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Characterization of the α-Amylases Synthesized by Aleurone Layers of Himalaya Barley in Response to Gibberellic Acid

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ABSTRACT

The gibberellic acid (GA_3)-induced α-amylases from the aleurone layers of Himalaya barley (*Hordeum vulgare* L. cv Himalaya) have been purified by cycloheptaamylose-Sepharose affinity chromatography and fractionated by DEAE-cellulose chromatography. Four fractions (α-amylases 1–4) were obtained which fell into two groups (A and B) on the basis of a number of characteristics. Major differences in serological characteristics and in proteolytic fingerprints were found between group A (α-amylases 1 and 2) and group B (α-amylases 3 and 4). Also, the lag time for appearance of group B enzyme activity was longer than for group A, and the appearance of group B required higher GA_3 levels than group A. The components of each group behaved similarly, although differences in proteolytic fingerprints were detected.

These results together with those from other studies indicate that GA_3 differentially controls the expression of two α-amylase genes or groups of genes giving rise to two groups of α-amylases with many different properties.

A multiplicity of α-amylase isozymes has been detected in germinating barley grain and in isolated barley endosperm treated with GA_3. The numbers of α-amylases reported vary from 2 to 18 which represents the enzymes detected using different techniques in whole grain, de-embryonated grain, and isolated aleurone layers of different species of *Hordeum* and of different cultivars of the same species (1, 2, 8, 14, 16, 17, 20, 27). Little is known of the significance of the multiplicity of α-amylases but they are known to differ between different genotypes in a number of ways. Heterogeneity in charge characteristics has been shown by gel electrophoresis, ion exchange chromatography, and isoelectric focusing. Also, within individual cultivars of barley, differences have been found in pH optima; in sensitivity to sulfhydryl reagents, chelating agents, and low pH; in requirement for calcium ions; and in serological type (1, 2, 8, 13, 14, 18, 21, 27, 28).

In this report, we extend our previous studies (13, 14) on the α-amylases synthesized and secreted by isolated aleurone layers of *Hordeum vulgare* L. cv Himalaya following GA_3 treatment. By partial fractionation of purified α-amylase, we have obtained evidence which indicates that the heterogeneity is associated with

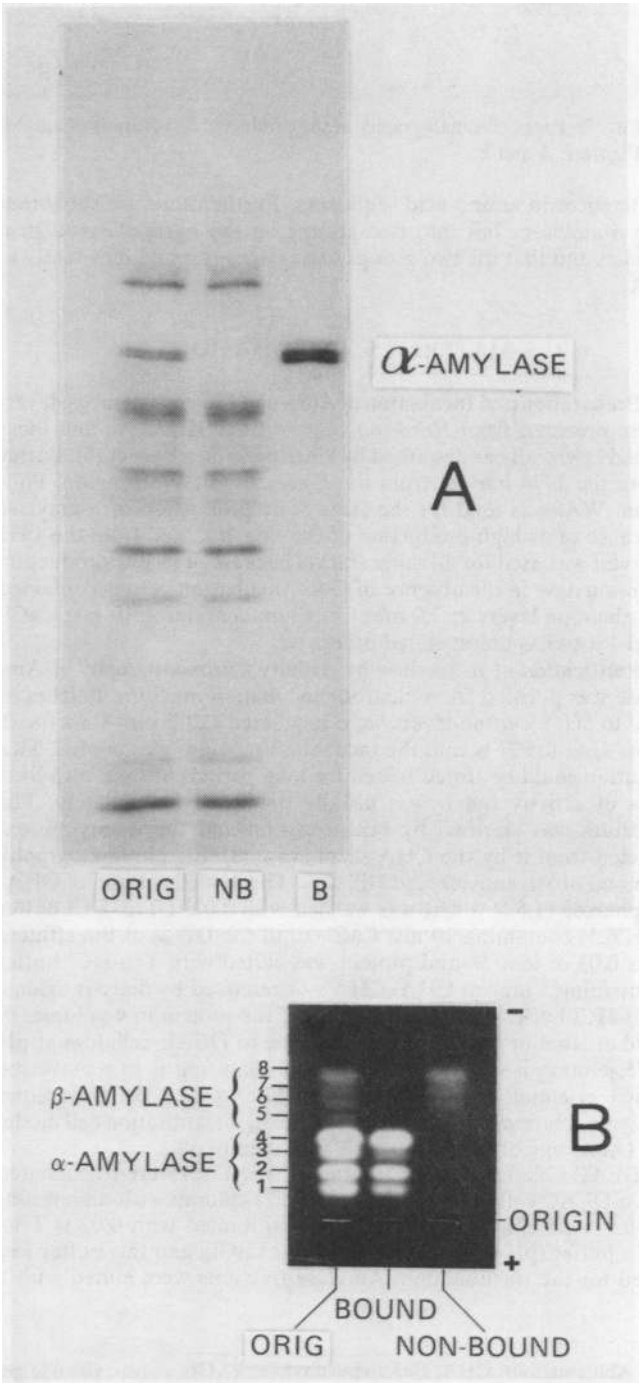

FIG. 1. Purification of α-amylase by CHA-Sepharose affinity chromatography. A, The total protein released from barley aleurone layers (ORIG) was passed through a CHA-Sepharose column, the bound (B) and non-bound (NB) fractions were recovered and fractionated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R. B, The same protein fractions as in A were subjected to agar gel electrophoresis, and the amylases were detected as described in "Materials and Methods."

