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STUDIES ON ALMOND EMULSIN β-D-GLUCOSIDASE

I. ISOLATION AND CHARACTERIZATION OF A BIFUNCTIONAL ISOZYME

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Summary

A β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) isozyme has been isolated from almond emulsin. The isolated enzyme is a glycoprotein and migrates as a single band on Sephadex G-200 filtration, CM 52 ion exchange chromatography, polyacrylamide gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focussing. The glucosidase and galactosidase activities traverse together during Sephadex G-200 gel filtration. Polyacrylamide gels stained specifically for the 2 enzymes reveal that the two activities comigrate. The molecular weight of the isozyme has been found to be 135 180 ± 770, and that of its protomers to be 65 150 ± 650.

Introduction

Glycosidases have been studied from a variety of sources and observed to exhibit different stereospecificity for substituents at the C-4 position of the pyranoside substrates. The enzyme, β-D-galactosidase (EC 3.2.1.23) that is found in *Escherichia coli* utilizes only β-D-galactopyranosides as substrates [1,2] while β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from yeast attacks only β-D-glucopyranosides [3]. The *E. coli* galactosidase is not inhibited by glucose [1] and the yeast glucosidase is not affected by galactose. Almond emulsin contains at least four isozymes of glucosidase differing in their ion-exchange properties [4]. The almond emulsin glucosidases have been purified to various degrees and these preparations have been demonstrated to hydrolyze β-D-glucopyranosides as well as β-D-galactopyranosides [5,6]. Two of these isozymes have been separated and each assayed for its glucosidase and galactosidase activities [6]. The ratio of the rate of hydrolysis of the two glycosides by one of the isozymes was observed to be different from a corresponding ratio obtained employing the other isozyme. To date, it

has not been clearly demonstrated whether the dual activity of the enzyme is due to different proteins or to polypeptide chains of a single type. This communication reports isolation of one of the almond emulsin glucosidase isozymes to near homogeneity, characterization of the protein and investigation into the possibility of existence of a bifunctional enzyme.

Materials and Methods

Reagents

Almond emulsin, phosphorylase, bovine serum albumin, catalase, glyceraldehyde-3-phosphate dehydrogenase, yeast alcohol dehydrogenase, liver alcohol dehydrogenase, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-galactopyranoside, fructose 1,6-diphosphate and Diazo Blue B were purchased from Sigma Chemical Company. Aldolase and ovalbumin were obtained from Pharmacia Fine Chemicals Inc. The reagents *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-D-galactopyranoside were obtained from Pierce Chemical Co., and NAD from Calbiochem. High purity sodium dodecyl sulfate was obtained from BDH Chemicals Ltd. The cellulose derivatives, CM 52 and DE 32 were purchased from Reeve-Angel. Ampholine, pH 3.4—10, was obtained from LKB Instruments, Inc. All other chemicals were obtained from standard commercial sources.

Enzyme assays

Glucosidase assays were carried out by monitoring the increase in absorbance at 400 nm due to appearance of *p*-nitrophenol resulting from the enzyme catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside. For the β-D-galactosidase assays *p*-nitrophenyl-β-D-galactopyranoside was used as a substrate in an analogous procedure. Similarly, the corresponding α-glycosides were also employed for estimation of α-glycosidase activities of the enzyme preparations. All assay solutions contained 20 mM of the specified glycoside and 50 mM sodium acetate pH 6.0. The assays were performed at room temperature. The extinction coefficient of *p*-nitrophenol was determined under the conditions of the assays to be 1406 $M^{-1} \cdot cm^{-1}$. One unit of enzyme activity was defined as the amount of the enzyme required to convert one μmole of the substrates into products per minute.

Catalase was assayed by the method of Beers and Sizer [7] and aldolase, as described by Jagannathan et al. [8]. Alcohol dehydrogenase activity was measured by a procedure modified from that of Vallee and Hoch [9]. Reaction mixtures containing 50 mM sodium phosphate, 1 M ethanol, 5 mM NAD and 2 mM hydrazine sulfate were used. The pH of these solutions was adjusted with sodium hydroxide to 7.5. The alcohol dehydrogenase activity was measured by monitoring the change in absorbance at 340 nm.

Protein estimation

Protein content of the solution was estimated either by measuring absorbance at 280 nm or by the method of Lowry et al. [10].

