Journal of Chromatography, 478 (1989) 159–167
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 636

PREPARATION OF ADSORBENTS FOR AFFINITY CHROMATOGRAPHY USING TSKGEL TRESYL-TOYOPEARL 650M

KOJI NAKAMURA*, KIYOHIRO TOYODA and YOSHIO KATO
Central Research Laboratory, Tosoh Corporation, Tonda, Shin-nanyo, Yamaguchi (Japan)
and
KIYOHITO SHIMURA and KEN-ICHI KASAI
Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa (Japan)
(First received January 24th, 1989; revised manuscript received May 23rd, 1989)

SUMMARY

The optimum conditions for the coupling of proteins were investigated using TSKgel Tresyl-Toyopearl 650M. They were dependent on the proteins coupled. For example, when soybean trypsin inhibitor was coupled at pH 8 the coupling was completed within 1 h and the subsequent adsorption capacity for trypsin was maximal. Longer coupling times decreased the adsorption capacity due to multi-point attachment. The adsorbents obtained were successfully used for affinity chromatography in a short time.

INTRODUCTION

The preparation of adsorbents for affinity chromatography requires suitable matrices and effective coupling methods for attachment of ligands. Beaded agarose activated by cyanogen bromide is the most popular and versatile solid support for affinity matrices. Though the cyanogen bromide method discovered by Axen *et al.*[1] has contributed to the recent development of affinity chromatography, it has some problems such as the labile linkage between the ligands and the matrix[2,3] and the introduction of charged species[4,5]. In order to overcome these problems, Nilsson and Mosbach[6] developed a procedure using organic sulphonyl chlorides as activating agents. They activated agarose with tresyl chloride under relatively mild conditions for a short period of time. This yielded predominantly primary hydroxyl activation and the result was an excellent matrix for coupling proteins and affinity ligands. However, agarose is not a completely satisfactory matrix especially for industrial use and high-performance affinity chromatography because of its poor mechanical strength.

Very recently, a new support for affinity chromatography has become commercially available under the trade-name of TSKgel Tresyl-Toyopearl 650M. According to the supplier, it is prepared by introducing tresyl groups into TSKgel Toyopearl HW-65 (44–88 μm), which is a hydrophilic resin-based material of large pore size employed for gel filtration[7]. This paper describes the preparation of adsorbents for affinity chromatography using TSKgel Tresyl-Toyopearl 650M.

0021-9673/89/$03.50

EXPERIMENTAL

Materials

Soybean trypsin inhibitor (STI), bovine trypsin, porcine trypsin, concanavalin A (Con A) and crude peroxidase were from Sigma. Purified peroxidase (RZ:3.48) was from Toyobo (Osaka, Japan). Human immunoglobulin (IgG) and human serum were from Miles, protein A from Repligen. TSKgel Tresyl-Toyopearl 650M was from Tosoh.

Coupling of proteins to Tresyl-Toyopearl

Proteins (5–80 mg) were dissolved in 4 ml of an appropriate coupling buffer at different pH values (0.1 *M* phosphate buffer containing 0.5 *M* NaCl for pH 6 and 7, 0.1 *M* carbonate buffer containing 0.5 *M* NaCl for pH 8 and 9) and mixed with 0.4 g dried Tresyl-Toyopearl (1 g dried Tresyl-Toyopearl gives a final gel volume of about 5 ml) at 4 or 25°C for 0.5–16 h. After washing three times with 20 ml of coupling buffer, the unreacted tresyl groups were deactivated by resuspending the gel in 10 ml of 0.1 *M* Tris–HCl (pH 8.5) for 1 h. Protein contents were determined by amino acid analysis.

Determination of adsorption capacity

The adsorption capacity of immobilized STI, Con A and protein A were determined by passing 10 mg of purified trypsin, peroxidase and human IgG, respectively, through each column (50 mm × 5 mm) equilibrated with the appropriate buffer [0.05 *M* Tris–HCl buffer containing 0.5 *M* NaCl and 20 m*M* $CaCl_2$ (pH 7.5) for the STI column, 0.1 *M* acetate buffer containing 0.5 *M* NaCl and 1 m*M* $MgCl_2$, $MnCl_2$ and $CaCl_2$ (pH 6.0) for the Con A column, 0.1 *M* phosphate buffer (pH 7.0) for the protein A column]. The excess of protein was removed with the equilibration buffer. Desorption of protein was achieved by an appropriate buffer [0.1 *M* acetic acid containing 0.5 *M* NaCl (pH 3.0) for the STI column, 0.11 *M* mannose in equilibration buffer for the Con A column, 0.1 *M* glycine–HCl (pH 2.2) for the protein A column]. The amounts of protein were calculated from the volume and absorption at 280 nm (trypsin and human IgG) and 403 nm (peroxidase).

