

# **Effect of $\alpha$ -amylase pretreatment on protein extraction from defatted roselle seed**

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## **Abstract**

Roselle (*Hibiscus sabdariffa* L.) seed, commonly discarded as waste, is known as a good source of protein. Roselle seed protein is mostly composed of albumin, globulin and glutelin, covering 82.31% of total nitrogen in roselle seed, and soluble in water, salt and dilute alkalizes solution, respectively. Roselle seed protein was reported to be highly soluble (90-95%) at pH 9. However, oligosaccharides and phytic acids may extensively bind to protein and hence could significantly interfere protein isolation. Based on other studies,  $\alpha$ -amylase (E.C. 3.2.1.1) can be used to release the bound protein into solution and it has higher effect than other carbohydrates on the extraction of protein. Thus, it is advantageous to isolate protein from defatted roselle seed by using water and salt solution at pH 9. Two-steps protein extraction using deionized water and salt solution at pH 9 resulted in 52.24% protein yield. This study found that the addition of  $\alpha$ -amylase pretreatment (1 800 unit/g DRSF for 6 h) resulted in 72.18% protein yield. Molecular weights of roselle protein, which has never been reported, range from slightly below 22 kDa to 95 kDa. Methionine is the main limiting amino acid. Roselle protein concentrate obtained in this study is rich in glutamic acid (28.78%), arginine (10.21%), aspartic acid (10.11%) and leucine (6.36%)

**Keywords:**  $\alpha$ -amylase pretreatment, native-PAGE, protein extraction, roselle seed, SDS-PAGE

## INTRODUCTION

*Hibiscus sabdariffa* L. (roselle) is an annual plant that belongs to the family Malvaceae and is native to Africa and tropical areas such as India, Malaysia, Vietnam, Eastern Taiwan, and Thailand. Lately, roselle has become one of the interesting crops in Vietnam, about 400 ha of roselle are planted for medical purpose by The National Pharmaceutical Company No.2 (Codupha) (Hoàng, 2010). Roselle fruit comprises the calyx, which has been known as a good source of antioxidants and has wide applications in pharmaceutical and food industries. Roselle seeds, a waste from calyx production, were reported to be high in protein (31.02%), lipid (21.60%), and carbohydrates (36.37%) (El-Adawy & Khalil, 1994). The seed is considered as an excellent feed for livestock. In some countries, some products are created from roselle seed such as Furundu in Sudan (Yagoub et al., 2004) and Bikalga in Africa (Parkouda et al., 2008). In the other parts of Africa, ground roselle seeds are mixed in food and sometimes roasted as a substitute for coffee (Morton, 1987). A study on the biological value of roselle seed protein isolated indicated that roselle protein is suitable for human and animal nutrition (Al-Numair & Ahmed, 2008). High protein and dietary fiber contents in roselle seed showed a potential of lowering the total cholesterol and low density lipoprotein cholesterol levels in rat (Hainida et al., 2008b). However, after being removed from the calyx, most roselle seeds are discarded by manufacturers.

Studies on roselle protein showed that roselle seed has the potential as a food supplement for human and livestock after it is processed to reduce the negative effect of oligosaccharides and anti-nutrients in its protein content (Anhwange et al., 2006; Halimatul et al., 2007). The essential amino acids of roselle seed protein contain high level of lysine and tryptophan, thus roselle seed protein could be developed as an ingredient in functional food products (Hainida et al., 2008a). Therefore, with the increase in world protein demand, roselle seed can be a potential source of high quality

protein. After the oil removal, defatted roselle seed could be utilized as a great source of protein.

To date, there is only one published work on roselle protein isolate which reported the effect of factors such as pH, salt type and salt concentration on protein extraction from defatted roselle seed (Abu-Tarboush, 1995). A maximum protein yield of 66.20% was achieved at pH 11. However, high pH used may damage and change the quality of protein isolate. According to El-Adawy and Khalil (1994), roselle seed protein mostly comprises albumin, globulin and glutelin, account for 82.31% of total nitrogen in roselle seed. They also reported that roselle protein is highly soluble (90 - 95%) at pH 9. Thus, it is advantageous to isolate protein from defatted roselle seed by using water and saline solution at pH 9.

