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Improved method of wheat starch isolation for friabilin analysis

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A b s t r a c t. Starch granules were isolated from two types of wheat grain: *Triticum durum* (one sample) and *Triticum aestivum* (two varieties). Prime starch was fractionated according to the method described by Wolf and by a modification to this assay, which we developed. The modification entailed changing some processing parameters for the fractionation of prime starch *ie* starch-to-water ratio and centrifugation speed. From the fractions so obtained, the crude protein content, the presence of friabilin (SDS-PAGE electrophoresis), and the share of A- and B-type starch granules as well as non-starch particles (DIA of SEM pictures) were determined. The proposed modification enabled easy separation of all fractions, which was necessary to verify the hypothesis that friabilin is bound to the surface of starch granules. It was found that the presence of friabilin on the surface of *T. durum* or *T. aestivum* starch granules is related to the method of isolation and fractionation employed; therefore, caution should be exercised when reporting a negative finding.

K e y w o r d s: wheat, hardness, starch granules, friabilin, fractionation

INTRODUCTION

Concerning cereal technology, especially in wheat processing, one of the most important properties describing technological usability is grain hardness/softness. Hardness of wheat kernels is an important factor when milling as it influences, amongst others, the yield, particle diameter, density of the prepared flour and starch damage. All of these mentioned factors are of great importance to the quality and end use properties of flours, because they affect the functional properties of resultant products. This is why the diversification/classification of wheat grain for different technological purposes is necessary (Pomeranz and Williams, 1990).

The molecular basis of wheat endosperm texture has been the subject of numerous investigations. The results have shown that the protein portion of wheat is crucial to the hardness/softness of the endosperm (Barlow *et al*., 1973; Greenwell and Schofield, 1986; Glenn and Saunders, 1990; Malouf *et al*., 1992). In numerous papers, special attention has been paid to the polypeptide friabilin (Sulaiman and Morrison, 1990; Morris *et al*., 1992; Oda *et al*., 1992; Bettge *et al*., 1995). This polypeptide, which has a molecular weight of 15-kDa, can be isolated from purified starch granules; that is, those previously isolated from soft wheat grain (Malouf *et al*., 1992; Morrison *et al*., 1992; Greenblatt *et al*., 1995). From literature, there have appeared also broader terms connected with friabilin, namely grain softness protein (GSP) (Jolly *et al*., 1993; 1996; Rahman *et al*., 1994) and starch granule protein (SGP) (Morris *et al*., 1992; Oda *et al*., 1992; Oda and Schofield, 1997). Detailed biochemical analyses of the 15-kDa fraction connected with SGP have revealed that it also contains -amylase inhibitors (Oda and Schofield, 1997). While using two-dimensional electrophoresis *ie* non-equilibrium pH gradient electrophoresis [NEPHGE] × Tricine-SDS-PAGE, Oda (1994) found four different friabilin fractions; that is, A, B, C and D, which were characterized as having identical molecular weights. Further GSP investigations have revealed that its two basic fractions are puroindoline A and B (Oda, 1994). These low-molecular weight cysteine-rich proteins were purified by Blochet *et al*. (1990) and characterized to possess hydrophobic domains (rich in tryptophan) in their structure. Giroux and Morris (1998) reported that a mutation *ie* Gly46 on Ser46 in the gene encoding of puroindoline B, or the absence of a puroindoline A gene, is manifested by

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phenotype hardness of wheat grain and thereby influences its technological quality/destination.

From research on friabilin, the crucial step has been the isolation and purification of starch granules. When comparing some of the commonly used assays (Table 1), there are evident differences in the analytical procedures (Wolf, 1964; Malouf *et al*., 1992; Bettge *et al*., 1995; Sulaiman and Morrison, 1990). Variations in the methods employed include the way of break-up, hydration, maceration, gluten, non-starch particles and starch separation techniques, drying parameters, and dehydration by acetone or lyophilisation. As a specific example, in the method proposed by Wolf (1964), filtration through a 70 μm sieve, which enables separation of prime starch (PS) from non-starch particles greater than 70 μm (F70), was applied. Whereas in the method proposed by Bettge *et al*. (1995) this step was replaced with decantation of gluten and bran particles from starch slurry. In addition, the methods proposed by Wolf (1964) and Sulaiman and Morrison (1990) include a step for the separation of a slow sedimenting fraction (SSF) from the main starch fraction (MSF) as well as fractionation of starch granules of A-type *ie* starch granules with a diameter larger than 10 μm and low level of proteins and B-type *ie* starch granules with a diameter less than 10 μm, but containing higher level of proteins. In the method proposed by Malouf *et al*. (1992) both steps mentioned above were omitted.

