Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1034 (2004) 155–159
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Ion exchange using poorly activated supports, an easy way for purification of large proteins

Benevides C.C. Pessela [a], Roberto Munilla [a], Lorena Betancor [a], Manuel Fuentes [a], Alfonso V. Carrascosa [b], Alejandro Vian [b], Roberto Fernandez-Lafuente [a,*], Jose M. Guisán [a,*]

[a] *Departamento de Biocatálisis, Instituto de Catálisis CSIC, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain*
[b] *Department of Microbiology, Instituto de Fermentaciones Industriales (CSIC), C/Juan de la Cierva 3, 28006 Madrid, Spain*

Received 4 November 2003; received in revised form 26 January 2004; accepted 29 January 2004

Abstract

Ion-exchange chromatography using commercial ionic supports is a commonly used technique for protein purification. However, selective adsorption of a target protein from a given extract onto commercial ion exchangers seems to be quite complex since they are designed to adsorb the maximum percentage of proteins with the opposite charge. In this paper, ion-exchanger supports with different activation degrees (from 1 to 40 μmol of amino groups per g of agarose) have been prepared and used for the purification of large proteins. These kinds of proteins have large surfaces to interact by many points with the support. Therefore, it was possible to purify large proteins as β-galactosidase from *Thermus* sp. strain T2 from a crude extract from *Escherichia coli* or bovine liver catalase from a commercial preparation, with tailor-made ion-exchanger supports. A simple step of adsorption/desorption on lowly activated supports rendered both enzymes rather pure as confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Moreover, this strategy makes also easy the desorption step that requires rather low NaCl concentrations, which may become a serious problem for desorption of large proteins when using conventional supports, due to their ability of generating a very strong adsorption.

Keywords: Stationary phases, LC; Adsorption; Ion exchangers; Proteins

1. Introduction

Ion-exchange chromatography using commercial ionic supports (DEAE, CM, Mono Q, etc.) is a widespread technique used for protein purification [1–7]. It is a simple and rapid method for protein separation which is usually based on the selective desorption of the proteins that have been adsorbed with different strength to the support [2,8–11].

In order to improve the purification efficiency, it would be very advantageous to have the possibility of selectively adsorb a target protein. This selective adsorption would not only greatly reduce the size of the column but also simplify the desorption process. It would even be possible to use batch processes.

Commercially available ion exchangers are normally highly activated with very strong ionic groups (DEAE-agarose, Q-Sepharose, etc.) so that they can adsorb the maximum percentage of proteins from a given extract (near 80% of the proteins of a crude extract from *Escherichia coli* is adsorbed on DEAE) [12,13]. However, selective adsorption of a target protein onto these kind of matrices seems to be quite complex.

In this paper, we will focus on the purification of large proteins, having large surfaces to interact with large areas of the support. Moreover, this large area of interaction may promote a very intense multipoint adsorption, which could make difficult their further desorption.

In order to design a selective adsorption on ionic supports of these large proteins, one could take advantage of the fact that proteins only become adsorbed on the chromatographic matrix by interacting at several sites on the surface of the protein molecule and support [14].

Then, as the adsorption process requires the simultaneous interaction between several groups of the protein and the

* Corresponding authors. Tel.: +34-91-585-48-09; fax: +34-91-585-47-60.
E-mail addresses: rfl@icp.csic.es (R. Fernandez-Lafuente), jmguisan@icp.csic.es (J.M. Guisán).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.01.061

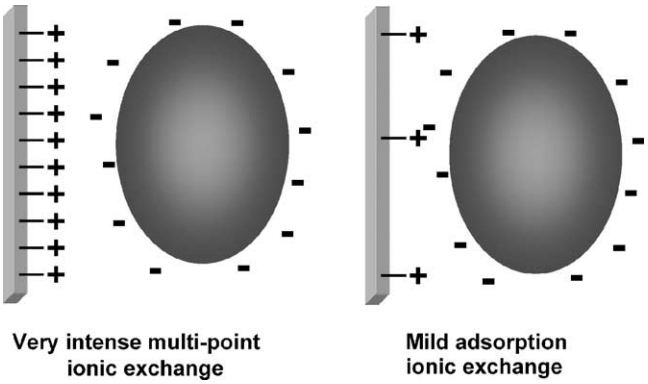

Fig. 1. Adsorption mechanism of proteins on differently activated anion-exchange supports.

support only large enough proteins (that is, covering a large area of the support) could be adsorbed on the matrix when using very lowly activated supports.

