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Effect of Recovery Method on Different Property of Mustard Protein

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Abstracts: Mustard protein was extracted in alkaline solution and detoxification with activated carbon treatment. The protein was extracted from solution by three different methods and the effects of recovery method on yield and removal of anti-nutritional factors were evaluated. Heat coagulation by steam injection resulted in a good yield with maximum removal of anti-nutritional factors but resulted in a protein isolate with a lower solubility (20-40%) at different pH compared to other methods. Incorporation of an enzyme modification step before spray drying, using Alcalase enzyme improved solubility of protein to 60-75%. Recovery of protein by heat coagulation reduced all functional properties of isolated protein but except foam stability, partial hydrolysis (DH= 9.4 ± 0.1) of isolated protein improved all functional properties. The SEM of isolated protein showed spherical particles ranged 1-20 µm in diameter but in protein hydrolysate majority of them were less than 5 µm in diameter. The SDS-PAGE pattern of meal protein showed 3 major bands with similar molecular weight but the protein hydrolysate did not show any bands.

Key words: Mustard Protein, Recovery method, Protein hydrolysate, SDS-PAGE pattern, Functional Properties

INTRODUCTION

In view of world-wide protein shortage, oilseeds are becoming of increasing importance as a source of edible proteins. Rapeseed, which is a major oilseed crop of temperate zones, has so far found little application in the production of protein. The seed contain about 42% oil, which is widely used as edible oil. The defatted meal contains up to 40% protein which is used in animal feeds or as an organic fertilizer. The protein has a well balanced amino acid composition [1] and compare favorably with soy proteins and the FAO pattern of essential amino acid requirement of human adults. Mustard/rapeseed proteins are rich in lysine and methionine, which are limiting essential amino acids in most of the cereals and other vegetable proteins [2].Therefore, rapeseed/mustard protein, may be considered as a potential source of food protein. The use of rapeseed meal as a protein source in livestock and human food is limited due to the presence of glucosinolates and other undesirable compounds such as phytates, phenolics and hull. These toxic and anti-nutritional components have to be removed completely before rapeseed protein could be used for human consumption [3]. Processes for the preparation of protein isolate from mustard, rapeseed and canola by different treatments to reduce anti-nutritional constituents have been developed and patented [4-8]. In our previous work a process for the production of mustard protein isolate, with reduced toxic and antinutritional constituents for food and feed purposes, has been developed and reported [9]. This method was based on steam injection heating to recover protein from activated carbon treated protein solution. The steam injection heating reduces solubility of protein and has negative effects on functional properties of protein isolate. Therefore, the objective of present study was to compare different recovery methods in view of yield and removal of anti-nutritional factors and to modify the previous method accordingly.

MATERIALS AND METHODS

Materials: Mustard seeds of the variety T-59 were purchased from Karnataka State Seeds Corporation Limited, Karnataka, India. Enzyme thioglucosidase (EC 3.2.3.1) and low molecular weight standards obtained

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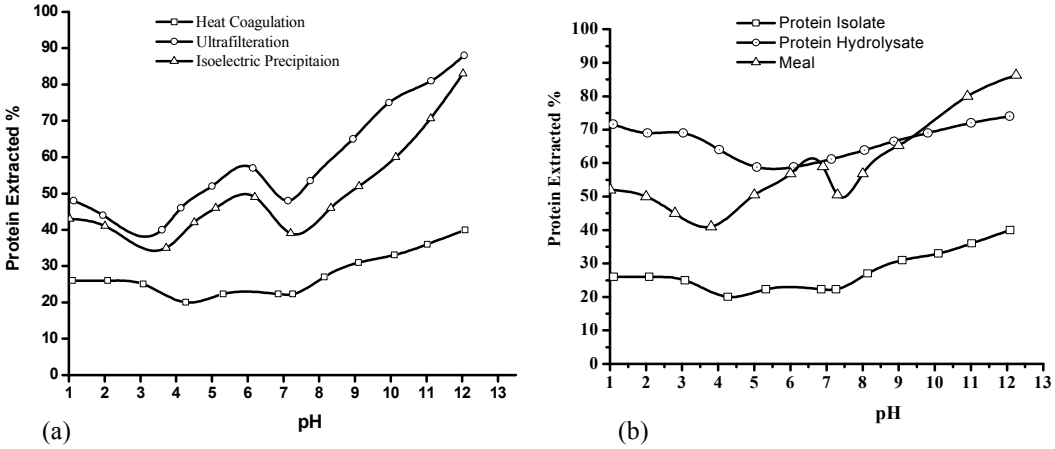

