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The Peptide Pools of Germinating Barley Grains: Relation to Hydrolysis and Transport of Storage Proteins[1]

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ABSTRACT

A quantitative procedure for purifying small peptides from plant tissues, involving both ion-exchange and gel-exclusion chromatography, is described. Peptides were quantified and characterized by using the fluorescence reagents dansyl chloride and fluorescamine. Large pools of small peptides and amino acids have been identified in both the endosperm and embryo of germinating barley grains. The peptide pool of the endosperm increases during the first 3 days of germination, subsequently decreasing, an observation compatible with a role for peptides as intermediates in the breakdown of the storage proteins and their transfer to the embryo. The amino acid composition of these peptides indicates that all the major classes of storage protein contribute to the pool. The concentration of peptides produced in the endosperm during germination is sufficient for the efficient operation of the peptide transport system of the scutellar membrane characterized previously (Higgins and Payne, Planta 136: 71-76, 1977; Planta 138: 211-215 and 217-221, 1978). Data presented here indicate that peptides play at least as important a role as amino acids in the transfer of stored nitrogen from the endosperm to the embryo during germination.

Recently, a peptide transport system with broad specificity for small peptides has been identified in the scutellum of germinating barley embryos (11-16, 28-30). It seems likely that this transport system plays a role in the transfer of stored nitrogen from the endosperm to the embryo during germination. However, in order to demonstrate the physiological importance of such a system it is necessary to show that the appropriate substrates are available *in vivo*. Although there is some evidence for the presence of a pool of peptides in the endosperm of germinating cereal grains (reviewed in ref. 11), an unambiguous demonstration has never been achieved. This study was undertaken in an attempt to identify and to characterize the peptide pools of germinating barley grains and to relate any findings to the function *in vivo* of the peptide transport system.

MATERIALS AND METHODS

Barley grains (*Hordeum vulgare* L. cv. Maris Otter, Winter), obtained from the National Seed Development Organization Ltd., Newton Hall, Cambridge, U. K., were wetted for 10 s in aqueous ethanol (70% v/v), surface sterilized with NaOCl (1% w/v available chlorine), and germinated on 'Alkathene' polyethylene granules (I. C. I. Ltd.) at 28 C in a dark spray room, with water misting for 5 min every hour.

TISSUE EXTRACTION

After appropriate periods of germination the embryos and endosperms from 60 seedlings were separated, blotted dry, and weighed. Tissues were rapidly ground in 1 ml acetic acid (5 N) and the volume made up to 12 ml (9 ml for embryos) with acetic acid (5 N) preheated to 100 C. Extraction was at 100 C for 20 min, in a stoppered tube to prevent evaporation. Extracts were centrifuged for 10 min at 1,500*g*, the supernatant solutions recovered and evaporated to dryness on a rotary evaporator. The residues were dissolved in 0.01 N HCl (half the original volume) and the pH adjusted to 2.0 with 5 N HCl. Undissolved material was removed by centrifugation (2 min, 10,000*g*) and the extracts stored at −20 C until analyzed (up to 1 month).

PURIFICATION OF EXTRACTS

A column (bed volume 9.5 ml) of Dowex 50W-X8 standard H^+ ion exchange resin (B.D.H. Ltd., Poole, Dorset, U.K.) was thoroughly washed with deionized H_2O, equilibrated with 0.01 N HCl, and 1 ml extract added. Unbound material was eluted with three bed volumes of 0.01 N HCl, followed by two bed volumes of deionized H_2O, and discarded. Compounds bound to the resin (including amino acids and peptides) were eluted with four bed volumes of 3 M ammonia. Eluate was evaporated to dryness in a rotary evaporator at 40 C, diluting with several volumes of deionized H_2O before complete evaporation was achieved to prevent alkaline hydrolysis of peptide bonds. The residues were redissolved in 1.5 ml PBS[3] and any undissolved material removed by centrifugation (3 min, 10,000*g*).

SEPARATION OF PEPTIDES

Samples (1.0 ml) of each purified extract were fractionated by gel-filtration on a Sephadex G-15 chromatography column (1.5 × 90 cm) with PBS as an eluent. A constant flow rate of 33.6 ml/h was maintained with a peristaltic pump. Fractions of 2.8 ml were collected, referred to below as G-15 fractions. The column was calibrated by determining the elution volumes of a number of small, defined peptides (200 μl, 1 mM) and amino acids (200 μl, 20 mM), by using a Uvicord III 2089 dual channel spectrophotometer (KB Ltd.) to monitor the A_{206} of the effluent.

ANALYSIS OF G-15 FRACTIONS

Four independent methods were used to identify and characterize the amino acids and peptides eluted in each G-15 fraction.

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[3] Abbreviations: PBS, phosphate buffered saline (0.1 M NaCl in deionized water, 0.01 M sodium phosphate buffer, pH 7.0); GAB, γ-aminobutyric acid; DNS, dansyl; TNBS, 2,4,6-trinitrobenzene-1-sulfonic acid.

A_{206}: Peptide Bonds. A_{206} (1 cm pathlength) of each fraction was measured with a Unicam SP 500/Gilford 2000 spectrophotometer, using dialanine as a standard.

Fluorescamine: Primary Amino Groups. Samples (normally 200 μl) of the G-15 fractions were mixed with 2.5 ml sodium tetraborate (0.1 M in deionized H_2O, pH 7.0). Fluorescamine (4-phenyl-spiro-[furan-2(3H), 1'-phthalan]-3,3' dione: 0.5 ml; 0.15 mg/ml in acetone) was added while vortexing rapidly, and the fluorescence yield determined using excitation and emission wavelengths of 390 and 480 nm, respectively (Perkin-Elmer 1000 Fluorescence Spectrophotometer). Diglycine was used to calibrate the assay.

