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Rapid ion-exchange chromatography for preparative separation of proteins

IV. Application to bovine carbonic anhydrase III from skeletal muscle

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ABSTRACT

Bovine muscle carbonic anhydrase III was purified to homogeneity by the strategy of rapid ion-exchange chromatography. The ionic exchanger used was CM-cellulose, and this is the first application of this technique on a cation exchanger. Nitrogen gas was used to pressurize the chromatographic column to accelerate the elution. The results show that proteins with high isoelectric points can also be purified in this way. The procedure is very time-saving compared with conventional chromatography, reducing the elution time five- to ten-fold. The proteins are in addition protected against oxidation by air.

INTRODUCTION

Carbonic anhydrase (CA) III (EC 4.2.1.1) is the most abundant soluble protein in red fibres of skeletal muscle [1]. The physiological function of this enzyme is to facilitate the diffusion of carbon dioxide from the muscle cells to the blood capillaries [2]. CA III can be isolated from mammalian muscle by conventional techniques, including cation-exchange and gel-filtration chromatography [3]. These purification procedures are, however, time-consuming (they take *ca.* 2 weeks to perform), and the high cysteine content of the enzyme makes it susceptible to air oxidation. These problems were overcome by applying nitrogen gas pressure to the column during both the sample application and the chromatographic procedure. The resulting rapid ion-exchange chromatography approach, with an anion exchanger (DEAE-cellulose), has previously been found to be a successful method for preparing porcine and bovine CA from blood [4]; the technique has also been used to purify ribulose-1,5-bisphosphate carboxylase from spinach leaves [5] and coagulation factors from blood [6]. In these cases, the chromatographic times were reduced by about a factor of ten. By introducing a cation-exchange resin (CM-cellulose), this technique can also be used to purify proteins with high isoelectric points, such as CA III.

EXPERIMENTAL

Calcium phosphate gel used for batch-wise adsorption chromatography

The gel was prepared mainly according to the method of Keilin and Hartree [7], as described by Colowick [8]. Using a magnetic stirrer, 0.60 l of 2.2 *M* $CaCl_2$ and 0.64 l of distilled water were mixed with 0.60 l of 14 m*M* Na_3PO_4. The pH was adjusted to 7.4 with 1 *M* acetic acid, and the suspension was stirred for 1 h. The slurry was centrifuged (3200 *g*,

10 min), and the precipitate was carefully mixed with distilled water and centrifuged as before. The washing procedure was repeated until the conductivity was the same as in 20 m*M* sodium phosphate (pH 7.5). The gel was finally suspended in 0.60 l of the same buffer.

Chromatographic resins

CM-Cellulose CM-23 (Whatman), a fibrous resin that allows high flow-rates and resists moderate pressures, and Sephadex G-100 (Pharmacia) were used.

Rapid ion-exchange chromatography equipment

The equipment used for rapid ion-exchange chromatography is very simple and has previously been depicted (see Fig. 1 in ref. 4). In the present application, a nitrogen gas cylinder was used to pressurize the chromatographic equipment. Two 2.5-l plastic bottles with screw-caps were connected to the nitrogen cylinder and, by means of a siphon, to each other. A glass column was joined to one of the bottles, and a clamp was placed above the column stopper to keep it in position. All connections were made with poly(vinyl chloride) tubing. This chromatographic set-up was used for gradient elutions. One of the bottles and the siphon were omitted during packing of the column, equilibration, sample application and isocratic elution. After the column had been packed the surface of the gel was covered with a filter paper. All chromatographic steps mentioned above were run at the same flow-rate.

Crude enzyme purification

Fresh, lean skeletal muscle from cattle (*e.g.*, from the flank) was obtained at a local slaughterhouse. The muscle was freed from fat and connective tissue, 1.5 kg were minced, and 3 l of 20 m*M* sodium phosphate buffer (pH 7.5), containing 0.5 m*M* EDTA and 1 m*M* dithiothreitol (DTT), were added. The suspension was homogenized in a commercial Waring blender for five 1-min intervals. The suspension was carefully chilled and exposed to a powerful stream of nitrogen, when the blender was off. The homogenate was then centrifuged (3200 *g*, 1 h), and any fat floating on top of the supernatant was discarded. A volume of 170 ml of calcium phosphate gel containing 0.5 m*M* EDTA and 1 m*M* DTT was added to each litre of solution, and this mixture was stirred under nitrogen for 30 min. The gel was removed by centrifugation (3200 *g*, 20 min), and the supernatant was dialysed overnight against 3 m*M* sodium phosphate buffer (pH 6.8), containing 0.5 m*M* EDTA and 1 m*M* DTT, in a weak flow of nitrogen gas. The purification was performed in a cold room (+4 to +8°C).

