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Short Communication

Evaluation of several affinity chromatographic supports for the purification of maltose-binding protein from *Escherichia coli*

Yolande Kroviarski

INSERM U 76, INTS, 6 Rue Alexandre Cabanel, 75739 Paris Cedex 15 (France) and Centre de Recherche sur les Enzymopathies de l'Association Claude Bernard, Hôpital Beaujon, 92118 Clichy (France)

Sylvie Cochet

INSERM U 76, INTS, 6 Rue Alexandre Cabanel, 75739 Paris Cedex 15 (France)

Pierre Martineau

CNRS URA 1444, Institut Pasteur, 25 Rue de Docteur Roux, 75015 Paris (France)

Jean Pierre Cartron and Olivier Bertrand*

INSERM U 76, INTS, 6 Rue Alexandre Cabanel, 75739 Paris Cedex 15 (France)

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ABSTRACT

To obtain affinity adsorbents with good mechanical resistance, suitable for the purification of maltose-binding protein (MBP) from *Escherichia coli* and genetically engineered proteins fused to MBP, a series of supports were prepared by grafting amylose on to agarose by different chemistries. Their capacities for MBP and their abilities to be used at relatively high flow-rates were examined. Efficient supports were most conveniently prepared by coupling amylose to epoxy-activated agarose in an aqueous–organic mixture.

INTRODUCTION

Gene fusion can provide an elegant solution to some purification problems [1]: if the gene for the protein of interest is fused to a DNA sequence coding for a protein or a peptide endowed with a particular property, such as a peculiar charge pattern [1–3], the propensity to make hydrophobic interactions [4,5] or an affinity for a specific ligand [6–9], the fusion protein can be rapidly purified by taking advantage of this property.

Fusion to maltose-binding protein (MBP) [10–14], for example, has the advantage that the fusion protein can be purified on reticulated amylose, for

* Corresponding author.

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which MBP has a significant affinity [15]. Fusion to the periplasmic MBP allows the recombinant protein to be exported to the periplasmic space. Targeting recombinant proteins to the periplasmic space has the following advantages: the periplasmic space is favourable to protein folding and disulphide bond formation, whereas cytoplasmic compartment is not [16,17]; it has been demonstrated that (pre) proinsulin was more stable when secreted in the periplasm than when expressed in cytoplasm, suggesting that proteolysis was lower in periplasm [18]; and periplasmic space contains no DNA, fewer proteins at lower concentrations than does the cytoplasm, periplasmic contents can be easily recovered by osmotic shock [19], hence export of the protein of interest to the periplasmic space provides for easier protein purification.

Despite its obvious advantages, the fusion of recombinant proteins to MBP seems to be confined to the research laboratory. One cause of this apparent lack of success in the large-scale production of recombinant proteins could be the poor mechanical resistance of the reticulated amylose which is generally used as the chromatographic support for MBP purification. Reticulated amylose can be used only at low flow-rates, and in fact often has to be used in a batch mode.

This study was carried out to prepare and test supports for MBP affinity chromatography that had better mechanical properties than reticulated amylose.

EXPERIMENTAL

Chemicals

Maltose, amylose from potato (Ref. No. A-0512) or from corn (Ref. No. A-7043) were purchased from Sigma (St. Louis, MO, USA), Sepharose 6B from Pharmacia (Uppsala, Sweden) and epichlorohydrin (gold label), hydrazine hydrate, and dimethyl sulphoxide (DMSO) from Aldrich (Milwaukee, WI, USA). Other chemicals were purchased from Merck (Darmstadt, Germany).

Enzyme-linked immunosorbent assay (ELISA) for MBP

ELISA was performed using a standard protocol for sandwich-type assays. Plates were coated with a monoclonal antibody directed against MBP and serial dilutions of MBP-containing material were incubated in the plate wells. The plates were washed and the bound MBP was determined using either an in-house prepared or a commercial (New England Biolabs, Beverly, MA, USA) rabbit anti-MBP antiserum. MBP purified by affinity chromatography was used as a standard.