Moreover, it may be expected that large proteins may be strongly adsorbed onto conventional matrices since their large external surface permits a great area of interaction with the support (Fig. 1). Thus, a very large number of ionic bonds may be formed and further desorption of the protein may be quite difficult (requiring very high ionic strength, drastic pH values, etc.). Again, the use of poorly activated supports might avoid these problems.

Thus, in this study we have explored the advantages of using lowly activated anion-exchange supports for the purification of large proteins.

2. Materials and methods

2.1. Materials

Agarose (6% cross-linked agarose beads, 6BCL) was a gift from Hispanagar (Burgos, Spain). *o*-Nitrophenyl-β-D-galactopyranoside (*o*-NPG), ethylenediamine, was from Sigma (St. Louis, MO, USA). β−Galactosidase from *Thermus* sp. strain T2 was produced as published elsewhere [15]. Catalase from bovine liver was purchased from Fluka (Buchs, Switzerland), and sodium periodate was from Merck (Darmstadt, Germany).

2.2. Preparation of monoaminoethyl-N-aminoethyl (MANAE)-agarose

The protocol was similar to the previously described [16], but using glyoxyl agarose [17] with different activation degrees. The 10 ml of agarose (4BCL) containing the desired amount of glyoxyl groups were suspended in 90 ml 1 M ethylenediamine pH 10.05 and gently stirred for 2 h. Then, 1 g of solid $NaBH_4$ was added and the support was reduced for 2 h. The reduced gels, MANAE-agarose, were filtered and sequentially washed with 100 ml of 0.1 M sodium acetate, 1 M NaCl at pH 5.0, with 100 ml of 0.1 M sodium hydrogencarbonate buffer, 1 M NaCl at pH 10.0 and finally with 500 ml of deionized water. The full conversion of glyoxyl to MANAE groups permitted that the concentration of amino groups corresponded to the initial concentration of glyoxyl group of the agarose [16].

2.3. Determination of enzyme activities

2.3.1. β-Galactosidase from Thermus sp. strain T2

Activity was followed spectrophotometrically by 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; 7.91 mM citric acid; 2.99 mM potassium biphosphate; 10.84 mM potassium phosphate; 19.43 mM potassium hydroxide; 4.08 mM magnesium chloride; 5.1 mM calcium chloride; 3.33 mM sodium carbonate) at 25 °C. The β-galactosidase activity is given in μmol of substrate hydrolyzed per min and per mg of protein under the described conditions. Experiments were carried out at least in triplicate and experimental error was never over ±5%.

2.3.2. Catalase from bovine liver

Catalase activity was determined spectrophotometrically by monitoring the decomposition of H_2O_2, via measurement of the change in the absorbance at 240 nm [18]. 2.9 ml of a 35 mM H_2O_2 solution in 50 mM sodium phosphate buffer pH 7.0 were incubated with 0.2 ml of enzyme solution. All the measurements were carried out at 25 °C.

One catalase unit was defined as the amount of enzyme that decomposes 1 μmol of hydrogen peroxide per min under the previously described conditions.

2.4. Protein adsorption on aminated supports

The 2 ml of anion-exchange support were suspended in about 10 ml of protein suspensions (1–3 mg of protein per ml of 5 mM sodium phosphate buffer at pH 7.0 and 25 °C).

To prevent diffusion limitations in activity determinations, standard experiments were performed using only about 10 IU of enzyme/ml packed support. During adsorptions, samples were withdrawn from the supernatant and the suspension and enzyme activities determined. After 1 h, the matrix with the adsorbed proteins were washed with an excess of distilled water and stored at 4 °C.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Experiments of SDS-PAGE were performed as described by Laemmli [19]; 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 markers from Pharmacia were used (M_r, 14 000–94 000).

2.6. Desorption of proteins or enzymes adsorbed on the supports

The 2 ml of immobilized enzymes were suspended in 5 ml of 50 mM of sodium phosphate buffer at pH 7.0. Different amounts of solid NaCl were then added to increase the ionic strength, and samples were taken from supernatant 30 min after addition of NaCl at room temperature longer incubation times, up to 4 h, did not result in significant increments in the desorbed proteins). Desorbed protein was checked via enzyme activity or Bradford [20] determination released from the support to the supernatant and compared to the activity of the suspension (which in all cases remained constant throughout the experiment). A reference solution with soluble enzyme was submitted to the same treatment to detect any possible effect of the NaCl upon activity of the enzyme. To ensure full desorption, in some instances the support was boiled in the presence of 9 M guanidine [13].

