Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography A, 986 (2003) 275–290
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Direct chromatographic capture of enzyme from crude homogenate using immobilized metal affinity chromatography on a continuous supermacroporous adsorbent

Pär Arvidsson[a], Fatima M. Plieva[b], Vladimir I. Lozinsky[b], Igor Yu. Galaev[a], Bo Mattiasson[a,*]

[a]*Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-22100 Lund, Sweden*

[b]*A N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, 28 Vavilov St, 119991 Moscow, Russia*

Received 25 July 2002; received in revised form 11 November 2002; accepted 14 November 2002

Abstract

A continuous supermacroporous matrix has been developed allowing direct capture of enzyme from non-clarified crude cell homogenate at high flow-rates. The continuous supermacroporous matrix has been produced by radical co-polymerization of acrylamide, allyl glycidyl ether and *N,N′*-methylene-bis(acrylamide) which proceeds in aqueous solution of monomers frozen inside a column (cryo-polymerization). After thawing, the column contains a continuous matrix having interconnected pores of 10–100 μm size. Iminodiacetic acid covalently coupled to the cryogel is a rendering possibility for immobilized metal affinity chromatographic purification of recombinant His-tagged lactate dehydrogenase, $(His)_6$-LDH, originating from thermophilic bacterium *Bacillus stearothermophilus*, but expressed in *Escherichia coli*. The large pore size of the adsorbent makes it possible to process particulate-containing material without blocking the column. No preliminary filtration or centrifugation is needed before application of crude extract on the supermacroporous column. A total of 210 ml crude homogenate, 75 ml of it non-clarified, was processed on a single 5.0 ml supermacroporous column at flow speeds up to 12.5 ml/min without noticeable impairment of the column properties. Mechanically the cryogel adsorbent is very stable. The continuous matrix could easily be removed from the column, dried at 70 °C and kept in a dry state. After rehydration and reinsertion of the matrix into an empty column, $(His)_6$-LDH was purified as efficiently as on the newly prepared column. The procedure of manufacturing the supermacroporous continuous cryogel is technically simple. Starting materials and initiators are cheap and available and are simply mixed and frozen under specified conditions. Altogether these qualities reveal that the supermacroporous continuous cryogels is a very interesting alternative to existing methods of protein purification from particulate-containing crude extracts.

Keywords: Immobilized metal affinity chromatography; Affinity adsorbents; Enzymes

1. Introduction

A critical element of modern process biotechnology is the separation and purification of the target

*Corresponding author. Tel.: +46-46-222-8264; fax: +46-46-222-4713.
E-mail address: bo.mattiasson@biotek.lu.se (B. Mattiasson).

0021-9673/02/$ – see front matter
doi:10.1016/S0021-9673(02)01871-X

product from a fermentation broth or cell rupture supernatant. As it represents the major manufacturing cost, competitive advantage in production will depend not only on innovations in molecular biology sciences but also on innovation and optimization of separation and down-stream processes. Chromatography, both analytical and large-scale chromatography of many kinds, is predominant now in down-stream separations. Traditional packed-bed chromatography with immobile stationary phase, however, despite its elegance and its resolving power suffers from a main drawback—incapability of processing particulate containing solutions, e.g. cell suspensions or non-clarified crude cell homogenates. Particulate material is trapped in between the beads of the chromatographic carrier resulting in increased flow resistance of the column and finally complete blockage of the flow. To circumvent this drawback, an expanded-bed chromatography has been proposed where the upstream flow lifts the beads of the carrier making the distance large enough for particulate material to pass through without being trapped. Properly designed density and size allows only the beads to have a restricted movement around a stationary position rather than uncontrolled movement in the whole column volume (as in fluidized bed reactors). Hence, a flow regime close to a plug flow is maintained in the column. For a detailed review of the recent advances in expanded bed chromatography see the special issue of *Bioseparation* on Expanded Bed Chromatography (vol. 8, No. 1/5, 1999). However, with all it advantages expanded bed chromatography requires special types of columns and equipment and cannot be fitted in traditional packed-bed chromatographic systems. Another possible approach is to use magnetic affinity beads, where the non-clarified crude cell homogenate is suspended in a given volume and mixed with affinity ligands immobilized on small particles of a ferromagnetic support [1,2]. The magnetic particles with bound target protein can then simply be separated by holding a magnet to the side of the vessel while removing the rest of the suspension. A disadvantage of the method is that because of limits in capacity of magnets, only small volumes and amounts can be processed. Also, one round of batch capturing can be considered corresponding to one theoretical plate. In the continuous bed system described in this paper, the height equivalent to a theoretical plate (HETP) was about 0.1 cm that gives ca. 45 plates in a 4.5 cm column.

It is attractive to have a packed-bed chromatographic carrier with pores large enough to accommodate cell debris and even the whole cells without being blocked. As such, large (on molecular scale) objects as cells and cell debris have a negligible diffusivity, the transport of them inside the column can be only convective. Hence, the desired bed should have a continuous system of interconnected pores rather than to be composed of porous beads because in the latter case the convective flow is mainly restricted to the inter-particle volume inside the column. Just that very structure is inherent to the so-called *cryogels*, i.e. the polymeric gels formed in moderately frozen media [3].

This paper presents the use of columns produced from supermacroporous continuous (monolith) poly(acrylamide) cryogels modified with the residues of iminodiacetic acid (IDA) for the direct capture of His-tagged recombinant lactate dehydrogenase, $(His)_6$-LDH, from crude cell homogenate using immobilized metal affinity chromatography (IMAC).

2. Materials and methods

2.1. Materials

N-2-Hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid (HEPES) was bought from BDH (Poole, UK). Copper sulphate, sodium pyruvic acid (pyruvate), bicinchoninic acid solution (BCA), EDTA–tetrasodium salt, ethanolamine, 1,4-butanediol digycidyl ether, imidazole and β-NADH were purchased from Sigma (St. Louis, MO, USA). High salt LB-Broth, micro agar and sodium ampicillin were of the brand Duchefa (Haarlem, The Netherlands) and bought from Saveen Biotech (Malmö, Sweden) along with isopropyl β-D-thiogalactopyranoside (IPTG). Recombinant strain of *Escherichia coli* TG1 with pUC $(His)_6$-LDH was a gift from Professor Leif Bülow, Department of Pure and Applied Biochemistry, Center for Chemistry and Chemical Engineering, Lund University. Sepharose CL-6B was a product of

Table 1
Preparation of continuous supermacroporus adsorbent. Conditions of cryopolymerization: Total co-monomer concentration 3% (w/w), temperature of freezing −7.0 °C; 24 h at −7.0 °C, thawing at room temperature

AAm/MBAAM (mol/mol)	AAm (g)	MBAAm (g)	TEMED (μl)	APS (mg)	Water (ml)
30:1	0.84	0.06	15.0	7.5	30
20:1	0.81	0.09	15.0	7.5	30
10:1	0.74	0.16	15.0	7.5	30
5:1	0.62	0.28	15.0	7.5	30

Amersham Bioscience (Uppsala, Sweden). Tyrosine, potassium carbonate and sodium chloride were supplied by Merck (Darmstadt, Germany). Tris was purchased from USB (Cleveland, OH, USA).

Acrylamide (AAm, more than 99.9% purity, electrophoresis reagent), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were from Bio-Rad (Hercules, CA, USA), *N*,*N*′-methylene-bis(acrylamide) (MBAAm) was from Acros (Geel, Belgium); ally glycidyl ether (AGE, 99%) was from Aldrich, DA was from Fluka (Buchs, Switzerland). Albumin fraction V from bovine serum (BSA) for HETP determination was from Merck, (Darmstadt, Germany) while albumin fraction V used as protein standard for BCA assay was purchased from ICN Biomedical (Aurora, OH, USA). Blue Dextran was from Amersham Biosciences.

2.2. Methods

2.2.1. Cryogenic co-polymerization of AAm with MBAAm

Monomers (Table 1) were dissolved in deionized water and the mixture was degassed under vacuum (water pump aspirator) for about 5 min to eliminate soluble oxygen. The gels were produced by free radical polymerization initiated by TEMED and APS. After addition of TEMED (1.2% of the total AAm + MBAAm mass) the solution was cooled in an ice bath for 2–3 min. Then APS (0.83% of the total AAm + MBAAm mass) was added and the reaction mixture was stirred for 1 min. Five millilitres of the reaction mixture was poured into plastic 5-ml syringes (I.D. 12.4 mm) with closed outlet at the bottom. The solution in the syringes was frozen within 5–7 min at −10 °C. The frozen samples were kept at −10 °C for 24 h and then thawed at room temperature. The cryogel matrix prepared in each syringe was washed by passing 200 ml water at a flow-rate of 1 ml/min.

