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Journal of Chromatography B, 668 (1995) 13–19

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Protein A immobilized polyhydroxyethylmethacrylate beads for affinity sorption of human immunoglobulin G

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First received 25 October 1994; revised manuscript received 16 January 1995; accepted 24 January 1995

Abstract

Protein A immobilized polyhydroxyethylmethacrylate (PHEMA) microbeads were investigated for the specific removal of HIgG from aqueous solutions and from human plasma. PHEMA microbeads were prepared by a suspension polymerization technique and activated by CNBr in an alkaline medium (pH 11.5). Protein A was then immobilized by covalent binding onto these microbeads. The amount of immobilized protein A was controlled by changing pH and the initial concentrations of CNBr and protein A. The maximum protein A immobilization was observed at pH 9.5. Up to 3.5 mg protein A/g PHEMA was immobilized on the CNBr activated PHEMA microbeads. The maximum HIgG adsorption on the protein A immobilized PHEMA microbeads was observed at pH 8.0. The non-specific HIgG adsorption onto the plain PHEMA microbeads was low (about 0.167 mg of HIgG/g PHEMA). Higher adsorption values (up to 6.0 mg of HIgG/g PHEMA) were obtained in which the protein A immobilized PHEMA microbeads were used. Much higher amounts of HIgG (up to 24.0 mg of HIgG/g PHEMA) were adsorbed from human plasma.

1. Introduction

Protein A is a cell wall protein of *Staphylococcus aureus* of molecular mass 42 000 with a strong, specific affinity for the Fc regions of immunoglobulins [1]. Due to this specificity, immobilized protein A matrices have a number of applications including isolation and purification of immunoglobulins, purification of monoclonal antibodies, isolation of immune complexes, and affinity chromatography of cells [1–3].

Therapeutic application of immobilized protein A matrices was first demonstrated by Bansal et al. [4] in their original publication describing the modality in perfusion of autologous plasma over *Staphylococcus aureus* or its active component, i.e. protein A. Thereafter, others have investigated antitumor responses in animal models and in human clinical trials [5–9]. Orlin and Berkman [10] have successfully utilized immobilized protein A matrices for the removal of antibodies in the cases of hemophilia and Goodpasture's syndrome, without depleting serum proteins other than IgG. Nilsson and coworkers [11,12] have used protein A linked agarose beads for removal of IgG from patients with chronic lymphocytic leukemia and autoimmune hemolytic anemia. Recently, Christie and Howe [13] have reported the treatment of refrac-

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0378-4347/95/$09.50
SSDI 0378-4347(95)00047-X

toriness to platelet transfusion by protein A column therapy.

Füglistaller reported a comparison of immunoglobulin binding capacities and ligand leakage values of several matrices [14]. The solid-phase matrices used for protein A immobilization are generally agarose, cellulose or controlled-pore glass, with cyanogen bromide as the common reagent for activation [15].

In the present study we have attempted to prepare a bioaffinity sorbent containing protein A for the selective adsorption of immunoglobulins (IgG) from human blood or plasma. We have prepared polyhydroxyethylmethacrylate (PHEMA) microbeads as the basic solid-phase matrix, which is one of the most widely used hydrophilic polymers in biomedical applications [16–19], by considering possible applications of these sorbents in direct hemoperfusion extracorporeal therapy, in which blood compatibility is one of the main concerns [20,21]. PHEMA microbeads were produced by a suspension polymerization technique. The hydroxyl groups on these microbeads were activated by CNBr, and then protein A molecules were covalently coupled to the microbeads through the active sites. HIgG adsorption on the protein A immobilized PHEMA microbeads from aqueous solutions containing different amounts of HIgG and at different pH, and also from human serum are reported here.

2. Experimental

2.1. Preparation of PHEMA microbeads

The basic monomer, 2-hydroxyethylmethacrylate (HEMA), was purchased from Sigma (St. Louis, MO, USA), and was purified by vacuum distillation under a nitrogen atmosphere. The comonomer ethyleneglycoldimethacrylate (EGDMA, Merck, Germany) was used as the crosslinking agent. The polymerization initiator was 2,2′-azobisisobutyronitrile (AIBN) (BDH, UK). The dispersion medium was a saturated aqueous solution of magnesium oxide (MgO) (Sigma).

