Plant Physiol. (1986) 81, 823–829
0032-0889/86/81/0823/07/$01.00/0

Acid Carboxypeptidases in Grains and Leaves of Wheat, *Triticum aestivum* L.

Received for publication November 22, 1985 and in revised form February 24, 1986

LEENA MIKOLA
Department of Biology, University of Jyväskylä, SF-40100 Jyväskylä, Finland

ABSTRACT

Extracts of resting and germinating (3 days at 20°C) wheat (*Triticum aestivum* L. cv Ruso) grains rapidly hydrolyzed various benzyloxycarbonyldipeptides (Z-dipeptides) at pH 4 to 6. Similar activities were present in extracts of mature flag leaves. Fractionation by chromatography on CM-cellulose and on Sephadex G-200 showed that the activities in germinating grains were due to five acid carboxypeptidases with different and complementary substrate specificities. The wheat enzymes appeared to correspond to the five acid carboxypeptidases present in germinating barley (L Mikola 1983 Biochim Biophys Acta 747: 241–252). The enzymes were designated wheat carboxypeptidases I to V and their best or most characteristic substrates and approximate molecular weights were: I, Z-Phe-Ala, 120,000; II, Z-Ala-Arg, 120,000; III, Z-Ala-Phe, 40,000; IV, Z-Pro-Ala, 165,000; and V, Z-Pro-Ala, 150,000. Resting grains contained carboxypeptidase II as a series of three isoenzymes and low activities of carboxypeptidases IV and V. During germination the activity of carboxypeptidase II decreased, those of carboxypeptidases IV and V increased, and high activities of carboxypeptidases I and III appeared. The flag leaves contained high activity of carboxypeptidase I and lower activities of carboxypeptidases II, IV, and V, whereas carboxypeptidase III was absent.

Umetsu *et al.* (19) observed that the bulk of the acid carboxypeptidase activity present in wheat bran could easily be crystallized. The crystalline material was fractionated by chromatography on CM-cellulose using Z-Glu-Tyr[1] as substrate and four activity peaks were obtained. The most abundant isoenzyme was isolated in pure form and shown to be a dimeric glycoprotein with mol wt 118,000. Later, the same authors studied the catalytic properties of this enzyme in greater detail (20) and showed that the four isoenzymes were immunologically identical (21). Earlier, Preston and Kruger (16, 17) had studied the acid carboxypeptidase activity present in germinating wheat grains using hemoglobin as substrate. Chromatography of this activity on CM-cellulose yielded two enzyme fractions which, however, differed in both catalytic and molecular properties from the enzyme isolated from resting grains (19). Various parts of senescing wheat shoots also contain high acid carboxypeptidase activity when assayed with Z-Phe-Ala as substrate (6, 23).

Acid carboxypeptidase activity is present also in germinating grains of barley (10), and chromatography on DEAE-cellulose, hydroxylapatite, and Sephadex G-200 has shown that this activity is due to five carboxypeptidases with different and complementary substrate specificities (11); these enzymes have been designated barley carboxypeptidases I to V. The most striking difference between their specificities is that the enzymes I, II, and III show no activity against substrates of the type Z-Pro-X, whereas the enzymes IV and V are prolylcarboxypeptidases requiring a proline residue in the penultimate position. Barley carboxypeptidases I (1, 22) and II (2) have been isolated and thoroughly characterized. The results show that carboxypeptidase II isolated from germinating barley (2) is remarkably similar to the carboxypeptidase purified from wheat bran (19).

In other respects the carboxypeptidases present in the grains of wheat and barley seemed dissimilar. The aim of this study was to determine whether these differences are real or due to the different methods of assay and fractionation used. Second, once suitable fractionation methods were available, they were used to determine what kind of carboxypeptidases is responsible for the high activities present in wheat leaves. The main results of this study have been reported previously in abstract form (13).

MATERIALS AND METHODS

Plant Materials. Grains of a Finnish spring wheat (*Triticum aestivum* L. cv Ruso) were obtained from the State Seed Testing Station, Helsinki, Finland. A sample of Canadian hard red spring wheat cv Neepawa was kindly provided by Dr. Ken Preston, Canadian Grain Commission, Winnipeg. For experiments involving germination the grains were surface-sterilized with 1% NaOCl solution (12) and allowed to germinate under aseptic conditions on agar gel in the dark (10) at 20°C usually for 3 d. Flag leaves of Ruso wheat were collected from a local farm about 2 weeks after anthesis and stored at −18°C for up to 6 months.

Preparation of Extracts for Carboxypeptidase Assays. Ten grains or grain parts were used for each extract. The sprouts of germinating grains were removed with a scalpel, and the grains (endosperm + scutellum) or separated endosperms and scutella were homogenized in 3 ml of cold 0.2 M sodium acetate buffer (pH 4.4) in a Potter-Elvehjem homogenizer with ground glass surfaces (cooled in an ice-water bath) until the suspension appeared homogeneous (1–6 min depending on material). The endosperms of grains germinated for up to 2 d were first cut to pieces with scissors to facilitate homogenization. Dry resting grains were first ground to a fine powder in a mortar mill, an amount of powder corresponding to 10 grains (about 350 mg) was suspended in 3 ml of the extraction buffer, and the suspension was homogenized as described above. The homogenates were centrifuged (29,000*g* for 15 min at 5°C), and 2-ml samples of the clear extracts were run through small columns of Sephadex G-25 (PD-10 prepacked disposable columns, Pharmacia) equilibrated with 50 mM sodium acetate buffer (pH 4.4); the large molecular fractions were collected and used for the enzyme assays.

Preparation of Extracts for Column Chromatography. Resting dry grains were ground to a fine powder in a mortar mill, and a sample corresponding to 300 grains (about 10.5 g) was suspended

[1] Abbreviations: Z, *N*-benzyloxycarbonyl; TNBS, 2,4,6-trinitrobenzene sulfonic acid; M_r, molecular mass.

in 90 ml of cold 0.2 M sodium acetate buffer (pH 4.4). The suspension was homogenized in batches of about 15 ml in a Potter-Elvehjem glass homogenizer (cooled in ice water) for 5 to 10 min. The homogenates were combined and the extract was separated by centrifugation (29,000*g* for 15 min at 5°C).

