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# Effects of poly(ethylene glycol) and salt on the binding of α-amylase from the fermentation broth of *Bacillus amyloliquefaciens* by $Cu^{2+}$-β-CD affinity adsorbent

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ABSTRACT

α-Amylase from *Bacillus amyloliquefaciens* was purified by the immobilized metal ion affinity adsorbent, $\beta\text{-CD}_{cl}$-IDA-$Cu^{2+}$. The adsorbent was prepared by reacting the cross-linked β-cyclodextrin (β-CD) with the ligand, iminodiacetic acid (IDA). The copper ion was further linked to the adsorbent. Poly(ethylene glycol) (PEG) was added to the fermentation broth to improve the adsorption efficiency of the adsorbent toward α-amylase. The effort was to provide hydrophobic interactions with the impurities which might interfere with the adsorption of α-amylase. It also provided a polymer shielding effect to prevent non-specific interactions. With the addition of PEG, the adsorption efficiency could be increased to 98%. Imidazole containing a phosphate buffer and NaCl was used to elute the bound α-amylase. By consecutive adsorption/desorption steps, up to 81% of the α-amylase activity could be recovered. Regarding the reutilization of the affinity adsorbents, α-amylase could be adsorbed and desorbed six times consecutively without a significant loss of α-amylase activity.



## 1. Introduction

α-Amylase (EC 3.2.1.1) is a very important enzyme because of its applications to textile, food, and pharmaceutical industries. It is an endo-acting amylase that hydrolyzes the α-1,4 glucosidic bonds of linear amylose and the α-1,6 glucosidic linkages of branching amylopectin. Amylose and amylopectin are the major components in starch and related substrates.

Purification of enzymes from the fermentation broth is generally carried out by chromatographic techniques after crude isolation. Immobilized metal ion affinity chromatography (IMAC) is a commonly used chromatographic method because of its specific affinity performed by the coordination of the transition metal ions with the exposed amino residues, such as histidine, cysteine and tryptophan, of the protein (Gaberc-Porekar & Menart, 2001). IMAC in the packed-bed system cannot be applied directly to purify enzymes or proteins from fermentation broths because the system easily leads to clogging and fouling. Additionally, before the use of a packed-bed system, the removal of whole cells or cell debris is required. Batch affinity adsorption or expanded bed chromatography could overcome the obstacles caused by the packed-bed system and reduce the required purification steps for the clarification of the fermentation broth without centrifugation and microfiltration (Amritkar, Kamat, & Lali, 2004). Although expanded bed chromatography can be directly used to purify enzymes from the cell broth or the cell debris contained broth, the operation cost is comparably higher than the others. Immobilized metal ion affinity adsorbents, $\beta\text{-CD}_{cl}$-IDA-$Cu^{2+}$, prepared in our previous work (Liao & Syu, 2005) were therefore used to purify α-amylase secreted from *Bacillus amyloliquefaciens* in this work.

The complexity in the fermentation broth results in undesired adsorption by the adsorbents. The separation method such as precipitation or the aqueous two-phase system (ATPS) is required to reduce the complexity of the fermentation broth. The combination of ATPS and IMAC was used for purification of GFPuv from the extracts of *Escherichia coli* (Li & Beitle, 2002). In addition to the crude separation step before further adsorption, improving the specific selectivity toward the target protein or enzyme is a useful alternative to purify proteins or enzymes from its fermentation broth. Armisén et al. (1999) found that the selective adsorption could be achieved by varying the ligand concentration, spacer arm length and type of metal ions. By adding polymers (Li, Agrawal, Sakon, & Beitle, 2001) to the solution, the affinity ligand could be protected from the undesired adsorption of contaminants. The approach focused on the preparation of the adsorbent. It took a lot of effort to find the optimal condition for specific adsorption of the desired protein or enzyme. Therefore, adding salts or polymers during the adsorption stage to improve the specific selectivity for the desired protein or enzyme is a simple and inexpensive strategy. The polymer molecules could be introduced to dye-affinity chromatography before the protein solution is loaded. By this way, it can form steric hindrance to prevent or reduce the non-specific

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interaction caused by the substances in the fermentation broth. This is called polymer shielding, which was proposed by Galaev et al. (Galaev, Garg, & Mattiasson, 1994; Garg, Galaev, & Mattiasson, 1996; Kumar, Galaev, & Mattiasson, 2000). They used polymers such as poly(vinyl pyrrolidone) (PVP), poly(vinyl alcohol) (PVA), and poly(vinyl caprolactam) (PVCL) to create polymer shields on the affinity dye, Cibacron Blue F3GA. The dextran-coated IMAC was studied by Mateo et al. to prevent undesired multipoint adsorptions. The exposed $Zn^{2+}$ on the IMAC showed strong one-point adsorption (Mateo et al., 2001).

