Amylases from Aleurone Layers and Starchy Endosperm of Barley Seeds

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ABSTRACT

Amylases from incubated aleurone layers or from starchy endosperm of barley seeds (Hordeum vulgare L. cv. Himalaya) were investigated using acrylamide gel electrophoresis and analytical gel filtration with Sephadex G-200. Electrophoresis of amylase from aleurone layers yields seven visually distinct isozymes with an estimated molecular weight of 43,000. Because each isozyme hydrolyzes β-limit dextrin azure and incorporates calcium-45, they are α-amylases. On Sephadex G-200, amylase from the aleurone layers is separated into seven fractions ranging in estimated molecular weights from 45,000 to 3,000. Little or no activity is observed when six fractions are subjected to electrophoresis. Electrophoresis of only the fraction with the estimated molecular weight of 45,000 gave the seven isozymes. The amylases are heat labile and cannot be stabilized by the presence of substrate or by the protease inhibitor, phenylmethylsulfonylfluoride. Electrophoresis of amylase from the starchy endosperm yields nine β-amylases. Four of these β-amylases are isozymes with an estimated molecular weight of 43,000. The other five forms of β-amylase represent molecular aggregates of the four basic β-amylase monomers. A dimer, a tetramer, and an octamer of β-amylase can be identified with estimated molecular weights of about 86,000, 180,000 and 400,000, respectively. These estimated molecular weights were confirmed on Sephadex G-200. There are five additional fractions of β-amylase with estimated molecular weights ranging from 30,000 to 4,000. These fractions are not observed electrophoretically.

MATERIALS AND METHODS

Preparation and Incubation of Aleurone Layers. Half seeds of barley (Hordeum vulgare L. cv. Himalaya) were sterilized in 1% (w/v) calcium hypochlorite for 20 min and imbibed on sterile moist sand for 3 days. The aleurone layers were separated from the bulk of the starchy endosperm. Residual starchy endosperm was removed from the aleurone layers in the following manner. Each aleurone layer was placed in a drop of sterile water on a depression slide under a dissecting scope. Using the blunt edge of a small scalpel, the starchy endosperm was removed from the aleurone layer. The aleurone layers were washed twice in sterile water before being placed in the incubation medium. Ten aleurone layers were incubated in 2 ml of sterile medium in a 25-ml Erlenmeyer flask at 25°C on a metabolic shaker for 16 hr. The incubation medium for the aleurone layers was 2 mM acetate buffer, pH 5.0, with and without 20 mM CaCl₂ and 1 μM gibberellic acid. The colorimetric assay for amylase was performed as described by Bilderback (2).

Preparation and Incubation of the Starchy Endosperm. The aleurone layer was removed from dry half-seeds with a razor blade. The starchy endosperm was sterilized in 1% (w/v) calcium hypochlorite for 20 min and imbibed on sterile moist sand for 3 days. Five starchy endosperms were incubated in a similar manner to the aleurone layers in medium from which calcium and GA₃ were deleted.

Determination of α- and β-Amylase. A substrate specific for α-amylase, β-limit dextrin azure was prepared, and the assay for amylase was followed as described by Bilderback (3). An aliquot of enzyme (0.1 ml) was added to 0.5 ml of 2% (w/v) β-limit dextrin azure suspended in 0.05 M sodium acetate buffer, pH 4.8, and incubated at 30°C for 2 hr. The substrate was kept in suspension by shaking every 5 min. The reaction mixture was diluted and passed through a Millipore filter into a spectrophotometer tube. The absorbance was read at 595 nm.

Acrylamide Electrophoresis. A solution containing 8, 10, or 12% (w/v) polyacrylamide (Cyanogum 41 gelling agent, Fisher Scientific Co.) and 0.75% (w/v) ammonium persulfate was subjected to a vacuum of about 700 mm Hg for 2 to 4 min to remove excess oxygen. These solutions were gelled between glass plates by adding 0.2% (w/v) N,N,N,N-tetramethyl-ethylenediamine.

