Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1055 (2004) 93–98
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Selective and mild adsorption of large proteins on lowly activated immobilized metal ion affinity chromatography matrices Purification of multimeric thermophilic enzymes overexpressed in *Escherichia coli*

Benevides C.C. Pessela[a,b], Rodrigo Torres[a,1], Manuel Fuentes[a], Cesar Mateo[a], Roberto Munilla[a], Alejandro Vian[a,b], Alfonso V. Carrascosa[a,b], Jose L. Garcia[a,c], Jose M. Guisán[a,2], Roberto Fernandez-Lafuente[a,*,2]

[a] *Departamento de Biocatálisis, Instituto de Catálisis-CSIC, Campus Universidad Autónoma Madrid, Cantoblanco, 28049 Madrid, Spain*
[b] *Departamento de Microbiología de los Alimentos, Instituto de Fermentaciones Industriales, C/Juan de la Cierva 3, 28006 CSIC, Madrid, Spain*
[c] *Centro de Investigaciones Biológicas, Departamento de Microbiología Molecular, C/Ramiro de Maetzu 9, 28040 CSIC, Madrid, Spain*

Received 15 March 2004; received in revised form 4 August 2004; accepted 23 August 2004

Abstract

A strategy to selectively adsorb large proteins on immobilized metal ion affinity chromatography supports is presented. It is based on the fact that large proteins have a large surface that permits the long distance interaction with groups placed quite far apart (very dispersed onto the support surface) in the support, therefore, even using lowly activated supports, these proteins may be able to yield multiple interactions with the support, which is not possible for smaller proteins. This has been shown using a crude extract from *Escherichia coli*, where only large proteins were adsorbed on supports having 0.25 μmol of metallic groups/g of support. Then, these lowly activated supports have been used for purifying multimeric enzymes from thermophilic organisms (α- and β-galactosidases from *Thermus* sp. strain T2) cloned and over-expressed in mesophilic ones. A previous heating step of the crude extract destroyed the quaternary structure of all multimeric enzymes from the host (*E. coli*). Thus, the only large protein remaining in the supernatant of this heated extract are the cloned multimeric thermophilic enzymes, permitting their very simple purification by using only one chromatographic step.

Keywords: Galactosidases; Metal ion affinity; Multimeric enzymes

1. Introduction

Protein adsorption on immobilized metal ion affinity chromatography (IMAC) matrices is a well-developed technique that consists in the use of matrices with immobilized metallic ions to purify proteins by adsorption through some amino acid residues such as histidine, cysteine or tyrosine [1–5]. It is a simple and rapid method for protein purification, which is usually based on the selective desorption of the proteins that have been adsorbed with different strength to the support [5–11]. In fact, most of the commercially available IMAC supports are designed for the adsorption of most of the proteins, and thus they are usually highly activated. In these matrices, more than 90% of the proteins of a crude extract from *Escherichia coli* are adsorbed [12,13], showing almost universal adsorption of proteins on this kind of supports.

* Corresponding author. Tel.: +34 91 585 48 09; fax: +34 91 585 47 60.
E-mail addresses: jmguisan@icp.csic.es (J.M. Guisán), rfl@icp.csic.es (R. Fernandez-Lafuente).
[1] Permanent address: Escuela de Química, Facultad de Ciencias, Edificio Camilo Torres, Universidad Industrial de Santander, Bucaramanga, Colombia.
[2] Co-corresponding authors.

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.141

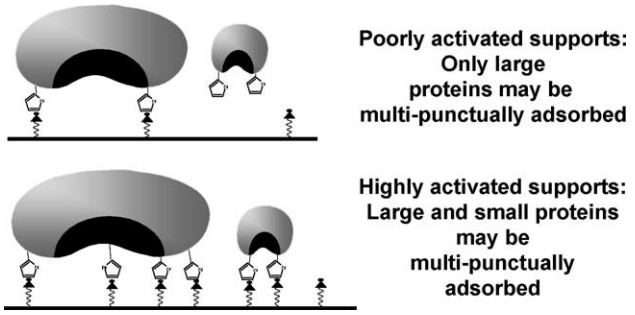


Fig. 1. Adsorption mechanism of proteins onto differently activated Me^{2+}-IDA supports.

