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Content of free amino groups during postharvest wheat and flour maturation in relation to gluten quality

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ABSTRACT

The objective of this study was to monitor the changes in the content of free amino groups during postharvest wheat and flour maturation. The content of free amino groups of wheat flour was analysed immediately after wheat harvest, after 50 days of wheat storage and after 14 days of flour storage varying by wet gluten samples incubation temperatures and incubation times (0, 90 or 135 min at 30 °C and after that 180 min at 37 °C). The results were observed in relation to wheat-bug damaged kernels content, gluten index values, proteolytic activity and electrophoretic properties of gliadins and glutenins. The content of free amino groups increased during postharvest wheat and flour maturation periods. Proteolytic activity values were the highest 50 days after the wheat storage. The electrophoretic determination indicated a macromolecular redistribution of the gluten proteins from the moment of the wheat harvest until the moment of flour stabilisation.

1. Introduction

The study of the biochemical changes in wheat gluten proteins during wheat postharvest maturation as well as the study of the after-stabilisation of flour milling dates back to early 1940s (Bayfield, Anderson, Geddes, & Hildebrand, 1943; Jones & Gersdorff, 1941; Shellenberger, 1939), continues in the early 1970s (Evers & Redman, 1973; Pyler, 1973; Skupin & Warchalewski, 1971) and still remains a topic of interest. The wheat postharvest maturing begins immediately after harvest, continues during storage, and depends on time, ambient conditions of storage and the grain moisture content. During the wheat maturation a number of biochemical and colloidal changes occur until the final technological maturity is reached. Newly harvested wheat has poor milling and baking quality, therefore, the postharvest maturation of wheat and wheat flour is a necessary part of improvement of their technological quality (Chen & Schofield, 1996; Wang & Flores, 1999).

After the milling of mature wheat, the maturation process continues in the wheat flour. Pyler (1973) described the complex biochemical changes during the flour maturation which started 4–5 days after milling and lasted for approximately 3 weeks.

Mature wheat flour has higher water absorption, better mixing tolerance, improved rheological properties, greater gas retention capability and produces bread with greater loaf volume (Miś, 2003; Wang & Flores, 1999).

The above mentioned complex biochemical changes include several groups of enzymes present in wheat grain and flour. These are: amylases, proteases, oxygenases, polyphenol oxidases and peroxidases. Proteases are concentrated in the endosperm, germ and aleurone layer (Evers & Redman, 1973). In contrast to protease, peptidase was found largely in the endosperm (Kruger, 1973). Bleukx, Roels, and Delcour (1997), in their research on the presence and activities of proteolytic enzymes in vital wheat gluten, concluded that one or more aspartic and serine endoproteases were causing gluten hydrolysis. Each time when a peptide bond is hydrolysed a free amino group and a free carboxyl group are released. The progress of hydrolysis is determined on the basis of the increase in the concentration of these groups. The measurement of the free amino groups content in wheat proteins is directly proportional to the degree of hydrolysis of proteins (Nielsen, Petersen, & Dambmann, 2001), i.e., to the degradation of the polymeric structure of protein up to the water-soluble free amino acids and small peptides (Aja, Pérez, & Rosell, 2004).

Although these enzymes are inactive during grain and flour storage after finished maturation process, when water is added they become active and play a significant role in determining the functional attributes of the flour. Lin, Lookhart, and Hoseney

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(1993) reported the positive effect of proteolytic enzymes on wheat dough rheological properties in production of fermented products. To our knowledge, in the available literature of recent date there are no similar data on monitoring biochemical changes during wheat postharvest and wheat flour maturation through the concentration of free amino groups in wet gluten. The exception to this assertion is the study of Zhao, Li, Liu, Liu, and Li (2012) who investigated the changes in the amount of free amino groups in the frozen gluten samples stored from 0 to 120 days. The negative effects of proteolytic enzymes activity in durum wheat products were described by Petrova (2002) and Olanca, Ozay, and Koksel (2009). Pérez, Bonet, and Rosell (2005) reported the increase in the amount of free amino groups during incubation of gluten of damaged wheat as a consequence of an activity of insect protease (saliva *Eurygaster* spp.).

The aim of this study was to determine the status of free amino groups in two different maturing periods of the harvested wheat and flour obtained from it. For the determination of free amino groups content, two temperatures were chosen (30 and 37 °C) and different incubation periods of the wet gluten on those temperatures (0, 90 or 135 min at 30 °C and after that 180 min at 37 °C). The temperature of 30 °C was chosen in order to simulate the measurement conditions of the rheological properties of the dough on the extensograph and the duration of processing of the dough in practise. The second temperature, 37 °C, was chosen as the optimum temperature for proteolytic enzyme activity, in order to determine the potential of the content of free amino groups as an indicator of the proteolytic activity.

