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Purification of S-oxynitrilase from *Sorghum bicolor* by immobilized metal ion affinity chromatography on different carrier materials

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ABSTRACT

The purification of the hydroxynitrile lyase (EC 4.1.2.11, S-oxynitrilase) from *Sorghum bicolor* is compared using different strategies. A new procedure is presented, which exploits the affinity of S-oxynitrilase towards metal ions as a key step in purification. The metal ions are immobilized by chelators on different carrier materials, *e.g.* Sepharose beads, microporous membranes or poly(ethylene glycol). A systematic examination demonstrates the excellent potential of immobilized metal affinity chromatography as a preparative separation method.

INTRODUCTION

In 1975, Porath *et al.* [1] reported a new method for the purification of proteins, making use of the selective retention of proteins on transition metal ions chelated to an insoluble matrix, such as agarose. The interaction occurs between the side-chains of particular amino acids (histidine, cysteine) exposed on the surface of the protein and divalent metal ions of copper, cobalt, nickel and zinc. An increasing number of papers have shown the importance of this technique, immobilized metal ion affinity chromatography (IMAC), for the purification of proteins [2], peptides [3], and whole cells [4]. The principles and applications of IMAC have been reviewed recently by Kagedal [5], Hemdan *et al.* [6] and Arnold [7]. Affinity extraction uses aqueous two-phase systems for the purification of macromolecules, often employing pseudospecific ligands such as triazine dyes [8]. Suh and Arnold [9] described the synthesis of poly(ethylene glycol-iminodiacetic acid) (PEG-IDA) and used this affinity ligand for metal affinity extraction or metal affinity precipitation of model proteins [10]. Hence, it was of interest to us to explore metal affinity interactions for the purification of enzymes from complex crude extracts.

This paper describes a fast purification scheme for the S-oxynitrilase from *Sorghum bicolor*. This enzyme catalyses the decomposition of S-cyanohydrins, the natural substrate being *p*-hydroxymandelonitrile. The S-oxynitrilase is of interest for the synthesis of chiral cyanohydrins, which are important educts for organic synthesis [11]. The enzyme has been isolated from seedlings of *Sorghum*, where it is present in rather low amounts [12]. We report, for the first time, an application of immobilized metal ion affinity extraction and the new IDA membrane for the purification of a native enzyme directly from plant extracts.

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EXPERIMENTAL

Chemicals

Inorganic salts, sodium acetate and Coomassie brilliant blue were obtained from E. Merck (Darmstadt, Germany). DL-*p*-Hydroxymandelonitrile was purchased from Aldrich (Steinheim, Germany). PEG samples (400, 1550, 2000 and 20 000) were from Chemische Werke Hüls (Marl, Germany). The chelating-Sepharose Fast Flow (FF) was purchased from Pharmacia (Uppsala, Sweden). The IDA membrane (Sartobind-IDA, SM 178 72, pore size 0.45 μm) and the anion-exchange membrane (Sartobind-Q, SM 178 73, pore size 0.45 μm, capacity 0.8 mg of bovine serum albumin per cm^2 membrane) were gifts from Sartorius (Göttingen, Germany).

Analytical procedures

The protein concentration was determined according to Bradford [13], using bovine serum albumin as the standard. For the S-oxynitrilase assay, a 0.001 *M* solution of *p*-hydroxymandelonitrile was freshly prepared in 0.05 *M* sodium citrate buffer (pH 3.75) and 1 ml of the substrate solution was mixed with 0.025 ml of a protein solution to be tested. The absorbance was followed at 285 nm in a Shimadzu UV-160 spectrophotometer (Shimadzu, Düsseldorf, Germany). A molar absorption value of 16.3 cm^{-1} mol^{-1} was used to calculate the enzyme activity. One unit is defined as the amount of enzyme that catalyses the decomposition of 1 μmol of substrate per minute under these conditions.

Gel electrophoresis was performed under native conditions in a 10–15% polyacrylamide gradient gel in a Phast system following the instruction of the manufacturer (Pharmacia, Uppsala, Sweden). The gel was stained with Coomassie brilliant blue R-250.

Enzyme preparation

Seeds of *Sorghum bicolor* were a gift from Semundo Saatzucht (Hamburg, Germany). Dark-grown shoots were harvested after seven days, frozen in liquid nitrogen and ground to powder [12]. The frozen plant material was suspended in 0.05 *M* potassium phosphate buffer (pH 7.5) with an Ultraturrax (IKA, Staufen, Germany). This suspension was centrifuged and the supernatant used for the following experiments.

