Journal of Molecular Catalysis B: Enzymatic 63 (2010) 188–193

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Chemical modification of stem bromelain with anhydride groups to enhance its stability and catalytic activity

Yong Xue[a], Cheng-Yao Wu[a], Christopher J. Branford-White[b], Xin Ning[c], Hua-Li Nie[a,*], Li-Min Zhu[a,*]

[a] College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, 2999 North Remin Road, Songjiang University City, Shanghai 201620, China
[b] Institute for Health Research and Policy, London Metropolitan University, 166-220 Holloway Road, London, UK
[c] Kimberly-Clark Co., Ltd, Shanghai 201620, China

ARTICLE INFO

Article history:
Received 19 June 2009
Received in revised form 14 January 2010
Accepted 21 January 2010
Available online 29 January 2010

Keywords:
Stem bromelain
Chemical modification
Pyromellitic dianhydride
Poly(maleic anhydride)
Stability
Activity

ABSTRACT

Chemical modification of lysine residues in stem bromelain was carried out using two novel reagents pyromellitic anhydride acid and poly(maleic anhydride). About 60% and 57% of the residues in bromelain were found to be modified by pyromellitic anhydride and poly(maleic anhydride), respectively. The modification brought about enhancement of thermal stability and the resistance to alkali and the surfactant. The optimum pH shifted from 7 to 9. Thermodynamic parameters, ΔH^*, ΔG^* and ΔS^*, were determined as a function of temperature. The kinetic constants K_m of the modified enzymes were determined as 0.4092×10^{-2} and 0.2825×10^{-2} mol l^{-1}, respectively. SDS-PAGE profiling revealed a major bands of native and modified enzyme with molecular weights of 26 and 28.5 kDa. The results of FT-IR studies suggested that the modification caused only local structural changes. These results provide guidance for future development of stable protein formulations.

1. Introduction

Stem bromelain (EC 3.4.22.32) is a basic, 23.8 kDa thiol proteinase obtained from stem of pineapple plant (*Ananas comosus*) [1]. Bromelain is well studied due to its relatively non-specific action on proteins and has antiedemic, anti-inflammatory and coagulation inhibitory potential [2]. A wide range of therapeutic benefits has been claimed for bromelain, such as reversible inhibition of platelet aggregation, angina pectoris, bronchitis and enhanced absorption of drugs, particularly antibiotics [3]. To date, its use in pharmacological or industrial applications is limited due to commercial availability, costs, sensitivity to pH and temperature. Therefore, greater understanding of the molecular mechanisms relatives to enzyme stability would be of interest.

A number of chemical methods have been described in the literature whereby the stability and activity of enzymes can be changed [4–6]. Theses include the introduction of hydrophilic and hydrophobic groups, cross-linking with chemicals and chemical modification of the amino acid side-chains of enzymes. Covalent chemical modification of proteins is widely used as a tool for studying localization of individual amino acids, their participation in the maintenance of the native conformation and for their stabilization [7]. In a number of cases, dramatic stabilization has been achieved [8,9]. Stem bromelain has been sequenced completely at the amino acid level and has been shown to be a member of the papain family [10]. When the amino acid sequence of bromelain is compared with papain and other cysteine peptidases, a number of differences that must affect the geometry of the active site are apparent [11]. Stem bromelain exists as a single polypeptide chain with 212 residues and has 15 lysine residues [12]. The catalytic residues are Cys-25 and His-159. Unlike other papain family members, bromelain is reported to lack Asn-175 and two adjacent residues and the mutation of the highly conserved Ser-176 to Lys [13]. As these lysine residues are not involved in catalysis, they provides targets opportunity for chemical binding. Several authors described the modification of ε-amino groups of lysine residues in papain [14,15]. The chemical modification of papain with polyethylene glycol (PEG) can be utilized to changed their molecular properties or confer new molecular functions [16]. Khaparde and Singhal found that succinyl papain is a potential alternative to the alkaline proteases used in detergents [17]. A preliminary study on stem bromelain modification was earlier carried out by Anwar et al. [18], and compared with papain, bromelain contains more lysine residues, so chemical modification of this amino acid could result in drastic changes in physicochemical characteristics.

This paper described the modification of stem bromelain using two novel modifiers, pyromellitic dianhydride and poly(maleic

* Corresponding authors. Tel.: +86 21 67792655; fax: +86 21 67792655.
E-mail addresses: niehuali2004@126.com (H.-L. Nie), lzhu@dhu.edu.cn (L.-M. Zhu).

1381-1177/$ – see front matter.
doi:10.1016/j.molcatb.2010.01.018

anhydride), which contained both larger molecular weight and more carboxylic groups. The structure of native and modified stem bromelain were studied using FT-IR. The molecular mass of enzymes was characterized. In addition, we also studied the effect of temperature, pH medium and the chemical effect on the native and modified enzymes in enzyme kinetics.

