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In Vitro Binding Capacity of Bile Acids by Defatted Roselle (*Hibiscus Sabdariffa L.*) Seed Protein Hydrolysates

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Abstract

This research aimed to produce bile acid binding peptides from Roselle seed proteins. Roselle seed protein was digested using different proteases, Alcalase, Flavourzyme and by treatment sequentially with Alcalase and Flavourzyme. Bile acids binding capacity was analyzed *in vitro* using hydrolysates obtained after hydrolysis of Roselle seed protein with different proteases. Some crystalline bile acids like sodium glycocholate, sodium cholate and sodium deoxycholate were individually tested using HPLC to see which enzymes can release more peptides with high bile acid binding capacity. All hydrolysates exhibited significantly stronger bile acid binding capacity (from 11,55±1,80 to 61,56±0,79%) and all crystalline bile acids tested were highly bound by Atorvastatine up to 83,17±0,69%, a positive control well known as a cholesterol-reducing agent. The bile acids binding capacity of the hydrolysates was almost preserved after gastrointestinal proteases digestion. The results showed that Roselle seed protein hydrolysates may be used as a potential cholesterol-reducing agent.

Keywords: Roselle-seeds, protein- hydrolysates, bile-acids, binding-capacity

1. Introduction

Protein is a food substance essentially required by the body that cannot be substituted by other compounds in the whole body tissues for its nitrogen contents; hence it must be provided in food. The population in developing countries generally obtains protein from cereals [1]. The development of new foods made from low-cost agricultural by-products is of great nutritional and economical. An example of this is the production of protein isolates and hydrolysates from defatted flours from oilseed crops such as rapeseed [1–2] or sunflower [3].

Defatted Roselle, a byproduct of the calices production, is rich in proteins, minerals and has a balanced amino acid composition [4–5].

Protein is required for normal growth, production, and health, thus an ideal protein must possess an amino acid composition in line with the human and animal requirements. Peptides derived from *in vivo* digestion of whole proteins by hydrolysation with bacterial proteases before ingestion have been reported to carry specific bioactivities. The identification and synthesis of these bioactive peptides have received considerable attention in recent years [6–8]. Hydrolysis of the proteins may have health benefits, such as hypocholesterolemic effect which is attributed to the suppression of both cholesterol adsorption and cholesterol micellar solubility, and inhibition of re-adsorption of bile acids [6].

Cholesterol is a water-insoluble molecule and the absorption of intestine is complexity similar to other lipids, including a previous step of micellar solubilisation [8]. It has been suggested

that hypocholesterolemic peptides inhibited micellar solubility of cholesterol, decreasing *in vivo* cholesterol absorption [9]. This class of peptides has been produced after plant [7, 10-11] and animal [9] protein hydrolysis. Many proteins hydrolysate are capable of hypocholesterolemic activity. Reported proteins that are sources of such hypocholesterolemic peptides include milk protein [9], soy protein [7] and sunflower protein [12]. Numerous studies have demonstrated that peptides may influence bile acids and cholesterol metabolism. Bile acids are the major metabolites of cholesterol and facilitate its elimination in the feces by the formation of micelles that solubilize the cholesterol in the bile. Within the intestinal lumen, bile acids interact with lipases and assist the lipolysis and absorption of fats, including fat soluble vitamins. In healthy people, only small quantities of bile acids are found in the peripheral circulation and urine [9]. Thus, depletion of bile acids from the circulation would promote cholesterol conversion into additional bile acids, thereby leading to significant reductions of liver and serum LDL cholesterol levels [10].

Bile acid binding by dietary fiber is well reported. Thus, various studies have been carried out on bile acid binding from different materials such as sunflower protein [12], lupin protein isolates and their hydrolysates, rapeseed protein isolates [13,14].

However, few reports are available on peptides with bile acid binding capacity. Some researchers reported the bile acid binding activity of buckwheat protein [15] and the binding of bile acids by lupin protein isolates and their hydrolysates [13]. Some studies have found that the protein hydrolysate has higher binding potential than the protein itself [16, 17].

Series of studies have been carried out on hypocholesterolemic activity from different plant protein hydrolysates including soy protein [7], sunflower protein [12], lupin protein [13], rapeseed protein [14] and buckwheat protein [15]. However, to date, not a single research on bile acid binding peptides from defatted Roselle protein has been reported.

