

EFFECT OF TRANSGLUTAMINASE AND NEUTRASE ON THE PROPERTIES OF PROTEIN ENRICHED RICE FLOUR

Livia PĂTRAȘCU, Iuliana APRODU, Ina VASILEAN, Iuliana BANU*

Dunarea de Jos University of Galati, Faculty of Food Science and Engineering,
Domneasca St. 111, 800201 Galati, Romania

*Corresponding author e-mail: iuliana.banu@ugal.ro

Abstract

One way to improve the functionality of the proteins within different food matrices consists on applying different enzyme treatments. The present study aimed at investigating the effect of transglutaminase catalyzed cross-linking and Neutrase catalyzed hydrolysis on the rheological properties of egg, gluten and soy protein derivatives, and on the thermo-mechanical performance of the proteins - whole rice flour mixtures. Tests performed on 15% protein suspensions indicated that the rheological behaviour varied significantly with the substrate and type of enzyme treatment. The controlled enzyme treatment improved both the consistency and strength of the egg and soy proteins based suspensions. Moreover, the values of G'' , G' and flow threshold values increased significantly after the enzymes treatment. On the other hand, lower viscosity and stability were observed when investigating the effect of the enzymes on the rheological behavior of gluten suspension. Further tests were meant to investigate the effect of transglutaminase and Neutrase addition on the thermo-mechanical behavior of the protein containing doughs based on whole rice flour. Addition of both enzymes to the flour mixtures resulted in significant changes in the Mixolab curves describing in particular the behaviour of the proteins at increasing temperature, but also starch gelatinization and retrogradation.

Key words: *gluten, egg proteins, soy proteins, transglutaminase, Neutrase.*

INTRODUCTION

The enzyme assisted bio-processing of flour can be successfully applied for improving dough processability and final quality of the bakery products. Applications on gluten free matrices are more challenging with respect to the wheat based ones, because of the lack of viscoelastic properties specific to the gluten network. Due to the low allergenic potential, pleasant sensory characteristics and nutritional benefits, rice flour was nominated as the most suitable cereal for making gluten free products (Gujral and Rosell, 2004; Marco and Rosell, 2008a). In order to overcome drawbacks like difficulties in retaining the CO₂ generated through fermentation, the addition of gums, starches and hydrocolloids to the gluten free bakery products has been proposed, resulting in very low protein contents and deficit of lysine. Therefore selection of appropriate protein sources, with balanced amino acids profile and appropriate functionality, plays a key role in obtaining bread products with desired quality. The baking performance of the gluten free batters highly depends on the particular

formulation used, because different components of the mixtures can interact to different extent to each other (Hager and Arendt, 2013; Matos and Rosell, 2013). In particular, depending on the source, proteins can alter water distribution within the batter and weaken the interactions between hydrocolloids and starch matrix (Crockett et al., 2011; Renzetti and Rosell, 2016).

One way to improve the proteins behavior in the gluten free mixtures, such as to resemble the network like structure, rely on the use of different protein modifying enzymes. Starting from the main elements defining the baking functionality of different batters, Renzetti and Rosell (2016) provided a nice comparative overview focusing on the use of enzymes for enhancing the functionality of proteins from both gluten-free flours used as basis in different mixtures, and those arising from different supplements.

Transglutaminase (TG) and different oxidases such as glucose oxidase, lipoxygenase, sulphhydryl oxidase, polyphenoloxidase and peroxidase, catalyzing direct or indirect cross-linking of proteins appeared to be effective

alternatives for generating effective protein networks in the gluten free matrices (Renzetti and Rosell, 2016). In case of TG, whose activity depends on the accessibility of glutamine and lysine residues in the proteins (Houben *et al.*, 2012), the enzyme concentration has to be accurately adapted to the substrate, such as to avoid altering the quality of the final product. For instance, Gujral and Rosell (2004) identified the optimum bread volume and crumb softness for the medium tested enzyme concentration of 1.0 (w/w), although the viscous (G'') and elastic (G') moduli of the rice flour based doughs displayed progressive enhancement at even higher enzyme concentration. Advanced protein cross-linking might cause excessive tightening of the dough structure, impeding the expansion during proofing.

