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Characterisation of the wheat Mr 15000 "grain-softness protein" and analysis of the relationship between its accumulation in the whole seed and grain softness

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Abstract. The Mr 15000 protein associated with water-washed wheat starch granules from soft wheats was shown to be heterogeneous: it could be divided into a fraction containing one or more α-amylase inhibitor subunits and a fraction largely composed of a previously uncharacterised polypeptide(s) referred to as the "grain-softness protein" (GSP). The major N-terminal sequence and sequences of peptides derived from protease digests of GSP are reported. An antiserum specific for GSP was used to show that GSP accumulated in both hard and soft wheat grains, but the GSP in soft grains associated more strongly with starch granules than the GSP in hard grains. A positive correlation between grain softness and accumulation of GSP in the seed was demonstrated for a range of cultivars. This differs from the qualitative relationship, based on the isolated starch fraction, between GSP and grain softness that has already been reported. Analysis of wholemeal extracts with the antiserum demonstrated that the accumulation of GSP in the seed was dependent on the short arm of chromosome 5D, which also encodes the *Ha* locus. In addition, examination of near-isogenic lines differing in hardness indicated that the gene(s) controlling GSP was (were) linked with the *Ha* locus. The findings indicate that GSP may be the product of the *Ha* locus and thus be the major factor that determines the milling characteristics of bread wheats.

Key words: Grain softness – Friabilin – Milling quality – *Triticum aestivum* – Seed storage-proteins

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Introduction

One of the major properties used to classify wheat for marketing is the softness or hardness of the grain. The crushing of a soft seed releases the endosperm cell contents as an amorphous fine powder, including many single starch granules. A hard seed is more difficult to crush and produces a coarser powder composed of angular aggregates of starch and protein, many of which are the entire contents of a single or aggregated group of cells (Biffen 1908; Greer and Hinton 1950; Greer et al. 1951).

The genetic basis of grain hardness is well established. Symes (1965) showed that in Australian wheats one major locus was involved and he subsequently produced near-isogenic pairs of soft and hard lines from the cultivars Falcon and Heron (Symes 1969). Mattern et al. (1973) and Law et al. (1978) showed that the softness/hardness locus, *Ha*, is on the short arm of chromosome 5D. In addition, chromosome 5A may carry a minor locus (Morrison et al. 1989). While the environment has been shown to modulate the penetrance of the *Ha* locus (e.g., Parish and Halse 1968; Stenvert and Kingswood 1977), it rarely seems to completely override it (Glenn and Saunders 1990; Jolly and Glenn, unpublished data).

The biochemical basis of grain softness is largely unknown. However the presence of "free" polar lipids in the endosperm has been correlated with grain softness (Morrison et al. 1984, 1989), and much recent work has focussed on an Mr 15,000 polypeptide that is readily identifiable by SDS-PAGE in extracts of water-washed starch granules from soft wheats but is absent, or nearly so, in extracts of water-washed starches from hard wheats (Greenwell and Schofield 1986; Schofield and Greenwell 1987; Morrison et al. 1992). The Mr 15,000 polypeptide is therefore a marker for grain-softness and

has been referred to as "friabilin" (Greenwell and Schofield 1989) or "GSP" (Jolly et al. 1990; Jolly 1991).

We have analysed the Mr 15,000 starch-granule polypeptide in detail, with the aim of establishing more clearly its relationship to the *Ha* gene. The Mr 15,000 band from starch was found to be heterogeneous, containing variable amounts of α-amylase inhibitor(s), but also always including a previously uncharacterised fraction – operationally defined as "GSP". This fraction was purified and partially characterised by amino-acid analysis. In contrast to earlier reports, which involved electrophoretic examination only of proteins associated with starch granules, GSP was found to occur in both hard and soft wheats. However, a positive correlation between the levels of GSP accumulated in the seed and seed softness was observed. Furthermore, the accumulation of GSP in *T. aestivum* seeds was dependent upon the presence of the short arm of chromosome 5D, and control of its accumulation was linked with *Ha* alleles. The possible role of GSP in models of the development of grain-softness is discussed.

Materials and methods

Wheat lines and accessions

Samples of grain from a number of sites used in the 1987 Australian Interstate Variety Trials were kindly donated by Mr. John Moss (Bread Research Institute of Australia, North Ryde, NSW). Other samples grown in randomised plots at Horsham, Victoria in 1983 were provided by Mr. Joe Panozzo (Victorian Crops Research Institute, Horsham, Victoria). Isogenic lines derived from cultivars Falcon and Heron (Symes 1969) and the parent cultivars were grown in plots at the Sydney University research station in Narrabri in 1988 with the co-operation of Dr. Frank Ellison. Seed of *Triticeae* species related to wheat was provided by Mr. Michael Mackay from the Australian Winter Cereals Collection (Tamworth, NSW). Seed of aneuploid stocks derived from the cultivars Chinese Spring and Gabo and the parent cultivars were provided by Dr. Robert McIntosh (Sydney University, Plant Breeding Institute, Cobbitty, NSW).

