JOURNAL OF combinatorial CHEMISTRY

Article

Direct Synthesis of Peptides on Convective Interaction Media Monolithic Columns for Affinity Chromatography

Karin Pflegerl, Ales Podgornik, Eva Berger, and Alois Jungbauer

J. Comb. Chem., **2002**, 4 (1), 33-37• DOI: 10.1021/cc0100060 • Publication Date (Web): 06 December 2001

Direct Synthesis of Peptides on Convective Interaction Media Monolithic Columns for Affinity Chromatography

Karin Pflegerl,[†] Ales Podgornik,[‡] Eva Berger,[†] and Alois Jungbauer*,[†]

Institute of Applied Microbiology, University of Agricultural Sciences, Muthgasse 18, A-1190 Vienna, Austria, and BIA Separations d.o.o., Teslova 30, SI-1000 Ljubljana, Slovenia

Received February 23, 2001

Solid-phase peptide synthesis was performed on glycidyle methacrylate-*co*-ethylene dimethacrylate monoliths using Fmoc chemistry. The native epoxy groups were amino-functionalized by reaction with ethylenediamine or ammonia ions. A peptide directed against human blood coagulation factor VIII was synthesized as a model peptide. Amino acid analysis revealed the correct amino acid ratio as present in the sequence. The ligand density of 5 μmol/mL was equal to that achieved with conventional peptide immobilization via epoxy groups. These supports were directly used as peptide affinity chromatography matrixes. The functionality of the CIM monolithic supports was proven by affinity chromatography of factor VIII. The ammonia-functionalized support performed with low hydrophobicity and did not show unspecific adsorption of proteins.

Introduction

Convective interaction media (CIM) monolithic supports are block polymers prepared by radical copolymerization of glycidyl methacrylate and ethylene dimethacrylate (GMA−EDMA) in the presence of porogens.[1] They are rigid, macroporous monoliths containing epoxy groups as functional groups that can be activated to allow immobilization of various ligands.[2] CIM monolithic supports have been used for the separation of proteins utilizing ion-exchange, reversed-phase, hydrophobic interaction, and affinity chromatography.[1,3−8] Separation of DNA and oligonucleotides[9,10] and the use of CIM monolithic supports for enzyme biocatalysis[11,12] have also been reported. CIM monolithic supports offer the advantage of high resolution practically unaffected by flow rate,[13,14] low back pressure due to high porosity and short column length, low nonspecific binding of biomolecules, and simple handling. For affinity chromatography purposes, the peptides were synthesized by solid-phase peptide synthesis on conventional polystyrene-based resins, cleaved, purified, and immobilized via the epoxy groups on CIM. In contrast, our goal was to create a matrix for affinity chromatography by direct solid-phase peptide synthesis[15] on CIM.

Several authors have shown recently that monolithic material can be used as a support for peptide synthesis.[16−18] Korolkov et al.[18] have also performed peptide synthesis on CIM monoliths to create ready-to-use matrixes for affinity chromatography. They used butoxycarbonyl chemistry and added styrene to reduce swelling and shrinking of the material during peptide synthesis. In the case of protein chromatography, hydrophobic moieties introduced by styrene can lead to high unspecific adsorption. Thus, such media are not suited for affinity chromatography and biospecific adsorption of protein on such media must be evaluated with care. Hydrophobic interaction can dominate the adsorption process, which can lead to false-positive results.

We used CIM columns prepared without the addition of styrene and Fmoc chemistry[19,20] for peptide synthesis. This allowed us to create an affinity matrix in one step, which exerts several beneficial features for the generation of peptide libraries. Peptide synthesis, screening for specific protein binders, and affinity chromatography on the same matrix become possible with such a support. Previous work had shown that the choice of matrix and spacer is very critical for optimal binding and elution in affinity chromatography.[8,21] The positioning of the peptide and the nature of the support is extremely crucial when peptides are immobilized on a solid phase. Because of steric hindrance and synergism between peptide conformation and the chemistry of the support, the selectivity of a ligand may change with the support. The possibility of using the same matrix for screening as for the affinity chromatography application would therefore be a definite advantage.

