HYDROPHOBIC AND BIOSPECIFIC CHROMATOGRAPHY IN THE PURIFICATION OF MALTODEXTRIN PHOSPHORYLASE FROM *E. COLI*

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1. Introduction

Maltodextrin phosphorylase from *E. coli* (EC 2.4.1.1) catalyzes the phosphorolytic cleavage of linear α(1→4) oligosaccharides [1,2]. Schwartz and Hofnung [2], who carried out an extensive study on the molecular properties of the enzyme, purified it 46-fold by a heat treatment step, an ammonium sulfate precipitation and chromatography on DEAE cellulose, achieving a specific activity of 4. 8 1. U./mg. The enzyme was reported to be essentially homogenous as judged by electrophoresis on polyacrylamide gels and by sedimentation in the ultracentrifuge [2].

In common with the animal and plant polysaccharide phosphorylases, the enzyme was found to contain PLP* [2]. But in contrast to phosphorylases from higher organisms which are polymeric [3,4] it was reported [2] on the basis of ultracentrifuge studies and PLP analyses that the bacterial enzyme has a monomeric structure (mol. wt of 130 000–147 000).

In view of the intriguing but yet unestablished role of PLP in polysaccharide phosphorylases and since this cofactor dramatically affects both the catalytic activity and the aggregation state of these enzymes [5–7] it seemed interesting to study the contribution of PLP to the structure and function of this simpler monomeric phosphorylase.

This paper describes a new method for the purification of the enzyme which makes use of hydrophobic [8,9] and biospecific affinity chromatography [10,11]. The procedure results in an overall 78-fold purification and yields an enzyme with a specific activity about 2.5-fold higher than the one reported previously. The enzyme was found to have a subunit mol. wt of 82 000 (by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate) and to contain 0.65–0.70 mol of PLP per 82 000 g of protein. These results, together with previous data on the molecular weight of the native, non-dissociated enzyme [2] and crosslinking experiments (Thanner and Palm, unpublished results) suggest that maltodextrin phosphorylase is a dimeric enzyme.

2. Materials and methods

Source of enzyme: *E. coli* K12 Hfr H8000 were grown in the nitrogen-limiting medium described by Chen and Segal [12]. The production of maltodextrin phosphorylase was induced to a higher level by growing the cells in the presence of maltose (5 g/1) and dextrin (8 g/1) [13]. Large scale preparations of the cells were carried out by Merck, Darmstadt. The enzyme was purified 2–4-fold by a heat treatment (51°C) and precipitation with $(NH_4)_2SO_4$ (30–60% at 4°C) following the procedure described by Schwartz and Hofnung [2]. This precipitate was then dialyzed at 4°C against a buffer composed of Tris–acetate (10 mM), EDTA (1 mM), pH 7.5 (buffer A).

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** *Abbreviations:* PLP: pyridoxal 5'-phosphate. Seph-C_n-NH_2: represents Sepharose 4B activated with CNBr and reacted with an α,ω-diaminoalkane n-carbon atoms long. Seph- SA-dextrin: represents Sepharose 4B activated with CNBr and reacted with a dextrin which had been previously coupled with sulfanilamide (see Methods).

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Assay: maltodextrin phosphorylase was assayed by the formation of glucose-1-P from maltodextrin and P_i [2,14] or by the release of P_i [15] from maltodextrin and glucose-1-P. Values of specific activity reported in this manuscript are given in I. U. and refer to the maltodextrin breakdown assay. It should be noted that the maltodextrin synthesis assay (release of P_i) yields specific activities that are 2.6-fold higher.

Protein concentrations were determined by the method of Lowry et al. [16] or monitored by absorbance at 280 nm.

Polyacrylamide gel electrophoresis on slabs was performed on 7.5% gels in the presence of sodium dodecylsulfate [17]. Samples were prepared by diluting an appropriate volume of the fraction to be tested with an equal volume of a 2-fold concentrated 'sample buffer' and heating for 3 min at 100°C.

Hydrophobic chromatography columns, Seph-C_n-NH_2 (n=0–12) were prepared by the procedures described elsewhere [9].

Dextrin-coated agarose (Seph-SA-dextrin) was prepared by a general procedure for binding of oligo- or polysaccharides onto agarose [18]. This procedure involves the preparation the corresponding glycosylamine of sulfanilamide (*N-p*-sulfamylphenyl-glycoside) and subsequent reaction with CNBr activated agarose. The dextrin-coated agarose used in the purification of *E. coli* phosphorylase was prepared as follows: dextrin (20 g) and sulfanilamide (15 g) were suspended in 50 ml of 0.25 NHCl and kept in a boiling water bath for 15 min [19]. After cooling, ethanol was added to a final concentration of 80% (v/v). The precipitate was resuspended in 10 ml water and reprecipitated by addition of 40 ml ethanol. The product was then exhaustively extracted with dry acetone and dried. The ratio of sulfanilamide to glucosyl residues in the product was found to be 1:45 [20,21]. This product (10 g) was allowed to react (24 hr at 3°C) with 100 g of CNBr activated Sepharose 4B [22] suspended in 100 ml of 0.25 M $NaHCO_3$, pH 9.8. The resulting agarose derivative was found to contain 0.8–1 μmol of sulfanilamide residues and 24-28 μmoles of glucose residues [23] per g of packed wet agarose.

