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Aromatic amino acids and their derivatives as ligands for the isolation of aspartic proteinases

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Abstract

Affinity chromatography was used to study an interaction of aspartic proteinases with immobilized aromatic amino acids and their derivatives. The following ligands were used: L-tyrosine, 3-iodo-L-tyrosine, 3,5-diiodo-L-tyrosine, L-phenylalanine, *p*-iodo-L-phenylalanine and *N*-acetyl-L-phenylalanine. With the exception of the last one, ligands were coupled directly to divinyl sulfone activated Sepharose 4B. For the preparation of immobilized *N*-acetyl-L-phenylalanine, divinyl sulfone activated Sepharose 4-B with linked ethylene diamine was used. Porcine pepsin was used for the evaluation of the capacity of the prepared affinity carriers. The capacity of the immobilized amino acid derivatives significantly increased in comparison with the non-derivatized amino acids. The prepared immobilized ligands were further used for the separation of human pepsinogens.

Keywords: Aromatic amino acids; Aspartic proteinases; Enzymes

1. Introduction

Aspartic proteinases are present in gastric juice of different species and are synthesized in gastric mucosa as inactive zymogens. Zymogens are converted to active enzymes by removing of N-terminal amino acid sequence, when they are released from gastric mucose to acid conditions in gastric juice [1]. Human gastric juice contains two major groups of aspartic proteinases [2]: pepsin A (pepsin I group, pepsin, EC 3.4.23.1) and pepsin C (pepsin II group, gastricsin, EC 3.4.23.3) that differ in their amino acid composition and in their immunological specificity. An occurence of multiple forms of zymogens is characteristic for this type of enzymes. Human pepsinogens were separated up to seven isoforms (five of pepsinogen A and two of pepsinogen C) using electrophoretic [3] or high-performance liquid chromatography (mostly ion-exchange chromatography) [4–6]

Content and changes in the ratios between individual isoforms of human aspartic proteinases and their zymogens were found to be important from the diagnostic point of view. Low concentration of pepsinogen A in serum was found to be marker of gastric cancer, similarly low ratio of pepsinogen A to pepsinogen C [7–9]. The change in the level of individual isoforms of pepsinogen in gastric mucosa was found in patients with gastric cancer and ulcer [10]. Estimation of the pattern of isoforms both the proteinases and the zymogens is important for the early diagnosis of gastric diseases. Serum pepsinogen A and pepsinogen C levels can be used as subclinical markers of gastric cancer. Methods based

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on biospecific interactions are very suitable for this purpose. Affinity chromatography on different types of ligand, which can differentiate between the individual pepsinogens, is one of these methods.

For the determination of the pepsin activity, different types of peptides of various lengths are used [11]. An advantage of *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine as a substrate is its ability to differentiate between pepsin A and pepsin C (pepsin and gastricsin) [11]. In our previous communication we have described a high affinity of porcine pepsin to the immobilized 3,5-diiodo-L-tyrosine [12,13]. In the present communication we have studied the interaction of porcine pepsin and human pep sinogens with different derivatives of aromatic amino acids immobilized to Sepharose.

2. Experimental

2.1. Chemicals

Porcine pepsin, 3-iodo-L-tyrosine, 3,5-diiodo, L-tyrosine, L-tyrosine, *p*-iodo-L-phenylalanine, L-phenylalanine, *N*-acetyl-L-phenylalanine and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide purchased from Sigma (St. Louis, MO, USA), divinyl sulfone (Fluka, Neu-Ulm, Switzerland), Sepharose (Pharmacia Biotech. Uppsala, Sweden).

Human pepsinogens were isolated from extract of human gastric mucosa. This extracts were obtained from resected parts of stomach from patients suffering from gastric diseases and prepared as described previously [14]. Individual enzymes were separated by methods according to Foltmann [15] using ion-exchange chromatography on DEAE cellulose.

2.2. Preparation of affinity carriers

2.2.1. Activation of Sepharose 4B with divinyl sulfone [16]

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

2.2.2. Coupling L-tyrosine and L-phenylalanine and their iodinated derivatives

The gel of divinyl sulfone 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 lysine 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 of N-acetyl-L-phenylalanine

The same procedure as described above was used for the coupling of ethylene diamine to DVS activated Sepharose: 2 g ethylene diamine (di hydrochloride) was coupled with 10 ml of the gel of activated Sepharose. 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.

