Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1084 (2005) 108–112
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Affinity chromatography of porcine pepsin A using quinolin-8-ol as ligand

Lenka Novotná[a,*], Martin Hrubý[b], Milan J. Beneš[b], Zdenka Kučerová[a]

[a] *Institute of Pathophysiology, First Faculty of Medicine, Charles University, U Nemocnice 5, 12853 Praha 2, Czech Republic*
[b] *Institute of Macromolecular Chemistry, AS CR, Heyrovsky Sq. 2, 16206 Praha 6, Czech Republic*

Available online 6 November 2004

Abstract

Stationary phase containing quinolin-8-ol immobilized on macroporous methacrylate support for the affinity chromatography of porcine pepsin A is described. Optimized chromatographic conditions for separation of porcine pepsin A on this stationary phase were found investigating the influence of pH, concentration, ionic strength and chemical composition of the used mobile phases. The stationary phase shows a good reproducibility of chromatographic analyses (relative standard deviation, ±2%), a high recovery (ca. 93%) and a satisfactory capacity (13 mg pepsin A/1 mL stationary phase) for porcine pepsin A. The obtained findings confirm the applicability of affinity chromatography on the stationary phase with immobilized quinolin-8-ol to the isolation and determination of porcine pepsin A.

Keywords: Porcine pepsin A; Affinity chromatography; Quinolin-8-ol

1. Introduction

Gastric juice of vertebrates contains proteinases that digest dietary proteins. Gastric juice proteinases are classified according to their enzymatic and immunochemical properties to the following four groups: pepsin A (EC 3.4.23.1), pepsin B (EC 3.4.23.2), pepsin C (EC 3.4.23.3) and chymosin (EC 3.4.23.4). Pepsin A, pepsin B, and pepsin C are present in the gastric juice of adult vertebrates, while chymosin is present exclusively in neonates [1]. The concentration and ratio of individual types of pepsins varies among species. For example, the ratio pepsin A/pepsin C in human gastric juice is approximately 3:1 while porcine gastric juice contains only very small amounts of pepsin C in comparison with pepsin A [2]. The similarity between human and porcine pepsins A is greater than similarity between human pepsin A and human pepsin C [3].

Gastric proteinases are secreted by the cells of the gastric mucosa as inactive precursors, pepsinogens (designed as PGA, PGB, PGC and prochymosin). The acid environment of gastric juice converts pepsinogens to the active forms, pepsins [4]. The human gastric mucosa and human blood serum consist of PGA and PGC. The levels of serum PGA and PGC seem to be associated with gastric diseases. In duodenal ulcer patients, elevated serum PGA and in gastric cancer patients low serum PGA were found, compared with the healthy controls [2,5].

Affinity chromatography has been used for the effective isolation of pepsins from various sources. For this purpose, antibodies [6], substrates [7–9], and inhibitors [10–12] bound to a solid support were used. Previously our laboratory used a stationary phase with bound synthetic inhibitors containing D-amino acids [13] and derivatives of aromatic amino acids for the same purpose [14]. Phosphoproteins (pepsin A) and peptides were also separated using immobilized metal affinity chromatography (IMAC) mostly with immobilized Fe(III) [15–19] and also with Ga(III) [20].

The present paper describes the preparation of the stationary phase with quinolin-8-ol as a ligand covalently bound to a macroporous methacrylate support and its utilization for the isolation and determination of pepsin A. As pepsin A model porcine pepsin A was chosen due to its similar chemical, physical and biological features to those of human pepsin A.

* Corresponding author. Tel.: +420 224 965 910; fax: +420 224 912 834.
E-mail address: lenka.novotna@lf1.cuni.cz (L. Novotná).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.058

2. Experimental

2.1. Chemicals

Porcine pepsin A was purchased from Sigma, St. Louis, USA. *N*-Acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine, quinolin-8-ol and 41% aqueous methylamine were purchased from Fluka Chemie, Buchs, Switzerland. The other chemicals were bought from LachNer, Neratovice, Czech Republic and from Ing. Petr Lukes Company, Uhersky Brod, Czech Republic.

2.2. Preparation of affinity stationary phase

See Fig. 1 for reaction scheme. The starting macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) with reactive epoxide units I, bead size 5–9 μm, was prepared according to [21]. The adsorbent with unit III was prepared by a modified procedure according to [22].

