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Separation of Nattokinase from *Bacillus subtilis* Fermentation Broth by Expanded Bed Adsorption with Mixed-mode Adsorbent

Miao-Hua Lu, Dong-Qiang Lin, Yuan-Chun Wu, Jun-Xian Yun, Le-He Mei, and Shan-Jing Yao*

Department of Chemical and Biochemical Engineering, Zhejiang University, Hangzhou 310027, China

Abstract Mixed-mode hydrophobic/ionic matrices exhibit a salt-tolerant property for adsorbing target protein from high-ionic strength feedstock, which allows the application of undiluted feedstock *via* an expanded bed process. In the present work, a new type of mixed-mode adsorbent designed for expanded bed adsorption, Fastline PRO®, was challenged for the capture of nattokinase from the high ionic fermentation broth of *Bacillus subtilis*. Two important factors, pH and ion concentration, were investigated with regard to the performance of nattokinase adsorption. Under initial fermentation broth conditions (pH 6.6 and conductivity of 10 mS/cm) the adsorption capacity of nattokinase with Fastline PRO was high, with a maximum capacity of 5,350 U/mL adsorbent. The elution behaviors were investigated using packed bed adsorption experiments, which demonstrated that the effective desorption of nattokinase could be achieved by effecting a pH of 9.5. The biomass pulse response experiments were carried out in order to evaluate the biomass/adsorbent interactions between *Bacillus subtilis* cells and Fastline PRO, and to demonstrate a stable expanded bed in the feedstock containing *Bacillus subtilis* cells. Finally, an EBA process, utilizing mixed-mode Fastline PRO adsorbent, was optimized to capture nattokinase directly from the fermentation broth. The purification factor reached 12.3, thereby demonstrating the advantages of the mixed-mode EBA in enzyme separation.

Keywords: expanded bed adsorption, mixed-mode adsorbent, nattokinase, separation

INTRODUCTION

Expanded bed adsorption (EBA) is an innovative chromatography method, which allows for the adsorption of target proteins directly from unclarified feedstock, *e.g.* culture suspensions, cell homogenates, or crude extracts. Using specially-designed adsorbents and columns, the EBA technique involves the combination of solid-liquid separation with an adsorption step in a single unit operation. This technique was designed to increase overall yield and reduce operational time, as well as requiring less capital investment and consumables [1,2]. The most important property of the expanded bed is the perfectly classified fluidization of the adsorbent, which increases the void fraction to allow for the introduction of particle-containing feedstock, without the risk of blocking the bed [3]. The feasibility of this technique has been demonstrated with a variety of expression systems (*E. coli*, yeast, and mammalian cells) [4].

In real applications, EBA processes encounter complex feedstock, which can contain culture medium, cell excretions, or intercellular compositions. Therefore, ligand chemistry is one extremely relevant factor in terms of the efficient capture of target biomolecules. Ion exchange ligands are widely used in EBA processes. However, conventional ion exchange ligands, such as SP, DEAE, or Q, do not usually bind protein efficiently at increased conductivities (above 5 mS/cm). In most feedstock based on high-density cell cultures, conductivities are in a range between 10~30 mS/cm. Therefore, several folds of dilution are necessary to effect the efficient capture of the target protein. Dilution results not only in decreased adsorption efficiency and longer processing time, but also in increased buffer consumption and waste. This is certain to significantly impact process productivity and economics. The achievement of selective adsorption at a high ionic strength would thus significantly reduce the cost of primary capture in the downstream process [5]. The perfect solution, of course, would be to develop a new ligand with salt-tolerant ability. Burton *et al.* described mixed-mode hydrophobic/ionic matrices for the adsorption of chymosin from high-ionic strength feedstock [6-8]. The mixed-mode ligands exhibit multi-modal functionality, in which the charged group performs ion exchange function and the hydrophobic side chain contributes the salt tolerance. High binding capacities are achieved at moderate conductivities (15~30 mS/cm), allowing for the application of undiluted feedstock in an expanded bed process.