Dansyl Chloride: Primary Amino Groups. Free amino acids in each G-15 fraction were determined by reacting samples (20–400 μl) with dansyl chloride, separating the derivatives on polyamide sheets by two-dimensional chromatography and quantifying each amino acid as described previously (13).

"Bound" amino acids (released by acid hydrolysis: presumptive peptides) were determined by dansylation as above. Hydrolysis of freeze-dried samples (20–400 μl) of each column fraction was with HCl (50 μl: 6 N) at 105 C for 16 h in sealed tubes. HCl was removed *in vacuo* over NaOH prior to dansylation.

N-terminal amino acids were determined as above, except that hydrolysis was after dansylation but prior to chromatographic separation. Because of the labile nature of the NDS-proline bond (10), N-terminal prolyl residues were determined after only 4.5 h hydrolysis.

TNBS Assay: Primary Amino Groups. TNBS reacts equally with the α-amino groups of amino acids and peptides. However, in the presence of Cu^{2+} ions the reaction with peptides is specifically inhibited (2; unpublished results). The assay can, therefore, be used to distinguish between amino acids and peptides. Assay conditions were adapted from Binkley *et al.* (2). Samples of the G-15 column fractions (1.1 ml; 10–200 nmol free amino groups) were mixed with 0.9 ml of sodium tetraborate (0.13 M) and equilibrated at 37 C. A freshly prepared solution of TNBS (250 μl; 4 mg/ml in water, also at 37 C) was added and thoroughly mixed. After exactly 30 min further incubation at 37 C the A at 420 nm was measured (Hilger-Watts Uvispek H700 spectrophotometer). For assays in the presence of Cu^{2+} ions 50 μl of $CuSO_4$ (24 mM) replaced 50 μl of the tetraborate buffer. Glycine was used to calibrate the assay.

TISSUE WATER CONTENT

Tissue dry weights were determined after drying to constant weight at 75 C under vacuum. Water content was considered to be the difference between fresh and dry weights.

AUTOMATIC AMINO ACID ANALYSIS

Amino acid compositions of ungerminated barley meal and various G-15 fractions were obtained from acid hydrolysates (6 N HCl, 22 h, 105 C), using a Locarte amino acid analyzer.

SEPARATION OF POLYPEPTIDES AND PROTEINS

A Sephadex G-50 superfine column (1.6 × 40 cm) was equilibrated with PBS at a constant flow rate of 14.1 h. One-ml samples were added to the column and eluted with PBS. The A of the effluent was monitored continuously at 206 nm as described above. The column was calibrated with three standard peptides, pentaalanine (200 μl, 1 mM), insulin α-chain (200 μl, 5 mg/ml), and insulin β-chain (200 μl, 5 mg/ml).

RESULTS AND DISCUSSION

METHODOLOGY

Extracts were made of the endosperm from 1- to 6-day seedlings and of the embryos from 1- to 3-day seedlings. In ungerminated grains (day 0) it proved impossible to separate the embryo and endosperm. Consequently, in addition to the complete grain, the separated proximal and distal half-grains (including and excluding the embryo, respectively) were extracted for comparison.

G-15 Column Calibration. A linear relationship was shown to exist between K_d (fraction of internal column volume in which peptide elutes) and $\log_{10}$ molecular weight for a number of small, defined peptides (results not presented). This relationship can be used to predict the chain lengths of peptides expected to elute in each G-15 fraction: none in the void volume (fractions 1–16), proteins and polypeptides of more than six residues in fractions 17 to 20, peptides of six or less residues in fractions 21 to 27, and amino acids in fractions 28 to 30. The latter three groups of fractions will be referred to as the polypeptide, peptide, and amino acid fractions, respectively.

Efficiency of Extraction. Acetic acid (5 N) has been shown to be an efficent extractant for amino acids and small peptides from barley embryos, complete recovery being achieved (11). As an overall check on the efficiency of extraction and purification, three peptides, dialanine, pentaglycine, and phenylalanylglutamic acid, were added to the acetic acid extractant. This was used to prepare an extract of 3-day endosperm tissue which was taken through the complete purification and separation procedure. Dansylation of the final G-15 fractions showed better than 85% recovery of each peptide. Thus, any amino acids and small peptides extracted would seem to be representative of the pools present *in vivo* .

Polypeptide Separation. Figure 1 shows the levels of three representative amino acids, glycine, proline and GAB, in each G-15 fraction of a 2-day endosperm extract, determined by using dansyl chloride both before and after acid hydrolysis. These results (together with similar results obtained for all other amino acids) illustrate the efficient separation of free and bound amino acids by gel-exclusion chromatography. Apart from the following exceptions, bound amino acids (presumptive peptides) only elute in the polypeptide and peptide fractions (fractions 17–21 and 21–27, respectively) and free amino acids only in fractions 28 to 31. Glutamic acid, aspartic acid, and lysine eluted rather early, peaking in fractions 25 to 26. Although the free and bound forms of these amino acids can be separated by the dansyl chloride technique, the presence of these free amino acids in the peptide fractions must be considered when interpreting the results of other assays. In addition, tyrosine, histidine, and phenylalanine were retarded, eluting in fractions 32 to 36, presumably because of hydrophobic interactions with the dextran gel (24). These fractions also contained large amounts of an unknown dansyl-reactive compound, precluding the accurate estimation of the free form of these amino acids.