The polymer I (50.0 g, 150 mmol epoxide) was reacted with 41% aqueous methylamine (1.00 mL, 11.8 mmol) in methanol (150 mL) for 7 days at room temperature with occasional shaking. The polymer was then filtered off, washed with water and left overnight under 10% aqueous sulfuric acid. The resulting polymer was then subsequently washed with water, 2% aqueous sodium hydroxide and water and dried in air. The IR spectrum showed no residual epoxide groups in the product II (908 cm^{-1}).

The polymer II was reacted with quinolin-8-ol (50 g, 344 mmol) and formaldehyde (30 mL of 36% aqueous solution, 388 mmol) in methanol (500 mL) with the addition of a drying agent, anhydrous sodium sulfate (100 g), with occasional shaking at room temperature for 10 days. The polymer was then filtered off, subsequently washed with water, methanol, aqueous HCl (1 M) and water again and stored under water before packing into column. The equilibrium saturation capacity for Cu^{2+} in acetate buffer (1 M, pH 4.75) is 0.185 mmol per gram of dry matrix of IV.

2.3. Affinity chromatography of porcine pepsin A

Affinity chromatography of porcine pepsin A was performed using HPLC system (Ecom, Prague, Czech Republic). The steel column (250 mm × 4 mm i.d.) was packed with the quinolin-8-ol stationary phase and washed with the equilibration mobile phase. Pepsin A was dissolved in this mobile phase. Aliquots of 0.25 mL were applied onto the column. Unadsorbed substances were eluted using equilibration mobile phase. Different mobile phases were used for the elution of the adsorbed enzyme (flow rate, 1 mL/min). The analyses were monitored using a UV detector set at 280 nm. In the course of the analyses, 1 mL fractions were collected. The pH value of each fraction was adjusted with 1 M HCl to pH 2. Proteolytic activity of each fraction was determined according to the Anson and Mirsky method [23] as trichloracetic acid-soluble peptides using hemoglobin as a substrate. One unit will produce a ΔA_{280} of 0.001/min at pH 2.0 at 37 °C.

Chromatographic conditions for the sorption of pepsin A on the stationary phase containing quinolin-8-ol were found and optimized. The following compositions of the starting mobile phases were used: 0.01–1.00 M acetate buffer, pH 3.5–6.0, containing 0.0–1.0 M NaCl; 0.05 M phosphate buffer, pH 5.6. The adsorbed protein was eluted under different conditions: 0.03–1.00 M acetate buffer, pH 6.0, containing 0.0–1.0 M NaCl or 20 mM *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine; 0.05 M phosphate buffer, pH 5.6, containing 1.0 M NaCl.

The determination of the capacity of the stationary phase with immobilized quinolin-8-ol for porcine pepsin A was

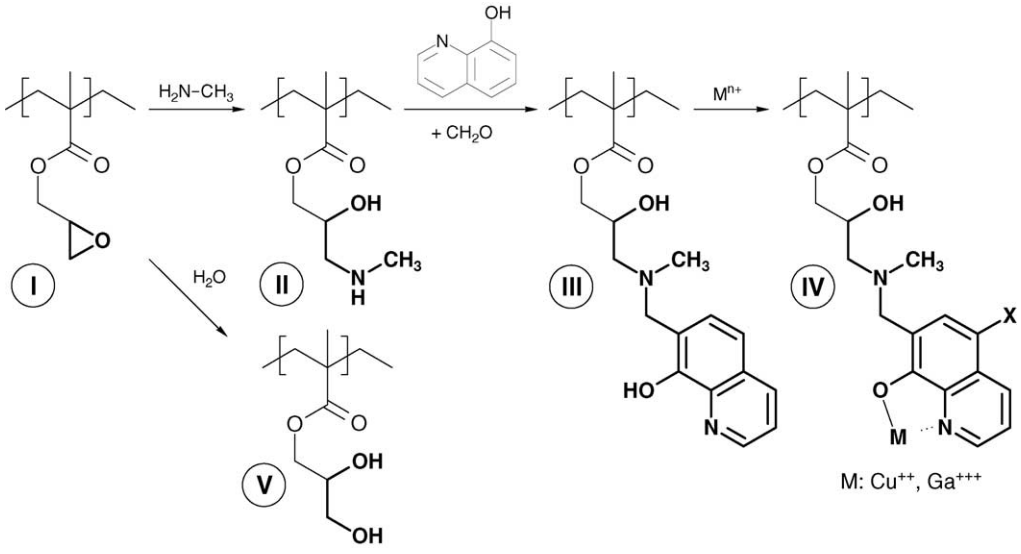

Fig. 1. Reaction scheme: preparation of stationary phase.