The gel was suspended in the mixed solution containing *N*-acetyl-L-phenylalanine (250 mg in 2.5 ml distilled water) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (200 mg in 5 ml distilled water). The suspension was shaken for 24 h at room temperature and again washed with distilled water.

2.2.4. Iodination of immobilized L-tyrosine, L-phenylalanine 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.

2.3. Affinity chromatography of porcine pepsin

The affinity column was equilibrated with the starting buffer (0.05 *M* acetate buffer pH 3.5).

Porcine pepsin (Sigma) (1 mg/1 ml of starting buffer) was applied to a 1 ml column (26×7 mm I.D.). Non-adsorbed proteins were eluted with the starting buffer. For the elution of adsorbed enzyme, 0.05 *M* acetate buffer pH 5.4. (at a flow-rate of 0.3 ml/min, 3 ml fractions were collected). For the immobilized *N*-acetyl-L-phenylalanine, the gradient of pH (from pH 5.4 to 6.4; 0.05 *M* acetate buffer pH 5.4–0.05 *M* sodium acetate pH 6.4) and then 0.05 *M* sodium acetate pH 6.4 containing 1 *M* NaCl were used. Each fraction was immediately mixed with 1 *M* HCl (0.5 ml) after the elution. The proteolytic activity in individual collected fractions was determined by the Anson and Mirsky method [17].

For the determination of capacity, the solution of porcine pepsin (200 mg/50 ml of 0.05 *M* acetate buffer pH 3.5) was applied to the affinity column (volume of 1 ml) till the proteolytic activity was determined in the eluate. Capacity was expressed in milligrams of the enzyme adsorbed to the affinity carrier.

2.4. Affinity chromatography of human pepsinogens

The affinity column was equilibrated with the starting buffer (0.05 *M* acetate buffer pH 5.5). Human pepsinogens in 0.05 *M* acetate buffer pH 5.5 were applied to a 1 ml column (26×7 mm I.D.). Non-adsorbed proteins were eluted with the starting buffer. For the elution of adsorbed enzyme 0.05 *M* phosphate buffer pH 6.8, or pH 6.2 (for iodinated L-tyrosine-Sepharose) were used (at a flow-rate of 0.3 ml/min, 3 ml fractions were collected). Proteolytic activity was determined after activation with HCl solution by the Anson and Mirsky method [17].

2.5. Analytical methods

The amount of coupled ligands in the prepared affinity carriers was determined by absorption measurement at 290 nm for L-tyrosine and its derivatives, at 240–250 nm for L-phenylalanine and its derivatives) after the gel solubilization in 50% glycerol.

The iodine content was determined by combustion microanalysis according to Schoniger [18] followed by the iodometric titration.

Proteolytic activity was determined by the method of Anson and Mirsky [17] (one unit will produced ΔA_{280} 0.001/min at pH 2 and 37 °C measurement as trichloracetic acid–soluble peptides using hemoglobin as substrate.

3. Results

3.1. Preparation of immobilized derivatives of L-tyrosine

Divinyl sulfone activated Sepharose 4B was used for the immobilization of L-tyrosine and L-phenylalanine and their iodinated derivatives. L-Phenylalanine, *p*-iodo-L-phenylalanine, L-tyrosine, 3-iodo-L-tyrosine and 3,5-diiodo-L-tyrosine were coupled directly to the activated matrix via their free amino groups. For the preparation of immobilized *N*-acetyl-L-phenylalanine, first ethylene diamine was linked to divinyl sulfone activated Sepharose; then carbodiimide reaction was used for coupling the ligand.

The prepared affinity gels were analyzed by means of measurement of UV absorption spectra and by the determination of iodine content. The content of immobilized *N*-acetyl-L-phenylalanine via the ethylene diamine spacer arm was about three to five times lower than that of the ligands coupled directly to the divinyl sulfone activated carrier (Table 1).

