Journal of Chromatography, 26 (1983) 206–209
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,662

Note

New method for the purification of 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (E.C. 2.7.6.3) from *Escherichia coli*

Application of hydrophobic interaction chromatography and flat-bed isoelectric focusing

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(Received December 30th, 1982)

The enzyme 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (kinase, E.C. 2.7.6.3) from *Escherichia coli* has been purified by the classical separation techniques, gel filtration and ion-exchange chromatography[1,2]. In this paper we report methods for further purification of this enzyme based on hydrophobic properties and isoelectric characteristics of the enzyme.

Following the classical procedure of Richey and Brown[1] including the gel filtration step (fraction I, containing proteins with M_r of about 20,000), the enzyme was further purified in two consecutive steps: (1) hydrophobic interaction chromatography (HIC) on a phenyl-Sepharose CL-4B column (fraction II) and (2) flat-bed isoelectric focusing (flat-bed IEF) in a granulated gel slab (fraction III).

EXPERIMENTAL

All enzyme purification steps were carried out at 4°C unless stated otherwise.

Fraction I was adjusted to the salt concentration of a starting buffer, 10 m*M* Tris–HCl (pH 8.1) (buffer A) + 3 *M* NaCl, and applied to a phenyl-Sepharose CL-4B column (Pharmacia). After elution of unbound and inactive material with starting buffer, bound kinase was eluted with the aid of a decreasing salt gradient of 3 *M* NaCl in buffer A to pure buffer A (for details see Fig. 1).

In the case of HIC, enzyme activity was determined by following the decrease of the fluorescent substrate, 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine, by a thin-layer chromatographic (TLC) method[3] and expressed as area under the curve in arbitrary units (TLC-scanning). In the case of flat-bed IEF, enzyme activity was determined from the increase of the likewise fluorescent reaction product.

Fractions with kinase activity were pooled, dialyzed against buffer A by an ultrafiltration procedure (PM-10 membrane, Amicon Corp.) and lyophilized (fraction II). Fraction II was further purified by flat-bed IEF in a shallow pH gradient stabilized by an agarose-Sephadex matrix (1.5 × 100 × 180 mm) according to ref. 4. For a better sample application, a trough (2 × 60 mm, depth 0.8 mm) was cut out. The gel containing a 3% carrier ampholyte mixture of Servalyt AG 3-5 and 2-11 (1:1) was

subjected to a prefocusing step of 3200 V · h (TLE-double chamber, Desaga) at 15°C. An aliquot of Fraction II was dissolved in about 150 µl of a 3% solution of Servalyt AG 5-8 (mean pH 7.5). This solution was transferred into the through and isoelectric focusing was carried out at 12,800 V · h and 15°C.

After focusing a contact print was prepared and stained for protein identification according to ref. 5. The gel was divided into segments of width 7 mm with the aid of a grid, and the pH gradient was determined on the gel surface at room temperature with a glass micro-electrode (Ingold No. 403-6298-K7). The gel segments were scraped off, and the material was mixed and homogenized in 500 µl of 0.4 *M* Tris–HCl buffer, pH 7.9, squeezed by centrifugation (Eppendorf centrifuge 3200) and the supernatant analyzed for enzyme activity according to ref. 3. For more details concerning the enzyme elution and removal of carrier ampholytes see ref. 6.

The purification procedure was monitored by isolelectric focusing in ultrathin-layer polyacrylamide gels[7].

RESULTS AND DISCUSSION

Many proteins have hydrophobic sites on their surfaces[8,9]. The first stage of the purification of the enzyme was based on this property.

