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Mobilization of Proline in the Starchy Endosperm of Germinating Barley Grain*

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Abstract. In germinating grains of barley, *Hordeum vulgare* L. cv. Himalaya, free proline accumulated in the starchy endosperm during the period of rapid mobilization of reserve proteins. When starchy endosperms were separated from germinating grains and homogenized in a dilute buffer of pH 5 (the pH of the starchy endosperm), the liberation of proline continued in these suspensions. The process was completely inhibited by diisopropylfluorophosphate, indicating that it was totally dependent on serine carboxypeptidases. The carboxypeptidases present in the starchy endosperms of germinating grains were fractionated by chromatography on DEAE-cellulose. Four peaks were obtained, all with different activity spectra on the seven carbobenzoxydipeptides (Z-dipeptides) tested. Two of the peaks corresponded to previously known barley carboxypeptidases; these as well as a third peak hydrolyzed substrates of the types Z-X-Y and Z-X-Pro (X and Y denote any amino acid residue except proline). The fourth peak corresponded to a proline carboxypeptidase specific for substrates of the Z-Pro-X type. Apparently, in the hydrolysis of longer proline-containing peptides there must be sequential cooperation between the two carboxypeptidase types. The carboxypeptidases in extracts of starchy endosperms also liberated proline from the peptides Ala-Ala-Ala-Pro and Ala-Ala-Pro while Ala-Pro and Pro-Ala were not attacked. The dipeptides, however, were rapidly hydrolyzed around pH 7 by extracts prepared from the scutella of germinating grains. It is concluded that one part of the proline residues of the reserve proteins is liberated in situ in the starchy endosperm through the combined action of acid proteinases and carboxypeptidases, while another part is taken up in the form of small peptides by the scutellum, where proline is liberated by amino- and/or dipeptidases in some "neutral compartment".

Key words: Endosperm – Germination (seeds) – *Hordeum* – Proline – Reserve mobilization (seeds) – Seed germination.

Introduction

In germinating seeds, reserve proteins are hydrolyzed to free amino acids (and partly converted to glutamine and/or asparagine and sucrose) before transport to the growing tissues of the seedling. In barley grains proline is one of the most abundant amino acids, comprising about 12% of the amino acid residues in the total protein and about 20% in the main reserve protein, hordein (Shewry et al. 1978). Proline is also by far the most abundant free amino acid in germinated barley (malt) and it is rapidly liberated during autolysis of malt at 65° C (brewery mashing) with a pH optimum near 5 (Jones and Pierce 1967). However, none of the well-known barley peptidases (Kolehmainen and Mikola 1971; Moeller et al. 1970; Sopanen 1976; Sopanen and Mikola 1975; Visuri et al. 1969) has been shown to possess even moderate activity on peptides containing proline. In this report we describe the results of an investigation made to find out which enzymes liberate proline in germinating barley and in which parts of the grain the reactions occur.

Materials and Methods

Plant Material. Grains of a huskless variety of barley *Hordeum vulgare* L. cv. Himalaya were obtained from the Agronomy Club,

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Abbreviations: DFP = diisopropylfluorophosphate; DTT = dithiothreitol; TNBS = 2,4,6-trinitrobenzenesulphonic acid; Z- = N-carbobenzoxy; TLC = thin layer chromatography.

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Washington State University, Pullman, Washington. The grains were surface-sterilized by treatment with 1% sodium hypochlorite solution for 20 min, washed once with water, immersed in 10 mM HCl for 10 min to destroy remaining hypochlorite (Abdul-Baki 1974), and finally washed 8 times with water. The grains were allowed to germinate on agar gel at 16° C in the dark (Mikola and Kolehmainen 1972).

Determination of the Distribution of Total Nitrogen. The grains were allowed to germinate for 1 to 7 days and the embryo/seedling, including the scutellum, was separated from the endosperm by careful dissection from batches of 70 to 180 seeds; the "0-day" seed parts were separated from seeds imbibed in a shallow layer of water for about 6 h at 25° C. The separated grain parts were dried at 105° C for 18 h, weighed, and ground to a fine powder with a mortar mill. Nitrogen contents were determined by the Kjeldahl method using 10 to 40 mg samples of the powders.

