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Characterization of *p*-aminobenzamidine-based sorbent and its use for high-performance affinity chromatography of trypsin-like proteases

Koji Nakamura[a,*], Takao Suzuki[a], Masazumi Hasegawa[a], Yoshio Kato[a], Hiroo Sasaki[a], Kuniyo Inouye[b]

[a]*Tosoh Corporation, Nan-yo Research Laboratory, 4560 Kaisei, Shin-nan-yo, Yamaguchi 746-8501, Japan*
[b]*Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*

Abstract

An affinity sorbent, hydrophilic polymer-based carrier of different pore size (Toyopearl) with immobilized *p*-aminobenzamidine (ABA), has been prepared. Its basic properties and some applications for protein purification were studied. ABA, which is a synthetic inhibitor for trypsin-like proteases, was covalently immobilized to Toyopearl by reductive amination. The ligand density and binding capacity for porcine trypsin varied depending on the pore size of Toyopearl. The maximum binding capacity of the immobilized *p*-aminobenzamidine Toyopearl (ABA-Toyopearl) for trypsin was more than 40 mg/ml gel. ABA-Toyopearl thus obtained was very stable below pH 8 and was successfully used for high-performance affinity chromatography of trypsin-like proteases such as trypsin, thrombin, tissue-type plasminogen activator or urokinase in a single step at 25 °C.

Keywords: Affinity adsorbents; Aminobenzamidine; Enzymes; Enzyme inhibitors

1. Introduction

Amidine derivatives are known to inhibit trypsin-like proteases due to interaction between amidine moiety and the active site of the enzymes [1–3]. Consequently, amidine derivatives, such as *p*- or *m*-aminobenzamidine, have been used as affinity ligands for removal of proteases and purification of trypsin and trypsin-like proteases such as plasmin, thrombin, urokinase, enterokinase, etc. [4,5]. Because of the tendency of trypsin and trypsin-like proteases to undergo autodegradation during purification and storage [6], a rapid and simple method for purification is favored. However agarose material used for such affinity chromatography of trypsin-like proteases cannot withstand high flow-rate. Therefore chromatography must be carried out at 4 °C. The purpose of this study is to prepare a new affinity adsorbent for high-performance affinity chromatography (HPAC) of trypsin-like proteases. We immobilized aminobenzamidine (ABA) onto Toyopearl with different pore size, which is rigid enough to be used for rapid purification, in order to study the effect of pore size on binding capacity for trypsin and the

*Corresponding author. Fax: +81-834-639-924.
E-mail address: k_naka@tosoh.co.jp (K. Nakamura).

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purification of trypsin-like proteases in a single step at 25 °C.

2. Experimental

2.1. Materials

Toyopearl HW-50C, 55C and 65C (50–100 μm) were obtained from Tosoh (Tokyo, Japan). Sodium acetate, acetic acid, sodium chloride, Tris(hydroxymethyl)aminomethane and calcium chloride were obtained from Kishida (Osaka, Japan). Pyridine borane and sodium metaperiodate were obtained from Wako (Osaka, Japan). *p*-Aminobenzamidine (ABA) dihydrochloride, porcine trypsin and bovine thrombin were obtained from Sigma (Tokyo, Japan). Benzoyl-L-arginine ethyl ester (BAEE) was obtained from Nakarai (Kyoto, Japan). Tissue-type plasminogen activator (t-PA) (trade name Activacin) was obtained from Kyowa Hakko (Tokyo, Japan). Urokinase (UK) was obtained from Mitsubishi Welpharma (Tokyo, Japan). A molecular mass marker kit (Broad Range) was obtained from Bio-Rad Labs. (Hercules, CA, USA). Coomassie Brilliant Blue R-250 was obtained from ICN Biomedicals (Aurola, OH, USA). Pyr–Gly–Arg–MCA (L-pyroglutamyl–glycyl–L-arginine 4-methylcoumaryl-7-amide) and Boc–Val–Pro–Arg–MCA (*tert*.-butyloxycarbonyl–L-valyl–L-prolyl–L-arginine 4-methylcoumaryl-7-amide) were obtained from the Peptide Institute (Osaka, Japan).