Iodination of L-tyrosine-Sepharose and L-phenylalanine-Sepharose was performed by the standard chloramine method. The substitution degree was higher in the case of immobilized L-tyrosine than that of L-phenylalanine. The same conditions were used for iodination of 3,5-diiodo-L-tyrosine-Sepharose to

Table 1
Ligand content in the prepared affinity gels

Affinity gel	Ligand content[a] μmole/ml of gel	Iodine content % w/w
L-Phe-Sepharose	15.0	0
p-Iodo-L-Phe-Sepharose	14.5	8.0
Iodinated L-Phe-Sepharose	15.0	5.9
N-acetyl-L-Phe-Sepharose	3.0	0
L-Tyr-Sepharose	9.3	0
3-Iiodo-L-Tyr-Sepharose	4.9	9.7
3,5-Diiodo-L-Tyr-Sepharose	11.0	7.7
Iodinated L-Tyr-Sepharose	9.3	8.7

[a] Based on the absorbance measurements.

show the effect of this reaction on Sepharose and the spacer arm resulting from the divinyl sulfone activation.

3.2. Affinity chromatography of porcine pepsin

Porcine pepsin was used to evaluate the capacity of the prepared gels. The capacity was determined as an amount of the enzyme adsorbed to 1 ml of the carrier under the same conditions (Fig. 1). Porcine pepsin was desorbed from all prepared affinity gels by an increase of pH (from pH 3.5 to 5.4), with an exception of *N*-acetyl-L-phenylalanine-Sepharose; in this case it was necessary to use for the elution the solution of pH 6.4 containing 1 *M* NaCl. The course of chromatography was followed by the determination of proteolytic activity in elution fractions. The recovery of the loaded enzyme was about 95%. The highest capacity for porcine pepsin was found for the immobilized *N*-acetyl-L-phenylalanine. This ligand linked via its carboxyl group to Sepharose and containing acetylated amino group adsorbed more than 20 times of the enzyme in comparison with L-phenylalanine-Sepharose containing the ligand coupled via amino group. Affinity chromatography of porcine pepsin on immobilized *N*-acetyl-L-phenylalanine is shown in Fig. 2.

Out of immobilized iodinated derivatives of L-tyrosine and L-phenylalanine, the high capacity of porcine pepsin was determined in the case of 3,5-diiodo-L-tyrosine and *p*-iodo-L-phenylalanine. 3-Iodo-L-tyrosine possessed significantly lower affinity for the enzyme in comparison with monoiodo derivative of L-phenylalanine. The capacity of iodinated L-tyrosin-Sepharose did not significantly differ from that of immobilized 3,5-diiodo-L-tyrosine. The low capacity of iodinated L-phenylalanine-Sepharose is probably caused by a lower degree of substitution.

