

EFFECT OF NATIVE ENZYMES ON THE QUALITY OF LIPIDS IN EXPERIMENTALLY-STORED RAPESEED

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Activity and immunoreactivity of lipase, activity of lipoxygenase, acid and peroxide values were determined during the storage of rapeseed with 6, 10, 14, and 18% moisture content. The most intensive changes in lipase activity were observed during storage of rapeseed with 10% moisture content and ranged from 18.73 to 55.85 A.U./mg protein as compared to stored rapeseed with 6, 14, and 18% moisture content. During the storage there were also observed changes in lipase immunoreactivity, lipoxygenase activity, acid and peroxide values, however none of the relationships between enzymes and quality of rapeseed lipids was found to be statistically significant and therefore it can be concluded that the processes taking place during storage of rapeseed with varied moisture content show complexity and the enzymes examined in the experiment can appear to be not only factors affecting the quality of the lipid fraction.

INTRODUCTION

Rapeseed is considered as an important source of edible vegetable oil, in which nonpolar lipids account for 96% of total lipids with a predominant fraction of triglycerides (92%) and much less abundant fractions of diglycerides and monoglycerides [Zadernowski & Sosulski, 1979].

Lipase (triacylglycerol hydrolase EC 3.1.1.3) and lipoxygenase (linoleate oxidoreductase EC 1.13.11.12) are the enzymes participating in reactions of biochemical transformations of lipids and thus their activities can affect the quality of raw materials and foodstuffs. At first, lipase catalyses the hydrolysis of triacylglycerols to fatty acids and next free polyenoic fatty acids containing *cis,cis*-1,4 pentadiene structure are oxidized by lipoxygenase. Although lipoxygenase can oxygenate storage triacylglycerols, it is known that free polyenoic fatty acids are preferred substrates [Gardner, 1995]. As a result of lipoxygenase activity hydroperoxide derivatives are generated which may undergo further rearrangements, be degraded to secondary products and react with other compounds, thus leading to the production of off-flavours, fat deterioration and lowering the nutritional value of foodstuffs.

Lipases are the enzymes which play an essential role in early seedlings growth of oil seeds, when the reserve triacylglycerols in the storage tissues are intensively mobilised and hydrolysed to fatty acids. Lipase activity generally is not detected in dry seeds, however it may also be active, to some extent only, in resting seeds at appropriate humidity conditions, raised temperature, and in damaged seeds. The production of polyclonal antibodies raised against castor bean lipase [Hills & Beevers, 1987] as well against pancreatic lipase [Bel-

guith *et al.*, 2001] and their use to detect enzyme presence in seeds have been reported. Rapeseed lipase activities have been found in both lipid bodies and microsomes and are characterised by varied biochemical properties regarding pH optimum, kinetics and substrate specificity depending on fatty acids occurring in triacylglycerol [Hills & Murphy, 1988]. The microsomal lipase activity comprises 75-80% of the total extracted [Hills & Murphy, 1988].

As far as rapeseed lipoxygenase is concerned, its activity has not been detected in a number of experiments [Angelo *et al.*, 1979; Fauconnier *et al.*, 1995], however there are literature data which report on the activity of lipoxygenase in rapeseed. Khalyafa *et al.* [1990] have purified and characterised canola seed lipoxygenase, and Meshedani *et al.* [1990a,b] purified two lipoxygenase isozymes from rapeseed and examined the possibilities of their inactivation.

The aim of this work was to determine the effect of lipase and lipoxygenase on the quality of lipids (expressed as acidic and peroxide values) during the storage of rapeseed with 6, 10, 14, and 18% moisture content.

MATERIALS AND METHODS

Materials

Seeds of rape var. Bolko were obtained from Olsztyn Seed Central – OLZNASCN, Olsztyn, Poland.

Storage

The moisture content of rapeseed was adjusted to 6, 10, 14 and 18% overnight according to the method of Meshedani *et al.* [1990a, b]. The seeds with 6 and 10% moisture con-

tent were stored for 56 days and those with moisture content of 14 and 18% for 21 days at the temperature 0-4°C. The samples were harvested at regular intervals in order to determine the activity and immunoreactivity of lipase, lipoxygenase activity, and quality of an extracted lipid fraction.