Enzyme purification

Almond emulsin was employed as a source of β-D-glucosidase and all operations in the purification were carried out in a cold room, at 4° C. The purification procedure consisted of chromatography on carboxymethyl and diethylaminoethyl derivatives of cellulose followed by Sephadex G-100 gel filtration. The first step consisted of application of extremely large amounts of the almond emulsin to CM 52 columns at pH 3.6. In a typical experiment 3.6 g of the emulsin were suspended in 90 ml of 0.1 M sodium acetate pH 3.6 and applied to a column containing 30 ml of CM 52, pre-equilibrated with the application buffer. In various experiments, 2.5—3.5 ml of a solution containing the emulsin (40 mg/ml) were applied per ml of the settled cellulose. Washing with the same buffer resulted in elution of the enzyme. Eluate fractions with the highest specific activities were pooled and applied to a larger CM 52 column pre-equilibrated with the same buffer. The second column contained 3 ± 0.5 times the volume of cellulose of the first column. After application of the enzyme, the column was washed with the 0.1 M sodium acetate, pH 3.6 buffer. Elution was then performed using 0.1 M sodium acetate pH 3.6—0.15 M sodium acetate pH 6.0 gradient. Eluate fractions with the highest specific activities were pooled and concentrated by ultrafiltration through an XM 50 Amicon membrane at 50 PSI nitrogen pressure. The concentrated pool was dialyzed against 400 volumes of 0.05 M sodium acetate pH 6.0 and applied to a DE 32 column pre-equilibrated with this buffer. The amount of cellulose employed was comparable to the amount of the CM 52 used in the first step. Application of the enzyme was followed by washing the column with the application buffer (0.05 M sodium acetate, pH 6.0). Elution was then performed using a 0.05—0.3 M sodium acetate pH 6.0 linear gradient. The peak activity fractions were pooled, concentrated by ultrafiltration, and applied to a Sephadex G-100 column (2.5 cm × 95 cm) pre-equilibrated with the 0.05 M sodium acetate pH 6.0 buffer. The eluates were concentrated and stored at 4° C.

Electrophoresis

Acrylamide gel electrophoresis was carried out at pH 8.9 by the method of Davis and Ornstein [11] using 7% acrylamide gels. Only the analyzer gels were used and the electrophoresis was carried out employing 2 mA current per gel. The sodium dodecyl sulfate gel electrophoresis was carried out according to Weber and Osborn [12] except that the protein samples were prepared by incubation at 100° C for 15 min rather than 2 h at 37° C.

Isoelectric focussing in 6% polyacrylamide gels was carried out by the procedure described by Wrigley [13]. The gels were prepared using the pH range 3.5—10 Ampholine. The electrode buffers used for the experiment were 0.2% sulfuric acid and 0.4% ethanolamine in the anode and the cathode tanks, respectively, and a pH gradient was established by application of a 200 V potential. Protein samples applied to the gels ranged from 25 to 200 μg. Electrofocussing was carried out at 100 V for 1 h followed by 200 V for 12 h. The gels were removed and stained as described by Wrigley [13]. The gels to be employed for measuring the pH gradient were placed in a freezer, sliced when frozen and placed in water for extraction. The pH of the water extracts of the gel sections was employed as a measure of the pH gradient.

Gels were stained for protein with Coomassie Blue, and destained and stored in a solution containing 7% acetic acid and 10% methanol [12]. Gels were stained for glycoprotein using the periodate-metabisulfite procedure [14]. The gels were placed in a 12% trichloroacetic acid solution for 30 minutes, rinsed in distilled water and transferred to a 1% perchloric acid solution (in 3% acetic acid). After 60 min, the gels were washed with distilled water and placed in a 0.5% sodium metabisulfite solution (containing 3% acetic acid). After 30 min, they were transferred to tubes containing 7% acetic acid for storage. The acrylamide gels were also stained for β-D-galactosidase activity by the method of Erickson and Steers [15] and for β-D-glucosidase by an analogous procedure. After the electrophoresis at pH 8.9, the gels were placed in a solution containing 20% ethanol and 0.025% of either 6-bromo-2-naphthyl-β-D-glucopyranoside or 6-bromo-2-naphthyl-β-D-galactopyranoside. After a period of 2 h, the gels were rinsed in distilled water and transferred to a freshly prepared 0.1% Diazo Blue B solution. After 2 min the gels were destained and stored in a solution containing 10% methanol and 7% acetic acid.

