

# In Vitro Binding of Puroindolines to Wheat Starch Granules

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## ABSTRACT

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Puroindoline (pin) preparations made from flours of hard and soft wheats contained a mixture of pin-a, 0.19/0.53  $\alpha$ -amylase inhibitor, and purothionins. Starch granule preparations from the same cultivars were treated with proteinase to remove surface proteins and incubated with solutions of the pin preparations. Binding of pin-a and purothionins but not the 0.19/0.53 inhibitor was observed with no apparent differences between the behavior

of the pin preparations or starch granule preparations from hard or soft types. No binding was observed when several other proteins (bovine serum albumin, total albumins, a commercial preparation of wheat  $\alpha$ -amylase inhibitors, and barley  $\beta$ -amylase) were incubated with the starch granules under the same conditions, indicating that in vitro binding can be used to study specific starch granule and protein interactions.

The demonstration by Greenwell and Schofield (1986) that grain softness in hexaploid wheat is associated with the presence of a group of  $M_r$  15,000 proteins (friabilins) on the starch granule surface has led to considerable interest in starch granule-associated proteins and their role in determining grain texture. These studies have shown that friabilin consists of multiple components that include previously characterized inhibitors of  $\alpha$ -amylase (Morris et al 1994, Oda and Schofield 1997) and a related group of grain softness proteins (GSP) (Rahman et al 1994, Jolly et al 1996). However, most attention has focused on two major basic components that are identical to previously characterized tryptophan-rich, lipid-binding proteins called puroindolines (pin-a and pin-b) (Rahman et al 1994, Gautier et al 1994). The pins are encoded by genes close to or at the hardness locus (*Ha*) on chromosome 5D of bread wheat (Giroux and Morris 1997, 1998) and their absence from durum wheat is considered to be responsible for its ultrahard texture. Furthermore, Campbell et al (1999) have demonstrated that >60% of the variation for texture in a population of 78 recombinant inbred lines from a cross between hard and soft types could be explained by a pin-b marker. Morris and coworkers have proposed that hardness in bread wheat can result from one of four mutations that affect either the expression of the pin-a gene or the binding of the pin-b protein to starch (Giroux and Morris 1997 and 1998, Lillemo and Morris 2000). However, this hypothesis has not so far been confirmed in other laboratories (Turnbull et al 2000).

Work done so far has been purely correlative in approach, relating the presence or absence of proteins on water-washed starch granules to differences in protein sequence (revealed by polymerase chain reaction analysis) and grain texture. We have, therefore, developed an assay to determine starch binding in vitro and evaluated this by comparing the binding properties of puroindolines from hard and soft cultivars and a range of other proteins.

## MATERIALS AND METHODS

### Materials

The uncoupled monoclonal antibody to hexaploid wheat friabilin (as used in the Durotest P kit) was obtained from Rhone-diagnostics Technologies Ltd., Glasgow, UK. Bovine serum albumin (A7096),  $\beta$ -amylase (A7130), and wheat grain  $\alpha$ -amylase inhibitors (A1520) (Silano et al 1975, O'Donnell and McGeeney 1976) were

obtained from Sigma-Aldrich Co. Ltd., Poole, UK. Total wheat albumins were prepared by mixing 40 mg of flour from cv. Mercia or Riband with 1 mL of water and suspending in a sonic bath for 30 min. Pin-a and pin-b antibodies were obtained from Didier Marion (INRA, Nantes, France).

### Puroindoline Purification

Puroindolines were isolated using the Triton X-114 phase partitioning method of Blochet et al (1993). Puroindolines, along with some other hydrophobic proteins, are soluble in the nonionic detergent Triton X-114. These proteins can be separated away from other nonhydrophobic proteins due to the ability of Triton X-114 to separate into an aqueous and detergent phase at >20°C (Bordier 1981, Blochet et al 1993). The detergent-soluble proteins can then be removed by precipitation before further purification. In this scaled-down method, barley or wheat flour (100 g) was milled and extracted at 4°C overnight with 500 mL of 100 mM Tris/HCl buffer, pH 7.8, containing 5 mM EDTA, 100 mM potassium chloride, and 4% (w/v) Triton X-114. After centrifugation at 5,000  $\times$  g for 15 min, the supernatant was incubated at 37°C until phase separation occurred and then centrifuged at 5,000  $\times$  g for 15 min to complete the separation. The upper (detergent-free) phase was removed and replaced with an equal volume of Tris/HCl buffer without added Triton X-114 and the phase separation was repeated. The lower detergent phase was precipitated overnight at -18°C following the addition of 200 mL of ice cold diethylether and ethanol (1:3). After centrifugation at 2,000  $\times$  g for 15 min, the surface of the pellet was washed with more solvent, and the pellet was allowed to dry overnight. The pellet was resuspended in 5 mL of 50 mM acetic acid, centrifuged at 20,000  $\times$  g for 5 min, and the supernatant was loaded onto a column of Sephadex G50. Column fractions were analyzed by SDS-PAGE using 12.5% Laemmli gels and the samples containing  $M_r$  15,000 proteins were pooled and freeze-dried.