Starch preparation and starch-granule-protein extraction

Starch was prepared as described by Skerritt et al. (1990). Proteins associated with starch granules were extracted by suspending starch in 10× volumes of the extractant (1% SDS, unless stated otherwise), and stirring constantly on a magnetic stirrer for 1 h. The starch was pelleted at 5,000 g for 20 min and the pellet washed with the same solvent before a subsequent extraction with a different solvent was carried out. Supernatants were filtered through Whatman No. 1 paper and precipitated overnight at 4 °C with two volumes of acetone. Such precipitates contained both protein and carbohydrate.

Extraction of protein from crushed grains or flour and measurement of protein levels

Samples of wholemeal flours were extracted with SDS-PAGE sample buffer (50 mM Tris HCl, 3% SDS, 10% glycerol, 0.088% bromophenol blue, pH 7.5) at a ratio of 50 mg flour per ml of extractant, using sonication as described by Singh et al. (1990). The protein content of wholemeal flours was estimated by NIR at the Analytical Laboratory of the Bread Research Institute of Australia (Williams 1979). The protein content of extracts from starches or wholemeals was estimated with a dye-binding protein assay (BioRad), using protein extracted from wheat flour in 70% ethanol as a standard.

Analytical SDS polyacrylamide-gel electrophoresis (SDS-PAGE) and Western blotting

Slab gradient polyacrylamide gels (0.7 mm thick, 12.5–25% acrylamide) were prepared and run according to the procedure of Spencer et al. (1980), with the addition of 4 M urea to the gel buffers if the gel was to be blotted, since this improved the sensitivity of Western-blot analysis (data not shown). Blotting of polypeptides from unfixed SDS-PAGE gels was performed according to the procedure of Kyshe-Andersen (1984), using 0.2 μm pore-size nitrocellulose (Schleicher and Schuell). Reversible staining and immuno-detection of proteins followed standard procedures (Gallagher et al. 1992), with BSA (Boehringer) used as the blocking agent and goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega) as the secondary antibody. The chromogenic reagents were 4-nitro blue tetrazolium chloride (Boehringer) and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer).

Purification of GSP by preparative SDS-PAGE

GSP was extracted in bulk with 1% SDS from cultivar Rosella starch and purified by preparative SDS-PAGE. Initially, protein fractions were collected by continuous elution from 15% SDS-PAGE gels (Carpenter et al. 1986). Fractions containing only Mr 15,000 protein (as determined by analytical SDS-PAGE) were pooled, dialysed against 0.1% SDS and lyophilised. Later preparative gels were run as for analytical gels, but were stained with 0.1% Coomassie in 50% methanol, 20 mM Tris-HCl (pH 7.2), then destained in the same cocktail, without stain. This procedure stained GSP adequately, without exposing it to extremes of pH and without any observable loss of the unfixed protein from the gel. GSP was then eluted from excised gel slices, using a BioRad electro-eluter following the manufacturer's instructions.

Amino-acid sequencing of GSP

A sample of GSP (approximately 380 μg or 25 nmol of protein) purified by SDS-PAGE was dissolved at 50 °C for 30 min in 200 μl 0.5 M Tris-HCl, 2% SDS, pH 8.5 and reduced for 1 h with 20 μl 1M DTT under N_2. Alkylation was performed at room temperature for 30 min in the dark with 60 μl 3 M iodoacetic acid, then excess reagent was inactivated with DTT. The sample was dried, redissolved in 100 μl water and precipitated with 900 μl methanol at −20 °C for 16 h. The precipitate was dissolved in 400 μl 0.1 M ammonium bicarbonate buffer, pH 8.0. The S-carboxymethylated GSP (10 nmol) was digested with lysyl endopeptidase (Wako) and chymotrypsin (Worthington) and soluble peptides were fractionated as described by Kortt et al. (1991). The amino-terminal sequence of the intact GSP was determined with a gas phase sequencer (Applied Biosystems, USA) and peptides isolated from the digests were sequenced manually as described in Kortt et al. (1991).

Antibodies to GSP

Antiserum (asGSP1) was raised in a rabbit using GSP purified by SDS-PAGE. The antigen was resuspended in phosphate buffered saline at approximately 1 mg/ml and prepared for subcutaneous injections by mixing 200 μl with 200 μl Freund's complete adjuvant. After 4 weeks, a second injection containing 100 μl antigen and 100 μl Freund's incomplete adjuvant was