Fig. 1: Solubility profile of (a) Protein extracted by different recovery method and (b) dehulled defatted meal, protein isolate prepared by steam injection heating and protein hydrolysate (DH= 9.4 ± 0.1)

from Sigma Chemicals, USA; Alcalase was from NOVO Industries, Denmark. All other chemicals used were of analytical grade.

Methods

Preparation of Protein Solution: Defatted mustard meal was prepared as reported in our previous work [9]. Defatted mustard meal dispersed in 0.1 M NaCl in a ratio 1:15 (w/v) and was incubated at 37°C for 1 h. The pH was adjusted to 11 with the addition of 2 N NaOH. The dispersion was subjected to shaking for 30 min at room temperature before centrifuging at 1118 × g (Beckman centrifuge, Model: GS-15R, Rotor: F0850, Beckman Instruments Inc; PALO ALTO, California, USA) for 20 min. The pH of the supernatant was readjusted to 7.0 with 2 N HCl. Activated carbon granules (2% w/v) were added and kept for shaking for 1h and filtered.

Recovery of Protein from Solution: To compare the effect of protein recovery method on yield and removal of antinutritional factor in addition to steam injection heating that was used in our previous work [9], two alternative methods including: isoelectric precipitation and ultrafiltration were used as follow:

Recovery of Proteins by Isoelectric Precipitation: In isoelectric precipitation, two isoelectric pH 7.8 and 3.8 (obtained from nitrogen solubility curve of meal, Figure 1b) were used respectively. The solution after treating with activated carbon was adjusted to first isoelectric point (pH 7.8), using 1 N NaOH and the solution was centrifuged at 1118 × g and precipitated protein was recovered. The pH of supernatant re-adjusted to second isoelectric point (pH 3.8) using 1N HCl and solution was centrifuged again. The two precipitated protein were combined, washed and spray dried (Bowen Engineering Inc., New Jersey, USA). The inlet temperature of 150°C±5 and outlet temperature 110°C±5 was used.

Recovery of Proteins by Ultrafiltration: In ultrafiltration method, the protein solution after activated carbon treatment was passed through ultrafiltration unit (Watson Marlow, Model No.623S, Falmouth, Cornwall, TR 11 4Ru, England). Generally, a built-in peristaltic pump (Bradel Pump) drew solution from a sample container, pumped it through a Polysulfone membrane cartridge (Sartocon slice, 30S 1463901 E-SG with a nominal molecular weight cut-off of 10 KD). Pressure in the cartridge was controlled by a back-pressure valve at the outlet. The retentate was returned to the sample container. The sample flows continuously through ultrafiltration unit till its volume decrease to 1:10 original volume. The concentrated sample was diluted with water to 20% of solid material and spray dried.

Preparation of Protein Hydrolysate: The protein hydrolysate was prepared by the following method. The coagulated protein obtained by steam injection heating, after washing was dispersed in water to a solid content of 20% and adjusted to pH 8. Enzyme Alcalase (1ml/100g dry protein) was added and incubated at 50°C for 60 min [10]. After hydrolysis, the temperature was raised to 85°C for 10 min to inactivate enzyme and the solution was spray dried to obtain protein hydrolysate.

