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Effect of chymotrypsin digestion followed by polysaccharide conjugation or transglutaminase treatment on functional properties of millet proteins

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Abstract

Millet protein was solubilized by chymotrypsin; the soluble protein was conjugated to galactomannan under controlled conditions (60 °C, 76% RH) or polymerised by transglutaminase (TGase). SDS–PAGE patterns showed that the conjugated and polymerised proteins had higher molecular mass bands above the stacking gel. SDS–PAGE patterns also indicated that the digest was conjugated to galactomannan and polymerised by TGase. The free amino groups (OD_{340}) of the conjugated and polymerised digest were greatly reduced. Although the chymotrypsin digest was considerably insoluble between pH 2.0 and 5.0, galactomannan conjugate was completely soluble at all levels of pH. TGase polymer was slightly insoluble at pH 4.0. Galactomannan conjugate resisted heat-induced aggregation, even after heating at 90 °C for 20 min, while TGase polymer resisted heat-induced aggregation up to 70 °C, after which its solubility started to decline. The emulsifying properties of the conjugate and the polymerized proteins were greatly improved, compared to the native and chymotrypsin digests.
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Keywords: Millet; Chymotrypsin; Transglutaminase; Galactomannan; Maillard reaction

1. Introduction

Pearl millet (*Pennisetum typhoides*) is a staple food for a large segment of population in Asian and African countries, where it contributes a major part of dietary nutrients (Burton, Rachie, & Wallance, 1972). Millet protein (8.6–13.7%) was fractionated into albumin–globulin, prolamin, and glutelin fractions. The prolamin fraction constitutes the major storage protein of the grain (Monteiro, Virupaksha, & Rao, 1982). Therefore, its solubility was found to be very poor. Ramachandra, Virupakshaka, and Shadaksharaswamy (1978) reported that the insolubility of millet protein was mainly due to its amino acid composition. The large amounts of non-polar amino acid residues, such as proline and leucine, tend to be involved in hydrophobic bonding. Also as the presence of considerable amounts of amide groups in the side chains of amino acids such as glutamine, play an important role in stabilizing the protein structure and in promoting the association of protein molecules through hydrogen bonding. Although functionality of proteins has generally been improved by solubility, contradictory results were reported with respect to emulsifying properties (Akio, Taneyama, & Inami, 1980; McWatters & Holmes, 1979).

The modifications of proteins by enzymatic and chemical reagents have been extensively studied and have been shown to be very powerful tools for improving the functional properties of these macromolecules. The effect of the chemical and enzymatic deamidation of food proteins on the functional properties of proteins has been of great interest in the food industry (Shih, 1991). A number of molecular parameters such as mass, conformation, flexibility, net charge, and hydrophobicity of proteins, as well as interactions with other food components, have already

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been shown to play an important part in both their emulsifying and foaming properties (Nakai & Voutsinas, 1983). Transglutaminase as a polymerizer has been extensively studied (Babiker, 2000) and is known to catalyze the transfer reaction between an amide group in a protein bound-glutamine and an ε-amino group in a protein-bound lysine side chain, resulting in cross-links between the protein molecules. Treatment of protease and acid hydrolysate of gluten (Babiker, Fujisawa, Matsudomi, & Kato, 1996a) and soy protein (Babiker, Khan, Matsudomi, & Kato, 1996b) by transglutaminase was found to improve the functional properties of the protein. Soy protein-galactomannan conjugation was found to improve solubility at all levels of pH, heat stability and emulsifying properties (Babiker et al., 1999a). A study on an ovalbumin–glucose 6-phosphate conjugate (Kato, Aoki, Kato, Nakamura, & Matsuda, 1995) demonstrated that the solubility and heat stability of the modified protein were greatly improved.

Transglutaminase treatment and polysaccharide conjugation of wheat proteins greatly improved solubility, heat stability and emulsifying properties (Babiker, Mohamed, & Tinay, 1999b). Moreover, chitosan conjugation greatly affected the functional properties and bactericidal activity of lysozyme (Song, Babiker, Usui, Saito, & Kato, 2002) and gluten peptides (Babiker, 2000). In spite of the long history of cultivation of millet in the semi-arid tropics, information is scarce on its functional properties. In this paper an attempt was made to study the functional properties of millet protein and the effect of chymotrypsin digestion, followed by polysaccharide conjugation or transglutaminase treatment on the structure and functionality of the protein.