3. Results and discussion

In an attempt to select a suitable hydrophobic chromatography column for the purification of malto dextrin phosphorylase we used the exploratory kit of the Seph-C_n-NH_2 series described elsewhere [9,24]. Aliquots (0.1 ml containing 3 mg protein) of a crude extract which had been partially purified by $(NH_4)_2SO_4$ precipitation (see Materials and methods) were applied on each of the columns in the series. The percentage of protein as well as maltose phosphorylase activity in the excluded fractions were monitored and plotted as a function of the number of carbon atoms (n) in the hydrocarbon side chains in the agarose beads.

Under the conditions of the experiment described in fig.1, maltodextrin phosphorylase was retained already on Seph-C_4-NH_2, but the same column retained also about 70% of the protein content in this particular mixture. The enzyme was also retained by higher members of the series (n = 4–12) and could be eluted from all these columns by adding NaCl (final concentration 1 M) to the eluting buffer. However, the percentage of total protein eluted by 1 M NaCl decreased with increasing n. It seemed therefore

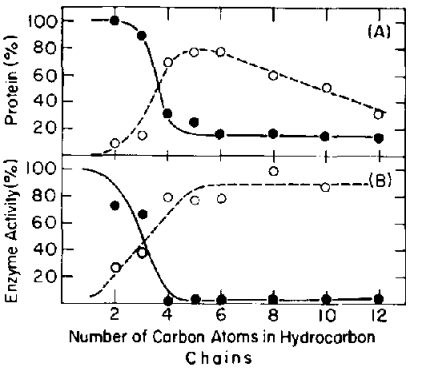

Fig.1. Selecting an ω-aminoalkylagarose for purification of maltodextrin phosphorylase. A kit of Seph-C_n-NH_2 columns (n = 2–12) was equilibrated at 20°C with a buffer composed of Tris–acetate (10 mM), EDTA (1 mM), pH 7.5. Aliquots (0.1 ml, containing 3 mg protein) of the crude extract (after precipitation with $(NH_4)_2SO_4$ and dialysis against the same buffer) were applied on each of the columns (0.4 × 5 cm) which were washed off with the same buffer. Fractions of 2.5 ml were collected (–•–). Subsequently the columns were eluted with a solution of NaCl (1 M) in the same buffer and another set of fractions (2.5 ml each) were collected (—○—). The protein absorption and elution profiles (A) were determined by the method of Lowry et al. [16] and the maltodextrin phosphorylase profiles (B) were monitored by the assay described under Materials and methods.

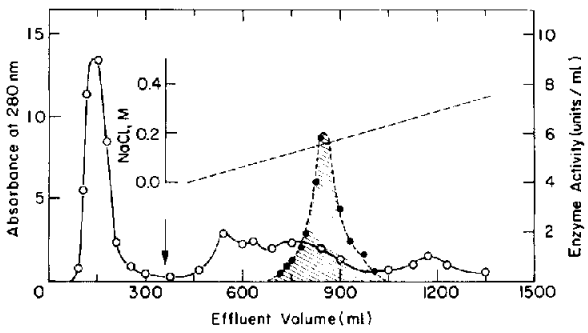

Fig.2. Preparative purification of maltodextrin phosphorylase on Seph-C_{10}-NH_2. A sample (65 ml) of the dialyzed $(NH_4)_2SO_4$ precipitate containing 2 g protein was applied on the column (3.5 × 8 cm) which was equilibrated at room temperature with a buffer composed of Tris–acetate (10 mM), EDTA (1 mM), and 2-mercaptoethanol (5 mM), pH 7.5. Unadsorbed protein was washed off with the same buffer and then (arrow) a linear NaCl gradient (up to 0.5 M, in the same buffer) was applied (400 ml in each reservoir). (–○–), Absorbance at 280 nm; (—●—), maltodextrin phosphorylase activity; (----) NaCl concentration.

that in this case, columns such as Seph-C_{10}-NH_2 or Seph-C_{12}-NH_2 could be efficient in the purification of this enzyme, since under the loading conditions they bind all the maltodextrin phosphorylase (together with about 80% of the protein in the mixture) but upon elution with 1 M NaCl they release essentially all the enzyme but only part (30 or 50%) of the protein. Further resolution could be achieved if, instead of the abrupt increase in ionic strength, a shallow salt gradient was applied. In the preparative scale experiment depicted in fig.2 an 8-fold purification of the enzyme was achieved in one step, with about 80% yield.

