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Separation and Properties of α and β-Galactosidase and other Glycosidases from Jack Bean Meal

by

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Glycosidases are very widely distributed in Nature. α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) occurs in vegetal sources such as coffee beans (4, 11, 28), watermelon seeds (24), *Phaseolus vulgaris* (12), sweet almond (17), actinomycetes (22) and higher fungi (14); it has also been detected in rat uterus (2).

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) has been found in many animal materials from mammals (3, 8, 20), *Helix pomatia* (9), and from alfafa seed (19) and several microorganisms (7, 21, 23, 25). The protozoa *Trichomonas foetus* contains α and β-galactosidase (27).

This paper describes a partial purification and some properties and the α and β-galactosidases studied in jack bean meal. A preliminary communication has appeared (1).

Experimental Procedure

Materials. The meal from seeds of a tropical American plant *(Canavalia ensiformis)*, jack bean meal, was obtained through Sigma Co.

Bio-Gel-P-200, Sephadex-G-200, CM-, DEAE- and TEAE-cellulose, the main part of the nitrophenylglycosides employed and other chemicals were commercial products. *p*-Nitrophenyl-α-L-fucoside, *p*-nitrophenyl-α-D-fucoside, *p*-nitrophenyl-β-D-fucoside and fucone-γ-lactone were generous gifts of Prof. J. CONCHIE.

Methods. Enzyme preparations. All the procedures were carried out between 0° and 4° C. 100 g of jack bean meal were extracted with 400 ml of water

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overnight by constant stirring. After centrifugation at 8,000 r.p.m., 10 min., the supernatant was decanted; the residue was washed with a small amount of water; the mixture was centrifuged again and the supernatants were combined; solid ammonium sulfate was added to the resultant liquid, 375 ml, to obtain a 40 % saturation. After 4 hours of stirring, it was centrifuged; the supernatant was decanted and more ammonium sulfate was added until 60 % saturation. After standing one night, the precipitate was separated by centrifugation, dissolved in 0.05 M citrate buffer (pH = 4.6) and dialysed against the same buffer. (This preparation is named «crude enzyme».)

Crude enzyme was fractionated using Bio-Gel-P-200 or Sephadex-G-200. Fractionations with calcium phosphate gel, CM-, DEAE- and TEAE-cellulose were also carried out. Furthermore, the dialysed solution was fractionated, with better result, using ethanol at —15° to obtain fractions precipitating between 0 to 33, 33 to 50 and 50 to 70 % ethanol.

Enzyme assays. Specific activity was defined as micromoles of *p*-nitrophenol liberated from *p*-nitrophenylglycoside per mg of protein per min, at the optimal conditions of pH and temperature.

The reaction mixture was carried out with 0.3 μmoles of *p*-nitrophenylglycoside (in 0.3 ml), 1.1 ml of 0.05 M citrate buffer and 0.1 ml of enzyme solution. After incubation at 25° for 5 min, the reaction was stopped by addition of 1.5 ml of 0.2 M Na_2CO_3 and the *p*-nitrophenol was determined in a Zeiss MQ 2 or in a Beckman DB-G spectrophotometer at 400 mμ.

In several cases fetuin, hog submaxillary mucin, seromucoid and γ-globulin were also employed as substrates.

The peptidase activity was assayed by the colorimetric procedure of DAVIS and SMITH (5).

The conversion of L-fucose to L-fuculose was performed by a mutant of *Escherichia coli* [a gift of Prof. S. S. COHEN (10)].

Analytical methods. Protein was determined by the LOWRY *et al.* method (18) or estimated by absorption at 280 and 260 mμ.

Sialic acids were determined by the WARREN method (26); fuculose, by the DISCHE and BORENFREUND method (6).

Descending paper chromatography was performed with Schleicher & Schüll 2043b paper and 1-butanol-pyridine-0.1 N HCl (5:3:2) for 50 h; reducing sugars were detected by the $AgNO_3$ reagent.

