The Isoelectric Focusing Electrophoresis of Wheat β-Amylases

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ABSTRACT

The different forms of wheat β-amylase—free and bound β-amylase from mature wheat and β-amylase from germinated wheat—are analyzed by means of isoelectric focusing electrophoresis. The results demonstrate the heterogeneity of wheat β-amylases: the free enzyme has isoelectric points at pH values of 4.8, 4.9, 5.6, and 5.8. After storage of wheat extracts, only the components at pH 4.8 and 4.9 are left. By fractionation with ammonium sulfate precipitation, free enzyme can be purified as two components: one with pI 4.8 and 4.9, and the other with pI 5.8. The bound enzyme is almost homogeneous; its pI is 5.0. The β-amylase from germinated wheat has pI values of 5.5 and 5.8. The results suggest that the base structure of the different components is the same.

In mature wheat there are two types of β-amylases: free (extractable with salt solutions) and bound (latend β-amylase, extractable with reducing agents). During germination, the amount of bound enzyme decreases and the amount of free β-amylase increases. It has been assumed that the increase in free β-amylase is due to release of bound β-amylase by scission of disulfide bonds.

The heterogeneity of these β-amylase components has been demonstrated by various analytical methods (1-6).

It is obvious that the free β-amylase exists as at least two major components having different properties in ion-exchange chromatography, in electrophoresis, and in exclusion chromatography (1-6). Reducing agents and storage can reduce the number of components (3,4).

The bound β-amylase is more homogeneous than the free, and some components of free β-amylase are identical with the components of bound β-amylase (5).

The β-amylase from germinated wheat is antigenically identical with the free β-amylase, which means that they have the same antigenic determinants in their protein structure (3,4). However, they have different properties in electrophoresis and in ion-exchange chromatography (3,5).

The aim here is to further investigate the relationship between the constituents of free and bound β-amylases from mature wheat and β-amylase from germinated wheat, by means of isoelectric focusing, and to compare these results with those obtained earlier using exclusion chromatography and immunoelectrophoresis (3,4).

MATERIAL AND METHODS

In this study, experiments were made with *Triticum vulgare* of the variety Capelle. The germination of grain samples was carried out by a method similar to that employed in previous studies (3). The grains were used 7 days after steeping. Before being soaked for germination, the grains were washed with ether.

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Extraction

The free β-amylase from mature seeds was extracted from acetone-defatted flour with 0.066M phosphate buffer containing 0.5M sodium chloride, as in the method described earlier (7).

The bound β-amylase was extracted with phosphate buffer containing 0.5% mercaptoethanol, after the free β-amylase had been washed away with phosphate buffer.

The β-amylase from germinated wheat was extracted with phosphate buffer after removal of stems and rootlets and homogenization with sand.

All extracts were desalted with Sephadex G-25 (in water) before focusing.

Ammonium Sulfate Precipitation

The procedure was carried out at 4°C. by progressive addition of solid ammonium sulfate to the extract of free β-amylase, and centrifugation after the solution had been stirred for 30 min. The pellet was solubilized in the extracting solution without sodium chloride. The final volume corresponded to a fourfold concentration of the initial extract. Fractions were analyzed in Sephadex G-100 (4). The active peaks were collected and desalted in Sephadex G-25 for focusing.

β-Amylase Determination

β-Amylase determination was carried out according to Kirsop's method modified as described earlier (7): the reducing sugars were determined colorimetrically, with 3,5-dinitrosalicylic acid as reagent.

Isoelectric Focusing Electrophoresis

Focusing was performed according to the method described by Vesterberg and Svensson (8). The volume of the column was 110 ml. Glycerol (50% w./v.), to give a density gradient, and synthetic ampholytes (ampholine pH 4 to 6, LKB, as a 1% solution) were mixed with the sample solution and transferred into the column from an LKB gradient mixer. The electrofocusing time was 70 hr., voltage 500 v. The elution rate was 0.3 ml. per min.; the volumes of the fractions collected were 1.5 ml. The pH was measured at room temperature (by a combination electrode), and β-amylase activity was determined from the fractions.

RESULTS

The isoelectric focusing resolves the free β-amylase from mature wheat into many peaks. The pI for the main peaks are 4.8, 4.9, 5.6, and 5.8 (Fig. 1). The area under pH 4.8 and 4.9 represents 50% of the total focused activity.

