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Purification and determination of glutamine synthetase by high-performance immunoaffinity chromatography

J. Alhama, J. López-Barea, F. Toribio* and J. M. Roldán

Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Avda. Medina Azahara, Universidad de Córdoba, 14071 Córdoba (Spain)

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ABSTRACT

High-performance immunoaffinity chromatography (HPIAC) with anti-glutamine synthetase polyclonal antibodies bound to epoxy-activated silica was used to purify and determine this enzyme from the cyanobacterium *Synechocystis*. A single-step HPIAC procedure with cell-free extracts yielded electrophoretically homogeneous glutamine synthetase. In the determination of glutamine synthetase by HPIAC a linear response in the range 10–60 μg of enzyme was observed. Recoveries of 70% of the loaded enzymatic activity and 100% of protein were obtained. The determination of glutamine synthetase protein by HPIAC was compared with that obtained by rocket immunoelectrophoresis. The chromatographic method is proposed as a possible alternative to other immunochemical quantitative techniques, particularly when non-limiting amounts of samples are available.

INTRODUCTION

Affinity chromatography is a well known procedure for the purification of biomolecules and its principles and applications have been reviewed in detail [1,2]. The biospecificity of conventional affinity chromatography (low pressure) has been successfully combined with the fast operation, resolution and sensitive detection of high-performance liquid chromatographic (HPLC) systems [3]. Higher selectivity and specifity can be achieved by using antibodies or antigens as immobilized ligands, which adds the advantages of immunochemical reactions to the separation process. The resulting technique is known as high-performance immunoaffinity chromatography (HPIAC) [4].

Epoxy-activated silica has been widely used as a support in high-performance affinity chromatography (HPAC) owing to its porosity and mechanical rigidity, allowing work at the pressures and flow-rates usual in HPLC. The oxirane groups present in epoxysilica react with nucleophiles such as amino [5], hydroxyl [6], thiol[7] and carboxy [8] in the pH range 3–7.5 to form stable covalent bonds. In general, the coupling to epoxysilica of ligands with such functionalities can be easily carried out, rendering this support advantageous for many applications. Thus, we have recently used this support to immobilize a pyridine nucleotide analogue for one-step purification of $NADP^+$-dependent dehydrogenases from different cell-free extracts [9]. This affinity matrix is also suitable for immobilization of antigens or antibodies for analytical and preparative separations, which permit rapid isolation and high recovery of biologically active molecules.

In this work, HPIAC with linked polyclonal antibodies was used for the determination of glutamine synthetase (GS), an enzyme that catalyses the incorporation of ammonia into amino acids in a wide variety of organisms. In bacteria and cyanobacteria, GS has a molecular weight of 600 000 dalton with twelve identical 50 000-dalton subunits [10,11], while the eukaryotic enzyme has eight subunits of 45 000–48 000 dalton [12,13]. GS has been purified from many organisms by conventional affinity chromatography [13–15] but, as far as we know, no data exist on the use of HPLC or HPIAC for the purification of this enzyme. Based on the results re-

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ported here, we propose HPIAC for the one-step purification of GS, and as an excellent alternative to other non-chromatographic immunoquantitative procedures.

EXPERIMENTAL

Chemicals

Salts for the mobile phases and acetonitrile were purchased from Merck (Darmstadt, Germany). Tris, phenylmethylsulphonyl fluoride (PMSF), EDTA and 3-(N-morpholino)propanesulphonic acid (MOPS) were obtained from Sigma (St. Louis, MO, USA). Purified glutamine synthetase was a gift from Drs. Candau and Florencio (Universidad de Sevilla). Freund's adjuvants were from Difco Labs. All other chemicals used were of the highest purity available.

Equipment

HPIAC was performed using a Beckman liquid chromatographic system equipped with two Model 110B pumps, an Altex 210A injection valve with a 2.0-ml loop, a Model 163 variable-wavelength UV detector and a Model 406 analogue interface module. Integration of chromatograms was performed in a AT-compatible computer with the Beckman System Gold software. An Ultraffinity-TM prepacked epoxy-activated silica analytical column (5 cm × 0.45 cm I.D.) from Beckman was used for coupling anti-GS antibodies. High-purity water was obtained using a Milli-Q system (Millipore).

