Modification of Wheat β-Amylase by Proteolytic Enzymes[1]

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ABSTRACT

Two β-amylase components, I and II, were separated from wheat flour by *N,N*-diethyl aminoethyl-cellulose and CM-cellulose ion-exchange chromatography. Component I contained three main forms and component II contained one form when analyzed by polyacrylamide slab electrofocusing. A papain or a malted wheat extract converted the three β-amylases in component I to five forms with more basic pI values and the one β-amylase in component II to three forms with pI values identical to those arising from degradation of component I. The new β-amylases resulting from proteolytic attack were identical in pI values to β-amylases in germinating wheat, thus indicating that the mechanism of formation of such enzymes is via limited proteolytic degradation.

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Wheat β-amylase is of considerable importance to cereal chemists because of its synergistic role with α-amylase in breaking down starch and complex dextrins during the breadmaking process. As a consequence, substantial research has been done on this enzyme. It was isolated and well characterized by Tipples and Tkachuk (1965, 1966) and Kato et al (1974). Part of the enzyme is bound to glutenin and can be released from the bound form by either thiols or proteolytic enzymes (Kruger 1970, Rowsell and Goad 1962b). Levels of the free form of the enzyme increase during germination. This release, however, occurs not by de novo synthesis (Rowsell and Goad 1962a) but by release of the preexistent bound enzyme. In addition, it has been observed that the major form of the enzyme is converted to an electrophoretically less mobile form during germination, as visualized by polyacrylamide slab electrophoresis at pH 8.9 (Kruger 1972). How this transformation occurs has not been established, but proteolytic enzymes, which also are synthesized as germination proceeds (Kruger 1973, Mounfield 1936), could be responsible.

This article investigates this possibility by examining the effects of both papain and a proteolytic extract from malted wheat on purified wheat β-amylases. Changes were detected by flat bed isoelectric focusing on polyacrylamide gels.

MATERIALS AND METHODS

Straight grade wheat flour, var. Neepawa, was used to isolate the β-amylase. The flour contained negligible α-amylase activity. Papain was a sample from S. B. Penick and Company, New York.

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Purification of β-Amylase

Extraction of β-Amylase. Flour (1,000 g) was slowly added to 2,000 ml of 1% sodium chloride and stirred. After an additional 2-hr stirring at room temperature, the mixture was centrifuged at 30,000× *g* at 4°C for 20 min and the supernatant of approximately 1,370 ml was saved. All subsequent centrifugations were done as described.

Ammonium Sulfate Fractionation. Solid ammonium sulfate to 0.2 saturation was added to the above extract and the mixture was stirred for 3 hr. After centrifugation and after the precipitate was discarded ammonium sulfate was added to 0.65 saturation and the mixture stirred for another 3 hr. The mixture was centrifuged and the precipitate dissolved in cold 0.2*M* Tris-HCl, pH 8.0, containing 0.001*M* EDTA. Before ion-exchange chromatography, the extract was dialyzed against the buffer and any precipitate removed by centrifugation. The final volume of liquid was approximately 200 ml.

Ion-Exchange Chromatography

This process was similar to one described previously (Kruger 1970). Ion-exchange chromatography at basic pH was done on a 2.1 × 45-cm column of microgranular *N,N*-diethyl aminoethyl (DEAE) cellulose equilibrated with 0.2*M* Tris-HCl, pH 8.0, containing 0.001*M* EDTA. Forty milliliters of extract was applied to the column and the proteins were eluted with a linear salt gradient prepared from a two-chambered device. The first chamber contained 800 g of stirring 0.2*M* Tris-HCl, pH 8.0, containing 0.001*M* EDTA, into which flowed the contents of the second container, 800 g of the same buffer containing 0.21*M* sodium chloride.

