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# Interaction of pepsin with aromatic amino acids and their derivatives immobilized to Sepharose☆

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**Abstract**

The interaction of porcine pepsin A with immobilized derivatives of aromatic amino acids was investigated. Divinyl sulfone-activated Sepharose was used to immobilize *N*-acetyl-L-phenylalanine and 3,5-diiodo-L-tyrosine via their free carboxyl groups and L-tyrosine via its amino group. Immobilized L-tyrosine was iodinated after coupling. The optimum conditions for the separation of porcine pepsin A using the prepared affinity carriers were studied and the following parameters were established: enzyme recovery, reproducibility of analyses, capacity and dependence of the elution peak area on the concentration of the loaded enzyme. The ability of the prepared affinity carriers to retain various types of proteins was compared under optimum conditions for porcine pepsin A separation. While immobilized 3,5-diiodo-L-tyrosine and iodinated L-tyrosine-Sepharose adsorbed relatively high amounts of bovine serum albumin and ovalbumin, only negligible amounts of these proteins were adsorbed to immobilized *N*-acetyl-L-phenylalanine. The behavior of porcine pepsin A was the same as its complex with pepstatin A on the prepared affinity carriers, indicating that the enzyme active site is not involved in the studied interaction.
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*Keywords:* Porcine pepsin A; Affinity chromatography; Immobilized derivatives of aromatic amino acids

## 1. Introduction

Affinity chromatography is a unique separation method based on the biospecific recognition of an immobilized ligand by proteins. Protein isoforms differing only slightly in binding properties might be separated by affinity-based techniques. Such slight differences in binding properties can be important to the physiological function of the protein isoforms. Gastric aspartate proteinases are such proteins.

Affinity-based separation techniques represent a valuable tool not only for the purification of proteins, but also, for the separation of various proteins differing only slightly in their binding properties, for analytical purposes, for studies of the structure of an active protein or binding site and in many other applications. The term 'affinity separation technique' includes a broad spectrum of different types of protein adsorption interactions with immobilized ligands from strictly specific to interactions whose mechanism is not fully understood. A choice of suitable ligand is evidently the most important factor in the successful application of these techniques. However, there are other components of the system used that influence the affinity separation, e.g. the nature of the matrix, matrix activation, spacer arm properties, mechanism of ligand coupling, etc. In the case of gastric mucosa aspartate proteinases, only a limited number of ligands have been reported to interact with these enzymes that could be successfully used in separation processes: antibodies, poly-lysine, peptide inhibitors [1,2], or substrate-derived analogs [3–5].

Two major groups of aspartate proteinases, pepsin A (EC 3.4.23.1) and pepsin C (EC 3.4.23.3) are present in human gastric juice [6]. They are synthesized in gastric mucosa in the

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form of inactive zymogens, pepsinogens and converted to active enzymes in the gastric juice [7]. These enzymes and their corresponding zymogens are of great medical and pharmaceutical interest; changes in their levels are associated with some gastric diseases [8,9]. It was proved that serum levels of pepsinogens reflect the morphological and functional status of the gastric mucosa. A low level of pepsinogen A or low ratio of pepsinogen A/pepsinogen C in serum was found to be markers for gastric cancer [9–12]. Determination of the content and mutual relationship of individual forms of human pepsins and their zymogens were found to be important from a diagnostic point of view [8,9,13–16].

In our previous work [3–5], we used immobilized derivatives of aromatic amino acids linked to divinyl sulfone-activated Sepharose to study the binding properties of porcine and human pepsins and their zymogens. The capacity of affinity carriers containing ligands coupled via free carboxyl group for porcine pepsin A was found to be higher than that of ligands using an amino group for the linkage [3,5].

