THE DISTRIBUTION OF CARBOXYPEPTIDASES IN ANATOMICAL TISSUES OF DEVELOPING AND GERMINATING WHEAT KERNELS[1]

J. E. KRUGER and K. PRESTON, Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Manitoba R3C 3G9

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ABSTRACT

The anatomical distribution of carboxypeptidase activity during the growth and maturation of HRS wheat kernels indicated that the activity was mainly concentrated in the pericarp and endosperm tissues. In the pericarp, the activity rose and fell during development. By contrast, activity in the endosperm tissue progressively increased throughout growth. Elution profiles following ion-exchange chromatography on CMC of pericarp and of green layer and aleurone tissue were identical and showed the presence of only one active peak eluted at low ionic strength. In contrast, endosperm tissue contained two major active peaks eluted at higher ionic strength. Upon germination, carboxypeptidase components identical to those present in immature wheat were found. No distinct separation of the three active peaks into the different tissues was observable except in the embryo, which contained only the component eluted at low ionic strength. Chromatography profiles with CBZ-phe-ala or hemoglobin as substrate were very similar.

The wheat kernel is known to contain numerous proteolytic enzymes (1–11). These are located in different anatomical parts of the kernel and the levels of their activities depend upon the physiological state of the seed. For example, it has been shown that during the growth and maturation of wheat there are proteolytic enzymes in the outer layers of the kernel which break down azocasein and hemoglobin (12–15). The levels of these enzymes increase until approximately 24 days after anthesis and then rapidly decline (14,15). In the endosperm, on the other hand, there are hemoglobin-degrading proteolytic enzymes which increase throughout growth and maturation (15). This increase is not observed with azocasein as substrate, and indicates that proteolytic enzymes in different tissues have different specificities. Upon germination of steeped wheat, the levels of the enzyme breaking down hemoglobin increase approximately two- to threefold after 3 days' germination, whereas the azocasein-degrading enzyme increases four- to sixfold (14,16).

Recently, we observed that the major proteolytic enzymes which degrade hemoglobin may be carboxypeptidases. Thus, purified preparations of hemoglobinases from malted wheat extracts contained quite strong carboxypeptidase activity toward the substrate carbobenzoxy-phenylalanyl-L-alanine (CBZ-phe-ala), and liberated only amino acids from hemoglobin (unpublished data). The present study was undertaken, therefore, to obtain more information on the carboxypeptidase system present in the wheat kernel during development and germination, and to assess its relation to the major hemoglobin-degrading enzymes in wheat.

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MATERIALS AND METHODS

A hard red spring wheat variety (*Triticum aestivum* L., cv. Neepawa) was grown on Canada Department of Agriculture experimental plots at Glenlea, Manitoba. Following anthesis, samples were harvested at various stages of kernel development up to full maturity. Excised heads were stored in a freezer until use.

For germination of wheat kernels, 100 kernels of wheat were placed in a 13 × 13.5-cm covered sample dish containing two 13 × 13.5-cm sheets of germination paper which had been moistened with 16 ml water. The sample dishes were placed in a moisture cabinet in the dark at 23° C and removed after 72 hr.

Dissection Techniques

Dissections performed were similar to those described previously (17). The tissues separated were pericarp, green layer combined with aleurone, endosperm, and embryo (with scutellum attached).

Extraction of Carboxypeptidase

For ion-exchange chromatography, kernels were homogenized in a Virtis 45 homogenizer with cold 0.2*M* acetate buffer, pH 4.4 (3:1v/wt), and then stirred for 60 min. The extracts were centrifuged 15 min at 25,000 × *g*. Dissected tissues were extracted by grinding in a mortar and pestle with a small amount of sand, followed by centrifugation as above.

For enzymatic determination, the pH of the extractant buffer was raised to 5.7.

