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Impact of transglutaminase treatment on properties and *in vitro* digestibility of white bean (*Phaseolus vulgaris* L.) flour

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ABSTRACT

Common beans (*Phaseolus vulgaris* L.) are rich in nutrients and have significant amounts of proteins and complex carbohydrates, besides to be rich in unsaturated fatty acids and dietary fibres. Consumption of beans could be improved by processing them into flour. In this study the effect of microbial transglutaminase (TG) on the structure, physical (colour parameters, moisture, water holding capacity), thermal properties and in *vitro* digestion of undehulled (WB) and manually dehulled (SB) flour samples from white common beans (*P. vulgaris L.*) was evaluated. Flour samples were incubated in the absence and presence of TG (WB/TG and SB/TG). We observed that the enzyme is able to catalyse the formation of polymers, suggesting that the proteins occurring in the bean flour act as TG substrates. Microstructure of samples was examined by Scanning Electron Microscopy (SEM), while thermal properties were studied by Differential Scanning Calorimetry. Microstructural results showed that the TG-treated samples possess a more compact structure, made of starch granules surrounded by proteins that, presumably, contain TG-catalysed polymers. Moreover, TG treatment had a major impact on colour, water holding capacity (WHC) and thermal properties. In particular, WB and SB samples presented a darker colour than WB/TG and SB/TG samples, while the latter showed reduced WHC that was only 30% and 37% of WB and SB samples, respectively. The transition enthalpy (ΔH) in the temperature range from 57 to 70 °C (WB, WB/TG) and from 60 to 68 °C (SB, SB/TG) followed the order: WB/TG > WB and SB/TG > WB, respectively. *In vitro* digestion experiments indicate that the presence of isopeptide bonds decreased the digestibility of TG-treated flour samples.

1. Introduction

Legumes are an inexpensive source of proteins with a high nutritional profile and, after cereals, the next most important food source for humans (Butt & Batool, 2010). In particular, white beans (*Phaseolus vulgaris L.*) are widely grown and consumed in developed as well as developing nations of the world, supplying significant amounts of protein, starch, unsaturated fatty acids, dietary fibre, mainly soluble fibre, besides being an excellent source of some minerals (iron and zinc) and vitamins (Villavicencio, Mancini-Filho, & Delinceé, 2000; Kutos, Golob, Kac, & Plestenjak, 2003). Despite these advantages, beans have some undesirable characteristics that limit their acceptability or nutritional value, such as hard-to-cook phenomenon, antinutrients or antinutritional factors or limitation in some amino acids of high biological value (Barampama & Simard, 1995; Corrêa et al., 2010).

Physical–chemical properties of beans change with postharvest handling and storage. If the latter are not properly realized, quality of seeds is negatively affected thus reducing consumer acceptance (Njintang, Mbofung, & Waldron, 2001). Consumption of beans could be improved by processing them into flour (Dzudie & Hardy, 1996). The functional properties of legume flour samples mainly depend on proteins, carbohydrates and other components. Proteins are one of the main classes of food components; hence, their modification *via* chemical, physical or enzymatic methods is an alternative available for the improvement and/or development of new functional properties (Gaspar & de Góes-Favoni, 2015).

Crosslinking enzymes have been proposed as processing aids for modifying functional properties of many protein-based food (Mariniello et al., 1993; Persico et al., 1992; Porta, Mariniello, Di Pierro, Sorrentino, Giosafatto, 2011). In particular, the enzyme microbial transglutaminase (TG) catalysing the formation of an ε-(γ-glutamyl) lysine isopeptide bond between glutamines and lysines of proteins, has been investigated to improve physical and biological properties of food proteins, i.e. gelation, viscosity, emulsification and foaming (Giosafatto et al., 2012;

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Mariniello et al., 2014; Mariniello & Porta, 2005; Porta, Giosafatto, Di Pierro, Sorrentino & Mariniello, 2011).

Moreover, some authors (Monogioudi et al., 2011; Stanic et al., 2010) describe the effect of enzymatic protein crosslinking on protein digestibility. In fact, it has been assumed that enzymatic modification of proteins leads to firmer matrices that are digested to a lower extent. Hence, food structures with higher satiety effects could be produced *via* enzymatic crosslinking.

The objective of the present paper was to study the effect of TG on the microstructure and on some physical and functional properties of bean flour. Moreover, the *in vitro* protein digestibility of white beans following the enzyme treatment was evaluated.

2. Materials and methods

2.1. Materials

White beans (*P. vulgaris L.*) were provided by Select (Agria, San Giuseppe Vesuviano, Italy) and stored at room temperature until processed. TG enzyme (Activa WM, Ajinomoto, Japan) was provided by Prodotti Gianni, Italy. α-amylase (product A1031). Pepsin from porcine gastric mucosa (product P6887), and all other reagents were purchased from Sigma Chemical Company (Pool, Dorset, UK). Electrophoresis reagents were from Bio-Rad (Segrate, Milano, Italy). All chemicals were of analytical grade.