Sepharose activated with divinyl sulfone, originally developed for cross-linking agarose gel [17], was found to be a suitable matrix for coupling various types of ligands (e.g. saccharides [18–20], peptides and proteins [21,22], or L-glyceryl phosphorylcholine [23]). Divinyl sulfone-activated Sepharose was also used for the preparation of thiophilic carriers (T-gels) [24]. Thiophilic gels have proved to be a useful tool in the selective purification of immunoglobulins from various sources (e.g. Refs. [25–33]), but it could be also employed in the purification of other proteins [26,33–35]. The thiophilic adsorption of proteins is based on an interaction with a sulfone group close to a thioether group of the T-gel ligand. Other nucleophilic atoms (O or N) may sometimes substitute a sulfur atom [26,36,37]. Carriers with sulfone-aromatic ligands were found to possess thiophilic properties and are useful in immunoglobulin separation [38–40]. A similar mechanism might participate in binding aspartate proteinases to carriers containing aromatic amino acids immobilized to divinyl sulfone-activated Sepharose [3–5].

This paper describes the results of a detailed study on the interaction of porcine pepsin A with derivatives of aromatic amino acids immobilized via a carboxyl or amino group to Sepharose activated with divinyl sulfone.

## 2. Experimental data

### 2.1. Chemicals

Porcine pepsin A (EC 3.4.23.1), L-tyrosine, *N*-acetyl-L-phenylalanine, pepstatin A, ovalbumin (albumin from chicken egg), α-chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), ethylenediamine dihydrochloride, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, divinyl sulfone, Sepharose 4B and *N*,*N*-dimethylformamide were purchased from Sigma–Aldrich (Praha, Czech Republic). Bovine serum albumin was obtained from Serva (Heidelberg, Germany).

The other chemicals were obtained from Ing. Petr Lukes (Uhersky Brod, Czech Republic).

### 2.2. Preparation of affinity carriers

#### 2.2.1. Activation of Sepharose 4B with divinyl sulfone [17]

Sepharose 4B (10 mL) was washed with distilled water (500 mL), then suspended in 0.2 M carbonate buffer (pH 10.7, 10 mL) and divinyl sulfone (1 mL) was added. The suspension was shaken at room temperature for 70 min. The gel was then washed with distilled water (500 mL) and 0.2 M carbonate buffer (pH 10.7, 200 mL).

#### 2.2.2. Coupling of ethylenediamine or L-tyrosine

Immediately after activation, divinyl sulfone-activated Sepharose 4B (10 mL) was mixed with 0.2 M carbonate buffer (pH 10.7, 10 mL) containing ethylenediamine dihydrochloride (2 g) or L-tyrosine (1 g) and the mixture was shaken at room temperature overnight. Afterwards the gels were washed with distilled water (500 mL) and 0.2 M carbonate buffer (pH 10.7, 200 mL). The prepared gels were then mixed with glycine solution (100 mg of glycine in 10 mL of 0.2 M carbonate buffer, pH 10.7) and shaken at room temperature for 2 h. Finally the gels were washed sequentially with distilled water (500 mL), 0.2 M carbonate buffer (pH 10.7, 200 mL) and distilled water (500 mL).

#### 2.2.3. Coupling of N-acetyl-L-phenylalanine or Boc-3,5-diiodo-L-tyrosine

A divinyl sulfone-activated gel containing an ethylenediamine spacer (10 mL) was suspended in the mixed solution containing the ligand (215 mg in 6 mL distilled water; *N*-acetyl-L-phenylalanine or Boc-3,5-diiodo-L-tyrosine) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (228 μL). The suspension was shaken for 24 h at room temperature and again washed with distilled water. An affinity carrier containing immobilized *N*-acetyl-L-phenylalanine was designated carrier A.

#### 2.2.4. Removal of protective Boc group from Boc-3,5-diiodo-L-tyrosine-Sepharose 4B

A gel containing immobilized Boc-3,5-diiodo-L-tyrosine was 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 (carrier B).

#### 2.2.5. Iodination of immobilized L-tyrosine to Sepharose 4B

L-Tyrosine-Sepharose 4B was washed with phosphate buffered saline, pH 7.4 (PBS) (200 mL) and mixed with PBS solution (7.8 mL) containing potassium iodide (2.4 g) and chloramine B (0.6 g). After 2 min, PBS solutions (7.2 mL) of sodium metabisulfite (0.1 g) and potassium iodide (2.4 g) were added. The prepared gel (carrier C) was washed with distilled water (500 mL).

