# α-AMYLASE I FROM MALTED BARLEY—PHYSICAL PROPERTIES AND ACTION PATTERN ON AMYLOSE[1]

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**ABSTRACT**

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α-Amylase I hydrolyzed amylose to a mixture of maltodextrins. Dextrins smaller than $G_7$ were hydrolyzed slowly. The enzyme had a molecular weight of 52,000, an isoelectric point of pH 5.1, an activation energy of 9.7 kcal/mol between 20 and 50° C, an optimum temperature of 55° C, and an optimum pH of 5.5. In addition, the enzyme showed unusually high stability at pH 3.6.

Malted barley α-amylase has been the subject of much study over many years and some properties of the enzyme have been well documented (1−5). More recently, the use of protein separation techniques such as discontinuous electrophoresis and isoelectric focusing, which have high resolving power, have shown that the enzyme is not a single entity but a complex mixture of α-amylases (6−16). Widely different estimates of the number of components in this mixture have been made (6−18). This variability is no doubt caused by a number of factors such as the barley cultivar used, the malting or germination method used (eg, whole kernels, half kernels, dissected tissues, germination on filter paper, incubation in an aqueous medium), and the method used to separate and detect the α-amylases. In most of these reports, individual α-amylase components were not isolated, purified, or extensively characterized.

To determine if these α-amylase components differ in properties other than electrophoretic mobility, a program has been started to purify and characterize the main components. Reports on the detection, identification, and purification of one of the components of malted barley α-amylase have been published previously (18,19).

This article describes some of the properties of α-amylase I from malted barley and compares these properties with those of other cereal α-amylases.

## MATERIALS AND METHODS

### Preparation of α-Amylase I

α-Amylase I was isolated from malted Conquest barley and purified as described previously (19).

### α-Amylase Activity

α-Amylase activity was determined as described previously (20). The β-limit dextrin of waxy maize starch was used as substrate.

### Amylose

Linear amylose was prepared from potato starch as described previously (21).

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**β-Amylase**

Crystalline sweet potato β-amylase was obtained from Sigma Chemical Company (St. Louis, MO).

**Hydrolysis of Amylose by α-Amylase**

*Production of Maltodextrins.* A digest was prepared containing α-amylase I (4,000 Iodine Dextrin Color (IDC) units) and amylose (30 mg) in a total volume of 260 ml of acetate buffer (0.001*M*, pH 5.5, 0.001*M* $CaCl_2$). The digest was incubated at 35° C and after 1/4, 1/2, 1, 1-1/2, 2-1/2, 24, and 48 hr, portions (30 ml) were removed, boiled for 5 min, deionized with Amberlite® monobed resin, and freeze-dried. A further portion of α-amylase (4,000 units) was added to the digest after 24 hr. The freeze-dried samples were dissolved in water (0.5 ml) and spotted on Whatman No. 1 chromatography paper together with a standard sugar mixture containing glucose ($G_1$), maltose ($G_2$), maltotriose ($G_3$), and maltotetrose ($G_4$). The sugars were separated by descending chromatography for 44 hr at 20° C with the solvent system *n*-propanol/ethyl acetate/water (14:2:7). A solution of amylo-1,6-glucosidase (5 mg/ml) was then sprayed on the paper and allowed to dry. Sugar spots were developed by Trevelyan's (22) method.

*Percentage of Conversion to Apparent Maltose.* To determine the percentage of conversion to apparent maltose, the following digests were prepared:

1. One milliliter of amylose solution (625 μg), 9 ml of buffer (0.01*M* acetate, pH 5.5, 0.001*M* $CaCl_2$), and α-amylase (0.02 ml, 170 units).
2. Same as above but containing sweet potato β-amylase (0.005 ml, 81 units) instead of α-amylase.

The digests were incubated at 35° C. After 1/4, 1/2, 1-1/2, 2, 3, and 24 hr, 1-ml portions of both digests were assayed for reducing power with neocuproine reagent (23). The concentration of amylose solutions was determined as described previously (24). Results were expressed as percentage of conversion of amylose into maltose.

