Association of Bound Beta-Amylase with Protein Bodies in Barley[1]

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ABSTRACT

Association of bound beta-amylase activity with the protein bodies of barley has been detected by immunochemical analysis. The enzyme was released from its bound state with beta-mercaptoethanol. The free beta-amylases of barley and the bound enzyme associated with the protein bodies have the same antigenic structure, as shown by double diffusion.

The early investigations of Sandegren and Klang (1) and Pollock and Pool (2) established that there are two forms of beta-amylase in the barley grain: 1) free beta-amylase, extractable from the barley by salt solutions, and 2) latent beta-amylase, extractable only with reducing agents. Nummi et al. (3) showed that free beta-amylase from barley consists of at least four different components of different molecular size; the enzyme with the smallest size (A_1) is the stable form.

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The latent, or bound, beta-amylase, when extracted with reducing agents, has the same molecular size as the basic unit (A_1) of the free enzyme.

The same relationship was shown for the sole beta-amylase found in barley malt, the free form. The free beta-amylases of barley and malt and the bound form of barley all have the same antigenic structure (4), but there is some heterogeneity apparent in their electrophoretic migrations. Beta-amylase from malt migrates more slowly than do those from barley.

The recent isolation of intact protein bodies from ungerminated barley showed two acid hydrolases, phytase and protease, associated with these particles (5). BAPA-ase[3], a proteolytic enzyme with a pH optimum at 8.6, was absent. The finding that only acid hydrolase activity may be associated with the particles suggested a possible lysosomal-like nature of the protein bodies. De Duve (6) found 12 to 14 acid hydrolases in animal lysosomes. There have been several reports on association of lysosomal enzymes with spherosomes (7,8), and protein bodies (9,10) of seeds, but until now no single plant organelle has been found to contain all of the enzymes found by De Duve in the animal lysosomes.

In our continuing investigations for possible lysosomal enzymes associated with barley protein bodies we have employed the IEA technique of Grabar and Williams (11) to characterize these proteins. In this report we present evidence for the association of the bound beta-amylase with isolated protein bodies. The enzyme was released from its bound state with reducing agents in the same manner that it is released in crude barley extracts (3).

MATERIALS AND METHODS

Seed Source

Barley seeds, two-row *Hordeum vulgare*, var. Kenia, and malt from Kenia barley were a gift from the Carlsberg Brewery Research Laboratory, Copenhagen. All samples were stored at 4°C.

Isolation of Protein Bodies

Intact protein bodies were isolated as previously described (5).

Extraction of Protein from Tissues

One gram of seed material was ground in a mortar and extracted with 3.0 ml. of 0.1M phosphate buffer, 0.4M NaCl, pH 6.6. The extracts were centrifuged for 20 min. at 20,000 × g and the supernatant solutions were used directly for the immunoassays.

Specific Enzyme Characterization

After the immunoprecipitates had been washed with NaCl and water, the lines containing amylase activity were characterized by the starch-iodine test according to Daussant (12).

Release of Bound Beta-Amylase with Beta-Mercaptoethanol

To determine if the amylase activity associated with the protein bodies was the

[3]Abbreviations used: IEA, immunoelectrophoretic analysis; BAPA-ase, the peptide hydrolase which acts on alpha-N-benzoyl-DL-arginine-p-nitroanilide; EA, electrophoretic analysis.

bound beta-amylase, the isolated protein bodies were treated as follows: 5 mg. of isolated protein bodies were ground in a mortar in the cold with 0.250 ml. of the phosphate buffer. Half of this extract was treated for 2 hr. with beta-mercaptoethanol (final concentration was 0.02M), and the other half was used as a control. The extracts were applied to immunoassays without centrifugation.

Preparation of Immunsera

Serum against total barley proteins was prepared by subcutaneous injections of the protein into rabbits seven times at weekly intervals (13). Fourteen days after the last injection the animals were bled. The antigens for injection consisted of a crude freeze-dried albumin-globulin preparation in Freund's complete adjuvant. The specific anti-alpha-amylase serum was a gift from J. Daussant, The Pasteur Institute, Paris. Antisera were stored at -20°C. until used.

Immunochemical Analysis

IEA was carried out according to Grabar and Williams (11) as modified by Scheidegger (14) with the LKB apparatus. The proteins were first separated by electrophoresis in a 1.25% agarose gel buffered with 0.05M veronal, pH 8.2, and electrophoresis was carried out for 6 hr. at 4°C. in a gradient of about 6 v. $cm.^{-1}$ For the Abelev analyses by double diffusion (15), the same gels and buffers were used. The immunodiffusions were complete after 3 days at 4°C. Where two bands join to form a single line, it signifies antigenic identity between the two proteins forming the precipitates. When the lines cross, however, the proteins in question differ in their antigenic structures.

