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THE ASSESSMENT OF LYSOSOMAL GLYCOSIDASES IN NORMAL SKIN

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SUMMARY

A new, reproducible method for the extraction and assay of four lysosomal glycosidases, chitobiose acetamidodeoxyglucohydrolase, EC 3.2.1.29 (chitobiase), β-D-galactoside galactohydrolase, EC 3.2.1.23 (β-galactosidase), β-D-glucuronide glucuronohydrolase, EC 3.2.1.31 (β-glucuronidase) and β-D-mannoside mannohydrolase, EC 3.2.1.25 (β-mannosidase) in skin is reported. Skin biopsies from normal human skin were frozen, sectioned on a cryostat at 6-μm intervals and homogenized for 2 min. Kinetic characteristics were evaluated, and the enzymes were demonstrated to be stable under the assay conditions utilized. Assays were then performed and the results reported as μmoles of product released per g of soluble protein per min of incubation. All results gave specific activities 8–10-fold higher than those previously reported.

INTRODUCTION

Lysosomal enzymes are present in many different mammalian tissues including liver, kidney, spleen, leucocytes, synovial tissue and skin. Since skin is the most readily available of all solid tissues, it would be very helpful to have a rapid and reproducible method for the accurate and reproducible assessment of lysosomal enzyme activity. However, because of its high content of collagen and other insoluble connective tissue components, skin has proven difficult to work with. Initial demonstrations of the presence of lysosomal enzymes in skin were done utilizing histochemical techniques[1–3]. More recently, quantitative assays have been devised utilizing either skin homogenates[4] or lyophylized skin[5]. The purpose of the present study is to report on a new technique for evaluating the lysosomal glycosidases of skin, specifically chitobiose acetamidodeoxyglucohydrolase, EC 3.2.1.29 (chitobiase), β-D-galactoside galactohydrolase, EC 3.2.1.23 (β-galactosidase), β-D-glucuronide glucuronohydrolase, EC 3.2.1.31 (β-glucuronidase) and β-D-mannoside mannohydrolase, EC 3.2.1.25 (β-mannosidase).

MATERIALS AND METHODS

Chemicals

p-Nitrophenyl-β-glucuronide was obtained from Calbiochem, La Jolla, Calif., U.S.A., *p*-nitrophenyl-β-*N*-acetylglucosaminide, *p*-nitrophenyl-β-galactopyranoside, *p*-nitrophenyl-β-mannopyranoside and bovine serum albumin were obtained from Sigma, St. Louis, Mo., U.S.A.

Enzyme preparation

Skin was obtained from the deltoid region of the shoulder using a standard 4-mm punch biopsy technique. The biopsy was dissected free of any fatty tissue, washed in 0.15 M KCl to remove any adherent blood or "tissue juice" and frozen at −20 °C. The frozen biopsy specimens, which maintained full activity for at least 7 days, were then sectioned using an International Harris Cryostat, Model C.T.D., using a 6-μm micrometer setting. The sections were placed in 1.0 ml of 0.15 M KCl and homogenized at high speed for 2 min using a Virtis 23 homogenizer. The suspension and wash (0.15 M KCl), approx. 2 ml, were then centrifuged at 14 500 × *g* for 20 min at 4 °C in a Servall RC-2. The supernatant was removed and used as the source of all enzyme preparations.

Enzyme assay conditions

The assay conditions for each enzyme are shown in Table I. The incubation mixtures for chitobiase, β-galactosidase and β-glucuronidase were maintained at 37 °C for 2 h. The reactions were terminated by the addition of 50 μl of 10% NaOH. The assay is based on the enzymatic release of *p*-nitrophenol which, in contrast to the intact glycoside, forms a chromophore under alkaline conditions. Absorbance at 400 nm was measured in a Beckman DU-2 spectrophotometer. The incubation mixture for β-mannosidase was also maintained at 37 °C for 2 h. This reaction mixture was stopped by the addition of 250 μl of 2% Na_2CO_3 in 0.1 M NaOH. The remainder of the assay was done as described for the other enzymes. Enzyme and substrate controls were done with each experiment.

Specific activity

The specific activity of the enzymes is defined as the μmoles of *p*-nitrophenol released/min per g of soluble protein under the appropriate optimal conditions. Protein was measured using the Folin–Ciocalteu method (Lowry method)[7].

RESULTS

Preparation of the enzyme

Initial experiments were carried out using cadaver skin. All final experiments were done using normal human skin. The skin was cut into small pieces and then placed in a solution of either 0.25 M sucrose or 0.15 M KCl. This suspension was homogenized for at least 1 min, centrifuged and then was assayed for enzyme activity. Using this method, it was soon apparent that the size of the skin particles influenced the amount of enzyme liberated and, consequently, reproducibility. The smaller the initial fragments obtained, the greater was the amount of enzyme liberated. There-

TABLE I

OPTIMAL ENZYME ASSAY CONDITIONS

All assays were performed with a final volume of 250 μl and incubated at 37 °C for 120 min.