2.2.2. Cryogenic co-polymerization of AAm with AGE and MBAAm

Monomers (Table 2) were dissolved in deionized water and the mixture was degassed for about 5 min, AGE was poured into the solution. After adding TEMED (1.0% of the total AAm + AGE + MBAAm mass) the solution was cooled in an ice bath for 2–3

Table 2
Epoxy activated supermacroporous continuous adsorbents. (AAm + AGE)/MBAAm = 5.3:1 AGE/AAm = 0.1 mol/mol; conditions of cryopolymerization: freezing at −12 °C, storage 24 h at −12 °C, thawing at room temperature

Total concentration of co-monomers in initial mixture, (%, w/w)	Gel matrix prepared	Water flow-rate (hydrostatic pressure ca. 0.01 MPa) (cm/h)	Content of epoxy groups (μmol/ml of gel)
3.5	Good sponge like gel, rather weak	1000[a]	14
4.4	Good sponge like gel	1070	23
5.0	Good sponge like gel, rather dense	750	24
6.0	Dense sponge like gel	630	25

[a] The gel matrix was compressed to about 80% of its initial height.

min. APS (0.98% of the total AAm+AGE+MBAAm mass) was added and the reaction mixture was stirred for 1 min and then poured in 5 ml portions into plastic 5-ml syringes with closed outlet at the bottom. The solution in the syringes was frozen within 6–8 min at −12 °C. The frozen samples were thawed at room temperature after keeping them frozen at −12 °C for 24 h. The cryogel matrix prepared in each syringe was washed by passing 200 ml water at a flow-rate of 1 ml/min.

2.2.3. Coupling of IDA–ligand to cryogel

Fifty millilitres 0.5 *M* Na_2CO_3 followed by 50 ml 1.0 *M* Na_2CO_3 solutions were passed through the gel matrix at a flow-rate of 1 ml/min. The IDA solution (0.5 *M* in 1.0 *M* Na_2CO_3, pH 10.0) was applied to the column at a flow-rate of 1 ml/min in a recycle mode during 24 h at room temperature. After that, the modified cryogel in the column was washed with 0.5 *M* Na_2CO_3 (100 ml) and then with water until pH was neutral. Cu^{2+} was bound to the IDA–Sepharose by passing 0.5 *M* $CuSO_4$ (dissolved in distilled water) through the gel.

2.2.4. Epoxy activation of Sepharose

Twenty millilitres of Sepharose CL-6B was thoroughly washed with distilled water and then suspended in 20 ml 0.6 *M* NaOH containing 38 mg sodium borohydride. Under agitation, 20 ml 1,4-butanediol diglycidyl ether was slowly added, then the suspension was incubated overnight on a shaking table, 120 rpm at room temperature. Excess reagent was removed by extensive washing of the resultant gel beads with distilled water.

2.2.5. Preparation of Cu^{2+}–iminodiacetate Sepharose (Cu^{2+}–IDA–Sepharose)

Twenty millilitres of epoxy activated Sepharose CL-6B was suction dried in a sintered glass funnel and transferred to a 250 ml shaking flask containing 2.5 g imininodiacetic acid (IDA) in 20 ml 2 *M* potassium carbonate. The flask was placed in a 60 °C shaking incubator overnight. The gel suspension was then filtered and washed with 1 liter 1 *M* NaCl followed by 1 liter distilled water. The excessive reactive groups were blocked by suspending the gel in 15 ml 1 *M* ethanolamine–HCl solution pH 9.0 and incubating the gel suspension on a shaking table at room temperature for 4 h followed by filtering and washing with 1 liter 1 *M* NaCl and 1 liter distilled water. The resulting IDA–Sepharose gel was degassed and 5.9 ml was packed in a column (I.D. 1.0 cm). Finally Cu^{2+} was bound to the IDA–Sepharose by passing 0.5 *M* $CuSO_4$ (dissolved in distilled water) through the packed gel.

2.2.6. Ligand density

The amount of immobilized IDA was determined for both IDA–cryogel and IDA–Sepharose by assaying the amount of bound copper ions at saturation assuming a stoichiometric ratio. The adsorbents were saturated with Cu^{2+} by passing 0.5 and 0.1 *M* $CuSO_4$ solution, respectively, through the columns at a flow-rate of 0.5 ml/min and were then thoroughly washed with water. The Cu^{2+} was then eluted from the columns with 0.1 *M* EDTA pH 7.6 and determined spectrophotometrically as absorbance of Cu^{2+}-complex formed in 0.1 *M* EDTA solution, pH 7.6 at λ_{max} 730 with $\varepsilon_{730}=46.8\ M^{-1}\ cm^{-1}$. After elution, both gels were washed with water and then dried at 70 °C for 4 days to establish their dry mass. Before drying, the wet mass of the IDA–cryogel was determined by weighing the syringe before and after removal of the gel.

2.2.7. Characterization of continuous supermacroporous cryogel matrices

The degree of swelling of the cryogels ($S_{w/w}$) was determined as follows: samples of gels were washed on porous filter until washing was clear, sucked dry and then transferred to pre-weighed vials and weighed ($m_{wet\ gel}$). After drying to constant mass in the oven at 60 °C, the mass of dried samples was determined ($m_{dry\ gel}$) The degree of swelling was calculated as:

$$S_{w/w} = (m_{wet\ gel} - m_{dry\ gel})/m_{dry\ gel}.$$

The flow-rate of water passing through the columns was measured at the constant hydrostatic pressure equal to 100 cm of water-column corresponding to a pressure of ca. 0.01 MPa. For each sample at least three measurements were done.

HETP analysis was performed using solutions of tyrosine (molecular mass, M_r 181), BSA (M_r 69 000) and Blue Dextran (M_r 2 000 000) as tracers. When

studying epoxy-containing continuous cryogel, epoxy groups were first blocked by passing 0.1 *M* ethanolamine solution, 0.1 *M* sodium carbonate buffer, pH 9.5 through the column during a 4 h recycle mode. Tyrosine and BSA were loaded in the 100 m*M* Tris–HCl buffer, pH 7.0, while Blue Dextran was loaded in the same buffer containing 1.0 *M* NaCl. The addition of salt in the latter case proved to be necessary to eliminate some non-specific interactions occurring between Blue Dextran and the activated continuous cryogel matrix with blocked epoxy groups. Chromatographic peaks were obtained by injecting 2 ml of the tracer solution at flow-rates 0.1–15 ml/min corresponding to superficial velocities from 4.8 to 750 cm/h. The total plate number was calculated according to the formula $N = 5.55 \cdot (t_R / w_{1/2})^2$, where t_R is retention time and $w_{1/2}$, is the width of a chromatographic peak at half of its height.

2.2.7.1. Cultivation and recovery of recombinant E. coli containing thermostable $(His)_6$-LDH

Recombinant strain of *E. coli* TG1 with pUC $(His)_6$-LDH (LDH originating from the thermophile *Bacillus stearothermophilus*) was cultivated on agar plates with ampicillin overnight at 37 °C. Five single colonies were transferred to five test tubes, each containing 10 ml LB medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) and 100 mg/l ampicillin and these were cultivated at 37 °C in a shaking incubator at 200 rpm overnight. The 10 ml cultivations were then used for inoculation of five 1-liter shaking flasks, each one containing 200 ml LB medium and 100 mg/l ampicillin. The cultivations in the five flasks were carried out at 37 °C in a shaking incubator at 120 rpm. When the optical density at 600 nm reached 0.7, IPTG was added to a final concentration of 48 mg/l to induce $(His)_6$-LDH gene expression. Another portion of ampicillin was also added to gain fresh 100 mg/l in the solution for stimulation of bacteria to keep the plasmid containing the $(His)_6$-LDH and ampicillin resistance genes. After $3\frac{1}{2}$ h cells were harvested by centrifugation at 16 200 *g* for 10 min. Pellets were washed with 50 m*M* Tris–HCl pH 7.0 and recentrifuged at 16 200 *g* for 10 min. After resuspension in 50 ml of the same buffer, the cells were disrupted by sonication six times of 1 min, each treatment followed by 1 min of interruption. The cells were kept in an ice bath and the sonicator was set to 0.5 cycles per second, maximum sonic intensity 105 W/cm^2 with the chosen probe (ca 1.5 cm diameter), 20% amplitude the first run, 40% the second run and 60% the last four runs. A portion of 25 ml cell suspension was sonicated each run. After disruption, the suspension was divided in two fractions, in one of these the cell debris was removed by centrifugation at 21 800 *g* (clarified crude extract) and the other fraction was not centrifuged (non-clarified crude extract). Both kinds of extract were divided into smaller fractions and stored at −20 °C.