However, a more selective adsorption of the target may have some advantages compared to selective desorption [12,13]. The mechanism of protein adsorption on IMAC matrices may give some keys to achieve this. Natural proteins only become adsorbed on the chromatographic matrix by the simultaneous interaction of several His, Cys or Tyr residues with several groups on the support surface [13–15]. Thus, only proteins having a cluster with several His or a poly-His tag may be adsorbed on these supports by interaction on just one ion metal chelate residue [5].

This multipoint mechanism of natural proteins adsorption on IMAC matrices opens some new opportunities for the purification of large proteins from small ones, via selective adsorption of the large ones. Large proteins, having bigger surfaces, might be able to interact with larger areas of the support, giving long distance multi-interactions. Then, as the adsorption process requires the simultaneous interaction between several groups of the protein and several groups of the support, only large enough proteins (those covering a large area of support) could be adsorbed on the matrix when using very lowly activated supports (Fig. 1).

Moreover, this large interaction area may permit a very intense multipoint adsorption using highly activated supports, which could make difficult the further desorption of these large proteins (Fig. 2). Again, this problem could be solved by using lowly activated supports.

Here, we will try to explore the advantages of this strategy, by using as model enzymes some multimeric thermophilic proteins over-expressed in *E. coli*.

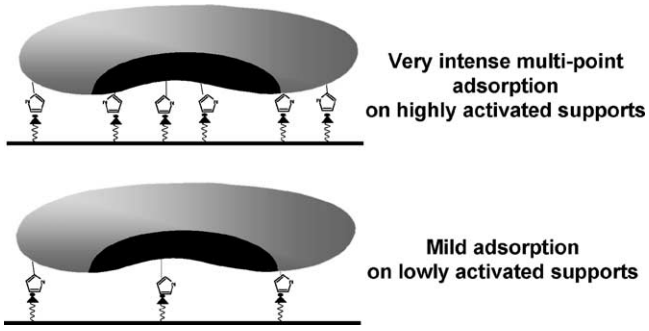


Fig. 2. Adsorption strength of large proteins onto differently activated Me^{2+}-IDA supports.

2. Materials and methods

2.1. Materials

Crosslinked 4% agarose beads were kindly donated by Hispanagar (Burgos, Spain). Weight of agarose was referred to vacuum dried ones (with the interparticle water eliminated). Agarose beads activated with 40 μmol of epoxy groups/g of support were produced using epichlorhydrin as previously described [16–18]. β-Galactosidase and α-galactosidase from *Thermus* sp. strain T2 ($pBGT_1$ and $pAGT_1$) were both over-expressed in *E. coli* and produced as published elsewhere [19,20]. Iminodiacetic acid disodium salt monohydrate (IDA) and copper sulphate(II) 5-hydrate were purchased from Fluka (Buchs, Switzerland). *o*-Nitrophenyl-β-D-galactopyranoside (*o*-NPG) and *p*-nitrophenyl-α-D-galactopyranoside (*p*-NPG) were obtained from Sigma (St. Louis, MO, USA). Epichlorohydrin and imidazole were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of different iminodiacetic acid-agarose supports

The preparation of IDA supports from epoxy agarose activated with 15 μmol of epoxy groups/g of support was carried out as follows: 7 g of vacuum dried epoxy agarose beads were suspended in a solution of sodium borate buffer 0.1 M containing 0.4 M of IDA (18 mL) and adjusted to pH 8.5 with NaOH. The flask was gently stirred at 25 °C for different time periods (from 5 to 120 min) to produce IDA-agarose beads with different activation degrees (0.25, 2, 4, 8, and 15 μmol of IDA groups/g of support) [16]. The amount of IDA groups/g of support was determined by titration of the ionisable groups present in the support by using a pH-stat. Epoxy agarose was used as a reference. Twenty-five micromol of NaOH was used as titrating agent. The remaining epoxy groups of the supports were destroyed by incubating the agarose-epoxy-IDA-supports with 0.5 M HCl for 4 h at room temperature. The derivatives were then washed and equilibrated with sodium phosphate buffer 0.5 M at pH 7.0, and finally with distilled water and stored at 4 °C.

