## ORIGINAL PAPER

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# Wheat endosperm hardness. Part II. Relationships to content and composition of flour lipids

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Abstract In Part I we analysed hardness and colour of wheat endosperm and stated that these features are quite well correlated among kernels of individual varieties. In order to enhance knowledge of the biochemical basis of endosperm hardness, this study aimed at determining how the content and the composition of free and starch lipids influence it. Wheat samples (used previously in Part I) were milled in a way that reduced the number of nonendosperm particles in flour. Simple linear correlation coefficients between endosperm hardness and its lipid composition indicated that hardness was positively correlated with the content of free glycolipids  $(r=0.82)$  and negatively with the content of surface lipids of starch, especially with their non-polar fraction  $(r=0.83)$ . The typical feature of harder wheat varieties was a substantially higher content of oleic acid in lipids of the starch surface.

Keywords Wheat · Endosperm hardness · Free lipids · Starch lipids

Abbreviations -NL: Non-polar lipid  $\cdot$  -GL: Glycolipid  $\cdot$  $-PhL$ : Phospholipid  $\cdot$  -FNL: Free non-polar lipid  $\cdot$  $-FGL:$  Free glycolipid  $\cdot$  -*FPhL*: Free phospholipid  $\cdot$ -STPhL: Starch total phospholipid · -SSL: Starch surface lipid · -SSPL: Starch surface polar lipid · -SSNL: Starch surface non-polar lipid  $\cdot$  -TLC: Thin layer chromatography · -TAG: Triacylglycerols · -PN: Polish Standard

## **Introduction**

Studies into biochemical determinants of the structure of wheat endosperm, reviewed by Turnbull and Rahman [1], have linked hardness with the occurrence of low-molecular-weight proteins, referred to as puroindolines a and b, on the surface of starch. Their synthesis in the kernel is controlled by genes located on a short arm of chromosome 5D [2]. A majority of hard wheats have a glycineto-serine mutation in puroindoline b, or they are devoid of puroindoline a [3, 4]. The presence of leucine and tryptophane, instead of proline and arginine in puroindoline b also determines the occurrence of a soft phenotype of wheat [4, 5].

Wheat lipids are also found to influence endosperm hardness but this relationship has not been fully recognised so far. Panozzo et al. [6] found that flours of extraction rate in the range 72.4–73.7% from hard grained wheats grown in Australia demonstrate higher levels of free lipids. At the same time Morrison et al. [7] stated that harder wheat lines had less free polar lipids. They discovered also that the level of free polar lipids is controlled by at least two genes (one of these genes could be allelic to Ha, or is closely linked to it) and that wheat lines lacking the short arm of chromosome 5D were much harder. In summing up these observations, it may be assumed that flours of harder wheat varieties contain more free non-polar lipids (FNLs). Their origin is worth considering. Most of the FNLs of grain and flour are represented by storage triacylglycerols (TAGs). Their sources are oil bodies, the so-called spherosomes. A majority of spherosomes are located in the aleurone layer and germ and their concentration in starchy endosperm is considerably lower [8]. Higher contents of free lipids in flour from harder wheat grains [6, 7] can be explained by its larger "contamination" with non-endosperm lipids dependent on milling conditions [9]. Forming explicit conclusions on the impact of lipids on endosperm hardness without a detailed control of the milling process is difficult. Correlation of these features should be performed only for "pure" endosperm flour that can be distinguished, for example, by ash content [10].

More accurate results are obtained in an analysis of starch lipid profile. Greenblatt et al. [11] have pointed to quantitative and qualitative diversification of lipids oc-

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curring on the starch surface of wheat grains with different hardnesses. Starch isolated from wheat with soft endosperm characteristics contains more lipids, especially polar ones. Previously, the same conclusion had been drawn by Matsoukas and Morrison [12], who demonstrated that these lipids were represented by lysophospholipids. The surface of soft wheat starch has also been demonstrated to contain substantially more puroindolines [11, 13]. Still, it is not only the artefact of starch elution with water, as was previously assumed, since Darlington et al. [14] have also found that differentiation in dryisolated starch. Interactions of these components may be facilitated by the presence of unique tryptophan-rich domains of high affinity for binding lipids in the structure of puroindolines [15]. Higher contents of glycine, leucine, and tryptophan in puroindoline b of soft wheat [4, 16] enhance the hydrophobicity of that protein, and thus may intensify lipid–protein interactions on the surface of starch.

