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Hydroxyapatite-based immobilized metal affinity adsorbents for protein purification

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Abstract

The employment of metal ion-charged hydroxyapatite for the one-step purification of poly(His)-tagged recombinant proteins was investigated. Fe(III) showed the highest selectivity toward the poly(His)-tagged D-hydantoinase and the best operation stability. The optimal selectivity was observed in 20 mM pH 8.0 buffer containing 150 mM NaCl and 50 mM NaF. The adsorbed poly(His)-tagged enzyme could be quantitatively recovered from hydroxyapatite with 150 mM pH 8.0 phosphate buffer. The capacity of Fe(III)-loaded hydroxyapatite for poly(His)-tagged D-hydantoinase was 4.9 mg/g hydroxyapatite, comparable to commercial agarose-based Ni-NTA adsorbents. Under optimal conditions, a D-hydantoinase preparation with a purity above 95% from crude cellular lysate could be obtained with the one-step purification process employing Fe(III)-loaded hydroxyapatite. The application of Fe(III)-loaded hydroxyapatite for the purification of poly(His)-tagged *N*-acetyl-D-glucosamine 2-epimerase under denaturing conditions was also demonstrated. These results demonstrate that hydroxyapatite is a promising adsorbent for immobilized metal affinity chromatography.

Keywords: Immobilized metal affinity chromatography; Hydroxyapatite; Protein purification

1. Introduction

Immobilized metal affinity chromatography (IMAC) has been widely used as a group specific affinity method for the purification of proteins since its introduction more than two decades ago [1]. The high selectivity of IMAC for recombinant proteins containing polyhistidine tags [2] and the introduction of many commercial IMAC adsorbents have made IMAC a standard technique for the purification of poly(His)-tagged recombinant proteins in molecular biology research [3,4]. In addition to high selectivity, IMAC exhibits the advantages of high binding capacity and high recovery yield due to the use of mild, non-denaturing elution conditions, compared to other biospecific chromatographic methods.

Immobilized metal affinity adsorbents have also been used as the matrices for the immobilization of enzymes [5,6]. In one of our recent studies, the utilization of a silica-based immobilized metal affinity adsorbent in an integrated enzyme purification and immobilization process for biotransformation was reported [7].

In light of its potentials for the downstream processing of poly(His)-tagged recombinant proteins, various IMAC-based processes have been developed, including immobilized metal affinity membranes [8,9] and immobilized metal affinity aqueous two-phase systems [10–12]. Detailed studies concerning the behavior of proteins in IMAC have also been conducted [13–15].

Although IMAC has been routinely used on bench scales for the recovery and purification of poly(His)-tagged recombinant proteins, successful applications of IMAC on industrial scales are still to be exploited due to process and safety considerations. Most of the commercially available

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polysaccharide-based IMAC adsorbents are generally of low mechanical strength and are subjected to biodegradation. The possible leaching of heavy metal from the matrices can also deem IMAC inappropriate for the purification of proteins for therapeutic or food applications. To address these issues, researches focusing on the development of novel metal chelators with high metal affinity [16] and alternative supports exhibiting better flow characteristics and mechanical and biological resistances have been reported [17].

Hydroxyapatite, $Ca_{10}(PO_4)_5(OH)_2$ [18], has been used for the chelation of heavy metals [19–21] and purification of proteins [22] and nucleic acids [23]. Due to the presence of crystal calcium ions and oxygen atoms associated with crystal phosphates on its surface, hydroxyapatite provides versatile interactions with proteins side chains under different pH and thus has been used as the medium in chromatographic columns for protein separation. The mechanisms for proteins adsorption on hydroxyapatite has been investigated [24–26]. Due to its excellent biocompatibility, hydroxyapatite has also been used as the carrier for drug delivery [27] and implants [28,29]. Recently, Lindqvist and coworkers reported the use of metal ion-loaded hydroxyapatite as the matrix for the purification of polyhistidine-tagged green fluorescent protein (GFP) [30]. It was demonstrated that metal ion-loaded ceramic hydroxyapatite exhibited high selectivity for the target protein. Compared to the conventional polysaccharide-based IMAC adsorbents, ceramic hydroxyapatite is easy to prepare and exhibits excellent flow characteristics and stability and is, thus, a promising substitute for conventional IMAC adsorbents.

In this study, we investigated the use of hydroxyapatite for the purification of two model poly(His)-tagged enzymes, D-hydantoinase [EC 3.5.2.2] and *N*-acetyl-D-glucosamine 2-epimerase (2-epimerase). D-Hydantoinase can catalyze the conversion of D,L-*p*-hydroxyphenylhydantoin to *N*-carbamoyl-D-hydroxyphenylglycine, an intermediate for the production of D-hydroxylglycine for the synthesis of semi-synthetic antibiotics such as amoxicillin and cefadroxil [31,32]. 2-Epimerase is one of the key enzymes for the enzymatic production of sialic acid [33], a major determinant of carbohydrate-receptor interactions in many systems pertinent to human health and diseases [34]. Parameters that might affect the selectivity and capacity of the metal-loaded hydroxyapatite for these two poly(His)-tagged recombinant proteins were systematically investigated. The application of hydroxyapatite for protein purification under denaturing conditions was also studied.