Amylase assay

Amylase was measured using a chromogenic *p*-nitrophenyl maltoheptaoside substrate, according to the vendor's recommendations (Sigma).

Gel electrophoresis

Gel electrophoresis was performed according to Laemmli [20] using 10% gels. They were stained with Coomassie Brilliant Blue.

Preparation of reticulated amylose

The procedure of Ferenci and Klotz [15] was followed exactly.

Grafting of amylose to agarose by epoxide coupling

Activation of agarose with epichlorohydrin. The activation procedure of Porath and Larsson [21] was used, with minor modifications. Sepharose 6B (10 ml) was exhaustively rinsed with water, transferred into a vessel with a minimum amount of water and 5 ml of 2 *M* NaOH were added, followed by 0.5 ml of epichlorohydrin and 20 mg of $NaBH_4$. Further aliquots of 1.2 ml of 2 *M* NaOH and 0.6 ml of epichlorohydrin were added every 30 min (five times) and the gel suspension was tumbled for 16 h at room temperature. This epoxide-activated gel was immediately used for coupling.

Coupling of amylose to epichlorohydrin-activated agarose in aqueous medium. A 2% (w/v) amylose solution in water was prepared by adding hot water to the amylose in small portions and warming the suspension carefully in a microwave oven until it had completely dissolved.

A 10-ml aliquot of epoxy-activated gel was rinsed with water on a fritted disc and transferred into a vessel with a minimum amount of water, then 80 ml of the 2% amylose solution, 2.3 g of Na_2CO_3 and 55 mg of $NaBH_4$ were added and the gel suspension was shaken for 36 h at 37°C.

Coupling of amylose to epichlorohydrin-activated agarose in aqueous–organic mixture. A 10-ml ali-

quot of epoxide-activated gel was rinsed with water on a fritted disc and then with 20 ml of DMSO–water (20:80, v/v) and with 100 ml of DMSO–water (50:50, v/v). It was then transferred into a vessel with a minimum amount of DMSO–water (50:50, v/v) and 40 ml of amylose solution in DMSO were added (several amylose concentrations were used; see Results and Discussion), then 30 ml of water and 48 mg of $NaBH_4$ were sequentially added. The pH of the gel suspension was increased to 12 with 2 *M* NaOH and the gel suspension was incubated at 37°C with gentle mechanical agitation for 36 h. The gel was then rinsed with DMSO–water (70:30, v/v), DMSO–water mixtures of decreasing DMSO concentration and finally with water alone.

Preparation of a hydrazide derivative of agarose. A 10-ml volume of epoxide-activated gel was rinsed with water and incubated with 15 ml of hydrazine hydrate and 0.3 mg of $NaBH_4$ for 90 min at 40°C, and finally rinsed exhaustively with water.

Coupling of amylose to a hydrazide derivative of agarose in aqueous medium. The technique was derived from the procedures of Wilchek and Lamed [22] as follows: A 10-ml volume 2 *M* of sodium acetate buffer (pH 5) was added to 200 ml of a 2% aqueous solution of amylose, followed by 10 ml of the hydrazide derivative of agarose (prepared as above). The gel suspension was tumbled for 36 h at room temperature. The gel was then washed on a Büchner funnel with 1 *M* NaCl solution and water (using prewarmed solution and glassware), and finally with 0.5 *M* Tricine–HCl buffer (pH 8.0). The gel was suspended in the latter buffer and four additions of 100 mg of $NaBH_4$ were made at 60 min intervals. The gel was finally rinsed with water.

Coupling of amylose to a hydrazide derivative of agarose in aqueous–organic mixture. A 4-g amount of amylose was added to 40 ml of DMSO and dissolved by briefly warming at 60°C. The hydrazide of agarose was gradually brought to DMSO–water (50:50, v/v) by successive 30-min incubations in solutions of increasing DMSO content and filtering through a Büchner funnel. A 10-ml portion of the gel was added to the amylose solution in DMSO. Water and 2 *M* sodium acetate buffer (pH 5.0) were then added to bring the final concentrations of sodium acetate and DMSO to 0.1 *M* and 50%, respectively. The gel to total reaction volume ratio was 1:8.