Glucosinolates: The isothiocyanates and oxazolidine thiones, the hydrolysed product of glucosinolates, were estimated as described by Wetter and Youngs [11] and expressed as mg of 3-butenyl isothiocyanate and 5-vinyloxazolidine -2 thione per g of meal or isolate.

Phytic Acid: The procedure of Thompson and Erdman [12] was used in phytic acid estimation by converting to ferric phytate and the phosphorus content was analysed by method of Taussky and Shorr [13]. Phytic acid content was derived from the phytate phosphorus values by multiplication using a factor of 3.55.

Phenolics: Total phenolics were extracted and estimated as tannic acid equivalents according to the method described by Deshpande *et al*. [14].

Protein Solubility: The protein solubility was determined according to the method of Mattil [15].

Degree of Hydrolysis: The degree of hydrolysis in protein hydrolysate was determined according to the method of Adler-Nissen [16].

Scanning Electron Microscopy (SEM): Scanning electron microscopic (SEM) studies of the meal, protein Isolate and hydrolysate were carried out using Scanning Electron Microscope (LEO 435 VP, Cambridge, UK). Before loading the sample into the system, it was coated with gold using Poloron SEM coating system E-5000. The coated sample was loaded on the system and the image was viewed under 20 kV potential using secondary electron image. The image was captured using 35 mm Ricoh Camera.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Whole mustard meal, protein isolate prepared by steam injection heating and protein hydrolystae were extracted and fractionated by SDS-PAGE according to the method of Laemmelli [17] with a 5% (w/v) stacking gel and 12% (w/v) separating gel. Vertical slab gel electrophoresis was carried out in a BROVIGA mini model electrophoresis unit (BROVIGA; Balaji Scientific Services, Chennai, India), at 25 ± 2 °C. Equal amounts of proteins were loaded on to each lane of gel and run for 3 hr at 50 mA in a 1 mm thick gel. The gels were stained with Coomassie blue. The approximate molecular weight of the hydrolysate was determined using low molecular weight standards obtained from Sigma Chemical company, St. Louis, USA.

Functional Properties: Water absorption capacity (WAC) of samples was determined according to the method of Sosulski [18], fat absorption capacity (FAC) by the method described by Sosulski *et al*. [19], foam capacity (FC) and foam stability (FS) were determined by the method of Lawhon and Carter [20] and emulsion capacities (EC) of samples were determined by the procedure of Beuchat *et al*. [21].

Statistical Analysis: Data and Statistical analysis were performed using scientific graphic and analysis computer software OriginPro (version 7) supplied by Origin Lab Corporation, Northampton, MA, USA and data was expressed as Mean ± standard deviation of three experiments.

RESULTS AND DISCUSSION

Table 1 shows the data on recovery of protein by different methods. In all the cases the protein after recovery was washed and spray dried except the ultrafiltration where the protein solution after concentration was spray dried without washing. The yield of protein by isoelectric precipitation was 45 and was low compared to other two methods. The recovery of protein by alkaline extraction and isoelectric precipitation was reported in the range of 49-56% [22]. Recovery of protein by heat coagulation and ultrafiltration gave higher yields of 60 and 65%, respectively. However, in the ultrafiltration method, the removal of phytates and phenolics was 90 and 78.2%, respectively and was much lower than other methods with lower protein content of 56.2%. Washing step before drying would help to maximize removal of anti-nutritional factors. However, practically it is not possible to wash ultrafiltered protein concentrate before drying. Removal of isothiocyanate and oxazolidinethione in all methods were high mainly due to treatment with activated carbon which remove these hydrolysis products, as reported by Woyewoda *et al*. [23]. Among the methods used to recover proteins, heat coagulation was suitable in terms of purity of protein, yield and removal of anti-nutritional factors. Heat coagulation almost completely eliminated the isothiocyanate and oxazolidinethione. In addition, 99% of phytates and 98.7% phenolics were removed in the protein. The purity of the protein was higher (95%) compared to other methods (Table 1). The protein solubility profiles of isolated protein prepared from dehulled meal by different recovery methods are