2.2.2. Preparation of Cu^{2+}-chelate agarose supports

Two grams of IDA-agarose beads (with different activation degrees) were incubated in an aqueous solution of $CuSO_4(II)\cdot 5H_2O$ (5 mg/mL) [21] for 2 h at room temperature. Finally, the supports were thoroughly washed with distilled water.

2.2.3. Copper determination in Cu-chelate agarose supports

The amount of copper ions present in Cu-chelate-agarose supports were determined by ICP atomic emission spectroscopy in a Perkin-Elmer 3300 DV/optima spectropho-

tometer. Cu-IDA-agarose supports were initially dried at 110 °C during 24 h. Then, 100 mg of each dried sample was added to 2 mL of nitric acid plus 1 mL of HCl and 0.5 mL of H_2O_2. The mixture was left in a closed PTFE/microwave reactor during 55 min scanning from 0 to 500 W. Finally, the samples were refilled to a final volume of 10 mL with deionised water. The amount of copper was registered at 324.6 nm.

2.3. Determination of enzyme activities

2.3.1. β-Galactosidase from Thermus sp. strain T2 ($pBGT_1$)

Activity was determined spectrophotometrically by following the increase in the absorbance at 405 nm caused by the hydrolysis of *o*-NPG.

The reaction medium was 13.3 mM *o*-NPG, dissolved in Novo buffer pH 6.5 (2.7 mM sodium citrate; 7.9 mM citric acid; 2.9 mM potassium hydrogenphosphate; 10.8 mM potassium phosphate; 19.4 mM potassium hydroxide; 4.1 mM magnesium chloride; 5.1 mM calcium chloride; and 3.3 mM sodium carbonate) at 25 °C. One unit of activity is defined as the amount of enzyme, which hydrolyzed 1 μmol of substrate per min and per mg of protein under the described conditions (molar extinction coefficient of *o*-nitro-phenol (*o*-NP) under those conditions was 3100 M^{-1} cm^{-1}). Experiments were carried out at least in triplicate and experimental error was never higher than ±5%.

2.3.2. α-Galactosidase from Thermus sp. strain T2 ($pAGT_1$)

Activity was followed spectrophotometrically by the increase in the absorbance at 405 nm caused by the hydrolysis of *p*-NPG. The reaction medium was 13.3 mM *p*-NPG, dissolved in 50 mM sodium phosphate buffer pH 7.0 at 25 °C. One unit of α-galactosidase activity is defined as the amount of enzyme, which release 1 μmol of *p*-nitro-phenol per min and per mg of protein under given assay conditions (molar extinction coefficient of *p*-nitro-phenol (*p*-NP) under these conditions was 10310 M^{-1} cm^{-1}). Experiments were carried out at least in triplicate and experimental error was never higher than ±5%.

2.3.3. Protein adsorption on Cu-IDA-agarose supports

Different protein solutions were diluted 10-fold in 5 mM sodium phosphate buffer, 100 mM NaCl at pH 7. The salt was included to prevent unspecific ionic interactions between proteins and the support. Ten microlitres of these protein solutions (0.7 mg/mL of protein concentration) were mixed with 2 mL of Cu-IDA-agarose. The incubation was carried out at 25 °C and under constant stirring.