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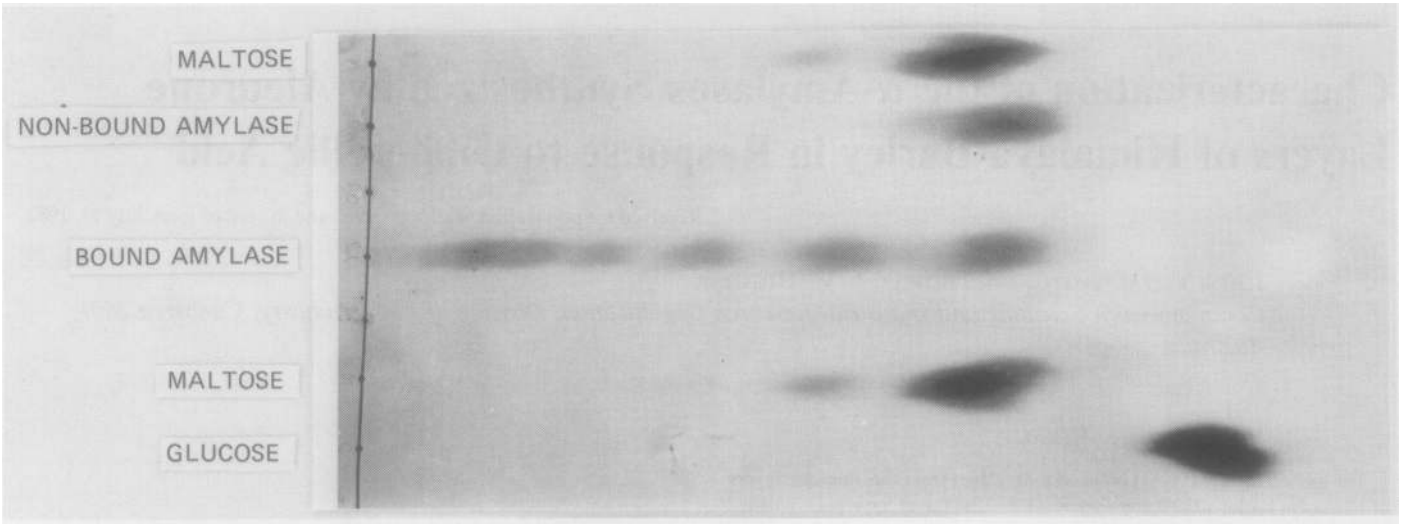

FIG. 2. Paper chromatography of the products of hydrolysis of amylose by the CHA-Sepharose bound and non-bound amylase fractions referred to in Figure 1, A and B.

differences in amino acid sequences. Furthermore, we show that the α-amylases fall into two groups on the basis of serological studies and that the two groups of enzymes respond differently to GA_3.

MATERIALS AND METHODS

Preparation and Incubation of Aleurone Layers. Aleurone layers were prepared from *Hordeum vulgare* L. cv Himalaya and incubated essentially as described by Chrispeels and Varner (5). Barley from the 1974 harvest from the University of Washington, Pullman, WA, was used for the large scale production of α-amylase because of its high production of enzyme, but seed from the 1972 harvest was used for all other studies because of its low production of α-amylase in the absence of GA_3. Incubation vessels contained 20 aleurone layers in 2.0 ml of medium containing 10 mM $CaCl_2$ and 1 μM GA_3 unless stated otherwise.

Purification of α-Amylase by Affinity Chromatography. α-Amylase was purified from aleurone incubation medium. Batches of 400 to 500 aleurone layers were incubated (20 layers/flask) with 1 μM GA_3 for 27 h, and the incubation medium was pooled. This solution could be stored frozen for long periods of time with little loss of activity but it was usually processed immediately. The medium was clarified by centrifugation and the α-amylase extracted from it by the CHA[1]-Sepharose affinity chromatography method of Silvanovich and Hill (25). The loaded column of CHA-Sepharose (1.5 × 4 cm) was washed with 0.02 M Tris-HCl buffer (pH 6.5) containing 10 mM $CaCl_2$ until the OD_{280} of the effluent was 0.03 or less. Bound protein was eluted with Tris-HCl buffer containing 5 mg/ml CHA. CHA was removed by dialysis against Tris-HCl buffer or by precipitation of the protein in 4 volumes of cold ethanol or by binding the enzyme to DEAE-cellulose at pH 7.75, eluting it with 0.4 N NaCl, and dialyzing it to remove the NaCl essentially as described by Okita *et al.* (23). Sometimes enzyme solutions were concentrated in an ultrafiltration cell model 12 (Amicon Corp., MA) using PM10 membranes.