### Starch Isolation

Starch granules were isolated using a method adapted from Wolf (1964). Flour was made into a dough ball by adding water and mixing. The starch was then washed away from the gluten and left overnight at 4°C to sediment. Excess water was removed by centrifugation (5,000  $\times$  g, 10 min), and the resulting starch pellet was resuspended in a solution of Proteinase K (5 mg/100 mL) and incubated at 37°C until all surface protein was removed. The starch was then washed thoroughly and freeze-dried.

### Starch-Protein Binding

Starch (30 mg) was preincubated with proteinase inhibitor (100  $\mu$ M phenylmethylsulfonyl fluoride [PMSF] in 1 mL of 0.1M Tris/HCl buffer, pH 5.5) for 1 hr at room temperature with constant mixing, to inhibit any residual Proteinase K. Samples were centrifuged and the pellet was washed in 0.1M Tris/HCl buffer (pH 5.5). The washed, pelleted samples were subsequently incubated with puro-

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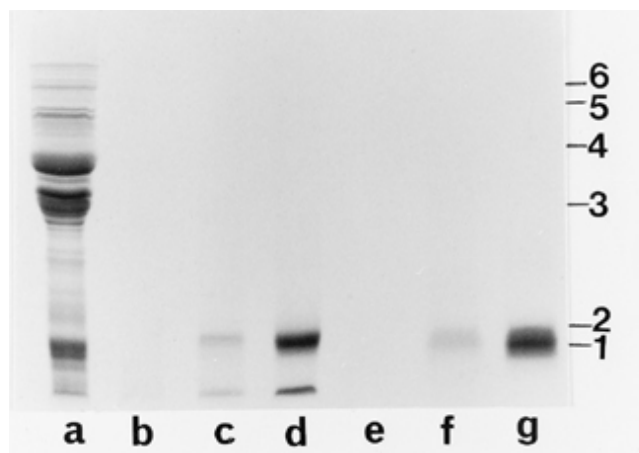
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indolines or other proteins (0.5 mg/mL in 0.1M Tris/HCl buffer, pH 5.5) overnight at 4°C. After incubation, the pellets were again washed for 30 min with buffer and retained for SDS-PAGE.

To investigate the effect of puroindoline concentration on starch binding, the above method was used with protein concentrations of 0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/mL. Starch samples (30 mg) were incubated with 1 mL of 0.1M Tris/HCl buffer, pH 5.5, containing the pin preparations at 4°C overnight. Samples were then centrifuged and the pellets washed with 0.1M Tris/HCl buffer, pH 5.5. The protein contents of the combined supernatants and washings were measured on triplicate aliquots using the Coomassie Plus protein assay reagent (Pierce product 23236).

### SDS-PAGE

Starch and pin samples were suspended in sample buffer (62.5 mM Tris/HCl, pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 0.002% [w/v] bromophenol blue for nonreduced samples, with the addition of 5% [v/v] 2-mercaptoethanol for reduced samples) to give a final

