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Journal of Chromatography B, 800 (2004) 109–114
JOURNAL OF CHROMATOGRAPHY B
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Affinity chromatography of porcine pepsin and pepsinogen using immobilized ligands derived from the specific substrate for this enzyme

Jana Frýdlová [a], Zdenka Kučerová [a,*], Marie Tichá [b]

[a] *Department of Pathophysiology, Institute of Pathophysiology, First Faculty of Medicine, Charles University, U nemocnice 5, 128 53 Praha 2, Czech Republic*
[b] *Department of Biochemistry, Charles University, Albertov 2030, 128 40 Praha 2, Czech Republic*

Abstract

Affinity chromatography of porcine protease and its zymogen was carried out on immobilized components of specific substrate used for the pepsin determination. For the immobilization of *N*-acetyl-L-phenylalanine and iodinated derivative of L-tyrosine, divinyl sulfone activated Sepharose was used. Ligands with blocked amino group and free carboxyl one were linked to Sepharose via ethylene diamine spacer using carbodiimide reaction. Conditions of affinity chromatography of porcine pepsin and pepsinogen on the prepared carriers were optimized: the effect of pH, ionic strength and a nature of the buffers used on adsorption of the enzyme and zymogen to an affinity carrier, as well as their elution was studied. The following parameters were taken into consideration: capacity of the prepared affinity matrices, reproducibility of experiments and the enzyme stability. Pepsin was adsorbed to both immobilized ligands at pH 3.5–4.0; for the elution of the enzyme it was necessary to increase ionic strength (up to 0.5 M). For the adsorption of pepsinogen pH 5.2 was found to be optimum, for its desorption, an increase of ionic strength was used.

Keywords: Porcine pepsin; Porcine pepsinogen

1. Introduction

Gastric mucosa of different species contains of aspartic proteinases. An occurrence of different forms of these hydrolases as well as of their zymogens is characteristic for this type of enzymes. They are secreted as pepsinogens and transformed to pepsins at acid pH. The presence and relative concentration of pepsin A (EC 3.4.23.1 pepsin) and pepsin C (EC 3.47.23.3, gastricsin) vary according to vertebrate species, genetic variation, and, at least in human, with different gastric diseases as well [1,2]. Content and changes in the ratios between individual 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 [1–3]. The changed of level of individual pepsinogen in gastric mucosa was found in patients with gastric cancer and ulcer [4,5].

Pepsin is an endopeptidase with a broad specifity. Pepsin hydrolyzes N- and C-terminally blocked synthetic dipeptides like Glu–Tyr or Phe–Phe but with poor efficiency. However, assays using synthetic peptides offer the possibility of distinguishing between different enzymes or enzyme forms. *N*-Acetyl-L-phenylalanine-3,5-diiodo-L-tyrosine represents such example. This substrate is used for the determination of pepsin activity in the mixture with gastricsin: gastricsin hydrolyzes the substrate at a rate of three orders of magnitude lower than that of pepsin [6].

In our previous communication we have described a high affinity of porcine pepsin to the immobilized 3,5-diiodo-L-tyrosine [7–9]. Results of our studies [8,9] have further shown that there exist a difference in binding of pepsin and its zymogen to immobilized aromatic amino acid derivatives coupled via amino or via car-

* Corresponding author. Fax: +420-224-912-834.
E-mail address: zkuce@lfl.cuni.cz (Z. Kučerová).

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doi:10.1016/j.jchromb.2003.07.007

boxyl group to an inert matrix. In the present communication, we have studied optimum conditions of an interaction of porcine pepsin and its zymogen with immobilized ligands derived from the enzyme substrate: *N*-acetyl-L-phenylalanine-3,5-diiodo-L-tyrosine.

2. Experimental

2.1. Chemicals

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

2.2. Preparation of affinity carriers

2.2.1. Activation of Sepharose 4B with divinyl sulfone [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 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. Ligand coupling

In the first step, ethylene diamine was coupled 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 of 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 ligand (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.

