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Primary recovery of miraculin from miracle fruit, *Synsepalum dulcificum* by AOT reverse micellar system

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ABSTRACT

Miracle fruit, *Synsepalum dulcificum*, contains a glycoprotein known as miraculin. After consuming this glycoprotein, sour foods taste sweet and the effect lasts for up to 4 h. With increasing demand for natural and "low-calorie" sweeteners, the use of miraculin as an additive is increasing enormously in the food, medicine and cosmetic industries. In this study, we used reverse micelles formed from a sodium di (2-ethylhexyl) sulfosuccinate (AOT)/isooctane system to purify miraculin from *S. dulcificum*. We studied factors affecting purification performance, such as surfactant (AOT) concentration and the pH of the crude during forward extraction. During backward extraction, we examined the effects of NaCl concentration, the pH of the aqueous phase and addition of isopropanol. We found that 0.1 mol/L AOT/isooctane solution mixed with crude extract at pH 8 during the forward extraction stage and 0.5 mol/L NaCl solution at pH 11 during the backward stripping stage were optimal purification conditions, from which 22% miraculin was recovered with a purity of 94.8%.

1. Introduction

Synsepalum dulcificum, a shrub native to tropical West Africa, produces red berries that have the unusual ability to modify a sour taste into a sweet taste (Wong & Kern, 2011). William Freeman Daniell (1852, cited in Kurihara & Beidler, 1968) first reported this unusual property of miracle fruits in the scientific literature. The active ingredient in the berries, miraculin, is a taste-modifying protein that causes the sour taste components such as citric and ascorbic acids to be perceived as sweet after consumption in the mouth (Zhang & Sun, 2001). The mechanisms behind the sweet-inducing activity of miraculin have not yet been identified but histidine residues in miraculin have been linked to its taste-modifying activity (Ito et al., 2010; Paladino, Colonna, Facchiano, & Costantini, 2010). Twenty micrograms of chromatographically purified miraculin produces a marked increase in sweetness of lemon and concomitantly a marked diminution of sourness (Giroux & Henkin, 1974). However, the activity of miraculin is prone to be destroyed when the solution is boiled or exposed to a high concentration of organic solvents at room temperature. The activity was also decreased at high pH (pH > 12) and is greatly decreased (pH < 2.5) (Kurihara & Beidler, 1968). Although a lot of experiments have been carried out to explore the structure and mechanism of miraculin and to study the actual function of miraculin, the purification procedures for miraculin nowadays are thought to be labor-intensive, time-consuming and costly.

Miraculin has been purified using various solvents, from polar to non-polar and from non-polar to polar in succession (Inglett, Dowling, Albrecht, & Hoglan, 1965). However, the solvent extraction method is tedious and the purity of miraculin is relatively low. High purity miraculin was first obtained by ion-exchange chromatography, in which it has been characterized as a basic glycoprotein (Kurihara & Beidler, 1968). Theerasilp and Kurihara (1988) also

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claimed that 97% of miraculin can be purified by ammonium sulfate fractionation, followed by two chromatographic steps (CM-Sepharose ion-exchange chromatography and ConA-Sepharose affinity chromatography). Although relatively high purity of miraculin can be obtained, this method is considered to be time-consuming, inefficient and costly. This is because of the ultrafiltration following ammonium sulfate fractionation and both chromatographic steps. Miraculin purified by ion-exchange column chromatography contains 63 g/L carbohydrate and 144 g/L nitrogen, and the molecular weights are 43 and 28 kDa in dimeric and monomeric forms, respectively (Zhang & Sun, 2001). The amino acid and carbohydrate compositions of miraculin have also been identified (Theerasilp et al., 1989). Recently, immobilized-metal affinity chromatography (IMAC) has been used to purify miraculin from native miracle fruit and transgenic tomato fruit (Duhita, Hiwasa-Tanase, Yoshida, & Ezura, 2009, 2011). Although IMAC is a simple and efficient method, chromatographic separations are considered to be expensive and difficult to scale-up beyond laboratory scale (Zainuddin, Mohamed, & Siti, 2007). Thus, an efficient purification method such as reverse micelle system was established to overcome this problem.

Reverse micelles are water-in-oil microemulsion droplets stabilized by surfactants in polar solvents, and have been widely studied for their potential to extract proteins in liquid–liquid extraction processes (Krei & Hustedt, 1992; Luisi, Giomini, Pileni, & Robinson, 1988; Lye, Asenjo, & Pyle, 1994). This method has been widely noted for its low energy requirements and also shows great potential for large-scale application and use in continuous separation of biological substances (Zainuddin et al., 2007). Many proteins have been successfully extracted by reverse micelles without excessive loss of bioactivity (Krei & Hustedt, 1992; Liu, Xing, Shen, Yang, & Liu, 2004; Shiomori, Ebuchi, Kawano, Kuboi, & Komasawa, 1998; Zhao et al., 2010). In the reverse micelles method, the protein is first extracted from the aqueous phase into the reversed micelle phase under certain conditions (forward extraction). The protein is then recovered from the organic phase by extracting the reversed micelle phase with a second aqueous phase (backward stripping) (Dekker, Hilhorst, & Laane, 1989).

