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High resolution of multiple forms of rabbit reticulocyte hexokinase type I by hydrophobic interaction chromatography

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Abstract

Hydrophobic interaction chromatography (HIC) has been employed extensively in the separation of proteins by elution using a descending salt gradient, with and without the use of detergents or denaturing agents. In this study, a new hydrophobic interaction chromatographic support, Toyopearl Phenyl 650 S, was investigated in order to examine the distribution of multiple forms of rabbit reticulocyte hexokinase type I. These distinct forms of the enzyme, designated hexokinase Ia, Ia* and Ib, show similar kinetic and physical properties, similar molecular masses (ca. 100 000) and a different intracellular distribution. The results obtained using Toyopearl Phenyl 650 S of 20–50-μm particle diameter show that this HIC support allows very high resolution, comparable to that obtainable with HIC–HPLC columns but with the advantage of charging a higher amount of starting material even with a high protein concentration. These characteristics render Toyopearl Phenyl 650 S suitable for analytical and preparative purposes. Further, in the separation of multiple forms of rabbit reticulocyte hexokinase, the HIC method was shown to be superior to RP-HPLC, making possible the efficient separation of proteins with high molecular mass and their recovery in active forms. The Toyopearl Phenyl 650 S column was also shown to be more efficient than the ion-exchange chromatographic media previously used, allowing a quicker analysis of the multiple forms of rabbit reticulocyte hexokinase under different biological conditions.

1. Introduction

Data published in recent years have clearly shown that the occurrence of multiple forms of enzymes is not unusual but, rather, is a common phenomenon. Hexokinase exists in mammalian tissues as four isoenzymes with distinct properties and tissue distributions [1,2]. It is commonly accepted that red blood cell hexokinase is mainly of type I, and the presence of sub-types has also been described [3]. Multiple forms of red blood cell hexokinase were first reported by Eaton et al. [4], who observed several electrophoretic bands. Many workers have confirmed the existence of two or more distinguishable forms of hexokinase in different mammalian erythrocytes [5–19]. Hexokinase in rabbit reticulocytes is present in three distinct forms, which we designated hexokinase Ia, Ia* and Ib [20]; hexokinase Ia and Ib are present in soluble form, whereas hexokinase Ia* is mainly bound to the mitochondria. In fact, in rabbit erythrocytes

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only the soluble forms Ia and Ib are present whereas hexokinase Ia*, associated with mitochondria, is detectable only in whole blood samples containing reticulocytes [20]. Further, these multiple forms are unstable and glucose, fructose, glycerol and sulphydryl protecting agents are required for the maintenance of their activity during purification procedures. All of these sub-types have high molecular masses (ca. 100 000), differ very little in their isoelectric points and show similar kinetic and physical properties [21].

For these reasons, the chromatographic profile of rabbit reticulocyte hexokinase can be considered of interest in testing the performance of new chromatographic media. Further, the use of the haemolysate allows us to test the efficiency using a real sample with very high protein concentrations. Usually, a mixture of standard pure proteins, such as albumin, cytochrome *c*, lysozyme and myoglobin, is used to test the performance of new chromatographic supports [22]. Although it is possible to gain some information under these "ideal" conditions, the situation can change significantly when a real sample is used, as in the case of haemolysates, tissue homogenates and bacterial lysates. In this paper, we report the rapid and high resolution of rabbit reticulocyte hexokinase sub-types obtained using a new hydrophobic interaction chromatographic support, Toyopearl Phenyl 650 S. This chromatographic matrix permits the efficient elution of proteins according to their different hydrophobicities without the use of detergents or denaturing agents. This allows the separation of proteins under conditions suitable for maintaining biological activity, a characteristic of fundamental importance above all when the proteins under examination are present in very small amounts and in multiple forms. Further, the chemical and physical properties of this support permit the use of flow-rates even higher than those used with HIC–HPLC columns, thus allowing rapid and complete protein separations. Therefore, this support is particularly suitable for the investigation of pattern modifications of enzymes under different biological conditions.

2. Experimental

2.1. Chemicals and reagents

Coenzymes, enzymes, substrates and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). Toyopearl Phenyl 650 S and M and TSKgel Phenyl-5 PW were obtained from Tosohaas Technical Center (Woburn, MA, USA). All other reagents were of analytical-reagent grade.

2.2. Instrumentation

A Kontron Uvikon 860 spectrophotometer was used for optical measurements. The HPLC system consisted of two Model 112 pumps (Beckman, Berkeley, CA, USA), a Model 340 dynamic gradient mixer (Beckman) and a Model 420 gradient controller (Beckman). A Minipuls 2 peristaltic pump (Gilson, Molsheim, France) was used for packing the columns.

2.3. Preparation of Toyopearl Phenyl columns

Toyopearl Phenyl 650 S (20–50-μm particles) and 650 M (40–90-μm particles) had to be separated from fines by decantation, as the presence of fines in suspension can affect the resolution. The required amount of resuspended resin was transferred into a beaker and distilled water was added (four times the resin volume). This solution was mechanically stirred gently and allowed to settle for 60–90 min. The supernatant containing the fines was removed by suction using a water pump. This procedure had to be repeated 2–3 times. For the final decantation, the resin was resuspended in the packing solvent (in our case the equilibrating buffer). Air bubbles were removed by leaving the slurry for 2–3 min in an ultrasonic bath. The settled gel was then resuspended in an equal volume of equilibrating buffer, poured into the appropriate column and packed, using a peristaltic pump, at a flow-rate of 0.5 ml/min.