Several additional lines of evidence also indicate that separation is based upon molecular weight. (*a*) Known peptides, applied to the G-15 column in the presence of endosperm extract, elute in the expected fractions (results not presented). (*b*) The average chain length of peptides eluted in each fraction, estimated from the ratio of N-terminal amino groups to the total bound amino acids (see below), corresponds well with the values predicted from their elution position on the column. (*c*) In a typical protein, lysine and tyrosine residues will be mainly located within the polypeptide chain, rather than at the N-terminus, and thus, N-terminal analysis will result in a high ratio of DNS-*O*-tyrosine and DNS-ε-lysine relative to their *bis*-derivatives. However, if the protein has been randomly cleaved to small peptides this ratio will be much smaller. The absence of any detectable DNS-*O*-tyrosine, and the presence of less DNS-ε-lysine than *bis*-DNS-lysine in the peptide fractions (Table I), is a good indication that no large polypeptides/proteins are present. (*d*) The compounds eluting in the endosperm polypeptide fractions were pooled, evaporated to dryness, redissolved in 1.0 ml PBS and separated on a Sephadex G-50 column. Figure 2 shows the A_{206} profile of column effluent during elution of the

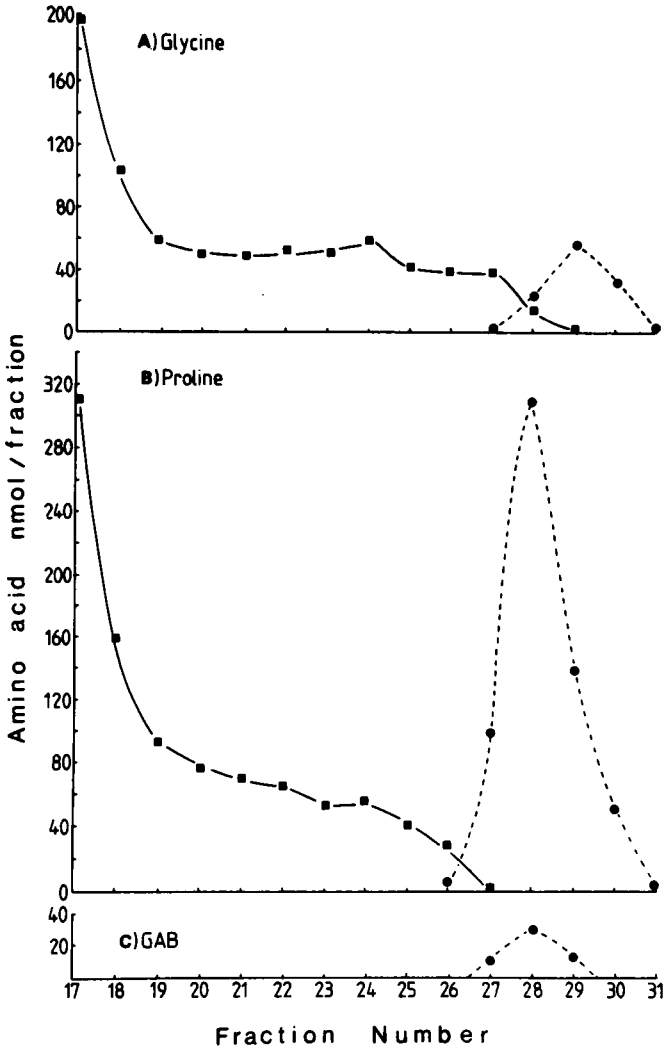


FIG. 1. Separation of free and bound amino acids by gel-exclusion chromatography. A 2-day endosperm extract was purified and fractionated on a Sephadex G-15 column. The levels of free (●-----●) and bound (■——■) amino acids in each fraction were estimated by using dansyl chloride.

polypeptide fractions from a 3-day endosperm extract. Comparison with the peptide standards shows that the polypeptide fractions actually contain peptides of 6 to 20 residues; the average chain length is about 10 residues. The polypeptide fractions of 1- and 6-day endosperm extracts showed a similar size distribution (results not presented). Clearly small peptides are not retarded during elution through the column.

Thus, separation of peptides by gel-exclusion chromatography is effective: only peptides with six or less residues elute in the peptide fractions (fractions 21–27) and only free amino acids in fractions 28 to 32.

Fluorescamine Assay. Under the conditions employed fluorescamine reacts with the N-terminal amino groups of peptides yet shows no appreciable reaction with free amino acids (11, 23; unpublished results). However, proline, prolyl peptides, and other secondary amines can interfere in the reaction between fluorescamine and primary amines. As proline is one of the most abundant amino acids in barley storage proteins (7), as well as being the most important free amino acid (20; Table II), this is a potential problem here. Essentially the same fluorescence yields were obtained for a given amount of dialanine, whether the reaction was in the presence or the absence of a 200-μl sample of any polypeptide or peptide fraction (results not presented), showing any interference from secondary amines to be negligible. In the amino acid fractions, however, the high levels of free proline did interfere with the assay, precluding its application to these fractions.

PEPTIDE AND AMINO ACID QUANTITATION

Peptides. The total size of the pool of bound amino acids in each G-15 fraction was determined using dansyl chloride, summing the values obtained for each individual amino acid (Figs. 3, 4). This is the most reliable estimate of the amounts of peptide in each fraction, if it is assumed that all amino acids released by hydrolysis are peptide bound. The close agreement obtained between the concentrations of bound amino acids (estimated by dansyl chloride) and the number of peptide bonds (estimated by 206 nm *A*) in each G-15 fraction, indicates that the bound amino acids are indeed peptides (Figs. 3, 4).

Use of both fluorescamine and TNBS gave very similar estimates of the free amino groups present in each fraction; a similar value was also obtained for the number of N-terminal amino acids (Figs. 3, 4). Thus, amino acid-containing compounds seem to account for all the free amino groups in the peptide fractions. Assuming the bound amino acids are all peptide-bound the average chain length of the peptides in each fraction, calculated by dividing the number of amino acids by the number of free amino groups, corresponds well with that predicted from calibration of the G-15 column (data not shown). Thus, it seems reasonable to assume that the bound amino acids determined with dansyl chloride are indeed peptide-bound. The excellent agreement between the experimentally derived and the predicted peptide chain lengths in each fraction seems unlikely to arise by chance in 10 separate extracts. In addition, there is considerable independent evidence that peptides do indeed elute from the G-15 column in the fractions predicted on the basis of their molecular size (see above).