It is evident that many different techniques for starch preparation are employed. Thus, the following basic questions, is friabilin associated with the surface of B-type starch granules (Sulaiman and Morrison, 1990) and is friabilin instead the component of a separate fraction which possesses rheological properties similar to those of the B-type starch granule fraction, need to be addressed, as they still remain without satisfactory answers.

The present work is an attempt to demonstrate how some steps of the separation procedure can be modified/improved. Data so obtained will hopefully give some answers, or at least provide a better insight into the two questions posed above.

T a b l e 1. Differences in the methods of starch isolation

Stage	Wolf (1964)	Malouf (1992)	Bettge (1995)	Sulaiman (1990)
Grain grinding	milling	milling	crushing	crushing
Sieving	x	x	–	–
Hydration	room temp. water 1-2 h	room temp. water 10 min	room temp. 0.1M NaCl 0.5 h	4°C water 6 h
Maceration	–	–	–	x
Filtration through a 75 μm sieve	–	–	–	x
Separation of gluten and non-starch compounds by centrifugation using an 80% (w/v) solution of CsCl	–	–	–	x
Gluten washing in water stream	x	x	x	–
Separation of starch slurry and non-starch particles of gluten and brain by decantation	–	–	x	–
Filtration through a 70 μm sieve	x	–	–	–
Fractionation on MSF and SSF or A-and B-starch granule fractions	x	–	–	x
Drying at 40-45°C	x	–	–	–
Dehydration with acetone, followed by drying at 22°C	–	–	x	x
Lyophilisation	–	x	–	–

x - applied, – - not applied.

EXPERIMENTAL

Materials

Wheat grains from *Triticum aestivum* of different hardness classes were examined. These included Torka, classified by Single Kernel Characterization System (SKCS) as hard (Hardness Index [HI] of 71.3), and MIB-496 classified as soft (HI 24.2), from The Research Centre for Cultivar Testing, Słupia Wielka. *Triticum durum* (Canadian commercial), which is classified as hard (HI 92.2), was obtained from a pasta manufacturer, Danuta Limited, Gdańsk, Poland.

All reagents used were of at least analytical grade.

To avoid repetitions of full terms for the fractions so obtained and analyzed, the following abbreviations are used:

F70 – the non-starch fraction with particle sizes over 70 μm
PS – prime starch (not purified)
MSF – main starch fraction
SSF – slow sedimenting fraction
LF – lower fraction
UF – upper fraction

Isolation of prime starch (Fig. 1)

After preliminary cleaning in an air/sieving separator SZD (ZPBP, Poland), wheat grains were ground using a laboratory roll mill SZD (ZPBP, Poland) with the bran fraction collector. Bran fractions with a particle size greater than 500 μm were discarded. Prime starch (PS) was isolated from the resultant flour and purified according to the dough-ball method of Wolf (1964). Briefly, 10 g of flour and 5.5 ml of water were mixed and formed into a round piece of dough which was then hydrated in water for 20 min. Starch isolation was then performed using 200 ml of water. The starch slurry was filtered through a 70 μm sieve (NAGEMA, Germany), resulting in two fractions: non-starch particles with sizes over 70 μm remaining on the sieve (marked as F70), and a non-purified liquid fraction with PS as its main component. Fraction F70 was purified from occasionally appearing dough or gluten particles, frozen at -70°C and then lyophilized. The slurry containing PS was cooled to 3±1°C and centrifuged at 2300×g at 4°C for 10 min. The supernatant was discarded; the PS sediment *ie* the pellet, was subjected to fractionation procedures and a parallel sample was frozen at -70°C and then lyophilized.

Prime starch (PS) fractionation

Fractionation of the PS so obtained was performed by two different methods using distilled water at 3±1°C.

