ELSEVIER

Journal of Chromatography A, 760 (1997) 81–87

JOURNAL OF CHROMATOGRAPHY A

Review

Large-scale high-performance liquid chromatography of enzymes for food applications

Jamel S. Hamada

U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA 70179, USA

Abstract

Processes for the production of food-grade enzymes must be cost-effective. Fortunately, most enzymes manufactured for food applications are not required to be highly purified. However, enzymes are considered additives and thus the concentration of DNA and other extraneous materials must be within FDA guidelines. Developing efficient, low-cost, large-scale HPLC methods for food applications is becoming more feasible with the introduction of new HPLC tools and technologies. Enzymes can be successfully separated from cell extracts by size-exclusion and ion-exchange HPLC using optimal gradient elution programs for the latter. Large-scale HPLC methods developed for the purification of some significant food enzymes are discussed in this paper.

Keywords: Reviews; Food analysis; Enzymes

Contents

1. Introduction

Many chemical processes that are traditionally carried out by direct chemical means can also be accomplished using enzyme catalysis. In food processing, the use of enzymes provides several advantages over using chemicals including rapid reaction rate, mild conditions and, most importantly, high

PII S0021-9673(96)00647-4

specificity. This will result in reduction of the cost of energy, labor and/or machinery required in the manufacturing process, increased processing efficiency and compliance with regulatory standards. Other attractive features of enzymes are that most enzymes can be produced in large quantities, with each having appropriate physical, chemical and catalytic properties. The cost of enzyme production is reasonable if one uses microbial fermentation or possibly biotechnological techniques [1].

Enzymes catalyze many important reactions in the food industry such as starch hydrolysis to produce maltose syrups and high fructose syrups. Enzymes are used in almost every major aspect of food processing such as baking, brewing, cheesemaking, juice purification, and meat tenderizing, as well as amino acid production and protein hydrolysis. Cheetham [2] presented an excellent review on the application of enzymes in the food industry. Endogenous enzymes play a major role in the conversion of many food constituents and ingredients during food processing. However, some endogenous process are relatively slow to reach the desired rate, and it becomes necessary to add pure exogenous enzymes or enzyme-rich fractions for the effective enzymatic conversions [3]. Accordingly, for these processes exogenous enzymes must be commercially available in concentrated and sufficiently purified form.

Most of the enzymes used in the food industry are carbohydrate hydrolases. These include α-amylase, dextranase, lactase, invertase, glucose isomerase and juice refining enzymes. The industrial use of some of these enzymes is growing dramatically. For instance, the use of lactase (β-galactosidase) to hydrolyze lactose has increased tremendously in the last few years both in food processing and in medicine to alleviate lactose intolerance. Lactose can be hydrolyzed by acid, but color is easily generated and the ion-exchange resins used for downstream processing are fouled. Many of these problems can be eliminated by the use of immobilized β-galactosidase [2].

Because of the relatively low value of food ingredients, which are produced in high bulk, compared to pharmaceutical materials for instance, processing enzymes must be cheap relative to total costs. Further, amounts of DNA and other extraneous materials must be within the ranges specified by the US Food and Drug Administration (FDA). Enzymes have been universally regarded as being safe, but their regulation for food application varies among countries. In the UK, there are no specific controls on the use of enzymes in food production, in which they are classed as food processing aids [2]. In the United States, enzymes are considered as additives and accordingly their use in foods and their processing is regulated by FDA. Therefore, a need exists for preparative methods that yield large quantities of nucleic-acid free enzymes from cell extracts. Most enzymes manufactured for industrial use are required to be stable but do not need to be highly purified. Since these enzymes need to be economically prepared at a food-grade level, high-performance liquid chromatography (HPLC) and other relatively expensive techniques must be avoided unless their use is justified. However, when very pure enzymes are needed, particularly for enzyme immobilization and prolonged use, HPLC can be a useful tool.

In this review, we investigate the use of HPLC in large-scale preparation of significant food enzymes and highlight the successful isolation of some microbial and plant enzymes for industrial applications.