In this paper, we show possible methods for direct peptide synthesis on CIM columns and the suitability of the prepared matrixes for protein affinity chromatography. We chose peptide no. 35[8] with the sequence NH_2-Glu-Tyr-Lys-Ser-Trp-Glu-Tyr-Cys-COOH as the model. It was derived from a combinatorial library on cellulose membranes prepared by spot synthesis.[22] Peptide no. 35 performs binding of human plasma derived blood coagulation factor VIII (pdFVIII) as well as recombinant factor VIII. In plasma, blood coagulation factor VIII (FVIII) is complexed with the von Willebrand factor (vWF).[23] This complex functions as a cofactor for the activation of factor X by activated factor IX in the extrinsic blood coagulation cascade. The FVIII molecule consists of a 80 kDa chain linked by a metal bridge to a heavy chain varying in size from 90 to 200 kD because of proteolysis from the C-terminus. The FVIII−vWF complex tends to

* To whom correspondence should be addressed. E-mail: jungbaue@hp01.boku.ac.at. Fax: +43 1 36 97615.

[†] University of Agricultural Sciences.

[‡] BIA Separations d.o.o.

10.1021/cc0100060 CCC: $22.00
Published on Web 12/06/2001

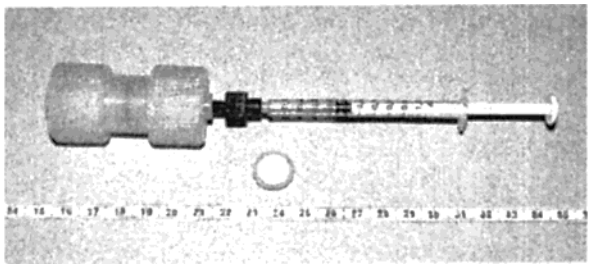

Figure 1. CIM column and cartridge adapted for solid-phase peptide synthesis (metric scale in centimeters).

show unspecific adsorption on several affinity matrixes.[24] Nevertheless, pdFVIII and its peptide ligand no. 35 were chosen as a model system because it can be considered as a worst case protein for affinity purification.

Results and Discussion

Solid-phase peptide synthesis on beaded particles takes place on the surface and also inside the particles in the small pores. This requires that the polymers swell in the applied solvents so that the reactants can reach all the active groups by diffusion, even when the peptide had already been grown to a large molecule. The swelling properties of a CIM column were evaluated before synthesis. The disk was immersed in the respective solutions for the same time and in chronological order as it would be exposed to during peptide synthesis, deprotection procedures, and solvent exchange. After the respective time, the dimensions of the disk were measured with a vernier caliper. The extent of swelling did not increase the back pressure.

All synthesis procedures were performed with the CIM column mounted to a cartridge (see Figure 1). Solvents and amino acid solutions were injected with a syringe. It is also possible to mount the CIM column to the reaction vessel of an automated peptide synthesizer. Side chain deprotection was carried out with the CIM column immersed in the deprotection solution in a beaker because the cartridge was not stable to trifluoroacetic acid (TFA). For such an operation a Teflon or stainless steel device would be suggested.

A handle for peptide attachment can be formed easily by hydrolysis of epoxy groups to adjacent hydroxyls. Esterification of an αN-Fmoc-protected amino acid derivative to these hydroxyl groups yields an amino-functionalized support. But the range of running conditions for chromatography is limited in this case because these esters are not stable to strong alkaline conditions. In an industrial environment sanitization procedures with sodium hydroxide are required.

Therefore, we used CIM EDA columns, which were amino-functionalized by reaction of the epoxy groups with ethylenediamine (EDA). On this handle peptide no. 35 directed against FVIII was synthesized using Fmoc chemistry as described in the Experimental Section. The expected amino acid ratio for peptide no. 35 of 2:2:1:1 Tyr/Glu/Lys/Ser was determined by amino acid analysis, and a peptide ligand density of 5 μmol/mL was calculated. This corresponds to the same ligand density of peptide no. 35 as achieved by conventional immobilization via epoxy groups.[8] A blank column was prepared by acetylation of the amino groups of a CIM EDA column. The functionality of the

