Journal of Chromatography, 577 (1992) 267–273
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6342

Use of biospecific interactions of collagen, fibronectin and their fragments in affinity chromatography☆

B. A. Klyashchitsky*, V. Kh. Mitina, N. A. Frantsuzova, Yu. M. Krasnopolsky and V. I. Shvets

Institute of Biological and Medical Chemistry, Russian Academy of Medical Sciences, Moscow (Russia)

(First received September 24th, 1991; revised manuscript received February 24th, 1992)

ABSTRACT

Various aspects of the application of fibronectin–collagen biospecific interactions in affinity chromatography are described. A new biospecific method for one-stage isolation of collagen peptides containing fibronectin-binding sites is proposed. The α_1CB_7-peptide of type-I collagen cyanogen bromide cleavage was isolated by means of affinity chromatography on adsorbents containing an immobilized gelatin-binding domain (45 000 relative molecular mass) of fibronectin. The method gives highly purified preparations of α_1CB_7-peptide. This peptide, as well as some other collagen molecular fragments (α-chains, β-components, α_1CB_8-peptide), were immobilized on Sepharose, and the properties of such affinity adsorbents obtained were studied. Adsorbents with immobilized α-chains and α_1CB_7-peptide had a fibronectin-binding capacity 1.5–2.0 times higher than commercial gelatin-Sepharose. Large-scale production of highly purified fibronectin from human plasma, using affinity chromatography on immobilized individual α-chains of collagen, was developed.

INTRODUCTION

Biospecific interactions between fibronectin (Fn) and collagen (Col) play an important role in vital processes [1]. This interaction has been widely exploited to assist the isolation of Fn from various sources by affinity chromatography (AC) on immobilized denatured Col (gelatin) [2]. Chromatography on gelatin-Sepharose (**1**) suffers from various shortcomings: (a) the necessity to carry out additional purification steps to obtain an electrophoretically homogeneous Fn; (b) insufficient stability of affinity adsorbents based on Agarose matrix; and (c) the relatively small capacity of the sorbent.

A reversed version of AC, based on biointeractions of Col and Fn, is also known [3]. It consists of the isolation of different types of Col by chromatography on immobilized gelatin-binding domains of Fn. So, the biospecific system "Fn-Col" is a versatile and convenient model for the study of various aspects of the AC method.

This paper describes the preparation, properties and application of affinity adsorbent with immobilized polypeptide fragments of the Col molecule: α_1-chain-Sepharose (**2**), α_2-chain-Sepharose (**3**), β_{11}-component-Sepharose (**4**), β_{12}-component-Sepharose (**5**), α_1CB_7-Sepharose (**6**), and α_1CB_8-Sepharose (**7**). A biospecific method for the one-step isolation of Col peptides containing the Fn-binding site, by AC on the immobilized gelatin-binding 45 000 relative molecular mass (M_r) of Fn (D45), is also reported.

☆ Presented at the *8th International Symposium on Affinity Chromatography and Biological Recognition, Yokohama, September 24–28th, 1991.*

0378-4347/92/$05.00

EXPERIMENTAL

Sepharose 4B, Sepharose CL-4B and Sephadex G-25 (Pharmacia-LKB, Uppsala, Sweden), type I rat-skin Col, phenylmethylsulphonyl fluoride (PMSF), cyanogen bromide (CNBr), sodium dodecylsulphate, Coomassie Brilliant Blue G-250 and cellulose CM-52 (Servacel) (Serva, Heidelberg, Germany), gelatin of type I (Sigma, St. Louis, MO, USA), α-chymotrypsin and soybean trypsin inhibitor (Reanal, Budapest, Hungary), and sodium cyanoborohydride (Fluka, Buchs, Switzerland) were used. CNBr peptides of Col (α_1CB_6, α_1CB_7 and α_1CB_8) were prepared as described previously [4]. To quantify Fn in fractions, the absorbance was read at 280 nm in each fraction and the concentration of Fn was determined using an absorbance value of 12.8 for 10 mg/ml [5].