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

2.3. Affinity chromatography of porcine pepsin

Affinity chromatography of porcine pepsin was performed using Bio-Scale MT2 column (52 mm × 7 mm) (Bio-Rad Labs., Hercules, CA, USA) filled with the prepared carriers. The affinity column was equilibrated with the starting buffer. Porcine pepsin (Sigma) (1–5 mg/1 ml of starting buffer) was applied to a column and non-adsorbed proteins were eluted with the starting buffer. For the elution of adsorbed enzyme different buffer soluions were tested (at a flow rate of 1 ml/min, 1 ml fractions were collected). Each fraction was immediately after the elution mixed with 1 M HCl (0.05 ml). The proteolytic activity in elution fractions was determined by Anson and Mirsky method [11]. The following composition of the starting buffer was examined: 0.02–1 M acetate buffer, pH 3.0–6.0. Adsorbed proteins were eluted under different conditions: 0.05–0.1 M acetate buffer, pH 6.7; 0.05–0.1 M phosphate buffer, pH 6.9; 0.05–0.1 M MES, pH 6.7 and all these buffers containing 0.5–1 M NaCl.

For the determination of capacity, the solution of porcine pepsin (200 mg/200 ml of 0.05–0.1 M acetate buffer, pH 3.5–6.0) was applied to the affinity column (volume of 2 ml) till the proteolytic activity was determined in the eluate. Capacity was expressed in mg of the enzyme adsorbed to the affinity carrier.

For the determination of an amount of adsorbed protein to affinity carriers, calibration curve was constructed: the dependence of peak area on an amount of enzyme (1–5 mg) applied to the affinity carrier.

2.4. Affinity chromatography of porcine pepsinogen

The affinity column was equilibrated with the starting buffer. Porcine pepsinogen in starting buffer was applied to a 1 ml column (26 mm × 7 mm i.d.). Non-adsorbed proteins were eluted with the starting buffer. For the elution of adsorbed enzyme different buffer solutions were tested (at a flow rate of 0.3 ml/min, 3 ml fractions were collected). The following composition of the starting buffer was examined: 0.02–1 M acetate buffer, pH 5.0–6.2. Adsorbed proteins were eluted under different conditions: 0.05–0.1 M acetate buffer, pH 5.2–6.7; 0.05–0.1 M phosphate buffer, pH 6.9; 0.05–0.1 M MES, pH 6.7 and all these buffers containing 0.5–1 M NaCl.

Proteolytic activity was determined after activation with HCl solution by Anson and Mirsky method [11].

2.5. Analytical methods

The amount of coupled ligands in the prepared affinity carriers was determined by absorption measurement at 290 nm for 3,5-diiodo-L-tyrosine and at 240–250 nm for *N*-acetyl-L-phenylalanine after the gel solubilization in 50% glycerol.

Proteolytic activity was determined by the method of Anson and Mirsky [11] as trichloracetic acid—soluble peptides using hemoglobin as a substrate. One unit will produce $\Delta A_{280} = 0.001\,\text{min}^{-1}$ at pH 2 at 37 °C.

3. Results and discussion

3.1. Preparation of immobilized amino acid derivatives

We have prepared two affinity carriers containing immobilized both components of the enzyme substrate used for the

Table 1
Ligand content in the prepared affinity gels and their capacity for porcine pepsin and pepsinogen

Affinity gel	Ligand content (μmol/ml of gel)[a]	Capacity of gel for pepsin[b]	Capacity of gel for pepsinogen[b]
N-Acetyl-L-Phe-Sepharose	4.5	76	66
3,5-Diiodo-L-Tyr Sepharose[c]	4.7	50	84

[a] Based on the absorbance measurements.
[b] Capacity expressed as mg of the enzyme adsorbed to 1 ml of a carrier.
[c] Coupled to Sepharose via carboxyl group.