Poly(ethylene glycol) (PEG) is a water soluble and biocompatible polymer. It bears ethylene groups responsible for attracting hydrophobic proteins. PEG is usually used in purification methods such as affinity partition and precipitation because of its steric exclusion effect and amphiphilic property. α-Amylase was separated from the fermentation broth of *Bacillus subtilis* by the PEG 4000/phosphate/NaCl system (Schmidt, Andrews, & Asenjo, 1996; Schmidt, Ventom, & Asenjo, 1994). In addition to the purification application, PEG also proved to be able to refold proteins (Cleland, Hedgepeth, & Wang, 1992) and protect enzymes from inactivation (Longo & Combes, 1999).

Despite the coordinated complexes formed in IMAC, hydrophobic and electrostatic interaction take place simultaneously. Ionic strength is usually regarded as an important factor to mediate the interactions between desired proteins and metal ions. Trace amounts of metals and potassium phosphate were added into the fermentation broth to promote the metabolism of *Bacillus amyloliquefaciens* for the production of α-amylase and maintain the pH of the solution as well. While studying the effect of salt on the adsorption of α-amylase, the removal of the existing salts is equally important. However, it is impractical to remove the salts from the fermentation broth. Consequently, the salt effect was studied on the desorption, which was carried out by the competitive agent imidazole.

In this study, PEG was added into the fermentation broth of *Bacillus amyloliquefaciens*. The addition of PEG to the α-amylase fermentation broth was to form an aqueous two-phase system for facilitating the purification of α-amylase from the broth. The metal ion affinity adsorbent, β-$CD_{cl}$-IDA-$Cu^{2+}$, were used to selectively bind α-amylase from the PEG containing fermentation broth. Cross-linked β-cyclodextrin was used as the support matrix instead of the other polysaccharides because it was difficult to be hydrolyzed by the target enzyme, α-amylase. Although purification of the enzyme by immobilized metal ion affinity adsorbents was investigated extensively, no literature has discussed the influence of PEG and NaCl on the purification of α-amylase directly from the fermentation broth without pretreatment. The possible mechanism responsible for promoting adsorption induced by PEG was proposed in this study. Adsorption kinetics and isotherm were investigated. Adsorption equilibrium time and appropriate amounts of adsorbents were also obtained. In addition, the effect of NaCl on the desorption of α-amylase and the reutilization of the affinity adsorbents were studied.

## 2. Experimental

### 2.1. Chemicals

Poly(ethylene glycols) (PEGs) with molecular weights of 1000, 4000 and 6000 were purchased from Panreac, Inc. (Barcelona, Spain). PEGs of molecular weights of 1000 and 10,000 were from Ferak, Inc. (Berlin, Germany). Dextrin from potato starch and dextrans with molecular weights of 18,100 and 40,000 from *Leuconostoc mesenteroides* were obtained from Fluka, Inc. (Buchs, Switzerland) and Sigma, Inc. (St. Louis, MO, USA), respectively. Ethylenediaminetetraacetic acid (EDTA) was purchased from JT Baker, Inc. (Phillipsburg, NJ, USA). β-Cyclodextrin (β-CD) hydrate and epichlorohydrin (EPI) were purchased from Acros, Inc. (New Jersey, USA) and Fluka, Inc. (Buchs, Switzerland), respectively. All chemicals were of analytical grade without further purification.

### 2.2. Cultivation condition for the secretion of α-amylase

*Bacillus amyloliquefaciens* (ATCC 23350, CCRC 10268) was purchased from the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsin-Chu, Taiwan. It was maintained on an agar slant and kept at 4 °C. The medium composition for shaken culture was 1.0 g/L yeast extract, 9.0 g/L $K_2HPO_4$, 2.0 g/L $KH_2PO_4$, 5.0 g/L $(NH_4)_2SO_4$, 1.0 g/L trisodium citrate, 9.0 mg/L $CaCl_2{\cdot}2H_2O$, 45 mg/L $FeSO_4{\cdot}7H_2O$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 1.0 mg/L $MnSO_4{\cdot}H_2O$ and 1.0 mg/L $ZnSO_4{\cdot}7H_2O$ (Syu & Chen, 1997). Cells were pre-cultivated with a working volume of 100 mL, respectively. The carbon sources used in pre-cultures and shake cultures were 2.0 g/L glucose and 10 g/L maltose, respectively. Twelve milliliters from the second pre-culture was inoculated into a fresh medium of 600 mL. It was then carried out at 37 °C for 40 h. The fermentation broth was collected afterwards. The cells and cell debris were removed by centrifugation at approximately 400*g* force for 20 min. Thus, the centrifuged supernatant was filtrated through a 0.45 μm cellulose acetate filter paper (Advantec MFS, Inc., Pleasanton, CA, USA). A clarified fermentation broth was thus obtained.

### 2.3. Preparation of the immobilized copper ion affinity adsorbents

The immobilized copper ion cross-linked β-cyclodextrin was used as the affinity adsorbent and is notated as β-$CD_{cl}$-IDA-$Cu^{2+}$ (Liao & Syu, 2005). β-Cyclodextrin (β-CD) was cross-linked by epichlorohydrin (EPI), and then was coupled with iminodiacetic acid (IDA) (Alfa Aesar, Ward Hill, MA, USA). It was further chelated with the copper ion from $CuSO_4$. Afterwards, the adsorbents were ground and sieved to a particle size in the range of 200–500 mesh, which corresponds to 25–74 μm.

