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MULTIPLE CARBOHYDRATE-CLEAVING SPECIFICITIES IN HUMAN ACIDIC AND NEUTRAL GLYCOSIDASES

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Summary

The common identity of human acidic β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and β-D-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) as one enzyme and that of acidic β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), β-D-fucosidase (no allotted EC number) and α-L-arabinosidase (α-L-arabinofuranoside arabinohydrolase, EC 3.2.1.55) as another enzyme is indicated by similar binding patterns of glycosidase activities of each enzyme to various lectins, by similar ratios between their intra- and extracellular levels in normal and I-cell fibroblasts and by their deficiencies in liver tissues from patients with Gaucher disease and GM_1 gangliosidosis, respectively. A third enzyme, neutral β-D-galactosidase, purified to homogeneity from human liver has been shown to possess all these five glycosidase activities at neutral pH. These neutral enzymic activities were not bound by any of the lectins examined and found to be reduced in liver and spleen of a patient with neutral β-D-galactosidase deficiency. An additional form of β-D-xylosidase with optimal activity at pH 7.4 was bound by the fucose-binding lectin from *Ulex eurpaeus* while no binding was observed for the acidic (pH 4.8) and neutral (pH 7.0) β-D-xylosidase activities of the multiple glycosidase enzymes.

Introduction

Glycosidases are the enzymes responsible for the catabolism of carbohydrate-containing compounds. Multiple forms of specific glycosidases have been described in human tissues. Some of these enzyme forms have been proposed to exhibit more than one carbohydrate-cleaving specificity.

Öckerman [1] separated β-D-galactosidase (β-D-galactose galactohydrolase,

EC 3.2.1.23) activity of human liver into three forms, one of which was associated with β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and β-D-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) activities. In a later study [2], homogenates of liver from three patients with Gaucher disease were shown to be profoundly deficient in β-D-fucosidase (no allotted EC number) as well as β-D-glucosidase activities. Recently, Chiao et al. [3] described a case of subacute neuropathic Gaucher disease in which liver, kidney and fibroblasts were shown not only to be glucocerebrosidase (D-glucosyl-*N*-acylsphingosine glucohydrolase EC 3.2.1.45) deficient [4] but also greater than 90% deficient in the activities of soluble β-D-glucosidase, β-D-xylosidase and β-D-galactosidase. Two isozymes of membrane-bound β-D-glucosidase were identified in cultured lymphoid cells and peripheral blood leukocytes but only the acidic form could be detected in cultured skin fibroblasts and only this form was severely reduced in the respective cells of patients with Gaucher disease [5].

A form of β-D-galactosidase which is deficient in GM_1 gangliosidosis has been purified from human liver and found also to possess β-D-fucosidase and α-L-arabinosidase (α-L-arabino-furanoside arabinohydrolase, EC 3.2.1.55) activities [6]. Another form of human liver β-D-galactosidase with a neutral pH optimum could not be separated from the neutral form of β-D-glucosidase by starch gel electrophoresis or gel filtration and both activities were similarly affected by Cl^- and heat inactivation [7]. Association of one form of β-D-galactosidase from human kidney with β-D-glucosidase activity was suggested on the basis of inhibition by lactones and their absence from urine [8]. Finally, common identity of five neutral glycosidases namely β-D-galactosidase, β-D-glucosidase, β-D-xylosidase, β-D-fucosidase and α-L-arabinosidase has been shown by competitive substrate inhibition in an enzyme isolated from human liver [2].

In the present study, we further examined the possible association of several glycosidase activities to acidic and neutral β-D-glucosidases and β-D-galactosidases. The existence of three multiple glycosidase enzymes was suggested on the basis of the following criteria: (1) Levels of glycosidase activities in tissues from patients with Gaucher disease, GM_1 gangliosidosis and neutral β-D-galactosidase deficiency. (2) Binding profiles of six different lectins. (3) Ratios between intra- and extracellular levels in normal and I-cell fibroblasts. (4) Specific activities in purified enzyme preparations.

