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Wheat gluten proteolysis by enzyme preparations of directional action

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Key words: Wheat gluten; endoprotease; exoprotease; degree of enzymatic hydrolysis; SDS-PAGE.

Abstract

A comparative study of enzymatic hydrolysis of dry wheat gluten was carried out by exo- protease and endoprotease enzyme praparations. Gluten of different rheological properties was conditionally divided into three groups on the basis of the compressive deformation index (H_{def}) determined with the IDK-1 instrument: short-cleaved (H_{def} 40 instr. units), good (H_{def} 65 instr. units) and weak gluten (H_{def} 80 instr. units). The optimal conditions for hydrolysis of different quality gluten by endoprotease enzyme praparations (Neutrase® 1.5MG, Protamex®) and exoprotease enzyme preparation (Flavourzyme® 500MG) were determined. The degree of proteolysis was assessed by the number of released amine nitrogen. It was shown that the stronger gluten is, the greater amount of amine nitrogen is formed under the influence of endoprotease (Neutrase® 1.5 MG, Protamex®), the weaker it is, the more nitrogen is formed under the influence of exoprotease (Flavourzyme® 500 MG). The most effective parameters of hydrolysis (time, concentration of the enzyme preparation, temperature, pH) revealed by us were specified by electrophoresis in polyacrylamide gel. The degree of hydrolysis necessary for achieving a certain molecular mass of peptides when used in breadmaking was determined. Thus, a process of hydrolysis of dry wheat gluten, aimed at expanding the range of its uses for improving and enriching wheat bread, was developed. We established that the functional properties of dry wheat gluten can be regulated by the change of the enzymatic hydrolysis time in the presence of the enzyme preparation Protamex®.

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Introduction

Wheat gluten as a protein typically insoluble in neutral pH environment is widely used to improve baking properties of wheat flour. Applications of gluten can be expanded by modification of its functional properties, among which the most important are enzymatic and chemical methods (Mimouni *et al.*, 1994; Babiker *et al.*, 1996; Babiker *et al.*, 1999; Larré *et al.*, 2000; Linares *et al.*, 2000; Popineau *et al.,* 2002; Surowka *et al.*, 2004). Native gluten can be added increased solubility, foaming and fatemulsifying properties, depending on the degree of hydrolysis of proteins and the value of the molecular weight of produced peptides (Kong *et al.,* 2007; Masson *et al.,* 1986; Drago and González, 2001). In breadmaking wheat flour is used with different rheological properties of gluten: resilience, flexibility, extensibility, therefore when using dry wheat gluten the improving effect will depend on the degree of hydrolytic cleavage, which, in its turn, is determined both by various methods of pre-treatment (Cui *et al.,* 2009; Peňas *et al.,* 2006; Sia *et al.,* 2007; Zhang *et al.,* 2012), and the use of different types of proteolytic enzyme preparations 9 (Kong *et al.*, 2007). The use of enzymes is determined by their selectively, mild reaction conditions and high conversion rates. They are harmless, for this reason, new opportunities for using enzymes in food industry for improving the quality and obtaining protein ingredients are being actively sought for in the world. Although a great number of researches are devoted to enzymatic treatment and modification of plant proteins (Mimouni *et al.,* 1994; Babiker *et al.*, 1996; Babiker *et al.,* 1999; Larré *et al.*, 2000; Linares *et al.*, 2000; Popineau *et al.,* 2002; Surowka *et al.*, 2004) it should be remembered that hydrolyzed product quality in each case is an individual process depending on the type of substrate and enzyme and always requires new experimental studies.

Several studies focused on physicochemical and functional properties of protein hydrolysates of gluten obtained with the maximum degree of hydrolysis, for example, down to 15.8 % (Kong *et al.*, 2007) or even lower molecular weight of peptides (Masson *et al.*, 1986). However, little is known about enzyme proteolysis of gluten of different rheological properties. These data are essential to increasing the efficiency of the process of hydrolysis and applying the obtained hydrolysates depending on their physicochemical characteristics (solubility, molecular weight of components etc.) and functional properties (foaming, emulsifying etc.) of their proteins. These data are needed to more precisely control protein properties with different types of proteases and, consequently, the expansion areas of their application in bakery, confectionery and other sectors of food and non-food industries.

The investigation of the process of enzyme hydrolysis of gluten and substantiation of targeted modification is of both theoretical and practical importance for studying the processes of deep processing of wheat grain and for biochemistry in general.

The aim of this work was to investigate protein hydrolysis of dry wheat gluten of different rheological properties under the influence of enzyme preparations containing endo- and exoproteases with the purpose of using hydrolysates as improvement in the production of dietary bread with high protein content. To this end the conditions of limited proteolysis were investigated using dry wheat gluten of good quality, weak and short-cleaved, as substrate of industrial production.

Materials and methods

Materials

In this study we used 11 dry wheat gluten samples obtained from the BM Company (Kazakhstan), which complied with the quality standards. The chemical composition of dry wheat gluten complied with the Codex Alimentarius requirements; the samples contained 75.6–81.1% of protein.

Dry wheat gluten was estimated by the yield of wet regenerated gluten, hydration capacity, and compressive deformation (Kazakov, 1987).

Endoprotease preparation Neutrase® 1.5 MG (EC 3.4.24.28, from *Bacillus amyloliquefacien,* 1.5 AU/g) and Protamex® (EC 3.4.24.28, from *Bacillus subtilis,* 1.5 AU/g) and exoprotease preparation Flavourzyme® 500 MG (EC 3.4.11.1 from *Aspergillius orysae,* 500 LAPU/g) with proteolytic activities of 105, 125 and 85 U/g protein, respectively, were obtained from Novozymes (Denmark). Proteolytic activity of enzyme preparations was determined according to Anson (Rukhlyadeva and Polygalina, 1981).

Analysis of the degree of hydrolysis of gluten proteins

The degree of hydrolysis of gluten proteins was determined by the formula *Dh = Cs/Cc * 100 %,* where C_s is the content of nitrogen in the sample (%) and C_c is the content of amine nitrogen in the completely hydrolyzed sample (%) (Melnick *et al.*, 1946). The content of nitrogen was determined after complete hydrolysis of an aliquot (100 mg) of a sample in 10 ml of 6 M HCl. The sample was incubated in a constant-temperature thermostate in sealed ampoules at 110°C for 24 h, after which the amine nitrogen was determined by titration with formaldehyde (Pleshkov, 1985) using pH-meter.