For the extraction of germinating grains, the sprouts were removed from 300 grains, and the grains were crushed in 15 ml of cold 0.2 M sodium acetate buffer (pH 4.4) using a chilled mortar and pestle. To this mixture were added 75 ml of the same buffer and the further homogenization and separation of the extract were done as described above.

Extracts from flag leaves were made from 24 g of frozen leaves. Batches of 2 g of leaves were cut into small pieces and added to a mortar kept on crushed ice, 2 g of acid-washed sand, 0.2 g of insoluble PVP (Sigma, P-6755), and 10 ml of 0.2 M sodium acetate buffer (pH 4.4) containing 10 mM sodium metabisulfite (5) were added, and the mixture was ground with a pestle until it appeared homogeneous (about 10 min). Twelve homogenates were combined and the extract was separated by centrifugation (29,000*g* for 15 min at 5°C).

For chromatography on CM-cellulose the extracts were extensively dialyzed against 25 mM sodium acetate buffer (pH 4.8) at 5°C. For chromatography on Sephadex G-200 the extracts were concentrated to about 8 ml in an Amicon ultrafiltration cell using a PM-10 membrane; the concentrated extract was dialyzed against column buffer.

Column Chromatography. All chromatographic runs were done in the cold using water-jacketed columns with circulating water thermostated to 5°C. CM-cellulose (Whatman CM32), DEAE-cellulose (Whatman DE32), and Sephadex G-100 and G-200 (Pharmacia) were handled according to the manufacturers' instructions. Estimations of mol wt, based on elution positions on Sephadex G-200, were made as in (11) except that the experiments were made at 5°C instead of 20°C.

Carboxypeptidase Assays. Z-Dipeptides used as substrates were obtained from Bachem Feinchemikalien A.G., Bubendorf, Switzerland; all the amino acid residues were of L-configuration. The assays were made essentially as described previously (10). The substrate concentration was 1.82 mM, the reaction temperature was 30°C, and the liberated C-terminal amino acids were determined with the TNBS-reagent. The pH optimum for the hydrolysis of each substrate was determined using the unfractionated extract of germinating grains. The final assays were done in 50 mM sodium acetate buffers containing 0.5 mM EDTA; the reaction pH was 5.2 for Z-Phe-Ala and Z-Ala-Phe, 5.7 for Z-Phe-Phe and Z-Ala-Arg, 4.8 for Z-Phe-Glu, and 4.4 for Z-Pro-Ala, Z-Pro-Met, and Z-Gly-Pro-Ala. The reaction time was usually 100 min for the five first-mentioned substrates and 18 h for the substrates with a penultimate proline residue. The standard curves for the TNBS-reaction of each C-terminal amino acid in the appropriate buffer were determined separately (the differences were within ± 10% with respect to L-alanine) and the activities are given as nanomoles of substrate hydrolyzed/min at 30°C.

Hydrolysis of Hemoglobin. A 2% (w/v) solution of acid-denatured bovine hemoglobin in sodium acetate-HCl buffer of pH 4.0 (ionic strength 0.1) was prepared as described by Preston (15) except that no 2-mercaptoethanol was added. Substrate solution (400 μl) was incubated with enzyme sample (50 μl) for 60 to 120 min at 30°C. The enzyme reaction was terminated by adding 150 μl of 15% (w/v) TCA-solution, and the tubes were kept in an ice-water bath for 15 min before the precipitates were removed by centrifugation. The amounts of TCA-soluble free amino groups in the supernatants were estimated with TNBS using a further modification of the method described by Shutov *et al.* (18). TNBS-reagent was prepared by mixing 1 volume of freshly prepared 0.3% (w/v) TNBS in water with 4 volumes of 4% (w/v) borax solution in 0.15 N NaOH. One-half ml of the reagent was mixed with 150 μl of sample, the reaction was allowed to proceed for 60 min at 30°C, and it was terminated by adding 2.5 ml of 0.5 N HCl. The *A* at 340 nm were read against a reagent blank. The standard curve was made with L-alanine and the activities are expressed as nanomoles of TCA-soluble amino groups liberated/min at 30°C.

RESULTS AND DISCUSSION

Effects of pH on the Hydrolysis of Z-Dipeptides. The pH dependence of the hydrolysis of a number of Z-dipeptides was determined using unfractionated extracts of germinating grains as enzyme source. The pH optima were between pH 4.4 and 5.8; the results for six Z-dipeptides later found to be suitable substrates for different wheat carboxypeptidases are shown in Figure 1. The pH optima are similar to those previously obtained for unfractionated extracts of germinating barley (11).

Changes of Carboxypeptidase Activity during Germination. The carboxypeptidase activity of endosperms was already high in resting grains (Fig. 2). During germination the activity against

