

Biochemical Characterization of γ -75k Secalins of Rye

II. Disulfide Bonds

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ABSTRACT

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Aggregated γ -75k secalins were isolated by preparative reversed-phase (RP) HPLC of the prolamin fraction of rye flour Danko and were digested with thermolysin. The resulting peptides were pre-separated by gel permeation HPLC into eight fractions (G1–G8). Peptides that were linked by disulfide bonds (cystine peptides) were identified by means of differential chromatography (RP-HPLC before and after the reduction of disulfide bonds). The cystine peptides present in fractions G3–G7 were isolated by preparative RP-HPLC and characterized by sequence analysis and, in parts, by mass spectrometry. Accordingly, the eight cysteine residues of the C-terminal domain of γ -75k secalins were linked in the same positions as the intramolecular disulfide bonds of γ -gliadins of wheat. The cysteine

residue located at position 12 of the N-terminal domain and characteristic for γ -75k secalins was linked by an intermolecular disulfide bond with a corresponding residue of the same protein type. This cysteine residue is likely to be responsible for the aggregative nature of γ -75k secalins similar to a cysteine residue in the N-terminal domain of LMW subunits of wheat glutenin. In contrast to LMW subunits of glutenin, γ -75k secalins do not possess an additional cysteine residue in the C-terminal domain that forms a second intermolecular disulfide bond. Therefore, the polymerization of γ -75k secalins is limited and the formation of large gluten-like aggregates of rye storage proteins is restricted.

Disulfide bonds play a key role in determining structure and rheological properties of wheat gluten. Previous studies have demonstrated that the C-terminal domains of α - and γ -gliadins and the LMW subunits of glutenin contain three or four homologous intramolecular disulfide bonds (Köhler et al 1993; Müller and Wieser 1997). Two further cysteine residues, one located in the N-terminal domain and the other in the C-terminal domain, are unique to LMW subunits and responsible for the aggregative character of this protein type. HMW subunits of glutenin are also involved in both inter- and intramolecular disulfide bonds (Shewry and Tatham 1997) and, together with LMW subunits, they form large protein aggregates. Though partially homologous to gluten proteins, the storage proteins (secalins) of rye are not able to develop comparably high amounts of protein aggregates (Gellrich et al 2003) and are, therefore, not able to form a gluten-like protein mass. A different disulfide structure of secalins could be responsible for it, but disulfide bonds of secalins have not yet been determined. The aim of the present work was, therefore, to investigate disulfide linkages in aggregated secalins.

MATERIALS AND METHODS

Preparation of Protein Fraction BC

Nondefatted flour (45 g) of the rye cultivar Danko was extracted stepwise three times with 400 mL of 0.4 mol/L NaCl + 0.067 mol/L H₂NaPO₄ (pH 5.7) and three times with 400 mL of 60% (v/v) ethanol. Each extraction step was performed by homogenization using an Ultra Turrax homogenizer for 5 min at room temperature (RT \approx 20°C) followed by centrifugation for 10 min at 11,000 \times g and RT. The alcoholic extracts (prolamin fraction) were combined, dialyzed against 0.1 mol/L acetic acid and distilled H₂O, and freeze-dried. The obtained proteins (400 mg) were redissolved in 20 mL of 60% (v/v) ethanol, filtered through a 0.45- μ m membrane and chromatographed under the following conditions: column: Nucleosil 300-5-C₈ (4.6 \times 240 mm) (Macherey-Nagel, Düren, Germany); temperature: 50°C; elution solvents: (A) 0.1% (v/v) trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% (v/v) TFA; gradient: linear, 0 min 20% B, 30 min

60% B; injection volume: 500 μ L (500 μ L of 0.1% v/v TFA injected before and after sample injection); flow rate: 1 mL/min; detection: UV absorbance at 230 nm. The column effluent corresponding to fraction BC (Fig. 1) was collected and freeze-dried.

