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In vivo Evaluation of Cross-Linked Milk and Wheat Proteins Mediated by Microbial Transglutaminase in White Wistar Rats

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Abstract: The present study was designed to evaluate the *in vivo* nutritional quality of the modified proteins of milk and wheat by cross-linking with microbial transglutaminase (TGase). White Wistar rats were divided into six groups receiving diets that contained casein, cross-linked milk protein, milk protein, cross-linked wheat protein, wheat protein, or a protein free diet. Results showed that cross-linked milk and wheat proteins can support growth, with the animals showing a positive nitrogen balance. Protein true digestibility was similar between casein and non-cross-linked milk protein diets. It was also observed that milk and wheat proteins were not affected by cross-linking concerning several quality parameters: protein efficiency ratio, food efficiency ratio, food transformation index, apparent nitrogen digestibility, true digestibility, biological value, net protein utilization, net protein ratio and protein retention efficiency. Based on these results, it can be suggested that the use of microbial TGase does not affect the nutritional quality of milk or wheat proteins, while improving their physicochemical properties.

Key words: Protein quality, milk proteins, wheat proteins, protein cross-linking, transglutaminase

INTRODUCTION

Foods are multi-components matrices of complex structures. The macromolecular structures of foods influence their mechanical and physical properties, chemical and microbiological stability, sensory properties and nutrition value. The nutritional and sensory properties of food play a major role in product quality because they are recognized by consumers and are the main factors behind food choices (Dickinson, 1997) and many of these nutritional and sensory properties are determined by proteins (Sakamoto *et al*., 1994). The nutritional quality of a protein is mainly due to its essential amino acid composition and digestibility. Functional properties such as solubility, water-holding capacity, viscosity, gelation, coagulation, emulsification and foaming, will all influence the food characteristics (Panyam and Kilara, 1996).

Modifications of food proteins can be achieved by chemical and enzymatic methods that have been shown to be powerful tools for improving the functional properties of the final product (Gerrard, 2002). The use of enzymes to modify the functional properties of food proteins is an area that has attracted considerable interest because consumers perceive enzymes to be more natural than chemicals (Singh, 1991). Protein functionality can be modified by intra- or intermolecular cross-linking (Jong and Koppelman, 2002). The cross-linking of food proteins by enzymatic reactions produces substantial changes in their structures and can modify many properties of the food such as texture,

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viscosity, solubility, water-holding capacity, thermal stability, emulsification and gelation (Kuraishi, 2000). Transglutaminase (TGase) is an enzyme that has received much attention for its ability to catalyze the cross-linking reaction in proteins (Kuraishi *et al*., 2001).

Transglutaminase (TGases) (protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyses acyl-transfer reactions between the γ-carboxyamide groups of peptide-bound glutamine residues and a variety of primary amines (Motoki and Seguro, 1998). When the ε-amino group of a peptide bound lysine residue acts as substrate, the two peptide chains are covalently linked through an ε-(γ-glutamyl)lysine bond. Thus, these enzymes are capable of introducing covalent intra- or intermolecular cross-linking of the proteins. TGases have been found in animal, plant tissues and microorganisms (Zhu *et al*., 1995). Microbial TGase has widespread and growing applications in food processing industry (Kuraishi *et al*., 2001). The enzyme has been used for improving the functional properties of several proteins including meat, soybean, milk and wheat proteins (Kuraishi *et al*., 2001; Motoki and Seguro, 1998; Zhu *et al*., 1995). Among dairy proteins, casein has shown to be a good substrate for TGase (Bonisch *et al*., 2006). It has been shown that the microstructure of yogurt can be improved by treatment of milk with TGase (Lauber *et al*., 2000) and the cross-linking of milk proteins by TGase appears to be an acceptable alternative to the addition of extra protein or stabilizer in the production of non-fat yogurt (Ozer *et al*., 2007). The treatment of milk with TGase improves its heat stability, probably by preventing dissociation of k-casein from the micelles (O'Sullivan *et al*., 2002). Wheat gluten was also shown to be cross-linked by TGase and the action of the enzyme reinforces the protein network structure causing the change of viscoelastic properties of the dough (Larré *et al*., 2000). TGase applications increased volume and improved structure of breads and dough stability (Gerrard *et al*., 1998) and also improved baking qualities of weak wheat flours (Basman *et al*., 2002).