Extraction of Proline from Grain Parts. Scutella (embryo proper/seedling removed) and endosperms were separated by careful dissection from seeds germinated for 1 to 7 days. The aleurone layer and starchy endosperm could be satisfactorily separated after 4 days' germination. The separated aleurone layers were immediately rinsed with distilled water for about 5 s and excess moisture was removed with absorbent paper; the scutella from grains germinated for 3 to 7 days were treated in a similar way.

In the extraction, use was made of the fact that proline is highly soluble in 70% ethanol. Ten seed parts were homogenized in 5 ml of 70% ethanol at 25° C in a Potter-Elvehjem homogenizer with ground-glass surfaces until the suspension appeared homogeneous (1 to 10 min). The suspensions were centrifuged at 25° C without further agitation, and the clear extracts were used for the proline assays.

Determination of Free Proline. The isatin method (Elliott and Gardner 1976) was modified by adding Zn^{2+} to the reaction mixture and the measuring solution to stabilize the blue, light-sensitive color given by proline with isatin (Zn^{2+} has been used for this purpose in some isatin sprays for TLC plates). In the routine assay the following solutions were mixed in 10 ml test tubes of brown glass: 100 µl of sample in 70% ethanol, 100 µl of disodium citrate-HCl buffer of pH 3.9 (prepared by adding 0.2 M HCl to 0.2 M disodium citrate) containing 0.2% $ZnCl_2$ (w/v), and 250 µl of 0.075% isatin in acetone. The tubes were kept in a boiling water bath for 15 min, during which time the contents of all tubes evaporated to dryness. The tubes were then transferred to racks covered with aluminium foil, and the following solutions were added: 0.5 ml phenol saturated with water, 1 ml 50 mM $ZnCl_2$ in 5 mM HCl, and 2 ml acetone. The tubes were agitated with a Vortex mixer to dissolve all the solids and the absorbances were read at 595 nm against a reagent blank. The color was practically stable for at least 1 h. The reaction was not completely specific for free proline because peptides with N-terminal proline gave a similar although less intensive color. However, separations with TLC followed by staining with isatin showed that with the extracts studied the contribution of peptides to the total color was negligible. This method worked well with the extracts of all grain parts, but some interfering substances present in extracts of shoots and rootlets caused low recoveries of proline from these organs.

Assays of Carboxypeptidase Activity. In the assays of the enzymatic hydrolysis of Z-Ala-Pro and Z-Phe-Pro, the substrate concentration was 8 mM and the reaction pH was 5.2 or 5.7, respectively. The proline liberated in the reaction was determined by the isatin reaction. The details of the procedure were as follows: One hundred µl of 20 mM substrate solution (sodium salt, pH about 6) were mixed with 100 µl of 0.1 M sodium citrate buffer, pH 5.2 or 5.7, in 10·100 mm centrifuge tubes, and the tubes were allowed to equilibrate at 30° C. The reactions were started by adding 50 µl suitably diluted enzyme solution, allowed to proceed for 2 h at 30° C, and terminated by adding 500 µl absolute ethanol. The tubes were stoppered and allowed to stand at room temperature for 15 to 30 min, after which the precipitated proteins were removed by centrifugation (4,700 *g*, 15 min, 25° C). Free proline was determined from 100 µl aliquots of the supernatants as described above.

The hydrolyses of Z-dipeptides having C-terminal α-amino acid residues were assayed as described previously (Mikola and Kolehmainen 1972). The reaction mixtures contained 1.82 mM substrate; 50 mM sodium acetate buffer, pH 5.2 for Z-Phe-Ala, pH 5.7 for Z-Phe-Phe, and pH 5.0 for Z-Pro-Trp, Z-Pro-Met, and Z-Pro-Ala; and 0.45 mM EDTA. The reaction times at 30° C were from 2 to 18 h depending on the substrate, and the amino acids liberated were assayed by the TNBS-reaction.