As a second purification step we made use of the biospecific recognition of the enzyme for its substrate maltodextrin. A series of affinity chromatography columns was prepared by binding of oligo- or polysaccharidies (maltose, amylodecaose, dextrin or glycogen) onto Sepharose 4B. This was achieved by reaction of the saccharide with sulfanilamide to yield its *N*-glycosylamine derivative which was then coupled through its amide nitrogen to CNBr-activated agarose [18]. It should be noted that saccharides bind to CNBr-activated agarose also through their hydroxyl groups [8], therefore there is some heterogeneity in the mode of binding of the saccharides to the agarose (especially in the case of polysaccharides which contain many hydroxyl groups that compete with the amide nitrogen). Fig.3 illustrates the biospecific purification

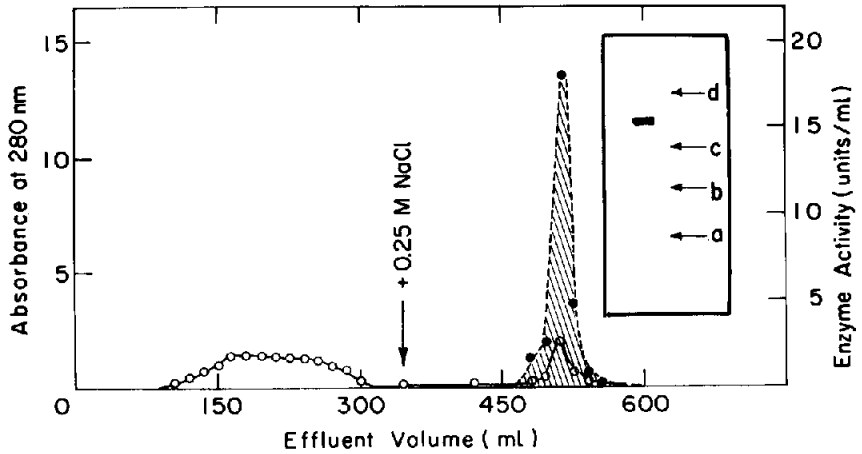

Fig.3. Biospecific purification of maltodextrin phosphorylase on Seph-SA-dextrin. A column of the dextrin-coated agarose (4 × 11 cm) was equilibrated at room temperature with the buffer described in the legend to fig.2. A sample of 140 ml (30.3 mg protein) obtained after chromatography on Seph-C_{10}-NH_2 which was previously dialyzed against the same buffer was applied on the column. Unadsorbed protein was washed off and then the enzyme was eluted with the same buffer including 0.25 M NaCl (arrow). (—●—), Absorbance at 280 nm; (—○—) maltodextrin phosphorylase activity. Inset: gel electrophoresis of the purified enzyme (1.1 μg) in the presence of sodium dodecylsulfate. Arrows indicate the migration of marker proteins with the appropriate subunit mol. wt given in parentheses a) pepsin (35 000). b) Heavy chain of bovine γ-globulin (50 000). c) Bovine serum albumin (66 000). d) Rabbit muscle glycogen phosphorylase (100 000).

Table 1
Purification of maltodextrin phosphorylase

Purification step	Sp. act. units/mg	Purification factor[a]	Yield of enzyme (%)[a]
Crude extract	0.15	–	–
$(NH_4)_2SO_4$ precipitate	0.35	2.3	80
Seph-C_{10}-NH_2	2.80	8.0	79
Seph-SA-Dextrin	11.70	4.2 (78)[b]	66 (52)[b]

[a] Calculated for each purification step.
[b] Values in parentheses represent the overall purification and yield achieved.

of maltodextrin phosphorylase on Seph-SA-dextrin which results in an essentially pure enzyme with a specific activity of 11.7 units/mg (about 2.5-fold higher than the one reported previously [2]. It should be emphasized, however, that some preparations (not necessarily the ones with lower specific activity) contain one to two additional minor bands in gel electrophorsis in the presence of sodium dodecylsulfate. Such a preparation is depicted in the inset to fig.3. The specific activities, purification factors and yields obtained in each step of the procedures given above are summarized in table 1.

This paper illustrates the usefulness of the exploratory kits for hydrophobic chromatography [9,24] and provides an example of the considerations to be made in the choice of the most suitable column out of a homologous series. In this case, the column of choice was not the one with the shortest hydrocarbon chains that retained the enzyme, since this column also retained a high percentage of the protein content of this particular mixture. A higher member of the series proved to be more effective, since it was possible to elute all of the enzyme from it together with only a relatively low amount of other proteins.

A prominent feature of our purified enzyme preparation lies in the fact that the subunit mol. wt of the enzyme as determined by gel electrophoresis in the presence of sodium dodecylsulfate was found to be 82 000 with a PLP content of 0.65–0.70 mol per 82 000 g protein. These results, together with previous data on the mol. wt of the non-dissociated enzyme (13 000–147 000 [2]) and the fact that this enzyme can be crosslinked by reaction with suberimidate [25] to yield a non-dissociable dimer with a mol. wt of approx 150 000 [18] suggest that native maltodextrin phosphorylase is a dimer.

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