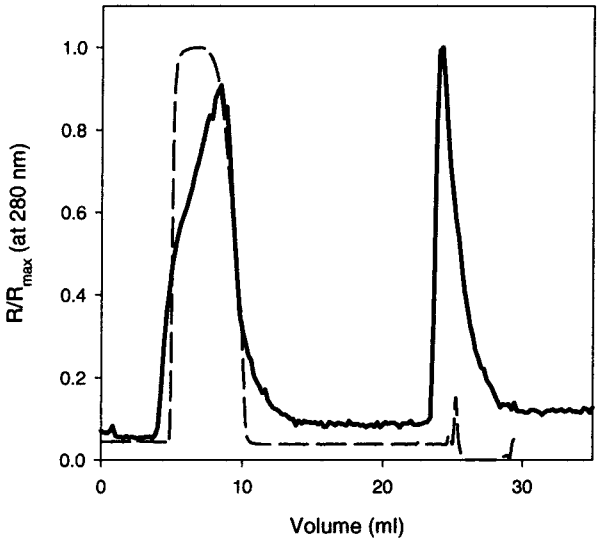

Figure 2. Peptide affinity chromatography of pdFVIII from a preparation containing the FVIII−vWF complex. The behavior of an affinity support consisting of peptide no. 35 synthesized on a CIM EDA column (solid line) was compared to that of a blank CIM EDA column (dashed line). Unreacted amino groups of both supports had been acetylated. Bound FVIII was eluted with 2 M guanidine hydrochloride, 5 mM calcium chloride dihydrate, and 0.1% Tween 20 and immediately transferred to a stabilization buffer. The absorbance data were normalized with respect to the maximum response value.

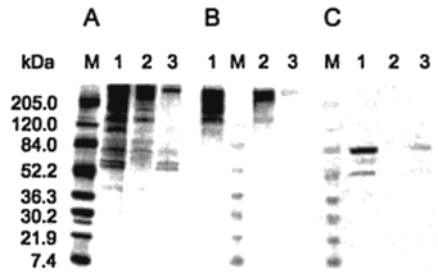

Figure 3. SDS−PAGE (A) and Western blots (B, C) of starting material, flow-through, and eluate of affinity chromatography of pdFVIII with an affinity support consisting of peptide no. 35 synthesized on a CIM EDA column. Part B was developed with antibody 0082 against vWF, and part C was developed with antibody 038 against FVIII (light chain). Lane 1, starting material; lane 2, flow-through; lane 3, eluate; M, molecular weight marker.

supports was tested by injection of a pdFVIII preparation containing the FVIII−vWF complex. A total of 200 IU of pdFVIII in a concentration of 50 IU/mL was loaded onto the peptide no. 35 CIM column and on the blank column. A comparison between a blank and a peptide column allows the evaluation of specific binding. Bound FVIII was eluted with 2 M guanidine hydrochloride with 5 mM calcium chloride dihydrate and 0.1% Tween 20. Immediately after elution, the buffer was exchanged to running buffer containing sucrose and glycine (stabilization buffer) to stabilize the labile FVIII molecule. Figure 2 shows the superimposed chromatograms of these affinity chromatography runs. SDS−PAGE and Western blot (see Figure 3) show that the flow-through from the peptide column contains only vWF, but no FVIII. Almost all loaded FVIII was found in the eluate. In contrast, the blank column also retained protein unspecifically (see lane A3 of Figure 4). Since the protein bands are only visible in SDS−PAGE, they must correspond to

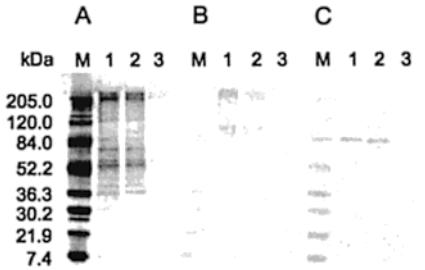

Figure 4. SDS−PAGE (A) and Western blots (B, C) of starting material, flow-through, and eluate of affinity chromatography of pdFVIII with a blank CIM EDA column. Part B was developed with antibody 0082 against vWF, and part C was developed with antibody 038 against FVIII (light chain). Lane 1, starting material; lane 2, flow-through; lane 3, eluate; M, molecular weight marker.

fragments that have lost their epitopes for the respective specific antibodies. In principle, ethylenediamine should ensure minimal steric effects and almost no unspecific interactions with most proteins. For some pdFVIII fragments this is obviously not the case.

We decided therefore to avoid a spacer like ethylenediamine. In the channels of CIM monoliths, there is no restricted diffusion, and mass transport is mainly accomplished by convection.[13] In addition, amine groups are not buried by the polymer backbone. Because they are ideally exposed on the surface of the polymethacrylate chains, a long spacer should not be necessary for suitable presentation even of small peptide affinity ligands.