Fraction analysis during chromatography was carried out at wavelengths of 230 nm (for collagen and its fragments) and 280 nm (for Fn).

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulphate (SDS) [6] was performed on gels of 5% (for Fn) and 10% (for polypeptide fragments of Col) acrylamide.

For AC of Fn and CNBr peptides of Col, 0.05 *M* Tris–HCl buffer containing 0.15 *M* NaCl, 0.5 m*M* PMSF and 0.02% sodium azide (pH 7.4) (buffer A) was used.

Type I Col was isolated from rat skin by the method described previously [7]. Col and its fragments were quantified on the basis of the hydroxyproline content in samples [8]. Collagen α-chains and β-components were separated as described previously [9]. The yields were as follows: α_1-chain, 18.92 mg; α_2-chain, 28.8 mg; β_{11}-component, 16.16 mg; β_{12}-component, 33.9 mg; the mixture of α_1-chain and β-component (uncompletely resolved), 14.5 mg. The recovery of total protein was 80.2%.

The D45 gelatin-binding domain of Fn was isolated by the partial proteolysis of Fn with α-chymotrypsin [3].

The affinity sorbents **1–7** were prepared by the standard CNBr method. Some properties of these sorbents are given in Table I. To prepare large amounts of the sorbents, the method of Sepharose activation with cyano-group transfer [10] was used.

D45-Sepharose CL-4B (**8**) was prepared by the same method (2.5 g of CNBr per 10 ml of packed gel). The D45 domain was immobilized with 20 mg of protein per 10 ml of activated gel.

DL-Malic acid dihydrazide

Hydrazine hydrate (500 ml, 10.3 mol) was gradually added to a solution of 100 ml (0.758 mol) DL-malic acid dimethyl ester in 100 ml of methanol at 4°C under stirring. The mixture was stirred for 16 h at 20°C. Then the residue was separated, washed with cold methanol, and dried over P_2O_5 *in vacuo*. The yield of DL-malic acid dihydrazide was 110.6 g (90%), m.p. 176–177°C.

TABLE I

COMPARATIVE PROPERTIES OF THE SORBENTS CONTAINING THE IMMOBILIZED FRAGMENTS OF COLLAGEN

Sorbent No.	Ligand	Content of ligand (mg/ml of gel)	Fn-binding capacity		
			mg/ml of gel	mg/mg of ligand	mol/mol of ligand
1	Gelatin	0.59	0.77	1.3	–
2	α_1-Chain	0.61	1.7	2.8	0.64
3	α_2-Chain	1.45	2.58	1.78	0.4
4	β_{11}-Fragment	1.68	2.16	1.29	0.59
5	β_{12}-Fragment	1.37	1.5	1.1	0.5
6	α_1CB_7	0.61	1.5	2.46	0.14
7	α_1CB_8	0.86	0.12	0.14	0.0075

D45-hydrazidomaloyl-Sepharose CL-4B (9) (Fig. 1)

A mixture of 10 mg of D45 in 15 ml of 0.2 *M* sodium phosphate (pH 6.0) and 1.5 mg $NaIO_4$ was stirred for 1 h at 20°C in the dark. The solution was applied to a 50 cm × 1.8 cm I.D. column packed with Sephadex G-25, equilibrated with the same buffer; the flow-rate was 10 ml/h. The void volume fraction (22 ml) containing the oxidized domain was stirred for 3 h at 20°C with 10 ml of hydrazidomaloyl-Sepharose (**10**), which had been prepared by coupling DL-malic acid hydrazide with CNBr-activated Sepharose CL-4B, and prior to chromatography was washed with 100 ml of 0.2 *M* sodium phosphate (pH 6.0). Then, sodium cyanoborohydride (10 mg) was added and the suspension was stirred for 16 h at 4°C. The gel was filtered off, washed with 200 ml of the above buffer and incubated with 10 ml of 1 *M* ethanolamine in the presence of 10 mg of sodium cyanoborohydride for 2 h at 20°C and pH 6.0. The gel was washed with 200 ml of 0.2 *M* sodium phosphate buffer containing 0.5 *M* NaCl (pH 6.0), and then with 50 ml of 0.1 *M* sodium acetate containing 1 *M* NaCl (pH 4.0) and 50 ml of 0.1 *M* sodium borate containing 1 *M* NaCl (pH 9.0) (the last two washings were repeated three times). Sorbent **9** thus prepared was washed with water and stored as an aqueous suspension in the presence of 0.02% sodium azide.

