Simple and Rapid Method for Purifying Low Molecular Weight Subunits of Glutenin from Wheat[1]

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ABSTRACT

A method for the preparation of low molecular weight glutenin subunits (LMW-GS) of bread wheat without contamination by high molecular weight glutenin subunits (HMW-GS) or gliadins is described. Using a simple protocol based on the selective precipitation by acetone, two fractions are obtained that correspond to HMW-GS and LMW-GS. The protein fractions can be obtained either reduced or reduced and alkylated. The protocol can be scaled up to obtain large quantities of LMW-GS.

Low molecular weight glutenin subunits (LMW-GS) are an important but relatively little known class of wheat proteins representing about 70% of glutenins and 20–30% of total proteins (Payne and Corfield 1979). They are polypeptides with a molecular mass of less than 60,000 kDa containing glutenins, large polymers linked by disulfide bonds or by noncovalent association between LMW-GS and HMW-GS (see review by Melas et al 1993). The first studies on glutenin subunits dealt almost exclusively with HMW-GS. Payne et al (1979) demonstrated their allelic variation and their technological importance. Studies on LMW-GS started later when Jackson et al (1983) researched common wheats and Autran and Berrier (1984) researched durum wheats. The importance of LMW-GS on dough quality has been shown only recently (Gupta and Shepherd 1987, 1988; Gupta et al 1990a,b).

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There have been a few attempts to purify LMW-GS, but this presented considerable difficulties because of the heterogeneous and insoluble nature of LMW-GS and their strong tendency to aggregate. Tatham et al (1987), encouraged by the work of Danno et al (1978), tried to use the difference in solubility between LMW-GS and HMW-GS in a 70% ethanol solution at different pH levels to separate HMW-GS from the other proteins, but LMW-GS remained contaminated by gliadins, albumins, and globulins. Wieser et al (1989) used a similar approach and managed to partly solubilize LMW-GS in a 70% ethanol solution at pH 7.6. Marchylo et al (1989) used still another procedure for the separation of HMW-GS by precipitation with 60% propanol, but LMW-GS remained contaminated by gliadins in the supernatant. Burnouf and Bietz (1984) took advantage of the hydrophobic tendencies of LMW-GS to separate them from HMW-GS with reversed-phase high-performance liquid chromatography (RP-HPLC), but they were only able to obtain very small quantities of purified proteins.

The method proposed in this study is based on the selective precipitation of LMW-GS by acetone and has the advantage of being simple and quick. Large quantities of the protein fraction corresponding to all LMW-GS in either a reduced or a reduced and alkylated state can be obtained.

MATERIAL AND METHODS

Extraction and Fractionation of Proteins

Proteins were extracted following the protocol established by Singh et al (1991) using ground grains and flour from two French wheat cultivars, Andain and Davidoc (11.6 and 11.8% protein, respectively).

Extraction solution A was 50% (v/v) isopropanol; solution B was 50% (v/v) isopropanol containing 0.08*M* Tris-HCl buffer, pH 8.0, without removing oxygen.

A 60° C oven was used for extraction, reduction, and alkylation. Albumin, globulin, and gliadin fractions were eliminated by suspending 300 mg of ground flour in 15 ml of solution A. Extraction lasted 30 min, with one agitation of the tubes (Heidolph vortex agitator) after 15 min. After centrifugation (5 min, 40,000 × *g*, 20° C), the supernatant was removed. Using a spatula and agitator, the residue was resuspended in 15 ml of solution A. Extraction was repeated as before, and the supernatant was removed. The residue was given a final wash in 7.5 ml of solution A.

The glutenins were extracted from the last residue (R) obtained. For the preparation of reduced and alkylated proteins, the residue (R) was resuspended in 1.5 ml of solution B containing 1% (w/v) dithioerythritol. After 30 min of reduction at 60° C and 5 min of centrifugation (40,000 × *g*, 20° C), the supernatant was recovered; 1.5 ml of solution B containing 1.4% 4-vinylpyridin was added. The supernatant (R-A) was recovered after 30 min of alkylation at 60° C and centrifugation (40,000 × *g*, 20° C).