Amino Acids. Because most amino acids have no measurable *A* at 206 nm, and the high levels of free proline interfere with analysis with fluorescamine, only the TNBS and the dansyl chloride methods were used to assay the free amino acids in the G-15 fractions (Figs. 3 and 4). Both methods gave very similar results. The somewhat lower values obtained with TNBS in certain fractions reflects the lack of reaction of this reagent with proline. It should be noted that an unidentified, non-amino, 206 nm-absorbing compound(s) also eluted in the amino acid fractions.

AMINO ACID COMPOSITIONS

Dansyl chloride analysis provides considerable information on the compositions of the free and bound amino acid pools. However, arginine was difficult to quantify as it chromatographs close to the solvent front. In addition, glutamine and asparagine were not detected in any fraction because of their deamidation to the corresponding acids. Thus, the acid/amide pools have been considered together.

Endosperm Peptides and Polypeptides. The amino acid compositions of the endosperm peptide and polypeptide fractions are presented in Table I. These compositions did not vary during germination (results not presented). The compositions of the isolated peptide and polypeptide fractions are very similar. However, they differ from the composition of the total seed protein (Table I). These differences seem to be real: (*a*) The compositions of the polypeptide/peptide fractions is the same whether estimated using dansyl chloride or an automatic amino acid analyzer (Table I). (*b*) Similar compositions were obtained independently for the endosperm at seven different stages of germination. (*c*) The differences are not simply due to the removal of free amino acids, as only 2% of the total amino acids of the ungerminated grain are present in an unbound form (calculated from Table III, assuming

Table I. *Amino Acid Compositions of the Peptide Pools of Barley Endosperm and Embryo*

All values are percentages of the total amino acids recovered.

Amino Acid	Ungerminated Whole Grains[a]	Endosperm[b]				Embryo[b]	
		Polypeptide Fractions[a]	Polypeptide Fractions[c]	Peptide Fractions[c]	N-Terminal Amino Acids in Peptide Fractions[c]	Polypeptide Fractions[c]	Peptide Fractions[c]
Ala	4.6	8.6	8.9	8.0	12.0	8.7	8.2
Gly	4.9	15.5	16.1	19.9	11.6	15.5	24.3
Glx	28.4	15.9	15.8	12.9	13.0	15.1	13.3
Asx	7.1	8.7	8.7	8.4	9.7	10.7	6.8
Ser	5.0	7.2	6.7	7.4	8.1	6.0	9.3
Thr	3.2	5.1	4.6	5.4	7.0	3.1	4.1
Pro	11.7	13.9	15.5	13.6	13.5	11.5	11.5
Val	5.4	5.9	5.4	6.5	9.4	6.6	5.1
Cys	—[d]	—	ND[e]	ND	ND	ND	ND
Met	1.7	—	ND	ND	ND	ND	ND
Leu	8.0	5.5	5.3	5.9	6.3	5.1	3.5
Ile	3.8	3.1	2.5	3.4	4.6	3.0	2.6
Phe	6.3	1.9	1.9	1.3	ND	2.2	0.8
His	2.6	2.0	2.6	3.4	ND	3.2	3.9
Lys							
bis	4.0	5.5	ND	ND	2.6	ND	ND
ϵ-			3.7	2.7	2.0	4.0	3.9
Tyr							
bis-	2.8	1.2	ND	ND	ND	ND	ND
O-			1.3	0.5	ND	3.5	1.7
GAB	ND	—	ND	ND	ND	ND	ND

[a] Obtained by using an amino acid analyzer.

[b] Average of the amino acid compositions at each stage of germination: compositions did not vary during germination.

[c] Obtained by using dansyl chloride.

[d] (—), Not determined.

[e] Not detected.

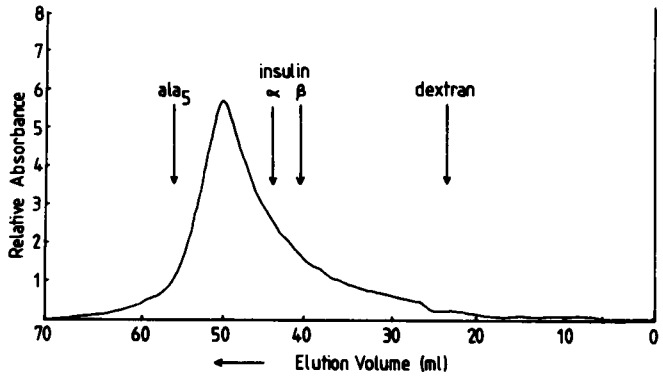


FIG. 2. Separation of endosperm polypeptide fractions by gel-exclusion chromatography. The pooled polypeptide fractions from an extract of 3-day endosperm tissue were passed through a Sephadex G-50 column and the *A*bsorbance of the effluent monitored continuously at 206 nm. The positions at which blue dextran and various peptide standards elute are also indicated.

a grain protein content of 10%). (*d*) The nondigestible proteins (*e.g.* in the testa and pericarp) cannot account for this difference, as the "average" amino acid composition of the barley storage proteins is very similar to the total composition of the grain (7).