The two fractions of free β-amylase obtained in exclusion chromatography (4) can be separated pure from each other by means of ammonium sulfate precipitation. With 20% of ammonium sulfate saturation, fraction II—with higher molecular weight—is precipitated; and with 30 to 50% saturation, fraction I—with lower molecular weight—is precipitated. Component II focuses at pH 5.8 and represents the labile part of the free enzyme, because 58% of its activity is lost during focusing. Component I focuses at pH 4.8 and 4.9 without loss in activity (Fig. 1).

The lability of free β-amylase focused on the more basic pH-area is demonstrated also when the solution of the free β-amylase is stored and focused thereafter (Fig. 2). The only components left are focused at pH 4.8 and 4.9.

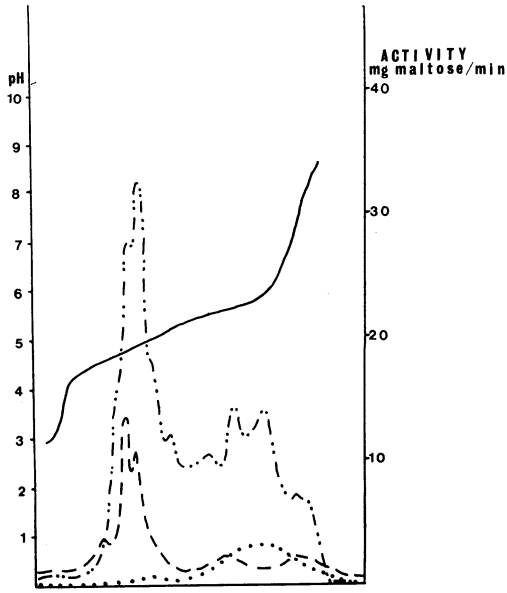

Fig. 1. Isoelectric focusing electrophorese of: the free β-amylase from mature wheat (_..._..._..); fraction I (– – – –); fraction II (. . . .); pH-gradient (————).

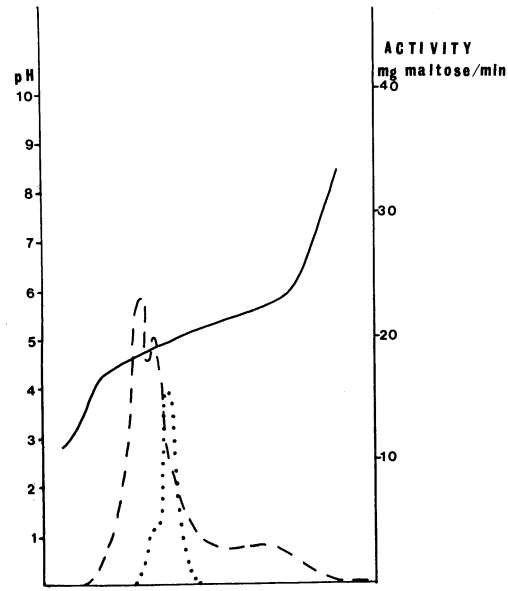

Fig. 2. Isoelectric focusing electrophoresis of: stored free β-amylase (– – – –); bound β-amylase (. . . .); pH-gradient (_____).

The bound β-amylase is the most homogeneous of the analyzed components: it focuses almost as a single peak at pH 5.0 (Fig. 2).

The β-amylase from germinated wheat is still heterogeneous, but displacement towards alkaline pH is obvious: the main peaks are focused at pH 5.5 and 5.8. Only 10% of activity focuses at pH 4.8 and 4.9 (Fig. 3).

DISCUSSION

Isoelectric focusing electrophoresis demonstrates the heterogeneity of free and bound β-amylases from mature wheat and of β-amylase from germinated wheat.

These results confirm the knowledge of wheat β-amylases obtained earlier by other electrophoretic methods. However, the resolving power of focusing is stronger than that of older methods.

The free β-amylase seems to be the most polydisperse of the components studied. The main components have peaks at pH values of 4.8, 4.9, 5.2, 5.4, 5.6, and 5.8. Immunoelectrophoresis gives a similar picture of free β-amylase: an elongated arc which can be due to the presence of the same antigenic group on molecules of different electrophoretic mobilities (3). These findings correlate well with the four to five β-amylase components found in free β-amylase by ion-exchange chromatography (1,5).