Although, these studies show the improvement of functional properties of milk and wheat proteins by microbial TGase cross-linking, information about changes in nutritional properties has not been reported. The introduction of covalent cross-links between proteins in food produces substantial changes in their structures and therefore is expected to have some effects on nutritional quality of the final product (Gerrard, 2002). The formation of covalent bond of amino acids in the same or in another protein molecule may decrease digestibility and biological availability of essential amino acids that involved in the cross-linking reaction (Erbersdobler, 1989). Therefore, the present study was conducted to investigate the nutritional effects of cross-linked milk and wheat proteins mediated by microbial TGase in White Wistar rats as a model of biological system.

MATERIALS AND METHODS

Materials

Commercial milk powder was obtained from Eleva SA (Rio Grande do Sul, Brazil), while commercial wheat flour was bought from Bunge Alimentos SA (Santa Catarina, Brazil). Microbial TGase was kindly provided by Ajinomoto Inc. (Tokyo, Japan), with a declared enzymatic activity of 100 U g^{-1}. All reagents were of analytical grade.

Texture Evaluation

A study was performed to determine the effect of TGase reaction time on texture of samples. The mixtures was mixed with water to the following proportions: 50 g milk powder:25 mL water; 50 g wheat flour:38 mL water. Milk powder and wheat flour samples were incubated with 1 % (w/w) of TGase at 37°C for an incubation time of 0, 1, 2, 3 and 4 h. Control samples were incubated without enzyme. The texture was determined using a Texture Analyzer TA-XT2 (Stable Micro Systems Ltd., New York, USA). Breaking force (g) was determined at room temperature using a cylindrical stainless

steel probe with a pressing surface of 962 mm^2 and the following conditions: measurement time, 14 sec; penetration speed, 0.5 mm sec^{-1}; penetration distance, 7 mm into surface. Gel strength was carried out in triplicate and expressed as kg m^{-2} of probe area.

Protein Sample Preparations

The cross-linking of both milk and wheat proteins was prepared by mixing either milk powder or wheat flour with 1 % (w/w) of TGase powder preparation. The preparations above described were incubated at 37°C for 2 and 3 h, respectively. These samples were designated Cross-linked Milk Protein (CMP) and Cross-linked Wheat Protein (CWP), respectively. The milk powder and wheat flour were incubated at 37°C for 2 and 3 h, without TGase and these samples were designated, respectively, Milk Protein (MP) and Wheat Protein (WP). All four samples were dried in an air circulation oven at 45°C and powdered in a food micro-homogenizer to pass through a 60-mesh sieve. These flours were stored at 4°C for the subsequent chemical analysis and for the preparation of respective diets.

Diets Composition of Rats

Table 1 shows the compositions of the tested protein diets (CMP, MP, CWP and WP). One standard protein diet (casein) and one protein free diet were used as controls to estimate the endogenous nitrogen excretion of the rats. Standard and test diets were adjusted to 10% protein content, whereas the non-protein diet was devoid of protein. In addition to the protein sources, the diets contained vitamin mix (Roche, Brazil) and mineral mix, prepared according to AIN-93G as published by Reeves *et al*. (1993). Cellulose, casein, corn starch, sucrose, L-cystine and choline bitartrate were bought from Farmaquimica (São Paulo, Brazil), while soybean oil was purchased from Bunge (Gaspar, Brazil). For the preparation of the diets, the ingredients were mixed and passed through a 60-mesh sieve to ensure uniform distribution of minerals and vitamins. All diets were analyzed for their moisture, protein, lipid and ash contents by standard chemical procedures.

Experimental Design for Rats Feeding Trials

All rats experiments were approved by the Committee of Bioethics of the University (see note at the end of this work). Young, 21-25 days old white male Wistar rats weighting 54.5±5.8 g were obtained from the Central Animal House, Pelotas Federal University, Rio Grande do Sul, Brazil. The rats were randomly divided into six groups, each consisting of six individuals that were separately

Table 1: Composition of the experimental diets

			Test diets			
Ingredients (g kg^{-1})	Basal diet	Casein diet	CMP	MP	CWP	WP
Casein	-	123.5	37.1	37.1	37.1	37.1
CMP flour	-	-	290.0	-	-	-
MP flour	-	-	-	305.3	-	-
CWP flour	-	-	-	-	658.5	-
WP flour	-	-	-	-	-	675.7
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Soybean oil	70.0	70.0	34.4	28.1	59.1	59.9
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Salt mixture[a]	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin mixture[a]	10.0	10.0	10.0	10.0	10.0	10.0
L-cystine	-	3.0	-	-	-	-
Choline bitartrate	-	2.5	-	-	-	-
Corn starch	735.0	606.0	443.5	434.5	50.3	32.3

