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Influence of Soy Protein Hydrolysates on Thermo-Mechanical Properties of Gluten-Free Flour and Muffin Quality

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Abstract: The influence of protease-assisted hydrolysis on the impact exerted by the soy protein isolate on the thermo-mechanical behavior and baking performance of the gluten-free composite flour, consisting of a mixture of rice and quinoa flours, was investigated. The mPAGE analysis revealed that soluble fractions of the hydrolysates, obtained with bromelain, Neutrase or trypsin, concentrated the peptides with a molecular weight lower than 20 kDa, whereas the insoluble ones retained higher molecular weight fragments. The influence of the separate and cumulative addition of the soluble and insoluble soy peptide fractions on the thermo-mechanical properties of dough was tested by means of a Mixolab device. Regardless of the enzyme used for hydrolysis, the addition of the soluble peptide fraction to the gluten-free composite flour resulted in delayed starch gelatinization, whereas the insoluble one caused a considerable increase in the dough consistency. The most important improvements in the dough behavior were observed when supplementing the gluten-free flour with 10% soy protein hydrolysates obtained with bromelain and trypsin. The gluten-free muffins enriched in soy protein hydrolysate exhibited important differences in terms of moisture, height and specific volume, compared to the control. Moreover, the ABTS- and DPPH-based methods indicated that protein hydrolysate addition caused a significant improvement in the antioxidant activity (by at least 38% and 23%, respectively) compared to the control. In conclusion, soy protein hydrolysate might be successfully used for increasing both the protein content and the antioxidant activity of the muffin samples.

Keywords: gluten-free composite flour; protein hydrolysis; rheological properties; muffins

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1. Introduction

The popularity of the gluten-free diet increased significantly in the last years, gluten-free products being popular not only among people who are intolerant or allergic to gluten but also among those who chose to have a healthy lifestyle [1]. The gluten-free diet has an important role in alleviating undesired or life-threatening symptoms in celiac or gluten-allergic patients, but careful attention should be paid to the long-term consequences caused by the nutritional limitations of the gluten-free diet. Considering the rather unique functionality of gluten in the breadmaking process and the quality of the final products, identifying a suitable gluten-free formulation for mimicking regular food products' quality, such as to meet the market demands, is challenging for food specialists. A widely used strategy for developing gluten-free products involves the use, as main ingredients, of gluten-free flours from cereals or pseudocereals and eventually of starches, proteins and hydrocolloids [1]. Do Nascimento et al. [2] pointed out that most commercially available gluten-free products are based on cheap ingredients, like rice and or corn flours, eventually combined with starch of different origins such as cassava and potato. Gluten-free products have higher amounts of fats, for improving the mouthfeel, carbohydrates and sodium, and are generally poor in high-quality proteins or fibers compared to regular products [1,3].

Because of the high digestibility, hypoallergenic properties, mild taste and white color [4], rice flour was used in the present study for developing gluten-free muffins. In order to balance the nutritional profile of the final product, quinoa flour was chosen to be used in ad-mixture with the rice flour. The quinoa contents of protein (14.12%), lipid (6.07%), ash (2.7%) and fiber (7.0%) are significantly higher compared to rice (6.81, 0.55, 0.19 and 2.8%, respectively) and most of the grains [5]. In particular, quinoa is acknowledged as a good source of high-quality proteins which are rich in amino acids deficient in cereals, such as lysine, methionine, histidine and threonine [6], of unsaturated fatty acids, like alpha-linoleic, oleic and linolenic acids which represent 87–88% of the total fatty acids [7] and dietary fibers [5]. Moreover, quinoa is rich in vitamins, mainly pyridoxine, folic acid and vitamin E, and minerals like calcium, iron, magnesium and potassium, in amounts considered sufficient for a balanced diet [5,6].

In order to compensate the low protein content in gluten-free mixtures, which do not usually meet the required daily dietary amounts and affect the quality of the backed products [3], different studies focused on identifying suitable sources of proteins for fortifying the gluten-free product. Among protein sources, legumes attained the interest of researchers, and soy is of particular importance because of the good availability, low cost and multiple benefits associated with the high biological value and good functional properties [3]. Moreover, different studies highlighted the possibility of enhancing the technological functionality of these proteins by using exogenous enzymes, in addition to improving the physiological properties because of the release of the encrypted peptides with antioxidant activity, antihypertensive and anticancer properties, hypocholesterolemic effects, etc. [8–10]. Unlike other physiologically active compounds, the use of peptides for providing potential health benefits to the host, through diet, is highly desired, because of the low costs, good absorption, avoidance of safety issues and contribution to nutritional value [10,11].

The aim of the study was to investigate the impact of soy protein hydrolysates' addition on the rheological properties of the gluten-free dough and on the properties of the muffins. The soy protein hydrolysates prepared using three different exogenous enzymes, namely bromelain, Neutrase and trypsin, were freeze-dried and were further added to the gluten-free composite flour consisting of whole rice (RF) and quinoa flour (QF). In addition to the influence of the total protein hydrolysates, the contribution of the soluble and insoluble fractions of soy protein hydrolysate to the rheological behavior of the gluten-free dough was investigated.

2. Materials and Methods

2.1. Materials

The commercial whole rice flour (RF; origin Greece) (moisture 11.2%, proteins 7.1%, fats 2.8%, fibers 4.6%) and quinoa flour (QF; origin Peru) (moisture 9.2%, proteins 14.0%, fats 6.1%, fibers 7.0%) distributed by Solaris Plant S.R.L. (Bucharest, Romania) and the soy protein isolate (SPI; Supro® XT 221D IP, distributed by KUK Romania, Voluntari, Romania) (moisture 4.8%, proteins 87.1%) were used in the study. Enzymes of various origins were selected for preparing the protein hydrolysates: bromelain of vegetal origin (Carl Roth, Karlsruhe, Germany), Neutrase 5.0 BG of microbial origin (Novo Nordisk, Bagsværd, Denmark) and trypsin of animal origin (Merck, Darmstadt, Germany).

1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

2.2. Composition Analysis

The analysis of the gluten-free flours' composition was conducted using the following methods: SR ISO 712:2005 for moisture content [12], the semimicro-Kjeldahl method (Raypa Trade, R Espinar, SL, Barcelona, Spain) for protein content (nitrogen-to-protein conversion

factor of 5.95 for rice flours and 6.25 for quinoa flour) and the Soxhlet method (SER-148; VELP Scientifica, Usmate Velate, Italy) for fat content.

2.3. The Hydrolysis of the Soy Protein Isolate

The hydrolysis of the soy protein suspensions (12% *w*/*v*) with bromelain (0.5 g/100 g proteins), Neutrase (1 g/100 g proteins) and trypsin (1 g/100 g proteins) was carried out for 68 h at 50 °C while continuously shaking (SI300R; Jeio Tech, Chalgrove, UK) at 100 rpm, as indicated in [10]. After enzymes' inactivation through heating for 5 min at 90 °C, half of each protein hydrolysate was directly subjected to freeze drying (CHRIST Alpha 1–4 LD plus, Osterode am Harz, Germany), and the resulting hydrolyzed SPIs (hSPIs) were coded hSPI-B, hSPI-N and hSPI-T; the other half was first centrifuged at 14,000 rpm for 10 min followed by freeze drying the soluble fractions concentrated in the supernatants (coded sSPI-B, sSPI-N and sSPI-T) and the insoluble ones separated in the pellets (coded rSPI-B, rSPI-N and rSPI-T).

2.4. mPAGE Analysis

The soy protein isolate and hydrolysates (hSPI, sSPI and hSPI) were diluted with distilled water, homogenized with mPAGE 4× LDS Sample Buffer (Merck, KGaA, Darmstadt, Germany), treated at 95 °C for 5 min and run in the denaturing mPAGE® Lux Casting Bis-Tris polyacrylamide gels (Merck, KGaA, Darmstadt, Germany). The concentrations of the separation and stacking gels were 12% and 5%, respectively. A volume of 10 µL (concentration of 4 µg/µL) of each sample was loaded on the stacking gel and run at 90 V for 80 minutes in the running buffer (Bio-Rad, Hercules, CA, USA). The protein bands were fixed with 50% methanol/10% acid acetic (*v*/*v*), stained in 0.1% *w*/*v* Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MI, USA) and then de-stained in methanol/acetic acid/water (40:20:140). The Precision Plus Proteins Dual Xtra Prestained marker (Bio-Rad, Hercules, CA, USA) was migrated in parallel with the samples.