The present work was undertaken to determine the best enzyme for hydrolyzing defatted Roselle protein with the purpose of generating more peptides with higher bile acid binding capacity. Also, the stability after gastrointestinal protease digestion was investigated. The defatted Roselle seed protein hydrolysis by proteases could suggest its potential application as a cholesterol-reducing agent for hypercholesterolemic patients.

2. Material and Methods

2.1 Material

Seeds of *H. sabdariffa* were obtained from Koutiala, Southern region of Republic of Mali. All enzymes used were of food grade. Alcalase and Flavourzyme were provided by Novo Nordisk (Bagsvaerd, Denmark). Sodium Glycocholate, sodium cholate, sodium Deoxycholate were from Sigma Chemical Co. All the other chemicals used in the experiments were obtained from commercial source and were of analytical grade.

2.2 Methods

2.2.1 Proximate analysis

The proximate analysis of defatted Roselle seed protein was determined according to AOAC [19]. The moisture content was determined by drying in an oven at 105ºC until a constant

weight. Ash was determined by weighing the incinerated residue at 550ºC for over 8-12h. Total crude protein (N×6.25) content was determined using the kjeldahl method. The fat was determined by gravimetric method after Soxhlet extraction.

2.2.2 Preparation of defatted Roselle seed protein

The defatted Roselle seed flour was mixed with distilled water at a ratio of 1:10 (v/w). After adjusting pH to 11.5 with 1.0 mol/L NaOH, the mixture was incubated with stirring in water bath at 50 °C for 40 min. The supernatant collected after centrifugation, and adjusted to pH 3.5 with 1.0 mol/L HCl to precipitate the proteins, and centrifuged at 4500 rpm for 15 min. The precipitates were washed several times with distilled water, dispersed in a small amount of distilled water, and adjusted to pH 7.0 by 0.1 mol/L NaOH and 0.1 mol/L HCl. The dispersed product was freeze-dried and stored for further experiments.

2.2.3 Enzymatic hydrolysis of defatted Roselle seed protein

The freeze-dried defatted Roselle seed protein was dissolved in the distilled water at a concentration of 5% (w/v) before hydrolysis with two different enzymes independently (Alcalase and Flavourzyme) and by the treatment sequentially with Alcalase and Flavourzyme. Homogenization was carried out for each enzyme for 30 min in order to adjust the appropriate pH (through addition of 0.5 mol/L NaOH) and temperature (Table 1). After optimum condition was reached, the reaction was initiated by adding each of the different enzymes with continuous stirring. Hydrolysis was carried out for 60 and 90 min, the pH of the mixture was kept constant by adding continuously 0.5 M NaOH solution to the reaction mixture. Then, the mixture was heated at 85 °C for 10 min to inactivate the enzyme and centrifuged by freezing centrifugation (ZOPR-52D, Hitachi Koki Co, Japan) at 10000 rpm for 25 min. The supernatant was freeze-dried and stored in the desiccator for further experiments.

Table 1: Optimum condition of used proteases

Enzyme	Conditions			
	Temperature (˚C)	pH value	Hydrolysis time (min)	
Alcalase	60	8.5	60	90
Flavourzyme	50	6.0	60	90

2.2.4 Enzyme Selection

The selected enzymes (Alcalase 2.4 L, and Flavourzyme) were evaluated for their suitability in hydrolyzing defatted Roselle seed protein and their ability to derive peptides with hypocholesterolemic effect in many reported studies.

