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β-GALACTOSIDASE ACTIVITY OF HUMAN SKIN FIBROBLASTS

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SUMMARY

The β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) activity of human skin fibroblasts from normal individuals was investigated. Based on the Lineweaver–Burk plots two K_m values were obtained, one at $4.12 \cdot 10^{-4}$ M and the other $5 \cdot 10^{-5}$ M, suggesting the presence of at least two enzyme species. The effects of NaCl stimulation, *p*-chlorophenylmercuriosulfonate inhibition and heat denaturation supported the hypothesis of 2 distinct enzyme populations.

INTRODUCTION

Numerous investigations have revealed the presence of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) activity in a variety of mammalian tissues[1–7]. Column-chromatographic and electrophoretic separations have indicated the presence of several different species of this enzyme. At least three different forms of β-galactosidase, which differ from one another both with respect to pH optimum and relative ability to cleave a synthetic substrate or lactose[8] have been reported in intestine. Particle-free extracts of human liver, when applied to Sephadex G-150 columns, also indicated the presence of at least 3 distinct peaks of β-galactosidase activity[9]

The activity of β-galactosidase is most conveniently assayed by the release of either a chromgenic compound (*o*- or *p*-nitrophenol) or a fluorogenic product (4-methylumbelliferone) from the corresponding β-galactoside. Acetate– and citrate–phosphate buffers at varying ionic strength have been employed in assays for this enzyme. The enzyme activity has been reported to have pH optimum varying from 3.0 to 5.0 depending upon the tissues and experimental conditions employed. A diminution of β-galactosidase activity using these synthetic substrates has been reported in G_{M_1}-gangliosidosis[10–12] and Hurler's disease[7,13].

Difficulty in obtaining classical K_m values for the mammalian enzyme has been reported due to atypical plots of the data[14,15]. Ho and O'Brien[16] have demonstrated an improvement of these kinetics by NaCl, with a lowering of the K_m and no change in V, in extracts of human liver.

This paper describes several observations suggesting the presence of several β-galactosidases of human skin fibroblasts grown in culture.

MATERIALS AND METHODS

Skin biopsy samples obtained from normal adults were grown and harvested as previously described[17] and these samples were provided by Dr A. Milunsky. The cells were suspended in 1 ml distilled water and sonicated for two 30-s bursts employing a Bronson Model 125 Sonifier with a semi-micro tip.

The assay procedure was based upon the release of 4-methylumbelliferone measured fluorimetrically[18] from 4-methylumbelliferyl-β-D-galactoside (Koch–Light or Pierce Chemicals). Each tube contained 10 μmoles of citrate–phosphate buffer (pH 4.0), 0.5 μmoles 4-methylumbelliferyl-β-D-galactoside and 10–20 μg enzyme protein in a total volume of 0.2 ml. The tubes were incubated in air for 1 h at 37 °C with shaking and 0.7 ml of 2.75% trichloroacetic acid, 0.45 ml 0.5 M NaOH and 0.75 ml 0.25 M glycine buffer (pH 10.2) added. The 4-methylumbelliferone released was measured fluorimetrically in an Amino–Bowman spectrofluorimeter with the excitation wavelength at 366 nm and the emmission wavelength at 466 nm. The citrate–phosphate buffers were prepared as described[19] at a 5-fold increase in final concentration. D-Galactonolactone was a commercial product and D- and L-fuconolactone were prepared and partially converted to the (1→5) lactones according to the procedure of Levvy and Conchie[20].

RESULTS

Initial experiments were designed to examine the effect of ionic strength upon the pH–activity curve of β-galactosidase activity in fibroblast extracts. Using a buffer concentration of 10–25 μmoles/ml a broad peak from approximately pH 3 to 4.5 was obtained without a sharp discrete peak. However, when the buffer concentration was increased to 50 μmoles/ml, a symmetrical curve was obtained with a discrete optimum at pH 4.0–4.5. This is shown in Fig. 1 along with the effect of NaCl addition at a final concentration of 0.125 M. It is apparent that in the presence of salt there is a 50% increase in activity at pH 4.0, nearly a 100% increase at pH 5.0 and a 50% reduction at pH 3.5. This stimulation by NaCl was further examined in detail as shown in Fig. 2. The concentration required for maximum stimulation was found to be approximately 0.125 M. That this was not merely due to a non-specific increase in ionic strength is indicated by the data, obtained by further increasing the quantity of the buffer in the reaction mixture. It is also apparent from Fig. 2 that an inhibition occurs at higher NaCl concentration.