2. Materials and methods

2.1. Materials

Millet seeds were obtained from an Agricultural Research Corporation in Sudan. Chymotrypsin (52 units/mg), was purchased from the Sigma Chemical Co. Tranglutaminase was obtained from Taiyo Chemical Co. Glactomannan was obtained by dialyzing a mannase hydrolysate of guar gum (average molecular weight of 15000 from Sigma Chemical Co). Unless otherwise stated, all reagents used in this study were reagent grade.

2.2. Preparation of millet protein

The protein was prepared from millet flours by extraction in an alkaline medium by the method of Iwabuchi and Yamaguchi (1987). A sample of defatted meal (100 g) was extracted once with 2 l of 0.3M Tris–HCl buffer (pH 8) containing 10 mM 2-mercaptoethanol (2-ME) at 20 °C. After centrifugation (8000 rpm), the supernatant was acidified to pH 4.8 with 2 N HCl and then centrifuged. The precipitate protein was dissolved in water at 4 °C and the pH adjusted to 8. After centrifugation (8000 rpm), the clear supernatant was dialyzed against distilled water for 24 h at 4 °C and then freeze-dried.

2.3. Preparation of chymotrypsin digests

A freeze-dried sample (2 g) of millet protein was suspended in 200 ml of 0.05 M Tris–HCl (pH 8.0) containing 0.05% sodium azide, and then 20 mg of chymotrypsin were added. The mixture was incubated at 37 °C for 6 h. After incubation, chymotrypsin was inactivated by heating at 100 °C for 3 min. The digested mixture was centrifuged (8000 rpm) to precipitate a small amount of undigested protein; the supernatant was dialyzed against distilled water for 24 h at 4 °C and then freeze-dried.

2.4. Galactomannan conjugation

Chymotrypsin digest and galactomannan were mixed in the weight ratio of 1:4. The mixture was dissolved in distilled water in 10% (w/v) concentration and freeze-dried. Powdered mixture was dry heated at 60 °C and 79% relative humidity in a desiccator containing saturated KBr solution for 7 days.

2.5. Transglutaminase (TGase) treatment

Chymotrypsin digest of the protein (10 mg/ml) was dissolved in 0.1 M phosphate buffer (pH 7.5) and then reacted with TGase (0.2 mg/ml). The mixture was incubated at 55 °C for 60 min. The enzyme was inactivated by *N*-ethylmaleimide (0.1 ml; 0.1%) (Babiker, 2000). The treated sample was dialyzed against distilled water and then freeze-dried.

2.6. Changes in free amino groups

Changes in free amino groups of 0.1% protein solutions were determined by spectrophotometric assay (OD_{340}) using *o*-phthaldialdehyde, as described by Church, Swaisgood, Porter, and Catignani (1983).

2.7. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done, using the method of Laemmli (1970), with 15% acrylamide separating gel and 3% acrylamide stacking gel containing 0.1% SDS. Samples (20 μ, 0.2%) were prepared in a Tris–glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a current of 10 mA for 5 h in electrophoretic Tris–glycine buffer containing 0.1% SDS. After electrophoresis, the gel sheets were stained for proteins and carbohydrate with 0.2% Coomassie brilliant blue-R250 and 0.5% periodate-fuchsin solution (Zacharius, Zell, Morrison, & Woodlock, 1969), respectively. Protein stain was destained with 10% acetic acid containing 20% methanol.

2.8. Measurement of solubility

Protein solubility was determined by the method of Akio et al. (1980), with a slight modification. Sample solutions (0.2%) at various pH values (pH 2–3, 0.05 M citrate buffer; pH 4–5, 0.05 M acetate buffer; pH 6–8 phosphate buffer; pH 9–11, 0.05 M carbonate buffer and pH 12, 0.05 M NaOH slightly adjusted with 0.05 M HCl) were centrifuged at 8000 rpm for 20 min and the protein content in the supernatant was measured. The protein solubility was indicated by the ratio of the protein concentration in the supernatant to that in the original protein dispersion.

2.9. Heat stability

Heat stability was determined by the method described by Kato et al. (1995). The samples were dissolved at a protein concentration of 2 mg/ml in 50 mM Tris–HCl buffer (pH 7.0) and heated at 50–90 °C for 20 min. Aggregates were precipitated by, centrifugation at 8000 rpm for 20 min, and their absorbance at 280 nm was measured. Soluble protein was determined and expressed as a percentage of total protein solution.