Method 1 (Wolf's method – Fig. 2)

From the PS sediment *ie* the pellet, the upper, loosely-packed slower sedimenting layer (SSF), which is characterised by a pale yellow-brownish colour, was carefully removed using a spatula, ensuring that there was no disturbance of the white, very compact lower layer; that is, the main starch fraction (MSF). The recovered SSF was transferred to a clean tube, frozen at -70°C and then lyophilised. The MSF was purified of SSF twice by re-suspension in 80 ml of water and centrifugation (2300×g, 4°C, 10 min), as discussed above.

Method 2 (Modified Wolf's method – Fig. 3)

The PS sediment was washed twice with distilled water (80 ml), centrifuged (2300×g, 4°C, 10 min) and the supernatant discarded. To the resultant pellet, 0.5 ml of water was added followed by mixing with a spatula to obtain a semi-liquid slurry; the contents were transferred to a 8 ml centrifuge tube (the starch slurry occupied as much as 7 ml). The samples were centrifuged at 10,000×g at 4°C for 10 min. After centrifugation, the tube containing the pellet was frozen at -70°C and then lyophilized. The test tube contents were carefully removed (the lyophilizate has a smaller diameter than the tube itself) and divided using a precision knife along the slightly marked gap between the lower (LF) and the upper fraction (UF).

The lyophilisates of the F70, PS, MSF, SSF, LF and UF fractions were carefully ground in a mortar and kept for further analyses.

Protein analyses

The crude protein contents of F70, PS, MSF, SSF, LF and UF fractions were determined by the Kjeldahl method using a KJELTEC SYSTEM supplied by Tecator, and the conversion factor, %N × 5.7.

Protein extraction was conducted with a mixture of 2-propanol and 0.1M NaCl (1:1, v/v) according to the method of Greenblatt *et al*. (1995). The samples of lyophilized fractions F70, PS, MSF, SSF, LF and UF (80 mg each) were suspended in 800 μl of the extraction mixture and extracted at room temperature for 30 min with occasional shaking. The suspension was centrifuged (10 000×g, 5 min) and the supernatant was decanted into new microtubes with the following volumes: F70 – 400 μl; UF – 600 μl; and PS, MSF, SSF, and LF – 700 μl. Proteins were precipitated with 700 μl acetone for 18 h at -20°C. After centrifugation, as described above, the supernatant was discarded and the sediment was washed using cold (-20°C) acetone. This procedure was repeated twice and the final sediment was subjected to drying under a stream of nitrogen.

SDS-PAGE electrophoresis

SDS-PAGE separation of proteins was achieved according to Laemmli (1970) and Hames (1990). The extracts were dissolved in a buffer containing 62.5 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS,