The additional aim was to relate the content of free amino groups with the selected quality parameters of the wheat/flour protein complex which included the gluten index at 30 and 37 °C, and the proteolytic activity.

2. Materials and methods

2.1. Samples

Three wheat varieties (*Triticum aestivum*) Pobeda (Pob), Zvezdana (Zve) and Apache (Ap) grown in 2011 in three areas in Serbia: Bačka Topola (BT), Sremska Mitrovica (SM) and Vršac (VR) were selected for the study. Pobeda and Zvezdana were bred by the Institute of Field and Vegetable Crops, Novi Sad, Serbia, whereas Apache was bred by Limagrain, Chappes, France. The measurements of temperature and precipitation at meteorological stations in the three examined areas were observed from the beginning of May until the time of harvest (8–9 July). During May and June, the VR area had sufficient precipitation and the smallest number of days with a maximum temperature higher than 30 °C; two times lower compared to the BT area and SM. The greatest number of days with a temperature higher than 30 °C was recorded at the SM area, while the BT area was characterised by a combination of two unfavorable impacts on the wheat development – drought and high temperature.

The appearance of wheat bug damage and black point kernels, kernels infected by Fusarium and broken kernels, was registered at all areas but the impact of climatic conditions on the amount and composition of impurities in the wheat could not be determined since the crops were treated with herbicides, fungicides and insecticides.

The samples were stored in craft paper bags under laboratory conditions (22 °C, 70% RH) for 50 days. The samples were cleaned and tempered and milled using a Bühler MLU 202 (Bühler, Uzwil, Switzerland) according to AACC methods (1999) immediately after harvest (point 1), and after 50 days of wheat storage (point 2). Flour obtained after 50 days of wheat storage was also analysed after 14 days of storage at the above mentioned conditions (point 3). Therefore, the samples originated from point 1 were regarded as freshly harvested wheat, samples originated from point 2 as matured wheat, while samples originated from point 3 were regarded as matured flour. The storage periods 1–2 and 2–3 present wheat postharvest maturation and flour maturation, respectively.

2.2. Content of wheat-bug damaged kernels

The content of wheat-bug damaged kernels (WBDK) was determined according to the ICC standard method 102/1 (ICC, 1972) in two replicates (SD = 0.08).

2.3. Free amino groups content

The content of free amino groups was determined according to the procedure described by Pérez et al. (2005) from wet gluten washed out from flour samples (obtained as described in Section 2.1) according to standard ICC method 106/2 (ICC, 1984). The determination of free amino groups was performed for different incubation times and temperatures in flour from freshly harvested wheat (point 1), flour from matured wheat (point 2) and matured flour (point 3), as presented in Table 1.

Every treatment was applied on flour samples of each examined wheat variety (Pob, Zve and Ap) from all three areas (BT, SM and VR) in all three test points (1, 2 and 3).

The determination of free amino groups was carried out in four replicates, where the results were calculated against a serine standard curve. The spectrophotometric readings were performed at 340 nm (GBC CINTRA 303UV/VIS) (for $SD_{(I)} = 0.03$, $SD_{(II)} = 0.02$, $SD_{(III)} = 0.03$, $SD_{(IV)} = 0.03$, $SD_{(V)} = 0.03$, $SD_{(VI)} = 0.03$).

2.4. Gluten index

Gluten index was measured at 1, 2 and 3 points in two different ways: according to the ICC standard method No 155 (ICC, 1994) (GIS) and by modified method (GIM) which includes incubation of dough ball at 37 °C for 90 min (Torbica, Antov, Mastilović, & Knežević, 2007). All measurements were performed in duplicate ($SD_{(GIS)} = 1.09$, $SD_{(GIM)} = 3.62$).

2.5. Proteolytic activity

The protease assay was performed for flour samples from wheat (point 1 and 2) and maturated flour samples (point 3) stored in defined periods, as described by Rani, Prasada Rao, Leelavathi, and Haridas Rao (2001). The proteolytic activity (PA) was measured by using azocasein as substrate. All spectrophotometric readings were performed at 440 nm (GBC CINTRA 303UV/VIS) in duplicate (SD = 0.06). One unit of activity is defined as the change in absorbance by 1.0 unit.

Table 1
Plan of experiment.