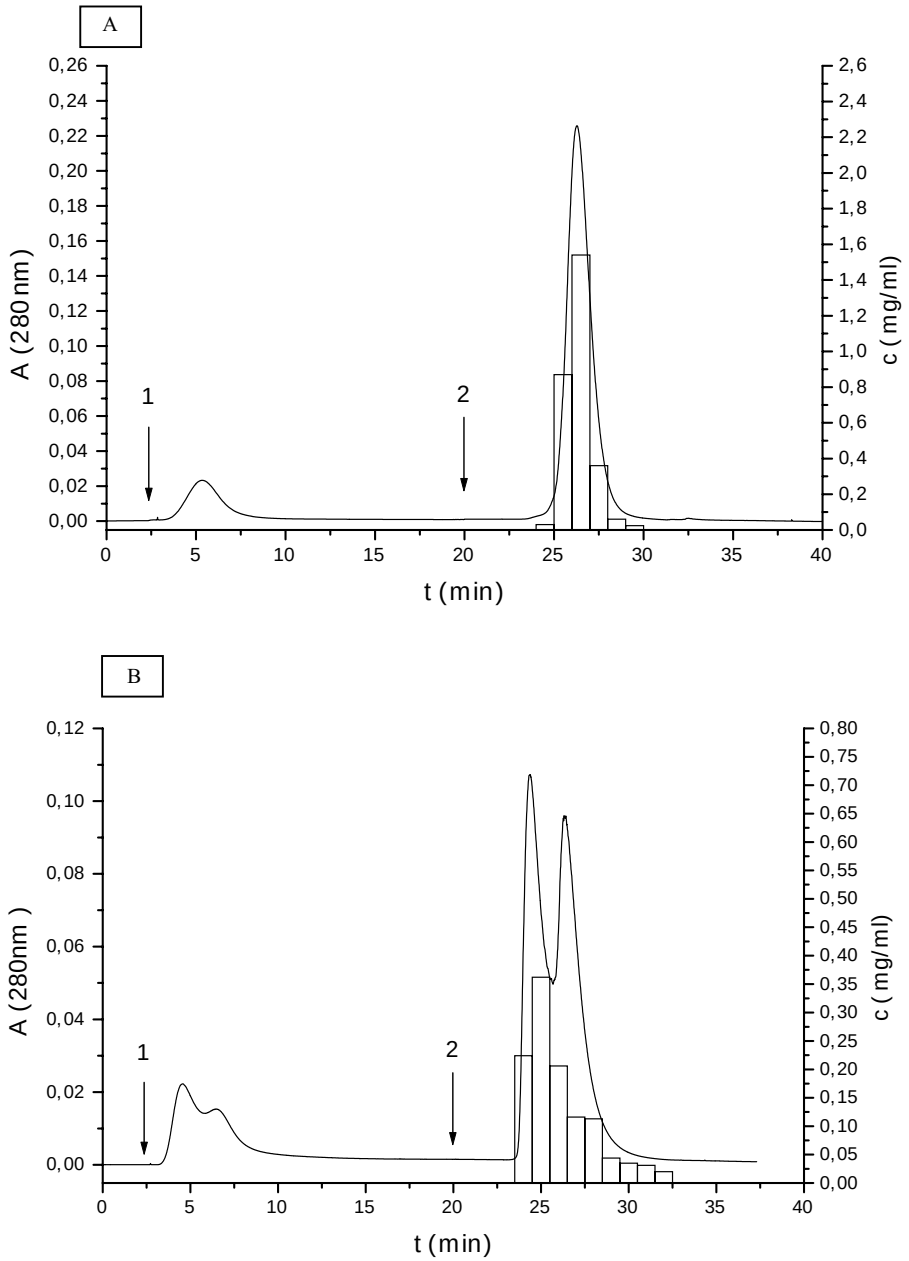

Fig. 1. Affinity chromatography of porcine pepsin on immobilized *N*-acetyl-L-phenylalanine (A) and 3,5-diiodo-L-tyrosine (B). (A) (1) 0.1 M acetate buffer, pH 3.7; (2) 0.1 M acetate buffer, pH 3.70 containing 0.5 M NaCl. (B) (1) 0.05 M acetate buffer, pH 6.0; (2) 0.05 M acetate buffer, pH 6.0 containing 1 M NaCl. c: concentration of active porcine pepsin A (mg/ml) as determined from the linear dependence of peak area vs. amount of applied enzyme.

Table 2
Optimum conditions of affinity chromatography of porcine pepsin and pepsinogen on immobilized *N*-acetyl-L-phenylalanine and 3,5-diiodo-L-tyrosine

Enzyme	Immobilized ligand	Starting buffer	pH of starting buffer	Eluting buffer	pH of eluting buffer
Pepsin	*N*-Acetyl-L-phenylalanine	0.1 M acetate	3.7	0.1 M acetate + 0.5 M NaCl	3.7
	3,5-Diiodo-L-tyrosine	0.05 M acetate	6.0	0.05 M acetate + 1 M NaCl	6.0
Pepsinogen	*N*-Acetyl-L-phenylalanine	0.02 M acetate	5.2	0.02 M acetate + 0.5 M NaCl	5.6
	3,5-Diiodo-L-tyrosine	0.02 M acetate	5.2	0.02 M acetate + 0.5 M NaCl	5.6