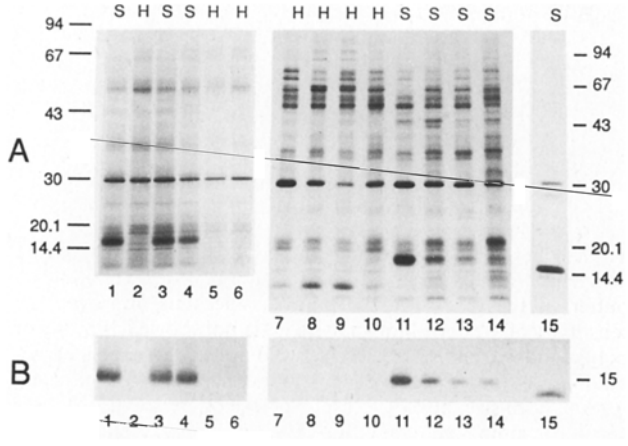

Fig. 1. Proteins extracted from water-washed starch granules with 1% SDS, separated by SDS-PAGE and (**A**) stained with Coomassie-R250 or (**B**) enzyme-linked immuno-stained with second bleed asGSP1 (see Fig. 2), diluted 1,500×. *Lanes for* **A** *and* **B**: *lanes 2 and 5–10* are hard wheats. *Lanes 1, 3, 4 and 11–15* are soft wheats. (*1*) "soft Heron", (*2*) "hard Heron", (*3*) cultivar Heron, (*4*) "soft Falcon", (*5*) "hard Falcon", (*6*) cultivar Falcon, (*7*) Cook, (*8*) Halberd, (*9*) Eagle, (*10*) Wilgoyne, (*11*) Rosella, (*12*) Matong, (*13*) Mokoan, (*14*) Egret, (*15*) Rosella – unreduced. For *lanes 1–6*, extracts equivalent to 250 mg of starch were loaded per lane. For lanes *7–15*, 60 µg of protein was loaded per lane. All samples were reduced with DTT, except for *lane 15*. The mobilities of molecular weight markers (Mr ×1,000) are shown at the right

administered. The rabbit was bled from an ear vein 10 and 30 days later. Antibodies to prolamins that were present in asGSP1 were reduced by incubating 1 ml of the antiserum, diluted to 10 ml with TBS, overnight at 4 °C with 1 cm × 8 cm strips of nitrocellulose previously soaked in 70% ethanol extracts of flour from durum grain (*T. turgidum*, var. durum), since durum grains do not contain any detectable GSP (see Results). The "pre-adsorbed" antiserum was then stored at 4 °C with 0.01% sodium azide as preservative.

Antibodies to α-amylase inhibitors

An antiserum (asAI) was kindly donated by Dr. Peter Shewry (Long Ashton Research Station, UK). It was raised in a rabbit against purified 0.28 α-amylase inhibitor (Kashlan and Richardson 1981). Samples of 0.28 and 0.31 α-amylase inhibitors purified by Dr Michael Richardson (Department of Botany, University or Durham, UK) were also supplied by Dr Peter Shewry.

Quantification of GSP in Western blots

Purified GSP standards were mixed with protein extracted from durum seeds as a carrier and run in at least three SDS-PAGE gel lanes of each blot. After immunostaining of GSP on the SDS-PAGE blots, the nitrocellulose was made transparent by soaking in paraffin oil and staining intensity was measured with an LKB laser densitometer. Only sample results that fell within or very close to the range of the standards on the same gel were used.

Measurement of seed-hardness

The hardness of samples from the Australian Interstate Variety Trials was measured by the Analytical Laboratories of the Bread Research Institute of Australia using NIR (Williams 1979), according to standard procedures. The NIR units were converted to particle size index (PSI) units using a conversion table based on extensive empirical data collected by Mr. John Moss (Bread Research Institute of Australia, North Ryde, NSW). The hardness of smaller sample sets was estimated by the particle size index (PSI) as described by Symes (1965). Hard wheats fall in the 10–15 PSI range, while soft wheats fall in the 18–30 PSI range.

Results

The relationship between Mr 15,000 polypeptide associated with starch granules and seed-softness

Starch was purified from the doughs of a number of cultivars by water-washing. As originally observed by Greenwell and Schofield (1986), Mr 15,000 polypeptides were detected in extracts of starch granules prepared from soft wheats, but were absent, or nearly so, in extracts of starch granules prepared from hard wheats (Fig. 1 A). Variation in other starch granule polypeptides was independent of grain-softness. GSP was not covalently associated in complexes because it was detectable at about Mr 15,000 with or without sample reduction (lanes 11 and 15). However, the slight increase in the Mr of GSP in the reduced form versus the non-reduced form indicates that it probably contains intra-molecular disulphide bonds.

Characterisation of asGSP1

The antiserum (asGSP1) raised against GSP purified by SDS-PAGE bound Mr 15,000 polypeptides on blots of starch-granule proteins from soft wheats, but bound little or no protein on blots of starch-granule proteins from hard wheats (Fig. 1 B).

The antiserum also contained antibodies that bound whole-seed proteins in addition to Mr 15,000 protein (Fig. 2). The cross-reactivity could be largely removed from the second bleed by pre-adsorbing the antiserum against prolamins, while the reactivity to GSP remained little changed by this process (Fig. 2). The cross-reactivity of the antiserum was therefore probably due to pre-immunity in the rabbit. This is a common occurrence, since prolamins present in most laboratory rabbit feeds can induce antibodies (Coombs et al. 1983). asGSP1 also bound a series of "streaky" bands with relative masses of between 45,000 and 67,000 (Fig. 2). These bands were artefacts due to the use of β-mercaptoethanol or DTT since they could be removed by the addition of iodoacetamide (data not shown) to remove excess reducing agent prior to electrophoresis (Hashimoto et al. 1983; Görg et al. 1988).