2.5. The Preparation of the Gluten-Free Flour Mixtures

The gluten-free composite flour consisting of equal parts of RF and QF was used as the basis for preparing dough. In order to increase the total protein content of the gluten-free composite flour, the SPI hydrolysates were further used to replace 10% of RF in the mixture. A total of nine protein-enriched gluten-free flour mixtures were prepared by incorporating the whole SPI hydrolysates (hSPI-B, hSPI-N and hSPI-T), the soluble fractions (sSPI-B, sSPI-N and sSPI-T) or the insoluble ones (rSPI-B, rSPI-N and rSPI-T) and were further used for testing the thermo-mechanical behavior of the dough. The composite flours supplemented with hSPI-B, hSPI-N and hSPI-T, which exhibited the most promising thermo-mechanical behavior, were further used for preparing muffins. The supplementation level of the gluten-free composite flour with soy proteins or peptides was established upon running preliminary tests, so as to ensure that about half of the total protein content of the final mixtures originate from soy. The composite flour consisting of equal parts of RF and QF was used as the control.

2.6. The Thermo-Mechanical Behavior of the Gluten-Free Flour Mixtures

The Mixolab device (Chopin Technology, Villeneuve La Garenne, France) was used to monitor the rheological properties of the gluten-free dough. The Mixolab curves recorded using the Chopin+ protocol include five distinct phases (Ph) describing the behavior of the dough subjected to double kneading and a temperature constraint. In the first phase (Ph I), ranging from 0 to 480 s, the dough is kneaded at a constant temperature of 30 °C; in the next 900 s, defining the second phase (Ph II), the dough is heated from 30 to 90 °C; in the third phase (Ph III), which lasts 420 s, the dough is kept at a constant temperature of 90 °C; the dough is afterwards cooled down from 90 to 50 °C over 600 s in the fourth phase (Ph IV); and finally, in the fifth phase (Ph V), is kept for 300 s at 50 °C. A specific parameter is marked in each phase on the Mixolab curve, as follows: C1 is the maximum

consistency of the dough registered during Ph I; C2 is the minimum dough consistency associated with the weakening of the proteins, measured during Ph II; C3 is the maximum consistency which measures gelatinization that occurred during Ph III; C4 is the minimum dough consistency indicating the stability over heating in Ph IV; and C5 is the maximum consistency providing an indication on starch retrogradation during Ph V. The dough consistency values registered at the end of Ph I (after 8 min), Ph II (after 23 min), Ph III (after 30 min), Ph IV (after 40 min) and Ph V (after 45 min) were termed C-I, C-II, C-III, C-IV and C-V, respectively. All experiments were conducted using a modified protocol which uses a dough weight of 90 g, and a water absorption (WA) of 65% was selected for preparing all samples. The measurements were performed at least in duplicate.

2.7. Muffin Preparation

The gluten-free mixtures supplemented with hSPI, which presented the best thermo-mechanical behavior, as indicated by the Mixolab measurements, were further used to prepare muffins. In agreement with Singh et al. [13] with slight modification, the batter formulations used to prepare the muffin samples were obtained by mixing the following ingredients: gluten-free mixtures (100 g), refined white sugar (65 g), sunflower oil (40 g), baking powder (5 g), salt (1g) and xanthan (0.5 g), as indicated in Table 1. The ingredients were well mixed using a Braun mixer (De'Longhi, Neu Isenburg, Hessen, Germany), as indicated by Banu et al. [14], and after pouring 48 g of each batter into the paper cups placed in the muffin baking trays, the backing was performed at 180 °C for 20 min using an electric oven (Electrolux, Stockholm, Sweden). The samples were stored in the paper cups at room temperature until further characterization.

Table 1. Formulation of batter used to obtain gluten-free muffins (M—control muffin; M-SPI—muffins enriched with soy protein isolate; M-hSPI-B—muffins enriched with soy protein hydrolysate produced with bromelain; M-hSPI-N—muffins enriched with soy protein hydrolysate produced with Neutrase; M-hSPI-T—muffins enriched with soy protein hydrolysate produced with trypsin).

Ingredients	Muffin Samples				
	M	**M-SPI**	**M-hSPI-B**	**M-hSPI-N**	**M-hSPI-T**
Rice flour, g	50	40	40	40	40
Quinoa flour, g	50	50	50	50	50
SPI, g	-	10	-	-	-
hSPI-B, g	-	-	10	-	-
hSPI-N, g	-	-	-	10	-
hSPI-T, g	-	-	-	-	10
Sugar, g	65	65	65	65	65
Oil, mL	40	40	40	40	40
Baking powder, g	5	5	5	5	5
NaCl, g	1	1	1	1	1
Xanthan gum, g	0.5	0.5	0.5	0.5	0.5
Water, g	85	85	85	85	85

The weight loss during baking (WLB) was calculated as follows:

$$WLB = \frac{m_B - m_F}{m_B} 100 \quad (1)$$

where m_B is the mass of the batter used to obtain the muffin, and m_F is the mass of the sample upon cooling to room temperature.

2.8. Muffin Characterization

The muffin samples were characterized by assessing the main physico-chemical characteristics within 24 h after samples cooling to room temperature.

The moisture content was determined using the method SR 91:2007 [12]. The muffin height was estimated by measuring, with a caliper, the distance between the flat bottom surface to the highest point of each sample. The specific volume (cm^3/g) of the muffins was determined by the rapeseed displacement method (SR 91:2007, [12]).

The texture of the muffin samples was assessed by measuring the firmness of the crumb with the MLFTA apparatus (Guss, Strand, South Africa) and a probe of 7.9 mm in diameter. For each tested formulation, two samples were penetrated for 25 mm in three different points, at a penetration speed of 5 mm/s and a trigger threshold force of 20 g [15].

The CIElab color parameters of the muffin crust in crumb, in terms of the brightness/darkness (L*), redness/greenness (a*) and yellowness/blueness (b*), were measured using the Chroma Meter CR-410 (Konica Minolta Business Solutions Europe GmbH) colorimeter. In agreement with Matos et al. [16], in order to evaluate the effect of soy proteins' or peptides' addition on the color appearance parameter of the muffin samples, the chroma (C*) and hue angle (H) were calculated using Equations (2) and (3):

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$H = \tan^{-1}(b^*/a^*) \text{ for quadrant I } (+a^*, +b^*) \quad (3)$$

The antioxidant activity of the muffin samples was determined, as described by [17], using the $ABTS^+$ and DPPH radical scavenging activity (ABTS-RSA and DPPH-RSA, respectively) methods. In short, the muffin samples were subjected to extraction in 80% aqueous methanol solution, for 2 h at 23 ± 2 °C. The extracts obtained upon centrifugation for 15 min at 10,000 rpm were further used for measuring the antioxidant activity. ABTS-RSA was determined by measuring the absorbance of a mixture consisting of a 40 µL extract and 2.96 mL $ABTS^{\cdot+}$ solution, at a wavelength of 734 nm, immediately and after 6 min. DPPH-RSA was determined by measuring the decrease, over 20 min, of the absorbance, at a wavelength of 515 nm, of a mixture consisting of a 100 µL extract, 250 µL DPPH solution and 2.1 mL of 80% aqueous methanol solution. In the case of each method, a Trolox standard curve was prepared, and the antioxidant activity was expressed as µmol Trolox/g d.w.

The baking experiment was carried out in duplicate, and the measurements were performed at least in triplicate.

2.9. Statistical Analysis

The statistical analysis was carried out using the Minitab 19 (Minitab LLC, State College, PA, USA) software. The ANOVA method and the post hoc test, based on the Tukey method, when $p < 0.05$ were considered to identify significant differences between results.