One would not necessarily expect the amino acid composition of the polypeptide fractions to resemble the overall amino acid composition of the grain as no attempt was made to extract a representative sample of large polypeptides/proteins. However, complete extraction of small peptides was achieved (see above): the differences in composition here must therefore reflect a physiological process. The most important differences between the amino acid compositions of the endosperm peptides and the total seed protein are their very low levels of glutamate and the high levels of glycine. Interestingly, peptides isolated from wheat flour are also reported to have relatively high levels of glycine and low levels of glutamic acid (9). It seems unlikely that these differences arise because of selective degradation of specific storage protein fractions, as comparison of the amino acid compositions of the polypeptide/peptide fractions with the compositions of each of the major barley storage proteins (7) shows that all the major storage proteins of barley contribute to these fractions. For the smaller peptides the unexpectedly high levels of peptide-bound glycine may reflect the relatively slow rate at which glycyl-peptides are transported from the endosperm by the scutellar transport system (14, 15, 30). However, the most probable explanation for these differences is the specificity of the proteases and peptidases of the endosperm. Two main types of enzyme operate in the endosperm. Endopeptidases cleave the proteins to smaller peptides that can then be attacked by carboxypeptidases (6, 22). It seems likely that bonds including glycine residues are particularly resistant to attack by barley carboxypeptidases and thus the proportion of glycine in the peptide fractions is high. This is also supported by the low levels of free glycine observed in the endosperm (Table II) and produced during endosperm breakdown in degermed malt where metabolic removal of free glycine is unlikely (18). Similarly, the rapid removal of glutamate/glutamine by carboxypeptidases would account for the low levels of these amino acids in the peptides.

Little is known about the specificities of barley carboxypepti-

Table II. *Compositions of the Free Amino Acid Pools of Barley Embryo and Endosperm*

Values were determined by using dansyl chloride analysis.

Amino Acid \ Tissue	Whole Grain[a]	Distal Half-grain[a]	Proximal Half-grain[a]	Endosperm[a]						Embryo[a]			Embryo[b]		
Days of Germination	0	0	0	1	2	3	4	5	6	1	2	3	1	2	3
Ala	13.4	14.2	11.5	14.3	13.0	13.3	11.3	11.9	16.3	12.0	8.1	9.2	24	67	92
Gly	3.9	6.0	5.6	7.7	5.1	4.4	4.0	7.4	7.7	6.5	3.5	3.1	13	29	31
Glx	7.3	9.0	8.4	18.5	6.9	4.8	6.1	5.0	8.9	9.5	4.5	8.9	19	37	89
Asx	21.5	20.3	19.6	14.1	7.4	4.8	7.4	4.9	6.2	5.0	19.9	16.2	10	165	162
Ser	3.7	5.8	2.2	4.6	4.8	3.9	3.3	3.1	3.9	4.5	4.8	4.5	9	40	45
Thr	1.8	1.7	1.6	2.2	2.8	2.3	2.3	1.8	3.5	2.5	3.5	3.7	5	29	37
Pro	17.9	19.8	19.3	17.2	27.5	38.0	31.4	30.4	16.7	24.6	10.8	11.8	49	90	118
Val	5.4	5.9	6.6	5.5	8.2	8.8	10.0	9.0	8.3	9.5	12.7	12.3	19	105	123
Cys	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND	0.1	0.7	0.6	0.2	6	6
Met	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Leu	3.1	3.4	2.1	4.6	10.2	7.6	8.3	8.2	7.3	3.0	12.4	10.0	6	103	100
Ile	2.3	3.5	1.9	2.0	5.1	4.1	5.7	4.5	3.8	4.0	8.1	5.6	8	67	56
Lys	2.1	2.8	2.5	2.2	5.0	3.8	4.8	4.3	5.8	6.0	6.0	4.1	12	50	41
GAB	4.0	3.1	5.6	3.9	2.6	3.3	4.1	8.6	10.6	4.5	3.6	8.0	9	30	80

[a] Expressed as a percentage of the amino acids recovered.
[b] Expressed as nmol/embryo.
[c] Not detected.

dases, although these enzymes do have restricted activity on peptide bonds involving proline (1, 26, 31). The fact that proline residues were found not to accumulate in barley peptides indicates that either carboxypeptidase activity is sufficient to release free proline (the carboxypeptidases of wheat will apparently cleave bonds involving proline, ref. 25), or that a specific enzyme(s) for such bonds exists. Some evidence in favor of such an enzyme has been presented (19).

The N-terminal residues of the endosperm peptides, although deficient in glycine, are otherwise similar in composition to the total peptide pool (Table I). Again the composition remained constant during germination (results not presented). Thus, there is no evidence for the development of specific enzymic activities during germination: protein/peptide cleavage remains uniform.

Embryo Peptides and Polypeptides. Embryo peptide and polypeptide compositions resemble the amino acid compositions of the proteins from barley seedlings (Table I) (20, 32), although they are higher in glycine and proline and low in lysine. Explanations analogous to those discussed above for the endosperm proteins may account for these differences, although it is also possible that the young embryos studied here have different compositions than do more mature seedlings.

Free Amino Acid Pools. Unlike the peptide pools, the compositions of the free amino acid pools of the embryo and endosperm vary considerably as germination proceeds (Table II). During germination, GAB increases more rapidly than any other free amino acid in both the embryo and the endosperm. The accumulation of this amino acid in barley is well documented (3). Changes in the proportions of other amino acids, particularly, of proline, glutamic acid, and alanine, are known to depend on the conditions in which the grain is germinated (see 11).

The compositions of the free amino acid pools are very different from the equivalent peptide pools. Proline, leucine, and valine constitute particularly high proportions of the free amino acid pools compared with the peptide pools, whereas free glycine and glutamate/glutamine are relatively scarce. Presumably the amount of any particular amino acid in the endosperm reflects a balance between its rate of release by carboxypeptidases and its transfer to the embryo. In the embryo, metabolism will also be important.