It was of interest to examine the effect of varying the amount of substrate added in the presence or absence of NaCl. At all substrate concentrations employed, NaCl caused an increase in measurable β-galactosidase activity as shown in Fig. 3. Both curves are unremarkable and no inhibition by high substrate concentration was observed and the absence of an S-shaped curve indicated the lack of allosterism. Lineweaver–Burk plots prepared from the experimental data are shown in Fig. 4 and it is obvious that all the experimental values do not fit on a single straight line. As a result, two K_m values can be obtained, one at $4.12 \cdot 10^{-4}$ M and the other at $5 \cdot 10^{-5}$ M,

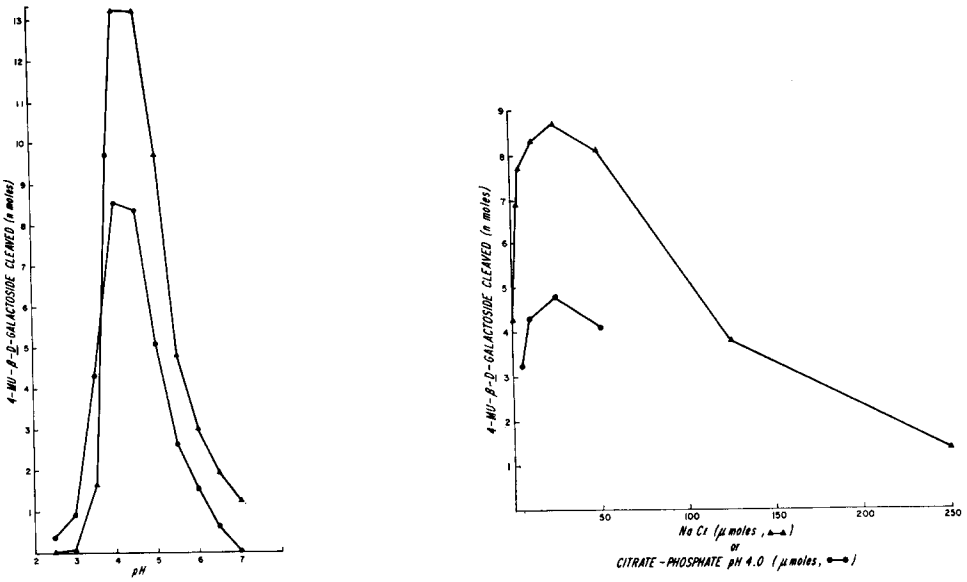

Fig. 1. pH–activity curve of skin fibroblast β-galactosidase activity. Citrate–phosphate buffers at a final concentration of 0.05 M were employed at the pH values indicated, NaCl was added at 0.125 M final concentration and 4-methylumbelliferyl(4-MU)-β-D-galactoside was present at 2.5 μmoles/ml final concentration. The final volume was 0.2 ml. The quantity of 4-methylumbelliferone released was determined as previously described. ▲—▲, NaCl present; ●—●, NaCl absent.

Fig. 2. Effect of increasing concentrations of NaCl (▲—▲) and citrate–phosphate buffer, pH 4.0, (●—●) on β-galactosidase activity of fibroblast extracts. The amount added is in terms of μmoles per incubation tube of 0.2 ml final volume. 4-mu, 4-methylumbelliferyl.