Ion-exchange chromatography at acidic pH was performed on a 2.1 × 45-cm column of carboxymethyl (CM) cellulose equilibrated with 0.03*M* sodium acetate, pH 4.6, containing 0.001*M* EDTA. The proteins were eluted with a two-chambered gradient vessel

consisting of 800 g of stirring 0.03*M* sodium acetate, pH 4.6, into which flowed 800 g of the same buffer, containing 0.086*M* sodium chloride. Proteins were detected and collected with a LKB Recychrom System. After chromatography, fractions containing β-amylase were concentrated to small volumes using an Amicon ultrafiltration apparatus equipped with a PM-10 membrane.

β-Amylase Activity. β-Amylase activity was determined by measuring the liberation of reducing sugars from 1% soluble starch Merck at pH 4.6 and 23°C using the Neocuproin method of Dygert et al (1965) and automated on a Technicon AutoAnalyzer (Technicon Corp., Chauncey, NY). Activity was expressed in terms of the milligrams of maltose liberated per minute at 23°C.

Polyacrylamide Slab Electrofocusing and Detection of Isozymes. MacGregor's procedure (1976) was followed. Initial experiments between pI 3.5 and 10 indicated that all β-amylase components were present in the pI 4–6 part of the gel, and a pI 4–6 ampholine gradient was therefore used for all later experiments. Amylase isozymes were located by the method of MacGregor et al (1974) using either β-limit dextrin or starch, respectively, in the polyacrylamide plates. Comparison of plates containing β-limit dextrin, which detected only α-amylases, with the starch plates, which detected both α- and β-amylases, indicated the location of the β-amylase isozymes.

RESULTS

Purification of β-Amylases

Ion-exchange chromatography on DEAE-cellulose of an extract of wheat flour purified by ammonium sulfate fractionation indicated presence of two β-amylase components (Fig. 1). This result is similar to that obtained previously (Kruger 1970). Addition of thioglycerol ($10^{-3}M$) to extracts and buffers used in chromatography had no effect on interconversion between the two forms of the enzyme. Thiol did appear to have a stabilizing effect, however, and was added routinely in later purifications.

Components I and II were further purified by chromatography on CM-cellulose and the separations obtained are shown in Fig. 2. There was no further separation into additional components, and the protein and activity profiles mirrored each other very closely. This confirmed studies, using a similar isolation procedure (Kruger 1970), which indicated that two discrete β-amylase components were present in wheat flour, as detected by ion-exchange chromatography and polyacrylamide slab electrophoresis at pH 9.0.

In the present study, analysis by polyacrylamide slab electrofocusing between pH 4 and 6 indicated that component I could be differentiated further into three main components with pI values of 4.65, 4.70, and 4.80, as determined by pH electrode readings on the surface of the gel. Component II consisted of a single component with a pI value of 4.40. Component II also contained traces of the three forms of component I, and the amount appeared to increase slightly after 2–3 weeks of storage, indicating some possible interconversion.

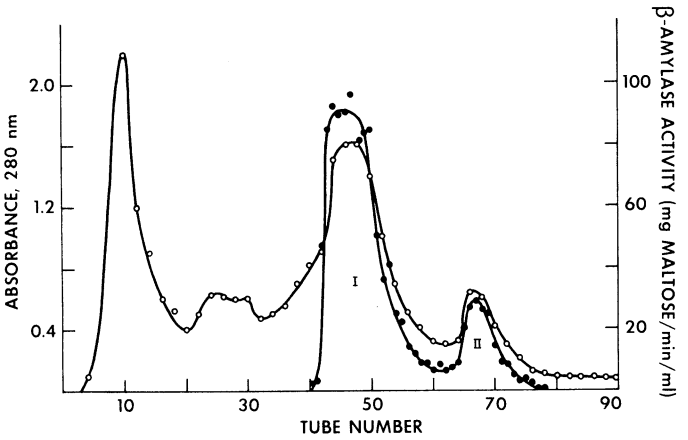

Fig. 1. Chromatography on DEAE-cellulose of a water-soluble β-amylase extract. O = protein, ● = β-amylase activity.