Affinity chromatography

All chromatographic measurements were performed at 4 or 25°C with a CCPM pump (Tosoh) equipped with a variable-wavelength UV detector Model UV-8000 (Tosoh).

Measurement of trypsin activity

Trypsin activity was measured with benzoylarginine ethyl ester as a substrate, essentially according to the method of Schwert and Takenaka[8].

Measurement of peroxidase activity

Peroxidase activity was measured by the change in absorbance at 460 nm due to the oxidation of *o*-dianisidine in the presence of peroxidase and H_2O_2 as described by Shannon *et al.*[9].

Electrophoresis

Electrophoretic analysis of the fraction was performed in slabs of a 4–20% polyacrylamide gradient gel (Tefco, Tokyo, Japan) according to the manual supplied.

RESULTS AND DISCUSSION

Coupling of STI at different pH values

Table I shows the results of coupling of STI at different pH values. At pH 6 the coupling reaction was very slow and the coupling yield was only 12% after 16 h. At pH 7 the coupling yield was increased with coupling time, and the adsorption capacity for trypsin was maximal after 16 h. At pH 8 the coupling of STI was completed in 1 h and the adsorption capacity was maximal. However, longer coupling times decreased the adsorption capacity. At pH 9, although the coupling reaction was also completed in 1 h, the adsorption capacity was constant. Amino acid analyses indicated that tyrosyl, histidyl and lysyl residues of STI were coupled to Tresyl-Toyopearl and the lysyl residue was more reactive at higher pH values.

Immobilization of protein is generally thought to occur via several bonds per protein molecule (if the protein has many available functional groups). The coupling of STI at higher pH values (pH 8 or 9) might favour the multi-point attachment which decreases the biochemical activity due to distortion of STI or to steric hindrance of its binding site.

Effect of ligand concentration

Table II shows the effect of the ligand concentration on the amount coupled and

TABLE I

COUPLING OF STI AT DIFFERENT pH VALUES

STI applied: 5 mg/ml gel. Temperature: 25°C.

	Time (h)					
	0.5	*1*	*2*	*4*	*8*	*16*
pH 6						
Coupled STI (mg/ml gel)	0.2	0.3	0.4	0.5	0.5	0.6
Coupling yield (%)	4.0	6.0	8.0	10	10	12
Adsorption capacity (mg/ml gel)	0	0.1	0.1	0.1	0.2	0.3
pH 7						
Coupled STI (mg/ml gel)	0.5	0.9	1.4	2.4	3.3	4.1
Coupling yield (%)	10	18	28	48	66	82
Adsorption capacity (mg/ml gel)	0.2	0.5	0.7	1.1	1.5	2.0
pH 8						
Coupled STI (mg/ml gel)	4.5	4.9	5.0	5.0	5.0	5.0
Coupling yield (%)	90	98	100	100	100	100
Adsorption capacity (mg/ml gel)	2.1	2.1	1.5	1.3	1.1	1.0
pH 9						
Coupled STI (mg/ml gel)	4.7	4.8	5.0	5.0	5.0	5.0
Coupling yield (%)	94	96	100	100	100	100
Adsorption capacity (mg/ml gel)	1.5	1.5	1.4	1.5	1.4	1.5

TABLE II
EFFECT OF THE AMOUNT OF STI
Coupling buffer: 0.1 *M* carbonate + 0.5 *M* NaCl (pH 8). Temperature: 25°C.