For the preparation of reduced fractions, the residue (R) was resuspended in 1.5 ml of solution B containing 1% (w/v) dithioerythritol. After 30 min of reduction at 60° C and 5 min of centrifugation (40,000 × *g*, 20° C), the supernatant (R-NA) was recovered; 1.5 ml of solution B was added.

Pure acetone (2 ml) was added to the 3 ml of supernatant (R-A and R-NA) to give a final concentration of 40% (v/v). After a 10-min rest at 20° C and 5 min of centrifugation (40,000 × *g*, 20° C), a first residue composed mainly of HMW-GS subunits was recovered. The concentration of the supernatant (5 ml) was then increased to 80% (v/v) acetone by adding 10 ml of pure acetone. After 5 min of centrifugation (40,000 × *g*, 20° C), a second residue was obtained containing uniquely LMW-GS.

Electrophoresis

The acetone precipitates were dried and resuspended in 500 µl of the extraction solution (Tris/HCl 0.06*M*, pH 6.8, sodium dodecyl sulfate 2%, 2-mercaptoethanol 5%). The resulting solution (5 µl) was loaded on top of a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Gupta and MacRitchie (1991). Migration lasted 3 hr at an intensity of 20 mA per gel (140 × 115 × 0.75 mm) at 18° C. Gels were stained with Coomassie Brilliant Blue R250 (0.05% in 12%, w/v, trichloroacetic acid solution) and destained with 10% trichloroacetic acid solution according to Chrambach et al (1967).

Densitometric Analysis

After coloring and drying the gel, the electrophoretic diagrams were analyzed using an Ultroscan 2202 laser densitometer (LKB, Bromma, Sweden).

Data were acquired and processed to determine the percentages of the different fractions (LMW-GS, HMW-GS) using Spectra Station software (Spectra-Physics USA, San Jose, CA).

Nitrogen Determination

The amount of nitrogen in flour was determined using the Kjeldahl method in duplicate. Protein content was calculated using the conversion coefficient of 5.7. The amount of glutenin extracted and acetone precipitated was measured by weighing the dry residue. Because Tris was used for protein extraction (solution B), no nitrogen determination could be performed on the extracts and precipitates by acetone.

Amino Acid Composition

Proteins were hydrolyzed by 6*N* hydrochloric acid (1 ml of acid per milligram of protein) for 48 hr at 112° C.

