Multiple Forms of Acidic Endopeptidase from Germinated Barley

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ABSTRACT

An endopeptidase preparation from germinated barley Hordeum vulgare L., cv. Trophy, purified by affinity chromatography and density-gradient electrofocusing, consisted of three or four components. The preparation was only partly resolved by electrofocusing, with evidence of three possible components (pI 4.15, 4.28, and 4.37). Gel filtration on Sephadex G-75 yielded an asymmetrical peak, the major part of which corresponded to a molecular weight of 14,100, with evidence of one larger and two smaller components. The activity of the preparation was sulfhydryl-dependent; cysteine was the most effective of several sulfhydryl compounds tested. The preparation was sensitive to O2 in the absence of metal chelating agents and was inhibited by sulfhydryl reagents. It showed very narrow concentration tolerances for both cysteine and a substrate, N,N-dimethylhemoglobin. The Km value on N,N-dimethylhemoglobin at pH 3.8 was 0.064 to 0.067% (w/v) substrate; Vmax was 0.80 to 0.83 Amax per hour. Normal enzyme activity and molecular-size distribution were observed when the endopeptidases were extracted in the inhibited state and subsequently reactivated, thus ruling out the possibility that the enzymes might be autolytic artifacts that arose during extraction and purification.

Germinated barley and malt contain several endopeptidases (3, 11, 15), most of which are sulfhydryl-dependent (12). Earlier work of this laboratory showed that two of these enzymes from germinated barley, which are active on hemoglobin as substrate at pH 3.8, can be separated from one another by adsorption on and elution from CM cellulose at pH 5.5, followed by gel filtration on Sephadex G-100. The remaining enzymes, those that were not adsorbed on CM-cellulose at pH 5.5, were more acidic in character. They were quite similar to one another in their gel-filtration characteristics (3), therefore they could not be separated. Separation methods with higher resolving power were required to purify these enzymes for more detailed study.

An obstacle to the study of most of the cereal proteinases is their relative liability under conditions often required for enzyme purification (3, 12). Techniques that are comparatively rapid or that present conditions favorable for retention of enzyme activity are therefore essential. Two such procedures, affinity chromatography and density-gradient electrofocusing, have been used in the present study of the acidic endopeptidases. The results show that these enzymes comprise a group of similar sulfhydryl-dependent endopeptidases that are unusually small and possess rather low isoelectric points.

MATERIALS AND METHODS

Materials. Barley (Hordeum vulgare L., cv. Trophy) was germinated and lyophilized as previously described (2). All buffers and reagent solutions were prepared in glass-distilled water. Analytical-grade reagents or the purest grades available from commercial sources were used. Hemoglobin-Sepharose was prepared according to Chua and Bushuk (4), with modifications according to Cuatrecasas (7), from twice-crystallized human hemoglobin (Miles Laboratories) and Sepharose 4B (Pharmacia Fine Chemicals). Hordein was prepared from H. distichum L., cv. Piroline, according to Pollack et al. (22). N,N-dimethylhemoglobin was prepared according to Lin et al. (16).

Preparation of Endopeptidases. Lyophilized germinated barley was finely ground in a Labconco mill. Two hundred g were extracted at ice-bath temperature with 300 ml of 0.1 N acetic acid containing 1 mm cysteine. The pH of the slurry was adjusted to 4.1 to 4.2 by the slow addition of glacial acetic acid with constant stirring. After 30 min, the mixture was centrifuged for 1 hr at 14,000 g.

Affinity chromatography was performed according to the procedure of Chua and Bushuk (4), except that the attachment of the enzyme was accomplished at pH 5.2 rather than pH 5.5. This change in the conditions provided some extra protection for the endopeptidases under study and ensured exclusion of the more anionic endopeptidase activity known to be present in germinated barley (3). The supernatant from the extraction was divided into two equal portions. Each of these was dialyzed at 2 C against two 1300-ml portions of 50 mm sodium acetate buffer, pH 5.2, containing 1 mm cysteine, for 16 hr on a revolving wheel (1 rpm), which operated intermittently: 1 min on, 6 min off. The retentate (about 250 ml) was then centrifuged for 15 min at 14,000 g, decanted, and applied by upward flow to a 2.5- x 35-cm column of hemoglobin-Sepharose with a pump at 80 ml/hr. This was followed by buffer of the same composition as that used for dialysis, until the optical absorbance at 280 nm of the column eluate had decreased to a constant value. Absorbance was measured with a Vanguard
flow-monitoring apparatus equipped with a flow cell of 40 
\( \mu l \) capacity. The acidic endopeptidase fraction was then eluted with 0.1 M acetic acid, which was 1 mm with respect to cysteine, pH 3.0. Eight-ml fractions were collected. Three-tenths ml of 4 M sodium acetate buffer, pH 4.5, was placed in each tube receiving the acidic eluate, to increase the pH to 4.1 and to lessen the chance of denaturing the enzymes.