Electrophoresis was done in a vertical gel apparatus with a continuous tris-glycine buffer, pH 8.8. Enzyme samples from the incubation media were absorbed onto small paper wicks. To monitor the progress of electrophoresis, bovine serum albumin stained with 1% (w/v) amido blue-black was absorbed onto a wick. The wicks were inserted into slits cut in the gel. Electrophoresis was accomplished with a voltage of 13 v/cm for 6 to 15 hr at 17°C. The wicks were removed after 1 hr. The disc electrophoretic method of Hedrick and Smith (8) was used.
without modification to separate size and charge isomers and to estimate mol wt. After electrophoresis, the gels were agitated in a starch solution of 2% (w/v) Lintner starch in 40 mM sodium phosphate buffer, pH 7.0, at 25 C from 5 to 12 hr. Bands of amylolytic activity were visualized by immersing the gel in I-K1 solution for 20 to 30 min.

Incorporation of Radioactive Calcium. Five aleurone layers were incubated for 16 hr in 0.5 ml of medium which was 2 mM acetate buffer, pH 5.0, 20 mM CaCl2, and 0.1 mM GAs. To this medium was added 1 mc of calcium-45. An enzyme sample from the incubation medium was absorbed onto a paper wick and electrophoretic separation was accomplished. The region with amylolytic activity was cut into 1.0-mm segments. Each segment was dehydrated and counted by scintillation.

Determination of Protease Activity. After electrophoresis, the length of the gel was cut into 4.0-mm segments. Each segment subsequently was cut into six portions. The six portions of each segment were placed in 1.0 ml of a saturated solution of gliadin in 0.05 M sodium acetate buffer, pH 4.8. For 24 hr, 1.0 ml of 0.5% w/v ninhydrin was added. The color was developed and the absorbance was read at 580 nm.

Analytical Gel Filtration. Sephadex G-200 (Pharmacia Fine Chemicals, 100–200 μ) was allowed to swell in 0.05 M sodium acetate buffer, pH 4.8, for 5 days at 4 C. A column (1.5 × 70 cm) was prepared according to the procedure of Andrews (1). The column was equilibrated 2 to 3 days in buffer before use. Eluents were collected in 2-ml fractions. The flow rate was 20 ml/hr. The column void volume (V0) was determined by reading blue dextran 2000 (Pharmacia Fine Chemicals) at 620 nm. Phenol red was used to determine the internal volume and was read at 500 nm. The protein standards were: horse heart cytochrome c (12,400), chymotrypsinogen (25,000), horse radish peroxidase (49,000), bovine serum albumin (67,000), and ox liver catalase (240,000). Cytochrome c was read at 412 nm. The other proteins were monitored at 280 nm.

The soluble starch substrate was prepared in 0.05 M sodium acetate buffer, pH 4.8, according to the method outlined by Bilderback (2). An aliquot of the column fraction (0.5–1.0 ml) was added to 0.5 ml of the starch solution in a test tube, and the reaction was allowed to proceed for a suitable time (5 min–24 hr.). Starch hydrolysis was stopped with 1.0 ml of an iodine reagent prepared according to the procedure of Bilderback (2). Absorbance was read at 620 nm. The change in absorbance was proportional to the amount of amylase present.

RESULTS

Amylases from the Aleurone Layer. Amylase from the aleurone layers can be separated electrophoretically into seven bands of activity which reside in three distinct zones (Fig. 1). After the gel has been incubated in Lintner starch for 5 hr, bands 1 and 2 of zone I can be visualized as broad ill-defined bands of relatively low activity. The greatest amount of activity resides in bands 3 and 4 of zone II. Bands 5, 6, and 7 of zone III are well defined but visually contained little activity. When gibberellic acid is removed from the incubation medium, the seven bands can still be visualized. However, the relative intensity of the bands is greatly reduced.

If each of the seven bands are removed from the zymogram, and individually incubated with an α-amylase specific substrate, β-limit dextrin azure, substantial hydrolysis of the substrate occurs. All seven bands of activity can be considered to be forms of α-amylases.

As much as net charge and mol wt of the protein can influence its electrophoretic separation in acrylamide gel (4), the concentration of the gel was varied to determine whether the α-amylases differ only in net charge or also in mol wt. After the electrophoretic front as indicated by stained serum albumin had traversed the same distance in 8, 10, and 12% gel, the relative mobilities of the seven α-amylases remained unchanged. Presumably, the seven α-amylases are similar in mol wt but differ only in net electrical charge. The more slowly migrating amylases (bands 5, 6, and 7) seem not to be molecular aggregates of any of the four more rapidly migrating amylases.