### 2.3. Affinity chromatography of porcine pepsin A

Affinity chromatography was performed using a BioLogic System with a 1 mL sample loop and Bio-Scale MT 2 High-Resolution Column (52 mm × 7 mm i.d.; Bio-Rad, Hercules, CA, USA). The column was packed with the prepared carriers (carrier A, B or C) and washed with the starting buffer. Protein

(1 mg) or a mixture of porcine pepsin A and pepstatin A (1 mg of enzyme + 1 mg of inhibitor) was dissolved in the starting buffer (1 mL), filtered through a 1 μm pore size PTFE membrane filter (Whatman, Maidstone, UK) and applied to the column. Non-adsorbed proteins were washed out with the starting buffer (25–100 mM acetate buffer, pH 3.7–4.0 and 6.0). The adsorbed proteins were eluted using the elution buffer (50–100 mM acetate buffer, pH 3.7 or 6.0, containing 0.5 or 1.0 M NaCl or 50 mM phosphate buffer, pH 6.0, containing 0.3 M NaCl). Alternatively, 25–100 mM acetate buffer (pH 3.7–6.0) containing either *N*-acetyl-L-phenylalanine or 3,5-diiodo-L-tyrosine (1 mg dissolved in 2 μL *N*,*N*-dimethylformamide per 1 mL of the buffer) was used. The effluent was monitored at 280 nm and fractions (1 mL) were collected at a flow rate of 1 mL/min. Over the course of porcine pepsin A analyses, the pH value of each fraction was adjusted to pH 2 with 1 M HCl. The same conditions were used to study the adsorption of bovine serum albumin, ovalbumin, trypsin or α-chymotrypsin to the prepared affinity carriers.

### 2.4. Analytical methods

The proteolytic activity of porcine pepsin A in each collected fraction was determined using the method of Anson and Mirsky [41] as trichloroacetic acid-soluble peptides, using hemoglobin as a substrate. One unit will produce a $\Delta A_{280}$ of 0.001/min at pH 2.0 at 37 °C.

The protein content was determined by bicinchoninic acid assay [42] (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL, USA).

## 3. Results

### 3.1. Preparation of immobilized aromatic amino acid derivatives

The activation of Sepharose 4B with divinyl sulfone was used to couple the following derivatives of aromatic amino acids: *N*-acetyl-L-phenylalanine, 3,5-diiodo-L-tyrosine with a protected amino group (Boc-derivative) and L-tyrosine. L-Tyrosine was linked to the activated agarose matrix via its free amino group. For the immobilization of the other two ligands (that is *N*-acetyl-L-phenylalanine, 3,5-diiodo-L-tyrosine with protected amino group) via their free carboxyl groups, ethylene diamine was first coupled to divinyl sulfone-activated Sepharose 4B. Afterwards, the ligands were linked using a carbodiimide reaction. The reactions used for coupling the ligands are shown in Fig. 1. After immobilizing the aromatic amino acid derivatives, L-tyrosine-Sepharose 4B was further modified by iodination performed via the standard chloramine method, while Boc-3,5-diiodo-L-tyrosine-Sepharose 4B was hydrolyzed to remove the Boc protective group.

### 3.2. Affinity chromatography of porcine pepsin A

The interaction of porcine pepsin A with the prepared affinity carriers was compared. The results are shown in Fig. 2(a–c). The enzyme was adsorbed to affinity carriers A and C at pH 3.7–4.0 and at ionic strength 25–100 mM. In contrast, an increase in pH to 6.0 was required for porcine pepsin A adsorption to carrier B. Under these conditions, porcine pepsin A was completely adsorbed to all prepared affinity carriers. No