2. Experimental

2.1. Microorganism and growth conditions

Escherichia coli BL21(DE3) harboring pET 36 encoding D-hydantoinase gene from *B. caldolyticus* was grown in 500 ml basal medium containing 100 μg/ml ampicillin at 37°C as described earlier [31,32]. The overexpression of D-hydantoinase was induced by adding IPTG into the culture to a final concentration of 0.02 mM and downshifting temperature to 27 °C when the optical density of the culture measured at 600 nm reached 1.0. *E. coli* NovaBlue harboring pQEAnaCH1 encoding *N*-acetyl-D-glucosamine 2-epimerase gene from porcine kidney was grown in 500 ml LB medium containing 100 μg/ml ampicillin at 37 °C. The overexpression of epimerase was induced by adding IPTG into the culture to a final concentration of 1 mM and downshifting temperature to 30 °C when the optical density of the culture measured at 600 nm reached 0.6. The induced culture was harvested 10 h after induction by centrifugation at 10,000 × *g* for 10 min. Cell pellets collected were resuspended in 20 mM Tris pH 8.0 buffer and were subsequently lysed by ultrasonication. The crude cell lysate or insoluble aggregates thus obtained upon centrifugation were used as the raw material for protein purification.

2.2. Preparation of metal ion-loaded hydroxyapatide

Pre-determined amount of hydroxyapatite (88–177 μm, Wako Chemicals, Japan) was rinsed sequentially with deionized water, 100 mM pH 4.5 sodium acetate buffer and 1 M sodium chloride solution [30]. The rinsed hydroxyapatite was then submerged at room temperature for 30 min in 50 mM $CuSO_4$ (Junsei Chemical, Japan), $FeCl_3$ (Hayachi Chemical, Japan), $Co(NO_3)_2$ (Wako), or $ZnSO_4$ (Hayachi Chemical, Japan) solutions depending on the type of metal ions desired. The metal ion-loaded hydroxyapatite was then washed sequentially with 1 M NaCl solution and 5 mM, pH 6.0 Na_2HPO_4 buffer.

2.3. Purification of recombinant proteins with metal ion-loaded hydroxyapatite

Metal ion-loaded hydroxyapatite (50 mg) was added to 1.5 ml of cell lysate at pH 7.5 containing 100 mM NaCl and 50 mM NaF. Unless specified otherwise, all adsorption steps were conducted at 4 °C for 1 h. The hydroxyapatite was then removed by centrifugation. The supernatant collected was subjected to protein assay for residual protein concentration and SDS–PAGE analysis for purity analysis. To establish optimal adsorption pH, metal ion-loaded hydroxyapatite was added to cell lysate in 20 mM Tris buffer at pH ranging from 6.0 to 8.0. The protein-loaded hydroxyapatite was then eluted with 150 mM pH 8.0 phosphate buffer following rinsing with equal volume of 100 mM pH 7.0 phosphate buffer. The *spent* cell lysate and eluted products were all subjected to protein assay and SDS–PAGE analysis. To optimize the type and concentration of salts for enzyme adsorption, NaF and/or NaCl at concentrations up to 300 mM were included in the cell lysate. The buffers and other conditions employed during adsorption, washing, and elution were identical to what were described above. To investigate the effect of eluent components on protein recover, *imidazole* and phosphate at concentrations

up to 300 mM were included in washing buffer and/or elution buffers.

2.4. Purification of enzymes under denaturing conditions

2-Epimerase inclusion bodies collected by centrifugation at 12,000 × *g* for 15 min upon cell lysis with pH 8.0, 50 mM Tris buffer containing 100 mM NaCl and 1 mM EDTA were solublized with 20 mM pH 7.5 phosphate buffer containing 150 mM NaCl, 50 mM NaF, 0.072 mM 2-mercaptoethanol (Sigma) and 6 M guanidine hydrochloride (Sigma) at 4 °C for 24 h and recovered by centrifugation. Fifty micrograms of Fe(III)-charged hydroxyapatite was added into 1 ml of the solublized 2-epimerase inclusion body solution and incubated at 4 °C for 1 h. The protein-loaded hydroxyapatite was then eluted with 150 mM phosphate buffer containing 0.072 mM 2-mercaptoethanol and 6 M guanidine hydrochloride following washing with the 20 mM pH 7.5 phosphate adsorption buffer. The spent inclusion body solution and the eluted products were subjected to protein assay and SDS–PAGE analysis.

2.5. Analysis

Protein concentrations of all samples were analyzed by the Bradford method with protein dye (Bio-Rad) at 595 nm. The purity of each protein preparations was analyzed by SDS–PAGE with 12% polyacrylamide gel stained with Commassie blue and quantified by densitometry. For samples containing high concentration of salt and/or guanidine hydrochloride, dialysis was performed before the samples were subjected to SDS–PAGE anaysis. The activity of D-hydantoinase was determined by the conversion of D,L-*p*-hydroxyphenylhydantoin (D,L-HPH) to *N*-carbamoyl-D-hydroxyphenylglycine (*cp*-HPG) with HPLC [31,32]. Pre-determined amount of purified D-hydantoinase was added to a substrate solution containing 1 mg/ml D,L-HPH in 50 mM pH 8.0 sodium phosphate buffer. The reaction as allowed to proceed for 1 h at 60 °C. Concentrations of D,L-HPH and *cp*-HPG in the reaction mixtures were analyzed by HPLC with a JASCO HPLC system equipped with a C18 reverse phase column (LiChrCart, 4.6 mm × 250 mm, Merck) with a mobile phase of 5% acetonitrile in 0.01% phosphoric acid solution at a flow rate of 0.5 ml/min [31,32]. The absorbance of the eluent from the column was monitored with an UV detector at 210 nm.