PHEMA microbeads were prepared by a suspension polymerization technique [20]. Polymerization was carried out in an aqueous dispersion medium containing magnesium oxide which was used to decrease the solubility of the monomer, HEMA in the medium. The monomer phase containing HEMA, EGDMA and AIBN was added to the dispersion medium within a laboratory type reactor (i.e., a two-neck flask with a volume of 500 ml) equipped with a blade-type stirrer. In order to produce polymeric microbeads of about 200 μm in diameter and with a narrow size distribution, the HEMA/EGDMA ratio, the monomer phase/dispersion phase ratio, the amounts of EGDMA and AIBN, and the agitation speed were 1:3 (v:v), 1:10 (v/v), 0.33 (mol EGDMA/mol HEMA), 0.0015 (mol AIBN/mol HEMA), 600 rpm, respectively. Polymerization was carried out at 70°C for 3 h and then at 90°C for 1 h. After cooling, the polymeric microbeads were separated from the polymerization medium by filtration, and the residuals (e.g. monomer, MgO, etc.) were removed by a cleaning procedure given in detail elsewhere [20]. Briefly, a fixed-bed column was filled with microbeads and washing solutions (i.e. a dilute HCl solution, and a water–ethanol mixture) were recirculated through the system which includes also an activated carbon column, until the microbeads were clean. The purity of the microbeads was followed by observing the change in optical density of the samples taken from the liquid phase in the recirculation system, and also from DSC thermograms of the microbeads obtained by using a differential scanning calorimeter (Mettler, Switzerland).

2.2. CNBr activation

Prior to the activation process, PHEMA microbeads were kept in distilled water for about 24 h and washed on a glass filter with 0.5 *M* NaCl solution and water in order to remove impurities. Cyanogen bromide (CNBr, Sigma) aqueous solutions (100 ml) with different initial concentrations (5–50 mg CNBr/ml distilled water) were prepared. The pH of this solution

was quickly adjusted to 11.5 with 2 *M* NaOH while it was magnetically stirred. One gram of PHEMA microbeads was then added to this solution and the activation procedure was continued for 60 min at a constant pH of 11.5. After the activation reaction, in order to remove the excess activation agent, the PHEMA microbeads were washed with 0.1 *M* $NaHCO_3$ and any remaining active groups (e.g. isourea) on the surfaces were blocked by treatment with ethanol amine (pH 9.1) and $FeCl_3$ solution for 1 h. Then, the activated PHEMA microbeads were washed four times with distilled water containing 0.5 *M* NaCl.

2.3. Protein A immobilization

Protein A (Cat. No: 6650 from *Staphylococcus aureus*) was purchased from Sigma. One gram of the freshly CNBr activated PHEMA microbeads was magnetically stirred (at 50 rpm) at a constant temperature of 20°C for about 4 h (i.e. equilibrium time) with 50 ml of a protein A solution. In order to observe the effects of the CNBr initial concentration and pH on covalent coupling of protein A to the CNBr activated PHEMA microbeads, the CNBr initial concentration and the medium pH were varied between 5 and 50 mg/ml and 8.5 and 10.5, respectively. The initial concentration of protein A was 0.2 mg/ml. To study the effect of the protein A concentration on coupling, the initial concentration of protein A was varied between 0.05 and 0.40 mg/ml in which the pH of the solution (containing 0.1 *M* $NaHCO_3$ + 0.5 *M* NaCl) was 9.5.

After coupling, the protein A immobilized PHEMA microbeads were washed with 0.0625 *M* borate buffer + 0.15 *M* NaCl (pH 8.8), with 2 *M* urea + 0.15 *M* NaCl, and finally with 0.1 *M* $NaHCO_3$ + 0.5 *M* NaCl (pH 9.5). The amount of protein A immobilized on the CNBr activated microbeads was determined by measuring the decrease of protein A concentration and also by considering the protein A molecules adsorbed non-specifically (the amount of protein A adsorbed on the plain PHEMA microbeads), by the modified Lowry method [20,22].

2.4. HIgG adsorption from HIgG solution

Adsorption of human IgG (HIgG, Sigma, Cat. No: 160101) on the protein A immobilized PHEMA microbeads was studied batch-wise. One gram of PHEMA microbeads containing protein A was incubated with 50 ml of HIgG solution at 20°C for 4 h (i.e. equilibrium time). In order to study the effects of CNBr concentration and pH on the covalent coupling of HIgG to protein A immobilized PHEMA microbeads, the CNBr concentration and the pH of the solution were varied between 5 and 50 mg/ml and 6.5 and 9.0, respectively. The initial concentration of HIgG was 0.20 mg/ml solution.

