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Calcined layered double hydroxides as a "biomolecular vessel" for bromelain: Immobilization, storage and release

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Abstract

Calcined layered double hydroxide (LDO) has been used as the novel biomolecular vessel for the immobilization, storage as well as release of bromelain. The immobilization is attributed to physical adsorption without affecting the structure of LDO, and the optimum initial amount of bromelain was 20 ml of 4 mg/ml for 1 g LDO and the immobilized enzyme activity express was 33.4% at pH 6.5. The adsorption isotherm of bromelain/LDO can be well described with the Langmuir model with a R^2 of 0.9946, a maximum adsorption amount of 157 mg/g and the Langmuir adsorption equilibrium constant of 0.07 ml/mg. Significant improvements have been found in the stability of immobilized bromelain upon heat treatment (increase in residual activity and denaturation temperature) as well as storage time compared with those of free bromelain. In addition, the release maximum of bromelain reached to 70% and the residual activity maintained 81% in water solution at pH 7.

Keywords: Biomolecular vessel; Bromelain; Layered double oxides; Layered double hydroxide; Calcination

1. Introduction

Enzymes are excellent catalysts with superb chemo-, regio-, stereo-, and chiral selectivities, but their use in chemical or industrial applications is severely limited for their insufficient availability, high price, sensitivity to pH/temperature, and their instability in organic solvents. Proteins/enzymes bound to solids, however, can overcome some of these problems. Among various solids used for enzyme binding, inorganic materials are extraordinarily interesting due to their hydrophilicity, thermal stability, chemical inertness, and their use as industrial catalysts or as catalyst support [1,2].

Layered inorganic solids such as LDHs (layered double hydroxides, which have the general formula $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}(X^{n-})_{x/n}\cdot mH_2O$, and consist of positively charged hydroxide sheets and interlayer guest anions and water molecules) [3], have attracted much attention as the host for biomolecules binding among the inorganic materials, due to the reason that the layers of these solids can be expanded to accommodate biomolecules of different sizes. LDHs are chemically inert, thermally stable, and they can be prepared in a crystalline form in large quantities. A number of biomolecular have been intercalated into LDHs, such as L-phenylalanine [4], deoxycholic acid [5], pectin, xanthan gum, κ-carrageenan, ι-carrageean [6] and alginate [7], DNA, mononucleotides, ATP, etc. [8], to study the physi-chemical properties of the hybrid composites. However, it is generally difficult for the intercalation of larger enzymes/proteins within the sheets of LDHs, since this process will be kinetically limited by the slow diffusion of the biomolecular guests. Therefore, calcination products of LDHs, known as layered double oxides (LDO), have been paid more attention owing to their larger surface area and less diffusion resistance than those of LDHs. Moreover, LDO have the advantage of porous structure and both of abundant acid and basic sites [9,10] to bind with an enzyme. Ren et al. have investigated LDO as potential support for Penicillin G acylase (PGA), and the results showed that immobilization of the enzyme lead to the increase in its acid resistance [11]. Such improvements in the physical, chemical, and catalytic properties of bound enzymes are welcome changes for biocatalytic applications.

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Enzymes bound to solid surfaces are often less active than the corresponding enzymes in aqueous solutions, but improved stability of the bound enzyme can more than compensate for this loss [12]. The decrease in the activity of the bound enzymes is often attributed to the loss of enzyme structure, resulting from the unfavorable interactions between the enzyme and the surface functions of the solid matrix. Such enzyme-solid interactions also play a major role in the denaturation, stability, refolding, and degradation of the bound enzyme.

Bromelain is a mixture of proteolytic enzymes that is derived from the stem of the pineapple plant, *Ananas comosus*. Others have previously shown that bromelain proteolytically removes certain cell surface molecules that affect lymphocyte migration and activation. In addition, bromelain treatment markedly affects the production of cytokines and inflammatory mediators by isolated leukocytes or colon epithelial cells in vitro [13]. These effects require that the bromelain be proteolytically active, therefore it is chosen as a representative example for current study. In this work, properties of bromelain upon immobilization, storage as well as release from LDO have been investigated to testify the possibility of LDO as a novel biomolecular vessel. Significant improvements have been found in the stability of immobilized bromelain upon heat treatment (increase in residual activity and denaturation temperatures) as well as storage time compared with those of free bromelain. In addition, the release behavior of the immobilized bromelain was studied in water solution, and the maximum release amount reached to about 70% and residual activity retained 81%. Therefore, LDO may have potential applications as the basis of a novel storage or delivery system for biomolecules or enzymes.

2. Experimental

2.1. Materials

Bromelain (EC 3.4.22.32, formerly EC 3.4.22.4; from pineapple stem; 800 units per mg protein) was purchased from Sigma, and chemicals including $Mg(NO_3)_2{\cdot}6H_2O$, $Al(NO_3)_3{\cdot}9H_2O$, NaOH, $NaNO_3$, CH_3COOH, Casein, etc., were of analytical grade and purchased from the Beijing Chemical Plant Limited.

