Effect of Different Heating Conditions on the Extractability of Barley Hordeins

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Summary The extractability of hordeins from barley grains was investigated after wet and dry heating conditions. It was found that the amount of hordeins extractable with 55% 2-propanol decreased in a time-dependent manner after barley grains were steamed (wet heating), whereas hordeins showed no effect from heating in an oven at 100°C for up to 120 min (dry heating). The result of SDS-PAGE analysis revealed that B-hordein decreased time-dependently in extractability with wet heating and had almost completely disappeared by 60 min, but C-hordein remained unchanged until 120 min. With the use of the hordein fraction prepared from the nonheated barley grains, it was confirmed that B-hordein suspended in boiling water lost solubility in 55% 2-propanol. The insolubilized B-hordein was redissolved by the addition of 2-mercaptoethanol to 1%, which suggested that the intermolecular disulfide bonds would play a significant role in the loss of solubility. On the other hand, C-hordein did not lose solubility from being heated under the same conditions.

Key Words B and C-hordeins, extractability, heating treatment, barley hordein

Heating is necessary to gelatinize and soften cereal starch. During the heating process, most of the proteins from cereals undergo denaturation. In regard to dough from wheat, it is reported that heat processing leads to an insolubilization by 80% of ethanol-extractable protein (1, 2). There is a report that cooked barley protein is less digestible than uncooked barley protein (3). This report suggests that protein preparation from barley grain will surely undergo denaturation by heating. However, heat-caused changes in physicochemical properties of barley proteins are not fully understood.

Hordeins, prolamins of barley, are characterized by an unusual amino acid composition, being rich in glutamine and proline and poor in basic and acidic amino acids. They comprise most barley protein and are roughly divided into four groups based on molecular weight and amino acid composition (4); A-, B-, C-, and D-hordeins have molecular weights of 20,000 or less, 32,400–45,000, 49,000–72,000, and 105,000 or more, respectively. It is noteworthy that B-hordein includes 8 cysteinyl residues, but C-hordein does not (5). The composition of these hordeins differs with maturity and variety (6). In general, B-hordein is the most abundant and C-hordein follows (7).

Hordeins are usually extracted with aqueous alcohols, such as 70% ethanol and 55% 1- or 2-propanol. The extractability is improved by the addition of 2-mercaptoethanol (2-ME) (8). Heating can cause denaturation and polymerization of proteins through the cleavage and reoxidation of disulfide linkage in protein structure. We investigated the effect of heating on the extractability of barley hordeins under wet- and dry-heating cooking conditions.

MATERIALS AND METHODS

Materials. Barley grain (Hordeum vulgare L) cultivar “Nozomi-nijo” was obtained from Kaneko Seeds Inc. (Maebashi, Japan). The grain was peeled and polished with a blender under chilled conditions to avoid a rise in grain temperature to above 40°C. The polished barley grains were recovered in a yield of 60% from the whole hulled ones and stored at 4°C until use. All the reagents commercially available were of analytical grade and used without further purification.

Heating of barley grains. Polished grains were heated in either a wet or a dry state. In wet heating, polished grains that had soaked in distilled water for 2 h at room temperature were allowed to stand in a steam bath (100°C) for the periods indicated in table or figure, then cooled to room temperature. The grains were dried in vacuo and ground into a flour with a coffee mill. In dry heating, polished grains were put in an oven adjusted to 100°C. They were cooled to room temperature and pulverized in the same manner as above.

Protein extraction. Flour samples were defatted with n-butanol and hexane, then air-dried. Defatted flour (1.5 g) was stirred with 30 mL of 5% (w/v) sodium chloride for 2 h at room temperature. The extract was centrifuged at 9,000×g for 20 min, and the resulting precipitate was extracted by stepwise elution for 2 h at 60°C with 55% (v/v) 2-propanol at 25°C, 55% 2-
propanol containing 1% (v/v) 2-mercaptoethanol (2-ME), and a mixture of 2% (w/v) sodium dodecyl sulfate (SDS) and 1% 2-ME in their order. The final residue was dried at room temperature. The protein contents in flour, extract, and insoluble residue were determined according to the micro- or macro-Kjeldahl method (N×5.7).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE).** The saline extract was dialyzed against distilled water, and the alcoholic extracts were deprived of solvent by evaporation. The protein samples were reduced by treatment with the pH 6.8 buffer containing 0.625 M Tris, 1% (w/v) SDS, 11% (v/v) glycerol, 5% (v/v) 2-ME, and 0.01% (w/v) bromophenol blue, pH 6.8, and heated in a boiling water bath for 2 min. After cooling, their aliquots (10 μL) were applied to a 12% SDS-PAGE gel. After electrophoresis with 25 mM Tris-glycine buffer of pH 8.9, containing 0.1% (w/v) SDS, the gel was stained with Coomassie Brilliant Blue R 250 and destained with 7% acetic acid.

