



Adsorption of histidine-containing dipeptides on copper(II) immobilized chelating resin from saline solution

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ABSTRACT

Adsorption of histidine-containing dipeptides such as carnosine (Car) was investigated using copper(II) immobilized cation exchange resins. Adsorption of Car was enhanced using Cu(II) immobilized resins, on the basis of metal affinity interactions. In particular, iminodiacetic acid chelating resin with immobilized Cu(II) (Cu-IDA) can adsorb Car from saline water. Car was adsorbed on Cu-IDA even in the presence of 1000 mM of NaCl. Adsorption of various amino acids on Cu-IDA was compared under same conditions. Histidine and the histidine-containing dipeptides were selectively adsorbed on Cu-IDA over other amino acids, both in the absence and in the presence of NaCl. Therefore, immobilized metal affinity adsorption is an efficient method for recovering histidine-containing dipeptides from saline water.

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1. Introduction

To date, a variety of functional foods and food supplements containing natural medicinal products are present on the market in line with people's increasing interest in health management. The naturally occurring histidine-containing dipeptides, carnosine (Car, β -alanyl-L-histidine) and anserine (Ans, β -alanyl-L-1-methylhistidine) (shown in Fig. 1) are two examples.

The histidine-containing dipeptides (abbreviated as HCDPs) are present in the muscle and brain of many animals and humans [1,2]. They act as cytosolic buffering agents, metal ion chelators, anti-oxidants, as well as having neurotransmitter functions [3–7]. HCDPs are also believed to be a potential treatment for Alzheimer's disease through the prevention of protein crosslinking [8]. Recently, Car has been found to protect against the zinc-induced death of cultured neurons, and has been the subject of a patent as a potential drug for ischemia-induced neuronal death and treatment/prevention for vascular type of dementia [9]. As such, HCDPs are attractive materials for food supplements.

HCDPs are contained in fresh meats and commercially available processed meat products [10–13]. As they are also contained in meat-derived extracts such as beef broth, chicken broth, and fish

broth [14,15], food extracts and food wastes which are eliminated during food processing might be candidates for cheap recovery of HCDPs. However, the food extracts obtained from processing contains other amino acids and inorganic salts. Therefore, separation techniques for recovering HCDPs from a mixture of amino acids and salts are required. Yanai et al. studied the isolation of HCDPs from chicken extract by membrane treatment [16]. Car and Ans were purified from a series of separation processes using an ultrafiltration membrane for removing proteins as an impurity followed by application of a nanofiltration membrane for recovering HCDPs as a retentate.

Ion exchange techniques are also effective for separation of amino acids and peptides; however, adsorption on ion exchange resins significantly decreases in saline water because of the inhibition of the adsorption by salts such as NaCl. In the present study, the adsorption of HCDPs using immobilized metal affinity resins from saline water was studied. Immobilized metal affinity chromatography (IMAC) has been developed as a selective separation tool for biomolecules [17–21]. The separation factor of IMAC is based on differences in the affinities between immobilized metal ions and functional groups of biomolecules. Transition metal ions such as Cu(II), Ni(II), Co(II), and Zn(II) immobilized to metal-chelating groups on adsorbents interact with amino acid side chains exposed on the surface of the protein molecules. Histidine residue shows the strongest affinity for the transition metal ions on the basis of the principles of hard and soft acids and bases (HSAB) [22]. IMAC is currently applied for the purification of recombinant proteins.

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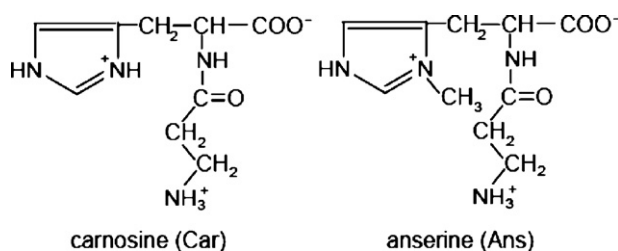


Fig. 1. Molecular structures of Car and Ans.

Recombinant proteins with fused peptide tags containing multiple histidine residues as metal affinity sites on their C- or N-terminus are selectively recovered using metal immobilized chelating gels often in a single chromatographic step [23–28].