**Effect of Temperature on Activity**

The effect of temperature on activity and stability of α-amylase I was determined as described previously (24). A temperature range of 4–70° C was used.

**Effect of pH on Activity**

The activity and stability of α-amylase I over the pH range of 3.5–8.5 were determined as described previously (24).

**Stability at pH 3.6**

Conquest barley malt (7.5 g) was extracted with tris hydrochloride buffer (20 ml, 0.04*M*, 0.01*M* $CaCl_2$, pH 8.0) in a VirTis homogenizer for 1 min. The extract was centrifuged (15,000 × *g* for 10 min), and the pH of one half of the supernatant solution was lowered to 3.6. At intervals, portions of the low pH extract were removed, brought to pH 5.5, and centrifuged (15,000 × *g* for 10 min). These extracts were analyzed for α-amylase and for α-amylase enzyme patterns by isoelectric focusing on polyacrylamide gel on a pH 4–8 gradient. Isoelectric focusing on polyacrylamide gel was performed as described previously (16).

**Determination of Isoelectric Point**

Determination of the isoelectric point was performed on an LKB 8101, 110-ml column, according to instruction leaflet 1-8100-E03 (LKB-Produkter AB, S-161 25 Bromma 1, Sweden). The following solutions were used:

| *Dense electrode solution* | | *Light electrode solution* | |
|---|---|---|---|
| Ethanolamine | 0.8 ml | Phosphoric acid | 0.3 ml |
| Sucrose | 24.0 g | Water | 30.0 ml |
| Water | 28.0 ml | | |
| *Dense gradient solution* | | *Light gradient solution* | |
| pH 4-6 ampholyte | 2.0 ml | pH 4-6 ampholyte | 1.0 ml |
| Sucrose | 27.0 g | Sucrose | 2.7 g |
| α-Amylase I (ca 50 μg) in 0.001*M* $CaCl_2$ | 2.0 ml | Water | 52.0 ml |
| Water | 33.0 ml | | |

The anode was at the top of the column. The run was continued for 66 hr, the column was emptied, and the contents were collected in 1-ml fractions. α-Amylase and pH determinations were made on each fraction.

**Molecular Weight**

The sodium dodecyl sulfate (SDS) electrophoresis method that Weber and Osborn (25) described was used with the modifications that Robyt et al (26) suggested.

## RESULTS AND DISCUSSION

The hydrolysis of amylose by α-amylase I took place in two distinct stages. In the first stage, hydrolysis was rapid and, after 30 min, the reducing power of the

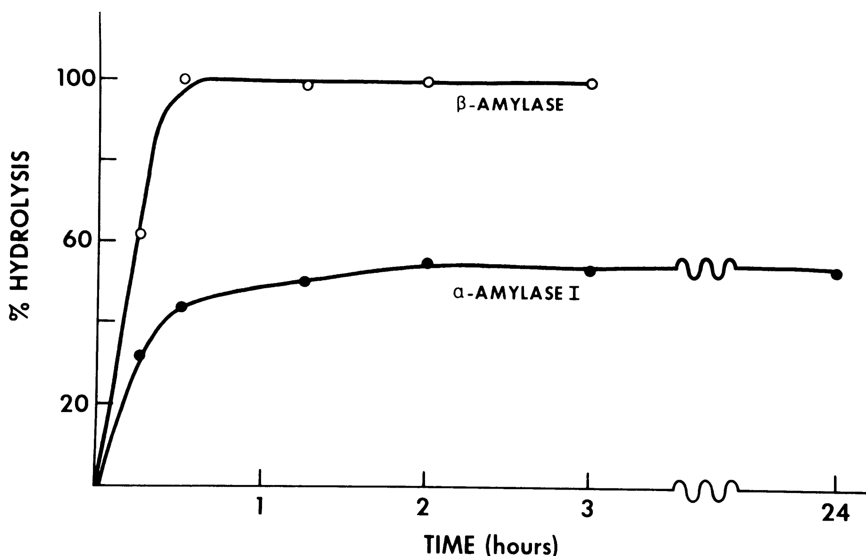


Fig. 1. Hydrolysis of linear amylose by α-amylase I and β-amylase. Results are expressed as percentage of conversion of amylose into apparent maltose.