Results

Purification of α and β-galactosidase. Jack bean meal has a very high β-N-acetylglucosaminidase activity, difficult to remove from the other glycosidases. It also contains α-mannosidase (15, 16). The separation between β-N-acetylglucosaminidase and galactosidase was achieved by filtration of the crude enzyme through Bio-Gel-P-200 or Sephadex-G-200; with Sephadex, β-Galactosidase was 40 fold purified, but the yield was poor.

Other procedures were also assayed, such as fractionations with DEAE- and TEAE-cellulose, and elution for 0.05 M to 1.0 M NaCl, but gave unsatisfactory results. Yet, CM-cellulose in columns of 18 × 1 cm (with citrate buffer, pH 4.6) permits the separation between β-galactosidase (which is eluted by 0.5 M NaCl) and β-N-acetylglucosaminidase (retained by the column).

The separation between α and β-galactosidase was attained by precipitation of the enzymic solution with ethanol at —15°. Fractions precipitating between 0 to 30, 33 to 50 and 50 to 70 % ethanol concentrations were prepared. The first fraction in enriched in α-galactosidase; β-galactosidase remains in the last supernatant, i.e. it is soluble in 70 % ethanol.

Fractionation of 1 ml of the dialysed

solution of the crude enzyme with calcium phosphate gel (dry weight 0.0035 g) gave only a partial resolution.

Properties: Effect of pH. Fig. 1 shows the differences between the optimal pH of the α and β-galactosidase. α-Galactosidase has an optimal pH around 6. β-Galactosidase has a broad optimum range between pH 4 to 5.

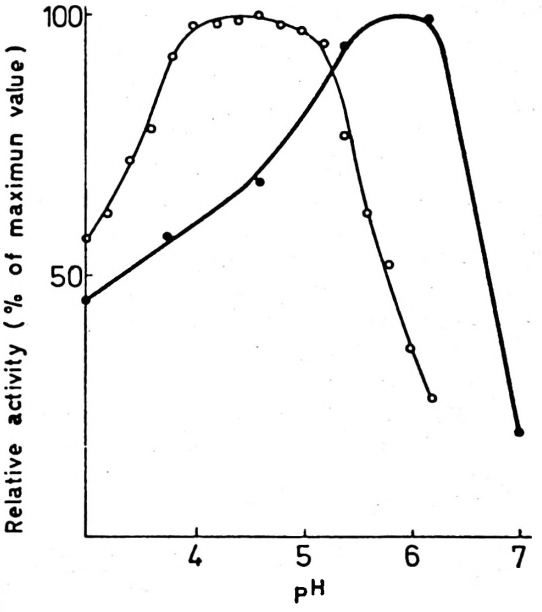

FIG. 1. Influence of pH on and galactosidase activity:

(○) α-D-galactosidase. (●) β-D-galactosidase.

Thermolability. The stability of both galactosidases seems to be different against heat. With preincubation at 62° during 15 to 30 min in water bath (at optimal pH in 0.05 citrate buffer), α-galactosidase is completely destroyed at 15 min, but β-galactosidase keeps 80 % of its original activity. (See Fig. 2.)

α and β-D-fucosidase activities were assayed in a similar manner. The results were parallel with α and β-galactosidase, respectively. β-N-Acetylglucosaminidase is

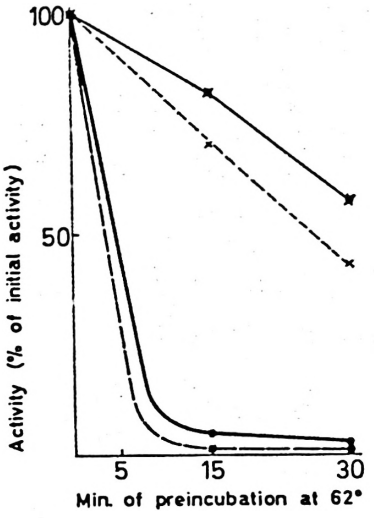

FIG. 2. Influence of heat on enzyme activities:

(●—●) α-D-galactosidase. (●- -●) α-D-fucosidase. (★—★) β-D-galactosidase. (★- -★) β-D-fucosidase.

destroyed in these conditions, after an increase of its activity at 2 ½ min of preincubation.