Enzymatic Hydrolysis

In preliminary experiments, fraction BC (1 mg) was incubated with thermolysin (Boehringer no. 161586) (40 μ g) in 2 mL of 0.1 mol/L Tris-HCl (pH 7.8 or 6.5) + 2 mmol/L CaCl₂ or with trypsin (Merck no. 24579) in 2 mL of 0.1 mol/L Tris-HCl (pH 7.8 or 6.5). The temperature was 37°C and the incubation time was 16 hr (thermolysin) and 8 hr (trypsin), respectively. The reaction was stopped by adjusting to pH 3.0 with 1 mol/L HCl. Aliquots of 500 μ L were then characterized by RP-HPLC. The preparative thermolytic and tryptic digestions were performed with 30 mg of fraction BC under corresponding conditions at pH 6.5.

Gel-Permeation Chromatography

The enzymatic hydrolyzates were separated by gel permeation (GP) HPLC under the following conditions: column: Superdex 30 (Pharmacia Biotech no. 9637121); temperature: RT; elution solvent: 0.06% (v/v) TFA; injection volume: 300–500 μ L; flow rate: 0.5 mL/min; detection: UV absorbance at 210 nm. The effluents containing different peptide fractions (G1–G8) were collected and freeze-dried.

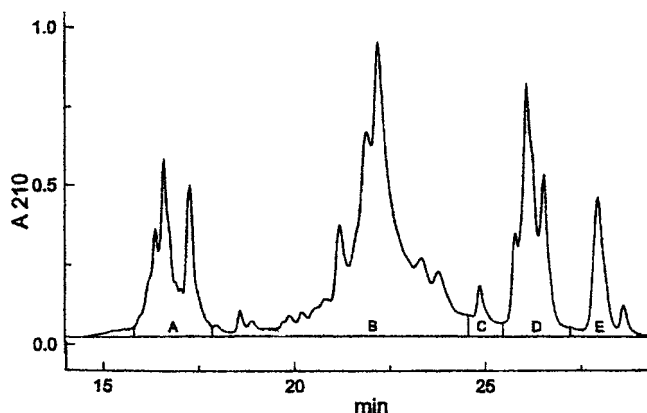


Fig. 1. Preparative RP-HPLC of the unreduced prolamin fraction of rye flour Danko on C₈ silica gel.

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Reversed-Phase HPLC

The analytical characterization of the enzymatic hydrolyzates, the analytical differential chromatography (before and after reduction of disulphide bonds) of peptide fractions G1–G8 and the preparative separation of peptides were performed under the following conditions: column: C₁₈ silica gel (5 μm, 12 nm), 4.6 × 240 mm (Thermoquest, Runcorn, UK); temperature: 50°C; elution system: (A) 0.01 mol/L of triethylammonium formate, pH 3.5 (TEAF), (B) TEAF/acetonitrile (60/40, v/v); gradient: stepwise linear, 0 min 20% B, 60 min 100% B (tryptic digest), and 0 min 2% B, 10 min 2% B, 130 min 80% B (thermolytic digest); injection volume: 50 μL (analytical runs) and 250–500 μL (preparative runs); flow rate: 1.0 mL/min; detection: UV absorbance at 210 nm. The rechromatography of peptides was performed under the same conditions, but with the elution system (A) 0.1% (v/v) TFA and (B) 0.1% (v/v) TFA + 99.9% (v/v) acetonitrile and a linear gradient of 0 min 5% B, 20 min 80% B.

Analytical Methods

Amino acid sequences of peptides were determined using a sequencing system Precise 492 (Applied Biosystems, Weiterstadt, Germany). Mass spectrometry of peptides was performed by means of a nanospray system (MSD/Protano A/S) and a LCQ mass spectrometer (Finnigan, Bremen, Germany).

