# Mixed Ion Exchange Supports as Useful Ion Exchangers for Protein Purification: Purification of Penicillin G Acylase from *Escherichia coli*

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A support having similar amounts of carboxymethyl and amino groups has been prepared and evaluated as an ion exchanger. It has been found that this support was able to adsorb a high amount of protein from a crude extract of proteins (approximately 55%) at pH 5. Moreover, it was able to adsorb approximately 60% of the protein that did not become adsorbed on supports bearing just one kind of ionic groups. The use of divalent cations reinforced the adsorption of proteins on these supports. These results suggest that the adsorption of proteins on supports bearing almost neutral charge is not driven by the existence of opposite charges between the adsorbent and the biomacromolecule but just by the possibility of forming a high number of enzyme−support ionic bonds. This support has been used to purify the enzyme penicillin G acylase (PGA) from *Escherichia coli*. PGA was not significantly adsorbed at any pH value on either amino- or carboxyl-activated supports, while it can be fully adsorbed at pH 5 on this new carboxyl-amino matrix. Thus, we have been able to almost fully purify PGA from crude extracts with a very high yield by using these new supports.

## 1. Introduction

Ion exchange chromatography is one of the most popular strategies used to purify proteins. The adsorption of proteins on these supports is via multipoint interaction among several ionic groups placed in the support and several ionic residues having the opposite charge and placed on the protein surface.[1−12] Moreover, it is assumed that anion exchangers will adsorb proteins bearing a net negative charge, while cation exchangers will adsorb only proteins having a positive net charge.[5]

However, it has been shown that a particular protein could be adsorbed under identical experimental conditions on a support coated with anionic or cationic exchangers, mainly if the support was coated with a polymer (e.g., polyethyleneimine or dextran sulfate),[13,14] suggesting that the requirement for a net charge in the proteins that was opposite to that of the support was not fully necessary. The existence of regions on the protein surface with many positive charges and other regions having many negative charges seems to be enough to adsorb the proteins on positively or negatively charged supports.

Thus, we can assume that to adsorb a protein on an ion exchanger it is necessary to establish multipoint ionic interactions between the support and the proteins but not necessary to have a net opposite charge between the support and the proteins. Following this idea, adsorption of some proteins could be obtained on ion exchangers bearing simultaneously a high number of positive and negative charges, if the protein surface also has many positive and negative charges able to form a "net" of ionic bridges between the enzyme and the support (Figure 1).

This kind of support may be easily prepared by chemical amination (to different degrees) of carboxymethyl agarose with ethylenediamine (at different percentages of modification of the carboxyl groups).[15,16] In this way, it is possible to have very different supports, a fully carboxyl support surface (the CM commercial supports), a fully aminated support (EDA support), and supports with a mixture of amino and carboxyl groups (CM−EDA supports), with a similar number of charges and therefore a negligible net charge.

The idea of using mixed-mode chromatography has been used previously in some instances to purify small analytes, e.g., combining hydrophobic and ionic groups or also cationic and ionic groups.[17] They may be produced in one column by mixed-bed, mixed-ligand (two or more different functionalized ligands on the same substrate particle), or mixed-mode packings (two or more types of interaction sites or modes on the same surface-attached moiety).[17,18] Several workers have designed systems to simultaneously separate anions and cations using a series arrangement of anion- and cation-exchange columns[19] or mixed beds of cation- and anion-exchange particles.[20] Another approach is to use the oppositely charged sites of zwitterionic ligands, either covalently bound to support particles[21] or dynamically sorbed from the mobile phase onto a $C_{18}$ stationary phase.[22,23] Much of this work has involved organic ions, for which dispersion as well as Columbic interactions come into play.[24]

Some work also has been done to simultaneously separate ionic and neutral organic analytes. Serial columns, mixed-bed,[25] and mixed-ligand approaches have been used[21] ($C_8$ cation