2.2. Immobilization of ABA

Immobilization of ABA onto Toyopearl by reductive amination was carried out with modifications to the procedure of Kanamon et al. [4]. A 10-g amount of epoxy-activated Toyopearl was suspended in 16 ml of 1 *M* sodium hydroxide at 80 °C for 4 h with stirring. After washing extensively with water, the gel was suspended in 16 ml of 0.4 *M* sodium metaperiodate at 40 °C for 1 h with stirring. After washing extensively with water, 10 g of formyl gel was suspended in 9 ml of 0.1 *M* sodium acetate buffer, pH 4.0, containing 200 mg of ABA dihydrochloride. The pH of the gel slurry was adjusted to 4.0 with 1 *M* sodium hydroxide. Then 250 μl of pyridine borane was added. The suspension was shaken for 24 h at 25 °C. The gel was washed successively with water and 0.1 *M* acetate buffer, pH 4.0 and stored at 4 °C.

2.3. Determination of immobilized ABA

The amount of immobilized ABA was determined by the difference of the peak area of ABA in suspension before and after immobilization reaction with high-performance liquid chromatography (HPLC). HPLC was carried out on a TSKgel G2000SWXL column (30 cm×0.78 cm I.D.) at 25 °C with a CCPM pump (Tosoh) equipped with a variable-wavelength UV detector Model UV-8000 (Tosoh) operated at 280 nm.

2.4. Determination of adsorption capacity for porcine trypsin

A 1.0-ml volume of each adsorbent was packed into a 12-ml Bond Elut empty reservoir column (GL Science, Tokyo, Japan). It was washed with 10 ml of equilibration buffer (0.05 *M* Tris–HCl containing 0.5 *M* sodium chloride and 0.02 *M* calcium chloride, pH 8.0). A 10-ml volume of porcine trypsin solution (10 mg/ml) was charged onto the column and equilibrated for appropriate time with every 15 min. stirring. After appropriate time, the column was washed with 10 ml of equilibration buffer. This washing procedure was repeated twice. Adsorbed trypsin was eluted with 3.0 ml of elution buffer (0.1 *M* acetate buffer, pH 3.0) and 3.0-ml fractions were collected. Trypsin content was measured spectrophotometrically (*E* 0.1% at 280 nm, 1.39 [7]).

2.5. Affinity chromatography

Affinity chromatography was carried out on a column (4 cm×0.6 cm I.D.) at 25 °C with a CCPM pump (Tosoh) equipped with a variable-wavelength UV detector Model UV-8000 (Tosoh) operated at 280 nm.

2.6. Determination of enzyme activity

Trypsin activity was measured with BAEE as a substrate by the method of Bergmeyer et al. [8]. UK

Table 1
Properties of ABA-Toyopearl

Name	Base material	Exclusion limit for PEO[a]	Ligand density (mg/ml gel)	Trypsin capacity (mg/ml gel)[c]
ABA-50	HW-50C[b]	1.8×10^4	13	7
ABA-55	HW-55C	1.5×10^5	8	40
ABA-65	HW-65C	1.0×10^6	4	24

[a] PEO: Polyethylene oxide.
[b] Particle size of C grade: 50–100 μm.
[c] Equilibration time: 15 min.

and t-PA activities were measured with Pyr–Gly–Arg–MCA as a substrate and thrombin activity was measured with Boc–Val–Pro–Arg–MCA as a substrate by the method of Morita et al. [9].

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out by the Laemmli method [10]. Electrophoresis was performed for 120 min at a constant current of 18 mA on 8–16% linear polyacrylamide gradient gel (1 mm thick) (Tefco, Tokyo, Japan). Detection was done by Coomassie Brilliant Blue R-250.

2.8. pH stability study

The pH stability of ABA-Toyopearl was tested in eight different pH solutions (pH 1.0; 0.1 *M* HCl, pH 2.0; 0.01 *M* HCl, pH 4.0; 0.1 *M* sodium acetate, pH 6.0; 0.1 *M* sodium phosphate, pH 8.0; 0.1 *M* Tris–HCl, pH 10.0; 0.1 *M* 1,3-diaminopropane–HCl, pH 12; 0.01 *M* NaOH, pH 13; 0.1 *M* NaOH) at 25 °C for appropriate time. A 1.0-ml volume of ABA-55 (see Table 1) was suspended with 5 ml of each solution. After treatment for an appropriate period, the stability was evaluated by measuring the binding capacity for trypsin.