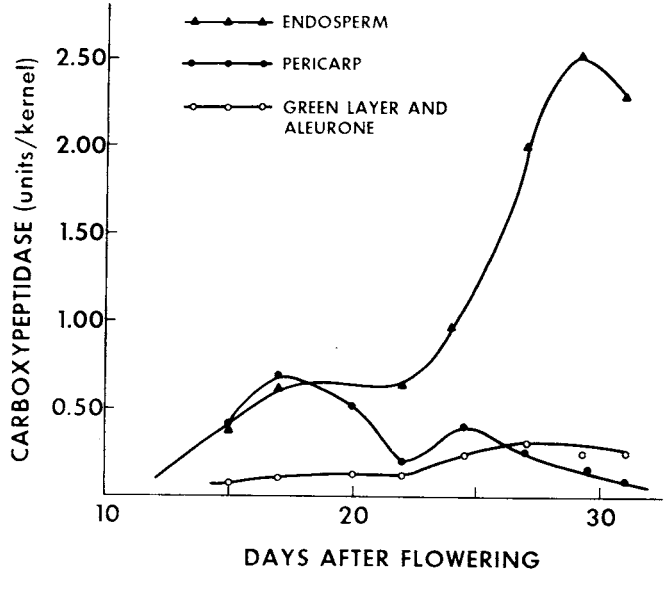

Fig. 1. Anatomical distribution of carboxypeptidase activity in wheat during kernel growth and maturation.

Ion-Exchange Chromatography

Extracts were first dialyzed against 0.025*M* acetate buffer, pH 4.4. The dialyzed extract was then chromatographed on a 1 × 30-cm column of Whatman CM32 carboxymethyl cellulose (CMC) pre-equilibrated with 0.025*M* acetate buffer, pH 4.4. The carboxypeptidase was then eluted from the column with a 400-ml linear gradient composed of 200 ml each of 0.025*M* and 0.40*M* acetate buffer, pH 4.4.

Proteolytic Activity Determinations

Carboxypeptidase. N-carbobenzoxy-L-phenylalanyl-L-alanine (Sigma Chemical Co.) was used as substrate at pH 5.7. The reaction was carried out by the method of Visuri *et al.* (18) at 35° C for 120 min. The alanine liberated was measured by the trinitrobenzenesulfonic acid method (19) at pH 9.6, and automated with a Technicon AutoAnalyzer (Technicon Corp., Chauncey, N.Y.). The system consisted of a Sampler II; proportioning pump II; 60° C constant-temperature bath with a standard 40-ft glass coil; colorimeter with a 420 nm filter; and a recorder, using absorbance paper. An aliquot of the enzyme mixture flowing at 0.23 cm/min met an air-segmented stream consisting of 0.017*M* 2,4,6-trinitrobenzenesulfonic acid (Pierce Chemical Co.), flowing at 0.60 cc/min combined with 0.5*M* borate buffer, pH 9.6, flowing at 1.60 cc/min. The stream

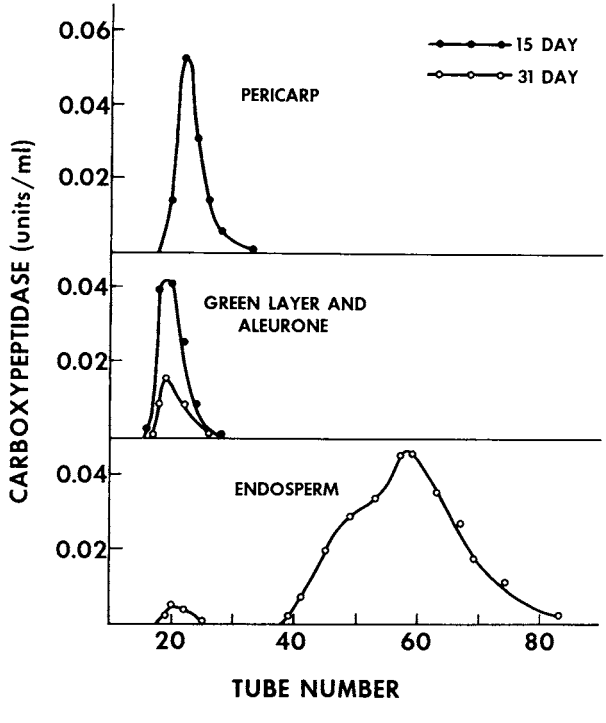

Fig. 2. CMC ion-exchange chromatography of carboxypeptidase in anatomical parts of immature wheat harvested at 15 and 31 days after flowering.

next passed through the 60° C temperature bath and the resulting increase in color measured at 420 nm. One unit of carboxypeptidase was defined as the liberation of one μmol of alanine/min at 35° C.

Hemoglobinase. This was determined by the automated fluorometric method of Preston (20). The method consists of incubating an extract with 2% acid denatured hemoglobin at pH 4.0 for 30 min at 40° C. The resulting peptides and amino acids are dialyzed and reacted with Fluorescamine (Pierce Chemical Co.) in acid to give an increase in fluorescence intensity (λex = 390 nm, λem = 480 nm). Activity is expressed in terms of the concentration (μmol/ml) of glycyl-glycine produced/min.

