Changes in the functional properties as a function of NaCl concentration of legumes protein isolate by transglutaminase cross linking

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Abstract: The effect of cross linking of the protein isolates of three legumes with the microbial enzyme transglutaminase (EC 2.3.2.13) on the functional properties at different NaCl concentration was studied. The reduction in the total free amino groups (OD340) of the polymerized protein showed that TGase treatment cross-linking the protein subunit of each legume. The solubility of the protein polymer of each legume was greatly improved at high concentration of NaCl. At 1.2 M NaCl the solubility of the native legumes protein was significantly decreased but after polymerization slightly improved. Cross linked proteins were less turbid on heating to higher temperature as compared to native proteins and the temperature at which the protein turns turbid also increased in the polymerized proteins. The emulsifying and foaming properties of the protein polymer were greatly improved at all concentrations of NaCl for all legumes.

Keywords: Transglutaminase, functional properties, NaCl, legumes, protein isolate

Introduction

Plant proteins play significant roles in human nutrition, particularly in developing countries where average protein intake is less than that required. Because of inadequate supplies of food proteins, there has been a constant search for unconventional legumes, as new protein sources, for use as both functional food ingredients and nutritional supplements (Onweluzo *et al.*, 1994). Proteins have unique surface properties due to their large molecular size and their amphiphilic properties. However, the industrial applications of food proteins are limited, because proteins are generally unstable with heating, organic solvents and proteolytic attack. Therefore, if proteins could be converted into stable forms, their applications would be greatly broadened. Modification of food proteins has been investigated to improve their physical functionality, i.e. gelation, viscosity, emulsification and foaming (Motoki and Seguro, 1998). The ability of transglutaminase (TGase; E.C. 2.3.2.13) to modify the functional properties of food proteins has been extensively reviewed (Motoki and

Seguro, 1998; Lorenzen, 2000; Kuraishi *et al.*, 2001). By acyl group transfer between the ε-amino group of lysine and the γ-carboxyamide group of glutamine residues in proteins/peptides, TGase catalyses the formation of an ε-(γ-glutamyl) lysine isopeptide bond. In the absence of free ε-groups, water acts as the acyl acceptor, resulting in the deamidation of glutamine to glutamic acid. Food proteins are often denatured during processing, so there is a need to understand the protein both as a biological entity with a predetermined function, and as a randomly coiled biopolymer. Protein cross-linking has profound effects on their structure which affects the functional attributes of these proteins. Food processing often involves high temperature as in baking and low pH as in beverage industry. Such conditions can result in the introduction of protein cross links producing substantial changes in the structure of proteins and which can be reflected in the final product profile (Gerrard, 2002). The formation of this cross link does not reduce the nutritional quality of the food, as the lysine residue remains available for digestion. Chemical and physical methods are commonly used.

Food proteins can have their functionality altered by temperature and other chemical means. Specific functional attributes could be obtained by enzymatic polymerization of proteins and such enzymatic reaction could be controlled for desired time to enhance the functionality to the desired level (Singh, 1991). Work on enzymes, especially mammalian and microbial transglutaminases have been employed to modify proteins for functionality. The covalent cross linking of proteins catalyzed by transglutaminases can cause significant changes in the size, conformation, stability and other properties of the proteins by enhancing protein–protein interaction. The enzymes have been used for modifying the functionalities of various proteins. In this study, an attempt was made to investigate the effect of transglutaminase cross linking on the functional properties of protein isolate of three legumes.

Materials and Methods

Materials

    Phaseolus (*Phaseolus vulgaris*), pigeon pea (*Cajanus cajan*) and cowpea (*Vigna unguiculata* (L.) were obtained from Khartoum North local market. Refined corn oil was brought from Bitar Company, Khartoum, Sudan. Transglutaminase was donated by Professor Akio Kato, Yamaguchi University, Faculty of Agriculture, Department of Biological Science, Japan. Unless otherwise stated all chemicals used in this study were reagent grade.