The active fraction prepared by affinity chromatography (70–75 ml) was dialyzed twice, against 1300 ml of 1% (w/v) glycerine containing 1 mm cysteine, for 16 hr in preparation for electofocusing. Electofocusing was performed for 60 to 65 hr in 1% (w/v) amlyophyte, pH 3 to 5, in a 440-ml LKB Ampholine column at 4 C. Voltage was applied to the column to maintain 6 W of power initially and 2 W during the later stages of separation. Final voltages ranged from 700 to 1000 V in different experiments. The column contents were removed at the end of the separation by displacement with water at 120 ml/hr. The contents were not monitored optically for protein (280 nm) with a flow cell, because this reduced the resolution of the sharp activity bands. Three-ml fractions were collected, assayed for proteolytic activity (see below), and the pH was determined using the expanded scale of a Radiometer-type PHM26 pH meter equipped with a combination electrode. The tip of the electrode was maintained at about 2 C, as were the fractions to be measured, the standard buffers, and the wash water for rinsing the electrode.

Narrow-range pH gradients were prepared by forming a pH gradient in the 110-ml LKB Ampholine column with a 3.3% (w/v) amlyophyte concentration (pH range 3–5) and without added sample. That portion of the resulting gradient from pH 4.0 to 4.6 was then used in a second run, with endopeptidase sample applied, to produce the desired gradient at 1% (w/v) ampholyte.

Several methods for removing the amylolyte and sucrose from the enzyme fractions were examined. A second passage through the affinity chromatography column was found to be either faster or less deleterious for the enzyme activity than dialysis, gel filtration on Sephadex G-25, or ion-exchange treatment (1), and was used as a routine step in preparing the endopeptidases.

**Proteolytic Assays.** Two methods were used to measure proteolytic activity. The method of Lin et al. (16) with N,N-dimethylhemoglobin as substrate was used at 35 C with 1 ml of about 0.075% (w/v) substrate in 0.1 M sodium acetate buffer, pH 3.8, incubated for 60 min with from 10 to 100 \( \mu l \) of enzyme. This procedure used TNBS to detect free amino groups. The results obtained, expressed as the change in absorbance at 340 nm per hr (\( \Delta A_{340} \)), were linear with respect to enzyme concentration for values as high as 0.75 with the purified preparation. Crude preparations from barley seedlings, which had been gel filtered on Sephadex G-25 to remove the high background of free amino groups, yielded enzyme-dilution curves with upward curvature at the higher enzyme concentrations. This was probably because exopeptidases were also present in the preparations.

The following method was employed with electofocused fractions, which contained high concentrations of free amino groups because of the presence of ampholyte: 3 ml of 0.7% (w/v) bovine hemoglobin in 0.1 M sodium acetate buffer, pH 3.8, was incubated for 1 hr at 35 C with 10 to 25 \( \mu l \) of enzyme. The reaction was stopped by adding 3 ml of 10% (w/v) trichloroacetic acid, and the mixture was held for 30 min at 35 C. After filtration through Whatman No. 40 filter paper, a 1-ml aliquot was neutralized with 0.1 ml 3 N NaOH and assayed by the procedure of Lowry et al. (17) for protein as a measure of the peptides released by the enzyme. Appropriate controls were included to determine background color, and the results are expressed as net absorbance at 800 nm (\( \Delta A_{800} \)). Results were not linear with enzyme content, but showed the decreased color yield with increased peptide concentration, typical of the Lowry procedure.