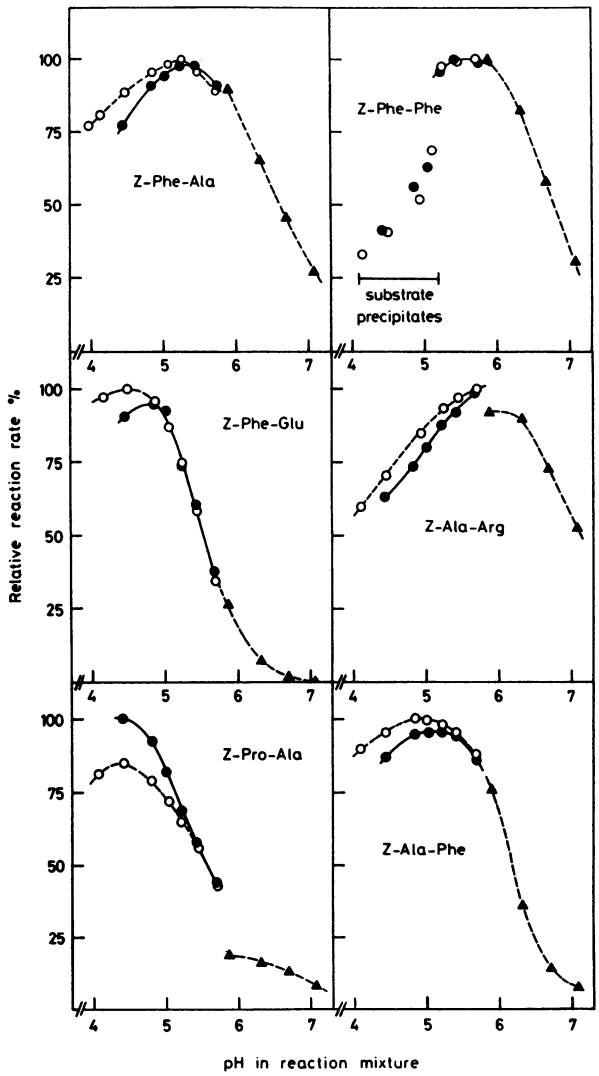

FIG. 1. Effects of pH on the hydrolysis of some Z-dipeptides by extracts of wheat grains germinated for 3 d at 20°C. The reaction rates were measured in 50 mM sodium acetate buffers (O---O), in sodium acetate buffers having a constant ionic strength of 0.05 and containing 0.5 mM EDTA (●——●), and in 50 mM sodium phosphate buffers (▲---▲).

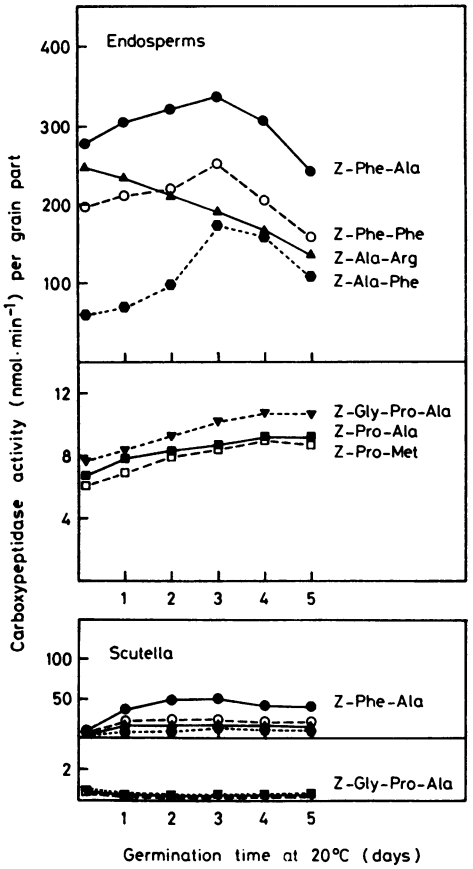

FIG. 2. Changes in carboxypeptidase activities in the endosperms and scutella of wheat grains (cv Ruso) during germination at 20°C. Extracts were prepared for the assays as described in "Materials and Methods." The average weight of an air-dry endosperm and scutellum separated from resting grains was 35 mg and 1.2 mg, respectively. Each point represents the mean of four extracts from two separate germination experiments.

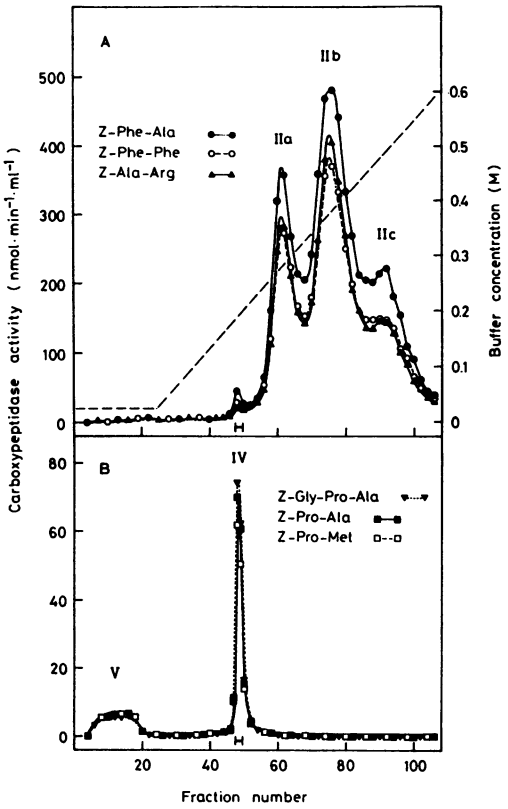

FIG. 3. Fractionation of the carboxypeptidase activity present in resting wheat grains (cv Ruso) by chromatography on CM-cellulose. An extract (80 ml) corresponding to 300 grains (10.6 g) was prepared as described in "Materials and Methods" and applied to a column (1.6 × 33.5 cm) of Whatman CM32 equilibrated with 25 mM sodium acetate buffer (pH 4.8) at 5°C. The column was washed with 40 ml of the starting buffer and eluted with a linear gradient of increasing buffer concentration (25–600 mM sodium acetate buffer, pH 4.8, total volume 500 ml). The flow rate was 30 ml/h, and 6-ml fractions were collected and assayed for their activities in the hydrolysis of the substrates shown in the figure. Roman numerals refer to the nomenclature suggested for barley carboxypeptidases (11). The bar under peak IV indicates the pool which was further analyzed by gel chromatography (Fig. 4).

Z-Ala-Arg (marker substrate for barley carboxypeptidase II) decreased while that against Z-Phe-Ala increased, reaching a maximum on the 3rd d of germination. The activities against the three prolylcarboxypeptidase substrates were only about 5% of those against the other substrates and they increased up to the 4th d of germination. These results suggest the presence of several enzymes with different specificities. All the substrates were also hydrolyzed by extracts of scutella. The relative activity against Z-Phe-Ala was higher than in endosperms, and there were no changes in the small prolylcarboxypeptidase activities.