2.10. Measurement of emulsifying properties

The emulsifying properties of samples were determined by the method of Pearce and Kinsella (1978). To prepare emulsions, 1.0 ml of corn oil and 3.0 ml of protein solution (0.2%) in 0.1 M phosphate buffer, pH 7.0, were shaken together and homogenized in an Ultra Turrax (Hansen & Co., Germany) at 12 000 rpm for 1 min at 20 °C. A 50 μl sample of emulsion was taken from the bottom of the container at different times and diluted with 5 ml of 0.1% sodium dodecylsulfate solution. The absorbance of the diluted emulsion was then determined at 500 nm. The emulsifying activity was determined from the absorbance immediately measured after the emulsion formation. The emulsion stability was estimated by measuring the half time of the initial turbidity of the emulsion.

3. Results and discussion

3.1. Effect of the protein modifications on the molecular mass and free amino groups

Millet protein was digested by chymotrypsin and then modified by galactomannan conjugation or transglutaminase (TGase) treatment. Fig. 1 shows the SDS–PAGE patterns of millet protein, chymotrypsin digests, galactomannan conjugate and TGase polymer. The SDS–PAGE pattern showed that the protein digest conjugated to galactomannan had higher molecular weight bands in the top of the stacking gel in both protein staining (Fig. 1a) and carbohydrate staining gels (Fig. 1b). The results indicated that the protein digest was covalently attached to galactomannan through the Maillard reaction between the ε-amino groups in the proteins and the reducing-end carbonyl groups in galactomannan during dry heating at 60 °C and 79% relative humidity for 7 days. The results obtained agree with those obtained previously for soy protein–galactomannan (Babiker et al., 1999b), chymotrypsin digested gluten–chitosan (Babiker, 2002) and lysozyme–chitosan conjugates (Song et al., 2002). The protein digest treated with TGase (Fig. 1a) also showed higher molecular bands in the top of the stacking gel. The results indicated that TGase catalyzed the transfer reaction between an amide group in the protein-bound glutamine and ε-amino group in a protein-bound lysine side chain, resulting in cross-links between the protein molecules (Babiker, 2000; Sergo, Kumazawa, Ohtsuka, Toiguchi, & Motoki, 1995; Sakamoto, Kumazawa, & Motoki, 1994, 1995). Further to confirm the attachment of polysaccharide and TGase polymerization of the protein digest, changes in the free amino groups of the protein before and after modification are shown in Fig. 2. The free amino groups of millet proteins ($OD_{340} = 0.18$) were greatly increased after chymotrypsin digestion ($OD_{340} = 0.36$). Polymerization of the protein digest greatly reduced the free amino groups and was found to be 0.08 and 0.03 for TGase and galactomannan polymers, respectively. Results revealed that the protein digest was cross-linked by TGase and polymerized by galactomannan as reported, for soy protein (Babiker, 2000).

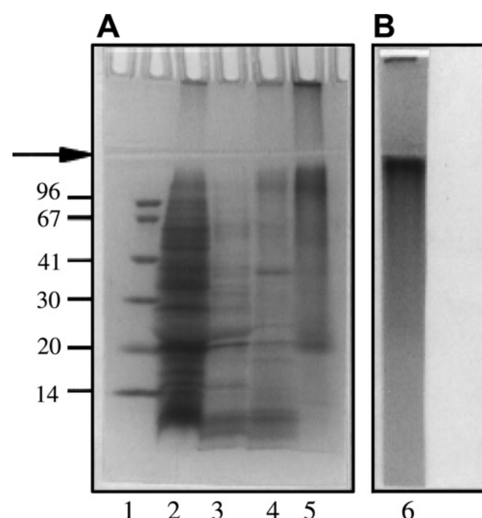

Fig. 1. SDS–PAGE patterns of millet protein modified with chymotrypsin, TGase treatment, and galactomannan conjugation. Panel A, Protein stain; Panel B, carbohydrates stain. Lane 1, molecular marker; lane 2, millet protein; lane 3, chymotrypsin digest; lane 4, TGase treated protein; lane 5, galactomannan conjugate heated for 7 days, lane 6, galactomannan conjugate. Arrows indicate the boundary between the stacking (upper) and separating (lower) gels.

3.2. Effect of the protein modification on the functional properties

The effect of various modifications on solubility of millet proteins at different pHs were investigated (Fig. 3). Millet

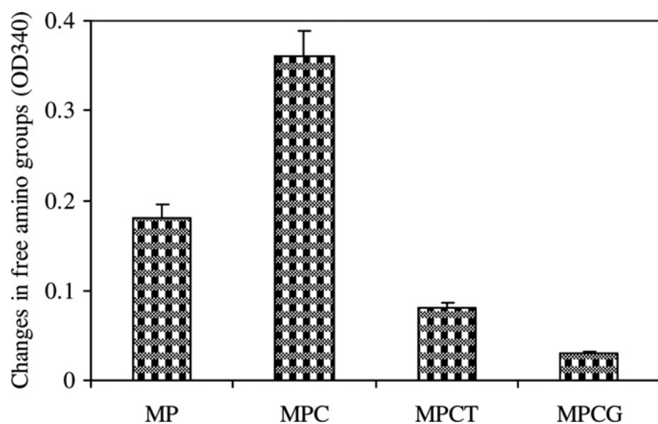

Fig. 2. Changes in free amino groups of millet protein (MP), chymotrypsin digested protein (MPC), MPC polymerized by TGase (MPCT) and MPC-galactomannan conjugate (MPCG). Error bars indicate the standard deviation of three replicates.

