IN VITRO STARCH BINDING EXPERIMENTS: STUDY OF THE PROTEINS RELATED TO GRAIN HARDNESS OF WHEAT

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Summary
Two friabilin components, puroindoline a and GSP-1 were expressed in *Escherichia coli*. Starch binding properties of the recombinant polypeptides and of friabilin extracted from wheat flour were compared *in vitro*. The produced proteins as well as native wheat friabilin bound to starch granules prepared from different (soft, hard and durum) wheat cultivars. Starch granules also bound specifically several wheat endosperm proteins other than friabilin.

Introduction
Grain hardness (endosperm texture) forms the fundamental basis of commercial differentiation of wheat cultivars. It is possible to differentiate ‘soft’ and ‘hard’ hexaploid wheats and ‘very hard’ durum wheat as three distinct qualitative classes. Texture determines flour particle size, starch damage, water absorption and milling yield. Therefore, grain hardness is an indicator of the suitability of a particular flour for a particular product. To the grower, texture is important as generally higher premiums are paid for the harder wheats (Morris, 2002).

The hardness of the grain is largely determined by the properties of the endosperm. The surface of the starch granules is covered by lipids and special proteins. Hard wheat starches prepared by water sedimentation have more material adhering to their surface than soft wheat starches prepared by the same method when examined by scanning electron microscopy (Barlow *et al*., 1973). It is generally accepted that the adhesion between the granules and the protein matrix is stronger in hard wheats than in soft wheats. During the milling of hard wheat, starch granules fragmentize. This fragmentation is called starch damage. Starch damage is the most important factor in determining water absorption of flour. It also affects the amount of carbohydrates available to yeasts, the fermentative activity, gas production, loaf volume and, as a result, baking quality. So far the most well characterized source of variation in grain hardness is the *Ha* (Hardness) locus located on the short arm of chromosome 5D of hexaploid wheat. The genes for puroindoline a, puroindoline b and GSP-1 (the three major components of the friabilin protein fraction) are tightly linked to the *Ha* locus. There is an unbroken linkage between mutations in any of the puroindoline genes and grain hardness. Friabilin is abundant on the surface of water-washed soft wheat starch granules, scarce on hard wheat starch and absent on durum wheat starch.

The biochemical mechanism governing endosperm texture is poorly understood. The cause of the strong adhesion between starch granules and protein matrix in hard and durum wheat cultivars is unknown. How friabilin located on the surface of the granules impairs the adhesion in soft wheats is also unclear. Detailed knowledge on the molecular background of grain hardness could help in producing cultivars specially fit for any one particular purpose.

Isolation of the components located at the starch-gluten interface and investigation of their interactions in *in vitro* experiments might help to elucidate the mechanism of adhesion between starch and matrix and the impairment of this adhesion by friabilin. There is no biochemical data available on interaction between matrix proteins and starch granules. Friabilin was proven to bind to the surface of starch granules in *in vitro* experiments (Bloch *et al*., 2001). Individual puroindoline proteins isolated by three consecutive chromatographic steps were shown to bind polar lipids, such as those present on the surface of starch granules (Dubriel *et al*., 1997). Extensive purification procedures might be avoided by heterologously expressing the individual components. Puroindoline a was produced earlier in *Pichia pastoris* (Issaly *et al*., 2001).

We aim to find the factors and mechanisms responsible for grain hardness/softness by investigation of the starch-lipid-friabilin-matrix protein complex. Here we present *in vitro* results on the interaction of native wheat friabilin and of *E. coli* expressed friabilin polypeptides with different types of starch granules. Starch granule binding by gluten components is also demonstrated.

Materials and methods

Plant material
Flour and starch samples used in this study were prepared from bread wheat cultivars Riband (soft) and Mercia (hard) and from durum wheat cultivars Martondur and Svevo.

Friabilin extraction
Wheat friabilin proteins were extracted using the Triton X-114 (TX-114) phase partitioning method of Blochet *et al.* (1993). The extracts were stored at 4°C.

Heterologous expression of friabilin proteins
Puroindoline-a gene was amplified from a cDNA library. Oligonucleotide 1 contained a sequence to replace the signal peptide sequence by a codon for Met and an *Nco*I restriction site was also added to the gene for cloning. Oligonucleotide 2 incorporated a *BamH*I restriction site next to stop codon of the gene. PCR fragment was cloned into pCR 2.1 TOPO vector and checked by DNA sequencing and was subcloned into pET11d expression vector. The clone was sequenced again and called PApET11d.

Part of a cDNA clone for GSP-1 (Grain Softness Protein) gene was amplified for cloning in pET17b vector and protein expression. Oligonucleotide 3 was designed to replace the sequence coding the N-terminal 40 amino acid residues (including the signal peptide) by a codon for Met and an *Nde*I restriction site was also added to the gene for cloning. Oligonucleotide 4 incorporated a *BamH*I restriction site next to stop codon of the gene. PCR fragment was cloned into pCR 2.1 TOPO vector and sequenced. After subcloning into pET17b expression vector, the construct was checked again by sequencing. This construct was called GSPUPpET17b.