All the activities are expressed as enzyme units, one unit corresponding to the hydrolysis of 1 µmol substrate min^{-1} at 30° C.

Determinations of the Enzymatic Hydrolysis of Peptides. Peptides (8 mM) were incubated with the enzyme solutions either in 40 mM sodium acetate buffer, pH 5.0, or in 40 mM sodium phosphate buffer, pH 7.1, at 30° C. Proline liberated from Ala-Pro, Ala-Ala-Pro, and Ala-Ala-Ala-Pro was determined by the isatin reaction and alanine liberated from Pro-Ala was determined by the TNBS-reaction as in the corresponding carboxypeptidase assays.

Estimation of the Internal pH of the Starchy Endosperm. The soft (strongly autolyzing, modified) proximal part of the starchy endosperm was separated from grains germinated for 3 to 5 days and macerated with 50 µl water in a small plastic vial. The pH of the thick slurry was measured with a semimicro surface electrode (Ingold LoT 403-M8). Next, 50 µl water were added, the slurry was further macerated, and the pH was recorded. This procedure was repeated once more. The three pH values never differed by more than 0.02 units and their mean was taken as the final value. For grains germinated for 6 to 7 days, the procedure was similar except that whole starchy endosperms were used and the water additions were 25 µl each.

Crude Enzyme Preparation for DEAE-Cellulose Chromatography. Starchy endosperms were separated from 500 grains after germination for 5 days and stored at −20° C. After being thawed, the material was homogenized in 15 ml of 5 mM acetic acid containing 50 mM 2-mercaptoethanol at about 5° C (Virtis 45 homogenizer, 6 times for 15 s). The volume of the slurry was then made up to 50 ml with the extraction solution, and its pH was adjusted to 4.9 with 1 M acetic acid. After 30 min at 5° C a clear extract was obtained by centrifugation (20,000 *g*, 15 min, 5° C), and the pH was adjusted to 6.5 with 1 M Tris-base. After about 18 h at 5° C the carboxypeptidases were precipitated with ammonium sulphate between 35% and 65% saturation [additions of solid $(NH_4)_2SO_4$, agitation for 30 min at 5° C, centrifugation as above]. The active precipitate was dissolved in 15 ml of 20 mM sodium phosphate buffer, pH 6.5, and the remaining ammonium sulfate was removed by gel filtration on Sephadex G-25 equilibrated with the same buffer (25° C).

Reagents. Peptides and Z-dipeptides were obtained from Bachem Feinchemikalien A.G., Bubendorf, Switzerland; all the amino acid residues were of the L-configuration. 2,4,6-Trinitrobenzenesulphonic acid was from Sigma Chemical Co. and isatin from E. Merck A.G. All normal reagents were of reagent grade.

Results and Discussion

Liberation of Proline In Vivo and In Vitro. In Himalaya barley germinating at 16° C the most rapid mobilization of endosperm reserve materials (decrease of dry weight and total nitrogen) occurred between the 3rd and 7th day (Fig. 1). During this time the amount of free proline remained approximately constant in the aleurone layer and scutellum, but increased greatly in the starchy endosperm (Fig. 2). This suggests that proline might actually be liberated in the starchy endosperm. To confirm this view some autolysis experiments were carried out with separated starchy endosperms. First, the pH of the autolyzing part of the starchy endosperm was determined and found to be remarkably constant at pH 4.9 to 5.0 between the 3rd and 7th day of germination (Table 1). Starchy endosperms were then separated from "4-day grains", macerated in dilute buffer, pH 5, and incubated in aseptic conditions at 30° C. A rapid liberation of proline occurred in these suspensions (Fig. 3), and it was completely inhibited by diisopropylfluorophosphate, a specific inhibitor of acid carboxypeptidases in barley (Mikola et al. 1971). These results indicate that at least a large part of the proline of the reserve proteins is liberated in the starchy endosperm, and that the process is completely dependent on carboxypeptidase activity. Presumably, the insoluble reserve proteins are first hydrolyzed to soluble peptides by the endosperm proteinases which are highly active at pH 5 (Sundblom and Mikola 1972) and some carboxypeptidases sequentially liberate proline among other amino acids from the C-termini of these peptides.