Time course and influence of temperature in the hydrolisis of p-nitrophenylglycosides. Fig. 3 expresses these results.

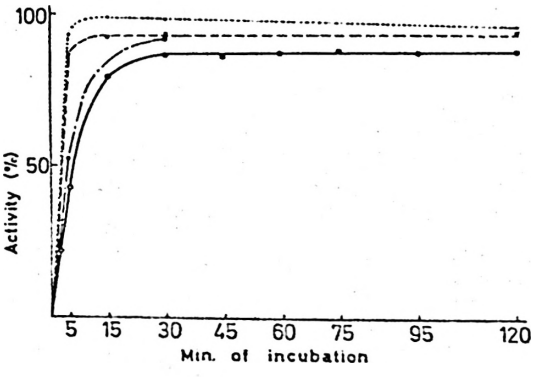

FIG. 3. Influence of temperature and time of incubation on the following activities:

(○) α-D-galactosidase, al 25°. (●) β-D-galactosidase, at 25°. (★) β-D-galactosidase, at 37°. (· · ·) β-D-galactosidase, at 50°.

β-N-acetylglucosaminidase gave practically the same values as β-galactosidase when incubated at 25, 37 and 50°.

On the other hand, the behaviour of β-fucosidase was different at different incubation temperatures as can be seen in Fig. 4.

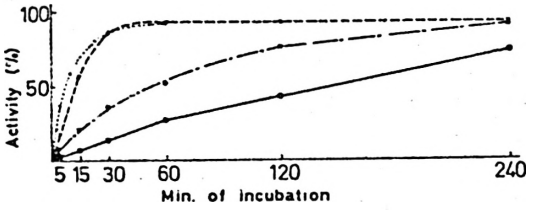

FIG. 4. Influence of temperature and time of incubation on the β-D-fucosidase activity:

(○) at 25°. (●) at 37°. (---) at 50°. (···) at 60°.

Effect of substrate concentration. Using seven different substrate concentrations ranging between 0.05 and 1.4 μmoles and the same time of incubation at optimal pH, the simple Lineweaver-Burk plots have been found to be linear for α and β-galactosidase activities.

Substrates specificities. p-Nitrophenyl derivatives were mainly employed as substrates. *o*-Nitrophenol was also liberated from *o*-Nitrophenyl-β-D-galactoside, but not as rapidly.

α-Galactosidase split natural substrates such as melibiose and raffinose. β-Galactosidase hydrolyses lactose. In both assays galactose was detected by paper chromatography.

p-Nitrophenyl-α-D-fucoside and *p*-nitrophenyl-β-D-fucoside were also hydrolysed by the crude enzyme preparation. On the other hand, no α-L-fucosidase activity was found when using *p*-nitrophenyl-α-L-fucoside.

This preparation was also employed on pig submaxillary gland mucin, fibroblastes and other substrates. We tried to determine the amount of fucose liberated by conversion of free fucose to fuculose, after treatment with *E. coli* isomerase enzyme and measuring the fuculose by the cysteine-carbazole assay (6). Little or no fucose appeared to be released with this assay. We could have detected less than 0.5 μg fucose liberated. The fucosidases apparently do not act on macromolecules.

Stability of α and β-galactosidase. The activity of the crude enzyme on *p*-Nitrophenylglycosides decreases to 25 % of the original after 17 months of storage at —5° and several freezings and thawings. The stability of ethanolic preparations and more pure fractions is considerably lower.

It seems that β-galactosidase keeps its activity in 0.05 M acetate (pH = 4.6) buffer better than α-galactosidase, while both activities decrease in a parallel manner when the buffer is 0.05 M citrate, at the same pH.

Inhibitors. Table I summarizes the results we obtained.