First rapid ion-exchange chromatography

The crude enzyme extract (2.9 l, 14.4 g of protein) was chromatographed on a CM-cellulose gel (38 × 4.0 cm I.D.) eluted with a linear gradient (10–30 m*M*) of two 2.5-l volumes of sodium phosphate buffer (pH 6.8), containing 0.5 m*M* EDTA and 1 m*M* DTT. Prior to the chromatography, the enzyme extract and the cellulose gel were equilibrated to the same ionic strength and pH as the 10 m*M* buffer. The flow-rate was *ca.* 20 ml/min, and the volume of the fractions was 25 ml. The fractions with enzyme activity were pooled and concentrated under nitrogen (Amicon Diaflo ultra-filters, PM 10).

Gel filtration

A column was filled with Sephadex G-100 (85 × 7.5 cm I.D.), and the concentrated enzyme solution (190 ml, 1.7 g of protein) from the rapid ion-exchange chromatography was applied. Elution was achieved with 3 l of 6 m*M* Tris–H_2SO_4 (pH 7.5), containing 0.5 m*M* EDTA and 1 m*M* DTT. The flow-rate was 2 ml/min, and the fraction volumes were 20 ml. The fractions with enzyme activity were pooled.

Second rapid ion-exchange chromatography

The pooled enzym sample (800 ml, 880 mg of protein) from the gel filtration was rechromatographed on the CM-23 resin in the same column as before. Prior to chromatography, the enzyme sample and the cellulose gel were equilibrated to the same ionic strength and pH as 6 m*M* Tris–H_2SO_4 (pH 7.5), containing 0.5 m*M* EDTA and 1 m*M* DTT. The elution was started with 1 l of the equilibration buffer, and this was followed by a linear gradient elution step with 6–15 m*M* of the same buffer (two 2.5-l volumes). Finally, an isocratic elution step was applied (1.5 l of the 15 m*M* buffer). The flow-rate and the fraction volumes were the same as during the first rapid ion-exchange chroma-

tography. The fractions with activity were pooled and concentrated.

Extra purification step

An extra ion-exchange chromatographic purification step was included when extremely pure preparations were needed. This chromatography was performed without applying any extra pressure to the column. A fraction of the concentrate (35 ml, 102 mg of protein) from the second rapid ion-exchange chromatography was then applied to a CM-23 column (33 × 1.1 cm I.D.). The enzyme sample and the column were equilibrated to the same ionic strength and pH as 6 m*M* Tris–H_2SO_4 (pH 7.5), containing 0.5 m*M* EDTA and 1 m*M* DTT. Elution was first performed with 70 ml of the equilibration buffer, followed by a linear gradient (two 50-ml volumes) of 6–15 m*M* of the same buffer and terminated with 200 ml of 15 m*M* buffer. The flow-rate was 2 ml/min, and the volume of the fractions was 1.4 ml. Fractions with activity were pooled and stored under nitrogen.

Activity measurements

The carbon dioxide hydration activity was determined by the colourimetric method of Rickli *et al.* [9]. The number of activity units (AU) was defined as $5 \times (t_0/t - 1)/V_e$, where t and t_0 are the times for change of indicator colour with and without enzyme, respectively, and V_e is the volume (ml) of enzyme solution added to the total assay solution of 5 ml.

Protein concentration determination

Protein concentrations were estimated spectrophotometrically using an $A_{280\,nm}$ of 2.07 for a 1 mg/ml solution of CA III [3].

Determination of isoelectric points

The p*I* values of bovine CA III and of myoglobin were determined with the aid of the PhastSystem (Pharmacia). A standard containing eleven proteins with p*I* values from 3.75 to 9.30 was used.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Electrophoresis was carried out in a Mini-PROTEAN II dual slab cell (Bio-Rad). The concentrations of the spacer gel and the separation gel were 4% and 15%, respectively. Separation of the proteins was performed for 40 min at 180 V. A 0.025 *M* Tris–glycine buffer (pH 8.3), containing 3.5 m*M* SDS, was used as electrode buffer. An electrophoresis calibration kit for molecular mass determination of low-molecular-weight proteins (Pharmacia) was used as a reference. The protein bands were stained with 0.1% Coomassie blue R-250 in methanol–acetic acid.

