ω-AMINOHEXYL-SEPHAROSE IN PURIFICATION OF LIVER GLYCOGEN PHOSPHORYLASE *b*

N. B. LIVANOVA, T. B. ERONINA, G. V. SILONOVA
A. N. Bakh Institute of Biochemistry of the USSR Academy of Sciences, Moscow 117071, USSR

and

E. V. RAMENSKY
Institute of Biological and Medical Chemistry of the USSR Academy of Medical Sciences, Moscow 119117, USSR

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1. Introduction

Liver glycogen phosphorylase exists in two interconvertible forms: inactive non-phosphorylated and active phosphorylated ones [1–3]. In contrast to the muscle enzyme, both forms of liver phosphorylase have molecular weights of about 185 000 and the two forms have been assumed to be dimers [4,5].

Both forms of liver phosphorylase have been isolated in highly purified states from the livers of dog [1], rabbit [5,6], pig [5] and rat [7].

The presence of particulate glycogen in liver extracts and the binding of phosphorylase to its polysaccharide substrate has been successfully used at the first step of phosphorylase purification [5,6]. The use of repeated chromatography on a DEAE-cellulose column as the next step in preparation of homogeneous liver phosphorylases *a* and *b* [4–6] does not appear to be satisfactory, because the yield is too low.

In this paper we report a novel method for the purification of rabbit liver glycogen phosphorylase *b* using ω-aminohexyl-Sepharose chromatography and electrofocusing. The subunit molecular weight has been determined by polyacrylamide gel electrophoresis with SDS.

2. Materials and methods

Sepharose 4B (Pharmacia) was activated within 2–3 min at pH 10.5–11 and 22°C by addition of 0.3 g CNBr to 10 g (wet weight) of Sepharose. CNBr was dissolved in acetonitrile and added to agarose beads suspended in a solution of 2 M sodium carbonate [8].

Coupling of activated Sepharose with α,ω-hexamethylendiamine was according to Shaltiel [9]. The amount of diamine coupled with CNBr-activated agarose was determined by titration of substituted Sepharose suspension in 0.25 M NaCl with 0.01 M HCl.

Phosphorylase activity was assayed following Illingworth and Cori [10]. The reaction mixture contained 20 mM β-glycerophosphate, 1 mM mercaptoethanol, 1 mM EDTA, 1% glycogen, 700 mM Na_2SO_4, 1 mM AMP and 18 mM or 100 mM glucose-1-phosphate; pH 6.5, the incubation at 30°C for 10 min.

Protein concentration was measured by Lowry's method [11] or spectrophotometrically using an absorbancy index (1% × cm^{-1}) of 13.2 [12].

Isoelectric focusing was done in an LKB 8100-1 analytic column; bed volume 110 ml, pH gradient 5–7, in sucrose density gradient 0–40%. The final

concentration of ampholines was 1%. A potential of 600 V for 67 h, at 4°C was used.

Disc gel electrophoresis was as described by Ornstein and Davis [13] and Davis et al. [14]. Electrophoresis in the presence of SDS was according to Weber and Osborne's method [15]. Protein was stained with 0.25% Coomassie Brilliant Blue in methanol/acetic acid/water and destained with 7% acetic acid.

3. Results and discussion

Fed adult rabbits were killed by decapitation. The livers were removed, washed, weighed and homogenized in a Waring Blendor in 4 vol cold 1 mM EDTA, pH 7.0, for 2 min. The homogenate was centrifuged for 15 min at 3000 × *g*, the supernatant was filtered through cheesecloth. The extract was centrifuged for 2 h at 62 000 × *g*. The glycogen pellet was collected and solubilized in buffer A (4 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol pH 7.2).

The suspension (50 ml, 100 mg of protein) was applied to a column of ω-aminohexylagarose (30 × 1.5 cm) equilibrated with buffer A pH 7.2, at 4°C. Unadsorbed protein and glycogen were washed off with the same buffer. Under loading conditions such a column binds all the phosphorylase *b* and about 40% of the other proteins of the mixture.

The column was then developed with a linear gradient of 0.5 l of buffer A to 0.5 l of buffer A containing 50 mM Na_2SO_4, at a constant flow rate of 9 ml/15 min regulated by a Microtechna pump. The eluate was monitored by absorption at 280 nm and by phosphorylase activity (fig.1). Fractions 79–98, containing phosphorylase *b*, were pooled and concentrated to 10 ml by pressure dialysis (Amicon Corp.) over N_2 gas using a PM-30 filter.