Methods
Protein isolates preparation
    The protein isolate was prepared by the method of Iwabuchi and Yamauchi (1987). A sample of defatted meal (100 g) was extracted once with 2 l of 0.03M Tris-HCl buffer (pH 8) containing 10 mM 2-mercaptoethanol (2-ME) at 20 °C. After centrifugation, the supernatant was acidified to pH 4.8 with 2N HCl and then centrifuged. The precipitated 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.

Transglutaminase treatment
    The protein isolate of each legume was dissolved in 0.1 M phosphate buffer (pH 7.5; 10 mg/ml) and then reacted with TGase (0.5 mg/ml). The mixture was incubated at 55 °C for 60 min. The enzyme was inactivated by N-ethylmaleimide (0.1 ml; 0.1%) (Kato *et al.*, 1991). The treated samples were dialyzed against distilled water and then freeze-dried.

Changes in free amino groups
    Changes in free amino groups of 0.1% protein solutions were determined by spectrophotometric assay (OD_{340}) using o-phthaldiadehyde as described by Church *et al*, (1983).

Measurement of solubility
    The samples of the native protein and that polymerized by transglutaminase (0.2%) were used for the determination of solubility at different NaCl concentration (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2M). Samples were dissolved in the buffer and shaken with a vortex mixer (Scientific Industries, adjusted on digit 4 to work on touch) for 10s, and the turbidity was measured at 500 nm. Values obtained are means of triplicate samples.

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. Protein turbidity was measured at 500 nm.

Measurement of emulsifying properties
    The emulsifying properties of the samples were determined by the method of Pearce and Kinsella (1978). To prepare emulsions, 3.0 ml of corn oil and 10.0 ml of protein solution (0.2%) in 0.1M phosphate buffer (pH 7.0) were shaken together and homogenized in an Ultra Turrax instrument (Hansen & Co., Germany) at 12 000 g for 1 min at 20 °C. Then the mixture was poured into centrifuge tubes and centrifuged at 2000 rpm for 5 minutes then poured into 50 ml measuring cylinders and stays a few minutes until the emulsified layer was stable. Emulsifying activity (EA) as a function of NaCl concentration (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2M) was determined according to the formula:

$$EA\% = \frac{\text{Height of emulsified layer}}{\text{Height of total content in the cylinder}} \times 100$$

For the emulsion stability, about 50 µl of the homogenized mixture was taken from the bottom of the container at different times and NaCl concentration and diluted with 5 ml of a 0.1% sodium dodecylsulfate solution. The absorbance of the diluted emulsion was then determined at 500 nm. The turbidity was plotted against the time at each NaCl concentration and then the emulsion stability was estimated by measuring

the half-time of the initial turbidity of the emulsion.

Measurement of foaming properties

Foaming capacity of the sample was determined by following the procedure described by Lawhon *et al.* (1972). About 2.0 gm of the sample were blended with 100 ml buffer at different NaCl concentration (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2M) in a Moulinex blender at "HI" speed for 2 minutes. The mixture was poured into a 250 ml measuring cylinder and the foam volume was recorded after 30 sec.

$$FC\% = \frac{\text{Volume after whipping} - \text{volume before whipping}}{\text{Volume before whipping}} \times 100$$

FC% was determined as a function of NaCl concentration (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2M). The foam stability (FS) was conducted according to Ahmed and Schmidt (1979). The FS was recorded at 15 minutes interval for the first 15 min after pouring the material in a cylinder. FS was determined using the following formula:

$$FS\% = \frac{\text{Foam volume after 15 min}}{\text{Initial foam volume}} \times 100$$

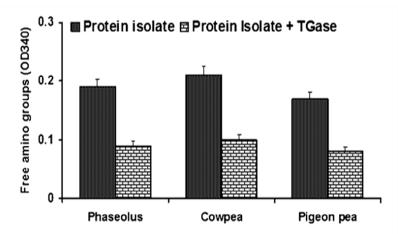FS% was determined as a function of NaCl concentration (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2M).