Before the chromatographic separation, the S-oxynitrilase was purified by extraction in an aqueous two-phase system. The composition of the first phase system was 20% PEG 1000 and 9% potassium phosphate (pH 7.0). The enzyme was extracted in the PEG-rich upper phase at room temperature. To generate the second phase system, 1.9% PEG 20000 and 8% potassium phosphate were added to the separated upper phase of system 1. Phases were mixed for 5 min on the overhead shaker and centrifuged for 1 min at 1000 *g* in order to accelerate phase separation. The volumes of the phases were noted, and samples were removed for enzyme assays and protein determination.

Determination of the partition coefficient

The partition coefficient, K, is defined as the ratio of the S-oxynitrilase activities in the upper and lower phase. The effect of the affinity of a protein for the metal–IDA-PEG complex was expressed in terms of $\Delta \log K$. $\Delta \log K$ was calculated as $\log K = \log K_{aff} - \log K_0$, where K_{aff} and K_0 are the partition coefficients of the protein in the presence and absence of metal–IDA-PEG in the system [1,4].

Preparation of copper–IDA-PEG

This PEG derivative was synthesized from $(Br)_2$-PEG (both terminal ends brominated) (M_r 20 000) and IDA according to a published procedure [9]. The tridentate IDA-PEG was mixed with excess copper(II) sulphate and extensively dialysed against distilled water before use. PEG of high M_r was chosen because the derivative can be retained by a dialysis membrane and exhibits a higher partition coefficient than PEG derivatives of lower M_r. Both factors help the handling and recovery of the affinity ligand.

Preparation of two-phase systems for affinity extraction

The aqueous two-phase systems used were

made up by mixing a stock solution of 20% sodium sulphate and 40% PEG 20 000 in appropriate amounts. The systems were buffered with 0.05 *M* sodium phosphate (pH 8.0) and mixed with metal (II)–IDA-PEG, water and protein solution to give the final concentrations indicated.

Each sample was equilibrated by overhead shaking for 5 min at 20°C. The separation of the phases was completed by centrifugation at 1000 *g* for 1 min.

Chromatographic procedures

The chelating-Sepharose FF was packed into a column (8 cm × 1 cm I.D.) and washed with water prior to loading with 30 m*M* copper(II) sulphate. The column was rinsed with water to remove any excess copper(II) ions and equilibrated with three column volumes of 0.05 *M* potassium phosphate buffer (pH 7.5). The flow-rate was 60 cm/h at ambient temperature. After sample application, the column was washed with 0.05 *M* sodium acetate (pH 4.5). For the elution of S-oxynitrilase, 0.05 *M* EDTA and 0.15 *M* NaCl (pH 7.0) were used. Fractions of 3 ml were collected, and the elution was monitored at 280 nm and by enzyme assay. The retention of the enzyme is expressed in V_e, where V_e is the volume of the eluate. The same procedure was used with the IDA membrane. Four membranes of 7.2 cm radius were packed into a filtration unit (Sartorius). The membranes were loaded with excess copper (II) ions. Washing and equilibration were identical with that of the gel. A flow-rate of 200 ml/h was employed. Fractions of 10 ml were collected, and the enzyme activity was determined.

Gel permeation chromatography

The S-oxynitrilase was separated from the copper(II)–EDTA complex by gel permeation chromatography. PD-10 columns from Pharmacia, were used, and 1 ml of the eluate from the IMAC column was applied. Equilibration of the column and elution of the enzyme were carried out following the instructions from the manufacturer.

Ion-exchange membrane (Sartobind-Q SM 178173)

If a larger volume of S-oxynitrilase solution had to be separated from the copper(II)–EDTA complex, the ion-exchange membrane was used instead of the gel permeation chromatography. To guarantee adsorption on the ion-exchange membrane, the solution was diluted 1:4 to lower the ionic strength. The S-oxynitrilase was bound to the membrane at pH 7.0 and eluted at pH 5.5 in 0.1 *M* sodium acetate–0.15 *M* NaCl.

After the affinity extraction by copper–IDA-PEG, the lower phase had to be desalted before it could be adsorbed on the ion-exchange membrane. Standard procedures (diafiltration, dialysis), were used for desalting. The S-oxynitrilase could be separated and recovered under the same conditions as described above.