RESULTS AND DISCUSSION

Preparation of Aggregated Secalins

The major portion of aggregated storage proteins (γ-75k and HMW secalins) of the rye flour Danko was previously shown to occur in the ethanol-soluble prolamins fraction (Gellrich et al 2003). This fraction was used as a starting material for the determination of disulfide bonds of aggregated secalins. The pH of solvents used

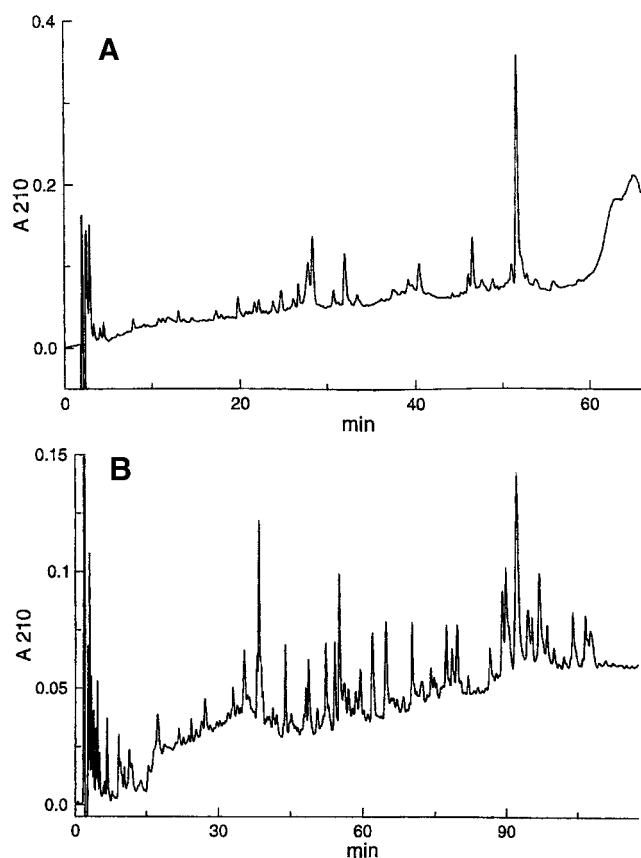


Fig. 2. RP-HPLC of the tryptic (A) and thermolytic (B) digest of prolamins subfraction BC on C₁₈ silica gel.

for the preextraction of the albumin-globulin fraction and the extraction of the prolamins fraction from Danko flour was decreased to 5.7 and 5.5, respectively, to minimize thiol-disulfide exchange reactions. The prolamins fraction obtained by the extraction with 60% ethanol was dialyzed, freeze-dried, and preparatively separated on C₁₈ silica gel. The chromatogram shown in Fig. 1 was in agreement with previous analytical studies (Gellrich et al 2003). The major subfraction BC containing the aggregated γ-75k and HMW secalins in a ratio of 17:1 was collected by numerous runs.

Isolation of Cystine Peptides

Fraction BC was digested with thermolysin and trypsin in parallel preliminary assays. RP-HPLC of the hydrolyzates demonstrated that the decrease from pH 7.8 (optimum for the enzymes) to pH 6.5 did not significantly change the peptide pattern; therefore, the lower pH was used for subsequent experiments. The chromatograms of the thermolytic and tryptic hydrolyzates shown in Fig. 2 revealed big differences. The digestion with thermolysin led to numerous major and minor peptides distributed over the whole range of elution, whereas the tryptic digest contained significantly less numbers and amounts of peptides, and the broad peak at the end of elution indicated either undigested proteins or large fragments. GP-HPLC of the hydrolyzates confirmed this presumption (Fig. 3): the thermolytic digest could be separated into eight peptide fractions (G1–G8), whereas the tryptic digest predominantly consisted of large components eluted within the void volume (G1). Accordingly, only the thermolytic digest was used for the preparation of peptides linked by disulfide bonds (cystine peptides). The peptide fractions G1–G8 obtained by GP-HPLC were analyzed by differential RP-HPLC (before and after reduction of disulfide bonds). Disappearance or height reduction of single peaks after reduction should indicate potential cystine peptides. The chromatograms of G1 and G8 did not show any peptide peak, and that of G2 did not change after reduction. G3–G7, however, contained peptides that changed their elution behavior after reduction (Fig. 4) and were, therefore, collected by several runs and purified by rechromatography (chromatograms not shown). Thereby, one to three pure peptides could be obtained by each rechromatography.