To prevent diffusion limitations that could alter the activity determinations, when using enzymes, the standard experiments were performed using only about 10 IU of activity/g of support. During the time course of the adsorptions, aliquots were withdrawn from the supernatant and the suspension and their activities and/or protein concentration determined as described. In all cases, the amount of proteins and/or enzymes adsorbed on the supports was considered as 100% for desorption calculations. After 1 h, the derivatives were washed with an excess of distilled water and stored at 4 °C.

2.3.4. Desorption of proteins adsorbed on IMAC supports

Two microlitres of support with adsorbed proteins were suspended in 5 mL of 5 mM sodium phosphate at pH 7.0. Proteins were desorbed by incubating the suspensions for 30 min at room temperature in the presence of different imidazole concentrations. Longer incubation times (up to 4 h) did not result in a significant increment in the quantity of desorbed proteins. The percentage of desorbed enzymes was followed via enzyme activity of the supernatant compared to the activity of the suspension (which in all cases remained constant throughout the experiment). As control, a solution with soluble enzyme was submitted to the same treatment to detect the effect (if any) of the imidazole upon the enzyme activity. The percentage of desorbed protein was calculated by determining the protein concentration and the expected protein adsorbed on the support. In some instances, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of

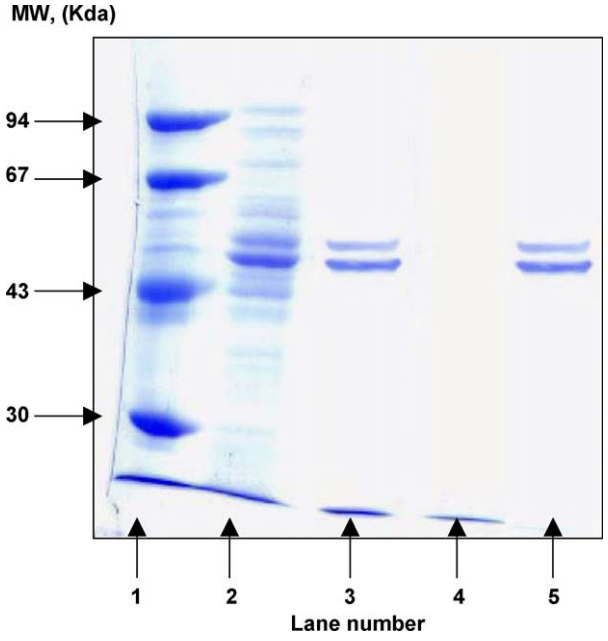


Fig. 3. SDS–PAGE gels of different samples of proteins from *Escherichia coli* (strain MC 2508). Experiments were performed as described in Section 2.2. Lanes: (1) molecular mass (Mw) markers (kDa, kilodalton); (2) *E. coli* crude extract (strain MC 2508); (3) proteins adsorbed on agarose activated with 0.25 μmol of Cu-IDA/g of support; (4) proteins that remain adsorbed on agarose activated with 0.25 μmol of Cu-IDA after desorption with 2 mM imidazole; and (5) proteins desorbed from lowly activated Cu-IDA-agarose using 2 mM imidazole.

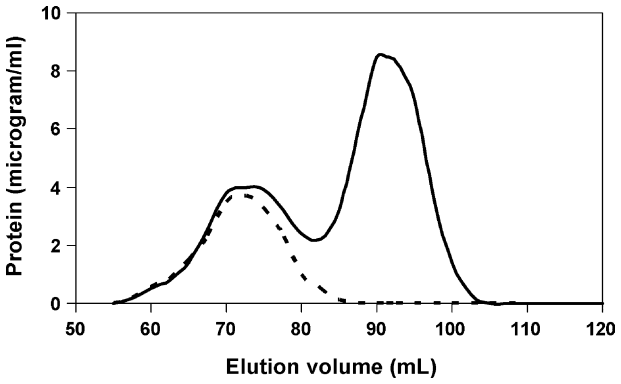


Fig. 4. Gel filtration analysis of different protein extracts from *Escherichia coli* (strain MC 2508). Samples were injected in a glass column containing 100 mL of 4BCL agarose. Flow rate was 0.5 mL/min. Other details are described in Section 2.2. *Escherichia coli* crude extract (solid line). *E. coli* proteins previously desorbed from agarose activated with 0.25 μmol of Cu-IDA/g of support (dashed line).

the support was performed to ensure the desorption degree of the proteins.