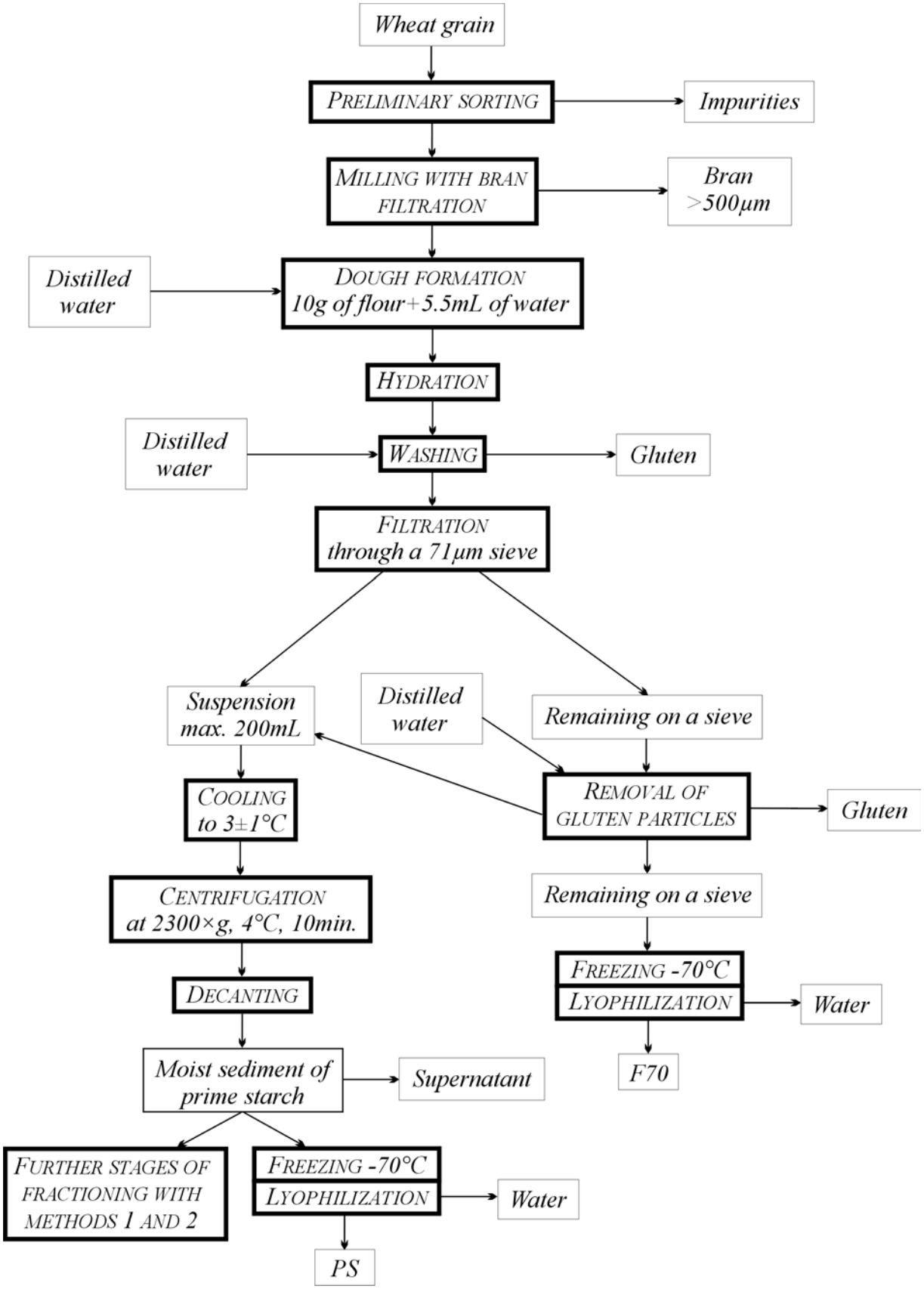

Fig. 1. Preparation of flour and isolation of prime starch.