Materials and Methods

Enzyme assays. Enzyme activities were assayed using 4-methylumbelliferyl glycosides (Research Products International, Elk Grove Village, IL, U.S.A.) as previously described [9]. Final substrate concentrations were as follows: 1 mM for β-D-galactoside and 2-deoxy-2-acetamide-β-D-glucoside; 2 mM for β-D-glucoside, β-D-xyloside, β-D-fucoside, α-L-arabinoside, α-D-glucoside, α-D-galactoside and α-D-mannoside; 5 mM for β-D-glucuronide. Glycosidase activities were assayed in 0.1 M citrate/phosphate buffer at varying pH values in order to determined the appropriate pH at which acidic and neutral forms could be measured in presence of each other. These values were chosen for maximal discrimination between acidic and neutral activities with retention of as high activity level as possible. For acidic activities, pH 4.3 was found suitable except

for β-D-glucosidase which was assayed at pH 4.0 and β-D-xylosidase at pH 4.8. Neutral activities were assayed at pH 6.2 except for β-D-glucosidase which was assayed at pH 6.5; β-D-xylosidase and β-D-fucosidase at pH 7.0 and the additional form of β-D-xylosidase at pH 7.4. Arylsulfatases A and B were assayed using *p*-nitrocatechol sulfate (Sigma, St. Louis, MO, U.S.A.) as described by Shapira and Nadler [10]. Glucocerebrosidase activity was assayed using *N*-[^{14}C]stearoyl glucosylceramide as previously described [11].

Protein determinations. Protein concentration were measured using the method of Bradford [12] with reagent kit supplied by Bio-Rad (Richmond, CA, U.S.A.); bovine γ-globulin served as standard.

Preparation of tissues. Human liver and spleen samples were obtained at autopsy from normal controls and patients who had been diagnosed as having infantile Gaucher disease, GM_1 gangliosidosis type I, or neutral β-D-galactosidase deficiency [13,14]; tissues were stored at −20°C prior to use. Samples were homogenized in 5 volumes of 20 mM sodium phosphate buffer, pH 7.0, sonicated three times for 20 s in an ice bath, then centrifuged at 26 000 rev./min for 30 min at 4°C on a Beckman Ultracentrifuge (Model L). Supernatants were assayed for enzymic activities.

Fibroblast cultures. Skin fibroblasts from five normal and three individuals diagnosed as having mucolipidosis II (ML II; I-cell disease) were obtained by skin biopsy and grown to confluency as previously described [15]. The medium was changed to Eagle's minimal essential medium (Gibco, Grand Island, NY, U.S.A.) 24 h prior to harvesting. Both medium and cells were collected, the medium was concentrated by negative pressure ultrafiltration and the cell pellets were washed and lysed [15]. The concentrated medium and cell lysates were assayed for enzymic activities. Intracellular levels of activity were expressed as nmol/h per mg cell protein. Levels of enzymic activity secreted into the medium were expressed relative to the intracellular activity as: medium activity (nmol/h) per intracellular activity (nmol/h).

Lectin-binding experiments. Immobilized lectins (2.0—2.2 mg lectin/0.5 ml agarose): concanavalin A, soy bean agglutinin, wheat germ agglutinin, *Ulex europaeus* agglutinin I, *Bandieraea simplicifolia* lectin and *Ricinus communis* agglutinin I (Vector, Novato, CA, U.S.A.) were mixed with 0.5 ml liver supernatant at 37°C for 1 h. Immobilized lectin with adsorbed proteins from the lysate was removed by passage through pasteur pipette columns containing glass wool. Eluates were assayed for enzymic activities. The extent of binding of activity by each lectin was calculated as: % bound = (initial activity − unbound activity)/initial activity (×100).

Purification and characterization of neutral β-galactosidase. Neutral β-D-galactosidase was purified from human liver as previously described [16]. The enzyme preparation was characterized as being a single species by isoelectric focusing, gel filtration and immunotitration with an antibody prepared to the purified enzyme. The enzyme preparation had been stored at −70°C prior to the present studies.

Results

Table I summarizes the assays of six lysosomal hydrolases in liver supernatants from two patients with GM_1 gangliosidosis type 1, one patient with

TABLE I

GLYCOSIDASE ACTIVITIES IN LIVER SAMPLES FROM FIVE NORMAL CONTROLS, TWO PATIENTS WITH GM_1 GANGLIOSIDOSIS TYPE I AND ONE PATIENT WITH INFANTILE GAUCHER DISEASE

Glycosidase activities are in nmol/h per mg protein.

