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Differential scanning calorimetry as tool in observing thermal and storage stability of recombinant bromelain

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Abstract: Knowledge about the thermal and storage behavior of produced protein is important for the purpose of storage, transport and shelve life during industrial application. Recombinant bromelain thermal and storage stability were measured and compared to the commercial bromelain using Differential Scanning Calorimetry (DSC). Recombinant bromelain is more stable than commercial bromelain at higher temperature but the stability was reduced after 7 days of storage at 4°C. Higher energy is needed to break the bond between amino acid chains in recombinant bromelain as shown by the enthalpy obtained, suggesting that recombinant bromelain has good protein structure and conformation compared to commercial.

Keywords: DSC, thermal stability, storage stability, recombinant bromelain

Introduction

The mechanisms of protein degradation by chemical or physical means have been extensively reviewed (Cueto *et al.,* 2003; Gao *et al.,* 2005). Protein structures are stabilized by non-covalent intramolecular interactions between amino acid side chains. The higher levels of protein structure are maintained by relatively weak non-covalent interactions. Disruption of these weak interactions can be caused by factors such as temperature (heat or cold), pressure, salt, pH, shear, surface interactions, and freeze-drying application. Thermal analysis is useful for detecting the effects of these factors on proteins or protein products.

Differential scanning calorimetry (DSC) has been widely used in studying the thermal stability of proteins and as one of the most sensitive technique for measuring the thermodynamic parameters of thermal protein unfolding (Chen and Oakley, 1995; Cordella *et al.,* 2003). DSC determines the calorimetric changes in proteins as a function of temperature. The thermal denaturation of proteins is attributed to the rupture of intramolecular hydrogen bonds. The denaturation temperatures are measures of the thermal stability of proteins. Their determination under controlled conditions can provide direct comparison of the thermal stability of the different proteins.

Equilibrium analysis of DSC thermograms corresponding to reversible unfolding of proteins provides information about the thermodynamics and mechanisms of the reversible unfolding. A kinetic model for the irreversible denaturation was applied to analyze heat capacity curves that are dependent on the DSC scan rate. Thus, information on the structure in solution and the stability of this respiratory protein was obtained (Cueto *et al*., 2003). DSC is also used to detect the glass transition temperature, Tg in protein solutions and lyophilized products. The determination of Tg is necessary in defining a freeze-drying cycle and storage temperature for the lyophilized products. The peak transition or denaturation temperature, Td, is a measure of thermal stability, while the enthalpy change (DH), measured as area under the endothermic peak, represents the proportion of undenatured protein in a sample, or extent of ordered structure (Charrier *et al.*, 2006). The sharpness of the transition peak, measured as width at half peak height (DT1/2), is an index of the cooperatively of the transition from native to denatured state (Amako and Xiong, 2001; Tang, 2007). Therefore, the aims of this study are to examine and characterize the thermal and storage stability of recombinant bromelain by DSC and compare it with the commercial bromelain.

Material and Methods

Vector construction

Bromelain gene was isolated and purified from *Ananas comosus* stem. The cDNA was synthesis from total RNA and cloned into the entry vector. *E. coli* BL21-AI was used as expression system as described previously (Ismail and Amid, 2008a; 2008b; Amid *et al*., 2011).

Purification of recombinant bromelain

Purification of the recombinant bromelain was carried out following the instruction of the manufacturer (Qiagen, Germany). The expressed recombinant bromelain was purified by affinity

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chromatography using a nickelnitrilotriacetic acid (Ni-NTA) agarose. Up to 15 mL of cell lysate was loaded onto the Ni-NTA column at a flow rate of 1 ml/min. The recombinant protein was eluted by elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole at pH 8.0).

Freeze drying

The purified recombinant bromelain was concentrated by spinning it at 7400 rpm, 30 min at 4°C using (Centrifugal Filter Unit, Millipore, USA) and kept at -80°C overnight. The freezed recombinant bromelain was plug into a freeze dryer (Labconco, USA) and left for 4 days until powderized.

Differential scanning calorimetry

Thermal analysis of freeze-dried powder was performed using differential scanning calorimetry (DSC) (Mettler Toledo, DSC822^e). Approximately, 5 mg sample was sealed in an aluminum pan and an empty pan was used as a reference. The lid of the aluminium pans was pierced just before analysis, allowing desorbed water to leave the pan. The powdered recombinant and commercial bromelain were heated from 29°C to 150°C at heating rate of 10ºC min^{-1}.

Enzymatic assay of bromelain

The protease activity towards Nα-CBZ-L-Lysine p-Nitrophenyl Ester (LNPE) was studied at 45°C and pH 4.6. The reaction was measured spectrophotometrically at 340 nm (Tecan, Switzerland) for the first five min. The assay was conducted in triplicate to confirm the precision of the measurement. The enzyme activity was calculated by applying equation (1).