Fractionation of Mr 15,000 starch-granule protein and analysis with antibodies

A minor fraction of the starch Mr 15,000 polypeptide which was extractable with 0.1 M NaCl contained an

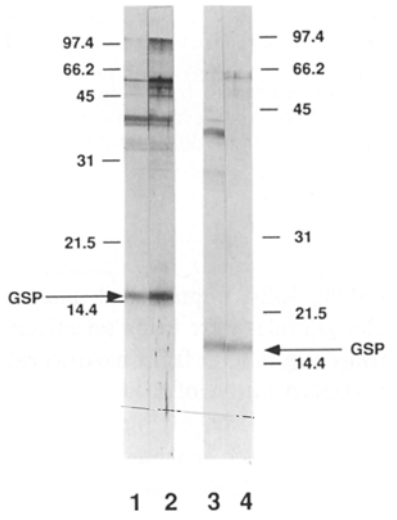

Fig. 2. Specificity and relative GSP-binding activities of asGSP1 determined by Western blotting. Cultivar Rosella wholemeal flour was extracted as described in Materials and methods. Strips (equivalent to about 1 mg of flour each) were immuno-stained with different dilutions of asGSP1. *Lanes:* (*1*) bleed 1, diluted 800×, (*2*) bleed 2, diluted 800×, (*3*) bleed 1, diluted 400×, (*4*) bleed 2, presorbed against a durum flour extract and diluted 1,000×. The mobility of molecular weight markers is given in kilodaltons. Note that *lanes 1 and 2* are from a different blot to *lanes 3 and 4*

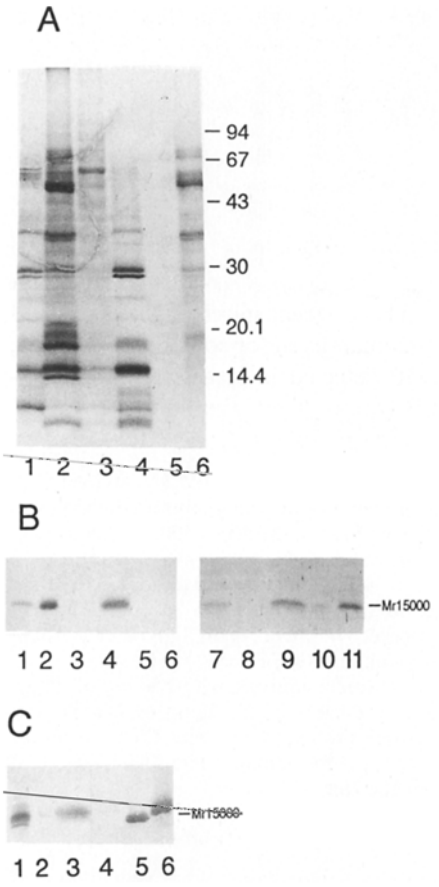

Fig. 3. Proteins extracted from (**A**) water-washed starch (cultivar Rosella) or (**B and C**) unfractionated wholemeal (Rosella) with different extractants, then separated by SDS-PAGE and stained with (**A**) Coomassie-R250 or (**B and C**) immuno-stained with asGSP1 (**B**) or with asAI (**C**). **A** *Lanes:* (*1–3*) sequential extractions of 0.5 g Rosella starch with (*1*) 0.1 M NaCl, (*2*) 1% SDS, (*3*) 1% SDS at 50 °C. (*4–6*) sequential extractions of another sample of Rosella starch with (*4*) 0.1 M Na-acetate, pH 4.4, (*5*) 0.1 M NaCl, (*6*) 1% SDS at 50 °C. Acetone precipitates equivalent to 250 µg dry weight were loaded in each lane. The relative positions of molecular weight markers (×1,000) are shown at right. **B and C** *Lanes:* (*1*) SDS-PAGE sample buffer-extract from 1 mg wholemeal flour, (*2*) acetone precipitate (200 µg) of 1% SDS-extract of starch, (*3*) acetone precipitate (400 µg) of 0.1 M NaCl extract of starch, (*4*) acetone precipitate (200 µm) of 1% SDS-extract of starch that had been pre-extracted with 0.1 M NaCl, (*5*) 0.31 α-amylase inhibitor (approximately 3 µg), (*6*) 0.28 α-amylase inhibitor (approximately 3 µg), (*7*) 0.1 M Na-acetate, pH 4.4-extract from 3.3 mg of wholemeal flour, (*8*) 0.01 M Na-acetate, pH 4.4-extract from 3.3 mg of wholemeal flour, (*9*) 0.1 M acetic acid-extract from 3.3 mg of wholemeal flour, (*10*) 0.1% SDS-extract from 1.1 mg of wholemeal flour, (*11*) 1% SDS-extract from 1.1 mg of wholemeal flour

α-amylase inhibitor (Figs. 3A, C) but its occurrence in starch-granule preparations was erratic and the abundance of α-amylase inhibitors in extracts of whole seeds was completely unrelated to softness (data not shown). The Mr 15,000 polypeptide fraction remaining after extraction with 0.1 M NaCl is here defined as "true" GSP, since this fraction was always present on starch from soft seeds (e.g., Fig. 1A). Comparison of lanes 1, 2 and 3 in Fig. 3B and C shows that although the binding of asAI to flour extracts was stronger than that of asGSP1, the reverse was observed with starch-extracts. This showed that the association of GSP with starch granules from soft wheats was much stronger than that of α-amylase inhibitors.