Preparation of Samples for Electron Microscopy

Pieces of aleurone layer from mature barley seeds were fixed in glutaraldehyde, then stained in 2% potassium permanganate in water for 30 min. The tissues were then washed well in water, dehydrated, and embedded for electron-microscopic analysis as described earlier (5).

RESULTS

IEA of proteins from the isolated protein bodies, followed by specific amylase staining (Fig. 1, A), showed that some form of amylase activity was associated with these particles. Subsequently, the isolated protein bodies were treated with beta-mercaptoethanol, a sulfhydryl-reducing agent. The reduced and original solutions were compared by simple electrophoretic analysis (Fig. 1, B) and by IEA (Fig. 1, C and D). Figure 1 (B and D) illustrates the effect of beta-mercaptoethanol on the bound enzyme activity. The electrophoretic mobilities of the beta-amylases from barley, reduced protein bodies, and barley malt are illustrated in the immunoelectrophoretic diagrams, Fig. 1, C, D, and E. The released amylase from protein bodies migrates more slowly than does the barley beta-amylase, but faster than the slowest of the two amylases in barley malt. It is evident, however, that all are localized in the same zone of migration. According to the antiserum used (anti-barley), the two lines with amylase activity detected in barley malt (Fig. 1, E) are both beta-amylases. The mobility of the long, slower-moving arc suggests that this one is the same as the beta-amylase described earlier by Daussant (4, 12). Two

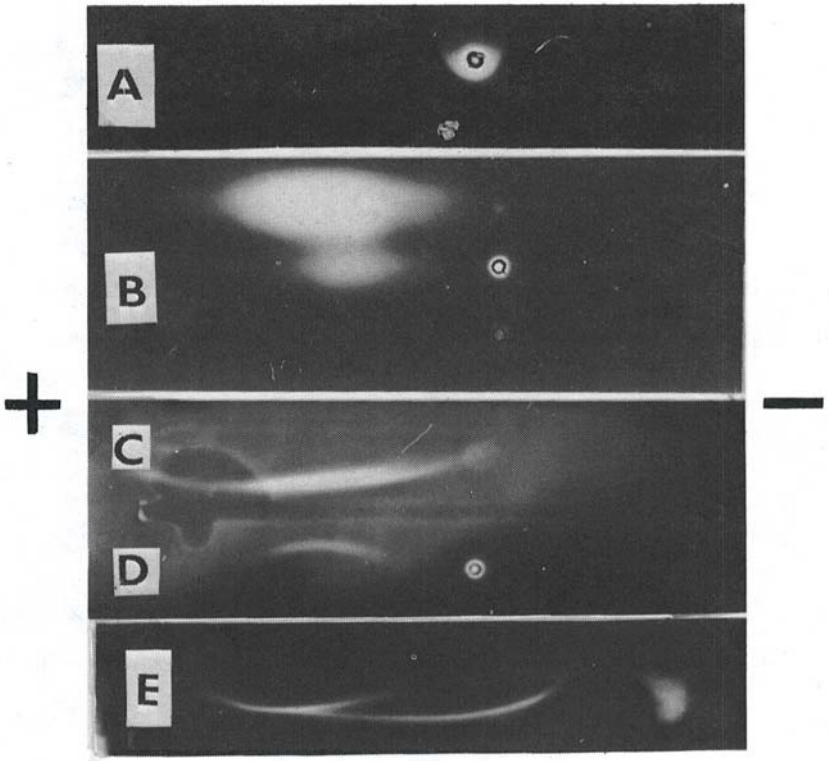

Fig. 1. Characterization of beta-amylases. Electrophoretic and immunoelectrophoretic analysis (IEA) of isolated protein bodies before and after treatment with beta-mercaptoethanol compared to barley and barley malt. A, IEA of protein bodies before reduction. B, EA comparison of dormant barley (upper well), reduced protein bodies (middle well), and untreated protein bodies (lower well). C, D, E, IEA of amylases of C, barley; D, reduced protein bodies, and E, barley malt. Immunserum anti-total barley is used for IEA of A and C to E.

components in barley malt with this activity have been reported earlier by Djurtoft (16).

Figure 2 demonstrates clearly that the beta-amylase released from the isolated protein bodies and the beta-amylases from the barley grains have the same antigenic structure, since no cross-reaction can be observed between the proteins (Fig. 2, A).

While this method used to identify amylase activity is not specific for beta-amylases only, it is well established that ungerminated, mature barley does not contain alpha-amylase activity (12). In addition, the antisera against these seed proteins do not contain anti-alpha-amylase antibodies. As further proof for the absence of alpha-amylase activity, the reduced protein body preparation was tested against a specific anti-alpha-amylase serum. The results, in Fig. 3, show that no line could be detected for the alpha-form (lower part of the picture).