2.2.7.2. Chromatography

In all experiments, the flow-rate was 1 ml/min unless stated differently. As running and dilution buffer 20 m*M* HEPES, 200 m*M* NaCl pH 7.0 was used for centrifuged homogenate and 20 m*M* HEPES, 200 m*M* NaCl, 10 m*M* imidazole for non-clarified homogenate. Clarified as well as non-clarified cell homogenates were thawed and diluted 10 times in respective type of running buffer prior to chromatography. Elution buffer was 20 m*M* EDTA, 50 m*M* NaCl pH 8.0, which also stripped both types of carriers from Cu^{2+}. For regeneration, the columns were washed with distilled water and then new Cu^{2+} was loaded by passing 0.5 or 0.1 *M* $CuSO_4$ (dissolved in distilled water) through the IDA–cryogel or IDA–Sepharose gel columns, respectively. For imidazole gradient experiments an ordinary gradient mixer was used generating a gradient of 0–200 m*M* imidazole in 20 m*M* HEPES, 200 m*M* NaCl pH 7.0. The chromatography processes were monitored using a LKB 2138 UVICORD S equipped with a 276 nm filter.

2.3. Analysis

2.3.1. $(His)_6$-LDH activity

The method used is based upon determining the decrease in optical density at 340 nm from oxidation of β-NADH, $\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$. Sample solutions were diluted to give a rate of 0.01–0.08 ΔA_{340}/min. In a 1.0 ml cuvette 0.965 ml of a reaction

mixture containing 0.225 m*M* β-NADH and 1.0 m*M* pyruvate in 0.2 *M* Tris–HCl pH 7.3 was mixed with 33 μl of appropriately diluted sample. The decrease of ΔA_{340}/min was recorded for 2 min. The activity in U (μmol/min) in the cuvette was calculated as $\Delta A_{340}/6.22$.

2.3.2. Protein content estimation with BCA

Standard solutions of 0, 200, 400, 600, 800 and 1000 μg/ml of albumin were prepared. One millilitre of a reaction mixture containing 1 part 4% (w/v) $CuSO_4$ and 50 parts BCA solution was mixed with 50 μl of appropriately diluted sample or standard. The preparations were incubated for 60 min in darkness at room temperature and then the optical density at 562 nm was read. The fractions after the imidazole gradient elution experiment were dialyzed three times for 3 h, each run against 2 liter 20 m*M* Tris–HCl pH 7.3 before being analyzed.

2.3.3. Turbidity

The optical density of fractions derived from non-clarified homogenate was measured at 450 nm. Cu^{2+}/EDTA at the concentrations used do not absorb light at this wavelength.

2.3.4. Preparation of a cryogel sample for microscopy

The sample was fixed in 2.5% glutaraldehyde in 0.15 *M* sodium cacodylate buffer overnight, post-fixed in 1% osmium tetroxide for 1 h. Then the sample was dehydrated stepwise in ethanol (0→50%→75%→96% →99.5%) and transferred to a critical point drier temperated to +10 °C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to +40 °C and the pressure to ca. 100 bar. Liquid CO_2 was transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage [4]. Release of the pressure at a constant temperature of +40 °C resulted in dried sample. Finally, it was coated with gold–palladium (40:60) and examined using a JEOL JSM-S600LV scanning electron microscope.

3. Results and discussion

3.1. Preparation and characterization of supermacroporous continuous cryogel matrices

3.1.1. Poly(acrylamide) cryogels

At first, the supermacroporous continuous chromatographic matrices have been produced by a direct cryogenic co-polymerization of AAm and MBAAm in a column in order to evaluate the feasibility of the physico–chemical properties of cryogel matrices for the bioseparation processes of interest, namely, for the flow-through procedures when using crude cell homogenates. The preparation of cryogels [from the Greek *κριοσ* (kryos) meaning frost or ice] was carried out essentially according to the earlier-described procedure [5]. The desired property of the cryogel, continuous supermacroporosity, is established by solvent crystallization, when the temperature is held below the freezing point of the solvent, which in this case is water. The monomers and initiators are concentrated in unfrozen micro zones of the apparently frozen system. The higher concentrations lead to increased reaction rates, even compared to homogeneous solution above the freezing point [6]. The polymerization reactions proceed in the unfrozen microzones. The crystals of reagent-free solvent grow during freezing, merge with other crystals until a continuous system of a frozen framework is created. Upon thawing after completed polymerization, the system consists of a monolithic matrix with continuous macroporous channels filled with liquid solvent (Fig. 1). Since the cavities are made by the frozen solvent, its crystals act as a pore-forming agent or porogen. When the matrix is ready, liquid solvent in the pores has a surface tension acting upon the wall of the gel matrix, making sharp angels from the freezing stage smoother. The walls of the polymer contain their own micropores [7]. Nevertheless, the combination of monolithic matrix structure and macroporosity, 10–100 μm in size [3], illustrated in Fig. 2, should make the system very attractive for biotechnology applications, especially since the macropores are not closed as in foam-like polymers, e.g. foam poly(urethane) rubber, but continuous all the way through the gel matrix.

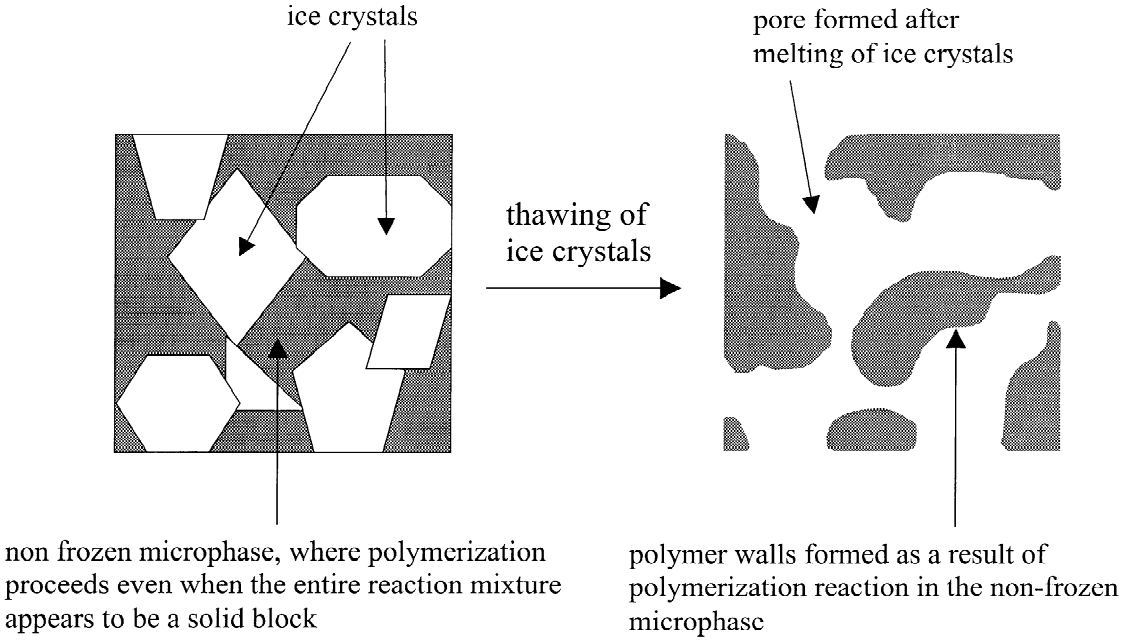

Fig. 1. Schematic presentation of cryopolymerization and formation of supermacroporous structure in a continuous polymer matrix.