Accumulation of GSP in the whole seed

Western blots of protein extracts from macerated whole seeds were probed with asGSP1 to examine the occurrence of GSP in unfractionated endosperm (e.g., Fig. 4). GSP was detected in all the *T. aestivum* cultivars screened, but generally at lower levels in the hard wheats (Figs. 4 and 5). Relationships between the apparent level of GSP in seeds and seed softness were estimated (Fig. 5) and a positive correlation was found between seed softness and the level of GSP accumulated (Figs. 5C, D). This was particularly apparent in Symes' (1969) near-isogenic lines, since the soft lines in both the cultivar Heron- and cultivar Falcon-derived isogenic pairs had an approximately four-fold higher apparent level of GSP than the hard lines, as was the case when the parent cultivars were examined. Genes controlling accumulation of GSP in the seed therefore remained coupled to a softness or hardness

allele through the seven cycles of meiosis that occurred in the derivation of the near-isogenic lines (Symes 1969). This is strong evidence for linkage between softness alleles and alleles controlling accumulation of GSP in the seed.

Dispersion of GSP during dough washing

The disparity between the levels of GSP detected in starch extracts versus whole-grain extracts suggested that the loss of GSP from hard-wheat starch as it was purified may have been artifactual. Even for soft-wheat varieties, the amounts of GSP detected in seeds were about 20-times higher than expected from the amount of GSP extractable from starch granules (compare Fig. 1 B with Fig. 4 taking note of the weights of materials extracted). Examination with asGSP1 of the gluten, water-soluble, and starch fractions produced when purifying starch showed that most GSP remained in the gluten fraction when starch was washed from doughs (Fig. 6). Starch that had been squeezed out of a dough but not well-washed (i.e., crude starch contaminated with gluten) contained readily-detectable GSP whether it originated from soft or hard wheat; although the crude starch from soft wheats still contained more (Fig. 6). Since small starch granules bind much more GSP than larger granules (Sulaiman and Morrison 1990; Jolly, unpublished data) it was possible that smaller granules were being selectively purified from soft-wheat doughs than from hard-wheat doughs, resulting in a concentration of GSP in starch preparations from soft wheats. However, comparison of the size distributions, measured by laser scattering (as described in Konik et al. 1991), of starch granules purified by dough-washing from hard and soft wheats argued against this explanation since the mean percentage of B granules in starch purified from soft wheats was 21.6 ($n=4$, SD = 5.1) while the mean for hard wheats was 19.5 ($n=4$, SD = 2.5). Therefore, the loss of GSP from the crude starch of hard wheats during washing was not an artifact due to selective loss of small starch granules, but reflected differences in the way GSP, starch, and the gluten components interacted in the soft and hard wheats. While GSP was completely extractable from starch in 0.1 M Na-acetate, pH 4.4 (Fig. 3 A, lane 4), this solvent was a poor extractant of GSP from flours (Fig. 3 B, lane 7). Solvents capable of penetrating storage-protein bodies (e.g., 0.1 M acetic acid and 1% SDS, Fig. 3 B) were required to extract GSP from flours more efficiently.

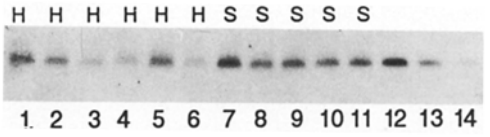

Fig. 4. GSP detected on Western blots by immuno-staining total-protein extracts from wholemeal flours with asGSP1. *Lanes:* (*1–6*) hard wheats, (*7–11*) soft wheats: (*1*) Cook, (*2*) Halberd, (*3*) Eagle, (*4*) Wilgoyne, (*5*) Oxley, (*6*) Miskle, (*7*) Rosella, (*8*) Matong, (*9*) Mokoan, (*10*) Egret, (*11*) Eradu, (*12–14*) *T. turgidum* var. durum + GSP standard (determined by BioRad dye-binding protein assay): (*12*) 2.6 µg GSP, (*13*) 1.3 µg GSP, (*14*) 0.7 µg GSP. GSP was immuno-stained with asGSP1, bleed 2, diluted 1,500×. Each lane contains an extract equivalent to 1 mg of wholemeal flour