Molecular weight estimation

Estimation of molecular weight of the protomers of the enzyme was carried out by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis [12]. Sephadex G-200 gel filtration was employed for estimation of size of the active enzyme. Sephadex G-200 was swollen and packed in 0.05 M sodium acetate pH 6.0, into a 2.7 × 98 cm bed. The various standards and the purified β-D-glucosidase were suspended in 3 ml of the column buffer. The amounts of the various enzymes applied were glucosidase 724 units, catalase 96 units, yeast alcohol dehydrogenase 2.35 units, liver alcohol dehydrogenase 2.65 units and aldolase 16 $\Delta A_{240\,nm}$/units. The column was eluted with the same buffer and 3.55 ± 0.15 ml fractions were collected. The eluate fractions were assayed for these enzymes and elution volume of the fraction with the highest activity of a particular enzyme was used as elution volume for the enzyme.

Results

Multiple forms of glucosidase of almond emulsin

Almond emulsin suspended in 0.05 M sodium acetate pH 3.6 was applied to a large CM 52 column. Application of a 0.05 M sodium acetate pH 3.6—0.5 M sodium acetate pH 4.9 gradient resulted in elution of three peaks bearing β-D-glucosidase activity (Fig. 1). The three glucosidases revealed different glucosidase/galactosidase ratios. The β-D-glucosidase/β-D-galactosidase ratios for the three peaks were 1.6, 2.7 and 6.8.

Enzyme purification

Glucosidase activity of almond emulsin ranged from 4.3—8.5 units/mg protein, in different batches of the almond emulsin. Application of an excess of the almond emulsin solutions to a short CM 52 column, followed by elution with the application buffer as described in the Materials and Methods, yielded an enzyme preparation containing 11.4—14.2 units of glucosidase/mg protein. The enzyme was applied to a long CM 52 column and eluted with the aid of a pH gradient as described in the Materials and Methods. Fractions containing the

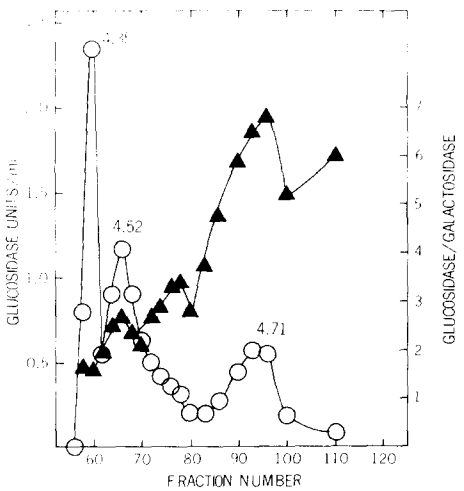

Fig. 1. β-D-Glucosidase isozyme pattern of almond emulsin. Almond emulsin (1.04 g) was suspended in 45 ml 0.05 M sodium acetate pH 3.6, and applied to a 2.5 × 40 cm CM-52 column. Elution was performed with a 0.05 M sodium acetate pH 3.6 to 0.5 M sodium acetate pH 4.9 gradient. Glucosidase activity is represented by (○———○), and glucosidase/galactosidase ratio (▲———▲). Numbers along the curve are the pH of the eluate fractions.

enzyme to be pooled had pH of 4.85 ± 0.20, and the fraction with the highest activity had a pH of 4.7. The fractionation on the second CM 52 column resulted in a further 5—7-fold purification. However, the yield at this step was extremely poor. Only half of the glucosidase activity was recovered. In another experiment, it was observed that more activity could be recovered if the elution was performed employing higher salt concentrations at pH > 4.8. However, as depicted in Fig. 1, this lead to elution of a variety of isozymes. Therefore, the purification procedure described in the Materials and Methods was employed routinely. Ultrafiltration of the CM 52 eluates through XM 50 Amicon membranes resulted in a further 1.4-fold purification without any loss of activity. The anion exchange chromatography at pH 6 employing DEAE cellulose (DE 32) resulted in a further 1.5—2-fold purification. The last step in the purification process consisted of gel filtration on a Sephadex G-100 column. This final step was employed to remove a high molecular weight protein contaminant (Fig. 2) from the preparations. The final β-D-glucosidase activity of these preparations was 210—244 units/mg protein. Results from a typical purification experiment are summarized in Table I.