Treatments labels	Test points	Sample incubation treatment			
		Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
NH2-I	1, 2, 3	–	–	–	–
NH2-II	1, 2, 3	30	90	–	–
NH2-III	1, 2, 3	30	135	–	–
NH2-IV	1, 2, 3	–	–	37	180
NH2-V	1, 2, 3	30	90	37	180
NH2-VI	1, 2, 3	30	135	37	180

2.6. Lab-on-a chip electrophoresis

The extraction of gliadins and glutenins from flour samples of freshly harvested wheat (point 1) and matured flour (point 3), was carried out according to the Osborn fractionation of the wheat protein and reduced by 2× treatment buffer (0.125 M Tris–Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol). The extracts were analysed using the Protein 230 Plus LabChip kit (Torbica, Živančev, Nikolić, Đorđević, & Nikolovski, 2010) by Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA) in duplicate (SD = 0.75–2.96 for molecular weights range from 14 to 220 kDa) (Živančev, Nikolovski, Torbica, Mastilović, & Đukić, 2013).

2.7. Statistical analysis

The effects of factors (storage period, gluten incubation temperature and gluten incubation time, variety and locality) on the free amino groups content and protein quality indicators were determined by ANOVA. Where the *F*-test for the ANOVA reached statistical significance ($p < 0.05$), the differences among specific means were assessed by Least Significant Difference (LSD) tests. The Principal Component Analysis (PCA) was used for the estimation of the relation between the content of free amino groups and selected quality parameters of wheat/flour samples. Statistical methods were performed using the StatSoft. Inc. (2013) STATISTICA (data analysis software system) version 12.

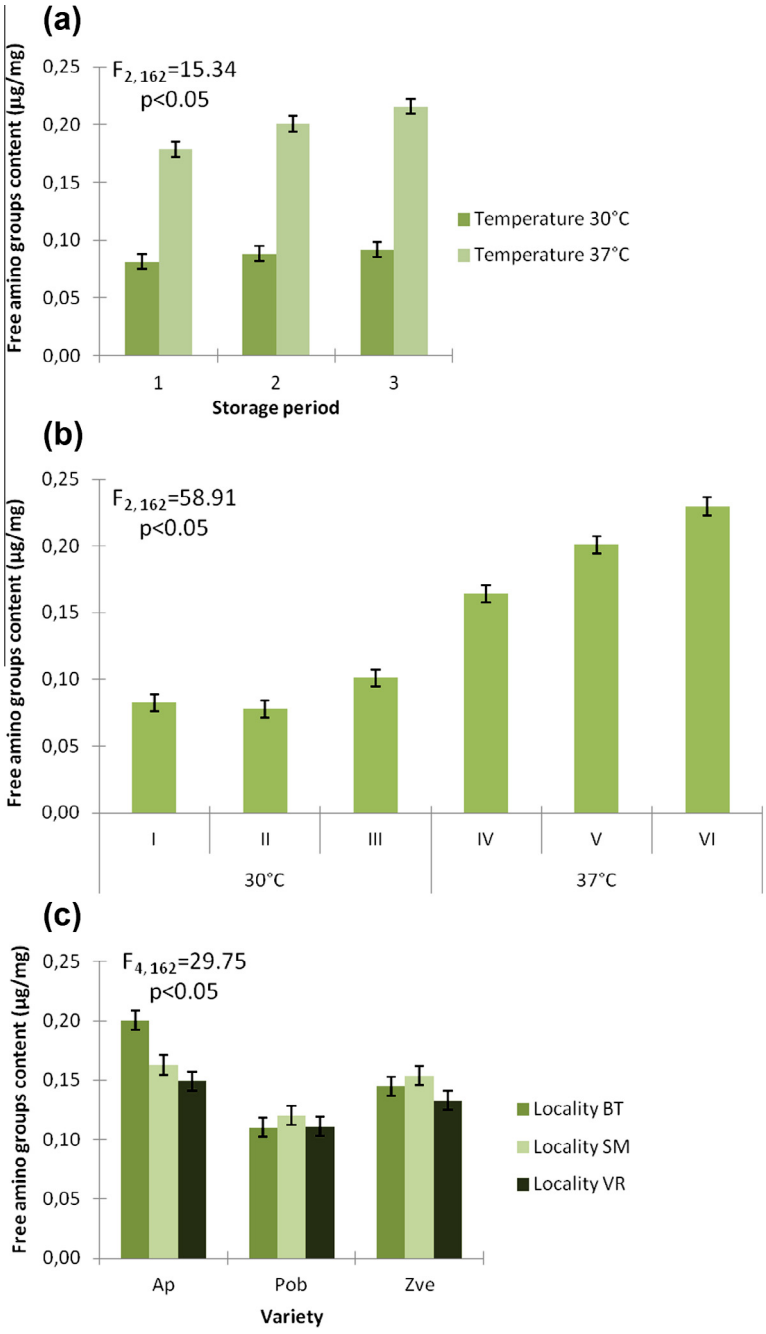

Fig. 1. Changes in free amino groups content during wheat and flour maturation as a function of two different gluten incubation temperatures (30 and 37 °C) (a), gluten incubation time (I, II, III, IV, V and VI) (b) and variety and locality (c). Measured values are the mean ± 0.95 LSD intervals.