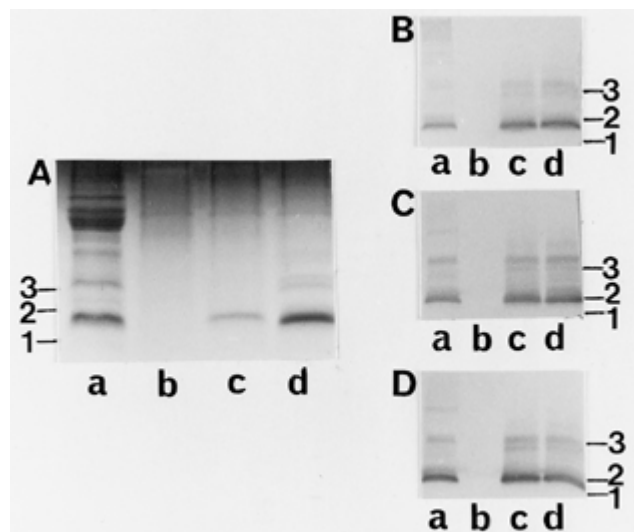


**Fig. 1.** Binding of a puroindoline (pin) preparation from cv. Mercia to starch granules from the same cultivar. SDS-PAGE separations (Laemmli system) are shown of (a) total flour proteins from cv. Mercia; (b) and (e) proteins recovered from proteinase-treated starch; (c) and (f) proteins recovered from starch granules after incubation with pin preparation; (d) and (g) pin preparations. Tracks a–d are separated under reducing conditions and tracks e–g without reduction. Similar results were obtained for Riband. Numbers 1–6 indicate the positions of  $M_r$  marker proteins: 1 = cytochrome c ( $M_r$  12,300), 2 = myoglobin ( $M_r$  17,200), 3 = carbonic anhydrase ( $M_r$  30,000), 4 = ovalbumin ( $M_r$  42,700), 5 = albumin ( $M_r$  66,200), and 6 = ovotransferrin ( $M_r$  76–78,000).

protein concentration of 0.5 mg/mL, heated at 100°C for 3 min, and centrifuged briefly. Samples (10  $\mu$ L) were loaded onto 12.5% SDS-polyacrylamide (Laemmli 1970) minigels (8  $\times$  7.3  $\times$  0.75 mm) or 16% Tris/Tricine (Shägger and von Jagow 1987) minigels. The gels were then either stained (0.1% Coomassie BB R250, 40% [v/v] methanol, 10% [w/v] trichloroacetic, destain in 0.5M sodium chloride) or used for western blotting.

### Western Blotting

Protein samples prepared in nonreduced SDS-PAGE sample buffer were used for western blotting. Proteins were transferred to nitrocellulose membrane using a Bio-Rad Trans-Blot cell. The membranes were blocked (5% [w/v] skimmed milk powder in Tris buffered saline (TBS) (20 mM Tris/HCl, 500 mM NaCl, pH 7.5) for 1 hr and the primary antibody was applied (Durotest-P diluted 1:1000, anti-pin a and b diluted 1:250), in TTBS (TBS and 0.05%



**Fig. 2.** Western blotting of puroindoline (pin) preparations from cv. Riband. SDS-PAGE separations (Tris/Tricine System under nonreducing conditions) of total proteins from flour of cv. Riband (tracks a), protease-treated starch granules from cv. Riband (tracks b), a pin preparation from cv. Riband (tracks d), and proteins recovered after incubation of the starch granules with the pin preparation (tracks c) were stained with Coomassie BB R250 (A) or used for western blotting with antibodies to pin-a (B), pin-b (C), or friabilin (D). Similar results were obtained for Mercia. Numbers 1–3 indicate the positions of  $M_r$  marker peptides obtained by cyanogen bromide cleavage of horse myoglobin: 1 =  $M_r$  8,159, 2 =  $M_r$  14,404, and 3 =  $M_r$  16,949.

**TABLE I**  
Comparison of N-Terminal Amino Acid Sequences<sup>a</sup>

	Position <sup>b</sup>										Yield <sup>c</sup>
	1	2	3	4	5	6	7	8	9	10	
15K Total Major	D/A	V	A	G	G	G	G	A	Q	Q	9
Minor	S	G	P	W	M	X	Y	P	X	X	...
Pin a	D	V	A	G	G	G	G	A	Q	Q	...
Pin b	E	V	G	G	G	G	G	S	Q	Q	...
0.19 Inhibitor	S	G	P	W	M	C	Y	P	G	Y	...
0.53 Inhibitor	S	G	P	W	M	C	Y	P	G	Q	...
15K Bound	D/A	V	A	G	G	G	G	A	Q	Q	8
5K Total	K	S	C	C	R/K	S/T	T	L	G	R	8
$\alpha$ -Purothionin <sup>d</sup>	K	S	C	C	R	S/T	T	L	G	R	...
$\beta$ -Purothionin	K	S	C	C	K	S	T	L	G	R	...
5K Bound	K	S	C	C	R/K	S/T	T	L	G	R	4.5

<sup>a</sup> Comparison of N-terminal amino acid sequences determined for the  $M_r$  15,000 (15K) and  $M_r$  5,000 (5K) bands present in total and bound puroindoline (pin) fractions from Riband with those reported for wheat puroindolines (Gautier et al 1994),  $\alpha$ -amylase inhibitors (Carbonero and Garcia-Olmedo 1999), and purothionins (Garcia-Olmedo 1999). Similar results were obtained for bands from cv. Mercia.