On the other hand, proteases are responsible for peptide bonds hydrolysis in proteins, being therefore effective in standard baking application for controlling gluten related properties of doughs. Concerning the gluten free products, Renzetti and Arendt (2009) reported improvement of the rheological properties of the batters and of the specific volume and crumb softness of breads obtained from brown rice flour treated with Neutrase (N) from *Bacillus amyloliquefaciens*. Moreover, Kawamura-Konishi *et al.* (2013) indicated the possibility of improving the quality of the rice flour based bread through treatment with different commercially available protease.

The aim of the present study was to estimate the impact of enzyme catalyzed protein hydrolysis and cross-linking on the rheological properties of protein derivatives, and their thermo-mechanical performance when introduced in a gluten free matrix. In particular the effect of TG and N addition on the properties of powdered eggs, soy protein concentrate and vital gluten alone or in admixture with whole rice flour was tested.

MATERIALS AND METHODS

Materials

The wholegrain rice flour (Solaris Plant SRL, Bucharest, Romania) was purchased from a local market (Galati, Romania). The proximate composition of the commercial wholegrain rice

flour was as follows: 13.46±0.11% moisture, 6.18±0.41% protein, 2.16±0.07% fat, and 0.99±0.17% ash.

The protein sources used as substrates for the enzyme treatments were: soy protein concentrate (Ubimedia S.R.L., Galati, Romania; 9.52±0.09% moisture and 74.28±0.47% protein), dried whole eggs powder (Agricola Bacau, Romania; 4.67±0.06% moisture and 49.39±1.51% protein), and vital gluten (SanoVita, Vâlcea, Romania; 6.62±0.04% moisture and 76.06±0.71% protein).

Commercial transglutaminase (ActivaTMTG, Ajinomoto Corporation Inc., Tokyo, Japan) and Neutrase 5.0 BG (Novo Nordisk, Denmark) were used in the experiment at levels recommended by the producers for bakery applications. Transglutaminase (TG) has a declared enzymatic activity of about 100 UE/g, and is active over large temperature (2–60°C) and pH (5–8) domains. Neutrase (N) has a declared activity of 5 UA/g and is active in the temperature and pH domains of 25–70°C and 5–8, respectively. The concentration of TG and N used in the experiment was 0.1g/g protein, and 0.001g/g protein, respectively.

Proximate composition

The moisture content was determined through the AACC 44-51 method, the protein content through the semimicro-Kjeldahl method (Raypa Trade, R. Espinar, S.L., Barcelona, Spain), the fat content by extraction with ether using a Soxhlet extractor (SER-148, VELP Scientifica, Usmate Velate (MB), Italy) and the ash content using SR ISO 2171: 2002 method.

Fundamental rheological measurements

Rheological properties of protein based suspensions of 15% concentration, treated with TG or N for 30 min at 50°C, were determined using an AR2000ex Rheometer (TA Instruments) equipped with Peltier control temperature jacket. The control samples for the rheological measurements consisted of protein suspensions with no enzyme addition. Due to different consistency of studied protein matrices – liquid like in case of egg suspension and solid like in case of soy protein isolate – distinct geometric systems were used. The egg based suspensions were tested in a double gap concentric cylinder (inner radius of 32 mm, outer radius of 35.03 mm, cylinder length of

54.95 mm and gap of 500 mm), while gluten and soy suspensions were tested with a single gap cylinder cup (inner radius of 28.01 mm, cylinder length of 42.01mm and gap of 5920 mm). The geometric systems were chosen such as to allow the best testing conditions, as it is known that choosing an adequate geometric system is crucial for correct rheological testing (Abu-Jdayil, 2003; Mori et al., 2006).

