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Reverse micellar extraction of bromelain from pineapple peel – Effect of surfactant structure

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ABSTRACT

Pineapple peel is generally disposed or used as compost. This study was focused on extracting bromelain from pineapple peel by using reverse micelles. It was found that gemini surfactant C_{12}-8-C_{12}·2Br (octamethylene-α,ω-bis(dimethyldodecylammonium bromide)) showed distinctive advantage over its monomeric counterpart DTAB (dodecyl trimethyl ammonium bromide); under optimized condition, the bromelain extracted with C_{12}-8-C_{12}·2Br reverse micelle had an activity recovery of 163% and a purification fold of 3.3, while when using DTAB reverse micelle, the activity recovery was 95% and the purification fold was 1.7. Therefore, the spacer of gemini surfactant should play a positive role in bromelain extraction and may suggest the potential of gemini surfactant in protein separation since it has been so far rarely used in relative experiments or technologies.

1. Introduction

Reverse micelles are aggregates of surfactant molecules with the head groups oriented toward the polar core and the hydrophobic tails into the nonpolar medium. They are characterized by the formation of water pools, located inside the micelles. Proteins can be solubilized into the water core, and hence be shielded from organic medium without losing significant biological activity (Martinek, Klyachko, Kabanov, Khmelnitsky, & Levashov, 1989; Stamatis, Xenakis, & Kolisis, 1999; Tonova & Lazarova, 2008). Now, reverse micellar extraction has been considered as an alternative to conventional separation and purification procedures for bioactive proteins since it has potential for continuous operation and is easy to scale up with no loss of native function/activity and high capacity of proteins (Gaikaiwari, Wagh, & Kulkarni, 2012a, 2012b; Harikrishna, Srinivas, Raghavarao, & Karanth, 2002; Hatton, 1989; Imm & Kim, 2009; Kadam, 1986; Kumar, Hemavathi, & Hebbar, 2011).

Two steps are included in liquid–liquid reverse micellar extraction process; a target protein is selectively solubilized into the organic phase (forward extraction) and subsequently stripped into the aqueous phase (backward extraction) by addition of fresh aqueous buffer, also called stripping solution (Dungan, Bausch, Hatton, Plucinski, & Nitsch, 1991; Harikrishna et al., 2002; Kumar et al., 2011; Nandini & Rastogi, 2009). Factors affecting the performance of reverse micelle system are rather complicated, including the nature and concentration of target protein, pH, the concentration and species of ions, type and concentration of surfactant, the composition of reverse micelles, and so on (Chen, Su, & Chiang, 2006; Ding, Cai, & Guo, 2015; Dong, Cai, Guo, & Xiao, 2013; Harikrishna et al., 2002; Hebbar & Raghavarao, 2007; Hebbar, Sumana, & Raghavarao, 2008; Imm & Kim, 2009; Noritomi, Kowata, Kojima, Kato, & Nagahama, 2006; Tonova & Lazarova, 2008; Xiao, Cai, & Guo, 2013). It has been generally considered that reverse micelle size should fit the target protein size and the transfer of protein between the two phases is primarily governed by electrostatic interaction between protein and surfactant; when using cationic surfactant, the forward extraction is generally carried out at pH higher than the isoelectric point (pI) of protein, while the backward extraction is always done at lower pH (Arshad et al., 2014; Chen et al., 2006; Harikrishna et al., 2002; Imm & Kim, 2009; Noritomi et al., 2006; Tonova & Lazarova, 2008). The hydrophobic interaction between the nonpolar domain of protein and surfactant also affects the reverse micelle extraction efficiency. It has been found that the recovery of ovalbumin from reverse micelle is more difficult than that of BSA (bovine serum albumin) due to the higher hydrophobicity of ovalbumin than BSA (Ding et al., 2015).

The application of reverse micelle in enzyme extraction from real biological system should be interesting and has been expected promising potential. Bromelain is a proteolytic enzyme and widely used in food, cosmetics and pharmaceutics. It is found in the tissues of plant family *Bromeliaceae*. Pineapple is the best known source of bromelain, present in its pulp, core, stem and peel, with

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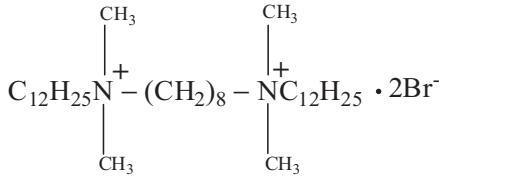
the molecular weight being ca. 23–36 kDa (Arshad et al., 2014). Bromelain is soluble in water but insoluble in organic solvent. Its isoelectric point is 4.6 when extracted from pulp or core, while 9.5 when obtained from stem or peel (Arshad et al., 2014; Hebbar et al., 2008). CTAB (cetyl trimethyl ammonium bromide) and AOT (di-2-ethylhexyl sodium sulfosuccinate) reverse micelles were used to extract bromelain from pulp, core and stem of pineapple, and CTAB was found more suitable (Hebbar et al., 2008; Hemavathi, Hebbar, & Raghavarao, 2007). When using CTAB, the extraction efficiency was ca. 20% and the activity recovery was 97% from pineapple pulp (Hemavathi et al., 2007) or 102–106% from core and stem (Hebbar et al., 2008), while the efficiency of AOT reverse micelle was very low (Hebbar et al., 2008; Hemavathi et al., 2007). The separated protein could be analyzed by SDS–PAGE electrophoresis (Gao, Liu, & Xiao, 2011; Li et al., 2012), based on which, the purity of the extracted bromelain seemed satisfactory (Hebbar et al., 2008). Fileti, Fischer, Santana, and Tambourgi (2009) carried out bromelain extraction from pineapple pulp by using BDBAC (benzil dodecyl bis(hydroxylethyl) ammonium chloride) reverse micelle and found the maximum purification factor could be ca. 3 (Fileti et al., 2009). However, how to get bromelain from pineapple peel with high activity has been still a problem.