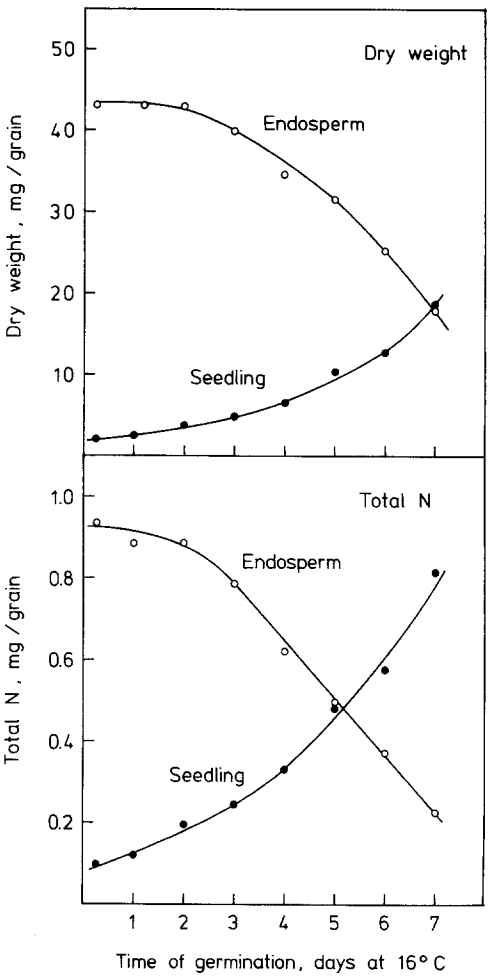

Fig. 1. Time course of the germination of Himalaya barley at 16° C. The grains were allowed to germinate in Petri dishes on 0.75% agar gel in the dark. The value for each point was obtained using a batch of 70 to 180 grains

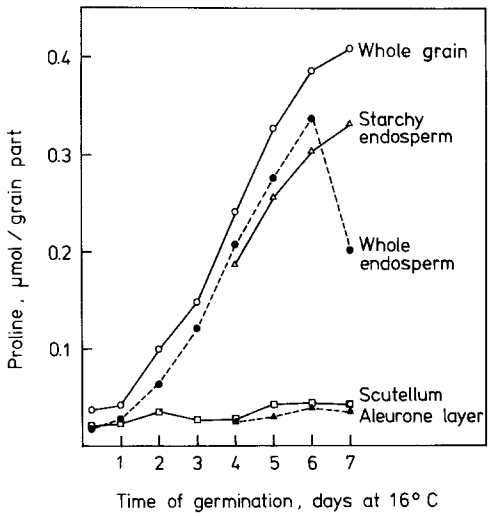

Fig. 2. Amount of free proline in different tissues of Himalaya barley during germination at 16° C. The points correspond to mean values of 3 extracts each prepared from 10 seed parts. The values for the starchy endosperm have been obtained by subtraction (whole grain – aleurone layer and scutellum). The low value for the whole endosperm on day 7 is at least in part due to losses of liquid starchy endosperm during the separation

Table 1. pH of the softened part of the starchy endosperm in grains of Himalaya barley germinating at 16° C. The figures are means of seven measurements each made from a macerate corresponding to 7 grains

Germination time, days	pH ± S.D.	Approximate fraction of the starchy endosperm used for the macerates
3	4.90 ± 0.04	2/5
4	4.93 ± 0.05	1/2
5	4.90 ± 0.05	2/3
6	4.99 ± 0.01	whole
7	5.02 ± 0.05	whole

Carboxypeptidases Present in the Starchy Endosperm. Extracts of germinating grains of Pirkka barley have