	Enzyme (μg of protein)	*Substrate*	*Buffer*
Chitobiase	5–25	2.0 μmoles of *p*-nitrophenyl-β-*N*-acetylglucosamine	25 μmoles of potassium citrate–citric acid, pH 4.0
β-Glucuronidase	5–25	1.5 μmoles of *p*-nitrophenyl-β-glucuronide	50 μmoles of sodium acetate–acetic acid, pH 4.0
β-Galactosidase	5–25	1.0 μmole of *p*-nitrophenyl-β-galactoside	50 μmoles of sodium acetate–acetic acid, pH 4.0
β-Mannosidase	5–25	1.5 μmoles of *p*-nitrophenyl-β-mannoside	25 μmoles of sodium acetate–acetic acid, pH 3.5

fore, in order to obtain smaller fragments of skin, a procedure which involved freezing the skin biopsy specimen and sectioning it with a microtome was carried out. It would have been desirable to weigh the samples at this point. However, because of the variable amount of "ice" transferred with each sample, we felt that this data would not be reliable. This suspension was then homogenized, centrifuged and assayed for enzyme activity. Table II shows the results of comparable experiments with and without the freezing and sectioning step. We also attempted to freeze–thaw specimens and then homogenize them with results comparable to the no-freezing data in Table II.

TABLE II

EVALUATION OF PREPARATIVE CONDITIONS

Typical results of single experiments using paired skin samples. Results are reported in μmoles of product released/g of soluble protein per min of incubation. The first experiment compares the results of freezing and sectioning and no-freezing step. The second experiment compares results obtained with 0.15 M KCl and 0.25 M sucrose after the initial freezing and sectioning procedure.

Enzyme	*Freezing and sectioning*	*No freezing*	*0.15 M KCl*	*0.25 M sucrose*
Chitobiase	15.0	3.1	17.4	12.5
β-Galactosidase	4.5	1.7	2.9	1.8
β-Glucuronidase	4.3	0.7	3.3	1.4

The optimal solution in which to suspend the enzymes was also investigated and, although 0.25 M sucrose did have significant activity, 0.15 M KCl was consistently more active as is shown in Table II.

Specific activity was optimal at 90–180 s of high-speed homogenization, using a Virtis 23 homogenizer (Table III). We chose to use 120 s of homogenization in all of our final experiments.

The final enzyme preparation could be kept at 0–4 °C for up to 48 h without significant loss of enzyme activity for any of the enzymes investigated.

The optimal buffer system was determined for each of the four enzymes. These results are presented in Fig. 1. Chitobiase and β-galactosidase have a peak activity around pH 4.0 using potassium citrate–citric acid buffer. β-Glucuronidase activity

TABLE III

THE EFFECT OF HOMOGENIZATION TIME ON ENZYME ACTIVITY

All experiments were performed as described under Methods in 0.15 M KCl following cryostat sectioning using a Virtis 23 homogenizer at high speeds. All results are expressed as spec. act. of the enzyme.

Enzyme	*Homogenization time (s)*					
	0	*30*	*60*	*90*	*120*	*180*
Chitobiase	4.7	10.8	16.6	36	36	33
β-Galactosidase	—	1.0	1.3	2.2	2.1	2.1
β-Glucuronidase	1.8	2.4	3.9	6.2	6.2	6.0
β-Mannosidase	—	—	1.0	1.7	1.8	1.8

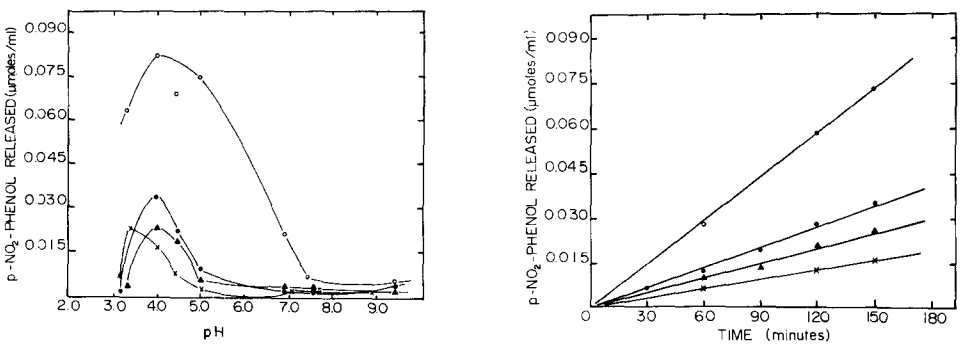

Fig. 1. All experiments were performed as described under Methods except for varying buffers and pH as shown on the abscissa. Buffers used in each experiment include: sodium acetate–acetic acid, pH 3.5 and 4.0; potassium citrate–citric acid, pH 3.2, 4.0 and 4.3; potassium phosphate at pH 6.9 and 7.4; ammonium acetate at pH 7.4; Tris buffer at pH 7.4 and glycine buffer at pH 9.2. The ordinate represents μmoles of *p*-nitrophenol released per ml of incubation mixture. Chitobiase (○—○) and β-galactosidase (●—●), both had optimum activities at pH 4 with potassium citrate–citric acid buffer. β-glucuronidase (▲—▲) activity was maximal at pH 4 with a sodium acetate–acetic acid buffer while β-mannosidase (×—×) had a pH optimum at 3.5 with sodium acetate–acetic acid buffer.