Earlier researches on the effect of enzyme-assisted hydrolysis on protein extraction from plant protein sources found that the hydrolysis of cell wall components resulted in an increase in the amount of extracted protein (Grossman et al., 2007; Mudgett et al., 1978). Defatted roselle seed generally contains 35.94% protein, 18.43% total dietary fiber, 2.37% phytic acid and 33.0% soluble carbohydrate (Abu-Tarboush & Ahmed, 1996; Anhwange et al., 2006; Hainida et al., 2008b). All oligosaccharides and phytic acids may extensively bind to protein and hence could significantly interfere with protein isolation. Yagoub et al. (2004) reported that roselle seed contains 28.96% total carbohydrates, and 63.23% of the total carbohydrates is starch. Enzymes which catalyze the hydrolysis of polysaccharide can be safely applied for the isolation of protein in food application.  $\alpha$ -Amylase (E.C. 3.2.1.1) has more significant effect than other carbohydrases on protein extraction from heat-stabilized defatted rice bran. It hydrolyze starch at  $\alpha$ -1,4-linkage and release the bound protein into solution (Tang et al., 2003).

The objective of this work was to investigate the effect of  $\alpha$ -amylase pretreatment on the extraction yield of protein from defatted roselle seed flour (DRSF).  $\alpha$ -Amylase

pretreatment was employed prior to solvent extraction using deionized (DI) water and NaCl solution at pH 9. Molecular weight and amino acid composition of the protein concentrate were also determined.

## **MATERIALS AND METHODS**

### **Materials**

Roselle seed was obtained from Binhthuan, Vietnam. The seed was sun-dried for 24 h, removed the impurities, ground and stored at 4 °C prior to use.  $\alpha$ -Amylase from *Aspergillus oryzae* was purchased from Sigma Aldrich (Switzerland). Amino acids standards were obtained from Sigma Aldrich (St. Louis, MO). All other chemicals used in this work were of analytical grade.

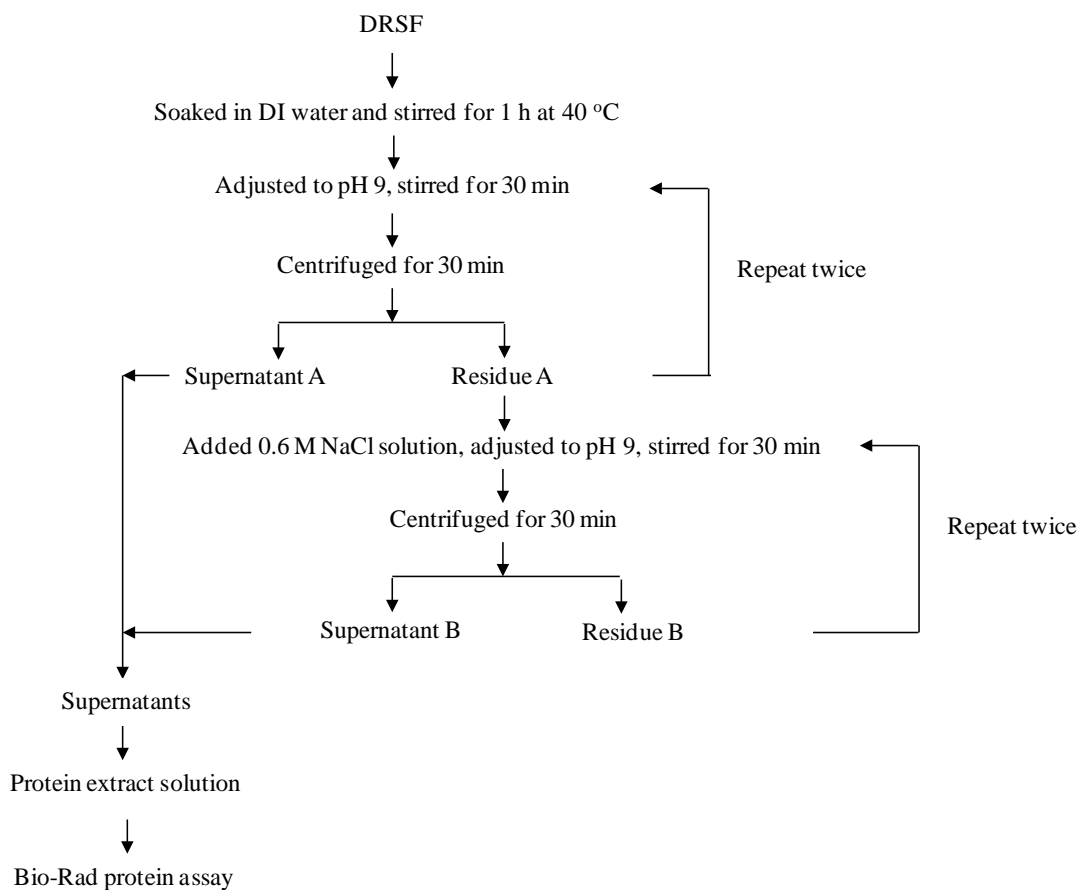
Roselle seed flour was defatted via soxhlet extraction with n-hexane as the solvent. The DRSF was stored at -20 °C prior to use.