2.7. Gel filtration

Gel-filtration analysis was 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. The eluting buffer was 50 mM sodium phosphate buffer, 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 at 280 nm (UV detector, Pharmacia). The eluted samples were collected in 1 ml aliquots and protein concentration was determined by Bradford's method [20].

3. Results and discussion

3.1. Adsorption of proteins on lowly activated supports

Different crude extracts (from *E. coli*, *A. turbidans*, etc.) were incubated with supports having very low content of amino groups (1 μmol/g). After 1 h of incubation, over 95% of the initial total protein remained un-adsorbed in the supernatant. Then, the support was recovered, washed, and incubated with 100 mM sodium phosphate buffer pH 7. This treatment allowed the full desorption of the proteins adsorbed on the support. The desorbed proteins were then analyzed in a gel filtration experiment and the results compared with those obtained using the crude extract. Fig. 2 shows the results achieved using an extract from *E. coli*. It is evident 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. Therefore, these results seem to support our starting hypothesis.

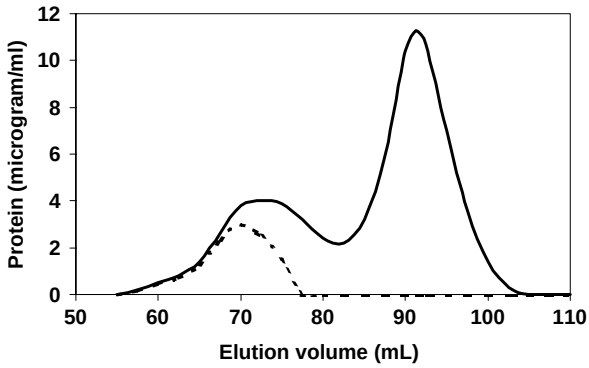

Fig. 2. Gel filtration analysis of different *E. coli* proteins. Two samples were analyzed, first the full crude and second the proteins desorbed from the poorly aminated support (1 μmol/g of support). This sample was injected in a glass column containing 100 ml of 4BCL agarose. Flow rate was 0.5 ml/min. Other details are described in Section 2. (—) Crude extract; (- - -) desorbed proteins from MANAE-agarose support (1 μmol/g).

3.2. Selective adsorption of large enzymes on aminated supports

A crude extract from *E. coli* with cloned and over expressed β-galactosidase from *Thermus* sp. strain T2, was offered to aminated agarose with different activation degrees (Fig. 3A). This enzyme is a multimeric protein of a large size (M_r, 70 000 each monomer with a predominant tetrameric form) [15]. Supports with the maximum activation (40 μmol/g) adsorbed most of the enzyme activity but also more than 70% of the bulk proteins. By decreasing the activation degree of the support, the amount of contaminant proteins adsorbed was highly decreased. In the case of the support containing 2.5 μmol/g, about 90% of the β-galactosidase activity and 20% of total protein were adsorbed. Moreover, when using supports with only 1 μmol of amino groups per g of agarose, 80% of the β-galactosidase activity was adsorbed with only 8% of total proteins, after 1 h of adsorption. The electrophoretic analysis of these results is shown in Fig. 3B. The purification of the β-galactosidase achieved was not very significant using highly activated supports, while a simple step of adsorption/desorption on lowly activated supports rendered a rather pure enzyme (the specific activity of the enzyme increased by a 20-fold factor).

The purification of the commercial catalase from bovine liver, a tetrameric enzyme of M_r, 240 000 [21] was also tried with the same method. Similar results to those of β–galactosidase were found with this enzyme. Fig. 4 shows again that the lower the activation degree of the support (1 μmol/g), the higher the purity degree achieved just by a selective adsorption of this large protein on the tailor-made support.

3.3. Desorption of β-galactosidase from Thermus sp. strain T2 from aminated supports

β-Galactosidase from *Thermus* sp. strain T2 can be strongly adsorbed on DEAE-agarose, in fact, a certain