According to Seguchi et al. [17], a change in protein content on the starch surface also proceeds spontaneously as a result of wheat flour ripening. The content of protein (especially of 22–150 kDa bands) on the starch surface increases three- to fourfold, which is accompanied by an increase in its hydrophobicity. It shows how important is giving information on the flour storage time and conditions prior to their fractionation (data often omitted).

This work is an attempt at answering what groups of free and starch lipids of wheat flour influence endosperm hardness. Until now relationships between the fatty acid characteristics of endosperm lipids and endosperm hardness have not been explored either; however, differences in their oxidisability can modify interactions between grain components.

### Materials and methods

Wheat samples. The wheat samples analysed in Part I (six Polish varieties: Zyta, Rysa, Banti, Elena, Sakwa, Mewa and one variety of Triticum durum as a hardness reference) were taken.

All wheat samples were milled in a Brabender Quadrumat Junior (Brabender OHG, Duisburg, Germany). To reduce the number of non-endosperm particles in flour, the following procedure was applied. Before milling, the grain moisture was tempered firstly to 14% (an appropriate amount of water was added, samples were shaken and allowed to equilibrate for 20 h) and wetted additionally to 14.5% half an hour before they were milled. Next, flours were sieved through 200  $\mu$ m mesh, which enabled us to obtain flours with ash contents ranging from 0.51 to 0.60% (Table 1). Extraction of free lipids and starch washing were done immediately after grain milling.

The moisture, protein and ash contents of flour were analysed according to Polish Standard PN-91/A-74010, PN-75/A-04018 and ISO-2171:1993, respectively.

Water-washed starch was prepared using the dough-ball method in an SZ-1 gluteniser (Research Institute of Bakery Industry, Bydgoszcz, Poland). Washing, after 1-h tempering of a dough-ball, was performed with the use of distilled water with a temperature of 20 °C. The collected starch suspension was passed through a 60- $\mu$ m screen (gluten remaining on screen was discarded). The filtrate was twice rinsed with pure water, centrifuged at  $2,000 \times g$  for 10 min and dried to circa 12%.

Table 1 Characteristics of flour samples

Variety	Flour yield	Flour ash	Protein content
	$(\%)$	$(\%)$	$(Nx5.7)$ (% of d.b.)
Durum Zyta Rysa Banti Elena Sakwa Mewa	$72.1^{\rm a}$ $67.6^{b}$ $69.4^{a,b}$ 66.1 <sup>b</sup> $71.1^{\rm a}$ 70.8 <sup>a</sup> $69.5^{a,b}$	0.60 <sup>a</sup> $0.52^{\rm b}$ $0.55^{\circ}$ $0.51^{\rm b}$ $0.59^{\rm a}$ $0.58^{a}$ 0.56 <sup>c</sup>	$13.12^a$ $12.96^{\rm a}$ $12.62^b$ 12.84 <sup>a,b</sup> $11.27^{\circ}$ $11.31^{\circ}$ $12.27^{\rm d}$

a<sup>-d</sup> Means with the same letter in the same column are not significantly different  $(P=0.05)$ 

Lipid analyses. The content of free lipids was analysed using the Soxhlet method with petroleum ether according to Polish Standard PN-73/R-66164. Starch surface lipids (SSLs) were extracted as described by Greenblatt et al. [11] with a propanol: water mixture (9:1 v/v) for 30 min at room temperature using a magnetic stirrer. The extract was next centrifuged at  $3,000 \times g$  for 10 min and evaporated under pressure to dryness at 40 °C. SPE prepacked columns (Bond Elut Glass-SI, 1000MG 6ML Varian) were used for lipid fractionation. Column conditioning and lipid separation were done according to Ohm and Chung [18] using a Baker vacuum manifold. The recovery of fractionated lipids was in the range 96– 98%. Total starch PhLs were also calculated by multiplying their phosphorus content (Polish Standard PN–88/A–86930) by 16.4 according to Shibanuma et al. [19].