Preliminary Fractionation Experiments. The five acid carboxypeptidases present in germinating barley grains were separated by DEAE-cellulose chromatography at pH 6.5 (11). This method, however, proved unsuitable for the fractionation of wheat carboxypeptidases. Chromatography on CM-cellulose, as used by Umetsu *et al.* (19), gave several partly overlapping peaks, and with a few minor modifications satisfactory separations were obtained with both resting and germinating grains.

Carboxypeptidases Present in Resting Grains. When extracts of resting wheat grains were fractionated by CM-cellulose chromatography, the activities on Z-Phe-Ala, Z-Ala-Arg, and Z-Phe-Phe were separated to three major and one minor peak (Fig. 3A). The elution curve was similar to the corresponding curve obtained by Umetsu *et al.* (19) in their fractionation of the crude crystalline carboxypeptidase from wheat bran. As the substrate specificities of the three major peaks were similar to that of barley carboxypeptidase II, the peaks were designated IIa, IIb, and IIc.

A part of the low prolylcarboxypeptidase activity eluted in the break-through fraction (Fig. 3B), while the other part eluted as a sharp peak coinciding with the minor peak acting on Z-Phe-Ala, Z-Ala-Arg, and Z-Phe-Phe. To determine whether the different activities were due to a single enzyme, the latter peak was chromatographed on Sephadex G-200 (Fig. 4). The activities against the three prolylcarboxypeptidase substrates eluted as a single symmetrical peak at a position corresponding to M_r 165,000; the other activities eluted later, showing that they were due to some different enzyme(s).

In contrast to barley carboxypeptidases IV and V (11), both prolycarboxypeptidase fractions of wheat exhibited similar activities against the three substrates used. As they also seemed to have similar mol wt (see below), they may represent a pair of isoenzymes. However, because their elution positions corresponded to the two distinctly different prolylcarboxypeptidases of barley (data not shown), they have been tentatively designated wheat carboxypeptidases IV and V.

Carboxypeptidases Present in Germinating Grains. CM-Cellulose chromatography of extracts of grains germinated for 3 d at 20°C (*cf.* Fig. 2) gave a sharp peak with high activity against Z-Phe-Ala, moderate activity against Z-Phe-Phe, and minimal activity against Z-Ala-Arg (Fig. 5A). This peak resembled barley

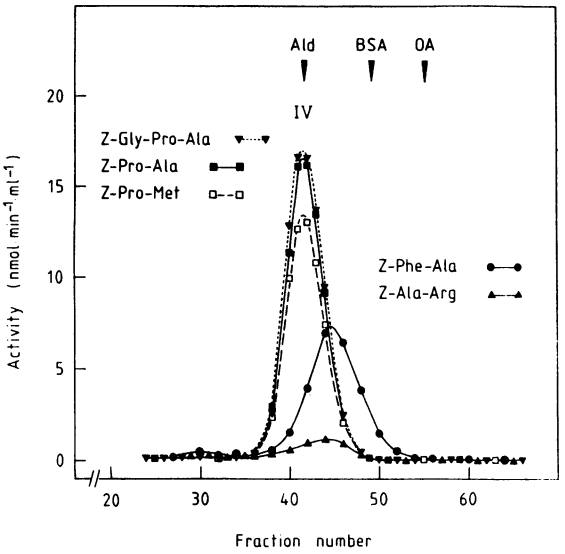

FIG. 4. Gel chromatography of partially purified carboxypeptidase IV. The fractions exhibiting highest prolylcarboxypeptidase activities in the CM-cellulose run presented in Figure 3 were combined, concentrated to 8 ml, dialyzed against 75 mM sodium phosphate buffer (pH 6.5) containing 0.1 mM DTT, and applied to a column (2.6 × 92 cm) of Sephadex G-200 equilibrated with the same buffer and thermostated at 5°C. Elution was done with the pH 6.5 buffer at a rate of 24 ml/h and 6-ml fractions were collected. Arrows indicate the elution positions of aldolase (Ald, M_r 158,000), BSA (M_r 67,000), and ovalbumin (OA, M_r 43,000) when these were run separately in the same column.

carboxypeptidase I both in substrate specificity and in elution position. Chromatography of pool 2 on Sephadex G-200 yielded a single peak of activity against Z-Phe-Ala and Z-Phe-Phe at a position corresponding to M_r 120,000.

Next, there was a peak corresponding to carboxypeptidase IIa of resting grains in both elution position and substrate specificity. When pool 3 was chromatographed on Sephadex G-200, a single activity peak corresponding to M_r 120,000 was obtained (Fig. 6A).

The third peak of activity in the CM-cellulose run (Fig. 5A) seemed to contain two enzymes, as the leading edge had a substrate specificity resembling carboxypeptidase II but the trailing edge showed highest activity against Z-Phe-Phe. The two enzymes were separated by chromatography on Sephadex G-200 (Fig. 6B); there was a faster eluting peak with high activity against Z-Ala-Arg and M_r of about 115,000 and a slower peak with high activity against Z-Phe-Phe, no activity against Z-Ala-Arg, and M_r of about 37,000. The former peak resembled carboxypeptidase IIb from resting grains (Fig. 3A) and the latter peak resembled barley carboxypeptidase III in substrate specificity,in elution position in the CM-cellulose experiment (not shown), and in molecular size (11).

The prolylcarboxypeptidase activities present in germinating grains eluted at the same positions (Fig. 5B) as those of resting grains (Fig. 3B). When pool 1 corresponding to carboxypeptidase V was concentrated and chromatographed on Sephadex G-200, activity eluted as a single symmetrical peak at a position corresponding to M_r 150,000.