2.2.5 *In vitro* bile acids binding capacity

The *in vitro* bile acid binding was executed through modification of the procedure previously reported by Kongo Dia-Moukala et al. [20]. Each bile acid (as substrate) was dissolved in 50 mmol/L phosphate buffer (pH 6.5) to make a 2 mmol/L bile acid solution which is in the same range of bile acid concentration in the human body (1.5-7 mmol/mL), and the pH was simulated to the physiological pH of duodenum. Then, ten milligrams of the peptides sample was added to each tube containing one milliliter of bile acid solution, and the individual substrate solution without samples was used as blank. The tubes were incubated for one hour at 37 °C in a shaking water bath. Mixtures were centrifuged in an ultracentrifuge (Model J-26XPI, Beckman, USA) at 10000 rpm for 30 min at 10°C. The supernatant was removed into a second set of tubes and frozen at -20°C for bile acid analysis. Bile acids were analyzed using HPLC (Model 1525, Waters, USA) on a symmetry C18 column (3.9×150mm i.d., 5µm particle size, Waters, USA), maintained at 30 °C. The injected sample volume was 10µL for each bile acids analysis. The bile acid was eluted with 40% acetonile + 60% of 0.05% H_3PO_4 at a flow rate of 1mL/min for 24min. The absorbance of the eluate was monitored continuously at 210 nm (Model 2996 PDA detector, Waters, USA) and quantified using standard calibration curves generated from the peak area responses of the standard solutions. Duplicate assays were conducted for each bile acid essay.
The percentage of bound bile acid was calculated using the Eq. (1).

$$\text{Bound (\%)} = [(Cc-Cs)/Cc] \times 100 \qquad (1)$$

Where, Cc and Cs represent bile acid concentrations in the control and in samples, respectively.

2.2.6 Stability against Gastric Protease *in Vitro*

The stability against *in vitro* gastric proteases was assessed based on the method described by Wu and Ding [21]. A 1% (w/v) hydrolysate solution in 0.1 M KCl–HCl buffer (pH 2.0) was treated with pepsin for 4 h in a rotary water bath at 37 °C. The pepsin-treated peptide was heated to boiling in a water bath for 15 min then adjusted to pH 7.0 with addition of 2 N NaOH. A 1 mL aliquot of the neutralized suspension was centrifuged at 10,000 g for 40 min and the supernatant portion was used to determine bile acid binding. The remaining portion of the suspension was further digested by 2% (w/w) pancreatin at 37 °C for 4 h, followed by enzyme inactivation by boiling for 15 min. The reaction solution was centrifuged at 10,000 g for 40 min. The supernatant portion was used for additional testing on bile acid binding capacity.

2.2.7 Statistical analysis

All analyses were carried out in triplicates and data were presented as mean ±SD. Analysis of variance (ANOVA) was carried out using software Minitab 18.1. Comparisons between means were done using a Fischer multiple-range test with a probability of $P<0.05$.

3. Results and Discussion

3.1 Proximate Composition

The proximate analysis of defatted Roselle seed protein extract is presented in Table 2. The protein extracted contained 69.34± 0.33% crude protein. There was a significant difference ($p<0.05$) in the crude protein content among the defatted Roselle seed flour and Roselle seed protein samples and this result is in agreement with that of rice endosperm protein [21] and quinoa seed protein [22]. The lipid content of defatted Roselle seed flour was 2.82± 0.13% and for protein extracted was 1.19± 0.12%. The decrease lipid content in the protein extracted might significantly increase stability towards lipid oxidation, which may also enhance product stability [24-26].

Table 2: Proximate composition of defatted Roselle seed protein (g/100 g, dry matter basis)

Composition (%)	Defatted Roselle seed protein
Moisture	7.14 ± 0.26
Ash	10.90 ± 0.13
Crude fat	1.19 ± 0.12
Crude protein (N × 6.25)	69.34 ± 0.33
Carbohydrate and others (by difference)	11.23

Values are means ± standard deviation of three determinations

3.2 *In vitro* Bile Acids Binding Capacity

Three bile acids were used to test the *in vitro* binding capacity of different hydrolysates comparatively to that of Atorvastatine.

3.2.1 Binding capacity of sodium glycocholate

As shown in Fig. 1, sodium glycocholate was bound by all our hydrolysates at different degree from 22.83±3.26% for the 90 min hydrolysate obtained by the sequentially treatment of the two used enzymes to 34.58±5.23% for the 90 min Alcalase Hydrolysate . Atorvastatine was used as positive control and had 79.49±0.33% of glycocholate binding capacity. Story and Krichevsky [27] reported that cholestyramine used as positive control in their study, bound glycocholate under various conditions to a degree of 74.2%.

In our study Atorvastatine used as positive control was binding by sodium glycocholate at 79.49% which was higher than to that obtained by Story and Krichevsky [27]. Alfalfa and wheat bran were shown to have a glycocholate binding capacity of 11.5% [27] and 100% [28], respectively; and the defatted soybean hydrolysate 0.2 to 8.5% [10]. All our tested hydrolysates showed higher glycocholate binding capacities than alfalfa and defatted soybean hydrolysates.