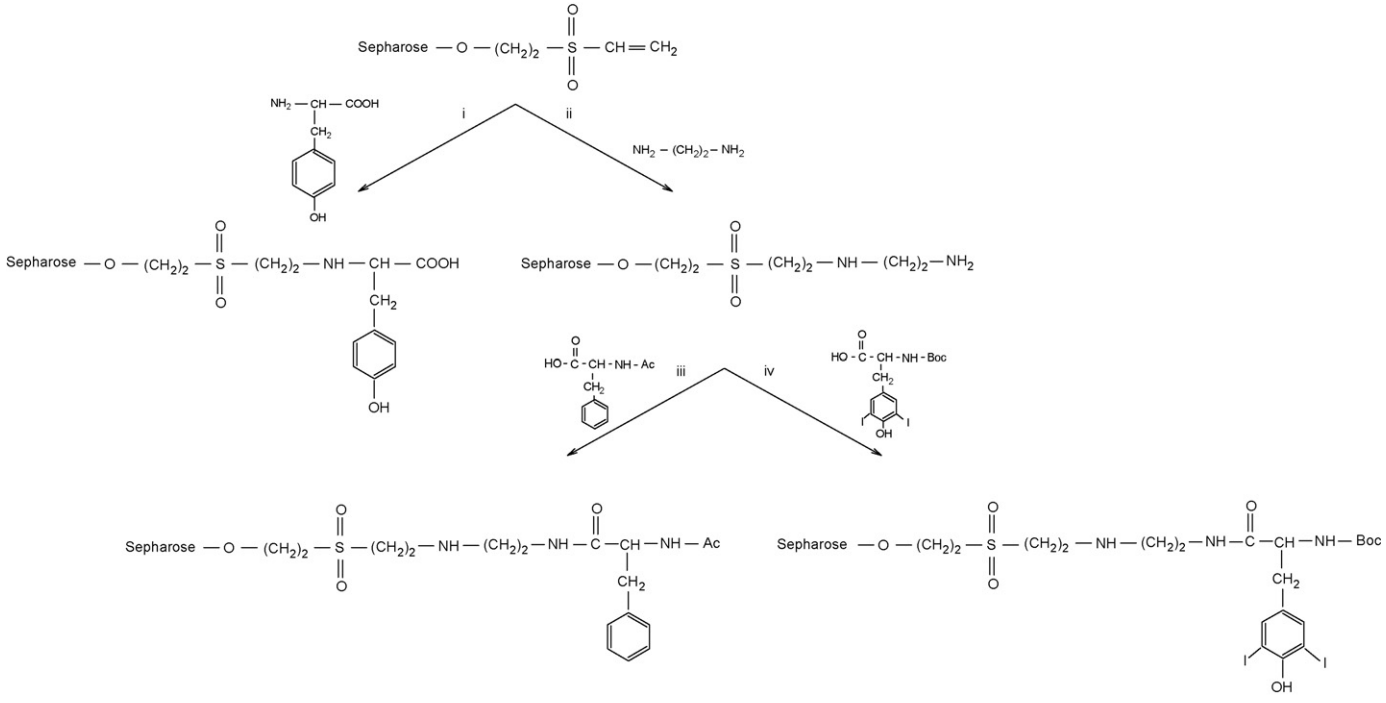


Fig. 1. Coupling of derivatives of aromatic acids to divinyl sulfone-activated Sepharose 4B. *Reaction conditions*: (i and ii) 0.2 M carbonate buffer, pH 10.7, at 20 °C for 18 h; (iii and iv) distilled water in the presence of carbodiimide at 20 °C for 24 h.