2.4. Chromatographic conditions

Triply-distilled water was prepared and used for preparing buffers, which were filtered through a 0.22-μm Millipore filter before HPLC analysis. The equilibrating solution used was 5 m*M* sodium potassium phosphate buffer (pH 8.1) containing 3 m*M* 2-mercaptoethanol (2-MSH), 3 m*M* KF, 1 m*M* dithiothreitol (DTT), 5 m*M* glucose and 30% (w/v) ammonium sulphate. Elution was performed using a descending gradient of ammonium sulphate. The profiles of the descending gradients used for separations are given on each chromatogram.

2.5. Hexokinase assay

Hexokinase (EC 2.7.1.1) activity was measured spectrophotometrically at 37°C in a system coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49), as described previously [23], except that 6-phosphogluconate dehydrogenase (EC 1.1.1.44) was omitted and the glucose-6-phosphate dehydrogenase concentration was increased to 0.5 IU/ml. One unit of hexokinase activity was defined as the amount of enzyme necessary to catalyse the formation of 1 μmol/min of glucose-6-phosphate at 37°C.

2.6. Protein determination

In the haemolysate, the haemoglobin concentration was determined spectrophotometrically at 540 nm with Drabkin's solution as described by Beutler [24]. Protein concentrations were determined spectrophotometrically at 280 nm in the course of elution of enzymes from the columns.

2.7. "In vivo" preparation of rabbit reticulocytes

Rabbit reticulocytes were obtained by the administration of phenylhydrazine to rabbits followed by the collection of blood samples at day 8 from the beginning of the treatment [19]. Using this procedure, the amount of reticulocytes in the whole blood usually ranged from 40 to 60%. The rabbit reticulocyte lysate used for the experiments came from different rabbits; therefore, the percentage of reticulocytes in the whole blood sample varied. This meant that the chromatographic profile of rabbit reticulocyte hexokinase was not necessarily the same from rabbit to rabbit.

2.8. Preparation of rabbit haemolysate

Whole blood was collected in heparin and immediately centrifuged at 1000 *g* for 10 min at 4°C. After removal of plasma and buffy coat, the red blood cells were washed twice with cold isotonic saline solution. The packed rabbit red blood cells were then lysed by the addition of an equal volume of 0.5% (v/v) Triton X-100 and the solution was left in ice for 30 min. The stroma were then removed by centrifugation at 11 000 *g* for 30 min.

3. Results and discussion

3.1. Separation of multiple forms of rabbit reticulocyte hexokinase by HIC

In mammalian red blood cells, hexokinase type I has been shown to be present in multiple forms [5–19]. Recently, using a new ion-exchange chromatographic support, Toyopearl DEAE 650 S, we had been able to separate three distinct forms of hexokinase from rabbit reticulocytes. It should be pointed out that, among the different anion exchangers commercially available, this chromatographic matrix was the only one which had allowed the complete separation of these three multiple forms [25]. Fig. 1A shows the hexokinase chromatographic profile of a rabbit blood sample with a content of reticulocytes ranging from 40 to 60%. As shown, the different forms of hexokinase can be eluted from Toyopearl DEAE 650 S as three distinct peaks, which we designated Ia, Ia* and Ib. In rabbit reticulocytes the hexokinase designated Ia* is mainly bound to the mitochondria [20]. A similar situation occurs with other tissues such as