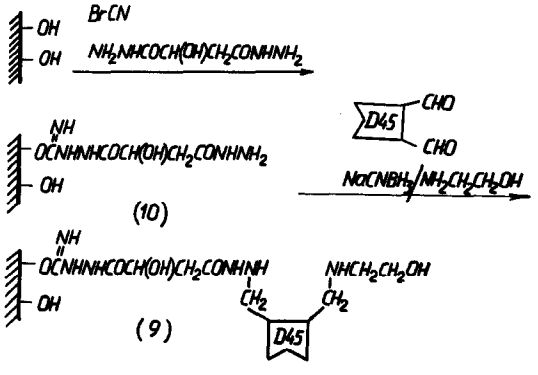

Fig. 1. Synthesis of D45-hydrazidomaloyl-Sepharose CL-4B (**9**).

*Affinity chromatography of the α_1CB_7-peptide on sorbents **8** and **9** (determination of the capacity of the sorbents)*

A solution of 5 mg of α_1CB_7 in 5 ml of buffer A (here and below without PMSF) was passed three times through a 4 cm × 0.8 cm I.D. column packed with 1 ml of sorbent **8** or **9**, equilibrated with the same buffer; the flow-rate was 20 ml/h. Then the column was washed with buffer A (15 ml) until absorption in the eluates at 230 nm disappeared. The α_1CB_7 was eluted with 6 ml of buffer A containing 4 *M* urea (Fig. 2a). The volume of the fractions was 0.5 ml. The α_1CB_7-containing fraction was dialysed against 0.1 *M* acetic acid and lyophilized. The yield of α_1CB_7 was 0.29 mg for sorbent **8** and 0.4 mg of sorbent **9**.

Affinity chromatography of α_1CB_6 and α_1CB_8 on sorbents **8** and **9** was carried out analogously.

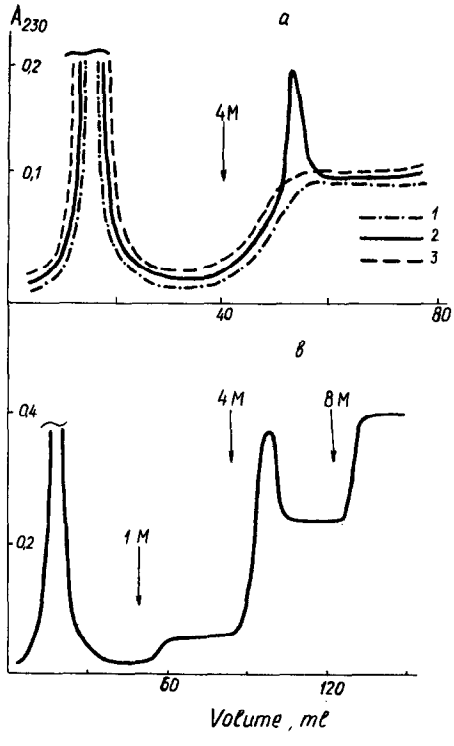

Fig. 2. Chromatography of (a) individual α_1CB_6 (1), α_1CB_7 (2) and α_1CB_8 (3), and (b) a mixture of the CNBr peptides of the α_1-chain on D45-Sepharose CL-4B (**8**).