[a]According to Reeves *et al*. (1993), Basal diet: Non-protein diet, Casein diet (control diet): Standard protein diet, CMP: Cross-linked Milk Protein, MP: Milk Protein, CWP: Cross-linked Wheat Protein, WP: Wheat Protein

housed in stainless steel metabolic cages designed for separate collection of faeces and urine. Cages were located in a room with 12 h light/dark cycle, at a temperature of 21±2°C, fitted with appropriate ventilation system. Food and water were given *ad libitum*.

Effect of Diets on Rats Growth

For growth studies, the rats were individually housed in stainless steel metabolic cages. One group of rats was fed a casein diet (standard diet), while the other four groups were fed test diets (CMP, MP, CWP and WP). The rats were weighed at the start of experiment and then again on every other day. Food and water were given ad libitum. Diets were given daily and the unconsumed feed was collected and weighed. The rats fed on different experimental and control diets were weighed for four weeks and the gain in weight during this period was recorded. The consumption of protein was calculated as nitrogen consumed, based on the content of nitrogen in the diets. Protein Efficiency Ratio (PER), Food Efficiency Ratio (FER) and Food Transformation Index (FTI) were calculated by the following formulas:

$$\text{PER} = \frac{[\text{Gain in body weight (g)}]}{[\text{Protein consumed (g)}]} \quad (1)$$

$$\text{FER} = \frac{[\text{Gain in body weight (g)}]}{[\text{Food consumed (g)}]} \quad (2)$$

$$\text{FTI} = \frac{[\text{Food consumed (g)}]}{[\text{Gain in body weight (g)}]} \quad (3)$$

The corrected protein efficiency ratio (C-PER) was calculated according to Chapman *et al.* (1959), where 2.5 is the standard value for casein:

$$\text{C} - \text{PER} = \frac{\text{PER} \times 2.5}{[\text{Determined PER for reference casein}]} \quad (4)$$

At the end of 4 weeks the final body weights of animals were recorded. After sacrifice, the liver, both kidneys and the spleen were carefully resected and immediately weighed. The weights of these organs were expressed per 100 g of final body weight.

Nitrogen Balance Experiments

The studies of nitrogen balance were carried out according to Miller and Bender (1955). Rats were individually housed in stainless steel metabolic cages. One group of rats was fed with non-protein diet (basal diet) and another on a casein diet (control diet). Four groups of rats were fed on test diets (CMP, MP, CWP and WP). The experiment was conducted for 14 days, which included an initial conditioning period of four days. During the last ten days, urine and faeces of each rat were separately collected. Food and water were given ad libitum and the change in body weight was recorded. The faeces were oven-dried at 100°C for 24 h. The dried samples were grounded to 20 meshes. The concentration of nitrogen in the urine and faecal powder was estimated by the microKjeldhal method. The non-protein diet group was used to measure the metabolic faecal nitrogen and the endogenous urinary nitrogen. Data obtained from this experiment were used to calculate Nitrogen Absorbed (NA), Nitrogen Retention (NR), Apparent Nitrogen Digestibility (AND), True Digestibility (TD), Biological Value (BV), Net Protein Utilization (NPU) and Net Protein Retention (NPR) and Protein Retention Efficiency (PRE), as described by Bender and Doell (1957), by employing the following formulas:

$$NA = NI - NF_1 \tag{5}$$

$$NR = NI - (NF_1 + NU_1) \tag{6}$$

$$AND = \frac{[NI - NF_1]}{NI} \times 100 \tag{7}$$

$$TD = \frac{NI - (NF_1 - NF_2)}{NI} \times 100 \tag{8}$$

$$BV = \frac{NI - (NF_1 - NF_2) - (NU_1 - NU_2)}{NI - (NF_1 - NF_2)} \times 100 \tag{9}$$

$$NPU = \frac{BV \times TD}{100} \tag{10}$$

$$NPR = \frac{[\text{Weight gain of test group} + \text{Weight loss of protein} - \text{free group}]}{[\text{Weight of test protein consumed}]} \tag{11}$$

$$PRE = NPR \times 16 \tag{12}$$

where, NI is nitrogen intake of animals fed test diet, NF_1 the excreted nitrogen in faeces of animals fed test diet, NF_2 the excreted nitrogen in faeces of animals fed non-protein diet (basal diet), NU_1 the excreted nitrogen in urine of animals fed test diet and NU_2 the excreted nitrogen in urine of animals fed non-protein diet.