Results and Discussion

Effect of transglutaminase (TGase) treatment on the free amino groups and solubility of legumes protein isolate.
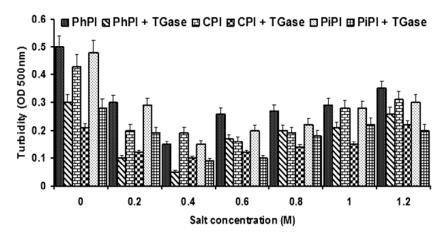
The native protein of each legume polymerized by TGase showed changes in free amino groups of the protein as shown in Figure 1. The free amino groups (OD_{340}) of the protein isolate of the three legumes were greatly reduced after TGase treatment. After TGase treatment about 49, 50 and 66% of the free amino groups of phaseolus, cowpea and pigeon pea, respectively were cross-linked. Results revealed that most of the protein molecules of each legume were cross-linked by TGase. The result indicated that TGase may have catalysed 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 (Sakamoto *et al.*, 1995; Sergo *et al.*, 1995). Similar results were obtained when soy protein and chymotrypsin digests were polymerized by TGase (Babiker, 2000).

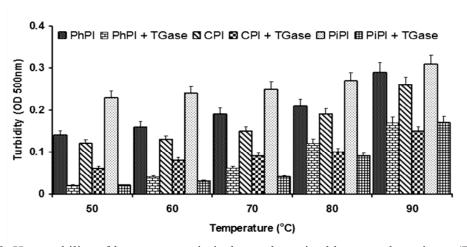The effect of NaCl concentration on solubility of the protein isolate of three legumes with and without TGase treatment was investigated (Figure 2). The results showed that the protein isolate of each legume had low solubility before addition of salt and at high salt concentration. However, after being polymerized by TGase, the protein isolate solubility was greatly improved even at high salt concentration. The solubility (OD_{500nm}) of the native proteins of the legumes was found to be 0.5, 0.43 and 0.48 and after TGase treatment was greatly reduced to 0.3, 0.21 and 0.28 for phaseolus, cowpea and pigeon pea protein isolates, respectively. Addition of NaCl greatly improved the solubility of the protein isolate of the legumes and further improvement was observed after polymerization of the protein isolate of all legumes. The improvement in the solubility due to TGase treatment of the protein isolate at different NaCl concentration is mainly due to the fact that the protein isolate had recognizable sites for TGase reaction and the resultant polymer, as shown in Figure 1, had lower level of free amino groups. Also, TGase treatment decreases the surface hydrophobicity of the proteins molecules and increases the electrostatic repulsion as a result of partial deamidation of glutamine and asparagine (Hassan *et al.*, 2007). Addition of NaCl at high concentration causes negatively charged chloride ions to interact with the positively charged proteins, thereby decreasing electrostatic repulsions and enhancing hydrophobic interactions. The increase in hydrophobic interactions would result in a higher tendency for the protein to form insoluble aggregates, thus decreasing solubility (Aluko and Yada, 1993). However, polymerization of the native protein isolate might increased the net negative charge on the protein, coupled with the salting-in effect of NaCl on the hydrophobic interactions dissociates the protein aggregates, and solubility increases. The solubility (OD_{500nm}) of the protein isolate of all legumes decreased as the heating temperature increased (Figure 3) and the turbidity was observed to reach 0.29, 0.26 and 0.31 for the native protein isolate and after polymerization the turbidity was decreased to 0.17, 0.15 and 0.17 at 90°C for phaseolus, cowpea and pigeon pea, respectively. Results indicated that TGase treatment was found to be effective in improving heat stability of legumes protein compared to the native protein. Proteins treated with transglutaminase form more compact structures which make it more heat stable. Similar increase in the thermal stability of oat globulin has been attributed to the formation of aggregates with compact network (Nai *et al.*, 2002). Transglutaminase often increase thermal stability by intramolecular or intermolecular interaction. Cross-linking of sodium caseinate with transglutaminase

Figure 1.   Changes in free amino groups of the protein isolate of selected legumes treated with transglutaminase (TGase). Error bars indicate the standard deviation of three replicates.