	Time (h)					
	0.5	*1*	*2*	*4*	*8*	*16*
5 mg						
Coupled STI (mg/ml gel)	4.5	4.9	5.0	5.0	5.0	5.0
Coupling yield (%)	90	98	100	100	100	100
Adsorption capacity (mg/ml gel)	2.1	2.1	1.5	1.3	1.1	1.0
10 mg						
Coupled STI (mg/ml gel)	5.3	6.7	8.2	8.5	9.0	9.0
Coupling yield (%)	53	67	82	85	90	90
Adsorption capacity (mg/ml gel)	2.7	3.3	3.0	2.6	2.4	1.8
20 mg						
Coupled STI (mg/ml gel)	7.4	9.8	12	14	14	14
Coupling yield (%)	37	49	60	70	70	70
Adsorption capacity (mg/ml gel)	4.2	5.1	43	3.9	3.6	3.4
40 mg						
Coupled STI (mg/ml gel)	8.3	12	15	15	17	17
Coupling yield (%)	21	30	38	40	40	40
Adsorption capacity (mg/ml gel)	5.4	5.4	5.4	5.2	4.8	4.8

the adsorption capacity for trypsin. Although the amount of protein coupled increased with increasing concentration, the proportion of the protein which coupled fell and the coupling was less efficient at high protein concentration. As for the trypsin capacity, the maximum was obtained in 1–2 h at each ligand concentration, but the capacity decreased with increasing coupling time. At the lowest concentration, it was decrased to 50% after 16 h, but at the highest concentration it was decreased to 89% after 16 h. This is because the multi-point attachment was prevented due to the high ligand concentration.

TABLE III
TEMPERATURE EFFECT
STI applied: 5 mg/ml gel. Coupling buffer: 0.1 *M* carbonate + 0.5 *M* NaCl (pH 8).

	Time (h)					
	0.5	*1*	*2*	*4*	*8*	*16*
25°C						
Coupled STI (mg/ml gel)	4.5	4.9	5.0	5.0	5.0	5.0
Coupling yield (%)	90	98	100	100	100	100
Adsorption capacity (mg/ml gel)	2.1	2.1	1.5	1.3	1.1	1.0
4°C						
Coupled STI (mg/ml gel)	0.7	1.2	2.3	3.2	4.1	4.9
Coupling yield (%)	14	24	46	64	82	98
Adsorption capacity (mg/ml gel)	0.4	0.7	1.3	1.5	1.6	2.1

TABLE IV
COUPLING OF CON A AT DIFFERENT pH VALUES
Con A applied: 15 mg/ml. Temperature: 25°C.

	Time (h)				
	1	*2*	*4*	*8*	*16*
pH 7					
Coupled STI (mg/ml gel)				2.4	4.1
Coupling yield (%)				16	27
Adsorption capacity (mg/ml gel)				0.5	0.9
pH 8					
Coupled STI (mg/ml gel)	7.0	9.3	12	13	13
Coupling yield (%)	47	62	80	88	88
Adsorption capacity (mg/ml gel)	2.9	4.5	4.8	5.6	6.2

Temperature effect

Table III shows that, although the coupling of STI was effectively completed in 1 h at 25°C, it took 16 h to complete the coupling at 4°C.

Coupling of other proteins

Con A and protein A were coupled at different pH values. The matrices were then assayed for the protein content and adsorption capacity.

Con A from Jack bean was coupled at pH 7 and 8 (Table IV). Both the coupling yield and the adsorption capacity for peroxidase were increased as the pH was increased from 7 to 8. However, Con A seemed to be aggregated at pH 9 as judged from gel filtration (data not shown).

TABLE V
COUPLING OF PROTEIN A AT DIFFERENT pH VALUES
Protein A applied: 2.5 mg/ml gel. Temperature: 25°C.

	Time (h)				
	1	*2*	*4*	*8*	*16*
pH 8					
Coupled STI (mg/ml gel)	1.0	1.4	1.6	1.8	1.9
Coupling yield (%)	40	56	64	72	76
Adsorption capacity (mg/ml gel)	3.3	2.7	1.9	1.7	1.3
pH 9					
Coupled STI (mg/ml gel)	1.8	1.9	2.0	2.1	2.1
Coupling yield (%)	72	76	80	84	84
Adsorption capacity (mg/ml gel)	1.7	1.3	0.8	0.5	0.5
pH 8[a]					
Coupled STI (mg/ml gel)	0.3	0.6	0.8	1.0	1.1
Coupling yield (%)	12	24	32	40	44
Adsorption capacity (mg/ml gel)	3.2	4.6	4.6	4.4	4.3

[a] 50 m*M* Tris–HCl + 0.5 *M* NaCl.

