BIOSPECIFIC AFFINITY CHROMATOGRAPHY OF SMOOTH MUSCLE GLYCOGEN PHOSPHORYLASE *b* ON GLYCOGEN–SEPHAROSE

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1. Introduction

Purification of biopolymers by affinity chromatography is often complicated by their nonspecific binding to affinity adsorbents [1,2]. The nonspecific effects were minimal when water-soluble branching and neutral polysaccharides were used as spacers in affinity adsorbents [3]. At the same time a polysaccharide–solid support system may be recommended as the biospecific adsorbent for proteins possessing the affinity to the immobilized polysaccharide.

This communication reports a new technique for the coupling of glycogen to Sepharose. The utilization of this adsorbent in a new procedure for the purification of smooth muscle glycogen phosphorylase *b* ('brain' isoenzyme) is also described.

2. Experimental

2.1. *Materials*

Sepharose 4B was purchased from Pharmacia Fine Chemicals. BrCN was obtained from Serva, AMP from Reanal and 1,6-diaminohexane from Koch-Light. Succinic acid dihydrazide, acetonitrile, $NaIO_4$, $NaBH_4$ were commercial grade reagents. Glycogen was isolated from rabbit liver according to [4].

2.2. *ω-Aminohexyl–Sepharose (AH–Sepharose)*

ω-Aminohexyl–Sepharose 4B was prepared as in [5]. ω-Aminohexyl group concentration was 1.3 μequiv./ml packed gel in contrast to the commercial AH–Sepharose samples (Pharmacia) which contain ~6–10 μequiv./ml.

2.3. *Glycogen–hydrazidosuccinyl–Sepharose (GH–Sepharose)*

Sepharose 4B was activated by modification of the methods in [6,7]. CNBr solution (4.5 g CNBr in 2.5 ml CH_3CN and 12.5 ml water) was added dropwise over 1–2 min at 4°C to suspension containing 30 ml packed Sepharose in 30 ml 5 M potassium phosphate buffer, pH 11.9. The slurry was vigorously stirred for 2–3 min at 4°C, the gel was washed with 0.5 l cold 0.1 M $NaHCO_3$ and the activated Sepharose was immediately suspended in solution containing 60 mmol succinic acid dihydrazide in 40 ml of water, pH 8. The slurry was stirred for 6 h at 20°C. Washed hydrazide-succinyl-Sepharose was mixed with a solution of 2.41 g glycogen pre-oxidized with $NaIO_4$ (1.5 h, pH 6.5–7.0, 5 equiv. $NaIO_4$/100 glycogen anhydroglucose residues) in 35 ml 0.1 M Na acetate, pH 5. The mixture was stirred for 16 h at 4°C. The gel was filtered, washed with water, 2 M NaCl and subsequently mixed with freshly prepared solution of 600 mg $NaBH_4$ in 30 ml 0.1 M $NaHCO_3$, pH 8.5. The slurry was stirred for 3 h at 4°C and washed with water.

2.4. *Isolation and partial purification of glycogen phosphorylase*

Cow uteri, 4 kg, were minced and extracted with 50 mM NaF–1 M EDTA, pH 7, fractionated with $(NH_4)_2SO_4$ and dialyzed. The insoluble protein–glycogen complex (234 mg protein) was obtained as in [8,9]. An assay following [10] was used to measure the phosphorylase activity. Protein concentration was measured by Lowry's method [11]. Disc-gel electrophoresis was carried out as in [12].

2.5. *Hydrophobic chromatography of glycogen phosphorylase on AH–Sepharose*

The above-mentioned glycogen–protein complex was solubilized in 70 ml 8 mM Tris–HCl, 1 M EDTA, 1 mM mercaptoethanol, pH 7.2 (buffer A) and dialyzed against the same buffer. The solution was applied to a column of AH–Sepharose (28 × 1.5 cm) equilibrated with buffer A at 4°C. Unbound protein and glycogen were washed off with the same buffer. The elution was performed with a linear gradient of 0.5 l buffer A – 0.5 l buffer A + 0.2 M Na_2SO_4 at 22 ml/h flow rate. The eluate was monitored by $A_{280\,nm}$ and by phosphorylase activity. Fractions containing this activity were pooled and concentrated to 50 ml by ultrafiltration (Diaflo cell, Amicon with XM 100 filter).