3. Results and discussion

3.1. Basic properties

Table 1 shows the basic properties of three kinds of ABA-Toyopearl. The ligand density decreased with the increase in pore size and this results in a lower surface area for small molecules. Although ABA-50 had the highest ligand density, the binding capacity for trypsin was fairly small. On the other hand, ABA-55, which has the second largest pore size of the three resins, had the highest binding capacity for trypsin possibly due to the largest surface area available to trypsin.

Fig. 1 shows the effect of equilibration time on the static binding capacity for trypsin. The binding capacity of ABA-50, which has the smallest pore size, increased with the equilibration time. On the other hand, the binding capacity of ABA-65, which has the largest pore size, remained constant between 5 and 120 min. These findings indicate that trypsin (M_r 25 100 [7]) can easily penetrate the pores of ABA-65 and interacts readily with the immobilized ABA at the internal pore surface, whereas it cannot access the pores of ABA-50.

3.2. Affinity chromatography of trypsin

Trypsin has been purified with *m*- or *p*-aminobenzamidine agarose at 4 °C because of the limitation of high flow-rates use of agarose and the tendency of trypsin to undergo autodegradation

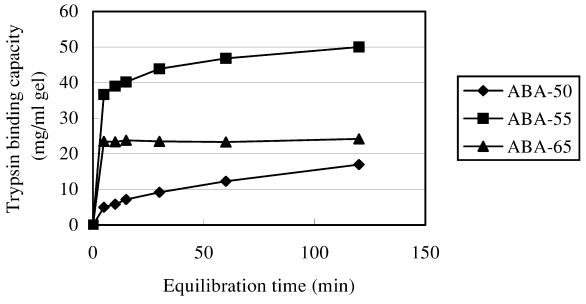

Fig. 1. Equilibration time vs. static binding capacity for trypsin.

during purification [6]. Toyopearl is a hydrophilic polymer-based material and has high physical stability and excellent flow characteristic [11]. We tried to purify trypsin with HPAC by using ABA-Toyopearl at 25 °C. Fig. 2 shows the purification of trypsin on three kinds of ABA-Toyopearl. With regard to chromatographic conditions, Tris–HCl buffer containing 0.5 *M* NaCl and 20 m*M* calcium chloride was used as an equilibration buffer due to the inhibition of electrostatic interaction between proteins and amidine functions on the surface of support. For elution of trypsin from the column, 0.1 *M* sodium acetate buffer (pH 3.0) containing no salts was used. Fig. 2 shows that almost identical chromatograms were obtained on ABA-65 and -55, but the peak area of flow-through on ABA-50 was slightly larger than