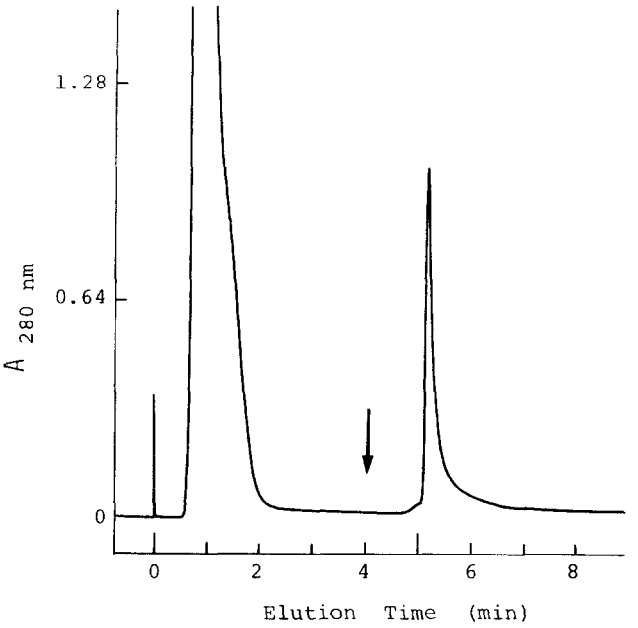

Fig. 1. Affinity chromatography of trypsin on STI-Toyopearl. Crude trypsin (5 mg in 500 μl) was loaded on the column (50 mm × 5 mm) previously equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.5) containing 0.5 *m* NaCl and 20 m*M* $CaCl_2$ at a flow-rate of 1 ml/min at 4°C. After washing the column with the equilibration buffer, elution of adsorbed trypsin with 0.1 *M* acetic acid containing 0.5 *M* NaCl was started at the time indicated by the arrow.

Protein A, which binds to the Fc region of several classes of immunoglobulins[10,11], was coupled at pH 8 and 9. Table V shows the coupling yield increased with coupling time. The adsorption capacity for human IgG is the amount eluted with 0.1 *M* glycine–HCl buffer (pH 2.2). However, when 100 μg of human IgG were loaded and eluted with the acidic buffer, the recovery of human IgG was lower than 80% and it was difficult to elute all the bound human IgG. This result was in good agreement with that reported by Nilsson and Mosbach[12] (immobilizing protein A with too many linkages makes it difficult to elute all of the bound IgG). In order to prevent multi-point attachment, immobilization of protein A was carried out using Tris as a coupling buffer. The results are also shown in Table V, which indicates that although the coupling yield was decreased, the adsorption capacity was increased. Also, in this case, the recovery of human IgG was quantitative.

Demonstration of affinity chromatography

Fig. 1 shows the purification of porcine trypsin on STI-Toyopearl. A sample of commercial crude trypsin (5 mg in 500 μl) was loaded onto a STI-Toyopearl column (5 cm × 5 mm) and desorption of the bound trypsin was carried out with 0.1 *M* acetic acid containing 0.5 *M* NaCl (pH 3.0). Two major peaks were obtained from STI affinity chromatography of trypsin. The first peak (unbound fraction) had no trypsin acitivity and the second peak (bound fraction) had 90% of trypsin activity. This bound fraction gave a single band corresponding to a molecular weight of 23 000 according to

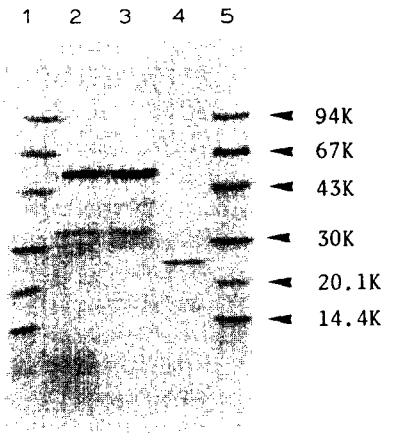

Fig. 2. SDS-polyacryl amide gradient gel (4–20%) electrophoresis. Lanes: 1 and 5 = low-molecular-weight marker (from Pharmacia); 2 = crude trypsin; 3 = unbound fraction; 4 = bound fraction. The gel was stained with 0.1% Coomassie Blue R250 in methanol–water–acetic acid (4:5:1, v/v/v). The anode is at the bottom. K = Kilodaltons.

sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see Fig. 2).

