Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1084 (2005) 207–213
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Preparation and testing of stationary phases and modified capillaries for affinity chromatography and affinity capillary electrophoresis of pepsin

Tereza Vařilová[a,*], Alice Vránková[a], Věra Pacáková[a], Marie Tichá[b], Karel Štulík[a]

[a] *Department of Analytical Chemistry, Charles University, Prague, Hlavova 2030/8, Praha 2, 12843 Czech Republic*
[b] *Department of Biochemistry, Charles University, Hlavova 2030/8, Praha 2, Czech Republic*

Abstract

Three stationary phases have been prepared for affinity liquid chromatography isolation and separation of porcine and human pepsin. The phases contain 3,5-diiodo-L-tyrosine (DIT) bound to the supports HEMA BIO VS, HEMA BIO E and EPOXY TOYOPEARL. These phases have been tested on a model sample of porcine pepsin A and applied to human pepsin. Fractions have been collected and the chymase activity determined in selected analyses. For affinity CE, capillaries have been prepared by modifying the wall with 3-aminopropyltriethoxysilane, followed either by direct binding of DIT, or by binding L-tyrosine that was subsequently iodated. The dissociation constant K_d has been determined for the pepsin–DIT complex from the changes in the electrophoretic mobilities.

Keywords: 3,5-Diiodo-L-tyrosine; Pepsin; Affinity liquid chromatography; Affinity capillary electrophoresis

1. Introduction

Pepsins are enzymes that belong among aspartate proteases; as most of them exhibit optimum activities in acidic media, they are also called acidic proteases. Pepsin is produced in vertebrates by stomach mucous membrane in the form of zymogene (pepsinogen) that is converted into pepsin at a low pH [1–4]. The presence and relative concentrations of pepsin A (EC 3.4.23.1, pepsin) and pepsin C (EC 3.4.23.3, gastricsin) vary in vertebrates in dependence on the species and genetic variation; in humans, they also vary during some stomach diseases [2]. The ratios among individual forms of human aspartate proteases and their zymogens constitute important diagnostic tools [2,4].

Pepsins A and C differ in their biochemical and immunological properties. Their molecular masses are around 35,000, or higher with pepsinogens (around 42,000) [5,6]. Pepsin A exhibits a high affinity toward 3,5-diiodo-L-tyrosine (DIT) [7,8]. The properties of porcine pepsin A are very similar to those of human pepsin, as can be seen in Table 1 [5,9].

Affinity chromatographic and electrophoretic techniques, based on a high selectivity of biospecific interactions and a high separation efficiency, play an important role in isolation of pepsins and pepsinogens and substantially extend the possibilities over other existing chromatographic methods used so far for pepsin isolation and analysis (see, e.g. references [10–13]). An increased affinity of pepsin toward substrates containing iodine-substituted tyrosine residues has already been observed [7,14,15]. This property has been used in preparations of affinity supports that permit selective adsorption of pepsin. However, low-pressure chromatography has been used. Capillary electrophoresis has been shown to be promising for porcine pepsin analysis [16–18]. This paper broadens the possibilities of pepsin isolation and characterization by describing the preparation of stationary phases for HPLC and of modified capillaries for affinity capillary electrophoresis.

* Corresponding author. Tel.: +420 221 951 229; fax: +420 224 913 538.
E-mail address: tereza.varilova@seznam.cz (T. Vařilová).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.022

Table 1
A comparison of the human and porcine pepsin properties [5,9]

	Theoretical molecular mass	Theoretical p*I*	Affinity to DIT	Affinity to *N*-acetyl-L-phe-D-phe-3,5-DIT	Affinity to ε-aminocaproyl L-phe-D-phe-OCH_3
Human pepsin A	34628	3.36	++	++	–
Human pepsin C	35461	3.59	++	+	–
Porcine pepsin A	34622	3.24	++	++	++

(++) strong interaction; (+) weak interaction; (–) not measurement.

2. Experimental

2.1. Chemicals

Porcine pepsin A (3100 units/mg; from porcine stomach mucosa), DIT (crystalline), ovalbumin and lysozyme (lyophilized powder, 50 000 units/mg protein) were purchased from Sigma Chemicals (St. Louis, USA); a sample of human pepsinogen and the Placer buffer (4 mL 0.1 mol/L $CaCl_2$ + 40 mL 0.2 mol/L CH_3COONa, pH 5.3) were provided by the Institute of Pathophysiology, First Faculty of Medicine, Charles University. The stationary phases Separon HEMA BIO 1000 E and Separon HEMA BIO 1000 VS were obtained from Tessek (Prague, Czech Republic) and the phase AF epoxy Toyopearl 650 M from Tossoh (Tokyo, Japan). Dried milk was a product of Nutricia (Opočno, Czech Republic); glycine was supplied by Reanal (Budapest, Hungary). All the other chemicals were products of Lachema (Brno, Czech Republic).