RESULTS

By using the strategy of rapid ion-exchange chromatography, a yield of 331 mg of bovine CA III was obtained from 1.5 kg of muscle in a considerably shorter time than with conventional techniques. The various purification steps are summarized in Table I.

TABLE I

PURIFICATION OF BOVINE MUSCLE CARBONIC ANHYDRASE[a]

Purification step	A_{280} (cm^{-1})	A_{420}/A_{280}	Spec. act.[b] (AU/A_{280})	Yield of activity (%)	Purity
First centrifugation	36.6	0.54	2.9	100	1
First rapid ion-exchange chromatography	18.9	1.06	31.1	45	11
Gel filtration chromatography	2.4	1.25	45.8	36	16
Second rapid ion-exchange chromatography	6.0	0.023	105	30[c]	37

[a] Starting material was 1.5 kg of fresh skeletal muscle.
[b] Specific activities were calculated as the number of activity units (AU) in the assay solution divided by A_{280}.
[c] The yield of purified enzyme was 331 mg.

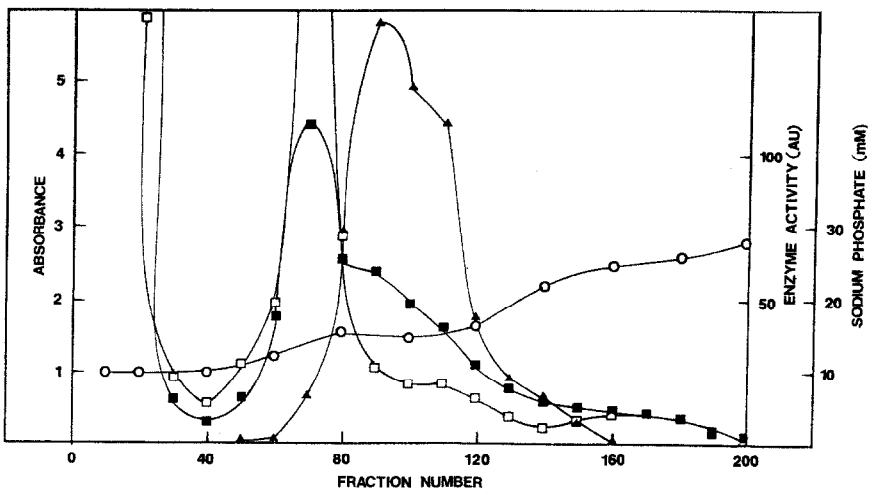

Fig. 1. First rapid ion-exchange chromatography of crude bovine CA III on a CM-cellulose CM-23 column. Gel bed dimensions, 38 × 4.0 cm I.D.; flow-rate, 20 ml/min (96 ml/h · cm^2); applied pressure, 0.2–0.3 kg/cm^2; volume of fractions, 25 ml; temperature, +4 to +8°C. Fractions 65–150 were pooled and concentrated. ■ = $A_{280\,nm}$; □ = $A_{420\,nm}$; ▲ = carbon dioxide hydration activity; ○ = buffer concentration. For further details, see Experimental.

Initial purification

To obtain an enzyme preparation in high yield, it is important that the bovine muscle tissue is not more than 2 days old: the older the tissue is, the more carbonic anhydrase seems to be associated to the muscle protein myoglobin, leading to separation problems and reduced yield of active enzyme. Furthermore, the oxidized fraction of the cysteines in the enzyme increases with time. To avoid oxidation by air, nitrogen gas was used in the initial purifica-