The present study aims to evaluate Cu(II) immobilized cation exchange resins for the adsorptive recovery of HCDPs from saline solution containing various amino acids and peptides. The metal immobilized resins, which interact with HCDPs via coordination bonding, are expected to function based on the metal affinity between the imidazole group of the HCDPs and Cu(II), with little interference from any coexisting inorganic ions. They should also possess selectivity for the HCDPs over other amino acids. Commercial polystyrene sulfonic acid resin as the strongly acidic cation exchange resin, carboxylic acid resin as the weakly acidic cation exchange resin, and iminodiacetic acid resin as the chelating resin were used for Cu(II) immobilization, in view of cost performance. Adsorption of Car on the cation exchange resins and the Cu(II) immobilized resins were demonstrated. Effect of pH and NaCl concentration coexisting in the solution were investigated. Adsorption selectivity of HCDPs over other amino acids and elution of HCDPs from the Cu(II) immobilized resin were also studied.

2. Experimental

2.1. Materials

Fig. 2 shows the molecular structures of the cation exchange resins used in this study. Commercially available cation exchange resins DIAION® SK-1B (abbreviated as SK), WK10 (abbreviated as WK), and CR11 (abbreviated as IDA) were kindly supplied by Mitsubishi Chemical Co., Japan. The conditionings of these cationic resins were conducted by washing them using 0.1 M of NaOH, 0.3 M of HCl, 0.3 M of NaCl, and finally distilled water.

Analytical grade amino acid reagents for the adsorption experiments were purchased and employed without further purification: i.e., L-carnosine (Car), L-anserine (Ans) (Sigma–Aldrich Co., USA), L-glutamic acid (Glu), glycine (Gly), L-cysteine (Cys), L-methionine (Met), L-tryptophan (Trp), L-phenylalanine (Phe), L-histidine (His), and L-arginine (Arg) (Wako Pure Chemical Industries, Japan). An aqueous mixed solution containing 0.25 mM of 17 amino acids (Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Lys, His and Arg) and 2 HCDPs (Car and Ans) were prepared using an amino

acids mixture standard solution (Wako Pure Chemical Industries, Japan) and analytical grade of Car and Ans. All other reagents were of reagent grade and were used as received.

2.2. Preparation of copper(II) immobilized resins

Cu-IDA was prepared by adsorption of Cu(II) ion on IDA via an ion exchange reaction as follows: An aqueous solution containing 10.0 mM of Cu(NO₃)₂ was prepared and the pH was adjusted to 4.3 using 5.0 mM of HEPES buffer and diluted hydrochloric acid. A 1.0-L portion of the aqueous solution and 5.0 g of IDA were mixed in a beaker and then stirred (150 rpm) at room temperature. A small amount of diluted aqueous NaOH solution was occasionally added into the mixture to keep the pH around 4. After 20 h, the adsorption reached equilibrium and the mixture was filtrated to collect the resin. The concentrations of Cu(II) in the remaining filtrate were analyzed using an atomic absorption spectrophotometer (PerkinElmer AAnalyst 100) to determine the amount of metal adsorbed. Cu(II) immobilized SK (abbreviated as Cu-SK) and Cu(II) immobilized WK (abbreviated as Cu-WK) were prepared in a similar manner, using SK and WK resins at pH 4.3 for SK or pH 6.0 for WK.

2.3. Adsorption test

Adsorption tests of Car using various resins were examined in a batchwise method as follows: An aqueous solution containing 0.25 mM of Car was prepared and the pH adjusted using 10.0 mM of HEPES buffer, diluted hydrochloric acid, and diluted sodium hydroxide. 15 mL of the aqueous solution and 30 mg of a resin were mixed in a stoppered glass sample tube and shaken in a thermostated shaker (120 rpm) at 30 °C. After 20 h at which time the adsorption had reached equilibrium, the mixture was filtrated to obtain the filtrate. To determine the percent adsorption of Car, the concentrations of Car in the aqueous solutions were analyzed using a Shimadzu Prominence HPLC Amino Acid Analysis System. The analysis system is based on a gradient reverse-phase HPLC using Shimadzu LC-20AB as a pump (flow rate: 0.6 mL min^{−1}) equipped with Shimadzu Shim-pack Amino Li as a column, followed by post-column fluorescence detection of OPA amino acid derivative with Shimadzu RF-10A_{XL} fluorescence detector (Ex: 350 nm and Em: 450 nm). The concentrations of Cu(II) ions eluted from the Cu-IDA resin were also analyzed using an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 100). Adsorption tests of different amino acids were also examined in a similar manner.