Therefore, an amino-functionalized monolith was generated simply by reaction of the native epoxy groups with ammonia, yielding a primary amine. Peptide no. 35 was again chosen as the model peptide and synthesized via a short spacer consisting of one β-Ala residue. The correct amino acid ratio and a ligand density of 5 μmol/mL was achieved again. An ammonia-activated column carrying one β-Ala residue was used as a blank. Both supports were acetylated. The performance as an affinity matrix was again checked by loading of 200 IU of pdFVIII. Superimposed chromatograms are shown in Figure 5. SDS−PAGE and Western blot revealed that all FVIII loaded on the peptide column was bound and eluted (see Figure 6). No protein was adsorbed onto the blank column (see Figure 7). Functionalization of the CIM epoxy groups with ammonia solution obviously provides suitable anchors for peptide synthesis and excludes unspecific adsorption of proteins during affinity chromatography. Acetylation also reduces the overall hydrophilicity of the support but does not cause unspecific adsorption of plasma proteins.

For further characterization of the reliability and reproducibility of the developed peptide synthesis procedure, seven peptide supports were investigated by amino acid analysis. In all cases a composition close to the theoretical could be found (see Table 1). The different yield is due to variations in coupling time. Rigorous peptide characterization besides amino acid analysis and functionality testing through affinity chromatography is not possible because the peptides are attached to the monolith without a cleavable linker to prevent leakage during chromatography procedures. Further characterization requires the development of novel analytical methods.

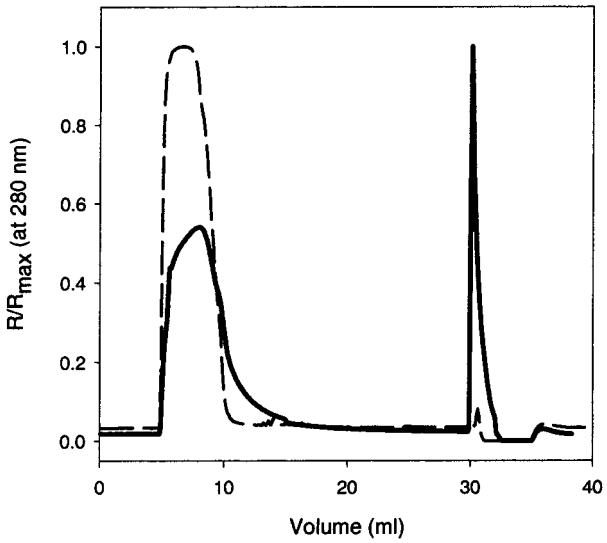

Figure 5. Peptide affinity chromatography of pdFVIII from a preparation containing the FVIII−vWF complex. The behavior of an affinity support consisting of peptide no. 35 synthesized on an ammonia-activated CIM column (solid line) was compared to that of a blank ammonia-activated CIM column (dashed line). Both supports carried a one β-Ala residue spacer. Unreacted amino groups of both supports had been acetylated. Bound FVIII was eluted with 2 M guanidine hydrochloride, 5 mM calcium chloride dihydrate, and 0.1% Tween 20 and immediately transferred to a stabilization buffer. The absorbance data were normalized with respect to the maximum response value.

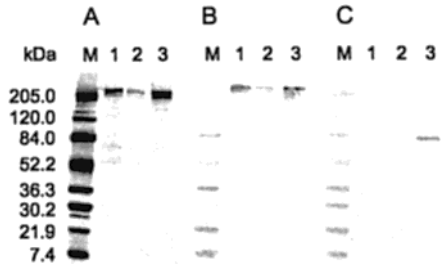

Figure 6. SDS−PAGE (A) and Western blots (B, C) of starting material, flow-through, and eluate of affinity chromatography of pdFVIII with an affinity support consisting of peptide no. 35 synthesized on an ammonia-activated CIM column. Part B was developed with antibody 0082 against vWF, and part C was developed with antibody 038 against FVIII (light chain). Lane 1, starting material; lane 2, flow-through; lane 3, eluate; M, molecular weight marker.