Gels were incubated for 60 h at 40°C with gentle mechanical agitation and rinsed on a prewarmed Büchner funnel with successively DMSO–water (70:30, 50:50 and 25:75, v/v), with distilled water and 1 *M* NaCl solution.

Determination of substitution levels of the various chromatographic supports

The amount of grafted amylose was determined by first hydrolysing amylose to glucose, by incubating an aliquot of gel in 1 *M* HCl for 60 min in a boiling water-bath. Sodium phosphate was added (final concentration 50 m*M*) to the hydrolysate and the pH was adjusted to 7.2 with 4 *M* NaOH. Glucose was then assayed using a commercial glucose oxidase kit (Merck).

Bacteria fermentation and preparation of MBP-containing crude extract

Bacteria of the ED9 *Escherichia coli* strain [23], harbouring a multicopy plasmid pPD1 (carrying a functional MalE gene coding for MBP [24]) were cultivated at 30°C in Luria broth. They were induced with 0.2% maltose and thereafter submitted to the osmotic shock procedure [19]. The osmotic fluid was made 20 m*M* in Tris–HCl (pH 8.0) and ammonium sulphate was added to 100% saturation. It was stored at 4°C. An aliquot of the suspension was centrifuged at 40 000 *g* for 30 min and the pellet was dissolved in 50 m*M* Tris containing 150 m*M* NaCl adjusted to pH 7.5 with HCl (buffer A). This solution was chromatographed on a Sephadex G-25 column equilibrated in the same buffer, to give the starting material for affinity chromatographic experiments.

Evaluation of the MBP capacity of the various supports by chromatography under overload conditions

The supports were sedimented in 1.1 cm I.D. columns to a height of 1 cm. The columns were equilibrated in buffer A. Several flow-rates were used (see Results and Discussion). Starting material was assayed for MBP by ELISA and diluted to 0.1 mg/ml MBP. A volume of 20 ml was loaded on to the column and the column was rinsed with 30 column volumes of buffer A. The retained MBP was eluted with 0.2 *M* maltose in buffer A. The amount of eluted MBP was determined by measuring the absorbance at 280 nm using $E = 14.7\ cm^{-1}\ (g/100\ ml)^{-1}$ [12].

Chromatography of MBP-containing extracts in normal (non-overload) conditions

Several supports were also used under normal conditions. The load was chosen (from data obtained under overload conditions) so as not to saturate the column. The procedure was otherwise the same as above. The amounts of MBP in pooled fractions (flow-through and rinse, and eluted proteins) were measured by ELISA.

RESULTS AND DISCUSSION

Substitution levels and capacities of the various preparations

Flow-rate capabilities. The degrees of amylose substitution of each chromatographic support are shown in Table I together with their capacities for MBP. Table I demonstrates that a series of efficient chromatographic supports for MBP purification were prepared. The capacities were similar to or better than those of the reference support, reticulated amylose, with a single exception.

As the ligand grafted on to the agarose is a high-molecular-mass polymer, it could possibly hinder diffusion of the solutes to the inside volume of beads. For this reason we carefully checked the effect of flow-rate on capacity using gels to which the amylose had been grafted by either reductive amination or epoxide coupling. The capacities of both types of gels for MBP did not decrease appreciably when the flow-rate was increased from 15 to 45 ml/h. Reticulated amylose is a very soft and compressible medium which cannot be used in large-volume columns or at high flow-rates (we could not pump at a flow-rate above 5 ml/h in our 1.1 cm I.D. column, of bed height 1 cm. In contrast, agarose can be used in columns and at flow-rates sized to the needs of the industrial production of proteins. The

TABLE I

CHARACTERISTICS OF VARIOUS AFFINITY CHROMATOGRAPHIC SUPPORTS FOR PURIFYING MBP

Column 1 shows the final amylose concentration in the coupling medium. Column 2: RAm indicates that the support was prepared by grafting amylose on to a hydrazide derivative of agarose and Epox that it was grafted on to epoxy-activated agarose. Aq. indicates that the coupling medium was purely aqueous and Aq.Org. that it contained DMSO. Column 3: figures given in parentheses are those obtained after further thorough washing of the gel with DMSO. The capacities for MBP were measured under overload conditions.