Analysis of the polypeptide composition of proteins

To determine the polypeptide composition of proteins, aliquots (0.2–0.5 g) of dry gluten samples were mixed with 20 ml of a buffer containing 62.5 mM Tris-HCl, 8 M Urea, 2% sodium dodecyl sulfate (SDS), and 0.01% of bromophenol blue (pH 6.8). The dispersion was shaked for 1 h at room temperature and left in the refrigerator for the night. The next day (15–17 h later) the samples were incubated at room temperature for 1h under shaking once again and then centrifuged at 12 000 rev min^{-1} for 20 min. The protein content in solutions was determined according to Kjeldahl in a BUCHI K-424 protein/nitrogen analyzer (Switzerland).

For reductive hydrolysis of proteins, we used a sample buffer supplemented with 5% 2-mercaptoethanol. The buffer was added to the pellet obtained by centrifugation after discarding the supernatant containing soluble proteins. The sample was incubated in a constant-temperature thermostat at 37°C for 2 h and then centrifuged at 12 000 rev min^{-1} for 20 min. The supernatant was used for analysis of the polypeptide composition by one-dimensional SDS-PAGE (Skazhennik *et al.,* 1981) in a polyacrylamide gel containing 10–20% acrylamide in the separating gel (pH 8.8) and 6 % acrylamide in the concentrating gel (pH 6.8). The electrode buffer contained Tris-glycine (pH 8.3) and 0.1% SDS. Electrophoresis was performed at 4–6°C for 6–8 h at a constant current of 25–30 mA. The protein content in samples was determined by the method of Bramhall (Bramhall *et al*., 1969) with some modification. Molecular-weight protein marker kits were purchased from Sigma (Germany). Phosphorilase b (MW 92 kD), bovine serum albumin (MW 66 kD), catalase (MW 60 kD), egg albumin (MW 45 kD), glyceraldehyde-3-phosphate dehydrogenase (MW 36 kD), carbonic anhydrase (MW 29 kD), trypsin inhibitor (MW 20 kD), cytochrome C (MW 12 kD) were used as the standards of molecular weight.

All the results presented concerning hydrolysis are the mean of triplicate experiments.

Results and discussion

Analysis of 11 wet regenerated gluten samples revealed distinctions in organoleptic and rheological characteristics. For this reason, these samples were conditionally divided into three groups on the basis of the compressive deformation index (H_{def}) determined with the IDK-1 instrument (Kazakov, 1987): samples of short-cleaved, good, and weak gluten with H_{def} values of 40, 65, and 80 instr. units, respectively. One sample of each type of gluten from each group was selected for investigations.

Hydrolysis parameters of dry wheat gluten of different rheological properties were determined using two endoprotease and one exoprotease preparations**.** Hydrolysis was monitored by using the previously developed method of amine nitrogen determination by formaldehyde titration with the

measurement of pH. Variable parameters included the concentration of the enzyme preparation, the concentration of gluten, the protein hydrolysis time, pH, and the enzyme treatment temperature.

Fig.s 1 and 2 show the effect of the concentration of the good-quality gluten (23 to 90%) and the effect of temperature (20 to 60°C**)** with different periods of hydrolysis with the endoprotease Neutrase® 1.5 MG at a dose of 0.3 U/g protein on the content of amine nitrogen. It can be seen that the largest amount of nitrogen evolved with gluten concentration of 37% at 50°C and hydrolysis time of 90–150 min.

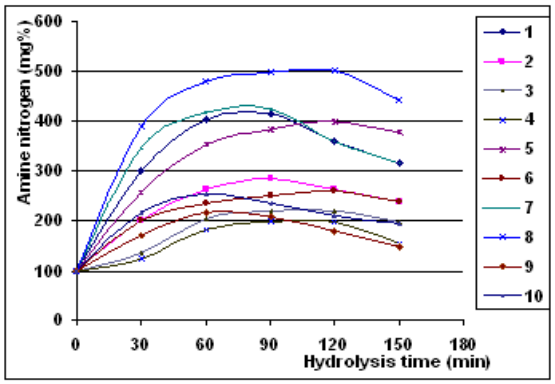


Fig. 1. Effect of concentration (%) of dry wheat gluten and hydrolysis time on the amine nitrogen content (mg %). Concentrations of dry wheat gluten: 1—90, 2—85, 3—80, 4—75, 5—47, 6—44, 7—42, 8—37, 9—25, and 10—23%.

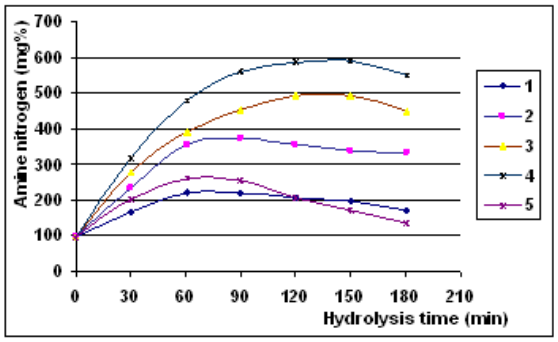


Fig. 2. Effect of temperature and hydrolysis time on the amine nitrogen content. Temperature (°C): 1—20, 2—30, 3—40, 4—50, and 5—60.

Hydrolysis with this endoprotease was most effective at pH 6.0–6.5 and hydrolysis time of 40–90 min.

The study of the effect of the concentration of the enzyme preparation in the range from 0.1 to 1.0 U/g protein on the amine nitrogen content under the above conditions showed that with an increase in the concentration of the enzyme preparation the content of nitrogen increased and reached its maximum (700 mg %) at the enzyme preparation concentration of 1.0 U/g protein and hydrolysis time of 30–60 min (Fig 3), which was 6–7 times higher than in the control.