RESULTS

Two new purification schemes for the S-oxynitrilase will be discussed. Both use the affinity of S-oxynitrilase for immobilized copper(II) ions on different carriers as key steps in separation.

Immobilized metal ion affinity chromatography

The first procedure is summarized in Table I. As the initial step, physical extraction in an aqueous two-phase system was applied. The enzyme could be separated from cell debris and recovered in the PEG-rich upper phase. Following the usual process design [15], a secondary phase system was then generated by adding salt and PEG 20 000 to the upper phase of the primary phase system. The conditions established by the addition of the PEG 20 000 and salt ensured that the enzyme was partitioned into the salt-rich lower phase. The lower phase was diluted 1:4, to reduce the high viscosity of the solution before sample application on the IMAC column.

Fig. 1 illustrates the chromatographic separation on a copper–IDA Sepharose FF column. S-oxynitrilase was applied in 0.05 *M* potassium phosphate (pH 7.5) on chelating-Sepharose loaded with copper(II). The major portion of the contaminating proteins were not retained by the col-

TABLE I

PURIFICATION OF S-OXYNITRILASE FROM *SORGHUM BICOLOR* (METHOD 1)

Purification step	Oxynitrilase activity (U)	Protein (mg)	Purification factor	Activity yield (%)	Specific activity (U mg^{-1})
Extraction	800	445.0	1.0	100	1.8
Two-phase system (20% PEG, 9% potassium phosphate pH 7.0)	639	106.5	3.3	80	6.0
IMAC column	524	2.6	111.0	65	200.1
(or IMAC membrane)	510	2.8	102.0	64	180.0
Gel permeation chromatography	481	1.8	150.0	60	270.0
(or anion-exchange membrane)	478	1.8	150.0	60	269.1

umn, and only traces of S-oxynitrilase were found in the wash. The protein peak eluted with 0.05 *M* sodium acetate (pH 4.5) contained little S-oxynitrilase activity. The majority of the S-oxynitrilase activity was eluted with 0.05 *M* EDTA –0.15 *M* NaCl (pH 7.0). In this case the absorbance at 280 nm is not useful for protein detection, because the copper(II)–EDTA complex has a high extinction at 280 nm. Besides treatment with EDTA, it was possible to elute S-oxynitrilase with 0.05 *M* sodium acetate buffer (pH 3.3)–1 *M* NH_4Cl. The recovery under these conditions, however, was lower than with the EDTA solution (data not shown).

A large volume of S-oxynitrilase solution could be purified on a newly available microporous IDA membrane. The copper–IDA membrane exhibited a capacity of 0.15 mg of S-oxynitrilase per cm^2. Four membranes (surface area 615 cm^2) were packed into a filtration unit. The membranes allowed a 200-fold increased flow-rate compared with the gel [16]. The bonding properties of the membrane were slightly weaker than those of the gel. The elution of S-oxynitrilase was possible with 0.05 *M* sodium acetate buffer (pH 3.7) or 0.05 *M* EDTA buffer (pH 7.0)–0.15 *M* NaCl (Fig. 2). Also in this case a higher yield was obtained with the EDTA buffer than by

