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Affinity chromatography of porcine pepsin on different types of immobilized 3,5-diiodo-L-tyrosine

H. Arnoštová[a], Z. Kučerová[b], I. Tišlerová[c], T. Trnka[c], M. Tichá[a,*]

[a]*Department of Biochemistry, Charles University, Albertov 2030, 128 40 Prague, Czech Republic*
[b]*Department of Pathophysiology, Charles University, U Nemocnice 5, 128 53 Prague, Czech Republic*
[c]*Department of Organic Chemistry, Charles University, Albertov 2030, 128 40 Prague, Czech Republic*

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Abstract

The preparation of affinity sorbents containing immobilized iodinated derivatives of L-tyrosine for the affinity chromatography of porcine pepsin is described. The ligand was coupled either to Sepharose 4B or bead cellulose after the divinylsulfone activation or to Sepharose 4B after the activation with 2,4,6-trichloro-1,3,5-triazine. The highest capacity for porcine pepsin was found in the case of 3,5-diiodo-L-tyrosine coupled to divinylsulfone-activated Sepharose.

Keywords: Immobilized amino acids; Pepsin; Diiodotyrosine; Enzymes

1. Introduction

Aspartic proteases are present in gastric juice of different species. An occurrence of different forms of these proteases as well as of their zymogens is characteristic for this type of enzyme. *N*-Acetyl-L-phenyl-L-tyrosine is used as a substrate for the determination of the activity of aspartic proteases. An exchange of L-tyrosine for 3,5-diiodo-L-tyrosine results in a significant increase of the affinity of pepsin (EC 3.4.23.1) to this substrate, while the affinities of other aspartic proteases are not changed. *N*-Acetylphenyl-3,5-diiodo-L-tyrosine is used as a substrate for the determination of the pepsin activity in the mixture with gastricsin; gastricsin (EC 3.4.23.3) hydrolyzes this substrate at a rate of three orders of magnitude lower than that of pepsin [1].

The estimation of the pattern of isoforms of aspartate proteases and their zymogens is important from the diagnostic point of view. Changes in the mutual ratios of individual isoforms are characteristic markers for some gastric diseases [2–4]. Methods based on biospecific interactions seem to be very suitable ones for the separation of the enzyme and its zymogen isoforms. There exist only a limited number of described ligands used for the affinity chromatography of pepsin and its zymogen: peptide inhibitors, poly-L-lysine, and antibody [5]. 3,5-Diiodo-L-tyrosine [6,7] represents another type of the ligand that might be suitable for the preparation of affinity carrier for the specific adsorption of pepsin and possible separation of its isoforms. The present

*Corresponding author. Fax: +420-2-2195-2331.

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communication describes the immobilization of iodinated derivatives of L-tyrosine to different supports.

2. Experimental

2.1. Chemicals

Porcine pepsin, 3,5-diiodo-L-tyrosine and L-tyrosine and their *tert*.-butoxycarbonyl (Boc) derivatives, potato phosphatase were purchased from Sigma (St. Louis, MO, USA), divinylsulfone was from Fluka (Neu-Ulm, Switzerland), Sepharose was from Pharmacia Biotech (Uppsala, Sweden), bead cellulose (Perlose MT 200) was from North Bohemian Chemical Works (Lovosice, Czech Republic). Dephosphorylation of porcine pepsin was carried out using potato acid phosphatase (EC 3.1.3.2) as described by Hynek et al. [8]. Dephosphorylated pepsin was a gift from H. Vaňková (Department of Pathophysiology, Charles University, Prague, Czech Republic). Dephosphorylation was proved by immobilized iron(III) metal chelate affinity chromatography of the chymotryptic digest of the enzyme (an absence of phosphopeptides) [9].

2.2. Preparation of affinity carriers

2.2.1. Activation of Sepharose 4B with divinylsulfone [10]

Sepharose 4B (10 ml) washed with distilled water (500 ml) was suspended in 0.2 *M* carbonate buffer, pH 10.7 (10 ml) containing divinylsulfone (1 ml). The suspension was shaken at room temperature for 70 min and then the gel was washed with distilled water (4×50 ml).