2.4. Elution test

Elution tests of Car from Cu-IDA using chelating reagents as eluents (acetic acid, iminodiacetic acid, citric acid, and imidazole) were examined in a batchwise method as follows: Adsorption of Car on 30 mg of Cu-IDA was examined in a similar manner to that described in the above section. After shaking at 120 °C for 20 h, the mixture was filtered and the residue containing Car-adsorbed

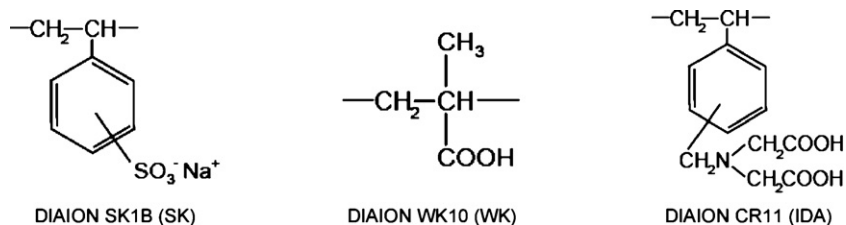


Fig. 2. Molecular structures of cation exchange resins.

Cu-IDA was collected. The recovered resin was mixed with 15 mL of aqueous solution containing the eluent (0–100 mM) and shaken at 30 °C for 20 h. After filtration of the mixture, the concentration of Car in the eluate as well as the concentrations of Cu(II) ions eluted from Cu-IDA was analyzed. On the basis of the mass-action law, the elution percentage of Car eluted from the adsorbent was calculated.

3. Results and discussion

3.1. Adsorption behavior of carnosine on copper(II) immobilized resins

In general, cation exchange resins adsorb amino acids based on electrostatic interactions. Fig. 3(a) shows the adsorption profiles of Car on the resins as a function of pH in the absence of NaCl. The strongly acidic cation exchange resin SK quantitatively adsorbs Car at pHs less than 6 under which Car exists as cationic species. As the functional groups of WK and IDA dissociate under weakly acidic conditions, WK and IDA adsorb Car only under a pH of around 6. The adsorption profiles of Car on the resins in the presence of NaCl (100 mM) are shown in Fig. 3(b). As the ion exchange reaction between cationic amino acid and the anionic functional group in the resins is inhibited by excess sodium ion, the adsorption of Car

significantly decreases. Car is not adsorbed on the cation exchange resins in the presence of NaCl under neutral conditions. Namely, it is difficult to directly recover Car using a cation exchanger from saline water.

Immobilized metal affinity adsorption was expected for the recovery of HCDPs from the saline water. The imidazole group of Car should strongly interact with the transition metal ion immobilized on the resin by coordination bonding, which is less affected by coexisting salts compared with electrostatic interactions. For metal affinity adsorption, Cu(II) was immobilized on each cation exchange resin. The amounts of Cu(II) immobilized on the resins by the adsorption procedure were 1.52 mmol g⁻¹ for Cu-SK, 1.43 mmol g⁻¹ for Cu-WK, and 1.36 mmol g⁻¹ for Cu-IDA. The amounts of adsorption are slightly smaller than those of the maximum adsorption capacities of the resins.

Fig. 4(a) displays the adsorption profiles of Car on Cu(II) immobilized resins as a function of pH in the absence of NaCl. As shown in Fig. 4(a), all of Cu(II) immobilized resins enhance the adsorption of Car compared with the original resins (see Fig. 3(a)). The strong acid ion exchange resin SK quantitatively adsorbs Car under conditions of pH 2–5, while the adsorption decreases under higher pHs. Cu-SK shows slightly higher adsorption than SK at neutral pH. Most Car molecules seem to be adsorbed on Cu-SK via electrostatic interactions, while a small amount of Car appears to be adsorbed via metal–ligand interactions. On the other hand, Cu-WK and Cu-IDA