Sephadex G-100 filtration eluate fractions were assayed for their β-D-glucosidase as well as β-D-galactosidase activities. The ratio of the two enzyme activities in these fractions was 3.33 ± 0.33. In order to ascertain whether further purification would lead to separation of the two activities, the Sephadex G-100 eluate was subjected to Sephadex G-200 filtration followed by ion-exchange chromatography on CM 52 carboxymethylcellulose as described in the footnote to Table I. After both the G-200 and CM 52 steps the β-glucosidase and β-galactosidase activities comigrated and, more importantly, the further purification steps did not alter the glucosidase/galactosidase activity ratio of the preparation.

The final specific activities thus obtained in the various preparations were

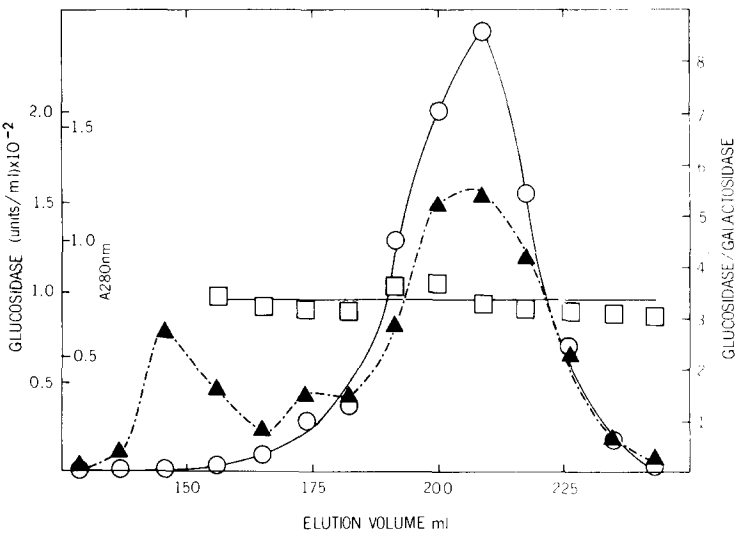

Fig. 2. Sephadex G-100 filtration, the last purification step of β-D-glucosidase. DE 32 pool fractions concentrated by ultrafiltration on XM 50 Amicon membrane containing 100 mg protein in 4.5 ml of 0.05 M sodium acetate pH 6.0 were applied to a 2.5 × 95 cm Sephadex G-100 column as described in the Materials and Methods. The various curves are: (○———○) β-D-glucosidase activity, (▲- - - - -▲)-absorbance at 280 nm and (□———□)-β-D-glucosidase/β-D-galactosidase ratio.

210—244 units glucosidase/mg protein and 64—78 units galactosidase/mg protein. The enzyme did not exhibit any detectable hydrolytic activity when > 80 μg of protein were used in assay solutions containing *p*-nitrophenyl-α-D-glucopyranoside or *p*-nitrophenyl-α-D-galactopyranoside as substrates. Routinely

TABLE I

PURIFICATION OF A β-D-GLUCOSIDASE FROM ALMOND EMULSIN

Step	Volume ml	Total activity (units)	Total protein (A_{280nm} · ml)	Specific activity	Fold purification
Almond emulsin	90	21 087	2 920	7.2	—
I CM 52 pool	66	15 464	1 261	12.2	1.7
II CM 52 pool	68	7 532	103	73.1	10.1
XM 50 concentrate	5.4	8 051	80.4	100	13.8
DE 32 pool	26	3 995	25.5	156.6	21.7
XM 50 concentrate	3.4	3 911	23.1	169.1	23.4
Sephadex G-100 pool *	—	2 377	11.3	210.2 **	29.1 ***

* 1.1 ml of the enzyme, out of a total of 3.4 ml obtained on XM 50 concentration of the DE 32 pool, was applied on a Sephadex G-100 column. However, the data have been normalized assuming all the enzyme had been used.