2.2. Bean flour sample preparation

White dried beans (*P. vulgaris L.*) were divided into two aliquots which were treated as undehulled (or whole beans, WB) and manually dehulled (or shelled beans, SB) respectively. Manual dehulling was done by soaking the seeds in cold water for 5 h, followed by vigorous hand-rubbing to detach the seed coats. The dehulled seeds were next dried in an oven at 65 °C for 24 h. Each bean samples were ground using a variable speed laboratory blender (LB20ES, Waring Commercial, Torrington, Connecticut, USA), so that the flour would pass through a 425 μm stainless steel sieve (Octagon Digital Endecotts Limited, Lombard Road, London, UK).

2.3. Protein determination

Protein determination was carried out by the Bio-Rad Protein Assay (Bio-Rad), using bovine serum albumin as standard (Bradford, 1976).

2.4. TG-mediated treatment of bean flour samples

Flour samples were prepared in distilled water at a concentration of 1 mg mL^{-1} and 100 μg were incubated at 37 °C for 2 h in the absence (WB and SB) and presence (WB/TG and SB/TG) of increasing amounts of TG (46, 92, 184, 368 U/g of bean flour) in Tris–HCl buffer 80 mM (pH 7.5). All samples were treated at the end of incubation at 100 °C for 15 min to achieve the double goal of stopping the enzymatic reaction and simulating the cooking process. Flour samples were dehydrated by a freeze-drier (alpha 1-2LD plus Christ, Germany). The temperature and pressure in the closed drying chamber were −51 °C and 250 Pa for 24 h, respectively. Then samples were collected and stored in polyethylene bags at 4 °C until used.

2.5. pH

The pH of samples (WB, SB, WB/TG and SB/TG) was measured by using a digital pHmeter (MP220, Mettler, Toledo) according to the AACC method (number 02–52.01, 1999). As expected, samples of flour were all characterized by the same pH values (6.8 ± 0.03).

2.6. Scanning Electron Microscopy analysis

Samples were dried at the critical point and coated with gold particles in an automated critical point dryer (model SCD 050, Leica Vienna). Microstructure of samples was examined by means of Scanning Electron Microscopy (SEM) (LEO EVO 40, Zeiss, Germany) with a 20 kV acceleration voltage and a magnification of ×2000.

2.7. Colour measurement

Colur of flour samples was measured with a tristimulus colourimeter (Minolta Chroma Metre model CR 300, Milan, Italy) with a circular measurement area ($D = 8$ mm). In the Hunter-Lab colourimeter, the colour of a sample is denoted by the three dimensions (chromatic coordinates) L (lightness), a ($+a$ red; $-a$ green) and b ($+b$ yellow; $-b$ blue), corresponding to the XYZ CIE lab system. The colourimeter was calibrated using a white standard plate ($L = 100/0$ white/black) at the beginning of each session (Romano, Giosafatto, Masi, & Mariniello, 2015). Chromatic coordinates L, a and b were reported as the average of six measurements on each sample. From the parameters determined, total colour difference (ΔE) was calculated by the equation:

$$\Delta E = \left[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2\right]^{1/2}. \quad (1)$$

2.8. Moisture content

The moisture content of each sample was determined by the AACC method (number 44–15.02, 1999). Three samples, weighing approximately 2 g, were dried for 24 h at 105 °C. Samples were removed from the oven, let cooling in a desiccator and weighted. This procedure was repeated until a constant weight was reached (usually four subsequent drying cooling cycles were sufficient). The dried samples weight was subtracted to the respective initial weight. The results were calculated as percentage of water per sample weight (%).

2.9. Water holding capacity

Water holding capacity (WHC) was determined by the method of D'Appolonia (1977) with some modifications. Five grammes of the samples were weighted into a 50 mL centrifuge tube to which 30 mL water were added. The slurry was stirred for 5 min and then allowed to stand for 30 min at 25 °C. The flour was then centrifuged at 4500 rpm for 25 min and the weight of free liquid measured. WHC was calculated as

$$\mathrm{WHC} = \frac{\text{weight of wet sample} - \text{weight of dry sample}}{\text{weight of dry sample (dry basis)}} x100. \quad (2)$$

Results were expressed as means and standard deviation of at least three independent experiments.

2.10. Thermal properties

Thermal characteristics of the samples were determined using a Differential Scanning Calorimeter (DSC Q200, TA Instruments, Milan, Italy). Samples of approximately 6 mg were hydrated in the Tzero aluminium hermetic pans at about 70% moisture content. The pan was closed with a lid and weighted. All samples were heated from 30 °C to 100 °C at 10 °C min^{-1} using an empty pan as the reference. The enthalpy (ΔH), onset temperature (To) and peak temperature (Tp) of endotherms were measured. Peak height index (PHI) was calculated as described by Krueger, Knutson, Inglett, and Walker (1987):

$$\mathrm{PHI} = \frac{\Delta H}{(\mathrm{Tp} - \mathrm{To})}. \quad (3)$$

Average values of three measurements were calculated for each sample.