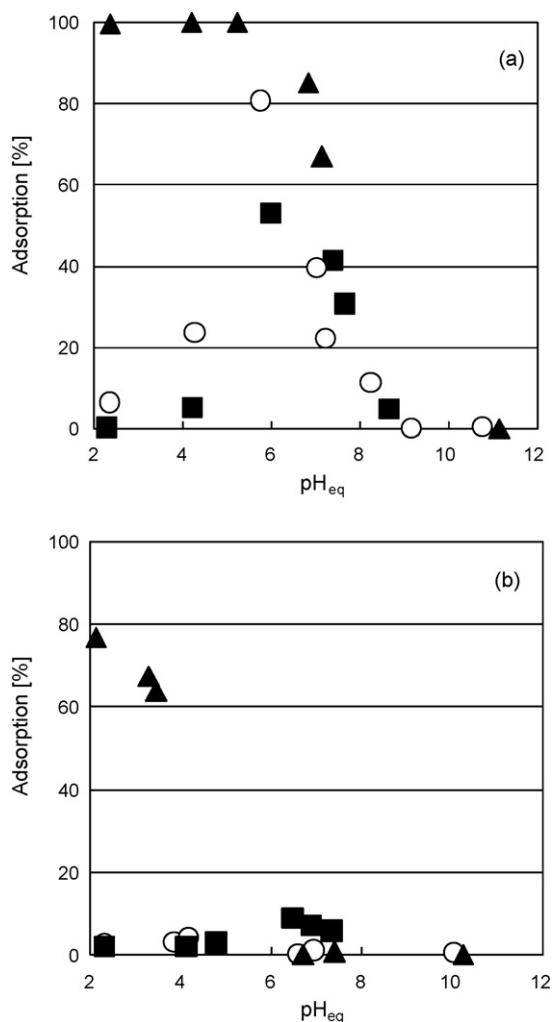


Fig. 3. Adsorption profiles of Car on cation exchange resins. [NaCl] = 0 (a) or 100 mM (b). Adsorbent, 30 mg; volume = 15 mL; [Car]_{ini} = 0.25 mM (circle: IDA; triangle: SK; square: WK).

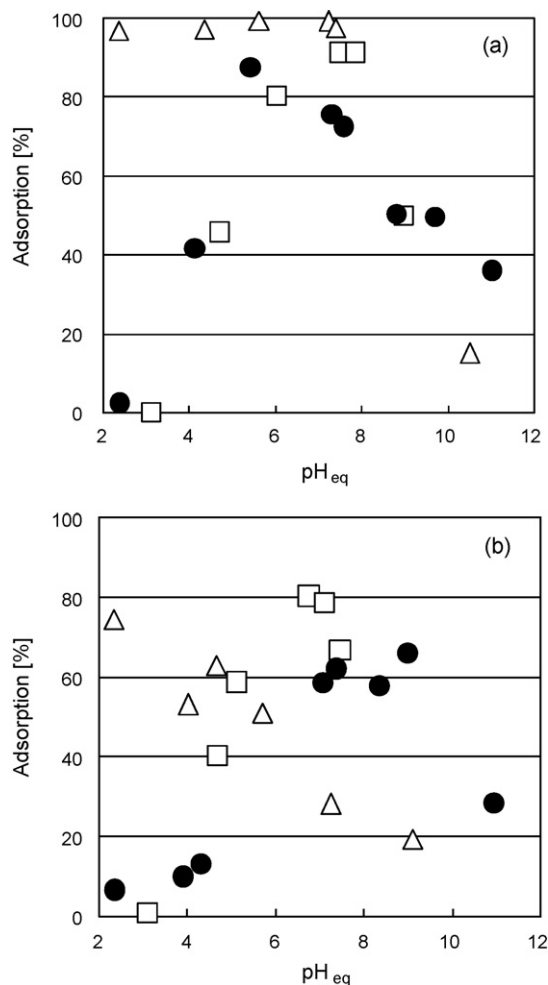


Fig. 4. Adsorption profiles of Car on Cu(II) immobilized cation exchange resins. [NaCl] = 0 (a) or 100 mM (b). Adsorbent, 30 mg; volume = 15 mL; [Car]_{ini} = 0.25 mM (circle: Cu-IDA; triangle: Cu-SK; square: Cu-WK).

show higher adsorption of Car than WK and IDA, respectively. Adsorptions of Car on Cu-WK and Cu-IDA under neutral pH are double compared with those on WK and IDA, respectively. Additionally, Cu-IDA adsorbs Car under basic conditions where IDA does not adsorb at all. Under such basic conditions, Car exists as an anionic species and is adsorbed on the Cu-IDA only through a coordination bond between Cu(II) ion and Car.

Fig. 4(b) shows the adsorption of Car on the Cu(II) immobilized resins in the presence of NaCl (100 mM). Adsorption of Car on Cu-SK in the presence of NaCl decreased compared with that on Cu-SK in the absence of NaCl. As the adsorption of Car on Cu-SK proceeds almost exclusively via electrostatic interactions, the adsorption is inhibited by the coexisting sodium ion. On the other hand, Car is relatively well retained on Cu-WK and Cu-IDA even in the presence of NaCl. The adsorptions on Cu-WK and Cu-IDA are maximized under neutral or weakly basic conditions. It should be noted that the adsorption of Car on the Cu(II) immobilized resins in the presence of NaCl is much higher than those on the resins without Cu(II) immobilization. Therefore it is suggested that the adsorption of Car on Cu(II) immobilized resins proceeds via metal affinity interactions. Car and Ans are found to be efficient copper-chelating reagents and it has been suggested that they may play a role in copper metabolism *in vivo* [29]. Car is adsorbed on the Cu(II) immobilized resins based on the chelating effect.

