated until all the bacteria from the five flasks were harvested. The multilayered pellets were then resuspended in 50 to 80 ml of phosphate buffer (50 mM), collected into one centrifuge tube, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was decanted

and the pellet was washed once again in 50 to 80 ml of phosphate buffer.

Cold-Shock Extraction

After centrifugation, the washed pellet was weighed by taring and 1 ml of Tris-Mg buffer (50 mM Tris, 0.2 M $MgCl_2$, pH 7.4) was added for every 0.2 g of cells. The bacterial suspension was alternately inculabed in a 30°C water bath for 10 minutes and incubated in an ice bath for 15 minutes for three cycles according to the procedure of Hoshino and Kageyama (1979). The suspension was then centrifuged at 10,000 rpm for 10 minutes. The supernatant (80 ml) was decanted and dialyzed against 2,000 ml CPA buffer, pH 6.0), 5 times (approximately 8 hours each 2-liter wash) to remove all traces of the $MgCl_2$. The crude periplasmic extract was then frozen at -20°C until use. These crude periplasmic extracts contained 2 to 7 mg protein per ml.

Buffers

Because a citrate concentration of 50 mM interferes with the absorbance reading at 230 nm, one of the wavelengths used to determine protein concentration by the method of Kalb and Bernlohr (1977), a CPA buffer with a lesser concentration of citrate than was used previously (Plano, 1984) was developed. CPA (citrate, phosphate, azide) buffer was prepared by adding 550 ml of dibasic sodium phosphate (50 mM) to 1,000 ml of citric acid (10 mM) to achieve a pH of 6.0. Deionized water (450 ml) was added to bring the total volume to 2,000 ml. Sodium azide (39 mg) was added to inhibit bacterial growth. Final

concentrations were 5 mM citric acid, 13.75 mM dibasic sodium phosphate, and 0.3 mM sodium azide. Stock solutions and buffer solutions were stored at 4°C.

TPA (Tris, phosphate, azide) buffer was prepared in the same manner. To 1,000 ml of Tris solution (10 mM), 550 ml of dibasic sodium phosphate (50 mM) solution, 450 ml of deionized water, and 39 mg of sodium azide were added. The pH was adjusted by adding HCL (4M) until a pH of 9.0 was obtained. Stock solutions and buffer solutions were stored at 4°C.

Concentration of Samples

Samples of partially purified MBP were concentrated using an Amicon Ultrafiltration Unit. A volume of 5 ml of sample was placed in the chamber with a 24 mm membrane filter in place. A magnetic stirrer was used to mix the sample while nitrogen (N_2) gas was passed through the chamber at 35 psi. Both sample and the collected effluent were kept cold by means of ice baths. More sample was added as needed until a final volume of 2-3 ml was achieved.

Protein Determination

Protein concentration was determined either by the colorimetric procedure of Lowry, Rosebrough, Farr, and Randall (1951) or by the spectrophotometric method of Kalb and Bernlohr (1977). Bovine serum albumin (BSA) was used as the protein standard for both assays. The UV method of Kalb and Bernlohr was used for all purified fractions to avoid loss of purified protein by the method of Lowry et al.

Mannitol Binding Protein Assay

MBP activity was determined by the nitrocellulose assay developed by Stinson, Cohen, and Merrick (1977) and modified by Eisenberg and Phibbs (1982). Protein solutions, up to 200 µg protein per ml, were mixed with 0.5 ml of ^{14}C-mannitol in CPA (2 nmoles mannitol/ml) and taken up in CPA buffer, pH 6.0, to 1 ml final volume. Specific activity of ^{14}C-mannitol was 53.4 Ci/mole. Immediately, the solution was filtered, under vacuum, through a pre-moistened 24 mm Millipore filter (type HA, 45 µm pore size), using a Millipore filtration appartus, and washed with 2 ml of CPA, pH 6.0. The filter was removed and transferred to a liquid scintillation vial to which was added 10 ml Aquasol (New England Nuclear). The vials were placed in an Isocap 300 liquid scintillation counter and counted for 10 minutes. A blank vial, a filter control vial without protein, and a ^{14}C-mannitol standard vial were included with each assay. One unit of MBP binding activity, corrected for low levels of protein-independent radioactivity that remained on the filter, 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 (SDS-PAGE)

Pouring the Gel

Slab gels for SDS-PAGE were poured according to the instructions given in the LKB Application Note 306 (Fehrnstrom & Moberg, 1977).

The mold used to cast the gel was made by placing a rubber gasket around the edges of the slot former and then placing the Gel-bond side of a glass plate (125x260x1 mm) on top of the gasket. The Gel-bond was attached to the glass plate following the procedure in the FMC Instruction Manual . . . (1985). A few drops of water were placed on the glass plate, the sheet of Gel-bond was placed squarely onto the plate with its hydrophilic side up, and a rubber roller was rolled over the plate from center out to remove excess water and any air bubbles. A second glass plate (125x260x3 mm) was placed over the first plate, metal clamps were put in place to hold the mold together, and the mold was placed upright in a plastic test tube rack.

A 10% acrylamide solution was prepared by deaerating a gel solution consisting of 33 ml of phosphate buffer stock solution (7.8 g $NaH_2PO_4 \cdot H_2O$, 18.6 g Na_2HPO_4, and 2.0 g SDS, final volume 1,000 ml, pH 7.2) and 29.7 ml of acrylamide solution (22.2 g acrylamide, 0.6 g N,N'-methylene biasacrylamide, final volume 100 ml). After deaeration, 3.2 ml of ammonium persulfate solution [1.5% (w/v) $(NH_4)_2S_2O_8$] and 0.05 ml tetramethylenediamine (TEMED) were added to the gel solution. Immediately, the gel solution was poured into the mold using a 10-ml pipette. The gel was allowed to stand until polymerized (approximately one hour). After polymerization, the gel was removed from the mold, wrapped in Saran Wrap, and retained at room temperature overnight, or longer, before use.