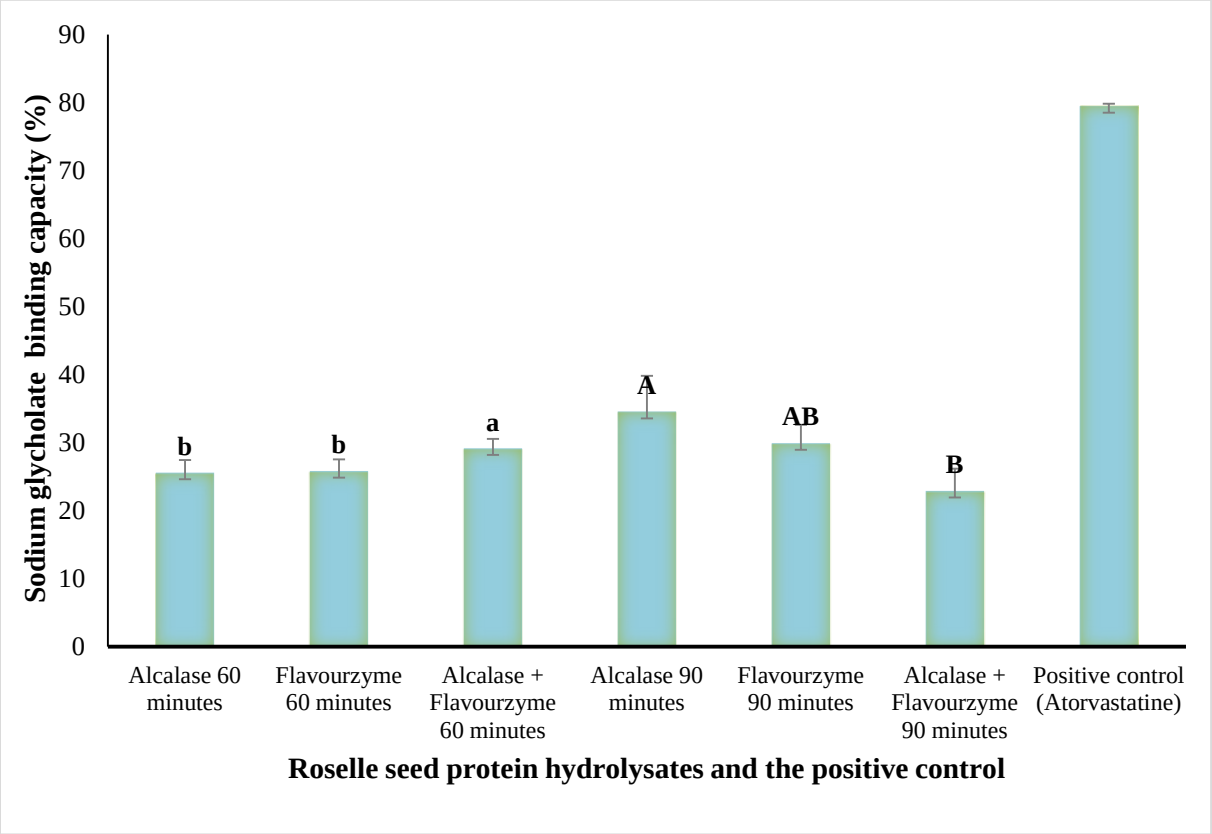

Fig. 1 Sodium glycocholate binding by different hydrolysates from defatted Roselle seed protein. Alcalase: Alca, Flav: Flavourzyme, sequentially treatement of the two enzymes: Alca+Flav and Atorvastatine (Atorv.) used as a positive control. Different lowercase indicate significant differences ($p < 0.05$). Different capital letters indicate significant differences ($p < 0.05$).