Conclusion

Irrespective of how the anchor was created, the developed strategy allowed peptide synthesis and affinity chromatography on the same support. The choice of the anchor is critical with regard to unspecific adsorption of proteins and leakage during affinity chromatography. Monoliths are used for fast chromatography because their mass transfer rate is very high.[21,25] The high mass transfer also may improve the synthesis. The washing procedure can be accelerated by working in a packed bed mode. The interstitial liquid is displaced by convection. Similar effects also improve the development of a library. Incubation of target molecules by pumping the molecule through the support allows the adjustment of a defined reaction time. The reaction time in an immersion process highly depends on the individual condition such as solvent exchange rate and support volume.

Table 1. Amino Acid Concentrations of Various Peptides Synthesized on CIM Monoliths

peptide	amino acid concentration[a] (μmol/g)							
	Glx[b]	Tyr	Lys	Ser	β-Ala	Leu	Val	Phe
Glu-Tyr-Lys-Ser-Trp-Glu-Tyr-Cys-β-Ala-NH_2-CIM[c]	16.7	16.5	8.9	7.9	19.1			
Leu-Val-β-Ala-NH_2-CIM[c]					27.3	26.8	22.8	
Glu-Tyr-Lys-Ser-Trp-Glu-Tyr-Cys-EDA-CIM[c]	23.3	16.1	10.4	9.9				
Glu-Tyr-Lys-Ser-Trp-Glu-Tyr-Cys-EDA-CIM[c]	24.7	19.5	8.9	8.0				
Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-β-Ala-EDA-CIM[c]	20.4		8.9	7.2	32.0			12.6
Glu-Tyr-Lys-Ser-Trp-Glu-Tyr-Cys-β-Ala-EDA-CIM[d]	2.8	2.3	0.7	1.0	16.6			
Glu-Tyr-Lys-Ser-Trp-Glu-Tyr-Cys-β-Ala-EDA-CIM[e]	2.3	1.5	0.6	0.7	22.6			

[a] Cys and Trp were not determined. [b] Gln or Glu. [c] 2 × 15 min coupling. [d] 5 min coupling. [e] 3 min coupling.

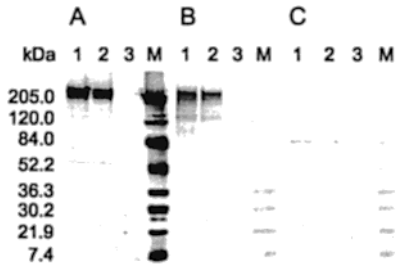

Figure 7. SDS−PAGE (A) and Western blots (B, C) of starting material, flow-through, and eluate of affinity chromatography of pdFVIII with a blank ammonia-activated CIM column. Part B was developed with antibody 0082 against vWF, and part C was developed with antibody 038 against FVIII (light chain). Lane 1, starting material; lane 2, flow-through; lane 3, eluate; M, molecular weight marker.

For screening purposes, the unbound material can be collected and analyzed. The bound material can be eluted and also subjected to analysis. The monoliths can be reused, which is considered an advantage compared to conventional libraries.

Experimental Section

General. A 25% ammonia solution, dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA), diisopropylamine (DIPA), piperidine, acetic anhydride, trifluoroacetic acid (TFA), and methanol were purchased from Merck, Vienna, Austria. *N*-Methylpyrrolidone (NMP) and dichloromethane (DCM) were obtained from Perkin-Elmer, Vienna, Austria. Buffer components, phenol, triisobutylsilane (TIBS), and dithiotreitol were purchased from Sigma-Aldrich, Vienna, Austria.

CIM Columns. CIM epoxy and CIM EDA disk monolithic columns are a trademark of BIA Separations, Ljubljana, Slovenia. They were supplied with polypropylene sealing rings. The dimensions of the CIM columns were 12 mm in diameter and 3 mm in height. One column had a mass of 0.16 g. Epoxy groups were aminated with 25% ammonia solution at 40 °C for 3 h following a protocol of Hermanson.[26] Then the CIM columns were washed with deionized water and with 1 M sodium chloride and then transferred to DMF for peptide synthesis.