2.2. Preparation of LDO

The precursor MgAl-NO_3-LDH was synthesized by the hydrothermal method reported previously [14]. The pH value of solution (200 ml) containing 0.08 mol $Mg(NO_3)_2{\cdot}6H_2O$ and 0.04 mol $Al(NO_3)_2{\cdot}9H_2O$ was adjusted to 10.0 with $NH_3{\cdot}H_2O$, and it was aged in an autoclave at 140 °C for 10 h. The precipitates were centrifuged, washed thoroughly with deionized and decarbon-dioxide water, and then dried at 60 °C for 24 h. Subsequently, LDO was obtained by calcination of MgAl-NO_3-LDH at 500 °C for 5 h.

2.3. Immobilization of bromelain onto LDO

The 0.1 M phosphate buffer solution (PBS) with pH 5.8 was added to the suspension including 0.5 g LDO under N_2 atmosphere until the pH value reached to 6.5. Ten milliliters of 4 mg/ml bromelain solution was then added to the stirred suspension and maintained at room temperature for 24 h. The immobilized bromelain was obtained by centrifugation, washed and dried in vacuum at ambient temperature for 10 h.

2.4. Determination of bromelain content

The amount of immobilized bromelain was determined from the concentration change of bromelain in solution before and after immobilization by measuring the absorbance photometrically at 275 nm. The immobilized bromelain was calculated by interpolation from a standard curve.

2.5. Measurement for the enzymatic activity

The caseinolytic determinations were carried out essentially according to the method reported previously [15], with minor modifications to solve the problem of the insolubility of LDO. The activities of free and immobilized bromelain were determined by the following way: 5 ml of enzyme solution containing 0.005 g of free bromelain or the immobilized bromelain was grinded for 10 min, and then it was added into a 5 ml of dilute solution of the enzyme which contained 2 mmol EDTA and 5 mm cysteine. After 10 min, 1 ml of above mixtures was hydrolyzed with 5.0 ml of 2.0 wt.% casein solution. The reaction mixture was maintained at 37 °C for 10 min, followed by termination with 5 ml of trichloroacetic acid solution containing 0.1 M trichloroacetic acid, 0.36 M sodium acetate and 0.32 M glacial acetic acid.

The absorbance of the solution or the supernatant at 275 nm was plotted versus the enzyme weight to determine the enzymatic activity. The relative activity (RA) was defined as the ratio of the hydrolytic activity of the immobilized enzyme to that of the free enzyme, which was used to evaluate the activity of the immobilized bromelain.

2.6. Stability measurements for the immobilized and free bromelain

The thermal stability of the immobilized bromelain was evaluated by measuring the residual activity (ZA) and denaturation of the enzyme. The solid enzymes exposed to various temperatures for 10 d were quickly cooled and assayed for their enzymatic activity at 37 °C immediately, and the denaturation exposed for 4 h was studied through monitoring the fluorescence spectrophotometer. The fluorescence spectra of bromelain (0.01 g) and bromelain/LDO (0.125 g) suspension in water were recorded at room temperature with 280 nm excitation. 338 nm emission is mostly due to the tryptophan residues present in the enzyme. The FT-IR absorbance at the amide I and II peak position of the enzyme samples was plotted as a function of temperature.

The storage stability of the free and immobilized bromelain was evaluated by placing bromelain at 25 °C for various periods of time and by assaying for the activity.

2.7. Release of bromelain

The release of bromelain was studied by putting the bromelain/LDO into the stirred pure water at pH 7.0, and the amount of bromelain released in solution at different time was determined with the method described in Section 2.4.

2.8. Techniques of characterization

Powder X-ray diffraction (XRD) measurements were performed on a Rigaku XRD-6000 diffractometer, using Cu Kα radiation (λ = 0.154 nm) at 40 kV, 30 mA, a scanning rate of 5° min^{-1}, a step size of 0.02° s^{-1}, and a 2θ angle ranging from 3° to 70°. The in situ Fourier transform infrared (FT-IR) spectra were recorded using a PERK$_1$N ELMER spectrometer configured with a CKW-2 accessory and an automated temperature controller in the range of 4000–400 cm^{-1} with 4 cm^{-1} resolution and heating rate of 5 °C min^{-1}. The standard KBr disk method (1 mg of sample in 100 mg of KBr) was used. The UV–vis spectra were collected at 275 nm in a Shimadzu U-3000 spectrophotometer. Fluorescence emission spectra were recorded on a RF-5301PC fluorophotometer in the range 300–420 nm with the excitation wavelength of 280 nm and slit widths of 5 nm. The low-temperature N_2 adsorption experiments were carried out using a Quantachrome Autosorb-1 system. The samples were outgassed at 200 °C for 4 h. The specific surface area of the sample was calculated using the BET method based on the N_2 adsorption isotherm.