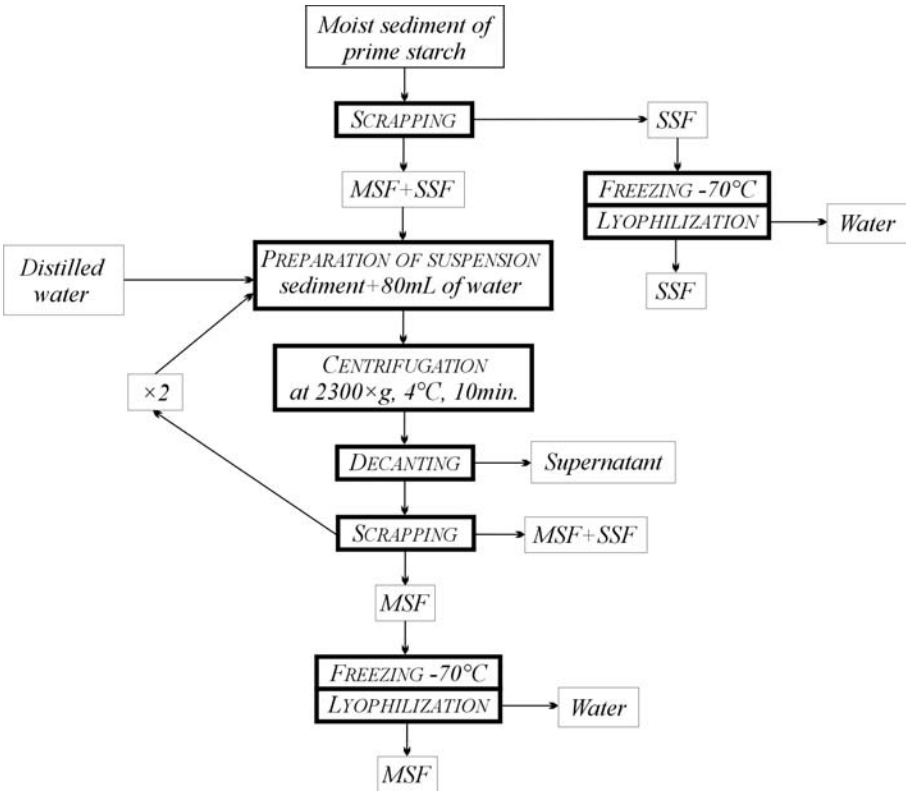

Fig. 2. Method 1 (Wolf, 1964).

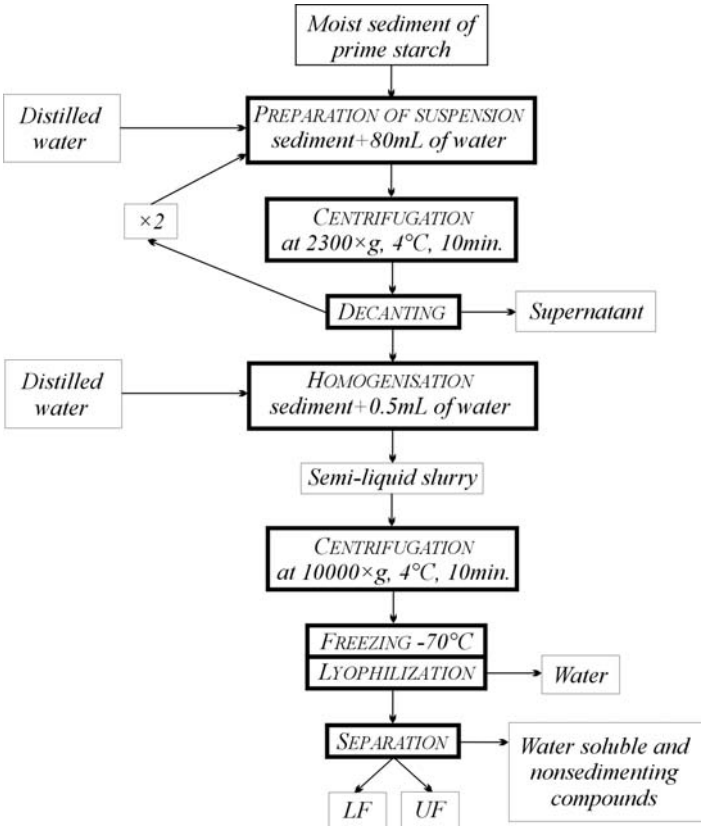

Fig. 3. Method 2 (modification of Wolf method).

0.002% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol. The quantities of reagent added to each sample were calculated based on the differences in the protein content of each fraction; that is, F70 – 0.1; PS – 1.2; GFS and LF – 4.0; SSF and UF – 0.8 mg preparation / μl of buffer. The solubilised samples (5 μl) were loaded onto polyacrylamide gels *ie* resolving gel 13.5% T/2.60% C and stacking gel 4% T/2.6% C. Electrophoresis was conducted at a constant voltage of 250 V and 15mA using a Flat Bed Apparatus (Model FBE-3000) supplied by Pharmacia. After the separation, the gel was fixed and silver stained according to Heukeshoven and Dernick (1985). Molecular-weight markers (SigmaMarker™ low range, M.W. 6 500-66 000) were obtained from the Sigma Chemical Company.

Scanning Electron Microscopy (SEM)

SEM analyses were performed using a JSM-5200 JEOL (Japan) scanning electron microscope. The samples were sprinkled on a specimen holder with Cu tape, gold coated in a vacuum evaporator *ie* JEE 400 and viewed at an accelerating voltage of 5kV at the magnification of 500× (F70) and 1000× for other fractions. Digital image analysis for particles of the analyzed fractions was performed using Olympus MicroImage 4.0 software (Sadowska *et al*., 1999).