2. Materials and methods

2.1. Materials

Stem bromelain (EC 3.4.22.32, 7 units/mg), albumin, 2,4,6-trinitrobenzenesulfonic (TNBS), pyromellitic dianhydride and poly(maleic anhydride) (M.W. = 500–1000) were purchased from Sigma–Aldrich (USA). L-Cysteine (Biological Reagent) and all the other reagents (analytical grade) were obtained from Sinopharm Chemical Reagent Co., Ltd, (Shanghai, China).

2.2. Chemical modification of stem bromelain

Chemical modification of stem bromelain was undertaken using standard procedures and reactions were carried out at pH 8.0. Stem bromelain was modified with 0.2–0.8 M pyromellitic dianhydride or poly(maleic anhydride) dissolved in dimethyl sulfoxide (DMSO) [19]. During the procedure, 0.1 ml of anhydride solution was added dropwise to 0.5 ml of bromelain (6.0 mg/ml) in 0.1 M phosphate buffer (pH 8.0). This immediately brought about a drop in pH and hence sodium hydroxide was gradually added to the mixture to maintain pH at 8.0. The reaction was allowed to proceed for 1.5 h at 25 °C. The samples were dialyzed exhaustively against distilled water at 4 °C.

2.3. FT-IR spectral measurements

A FT-IR spectrometer (Nicolet, NEXUS-670, USA) with 2 cm^{-1} resolution was used for sample analysis. The scans were repeated 200 times in spectral range from 4000 to 400 cm^{-1}. KBr were pressed to form a tablet.

2.4. Enzymatic assays

A given amount of native or modified stem bromelain and 1.6 ml 0.05 M Tris–HCl buffer pH 8.0 were placed in a tube, mixed with 0.2 ml bromelain activator consisting of 0.02 M ethylene diaminetetraacetic acid (EDTA) and 0.05 M L-cysteine pH 8.0, incubated for 10 min at 40 °C. This step reduces the disulphide bonds of cysteine within the protein. Then, 1 ml of 1% casein pH 8.0 as substrate was added. The reaction was carried out at 40 °C for 15 min and 3 ml of 5% trichloroacetic acid was added. The concentration of the acid-soluble digestion products in the supernatant was determined according to Lowry et al. [20]. One unit of enzyme activity was defined as the tyrosine content formed per minute at 40 °C and pH 8.0.

The relative activity (%) was the ratio between the activity of every sample and the maximum activity of samples.

2.5. Protein determination

Protein concentration was determined by the Bradford method [21], using the Coomassie protein assay reagent with bovine serum albumin as the standard.

2.6. Analysis of free amino groups

The number of free amino groups presented in stem bromelain before and after chemical modification was estimated using TNBS assay procedure of Habeeb [22]. The procedure involved the addition of 1 ml of a 0.1% (w/v) TNBS to 1 ml protein suspension. The samples were incubated in a 37 °C water bath for 2 h. SDS (1 ml, 10%) and HCl (0.5 ml, 1 M) were added to the protein solutions. Absorbance was measured at 335 nm in a UV-2102 PC spectrophotometer (UNICO, Shanghai). The degree of modification (DM) was calculated by the following equation:

$$DM\ (\%) = 1 - \frac{absorbance_c}{absorbance_o}$$

where the subscript c indicates the modified enzyme and o control (native) enzyme. Calibration curve was obtained using different concentrations of anhydrides. The activities of modified enzymes have also been studied by adding various amounts of anhydrides (0–0.8 M) at 40 °C. The residual activity was measured as above.

2.7. SDS-PAGE electrophores

Enzyme purity and molecular weight were analyzed by SDS-PAGE in a Mini Protean Electrophoresis Cell (Bio-Rad), with a 15% resolving and 10% stacking gels, respectively. The protein concentration of enzymes was adjusted with water to give a final concentration of 2 mg/ml. Samples were heated at 100 °C for 3 min prior to electrophoresis. Volumes containing 10 μg of native or modified bromelain were applied onto the gels. Proteins were stained using the Coomassie Blue staining technique. Molecular weight was estimated by comparison with standard markers.

2.8. Characterization of native and modified stem bromelain

2.8.1. Influence of temperature on the enzyme activity and stability

The optimum temperature of the native and modified bromelain was determined by carrying out the standard assay in Tris–HCl buffer. The temperature was varied from 30 to 90 °C at pH 8.0. The purified enzyme solutions were incubated for 1–4 h at the optimal temperature to assess the thermal stability of the enzyme. Heated samples were cooled immediately in ice water, and the residual enzymatic activity was determined.