An electron micrograph of ungerminated barley aleurone layer is shown in Fig. 4. The photograph shows several protein bodies surrounded by rings of

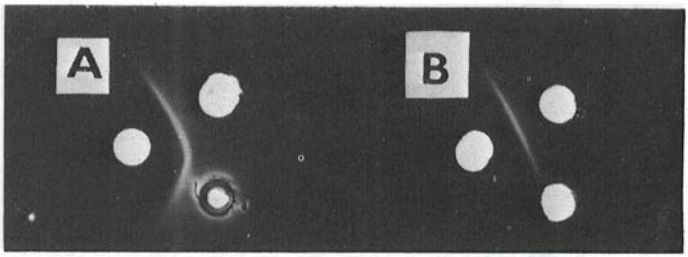

Fig. 2. Double diffusion according to Abelev. A and B, upper right wells: barley; A and B, left center wells: anti-total barley; lower right wells: in A, reduced protein bodies; in B, phosphate buffer. The lines are characterized by amylase activity.

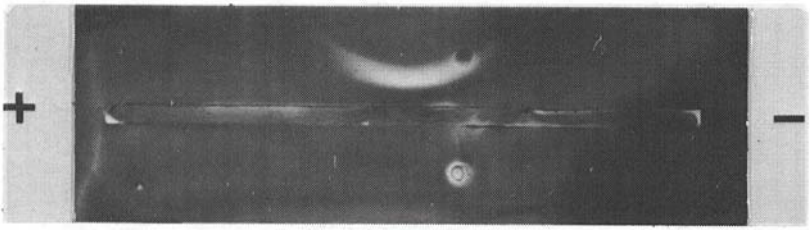

Fig. 3. IEA characterization of alpha-amylase with specific anti-alpha-amylase serum. Upper well: barley malt. Lower well: reduced protein bodies.

spherosomes. Adjacent to some of the protein bodies are electron-dense structures which stain heavily in $KMnO_4$.

DISCUSSION

Finding of beta-amylase activity associated with protein bodies is unusual, because the substrate for beta-amylase is starch, and here it seems to be bound to a protein storage particle. This naturally raises the question of the source of substrate; starch grains are generally assumed to be present in the endosperm only. However, the very dense particles next to some protein bodies (Fig. 4) could possibly be starch grains, or phytic acid deposits.

Buttrose (17) has made a thorough study by electron microscope of $KMnO_4$-stained starch grains in barley endosperm. He found that $KMnO_4$ treatment appeared to preserve starch in the electron beam to some extent, giving the starch a much higher contrast. It then showed a distinct spongy texture. (This spongy texture would probably be brittle when sliced by the microtome, and could result in uneven "waves" through the $KMnO_4$-stained areas rather than uniform slices, such as those in Fig. 4.) He also suggested that barley proplastids might be the synthesizing center supplying starch granules. Therefore, it is conceivable that these dense structures could be small starch grains in the aleurone layer.

The other possibility, that these are phytic acid deposits, likewise has some basis for support. Phytic acid has been reported to bind to beta-amylase (18) to form heterogeneous aggregates, and the phytase which acts on this substrate is also