2.2.2. Coupling 3,5-diiodo-L-tyrosine, L-tyrosine and glycine

The gel of divinylsulfone-activated Sepharose (immediately after the activation) (5 ml) equilibrated with 0.2 *M* carbonate buffer, pH 10.7 was mixed with the solution of the ligand (500 mg in 10 ml of the same buffer) and shaken at room temperature for 20 h. The gels were washed first with distilled water and finally with 0.2 *M* carbonate buffer, pH 10.7. Then the gels were suspended in glycine solution (100 mg glycine in 10 ml 0.2 *M* carbonate buffer, pH 10.7), shaken for 2 h at room temperature and again washed with distilled water, 0.05 *M* citrate buffer, pH 3.0 and distilled water.

2.2.3. Coupling Boc-3,5-diiodo-L-tyrosine and Boc-L-tyrosine

The gel of divinylsulfone-activated Sepharose (10 ml) was equilibrated with the mixture of 0.2 *M* carbonate buffer, pH 10.7–dimethylformamide (1:1) and mixed with the ligand solution in 50% dimethylformamide (400 mg in 14 ml). The suspensions were shaken for 20 h at room temperature and washed with the mixture of 0.2 *M* carbonate buffer, pH 10.7–dimethylformamide (1:1) and then with distilled water. The glycine deactivation was carried out as in the case of 3,5-diiodo-L-tyrosine.

For removal of Boc substituents, gels were suspended in distilled water (15 ml) containing trifluoroacetic acid (1.5 ml), shaken for 30 min at room temperature and then washed with distilled water.

2.2.4. Iodination of immobilized L-tyrosine and 3,5-diiodo-L-tyrosine to Sepharose

L-Tyrosine-Sepharose or glycine-Sepharose (10 ml) equilibrated with phosphate-buffered saline, pH 7.4 (PBS) was mixed with the PBS solution of KI (2.4 g in 3.6 ml) and suspension of chloramine B in PBS (0.6 g in 4.2 ml). After 2 min, the PBS solutions of sodium metabisulfite (0.1 g in 3.6 ml) and of KI (2.4 g in 3.6 ml) were added and gels were washed with distilled water.

2.2.5. Activation of bead cellulose and 3,5-diiodo-L-tyrosine coupling

Conditions for the activation of bead cellulose with divinylsulfone and 3,5-diiodo-L-tyrosine coupling were the same as in the case of the Sepharose carrier described above.

2.2.6. Activation of Sepharose 4B with 2,4,6-trichloro-1,3,5-triazine and 3,5-diiodo-L-tyrosine coupling

Procedure described by Lenfeld et al. [11] for the activation of cellulose was used.

To the suspension of Sepharose 4B washed with distilled water (20 ml), acetone (20 ml) was added. After 30 min shaking at room temperature, 10% NaOH (0.7 ml) was added and again shaken for 1 h.

The solution of 2,4,6-trichloro-1,3,5-triazine in ice-cooled acetone (705 mg in 8.5 ml) was mixed with the gel suspension and kept for 45 min in ice-cooled bath.

Gel suspension was washed with ice-cooled acetone and distilled water and mixed with 3,5-diiodo-L-tyrosine solution in 0.05 *M* borate buffer, pH 9.1 (207 mg in 36 ml). After shaking for 4 h at room temperature, the gel was washed with distilled water.

2.3. Affinity chromatography of porcine pepsin

Affinity chromatography of porcine pepsin was performed using the BioLogic System and a Bio-Scale MT2 column (52×7 mm) (Bio-Rad Labs., Hercules, CA, USA) filled with the prepared carriers.

The affinity column was equilibrated with the starting buffer (0.05 *M* acetate buffer, pH 3.5). Porcine pepsin (Sigma) (2, 5 and 10 mg in 1 ml of starting buffer) was applied to the affinity column. Non-adsorbed proteins were eluted with the starting buffer (50 ml) at the flow-rate of 1 ml/min. For the elution of adsorbed enzyme, 0.05 *M* phosphate buffer, pH 6.8 (50 ml) was used. The eluent was monitored at 280 nm and fractions (2.5 ml) were collected. Each fraction was immediately after the elution mixed with 1 *M* HCl (0.5 ml). The proteolytic activity in individual collected fractions was determined by the Anson and Mirsky method [12].