Table 1: Effects of recovery methods on yield and removal of anti-nutritional factors

Parameter [a]		Meal	Ultrafiltration	Isoelectric precipitation	Heat coagulation
Protein (%)		48.0±0.5	56.2±0.8	88±0.5	95±0.5
Yield of Protein (%) [b]		-	65	45	60
Isothiocyanates	amount(mg/g)	18.75±0.3	0.68±0.07	0.68±0.07	0.19±0.05
	Removal (%) [c]	-	98±0.3	98±0.5	99.5±0.1
5-Vinyl-Oxazolidine-2-thione	amount(mg/g)	13.75±0.35	0.33±0.01	0.33±0.01	ND*
	removal(%) [c]	-	98.8±0.2	98.8±0.4	100%
Phytates	amount (%)	5.6±0.1	1.1±0.03	0.54±0.02	0.14±0.03
	Removal (%) [c]	-	90±0.5	95±0.5	99±0.3
Phenolics	amount (%)	2.12±0.06	0.90±0.03	0.18±0.02	0.12±0.02
	Removal (%) [c]	-	78.7±0.5	96±0.3	98.7±0.3

[a]Mean ± SD of three determinations [b]As % of Total Protein

[c] Compare to starting meal using protein% * Not Detectable

Table 2: Functional properties of mustard meal, protein isolate and protein hydrolysate

Parameter [B]	Meal	Protein Isolate [A]	Protein hydrolysate (DH= 9.4 ± 0.1)
Water absorption capacity (g /100g)	275±5 [a]	21±5 [c]	260±4 [b]
Fat absorption capacity (ml/100g)	180±7 [a]	90±5 [c]	130±6 [b]
Emulsion Capacity (ml/g)	62±1 [a]	31±0.5 [c]	47±0.5 [b]
Foam capacity (%)	110±5 [a]	60±5 [b]	90±3 [c]
Foam stability at 30 min (%)	100±4 [a]	50±3 [b]	45±3 [b]

A Prepared by steam injection heating

[B] Values are Means ± SD of three determinations. Values Followed by different letters for each row are significantly different ($p<0.05$).

presented in Figure 1a. The use of heat coagulation for recovery of protein resulted in a protein isolate with a lower solubility (20-40%) at different pH compared to other methods. At pH 12 more than 85% of protein was soluble in the case of ultrafiltration and isoelectric precipitation methods (Figure 1a). Yousuf *et al*. [24] have reported that the roasting of Nigerian benniseed and Bambara groundnut at 80 and 120°C decreased the nitrogen solubility. Proteolytic enzymes have been widely used for modification of food proteins to improve their functional properties [10, 25]. In the present study enzyme modification has been used to improve the functional properties of heat coagulated mustard protein isolate. The enzyme Alcalase was used for partial hydrolysis of protein isolate. The optimal condition for hydrolysis was temperature 50° C, time 60 min, pH 8 [10]. The E/S ratio of 1:100 (v/w dry protein) was selected based on the optimum solubility of hydrolysate and to improve other functional properties. Figure 1b shows the protein solubility profile of meal protein hydrolysate (DH 9.4 ± 0.1) compared to protein isolate and dehulled defatted meal at different pH. The solubility of protein isolate prepared from meal by steam injection heating was in the range of 20-40% which increased to 60-75% on hydrolysis. Increased protein solubility with enzymatic hydrolysis, is well-documented [26]. The increase in solubility is due to the smaller size of peptides and higher hydrophilicity of the hydrolysates [27].