** Ratio of the specific activity β-D-glucosidase to β-D-galactosidase was 3.33 ± 0.33.

*** Chromatography of the preparation on Sephadex G-200 yielded a single β-D-glucosidase peak. β-D-Galactosidase activity comigrated with this peak. Sephadex G-200 fractions were pooled, concentrated by ultrafiltration through an Amicon XM-50 membrane and dialyzed against 0.1 M sodium acetate pH 3.6 buffer. The dialyzed enzyme was chromatographed on a CM-52 column by a procedure analogous to that for the II CM-52 column. The two activities β-D-glucosidase and β-D-galactosidase co-migrated. The last two steps did not alter the β-D-glucosidase to β-D-galactosidase ratio. Sodium dodecyl sulfate gel electrophoresis on the preparation revealed that the minor band in Fig. 3B had been diminished substantially in its amount.

0.1—1.6 μg of this protein were employed in assay solutions when β-D-glycoside substrates were used.

The protein obtained by this procedure had λ_{max} = 277 nm. The following extinction coefficients were measured $\epsilon_{277nm,\ 1\%}$ = 7.25, $\epsilon_{280nm,\ 1\%}$ = 7.06, $\epsilon_{290nm,\ 1\%}$ = 4.0, using 1 cm path-length cells.

Polyacrylamide gel electrophoresis

The purified enzyme preparation was subjected to polyacrylamide gel electrophoresis at pH 8.9. Only one major protein band, migrating with a relative mobility of 0.55 ± 0.01, was observed (Fig. 3A). Upon staining these gels for glycoproteins by the periodate-metabisulfite procedure, a reddish brown band migrating with a relative mobility of 0.55, was observed. The gels were also stained for β-D-glucosidase activity as described in the Materials and Methods. A faint purple band migrating with a relative mobility of 0.55 ± 0.02 was

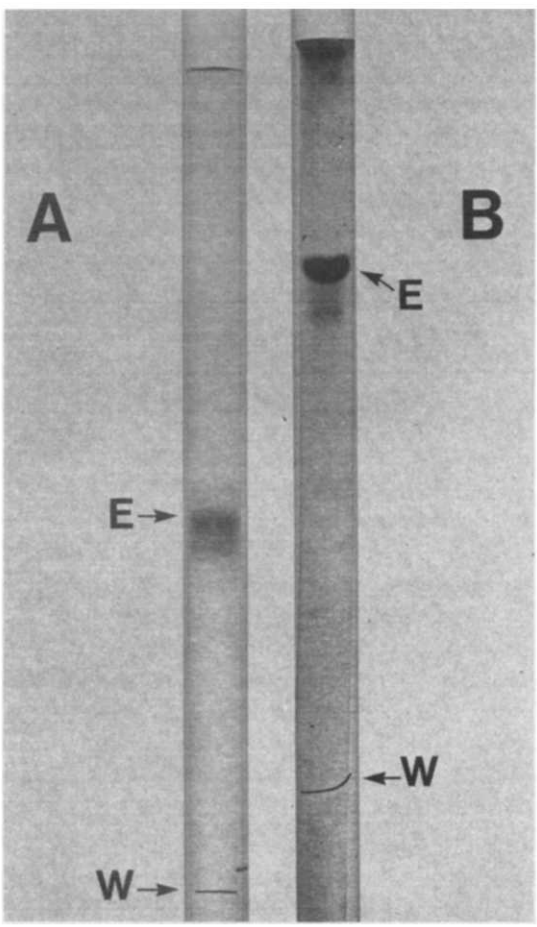

Fig. 3. Gel electrophoresis of the purified glucosidase. A piece of wire, W, was introduced to mark the position of the marker dye front and the gels were stained with Coomassie blue. Enzyme band has been labelled, E. A. Polyacrylamide gel, 8 μg protein was applied. B. Sodium dodecyl sulfate polyacrylamide gel, 40 μg protein was applied.

observed. Gels stained for β-D-galactosidase activity showed a similar band with the same relative mobility.

Isoelectric focussing

The enzyme preparation obtained by purification as described at the bottom of Table I was employed for isoelectric focussing in polyacrylamide gels. The pH gradient was linear over the range 4.0—8.5 and a single protein band, corresponding to p*I* = 7.3, was observed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The enzyme, purified by the procedure described above, was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in Materials and Methods. Only one major protein band, relative mobility = 0.30 ± 0.02, was observed (Fig. 3B). Phosphorylase, catalase, ovalbumin and glyceraldehyde-3-phosphate dehydrogenase were used as standards and a plot of relative mobility vs. log (molecular weight) is depicted in Fig. 4. The protomer molecular weights of these proteins were obtained from Weber and Osborn [12]. The plot in Fig. 4 shows a coefficient of variation equal to 0.01. Molecular weight of the enzyme band was estimated to be 65 150 ± 650.