For the comparison of capacity of the prepared affinity carriers, the percentage of adsorbed enzyme after the elution and non-adsorbed proteins were evaluated on the basis of peak areas. Porcine pepsin (5 mg) was applied to each of the affinity carriers.

2.4. Analytical methods

The presence of coupled L-tyrosine and its derivatives in the prepared affinity carriers was shown by UV spectra measurement in the region of 220–300 nm after the gel was made transparent in 50% glycerol.

The iodine content was determined by combustion microanalysis according to Schoniger [13] followed by the iodometric titration.

2.5. Nuclear magnetic resonance (NMR) spectra measurement

NMR spectra were recorded on a Varian ^{UNITY}INOVA 400 (proton frequency 400 MHz) instrument in hexadeuteriodimethylsulfoxide at 40°C. Signal of solvent was used as reference at $\delta=2.50$ for protons and $\delta=39.50$ for carbon signals. Chemical shifts are given in ppm, coupling constants in Hz. The assignment was checked by correlation experiments. Correlation spectroscopy (COSY) experiments were recorded in absolute value mode using standard two pulse sequence. Heteronuclear single quantum correlation spectroscopy (HSQC) and Heteronuclear multiple bond correlation (HMBC) were performed as gradient experiments. HSQC phase sensitive, HMBC in absolute value mode. All two-dimensional experiments were recorded with spectral windows 5000 Hz for proton and 25 000 Hz for carbon.

3. Results

3.1. Preparation of immobilized derivatives of L-tyrosine

Sepharose 4B was activated either with divinylsulfone or with 2,4,6-trichloro-1,3,5-triazine, bead cellulose with divinylsulfone. Divinylsulfone-activated Sepharose 4B was used for coupling: 3,5-diiodo2-L-tyrosine, L-tyrosine, Boc-3,5-diiodo-L-tyrosine, Boc-L-tyrosine and glycine. Iodination of L-tyrosine-Sepharose and glycine-Sepharose was performed by the standard chloramine method. Besides that, 3,5-diiodo-L-tyrosine was further coupled to divinylsulfone-activated bead cellulose or to 2,4,6-trichloro-1,3,5-triazine-activated Sepharose 4B.

The prepared affinity gels were analyzed by means of the measurement of UV absorption spectra in the region 220–400 nm and the determination of iodine content (Table 1). Since direct NMR analysis of the product of the reaction of divinylsulfone-activated Sepharose with 3,5-diiodo-L-tyrosine was impossible owing to limited solubility of the product in common NMR solvents, the model experiment with divinylsulfone and 3,5-diiodo-L-tyrosine was carried out. The product was studied by usual NMR meth-

Table 1
Analysis of the prepared affinity gels based on divinylsulfone-activated matrices

Affinity gel	UV absorption spectra (maximum) (nm)	Iodine content (%, w/w)
DIT-Sepharose	287	7.7
Tyr-Sepharose	276	0
Iodinated Tyr-Sepharose	288	8.7
Iodinated Gly-Sepharose	–[a]	0
Boc-DIT Sepharose	–[a]	0
Boc-Tyr Sepharose	–[a]	0
DIT-cellulose	n.d.	1.9

[a] No absorption in the region 240–320 nm.
DIT, 3,5-Diiodo-L-tyrosine.
n.d., Not determined.

ods. HMBC experiment exhibits correlation between CH_2-A carbon and CH-A proton, which proves that iodotyrosine, is bound to divinylsulfone by nitrogen atoms. ^{1}H-NMR spectrum (400 MHz, $C^2H_2SOC^2H_3$): 2.34 dd, 2H, J_1=13.9, J_2=7.5 and 2.68 dd, 2H, J_1=13.9, J_2=6.7 (2×Ar-C$\underline{H}_2$); 2.92 m, 2×S-C$\underline{H}_2$); 2.95 m, 2H (2×N-C$\underline{H}$); 3.00 m, 2H and 3.10 m, 2H (2×N-C$\underline{H}_2$); 7.25 s 4H (4×Ar-H). ^{13}C-NMR spectrum (100 MHz, $C^2H_2SOC^2H_3$): 34.03 t (Ar-C$\underline{H}_2$); 47.50 t (N-C$\underline{H}_2$); 51.96 t (S-C$\underline{H}_2$); 72.49 d (N-C$\underline{H}$); 89.93 s (I-$\underline{C}$); 122.67 s.