Amino Acid Sequences of Cystine Peptides

The isolated potential cystine peptides were analyzed by automatic sequence analysis and, in part, by mass spectrometry (Table I). Two or three amino acid residues were released by each degradation step of sequencing. The assignment of these residues to the sequence of each peptide and the identification of the location within the proteins could easily be done by comparison with previous sequence studies on γ-75k secalins P1 and P2 isolated from Danko (Gellrich et al 2004), with the complete DNA sequence of γ-75k secalin gSec 2A (Murray et al 2001), and with disulfide bonds of γ-gliadins from wheat (Müller and Wieser 1997). Al-

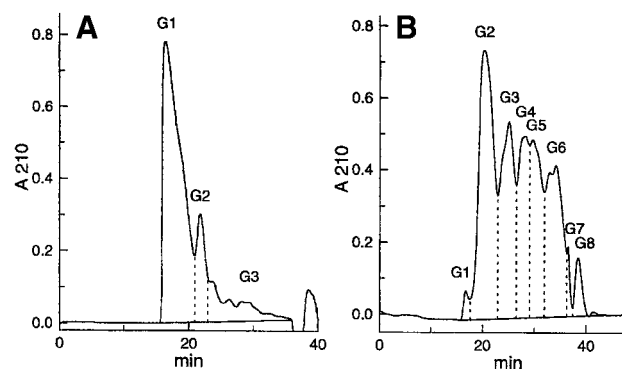


Fig. 3. GP-HPLC of the tryptic (A) and thermolytic (B) digest of prolamins subfraction BC on Superdex 30.

together, the sequences of 16 cystine peptides consisting of two or three cysteine peptides could be determined. Among these, 14 cystine peptides were derived from γ -75k secalins representing the linkages of cysteine residues C^d/C^a (G3-28d, G3-29c, G3-30c), C^c/C^{f1}C^{f2}/C^y (G3-15c, G3-16b, G3-19, G4-4c, G4-12f), C^d/C^e (G4-31c, G5-9c, G5-40c) and C^w/C^z (G6-41d, G6-45e, G7-30c) (Fig. 5). Because of the strict homology of the linkage types C^c/C^{f1}C^{f2}/C^y, C^d/C^e, and C^w/C^z with those of γ -gliadins, they are likely to be intramolecular as in γ -gliadins (Müller and Wieser 1997). Because peptides G3-28d, G3-29c and G3-30c revealed only one sequence, but disappeared in the HPLC pattern after reduction, it could be assumed that cysteine residue C^a corresponding to position 12 within the protein sequence formed an intermolecular linkage between two γ -75k secalins. The presence of the sequences VYVPRQC (peptides G6-41d and G7-30c) and IYVPRQC (peptide G6-45e) indicated the heterogeneity of the γ -75k secalin family. Altogether, the results demonstrated that all cysteine residues occurring in P1 and P2 have been acquired by at least one cystine peptide except C^b of P2 (Gellrich et al 2004).

One fragment peptide (VRPDCSN) of two cystine peptides (G5-13e, G6-25a) was derived from γ -40k secalins (unpublished). Because the second fragment peptide (LQC) occurred in both γ -40k and γ -75k secalins, it remained unclear whether the two cystine peptides represented an intermolecular linkage between both secalin types (C^w γ -75k/C^z γ -40k) or a linkage of γ -40k secalins (C^w γ -40k/C^z γ -40k) either intra- or intermolecular. Linkages of HMW secalins could not be detected obviously due to their low amount in fraction BC.