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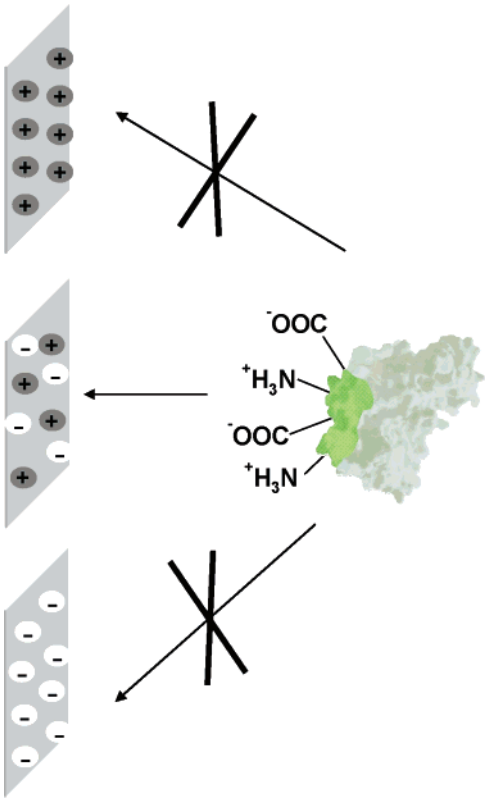


**Figure 1.** Proposed mechanism of adsorption of proteins on ionic neutral supports.

exchange)[26]. A few groups have deliberately designed multisite or mixed-mode columns to separate charged and uncharged analytes in one run.[21,22]

However, we have found just one paper referring to the simultaneous use of cation and anion exchangers to purify proteins, and that was proposed using a mixture of fully cationic and fully anionic membranes,[27] not a particle bearing both groups as proposed in this paper. Thus, this chromatography may become a new concept in the protein chromatography area, if matrixes bearing a high density of mixed cationic and anionic groups are able to adsorb proteins under conditions where those proteins are not able to become adsorbed on standard ionic supports (having a similar amount of ionizable groups but only positive or negative).

## 2. Materials and Methods

**2.1. Materials.** Carboxymethyl (CM) agarose 4BCL, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI), 2,4,6-trinitrobenzenesulfonic acid (TNBS), bovine serum albumin (BSA) grade X, sodium borohydride, aspartic acid, nickel chloride, manganese chloride, and chrome trichloride were from Sigma Chemical (St. Louis, MO). Trimethylaminoborane was purchased by Fluka (Buchs, Swizerland). Penicillin G acylase and penicillin G were kindly donated by Antibioticos (Leon, Spain). Protein extracts from *Escherichia coli* (nucleic-acid-free) were a kind gift from Professor Berenguer (Centro de Biología Molecular, Universidad Autónoma de Madrid). Other reagents were analytical grade. Ethylenediamine (EDA) supports were prepared as previously described.[15]

**2.2. Methods.** All experiments were performed at least in triplicate, and experimental error was never over 10%.

*2.2.1. Preparation of CM−EDA Agarose.* CM−EDA agarose was prepared by partial modification of CM agarose with EDA in the presence of EDCI.[15,16]

Approximately 10 g of CM agarose was suspended at 20 °C in 1.5 L of 1 M EDA adjusted with concentrated HCl at pH 4.75. Solid EDCI was then added to a concentration of 1 mM, and the reaction was allowed to proceed for 90 min, washed, and incubated in the presence of 0.5 M hydroxylamine pH 8 for 12 h. Finally, the supports were washed with distilled water. This permitted modification of 45−55% of the CM groups in the support.[15,16] A reference matrix subjected to a similar treatment but in the absence of EDA was prepared to discard any effect of the EDCI modification on the support in the observed results.

The amount of primary amino residues was determined by using TNBS.[28] Approximately 350 mg of CM−EDA agarose was added to 3 mL of 0.1% (w/v) TNBS, and the mixture was incubated for 30 min at room temperature. Then, the modified support was washed with distilled water and with sodium bicarbonate at pH 9, and the absorbance at 630 nm was determined. As a reference, inert agarose was treated in a similar way. Moreover, titration of amino and carboxyl groups using a autopH-stat was performed (in 1 M NaCl) using an automatic titrator (DL 50 Mettler Toledo).

*2.2.2. Determination of the Activity of Penicillin G Acylase.* Enzyme activity was evaluated using an automatic titrator (DL 50 Mettler Toledo) to titrate the release of phenylacetic acid produced by the hydrolysis of 10 mM penicillin G in 0.1 M sodium phosphate/0.5 M NaCl at pH 8 and 25 °C. A 100 mM NaOH solution was used as the titrating reagent. One international unit (IU) of penicillin G acylace (PGA) activity was defined as the amount of enzyme that hydrolyzes 1 $\mu$mol of penicillin G per minute at pH 8 and 25 °C.