***Corresponding author**
Tel: +86-571-8795-1982 Fax: +86-571-8795-1015
e-mail: yaosj@che.zju.edu.cn

Hamilton *et al.* reported the direct product sequestration of an extracellular protease from a microbial batch culture with the mixed-mode EBA process, which signifycantly increased the product yield and enzyme specific activity [9].

Intravascular thrombosis is one of the main causes of a wide variety of cardiovascular diseases. Thrombolytic therapy has been extensively investigated as a possible means of medical treatment. Nattokinase, a potential thrombolytic agent, was purified from *Bacillus subtilis* isolated from the traditional Japanese food, natto [10]. Nattokinase not only hydrolyzes the thrombi *in vivo*, but also converts plasminogen to plasmin [11]. Oral administration of nattokinase enhanced fibrinolytic activity in the plasma, and induced the production of tissue plasminogen activator [12]. In addition, this fibrinolytic activity in the blood could persist for more than 3 h [13]. These results suggest that nattokinase may be a potential natural agent for oral thrombolytic therapy. A particular strain of *Bacillus subtilis* was isolated from Japanese natto in our group, which evidenced high extracellular nattokinase productivity. After optimizing culture medium and fermentation conditions, the concentration of the product reached more than 2,000 U/mL fermentation broth by feed-batch fermentation in a 3-L reactor [14,15]. The conductivity of the fermentation broth was about 10 mS/cm, which hindered the direct application of the ion exchanger [16-18]. In the present work, a new type of mixed-mode EBA adsorbent, developed by Upfront Chromatography A/S, Fastline PRO, was used to challenge the high ionic fermentation broth of *Bacillus subtilis*. The selective adsorption of nattokinase under different operation conditions was investigated and evaluated. Also, we discussed an optimized EBA process for efficient capture of nattokinase directly from high ionic fermentation broth.

MATERIALS AND METHODS

Strain and Adsorbent

The *Bacillus subtilis* HL-1 strain was screened from natto, and stored in our laboratory. The mixed-mode adsorbent, Fastline PRO, was provided as a gift by Upfront Chromatography A/S (Denmark). The adsorbent is composed of high-density phase tungsten carbide, surrounded by cross-linked agarose derivativized with a mixed-mode ligand. The particle size is 20~200 μm, and the density is in the range of 2.5~3.5 g/cm^3 [19].

Batch Adsorption Experiments

In the batch adsorption experiments, clarified fermentation broth was incubated with some of the adsorbent under defined fluid phase conditions (pH, conductivity, *etc*). Prior to the experiments, the adsorbents were washed with appropriate buffers, and collected using a vacuum pump. 5 mL of fermentation broth was added to a series of shake flasks, each containing a selected amount of adsorbent. The flasks were then sealed and incubated with gentle agitation (100 rpm) for 8 h at ambient temperature (20~22°C). Samples were then removed, and enzyme activity in the sample was determined. The adsorption capacities for different fluid phase conditions could be calculated from the difference between initial enzyme activity and equilibrium enzyme activity, after which the isotherm curves were obtained. Analysis of adsorbent-free controls confirmed that loss of protein and enzyme activity could be negligible over the time scale of the experiments.

Packed Bed Adsorption

Packed bed adsorption was performed at ambient temperature using a FPLC system (Amersham Biosciences, Sweden). Fastline PRO was packed into a 12 mL volume in an XK-16 column (Amersham Biosciences, Sweden). Clarified fermentation broth was obtain with the high-speed centrifugation at 14,000 rpm, and was loaded into the column at a flow rate of 30 cm/h. Elution fractions were assayed for protein concentration and nattokinase activity.

Expanded Bed Adsorption

A homemade column for EBA (d_{int} = 0.02 m, h = 1 m) was used. A small amount of glass ballotini (0.3 mm diameter, < 5% total sedimented bed height) was added in order to improve flow distribution at the column inlet. A movable adapter was employed to adjust the position of the liquid outlet to the top of the expanded bed. The fluid was transported with a peristaltic pump (Longer Precision Pump Co. Ltd, Baoding, China). Proper vertical column alignment was confirmed in all experiments. The experiments were performed at ambient temperature. The bed expansion was measured as H/H_0, where H was the expanded bed height, and H_0 was the sedimented bed height. A UV detector (Knauer GmbH, Berlin, Germany) was used to monitor protein concentrations at the outlet of the expanded bed.