Disulfide Structure of γ -75k Secalins

The disulfide structure of γ -75k secalins postulated by Murray et al (2001) was substantiated by the present experiments. The structure of the C-terminal domain (divisions III, IV and V in Fig. 5) is closely related to that of monomeric γ -gliadins showing four homologous disulfide bonds and is similar to that of α -gliadins and LMW subunits of glutenin with three homologous disulfide bonds (Müller and Wieser 1997). In contrast to monomeric α - and γ -gliadins and analogous to LMW subunits and glutenin-

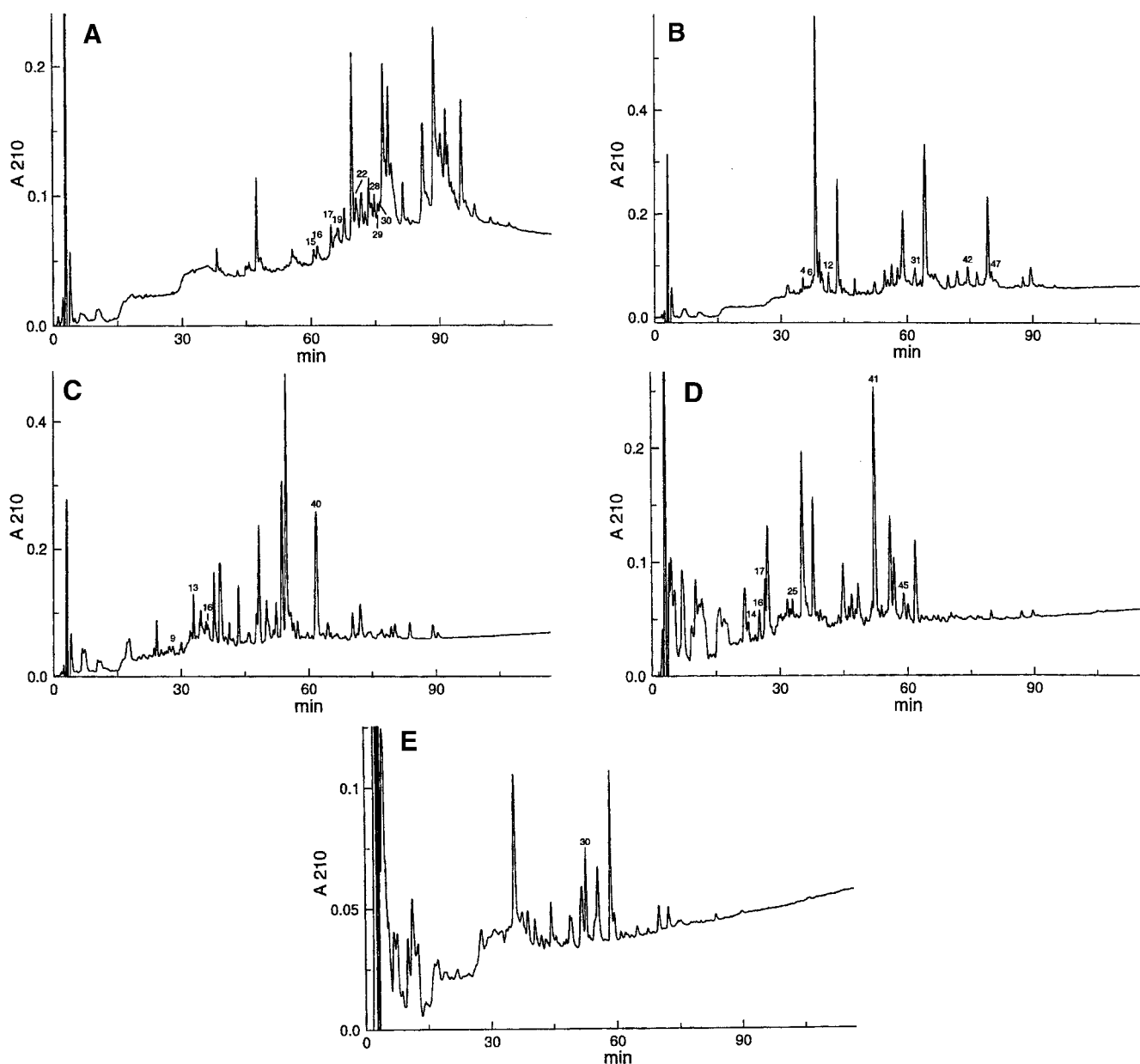


Fig. 4. RP-HPLC of peptide fraction G3 (A), G4 (B), G5 (C), G6 (D) and G7 (E) on C₁₈ silica gel (peaks marked with a number disappeared after reduction of disulfide bonds).