To study the effects of the amount of protein A on adsorption of HIgG, the amount of protein A on the PHEMA microbeads was varied between 2.00 and 2.89 mg protein A/g PHEMA, in which the pH of the adsorption medium and the initial concentration of HIgG were 7.4 and 0.20 mg/ml solution, respectively. To study the effects of the initial concentration of HIgG on adsorption, the initial concentration of HIgG was varied between 0.05 and 0.40 mg/ml. Here, the amount of protein A on the PHEMA microbeads was constant (2.71 mg protein A/g PHEMA), and the pH of the medium was 7.4.

After the HIgG adsorption the PHEMA microbeads were washed with 0.0625 *M* borate buffer + 0.15 *M* NaCl (pH 8.8), with 2 *M* urea + 0.15 *M* NaCl, and finally with 0.1 *M* $NaHCO_3$ (pH 9.5) + 0.5 *M* NaCl in order to remove the non-specifically adsorbed HIgG molecules. The amount of HIgG adsorbed through protein A on the PHEMA microbeads was determined with the modified Lowry method by measuring the decrease in the HIgG concentration and also by considering the non-specifically adsorbed HIgG molecules (the amount of HIgG adsorbed on the plain PHEMA microbeads) [20,22].

2.5. HIgG adsorption from serum

Adsorption of HIgG from human plasma on the protein A immobilized PHEMA microbeads was studied batch-wise. Fresh human blood was used in all experiments. Blood samples were

centrifuged at 500 g for 30 min at room temperature to separate plasma. A 0.2-g amount of PHEMA microbeads containing protein A was incubated at 20°C for 20 min with 2 ml of human plasma. In order to study the effects of the amount of immobilized protein A on the adsorption of HIgG, PHEMA microbeads containing different amounts of protein A (between 2.0 and 2.89 mg protein A/g PHEMA) were used. The amount of HIgG adsorbed through protein A on the PHEMA microbeads was determined by a solid-phase enzyme-linked immunosorbent assay method (ELISA) by measuring the decrease in the HIgG concentration in the plasma [23]. The initial plasma concentration of HIgG was 10.5 mg/ml solution.

3. Results and discussion

3.1. Protein A immobilization

In the first part of this study, the effects of medium pH, initial concentrations of CNBr and protein A on the immobilization of protein A onto the PHEMA microbeads were investigated in batch adsorption–equilibrium studies.

In order to establish the optimal pH for protein A immobilization, coupling studies were performed at pH values between 8.5 and 10.5 [20]. The maximum protein A immobilization was achieved around pH 9.5, which was assumed to be the optimal pH and which was used in the later part of this study. Similar pH values were also proposed and applied for protein A coupling by others [14,15,24–27].

Yields of protein A immobilization on conventional matrices reported in the literature vary over a wide range from about 0.45 mg protein A per gram of controlled-pore glass [25] up to 8.0 mg protein A/g matrix (i.e. surface modified cellulose fibers) [26]. Recently very high immobilization yields up to 32.8 mg/g wet pellets of S-layer microparticles (an unconventional matrix) from the Gram-positive *Clostridum thermohydrosulfuricum* L11-69 were also reported [27]. In our studies, the maximum ligand incorporation achieved was 3.5 mg protein A/g PHEMA over the range of CNBr concentration used; this is similar to the immobilization yields found for conventional matrices.

The initial concentration of the activating agent, i.e. CNBr, in the activation medium was varied between 5 and 50 mg/ml in order to change the number of activated sites on the PHEMA surfaces. These PHEMA microbeads with different degrees of activation were then incubated with protein A aqueous solutions at pH 9.5 [20]. Note that the non-specific adsorption (adsorption on the plain PHEMA microbeads) was less than 0.12 mg protein A/g PHEMA. The amount of protein A immobilized on the microbeads increased by increasing the initial concentration of CNBr, up to 30 mg/ml; however, above this value, the effect was less pronounced.

Note that an increase in the CNBr initial concentration corresponds to a larger number of activated sites on the surface of the carrier. Therefore, as expected, higher amounts of protein A are coupled on the CNBr activated PHEMA microbeads with a higher number of activated sites [24–27]. It should be noted that there is always a maximum capacity which depends on the number of functional groups on the matrix and the size of the ligand molecules. However, the key factor in the performance of ligands immobilized on a solid surface is the ligand mobility after coupling rather than the total number of ligands available for coupling. Binding the protein A molecules tightly to the surface would certainly reduce the effective utilization of the active sites on the protein A molecules [2,15,24,28].