2.11. In vitro gastric digestion model

The simulation of human digestion was carried out according to Giosafatto et al. (2012) and as described by Romano et al. (2015a) mimicking physiological conditions. Briefly, the oral phase was carried out by incubating both WB and SB flour (5.2 mg) modified and not by TG (368 U/g) for 2 min at 170 rpm and 37 °C with human α-amylase dissolved (150 U/mL) in 4 mL of Simulated Salivary Fluid (SSF, 0.15 M NaCl, 3 mM $CO(NH_2)_2$, pH 6.9).

The gastric phase was done according to Giosafatto et al. (2012) with modifications. Aliquots (100 μL) of Simulated Gastric Fluid (SGF, 0.15 M NaCl, pH 2.5) were placed in 1.5 mL microcentrifuge tubes and incubated at 37 °C. 100 μL of oral samples, the pH of which was adjusted to 2.5 with 6 M HCl, were added to each of the SGF vials to start the digestion reaction. Protein samples were tested as pepsin substrate in the physiological ratio equal to 20:1 (w/w). At intervals of 1, 2, 5, 10, 20, 40, 60 min 40 μL of 0.5 M ammonium bicarbonate (NH_4HCO_3) were added to each vial to stop the pepsin reaction. The control was set up by incubating the sample for 60 min without pepsin. Three sets of experiments were carried out.

2.12. SDS-PAGE

5 μL of sample buffer (15 mM Tris–HCl, pH 6.8, containing 0.5% *w*/*v* SDS, 2.5% *v*/v glycerol, 200 mM β mercaptoethanol, and 0.003% *w*/*v* bromophenol blue) were added to aliquots of 20 μL of any sample and analysed by 12% SDS-PAGE, as described by Laemmli (1970). Electrophoresis was performed at constant voltage (80 V for 2–3 h), and the proteins were stained with Coomassie Brilliant Blue R250. Bio-Rad Precision Protein Standards were used as molecular weight markers.

2.13. Image analysis

The SDS-PAGE gel images were acquired using Bio-Rad ChemDoc Imager. The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1). Densitometry was performed by calculating the percentage of average intensity of unmodified phaseolin (for polymerization assay) and of high molecular weight polymers (≥250 kDa) (for digestion experiments) normalized to control samples ("C"). Densitometry was carried out on three different sets of experiments.

2.14. Statistical analysis

All experimental results are reported as means and standard deviation of at least three independent experiments. t-Student test was performed in order to evaluate the effect of TG on experimental results of flour types (WB and SB). Significance of differences was defined at $P \leq 0.05$. All statistical analyses were performed using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA, 2010).

3. Results and discussion

3.1. TG-mediated crosslinking of bean flour proteins

It was already demonstrated that phaseolin, the main storage omotrimeric glycoprotein present in the bean seeds, whose monomer possesses a molecular mass of about 50 kDa, acts as an effective substrate of TG (Mariniello, Giosafatto, Di Pierro, Sorrentino, & Porta, 2007; Mariniello et al., 2007; Giosafatto et al., 2014a,b). Both intermolecular and intramolecular bonds were formed among different and within the same protein molecule, respectively. In particular, the intramolecular crosslinks led to more compact structure with a reduced hydrodynamic radius of phaseolin molecule (Mariniello et al., 2007). In the present work we want to verify whether other bean flour proteins, beside phaseolin, act as TG substrates. For this purpose, both SB and WB samples were incubated in the presence of increasing amounts of TG. At the end of incubation the extent of polymerization was evaluated by SDS-PAGE. The extent of crosslinking was assessed on the basis of the increased smearing in the gel lanes, the appearance of high molecular weight polymers, some of which enable to enter the stacking gel, and the concomitant disappearance of the flour protein bands. The lane 1 of Fig. 1 (Panel A) of both gels shows that the electrophoretic pattern of proteins from WB and SB incubated in the absence of the enzyme is the same. This suggests that the soaking used for treating the SB beans did not affect the protein profile. Moreover, as shown in Fig. 1, modification of the protein bands occurred even at the lowest concentrations of the enzyme (46 U/g, lane 2) showing that the glutamine and lysine reactive residues in protein flour were accessible to the enzyme. Moreover, by using the highest amount of the enzyme (368 U/g, lane 5) the protein polymerization was greatly enhanced. Since polymerization occurred in both samples, we can assess that the coat does not affect flour protein capability of acting as TG substrates. However, extensive polymerization is achieved already with 46 U/g only in SB samples, suggesting that the presence of the coat influences enzyme accessibility to substrates. Densitometry analysis (Fig. 1, Panel B) has demonstrated that in WB samples, the relative quantity of unmodified phaseolin was equal to 74 ± 5.6%, 61% ± 0.1% and 32.5 ± 17% by using 46, 92 and 184 U/g of bean flour, respectively, while in SB samples the relative quantity values equal to 44 ± 3.6%, 7.6 ± 5% and 7.9 ± 1.5% were observed. Using the highest amount of enzyme (368 U/g) the proteins from WB and SB were modified at the same extent, since densitometry analysis assessed that the amount of unmodified phaseolin in both WB and SB sample was very similar, being equal to 9.7 ± 3.4% and 5.2 ± 1.8%, respectively.