The main objective of this study was to investigate the feasibility of using the reverse micelle extraction method to extract miraculin from *S. dulcificum*. The effects of various factors that might influence performance were evaluated, such as crude pH, surfactant concentration during forward extraction and pH, isopropanol concentration, and salt concentration in the aqueous phase during backward stripping. The significant factors were also optimized to enhance extraction yield and product purity.

2. Materials and methods

2.1. Miracle fruits

Fresh miracle fruits, *S. dulcificum*, were obtained from a local farm (Nilai Nursery, Nilai, Malaysia). The skin and seeds of the fruits were separated manually using a knife and the pulp was freeze-dried, then ground into a fine powder using a blender. The pulp powder was kept at −30 °C prior to use in the extraction and purification procedures.

2.2. Chemicals

Sodium di (2-ethylhexyl) sulfosuccinate (AOT) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. Isooctane was purchased from Systerm-ChemAR (Selangor, Malaysia). Bradford reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Sodium chloride was purchased from Amresco (Solon, OH, USA). Isopropanol was obtained from R&M Chemicals (Essex, UK). Miraculin standard (~95% purity) was purchased from American Peptide Co. (Sunnyvale, CA, USA).

2.3. Preparation of miraculin extract

The extraction of miraculin was carried out according to the method described by Theerasilp and Kurihara (1988) with some modifications. In this method, 4 g of lyophilized pulp powder was suspended with 40 mL of water and homogenized for 2 min. The homogenate was centrifuged at 12,000 × g for 30 min. After discarding the supernatant, the sediment was homogenized for 2 min in 30 mL of 0.5 M NaCl solution (pH 6.8). The homogenate was clarified by centrifugation at 12,000 × g for 20 min and the colorless supernatant at pH 3 was stored at −30 °C.

2.4. Forward extraction

We performed the forward extraction and backward stripping according to the method described by Liu et al. (2004) with some modifications. Briefly, the organic phase was prepared by dissolving various concentrations of AOT (0.03, 0.05, 0.1 and 0.2 mol/L) in isooctane. The pH of the crude was adjusted with either 1 mol/L NaOH or 1 mol/L HCl. The isoelectric point (pI) of miraculin is 9 and miraculin was reported to be stable during storage between pH 2.5 and 12 (Kurihara & Beidler, 1968). Based on this information, the pH of the crude in the aqueous phase was adjusted to various pH values ranging from 3 to 10 to avoid miraculin precipitation and loss of activity during the experiments. Equal volumes (0.5 mL each) of aqueous and organic solutions were mixed gently in a tube and the mixtures were then shaken mechanically for 10 min. The mixtures were then centrifuged at 4000 × g for 5 min to reach a clear separation of the two phases.

2.5. Backward stripping

The reversed micellar solution with loaded protein was added to an equal volume of aqueous solution which consisted of 0.02 mol/L phosphate buffer at the required pH (7, 8, 9, 10 and 11) in a tube. The required concentrations of NaCl (0, 0.5, 1, 1.5 and 2 mol/L) and isopropanol (0, 50, 100, 150 and 200 mL/L) were also added to the tube. The organic phase and fresh aqueous phase were shaken for 20 min. The mixtures were then centrifuged at 4000 × g for 5 min to reach a clear separation of the two phases. The total protein in the stripping aqueous solution was determined.

2.6. Total protein assay

The total protein concentration in the crude sample was determined using the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. Ten μL of the sample was added to 200 μL of the diluted dye reagent (1 part dye reagent concentrate with four parts distilled, deionized water) in a microtiter plate and incubated at room temperature for at least 5 min. The absorbance was measured at 595 nm against a reagent blank.

2.7. Reverse-phase high performance liquid chromatography analysis

The miraculin concentration in the sample was analyzed using reversed phase high performance liquid chromatography (RP-HPLC, Separations Module 2695, Waters, Milford, MA) on a C-18 column (7 μm, 8 × 300 mm) according to the method described by Duhita et al. (2009) with some modifications. Sample (40 μL) was injected into the column and equilibrated with 1 mL/L trifluoroacetic acid (TFA) in water. The column was eluted using a linear

gradient of acetonitrile with increasing concentration from 150 mL/L to 700 mL/L, and the flow rate was fixed at 1 mL/min. The absorbance of the sample was read at 280 nm. Miraculin standards were prepared at concentrations ranging from 100 to 1000 mg/L. The relationship between miraculin concentration (mg/L) and peak area (AU min) was observed as 0.00013 mg miraculin/L/peak area. The purity of the peak was analysed based on the percentage of total peak area using Empower software (System Software, Waters Co.) for data acquisition and analysis.