The results of functional properties of meal, protein isolate prepared by steam injection heating and protein hydrolyste are presented in Table 2. The results showed that the WAC of dehulled defatted mustard was 275 g/100g and reduced to 210g/100g in protein isolate while increased to 260 g/ 100g after hydrolysis. The decrease in WAC of protein isolate may be due to heat treatment used for coagulation of protein. The lower water holding capacity has been reported in heat treated oilseed protein by Khalil *et al*. [28]. Improved WAC as a result of enzyme hydrolysis has been reported for canola/rapeseed [29]. The mustard meal showed higher FAC of 180 ml/100g sample compared to protein isolate (90 ml/100g) and upon enzyme modification the FAC value improved to 130 ml/100g. Vioque *et al*. [29] reported that rapeseed protein hydrolysate showed higher oil holding capacity than the original protein isolate. The FC and FS were reduced from 110 and 100% in the meal to 60 and 50% in protein isolate, respectively. However, enzyme hydrolysis slightly improved the foam capacity of protein

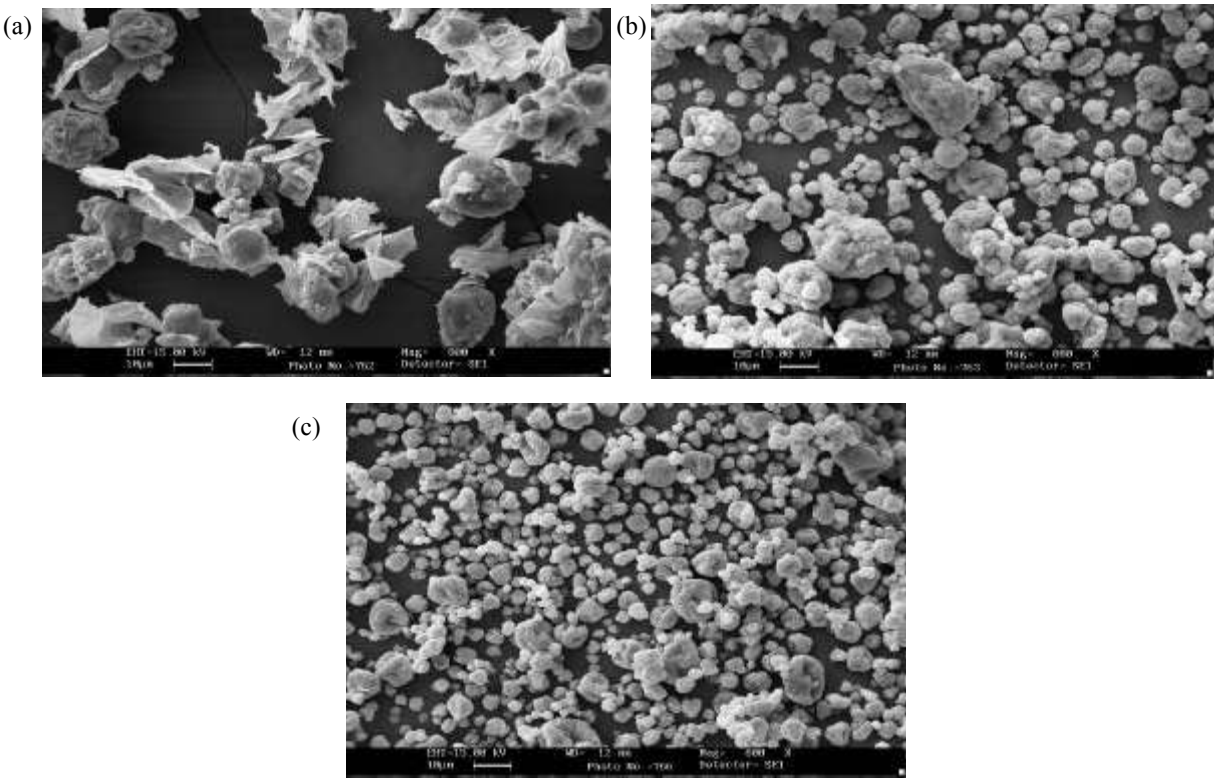

Fig. 2: SEM of (a) Meal, (b) Protein Isolate and (c) protein hydrolysate

hydrolysates, but reduced the foam stability. It has been reported that limited proteolysis may improve foaming capacity but decreases foam stability. Apparently, hydrolysates are capable of foaming but lack strength to maintain the foam as a result of reduction in peptide size [29]. Mustard meal showed higher EC compared to protein isolate and hydrolystae. The EC of protein isolate were much lower than the original meal with a reduction of 50%. However, the enzyme hydrolysis improved the EC of protein isolate. The low EC in protein isolate may be due to heat treatment used during coagulation of protein. It has been reported that heat processing markedly decreased the EC of winged bean flour [30]. Generally limited hydrolysis improves the emulsifying properties of proteins owing to the exposure of hydrophobic amino acid residue that may interact with the oil while the hydrophilic residues interact with water [29].