Statistical Analysis

Experimental data were analyzed by one-way ANOVA and Tukey's highly significant difference test. Differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Texture Evaluation of Proteins Samples

The maximal gel strength of milk and wheat proteins was obtained at 2 and 3 h, respectively. Results show that gel strength for the milk powder (Fig. 1A) is similar ($p>0.05$) for incubation times of 2, 3 or 4 h and for wheat flour samples the gel strength (Fig. 1B) is similar ($p>0.05$) only after 3 and 4 h of incubation with the enzyme. The susceptibility of a protein to TGase-induced cross-linking depends on the macromolecular structure of the protein. Earlier studies have shown that wheat gluten proteins can be cross-linked by TGase, despite their low lysine content because of their high glutamine content (Larré *et al*., 2000). Among the milk proteins, the casein fraction has been shown to be a good substrate for TGase (Bonisch *et al*., 2006).

Proximate Chemical Composition of Protein Samples

The protein content was 1.20% higher in CMP flour than in MP flour and only 0.27% higher in CWP flour than in WP flour (Table 2). Some researchers have shown that the protein content increased after the cross-linking with TGase. Ahn *et al*. (2005) observed that after the treatment with TGase, the protein contents of wheat, barley and soy flours increased 0.6, 0.8 and 1.6%, respectively. Similarly, Rosell *et al*. (2003) found that the wet gluten content of wheat flours was slightly increased with TGase treatment due to the polymerization of proteins.

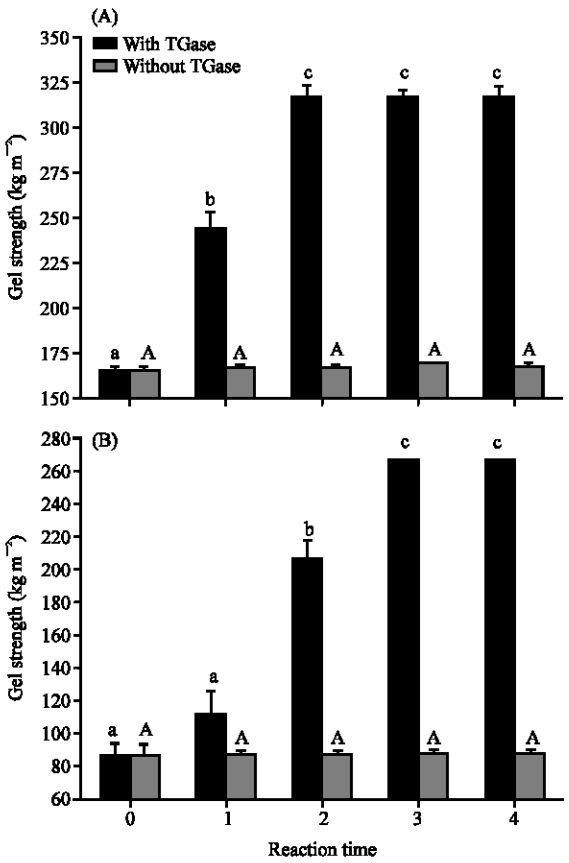

Fig. 1: Texture of non-crosslinked and cross-linked samples of milk powder and wheat flour modified by TGase (measured by gel strength). (A) milk protein and (B) wheat protein. Equal letter(s) indicate that samples do not significantly differ ($p>0.05$) (capital caps: samples without enzymatic treatment; lower: enzymatically treated samples)

Table 2: Chemical composition (%) of non-crosslinked and cross-linked milk protein and wheat flour

Samples	Moisture	Protein	Lipids	Ash
CMP	13.73±0.04	24.13±0.02	12.28±0.13	5.33±0.03
MP	10.64±0.02	22.93±0.12	13.72±0.09	5.56±0.02
CWP	10.98±0.01	10.63±0.11	1.65±0.06	0.49±0.05
WP	11.55±0.03	10.36±0.07	1.49±0.03	0.59±0.09

Values are means±SD of triplicate analysis, CMP: Cross-linked Milk Protein, MP: Milk Protein, CWP: Cross-linked Wheat Protein, WP: Wheat Protein