Extraction of enzymes

Lipases

Lipase extraction was carried out according to the method of Lin & Huang [1983]. Ten mL of extraction solution (0.05 mol/L TRISHCl, pH 7.5 containing 1 mmol/L EDTA, 10 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L DTT, 0.15 mol/L TRICINE) were added to two grams of material and then the mixture was homogenized on Ultraturax T25 (IKA Labortechnik) for 1.5 min at 24,000 rpm and temperature of 0-4°C, and centrifuged (10,081 ×g for 20 min). The obtained supernatant was used to determine lipase activity.

Lipoxygenases

Lipoxygenase was extracted with 0.05 mol/L phosphate buffer, pH 7.0, at a ratio of 1:5 (w/v). The material suspended in the phosphate buffer was homogenized using an MPW120 Homogenizer for 5 min at 5,000 rpm and temperature of 0-4°C. The extraction was carried out for 1 h with mixing every 10 min at a temperature of 0-4°C. Then, the mixture was centrifuged (24,000 ×g) for 20 min. The activity of lipoxygenase was measured in the obtained supernatant.

Protein content determination

Protein in extracts was determined with the Bradford's method [Bradford, 1976]. Bovine serum albumin (BSA) was used as a standard protein.

Enzymatic activity determination

Lipase activity

The activity of lipase was determined by the diffusion method in solidified agar according to Lawrence *et al.* [1967] using 1,2,3-tributylglycerol (SIGMA) as a substrate. The substrate was prepared by adding the emulsion of tributylglycerol in 0.1 mol/L TRISmaleate buffer, pH 8.5, to a hot solution of agar. One mL of the agar mixture was spread on a microscopic slide. A well of 5 mm in diameter in the solidified agar mixture was bored and 5 μL of the enzymatic extract was added. After incubation at 30°C for 16 h the diameter of the zone of clearing was measured. A logarithmic increase in the clearing zone by one unit was accepted as lipolytic activity unit (A.U.) according to Lawrence *et al.* [1967]. Results were given in conversion to the specific activity (A.U./mg protein).

Lipoxygenase activity

Determination of lipoxygenase activity was proceeded by the preparations of substrate and reaction mixture emulsion. Linoleic acid emulsion (substrate) was prepared according to the Surrey's method [Surrey, 1964] with modification by Shiiba *et al.* [1991] in nitrogen atmosphere from: 0.1 mL of *cis,cis*-9,12-octadecadienoic acid, 2.5 mL of 0.05 mol/L

acetate buffer (pH 7.0), 0.12 mL of Tween 20 and 0.32 mL of 1.0 mol/L solution of NaOH. The volume of the prepared emulsion was filled up to 50 mL with 0.05 mol/L of acetate buffer.

The reaction mixture contained 2.5 mL of reaction buffer, 0.09 mL of substrate emulsion and the addition of the enzymatic extract. Lipoxygenase activity was determined according to the Zimmerman & Vick's spectrophotometric method [Zimmerman & Vick, 1970] at a wavelength of 234 nm at 25°C. The amount of enzyme which caused an increase in absorption by one unit within one minute was accepted as one activity unit of lipoxygenase. Results were given in conversion to the specific activity (A.U./mg protein).

Lipase immunoreactivity

Immunogen

Lipase for the production of polyclonal antibodies against rapeseed native lipase was purified from seedlings of rapeseed of low erucic Bolko variety as previously described [Grabska *et al.*, 1997].

Antibodies production

Antibodies were produced by three rabbits. Immunogen prepared for the first immunization contained 0.5 mL of an antigen solution in 0.9% sodium chloride (1.0 mg/mL) emulsified with an equivalent volume of Freud's complete adjuvant. Next 3 immunizations were made at monthly intervals in the presence of Freud's incomplete adjuvant, and the last two immunizations were without adjuvant with the same volumes and concentrations of antigen as described previously. All immunization injections were given subcutaneously and intramuscularly in quadruple. The production of antibodies and an increase in their titre were controlled using the indirect ELISA method by taking blood samples from the marginal vein of rabbit 2-3 days prior to the subsequent scheduled immunization.