2.2. Apparatus

Liquid chromatographic measurements were carried out using a DIONEX instrument (Westmont, USA) provided with a gradient pump DIONEX P580 and a UV/VIS detector 170S/340S. The samples were injected through a RHEODYNE valve (Cotati, USA) with 10 and 100 μL loops. The data were handled by the Chromeleon 6.01 software (DIONEX).

A CRYSTAL CE SYSTEM ATI UNICAM (Cambridge, UK), Model 310, was used for capillary electrophoresis. The Chromatography Data Handling System Unicam 4880, version 1.1, was employed. The fused-silica capillary parameters: unmodified fused silica capillary from CACO (Bratislava, Slovak Republic), 75 μm i.d.; total length, L_T, 63 cm; length to the detector, L_D, 47 cm; and the same capillary modified by DIT, L_T 61 cm, L_D 47 cm. The capillary modified by a hydrophilic film of polyvinylalcohol (PVA) was obtained from Agilent Technologies (Palo Alto, USA) and its parameters were 75 μm i.d.; L_T, 87 cm; L_D, 75 cm.

2.3. Preparation of the stationary phase—binding of DIT

The procedure is based on references [4,19]: an amount of 3.5 g of a support containing either vinylsulphonic or epoxy active groups was rinsed with distilled water and mixed with the affinity ligand, 3,5-diiodo-L-tyrosine (500 mg of DIT suspended in 10 mL of a 0.2 mol/L carbonate buffer, pH 10.7). The suspension was shaken overnight at ambient temperature, washed three times with 50 mL distilled water, once with 50 mL of 0.2 mol/L carbonate buffer of pH 9.0 and once with 50 mL of 0.1 mol/L acetate buffer of pH 4.0. A small sample was collected for elemental analysis for iodine. An amount of 100 mg of glycine was dissolved in 10 mL of 0.2 mol/L carbonate buffer, pH 9.0, and added to the suspension to block the residual active groups of the support. The mixture was shaken overnight at room temperature, washed three times with 50 mL of distilled water, once with 50 mL of 0.1 mol/L acetate buffer of pH 4.0 and finally, once with 50 mL of distilled water. The stationary phase was packed into an 80 cm × 8 cm stainless steel column in the Watrex Company, Prague.

Reaction between the vinylsulphonic active groups and 3,5-diiodo-L-tyrosine corresponds to the scheme given in Fig. 1.

Three stationary phases were prepared, containing 3,5-diiodo-L-tyrosine bound to three different supports HEMA BIO VS (column no. 1 and 2; prepared twice), HEMA BIO E (column no. 3) and EPOXY TOYOPEARL (column no. 4). The amount of iodine was determined by elemental analysis and the amount of 3,5-diiodo-L-tyrosine was calculated from it (see Table 2). It can be seen from this table that the DIT amount bound to the epoxy activated supports (no. 3 and 4) is greater, by about 10 mg per g of support, than that bound to the divinylsulphone activated supports (no. 1 and 2). These differences follow from different numbers of the active functional groups.

2.4. Chromatographic conditions

The column was first washed with the starting mobile phase (0.05 mol/L acetate or Britton–Robinson buffer, both of pH 3.5) until the baseline stabilized (35–45 min). Sample was

Table 2
Amounts of DIT bound to the individual supports

Column	Affinity stationary phase	Amount of DIT (mg per g of support)
No. 1	HEMA BIO VS-DIT	48
No. 2	HEMA BIO VS-DIT	51
No. 3	HEMA BIO E-DIT	61
No. 4	EPOXY TOYOPEARL-DIT	64

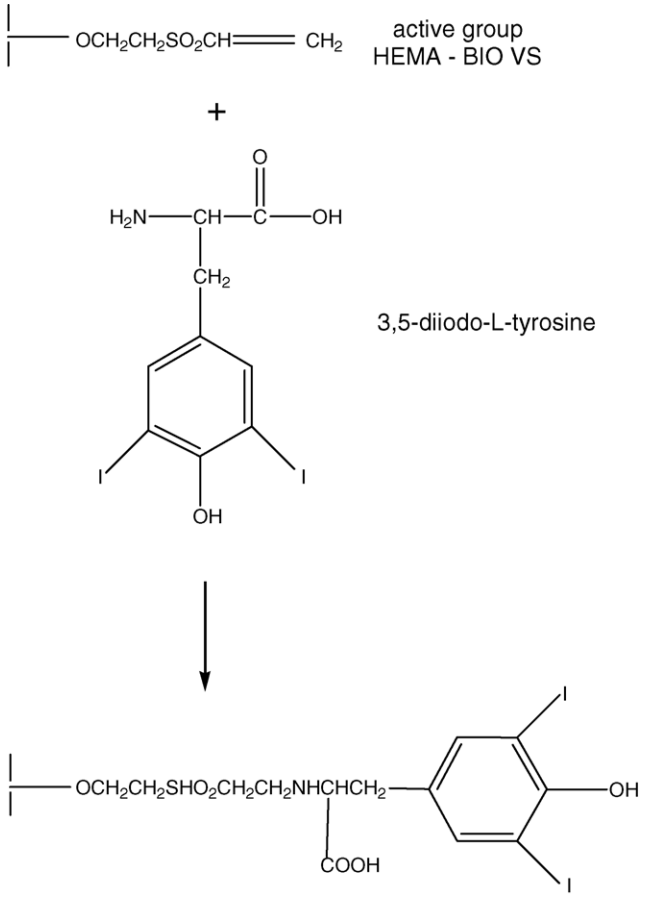

Fig. 1. Scheme of the vinylsulphone group reaction with 3,5-diiodo-L-tyrosine.