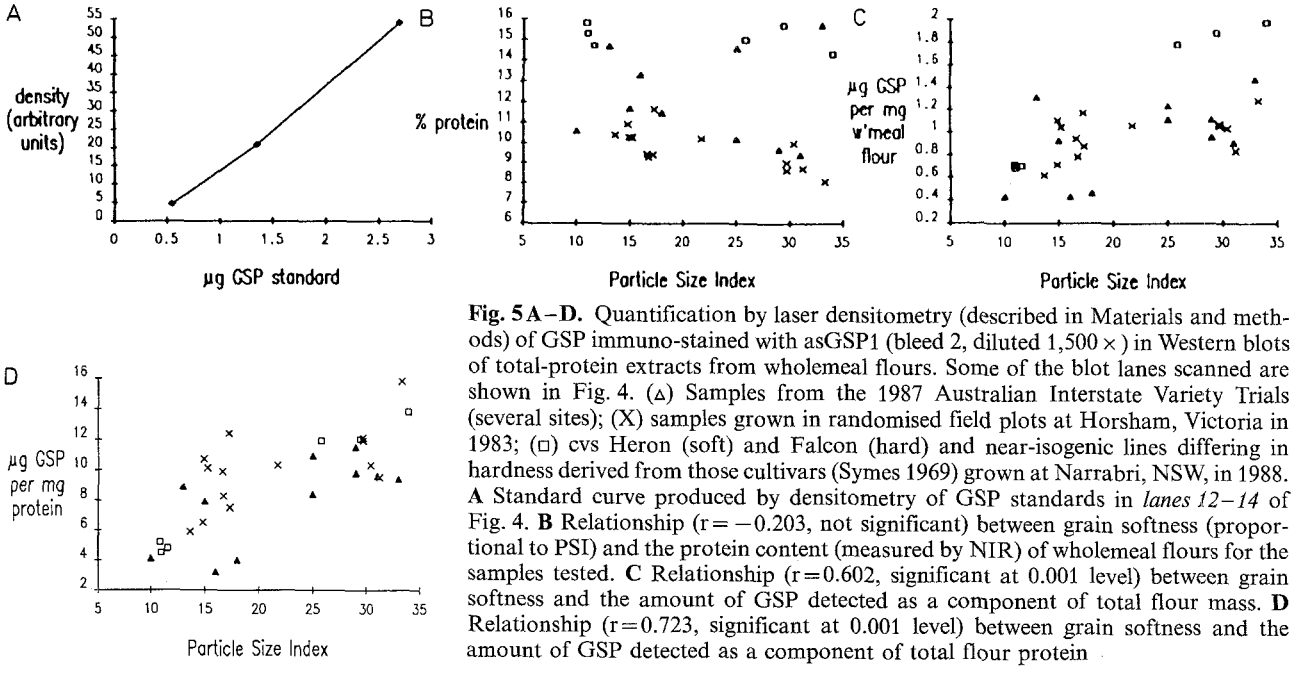

Fig. 5 A–D. Quantification by laser densitometry (described in Materials and methods) of GSP immuno-stained with asGSP1 (bleed 2, diluted 1,500×) in Western blots of total-protein extracts from wholemeal flours. Some of the blot lanes scanned are shown in Fig. 4. (△) Samples from the 1987 Australian Interstate Variety Trials (several sites); (X) samples grown in randomised field plots at Horsham, Victoria in 1983; (□) cvs Heron (soft) and Falcon (hard) and near-isogenic lines differing in hardness derived from those cultivars (Symes 1969) grown at Narrabri, NSW, in 1988. **A** Standard curve produced by densitometry of GSP standards in *lanes 12–14* of Fig. 4. **B** Relationship ($r=-0.203$, not significant) between grain softness (proportional to PSI) and the protein content (measured by NIR) of wholemeal flours for the samples tested. **C** Relationship ($r=0.602$, significant at 0.001 level) between grain softness and the amount of GSP detected as a component of total flour mass. **D** Relationship ($r=0.723$, significant at 0.001 level) between grain softness and the amount of GSP detected as a component of total flour protein

Chromosomal control of GSP accumulation

Total-protein extracts of seeds from a set of chromosome-engineered lines derived from cultivars Chinese Spring (soft) and Gabo (hard) were assayed for GSP by immuno-blotting. The Chinese-Spring set represented deletions of every chromosome or chromatid except for chromosome 4A and the short arm of chromosome 2A. The use of seed-extracts overcame the potential problem of artifacts due to the study of purified starch granules. GSP was readily detectable in extracts of all seeds except those in which chromosome 5D or its short arm was missing (e.g., Fig. 7). GSP was completely indetectable in seeds of Gabo where the short arm of chromosome 5D was deleted, but sometimes very faintly detected in Chinese Spring lines where it was deleted. This demonstrates that the major gene(s) controlling accumulation of, and therefore probably coding for, GSP is on the short arm of chromosome 5D. The inconsistent detection of GSP in Chinese-Spring lines lacking chromosome arm 5DS may have been due to the presence of diverged or poorly-expressed GSP genes on other chromosomes.

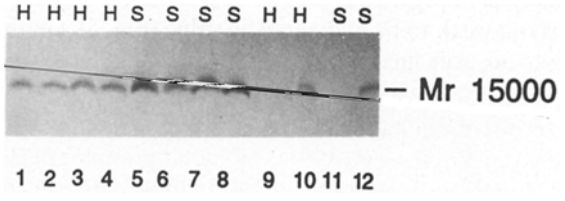

Fig. 6. Distribution of GSP to the crude starch, water-soluble, and gluten fractions during dough-washing. *Lanes: (1–4)* extracts of crude starches from hard cultivars: *(1)* Eagle, *(2)* Wilgoyne, *(3)* Cook, *(4)* Halberd; *(5–8)* extracts of crude starches from soft cultivars: *(5)* Rosella, *(6)* Egret, *(7)* Matong, *(8)* Mokoan; *(9 and 11)* water solubles, *(10 and 12)* gluten: *(9 and 10)* Cook, *(11 and 12)* Rosella. SDS-PAGE sample buffer-extracts equivalent to 200 µg of crude starch-granule protein, 10 mg of water solubles and 1 mg of powdered gluten were separated by SDS-PAGE and blotted. GSP was detected by enzyme-linked immuno-staining with asGSP1, bleed 2, at a dilution of 1,500×. Further washing of the crude starches to produced well-washed starch gave the results shown in Fig. 1