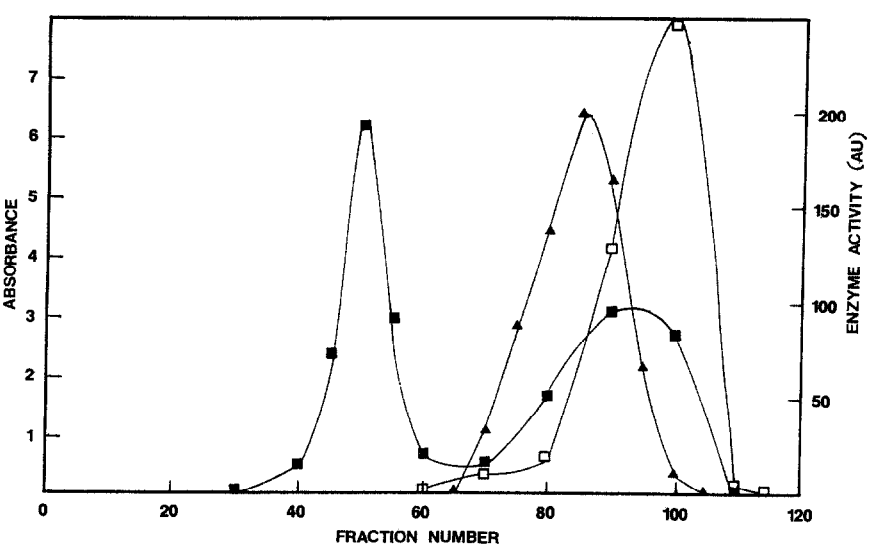

Fig. 2. Gel filtration chromatography of the concentrated bovine CA III solution from the first rapid ion-exchange chromatography on a Sephadex G-100 column. Gel bed dimensions, 85 × 7.5 cm I.D.; flow-rate, 2 ml/min (2.7 ml/h · cm^2); volume of fractions, 23.5 ml; temperature, +4 to +8°C. Fractions 65–98 were pooled. ■ = $A_{280\,nm}$; □ = $A_{420\,nm}$; ▲ = carbon dioxide hydration activity. For further details, see Experimental.

tion, as well as in several steps throughout the preparation. To further decrease the oxidation potential, DTT and EDTA were added to all solutions. The DTT-containing solutions should be as fresh as possible.

It is essential that all visible fat is discarded, otherwise the flow properties of the first ion-exchange column will be impaired owing to increased back-pressure.

First rapid ion-exchange chromatography

The first ion-exchange chromatography (Fig. 1) was completed in less than 4 h; this can be compared with conventional chromatography, which takes more than 24 h to perform [3]. During this step, CA III was separated from a large amount of contaminating substances. To facilitate the elution of myoglobin, with a determined p*I* of 7.0, the pH of the buffer was increased from 6.6 in the original preparation [3] to 6.8.

Gel filtration

Gel filtration (Fig. 2) was the most time-consuming preparation step. During this procedure CA III should be separated from proteins with higher molecular weights. However, CA and myoglobin (with molecular weights of 29 300[3] and 16 900[10], respectively, both calculated from amino acid sequence) cochromatographed to a great extent. The phosphate buffer (pH 6.6) previously used during this step [3] was replaced by a Tris buffer (pH 7.5), to avoid the need for a buffer change for the second rapid ion-exchange chromatography.

Second rapid ion-exchange chromatography

The p*I* value of bovine CA III was determined to be 9.0. During the second rapid ion-exchange chromatography (Fig. 3), a pH value of 7.5 was chosen between the p*I* values of CA III and the contaminating myoglobin. At low ionic strength, CA III was bound to the cation exchanger, whereas the negatively charged myoglobin was eluted during the first isocratic elution step.

The purity of the enzyme

To analyse the purity of bovine CA III in the various purification steps, SDS–PAGE was used (Fig. 4). Some minor bands, in addition to the predominant CA III band, appeared after the second rapid ion-exchange chromatography (Fig. 4, lane 5), indicating the presence of a small amount of contaminating proteins, probably mainly myoglobin. This protein has an absorbance maximum at 420 nm, hence the A_{420}/A_{280} ratio can be used as an