Amino acids were separated on a Dionex DC-6A cation

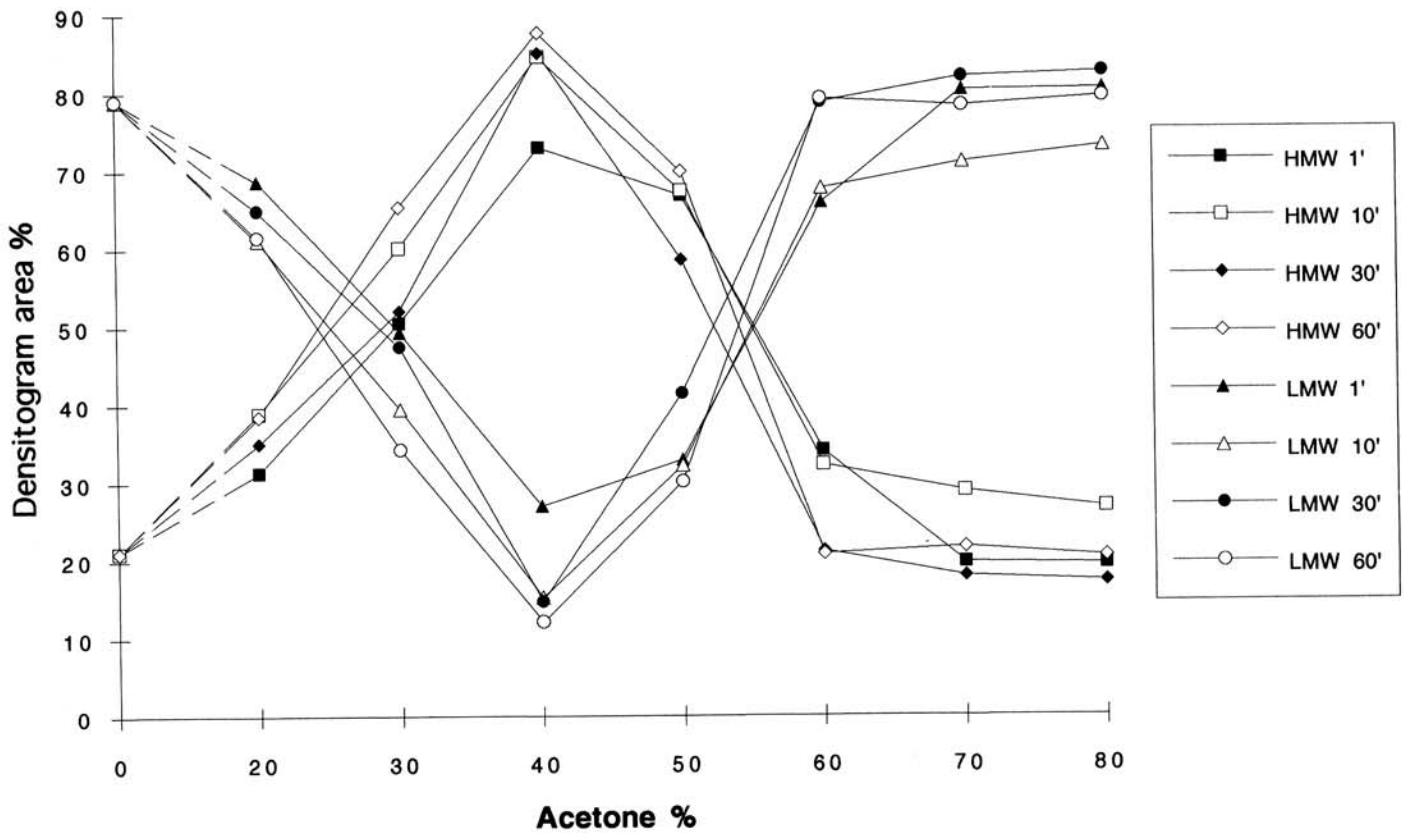

Fig. 1. Percentage of densitogram area representing high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) against the quantity of acetone added at 20° C. Proteins (0.4 mg) were precipitated by acetone concentrations of 20–80%. Contact time between acetone and glutenin extract was fixed at 1, 10, 30, and 60 min.

exchange column according to the Moore and Stein (1954) method with modifications (Benson et al 1967, Benson 1972).

RESULTS AND DISCUSSION

Precipitation by adding pure acetone was used previously to recuperate protein fractions from a solution (Scopes 1987, Dachkevitch 1989). During preliminary experiments, it was observed that all LMW-GS and HMW-GS subunits were precipitated at acetone concentrations equal to or greater than 80% (v/v). There was only partial precipitation at weaker concentrations, and the respective proportions of LMW-GS and HMW-GS subunits in the precipitate varied significantly according to the acetone concentration used. Therefore, we analyzed the effect of various test parameters such as increasing concentrations of acetone, contact time, temperature, and sample size to find an optimal method for the complete separation of LMW-GS and HMW-GS subunits.