In our studies the maximum amount of protein A immobilized was 3.5 mg protein A/g PHEMA over the CNBr concentration range studied. However, to eliminate the possible steric hindrance to ligand accessibility, and also by considering the toxicity of CNBr, an initial CNBr concentration of 30 mg/ml was chosen as an optimal value; this gives a ligand immobilization of 2.71 mg protein A/g PHEMA.

3.2. HIgG adsorption

Protein A affinity chromatography is a well known and popular method of purifying im-

munoglobulins as mentioned before [2]. In response to the increasing demand many companies have offered protein A immobilized affinity materials with different capacities ranging from 0.5 to 20 mg IgG/ml gel (i.e. matrix) [14,24]. Comparatively higher IgG binding values up to 45.6 mg IgG/g affinity microparticles were recently reported by Weiner et al. [27]. The performance of the affinity matrix depends on the activity of the immobilized ligand (i.e. protein A), which is not a linear function of ligand surface concentration. An optimal ligand surface concentration is expected in which the interaction ratio between protein A and immunoglobulin molecules is maximum. Solid-phase variables such as spacer arm length, ligand concentration, coupling method, and matrix porosity control the availability of the active sites on the protein A molecules after immobilization, while the liquid-phase variables such as pH and ionic strength determine the conformation and degree of ionization of the immunoglobulin molecules at which favorable interaction with immobilized protein A molecules will take place [2,14,15,24–27].

In this study, the effects of medium pH, surface concentration of the ligand (i.e. protein A), and initial concentration of HIgG in the incubation medium on the adsorption of HIgG molecules onto the plain and the protein A immobilized PHEMA microbeads were studied in batch experiments.

In the first group of experiments we changed the pH of the incubation medium between 6.5 and 9.0, and looked at HIgG adsorption from aqueous solutions onto the protein A immobilized PHEMA microbeads (containing 2.71 mg protein A/g PHEMA). Note that the initial concentration of HIgG in the incubation solution was 0.2 mg/ml solution. Fig. 1 shows the effect of pH on HIgG adsorption. The maximum amount of HIgG coupled with protein A molecules on the PHEMA microbeads was 6.0 mg HIgG/g PHEMA at pH 8.0. Significantly lower adsorption capacities were obtained below and above pH 8.0. Ey et al. [29] reported an enhanced binding of IgG to protein A at a pH of 8.0–9.0 [29], while Hou et al. [26] showed that the pH most favorable for adsorption of immunoglobulin molecules by protein A immobilized on their composite matrix was around 7.0. These differences may be attributed to differences in the environmental conditions, such as ionic strength of the medium.

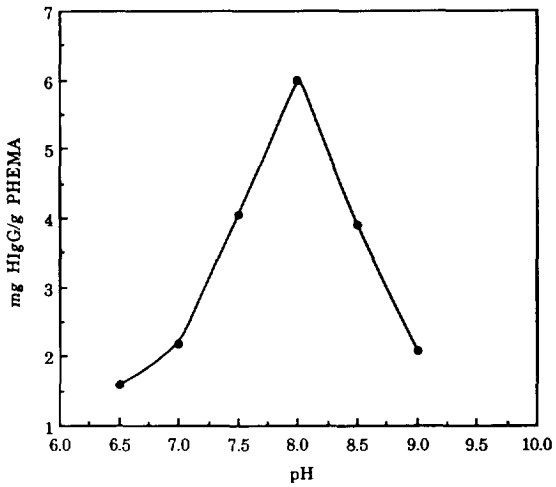

Fig. 1. Effect of pH on HIgG adsorption on protein A immobilized PHEMA microbeads.