The rheological behavior of all studied samples was evaluated by the means of low amplitude dynamic shearing tests - strain sweep, frequency sweep and temperature ramp. The registered parameters were storage modulus (G') and loss modulus (G'').

For strain sweep tests the controlled parameter was the oscillatory frequency of 1 Hz, while increasing strain from 0.01 to 100%. System temperature was set to 20°C. This test was used to identify the linear viscoelastic region (LVR) for all investigated samples.

The dynamic frequency sweeps were further performed in the 0.1 - 100 Hz domain, at a constant strain of 0.3% (determined to be within the LVR). The test provides information about structural assembly of the tested viscoelastic material as a function of the time scale of the applied deformation. A viscoelastic material will flow over long times and bound over short times of applied deformation. For each sample the frequency dependency was determined with power law equation: $\eta = k \times \omega^{(n-1)}$, using the TA Rheology Advantage Data Analysis Software V4.8.3., where η - viscosity (Pa·s), k - consistency index, ω - frequency (Hz), and n - frequency index.

Rheological behavior of studied suspensions as a function of system temperature was determined by ramping up the temperature from 20 to 90°C (110°C in case of SPI) with a rate of 1.5°C/min in *quasi-static* conditions (0.3% strain, 1 Hz). The temperature was then maintained for 10 min at 90/110°C. Water evaporation was avoided by covering the immersion cup with a solvent trap cover.

Empirical rheological measurements

The thermo-mechanical behavior of the mixtures consisting of 85% wholegrain rice flour and 15% of vital gluten, powdered eggs or soy protein concentrate supplemented with TG or N was assessed by means of Mixolab

Chopin (Tripette & Renaud Chopin, Villeneuve La Garenne, France), using the Chopin+ protocol, as previously described by Patrascu et al. (2016). In case of the samples supplemented with TG, the mixtures were kneaded in Mixolab tank with the appropriate amount of water to get doughs with maximum torque of 1.1 Nm, which were then left to rest for additional 30 minutes, after which the test was restarted.

The parameters recorded from the Mixolab curves give indications about proteins functionality (minimum C2 (Nm) torque is related to protein weakening while subjecting the sample to mechanical work and heating at 4°C/min) and starch behavior (the C3 (Nm) torque is related to starch gelatinization, C4 (Nm) gives indication on the stability of the starch gels at high temperature (90°C), and C5 (Nm) is connected to starch retrogradation when cooling the dough to 50°C) (Collar et al., 2007).

RESULTS AND DISCUSSIONS

Effect of enzyme addition on the rheological properties of the protein suspensions

The strain sweep test was used to differentiate between three structure characteristics of the investigated viscoelastic materials, as described in Patrascu et al. (2016) - linear viscoelastic region characterized by constant G' and G'' values, transition phase when G' values starts to decrease while the tested material still shows a solid like behavior ($G' > G''$), and the yield point at G'/G'' intersection when the onset of flow occurs - material enters the viscous domain and phase angle (δ) exceeds 45°. In the case of egg and soy proteins based suspensions the enzyme treatment determined the increase of samples elasticity. The strain values corresponding to G'/G'' cross-over, marking the phase inversion and the beginning of flow, significantly increased after enzyme treatment ($p < 0.05$) (Table 1). It is known that TG addition determines proteins crosslinking, improving their mechanical properties (Marco and Rosell, 2008).

Regardless of the enzyme treatment, gluten based suspensions registered decreased consistency (lower G' values) compared to the control sample. Moreover, the enzyme treatment significantly affected samples

resistance ($p < 0.05$), *i.e.* lower yield point values were obtained (Table 1).

Regarding N effect on the behavior of gluten suspension during the strain sweep tests, the obtained response can be explained by advanced peptide bonds breakdown leading to higher concentration of soluble proteins. As for the poor rheological characteristics obtained for

gluten suspension treated with TG, the behavior can be due to the low lysine content.