DEAE-Cellulose Chromatography. Amylases were fractionated on a DEAE-cellulose (Whatman DE52) column with dimensions of 0.9 × 25 cm. The column was equilibrated with 0.02 M Tris-HCl buffer (pH 6.5) containing 10 mM $CaCl_2$, and this buffer was used for the fractionation. Amylase fractions were eluted with a

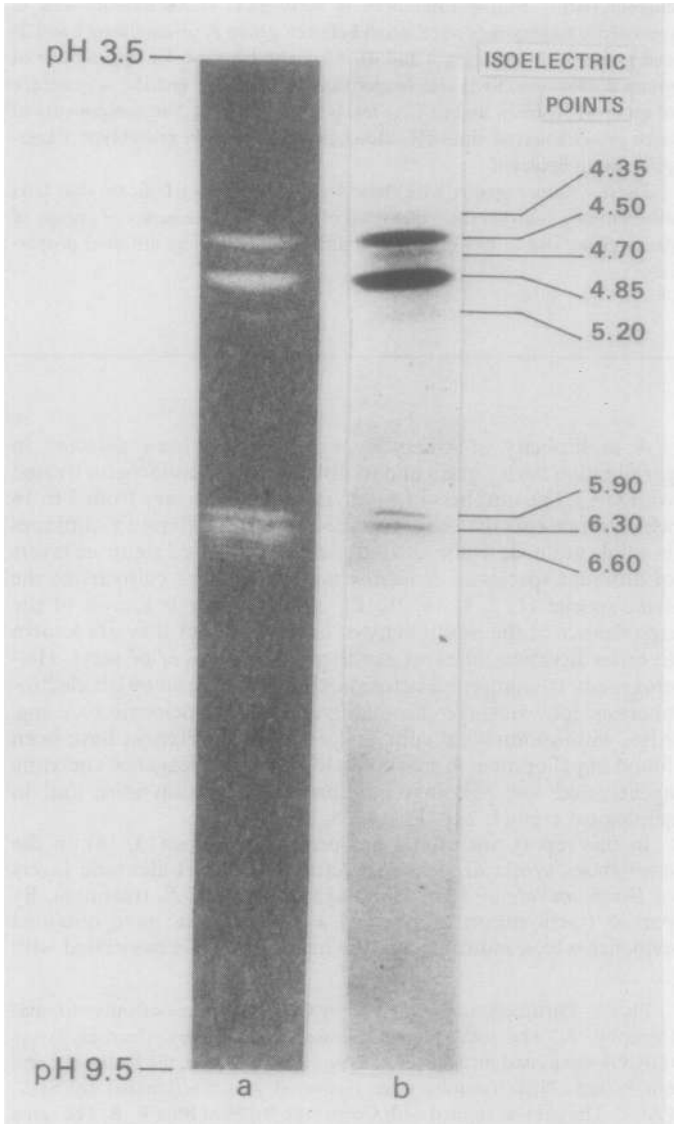

FIG. 3. IEF of α-amylase purified by CHA-Sepharose chromatography. After detection of the zones of α-amylase activity with lyosin red-impregnated paper (a), the gel was fixed and stained with Coomassie Brilliant Blue R (b).

[1] Abbreviations: CHA, cycloheptaamylose; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; SAP, *Staphylococcus aureus* protease.

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linear 0 to 0.4 M NaCl gradient. Buffers were prepared and columns equilibrated and run at room temperature (22°C). Amylase activity was monitored by the assay of Chrispeels and Varner (5).

Purification of α-Amylase Components by DEAE-Cellulose Chromatography. α-Amylase, which had been purified by CHA-Sepharose affinity chromatography, was dialyzed against the DEAE-cellulose chromatography buffer and then fractionated by DEAE-cellulose chromatography. Each of the α-amylase components was purified by repeated chromatography on DEAE-cellulose. The central fractions of each component from the first cycle were pooled and dialyzed against DEAE-cellulose chromatography buffer to reduce the salt concentration. Each preparation was then subjected to DEAE-cellulose chromatography as before. Most of the activity in each preparation eluted as a single peak, the central fractions of which contained only one α-amylase when examined by agar gel electrophoresis. α-Amylase 3 was depleted during extensive washing of the loaded CHA-Sepharose column and consequently CHA-purified α-amylase preparations were often very low in this component. It was usually purified by first fractionating the total incubation medium proteins by DEAE-cellulose chromatography as shown in Figure 4A. The central fractions of α-amylase 3 were repurified on DEAE-cellulose, and the α-amylase in the central fractions was extracted by CHA-Sepharose affinity chromatography using a brief column-washing procedure.

Amylose Hydrolysis. The products of amylose hydrolysis by various enzyme solutions were examined as described previously (14) using the paper chromatography method of Robyt and French (24).

Electrophoretic Techniques. The procedures for agar gel electrophoresis and detection of amylase activity on these gels have been described previously (14). SDS-PAGE was performed on gradient gels containing 12.5 to 20% acrylamide unless otherwise stated using the methods of Laemmli and Favre (15) as modified by Spencer *et al.* (26), and protein bands were stained with Coomassie Brilliant Blue R. IEF was performed in a LKB Multiphor apparatus on LKB-prepared gels of pH range 3.5 to 9.5. After amylase detection (see below), the gels were fixed and stained according to the maker's instructions. Isoelectric points of α-amylase were determined with the aid of a mixture of proteins of known isoelectric points (Pharmacia, Uppsala, Sweden).

Detection of α-Amylase Activity on IEF Gels. Amylase activity on IEF gels was detected using the lyosine red-impregnated paper method of Burdett *et al.* (4). A 1.5 or 2% lyosine red (Reliable Chemical Co., St. Louis, MI) solution was used to prepare the paper in order to enhance the contrast between the white bands of amylase activity and the pink background. When the white bands had developed sufficiently (usually about 10 min), the paper was flooded with 0.1 N HCl to inactivate amylase and peeled from the IEF gel. The paper was then allowed to dry overnight on an absorbent backing. The IEF gel was fixed immediately after α-amylase detection and stained to detect protein bands as described above.