Affinity chromatography of a mixture of the CNBr peptides of the α_1-chain on sorbent ***8***

A lyophilized mixture of the CNBr peptides (30 mg), obtained as described in ref. 4, was dissolved in 10 ml of buffer A (here and below without PMSF). The solution was passed three times through a column packed with 10 ml of sorbent **8** for 1 h; the flow-rate was 30 ml/h. Circulation of the solution and subsequent chromatography were carried out at 37°C. The column was washed with 100 ml of buffer A and 50 ml of buffer A containing 1 *M* urea, and the α_1CB_7-peptide was eluted with 25 ml of buffer A containing 4 *M* urea. The yield of α_1CB_7 was 2.94 mg. The peptide obtained was identical with the α_1CB_7-peptide prepared by ion-exchange chromatography of a mixture of the CNBr peptides of the $\alpha_1$chain [4].

Chromatography of the CNBr peptides of the α_1-chain on sorbent **9** was conducted under analogous conditions.

Affinity chromatography of Fn on α_1CB_7-Sepharose ***6***

A mixture of 30 ml of a human plasma solution (lyophilized plasma from 60 ml of blood was dissolved in 30 ml of buffer A) and 5 ml of sorbent **6** was incubated for 1 h at 20°C. Then the sorbent was packed into a 3 cm × 1.2 cm I.D. column, which was washed with 20 ml of buffer A at 20°C; the flow-rate was 30 ml/h. The sorbent was washed with 15 ml of buffer A containing 1 *M* urea, and Fn (7.5 mg) was eluted with buffer A containing 4 *M* urea (7–10 ml). Finally, the column was washed with buffer A containing 8 *M* urea (10 ml).

Chromatography of Fn on other sorbents (**1–5** and **7**) was carried out under analogous conditions.

RESULTS AND DISCUSSION

To obtain effective new affinity sorbents for the isolation of Fn, we developed a method based on fragmentation of a high-molecular-mass ligand and using the fragment bearing the site responsible for binding with biopolymer to be purified.

It is known that the Fn binding site is localized both in the separated α_1-chains of type I Col and in its CNBr peptides (α_1CB_7 and α_2CB_5). Quantitative aspects of the interaction between Fn and Col molecular fragments have been studied insufficiently. Only recently the dissociation constant of the complex Fn–α_1-chain ($1.3 \cdot 10^{-8}$ *M*) has been determined [11]. It was also shown that Fn binds equally strongly to α_1CB_7-peptide and α_1-chains.

To screen sorbents for optimal AC of Fn, new sorbents (**2–7**), with the immobilized type I Col fragments containing the Fn-binding site, were synthesized. The sorbents were prepared by the CNBr method. The study of the properties of such sorbents is also of interest for investigation of the mechanism of the interaction of Col with Fn and other biopolymers with affinity for Col, *e.g.* with collagenases. In this connection, the sorbents prepared are more versatile than gelatin-Sepharose (**1**) because, in contrast to the latter, they can find application both in the isolation of Fn and in the purification of animal collagenase [12].

Previously denatured rat-skin type I Col was separated to α-chains and β-components by ion-exchange chromatography on cellulose CM-52 [9]. The α_1CB_6-, α_1CB_7- and α_1CB_8-peptides were isolated from a mixture of eight CNBr peptides by ion-exchange chromatography on cellulose CM-52, after fragmentation of the α-chain by CNBr in 70% formic acid under a nitrogen atmosphere. The fractions containing individual CNBr peptides were desalted on Sephadex G-25 and lyophilized.

Collagen peptides containing the Fn-binding site are of considerable interest, not only for the study of the mechanism of functioning components of a connective tissue and an extracellular matrix but also in applied aspects, *e.g.* for use as ligands in affinity sorbents for the isolation and purification of biopolymers with an affinity for Col.