PEPTIDE CHAIN LENGTH

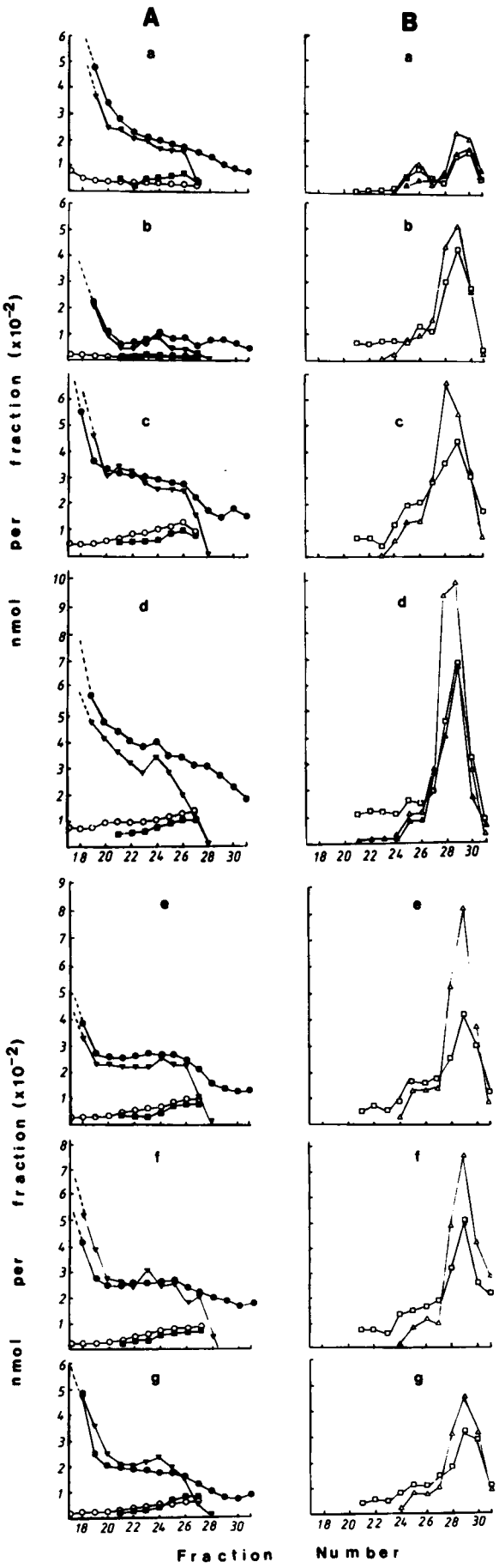
At any particular stage of germination all small peptides are present in similar amounts in both the endosperm and embryo: there is no preponderance of di- or tripeptides. Thus, although small peptides are produced in the endosperm during germination they do not accumulate and may even decline, despite the apparent absence of peptidase activity (22). This is a good indication that peptides are being transported to the embryo.

PEPTIDE AND AMINO ACID POOL SIZES

Assuming that all bound amino acids are conjugated in peptide linkages, the total peptide (fractions 21–27) and amino acid (fractions 28–32) pools in both the endosperm and embryo can be calculated for each stage of germination (Table III). The pool sizes vary considerably during germination (Fig. 5).

Considerable quantities of peptides are present in ungerminated barley grains. Comparison of the peptides present in the proximal and distal half-grains indicates that most of these peptides are endospermal (especially considering that the embryo actually occupies less than 30% of the proximal half-grain) and that free amino acids are at higher concentrations in the embryo than in the endosperm (Table III). The quantities of peptides in the endosperm decrease during the first 24 h of germination. This may be a result of the movement of peptides to the embryo via the peptide transport system, known to be active after only a few hours of germination (11, 28). In addition, the overall loss of peptides from the grain (embryo plus endosperm) between days 0 and 1 indicates that peptidases are active at very early stages of germination.

The peptide and amino acid pools of the endosperm increase to a maximum after 2 to 3 days of germination, and subsequently decline (Fig. 5). Similar changes in the soluble nonprotein nitrogen fractions (probably peptides) and the free amino acid pools have been reported in barley (8) and maize (17) endosperm. The peptide pool reaches its maximum size before the free amino acid pool. This might be expected if free amino acids are produced from peptides by hydrolysis. Although the total pool of peptides per endosperm decreases in size between days 2 and 6 of germination, the amount of peptide per g dry weight actually increases (Table



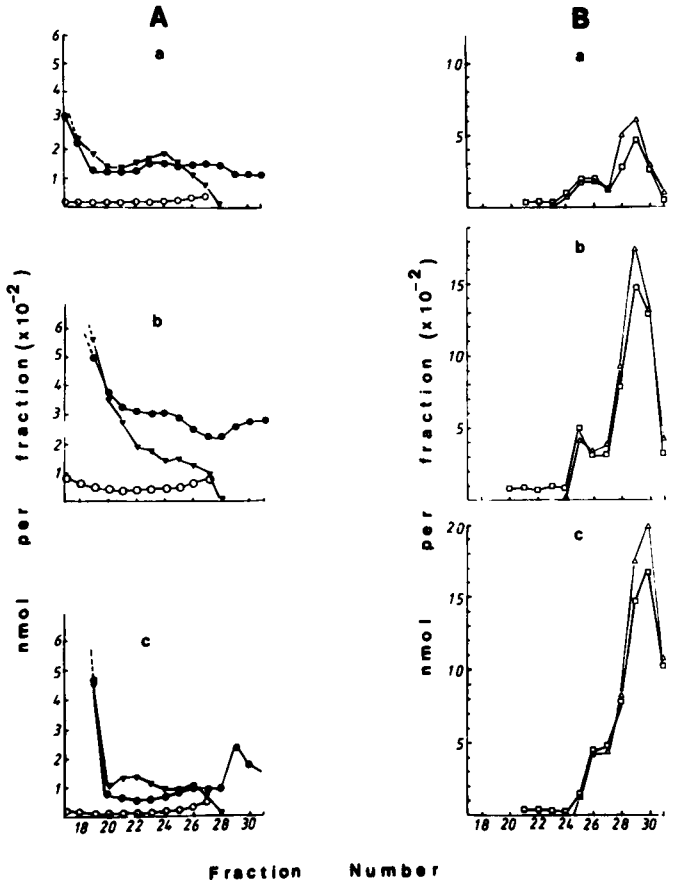


FIG. 4. Separation of peptides (A) and amino acids (B) from embryo extracts of barley grains germinated for varying periods of time (a–c are days 1–3, respectively). Peptides and amino acids eluting in each fraction were assayed in various ways. (A), Absorbance at 206 nm (nmol of peptide bond per fraction, ●); dansyl chloride after acid hydrolysis (nmol of bound amino acids per fraction, ▼); fluorescamine (nmol of free amino groups per fraction, ○). (B), Dansyl chloride (nmol of free amino acids per fraction, △); TNBS (nmol of free amino groups per fraction, □). Note the difference in scales between A and B.