digest was equivalent to a 45% conversion of the amylose into maltose (Fig. 1). During the subsequent 23-1/2 hr, amylose hydrolysis was slow so that the maximum hydrolysis obtained was only 55% apparent conversion into maltose. The rapid, complete hydrolysis of amylose into maltose by β-amylase showed that the amylose was completely linear and did not contain structural

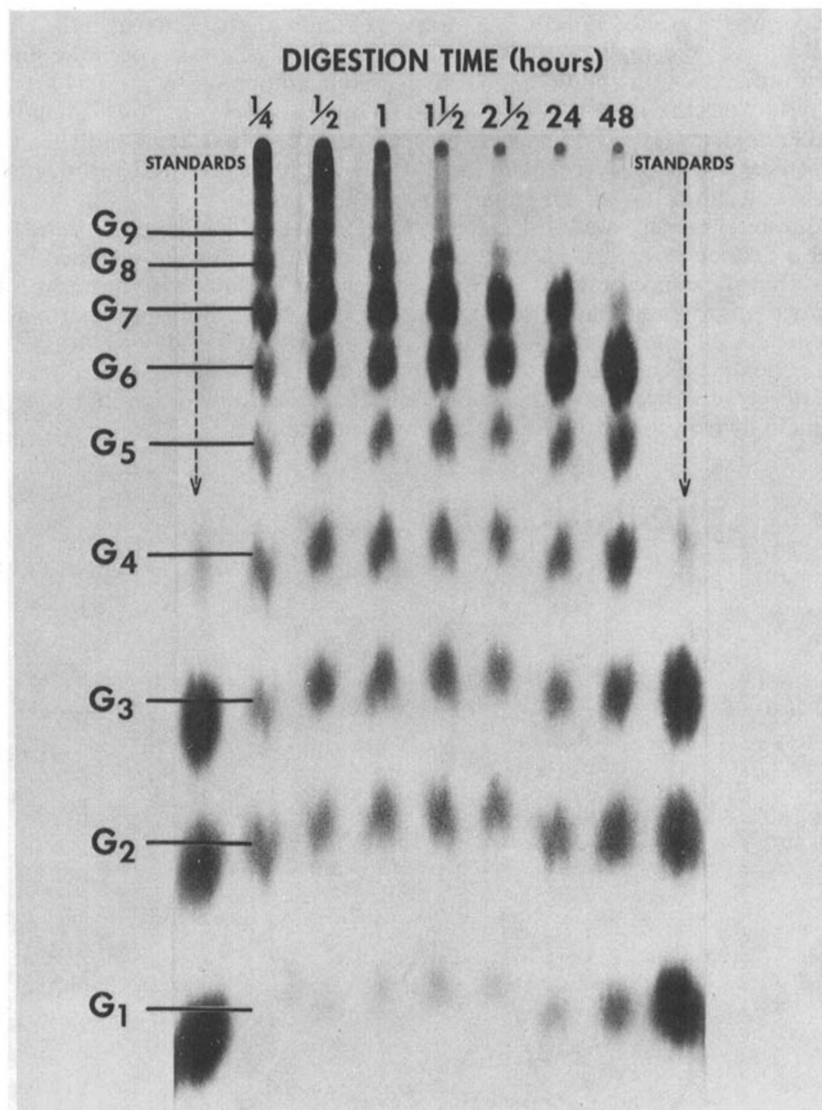


Fig. 2. Products of hydrolysis of linear amylose by α-amylase I. $G_1$ represents glucose; $G_2$, maltose; $G_3$, maltotriose; $G_4$, maltotetrose; $G_5$, maltopentose; $G_6$, maltohexose; $G_7$, maltoheptose; $G_8$, maltooctose; $G_9$, maltononose.

modifications that would block α-amylase action.