then injected and the starting mobile phase was further passed to elute non-adsorbed substances. Adsorbed substances were then eluted with the elution mobile phase (0.05 mol/L acetate buffer of pH 5.6, a phosphate buffer of pH 6.8 or a Britton–Robinson buffer of pH 6.8). A pH gradient elution was used with various gradient slopes, from 100% starting buffer to 100% elution buffer. The column was washed with the starting buffer after each analysis for baseline stabilization (25–35 min). Flow rate used was 0.5 mL min^{-1} and the detection wavelengths were 214 nm [16] and 280 nm [4].

The chymase activity (determination of the sum of proteases using milk curdling) was determined in some analyses [20]. Fractions of 1 mL were collected, the time of the beginning of milk curdling was measured and the reciprocal of the curdling time for 70 μL of the given elute fraction was plotted against the time of the fraction collection.

2.5. Human pepsin sample preparation

Homogenate was filtered through cotton wool. A 9 mm × 20 mm DEAE-Separon column with a volume of 1 mL was washed with 50 mL 0.05 mol/L Tris–HCl buffer of pH 7.3, allowed to swell for 1 h and then washed again with 30 mL of the same buffer. A 1 mL volume of the homogenate sample was applied to the column; three 10 mL portions of the above buffer were passed through the column and pepsinogen was then eluted with 10 mL of a 0.1 mol/L acetate buffer of pH 5.6 containing 0.5 mol/L NaCl. The first 5 mL of the eluate was dialyzed overnight against the 0.1 mol/L acetate buffer of pH 5.6. A new portion of the buffer was used after 2 h of dialysis. After dialysis, the sample was concentrated at a decreased temperature (refrigerator) from a volume of 5 to 1 mL in a Vivapore Concentrator vessel with a 7500 MWCO PES membrane. Finally, the pepsinogen was activated for about 45 min to yield pepsin using 1 mol/L HCl to attain a pH about 3.5. Sample was then injected onto the affinity column.

2.6. Preparation of silica capillary modified with 3,5-diiodo-L-tyrosine

The silica capillary was first washed at 1000 mbar for 10 min with 1 mol/L NaOH, for 10 min with deionised water, for 5 min with acetone and then air was passed for 5 min. Capillary was then activated by filling it with a solution of 3-aminopropyltriethoxy silane for 20 min, washing it with methanol, passing air through it and then heating it to 45 °C for 12 h. Two procedures were used to bind DIT to the amino groups thus formed.

The first procedure involved binding of L-tyrosine followed by its iodation. A 5% solution of BOC-L-tyrosine hydoxysuccinimide ester in dimethylformamide (DMF) was aspirated into the capillary and left there for 12 h at ambient temperature. Capillary was then washed with DMF and dichloromethane. It was washed with anhydrous trifluoroacetic acid (TFA) for 1 h at a pressure of 1000 mbar and ambient temperature to remove BOC, washed with tetrahydrofuran (THF) and with deionised water. The bound tyrosine was iodated by filling the capillary with a solution of 6.6 g of potassium iodide and 2.2 g of chloramine B in 10 mL of phosphate-buffered saline (PBS). Reaction was stopped after 5 min by washing the capillary with a solution of 50 mg of sodium pyrosulphite in 1.1 mL of PBS and finally with water. The other procedure was direct binding of DIT to the activated capillary wall: a solution containing 0.5 mL of DMF, 20 mg of BOC-diiodotyrosine and 15.5 mg of dicyclohexylcarbodiimide (DCC) was introduced into the capillary and left for 12 h at ambient temperature. Capillary was then washed with DMF, dichloromethane and was finally treated with a 50% solution of TFA in dichloromethane at ambient temperature for 1 h at a pressure of 1000 mbar. Afterwards, capillary was washed with dichloromethane, acetone and air was then passed through it.

Hydrophilically modified capillary was maintained in a reproducible state by washing with deionised water (1000 mbar), 10 mmol/L phosphoric acid and methanol. Prior to a several-day break in the measurements, the capillary was washed with deionised water for 20 min, with methanol for 5 min, and then dried by air. The diiodotyrosine-modified capillary was only washed with deionised water.

2.7. Electrophoretic conditions

The capillary was washed (1000 mbar) with deionised water and the separation buffer (0.025 or 0.05 mol/L phosphate buffer, pH 3.0, 6.8 or 8.5) at the beginning of measurement and washed with the separation buffer between runs. The detection wavelength was 214 nm [16].