Effect of Acetone Percentage on the Precipitation of Different Glutenin Subunits

The soluble properties of LMW-GS and HMW-GS were studied according to the percentage of acetone used as a precipitation agent. For this, equal volumes of reduced and alkylated (R-A), or reduced only (R-NA), supernatants were placed in a series of seven tubes. Acetone was added to give concentrations of 20, 30, 40, 50, 60, 70, and 80%. After 10 min of rest at 20°C and centrifugation (40,000 × *g*, 20°C), the different precipitates were resuspended in the extraction solution Tris-HCl-SDS-2-ME (0.4 mg per 100μl) and analyzed by SDS-PAGE. Acetone was added to the supernatants to achieve an 80% concentration to precipitate all the proteins. The precipitates were analyzed by electrophoresis as described above. The quantitative composition of LMW-GS and HMW-GS in fractions separated using different concentrations of acetone was determined by electrophoregram densitometry. Figure 1 shows the influence of acetone concentration on LMW-GS and HMW-GS fractions recovered in the different precipitates obtained from an R-A extract. Compared to the initial extract, which before acetone precipitation contained 20% HMW-GS and 80% LMW-GS, Figure 1 shows that the proportion of HMW-GS in the precipitates increases to a maximum value at 40% acetone, after which it decreases. The opposite is true for LMW-GS, where proportion decreases in the precipitates to achieve a minimum value at 40% acetone, after which it increases to reach 80% acetone, a value equivalent to that of the initial extract. It is thus a priori that a 40% acetone concentration gives the best separation of LMW-GS and HMW-GS subunits.

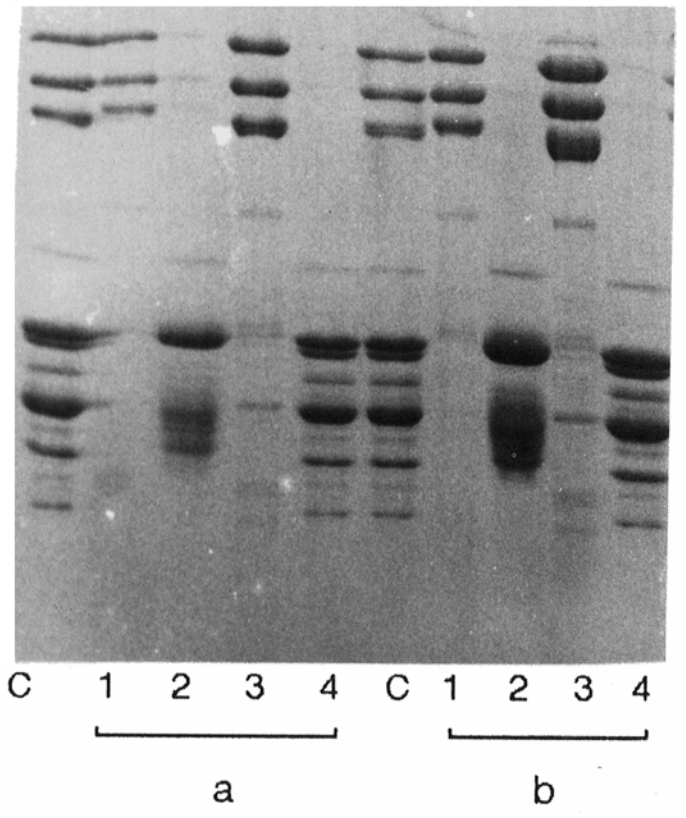

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% acrylamide) of high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). Reduced (lanes 1 and 2) and reduced and alkylated (lanes 3 and 4) fractions precipitated by a 40% acetone concentration (lanes 1 and 3). Supernatants made up to 80% acetone (lanes 2 and 4). Fractions 1 and 3 = HMW-GS. Fractions 2 and 4 = LMW-GS. Aliquots of 5 μl (15 μg for a), and 10 μl (30 μg for b). 15 μg of Andain glutenin control extract (C) was also loaded.

Effect of Contact Time and Temperature

The effect of contact time on extract (R-A) precipitation was tested for times between 1 and 60 min. These results are also shown in Figure 1. The behavior of glutenin subunits according to the acetone percentage did not change. In particular, no significant change was observed at over 10 min of contact time.