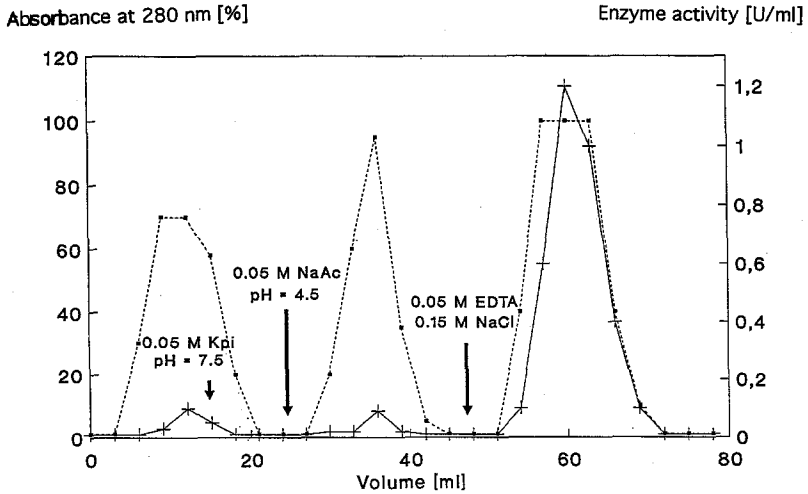

Fig. 1. Purification of S-oxynitrilase on a copper(II)-loaded chelating-Sepharose FF column. A sample of 15 ml was applied to the column, equilibrated in 0.05 *M* potassium phosphate buffer (pH 7.5). The column was washed with 10 ml of starting buffer and 25 ml of 0.05 *M* sodium acetate (pH 4.5) buffer, and the protein was eluted with 0.05 *M* EDTA–0.15 *M* NaCl (pH 7.0) at a flow-rate of 1.0 ml/min. Protein was monitored at 280 nm (dotted line). The enzyme activity was assayed in the fractions (continuous line).

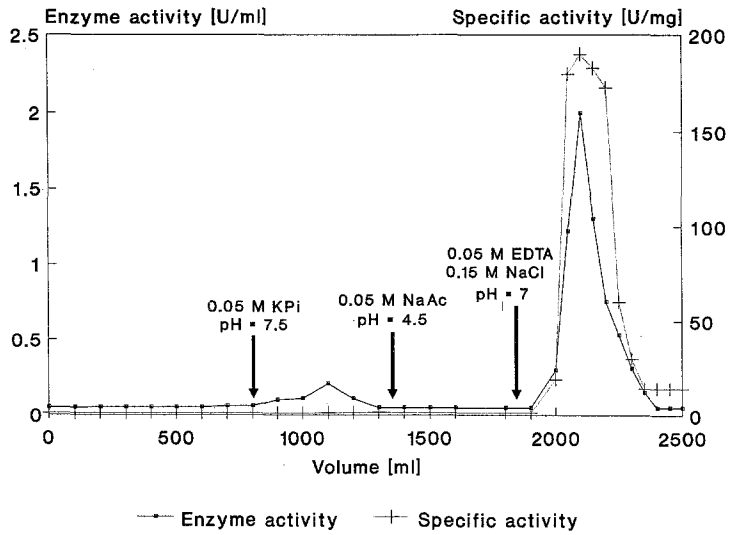

Fig. 2. Chromatography of S-oxynitrilase on copper(II)–IDA membrane. A sample of 800 ml of S-oxynitrilase was applied to the membrane. Equilibration, washing and elution conditions as in Fig. 1.

elution with the pH shift (data not shown). To separate the S-oxynitrilase from the copper(II)–EDTA complex, gel permeation chromatography or the ion-exchange membrane could be used. The specific activity of the preparation was 270 U/mg, which is similar to published values [12]. Only one protein band was detected by gel electrophoresis.

Immobilized metal ion affinity partitioning

The second purification procedure employed affinity extraction as the first step to purify the S-oxynitrilase (method A). The partition coefficient in PEG–sodium sulphate systems is very small for the S-oxynitrilase, reflecting the proteins' preference for the salt-rich phase (Table II). However, when a small amount of the affinity ligand copper–IDA-PEG was added, the partitioning increased dramatically by more than a 100-fold (Fig. 3). Ligand-exchange binding of S-oxynitrilase to the chelated transition-metal ions is very sensitive to the pH of the medium because it requires an unprotonated nitrogen atom as a donor. The magnitude of such pH effects could be measured exactly by titration experiments (Fig. 4). At a pH value below 5 the binding was weakened and the partition coefficient decreased.

The procedure described was used for the purification of S-oxynitrilase from the homogenate after cell disintegration. The first step consisted

TABLE II

PURIFICATION OF S-OXYNITRILASE FROM *SORGHUM BICOLOR* (METHOD 2A)

Purification step	Oxynitrilase activity (U)	Protein (mg)	Purification factor	Activity yield (%)	Specific activity (U mg^{-1})
Extraction	500	312.5	1	100	1.6
1. Two-phase system (0% copper–PEG-IDA)	461	106.5	2.6	92	4.1
2. Two-phase system (2.8% copper–IDA-PEG)	419	33.5	7.8	84	12.5
3. Two-phase system (pH shift to 4)	373	9.3	25.0	75	40.0
Gel permeation chromatography	320	1.2	167.5	64	268.0
(or anion-exchange membrane)	301	1.12	168.0	60	269.0