2. Efficient large-scale HPLC methodolgy

Developing efficient, low-cost, large-scale HPLC methods for food applications has become more feasible with the introduction of new HPLC materials and techniques. Preparative HPLC provides high recovery and reduced separation time and elution volumes. The superior performance of HPLC columns is derived from the small size, rigid particles of the derivatized macroporous supports leading to high pressure and usable flow-rates [4]. The success of preparative liquid chromatography relies on the intrinsic characteristics of the packing materials [5]. The introduction of many new and improved sorbents that are specific for protein separation on a preparative scale permits an optimized selection for a particular separation purpose [5]. Speed, practicality, stability and yield are among the main factors that should be considered when one uses preparative chromatography. In research, some of the advantages of preparative HPLC are purity, maximum quantity and speed. However, among the critical issues in process-scale liquid chromatography are economics,

process engineering and scaling-up to purify hundreds or thousands of kilograms of product per day [6]. The industrial HPLC separation of significant food enzymes is often unreported because of proprietary considerations. Most new enzymatic procedures addressed here for large-scale HPLC are limited to basic research and published articles on methods developed for process-scale chromatography. The extent to which any large-scale HPLC method for food enzymes can be used by industry will depend on demonstrated practicality and economic viability.

3. Isolation and purification of food enzymes

3.1. Carbohydrate hydrolases

The need for purification of enzymes, particularly those which are derived from microorganisms, for food applications is growing tremendously. Numerous publications are reported every year on the purification, characterization and sometimes the structural analysis of various enzymes. In 1995, many investigators reported their work for chromatographic separations of significant enzymes, particularly those from microbial sources. For instance, the purification of α-amylase [7,8], NADPH-dependent D-xylose reductases [9], acidic chitinase [10], endo-1,4-beta-D-glucanase [11,12] and protease-resistant cellulase [13] was reported last year alone.

Annous and Blaschlk [7] reported the isolation and characterization of α-amylase derived from *Clostridium acetobutylicum* ATCC 824 in a starch media. The extracellular enzyme was purified to homogeneity from the bacterial culture supernatant using ammonium sulfate fractionation, anion-exchange chromatography and size-exclusion HPLC. Abdel-Naby et al. [14] purified the extracellular chitinase from *Aspergillus carneus* by combination of ammonium sulfate precipitation, size-exclusion chromatography through Sephadex G-100 and preparative ion-exchange HPLC. However, preparative ion-exchange HPLC alone was effective in the isolation of chitinase isoenzymes. Araki et al. [10] reported the use of quaternary ammonium ion detergent for the separation of yam chitinase from viscous tissue extract. For the purification of cellulases from *Streptomyces* strain A20C, Curioni et al. [15] used size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, preparative isoelectric focusing, hydrophobic chromatography and separation on hydroxylapatite. They reported that five microbial cellulolytic enzymes were separated in one step using electroendosmotic preparative electrophoresis. However, the recovery of enzyme activity was poor ranging from 32 to 47%.

3.2. Proteases

Proteases hydrolyze peptide bonds in proteins to modify their structure. The greatest commercial use of proteases is in the laundry detergent industry to help remove protein-based stains such as blood and egg from clothing. The early use of proteases in the food industry has been in the preparation of whipping formulations and milk replacers. Food proteins are hydrolyzed to improve dispersibility and nutritional availability without significantly affecting flavor, color and nutritional value. Despite the continued introduction of new proteases for food applications, very little appeared in the literature regarding their separation and downstream processing. The new proteases possess special characters including hyperactivity at certain pH values and their cleavage potential at certain sites such as at the hydrophobic or neutral residues. Bedi [16] partially purified four gelatin cleaving proteases from culture media of *Porphyromonas gingivalis* by ion-exchange, size-exclusion and chromatofocusing chromatography. In addition to their cleavage specificity, these four proteases differed also in their subunits' molecular weights, charge characteristics and inhibition profiles.

3.3. Transglutaminases

Transglutaminase (TGase) catalyzes the incorporation of primary amines into proteins and polypeptides. Folk and Finlayson [17] and more recently Lorand and Conrad [18] discussed the various aspects of TGases and focused on their biological roles.This enzyme is used primarily for cross-linking food proteins and for the covalent attachment of essential amino acids to nutritionally inferior proteins. Hamada [19] reviewed the potential uses of

TGases in food processing. Nio et al. [20,21] used DEAE-cellulose column chromatography and sequential ammonium sulfate precipitation to purify guinea pig liver TGase 60-fold with 45% recovery. Researchers from the same laboratory [22–24] reported the production of TGase from the microbial source *Streptoverticillium* sp. The isolated enzyme is of great interest to the food processor since the enzyme can be produced in larger quantity for industry by large-scale fermentation methods. The culture filtrate of *Streptoverticillium* strain was purified by ultrafiltration, Amberlite CG-50 (twice) and blue sepharose. This increased the specific activity of the enzyme from 0.13 to 22.6 units with 42% yield.