Peptide Mapping of Purified α-Amylase. Peptide mapping of the purified α-amylase fractions was carried out using the procedure of Cleveland *et al.* (6) as described by Spencer *et al.* (26). A number of enzyme concentrations were used in each case. We

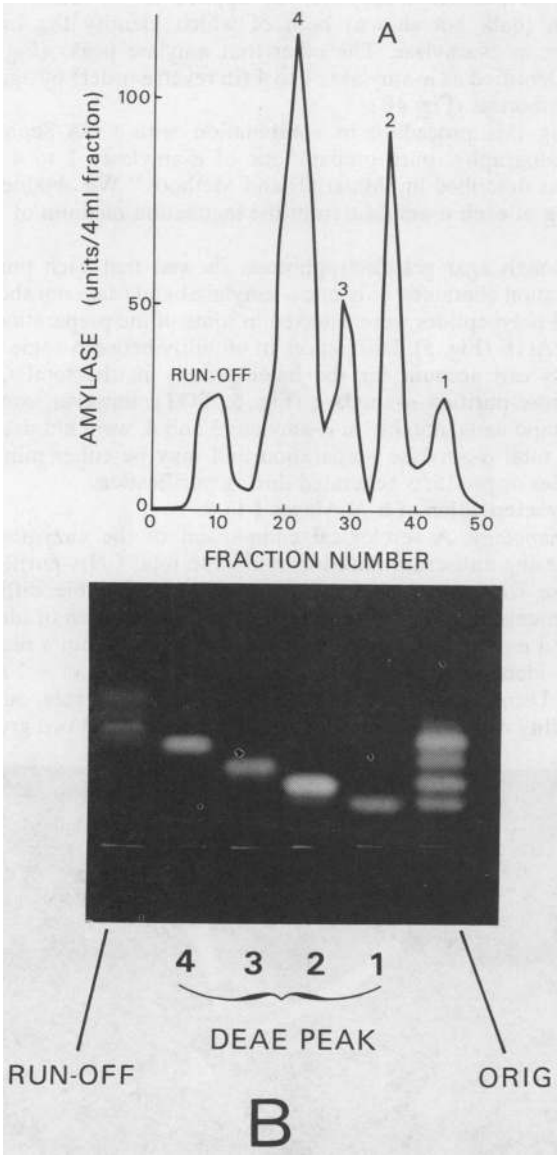

FIG. 4. Fractionation of total amylase in the aleurone incubation medium by DEAE-cellulose chromatography. A, The DEAE-cellulose profile of amylase activity. Total incubation medium protein was applied to the column. The first peak (run-off) emerged from the column before the salt gradient was begun. The next four peaks were identified as α-amylases 1 to 4 as shown in B. B, The central fractions of each peak of amylase activity shown in A were subjected to agar gel electrophoresis, and the zones of amylase activity were detected.

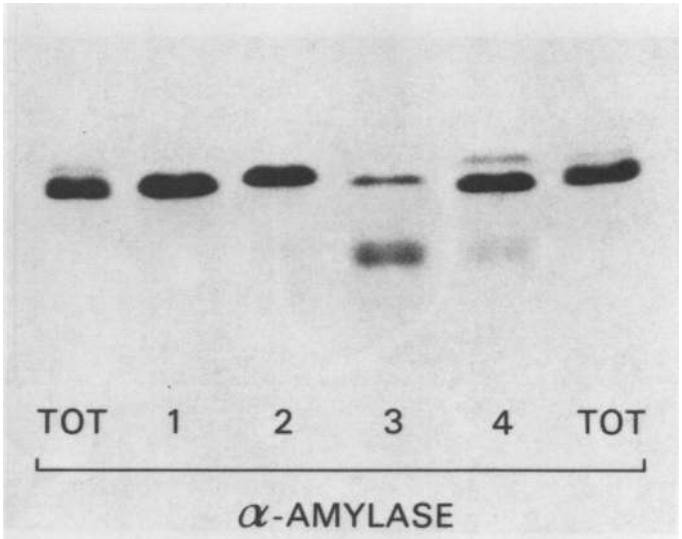

FIG. 5. SDS-PAGE of α-amylase purified by CHA-Sepharose chromatography (TOT) and of the component α-amylases 1 to 4 purified by DEAE-cellulose chromatography.

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have shown the results of only one concentration in which digestion of the parent polypeptides was 75 to 90% complete.

RESULTS

Purification of α-Amylase. Aleurone layers (400–500) were incubated for 27 h with GA_3, and the proteins released into the medium were fractionated first by CHA-Sepharose affinity chromatography and then by SDS-PAGE (Fig. 1A). The original medium contained about 15 major polypeptides (Fig. 1A, ORIG). The CHA column bound three polypeptides, two major components and one minor, with very similar (mol wt, ~44,000) mobilities on polyacrylamide gel (Fig. 1A, B) and the remaining polypeptides of the original medium were recovered in the fraction which did not bind to the column (Fig. 1A, NB). The bound fraction contained 4 to 6 mg of protein and about 80% of the original amylase activity.