The supermacroporous continuous matrix was produced by cryopolymerization of AAm with MBAAm (Table 1). The concentration of initiator (0.83%, w/w, APS to total mass (of AAm+ MBAAm) was chosen to produce the poly-(acrylamide) gel at room temperature in about 60 min. This gelation time was long enough to freeze the reaction system without hazard that the gel-formation will proceed yet before freezing, which, in its turn, required no more than 7–10 min under cooling conditions employed (see Materials and methods). One should mention that the appearance of the cryopolymerized AAm-based gels is completely different as compared to the gels produced from exactly the same reaction mixture in a liquid state. Poly(acrylamide) gels synthesized at room temperature are similar to those gels used in electrophoresis. These gels are transparent and rather brittle. Contrary, cryopolymerized gels are opaque, sponge like and elastic. When removed from the syringe, these gels can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of gel was submerged in water, it soaked in water and within 1–2 s restored its original size and shape.

It is known that the higher the temperature during freezing, the bigger the pore size of cryogels prepared [6]. However, there is a risk that the system to be frozen will be in 'overcooled' condition. So the temperature should be low enough to ensure freezing. The monomer solution in 5-ml syringes (I.D. 12.4 mm) was frozen within 10 min at −7 °C. The flow-rate of water through the gel matrix (hydrostatic pressure ca. 0.01 MPa) was decreasing sharply with decreasing MBAAm content in the polymerization mixture (Fig. 3a). A high flow-rate through the gel matrix is a simple way of estimating superporosity. The continuous gel matrix with a very high flow-through (or low flow resistance) was obtained when the molar ratio between vinyl and divinyl components in the reaction mixture were in the range of 5:1–10:1. The less cross-linked cryogels [obtained with smaller content of the cross-linker (MBAAm) in the initial mixture], the more swelling in water. As a result the size of macropores is decreased and the hydrodynamic resistance of the macroporous matrix

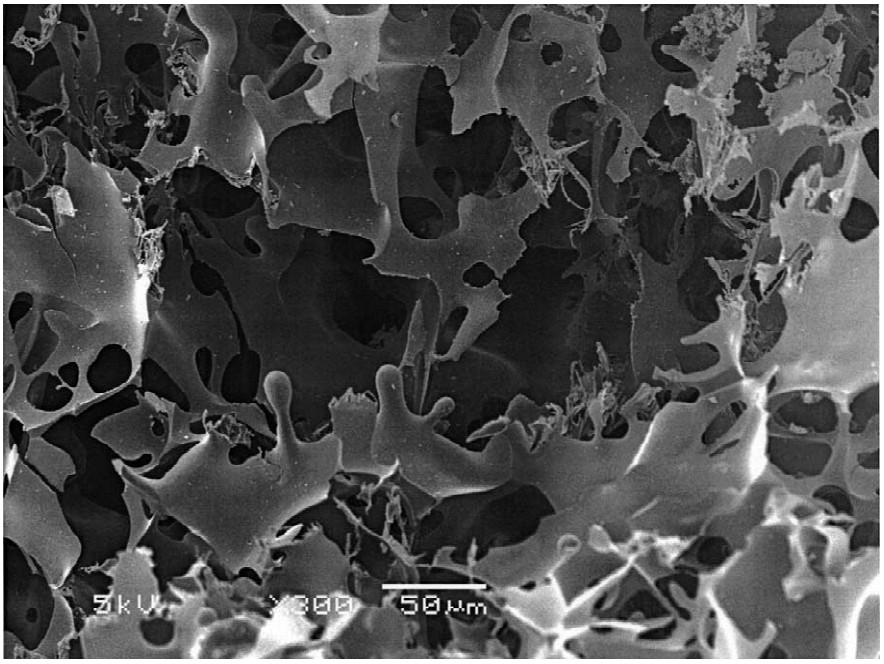

Fig. 2. Scanning electron microscope photograph of cryopolymerized poly(acrylamide) matrix. The sample was fixed in 2.5% glutaraldehyde in 0.15 *M* sodium cacodylate buffer overnight, postfixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol and critical point dried (see Materials and methods). The dried sample was coated with gold–palladium (4:60) and examined using a JEOL JSM-S600LV scanning electron microscope. Cracks and crumbled material are artifacts of cutting and drying.

is increased. At the same time, the higher the MBAAm content in the reaction mixture the more rigid matrix was obtained, allowing higher flow-through rates.

The drop in freezing temperature to −10 °C did not practically affect the flow-rate through the column (Fig. 3a, open circles) while freezing the reaction mixture at −15 °C resulted in the matrices with smaller size of macropores and consequently rather low flow-rates (Fig. 3a, closed triangles). There were practically no differences in flow-rates through the matrices obtained at −15 °C at different AAm/MBAAm ratios (all ratios in mol/mol).

Characteristics of the matrices depended on the total concentration of the co-monomer in the initial mixture. Matrices prepared from 2% (w/v) solutions of monomers (AAm/MBAAm ratio 10:1) were sponge-like and rather soft. As a consequence of the flow they were compressed to 90% of their initial height when flow-through rate was determined (hydrostatic pressure, ca. 0.01 MPa). Rather dense gel matrices with high flow-through rate (900–1500 cm/h) were obtained under the same conditions when using 3–4%(w/v) solutions of monomers.

3.1.2. Epoxy-containing poly(acrylamide) cryogels

AGE was selected for co-polymerization in various ratios to acrylamide in order to insert reactive epoxy groups in the cryogel. AGE itself does not polymerize under normal free radical polymerization conditions: 3% (w/v) aq. solution of AGE in the presence of TEMED and APS (1% to AGE mass). The AGE solubility in water (50 g/l at 20 °C, Merck Eurolab Catalogue) seemed feasible for the copolymerization of AGE with AAm. The concentration of APS (0.98–1.0%, w/w, APS to total mass of AAm + AGE + MBAAm) was chosen to produce gel at room temperature in 50–60 min. AGE content in the initial reaction mixture more then 0.2 (ratio AGE/AAm) gave a rather brittle and easily broken cryogel matrix. Further on, the AGE/AAm ratio of 1:10 was used in all experiments. The monomer

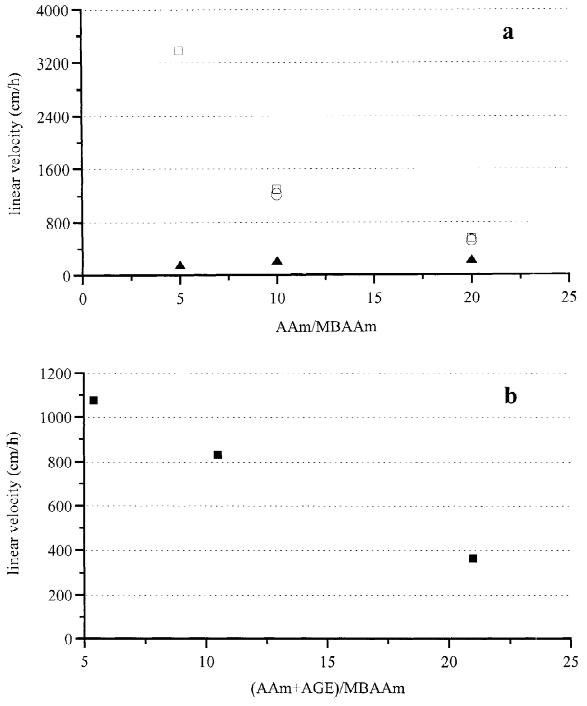

Fig. 3. (a) Dependence of the water flow-rate (hydrostatic pressure, ca. 0.01 MPa) through continuous supermacroporous columns (I.D. 12.4 mm) prepared from co-monomer mixture with different AAm/MBAAm ratio. Cryopolymerization conditions: Temperature of freezing −7.0 °C, storage 24 h at −7.0 °C (open squares); temperature of freezing −10.0 °C, storage 24 h at −10.0 °C (open circles); temperature of freezing −15.0 °C, storage 24 h at −15.0 °C (closed triangles), thawing at room temperature for all cases. Total concentration of comonomers (AAm+MBAAm) 3% (w/v). The average results of parallel measurements on three different columns produced under the same conditions are presented. (b) Dependence of the flow-rate of water (hydrostatic pressure, ca. 0.01 MPa) through continuous supermacroporous columns (I.D. 12.4 mm) prepared from co-monomer mixture with different (AAm+AGE, 10:1)/MBAAm ratio. Conditions of cryopolymerization: Temperature of freezing −12.0 °C, storages at −12 °C for 5 h, then overnight at −18.0 °C, thawing at room temperature. Total concentration of monomers (AAm+MBAAm) 4.0% (w/v). The average value of measurements performed on three columns in parallel is presented.

solution in 5-ml syringes was frozen within 10 min at −12 °C. As for epoxy activated supermacroporous continuous matrix, the flow-rate depended less on the ratio (AAm+AGE)/MBAAm in the initial reaction mixture (Fig. 3b) than on the ratio of vinyl/divinyl components for plain cryogel. Supermacroporous gels with high flow-rate were prepared when (AAm+AGE)/MBAAm was in the range 5:1–10:1 (mol/mol).