Similar observations were made by Renzetti et al. (2008) when studying the effect of transglutaminase on some gluten free flours, who declared that the weakening of corn flour structure can be due to the lysine deficiency of corn proteins.

Table 1. The yield point of the egg, gluten and soy protein based suspensions during strain sweep test

	Enzyme treatment		
	Control*	TG	N
Dried whole eggs	1.88±0.18%	4.29±0.79%	4.44±1.03%
Vital Gluten	38.2±1.24%	19.74±2.30%	10.98±0.82%
Soy protein isolate	3.84±0.08%	38.40±1.18%	83.37±1.56%

*Control – no enzyme treatment

The viscoelastic characteristics of egg, gluten and soy protein based suspensions as a function of time scale of the applied deformation are presented in Figure 1.

The egg based suspensions showed a frequency dependent viscoelastic response, with alternate prevailing of elastic ($G' > G''$) or viscous

moduli ($G'' > G'$) resembling plastic behavior. Enzymatic treatment of egg proteins determined an increase in consistency when compared to control sample.

Cold enzymatically set gels were obtained when treating the sample with TG.

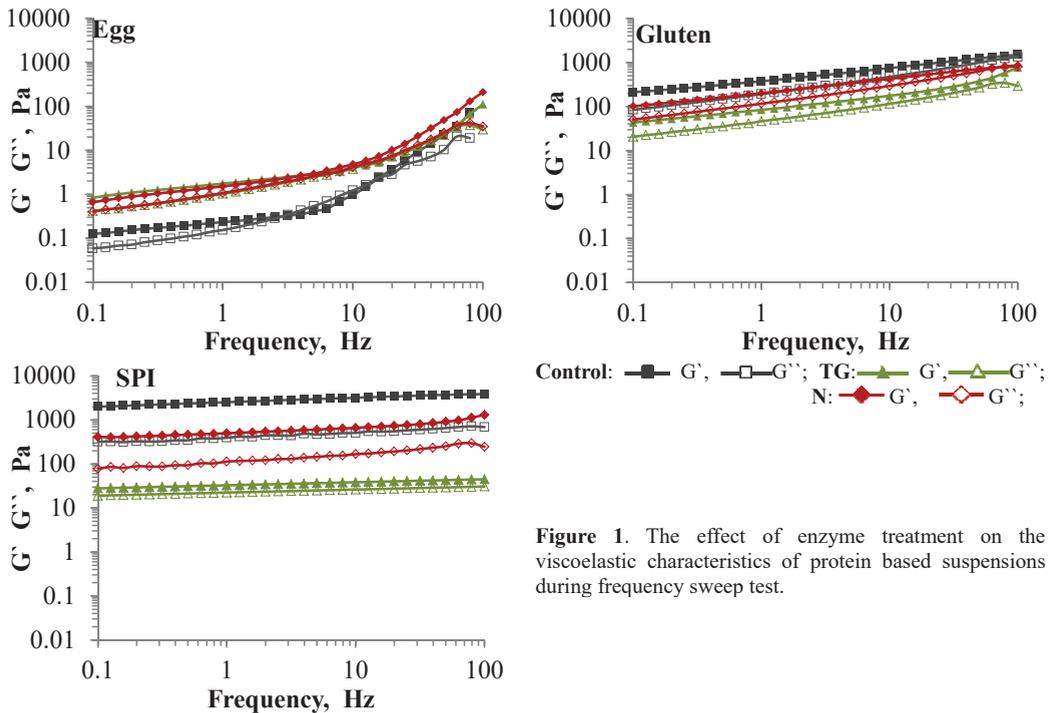


Figure 1. The effect of enzyme treatment on the viscoelastic characteristics of protein based suspensions during frequency sweep test.