Glycosidase	Normal controls activity (range)	GM_1 gangliosidosis activity (% of control)	Gaucher disease activity (% of control)
β-D-Galactosidase	138 (132—143)	1.07 (0.78)	121 (87.7)
β-D-Fucosidase	29.5 (29.0—30.0)	1.72 (5.83)	34.9 (118.3)
α-L-Arabinosidase	31.1 (23.5—44.9)	0.50 (1.61)	26.0 (83.6)
β-D-Glucosidase	6.48 (4.6—7.65)	4.8 (74.1)	1.32 (20.4)
β-D-Xylosidase	1.43 (1.17—1.60)	1.51 (105.6)	0.22 (15.4)
α-D-Mannosidase	146 (118—180)	128 (87.7)	175 (119.9)

infantile Gaucher disease and three normal controls. In addition to the deficiency of β-D-galactosidase activity (0.78% of control) for the GM_1 gangliosidosis livers, β-D-fucosidase and α-L-arabinosidase activities were also markedly reduced; β-D-glucosidase, β-D-xylosidase and α-D-mannosidase activities were near normal or normal. The liver of the patient with Gaucher disease showed a reduction of activity of β-D-glucosidase activity; under the assay conditions of these experiments, 20.4% of normal activity was observed although assays using radiolabelled natural substrate, glucosylceramide, demonstrated a much more marked deficiency of glucocerebrosidase activity (4.5% of control). In addition to the reduction of β-D-glucosidase activity, β-D-xylosidase activity was also significantly reduced to 15.4% of control: β-D-fucosidase, β-D-galactosidase, α-L-arabinosidase and α-D-mannosidase activities were within the normal range.

Table II presents the data for the assay of seven hydrolases in a spleen supernatant from a patient with neutral β-D-galactosidase deficiency [13] and three normal controls. These activities were assayed at the neutral and acidic pH values described in Materials and Methods. Because of the extensive overlap of the acidic activities into the neutral pH region of β-D-galactosidase [7], the data are best expressed as the ratio of enzymic activity observed at neutral pH to the activity observed at acidic pH. The five neutral activities: β-D-galactosidase, β-D-glucosidase, β-D-xylosidase, β-D-fucosidase and α-L-arabinosidase were

TABLE II

RATIO OF NEUTRAL TO ACIDIC GLYCOSIDASE ACTIVITIES IN SPLEEN FROM THREE NORMAL CONTROLS AND A PATIENT WITH NEUTRAL β-D-GALACTOSIDASE DEFICIENCY

Glycosidase	Normal controls mean (range)	Neutral β-D-galactosidase deficiency
β-D-Galactosidase	0.27 (0.23—0.31)	0.10
β-D-Fucosidase	0.45 (0.41—0.48)	0.06
α-L-Arabinosidase	0.25 (0.24—0.28)	0.09
β-D-Glucosidase	2.15 (1.87—2.46)	0.11
β-D-Xylosidase	0.58 (0.56—0.61)	0.19
α-D-Mannosidase	1.73 (1.65—1.84)	1.59
β-D-Glucuronidase	1.35 (1.24—1.44)	1.28

found to be reduced in the patient with neutral β-D-galactosidase deficiency, the ratio of neutral to acidic glycosidase activities was 2.7—19 fold lower than that of normal controls. The most extensive reduction was for β-D-glucosidase activity which has a 2 fold higher neutral activity than acidic activity in the normal spleen supernatant. Two other hydrolases, α-D-mannosidase and β-D-glucuronidase showed no alterations in the ratio of neutral to acidic activities in affected spleen compared to the controls. Similar findings were obtained in a comparison of β-D-galactosidase-deficient liver and normal controls although more overlap between acidic and neutral activities was observed.

The data in Tables I and II, in addition to the results of other investigators, suggested that these hydrolases may be placed in three groups which may represent three distinct enzyme proteins. Thus, the behavior of these three groups of enzymic activities in several experimental systems were examined. Group A consisted of acidic β-D-glucosidase and β-D-xylosidase activities; group B consisted of acidic β-D-galactosidase, β-D-fucosidase and α-L-arabinosidase, activities, and group C consisted of neutral β-D-galactosidase, β-D-glucosidase, β-D-xylosidase, β-D-fucosidase and α-L-arabinosidase activities.

Table III presents the data for the binding of group A and B activities and four other hydrolase activities to six immobilized lectins of differing carbohydrate specificities. Titration experiments using 0.5 ml liver supernatants and increasing amounts of immobilized lectins have shown that the quantity of each lectin (2.0—2.2 mg) in 0.5 ml of the agarose-bound preparations is in excess to the glycosidase activities tested. The binding of both group A activities were nearly identical for all six lectins and differed in their profiles from the four controls as well as from group B activities. Similarly, group B activities were all bound to the same extent by each lectin. These activities also differed in their profile from both group A and the controls, although some resemblance in the profiles are observed to α-mannosidase; this latter activity is unaffected in GM_1 gangliosidosis (Table I) where group B activities are all deficient. No binding of group C activities to any of the lectins was observed although when β-D-xylosidase activity was assayed at a more alkaline pH (7.4)

TABLE III

PERCENT BINDING OF HUMAN LIVER ACIDIC HYDROLASES BY IMMOBILIZED LECTINS

Con A, concanavalin A; SBA, soybean agglutinin; WGA, wheat germ agglutinin; UEAI, *U. europaeus* agglutinin I; BSL, *B. simplicifolia* lectin; RCAI = *R. communis* agglutinin I.