Units/mL enzyme =

$$\frac{(\Delta A340nm/min\ Test - \Delta A340nm/minBlank)(280)(df)}{(0.6212)(10)} \quad (1)$$

where :

280 = volume (in microliters) of assay

df = dilution factor

0.6212 = milimolar extinction coefficient of p- nitrophenol at 340 nm

10 = volume (in microliters) of enzyme used

Results and Discussion

A stable and correctly folded protein is an absolute requirement for a successful biotherapeutic. All biological processes depend on proteins being stable and in the appropriate folded conformation. The thermal and storage behavior of recombinant bromelain was investigated by DSC. DSC directly measures the thermal stability and unfolding of a protein.

Temperature stability

Figures 1 and 2 show the glass transition temperature, *Tg* for recombinant and commercial bromelain for day 1 and after 7 days of storage in 4°C. The *Tg* is defined as the temperature at which an amorphous system changes from a hard or glass to the rubbery state. The high *T*g value indicate the greater the product stability to high temperature while the low *T*g value indicate the vulnerability of the product during processing, handling and storage in response to temperature. The higher *Tg* of recombinant bromelain (98.19°C and 94.87°C) showed higher thermal stability compared to commercial bromelain (90.61°C and 90.53°C) at day 1 and day 7 (Figures 1 and 2). A ligand will bind to a protein only if the resulting complex is more stable than the non-liganded protein. Binding can occur to the native, folded protein which stabilizing the native states or it can destabilize the native protein by binding to the

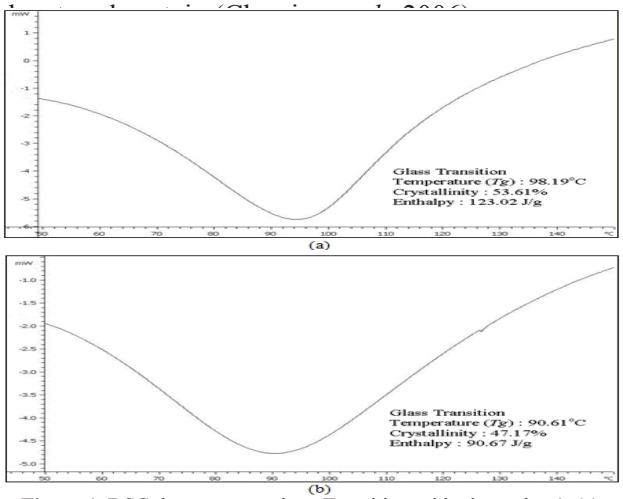

Figure 1. DSC thermograms show Transition midpoint at day 1. (a) Recombinant bromelain (b) Commercial bromelain

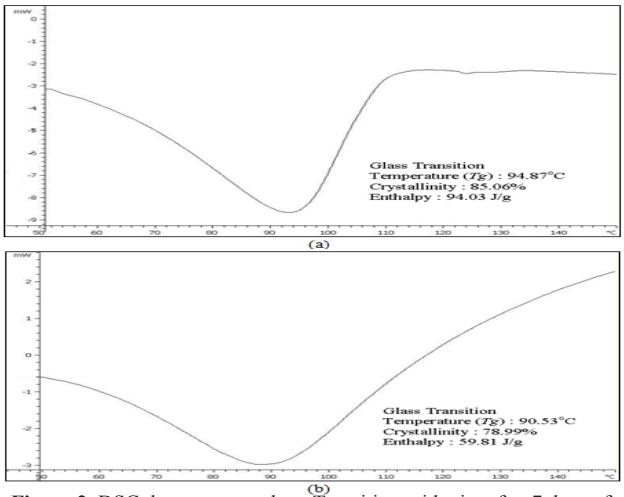

Figure 2. DSC thermograms show Transition midpoint after 7 days of storage at 4°C. (a) Recombinant bromelain (b) Commercial bromelain

The use of enzyme in industrial processes may require reaction to be conducted at high temperature in order to improve productivity and the the results obtained suggested that recombinant bromelain could be considered as potential candidate for various industrial applications. In DSC, the protein is heated at a constant rate and the heat change associated with thermal denaturation is detectable. The process absorbs energy from the surrounding in the form of heat which describes the endothermic process. The amount of heat absorbed is measured by DSC in the form of unfolding enthalpy due to heat denaturation (Chen and Oakley, 1995).

Higher enthalpy was observed by recombinant bromelain where 123.02 J/g and 94.03 J/g were needed to denature the protein structure and conformation at day 1 and day 7 respectively (Table 1). Enthalpy changes observed in Table 1 may be associated with molecular changes as a result of protein unfolding. The enthalpy value is correlated with the net content of the ordered secondary structure of a protein. The changes are due to a combination of endothermic reactions, such as disruption of hydrogen bonds and exothermic reactions, such as disruption of hydrophobic interactions (Li *et al.,* 2008). This showed higher amount of heat needed to break the bond between the amino acid chains in recombinant bromelain compared to commercial, explained that recombinant bromelain can maintained its structure and stably folded although exposing to higher temperature.