Proteins were expressed in Origami B (DE3) as host cell. For SDS-PAGE and Western blot analysis, bacteria were directly suspended in protein loading dye. For starch binding experiments, bacterial pellet was resuspended in 8M urea, 50mM Tris-HCl (pH 6.8) and incubated for 4 h at room temperature with constant agitation. Sample was centrifuged and the supernatant was dialyzed overnight against 4M urea, 50mM Tris-HCl (pH 6.8) at 16ºC. The resulting solution was dialyzed for 8 h against 50mM Tris-HCl (pH 6.8) at 4ºC, changing the solution once. The final solution was used promptly for starch binding experiments, or mixed with protein loading dye.

Starch binding experiments
Starch binding experiments were performed using a method adapted from Bloch *et al*. (2001).

Separation and identification of proteins
Protein loading dye was added to the samples. After heating at 100ºC for 2 min, samples were loaded on 15% Tris-Tricine (Schägger and von Jagow, 1987) minigels and proteins were separated under reducing conditions. Gels were then either Coomassie stained or used for Western blotting. Fermentas Prestained Protein Ladder was used as molecular weight standard.

Cation exchange FPLC
The pH of the TX-114 extract was set to 4.9 by addition of sodium acetate. The sample was then applied to a Mono-S cation exchange column (Pharmacia). Proteins were eluted with a 50 mM to 1 M sodium acetate (pH 4.9) concentration gradient, which contained 20% (v/v) acetonitrile, and protein in the column effluent was monitored by absorbance at 280nm.

Results and discussion

Grain hardness is caused by strong adhesion between gluten and starch granules. Proteins present at the matrix-starch granule interface play a crucial role in this adhesion. Surface protein composition of starch granules prepared from wheat cultivars Riband (soft), Mercia (hard) and Svevo (durum) was checked after water washing and after washing five times with 50 mM sodium acetate (pH 4.0) buffer. Proteins present on sodium acetate-washed starch and on water washed starch showed similar patterns when analyzed by SDS-PAGE, although the amount of protein was significantly lower after sodium acetate treatment. There were several bands visible in the 33 to 54 kDa molecular weight region. The corresponding proteins in similar extracts are considered to be storage proteins remaining adsorbed to the surface of starch granules after starch extraction. Their presence is generally believed to be artifactual, caused by binding to the granules during the isolation/extraction process (Baldwin 2001). To our knowledge, there is no experimental evidence underlying the latter statement. It is worth to note, that the amount of protein extractable from the surface of starch granules was smallest in case of soft wheat, intermediate in case of hard wheat and most in case of durum wheat. These findings are in accordance with earlier scanning electron microscopic results (Barlow *et al*., 1973). Only the amount of friabilin followed an opposite pattern.

To test the protein binding properties of the different types of starch granules, TX-114 extract of Riband flour was incubated with different starch granules. SDS-PAGE analysis of the eluates showed (Fig. 1.) that all three types of starch granules used in this study bound friabilin selectively, but also had affinity for other proteins present in the preparation. The additional bands visible in the eluates indicate that there are several other TX-114 soluble proteins in Riband flour that bound to starch granules in our assay. Not all the proteins in the TX-114 extract bound to starch granules and the relative amount of the bound components does not correspond to the amounts present in the original TX-114 extract. These results suggest that binding was specific. The band observed at approx. 60 kDa probably corresponds to GBSS I, a crucial enzyme of starch synthesis, located on the surface of and inside the starch granules in wheat endosperm. The bands in the 33-54 kDa region might correspond to storage proteins that are identical to the ones present on the untreated surface of starch granules isolated by water washing.

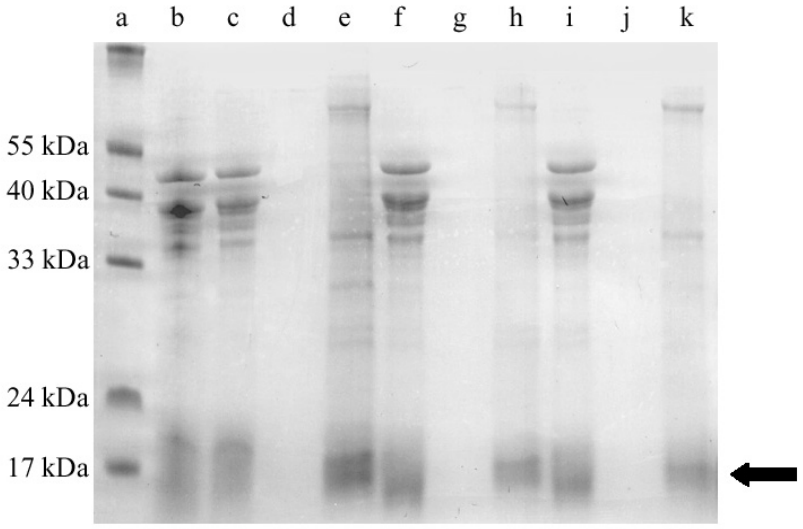

Figure 1: Binding of a TX-114 extract of cultivar Riband to different starch granules. SDS-PAGE separations are shown of (a) molecular weight standard; (b) TX-114 extract; (c, f and i) flow through; (d, g and j) washing; (e, h and k) eluate. Starch columns were prepared from cv. Riband (c-e), cv. Mercia (f-h), cv. Martondur (i-k). The arrow indicates friabilin.