2.8.2. Influence of pH on the enzyme activity

The effects of pH on the activities of the native and modified enzyme were determined by carrying out the standard assay in Tris–HCl buffer. The enzyme was dissolved in buffers within the pH range 6.0–11.0 (pH 6.0–10.0, Tris–HCl; pH 11.0, Tris–NaOH) and incubated for 20 min at 40 °C. The residual activity was measured and casein was used as the substrate.

2.8.3. Influence of surfactant on the enzyme activity

For each sample, a measured amount of SDS in distilled water was used to give a final concentration of 5–25 mg/ml. A suitable dilution of the native and modified enzymes were prepared in the SDS solution respectively and then assayed for the proteolytic activities at 40 °C. The residual activity was measured as above.

2.9. Kinetics of thermal inactivation and estimation of the inactivation energy

In order to study the thermal inactivation kinetics of native and modified bromelain, the enzymes were incubated at 60, 65, 67, 70, 72 °C. Thermal inactivation of enzymes can be described by a first-order reaction:

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \quad (1)$$

where C_t is the enzyme activity in the absence of the substrate at treatment time t, C_0 is the initial enzyme activity, and k is the inactivation rate constant at the temperature studied. The inactivation rate constant k can be estimated by linear regression analysis of the natural logarithm of residual activity versus treatment time. Half-life is the time where the residual activity reaches 50%, which can be described by Eq. (2)

$$t_{1/2} = \frac{\ln(2)}{k} \tag{2}$$

2.10. Estimation of thermodynamic parameters

The Arrhenius equation is the most common mathematical expression to describe the temperature effect on the inactivation rate constants and the dependence is given by the activation energy (E)

$$\ln(k) = \ln(A) - \frac{E}{RT} \tag{3}$$

where A is the Arrhenius constant, E the activation energy. The activation energy can be calculated by linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature. The other thermodynamic parameters, namely the enthalpy (ΔH^*), the Gibbs free energy (ΔG^*) and the entropy of inactivation (ΔS^*) were calculated as shown in Eqs. (4)–(6)

$$\Delta H^* = E - RT \tag{4}$$

$$\Delta G^* = -RT \ln\left(\frac{kh}{kT}\right) \tag{5}$$

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T} \tag{6}$$

where T is the average temperature (339.8 K in this study).

2.11. Determination of kinetic constant

Experiments for the determination of kinetic parameters, the maximum rate (V_{max}) and the Michaelis constant (K_m), were performed at 40 °C. The substrate concentration was varied between 2 and 10 mg/ml under the same conditions as described in "enzymatic assays".

3. Results and discussion

3.1. Determination of modification degree and catalytic activity

The modification degree obtained for both pyromellitic stem bromelain (PB) and polymaleic stem bromelain (PMB) are summarized in Fig. 1A and B, respectively. The DM increased with the concentration of anhydrides, it was observed that pyromellitic stem bromelain has minimum numbers of exposed ε-amino groups, about 60% lysine residues of the enzyme were modified by the present method. In addition, the influence of the modifiers on the enzyme activities were also reported in Fig. 1A and B. The enzymatic activities of modified stem bromelain decreased with the increased of the DM, the enzyme using higher molecular weight modifier showed lower activity. However, the modified bromelain did not lose its activity significantly. This observation suggested that among different anhydrides those with small molecular size are preferentially modified during the cross-linking reaction and the amino acid at the active site causes no detectable change in the tertiary structure of the enzyme. For PB and PMB, a slight increase in DM was observed from 0.4 to 0.8 M (concentration of the anhydrides), but the activities decrease apparently. Therefore, in consideration of both DM and activities, 0.4 M anhydride was an appropriate concentration for modification.

