Characterization by HPLC, MALDI mass spectrometry, and Edman degradation of a glutenin dimeric protein.

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ABSTRACT

The elasticity of wheat flour dough is determined by the ability of certain gluten proteins to form intermolecular disulfide bridges that link the proteins into a polymeric system. Gluten has two main fractions: the glutenin proteins have the capability of forming both intermolecular and intramolecular disulfide bonds whereas the gliadin proteins usually have an even number of cysteines that form only intramolecular disulfide bonds. However, nucleotide sequencing has indicated that some modified gliadins have an odd number of cysteine residues, which should cause them to be incorporated into the glutenin polymers. We have isolated by preparative HPLC a dimeric protein form having a gamma gliadin type of sequence for the constituent monomers and having an odd number of cysteine residues. Although glutenin polymers have a large average molecular weight in the millions, this dimer may be considered the simplest glutenin molecule. We have demonstrated by MALDI mass spectrometry and Edman degradation that the intermolecular cysteine bond between the two proteins occurs in the N-terminus of the molecule at position 26. The presence of proteins in glutenin that are capable of forming only a single intermolecular bond means that such proteins should act to terminate the growing polymer chains and lower the molecular weight distribution of the growing polymer. Larger amounts of such chain terminators in a cultivar might decrease dough elasticity.

These studies were carried out as part of a project directed toward the disulfide cross-linkages between glutenin proteins.

1.) Gluten proteins that were soluble in 0.1 normal acetic acid were extracted from wheat flour and after concentration by freeze-drying 100 mg portions of protein were dissolved in 6 ml of 0.1 N acetic acid and applied to a BioGel P-100 column.

2.) The column was eluted with 0.1 N acetic acid and the fractions ere screened for the presence of glutenin polymers using native and reducing SDS gel electrophoresis.

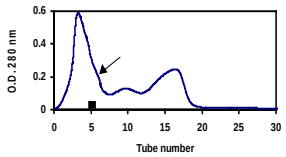

Fig. 1) BioGel P-100 gel filtration of acid soluble gluten proteins. The arrow indicates the region of the elution profile that was further examined. Molecules eluting from the column in this region have a molecular weight, as determined by SDS gel electrophoresis, of about 60kD.

The fractions of interest from the BioGel P-100 column were further separated using a Vydac semipreparative C-18 column. Concentration of the sample was effected either by evaporation using a SpeedVac or by direct injection of BioGel P-100 fractions in volumes from 4 to 20 ml of 0.1 N acetic acid onto the Vydac semipreparative column.

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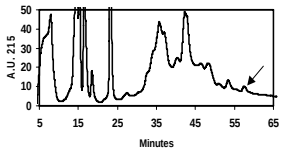


Fig. 2.) HPLC elution profile resulting from the injection the fraction from the BioGel P-100 column. Gradient was from 70% solvent A to 70% solvent B in 55 minutes . Flow rate was 1.5 ml per minutes, both solvent A and solvent B were 0.1% in trifluoroacetic acid. Solvent B was 6.75 parts acetonitrile, 2.75 parts 2-propanol, and 1 part water.

The fraction indicated by the arrow was collected from a number of HPLC runs. All subsequent HPLC manipulations were carried out using a Higgens 2.1mm x 2 cm guard column to minimize losses.

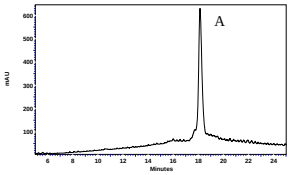


Fig. 3) Rechromatography of the peak indicated by the arrow in figure 2. The gradient was from 90% of solvent A to 90% of solvent B in 20 minutes starting at five minutes after injection. The column was eluted at a flow rate of 1 ml per minute, using 0.1% TFA as the initial solvent and a one-to-one mixture of acetonitrile/ 2 propanol that was 0.1% in TFA. The column was a 2.1 mm x 2 cm Higgins guard cartridge. Detection was at 215 nm

Ten percent of peak A from Fig. 3 was used for mass determination by MALDI mass spectrometry.

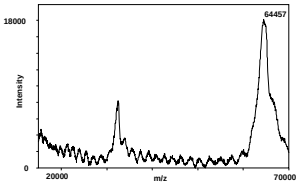


Fig. 4.) Mass spectrum of the HPLC purified component A from figure 3. Data were obtained using a Bruker Reflex II operated in the positive ion mode. Samples of the dissolved protein were mixed with an equal volume of saturated matrix (3,5-dimethoxy-4-hydroxy cinnamic acid) prepared in 30% acetonitrile which was 0.1% TFA. Approximately 0.5 microliters of the sample was spotted on the MALDI target and allowed to dry at room temperature before analysis.

After reduction and alkylation the decrease in mass indicated (Fig. 5) that the starting material was in the form of a disulfide-linked dimer.

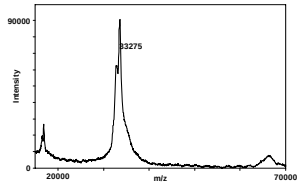


Fig. 5.) Reduction and alkylation of the glutenin peak (A) from Figure 3 was carried out by dissolving the sample in 50 microliters of 6M guanidine hydrochloride which was 0.1M in Tris-HCl, pH8.2 and contained 4 mg per ml of dithiothreitol and allowing reduction to proceed at room temperature for 30 minutes. Alkylation of the reduced protein was carried out by adding 1.4 equivalents of 4-vinylpyridine to DTT thiol groups and allowing the reaction to proceed for 30 minutes at room temperature. Alkylation was terminated by acidification with 88% formic acid. The reduced and alkylated components were immediately diluted fivefold with HPLC running buffer and purified by reverse phase chromatography on a Higgins 2.1 mm x 2 cm guard column (Fig. 3) and analyzed by mass spectrometry as described in Fig. 4.