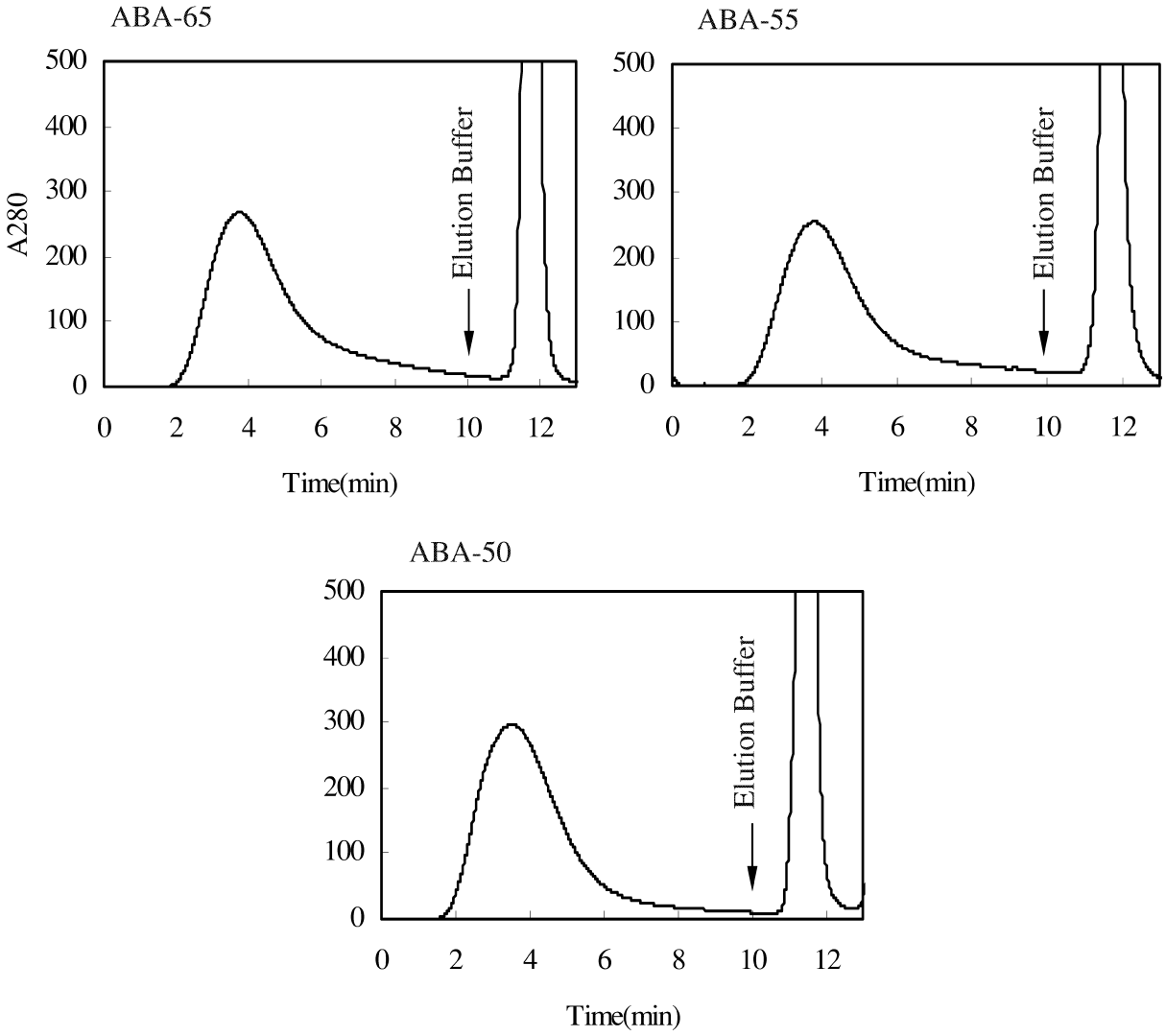

Fig. 2. Chromatograms of trypsin on ABA-Toyopearl. Each column (4 cm×0.6 cm I.D.) was equilibrated with Tris–HCl buffer (0.05 *M*, pH 8.0) containing 0.5 *M* NaCl and 0.02 *M* calcium chloride at a flow-rate of 1.0 ml/min at 25 °C. A 1-ml volume of 10 mg/ml trypsin solution was applied, the column washed with Tris–HCl buffer (0.05 *M*, pH 8.0) containing 0.5 *M* NaCl and 0.02 *M* calcium chloride for 10 min and the bound protein then eluted with sodium acetate buffer (0.1 *M*, pH 3.0).

that of ABA-65 or -55. The flow-through fractions (7 ml) and the acid fractions (3 ml) were collected and checked for trypsin activity. The acid fractions from ABA-65 or -55 retained 100% of trypsin activity whilst that from ABA-50 retained only 88% with the remaining activity present in the flow-through fraction. The absorbance at 280 nm of the acidic fractions were also measured to compare the protein recovery. The absorbance of acidic fractions from ABA-65 and -55 was both 0.36 but that from ABA-50 was 0.31. The results are shown in Fig. 1, however the flow-rate of 1 ml/min could possibly be too fast for ABA-50.

3.3. Affinity chromatography of thrombin

Thrombin is also inhibited by amidine derivatives and has been purified with ABA adsorbents. Thrombin has usually been purified in the coldroom at 4 °C because of much lower stability than trypsin. Elution buffers for trypsin-like proteases on ABA adsorbents are acidic solutions such as HCl or acetic acid at around pH 3. These acid buffers cannot be used for thrombin purification because they will destroy thrombin. Therefore 20–50 m*M* *p*-aminobenzamidine or benzamidine in equilibration buffer has been used for elution of thrombin [3,12]. HPAC was selected for thrombin purification using acid elution. The acid fractions were neutralized immediately with 1 *M* Tris–HCl (pH 9.0) in order to prevent denaturation of thrombin at low pH. Fig. 3 shows the purification of thrombin on a column of ABA-55. The flow-through fractions showed no thrombin activity and the eluted fractions with acidic buffer contained 85% of activity. Fig. 4 shows SDS–PAGE of the fractions indicating that the purity of the eluted protein is comparable to that of the commercially available pure thrombin sample.