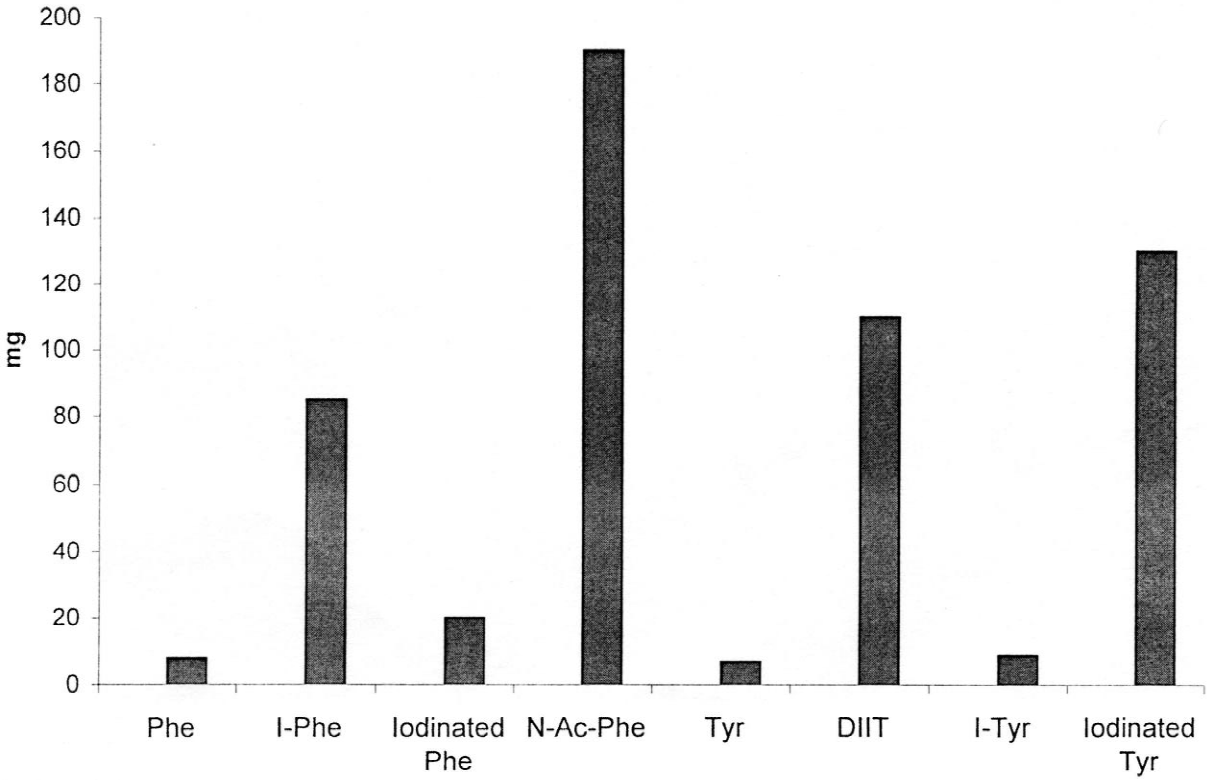

Fig. 1. Capacity of the prepared affinity carriers for porcine pepsin capacity expressed as milligrams of the enzyme adsorbed to 1 ml of a carrier. Phe–L-phenylalanine, I-Phe–*p*-iodo-L-phenbylalanine, Iodinated Phe–iodinated L-phenylalanine-Sepharose, N-Ac-Phe–*N*-acetyl-L-phenylalanine, Tyr–L-tyrosine, DIIT–3,5-diiodo-L-tyrosine, I-Tyr–3-iodo-L-tyrosine. Iodinated Tyr–iodinated L-tyrosine-Sepharose.

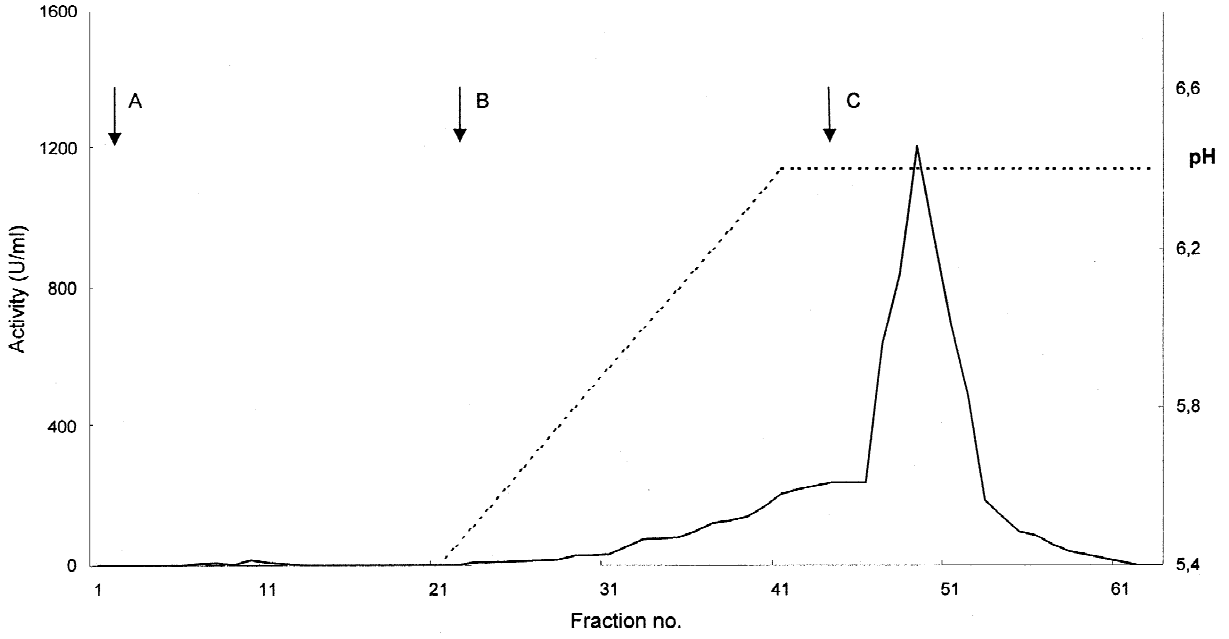

Fig. 2. Affinity chromatography of porcine pepsin on *N*-actyl-L-phenylalanine-Sepharose (A) 0.05 *M* acetate buffer, pH 3.5, (B) gradient of pH: 0.05 *M* acetate buffer pH 5.4–0.05 *M* sodium acetate pH 6.4, (C) 0.05 *M* sodium acetate pH 6.4 containing 1 *M* NaCl.