2.6. *Bioaffinity chromatography of glycogen phosphorylase* b *on GH–Sepharose*

The solution of the enzyme after AH–Sepharose chromatography (50 ml, 28 mg protein) was dialysed against 10 mM Tris–acetate, pH 7.4 (buffer B) and applied onto a column of GH–Sepharose (26 × 1.5 cm) equilibrated with buffer B at 4°C, 10 ml/h flow rate, fraction vol. 10 ml. The unadsorbed protein was washed off with buffer B at 15 ml/h flow rate. The column was then developed with buffer B containing 10 mM AMP at 10 ml/h flow rate. The fractions with phosphorylase activity were pooled and concentrated as in section 2.5.

3. Results and discussion

Earlier we described the purification of glycogen phosphorylase *b* from smooth muscle ('brain' isoenzyme, protophosphorylase) using conventional methods [8,9]. Now we used affinity chromatography on immobilized glycogen as the final stage in the purification procedure. The preceding step was the AH–Sepharose chromatography.

Hydrophobic chromatography of rabbit liver phosphorylase *b* with AH–Sepharose resulted in a highly purified enzyme [5]. The analogous chromatography of the smooth muscle enzyme was less effective. The elution pattern is depicted in fig.1. After AH–Sepharose chromatography the preparations of the enzyme usually contained several bands on disc electrophoresis (fig.2a). Therefore further purification was necessary.

Glycogen–aminoalkyl–Sepharose for bioaffinity chromatography of glycogen phosphorylase from skeletal muscle was attempted [13]. A nonspecific binding of the enzyme to hydrophobic spacers was found. However, the adsorbent with a short aminoethyl spacer was ineffective. It would be reasonable to suggest that in this case the adsorbent's ineffectiveness was a result of a considerable structural modification of the glycogen molecule after its treatment with CNBr. Therefore in the present study we used a milder method of controlled periodate oxidation for glycogen activation. Subsequent coupling of periodate oxidized

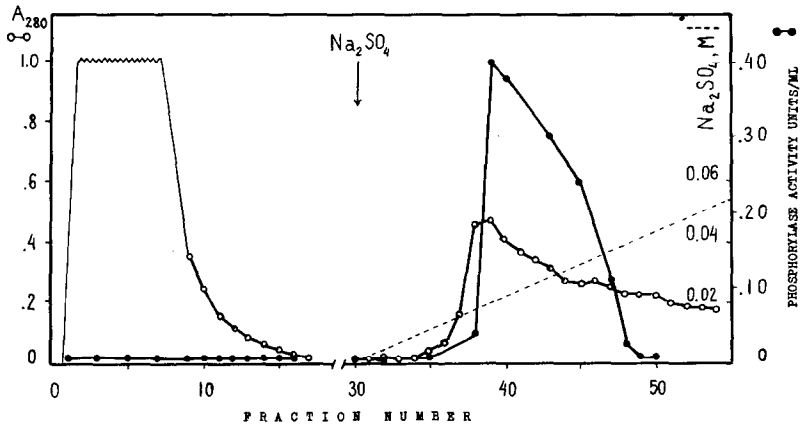

Fig.1. Elution pattern of smooth muscle phosphorylase *b* from AH–Sepharose column chromatography. For conditions see section 2.

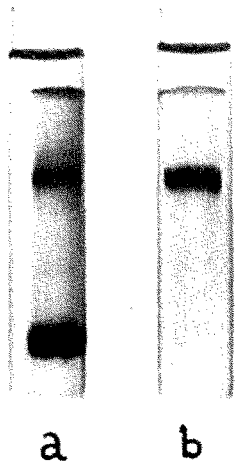

Fig.2. Disc gel electrophoresis of smooth muscle phosphorylase *b* in 7% gel followed by staining with 0.25% Coomassie dye. (a) Phosphorylase *b* after AH–Sepharose chromatography and (b) phosphorylase *b* after GH–Sepharose chromatography.

glycogen to hydrazido–succinyl–Sepharose made it possible to obtain an adsorbent devoid of charged groups at physiological pH values [14]. Such a GH-Sepharose column specifically adsorbed partially purified phosphorylase *b* from smooth muscles. The major proportion of other proteins passed through the column in the washings (fig.3). The elution of the phosphorylase with the Na_2SO_4 gradient in 0.4 M imidazole–citrate buffer containing 0.05 M mercaptoethanol and 1 mM EDTA, pH 7.4, or with 0.1 M glucose-6-phosphate in buffer B gave the electrophoretically homogeneous enzyme but with a poor yield. The quantitative desorption of non-homogeneous enzyme could be achieved when 0.4% glycogen solution was used for elution. When the column was developed with buffer B + 10 mM AMP, an activator of phosphorylase *b*, only one protein band could be detected after disc electrophoresis of active fractions (fig.2b).