($\underline{C}_{(Ar)}$-C); 137.92 d ($\underline{C}_{(Ar)}$-H); 163.42 s ($\underline{C}_{(Ar)}$-OH); 174.92 s ($\underline{C}$OOH). Results of NMR analysis correlate also with indirect proof based on coupling of Boc derivatives of L-tyrosine and 3,5-diiodo-L-tyrosine (Table 1). It can be concluded, that L-tyrosine and its derivatives are linked to divinylsulfone-activated Sepharose only via amino groups.

3.2. Affinity chromatography of porcine pepsin

For comparison of the capacity of the prepared carriers, an amount of porcine pepsin adsorbed under the same conditions was determined using the measurement of absorbance and proteolytic activity (Fig. 1). Under the used experimental conditions, the enzyme activity was fully adsorbed on immobilized 3,5-diiodo-L-tyrosine and on iodinated L-tyrosine-Sepharose. The recovery of loaded enzyme was about 95%.

Sorbents based on divinylsulfone-activated Sepharose possessed better properties than those based on triazine-activated Sepharose. Affinity chromatography on 3,5-diiodo-L-tyrosine bound to triazine-activated Sepharose yielded elution curves with broader and asymmetrical peaks and the capacity of this carrier was low.

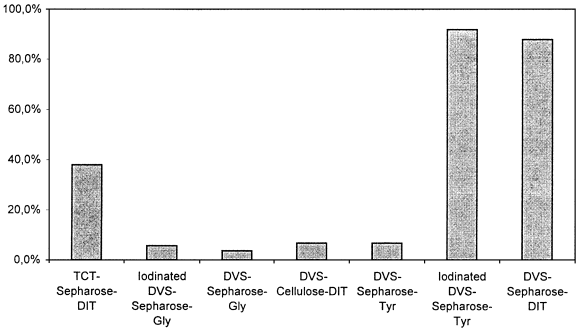

Fig. 1. Capacity of the prepared affinity carriers for porcine pepsin. %, Percentage of adsorbed enzyme to the affinity carriers; [5 mg of porcine pepsin in 1 ml of 0.05 *M* acetate buffer, pH 3.5 was applied to affinity column filled with prepared carriers (52×7 mm); adsorbed enzyme was eluted with 0.05 *M* phosphate buffer, pH. 6.8]; DV, divinylsulfone; TCT, 2,4,6-trichloro-1,3,5-triazine; DIT, 3,5-diiiodo-L-tyrosine.

The highest capacity for the porcine pepsin was observed in the case of 3,5-diiodo-L-tyrosine coupled to divinylsulfone-activated Sepharose. Pepsin was adsorbed to 3,5-diiodo-L-tyrosine due to the presence of iodine substituents (Fig. 2A and B); less than 10% of pepsin was adsorbed to L-tyrosine-Sepharose. The enzyme was not adsorbed to the carriers obtained by coupling Boc derivatives of 3,5-diiodo-L-tyrosine and L-tyrosine in agreement with the obtained analytical data (no ligand was coupled).

Pepsin was separated by affinity chromatography on iodinated L-tyrosine-Sepharose into two active fractions. Results are different from those obtained with 3,5-diiodo-L-tyrosine-Sepharose (Fig. 2B and C). This fact suggests that the resulting derivative of iodination of L-tyrosine residues is not homogeneous 3,5-diiodo-L-tyrosine. Pepsin is not adsorbed to iodinated glycine-Sepharose indicating that iodination of inert matrix or spacer arm is not the reason of different behavior of pepsin on affinity gels. The presence of two isoforms in porcine pepsin is not due to a partial dephosphorylation of the porcine protease: the behavior of native and dephosphorylated enzyme on iodinated L-tyrosine-Sepharose was very similar.