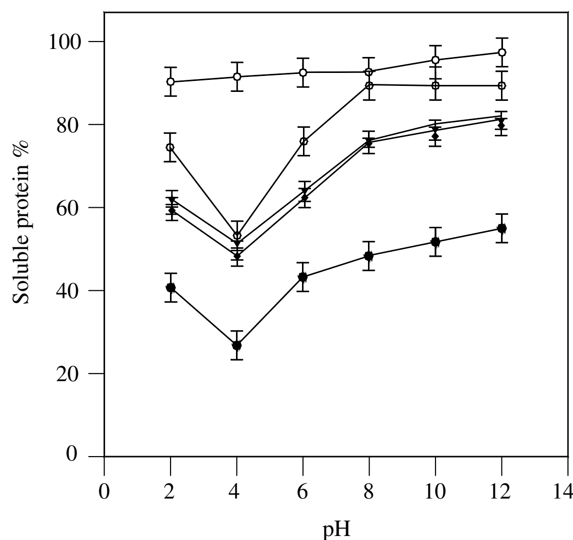

Fig. 3. Solubility of millet protein digested by chymotrypsin and modified by transglutaminase (TGase) or galactomannan conjugation. (●) native, (◆), chymotrypsin digest, (⦶) TGase-treated sample, (▼) protein–galactomannan/mixture, (○) protein–galactomannan conjugate. Error bars indicate the standard deviations ($n = 3$).

protein solubility was very poor at pH 4 (30%) and it increased slightly as the pH increased, with a maximum solubility of 50% at pH 12. Chymotrypsin digestion caused an improvement in solubility to 55% and 78% at pH 4 and 12, respectively. Chymotrypsin digest mixed with polysaccharide without dry heating gave results similar to those obtained for the digest. Dry heating of the mixture at 60 °C and 79% relative humidity for 7 days improved solubility of the protein at all level of pHs and was found to be 88% and 95% at pH 4 and 12, respectively. The improvement in the solubility at various pH levels is apparently due to the attachment of galactomannan to the protein through the Maillard reaction between amino groups in millet protein and the reducing-end carbonyl group in galactomannan, which buried the hydrophobic residues of the protein. Similar results were reported when chymotrypsin-treated gluten was conjugated with chitosan (Babiker, 2002). TGase treatment also caused improvement in solubility to 60% and 90% at pH 4 and 12, respectively. The improvement in solubility is likely to be due to an increase in the electrostatic repulsion, as a result of reduction in hydrophobic residues of the protein. Moreover, it is probable that TGase can deamidate the glutamine residues without cross-linkage, which might contribute also to solubility improvement (Motoki, Seguro, Nio, & Takinami, 1986). Heat stabilities of millet protein, chymotrypsin digest, galactomannan mixture and conjugate and TGase polymer are shown in Fig. 4. The solubility of millet protein decreased as the heating temperature increased and was found to be in a range between 35% and 47%. For both chymotrypsin digests and polysaccharide mixture the solubility slightly decreased as the heating temperature increased and was found to be 52% when heated at 90 °C. When the mixture was dry heated, the resultant conjugate was completely soluble even after heating at 90 °C for 20 min (95–90%). TGase polymer substantially resists heat-induced aggregation up to 70 °C after which its solubility slightly decreased and was found to be in the range between 65% and 90%. Similar results were reported for a soy protein–galactomannan conjugate (Babiker et al., 1999b) and an ovalbumin glucose 6-phosphate modification (Kato et al., 1995).

The effect of various modifications of millet protein on the emulsifying properties was investigated (Fig. 5). The emulsifying properties of millet protein were very poor while that of chymotrypsin digest was slightly improved.