Elution of Cu(II) from the immobilized resin during the adsorption of HCDPs should be minimized with a viewpoint to the continuous use of the adsorbent. Fig. 5 exhibits the elution of Cu(II) from the immobilized resins as a function of pH. As shown in Fig. 5, Cu(II) ion immobilized on Cu-IDA is not eluted at all at neutral pH. Moreover, the amount of elution of Cu(II) ion from Cu-IDA under acidic conditions is smaller than from Cu-SK and Cu-WK. Namely, the chelating resin IDA is the most preferable for holding Cu(II) ion. The iminodiacetic acid group is one of the most commonly used chelating groups for IMAC. On the basis of these results, Cu-IDA is a desirable adsorbent for histidine-containing dipeptides from saline water using immobilized metal affinity adsorption.

Fig. 6 exhibits the effect of NaCl concentration on the adsorption of Car on IDA and Cu-IDA. Adsorption of Car on the chelating resin IDA significantly decreased by adding NaCl. The adsorption percentage of Car in the presence of 50 mM of NaCl was only 1.3%. Thus the chelating resin was not efficient for the adsorption of the dipeptide from saline solution. In contrast, adsorption of Car on Cu-IDA slightly decreased after adding NaCl and was retained even

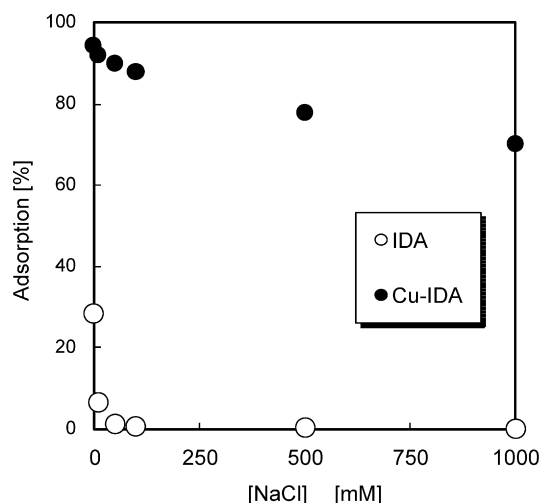


Fig. 6. Effect of salt concentration for the adsorption of Car on IDA and Cu-IDA. pH_{ini} 8.0; adsorbent, 30 mg; volume = 15 mL; $[\text{Car}]_{\text{ini}} = 0.25 \text{ mM}$; $[\text{NaCl}] = 0\text{--}1000 \text{ mM}$.

in the presence of 1000 mM of NaCl. The metal affinity adsorption is based on the coordination of ligand against the immobilized metal ion. As the metal affinity interactions are insensitive to elevated salt concentrations, the inhibition of Car adsorption by NaCl is quite small [30,31]. Namely, the immobilized metal affinity adsorption is effective for the recovery of HCDPs especially from saline solution.

Fig. 7 shows the adsorption isotherm of Car on Cu-IDA at varying equilibrium concentrations at pH 6.0. As shown in the figure, the amount of Car adsorbed on Cu-IDA increased with an increase in the equilibrium concentration of Car. The equilibrium experimental data were correlated both with the Langmuir isotherm model and the Freundlich isotherm model, in order to understand the adsorption manner and to determine the maximum adsorption capacity of Car on Cu-IDA (q_{max} [mmol g^{-1}]). The Langmuir model assumes a monolayer adsorption as shown in the following mathematical expression:

$$\frac{C_e}{q} = \frac{C_e}{q_{\text{max}}} + \frac{1}{q_{\text{max}}K_L}$$

where q denotes the amount of Car adsorbed [mmol g^{-1}], C_e denotes the equilibrium concentration of Car in the aqueous solution, and K_L denotes adsorption equilibrium constant [L mmol^{-1}],

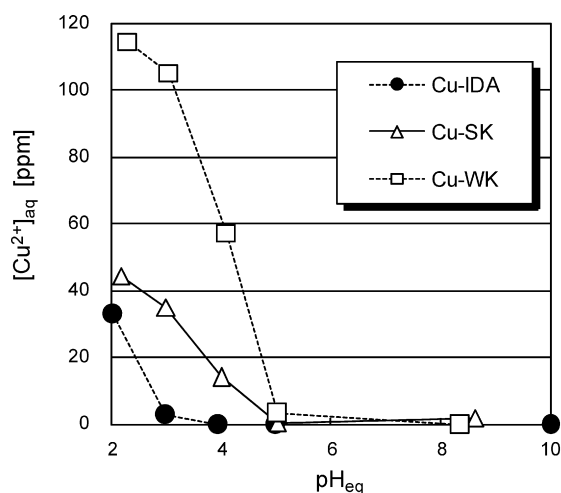


Fig. 5. Elution of Cu(II) from the immobilized cation exchange resins as a function of pH. Adsorbent, 20 mg; volume = 15 mL (circle: Cu-IDA ($q = 1.36 \text{ mmol g}^{-1}$); triangle: Cu-SK ($q = 1.52 \text{ mmol g}^{-1}$); and square: Cu-WK ($q = 1.43 \text{ mmol g}^{-1}$)).