When the remainder of Peak A from Fig. 3 was subjected to cyanogen bromide fragmentation the pattern shown in Fig. 6 was obtained. Sequencing of the fractions indicated that the early eluting peak (B) exhibited only the N-terminal sequence of the protein.

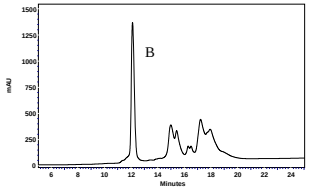
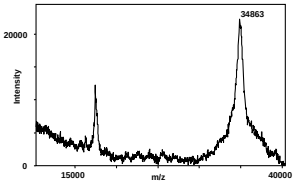


Fig. 6) HPLC separation of cyanogen bromide fragmentation of Peak A from Fig. 3. The cyanogen bromide fragmentation was carried out by dissolving the protein in 50 µl of 6M guanidine hydrochloride which was 0.1 M in HCl. Ten µl of 5M cyanogen bromide in acetonitrile was then added and the reaction allowed to proceed in the dark overnight at room temperature. The reaction mixture was then diluted fivefold with 0.1% TFA and injected onto the HPLC column. Chromatography conditions were identical to those described in Fig. 3.



MALDI mass spectrometry of the N-terminal fragment (B) from Fig. 6 showed that it consisted of one component with the mass of 34 863. Reduction with dithiothreitol of a portion of fragment B from Fig. 6 followed by MALDI mass spectrometry (Fig. 7) revealed that it had a mass of 17,524. Following reduction and alkylation of another portion of fragment B from Fig. 6 was shown by mass spectrometry (Fig. 8) to have a mass of 17,638. The difference of 114 mass units (105 expected from 4-vinylpyridine) suggested that the N-terminal fragment contained a single cysteine crosslink.

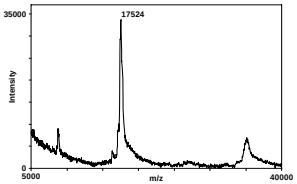


Fig. 7 Mass spectrum of reduced peak B from Fig.6. Reduction was as described for Fig.5, and mass spectrometry was as described in Fig. 4.

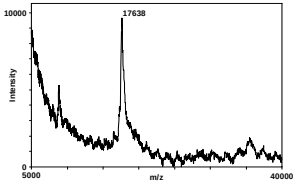


Fig. 8 Mass spectrum of reduced peak B from Fig. 6. Reduction was as described for Fig. 5 and mass spectrometry as described in Fig. 4.

Edman degradation of the reduced and alkylated N-terminal cyanogen bromide fragment (B) from Fig. 6 revealed the presence of a pyridyethyl group at position 24. Sequence interpretation was complicated by the occurrence of an ASP-PRO cleavage at position 3.

The expected sequence, based upon previous sequencing experiments in our laboratory was:

N M Q V D P S G Q V Q W P Q Q Q P F P Q P Q Q P F -

After cyanogen bromide fragmentation the following sequences were obtained for the alkylated peak B from Fig. 6 :

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                    1         2
          1    5    0    5    0    5
1⁰        Q V D P S G Q V Q W P Q Q Q P F P Q P Q Q P F C Q
2⁰        P S G Q V Q W P Q Q Q P F P Q P Q Q P F C -
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Discussion

The N-terminal sequence of the protein which we were investigating was similar to that of a nucleotide sequence reported by Scheets and Hedgcoth(1988) that possessed an odd number of cysteine residues. Eight of the nine cysteine residues reported in that molecule have been shown to be involved in intramolecular disulfide bridges whereas the extra cysteine residue located at position 26 in the amino acid sequence is believed to form an intermolecular disulfide bond which would allow it to be incorporated into the glutenin polymer fraction. The results of our investigation indicate that the cysteine residue at position 26 in the native molecule is involved in intermolecular disulfide bond formation.

The presence of proteins in glutenin that are capable of forming only a single intermolecular bond means that such proteins should act to terminate the growing polymer chains. It is likely that the glutenin protein reported upon here is able to crosslink to other types of glutenin molecules and disrupt extended polymer formation. Larger amounts of such chain terminators in a cultivar might decrease dough elasticity(D'Ovidio et. al. 1995) The formation of the γ-γ dimer described here is a special case that may not be typical. Most of the γ– type subunits are probably linked to other types of glutenin subunits in the glutenin polymers.

References

D'Ovidio, R., Simeone, M., Masci, S., Porceddu, E., Kasarda, D. "Nucleotide Sequence of a γ-Type Glutenin Gene from a Durum Wheat: Correlation with a γ-Type Glutenin Subunit from the Same Biotype. Cereal Chem. (1995) 72(5):443-449.

Scheets K., Hedgcoth C. "Nucleotide sequence of a gamma gliadin gene: comparisons with other gamma gliadin sequences show the structure of gamma gliadin genes and the general primary structure of gamma gliadins" Plant Sci. 57 (1988) 141-150.