To confirm and quantify the observation that the α-amylases were of similar mol wt, differing only in electrical charge, the disc electrophoretic method of Hedrick and Smith (8) was used. When α-amylase from aleurone layers are placed on 6, 7, and 8% (w/v) discs gels, complete electrophoretic separation cannot be achieved. Only two bands of activity can be visualized which corresponded to zones I and II. The individual bands within zones I and II cannot be completely separated. The faint bands of zone III cannot be observed on the small disc gels. Regardless of the incomplete separation on disc gels, a plot of the log of the relative mobilities of the two zones against the gel concentration yields two parallel lines (Fig. 2). These two zones of α-amylase activity differ only in respect to charge and not mol wt. The mol wt is estimated to be about 43,000.

If an enzyme sample from incubated aleurone layers is heated at 70 C for 30 min before electrophoresis, no bands of activity are observed on the zymogram. To further study this phenomenon, amylase was incubated with an α-amylase specific substrate, β-limit dextrin azure (Fig. 3A). When an aliquot of α-amylase is incubated for 2 hr with the substrate, a large increase in absorbance is observed. However, little or no absorbance is observed when amylase is heated for 30 min. This heat instability is not due to a lack of substrate. When the α-amylase found in the starchy endosperm is heat-treated, little or no

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Fig. 1. A 10% acrylamide gel zymogram of α-amylases from barley aleurone layers.
enzymatic activity is observed (Fig. 3B). Similar results are obtained when aleurone layers are incubated in medium containing the protease inhibitor, phenylmethylsulfonylfluoride, at concentrations of 50 and 100 μg/ml.

If the aleurone layers are removed from the incubation medium after 16 hr, and the incubation medium is made 50 mM with respect to EDTA, bands 5, 6, and 7 are absent from the zymogram. Bands 1 and 2 are reduced in relative activity and bands 3 and 4 appear to be unaffected.

Calcium is not required in the incubation medium for the appearance of bands 3 and 4 on the zymogram. However, bands 1, 2, 5, 6, and 7 are greatly reduced in activity or absent when calcium is deleted from the incubation medium. When aleurone layers are incubated in "Ca", nine peaks of radioactivity are observed (Fig. 4). Four peaks of radioactivity are associated with the two broad bands of amylolytic activity in zone I. The remaining five peaks of radioactivity correspond to the five bands of α-amylase. However, the major peak of radioactivity associated with band 4 is centered at the trailing edge of the band of enzymatic activity. Because Sundblom and Mikola (13) have reported that aleurone layers produce calcium-dependent proteases, the zymogram was assayed for proteolytic activity. Proteases were found which closely corresponded to the two additional peaks of radioactivity in zone I. A third protease was localized at the trailing edge of the fourth α-amylase. If the gels were agitated in 50 mM EDTA prior to the proteolytic assay, no proteases can be observed. Apparently, these three peaks of radioactivity represent calcium containing proteases released from the aleurone layer.

When an enzyme sample from aleurone layers is eluted through a Sephadex G-200 column, seven fractions ranging in estimated mol wt from 45,000 to 3,000 are obtained (Fig. 5). The fraction with an estimated mol wt of 45,000 differs substantially from the remaining lighter fractions. This fraction is unable to rapidly hydrolyze soluble starch. An incubation period of 2 to 6 hr is required. The fractions with estimated mol wt of 35,000, 30,000, 25,000, 14,000, 8,000, and 3,000 rapidly hydrolyze the soluble starch substrate. All fractions hydrolyze β-limit dextrin azure and can be considered α-amylases. When these individual fractions are subjected to electrophoresis and incubated in Lintner starch for 10 hr, only the fraction with an estimated mol wt of 45,000 yielded the seven previously visualized isozymes. Fractions with estimated mol wt of 14,000 and 8,000 gave weak bands which migrated at the trailing edge of band 4. All other fractions (35,000, 30,000, 25,000, and 3,000) cannot be visualized on the zymogram.

**Amylases from the Starchy Endosperm.** When an enzyme sample from the starchy endosperm imbibed with aleurone layers is incubated with the α-amylase specific substrate, β-limit dextrin azure, a significant increase in the absorbance is observed (Fig. 3C). The starchy endosperm is contaminated with α-amylase from the aleurone layers. If an aliquot of amylase of comparable specific activity from imbibed starch endosperm without aleurone layers is incubated with the α-amylase specific substrate, essentially no α-amylase activity is observed. The amylases from the starchy endosperm are β-amylases.