3. Results and discussion

3.1. Purification of D-hydantoinase with metal ion-loaded hydroxyapatite

3.1.1. Optimal metal ion

The effect of type of metal ion chelated on hydroxyapatite on the adsorption of poly(His)-tagged D-hydantoinase

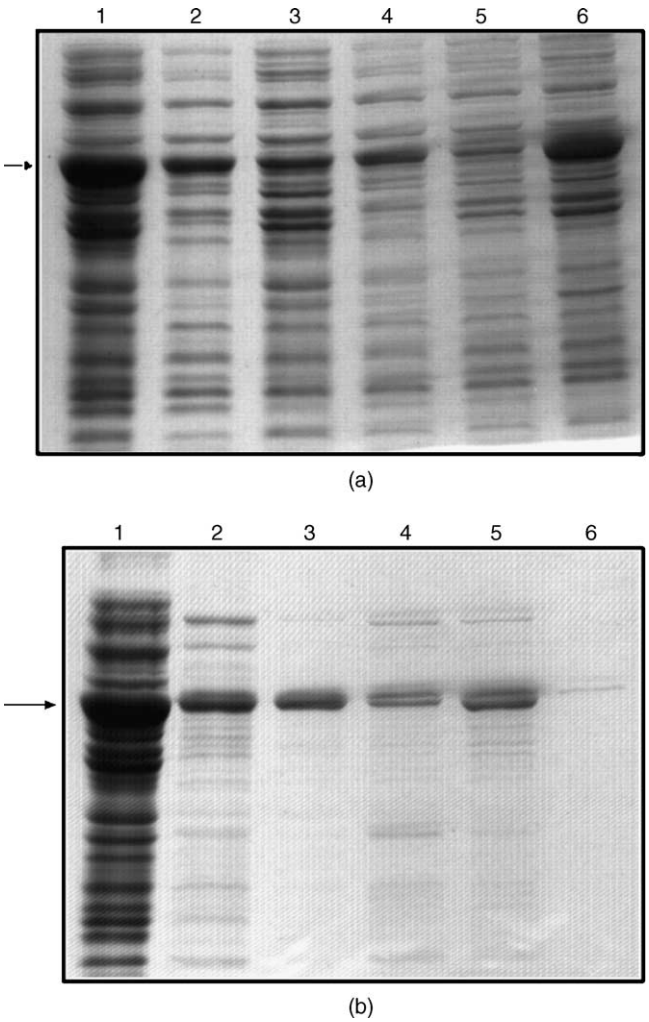

Fig. 1. Efficiency of protein adsorption of hydroxyapatide (HAP) loaded with different metal ions. (a) SDS–PAGE analysis of flow through from column containing HAP loaded with different ions. Lane 1: crude cell lysate, lane 2: Cu(II), lane 3: Fe(III), lane 4: Zn(II), lane 5: Co(II), and lane 6: control-HAP without metal ions, and (b) SDS–PAGE analysis of eluted products from column with HAP loaded with different metal ions. Lane 1: crude cell lysate, lane 2: Cu(II), lane 3: Fe(III), lane 4: Zn(II), lane 5: Co(II), and lane 6: control-HAP without metal ions. Elution was performed with 150 mM pH 8.0 phosphate buffer following washing with 100 mM pH 7.0 phosphate buffer.

was shown in Fig. 1. The efficiency of Ni(II), which is commonly used in many IMAC systems, for protein adsorption in the hydroxyapatite system was not studied because both our preliminary results (data not shown) and Lindqvist and coworkers' work have found that Ni-loaded exhibits poor binding properties toward poly(His)-tagged proteins [30]. In Fig. 1a, the spent cell lysates after contacting with different metal ion-charged hydroxyapatite were analyzed. It is evident that metal ion-loaded hydroxyapatite is capable of selectively adsorbing poly(His)-tagged proteins. While significantly lower protein adsorption was observed with control hydroxyapatite without metal ion, lane 6, all metal ion-loaded hydroxyapatites exhibited adsorption capability toward poly(His)-tagged proteins, lanes 2–5. It was

observed the spent cell lysate from Fe(III)-loaded hydroxyapatite, lane 3, contained the highest amounts of residual contaminating cellular proteins, indicating that Fe(III) exhibited the lowest protein adsorption capacity for contaminating proteins and thus probably the highest adsorption selectivity toward the poly(His)-tagged D-hydantoinase. Results of SDS–PAGE analysis on the elution products, Fig. 1b, indeed indicated that the Fe(III)-loaded hydroxyapatite exhibited the highest specificity toward poly(His)-tagged D-hydantoinase. While the eluted products from Cu(II), Zn(II), and Co(II)-load hydroxyapatite contained visible amounts of contaminating proteins, only trace amount of contaminating proteins was observed in the eluted product from Fe(III)-loaded hydroxyapatite, lane 3. It is also noteworthy that Cu(II) and Zn(II)-load hydroxyapatite exhibited the highest, lane 2, and the lowest, lane 4, binding capacities toward D-hydantoinase, respectively. These results contradict with Lindqvist and coworkers' findings showing that Zn(II)-load hydroxyapatite exhibited the superior affinity over other metal ions toward both poly(His)-tagged GFP and untagged GFP [30]. Nevertheless, it is consistent with Porath and coworkers' results indicating that Zn(II) exhibits weak affinity [35].

The fact that *very little* poly(His)-tagged D-hydantoinase was recovered from the control experiment, hydroxyapatite without chelated metal ions (lane 6), indicates that the adsorption of poly(His)-tagged D-hydantoinase by the metal ion loaded hydroxyapatite is likely to be mediated by the formation of coordinated complex between the *imidazole* groups on protein surface and the chelated metal ions, instead of ionic interactions between the charges groups on protein and hydroxyapatite surfaces. Schematic representation of the binding of poly(His)-tagged proteins to the surface of metal ion-charged hydroxyapatite is shown in Fig. 2.