4. Practical aspects of large-scale HPLC separation

4.1. Supplemental pre-HPLC procedures

The use of pre-HPLC purification methods such as gel adsorption, centrifugation, ultrafiltration and fractional precipitation should be encouraged since they have been shown necessary for enhancement of the HPLC separation in so many cases. Baetselier et al. [25] described the scale-up, purification and characterization of a recombinant *Aspergillus niger* glucose oxidase (GO). GO has a number of industrial applications, such as removal of O_2 from beverages, removal of glucose from powdered eggs, as an H_2O_2 source in food preservation and in sensors for glucose determination. Recombinant GO from *Saccharomyces cerevisiae* was secreted as a hyperglycosylated product with a higher molecular weight than that extracted from *A. niger*. The authors took advantage of the molecular weight in their purification scheme using only cross-flow filtration that recovered more than 80% of product with greater than 95% purity. GO was free of detectable levels of major impurities such as catalase, amylase and cellulase found in commercial GO preparations. On the other hand, some enzymes require extensive purification procedures due to the close similarity in the structure of their component fractions. Two examples of this kind of difficulty are cited for a cereal Bowman-Birk type trypsin inhibitor from seeds of Jobs' tears (*Coix lachryma-jobi L.*) [26] and *Bacillus circulans* peptidoglutaminase (PGase) [27]. Large-scale purification methods such as precipitation, gel adsorption and ultrafiltration were ineffective in increasing PGase specific activity by more than 2-fold. Therefore, to obtain a large quantity of high purity PGase from *B. circulans* cell extract in high yields, that is void of all nucleic acids and other contaminants, preparative chromatography must be used.

4.2. Polyethylene glycol (PEG) precipitation as a supplemental tool in ion-exchange HPLC separation

Polyethylene glycol (PEG) was used for the precipitation of acid phosphatases (APases) from the crude extracts of soybean [3] and *Aspergillus ficuum* [28] before their HPLC separation, according to the method of Miekka and Ingham [29] and Ingham [31] after modification(see also [30]). After PEG fractionation, each APase of *A. ficuum* [28] or soybean [3] was separated by anion-exchange HPLC (Fig. 1). Use of PEG reduced separation time in half, allowed ten-fold increase in sample load and resulted in increased recovery and many fold-purification [28]. The use of PEG prior to ion-exchange chromatography seems more attractive than either recycle or peak shaving since it ends overlapping between APases when cell extracts are separated only by ion-exchange HPLC. Prior PEG fractionation improves resolution by maximizing the separation factor (α) between the APase and other protein components [28]. Maximizing α between components is the single most significant step that can be taken to optimize a preparative liquid chromatography separation [32].

4.3. Special futures of ion-exchange HPLC separation

Electrophoresis and ion-exchange HPLC are the techniques usually used for the successful fractionation of complex mixtures of closely related proteins or enzymes called isoenzymes. Ion-exchange HPLC, however, has larger resolving power since it depends

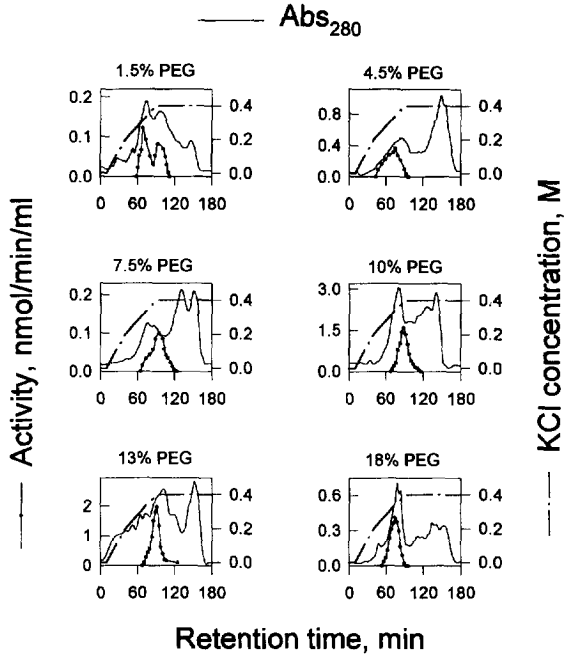

Fig. 1. Anion-exchange separation of APases of PEG precipitates obtained from soybean cell extract. Salt gradient was from 0 to 80% 0.5 *M* potassium chloride in 0.1 *M* Tris-HCl buffer, pH 8.0 (—.—) and protein detection was at 280 nm (———). Activity of enzyme fractions (—○—) was expressed as phosphorus released from nitrophenol phosphate [from [3] with permission].