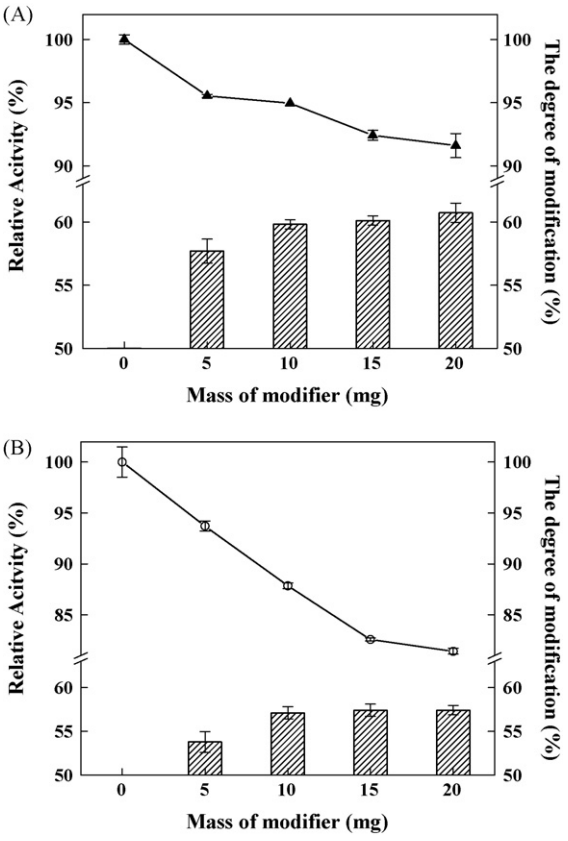

Fig. 1. Numbers of acylated primary amino groups in the stem bromelain molecule. Modifying agent: (A) pyromellitic anhydride; (B) poly(maleic anhydride). Enzyme activity and modified degree were determined under standard conditions of temperature and pH. Indicated values are means of three experiments.

3.2. FT-IR characterization

FT-IR spectroscopy has been proven to be a powerful tool for providing conformational and structure dynamics information of proteins. Comparison of FT-IR spectra of the enzyme before and after modification was shown in Fig. 2. Higher absorptions in the region from 3381 to 3423 cm^{-1} corresponding to –OH stretching of the –OH groups present in NB, PMB and PB. A relatively higher absorption at the characteristic peak (1649 cm^{-1}) and (1517 cm^{-1}) of amide I and amide II was observed after the chemical modifica-

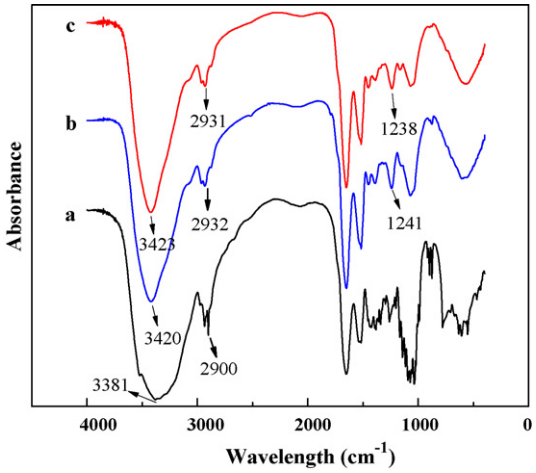

Fig. 2. FT-IR spectra of (a) NB, (b) PB, (c) PMB.

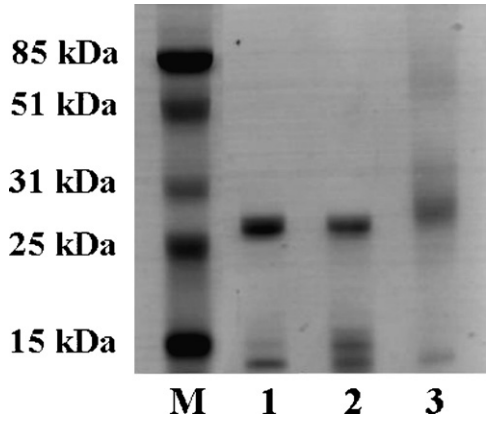

Fig. 3. SDS gel electrophoresis of native and modified stem bromelain. Lane M: molecular weight markers; lane 1: pyromellitic stem bromelain; lane 2: native stem bromelain; lane 3: polymaleic stem bromelain.

tion. Here the peak at 1238 and 1241 cm^{-1} is attributed to the C–O stretching of the ester group. These phenomena demonstrated that the reactions between anhydride acid and amino groups produced amide groups and chemical cross-linking.

3.3. Molecular weight analysis

The molecular weight of the enzyme was examined by SDS-PAGE electrophoresis. In lane 2 of the Coomassie stained gel (Fig. 3), native bromelain migrated as a single band corresponding to a molecular mass around 26 kDa, which lie close to the reported range of the molecular weight (23.8 kDa) of stem bromelain. Lane 3 represents the PMB, which showed a slower migration with a rather diffused band and apparent an average molecular mass of the protease, about 28.5 kDa. This could be explained by the reduction of the number of positive charges on the surface of the protein. The PAGE pattern of pyromellitic stem bromelain exhibited 2 or 3 bands, 1 of which predominated. These results show that the primary amino groups of stem bromelain are not acylated equally and consequently there may be some variances between the enzyme molecules after modification, resulting in different PAGE patterns.