As shown in Fig. 1 the maximum kinase activity is eluted from the phenyl-

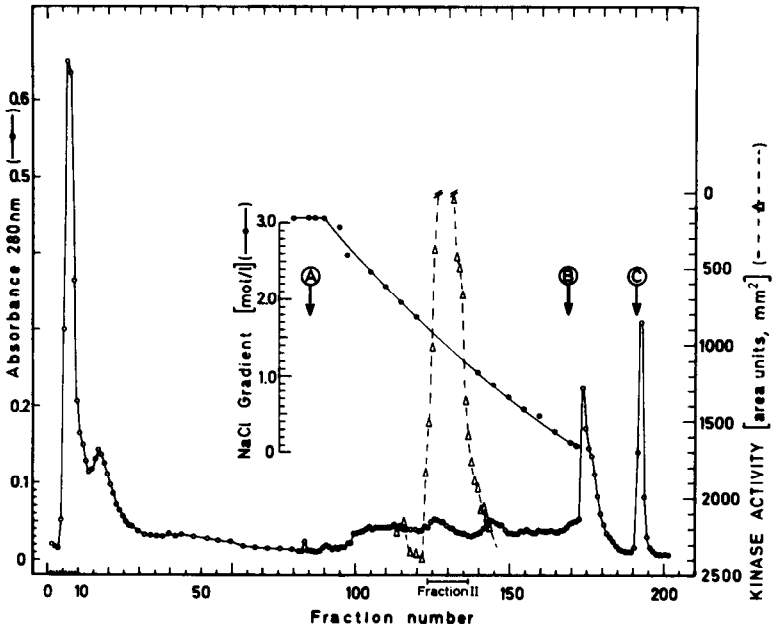

Fig. 1. Hydrophobic interaction chromatography of kinase activity on a phenyl-Sepharose CL-4B column (2 × 9 cm). Sample: fraction I (Sephadex G-100), 4.5 mg protein/ml in a total volume of 21.5 ml starting buffer (3 *M* NaCl in buffer A). Flow-rate: 12 ml/cm² · h. Fractions: 8.7 ml. A, Commencement of gradient of 3 *M* NaCl in buffer A to pure buffer A (250 ml each); B, pulse of buffer A; C, pulse of twice distilled water. Kinase activity (substrate) was determined as indicated in ref. 3. Fractions 123–136 were pooled and treated as indicated in Experimental (fraction II).

Sepharose CL-4B column by lowering the salt concentration from 3 *M* to about 1.4 *M* NaCl in buffer A. As regards the amount of proteins[10] in fractions I and II, respectively, the degree of purification by the HIC technique was about 70–100 fold. When the more hydrophobic matrix octyl-Sepharose CL-4B was tried instead of phenyl-Sepharose little kinase activity was recovered in the presence of buffer A and 20% ethylene glycol. Using twice distilled water most of the enzyme activity could be regained. Thus the kinase protein seems to exhibit at least one hydrophobic site.

A very efficient further purification step was achieved when fraction II was subjected to preparative flat-bed IEF (Fig. 2) to yield fraction III. This is seen by comparing the protein patterns of fractions II and III, obtained by the highly selective ultrathin-layer IEF technique (Fig. 3). The enzymatically inactive main band of fraction II centered at pH 4.8 (compare contact print at the top of Fig. 2) could clearly be separated from the proteins of segments 10–12 containing the bulk of activity (Fig. 2). Flat-bed segment 11 representing the peak of kinase activity within a range p*I* 4.55–