According to observations made by Tunick (2011) and Alting et al. (2004), gels with a perfectly crosslinked structure present a minimal frequency dependency and are more solid like due to the formation of a covalent network. The TG egg proteins based suspension presented a weak gel like structure (frequency index $n = 0.664$). Gluten and soy protein based suspensions, both controls and enzymatically treated ones, as presented in Figure 1, showed a dominant elastic response, specific to solid like materials. However, the frequency dependency behavior

was more evident in case of the gluten suspensions. The lowest frequency dependency was observed in the case of soy protein based sample treated with TG. In addition, the frequency index of this sample was very low ($n = 0.06$), being characteristic to perfectly crosslinked structures.

When studying temperature dependent viscoelastic behavior of targeted suspensions in *quasi-static* conditions, it was observed that the enzyme treatment influenced mainly the egg and soy protein based samples (Figure 2).

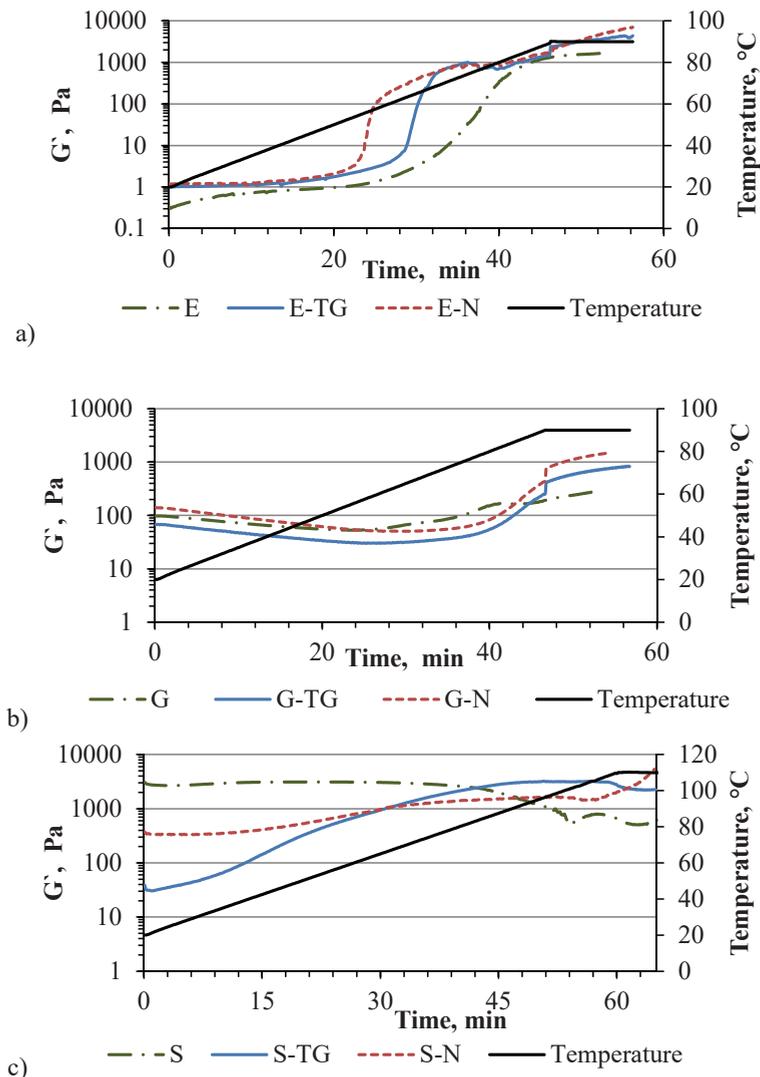


Figure 2. Rheological behavior of TG and N treated egg (a), gluten (b) and soy (c) protein suspensions during oscillatory temperature ramp test.