Iodination of 3,5-diiodo-L-tyrosine-Sepharose did not result in a change of the enzyme affinity to the gel (not shown).

3.3. Affinity chromatography of human pepsinogens

The prepared gels were used to study the different binding human pepsinogens A and C to the immobilized ligands. Human pepsinogens were loaded to affinity gels at pH 5.5 and desorbed at pH 6.8; only in the case of iodinated L-tyrosine-Sepharose, pH 6.2 was used. Results are summarized in the Table 2. Pepsinogen A interacted only with immobilized *N*-acetyl-L-phenylalanine and iodinated L-tyrosine-Sepharose. Pepsinogen C was adsorbed, besides these gels, also to immobilized *p*-iodo-L-phenylalanine and 3,5-diiodo-L-tyrosine. No interaction was observed with immobilized amino acids (L-phenylalanine and L-tyrosine) and 3-iodo-L-tyrosine. The course of affinity chromatography of a mixture of human pepsinogen A and C on 3,5-diiodo-L-tyrosine-Sepharose and iodinated L-tyrosine-Sepharose is shown in Fig. 3.

4. Discussion

Immobilized amino acids are very versatile affinity media. Their use in the isolation of proteins and enzymes is well established. There has been increasing interest in the use of this resin to serum protein separation [19]. For the isolation of proteinases, they might represent a suitable and simple affinity carrier lacking peptide bonds. Immobilized L-phenylalanine has been used for such purposes [20,21]. Our study has been focused on the possibility of differentiation

Table 2
Adsorption of human pepsinogen A (PGA) and pepsinogen C (PGC) to the prepared affinity gels

Affinity gel	PGA	PGC
L-Phe-Sepharose	–	–
p-Iodo-L-Phe-Sepharose	–	X
Iodinated L-Phe-Sepharose	–	–
N-Acetyl-L-Phe-Sepharose	X	X
L-Tyr-Sepharose	–	–
3-Iiodo-L-Tyr-Sepharose	–	–
3,5-Diiodo-L-Tyr-Sepharose	–	X
Iodinated L-Tyr-Sepharose	X	X

X, interaction of the zymogen with immobilized ligand.