The SEMs of meal, protein isolate and hydrolysate prepared from meal are presented Figure 2a, 2b and 2c, respectively. The isolated protein (Figure 2b) contain mostly spherical particles 1-20 μm in diameter probably contained the protein bodies and smaller particles seems to be due to rupture of larger protein bodies during isolation process. The reduction in the size of protein bodies in protein isolate compared to meal (Figure 2a) may be due to the effect of heat treatment. The particle size of protein hydrolysates (Figure 2c) is around 1-10 μm and majority of them were less than 5 μm in diameter. It has been reported that the most evident change observed in hydrolysates with respect to the protein isolates is the reduction in the molecular weight of proteins as a direct consequence of protease activity [10].

The SDS-PAGE pattern of total protein from dehulled defatted meal, protein isolate and protein hydrolysate is given in Figure 3. The meal protein resolved into 3 major and 8 minor bands. The molecular weights of the major bands were 34,000; 28,000; and 20,000 Da. The minor bands had the molecular weights ranged from 33,000 to 60,000 Da. On the other hand, the protein isolate showed 3 major bands with similar molecular weight to that of total proteins from meal. However, the protein hydrolysate did not show any bands. This may be due to enzyme hydrolysis of protein which decreased the molecular weight of proteins. This result is consistent with smaller size of protein hydrolysate as seen in SEM of this fraction (Figure 2c). Gururaj Rao and Narasinga Rao [31] have reported that in the SDS-PAGE pattern of mustard and rapeseed protein 3 major bands corresponding to

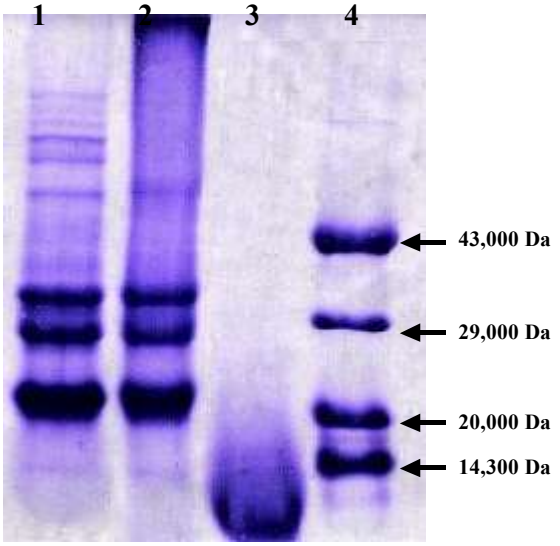

Fig. 3: SDS-PAGE pattern of (1) dehulled, defatted meal; (2) protein isolate; (3) protein hydrolysate and (4) markers

molecular weight of 34,000; 27,000; and 20,000 Da. were prominent. Prakash and Narasinga Rao [32] have reported that the 12S protein of rapeseed was separated into 8 subunits with molecular weights ranged from 11,000 to 70,000 Da.

CONCLUSIONS

Among the methods used for recovery of proteins, heat coagulation by steam injection heating resulted in better yield with maximum removal of anti-nutritional factors. However protein isolate showed lower solubility and poor functional properties. Incorporation of an enzymatic hydrolysis step yield protein with better functional properties and high solubility at all pH values.

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