*2.2.3. Adsorption of Proteins on Different Matrixes.* Adsorptions were performed in 5 mM sodium phosphate buffer at different pH values. The protein concentration of the supernatant phase was determined by Bradford's method.[29] When studying enzymes, adsorption was also checked by determination of PGA activity of the supernatant and the suspension.

After 2 h, the immobilized preparations were washed with an excess of distilled water and stored at 4 °C. Furthermore, most experiments were performed using only 10 units of the enzyme per milliliter of the support to prevent diffusion limitations that could complicate the understanding of the immobilization effects on enzyme activity. When using crude protein extracts, the maximum amount of protein offered was 5 mg per milliliter of support.

In some instances, $MgCl_2$ up to a concentration of 10 mM was added.

*2.2.4. Desorption of Adsorbed Proteins from Ion Exchangers.* The composites containing the adsorbed protein were resuspended in 5 mM sodium phosphate at different pH values. Then, increasing concentrations of NaCl were added, and samples were taken from the supernatant 30 min after the NaCl addition. Longer incubation times (up to 3 h) did not result in a significant increase in the amount of desorbed protein. The protein concentrations were determined by Bradford's method.[29] When studying enzymes, desorption was checked via enzyme activity determinations. A reference solution with soluble enzyme was subjected to the same treatment to detect any possible effect of the NaCl upon activity of the enzyme. The percentage of desorbed enzyme is referred to the amount of adsorbed protein. Additionally, adsorption of proteins on inert agarose (treated or untreated with EDCI) was studied, although significant adsorption of proteins on these supports was never found to take place.

*2.2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Analysis.* Samples of the proteins adsorbed on the different matrixes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli,[30] using 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 5% polyacrylamide. Gels were stained by silver and/or Coomasie. Low molecular weight markers from Pharmacia were used (14−94 kDa).

## 3. Results

**3.1. Characterization of the Supports.** EDA and CM supports presented 110−120 $\mu$mol of amino or CM groups per

**Table 1.** Percentage of Adsorbed Proteins on Different Ion Exchange Supports under Different Conditions

| support | pH | % adsorbed proteins |
|---|---|---|
| EDA | 7 | 80 |
| CM | 7 | 18 |
| CM−EDA | 7 | 20 |
| EDA | 5 | 35 |
| CM | 5 | 50 |
| CM−EDA | 5 | 55 |

gram of support. We prepared six different CM−EDA supports following the protocol proposed in the Materials and Methods section. The protocol showed great reproducibility of the modification degree, although in most cases a slight excess of the CM or EDA groups was found. In fact, the six samples ranged from 47% to 52% amidation. For further studies, experiments were performed using all six preparations to determine if this small excess of positive or negative charges presented any effect on the behavior for the chromatographic matrixes.

**3.2. Adsorption of a Protein Crude Extract from *E. coli* on Different Ion Exchangers.** A crude protein extract from *E. coli* was offered to CM, EDA, and the six samples of CM−EDA agarose at pH 7 or 5. At pH 7, adsorption of the proteins was approximately 20% on both CM and all CM−EDA supports, while 80% of the proteins became adsorbed on EDA supports (Table 1). At pH 5, the adsorption on the EDA support was reduced to 35%, while CM agarose adsorbed 50% of the proteins and all CM−EDA gave an adsorption slightly over 50% (Table 1), with no significant differences among the six different matrixes (less than 5%). This last result suggested that the slight positive or negative net charge on the supports does not have any significant effects on the adsorption capacity of the support. CM support treated with EDCI as described in the Materials and Methods section behaves very similarly to nonmodified CM agarose.

Some of the proteins adsorbed on CM or EDA supports at pH 5 could, after being desorbed and offered to the CM−EDA supports, become adsorbed on this new support (40% and 50%, respectively), and very interestingly, approximately 60% of the proteins that were not adsorbed either on CM or EDA supports could be adsorbed on CM−EDA supports. Thus, some of the proteins adsorbed on CM−EDA supports could be adsorbed on EDA or CM supports, while other percentages of proteins could not be adsorbed on supports presenting just one kind of ionic group.