	Enzymic activity	Con A	SBA	WGA	UEAI	BSL	RCAI
Control	α-D-Galactosidase	96	81	85	3	17	4
	α-D-Mannosidase	90	25	81	20	81	64
	β-D-Hexosaminidase	95	3	85	3	13	6
	Arylsulfatase A	78	4	25	9	9	8
Group A	β-D-Glucosidase	83	18	34	4	44	18
	β-D-Xylosidase	88	19	40	5	41	19
Group B	β-D-Galactosidase	91	30	76	11	56	33
	α-L-Arabinosidase	93	29	80	10	59	33
	β-D-Fucosidase	91	31	76	13	61	34

TABLE IV

INTRACELLULAR LEVELS OF ACIDIC HYDROLASE ACTIVITIES IN I-CELL FIBROBLASTS

Normal fibroblasts, $n = 5$; I-cell fibroblasts, $n = 3$.

	Enzymic activity	% of normal	
		Mean	Range
Controls	β-D-Hexosaminidase	15	12— 17
	Arylsulfatase A	11	9— 13
	Arylsulfatase B	10	8— 11
	α-D-Glucosidase	79	75— 83
	α-D-Mannosidase	19	18— 21
	β-D-Glucuronidase	23	17— 25
Group A	β-D-Glucosidase	110	99—115
	β-D-Xylosidase	130	117—140
Group B	β-D-Galactosidase	5	4— 7
	α-L-Arabinosidase	6	5— 7
	β-D-Fucosidase	6	5— 8

than that used in the assay in Table II, 50% of the activity was found to be bound by *U. europaeus* agglutinin.

Table IV demonstrates the intracellular levels of various hydrolase activities in cultured skin fibroblasts from three unrelated patients with mucolipidosis II (I-cell disease). The data are expressed as the percent of normal specific activity (nmol/h per mg protein) based in the data from five control cell lines. Both group A activities were found to be near or above control levels: group B activities were nearly identical in their intracellular deficiency. Six other hydrolases are presented to demonstrate the range of intracellular deficiency of hydrolase activity in ML II fibroblasts. Neutral hydrolase activities (Group C) are extremely low in skin fibroblasts and not readily amenable to this type of analysis.

Table V presents the levels of acid hydrolase activities secreted into the

TABLE V

SECRETION OF ACIDIC GLYCOSIDASE ACTIVITIES INTO MEDIA FROM NORMAL AND I-CELL FIRBOBLASTS

Normal fibroblasts, $n = 3$; I-cell fibroblasts, $n = 3$

	Enzyme activity	% of intracellular activity (mean (range))	
		Normal	I-cell
Controls	β-D-Hexosaminidase	62 (52—72)	3 600 (2 900— 4 200)
	α-D-Mannosidase	41 (36—45)	10 200 (9 300—11 100)
	β-D-Glucuronidase	53 (45—60)	1 800 (1 300— 2 100)
Group A	β-D-Glucosidase	Undetectable	Undetectable
	β-D-Xylosidase	Undetectable	Undetectable
Group B	β-D-Galactosidase	2 (1—4)	225 (150—275)
	α-L-Arabinosidase	3 (1—4)	250 (175—325)
	β-D-Fucosidase	4 (3—5)	285 (175—360)

TABLE VI

GLYCOSIDASE ACTIVITIES OF NEUTRAL β-D-GALACTOSIDASE PURIFIED FROM HUMAN LIVER

Glycosidase	Enzyme activity (μmol/h per mg protein)
β-D-Galactosidase	76.50
β-D-Glucosidase	15.50
β-D-Xylosidase	1.14
β-D-Fucosidase	0.62
α-L-Arabonisidase	2.85
α-D-Mannosidase	0
β-D-Glucuronidase	0
β-D-Hexosaminidase	0

media over a 24 h period from both I-cell and normal fibroblasts. Both group A activities were undetectable in the media of I-cells, while the three group B activities were secreted to the same extent. Three other hydrolases are presented as examples of the variability in secretion of lysosomal enzymes into the media. Data showing the similarities within each group and between the groups of hydrolases were observed for the secretion from normal cells, although the extent of secretion was markedly less than for I-cell.