Methods of fractionation according to molecular size (exclusion chromatography, disc electrophoresis) indicate the presence of only two components (4,6). These two fractions were separated by means of precipitation with ammonium sulfate. According to exclusion chromatography, fraction I has a

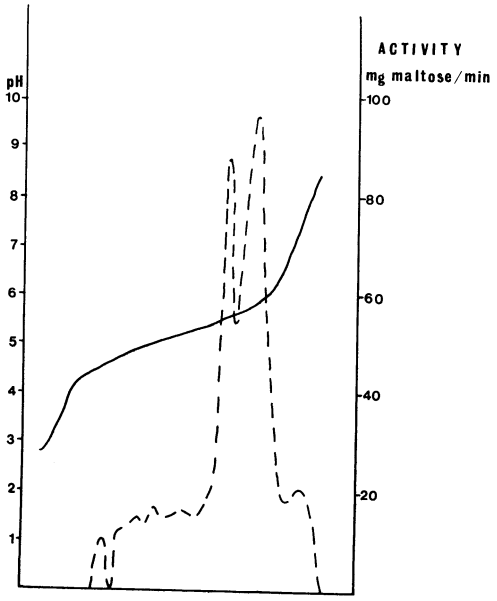

Fig. 3. Isoelectric focusing electrophoresis of: β-amylase from germinated wheat (– – – –); bopH-gradient (_____).

smaller molecular weight than does fraction II (4). In focusing, fraction I has pI values of 4.8 and 4.9, and fraction II has a pI of 5.8.

Immunoelectrophoresis confirms the focusing results of the free β-amylase fractions: fraction I is more acid than fraction II. Fraction I seems to be the main, stable form of free β-amylase, because treatment with reducing agents converts fraction II to fraction I (3). Fraction I also represents 40% of the activity of the free β-amylase in ammonium sulfate precipitation and 50% in focusing (Fig. 1).

The lability of fraction II is observed also when stored free β-amylase is focused: only the peaks at pH 4.8 and 4.9 are left after storage of the extract (Fig. 2). Immunoelectrophoresis and exclusion chromatography confirm the identicality of fraction I and the component left in stored free β-amylase solution.

The very large number of components of free β-amylase obtained when analyzed by ion-exchange chromatography or by focusing—as compared to the two obtained by exclusion chromatography—are not in contradiction, because groups causing different ionic properties in proteins do not necessarily change their molecular weights.

The isoelectric points reported earlier (2) for wheat β-amylase (4.1, 4.4, and 4.9) are a little lower than those obtained in this study (4.8, 4.9, 5.6, and 5.8). It has been stated, however, that in general, pI's obtained from moving-boundary electrophoresis is lower than pI's obtained in stationary electrophoresis, because in moving-boundary experiments buffer ions form complexes with the proteins which are not formed in stationary experiments (8). The isoelectric points obtained from stationary electrophoresis thus represent the isoionic points of the proteins, which are the pI values in the absence of complex-forming ions.

The focusing of bound β-amylase shows that it is the most homogeneous of the β-amylase components tested (Fig. 2). The difference between the pI values of free β-amylase (4.9) and of bound β-amylase (5.0) is very small. Considering the errors occurring in the measurements, one can assume that bound β-amylase coincides with one of the constituents of free β-amylase. The fact that bound β-amylase moves in immunoelectrophoresis like fraction I of free β-amylase, is identical immunologically with it, and has the same molecular weight according to the exclusion chromatography confirms this assumption. It has also earlier been stated that bound β-amylase and free β-amylase behave similarly in ion-exchange chromatography and in disc electrophoresis (5).

β-Amylase from germinated wheat is clearly more alkaline than free β-amylase from mature wheat (Fig. 3). This is confirmed by immunoelectrophoresis (3). Despite its different electrophoretic mobility, β-amylase from germinated wheat is immunologically identical with the free β-amylase (3), and has the same molecular weight in exclusion chromatography as fraction I. This suggests that some changes in the number of ionic groups of β-amylase do occur during germination. This is shown also in ion-exchange chromatography where β-amylase from germinated wheat behaves quite differently than the free β-amylase from mature wheat (5).

The difference between the free and the bound β-amylases from mature wheat is the greater polydispersity of the free enzyme. This could be explained by the presence of some molecules, e.g., peptides or polyphenols, firmly associated with free β-amylase and affecting its ionic properties, and which are released during preparation of the bound β-amylase with reducing agents.

The difference between free β-amylases from mature and germinated wheats is

that β-amylase from germinated wheat is more homogeneous and more basic than β-amylase from mature wheat. It has been shown that new molecules of antigenic enzyme are not synthesized during germination (9). Thus the cause of the changed electrophoretic properties of β-amylases during germination is unknown.

It seems that the main portions of the different β-amylase constitutents studied are identical. They have the same immunological structures and the same molecular weights. The differences in electrophoretic behavior remain to be investigated.

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