### 2.4. Analysis of α-amylase activity and protein concentration

α-Amylase activity was analyzed from the hydrolysis of the starch substrate. The substrate solution was 5 mL of 1.0% (w/v) potato starch with a 0.1 M phosphate buffer of pH 7.0. The substrate solution was placed at 37 °C. Later, 0.5 mL of enzyme solution was added into the solution. After 10 min, an HCl solution was added to stop the reaction. About 0.5 mL of the resultant mixture was added to 4.9 mL of deionized water. Then, the solution was added to 50 μL of iodine solution. The mixture was measured in a spectrophotometer at 580 nm. The α-amylase activity was defined by the following formula,

$$\text{Activity (U/ml)} = D \cdot (R_0 - R)/(R_0 - R_{blank}) \quad (1)$$

where $R_0$ is the absorbance of the phosphate solution; $R_{blank}$ is the absorbance of an iodine solution; and $D$ is the dilution factor. Thus, one unit (U) of α-amylase was defined as the amount of enzymes that hydrolyzed 1.0 mg of starch in a 1.0 mL enzyme solution for 10 min at 37 °C and pH 7.0.

Protein concentration was determined using the Bio-Rad protein microassay kit (Hercules, CA, USA) with bovine serum albumin as the standard. Diluted dye reagent of 5 mL from the assay kit was added into a sample solution of 100 mL. The resultant solution was incubated at room temperature for 5 min. It was then placed in a spectrophotometer for the measurement of absorbance at the wavelength of 595 nm. Thus, the calibration of protein concentration versus absorbance was established.

### 2.5. Adsorption and desorption of α-amylase by β-$CD_{cl}$-IDA-$Cu^{2+}$ affinity adsorbent

The adsorption performance (%) was determined from the difference of α-amylase activities expressed as follows.

$$\text{Adsorption}\ (\%) = \frac{E_0 - E}{E_0} \times 100\% \quad (2)$$

where $E_0$ and $E$ are the initial and residual α-amylase activities after adsorption (for 30 min), respectively. It should be noted that the deactivation of amylase was experimentally proven to be negligible within the period of operation.

The affinity adsorbents of 100 mg prepared as described above, were placed in 5 mL of α-amylase fermentation broth at 37 °C with mild agitation. After 30 min, the adsorbents, assumed to be bound with proteins, were collected by filtration with a 0.45 μm filter for the following desorption studies. 900 mg of PEG 4000 was dissolved in 6 mL of the fermentation broth to make up a PEG concentration of 15% (w/v). Hundred milligrams of β-$CD_{cl}$-IDA-$Cu^{2+}$ affinity adsorbents prepared likewise was suspended in 5 mL of the PEG contained fermentation broth for the adsorption of α-amylase. Effects of the PEG composition, PEG molecular weight, adsorption time and amount of affinity adsorbents on the adsorption performance were all investigated in this work.

The protein bound adsorbents were eluted with 5 mL of 200 mM imidazole of pH 7.0 (Riedel-deHaën, Seelze, Germany) containing 200 mM NaCl, while the desorption of α-amylase from the beads was carried out at 37 °C for 30 min. The filtrate was obtained through a 0.45 μm filter and the desorbed activity could be measured. The desorption performance (%), the specific desorbed activity (U/μg) and the enzymatic activity recovery (%) were calculated as follows:

$$\text{Desorption performance}\ (\%) = \frac{\text{Desorbed activity}}{\text{Adsorbed activity}} \times 100\% \quad (3)$$

$$\text{Desorbed specific activity}\ (\text{U}/\mu\text{g}) = \frac{\text{Desorbed activity}\ (\text{U/mL})}{\text{Desorbed protein concentration}\ (\mu\text{g/mL})} \quad (4)$$

$$\text{Enzymatic activity recovery}\ (\%) = \frac{\text{Desorbed activity}}{\text{Initial activity}} \times 100\% \quad (5)$$

After operation through consecutive adsorption and desorption batches, the adsorbents β-$CD_{cl}$-IDA-$Cu^{2+}$ were eluted with 5 mL of 0.2 M cupric sulphate. Regeneration of the adsorbents was carried out at 37 °C for 30 min, and then the adsorbents were collected by filtration. Before reusing the adsorbents, 5 mL of deionized water was applied to remove the excess copper ions which might be left on the surface of the adsorbents from the previous treatment. This washing procedure was also carried out at 37 °C.

## 3. Results and discussion

Dextrans of different molecular weights were added into the fermentation broth for the observation of their influence on the adsorption of α-amylase. All the additives in groups A and B (Table 1) were in the concentration of 10% and 15%, respectively.

Different amounts of PEG with a molecular weight of 4000 (300 mg, 600 mg, 900 mg, 1.2 g, and 1.5 g) were added to 6 mL of the fermentation broths to make up 5%, 10%, 15%, 20%, 25% (w/v) PEG concentrations. The broth without PEG was used as the control. β-$CD_{cl}$-IDA-$Cu^{2+}$ of 100 mg was then applied to the fermentation broth and agitated for 30 min. The initial activities of the fermentation broths with different amounts of PEG (with a molecular weight of 4000) and the residual activities of the PEG contained fermentation broths after adsorption by the affinity adsorbents were determined.