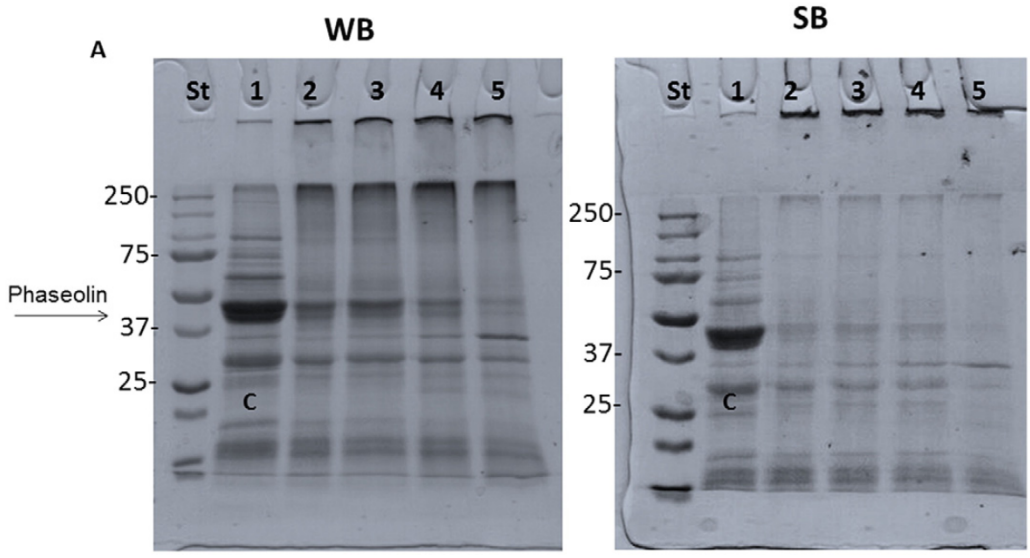
3.2. Microstructural characteristics of flour

In order to study the effects of TG on microstructural properties of flour samples incubated in the absence (WB and SB) and presence (WB/TG and SB/TG) of TG, the characterization of the microstructure by means of SEM was performed.

SEM images of flour samples are shown in Fig. 2. Morphological features captured through SEM of WB and SB samples showed the globular structures reported as starch granules, spherical proteins and lipid bodies (Fig. 2a, c, respectively). In general, the size of starch granules is larger than that of lipid and protein bodies (Hsieh, Swanson, & Lumpkin, 1999; Young, Pattee, Schadel, & Sanders, 2004), thus, the larger globular structures found in the SEM images (Fig. 2) could possibly be starch granules, varying in shape from ovoid to spherical, with heterogeneous sizes ranging from 19 to 30 μm (Romano, Giosafatto, Masi, & Mariniello, 2015). The microstructures of the formed TG- treated samples, WB/TG and SB/TG (Fig. 2b, d respectively), were distinctly different from their initial flour samples.

In particular, the starch granules in WB (Fig. 2a) are present in the cotyledon cells and are embedded in the protein matrix of the cellular contents, as reported by the microstructural study of Berg, Singh, Hardacre, and Boland (2012) on navy bean flour and starch. The addition of TG produced an evident crosslinking in WB/TG sample (Fig. 2b). WB/TG showed a more branched structure with heterogeneous and irregular particles, which represent the protein bodies or fragments of protein matrix, mineral and fibre components, as reported in different legumes by other authors (Aguilera, Esteban, Benitez, Molla, & Martin-Cabrejas, 2009; Ma et al., 2011). Instead, the starch granules in WB/TG were not visible and discernible. It seems that apparently TG has reinforced the protein–protein interactions, if it is compared with WB.

The starch granule structure in SB (Fig. 2c) was well visible and hydrated. Strands of protein bodies or fragments of protein matrix were also observed on the top surface of starch granules. SB/TG (Fig. 2d)



B

	TG (U/g)	46	92	184	368
SB	Relative quantity of unmodified phaseolin (%)	44±3.6	7.6±5	7.9±1.5	5.2±1.8
WB	Relative quantity of unmodified phaseolin (%)	74±5.6	61±0.1	32.5±17	9.7±3.4

Fig. 1. TG-mediated modification of whole bean (WB) and shelled bean (SB) flour samples. (A) 100 μg of flour proteins were incubated for 2 h at 37 °C in the absence (lane 1, C) and presence of increasing amount of TG (from lane 2 to 5: 46, 92, 184, 368 U/g of bean flour). St, Molecular weight standards, Bio-Rad. (B) Densitometry analysis of unmodified phaseolin remaining after TG treatment of bean flour proteins.