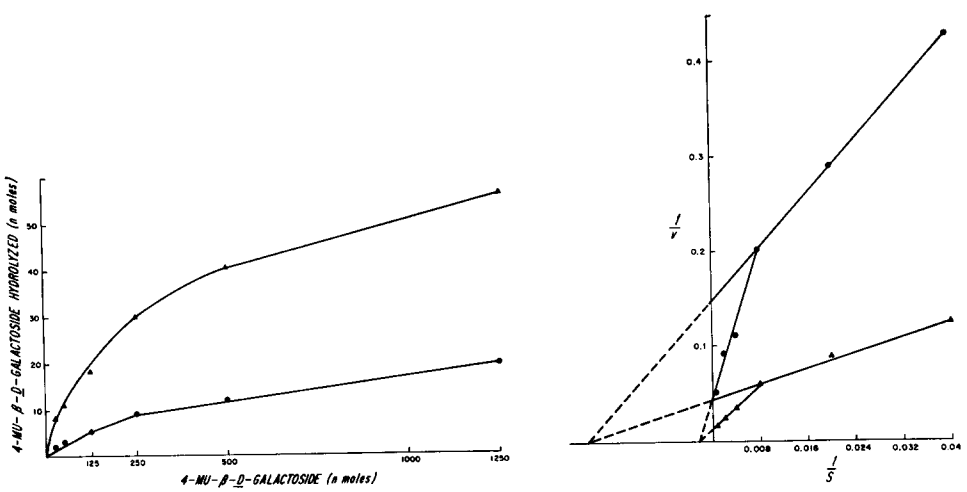

Fig. 3. Effect of increasing substrate concentration in the absence (●—●) and presence (▲—▲) of 0.125 M NaCl. Citrate–phosphate, pH 4.0, 0.05 M was the buffer employed. 4-MU, 4-methylumbelliferyl.

Fig. 4. Lineweaver–Burk plots of the effect of varying substrate concentration in the presence (▲—▲) and absence (●—●) of NaCl. The data are derived from Fig. 3.

which will be referred to as low and high affinity activities, respectively. There are no differences in the K_m values obtained after extrapolating the plots to the abcissa derived from samples incubated either in the absence or presence of NaCl. Three different V values are obtained along the ordinate, 6.89, 23.59 and 100 μmoles/h, which indicated an approximate 4-fold increase in V by the addition of NaCl. This increase in the reaction rates at higher substrate concentration has been ascribed to three conditions: (a) substrate synergism; (b) two enzymes acting on the same substrate; (c) an inflection point at the critical micellar concentration.

Since other investigators have indicated the presence of several β-galactosidases in mammalian tissues this possibility was further investigated. The operating hypothesis was that two enzymes with different affinities were present in the fibroblast extracts. The data presented in Figs 1 and 2 were obtanied with a substrate concentration of 2.5 μmoles/ml, a level at which the activity of several enzymes could presumably be measured. The pH–activity curve was reinvestigated using a lower substrate concentration of 0.125 μmole/ml which should preferentially measure the high affinity activity. The data provided in Fig. 5 illustrates the results of these determinations both in the presence and absence of 0.125 M NaCl. Two differences are apparent when this curve is compared to Fig. 1. There is a sharp pH optimum at 4.5 and the addition of NaCl causes a 3-fold increase in measured activity. The effect of varying the amount of NaCl was also investigated in the presence of low substrate concentration. The maximal activation of β-galactosidase activity was obtained at only 1/10 the quantity of NaCl required in the presence of high substrate concentration, as seen in Fig. 6. In addition, no inhibition was evident with high NaCl additions.

The inhibitory effect of p-chlorophenylmercuriosulfonate on fibroblast β-

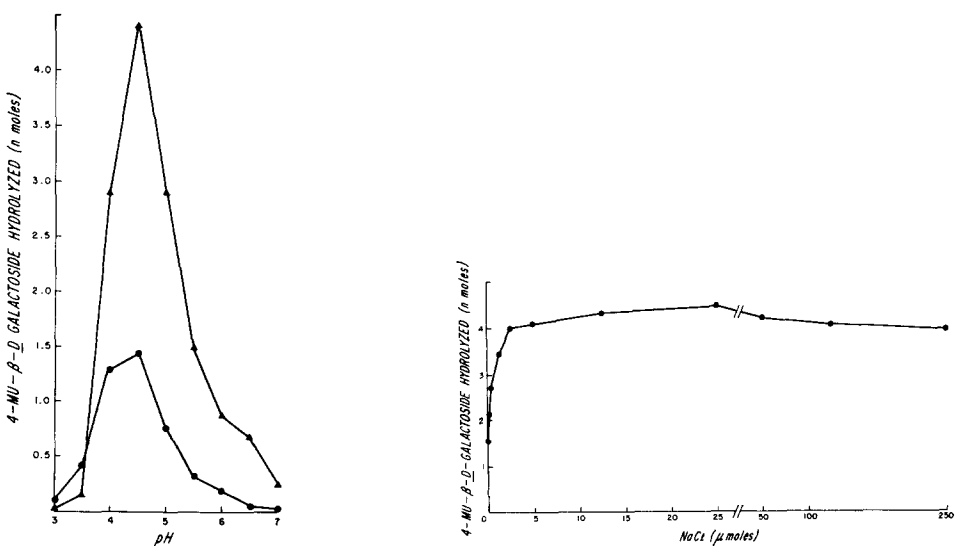

Fig. 5. pH–activity curve at low 4-methylumbelliferyl(4-MU)-β-D-galactoside concentration. The incubations were identical to those provided in Fig. 1.