As in the case of AAm/MBAAm cryogels, characteristics of the matrices depended on the total concentration of the co-monomers in the initial mixture (Table 2). Cryogels prepared from monomer solutions with less than 3% (w/v) were rather weak. The matrix prepared from 3.5% (w/v) co-monomer solution compressed up to 20% at a flow-rate generated by a hydrostatic pressure of ca. 0.01 MPa. The gel matrix prepared from 4.4% (w/v) and 5% (w/v) of monomer solutions withholds these pressures without any visible changes. A matrix that was prepared from 6% solutions of co-monomers resulted in a rather dense cryogel.

The supermacroporous gel matrices prepared by cryopolymerization swell much less as compared to the ordinary AAm-based gels prepared at the same monomer/initiator/activator concentration at room temperature (Fig. 4). The swelling degree of the AAm/MBAAm cryogels (3–5 g of water per gram of dry polymer) and epoxy-containing cryogels (5–6 g of water per gram of dry polymer) corresponded to the swelling degree of ordinary AAm/MBAAm-gels prepared from much higher monomer concentrations of 10–15% (v/w) [8]. This is due to the fact that the proper gel framework (pore walls) of macroporous cryogels is composed from the high-concentrated polymer network possessing low swelling extent.

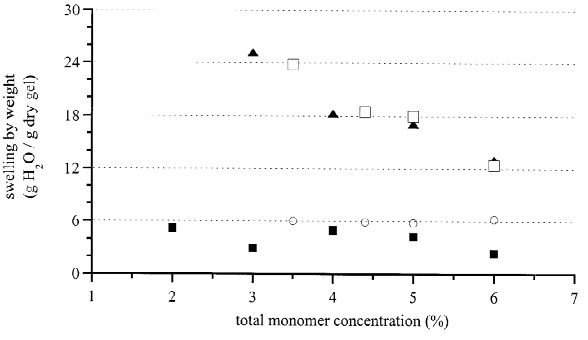

Fig. 4. The degree of swelling, $S_{w/w}$, of cryogels (closed squares) prepared by cryopolymerization for 24 h at −10 °C and epoxy-activated cryogels (open circles) prepared by cryopolymerization for 24 h at −12 °C. For comparison, the degree of swelling of gels prepared by the polymerization of the same reaction mixture at room temperature are presented (closed triangles and open squares for the gel and epoxy-containing gel, respectively).

So, the supermacroporous sponge-like epoxy-containing gel matrix (flow-rate 1000–1200 cm/h) with high concentration of reactive epoxy groups (20–23 μmol/ml of gel) was obtained when total concentration of co-monomer (AAm + AGE + MBAAm) is 4–5% and vinyl/divinyl (AAm + AGE, 10:l)/MBAAm ratio lies in the range of 5:1–10:1. The column that was used in LDH-chromatography experiments had a total concentration of 4.4% comonomer and the (AAm + AGE, 10:1)/MBAAm ratio was 5.4:1.

Due to the large pore size and interconnected pore-structure, the continuous chromatographic poly(acrylamide) matrix has a very low flow resistance when the molar ratio between vinyl and divinyl component in the reaction mixture was in the range of 5:1–10:1. Water flows freely through the 4.5 cm high column at flow-rates about 750–2000 cm/h, (hydrostatic pressure ca. 0.01 MPa).

It is worthwhile to point out for comparison that HPLC operates at flow-rates of 300–1700 cm/h at excessive pressures of 2–10 MPa [9] and recently developed expanded bed chromatography at flow-rates of 200–400 cm/h at excessive pressure about 0.01 MPa [10]. Thus, it is reasonable to foresee that after an appropriate optimization, continuous supermacroporous matrices produced by the cryotropic gelation technique would allow chromatographic process at flow-rates comparable with those in HPLC and exceeding those used in expanded bed chromatography, while using only minimal pressures typical for low pressure protein chromatography. Flow rates up to 2000 cm/min were reported for the continuous matrices based on the copolymer of glycidyl methacrylate and ethylene dimethacrylate, so called CIM disks (BIA Separation, Slovenia) [11]. However, it should be noticed that these disks are only about 2 mm thick [12].

The large and rather uniform pore size in the continuous cryogel matrices was confirmed by measuring HETP values at different flow-rates and for compounds of different sizes, namely tyrosine (M_r, 181), BSA (M_r 69 000) and Blue Dextran (M_r 2 000 000). The elution volume and HETP were essentially the same for all the above mentioned substances. Only a slight increase in HETP was detected when the flow-rate was increased more than 10-fold (Fig. 5a and b).

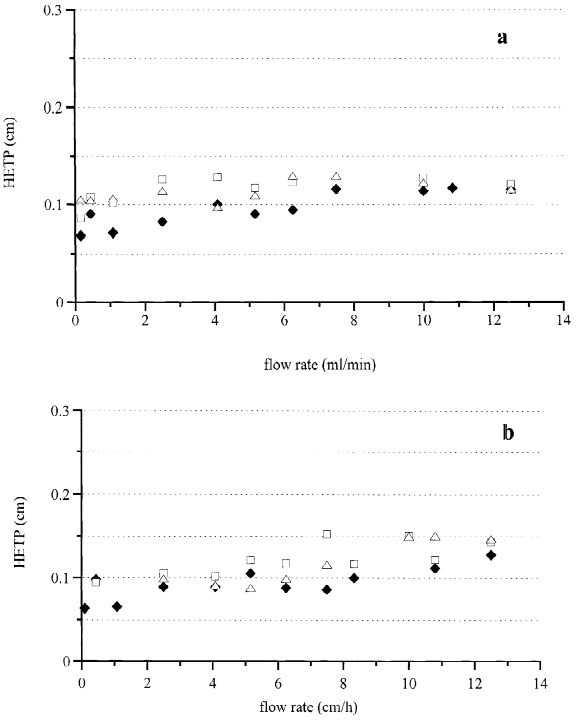

Fig. 5. Height equivalent to theoretical plate (HETP) variation with flow-rate for tyrosine (closed rhombs), bovine serum albumin (squares) and Blue Dextran (open triangles) for the AAm/MBAAm column (a) and (AAm + AGE)/MBAAm column (b). AAm/MBAAm column: total monomer concentration (AAm + MBAAm) 3%, AAm/MBAAm ratio 10:1, temperature of freezing −10.0 °C, storage 24 h at −10.0 °C, thawing at room temperature. Epoxy-containing column (with blocked epoxy groups): total monomer concentration (AAm + MBAAm) 4.0%, (AAm + AGE, 10:1)/MBAAm 5.4:1, temperature of freezing −12.0 °C, storage 24 h at −12.0 °C, thawing at room temperature.

3.1.3. Continuous cryogel matrices for metal chelate affinity chromatography of LDH

The good chromatographic performance of the column was also demonstrated by the sharp elution profile of $(His)_6$-LDH from crude extract (Fig. 6a). Visually, reasonably flat concentration profiles, both when Cu^{2+} ions were loaded on the IDA-coupled supermacroporous continuous column and when bound Cu^{2+} ions were eluted with EDTA solution, were observed as a blue color on a white background. These facts indicate a homogeneous structure of the matrix with no channels, cracks etc.