3. Results and Discussion

3.1. mPAGE Analysis of Soy Protein Hydrolysates

In agreement with our previous study, Brumă et al. [10], bromelain, Neutrase and trypsin ensured different hydrolysis degrees (HDs) of soy protein isolate. Among all the tested enzymes, bromelain was the most efficient in hydrolyzing the soy proteins (HD of 10.3%), being followed by Neutrase (HD of 9.7%) and trypsin (HD of 1.9%). The electrophoretic analysis was employed to check the molecular weight distribution of the resulting peptides, separated through centrifugation into soluble and insoluble fractions. The mPAGE analysis revealed that the SPI used in the study has high amounts of 7S (β-conglycinin) and 11S (glycinin), which are the major globulins found in soybeans [18]. Figure 1 (lane SPI) emphasizes the specific subunits of the main globulins found in soybeans: α′ (~75 kDa), α (~72 kDa) and β (~53 kDa) subunits of β-conglycinin, together with the acidic (33–42 kDa) and basic subunits (~22 kDa) of glycinin [19,20].

Analyzing the results presented in Figure 1, one can observe that the soluble fraction of soy protein hydrolysates retained only the peptides with molecular weights lower than 20 kDa (lanes sSPB, sSPN, sSPT). On the other hand, the insoluble fractions (residuals)

of soy protein hydrolysates retained higher molecular weight fragments (Figure 1, lanes rSPB, rSPN, rSPT), probably resulting from the partial digestion of the main soy proteins (lane SPI in Figure 1). The smearing in the rSPB, rSPN and rSPT lanes (Figure 1) might also suggest the rather low specificity of exogenous proteases used in this study. In agreement with our observations, Yang et al. [21] reported the reducing of the number of protein bands as a consequence of the Alcalase-assisted hydrolysis of the α′ and α subunits of β-conglycinin, together with the decrease in the antigenic properties. Moreover, Wen et al. [22] emphasized that soy protein hydrolysis with Alcalase and Neutrase resulted in 84 peptides with immunoregulatory activities and other positive effects on human health. In addition to modulating the functional properties of the soy protein isolate, enzyme-assisted hydrolysis might also allow for the reduction in allergenic properties. Various studies assigned the allergenic properties of soybeans to various 7S and 11S globulins [23,24] or to proteins located in soybean hulls [25,26]. The major soybean protein allergens are considered to be Gly m 4 (17 kDa), Gly m 5 (β-conglycinin) and Gly m 6 (with five subunits having polypeptides of 20 kDa and 40 kDa) [26,27]. Some other proteins responsible for allergenic activities were described as Gly m1, Gly m 2, Gly m 3 or 2S-globulin fraction, having lower molecular weights of 7 kDa, 7.5 kDa, 8 kDa and 12–15 kDa, respectively [19,20]. To reduce or eliminate the allergenicity of foods based on soybean proteins, different thermal and non-thermal treatments were applied. Among these treatments, enzyme-assisted hydrolysis appears very efficient for altering the structure of allergenic proteins. This effect of allergenic potential reduction associated with hydrolysis with exogenous enzymes is of particular interest in the case of those proteins which are resistant to in vivo digestion, as is the case of soy proteins [10]. The results presented in Figure 1 suggest that the separation of the soluble fraction of the soy protein hydrolysates obtained with bromelain or Neutrase might allow for obtaining soy protein ingredients devoid of such allergens.

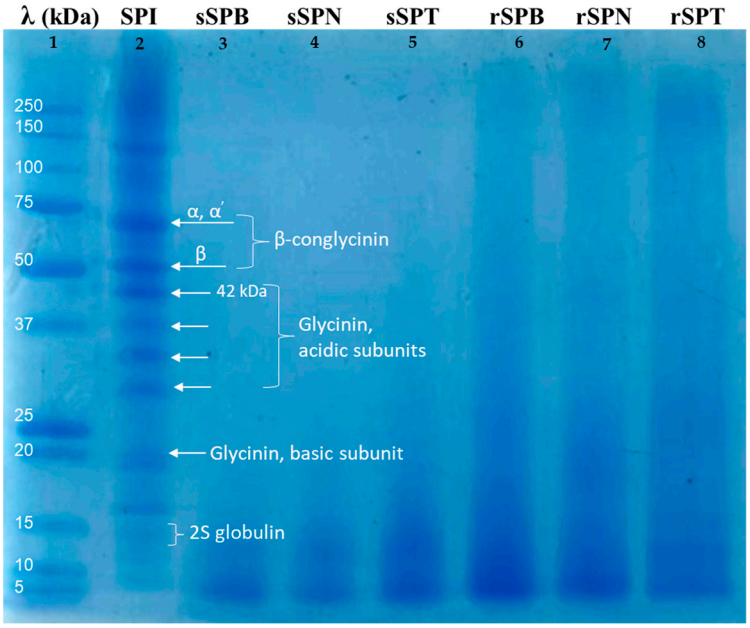

Figure 1. The mPAGE profile of the soy protein isolate (SPI, lane 2) and of the soluble and insoluble fractions of the hydrolysates obtained with bromelain (sSPI-B and rSPI-B, lanes 3 and 6), Neutrase (sSPI-N and rSPI-N, lanes 4 and 7) and trypsin (sSPI-T and rSPI-T, lanes 5 and 8). Lane 1—Dual Xtra marker (Bio-Rad, Hercules, CA, USA). The arrows on lane 2 indicate the subunits of β-conglycinin [20], glycinin [19] and 2S-globulin [20].

3.2. The Influence of SPI Addition on the Thermo-Mechanical Properties of the Gluten-Free Mixtures

In order to investigate the influence of the addition of soy protein isolate and hydrolysates on the rheology of the dough based on gluten-free composite flour consisting

of whole rice flour and quinoa flour, the evolution of the doughs' consistency over the entire Mixolab curves was considered (Figures 2 and 3). Particular attention was paid to the consistency values at the end of each phase of the Mixolab curves (Table 2), as well as to the specific parameters highlighted by the Mixolab software (version 4.1.2.10) (Table 3).

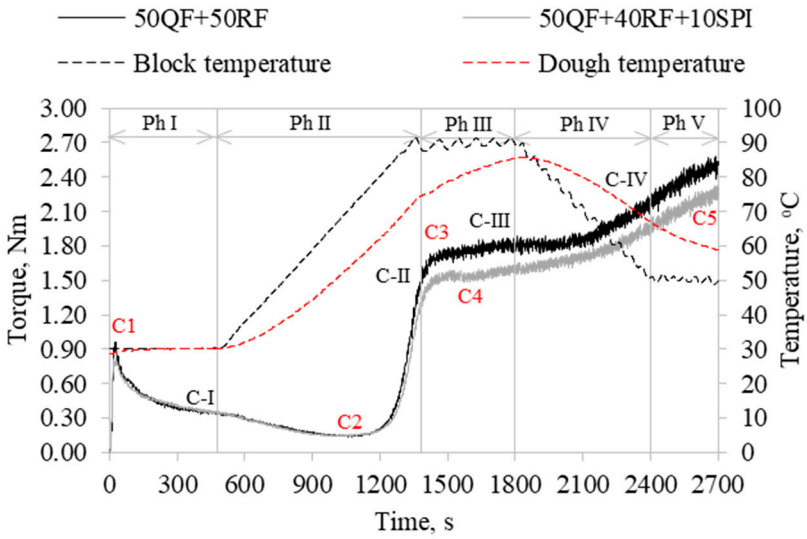

Figure 2. Mixolab curve of dough prepared mixtures of quinoa flour (QF), rice flour (RF) and soy protein isolate (SPI).

Table 2. The influence of gluten-free flour supplementation with soy proteins or peptides on the dough consistency measured at the end of each Mixolab phase.