In their studies on wheat carboxypeptidases Preston and Kruger (9, 16) assayed the activity using hemoglobin as substrate. The hemoglobin-hydrolyzing activity of the fractions of the present experiment is shown in Figure 5C. The two main peaks possibly correspond to the two carboxypeptidase fractions described by Preston and Kruger (16, 17). According to the results presented in Figures 5A and 6, the former peak corresponds to carboxypeptidase IIa, whereas the latter peak corresponds to a

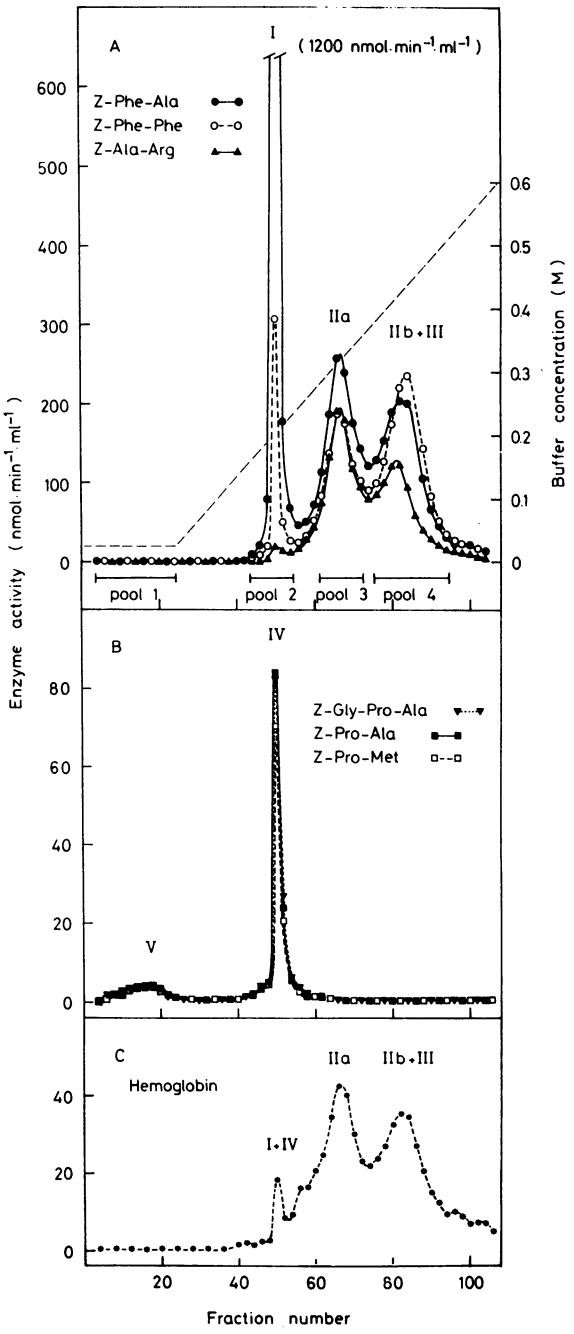

FIG. 5. Fractionation of the carboxypeptidase activity present in germinating wheat grains (cv Ruso) by chromatography on CM-cellulose. An extract (93 ml) corresponding to 300 grains germinated for 3 d at 20°C was prepared as described in "Materials and Methods" and applied to a column (1.6 × 34 cm) of Whatman CM32 equilibrated with 25 mM sodium acetate buffer (pH 4.8) and thermostated at 5°C. The column was eluted first with 27 ml of the starting buffer and then with a linear gradient of increasing buffer concentration (25–600 mM sodium acetate buffer, pH 4.8, total volume 500 ml) at a flow rate of 30 ml/h. Fractions (6 ml each) were assayed for their carboxypeptidase activities against Z-peptides (A, B) and for their ability to liberate TCA-soluble amino groups from hemoglobin at pH 4 (C). Pools 1 to 4 were used for estimation of mol wt (see text and Fig. 6).

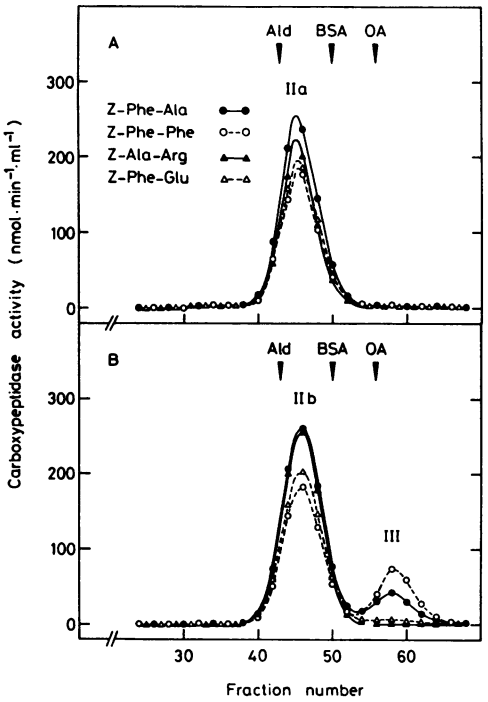

FIG. 6. Gel chromatography of wheat carboxypeptidase fractions on Sephadex G-200. Pools 3 (top) and 4 (bottom) from the CM-cellulose experiment shown in Figure 5 were concentrated to 9 ml each and dialyzed against 75 mM sodium phosphate buffer (pH 6.5) containing 0.1 mM DTT. Samples of 8 ml were run on a column (2.6 × 92 cm) of Sephadex G-200. Experimental conditions and M_r reference proteins were as described under Figure 4.

mixture of carboxypeptidases IIb and III. In addition there is a small sharp peak at the elution position of carboxypeptidases I and IV.

Corresponding CM-cellulose chromatography was done with Canadian Neepawa wheat germinated for 3 d. The results were very similar to those presented in Figure 5, A and B, except that peak III was more prominent. Comparative experiments were also made with germinating Himalaya barley. Barley carboxypeptidases I to V were readily identified on the basis of their substrate specificities; the elution position of each enzyme was close to that of the corresponding wheat carboxypeptidase.