Biomass Pulse-response Experiments

The adsorbent (10~12 cm sedimented bed height) was fluidized with a buffer, and the expanded bed was allowed to stabilize for at least 30 min. The top adapter was relocated to within a few mm above the top of the expanded bed. A pulse of biomass suspension was applied to the mobile phase, using a three-way injection port. The pulse volume was equal to 80% of the sedimented bed volume. Cell concentration was monitored by the absorbance at 600 nm both before and after passing through the column. Comparing the area of the biomass peak before (A_{before}) and after column passage (A_{after}) yielded the fraction of biomass lost due to adsorption in the column, thus also providing a 'cell transmission index', $P = A_{after}/A_{before}$, which quantifies the extent to which cell/ adsorbent interaction took place in a given trial. A linear correlation of the signal at 600 nm with the concentration of cells in the sample was obtained in the range of con-

centrations under study. The detailed experimental set-up is described elsewhere [20,21].

Enzyme Analysis

Enzyme activity was assessed via the standard fibrin plate method [22]. Standard urokinase was used as a reference of fibrinolytic activity. The protein concentration was measured by Bradford's method [23], with bovine serum albumin as the standard protein.

Zeta Potential Measurement

Cells were suspended at an appropriate concentration in buffers at different pHs. The appropriate cell concentration was about 0.02% in wet weight basis, corresponding to an OD_{600} at a range of 0.05~0.1. The instrument used was a Zetasizer Nano ZS (Malvern Instruments, UK). Zeta potentials were calculated from the electrophoretic mobilities, using Smoluchowski's equation. Measurements were usually carried out in triplicate. The standard deviation of the mean zeta potential values was in the range of ± 1 mV.

RESULTS AND DISCUSSION

Batch Adsorption of Nattokinase with Fastline PRO

Influence of pH

As normal ion exchangers, mixed-mode adsorbents possess ionic ligands. Therefore, variations in pH are the first important factors to influence target protein adsorption. The static batch adsorption behaviour of nattokinase with Fastline PRO was assessed at different pH values with clarified fermentation broth. The isotherm adsorption curves at pH values of 6.6, 7.5, 9.5, and 10.5 are shown in Fig. 1, and the parameters of Langmuir's correlation are also listed in Table 1. The pH of the fermentation broth was about 6.6, and the activity of nattokinase in the fermentation broth was about 2,000 U/mL. At the initial pH of 6.6, Fastline PRO exhibited profound nattokinase adsorption ability, with a maximum capacity of 5,350 U/mL adsorbent, and an efficient capacity of about 4,000 U/mL adsorbent under fermentation broth conditions. As compared to normal EBA adsorbent, Streamline SP exhibited a complete absence of nattokinase adsorption under initial fermentation conditions, due principally to high ionic concentrations in the fermentation broth. After 5-fold dilution, the equilibrium adsorption was still only about 100 U/mL adsorbent, which is still far too low for efficiency with regard to a real application [16]. The unique property of high adsorption capacity under high ionic strength indicated Fastlien PRO as an ideal mixed-mode adsorbent. With pH increases, the nattokinase adsorption ability decreased significantly, particularly for pH values above the isoelectric point of nattokinase (pH 8.6), as shown in Fig. 1. After applying Langmuir's correlation, results demonstrated that the maximum adsorption capacity of Q_m decreased, and equilibrium constant

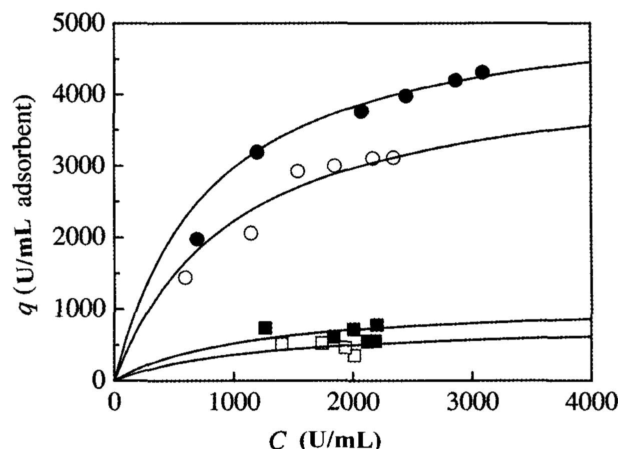

Fig. 1. Isotherm adsorption of nattokinase with Fastline PRO at different pH values and Langmuir's correlation. pH 6.6 (●); pH 7.5 (○); pH 9.5 (■); pH 10.5 (□).