HIgG adsorption capacities of the PHEMA microbeads containing different amounts of protein A (between 2.00 and 2.89 mg protein A/g PHEMA) were investigated at pH 8.0. The initial concentration of HIgG in the incubation solution was 0.2 mg/ml. Table 1 shows the effects of protein A surface concentration on HIgG adsorption onto PHEMA microbeads. When the surface protein A concentration (i.e. the number of protein A molecules per unit

Table 1
HIgG adsorption onto protein A immobilized PHEMA

Amount of protein A immobilized (mg)[a]	Amount of HIgG adsorbed (mg)[a]	Weight ratio of HIgG/protein A
2.00 ± 0.01	4.750 ± 0.07	2.375
2.42 ± 0.03	5.740 ± 0.06	2.371
2.71 ± 0.05	5.980 ± 0.01	2.200
2.79 ± 0.02	6.125 ± 0.03	2.195
2.82 ± 0.04	6.250 ± 0.02	2.216
2.89 ± 0.02	6.300 ± 0.05	2.179

[a] The average and the standard deviation of three parallel studies.

surface area) increased the amount of HIgG adsorbed onto PHEMA microbeads, first increased and then reached an almost constant value. This maximum HIgG adsorption capacity was 6.0 mg/g PHEMA. This behavior may be explained by considering a possible steric hindrance effect exerted by the high ligand surface concentration. Table 1 gives the mass ratio of HIgG/protein A adsorbed onto PHEMA microbeads containing different amounts of protein A. Note that the changes in the mass ratios also reflect changes in the ratios of the numbers of the interacting molecules which indicates that the interaction ratios are lower at higher ligand concentrations, most likely due to steric hindrance: this was also stated by others [2,14,15,24–27].

HIgG adsorption onto the plain PHEMA and PHEMA microbeads containing 2.71 mg protein A/g PHEMA from aqueous solutions containing different amounts of HIgG (0.05–0.4 mg/ml) was studied at a constant pH of 8.0. Fig. 2 gives the adsorption data on the plain PHEMA microbeads (0.1 mg HIgG/g PHEMA). Specific adsorption (i.e. adsorption of HIgG molecules onto the PHEMA microbeads through protein A molecules) was significant (up to 6.0 mg HIgG/g PHEMA), and increased with increasing initial concentration of HIgG in the incubation medium. As expected, the amount of HIgG coupled to PHEMA microbeads via protein A molecules almost reached a plateau value around 0.2 mg/ml, possibly due to the steric hindrance effect mentioned before.

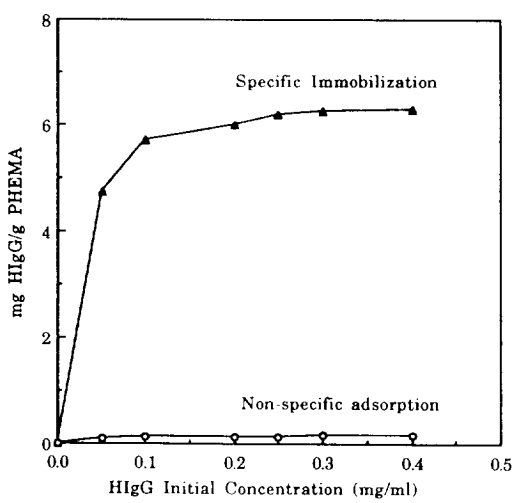

Fig. 2. Effect of HIgG initial concentration on HIgG adsorption through protein A immobilized onto PHEMA microbeads.

HIgG adsorption from human plasma obtained from a healthy donor was also investigated. The initial concentration of HIgG in this plasma was 10.5 mg/ml. The PHEMA microbeads containing different amounts of protein A (2.00–2.89 mg/g PHEMA) were incubated with the plasma. Table 2 gives the adsorption data. As seen here, very low non-specific adsorption (0.22 mg HIgG/g PHEMA) of HIgG onto the plain PHEMA microbeads was observed. There was a pronounced adsorption of HIgG (up to 24 mg/g PHEMA), as expected from the earlier studies presented above. The adsorption of HIgG onto the PHEMA microbeads containing 2.71 mg protein A/g PHEMA from human plasma was approximately 3-fold higher (18.89 mg HIgG/g PHEMA) than that obtained in the studies where aqueous solutions were used. This cannot be due to the high initial concentration in the plasma because there is a plateau value (6.0 mg HIgG/g PHEMA) for HIgG adsorption from aqueous solutions (at pH 8.0) as shown in Table 1. This is possibly due to the conformation of the HIgG molecules in their native medium (i.e. plasma pH 7.4) which may be more suitable for interaction with the protein A molecules adsorbed onto the PHEMA microbeads.

Table 2
HIgG adsorption from human plasma

Amount of protein A immobilized (mg/g)[a]	Amount of HIgG adsorbed (mg/g)[a]
0.00	0.22 ± 0.04
2.00 ± 0.01	14.23 ± 1.25
2.71 ± 0.05	18.89 ± 1.36
2.89 ± 0.02	24.00 ± 2.19

[a] The average and the standard deviation of three parallel studies.