The nature of the bound fraction was examined further because of its heterogeneity on SDS-PAGE and because it was not known whether CHA-Sepharose bound β-amylase as well as α-amylase. Samples of each of the fractions shown in Figure 1A were fractionated by agar gel electrophoresis, and the gel was stained to detect amylase activity (Fig. 1B). This technique permits the resolution of eight amylase bands (Fig. 1B, ORIG), four of which (1–4) have been identified previously as α-amylases and the other four (5–8) as β-amylases (14). Only the α-amylases bound to the CHA-Sepharose column, while the β-amylases appeared in the fraction which did not bind to the column (Fig. 1B, bound and non-bound, respectively). Additional proof of the identity of the CHA-bound and non-bound amylases was obtained by examination of the products of amylose hydrolysis by the fractions. Fractionation of the hydrolysis products by paper chromatography (Fig. 2) showed that CHA-bound amylase gave rise to a range of glucose polymers (although glucose itself was not produced) identified previously as being characteristic of α-amylase action (14), whereas the non-bound fraction liberated maltose which is characteristic of β-amylase. The CHA-Sepharose column therefore discriminates between α- and β-amylase, and the bound protein contains only α-amylase.

The heterogeneity of the CHA-bound fraction, although not due to the presence of β-amylase, could be due to contaminating proteins without α-amylase activity. To resolve this, the bound protein was fractionated by IEF over the pH range 3.5 to 9.5. α-Amylase activity was detected with lyosine red-impregnated paper, and then the gel was fixed and stained with Coomassie Blue. IEF resolved the CHA-Sepharose-bound protein into a number of Coomassie Blue-stained components, all of which exhibited α-amylase activity (Fig. 3). Some components were detected by α-amylase activity but not by Coomassie Blue. It is also evident from IEF studies that the α-amylases fall into two well-separated groups, one with isoelectric points in the range of 4.5 to 5.1 and the other in the range of 5.9 to 6.6.

Fractionation of α-Amylase by DEAE-Cellulose Chromatography. α-Amylase was fractionated preparatively by ion-exchange chromatography. The total protein released into the incubation medium of GA_3-treated aleurone layers was fractionated by DEAE-cellulose column chromatography at pH 6.5 using a linear salt gradient. Five amylase components were resolved (Fig. 4A). The first peak of activity did not bind to the column (Fig. 4, run-off) and was identified by agar gel electrophoresis as β-amylase (Fig. 4B; compare with Fig. 1B). In addition, the run-off peak of amylase was not present in heated aleurone incubation medium (under which conditions β-amylase is heat labile) or when CHA-Sepharose purified α-amylase was applied to the ion-exchange column (data not shown) both of which identify the run-off amylase as β-amylase. The other four amylase peaks (Fig. 4A) were identified as α-amylases 1 to 4 (in reverse order) by agar gel electrophoresis (Fig. 4B).

Using this procedure in combination with CHA-Sepharose chromatography, pure preparations of α-amylases 1 to 4 were made as described in "Materials and Methods." We obtained 1.5 to 2 mg of each α-amylase from the incubation medium of 1,500 layers.

Although agar gel electrophoresis showed that each purified preparation contained only one α-amylase band (data not shown), several polypeptides were detected in some of the preparations by SDS-PAGE (Fig. 5). Differences in mobility between some components can account for the heterogeneity in the total CHA-Sepharose-purified α-amylase (Fig. 5, TOT); however, some of the components, notably in α-amylase 3 and 4, were not detected in the total α-amylase preparation and may be either minor α-amylases or products generated during purification.

Characterization of α-Amylases 1 to 4.

Immunology. A serological comparison of the enzymes was made using antiserum raised in rabbits to total CHA-purified α-amylase from aleurone incubation medium. Double diffusion experiments (Fig. 6) showed that there was a reaction of identity between α-amylases 1 and 2 and between 3 and 4, but a reaction of non-identity between members of the two groups (1 + 2 and 3 + 4). Using methods such as these, we cannot rule out the possibility of some minor cross reaction between the two groups.