on both the charge density and the distribution of charges on the protein surfaces, i.e., charge anisotropy [33]. PGases was separated directly from *B. circulans* extract (20–30 mg) in one peak with an 8-fold purification on a 43-ml anion-exchange column at 2 cm/min in 35–40 min [27]. More than 65% of the cell extract proteins were eluted after the PGase peak. The last peak of the chromatogram contained all the nucleic acids of the injected cell extract and thus can be easily separated from the PGase peak. This separation method is the most suitable for the scale-up process since it appears to meet the requirements of purity, yield, speed, and other economic aspects for successful production of PGase for potential modification of food proteins in industrial reactors. Scale-up of ion-exchange processes is usually achieved by increasing the column diameter while maintaining the column bed height and linear flow-rate constant [32,34,35].

4.4. Practical aspect of size-exclusion separation

Size-exclusion chromatography separates molecules on the basis of their molecular size. The use of this technique has been shown to be very effective in the isolation of many enzymes. The separation of 2 mg of the proteins of *Bacillus circulans* on a preparative gel column [27] is presented in Fig. 2A. Size-exclusion HPLC gave 9 peaks, of which the fourth peak had PGase activity with 90% yield and a 250-fold increase of the specific activity. Runs of 20–30 mg loads (Fig. 2B) gave only 6 overlapping peaks. Although the yield was not dramatically affected compared to the yield of the runs of lower loads, the separation efficiency was inferior: The specific activity of the PGase peak increased only 60 to 100-fold and this peak contained about 20–35% of the nucleic acids (Fig. 2B). The % area and % protein for the peaks of each chromatogram are independent of flow-rate and protein load. However, protein load and flow-rate significantly affectes V_e [36]. Vlaanderen et al. [37] investigated the effect of

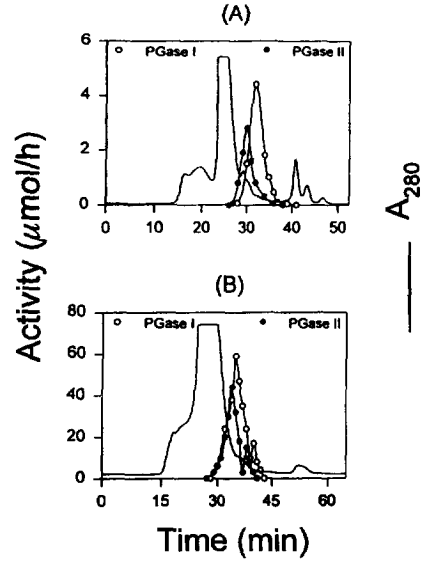

Fig. 2. Size-exclusion of *B. circulans* proteins on acrylate gel using 2 mg (A) and 20 mg (B) of cell extract proteins. Activities of peptidoglutaminase (PGase) I (—○—) and II (—●—) were evaluated by determining the ammonia released from CBZ-L-glutamine and t-BOC-L-glutaminyl-L-prolamine, respectively [from [27] with permission].

protein load and flow-rate on the HPLC fractionation of crystallins on Superose-6 having a fractionation range of 1.0×10^4 to 4×10^6. The authors did not observe any effects of flow-rates ranging from 0.15 to 0.5 ml/min at protein load of 4.4 mg. However, they found that lowering protein load of injected sample decreased the V_e value and subsequently the determination of M_r. Decreasing protein injection from 175 to 1.75 mg/ml shifted the M_r of one of the peaks from 220 000 to 130 000.

5. Reversed-phase and affinity HPLC

Reversed-phase chromatography separates on the basis of hydrophobicity. Because of resolution, reversed-phase HPLC is an excellent and widely used technique for the analysis and laboratory purification of biomolecules, particularly peptides and proteins. Reversed-phase HPLC is able to separate polypeptides of nearly identical sequences, not only for small peptides, but even for much larger proteins [38–40]. Although reversed-phase HPLC can purify proteins and peptides that other chromatographic methods are not capable of [41], its usefulness in enzyme purification is limited. This because of its tendency to disrupt tertiary structure of proteins leading to some loss of biological activity [42,43]. Disruption of protein tertiary structure is caused by the hydrophobic solvents used for elution and/or the interaction of the proteins with the hydrophobic surface of the packing material [42,43].