Ten days after the last immunization the rabbits were exsanguinated. Blood was incubated for 1 h at 30°C and then centrifuged at 1500 ×g for 20 min. Thus obtained serum IgG antibodies were used to determine the immunoreactivity of lipase during storage of rapeseed with different moisture content.

ELISA method

Competitive ELISA was used to determine the immunoreactivity of lipase during storage. The microtitre plate was coated with 100 μL/well of pure antigen, and incubated for 12-18 h at 4°C. The plate was then washed four times with 10 mmol/L phosphate buffer of pH 7.4, containing 0.5% Tween 20. This washing system was used after each analytical step. Residual free binding sites were blocked with 150 μL/well of 1.5% gelatine in coating buffer for 30 min at 25°C. Next, the solution of rabbit antibodies (50 μL/well) and the sample examined (50 μL/well) were added at the same time to the antigen-coated and gelatine blocked well. The plate was then incubated for 1 h at 37°C. After washing, the plate was incubated for 1 h at 37°C with 100 μL/well of peroxidase-conjugated goat anti-rabbit immunoglobulin, followed by washing and addi-

tion of *o*-phenylene-diamine dihydrochloride in 9 mmol/L citrate buffer of pH 5.0. After incubation for 30 min, 100 μ L/well of 4 mol/L sulphuric acid was added to stop the reaction. Absorbance was read at 492 nm on an automatic plate reader. The results were calculated statistically by the use of Immunofit™ EIA/RIA. The lipase immunoreactivity was calculated from the standard curve and expressed in μ g/mL. The results were expressed as mean value and standard deviation.

Determination of extracted lipids quality

Lipids extraction

The lyophilised material was ground and then extracted with petroleum ether at the ratio of 1:5 (w/v) by shaking for 3 h. The solvent was removed using a rotary evaporator and so obtained lipid samples were subjected to further analyses.

Acid value

Acid value was determined according to the Polish Standard [PN 74/R-66165], and expressed in mg KOH/g lipids.

Peroxide value

Peroxide value was determined according to the Polish Standard [PN 84/A-86918] and expressed in miliequivalents O₂/kg lipids.

Statistical analysis

Each measurement was performed in triplicate and the experiment was repeated twice, and since the results of these

experiments are similar, the results of only one experiment are shown in this manuscript. The results were presented as mean value and standard deviation. Differences among the mean values were tested using the Least Significant Difference (LSD). Values were considered significant at $p < 0.05$.

General linear model procedure was used to analyse correlations between enzymes and lipid fraction quality.

RESULTS

In the experiment, the activity and immunoreactivity of lipase, lipoxygenase activity as well acid and peroxide values were determined in stored rapeseed with 6, 10, 14 and 18% moisture content. It was found that lipase activity ranged from 16.40 ± 0.500 to 23.20 ± 1.743 A.U./mg protein, from 18.73 ± 1.570 to 55.85 ± 0.790 A.U./mg protein, from 18.02 ± 0.190 to 23.78 ± 1.524 A.U./mg protein and from 19.72 ± 0.746 to 21.66 ± 0.460 A.U./mg protein during storage of rapeseed with respectively 6, 10, 14, 18% moisture content (Figure 1). During the storage, there were observed changes in lipase immunoreactivity (Figures 1), however the highest activity of lipase did not correspond with the highest enzyme immunoreactivity, thus while comparing the changes of lipase activity and enzyme immunoreactivity it was found that they were characterised by a negative relationship during the storage of rapeseed with 6 and 10% moisture content and by a positive relationship during the storage of rapeseed with 14 and 18% moisture content, however none of these relationships was found to be statistically significant.