3. Results and discussion

3.1. The immobilization of bromelain onto LDO

FT-IR spectra, in the 4000–400 cm^{-1} wavenumber range at room temperature, of LDO, free bromelain and bromelain/LDO are shown in Fig. 1. The spectrum of LDO (Fig. 1a) displays absorption bands at 3579 and 1645 cm^{-1} owing to the O–H stretching and flexural oscillations of OH^- group, respectively. The 1382 cm^{-1} band is assigned to absorption of NO_3^- group. Although heat treatment of LDH at 500 °C leads to its transformation to LDO, it still remains the basic layered structure. Based on the crystal structure of LDHs, each OH-group in the LDH layer is bonded to three metal cations [16]. Therefore, the superposition of bands belonging to $3Mg^{2+}$–OH, $2Mg^{2+}Al^{3+}$–OH, $Mg^{2+}2Al^{3+}$–OH and $3Al^{3+}$–OH resulted in the broad band at 3000–3750 cm^{-1} (Fig. 1a) [17]. For the free bromelain (Fig. 1b), the band around 3400 cm^{-1} is attributed to the N–H stretching mode of the amidic binding of the enzyme, while the bands at 1650 and 1535 cm^{-1} arise from the C=O stretching (amides I) and N–H bending (amides II) vibrations, respectively. The band around 2934 cm^{-1} corresponds to the C–H stretching of the CH_2 groups, and bands at 500–700 cm^{-1} are assigned to absorption of deformation vibration belonging to amide in the area of crystallization. Compared with LDO, spectrum of bromelain/LDO (Fig. 1c) displays several new absorption peaks, such as the band around 2954 cm^{-1} due to the C–H stretching of the CH_2 groups, 1533 cm^{-1} belonging to N–H bending (amides II) vibrations, and 500–700 cm^{-1} owing to absorption of deformation vibration of amide in the area of crystallization. Furthermore, there was a strong band at 1651 cm^{-1} resulting from the superposition of the absorption of LDO at 1645 cm^{-1} and of bromelain at 1650 cm^{-1}. The results above indicate the occurrence of the immobilization of bromelain onto LDO. No obvious shift in the bands corresponding to the C=O stretching and N–H bending vibrations of bromelain after immobilization can be observed (1650–1651 and 1535–1533 cm^{-1}), implying a weak interaction between the bromelain and LDO. Moreover, compared with LDO, the spectrum of bromelain/LDO (Fig. 1c) showed a relatively narrow absorption peak at 3000–3700 cm^{-1}, and the O–H stretching absorption of hydroxyl group shifted to low frequency from 3579 cm^{-1} of LDO (Fig. 1a) to 3438 cm^{-1} of bromelain/LDO (Fig. 1c). The immobilization of bromelain occurs through the formation of hydrogen bonding between –OH groups on the surface of LDO and $–NH_2$ of bromelain.

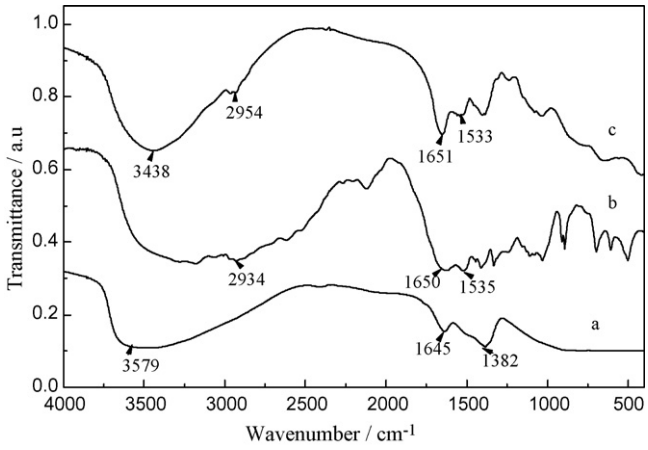

Fig. 1. FT-IR spectra (4000–400 cm^{-1}region) of (a) LDO, (b) free bromelain and (c) bromelain/LDO at room temperature.

Fig. 2 shows the XRD patterns of the precursor MgAl-NO_3-LDH, LDO, bromelain/LDO, and LDO-PBS, respectively. As shown in Fig. 2a, for the case of Mg/Al = 2, the LDH precursor displays the characteristic X-ray powder diffraction pattern of a layered hydrotalcite-like material. After calcinations at 500 °C, the layered structure disappeared and the XRD pattern (Fig. 2b) resembles that of poorly crystalline magnesium oxide as has been reported elsewhere [18]. When the LDO was dispersed in an aqueous solution containing PBS and bromelain under N_2 atmosphere for 24 h, the XRD pattern of bromelain/LDO (Fig. 2c) is rather similar to that of the pristine LDO except that the intensity of reflections both at 43.22° and 62.58° 2θ decreased and their half peak breadth widened attributed to the decrease in the crystallinity after the immobilization of bromelain onto the surface of LDO. In order to investigate the influence of PBS during the immobilization of bromelain, a comparison study was carried out by dispersing LDO in a PBS solution without bromelain under N_2 atmosphere. From