Effect of Diets on Rats Growth

Figure 2 shows the cumulative body weight gain of the rats fed on control and test diets during the 28 days of treatment, with a linear increase of the body weight been observed for all groups. Rats fed on control diet (casein diet) grew faster than rats fed on other diets, while the growth rate of rats fed on Wheat Protein (WP) was the lowest. However, rats fed on either Cross-linked Milk Protein (CMP) or Milk Protein (MP) grew at a rate that was not significantly different ($p<0.05$) from casein diet (Table 3). Rats fed on either Cross-linked Wheat Protein (CWP) or Wheat Protein (WP) had growth rates not significantly different (Table 3). As shown in Fig. 2, the body weight gain of rats fed on cross-linked protein diets was slightly higher than the respective non-cross-linked diet.

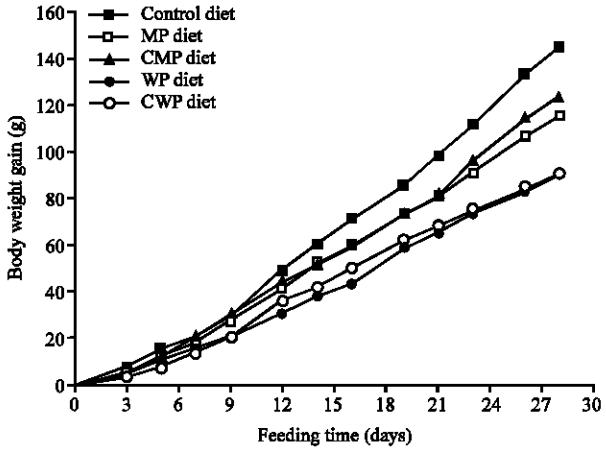

Fig. 2: Body weight gain of rats fed experimental diets. Control diet (Casein diet): Standard protein diet; MP diet: Milk Protein diet; CMP diet: Cross-linked Milk Protein diet; WP diet: Wheat Protein diet; CWP diet: Cross-linked Wheat Protein Diet

Table 3: Effects of feeding experimental diets on the growth performance of rats

		Test diets			
Parameters	Casein diet	CMP	MP	CWP	WP
Food intake (g)	379.67±70.22^{a}	397.77±28.91^{a}	373.80±51.29^{a}	370.07±32.02^{a}	376.62±12.99^{a}
Total weight gain (g)	145.35±26.60^{a}	123.81±12.43^{a}	115.41±20.43^{ac}	91.06±6.54^{bc}	91.19±7.77^{bc}
PER	3.78±0.22^{a}	3.32±0.18^{b}	3.31±0.22^{b}	2.34±0.30^{c}	2.38±0.16^{c}
C-PER	2.50±0.00^{a}	2.20±0.12^{b}	2.19±0.15^{b}	1.55±0.20^{c}	1.58±0.10^{c}
FER	0.38±0.02^{a}	0.31±0.02^{b}	0.31±0.02^{b}	0.25±0.03^{c}	0.24±0.02^{c}
FTI	2.61±0.16^{a}	3.22±0.17^{b}	3.26±0.21^{b}	4.08±0.47^{c}	4.15±0.27^{c}

Values are means±SD of six rats in each group throughout 28 days of experimental period, Means with different superscripts in the same horizontal row are significantly different ($p<0.05$); Casein diet (control diet): Standard protein diet, CMP: Cross-linked Milk Protein, MP: Milk Protein, CWP: Cross-linked Wheat Protein, WP: Wheat Protein, C-PER: Based on values of 2.5 as standard for casein. PER = protein efficiency ratio, C-PER: Corrected protein efficiency ratio, FER: Food Efficiency Ratio, FTI: Food Transformation Index

Food intakes after 28 days on the CMP and casein diets were the highest, 397.77 and 379.67 g per rat, respectively. However, these values did not significantly differ ($p>0.05$) from the other three diets where food intake ranged from 370.07 to 376.62 g per rat. The results of food intake for the rats fed on CMP and CWP diets, 397.77 and 370.07 g per rat, respectively, were similar to the values obtained by Seguro *et al*. (1996) for the rats fed with diets containing cross-linked casein.