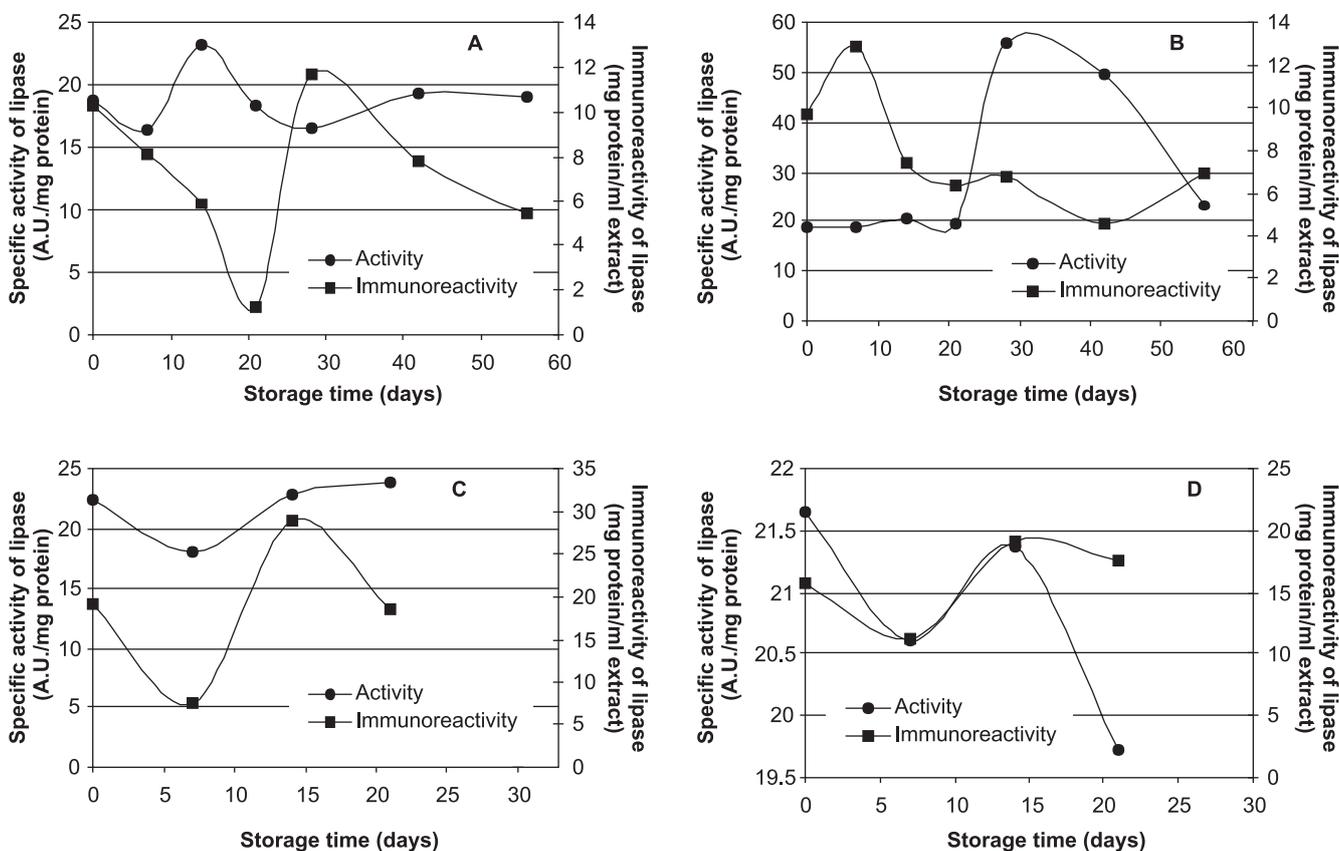


FIGURE 1. Changes in activity and immunoreactivity of lipase during the storage of rapeseed with 6%(A), 10%(B), 14%(C) and 18%(D) moisture contents.