The chelating strength of hydroxyapatite toward different metal ions is different. It was observed that Cu(II) and, to a less extent, Co(II), were displaced from hydroxyapatite with 50 mM *imidazole*, a displacer frequently used for the recovery of protein from IMAC adsorbents. On the contrary, only minimal amount of Fe(III) was eluted from hydroxyapatite even with 300 mM imidazole, indicating that hydroxyapatite exhibits the strongest chelating strength toward Fe(III). In light of this finding, Fe(III)-loaded hydroxyapatite was used for all the subsequent experiments, even though the Cu(II) exhibits the higher adsorption capacity toward the targeted enzymes.

3.1.2. Optimal adsorption pH

To identify the optimal pH for the adsorption of D-hydantoinase on Fe(III)-loaded hydroxyapatite, the adsorption was conducted at pH ranging from 6.0 to 8.0 and the adsorbed proteins were eluted with the same eluting buffer, Fig. 3. It was observed that while the amount of proteins, ca. 4.0 mg/g hydroxyapatite, recovered from the adsorbents was relatively independent of the pH, the amount of total proteins adsorbed decreased significantly with pH. At pH 8.0 the amount of total proteins adsorbed was the lowest,

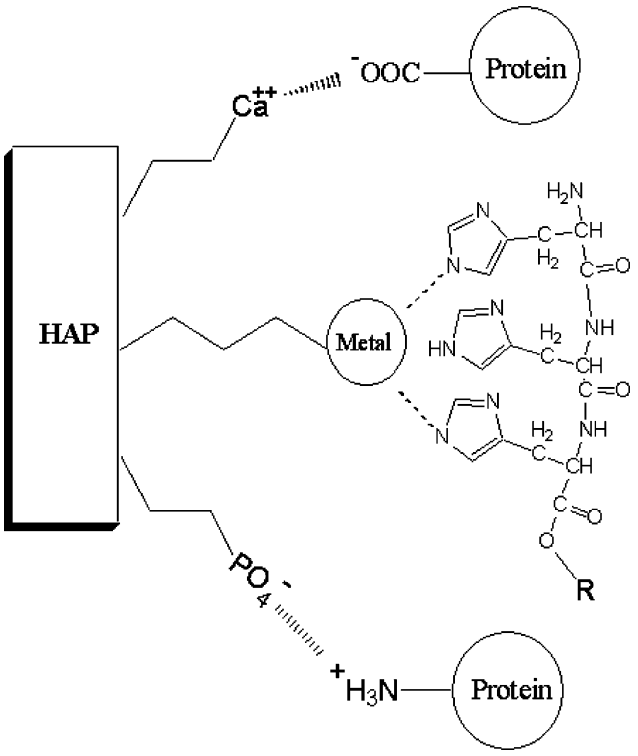

Fig. 2. Schematic representation of the adsorption of poly(His)-tagged proteins on the surface of metal ion-charged hydroxyapatide. The chelation of metal ion is mediated by clusters of negatively charged oxygen atoms associated with crystal phosphates on the hydroxyapatide surface. The calcium sites and phosphate sites can also participate in the adsorption of proteins via nonspecific ionic interactions.

indicating better selectivity toward targeted proteins at this pH. This observation was further supported by the results of D-hydantoinase activity analysis, which showed that the eluted products obtained at an adsorption pH of 8.0 gave

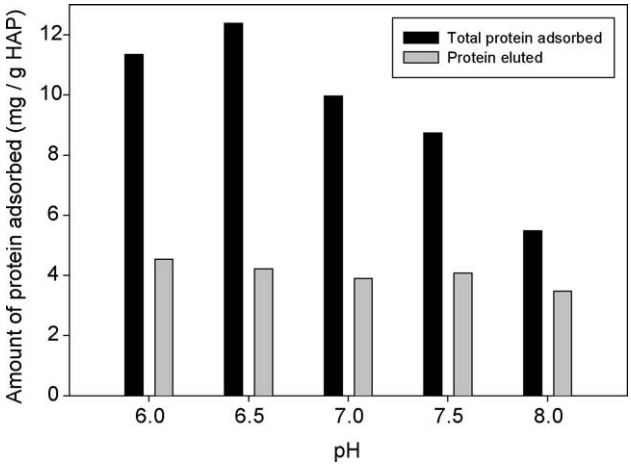

Fig. 3. Effect of pH on protein adsorption with Fe(III)-charged HAP. The amount of total protein adsorbed was quantified by subtracting the amount of proteins in the flow through from the amount of proteins in the crude cell lysate. Elution was performed with 150 mM pH 8.0 phosphate buffer following washing with 100 mM pH 7.0 phosphate buffer. Protein concentration was determined by Bradford method.

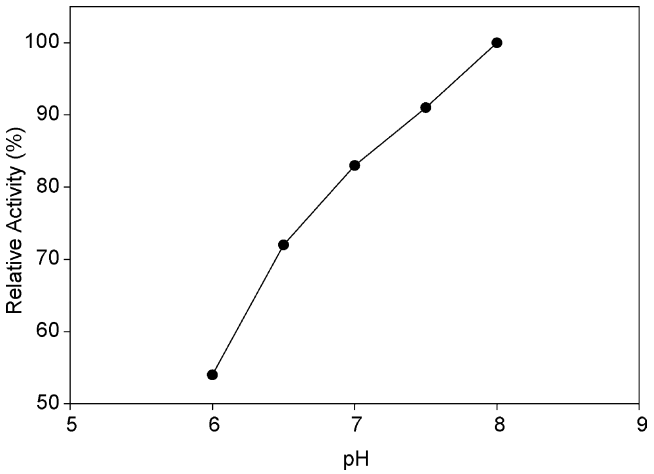

Fig. 4. Effect of adsorption pH on the relative specific D-hydantoinase activity of protein products eluted from Fe(III)-charged HAP. All the protein preparations were obtained by eluting with pH 8.0150 mM phosphate buffer. D-Hydantoinase activity was determined by HPLC. The specific D-hydantoinase activity of the eluted products obtained from adsorption conducted at pH 8.0 was used as the reference.