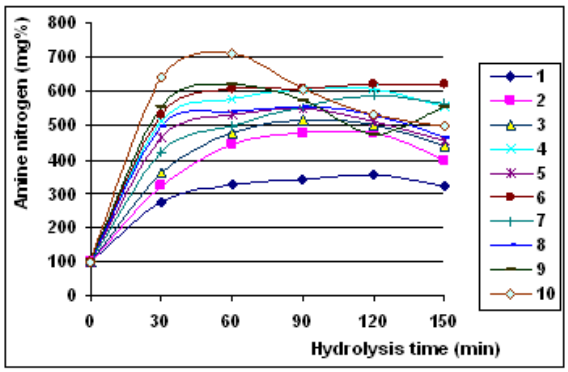


Fig. 3. Effect of Neutrase® 1.5 MG concentration (U/g protein) on the amine nitrogen content: 1—0.1, 2—0.2, 3—0.3, 4—0.4, 5—0.5, 6—0.6, 7—0.7, 8—0.8, 9—0.9, and 10—1.0.

Table 1 summarizes the parameters of gluten hydrolysis required for maximum accumulation of amine nitrogen at concentrations of 0.3, 0.6, and 1.0 U/g protein.

Table 1. Parameters of proteolysis of dry wheat gluten with Neutrase 1.5 MG.

Preparation	*Moisture*	*pH*	*Temperature (°C)*	*Hydrolysis time*	*Amine nitrogen*

concentration (U/g)	*(%)*			*(min)*	*(mg%)*
0.3	63	6.8-7.0	50	90	540
0.6	63	6.8-7.0	50	60	610
1.0	63	6.8-7.0	50	40	700

Then these parameters were used to determine the optimum regime of gluten hydrolysis by the influence of second endoprotease preparation, Protamex®, using the mathematical planning method. The factors were time (X) and temperature (Y), the function was the amine nitrogen (Z). The experiments were performed at pH 6.5–6.8 (Const) for three concentrations of the enzyme preparation (0.3, 0.6, and 1.0 U/g). As a result, we obtained an equation for predicting the content of amine nitrogen depending on the factors studied (Fig 4).

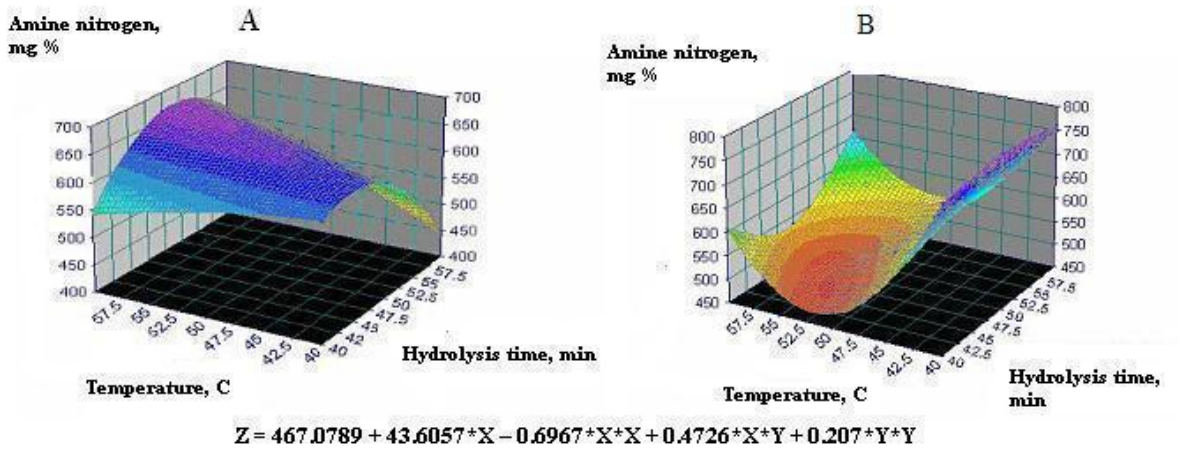


Figure 4.

Fig. 4. Dependence of the amine nitrogen content on temperature and hydrolysis time of dry wheat gluten: A— Protamex® concentration 0.3 U/g, B— Protamex® concentration 1.0 U/g.

Data processing with Matematika and Table Curve software made it possible to determine the optimum hydrolysis parameters for the extremum points of the maximum amine content for the good-quality gluten (Table 2). At the concentration of the enzyme preparation (1.0 U/g, as compared to the concentration 0.3 U/g) the amine nitrogen content was greater by only 8.5%, whereas at concentrations 0.3 and 0.6 U/g it was nearly the same. With allowance for these data, we decided to use the concentration 0.3 U/g in further experiments.

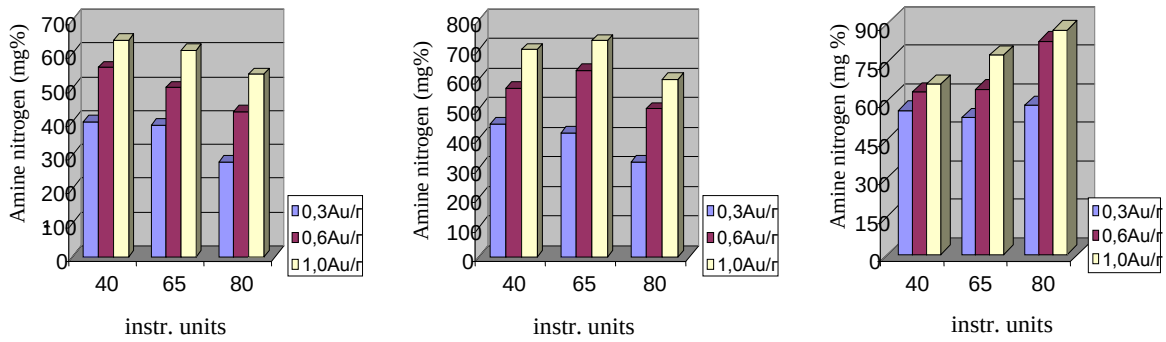
Table 2. Optimal parameters of hydrolysis of dry wheat gluten with Protamex.

Enzyme concentration (U/g)	*Temperature (°C)*	*Hydrolysis time (min)*	*pH*	*Amine nitrogen (mg%)*
0.3	50.0	48.6	6.5	575
0.6	50.6	48.8	6.5	581
1.0	50.0	50.0	6.5	628

We also determined the optimum parameters of good-quality gluten hydrolysis by the enzyme preparation Flavourzyme® 500 MG: pH 6.5; temperature, 50°C; and the hydrolysis time, 55, 43, and 28 min at Flavourzyme® 500MG concentrations of 0.3, 0.6, and 1.0 U/g, respectively.