In an 8% acrylamide gel, β-amylase from the starchy endosperm separates into eight bands of activity (Fig. 6). When the concentration of acrylamide is increased to 10%, nine bands of activity can be visualized (Fig. 6). The additional band results from the differentiation of the relatively broad band 2 into two
zymograms of Physiol. components minor four bands. The discrete bands seeds. cannot be separation amylases soluble with position of the amylases with relatively constant mol wt was 20 ml/hr, and 2-ml fractions were collected. Vc indicates the position of the void volume marker (blue dextran 2000). Estimated mol wt of the major fractions are shown on the figure. ✓ fractions incubated with soluble starch for 6 hr; ○: fractions incubated with soluble starch for 5 min.

FIG. 5. Sephadex G-200 column elution patterns of α-amyrase from barley aleurone layers. Sample volume was 2 ml. The column was eluted with 0.05 M sodium acetate buffer, pH 4.8. Flow rate was 20 ml/hr, and 2-ml fractions were collected. Vc indicates the position of the void volume marker (blue dextran 2000). Estimated mol wt of the major fractions are shown on the figure. ✓ fractions incubated with soluble starch for 6 hr; ○: fractions incubated with soluble starch for 5 min.

Fig. 6. Schematic representation of 8 and 10% acrylamide gel zymograms of β-amylases from the starchy endosperm of barley seeds.
discrete bands of activity. The majority of activity resides in four bands. The remaining five bands appear to be relatively minor components of the enzyme sample. Good electrophoretic separation cannot be obtained on 12% gel.

If, after electrophoresis, the gel is flooded with p-chloromercuribenzoate, only bands 2a and 4 are visible. All other bands are absent from the zymogram.

By increasing the concentration of acrylamide from 8 to 10%, the amylases are separated into two distinct classes: (a) those amylases with relatively constant mobilities, and (b) those amylases with delayed mobilities (Fig. 6). The mobilities of bands 1, 2a, 3, and 4 are essentially unchanged. Apparently, these amylases are similar in mol wt but differ in net electrical charge. Bands 2b, 5, 6, 7, and 8 are considerably delayed in the 10% acrylamide gel. These delayed amylases differ from those amylases with constant mobilities not only in net electrical charge but also in size or mol wt. From their relative positions on the zymogram, bands 5, 6, 7, and 8 can be large protein moieties.

When the β-amylases were placed on 6, 7, and 8% disc gels, complete electrophoretic separation could not be accomplished. One broad band and three narrow bands of activity were visualized on the small disc gels. The broad band corresponds to the first four bands of activity on the large vertical gel. The second and third bands on the disc gel corresponded to bands 5 and 6, respectively. The fourth band on the disc gel contained the small bands 7 and 8.

When the log of the relative mobilities of these four bands of activity are plotted against the gel concentration, nonparallel lines result (Fig. 2). When these nonparallel lines are extended, they intersect at a gel concentration of 4.3%. Hedrick and Smith (8) have shown that nonparallel lines intersecting at gel concentrations other than 0% are characteristic of proteins which differ in both charge and mol wt. From the slope of the lines, the mol wt of the four bands of activity are estimated to be 43,000, 86,000, 180,000, and 400,000, respectively. These estimated mol wt are confirmed when β-amylase from the starchy endosperm is eluted through Sephadex G-200 (Fig. 7).

In addition to these four fractions, five additional fractions were observed with estimated mol wt of 30,000, 16,000, 13,000, 8,000, and 4,000. These five fractions cannot be observed electrophoretically.

When fractions with estimated mol wt of 400,000, 186,000, and 86,000 are pooled and eluted through Sephadex G-200 for a second time, a fraction with an estimated mol wt of 45,000

FIG. 7. Sephadex G-200 column elution patterns of β-amyrase from the starchy endosperm of barley seeds. Sample volume was 2 ml. The column was eluted with 0.05 M sodium acetate buffer, pH 4.8. Flow rates were 20 ml/hr and 2-ml fractions were collected. Vc indicates the position of the void volume marker (blue dextran 2000). Estimated mol wt of the major fractions are shown on the figure.

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is observed. The heavier fractions appear to be molecular aggregates of a monomer with an estimated mol wt of 45,000.