Effect of Papain

One part of a solution containing 100 mg of papain per milliliter of 0.02*M* acetate buffer, pH 4.5, was incubated with nine parts by volume of purified β-amylase component I solution for 3 hr at 37°C. Large changes occurred in the isoelectric focusing profile, as shown in Fig. 3. The three β-amylase forms were converted to five forms with more basic pI values. A heat-treated papain extract and various thiols failed to effect the conversion, indicating that enzymic cleavage was responsible for the change. Detection of enzymic activity indicated that it was retained by all forms. A number of minor forms also were found. Such forms were not evident, however, if the incubation time was reduced, indicating that they did not constitute a large portion of the β-amylase. Incubation of the papain and the β-amylase extract past the 3-hr incubation up to 16 hr at room temperature did not change the protein pattern or decrease the enzymic activity significantly, as visualized on the electrofocused gel, indicating that the converted components were stable species.

Component II from ion-exchange chromatography was also treated with papain. As shown in Fig. 4, three components with more basic isoelectric points resulted, all having enzymic activity. An extract of component I treated with papain is also shown, to illustrate that the three components corresponded in pI value to amylases β-5, β-7, and β-9, which resulted from enzymic degradation of component I.

To determine if the enzymic changes in β-amylase resulted in a noticeable change in molecular weight, β-amylase components I and II and their papain degradation products were passed through a 2.2 × 90-cm column of Biogel P-100. There were no discernible differences in elution volumes, indicating that drastic changes in molecular weight had not occurred.

Effect of Malted Wheat Proteolytic Enzymes

An extract of germinated wheat was prepared by stirring five-day ground germinated wheat with 0.05*M* acetate buffer, pH 4.6 (2:1, w/v), for 2 hr followed by centrifugation at 3,000 × *g*. One part of the extract was mixed with nine parts of β-amylase components I and II, respectively, and incubated as in the papain experiments described. Electrofocusing betwen pH 4 and 6 of the extracts indicated that conversions identical to the papain experiments occurred. A heat-treated wheat extract (90°C for 15 min) did not cause any change in the β-amylases, indicating that proteolytic enzymes were probably responsible for the change. The proteolytic system in wheat is a complex system of exo-and endo-type proteases. Recent studies have shown that one of the major proteolytic enzymes in the endosperm of wheat is a carboxypeptidase (Kruger and Preston 1977). A preparation of the purified carboxypeptidase (Preston and Kruger 1976), however, was tested as above and found to have no effect.

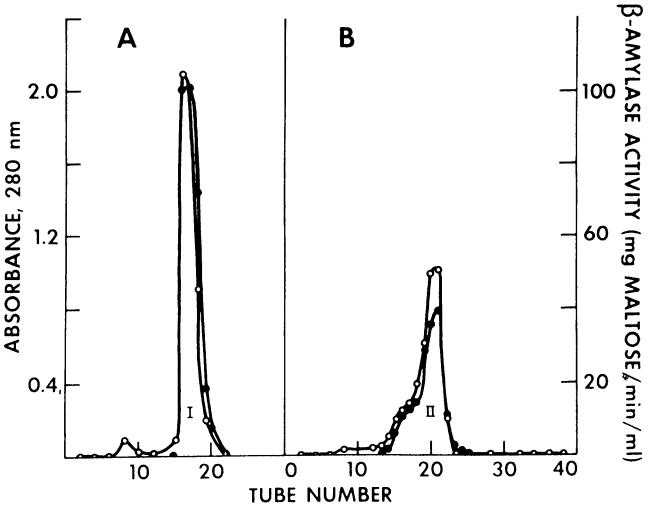

Fig. 2. Chromatography on CM-cellulose of component I (left) and component II from DEAE-cellulose ion-exchange chromatography. O = β-amylase activity.