Cell culture

Synechocystis sp. PCC 6803 cells were grown at 37°C as described previously [16]. The medium was supplemented with 20 m*M* $NaNO_3$ as a nitrogen source. Cells growing at mid-log phase were harvested by centrifugation at 19 000 *g* for 25 min. The cells were washed with 50 m*M* MOPS buffer (pH 7) containing 1 m*M* EDTA and 1 m*M* PMSF (5 ml buffer/g wet cells) and centrifuged at 19 000 *g* for 10 min. Both processes were repeated three times. The pellet was then resuspended with buffer as described above and sonicated for 6 × 30 s at 1-min intervals. Cellular debris was removed by centrifugation at 39 000 *g* for 20 min. The cell-free extract contained 2.6 U GS/mg protein.

Enzyme activity and purification

The transferase activity of GS was determined as described previously [17]. One unit of activity is the amount of enzyme that catalyses the formation of 1 μmol of γ-glutamylhydroxamate per minute. The enzyme from *Synechocystis* was purified by ion-exchange and affinity chromatography on 2′,5′-ADP-Sepharose [15]. The elution from the affinity column was carried out by a pulse of ADP. Protein concentration was determined by the bicinchoninic acid assay [18] using bovine serum albumin as a standard.

Immunization and purification of antibodies

Purified GS was subjected to disc gel electrophoresis and its activity was localized on the gels. Gel sections containing GS were pooled, disrupted and emulsified in 4 ml of a mixture containing equal volumes of phosphate-buffered saline (PBS) and Freund's complete adjuvant and injected subcutaneously into a non-immunized rabbit. Bleeding and further booster injections were performed as reported previously [19]. After centrifugation and inactivation of complement, the immunoglobulin G (IgG)-enriched fraction was purified by chromatography on protein A-Sepharose according to Campbell and Remmler [20].

Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in slab gels according to Laemmli [21]. Acrylamide concentrations were 10% and 4% (w/v) for running and stacking gels, respectively. Rocket immunoelectrophoresis was carried out as described by Weeke [22], in agarose plates containing, per millilitre of gel, 21 μg of protein A-purified IgG-rich fraction from anti-GS serum. Samples of crude extracts (9 μl) were run overnight at 10°C and 10 V/cm. Proteins were stained with Coomasie Blue R250.

Coupling of antibodies to the epoxysilica column

The IgG fractions eluted from the protein A-Sepharose column were pooled and dialysed overnight against 0.5 *M* potassium phosphate buffer (pH 7) (coupling buffer). For the coupling, a solution containing 9.34 mg of protein in 12 ml was

used. The Ultraffinity-EP column was connected to an HPLC pump primed with coupling buffer. The column flow rate was maintained at 1 ml/min until liquid eluted from the column end, then it was changed to 0.2 ml/min and recycled for 36 h at 35°C. Subsequently the column was extensively washed with coupling buffer to remove unbound antibodies. Removal of ionically bound ligand was carried out, under identical conditions, by washing the column with 1 *M* KCl for 1 h at 1 ml/min and then with 20 m*M* KCl to eliminate hydrophobically bound antibodies. The remaining unreacted oxirane groups on the silica support were deactivated by pumping 0.5 *M* glycerol in 1 *M* potassium phosphate buffer (pH 4.5) at 0.2 ml/min for 8 h at 35°C. After washing with equilibrating buffer [50 m*M* MOPS (pH 7.0)–1 m*M* EDTA], the column was ready for use. When not in use, the column was stored at 4°C.

Chromatographic procedure

The silica anti-GS column was equilibrated with a loading buffer containing 50 m*M* MOPS, 250 m*M* KCl and 1 m*M* EDTA (pH 7). Samples (2 ml) were injected at a flow rate of 1 ml/min. After extensive washing with loading buffer, a pulse of 1 *M* KCl in loading buffer (2.0 ml) was applied to elute non-specifically retained compounds. Elution of GS was performed at 0.5 ml/min with a 2-ml pulse of loading buffer supplemented with 10% of acetonitrile and 2.5 *M* $MgCl_2$. GS transferase activity was measured in the 0.5-ml fractions collected. The influence of eluents on the baseline at 280 nm was corrected. The samples were filtered through 0.20-μm membrane hydrophilic filters before injection into the immunoaffinity column. The mobile phases were degassed under vacuum for 30 min in an ultrasonic bath and filtered before use. All chromatographic procedures were performed at room temperature.