In these two cases, the G' vs. applied temperature curves presented a different trend with respect to the control. Thus, if control soy protein suspension presented at low temperatures rather constant values for G' which started to decrease after 50°C, the enzyme treated ones registered a constant and accentuated increase of storage modulus from the beginning of the test. Obtained results are similar to those reported by Tang et al. (2006) when studying the effect of transglutaminase on glycinin and β -coglycinin. They observed that during temperature increase from 25 to 90°C, the G' values constantly increased, most probably due to interactions between β subunits of β -coglycinin and BS subunits of glycinin. It was also observed that enzymatic treatment influenced the temperature values characteristic to proteins denaturation. The phenomenon is usually marked by the presence of inflection points in the G' curve. In this respect, it could be observed that enzymatic treatment of egg based suspensions led to a pronounced decrease of temperature domains associated with protein denaturation and gel formation. In case of the enzyme treated gluten and soy protein based samples, the temperature domains associated to sol-gel transition were rather within those of the corresponding control samples. These results indicate lower thermal stability of the gluten and soy protein aggregates and hydrolysates with respect to the native proteins.

Effect of enzyme addition on the thermo-mechanical properties of whole rice flour enriched with proteins

The Mixolab apparatus was further used to test the influence of TG and N on the thermo-mechanical behavior of the doughs, consisting on whole rice flour (85%) and protein derivatives (15%). Preliminary tests were carried out on whole rice flour with no protein addition, such as to get an overview on the susceptibility of the rice proteins to cross-linking and hydrolysis reactions catalyzed by TG and N, respectively. Regardless of the enzyme added, the water needed to get a maximum torque value of about 1.1 Nm (C1) at 30°C for the whole rice flour was 62%. In case of the samples supplemented with transglutaminase, after 30 min of enzyme reaction at 30°C the Mixolab test indicated an

increase of the development time (from 1.38 to 1.92 min) required to get the maximum torque value. These results are in agreement with the observations of Marco and Rosell (2008a), suggesting higher resistance of the dough to kneading, as a consequence of the TG catalyzed cross-links formed between rice proteins. Mainly albumin-globulin and glutelin fractions, representing about 15.5% and 77.8%, respectively of total proteins in the rice flour, were reported to be involved in the cross-linking reactions catalyzed by TG (Marco et al., 2007). As a result of the increase of proteins molecular weight in the rice flour, a progressive improvement of the dynamic rheological properties of the doughs with the raise of TG concentration was observed by Gujral and Rosell (2004). Further heating and cooling of the samples over the entire Chopin+ test, which simulates the breadmaking process, resulted in rather similar values of the specific minimum and maximum torques. The C2 torque was higher in the sample treated with TG (0.789 Nm) with respect to the whole rice flour sample with no enzyme addition (0.761 Nm) (Figure 3). On the other hand, addition of the N caused the significant reduction of the C2 to 0.542 Nm, whereas all other Mixolab parameters were similar to the whole rice flour samples with no enzyme addition (Figure 3).

The influence of powdered eggs, soy protein concentrate and vital gluten addition of the thermo-mechanical properties of whole rice flour was discussed in detail elsewhere (Patraşcu et al., 2016). Therefore, only the characteristics that help understanding the effect of TG and N catalyzed reactions will be further presented. Protein addition to the whole rice flour affected the water absorption required to get the constant C1 torque of 1.1 Nm, corresponding to the optimum dough consistency (Patraşcu et al., 2016). For each type of investigated protein product no variation of the water absorption was considered when testing the effect of enzyme addition. Addition of TG to the protein enriched whole rice flour based samples caused no significant changes of the Mixolab curve parameters. Our results comply with the observation of Marco and Rosell (2008b) who studied the effect of TG on the rheological properties of a mixture consisting on rice flour

and egg proteins, and reported no significant differences in RVA parameters - peak viscosity, gel stability and starch retrogradation. The most significant changes induced by TG addition were observed in samples with soy protein

concentrate, especially at temperatures over 61°C, when the highest C3, C4 and C5 values (3.01, 3.34 and 5.61 Nm, respectively) were registered in the Mixolab curves (Figure 3).