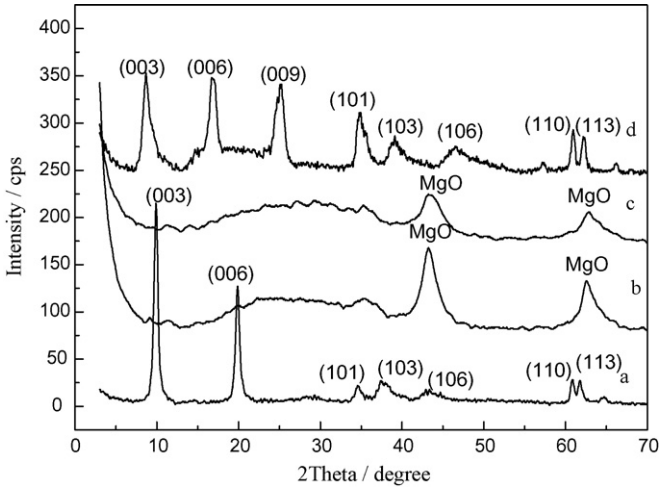

Fig. 2. Powder X-ray diffraction patterns for (a) pristine MgAl-NO_3-LDH, (b) LDO, (c) bromelain/LDO and (d) LDO-PBS.

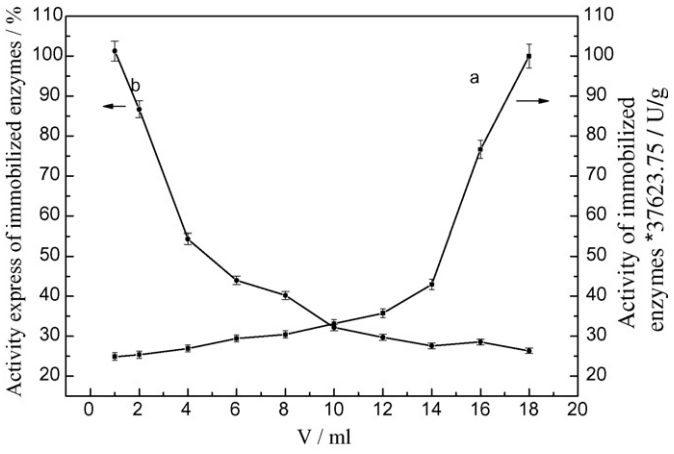

Fig. 4. The relationship between the activity as well as the activity express of the immobilized enzyme and the initial amount of bromelain: (a) activity, (b) activity express of the immobilized bromelain. Indicated values are means of three experiments, and error bars represent the standard errors of means.

Fig. 2d, the characteristic pattern of MgAl-PO_4-LDH [19] was observed. Therefore, the comparison between the XRD pattern of bromelain/LDO and that of the LDO-PBS indicates that the immobilization of bromelain on the surface of LDO prevents the regeneration of layered PO_4-LDH structure under the experimental conditions.

In order to find the optimal immobilization condition of bromelain on the surface of LDO, effects of both pH and initial amounts of bromelain on the activity of immobilized bromelain were examined.

The effect of pH on the activity of immobilized bromelain was studied by adjusting the value of solution pH in the range 5.0–7.5 with PBS, taking into account the optimum pH range for free bromelain itself with maximum activity express in the reaction with casein substrates (6.0–7.0). As shown in Fig. 3, the activity of immobilized bromelain has the highest value at pH 6.5, which accords well with the optimum pH of free bromelain. Thus, in all the following experiments, the pH value of reaction system was maintained at 6.5, unless otherwise mentioned.

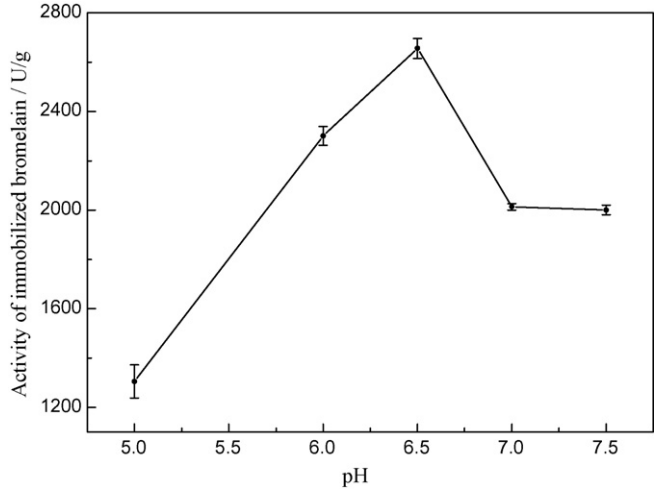

Fig. 3. Effect of pH on the activity of immobilized bromelain. Indicated values are means of three experiments, and error bars represent the standard errors of means.