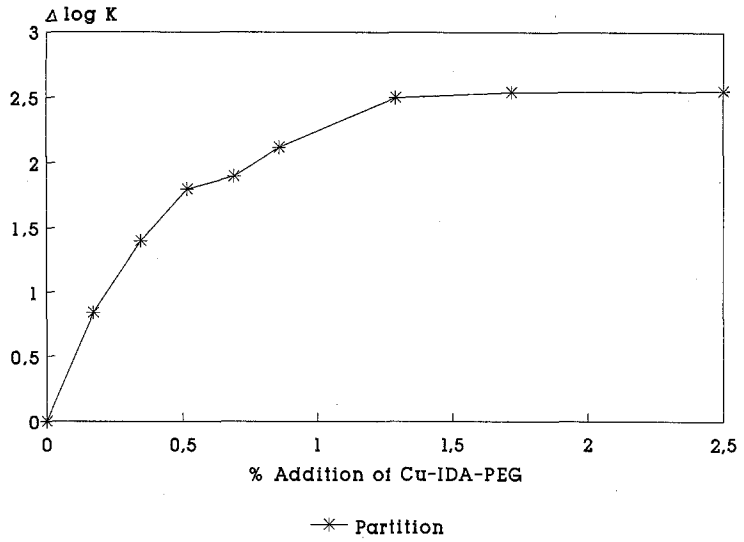

Fig. 3. Effect of copper–IDA-PEG concentrations on partitioning of S-oxynitrilase. The two-phase systems were composed of 18% PEG 20 000, 7.2% sodium sulphate, 0.1% potassium phosphate (pH 8.0) and 0.05 mg of S-oxynitrilase. The concentration of the modified polymer is expressed as a percentage of the total weight of the system.

of physical extraction in an aqueous two-phase system in the absence of copper–IDA-PEG (Fig. 5, method A). It has the advantage of separating the enzyme from peptides, amino acids and other small molecules, which interfere with the binding between metal ion and enzyme. The desired enzyme was partitioned into the salt-rich lower phase, which was separated.

A secondary phase system was then generated by adding copper–IDA-PEG and PEG to the lower phase of the primary phase system. The partition of S-oxynitrilase readily changed in response to the affinity ligand, and 84% of the activity was found in the PEG-rich upper phase. With a third extraction step after a shift to pH 4 the copper–IDA-PEG binding to the enzyme was

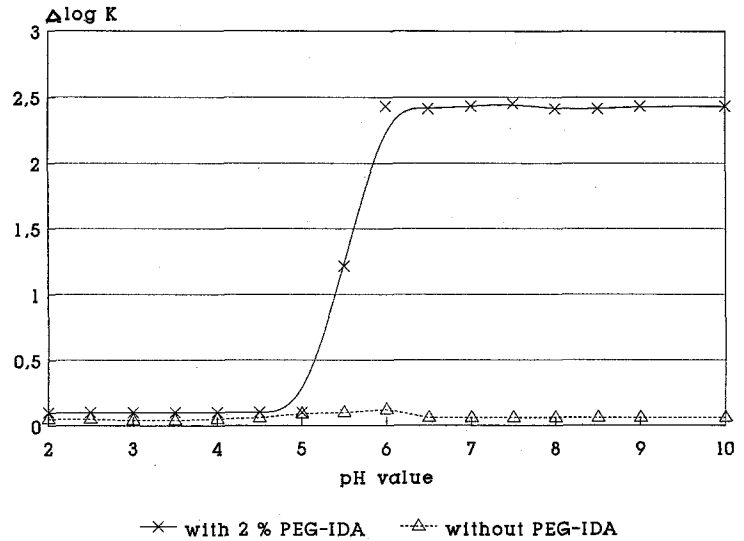

Fig. 4. Effect of pH on affinity partitioning of S-oxynitrilase. Composition of the systems: 2% copper–IDA-PEG, 18% PEG 20 000, 7.2% sodium sulphate, 0.1% potassium phosphate and 0.05 mg of S-oxynitrilase.