Sample/Phase	Phase I 0–480 s 30 °C C-I, Nm	Phase II 480–1380 s 30–90 °C C-II, Nm	Phase III 1380–1800 s 90 °C C-III, Nm	Phase IV 1800–2400 s 90–50 °C C-IV, Nm	Phase V 2400–2700 s 50 °C C-V, Nm
50QF + 50RF	0.34 ± 0.01 ^{d}	1.38 ± 0.02 ^{d}	1.86 ± 0.02 ^{b}	2.18 ± 0.02 ^{a}	2.51 ± 0.02 ^{b}
50QF + 40RF + 10SPI	0.34 ± 0.01 ^{d}	1.30 ± 0.02 ^{e}	1.58 ± 0.02 ^{e}	1.98 ± 0.02 ^{c}	2.27 ± 0.01 ^{d}
50QF + 40RF + 10sSPI-B	0.04 ± 0.01 ^{f,g}	0.32 ± 0.01 ^{i}	1.23 ± 0.02 ^{h}	1.51 ± 0.02 ^{e}	1.73 ± 0.01 ^{h}
50QF + 40RF + 10sSPI-N	0.06 ± 0.01 ^{f}	0.27 ± 0.01 ^{j}	1.23 ± 0.02 ^{h}	1.45 ± 0.02 ^{f}	1.62 ± 0.02 ^{i}
50QF + 40RF + 10sSPI-T	0.02 ± 0.01 ^{g}	0.26 ± 0.01 ^{j}	1.29 ± 0.02 ^{g}	1.53 ± 0.02 ^{e}	1.79 ± 0.02 ^{g}
50QF + 40RF + 10rSPI-B	0.75 ± 0.02 ^{b}	1.48 ± 0.02 ^{c}	1.73 ± 0.01 ^{c}	2.00 ± 0.02 ^{c}	2.40 ± 0.02 ^{c}
50QF + 40RF + 10rSPI-N	0.59 ± 0.01 ^{c}	1.62 ± 0.02 ^{b}	1.74 ± 0.01 ^{c}	1.90 ± 0.02 ^{d}	2.53 ± 0.02 ^{b}
50QF + 40RF + 10rSPI-T	0.99 ± 0.02 ^{a}	1.78 ± 0.02 ^{a}	1.92 ± 0.02 ^{a}	1.53 ± 0.01 ^{e}	2.74 ± 0.01 ^{a}
50QF + 40RF + 10hSPI-B	0.24 ± 0.01 ^{e}	0.90 ± 0.02 ^{g}	1.53 ± 0.01 ^{f}	1.89 ± 0.02 ^{d}	2.16 ± 0.01 ^{e}
50QF + 40RF + 10hSPI-N	0.23 ± 0.01 ^{e}	0.82 ± 0.02 ^{h}	1.51 ± 0.01 ^{f}	2.16 ± 0.01 ^{a}	2.05 ± 0.02 ^{f}
50QF + 40RF + 10hSPI-T	0.35 ± 0.01 ^{d}	1.07 ± 0.02 ^{f}	1.68 ± 0.02 ^{d}	2.06 ± 0.02 ^{b}	2.39 ± 0.02 ^{c}

QF—quinoa flour; RF—rice flour; SPI—soy protein isolate; sSPI, rSPI and hSPI—soluble, insoluble and total soy protein hydrolysate fraction, respectively, obtained with bromelain (B), Neutrase (N) and trypsin (T). Different superscript letters associated with values in same column indicate significant differences among results, as resulted from ANOVA and Tukey post hoc test at $p < 0.05$.

SPI addition to the composite flour resulted in no changes in the Ph I and Ph II of the Mixolab curve (Figure 2). The dough consistency increased rapidly in the first 30 s of kneading and decreased at a faster rate in the phase run at 30 °C, from 0.80 Nm to 0.34 Nm in 480 s, and more slowly in the next 600 s, from 0.34 (C-I) to 0.15 Nm (C2), in Ph II when the temperature increased constantly (Tables 2 and 3).

The main changes caused by the replacement of 10% RF by SPI can be observed in Ph III, IV and V of the Mixolab curves (Figure 2). The addition of SPI lowered the values of the

dough consistency at the beginning and end of each of the phases III–V. This behavior can be attributed to starch dilution in the dough system, caused by SPI incorporation. Under these conditions, the water released by the dough system as a result of protein weakening at increasing temperature is higher, therefore causing the dough consistency decrease [28].

Table 3. The influence of gluten-free flour supplementation with soy proteins or peptides on the Mixolab parameters of dough.

Sample/Parameter	C1, Nm	C2, Nm	C3, Nm	C4, Nm	C5—C-III, Nm
50QF + 50RF	0.88 ± 0.02 ^{d}	0.15 ± 0.01 ^{e}	1.74 ± 0.02 ^{b}	1.83 ± 0.02 ^{a}	0.65 ± 0.002 ^{e}
50QF + 40RF + 10SPI	0.80 ± 0.02 ^{e}	0.15 ± 0.01 ^{e}	1.58 ± 0.02 ^{c}	1.60 ± 0.02 ^{c}	0.69 ± 0.002 ^{c,d}
50QF + 40RF + 10sSPI-B	0.16 ± 0.01 ^{j}	0.01 ± 0.01 ^{f,h}	1.15 ± 0.01 ^{e}	1.18 ± 0.02 ^{e}	0.50 ± 0.008 ^{h}
50QF + 40RF + 10sSPI-N	0.23 ± 0.01 ^{i}	0.03 ± 0.01 ^{f}	1.16 ± 0.01 ^{e}	1.18 ± 0.02 ^{e}	0.39 ± 0.002 ^{i}
50QF + 40RF + 10sSPI-T	0.18 ± 0.01 ^{j}	0.00 ± 0.01 ^{h}	1.17 ± 0.01 ^{e}	1.20 ± 0.02 ^{e}	0.50 ± 0.001 ^{h}
50QF + 40RF + 10rSPI-B	1.96 ± 0.02 ^{b}	0.30 ± 0.01 ^{b}	1.71 ± 0.02 ^{b}	1.66 ± 0.01 ^{b}	0.67 ± 0.013 ^{d}
50QF + 40RF + 10rSPI-N	1.56 ± 0.02 ^{c}	0.27 ± 0.01 ^{c}	1.70 ± 0.02 ^{b}	1.64 ± 0.01 ^{b}	0.79 ± 0.003 ^{b}
50QF + 40RF + 10rSPI-T	2.17 ± 0.02 ^{a}	0.42 ± 0.02 ^{a}	1.91 ± 0.02 ^{a}	1.83 ± 0.02 ^{a}	0.82 ± 0.002 ^{a}
50QF + 40RF + 10hSPI-B	0.46 ± 0.01 ^{h}	0.11 ± 0.01 ^{f}	1.45 ± 0.02 ^{d}	1.44 ± 0.02 ^{d}	0.63 ± 0.002 ^{f}
50QF + 40RF + 10hSPI-N	0.71 ± 0.01 ^{f}	0.10 ± 0.01 ^{f}	1.44 ± 0.02 ^{d}	1.44 ± 0.02 ^{d}	0.55 ± 0.010 ^{g}
50QF + 40RF + 10hSPI-T	0.50 ± 0.01 ^{g}	0.18 ± 0.01 ^{d}	1.55 ± 0.02 ^{c}	1.57 ± 0.02 ^{c}	0.71 ± 0.006 ^{c}

QF—quinoa flour; RF—rice flour; SPI—soy protein isolate; sSPI, rSPI and hSPI—soluble, insoluble and total soy protein hydrolysate fraction, respectively, obtained with bromelain (B), Neutrase (N) and trypsin (T). Different superscript letters associated with values in same column indicate significant differences among results, as resulted from ANOVA and Tukey post hoc test at $p < 0.05$.

Regardless of SPI addition, dough consistency increased over Ph III: the increase rate was lower for the sample with SPI addition (from 1.30 to 1.66 Nm) compared to the control dough (from 1.38 to 1.88 Nm). On the other hand, during Ph IV when the temperature dropped from 90 to 70 °C (from 1800 to 2100 s), the consistency of the sample with SPI increased by 0.13 Nm (from 1.58 to 1.71 Nm), whereas the control registered no important variation in the consistency (from 1.86 to 1.87 Nm) (Table 2). This behavior could be due to the aggregation of soy proteins during heating. The main soybean proteins, glycinin and β-conglycinin, dissociate into monomers with increasing temperature, exposing a number of hydrophobic patches which might lead to the formation of aggregates and ordered gel structures [29].