2.8. SDS-PAGE and silver staining

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Bio-Rad electrophoresis unit as described by Laemmli (1970). The acrylamide gel was prepared as a 120 mL/L resolving gel and a 45 mL/L stacking gel. Protein samples recovered from the top phase were concentrated and precipitated using 100 mL/L trichloroacetic acid (TCA) solution, which removed the salts that affect the electrophoresis process. The pellets were resuspended in denaturing buffer (0.1 mol/L Tris–HCl pH 6.8, 40 g/L SDS, 100 mL/L 2-mercaptoethanol, 200 mL/L glycerol and bromophenol blue). The electrophoresis was run at 110 V and 36 mA for 75 min. The gel was stained with a buffer solution consisting of 0.5 mL/L Coomassie Brilliant Blue G-250, 300 mL/L methanol and 100 mL/L acetic acid. After destaining, protein bands were visualized using the same buffer solution in the absence of Coomassie Brilliant Blue. The gel was then stained with a PageSilver™ silver-staining kit (Fermentas, St. Leon-Rot, Germany).

2.9. Miraculin sensory analysis

The taste-modifying activities of miraculin were evaluated by five subjects by tasting 0.2 mL of partially purified miraculin solution and held in mouth (tongue) for 5 min. Subsequently, each subject expectorated out the partially purified miraculin solution, washed the mouth with distilled water and sipped 5 mL of 0.02 mol/L citric acid and finally evaluated the presence of taste-modifying activities in the purified miraculin.

2.10. Definition

$$\text{Specific miraculin in crude} = \frac{\text{Miraculin in crude extract}}{\text{Total protein in crude extract}}$$

$$\text{Specific miraculin in back extraction aqueous phase} = \frac{\text{Miraculin in back extraction aqueous phase}}{\text{Total protein in back extraction aqueous phase}}$$

$$\text{Purification factor} = \frac{\text{Specific miraculin in back extraction aqueous phase}}{\text{Specific miraculin in crude}}$$

$$\text{Yield of miraculin } (\%) = \frac{\text{Miraculin in back extraction aqueous phase}}{\text{Miraculin in crude}} \times 100$$

$$\text{Yield of total protein } (\%) = \frac{\text{Total protein in back extraction aqueous phase}}{\text{Total protein in crude}} \times 100$$

3. Results and discussion

3.1. Effect of AOT concentration during forward extraction

The effect of AOT concentration on protein recovery by reverse micelles is shown in Fig. 1a. AOT based reverse micellar phases was selected in the present study as the results indicated that miraculin can be easily extracted into reverse micelle when AOT was used as surfactant. This could be due to AOT which is an anionic surfactant, is suitable for purifying proteins with low molecular weight and high isoelectric point. On the other hand, cationic surfactant such as CTAB generally used to separate proteins with high molecular weights and low isoelectric points was not suitable in this study (Yin et al., 2011). Thereof, in the present study, AOT was used for separation of miraculin which also has low molecular weight (only 28 kDa) and high isoelectric point value of 9. A similar result was reported by Li et al. who used AOT to separate low molecular weight and high isoelectric point proteins such as resistance-like protein P–B and pentatricopeptide repeat containing protein (Li et al., 2012).

Protein recovery was increased marginally from 37% to 50% as AOT concentration increased from 0.03 to 0.2 mol/L, while miraculin recovery was increased from 47% to 63% as AOT concentration increased from 0.03 to 0.1 mol/L, and remained constant (63%) at 0.2 mol/L. An increase in AOT concentration leads to an increase in the aggregation number of AOT and the size of reverse micelles (Goklen & Hatton, 1987; Pires, Aires-Barros, & Cabral, 1996). Subsequently, the increase in size would lead to a decrease in steric hindrance of reverse micelles (Liu et al., 2004). These effects contributed to the increasing amount of protein extracted. For AOT concentrations ranging from 0.1 to 0.2 mol/L, miraculin recovery remained constant, while protein recovery increased from 49% to 54%, indicating that the latter increase was caused by impurities. A similar trend was reported by Liu et al. (2004) and Zhao et al. (2010), for reverse micelles extraction of nattokinase and soybean protein, respectively. The highest purification factor (1.27) was obtained at 0.1 mol/L AOT, which was further confirmed by the HPLC result, giving a purity of 44%. Therefore, 0.1 mol/L AOT was used in subsequent experiments.