In general, the adsorbed amount of protein on the affinity adsorbent is a function of time. The adsorbed amount increases with time until the adsorption equilibrium is achieved. Samples were taken at different time intervals. The adsorption time profile of α-amylase from the PEG containing fermentation broth by β-$CD_{cl}$-IDA-$Cu^{2+}$ was obtained (data not shown). The adsorption kinetics was used to determine the adsorption equilibrium time. The adsorption time profile of α-amylase from the PEG containing fermentation broth by β-$CD_{cl}$-IDA-$Cu^{2+}$ was obtained. In Fig. 1, the adsorbed activity of α-amylase is plotted against the increased amount of adsorbents.

The comparison of different desorption agents is made in Table 2. The adsorption as well as desorption performance compared from the fermentation broth and the PEG contained broth is evaluated in Table 3. In order to evaluate the enhancing effect, PEG was added in the fermentation broth. The results with added PEG and

**Table 1**
Adsorption results of α-amylase from the fermentation broths containing different additives in different concentrations.

| Additives | | Initial activity, $A_0$ (U/mL) | Residual activity, $A$ (U/mL) | Adsorption (%) |
|---|---|---|---|---|
| A (10%) | Without additive | 424 | 190 | 55 |
| | Dextran 18,100 | 393 | 96 | 76 |
| | Dextran 40,000 | 423 | 101 | 77 |
| | PEG 6000 | 429 | 29 | 94 |
| B (15%) | Without additive | 353 | 167 | 53 |
| | PEG 1000 | 332 | 9.59 | 97 |
| | PEG 4000 | 355 | 5.90 | 98 |
| | PEG 10,000 | 357 | 7.00 | 98 |
| PEG 4000 | Without PEG | 340 | 170 | 50 |
| | 5% | 350 | 56.0 | 84 |
| | 10% | 340 | 10.2 | 97 |
| | 15% | 320 | 6.4 | 98 |
| | 20% | 310 | 15.5 | 95 |
| | 25% | 310 | 15.5 | 95 |

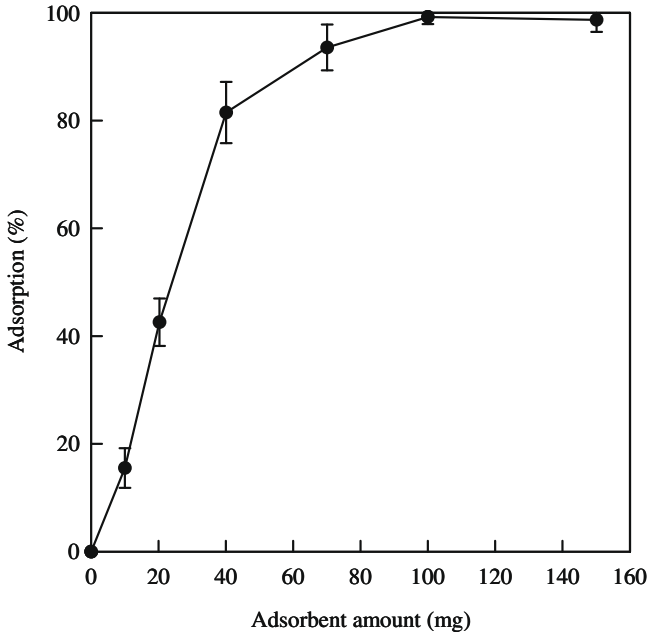


**Fig. 1.** Adsorption efficiency of α-amylase versus the amount of adsorbent. β-$CD_{cl}$-IDA-$Cu^{2+}$ was used as the adsorbent. The initial α-amylase activity in 15% (w/v) PEG contained fermentation broth was 395 U/mL.

**Table 2**
Desorption of α-amylase and re-adsorption from 15% (w/v) PEG contained fermentation broths.

| Desorption agent[a] | EDTA | Imidazole |
|---|---|---|
| Desorbed activity[b] (U/mL) | 165 | 233 |
| Desorption (%) | 47.12 ± 5.58 | 66.32 ± 1.34 |
| Protein concentration (μg/mL) | N.D.[c] | 5.07 |
| Desorbed specific activity (U/μg) | N.D. | 45.92 ± 0.093 |

[a] Desorption agents: 200 mM in 100 mM pH 7.0 phosphate buffer, the pHs were adjusted by NaOH or by HCl to pH 7.0.
[b] Initial specific α-amylase activity in the fermentation broth was 25.48 U/μg and the adsorbed activity from the 15% PEG contained fermentation broth was 351 U/mL.
[c] N.D., no data.

without PEG are compared and shown in Fig. 2. In Fig. 2, the affinity adsorbents were applied for three times from repeated adsorption and desorption. Obviously, the adsorption efficiency was decreased more as it was operated for more times. The effect of salt on the desorption capacity is plotted in Fig. 3. The adsorption/desorption of α-amylase from the PEG contained fermentation broth could be repeatedly operated by using the regenerated affinity adsorbents, as shown in Fig. 4.