possessed a more compact structure covered by irregular particles, as observed in WB/TG. In general, microscopy observations indicated that when TG (368 U/g) was added, bean flour constituents (starch granules and proteinaceous matrix) seemed to be integrated in a compact structure (Fig. 2b, d), which might be attributed to the formation of TG-catalysed heteropolymers. A more compact structure was found

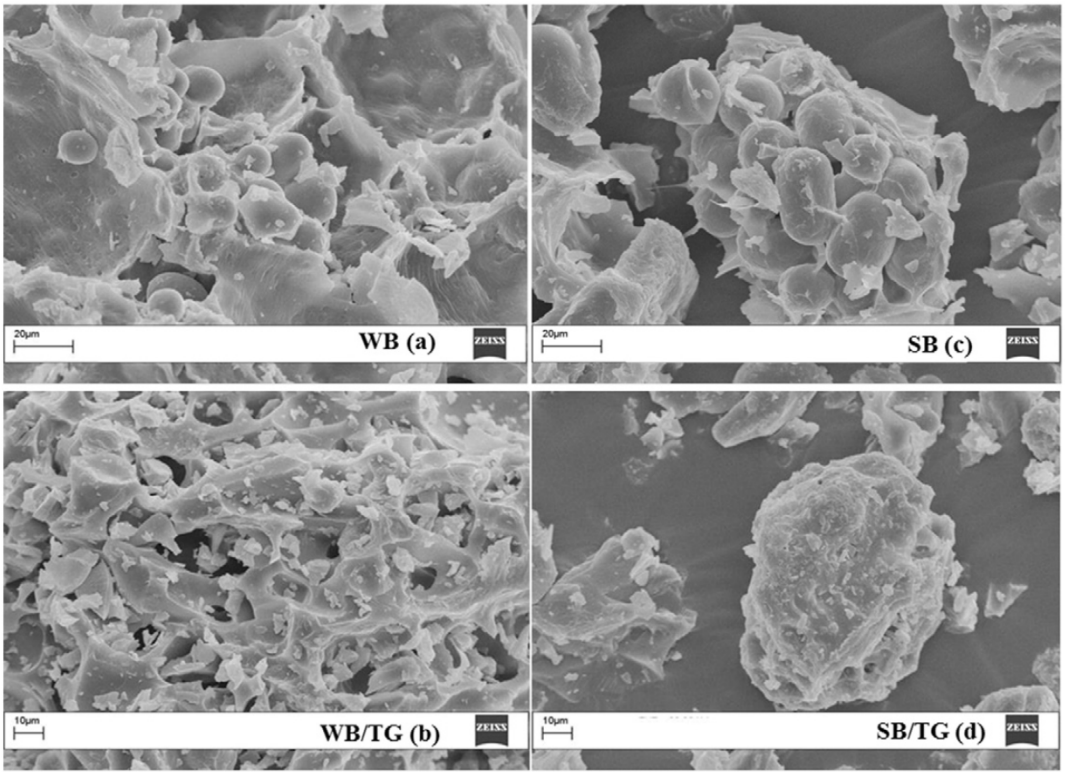


Fig. 2. Representative scanning electron micrographs of samples: whole beans flour (WB) incubated in the absence (a) and presence (b) of 368 U of TG/g of bean flour TG; shelled beans flour (SB) incubated in the absence (c) and presence (d) 368 U of TG/g of bean flour of TG.

also by Bonet et al. (2006) that studied the glucose oxidase effect on wheat flour dough at molecular level.

3.3. Physical and functional properties

As physical and functional properties, the colour parameters, moisture content and WHC were investigated. The examined parameters are shown in Table 1. To highlight the effects of TG on physical–chemical properties of flour samples incubated in the absence and in presence of TG, Table 1 lists parameters into two distinct blocks: the first one for WB and WB/TG and the second for SB and SB/TG.

For each type of flour, the colour analysis showed significant differences ($P < 0.05$) between samples with regard to colour values (L, b and ΔE). The lightness (L) of WB was lower (90.4) than WB/TG, a values were not different ($P < 0.05$) between samples, while the yellow (b) and the total colour difference (ΔE) values of WB were significantly higher ($P < 0.05$) than WB/TG. The whiteness improves with TG treatment, indicating that flour colour improvement is chiefly due to reduction in yellowness, which was possibly due to modification of pigments (carotenoids) or denaturation of proteins in bean flour system. The similar whitening effect of TG on SB/TG samples was observed: b and ΔE values (9.4 and 12.4, respectively) of SB was significant greater ($P < 0.05$) than SB/TG ones (7.7 and 10.3, respectively), while L value (91.9) was less (93.2).

In conclusion, higher L and b values of TG-treated counterpart (WB/TG and SB/TG) indicated their lighter colour as compared to their flour samples (WB and SB). The effect of TG on flour samples was similar in WB and SB, but dependent by different coat and pigment contents of flour samples. Coat layers are the chief source of bioactive compounds and pigments in cereal and legumes, thus any whiteness effect of enzyme system is expected to be greatest in the whole system (Okot-Kotber, Liavoga, Yong, & Bagorogoza, 2001; Lamsal & Faubion, 2009).