RESULTS AND DISCUSSION

The high affinity and specificity of the antigen–antibody interactions turn HPIAC into an efficient analytical technique. The main aim of this work was to use HPIAC for the determination of GS in complex protein mixtures. Polyclonal antibodies used for coupling to epoxy-activated silica were obtained by protein A-Sepharose chromatography of an anti-GS serum. Monospecifity of the purified antiserum, when challenged with crude extracts of *Synechocystis*, was demonstrated by immunoelectrophoretic procedures (not shown).

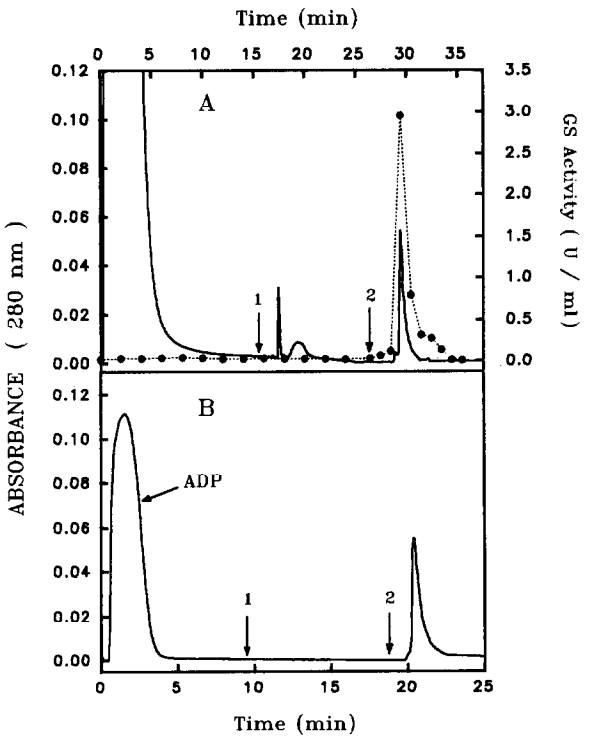

Fig. 1. HPIAC elution pattern of *Synechocystis* glutamine synthetase in (A) cell-free extracts and (B) conventionally purified preparations. (A) A *Synechocystis* crude extract sample (2 ml) containing 2.4 mg of total protein was chromatographied as described under Experimental. (B) A sample (2 ml) of *Synechocystis* glutamine synthetase purified by conventional chromatography, containing 50 μg of protein, was processed as described under Experimental. Solid lines, absorbance at 280 nm; ●, GS transferase activity. Times for different pulses are indicated with arrows: (1) 1 *M* KCl; (2) 10% acetonitrile in 2.5 *M* $MgCl_2$.

The effects of different factors such as ionic strength, type of buffer and pH on the binding of GS from *Synechocystis* to the anti-GS column were studied. Complete binding of up to 60 μg of GS was achieved using 50 m*M* MOPS buffer containing 250 m*M* KCl and 1 m*M* EDTA. Consequently, this solution was selected as the equilibrating buffer in the subsequent chromatography. On the other hand, different GSs antigenically related to the enzyme from *Synechocystis* were not effectively bound by the antibodies coupled to the column. Thus, the en-

zyme from the cyanobacterium *Synechococcus*, which presented high degree of cross-reactivity with our anti-GS IgGs in double immunodiffusion tests, was less than 20% retained by the HPIAC column under our experimental conditions. Negligible binding to the immunoaffinity column was also observed when injecting crude extracts of other cyanobacteria (*Calothrix*) or a photosynthetic bacteria (*Rhodobacter*). This restricted binding capacity corroborates the high specificity of immunochemical interaction. Thus, GSII isoenzyme from *Rhizobium* does not cross-react with polyclonal anti-GSI antibodies, illustrating differential recognition of isoenzymes from the same organism [23].

In most instances, values lower than 10^{-8} are typical for dissociation constants of immuno complexes in free solution [24]. However, the disruption of immobilized complexes can be achieved using different conditions, such as low-pH buffers, high salt concentrations (chaotropics), organic solvents or mixtures (organics plus salts). Proteins have to be treated gently in order to avoid denaturation; therefore, the elution conditions are specific for each case, depending on the particular affinity of antibodies towards the antigen to be purified. A wide range of conditions have been used to elute GS from the HPIAC polyclonal IgG column. Thus, acidic buffers such as acetic acid (pH 2.7) or glycine–HCl (pH 2.2) and organics such as 50% ethylene glycol or 10–30% acetonitrile were not effective for the disruption of the antigen-immobilized antibody complex, while $MgCl_2$ (2.5–3 *M*) showed a partial efficiency for enzyme elution. However, the GS retained in the column was completely eluted with 10% acetonitrile in 2.5 *M* $MgCl_2$, and this eluent was routinely used in the HPIAC experiments.