the highest specific activity, Fig. 4. This result is consistent with the general practice in IMAC adsorption. It is generally recommended to conducted protein adsorption with IMAC adsorbents at pH above the pK_a of histidine, which could strengthen the binding between poly(His)-tagged proteins by deprotonating the imidazole group of the histidine residues and thus eliminating the possible repulsive forces between them and the chelated metal ions.

3.1.3. Effect of salt on adsorption selectivity

It is generally desirable to include high concentration of salt during protein adsorption in IMAC to render higher selectivity by attenuating nonspecific adsorption mediated by ionic interactions. The effect of the inclusion of NaCl

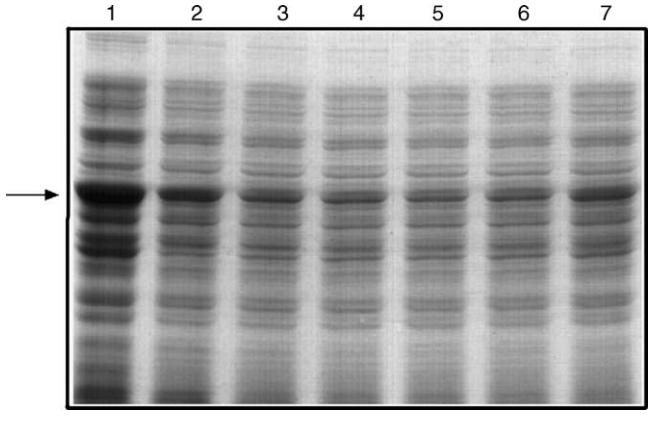

Fig. 5. Effect of NaCl concentration on protein adsorption with Fe(III)-charged HAP. SDS–PAGE analysis of flow through from column containing Fe(III)-charged HAP. Adsorption of proteins was performed at a NaCl concentration of 0 mM (lane 2), 100 mM (lane 3), 150 mM (lane 4), 200 mM (lane 5), 250 mM (lane 6), and 300 mM (lane 7). Crude cell lysate (lane 1) was included as the control.

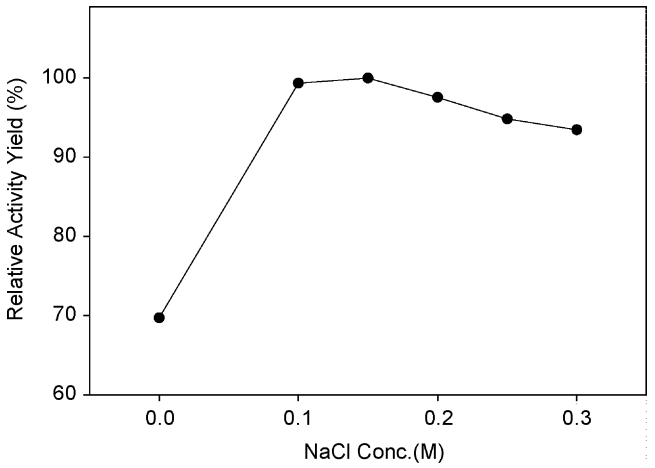

Fig. 6. Effect of NaCl concentration in adsorption buffer on the D-hydantoinase activity of protein products eluted from Fe(III)-charged HAP. D-Hydantoinase activity was determined by HPLC.

and/or NaF in the adsorption buffers on adsorption selectivity was thus investigated. In addition to the phosphate groups used to chelate metal ions, there are two other types of potential protein adsorption sites, calcium and hydroxyl, which might mediate the nonspecific adsorption of proteins. The effect of NaCl concentration in the adsorption buffer was shown in Fig. 5. The inclusion of NaCl promoted the specific adsorption of poly(His)-tagged enzymes as well as the nonspecific adsorption of contaminating protein, as evident by the decrease in band intensities of the adsorbed cell lysates in SDS–PAGE. This observation was further supported by the results of D-hydantoinase activity analysis, which indicated that the highest enzyme activity was obtained in the presence of 150 mM NaCl, Fig. 6. This result is somewhat unexpected as there is no potential hydrophobic interactions that would be promoted by high salt concentration. The level of enhancement in protein adsorption mediated by the presence of NaCl for poly(His)-tagged

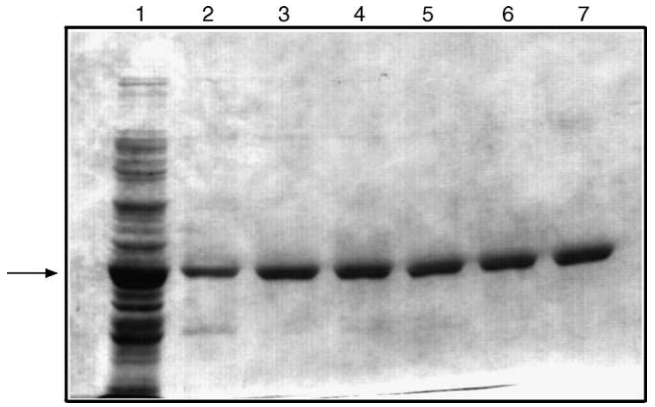

Fig. 7. Effect of NaF concentration on protein adsorption with Fe(III)-charged HAP. SDS–PAGE analysis of protein products eluted from column containing Fe(III)-charged HAP with adsorption performed at a NaF concentration of 0 mM (lane 2), 50 mM (lane 3), 100 mM (lane 4), 150 mM (lane 5), 500 mM (lane 6), and 250 mM (lane 7). Crude cell lyaste is the control.