Table VI presents the results of the assays of purified neutral β-D-galactosidase for eight neutral hydrolase activities. All previously designated group C activities were present in the purified preparation while the three other activities were not similarly found despite very long incubations at 37°C (up to 24 h). Although β-D-galctosidase and β-D-glucosidase activities were markedly higher than the other three activities, these latter activities were found at appreciable levels.

Discussion

A number of different criteria have been used to demonstrate three multiple glycosidase enzymes: one enzyme with activity at acidic pH towards β-D-glucoside and β-D-xyloside (group A), another enzyme capable of hydrolyzing at acidic pH β-D-galactoside, β-D-fucoside and α-L-arabinoside (group B) and a third one possessing activity at neutral pH towards all five glycosides mentioned above (group C). The similar behaviour within the same group of glycosidases suggests that these activities are likely representing a single enzyme protein. The inability to differentiate between two or more enzymic activities by separation methods such as chromatography and electrophoresis or on the basis of the effect of external conditions such as temperature, pH, ionic strength and denaturating agents provides only circumstantial evidence that the activities are due to the same protein. However, in a single study, a combination of several parameters that reflect different properties of enzyme proteins provides a better basis for such indications. The coexistence of several activities in an enzyme protein purified to homogeneity is by itself a good criterion for multiple substrate specificity. Norden et al. [6] have demonstrated purified GM_1 β-D-galactosidase to hydrolyze β-D-fucoside and α-L-arabinoside as well as

β-D-galactoside. In the present study, we have found that purified neutral β-D-galactosidase also possesses β-D-glucosidase, β-D-xylosidase, β-D-fucosidase and α-L-arabinosidase activities. The deficiency within a given inherited disease of enzymic activities other than the primary one also provides an indication for a multiple substrate enzyme. We based our suggestion for multiple carbohydrate-cleaving specificities on the studies of glycosidase activities in tissues from patients with an infantile form of Gaucher disease, GM_1 gangliosidosis type I and neutral β-D-galactosidase deficiency. The latter condition is probably a benign deficiency [14] and although there are no indications for its inheritance, the levels of the enzyme activity were found to be severely reduced in the liver, spleen and skin fibroblasts of the patient we studied [13].

In the case of Gaucher disease, contradictory data have been reported [1—3] with respect to which enzymic activities are deficient in addition to glucocerebrosidase and acidic β-D-glucosidase activities. The different results may be explained by heterogeneity of the disease with mutations that affect differently the substrate specificity of the deficient enzyme. The relationship between the insoluble glucocerebrosidase and the soluble acidic β-D-glycosidase is not clear and determination of glycosidase activities in appropriate tissues of persons heterozygous for Gaucher disease may help to clarify these puzzling findings.

Most human tissues contain acidic and neutral glycosidase activities in the presence of each other. Overlapping of apparently independent acidic and neutral activities occurs over a quite broad range of pH values. Furthermore, the range and optimal pH of different activities of multiple substrate enzyme are not necessarily identical. Van Hoof and Hers [17] have shown for human liver acidic β-D-galactosidase and α-L-arabinosidase activities that although they are associated and probably represent a single enzyme, their pH curves are not superimposable on each other. The choice of appropriate pH for a specific acidic or neutral enzymic activity should be based on the pH profile of this activity in order to minimize as much as possible the contribution of the other neutral or acidic activity, as we have done in the present study. The possible presence of an additional form of human liver β-L-xylosidase with pH optimum at 7.4 was first suggested by the pH profile of this activity and later on the basis of the exclusive binding of this form to *U. europaeus* agglutinin I. An isolated deficiency of β-D-xylosidase has been reported [18] in cultured lymphocytes of a 9 month-old girl suffering from deafness and blindness. However, this deficiency has been demonstrated only at acidic pH and it would be difficult to relate it to an enzyme form with optimal activity at pH 7.4.

Lectins exhibit a variety of carbohydrate specificities; they have different affinities to different glycoproteins according to the number, accessibility and orientation of carbohydrate residues in the glycoprotein molecules [19]. The differences found among human glycosidases in their binding to lectins [9] and the variability found in the intracellular and extracellular levels of glycosidases in normal and I-cell disease fibroblasts [20] make these two systems suitable for comparative study of glycosidases and thus, help to indicate which activities are likely to be associated.

The data presented here support the existence of multiple carbohydrate-cleaving specificities in human acidic and neutral glycosidases and suggest that in the case of acidic β-D-glucosidase and β-D-galactosidase, the associated

deficiencies of other glycosidases may play a role in the pathogenesis of Gaucher disease and GM_1 gangliosidosis.

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