3.3.2 Binding capacity of sodium cholate

Sodium cholate was bound by all our hydrolysates at different level from 11.55±1.80% for the 90 min hydrolysate obtained by the sequentially treatment of the two used enzymes, to 33.25±5.03% for the 90 minutes Alcalase Hydrolysate, while Atorvastatine, used as positive control had 77.03±1.37% (Fig. 2). This value was much lower than that recorded with sodium glycocholate 79.49%. Alcalase hydrolysate showed much higher cholate binding capacity than all the tested hyrolysates 33.25%, but lower than that observed in our positive control Atorvastatine with 77.03% (Fig. 2). Camire and Camire et al. [28-29] reported cholate binding by cholestyramine, three types of raisins, wheat bran and various types of potato peels at a cholate concentration of 12.5 mM. Their work indicated cholate binding of 75%, 15-20%, 10% and 1.9-8.1%, respectively. Alcalase, Flavourzyme and our 60 min hydrolysates obtained by the sequentially treatment of the two used enzymes exhibited higher cholate binding capacities than three types of raisins, wheat bran and various types of potato peels. The cholate binding capacity of our positive control Atorvastatine was higher than that of cholestyramine by [28-29] in their studies.

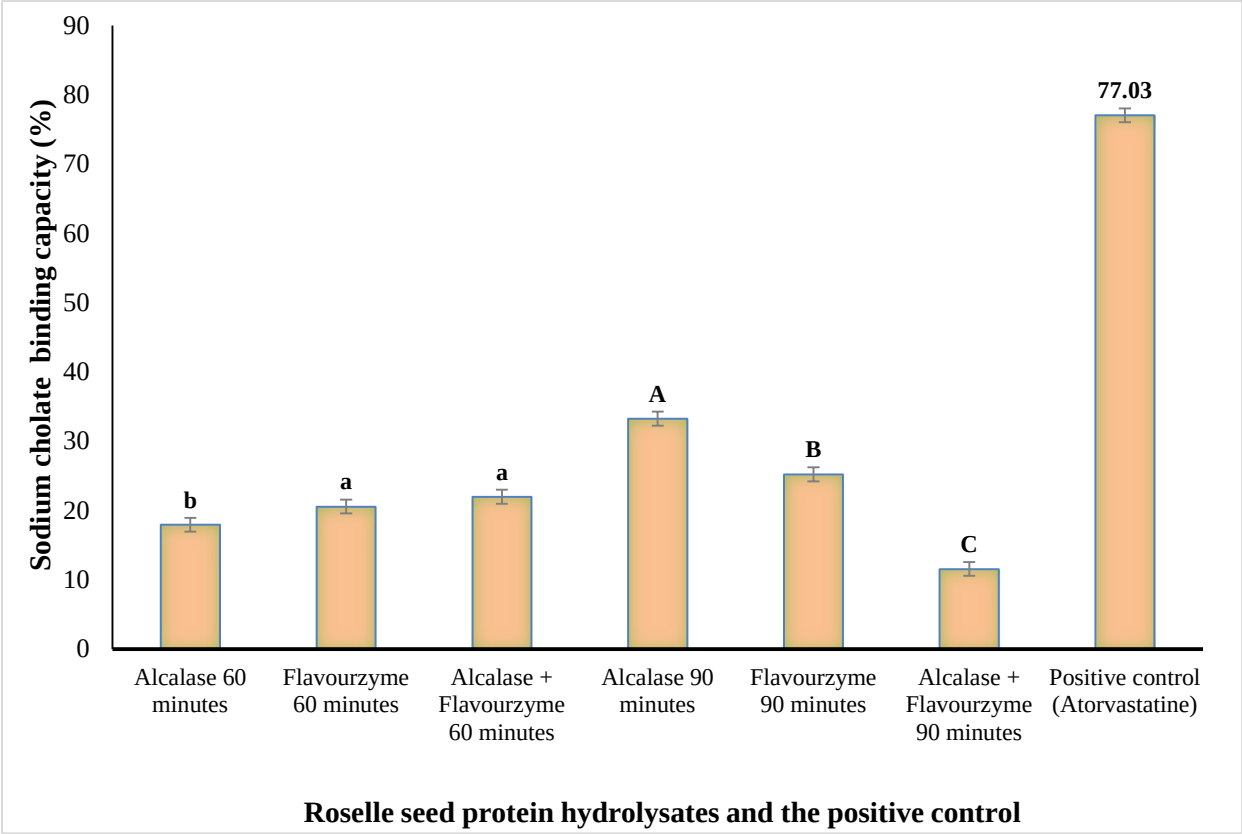

Fig. 2 Sodium cholate binding by different hydrolysates from defatted Roselle seed protein. Alcalase: Alca, Flav: Flavourzyme, sequentially treatement of the two enzymes: Alca+Flav and Atorvastatine (Atorv.) used as a positive control. Different lowercase indicate significant differences ($p < 0.05$). Different capital letters indicate significant differences ($p < 0.05$).