To simplify the preparation of the α_1CB_7-peptide, we studied its possible isolation from a mixture of CNBr peptides of the α_1-chain by chromatography on sorbents containing the D45 do-

main of Fn. Sorbents of two types were synthesized, namely D45-Sepharose CL-4B (**8**) and D45-hydrazidomaloyl-Sepharose CL-4B (**9**). We expected such a method to be successful because, of the eight CNBr peptides of the α_1-chain, only α_1CB_7 will specifically interact with immobilized D45. D45 was prepared by the partial proteolysis of Fn with α-chymotrypsin.

Sorbent **8** was prepared by the standard CNBr method. Cross-linked Sepharose was chosen as a matrix because of its higher stability, in view of the necessity to carry out subsequent isolation of α_1CB_7 at 37°C. The direct coupling of D45 with Sepharose CL-4B can induce undesirable conformational changes in the domain molecule, resulting in a reduction of its affinity for α_1CB_7 and in the appearance of steric hindrance to its interaction with α_1CB_7. These undesirable effects may be avoided by immobilization of D45 via its carbohydrate moiety.

It is known [13] that most of carbohydrate side-chains in Fn are located in the gelatin-binding domain. In addition, it was established that the carbohydrate moiety of Fn does not take part in its binding to Col. Therefore, one could expect immobilization of D45 via its carbohydrate chains to be useful for the preparation of a more effective affinity sorbent for isolation of Col and collagenous peptides. The analogous immobilization of glycoproteins on hydrazido sorbents via a carbohydrate moiety of biopolymers has been described [14].

In the synthesis of sorbent **9**, D45 was oxidized by sodium periodate for 1 h at pH 6.0. The protein fraction containing the oxidized domain was separated from low-molecular-mass components by chromatography on Sephadex G-25, and then incubated with hydrazidomaloyl-Sepharose CL-4B (**10**) for 3 h at 20°C. Sorbent **10** was prepared by the coupling of malic acid dihydrazide to CNBr-activated Sepharose CL-4B. In comparison with widely used hydrazidosuccinyl (or hydrazidoadipoyl)-Sepharose, sorbent **10** is more hydrophilic. Coupling of the oxidized D45 to sorbent **10** resulted in formation of sorbent **9** (Fig. 1). The sorbents **8** and **9** contained 1.4 and 0.9 mg of D45 per ml of packed gel, respectively.

At the next stage, a model study of the separate chromatography of gelatin and the individual CNBr peptides of the α_1-chain on sorbents **8** and **9** was carried out. The chromatographic conditions were the same as for the chromatography of Fn on gelatin-Sepharose: the sorbents were equilibrated with buffer A (without PMSF) and successive elution was performed with solutions of 1, 4 and 8 *M* urea in the same buffer [2]. The fractions were analysed by absorption at of 230 nm. Chromatography was carried out at 37°C. It can be seen (Fig. 2a) that gelatin and α_1CB_7 were retained by the sorbents under saturation conditions and eluted with a 4 *M* urea solution, whereas α_1CB_6 and α_1CB_8 were not bound to the sorbents and were eluted with the starting buffer. These data indicate the biospecific character of gelatin and α_1CB_7 binding to the immobilized D45. Neither gelatin nor the α_1CB_7-peptide adsorb on unmodified Sepharose CL-4B or on hydrazidomaloyl-Sepharose CL-4B (**10**). The gelatin-binding capacities of sorbents **8** and **9** average 0.34 and 0.61 mg/mg of immobilized D45, respectively, whereas the α_1CB_7-binding capacity was 0.21 and 0.44 mg/mg of ligand. Thus, sorbent **9** was twice as effective as sorbent **8** prepared by direct coupling of D45 to CNBr-activated Sepharose.

The model investigations performed were the basis for the elaboration of a method for one-step isolation of the α_1CB_7-peptide from a mixture of CNBr peptides of the α_1-chain by affinity chromatography on sorbents **8** and **9** (Fig. 2b). The peptide mixture was applied to small columns packed with sorbents **8** and **9**, which were equilibrated with buffer A (without PMSF) at 37°C. The flow-rate was kept at 60 ml/h. Unbound peptides were washed off with 1 *M* urea in the starting buffer, after which homogeneous α_1CB_7 was eluted with 4 *M* urea in the same buffer. Finally, the sorbent was washed with 8 *M* urea in the same buffer.