Relationship of β-Amylase Components

Results of using polyacrylamide slab electrophoresis at alkaline pH have shown that the β-amylase components in ungerminated wheat kernels disappear during the germination process, and electrophoretically less mobile forms appear (Kruger 1972). In the present study, isoelectric focusing between pH 4 and 6 was used to follow the changes in β-amylases during germination. In ungerminated samples, β-amylase components 1–4 were present, and α-amylase activity was not detectable. As germination proceeded, α-amylase formed, as did a number of β-amylase compo-

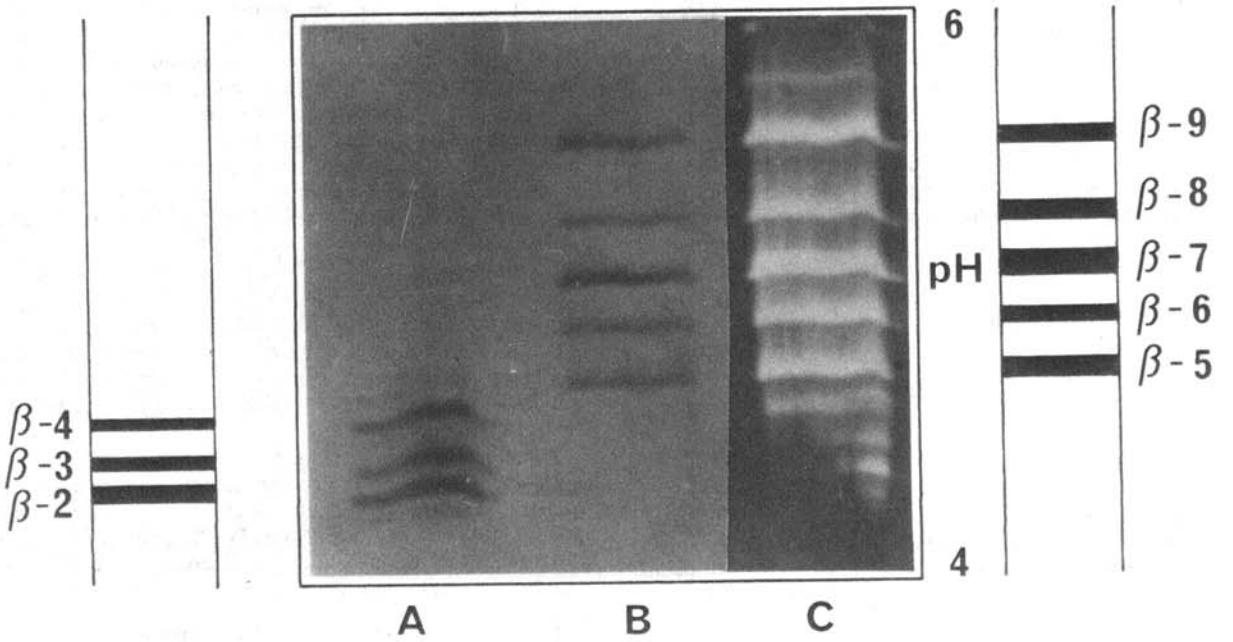

Fig. 3. Isoelectric focusing on a polyacrylamide slab between pH 4 and 6 of **A)** β-amylases present in component I, as detected with a protein stain; **B)** β-amylases arising from papain degradation of component I, as detected with a protein stain; and **C)** β-amylases arising from papain degradation of component I, as detected enzymatically with starch substrate. Schematics for the transformation are shown on the left and right of the electrofocused gels.