3.4. Effect of temperature on enzyme activity

As to be expected, the enzyme activity of native and chemical modified stem bromelain increased gradually with increasing temperature and the maximum activity was obtained at 60 °C (Fig. 4). This confirmed that the enzyme favored higher temperature as could be observed from the activity data at 50, 60 and 70 °C, While at temperature above 60 °C, the activity decreased sharply with the increase of temperature. At 80 °C PMB showed 34% of maximum activity, which was 20% more than the activity of NB. When the enzymes were kept at 90 °C, the UB and PB were thoroughly inactivated, however, about 15% of the enzyme activity exhibited by PMB could be seen from the data. The results indicated that the PMB showed good heat resistance. The modification of the amino groups of the enzyme, mainly on the lysine residues, altered the protein positive charge considering that anhydrides was linked to about 60% of the total amino groups of bromelain. Thus, the greater stability of modified enzymes may arise from the neutralization of positive charges leading to decreased charge repulsion within the polypeptide [23]. It has also been shown that the thermal stability of the native and modified enzymes is partly due to the presence of a large number of cysteine residues in the polypeptide chain [24].

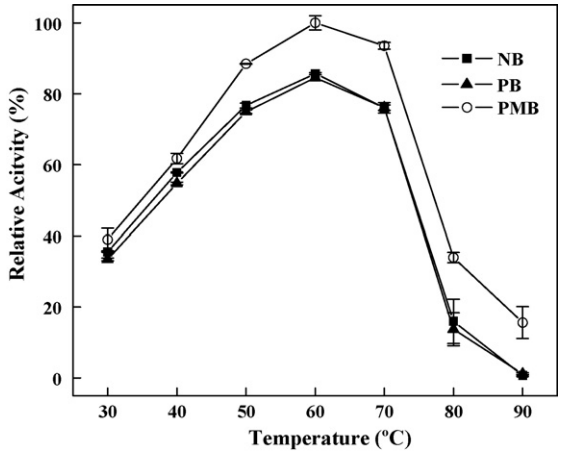

Fig. 4. Effect of temperature on native and modified stem bromelain activities. Enzyme activity was determined under standard conditions of pH and substrate concentration. 100% relative activity corresponds to the maximum activity of samples. Indicated values are means of three experiments.

3.5. Effect of pH on enzyme activity

pH value was a very important parameter to investigate in this study. The tertiary structure of the enzyme depends on pH and the enzyme can be denatured at extremes of pH [25]. Hence, the main objective of bromelain modification was to improve the stability of enzyme in extreme environmental conditions in terms of pH, detergent. The variation of the relative activity of the native and modified stem bromelain at different pH values is shown in Fig. 5, which indicated that their optimum pH were 7.0, 8.0 and 9.0, respectively. The PB and PMB were able to adapt to a wider pH region comparing to NB, displaying over 90% of their activities in the pH range 7.0–11.0. The optimum pH value of NB shifted with 1–2 units towards the alkaline region after chemical modification. It suggested that the acid anhydride reacted with the lysine groups of bromelain lead to a net overall anionic charge. Enveloping the enzyme with negatively charge groups might have caused a localized lowering of pH which respects to the surroundings [26]. Moreover, the poly(maleic anhydride) has more free carboxyl groups after hydrolyzing, and the acid limbs attaching to the enzyme can be effectively used to adjust the alkaline condition.

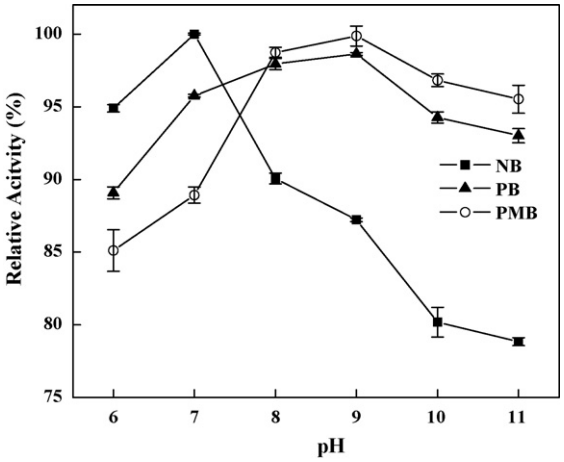

Fig. 5. Effect of pH on native and modified stem bromelain activities. Enzyme activity was determined under standard conditions of temperature and substrate concentration. 100% relative activity corresponds to the maximum activity of samples. Indicated values are means of three experiments.

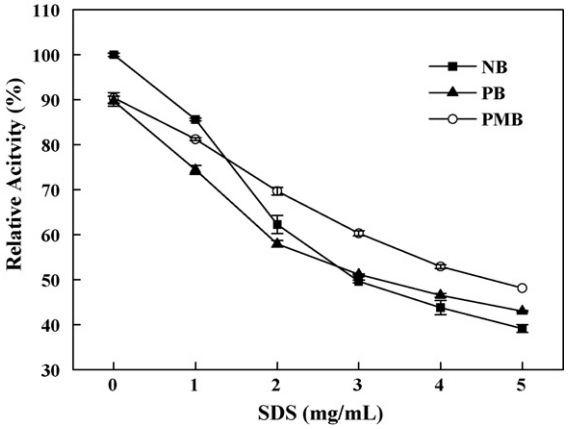

Fig. 6. Effect of surfactant on native and modified stem bromelain activities. Enzyme activity was determined under standard conditions of temperature, pH and substrate concentration. 100% relative activity corresponds to the maximum activity of samples. Indicated values are means of three experiments.