3. Results and discussion

The biochemical changes in the proteins during wheat postharvest and flour maturation were estimated on the basis of the content of free amino groups, gluten index, relative amounts of gliadins and glutenins and flour proteolytic activity. These parameters depend on wheat variety, the production year and the conditions of the harvest and also the milling technology, are important for processing of the flour in bakery and the quality of the final products (Johansson et al., 2013).

3.1. Content of wheat-bug damaged kernels

The insect protease might have promoted the gluten breakdown, favouring the accessibility to some amino groups previously hidden in the polymeric structure and, as a consequence, the release of diverse polypeptides (Pérez et al., 2005). In the samples from our study the wheat-bug damaged kernels (WBDK) ranged from 0.30% to 1.32%. According to Serbian regulation (Regulation of methods of physical and chemical analysis for quality control of grain, milling and bakery products, pasta and quickly frozen dough, 1988) the allowed content of WBDK is 2%, while according to the U.S. Sample Grade Criteria (Grain inspection handbook, 2013) the allowed number of insect damaged kernels in 1000 g of wheat kernels is 32. In our study, the highest average value of WBDK was registered in the VR area and the lowest content in the SM area.

3.2. Content of free amino groups of gluten during incubation at 30 and 37 °C

The influence of individual factors as well as the simultaneous influence of selected factors (storage periods (1–2, 2–3), gluten incubation temperature, gluten incubation time, varieties and localities) on the content of free amino groups, is shown in Fig. 1a–c and Supplementary Table 1.

The content of free amino groups significantly increased ($p < 0.05$) during postharvest wheat and flour maturation (0.130–0.154 μg/mg). The content of free amino groups increased with the increase in incubation temperature of gluten from 30 °C (0.081–0.092 μg/mg) to 37 °C (0.179–0.215 μg/mg) ($p < 0.05$) (Fig. 1a and b). The influence of incubation at 30 °C was obvious only after 135 min, while the values of free amino groups content at 37 °C for all tested points were significantly higher with the prolongation of incubation time (0.164–0.230 μg/mg) ($p < 0.05$) (Fig. 1b). The increase in the content of the free amino groups ($p < 0.05$) was observed for IV, V and VI sample treatment (Fig. 1b) These results were in accordance with Aja et al. (2004), who showed that under the same conditions of wet gluten incubation, a significantly higher amount of free amino acids and small peptides occurs after 180 min of gluten incubation at 37 °C, as a consequence of the gluten hydrolysis under the optimal conditions for the proteolytic enzymes activity.

During the total period of storage the highest content of free amino groups was observed in the third point, i.e., after the completed biochemical processes in wheat and flour at 30 and 37 °C

