PURIFICATION OF HORDEIN POLYPEPTIDES BY COLUMN CHROMATOGRAPHY USING VOLATILE SOLVENTS

by

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Hordein polypeptides C2 and B1 have been purified using gel filtration and ion exchange chromatography in volatile solvents. They are different in amino acid composition, the B1-hordein containing lower amounts of glutamine/glutamic acid and proline. By digestion with carboxypeptidase Y the C-terminal sequence Gly-Val-COOH was deduced for the B1 polypeptide whereas no clear sequence could be established from the amino acids Ser, Val, Ile and Met which were released from the C2-hordein. This indicates possible heterogeneity of the C2 component.

1. INTRODUCTION

Hordein, the alcohol-soluble prolamin of the barley endosperm, is characterized by a high content of glutamine and proline. This group of reserve proteins can be separated into a number of discrete polypeptide bands by electrophoresis in the presence of sodium dodecyl sulfate (SDS). The polypeptides of the cultivar Bomi range in molecular weight from about 27 to 67 kD and chymotryptic peptide mapping reveals structural homology as well as distinct differences among the separated bands, designated as hordein B1 to B3 and hordein C1 to C5 (4). Some of these

polypeptides display heterogeneity, when analyzed in an additional isoelectric focusing dimension (9, 13). Cloning of a double stranded DNA complementary to one messenger RNA molecule revealed the latter to code for a B1-hordein polypeptide which is partly homologous to the B2-hordein (1). According to these results the various hordein polypeptides are translated from different messenger RNA molecules. For the further analysis of the organization and expression of the hordein genes additional information on the primary structure of the different hordein polypeptides is required. Techniques to separate

the various hordein polypeptides on a preparative scale have therefore been explored.

Attempts to fractionate hordein by free-flow electrophoresis (16) or gel filtration in the presence of urea (8) have yielded cross-contaminated fractions, with the possible exception of one α -hordein (8). This corresponds to a A-hordein with the presently used nomenclature (see MIFLIN and SHEWRY, 10). The A-fraction, however, is no longer considered to be hordein proper as it is not deposited in endosperm protein bodies (5). Purifying hordein polypeptides by one- or twodimensional polyacrylamide gels gives very pure fractions in low yield (4, 9).

I have developed fractionation methods capable of providing large quantities of pure polypeptides, suitable for further analysis. The rationale of this approach was to purify hordein polypeptides in columns with different stationary phases using alcohols and volatile salts as mobile phases in order to simplify retrieval of the isolated fractions.

2. MATERIALS AND METHODS

2.1. Isolation of hordein from barley meal

Barley seeds (*Hordeum vulgare* L.) cv. Bomi were ground in an Udy Cyclone mill. The meal (100 g) was stirred at room temperature for 30 min in 1 litre of a solution containing 1% mercaptoethanol, 1 mM-phenylmethylsulfonyl-fluoride, 1 mM-EDTA and 0.1% (w/v) polyvinylpyrrolidone 10,000. The suspension was then centrifuged for 10 min at 13,000 rpm in a Sorvall GSA rotor. The pellet was stirred vigorously for 3 hours at 60 °C under protective gas containing a chloro-fluoro carbon compound (Protectan, Tetenal-Werke, Hamburg, Germany) in 1 litre of 55% isopropanol, 2% mercaptoethanol. The suspension was centrifuged at room temperature under the above conditions. The supernatant was clarified by filtration and dialyzed extensively against 100 mM-mercaptoethanol in the coldroom to precipitate the hordein polypeptides. The dialysis residue was centrifuged at 4 °C for 30 min at 13,000 rpm in the Sorvall GSA rotor. The sediment was stirred in 5 litres 55% isopropanol, 100 mM-mercaptoethanol as described above until it had completely dissolved. The solution was stored at 4 °C or at -18 °C under Protectan gas. The hordein

precipitated under these conditions and was redissolved by heating to 60 °C before use. All columns were run at room temperature.

2.2. Gel filtration on Sephadex LH60

Total hordein (up to 25 ml) was chromatographed on a column (2.6 × 30 cm) of Sephadex LH60 (Pharmacia), equilibrated with 55% isopropanol, 0.1% mercaptoethanol. The proteins were eluted under 1m of hydrostatic pressure. The effluent from the column was monitored at 280 nm.

2.3. Gel filtration on Sephacryl S300

50 ml of hordein solution was applied to a column (5 cm × 90 cm) of Sephacryl S300 (Pharmacia) and eluted with 55% isopropanol, 0.1% mercaptoethanol at a flow rate of 40 ml · hour⁻¹. Afterwards the column was washed with 15 ml of 1% acetic acid in column medium to remove bound material.

2.4. Ion exchange chromatography

Hordein was dialyzed against 60% isopropanol, 20 mM-ammonium bicarbonate, 0.1% mercaptoethanol, pH 8.8, and loaded on a 2.6 cm × 10 cm column of DEAE-Sephacryl CL-6B (Pharmacia), equilibrated with the same buffer. The column was washed at 60 cm hydrostatic pressure with starting buffer until the absorption at 280 nm wavelength had returned to the initial baseline level. The bound proteins were then eluted with a pH-gradient produced with increasing concentrations of ammonium acetate in 60% isopropanol, 0.1% mercaptoethanol until a concentration of 10 mM-ammonium acetate, pH 7.2, was reached. The pH of the starting and limiting buffer was measured with a glass electrode prior to the addition of mercaptoethanol.

