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POLYMORPHISM OF SOME GLYCOSIDASES FROM BARLEY

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; α-L-arabinosidase; β-fucosidase; β-galactosidase; β-glucosidase; isoenzymes.

Abstract — Barley grain extract displayed α-L-arabinosidase, β-fucosidase, β-galactosidase and β-glucosidase activities. Some of the glycosidases were separated from one another by Sephadex gel-filtration and CM-cellulose chromatography. The glycosidase types were more varied than reported by earlier workers. The multifunctional nature of some of the enzymes was demonstrated by inhibition studies, gel electrophoresis, pH and thermal stability studies.

INTRODUCTION

In general, glycosidases have specific configurational requirements for the glycone residues of their substrates. However, some glycosidases are known to tolerate changes at C-6 of the glycone. Thus a β-galactosidase will also hydrolyse β-D-fucoside and α-L-arabinoside in addition to a β-D-galactoside [1]; however, a β-D-glucoside is unaffected. Similar examples of specificity are known for α-galactosidases [2] and β-glucosidases [3].

Substrate specificity studies (with respect to C-4 and C-6) of β-glucosidase, β-galactosidase and β-fucosidase from barley and limpet showed that the former two were different enzymes and that β-glucosidase also displayed β-fucosidase acitvity [4]. It was also demonstrated that in barley α-L-arabinosidase activity was associated with β-galactosidase but not with β-glucosidase or β-fucosidase [5]. In emulsin, all three activities and α-L-arabinosidase activity resided in the same protein indicating a lack of specificity for C-4 and C-6 of the substrate [5]. These conclusions were drawn on the basis of ammonium sulphate fractionation, pH stability and inhibition studies.

This paper describes distinct separation of some glycosidases by column chromatographic methods. It also indicates the apparent multifunctional behaviour of some of the fractionated enzymes.

RESULTS AND DISCUSSIONS

Enzyme fractionation

The isolation procedure was an adaptation of the method described by Conchie and co-workers [4].

Sephadex G-75 gel filtration

The $(NH_4)_2SO_4$ fraction (5 ml containing 60 mg protein) was passed through a Sephadex G-75 column (95 × 2.6 cm), equilibrated with McIlvaine buffer, pH 5.5 containing 0.1 M KCl. The elution (30 ml/hr and 3 ml/fraction) pattern of various enzyme activities is shown in Fig. 1. Activities could be separated into two peaks, A and B. The apparent MWs of peak A and B, using Sephadex G-75 were 65000 and 48000 (assuming that the proteins are globular; a ±10% error would be expected). These results were confirmed with Sephadex G-100 gel filtration. Peak A displayed β-galactosidase, β-fucosidase, α-L-arabinosidase and β-glucosidase activity and peak B showed only the latter three. The ratio of activities is compared in Table 1. It is apparent from Fig. 1 that β-galactosidase, β-glucosidase and α-L-arabinosidase exist in two molecular forms. In peak A β-fucosidase and α-L-arabinosidase activities could also be due to β-galactosidase; this has been shown in the case of some β-galactosidases [7]. On the other hand, β-fucosidase in peak B could be an independent enzyme (because of its predominant activity). These possibilities are closely examined in further separation steps. (The recoveries of individual enzymes in this and in future steps were not estimated because of the multifunctional nature of some enzymes.)

CM-cellulose chromatography of peak A

Peak A was concentrated by ultrafiltration and passed through a carboxy methyl cellulose (CM-52, Whatman) column (3 × 2.5 cm) equilibrated with McIlvaine buffer, pH 4.5. Elution was carried out (12 ml/hr) by a stepwise change in pH of the buffer. A major active fraction, A1, was eluted at pH 5 and a small peak, A2, at pH 6. Fraction A1 showed the order of activities as, β-galactosidase > β-glucosidase > β-fucosidase ≃ α-L-arabinosidase. The equal proportion of the latter two activities (see Table 1) is rather surprising; β-galactosidase is known to display both of these activities but α-L-arabinoside is hydrolysed at a slower rate than a β-fucoside. Peak A2 displayed only β-glucosidase activity.

All three activities of peak A1 except for β-glucosidase were completely inhibited by 3 mM D-galactono-(1→5)-lactone under the normal assay conditions (competitive inhibition; $K_i = 3 \times 10^{-4}$ M at pH 5.1 with respect to *p*-nitrophenyl β-D-galactoside). On polyacrylamide gel electrophoresis, the three activities migrated towards the