Discussion

When this study was started, its first aim was the search for a fucosidase which could be employed to liberate fucose from its natural compounds. We have found practically no α-L-fucosidase activity. α-D-Fucosidase and β-D-fucosidase activities were detected; but, in all cases, accompanied by α-D-galactosidase and β-D-galactosidase, respectively. We have been unable to separate both fucosidases from galactosidase activities. The behaviour against heat (62°) and inhibitors show a remarkable parallelism for α-D-galactosidase and α-D-fucosidase activities, and β-D-galactosidase and β-D-fucosidase activities. The structural configurations of α and β-D-fucopyranose are very similar to those of α and β-D-galactopyranose. LEVVY and MCALLAN (13) have found that β-D-fucosidase and β-D-galactosidase activities from

TABLE I

Influence of inhibitors against α and β-galactosidase and β-D-fucosidase activities

Inhibitor (Concentration 90 μM)	% of inhibition against		
	α-D-galacto-sidase	β-D-galacto-sidase	β-D-fuco-sidase
D-galactose	97	97	40
L-galactose	68	42	54
γ-D-galactone lactone	69	100	100
D-galacturonic acid	20	0	—
α-methyl-β-D-galactoside	87	93	49
Methyl-β-D-thiogalactoside	48	79	36
N-acetyl-D-glucosamine	0	22	30
N-acetyl-D-galactosamine	9	9	36
D-glucosamine	7	51	39
D-galactosamine	16	51	56
α-L-fucose	0	8	34
D-fucone-γ-lactone	—	84	58
Fucoidin	—	18	—
D-glucose	14	21	34
D-mannose	12	30	35
D-fructose	0	4	39
D-ribose	0	13	20
2-deoxy-D-ribose	24	6	34
D-xylose	92	33	0
L-arabinose	69	38	34
L-rhamnose	11	0	16
δ-D-glucono lactone	15	62	92
D-glucurono-lactone	0	0	0
α-methyl-D-glucoside	6	0	—
Methyl-β-D-glucoside	0	0	—
α-methyl-D-mannoside	0	0	—
Methyl-β-D-xylopyranoside	0	5	—
Lactose	17	61	—
Melibiose	96	81	—
Sucrose	5	0	—
Maltose	8	22	—
Raffinose	88	39	—
N-acetyl-lactosamine (45 μM)	37	46	—
EDTA (Na) (45 μM)	8	0	19

ox liver and rat epididymis were not easily separated. It is then possible that the two activities are carried out by the same enzyme.

As can be deduced from the introduction, α-D-galactosidases are mainly distributed in the vegetal kingdom, and β-D-galactosidases have been found in it and, probably with a wider distribution, in animal tissues. We have found β-D-galactosidase in calf serum and in mouse fibroblast membrane preparations and β-D-N-acetylglucosaminidase with a higher activity.

Jack bean meal is a rich source of urease, glycosidase, peptidases, esterases, etc. We have found no neuraminidase in it.

Summary

α and β-Galactosidases were extracted from jack bean meal; they were partially purified by precipitation with ammonium sulfate at 60 % saturation and fractionation with Bio-Gel-P-200 or Sephadex-G-200; the purification factor was about 40. Other fractionations with CM-, DEAE- and TEAE-cellulose, calcium phospate gel and ethanol were also investigated; the last procedure gave the best possibility of separation between α-galactosidase (which is found in the 0-30 % fraction) and β-galactosidase (which remains in the supernatant of the 70 % fraction).

Using *p*-nitrophenyl-α-D-galactopyranoside as substrate, the optimal pH for α-galactosidase, in citrate buffer, was 6.2 at 25°; for β-Galactosidase, in the same conditions, it was 4.6 using the β-nitrophenyl derivative. α-Galactosidase split natural substrates such as melibiose and raffinose; β-galactosidase hydrolyses lactose.

α-D-Fucosidase and β-Fucosidase activities were detected in enzymic preparations, parallel with α and β-D-galactosidase, using *p*-nitrophenyl-α and β-fucoside as substrates. We could not separate either fucosidases from the respective galactosidase activities.

With preincubation at 62° for 15 min, α-D-galactosidase is completely destroyed, but β-D-galactosidase retains 80 % of its original activity. Both galactosidases were completely inhibited by D-galactose and γ-galactono-lactone, and β-D-galactosidase was inhibited by D-xylose as well.

β-D-Galactosidase and β-D-N-acetylglucosaminidase activities were also detected in calf serum and in mouse fibroblasts.

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