Peptide Synthesis. Peptide synthesis was carried out following a protocol of Frank.[22] CIM columns were mounted in a cartridge designed for chromatography and were washed by injection of DMF prior to synthesis. Peptide synthesis was performed by injecting 0.3 M solutions of amino acid pentafluorophenyl esters with N-terminal Fmoc protection (Novabiochem, Laeufelfingen, Switzerland) in NMP. Side chain protection groups were trityl for Cys, *tert*-butyl for Glu, Ser, Thr, and Tyr, and butoxycarbonyl for Lys. Fmoc-β-Ala-OH was activated with 1 equiv of PyBOP (Novabiochem) and 2 equiv of DIPEA. Two 0.4 mL portions of the respective amino acid solutions were injected into the CIM monolithic column to react for 15 min each. Surplus amino groups were acetylated with 2% acetic anhydride, 1% DIPA, and 97% DMF before Fmoc deprotection with 20% piperidine in DMF. Free amino terminal ends were also acetylated before side chain deprotection. Deprotection was performed with 50% TFA, 4% DCM, 1% phenol, 3% TIBS, and 2% H_2O for 30 min and with 90% TFA, 3% TIBS, 2% H_2O, 1% phenol, 4% DCM for 120 min. After intensive washing with DCM, DMF, and methanol, the columns were transferred to the aqueous running buffer system for chromatography or stored in 20% methanol in water at 4 °C.

Amino Acid Analysis. Since the synthesized peptides were acetylated, the N-termini were not accessible for peptide sequencing to corroborate the right sequence and to quantify ligand density. Alternatively, the peptide support was dried, ground up, and subjected to amino acid hydrolysis with 6 M hydrochloric acid at 115 °C for 20 h. The resulting amino acids were analyzed on a 5 μm Hypersil column (Forschungszentrum Seibersdorf, Vienna, Austria) of 4 mm in diameter and a length of 250 mm, using a Hewlett-Packard HP 1050 series system with a Shimadzu RF 535 variable-wavelength fluorescence detector. Precolumn derivatization was performed with *o*-phthalaldehyde.[27,28] Cys and Trp cannot be detected by this method because these amino acids are destroyed during hydrolysis.

Affinity Chromatography. Chromatography experiments were performed on a ProSys workstation (Biosepra, USA) or on a FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden) with a UVM-II monitor (Amersham Pharmacia Biotech) detecting protein absorbance at 280 nm. The CIM columns were mounted in the appropriate cartridge, connected to the respective chromatography system, and equilibrated with the running buffer consisting of 10 mM Hepes, 5 mM calcium chloride dihydrate, 100 mM sodium chloride, and 0.1% Tween 20, pH 7.4. The flow properties of the CIM columns were checked by acetone pulse experiments (data not shown).

Lyophilized pdFVIII preparation Octanate (Octapharma, Vienna, Austria), lot no. 935581A120/U or 910528B120/U, was reconstituted in water to a concentration of 50 IU/mL. A total of 200 IU was loaded onto each CIM column carrying the respective peptide. Elution was performed with 2 M guanidine hydrochloride, 5 mM calcium chloride dihydrate,

and 0.1% Tween 20. A flow rate of 160 cm/h was applied. Flow-through and eluates were collected for analysis by SDS−PAGE and Western blot. The eluates were desalted into running buffer containing 2% sucrose and 10 mM glycine using Sephadex G25 medium packed in a XK column (Amersham Pharmacia Biotech) of 16 mm in diameter and 120 mm in length.

SDS−PAGE and Western Blot. Electrophoretic separation of proteins was performed with a Xcell Mini-Cell system (Novex, San Diego, CA) in 4−20% Tris-glycine Novex precast gels (Invitrogen, Groningen, The Netherlands) under reducing conditions. A 2% dithiotreitol sample was used as the reducing reagent. Prestained broad range SDS−PAGE-standards were from Bio-Rad Laboratories, Vienna, Austria. Electrophoretically separated proteins were either silver-stained or transferred to a 0.2 μm of Protran nitrocellulose membrane (Merck) using the Xcell Mini-Cell system. After the membrane was blocked with 3% skim milk powder in washing buffer (phosphate-buffered saline with 0.1% Tween 20), monoclonal antibody 038 (Biomedica, Vienna, Austria) was used to probe for FVIII. Bound MAb 038 was detected by an alkaline phosphatase conjugated antimouse monoclonal antibody (Sigma-Aldrich) with nitroblue-tetrazoliumchloride and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) as substrates. Anti-vWF peroxidase conjugate (DAKO, Vienna, Austria) and HRP color development reagent DAB (Bio-Rad) were used to probe the vWF.