Table 1. Summary of DSC results for recombinant and commercial bromelain at day 1 and after 7 days of storage

Duration Time	Day 1		A week (Day 7)	
Proteins	Recombinant Bromelain	Commercial Bromelain (Merck, USA)	Recombinant Bromelain	Commercial Bromelain (Merck, USA)
Tm (transition midpoint) (°C)	98.19	90.61	94.87	90.53
Crystallinity (%)	53.61	47.17	85.06	78.99
Enthalpy (ΔH) (J/g) – 100%	123.02	90.67	94.03	59.81
Enzyme Activity	57.39	93.62	41.59	89.15

The enthalpy change was measured when all the protein is crystallized (100% crystallization). Crystallinity property is related to enthalpy due to unfolding of the protein. The protein will crystallize more rapidly when the protein absorbs more energy (Li *et al.,* 2008). Therefore, higher percentage of crystallinity was observed when higher amount of enthalpy change absorbed for the protein to denature (Table 1).

From the existing knowledge from other drying techniques it can be expected that the presence of stabilizers such as certain sugars is necessary for successful drying and storage stability of the dried proteins (Wang, 2000). Previous studied by Melo *et al.* (2001) shows that most protein are stabilized in the presence of sugar such as trehalose and sucrose. Remarkably, sucrose and trehalose, had a strong impact on the particle morphology and showed distinct physical characteristics (Wang, 2000; Melo *et al.,* 2001).

Kaushik and Bhat (2003) reported on the thermal stabilization of five proteins differing in their various physico-chemical properties with sugar, and that sugar is a universal protein stabilizer, and can be used to increase the stability of many industrially and therapeutically important enzymes without fail. However Habib *et al.* (2007) reported that both kinetic and thermodynamic measurements on the stability of bromelain under thermal stress revealed that this enzyme is less stable in the presence of sucrose or trehalose. It appears that 1 M trehalose inactivated bromelain more than sucrose at temperature 60°C. Sucrose showed a stable system during the crystalline phase compared to trehalose which forms a heterogeneous system, locally differing in water content or in the presence of crystalline phase. This could also make trehalose an unstable matrix and changes of the formulation matrix which influence the protein conformation (Allison *et al.,* 2000). Bromelain contains a single oligosaccharide moiety per molecule which interacts largely with the sugar. The binding of sugars to the folded enzyme (native) is expected to be more than to the unfolded ones (denatured). Other stabilizers which exhibited a higher stabilizing effect and preserved 75% of enzyme activity of the enzymes are glycerol and sorbitol (Li *et al.,* 2008). The availability of sugar such as sucrose in unpurified commercial bromelain results in osmolyte exclusion and preferentially hydration of the denatured bromelain and appears to be the reason for its destabilization.

Storage stability

In terms of storage stability study, stable glass transition temperature, *Tg*, commercial bromelain was observed after 7 days storage of the enzyme. Meanwhile, the *Tg* of recombinant bromelain is reduced to 94.87°C after 7 days storage in 4^0C (Figures 1 and 2). The *Tg* of recombinant bromelain was reduced from 98.19°C to 94.87°C after 7 days of storage simultaneously indicated by the reduction in the enzyme activity (Table 1). Commercial bromelain exhibited higher storage stability compared to recombinant when the *Tg* is maintained at 90°C after

7 days of storage (Devakate *et al.,* 2009). This is due to the addition of stabilizer and other components in commercial bromelain since it is commercially manufactured and marketing in all over the world. Unfortunately, these components addition may decrease the bond between amino acid chains in commercial bromelain and easily break and denatured at lower temperature compared to recombinant explained the lower thermal stability of commercial bromelain (Arroyo-Reyna and Hernandez-Arana, 1995; Charrier *et al.,* 2006; Tang, 2007; Li *et al.,* 2008).

The stability of protein is strongly associated with the glass transition temperature (*T*g), which depends on the storage conditions such as water activity, humidity and temperature (Ross and Karel, 1991). Hence, the *T*g can be set as a reference parameter to characterize the properties, quality, stability and safety of the food systems. The powder samples of recombinant and commercial bromelain stored at 4°C shows the loss in activities by recombinant and commercial bromelain are 27.5% and 4.7% respectively (Table 1). Thus, the inactivation rate was found to be strong function of storage temperature and time. Besides that, previous study (Hale *et al.,* 2005) shown that proteinase activity of pineapple fruit is higher than stem bromelain. This would suggest that higher activity of commercial bromelain is due to the combination of fruit and stem bromelain whereas recombinant bromelain is purely enzyme extracted from pineapple stem.

Devakate *et al.* (2009) found the glass transition temperature of freeze-dried bromelain is 61°C, whereas Arroyo-Reyna and Hernandez-Arana (1995) recorded the maximum of the heat capacity curve occurred at 59.3°C. This is in agreement with the transition midpoint, Tm value observed in circular dichroism experiment (59.6°C) performed under 1°C/min heating rate. From DSC study, it can be concluded that in order to avoid any structural changes and protein denaturation in recombinant bromelain lypholization especially in spray drying process, the bulb temperature of outlet drying air should be less than 100°C. Thus, our study shows that the recombinant bromelain exhibited higher thermal stability and lower storage stability compared to commercial. Future studies on the recombinant bromelain formulation are needed to improve the storage stability of the enzyme.

Conclusions

The ability of DSC to detect differences in *Tg* in complex protein formulations is extremely valuable in protein characterization and formation screening. It can be used to evaluate protein stability and in process development is gaining recognition.

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