The relationship between the activity as well as the activity express of the immobilized enzyme and the initial amount of bromelain are shown in Fig. 4. There was a tendency of the activity of immobilized bromelain coupling to increase with the initial amount of bromelain, however, the activity express of immobilized bromelain followed decrease due to possible effects of steric hindrance at higher immobilized densities. Similar conclusion has been reported by other researchers in the study of immobilization of β-fructofuranosidases on methacrylamide-based polymeric beads [20]. Based on the results above, the optimum amount of the initial enzyme was chosen as 10 ml of 4 mg/ml bromelain solution for 0.5 g dry LDO.

In order to get further study of the immobilization of bromelain onto LDO, adsorption isotherm was conducted by filling with 4 mg/ml aqueous solution of bromelain ranging in volume from 0.0 to 20 ml into 0.5 g LDO solid in the system of immobilization of bromelain. The concentration of bromelain in the solutions, before and after adsorption experiments respectively, was determined by UV–vis spectra at the wavelength of 275 nm. The amount of bromelain adsorbed by LDO was obtained from the difference between the initial (C_i) and equilibrium concentrations (C_e), per gram of LDO adsorbent: $Q = (C_i - C_e) \times V/m$. The adsorption isotherm was obtained by plotting the amount of bromelain adsorbed (Q) versus the adsorbate concentration (C_e) in the equilibrium solution (as shown in Fig. 5). Three models, i.e., the Langmuir, the Freundilich, and the linear model were used to fit the experimental data, respectively. The experimental conditions, the estimated model parameters and regress coefficients (R^2) are reported in Table 1 for all adsorption experimental runs. As can be seen from Table 1, the regress coefficients (R^2) for the Langmuir model (0.9946) are larger than that of Freundilich and the linear model, and the experimental q_e values agree well with the calculated ones obtained from the Langmuir model. This indicates that the Langmuir model can be used to describe the adsorption of bromelain by LDO satisfactorily. The

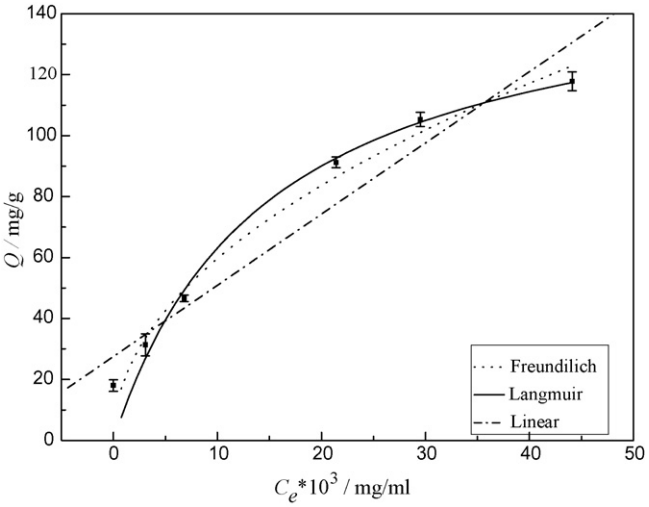

Fig. 5. The adsorption isotherms of bromelain on LDO fitted by the Langmuir, the Freundilich, and the linear model, respectively. Indicated values are means of three experiments, and error bars represent the standard errors of means.

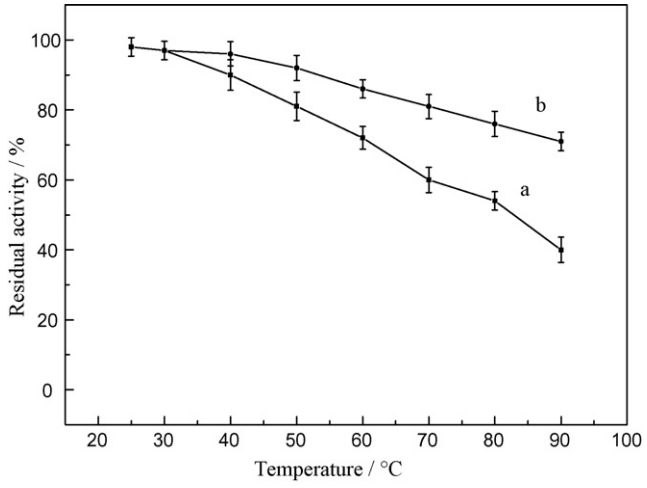

Fig. 6. Effect of the heat treatment at different given temperatures for 10 days on the residual activity of (a) free bromelain, and (b) bromelain/LDO. Indicated values are means of three experiments, and error bars represent the standard errors of means.

Langmuir adsorption equation is shown in Table 1, where q_m is the maximum amount of adsorbed bromelain per gram of adsorbent (mg/g) and K is the Langmuir adsorption equilibrium constant (ml/mg). From the results of fitting, the values of q_m and K could be estimated as 157 mg/g and 0.07 ml/mg, respectively.