**DISCUSSION**

Using agar gel electrophoresis, Frydenberg and Nielsen (7) were able to separate seven forms of amylase from germinating barley seeds. Five forms were identified by their physical and chemical properties as α-amylases. Two amylases could not be characterized. Jacobsen et al. (9) observed only four α-amylases when an enzyme sample from aleurone layers treated with GA₃ were separated by agar gel electrophoresis. They suggested several reasons for the absence of the α-amylases observed by Frydenberg and Nielsen (7). First, the missing amylases simply could not be resolved by their methods. Second, the appearance of the α-amylases could be dependent upon some factors in the starchy endosperm, or they could arise from the hybridization of α- and β-amylases.

In this study, seven isozymes of α-amylase from aleurone layers could be visualized on the acrylamide gel zymogram. Because the aleurone layers are completely free of any starchy endosperm, the appearance of any of the amylases is not dependent upon factors from the starchy endosperm. Therefore, the possibility that some amylases are heavier molecular hybrids between α- and β-amylases is eliminated.

All seven isozymes of α-amylase have an estimated mol wt of 45,000. This estimated mol wt compares favorably with the value of 42,000 published by Stoddart (12). However, these values are considerably less than the mol wt of 60,000 reported for crystalline barley α-amylase (11).

The isozymes of α-amylase observed on the zymogram represent only a small portion of the total amylolytic activity secreted by the aleurone layers into the surrounding medium. The majority of activity resides with fractions with mol wt ranging from 35,000 to 3,000. The fact that these fractions cannot be easily visualized electrophoretically suggests a structural instability of these fractions. These forms of amylase could result from the partial hydrolysis by proteases released from the aleurone layers. On the other hand, Filner and Varner (6) observed no low mol wt activity when crude α-amylase was subjected to isopycnic equilibrium centrifugation. The low mol wt Sephadex fractions of α-amylase might be spurious.

α-Amylase secreted by the aleurone layers into the surrounding medium are heat labile. These results were contrary to the published reports of a number of workers (7, 14, 16). Structural instability of the amylases resulting from proteolytic activity could be responsible for this observed heat lability.

β-Amylases from barley have not been extensively investigated. Frydenberg and Nielsen (7) found two amylases from germinating barley seed which had characteristics of β-amylase. Jacobsen et al. (9) found four β-amylases in the incubation medium surrounding aleurone layers. When an enzyme sample from the starchy endosperm alone was placed on agar gel, no electrophoretic separation could be achieved (9). Stoddart (12) and Bilderback (2) independently reported that six to eight amylases from the starchy endosperm could be electrophoretically separated on acrylamide gel. These amylases were characterized by Stoddart (12) as β-amylases.

In the present study, nine β-amylases are separated electrophoretically from starchy endosperm completely isolated from the aleurone layer. Of these nine β-amylases, four forms are isozymes with an estimated mol wt of about 43,000. The remaining β-amylases appear to be dimers (86,000), tetramers (180,000) and octamers (400,000) of the basic β-amylase monomers. Multimeric forms of β-amylase have also been isolated from sweet potato (15). Using gel filtration methods, Stoddart (12) found that the mol wt of barley β-amylases fell in a wide range between 45,000 and 400,000. However, Stoddart (12) believed that the heavier components were not molecular aggregates but consisted of a single enzyme species with varying amounts of nonenzymatic protein associated with it.

In addition to these β-amylases, there are additional components with mol wt ranging from 30,000 to 4,000. Cooper and Pollock (5) reported that commercial malt syrup has low mol wt forms with β-amylase activity.

On the zymogram, only two β-amylases are insensitive to p-chloromercuribenzoate. Two β-amylase monomers require free sulfhydryl groups for activity and two monomers do not. It is of interest that the molecular aggregates of β-amylase all require free sulfhydryl groups for activity.

Jacobsen et al. (9) found that prior treatment of the starchy endosperm with a proteolytic enzyme was necessary for separation of β-amylases by agar gel electrophoresis. With acrylamide gel electrophoresis, proteolytic treatment was not necessary for separation of the β-amylases. In fact, Bilderback (2) reported that proteolytic treatment actually reduced the number of β-amylases from eight to four. Apparently, proteolytic enzymes do not only release latent β-amylase from glutenin (10) but also reduce the large molecular aggregates of β-amylase to the basic monomeric constituents.

**LITERATURE CITED**