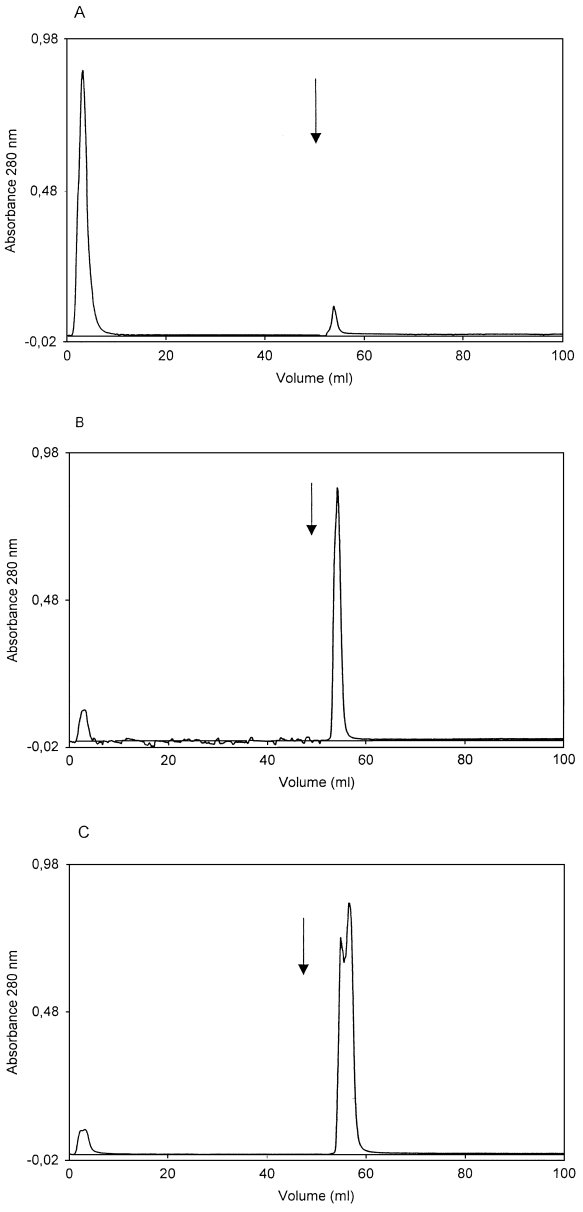

Fig. 2. Affinity chromatography of porcine pepsin on different types of ligands coupled to divinylsulfone-activated Sepharose 4B. (A) L-Tyrosine, (B) 3,5-diiodo-L-tyrosine, (C) iodinated immobilized L-tyrosine residues to Sepharose [5 mg of porcine pepsin in 1 ml of 0.05 *M* acetate buffer, pH 3.5 was applied to affinity column filled with prepared carriers (52×7 mm); an arrow indicates the start of elution with 0.05 *M* phosphate buffer, pH 6.8].

The low capacity of the prepared affinity carrier based on bead cellulose corresponded to the low substitution degree of the carrier with the ligand, in comparison with the Sepharose carriers (Table 1). The reaction of cellulose with divinylsulfone under the used conditions probably resulted in a low activation degree of the carrier.

4. Discussion

Divinylsulfone-activated Sepharose originally developed for cross-linking of agarose gel [10] was found to be suitable matrix for coupling different types of ligands (e.g., saccharides [14,15], peptides and proteins [16,17]. Besides that, divinyl-activated Sepharose was used for the preparation of thiophilic adsorbents (T-gels) [18,19]. Coupling 3,5-diiodo-L-tyrosine to divinylsulfone-activated Sepharose described in the present paper yielded an affinity carrier with a high capacity for porcine pepsin. The different affinity of aspartic proteases to *N*-acetylphenyl-3,5-diiodo-L-tyrosine used for their determination can be used for their separation as well for the separation of individual isoforms.