Nature of the observed protein bands was investigated by Western blot analysis. Using an anti-friabilin serum it was confirmed that Riband friabilin bound to all three types of starch granules. Binding of friabilin to soft and hard wheat starch granules *in vitro* was demonstrated earlier by Bloch *et al*. (2001). Binding of friabilin to durum wheat starch granules was not reported

before. Analysis of the eluates with anti-storage protein serum showed more bands in the eluates than Coomassie staining of SDS-PAGE gels. The results suggest that several storage protein components bind the granules. To test the starch binding ability of storage proteins, 70% ethanol extract (containing mainly gliadins) of Riband flour was used for binding to starch prepared from cv. Riband. SDS-PAGE analysis showed faint bands in the eluate at the 33-54 kDa region. This confirms that several wheat storage proteins are capable of starch binding *in vitro*.

It is possible that lipids bound to starch granules affect the binding of proteins. According to Greenblatt *et al*. (1995), propan-2-ol and water (90:10) is effective in removing bound polar lipids from starch. Following their procedure the three different types of starch granules were treated with the solvent and then used for starch binding experiments with Riband TX-114 extract. SDS-PAGE patterns of the eluates were similar to patterns without propan-2-ol treatment, but the amount of bound protein was significantly lower. Therefore, it is possible that starch bound polar lipids have a role in the granule-protein interaction, but further investigation is necessary to confirm these results and elucidate the role of lipids.

To be able to characterize the individual components eluted in the starch granule binding assay, we attempted to isolate them from the TX-114 extract by cation exchange FPLC. We were unable to completely purify the individual components or to identify the fraction containing the M_r≈25000-54000 proteins observed in the eluates after starch binding. Friabilin purified by FPLC bound to the granules. This suggests that the chromatographic procedure used in this study does not affect the functionality of the proteins. Further investigation is needed to characterize the additional bound proteins and their interactions with starch granules and friabilin.

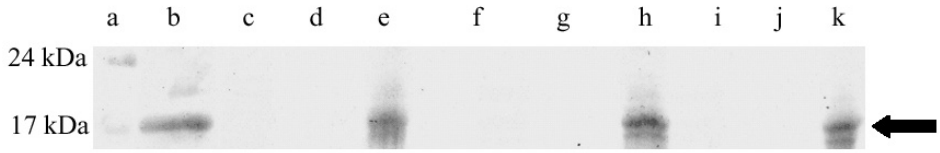

Figure 2: Binding of expressed GSPP to different starch granules. SDS-PAGE separations are shown of (a) molecular weight standard; (b) urea extract; (c, f and i) flow through; (d, g and j) washing; (e, h and k) eluate. Starch columns were prepared from cv. Riband (c-e), cv. Mercia (f-h), cv. Martondur (i-k). The arrow indicates GSPP.

To avoid the difficulties of separation of different proteins extracted from wheat samples, two friabilin polypeptides were expressed in an *E. coli* system. Friabilins are synthesized as preproproteins in wheat endosperm. After the removal of the signal peptide, the maturing proteins are cleaved again at both the N- and C-terminals *in vivo*. Therefore, sequence of the cDNA clones was modified as follows: puroindoline a construct (PA) codes for a protein lacking the signal peptide only. GSP-1 construct (GSPP) codes for a protein completely processed at the N-terminal (designed according to Douliez *et al*., 2000). SDS-PAGE analysis of proteins produced heterologously by *E. coli* showed an additional band at approx. 15 kDa, when compared to the negative control. Western blot analysis using an antiserum raised against wheat friabilin also showed an extra band at approx. 15 kDa. Identity of the produced GSPP polypeptide was also confirmed by MALDI-TOF MS. Urea extracts of the bacterial cultures producing the heterologous proteins were assayed for starch binding. SDS-PAGE (Fig. 2.) and Western blot analyses showed that both PA and GSPP are capable to bind to wheat starch granules (soft, hard and durum) *in vitro*. We found that the expressed proteins show a functional trait similar to native wheat friabilin. The system is, therefore, suitable for exploring the interactions of the starch granule surface with recombinant polypeptides. The analysis of mutant and modified proteins produced using protein engineering may assist to clarify the molecular basis for friabilin binding and grain texture.

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