Fatty acid composition of free and SSLs was analysed according to PN-EN-ISO-5508:1996. The conditions of separation were as follows: CHROM 5 GC with FID detector; glass column packed with GP3% SP-2310/2% SP-2300 on 100/120 Chromosorb W AW; and injection, oven and detector temperatures were 230, 210 and 250 °C, respectively. Fatty acids methyl esters were prepared according to Zadernowski and Sosulski [20] using a mixture of chloroform:methanol:sulphuric acid (90:10:1, v/v/v). Identification of each fatty acid was done by comparing with standards.

Statistics. The experimental results were analysed using Statistica 6.0 software (StatSoft, Tulsa, USA). Analysis of variance with Duncan tests was performed on the basis of triplicate measurements. Values of endosperm hardness presented in Part I were utilised for determination of correlation coefficients with composition of extraction flours. All calculations were done at a significance level of P<0.05

#### Results and discussion

#### Free lipids

The content of total free lipids was in the range of 838– 1,407 mg in 100 g of flour (Table 2). Flours from harder varieties contained more free lipids, which confirmed the results of Panozzo et al. [6]. Skarsaune et al. [21], Davis et al. [22] and Bekes et al. [23] also stated that hard varieties, especially of red appearance, are richer in this component. Non-polar fractions dominated in the lipid composition and their proportion was 70.5–78.2%. The percentage of glycolipids (GLs) and PhLs in the composition of free lipids ranged from 10.1 to 12.8 and from 9.6 to 19.2%, respectively. The high content of NLs in kernels proves their full maturity, as immature cereal grain is characterised by the prevalence of structural lipids, mainly PhLs [24]. During the final dehydration step of

Table 2 Content and composition of wheat extraction flour free lipids



 $a-e$  Means with the same letter in the same column are not significantly different (P<0.05)

Table 3 Fatty acids compositions of wheat extraction flour free lipids

Variety	Fatty acid $(\% )$						
	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Others
Durum	$16.19^a$	$0.06^{\rm a}$	$0.35^{\rm a}$	$20.22^{\rm a}$	$58.35^{\rm a}$	4.16 <sup>a</sup>	$0.67^{\rm a}$
Zyta	$16.79^{\rm a}$	0.31 <sup>a</sup>	$0.44^{\rm a}$	$12.43^{b,d}$	$64.62^b$	$4.95^{\rm a}$	$0.46^{\rm a}$
Rysa	16.08 <sup>a</sup>	$0.38^{a}$	$0.53^{\rm a}$	$13.89^{b,c}$	$63.78^{b,c}$	4.68 <sup>a</sup>	$0.66^{\rm a}$
Banti	$17.10^a$	$0.43^{\rm a}$	$0.55^{\rm a}$	$14.87^{\circ}$	$62.61^{\circ}$	$3.76^{\rm a}$	$0.68^{\rm a}$
Elena	$17.26^{\rm a}$	$0.03^a$	$0.56^{\rm a}$	12.74 <sup>b</sup>	$64.58^{b}$	4.04 <sup>a</sup>	0.79 <sup>a</sup>
Sakwa	$17.71^{\rm a}$	$0.20^a$	$0.38^{a}$	$11.81^d$	$65.05^{b}$	$4.58^{a}$	$0.27^{\rm a}$
Mewa	$17.14^a$	$0.13^a$	$0.52^{\rm a}$	12.01 <sup>d</sup>	$64.86^{b}$	$4.65^{\rm a}$	$0.69^{\rm a}$