Fig. 6. Effect of increasing NaCl on β-galactosidase activity at low substrate concentration. Citrate–phosphate: 50 μmoles/ml, pH 4.5; 4-methylumbelliferyl(4-MU)-β-D-galactoside. 0.125 μmole/ml.

galactosidases was described previously[17]. Studies were undertaken to determine if any quantitative differences were apparent when fibroblast extracts were assayed under conditions of high and low affinity and these results are presented in Table I. The addition of 50 nmoles of *p*-chlorophenylmercuriosulfonate under low and high affinity conditions caused inhibition of 36% and 62.5%, respectively. The presence of 125 μmoles NaCl increased the inhibition to 70% in the former case and reduced it to only 13% with the latter.

TABLE I

EFFECT OF *p*-CHLOROPHENYLMERCURIOSULFONATE ON β-GALACTOSIDASE ACTIVITY

Low affinity conditions: 2.5 μmoles/ml 4-methylumbelliferone-β-D-galactoside, 50 μmoles/ml citrate–phosphate buffer, pH 4.0. High affinity conditions: 0.125 μmoles/ml 4-methylumbelliferone-β-D-galactoside, 50 μmoles/ml citrate–phosphate buffer at pH 4.5.

Additions	*Low affinity conditions*		*High affinity conditions*	
	β-Galactosidase activity (nmoles)	*% Inhibition*	*β-Galactosidase activity (nmoles)*	*% Inhibition*
None	3.8	—	1.6	—
50 nmoles *p*-chlorophenylmercuriosulfonate	2.4	36	0.6	62.5
125 nmoles *p*-chlorophenylmercuriosulfonate	0.75	81	0.17	90
125 nmoles NaCl	9.2	—	4.1	—
125 nmoles NaCl + + 50 nmoles *p*-chlorophenylmercuriosulfonate	2.7	71	3.6	13
125 nmoles NaCl + + 125 nmoles *p*-chlorophenylmercuriosulfonate	0.8	92	0.175	96

The heat stability of β-galactosidase activity of skin fibroblasts was studied by incubating aliquots for 15 and 30 min at 42 °C in buffer, either in absence or presence of 0.125 M NaCl while another set of tubes received the NaCl at the end of the heating procedure. The samples were then assayed both under the low and high affinity conditions. The results presented in Table II indicates that the enzyme species assayed under low affinity conditions were inactivated to a greater degree than the high affinity conditions, 80% *vs* 53% at 15 min heating. The presence of 0.125 M NaCl during the heating procedure resulted in nearly a complete loss of activity. The addition of NaCl after the heat treatment, however, reduced the degree of inactivation in both cases. This sparing effect was more marked with the high affinity conditions.

An aliquot of the cell extract containing 4.6 mg protein was fractionated on a Sephadex G-150 column (1.5 cm × 20 cm bed volume) according to the published procedure for the human liver enzyme activity[9] and two peaks of activity were obtained (Fig. 7). A small peak appeared just after the V_0 and the other larger peak shortly thereafter. The smaller early peak was found to be stable to storage and have a K_m of $4 \cdot 10^{-4}$ M. The second peak was unstable and rapidly lost detectable activity on storage. This activity could be partially restored upon the addition of serum

TABLE II

HEAT STABILITY OF β-GALACTOSIDASE ACTIVITY

Low affinity conditions: 2.5 μmoles/ml 4-methylumbelliferone-β-D-galactoside; 50 μmoles/ml citrate–phosphate buffer, pH 4.0. High affinity conditions: 0.125 μmoles/ml 4-methylumbelliferone-β-D-galactoside; 50 μmoles/ml citrate–phosphate buffer at pH 4.5.