3.4. Affinity chromatography of t-PA and UK

Both t-PA and UK, which are types of trypsin-like proteases, have been widely used as effective thrombolytic agents for medication of acute myocardial infarction, peripheral arterial occlusion, and acute cerebral infarction [13–15]. We applied HPAC using ABA-Toyopearl to the purification of these enzymes.

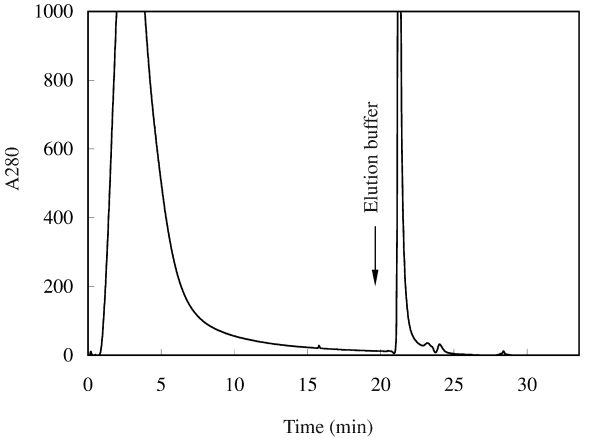

Fig. 3. Chromatogram of crude bovine thrombin on ABA-55. A column (4 cm×0.6 cm I.D.) was equilibrated with Tris–HCl buffer (0.05 *M*, pH 8.0) containing 0.5 *M* NaCl at a flow-rate of 1.0 ml/min at 25 °C. A 1-ml volume of thrombin solution was applied, the column washed with Tris–HCl buffer (0.05 *M*, pH 8.0) containing 0.5 *M* NaCl until 20 min and the bound protein then eluted with sodium acetate buffer (0.1 *M*, pH 3.0).

Fig. 5 shows the purification of t-PA and UK on a column of ABA-55. These enzymes are used as pharmaceutical products. Although the flow-through fractions of UK sample contained a large amount of impurity, this is human serum albumin added to UK during manufacture. Both flow-through fractions contained no enzymatic activity and the acidic fractions of t-PA and UK retained 75 and 84% of activity, respectively.

3.5. pH stability study

Fig. 6 shows the pH stability study of ABA-55 over time. Generally speaking, the coupling method by reductive amination is considered to be a stable bonding even in alkaline condition. But at pH 10 or higher, the binding capacity for trypsin was decreased over time. This could be due to the instability of the ligand itself. The ligand is first hydrolyzed to *p*-aminobenzamide and then further to *p*-aminobenzoic acid under alkaline condition [16]. Below pH 8, ABA-55 was stable at 25 °C for 4 weeks and there were no observable changes of the binding capacity for trypsin at 4 °C during one and a half years.