<sup>b</sup> Standard single letter abbreviations for amino acids.

<sup>c</sup> Yield pmol/gel track can be used as an approximate estimate of the proportions of  $M_r$  15,000 and  $M_r$  5,000 bands within the total and bound fractions.

<sup>d</sup>  $\alpha$ -Purothionin consists of  $\alpha_1$  and  $\alpha_2$  forms with S and T, respectively, at position 6.

[v/v] Tween, 1% [w/v] bovine serum albumin [BSA]) for 1 hr. The secondary antibody (Sigma-Aldrich anti-mouse alkaline phosphatase conjugate, 1:6250 in TTBS with 1% [w/v] BSA for the friabilin antiserum and Sigma-Aldrich anti-rabbit alkaline phosphatase for the pins) was applied for ≈1 hr. The blots were washed thoroughly in TTBS and TBS and developed.

### N-Terminal Amino Acid Sequencing

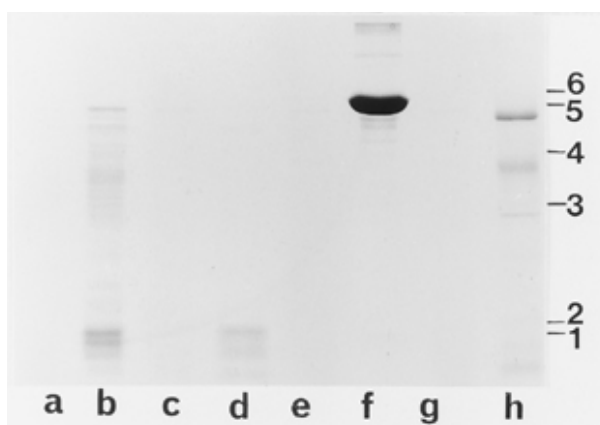
Purified  $M_r$  15,000 proteins were separated on 16% Tris/Tricine gels and blotted onto Problott polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, Foster City, CA). Protein bands were visualized using 0.1% (w/v) Coomassie BB R-250 in 40% (v/v) methanol with 1% (v/v) acetic acid. Stained protein bands were excised from the membrane and N-terminal amino acid sequences were determined by Mike Naldrett at the John Innes Centre, Norwich, using a pulsed-liquid amino acid sequencer (model 477A, Applied Biosystems) equipped with online phenylthiohydantoin (PTH) amino acid analyzer (model 120A).

## RESULTS

### Preparation of Starch and Puroindolines

Starch granules were prepared from the hard winter wheat cv. Mercia and the soft winter wheat cv. Riband, treated with Proteinase K to remove surface-bound proteins and then washed and treated with the proteinase inhibitor PMSF. This strategy was adopted as an alternative to washing because Seguchi and coworkers have demonstrated that it is difficult to remove all of the surface proteins from wheat starch without using solvents such as SDS and 2-mercaptoethanol, which could be expected to affect the starch granule surface (Seguchi 1986, Seguchi and Yamada 1989). Washing the granules with SDS-PAGE loading buffer followed by SDS-PAGE showed no major surface-associated proteins (Fig. 1, tracks b and e), whereas western blotting with antibodies to pin-a, pin-b, and friabilin showed no immunoreactive proteins (Fig. 2).

Pin preparations were made from the same flour samples of Riband and Mercia following the procedure of Blochet et al (1993). SDS-PAGE under nonreducing conditions (Fig. 1, track g) showed a group of bands at  $M_r$  ≈15,000. On reduction an additional band of lower  $M_r$  (≈5,000) was observed (Fig. 1, track d). There were no obvious differences in the patterns or relative proportions of