Fig. 3 shows the purification of peroxidase on Con A-Toyopearl. A crude peroxidase (5 mg in 0.5 ml) dissolved in equilibration buffer was loaded onto a Con A-Toyopearl column (5 cm × 5 mm). Desorption of the bound peroxidase was carried

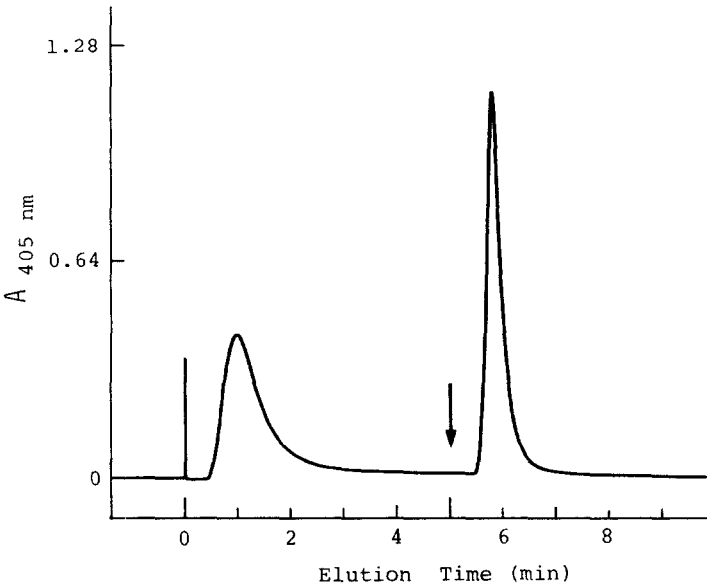

Fig. 3. Affinity chromatography of peroxidase on Con A-Toyopearl. Crude peroxidase (5 mg in 0.5 ml) was loaded on the column (50 mm × 5 mm) previously equilibrated with 0.1 *M* acetate buffer (pH 6.0) containing 0.5 *M* NaCl and 1 m*M* $CaCl_2$, $MnCl_2$ and $MgCl_2$ at a flow-rate of 1 ml/min at 25°C. After washing the column with the equilibration buffer, elution of adsorbed peroxidase with 0.11 *M* mannose in equilibration buffer was started at the time indicated by the arrow.

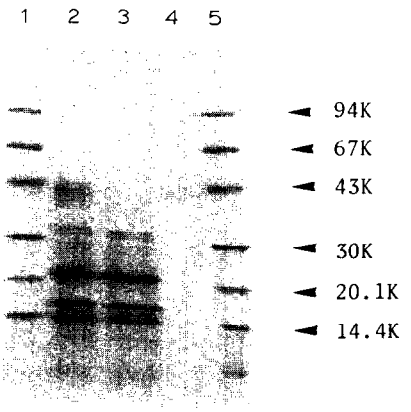

Fig. 4. SDS-polyacrylamide gradient gel (4–20%) electrophoresis. Lanes: 1 and 5 = low-molecular-weight marker (from Pharmacia); 2 = crude peroxidase; 3 = unbound fraction; 4 = bound fraction. Conditions as in Fig. 2.