2.3.5. Protein determination

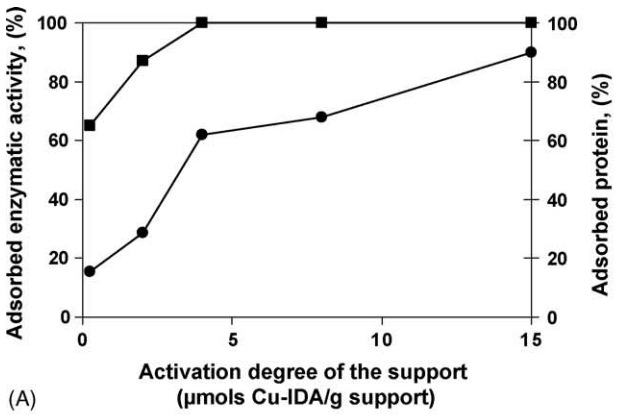
Protein concentrations were determined by the Bradfords' method [22]. Bovine serum albumin (BSA) was used as a reference.

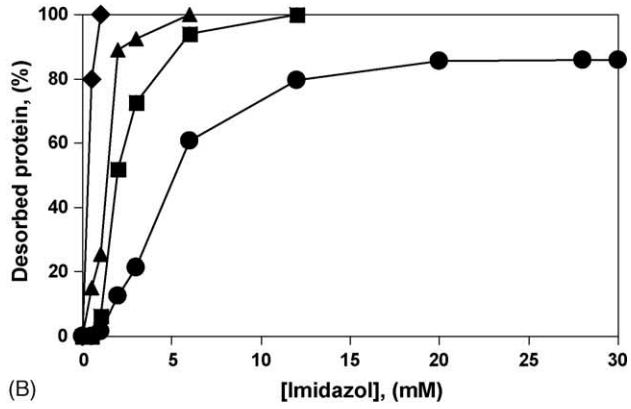
2.3.6. Gel-filtration

Gel-filtration analyses were performed using a glass column packed with agarose 4BCL (column bed volume: 100 mL). The column was previously equilibrated with 500 mL of the elution buffer (50 mM sodium phosphate at pH 7.0). All separations were carried out at 25 °C with a flow rate of 0.5 mL/min employing an isocratic pump (Pharmacia) and detecting the absorbance of the eluted proteins at 280 nm (UV detector, Pharmacia). The eluted samples were collected in 1 mL aliquots.

2.3.7. SDS–PAGE analysis

SDS–PAGE experiments were performed as described by Laemmli [23]; in a SE 250-Mighty small II electrophoretic unit (Hoefer Co.) using gels of 12% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. Gels were stained with the Coomassie brilliant blue method. Low-molecular mass marker kits from Pharmacia were used (M_r = 14 000–94 000).