The concentrated solution passed through a column of Sephadex G-50 that had been equilibrated with bidistilled water. Then this highly purified phosphorylase *b* preparation, with a specific activity of 18 ± 2 units/mg (at 18 mM glucose-1-phosphate), was electrofocused. After electrofocusing the column was eluted with a peristaltic pump. Fraction volume was 3 ml, for each fraction the pH was determined immediately with a pH-262 pH-meter and the A_{280} using an SF-4A spectrophotometer.

The fractions were assayed for phosphorylase activity after removing ampholines by passing through a column of Sephadex G-50 that had been equilibrated with 4 mM Tris–HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 6.8. Fractions containing phosphorylase *b* were pooled, concentrated by ultrafiltration and stored under toluene vapour at 4°C. Storage for at least 1 month did not affect enzyme specific activity.

Figure 2 shows a typical electrofocusing experiment.

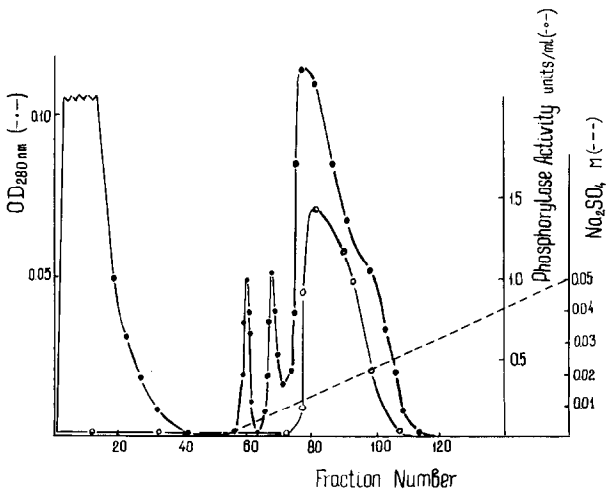

Fig.1. Elution pattern of liver phosphorylase *b* from ω-aminohexyl-Sepharose column chromatography. Conditions as indicated in the text.

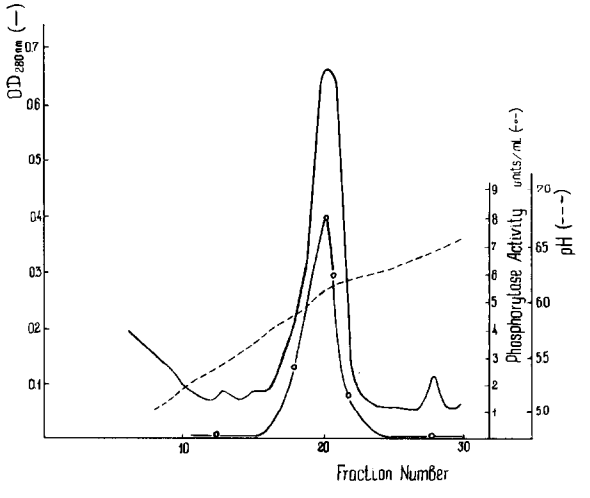

Fig.2. Narrow range electrofocusing of 7 mg of liver phosphorylase *b*.

Table 1
Purification of rabbit liver glycogen phosphorylase *b*[a]

Fraction	Total protein (mg)	Total units[b]	Sp. act. (units/mg)	Yield (%)
Liver extract	12 760	191	0.015	
Glycogen-enzyme pellet	275	264	0.96	100
ω-Aminohexyl-Sepharose peak fractions	12.8	230	18.00	87
Isoelectric focusing peak fractions	7.7	154	20.0	58

[a] Starting material, 200 g of fresh rabbit liver.
[b] Phosphorylase units measured as described in text, at 18 mM glucose-1-phosphate

There was one main peak with high phosphorylase activity (20 ± 2 units at 18 mM of glucose-1-phosphate) and several minor peaks, devoid of phosphorylase activity. The isoelectric point of the main peak was 6.2.

Results of the purification procedure are summarised in table 1. The first and very effective step is the separation of enzyme–glycogen complex by centrifugation. Previous authors [5,6] had collected the glycogen pellet by centrifugation for 2 h at 35 000 × *g* and about 70% of the phosphorylase remained in the supernatant. We have that after the separation of protein–glycogen complex by centrifugation for 2 h at 62 000 × *g* only a neglegible amount of phosphorylase remains in the supernatant solution.

It is noteworthy that the total phosphorylase activity of the glycogen pellet is higher than the total activity of liver extract. The latter probably contains a phosphorylase inhibitor that does not bind to glycogen and remains in the supernatant. Addition of supernatant to the glycogen pellet and to purified liver or muscle phosphorylases *b* and *a* causes a noticeable inhibition of activity; preincubation of supernatant with trypsin showed that this inhibitor is trypsin-sensitive. The nature and the mode of action of this inhibitor are under study now.

