PURIFICATION OF THE LACTOSE SPECIFIC FACTOR III OF THE STAPHYLOCOCCAL PEP DEPENDENT PHOSPHOTRANSFERASE SYSTEM*

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1. Introduction

The PEP dependent phosphotransferase system in bacteria is involved in the accumulation of certain carbohydrates into the bacterial cell [1, 2].

The system has been investigated in detail in *E. coli* [1]. In *S. aureus* an additional component which is sugar specific like the membrane bound component designated factor III has been demonstrated [3]. A mutant missing this new cytoplasmic component was isolated and used to perform a very convenient colorimetric assay for the factor III specific for lactose [4]. The function of this new factor in the PEP dependent phosphotransferase system is not yet clear. In order to obtain information about the physiological significance of the component we extensively purified this protein. The purification procedure including some preliminary characterization of the protein will be reported in this communication.

2. Materials and methods

2.1. *Cell-free extract*

30 g of staphylococcal cells (strain S 305 A, constitutive for lactose utilisation) were mixed with 40 ml of standard buffer pH 7.5 (tris-HCl 0.1 M, 0.1 M NaCl, 0.01 M $MgCl_2$, 10^{-4} M EGTA), 1 mg DNase and 90 ml wet glass beads 0.3 mm ϕ. This suspension was shaken in a Buehler cell homogeniser for 45 min at 13°. The turbidity measurement of the cell suspension after the disruption procedure indicated that 80% of the cells were broken. Cell debris and whole cells were sedimented at 10,000 *g* for 30 min. Cell membranes were removed by centrifugation at 150,000 *g* for 90 min; 3 batches of 30 g of bacteria were ground to obtain the cell-free extract.

2.2. *Factor III assay*

2 g of staphylococcal cells (wet paste) strain 714 G (a mutant missing factor III specific for lactose) were suspended in 2 ml of standard buffer containing 0.1% mercaptoethanol and 100 μg DNase and disrupted with a Branson sonifier in the presence of 2.5 g of glass beads (0.1 mm ϕ) for 4 min (cooling with solid CO_2–acetone mixture). The beads were removed by decanting, cell debris by centrifugation at 20,000 *g*.

The following reaction mixture was incubated at 37° (solvent: standard buffer):

0.1 ml extract of 714 G
0.1 ml PEP 5×10^{-2} M
0.1 ml *O*-nitrophenyl-β-D-galactoside 2×10^{-2} M
0.1 ml sample of FIII in buffer

After incubating for 10 min the reaction was stopped by adding 2 ml of 0.5 M Na_2CO_3 solution. The absorbance was measured at 405 nm. Linearity of the assay was observed up to an A of 0.6 at 405 nm (A = 0.6 ⩾ 10 μg of purified factor III). One unit of factor III corresponds to 1 μM of *o*-nitrophenol released in 10 min at 37° under the above conditions.

2.3. *Protein determination*

Protein concentrations were estimated according to Lowry et al. [5].

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2.4. *Heat denaturation*

The cell-free extract was heated to 58° for 10 min, then cooled to 4°. Denatured protein was sedimented at 20,000 *g* for 30 min. The supernate contains factor III activity.

2.5. *Acid precipitation*

The supernate of the heat denaturation step was adjusted with acetic acid to pH 4.8 at 0°. The precipitate was removed by centrifugation and contained the factor III activity. It was dissolved in tris buffer 0.05 M. The pH was adjusted to 7.5.

2.6. *Chromatography on DEAE cellulose*

The dissolved acid precipitate was applied to a DEAE cellulose column 2.5×20 cm (Whatman DE 23). A linear gradient of 0.1–0.6 M NaCl in 0.05 M tris-HCl pH 7.5 (total gradient volume 1 l) was applied. Fractions of 11 ml were collected.

2.7. *Chromatography on Sephadex G 100*

The eluate from DEAE cellulose tube no. 30–50 were pooled and concentrated in the Amicon pressure dialysis apparatus (dialysis membrane PM 10) to a small volume of nearly 5 ml, 2.5 ml of which were applied to a G 100 column 2.5×90 cm (elution buffer: 0.05 M tris-HCl pH 7.5, 0.1 M NaCl, 10^{-4} M EGTA). The G 100 column has been calibrated with standard proteins (fig. 4) according to Andrews [6].

2.8. *Acrylamide disc gel electrophoresis*

Electrophoresis at pH 9.3: The procedure of Hjerten et al. [7] using tris-glycine buffer pH 9.3 gel concentration 5% without stacking gel was used. After purification factor III could be made visible as a turbid band in the gel by dipping it in acetate buffer pH 4.5. This band was cut out and after disintegrating the gel slice in tris buffer pH 8.3, factor III activity could be demonstrated. To detect impurities the gels were routinely stained with Coomassie blue and destained in 7.5% acetic acid 5% methanol.

Electrophoresis at pH 7.0. The gels containing 5% acrylamide and sodium phosphate buffer 0.05 M pH 7 were prepared. Again no stacking gel was used. The sample was applied in 0.01 M phosphate buffer.