These proteins adsorbed on CM−EDA became adsorbed on a support where the net charge was negligible (in some cases very slightly positive, in others very slightly negative) compared to that in either fully positively or negatively charged supports (bearing a similar global number of similar ionizable groups). Moreover, the adsorption on this kind of supports increased under pH values where most proteins presented a much reduced net charge but where the total number of charged groups may be maximal. That is, adsorption of some proteins seems to be achieved on ion exchangers simultaneously bearing a high number of positive and negative charges, if the protein surface has simultaneously also many positive and negative charges. This suggested that this "neutral" ionic support could be able to adsorb proteins via a different mechanism than the conventionally assumed one based on opposite net charges of the proteins and the support.

The addition of 200 mM NaCl during adsorption almost suppressed the adsorption of proteins on these supports.

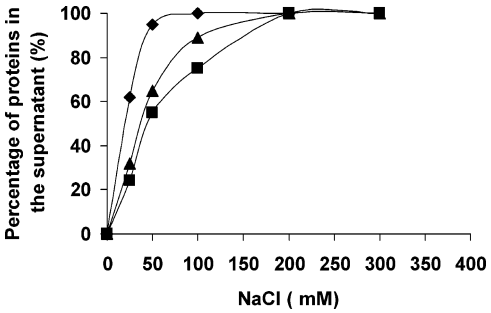


**Figure 2.** Desorption of adsorbed proteins at pH 5 on different ion exchange supports. Other specifications are described in the Materials and Methods section: diamonds, CM−EDA; squares, CM; triangles, EDA.

**Table 2.** Percentage of Adsorbed Proteins on Different Ion Exchange Supports at pH 5 in the Presence of $MgCl_2$

| support | pH | % adsorbed proteins |
|---|---|---|
| CM | 7 | 25 |
| CM−EDA | 7 | 40 |
| CM | 5 | 61 |
| CM−EDA | 5 | 70 |

**3.3. Desorption of Proteins Adsorbed on CM−EDA Supports.** Proteins adsorbed on these supports at pH 5.0 could be readily desorbed at pH 5 by just a slight increase in the ionic strength; using 50 mM NaCl, more than 90% of the proteins become desorbed from the CM−EDA support (Figure 2). CM or EDA supports absorbed the proteins more strongly, requiring the use of much higher ionic strengths to release all of the adsorbed proteins.

**3.4. Effects of Polyvalent Cations during Adsorption.** Polyvalent cations have been reported to increase the adsorption strength on cation exchangers by forming salt bridges between negatively charged groups in the support and in the protein, converting repulsion forces into new ionic bridges.[31] Table 2 shows that the presence of 10 mM $Mg^{2+}$ promoted an increase in the percentage of proteins that become adsorbed to CM−EDA supports, so that now approximately 40% of the proteins may be adsorbed on CM−EDA at pH 7 and approximately 70% at pH 5. The presence of $Mg^{2+}$ ions also increases the adsorption of proteins on CM agarose, although in a more reduced way.

**3.5. Purification of PGA by Using CM−EDA Supports.** As a model enzyme, we have tried the purification of PGA from *E. coli* using these new supports. PGA is an enzyme whose physiological role remains unclear, although it has been suggested that it is involved in the assimilation of aromatic compounds as carbon sources.[32,33] However, the enzyme has great interest in the pharmaceutical industry, mainly related to the production of semisynthetic $\beta$-lactam antibiotics.[34,35] The enzyme has been crystallized and characterized.[36−38] This enzyme is produced as a monomer that suffers autoproteolysis to yield the active two-subunit form.[39]

The enzyme PGA presents some protein subpopulations with a pI ranging between 6.2 and 6.8.[38] Thus, at first glance the enzyme should be adsorbed at pH 7−8 on EDA supports and at pH 5 on CM. However, PGA is not significantly adsorbed on either EDA or CM supports at pH values ranging from 5 to 9 (Figure 3). However, PGA becomes fully adsorbed at pH 5 on the different samples of CM−EDA supports (Figure 3). Figure 3 shows that an increase in the pH value promoted a decrease in the adsorption of the protein. Thus, at pH 5.5 more than 90% of PGA is still adsorbed on the support, and this number progressively decreases until pH 8, where PGA adsorp-