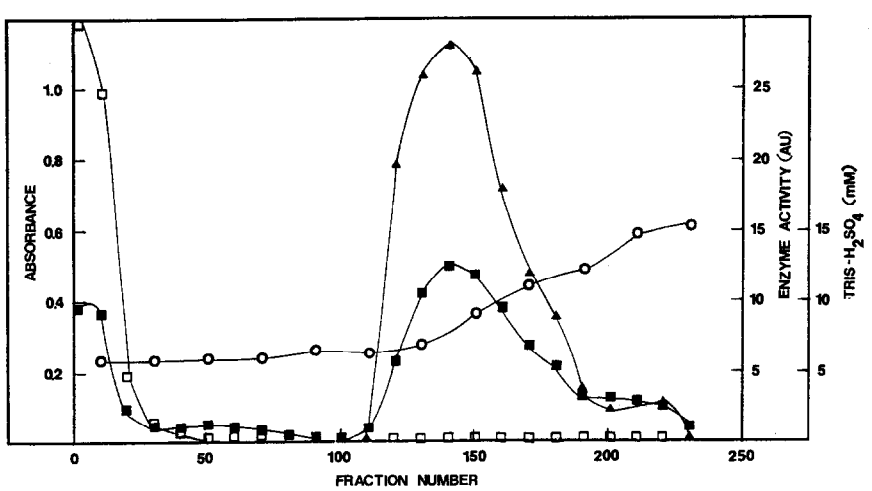

Fig. 3. Second rapid ion-exchange chromatography of the pooled fractions from the gel filtration chromatography. The resin, the bed dimensions, the flow-rate and the temperature were the same as in Fig. 1. Volume of fractions, 23 ml. Fractions 110–228 were pooled and concentrated. ■ = $A_{280\ nm}$; □ = $A_{420\ nm}$; ▲ = carbon dioxide hydration activity; ○ = buffer concentration. For further details, see Experimental.

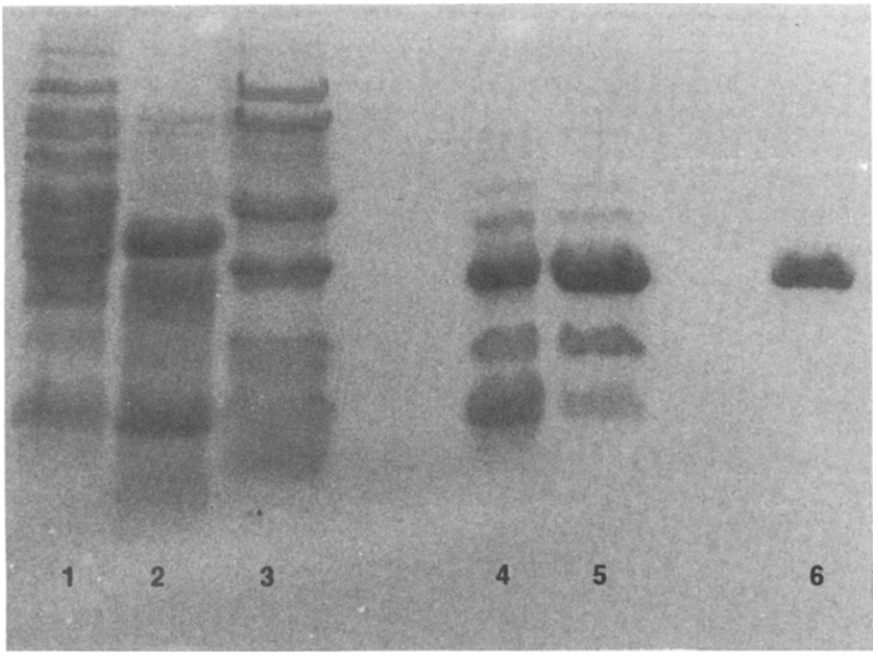

Fig. 4. SDS–PAGE. The samples in the respective lanes, with the amount of protein applied given within parentheses, are from: 1 = the centrifuged homogenate (20.5 μg); 2 = first rapid ion-exchange chromatography (20.9 μg); 3 = the reference calibration kit (30.0 μg); 4 = gel filtration chromatography (11.8 μg); 5 = second rapid ion-exchange chromatography (5.2 μg); 6 = the extra ion-exchange chromatography (5.8 μg). For further details, see Experimental.