ω-Aminoalkyl-Sepharoses are very effective in the purification of several proteins [16]. Their retention and discrimination power may involve different types of interactions, however, hydrophobic interactions seem to play a key role in the resolution. Shaltiel and Er-El [9] showed, using a series of Sepharoses substituted with homologous α,ω-diaminoalkanes, that

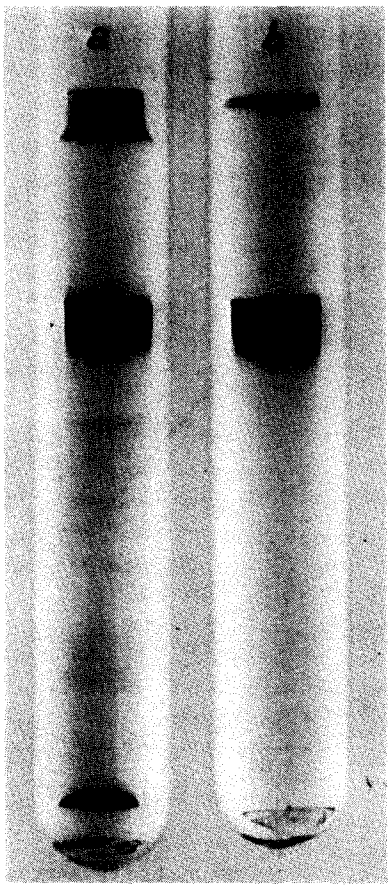

Fig.3. Disc gel electrophoresis of purified phosphorylase *b* (30 μg) in 7% gel followed by staining with 0.25% Coomassie Brilliant Blue. (a) Phosphorylase *b* after aminohexylagarose chromatography and (b) Phosphorylase *b* after electrofocusing.

ω-aminohexyl-Sepharose is the most appropriate for adsorbing rabbit muscle phosphorylase *b*. The binding properties of alkylamine-Sepharose columns depend both on the length of the hydrocarbon side chains and on the degree of substitution with alkylamine groups [17].

We varied the degree of Sepharose substitution by varying the cyanogen bromide concentration in the activation mixture and found that, under identical conditions of pH, ionic strength, buffer composition and temperature, the ability of ω-aminohexyl-Sepharose to retain liver phosphorylase *b* strongly depends on the degree of substitution. The affinity for phosphorylase *b* increases with increasing of hydrophobicity of the Sepharose. We found that optimal retention and discrimination power resulted when the activation mixture contained 0.3 g of CNBr per 10 g of Sepharose and 1.3 ± 0.1 μequiv of ω-hexylamino groups were coupled per milliliter of packed Sepharose.

A preparation carried to the last stage of purification appeared to be homogeneous: it sedimented as a single component in the analytical ultracentrifuge and produced a single band in acrylamide gel electrophoresis (fig.3). Preparations after aminohexyl-Sepharose chromatography usually produced one main band and several minor bands by disc gel electrophoresis (fig.3). The activity occurring only in the main band.

The specific activity of the enzyme after electrofocusing was 35–40 units/mg, at 100 mM glucose-1-phosphate, which is higher than the 25 units/mg obtained by Wolf et al. [4].

The mol. wt of liver phosphorylase *b* subunits was estimated by Na-dodecylsulfate gel electrophoresis to be 100 000 (fig.4).

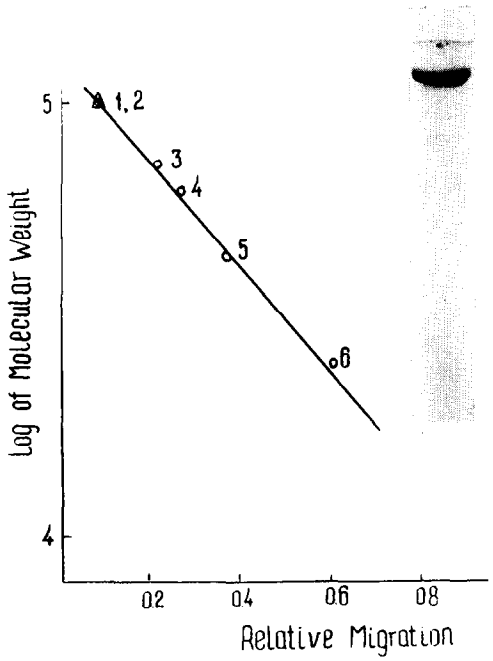

Fig.4. Subunit molecular weight of rabbit liver phosphorylase *b* by Na dodecylsulphate gel electrophoresis in 10% gel. (1,2) Muscle and liver glycogen phosphorylase (100 000); (3) bovine serum albumin (68 000); (4) catalase (60 000); (5) ovalbumin (43 000); (6) chymotrypsinogen (24 000). The electrophoregram of the liver phosphorylase *b* (10 μg) is shown in the insert.

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