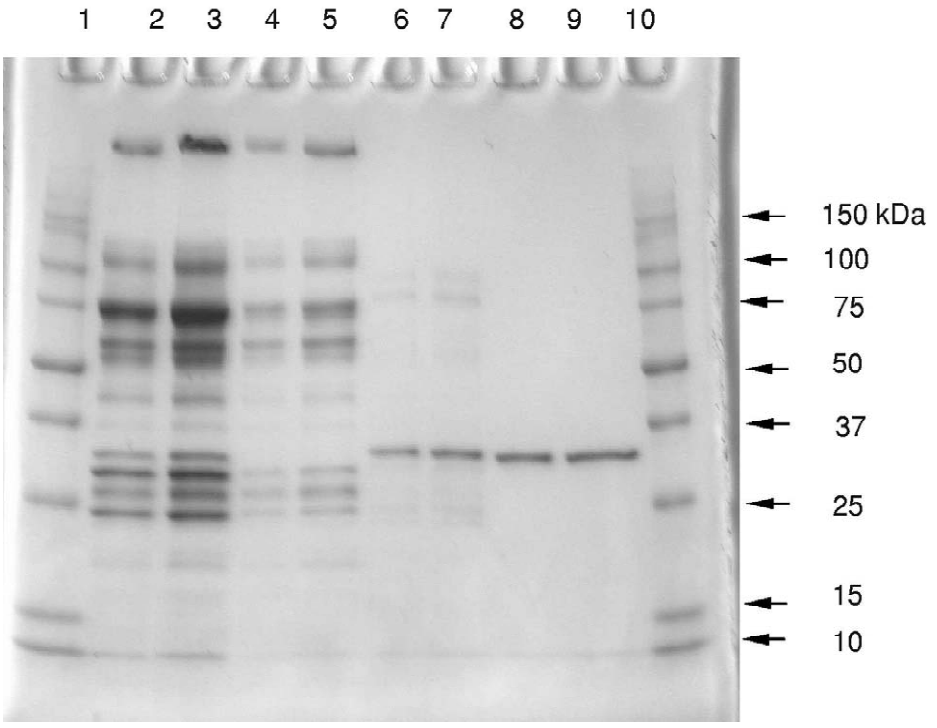

Fig. 4. SDS–PAGE analysis of the purity of each fraction in Fig. 3 eluted from ABA-55 and stained with Coomassie Brilliant Blue G-250. Lanes: 1 and 10, molecular mass markers; 2 and 3, applied samples; 4 and 5, flow-through fractions; 6 and 7, acid fractions; 8 and 9; pure bovine thrombin. kDa=Kilodaltons.

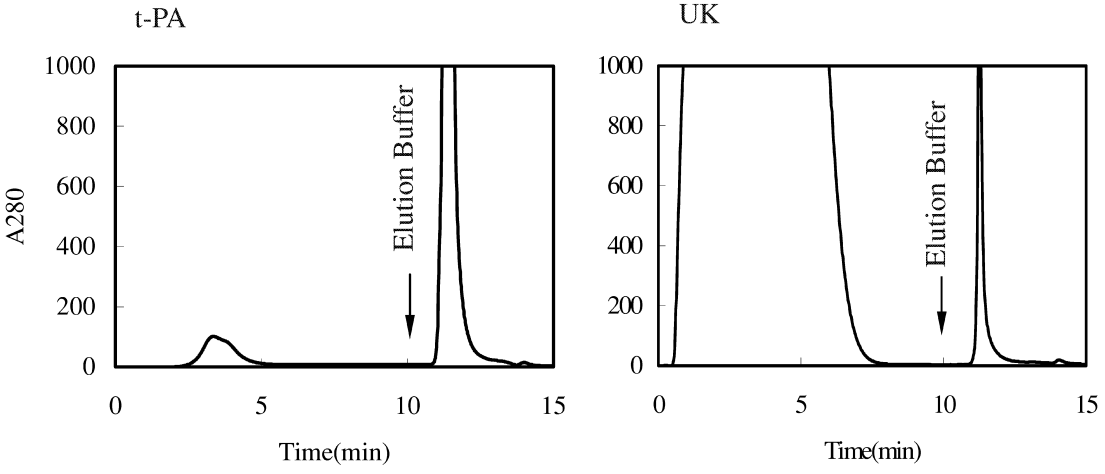

Fig. 5. Chromatograms of t-PA and UK on ABA-55. A column (4 cm×0.6 cm I.D.) was equilibrated with Tris–HCl buffer (0.05 *M*, pH 8.0) containing 0.5 *M* NaCl at a flow-rate of 1.0 ml/min at 25 °C. 600 000 units of t-PA or 30 000 units of UK were applied, the column washed with Tris–HCl buffer (0.05 *M*, pH 8.0) containing 0.5 *M* NaCl until 10 min and the bound protein then eluted with sodium acetate buffer (0.1 *M*, pH 3.0).

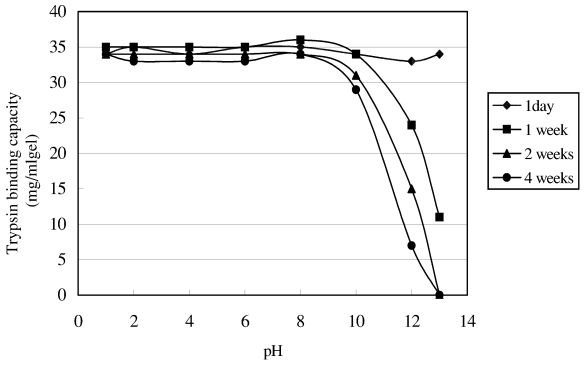

Fig. 6. pH stability study with ABA-55.

4. Conclusion

It was demonstrated that it is important to optimize the pore size of base material when preparing an affinity adsorbent. Optimized ABA-Toyopearl seems to be a suitable adsorbent for HPAC of trypsin and trypsin-like proteases because of high binding capacity and high chemical stability. There were no measured changes to the binding capacity for trypsin at 4 °C over one and a half years.

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