Table 1. Parameters correlated by Langmuir equation for nattokinase adsorption with Fastline PRO at different pHs

pH	6.6	7.5	9.5	10.5
Q_m (U/mL adsorbent)	5,350	4,450	1,100	800
K (U/mL)	800	1,000	1,100	1,200

K increased, as a result of pH increases. This indicated that the initial pH condition of the fermentation broth was suitable for the adsorption of nattokinase by Fastline PRO.

Influence of Salt Concentration

Ionic strength is another important factor with regard to mixed-mode adsorbents. Salt-tolerant properties are predicated on the ligand chemistry of mixed-mode adsorbents. Some mixed-mode ligands exhibited total independence of ionic strength, and some mixed-mode ligands evidenced slight dependence. The chemical structure of the ligand of Fastline PRO was unknown, so the influence of salt concentration on adsorption characteristics should be investigated before application. The static adsorption behaviour of nattokinase with Fastline PRO was studied under different salt concentrations at pH 6.6, using clarified fermentation broth. The isotherm adsorption curves are shown in Fig. 2. We found that increases in salt concentration caused the adsorption ability of nattokinase to decrease slightly. At the enzyme concentration of 2,000 U/mL, the adsorption capacity decreased from 3,700 U/mL adsorbent for the initial fermentation broth, to 3,100 U/mL adsorbent when 200 mM NaCl was added, and 2,200 U/mL adsorbent when 400 mM NaCl was added. Fastline PRO continued to manifest corresponding high nattokinase adsorption levels under high salt concentration, especially as compared with ion exchangers (nearly no adsorption with Streamline SP under same conditions [16]). This constituted further

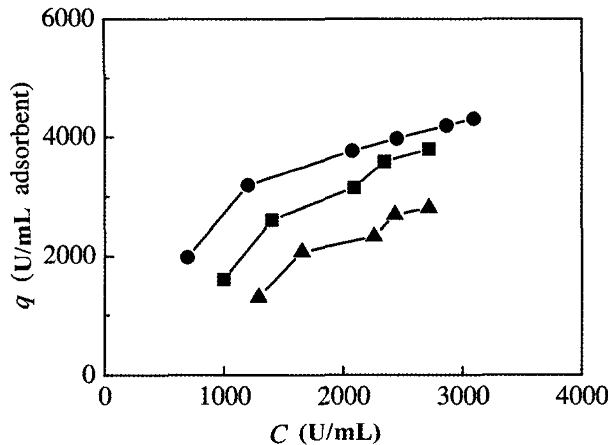

Fig. 2. Effect of ion strength on the static batch adsorption of nattokinase with Fastline PRO at pH 6.6. Fluid phases: initial fermentation broth (●); 200 mM NaCl addition (■); 400 mM NaCl addition (▲).

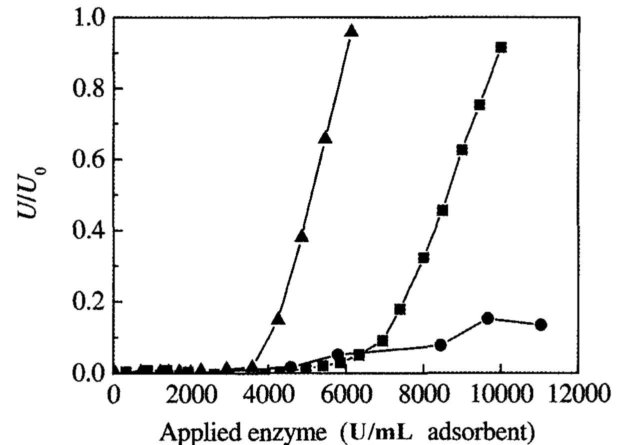

Fig. 3. Effects of ion strength on the breakthrough curves of nattokinase with Fastline PRO packed bed at pH 6.6. Conductivities of Fluid phases: 3 mS/cm (●); 10 mS/cm (■); 33 mS/cm (▲).