### **Protein extraction**

#### *Protein extraction without $\alpha$ - amylase pretreatment*

Figure 1 is the flowchart of protein extraction. DRSF (5 g) was soaked in DI water (100 mL) at 40 °C. The pH of slurry was adjusted to 9 using 1 mol/L NaOH and continuously stirred for 1 h. The slurry was then centrifuged (4 500 g) for 30 min. Residue obtained (residue A) was re-extracted once with DI water under the same conditions as stated above.

After the extraction using DI water, residue A was then re-extracted twice by using 0.6 mol/L NaCl for 30 min and centrifuged (4 500 g) for 30 min. All supernatants were pooled together before subjected to Bio-Rad protein assay.



**Figure 1.** Flowchart of protein extraction

*Protein extraction with  $\alpha$ - amylase pretreatment*

A fixed amount (1 400, 1 800, 2 200 or 2 600 unit/g DRSF) of  $\alpha$ -amylase was added to a mixture of DRSF (5 g) and DI water (100 mL) at pH 6.25 and 50 °C (Tang et al., 2003). The mixture was stirred for a predetermined time (4, 6, 8 or 10 h). The treated-slurry was centrifuged (4 500 g) for 30 min and the supernatant was collected. The residue was extracted according to procedures described in Figure 1.

The yield of protein extraction was calculated by the equation (1).

$$\text{Yield of protein extraction (\%)} = \frac{\text{Amount of protein extracted}^a \text{ (g)}}{\text{Initial amount of protein in DRSF (g)}} \times 100 \quad (1)$$

<sup>a</sup> In process with pretreatment, the amount of protein extracted must be deducted with the amount of enzyme used.

Concentration of the protein in extract solution (supernatant) was determined by using Bio-Rad protein assay.

### **Molecular weight of roselle protein**

Supernatant obtained from protein extraction without using  $\alpha$ -amylase pretreatment was adjusted to pH 4, allowed to stand for 1 h at 4 °C to precipitate completely and centrifuged (4 500 g) for 30 min. The residue as wet protein concentrate was rehydrated and then freeze dried to obtain the roselle protein concentrate (RPC).

Sample for SDS-PAGE was prepared by dissolving RPC in DI water (10 mg/mL). SDS-PAGE gel electrophoresis was performed on 12% acrylamide resolving gel and 5% acrylamide stacking gel. Reduction of protein was performed with 2-mercaptoethanol at 100 °C for 10 min. After the reduction step, the RPC sample containing 80  $\mu$ g protein was applied onto the 12% acrylamide gel. Ten microliters of protein sample were loaded to the gel. Pre-electrophoresis was carried out at 70 V for 20 min and 110 V for 90 min. Protein bands were fixed in the gel by using acetic acid in ethanol and coomassie blue stain. For native PAGE, RPC was loaded on the gel without heating under non-denaturing and non reducing conditions.