Thus, we determined the parameters of proteolysis of proteins contained in good-quality gluten in the case of enzymatic modification of the latter by the endo- and exoprotease.

These values were then used to modify two other samples of dry wheat gluten at different enzyme concentrations (Fig 5). It was found that the content of amine nitrogen formed under the treatment with both Neutrase® 1.5 MG and Protamex® directly depended on the gluten strength. Conversely, the content of amine nitrogen produced during hydrolysis with the exoprotease Flavozyme® 500 MG increased with a decrease in the strength of gluten. This consistent pattern was observed at all three concentrations of enzyme preparations.



Neutrase 1.5 MG Protamex Flavozyme 500 MG

Fig. 5. Dependence of the amine nitrogen content on the compressive deformation index of gluten.

At the next stage of the study, we determined more accurately the hydrolysis time of dry wheat gluten for three concentrations (0.3, 0.6, and 1.0 U/g) of endoproteases (Neutrase® 1.5 MG and Protamex®) and the exoprotease (Flavozyme® 500 MG) at optimal values of moisture, pH, and temperature with allowance for accumulation of 400 mg% of amine nitrogen (Table 3). Comparative analysis of the results showed that Protamex® was more effective than Neutrase® 1.5 MG, since the same amount of amino nitrogen in our chosen concentrations of enzyme preparations 0.3 and 0.6 U/g, respectively, was achieved in less than 11-41 min at Protamex®, than Neutrase® 1.5 MG.

Table 3. Hydrolysis time determined for dry wheat gluten of different quality.

Enzyme preparation (U/g)		***Compressive deformation index H_{def} (instr. units)***		
		40	***65***	***80***
		Hydrolysis time (min)		
Protamex	0.3	35	45	80
	0.6	24	25	33
	1.0	18	15	20
Neutrase 1.5 MG	0.3	40	50	90
	0.6	30	28	40
	1.0	20	18	25
Flavourzyme 500 MG	0.3	80	55	26
	0.6	60	43	23
	1.0	35	28	20

Determination of the degree of hydrolysis of proteins showed that, in the case of Protamex®, it was somewhat higher than in the case of Neutrase® 1.5 MG. For the weak gluten, the degree of hydrolysis of gluten was higher in the presence of exoprotease Flavozyme® 500 MG; for the short-cleaved gluten, in

the presence of endoproteases. In general, the degree of gluten hydrolysis varied from 1.5 to 3.4%,

If the enzyme preparation was selected optimally, the completeness of hydrolysis of the weak gluten was higher than that of the good or short-cleaved gluten. Thus, our data confirmed the assumption that endoprotease-catalyzed hydrolysis is more effective for the short-cleaved and good gluten whereas exoprotease-catalyzed hydrolysis is more effective for the weak gluten. The amount of amine nitrogen evolved as a result of hydrolysis depended not only on the type of proteinase but also on the gluten quality (Table 4).

Table 4. Degree of hydrolysis of dry wheat gluten of different quality.

Enzyme preparation	***Compressive deformation index H_{def} (instr. units)***								
	40			**65**			**80**		
	Concentration (U/g)			***Concentration (U/g)***			***Concentration (U/g)***		
	0.3	**0.6**	**1**	**0.3**	**0.6**	**1**	**0.3**	**0.6**	**1**
Neutrase 1.5 MG	1.56	2.11	2.68	1.37	1.76	2.18	1.07	1.61	2.14
Protamex	1.72	2.21	2.89	1.4	2.18	2.57	1.15	1.84	2.24
Flavourzyme 500 MG	2.1	2.5	2.62	1.76	2.14	2.6	2.86	3.22	3.41

The data indicate that the degree of hydrolysis under the influence of the enzyme preparations Neutrase® 1.5 MG and Protamex® is 1,07-3,41%. This corresponds to previously reported data obtained by Kong *et al.* (2007), except for the fact that Neutrase® 1,5 MG is more effective than Protamex®. This may be explained by differences in the choice of the concentration of the enzyme preparation and the differences in rheological properties of the original gluten.

Determination of molecular weight (MW) of polypeptides in hydrolysates of dry wheat gluten of different rheological properties by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) for different concentrations of the enzyme preparations at amine nitrogen content of 400 mg% showed that the spectra of single-chain polypeptides in the control samples of weak and good gluten were the same, which apparently testified to the common origin of varieties. In 1974 Russian scientists (Prishchep *et al.*,1974) showed that the electrophoretic spectra of proteins of wheat of the same variety, but with different rheological properties of gluten, are the same.

Electrophoresis of polypeptide hydrolysates produced by the endoprotease Protamex

Gradient electrophoresis in 10–20% polyacrylamide gel (Fig 6) showed that the control samples of gluten of different quality contained 10 or 11 components whose MW varied from 11 до 120 kDa, whereas the hydrolysates produced by the endoprotease Protamex® contained from 6 to 11 polypeptides; i.e., proteins of hydrolysates were less heterogenous. The amount of proteins with MW > 120 kDa that did not enter the gel was much smaller.