Fractionation of Carboxypeptidases Present in Mature Flag Leaves. In the study of Waters *et al.* (23), carboxypeptidase activity in the flag leaves of an Australian cultivar of wheat was maximal about 2 weeks after anthesis. Flag leaves of the Finnish Ruso wheat were collected at this stage and their carboxypeptidases were fractionated by chromatography on CM-cellulose. Some activity resembling carboxypeptidase I eluted in the breakthrough fraction (Fig. 7A); this peak may be due to binding of carboxypeptidase I to the abundant soluble proteins present in the leaves. Then there followed a very high peak, the elution position and specificity of which corresponded to carboxypeptidase I. This peak was preceded by a smaller peak with the same specificity; whether this peak represents an isoenzyme or is due to complex formation remains to be clarified. After peak I there was a peak with all the properties of grain carboxypeptidase IIa. The leaves contained the same two prolylcarboxypeptidases (Fig. 7B) as grains but peak IV was much higher.

In the hydrolysis of hemoglobin (Fig. 7C) the breakthrough fraction showed relatively high activity. This is probably due to cooperation between proteinases and carboxypeptidases, as Frith *et al.* (7, 8) have shown that wheat leaves contain high acid proteinase activity, of which the major fraction has a pI below

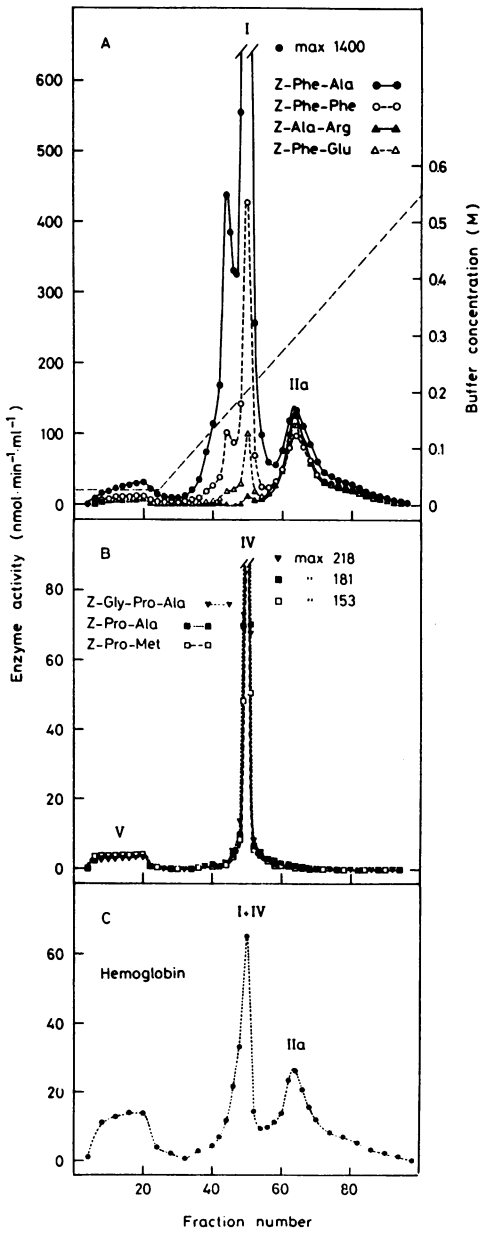

FIG. 7. Fractionation of the carboxypeptidase activity present in flag leaves of wheat (cv Ruso) by chromatography on CM-cellulose. Leaves were harvested about 2 weeks after anthesis, stored at −18°C, and extracted as described in "Materials and Methods." A dialyzed extract (100 ml) corresponding to 23 g of leaves was applied to a column (1.6 × 34 cm) of Whatman CM32 equilibrated with 25 mM sodium acetate buffer, pH 4.8; the column was washed with 20 ml of the starting buffer and eluted with a buffer gradient as described under Figure 5. Activities against the substrates shown in the figure were determined from 6-ml fractions of the eluate.

pH 4.8. The two other activity peaks obtained with hemoglobin substrate coincided with carboxypeptidases I + IV and carboxypeptidase IIa.

Hydrolysis of Various Z-Dipeptides by Carboxypeptidases I, II, and III. Preliminary substrate specificity tests showed that Z-Ala-Phe was rapidly hydrolyzed by carboxypeptidase III separated as shown in Figure 6B. As a further test an unfractionated extract of germinating grains was directly chromatographed on Sephadex G-100 (Fig. 8). Carboxypeptidase III was eluted after the main peak, apparently containing carboxypeptidases I, IIa, and IIB, at a position corresponding to M_r 40,000. The activity

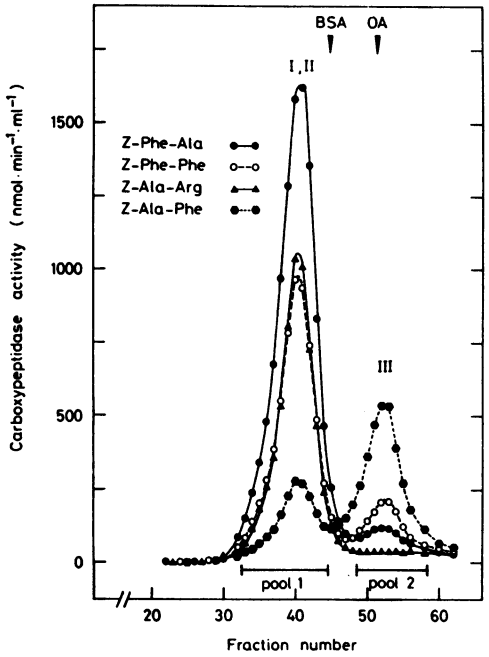

FIG. 8. Separation of wheat carboxypeptidase III from carboxypeptidases I and II by chromatography on Sephadex G-100. An extract (90 ml) made from 300 grains germinated for 3 d at 20°C was concentrated to 8.3 ml and dialyzed against 0.1 M sodium acetate buffer (pH 4.8). An 8-ml sample was run on a column (2.6 × 96 cm) of Sephadex G-100 equilibrated and eluted with the same pH 4.8 buffer. The column was operated at 5°C at a flow rate of 24 ml/h and 6-ml fractions were collected. Arrows indicate the elution positions of BSA (M_r 67,000) and ovalbumin (OA, M_r 43,000) run separately in the same column.

curves show that Z-Ala-Phe is a relatively good marker substrate for carboxypeptidase III.