In the last phase, the dough consistency increase in the two samples was rather similar: 0.29 Nm (from 1.98 to 2.27 Nm) for the sample with SPI and 0.33 Nm (from 2.18 to 2.51 Nm) for the control sample (Table 2).

Marco and Rosell [29] mentioned that dough consistency during heating and cooling depends on the amount of soy protein isolate substituting rice flour, and Bonet et al. [30] emphasized the importance of soy protein processing conditions on the properties of SPI. When adding 13% SPI to rice flour, Marco and Rosell [29] obtained a decrease in C3 and C4 values and an increase in C5 and (C5–C4) values. On the other hand, Patrascu et al. [28] reported a decrease in C3, C4 and C5 values when incorporating 15% SPI into rice flour; a similar trend was observed by Nogueira et al. [31] when SPI was added to the wheat flour.

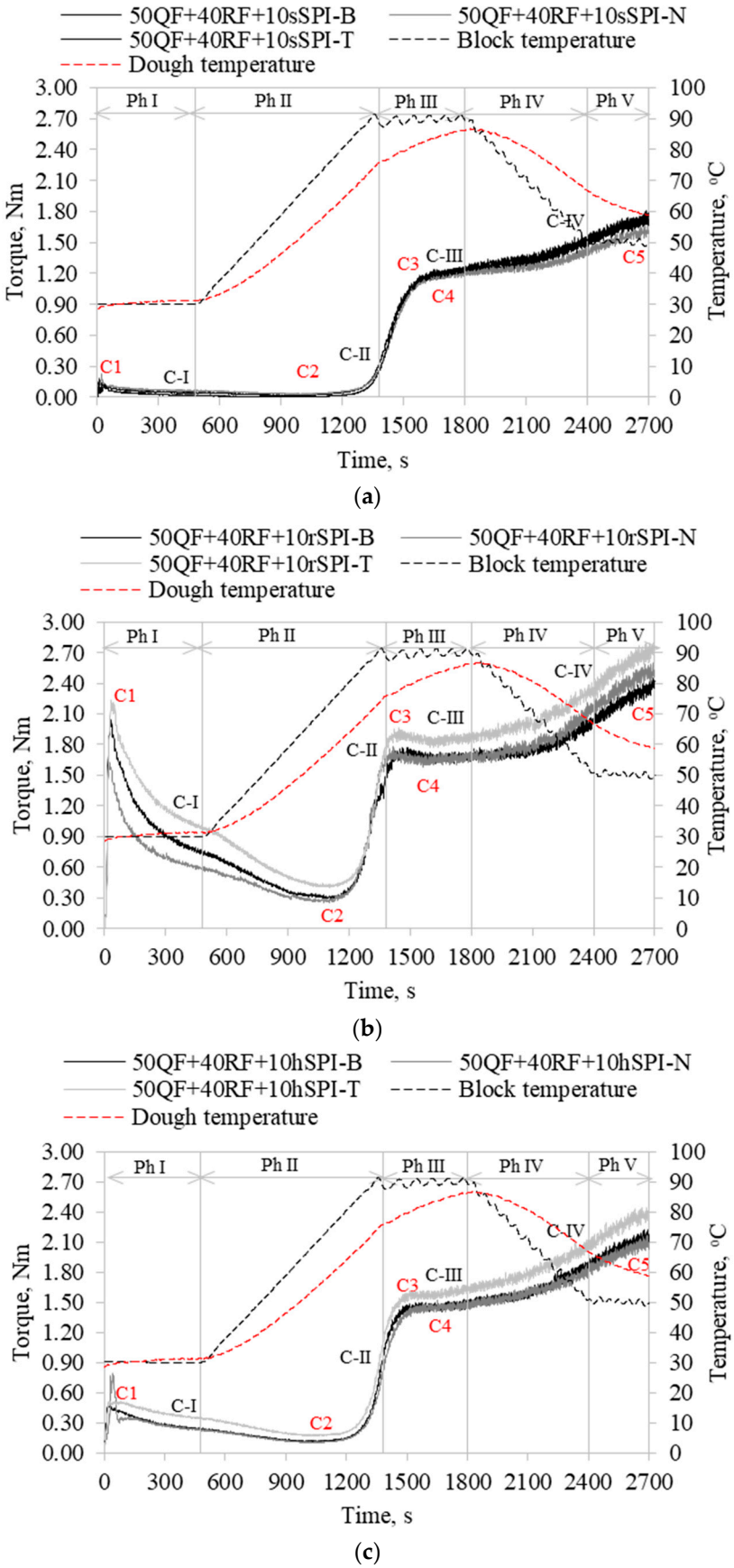

Figure 3. Mixolab curve of dough prepared mixtures of quinoa flour (QF), rice flour (RF) and (**a**) soluble fraction of soy protein hydrolysates (sSPIs), (**b**) insoluble fraction of soy protein hydrolysates (rSPIs) and (**c**) total soy protein hydrolysates (hSPIs) obtained with bromelain (B), Neutrase (N) and trypsin (T).

3.3. The Influence of Soluble Soy-Derived Peptides' Addition on the Thermo-Mechanical Properties of the Gluten-Free Mixtures

Regardless of the exogenous enzyme used for SPI hydrolysis, the addition of sSPI to the composite flour notably reduced the consistency of the dough during Ph I and Ph II (Figure 3). It is known that the protein from quinoa contains mainly globulins and albumins, stabilized by disulfide bonds, the cysteine residues from these fractions playing, therefore, an important role in protein contribution to the overall behavior of the dough matrix [32]. In the composite flour, this behavior is balanced by the presence of high-molecular-mass glutelins, which are the proteins prevailing in the rice flour [33]. The addition of sSPI, containing low-molecular-mass peptides (Figure 1), disturbed the protein network of the dough obtained from the composite flour. Guo et al. [34] reported a deterioration of the protein network in the dough prepared from wheat flour supplemented with soy protein hydrolysates. The authors speculated that the interactions established between the soy peptides and the low-molecular-weight glutenin fractions and gliadin involved disulfide bonds, therefore interfering with the formation of the typical protein network within the dough. Although the number of hydrogen and disulfide bonds was reported to increase, the addition of soy protein hydrolysate weakened the gluten network. Schmiele et al. [35] noted that soy protein hydrolysates could modify ionic and hydrophobic interactions and covalent and hydrogen bonding, preventing the complete hydration of proteins during dough formation.

Another possible explanation for the decrease in dough consistency in Ph I and Ph II could be related to the fact that peptides with low molecular weight have a low water binding capacity. Lamsal et al. [36] reported the decrease in the apparent viscosity of the soy protein hydrolysate obtained with bromelain as a result of the high solubility of the low-molecular-weight peptides formed. Similar observations were collected by Tsumura et al. [37] for the soy protein hydrolysate obtained with papain. Taking into account that all Mixolab measurements were carried out at the same WA of 65%, the excess of the water in the system significantly reduced the consistency of the soy peptide-rich dough samples.

The minimum consistency of the dough samples enriched in sSPI ranged between 0 and 0.03 Nm and was registered after 1080 s at temperature of 55 °C. When compared with the control and the dough supplemented with SPI, one can observe that sSPI addition delayed the start of starch gelatinization (Figure 3). Thus, after 1380 s of mixing and reaching the maximum heating temperature specific to the Chopin+ protocol, the C-II of the soy peptide-enriched doughs was 0.32, 0.27 and 0.26 Nm, for samples supplemented with sSPI-B, sSPI-N and sSPI-T, respectively (Table 2). The gelatinization maximum was reached only after 1620 s, at a temperature of 83 °C, the C4 of the samples ranging between 1.15 and 1.17 Nm (Table 3). At the end of Ph III, the dough reached a temperature of 87 °C, and a C-III of 1.23 Nm was recorded for the samples with sSPI-B and sSPI-N and of 1.29 Nm for the sample with sSPI-T (Table 2). Li et al. [38] investigated the possibility of improving the quality of steamed bread by supplementing flour with soy protein hydrolysate obtained with pepsin. They observed that the addition of soy protein hydrolysate interfered with the orderly structure of the starch granules, therefore affecting the swelling properties and causing the increase in the gelatinization temperature and the decrease in the gelatinization enthalpy.