3.2. Effect of crude pH during forward extraction

The effect of crude pH on the partitioning behavior of reverse micelles is shown in Fig. 1b. As the pH of the crude increased from 3 to 6, protein recovery significantly decreased from 50% to 32%. Protein recovery was further decreased to 17% at pH 7 and declined slightly to 16% at pH 8. Protein recovery at crude pH 10 was only 1.5%. A similar trend was also observed with a change in pH from 3 to 6, where protein recovery decreased from 61% to 25%. Miraculin recovery was stable at pH values ranging from 6 to 8 and miraculin was not recovered at pH 10. Because of the rapid decrease in

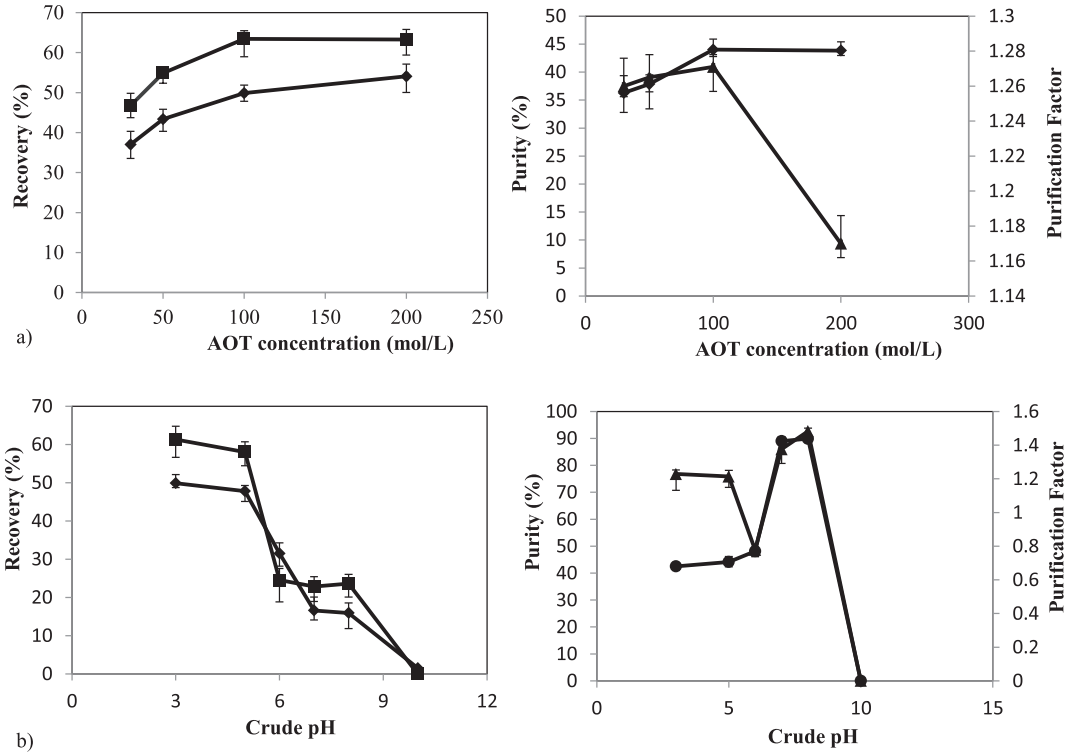

Fig. 1. (a) Effect of different AOT concentrations with crude at pH 3 and (b) different crude pH values on purification efficiency of miraculin in the forward extraction stage. The results were expressed as the means of triplicate readings with standard deviations represented in error bars. Symbols: protein recovery (◆) miraculin recovery (■) purification factor (▲) purity (●).

miraculin recovery, the purification factor was lowest at pH 6 (0.778), while the highest purification factor was obtained at pH 8 (1.48). From HPLC analysis, the purity obtained at pH 6 was 48% and the purity was increased markedly to 89% and 90% at pH 7 and 8, respectively. From the HPLC chromatogram, the removal of main protein contaminants was observed at pH 7 and 8 (Fig. 2A).