2.9. *Electrophoresis in sodium dodecylsulfate*

The method of Weber and Osborn [8] was used without modification. The molecular weight of the polypeptide chain has been estimated using the calibration proteins as internal standards (fig. 4B). The acrylamide concentration in the gel was 5%.

3. Results and discussion

The purification of factor III specific for lactose is summarized in table 1. The purification steps include a heat denaturation, an acid precipitation, a DEAE cellulose chromatography (fig. 1a), a Sephadex G 100 chromatography (fig. 1b). The resulting material migrated as a single band in the disc gel electrophoresis at various pHs and in sodium dodecylsulfate (fig. 2). Further evidence for the purity of the enzyme was obtained by comparing the protein patterns obtained after disc electrophoresis of the extracts of an uninduced and a constitutive culture (strains S 5601 and S 305 A). The band missing in the uninduced strain is visible in the constitutive one (fig. 3). The heavy band migrating slower than the factor III band contains the staphylococcal 6-phospho-β-galactosidase which is produced in large amounts by the strain constitutive for lactose metabolism. The molecular weight estimated by gel filtration was found to

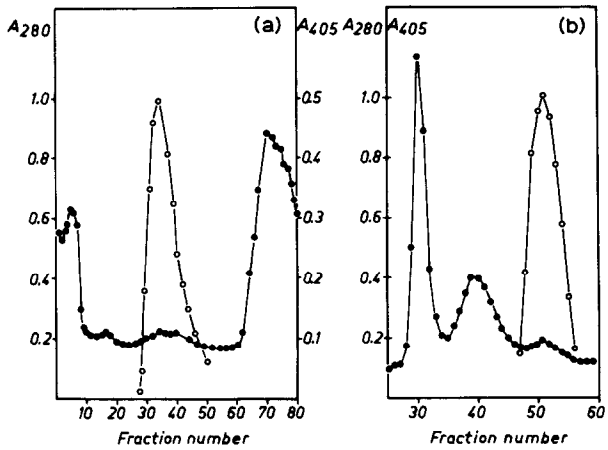

Fig. 1. (a) Gradient elution of factor III from the DEAE cellulose column. Absorbance at 280 nm (●——●), factor III activity (A at 405 nm) 0.2 ml was tested for FIII activity (○——○). (b) Chromatography of the eluate from the DEAE cellulose column on Sephadex G 100. Symbols as in (a). 0.1 ml was tested for FIII activity.

Table 1
Purification of factor III specific for lactose.

Step	Volume (ml)	Total protein mg	Total units	Units per mg	Overall purification	Yield (%)
Cell-free extract	200	9900	2850	0.28	–	100
Heat denaturation	200	2020	2550	1.25	5	90
Acid precipitation	40	400	1540	3.8	15	54
Gradient elution from DEAE cellulose	10	91	1225	13.5	50	43
Chromatography on Sephadex G 100	4.8	9.6	300	31	110	11*

* Half of the activity of factor III eluted from the G 100 column was pooled and concentrated.

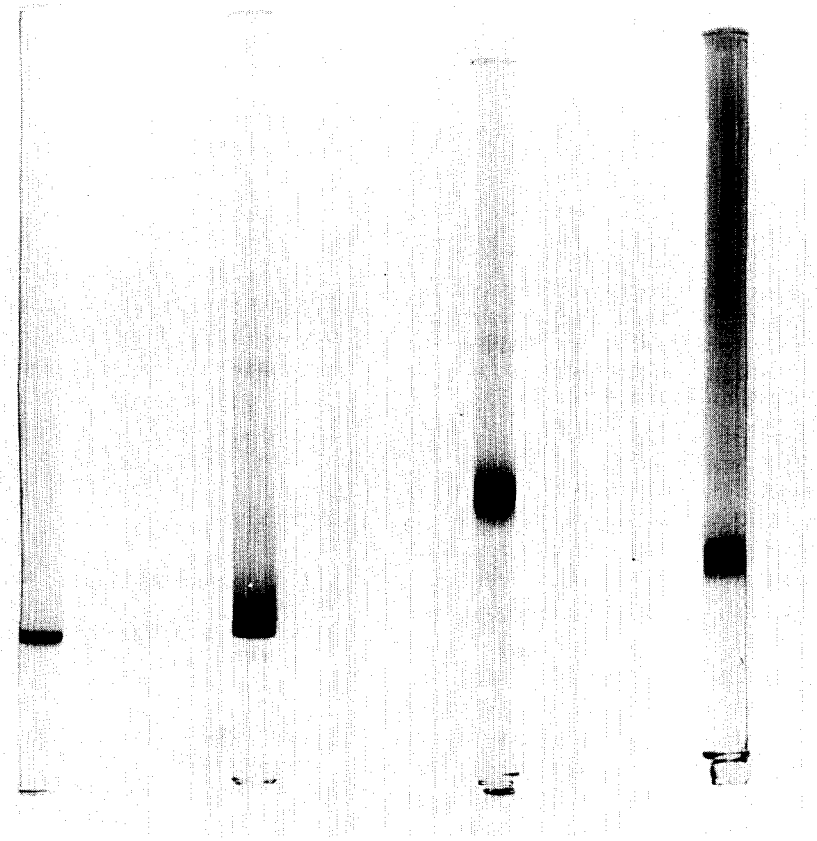

Fig. 2. Acrylamide gel electrophoresis of purified factor III. Gels from left to right: Anode at the bottom, 4 μg of factor III; 20 μg, pH 9.3 tris-glycine buffer 0.4 M; 20 μg, pH 7 sodium phosphate buffer 0.05 M; 10 μg, 0.1% sodium dodecylsulfate in 0.1 M sodium phosphate pH 7.2.