Fig. 1A shows the elution profile of protein and GS activity after chromatography of a *Synechocystis* crude extract. Traces of unspecifically adsorbed proteins were washed out with a 2-ml pulse of 1 *M* KCl. Elution of GS protein and activity was performed with a 2-ml pulse of 10% acetonitrile–2.5 *M* $MgCl_2$. The chromatographic behaviour of purified *Synechocystis* glutamine synthetase is shown in Fig. 1B: a well defined peak was again observed after the pulse of elution buffer, whereas no protein was detected, in this instance, after washing whith 1 *M* KCl. As the enzyme had been purified by affinity chromatography (2′,5′-ADP-Sepharose) and eluted by ADP, unretained ADP yielded a peak, a marker of the dead volume of the HPLC tubing.

Specificity of the bound antibodies was further corroborated by SDS-PAGE of samples obtained by concentrating the GS activity-containing fractions eluted from the HPIAC column (Fig. 2). Thus, one densely stained band was obtained after electrophoresis of GS purified by HPIAC of *Synechocystis* crude extracts (lane E). The same band was observed with purified GS, either before (lane B) or after (lane C) HPIAC processing. Comparison of lanes D, E and F confirms the power of the HPIAC for one-step purification of *Synechocystis* glutamine synthetase, although this was not the main aim of this work. By comparing with the molecular weight markers, an approximate value of 55 000 dalton can be inferred for the *Synechocystis* GS subunit, which is close to that previously reported [25] for the enzyme monomers of 52 000 dalton.

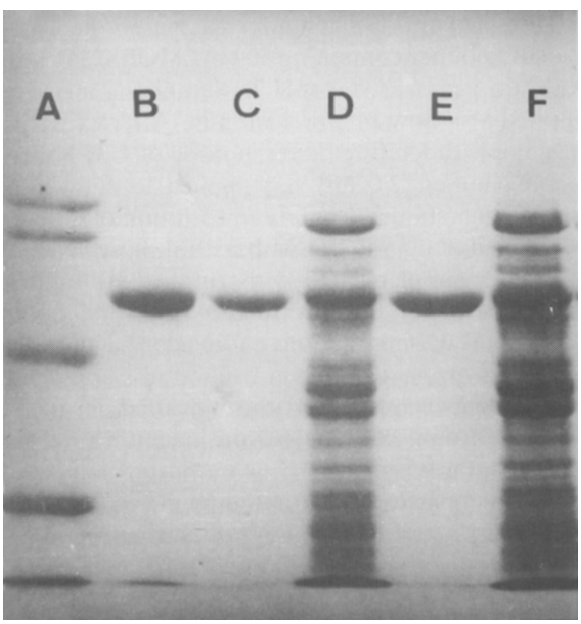

Fig. 2. SDS-PAGE of crude extracts and purified GS from *Synechocystis*. Conditions as indicated under Experimental. (A) 35 μg of a standard mixture containing the markers carbonic anhydrase (30 000), ovoalbumin (45 000), bovine serum albumin (67 000) and ovotransferrin (78 000 dalton); (B) 25 μg of GS purified by conventional affinity chromatography; (C) 15 μg of purified GS further processed by HPIAC; (D) and (F) *Synechocystis* crude extracts (108 and 162 μg of protein, respectively); (E) 22 μg of GS, purified by one-step HPIAC from *Synechochystis* crude extract.

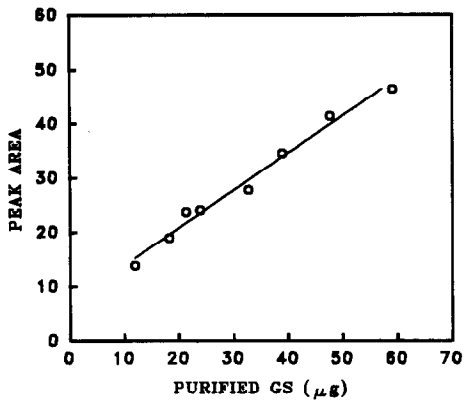

Fig. 3. HPIAC calibration graph for purified glutamine synthetase. Samples (2 ml) containing different amounts of purified GS were chromatographed according to the procedure described under Experimental. GS elution peak areas are expressed in arbitrary units.