3.2. The storage properties of immobilized bromelain

3.2.1. Effect of temperature on thermal stability

The thermal stability of immobilized enzymes is one of the most important criteria considered before their utilization in applications. The activity of immobilized enzymes is more resistant against heat treatment and denaturing agents than that of the soluble form. The effect of temperature on the stability of the immobilized bromelain on LDO was studied, and Fig. 6 illustrates the residual activity of both free and immobilized bromelain with casein hydrolysis at 37 °C after the preheat treatment for 10 days at different given temperatures. It can be seen that the immobilized bromelain is more stable than that of the free one in the higher temperature rang. For instance, immobilized enzyme retained 92% of its initial activity after heat treatment at 50 °C, while the corresponding free bromelain was 81%. This indicates that the immobilization of bromelain on LDO can improve its thermal stability remarkably and thus can be preserved at higher temperature.

The FT-IR spectroscopy of enzymes provides direct method to monitor the temperature induced enzyme denaturation, for the amide I/amide II vibrational bands of the proteins/enzymes are sensitive to their structural changes [21]. Upon denaturation, the amide I and amide II bands shift to higher and lower frequency, respectively [22], and the peak positions are estimated using the first or the second derivative method, as needed [23].

Table 1
The equation and correlation coefficients estimated from NLLS method for different isotherms

Isotherm model	Equation	R^2	K	
Freundilich	$Q = KC^{1/n}$	0.9858	19.5	n: 2.05
Linear	$Q = b + KC$	0.9347	2.34	b: 27.5
Langmuir	$C/Q = (1/q_mK) + (C/q_m)$	0.9946	0.07	q_m: 157

The in situ FT-IR spectra of both bromelain and bromelain/LDO at various temperatures are displayed in Fig. 7 (only a few spectra are shown for clarity). As can be observed from Fig. 7A, the absorption bands at 1650 and 1535 cm^{-1} (at 30 °C) are characteristic of the amide I and amide II of free bromelain, respectively. With increasing the temperature to 80 °C, the amide I band moved to high frequency by 7 cm^{-1} (from 1650 to 1657 cm^{-1}), and the amide II band shifted to low frequency by 2 cm^{-1} (from 1535 to 1533 cm^{-1}). This indicates the occurrence of denaturation of free bromelain. However, no obvious shift in the amide bands of bromelain/LDO can be observed upon increasing the temperature from 30 to 100 °C. The FT-IR spectra indicate that the denatured temperature for the immobilized bromelain is much higher than that of the corresponding free bromelain. Moreover, the hydrogen bonding between bromelain and LDO might play an important role on the immobilization, for weak interaction may be more favorable to retain the native structure of enzyme [24], while strong interactions between the enzyme and the matrix results in the distortion of the enzyme structure and thus the decrease in its stability [25].

The improvement in thermal stabilities for the immobilized bromelain was also verified by fluorescence studies. It is has been reported that excitation in the 280–310 nm absorption bands of proteins usually results in fluorescence from the Trp residues in the 310–390 nm region [26]. This is a convenient marker for protein, representing as large decreases or red shifts in fluorescence when denaturation of proteins occurs [27,28]. These changes are, most often, due to the exposure of the Trp residues that are buried in the protein, and they may also be due to the changes in the proximities of specific residues that may act as fluorescence quenchers [24].

The fluorescence emission spectra of free bromelain and bromelain/LDO recorded after heat treatment at various tem-

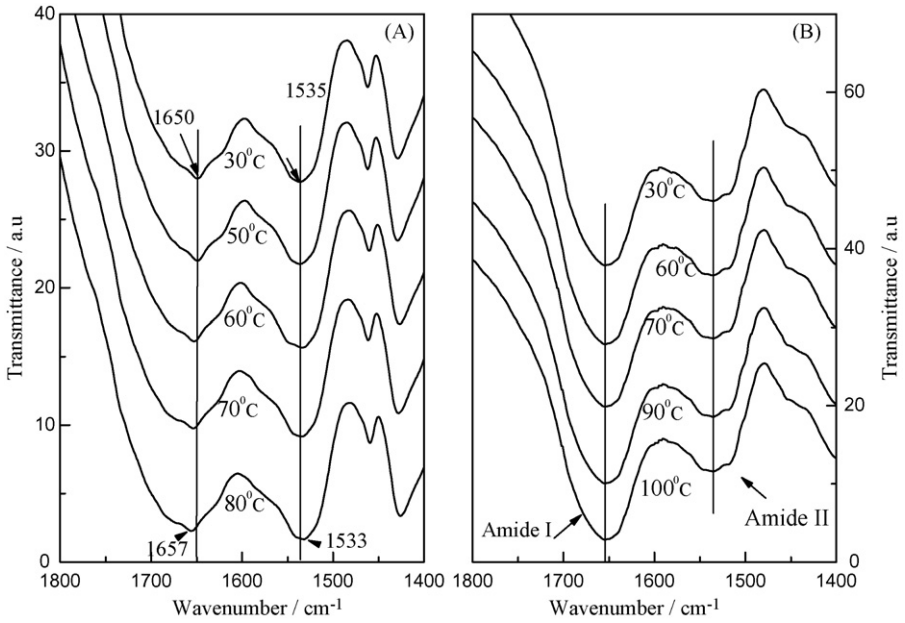

Fig. 7. In situ FT-IR spectra of (A) bromelain and (B) bromelain/LDO recorded at various temperatures (only a few spectra are shown for clarity).