Treatment	*NaCl added (M)*	*Low affinity conditions*		*High affinity conditions*	
		β-Galactosidase activity (nmoles)	*% Inhibition*	*Galactosidase activity (nmoles)*	*% Inhibition*
None	—	2.4	—	0.55	—
None	0.125	6.3	—	2.275	—
15 min at 42 °C	—	0.5	80	0.262	53
30 min at 42 °C	—	0.28	89	0.207	63
15 min at 42 °C	0.125	0.55	92	0.337	86
30 min at 42 °C	0.125	0.25	96	0.205	91
15 min at 42 °C	0.125	3.25	49	1.83	20
30 min at 42 °C	0.125	1.72	73	1.36	41

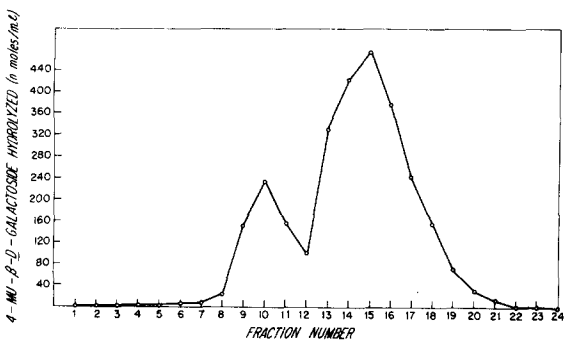

Fig. 7. Fractionation of human skin fibroblast β-galactosidase activity on Sephadex G-150 column eluted with 0.01 M phosphate buffer (pH 7.6)–0.01 M EDTA in 0.4 M NaCl. 4-MU, 4-methylumbelliferyl.

albumin to the incubation mixtures. The K_m for this more unstable fraction appeared to be in the order of 10^{-4} M.

D-Galactonolactone was found to be a competitive inhibitor with a K_i of $2.5 \cdot 10^{-4}$ M. D-Fuconolactone, a reported inhibitor of ox liver β-galactosidase[14] caused no detectable inhibition at $1 \cdot 10^{-2}$ M.

DISCUSSION

Fluharty *et al.*[13] have reported the presence of two electrophoretically distinct β-galactosidase-containing bands to occur in human skin fibroblasts. Ho and O'Brien[21] have reported 2 separate β-galactosidases from the soluble fraction obtained by hypotonic extraction of human liver, based upon heat denaturation and NaCl stimulation. The results of the present study suggests the presence of at least two β-galactosidases in human skin fibroblasts. Based on the kinetics of Lineweaver–Burk plots these have been operationally referred to as low and high affinity forms. The presence

of separate enzyme species is also suggested by differences in (a) degree of stimulation by NaCl (Figs 1 *vs* 5, and 2 *vs* 6), (b) inhibition by *p*-chlorophenylmercuriosulfonate (Table I) and (c) heat stability (Table II).

Ho and O'Brien[16] reported the ability of NaCl to stimulate the soluble β-galactosidase activity of human liver and indicated this specifically occurred at pH 4.5. The results with the fibroblast extracts indicates a general stimulation by NaCl at all pH values examined.

Several investigators have commented upon the difficulty of obtaining K_m values for mammalian β-galactosidases[14,15]. The general reported observation appears to be a change in the slope with a downward inflection at higher substrate concentration as shown in Fig. 4. Ho and O'Brien[16] have reported that with the solubilized human liver enzymes this change disappears when NaCl is added to the incubation mixtures. The fibroblast extract activity shows this phenomena both in the presence and absence of NaCl.

The data assembled for human skin fibroblasts appears to indicate that the pH optimum is 4 or 4.5 and this activity is stimulated by the presence of 0.125 M NaCl. The observation obtained suggests, but does not prove unequivocally, that there are two distinct species of β-galactosidase activity. It should be noted that three peaks of activities have been obtained for both human liver and rat intestinal homogenates. In the studies on the intestine one of these peaks has a pH optimum close to neutrality with relatively high activity towards lactose and low activity towards the unnatural synthetic β-galactosides[8]. The third peak from human liver appears to be associated with β-glucosidase activity and has a pH optimum near 5.6 (ref. 22) and is also correlated with one of the three bands seen on electrophoresis[21]. It should be noted that Fluharty *et al.*[13] were only able to detect two bands on electrophoresis of human skin fibroblasts, supporting the present finding of only two peaks on Sephadex column chromatography.

The use of 4-methylumbelliferyl- and *p*-nitrophenyl-β-D-galactosides have been useful in detecting the enzyme defect in G_{M_1}-gangliosidosis and Hurler's syndrome. Operationally one assays samples under optimal conditions of pH, ionic strength, substrates *etc.* in order to yield maximal hydrolysis obtained with control samples. It is conceivable that in certain metabolic diseases it will be necessary to adapt the assay conditions so that only certain species of the hydrolytic enzyme activities are detected.

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