2.8. Determination of the dissociation constant K_d

The dissociation constant of the porcine pepsin complex with DIT was determined from the mobility changes in a bare silica capillary [21–23]. DIT (ligand) plus thiourea (marker) were injected into the running buffer of 0.25 mol/L phosphate buffer, pH 3.0, containing porcine pepsin at increasing concentrations. The porcine pepsin concentration ranged from 0 to 14.3 μmol/L. UV detection at 214 nm was used.

3. Results and discussion

3.1. Stationary phases with immobilized DIT

Support activated by the manufacturer was used for preparation of stationary phases. The affinity ligand, 3,5-diiodo-L-tyrosine, was bound to the active vinylsulphone- and epoxy groups via amino group by the reaction given in Fig. 1. The ligand amount was calculated from the iodine content (see Table 2).

The stationary phases prepared were tested using porcine pepsin A samples. The effects of the buffer compositions and of the slope of the elution pH gradients were studied. Two UV detection wavelengths were tested, 214 and 280 nm. An optimum separation was attained for the 0.05 mol/L acetate buffer of pH 3.5 (starting) and pH 5.6 (eluting); detection at 214 nm was more sensitive. A pH gradient from 0 to 100% of the eluting buffer within 30 or 50 min was used.

The course of the affinity chromatography of porcine pepsin A in an acetate buffer on HEMA BIO VS-DIT (column no. 1) can be seen in Fig. 2. The enzyme was adsorbed at pH 3.5 and eluted by changing the pH to a value of 5.6. The course of the chymase activity is given in Fig. 3. It can be seen that the peaks eluted at the dead time (no. 1 and 2) did not exhibit chymase activity; therefore, these peaks probably corresponded to impurities present in the commercial porcine pepsin A preparation, or they were system peaks. Peak at time $t = 28$ min (no. 3) exhibited a chymase activity and thus corresponded to pepsin.

Dependence of the area of the porcine pepsin A peak on the pepsin concentration was obtained for all the stationary phases. It was observed that the peak area linearly increases with increasing pepsin concentration only to a concentration of 3.0 mg/L; the binding sites are then saturated and the area increases more slowly (Fig. 4).

The repeatability of measurement was found for HEMA BIO E-DIT (column no. 3) from a comparison of the peak areas for a series of six measurements performed with a new column and for another series of six measurements with the column after two months of its use. The coefficients of variation 0.9 and 6.2% demonstrate a limited life-time of the affinity stationary phase, as the repeatability substantially deteriorated after two months of use of the column.

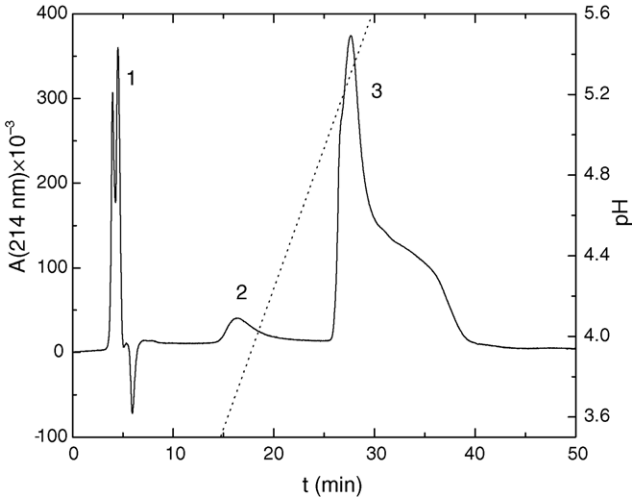

Fig. 2. Affinity HPLC of porcine pepsin on HEMA BIO VS-DIT (column no. 1)—protein elution: (1, 2) impurity; (3) porcine pepsin. Starting mobile phase, 0.05 mol/L acetate buffer, pH 3.5; elution mobile phase, 0.05 mol/L acetate buffer, pH 5.6; pH gradient elution from the 15th to 30th min (dashed line); flow rate 0.5 mL/min; sample concentration 1.0 mg/mL.

To verify specificity of the affinity binding of pepsin to 3,5-diiodo-L-tyrosine, a mixture of pepsin, ovalbumin and lysozyme was injected onto the column (Fig. 5). It can be seen that only pepsin interacts with DIT, because ovalbumin and lysozyme are not retained.