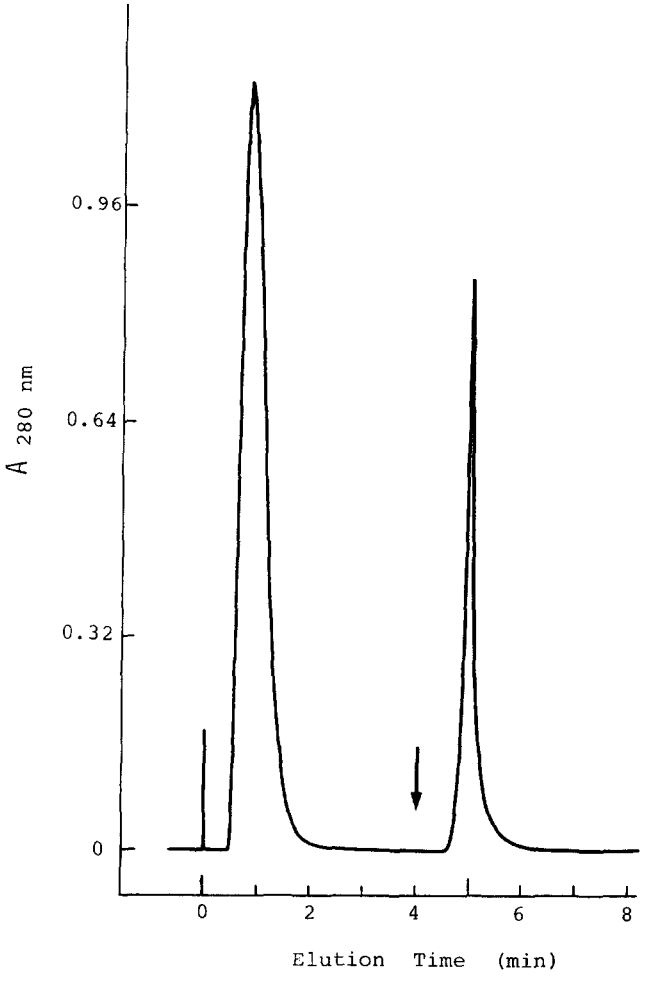

Fig. 5. Purification of human IgG on protein A-Toyopearl. Human serum (50 μl) was loaded on the column (50 mm × 5 mm) previously equilibrated with 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min at 25°C. After washing the column with the equilibration buffer, elution of adsorbed IgG with 0.1 *M* glycine–HCl buffer (pH 2.2) was started at the time indicated by the arrow.

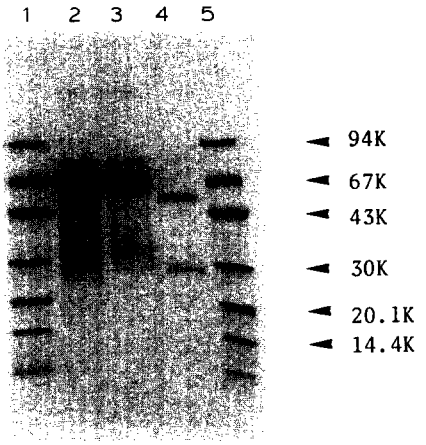

Fig. 6. SDS-polyacrylamide gradient gel (4–20%) electrophoresis. Lanes: 1 and 5 = low-molecular-weight marker (from Pharmacia); 2 = human serum; 3 = unbound fraction; 4 = bound fraction. Conditions as in Fig. 2.

out with 0.11 *M* mannose. The bound fraction had 70% of peroxidase activity and was also subjected to SDS-PAGE. Fig. 4 shows this fraction has several protein bands with a molecular weight of 42000. However, this fraction gave a single peak in reversed-phase chromatography (data not shown).

Fig. 5 shows the purification of human IgG on protein A-Toyopearl. Human serum (50 μl) was loaded onto a protein A-Toyopearl column (5 cm × 5 mm) equilibrated in 0.1 *M* phosphate buffer (pH 7.0). Desorption of the bound IgG was performed with 0.1 *M* glycine–HCl buffer (pH 2.2). Judging from SDS-PAGE, the bound fraction contained only the light and heavy chain of human IgG (see Fig. 6). As demonstrated above, Tresyl-Toyopearl can be successfully used for affinity chromatography in a short time.

REFERENCES

1 R. Axen, J. Porath and S. Ernback, *Nature (London)*, 215 (1967) 1302.
2 J. Ludens, I. R. De Vries and D. D. Fanstiel, *J. Biol. Chem.*, 247 (1972) 7533.
3 V. Sica, E. Nola, I. Parikh, G. A. Puca and P. Cuatrecasas, *J. Biol. Chem.*, 248 (1973) 6543.
4 R. Lamed, Y. Levin and A. Oplatka, *Biochim. Biophys. Acta*, 305 (1973) 163.
5 A. H. Nishikawa and P. Bailon, *Arch. Biochem. Biophys.*, 168 (1975) 576.
6 K. Nilsson and K. Mosbach, *Biochem. Biophys. Res. Commun.*, 102 (1981) 449.
7 Y. Kato, K. Komiya and T. Hashimoto, *J. Chromatogr.*, 247 (1982) 184.
8 G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, 16 (1955) 570.
9 L. Shannon, E. Kay and J. Y. Lew, *J. Biol. Chem.*, 244 (1966) 2166.
10 J. J. Langone, *J. Immunol. Methods*, 55 (1982) 277.
11 W. L. Bigbee, M. Vanderlaan, S. S. N. Fong and R. H. Jensen, *Mol. Immunology*, 20 (1983) 153.
12 K. Nilsson and K. Mosbach, *Methods Enzymol.*, 104 (1984) 56.