A semiquantitative assay procedure with gelatin as substrate was used with samples that were known to contain high concentrations of exopeptidases. A 7% (w/v) solution of U.S.P. gelatin in 0.1 M sodium acetate buffer, pH 4.1, containing 0.1 mm EDTA and 0.1 mm cysteine, was allowed to gel in a layer about 5 mm thick in a plastic box equipped with a cover. Aliquots (20 \( \mu l \)) of the fractions obtained from gel-filtering, freshly prepared seedling extractions on Sephadex G-75 were placed on the surface of the gel and incubated with the cover closed at 12 C for 20 hr. The fractions containing gelatinase activity, that is, true endopeptidases, were detectable by the depressions in the surface of the gel caused by liquefaction.

Carboxypeptidase activity was determined with Z-Glu-Tyr as substrate. This compound is hydrolyzed by two of the known carboxypeptidases of barley (19). One ml of solution containing 6 \( \mu l \) of Z-Glu-Tyr in 0.1 M sodium acetate, pH 5.2, was treated with 10 to 25 \( \mu l \) of enzyme for 1 hr at 35 C. The rest of the procedure was the same as that of Lin et al. (16) for endopeptidase activity using TNBS.

**Molecular Weight Determination by Gel Filtration.** Estimations of molecular weight were made with a 2.5- × 36-cm column of Sephadex G-75, which was pumped by upward flow at 19.8 ± 0.2 ml/hr, with 50 mm sodium acetate buffer, pH 4.5, which contained 0.1 NaCl and 1 mm cysteine. The standard proteins were aldolase, chymotrypsinogen A, and ribonuclease A. Molecular weights were estimated by use of K,, values.

**Enzymic Properties.** The effects of various reagents on the purified endopeptidase system were determined by incubating a suitable aliquot (10–100 \( \mu l \)) of the enzyme for 15 min with 100 \( \mu l \) of the reagent solution. This was usually done at pH 4.2 to 4.5 at 35 C, although where the effects of O, and N, atmospheres was studied, the sample was held at 2 C for longer lengths of time.

Tests for the hydrolysis of various proteins by the endopeptidase were conducted as follows: 1 ml of substrate solution containing 6.9 mg of protein in 0.1 M sodium acetate, pH 3.8, and 100 \( \mu l \) of 30 mm thiglycolic acid were treated with 100 \( \mu l \) of enzyme for 1 hr at 35 C. The reaction was stopped by placing the tube in a boiling water bath for 6 min. Ten-\( \mu l \) aliquots of the filtered reaction mixture were spotted on silica Gel G (E. Merck AG) TLC plates, chromatographed with BuOH-HOAc-H_2O (4:1:1) air-dried overnight, and sprayed with Moffat-Lytle reagent (20) to detect peptides and amino acids.

Protein was determined by the Lowry method (17) using crystalline bovine serum albumin as standard. Amino acid analyses were performed on a Beckman 121 automatic amino acid analyzer. Disc electrophoresis was performed according to Davis (8) at 3 ma/tube for 95 min. The gels were stained with Amido Black 10B for 60 min and destained electrophoretically at 10 ma/tube. Ultrafiltration experiments were performed with an Amicon microultrafiltration apparatus, using a PM10 membrane, under 2.4 atm of purified nitrogen, and with the apparatus described by Craig (5), equipped with Visking 5/2 or Arthur H. Thomas 6.3-mm dialysis tubings.

**RESULTS**

**Purification.** Because of the liability of some of the barley endopeptidases, the affinity-chromatographic procedure of Chua and Bushuk (4) offered advantages over methods used previously to purify these enzymes. The chromatographic
agent is hemoglobin attached covalently to agarose gel, presumably via free amino groups of the protein. The purification is based on the use of a substrate-support matrix combination at suboptimal pH for enzyme activity, pH 5.2 in our experiments, rather than the more commonly used enzyme inhibitor-support matrix combination (6). The lack of known competitive inhibitors for the barley endopeptidases precluded the use of the more desirable inhibitor-matrix combination.

Figure 1 is an affinity chromatographic pattern obtained with a 2.5- x 36-cm column of hemoglobin-Sepharose, in which the enzyme was eluted with 0.1 M acetic acid containing 1 mM cysteine. Not all of the proteolytic activity was retained by the column during the washing with pH 5.2 buffer. Some appeared in the initial peak, along with the extraneous proteins. This did not appear to be a result of overloading the column, because the activity eluted in this manner was proportional to the amount of enzyme extract applied to the column. The nature of the unadsorbed activity was not studied; however, it was found only in the more basic protein fraction when the barley seedling proteins were partitioned on CM-cellulose at pH 5.5 (3) before affinity chromatography.