Body weight gain on the control group (casein diet) was the highest (145.35 g per rat) after 28 days of feeding trial, but the difference was not significantly ($p>0.05$) different from CMP and MP diets (123.81 and 115.41 g per rat). The body weight gain was significantly lower ($p<0.05$) in the groups fed on wheat protein and cross-linked wheat protein. The cross-linking reaction by TGase of the milk and wheat proteins showed no effect on the food intake and body weight gain of the rats after 28 days. Our results suggest that cross-linked milk and wheat proteins could support the growth of young rats similarly to the non-cross-linked proteins. Moreover, the cross-linked milk protein supports the growth of the test rats similarly to the standard casein diet.

The casein diet produced a PER of 3.78, which was significantly higher ($p<0.05$), than cross-linked and non-cross-linked milk protein diets. The PER of these three formulations were significantly higher ($p<0.05$) than the PER of the cross-linked and non-cross-linked wheat proteins (2.34 and 2.38, respectively), probably due to limiting amino acids in the wheat gluten, a lysine-poor protein. Protein

Table 4: Relative weight of the organs of rats fed experimental diets

		Test diets			
Organs	Casein diet	CMP	MP	CWP	WP
Liver (%)	3.97±0.42	3.95±0.40	3.84±0.25	3.55±0.27	3.70±0.44
Right kidney (%)	0.44±0.05	0.42±0.03	0.45±0.05	0.44±0.04	0.42±0.02
Left kidney (%)	0.42±0.04	0.42±0.04	0.43±0.04	0.43±0.02	0.41±0.02
Spleen (%)	0.29±0.03	0.26±0.05	0.28±0.05	0.26±0.02	0.27±0.02

Data are reported as mean±SD (n = 6) based on the percentage of the organ weight in relation to total body weight at the end of 28 days of feeding trial. Data were analyzed by one-way ANOVA and no significant differences were observed ($p>0.05$), Casein diet (control diet): Standard protein diet, CMP: Cross-linked Milk Protein, MP: Milk Protein, CWP: Cross-linked Wheat Protein, WP: Wheat Protein

quality, weight gain and PER are inter-related. The better the protein quality, the higher the weight gain and the higher would be the PER (Sgarbieri, 1996). The C-PER values of four test diets 1.55 (CWP), 1.58 (WP), 2.19 (MP) and 2.20 (CMP) were significantly lower ($p<0.05$) than the casein diet (2.50) The differences between the PER and C-PER of the cross-linked and non-cross-linked proteins were not significantly different ($p>0.05$), so the formation of ε-(γ-glutamyl)lysine cross-links in milk and wheat proteins did not appear to have any influence on body weight gain. Seguro *et al*. (1996) studied the bioavailability of the ε-(γ-glutamyl)lysine moiety in cross-linked casein in rats and obtained the C-PER value of 2.47 for the diet containing cross-linked casein.

The FER (food efficiency ratio) was the highest for the rats fed on casein diet (0.38). This value differed significantly ($p<0.05$) from the milk protein diets and from the cross-linked and non-cross-linked wheat protein diets. The Food Transformation Index (FTI) was higher for rats fed on casein diet. This group required less feeding to increase weight gram (2.61) compared to the rats fed on CMP (3.22), MP (3.26), CWP (4.08) and WP (4.15) diets. Statistical analysis showed a similar trend to the food efficiency ratio. The polymerization of milk and wheat proteins by TGase treatment did not affect the FER and FTI of the animals.

Concerning the relative weight of the organs (liver, kidneys and spleen) of rats fed on the different diets, no differences ($p>0.05$) were observed among selected organs (Table 4) and they were in the normal range of weight expected for healthy rats. Moreover, no visual macroscopic abnormalities were observed in the studied organs.

Protein cross-linking can have a profound effect on the structure and function of proteins in foods, so it could affect their nutritional qualities (Friedman, 1999). However, our results for body weight gain, PER, FER and FTI, especially for the milk proteins, have shown good nutritional quality of the cross-linked proteins. According to Sgarbieri (1996), when experimental animals gain weight and other indexes as FER and PER are good, it is an excellent indicator that the fed diets are of high quality.

Nitrogen Balance Experiments

The nitrogen consumed, nitrogen absorbed and nitrogen retained were similar for casein and the test groups (Table 5). The nitrogen retained was positive in all groups, showing that the amount of nitrogen intake was higher than the faecal and urinary excretions. The cross-linking of milk and wheat proteins increased the content of both nitrogen absorbed and nitrogen retained by the rats. Nitrogen excreted in faeces was lower ($p<0.05$) for rats fed with control and MP diets. However, the nitrogen excreted in urine was higher ($p<0.05$) for rats on both wheat proteins diets, probably due to limiting amino acids (or should be low energy content? By referring to the chemical composition results of Table 2, the lipid and ash contents of CWP and WP are much lower than CMP and MP) in the wheat gluten, causing deamination of most of amino acid content for energy production (Banks *et al*., 1964).