The course of the affinity chromatography of human pepsin on the EPOXY TOYOPEARL-DIT (column no. 4) is

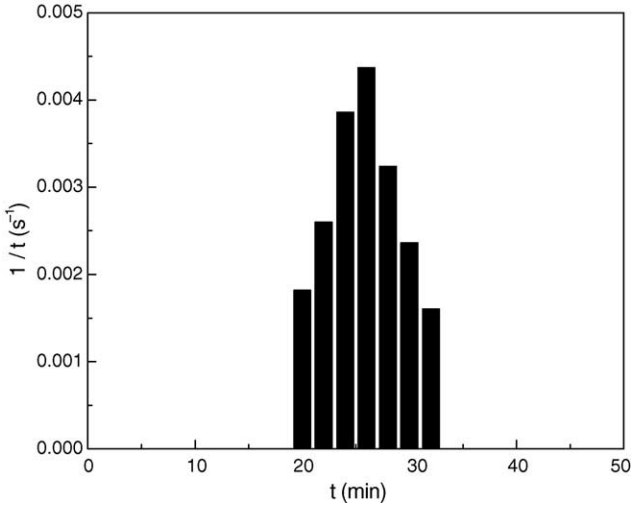

Fig. 3. Profile of chymase activity of porcine pepsin (samples taken during analysis on HEMA BIO VS-DIT (column no. 1)): *x*-axis, time of analysis; *y*-axis, reciprocal time corresponding to precipitation milk casein.

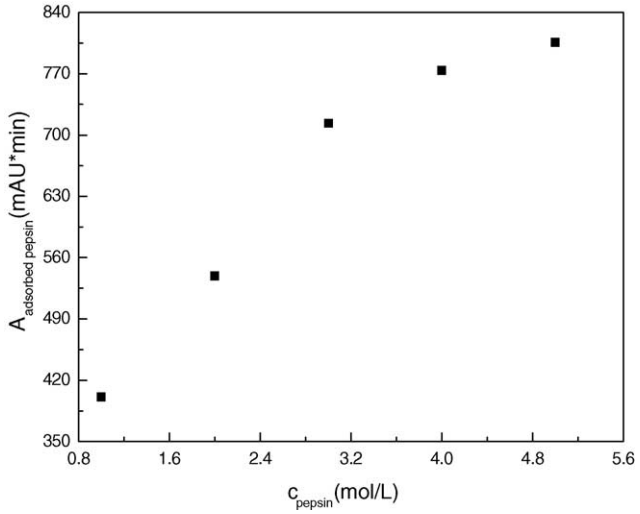

Fig. 4. Dependence of amount of adsorbed porcine pepsin A on its concentration on applied HEMA BIO VS-DIT (column no. 1). Amount of adsorbed enzyme expressed in the value of area (mAU* min) of pepsin peak. Experimental conditions as in Fig. 2.

shown in Fig. 6. It is evident that the behaviour of the model porcine pepsin A and of human pepsin is analogous (human pepsin elutes faster: its retention time is 26.2 min, whereas that of porcine pepsin A is 46.6 min). The chymase activity was measured for the fractions collected during the whole analysis. The peak located at the dead time (no. 1) exhibits no chymase activity and apparently corresponds to non-sorbing substances present in the human pepsin sample. The elution peak (no. 2) exhibits chymase activity whose maximum corresponds to the peak apex, i.e., it corresponds to pepsin. For

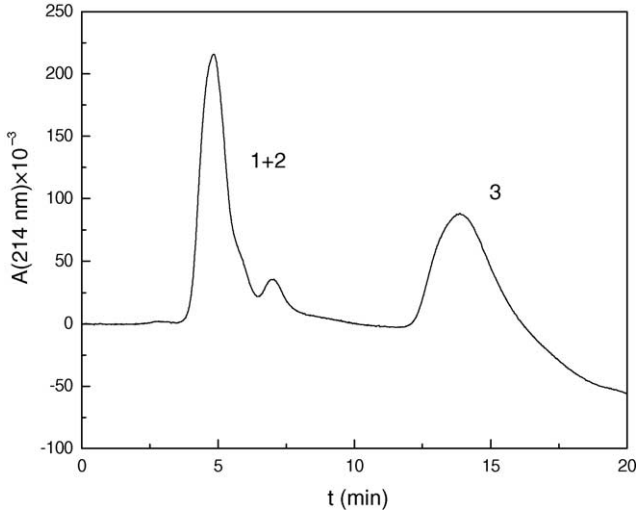

Fig. 5. Affinity chromatography of porcine pepsin, ovalbumin and lysozyme on HEMA BIO E-DIT (column no. 3): (1, 2) ovalbumin and lysozyme; (3) porcine pepsin. Starting mobile phase, 0.05 mol/L acetate buffer, pH 3.5; elution mobile phase, 0.05 mol/L acetate buffer, pH 5.6; pH gradient elution from the 15th to 30th min; flow rate 0.5 mL/min; sample concentration: ovalbumin 0.5 mg/mL, lysozyme 0.5 mg/mL, pepsin 0.5 mg/mL.