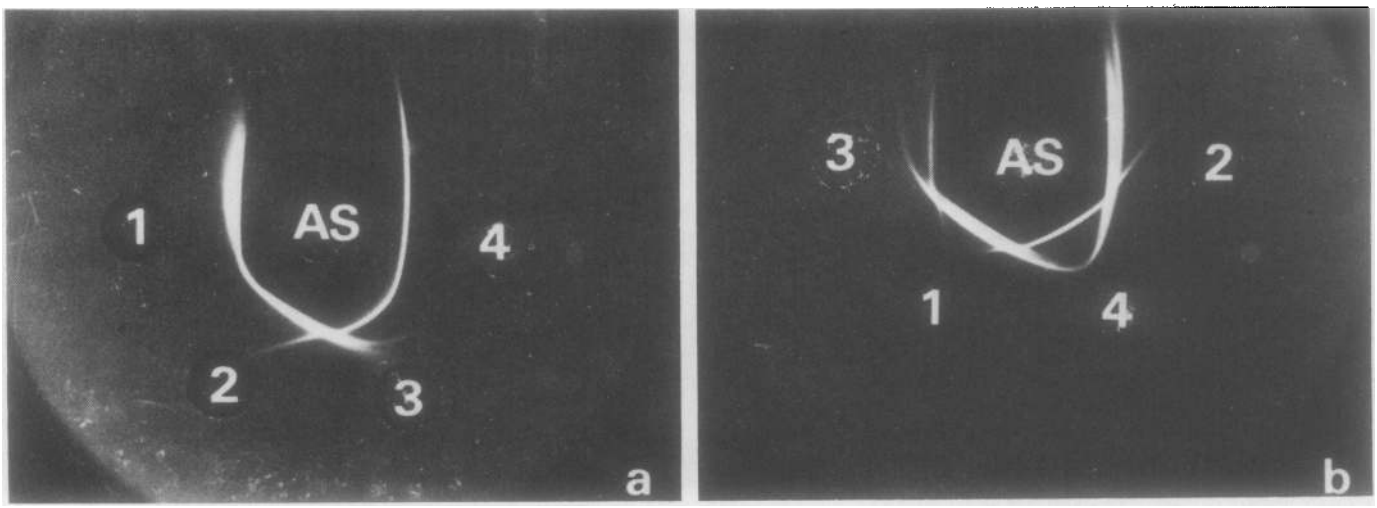

FIG. 6. Serological comparison of α-amylases 1 to 4 on Ouchterlony plates. Antiserum containing antibodies against all α-amylases (AS) was placed in the central well of both plates, and the α-amylases were placed in two different arrangements (a and b) in the outer wells.

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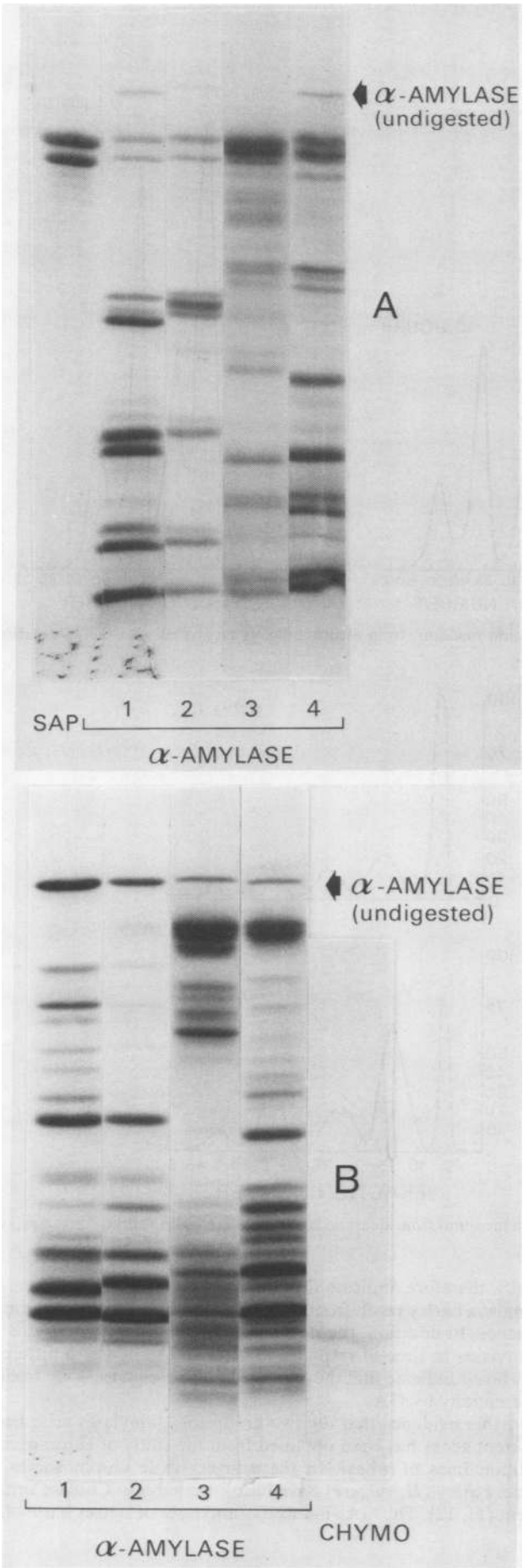

Peptide Mapping. Samples of each α-amylase were digested with SAP and with chymotrypsin, and the products of proteolysis were fractionated by SDS-PAGE (Fig. 7). The peptide profiles of α-amylases 1 and 2 using SAP (Fig. 7A) showed a number of similarities as well as some differences. Similarly, α-amylases 3 and 4 had a number of peptides in common but also there were many differences. There appear to be few similarities between the two groups (1 + 2 and 3 + 4). These results were confirmed using chymotrypsin (Fig. 7B). There were many similarities and some differences between α-amylases 1 and 2 and between 3 and 4, but there was little similarity between the two groups. In general, therefore, these results indicate that there are extensive differences in amino acid sequence between the serologically distinct α-amylase groups and few but still substantial differences between the enzymes in each group.

Responses of α-Amylases 1 to 4 to GA_3.

Time of Appearance. Batches of 160 aleurone layers were incubated with 1 μM GA_3 (20 layers/flask) for various periods of time up to 24 h. After incubation, the layers for each batch were pooled and ground in their own incubation medium so that the homogenates contained total amylase. The amylases were then fractionated by DEAE-cellulose chromatography (Fig. 8). Freshly isolated aleurone layers (0 h) contained very small amounts of α-amylases 1 and 2. During incubation with GA_3, the amounts of these enzymes steadily increased in parallel. α-Amylases 3 and 4 could not be detected by 8 h, but by 13 h they had appeared and thereafter they increased rapidly in parallel. Together α-amylases 3 and 4 made an increasing contribution to total α-amylase activity with time, increasing from 0 at 8 h to about 60% at 24 h. At 24 h, the major α-amylase was 4 which contributed 44% of the total activity.