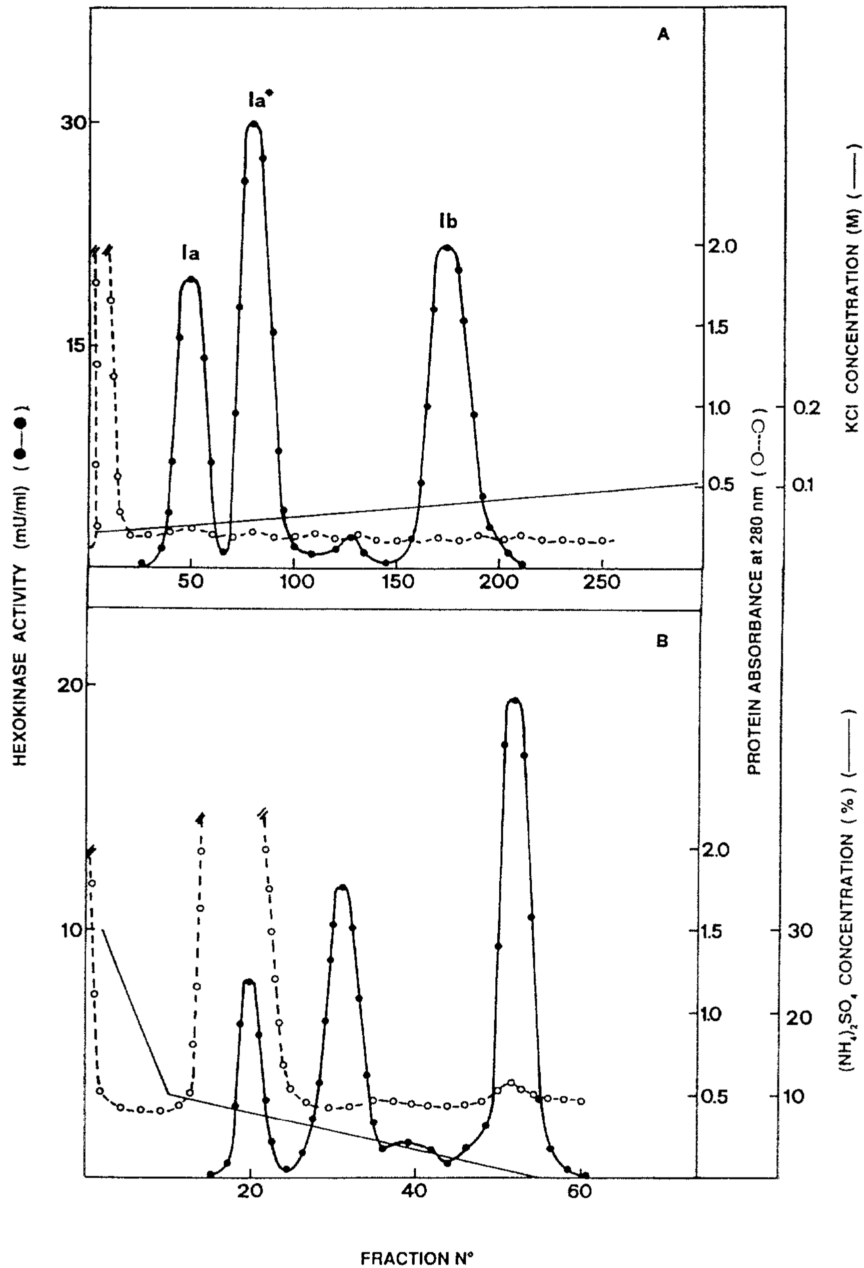

Fig. 1. Separation of multiple forms of hexokinase from rabbit reticulocytes. (A) Toyopearl DEAE 650 S column (15 cm × 1 cm I.D.). A 12-ml sample of haemolysate with a protein concentration of 50 mg/ml was charged on to the column. The column was equilibrated in 5 m*M* sodium potassium phosphate buffer (pH 8.1) containing 3 m*M* KF, 3 m*M* 2-MSH, 1 m*M* DTT and 5 m*M* glucose and operated at 0.3 ml/min at 4°C. The elution of the hexokinase activity was obtained using a 200-ml linear gradient of KCl from 40 to 200 m*M* in the same equilibrating buffer. Fractions of 0.7 ml were collected and assayed for hexokinase activity. (B) Toyopearl Phenyl 650 S column (5 cm × 1.2 cm I.D.). A 500-μl sample of haemolysate with a protein concentration of 190 mg/ml was charged on to the column. The column was equilibrated in the buffer described under Experimental and operated at 0.5 ml/min at room temperature. The hexokinase activity was eluted using a two-step descending gradient of ammonium sulphate from 30 to 10% in 15 min and from 10 to 0% in 90 min. Fractions of 1 ml were collected and assayed for hexokinase activity.

the brain [26–29] and in transformed cell lines, where it has been shown that the increase in hexokinase activity involves mainly the form bound to the mitochondria [30,31]. The binding of the enzyme to these organelles, in the rat brain, is due the presence of nine additional N-terminal hydrophobic amino acids [32]. It is reasonable to assume that a similar situation can occur in rabbit reticulocytes, making the hexokinase Ia* more hydrophobic than hexokinase Ia and Ib, which are present in soluble form. A simple method to distinguish between the bound and soluble forms of the enzyme would be useful in order to study the properties of hexokinase Ia*.

Fig. 1B shows the chromatographic profile obtained using a Toyopearl Phenyl 650 S column. The result is very interesting, not only because this HIC support allows the elution of the more hydrophobic form Ia*, but also because a complete separation of the soluble forms Ia and Ib, which are eluted in the first part of the chromatogram, was possible. However, comparing the two chromatographic profiles shown in Fig. 1, we can observe that, using Toyopearl DEAE 650 S, the haemoglobin is immediately eluted from the column with the equilibrating buffer. With Toyopearl Phenyl 650 S, in contrast, only a small amount of the haemoglobin is removed by washing and most of this protein is eluted concomitantly with hexokinase Ia. In any case, the HIC matrix permits a quick separation of reticulocyte hexokinase sub-types by means of a descending gradient of ammonium sulphate and without the use of detergents or denaturing agents. Further, these experimental conditions allow the recovery of the sub-types of the enzyme in their active form.

3.2. *Identification of multiple forms of hexokinase from rabbit reticulocytes using Toyopearl Phenyl 650 S columns*

The identification of the three distinct forms of rabbit reticulocyte hexokinase eluted by HIC was performed by charging each peak separated by ion-exchange chromatography on to the Toyopearl Phenyl 650 S columns. The chromatographic profiles of each form (Fig. 2A–C) show that their elution times are in agreement with the complete isozymic pattern obtained by charging a real sample of rabbit reticulocyte lysate on to the column (Fig. 2D). On the basis of these results, hexokinase Ia is the least hydrophobic form, whereas hexokinase Ib shows an intermediate hydrophobicity and, as expected, hexokinase Ia* is the most hydrophobic form. Hexokinase Ia* is mainly bound to the mitochondria, and previous studies have shown that this form of the enzyme sediments in density gradients with these organelles [33]. Only the addition of glucose-6-phosphate or detergents such as saponin or Triton X-100 causes its solubilization.