For each type of flour (WB and SB), there were no significant differences ($P > 0.05$) in the moisture content between flour samples and their TG- treated counterpart (WB/TG and SB/TG, respectively). Despite moisture results, significant differences ($P < 0.05$) in WHC values were observed for both WB and SB. WHC is the ability of a protein matrix to absorb and retain bound, hydrodynamic, capillary, and physically entrapped water against gravity (Damodaran & Paraf, 1997). The study of effect of TG on WHC of flour samples (Table 1) is important for a potential use in food and industrial applications of TG-treated samples as thickeners or colloidal stabilizers. Table 1 shows that the WHC of WB and SB were significantly ($P < 0.05$) higher than those of WB/TG and SB/TG. In particular, use of TG reduced the WHC values of WB and SB. The decrease in WHC of WB/TG and SB/TG could be due either to loss of solubility or protein aggregation, thus decreasing the surface area exposed to the water phase. WHC results agree with microstructural characteristics of flour samples (Fig. 2). In fact, the differences of structural characteristics of different samples may account for the differences of WHC. The higher WHC of WB and SB may be attributed to their more open microstructure (Fig. 2a, c) that enhances interaction with water when compared to the more compact microstructure of WB/TG (Fig. 2b) and SB/TG (Fig. 2d). The space within the protein network in foods is the most important factor for WHC, and the TG- treatment causes either a loss of solubility or excessive protein aggregation and a very tight protein network, with concomitant space reduction. To our knowledge, no study has reported the effect of TG on WHC of bean flour, while several authors have studied how TG influences the WHC of buckwheat flour and of whole rice bread (Renzetti, Dal Bello, & Arendt, 2008), as well as of wheat gluten (Wang, Zhao, Yang, Jiang, & Chun, 2007).

3.4. Thermal properties

Thermal analysis is a valuable tool for studying the effect of thermal processing on vegetable proteins (Ma, 1990) and the phase transition of starch (Laaksonen & Roos, 2000). It is well known that the cooking processes cause some structural changes on starch and protein network of foams (Barbiroli et al., 2013; Romano, Di Luccia, Romano, Sarghini, & Masi, 2015). The thermal interactions between starch and protein network and the effect of TG on thermal properties of flour samples were studied by means of a Differential Scanning Calorimeter, and related characteristics (ΔH — transition enthalpy, To — transition onset temperature, Tp — transition peak temperature and PHI — Peak height index) are summarized in Table 2.

Flour samples showed similar thermal behaviour, but details of the thermal properties differed. When flour samples were heated in the presence of excess water (70%), a single endothermic transition, corresponded mainly to the gelatinization transition of the starch, was observed in the Differential Scanning Calorimeter profiles of all tested flour samples (data not shown). The observation of a single endotherm is to be expected considering the level of moisture (70%) at which samples were scanned. The water content in a food system has great influence on the gelatinisation behaviour of starch and similarly water is a major factor determining the thermal stability of proteins (Henshaw, McWatters, Akingbala, & Chinnan, 2003). According to Biliaderis, Maurice, and Vose (1980) two endothermic transitions were exhibited by native starches of some legumes when heated at water contents between 45 and 48% w/w and a single endotherm was observed when the water content was increased. According to literature, the range of starch gelatinization temperatures of bean flour from different kidney bean cultivars (*P. vulgaris L.*) is: 60.9–73.3 °C, respectively for To and Tp (Wani, Sogi, Wani, & Gill, 2013). Values obtained in this experiment fall within this range (Table 2).

The results presented in Table 2 show that To, Tp and ΔH varied significantly ($p < 0.05$) among WB and WB/TG. After TG-treatment, WB samples exhibited an increase of To from 57 to 59.3 °C, Tp from 65.2 to 69.7 °C, and ΔH from 1.1 to 1.4 J/g. The increased of To and Tp of samples indicate increased resistance of the starch to gelatinization and reflect an increase in the energy needed for starch gelatinization (Hayakawa & Breene, 1982; Kaur & Singh, 2007). Moreover, WB/TG thermal results suggest that the double helices of starch granules that melt during gelatinization are strongly associated within their native structure, in agreement with microstructural results of flour samples (Fig. 2a, c).

Table 1
Effect of TG on properties of bean flour samples, expressed as means ± s.d.

Parameter	WB	WB/TG[§]	SB	SB/TG[§]
Colour values				
Lightness (L)	90.35 ± 0.2	92.79 ± 0.8*	91.94 ± 0.1	93.16 ± 0.7*
Redness (a)	0.74 ± 0.03	0.72 ± 0.07	0.13 ± 0.04	0.12 ± 0.10
Yellowness (b)	11.35 ± 0.1	8.78 ± 0.3*	9.40 ± 0.4	7.71 ± 0.3*
ΔE	14.92 ± 0.2	11.40 ± 0.7*	12.39 ± 0.3	10.31 ± 0.6*
Moisture (%)	4.41 ± 0.3	4.09 ± 0.5	3.82 ± 0.6	3.28 ± 0.5
WHC (%)	171.20 ± 6.9	52.60 ± 2.1*	129.42 ± 2.4	48.94 ± 0.9*

For each type of flour (WB and SB), significant differences (t-Student) were shown in experimental results (: $p < 0.05$).*
§ 368 U of TG were used for gr of bean flour.