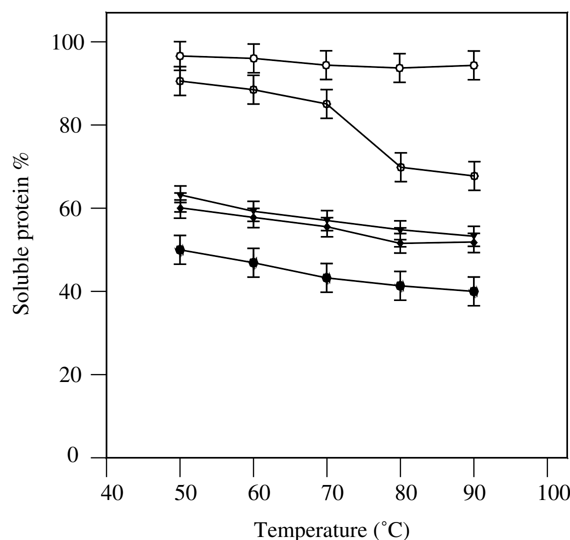

Fig. 4. Heat stability of millet protein digested by chymotrypsin and modified by transglutaminase (TGase) or galactomannan conjugation. (●) native, (◆) chymotrypsin digest, (⦶) TGase-treated samples, (▼) protein–galactomannan mixture, (○) protein–galactomannan conjugate. Error bars indicate the standard deviations ($n = 3$).

Dry heating of the protein polysaccharide mixture greatly improved the emulsifying properties. TGase treatment, on the other hand, also caused an improvement in the emulsifying properties. The emulsifying activity of millet protein, which is estimated by the turbidity of the emulsion measured immediately after emulsion formation was increased in the range between 0.48 and 0.90 after various modifications (Table 1). The emulsion stability (the half time of the initial turbidity) was found to be 2.0, 4.0, 6.0, 3.0 and 10.0 min for millet protein, chymotrypsin digest, TGase polymer, galactomannan mixture and conjugate, respectively (Table 1). The results revealed that the emulsion stability of millet protein was greatly improved by polysaccharide conjugation. It has been reported that the hydrophobic residues of millet protein may be anchored to the surface of oil droplets in an emulsion and the hydrophilic attached polysaccharide oriented to the water phase may cover the oil droplets, to inhibit their coalescence. Thus, a stable emulsion may be formed in the presence of the protein-polysaccharide conjugate. Similar results were reported for soy protein–galactomannan (Babiker et al., 1999b) and chitosan–lysozyme conjugates (Song et al., 2002).

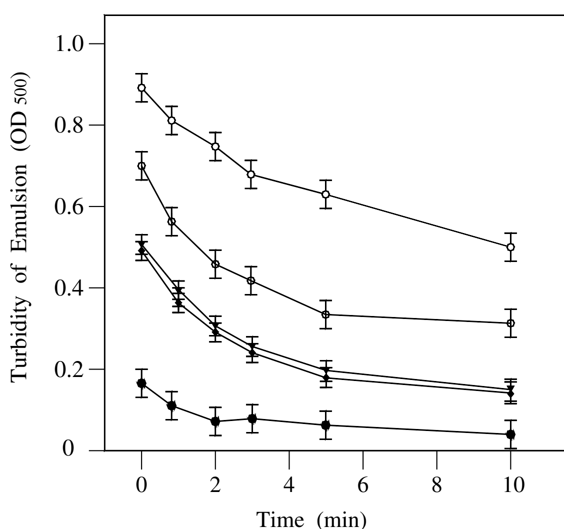

Fig. 5. Emulsifying properties of millet protein digested by chymotrypsin and modified by transglutaminase (TGase) or galactomannan conjugation. (●) native, (◆) chymotrypsin digest, (Ⓘ) TGase-treated samples, (▼) mixture, (○) conjugate. Error bars indicate the standard deviations ($n = 3$).

Table 1
Emulsifying properties of millet protein digested by chymotrypsin and modified by transglutaminase (TGase) or galactomannan conjugation

Samples	Emulsifying property (OD_{500})	Emulsion stability (min)
Native millet proteins	0.17 (±0.06)	2.00 (±0.10)
Chymotrypsin digest	0.48 (±0.12)	4.00 (±0.21)
TGase treatment	0.68 (±0.08)	6.00 (±0.51)
Galactomannan mixture	0.50 (±0.05)	3.00 (±0.10)
Galactomannan conjugate	0.90 (±0.21)	10.0 (±0.63)

Values are means (± SD).

In conclusion, the modification of millet protein by polysaccharide conjugation is a powerful tool in making dramatic improvements in the functional properties of food proteins. This method is promising for industrial application because it is naturally occurring reaction. In spite of only slight improvement in the functional properties by TGase compared to polysaccharide conjugation, it is also a promising tool for industrial application.

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