**Thermal treatment of hordeins from nonheated barley grains.** Defatted barley flour from nontreated grains was washed with 5% NaCl, followed by extraction with 55% 2-propanol to prepare the hordeins. After dialysis against distilled water, the hordein fraction was collected by centrifugation. To investigate the effect of heating in a soluble state, the hordeins were dissolved in 55% 2-propanol and poured in small portions into test tubes (15×150 mm) with sealed caps, which were heated for 0, 15, 30, 60, and 120 min, respectively, at 90°C. After cooling to room temperature, the supernatant was collected by centrifugation at 9,000×g for 20 min. On the other hand, suspensions of hordeins in water were heated at 90°C under the same conditions and cooled to room temperature, to which 2-propanol was added up to a final concentration of 55% (v/v), followed by centrifugation at 9,000×g for 20 min. The protein content in the supernatant was determined by the micro Kjeldahl method, and the protein composition was analyzed by SDS-PAGE in a reductive state.

**RESULTS**

**Extractability of barley proteins obtained from grains after dry and wet heating (g/100 g grain).**

| Fraction | Control (Unheated) | Heating condition |
|----------|--------------------|------------------|
|          | Dry                | Wet              |
| (Grain)  | 8.61±0.37 (100)    | 8.59±0.42 (100)  |
| 5% NaCl  | 2.20±0.25 (26)     | 1.40±0.09 (16)*  |
| 55% 2-Propanol | 2.26±0.18 (26) | 2.06±0.09 (24)  |
| 55% 2-Propanol+1% 2-ME | 1.30±0.24 (15) | 1.65±0.09 (19)  |
| 2% SDS+1% 2-ME | 2.00±0.10 (23) | 2.05±0.25 (24)  |
| Residue  | 1.06±0.32 (12)     | 1.82±0.31 (21)*  |
| (Total)  | 8.81±0.94 (102)    | 8.99±0.74 (105)  |
|          | 8.57±0.48 (100)    | 0.71±0.14 (8.3)* |
|          | 0.61±0.15 (7.1)*   | 2.58±0.24 (30)* |
|          | 2.95±0.28 (34)*    | 1.92±0.24 (22)* |
|          | 8.77±0.85 (102)    |                  |

Grains heated under dry or wet conditions for 2 h were milled, defatted, and extracted stepwise with indicated solvents as described in Materials and Methods. Protein was determined by the micro-Kjeldahl method. Data are expressed as mean±SD (n=4). *Significantly different at p<0.05 from the control in the same row. Numbers in parentheses represent percent of recovery relative to the value for flour.

The barley flour of grains with dry or wet heat treatment was defatted and then extracted successively with 5% NaCl, 55% 2-propanol, and 55% 2-propanol containing 1% 2-ME and 1% SDS containing 1% 2-ME. As shown in Table 1, the amount of protein extractable with 5% NaCl decreased to 0.71 g/100 g grain after wet heating, and to 1.40 g/100 g grain after dry heating in comparison with 2.2 g/100 g grain in the control. The extracted proteins were mainly albumin and globulin. The amount of proteins extractable with 55% 2-propanol was 0.61 g/100 g grain after wet heating, in contrast to 2.06 g/100 g grain after dry heating. Prolamin is generally considered to be heat-stable. It is noteworthy that in this connection, wet heating appreciably brought about the denaturation of prolamin. Most insoluble proteins in globulin and prolamin fractions were converted to soluble ones by treatment with 2-ME. The increased protein in 55% 2-propanol plus 1% 2-ME fraction has certainly arisen from the dissolution of prolamin not extractable with 55% 2-propanol. The pH value of grains changed neither with dry heating nor wet heating (data not shown), indicating that the protein extractability was not affected by pH.