Divinylsulfone activation was also used to couple 3,5-diiodo-L-tyrosine to cellulose; however, the capacity of the obtained carrier was low, due to the low substitution degree. The reaction of cellulose with divinylsulfone under the used conditions probably did not result in a high activation of the carrier.

The different behavior of 3,5-diiodo-L-tyrosine-Sepharose and iodinated L-tyrosine-Sepharose in pepsin binding might be explained by not complete iodination of L-tyrosine residues in the affinity carrier. Our results have shown that 3,5-diiodo-L-tyrosine and L-tyrosine are linked to divinylsulfone-activated Sepharose only via amino group. As well as the effect of iodination on the carrier with the spacer arm was eliminated: in affinity chromatography on iodinated glycine-Sepharose no pepsin was adsorbed and the course of chromatography on iodinated 3,5-diiodo-L-tyrosine-Sepharose was the same as that on the carrier prior iodination (results not shown).

Affinity chromatography on iodinated L-tyrosine-Sepharose showed the presence of at least two proteolytically active components in the enzyme

preparation. Porcine pepsin A is *O*-phosphorylated [20]. Our experiments with dephosphorylated enzyme did not show that the heterogeneity of porcine pepsin could be caused by a partial dephosphorylation. The existence of multiple forms of porcine pepsin differing in their affinity to 3,5-diiodo-L-tyrosine could be be explained by the presence of other aspartic protease in the commercial enzyme preparation or by the presence of partially degraded pepsin molecules.

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References

[1] A.P. Ryle, in: H.V. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Vol. 5, Verlag Chemie, Weinheim, Deerfield Beach, 1984, p. 99.
[2] S.C. Huang, K. Miki, C. Furihata, M. Ichinose, A. Shimizu, H. Oka, Clin. Chim. Acta 175 (1988) 37.
[3] M.T. Hallisey, J.A. Dun, J.W.L. Fielding, Scand. J. Gastroenterol. 29 (1994) 1129.
[4] B. Foltmann, A.L. Jensen, Eur. J. Biochem. 128 (1982) 63.
[5] J. Turková, in: Bioaffinity Chromatography, Journal of Chromatography Library, Vol. 55, Elsevier, Amsterdam, 1993, p. 405.
[6] E. Tonková, M. Tichá, Z. Kučerová, Int. J. Biochromatogr. 4 (1998) 35.
[7] Z. Kučerová, M. Beneš, J. Lenfeld, Int. J. Biochromatogr. 3 (1997) 177.
[8] R. Hynek, V. Kašicka, Z. Kučerová, J. Káš, J. Chromatogr. B 681 (1995) 37.
[9] H. Vaňková, personal communication.
[10] J. Porath, T. Laas, J.C. Jansen, J. Chromatogr. 103 (1975) 49.
[11] J. Lenfeld, M.J. Beneš, Z. Kučerová, React. Funct. Polym. 28 (1995) 61.
[12] M.C. Anson, A.E. Mirsky, J. Genet. Physiol. 16 (1932) 59.
[13] W. Schoniger, Microchim. Acta (1955) 123.
[14] M. Fouillet, M. Levi-Strauss, V. Gindicelli, D. Lutomski, D. Bladier, M. Caron, R. Jourbet-Caron, J. Chromatogr. B 706 (1998) 167.
[15] S.K. Nadimpalli, V.L. Zerramalle, A. Hille-Rehfeld, K. von Figura, Comp. Biochem. Physiol. B – Biochem. Mol. Biol. 123 (1999) 261.
[16] P. Pauw, C. Neyt, E. Vanderwinkel, R. Wattiez, P. Falmagne, Protein Expr. Purif. 6 (1995) 371.
[17] J.D. Cornillot, M. Caron, R. Jourbet-Caron, D. Bladier, Int. J. Biochem. 24 (1992) 1585.
[18] J. Porath, M. Belew, Trends Biotechol. 3 (1987) 2251.
[19] J.A. Scoble, R.K. Scopes, J. Chromatogr. A 787 (1997) 47.
[20] J. Tang, P. Sepulveda, J. Marciniszyn, K.C.S. Chen, Proc. Natl. Acad. Sci. USA 70 (1973) 3437.