performed by the application of the porcine pepsin A solution (50 mg/50 mL) onto the column until a proteolytic activity was determined in the eluate. The capacity was expressed in mg of porcine pepsin A adsorbed to 1 mL volume of the stationary phase containing quinolin-8-ol as a ligand.

The dependence of the elution peak area on the concentration of applied porcine pepsin A (0.25–4.00 mg/mL) was constructed under established optimized conditions. The reproducibility of the chromatographic analyses was determined (the same analyses were performed 10 times).

3. Results and discussion

3.1. Preparation of stationary phase with immobilized quinolin-8-ol

The quinolin-8-ol ligand was attached (see Fig. 1) to the matrix by aminolysis of the epoxide groups of I with aqueous methylamine and subsequent Mannich condensation reaction of the secondary amine intermediate II with aqueous formaldehyde and quinolin-8-ol using a modified procedure according to [22]. Smaller beads suitable for HPLC were functionalized at the molar ratio methylamine/epoxide 1/12.7 to obtain a lower concentration of the ligand on level appropriate for affinity chromatography. The support with the ligand content as high as 1.46 mmol g^{-1} dry matrix (used earlier for sorption of phenols and toxic oxoanions) adsorbed proteins irreversibly. The epoxide polymer I was, therefore, reacted with a limited amount of methylamine and the remaining epoxide groups were then hydrolyzed under acidic catalysis to V. The following Mannich condensation reaction leading to sorbent III was then performed with great excess of low-molecular-weight reagents to ensure complete conversion of secondary amine groups on polymer II.

Table 1
Optimized chromatographic conditions for porcine pepsin A on the stationary phase containing immobilized quinolin-8-ol

	Adsorption (acetate buffer)	Elution (acetate buffer containing NaCl)
Buffer concentration (M)	0.05	0.05
pH	6.0	6.0
NaCl concentration (M)	–	1.0

3.2. Affinity chromatography of porcine pepsin A

Several pepsin separation methods was described using low and medium-pressure affinity stationary phases based on bead cellulose [9,24] or Sepharose 4B [8,9,25]. In this study, we prepared a high-pressure affinity stationary phase based on a methacrylate support.

The sorbent III with quinolin-8-ol moieties was used for the affinity chromatography of pepsin A. As pepsin A is a phosphoprotein, we started sorption experiments with stationary phase IV in Ga^{3+} form using the IMAC mode. Pepsin A was bound quantitatively on Ga^{3+}-quinolin-8-ol. Surprisingly, pepsin A was bound specifically also on a quinolin-8-ol moiety itself; therefore, stationary phase III (without metal) was used for other experiments. This stationary phase was characterized by the determination of capacity for pepsin A. Stationary phase (1 mL) adsorbed 13 mg of pepsin A.

Chromatographic conditions for the separation of porcine pepsin A on the prepared stationary phase were found and optimized. The analysis of pepsin A under optimized conditions is shown in Fig. 2. By increasing pH of the mobile phase, the adsorption of pepsin A on the stationary phase increased. (Ultimate pH is 6.0 through the denaturation of pepsin A.) The enzyme was completely adsorbed at pH 5.5–6.0 and at ionic strength lower than 0.06 M. By increasing ionic strength above 0.06 M the adsorption significantly decreased. Therefore, the participation of hydrophobic interaction in pepsin