Affinity chromatography is also considered to be the most specific and efficient separation procedure for the laboratory-scale separation of proteins because of the absolute dependence of affinity interaction on biological recognition rather than on physicochemical properties [44]. However, its use as a preparative tool for protein and other macromolecules has been limited because of high cost and the instability of affinity ligands. Unfortunately, these limitations can neither be ignored nor overcome in large-scale purification of enzymes for food use.

6. Comparison of preparative HPLC methods

Ion-exchange chromatography is widely used for downstream processing of useful proteins [35] but size-exclusion fractionation of proteins is also a desirable technique that can be scaled up to process level. The relatively high purity and the excellent resolution achieved with size-exclusion are sometimes offset by overlapping with parts of the nucleic acids and its lack of speed. Because nucleic acids are heterogenous and contain molecules with widely different sizes, they can be eluted into more than one peak on size-exclusion with late elution of the peak containing nucleic acids with low-molecular-masses [45].

In recent years, ion-exchange HPLC has been widely accepted as an effective preparative technique for the separation of complex mixtures of proteins [33]. Ion-exchange HPLC methods are preferred to the corresponding reversed-phase HPLC methods because the latter may lead to enzyme inactivation as well as deterioration of performance with large sample loads, and poor scaling properties and recovery, especially with very hydrophobic proteins [46]. New HPLC column technology has led to the development of rigid inorganic supports that withstand high pressure. Ion-exchange columns provide highly reproducible, selective and rapid separations [5,47]. Due to the high negative charges in DNA phosphate groups, anion-exchange separation can be the most effective technique for the removal of DNA contaminant from protein preparations [27,45,48]. Therefore, a purification protocol utilizing ion-exchange HPLC will impact the economy of large-scale techniques since expensive chromatographic separations, and chemical or enzymatic treatments for the removal of nucleic acids from cell extracts are not needed.

References

[1] J.S. Hamada, Crit. Rev. Food Sci. Nutr., 34 (1994) 283.
[2] P.S. Cheetham, in A. Wiseman (Editor), Handbook of Enzyme Biotechnology, Ellis Horwood Limited, Chichester, West Sussex, England, 1986, p. 274.
[3] J.S. Hamada, J. Am. Oil Chem. Soc., 73 (1996) 1143.
[4] M.D. Scawen and J. Melling, in A. Wiseman (Editor), Handbook of Enzyme Biotechnology, Ellis Horwood Limited, Chichester, West Sussex, England, 1986, p. 15.
[5] E. Boschetti, J. Chromatogr. A, 658 (1994) 207.
[6] M.D. Weiss, Ind. Chemist, August issue (1987) 16.