Molecular weight of the active enzyme

Molecular weight of the active enzyme was estimated by means of Sephadex G-200 filtration in 0.05 M sodium acetate pH 6.0. Catalase, aldolase, yeast alcohol dehydrogenase and liver alcohol dehydrogenase were employed as stan-

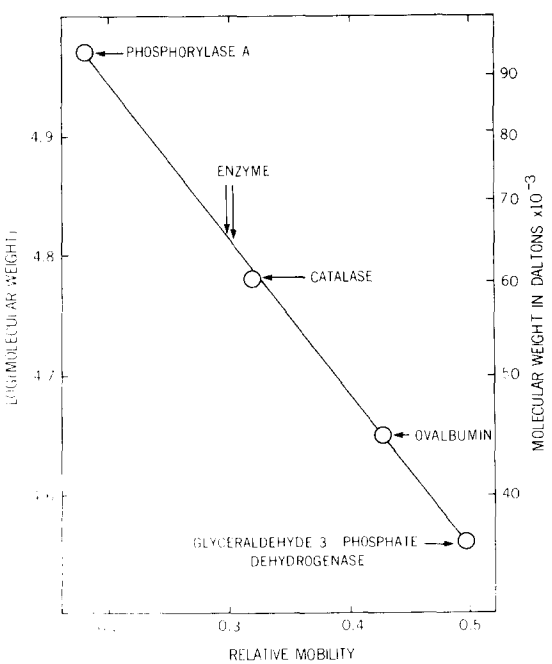

Fig. 4. Standard curve for molecular weight estimation by sodium dodecyl sulfate gel electrophoresis. Arrows indicate the position of the enzyme. See text for details.

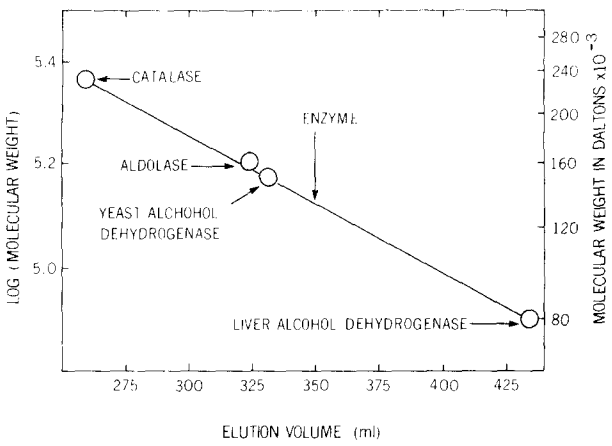

Fig. 5. Standard curve for molecular weight estimation by Sephadex G-200 gel filtration. Arrow indicates the position of the enzyme. See text for details.

dards. Molecular weights of the enzymes, as given by Klotz and Darnall [16], have been used in the plot of log (molecular weight) vs. elution volume (Fig. 5). This plot shows a coefficient of variation equal to 0.006. The elution volume of the purified β-D-glucosidase corresponded to a molecular weight of 135 180 ± 770.

Discussion

Almond emulsin has been reported to contain a variety of glucosidase isozymes. These isozymes have been observed to differ in their ion-exchange behaviour [4] and in their ratios of β-D-glucosidase/β-D-galactosidase activity [5]. In Fig. 1, we demonstrated that at least three isozymes are present in the emulsin. The number of isozymes may be more than three if any of these peaks represents a convolution of elution profiles of more than one isozyme. The three isozymes differ in their affinity for the cation exchanger carboxymethyl cellulose. The ratios of their β-D-glucosidase/β-D-galactosidase activity range from 1.6 to 6.8. A procedure for isolation of one of these isozymes to near homogeneity has been described in this communication. The isozyme isolated from the CM 52 column (cf. Fig. 2) had a pH = 4.7 and probably corresponds to a fraction in the pH 4.71 peak of Fig. 1. The specific activity of the isolated enzyme does not correspond to the activity of the pH 4.71 peak of Fig. 1 since that peak is a convolution of more than one isozyme. The overall yield of the enzyme, is only 11%. This is mainly due to the fact that the β-D-glucosidase activity in the emulsin is due to many isozymes, only one of which is predominant in the purified preparation. The second reason, of course, is that a large number of purification steps were required for removal of these contaminating isozymes. The purified preparation contains only one major protein band as determined from scans of the polyacrylamide gel electrophoresis in the absence, and presence, of sodium dodecyl sulfate. An isoelectric focussing experiment also showed the presence of only one band. The enzyme is a glycoprotein as demonstrated by staining of the polyacrylamide gels by the periodate-metabi-