The dough consistency continued to increase until the end of Ph IV, but also over the entire Ph V (Figure 3). The strength of the gel was higher in the case of dough with sSPI-T, followed by sSPI-B and sSPI-N. It should be noted that trypsin ensured the lowest hydrolysis degree among all enzymes used for soy protein hydrolysis [10]. Moreover, as indicated by the SDS-PAGE analysis, sSPI-T included higher-molecular-weight peptides compared to sSPI-B and sSPI-N (Figure 1). Ashaolu [39] mentioned the importance of the size of the peptide molecules within protein hydrolysates and appreciated that lowering the molecular weight can affect the strength of the gels in which they are incorporated. Anyway, the degree of hydrolysis and the nature of the enzyme used for hydrolysis are critically important. In this respect, Hou and Zhao [40] observed that soy protein hydrolysates have

better gel-forming properties than the original protein isolate but also the fact that gel properties depend on the enzyme used for hydrolysis and the degree of hydrolysis. The authors reported that the protein hydrolysate obtained with Neutrase allowed for gels with higher strength compared to those obtained with trypsin. In addition, it is mentioned that at lower degrees of hydrolysis, the strength of the gel is higher. Huang et al. [41] observed that the peptide fraction with low molecular weight (0.5–10 kDa), separated from the hydrolysate obtained from soy protein isolate, allowed for obtaining gels at temperatures above 65 °C, and the obtained gel had high strength compared to the control.

3.4. The Influence of the Insoluble Soy-Derived Peptides' Addition on the Thermo-Mechanical Properties of the Gluten-Free Mixtures

The addition of rSPI considerably increased the consistency of the dough in Ph I and Ph II, compared to the control and dough sample supplemented with SPI (Figure 3). The abrupt drop in the dough consistency in the first 8 minutes while kneaded at 30 °C can be attributed to the slower formation of the dough network. The consistency at the end of Ph I (C-I) was 0.99, 0.75 and 0.59 Nm, while the minimum C2 consistency reached in Ph II was 0.42, 0.30 and 0.27 Nm for the dough samples supplemented with rSPI-T, rSPI-B and sSPI-N, respectively (Table 3). The dough sample prepared with the residue of the hydrolysate obtained with trypsin, which also had the lowest degree of hydrolysis, exhibited the highest consistency after 480 s of kneading at 30 °C (C-I), the steepest consistency drop (C1–C2 of −1.75 Nm) and the highest consistency value (C2) measured at 55 °C (Table 3). The dough samples supplemented with rSPI-B and rSPI-N recorded consistency decreases of −1.66 Nm and −1.29 Nm, respectively. All dough samples supplemented with rSPI had higher C2 values compared to the control and the sample with SPI. The peptide profile of the three hydrolysates used to supplement the gluten-free dough and their water related properties can explain the different consistency variations in the dough [36]. We can assume that the peptides from rSPI have a higher water binding capacity [39], compared to the sample with and without the addition of SPI, but the development of the dough network is more difficult and the stability lower. However, the higher C2 values registered for the samples with rSPI may indicate a greater stability of the protein network upon heating, as a result of molecular interactions through hydrogen bonding and hydrophobic contacts, involving the proteins of rice and quinoa and soy peptides, which improved the resistance of the dough to kneading and heating.

The addition of rSPI to the gluten-free composite flour did not delay starch gelatinization, as observed in case of the addition of soluble soy peptides, but increased the consistency of the gel compared to the control and the sample with SPI. The highest maximum dough consistency of 1.91 Nm, associated with starch gelatinization at 76 °C, was measured in the case of the sample supplemented with rSPI-T derived from the hydrolysate with the lowest degree of hydrolysis, whereas the addition of rSPI-N and rSPI-B to the gluten-free composite flour resulted in the maximum gelatinization values of 1.70–1.71 Nm, obtained at 78 °C (Table 3). As the temperature of the dough increased to 81 °C for the sample with rSPI-T, and to 83 °C for samples with rSPI-N or rSPI-B, the consistency decreased by 0.08 Nm and 0.05–0.06 Nm, respectively. This downward trend was noticed only when supplementing the gluten-free flour with the rSPI fraction of the hydrolysates. When studying the gelling properties of the soy protein hydrolysates obtained with bromelain, Lamsal et al. [36] noted that, although they present gelling capacity, the gel resistance is reduced. The authors assigned the reduced resistance of the gels to the limited hydrophobic interactions and sulfhydryl exchange reactions established between peptides during gelation.

At the maximum dough temperature of 85–87 °C, the consistency reached a higher value in the case of rSPI-T (C-III of 1.92 Nm), compared to rSPI-B and rSPI-N (C-III of 1.74–1.73 Nm) (Table 2). Further on, at the end of Ph V, the consistency of the dough sample supplemented with rSPI-T reached 2.74 Nm, and for the samples with rSPI-N and rSPI-B, with a higher degree of hydrolysis, the consistency reached 2.53 and 2.40 Nm, respectively (Table 2). The difference between the maximum consistency of the dough, recorded at

the end of Ph III, and the consistency at the end of Ph V, which can be considered an indicator for starch degradation, had the lowest value of 0.67 Nm, when supplementing the gluten-free composite flour with the residue fraction of the soy protein hydrolysate with the highest degree of hydrolysis (rSPI-B). In the case of the dough with rSPI-N and rSPI-T, the C5-C-III difference was 0.79 and 0.82 Nm, respectively (Table 3).

3.5. The Influence of the Total Soy Protein Hydrolysate Addition on the Thermo-Mechanical Properties of the Gluten-Free Mixtures

The addition of hSPI produced the smallest changes in the dough behavior during mixing at 30 °C and during heating up to 55 °C, compared to the control and the sample supplemented with SPI. The curves obtained for the samples supplemented with hSPI-B and hSPI-N showed almost identical consistency values in the mentioned temperature range but lower compared to the control and the sample supplemented with SPI (Figure 2). Thus, the C-I consistency at the end of Ph I was 0.23–0.24 Nm, and the minimum C2 consistency of 0.10–0.11 Nm was recorded at 55 °C. In contrast, the dough sample with hSPI-T presented a C-I of 0.35 Nm and a minimum C2 consistency of 0.18 Nm, values extremely close to the control and the sample supplemented with SPI (Table 2). Practically, among all studied peptide-enriched dough samples, the dough including soy protein hydrolysate with the lowest degree of hydrolysis (hSPI-T) altered to the lowest extent the interactions between the main macromolecules of the rice and quinoa flours, which are essential for the dough system.

Zhang et al. [42] investigated the effect of wheat flour supplementation with soy protein hydrolysates obtained with Neutrase and reported a decrease in the dough stability, an increase in dough softening and the farinographic number and the obtained dough being more resistant and less extensible and elastic. The authors also noted the decrease in the content of high-molecular-weight protein fractions with the addition of protein hydrolysate, suggesting that the peptides from the hydrolysate formed disulfide bonds with the wheat protein fractions, interfering with glutenin polymerization during dough formation. Li et al. [38] reported a decrease in intermolecular and anti-parallel β-sheets and an increase in β-turns upon the addition of soy protein hydrolysate, stating that the intermolecular β-sheets are an indicator of protein polymerization. The same authors noted that during fermentation, the content of α-helices and β-turns decreased, but the content of anti-parallel β-sheets increased.