The crude pH, which affects the aqueous phase pH, determines the ionization state of the surface-charged groups on the protein molecule. Attractive electrostatic interactions between the protein molecule and the surfactant head groups, which form the internal surface of the reversed micelle, will occur if the overall charge of the protein is opposite to the charge of the surfactant head groups (Dekker et al., 1989). AOT, which is an anionic surfactant, forms a micellar structure, with the negative charge created by the surfactant head groups (Goklen & Hatton, 1987). When the pH is lower than the protein's pI value, the protein will provide a positive charge at its surface, resulting in the development of electrostatic interactions between the charged amino acid residues on the protein surface and the electrical double-layer created by the surfactant head groups (Lye et al., 1994). Clearly, these interactions occurred when crudes of pH 3 to 8 (lower than the miraculin pI), were used, causing miraculin to be extracted into the solvent phase in the reverse micelle. Otherwise, miraculin would be stripped from reverse micelle, which is the main reason why miraculin could not be extracted when crude at pH 10 was used. The results from this study also showed that the pI values of most protein contaminants were below 7 or 8, and the relative values of pI and pH determined the amount of protein recovery using AOT as a surfactant (Zhou & Weng, 2006).

In accordance with the general conclusions from the forward extraction studies, the condition of 0.1 mol/L AOT/isooctane mixed with crude at pH 8 was applied for subsequent studies on backward transfer.

3.3. Effect of isopropanol concentration during backward stripping

According to previous work, accomplishing backward extraction of protein from the solvent phase to the aqueous phase may be difficult. It was reported that the rate of stripping is about three orders of magnitudes slower than forward extraction (Dungan, Bausch, Hatton, Plucinski, & Nitsch, 1991). Considering the traditional method of backward extraction for nattokinase stripping, Liu et al. (2004) found that backward extraction was rather difficult when changing the pH and salt concentration. When different concentrations of isopropanol were added to the aqueous phase, 150 mL/L isopropanol strongly promoted the backward transfer of nattokinase (2004). A similar result was reported by Carlson and Nagarajan (1992), where nearly complete backward transfer of porcine pepsin and 70% backward transfer of bovine chymosin were obtained after the addition of 100–150 mL/L isopropanol. When small amounts of alcohols are added, they may affect the micellar–micellar interactions in the reverse micelle system. The hydrophobic hydrocarbon group suppresses the intermicellar attractive interaction in proportion to their carbon chain length, while the hydrophilic hydroxyl group enhanced the interaction (Hong & Kuboi, 1999). The addition of isopropanol probably leads to an increase in the attractive interactions between reverse micelles and the arrangement of AOT molecules in isooctane, and this causes instability in the reverse micelles and also the exclusion of protein from reverse micelles (Liu et al., 2004).

Although protein recovery increased slightly from 15% to 17% with increasing isopropanol concentrations from 0 to 200 mL/L, miraculin recovery (ranging from 23% to 24%) was not significantly improved (Fig. 3a). In addition, the purity of miraculin (ranging from 44% to 45%) as shown by HPLC chromatography was not significantly improved. Results from this study showed that nearly all proteins and miraculin were stripped without addition of