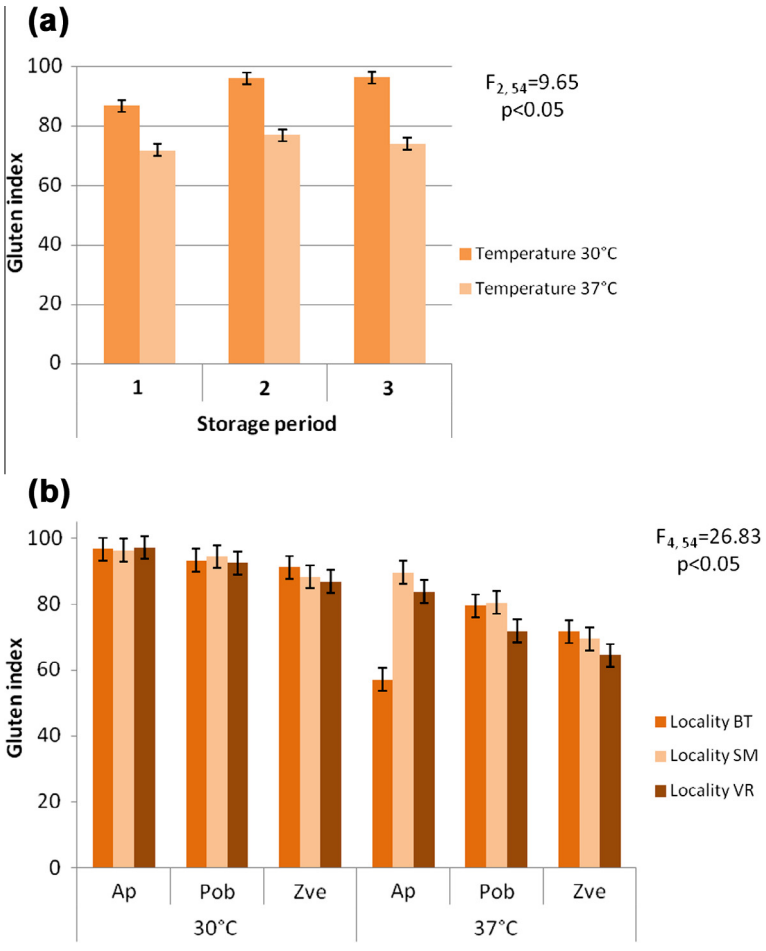

Fig. 2. Effects of (a) the storage period and gluten incubation temperature, (b) variety and locality on the gluten index. Measured values are the mean ± 0.95 LSD intervals.

(Fig. 1a). Referring to the claim of Shelke, Hoseney, Faubion, and Curran (1992) that the cake-baking quality of the flour improved with both wheat and flour maturity, it can be assumed that the increase in the content of free amino groups is a factor that affects the formation of the final quality of the flour. In the present study, the increase in the content of free amino groups was more obvious during the wheat postharvest maturation in comparison to the period of flour maturation. That was in accordance with the claims of Shelke et al. (1992) who found that the flour milled from freshly harvested soft wheat changed rapidly immediately after milling.

According to Fig. 1c, the big difference between Pob and Ap varieties in their free amino groups content is clear. The greatest impact of the growing area was noticed for variety Ap and the lowest was for variety Pob.

3.3. Gluten index determination

The standard gluten index values determined without previous incubation increased during postharvest wheat maturation ($p < 0.05$), and then stay constant during flour maturation period (14 days) (Fig. 2a). These results were in accordance with the results of Miś (2003), who reported no major changes in the characteristics of gluten during four weeks of flour storage, unlike the longer time of storage which influenced the rheological properties of gluten as determined by GIS. The GIM values obtained with previous incubation were lower ($p < 0.05$) than those obtained without incubation (Fig. 2a) (Aja et al., 2004; Torbica et al., 2007), which was expected as a consequence of applying the optimum temperature for proteolytic enzyme activity; the highest value was in the point 2. The varieties had almost the same GIS values while the differences between varieties were evident for the GIM values. Ap exhibited the highest, while Zve the lowest values of GI (Fig. 2b). Regarding the area, SM compared with the other two areas, had the highest GIM values which were in accordance with lowest content of WBDK (Fig. 2b). The obtained results complied with the results of Har Gil, Bonfil, and Svoray (2011), which indicated that the genotype, climatic factors and agricultural practices applied had an important impact on the values of GI. All samples reached their maximum at the end of the first period (point 2) of maturation (data not shown) and stayed constant until the end of flour maturation (point 3). Only variety Pob showed the changes in GIM values during the tested period.

3.4. Proteolytic activity determination

On the basis of previously reported results of Pérez et al. (2005), it was assumed that the time period of 180 min would be sufficient to observe the clear distinction between different gluten qualities in regard to free amino groups content.

The proteolytic activity of tested samples was in the range of 1.75–3.05 U/g flour (data not shown). The obtained values of the total proteolytic activity were significantly higher ($p < 0.05$) after