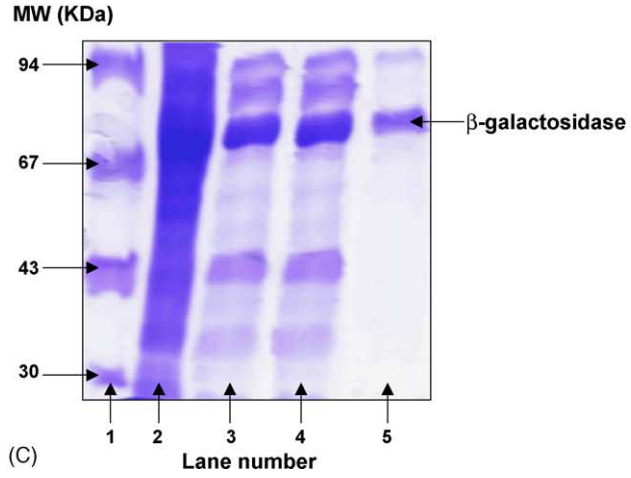


Fig. 5. (A) Adsorption of a protein extract containing β-galactosidase from *Thermus* sp. on Cu-IDA-agarose support with different activation degrees. Incubation was performed in 5 mM sodium phosphate buffer at pH 7.0 and 25 °C during 1 h. Hundred percent was considered the total amount of proteins adsorbed on the supports. Experiments were done by triplicate and data points were averaged. Other specifications as described in Section 2.2. (■) β-Galactosidase activity adsorbed on different Cu-IDA-agarose supports; (●) proteins adsorbed on different Cu-IDA-agaroses. (B) Imidazole-promoted desorption of β-galactosidase from *Thermus* sp. strain T2 (pBGT1) from different agarose–metal chelate supports. Adsorbed enzymes were incubated 30 min with increasing concentrations of imidazole as described in Section 2.2. (♦) Enzymatic activity released from supports activated with 0.25 μmol Cu-IDA/g; (▲) enzymatic activity released from supports activated with 4 μmol Cu-IDA/g; (■) enzymatic activity released from supports activated with 8 μmol Cu-IDA/g and (●) enzymatic activity released from supports activated with 15 μmol Cu-IDA/g. (C) SDS–PAGE (polyacrylamide 12%) analysis of different preparations from a crude extract from *E. coli* containing the enzyme β-galactosidase (pBGT1) from *Thermus* sp. strain T2. Lanes: (1) molecular mass markers; (2) crude extract; (3) soluble proteins after incubation at 70 °C for 20 min; (4) proteins from lane 3 adsorbed on agarose activated with 15 μmol of Cu-IDA/g of support; and (5) proteins from lane 3 adsorbed on agarose activated with 0.25 μmol of Cu-IDA/g of support.

3. Results and discussion

3.1. Adsorption of proteins on lowly activated supports

Crude extracts (from *E. coli*) were incubated with supports having very low content of Cu-IDA groups (0.25 μmol/g of support). After 1 h of incubation, over 90% of the initial total protein remained unadsorbed in the supernatant. Then, the support was recovered, washed, and incubated with 10 mM imidazole. This treatment allowed full desorption of the proteins adsorbed on the support (Fig. 3). The desorbed proteins were then analysed in a gel filtration experiment and the results compared with those obtained with the crude extract. Fig. 4 shows that the proteins adsorbed on the lowly activated support correspond to the fraction of proteins with larger molecular size. The same results were obtained when using other protein extracts. In fact, the SDS–PAGE analysis of the recovered protein showed only two main protein bands (Fig. 3). This high selectivity may be related to the topographic requirements for the adsorption of proteins on IMAC-supports. In fact, not only it is necessary a large protein, but also that the His, Cys, and/or Tyr residues (not very frequent in the protein surface) had to be in the right relative distance to simultaneously interact with this lowly activated support.

3.2. Selective adsorption of multimeric thermophilic enzymes over-expressed in mesophilic organisms

Heating of crude preparations of mesophilic organisms promotes the precipitation or destruction of most of the quaternary structure of multimeric proteins in these preparations [24]. Therefore, we have used this strategy, coupled to the use of lowly activated IMAC supports, to purify two large multimeric enzymes cloned and over-expressed in *E. coli*. The β-galactosidase and the α-galactosidase from *Thermus* sp. strain T2. The first enzyme is a tetrameric one with monomers of 67 kDa [25], the second one presents a hexameric structure [26].