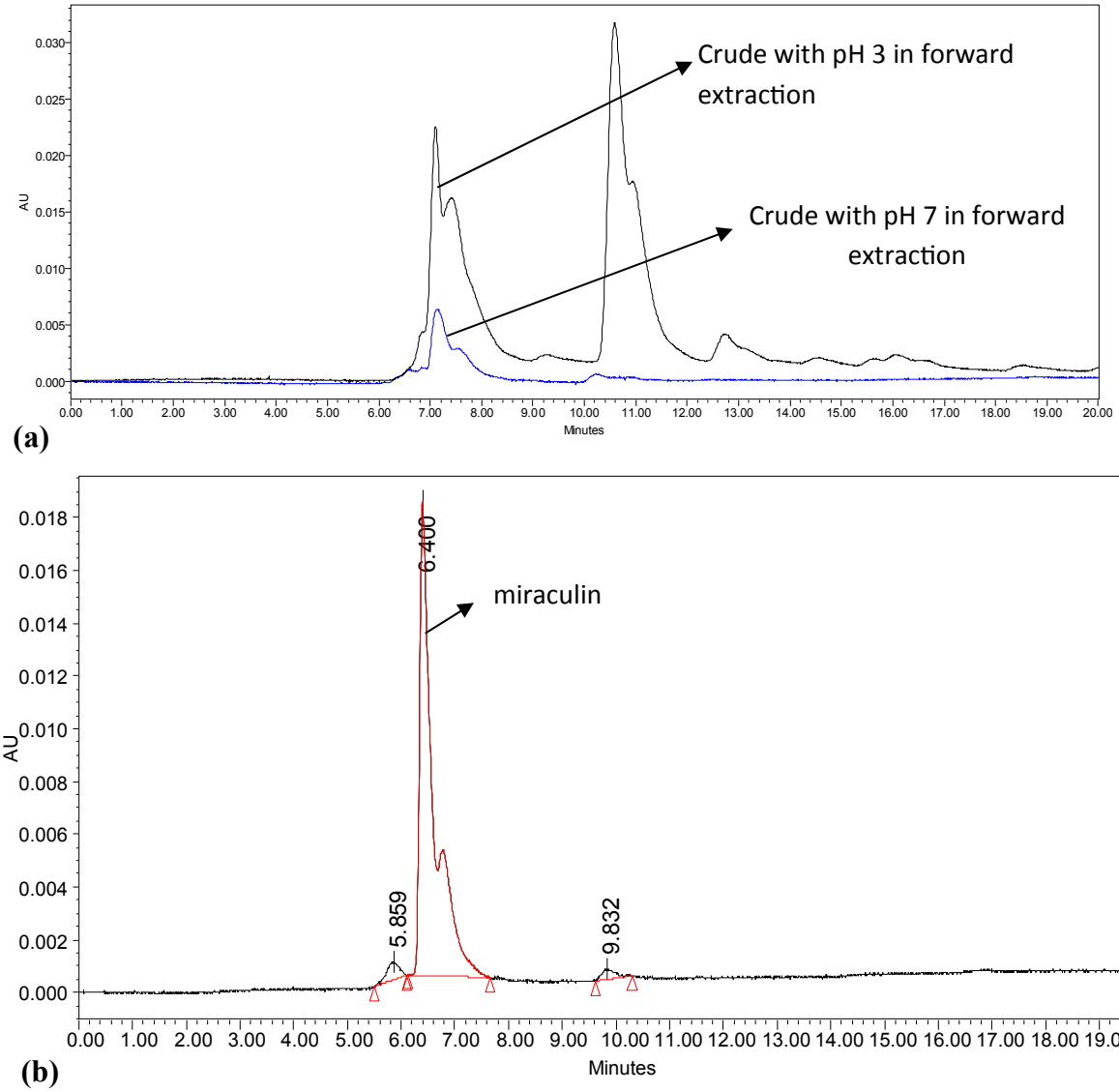

Fig. 2. (a) HPLC chromatography of miraculin-modified crude at pH 3 and pH 7 during forward extraction stage. (b) RP-HPLC chromatogram of miraculin extraction using reverse micelles under optimal conditions. Separation conditions: stationary phase: C-18 column (7 μm, 8 × 300 mm). Mobile phase A: 1 mL/L trifluoroacetic acid (TFA); mobile phase B: 1000 mL/L acetonitrile. Linear gradient with increasing mobile phase B concentration from 150 mL/L to 700 mL/L, and the flow rate was fixed at 1 mL/min.

isopropanol, or that isopropanol had little effect in this case. Thus, isopropanol was not added in subsequent experiments.

3.4. Effect of pH during backward stripping

The mechanism by which pH affects extraction performance during backward stripping was similar to that observed for forward extraction. Briefly, the electrical interactions between the charged AOT head groups and the protein surface implied that the pH in the added aqueous phase should be higher than the pI of miraculin. The same negative charge on the protein surface and on the surfactant head groups would lead to the stripping of protein from solvent phase to aqueous phase. The results collected and shown in Fig. 3b also indicate a similar conclusion. No miraculin was observed through HPLC under pH 7 and 8, and little miraculin was stripped at pH 9 (miraculin recovery was 1.2%), with a purification factor of 0.1. Nevertheless, miraculin recoveries markedly increased to 22% and 23% when the pH values of the modified aqueous phase were adjusted to pH 10 and 11, respectively. Protein recovery and the purification factor at pH 10 were close to that at pH 11. However, the purity indicated by HPLC at pH 11 was 94%, which was relatively higher than that obtained at pH 10 (90%). Thus, pH 11 was considered the optimal pH of the aqueous phase during backward extraction.

3.5. Effect of salt concentration during backward stripping

Fig. 3c illustrates the effect of salt concentration during backward stripping on the performance of protein and miraculin extraction. The protein recovery increased from 13.5% to 16.5% with the addition of NaCl in the range 0.5–2 mol/L. Change in salt concentration affects both the size and hydrophobicity of reverse micelles and hydrophobicity of proteins (Andrews, Pyle, & Asenjo, 1994). The increase in ionic strength with increasing NaCl concentration led to a decrease in electrostatic interactions between AOT reverse micelles and protein, which promoted backward transfer (Liu et al., 2004). When increasing the NaCl concentration, the miraculin recovery did not follow the same trend as protein recovery but remained constant at about 22%. This strongly implies that miraculin had been completely stripped under this condition.