III). Assuming a seed protein content of 10%, on day 2 about 3% of the endosperm nitrogen is present as peptide or amino acid. This proportion reaches 15% by day 6, as nitrogen is removed from the endosperm.

Peptides are also detected in the embryo. These may arise from the movement of peptides into the embryo from the endosperm. Alternatively, they may be produced within the embryo from prematurely terminated proteins, or more probably from the degradation of storage proteins known to be present in the scutellum (5). Unlike the peptide pool, the free amino acid pool of the embryo increases very rapidly during germination. Similar increases have been reported elsewhere (8, 17, 20). However, al-

FIG. 3. Separation of peptides (A) and amino acids (B) from endosperm extracts of barley grains germinated for varying periods of time (a–g are 0–6 days, respectively). Various methods were used to assay the peptides and amino acids eluting in each fraction. Absorbance at 206 nm (nmol peptide bond per fraction, ●); dansyl chloride analysis after acid hydrolysis (nmol of bound amino acids per fraction, ▼); fluorescamine analysis (nmol of free amino groups per fraction, ○); N-terminal analysis (nmol of N-terminal amino acids per fraction, ■). (e–g), Dansyl chloride analysis (nmol of free amino acids per fraction, △); TNBS assay (nmol of free amino groups per fraction, □); TNBS assay in the presence of Cu^{2+} ions (nmol of free amino groups (nonpeptide) per fraction, ▲).

Table III. *Peptide and Amino acid Pool Sizes of Germinating Barley Grains*

Tissue	Whole Grain	Distal Half-grain	Proximal Half-grain	Endosperm						Embryo		
Days of Germination	0	0	0	1	2	3	4	5	6	1	2	3
Peptides												
nmol/grain	69	64	45	20	117	116	96	83	62	34	62	36
μmol/g dry wt	2.0	2.2	1.9	0.6	4.2	5.0	5.6	6.2	7.8	26.0	13.9	14.4
μmol/ml cell fluid	19.5	20.8	18.4	0.9	4.2	3.4	3.1	2.4	1.9	6.6	1.3	1.1
Free amino acids												
nmol/grain	271	196	185	155	399	547	402	313	205	197	829	996
μmol/g dry wt	7.9	6.5	7.8	4.1	14.2	21.0	23.6	23.5	26.0	154	187	40
μmol/ml cell fluid	76.2	63	75	6.2	14.2	14.5	12.9	9.2	6.3	39	13	30

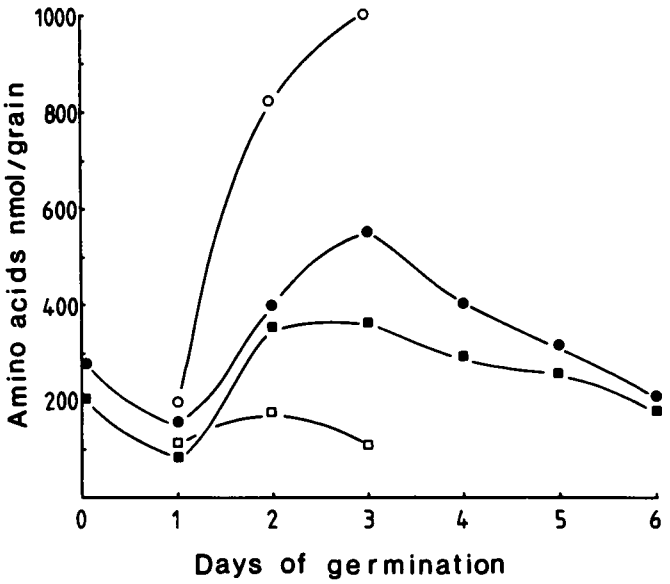


FIG. 5. Variations in the size of the peptide and free amino acid pools of barley tissues during germination. Peptides (□, ■) and amino acids (○, ●) in the embryo (□, ○) and endosperm (■, ●) were estimated using dansyl chloride. Peptide concentrations are expressed as nmol of peptide-bound amino acids per grain.

though the total amount of amino acids per embryo increases markedly, the concentration in the cell fluid remains relatively constant (Table III).

Similar amounts of free and bound amino acids are found in the endosperm. This indicates that peptides might be at least as important as amino acids in the transport of nitrogen from the endosperm to the embryo. The concentration of peptides in the endosperm reaches 2 to 4 mM during germination (Table III). This value is a minimum as it is unlikely that the peptides are distributed evenly throughout the endosperm *in vivo*: higher concentrations may be reached adjacent to the scutellum. These peptide concentrations are very close to the K_t values obtained for the scutellar transport system (*e.g.* 1.9 mM for trialanine; unpublished results) and the K_m of the embryo peptidases (27). Thus, peptides are present in the endosperm at adequate concentrations for the efficient operation of the peptide transport system.