When compared with the sample supplemented with SPI, the addition of sSPI delayed starch gelatinization, causing an important reduction in the gelatinization maximum and the decrease in starch retrogradation; rSPI facilitated the faster achievement of the gelatinization maximum by approximately 120 s while increasing starch retrogradation, whereas hSPI addition resulted in delaying the maximum gelatinization by about 60–120 s. The maximum dough consistency and starch retrogradation varied with the nature of the enzyme used for soy protein hydrolysis and consequently with the degree of hydrolysis. Thus, flour supplementation with hSPI-T, which presented the lowest degree of hydrolysis among all investigated samples, resulted in a gelatinization maximum (C3 of 1.55 Nm after 1500 s of Chopin+ protocol) comparable to the dough with SPI (C3 of 1.58 Nm after 1560 s) (Table 3). The samples prepared with hSPI-B and hSPI-N exhibited significantly lower gelatinization maxima, a C3 of 1.44–1.45 Nm being recorded at 1620 s (Table 3). The slight delay in starch gelatinization could be attributed to the effect of soluble peptides present in the hydrolysate, which produced important changes in the behavior of the dough in which SPIH was incorporated.

The dough consistency continued to increase throughout Ph III, IV and V (Figure 3, Table 2). The starch retrogradation of the dough samples with hSPI-B and hSPI-N was significantly lower compared to the control ($p < 0.05$), whereas the starch retrogradation behavior of the dough supplemented with hSPI-T was similar to the sample with SPI (Table 3). Taking into account the overall behavior of the dough supplemented with soy protein hydrolysates, over the five phases of the Mixolab curves (Figure 3), it can be

concluded that hSPI-B and hSPI-N ensured the most important improvements to the dough, compared to the sample with SPI. In agreement with our results, Schmiele et al. [35] reported a decrease in C3, C4 and C5 upon the addition of soy protein hydrolysate to wheat flour. However, the authors noted that the presence of protein hydrolysate accelerated starch gelatinization, compared to the control sample and reduced (C5–C4). As in the case of the dough samples prepared with hSPI-B and hSPI-N in the present study, Schmiele et al. [35] mentioned the anti-retrogradation effect exerted by protein hydrolysates on starch, a very important aspect for preventing the staling of bakery products.

3.6. The Influence of Soy Protein Hydrolysate Addition on the Quality of Muffins

The gluten-free flour mixtures supplemented with SPI and hSPI were further used for preparing muffins (Figure 4).

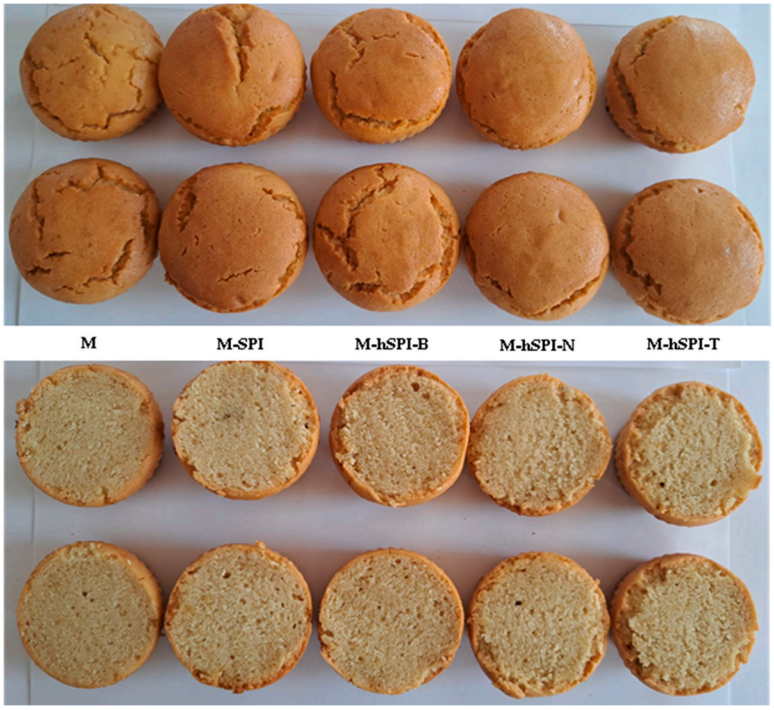

Figure 4. The appearance of the upper surface and crumb of the muffin samples (M—control muffin; M-SPI—muffins enriched with soy protein isolate; M-hSPI-B—muffins enriched with soy protein hydrolysate produced with bromelain; M-hSPI-N—muffins enriched with soy protein hydrolysate produced with Neutrase; M-hSPI-T—muffins enriched with soy protein hydrolysate produced with trypsin). Images were taken with the Canon PowerShot G16 digital camera (Canon Inc., Tokyo, Japan).

Among all investigated muffin formulations, the samples supplemented with M-hSPI-B or M-hSPI-N exhibited the lowest values of weight loss during baking, suggesting the good water holding capacity of the products and finally the advantageous production process [15]. Analyzing the results presented in Table 4, one can observe that flour supplementation with SPI resulted in a significant improvement in the specific volume of the muffins. On the other hand, the specific volume of the hSPI-supplemented muffins decreased significantly compared to the control ($p < 0.05$), as a result of the reduced ability of the network to retain the CO_2 released by the baking powder during baking. The total height of the muffins was significantly influenced by the soy protein or hydrolysate addition ($p < 0.05$). The largest height values were registered in the case of the samples supplemented with SPI, hSPI-B or hSPI-T (Table 4). Regardless of soy protein isolate or hydrolysate addition, no important differences were noted in terms of the firmness of the final product.

Table 4. The influence of gluten-free flour supplementation with soy proteins/peptides on the quality characteristics of the muffins (M—control muffin; M-SPI—muffins enriched with soy protein isolate; M-hSPI-B—muffins enriched with soy protein hydrolysate produced with bromelain; M-hSPI-N—muffins enriched with soy protein hydrolysate produced with Neutrase; M-hSPI-T—muffins enriched with soy protein hydrolysate produced with trypsin).

Muffin Sample	Weight Loss during Baking, g/100 g	Moisture, g/100 g	Height, mm	Specific Volume, cm^3/g	Firmness, N
M	$9.90 \pm 0.14^{a,b}$	20.70 ± 0.05^{a}	3.00 ± 0.00^{c}	159.48 ± 1.43^{b}	6.49 ± 0.89^{a}
M-SPI	10.50 ± 0.14^{a}	20.10 ± 0.02^{c}	3.40 ± 0.00^{a}	181.73 ± 0.59^{a}	6.63 ± 0.66^{a}
M-hSPI-B	9.80 ± 0.00^{b}	20.66 ± 0.05^{a}	$3.25 \pm 0.07^{a,b}$	152.84 ± 0.20^{c}	5.60 ± 0.77^{a}
M-hSPI-N	9.70 ± 0.14^{b}	20.36 ± 0.05^{b}	$3.10 \pm 0.00^{b,c}$	151.93 ± 2.14^{c}	5.74 ± 0.20^{a}
M-hSPI-T	$10.20 \pm 0.28^{a,b}$	20.28 ± 0.02^{b}	$3.25 \pm 0.07^{a,b}$	136.99 ± 0.31^{d}	7.00 ± 0.21^{a}

Different superscript letters associated with values in same column indicate significant differences among results, as resulted from ANOVA and Tukey post hoc test at $p < 0.05$.

Taking into account that color is a sensory attribute decisive for the consumers' choice, the color characteristics of the muffin samples were measured both on the crust and crumb (Table 5). Taking into account the muffins are baked products, the color properties are due both to the individual contribution of the ingredient used for preparation and to their interactions, especially during baking [13,43]. No important differences were observed between samples in terms of crust lightness, except for the muffin supplemented with M-hSPI-T that registered a significant increase in L* (Table 5). The crust of all muffins presented positive a* values, the red color being more intense in the case of the samples with SPI and hSPI-B ($p < 0.05$). No important differences were noted between samples in terms of yellowness, C* and H angle (Table 5). A different trend was noticed in the color parameters of the crumb when supplementing the gluten-free flour formulations with soy protein isolate or hydrolysates (Table 5). The hSPI-enriched samples presented a significantly lower L* and more intense yellow shades compared to the control and muffins with SPI. Moreover, hSPI addition resulted in significantly higher C* values ($p < 0.05$), as a measure of color purity in the CIELAB space [44].