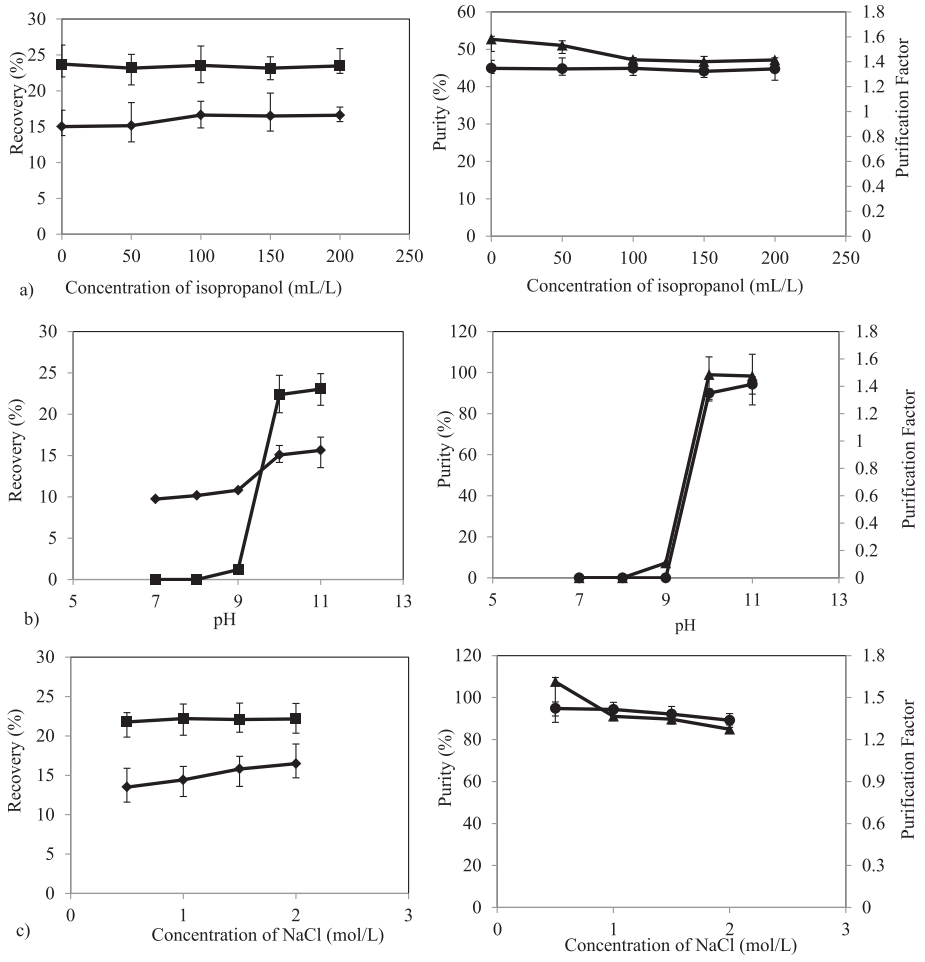

Fig. 3. (a) Effect of different isopropanol concentrations (b) different pH values (c) different salt concentrations in the aqueous phase on purification efficiency of miraculin in the backward extraction stage. The results were expressed as the means of triplicate readings with standard deviations represented in error bars. Symbols: protein recovery (◆) miraculin recovery (■) purification factor (▲) purity (●).

This result also led to a decrease in purification factor from 1.6 to 1.3, confirmed by purity analysis using HPLC (reduced from 95% to 89%).

A turbid aqueous phase was observed during the stripping stage when NaCl was omitted (data not shown). This phenomenon has been observed by many researchers for soluble and non-soluble proteins in aqueous buffers. However, this cloudy aqueous phase may affect protein release. For example, Marcozzi, Correa, Luisi, & Caselli (1991) found that α-chymotrypsin could not be purified at very low ionic strength (NaCl/KCl concentrations $\leq$ 0.01 mol/L) because the solution became cloudy. During pepsin purification, the lower phase was cloudy when salt was not added to the aqueous system (Carlson & Nagarajan, 1992). However, an increase in salt concentration resulted in the formation of a clear lower phase, which did not promote the release of pepsin. During lactoferrin purification, the phases turned cloudy and the extraction efficiency was greatly reduced in the system without the addition of NaCl (Anjana, Kumar, Sirivansh, Suryaprakash, & Kumar, 2010). This behavior suggests that NaCl should be used during backward stripping. Under different NaCl concentrations (0.5–2 mol/L), miraculin recovery varied from 21.8% to 22.2%.