The hydrolysis products of a similar amylose-α-amylase I digest were analyzed by paper chromatography (Fig. 2). Because the paper was sprayed with amylo-1,6-glucosidase solution to reduce all dextrins to glucose before the dextrin spots were made visible with silver nitrate, the relative concentrations of the dextrins may be tentatively determined from the intensities of the spots on the paper. Again, a two-step degradation reaction was found. During the initial 30 min, amylose was degraded rapidly to a mixture of maltodextrins in which the larger members ($G_6$ and higher) predominated. As hydrolysis proceeded, the higher dextrins disappeared and $G_6$ and $G_7$ increased in amount until, after 24 hr, they not only were the largest dextrins present but also were the most abundant. Smaller dextrins ($G_2$–$G_5$) also were present from the initial stages of hydrolysis. Only traces of glucose were found until after 1-1/2 hr of hydrolysis; even after 24 hr, the concentration of this sugar was relatively small.

Additional enzyme was added to the amylose-α-amylase I digest after 24 hr. After a further 24-hr period of incubation, only a trace of $G_7$ remained in the digest, but the concentrations of $G_1$–$G_6$ increased. Presumably, the conversion of amylose into apparent maltose increased also, but this was not determined.

These results show that α-amylase I rapidly hydrolyzed amylose to $G_7$ and smaller dextrins. Hydrolysis of $G_7$ was slow, and that of $G_6$ and smaller dextrins was still slower. Obviously, a large amount of enzyme would be required to effect complete hydrolysis of amylose to glucose and maltose.

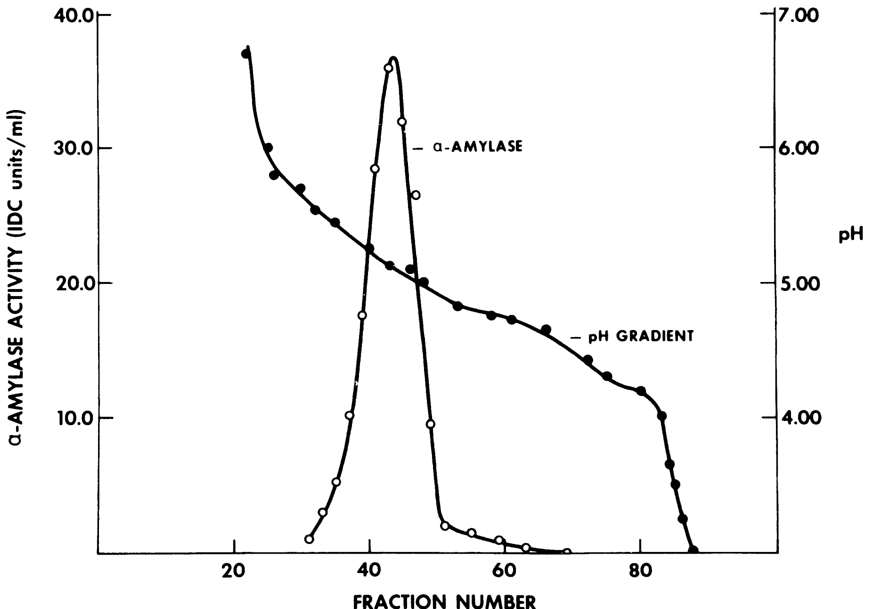


Fig. 3. Isoelectric focusing of α-amylase I on pH 4–6 gradient.

Similar results have been obtained for all higher plant $\alpha$-amylases examined thus far (27). The dextrin pattern obtained after 48-hr hydrolysis of amylose (Fig. 2) is characteristic of the action of cereal $\alpha$-amylases on amylose (27). Quantitative data on the liberation and hydrolysis of individual maltodextrins by $\alpha$-amylase I is required before a detailed comparison can be made of the action pattern of this enzyme with the action patterns of other $\alpha$-amylases.