Table 2
Effect of G on thermal properties of the analysed samples[1]: ΔH, transition enthalpy; To, onset temperature; Tp, peak temperature; PHI, peak height index.

Parameter	WB	WB/TG[§]	SB	SB/TG[§]
To (°C)	56.97 ± 1.18	59.33 ± 1.17*	60.90 ± 1.11	59.84 ± 1.89
Tp (°C)	65.17 ± 1.81	69.71 ± 1.40*	67.23 ± 2.10	67.61 ± 1.21
ΔH (J/g)	1.12 ± 0.02	1.36 ± 0.03*	1.00 ± 0.04	1.45 ± 0.02*
PHI	0.14 ± 0.01	0.13 ± 0.01	0.16 ± 0.01	0.19 ± 0.02*

For each type of flour (WB and SB), significant differences (t-Student) were shown in experimental results (: $p < 0.05$).*
§ 368 U of TG were used for gr of bean flour.

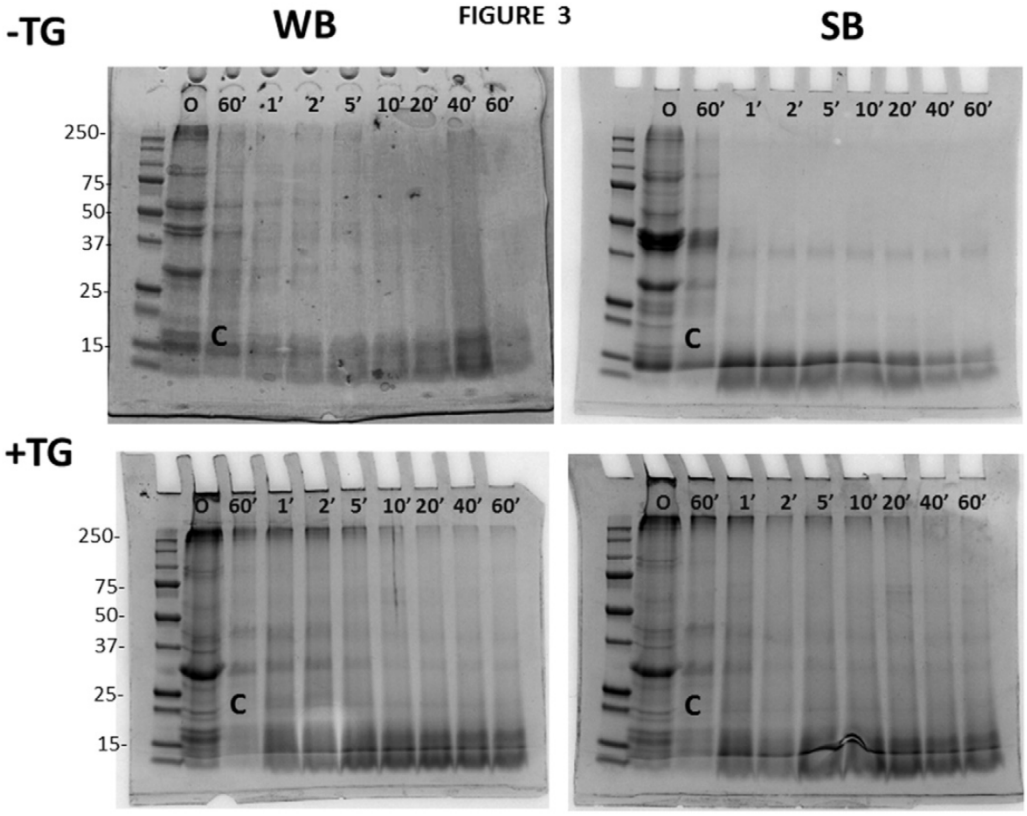


Fig. 3. SDS-PAGE analysis of whole bean (WB) and shelled bean (SB) flour samples, treated (lower panels) or untreated (upper panels) with TG, subjected to oral ("O") and gastric "*in vitro*" digestion, at different times (minutes). Lane "C" corresponds to the samples incubated in the absence of pepsin. St, Molecular weight standards, Bio-Rad.

The To and Tp of SB were unaffected by TG treatment (Table 2), while ΔH and PHI varied from 1.0 to 1.5 J/g and from 0.16 to 0.19, respectively. The highest value of ΔH of SB/TG may be due to a less competition of protein and starch for water due to the very compact structure of this protein network (Fig. 2d) and their less ability to absorb and retain entrapped water in agreement with WHC results (Table 1).

In conclusion, the thermal properties of TG-treated flour were significantly different from their normal counterparts and ΔH of flour was the most discriminating thermal property.