 $^{\text{a-d}}$  Means with the same letter in the same column are not significantly different (P<0.05)

grain maturation, the lamellar biomembranes of starchy endosperm, composed partly of polar lipids, become nonlamellar, mainly hexagonal and/or cubic [25]; lipids in that shape can probably be extracted by non-polar solvents. However, the content of the non-polar fraction ranging from 70 to 78% can indicate that non-endosperm lipids were not removed completely. According to Hargin et al. [8], the non-polar fraction constitutes up to around 50% of free endosperm lipids. Another explanation is that the grain of wheat cultivated in Poland is richer in endosperm spherosomes. Hence, the final determination of the effect of endosperm lipids on endosperm hardness should be based on preparing degermed and pearled wheat endosperm samples, as was manually done by Hargin et al. [8].

The lower content of free polar lipids in harder wheat reported by Morrison et al. [7] was not confirmed. On the contrary, the experimental harder wheat demonstrated substantially higher contents of GLs at relatively equal levels of PhLs (milligrams/100 g of flour). According to Ohm and Chung [26], the effect of multiple GL fractions on endosperm hardness of HWW wheat is inverse, but a higher content of digalactosyldiglyceroles, dominant in wheat GL, corresponds to increased hardness.

An analysis of fatty acid composition of free lipids has not revealed any significant diversification in Triticum aestivum wheats (Table 3), which indicates no effect of that feature of free lipid structure on the hardness of endosperm. The fatty acids of durum wheat contained more oleic and less linoleic acid, which is typical of that wheat variety [22, 27]. Apart from genetic factors, the fatty acid composition is also determined by grain maturity (the concentrations of linolenic, palmitic and stearic acids decrease gradually in favour of linoleic acid [24]) and climatic conditions (high temperature and low rainfalls enhance synthesis of less saturated acids [22]).

#### Starch lipids

The analysis included both total starch PhLs (after sample ashing and spectrophotometric determination of phosphorus) and the content of lipids extracted with a propanol–water mixture at room temperature according to Greenblatt et al. [11]. The latter fraction is mainly composed of surface lipids, as complete extraction of starch lipids requires the application of a mixture of hot solvents [28, 29].

The total content of PhLs ranged from 689 to 853 mg/ 100 g of starch (Table 4); however, it was found to be higher in the visually mealy varieties. Higher levels of lipids have been generally associated with the B-type granules [12, 30] and also cooler ambient temperatures during the grain-filling period [12]. At the same time, it is well documented that a higher percentage of small starch granules is typical of harder wheat [30, 31, 32]. This apparent inconsistency may be explained by the fact that the starch isolated from harder wheat varieties contained slightly less total lipids and they were mostly constituted by internal lipids (measured as total starch phosphorus). A high yield of lipid extraction from starch of softer varieties points to their high percentage on the surface of starch granules or to higher extractivity with the use of a cold propanol:water mixture or to higher adsorption of free lipids of flour on the granules' surface during waterwashing of starch. The latter phenomenon is indicated by the occurrence of triglycerides in the surface of starch, which are not membrane components of amyloplasts [33].

Table 4 Content and composition of starch lipids. STPhL Starch total phospholipids, SSL starch surface lipids, PhL phospholipids,  $TL$  total lipids,PL polar lipids, NL nonpolar lipids, TAG triacylglycerols,



<sup>a–d</sup> Means with the same letter in the same column are not significantly different (*P*<0.05) <sup>e</sup> Calculated according to Shibanuma et al. [19]