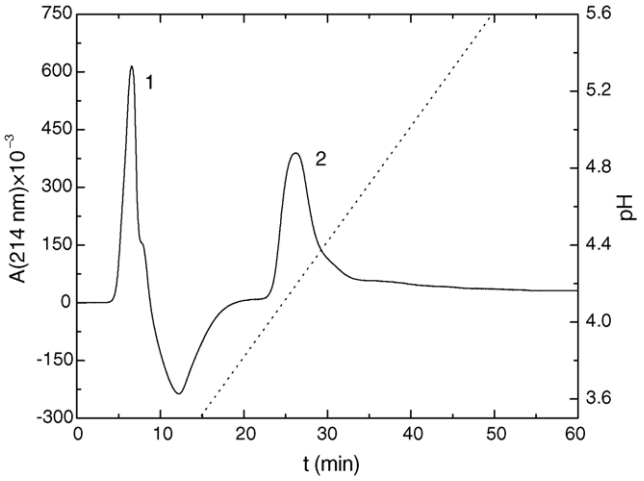

Fig. 6. Affinity chromatography of human pepsin on EPOXY TOYOPEARL-DIT (column no. 4): (1) non-sorbing material; (2) pepsin. Experimental conditions as in Fig. 2 except for the pH gradient: elution from 15th to 50th min.

the column EPOXY TOYOPEARL-DIT (column no. 4), the time of the elution pH gradient from pH 3.5 to pH 5.6 had to be prolonged (from 15th to 50th min).

3.2. Affinity capillary electrophoresis

Affinity capillary electrophoresis has become a popular method for studying protein–ligand interactions [24,25]. An interaction of pepsin with DIT has been studied by ACE in this paper.

Commercial capillary hydrophilically modified with PVA has been recommended for separation of peptides and proteins [17]. An electropherogram of porcine pepsin in this capillary is shown in Fig. 7. A partial separation of porcine pepsin A can be seen in Fig. 7. It was reported that separated peaks correspond to pepsin glycoforms [17,18] or that they reflect the autodigestion of porcine pepsin [16].

We prepared DIT-modified capillaries for ACE, using the procedure described in Section 2. DIT was bound to the capillary by two different procedures and the capillaries were tested with porcine pepsin. We found that reproducible results were only obtained with the capillary prepared by binding L-tyrosine to the wall and subsequent iodation. A CE analysis of porcine pepsin A in this capillary is given in Fig. 8. Peak of porcine pepsin A is separated into several isoforms. A comparison with Fig. 7 reveals that the capillary with immobilized DIT yielded a better separation and a shorter migration time.

3.3. Determination of the dissociation constant of the DIT–porcine pepsin complex

Dynamic equilibrium affinity electrophoresis was used in this paper for determining the dissociation constant [22].

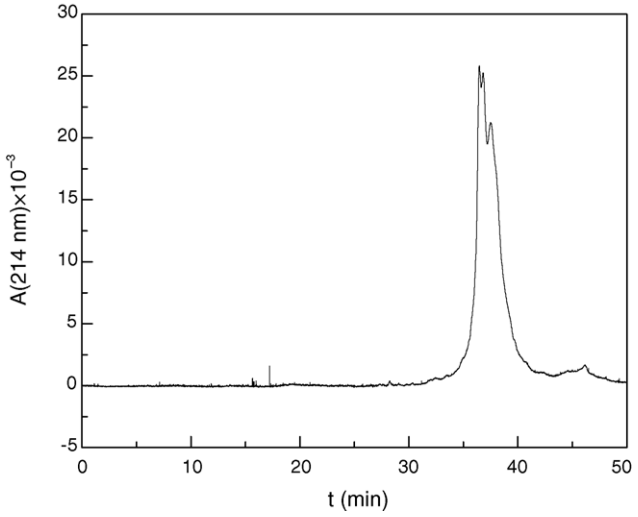

Fig. 7. Affinity capillary electrophoresis of porcine pepsin A on capillary modified PVA. Separation buffer, 0.025 mol/L phosphate buffer, pH 6.8; voltage, 13 kV; pneumatic sampling, 10 mbar for 20 s; sample concentration, 57.1 μmol/L.

Receptor peak migration shifting as a function of the concentration of the ligand in the separation buffer was used. Eq. (1) links mobility shifts ($\Delta\mu$) with ligand concentration [L] and dissociation constant K_d:

$$\Delta\mu = \Delta\mu_{max} - K_d\left(\frac{\Delta\mu}{[L]}\right) \quad (1)$$

Since $\mu = L_D/Et$, where L_D is the migration distance to the detector, E the field intensity, and t is the migration time, Eq. (1) can be transformed into a practical form employing the difference in the migration times, Eq. (4), provided that the values of E and L_D are the same in all the experiments, and correcting t-values by t_m-values obtained from non-interacting marker molecule [22]:

$$\Delta\mu = \frac{I}{E}\left[\left(\frac{1}{t} - \frac{1}{t_m}\right) - \left(\frac{1}{t_0} - \frac{1}{t_{m0}}\right)\right] \quad (2)$$

$$\Delta\mu = \frac{I}{E}\left[\Delta\left(\frac{1}{t}\right)\right] \quad (3)$$

$$\Delta\left(\frac{1}{t}\right) = \Delta\left(\frac{1}{t}\right)_{max} - K_d\left(\frac{\Delta(1/t)}{[L]}\right) \quad (4)$$

where t_{m0} and t_0 are the migration times of a marker and a receptor for an electrolyte without ligand ($[L] = 0$) and t_m and t correspond to the migration times of a marker and a receptor in an electrolyte with a ligand concentration ($[L] \neq 0$).