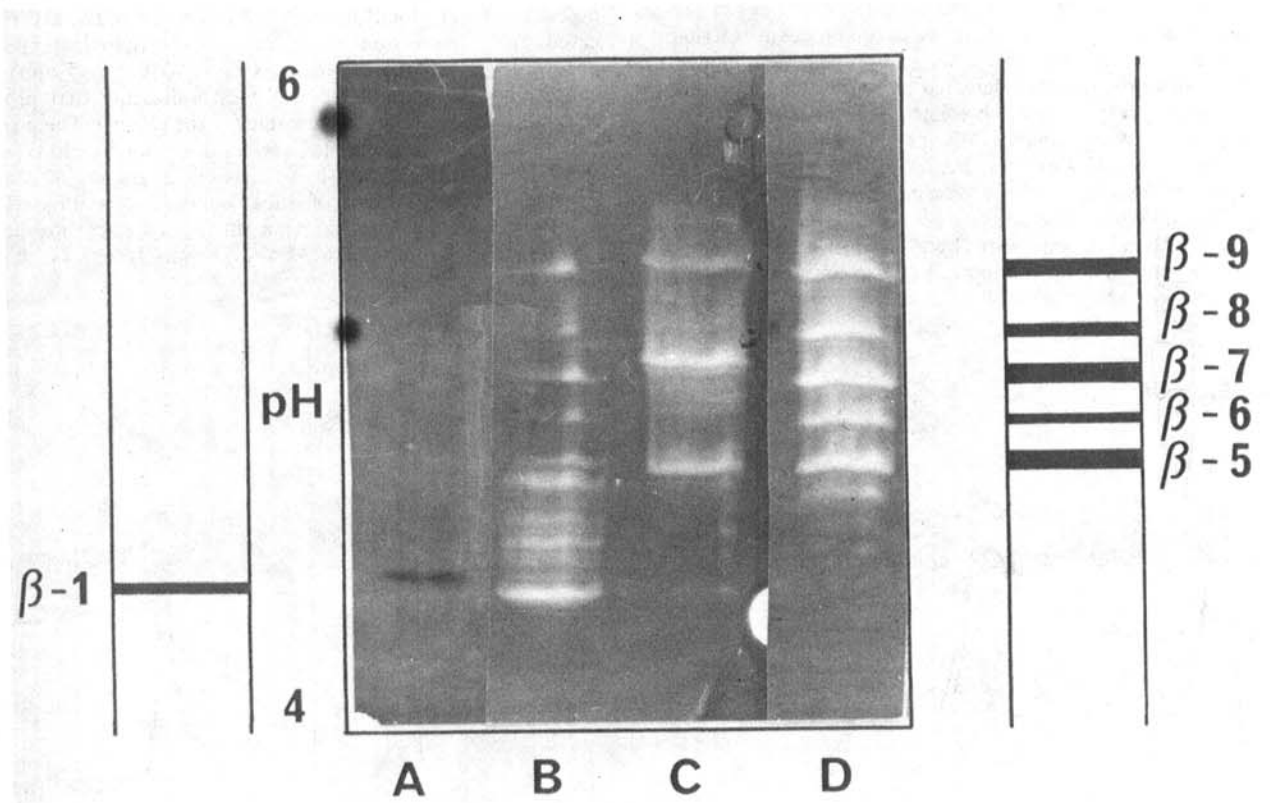

Fig. 4. Isoelectric focusing on a polyacrylamide slab between pH 4 and 6 of **A)** β-amylases in component II, as detected with a protein stain; **B)** β-amylases in component II, as detected enzymatically with starch; **C)** β-amylases arising from papain degradation of component II, as detected enzymatically with starch substrate; and **D)** β-amylases arising from papain degradation of component I, as detected enzymatically with starch substrate. Schematics for the transformation are shown on the left and right of the electrofocused gels.