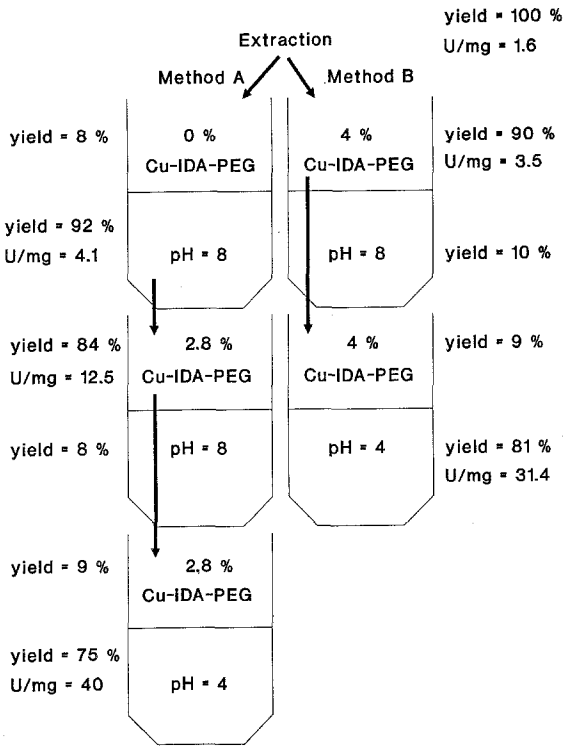

Fig. 5. Two-phase systems for purification of S-oxynitrilase. Composition of the systems with variable concentration of copper–IDA-PEG and different pH values (18% PEG 20 000, 7.2% sodium sulphate and 8% powdered plant material).

broken, and the S-oxynitrilase was recovered in the salt-rich upper phase. The affinity ligand also remained in the upper phase and could be separated and collected.

The calculated specific enzyme activity after the three consecutive extraction steps varied considerably. This result is mainly due to uncertainties of the protein determination at low levels and in the presence of high salt concentrations. Gel electrophoresis indicated the presence of only a small amount of contaminating proteins (data not shown). After gel permeation chromatography or ion-exchange membrane adsorption, however, the enzyme was found to be homogeneous.

In a second experiment (Fig. 5, method B) the first physical extraction of the enzyme was omitted. In this case it was necessary to add 30% more of copper–IDA-PEG to the affinity extraction system, in order to enhance the partitioning to values similar to those achieved with method A. This finding reflects the competition of amino acids, peptides or other proteins for ligand binding. After reextraction using a pH shift followed by gel permeation chromatography or ion-exchange membrane adsorption, pure S-oxynitrilase was obtained (Table III).

DISCUSSION

In this study excellent feasibility of immobilized metal ions on different carriers was demonstrated for the purification of the S-oxynitrilase.

Agarose is the best known carrier of the IDA ligand for IMAC. In our study we found that this matrix exhibited the best binding properties, but had a comparatively low flow-rate. The newly available IDA membrane provided a good alternative to the chelating-Sepharose FF. Advantages of the membrane are the high flow-rate, the easy handling and the fast cycle times. The binding between S-oxynitrilase and chelated copper (II) on the membrane is somewhat weaker, as evi-

TABLE III

PURIFICATION OF S-OXYNITRILASE FROM *SORGHUM BICOLOR* (METHOD 2B)

Purification step	Oxynitrilase activity (U)	Protein (mg)	Purification factor	Activity yield (%)	Specific activity (U mg^{-1})
Extraction	500	312.5	1	100	1.6
1. Two-phase system (4% copper–PEG-IDA)	450	129.0	2.2	90	3.5
2. Two-phase system (pH shift to 4)	404	12.9	7.8	81	31.4
Gel permeation chromatography	345	1.3	163.0	69	261.0
(or anion-exchange membrane)	321	1.2	168.0	65	259.0

denced by the desorption conditions. The capacity per milligram of wet carrier was lower than with the column material, but improvements may still be possible. The aqueous two-phase systems, in conjunction with metal affinity extraction, should be an interesting approach for large-scale purification.

Previously published methods involved ammonium sulphate precipitation and salting-out chromatography, and required more than twice the time. The use of aqueous two-phase systems, with or without an affinity ligand, together with the modified microporous membranes offers easy scale-up, low labour costs and fast purification.

The immobilized IDA-metal complex is a very good general affinity ligand with respect to its high stability, relatively low costs and high capacity compared with other more "biospecific" ligands. The application of various genetic engineering techniques has been reported by different groups [17–19] to modulate protein binding to immobilized metal ions. Depending on the scale of operation, different carriers may be used with advantage to design an efficient purification process based on ligand-exchange interactions.

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