### **Amino acids analysis**

The amino acids composition of RPC was analyzed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). RPC sample containing about 40 mg protein was hydrolyzed for 24 h at 110 °C using 6 N HCl (Hainida et al., 2008a). After hydrolysis,  $\alpha$ -aminobutyric acid (10% in sample) was added into the mixture as an internal standard. The total volume was adjusted to 50 mL with DI water. The mixture was successively filtered through a Whatman filter paper No.1 and a polyvinylidene difluoride (0.22  $\mu$ m) prior to derivatization of amino acids. Amino acid

sample (2  $\mu\text{L}$ ) was added to a mixture of  $\text{Na}_2\text{CO}_3$  solution (10  $\mu\text{L}$ , 0.5 M, pH 9.2), dansyl chloride (10  $\mu\text{L}$ , 10 mg/mL freshly prepared in acetone) and 8  $\mu\text{L}$  DI water. The reaction was carried out at 60  $^\circ\text{C}$  for 2 h, then stopped by adding 0.167 mol/L ethanolamine (10  $\mu\text{L}$ ) and incubated for another 30 min. DI water (160  $\mu\text{L}$ ) was added to the reacted solution and the mixture was centrifuged (24 000 g) for 15 min. The supernatant was collected and subjected to LC-ESI-MS analysis. Amino acid standards were derivatized as described above and used to determine the concentrations of amino acids.

The LC-ESI-MS system consists of an ultra-performance liquid chromatography system (Ultimate 3 000 RSLC, Dionex) and an electrospray ionization source of quadruple time-of-flight mass spectrometer (maXis HUR-QToF system, Bruker Daltonics). Separation was performed with reversed-phase liquid chromatography on a BEH C18 column (2.1 x 100 mm, Waters). Gradient elution was performed using mobile phase A (0.1% formic acid in DI water) and mobile phase B (0.1% formic acid in acetonitrile) with the following program: 0 - 0.5 min, 1% B; 0.5 – 5.5 min, 1-60% B; 5.5 – 6.0 min, 60-90% B; 6.0 – 7.5 min, 90% B; 7.5 - 8.0 min, 90-1% B. The flow rate was set at 0.4 mL/min with an injection volume of 2  $\mu\text{L}$ .

### **Protein, starch, fiber and ash analyses**

The Kjeldahl method was employed to determine the protein contents in DRSF and RPC. Starch and fiber content were measured following the method of Fabian et al. (2011). The ash content was determined according to Official Methods and Recommended Practice of the AOCS Ba 5a-49 (AOCS Official Method Ba 5a-49, 1997).



## **Statistical analysis**

Each experiment was performed at least in duplicate and statistical parameters determined were expressed as mean  $\pm$  standard deviation. All results were used for the statistical analysis using Minitab® 15.1.0.0.

## **RESULTS AND DISCUSSION**

### **Defatted roselle seed protein extraction**

Dry roselle seed contains 20.94% of crude oil. After defatting, hydrophilicity of the DRSF increases. This facilitates protein extraction by allowing water to enter the DRSF easier, thus causing particles of the DRSF to swell. Therefore, soaking is an important step for protein extraction as well as a required step for enzyme pretreatment of the DRSF.

The significant increase in the yield of protein extract ( $P = 0.045$ ) was observed (Table 1) by adjusting pH to 9. Regarding the aforementioned roselle protein composition and their solubility characteristics, 90-95% of roselle protein was soluble in water, saline and alkaline solution at pH 9 (El-Adawy & Khalil, 1994). Higher pH ( $\text{pH} > 9$ ) may change its nutritional characteristics because at alkaline conditions, cysteine and serine residues of protein may be converted to dehydroalanine, which may form lysinoalanine with the  $\epsilon$ -amino groups of lysine, and thereby it may turn the protein into a toxic compound and lose its nutritional value (Ansharullah et al., 1997). In the first step of extraction, DI water was employed to obtain albumin and some globulin since salts may occur naturally in plant tissues (Fabian et al., 2010). The residue was then extracted with saline solution which yielded globulin. An increase in protein yield may result from the pH adjustment to 9 (glutelin is highly soluble at pH 9) and due to increase of the albumin and globulin yields (Abu-Tarboush, 1995). However, the yield of protein extraction without  $\alpha$ -amylase pretreatment is low (52.24%).

Abu-Tarboush (1995) reported a protein extraction yield of 66.20% by using 0.4 mol/L CaCl<sub>2</sub> solution. This high yield may be ascribed to the Kjeldahl method used. The Kjeldahl method tends to over-estimate protein content as it also measured other nitrogen not associated with amino acids. On the other hand, Bio-Rad protein assay used in this study is a simple and accurate procedure to determine concentration of protein in solution (Kamizake et al., 2003; Owusu-Apenten, 2002).