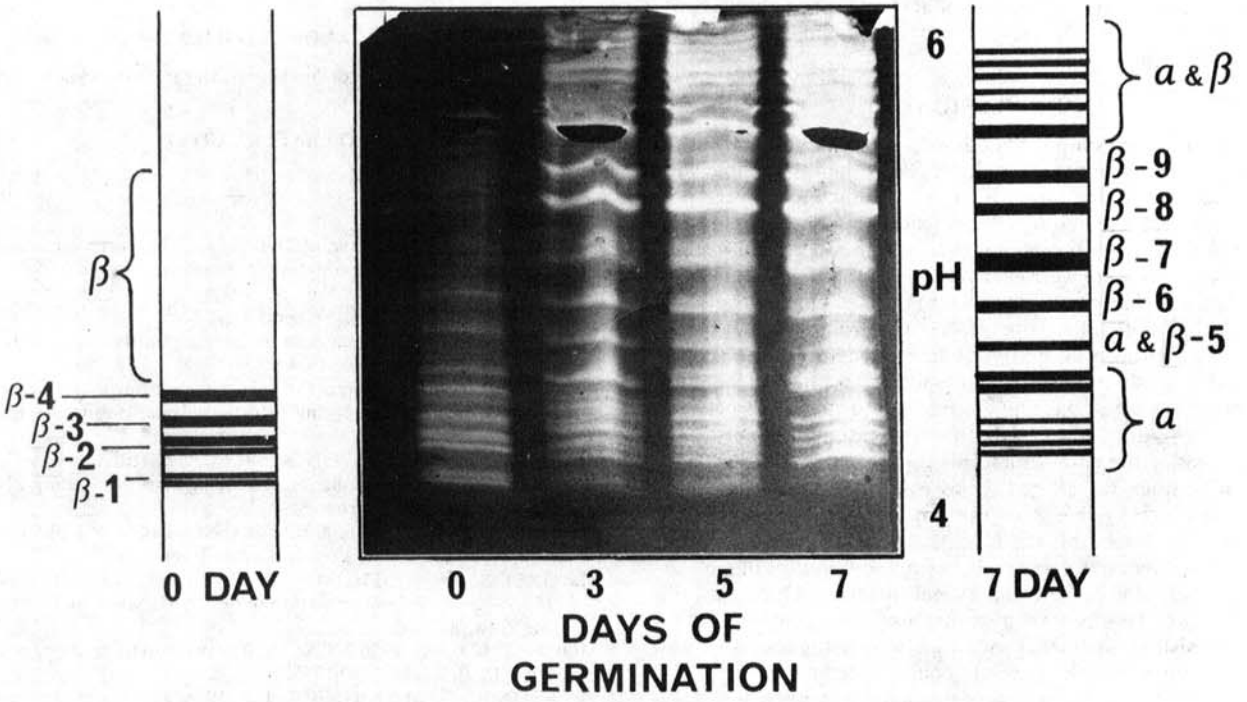

Fig. 5. Isoelectric focusing on polyacrylamide slabs of amylases in germinating wheat at 0, 3, 5, and 7 days, as detected with a starch substrate plate. A β-limit dextrin substrate plate was used to differentiate α- and β-amylases. Schematics for 0 and 7 days' germination are shown on the right and left respectively.