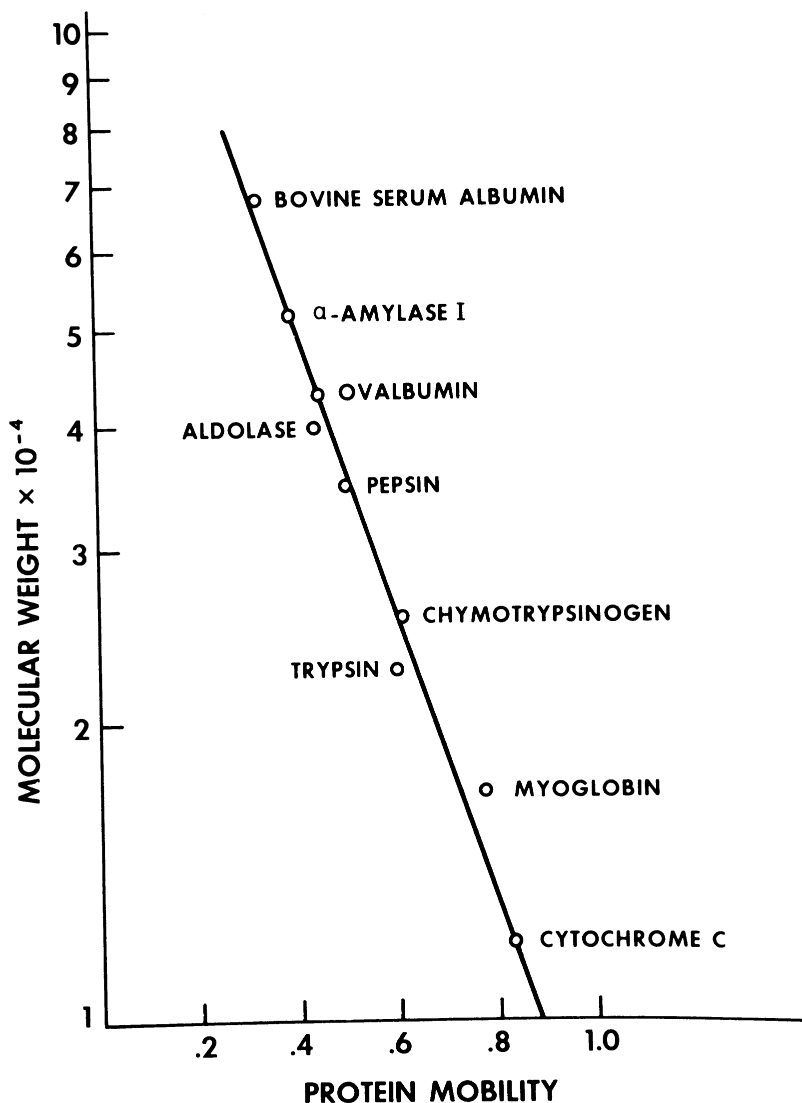


Fig. 4. Determination of molecular weight of $\alpha$-amylase I by SDS-polyacrylamide gel electrophoresis.

The isoelectric point of α-amylase I was 5.1 (Fig. 3). This value is much lower than the range of pH 6.2–6.4 found for the isoelectric point of the α-amylase II complex[2] of malted barley, but is similar to results obtained for the α-amylases present in immature wheat (28) and immature barley (24). These enzymes were readily separated by ion-exchange chromatography at pH 4.75 because of the large difference in isoelectric points of α-amylases I and II (18).

Extensive loss of α-amylase I activity occurred during isoelectric focusing of the enzyme in a pH 4–6 gradient. In later studies on other α-amylase components, the addition of 0.001*M* $CaCl_2$ to all solutions appeared to stabilize the enzymes during isoelectric focusing runs. Presumably, α-amylase I activity could be stabilized in the same way.

The molecular weight of α-amylase I, as determined by the SDS-polyacrylamide gel method, was 52,000 (Fig. 4). This was the average value from three separate determinations. The value is higher than reported values (42,000–46,000) for immature barley (24) and for several other cereal α-amylases (27), but is similar to the molecular weight range (52,000–54,000) of α-amylases from immature wheat (28).

[2]Unpublished data, A. W. MacGregor.