**Table 1.** Yield of protein extraction with and without pH adjustment

Solvent	Yield of protein extraction (%) <sup>a</sup>	
	Without pH adjustment	pH 9
DI water	20.25±1.47	25.32±2.01
0.6 mol/L NaCl	26.47±1.32	26.92±1.66
Total	46.72	52.24

<sup>a</sup> Average of two independent experiments

Compositions (on dry weight basis) of the DRSF and the RPC are shown in Table 2. The DRSF contains high amount of protein (38.18%), starch (22.25%) and fiber (27.92%). Hainida et al. (2008a) and Abu-Tarboush (1995) reported similar protein contents in roselle seeds from Malaysia and Sudan, respectively. However, DRSF from Saudi Arabia with higher protein contents of 50.60% and 50.63% were reported by Al-Numair and Ahmed (2008) and Abu-Tarboush et al. (1997), respectively. On the other hand, Samy (1980) reported a smaller protein content of 27.50% in roselle seed from Egypt. In the current study, the protein content in RPC obtained by acid precipitation was 86.99% with a protein yield of 72.18%. Al-Numair and Ahmed (2008) reported a higher protein content in RPC (94.12%). However, they did not mention about the protein extraction yield.

**Table 2.** Compositions of DRSF and RPC (%)

	DRSF	RPC <sup>a</sup>
Protein	38.18±0.20	86.99±0.06
Starch	22.25±0.18	3.61±0.11
Fiber	27.92±0.11	4.22±0.10
Ash	8.50±0.04	4.90±0.06

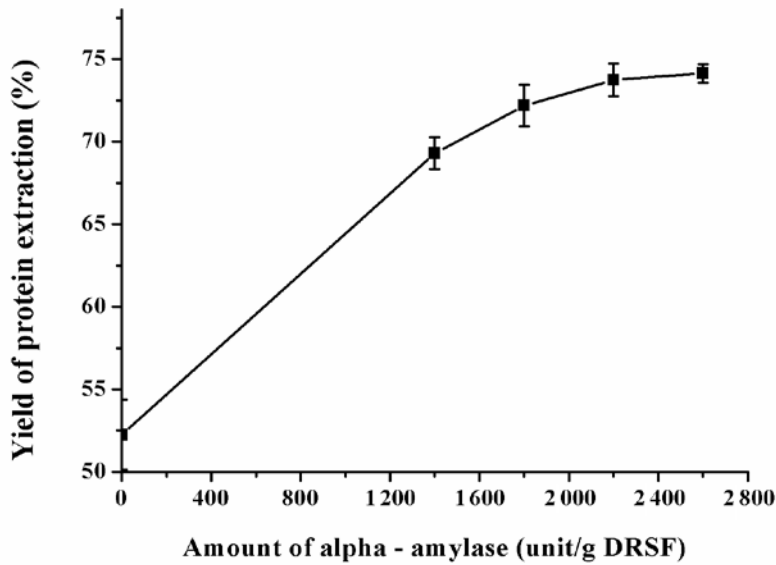
<sup>a</sup> The composition was measured from RPC, which was obtained in optimum condition of  $\alpha$ -amylase pretreatment (1 800 unit  $\alpha$ -amylase/g DRSF; 6 h hydrolysis time)

### Effect of $\alpha$ -amylase amount

In this study,  $\alpha$ -amylase was employed to enhance protein extraction yield. Tang et al. (2003) reported that  $\alpha$ -amylase was more effective than other carbohydrates for protein extraction. As shown in Figure 2, the yield of protein extraction increased to 72.18% with  $\alpha$ -amylase pretreatment (1 800 unit/g DRSF, 6 h) which is significantly ( $P = 0.001$ ) higher than that without  $\alpha$ -amylase pretreatment (52.24%). Tang et al. (2002) reported that the protein yield of heat-stabilized defatted rice bran can be increased from 11.7% to 37.3% by using higher amount of  $\alpha$ -amylase (2 200 unit/g heat-stabilized defatted rice bran). The higher amount of food-grade  $\alpha$ -amylase required in the work of Tang et al. (2003) was caused by contamination of enzyme by protease.