3.6. Effect of chemical on enzyme activity

Enzymes are usually inactivated by the addition of surfactants to the reaction solution. The effect of SDS on the stability of enzymes was also studied (Fig. 6). The PMB showed significant resistance to the surfactant tested. In addition, an enhancement in the PB activity was observed in the presence of SDS. In this study, the enzyme activity of the sample without any SDS was taken as control. After the enzyme was incubated with 5 mg/ml SDS for 30 min at 40 °C, the PMB still retained about 48% of its activity. For NB, the strong surfactant at 4 and 5 mg/ml caused a significant inhibition of 57% and 61%, respectively. The anionic surfactants usually affected negatively on the enzyme activity [27] and so the modification of bromelain caused an increase in the enzyme stability against the anionic surfactants. The change in activity may be due to the alkaline pH which was caused by the detergent favoring the modified bromelain.

3.7. Kinetics and associated parameters

3.7.1. Inactivation kinetics and estimation of thermodynamic parameters

The process whereby the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds is defined as inactivation [28]. Inactivation rate constants of the enzymes, were presented in Table 1 at 60, 65, 67, 70 and 72 °C. The thermal stability of the enzymes decreased with increasing temperature. Beyond 65 °C, enzymes activities were severely inhibited. Likewise, the half-life values estimated using these constants and Eq. (2) were presented in the same table, PMB showed maximum half-life of 34.64 min at 60 °C and with increasing temperature half-life decreased sharply. However, the half-life of PMB at 72 °C was 1.61 and 1.43 times higher than the half-life values of NB and PB, respectively.

The dependence of the inactivation rate constants with temperature fitted adequately the Arrhenius equation (Fig. 7). The changes in enthalpy (ΔH^*) and entropy (ΔS^*) are calculated using transition state theory according to Eqs. (4)–(6) for the thermal inactivation of native and modified stem bromelain (Table 2). A positive ΔH^* and ΔS^* were determined in the temperature ranges studied. With increase of molecular weight, a slight decrease in ΔH^* and a marked in ΔS^* were observed. This suggested thermal denaturation of the enzymes, possibly due to disruption of non-covalent linkages, including hydrophobic interactions [29]. Thermo stabilization of enzymes is mostly accompanied by a decrease in ΔS^* and an increase in ΔG^* [30]. Thus the increase in ΔS^* indicated that there was an increase in the number of protein molecules in transition activated stage. Moreover, solvent and structural effects are reported to be the two major factors influencing the numerical values of ΔH^* and ΔS^*. Particularly, ΔS^* are known to provide information regarding the degree of compactness of protein molecule [31]. Also the increase in the ΔH^* with respect to molecular weight decrease revealed that the conformation of the enzyme was altered, chemical modification by anhydrides introduces carboxylic groups to each amino group modified, thus decreasing the flexibility of an external loop. This would signify that the bromelain modified by poly(maleic anhydride) would be more readily activated under the condition employed. The use of enzyme in industrial processes may require reactions to be conducted at high temperature in order to improve productivity, and the above issues suggested that PMB could be considered as a potential candidate for various industrial applications.

Table 1
Kinetic parameters of thermal inactivation of native and modified stem bromelain.

Temperature (°C)	Enzyme	k (min^{-1})	R^2	$t_{1/2}$ (min)
60	NB	0.02652	0.9959	26.14
	PB	0.02133	0.9706	32.50
	PMB	0.02001	0.9716	34.64
65	NB	0.06932	0.9907	9.99
	PB	0.06355	0.9926	10.91
	PMB	0.04581	0.9879	15.13
67	NB	0.10450	0.9958	6.63
	PB	0.09480	0.9914	7.31
	PMB	0.06162	0.9913	11.25
70	NB	0.14242	0.9981	4.87
	PB	0.12962	0.9789	5.35
	PMB	0.08917	0.9991	7.77
72	NB	0.17146	0.9796	4.04
	PB	0.15290	0.9841	4.53
	PMB	0.10684	0.9938	6.49

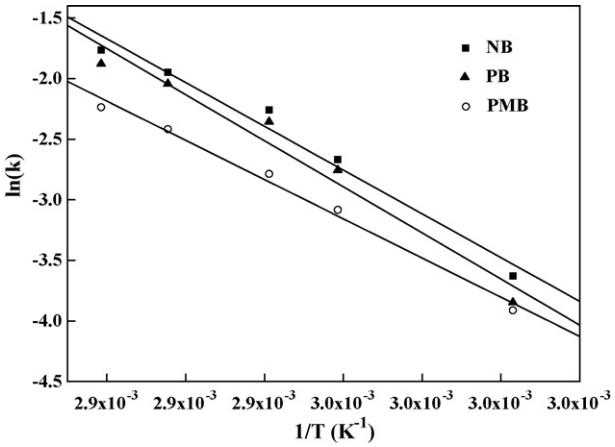

Fig. 7. Arrhenius plots for thermal inactivation for native and modified stem bromelain.