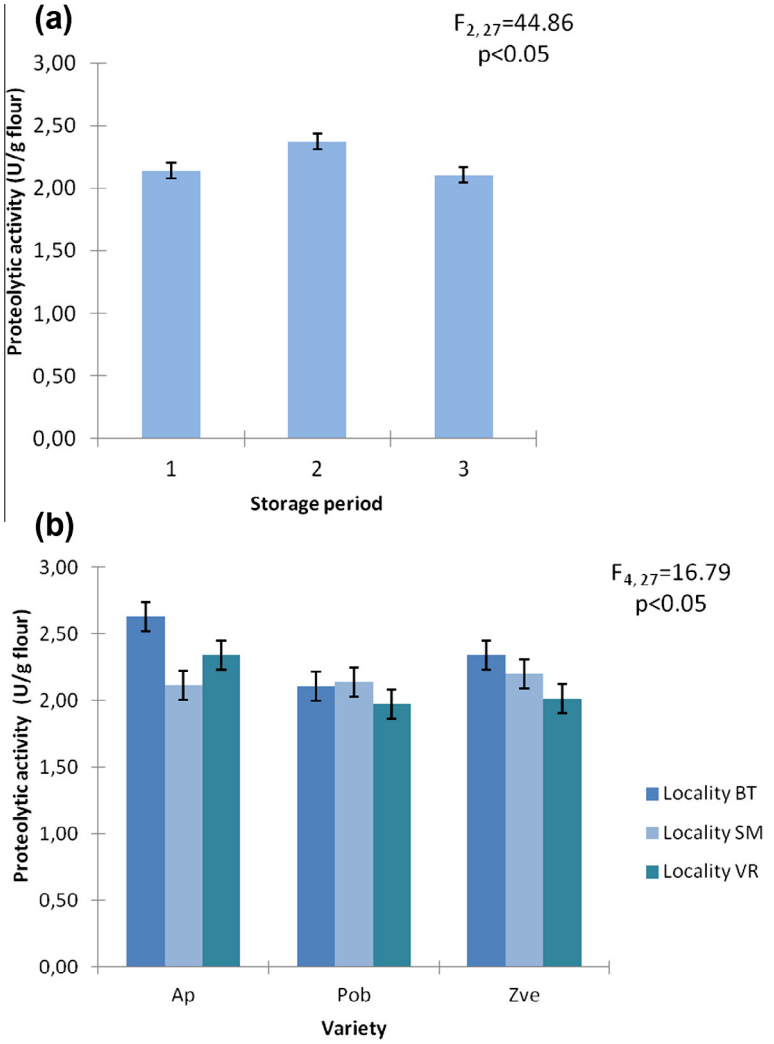

Fig. 3. Effect of the storage period (a), variety and locality (b) on the proteolytic activity values. Measured values are the mean ± 0.95 LSD intervals.

50 days of wheat maturation (point 2) than those immediately after harvest (point 1) and after 14 days of flour maturation (point 3) (Fig. 3a). In the period of storage from point 1 to point 2 the samples were stored in the grain form, while from point 2 the storage continued in the form of flour, which due to the removed aleurone layer exhibited a significantly reduced level of acting enzymes. At the end of the second phase of storage (from point 2 to point 3), a decreasing trend of the overall proteolytic activity was observed compared to their initial values (Fig. 3a). Fig. 3b shows the proteolytic activity depending on varieties and the areas from which they originated. No significant differences between the proteolytic activity of varieties in the area of SM were observed. On the other hand, the Ap variety in the areas of BT and VR exhibited the highest total proteolytic activity. Regarding the values of free amino groups content, gluten index and proteolytic activity (Figs. 1c, 2b, and 3b), variety Pob manifested very small differences in relation to the origin of the samples, while variety Ap showed statistically significant difference between the values of the same indicators depending on the area. Those findings imply that the varieties had a bigger influence on the values of the determined parameters than the climate conditions.

3.5. State of the protein complex

The relative amounts of the analysed glutenins and gliadins fractions at the beginning and in the end of wheat and flour maturation (from point 1 to point 3) were statistically significantly different ($p < 0.05$). During the tested period, a clear trend in the relative amount changes was not observed in both cases (glutenins and gliadins). The exceptions were Glu 40–80, Glu 80–120 and Gli > 120 kDa fractions (Supplementary Figs. 1 and 2). Thus, it can be presumed that from the moment of the wheat harvest until the moment of flour stabilization, changes on the macromolecular level of the protein complex structure took place (Supplementary Figs. 1a,b and 2a,b). Hence, glutenins showed greater stability than gliadins in respect to the total amount of fractions that participated in macromolecular redistribution. This also indicates that proteolytic enzymes were more susceptive to gliadins than high molecular weight glutenins, which is in agreement with Rosell, Aja, Bean, and Lookhart (2002). During flour maturation, the proteolytic activity decreases as a result of flour aeration. Namely, specific sulfhydryl blockings and oxidising agents such as oxygen exhibits an inhibitory effect on the sulfhydryl nature of the proteolytic