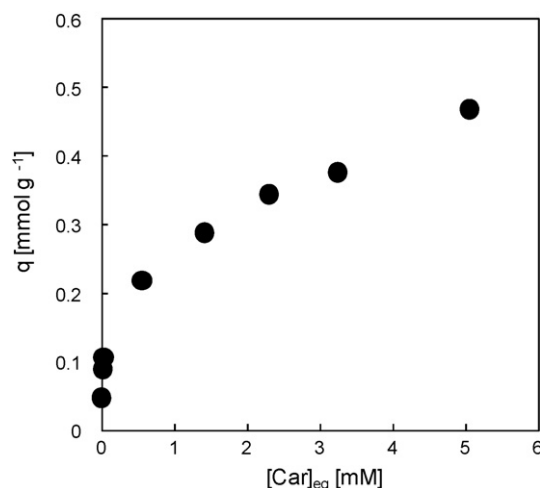


Fig. 7. Adsorption isotherm of Car on Cu-IDA at 30 °C. pH_{ini} 6.0; adsorbent, 30 mg; volume = 15 mL; $[\text{Car}]_{\text{ini}} = 0.1\text{--}6.0 \text{ mM}$.

Table 1

The Langmuir and Freundlich constants for adsorption of Car on Cu-IDA calculated from the results in Fig. 7.

Langmuir constants			Freundlich constants		
q_{\max} (mmol g ⁻¹)	K_L (L mmol ⁻¹)	R^2	n	K_F	R^2
0.498	2.67	0.959	3.14	0.293	0.987

respectively. On the other hand, the Freundlich model is the empirical relationship for heterogeneous surface energy system [32], shown in the following mathematical expression:

$$q = K_F(C_e)^{1/n}$$

where K_F and n are the Freundlich equilibrium constants.

The Langmuir and the Freundlich constants for Car adsorption on Cu-IDA as well as the correlation coefficients (R^2) are summarized in Table 1. The q_{\max} value based on the Langmuir equation was calculated as 0.498 [mmol g⁻¹]. However, the correlation coefficient for the Freundlich model is higher than that of the Langmuir model. It is likely that HCDPs are adsorbed on Cu(II) immobilized resin mainly via coordination bonding between imidazole group of HCDPs and Cu(II), however, the higher correlation coefficient for the Freundlich model suggests that the adsorption of Car on Cu-IDA is partly heterogeneous. Further investigation is required, in order to clarify the detailed adsorption manner completely.

3.2. Adsorption selectivity of amino acids on copper(II) immobilized iminodiacetic acid resin

The adsorption selectivity of HCDPs and amino acids using Cu-IDA was studied for the selective recovery of HCDPs from a mixed solution such as food extracts. Adsorptions of various amino acids on Cu-IDA were individually examined under various pHs both in the absence (Fig. 8(a)) and presence (Fig. 8(b)) of NaCl. As shown in Fig. 8(a), His and the histidine-containing dipeptides Car and Ans, are selectively adsorbed on Cu-IDA over other amino acids at wide pH ranges. As the adsorption is mainly based on a coordination bond between the metal ions and the amino acid, His, which forms the strongest complex with Cu(II) ions, showed the highest adsorption [33,34]. The extent of the adsorption of the basic amino acid Arg is next after those of His and HCDPs. The adsorptions of other amino acids are much smaller compared to those of His and HCDPs because the metal affinity of Cu(II) for neutral and acidic amino acids is much smaller compared to those for His and HCDPs.