evidence that Fastline PRO functions as a sort of mixed-mode adsorbent. On the other hand, the decreasing capacity observed with increasing salt concentrations indicated that the electrostatic interactions between nattokinase and the ionic ligand group of Fastline PRO played an important role with regard to nattokinase adsorption. The hydrophobic interactions occurring between nattokinase and the hydrophobic ligand group might exert an ancillary function, which could bolster salt tolerance. Our results indicated that the initial ionic conditions of the fermentation broth were suitable for the adsorption of nattokinase with Fastline PRO.

Packed Bed Adsorption of Nattokinase with Fastline PRO

Influence of Ionic Strength on the Dynamic Adsorption

As mentioned above, the ionic strength of the mobile phase had great influence on nattokinase adsorption at pH 6.6. With a 0.016-m diameter Fastline PRO packed bed, we attempted to assess the dynamic adsorption capacities of nattokinase under different conductivities. Three conductivities were chosen for this set of experiments. The initial broth was set at a conductivity of 10 mS/cm. The low-conductivity feedstock, at 3 mS/cm, was prepared by desalting clarified fermentation broth, and the high-conductivity feedstock, at 33 mS/cm, was constructed by the addition of NaCl to the clarified fermentation broth. The breakthrough curves of nattokinase under three conditions are shown in Fig. 3. At a conductivity of 10 mS/cm, the breakthrough curve demonstrated high nattokinase adsorption capacity, which was consistent with the results of static adsorption experiments. Lower conductivity induced higher adsorption capacity. The dynamic adsorption capacity of about 3,000 U/mL adsorbent could be obtained even at the relatively high conductivity of 33 mS/cm, confirming the salt-tolerant ability of the mixed-mode Fastline PRO adsorbent. The results of packed-bed dynamic adsorption trials indicated that it should be possible to use Fastline PRO for capturing nattokinase directly from high-ionic strength *Bacillus subtilis* fermentation broth under initial pH.

Influence of pH and Ionic Strength on the Elution of Nattokinase

The efficient elution of target proteins from adsorbents is the hallmark of a successful chromatographic separation process. Two important factors, pH and ionic strength, were investigated in detail. The influence of pH on the elution of nattokinase from Fastline PRO mixed-mode adsorbent is shown in Fig. 4. The isoelectric point of nattokinase is about 8.6, thus the elution solutions were set to pHs of around 8.6. Examples include the pH 7.5 solutions with sodium phosphate, pH 9.5 and pH 10.5 solutions with glycine-NaOH buffer, and pH 12.6 solution with diluted NaOH. We found that at pH 7.5, lower than nattokianse's isoelectric point, nattokianse could not be eluted from Fastline PRO, due largely to the strong electrostatic attraction between the positively-charged nattokianse and the negatively-charged mixed-mode ligand in the Fastline PRO. At pH 9.5, slightly higher than nattokianse's isoelectric point, we found that nattokianse could be efficiently eluted, with a perfect elution peak, due to the electrostatic repulsion between the negatively-charged nattokianse and the negatively-charged ligand of Fastline PRO. With increases in pH to 10.5 and 12.6, no more nattokinase could be eluted from the Fastline PRO column. This meant that at higher than reasonable pH values, pH increases would not result in the increases of elution yield. The influence of ionic strength on the elution of nattokinase from mixed-mode Fastline PRO adsorbent was also investigated, and results are shown in Fig. 5. It was discovered that increases in NaCl concentration did not enhance the nattokinase elution yield. Therefore, glycine-NaOH buffer at pH 9.5 without