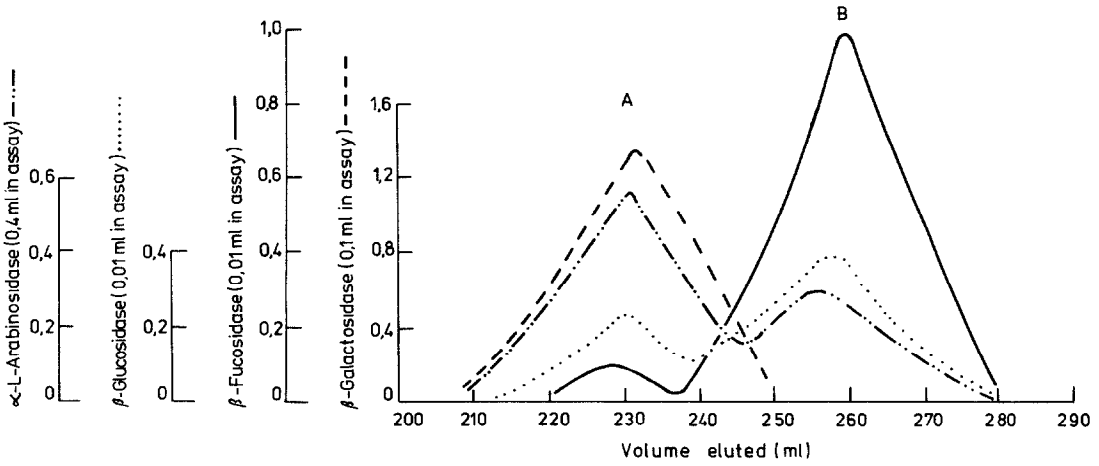

Fig. 1. Sephadex G-75 gel filtration of a barley enzyme-preparation. Enzyme activities are expressed in the form of A at 405 nm as measured in the assay. Other details are described in the text.

anode as a single protein band. On the other hand, methyl β-D-glucoside exclusively inhibited β-glucosidase (1 mM in assay) without affecting any of the other activities (competitive inhibition; $K_i = 5 \times 10^{-3}$ M at pH 4.5). The electrophoretic mobility of β-glucosidase was higher than the band displaying the three activities. This shows an independent identity of β-glucosidase; the other activities of peak A1 were apparently due to a single enzyme. The pH optimum and K_m of the β-glucosidase were 4.5 and 0.2 mM with *p*-nitrophenyl β-D-glucoside. The three associated activities displayed a common pH optimum of 5.1 against their corresponding *p*-nitrophenyl substrates. In conclusion it may be said that peak A1 consists of a β-glucosidase and an enzyme displaying β-galactosidase, β-fucosidase and α-L-arabinosidase activities.

The β-glucosidase of peak A2 had a pH optimum of 4 and K_m of 0.5 mM with *p*-nitrophenyl-β-D-glucoside. The enzyme was 50% inhibited by 0.35 mM methyl β-D-glucoside (competitive inhibition; $K_i = 2.5 \times 10^{-4}$ M at pH 4). It seems that the β-glucosidase activity of peak A2 is different from that of peak A1.

CM-cellulose chromatography of peak B

The low MW peak B (as obtained from Sephadex gel-filtration) was further resolved into three active peaks B1, B2 and B3 eluted at pH's 4.8, 5 and 6 respectively. Peak B1 displayed β-fucosidase and β-glucosidase activity, B2 showed α-L-arabinosidase and β-galactosidase activity and B3 had β-fucosidase and β-glucosidase activity.

Both activities of B1 had pH optimum of 5.6 and were inhibited by methyl β-D-glucoside (competitive inhibition; $K_i = 7.5 \times 10^{-4}$ M at pH 5.6) and D-fucono-(1→5)-lactone (competitive inhibition; $K_i = 2 \times 10^{-5}$ M at pH 5.6). On gel electrophoresis the two activities were shown by a single protein band. Incubation of the enzyme preparation at pH 5.6 and 60° caused 40 and 65% inactivation in 20 and 30 min respectively of both the activities. Further, the activities were inhibited by 25% at pH 3.5 and 37° in 2 hr. Therefore, it seems likely that both the activities (a predominant β-fucosidase; see Table 1) were due to a single enzyme.

Peak B2, on the other hand, seemed to be an α-L-arabinosidase also displaying β-galactosidase activity.

Table 1. Relative levels of various glycosidase activities at different stages of separation

Fractionation step	Relative amounts of enzyme activity (arbitrary units) at each stage of separation			
	β-Galactosidase	β-Glucosidase	β-Fucosidase	α-L-Arabinosidase
1. Crude extract	1	4.80	5.50	0.30
2. $(NH_4)_2SO_4$ fraction (35–80%)	1	4.75	5.45	0.30
3. Sephadex G-75 gel filtration (Fig. 1)				
Peak A	1	1.43	0.14	0.08
Peak B	absent*	1	3.33	0.01
4. CM-52 Chromatography of A (elution at pH 5–6)				
Peak A1	1	0.25	0.125	0.125
Peak A2	absent	present	absent	absent
5. CM-52 Chromatography of B (elution at pH 4.8–6)				
Peak B1	absent	1	4.4	absent
Peak B2	1	absent	absent	1.4
Peak B3	absent	1	2.3	absent

*There was no measurable β-galactosidase activity at this step. This, however, was detected in peak 2 of step 5 which may be due to further concentration and enrichment of the enzyme preparation.