It should be noted that His, Car, and Ans are selectively adsorbed on Cu-IDA even in the presence of 100 mM of NaCl as shown in Fig. 8(b). On the other hand, the adsorption of Arg on Cu-IDA was decreased in the presence of 100 mM NaCl, probably because the basic amino acid Arg is adsorbed mainly via electrostatic interactions. In contrast, adsorptions of Cys, Met and Phe were increased in the presence of NaCl [35]. The reason is not clear, however, the metal affinity interactions and hydrophobic interactions between these amino acids and Cu-IDA would be stronger under higher salt concentrations. Table 2 summarizes adsorption of amino acids and

Table 2

Adsorption of amino acids and HCDPs on Cu-IDA under neutral pH condition (single component). pH 7.29–7.40; [NaCl] = 0 or 100 mM; adsorbent, 30 mg; volume = 15 mL; [amino acid]_{ini} = 0.25 mM.

[NaCl] (mM)	Adsorption of amino acids and HCDPs (%)									
	Glu	Gly	Cys	Met	Trp	Phe	His	Ans	Car	Arg
0	0	4.0	17.2	5.5	29.7	5.8	82.9	74.1	75.6	37.3
100	0	3.2	40.0	20.9	0	34.9	90.1	50.2	58.5	3.1

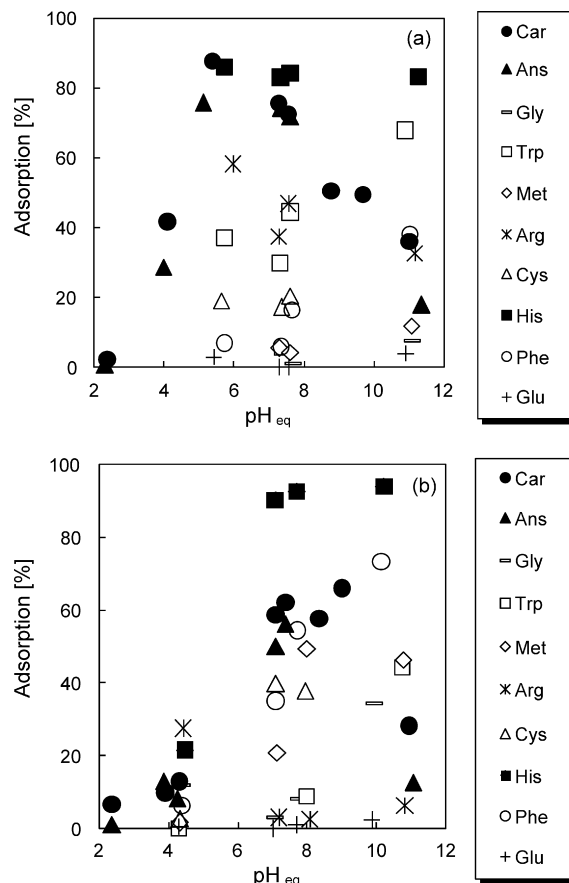


Fig. 8. Adsorption profiles of amino acids and HCDPs on Cu-IDA as a function of pH (single component). [NaCl] = 0 (a) or 100 mM (b). Adsorbent, 30 mg; volume = 15 mL; [amino acid]_{ini} = 0.25 mM.

HCDPs on Cu-IDA under neutral pH condition. His and HCDPs are selectively adsorbed on Cu-IDA over other amino acids under the commonly used neutral pH condition, both in the absence and the presence of salts.

Fig. 9 shows the adsorptions of amino acids and HCDPs on Cu-IDA from a mixed solution containing Car, Ans, and 17 amino acids at around pH 7. His, Car, and Ans are selectively adsorbed on Cu-IDA both in the absence and the presence of NaCl. The adsorption percents of most of acidic and neutral amino acids on Cu-IDA are less than 10%. Basic amino acids Lys and Arg are also adsorbed on Cu-IDA, however, the adsorption of these basic amino acids are decreased in the presence of NaCl. The adsorption percents of Car and Ans in the presence of NaCl are also decreased but appreciably retained. Similarly to the results in Fig. 8(b), the adsorption of Cys

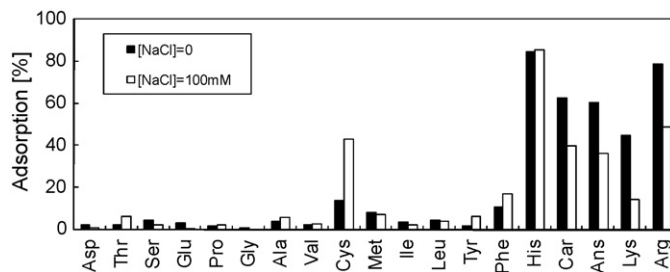


Fig. 9. Adsorptions of amino acids and HCDPs on Cu-IDA (multi component). pH_{eq} 6.8–7.0; adsorbent, 30 mg; volume = 15 mL; [amino acid or HCDP]_{ini} = 0.1 mM; [NaCl] = 0 or 100 mM.