D-hydantoinase was probably higher than that for the nonspecific adsorption of contaminating proteins because the specific enzyme activities of the eluted products with NaCl were at least 24% higher than that of the control (data not shown).

The effect of NaF concentration in the adsorption buffer was also investigated, because it had been found that NaF could inhibit the nonspecific binding between the carboxyl groups of proteins and calcium ions on hydroxyapatite [24–26]. It was observed that the amount of D-hydantoinase recovered was increased in the presence of 50 mM NaF, lane 3, Fig. 7. However, further increase in NaF concentration did not improved the activity recovery yields or purities of the eluted products.

3.1.4. Optimal elution conditions

Low concentration of imidazole has been frequently used for the removal of proteins adsorbed via nonspecific interactions on IMAC adsorbents before the targeted proteins are eluted. The inclusion of 20 mM imidazole in the washing buffer was thus used to investigate the efficiency of contaminating protein removal from hydroxyapatite, Fig. 8a. Small amount of contaminating proteins accompanied by an even higher amount of D-hydantoinase was desorbed from hydroxyapatite, lane 3. Phosphate, previously used at a concentration of 50 mM to eluted poly(His)-tagged proteins from hydroxyapatite [30], was used to substitute for imidazole in the washing buffer, Fig. 8b. It is apparent that with 10 mM phosphate at pH 8.0 the majority of the targeted proteins were eluted together with some contaminating proteins, lane 3. In an effort to limit the use of imidazole, phosphate buffers at different concentrations and pHs were used to elute contaminating proteins and elute targeted proteins, Fig. 8c. It was found that while the washing strength of 100 mM pH 7.0 phosphate buffer, lane 3, Fig. 8c, was equivalent to that of 20 mM pH 8.0 Tris buffer containing 300 mM NaCl and 10 mM phosphate, lane 3, Fig. 8b, the elution strength of 150 mM pH 8.0 phosphate buffer, lane 4, Fig. 8c, was much higher than that of Tris buffer containing 300 mM imidazole, lane 4, Fig. 8b. Results of mass balance analysis, data not shown, indicated that not all of the D-hydantoinase adsorbed was washed and eluted with 300 mM imidazole, Fig. 8a and b. A denaturing elution protocol involving boiling the eluted hydroxyapatite with SDS–PAGE sample buffer containing 10% SDS at 100 °C for 10 min was used to dislodge proteins remained on hydroxyapatite after elution [36], Fig. 9.