For more extensive specificity experiments pool 2 from Figure 8 was used as "pure" carboxypeptidase III, and carboxypeptidases I, IIa, and IIb were obtained by CM-cellulose chromatography from the corresponding pool 1. The activities of the separated carboxypeptidase fractions as well as those of unfractionated extracts of resting and germinating grains were tested using 17 Z-dipeptides (Table I).

Z-Phe-Ala was hydrolyzed by all the three enzymes, but it still seems the best marker substrate for carboxypeptidase I, especially in germinating seeds (Fig. 5A) and in leaves (Fig. 7A), where this enzyme is abundant.

Carboxypeptidase IIa rapidly hydrolyzed Z-Ala-Arg, Z-Phe-Glu, and Z-Tyr-Glu, all of which are poor substrates for carboxypeptidases I and III. Z-Ala-Arg appears to be the best marker substrate for this enzyme. The specificity of carboxypeptidase IIb was tested with 13 of the substrates shown in Table I at pH 5.5. The results were, within experimental error, the same as those for carboxypeptidase IIa. Moreover, the results in Table I show that the unfractionated extract of resting grains possessed an activity spectrum closely resembling that of carboxypeptidase II. This confirms the earlier conclusion that the three isoenzymes of carboxypeptidase II (Fig. 3A) are responsible for all the carboxypeptidase activity present in resting grains except for the low prolylcarboxypeptidase activity due to carboxypeptidases IV and V.

The substrate most rapidly hydrolyzed by carboxypeptidase III was Z-Ala-Phe, and high reaction rates were also observed for all Z-dipeptides in which both amino acid residues had strongly hydrophobic side chains.

Corresponding specificity tests were also performed with carboxypeptidases I, IIa, IIb, and III separated from germinating grains of Neepawa wheat and with carboxypeptidase I separated from the flag leaves of Ruso wheat. The results were practically the same as those shown in Table I.

Earlier studies on the specificity of barley carboxypeptidase I (1, 22), barley carboxypeptidase II (2), and wheat carboxypeptidase II (19) were made using different sets of substrates and/or different assay pH. However, comparison of these data with those in Table I suggests that the specificities of barley carboxypeptidases I and II are close to those of the corresponding wheat enzymes.

Z-Phe-Ala is the Z-dipeptide most rapidly hydrolyzed by extracts of most plant tissues (for references, see Mikola and Mikola [14]). However, the results shown in Table I and the fractionation diagrams indicate that Z-Phe-Ala is not a reliable substrate for measuring the overall carboxypeptidase activity because its rate of hydrolysis may largely depend on the abundance of carboxypeptidase I. Instead, Z-Phe-Leu seems a good choice for a single substrate as it is relatively rapidly hydrolyzed by all the three enzymes. However, carrying out the assays with the four substrates, Z-Phe-Ala, Z-Ala-Arg, Z-Ala-Phe, and Z-Pro-Ala gives more detailed information of activity changes taking place for example during germination.

CONCLUDING REMARKS

The results of this study show that germinating wheat grains contain five acid carboxypeptidases. Three of the enzymes hydrolyze various Z-dipeptides provided that there is not a proline residue in the penultimate position, whereas the remaining two appear to be prolylcarboxypeptidases acting only on substrates of the type Z-Pro-X. Each of these enzymes resembles one of the five acid carboxypeptidases present in germinating barley grains. Therefore, they have been designated wheat carboxypeptidases I to V in accordance with the nomenclature earlier suggested for the barley enzymes (11). Suitable substrates and approximate mol wt of the different wheat carboxypeptidases are: I, Z-Phe-Ala, 120,000; II, Z-Ala-Arg, 120,000; III, Z-Ala-Phe, 40,000; IV, Z-Pro-Ala, 165,000; and V, Z-Pro-Ala, 150,000. The only major difference between the two species concerns carboxypeptidase V. In barley this enzyme had an M_r of 95,000 and it hydrolyzed Z-Gly-Pro-Ala about twice as rapidly as Z-Pro-Ala (11); in wheat the corresponding enzyme had an M_r of about 150,000 and it hydrolyzed the two substrates at about the same rate.

The two major carboxypeptidases differed in their physiological behavior. Carboxypeptidase II was abundant in resting grains and decreased during germination. Carboxypeptidase I, in contrast, was absent from resting grains but attained high activity during germination. Secondly, carboxypeptidase I was much more abundant in leaves.

Corresponding differences are apparent in barley. In germinating grains the activity against Z-Phe-Ala increased more than that against Z-Phe-Phe (10), suggesting that the main change during germination is an increase of carboxypeptidase I. This enzyme was also the dominant carboxypeptidase in young barley seedlings (L Mikola, unpublished observations). Two acid carboxypeptidases acting on Z-Phe-Ala were detected also in rice (3, 4). One of the enzymes (M_r 110,000) was present in resting grains and decreased during germination. The other enzyme (M_r 120,000) was absent from resting grains, appeared during germination, and was present in young shoots and mature leaves. The accumulated evidence suggests that enzymes corresponding to carboxypeptidases I and II of barley and wheat possibly occur in all cereals and that the physiological roles of the two enzymes are at least to some extent different.

Acknowledgments—I am grateful to Ms. Pirjo Kauppinen for expert technical assistance, to Dr. Ken Preston for providing seeds of Neepawa wheat, and to Dr. Juhani Mikola for useful suggestions concerning the manuscript.