Amylose concentration in coupling medium (%, w/v)	Activation method	Substitution (mg/ml gel)	MBP capacity (mg/ml gel)		
			5 ml/h[d]	15 ml/h[d]	45 ml/h[d]
	Reticulated amylose		0.68		
1.8[a]	Aq./RAm	31.3 (12.0)	0.90	0.94	0.60
1.8	Aq./RAm	26.7 (12.7)		0.81	
1.8	Aq./Epox	21		0.61	
5	Aq.Org./RAm	4.5		0.67	
5	Aq.Org./RAm	2.7		0.90	
5[b]	Aq.Org./RAm	3.9		0.70	
0.5	Aq.Org./Epox	3.0		1.17	
1	Aq.Org./Epox	2.5		1.05	
2[a]	Aq.Org./Epox	10.8	0.51	1.21	1.0
2	Aq.Org./Epox	10.2		0.54	
2	Aq.Org./Epox	9.9		0.82	
3	Aq.Org./Epox	11.4		0.86	
5[c]	Aq.Org./Epox	n.d.		n.d.	

[a] Support used for experiments illustrated by Table II and Fig. 1.
[b] Amylose from corn.
[c] At the end reaction the beads had coalesced into an intractable mass.
[d] Flow-rates.

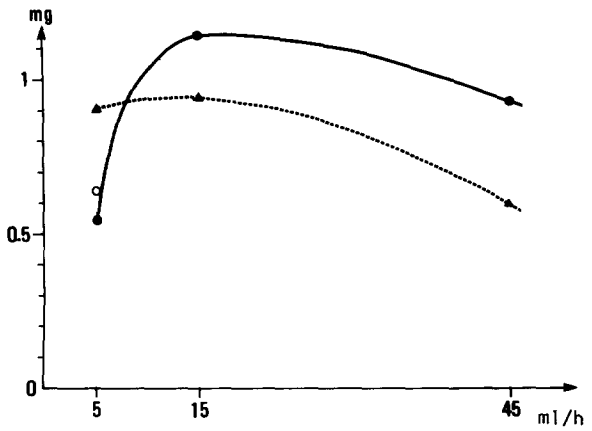

Fig. 1. Capacity of amylose–agarose for MBP. Capacities were measured under overload conditions at various flow-rates. Solid line = Support prepared by grafting amylose on to epoxide-activated gel in aqueous–organic medium. ● = Results obtained with periplasmic extract; ○ = obtained with purified MBP. Dotted line = support prepared by grafting amylose on to a hydrazide derivative of agarose in aqueous medium.

results in Fig. 1 demonstrate that full benefit can be taken of the mechanical resistance of agarose to purify MBP at relatively high flow-rates.

Fig. 1 shows that the capacity of the amylose epoxy agarose gel was higher at a flow-rate of 15 ml/h than at 5 ml/h. This paradoxical result is not related to the presence of amylase in the starting material. It was confirmed by activity measurements that some amylolytic activity was effectively present in the starting material for chromatography, but the same value for the gel capacity was found when purified MBP (devoid of any amylase activity) was used (Fig. 1).

The capacities shown in Table I were obtained under overload conditions, but satisfactory performance was obtained under conditions closer to the usual operating conditions (Table II). About one tenth of the MBP loaded was found in the breakthrough fractions with all three supports tested. Others have also noticed that small, but definite, amounts of MBP-containing fusion proteins were released from the column during sample loading on to reticulated amylose [12,14]. These supports allowed pure MBP [as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)] to be prepared in a single chromatographic step (Fig. 2).