peratures for 4 h are shown in Fig. 8. The emission spectrum of free bromelain at 25 °C in water shows a maximum at 338 nm (280 nm excitation), which is indicative of the presence of tryptophan residues. Bromelain contains five tryptophan residues [29] and three of them may be buried in the hydrophobic core and other two may be located near the surface of the molecule [30]. The free bromelain denatured as the temperature increased to 70 °C, which was accompanied by the quenching of fluorescence at 338 nm, as well as the growth of a new peak at 387 nm (Fig. 8). A similar thermal denaturation has been reported earlier for cyt c by Chaudhari [31]. The emission spectra of bromelain/LDO, in contrast, display a singe peak at 338 nm (280 nm excitation), and no significant changes in the position of the fluorescence maximum were observed on increasing the temperature from 25 to 100 °C. The decrease in the intensity of the band might be attributed to the presence of a non-native stable intermediate state similar to the molten globule formation, which has been reported previously for bromelain at low pH [30]. The improvement in the stability of immobilized bromelain testified by fluorescence spectroscopy is in accordance with the results obtained by in situ FT-IR presented above.

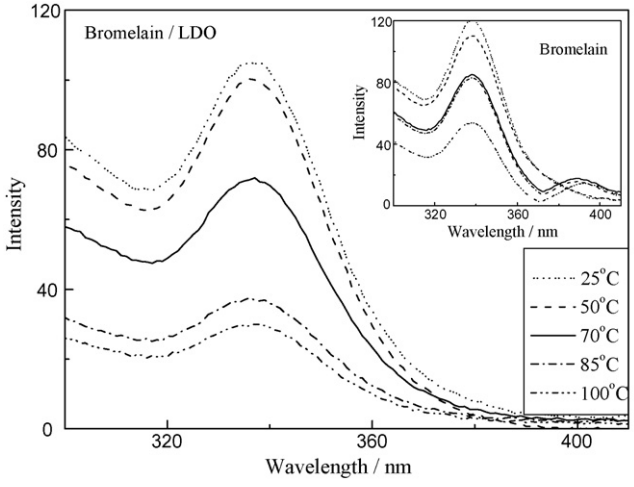

Fig. 8. The fluorescence spectra of bromelain/LDO and free bromelain recorded after heat treatment at various temperatures for 4 h.

3.2.2. *Effect of storage time on residual activity*

It was found in this study that no significant loss in the enzymic activity was observed for the solid and dry immobilized bromelain after storage in vacuum vessel at room temperature for as long as 3 months, whereas the corresponding free bromelain lost more than 60% of its initial activity under the same conditions. The higher storage stability of the immobilized bromelain can be attributed to the prevention of autodigestion and thermal denaturation as a result of the fixation of bromelain molecules on the surface of LDO.

3.3. *The release of bromelain from LDO*

One of the most exploited properties of calcined LDHs is its "memory effect", i.e., it can reconstruct the original layered structure if being calcined at moderate temperatures (300–500 °C), and then equilibrated with water vapor (forming meixnerite like materials, with interlayer hydroxyl anions), CO_2 (forming hydrotalcite-like materials), or immersed in a solution containing different anions. Generally, LDHs have greater affinities for multivalent anions than for monovalent anions. For example, CO_3^{2-} is preferentially adsorbed and not readily replaceable by other anions [32,33]. As a result, the release of bromelain from LDO was studied based on the "memory effect" of LDO in this work. The release process was carried out in water solution without adding any other agent or under nitrogen atmosphere. Fig. 9 displays the XRD patterns of bromelain/LDO before and after the release reaction along with time.