RESULTS AND DISCUSSION

Sieving of the starch slurry through a 70 μm mesh resulted in a satisfactory separation of starch from non-starch components (F70). The crude protein content in F70 fractions obtained from the different wheat types varied from 9.20 (Torka var.) to 15.87 dm % (MIB-496 var.) (Table 2). The PS, MSF and LF fractions were characterized with a similar content of protein irrespective of the wheat types examined, while SSF and UF from durum wheat were much lower in protein.

T a b l e 2. Crude protein content in different fractions of wheat grains

	Protein content (% dry weight)		
Symbol	Durum	Torka	MIB-496
F70	12.68±0.83	9.20±1.49	15.87±0.46
PS	0.46±0.01	0.50±0.02	0.52±0.01
MSF	0.29±0.01	0.23±0.06	0.26±0.04
SSF	1.19±0.08	1.70±0.30	1.68±0.08
LF	0.32±0.01	0.30±0.01	0.33±0.03
UF	2.61±0.72	5.81±1.71	6.96±0.43

The modification to Wolf's method proposed in this work led to easy fractionation of PS into two diverse fractions, namely LF and UF; each differed distinctly in their protein content. In comparison to SSF, the crude protein content increased markedly in the UF fraction by 119, 242 and 314% for durum wheat, Torka and MIB-496, respectively (Table 2).

The results of digital image analysis of SEM micrographs for different fractions, as presented in Fig. 4, show that the starch granules of A- and B-type are still present in F70 (Fig. 5a); however, their share is only minimal as compared to PS (Fig. 5b). In the former, the predominant structures were irregularly shaped non-starch components. The fractionation of PS into MSF (Fig. 5c) and SSF (Fig. 5d), as viewed under the microscope, resulted in a clear separation of both granules fractions. In comparison to PS, the number of A-type granules in MSF was about 400% more, which was accompanied by a reduction in the number of B-type starch granules and non-starch particles by *ca*. 40 and 90%, respectively. On the other hand, the SSF was affected only very slightly by the separation procedure employed. A decrease by a mere 13% for non-starch

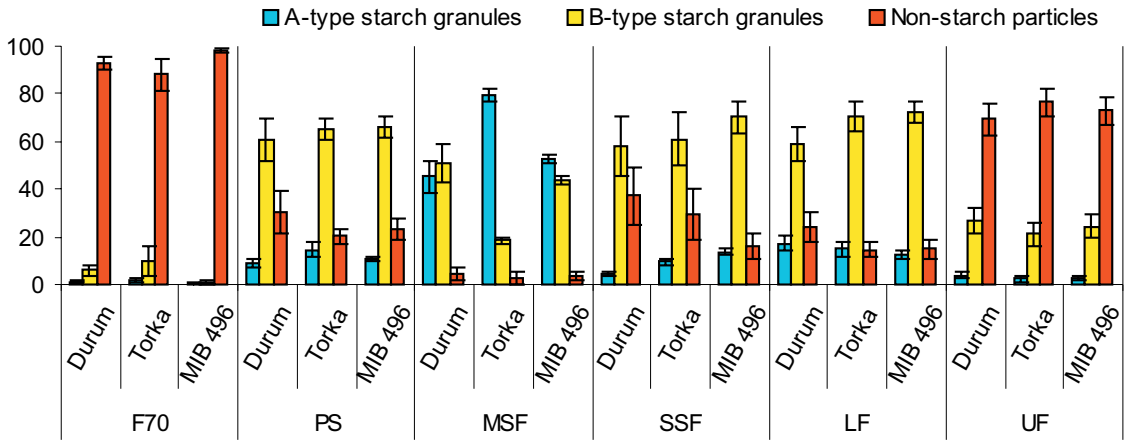

Fig. 4. Digital image analysis of SEM pictures for A- and B-type starch granules and non-starch particles (% of observed objects, average from 10 pictures analyzed).