Electrophoresis Procedure

The SDS-PAGE was performed by the procedure of Weber and Osborn (1969) as modified by LKB Application Note 306 (Fehrnstrom & Moberg, 1977). The slab gel was pre-electrophoresised for 30 minutes at 90 mA. Lyophilized protein samples were dissolved in 0.5 ml of sample buffer (5 ml phosphate buffer stock solution, 1 ml 2-mercaptoethanol, 1 g SDS, final volume 100 ml). A 250 µl aliquot of this protein sample was incubated in a boiling water bath for two minutes and then chilled. Ten µl of bromophenol blue (0.25% w/v) and 10 µl 2-mercaptoethanol were added and mixed before pipetting into 10 µl wells in the gel. After all the samples had been applied, the slab gel was electrophoresised at 20 mA for the first 10 minutes and then the current was increased to 90 mA. The gel was run at 90 mA until the tracking dye (bromophenol blue) reached the end of the gel (7-8 hrs). The gel was then placed in a fixing solution (57 g trichloroacetic acid - TCA, 17 g sulfosalicylic acid, 150 ml methanol, and 350 ml deionized water) for 30 minutes. The gel was next stained for one hour in Coomassie blue (1.25 g Coomassie blue R, 230 methanol, 40 ml acetic acid, and 230 ml deionized water). The gel was destained in destaining solution (870 ml deionized water, 50 ml methanol, and 80 ml acetic acid) until the gel background became just clear.

Chemicals

D-(1-^{14}C)-mannitol (53.4 mCi/mmole) was purchased from New England Nuclear. All other reagents used were either analytical or reagent grade and purchased from the usual commercial vendors.

RESULTS

Dialysis of MBP Against CPA, pH 8.3

Mannitol binding protein (MBP) has an isoelectric point (pI) of 8.3 (Eisenberg & Phibbs, 1982). At its pI, a protein should precipitate out of solution (Alden & Hughes, 1955). Therefore, a sample of crude periplasmic extract (92 ml), designated as Fraction I (FI), was dialyzed against 2,000 ml CPA, pH 8.3. After four changes of dialysis wash (8-10 hrs each), the dialysate was centrifuged at 10,000 rpm for 30 minutes. The supernatant (FIs) was decanted and saved for further assays. The pellel (FIp) was resuspended in 1 ml of CPA, pH 6.0. Protein and binding assays were performed on both fractions (Table 1). The FIs fraction had both a larger protein concentration and a higher binding activity, indicating that more MBP was in the supernatant than in the pellet. This result was opposite of what was desired for purification of MBP by precipitation at it pI.

Divalent cations have also been used to precipitate proteins (Alden & Hughes, 1955). A sample (20 ml) of crude periplasmic extract (FI) was dialyzed against 1,000 ml CPA, pH 7.0, with 1 M $MgCl_2$. After 24 hours, the dialysate was centrifuged at 10,000 rpm for 10 minutes. The supernatant (FIs) was decanted (20 ml) and then dialyzed against 1,000 ml CPA, pH 8.3. The pellet (FIp) was resuspended in 1 ml CPA, pH 6.0, and saved for further assays. After two dialysis wash changes (8-10 hrs each), the FIs dialysate was centrifuged at 15,000 rpm for 10 minutes. The supernatant (FIIs) was decanted and saved for further

Table 1

Effect of Dialysis Against CPA, pH 8.3 on MBP Activity

Fraction	Volume	Protein Concentration	Binding Activity[1]	Specific Activity[2]
Dialysate Supernatant[3] (FIs)	92 ml	2.32 mg/ml	38.9/ml	16.7
Dialysate Pellet[3] (FIp)	1 ml	1.86 mg/ml	23.9/ml	12.8

[1] 1 unit of activity = 1 pmole ^{14}C-mannitol bound per filter

[2] specific activity = units of activity per mg protein

[3] crude periplasmic extract dialyzed against CPA, pH 8.3 and centrifuged at 10,000 rpm for 30 minutes

assays. The pellet (FIIp) was then resuspended in 1 ml CPA, pH 6.0. Protein and binding assays were performed on all of the fractions (Table 2). Both supernatants, FIs and FIIs, had a larger concentration of protein and a higher binding activity than the pellet fractions, FIp and FIIp.

It was discovered (established later in this study) that MBP loses its mannitol binding activity at its pI (to be discussed in more detail in a later section). A fraction of crude periplasmic extract (FI) was divided into two equal (10 ml) portions. The first portion, designated Fraction I, pH 8.3 (FI, 8.3), was dialyzed exclusively against CPA,

Table 2

Effect of Dialysis Against 1 M Magnesium Chloride and Against CPA, pH 8.3 on MBP Activity

Fraction	Volume	Protein Concentration	Binding Activity[1]	Specific Activity[2]
Dialysate Supernatant[3] (FIs)	20 ml	3 mg/ml	22.8/ml	7.6
Dialysate Pellet[3] (FIp)	1 ml	1 mg/ml	16.7/ml	16.7
Dialysate Supernatant[4] (FIIs)	20 ml	0.8 mg/ml	48.7/ml	60.9
Dialysate Pellet[4] (FIIp)	1 ml	0.1 mg/ml	2.4/ml	24.0

[1] 1 unit of activity = 1 pmole ^{14}C-mannitol bound per filter

[2] specific activity = units of binding activity per mg protein

[3] dialysis against CPA, pH 7.0 with 1 M $MgCl_2$ and centrifugal at 10,000 rpm for 30 minutes

[4] dialysis against CPA, pH 8.3 and centrifuged at 10,000 rpm for 30 minutes

pH 8.3. The second protion, designated Fraction I, pH 8.3/pH 6.0 (FI, 8.3/6.0), was dialyzed overnight against 1,000 ml CPA, pH 8.3 and then dialyzed against 1,000 ml CPA, pH 6.0. If the loss of binding activity is a reversible phenomenon, then the fraction FI, 8.3/6.0 would be expected to have greater binding activity than fraction FI, 8.3. However, that is not the case (Table 3); both fractions

Table 3

Lack of Reversible Binding Activity of MBP

Fraction	Volume	Binding Activity[1]
FI, 8.3[2]	10 ml	8.1/ml
FI, 8.3/6.0[3]	10 ml	5.8/ml

[1] 1 unit of activity = 1 pmole ^{14}C-mannitol bound per filter

[2] dialyzed exclusively against CPA, pH 8.3

[3] dialyzed first against CPA, pH 8.3 and then against CPA, pH 6.0

have similar amounts of binding activity. Therefore, the loss of MBP activity is not a reversible event.