Table 5 Fatty acids compositions of starch surface lipids

Variety	Fatty acid $(\%)$						
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$		
Durum Zyta Rysa Banti Elena Sakwa Mewa	49.76 <sup>a</sup> $40.77^{\rm b}$ $40.22^b$ 47.18 <sup>a</sup> $41.80^{b}$ $47.41^{\rm a}$ $46.06^{\rm a}$	$2.80^{a,d}$ $2.35^{\rm a}$ $0.99^{b,c}$ $0.26^{\rm b}$ $2.10^{a,c}$ $3.58^{d}$ $0.52^b$	$22.43^a$ 16.99 <sup>b</sup> 12.99 <sup>c</sup> $18.54^{b}$ $11.63^{\circ}$ $11.07^{\circ}$ $12.53^{\circ}$	$24.85^{\rm a}$ $39.23^{b}$ $45.08^{\circ}$ $33.51^{\rm d}$ 44.11 <sup>c</sup> 37.00 <sup>b</sup> $40.55^{b}$	$0.16^a$ $0.66^{\rm a}$ $0.72^{\rm a}$ 0.51 <sup>a</sup> $0.36^{\rm a}$ $0.94^{\rm a}$ $0.44^{\rm a}$		

<sup>a–d</sup> Means with the same letter in the same column are not significantly different  $(P<0.05)$ 

The surface lipid composition was dominated by polar lipids (74.63–89.06% of total lipids), especially by PhLs (results of a TLC analysis; not shown) that are probably amyloplast membrane remains [34]. The remaining part of that fraction was formed by NLs, mainly triglycerides. The fact that triglycerides represent a major fraction of surface lipids of wheat starch was also emphasised by Buléon et al. [34]. The fatty acid composition of starch lipids included three main acids: palmitic, linoleic and oleic (Table 5). A similar acid composition of wheat starch was reported by Morrison [35], Yasui et al. [36] and Błaszczak et al. [29]. The contribution of palmitic acid varied in the range 40.22–49.76%, irrespective of endosperm hardness. Different proportions were, however, noted for acids with a chain length of 18 carbon atoms. The most typical feature of harder varieties (durum, Zyta and Banti) was a substantially higher content of oleic

acid. A higher contribution of more oxidation-susceptible linoleic acid on the surface of starch granules of softer varieties is likely to initiate reactions with sulphur-rich puroindolines [15, 25], which may lead to the formation of new lipid–protein structures on the surface of starch.

#### Correlations with endosperm hardness

Simple linear coefficients between endosperm hardness and its lipid composition and protein content (Table 6) indicate that hardness (measured as the mean force for dominant samples) is positively correlated with the content of FGLs  $(r=0.82)$  and negatively correlated with the content of surface lipids of starch (SSLs), especially with their non-polar fraction  $(r=-0.83)$ . Simultaneously, only an increasing tendency was observed for endosperm hardness with increased protein content in flour. A similar, unequivocal relationship between wheat hardness and protein content was reported, for example, by Martin et al. [4].

To conclude, it is well known that the difference in hardness of wheat endosperm between soft and hard varieties depends on the strength of interaction between the starch granules and the protein matrix [37, 38, 39, 40]. The range of those interactions is considerably higher in harder wheats, which are rich in small starch granules. It may result from the fact that the surface area of B-granules, typical of hard endosperm and estimated at  $0.7 \text{ m}^2$ per gram of starch, is about three times higher than that for A-granules [41]. Higher contents of lipids and low-

Table 6 Simple linear correlation coefficients between endosperm indentation hardness  $(F_{\text{max}})$  and contents of selected groups of lipids and protein of extraction flour. At sample size  $n=7$  for  $P<0.05$ , critical value of correlation coefficient is 0.76. FNL Free non-polar lipids, FGL free glycolipids, FPhL free phospholipids, STPhL starch total phospholipids, SSL starch surface lipids, SSPL starch surface polar lipids, SSNL starch surface non-polar lipids



<sup>a</sup> Values of  $F_{\text{max}}$ -average and  $F_{\text{max}}$  for main fraction are presented in Part I

molecular-weight proteins on the surface of starch granules from wheat with soft endosperm characteristics may indicate their higher hydrophobicity. Nevertheless, this hypothesis should be proven in further studies. The variability of the fatty acid composition of SSLs reported in this study should also be confirmed.

Further studies should limit the possibility of endosperm lipid contamination with fractions originating from the germ and aleurone layer through, for example, kernels degerming and pearling before milling. In the case of starch lipids, starch dry-isolation or defatting preventing selective adsorption of free lipids on starch surface during its isolation is more advisable.

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