2.5. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate

Slab gels of 1 mm thickness containing 12.5% acrylamide or 15% acrylamide with 2 M-urea were run in Laemmli's buffer system (6). The ratio of acrylamide to bisacrylamide was 37.5:1.

The samples were dried in vacuo in the presence of 250–500 µg SDS and dissolved in Laemmli's sample buffer, heated to 100 °C for 1–2 min and run at 20–25 mA constant current over night. Gels were fixed in 10 % trichloroacetic acid and stained in a solution containing 0.1 %–0.2 % Coomassie blue R 250, 50 % Methanol and 10 % acetic acid. Gels were partially destained in the same solution without Coomassie blue and then in 10 % trichloroacetic acid (J. INGVERSEN, personal communication). This greatly enhanced the staining intensity of the C-hordein.

2.6. Amino acid analysis

Samples were hydrolyzed in evacuated ampoules with 6N HCl containing 0.1 % phenol for 24 hours at 110 °C, dried and analyzed on a Durrum D500 analyzer. Cysteine and methionine were determined using performic acid oxidised samples (3).

2.7. Carboxypeptidase Y digestion

Carboxypeptidase Y was kindly supplied by Dr. IB SVENDSEN. Digestions were made in 0.1 M-N-ethylmorpholine acetate buffer, pH 7.0, containing 0.5 % SDS (7) and norleucine as an internal standard. Incubation temperature was 30 °C. Enzyme-protein ratios are given in the legend to Figure 5. The reaction was stopped by acidification with HCl.

The samples were dried in vacuo, resuspended in citrate buffer, centrifuged and the supernatants were run on the amino acid analyzer.

3. RESULTS

3.1. Gel filtration on Sephadex LH60

To purify hordein from low molecular weight contaminants, gel filtration in Sephadex LH60 was employed using 55 % isopropanol, 0.1 % mercaptoethanol as eluent. An elution profile of a typical separation is shown in Figure 1. The peak representing the excluded volume is skewed. This is caused by a slight retardation of the B-hordein polypeptides (see Figure 2). Since the manufacturer states a fractionation range of up to about 20 kD, this retardation of the more than 27 kD B-hordein is thought to be due to adsorption effects.

Figure 1. Elution profile of hordein chromatographed on Sephadex LH 60. Fractions of 12 ml were collected.

The arrow points to a shoulder in the peak which elutes with the excluded volume.

3.2. Gel filtration on Sephacryl S300

For fractionation according to molecular size, Sephacryl S300 was chosen as stationary phase, again with 55 % isopropanol, 0.1 % mercaptoethanol as eluant. As can be seen in Figure 2, the individual hordein polypeptides did not elute in the order of decreasing molecular weight, as determined by electrophoresis in the presence of sodium dodecyl sulfate. The B1 polypeptide was adsorbed on the column. The adsorbed material could be released from the column by washing with a small volume of 1 % acetic acid in the eluant, as shown in Figure 3.

Two polypeptide size classes could be obtained from this column in purified form: An early eluting C2-hordein, which possibly consists of two polypeptide chains (see fractions 16–20 in Figure 2) as well as B1-hordein from the acetic acid wash (fractions 18–21 in Figure 3). These two fractions were used for further analysis.

3.3. Ion exchange chromatography

The approach employing high concentrations of isopropanol as chromatographic solvent appears also useful for separation of hordein polypeptides by ion exchange chromatography. With DEAE Sepharose CL-6B as adsorbent, volatile salts (ammonium bicarbonate and ammonium acetate) were chosen as ionic compo-

Figure 2. (a) Elution profile of hordein chromatographed on Sephacryl S300. Fractions of 13 ml were collected. (b) Gel electrophoretic analysis of the fractions from the column (cf. 3.2). The right lane contains total hordein (cf. 2.1) for comparison. The designation of the polypeptides is according to HOLDER and INGVERSEN (4). Note the double band in lane 20.

nents of the eluant. A separation is shown in Figure 4. The B1 polypeptide is not bound to the column and elutes in three peaks of high purity. This elution behaviour is consistent with the alkaline isoelectric point as determined by isoelectric focusing and the three peaks possibly represent the isoelectric variants described by MIFLIN & SHEWRY (10). Some of the C-hordein polypeptides are eluted with a pH-gradient using 10 mM-ammonium acetate, pH 7.2 as a limiting buffer.

3.4. Amino acid analysis

Amino acid analysis of the purified B1 and C2 polypeptides give quite different compositions for these two proteins (Table I). The C2-hordein contains higher amounts of glutamic acid/ glutamine and proline, but lower amounts of threonine, valine, methionine, isoleucine, leucine and lysine which are essential for mammalian nutrition. Phenylalanine is the only essential amino acid which is lower in the B1 polypeptide (tryptophane has not been determined). Particu-

larly striking is the low lysine content of the C hordein, amounting to about 1 lysine residue per chain.