An additional purification step was required to separate the mixture of enzymes that was eluted from the hemoglobin-Sepharose column, as well as to remove hemoglobin peptides that were eluted with the enzymes. Electrofocusing in liquid medium was used for this purpose, because most of the barley endopeptidases are maximally stable in the region of their isoelectric points, and the relatively large volumes required for preparative purposes precluded the use of methods involving acrylamide gel. Certain exopeptidases that were bound by the hemoglobin during affinity chromatography were also removed by electrofocusing. Among them was a carboxypeptidase that was active on Z-Glu-Tyr. After electrofocusing, the endopeptidase preparation possessed no carboxypeptidase activity on this substrate at pH 5.2. In addition, this method facilitates a precise measurement of pi values of the resolved proteins, and it is conducive to very sharp resolution of the individual components. Figure 2 is an electrofocusing pattern of the endopeptidase activity prepared by affinity chromatography. Three possible components, indicated by the light lines, approximate the total amounts of activity in each fraction. These peaks correspond to pi values of 4.15, 4.28, and 4.37. Potentials of 700 to 1000 v produced evidence of at least three components in the patterns, but experiments in which a potential of only 450 v was used revealed little evidence of such heterogeneity, other conditions being the same. Apparently, the tendency for diffusion or association of the individual endopeptidases is overcome by the higher electrical field. In this respect these enzymes appear to be particularly responsive to variations in the applied potential.

Improved resolution of the three components was attempted by the use of an ampholyte of narrow pH range (4.0-4.6). The pattern obtained under these conditions was poorly resolved; however, it contained three major peaks of activity at pH 4.19, 4.29, and 4.33, all of which are within experimental error of the values shown in Figure 2.

The preparations obtained after the second passage through the hemoglobin-Sepharose column consisted of 35 to 55 ml of solution with a $\Delta A_{280}$ value of 25 to 35 per ml and a protein content of 0.16 to 0.25 mg/ml. Because of the marked liability of most of the barley endopeptidases and the presence in the seedling extracts of relatively large amounts of the more basic enzymes, which were removed by affinity chromatography, the calculated purification amounted to only 10- to 20-fold (5–10% recovery).

**Disc Electrophoresis of Purified Endopeptidase.** The purity of the endopeptidase preparations was examined by disc electrophoresis at pH 9.5 in 7.5% polyacrylamide gel (8). The pattern obtained with 90 $\mu$g of protein consisted of a dense, somewhat broad band, believed to be the endopeptidases, preceded by four very faint, thin bands. Since the enzyme preparation represented a rather narrow cut (pH 4.1–4.4) from an electrofocusing run, any disc electrophoretic components differing appreciably in mobility from the endopeptidase most probably represent materials that were adsorbed on or in some way combined with the acidic enzymes during electrofocusing.

**Determination of Molecular Weight by Gel Filtration.** In several experiments in which gel filtration of the purified endopeptidase preparation was performed with Sephadex G-75 columns, the activity was eluted as an asymmetrical peak (Fig. 3). Replications of the experiment confirmed the asymmetry, and it was highly reproducible. From these elution profiles, it is apparent that, on the basis of molecular size or shape, there are more than three components present in the endopeptidase preparation.

The molecular weight of the major component of the endo-
peptidase mixture was obtained by calibrating the column with several globular proteins of known molecular weight. The elution volume of the tube containing maximal proteolytic activity corresponded to a molecular weight of 14,100. The shape of these peaks, as in Figure 3, indicate there are at least two components of larger elution volume and one of smaller elution volume.

Ultrafiltration of the Enzyme Complex. Ultrafiltration of the endopeptidase preparation was of interest, because this technique is of considerable value in the purification or concentration of enzymes (5) and would be particularly valuable for purifying small enzymes. Two brands of commercial dialysis tubing and the Amicon PM10 membrane were used. Recovery of activity in the ultrafiltrates obtained with each of these membranes was only 30 to 40%. Protein recoveries of 80 to 85% in the ultrafiltrates indicated rapid passage of the enzyme, but it was apparently inactivated in the process. No evidence of activity loss caused by passage of the endopeptidase through dialysis tubing was observed during dialysis. This is probably a reflection of the smaller average pore size in the unstretched membrane during dialysis.