First, the adsorption of the β-galactosidase after thermal precipitation of the mesophilic multimeric proteins was studied using supports with different Cu-IDA concentrations. When using supports with only 0.25 μmol/g of support, about 65% of the β-galactosidase activity was adsorbed after 24 h, accounting around 15% of the proteins contained in the crude preparation. If higher activation degree supports were used (15 μmol/g of support), the amount of adsorbed proteins increased up to 90%, suggesting a much lower selectivity towards the target protein (Fig. 5A). The enzyme adsorbed on the poorly activated support was very easily released (by just using 3 mM imidazole), on the contrary when using supports with higher activation degree, desorption became more difficult. In fact, a certain percentage of enzyme remained adsorbed on the support activated with 15 μmol Cu-IDA/g of support even at concentrations of imidazole of 2 mM (Fig. 5B). A single protein band could be stained in the SDS–PAGE obtained from this lowly activated support (Fig. 5C).

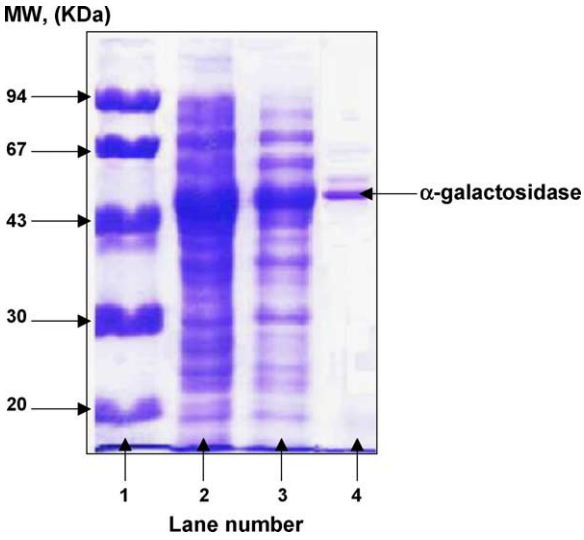


Fig. 6. SDS–PAGE (polyacrylamide 12%) analysis of different preparations from a crude extract from *E. coli* containing the enzyme α-galactosidase from *Thermus* sp. strain T2. Samples of supernatant were obtained after boiling the supports with the adsorbed proteins in the presence of SDS as described in Section 2.2. Lanes: (1) low-molecular-mass markers; (2) crude extract of α-galactosidase from *Thermus* sp. strain T2; (3) crude extract of α-galactosidase from *Thermus* sp. strain T2 after incubation at 70 °C during 20 min; and (4) proteins released from 0.25 μmol Cu-IDA-chelate support desorbed with 2 mM imidazole.

Similar results (in terms of adsorption selectivity and adsorption strength) were achieved using the α-galactosidase (Fig. 6).

The lowly activated supports permits to adsorb around 10 mg of protein/wet gram of support.

4. Conclusions

The results shown in this manuscript seem to confirm that the use of tailor-made IMAC matrices may permit a rapid and easy purification of large proteins by their selective adsorption on poorly activated supports. This strategy also makes easy the desorption step, that may become a serious problem for desorption of large proteins when using conventional IMAC supports (with much higher metal ion chelate density), due to their ability to generate a much more intense multipoint interaction between the proteins and the support. These results, together to similar ones obtained using anion exchanger matrices [27], suggest that any support that requires a multipoint process to adsorb the proteins may be used to separate large from small proteins. In fact, the use of several of these lowly activated supports could permit the purification of most of the multimeric proteins.

In the specific case of multimeric enzymes from thermophilic organisms cloned and overexpresed in mesophilic organisms, the simple heating of the crude containing the

multimeric thermophilic enzyme and their further adsorption in lowly activated supports permits a rapid and simple purification of these multimeric proteins.