Test were performed at two different temperatures, 20 and 0°C. At 0°C (results not shown), the acetone concentration for maximum solubility of LMW-GS was 30%, versus 40% at 20°C. This indicates that when the temperature varies, the interaction of proteins with their solvent differs. However at 0°C, the difference in solubility between LMW-GS and HMW-GS was rapidly reduced by an increase in contact time. Because the differences in solubility between LMW-GS and HMW-GS were less at 0°C than they were at 20°C, the last temperature was used in the protocol.

Effect of Successively Increasing Concentrations of Acetone

In another series of experiments, a propanol extract was precipitated by a 20% acetone concentration. After the precipitate had been recovered, the supernatant was made up to a concentration of 30% acetone and then successively increased by 10% (using a rest time of 10 min) until it reached a concentration of 80%. All the precipitates and supernatants were then analyzed by SDS-PAGE to determine the respective proportions of LMW-GS and HMW-GS (results not shown).

The HMW-GS were no longer present in the supernatant at acetone concentrations higher than 50%, whether the series of precipitations were performed at 20 or 0°C. By contrast, LMW-GS appeared in precipitates obtained from acetone concentrations of 20–70%. No glutenin subunit appeared in supernatants obtained above 70% acetone. The LMW-GS was thus totally free of HMW-GS in the precipitates obtained from 60% acetone concentrations.

TABLE I
Amino Acid (mol%) Composition of Reduced Fractions Precipitated with 40% Acetone[a]

Amino Acid	Fraction 1[b]	2[c]	3[d]	4[e]
Asp	1.9	1.69	1.2–1.9	0.7–1.5
Thr	3.33	3.77	3.0–3.7	1.8–2.9
Ser	6.82	6.43	7.0–8.7	8.0–9.5
Glu	31.3	35.33	31.0–34.5	38–41.9
Pro	11.46	16.76	10.8–12.8	14.0–16.2
Gly	17.15	3.63	17.5–21.4	2.3–3.2
Ala	4.78	3.51	3.0–3.8	1.7–2.3
Val	4.92	5.08	1.9–2.9	3.8–5.0
Ile	1.57	4.69	1.0–1.9	3.6–4.4
Leu	5.25	7.84	3.7–4.9	5.3–7.5
Tyr	3.71	1.64	4.1–6.5	1.0–1.6
Phe	1.37	4.45	0.9–1.8	3.8–5.5
Lys	1.68	1.02	0.9–1.4	0.2–0.6
His	1.24	2.39	0.7–1.6	1.3–1.8
Arg	2.23	1.77	1.5–2.3	1.5–2.1

[a] Cys, Met, and Trp residues not determined.
[b] High molecular weight glutenin subunits (HMW-GS).
[c] Low molecular weight glutenin subunits (LMW-GS).
[d] Average composition of amino acids for HMW-GS (Moonen et al 1985).
[e] Average composition of amino acids for LMW-GS (Wieser et al 1990).

Furthermore, partial separation was observed between LMW-GS types B and C. The B subunits were totally precipitated from 60% acetone concentrations (the 60% acetone supernatant containing only C subunits); C subunits were still present in the 70% acetone precipitate. This series of precipitations confirms that the optimal acetone concentrations for selective precipitation of HMW-GS to achieve maximum recovery of pure LMW-GS are, respectively, 40 and 80%.

Preparation of Larger Quantities of LMW-GS

Having established a protocol for producing pure LMW-GS, it was necessary to verify whether the differences in solubility of the subunits would also apply on a scale capable of producing large quantities of pure LMW-GS.