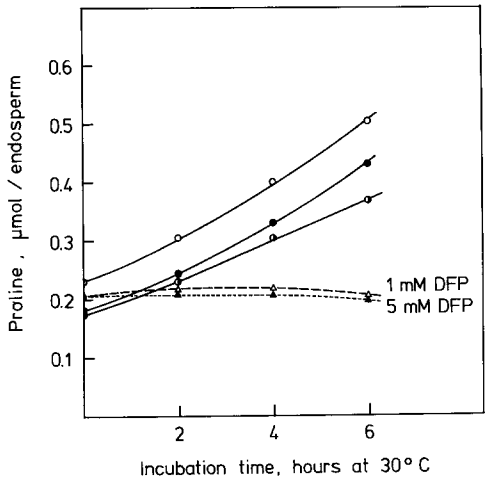

Fig. 3. Liberation of proline in vitro in suspensions of starchy endosperms at pH 5. Starchy endosperms were separated from 10 "4-day grains" and homogenized in 3 ml of 50 mM sodium acetate buffer of pH 5.0 containing 1 mM DTT. The suspensions were incubated under aseptic conditions at 30° C. DFP was added as a 500 mM solution in isopropanol. Each curve represents one suspension

Table 2. Hydrolysis of Z-dipeptides by extracts of starchy endosperms separated from barley grains germinated for 5 days at 16° C. Small portions of extracts prepared for DEAE-cellulose chromatography were purified by gel filtration in columns of Sephadex G-25 equilibrated with 20 mM sodium phosphate buffer of pH 6.5. The figures are mean values of 3 extracts given with S.D.

Substrate	pH	Reaction rate µmol min^{-1} 100 $grains^{-1}$	Relative rate
Z-Phe-Phe, 1.82 mM	5.7	10.2±3.4	100
Z-Phe-Ala, 1.82 mM	5.2	18.8±3.7	184
Z-Phe-Pro, 8 mM	5.7	0.60±0.24	6
Z-Ala-Pro, 8 mM	5.2	0.60±0.03	6
Z-Gly-Pro, 8 mM	5.0	<0.02	–
Z-Pro-Pro, 8 mM	5.0	<0.02	–
Z-Pro-Ala, 1.82 mM	5.0	0.20±0.03	2
Z-Pro-Met, 1.82 mM	5.0	0.20±0.03	2
Z-Pro-Trp, 1.82 mM	5.0	0.10±0.02	1

