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Separation of hexokinase activity using different hydrophobic interaction supports

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Abstract

Hydrophobic interaction chromatography (HIC) has been used extensively for the separation of proteins and peptides by elution using a descending salt gradient, with and without the use of detergents or denaturing agents. In this paper we compare different hydrophobic interaction chromatographic media for the separation of multiple forms of hexokinase from rabbit reticulocytes. Among the different hydrophobic chromatographic media tested (Toyopearl Phenyl 650S, Ether 650S and Butyl 650S) Toyopearl Phenyl 650S offered the best separation of multiple forms of hexokinase, probably due to its intermediate hydrophobicity. In order to establish the optimal experimental conditions, we evaluated the effects of different salts, and the results obtained demonstrated that among the antichaotropic salts, ammonium sulphate is the most suitable for the separation of hexokinase sub-types. The sample loading capacity of the three Toyopearl supports was investigated and the recovery of enzymatic activity obtained ranged from 60% to 90%, depending on the different salts and hydrophobic media used. The chromatographic profiles of hexokinase activity from various mammalian and fungal tissues also demonstrate that Toyopearl Phenyl 650S can be successfully employed for the separation of multiple forms of enzymes from different biological sources. © 1997 Elsevier Science B.V.

Keywords: Hexokinase; Enzymes

1. Introduction

In recent years, hydrophobic interaction chromatography (HIC) has been widely employed for the separation and purification of proteins and other biomolecules [1–3]. As compared to reversed-phase high-performance liquid chromatography (HPLC) [4], HIC offers interesting advantages due to the lesser hydrophobicity of bonded groups and to the mobile phase gradient of salts which allows protein separation with a high recovery of mass and biological activity under mild conditions [5,6], which is not possible under the denaturing conditions of HPLC [7,8]. The first chromatographic matrices used for HIC, uses hydrophobic ligands (alkyl or aryl groups) attached to agarose gels [9,10] which have a low physical stability. More recently, a number of silica- and polymer-based stationary phases were developed to provide greater chemical stability [11–13]. For the purification of biochemicals the use of ligands having intermediate hydrophobic characteristics should provide binding strength without causing the irreversible adsorption and denaturation during elution [14].

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Previously [15] we investigated the performance of the hydrophobic chromatographic support Toyopearl Phenyl 650S (20–50 μm particle size) in the rapid separation of multiple forms of hexokinase type I from rabbit reticulocyte lysates. These cells, in which the enzyme is present in at least three different molecular forms designated hexokinase Ia and Ib, present in soluble form, and Ia*, bound to the mitochondria [16], represent a good model to test the performance of a chromatographic medium. In fact, all three distinct forms of the enzyme possess high-molecular mass (100 000), differ very little in their kinetic, chemical and physical properties and also show different stability [17]. Furthermore, this system allows the efficiency of new chromatographic matrices to be tested utilizing real biological samples, such as cell lysates and tissue homogenates with high protein concentrations (160–190 mg/ml). Toyopearl Phenyl 650S allows high recovery of these forms, working at very high flow-rates and without the use of detergents or denaturing agents [15].

In this study, we compared the performances of three different chromatographic media, Toyopearl Phenyl, Butyl and Ether 650S by testing different equilibrating salts and their effect on protein retention. Finally, the validity of the strategy developed was tested by studying the chromatographic profiles of hexokinase multiple forms from some mammalian tissues, such as rat brain, human placenta and human red blood cells and from *Tuber borchii* mycelium and fruitbody.

2. Experimental

2.1. Chemicals and reagents

Coenzymes, enzymes, substrates and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). Toyopearl Phenyl 650S, Butyl 650S and Ether 650S (20–50 μm particle size) were obtained from Tosohaas Technical Center (Woburn, MA, USA). All other reagents were of analytical grade.

2.2. Instrumentation

A Kontron spectrophotometer (Model Uvikon 860) 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 Minipulse 2 peristaltic pump (Gilson, Molsheim, France) was used to pack the columns.