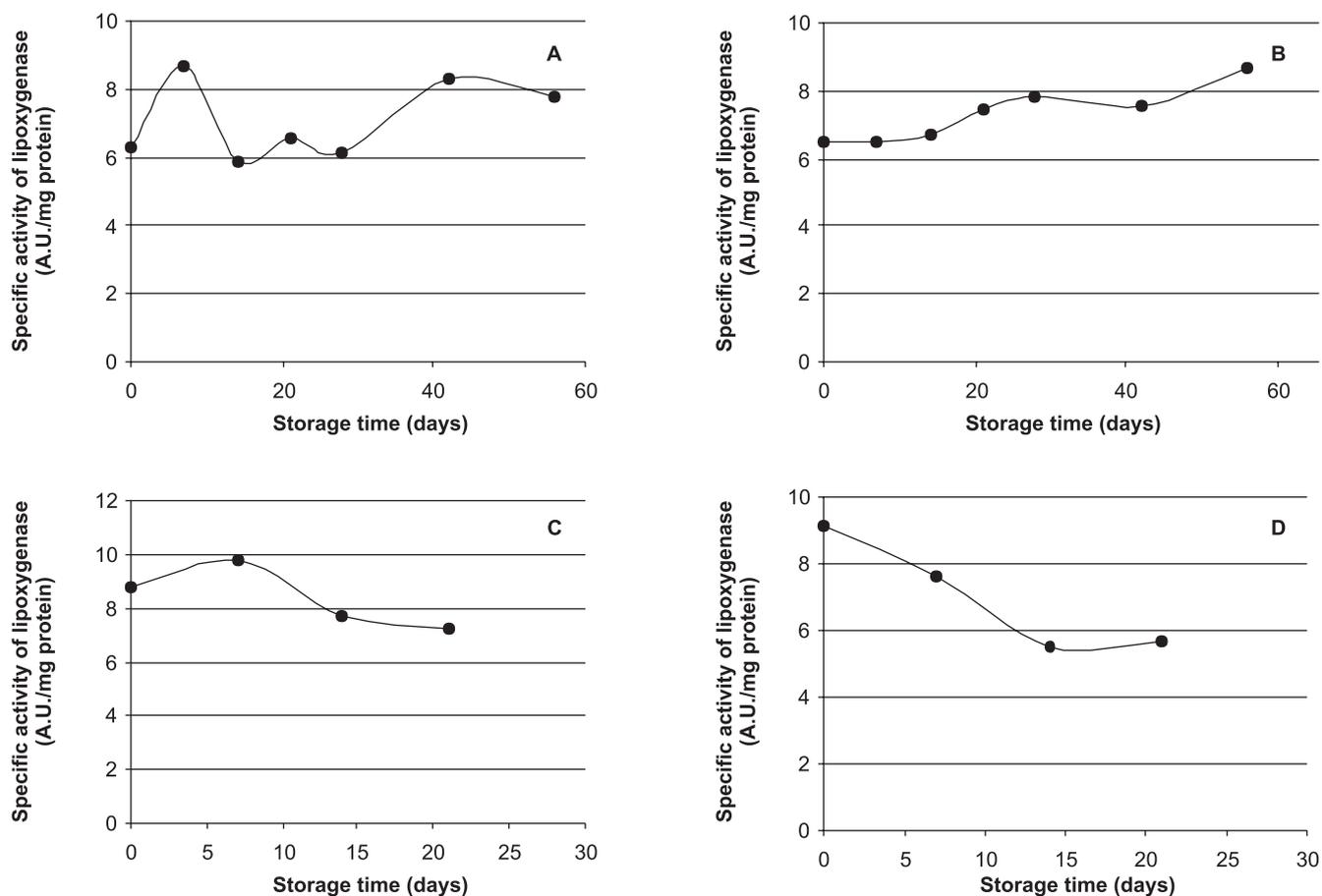


FIGURE 2. Changes in lipoxygenase specific activity during the storage of rapeseed with 6%(A), 10%(B), 14%(C) and 18%(D) moisture contents.