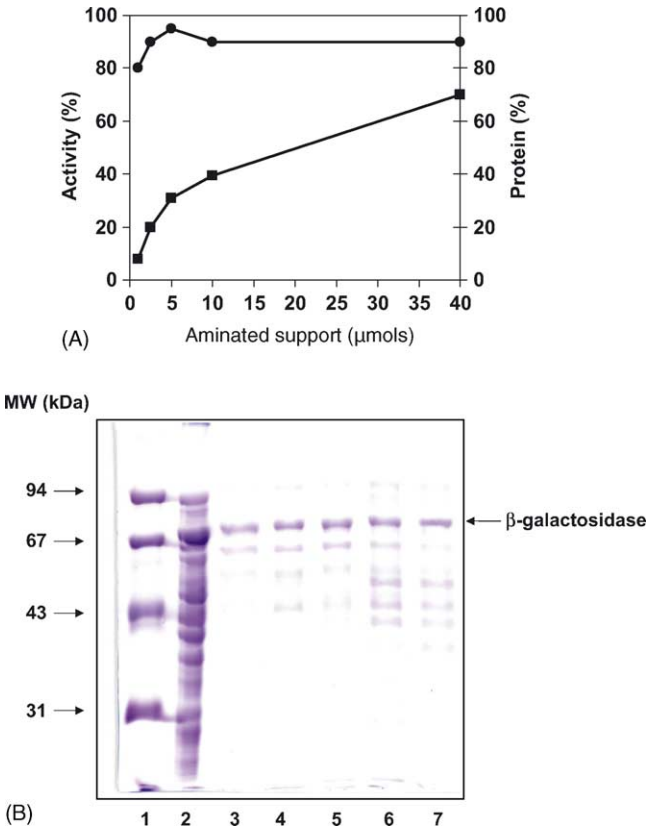

Fig. 3. (A) Adsorption of β-galactosidase from *Thermus* sp. strain T2 on different activated MANAE-agarose 4BCL. Incubation was performed at pH 7.0 and 25 °C during 1 h. Other specifications as described in Section 2.2. (●) β-Galactosidase activity adsorbed on different aminated supports; (■) other proteins from the extract adsorbed on different aminated supports. (B) Analysis by SDS-PAGE (12%) of proteins adsorbed/desorbed on different aminated supports. Lanes: 1, molecular marker; 2, crude extract of β-galactosidase from *Thermus* sp. T2; 3, all proteins adsorbed on 1 μmol aminated agarose support; 4, all proteins adsorbed on 2.5 μmol aminated agarose support; 5, all proteins adsorbed on 5 μmol aminated agarose supports; 6, all proteins adsorbed on 10 μmol aminated agarose support; 7, all proteins adsorbed on 40 μmol aminated agarose support. Experiments were performed as described in Section 2.2. MW, molecular mass; kDa, kilodalton.

percentage of protein cannot be desorbed from the support even using very drastic conditions [12]. We have studied the amount of NaCl needed to desorb the enzyme, associated to supports with increasing concentrations of MANAE groups. Fig. 5 shows that using highly activated MANAE-agarose, only 40% of the enzyme could be desorbed even using 1 M of NaCl. Similar results were observed when the pH value was 5.

However, by decreasing the activation of the support, the enzyme desorption was achieved with lower concentrations of NaCl. Using supports with 10 μmol/g, it was possible to recover 80% of the activity, while using supports activated with 5 μmol/g, 90% of the enzyme activity was desorbed with only 100 mM NaCl. When the enzyme was adsorbed on the lowly activated ion exchanger (1 μmol/g), full desorption of the enzyme occurred at very low ionic strength (50 mM NaCl).

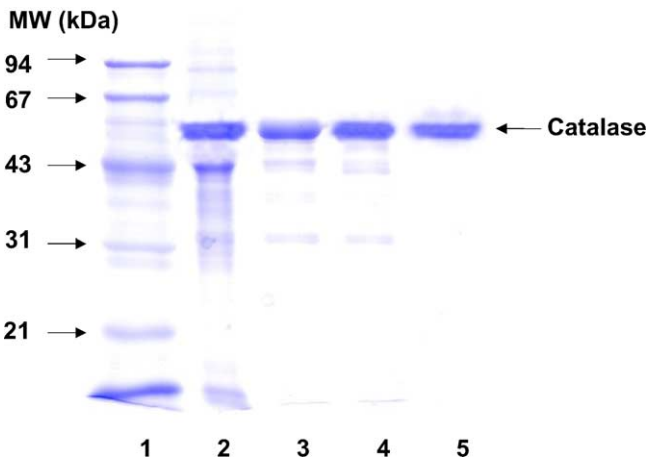

Fig. 4. SDS-PAGE (12%) gels of catalase adsorbed on differently activated amino supports. Lanes: 1, molecular marker; 2, commercial extract of bovine liver catalase; 3, commercial extract of bovine liver catalase adsorbed onto 5 μmol aminated agarose support; 4, commercial extract of bovine liver catalase adsorbed onto 10 μmol aminated agarose support; 5, commercial extract of bovine liver catalase adsorbed onto 1 μmol aminated agarose.