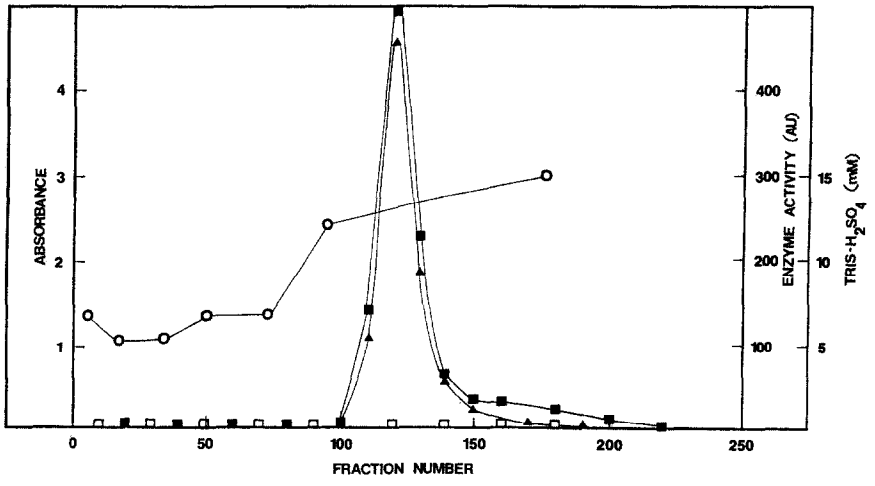

Fig. 5. Extra purification step of the bovine CA III from the second rapid ion-exchange chromatography on a CM-cellulose CM-23 column. Gel dimensions, 35 × 1.1 cm I.D.; flow-rate, 2 ml/min (126 ml/h · cm^2); no pressure applied; volume of fractions, 1.4 ml; temperature, +4 to +8°C. Fractions 110–130 were pooled. ■ = $A_{280\,nm}$; □ = $A_{420\,nm}$; ▲ = carbon dioxide hydration activity; ○ = buffer concentration. For further details, see Experimental.

index of myoglobin contamination of the CA III samples. A somewhat elevated A_{420}/A_{280} ratio was obtained for the enzyme solution from the second rapid ion-exchange chromatography, confirming the presence of some myoglobin impurities at this stage of the preparation.

If, for some purpose, an extremely pure enzyme is required, some of the protein solution from the second rapid ion-exchange chromatography can be rechromatographed on a CM-cellulose CM-23 resin (Fig. 5). The A_{420}/A_{280} ratio was decreased from 0.023 to 0.002 by this extra chromatographic step, and the SDS–PAGE analysis showed no contaminating bands, indicating that this treatment removed the last traces of myoglobin (Fig. 4, lane 6).

DISCUSSION

The results indicate the successful purification of bovine CA III by rapid ion-exchange chromatography performed on a CM-cellulose resin. This means that, in addition to anion exchangers [4–6], the "flush chromatography" technique can be used with a cation exchanger. Thus, the pH interval for using rapid ion-exchange chromatography has been considerably increased, and the method can therefore be applied even to proteins with high isoelectric points.

The nitrogen gas used to produce the rapid elution also protects the cysteine-containing bovine CA III from oxidation by air. The total preparation time, including all steps, is decreased from 2 weeks with conventional chromatography to only 5 days. If an ultra-pure enzyme is to be prepared, an extra ion-exchange step can be added, which will take another day.

Myoglobin can be difficult to separate from the CA III solution, since the two proteins seem to associate to each other. A similar phenomenon has also been noted for the closely related protein haemoglobin, which has been shown to associate to human CA I and II [11,12]. This problem is diminished by the shortened preparation time and by using very fresh muscle tissue. The purified enzyme is homogeneous, as judged by SDS–PAGE (Fig. 4), and the recovery of active enzyme is similar to that obtained using conventional methods.

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REFERENCES

1 A. M. Register, M. K. Koester and E. A. Noltmann, *J. Biol. Chem.*, 253 (1978) 4143–4152.
2 G. Gros and S. J. Dodgson, *Ann. Rev. Physiol.*, 50 (1988) 669–694.
3 P. Engberg, E. Millqvist, G. Pohl and S. Lindskog, *Arch. Biochem. Biophys.*, 241 (1985) 628–638.
4 N. Bergenhem, U. Carlsson and K. Klasson, *J. Chromatogr.*, 319 (1985) 59–65.
5 N. Bergenhem and U. Carlsson, *J. Chromatogr.*, 360 (1986) 279–281.
6 N. Bergenhem, U. Carlsson, L. E. Ellow and S. Rosén, *J. Chromatogr.*, 403 (1987) 383–387.
7 D. Keilin and E. F. Hartree, *Proc. Royal Soc. (London)*, B 124 (1938) 397–405.
8 S. P. Colowick, *Methods Enzymol.*, 1 (1955) 90–98.
9 E. E. Rickli, S. A. S. Ghazanfar, B. H. Gibbons and J. T. Edsall, *J. Biol. Chem.*, 239 (1964) 1066–1078.
10 K. K. Han, M. Dautrevaux, X. Chaila and G. Biserte, *Eur. J. Biochem.*, 16 (1970) 465–471.
11 L. Backman, *Eur. J. Biochem.*, 120 (1981) 257–261.
12 N. Bergenhem, U. Carlsson and C. Hansson, *Anal. Biochem.*, 134 (1983) 259–263.