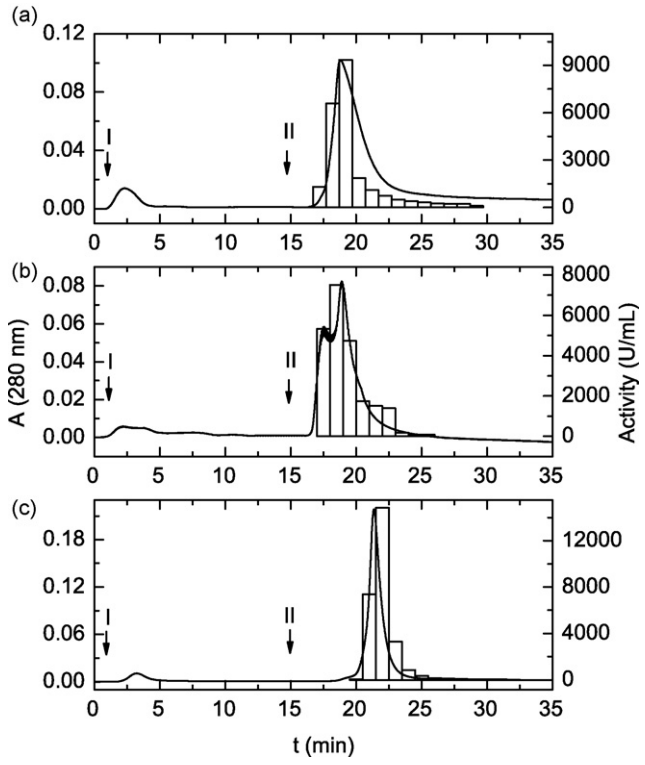


Fig. 2. Affinity chromatography of porcine pepsin A on carriers A (a), B (b) and C (c) under the optimum conditions. (a) I, 100 mM acetate buffer, pH 3.7; II, 100 mM acetate buffer, pH 3.7, containing 0.5 M NaCl; (b) I, 50 mM acetate buffer, pH 6.0; II, 50 mM acetate buffer, pH 6.0, containing 1.0 M NaCl; (c) I, 25 mM acetate buffer, pH 4.0; II, 50 mM phosphate buffer, pH 6.0, containing 0.3 M NaCl. Column graph: Activity, the enzyme activity determined by the Anson and Mirsky method [41]; solid line: *A* (280 nm), absorbance measured at 280 nm. Carrier A: *N*-acetyl-L-phenylalanine coupled via its free carboxyl group to divinyl sulfone-activated Sepharose 4B; carrier B: 3,5-diiodo-L-tyrosine coupled to divinyl sulfone-activated Sepharose 4B via its free carboxyl group; carrier C: L-tyrosine coupled via its free amino group to divinyl sulfone-activated Sepharose 4B and then iodinated.

proteinase activity was detected in the flow-through fractions (Fig. 2a–c).

In the case of carriers A and B, the enzyme was only eluted by the starting buffer (pH 3.7 or 6.0) after the addition of NaCl (0.5 or 1.0 M). Adding NaCl to the starting buffer (0.3–1.0 M NaCl) was not enough to elute porcine pepsin A adsorbed to affinity carrier C, an increase in the pH of the elution buffer to 6.0 was also required.

The non-specific adsorption of other proteins of various types to the studied affinity carriers was investigated under the optimum conditions for porcine pepsin A adsorption to each of the tested carriers. The behavior of the following proteins besides porcine pepsin A was examined: bovine serum albumin, ovalbumin, trypsin and α-chymotrypsin. The results obtained are presented in Fig. 3. Under the conditions used, high amounts of bovine serum albumin and ovalbumin were retained on carriers B and C. In contrast, carrier A was the most ideal from this point of view. Table 1 summarizes the amounts of the various proteins recovered from the prepared affinity carriers under the conditions used for the elution of porcine pepsin A. Under such conditions, no or negligible amounts of other proteins were