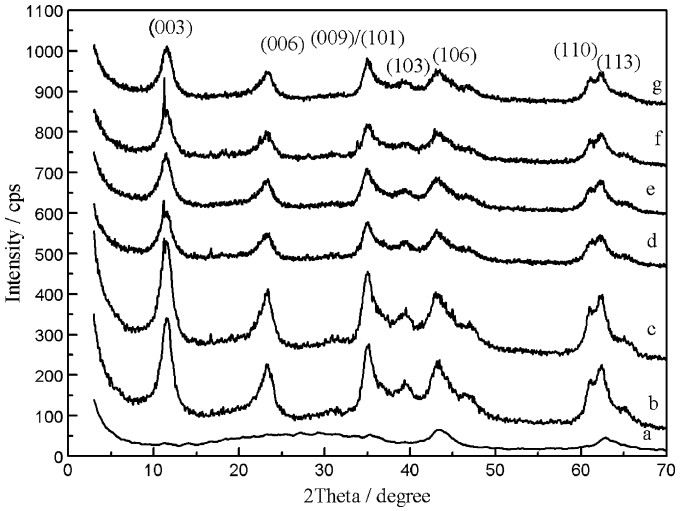

Fig. 9. Powder X-ray diffraction patterns for bromelain/LDO before (a) and after release of bromelain in water at 25 °C for 5 min (b), 10 min (c), 15 min (d), 20 min (e), 25 min (f) and 30 min (g), respectively.

The characteristic pattern according to MgAl-CO_3-LDH [34] was observed after 5 min release (Fig. 9b), including its (0 0 3), (0 0 6), (0 0 9), (1 0 1), (1 0 3), (1 0 6), (1 1 0) and (1 1 3) reflections, indicating the occurrence of the transformation from LDO to LDH. The XRD pattern after 10 min release (Fig. 9c) was rather similar to Fig. 9b, while the intensity of the reflections after 15 min (Fig. 9d) decreased significantly and almost maintained unchanged along with the release time until to 30 min (Fig. 9g). The results indicate that the MgAl-CO_3-LDH phase formed with higher crystallinity during the first 10 min release of bromelain from LDO, and after that the stacking sequence somewhat decreased.

To demonstrate the practicability, the release amount of bromelain in water at pH 7.0 over a period of 35 min was investigated. Fig. 10 displays the release percentage of the immobilized bromelain along with time. The release percentage had a tendency coupling to increase with time during the first 20 min, while decreased at 25 min and retained unchanged. The maximum release amount reached to 70% and the residual activity retained as 81% at 20 min. As a result, the optimal release time should be chosen as 20 min. The release of bromelain from LDO was associated to two possible reasons: (1) the significant decrease in the specific surface area during the reconstruction of LDH by hydration of LDO (from 182 to 20 m^2/g obtained by BET data), which has also been reported by other researchers [35]; (2) the electrostatic repulsion between the positively charged bromelain under the release condition and the host layer of LDHs. The unchanged release amount from 5 to 10 min (Fig. 10) might be owing to the phase transformation from LDO to LDH which has been confirmed by XRD (Fig. 9b and c), while the decrease after 20 min might be due to the lower release rate of bromelain as a result of the better dispersion of LDHs particles along with release time, and the release equilibrium reached at 25 min. Therefore, this work provides a novel biomolecular vessel for prospective application on the storage or delivery system for bromelain.

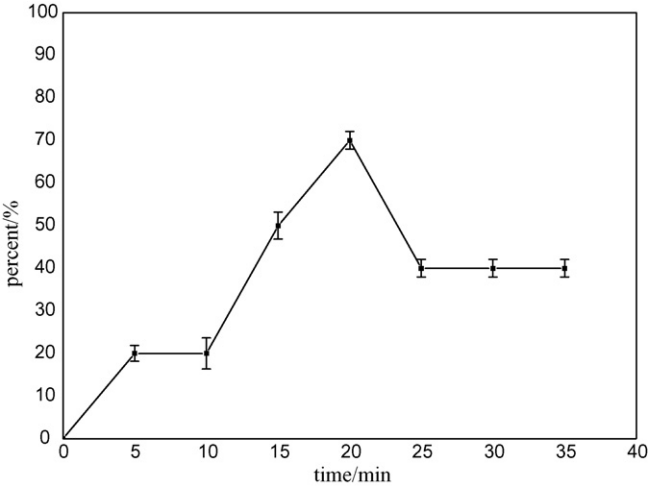

Fig. 10. Release profile of bromelain from LDO along with time. The release study was performed in water at ambient temperature. Indicated values are means of three experiments. Indicated values are means of three experiments, and error bars represent the standard errors of means.

4. Conclusions

LDO has been demonstrated to be an effective support for the immobilization, storage and release of bromelain. The optimum initial amount of bromelain was 20 ml of 4 mg/ml for 1 g LDO and the immobilized enzyme activity express was 33.4% at pH 6.5. The immobilization is attributed to physical adsorption without affecting the structure of LDO and the adsorption isotherm of bromelain/LDO can be well described with the Langmuir model with a R^2 of 0.9946, a maximum adsorption amount of 156.92 mg/g and the Langmuir adsorption equilibrium constant of 0.07 ml/mg. Both thermal stability of the immobilized bromelain and its storage time were improved significantly compared with that of free bromelain. Furthermore, the release behavior of bromelain from LDO was investigated based on the "memory effect" of LDHs. The release maximum of bromelain reached to 70% and the residual activity maintained 81%. Therefore, this work should be helpful for the development of LDO as the basis of a novel storage or delivery system for biomolecules or enzymes.

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