Kurokawa et al. [34] have reported the separation of mitochondria-bindable hexokinase from rat brain by HIC using a Phenyl-Sepharose column. Under their experimental conditions the enzyme present in soluble form was not adsorbed on the column, but rather was eluted with the equilibrating buffer, while the elution of the mitochondria-bindable hexokinase required the addition of a detergent such as Lubrol PX. In our case, Toyopearl Phenyl 650 S has the advantage of eluting the most hydrophobic form, Ia*, without the use of detergents or denaturing agents. Further, hexokinase Ia and Ib, present in soluble form, were adsorbed to the column and were eluted at the beginning of the gradient in two distinct peaks (Fig. 2D). The good separation of these sub-types was unexpected, as both are present in soluble form and show very similar molecular masses (ca. 100 000), kinetic properties (the K_m value of glucose was 0.04 mM for hexokinase Ia and 0.125 mM for hexokinase Ib; both enzymes have the same K_m value for MgATP, 0.05 mM) and differ very little in their isoelectric points [21].

Further evidence confirming that the identification of multiple forms of hexokinase was correct was also obtained by investigating the chromatographic profile of the enzyme in rabbit erythrocytes. The isozymic profiles obtained using Toyopearl DEAE 650 S (Fig. 3A) and Toyopearl Phenyl 650 S (Fig. 3B) columns only show the presence of the soluble forms Ia and Ib. This is due to the fact that during maturation

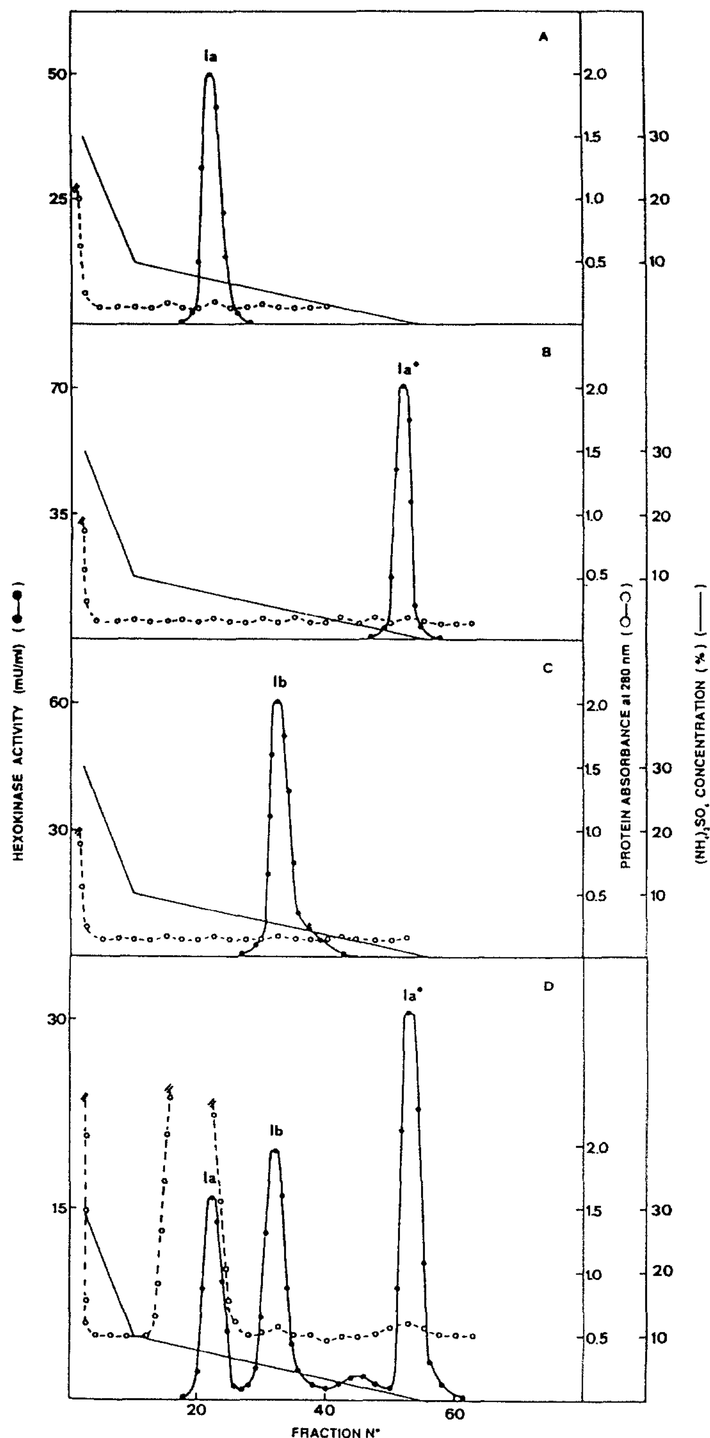

Fig. 2. Identification of multiple forms of hexokinase from rabbit reticulocytes. Hexokinase (A) Ia, (B) Ia* and (C) Ib, eluted from Toyopearl DEAE 650 S, were charged separately on to Toyopearl Phenyl 650 S columns. (D) Complete isozymic profile of rabbit reticulocytes. All chromatographic profiles were obtained using the experimental conditions as in Fig. 1B.