Acknowledgment. We gratefully acknowledge Octapharma Pharmazeutika Produktionsges.m.b.H., Vienna, for the support of this project and the kind donation of plasma-derived factor VIII, and we acknowledge BIA Separations, Ljubljana, for the donation of CIM columns. We thank T. Dalik, Institute of Chemistry, University of Agricultural Sciences, for amino acid analyses.

References and Notes

(1) Tennikova, T. B.; Belenkii, B. G.; Svec, F. *J. Liq. Chromatogr.* **1990**, *13*, 63−70.
(2) Josic, D.; Buchacher, A.; Jungbauer, A. *J. Chromatogr. B* **2001**, *752*, 191−205.
(3) Tennikova, T. B.; Svec, F. *J. Chromatogr.* **1993**, *646*, 279−288.
(4) Svec, F.; Frechet, J. M. *J. Chromatogr. A* **1995**, *702*, 89−95.
(5) Strancar, A.; Koselj, P.; Schwinn, H.; Josic, D. *Anal. Chem.* **1996**, *68*, 3483−3488.
(6) Josic, D.; Lim, Y. P.; Strancar, A.; Reutter, W. *J. Chromatogr. B* **1994**, *662*, 217−226.
(7) Abou-Rebyeh, H.; Josic, D.; Gottschall, K.; Schubert-Rehberg, K.; Korber, F. *J. Chromatogr.* **1991**, *566*, 351−359.
(8) Amatschek, K.; Necina, R.; Hahn, R.; Schallaun, E.; Schwinn, H.; Josic, D.; Jungbauer, A. *J. High Resolut. Chromatogr.* **2000**, *23*, 47−58.
(9) Giovannini, R.; Freitag, R.; Tennikova, T. B. *Anal. Chem.* **1998**, *70*, 3348−3354.
(10) Podgornik, A.; Barut, M.; Jancar, J.; Strancar, A.; Tennikova, T. *Anal. Chem.* **1999**, *71*, 2986−2991.
(11) Abou-Rebyeh, H.; Korber, F.; Schubert-Rehberg, K.; Reusch, J.; Josic, D. *J. Chromatogr.* **1991**, *566*, 341−350.
(12) Vodopivec, M.; Berovic, M.; Jancar, J.; Podgornik, A.; Strancar, A. *Anal. Chim. Acta* **2000**, *407*, 105−110.
(13) Hahn, R.; Jungbauer, A. *Anal. Chem.* **2000**, *72*, 4853−4858.
(14) Schuster, M.; Wasserbauer, E.; Neubauer, A.; Jungbauer, A. *Bioseparation*, **2001**, *9*, 259−268.
(15) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154.
(16) Hird, N.; Hughes, I.; Hunter, D.; Morrison, M. G. J. T.; Sherrington, D. C.; Stevenson, L. *Tetrahedron* **1999**, *55*, 9575−9584.
(17) Tripp, J. A.; Stein, J. A.; Svec, F.; Frechet, J. M. *Org. Lett.* **2000**, *2*, 195−198.
(18) Korolkov, V. I.; Platonova, G. A.; Azanova, V. V.; Tennikova, T. B.; Vlasov, G. P. *LIPS* **2000**, *5*, 1−9.
(19) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404−3409.
(20) Chang, C. D.; Meienhofer, J. *Int. J. Pept. Protein Res.* **1978**, *11*, 246−249.
(21) Hahn, R.; Amatschek, K.; Schallaun, E.; Necina, R.; Josic, D.; Jungbauer, A. *Int. J. Bio-Chromatogr.* **2000**, *5*, 175−185.
(22) Frank, R. *Tetrahedron* **1992**, *48*, 9217−9232.
(23) Lollar, P. *Mayo Clin. Proc.* **1991**, *66*, 524−534.
(24) Strancar, A.; Barut, M.; Podgornik, A.; Koselj, P.; Schwinn, H.; Raspor, P.; Josic, D. *J. Chromatogr. A* **1997**, *760*, 117−123.
(25) Iberer, G.; Hahn, R.; Jungbauer, A. *LC−GC* **1999**, *13*, 88−95.
(26) Hermanson, G. T.; Mallia, A. K.; Smith, P. K. *Immobilized Affinity Ligand Techniques*; Academic Press: San Diego, 1992; p 153.
(27) Jones, B. N.; Gilligan, J. P. *J. Chromatogr.* **1983**, *266*, 471−482.
(28) Schuster, R. *J. Chromatogr.* **1988**, *431*, 271−284.

CC0100060