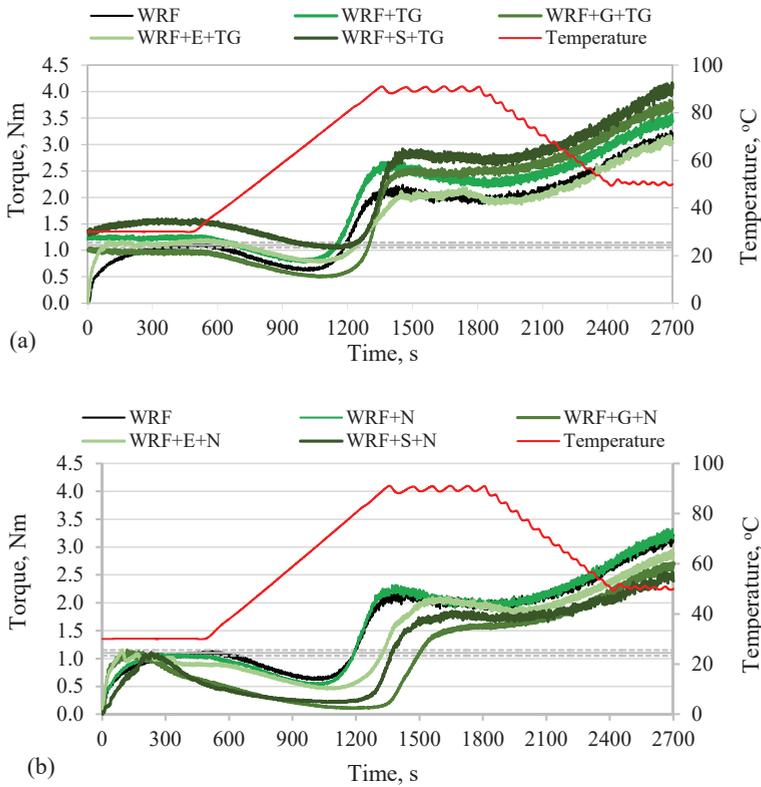


Figure 3. Thermo-mechanical behavior of the whole rice flour (WRF) supplemented with 15% vital gluten (G), powdered eggs (E) or soy protein concentrate (S) and treated with TG (a) or N (b)

These results might be due to the high molecular weight protein aggregates formation catalyzed by TG, resulting in a more continuous protein phase (Marco et al., 2007) in the investigated samples. Marco et al. (2008) and Tang et al. (2006) showed that TG is able to catalyze intermolecular cross-linking of proteins from soy and rice. Most of the cross-links involved β -conglycinin and glycinin from soy, and glutelin, albumin and globulin from rice (Renzetti and Rosell, 2016). In particular, the glycinin/ β -conglycinin ratio appears to be decisive for the properties of the TG cross-linked soy protein based gels (Tang et al., 2006). Finally, in case of the gluten containing sample, addition of TG resulted in the increase

of the C2, C4 and C5 values. A higher exposure of the lysine and glutamine residues was reported by Wang et al. (2007) when heating the gluten, therefore providing new sites recognized by TG. The TG catalyzed cross-links at thermal treatment might significantly contribute to defining the thermo-mechanical behavior of the dough in the regions of the Mixolab curve where starch is normally the main contributor. As shown by Wu and Corke (2005), gluten cross-linking might improve the water binding capacity due to the increase of overall hydrophobicity, therefore explaining the tendency observed in the torque values (Table 2).

Table 2. Effect of TG and N addition on the torque values of the whole rice flour (WRF) based batter samples supplemented with 15% gluten (G), powdered eggs (E) or soy protein concentrate (S). The torque values were assessed by Mixolab device during mixing and heating stages of the Chopin+ protocol.