The effect of  $\alpha$ -amylase amount on protein yield is shown in Figure 2. At 1 400 unit/g DRSF, the yield of protein extraction was 69.29% and the yield increased to 72.18%, 73.72% and 74.13% when 1 800, 2 200 and 2 600 unit of enzyme per gram DRSF was utilized, respectively. It can be seen that protein yield changes insignificantly ( $P = 0.192$ ) with increasing amount of  $\alpha$ -amylase after 1 800 unit/g DRSF. These results are supported by results from statistical analysis result. The result clearly indicates that

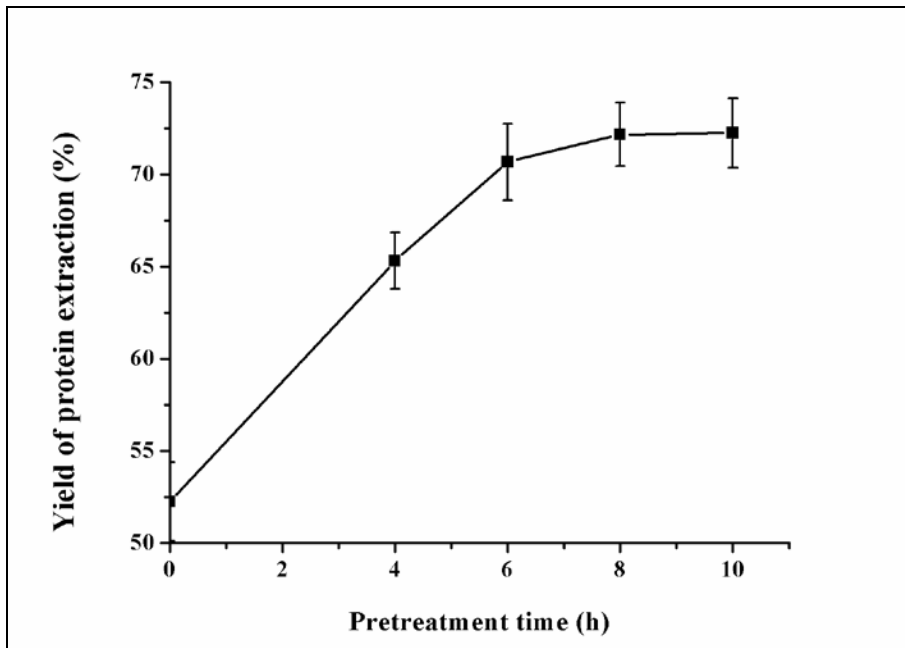
$\alpha$ -amylase had a strong effect in enhancing protein extraction from DRSF.



**Figure 2.** Effect of  $\alpha$ -amylase amount on protein yield, pretreatment time = 6 h

### Effect of pretreatment time

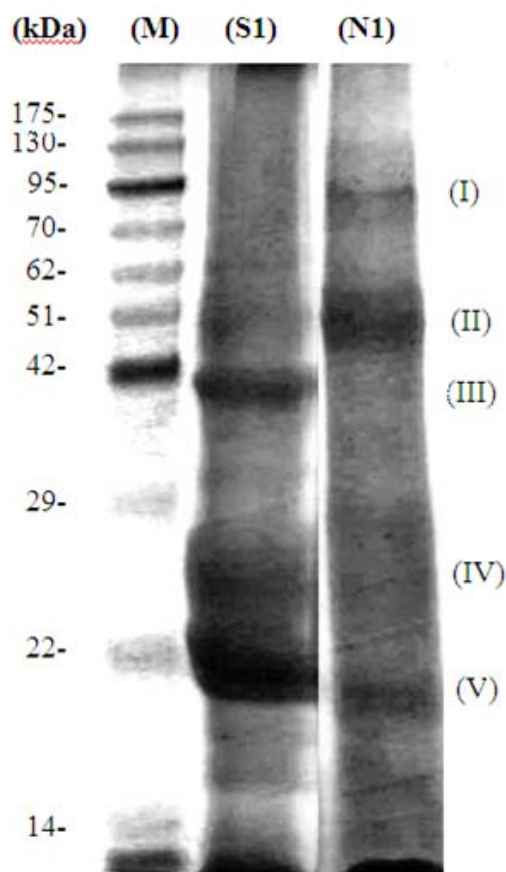
Figure 3 shows the effect of pretreatment time on the yield of protein extraction. A significant increase ( $P = 0.037$ ) of protein yield was observed when the hydrolysis time was increased from 4 h (65.31%) to 6 h (70.67%) while a slight increase occurred when the hydrolysis time was extended from 6 h to 10 h. Therefore, a pretreatment time of 6 h was employed in this study.