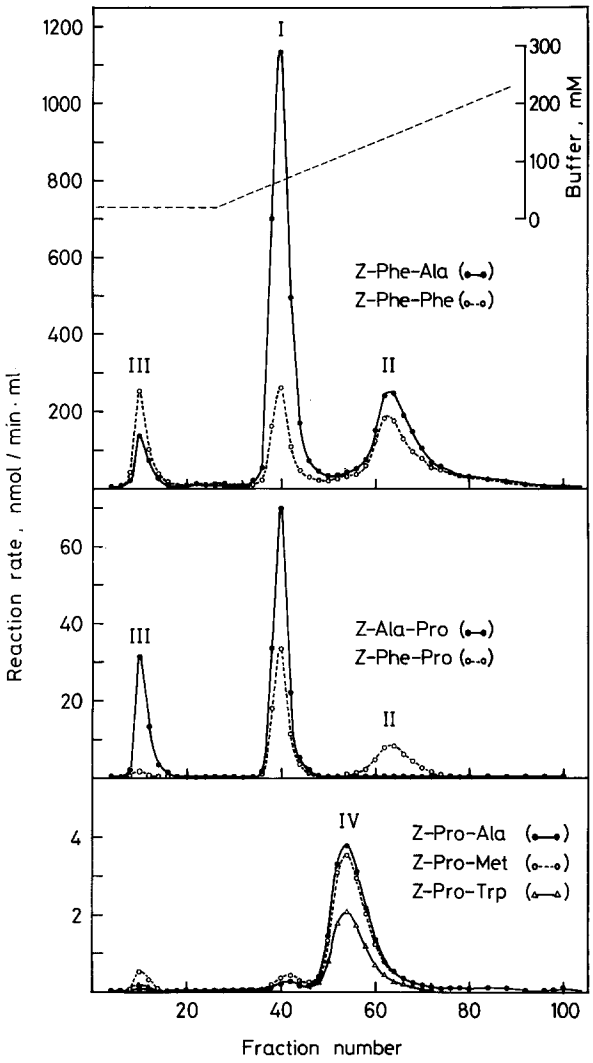

Fig. 4. Fractionation of the carboxypeptidases present in the starchy endosperm of germinating barley grains by chromatography on DEAE-cellulose. A crude enzyme preparation corresponding to 500 "5-day grains" (see Materials and Methods) was fed into a 1.6·35 cm column of Whatman DE-32 equilibrated with 20 mM sodium phosphate buffer of pH 6.5 and thermostated at 25° C. The column was washed with 110 ml of the starting buffer and eluted with a linear gradient of increasing buffer strength (20 to 300 mM sodium phosphate of pH 6.5, 500 ml). The elution rate was 30 ml h^{-1}, and carboxypeptidase activities were determined from 6 ml fractions of the eluate

been reported to have no activity on Z-Gly-Pro but to slowly hydrolyze Z-Pro-Trp (Visuri et al. 1969). We confirmed these findings with extracts of the starchy endosperms of "5-day grains" of Himalaya barley and further tested five other proline-containing Z-dipeptides (Table 2): Z-Phe-Pro, Z-Ala-Pro, Z-Pro-Ala, and Z-Pro-Met were all hydrolyzed while no reaction was detected for Z-Pro-Pro. To find out which carboxypeptidases were responsible for these activities, a method was developed for separating barley carboxypeptidases by chromatography on DEAE-cellulose. The result obtained with starchy endosperms of 5-day grains is shown in Fig. 4. There are four peaks, all with different activity spectra on the seven Z-dipeptides tested. The properties of each enzyme and their relationships to formerly studied carboxypeptidase activities of barley are discussed in some detail below. The numbers shown above the peaks correspond to a suggested extension of the nomenclature of barley carboxypeptidases used by Yabuuchi et al. 1972).

Peak I corresponds to the barley carboxypeptidase purified by Visuri et al. (1969) and later studied and called barley carboxypeptidase I by Yabuuchi et al. (1972). Recently Schroeder and Burger (1978) have shown that this enzyme is synthesized and secreted by separated aleurone layers of barley. The enzyme is characterized by very high activity on Z-Phe-Ala and moderate activity on Z-Phe-Phe. It also liberated proline from Z-Phe-Pro and Z-Ala-Pro (Fig. 4, middle). Our earlier conclusion that this enzyme does not liberate C-terminal proline (Visuri et al. 1969), which was based on the use of the single, poor substrate Z-Gly-Pro, was thus premature. On the other hand, it seems that this enzyme has no activity on substrates with a penultimate proline residue (Fig. 4, bottom). There is a small activity peak, but it is slightly behind peak I; moreover, in a corresponding run made at 5° C, this small peak as well as peak IV was retarded and clearly separated from peak I.

Peak II corresponds to the enzyme which Yabuuchi et al. (1972) separated from carboxypeptidase I by chromatography on DEAE-cellulose and called barley carboxypeptidase II. This enzyme is characterized by high activity on both Z-Phe-Ala and Z-Phe-Phe. Later, Yabuuchi et al. (1973) showed that it sequentially liberates Lys, Gly, Phe, and Val residues from the C-terminus of a natural dodekapeptide. It should be noted that this enzyme releases proline from Z-Phe-Pro but not from Z-Ala-Pro (Fig. 4, middle). Apparently it has no activity toward substrates of the Z-Pro-X type (Fig. 4, bottom).

Peak III possibly corresponds to the carboxypeptidase purified by Moeller et al. (1970) as the barley enzyme which liberates C-terminal tryptophane from α-casein; the use of different purification methods and substrates, however, leaves this question open. The properties of the enzyme as shown in Fig. 4 include a higher activity on Z-Phe-Phe than on Z-Phe-Ala, a relatively high activity on Z-Ala-Pro, and a definite but very low activity on the substrates with a penultimate proline residue.