In all instances described above, the supernatants had larger amounts of MBP than did the pellets. Obviously then, dialysis at its pI was not practical as a first step in purifying MBP activity. In view of the fact that MBP loses its activity at its pI (and the loss of activity is not reversible), it would not be desirable to dialyze MBP at pH 8.3 as an early step in purification of MBP activity.

Sodium Chloride Elution of MBP

A sample of crude periplasmic extract (FI) was loaded onto a CMC-50 cation exchange column. After washing with two bed volumes of CPA, pH 6.0, MBP was eluted from the column with two bed volumes

of a 150 mM sodium chloride (NaCl) solution. Two protein peaks were observed at A_{280} (Figure 1). When assayed for MBP activity, only the second peak showed any binding activity (Figure 1). These results confirm the previous findings of Plano (1984). The fractions comprising the first peak, tubes 4 to 22, were collected, pooled (40 ml), and dialyzed against 1,000 ml CPA, pH 6.0. This pooled fraction, designated Fraction IIA (FIIA), was divided into two portions with the larger portion frozen. The smaller portion was dialyzed against dionized water, lyophilized, and saved for SDS-PAGE analysis. The fractions comprising the second peak, tubes 54 to 60, were also collected, pooled (20 ml), and dialyzed against described above, and the larger portion saved for future use.

pH Gradient Elution of MBP

pH Gradient 6.0 to 9.0

The larger portion of FIIB was loaded onto a CMC-50 cation exchange column. The sample was washed with two bed volumes of CPA, pH 6.0, followed by elution via a pH gradient from 6.0 to 9.0. At least four peaks were observed at A_{280} (Figure 2). The first two peaks, designated Fraction IIIA (FIIIA), covered the pH range 6.2 to 7.0. The second two peaks, designated Fraction IIIB (FIIIB), covered the pH range of 7.5 to 8.7. The major peaks were tested for MBP binding activity. The elution at pH 8.0 was the only one to show MBP binding activity (Figure 2). Fractions comprising the first two peaks, tubes 19 to 27, designated FIIIA, were collected, pooled

Figure 1. Sodium Chloride Elution of Crude Periplasmic Extract (F1) on a Sephadex CMC-50 Cation Exchange Column (2.5 by 23 cm). The sample solution (75 ml) was loaded onto the column, which had been equilibrated with CPA buffer, pH 6.0. The column was next washed with 150 ml of CPA, pH 6.0. MBP was eluted from the column with 150 ml of NaCl (150 mM) in CPA. Tube 47 is the beginning of the elution by NaCl. The fraction volume was 5 ml and the flow rate was 40 ml per hour. Symbols: O - absorbance at 280 nm; ■ - binding activity (1 unit = 90 cpm/pmole ^{14}C-mannitol).