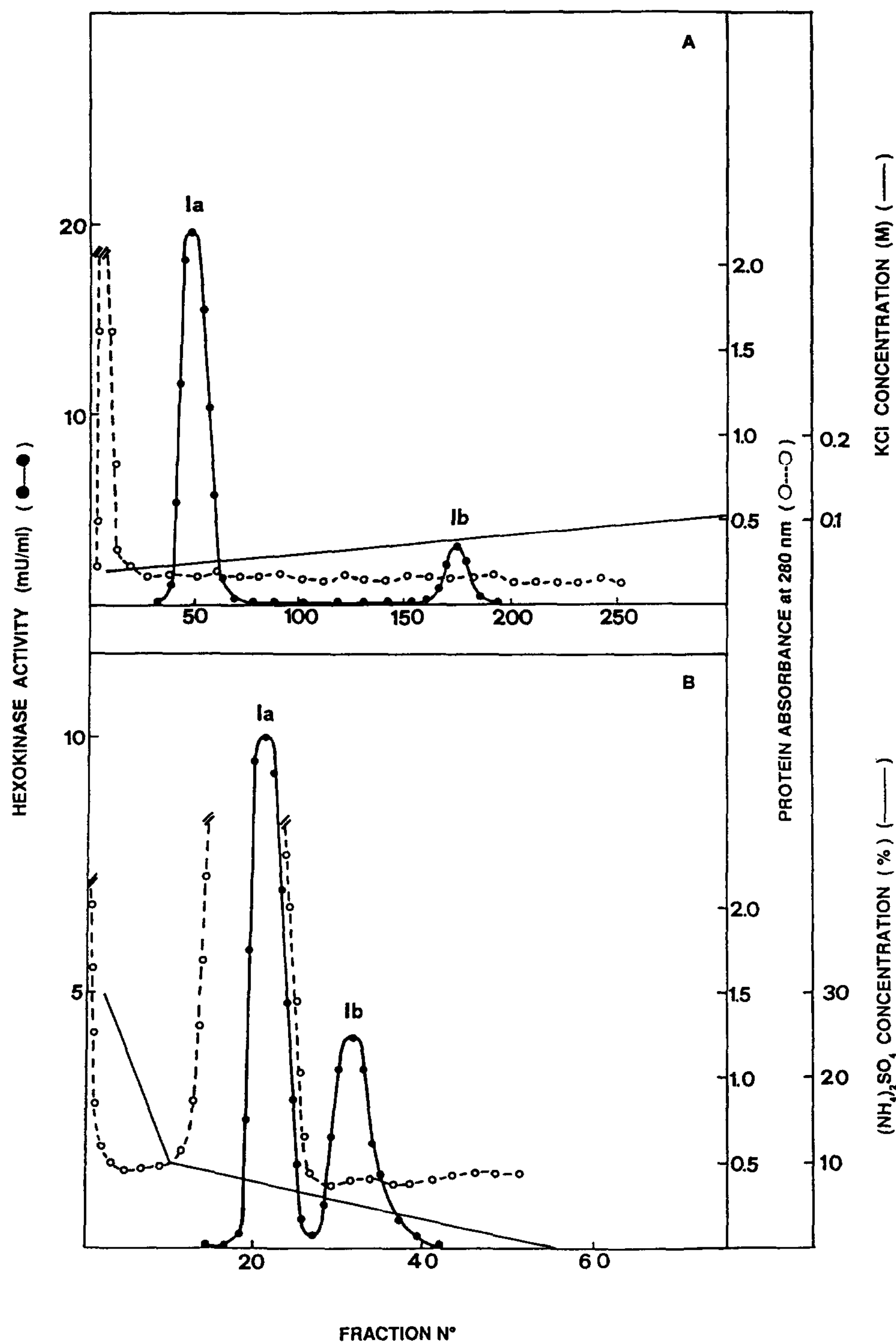

Fig. 3. Separation of multiple forms of hexokinase from rabbit erythrocytes using (A) a Toyopearl DEAE 650 S column and (B) a Toyopearl Phenyl 650 S column. All chromatographic profiles were obtained using the same experimental conditions as in Fig. 1.

of the reticulocytes to erythrocytes there is a significant change in the chromatographic profile of hexokinase. In fact, hexokinase Ia* is completely lost, and only hexokinase Ia and a small amount of hexokinase Ib are present in the erythrocytes. It is of interest that hexokinase Ib, which had previously been shown to undergo a faster decay than hexokinase Ia during the ageing of erythrocytes [18], is also more hydrophobic than hexokinase Ia.

3.3. Influence of flow-rates and salt gradients on resolution

In order to obtain more information on the behaviour of Toyopearl Phenyl 650 S, we investigated the effects of different flow-rates and salt gradients on the resolution of multiple forms of rabbit reticulocyte hexokinase. As regards the effects of flow-rates on the elution of proteins, we performed experiments at 0.5 ml/min (Fig. 4A) and 1.0 ml/min (Fig. 4B). As shown in Fig. 4B, it is possible to maintain a good resolution of multiple forms of hexokinase working at a flow-rate of 1 ml/min as used with HPLC columns. Further, we have shown that this HIC support can be used to separate hexokinase from other tissues even working at a flow-rate of 1.5 ml/min (data not shown). The results obtained show that the rigid methacrylic polymer structure of the Toyopearl HIC resins is mechanically stable, allowing the use of higher flow-rates than with other conventional media.

As another parameter affecting separation in HIC is the salt gradient, we also investigated its influence on the separation of multiple forms of hexokinase. Fig. 5A shows the elution of hexokinase activity using a two-step descending gradient of ammonium sulphate, from 30 to 10% in 8 min and from 10 to 0% in 45 min at a flow-rate of 1.0 ml/min. Fig. 5B shows the chromatographic profile obtained using a different gradient, from 30 to 0% ammonium sulphate in 120 min at a flow-rate of 1.0 ml/min. As shown, the two-step gradient allows a better resolution than a linear gradient with almost the same slope. Probably the use of a two-step gradient, with a drastic decrease in salt concentration in the first step, allows a better separation of soluble forms Ia and Ib. The use of a descending salt gradient of ammonium sulphate allows the elution of enzymes in their active forms. In fact, the solvent conditions in HIC generally stabilize tertiary and quaternary protein structures [35] whereas the use of organic solvents in RP-HPLC promotes the denaturation of proteins [36,37].