Table I. *Relative Rates of Hydrolysis of Some Z-Dipeptides at pH 5.5 by Unfractionated Extracts of Resting and Germinating Wheat Grains (cv Ruso) and by Different Carboxypeptidases Separated from Germinating Grains*

Extracts were prepared as described in "Materials and Methods." An extract from grains germinated for 3 d was first chromatographed on Sephadex G-100 (Fig. 8) to obtain a peak containing carboxypeptidase III. The main peak from the same run was chromatographed on CM-cellulose (*cf.* Fig. 5A) to separate carboxypeptidases I, IIa, and IIb. Peak fractions were used for activity determinations with 1.82 mM substrate in 50 mM sodium acetate buffer (pH 5.5) containing 0.5 mM EDTA.

Substrate	Hydrolytic Rate					
	Unfractionated extracts of		Separated carboxypeptidase fractions			
	Resting grains	Grains germinated for 3 d	I	IIa	IIb	III
	relative activity					
Z-Phe-Ala	100	100	100	100	100	100
Z-Phe-Gly	20	15	4	21	22	11
Z-Phe-Leu	169	141	63	172	174	305
Z-Phe-Met	178	140	59	145	162	109
Z-Phe-Phe	73	61	24	74	70	234
Z-Phe-Glu	36	26	2	46	—[a]	15
Z-Tyr-Glu	20	15	2	22	—	8
Z-Leu-Gly	2	1	<1	3	3	5
Z-Leu-Leu	33	32	21	33	35	135
Z-Leu-Phe	27	24	8	28	29	110
Z-Gly-Leu	<1	<1	<1	1	—	12
Z-Gly-Phe	<1	<1	<1	1	1	4
Z-Ala-Ala	3	10	16	4	5	63
Z-Ala-Phe	16	32	8	15	17	493
Z-Ala-Arg	80	58	1	72	73	19
Z-Glu-Phe	<1	1	3	3	—	44
Z-Glu-Tyr	<1	<1	1	4	4	10

[a] —, not determined.

LITERATURE CITED

1. BREDDAM K, S BECH SØRENSEN, M OTTESEN 1983 Isolation of a carboxypeptidase from malted barley by affinity chromatography. Carlsberg Res Commun 48: 217–230
2. BREDDAM K, S BECH SØRENSEN, M OTTESEN 1985 Isolation of carboxypeptidase II from malted barley by affinity chromatography. Carlsberg Res Commun 50: 199–209
3. DOI E, N KOMORI, T MATOBA, Y MORITA 1980 Some properties of carboxypeptidases in germinating rice seeds and rice leaves. Agric Biol Chem 44: 77–83
4. DOI E, N KOMORI, T MATOBA, Y MORITA 1980 Purification and some properties of a carboxypeptidase in rice bran. Agric Biol Chem 44: 85–92
5. DRIVDAHL RH, KV THIMANN 1977 Proteases of senescing oat leaves. I. Purification and general properties. Plant Physiol 59: 1059–1063
6. FELLER U, KH ERISMANN 1978 Veränderungen des Gaswechsels und der Aktivitäten proteolytischen Enzymen während der Seneszenz von Weizenblättern (*Triticum aestivum* L.). Z Pflanzenphysiol 90: 235–244
7. FRITH GJT, KHJ GORDON, MJ DALLING 1978 Proteolytic enzymes in green wheat leaves. I. Isolation on DEAE-cellulose of several proteinases with acid pH optima. Plant Cell Physiol 19: 491–500
8. FRITH GJT, LB SWINDEN, MJ DALLING 1978 Proteolytic enzymes in green wheat leaves. II. Purification by affinity chromatography, and some properties of proteinases with acid pH optima. Plant Cell Physiol 19: 1029–1041
9. KRUGER JE, K PRESTON 1977 The distribution of carboxypeptidases in anatomical tissues of developing and germinating wheat kernels. Cereal Chem 54: 167–174
10. MIKOLA J, L KOLEHMAINEN 1972 Localization and activity of various peptidases in germinating barley. Planta 104: 167–177
11. MIKOLA L 1983 Germinating barley grains contain five acid carboxypeptidases with complementary substrate specificities. Biochim Biophys Acta 747: 241–252
12. MIKOLA L, J MIKOLA 1980 Mobilization of proline in the starchy endosperm of germinating barley grain. Planta 149: 149–154
13. MIKOLA L, J MIKOLA 1984 Carboxypeptidases in grains and leaves of wheat. Plant Physiol 75: S-119
14. MIKOLA L, J MIKOLA 1986 Occurrence and properties of different types of peptidases in higher plants. *In* MJ Dalling, ed, Plant Proteolytic Enzymes, Vol I. CRC Press, Boca Raton, FL. In press
15. PRESTON KR 1975 An automated fluorometric assay for proteolytic activity in wheat. Cereal Chem 52: 451–458
16. PRESTON KR, JE KRUGER 1976 Purification and properties of two proteolytic enzymes with carboxypeptidase activity in germinated wheat. Plant Physiol 58: 516–520
17. PRESTON K, J KRUGER 1977 Specificity of two isolated wheat carboxypeptidases. Phytochemistry 16: 525–528
18. SHUTOV AD, DO NGOK LANH, IA VAINTRAUB 1982 Purification and partial characterization of protease B from germinating vetch seeds. Biochemistry (USSR) 47: 678–685
19. UMETSU H, M ABE, Y SUGAWARA, T NAKAI, S WATANABE, E ICHISHIMA 1981 Purification, crystallisation and characterisation of carboxypeptidase from wheat bran. Food Chem 7: 125–138
20. UMETSU H, E ICHISHIMA 1983 Action on peptides by wheat carboxypeptidase. Phytochemistry 22: 591–592
21. UMETSU H, K MORI, E ICHISHIMA 1984 Subunit structure and immunological properties of wheat carboxypeptidase. Phytochemistry 23: 2435–2438
22. VISURI K, J MIKOLA, T-M ENARI 1969 Isolation and partial characterization of a carboxypeptidase from barley. Eur J Biochem 7: 193–199
23. WATERS SP, MB PEOPLES, RJ SIMPSON, MJ DALLING 1980 Nitrogen redistribution during grain growth in wheat (*Triticum aestivum* L.). I. Peptide hydrolase activity and protein breakdown in the flag leaves, glumes and stem. Planta 148: 422–428