We also examined the protein composition of each fraction by SDS-PAGE in the absence or presence of 2-ME (Fig. 1). The protein patterns in each fraction were quite similar among lanes 1, 2, and 3. In the 5% NaCl-extracted fraction, most bands disappeared during wet heating (lane I-3). In regard to the 55% 2-propanol-extracted fraction, two polypeptides of about 37-kDa probably corresponding to B-hordein disappeared during wet heating, but polypeptides of about 50-kDa appeared with no considerable change (lane II-3). Also, B-hordein was extracted with 55% 2-propanol containing 2-ME (lane III-3). These results implied that wet heating would have caused an insolubilization of B-hordein (37-kDa band) through intermolecular S-S linkage in hordein, thereby making difficult its extraction by 55%
Influence of Heating on Extractability of Barley Hordeins

Fig. 1. SDS-PAGE of proteins in extracts from grains heated under dry and wet conditions. Protein extracts were prepared as described in Table 1. SDS-PAGE was performed with a 12% slab gel in a reductive state. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R 250 and destained with 7% acetic acid. I, extract with 5% NaCl; II, extract with 55% 2-propanol; III, extract with 55% 2-propanol containing 1% 2-ME; IV, extract with 2% SDS containing 1% 2-ME. Extracts from 1) unheated barley grains, 2) dry-heated grains, and 3) wet-heated grains. S, molecular-weight markers (prestained precision protein standard TM, Bio Rad).

Fig. 2. Effect of heating time of grains on the extractability of hordeins. A: extractability of hordeins. Barley grains were wet-heated for 0, 15, 30, 60, and 120 min at 100°C, from which flours were extracted with 55% 2-propanol to obtain soluble proteins, as described in Materials and Methods. Protein content was determined by the micro-Kjeldahl method. Data are expressed as a percentage of the protein content in extract from nonheated grains (time 0). B: SDS-PAGE pattern. Protein extracts (10 μL each) were subjected to SDS-PAGE under the same conditions as in Fig. 1.

Effect of wet heating time on the extractability

To examine the effect of heating time on hordein extractability, barley grains were wet-heated for 15, 30, 60, and 120 min, and protein recovery was measured by the micro-Kjeldahl method with the 55% 2-propanol extract after washing with 5% NaCl. The data were expressed as the ratio of a heated sample to an unheated one in protein recovery. The value decreased time-dependently and reached 27.4 ± 8.2% by a 30-min heating (Fig. 2A). SDS-PAGE also revealed that polypeptide of 37-kDa disappeared almost completely on the gel (Fig. 2B), indicating that B-hordein was so easily polymerized as to lose its solubility in 55% 2-propanol during wet-heating.

Effect of heating on hordeins in solution

 Hordeins extracted from nonheated barley grains were examined for changes in solubility in 55% 2-propanol during heating. The hordeins in 55% 2-propanol retained their solubility (Fig. 3A) together with no change in molecular distribution on SDS-PAGE (Fig. 3C) even after heating up to 120 min. When a suspension of hordein in water was heated at 90°C, their
solubility in 55% 2-propanol decreased to 38% 30 min after heating (Fig. 3B). The degree of decrease was at a similar level for up to 120 min. B-Hordein of about 37-kDa had almost disappeared 30 min after heating (Fig. 3D). Despite a complete disappearance of B-hordein, however, no change was observed for C-hordein 50-kDa or 65-kDa up to 120 min. It turned out from these results that B-hordein easily lost its solubility in 55% 2-propanol during heating in water, but the same didn’t hold for C-hordein. When hordeins treated with N-ethylmaleimide (NEM) were suspended in water, their solubility in 55% 2-propanol showed no change with heating (Table 2). A similar result was obtained with heated hordeins in water containing 2-ME.