Sample	Enzyme treatment	C2-C2 _E , Nm	C3-C3 _E , Nm	C4-C4 _E , Nm	C5-C5 _E , Nm
WRF	TG	-0.028	0.017	0.022	-0.035
	N	0.094	-0.113	-0.031	-0.070
WRF+S	TG	-0.053	-0.179	-0.646	-1.539
	N	0.742	0.828	0.779	1.363
WRF+E	TG	-0.049	-0.030	-0.02	0.039
	N	0.324	-0.011	0.142	0.215
WRF+G	TG	-0.028	0.034	-0.285	-0.267
	N	0.199	0.981	0.432	0.734

The addition of N to the whole rice flour based samples supplemented with 15% protein products caused important changes of the Mixolab curve with respect to the corresponding samples with no enzyme addition. Regardless of the type of protein used to supplement the whole rice flour, N caused the decrease of the C2 torque (Table 2). Moreover, the addition of N in the whole rice flour based samples supplemented with protein products modified the development time, the stability of the dough, and the speed of proteins softening. For instance, when N was added to the sample with powdered eggs the dough development time decrease from 8.77 min to 1.57 min, and the stability decreased from 11.6 min to 4.78 min. Similarly, a significant reduction of the development time from 9.37 min to 3.97 min, and of dough stability from 6.98 to 2.32 min was observed in case of adding N to the sample with soy protein isolate. Moreover, an increased protein softening, resulting in the decrease of the minimum C2 torque from 0.96 to 0.22 Nm, was associated to the hydrolysis of the soy proteins by N. In addition, a 4°C shift of the temperature associated to the C2 values (from 64.3 to 59.3°C) was registered in the Mixolab curves, suggesting lower thermal stability of the soy peptides after the hydrolysis catalyzed by N. Unlike soy proteins, hydrolysis of the vital gluten incorporated into whole rice flour was accompanied by the increase from 55.6 to 63.6°C of the temperature corresponding to the minimum C2 torque.

Regarding the torque values associated to starch behavior, except for the C3 which was higher when adding N to the powdered egg supplemented sample, all other torque values decreased in the samples where protein hydrolysis occurred (Table 2). Although acting

on protein substrates, N significantly altered the Mixolab parameters related to starch gelatinization, gel stability and starch retrogradation. For instance, when adding N to the dried eggs supplemented sample, the C4 decreased from 1.986 to 1.844 Nm, and C5 from 3.095 to 2.880 Nm. Even if the most significant decrease of the torque values due to the N catalyzed hydrolysis was registered in case of the samples with soy proteins (C3 from 2.63 to 1.80 Nm, C4 from 2.49 to 1.71 Nm, and C5 from 3.83 to 2.47 Nm), the thermo-mechanical parameters indicated that the mixture is suitable to be used for gluten free bread applications. Ragae and Abdel-Aal (2006) suggested that changes in starch behavior at thermal treatment might be a consequence of altering the starch - protein interactions, subsequent to the hydrolytic activity of proteases. Depending on the source and properties, when incorporated in certain matrices the protein products might prevent the starch granules to swell sufficiently. The proteins surrounding the starch granules confer rigidity to the starch paste, but proteins hydrolysis might induce viscosity decrease. Anyway, Renzetti and Arendt (2009) observed that lowering the viscosity of the batters based on rice flour, as a consequence of proteolytic enzymes addition can be correlated with the improvement of the volume of products.

CONCLUSIONS

The rheological behavior of the protein based suspensions highly depended on the source of proteins and type of enzyme used for the preliminary treatment. The Mixolab curves indicated that transglutaminase and Neutrase had no significant influence on the rice proteins. The most important changes in the

thermo-mechanical behavior of the enzyme treated samples were registered in the Mixolab zones mainly showing the protein behavior, in case of the soy protein containing samples. In addition to the protein source and composition, the cross-linking and hydrolysis reactions catalyzed by transglutaminase and Neutrase influenced the gelatinization and retrogradation of the starch from the whole rice flour.

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