Acid Carboxypeptidases in Grains and Leaves of Wheat, 

Triticum aestivum L. 

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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 benzoylcarbonyl

ylide peptides (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-Tyr1 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 design-

1 Abbreviations: Z, N-benzoylcarbonyl; TNBS, 2,4,6-trinitrobenzene sulfonic acid; Mr, 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 unfraccionated 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 unfraccionated 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 unfraccionated 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.](https://www.plantphysiol.org/)

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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.

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 \times 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).

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 Mr 165,000; the other activities eluted later, showing that they were due to some different enzymes(s).

In contrast to barley carboxypeptidases IV and V (11), both prolylcarboxypeptidase 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...
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).

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.

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).
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 proteases 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. 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 x 92 cm) of Sephadex G-200. Experimental conditions and Mr reference proteins were as described under Figure 4.

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 unfraccionated 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, 40,000. The activity
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-dipeptide 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 Mr 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 Mr 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 I was abundant in resting grains and decreased during germination. Carboxypeptidase I, in contrast, was absent from resting grains but attained high activity during germination. Second, 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 (I. Mikola, unpublished observations). Two acid carboxypeptidases acting on Z-Phe-Ala were detected also in rice (3, 4). One of the enzymes (Mr, 110,000) was present in resting grains and decreased during germination. The other enzyme (Mr, 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.

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Table 1. 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.

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