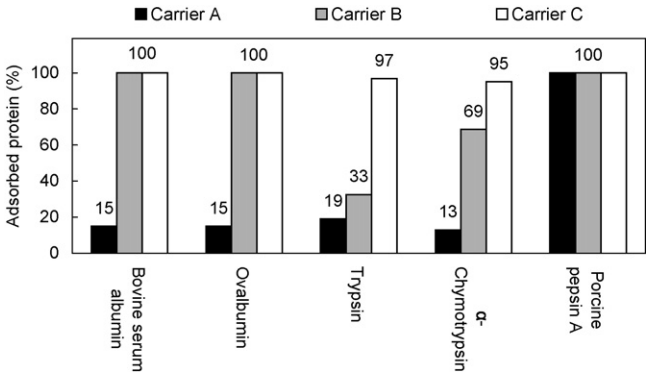


Fig. 3. Adsorption of proteins to carriers A, B and C under the optimum conditions for porcine pepsin A. Adsorbed protein: Amount of protein adsorbed to the affinity carrier expressed in percentage of applied protein (equal to 100%). Carrier A: *N*-Acetyl-L-phenylalanine coupled via its free carboxyl group to divinyl sulfone-activated Sepharose 4B; carrier B: 3,5-diiodo-L-tyrosine coupled to divinyl sulfone-activated Sepharose 4B via its free carboxyl group; carrier C: L-tyrosine coupled via its free amino group to divinyl sulfone-activated Sepharose 4B and then iodinated.

eluted from affinity carrier A, while almost 100% of the adsorbed porcine pepsin A was desorbed.

While the adsorption capacity of prepared affinity carriers B and C for porcine pepsin A was 50 and 42 mg/mL of the wet gel, respectively, that of carrier A was significantly higher (77 mg/mL) (Table 2). The linear dependence of bound porcine pepsin A on the applied enzyme was determined in the concentration range 125–5000 μg/mL for carrier A, 1000–5000 μg/mL for carrier B and 125–2000 μg/mL for carrier C, with regression coefficients ($R^2$) in the range 0.993–0.999.

### *3.3. Interaction of porcine pepsin A with immobilized ligands in the presence of pepstatin A*

The behavior of a mixture of porcine pepsin A and pepstatin A (inhibitor) on the prepared affinity carriers was compared with that of the enzyme without the inhibitor. The results obtained with carrier A are shown in Fig. 4. The presence of pepstatin A did not affect enzyme behavior. Porcine pepsin A was adsorbed

Table 1
Desorption of proteins from the prepared affinity carriers

| | Recovery (%) | | |
|---|---|---|---|
| | Carrier A | Carrier B | Carrier C |
| Bovine serum albumin | 0 | 71 | 0 |
| Ovalbumin | 0 | 75 | 69 |
| Trypsin | 2 | 15 | 3 |
| α-Chymotrypsin | 2 | 84 | 50 |
| Porcine pepsin A | 96 | 88 | 96 |

Optimum conditions for affinity chromatography of porcine pepsin A on carriers A–C were used (see Fig. 2). Recovery: Eluted amount of adsorbed protein from affinity carrier expressed in percent of applied protein (equal to 100%); carrier A: *N*-acetyl-L-phenylalanine coupled via its free carboxyl group to divinyl sulfone-activated Sepharose 4B; carrier B: 3,5-diiodo-L-tyrosine coupled to divinyl sulfone-activated Sepharose 4B via its free carboxyl group; carrier C: L-tyrosine coupled via its free amino group to divinyl sulfone-activated Sepharose 4B and then iodinated.

Table 2
Comparison of some characteristics of the prepared affinity carriers

| Characteristics | Carrier A | Carrier B | Carrier C |
|---|---|---|---|
| Reproducibility R.S.D. (%) | 2 | 4 | 5 |
| Capacity (mg/mL) | 76.5 | 49.7 | 41.5 |
| $R^2$ | 0.993 | 0.998 | 0.999 |
| Recovery (%) | (96 ± 1) | (88 ± 1) | (96 ± 2) |

Reproducibility: Expressed as R.S.D. (%) of elution peak area for ten repeated individual experiments; capacity: expressed in mg of porcine pepsin A adsorbed to 1 mL of wet carrier; $R^2$: regression coefficient of the linear dependence of the elution peak area on the applied amount of porcine pepsin A; recovery: eluted amount of adsorbed porcine pepsin A from affinity carrier expressed in percent of applied enzyme (equal to 100%); carrier A: *N*-acetyl-L-phenylalanine coupled via its free carboxyl group to divinyl sulfone-activated Sepharose 4B; carrier B: 3,5-diiodo-L-tyrosine coupled to divinyl sulfone-activated Sepharose 4B via its free carboxyl group; carrier C: L-tyrosine coupled via its free amino group to divinyl sulfone-activated Sepharose 4B and then iodinated.

and desorbed under the same conditions, both in the presence and absence of the inhibitor. In the presence of pepstatin A, porcine pepsin A was desorbed in the form of an inactive complex. The same results were obtained for carriers B and C (results not shown).