### 3.1. Influence of the presence of PEG on the adsorption of α-amylase fermentation broth

The proposed affinity adsorbent, β-$CD_{cl}$-IDA-$Cu^{2+}$, was proven to be able to bind α-amylase in our previous work. Although the approach of immobilized metal ion affinity adsorption is regarded as a very effective purification method for protein, usually other separation methods are required as well because of the complexity of the fermentation broth. It was believed that via an aqueous two-phase system, certain contaminants interfering with the affinity adsorption could be removed or reduced. A PEG/phosphate/NaCl aqueous two-phase system was considered (Schmidt et al., 1994) in this work. With the aid of β-$CD_{cl}$-IDA-$Cu^{2+}$ affinity adsorbents, the adsorption of α-amylase in the α-amylase-enriched top phase could be enhanced. PEG and sodium phosphate were added, respectively, to the fermentation broth. β-$CD_{cl}$-IDA-$Cu^{2+}$ affinity adsorbents were then suspended in the resultant broth for the adsorption of α-amylase. Through this process, the factors affecting the adsorption of α-amylase could be identified. The result revealed that the presence of PEG could enhance the adsorption of α-amylase. However, the effect induced by PEG was rather different from that of an aqueous two-phase system. The partition of the desired proteins and other contaminants to an opposite phase was a result of their different partitions. The contaminants mainly remained in the PEG contained fermentation broth. Microfiltration was also used to examine if the precipitation of α-amylase occurred in this system. The result shows that the filtrate of the PEG containing fermentation broth retained the majority of the activity. Therefore, the possibility of precipitation caused by PEG could be excluded.

As shown in groups A and B of Table 1, the presence of PEG in the fermentation broth, no matter which molecular weight it was, all significantly enhanced the adsorption performance of α-amylase from around 50% (55% in group A and 53% in group B) to higher than 90%. Addition of dextrans of molecular weights of 18,100 and 40,000, respectively, could only improve the adsorption performance by approximately 20% in both cases (from 55% to 76%), which were not as much as the result from the addition of PEG. Compared with α-amylase with a molecular weight of 50 kDa (Schmidt et al., 1996), dextran could be regarded as a small molecule that interfered with the specific adsorption of α-amylase by the affinity adsorbents. It implied that non-specific interaction was due to dextran and other metabolites (Alam, Hong, & Weigand,

**Table 3**
Adsorption and desorption of α-amylase from the unclarified fermentation broths using β-$CD_{cl}$-IDA-$Cu^{2+}$ as the adsorbent.

| | Unclarified fermentation broth | PEG contained unclarified fermentation broth |
|---|---|---|
| Initial activity (U/mL) | 380 | 371 |
| Residual activity (U/mL) | 344 | 39 |
| Adsorbed activity (U/mL) | 36 | 332 |
| Adsorption (%) | 9.53 | 90 |
| Desorbed activity (U/mL) | 78 | 301 |
| Desorption (%) | 100 | 91 |

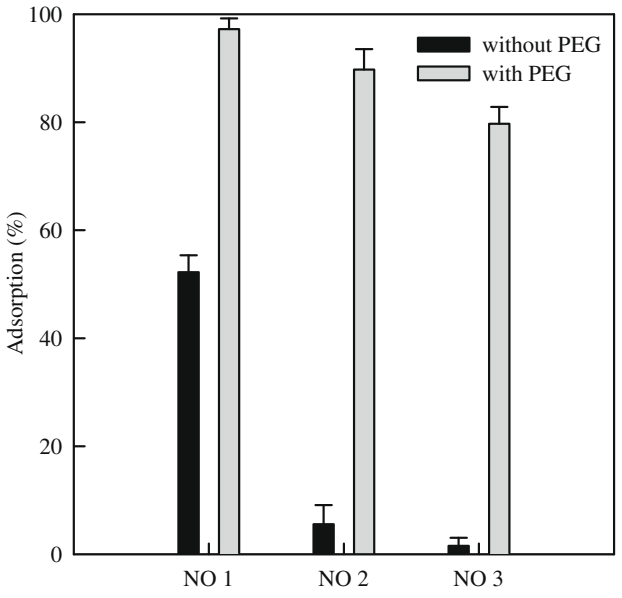


**Fig. 2.** Adsorption performances for α-amylase from three different fermentation batches. The conditions for these three batches of fermentation were the same.