2.3. Preparation of hexokinase samples

Rabbit reticulocytes were obtained from rabbits made anemic by phenylhydrazine administration [18]. Blood containing 65 to 70% reticulocytes was collected using EDTA as anticoagulant. The reticulocytes were washed twice with 0.9% NaCl (w/v). The buffy coat was removed by suction. The rabbit reticulocytes were hemolyzed for 30 min with an equal volume of 0.5% Triton X-100 (v/v) to solubilize the molecular form Ia* bound to the mitochondria. The red cell stroma were then removed by centrifuging the lysate at 13 000 *g* for 30 min. Human blood was drawn from normal subjects ranging in age from 20 to 30 years and collected in heparin. Human red blood cells lysed in 0.5% Triton X-100 (v/v) were obtained as described by Stocchi et al. [15]. Rat brain homogenate was prepared according to the method of Chou and Wilson [19] and the hydrophobic molecular form bound to the mitochondrial membranes was solubilized using 0.5% Triton X-100 (v/v) and 1 m*M* glucose-6-phosphate. Truffle mycelium and fruitbody homogenates were obtained using the procedure described in Ref. [20].

2.4. Chromatographic conditions

The Toyopearl Phenyl 650S, Ether 650S and Butyl 650S columns (5.0 cm×1.2 cm I.D.) were prepared using a peristaltic pump at a flow-rate of 0.5 ml/min as described by Stocchi et al. [15]. The settled gel was equilibrated using 5 m*M* sodium–potassium phosphate buffer, pH 8.1, containing 3 m*M* β-mercaptoethanol (β-MSH), 3 m*M* potassium fluoride (KF), 1 m*M* dithiothreitol (DTT), 5 m*M* glucose (washing buffer) plus various salts at different concentrations as described in the legends of the Figures. The hemolysates were diluted with 3 volumes of the equilibrating buffer reported in the figures to

obtain a hemoglobin concentration of 45 mg/ml. The hexokinase samples were loaded in cold room (4°C), after which the chromatographic profiles were obtained using a HPLC system at room temperature. The profiles of the descending gradients used for the 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 previously described [21], except that 6-phosphogluconate dehydrogenase (EC 1.1.1.44) was omitted and glucose-6-phosphate dehydrogenase was increased to 0.5 IU/ml. One unit of hexokinase activity was defined as the amount of enzyme necessary to catalyze the formation of 1 μmol of glucose-6-phosphate/min at 37°C.

2.6. Protein determination

The hemoglobin concentration in the hemolysates was determined spectrophotometrically at 540 nm with Drabkin's solution as described by Beutler [22]. In the homogenates of other tissues the protein concentrations were determined as described by Lowry et al. [23] using bovine serum albumin as standard. During the elution of the enzyme from the columns, protein concentration was determined spectrophotometrically at 280 nm.

3. Results and discussion

3.1. Comparison of various hydrophobic interaction chromatographic media

As previously shown [15], we used Toyopearl Phenyl 650S (20–50 μm particle sizes) column for the separation of the multiple forms of hexokinase. The chromatographic profile was obtained using 30% (w/v) ammonium sulphate as equilibrating salt, corresponding to a concentration of 2.3 *M*. Fig. 1A shows the good separation of rabbit reticulocyte hexokinase obtained using a decreasing gradient of ammonium sulphate in two steps: the first from 2.3 to 0.76 *M* in 8 min and the second from 0.76 *M* to 0 *M* in 45 min.

In order to check the performances of the different Toyopearl HIC media, we compared the performance of Phenyl 650S to those of Butyl 650S and Ether 650S in the separation of multiple forms of hexokinase from rabbit reticulocytes.

Fig. 1B shows the chromatographic profile obtained for rabbit reticulocyte hexokinase using the Toyopearl Ether 650S support. Among the three chromatographic media, Toyopearl Ether 650S is the least hydrophobic support and also the least selective. Under the same experimental conditions utilized for Toyopearl Phenyl 650S (Fig. 1A), the three sub-types of hexokinase were eluted in only one peak. Other gradients were tested to improve the separation of hexokinase multiple forms; the gradient slope between 1.5 *M* and 0.76 *M* ammonium sulphate in 70 min was able to separate the three molecular forms, as shown in the inset to Fig. 1B.