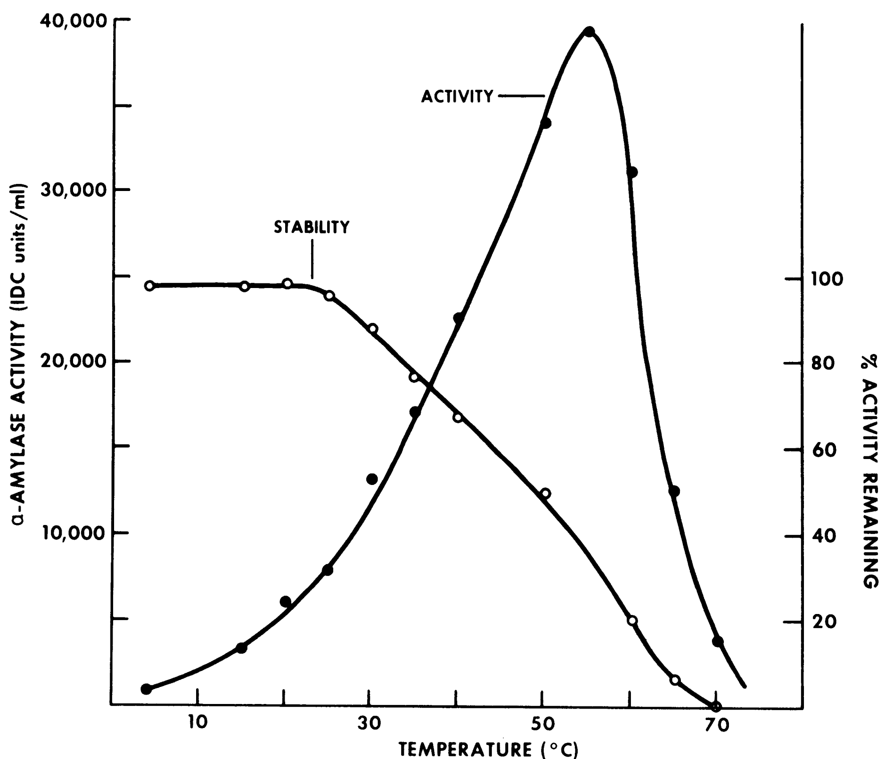


Fig. 5. Effect of temperature on activity and stability of α-amylase I. Stability of enzyme is expressed as percentage of original activity at 35° C that remains after 1 hr of incubation at various temperatures.

The temperature of maximum activity was 55° C (Fig. 5). This is similar to results reported for $\alpha$-amylase from immature barley (24), but is slightly higher than values reported for other cereal $\alpha$-amylases (27). These differences, however, are not significant. They could be caused by a variation in the purity of the enzyme preparations studied rather than by any inherent difference in the chemical or physical properties of the enzymes.

Although the enzyme was active at 55° C, it was unstable at this temperature and had lost 65% of its activity after 1 hr. The enzyme was unstable even at relatively low temperatures, and started to lose activity after 1 hr at 25° C. The commonly used temperature of 35° C for enzyme incubations was used for $\alpha$-amylase I, however, because it afforded reasonably high activity (2.5 times higher than at 20° C) and acceptably low loss in activity (about 4%) during the time required to perform an activity determination (usually 10 min).

Heat inactivation of $\alpha$-amylase I, and presumably of many other enzymes, is dependent on the state of purity of the enzyme. The stability of the enzyme decreases as the purity of the sample increases. This explains the high stability of $\alpha$-amylase I in a crude malt extract at 70° C and the complete inactivation at this temperature of a highly purified sample of the enzyme (Fig. 5). The high stability