Table 2
Thermodynamic parameter values of thermal inactivation of native and modified stem bromelain activities.

Enzyme	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (J mol^{-1} K^{-1})
NB	147 ± 2	78.7 ± 0.5	202
PB	155 ± 2	79.3 ± 0.6	224
PMB	132 ± 1	80.2 ± 0.3	152

Table 3
Kinetic constants of native and modified papain.

Enzyme	k_{cat}/(mol s^{-1} mol enzyme^{-1})	V_{max}/(mol s^{-1} g^{-1})	K_m/(mol l^{-1})	k_{cat}/K_m
NB	10.0	$(0.908 \pm 0.095) \times 10^{-2}$	$(0.526 \pm 0.063) \times 10^{-2}$	1.91×10^3
PB	7.11	$(0.592 \pm 0.064) \times 10^{-2}$	$(0.409 \pm 0.042) \times 10^{-2}$	1.74×10^3
PMB	7.35	$(0.574 \pm 0.092) \times 10^{-2}$	$(0.283 \pm 0.072) \times 10^{-2}$	2.60×10^3

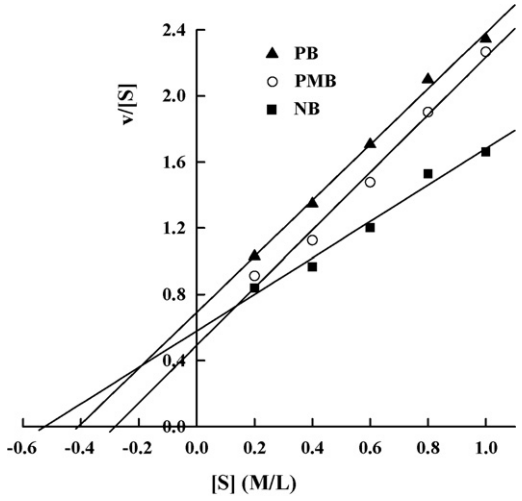

Fig. 8. Hanes–Woolf plots of native and modified stem bromelain.

3.7.2. Michaelis constant and maximal velocity

The experiments were carried out with low initial substrate reservoir concentration so that the Michaelis–Menten kinetics can be approximated by first-order kinetics [32]. Accordingly, the kinetic parameters of the enzyme were derived, including the maximum reaction rate (V_{max}) of the enzymatic reaction and the Michaelis–Menten constant (K_m). These parameters were obtained from Hanes–Woolf plot which can be written as:

$$\frac{[S]}{v} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}}$$

This expression avoids both misleading impression of the experimental error and the uneven distribution of the points by Lineweaver–Burk plot and the angular distortion of the errors of the Eadie–Hofstee plot [33]. When comparing the K_m values of NB with PB and PMB (Fig. 8), some differences in K_m values showed that the modified enzyme has greater affinity towards the substrate (Table 3). This may also be attributed to the enhancement of substrate-enzyme complex stability. In addition, NB has the highest k_{cat} value followed by PMB and the PB. The catalytic efficiency value (k_{cat}/K_m) provides an approximate but useful model to determine the enzyme efficiency with a view to industrial application. Among all the enzymes, PMB showed a highest catalytic efficiency (2.602×10^3) and the difference may be ascribed to the structural differences of the enzyme.

4. Conclusions

One of the primary objectives of enzyme engineering is to produce stable enzymes useful for various biotechnological applications and this can be achieved by various methods. In this study, two novel modifiers were used to modify bromelain. According to the TNBS experiments, this chemical modification of the enzyme resulted in the acylation of 8–9 of the 15 primary amino groups. The chemically modified enzymes exhibited remarkable stability for medium under condition and high temperatures, as well as good reusability. Furthermore, the UV–vis and FT-IR measurements demonstrated that there were minor structural change, indicating that the proposed chemical modification does not affect the enzyme inner layer and hence activity. It appears that this simple method may provide a very useful strategy for giving proteins some new and useful characteristics related to stability and catalytic activity.

Acknowledgments

The present investigation was supported by Grant 50773009 from NSFC, the Research Fund for the Doctoral Program of Higher Education (No. 20090075120001) and partly by Grant 08JC1400600 from Sci & Tec Commission of Shanghai Municipality.