Fig. 1C shows the elution profile of rabbit reticulocyte hexokinase obtained using Toyopearl Butyl 650S. This support is the most hydrophobic and most retentive of the three and under the same experimental conditions utilized for Toyopearl Phenyl 650S, the soluble forms Ia and Ib were incompletely separated and Ia* was recovered only after the addition of the detergent Triton X-100 (1% v/v) to the elution buffer. Other gradients were tested to improve the separation of hexokinase multiple forms (data not shown), but the results obtained confirmed the inadequacy of this support for our aim.

3.2. Influence of different salt gradients

Protein retention and selectivity in HIC are highly dependent upon the chemical nature of the salt used to promote binding [5,24,25]. Salts useful in this regard are termed antichaotropics and belong to the lyotropic series of ions [25,26].

We investigated the effect of different salts on the retention and resolution of hexokinase multiple forms using the three different chromatographic media. In particular, we utilized KCl and Na_2HPO_4 which have a low ability to enhance hydrophobic interactions and Na_2SO_4 and sodium citrate which promote a high retention of enzymes as they are at the beginning of the lyotropic series [25].

Toyopearl Ether 650S was unable to retain the

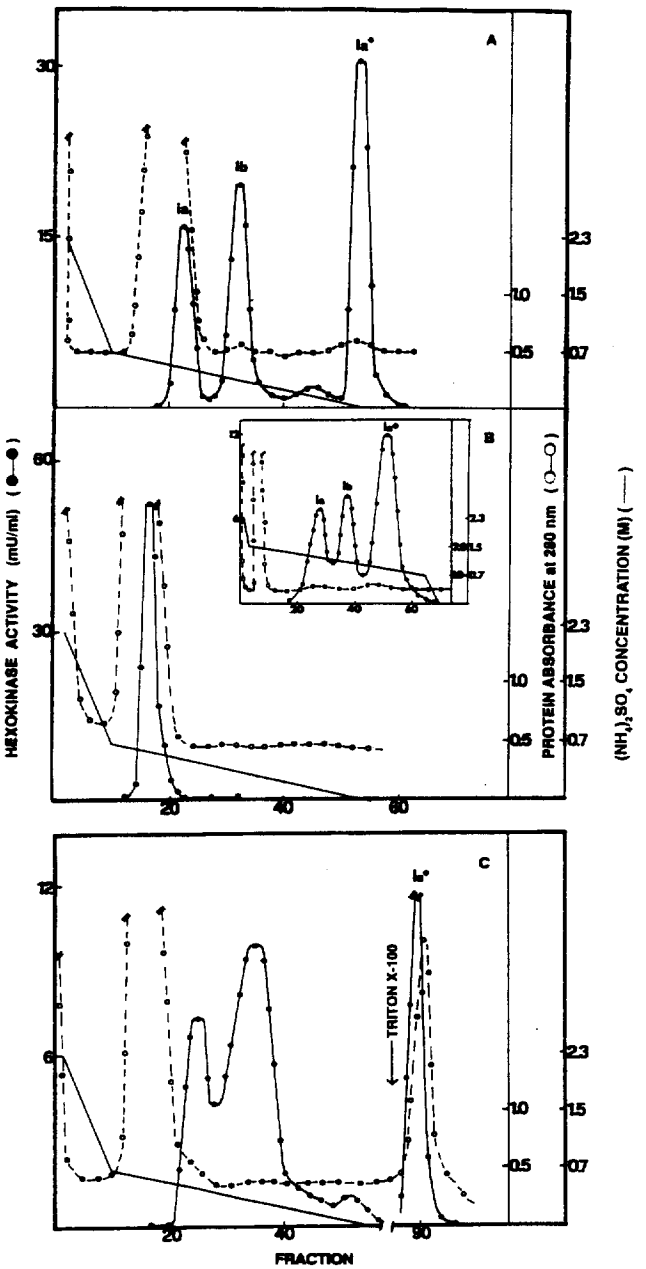

Fig. 1. Use of different HIC media for the separation of sub-types of rabbit reticulocyte hexokinase. A 250 μl sample of hemolysate with a hexokinase activity of 0.3 total units was diluted with 3 volumes of equilibrating buffer and charged in the columns. (A) Toyopearl Phenyl 650S column (5.0 cm×1.2 cm I.D.) equilibrated in 5 m*M* sodium–potassium phosphate buffer, pH 8.1, containing 3 m*M* KF, 3 m*M* β-MSH, 1 m*M* DTT, 5 m*M* glucose and 2.3 *M* ammonium sulphate. The elution profile was obtained using a decreasing gradient of ammonium sulphate in two steps: from 2.3 *M* to 0.76 *M* in 8 min and from 0.76 *M* to 0 *M* in 45 min. Fractions of 1.0 ml were collected and assayed for hexokinase activity. (B) Toyopearl Ether 650S column (5.0 cm×1.2 cm I.D.); the chromatographic profile was achieved using the same experimental conditions described for (A). The inset shows the chromatographic profile obtained utilizing a separating step from 2.3 *M* to 1.5 *M* ammonium sulphate in 5 min from 1.5 *M* to 0.76 *M* in 70 min and from 0.76 *M* and 0 *M* in 5 min. (C) Toyopearl Butyl 650S column (5.0 cm×1.2 cm I.D.); the chromatographic profile was achieved using the same experimental conditions described for (A), but the elution profile of HK Ia* was obtained only after the addition of 1% (v/v) Triton X-100 in the washing buffer.