3.6. Determination of the taste-modifying activities and purity of miraculin after reverse micelle treatment

The taste-modifying activities of purified miraculin were

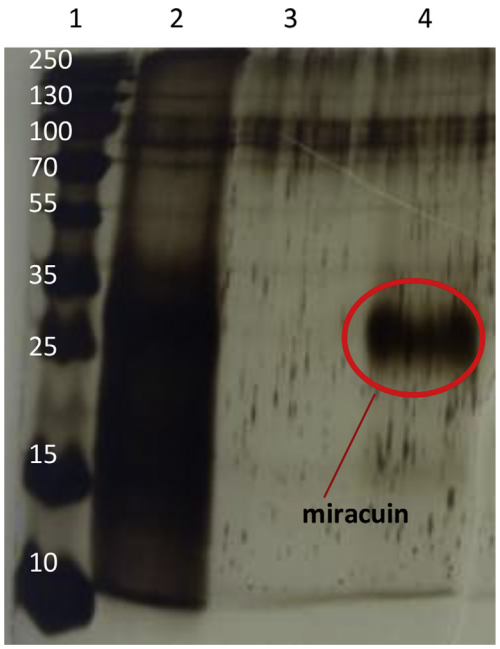

Fig. 4. SDS-PAGE silver-staining gel. Lane 1: protein marker, Lane 2: crude, Lane 3: blank, Lane 4: purified miraculin in the aqueous phase after reverse micelle treatment.

Table 1
Total protein content in crude and aqueous phase, miraculin content in crude and aqueous phase, specific miraculin in crude and aqueous phase, purification factor, recovery yield and purity of miraculin obtained under optimal reverse micelle conditions.

Total protein content in crude (mg/L)	Miraculin content in crude (mg/L)	Total protein content in aqueous phase (mg/L)	Miraculin content in aqueous phase (mg/L)	Specific miraculin in crude	Specific miraculin in aqueous phase	Purification factor	Yield (%)	Purity (%)
330.0 ± 11.6	190.6 ± 9.4	44.5 ± 2.8	41.5 ± 2.0	0.6 ± 0.0	0.9 ± 0.1	1.6 ± 0.1	22 ± 4	94.8

All experiments were performed in triplicates. ± is standard deviation of triplicate data. Specific protein, purification factor and yield were calculated with the average values.

determined by sensory analysis. The results showed that the purified miraculin successfully changed the sourness of citric acid into sweetness (data not shown). The purity of miraculin obtained from reverse micelles performed under optimal conditions was 94.8%, as determined by HPLC (Fig. 2B). High purity miraculin was eluted at retention times of 6.4 min, with some minor peaks found at retention times of 5.8 min and 9.8 min. The realization of recovery of miraculin in the aqueous phase after reverse micelle treatment in a partially purified form was confirmed by SDS-PAGE with silver staining (Fig. 4). Interestingly, a small peak appeared at a retention time of 7 min, near the major peak. This result differs from those reported by Theerasilp and Kurihara (1988) and Duhita et al. (2009), where only one peak was observed during HPLC. However, silver staining under reducing conditions proved that miraculin occupied the major band in the gel. The molecular weight of miraculin varies from 25 (Theerasilp et al., 1988) to 28 kDa (Zhang & Sun, 2001) under reducing conditions, indicating that miraculin was successfully purified in this study. This is probably because miraculin shows dimeric and tetrameric structures after reverse micelle treatment (where both tetrameric and dimeric miraculin in the crude state show taste-modifying activities (Igeta, Tamura, Nakaya, Nakamura, & Kurihara, 1991)), or protein aggregation may have occurred during HPLC, which is rather common in protein solutions (Grinberg, Blanco, Yarmush, & Karger, 1989).

Under optimal reverse micelle extraction conditions (crude at pH 8 as the aqueous phase and 100 mmol/L AOT/isooctane as the solvent phase during forward extraction; 0.5 mol/L NaCl solution at pH 11, without isopropanol, as the aqueous phase during backward stripping), the maximum purification factor, purity and total purified miraculin were 1.6, 94.8% and 41.5 mg/L, respectively (Table 1). Thus, reverse micelle extraction could be used as a partial purification step of the miraculin from miracle fruit. In this study, the functionality or structural state of the purified protein remains unknown as partially purified miraculin could not be accessed in functionality or structural study. This is a subject for future study after a further purification step such as size exclusion purification or gel-filtration is performed to obtain pure miraculin.

4. Conclusions

Reverse micelle extraction can be applied as a simple and convenient process for the purification of miraculin from miracle fruit, *S. dulcificum*. Optimization of purification parameters was required to improve total miraculin purified, purification factor and purity. Miraculin can be extracted under a wide range of crude extract pH values but pH 8 gave the highest purification factor and purity of miraculin. It should be noted that the amount of miraculin extracted at crude pH 3 was nearly three times that at pH 8, with a decreasing purification factor and purity. Optimizing pH had more effect than optimizing the salt concentration. During the backward stripping stage, it is not necessary to add isopropanol because similar miraculin recoveries were obtained using different isopropanol concentrations. Results of HPLC and SDS-PAGE silver staining analyses also indicated that high purity miraculin could be obtained by purification using reverse micelles.

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