**Separation of B- and C-hordeins**

The finding that only B-hordein was insolubilized during heating in water was actually applied to the preparative separation of B- and C-hordeins. Hordein preparations from barley flour by extraction with 55% 2-propanol after washing with 5% NaCl were resuspended at a level of 50 mg/10 mL in distilled water, heated for 1 h in a boiling water bath, and cooled to room temperature, to which 1.2-fold volumes of propanol were added. The solution was separated into supernatant and pellet by centrifugation; the former

| Table 2. Effect of sulfhydryl blocking or reducing agents on the extractability of hordeins after wet heating. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Heating time (min) | 0 | 30 | 60 | 120 |
| Untreated | 100 | 36 | 35 | 35 |
| NEM | 100 | 98 | 100 | 100 |
| ME | 100 | 98 | 97 | 98 |

Hordein was prepared from unheated barley flour (defatted).  
1Hordeins modified with N-ethylmaleimide (NEM) were suspended in distilled water and heated for an indicated period. The heated suspension was mixed with 1.2 volumes of 2-propanol to extract hordeins. 
2Hordeins were suspended in 0.5% 2-ME, heated as mentioned above, and centrifuged at 9,000×g for 20 min. The resulting precipitate was washed with distilled water, extracted with 55% 2-propanol. 
Nitrogen in the supernatant was determined by the micro-Kjeldahl method.
Influence of Heating on Extractability of Barley Hordeins

Fig. 4. SDS-PAGE of the soluble and insoluble fractions in 55% 2-propanol after the suspension was heated in water. Hordeins obtained by extraction with 55% 2-propanol and dialysis against water (lane a) were suspended in water (50 mg/10 mL) and heated for 1 h in a boiling water bath. After cooling to room temperature, the suspension was mixed with 1.2 volumes of 2-propanol and centrifuged to remove the supernatant (lane b). The residue was freshly resolved in 55% 2-propanol containing 1% 2-ME; the supernatant was collected by centrifugation (lane c).

was regarded as a semipurified source of C-hordein (Fig. 4, lane b), and the latter was again dissolved in 55% 2-propanol plus 1% 2-ME, followed by centrifugation. The resulting supernatant gave a double band corresponding to B-hordein (Fig. 4, lane c). Therefore this procedure serves as a convenient means to separate B- and C-hordeins in roughly pure forms.

DISCUSSION

Hordeins are major storage proteins in barley, but after they are heated their chemical properties are not fully understood. In the present study, we demonstrated that a significant decrease in 55% 2-propanol-extractable proteins arose from the heating of grains under wet conditions. On the other hand, the addition of 2-ME to some extent restored the extractability of insolubilized proteins when heated. This fact was confirmed by using hordeins extractable from nonheated barley flour; B-hordein deprived of solubility in 2-propanol by heating had its intrinsic nature restored in the coexistence of 2-ME, but C-hordein was not influenced by heating. This finding clearly shows that the heat-susceptibility of B-hordein to insolubilization in an aqueous solution is attributable to its structural characteristics.

In the present study, the heating procedures of polished grains reflect the actual cooking or processing; that is, wet heating is a model of boiled or steamed barley, the results being therefore compared with those of dry heating. The protein composition of barley may also affect the processing like D-hordein, which are responsible for malting quality (9). For example, B-hordein was polymerized by the heating of moistened grains. It was not expectantly detected in the 55% propanol extract from rolled barley, polished barley that had been humidified and pressed flat (data not shown). B-hordein is rich in cysteine, proline, and other hydrophobic amino acids (4, 5). It has recently been demonstrated that both hordeins of 34-kDa and 97-kDa are completely degraded during maiting barley grains, especially of cultivar Charriot (10). This suggests that the degradation or denaturation of B- and D-hordeins relative to C-hordein easily occurs under moist conditions.

Incidentally, B-hordein lost solubility in 55% 2-propanol after wet-heating, but C-hordein retained solubility, as mentioned above. Moreover, B-hordein showed no decrease in solubility by wet heating when sulfhydryl groups had been protected or blocked. These results strongly suggest that a decrease in the solubility of B-hordein is due to polymerization through the formation of an interdisulfide bond. Heat-treated B-hordein gave rise to simple cross-linking between sulfhydryl groups, because insoluble B-hordein returned to solubility by the addition of 2-ME. The interaction of B-hordein molecules is undoubtedly important in their polymerization. It seems quite likely that the polymerization of B-hordein is hardly affected by non-protein components such as lipid and carbohydrate.

The result of this study provides information about the separation procedure of B-hordein from other hordeins on a laboratory scale. Namely, the recovery experiment revealed that the 55% 2-propanol extract included B- and C-hordeins at a ratio of about 2:1 in a yield of almost 100% and that these hordeins could be separated solely by heat treatment in an aqueous solution and its subsequent extraction with 55% 2-propanol in the presence or absence of 2-ME. Consequently, this method is applicable at once to further investigation of the function of B- and C-hordeins.

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