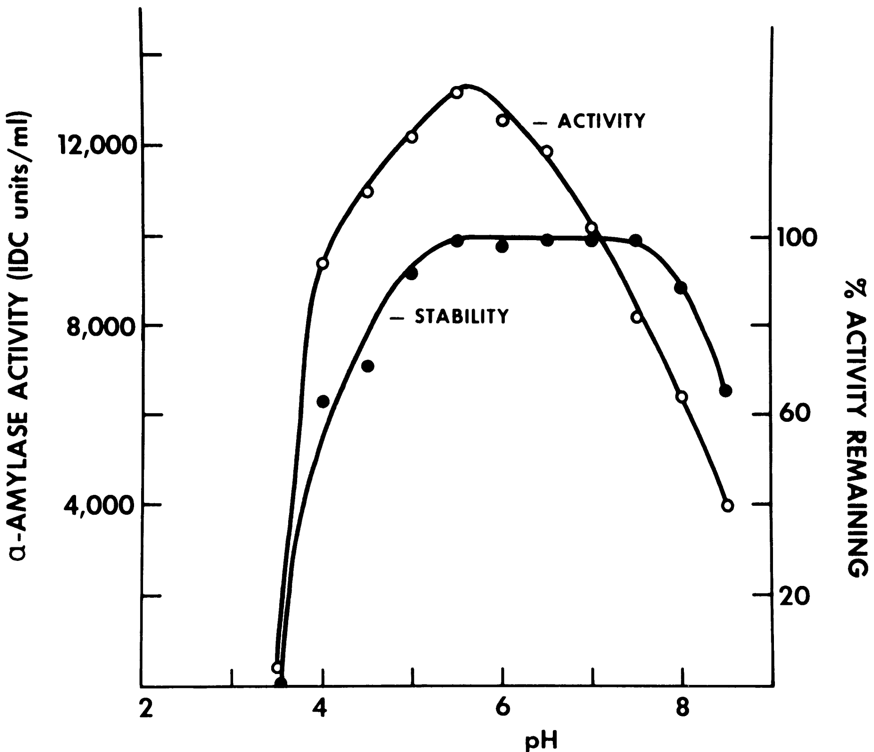


Fig. 6. Effect of pH on activity and stability of $\alpha$-amylase I. Stability of enzyme is expressed as percentage of original activity remaining after 1 hr at each pH.

reported for cereal α-amylases at 70° C applies only to impure preparations of these enzymes.

The apparent energy of activation of α-amylase I was calculated to be 9.7 kcal/mol between 20 and 50° C, which agrees with a value of 9.5 kcal/mol found for α-amylase from immature barley (24) and with values obtained for wheat α-amylases (28).

α-Amylase I had an optimum pH of 5.5 (Fig. 6). This is similar to values reported for other cereal α-amylases (5,24,28). The enzyme, however, showed unusually high activity and stability in the pH 4–5 region where most α-amylases from higher plants show a rapid loss in activity. In this respect, α-amylase I was more similar to the α-amylases from immature barley and wheat (24,28) than to the major α-amylases from malted cereals (5,28).