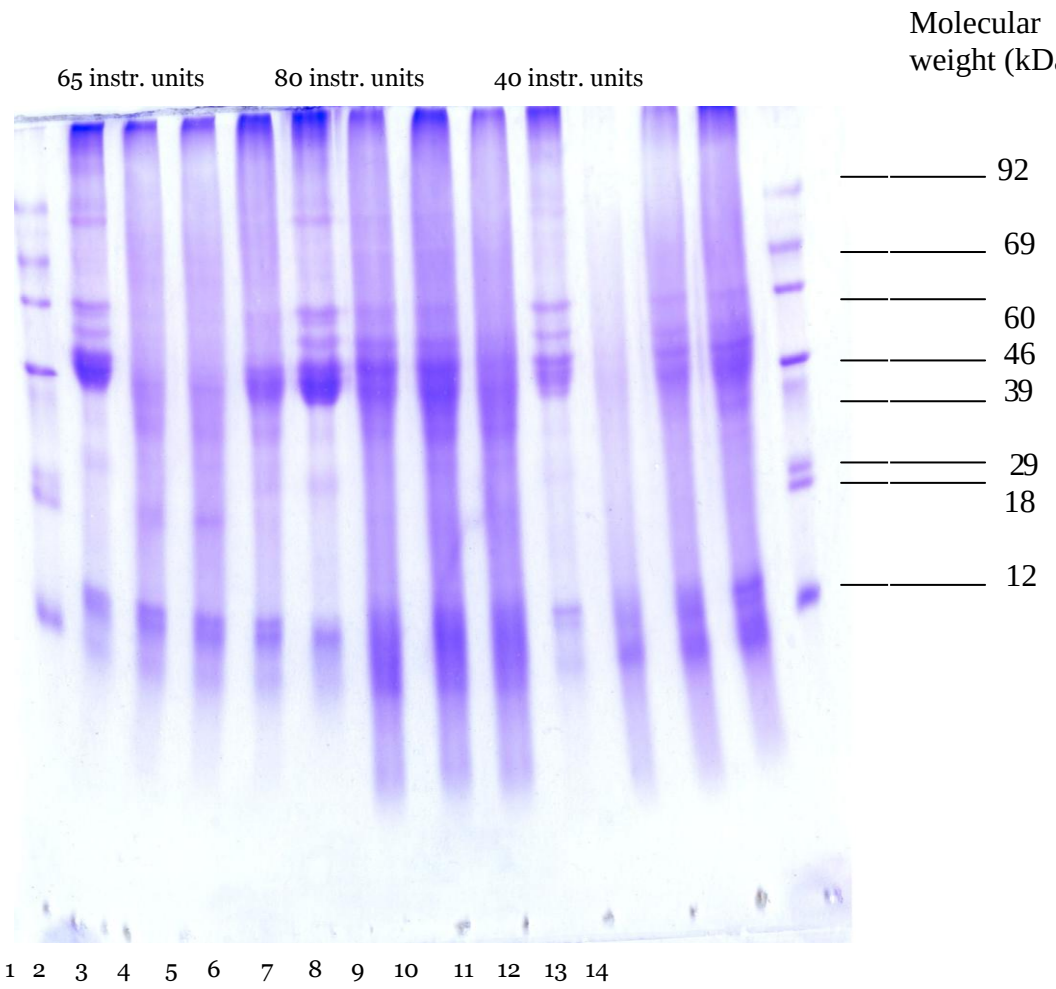


Fig. 6. Polypeptide composition of dry wheat gluten of different quality hydrolysed by the endoprotease Protamex® (PAGE in 10% polyacrylamide gel). Lanes 1 and 14 show molecular-weight markers. Dry wheat gluten (H_{def}, instr. units): 2—65, 6—80, 10—40. Hydrolysates: lanes 5, 9, and 13—enzyme concentration 0.3 U/g; lanes 4, 8, and 12—0.6 U/g; lanes 3, 7, and 11—1.0 U/g.

The compositions of polypeptides of hydrolysates of weak and good gluten differed from each other. Proteins with MW of 16, 18, 43, and 46 kDa were lacking in the hydrolysate obtained in the presence of 0.3 U/g the enzyme preparation. Hydrolysates produced by 0.6 and 1.0 U/g the enzyme preparation contained additional components with MW of 18, 43, and 46 kDa.

Thus, the composition of hydrolysates depends not only on the concentration of the enzyme preparation but also on the gluten quality. The higher degree of hydrolysis of the good gluten (1.76–2.18%) was accompanied by a more heterogenous composition of single-chain components (10 or 11); the lower degree of hydrolysis of the weak gluten (1.61–1.84%) was accompanied by a less heterogenous composition (6–9 components). These data confirmed the fact that endoprotease hydrolyses the gluten of good quality more completely versus the weak gluten.

It is known that the protein that remains at the start level and does not enter the gel contains polypeptides linked through S–S-bonds (Bietz and Wall, 1972). With allowance for this fact, we performed electrophoresis in the presence of mercaptoethanol, which breaks S–S bonds, in order to reveal distinctions in the composition of multi-chain polypeptides (Fig 7). High-molecular-weight proteins were absent at the start level in all samples. Therefore, the hydrolysate contained multi-chain polypeptides whose single-chain components were linked through S–S-bonds. Part of polypeptides coincided in MW with single-chain polypeptides;

others had different MW and were specific. Nevertheless, even after the break of S–S-bonds, the polypeptide spectra of control samples of weak and good gluten were the same, whereas the short-cleaved gluten had a different polypeptide composition. The main characteristics of MW before and after breaking S–S-bonds are summarized in Table 5.

Table 5. Characteristics of molecular weight of polypeptides before and after cleavage of dry wheat gluten of different quality (MCPs - multi-chain polypeptides, SCPs - single-chain polypeptides).

dry wheat gluten quality	0.3 U/g	0.6 U/g	1.0 U/g
Good	8 SCPs with MW 13–46 kDa; MCP with MW > 120 kDa consisting of 18 SCPs with MW 12–110 kDa.	7 SCPs with MW 13–-44 kDa; MCPs with MW > 120 kDa consisting of 13 SCPs with MW 12–92 kDa.	
Weak	8 SCPs with MW 13–46 kDa; MCPs with MW > 120 kDa consisting of 18 SCPs with MW 12–110 kDa.	9 SCPs with MW 13–86 kDa; MCPs with MW > 120 kDa consisting of 16 SCPs with MW 12–110 kDa.	9 SCPs with MW 12–86 kDa; MCPs with MW >120 kDa consisting of 4 SCPs with MW 12–22 kDa.
Short-cleaved	7 SCPs with MW 14–60 kDa; MCPs with MW > 120 kDa consisting of 13 SCPs with MW 12–110 kDa.	6 SCPs with MW 14–60 kDa; MCPs with MW > 120 kDa consisting of 9 SCPs with MW 12–60 kDa.	5 SCPs with MW 14–46 kDa; MCPs with MW > 120 kDa consisting of 4 SCPs with MW 12–22 kDa.