RESULTS AND DISCUSSION

Carboxypeptidase in Developing and Maturing Wheat

Kernels of wheat at various stages of growth and maturation were dissected into their anatomical parts and carboxypeptidase was determined on each part. The results are shown in Fig. 1. Carboxypeptidase activity was present in substantial amounts in all tissue except the embryo. The embryo did contain some activity but it was much lower relative to the other tissue and, consequently, was not shown in Fig. 1. Carboxypeptidase in the pericarp increased until approximately 15 days after flowering and then decreased. In the green layer and aleurone, the activity remained at a constant level and increased somewhat with final maturation. The most dramatic change in carboxypeptidase during kernel

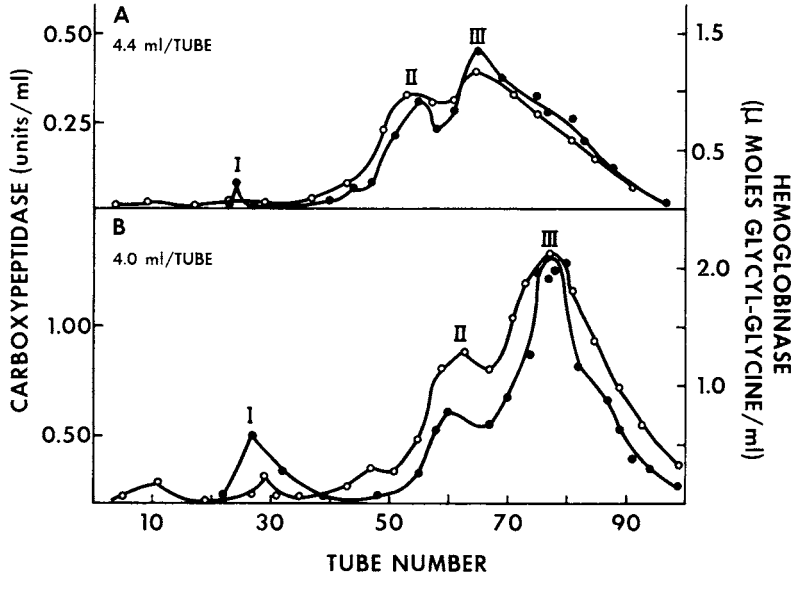

Fig. 3. CMC ion-exchange chromatography of carboxypeptidase and hemoglobinase in: A) sound, and B) Neepawa wheat germinated 72 hr. Closed circles = carboxypeptidase; open circles = hemoglobinase.

development was in the endosperm tissue. The activity was found to increase steadily, synchronous with increases in development of this tissue. This increase occurred at a time when the storage proteins of the wheat kernel were also being laid down. Evidently some mechanism must be present, such as enzyme compartmentalization or presence of inhibitors, which prevents the degradation of gluten proteins by carboxypeptidase. Such interactions, if they did occur under certain circumstances, might be expected to alter dramatically the quality of a wheat variety. As the activity in Fig. 1 is expressed in terms of activity per kernel, one must take into consideration that the dry weight of the endosperm increases during maturation. As a consequence, the activity per unit weight may be constant or even decreasing. At final kernel maturity, the endosperm contained the bulk of the carboxypeptidase activity.

CMC ion-exchange chromatography was carried out on dissected tissues of developing wheat harvested at 15 and 31 days after flowering. Elution profiles (Fig. 2) showed that the pericarp and the combined green layer and aleurone tissue had one main carboxypeptidase. The endosperm, on the other hand, had two main components with one component appearing as a shoulder on the second one. These carboxypeptidases were eluted at higher ionic strength than the carboxypeptidase present in the outer tissues. A small amount of the pericarp-like carboxypeptidase was also present in the endosperm tissue but it is possible that this was the result of a small amount of tissue contamination.