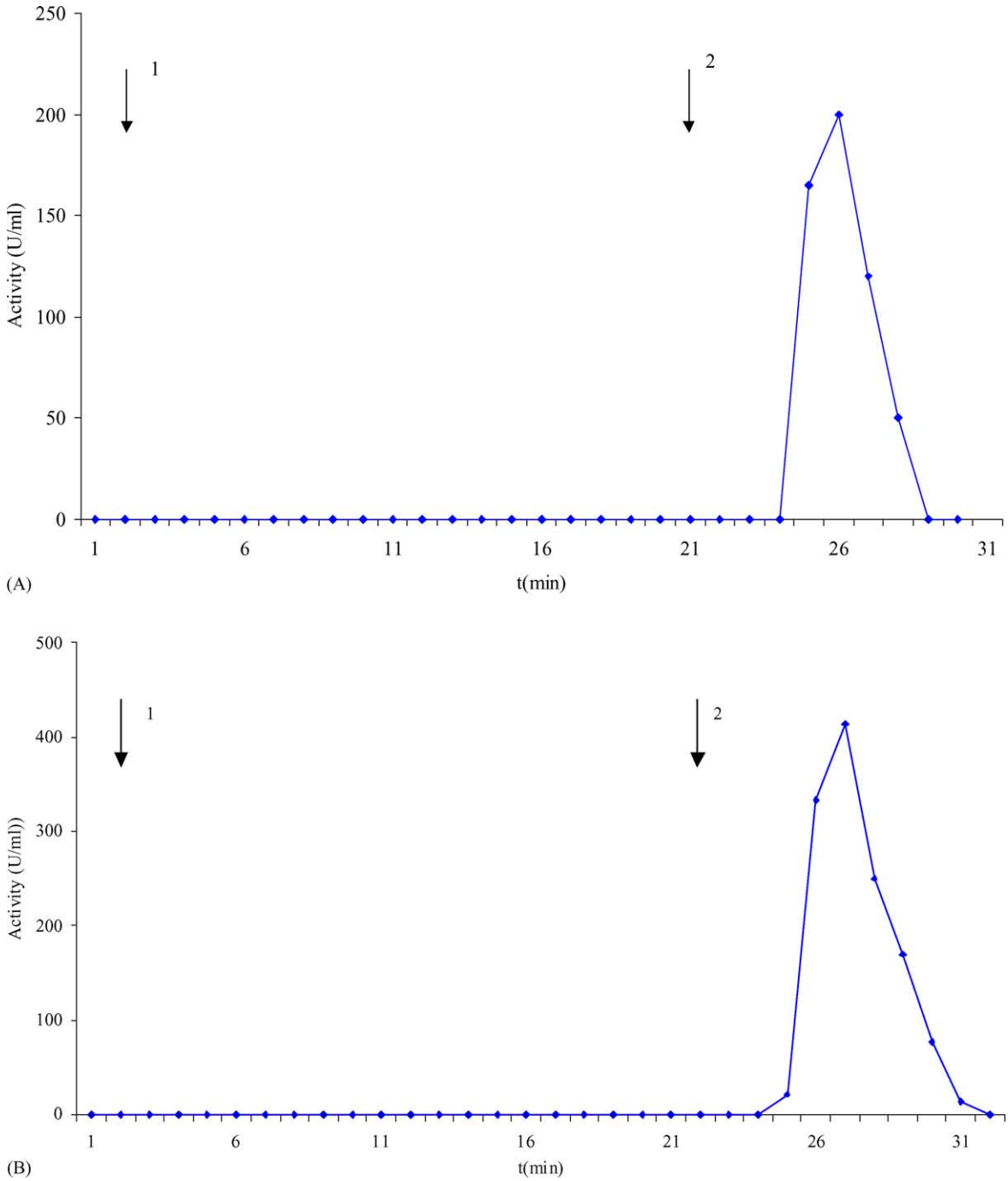

Fig. 2. Affinity chromatography of porcine pepsinogen on immobilized *N*-acetyl-L-phenylalanine (A) and 3,5-diiodo-L-tyrosine (B). (1) 0.02 M acetate buffer, pH 5.2; (2) 0.2 M acetate buffer, pH 5.6 containing 0.5 M NaCl.

determination of pepsin activity: *N*-acetyl-L-phenyl-alanine and 3,5-diiodo-L-tyrosine.

For the coupling of these ligands to Sepharose via free carboxyl group, it was necessary to prepare the ethylene diamine derivative of Sepharose. Ethylene diamine was coupled to divinyl sulfone activated Sepharose and for the immobilization of both ligands, carbodiimide reaction was used. In the case of 3,5-diiodo-L-tyrosine, BOC-protected derivative the amino acid was linked to the matrix. The protecting groups was removed by hydrolysis of the final product.