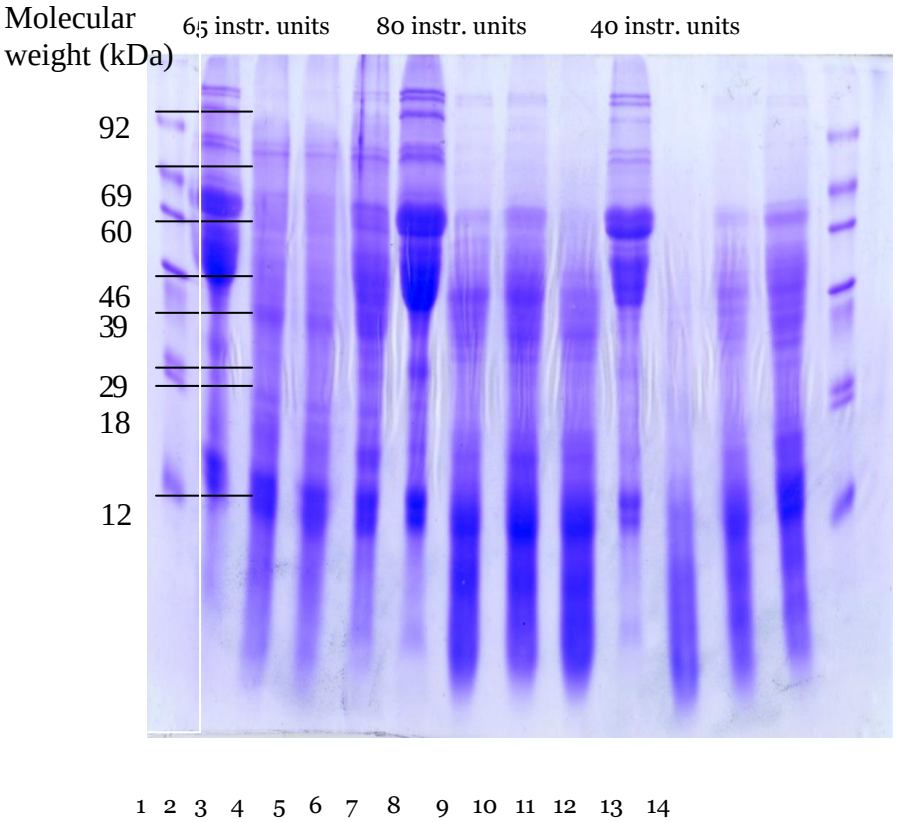


Fig. 7. Polypeptide composition of dry wheat gluten of different quality after breaking S–S bonds by the endoprotease Protamex®. Lanes 1 and 14 show molecular-weight markers. Dry wheat gluten c H_{def} (instr. units): 10—40, 2—65, 6—80. Hydrolysates: lanes 5, 7, and 13—enzyme concentration 0.3 U/g; lanes 4, 8, and 12—0.6 U/g; and lanes 3, 9, and 11—1.0 U/g.

Thus, depending on the concentration of the enzyme preparation, differences in the MW of polypeptides present in hydrolysates of all gluten samples were expressed at the level of both single-chain polypeptides and multi-chain polypeptides (depending on rheological properties of the gluten, largely at the level of multi-chain polypeptides).

The results obtained in this study confirmed the limited degree of proteolysis of dry wheat gluten.

Electrophoresis of polypeptide hydrolysates produced by the exoprotease

Molecular weights of single-chain proteins for which electrophoretic spectrum was obtained at different concentrations of exoprotease Flavozyme® 500 MG also varied from 18 to 110 kDa (Fig 8); however, the electrophoretic spectrum in the case of exoprotease-catalyzed hydrolysis was more heterogenous than in the case of endoprotease. This phenomenon was apparently due to the fact that proteins at the outer layers are more accessible for hydrolysis. At the same time, gluten samples were catalyzed less intensively, judging by the presence of proteins that did not enter the gel and remained at the start level.