An extraction was investigated with 4 g of Andain flour. Precipitation of glutenin subunits, reduced and alkylated using a 40% acetone solution, produced a 100-mg fraction containing mostly HMW-GS. A second precipitation at 80% acetone produced a 100-mg fraction containing pure LMW-GS, as revealed by SDS-PAGE electrophoregram (Fig. 2). What is more, the amino acid composition of reduced proteins (Table I) indicates that the 40% acetone precipitates, and those followed by 80% acetone precipitates, have an amino acid composition characteristic of HMW-GS and LMW-GS, respectively.

Physico-Chemical Base of Selective Acetone Precipitation

The selective precipitation of LMW-GS and HMW-GS by acetone is probably caused by differences in their molecular mass. According to Scopes (1987), the higher the molecular mass, the lower the percentage of acetone needed for precipitation. In principle, the acetone acts by reducing the activity of water, enabling electrostatic and Van der Waals forces to play their role. Our results might be explained by assuming that the larger the molecule size, the greater the chance that the surface will be charged, and therefore, the greater the chance of aggregation. It is also possible that the hydrophobic differences between the LMW-GS and HMW-GS subunits (as defined by the RP-HPLC elution order) helps to explain differences in their solubility in an organic solvent.

CONCLUSIONS

A protocol for the preparation of large quantities of reduced and alkylated LMW-GS has been established. A 3-hr process using extraction, agitation, and centrifugation produced 100 mg of 99% pure (as determined by densitometry) LMW-GS from 4 g of flour. Similar results have been obtained using reduced and nonalkylated glutenins.

A segregation between LMW-GS and HMW-GS was observed. LMW-GS fractions obtained from different varieties could be used in reconstitution experiments. For example, based on the possibility of reversible reduction-oxidation, as already shown by Békés et al (1992), the influence of different glutenin allelic types on the formation and protein complex properties of the dough could be determined.

The acetone method of precipitation can also be used as a prepurification step before fractionation of LMW-GS.

LITERATURE CITED

AUTRAN, J. C., and BERRIER, R. 1984. Durum wheats functional subunits revealed through heat treatments. Biochemical and genetic implications. Pages 175-183 in: Proceedings International Workshop on Gluten Proteins, 2nd. A. Graveland and J. H. E. Moonen, eds. TNO: Wageningen, The Netherlands.

BÉKÉS, F., GRAS, P. W., GUPTA, R. B., and WRIGLEY, C. W. 1992. Reversible reduction/oxidation of glutenin during dough mixing. Cereal Foods World 37:556.

BENSON, J. V., JR., 1972. Multipurpose resins for analysis of amino acids and ninhydrin positive compounds in hydrolysates and physiological fluids. Anal. Biochem. 50:477-493.

BENSON, J. V., JR., GORDON, M. J., and PATTERSON, J. A. 1967. Accelerated chromatographic analysis of amino acids in physiological fluids containing glutamine and asparagine. Anal. Biochem. 18:228-240.

BURNOUF, T., and BIETZ, J. A. 1984. Reversed-phase liquid chromatography of reduced glutenin, a disulfide-bonded protein of wheat endosperm. J. Chromatogr. 299:185-199.

CHRAMBACH, A., REIFELD, R. A., WYCKOFF, M., and ZACCARY, J. 1967. A rapid and sensitive method for the staining of proteins fractionated on polyacrylamide gels. Anal. Biochem. 20:150-154.

DACHKEVITCH, T. 1989. Etude des complexes protéiques de blé tendres par chromatographie liquide à haute performance de tamisage moléculaire (SE-HPLC): Relation avec la qualité technologique. Thése de doctorat d'état. Université des Sciences et Techniques des Languedoc: Montpellier, France.

DANNO, G., KANAZAWA, K., and NATAKE, M. 1978. Improved fractionation of constituent polypeptides from wheat glutenin. Agric. Biol. Chem. 42:11-16.

GUPTA, R. B., and MacRITCHIE F. 1991. A rapid one-step one-dimensional SDS-PAGE procedure for analysis of subunit composition of glutenin in wheat. J. Cereal Sci. 14:105-109.