3.4. Effects of different particle sizes on resolution

Conventional low-pressure chromatographic procedures usually give a lower resolution than those obtainable using HPLC columns. The efficiency of the Toyopearl Phenyl 650 S column (20–50-μm particles), packed using a peristaltic pump, was assessed by comparing the elution profiles obtained using an HIC–HPLC column. Fig. 6A shows the chromatographic profile of rabbit reticulocyte hexokinase obtained using a TSKgel Phenyl-5 PW HPLC column (10-μm particles). This HIC–HPLC support allows the adsorption of soluble forms Ia and Ib on the column with a very good resolution into sharp peaks. It is interesting that, as shown in Fig. 6B, the low-pressure Toyopearl Phenyl 650 S support allows a resolution comparable to that obtained with HPLC columns, but with the advantage of charging higher amounts of starting material. The amount of sample that can be charged on to the HPLC column is very small because the haemolysate has a high protein concentration (160–200 mg/ml). With rabbit erythrocytes, and also other mammalian red blood cells, the level of hexokinase activity is very low, 0.0003% (w/w) [21]. Therefore, using a TSKgel Phenyl-5 PW HPLC column, only analytical separations can be performed.

Further studies concerning the kinetic, chemical and physical characteristics of each form of hexokinase are limited by the amount of starting material which can be charged on to the column. In fact, using red blood cell haemolysates, the protein concentration ranges from 160 to 200 mg/ml; under these conditions, the amount of starting material that can be injected on to a TSKgel Phenyl-5 PW HPLC column ranges from

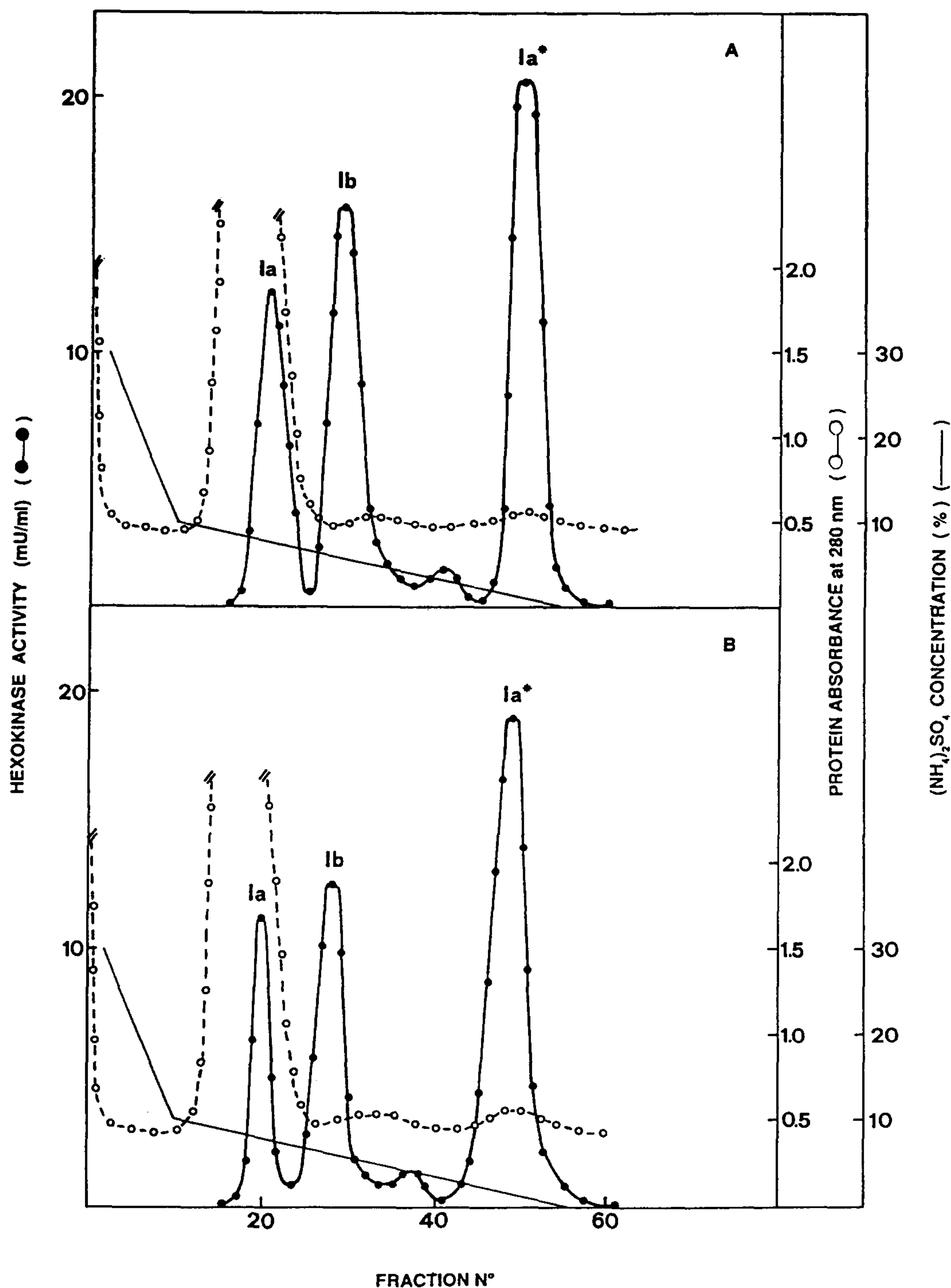

Fig. 4. Influence of flow-rates on the separation of multiple forms of hexokinase from rabbit reticulocytes. (A) Elution of hexokinase activity using the same experimental conditions as in Fig. 1B; (B) elution of hexokinase activity using a flow-rate of 1 ml/min; the descending gradient of ammonium sulphate was from 30 to 10% in 8 min and from 10 to 0% in 45 min.