Table 5. The influence of gluten-free flour supplementation with soy proteins/peptides on the color characteristics of the crust and crumb of muffin samples (M—control muffin; M-SPI—muffins enriched with soy protein isolate; M-hSPI-B—muffins enriched with soy protein hydrolysate produced with bromelain; M-hSPI-N—muffins enriched with soy protein hydrolysate produced with Neutrase; M-hSPI-T—muffins enriched with soy protein hydrolysate produced with trypsin).

Muffin Sample	L*	a*	b*	C*	H, °
Crust					
M	46.21 ± 1.94^{b}	10.48 ± 1.01^{c}	26.03 ± 0.68^{a}	28.07 ± 0.30^{a}	68.06 ± 2.96^{a}
M-SPI	45.10 ± 0.20^{b}	13.09 ± 0.41^{a}	26.31 ± 0.56^{a}	29.39 ± 0.39^{a}	63.54 ± 1.42^{a}
M-hSPI-B	44.85 ± 0.92^{b}	$12.50 \pm 0.07^{a,b}$	26.01 ± 0.67^{a}	28.85 ± 0.73^{a}	64.32 ± 0.76^{a}
M-hSPI-N	46.83 ± 0.46^{b}	$11.42 \pm 0.05^{b,c}$	26.62 ± 0.38^{a}	28.97 ± 0.41^{a}	66.78 ± 0.25^{a}
M-hSPI-T	49.87 ± 0.48^{a}	10.88 ± 0.24^{c}	26.56 ± 0.23^{a}	28.70 ± 0.00^{a}	67.72 ± 0.46^{a}
Crumb					
M	$54.86 \pm 0.17^{a,b}$	2.42 ± 0.17^{a}	18.32 ± 0.30^{c}	18.48 ± 0.39^{b}	82.48 ± 0.48^{a}
M-SPI	55.46 ± 0.23^{a}	2.45 ± 0.09^{a}	19.00 ± 0.09^{b}	$19.16 \pm 0.12^{a,b}$	82.66 ± 0.22^{a}
M-hSPI-B	$54.24 \pm 0.26^{b,c}$	2.70 ± 0.27^{a}	19.85 ± 0.38^{a}	20.03 ± 0.49^{a}	82.28 ± 0.75^{a}
M-hSPI-N	53.77 ± 0.57^{c}	2.66 ± 0.09^{a}	19.73 ± 0.26^{a}	19.91 ± 0.31^{a}	82.32 ± 0.20^{a}
M-hSPI-T	$54.84 \pm 0.73^{a,b}$	2.73 ± 0.01^{a}	19.85 ± 0.10^{a}	20.03 ± 0.04^{a}	82.17 ± 0.04^{a}

Different superscript letters associated with values in same column indicate significant differences among results regarding crust or crumb, as resulted from ANOVA and Tukey post hoc test at $p < 0.05$.

A diet rich in antioxidant compounds might help alleviate the prevalence of lifestyle and degenerative diseases [45]. The antioxidant activity of the muffin samples was measured using the DPPH and ABTS radical scavenging activity methods, and the results are presented in Figure 5. Both methods indicated that gluten-free composite flour supplementation with SPI resulted in a significant increase in the antioxidant activity ($p < 0.05$). Meanwhile, the increase was even higher when SPI was replaced by hSPI ($p < 0.05$). The DPPH-based method indicated no differences between muffins supplemented with hSPI, whereas the ABTS-based method suggested that samples with hSPI-B and hSPI-N exhibited the highest antioxidant activity ($p < 0.05$). Previous studies highlighted that possibility of using exogenous enzymes for enhancing the physiological roles of proteins, by releasing the encrypted bioactive peptides responsible for antioxidant properties, immunomodulatory activity, antimicrobial properties, hypocholesterolemic and antihypertensive effects, etc. [11]. The antioxidant activity was mainly assigned to the 2–20 amino acid long peptides with a molecular weight below 6 kDa [11]. Moreover, the exogenous enzyme used for protein hydrolysis, the propensity of hydrophobic amino acids and their location in the peptides highly influence the antioxidant activity. Brumă et al. [10] observed that the hydrolysis of soy protein isolate with bromelain, Neutrase and trypsin resulted in a significant increase in the antioxidant activity. Both DPPH-RSA and ABTS-RSA methods indicated that Neutrase released the highest number of peptides with antioxidant activity. They reported no important differences between samples treated with trypsin and bromelain in terms of ABTS-RSA. In agreement with our observations (Figure 5), Brumă et al. [10] measured higher radical scavenging activity when using the ABTS- compared to DPPH-based method.

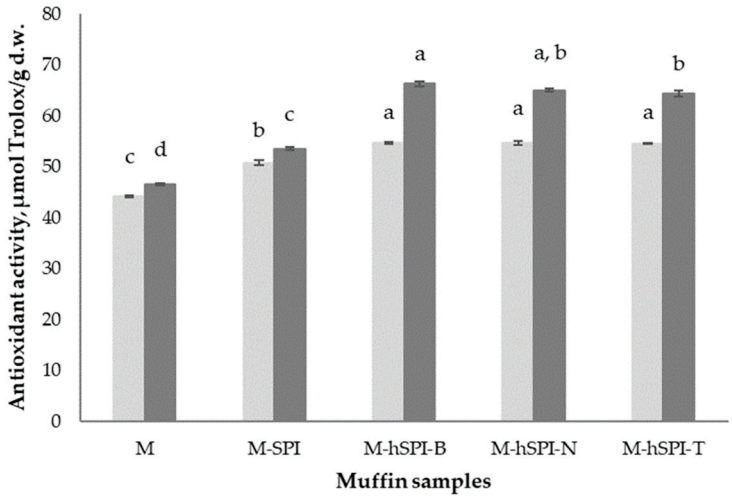

Figure 5. The antioxidant activity of the muffin samples (M—control muffin; M-SPI—muffins enriched with soy protein isolate; M-hSPI-B—muffins enriched with soy protein hydrolysate produced with bromelain; M-hSPI-N—muffins enriched with soy protein hydrolysate produced with Neutrase; M-hSPI-T—muffins enriched with soy protein hydrolysate produced with trypsin). The results of the DPPH- and ABTS-based assays are represented with light and dark gray, respectively. Different superscript letters associated with mean antioxidant activity values determined with the same method indicate significant differences at $p < 0.05$.

4. Conclusions

The gluten-free composite flour consisting of a mixture of rice and quinoa flour was used as the basis for supplementation with a 10% soy protein isolate of hydrolysate obtained with bromelain, Neutrase or trypsin. Empirical rheological measurements were employed to determine the thermo-mechanical behavior of the gluten-free doughs. The flour mixtures enriched with soluble soy peptide fractions, with a molecular weight lower than 20 kDa, exhibited delayed starch gelatinization. The addition of the insoluble soy peptide fraction resulted on the other hand in a dough consistency increase. Based on the

thermo-mechanical behavior, it was concluded that, among all tested formulations, the most important improvements in the dough behavior were observed in the case of supplementing the gluten-free flour with 10% soy protein hydrolysates obtained with bromelain and trypsin. The gluten-free muffins' supplementation with soy protein hydrolysate resulted in a more vivid color of the crumb and no important changes in terms of texture, but the specific volume was significantly lower compared to the sample with soy protein isolate. The antioxidant activity of the gluten-free muffins enriched with soy protein hydrolysate was significantly higher compared to the control. In conclusion, soy protein hydrolysate can be successfully used to obtain baked gluten-free products with an improved nutritional and bioactive profile.

Author Contributions: Conceptualization, I.A.; methodology, I.B., I.V. and L.G.-G.; software, I.A., L.D. and I.B.; validation, M.B., L.G.-G., L.D. and I.V.; formal analysis, I.A.; investigation, M.B., L.G.-G., I.V., I.B., L.D. and I.A.; resources, I.A. and L.G.-G.; data curation, L.G.-G., I.V. and I.A; writing—original draft preparation, I.B., L.G.-G., I.V., M.B. and I.A.; writing—review and editing, L.D. and I.A.; visualization, I.A. and L.G.-G.; supervision, I.A.; project administration, I.A.; funding acquisition, I.A. All authors have read and agreed to the published version of the manuscript.

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