GUPTA, R. B., and SHEPHERD, K. W. 1987. Genetic control of LMW glutenin subunits in bread wheat and association with physical dough properties. Pages 13-19 in: Proceedings International Workshop on Gluten Proteins, 3rd. R. Lásztity and F. Békés, eds. World Scientific: Singapore.

GUPTA, R. B., and SHEPHERD, K. W. 1988. Low-molecular-weight glutenin subunits in wheat: Their variation, inheritance and association with bread-making quality. Pages 943-949 in: Proc. Int. Wheat Genet. Symp., 7th. Cambridge.

GUPTA, R. B., BÉKÉS, F., and WRIGLEY, C. W. 1990a. Predicting values of LMW glutenin alleles for dough quality of bread wheat. Pages 615-620 in: Gluten Proteins 1990. W. Bushuk and R. Tkachuk, eds. Am. Assoc. Cereal Chem.: St Paul, MN.

GUPTA, R. B., MacRITCHIE, F., SHEPHERD, K. W., and ELLISON, F. 1990b. Relative contribution of LMW and HMW glutenin subunits to dough strength and dough stickiness of bread wheat. Pages 71-80 in: Gluten Proteins 1990. W. Bushuk and R. Tkachuk, eds. Am. Assoc. Cereal Chem.: St Paul, MN.

JACKSON, E. A., HOLT, L. M., and PAYNE, P. I. 1983. Characterization of high molecular weight gliadin and low molecular weight glutenin subunits of wheat endosperm by two-dimensional electrophoresis and the chromosomal location of their controlling genes. Theor. Appl. Genet. 66:29-37.

MARCHYLO, B. A., KRUGER, J. E., and HATCHER, D. W. 1989. Quantitative reversed-phase high-performance liquid chromatography analysis of wheat storage proteins as a potential quality prediction tool. J. Cereal Sci. 9:113-130.

MELAS, V., MOREL, M. H., and FEILLET, P. 1993. Les sous unités gluténines de faible poids moléculaire: des protéines d'avenir? Ind. Céréales 84:3-16.

MOONEN, J. H. E., SCHEEPSTRA, A., and GRAVELAND, A. 1985. Biochemical properties of some high molecular weight subunits of wheat glutenin. J. Cereal. Sci. 3:17-27.

MOORE, S., and STEIN, W. H. 1954. Modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211:907-913.

PAYNE, P. I., and CORFIELD, K. G. 1979. Subunit composition of wheat glutenin proteins isolated by gel filtration in a dissociating medium. Planta 145:83-88.

PAYNE, P. I., CORFIELD, K. G., and BLACKMAN, J. A. 1979. Identification of a high molecular weight subunit of glutenin whose presence correlates with breadmaking quality in wheats of related pedigree. Theor. Appl. Genet. 55:153-159.

SCOPES, R. K. 1987. Separation by precipitation. Pages 41-71 in: Protein and Purification, Principles and Practice, 2nd ed. Springler-Verlag: New York.

SINGH, N. K., SHEPHERD, K. W., and CORNISH, G. B. 1991. A simplified SDS-PAGE procedure for separating LMW subunit of glutenin. J. Cereal Sci. 14:203-208.

TATHAM, A. S., FIELD, J. M., SMITH, S. J., and SHEWRY, P. R. 1987. The conformations of wheat gluten proteins. II. Aggregated gliadins and low molecular weight subunits of glutenin. J. Cereal Sci. 5:203-214.

WIESER, H., SEILMEIER, W., and BELITZ, H.-D. 1989. Reversed-phase high-performance liquid chromatography of ethanol-soluble and ethanol-insoluble reduced glutenin fractions. Cereal Chem. 66:38-41.

WIESER, H., SEILMEIER, W., and BELITZ, H.-D. 1990. Characterization of ethanol-extractable reduced subunit of glutenin separated by reverse-phase high-performance liquid chromatography. J. Cereal Sci. 12:63-71.

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