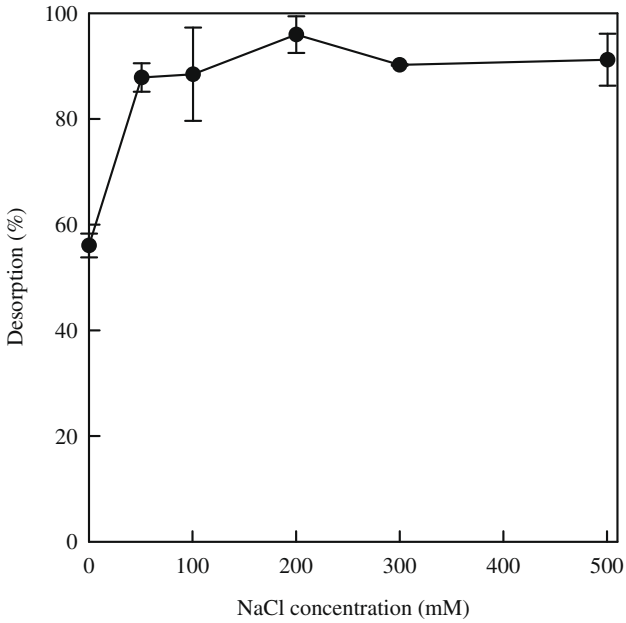


**Fig. 3.** Effect of NaCl concentration on the desorption of α-amylase. The bound α-amylase on the adsorbent was desorbed by 200 mM imidazole containing 100 mM phosphate buffers and 0–500 mM NaCl. The desorption was carried out at 37 °C for 30 min.

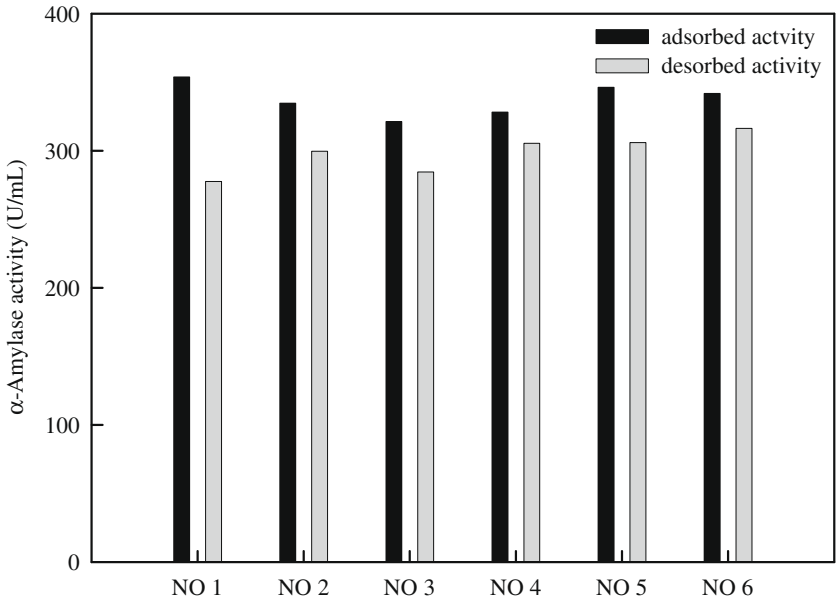


**Fig. 4.** Repeated use results of the affinity adsorbent β-$CD_{cl}$-IDA-$Cu^{2+}$ for α-amylase fermentation broth.

1989) present in the fermentation broth during the adsorption of α-amylase. A schematic representation of putative binding of α-amylase from the fermentation broth is shown in Fig. 5(a). It explained the binding that occurred between the species in the fermentation broth and the immobilized metal ion affinity adsorbents. Undesired proteins in the fermentation broth could also bind to the chelated metal ion affinity adsorbents. The small impurities in the fermentation broth could also enter the vacant surface of adsorbents much more easily and thus occupy the vacancy, which may hinder the specific binding of α-amylase. Therefore, the specific adsorption of the adsorbents towards α-amylase could be reduced. As shown in Fig. 5(b), the presence of polymers such as PEG and dextran not only reduced the stacking of impurities on the surface of the adsorbents but also formed polymer shielding which could further prevent the non-specific binding (Garg et al., 1996; Kumar et al., 2000).