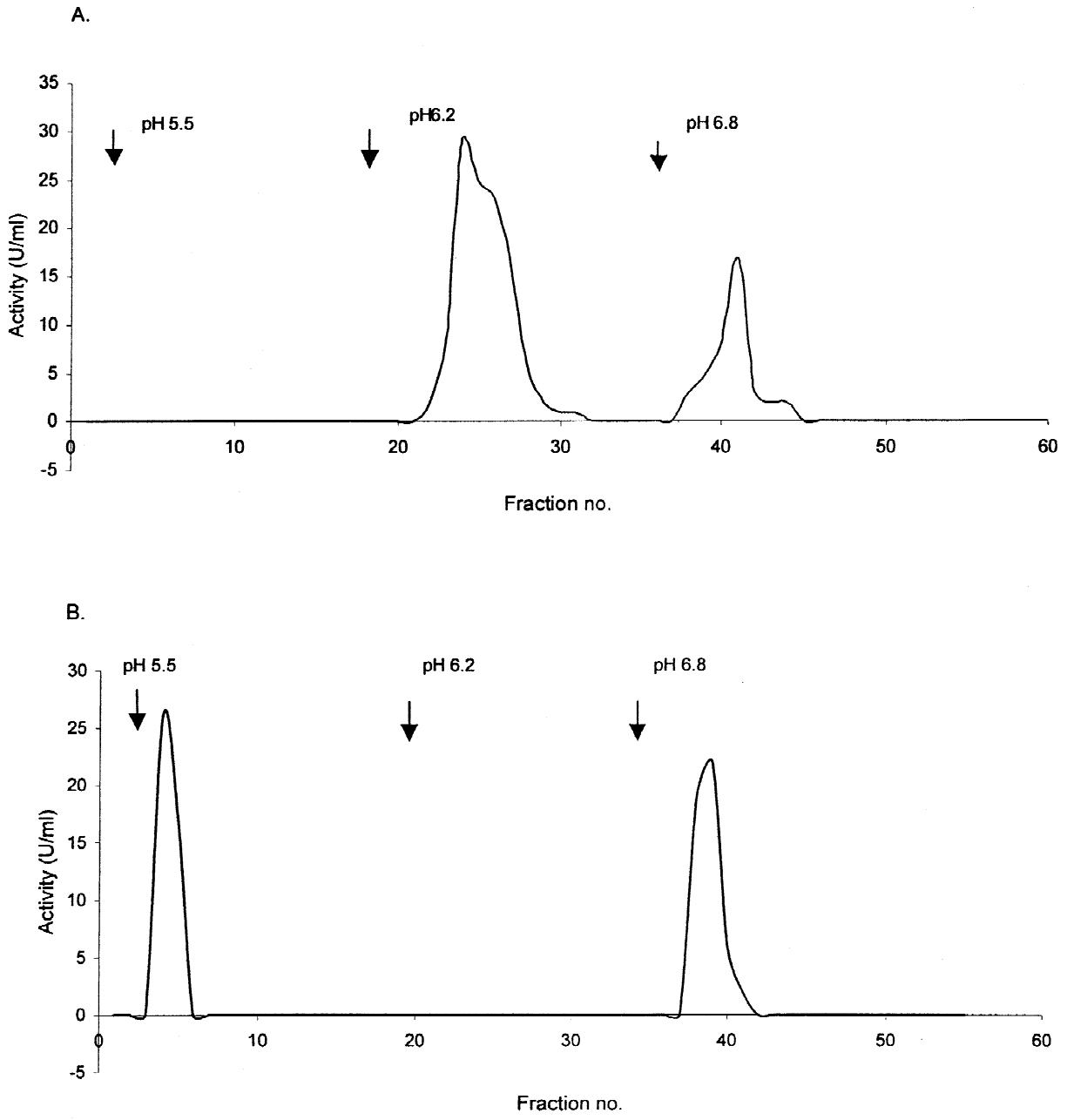

Fig. 3. Affinity chromatography of mixture of human pepsinogen A and C on iodinated L-tyrosine-Sepharose (A) and 3,5-diiodo-L-tyrosine (B).

between different types of pepsin, as well as between different type and isoforms of their zymogens. *N*-acetyl-L-phenylalanyl-3, 5-diiodo-L-tyrosine is a known substrate enabling to determine the pepsin activity in the mixture with gastric sin [11]. We have prepared immobilized different derivatives of L-tyrosine and L-phenylalanine to evaluate a contribution of different parts and components of the above-

mentioned substrate to the interaction with pepsin. Immobilization of 3,5-diiodo-L-tyrosine to three types of inert matrices and affinity chromatography of porcine pepsin was the subject of our previous communication [12]; the best results were obtained with coupling the ligand to divinyl sulfone activated Sepharose. On the basis of these results, we have chosen this activated matrix for the ligand immobilization. With an exception of *N*-acetyl-L-phenylalanine, all other ligands were directly linked to divinyl sulfone activated Sepharose, most probably via their free amino group, as was shown in our previous communication in the case of 3,5-diiodo-L-tyrosine [12]. For the coupling of *N*-acetyl-L-phenylalanine a new approach was used.

Our results have proved that a high affinity of pepsin to immobilized 3,5-diiodo-L-tyrosine is due to the presence of two iodine substituents in the ligand molecule. The capacity of immobilized mono-iodinated derivative (3-iodo-L-tyrosin) for porcine pepsin was very low and did not significantly differ from that of immobilized L-tyrosine. Human pepsinogens A and C did not interact with L-tyrosine-Sepharose and its 3-iodo derivative at all. However, pepsinogen A and pepsinogen C differ in their affinity to the immobilized 3,5-diiodo-L-tyrosine.