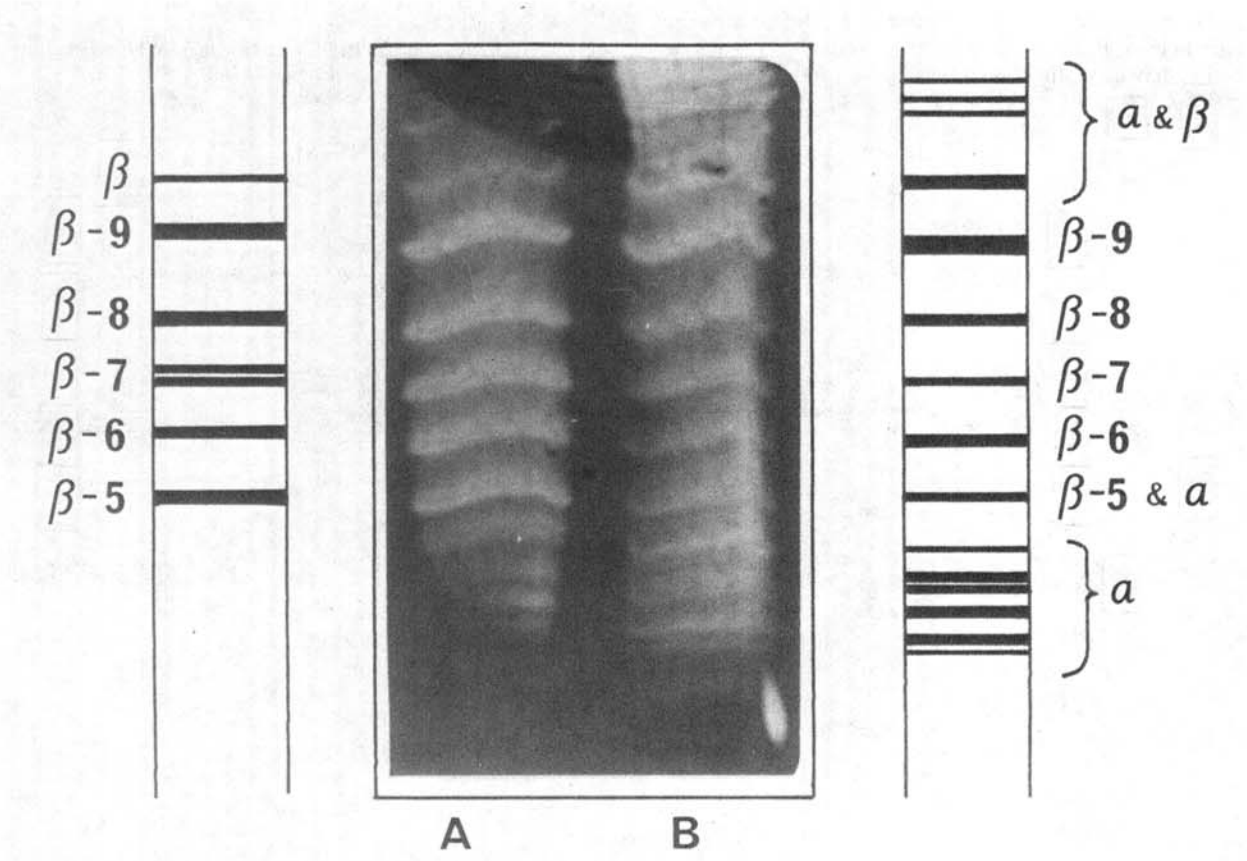

Fig. 6. Comparison of amylases resulting from papain degradation of component I (left) with those present in a 7-day sample of germinated wheat (right) as detected with a starch plate.

nents with higher pI values (Fig. 5).

Extracts of β-amylase components I or II that had been incubated 3 hr with papain or germinated wheat protease were compared with an extract of kernels germinated for seven days. As illustrated in Fig. 6, the five main products of enzymic breakdown are identical to five of the major β-amylase components formed during germination.

DISCUSSION

Isoelectric focusing on polyacrylamide slabs is able to achieve a better resolution of β-amylase multiple forms than is possible with ion-exchange chromatography. Four main components were present that were not affected by the presence of thiol. Upon incubation with proteolytic enzymes, however, changes occurred in their molecular structure that were evidenced by changes in their isoelectric points. Such changes did not result in loss of enzymic activity, indicating that the active site of the molecule was not being destroyed. Futhermore, even after 16 hr of incubation with proteolytic enzyme, five discrete components were present, rather than a multidisperse system of varying charge. The distribution between the altered forms did not vary with time, indicating that indiscriminant cleavages of the parent molecule did not occur. Futhermore, conversion between the altered forms could not be distinguished, indicating that irreversible changes from the parent molecules had taken place. In any event, the changes in molecular weight that occurred must have been very small, because differences in molecular weight could not be detected by gel filtration. Once enzymic cleavages occur, therefore, the remainder of the molecule must be extremely resistant to further cleavage, which suggests that the main part of the molecule is tightly bound and that ony a limited amount of enzyme-susceptible peptide chain protrudes.

The identity found between the β-amylase components degraded by proteolytic enzymes and the β-amylase components present in germinated wheat points to the mechanism of formation of the germinated components. As germination proceeds, the "free" and slowly released "bound" β-amylases are degraded by the newly synthesized proteolytic enzymes to components with higher pI values. The reason that this change is required by the germinating kernel is not known. It may simply be a by-product of proteolytic enzyme action, having no functional reason. Alternately, the structural change may make the β-amylase more efficient in breaking down starch and oligosaccharides. Comparison of properties such as K_m and V_M of ungerminated and germinated β-amylase components should establish if this is the case.

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