The proposed method for the preparation of the α_1CB_7-peptide allowed us to reduce the number of stages of the process in comparison with the known method [4] and to obtain the highly purified α_1CB_7-peptide within a short time. Re-

cently, an analogous approach was adopted to the isolation of nascent collageneous polypeptides using affinity chromatography on an immobilized gelatin-binding domain of Fn [15].

To study AC of Fn on sorbents **1–7**, their Fn-binding capacity was determined using small columns under standard conditions [2] at saturation of the sorbent with Fn (Fig. 3). The data obtained are shown in Table I.

The degree of purity of Fn prepared by chromatography on sorbents **2–7** exceeded 95%, *i.e.* the purity was higher than in the case of chromatography on gelatin-Sepharose (**1**).

The results show that the sorbents with the α-chains and the α_1CB_7-peptide possess a higher mass capacity (mg/mg of ligand) than the other sorbents. The mass capacity of the sorbents with the β_{11}- and β_{12}-components was comparable with that of gelatin-Sepharose (**1**), whereas the sorbent with the α_1CB_8-peptide, which does not contain the Fn-binding site, proved to be ineffective for the isolation of Fn.

The real efficiency of a ligand becomes obvious from the calculation of the molar ratio between the immobilized ligand and the adsorbed Fn. This calculation shows that in the case of sorbents **2–5** there are 0.4–0.6 mol of bound Fn per mol of the ligand. The β_{11}-component is known to consist of two covalently bound α_1-chains, whereas the β_{12}-component contains the covalently bound α_1- and α_2-chains. Thus, the molecular size of β_{11}- and β_{12}-fragments is twice that of individual α-chains; therefore, in relation to the molar capacity of the sorbents, α-chains are twice as effective as ligands for Fn than the β-components. One can suppose that only one of the two sites of the strong binding of Fn [16] in the β-components takes part in the biospecific sorption of Fn during AC.

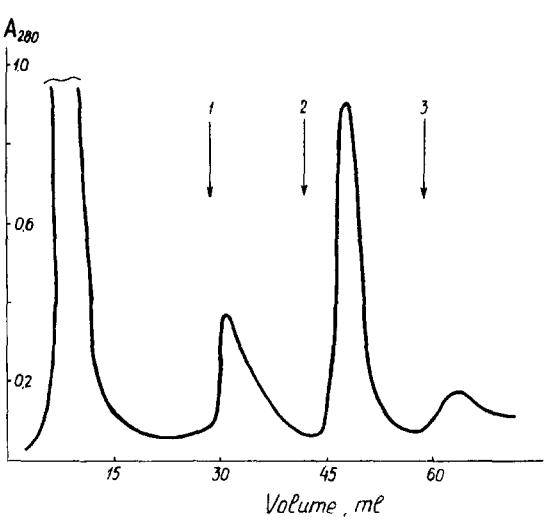

Fig. 3. Affinity chromatography of human plasma proteins on sorbent **6**. Arrows 1–3 indicate the beginning of elution with 1, 4, and 8 *M* urea in of buffer A, respectively (see Experimental for the complete procedure).

As regards the α_1CB_7-peptide, there are 0.14 mol of bound Fn per mol of α_1CB_7. The four- to five-fold reduction of the molar capacity of immobilized α_1CB_7, compared with that of the α-chains, may reflect the greater probability of conformational changes in the peptide and screening of its binding site. As a result, only every seventh molecule of immobilized α_1CB_7 binds an Fn molecule in the course of AC. Nevertheless, sorbent **6** has one of the highest mass capacities, because the molecular mass of α_1CB_7 is four times smaller than that of the α-chains, *i.e.* there are more moles of the immobilized ligand on sorbent **6** than on sorbents **2–5**. On the other hand, the molar capacity of α_1CB_8-Sepharose (**7**) was the least: 1 mol of the ligand binds only 7.5 nmol of Fn; in other words, there is only 1 mol of bound Fn per 130 mol of the immobilized α_1CB_8.