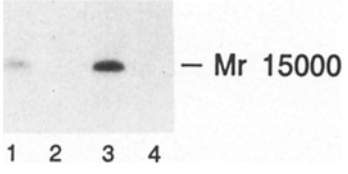

Fig. 7. Immuno-detection of GSP in chromosome-deficient lines. *Lanes: (1)* Gabo, hard; *(2)* Gabo, monotelosomic 5DL, hard; *(3)* Chinese Spring, soft; *(4)* Chinese Spring, ditelosomic 5DL, hard. Samples equivalent to 1 mg of flour were fractionated by SDS-PAGE and blotted. GSP was detected by enzyme-linked immuno-staining with asGSP1, bleed 2, diluted 1,500×

Whole-seed extracts of 21 *Triticeae* species were also assayed for GSP by immuno-blotting. GSP was detected in seed-extracts of *T. monococcum* (A), *T. urarta* (A). *Secale cereale* (R), *T. spelta* (ABD), *T. vavilovii* (ABD), *T. macha* (ABD), *T. compactum* (ABD), *T. uniaristatum* (Un), *T. sphaerococcum* (ABD), *T. tauschii* (D), *T. sharonense* (S1), *T. speltoides* (S), *T. bicorne* (Sb), *T. dichasians* (C), *T. comosum* (M) and *T. recta* (UMUMUn) accessions at levels comparable to *T. aestivum*. Within most of these species a range of GSP accumulation was observed as in *T. aestivum*. A trace amount of GSP or GSP-like protein was detected in *Hordeum vulgare* (H), *T. timopheevi* (AG), *T. umbellulatum* (U) and *T. neglecta* (UMUM) accessions, while no GSP was detectable in any *T. turgidum* var. durum (AABB) accessions or in one *T. vavilovii* accession. This shows that functioning GSP genes occur in genomes other than the D-genome and that grain-softness might be correlated with GSP accumulation in many *Triticeae* species.

Amino-acid sequencing of GSP

GSP was purified by preparative SDS-PAGE to homogeneity as determined by silver-staining of analytical SDS-PAGE gels (data not shown). However, amino-terminal sequencing showed that this material was nevertheless heterogeneous. The yields of the PTH-derivatives at each cycle indicated the presence of a major sequence and at

Table 1. Amino-acid sequences obtained for GSP. Residues in brackets are tentative

N-terminal amino acid sequence:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
E	V	G	G	G	G	G	S	Q	E	P	P	Q	E	R	K	L	N

Partial sequences of major peptides isolated from lysyl-endopeptidase digests of GSP:

(1)	V	I	Q	E	A	K		(2)	G	G	E	E	H	E	V	(3)	A	R	T	V	Q	T	A				
(4)	Q	L	Q	R	A	Q	S	(5)	S	Y	V	Y	E	Q		(6)	D	Y	V	X	E						
(7)	N	F	P	V				(8)	A	(L)	A	F	P			(9)	E	V	G	G	G	G	G	S	Q	E	P

Partial sequences of major peptides isolated from chymotrypsin digests of GSP:

(10)	S	Q	I	A	P	Q		(11)	R	G	Q	V	F	L		(12)	L	G	I	R			

least two minor sequences. Disparity between the yields and the amount of material sequenced also suggested that it was possible that some polypeptides were blocked at the N-terminus. The major sequences found in the analyses are presented in Table 1. Sequence analysis of the major peptides isolated from protease-digests of GSP confirmed the major N-terminal sequence found for intact GSP and provided partial sequences of 11 other peptides from GSP as shown in Table 1.

Discussion

Mr 15,000 polypeptides that remained on starch granules washed with 0.1 M NaCl have been operationally defined as grain-softness protein(s) (GSP). The accumulation of GSP has been correlated with grain softness using purified starch fractions (Greenwell and Schofield 1986; this paper) and now also for the first time using whole-seed extracts. The detection of multiple N-terminal amino-acid residues indicates that GSP consists of more than one type of polypeptide. However, these polypeptides have not been detectably separated by isoelectric focussing (data not shown). Attempts were made to analyse GSP heterogeneity further by reversed-phase HPLC, but the poor solubility of GSP hampered these attempts.