hexokinase activity with the four salts tested. In fact, 80% of hexokinase activity was recovered in the washing buffer and 20% of the activity retained was eluted in a single peak. In contrast to this, Toyopearl Butyl 650S was most retentive with all salts used. 50% of hexokinase activity was eluted during the descending gradient, but the remaining hexokinase was eluted only after the addition of the detergent Triton X-100 (1% v/v) to the elution buffer. Furthermore, the total recovery ranged from 60 to 70%.

The Toyopearl Phenyl 650S matrix is the most suitable support for the separation of rabbit reticulocyte hexokinase activity using a descending gradient of ammonium sulphate. In fact, the use of other salts does not permit to separate the three molecular forms of the enzyme. Fig. 2A and Fig. 2B show the elution profiles obtained using 2.3 *M* KCl and 0.76 *M* Na_2HPO_4, respectively. Using the KCl gradient, hexokinase is eluted at a higher salt concentration than with ammonium sulphate and only two forms of the enzyme can be separated; on the contrary, when the Na_2HPO_4 gradient is used hexokinase is eluted in only one peak. These two salts have a low ability to enhance hydrophobic interactions and the chromatographic profiles show that most of the hexokinase activity is not adsorbed to the column and is recovered during the washings in the equilibrating buffer.

Fig. 2C and Fig. 2D show the elution profiles obtained using 0.76 *M* Na_2SO_4 and 0.76 *M* sodium citrate in the starting buffer. The concentration of these salts was sufficient to promote a high retention of enzyme to the matrix because these compounds are at the beginning of the lyotropic series [25]. Although the total amount of hexokinase is adsorbed, it is not possible to separate the three molecular forms with these decreasing gradients. The recovery of hexokinase activity ranged from 65 to 75%.

The results obtained demonstrate that ammonium sulphate is the most suitable salt for the separation of sub-types of hexokinase, given the fact that the total activity is retained and the three molecular forms are eluted separately. In fact, ammonium sulphate is the salt most commonly used for HIC because its properties (high solubility, low ultraviolet absorbance, intermediate molal surface tension increment) make it able to retain and separate most proteins [27].