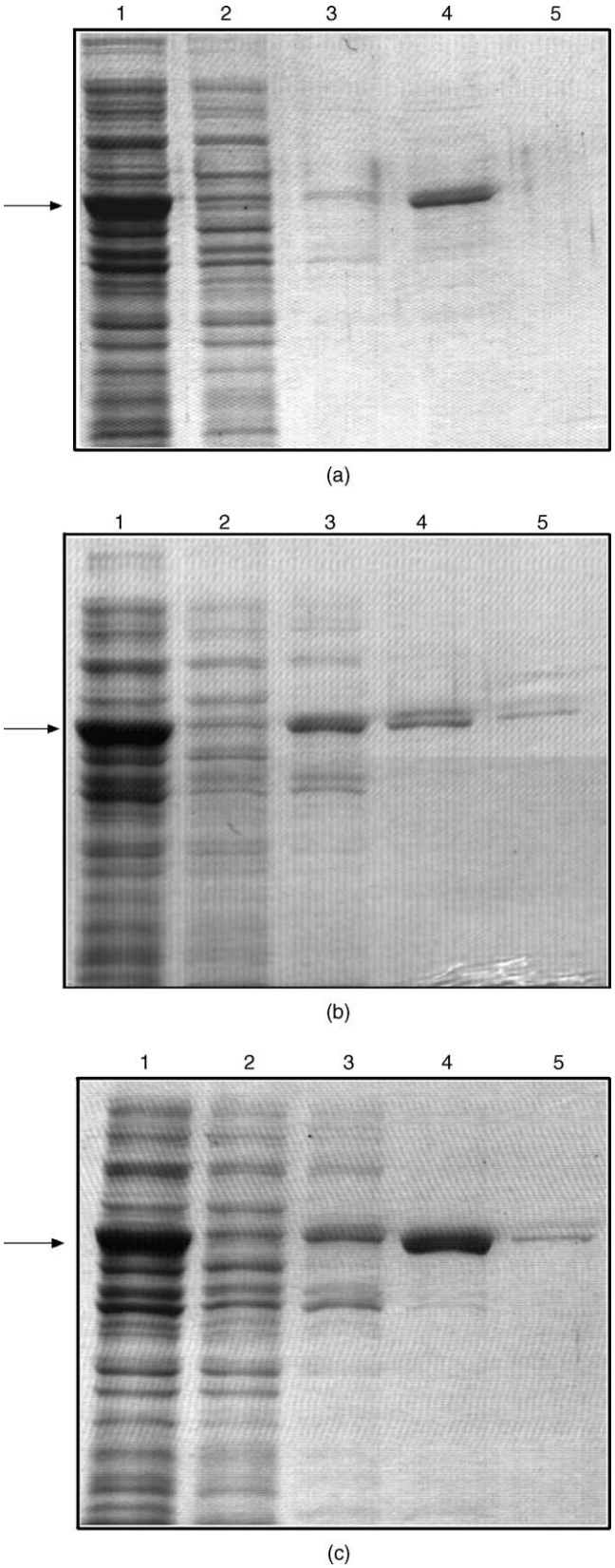

Fig. 8. Effect of washing and elution buffers on protein recovery. (a) Lane 1: cell lysate, lane 2: flow through, lane 3: wash with 20 mM pH 8.0 Tris–HCl, 300 mM NaCl, and 20 mM imidazole, lane 4: first elution with 20 mM pH 8.0 Tris–HCl, 300 mM NaCl, and 300 mM imidazole, and lane 5: second elution with 20 mM pH 8.0 Tris–HCl, 300 mM NaCl, and 300 mM imidazole, (b) lane 1: cell lysate, lane 2: flow through, lane 3: wash with 20 mM pH 8.0 Tris–HCl, 300 mM NaCl and 10 mM Na_2HPO_4, lane 4: first elution with 20 mM pH 8.0 Tris–HCl, 300 mM NaCl and 300 mM imidazole, and lane 5: second elution with 20 mM pH 8.0 Tris–HCl, 300 mM NaCl, and 300 mM imidazole, and (c) lane 1: cell lysate, lane 2: flow through, lane 3: wash with 100 mM pH 7.0 Na_2HPO_4, lane 4: first elution with 150 mM pH 8.0 Na_2HPO_4, and lane 5: second elution with 150 mM pH 8.0 Na_2HPO_4.

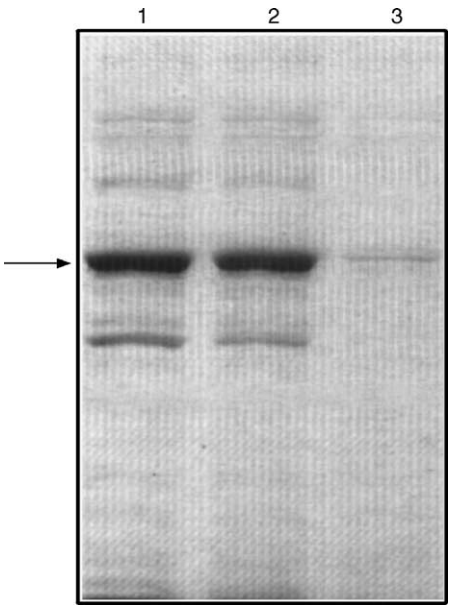

Fig. 9. Elution of spent adsorbents with SDS buffer with boiling. Lane 1: eluted products from adsorbent washed and eluted with buffer described in Fig. 8a lane 2: eluted products from adsorbent washed and eluted with buffer described in Fig. 8b, and lane 3: eluted products from adsorbent washed and eluted with buffer described in Fig. 8c.

It was observed that while large amounts of D-hydantoinase and contaminating proteins were eluted from hydroxyapatite previously eluted with 300 mM imidazole, negligible amount of proteins was eluted from hydroxyapatite previously eluted with 150 mM pH 8.0 phosphate buffer. This result further confirms that 150 mM pH 8.0 phosphate buffer is more effective than 300 mM imidazole in eluting poly(His)-tagged proteins from metal ion-loaded hydroxyapatite.

It has been previously described that the elution of poly(His)-tagged GFP could be achieved with phosphate, which can compete with the surface-bound phosphate on hydroxyapatite and thus leads to the elution of proteins, and was not affected by pH [30]. However, we found that the elution of protein with phosphate buffers was only effective at pH 8.0 not at pH 7.0. The difference in elution behavior at such pH range might imply the involvement of imidazole side chains of histidine residues, which has a pK_a of 6.04 and could vary between 6.0 and 8.0 depending on it's the adjacent microenvironment, in electrostatic interactions with surface-bound phosphate groups. We believe at pH 7.0 the dissociation of poly(His)-tagged proteins from the immobilized metal by phosphate ions was partially off-set by the attractive ionic interactions between the positively charged, protonated imidazole side chains of the poly(His)-tagged proteins and the negatively charged surface-bound phosphate groups whose chelated metal ions were stripped off by phosphate ions in the elution buffer and, thus, led to the low efficiency of protein elution. On the contrary, at pH 8.0 the ionic interactions mentioned above were nullified, due to the de-protonation of the imidazole side chains, and thus allowed the complete elution of poly(His)-tagged protein with phosphate.

Based on the results of protein assay and image analysis on SDS–PAGE gel, Fe(III)-loaded hydroxyapatite exhibited a capacity for D-hydantoinase of 4.9 mg/g hydroxyapatite, far superior to silica-based IMAC adsorbents [7] and comparable to that obtained with the other commercial agarose-based Ni-NTA adsorbents. Under optimal conditions, the Fe(III)-loaded hydroxyapatite can be use to purify D-hydantoinase from crude cellular extract in one-step with a purity above 95%, based on densitometry analysis on SDS–PAGE gels.