Both activities showed a pH optimum of 4.2 and were 50% inhibited by 2.35 mM L-arabino-(1 → 5)-lactone (competitive inhibition; $K_i = 7.8 \times 10^{-4}$ M at pH 4.2). The thermal stability of the two activities followed a similar pattern at 60° (35 and 43% inactivation in 20 and 30 min respectively) and were inhibited by 12% at pH 3.5 and 37° in 2 hr. They were not separable by gel electrophoresis and were located in a single protein band. β-Galactosidase activity, which was not detected in peak B, of the Sephadex gel-filtration pattern, appears in this sub-fraction; this may be due to concentration and further enrichment of the preparation.

Peak B3 also possessed β-fucosidase and β-glucosidase but the ratio of the activities was different from that of B1. This seems to be a different enzyme species as the pH optimum of the activities is 4.5 compared to 5.6 for B1. Both activities were inhibited either by methyl β-D-glucoside (competitive inhibition; $K_i = 1.1 \times 10^{-4}$ M at pH 4.5) or by D-fucono-(1→5)-lactone (competitive inhibition; $K_i = 5 \times 10^{-5}$ M at pH 4.5). On gel electrophoresis the two activities moved in a single protein band but the rate of migration was twice as fast as B1. Thermal inactivation showed 50 and 75% loss of activity in 20 and 30 min respectively at 60° and pH 4.5. This enzyme was slightly more unstable than B1 and lost 40% activity at pH 3.5 and 37° in 2 hr.

In conclusion it can be said that multiple molecular forms of glycosidases exist in barley and the types are more varied with respect to their action on specific substrates than was reported earlier [4, 5]. Thus, the enzymes which could be separated from each other were as follows (relative activities displayed by each enzyme are shown in Table 1): High MW enzymes (MW *ca* 65000): β-galactosidase/β-fucosidase/α-L-arabinosidase; β-glucosidase. Low MW enzymes (MW *ca* 48000): β-galactosidase/α-L-arabinosidase; two isoenzymes of β-fucosidase/β-glucosidase.

EXPERIMENTAL

Barley cv Ymer (200 g) was ground in a hand mill, washed with Me_2CO (1 l. at −10°), filtered and air dried. This treatment removed some colouring matter, lipids and fats. Powder was then homogenized in McIlvaine buffer [6], 0.1 M, pH 5.5 (500 ml), stirred for 1 hr at 4° and was strained through a double layer of cheese-cloth. The extract was centrifuged (10000 *g* for 30 min) and clear supernatant (370 ml) possessed the following activities (milliunits as shown in brackets): β-galactosidase (3700), β-glucosidase (17800), β-fucosidase (20400) and α-L-arabinosidase (1110). Protein content was 3.25 mg/ml. The enzyme extract was subjected to $(NH_4)_2SO_4$ fractionation; the protein fraction at 35–80% was isolated, suspended in McIlvaine buffer, pH 5.5 (20 ml) and dialysed 18 hr against the same buffer (1 l.) with two changes. The dialysed soln was turbid and cleared by centrifugation. It was then concd (final vol. 10 ml) by ultrafiltration (PM 10 membrane). 80–90% of the activities were recovered with an overall 10-fold purification; the ratio of various activities remained the same. Ultrafiltration aided in purification by discarding proteins of MW under 10000. Glycosidase activities were assayed by incubating the enzyme with the appropriate *p*-nitrophenyl glycopyranosides at 30° for 15 min. All solns were prepared in McIlvaine buffer (0.1 M) [6], the assay pH was optimal for each enzyme and the substrate concn in a total assay vol of 1 ml, was 1mM. The reaction was stopped with 5 ml 0.1 M Na_2CO_3. Appropriate blanks were also prepared. The yellow colour developed from the liberation of *p*-nitrophenol was estimated by A_{405} nm. The amount of enzyme taken in the assay was so adjusted as to obtain an A of 0.1–0.6 at 405 nm. Under these conditions the enzyme reaction followed a linear order. A unit of enzyme activity was the same as described earlier [8]. Protein was estimated by the method of ref. [9] using BSA as standard. The Sephadex column was calibrated according to the method of ref. [10]. Polyacrylamide gel electrophoresis was carried out by the method of ref. [11] at 4° using 7% gel and 0.05 M acetate buffer, pH 5.5. Electrophoresis was performed using a Shandon apparatus for 5 hr at 100 V and 3 mA/tube. Protein was located with Amido Schwarz [11] and enzyme activity, by cutting 2 mm pieces of the gel lengthwise and analysing them with specific substrates. Inhibition studies were carried out by pre-incubating the enzyme with the specific inhibitor for 10 min and the reaction was started by adding a measured amount of the substrate soln so that its concn in the final assay (1 ml) is 1 mM. The rest of the procedure was as for the enzyme assay. D-Galactono-(1→5)-lactone [12, 13], D-fucono-(1 → 5)-lactone [13] and L-arabino-(1→5)-lactone [13] were prepared according to the methods described elsewhere. Optimal pH's of the enzymes were determined using McIlvaine buffer (pH 2.7–7).

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