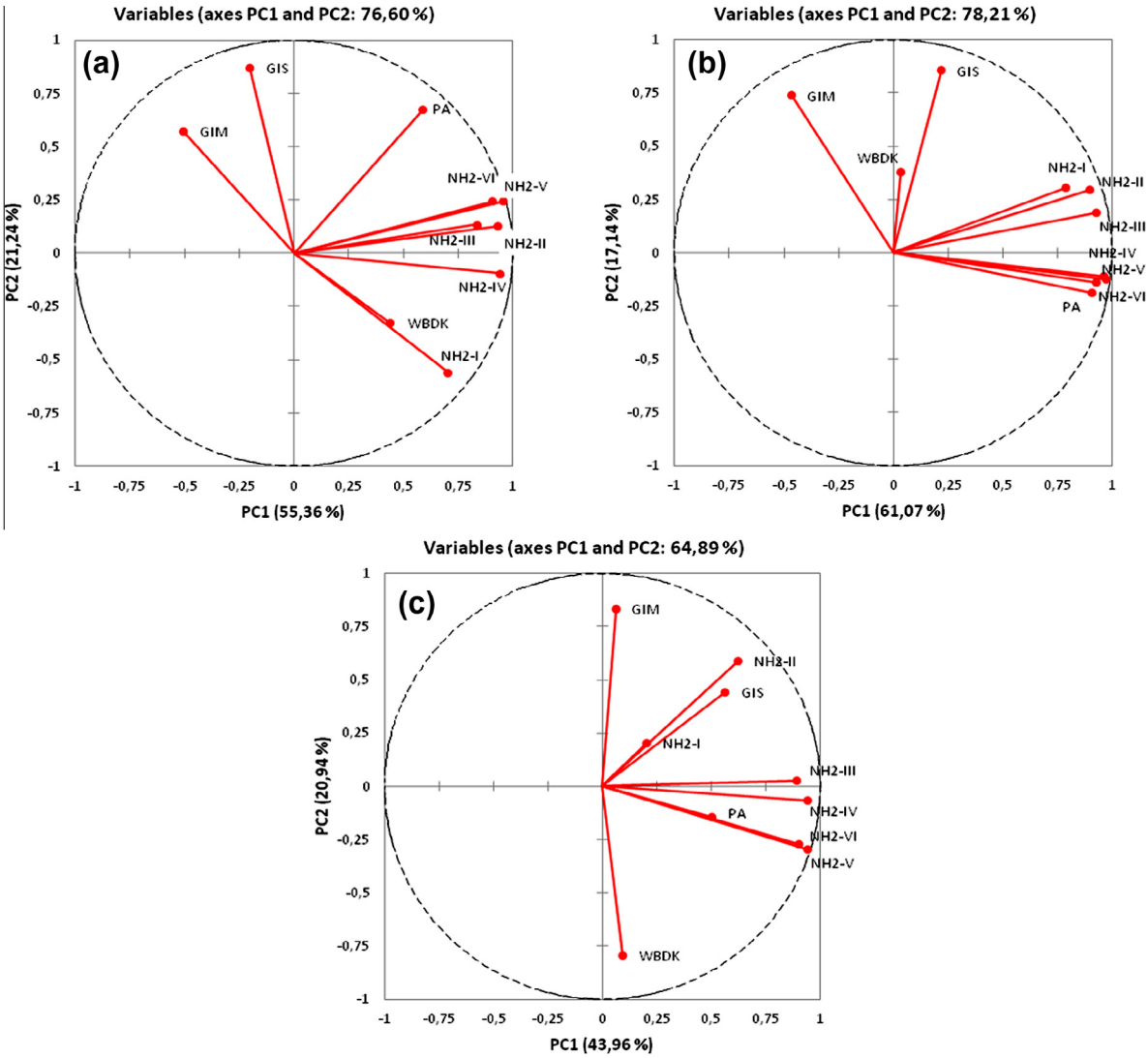

Fig. 4. Principal component loading plots for (a) flours from freshly harvested wheat, (b) flours obtained after 50 days of wheat maturation, and (c) flours after 14 days of its maturation for quality indicators: NH2/I–VI – free amino groups content at different time and temperature of gluten incubation (μg/mg); GIS – standard gluten index; GIM – modified gluten index at 37 °C; PA – proteolytic activity (U/g flour) and WBDK – wheat-bug damaged kernels (%).

enzymes of wheat grain (Skupin & Warchalewski, 1971), so it is possible that the final redistribution and polymerisation of the protein macromolecules occurred in this phase.

3.6. Principal Component Analysis (PCA) of flour quality data and content of free amino groups during wheat postharvest and flour maturation

Statistical analysis of obtained data included all the tested flour samples and the protein quality indicator values. In order to get an overview of the progress of the biochemical changes in proteins during wheat postharvest and flour maturation a Principal Component Analysis (PCA) was performed. The PCA was applied to visualise the relationships between all the measured variables and to present the results in plots that can be used for simple interpretation. The loading plot shows a projection of the variables in the factors space. When two variables are far from the center (close to the circle line), then, if they are close to each other, they are significantly positively correlated (r close to +1); if they are orthogonal, they are not correlated (r close to 0); if they are on the opposite side of the center, then they are significantly negatively correlated (r close to −1). From the resulting dependences, we have chosen to observe only the most important.