Figure 2. Solubility of legumes protein isolate polymerized by transglutaminase (TGase) at different NaCl concentration (M). PhPI, Phaseolus protein isolate; CPI, cowpea protein isolate; PiPI, Pigeon pea protein isolate. Error bars indicate the standard deviations (n = 3).

Figure 3. Heat stability of legumes protein isolate polymerized by transglutaminase (TGase) at different temperature. PhPI, Phaseolus protein isolate; CPI, cowpea protein isolate; PiPI, Pigeon pea protein isolate. Error bars indicate the standard deviations (n = 3).

resulted in the lower turbidity at 140 °C. This indicated that the cross linked products were more heat stable than the unmodified sodium caseinate (Flanagan *et al.*, 2003).

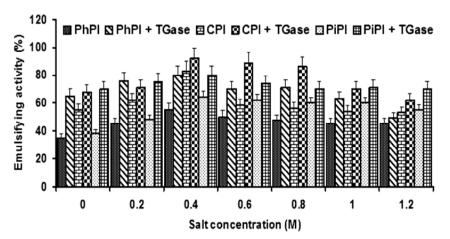*Effect of TGase treatment on physical functionality of legumes protein isolate*

The physical functionalities such as the emulsification and foaming properties of the native protein isolate of the legumes are poor and in order to improve these physical properties, the effect of TGase treatment was investigated. As shown in Figure 4, the emulsifying activity of the protein isolate polymers was improved. The emulsifying activity of the native protein isolate, which is estimated as a percentage of emulsion, was greatly improved as NaCl concentration increased up to 0.4M with a optimum concentration of 0.4M at which the emulsifying activity was found to be high for both native and polymerized protein isolate of all legumes. Further increase in NaCl concentration was observed to decrease the emulsifying activity for the native protein and even after polymerization. The emulsion stability (the half time of the initial turbidity) of legumes native protein isolate was 2, 4 and 2 min and after polymerization it increased to 4, 6 and 4 min for phaseolus, cowpea and pigeon pea protein isolate, respectively (Figure 5). Addition of NaCl improved the emulsion stability of the native protein isolate up to 0.4M and further improvement was observed after polymerization. Also it was observed that TGase treatment alleviated the effect of high concentration of NaCl. Similar results were reported by Olayide (2004). The improvement in the emulsifying properties is likely due to an increase in the negative charges which result from the hydrolysis of the amide groups in glutamine and asparagine, as reported for millet protein (Hassan *et al*, 2007). The results obtained show that polymerization of legumes protein was very effective in the improvement of the emulsifying properties. It has been reported that addition of NaCl increased both emulsifying activity and stability. However, further increase in ionic strength progressively reduced both emulsifying activity and emulsion stability. Increase in ionic strength up to 0.2 M encouraged unfolding of protein molecules and subsequent increase in protein solubility (Aluko and Yada, 1995). This increase in protein solubility enhanced a rapid migration to the oil–water interface and improved emulsifying activity of the protein. With further increase in ionic strength (>0.2 M), screening of the surface charges increased and this encouraged protein–protein interaction but reduced protein–oil interaction.