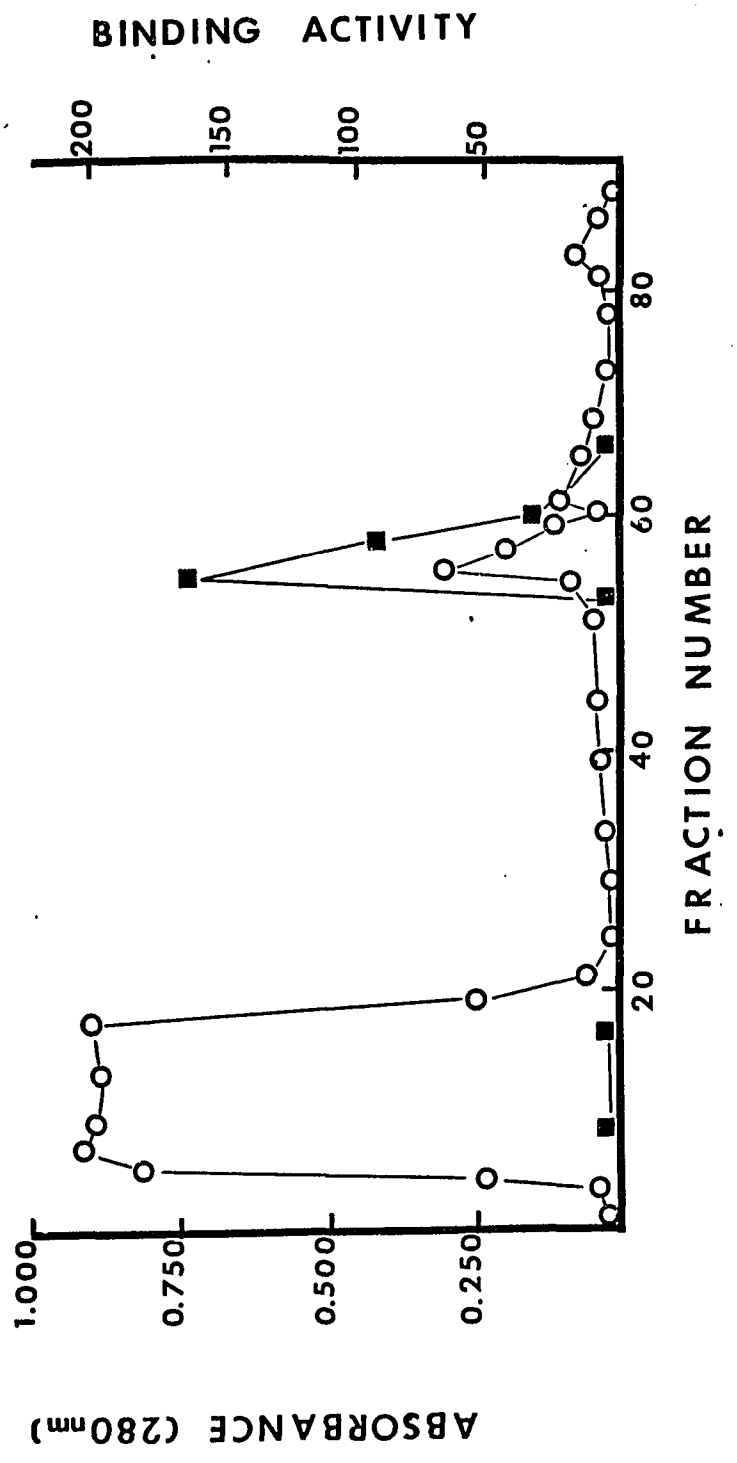
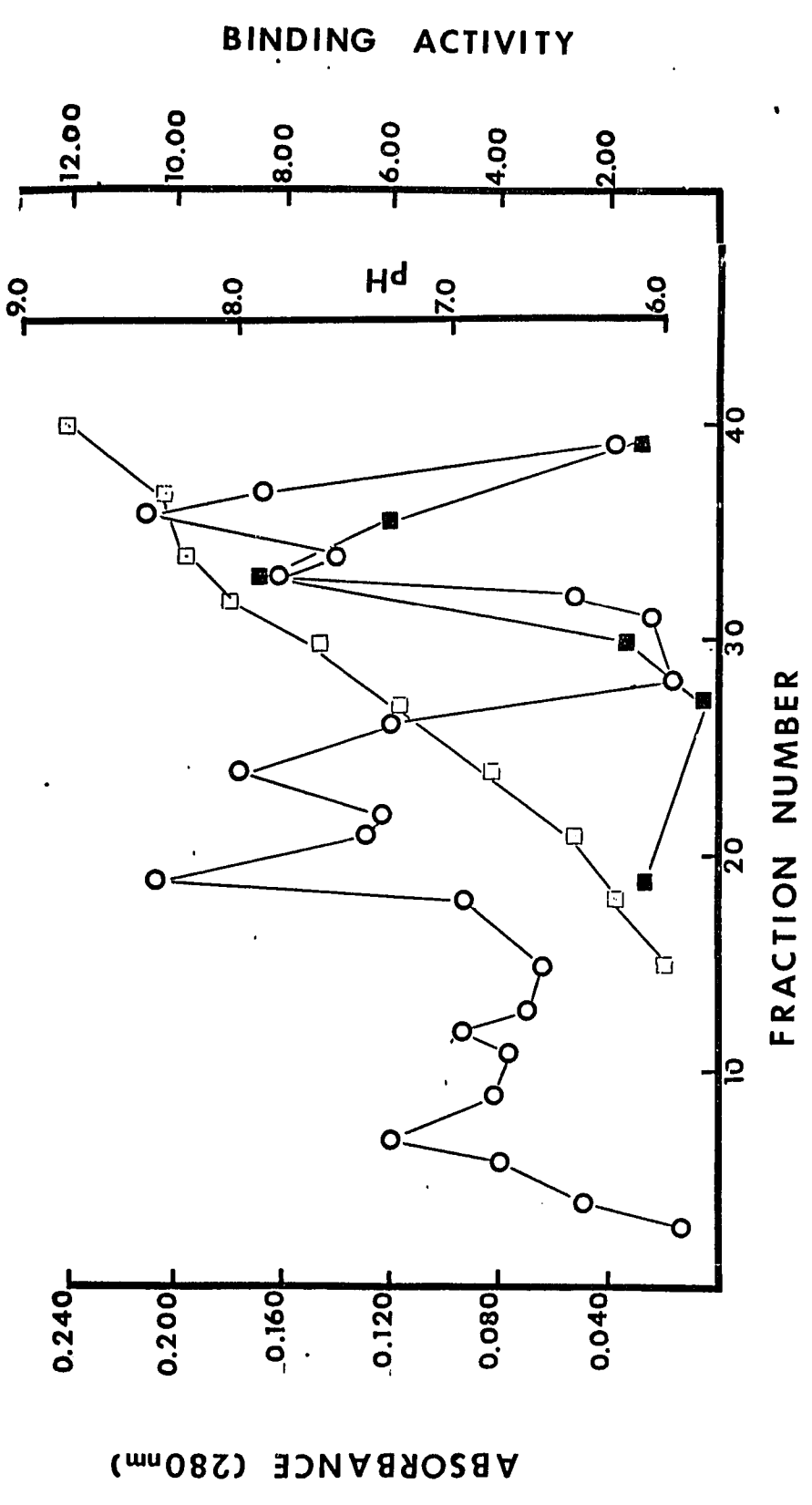


Figure 2. pH Gradient (6.0 to 9.0) Elution of Partially Purified MBP (FIIB) on a Sephadex CMC-50 Column (2.5 by 23 cm). The sample solution (15 ml) was loaded onto the column, which had been equilibrated with CPA, pH 6.0. After the column had been washed with 30 ml of CPA, pH 6.0, MBP was eluted from the column with a pH gradient consisting of 30 ml CPA, pH 6.0 and 30 ml TPA, pH 9.0. The fraction volume was 5 ml and the flow rate was 40 ml per hour. Symbols: O - absorbance at 280 nm; ■ - MBP binding activity; □ - pH.



(30 ml), and dialyzed against 1,000 ml CPA, pH 6.0. A small portion was prepared for SDS-PAGE analysis while the larger portion was frozen and saved. The fractions of the remaining peaks were dialyzed against CPA, pH 6.0 as soon as they came off the column. It had been discovered in a previous pH gradient (results to be discussed below) that MBP activity was rapidly lost if the fractions were retained at a pH of 8.3. The fractions, tubes 33 to 37, designated FIIIB, after CPA dialysis, were pooled, and then divided with the larger portion saved for future use and the smaller portion prepared for SDA-PAGE.