Peak IV apparently corresponds to the activity first observed with Z-Gly-Pro-Gly-Gly-Pro-Ala and Z-Pro-Trp (Visuri et al. 1969) and later shown to be more heat labile than the activities on the key substrates Z-Phe-Ala and Z-Phe-Phe (Mikola et al. 1972). The enzyme seems to be a proline carboxypeptidase, as it acts only on substrates with a penultimate proline residue. Enzymes of this type occur in mammalian tissues (see, Odya et al. 1978) and are typical serine carboxypeptidases with pH optima near 5 and complete inactivation by DFP. The activities on the three substrates (Fig. 4, bottom) are quite low compared to the activities of peaks I–III on their best substrates. However, the blocking of the imino group of the proline residue with the carboxybenzoxy group probably has a retarding effect on the reaction. With the corresponding enzyme of human kidney, natural oligopeptides are hydrolyzed about 20 times faster than the corresponding Z-dipeptides (Odya et al. 1978).

Table 3. Liberation of C-terminal amino acids from some peptides at pH 5.0 by an extract of starchy endosperms of grains germinated for 5 days at 16° C. Ten starchy endosperms were extracted with 3 ml of 50 mM sodium acetate buffer, pH 5.0, containing 1 mM DTT. Small-molecular compounds were removed by gel filtration in a column of Sephadex G-25 equilibrated with the same buffer containing 0.1 mM DTT

Substrate 8 mM	Reaction rate μmol min^{-1} 100 $grains^{-1}$	Relative rate %
Ala-Ala-Ala-Pro	2.87	100
Ala-Ala-Pro	0.80	20
Ala-Pro	0.01 (4.18)[a]	–
Z-Ala-Pro	0.40	14
Z-Pro-Ala	0.26	9
Pro-Ala	0.08 (8.57)[a]	3

[a] Rate given by a corresponding extract of "5-day scutella" at pH 7.1

In summary, there are three enzymes which liberate both proline and other amino acids from C-termini of peptides, provided that the penultimate residue is not proline, and one enzyme which requires a proline residue in the second position. Apparently, in the hydrolysis of longer peptides containing proline, there must be sequential cooperation between the two carboxypeptidase groups.

Action of the Carboxypeptidases on Oligopeptides. The cut-off limit of the carboxypeptidases was tested using extracts of starchy endosperms of 5-day grains (Table 3). C-Terminal proline was rapidly liberated at pH 5 from a tetrapeptide and more slowly from the corresponding tripeptide, while no reaction was observed for the corresponding dipeptide Ala-Pro. The hydrolyses of the tetra- and tripeptide were completely inhibited by 1 mM DFP. The dipeptide Pro-Ala was hydrolyzed very slowly; this reaction was not affected by DFP. These results suggest that the carboxypeptidases are able to liberate C-terminal proline from tripeptides and especially from larger peptides faster than from corresponding Z-dipeptides. Second, they suggest that the carboxypeptidases are not able to liberate proline from dipeptides. The slow hydrolysis of Pro-Ala is apparently due to some di- or aminopeptidase(s); whether it is an intrinsic constituent of the starchy endosperm or a "contaminant" due to leakage from other tissues during the dissection cannot be answered on the basis of the present data.

The results described above suggest that the hydrolysis of reserve proteins in the starchy endosperm by proteinases and carboxypeptidases leads to an accumulation of free proline and proline-containing di- and possibly tripeptides. The scutellum of the germinating grain, however, has the capacity for rapid, active uptake of small peptides including dipeptides with proline in either position (Higgins and Payne 1978; Sopanen et al. 1978). Moreover, the scutellum contains some peptidases with high activities on such peptides at pH 7.1 (Table 3). Therefore, it seems probable that a part of the proline derived from the reserve proteins is taken up by the scutellum in the form of small peptides, and that these peptides are hydrolyzed in some neutral compartment in the scutellum before further transport or metabolism.

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