3.2. Purification of 2-epimerase with metal ion-loaded hydroxyapatite

The purification of soluble 2-epimerase from cell lysate with Fe(III)-loaded hydroxyapatite was conducted. The conditions for adsorption, washing and elution were identical

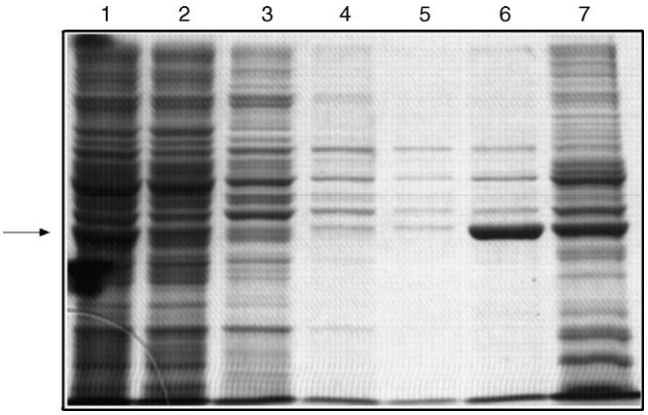

Fig. 10. Purification of soluble 2-eprimerase with Fe(III)-loaded hydroxyapatide. Lane 1: cell lysate, lane 2: flow through, lanes 3–5: wash with 100 mM pH 7.0 phosphate, lane 6: elution with 150 mM pH 8.0 phosphate, and lane 7: elution with SDS buffer with boiling.

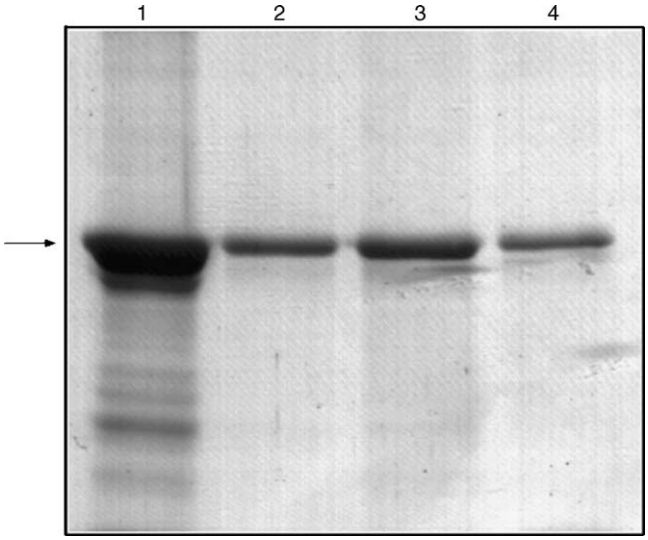

Fig. 11. Purification of 2-eprimerase with Fe(III)-loaded hydroxyapatide under denaturing condition. Lane 1: crude inclusion bodies, lane 2: flow through, lane 3: elution with 150 mM pH 8.0 phosphate, and lane 4: elution with SDS buffer with boiling.

to those used in Fig. 8c. Again high purity of 2-epimerase can be obtained following the protocol described in the previous section, Fig. 10. However, it was also found that about 50% of the adsorbed 2-epimerase together with some contaminating proteins could not be eluted from hydroxyapatite without using the aforementioned SDS-boiling method.

The possibility of employing metal ion-loaded hydroxyapatite for the purification of 2-epimerase under denaturing conditions was also investigated. 2-Epimerase inclusion bodies solubilized in 6 M guanidine hydrochloride was loaded onto Fe(III)-loaded hydroxyapatite column pre-equilibrated with adsorption buffer containing 0.072 mM 2-mercaptoethanol and 6 M guanidine hydrochloride. The protein-charged column was subsequently washed with the equilibrating buffer and eluted with elution buffer also containing 0.072 mM 2-mercaptoethanol and 6 M guanidine hydrochloride. It was found that higher purity of solubilized 2-epimerase could be recovered under denaturing conditions, Fig. 11. Not unlike the purification of soluble 2-epimerase, some adsorbed 2-epimerase could not be eluted without using the SDS boiling treatment.

4. Conclusions

The results of one-step protein purification with metal ion-loaded hydroxyapatite under both non-denaturing and denaturing conditions demonstrate that hydroxyapatite is a promising adsorbent for IMAC. However, the optimal conditions, including the type of metal ions, for the purification of poly(His)-tagged proteins with metal ion-charged hydroxyapatite may vary for different proteins. For example, while Zn(II) has been shown to exhibit superior binding selectivity toward poly(His)-tagged green fluorescent protein [30], in this study Fe(III) was found to be ideal candidate for the purification of poly(His)-tagged D-hydantoinase. Nevertheless, since it was also observed that Fe(III) exhibited superior operation stability on hydroxyapatite, we recommend that Fe(III) should be considered first as the ligand for the purification of poly(His)-tagged proteins with hydroxyapatite-based IMAC. The results of this study also suggest that the mechanisms of protein adsorption on metal ion-loaded hydroxyapatite might be more complicated than that involved in protein adsorption on conventional IMAC adsorbents. Although high concentration of *imidazole* in conjuction with acidic pH has been widely used to elute poly(His)-tagged proteins from IMAC adsorbents, large amount of poly(His)-tagged D-hydantoinase and contaminating proteins remained uneluted with this approach, implying the possible involvement of interactions in addition to the coordinate bonds between the adsorbents and proteins. In addition to the phosphate sites responsible for the chelation of metal ions, calcium and hydroxyl groups abundant on the surface of hydroxyapatite may also participate in the adsorption of proteins. The optimal pH of phosphate buffer for the elution of targeted proteins is critical for the success for protein purification with hydroxyapatite-based IMAC adsorbents and could vary depending on the nature of the targeted proteins. The utility of the metal ion-loaded hydroxyapatite for the purification of poly(His)-tagged protein under denaturing conditions was also demonstrated with *N*-acetyl-D-glucosamine 2-epimerase inclusion bodies solubilized in buffer containing 6 M guanidine hydrochloride. Hydroxyapatite, exhibiting superior flow characteristics as well as biological and physicochemical stability, is thus a promising substitute for conventional IMAC adsorbents for protein purification.

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