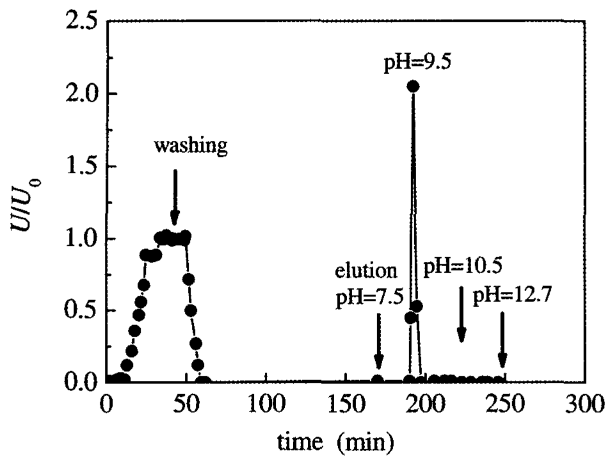

Fig. 4. Effect of pH on the elution of nattokinase with Fastline PRO packed bed.

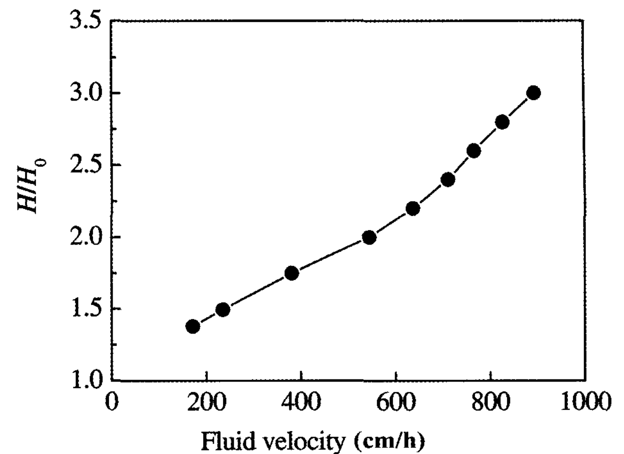

Fig. 6. Bed expansion of Fastline PRO expanded bed at varying fluid velocities.

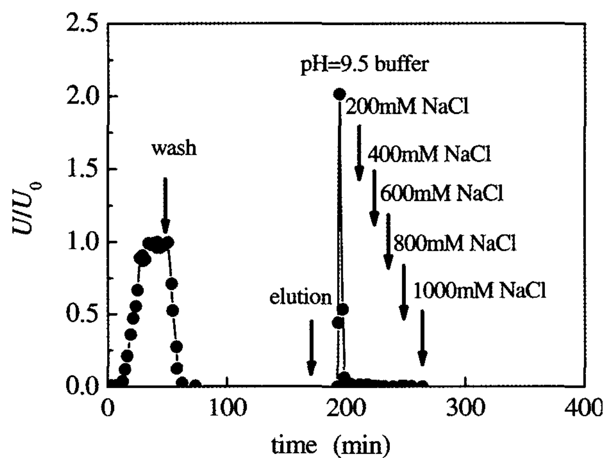

Fig. 5. Effect of ion strength on the elution of nattokinase with Fastline PRO packed bed.

any salt addition was selected as the optimized elution conditions.

Bed Expansion of Fastline PRO in Expanded Bed

Bed expansion is an important operational factor with regard to the determination of suitable fluid velocity and product throughput for EBA processes. Fastline PRO is a novel EBA adsorbent, so no data regarding the bed expansion of Fastline PRO have yet been collected. Therefore, we were compelled to study the bed expansion properties of Fastline PRO in an expanded bed under varying fluid velocities before EBA application. The results are shown in Fig. 6. Consistent with a normal bed expansion of 2~3, the fluid velocity of Fastline PRO expanded bed could be controlled in a range between 500~900 cm/h, which was significantly higher than that of the Streamline series adsorbents (about 300 cm/h). Because of the high enzyme activity in the fermentation broth (about 2,000 U/mL), the sample load volume was relative low (about 3 sedimented bed volumes) for the EBA process. Therefore, a relatively low bed expansion, of about 2, was selected in order to ensure efficient adsorption effects.