Acknowledgements

Part of these studies was performed by Dr. Adil Denizli in the laboratories of Kavetsky Institute, Ukranian Academy of Sciences, Kiev. The authors would like to thank Dr. V. Nicolaev and Dr. V.G. Sobko for their kind supervision.

References

[1] J.J. Langone, M.D.P. Boyle and T. Boras, *J. Immunol.*, 121 (1978) 327.
[2] J.J. Langone, *J. Immunol. Methods*, 55 (1982) 277.
[3] V. Ghetie and J. Sjoquist, in *Methods in Enzymology*, Vol. 108, Academic Press, New York, NY, 1984, p. 133.
[4] S.C. Bansal, B.R. Bansal, H.I. Thomas, J.E. Siegel, J.E. Rhodas, D.S. Copper and R.M. Terman, *Cancer*, 42 (1978) 1.
[5] D.S. Terman, T. Yamamoto and R.L. Tillquist, *Science*, 209 (1980) 1257.
[6] P.K. Ray, A. Idiculla and R. Mark, *Cancer*, 49 (1982) 1800.
[7] J.B. Young, J.C. Ayus and L.K. Miller, *Am. J. Med.*, 75 (1983) 75.
[8] J.J. Langone, C.H. Das, D. Bennett and D.S. Terman, *J. Immunol.*, 133 (1984) 1057.
[9] G.L. Messerchmidt, D.H. Henry and H.W. Snyder, *J. Clin. Oncol.*, 6 (1988) 203.
[10] J.B. Orlin and E.M. Berkman, *Blood*, 56 (1980) 1055.
[11] I.M. Nilsson, S. Jonsson, S.B. Sundquist, A. Ahlberg and S.E. Bergentz, *Blood*, 58 (1981) 38.
[12] I.M. Nilsson, S.B. Sundquist, R. Ljung, L. Holmberg, C. Freiburghaus and G. Bjorlin, *Scan. J. Haematol.*, 30 (1983) 458.
[13] D.J. Christie and R.B. Howe, *Transfusion*, 33 (1993) 961.
[14] P. Füglistaller, *J. Immunol. Methods*, 124 (1989) 171.
[15] G.T. Hermanson, A.K. Mallia, and P.K. Smith (Editor), *Immobilized Affinity Ligand Techniques*, Academic Press, London, 1992.
[16] A.S. Hoffman and C. Harris, *Am. Chem. Soc. Div. Polym. Chem., Polym. Prepr.*, 13 (1972) 740.
[17] S.D. Bruck, *J. Biomed. Mat. Res.*, 6 (1972) 173.
[18] J.D. Andrade (Editor), *Hydrogels for Medical and Related Applications*, American Chemical Society, Washington, DC, ACS Symp. Ser. No. 31, 1976.
[19] B.J. Tighe, in N.A. Peppas (Editor), *Hydrogels in Medicine and Pharmacy*, Vol. 2, CRC Press, Boca Raton, FL, 1987. p. 53.
[20] A. Denizli, Ph.D. Thesis, Hacettepe University, Ankara, Turkey, 1992.
[21] A. Denizli and E. Pişkin, *J. Chromatogr. B*, 1995 (in press).
[22] G.L. Petterson, *Anal. Biochem.*, 100 (1979) 201.
[23] E. Harlow and D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, NY, 1988.
[24] T.M. Phillips, N.S. More, W.D. Queen, T.V. Holohan, N.C. Kramer and A.M. Thompson, *J. Chromatogr.*, 317 (1984) 173.
[25] T.M. Phillips, N.S. More, W.D. Queen and A.M. Thomson, *J. Chromatogr.*, 327 (1985) 205.
[26] K.C. Hou, R. Zaniewski and S. Roy, *Biotech. Appl. Biochem.*, 13 (1991) 257.
[27] C. Weiner, M. Sara and U.B. Sleytr, *Biotechnol. Bioeng.*, 43 (1994) 321.
[28] P. Cuatrecass, M. Wilcek and C.B. Anfinsen, *Proc. Natl. Acad. Sci.*, 61 (1968) 636.
[29] P.L. Ey, S.J. Prowse and C.R. Jenkin, *Immunochemistry*, 15 (1978) 429.