3.2.3 Binding capacity of sodium deoxycholate

Under similar condition, Flavourzyme hydrolysate showed the higher percentage of bile acids binding with sodium deoxycholate than sodium glycocholate and sodium cholate (Fig. 3). Sodium deoxycholate was bound by Flavourzyme hydrolysate to a degree of 48.28±6.71% and 83.17±0.69% by Atorvastatine. Compared to Cholestyramine wich bound sodium deoxycholate over 99% [29], 92.5% [27] and 85% [28]. Our result (with Atorvastatine) was lower to those results. Alfalfa [27], raisins, wheat bran [28], and potato peels [29] showed 10.8%, 5-10%, 15%, and 10.6-18.9%, respectively. Compared to those samples, all our tested hydrolysates showed much higher deoxycholate binding capacities.

As demonstrated by previous work authors [30-34] our investigations confirmed that bile acids binding degree decreases with hydroxyl group increment in steroid ring structure. In addition we found that sodium cholate primary bile acid (three hydroxyls) was the less bound than sodium glycocholate a conjugation of sodium cholate with glycine, however sodium deoxycholate secondary bile acid (two hydroxyls) was the best bound. Numerous authors [10, 27, and 35] have reported a greater binding capacity of sodium deoxycholate than sodium cholate.

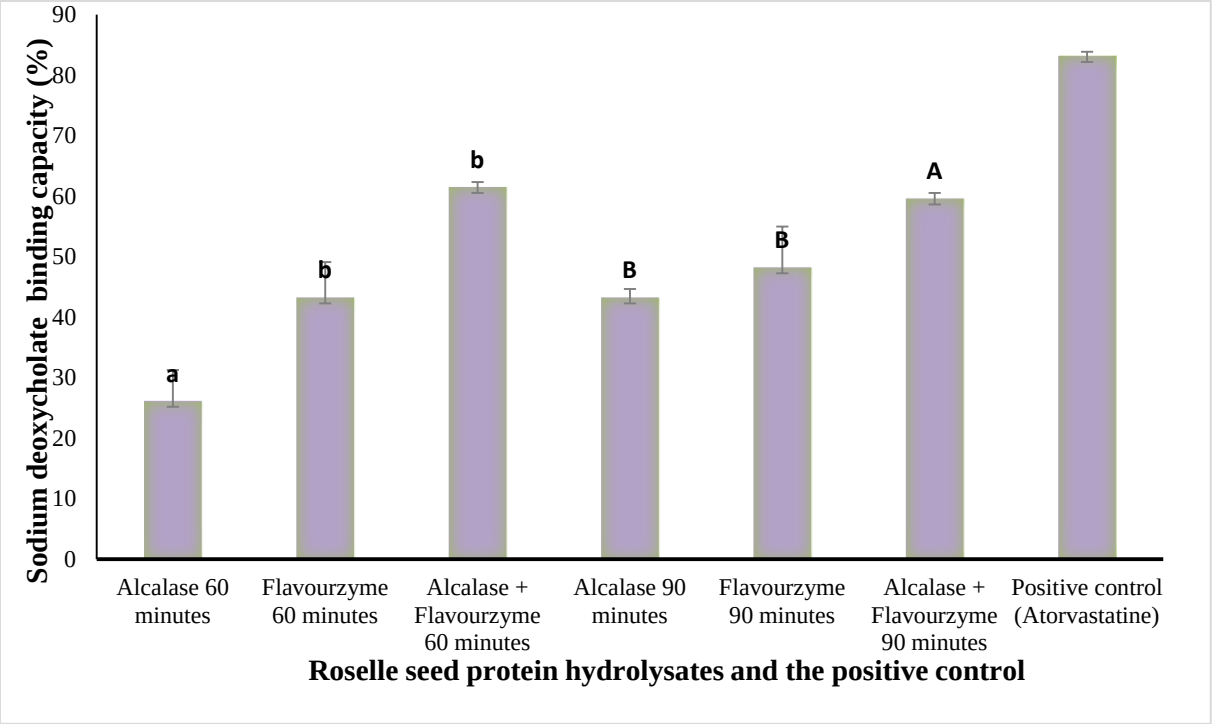

Fig. 3 Sodium deoxycholate binding by different hydrolysates from defatted Roselle seed protein. Alcalase: Alca, Flav: Flavourzyme, sequentially treatement of the two enzymes: Alca+Flav and Atorvastatine (Atorv.) used as a positive control. Different lowercase indicate significant differences ($p < 0.05$). Different capital letters indicate significant differences ($p < 0.05$).

Kern et al. [36] proposed that less sodium cholate, a trihydroxy bile acid was bound than dihydroxy bile acid because hydrophobic interactions are involved with binding.

The method reported here appears to be a satisfactory technique for measurement of the peptides bile acids binding capacity.

Atorvastatine, as expected, bound all the bile acids selected under the same treatment. The concentration of bile acids used and the percentage bound in the present study are in accordance with finding from previous studies.

As the results showed, all hydrolysates contained effective peptides capable to bind bile acids. Moreover, higher bile acid binding capacities were observed with the 60 min hydolysate obtained by sequentially treatement of the two enzymes, the 90 min Alcalase and 90 min Flavourzyme hydrolysates. It is suggested that the higher bile acid binding of the Flavourzyme hydrolysate in our study may be due to the use of physiological pH of 6.5 which is closer to the pH of the hydrophobic amino acids. Those hydrophobic amino acids can bind bile acids strongly via hydrophobic reactions, since the hydrophobic amino acids present a strong interaction with lipids (cholesterol, bile acids, others sterols and others lipids).