The first eluted amylase peak (---) is probably mostly β-amylase (see earlier). β-Amylase is not synthesized by aleurone but preexists in finite amounts in the starchy endosperm (22, 29), some of which adheres to isolated aleurone layers. Hormone treatment leads to solubilization of the β-amylase probably by proteolysis (14), which would account for the rapid rise in the amount of the run-off amylase fraction. The decrease in amount between 18 and 24 h is probably due to thermolability.

Response to Different Levels of GA_3. Batches of 60 aleurone layers were incubated (20 layers/flask) for 24 h with GA_3 concentrations increasing from 0.001 to 1 μM. The layers were ground in their own medium, and the amylases were fractionated by DEAE-cellulose chromatography. Figure 9 shows that, at 0.001 and 0.01 μM GA_3, only α-amylases 1 and 2 appeared. At 0.1 μM GA_3, α-amylases 3 and 4 appeared, and in 1 μM GA_3 they contributed about 65% of the total α-amylase activity. In these experiments, α-amylase 1 responded in parallel with 2, and 3 with 4.

DISCUSSION

In this study, we have fractionated the α-amylases synthesized by GA_3-treated aleurone layers from Himalaya barley into two major classes, each class containing at least two components. The classification is based on a number of criteria including charge characteristics, serological characteristics, peptide mapping, time course of production, and response to varying levels of GA_3. This extends earlier results (1, 13, 14) which, on the basis of serology, calcium requirement, and differential sensitivity to low pH and sulfhydryl reagents, indicated that the enzymes fall into two groups. The groups have been designated A (α-amylases 1 and 2) and B (α-amylases 3 and 4) (13).

The differences between the two groups of α-amylases, particularly in serological characteristics and peptide maps, indicate

FIG. 7. SDS-PAGE of the products of hydrolysis of α-amylases 1 to 4 by SAP (A) and by chymotrypsin (CHYMO) (B).

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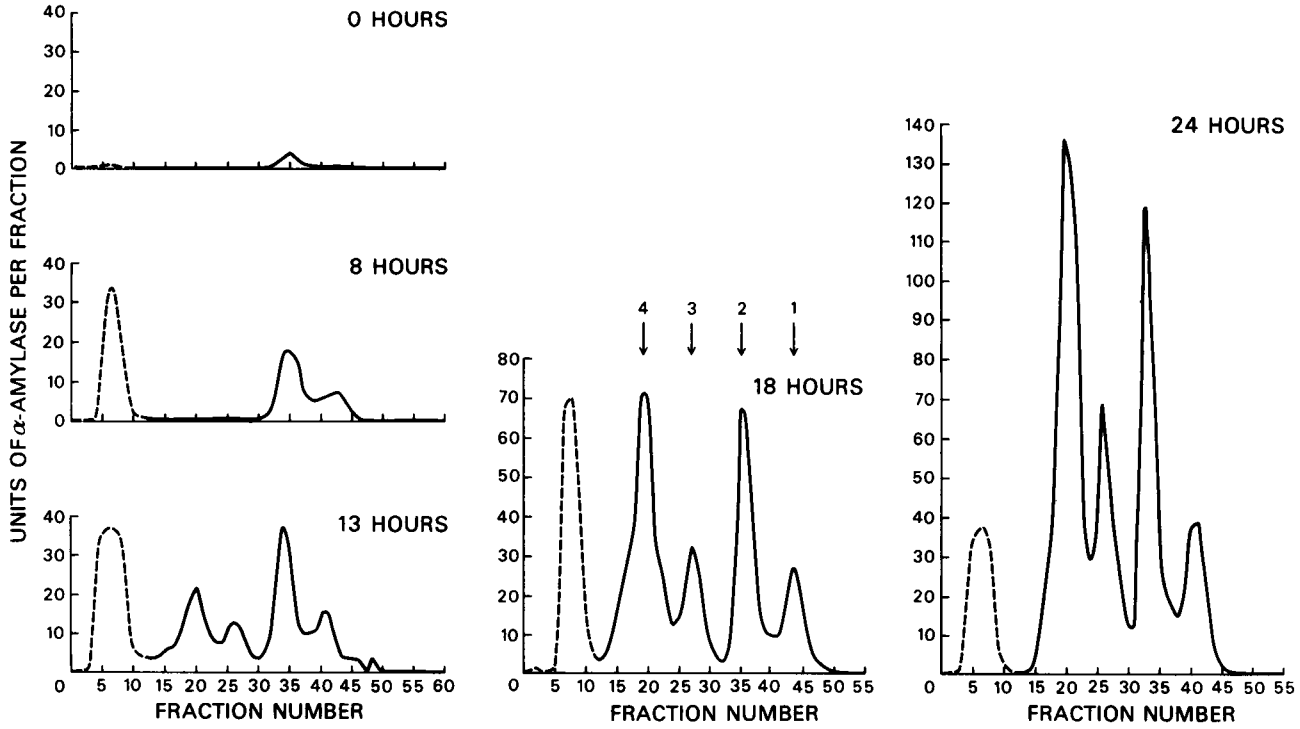

FIG. 8. DEAE-cellulose fractionation of the total amylase (tissue and incubation medium) from aleurone layers incubated with GA_3 for various times.