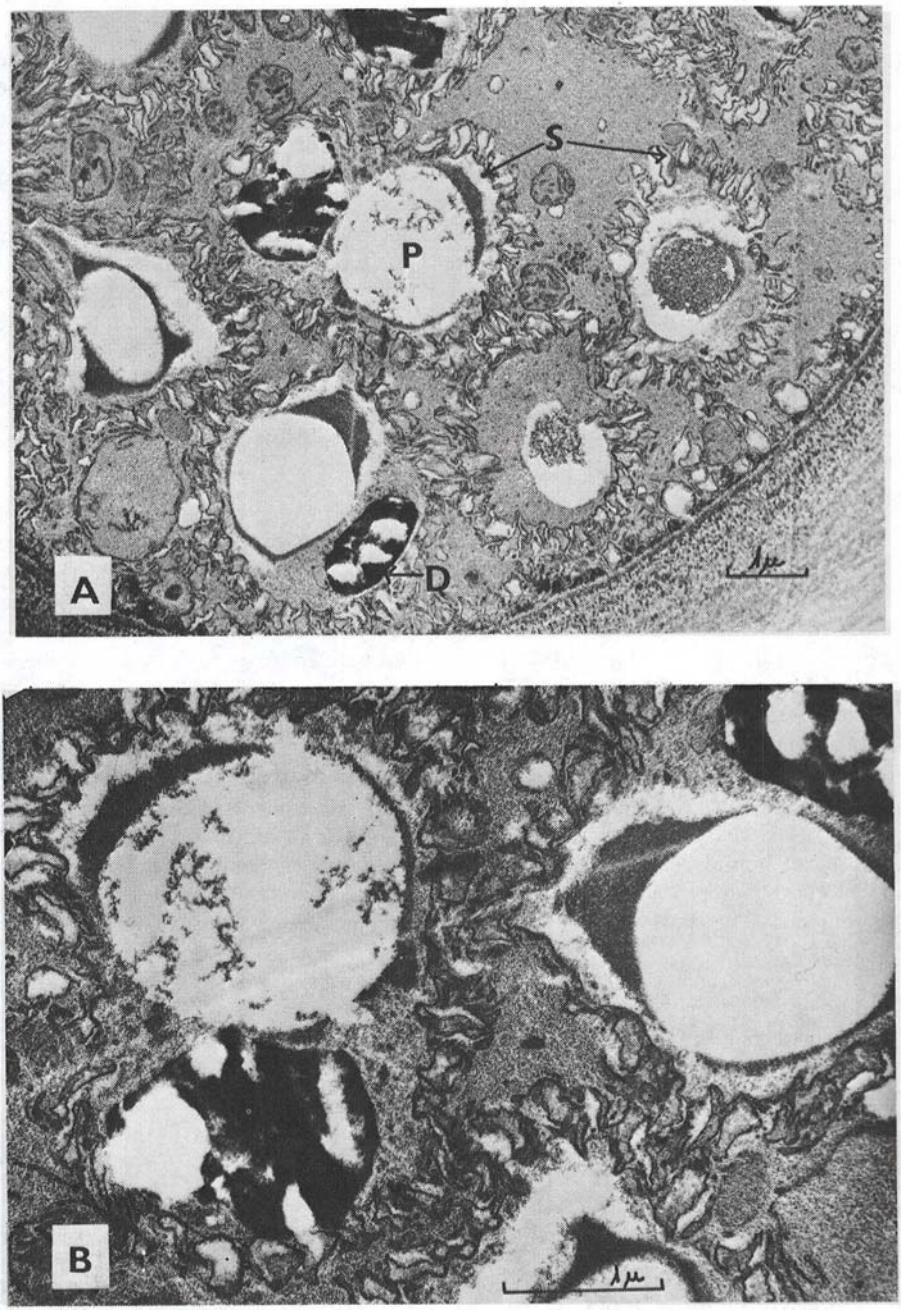

Fig. 4. Electron micrograph of barley aleurone layer stained with $KMnO_4$. A, low-power magnification showing protein bodies (P), dense particles (D), surrounded by spherosomes (S). B, higher magnification of one area of A.

associated with the protein bodies (5). Thus, there appears to be some evidence to support both possibilities.

The protein bodies isolated earlier (5) had 25 to 30% carbohydrate, but the form was unknown. It does not appear to be adhering starch grains, despite the possible proximity of starch grains to protein bodies suggested in Fig. 4, since careful examination of several preparations of protein bodies in the electron microscope (5) failed to show contaminating starch grains. This associated carbohydrate might also be considered as potential substrate for an amylase.

The fact that beta-amylases of larger molecular size in barley disappear during germination to form enzymes of a smaller basic unit, and the bound beta-amylase, when treated with reducing agents, retains the same basic size, has led to the proposal of a "splitting enzyme" system in germinating barley, which releases the bound enzyme without destroying its activity (3). Sulfhydryl plant proteases such as papain, ficin, and bromelain had the same effect on the larger beta-amylases as reducing agents or malting. Protein bodies of barley also contain a sulfhydryl protease (5), which could serve as the natural "splitting enzyme" proposed by Nummi et al. (3).

The mobility of the amylases from the reduced protein bodies, when compared to barley endosperm or malt (Fig. 1), shows that the enzymes are located in the same zone of electrophoretic migration. The Abelev double-diffusion test (Fig. 2) indicated that the enzymes in barley endosperm and reduced protein bodies are antigenically identical. While this evidence is not sufficient for it to be said that the protein bodies are the only sites of the bound beta-amylase, it does suggest these particles as perhaps a major site. The two lines with beta-amylase activity in Fig. 1, E, are noteworthy, since Nummi et al. (3) have found malt to contain only beta-amylase of the same molecular size as the basic unit of barley beta-amylase.

Lehninger (19) has described sophisticated enzyme systems not readily diffusible within the cell cytoplasm as "supramolecular assemblies" which are found attached to different types of membranes or to particulate elements. This binding of bound beta-amylase to protein bodies of barley, and the previous report of phytase and protease associated with these organelles (5), suggest that this system may be part of such a supramolecular organization as described by Lehninger.

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