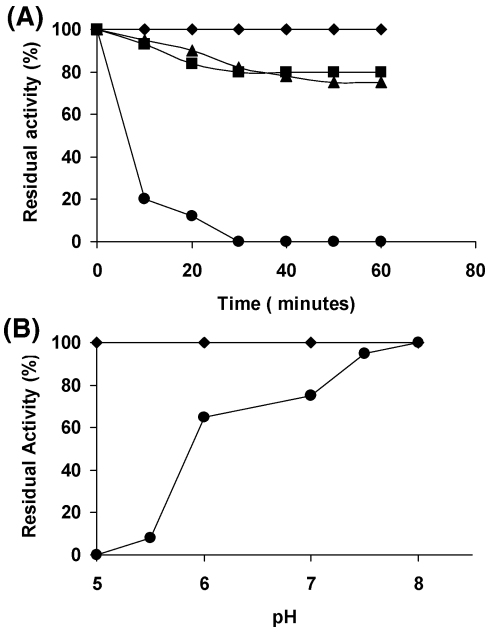


**Figure 3.** **(**A**)** Adsorption course of PGA at pH 5 on ionic exchangers: Diamonds: all suspensions; circles, supernatant of CM−EDA; triangles, supernatant of EDA; squares, of supernatant CM. **(**B**)** Ionic adsorption at different pH values of PGA on CM−EDA at different pH values: diamonds, activity in suspension; circles, activity in supernatant of CM−EDA. Other specifications as described in the Materials and Methods section.

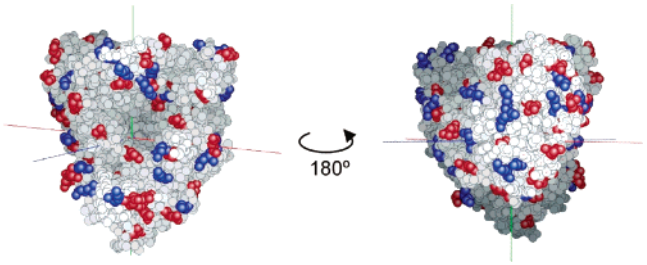


**Figure 4.** Surface charge distribution of PGA from *E. coli.* Positive (Arg, Lys) and negative (Glu, Asp) residues are colored in blue and red respectively. The figure was created with GRASP.[29]

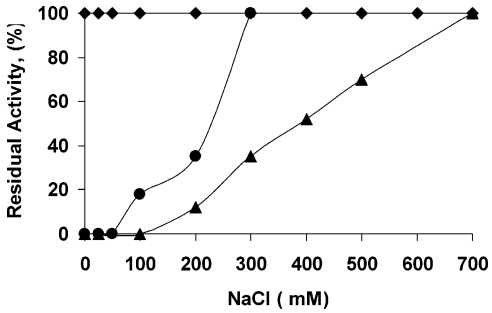


**Figure 5.** Desorption of PGA from *E. coli*, adsorbed on CM−EDA supports at growing ionic strengths: Diamonds: all suspensions; circles, PGA adsorbed on CM−EDA in 5 mM sodium acetate at pH 5; triangles, PGA adsorbed on CM−EDA in 5 mM sodium acetate/10 mM $MgCl_2$ at pH 5.

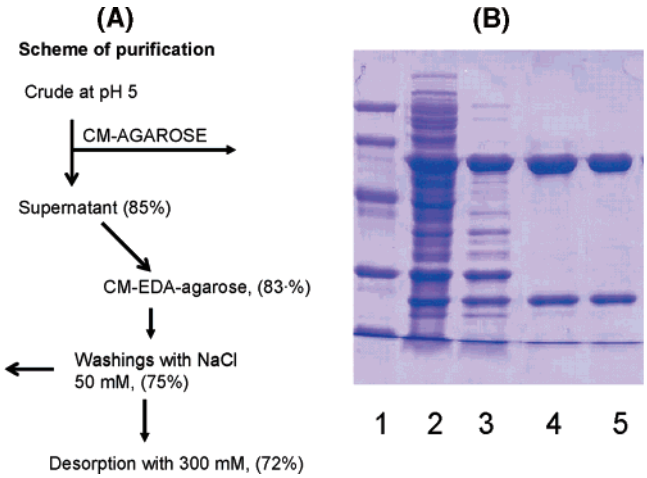


**Figure 6.** **(**A**)** Scheme of the purification of PGA using CM−EDA supports. **(**B**)** SDS-PAGE of the different steps in the purification of PGA using CM−EDA supports: lane 1, molecular weight markers; lane 2, crude extract; lane 3, proteins not adsorbed on CM agarose; lane 4, proteins that remain adsorbed on CM−EDA agarose after washing with 5 mM sodium phosphate/50 mM NaCl; lane 5, proteins desorbed from CM−EDA by washing with 5 mM sodium phosphate/300 mM NaCl.