It is apparent from the electrofocusing and gel filtration data (Figs. 2 and 3) that the individual enzymes comprising the acidic endopeptidase preparation are very similar in their net surface charge and molecular size or shape or both. The use of a narrow range ampholyte did not produce the desired resolution of the enzymes by electrofocusing. The only other method deemed to be of possible value in resolving the individual enzymes of the complex was gel filtration. In view of the disadvantages of sample dilution and additional time required for gel filtration on longer columns, as well as the demonstrated high resolving power of electrofocusing (23), the acidic endopeptidase mixture or complex was treated as a whole for subsequent study. Their response to inhibitors and activators (see below) indicates that these endopeptidases are also similar in respects other than surface charge and molecular size.

N,N-Dimethylhemoglobin as Substrate; Kinetic Properties. The use of N,N-dimethylhemoglobin as substrate for the barley endopeptidases, according to Lin et al. (16), was more sensitive and specific for the measurement of release of amino groups than methods used in previous work (3). However, unlike denatured hemoglobin used in the previous study, the methylated protein was inhibitory at concentrations as low as 0.1 to 0.125% (w/v) at pH 3.8. The optimal substrate concentration depended upon the particular preparation of substrate used and was usually 0.075% (w/v). For this reason the optimal concentration of each preparation of substrate was determined before its routine use.

A plot of S/ν versus S for the endopeptidase complex acting on N,N-dimethylhemoglobin and pH 3.8 yielded a Km value of 0.064% substrate, and the calculated Vmax was 0.80 A/hr. A Lineweaver-Burk plot of the same data produced very similar values, with a Km of 0.067% substrate and a Vmax of 0.83 A/hr.

Oxidation of Enzyme Sulfhydryl Groups. In the previous study of barley endopeptidase fractions prepared by partitioning on CM-cellulose, and gel filtration on Sephadex G-100 (3), there was little evidence for any marked enhancement of activity on dialysis of the more acidic fraction against various sulfhydryl compounds. This fraction appears to correspond to the endopeptidase complex of the present study. Because the previous experiments were carried out under N2, the oxidation of −SH groups by oxygen was probably limited to that caused by dissolved air in the solutions involved. For this reason the effects of O2 and N2 atmospheres, together with any interactions with sulfhydryl compounds, was of interest. The presence of 1 mM cysteine during dialysis in the presence of air had a strong protective effect on the enzyme. Some reversal of the inhibition caused by the absence of cysteine occurred; samples held under O2 retained only 40 and 30% of their original activity after 20 and 40 hr, respectively, and the addition of 0.1 μmole of cysteine after 40 hr caused a doubling in activity. Essentially no activity was lost in the samples held under N2.

The response of the endopeptidases to cysteine has a sharp maximum, which occurs at approximately 1 mM cysteine (Fig. 4). With increased concentration, there is a sharp decline in activity, and with 10 mM cysteine in the reaction mixture, there is less than 5% of the maximal activity.

Of five −SH compounds examined for their ability to activate the endopeptidase complex after it had been thoroughly dialyzed against acetate buffer, cysteine was the most effective (Table I). All of the compounds were tested at a concentration...
of 1 mm in the preincubation mixture. This corresponds to 0.17 mm in the proteolytic reaction mixture. A more detailed examination of each of the compounds tested probably would reveal some concentration differences that are not apparent from the data of Table I.

Because the oxidation of sulfhydryl groups does not occur directly by reaction with molecular oxygen, but is mediated by polyvalent metal ions (24), the inhibitory threshold for cysteine probably depends on the amount of contaminating metal ions that are present in a given preparation of enzyme, buffer, or substrate. For this reason the metal-chelating agent, EDTA, was examined for its effect on the endopeptidase in the presence of O₂. When the cysteine concentration was suboptimal, that is, 0.1 mm or less, EDTA produced an increase in activity over a wide concentration range. The maximal effect, which equaled that produced by about 1 mm cysteine, was maintained by concentrations from 1 mm to as low as 3 × 10⁻³ mm EDTA; below which level the activity dropped sharply. When EDTA was added to an enzyme that contained an inhibitory level of cysteine, for example, more than 2 to 3 mm, no increase in activity resulted. Thus either reagent is able to stimulate activity to the same extent, but there is a narrow tolerance for cysteine, whereas EDTA is optimal over a very broad range.