Results indicate that the True Digestibility (TD) was significantly lower ($p<0.05$) for both cross-linked milk (95.36%) and wheat proteins (94.45%) than the TD value obtained for rats on casein diet

Table 5: Nitrogen balance values of rats fed experimental diets

		Test diets			
Parameters	Casein diet	CMP	MP	CWP	WP
Daily food intake (g)	13.17±2.34^{a}	13.63±1.32^{a}	13.27±2.15^{a}	14.61±0.95^{a}	13.19±0.72^{a}
Daily body weight gain (g)	5.12±1.09^{a}	4.28±0.84^{ac}	4.31±1.13^{ac}	3.62±0.23^{bc}	3.12±0.37^{bc}
Nitrogen consumed (g)	3.00±0.53^{ab}	2.86±0.28^{ab}	2.77±0.45^{b}	3.48±0.23^{a}	2.99±0.16^{ab}
Nitrogen absorbed (g)	2.88±0.53^{a}	2.70±0.26^{a}	2.63±0.45^{a}	3.26±0.23^{a}	2.80±0.16^{a}
Nitrogen retained (g)	2.72±0.51^{a}	2.54±0.21^{a}	2.46±0.36^{a}	2.76±0.23^{a}	2.36±0.15^{a}
Faecal nitrogen (g)	0.12±0.01^{bc}	0.16±0.02^{ac}	0.14±0.01^{bc}	0.22±0.01^{a}	0.20±0.01^{a}
Urinary nitrogen (g)	0.16±0.06^{b}	0.16±0.11^{b}	0.17±0.12^{b}	0.50±0.01^{a}	0.44±0.08^{a}
AND (%)	95.98±0.79^{a}	94.52±0.57^{bc}	94.93±0.89^{ac}	93.76±0.53^{bcd}	93.18±0.46^{d}
TD (%)	96.81±0.64^{a}	95.36±0.58^{bc}	95.82±0.73^{ac}	94.45±0.49^{bd}	93.98±0.43^{d}
BV (%)	94.79±1.96^{a}	94.59±3.55^{a}	94.19±3.83^{a}	85.01±1.55^{b}	84.70±2.68^{b}
NPU (%)	91.76±1.74^{a}	90.20±3.38^{a}	90.23±3.27^{a}	80.29±1.49^{b}	79.59±2.41^{b}
NPR	4.35±0.27^{a}	3.88±0.39^{a}	4.02±0.40^{a}	2.78±0.12^{b}	2.85±0.19^{b}
PRE	69.60±4.27^{a}	62.12±6.29^{a}	64.25±6.37^{a}	44.54±1.96^{b}	45.52±3.05^{b}

Values are means±SD of six rats in each group throughout 10 days of experimental period, Means with different superscripts in the same horizontal row are significantly different ($p<0.05$), Casein diet (control diet): Standard protein diet, CMP: Cross-linked Milk Protein, MP: Milk Protein, CWP: Cross-linked Wheat Protein, WP: Wheat Protein, AND: Apparent Nitrogen Digestibility, TD: True Digestibility, BV: Biological Value, NPU: Net Protein Utilization, NPR: Net Protein Retention, PRE: Protein Retention Efficiency

(96.81%). TD and Apparent Nitrogen Digestibility (AND) followed a similar trend. The AND and TD were similar ($p>0.05$) between casein and non-cross-linked milk protein diets, but due to the cross-linking process, both parameters were significantly higher ($p<0.05$) for the rats fed on the casein diet than the rats fed on the CMP diet. The polymerization process of milk and wheat proteins by TGase have no influence on TD and AND of these proteins, since these nutritional parameters were no statistically different ($p>0.05$), between CMP and MP or between CWP and WP. Research on the *in vitro* degradation of ε-(γ-glutamyl)lysine show that after ingestion of cross-linked proteins, normal mammalian gastrointestinal digestive enzymes cleave them into amino acids but leave the ε-(γ-glutamyl)lysine dipeptide intact. The resistant ε-(γ-glutamyl)lysine dipeptide may be absorbed through the intestinal brush-border and transported to the kidney (Fink *et al*., 1980).