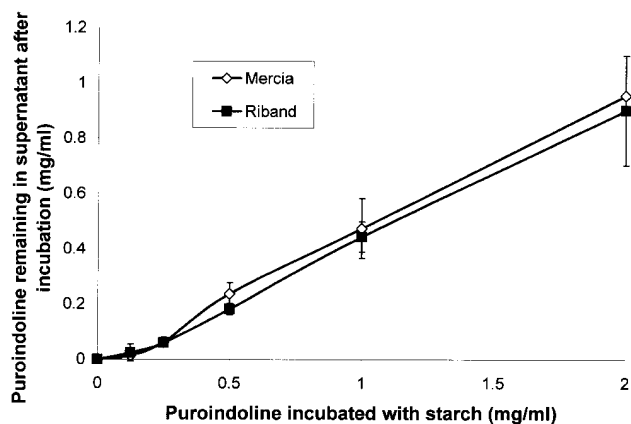


**Fig. 3.** Binding of control proteins to starch granules from cv. Mercia. SDS-PAGE separations (Laemmli system under reducing conditions) of protein preparations incubated with starch granules (b, d, f, and h) and proteins recovered from the starch granules after incubation with the preparations (a, c, e, and g). Tracks a and b are total albumins from flour of cv. Mercia, c and d are wheat  $\alpha$ -amylase inhibitors, e and f are bovine serum albumin, and g and h are barley  $\beta$ -amylase. Similar results were obtained for Riband. Numbers 1–6 indicate the positions of  $M_r$  marker proteins: 1 = cytochrome c ( $M_r$  12,300), 2 = myoglobin ( $M_r$  17,200), 3 = carbonic anhydrase ( $M_r$  30,000), 4 = ovalbumin ( $M_r$  42,700), 5 = albumin ( $M_r$  66,200), and 6 = ovotransferrin ( $M_r$  76–78,000).

components between the fractions from hard (Mercia, Fig. 1) and soft (Riband, not shown) wheats.

N-terminal amino acid sequencing was done on the reduced  $M_r$  15,000 bands from both Riband and Mercia. They gave similar results; only those for Riband are given (Table I). Two sequences were present in an approximate ratio of 1.2:1 (Table I). The quantitatively major sequence was identical to that of pin-a, except that alanine and aspartic acid were recovered at position 1. The minor sequence was identical to the 0.19 and 0.53  $\alpha$ -amylase inhibitors, allowing for our inability to positively identify residues at positions 6, 9, and 10. The presence of 0.19 and 0.53  $\alpha$ -amylase inhibitors is consistent with the results of Oda and Schofield (1997), who reported the presence of 0.19 and 0.53 inhibitors in a friabilin preparation from cv. Norin 61. Western blotting used antibodies to friabilin, pin-a, and pin-b. Similar results were obtained with the two cultivars; only those from Riband are shown (Fig. 2). All three antibodies reacted with bands of similar mobility in the pin preparation (Fig. 2, tracks d) and in a total protein fraction extracted from flour (Fig. 2, tracks a). Although the pin-b antibody reacted strongly with the pin preparation, no separate bands corresponding to pin-a and pin-b were observed and it is probable that cross reaction with pin-a was observed. We are unable to account for the absence of pin-b from our preparations.

The N-terminal amino acid sequence of the reduced  $M_r$  5,000 components from Mercia (not shown) and Riband (Table I) showed that they corresponded to purothionins, with similar yields of arginine and lysine at position 5, indicating approximately equimolar mixtures of  $\alpha$ - and  $\beta$ -purothionins (Garcia-Olmedo 1999). The recovery of some threonine at position 6 is consistent with the presence of this residue in  $\alpha$ 2-purothionin. Purothionins comprise only 40 amino acid residues with four intrachain disulfide bonds. Consequently, they migrate very quickly on SDS-PAGE in the non-reduced state and probably ran with the front on the Laemmli gel system used in Fig. 1. A Tris/Tricine system was therefore also used to separate the samples. This has been reported to resolve proteins of  $M_r$  5,000–20,000 (Schägger and von Jagow 1987) but still failed to show any low  $M_r$  bands corresponding to purothionins in the nonreduced pin preparations (not shown). However, the presence of purothionins in the unreduced pin preparations was demonstrated by N-terminal amino acid sequencing of the mixture of proteins. This demonstrated the presence of lysine in addition to serine and aspartic acid at position 1 and of lysine and arginine in addition to glycine and methionine at position 5, indicating the presence of purothionins a and b.



**Fig. 4.** Concentration-dependant binding of puroindoline preparations to starch granules. Starch granules from cvs. Mercia ( $\diamond$ ) and Riband ( $\blacksquare$ ) were incubated with puroindoline preparations from the same cultivars at concentrations of 0.125 to 2.0 mg/mL. Starch granules were then removed by centrifugation and the protein remaining in the supernatant determined. Results are the means of three experiments conducted on different days with standard deviations shown.