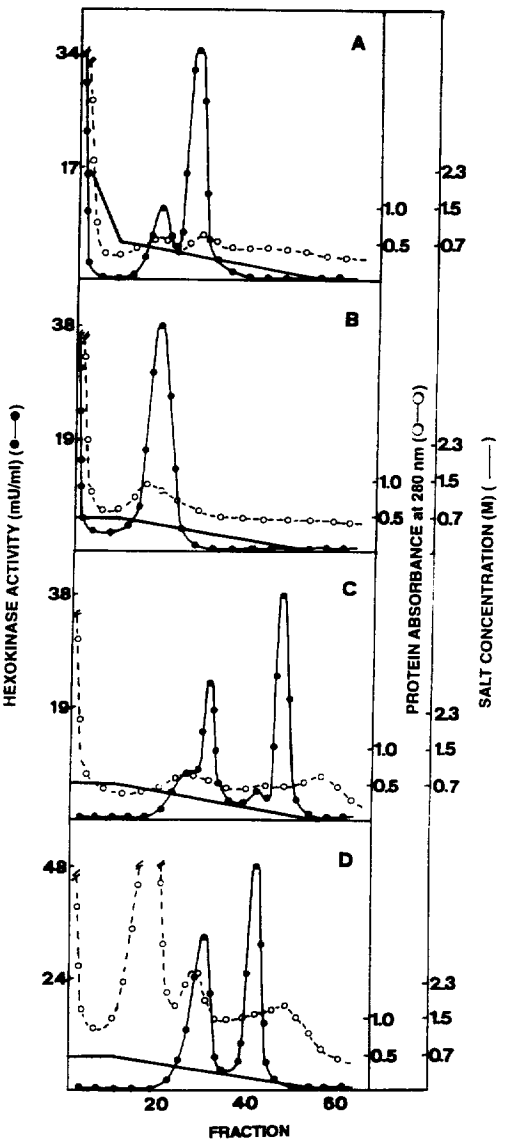

Fig. 2. Influence of different salts on the resolution and retention of rabbit reticulocyte hexokinase using the Toyopearl Phenyl 650S support. Hexokinase activity of 0.5 total units was charged onto the Toyopearl Phenyl 650S column (5.0 cm×1.2 cm I.D.) equilibrated in 5 m*M* sodium–potassium phosphate buffer, pH 8.1, containing 3 m*M* KF, 3 m*M* β-MSH, 1 m*M* DTT, 5 m*M* glucose and: (A) 2.3 *M* KCl; (B) 0.76 *M* Na_2HPO_4; (C) 0.76 *M* Na_2SO_4; (D) 0.76 *M* sodium citrate. The elution profiles were obtained using the following salt descending gradients: from 2.3 *M* to 0.76 *M* in 8 min and from 0.76 *M* to 0 *M* in 45 min (A); from 0.76 *M* to 0 *M* in 45 min (B, C, D). Fractions of 1.0 ml were collected and assayed for hexokinase activity.

3.3. Separation of hexokinase type I from different tissues using Toyopearl Phenyl 650S

Many data published in recent years have clearly shown that hexokinase exists in mammalian tissues as four isoenzymes that can present a further micro-heterogeneity [28]. The presence of sub-types of hexokinase type I was observed in rat liver [29], rat and rabbit brain [30], human and rabbit red blood cells [31] and human placenta [32]. Previously [15,33] we had used Toyopearl Phenyl 650S for the complete separation of hexokinase multiple forms from rabbit reticulocytes, erythrocytes and brain; in this study, in order to examine the performance of this chromatographic support, we analyzed the pattern of hexokinase from other sources (Fig. 3).

With the exception of human placenta (Fig. 3A), which presents two sub-types separable only by sodium dodecyl sulphate gel electrophoresis [32], we were able to discriminate among the multiple forms of hexokinase type I present in other mammalian tissues tested.

Fig. 3B shows the hexokinase pattern from human red blood cells. In earlier studies [34,35] using anion-exchange chromatography, a complicated isozymic pattern with the presence of three major incompletely separated forms had been demonstrated. The results obtained in the present study using the Toyopearl Phenyl 650S hydrophobic support show a very interesting and complex pattern of the multiple forms present.