[7] B.S. Annous and H.P. Blaschk, J. Ind. Microbiol., 13 (1994) 10.
[8] U.K. Tae, G.u. Bu, Y.J. Jae, M.B. Si, C.S. Yong, App. Environ. Microbiol., 61 (1995) 3105.
[9] S. Yokoyama, T. Suzuki, K. Kawai, H. Horitsu and K. Takamizawa, J. Fermen. Bioeng., 79 (1995) 217.
[10] T. Araki, M. Kuramoto and T. Torikata, Biosci. Biotechnol. Biochem., 59 (1995) 430.
[11] T.M. Alconada and M.J. Martinez, FEMS Microbiol. Letts., 118 (1994) 305.
[12] P. Christakopoulos, D. Kekos, B.J. Macris, M. Claeyssens and M.K. Bhat, J. Biotechnol., 39 (1995) 85.
[13] S. Akiba, Y. Kimura, K. Yamamoto and H. Kumagai, J. of Ferm. and Bioeng., 79 (1995) 125.
[14] M.A. Abdel Naby, N.M. El Shayeb and A.A. Sherief, Applied Biochem. Biotechnol., 37 (1992) 141.
[15] A. Curioni, A. Dal Belin Peruffo and M.P. Nuti, Electrophoresis, 9 (1988) 327.
[16] G.S. Bedi, Preparative Biochem., 25 (1995) 133.
[17] J.E. Folk and J.S. Finlayson, in C.B. Anfinsen, J.T Edsall and F.M Richards (Editors), Advances in Protein Chem, Vol. 31, Academic Press, New York, 1977, p. 1.
[18] L. Lorand and S. Conrad, Mol. and Cell. Biochem., 58 (1984) 9.
[19] J.S. Hamada, in B.J.F. Hudson (Editor), Biochemistry of Food Proteins, Elsevier Applied Science, Elsevier Science Publishers, London, 1992, p. 249.
[20] N. Nio, M. Motoki and K. Takinami, Agric. Biol. Chem., 50 (1986) 851.
[21] N. Nio, M. Motoki, and K. Takinami, Agric. Biol. Chem., 50 (1986) 1409.
[22] H. Ando, M. Adachi, K. Umeda, A. Matsura, M. Nonaka, H. Uchio, H. Tanaka and M. Motoki, Agric. Biol. Chem., 53 (1989) 2613.
[23] M. Nonaka, H. Tanaka, A. Okiyama, M. Motoki, H. Ando, K. Umeda and A. Matsura, Agric. Biol. Chem., 53 (1989) 2619.
[24] H. Tanaka, M. Nonaka and M. Motoki, Nippon Suisan Gakkaishi., 56 (1990) 1341.
[25] A. de. Baetselier, A. Vasavada, P. Dohet, Thi. V. Aha, M. de. Beukelaer, T. Erpicum, L. de. Clerck, J. Hanotier and S. Rosenberg, Biotechnol., 9 (1991) 559.
[26] M.B. Ary, P.R. Shewry and M. Richardson, FEBS Letts., 229 (1988) 111.
[27] J.S. Hamada, J. Chromatogr. A, 702 (1995) 163.
[28] J.S. Hamada, J. Chromatogr. A, 658 (1994) 371.
[29] S.I. Miekka and K.C. Ingham, Arch. Biochem. Biophys., 191 (1978) 525.
[30] A. Veide, C. Hassinen, D. Hallen, M. Eiteman, B. Lassen and K. Holmberg, in R.D Rogers and M.A. Eiteman (Editors), Proceedings of the American Chemical Society Symposium on Aquous Biophasic Separation, Plenum Publishers, New York, 1995, p. 133.
[31] K.C. Ingham, Meth. Enzymol., 182 (1990) 301.
[32] P.D. McDonald and B.A. Bidlingmeyer, in B.A. Bidlingmeyer (Editor), Preparative liquid Chromatography, J. Chromatography Library, Vol. 38, Elsevier Science Publishers B.V., Amsterdam, The Netherlands, 1987, p. 1.
[33] E. Karlsson and L. Ryden, in J.C. Janson and L. Ryden, (Editors), Protein Purification: Principles, High Resolution Methods and Applications, VCH Publishers, New York, 1989, p. 107.
[34] N.J. Little, R.I. Cotter, J.A. Prendergast and P.D. McDonald, J. Chromatogr., 126 (1976) 439.
[35] P.R. Levison, in G. Ganttsos and P.E. Barker (Editors), Preparative and Production Scale Chromatography, Marcel Dekker, New York, 1993, p. 617.
[36] J.S. Hamada, J. Chromatogr. A, (1996) (in press).
[37] I. Vlaanderen, R. Van Grondelle and M. Bloemendal, J. Liquid Chromatogr., 16 (1993) 367.
[38] J. Rivers, J. Chromatogr., 268 (1983) 112.
[39] M. Kunitani and D. Johnson, J. Chromatogr., 268 (1986) 313.
[40] Y.C. Liu, L.H. Sowdal and N.C. Robinson, Arch. Biochem. Biophys., 342 (1995) 135.
[41] N.C. Robinson, M.D. Dale and L.H. Talbert, Arch. Biochem. Biophys., 281 (1990) 239.
[42] K. Titani, T. Sasagawa, K. Resing and K.A. Walsh, Anal. Biochem., 123 (1982) 408.
[43] E.P. Kroeff, R.A. Owens, E.L. Campbell, R.D. Johnson, and H.I. Marks, J. Chromatogr., 461 (1989) 45.
[44] S.R. Narayanan, J. Chromatogr. A, 658 (1994) 237.
[45] P. Ng and G. Mitra, J. Chromatogr. A, 658 (1994) 459.
[46] M. Capel, D. Datta, R. Nierras and G.R. Cravent, Anal. Biochem., 158 (1986) 179.
[47] P.J. Flamion and J.P. Schreiber, Anal. Biochem., 147 (1985) 458.
[48] W. Jiscot, J.C. Van Hertrooij, J.W. Gebbinck, T.V. Velden-de Groot, D.J. Crommelin and E.C. Beuvery, J. Immunol. Methods, 124 (1989) 143.