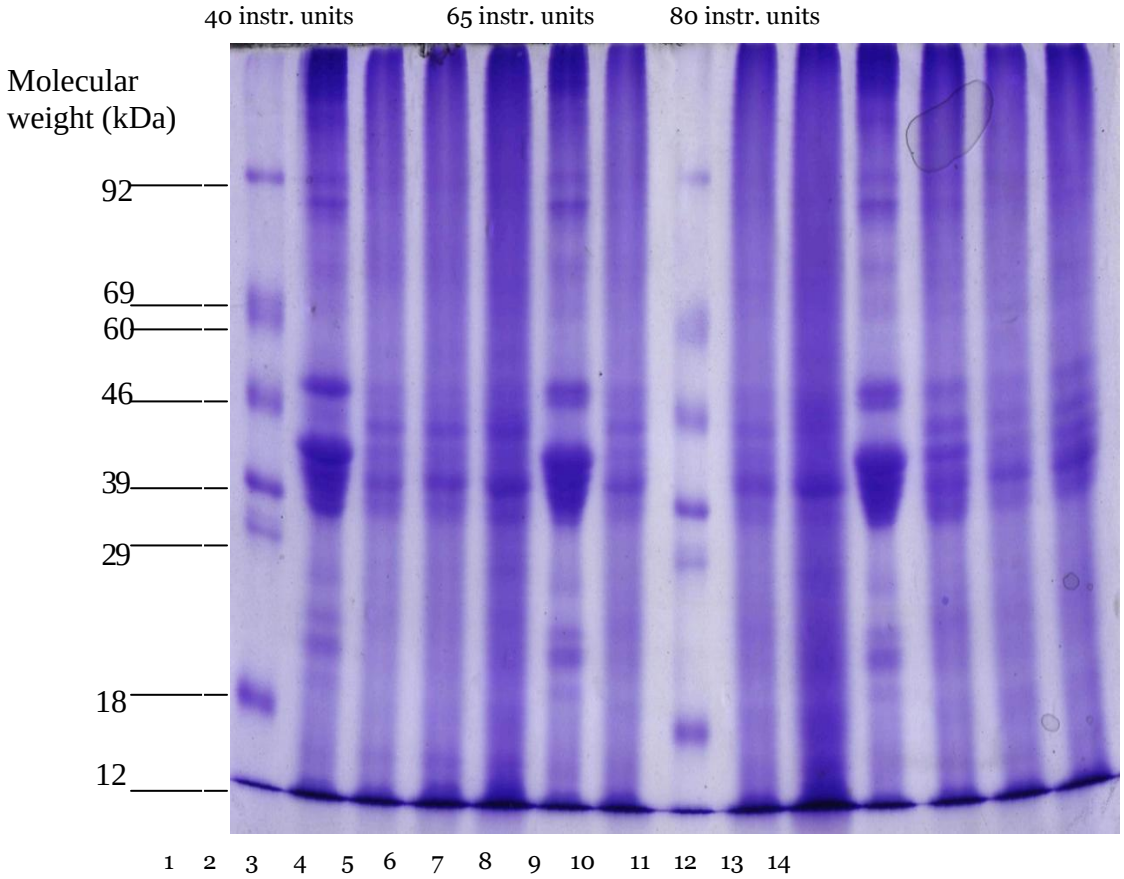


Fig. 8. Polypeptide composition of dry wheat gluten of different quality hydrolysed by the exoprotease Flavourzyme® 500 MG. Lanes 1 and 8 show molecular-weight markers. Dry wheat gluten with H_{def} (instr. units): lane 2—40, lane 6—65, and lane 11—80. Hydrolysates: lanes 3, 7, and 12—enzyme concentration 0.3 U/g; lanes 4, 9, and 13—0.6 U/g; and lanes 5, 10, and 14—1.0 U/g.

No significant differences among gluten of different quality were detected both at the level of single-chain polypeptides and polypeptides that were components of multi-chain polypeptides, except that the hydrolysate of the weak gluten contained higher quantities of low-molecular-weight proteins (16–22 kDa). This finding was suggestive of a higher degree of hydrolysis of initial proteins by exoprotease.

Thus, we showed that, although the quantity of amine nitrogen was the same (400 mg %), the polypeptide spectra of all samples differed from one another. Therefore, the amine nitrogen index should always be verified electrophoretically.

Comparison of these results with the data obtained for the endoprotease showed that protein spectra slightly differed from one another. Therefore, the polypeptide composition of gluten was also individual and depended on the enzyme preparation used.

Electrophoretic analysis of polypeptides in the presence of mercaptoethanol (Fig 9) showed that the spectrum of proteins obtained using the exoprotease Flavozyme® 500 MG was more heterogenous than that obtained using the endoprotease Protamex®. For example, the number of components in the hydrolysates of the control gluten samples treated with the exoprotease and endoprotease amounted to 22 or 23 and only 16–19, respectively. In the spectra of reduced control samples of gluten of different quality, no differences were detected, whereas the spectra of hydrolyzed proteins contained different numbers of single-chain components linked through S–S-bonds. No differences in the distribution patterns of molecular weights depending on concentrations of the enzyme preparation were detected for all three gluten samples, except for the hydrolysate of the good-quality gluten obtained at the concentration of 1.0 U/g the enzyme preparation. The latter was less heterogenous compared to the spectra of proteins obtained at concentrations of 0.3 and 0.6 U/g the enzyme preparation.

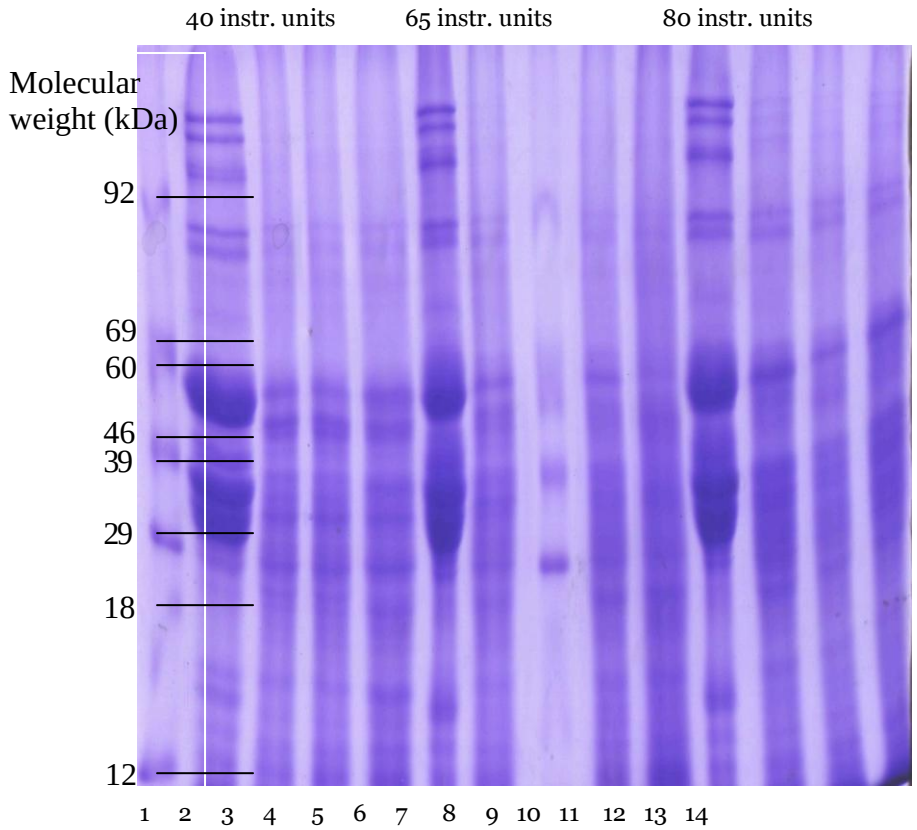


Fig. 9. Polypeptide composition of dry wheat gluten of different quality after breaking S–S bonds by the exoprotease Flavourzyme® 500 MG. Lanes 1 and 8 show the molecular-weight markers. Dry wheat gluten with H_{def} (instr. units): lane 2—40, lane 6—65, and lane 11—80. Hydrolysates: lanes 3, 7, and 12—enzyme concentration 0.3 U/g; lanes 4, 9, and 13—0.6 U/g; and lanes 5, 10, and 14—1.0 U/g.

The electrophoretic spectrum of hydrolysates of the weak gluten slightly differed in the set of polypeptides from the spectrum of the control sample: it included 20 polypeptides, the majority of which were present in trace amounts. The spectra of the short-cleaved and good-quality gluten only slightly differed from each other, compared to the control and hydrolysate of the weak gluten.

As in the experiments with endoprotease, the differences between the samples of hydrolyzed gluten of different qualities prepared with exoprotease enzyme preparation are more pronounced at multi-chain proteins than single-chain. Weak gluten protein hydrolysis proceeded more efficiently with exoprotease preparation, hydrolysis of short-cleaved and good gluten - with endoproteases.