tion becomes negligible. Figure 4 shows the distribution of charged residues (Glu and Asp in red, Lys and Arg in blue) on the PGA molecular surface as displayed by the GRASP program.[40] It is evident that both positively and negatively charged groups are closely mixed. This could explain why PGA becomes only adsorbed on very strong anion exchangers at pH 7 (e.g, Q-sepharose): The support needs to break the intramolecular ionic bridges in the protein.

The desorption of PGA adsorbed on CM−EDA supports at pH 5.0 reveals that PGA is quite strongly adsorbed, desorption starts at 100 mM NaCl, and full desorption of the enzyme required 300 mM NaCl (Figure 5). The adsorption and desorption of the PGA from CM−EDA in the presence of 10 mM $MgCl_2$ increased the adsorption strength: Now PGA starts to be desorbed from the support at 200 mM NaCl (Figure 5).

This strong adsorption of PGA on this new CM−EDA support permitted the development of a simple protocol to purify PGA: The crude protein extract containing the enzyme was offered to the CM support at pH 5, and then the nonadsorbed fraction was offered to CM−EDA agarose and washed with 50 mM NaCl, and then the PGA was desorbed almost fully pure from the support by washing the matrix with 300 mM NaCl. The yield was over 70%, and the final PGA presented more than 98% purity (Figure 6), only the $\alpha$ and $\beta$ subunits being visible in the SDS-PAGE gel.

## 4. Discussion

Supports bearing a high density of mixed positive and negative groups have proven to be able to adsorb proteins under conditions where the protein is not able to become adsorbed on standard ionic supports (bearing similar amounts of weak ionizable groups, but only positive or negative). Maximum adsorption of proteins occurs under conditions near the isoelectric points of enzymes. This fact suggests that this new kind of supports adsorbs proteins by forming a net of ionic bridges where both positive and negative groups in the support and protein contribute (Figure 1). The presence of divalent cations permitted the reinforcement of the adsorption of the proteins on these supports, very likely by permitting the formation of new salt bridges between the negatively charged groups in the supports and the negatively charged groups in the protein. In the case of PGA a very high degree of purification may be achieved by following a very simple purification protocol.

These new supports are a new tool for the purification of proteins based on ion exchange.