Ligand density for the IDA–cryogel used in this experiment was 23.3 μmol/ml gel or 23.3 m*M*. The

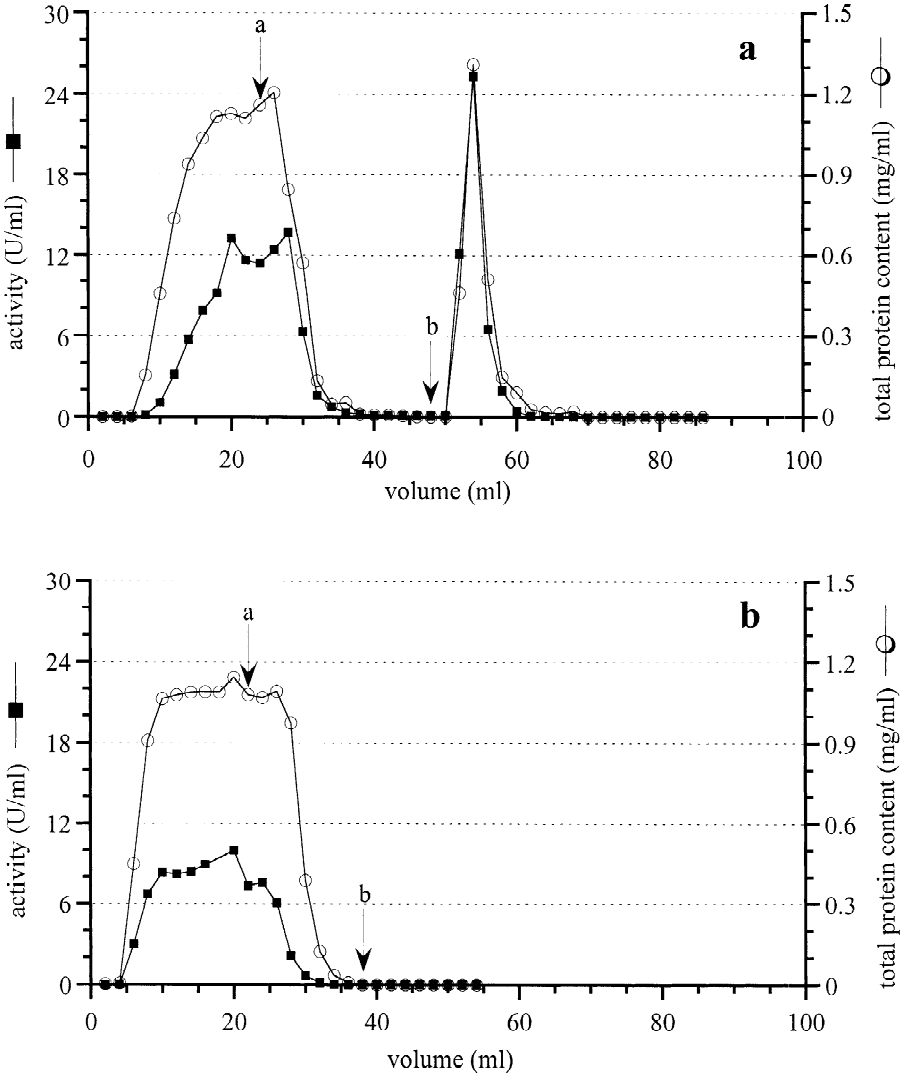

Fig. 6. (a) Breakthrough and elution profile for clarified crude extract upon the Cu^{2+}–IDA cryogel column. A 23 ml volume of clarified crude extract was applied at a flow-rate of 1 ml/min. Arrow **a** indicates the start of washing with 20 m*M* HEPES, 200 m*M* NaCl pH 7.0 and arrow **b** indicates elution with 20 m*M* EDTA, 50 m*M* NaCl pH 8.0. Closed squares represent LDH activity and open circles represent total protein. (b) Chromatography profile where neither $(His)_6$-LDH, nor other proteins bind to the ethanolamine-blocked epoxy activated column. A 20 ml volume of clarified crude extract was applied at a flow-rate of 1 ml/min. Arrow **a** indicates the start of washing with 20 m*M* HEPES, 200 m*M* NaCl pH 7.0 and arrow **b** indicates elution with 20 m*M* EDTA, 50 m*M* NaCl pH 8.0. Closed squares represent LDH activity and open circles represent total protein content.

wet mass of the 5.4 ml IDA–cryogel was 5.7 g and the mass (after drying was 170 mg or 31 mg/ml swollen gel. This gives a ligand density of 745 μmol/g dried polymer. Corresponding figures for the IDA–Sepharose were 67.3 μmol/ml gel or 67.3 m*M*. The 5.9 ml (packed volume) IDA–Sepharose had a dry mass of 0.38 g or 64 mg/ml giving a ligand density of 1050 μmol/g dried gel. Comparing these results shows that the density of available ligands of the IDA–cryogel is 35% of the IDA–Sepharose with respect to concentration in the wet columns.

Drying of the matrix at 70 °C did not affect the

binding capacity of rehydrated column towards $(His)_6$-LDH. The matrix was exposed to three subsequent cycles of drying and swelling. The last drying period was for 3 weeks, after which the matrix was put back into its column and a $(His)_6$-LDH purification was performed without any deterioration in quality. This may be a valuable commercial property.

3.2. $(His)_6$-LDH binding/elution on IDA–cryogel continuous column

E. coli cells used for the production of $(His)_6$-LDH have a size of 1×3 μm [13]. Hence cell debris and even whole cells were expected to pass rather easily through the pores of 10–100 μm size in the continuous poly(acrylamide) cryogel matrix.

As the chromatographic carrier developed is completely new, we evaluated first its chromatographic behavior as compared to the traditional carrier, IDA–Sepharose Fast Flow, using particulate-free (centrifuged) cell homogenate as a source of $(His)_6$-LDH. Under the conditions used, the cryogel continuous columns without ligand (epoxy activated column with epoxy groups blocked by ethanolamine) did not bind any detectable amount of $(His)_6$-LDH or proteins present in the homogenate clarified by centrifugation (Fig. 6b). When loaded with Cu^{2+} ions, the supermacroporous column bound efficiently $(His)_6$-LDH (Fig. 7), the latter was completely eluted with 20 m*M* EDTA solution (Fig. 6a). The breakthrough profiles of both $(His)_6$-LDH, prepurified by IDA–Sepharose Fast Flow chromatography and $(His)_6$-LDH from the clarified crude extract were very similar and clearly came later than the breakthrough of $(His)_6$-LDH from crude extract on the same type of column but containing no ligand (epoxy activated matrix with ethanolamine-blocked epoxy groups). The breakthrough profiles indicated that the column bound specifically $(His)_6$-LDH, which could be eluted later when using EDTA capable of removing metal ions off IDA ligands. The dynamic binding capacity of the column with respect to $(His)_6$-LDH was 7.9 U/ml matrix (at a breakthrough level of 7% of maximal) while static binding capacity at the column saturation was 26.6 U/ml matrix. Assuming specific activity of 60 U/mg protein for the $(His)_6$-LDH preparation purified using Cu^{2+}–IDA–Sepharose Fast Flow, one could calculate dynamic and static binding capacities of the developed continuous supermacroporous matrix as 0.13 and 0.44 mg/ml, respectively. Certainly, these values are less as

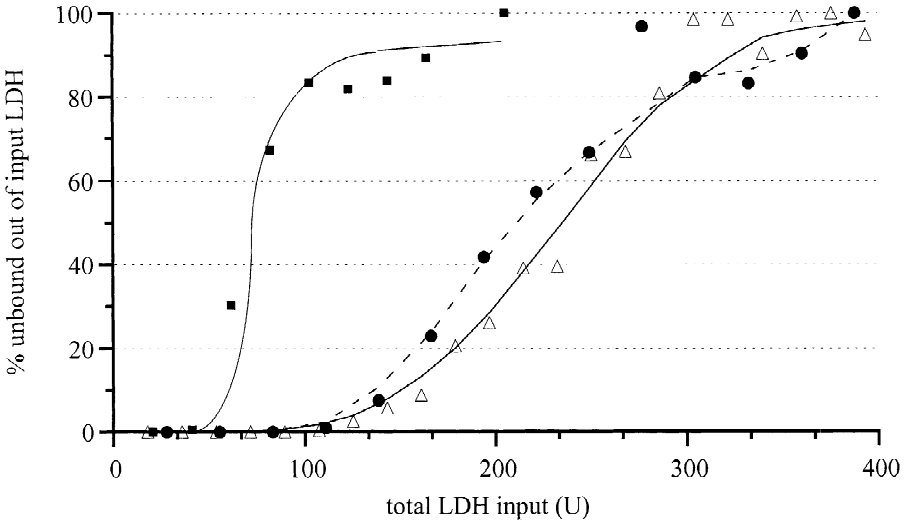

Fig. 7. Breakthrough profiles of clarified crude extract upon the ethanolamine-blocked epoxy activated column (closed squares and solid line), clarified crude extract upon the Cu^{2+}–IDA–cryogel column (open triangles and solid line) and purified, clarified crude upon the Cu^{2+}–IDA–cryogel column (open triangles and solid line) and purified, clarified crude upon the Cu^{2+}–IDA–cryogel column (closed circles and dotted line). Purification of clarified crude extract is described in figure legend 8a.

compared to what is achieved for beaded gels. One should take into account that the pore size like in the agarose gels is usually less than 0.1 μm, which is about two orders of magnitude less than in the continuous supermacroporous matrices developed. Hence the available surface for protein binding will be about four orders of magnitude less than in agarose gels. The main advantage of the continuous supermacroporous matrices is the possibility to directly process non-clarified homogenates, which will be demonstrated further on in the paper.