Influence of Biomass in the Fermentation Broth

One of the most important advantages of the EBA process is the capture of the target protein directly from the biomass-containing feedstock. However, the precondition for this is that the biomass (cell or cell debris) must not influence the stability of the expanded bed. A series of methods have been developed to evaluate the biomass/adsorbent interaction in the expanded bed [20,21,24,25]. In our present study, the influence of *Bacillus subtilis* cells in the fermentation broth was assessed using biomass-pulse response experiments. The biomass transmission index was near 1.0, indicating that there were no interactions, or only slight interactions, between the *Bacillus subtilis* cells and the Fastline PRO adsorbent. The zeta potential of *Bacillus subtilis* cells was determined at varying pH values. The results are shown in Fig. 7. Strong negative zeta potential at pH 6.6 indicated negatively charged *Bacillus subtilis* cell surfaces under initial fermentation broth conditions. Identical charges in the cell surfaces and the adsorbent would result in the cells not adsorbing to the adsorbent surface, instead passing freely through the void in the expanded bed. Therefore, an expanded bed with Fastline PRO was able to maintain the stability of the fermentation broth containing *Bacillus subtilis* cells at pH 6.6.

Expanded Bed Adsorption of Nattokinase with Fastline PRO

The breakthrough curve of Fastline PRO EBA was determined using clarified fermentation broth. Fig. 8 compares the breakthrough behaviour of the expanded bed with that of a packed bed. We found that the dynamic adsorption associated with an expanded bed was equiva-

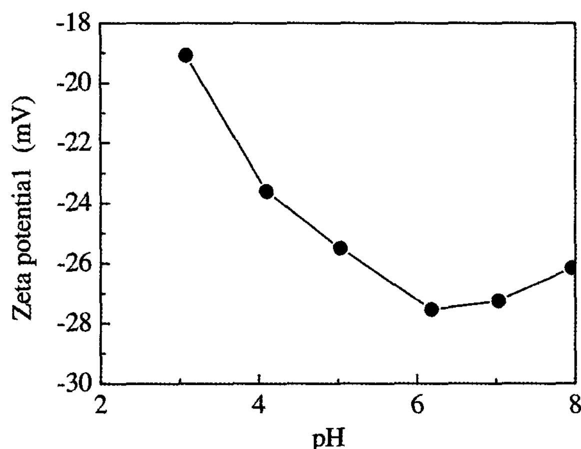

Fig. 7. Zeta potential of *Bacillus Subtilis* cells at varying pH.

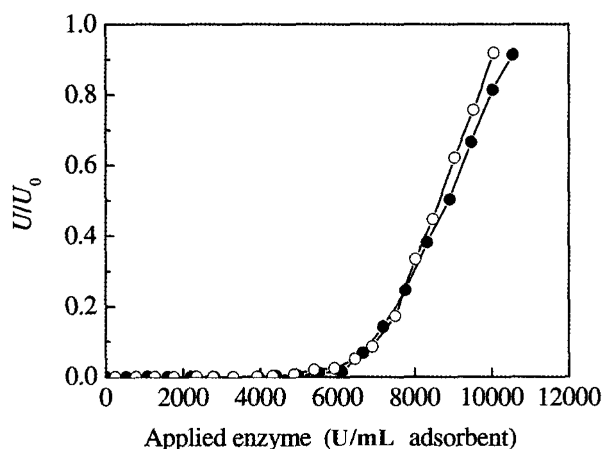

Fig. 8. Comparison of breakthrough behaviors of nattokinase with Fastline PRO expanded bed (●) and packed bed (○). Loading conditions: pH 6.6, conductivity 10 mS/cm, clarified fermentation broth.

lent to that exhibited by a packed bed, indicating that expanded bed with the Fastline PRO adsorbent could provide a stable classified fluidized situation for the efficient adsorption of target proteins. Then the expanded bed with the Fastline PRO was used to capture nattokinase directly from fermentation broth containing *Bacillus subtilis* cells. The wet weight of the cells was equal to 0.05 g/g fermentation broth. The initial enzyme activity of the nattokinase in the fermentation broth was 2,280 U/mL, protein concentration was 1.8 mg/mL, and the specific activity was 1,267 U/mg. The equilibrium buffer used was 50 mM sodium phosphate at pH 6.6, and the operation bed expansion ratio was measured at 2.0. The sample load was 132 mL, corresponding to about 3 sedimented bed volumes. After washing with 8 sedimented bed volumes of equilibrium buffer, microscopic observation indicated that there were no cells in the fluid phase. Then, glycine-NaOH buffer (pH 9.5) was used to elute nattokinase at the same fluid velocity as in the sample