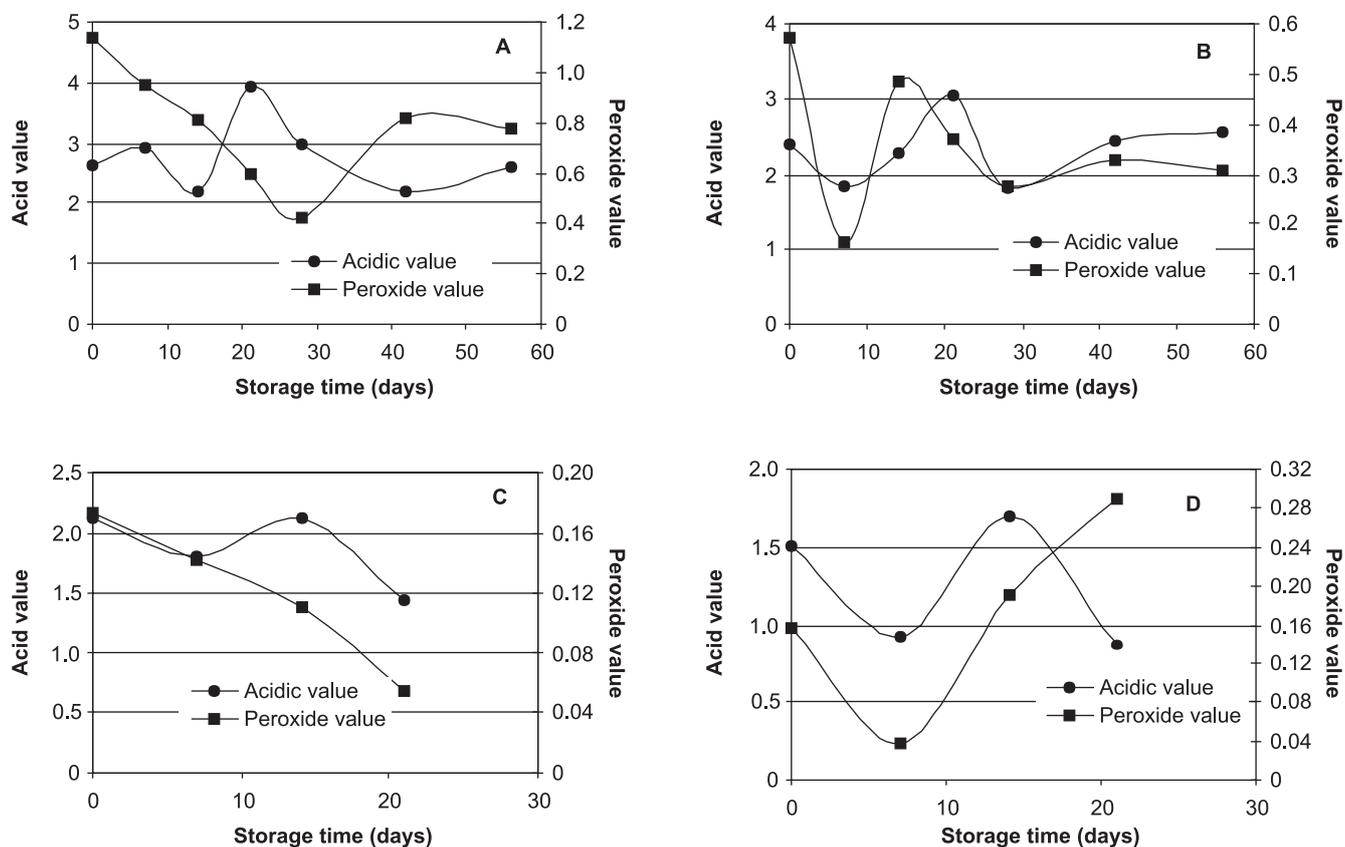


FIGURE 3. Changes in acid and peroxide values during the storage of rapeseed with 6%(A), 10%(B), 14%(C) and 18%(D) moisture contents.

TABLE 1. Correlation coefficient between activity and immunoreactivity of lipase, lipoxigenase activity, acid and peroxide values during the storage of rapeseed with 6%, 10%, 14% and 18% moisture content.

Cor-relation objects	Lipase activity				Lipase immunoreactivity				Lipoxigenase activity				Acid value				Peroxide value			
	6%	10%	14%	18%	6%	10%	14%	18%	6%	10%	14%	18%	6%	10%	14%	18%	6%	10%	14%	18%
Lipase activity	1.00	1.00	1.00	1.00	-0.336	-0.558	0.783	0.080	-0.371	0.409	-0.922	0.521	-0.534	-0.372	-0.082	0.878	0.203	-0.298	-0.498	-0.386
Lipase immunoreactivity	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-0.038	-0.626	-0.735	-0.584	-0.450	-0.476	0.412	0.529	0.146	-0.194	-0.228	0.836
Lipoxigenase activity	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-0.140	0.260	0.306	0.095	0.266	-0.317	0.774	-0.568
Acid value	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Peroxide value	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

The observed changes in lipoxigenase activity ranged from 5.90 ± 0.078 to 8.67 ± 0.321 A.U./mg protein, from 6.51 ± 0.261 to 8.68 ± 0.506 A.U./mg protein, from 7.24 ± 0.296 to 9.77 ± 0.523 A.U./mg protein and from 5.55 ± 0.210 to 9.15 ± 0.365 A.U./mg protein during storage of rapeseed with respectively 6, 10, 14, 18% moisture content (Figure 2).