Elution of bound $(His)_6$-LDH with imidazole gradient from both Cu^{2+}–IDA supermacroporous column and Cu^{2+}–IDA–Sepharose Fast Flow resulted in similar elution profiles (Fig. 8a and b) with two distinct fractions eluted. The first one containing lots of proteins with a relatively low enzyme activity while the second one contains highly active enzyme. Probably the first peak contains proteins with inherent histidine residues at the surface with native wild type lactate dehydrogenase being one of them [14]. To avoid binding of the proteins eluted in the first peak of the gradient elution, 10 m*M* imidazole was included in the running buffer when running chroma-

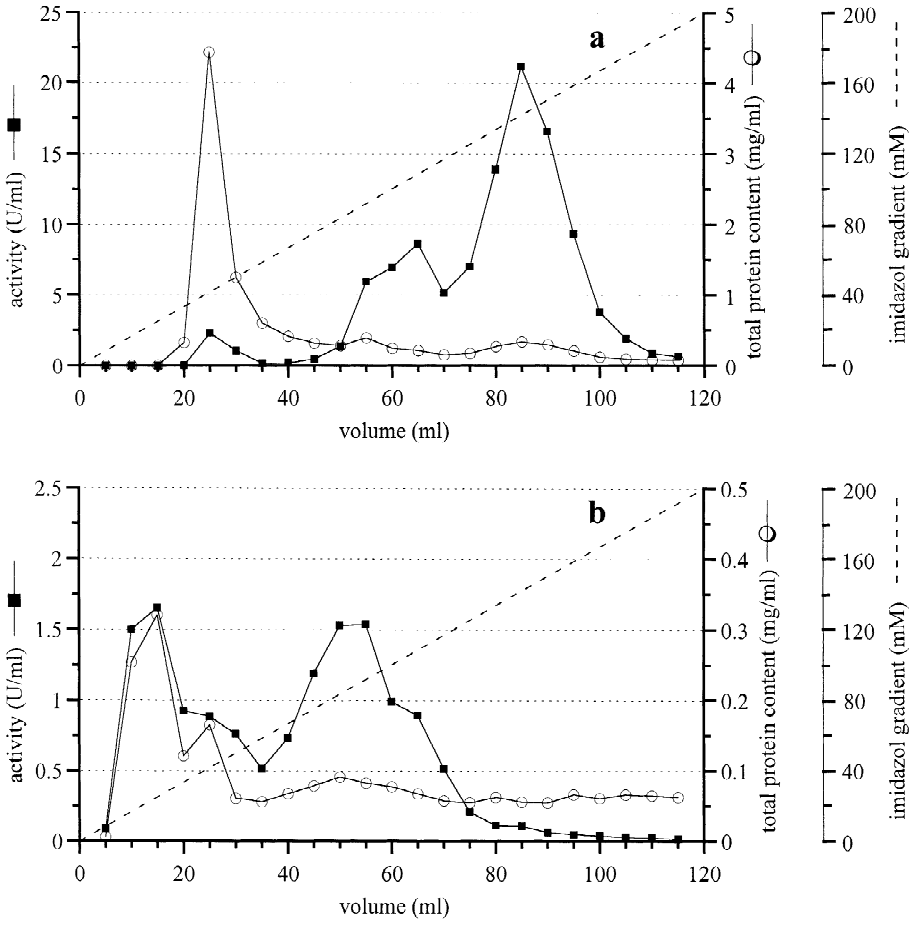

Fig. 8. Gradient elution of LDH from (a) 45 ml clarified crude extract on a 5.9 ml Cu^{2+}–IDA–Sepharose Fast Flow column and (b) 28 ml clarified crude extract on a 5.4 ml Cu^{2+}–IDA supermacroporous continuous column. Flow rate: 1 ml/min. Elution was performed with a 0–200 m*M* imidazole gradient containing 20 m*M* HEPES, 200 m*M* NaCl pH 7.0 followed by releasing of Cu^{2+} with 20 m*M* EDTA, 50 m*M* NaCl pH 8.0. Very low LDH activity was found in the EDTA fraction (data not shown). The two elution peaks (elution volume 55–100 ml) from the purification on Cu^{2+}–IDA–Sepharose were pooled and dialyzed three times 3 h against 2 liter 20 m*M* Tris–HCl pH 7.3 and were used as purified, clarified crude for one of the breakthrough experiments in Fig. 7.

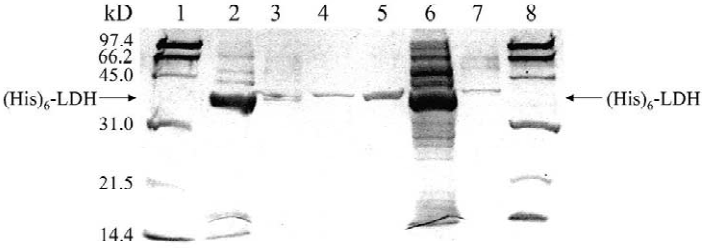

Fig. 9. Sodium dodecylsulphate–polyacrylamide gel. Lanes: 1 and 8 = molecular mass standards; 2 = clarified crude extract; 3 = elution peak fraction from Fig. 7; purification from clarified crude extract upon Cu^{2+}–IDA–cryogel; 4 = elution peak fraction at 85 ml from Fig. 8a; gradient elution from clarified crude extract upon Cu^{2+}–IDA–Sepharose Fast Flow column; 5 = elution peak fraction at 55 ml from Fig. 8b; gradient elution from clarified crude extract upon Cu^{2+}–IDA–cryogel 6 = non-clarified crude homogenate; 7 = elution peak fraction from Fig. 10; purification from non-clarified crude extract upon Cu^{2+}–IDA–cryogel. kD = kilodalton.

tography on non-clarified crude homogenate. Under these conditions 3.1-fold purification was achieved from peak fraction and a total recovery of about 90% from the entire elution volume. One should note, that high purification folds could not be expected in this case as $(His)_6$-LDH presents the main part of soluble proteins in the crude extract (Fig. 9).

The lower purification factor obtained from imidazole gradient elution on the continuous supermacroporous Cu^{2+}–IDA column (3.5) as compared to the Cu^{2+}–IDA Sepharose Fast Flow column (6.7) could be due to the lower ligand density on the continuous supermacroporous Cu^{2+}–IDA column. The lower the ligand density, the less adsorption/desorption events for the $(His)_6$-LDH could take place, resulting in a lower selectivity of the column. In the case of non-clarified crude homogenate, the highest purification factor obtained on the Cu^{2+}-IDA column was also slightly lower (3.1) than for clarified crude homogenate on the same column. Some components of the non-clarified crude could interact with continuous supermacroporous Cu^{2+}–IDA column decreasing the total purification factor for $(His)_6$-LDH.

3.3. Direct capture of $(His)_6$-LDH from crude extract using IDA-supermacroporous continuous column

The most critical part of the study was to evaluate

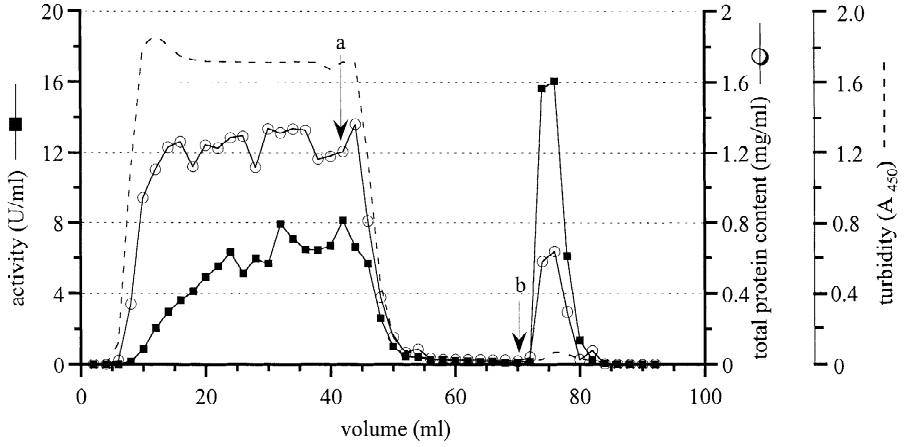

Fig. 10. Breakthrough and elution profile for non-clarified crude extract upon the Cu^{2+}–IDA–cryogel column. Forty millilitres of non-clarified crude extract diluted in flow buffer containing 10 m*M* imidazole were applied at a flow-rate of 1 ml/min. Arrow **a** indicates the start of washing with 20 m*M* HEPES, 200 m*M* NaCl, 10 m*M* imidazole pH 7.0 and arrow **b** indicates elution with 20 m*M* EDTA, 50 m*M* NaCl pH 8.0. Closed squares represent LDH activity and open circles represent total protein content.