Acknowledgments

This work was supported by grant O7G/002/2003 from the Comunidad Autónoma de Madrid and the EC Project (MATINOES G5RD-CT-2002-00752). The support of Universidad Industrial de Santander to R.T. and the helpful comments of MSc. Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

References

[1] R.J. Everson, H.E. Parker, Bioinorg. Chem. 4 (1974) 15.
[2] E. Hochuli, H. Doebeli, A. Schacher, J. Chromatogr. 411 (1987) 177.
[3] E. Hochuli, W. Bannwarth, H. Doebeli, R. Gentz, D. Stueber, Biotechnology (1988) 1321.
[4] V. Gaberc-Porekar, V. Menart, J. Biochem. Biophys. Methods 49 (2001) 335.
[5] G.S. Chaga, J. Biochem. Biophys. Methods 49 (2001) 313.
[6] L. Yung-Chuan, C.C. Chih-Chiang, S. Shing-Yi, J. Chromatogr. B 794 (2003) 67.
[7] V. Sandra, A.M. Everson, A.B. Sonia, Maria, Process Biochem. 37 (2002) 573.
[8] F.H. Arnold, Biotechnology 9 (1991) 150.
[9] P.R. Hari, W. Paul, C.P. Sharma, J. Biomed. Mater. Res. 50 (2000) 110.
[10] R.H. Clemmitt, H.A. Chase, Biotechnol. Bioeng. 67 (2000) 206.
[11] H. Rogl, K. Kosemund, W. Kühlbrandt, I. Collinson, J. Ferment. Bioeng. 86 (1998) 357.
[12] E. Ordaz, A. Garrido-Pertierra, M. Gallego, A. Puyet, Biotechnol. Prog. 16 (2000) 287.
[13] C. Mateo, G. Fernandez-Lorente, B. Pessela, A. Vian, A. Carrascosa, J.L. García, R. Fernandez-Lafuente, J.M. Guisan, J. Chromatogr. A 915 (2001) 97.
[14] K.L.M.C. Franken, H.S. Hiemstra, K.E. van Meijgaarden, Y. Subronto, J. den Hartigh, T.H.M. Ottenhoff, J.W. Drijfhout, Protein Exp. Purif. 18 (2000) 95.
[15] C.M. Zhang, S.A. Reslewic, C.E. Glatz, Biotech. Bioeng. 68 (2000) 52.
[16] P. Armisen, C. Mateo, E. Cortés, J.L. Barredo, F. Salto, B. Diez, L. Rodes, J.L. García, R. Fernandez-Lafuente, J.M. Guisan, J. Chromatogr. A 848 (1999) 61.
[17] H. Hubert, J. Porath, J. Chromatogr. 198 (1980) 247.
[18] L. Sundberg, J. Porath, J. Chromatogr. 90 (1974) 87.
[19] A. Vian, A.V. Carrascosa, J.L. Garcia, E. Cortés, Appl. Environ. Microb. 64 (1998) 2187.
[20] B.C.C. Pessela, M. Fuentes, M. Filho, A.V. Carrascosa, A. Vian, R. Fernandez-Lafuente, J.M. Guisán, J. Food Sci. (2004), submitted for publication.
[21] E.S. Hemdan, Y.-J. Zhao, E. Sulkowski, J. Porta, Proc. Nat. Acad. Sci. U.S.A. 86 (1989) 1811.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[23] U.K. Laemmli, Nature 277 (1970) 680.
[24] O.M. Poltorak, E.S. Chukhary, I.Y. Torshin, Biochemestry, Moscow 63 (1998) 360.
[25] B.C.C. Pessela, A. Vian, C. Mateo, R. Fernández-Lafuente, J.L. García, J.M. Guisan, A.V. Carrascosa, Appl. Environ. Microb. 69 (2003) 1967.
[26] M. Ishiguro, S. Kaneko, A. Kuno, Y. Koyama, S. Yoshida, G. Park, Y. Sakakibara, I. Kusakabe, H. Kobayashi, Appl. Environ. Microb. 67 (2001) 1601.
[27] B.C.C. Pessela, L. Betancor, R. Munilla, M. Fuentes, A.V. Carrascosa, A. Vian, R. Fernandez-Lafuente, J.M. Guisan, J. Chromatogr. A 1034 (2004) 155.