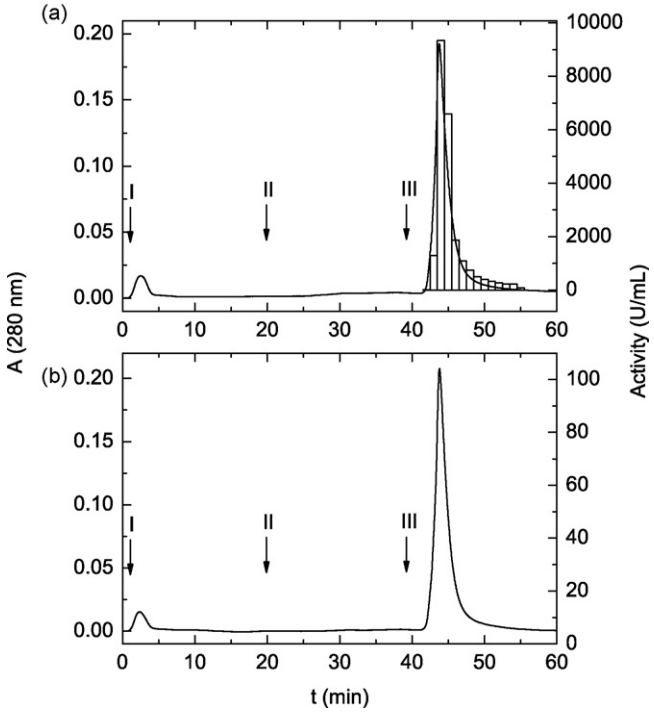


Fig. 4. Affinity chromatography of porcine pepsin A on carrier A in the absence (a) and presence (b) of pepstatin A (inhibitor). I: 100 mM acetate buffer, pH 3.7; II: *N*-acetyl-L-phenylalanine (1 mg + 2 μL *N*,*N*-dimethylformamide) in 100 mM acetate buffer, pH 3.7 (1 mL); III: 100 mM acetate buffer, pH 3.7, containing 0.5 M NaCl. Column graph: Activity, the enzyme activity determined by the Anson and Mirsky method [41]; solid line: *A* (280 nm), absorbance measured at 280 nm; carrier A, *N*-acetyl-L-phenylalanine coupled via its free carboxyl group to divinyl sulfone-activated Sepharose 4B. (a) Porcine pepsin A (1 mg) dissolved in 100 mM acetate buffer (pH 3.7, 1 mL) was applied to the affinity column; (b) porcine pepsin A (1 mg) and pepstatin A (1 mg) dissolved in 100 mM acetate buffer (pH 3.7, 1 mL) was applied to the affinity column.

The binding of porcine pepsin A to immobilized derivatives of aromatic acids was also not influenced by the presence of the corresponding free ligand. No enzyme was released with *N*-acetyl-L-phenylalanine solution from porcine pepsin A adsorbed to carrier A (Fig. 4). Similarly, 3,5-diiodo-L-tyrosine solution had no effect on porcine pepsin A adsorbed to carrier B or C (results not shown).

## 4. Discussion

The choice of ligands used in this study was based on a substrate (*N*-acetyl-L-phenylalanine-3,5-diiodo-L-tyrosine) that is used to differentiate pepsin A and pepsin C [43]: both components of the dipeptide were immobilized to Sepharose either via a carboxyl group (*N*-acetyl-L-phenylalanine and 3,5-diiodo-L-tyrosine) or amino group (L-tyrosine). Previous studies [5] showed that the pepsin interaction with ligands derived from aromatic amino acids depended on the manner of coupling.