The content of ligands immobilized to Sepharose was determine by the measurement of UV absorption spectra (Table 1).

3.2. Affinity chromatography of porcine pepsin and pepsinogen

Prepared affinity carriers were characterized by determination of capacity for porcine pepsin. The capacity was determined as the amount of enzyme adsorbed onto 1 ml of the carrier. Results are presented in Table 1.

Affinity chromatography of porcine pepsin on immobilized *N*-acetyl-L-phenylalanine and 3,5-diiodo-L-tyrosineis shown in Fig. 1A and B, respectively. The enzyme was adsorbed to both affinity carriers at pH 3.5–4.0 and at ionic strength lower than 0.05 M. Under optimum conditions (Table 2), porcine pepsin was completely adsorbed to the affinity columns, no proteinase activity was detected in the flow-through fraction (Fig. 1). The non-active fraction of proteins represented only small portion of proteins present in the sample of porcine pepsin.

The enzyme adsorbed to the immobilized ligands was not possible to elute only by increasing pH to 6.7 without an increase of ionic strength. For an elution pepsin from ligands immobilized via carboxyl group, it was necessary to increase ionic strength up to 0.5 M. A change of the buffer composition (0.1 M phosphate, 0.1 M acetate or MES buffers) did not affect the behavior of porcine pepsin in the affinity chromatography experiment. Much weaker interaction was observed in the case of porcine pepsin and immobilized L-tyrosine derivatives coupled to Sepharose via amino group [9]: in this case proteinase was eluted from the column on increasing the pH from 3.5 to 5.4.

The recovery of the loaded enzyme to both affinity carriers was approximately 96% for *N*-acetyl-L-phenylalanine-Sepharose and 88% for immobilized 3,5-diiodo-L-tyrosine. The reproducibility of results of affinity chromatography was high; there were performed 40 experiments in the course of 3 months: changes of the amount of adsorbed enzyme were not higher than ±6%. The linear dependence of an amount of adsorbed enzyme on an amount of loaded protein was determined in the range of 1–5 mg of pepsin per 2 ml of the affinity gel.

The presence or absence of BOC protecting group did not significantly influence the enzyme affinity to immobilized ligand (not shown).

It is interesting that porcine pepsin was separated by affinity chromatography on 3,5-diiodo-L-tyrosine immobilized via carboxyl group into two active fractions. Such separation of porcine pepsin was observed only in the case of affinity chromatography on iodinated L-tyrosine-Sepharose but not on immobilized 3,5-diiodo-L-tyrosine coupled via amino group [8]. It has been shown, that the presence of two isoforms is not due to a partial dephosphorylation of the porcine proteinase [8]. The existence of multiple forms of porcine pepsin differing in their affinity to 3,5-diiodo-L-tyrosine could be explained by the presence of other aspartic proteinases in the commercial enzyme preparation or by the presence of partially degraded pepsin molecules.

The prepared gels were further used to study the binding porcine pepsinogens to the immobilized ligands. Optimum conditions of the affinity chromatography of pepsinogens are given in Table 2. Porcine pepsinogen was loaded to affinity gels at pH 5.2 and desorbed by 0.2 M acetate buffer, pH 5.6, containing 0.5 M NaCl Results of affinity chromatography of porcine pepsinogen on immobilized *N*-acetyl-L-phenylalanine and 3,5-diiodo-L-tyrosine linked via carboxyl group is presented in Fig. 2A and B, respectively. Under optimum conditions no activity was eluted from both immobilized ligands by starting buffer.

4. Concluding remarks

(i) Immobilized 3,5-diiodo-L-tyrosine and *N*-acetyl-L-phenylalanine coupled via carboxyl group are suitable matrices to study binding properties of pepsin and its zymogen.
(ii) The presence of free non-substituted carboxyl group results in a decrease of porcine pepsin affinity to the immobilized ligand; porcine pepsinogen does not interact with amino acid derivatives coupled via amino group.
(iii) Porcine pepsinogen and especially pepsin are adsorbed to both prepared affinity carriers strongly, for their elution an increase of ionic strength is necessary.

Acknowledgements

This work was supported by the Grant No. 303/01/1216 of the Grant Agency of the Czech Republic and by the Ministry of Education of the Czech Republic, Grants No. MSM 113100001 and 111100003.

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