PCA was separately performed for period of postharvest wheat maturation and after-milling flour maturation. Fig. 4a shows PCA loading plot for freshly harvested wheat (point 1). The first two components explained 69.66% of the total variance in the biochemical indicators of protein properties determined immediately after wheat harvest (point 1). The first PC1 (50.34%) was related to the free amino groups content of wet gluten determined following the patterns listed in Section 2.3, with PA and with the content of wheat-bug damaged kernels (WBDK) (Fig. 4a). Karababa and Ozan (1998) and Hariri, Williams, and El-Haramein (2000) reported that wheat samples which have more than 5% bug damaged kernels showed significantly low-quality properties. Even though the content of infested kernels in the tested samples of freshly harvested wheat was less than 2%, it partly affected the increase of the content of free amino groups in wet gluten (treatment NH2-I) ($r = 0.678$, $p < 0.05$). The greatest impact of WBDK (1.32%) on the content of free amino groups (NH2-I, 0.10 μg/mg) was observed for variety Zve from the VR area (data not shown). The content of free amino groups determined after 90 min of gluten incubation at 30 °C and further prolongation for 180 min at 37 °C (NH2-V) was significantly correlated with proteolytic activity ($r = 0.74$, $p < 0.05$).

Fig. 4b shows the PCA loading plot for matured wheat (point 2). The first two components explained 86.51% of the total variance in the biochemical indicators of protein properties determined from flour obtained after 50 days of wheat maturation. The first PC1 which explained the most variance (67.84%) reflected the content of free amino groups determined following the treatments presented in Section 2.3 in addition to PA. The second component was closely related to GIM and GIS (Fig. 4b (loading plot)). The processes that occurred during wheat postharvest maturation affected the enhancement of correlation coefficients between the content of free amino groups and proteolytic activity (Fig. 4a and b). This is reflected in Fig. 4a and b by closely positioning the variables vectors. The content of free amino groups determined following the treatments that included previous gluten incubation (NH2-II, NH2-III, NH2-IV, NH2-V and NH2-VI) was significantly correlated with the proteolytic activity ($r = 0.73$, 0.81, 0.75, 0.91 and 0.95, respectively) ($p < 0.05$). On the basis of obtained results, it might be assumed that the determination of free amino groups as an indicator of the damage of the protein primary structure due to the proteolytic enzymes present, can be determined by selecting NH2-V and/or NH2-VI treatments ($r = 0.908$ and $r = 0.950$, respectively) which Fig. 4b clearly shows.

Fig. 4c shows the PCA loading plot for matured flour (point 3). The first two PCs explained 67.19% of the total variance in the biochemical indicators of protein properties determined from flour that was stored for 14 days as part of the maturation process. The first PC1 (46.97%) was associated with the content of the free amino groups determined following NH2-III, NH2-IV, NH2-V and NH2-VI treatments as well as with GIS. The difference in the correlation coefficients between the content of free amino groups (NH2-III, NH2-IV, NH2-V, NH2-VI) and proteolytic activity before ($r = 0.75–0.95$, $p < 0.05$) and after flour maturation process ($r = 0.17–0.53$, $p > 0.05$) could be explained by the decrease in the total proteolytic activity of the tested samples after flour maturation (Fig. 4c).

4. Conclusions

During the wheat and flour maturation the content of free amino groups increased, but GIS, GIM and proteolytic activity increased only during the wheat maturation. According to those findings it could be assumed that the increase in free amino groups content during the 50 days of wheat maturation is a consequence of increased proteolytic activity. After that period, the proteolytic activity decreased. Despite the increase in the content of free amino groups during flour maturation, the GIS values were stabile because gliadins were more susceptible to proteolytic enzymes as revealed using electrophoresis. During the flour maturation process of 14 days the proteolytic activity decreased, and the increase in the free amino groups content in the same period could be explained by changes on a macromolecular level of the wheat flour protein complex structure.

The analytical method, incubation time and temperature treatments applied for the determination of the content of free amino groups indicated an increase in free amino groups content with increasing the incubation temperature and time. The highest content was determined at 37 °C by V and VI sample treatments, suggesting that the damage of the proteins' primary structures, due to proteolytic enzymes activity, could be obtained by applying the treatments NH2-V and/or NH2-VI.

The present study generated knowledge on the changes of the free amino groups content during the chosen period of wheat and flour maturation. However, in order to obtain more reliable data further examinations should be conducted covering a wider range of wheat varieties from different production years.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.05.054.

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