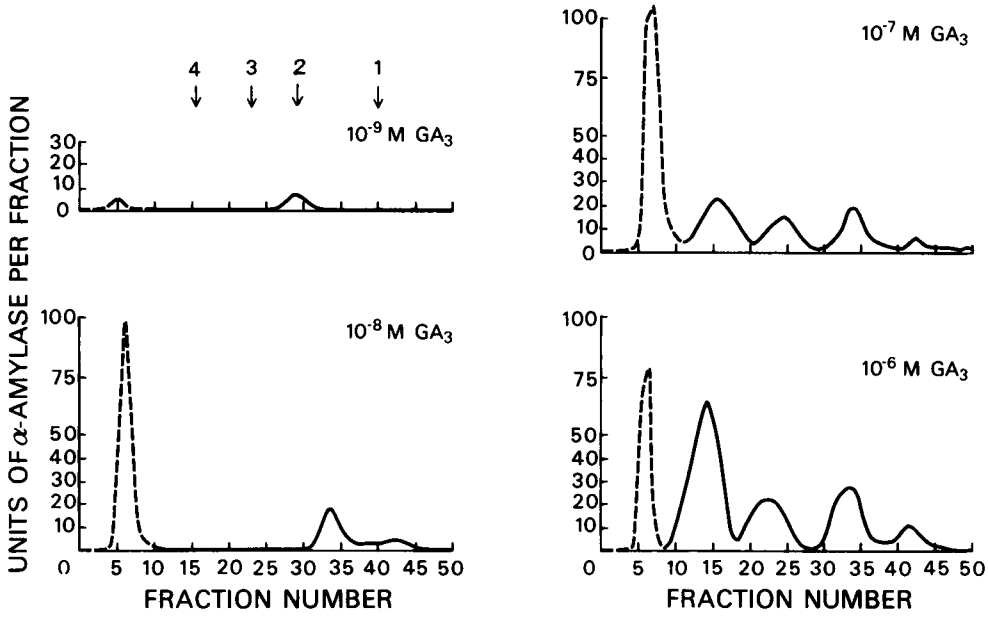

FIG. 9. DEAE-cellulose fractionation of the total amylase (tissue and incubation medium) from aleurone layers incubated with various concentrations of GA_3 for 24 h.

that the two enzyme groups contain polypeptides which differ considerably in amino acid sequence and which are probably the products of different genes (see below). Differences between α-amylases within groups are much smaller than between groups. These enzymes also differ in charge characteristics and peptide maps, and although they too may be products of different genes, these smaller differences may arise by posttranslational modification of a single polypeptide. Alternatively, the enzymes may represent different polypeptides derived from different mRNAs which are transcribed from a single gene. This is known to be the case for mouse liver and salivary gland α-amylases (10, 30). Our results, therefore, indicate that the α-amylases of the aleurone of Himalaya barley result from the expression of two genes or groups of genes. In addition, the differences between the two groups of α-amylases in time of response to GA_3 and response to different GA_3 levels indicate that the expression of these genes is controlled differentially by GA_3.

Further evidence that the two groups of α-amylases arise from different genes has been obtained from the study of chromosome addition lines of wheat. In these lines, single chromosomes of Betzes barley (*H. vulgare*) have been inserted into Chinese spring wheat (11, 12). The GA_3-induced α-amylases of Betzes barley fall

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into two isoelectric point groups, one group with isoelectric points ranging from pH 4.5 to 5.2 and the other from 5.9 to 6.6, and it has been shown that the gene(s) for the first group occur on chromosome 1 and the gene(s) for the second group on chromosome 6 (3). The present study shows that the A and B groups of Himalaya barley (also *H. vulgare*) fall into similar isoelectric point groups, and so it is likely that the genes for the A and B groups of Himalaya α-amylases also occur on different chromosomes. This would provide a cytological basis for the dissimilarities between the two enzyme groups. In addition, in *Hordeum spontaneum*, which also has two isoelectric point groups of α-amylases, the enzymes within each group behave genetically as though they arise from a single gene or from closely linked genes (3). Taken together, these studies indicate that the two genes or clusters of genes controlled by GA_3 occur on different chromosomes.

The occurrence of multiple α-amylases in barley is widespread. In addition to those mentioned above, electrophoretic studies have demonstrated that there is considerable variation between enzymes in different species of *Hordeum* and between different cultivars of the same species (8, 9, 21). In several cultivars of *Hordeum vulgare* (in addition to Himalaya), the enzymes have been found to fall into two well-separated isoelectric point groups (16, 27), and in some varieties (Conquest, Pirka, Ingrid, and Carlsberg 11) the isoelectric point groups are serologically different (2, 7, 19). In addition, it has been shown that, in two of these varieties (Carlsberg 11 and Ingrid), the two serologically different α-amylases arise asynchronously (2, 7). Such results indicate that the differences between the α-amylases of Himalaya may be widespread in *H. vulgare*. The occurrence of two isoelectric point groups of α-amylases in *H. spontaneum* and the genetic analysis of the enzymes (3) indicates that this species may conform to the *H. vulgare* pattern. On the other hand, *H. arizonicum*, *H. glaucum*, and *H. distichon* (syn. *H. vulgare*) have multiple α-amylases but no evidence of distinct isoelectric point groups has been found (20, 21). Further examination of these as well as other species of barley is required especially using serological techniques, and at present care must be exercised in extrapolating the results of this study of Himalaya barley to all *Hordeum* species.

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