In a previous pH gradient (6.0 to 9.0) elution (figure not shown), MBP binding activity was analyzed at two different times. The first assay was performed immediately after the fractions were eluted from the column. The second assay was performed twelve hours later. Both fractions showed strong binding activity immediately after elution (Table 4). However, after being retained at pH 8.2 and 8.25, respectively, the fractions lost their MBP binding activities, thereby indicating that MBP is denatured at a pH near its pI.

pH Gradient 7.5 to 9.5

A portion of FIIB was loaded onto a CMC-50 cation exchange column. The sample was washed with two bed volumes of CPA, pH 7.0, followed by elution via a pH gradient from 7.5 to 9.5. The peaks were observed by A_{280} (Figure 3). The first peak, designated Fraction IVA (FIVA), was a sharp peak which coincided with the washing of the FIIIB sample before the start of the pH gradient. The second peak was a small, broader peak covering the pH range of 7.8 to 9.1.

Table 4

Loss of Binding Activity in MBP

Fraction	pH	Binding Activity[1,2]	Binding Activity[1,3]
Fraction 40	8.20	31.4/ml	2.2/ml
Fraction 41	8.25	83.4/ml	2.7/ml

[1] 1 unit of activity = 1 pmole ^{14}C-mannitol bound per filter

[2] MBP assay performed immediately after elution

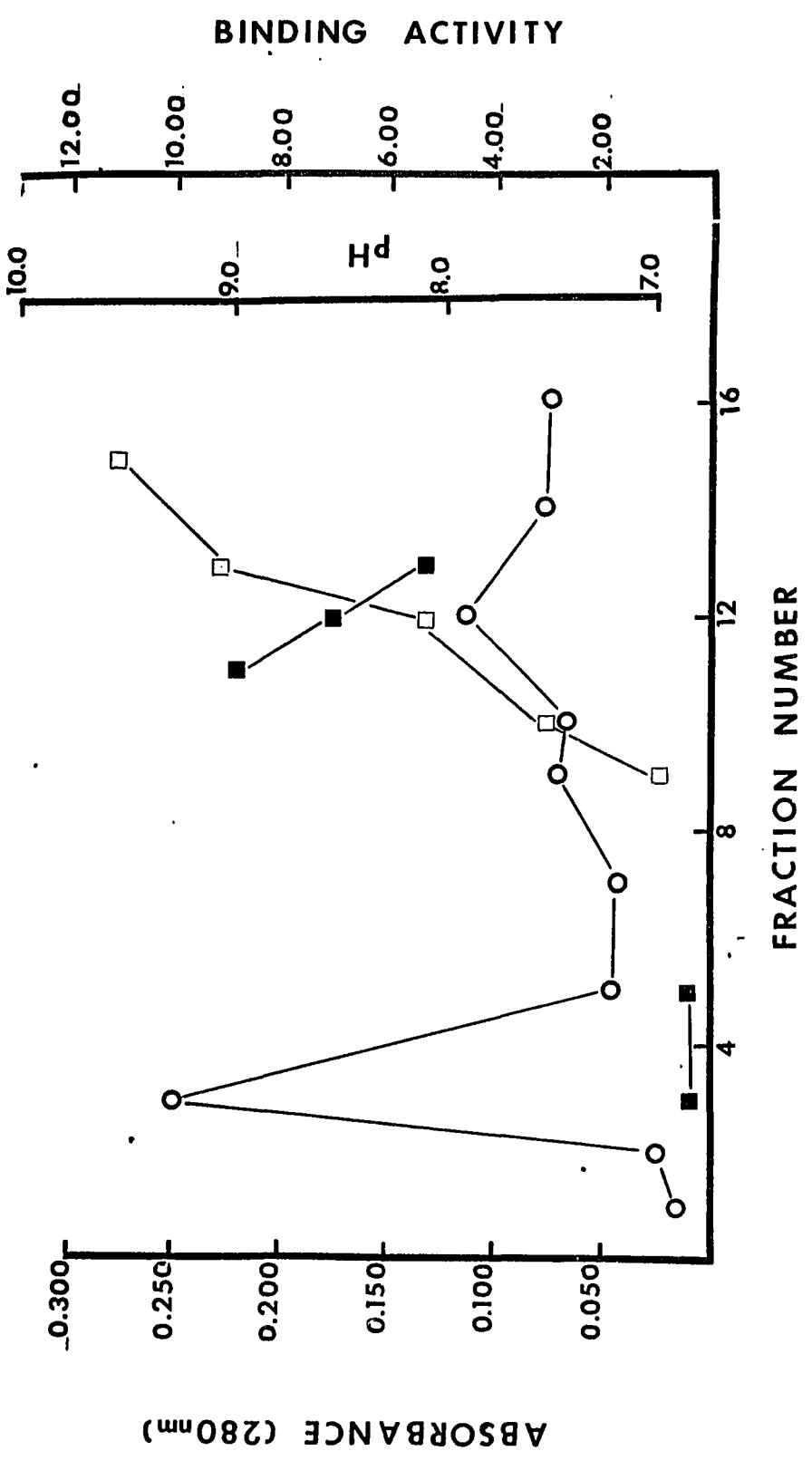
[3] MBP assay performed 12 hrs after elution

The two peaks were tested for binding activity. The second peak was the only one to show MBP binding activity (Figure 3). Tube 11, designated FIVB,11 and tube 12, designated FIVB,12, were separately dialyzed against 600 ml CPA, pH 6.0 and prepared for SDS-PAGE analysis. It should be noted that the lack of an MBP activity peak may be due to loss of MBP activity at increasing pH.

pH Gradient 7.5 to 8.5

A second portion of the FIIIB fraction was again loaded onto a fresh CMC-50 cation exchange column. The sample was washed with two bed volumes of CPA, pH 7.0, followed by elution via a pH gradient from 7.5 to 8.5. One major sharp peak, covering the pH range 7.5 to

Figure 3. pH Gradient (7.5 to 9.5) Elution of Partially Purified MBP (FIIIB) on a Sephadex CMC-50 Column (2.5 by 25 cm). The sample solution (10 ml) was loaded onto the column, which had been equilibrated with CPA, pH 6.0. After the column had been washed with 20 ml CPA, pH 7.0, MBP was eluted from the column with a pH gradient consisting of 25 ml CPA, pH 7.5 and 25 ml TPA, pH 9.5. Tube 8 marks the beginning of the pH gradient. The fraction volume was 4.5 ml and the flow rate was 15 ml per hour. Symbols: O - absorbance at 280 nm; ■ - MBP binding activity; □- pH.