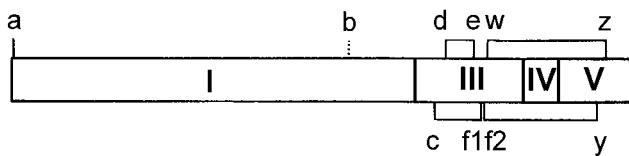


Fig. 5. Proposed disulfide bond structure of γ -75k secalins (designation of sequence divisions I-V according to Kasarda et al (1984) and of cysteine residues according to Köhler et al (1993)).

TABLE I
Amino Acid Sequences and Linkage Types of Cystine Peptides from Prolamin Fraction BC

| Peptide ^a | Sequences ^b | Linkage Type ^c | Mass ^d |
|----------------------|--|---------------------------------|-------------------|
| G3-28d, G3-29c | VQCPQQQPPFPQPOQ... | C ^a | - |
| G3-30c | VQCPQQQPPFPQPOQ... VNPSGQVQCPQOQ... | C ^a | - |
| G3-15c | VQCPQQQ... LNPCKN | C ^a | - |
| G3-16b, G3-19 | VMQQQCCQOQ... LQNLETCN LNPCKN | C ^{f1} C ^{f2} | - |
| G4-4c, G4-12f | VMQQQCCQOQ... VLQNLFTMCN LNPCKN | C ^{f1} C ^{f2} | - |
| G4-31c, G5-40c | VMQQQCCQOQ... MCN | C ^{f1} C ^{f2} | 1622.4, 1622.5 |
| G5-9c | IFPQSECC LQCCSP | C ^d | - |
| G6-41d, G7-30c | SECQ LQCCSP | C ^e | 1223.4, 1223.5 |
| G6-45e | LQC | C ^f | 1237.4 |
| G5-13e, G6-25a | VYVPRQC LQC IYVPRQC | C ^g | 1149.5, 1149.4 |
| | VRPDCSN | C ^h (γ -40k) | |

^a See Fig. 4.

^b One-letter codes for amino acids; ., incomplete sequence.

^c See Fig. 5.

^d -, Not determined.

bound γ -gliadins (Köhler et al 1993; Keck et al 1995), γ -75k secalins have an additional cysteine residue (C^d) in the N-terminal domain which is likely to be responsible for the aggregative nature of γ -75k secalins forming a disulfide bond with other unpaired cysteines in storage proteins (Murray et al 2001). Cysteine peptides G3-28d and G3-29c (Table I) indicate the formation of γ -75 secalin dimers. Contrary to LMW subunits, γ -75k secalins do not possess a further cysteine residue in division IV (C^b) which enables the polymerization of LMW subunits by a second intermolecular disulfide bond (Müller and Wieser 1997). Therefore, the γ -75k secalins such as P1 or gSec2A might act as chain

terminators (Kasarda 1989) preventing the formation of large aggregates. The role of a second cysteine residue (C^b) found in the N-terminal domain of P2 (Gellrich et al 2004) remains unclear.

CONCLUSIONS

The four disulfide bonds present in the C-terminal domain of γ -75k secalins are homologous with those of monomeric γ -gliadins of wheat and are, therefore, likely to be intramolecular. Analogous to glutenin-bound γ -gliadins and LMW subunits of glutenin, an additional cysteine residue is present in the N-terminal domain of γ -75k secalins which forms an intermolecular disulfide bond and thus is responsible for the aggregative nature of γ -75k secalins. In contrast to LMW subunits, γ -75k secalins do not possess an additional cysteine residue in the C-terminal domain that could form a second intermolecular disulfide bond. Therefore, the polymerization of γ -75k secalins and the formation of large gluten-like aggregates of rye storage proteins are restricted.

ACKNOWLEDGMENTS

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