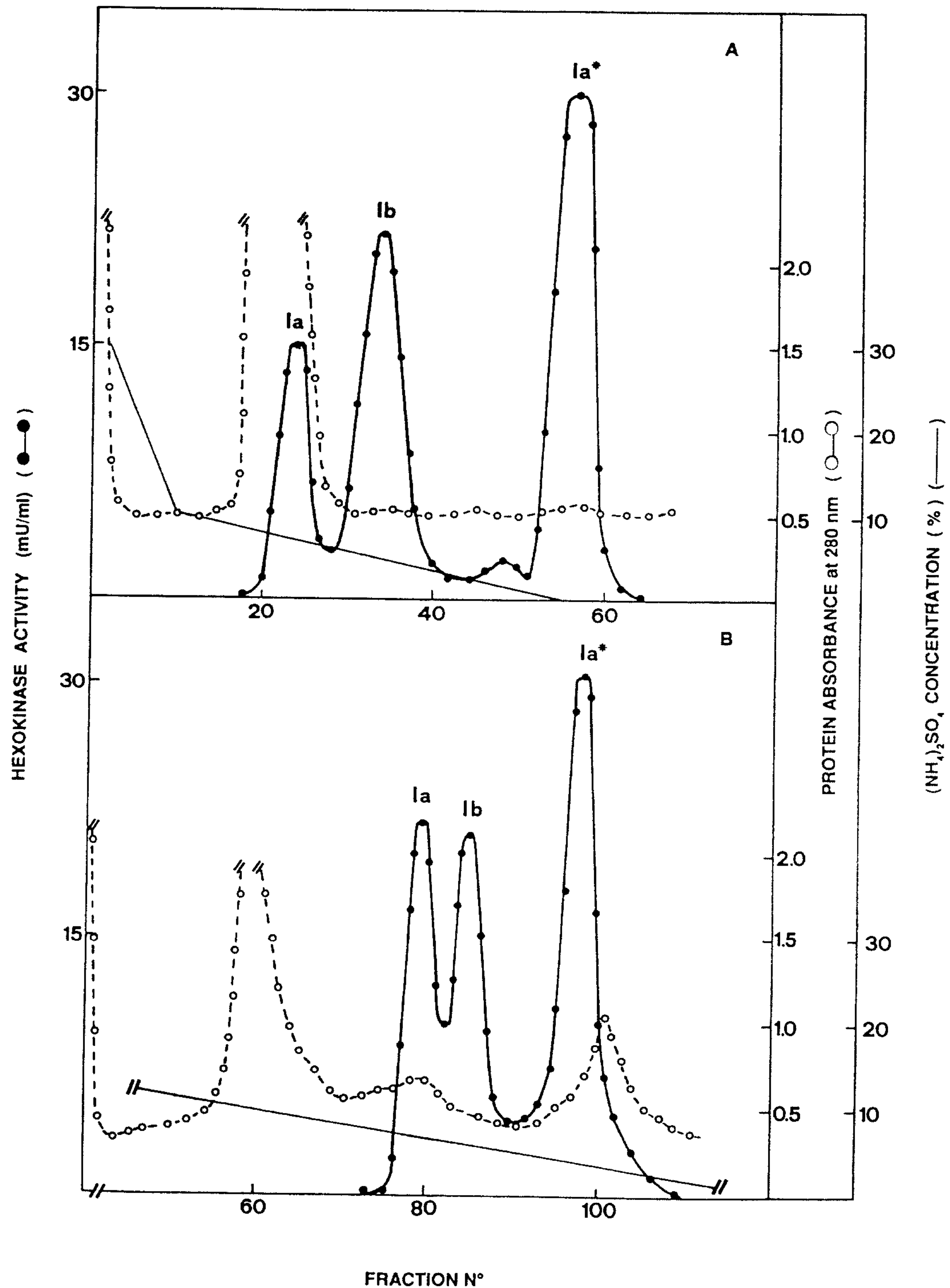

Fig. 5. Influence of the gradient on the separation of multiple forms of hexokinase from rabbit reticulocytes. (A) Elution of hexokinase activity using the same experimental conditions as in Fig. 4B; (B) elution of hexokinase activity using a one-step descending gradient from 30 to 0% ammonium sulphate in 120 min at a flow-rate of 1 ml/min.