There are conflicting data concerning α_1CB_8 affinity for Fn. For instance, it was shown that both α_1CB_7 and α_1CB_8 inhibited the binding of ^{125}I-labelled collagen to an Fn–anti-Fn immunoprecipitate at 4°C [17]. Earlier, it was noted [16] that the α_1CB_8-peptide, like the α_1CB_7-peptide, reversibly binds to Fn, the dissociation constant of both complexes being $2 \cdot 10^{-9}$ *M*. On the other hand, the α_1CB_8-, α_1CB_3- and α_1CB_6-peptides did not interact with the gelatin-binding domain of Fn, whereas the α_1CB_7-peptide biospecifically bound to this domain [18]. Our data indicate only a slight affinity of the immobilized α_1CB_8-peptide for Fn. The problem of the biospecific interaction of this peptide with Fn needs additional study.

The unique properties of Fn [13,19] need a

comprehensive biochemical and medical study, which would require sufficient amounts of highly purified Fn. Note that the use of sorbents containing the immobilized collagen polypeptide fragments is promising with regard to the preparative isolation of Fn. Among the sorbents tested, sorbents **2** and **4**, which contain the individual α-chains, are optimal in view of their great capacity and straightforward preparation in comparison with α_1CB_7-Sepharose (**6**).

A pilot approval and an industrial implementation of the proposed method for the isolation of Fn from human plasma using AC on the immobilized α-chains of Col have been carried out at the Kharkov Enterprise for Manufacturing Bacterial Preparation.

REFERENCES

1 A. J. D'Ardenne and J. O'D. McGee, *J. Pathol.*, 142 (1984) 235.
2 E. Engvall and E. Ruoslahti, *Int. J. Cancer*, 20 (1977) 1.
3 E. Engvall, M. L. Bell and E. Ruoslahti, *Collagen Rel. Res.*, 1 (1981) 505.
4 C. Black, D. M. Douglas and M. L. Tanzer, *J. Chromatogr.*, 190 (1980) 393.
5 M. W. Mossesson, A. B. Chen and R. M. Huseby, *Biochem. Biophys. Acta*, 386 (1975) 509.
6 U. K. Laemmli, *Nature*, 227 (1970) 680.
7 K. A. Piez, E. A. Eigner and M. S. Lewis, *Biochemistry*, 2 (1963) 58.
8 J. Bergman and A. Loxley, *Anal. Chem.*, 35 (1963) 1961.
9 W. T. Butler, K. A. Piez and P. Bornstein, *Biochemistry*, 6 (1967) 3771.
10 J. Kohn and M. Wilchek, *Appl. Biochem. Biotechnol.*, 9 (1984) 285.
11 K. C. Ingham, S.A. Brew and B. S. Isaacs, *J. Biol. Chem.*, 263 (1988) 4624.
12 P. A. McCroskey, J. F. Richards and E. D. Harris, Jr., *Biochem. J.*, 152 (1975) 131.
13 T. Vartio, *Med. Biol.*, 61 (1983) 283.
14 D. J. Shannessy and W. L. Hoffman, *Biotechnol. Appl. Biochem.*, 9 (1987) 488.
15 V. V. Bychkova and A. E. Berman, *Voprosy Med. Khimii*, N 6 (1988) 129.
16 D. F. Mosher, in T. H. Spaet (Editor), *Progress in Hemostatis and Thrombosis*, Vol. 5, Grune & Stratton, New York, 1980, p. 111.
17 D. F. Mosher, *Mol. Cell. Biochem.*, 58 (1984) 63.
18 M. L. Bell and E. Engvall, *Anal. Biochem.*, 123 (1982) 329.
19 F. P. Schena and G. Pertosa, *Nephron*, 48 (1988) 177.