Porcine pepsin A was adsorbed to all the prepared affinity carriers (denoted A–C), but under different conditions. While the pH of the starting buffer necessary for complete porcine pepsin A adsorption was 3.7–4.0 for carriers A and C, pH 6.0 was optimal for carrier B. Adsorption of the enzyme to carrier C was neither dependent on an increased ionic strength of the starting buffer, nor did it require the presence of lyotropic salts. The retained enzyme was desorbed either by increased ionic strength (carrier A and B) or by an increase in pH and ionic strength (carrier C).

The chromatographic behavior of porcine pepsin A in the presence and absence of pepstatin A on the prepared affinity carriers was compared. Pepstatin A is a peptide inhibitor of aspartate proteinases, which binds tightly to the active site of the enzyme [44,45]. In our experiments, the behavior of porcine pepsin A and its complex with pepstatin A on all affinity carriers (A–C) was the same: the enzyme and the inactive complex were completely adsorbed to the affinity columns under the same conditions; the same was true for the desorption conditions. This observation might indicate that the enzyme active site is not involved in the adsorption to the immobilized ligands. Other hydrophobic and/or aromatic residues of the enzyme probably participate in the studied interactions. This fact was confirmed by the observation that corresponding free derivatives of amino acids did not release the adsorbed enzyme.

The behavior of pepsin and its complex with pepstatin A on immobilized ligands that are based on derivatives of aromatic amino acids differs from that of the same enzyme and its complex on Phenyl-Sepharose (Table 3) [46]. The complex of porcine pepsin A with pepstatin A exhibited reduced binding to Phenyl-Sepharose in comparison with the enzyme in the absence of the inhibitor [46]. Phenyl-Sepharose is very often used as a carrier in hydrophobic chromatography. The usefulness of this carrier has also been reported in the separation of aspartate proteinases from various sources [46–48].

Aromatic amino acid derivatives were coupled to divinyl sulfone-activated Sepharose either directly via an amino group or via an ethylene diamine spacer arm. In both cases, the amino group is coupled with the vinyl group of the divinyl sulfone-

Table 3
Comparison of chromatographic behavior of porcine pepsin A on prepared affinity carriers and Phenyl-Sepharose

| | Protein bound (%) | | Reference |
|---|---|---|---|
| | Pepsin | Pepsin + pepstatin A | |
| Phenyl-Sepharose | 95 | 61 | [46] |
| A-Sepharose | 100 | 100 | This paper |
| B-Sepharose | 100 | 100 | This paper |
| C-Sepharose | 100 | 100 | This paper |

A: *N*-Acetyl-L-phenylalanine coupled via its free carboxyl group; B: 3,5-diiodo-L-tyrosine coupled via its free carboxyl group; C: L-tyrosine coupled via its free amino group and then iodinated.

activated agarose matrix. The structure of the resulting affinity carrier corresponds to the general formula M–O–$CH_2$–$CH_2$–$SO_2$–$CH_2$–$CH_2$–X–Y [38,49]. Where M is the agarose matrix, X, besides S, can also be O or N and Y is an aromatic or heteroaromatic compound. Studies on the use of these affinity carriers were mainly directed to the thiophilic adsorption separation of different antibodies from various sources. It has been shown that the presence of a thio-ether group is important for introducing selectivity for the given group of proteins [38,49]. N-containing aromatic thiophilic carriers were much less selective for antibodies than regular T-gels [28,50]. Porath and Oscarsson [51] suggested that such immobilized ligands are intermediates between thiophilic and hydrophobic carriers. The similarity of our affinity carriers to these structures might explain the observed strong adsorption of bovine serum albumin and ovalbumin to the carriers we prepared. The thiophilic adsorption properties of the carriers used probably affect their interaction with porcine pepsin A to a lesser extent.

More experiments will be necessary to explain the different behavior of porcine pepsin A and its complex with pepstatin A on immobilized derivatives of aromatic amino acids and Phenyl-Sepharose.

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