8.1, was observed by A_{280} (Figure 4). A second, less defined, peak, covering the pH range 8.3 to 8.5, was also observed by A_{280} (Figure 4). Both peaks were tested for binding activity. The first peak was the only peak to show MBP binding activity (Figure 4). Again, the activity "peak" for MBP was not coincident with the observed A_{280} peak and the loss of MBP activity at increasing pH obfuscates interpretation of these results. Fractions comprising the first peak, tubes 21 to 24, designated FVA, were collected, pooled (8 ml), and dialyzed against 600 ml CPA, pH 6.0. Fractions comprising the second peak, tubes 28 to 30, designated FVB, were also collected and pooled (6 ml) after dialysis against 600 ml CPA, pH 6.0. Both pooled fractions were dialyzed against deionized water in preparation for lyophilization. Unfortunately, these fractions were lost during the lyophilozation procedure and thus were not available for SDS-PAGE analysis.

SID-Polyacrylamide Gel Electrophoresis

The lyophilized samples were applied to a SDS-PAGE slab gel along with four standards: bovine serum albumin (MW 68,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 29,000), and trypsin inhibitor (MW 20,000) (Figure 5). The FI lane showed a variety of protein bands which would be expected from crude periplasmic extract. The FIIA, which showed no MBP binding activity, upon electrophoresis showed many proteins but showed very few bands between bovine serum albumin (BSA) and ovalbumin. Because the molecular weight (MW) of MBP was reported to be 45,000 as determined by SDS-PAGE (Plano, 1984), the MBP band would be expected to be between the BSA and ovalbumin

Figure 4. pH Gradient (7.5 to 8.5) Elution on a FIIIB Fraction of MBP on a Sephadex CMC-50 Column (2.5 by 20 cm). The sample solution (6 ml) was loaded on the column, which had been equilibrated with CPA, pH 6.0. After the column had been washed with 20 ml CPA, pH 7.0, MBP was eluted from the column with a pH gradient consisting of 25 ml CPA, pH 7.5 and 25 ml TPA, pH 8.5. Tube 17 marks the beginning of the pH gradient. The fraction volume was 2 ml and the flow rate was 15 ml per hour. Symbols: O - absorbance at 280 nm; ■ - MBP binding activity; □ - pH.