Fig. 2. All experiments were performed as described under Methods except for varying periods of incubation as noted on the abscissa. The ordinate represents μmoles of *p*-nitrophenol released per ml of incubation mixture. Chitobiase (○—○), β-galactosidase (●—●), β-glucuronidase (▲—▲) and β-mannosidase (×—×) activities were linear for at least 150 min.

is maximal with sodium acetate–acetic acid buffer at a final pH of 4.0 and β-mannosidase has a pH optimum of 3.5 with sodium acetate–acetic acid buffer.

All enzyme reactions were linear with respect to time (Fig. 2) and protein concentrations (Fig. 3) for at least 2 h. All reactions were also carried out at substrate saturation levels.

Following the establishment of the appropriate assay conditions for each of the four enzymes, specific activity was determined in normal human skin. The results of these experiments are presented in Table IV as range of activity and mean for biopsies from seven different individuals.

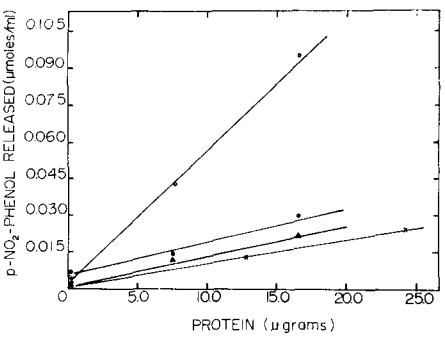

Fig. 3. All experiments were performed as described under Methods except for varying protein concentrations as shown on the abscissa. The ordinate represents μmoles of *p*-nitrophenol released per ml of incubation mixture. Chitobiase (○—○), β-galactosidase (●—●), β-glucuronidase (▲—▲) and β-mannosidase (×—×) activities were linear with respect to all protein concentrations used in the experiments.

TABLE IV

ENZYME ACTIVITY IN NORMAL SKIN

All results are expressed as spec. act. of the enzyme. S.D. represents the standard deviation from the average.

Enzyme	*Samples*	*Range*	*Average*
Chitobiase	7	15.0–29.2	22.7 S.D. ± 5.0
β-Galactosidase	7	3.0– 5.7	3.8 S.D. ± 0.85
β-Glucuronidase	7	3.8– 8.3	5.9 S.D. ± 1.6
β-Mannosidase	7	2.5– 3.3	2.9 S.D. ± 0.31

DISCUSSION

The initial work done on quantitating lysosomal enzymes in skin was done by Öckerman[4,8]. The results in the present study are approximately 10-fold higher for the three enzymes analyzed in both his work and in ours, (chitobiase, β-galactosidase and β-glucuronidase). Results similar to those of Öckerman were obtained by Goldberg *et al.*[6] in their normal subjects, again using the same three enzymes and by Clausen *et al.*[5] with β-galactosidase. Levin *et al.*[9] report levels of activity in skin only 25–50% lower than our values, but they were measuring μmoles of product released per g of tissue protein and not per g of soluble protein.

The higher values reported in this study seem most likely due to methodology. Öckerman has reported on two different enzyme preparations. In the first method[4], he freezes the skin in CO_2 and then homogenizes the sample. He notes that there are unbroken pieces of skin which he has to remove. A second method reported by Öckerman[8] involves homogenization of the skin biopsy in cold 0.25 M sucrose, centrifugation of the sample and then assay. Other workers[5] have immediately frozen their specimens, lyophylized them and then homogenized the specimens. Results in all of these studies, when expressed as μmoles of substrate released/g of soluble protein per min of incubation were all similar and as noted, 10–12% of those obtained in this study. The present method differs from those previously described

primarily in that our total biopsy specimen is cryostat-sectioned prior to homogenization. It is postulated that sectioning at 6-μm intervals disrupts nearly all cells and facilitates enzyme release.

The advantage of 0.15 M KCl over sucrose, which was used by Öckerman[4], may have also played a role in increased activity in our preparation.

The assay condition for each of the enzymes described did not vary greatly from previously reported data[4,5,8], with the pH optimum being similar both to previous reports on skin specimens and other tissues including liver, kidney, synovial membrane, synovial fluid and leukocytes.

The ease and reproducibility of this method will allow better quantitative evaluation of these acid hydrolases in skin specimens from various pathological conditions. Though we have not measured other soluble enzymes of the skin, there is no reason not to believe that this methodology might allow better quantitation of these enzymes also.

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