3.5. Carboxypeptidase Y digestion

Carboxypeptidase Y was used for C-terminal analysis in the presence of sodium dodecyl sulfate to keep the hordein polypeptides in solution. The time course of the digestion for the two purified hordein polypeptides is shown in Figure 5. A clear release pattern for the B1-hordein suggests the sequence -Gly-Val-COOH. The C2-hordein fraction displays more complicated kinetics, indicating that the carboxyterminal sequences of two different polypeptide chains may be present in this hordein preparation. This can also be seen in the electrophoretic patterns of Figure 2. Possible sequences could be -Val-Ser-COOH and -Ile-Met-COOH.

4. DISCUSSION

Three chromatographic fractionation methods for hordein polypeptides all based on isopropa-

Table I

Amino acid composition of hordein polypeptides C2 and B1.

Amino acid	Mole %	
	Residues/100	Residues
	Isolated hordein polypeptides	
	B1	C2
Cysteine	2.8	0.9
Aspartic acid	1.5	1.2
Threonine	1.8	1.2
Serine	2.9	2.1
Glutamic acid	34.2	41.1
Proline	16.8	27.4
Glycine	3.8	1.2
Alanine	3.0	1.0
Valine	6.1	1.6
Methionine	1.1	0.6
Isoleucine	4.1	3.7
Leucine	8.8	5.2
Tyrosine	2.5	2.5
Phenylalanine	4.7	8.0
Histidine	1.6	0.7
Lysine	1.0	0.2
Arginine	2.8	1.7

Figure 3. Release of polypeptides adsorbed to Sephacryl S300 with acetic acid (cf. 2.3).

(a) Elution profile. (b) Gel electrophoresis of eluted fractions. The right lane contains total hordein (cf. 2.1) for comparison.

nol-water solutions as eluants have been described. They allow the purification of the C2 and the B1 polypeptides. In addition, they may be useful in obtaining fractions enriched in other hordein polypeptides, which then may serve as starting

Figure 4. Ion exchange chromatography of hordein on DEAE Sepharose CL-6B. (a) Elution profile. The arrow designates the start of the gradient (cf. 2.4). (b) Gel electrophoresis of eluted fractions. The right lane contains total hordein (cf. 2.1) for comparison.

material for further purification (see Figures 2, 3 and 4).

It should be mentioned that the Sephacryl S300 separation depended strongly on the conditions described. For instance, inclusion of 1% acetic acid in the 55% isopropanol to

achieve elution in one step resulted in a greatly reduced resolution. The ion exchange method, on the other hand, provides flexibility for the choice of salt, pH and gradient shape to enrich for other hordein polypeptides.

Amino acid analysis shows that the isolated

Figure 5. Time course of carboxypeptidase Y digestion.

(a) Digestion of 2 mg B1 hordein with 9 μ g of carboxypeptidase Y in a total volume of 1.5 ml. Val ($\bullet$), Gly ($\circ$). (b) Digestion of 2.5 mg C2 hordein with 6 μ g of carboxypeptidase Y in a total volume of 2.5 ml. The identity of the released amino acids was confirmed by dansylation as described by POULSEN (12). Weak spots of glutamic acid and aspartic acid were found after hydrolysis for 4 hours in addition to the residues shown in the figure. Ser ($\blacktriangledown$), Val ($\bullet$), Met ($\blacksquare$), Ile ($\blacklozenge$).

B1 and C2 polypeptides are different in composition. The B1-hordein contains about 5 times as much lysine as the C2-hordein on a mole per cent basis. It is furthermore higher in content for almost all amino acids which are essential for human nutrition (section 3.4). To improve the nutritional value of barley it may therefore be useful to breed for mutants with an increased content of B1-hordein. Carboxypeptidase Y releases different C-terminal amino acids from the purified polypeptides. The results indicate one sequence -Gly-Val-COOH from the B1-hordein, but possibly two sequences for the C2-hordein, involving the amino acids Ser, Met, Val

and Ile. The presence of one of the two methionine residues in the C2-hordein close to the carboxyterminus is interesting, since I have not succeeded in producing fragments from the C2-hordein by treatment with cyanogen bromide (as judged by SDS gel electrophoresis). If this result is not caused by an artifact due to the highly hydrophobic nature of the hordein polypeptides, I would expect the other methionine residue to be located near the N- or C-terminus, and it becomes understandable that CNBr-cleavage does not alter the apparent molecular weight significantly.

The differences in total amino acid composition of the B1- and C2-hordein polypeptides and in the structure of the carboxy-terminus supports the conclusion reached by the analysis of the messenger RNA for the B1-hordein (1), namely that the different hordein polypeptides have distinct primary structures.

The B-hordein polypeptides and the C-hordein polypeptides are coded for by the *hor-2* and *hor-1* regions on chromosome 5, respectively (2, 11, 14, 15). Further studies will have to show whether the primary structure differences among the B-hordeins or among the C-hordeins in general are as large as those found here between the B1-hordein and the C2-hordein. This will provide a basis for rational improvement of the quality of the hordein proteins by plant breeding.

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