Bile acids, especially deoxycholate, are thought to be involved in the etiology and development of colorectal cancer [37]. The synthesis of bile acids from cholesterol is adjusted by their concentration in the liver [38]. The presence of the 90 min Flavourzyme hydrolysate in intestine can decrease not only enter hepatic circulation of bile acids, but also their contact with colorectal mucosa through the binding effect. Therefore, it’s possible for human to ingest some of Flavourzyme hydrolysate to prevent hypercholesterolemia, and colorectal cancer at the same time.

3.3 Stability of Bile Acid Binding by Flavourzyme Hydrolysate after Digestion by Gastrointestinal Proteases

The stability of Flavourzyme hydrolysate against gastrointestinal proteases in vitro was tested in order to predict the bile acid binding in vivo. Some food protein derived-bioactive-peptides, for example ACE-inhibitory peptides, failed to express bioactivity after oral administration to Spontaneously hypertensive rats due to the fact that they were hydrolyzed in the gastrointestinal tract to peptides or amino acids with reduced activity [39]. The bile acid binding of Flavourzyme hydrolysate was almost preserved after gastrointestinal proteases treatment (Table 3), suggesting that these hydrolysates may be resistant to digestion in the gastrointestinal tract. Previous reports have also shown that small peptides have low susceptibility to hydrolysis by gastric proteases [40, 41].

Table 3: Bile acid binding capacity after digestion by gastrointestinal proteases

Protease	Cholate	Glycocholate	Deoxycholate
Control	25.21± 0.43	29.96± 0.53	48.28± 0.35
Pepsin	24.99± 0.37	29.76± 0.48	48.10± 0.28
Pepsin+pancreatin	25.08± 0.39	29.86± 0.63	48.15± 0.25

Each measurement was carried out in duplicate. Values with the same letters denote no significant difference ($p<0.05$)

4. Conclusions

Defatted Roselle seed protein was effectively hydrolyzed by all the used proteases to obtain the peptides with strong hypocholesterolemic activity. In our study the higher bile acid binding was obtained with Flavourzyme hydrolysate. The results suggested that peptides with strong hypocholesterolemic activity can be obtained. The hydroysates can be useful as additive in food and pharmaceutical products. However, further study is undergoing to isolate the individual peptides responsible for the hypocholesterolemic activity of the hydrolysates and to identify their amino acid sequences, which will allow a better understanding of the peptide structure and functionality relationship.

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