sulfite procedure. This confirms the findings of the earlier workers who reported the presence of carbohydrates in their preparations [6,16,17].

The purified enzyme can hydrolyze β-D-glucopyranoside and β-D-galactopyranoside but not the corresponding α-anomers. A Sephadex G-100 filtration experiment (Fig. 2) of purified enzyme shows that the β-D-glucosidase/β-D-galactosidase activity ratio in various fractions is 3.33 ± 0.33, and, thus, constant within the limits of experimental error. Thus, the molecular weights of the enzymes responsible for the two activities are similar. Further purification using Sephadex G-200 and CM 52 did not alter this ratio nor resolve any other active components. Sodium dodecyl sulfate polyacrylamide gel electrophoresis reveals that proteins consisting of protomers of the same molecular weight must be responsible for the two enzymatic activities (Fig. 3B). The purified preparation showed a single protein band on isoelectric focusing. Polyacrylamide gel electrophoresis, again, shows a single major protein band. Finally, staining the gels specifically for the two glycosidases establishes that the same protein is responsible for the two activities.

We conclude that the two reactions are catalyzed by protein molecules of the same molecular weight and the same charge. Furthermore the polypeptides constituting the protein molecules also have the same molecular weight. However, the possibility that the two activities are due to proteins differing in the amount of carbohydrate attached to them or due to subtle differences in conformation, can not be completely ruled out. Heyworth and Walker [5] demonstrated that heating the partially purified almond emulsin preparations at 65° C lead to parallel inactivation of β-D-glucosidase and β-D-galactosidase. Thus, having been unable to show two distinct protein species by the widely varying techniques of DEAE-cellulose and CM 52 ion-exchange chromatography, Sephadex gel filtration, polyacrylamide gel electrophoresis, electrofocusing in polyacrylamide gels using an ampholine pH gradient, and sodium dodecyl sulfate polyacrylamide gel electrophoresis, we judge that two proteins, one a glucosidase and one a galactosidase, have a low probability of existence. This is in agreement with the suggestions made by previous workers [2,4—7,17,18] that the almond emulsin β-D-glucosidase and β-D-galactosidase activities are due to the same protein.

The enzyme in its active form has a molecular weight of 135 180 ± 770, as revealed by the Sephadex G-200 filtration experiments (Fig. 5). Relative mobility of the enzyme on electrophoresis in the sodium dodecyl sulfate-acrylamide gel indicates a molecular weight of 65 150 ± 650 for its protomers. The number of subunits constituting the active enzyme, therefore, is calculated to be 2.08 ± .04. Thus, the enzyme appears to be a dimer consisting of two subunits of the same molecular weight. The β-D-glucosidase isolated here differs in properties from other isozymes separated from almond emulsin. An isozyme of molecular weight 135 000 has been reported [17] but shows a glucosidase/galactosidase ratio of 9.8 ± 0.7 [6] while the isozyme reported here yields a value of 3.33 ± 0.33.

Conclusion

An isozyme of glucosidase isolated from almond emulsin, has been demonstrated to be a bifunctional protein capable of hydrolyzing β-D-glucopyrano-

sides as well as β-D-galactopyranosides. The active enzyme is a glycoprotein with a molecular weight of 135 180 ± 770, and consists of two identical subunits each with a molecular weight of 65 150 ± 650.

The influence of various inhibitors on the two activities of the enzyme, and other kinetic studies on the enzyme are reported in the accompanying paper to determine whether the same catalytic site on the protein is responsible for both β-D-glucosidase and β-D-galactosidase activities or whether two independent sites carry out these functions [19].

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