The maximum rate of nitrogen transfer from the endosperm to the embryo of barley grains during germination is about 150 μg nitrogen/grain/day, or 940 μg protein/grain/day (21). Assuming transfer occurs entirely as tripeptides, this corresponds to a maximum rate of transfer of about 2.9 μmol peptide/grain/day. The concentration of peptides in the endosperm is at least 3 mM (Table III). At this concentration the rate of trialanine uptake is about 5 μmol/g fresh wt·h, and most other peptides seem to be transported at similar rates (11). Thus, the rate of peptide transport is about 0.5 μmol/grain/h, or 12 μmol/grain/day. Peptide transport therefore occurs at a more than adequate rate to account for the complete transfer of nitrogen from the endosperm to the embryo during germination. Similar calculations show that the rather slower rates of amino acid transport (11, 14) are unable to account for this transfer.

The present results provide the first unambiguous demonstration of pools of small peptides in plant tissues. Although there have been a number of reports indicating that growing seedlings may contain such pools (*e.g.* 4, 8) these may be subject to other interpretations. It seems likely that the methods employed here could be applied with little or no modification to other tissues in order to establish the general existence and possible function of peptide pools in plant tissues.

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LITERATURE CITED

1. BAXTER ED 1978 Purification and properties of malt carboxypeptidases attacking hordein. J Inst Brew 84: 271–275
2. BINKLEY F, F LEIBACH, N KING 1968 A new method of peptidase assay and the separation of three leucylglycinases of renal tissues. Arch Biochem Biophys 128: 397–405
3. DIXON ROD, L FOWDEN 1961 γ-Aminobutyric acid metabolism in plants. 2. Metabolism in higher plants. Ann Bot 25: 513–530
4. DUKE SH, LE SCHRADER, MG MILLER, RL NIECE 1978 Low temperature effects on soybean (*Glycine max* (L.) Merr. cv. Wells). Free amino acid pools during germination. Plant Physiol 62: 642–647
5. DURE LS 1960 Gross nutritional contributions of maize endosperm and scutellum to germination growth of maize axis. Plant Physiol 35: 919–925
6. ENARI T-M, J MIKOLA 1977 Peptidases in germinating barley grain: properties localization and possible functions. *In* K Elliott, M O'Connor, eds, CIBA Foundation Symposium No. 50. Associated Scientific Publishers, Amsterdam, pp 335–352
7. FOLKES BF, EW YEMM 1956 The amino acid content of the proteins of barley grains. Biochem J 62: 4–11
8. FOLKES BF, EW YEMM 1958 The respiration of barley plants. X. Respiration and the metabolism of amino-acids and proteins in germinating grain. New Phytol 57: 106–131
9. GRANT DR, CC WANG 1972 Dialysable components resulting from proteolytic activity in extracts of wheat flour. Cereal Chem 49: 201–207
10. HARTLEY BS 1970 Strategy and tactics in protein chemistry. Biochem J 119: 805–822
11. HIGGINS CF 1979 Peptide transport of embryos of germinating barley (*Hordeum vulgare*). PhD thesis. University of Durham, Durham, England
12. HIGGINS CF, JW PAYNE 1977 Peptide transport by germinating barley embryos. Planta 134: 205–206
13. HIGGINS CF, JW PAYNE 1977 Characterization of active peptide transport by germinating barley embryos: effects of pH and metabolic inhibitors. Planta 136: 71–76
14. HIGGINS CF, JW PAYNE 1978 Peptide uptake by germinating barley embryos: uptake of physiological di- and oligopeptides. Planta 138: 211–215
15. HIGGINS CF, JW PAYNE 1978 Peptide transport of germinating barley embryos: evidence for a single common carrier for di- and oligopeptides. Planta 138: 217–221
16. HIGGINS CF, JW PAYNE 1978 Stereospecificity of peptide transport by germinating barley embryos. Planta 142: 299–305

17. INGLE J, L BEEVERS, RH HAGEMAN 1964 Metabolic changes associated with the germination of corn. Plant Physiol 39: 735–740
18. JONES M, JS PIERCE 1966 Biochemistry of amino acid production in germinating barley. Proc Am Soc Brew Chem 10: 10–17
19. JONES M, JS PIERCE 1967 Malt peptidases. J Inst Brew 73: 349–351
20. JONES M, JS PIERCE 1967 The role of proline in the amino acid metabolism of germinating barley. J Inst Brew 73: 577–583
21. METEVIER JR, JE DALE 1977 The utilization of endosperm reserves during the early growth of barley cultivars and the effect of time of application of nitrogen. Ann Bot 41: 715–728
22. MIKOLA J, L KOLEHMAINEN 1972 Localization and activity of various peptidases in germinating barley. Planta 104: 167–177
23. PERRETT D, JPW WEBB, DBA SILK, ML CLARK 1975 The assay of dipeptides using fluorescamine and its application to determining dipeptidase activity. Anal Biochem 68: 161–166
24. PORATH J 1960 Gel filtration of proteins, peptides and amino acids. Biochim Biophys Acta 19: 193–207
25. PRESTON K, J KRUGER 1977 Specificity of two isolated wheat carboxypeptidases. Phytochemistry 16: 525–528
26. RAY LE 1976 Large scale isolation and partial characterization of some carboxypeptidases from malted barley. Carlsberg Res Commun 41: 169–182
27. SOPANEN T 1976 Purification and partial characterization of a dipeptidase from barley. Plant Physiol 57: 867–871
28. SOPANEN T 1979 Development of peptide transport activity in barley scutellum during germination. Plant Physiol 64: 570–574
29. SOPANEN T, D BURSTON, DM MATTHEWS 1977 Uptake of small peptides by the scutellum of germinating barley. FEBS Lett 79: 4–7
30. SOPANEN T, D BURSTON, E TAYLOR, DM MATTHEWS 1978 Uptake of glycylglycine by the scutellum by germinating barley grain. Plant Physiol 61: 630–633
31. YABUUCHI S, E DOI, T HATA 1973 The mode of action of a carboxypeptidase from malt. Agric Biol Chem 37: 687–688
32. YEMM EW, BF FOLKES 1953 The amino acids of cytoplasmic and chloroplastic proteins of barley. Biochem J 55: 700–707