Porcine pepsin, as well as pepsinogen A and C did not show any significant affinity to immobilized L-phenylalanine (linked via amino group). Fungal proteinases that were retained by this affinity carrier belonged to seine proteinases [20,21]. It is interesting that Phenyl-Sepharose has been used for the affinity purification of proteinases from bacterial sources belonging to serine aspartate and metallo mechanistic classes [22]. *N*-acetyl-L-phenylalanine linked to Sepharose via carboxyl group, contrary to immobilized L-phenylalanine (coupled via amino group), exhibited the highest capacity for porcine pepsin out of all prepared affinity gels. A similar situation was observed in the case of both human pepsinogens. The high capacity of *N*-acetyl-L-phenylalanine-Sepharose is not related to an amount of immobilized ligands.

Substitution in the phenyl ring by iodine (immobilized *p*-iodo-L-phenylalanine) significantly increased their affinity for porcine pepsin and pepsinogen C in comparison with unsubstituted amino acid. A low increase of capacity after iodination of L-phenylalanine-Sepharose is probably caused by a low substitution degree due the low reactivity of L-phenylalanine residue. A quite different situation is in the case of iodination of L-tyrosine Sepharose. Iodinated L-tyrosin-Sepharose exhibited a high capacity for porcine pepsin and adsorbed both human pepsinogens, contrary to 3,5-diiodo-L-tyrosine that interacts only with pepsinogen C. This phenomenon is not fully understood; it was proved that it is not caused by iodination of the matrix or by the presence of not fully iodinated L-tyrosine residues.

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References

[1] M.S. Andreeva, A.E. Gutschina, Biochem. Biophys. Res. Commun. 32 (1979) 42.
[2] V. Richmond, J. Tang, R.E. Truco, R. Capputo, Biochim. Biophys. Acta 29 (1958) 453.
[3] I.M. Samloof, Gastroenterology 57 (1969) 659.
[4] C.J. Newton, B.R. Norman, W.H. Taylor, J. Chromatogr. 417 (1987) 391.
[5] A. Bank, A.W. Eriksson, G. Pals, J. Chromatogr. 571 (1991) 47.
[6] R. Hynek, J. Sajdok, Z. Kučerová, J. Káš, Sborník Lékařský 797 (1996) 135.
[7] M. Yoshikara, K. Sumi, Ann J. Gastroenterol. 93 (1998) 1090.
[8] I.M. Samloff, Gastroenterology 96 (1989) 586.
[9] G.N. Stemmermann, I.M. Samloof, A.M.Y. Nomura, L.K. Heilbrun, Clin. Chim. Acta 163 (1987) 191.
[10] M.T. Hallisey, J.A. Dun, J.W.L. Fielding, Scand. J. Gastroenterol. 29 (1994) 1129.
[11] A.P. Ryle, in: H.V. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Vol. 5, Verlage Chemie, Weinheim, Deerfield Beach, Basel, 1984, p. 99.
[12] Z. Kučerová, M. Beneš, J. Lenfeld, Int. J. BioChromatogr. 3 (1997) 177.
[13] H. Amoštová, Z. Kučerová, I. Tišlerová, T. Trnka, M. Tichá, J. Chromatogr. A 911 (2001) 211.
[14] Z. Kučerová, L. Korbová, J. Kohout, J. Šváb, Sbornik Lékařiský 94 (1993) 163.
[15] B. Foltmann, A.L. Jensen, Eur. J. Biochem. 128 (1982) 63.
[16] J. Porath, T. Laas, J.C. Jansen, J. Chromatogr. 103 (1975) 49.
[17] M.C. Anson, A.E. Mirsky, J. Genet. Physiol. 16 (1932) 59.
[18] W. Schoniger, Microchim. Acta (1955) 123.

[19] J. Turková, Bioaffinity Chromatography, Journal of Chromatography Library, Vol. 55, Elsevier, Amsterdam, 1993, p. 405.
[20] G. Larcher, J.P. Bouchara, V. Annaix, F. Symoens, D. Chabasse, G. Tronchin, FEBS Lett. 308 (1992) 65.
[21] G. Larcher, B. Cimon, F. Symoens, G. Tronchin, D. Chabasse, J.P. Bouchara, Biochem. J. 315 (1996) 119.
[22] M. Prescott, K. Peek, D.P. Veitch, R.M. Daniel, J. Biochem. Biophys. Methods 26 (1993) 51.