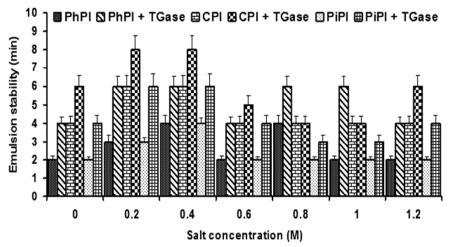The foaming properties of the legumes protein isolate were also improved after polymerization by TGase (Figures 6 and 7). Increase in concentration of NaCl favoured the foaming capacity up to 0.6M concentration, while further increase in concentration, from 0.8M to 1.2M reduced the foaming capacity of the native protein isolate of all legumes (Figure 6). The foaming capacity of the proteins was high at 0.4M concentration and after polymerization was further increased for all legumes protein isolate. The foam stability (Figure 7) of the protein was determined as a percentage of total whipping volume after the mixture was stands for 15 min. As shown in Figure 7, the foam stability of the protein isolate stand for 15 min was high when NaCl of a concentration of 0.2M was added. Transglutaminase treatment greatly improved the foam stability of the proteins of all legumes. Akintayo *et al*. (1999) reported initial increase in foam capacity and stability of pigeon pea protein concentrate up to 0.5 M NaCl protein solution, while further increase in ionic strength markedly reduced foam capacity and stability. The improvement of the foaming properties of the protein polymers reflects the importance of protein association or polymerization as a structural factor governing the foaming property. The higher emulsion and foaming attributes of the treated protein could have been due to increased ability to form a interfacial protein film, since its high molecular size and cross linked structure are more resistant to excessive denaturation than the native protein at the high speed of the homogenization used to make emulsions and foams. Moreover, reduced electrostatic repulsion as the result of decrease in the number of amino groups could have enhanced protein–protein interaction and therefore protein adsorption on the interface. Transglutaminase catalysed polymers of cowpea proteins were also found to form better foam and emulsion forming ability than the native protein, results that were attributed to increase in strengthening of the interfacial protein film by the polymerized proteins (Aluko and Yada, 1995).
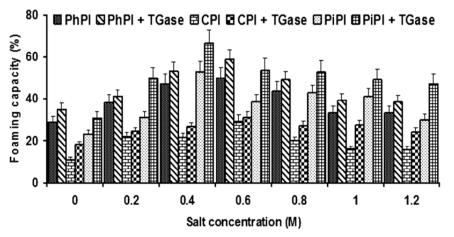
Conclusion

The protein–protein complexes obtained form cross linking of legumes protein isolate had improved functional properties in the presence of high salt concentration. The solubility and heat stability of the polymers was enhanced significantly than the unpolymerized proteins. Thus transglutaminase could be used to improve the functional attributes of proteins with varied applications in food products.
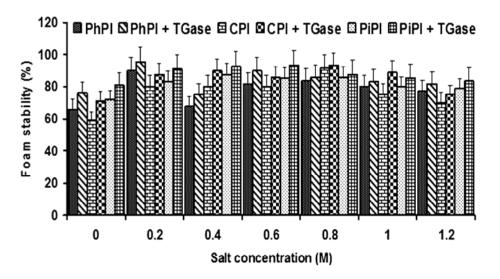
Figure 4. Emulsifying activity of legumes protein isolate polymerized by transglutaminase (TGase) at different NaCl concentration (M). PhPI, Phaseolus protein isolate; CPI, cowpea protein isolate; PiPI, Pigeon pea protein isolate. Error bars indicate the standard deviations (n = 3).

Figure 5. Emulsion stabilty of legumes protein isolate polymerized by transglutaminase (TGase) at different NaCl concentration (M). PhPI, Phaseolus protein isolate; CPI, cowpea protein isolate; PiPI, Pigeon pea protein isolate. Error bars indicate the standard deviations (n = 3).

Figure 6. Foaming capacity of legumes protein isolate polymerized by transglutaminase (TGase) at different NaCl concentration (M). PhPI, Phaseolus protein isolate; CPI, cowpea protein isolate; PiPI, Pigeon pea protein isolate. Error bars indicate the standard deviations (n = 3).

Figure 7. Foam stability of legumes protein isolate polymerized by transglutaminase (TGase) at different NaCl concentration (M). PhPI, Phaseolus protein isolate; CPI, cowpea protein isolate; PiPI, Pigeon pea protein isolate. Error bars indicate the standard deviations (n = 3).

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