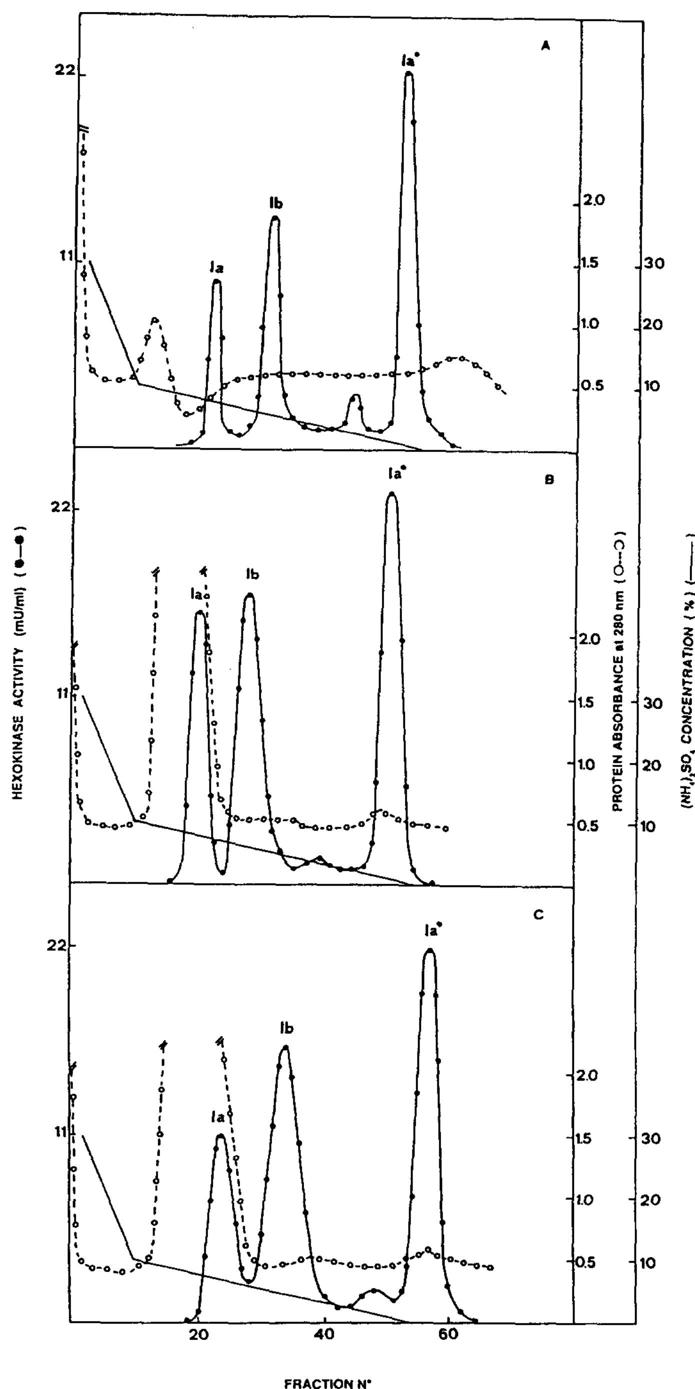

Fig. 6. Separation of multiple forms of hexokinase from rabbit reticulocytes using different chromatographic supports. (A) TSKgel Phenyl-5 PW (7.5 cm × 0.75 cm I.D.), particle size 10 μm; (B) Toyopearl Phenyl 650 S (5 cm × 1.2 cm I.D.), particle size 20–50 μm; (C) Toyopearl Phenyl 650 M (5 cm × 1.2 cm I.D.), particle size 40–90 μm. All chromatographic profiles were obtained using the same experimental conditions as in Fig. 4B.

100 to 500 μl. Further, after several injections of 500 μl of haemolysate, charged on to the column in separate aliquots of 100 μl, we observed a significant decrease in resolution due to the aspecific adsorption of material which remains bound to the column and is difficult to remove even by performing different regeneration procedures [0.1–0.2 *M* sodium hydroxide solution or 20–40% (v/v) acetic acid]. In order to overcome this inconvenience, we charged the haemolysate on to a guard column of TSKgel Phenyl-5 PW (1 cm × 0.6 cm I.D.). Under these conditions the hexokinase present in 500 μl of starting material is completely adsorbed, while most of the haemoglobin, which represents about 98% of the total proteins present in the haemolysate, is eluted using the equilibrating buffer. After removal of the bulk of the proteins, the guard column was connected to the analytical column and the elution of the distinct forms of hexokinase was obtained using a descending gradient of ammonium sulphate. Although this procedure prolongs the life of the analytical column, some proteins adsorbed on the guard column flow through the analytical column and, eventually, significantly affect the resolution. Given these results, it appears clear that the TSKgel Phenyl-5 PW HPLC column is very useful for analytical separations when injecting samples with low protein concentrations, whereas its use is not recommended when the starting material has a very high protein concentration (as with haemolysates, tissue homogenates, etc.).

In order to evaluate the influence of particle diameter on resolution, we compared the performance of Toyopearl Phenyl 650 S (20–50-μm particles) with that of Toyopearl Phenyl 650 M (40–90-μm particles). The chromatographic profile of rabbit reticulocyte hexokinase, shown in Fig. 6C, is very interesting, as the three molecular forms of hexokinase show a good resolution notwithstanding the fact that the peaks are slightly broader than those obtained using Toyopearl Phenyl 650 S (20–50 μm). The above results suggest that particle size does not significantly influence the resolution of hexokinase type I, even though Toyopearl Phenyl 650 S allows a better resolution with sharper peaks.

4. Conclusions

The use of Toyopearl Phenyl 650 S was found to allow a quicker and higher resolution of multiple forms of rabbit reticulocyte hexokinase Ia, Ia* and Ib than some other chromatographic methods [25]. The results reported in this paper also show that the low-pressure Toyopearl Phenyl 650 S support allows a resolution similar to that obtainable using a TSKgel Phenyl-5 PW HPLC column but with the advantage of charging higher amounts of starting material even with a very high protein concentration. These properties make this chromatographic support suitable for analytical and preparative purposes. Further, compared with other HIC supports, Toyopearl Phenyl 650 S allows the elution of different forms of hexokinase without the use of detergents or denaturing agents, thus allowing the recovery of proteins in their active form. A wide application of this technique in biochemical analysis and biotechnology is expected in the near future.

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