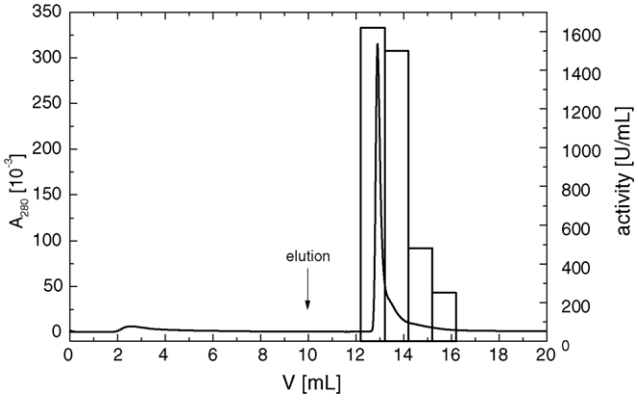

Fig. 2. Chromatography of porcine pepsin A under optimized conditions on the stationary phase containing immobilized quinolin-8-ol; sample: 0.25 mL of porcine pepsin A solution (concentration, 1.5 mg/mL); starting mobile phase: 0.05 M acetate buffer, pH 6.0; elution mobile phase: 0.05 M acetate buffer, pH 6.0, containing 1.0 M NaCl; solid line: absorbance (280 nm). Column graph: proteolytic activity.

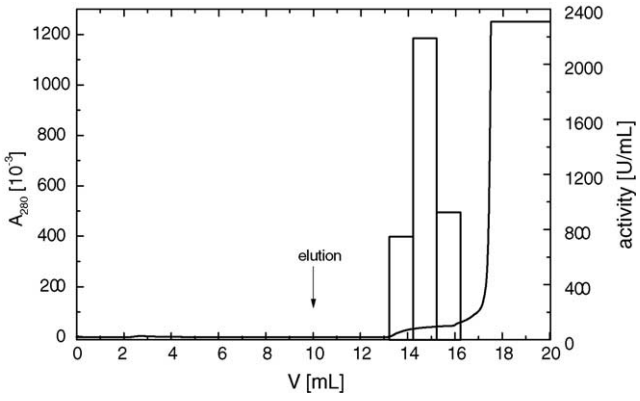

Fig. 3. Chromatography of porcine pepsin A on quinolin-8-ol; sample: 0.25 mL of porcine pepsin A solution (concentration, 1.5 mg/mL); starting mobile phase: 0.05 M acetate buffer, pH 6.0; elution mobile phase: 0.05 M acetate buffer, pH 6.0, containing 20 mM *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine; solid line: absorbance (280 nm). Column graph: proteolytic activity.

Table 2
Reproducibility

Experiment	Area (s)
1	6380
2	6200
3	6190
4	6160
5	6040
6	6340
7	6330
8	6280
9	6150
10	6210

An amount of adsorbed porcine pepsin A to stationary phase containing quinolin-8-ol as a ligand expressed in an area of protein peak. A volume of 0.25 mL of pepsin A solution (concentration, 1.5 mg/mL) was applied. The same analyses were performed 10 times.

A binding is not dominant. Under the optimized chromatographic conditions (Table 1), pepsin A was completely adsorbed to the stationary phase, no proteinase activity was detected in the eluted fractions (Fig. 2). The inactive fractions of proteins contained only small portion of contaminants present in the porcine pepsin A sample.

Pepsin A adsorbed to immobilized quinolin-8-ol was eluted increasing ionic strength up to 1.0 M. The buffer composition (0.05 M acetate or 0.05 M phosphate buffers) did not influence the adsorption of pepsin A on the stationary phase. Adsorbed pepsin A could also be eluted by the addition of its specific substrate *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine to mobile phase (Fig. 3). This result confirmed the affinity character of sorption of pepsin A on the stationary phase with quinolin-8-ol.

The recovery of the applied pepsin A was about 93%. This value is comparable with or higher than those reported for low- and medium-pressure affinity stationary phases (e.g. 88% [25]). The stationary phase exhibits a good reproducibility of analyses performed under optimized conditions. The same analyses were performed 10 times (Table 2). The average protein peak area was (6230 ± 100) s. Therefore, the relative standard deviation found was 2% compared with 4% reported in [25]. The operation life of this affinity stationary phase is long. It does not exhibit any changes in the amount of adsorbed pepsin A even after 140 performed analyses. A linear dependence of the protein peak area on the amount of adsorbed pepsin A was determined in the concentration range of porcine pepsin A solutions: 0.25–4.00 mg/mL (area = $4550c_{\text{pepsin}}$, $R^2 = 0.9962$). The examined high-pressure affinity stationary phase allows loading of lower concentration of pepsin A solution and considerable time saving in comparison with low- and medium-pressure stationary phases.