The high stability of α-amylase I at low pH was investigated further by treating

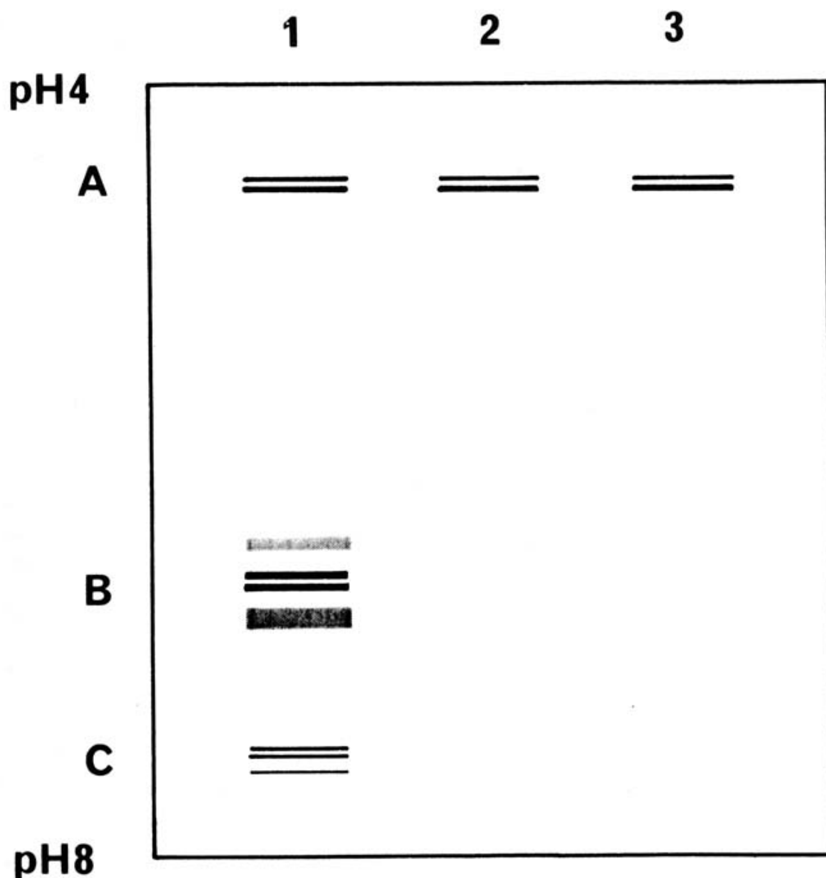


Fig. 7. α-Amylase zymogram obtained after isoelectric focusing on pH 4–8 gradient. 1) Conquest barley malt extract, 2) malt extract after 15 min at pH 3.6, 3) malt extract after 2 hr at pH 3.6, A) α-amylase I, B) α-amylase II, C) α-amylase III.

a malted barley extract at pH 3.6 and analyzing for residual α-amylases by isoelectric focusing on polyacrylamide gels (Fig. 7). The identity of the three groups of α-amylases (A, B, and C) in the original extract has been discussed in a previous publication (16). α-Amylase II (group B), the major α-amylase component, was completely inactivated after 15 min at pH 3.6. α-Amylase I, however, was still active after 2 hr of treatment.

The α-amylase activity of the pH 3.6 treated extract was determined at 0, 15, 30, 45, 60, and 120 min, and the percentage of original α-amylase activity was 1.2, 1.2, 1.1, 1.1, and 1.1, respectively.

These results confirm the gel findings that most of the α-amylase was denatured irreversibly within 15 min at pH 3.6, but a small amount of enzyme survived even after 2 hr. This residual activity was due to α-amylase I. These results suggest that α-amylase I represents only 1–2% of the total malt α-amylase. This seems to be low, because previous work has shown that α-amylase I can account for up to 10% of the total α-amylase, but the actual amount found was dependent on the cultivar used and the conditions of germination (29). α-Amylase I, however, survived the classical pH 3.6 treatment for inactivating plant α-amylases.

Similarities exist between α-amylase I and other cereal α-amylases described in the literature. The enzyme is similar in molecular weight and electrophoretic mobility to a new amylase found in barley and malt (30,31). To suggest at least a partial identify between the enzymes is tempting. More recent work (32), however, has shown that starch β-limit dextrin is 70% hydrolyzed to a mixture of glucose and maltose by the new amylase. This is not characteristic of a cereal α-amylase. The enzyme contains two components, however, and there is no evidence to show that both components have the same action pattern. Because of the apparent novel action pattern of the combined components, comparing the new amylase with other cereal amylases will be possible only when such evidence is available.

During immunochemical studies on barley and malt α-amylases, Daussant et al (33) partially characterized a minor α-amylase component present in germinated barley. That enzyme, called α-amylase I, was probably the same enzyme that has been described in this article.

α-Amylase I and the α-amylase from immature barley have similar molecular weights, isoelectric points, and stabilities at low pH. Previous work has shown that they also have similar chromatographic and electrophoretic properties (24). The identity or partial identity of these various α-amylases will be explored further using immunochemical techniques.

In summary, this report has confirmed the identity of α-amylase I by showing that the maltodextrin pattern obtained from an α-amylase I-amylose digest was similar to that generated by other α-amylases isolated from cereal grains. The enzyme had a low isoelectric point and an unusually high stability at pH 3.6. Electrophoretic properties of the enzyme were similar to those reported for the α-amylase present in immature barley and to a minor α-amylase component of malted barley that other workers described.

**Acknowledgment**

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