Purely aqueous vs. aqueous–organic synthesis mixtures. Amylose is poorly soluble in water; the solubility varies from batch to batch of amylose, purchased from the same supplier, and having the same reference number. It is extremely difficult to remove unbound amylose by filtration, because the fritted discs rapidly become clogged by amylose coming out of solution, even when prewarmed solutions and glassware are used. This problem is worse when the resin is prepared on a larger scale.

We suspected that the high glucose figures obtained with the gels prepared by reductive amination of a hydrazide derivative of agarose were in fact due to incomplete rinsing of the supports,

TABLE II

MBP CAPACITIES OF THREE PREPARATIONS OF AFFINITY CHROMATOGRAPHIC SUPPORTS

The amounts of MBP in the starting material and in the collected pools were determined by ELISA. The flow-rate was 5 ml/h for the reticulated amylose column and 15 ml/h for the others. Column dimensions: I.D. 1.1 cm and bed height 1 cm.

Preparation	MBP loaded (μg)	MBP found in breakthrough (μg)	MBP in maltose eluate (μg)
Reticulated amylose	250	19	220
Amylose agarose (Aq./Ram)[a]	250	27	220
Amylose agarose (Aq.Org./Epox)[a]	250	27	220

[a] See definitions in Table I.

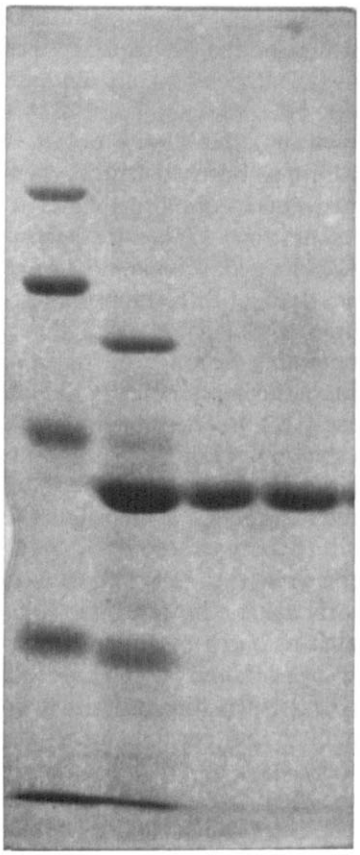

Fig. 2. SDS gel showing, from left to right molecular mass standards (from top to bottom, 94 000, 67 000, 43 000, 30 000), periplasmic extract and purified MBP obtained by chromatography on supports used to collect the data in Table II and Fig. 1.

which probably contained some precipitated amylose. One such gel was examined under a light microscope and it was found to contain both agarose beads and clumps of insoluble amylose (Fig. 3A). This gel sample was incubated in DMSO followed by careful rinsing in the same solvent on a Büchner funnel. It was then rinsed with DMSO–water mixtures of increasing water content and finally with water alone. This treatment significantly reduced the amylose content (Table I) with the loss of the peculiar appearance of the gel under the light microscope (Fig. 3B).

The difficulties associated with the low aqueous solubility of amylose led us to perform the coupling reaction in DMSO–water. The results (Table I) demonstrate that this method can be used to prepare satisfactory supports.

Grafting of amylose on to a hydrazide derivative of agarose vs. grafting on to epoxide-activated support

Several batches were prepared by reductive amination of amylose using a hydrazide derivative of agarose. The substitution levels routinely obtained using aqueous–organic coupling mixture were *ca.* 3 mg of amylose per ml of gel. The capacity for MBP was similar to that of the reticulated amylose, but, as already discussed, with much better flow-rate capabilities.