4. Conclusion

A stationary phase containning quinolin-8-ol covalently bound to macroporous methacrylate support was prepared according to Fig. 1 and used in the affinity chromatography of porcine pepsin A. The affinity mechanism of sorption was proved by the ability of specific substrate *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine to elute adsorbed pepsin A (Fig. 3). As sorption takes place at low and desorption, in contrast, at higher ionic strength, the participation of hydrophobic interaction is not dominant.

The chromatographic conditions for the separation of porcine pepsin A on the stationary phase were found and optimized (Table 1, Fig. 2). This stationary phase exhibits good reproducibility of analyses performed under optimized conditions (Table 2; relative standard deviation, ±2%), a high recovery (ca. 93%), low nonspecific interactions and a high capacity for porcine pepsin A (13 mg pepsin A/1 mL stationary phase). Also the operation life is long. It does not exhibit any changes even after 140 performed analyses.

The obtained findings confirm the possibility of using affinity chromatography on the stationary phase with immobilized quinolin-8-ol for the isolation and determination of porcine pepsin A.

Acknowledgements

This work was supported by the Ministry of Education of the Czech Republic (Grant No. MSM 111100003) and by the Academy of Sciences of the Czech Republic (Project KSK40501110).

References

[1] B. Foltmann, Pepsinogens in man: clinical and genetic advances, in: J. Kreuning, I.M. Samloff, J.I. Rotter, A.W. Eriksson (Eds.), Progress in Clinical and Biological Research, vol. 173, Alan R. Liss Inc., New York, 1984, p. 1.
[2] I. Gritti, G. Banfi, G.S. Roi, Pharmacol. Res. 41 (2000) 265.
[3] C. Richter, T. Tanaka, R.Y. Yada, Biochem. J. 335 (1998) 481.
[4] M. Fusek, V. Vetvicka, Aspartic Proteinases, CRC Press, Houston, 1995.
[5] I.M. Samloff, Gastroenterology 96 (1989) 586.
[6] M. Zoller, S. Matzku, W. Rapp, Biochim. Biophys. Acta 427 (1976) 708.
[7] Z. Kucerova, M. Ticha, J. Chromatogr. B 770 (2002) 121.
[8] E. Tonkova, M. Ticha, Z. Kucerova, Int. J. Biochromatogr. 4 (1998) 35.
[9] H. Arnostova, Z. Kucerova, I. Tislerova, T. Trnka, M. Ticha, J. Chromatogr. A 911 (2001) 211.
[10] J. Marciniszyn, J.A. Hartsuck, J. Tang, J. Biol. Chem. 251 (1976) 7088.
[11] J. Pohl, M. Zaoral, A. Jindra Jr., V. Kostka, Anal. Biochem. 139 (1984) 265.
[12] J. Turkova, K. Blaha, K. Adamova, J. Chromatogr. 236 (1982) 375.
[13] Z. Kucerova, J. Pohl, L. Korbova, J. Chromatogr. 376 (1986) 409.
[14] Z. Kucerova, M. Ticha, J. Chromatogr. B 770 (2002) 121.
[15] G. Muszynska, G. Dobrowolska, A. Medin, P. Ekman, J.O. Porath, J. Chromatogr. 604 (1992) 19.
[16] L.D. Holmes, M.R. Schiller, J. Liq. Chromatogr. Relat. Technol. 20 (1997) 123.
[17] P. Cao, J.T. Stults, J. Chromatogr. A 853 (1999) 225.
[18] M. Zachariou, M.T. Hearn, J. Chromatogr. A 890 (2000) 95.

[19] Z. Kucerova, P. Majercakova, J. Biochem. Biophys. Methods 49 (2001) 523.
[20] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883.
[21] F. Svec, J. Hradil, J. Coupek, J. Kalal, Angew. Makromol. Chem. 48 (1975) 135.
[22] M. Hruby, J. Hradil, M.J. Benes, React. Funct. Polym. 59 (2004) 105.
[23] M.C. Anson, A.E. Mirsky, J. Genet. Physiol. 16 (1932) 59.
[24] Z. Kucerova, M.J. Benes, J. Lenfeld, Int. J. Bio-Chromatogr. 3 (1997) 177.
[25] J. Frydlova, Z. Kucerova, M. Ticha, J. Chromatogr. B 800 (2004) 109.