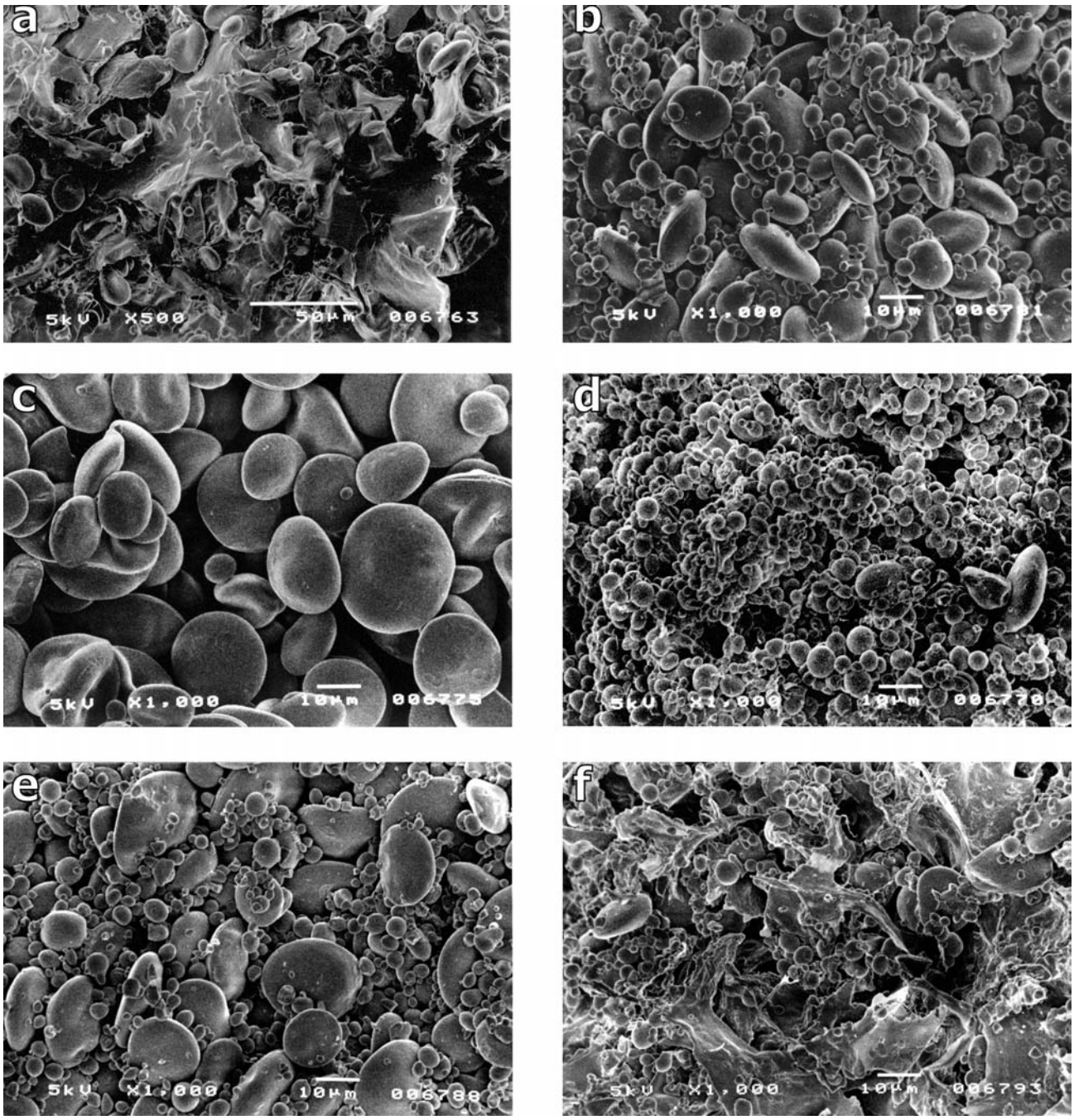

Fig. 5. SEM pictures of the fractions (Torka var.): a) F70, b) PS, c) MSF, d) SSF, e) LF, f) UF.

particles and a reduction in the share of A- and B-type starch granules by 19 and 2%, respectively, was also found.

In using the modified procedure, it was possible to separate PS into two different fractions: LF (Fig. 5e) and UF (Fig. 5f). A clear fractionation of starch and non-starch components of relatively high purity of preparations is visible. This supports the aforementioned results concerning the protein determination in both fractions (Table 2).