In our approach, 3,5-diiodo-L-tyrosine and thiourea (the eof marker) were injected as the sample while porcine pepsin A was dissolved in the phosphate buffer. The concentration of 3,5-diiodo-L-tyrosine was 2.3 mmol/L, that of thiourea was 13.1 mmol/L and the porcine pepsin concentration varied from 0 to 14.3 μmol/L. An electropherogram of DIT and thiourea in a bare silica capillary is shown in Fig. 9. The DIT peaks change in dependence on the addition of porcine pepsin A (Fig. 10). The dissociation constant is then estimated from the linearized plot with ($-K_d$) as the slope [22]. The slope of the straight line, $y = -6.10x + 203.1$, corresponds to the $-K_d$ which amounts to 6.1 μmol/L. No value has so far been published for K_d of pepsin–DIT. However, the results are similar to those obtained in our measurement of K_d of complexes of concanavalin A with other glycoproteins [26]. The values of 8.1 μmol/L for ovalbumin, 9.5 μmol/L for fetuin and

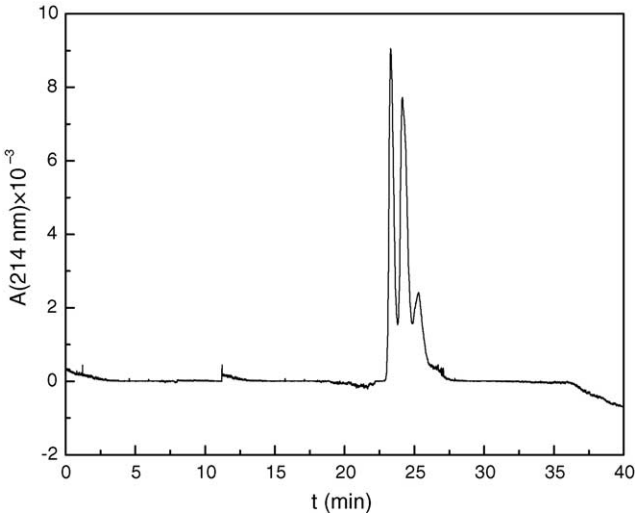

Fig. 8. Affinity capillary electrophoresis of porcine pepsin A on capillary modified with L-tyrosine with subsequential iodation. Separation buffer, 0.025 mol/L phosphate buffer, pH 6.8; voltage, 13 kV; pneumatic sampling, 10 mbar for 20 s; sample concentration, 57.1 μmol/L.

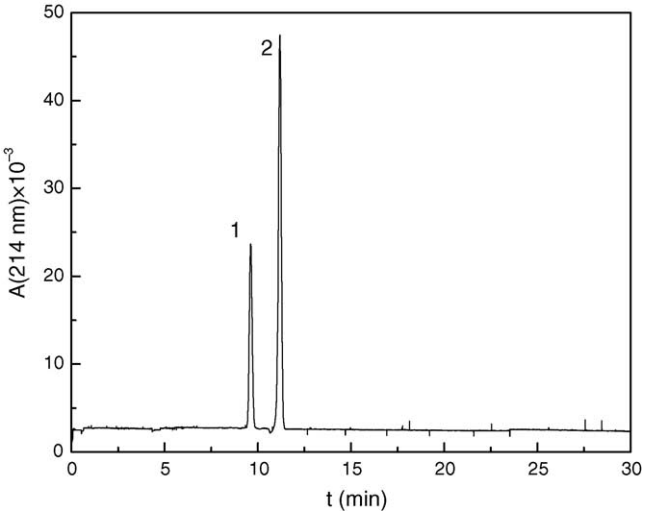

Fig. 9. Capillary electrophoresis of DIT and thiourea (sample) in present porcine pepsin A (dissolved in buffer) on silica capillary: (1) DIT; (2) thiourea. Separation buffer, 0.025 mol/L phosphate buffer, pH 3.0; voltage, 28 kV; pneumatic sampling, 10 mbar for 20 s; sample concentration: DIT 2.3 mmol/L, thiourea 13.1 mmol/L.

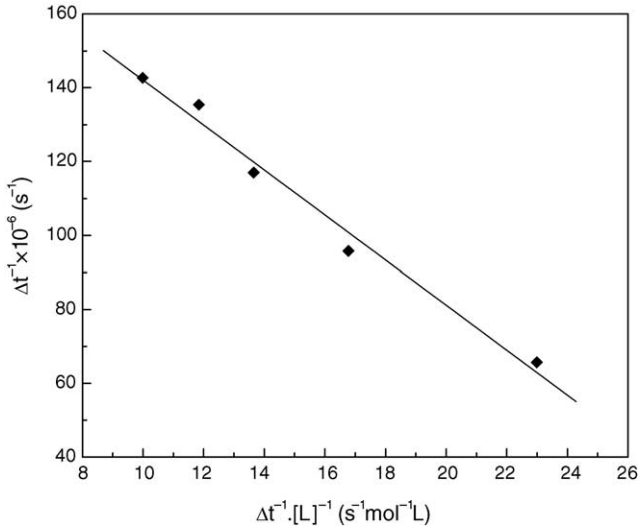

Fig. 10. Graphic dependence for determination of dissociation constant K_d of complex porcine pepsin A-DIT. Dependence of Δt on $K_d\Delta(\mu)/[L]$.