The results found in this paper are similar to those reported recently with hemoglobin as substrate (15). Hemoglobinase activity rose and fell in the outer

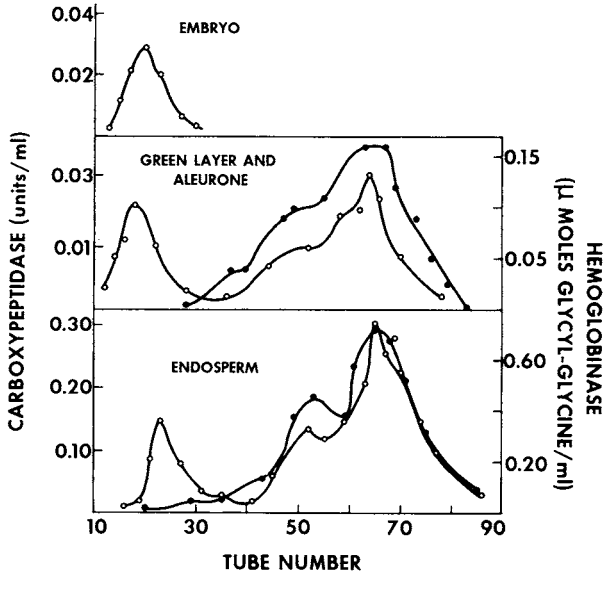

Fig. 4. CMC ion-exchange chromatography of carboxypeptidase and hemoglobinase present in anatomical parts of Neepawa wheat germinated 72 hr. Open circles = carboxypeptidase; closed circles = hemoglobinase.

pericarp layer but steadily increased in the endosperm. The CMC ion-exchange chromatography profiles were also very similar, except that an additional hemoglobinase, which eluted just following the peak initially eluted on ion-exchange chromatography, was present in the early developmental stages of the kernel.

Carboxypeptidase in Germinating Wheat

Upon germination of steeped wheat, carboxypeptidase activity increases approximately threefold (16). Such enzyme formation occurs either by new protein synthesis or by activation of proenzymes. The latter mechanism might occur by removal of carboxypeptidase inhibitors which appear to be present in wheat (15). In fact, evidence has been presented by Mikola and Kolehmainen (21) which indicates that this is probably the case with germinating barley. Therefore, elution profiles of extracts from germinated and ungerminated wheat were compared, following ion-exchange chromatography to determine if additional carboxypeptidase components, suggestive of new protein synthesis, were formed. The results found (Fig. 3) indicated that no additional components were present but that the existing three components increased in amounts. The elution profiles were also analyzed for hemoglobinase. It was found that the hemoglobinase profiles of peaks II and III mirrored the carboxypeptidase profile very closely in both the sound and 72-hr germinated samples. Peak I, on the other hand, was barely detectable by the hemoglobinase assay. Evidently, the specificity of this carboxypeptidase is different from the other two components, such that it is less able to degrade hemoglobin.

Examination by ion-exchange chromatography of the carboxypeptidase components present in the different anatomical parts of the kernel was also determined for 72-hr germinated wheat (Fig. 4). Activity in the pericarp was insufficient for determination of carboxypeptidase activity from ion-exchange columns. The embryo contained one carboxypeptidase component which corresponded to the component eluted at low ionic strength from the column (peak I) in whole wheat extracts. The green layer and aleurone as well as the endosperm contained all three components present in whole wheat extracts. This suggests that, if new protein synthesis is not responsible for increases in carboxypeptidase during germination, there must be substantial migration of the carboxypeptidase components between these tissues after 72 hr of germination.

From Fig. 4 it can be noted again that the first component eluted from the column, in the case of all tissues, had much less facility in breaking down hemoglobin compared to the second and third component. With the two components eluted at higher ionic strength, the carboxypeptidase and hemoglobinase profiles mirrored each other.

The similarity of profiles on ion-exchange chromatography with either CBZ-phe-ala or hemoglobin as substrate for immature, mature, and germinated wheat extracts suggests that the normally used Ayre-Anderson method with hemoglobin as substrate may be measuring very potent carboxypeptidase activity. As such, this method may not give a good indication of the gluten-softening enzyme in wheat. Such an enzyme would be expected to have an endo- rather than an exo- mode of attack. It is interesting to speculate, however, that the proteolytic enzyme system in wheat is similar to the amylase system where limited attack upon starch by α-amylase makes the remaining substrate more

susceptible to attack by β-amylase. Wheat carboxypeptidase may perform similarly to β-amylase in being responsible for a great deal of observable protein breakdown once some initial cleavages have been made by an endo- type of proteolytic enzyme. The present extraction condition does not extract all of the proteolytic activity from wheat (10,11,14) and it is possible that this endo-type of proteolytic enzyme remains insolubilized. Evidence for an insoluble proteolytic enzyme which breaks down gluten has been presented by Hanford (22). Moreover, a study by Grant and Wang (23), who used a more extensive extraction procedure than in the present study, has indicated that flour proteolytic enzymes acting on indigenous flour proteins and on hemoglobin can produce a significant level of free peptides in addition to high levels of free amino acids.

Acknowledgments

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