Gemini surfactant is made up of two hydrophilic head groups, two hydrophobic chains, and a spacer linking the two head groups via covalent bonds. It has been reported that the spacer shows an obvious effect on the interaction between protein and gemini surfactant in aqueous solution (Amiri et al., 2012; Mir, Khan, Khan, Rather, & Dar, 2010). However, gemini surfactant has been rarely used in protein extraction (Dong et al., 2013). In the present paper, we will use reverse micelles from gemini surfactant C_{12}-8-C_{12}·2Br (shown in Scheme 1A) and its counterpart monomer – conventional surfactant DTAB (dodecyl trimethyl ammonium bromide, shown in Scheme 1B) to extract bromelain from pineapple peel, with a purpose to study whether and how the spacer of gemini surfactant works in bromelain extraction. Since pineapple peel is generally disposed or used as compost, this study should be interesting and expected to lead to value addition to the peel.

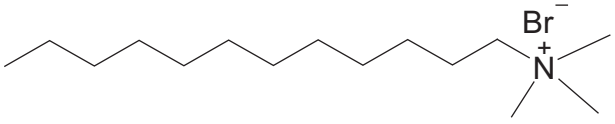
2. Materials and methods

2.1. Materials

Gemini surfactant C_{12}-8-C_{12}·2Br (Scheme 1A, Molecular Weight: 698.86) was prepared according to the reference (Zana, Benrraou, & Rueff, 1991). Its purity (at least 98%) was checked by NMR and elemental analysis (Zana et al., 1991). DTAB (Scheme 1B, Molecular Weight: 308.34) was bought from Amresco Co. Solon, OH (99% purity). *n*-Hexane and 1-hexanol were bought from Chinese Chemicals (analytical grade, Sinopharm chemical reagent Co. Ltd, Shanghai, China). Matured pineapple fruits (*Ananas comosus* L. Merryl, Produced in Hainan, P.R. China) used in this study were purchased from the local market. The water used was deionized.



A



B

Scheme 1. Structures of C_{12}-8-C_{12}·2Br (A) and DTAB (B).

The buffer used in the forward extraction was prepared by disodium hydrogen phosphate (10 mM)/citric acid (pH ⩽ 8.0) and glycine (10 mM)/sodium hydroxide (pH > 8.0). The stripping solution in the backward extraction was prepared by acetic acid/sodium acetate (10 mM, pH ⩽ 5.7) and disodium hydrogen phosphate/-sodium dihydrogen phosphate (10 mM, pH: 5.8–8.0).

2.2. Methods

2.2.1. Preparation of reverse micelle

The reverse micelle was prepared by known quantities of *n*-hexane, 1-hexanol, surfactant and water. The volume ratio of 1-hexanol to *n*-hexane is 1:9.

2.2.2. Preparation of crude extract

The peel was manually separated from the fruit. For the preparation of crude extract, a known quantity of peel was crushed along with extraction buffer (0.04 M sodium phosphate buffer of pH 6.0, containing 5 mM EDTA) at 1:1 ratio for 5 min and then filtered through a cheese cloth. The filtrate was centrifuged at 10,000 rpm (Allegra 64R centrifuge, Beckman Coulter, USA) for 25 min and the supernatant (i.e. crude enzyme extract) obtained was used for reverse micellar extraction experiments.

2.2.3. Reverse micellar extraction

There are two steps in the liquid–liquid reverse micellar extraction process: forward extraction and backward extraction. Aqueous (crude enzyme extract/buffer) and organic (reverse micelle) phases were mixed with a volume ratio of 1:1 and the mixture was vortexed for 25 min at room temperature. Phase separation was done by centrifuging at 14,000 rpm (Allegra 64R centrifuge, Beckman Coulter, USA) for 30–50 min. Backward extraction was carried out by mixing the organic phase obtained from forward extraction with an equal volume of a fresh aqueous phase, also termed as stripping phase (buffer of known pH). The mixture was then centrifuged at 14,000 rpm (Allegra 64R centrifuge, Beckman Coulter, USA) for 30–40 min, followed by phase separation. The aqueous phase obtained after backward extraction was analyzed for bromelain activity and total protein content.

2.2.4. Protein activity determination

Bromelain activity was determined according to the casein digestion unit (CDU) method using casein (0.6%) as substrate in the presence of cysteine and EDTA (Murachi, 1976). The assay was based on proteolytic hydrolysis of casein substrate. The absorbance of the clear filtrate (solubilized casein) at 275 nm was measured using UV–vis spectrophotometer (Shimadzu UV-160). One unit of bromelain activity was defined as 1 μg of tyrosine released in 1 min per ml of sample when casein was hydrolyzed under the standard conditions of 37 °C and pH 7.0 for 10 min.

2.2.5. Protein content measurement

Protein content in aqueous phase was determined by the dye binding method (Bradford method, using BSA as standard) (Bradford, 1976). The sample analyses were performed against respective blank solutions. The overall extraction efficiency (OEF, %), activity recovery (AR, %) and purification fold (PF) were