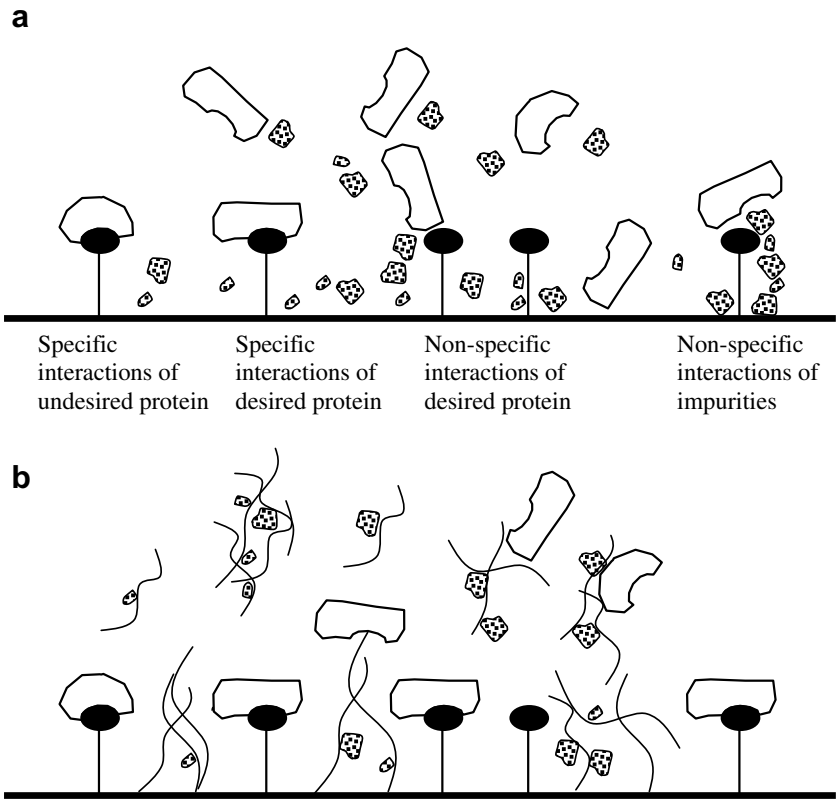


**Fig. 5.** A schematic representation of putative adsorption from (a) fermentation broth; (b) polymer containing fermentation broth. There are four interactions taking place simultaneously in (a) and the non-specific interactions can be reduced by the addition of polymers.

Although dextran was reported to be used as the immobilized metal ion affinity adsorbents for chromatography applications in the literature (Mateo et al., 2001), its relatively hydrophilic backbone, composed of sugar structures, could slightly attract hydrophobic compounds in the fermentation broth. Hence, adsorption of the α-amylase from the dextran containing fermentation broth was only around 76% (Table 1A). There was no significant adsorption of α-amylase from the PEG contained fermentation broth by β-$CD_{cl}$-IDA, which revealed that α-amylase was specifically bound to the chelated metal ion $Cu^{2+}$. The fermentation broths containing 15% (w/v) of PEGs with the different molecular weights of 1000, 4000 and 10,000, were applied to the adsorption of α-amylase. The adsorption efficiency was improved by the addition of PEG. The results from the added PEG were compared to that without PEG. The adsorption was increased from approximately 53% to 98%. As shown in group B of Table 1, more than 95% of α-amylase was adsorbed even though the molecular weight of PEG ranged widely from 1000 to 10,000. The adsorption of α-amylase was irrelative to the molecular weight of PEG when the added amount reached 15%. For the sake of convenience, the molecular weight of 4000 was chosen for the following experiments.

### 3.2. Effect of concentration of PEG with a molecular weight of 4000

The adsorption performances (%) with respect to different PEG concentrations (0%, 5%, 10%, 15%, 20%, and 25%) were under investigation. The results are shown in Table 1. The addition of PEG might stabilize or re-activate α-amylase, which resulted in a minor increase in the initial activity of α-amylase upon application with smaller amounts of PEG. However, the initial activity of α-amylase gradually decreased slightly with an increased PEG concentration. Nevertheless, the adsorption performance gradually increased to a maximum adsorption of approximately 98% and then decreased with the PEG amount accordingly. The maximum adsorption from the broth was reached with a PEG concentration of higher than 10%. Obviously, to add PEG into the fermentation broth, adsorption efficiency was significantly improved. PEG functions by promoting the adsorption of α-amylase (shown in Fig. 5 (a) and (b); (a) is to bind with the impurities in the fermentation broth by the hydrophobic interactions so as to form the network structures which are geometrically incongruent to reach the vacancy; and (b) is to protect the affinity chelated metal ions from non-specific interactions by means of the shielding effect of the PEG. The sufficient amount of PEG applied was assumed to meet these two effects. However, excess PEG amounts caused a higher viscosity of the broth and resulted in worse dispersion of adsorbents. Among all, 15% PEG showed superior performance. Hence, the amount of PEG added into the fermentation broth was set at 15% (w/v).

### 3.3. Adsorption kinetics and isotherm

The adsorption of α-amylase from the PEG contained fermentation broth by β-$CD_{cl}$-IDA-$Cu^{2+}$ took approximately 15 min to reach equilibrium, which could achieve more than 95% of adsorption. In the first minute, it could already reach 64% adsorption. Consequently, to secure the equilibrium, 30 min was determined as the adsorption time.

The adsorption isotherm of α-amylase from the PEG contained fermentation broth was obtained; however, instead of varying the α-amylase concentration, the adsorbent amount was varied from 10 to 150 mg. As shown in Fig. 1, obviously, the adsorbed activity of α-amylase increased with the increased amount of adsorbents. However, to further increase the amount to more than 100 mg, an extremely high as well as the highest adsorption efficiency of 99% was achieved. As a result, the required adsorbent amount was determined to be 100 mg.

### 3.4. Comparison of different desorption agents

The fermentation broths from three different batches were used for the adsorption study of α-amylase via the immobilized metal ion affinity adsorbents, β-$CD_{cl}$-IDA-$Cu^{2+}$. As shown in Fig. 2, the adsorption of α-amylase from the PEG contained fermentation broths decreased gradually between subsequent batches. Nevertheless, the adsorptions efficiency of α-amylase was significantly improved by the addition of PEG. In addition, the adsorption of α-amylase from the last-batch (third batch) of fermentation broth could be enhanced by adding a higher amount of PEG, say 25% (w/v). From such, the adsorption performance reached 94%.