An average of 70% of GS activity was recovered in the HPIAC process. This value does not take into account the slight inhibition promoted by the eluent on GS transferase activity. Futher, a linear relationship was obtained when the peak areas at 280 nm of eluted GS were plotted against the amounts of purified protein injected on to the column (Fig. 3). Linearity was also observed on injecting different dilutions of *Synechocystis* crude extracts (not shown), indicating a complete mass recovery of GS protein from the column under the elution conditions used [26,27]. This result opens up the possibility of determining GS in complex protein mixtures by using Fig. 3 as a standard.

Glutamine synthetase in *Synechocystis* cell-free extracts was determined by comparing rocket immunoelectrophoresis with HPIAC. Table I shows the results obtained. The amount of GS protein was determined by rocket immunoelectrophoresis in each cell-free extract sample by comparison with a standard of purified GS subjected to an identical process. A linear regression analysis of the data gave a correlation coefficient of 0.998. Additionally, HPIAC determination of GS protein in crude extracts was performed by measuring the specific peak area at 280 nm and comparing it with the calibration graph shown in Fig. 3. A correlation coefficient of 0.994 was calculated from the linear least-squares analysis of the data and a linear range (10–60 μg of GS) was found. The results obtained by both methods (averages 21.5 ± 0.5 and 24.1 ± 0.4 μg GS/mg total protein) allows HPIAC determination to be proposed as an alternative to non-chromatographic immunochemical quantitative methods. Rocket immunoelectrophoresis and the more sensitive enzyme-linked immunosorbent assay (ELISA) are able to detect minute amounts of antigens, starting from small amounts of protein mixtures, and allow several samples to be run at a time. However, both procedures consist of a series of steps which, in many cases, could result in a time-consuming overall process. Although the HPIAC determination described here requires larger amounts of samples (see Table I), further optimization of the HPIAC technique might shorten the time required, making it advantageous for the rapid, direct determination of antigens, particularly when non-limiting amounts

TABLE I

DETERMINATION OF GLUTAMINE SYNTHETASE IN CRUDE EXTRACTS FROM *SYNECHOCYSTIS*

Rocket immunoelectrophoresis				HPIAC			
Protein (μg)[a]	*h* (cm)	GS (μg)[a]	GS (μg)[b]	Protein (mg)[c]	Peak area[d]	GS (μg)[c]	GS (μg)[b]
4.88	1.7	0.11	24.00	1.20	24.11	25.33	21.25
6.97	2.3	0.17	24.31	1.55	30.04	33.74	21.71
8.13	2.6	0.20	24.39	2.17	38.04	45.10	20.80
9.76	3.1	0.24	24.50	2.35	42.50	52.01	22.13
12.20	3.6	0.28	23.51	2.53	44.70	54.55	21.57

[a] In 9 μl of crude extract.
[b] Per mg total protein.
[c] In 2 ml of crude extract.
[d] Expressed in arbitrary units.

of samples are available or in antigen-rich mixtures. On the other hand, immunoaffinity chromatography has been often used as a prepurification step, followed by determination by other HPLC techniques [28–30]. Additionally, the direct HPAC determination of proteins has been reported [27].

Non-specific binding to affinity chromatographic systems has been prevented by adding detergents such as Tween 20 to mobile phases [31] or by using a precolumn with linked non-immune γ-globulins [32]. In our case, accumulation of non-specifically adsorbed proteins was avoided by routine regeneration of the column with 30% acetonitrile after two or three runs. The column capacity appeared to remain constant after 50 runs.

Although the current trend in HPIAC is to use monoclonal antibodies, the results obtained with the polyclonal anti-GS system seem to be as reliable as those previously reported with monoclonals for other proteins [33]. Moreover, the production of this last type of antibodies is laborious and expensive and in many instances drastic elution conditions are needed [33,34]. The choice of monoclonal or polyclonal antibodies for a given HPIAC system could depend on a balance of the cost of producing the necessary amount of the ligand to be immobilized and the half-life of the column. In conclusion, we propose the use of HPIAC for the determination of different macromolecules in cell-free extracts, particularly when non-limiting amounts of samples are available.