Fig. 3C shows the chromatographic profile of rat brain hexokinase. This tissue contains two distinguishable sub-types of the enzyme, the most hydrophobic of which is bound to the mitochondria, whereas the other has lost this ability due to the removal of a very small peptide responsible for the interactions between hexokinase and the mitochondrial membrane [36]. In fact, as shown in the Figure, Toyopearl Phenyl 650S allows a rapid and very efficient separation of the two forms despite the fact that they differ very little in their chemical and physical properties [36].

In another study [20], we reported some biochemical characteristics of the fruit-bodies from three white truffle species, very interesting ectomycorrhizal Ascomycetous fungi belonging to the genus *Tuber*. In the present paper we compare the chromatographic profiles of *Tuber borchii* mycelium (Fig. 4A) and fruit-body (Fig. 4B). The hexokinase pattern present in the mycelium shows two distinct molecular forms of the enzyme while only one form is present in the fruit-body. This difference could be explained by the fact that fruit-body represents the terminal step of the truffle life cycle.

These findings indicate that this support can be used with success in the analysis of a large variety of

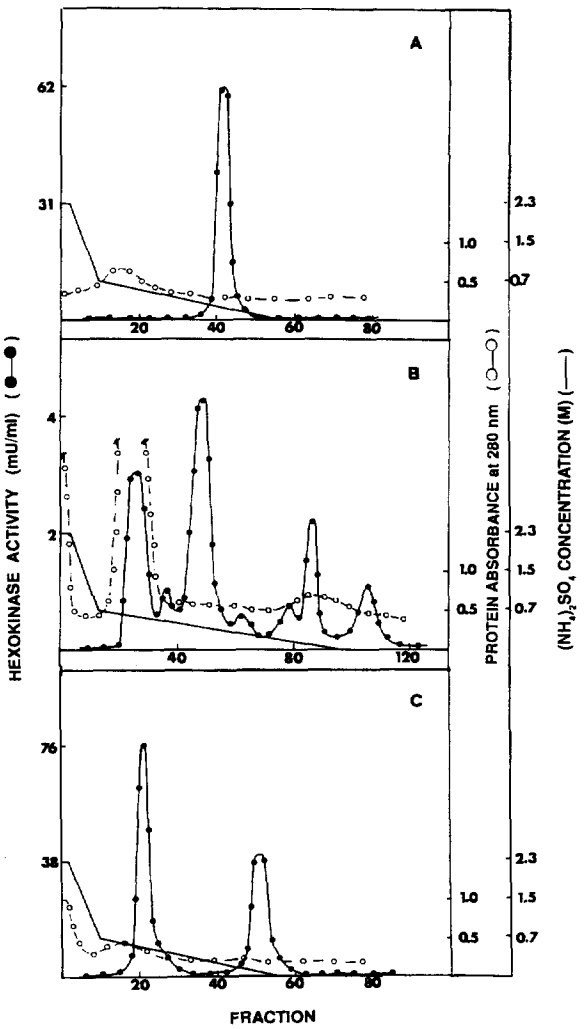

Fig. 3. Hexokinase pattern in different mammalian tissues. A Toyopearl Phenyl 650S column (5.0 cm×1.2 cm I.D.) was used, under the same experimental conditions described in Fig. 1A, to separate the hexokinase multiple forms from (A) purified human placenta; (B) human red blood cells; (C) rat brain.