The desorption agents applied afterwards could be buffers of a lower pH, competitive binding agents and chelating agents. The target proteins adsorbed by the adsorbents could be eluted via the electrostatic repulsion interaction between the metal ions and the target proteins. The interaction is induced by adjusting the pH to a lower level than the pI of the target proteins. The pI value of α-amylase, secreted from *Bacillus amyloliquefaciens*, was reported to be 4.9 (Schmidt et al., 1996). While lowering the pH, it must be noted that α-amylase could become inactive due to the low pH condition (Welker & Campbell, 1967). As shown in Table 2, in general, the competitive agent, a ligand exchange imidazole, and the strong chelating agent EDTA could be used as the desorption agents, respectively. The adsorbed α-amylase together with the $Cu^{2+}$ from the affinity adsorbents could be eluted by EDTA. The eluted $Cu^{2+}$ may interfere with the measurement of the protein concentration. Although EDTA had a strong chelating effect to cleave the binding between the ligand IDA and $Cu^{2+}$ of the adsorbent, the desorbed α-amylase activity was low (around 47% desorption). Therefore, imidazole, with superior desorption performance, was used for further investigation.

### 3.5. NaCl effect on desorption

It is a common approach to increase the hydrophobic interaction of proteins by adding salts to draw away water from the exposed hydrophobic areas. The presence of salt played the same role on desorption of the bound α-amylase from the β-$CD_{cl}$-IDA-$Cu^{2+}$ adsorbents. The interaction between the target enzyme and the copper ion complex could be formed and the surrounding water molecules could occupy the residual coordinate sites (Gaberc-Porekar & Menart, 2001). The kosmotropic ions interact strongly with water so as to destabilize the complexes (Fazal, Roy, Sun, Mallik, & Rodgers, 2001), and then imidazole could easily displace the bound proteins through the exchange of ligands. Hence, the desorption could be promoted by adding an appropriate amount of NaCl. As shown in Fig. 3, 95% of the desorption efficiency was achieved by the desorption agent, 100 mM phosphate buffer containing 200 mM imidazole and 200 mM NaCl. The α-amylase activity of 375 U/mL was eluted from the bound activity of 391 U/mL, which was adsorbed from the 15% PEG contained fermentation broth with an initial enzymatic activity of 429 U/mL. Therefore, recovery of α-amylase was 87%. Although NaCl promoted desorption of bound α-amylase from the adsorbents, the NaCl contained buffer was hardly able to elute the bound α-amylases due to the strong specific binding between the α-amylase and the metal ions.

In conclusion, β-$CD_{cl}$-IDA-$Cu^{2+}$ affinity adsorbent was successfully used to purify α-amylase from the clarified PEG contained fermentation broth. In order to reduce the steps of the downstream process, the fermentation broth without clarification was used directly after 40 h of the batch fermentation. The results (shown in Table 3) agreed with those obtained from the clarified fermentation broth. The presence of PEG in both clarified and unclarified fermentation broths did improve the adsorption of α-amylase through the utilization of β-$CD_{cl}$-IDA-$Cu^{2+}$. Furthermore,

α-amylase could be efficiently desorbed by a desorption agent as well. Consequently, 82% (90% × 91%) recovery in enzymatic activity was obtained by two steps of adsorption/desorption of α-amylase from the PEG contained unclarified fermentation broth.

### 3.6. Reuse of adsorbents

The affinity adsorbent β-$CD_{cl}$-IDA-$Cu^{2+}$ had the capacity of adsorption/desorption of α-amylase. However, the enzyme-bound adsorbents, upon desorption by a desorption agent, could no longer adsorb α-amylase from neither the fermentation broth or the PEG contained fermentation broth. The residual amount of desorption agent imidazole left on the adsorbents may likely prevent the rebinding of α-amylase. Hence, a buffer of low pH could be used to remove the bound imidazole by electrostatic repulsion. 0.2 M cupric sulphate was applied to elute the bound substances by its low pH and regenerated fresh cupric ions on the affinity adsorbents as well. Thus, after washing with deionized water, the adsorption/desorption of α-amylase from the PEG contained fermentation broth could be repeatedly operated six times by using the regenerated affinity adsorbents. Fig. 4 demonstrates the results. As shown in Fig. 5, there was no significant loss in both adsorbed and desorbed activities. This result demonstrated not only the stability of the adsorbents prepared, but also the feasibility for commercialization from easy and low-cost regeneration.

## 4. Conclusions

The immobilized copper ion affinity adsorbents, β-$CD_{cl}$-IDA-$Cu^{2+}$, were successfully applied to purify α-amylase from the fermentation broth by means of adding PEG and NaCl during the adsorption and desorption steps, respectively. The downstream process for α-amylase purification was remarkably reduced to only two steps, which were adsorption and desorption. The recovery efficiency of the α-amylase activity could be achieved to be above 80%. The α-amylase adsorption efficiency, carried out at 37 °C for 30 min by 100 mg of adsorbent, could reach a value of higher than 95% after adding PEG into the fermentation broth. Furthermore, this method was proven to be feasible by using three different fermentation batches. 200 mM imidazole containing 200 mM NaCl and 100 mM phosphate buffer could efficiently remove the bound α-amylase from β-$CD_{cl}$-IDA-$Cu^{2+}$ completely. In addition, the adsorbents could be reused after the easy and inexpensive regeneration step.

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