Thus, we were the first to show that endoprotease enzyme preparation Protamex® provides more significant differences in molecular weighs of polypeptides of hydrolysates of varying quality gluten than exoprotease, so it is reasonable to recommend it into practice as an effective preparation for the limited proteolysis and modification of the functional properties of wheat gluten.

Conclusioin

Many researchers (Popineau *et al*., 2002; Kong *et al*., 2007; Zhang *et al* ., 2012; Kammoun *et al*., 2003) have studied the hydrolysis of wheat gluten in order to increase its solubility and improve the various physico-chemical indicators of the surface-active properties. The use of proteolytic enzymes for this purpose is one of the most promising ways to modify the functional properties of both crude (Fedotova *et al*., 2009) and dry wheat gluten (Popineau *et al*., 2002; Zhang *et al* ., 2012; Prishchep *et al* ., 1974) due to the fact that safe techniques and mild condition were used (Mikulovich, 1991). Research results on the application of the hydrolytic enzyme preparations such as Neutrase®, Protease 2500S, Alcalasa® 2.4 L, Pepsin, Protamex® to modify the properties of gluten are published in the papers mentioned above. Researches have shown that the used enzymes hydrolyzed gluten for improvement its solubility (Kong *et al*., 2007) and surface-active properties, accompanied by changes in the structure of its proteins (Popineau *et al*., 2002; Zhang *et al* ., 2012). At the same time, it is known (Puchkova *et al*., 2005) that by the action of proteases gluten loses visco-elastic properties. In this case the use of hydrolyzed products for improving the properties of wheat flour and bread quality is impossible, if hydrolysates contain a large number of low molecular weight peptides (eg, MW lower than 5000-10000 Da, as obtained Zhang *et al.* (2012) with Alcalasa®).

Our task in the present research was to examine such conditions of limited proteolysis of dry wheat gluten, which would yield peptides with such molecular weight which can provide improved baking properties of flour if it is obtained from grain grown under high-contrast climatic conditions (high humidity and lower temperatures) (Kolpakova *et al.*, 2007)**.** We also took into account the fact that gluten can have different rheological properties (short-cleaved, good and weak gluten) and the hydrolysates can be applied along with increasing amounts of vegetable protein products, with a more balanced amino acid composition than gluten (soya flour, concentrates of amaranth, pea, etc.).

With an increased amount of protein in the dough (more than 5% by weight of flour), bread volume diminishes, porosity deteriorates elasticity of crumb products is reduced, while hydrolysates containing low-molecular nitrogen compounds (peptides, amino acids) may intensify the process of fermentation of the dough and reduce the negative effect of the influence of increased amounts of proteins on the quality of bread.

In previously published articles, the authors used dry (Popineau *et al*., 2002; Kammoun *et al*., 2003) or crude (Zhang *et al*., 2012; Fedotova *et al*., 2009) wheat gluten for proteolysis, but in all cases they did not take into account its initial rheological properties and the different effect of the endo- and exoprotease enzyme preparations on the development of the

parameters of proteolysis. Comparative study of the enzymatic hydrolysis of dry wheat gluten of different rheological properties (short-cleaved, good and weak gluten) by enzyme preparations containing endo- and exoproteases showed that the stronger gluten, the more amine oxide is formed by the action of endoproteases (Protamex®, Neutrase® 1.5 MG), and the weaker it is the more amino nitrogen is released as a result of the action of exoprotease (Flavourzyme® 500 MG). This suggests that the amount of amino nitrogen in hydrolysates depends both on the quality of the regenerated dry gluten and on the type of proteolytic enzyme preparations, so the protein hydrolysis time as well as the concentrations of the enzyme preparation for proteolysis will be different.

The results of determining the extent of proteolysis, calculated according to the amine nitrogen value demonstrate that the degree of hydrolysis of proteins of dry wheat gluten in the presence of Protamex was higher than in the presence of Neutrase® 1.5 MG for short-cleaved and good gluten. The degree of hydrolysis of weak gluten was higher in the presence of exoprotease Flavourzyme® 500 MG. These data were confirmed by the results of electrophoresis of the protein components obtained after hydrolysis of different quality gluten with mercaptoethanol and without it. Thorough analysis of protein electrophoregrams obtained in a gradient 10-20% acrylamide permits to conclude that differences in molecular weight of polypeptides for all hydrolysates are revealed at the level of both single-chain polypeptides and multi-chain polypeptides. But the dependence of component composition on the rheological properties of gluten was more pronounced at the level of multi-chain components.

The highest (3.41%) the degree of proteolysis was observed in weak gluten under the influence of exoprotease Flavourzyme® 500 MG. This hydrolysis resulted in the formation of 9 single-chain polypeptides with MW of 12-86 kDa proteins and multi-chain polypeptides with MW more than 120 kDa, consisting of 4 single-chain peptides with MW of 12-22 kDa. The degree of hydrolysis of short-cleaved gluten under the influence of endoprotease Protamex® was 2.89%. The hydrolysis was accompanied by the formation of 5 single-chain peptides with lower MW (14-46 kDa) and multi-chain polypeptides consisting of 4 single-chain peptides with MW of 12-22 kDa. Good gluten hydrolysates, obtained with Protamex ® with the degree of hydrolysis of 2.57% consisted of 7 single-chain peptides with MW from 13 to 44 kDa, but multi-chain polypeptides consisted of 13 single-chain peptides of MW 12-92 kDa.

Consequently, the molecular weight composition of the polypeptides of gluten hydrolysate obtained by us with the preparations Flavourzyme® 500 MG and Protamex®, at practically the same amount of amino nitrogen in the medium, depended on its rheological properties, the concentration and the type of enzyme preparations. Molecular weights of polypeptides are much lower than those at the degree of hydrolysis of 30%, achieved with the enzyme preparation Alcalasa® 2.4 L by other authors (Zhang *et al.,* 2012). In fact, this may be what is needed to improve the quality of bread from low quality flour or to improve the functional properties of gluten proteins for their application as foamers, emulsifiers and emulsion stabilizers.

Thus we have developed parameters of proteolysis of gluten with different rheological properties (weak, short-cleaved, good) with a degree of hydrolysis from 1.5 to 3.4% by the action of enzyme preparations Protamex ®, Neutrase® 1,5 MG and Flavourzyme® 500 MG for future research of using hydrolysates in food industry.

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