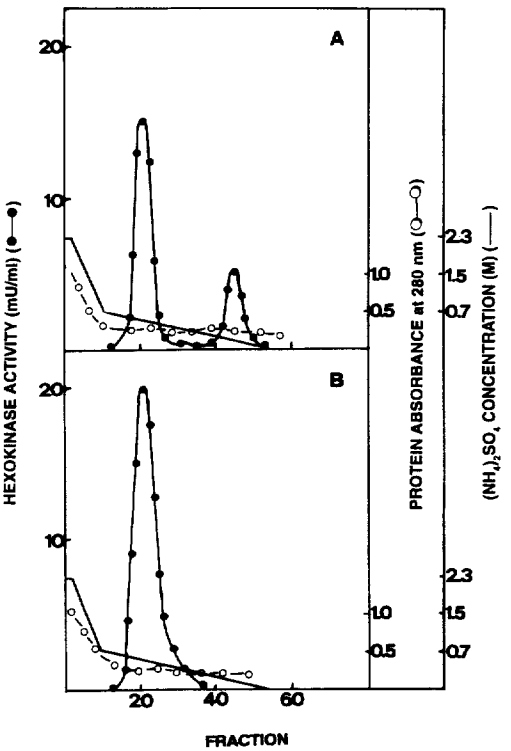

Fig. 4. Hexokinase pattern in *Tuber borchii* mycelium and fruit-body. A Toyopearl Phenyl 650S column (5.0 cm×1.2 cm I.D.) was used, under the same experimental conditions described in Fig. 1A, to separate the hexokinase from (A) *Tuber borchii* mycelium and (B) *Tuber borchii* fruit-body homogenates.

biological samples as well as hemolysates and tissue homogenates.

3.4. Protein loading capacity and pH effect

The protein loading capacity of Toyopearl Phenyl, Butyl and Ether 650S, equilibrated in 2.3 *M* ammonium sulphate, was tested using rabbit red blood cell hemolysate as starting material and monitoring the hemoglobin binding to the columns. The red cell hemolysate was diluted with equilibrating buffer to a protein concentration of 3.5 mg/ml and charged onto the hydrophobic Toyopearl columns (2.0 cm×0.78 cm I.D., corresponding to 1.0 ml of packing material). The total protein loading capacity is shown in Table 1. Toyopearl Butyl, the most hydrophobic support, has a sample loading capacity higher than Toyopearl Ether and Phenyl. Toyopearl Phenyl 650S, has an intermediate loading capacity, but is the most suitable support for the hexokinase separation.

Table 1
Protein loading capacity of different hydrophobic Toyopearl supports

Matrix	Loading capacity/ml of resin
Toyopearl Phenyl 650S	23 mg
Toyopearl Ether 650S	18 mg
Toyopearl Butyl 650S	28 mg

The protein loading capacity was tested using a rabbit reticulocyte hemolysate, diluted to a protein concentration of 3.5 mg/ml, charged onto a hydrophobic Toyopearl column (2.0 cm×0.78 cm I.D.).

Furthermore, we tested the effect of pH on the resolution of hexokinase multiple forms. Toyopearl Phenyl 650S can be successfully used over a wide range of pH without drastic effects on protein retention [37]. The separation can be performed at a pH ranging between 7.0 and 9.0 without any modification in the hexokinase profile (data not shown).

4. Conclusions

In the present paper, we report the performance of Toyopearl Phenyl 650S in the separation of hexokinase type I from different biological systems. This support was tested in comparison to two other hydrophobic interaction chromatographic media, Toyopearl Butyl and Ether 650S.

The intermediate hydrophobicity of Toyopearl Phenyl 650S makes it the most suitable medium for the separation of rabbit reticulocyte hexokinase multiple forms. In fact, the least hydrophobic support, Toyopearl Ether 650S, does not retain the hexokinase activity even at high ionic strength. In contrast Toyopearl Butyl 650S, the most hydrophobic support retained the enzyme very strongly even at low ionic strength, but the peaks eluted were very broad and this caused a decrease in resolution.

By varying the salts commonly used in HIC, it was shown that ammonium sulphate is the most suitable salt for the retention and separation of multiple forms of hexokinase.

Toyopearl Phenyl 650S has a sample loading capacity lower than that of Toyopearl Butyl 650S and the recovery of enzymatic activity ranged from

85 to 90%. Furthermore, the resin was also used to analyze multiple forms of hexokinase type I in different tissue samples, showing that it is possible to achieve a very good separation.

The results reported in this paper offer useful information for the hydrophobic separation of enzymes present in multiple forms which differ very little in stability, physical and kinetic properties and high-molecular mass. Furthermore, the suitability of Toyopearl Phenyl 650S for use in different biological systems makes it an important tool for the study of "in vivo" and "in vitro" modifications of proteins and enzymes.

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