The results of the purification procedure are summarized in table 1. Glycogen phosphorylase *b* was purified about 2500 times in relation to the crude myometrium extract and the recovery was 12%.

It should be noted that the adsorbent usually lost its ability to bind phosphorylase *b* after 1–2 purification cycles. Figure 3 shows that the phosphorylase activity appeared in the eluate when the column was washed with several volumes of the elution buffer. Then enzyme was eluted over about 30 h within 8–10 column volumes. It was suggested that during elution the phosphorolysis of glycogen side chains responsible for the enzyme binding could occur.

In order to prove this suggestion we attempted to digest native or periodate oxidized glycogen by phosphorylase in buffer B containing 10 mM commercial AMP for 30 h at 4°C. Indeed, glucose was detected in both solutions after ultrafiltration and acid hydrolysis.

It was established that the commercial AMP preparation contained 0.2 mol P_i/100 mol AMP. Taking into account the low capacity of the adsorbent (100 μg enzyme/1 ml gel), this amount of P_i was sufficient to inactivate the adsorbent during 1–2 purification cycles.

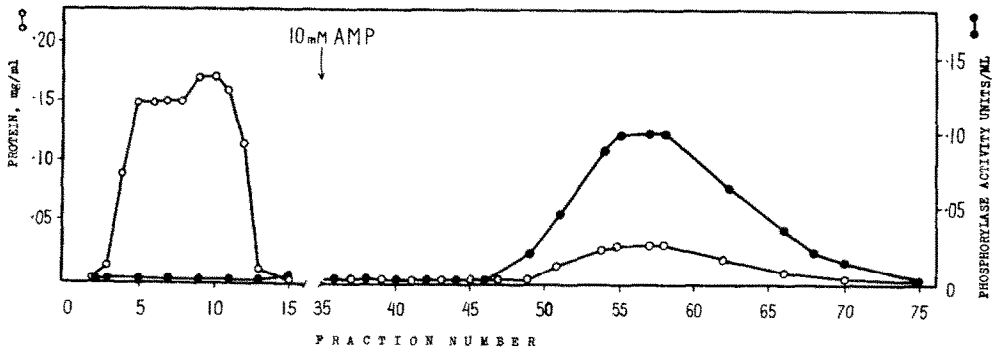

Fig.3. Elution pattern of smooth muscle phosphorylase *b* from GH–Sepharose column chromatography. For conditions see section 2.

Table 1
Purification of cow uterus glycogen phosphorylase *b*[a]

Fraction	Total protein (mg)	Total units[b]	Spec. act. (units/mg)	Purification (-fold)	Yield (%)
Myometrium extract[c]	83 125	133	0.0016	1	100
0–0.5 saturated $(NH_4)_2SO_4$	13 714	86	0.0063	4	65
Glycogen-enzyme pellet	234	47	0.20	125	35
AH–Sepharose peak fractions	28	28	1.0	625	21
GH–Sepharose peak fractions	4.0	16	4.0	2500	12

[a] Starting material, 4 kg fresh frozen cow uteri
[b] Phosphorylase units measured as μmol P_i/min at 30°C
[c] Three first purification steps as in [8,9]

Finally, when the column with adsorbed enzyme was washed with the buffer containing 10 mM recrystallized P_i-free AMP the eluate did not contain phosphorylase activity.

Thus it was proved that the removal of the enzyme from the GH–Sepharose was due to phosphorolysis of the immobilized glycogen. To determine a chromatographic process involving an irreversible reaction between an enzyme to be bound and an immobilized ligand, the term 'dynamic affinity chromatography' has been used [15]. The type of elution described here may be named 'dynamic biospecific elution'. Application of the dynamic biospecific elution makes it possible to study some aspects of enzymatic reactions.

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