**Figure 3.** Effect of  $\alpha$ -amylase pretreatment time on protein yield, amount of  $\alpha$ -amylase = 1 800 unit/g DRSF.

### **Molecular weight of roselle protein**

The SDS-PAGE patterns of protein in RPC are presented in Figure 4. Protein bands appear in the range from less than 22 kDa to 95 kDa and can be divided into 6 fractions. Based on the intensity of protein bands shown in Figure 4, it can be tentatively concluded that there are low molecular weight (fractions III, IV, V and VI) and high molecular weight (fractions I and II) protein in roselle seed. The high molecular weight (fraction I) of roselle protein was found in native PAGE (N1), but not in SDS PAGE (S1). The major fractions detected in native PAGE are I and II, while in SDS PAGE are III and VI. Heating prior to SDS-PAGE was highly likely to dissociate the high molecular weight into lower molecular weight subunits (Yadav et al., 2009). The high molecular weight fractions (I and II) in native PAGE may largely correspond to globulin as proposed by El-Adawy and Khalil (1994). Other fractions with low molecular weight in roselle protein represent albumin and glutelin.



**Figure 4.** Coomassie-stained SDS-acrylamide gel (12% acrylamide) containing RPC samples. M-Marker; S1 - SDS PAGE of RPC; N1 – native PAGE of RPC.

#### **Amino acids composition**

Table 3 lists the amino acid composition of the RPC as well as amino acid compositions of other seeds. It was found that the main amino acids in roselle seeds protein are glutamic acid (28.78%), arginine (10.21%), aspartic acid (10.11%) and leucine (6.36%). According to Sergio (Sergio, 2010), roselle protein contains high amounts of leucine, aspartic acid, and glutamic acid as common cereals protein. It was also noted that the amount of essential amino acids (29.56%) is considerably smaller than that of nonessential amino acids (67.91%). This result is in agreement with results of previous studies (Al-Numair & Ahmed, 2008; Al-Wandawi et al., 1984; Hainida et al., 2008b; Rao, 1996). However, lysine content obtained in this study is lower than those reported in previous studies. The limiting amino acid in RPC found in this work is

methionine, while Al-Numair and Ahmed (2008) and Al-Wandawi et al. (1984) reported that tryptophan was the limiting amino acid in roselle seed protein. This might be due to the variations of roselle seed origins.

**Table 3.** Amino acids compositions (%) of RPC, protein isolate obtained from previous study<sup>a</sup>

Amino acids composition	RPC	Roselle seed protein isolate <sup>a</sup>
<b>Essential</b>		
Lysine	3.84±0.13	5.10±0.41
Threonine	4.24±0.29	2.91±0.28
Valine	4.37±0.39	4.55±0.02
Methionine+Cystine	NA	3.89
Methionine	0.99±0.31	1.48±0.01
Cystine	NA	2.41±0.09
Isoleucine	3.25±0.23	3.01±0.17
Leucine	6.36±0.27	5.92±0.50
Phenylalanine+Tyrosine	NA	8.71
Phenylalanine	4.16±0.28	5.99±0.29
Tyrosine	NA	2.72±0.26
Histidine	2.35±0.19	1.80±0.18
Tryptophan	NA	0.76±0.18
<b>Non-essential</b>		
Arginine	10.21±0.34	9.58±0.26
Aspartic acid	10.11±0.56	10.28±0.29
Glutamic acid	28.78±0.50	24.00±0.59
Proline	4.29±0.17	4.30±0.20
Glycine	4.96±0.06	5.09±0.21
Alanine	4.05±0.36	5.56±0.06
Serine	5.51±0.25	4.70±0.21

NA, not analyzed.

<sup>a</sup> Source: Al-Numair and Ahmed (2008)

## CONCLUSIONS

$\alpha$ -Amylase pretreatment was effective in enhancing protein extraction yield from DRSF. Pretreatment time has a slight effect on protein yield, whereas protein yield strongly depends on the amount of  $\alpha$ -amylase used. Results of SDS-PAGE analysis indicate that the major roselle proteins have high molecular weight. Methionine is the limiting amino acid of protein obtained from roselle seeds cultivated in Vietnam. Further study on functional properties and antinutritional factors may be necessary to affirm the quality of RPC.

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