References

[1] P. Gupta, M. Saleemuddin, Biotechnol. Lett. 28 (2006) 917–922.
[2] P. Gupta, T. Maqbool, M. Saleemuddin, J. Mol. Catal. B-Enzym. 45 (2007) 78–83.
[3] H.R. Maurer, CMLS-Cell. Mol. Life. Sci. 58 (2001) 1234–1245.
[4] B. Castillo, R.J. Sola, A. Ferrer, G. Barletta, K. Griebenow, Biotechnol. Bioeng. 99 (2008) 9–17.
[5] G. Desantis, J.B. Jones, Curr. Opin. Biotechnol. 10 (1999) 324–330.
[6] M.Y.J. Lee, S. Dordick, Curr. Opin. Biotechnol. 13 (2002) 376–384.
[7] K. Khajeh, H.N. Manesh, B. Ranjbar, A.M. Movahedi, M.N. Gorgani, Enzyme Microb. Technol. 28 (2001) 543–549.
[8] E. Miland, M.R. Smyth, C.O. Fagain, Enzyme Microb. Technol. 19 (1996) 63–67.
[9] V.V. Mozhaev, N.S. Melik-Nubarov, V.Y. Levitsky, V.A. Siksins, K. Martinek, Biotechnol. Bioeng. 40 (1992) 650–662.
[10] A. Ritonja, A.D. Rowan, D.J. Buttle, N.D. Rawlings, V. Turk, A.J. Barret, FEBS Lett. 247 (1989) 419.
[11] A. Ritonja, A.D. Rowan, D.J. Buttle, N.D. Rawlings, V. Turk, A.J. Barrett, FEBS J. 247 (1989) 419–424.
[12] H. Ishihara, N. Takahashi, S. Oguri, S. Tejima, J. Biol. Chem. 254 (1979) 10715–10719.
[13] A.J. Barrett, N.D. Rawlings, M.E. Davies, W. Machleidt, G. Salvesen, V. Turk, Proteinase Inhibitors, North-Holland, Amsterdam, 1986, pp. 515–569.
[14] A. Szabo, M. Kotorman, I. Laczko, L.M. Simon, Process Biochem. 44 (2009) 199–204.
[15] B.G. Davis, Curr. Opin. Biotechnol. 14 (2003) 379–386.
[16] Y. Inada, M. Furukawa, H. Sasaki, Y. Kodera, M. Hiroto, H. Nishimkura, A. Matsushima, Trends Biotechnol. 13 (1995) 86–91.
[17] S.S. Khaparade, R.S. Singhal, Bioresour. Technol. 78 (2001) 1–4.
[18] T. Anwar, B. Ahmad, H. Younus, Biocatal. Biotransform. 25 (2007) 453–458.
[19] J.J. Roy, S. Sumi, K. Sangeetha, T.E. Abraham, J. Chem. Technol. Biotechnol. 80 (2005) 184–188.
[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[21] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[22] A.F.S.A. Habeeb, Anal. Biochem. 14 (1966) 328–336.
[23] F.Q. Guerrero, M.A.D. Vazquez, R. Tinoco, M.G. Suarez, B.E.G. Almendarez, R.V. Duhalt, C. Regalado, J. Agric. Food Chem. 56 (2008) 8058–8065.
[24] M. Ofelia, N.W. Krzysztof, M.O. Rosa, T.P. Violeta, Lebensm-Wiss. Technol.-Food. Sci. 41 (2008) 1372–1379.
[25] S. Kurniawati, J.A. Nicell, Bioresour. Technol. 99 (2008) 7825–7834.
[26] Y. Xue, H.L. Nie, L.M. Zhu, S.B. Li, H.T. Zhang, Appl. Biochem. Biotechnol. (2009), doi:10.1007/s12010-009-8588-x.
[27] J. Lalonde, E.J. Witte, M.L. Oconnell, J. Am. Oil Chem. Soc. 52 (1995) 53–59.
[28] G.S.N. Naidu, T. Panda, Biochem. Eng. J. 16 (2003) 57–67.
[29] H.N. Bhatti, M. Asgher, A. Abbas, R. Nawaz, M.A. Sheikh, J. Agric. Food Chem. 54 (2006) 4617–4623.
[30] I. Urabe, H. Nanjo, H. Okada, Biochim. Biophys. Acta 302 (1973) 73–79.
[31] T. Canan, D. Nergiz, G. Nihan, Food Chem. 111 (2008) 824–829.
[32] G. Narsimhan, Chem. Eng. J. 22 (1981) 101–105.
[33] K. Sangeetha, T.E. Abraham, J. Mol. Catal. B-Enzym. 38 (2006) 171–177.