Most of the characterised Mr 15,000 polypeptides in wheat grains are closely related members of a so-called 2S superfamily of proteins, usually called "CM"-proteins in wheat, which appear to serve dual roles as storage proteins and/or as inhibitors of exogenous hydrolases (for review see Garcìa-Olmedo et al. 1987). Since *T. turgidum* contains abundant amylase inhibitors and CM-proteins, the lack of binding of asGSP1 to durum seed-extracts showed that the major component of GSP is not closely related to any characterised CM-proteins, although the DYVXE peptide (Table 1) is similar to sequences in the CMd (barley), CM3 (wheat) and CM16 (wheat) albumins (see Garcìa-Maroto et al. 1990). Therefore, the determination of the complete sequences of GSP polypeptides will be required to establish their relationship to the 2S superfamily. All of the peptides in Table 1 were compared with protein databases (Swissprot, PIR and Genpeptide) using the GCG, version 6 software (Devereaux et al. 1984). Although many similarities, such as that mentioned above, to published sequences were found [the most significant similarity being a perfect match of the SQIAPQ peptide the to N-(5′-phosphoribosyl)-anthranilic acid isomerase and the indole-3-glycerol phosphate synthetase encoded by the *Escherichia coli* tryptophan operon – see Christie and Platt 1980], no protein or class of proteins with similarities to more than one or two GSP derived peptides were found. Oda et al. (1992) reported a similar N-terminal sequence for this protein.

Greenwell and Schofield (1986) concluded that GSP accumulation depended on chromosome 5D. However, as their methods only allowed for the detection of GSP associated with starch granules, it remained possible that in negative samples the GSP present had merely been lost from the starch fraction. Our results rule out that possibility and demonstrate clearly that the short arm of chromosome 5D controls GSP accumulation. Antiserum screening indicated that, generally, more GSP accumulates in soft wheats than in hard wheats. However, unlike the analysis of proteins from starch granules, the correlation is too weak to be of diagnostic value (Fig. 5). On the other hand, soft lines derived from cultivars Heron and Falcon (Symes 1969) accumulated GSP to much higher levels in the seed than did near-isogenic lines carrying the allelic hardness gene (Fig. 5 D). The genes that determine softness in Symes' near-isogenic lines are presumably *Ha* alleles. That is, the *ha* allele (hard) is linked with a genes(s) that causes GSP accumulation to be low, while the *Ha* allele (soft) is linked with a gene(s) that causes GSP accumulation to be high.

In many *Triticeae* species GSP, or GSP-like protein (as detected with asGSP1), was found to accumulate in the absence of the D-genome (as also found by Morrison et al. 1992 when they analysed starch extracts). This shows that GSP genes do occur elsewhere than chromosome 5D, probably on chromosome 5A and homoeologous chromosomes. In the polyploid species, such as *T. turgidum* (AABB) and *T. aestivum* (AABBDD), they are either not significantly expressed or have diverged sufficiently from the GSP gene on chromosome 5D for their products not to be recognised by asGSP1. Since the GSP in *T. monococcum* (soft) and other wheat species (Morrison et al. 1992) associates with starch, at least some non-5D-encoded GSPs are functionally similar to the GSP expressed in *T. aestivum*.

Barlow et al. (1973) suggested that grain hardness resulted from greater adhesion in the endosperm between starch granules and the surrounding matrix of storage protein. This conclusion was based on a microscale analysis of the hardness of endosperm components and upon electron- and light-microscope analysis of the way the endosperm fragmented (Simmonds 1972; Barlow et al. 1973; Simmonds et al. 1973). Schofield and Greenwell (1987) proposed that the presence of GSP at the starch/protein interface may act to reduce adhesion and thus produce a soft wheat. The absence of the protein at the interface thus results in a hard wheat by default. Their model was proposed in the absence of evidence that hard wheats produce significant amounts of GSP. By screening wholemeal extracts with an antiserum specific for GSP we have shown that GSP accumulates in both hard and soft wheats, and to levels much higher than indicated by analysis of the proteins associated with starch granules. However, these observations can still be accomo-

dated by a modification of the Greenwell-Schofield model by proposing that the GSP that associates with starch granules represents a unique fraction, found only in soft wheats, of the total (mostly gluten-associated) GSP-like proteins in the seed.

Alternatively, it can be proposed that the GSP detected in hard and soft wheats is identical, but the soft-grain phenotype requires a threshold level of GSP accumulation. If this is so, then the factors that determine the threshold level are not simple. Some wheats with apparently similar levels of GSP in the seed were shown to differ greatly in hardness. However, a clue may exist in the finding that the correlation between grain softness and GSP accumulation was improved if corrected for seed-protein content (Fig. 5). Stenvert and Kingswood (1977) hypothesized that increasing continuity in the storage-protein matrix resulted in increasing grain hardness, and more recent data has been consistent with this (Glenn and Saunders 1990). The poor extractability of GSP from lours in solvents incapable of extracting protein bodies indicates that much GSP in the seed is probably present in protein bodies. Thus, higher levels of storage-protein accumulation might raise the threshold required for the accumulation of GSP to result in a soft grain by "neutralising" an increasing proportion of the GSP present.

In conclusion it has been suggested above that GSP may be a mixture of proteins, all encoded by the short arm of chromosome 5D and possibly all by genes at the *Ha* locus. This implies that the *Ha* locus may not be a single gene, but a "minimum recombination unit" containing several genes that rarely recombine. In combination, the linkage between *Ha* and GSP alleles, the correlation between apparent levels of GSP in the seed and seed softness, and the marked difference in the interaction of GSP (or a GSP fraction) from hard and soft wheats with starch granules are strong circumstantial evidence that GSP is encoded at the *Ha* locus. However, further work is needed to prove this.

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