**Inhibition of Endopeptidase Activity by Sulfhydryl Reagents.** In view of the requirement of sulfhydryl groups in the endopeptidase reaction, it was anticipated that sulfhydryl reagents would be inhibitory. Both p-chloromercuribenzen sulfo- nate and bromate ion were completely inhibitory at 0.7 mm concentration in the preincubation mixture and at 0.09 mm in the reaction mixture. Iodoacetamide and oxidized glutathione produced only 50 and 80% inhibition, respectively, at the same concentrations.

**Activity with Various Proteins as Substrate.** Several purified proteins of known amino acid sequence were used to determine their susceptibility to attack by the barley endopeptidase complex. Examination of the proteolytic digests by thin layer chromatography showed that hemoglobin yielded four hydrolytic products, myoglobin yielded six, insulin seven, bovine serum albumin three, and protamine three. A preparation of hordein yielded five products under the same conditions. The low number of products produced from each of these substrates suggested that few, if any, contaminating exopeptidases were present in the enzyme preparation, as these are likely to produce more complex chromatographic patterns from the release of individual amino acids. As a further test of this point, ion exchange amino acid analyses of the hemoglobin-digest solutions were performed. There was 0.008 μmole of arginine/ml and no detectable histidine, tyrosine, or lysine. The C-terminal sequences of amino acids in the α- and β-chains of human hemoglobin are -- Lys-Tyr-Arg and -- Lys-Tyr-His, respectively (9), therefore, it appears that only traces of carboxypeptidases capable of acting on hemoglobin were present in the endopeptidase preparation. By the same token, aminopeptidase activity was evidenced by only 0.10 μmole of valine and 0.036 μmole of leucine/ml. These probably originated from the N-termini of the α- and β-chains of hemoglobin, which are Val-Leu-Ser -- and Val-His-Leu --, respectively (9). There was no detectable serine.

**Tests for Autolysis of Endopeptidase.** The number and relatively small size of the endopeptidases isolated in this study posed the question of whether or not they are degraded forms of a native enzyme. An endopeptidase could conceivably undergo autolysis during the extraction and purification operations, because the latter were performed in the presence of —SH compounds at a pH not far removed from optimal for hemoglobin hydrolysis. To check for autolysis, freshly prepared extracts were gel-filtered on Sephadex G-75 in the cold. The results obtained are shown in Figure 5. There were two main groups of proteolytic enzymes in the column eluate that were detected by the TNBS assay procedure. The first to be eluted was of relatively low activity, and the second was more heterogeneous and more active. Assays for gelatinase activity, which detect many if not all of the barley endopeptidases (11), are indicated by the bar in Figure 5. The enzymes capable of hydrolyzing gelatin were confined to the second or larger group of enzymes. The initial peak, therefore, appears to consist of exopeptidases, which do not register in the liquefaction of gelatin gels. Superimposed upon Figure 5 is the elution pattern obtained with the marker protein, purified RNAase A, under the same experimental conditions. The RNAase (mol wt = 13,700) has an elution volume that is very similar to that of the major endopeptidase peak obtained with the extract in question. Thus the low molecular weight endopeptidases are distinguishable within a matter of 6 or 7 hr after extraction and gel filtration in the cold.

In another type of experiment designed to check for autolysis, the plant extract was prepared in the same manner as above, except that 10 mm sodium tetrahydroxionate was included in the extraction solution, according to the technique of

![Figure 5. Gel filtration of freshly prepared barley seedling extract on Sephadex G-75 column (2.5 × 36 cm). Elution buffer: 0.1 M sodium acetate, 0.1 M NaCl, 0.1 M EDTA, 0.1 M cysteine, pH 4.7. Pump rate: 19.8 ml/hr; fractions: 4 ml. Heavy solid line indicates absorbance at 280 nm (——); proteolytic activity on N,N-dimethylhemoglobin at pH 3.8 (——). Shaded area is pattern of purified ribonuclease A (mol wt 13,700) produced under same experimental conditions. The horizontal bar indicates the region of effluent containing gelatinase.](image-url)
Fig. 6. Gel filtration of barley seedling extract prepared with 10 mM sodium tetrathionate. Gel filtration conditions were same as those for Figure 5, except that elution buffer contained 1 mM tetrathionate. Absorbance at 280 nm (•••); proteolytic activity on N,N-dimethylhemoglobin of fractions as eluted from column (---). Activity after aliquots of fractions were preincubated with an excess of 20 μmoles of cysteine over tetrathionate (----).