Satisfactory results were also obtained by simply grafting amylose on to an epoxide-activated gel. The highest capacities were obtained with these gels. The capacity for MBP is not correlated with the degree of amylose substitution. Although we have no proved satisfactory explanation, the amylose coverage of each support may be not equally accessible to MBP present in the mobile phase. Amylose can have either a random coil or a helical conformation, and it is not known if both conformations are satisfactory ligands for MBP. If they are not, a difference in the relative proportions of helix or random coil from batch to batch could explain variations of capacities for MBP. These possibilities will be tested in the near future. Nevertheless, most of the supports prepared were at least as efficient as, and some were much better than, the reticulated amylose reference support with regard to their capacities for MBP. However, the epoxy-activated agarose beads agglomerated into a single intractable mass if the amylose concentrations in coupling mixture was too high (*e.g.*, 5%).

CONCLUSIONS

Affinity chromatography on amylose or on starch can be traced back to the very early days of affinity chromatography. Ambard [25] used insoluble starch to adsorb amylase selectively from biological fluids and eluted the enzyme with glycogen or soluble starch. More recently, cross-linked starch has been used to purify amylases of bacterial and insect origin [26,27] and a glycosyl transferase from *Bacillus circulans* [28,29]. An amylose agarose composite has been used to purify lectins [30]. Amylose was immobilized on amino-silica and used to purify

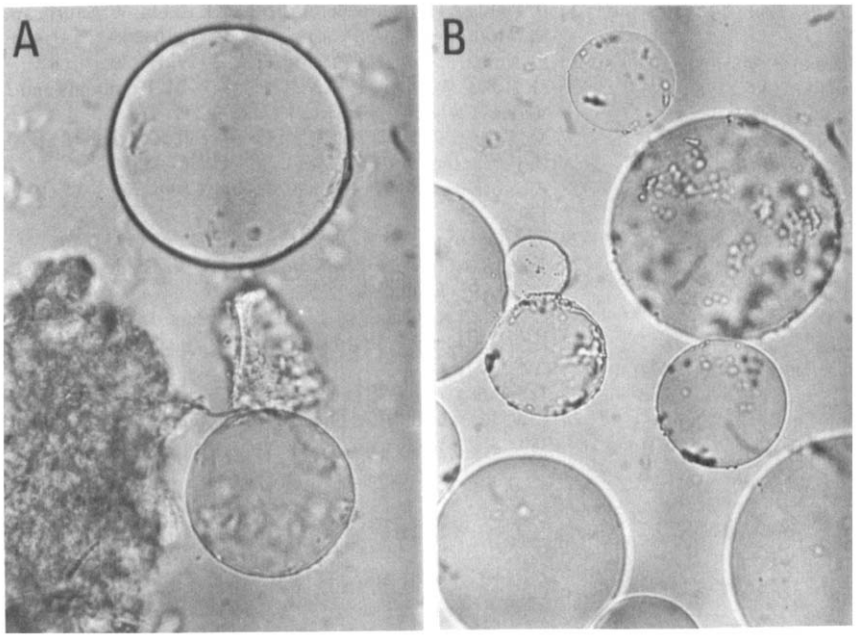

Fig. 3. Light microscope photographs of amylose–agarose prepared by reductive amination in purely aqueous medium. (A) The gel was washed only with aqueous solutions; (B) the gel was also rinsed with DMSO.

a bacterial amylase [31]. Cross-linked amylose has also been used to purify MBP and fusion proteins [10–15,32]. A reticulated composite containing both agarose and amylose and also amylose-coated silica have also been used to purify MBP but no details of their preparation are available [32].

Amylose was covalently grafted on to agarose and used to separate, in a population of *E. coli*, bacteria deficient for LamB (the outer membrane maltose transporter) from wild-type bacteria [33,34], but no attempt has been made to use it to purify MBP [33].

These results clearly demonstrate that amylose covalently grafted to agarose can be used to purify MBP efficiently. Unpublished results suggest that this support can also be used to purify recombinant fusion proteins to MBP. The relatively satisfactory mechanical resistance of agarose will make such supports suitable for production-scale chromatography. Several routes for synthesizing these supports have been evaluated. Epoxy activation of the agarose support, followed by coupling of amylose in aqueous–organic conditions, appears to be the most convenient and effective procedure.

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