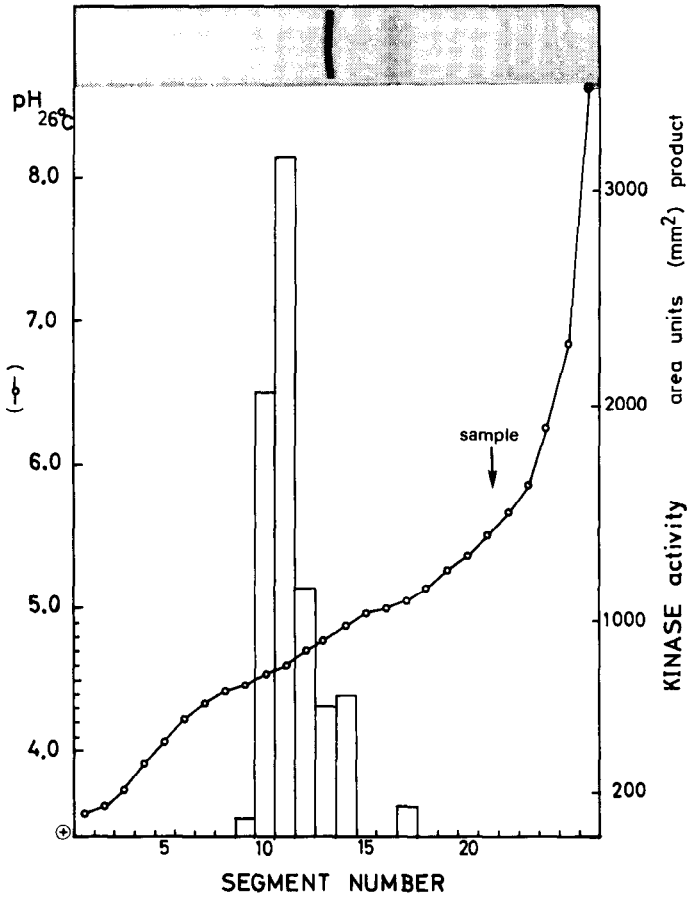

Fig. 2. Preparative isoelectric focusing of kinase fraction II in a 1.5 × 100 × 180 mm slab according to ref. 3. 3% carrier ampholyte mixture of Servalyt AG 3-5 and 2-11 (1:1); anode, 25 m*M* aspartic acid, 25 m*M* glutamic acid; cathode, 2 *M* ethanolamine, 25 m*M* arginine, 25 m*M* lysine; prefocusing, 3200 V · h at 15°C; sample, buffered solution of 2 mg protein (fraction II) applied in the trough (indicated by arrow); focusing, 12,800 V · h at 15°C; contact print stained for protein (Whatman No. 3 paper, Serva Blue G, see ref. 5. For method of determination of the pH gradient and enzyme activity (product) see Experimental. After the carrier ampholytes had been separated from the recovered fractions by gel filtration on PD-10 columns (Pharmacia) equilibrated with water, the samples were lyophilized (peak activity of segment 11 as fraction III).

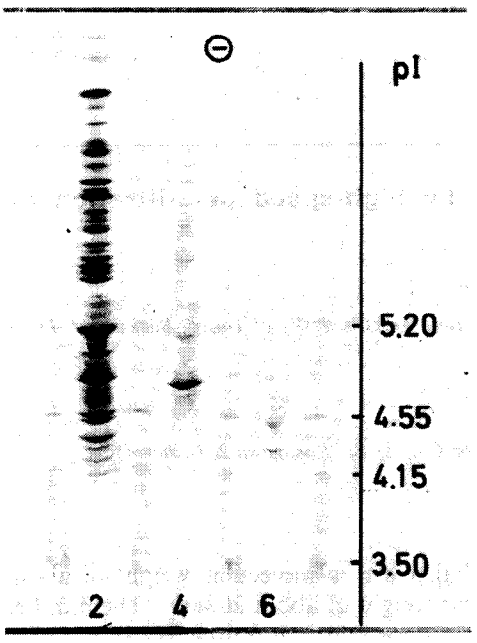

Fig. 3. Isoelectric focusing of kinase fractions in an ultrathin-layer polyacrylamide gel slab stained for protein[7]. Lanes: 1, 3, 5, 7; Pharmacia calibration proteins (low p*I* kit); 2 fraction I (13 μg protein); 4, fraction II (2 μg); 6, fraction III (less than 0.5 μg); corresponding p*I* values as indicated. Conditions: polyacrylamide gel 5% T/3% *C**, 0.1 × 50 × 90 mm; 3% carrier ampholyte mixture of Servalyt AG 3-5 and 2-11 (1:2); prefocusing, 80 V · h; focusing, 550 V · h at 4°C; anolyte and catholyte, see ref. 7.

4.65 reveals the proteins in two main and three side bands (Fig. 3, lane 6). Because of the very low protein content of fraction III the purification effect in this step could not be determined.

In flat-bed IEF it is important to note that the prefocusing conditions of the gel slab may be significant for the migration of the kinase activity in the pH gradient, since without sufficient prefocusing (at least 3000 V · h) this activity is scattered over a wide range from p*I* 4.5 to 5.6 with several focal points. Finally, the point of sample application (pH 5.5–6) may be decisive for the selectivity.

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*gel concentration $T = \frac{(a + b) \cdot 100}{V}$ %; cross-link $C = \frac{b \cdot 100}{a + b}$ %

where a = gram acrylamide, bn = gram cross-link (N,N-methylene bisacrylamide), V = volume of unpolymerized solution.