3.5. In vitro simulated gastric digestion

In order to evaluate the effect of TG on the digestion of bean proteins present in cooked flour samples, both oral and gastric *in vitro* digestion were performed and products analysed by SDS-PAGE (12%). Fig. 3 shows oral and gastric digestion of WB and SB (upper panels) and WB/TG and SB/TG (lower panels). Bean flour proteins (Fig. 3, lane "O″) comprises a mixture of proteins soluble at the SSF (pH 6.9). Upon addition of SGF, the pH of which is 2.5, some of these proteins precipitated (Fig. 3, lane C). This phenomenon was observed also by Smith, Pan, Bellido, Toole, and Gates (2015) studying the *in vitro* oral and gastric digestibility of gluten proteins. Thus, SGF soluble proteins were subjected to digestion comparing the results to the controls (lanes "C″) incubated in the absence of pepsin for 60 min. It is possible to observe that no differences were observed in terms of digestion between WB and SB (upper panels) since samples were promptly digested after 1 min incubation with pepsin. On the contrary, digestion was considerably reduced in TG-treated samples (lower panels). A tight and compact structure of proteins obtained through crosslinking by TG most probably explains the reduced digestion. Similar results were obtained by Giosafatto et al. (2012), studying the digestibility of TG-treated ovalbumin. Densitometry analysis of electrophoretic bands were then performed, and the results allowed to identify differences between the gastric digestion of WB/TG and SB/TG samples (Table 3). In particular, taking into account the relative density of TG-catalysed polymers having a molecular mass ≥ 250 kDa, the SB sample seems to be more rapidly digested. This is particularly true after 60 min gastric digestion where 34 ± 9% of polymers from WB/TG sample flour was still present, whereas the TG-catalysed polymers in SB samples represented only 14 ± 3% (Table 3). Likely this result is due to the fact that complex

Table 3
Densitometry analysis of TG[§]-mediated polymers following digestion of SB flour and WB flour.

	Incubation time (min)	C	1	2	5	10	20	40	60
SB	Relative quantity (%)	100	80 ± 6	66 ± 14	50 ± 9	42 ± 7	32 ± 2	29 ± 3	14 ± 3*
WB	Relative quantity (%)	100	89 ± 5	88 ± 2	87 ± 9	52 ± 8	47 ± 2	34 ± 8	34 ± 9*

"C" corresponds to the samples incubated for 60 min in the absence of pepsin.
For each type of flour (WB and SB), significant differences (t-Student) were shown in experimental results (: $p < 0.05$).*
§ 368 U of TG were used for gr of bean flour.

carbohydrates mainly found in the coat (Romano, Giosafatto, Masi, & Mariniello, 2015) exhibit negative effects on digestibility of TG-mediated crosslinked proteins. Since the proteolysis is dependent on the amino-acid sequence of a protein as well as on the susceptibility of peptide bonds, it may be possible that upon enzyme modification some of proteins undergo conformational changes that influence their binding to the sugars present only in WB samples and, thus, making some pepsin sites less accessible to hydrolysis.

The decreased digestibility of TG-treated bean flour proteins, especially in WB samples, could have an impact on the understanding of how food structure can affect digestibility and gastric emptying time. In addition, these results can be exploited in tailoring novel food matrices that, because of its decreased digestibility, could provide commercial products with controlled energy intake (Monogioudi et al., 2011). Moreover, the crosslinking treatment may have an impact on digestibility of starch of bean flour. In fact, Gan, Ong, Wong, and Easa (2009) have demonstrated that soy protein-based noodles modified by TG had stronger texture and, above all, the lowest glycemic index.

4. Conclusions

Our results demonstrated the influence of TG-treatment on the properties of *P. vulgaris* flour. The SEM observations of starch and proteins showed an evident effect of TG on structure of flour samples. In fact, when TG was added, bean flour constituents seemed to be integrated in a compact structure, which might be attributed to the formation of TG-catalysed heteropolymers. Whole beans flour (WB) and shelled beans flour (SB) presented a darker colour (lower *L* and *b* values) and higher water holding capacity (WHC) values than their TG-treated counterparts (WB/TG and SB/TG). In particular, use of TG reduced the WHC values of WB and SB.

TG had a significant effect on the thermal parameters of flour samples, which indicated an increase in resistance of the starch within the granules to gelatinization. In particular, thermal results of bean flour by Differential Scanning Calorimetry showed that the transition enthalpy ΔH was the most discriminating thermal property. The transition enthalpy could reasonably become an index of bean flour functionality. In addition, the TG through the formation of ε-(γ-glutamyl) lysine isopeptide bonds affects the gastric digestibility of the bean flour. Further studies will rely on the fate of TG-mediated polymers also in the small intestine, thus, let possible to design the development of novel foods that may enhance satiety.

Abbreviations

SB	shelled beans
SEM	Scanning Electron Microscopy
SGF	Simulated Gastric Fluid
SSF	Simulated Salivary Fluid
WB	whole beans
WHC	water holding capacity
TG	transglutaminase

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