During the storage, intensive changes were observed in acid and peroxide values (Figure 3). It has been found that both acid and peroxide values of the extracted lipid fraction of stored rapeseed show the decreasing tendency with an increasing moisture content. It has been found that the activity and immunoreactivity of lipase as well lipoxigenase activity do not exert any statistically significant effect on lipids quality expressed as acid and peroxide values (Table 1).

DISCUSSION

The quality of lipids in raw materials during storage is dependent on a number of factors and moisture content appears to be one out of those of high importance. The optimal moisture content for stored rapeseed is approx. 5-7%. Higher moisture content can affect adversely the activity of native enzymes present in raw materials and foodstuffs, which in turn induces unfavourable biochemical transformations of different native compounds.

In our experiment, it has been assumed that varied moisture content can change the environment of lipase and lipoxigenase and affect their activities towards native lipids occurring in rapeseed to different extent, and thus the effect of these enzymes on the quality of stored raw material can be determined.

As it has been found in a number of studies the medium conditions can affect the lipase activity to a high extent [Watanabe *et al.*, 2004; James *et al.*, 2003; Secundo *et al.*, 2004; Lima *et al.*, 2004; Yang *et al.*, 2003]. Physical environment has been proven to affect rapeseed lipase to a high extent and the rate of its inactivation, which is reported to be higher in aqueous environment than in the seed matrix [Ponne *et al.*, 1996]. Lipase has been found to be active in organic solvents and addition of water to the medium can induce conformational flexibility of lipase, change substrate recognition and enzyme enantioselectivity of lipase and thus affect the formation of substrate-enzyme complexes and reaction rate [Watanabe *et al.*, 2004]. The lack of a statistically significant correlation between lipase activity and its immunoreactivity, observed in our experiment, may result from the presence of two different isozymes in rapeseed and the cross-reactivity of lipase of the microsomal fraction with antibodies raised against lipid bodies lipase or the medium conditions may induce changes in lipase structure. It can be supposed that the varied moisture content in seed may contribute to intensive biochemical transformation within the epitope regions, thus can change the enzyme immunoreactivity. Moreover better relationships between activity and immunoreactivity of lipase in rapeseed with higher moisture content could be contributed to the fact that the anti-lipase antibodies applied in the study were raised against purified lipase of rapeseed seedlings which are characterised by a higher moisture con-

tent as compared to dry seeds. It seems likely to prove the hypothesis that moisture content may affect lipase structure and thus its activity and immunoreactivity.

It is accepted that preferable substrates for lipoxygenase are free unsaturated fatty acids, which can be released due to lipase activity [Gardner, 1995], so it can be assumed that there should be some correlation between the enzymes examined. No correlation between the enzymes found in our studies can be contributed to the presence of two different forms of lipase in rapeseed and further to some possible changes in their specific release of the substrate for lipoxygenase enzyme as well as the oleic and erucic acids which are considered to show an inhibitory effect on lipoxygenase activity [Rodriguez-Rosales *et al.*, 1998; Angelo *et al.*, 1979] and thus inhibit the lipoxygenase activity. Also the hydroperoxide products of lipoxygenase reaction can be prone to the activity of other enzymes present in the seed matrix, which can be activated to different extent due to differentiated moisture content and thus change their structure so they cannot be determined by the peroxide value assay.

CONCLUSIONS

The quality of lipids is considered to be highly dependent on the native enzymes activity. In our study no statistically significant correlation has been found between the activity of enzymes and the quality of extracted lipids of stored rapeseed expressed as acid and peroxide values. It can be concluded that the processes taking place during storage of rapeseed with varied moisture content show complexity and the enzymes examined in the experiment can appear to be not only factors affecting the quality of the lipid fraction.

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