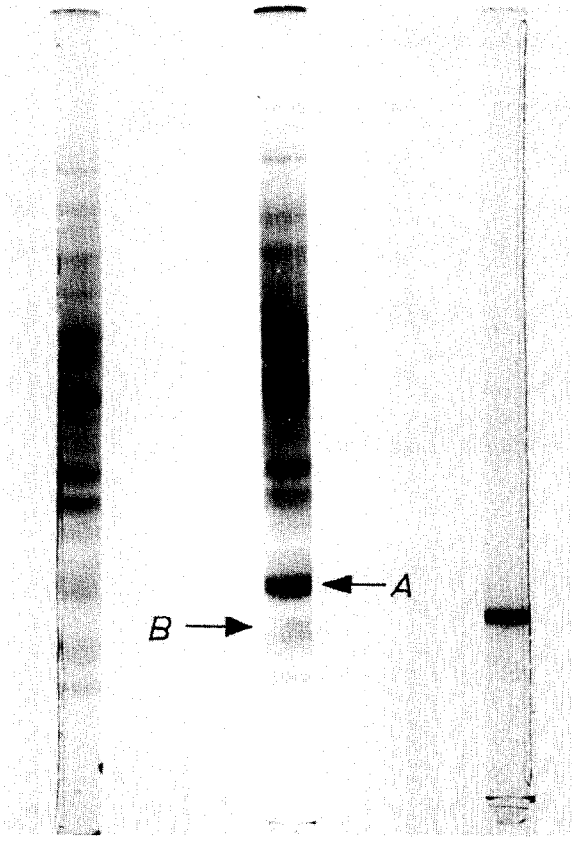

Fig. 3. Acrylamide gel electrophoresis of extracts of a culture constitutive for lactose metabolism and an uninduced culture. *Left gel*: extract of the uninduced culture; *middle gel*: extract of the constitutive culture; *right gel*: purified factor III. A → 6-phospho-β-galactosidase, B → factor III.

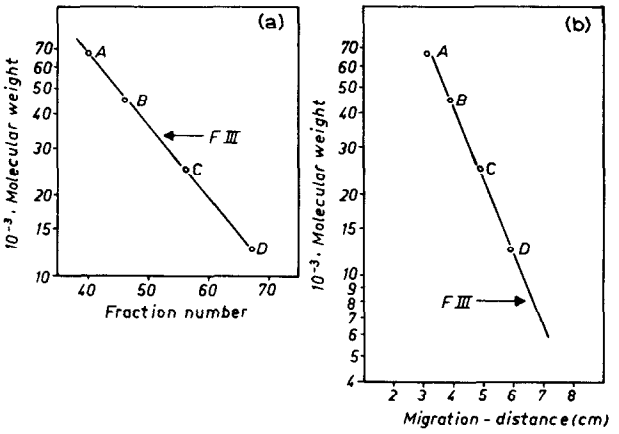

Fig. 4. (a) Estimation of the molecular weight of factor III by gel filtration on Sephadex G 100. The fraction number (1 fraction = 5 ml) was plotted against the logarithm of molecular weight. Protein standards: (A) bovine serum albumin 67,000, (B) ovalbumin 45,000, (C) chymotrypsinogen 25,000, (D) cytochrome C 12,500 → factor III. (b) Estimation of the molecular weight of the polypeptide chain by acrylamide gel electrophoresis in 0.1% sodium dodecylsulfate. The migration distance of the samples was plotted against the logarithm of the molecular weight. Standard proteins and symbols are the same as in (a).

be 33,000 ± 10% (fig. 4a). The treatment of the native protein with sodium dodecylsulfate dissociated the 33,000 molecular weight proteins into its protomeres (fig. 4b). The molecular weight of the protomere of 8000 ± 10% suggests that the native protein contains 4 polypeptide chains.

More detailed investigations about the subunits of the protein should reveal whether the protein is composed of identical or chemically different subunits.

The availability of homogeneous factor III specific for lactose may enable us to elucidate its physiological function by kinetic analysis of the PEP dependent phosphorylation reaction. We hope to be able to correlate the subunit structure of the protein withi its mechanism of action.

The genetic linkage of the gene for factor III to the other structural genes of the lactose metabolism in *S. aureus* [9] favours the concept that factor III is involved in the transfer of phosphate from the heat stable component HPr to the sugar.

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