The presence of purothionins in the pin preparations is not surprising in view of their well-established hydrophobic properties; their presence in similar preparations was reported previously (Blochet et al 1991, 1993; P. Greenwell, *personal communication*).

### Binding of Pins to Starch Granules

The ability of the isolated pins to bind to starch granules was initially determined by gently rotating 30 mg of starch granules in 1 mL of a 0.05% solution of pins in buffer at pH 5.5 overnight at 4°C. The starch granules were then recovered by centrifugation, washed with buffer (pH 5.5), and the surface-bound proteins eluted with gel loading buffer and separated by SDS-PAGE under nonreducing conditions. Similar results were obtained when the pins and starch granules were prepared from the same hard or soft cultivar (Mercia in Fig. 1, tracks c and f; Riband, not shown) or were exchanged between the cultivars (not shown). The unreduced protein fractions eluted from starch granules showed patterns similar to the total pin preparations (Fig. 1, tracks f and g) with a group of  $M_r$  15,000 components which reacted with antibodies to friabilin, pin-a, and pin-b (Fig. 2, tracks c and d). However, differences were observed with the reduced protein preparations. The fractions eluted from the starch granules appeared to contain a lower proportion of  $M_r$  5,000 components than the total preparations; this was confirmed by the relative recoveries of residues on amino acid sequencing (Table I). Furthermore, N-terminal amino acid sequencing of the  $M_r$  15,000 band showed only the pin-a sequence, with no residues corresponding to the 0.19 and 0.53  $\alpha$ -amylase inhibitors present in the total pin preparation.

These results indicate that the granules selectively bind pin-a but also have a lower affinity for purothionins. However, the similar results obtained with pins and starches from hard and soft cultivars raises the possibility that the binding was nonspecific. Therefore, similar experiments were conducted with several control protein fractions. These were a total albumin preparation prepared from wheat cv. Mercia or Riband and three fractions purchased from Sigma-Aldrich: BSA, a commercial preparation of  $\alpha$ -amylase inhibitors from wheat, and barley  $\beta$ -amylase (the latter reported to bind to starch granules in the mature barley grain) (Hara-Nishimura et al 1986). No significant binding of any of these fractions was demonstrated for Riband (Fig. 3). There is no evidence that purothionins bind to starch granules in vivo (Garcia-Olmedo 1999), and the in vitro binding demonstrated here may have no biological or functional significance.

### Effect of pH and Protein Concentration on Pin Binding

The binding experiments with pins and starch from cvs. Riband and Mercia were also conducted at pH 4 and pH 8, showing no obvious differences from the results obtained at pH 5.5 (not shown). The concentration dependence also was determined by using protein concentrations of 0.125–2.0 mg/mL. A linear relationship was observed with pins and starch from Riband and Mercia with no statistically significant differences between the two cultivars (Fig. 4).

## CONCLUSIONS

The proteinase-treated starch granules specifically bind pins, with a lower affinity for purothionins and no affinity for a range of control proteins. The system is, therefore, suitable for exploring starch granule surface and protein interactions and, specifically, the molecular basis for pin binding and grain texture.

An in vitro assay is particularly important for this work, because it will allow the analysis of mutant and modified proteins produced using protein engineering or chemical methods. Similarly, chemical modification could be used to alter the starch granule surface, meaning that competitive or synergistic interactions with other proteins or grain components (notably lipids) could be studied.

Our failure to detect any differences between the binding characteristics of pins from hard and soft cultivars of wheat could be the result of technical limitations. For example, partial denaturation of the pins could have occurred during purification, which could have altered the conformations of their starch binding sites. However, this would be more likely to have led to loss rather than to gain of starch binding properties. Similarly, it is unlikely that any biological activity is required for specific pin binding because the pins appear to be initially deposited in protein bodies (Dubreil et al 1998a) and only become associated with the starch granule surface when the protein bodies coalesce to form a continuous matrix during the later stages of grain maturation and desiccation. A more likely explanation is that one or more additional endosperm components are required to confer specificity to pin binding, possibly polar lipids. This is consistent with the observation of Greenblatt et al (1995) that removal of bound polar lipids from starch renders friabilin components extractable with aqueous salt solution. Those authors proposed that lipid-mediated interactions are important in determining the binding of friabilin to starch. The in vitro system described here should facilitate the analysis of such components and interactions.

## ACKNOWLEDGMENTS

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