whether it was possible to capture directly $(His)_6$-LDH from non-clarified homogenate. Fig. 10 presents a breakthrough profile of non-clarified cell homogenate through the column. First came the sharp breakthrough of turbidity shortly followed by a sharp protein profile. The breakthrough profile of $(His)_6$-LDH came later and was less sharp. The particulate material in the cell homogenate was removed from the column at the washing step and the EDTA-eluted fractions were transparent. The elution peak was rather symmetrical with minimal tailing. The particulate material was not accumulated in the column. Under six repetitive runs of $(His)_6$-LDH purification from non-clarified crude homogenate the column did not demonstrate any pronounced deterioration in performance. The recovery varied between 70 and 90% with the purification factor slightly decreasing from 2.3 to 2.1 (Fig. 11a). The above mentioned results were obtained at a flow-rate of 1 ml/min (50 cm/h). To evaluate the limits of the column performance, the repetitive purifications were carried out at increasing flow-rates up to 12.5 ml/min (625 cm/h). At this flow-rate, the column behaved nearly as well as at 1 ml/min (Fig. 11b). At 15 ml/min, pumping of the non-clarified homogenate resulted in the compression of the matrix and sharply increased flow resistance. Probably, the operational flow-rates can be increased even more in supermacroporous continuous matrices produced by cryopolymerization with further optimization of the cryotropic gel-formation procedure and selecting monomers capable of giving more rigidity than poly(acrylamide) networks.

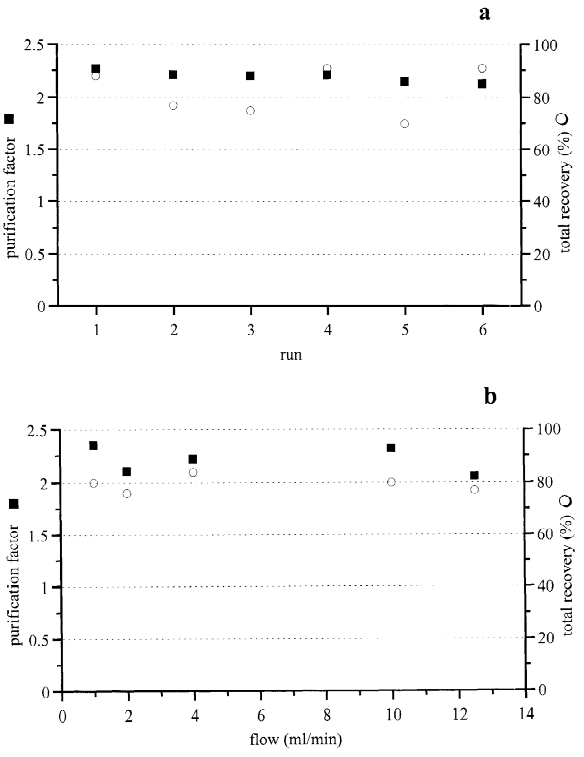

Fig. 11. (a) Repetitive purification of $(His)_6$-LDH at 1 ml/min flow-rate. Six cycles of 2 ml portions of non-clarified crude extract diluted in flow buffer containing 10 m*M* imidazole were applied to the Cu^{2+}–IDA–cryogel column and after washing with the same buffer the bound proteins were eluted with 20 m*M* EDTA, 50 m*M* NaCl pH 8.0. In each run two elution fractions were collected, the first was 9 ml and the second 15 ml. Closed squares represent purification factor of the first elution fraction and open circles represent total recovery of both elution fractions. (b) Repetitive purification of $(His)_6$-LDH at increasing flow-rate. Five cycles of 1 ml portions of non-clarified crude extract diluted in flow buffer containing 10 m*M* imidazole were applied to the Cu^{2+}–IDA–cryogel column but with different flow-rates: 1, 2, 4, 10 and 12.5 ml/min. After washing with the same buffer the bound proteins were eluted with 20 m*M* EDTA, 50 m*M* NaCl pH 8.0. In each run two elution fractions were collected, the first was 9 ml and the second 15 ml. Closed squares represent purification factor of the first elution fraction and open circles represent total recovery of both elution fractions.

4. Conclusion

A new technique, cryotropic gelation via co-polymerization of AAm(+AGE)/MBAAm mixtures in frozen aqueous media, has been developed to produce poly(acrylamide)-based supermacroporous continuous adsorbents carrying affinity ligands. Non-clarified crude cell homogenates can be processed directly on these adsorbents with no need for preliminary centrifugation or filtration. Due to the large pore size of 10–100 μm in the developed adsorbents, particulate material present in the crude passes easily through the continuous column without visible accumulation. For example, all experiments presented in this paper were carried out on the same column. About 135 ml of clarified and 75 ml of non-clarified crude homogenate were processed on the same 5.0 ml column in a total of 26 runs with no significant deterioration of the column performance. Relatively low capacity of the developed continuous supermacroporous adsorbents caused by the relatively low

surface area available for protein binding is compensated by the high flow-rates through the column, competitive with those achieved in expanded bed chromatography. The potential areas of application of continuous supermacroporous adsorbents are fast on-line analysis of fermentation processes, direct capture of labile products from suspensions and cell homogenates and chromatographic separation of large (at the molecular level) products, like plasmids, viruses, cell organelles and even whole intact cells.

Moreover, the technology of producing the continuous matrix is straightforward. The starting materials, AAm, MBAAm and initiators are essentially the same as used everyday in the practice of electrophoresis. These materials are available and cheap and this could make the cryopolymerized matrices suitable for single time use in the same way as electrophoresis gels.

Acknowledgements

The authors would like to thank the Swedish Institute for providing a fellowship to F.M.P. for her stay at the Department of Biotechnology, Lund University; INTAS project 00-00057 and Lund University, Faculty of Technology for financial support.

References

[1] M. Schuster, E. Wasserbauer, C. Ortner, K. Graumann, A. Jungbauer, F. Hammerschmid, G. Werner, Bioseparation 9 (2000) 59.
[2] X.-D. Tong, B. Xue, Y. Sun, Biotechnol. Progr. 17 (2001) 134.
[3] V.I. Lozinsky, F.M. Plieva, I.Yu. Galaev, B. Mattiasson, Bioseparation 10 (2001) 163.
[4] M.T. Postek, K.S. Howard, A.H. Johnson, K.L. McMichael, Scanning Electron Microscopy, Ladd Research Industries, Burlington, VT, 1980.
[5] V.I. Lozinsky, E.S. Vainerman, S.A. Ivanova, E.F. Titova, M.I. Shtil'man, E.M. Belavtseva, S.V. Rogozhin, Acta Polym. 37 (1986) 142.
[6] V.I. Lozinsky, S.A. Morozova, E.S. Vainerman, E.F. Titova, M.I. Shtil'man, E.M. Belavtseva, S.V. Rogozhin, Acta Polym. 40 (1989) 8.
[7] E.M. Belavtseva, E.F. Titova, V.I. Lozinsky, E.S. Vainerman, S.V. Rogozhin, Colloid Polym. Sci. 262 (1984) 775.
[8] K. Sun, A.H. Senon, Can. J. Chem. 43 (1965) 969.
[9] S. Lindsay, High Performance Liquid Chromatography, Wiley, Chichester, 1992.
[10] H.A. Chase, Trends Biotechnol. 12 (1994) 296.
[11] R. Hahn, A. Jungbauer, Anal. Chem. 72 (2000) 4853.
[12] C. Kasper, L. Meringova, R. Freitag, T.B. Teimikova, J. Chromatogr. A 798 (1998) 65.
[13] M.T. Madigan, J.M. Martinko, J. Parker, Brock Biology of Microorganisms, Prentice–Hall, Upper Saddle River, NJ, 2000.
[14] M. Mejàre, S. Ljung, L. Bülow, Protein Eng. 11 (1998) 489.