The Biological Value (BV) was higher (94.79%) for rats on the control diet but similar ($p>0.05$) to the values obtained for rats on the CMP diet (94.59%) and MP diet (94.19%). The BV values obtained for rats on wheat protein diets (84.70 % to WP and 85.01% to CWP) were similar ($p>0.05$), but lower ($p<0.05$) than the others. According to Whitney and Rolfes (1996), a protein with a BV of 70% or more can support human growth and tissue maintenance as long as energy intake is adequate. Since the BV of the milk and wheat cross-linked protein diets were, respectively 94.59 and 85.01%, these proteins could support growth and tissue maintenance. TGase action involves cross-linking of free primary amino groups of lysine with glutamine residues, but the results indicate that the biological value of the milk and wheat proteins is not reduced by this process. The protein nutritive value of a food reflects its ability to meet nitrogen and amino acid requirements assuring proper animal growth and maintenance. This ability is a function of several factors, including protein content, digestibility and amino acid composition (Cheftel *et al*., 1985). According to Seguro *et al*. (1996), the cross-linking by TGase is thought to protect nutritionally valuable lysine residues in food from various deteriorative reactions. Furthermore, the use of TGase potentially allows production of food proteins of higher nutritional quality, through cross-linking of different proteins containing complementary amino acids (Zhu *et al*., 1995).

The Net Protein Utilization (NPU) value was higher (91.76%) for rats on the control diet but similar ($p>0.05$) to the value obtained for rats fed on the cross-linked milk protein (90.20%) and milk protein (90.23%) diets. The NPU values obtained for rats on diets of CWP and WP, 80.29 and

79.59%, respectively, were similar ($p>0.05$), but lower ($p<0.05$) than the standard protein diet. The NPU has been suggested to be a more practical value than BV in protein quality evaluation. This is because digestibility is an important and integrated part of the nutritive value of a dietary protein source. NPU is a measure of both digestibility and BV of the amino acid mixture absorbed from food (Whitney and Rolfes, 1996). Thus the results indicate that both milk and wheat proteins nutritional qualities were not affected by the TGase-induced cross-linking.

Daily food intakes of animals on the control and test diets were not significantly ($p>0.05$) different. However, animals on the casein, CMP and MP diets gained body weight rapidly and had significantly ($p<0.05$) higher NPR than the animals on cross-linked and non-cross-linked wheat protein diets. The differences between the NPR values of the proteins cross-linked and non-cross-linked were non-significant ($p>0.05$), so the cross-linking process by microbial TGase in milk and wheat proteins had not influenced the availability of proteins to support both maintenance and growth.

The Protein Retention Efficiency (PRE) indicates the distinct superiority of the casein diet over the other diets, since this value for casein diet was the highest 69.60 and for test diets, it was ranging from 44.54 to 64.25. Casein is a pure protein source with well balanced amino acid profile and hence it is usually used as a standard protein diet (control diet) for comparison purpose. The results suggest that the PRE of milk and wheat proteins is not affected by the cross-linking process.

Based on the *in vivo* biological values examined, the milk and wheat protein quality cross-linked by TGase was not affected. Therefore, the results obtained in this study are contrast with those reported by Tang *et al*. (2006), who has reported the effect of *in vitro* digestibility of soy proteins cross-linked by microbial TGase. They suggested that this enzyme may negatively affect the nutritional properties of food proteins. According to them, the nutritive value of a protein can be evaluated by various *in vitro* and *in vivo* methods, but due to the simplicity and speed, the *in vitro* digestibility methods have been more widely used than *in vivo* methods. However, the biological evaluation of protein provides useful information regarding their overall quality and it is the best tool for judging the protein nutritional quality, since many factors can affect the quality of a specific protein *in vivo* (Sogi *et al*., 2005).

CONCLUSION

This study shows that milk and wheat proteins cross-linked by microbial TGase could support the growth of young rats. The polymerization process of these proteins by this enzyme did not negatively affect some nutritional parameters such as protein efficiency ratio, food efficiency ratio, food transformation index, apparent nitrogen digestibility, true digestibility, biological value, net protein utilization, net protein ratio and protein retention efficiency, indicating that the cross-linking process by TGase had no influence on the nutritional quality of milk and wheat proteins. These results are important because protein cross-linking is important for industrial applications in order to improve food texture, but these modifications should come without causing the loss of nutritional quality.

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