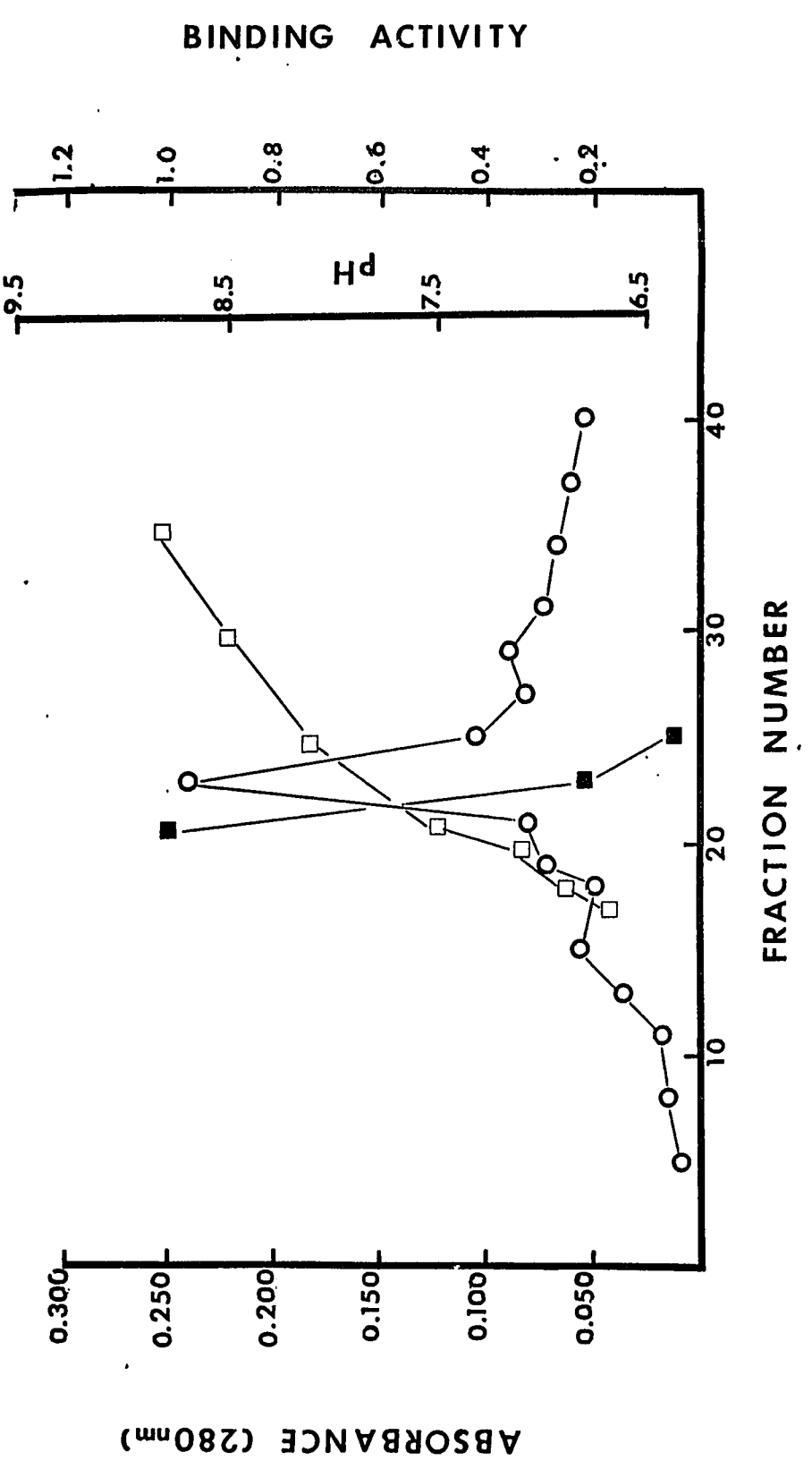
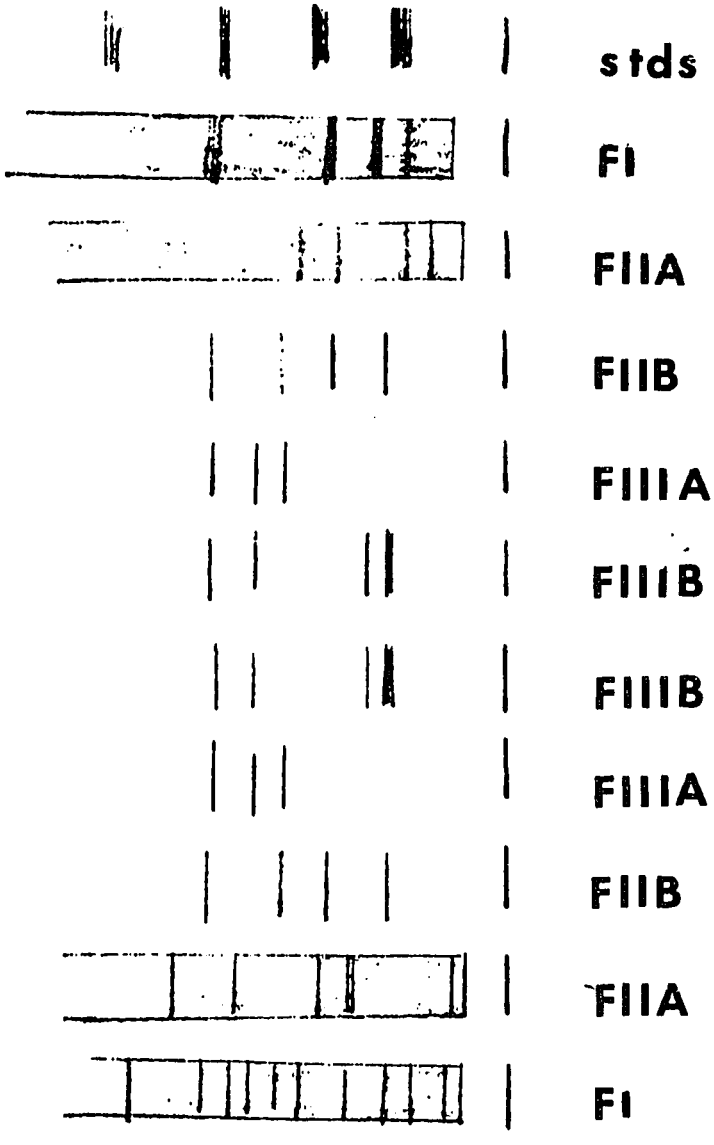


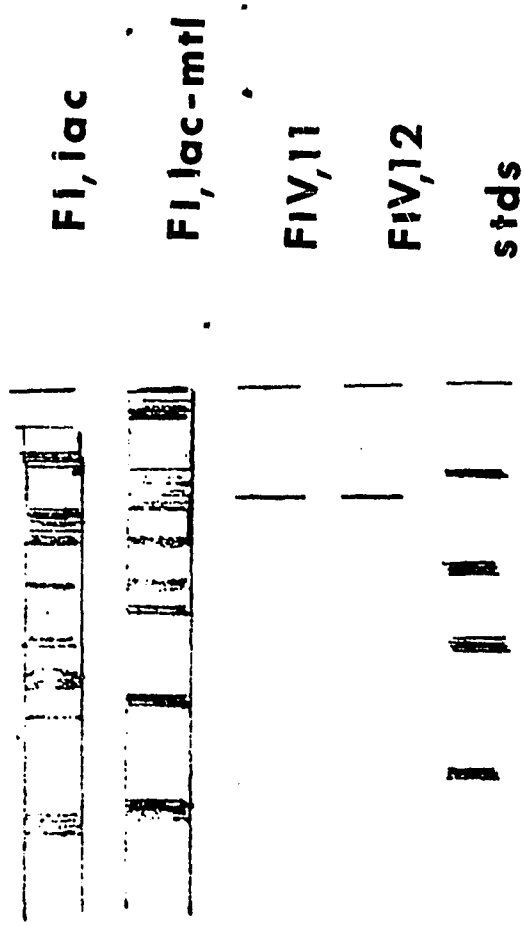
Figure 5. SDS-PAGE on Crude Periplasmic Extract (FI) and Partially Purified Fractions. The gels were prepared and run as described in Materials and Methods. The concentration of the standards were 1 mg/ml. The protein concentration of the fractions were as follows: FI 7 mg/ml; FIIA 7 mg/ml; FIIB 0.6 mg/ml; FIIIA 0.3 mg/ml; and, FIIIB 0.3 mg/ml.



bands. The FIIB lane faintly showed two bands between BSA and ovalbumin as well as a few other faint bands. The FIIIA lane showed one very faint band between BSA and ovalbumin and three other bands between ovalbumin and carbonic anhydrase. The FIIIB lane showed two heavy bands between BSA and ovalbumin.