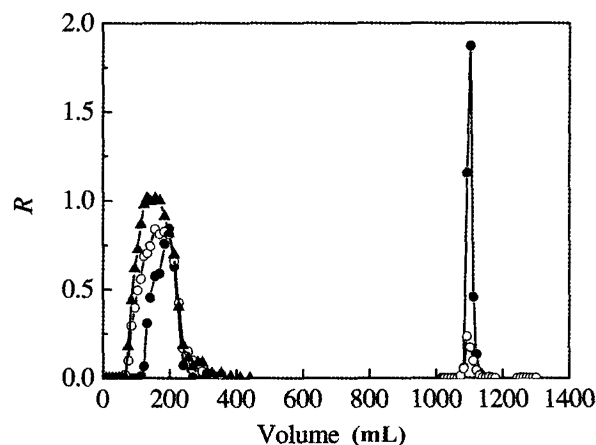

Fig. 9. Chromatograms of nattokinase (●), proteins (○) and cells (▲) separated by Fastline PRO expanded bed. Loading conditions: pH 6.6, conductivity 10 mS/cm, fermentation broth. Elution condition: pH 9.5 glycine-NaOH buffer.

loading. The expanding-mode elution without moving the top adaptor of the column simplified the EBA operation, thereby saving process time. Because we used the same fluid velocity in different steps of EBA process, the bed height changed slightly during the operation, from 25.2 cm in the equilibrium, to 31.4 cm for sample loading, 24.5 cm for the washing step, and 25 cm for elution. The chromatographic results of the nattokinase, total protein, and cells are shown in Fig. 9. Fastline PRO expanded bed adsorption was demonstrated to effect the direct and efficient capture of nattokinase from the *Bacillus subtilis* fermentation broth. The purification factor of one EBA unit could reach as high as 12.3, and the final specific activity of elution collection was 15,610 U/mg. The final recovery was about 47.3%. Hu developed an optimized separation process for nattokinase, consisting of a precipitation with 30~60% saturated ammonium sulfate solution, Sephadex G-75 gel filtration, and Sepharose CM Fast Flow ion exchange chromatography, with a purification factor of 6.3, and a total yield of 51.4% [17]. Hu also developed Streamline SP XL expanded bed adsorption, which resulted in the separation of nattokinase with a 4-fold dilution of the fermentation broth, in which the purification factor was 8.7, and the adsorption capacity was only about 450 U/mL adsorbent [16]. Therefore, the EBA process with Fastline PRO, as developed in the present work, demonstrated the advantage of mixed-mode EBA in enzyme separation.

CONCLUSION

A new kind of mixed-mode EBA adsorbent Fastline PRO was challenged to effect the direct capture of nattokinase from highly ionic fermentation broth containing *Bacillus subtilis*. The influences of pH and ion concentration on the adsorption performance of nattokinase on Fastline PRO adsorbent, as well as elution behaviors,

were investigated by batch and packed bed adsorption experiments, respectively. Our results indicated that the adsorption capacity of nattokinase with Fastline PRO was high at a pH of 6.6, and a highly ionic concentration of the initial fermentation broth. The adsorption processes were affected by both pH and ion concentration. The effective desorption of nattokinase was achieved by a step change from pH 6.6 to 9.5, across the isoelectric point of nattokinase. The biomass pulse response experiments indicated that there were no biomass/adsorbent interactions, or only very slight ones, occurring between *Bacillus subtilis* cells and Fastline PRO. Also, we found that a stable expanded bed could be maintained in feedstock containing *Bacillus subtilis* cells. Based on these results, an EBA process with mixed-mode Fastline PRO adsorbent was developed, which effected the direct capture of nattokinase from *Bacillus subtilis* fermentation broth. The purification factor reached as high as 12.3, demonstrating the integration advantage of EBA in enzyme separation.

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