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REFERENCES

1 P. D. G. Dean, W. S. Johnson and F. A. Middle, in D. Rickwood and B. D. Hames (Editors), *Affinity Chromatography: a Practical Approach*, IRL Press, Lancaster, 1985, p. 1.
2 P. Mohr and K. Pommerening, *Affinity Chromatography: Practical and Theoretical Aspects*, Marcel Dekker, New York, 1985, p. 1.
3 S. Ohlson, L. Hansson, P. O. Larsson and K. Mosbach, *FEBS Lett.*, 93 (1978) 5.
4 T. M. Philips, *Adv. Chromatogr.*, 29 (1989) 133.
5 M. Landt, S. C. Boltz and L. G. Butler, *Biochemistry*, 17 (1978) 915.
6 P. Vretblad, *Biochim. Biophys. Acta*, 434 (1976) 169.
7 P. C. Simon and D. C. Van der Jagt, *Anal. Biochem.*, 82 (1977) 334.
8 J. Turkova, K. Blaha, M. Malanikova, D. Vancurova, F. Svec and J. Kalal, *Biochim. Biophys. Acta*, 524 (1978) 162.
9 J. Alhama, J. López-Barea and F. Toribio, *J. Chromatogr.*, 586 (1991) 51.
10 E. R. Stadtman and A. Ginsburg, in P. D. Boyer (Editor), *The Enzymes*, Vol. X, Academic Press, New York, 3rd ed., 1974, p. 755.
11 M. J. A. M. Sampaio, P. Rowell and W. D. P. Stewart, *J. Gen. Microbiol.*, 111 (1979) 181.
12 B. J. Miflin, P. J. Lea and R. M. Wallsgrove, in J. Mora and R. Palacios (Editors), *Glutamine: Metabolism, Enzymology and Regulation*, Academic Press, New York, 1980, p. 213.
13 G. Tholey, S. Bloch, M. Ledig, P. Mandel and F. Wedler, *Neurochem. Res.*, 12 (1987) 1041.
14 S. Wcining and D. J. D. Nicholas, *Biochem. Int.*, 14 (1987) 337.
15 F. J. Florencio and J. L. Ramos, *Biochim. Biophys. Acta*, 838 (1987) 39.
16 M. Herdman, S. F. Delaney and N. G. Carr, *J. Gen. Microbiol.*, 79 (1973) 233.
17 B. M. Shapiro and E. R. Stadtman, *Methods Enzymol.*, 17a (1970) 910.
18 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. M. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.*, 150 (1985) 76.
19 A. López-Ruiz, J. M. Roldán, J. P. Verbelen and J. Diez, *Plant Physiol.*, 78 (1985) 614.
20 W. H. Campbell and J. L. Remmler, *Plant Physiol.*, 80 (1986) 435.
21 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
22 B. Weeke, in N. H. Axelsen, J. Kroll and B. Weeke (Editors), *Quantitative Immunoelectrophoresis, (Scand. J. Immunol.*, Vol. 2, Suppl. 1), Universitetsforlaget, Oslo, 1973, p. 37.
23 M. Rossi, R. Defez, M. Chivrazzi, A. Lamberti, A. Fuggi and M. Iaccarino, *J. Gen. Microbiol.*, 135 (1989) 629.
24 M. W. Steward and J. Steensgaard, *Antibody Affinity: Thermodynamic Aspects and Biological Significance*, CRC Press, Boca Raton, FL. 1983, p. 1.
25 A. Mérida, L. Leurentop, P. Candau and F. J. Florencio, *J. Bacteriol.*, 172 (1990) 4732.
26 A. L. Khurana, E. T. Butts and C. T. Ho, *J. Liq. Chromatogr.*, 12 (1989) 3019.
27 H. W. Jarret, *J. Chromatogr.*, 363 (1986) 456.
28 T. Hayashi, S. Sakamoto, I. Wada and H. Yoshida, *Chromatographia*, 27 (1989) 574.
29 L. S. Van Ginkel, H. Van Blitterswijk, P. W. Zoontjes, D. Van den Bosch and R. W. Stephany, *J. Chromatogr.*, 445 (1988) 385.
30 E. J. Trione, B. B. Krygier, J. M. Kathrein, G. M. Banowetz and L. A. Sayavedra-Soto, *Physiol, Plant.*, 70 (1987) 467.
31 T. Moreaux, M. T. Leidecker and C. Meyer, *Eur. J. Biochem.*, 179 (1989) 617.
32 R. J. Fido, *Plant Sci.*, 50 (1987) 111.
33 R. Sportman and G. S. Wilson, *Anal. Chem.*, 52 (1980) 2013.
34 N. E. Thompson, D. B. Aronson and R. R. Burgess, *J. Biol. Chem.*, 265 (1990) 7069.