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## References and Notes

(1) Rao, C. S. *Proc. Biochem.* **2001**, *37*, 247−256.
(2) Xiao-Dong, T.; Xiao-Yang, D.; Yan, S. *Biochem. Eng. J.* **2002**, *12*, 117.
(3) Lyddiatt, A. *Curr. Opin. Biotechnol.* **2002**, *13*, 95.
(4) Berger, J. L.; Lee, B. H.; Lacroix, C. *Biotechnol. Appl. Biochem.* **1997**, *25*, 29.
(5) Scopes, R. K. *Protein Purification: Principles and Practice*, 2nd ed.; Springer-Verlag: New York, 1987; p 126.
(6) Shih, Y.; Prausnitz, J.; Blanch, H. *Biotechnol. Bioeng.* **1992**, *40*, 1155.
(7) Ghose, S.; Chase, H. A. *Bioseparation* **2000**, *9*, 29.
(8) Baumeister, A.; Stephanie, V.; Fischer, L. *J. Chromatogr., A* **2003**, *1006*, 261.
(9) Shudong, S.; Yilun, Y.; Huang, X.; Meng, D. *J. Membr. Sci.* **2003**, *222*, 3.
(10) Anspach, F. B.; Curbelo, D.; Hartman, R.; Garke, G.; Deckwer, W.-D. *J. Chromatogr., A.* **1999**, *865*, 129.
(11) Iberer, I.; Schwinn, H.; Jungbauer, A. Buchacher, A. *J. Chromatogr., A* **2001**, *921*, 15.
(12) Kumar, A.; Yu, I.; Galaev, B.; Mattiasson, B. *J. Chromatogr., B: Biomed. Sci. Appl.* **2000**, *741*, 103.
(13) Fuentes, M.; Pessela, B. C. C.; Maquiese, J. V.; Ortiz, C.; Segura, R. L.; Palomo, J. M.; Abian, O.; Torres, R.; Mateo, C.; Fernández-Lafuente, R.; Guisán, J. M. *Biotechnol. Prog.* **2004**, *20*, 1134.
(14) Kopaciewicz, W.; Rounds, M. A.; Regnier, F. E. *J. Chromatogr., A* **1983**, *266*, 3.
(15) Fernández-Lafuente, R.; Rosell, C. M.; Rodríguez, V.; Santana, C.; Soler, G.; Bastida, A.; Guisán, J. M. *Enzyme Microb. Technol.* **1993**, *15*, 546.
(16) Muenter, M. M.; Stokes, K. C.; Obie, R. T.; Jezorek, J. R. *J. Chromatogr., A* **1999**, *844*, 39
(17) Afrashtehfar, S.; Cantwell, F. F. *Anal. Chem.* **1982**, *54*, 2422.
(18) Pietrzyk, D. J.; Senne, S. M.; Brown, D. M. *J. Chromatogr., A* **1991**, *546*, 101.
(19) Saari-Nordhaus, R.; Nair, L.; Anderson J. M., Jr. *J. Chromatogr., A* **1992**, *602*, 127.
(20) Pietrzyk, D. J.; Brown, D. M. *Anal. Chem.* **1986**, *58*, 2554.
(21) Hartwick, B. A.; Yu, L. W.; Floyd, T. R. *J. Chromatogr. Sci.* **1986**, *24*, 177.
(22) Hu, W.; Takeuchi, T.; Haraguchi, H. *Anal. Chem.* **1993**, *65*, 2204.
(23) Hu, W.; Haraguchi, H. *Anal. Chem.* **1994**, *6*, 765.
(24) Davis, P. J.; Ruane, R. J.; Wilson, I. D. *Chromatographia* **1993**, *37*, 60.
(25) Jezorek, J. R.; Wagner, K. A. *J. Chromatogr., A* **1997**, *776*, 221.
(26) Freitag, R.; Splitt, H.; Reif, O.-W. *J. Chromatogr., A* **1996**, *728*, 129.
(27) Carraway, K. K.; Koshland, D. E. *Methods Enzymol.* **1972**, *25*, 616.
(28) Snyder, S. L.; Sobocinski, P. Z. *Anal. Biochem.* **1975**, *64*, 84.
(29) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
(30) Laemmli, U. K. *Nature* **1970**, *277*, 680.
(31) Fuentes, M.; Maquiese, J. V.; Pessela, B. C. C.; Torres, R.; Grazú, V.; Fernández-Lafuente, R.; Guisán, J. M.; Mateo, C. *Enzyme Microb. Technol.* **2006**, *39*, 332.
(32) Merino, E.; Balbas, P.; Recillas, F.; Becerril, B.; Valle, F.; Bolivar, F. *Mol. Microbiol.* **1992**, *6*, 2175.
(33) Valle, F.; Balbás, P.; Merino, E.; Bolivar, F. *Trends Biochem. Sci.* **1991**, *16*, 36.
(34) Arroyo, M.; Mata, I.; Acebal, C.; Castillón, M. P. *Appl. Microbiol. Biotechnol.* **2003**, *60*, 507.
(35) Rajendhran, J.; Gunasekaran, P. *J. Biosci. Bioeng.* **2004**, *97*, 1.
(36) Duggleby, H. J.; Tolley, S. P.; Hill, C. P.; Dodson, E. J.; Dodson, G.; Moody, P. C. *Nature* **1995**, *373*, 264.
(37) McVey, C. E.; Walsh, M. A.; Dodson, G. G.; Wilson, K. S.; Brannigan, J. A. *J. Mol. Biol.* **2001**, *313*, 139.
(38) Kutzbach, C.; Rauenbusch, E. *Physiol. Chem.* **1974**, *353*, 45.
(39) Bruns, W.; Hoppe, J.; Tsai, H.; Brüning, H. J.; Maywald, F.; Collins, J.; Mayer, H. *J. Mol. Appl. Genet.* **1985**, *3*, 36.
(40) Nicholls, A.; Sharp, K. A.; Honig, B. *Proteins: Struct., Funct., Genet.* **1991**, *11*, 281.

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