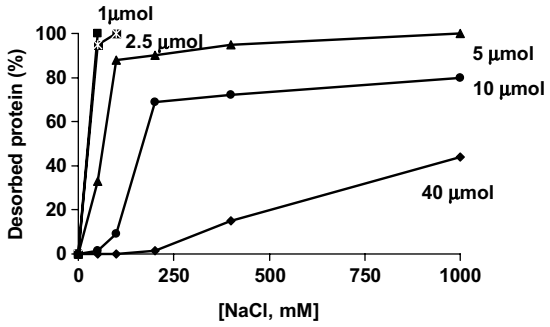

Fig. 5. Desorption of β-galactosidase from *Thermus* sp. strain T2. adsorbed on different aminated supports. Adsorbed enzyme was incubated at growing concentration of NaCl at pH 7.0 as described in Section 2.2. (■) Enzymatic activity released from 1 μmol aminated support (after 30 min of being adsorbed); (∗) enzymatic activity released from 2.5 μmol aminated support (after 30 min of being adsorbed); (▲) enzymatic activity released from 5 μmol aminated support (after 30 min of being adsorbed); (●) enzymatic activity released from 10 μmol aminated support (after 30 min of being adsorbed); (◆) enzymatic activity released from 40 μmol aminated support (after 30 min of being adsorbed).

4. Conclusions

The results shown in this manuscript seem to confirm that the use of tailor-made ion exchanger supports may permit a rapid and easy purification of large proteins by their selective adsorption on poorly activated supports. This strategy makes also easy the desorption step, that may become a serious problem for desorption of large proteins when using conventional supports, due to their ability of generating a very strong adsorption. The possibilities of this new technique to discriminate by high molecular size are very wide and will be subject of forth-coming papers.

Acknowledgements

This research has been funded by Comunidad de Madrid, (Projects 07G/002/2003) and Eu Project (MATINOES G5RD-CT-2002-00752). We thank R. Martinez (Novozyme) for the kind supply of enzymes, and Dr. J.L. Garcia (CIB-CSIC) for his support and interesting suggestions.

References

[1] C.S. Rao, Proc. Biochem. 37 (2001) 247.
[2] T. Xiao-Dong, D. Xiao-Yang, S. Yan, Biochem. Eng. J. 12 (2002) 117.
[3] A. Lyddiatt, Curr. Opin. Biotechnol. 13 (2002) 95.
[4] J.L. Berger, B.H. Lee, C. Lacroix, Biotechnol. Appl. Biochem. 25 (1997) 29.
[5] R.K. Scopes, Protein Purification: Principles and Practice, second ed., New York, 1987, p. 126.
[6] Y. Shih, J. Prausnitz, H. Blanch, Biotechnol. Bioeng. 40 (1992) 1155.
[7] S. Ghose, H.A. Chase, Bioseparation 9 (2000) 29.
[8] A. Baumeister, V. Stephanie, L. Fischer, J. Chromatogr. A 1006 (2003) 261.
[9] S. Shudong, Y. Yilun, X. Huang, D. Meng, J. Membrane Sci. 222 (2003) 3.
[10] F.B. Anspach, D. Curbelo, R. Hartman, G. Garke, W.-D. Deckwer, J. Chromatogr. A 865 (1999) 129.
[11] I. Iberer, H. Schwinn, A. Jungbauer, A. Buchacher, J. Chromatogr. A 921 (2001) 15.
[12] B.C.C. Pessela, R. Fernandez-Lafuente, M. Fuentes, A. Vian, J.L. Garcia, A.V. Carrascosa, C. Mateo, J.M. Guisan, Enzyme Microb. Technol. 32 (2003) 369.
[13] C. Mateo, O. Abian, R. Fernández-Lafuente, J.M. Guisan, Biotechnol. Bioeng. 68 (2000) 98.
[14] A. Kumar, I. Yu, B. Galaev, B. Mattiasson, J. Chromatogr. B 741 (2000) 103.
[15] 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.
[16] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida, J.M. Guisán, Enzyme Microb. Technol. 15 (1993) 546.
[17] J.M. Guisán, Enzyme Microb. Technol. 10 (1988) 375.
[18] H.E. Aebi, in: H.U. Bergmeyer (Ed.), third ed., Verlag Chemie, Weinheim, 1974, p. 672.
[19] U.K. Laemmli, Nature 277 (1970) 680.
[20] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[21] M.E. Percy, Can. J. Biochem. Cell Biol. 62 (1984) 1006.