9.5 μmol/L for acid-α-glycoprotein were obtained, respectively.

4. Conclusions

The results obtained demonstrate that the preparation of affinity stationary phases with 3,5-diiodo-L-tyrosine as the affinity ligand is reproducible, that these phases exhibit sufficient selectivity for pepsin and are applicable to practical samples, such as those of human or porcine pepsin. Their lifetime is limited to roughly two months; this is not particularly long but their use in practice is not prevented.

Of the two procedures tested for the preparation of CE capillaries modified with DIT, only the two-step synthesis involving the binding of L-tyrosine followed by its iodation produced usable capillaries. We compared analyses for porcine pepsin A on PVA modified capillary (commercially available) and DIT modified capillary (prepared by us); the capillary with immobilized DIT permitted a better separation and a shorter migration time.

The dissociation constant $K_d = 6.1$ μmol/L has been determined for the pepsin-3,5-diiodo-L-tyrosine complex using dynamic equilibrium ACE approach.

Acknowledgements

The authors are grateful to Dr. Kučerová from the 1st Faculty of Medicine of Charles University for valuable discussion and samples of pepsins. Financial support from the Grant Agency of the Czech Republic, No. 303/01/1216, is gratefully acknowledged.

References

[1] B.C. Bryksa, T. Tanaka, R.Y. Yada, Biochemistry 42 (2003) 13331.
[2] J. Frýdlová, Z. Kučerová, M. Tichá, J. Chromatogr. B 800 (2004) 109.
[3] M. Wells, B. Brown, J. Hall, Cell. Phys. Biochem. 13 (2003) 301.
[4] H. Arnoštová, Z. Kučerová, I. Tišlerová, T. Trnka, M. Tichá, J. Chromatogr. A 911 (2001) 211.
[5] Internet address, http://ca.expasy.org./cgi-bin/pi_tool (15 February 2003).
[6] I. Gritti, G. Banfi, G.S. Roi, Pharm. Res. 41 (2000) 265.
[7] Z. Kučerová, M. Tichá, J. Chromatogr. B 770 (2002) 121.
[8] R.A. da Silva Gomes, R.P. Batista, A.C. de Almeida, D.N. da Fonseca, L. Juliano, V. Hial, Anal. Biochem. 316 (2003) 11.
[9] J. Turková, Bioaffinity Chromatography, second ed., Elsevier, Amsterdam, 1993 (completely revised edition).
[10] J.Y. Fu, S.M. Wu, Ch.T. Chang, H.Y. Sung, J. Agric. Food Chem. 51 (2003) 1042.
[11] K. Peek, N.B. Roberts, W.H. Taylor, J. Chromatogr. 476 (1989) 491.
[12] J.S. Fruton, Q. Rev. Biol. 77 (2002) 127.
[13] A.T. Jones, K.K. Balan, S.A. Jenkins, R. Sutton, M. Critchley, N.B. Roberts, J. Clin. Pathol. 46 (1993) 254.
[14] E. Tonková, M. Tichá, Z. Kučerová, IJBC 4 (1998) 35.
[15] Z. Kučerová, M. Tichá, J. Sep. Sci. 26 (2003) 669.
[16] J. Haginaka, Y. Miyano, Y. Saizen, Ch. Seyama, T. Murashima, J. Chromatogr. A 708 (1995) 161.
[17] M. Gliges, M.H. Kleemiss, G. Schomburg, Anal. Chem. 66 (1994) 2038.
[18] J.P. Landers, R.P. Oda, B.J. Madden, T.C. Spelberg, Anal. Biochem. 205 (1992) 115.
[19] Affinity Chromatography—Principles and Methods, Pharmacia Fine Chemicals (bulletin), Uppsala, Sweden, Epoxy-activated Sepharose 6B.
[20] P. Mc Phie, Anal. Biochem. 73 (1976) 258.
[21] K. Shimura, K. Kasai, Anal. Biochem. 251 (1997) 1.
[22] N.H.H. Heegaard, S. Nilsson, N.A. Guzman, J. Chromatogr. B 715 (1998) 29.
[23] J. McKeon, L.A. Holland, Electrophoresis 25 (2004) 1243.
[24] Y.H. Chu, L.Z. Avila, J. Gao, G.M. Whitesides, Acc. Chem. Res. 28 (1995) 461.
[25] H. Kajiwara, Anal. Chim. Acta 383 (1999) 61.
[26] S. Hubená, Characterization of glycoproteins by capillary electrophoresis, Diploma thesis, Charles University, Prague, 1999.