The SDS-PAGE electrophoregram (Fig. 6) of PS, MSF and LF extracts of starches obtained from the wheat grains examined showed the absence of a polypeptide fraction with a molecular weight in the range of 6.5–20 kDa. In F70, a single band denoting a fraction corresponding to the 11-kDa marker and no diversification between the wheats employed was observed. In contrast, differences between the wheat grains were detected in SSF and UF, where an

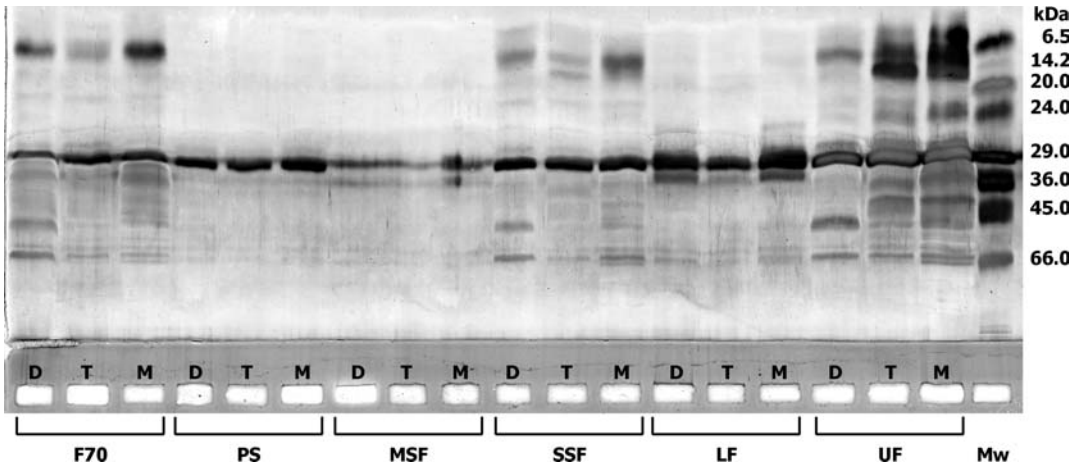

Fig. 6. Silver-stained electrophoregram of proteins from F70, PS, MSF, SSF, LF and UF from durum wheat (lanes D), hard wheat Torka (lanes T) and soft wheat MIB496 (lanes M) extracted with propan-2-ol:0.1M NaCl (ratio 1:1 v/v) and precipitated with acetone. Lane Mw contains molecular weight markers.

additional band corresponding to a 15-kDa polypeptide fraction appeared in extracts of starch isolated from soft kernels; that is, Torka and MIB-496. The absence of the 15-kDa band in durum wheat fractions may be indirect confirmation that the polypeptide was friabilin. Even when extracts from MSF and LF were loaded on the gel at quantities four times greater than those recommended by Greenblatt *et al*. (1995), no polypeptide fractions corresponding to the 15-kDa molecular weight marker were observed.

The share of A- and B-type starch granules as well as non-starch particles in MSF and LF might suggest that friabilin is not connected with B-type starch granules, but rather is a component of the non-starch fraction with rheological properties very similar to the B-type starch granule fraction; this is contrary to the hypothesis proposed by Sulaiman (1990).

Even though the separation of PS components was achieved during centrifugation in both methods, the MSF contained mostly starch granules of A-type when employing Wolf's original methodology, whereas SSF was composed mainly of B-type granules with non-starch particles. This suggests a separation mechanism in which the suspended particles are sedimented independently. The use of the proposed modification to Wolf's method ensures higher representativeness of the LF, which contains A- and B-type starch granules in proportions comparable to PS, while UF contains mainly non-starch components and a relatively small quantity of starch granules.

CONCLUSIONS

1. The presented modification to Wolf's method resulted in a fraction (UF) of higher quality for further analysis. The UF was characterized by higher protein and non-starch particles content and very limited number of starch granules than SSF.

2. Separation of fractions in Wolf's method is proceeded through manual scrapping of the upper layers whereas our modification is based on natural differences between the phase component properties appearing during freezing and lyophilization, which eliminates the subjective manual division and scrapping of the remaining fraction. This indicates that a complete separation and purification of starch fractions is crucial to the experiment results.

3. The presence of friabilin in SSF and UF, and lack thereof in MSF and LF, led to the conclusion that this polypeptide is not connected with the starch granule; rather, it is the component of non-starch particles which are accompanied by starch granules during gluten washing.

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