Williams and Whitaker (25), to inhibit sulfhydryl-dependent enzymes. The gel elution buffer contained 1 mM tetrathionate. Although the initial peak of proteolytic activity (Fig. 6) was not reduced in size by the tetrathionate treatment, the bulk of the endopeptidase activity in the second peak was about 75% inhibited. When the individual fractions were reasayed after preincubation of 20-μl aliquots of enzyme for 30 min in the presence of 40 μmoles of cysteine, that is, an excess of 20 μmoles cysteine over tetrathionate, the low molecular weight endopeptidase region was again detectable (Fig. 6). Furthermore, the activity levels and elution volumes are in close agreement with those observed in the absence of the inhibitor (Fig. 5).

DISCUSSION

The endopeptidase preparation described in this study obviously consists of several enzymes that are similar in size as well as in their surface charge makeup. At first consideration, they might be thought of as isozymes; however, since it is not known if all of them actually catalyze the hydrolysis of the same peptide bonds in the substrates used, they cannot be properly termed isozymes. Recent recommendations (13) also require that the term “isozyme” apply only to those multiple forms of enzymes arising from genetically determined differences in primary structure. The present endopeptidases are therefore referred to as “multiple forms.”

The acidic fraction of the endopeptidases was obtained in relatively pure form by the rapid and gentle treatment afforded by affinity chromatography on hemoglobin Sepharose. This procedure removes large quantities of extraneous proteins that are not bound to the peptide chains of hemoglobin at pH 5.2, including the more basic endopeptidases, but it does not remove all of the exopeptidases present in the barley seedling extract. Germinated barley contains three known carboxypeptidases (18), so more than one species of carboxypeptidase could conceivably become attached to the accessible carboxyl termini of the hemoglobin molecule. The presence of at least one carboxypeptidase was evidenced by the activity on Z-Glu-Tyr substrate in the acidic eluate from the column. This was removed by electrofocusing, and the final product was inactive with Z-Glu-Tyr as substrate. Only traces of exopeptidase activity were detected by amino acid analysis of the hemoglobin digest.

The principal assay method used is based on the detection of free amino groups by the formation of trinitrophenyl derivatives of peptides or amino acids after treatment of the enzyme digests with trinitrobenzene sulfonic acid. This detects the products of exopeptidase as well as endopeptidase action; however, by carrying out the assays at pH 3.8, exopeptidase action is minimized, since the carboxypeptidases of barley are either inactive or of greatly reduced activity at pH 3.8 (18).

The molecular weight of the major component of the acidic endopeptidases was 14,100. Although most of the endopeptidases from other sources that have been characterized are of relatively low molecular weight (10), the components of the present group appear to be some of the smallest enzymes thus far reported; only some of the ribonucleases appear to be smaller. Based on the data obtained with either freshly prepared or sulfhydryl-inhibited extracts, the low molecular weight of the preparation does not appear to be an artifact resulting from autolysis during extraction or purification. If the components of the preparation are not native forms as secreted by the aleurone (14), for instance, they could conceivably arise within the starch endosperm during seedling growth as products of a normal degradative process.

Some of the properties of the present preparation do not agree with those of the endopeptidase activity secreted by the barley aleurone under the influence of gibberellic acid reported by Jacobsen and Varner (14). Their preparation was stimulated by β-mercaptoethanol, whereas the present preparation was rather strongly inhibited by this particular —SH compound. Their preparation was inhibited only slightly by oxygen and was stimulated slightly by Ca2+ and Mn2+: our preparation was oxygen-sensitive, and EDTA was definitely stimulatory in the presence of suboptimal cysteine concentrations.

Palmer (21) has reported recently that the starchy endosperm cells of barley have an intercellular lamella of protein, which resembles glutelin (hordenin). In addition, he reported that the hemicellulose in the cell walls of the endosperm was not attacked by hemicellulase until this protein had been removed. These results suggest that the requirement for an endopeptidase to initiate the series of reactions that result in the degradation of the reserve protein and starch of the endosperm. From a purely functional standpoint, a relatively small endopeptidase molecule would obviously diffuse more rapidly and be more effective in penetrating the partly hydrolyzed, glutelin-like, intercellular milieu during the early stages of seedling growth.

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LITERATURE CITED

MULTIPLE FORMS OF BARLEY ENDOPEPTIDASE