SDS-PAGE was performed on crude periplasmic extract from cells grown only in lactate and on crude periplasmic extract from cells grown in lactate and mannitol. Both lanes show a variety of protein bands (Figure 6), confirming what was observed by Plano (1984). SDS-PAGE was also performed on fractions FIVB,11 and FIVB,12. Both lanes showed only one band in the same location between BSA and ovalbumin (Figure 6). Although this band is closer to the BSA band, implying a MW greater than 45,000 (as reported by Plano, 1984), the appearance of only one protein band in these fractions is very encouraging and represents the first data that has MBP activity associated with only one protein band in SDS-PAGE analysis.

Figure 6. SDS-PAGE on Crude Periplasmic Extracts and Partially Purified Fractions of MBP. The gel was prepared and run as described in Materials and Methods. The concentration of standards were 1 mg/ml. The protein concentration of the samples were as follows: FI (lactate) 0.4 mg/ml; FI (lactate+mannitol) 0.6 mg/ml; FIVB, 11 0.08 mg/ml; FIVB, 12 0.08 mg/ml.



DISCUSSION

MBP is a somewhat unusual periplasmic binding protein with a pI of 8.3, considering that most periplasmic proteins that have been studied in Gram-negative bacteria have acidic isoelectric points (Eisenberg & Phibbs, 1982). This unusual characteristic has been utilized in the attempts to purify MBP via pH gradient elution. As seen in Figure 2, several protein peaks appeared between the pH range of 6.0 to 7.0 as expected based on the isoelectric points of most periplasmic proteins. However, no MBP binding activity was observed. As the pH gradient became more alkaline, a single protein peak was seen which did have MBP activity. This peak was observed near the pI of MBP. On analysis by SDS-PAGE, however, four protein bands were seen (Figure 5), indicating only partial purification of MBP.

On elution by a more alkaline pH gradient (7.5 to 9.5), MBP binding activity was found in a fraction eluted from the column at a pH (7.8) lower than the pI (8.3) of MBP (Figure 3). MBP activity was also seen, however, in a fraction eluted from the column at a pH (8.2) near the pI of MBP (Figure 3). On analysis by SDA-PAGE, both fractions showed only one identical band (Figure 6) implying final purification of MBP. Because MBP activity is lost as pH increases, the lack of a MBP activity peak was not totally unexpected.

It is unfortunate that the fractions from the pH gradient (7.5 to 8.5) elution were not analyzed by SDS-PAGE. It would be

interesting to see if there is only one electrophorectic protein band in fraction FVA which showed a sharp protein peak at A_{280} (Figure 4) as well as MBP binding activity at a pH lower than 8.3. Based on the results from the previous pH gradient (7.5 to 9.5) elution and the resulting loss of MBP activity as pH increases, only one protein band would be expected.

The pH gradient elution purification scheme appears most promising (Table 5). The gels were stained with Coomassie blue which is sensitive to a protein concentration of 0.01 mg (Weber & Osborn, 1969). Silver stain procedures are more sensitive than Coomassie blue, detecting down to ng of protein (Morrisey, 1981). It may be advantageous to stain SDS-PAGE gels with silver stain to confirm that there is only one protein band in the FIV fractions (Figure 6). Because the FIV bands are faint, it is possible that there may be other protein bands in the fraction that are too dilute to be stained by the Coomassie blue, although concentrating the sample by lyophilization should have avoided that problem.

There are four steps in the present purification scheme (Table 5). These are: (a) cold-shock extraction of the periplasmic proteins; (b) partial purification of MBP by NaCl elution from a CMC-50 cation exchange column; (c) use of a broad pH gradient (6.0 to 9.0) to further purify MBP; and, (d) use of a more alkaline pH gradient (7.5 to 9.5) to obtain one protein band on SDS-PAGE.

Although pH gradient elution from CMC-50 columns appears to be a promising method for final purification of MBP, this method suffers from the irreversible loss of MBP activity as the pH approaches the pI

Table 5

Summary of Purification Scheme

Fraction	Volume	Protein Concentration	Binding Activity[1]	Specific Activity[2]
FI[3]	80 ml	2 mg/ml	277.8	138.9
FIIA[4]	40 ml	1.52 mg/ml	14.4	9.5
FIIIB[5]	20 ml	0.23 mg/ml	333.3	1424.3
FIIIA[6]	30 ml	0.029 mg/ml	0.88	30.3
FIIIB[7]	20 ml	0.026 mg/ml	15.96	613.8
FIVB,11[8]	4.5 ml	0.008 mg/ml	17.78	2222.5
FIVB,12[9]	4.5 ml	0.018 mg/ml	13.34	741.1

[1] 1 unit of activity = a pmole ^{14}C-mannitol bound per filter

[2] specific activity = units of binding activity per mg protein

[3] crude periplasmic extract dialyzed against CPA, pH 6.0

[4] first peak of 150 mM NaCl column; see Figure 1

[5] second peak of 150 mM NaCl column; see Figure 1

[6] pH(6.0-9.0) gradient column; peak covering pH range 6.8-7.2; see Figure 2

[7] pH(6.0-9.0) gradient column; peak covering 7.5-8.7 pH range; see Figure 2

[8] pH(7.5-9.5) gradient column; peak eluted at pH 7.8; see Figure 3

[9] pH(7.5-9.5) gradient column; peak eluted at pH 8.2; see Figure 3

of MBP. Inactive MBP, however, would be a useful product if it is devoid of any contaminating polypeptides. Thus, inactive MBP would be useful for establishing the amino acid sequence of MBP and could also be useful for preparing antibody against active MBP for another purification procedure using antibody mediated affinity chromatography (Wallace, Tallant, & Cheung, 1980).

The loss of MBP activity might be reduced by protecting the protein. Thus, using mannitol, during the purification procedures, as part of the buffer and gradients might protect the active site of the binding protein. Also, use of reducing agents such as dithiothreitol or β-mercaptoethanol might protect against loss of binding activity. Finally, other pH gradients buffer components may be less denaturing than those employed in this study. All of these possibilities should be considered as part of a continuation of any further studies on purification of MBP by pH gradient elution from cation exchange columns.

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