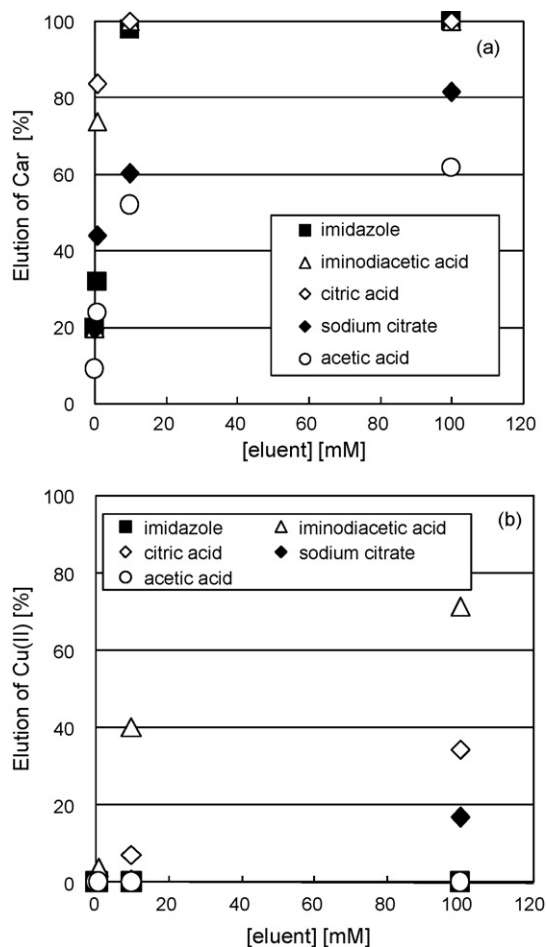


Fig. 10. Elution of Car from Cu-IDA as a function of eluent concentration (a) as well as elution of Cu(II) during elution procedure (b). Adsorbent, 30 mg; volume = 15 mL.

and Phe are increased in the presence of NaCl. On the basis of these results, it was confirmed that Cu-IDA is effective for the selective recovery of HCDPs from a mixed solution containing various amino acids and salts.

3.3. Elution of carnosine from copper(II) immobilized iminodiacetic acid resin

The HCDPs adsorbed on Cu-IDA should be eluted using eluents which can act as competitors for binding to Cu(II). In the present study, the elution of Car from Cu-IDA was examined using acetic acid, iminodiacetic acid, citric acid, sodium citrate, and imidazole. Fig. 10(a) exhibits the effect of the concentration of eluents on the elution of Car from Cu-IDA. The elution of Car increases with increasing concentration of the eluents. Imidazole is the most effective for the elution of Car from the Cu(II)-immobilized resin in the eluents examined and Car is quantitatively recovered in the presence of 10 mM of imidazole. Iminodiacetic acid, citric acid and sodium citrate also elute Car effectively. Acetic acid also acts as an eluent for Car, however, the elution percentage is relatively small compared with the other eluents. Fig. 10(b) shows the elution of Cu(II) from Cu-IDA accompanying the elution of Car. Cu(II) is eluted from the resin using the chelating reagents iminodiacetic acid and citric acid with the elution of Car. In contrast, Cu(II) is not eluted from the resin in the presence of 100 mM imidazole and acetic acid. Based on the results, imidazole and acetic acid are more favorable as an eluent for HCDPs.

Generally, imidazole is used as an eluent for His-tagged recombinant protein in IMAC. Imidazole is readily available in the biotechnology field, however, acetic acid may be more favorable for the elution of HCDPs from metal immobilized chelating resin in view of physiological safety issues as well as cost performance.

4. Conclusions

Immobilized metal affinity adsorption was found to be effective for the recovery of histidine-containing dipeptides from saline water. HCDPs are adsorbed on Cu(II) immobilized resin in the presence of high concentrations of NaCl while they are not absorbed on cation exchange resins under similar conditions. Iminodiacetic acid chelating resin is more favorable for the metal affinity adsorption of HCDPs compared with commercially available cation exchange resins from the viewpoint of a wider available pH range, less interference by salt, and less elution of the immobilized Cu(II). In addition, histidine and histidine-containing dipeptides were selectively adsorbed on the resin from a mix of amino acids. The HCDP adsorbed on the Cu(II) immobilized resin was quantitatively recovered using chelating reagents as eluents. In summary, immobilized metal affinity adsorption is available for constructing a simple purification process for HCDPs by elimination of an intermediate desalting process. HCDPs can be recovered selectively from food extracts containing various amino acids and salts.

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