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NEUTRAL α-MANNOSIDASE ACTIVITY IN HUMAN SERUM

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Summary

Two types of α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) with neutral pH optima exist in serum. The activity with an optimum between pH 6.0 and 6.4 is similar to α-mannosidase C, described earlier in tissues. The second activity, with a pH optimum between pH 5.2 and 5.8 is the dominant form in serum. These two forms can be differentiated from each other by gel-filtration, chromatography on DEAE-cellulose or chromatography on Concanavalin-A Sepharose. Using the chromatographic techniques, the serum type neutral activity co-elutes with the acidic forms of the enzyme. However, these two forms can be easily distinguished by effect of pH, heating or inhibition by the substrate methyl-α-D-mannopyranoside. The presence of the serum type α-mannosidase activity is discussed with respect to mannosidosis, a lysosomal storage disease.

Introduction

Several forms of α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) have been described in mammalian tissues. Of these, forms A and B have been shown to be acidic enzymes, with a pH optimum between 3.8 and 4.5 in tissues from humans, cattle, and rats [1—3]. A third enzyme form, called α-mannosidase C, has a pH optimum closer to neutral, between 6.0 and 6.5, and has also been demonstrated from the same sources. Subcellular centrifugation studies have shown that the acidic activity is lysosomal and that the neutral activity is located in the cytosol [4]. In addition to these forms, another α-mannosidase activity has been described in the Golgi membranes of rat liver. This is characterized by a pH optimum of 5.5, intermediate between the acidic and the neutral activity [5]. Mannosidosis, a lysosomal storage disease in humans and cattle, is attributed to a drastic reduction of both α-mannosidase A and B, the activity of α-mannosidase C being unaffected [2,6]. No information is at present available on the activity of Golgi α-mannosidase in the disease.

However, another neutral enzyme activity, with the same pH optimum as the Golgi membrane α-mannosidase is the dominant enzyme in both normal and mannosidosis serum [7]. This communication deals with this neutral type of enzyme from human serum.

Experimental Procedure

Fresh normal serum was stored at −20° C until analysed not more than a week later. In addition, normal and mannosidosis serum [7] had been stored at −20° C for 18 months. Normal human liver was obtained at autopsy 18 h after death and stored frozen for the same period as fresh serum.

Heat inactivation

2 ml of serum were heated at 60° C for timed intervals up to 60 min. Aliquots (400 μl) were removed, cooled in an ice-bath and analysed immediately at 37° C by incubating 50 μl enzyme with 50 μl citrate/phosphate buffer (100 mM citric acid, 200 mM Na_2HPO_4), pH 4.5 and 5.5, and 100 μl of a 2 mM 4-methylumbelliferyl-α-D-mannopyranoside (Koch-Light Laboratories, Colnbrook, England), dissolved in citrate/phosphate buffer. The incubation time was 3 h. After the reaction had been stopped by adding 3 ml of 200 mM glycine/NaOH buffer, pH 10.4, the fluorescence was measured as described before [1].

Chromatography

All work was performed at 4° C, unless otherwise stated. 2 ml of serum were heated at 60° C for 15 min, cooled and dialysed overnight against 10 mM NaH_2PO_4/NaOH buffer, pH 6.0. The sample was then centrifuged at 1000 × *g* for 5 min and 1 ml applied to a column of DEAE-cellulose (type DE-52, W.&R. Balston Ltd., Maidstone, England) [7]. The fractions eluted were assayed for α-mannosidase activity at pH 4.5 and 5.5. In addition, the fractions were analysed at pH 5.5 in the presence of 2.5 mM Co^{2+} (final concentration). Serum, which had not been heated, was also treated identically.

Ion-exchange chromatography was also carried out on serum which had been treated with neuraminidase from *Clostridium perfringens* type VI as described before [1]. The control sample was treated identically, except that neuraminidase was omitted.

For gel-filtration, 1 ml of serum was applied to a column (1.5 × 100 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), pre-equilibrated in 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.0, containing 150 mM NaCl. 2-ml fractions were collected at a flow rate of 15 ml per h and assayed as outlined in Fig. 1. Chromatography on concanavalin-A Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was carried out by applying 500 μl of serum or a supernatant prepared from a 10% (w/v) homogenate of liver in 25 mM Tris · HCl buffer, pH 8.0, to pasteur pipettes containing concanavalin-A Sepharose, pre-equilibrated with 25 mM Tris · HCl buffer, pH 8.0. The column was eluted with 5 ml of the buffer (denoted as Fraction 1), followed by a further 5 ml of the buffer containing 200 mM of methyl-α-D-mannopyranoside (Koch-Light). This fraction was denoted as Fraction 2. The two fractions were dialysed against

the Tris · HCl buffer before analysis at pH 4.5 and 5.5. The effect of heating these two fractions was also tested.

Studies with methyl-α-D-mannopyranoside were also performed on the most active fractions obtained after chromatography of serum on DE-52 or concanavalin-A Sepharose. The final concentration of the methyl-α-D-mannopyranoside was 200 mM.

Results

In serum heated at 60° C at timed intervals up to 60 min, the α-mannosidase activity at pH 4.5 was decreased by 10—20%, whilst the activity at pH 5.5 decreased by 60—80%. In each case, this loss of activity was observed during the first 15 min, the residual activities being stable to further heating. The activity of α-mannosidase from mannosidase serum at pH 5.5 decreased similarly as the neutral activity from normal serum.

Fig. 1. shows the profile for α-mannosidase after chromatography on DEAE-cellulose. In the control serum sample, four peaks of activity, designated as α-mannosidases A, B_1, B_2, and C were obtained. Peaks A and C had higher activi-

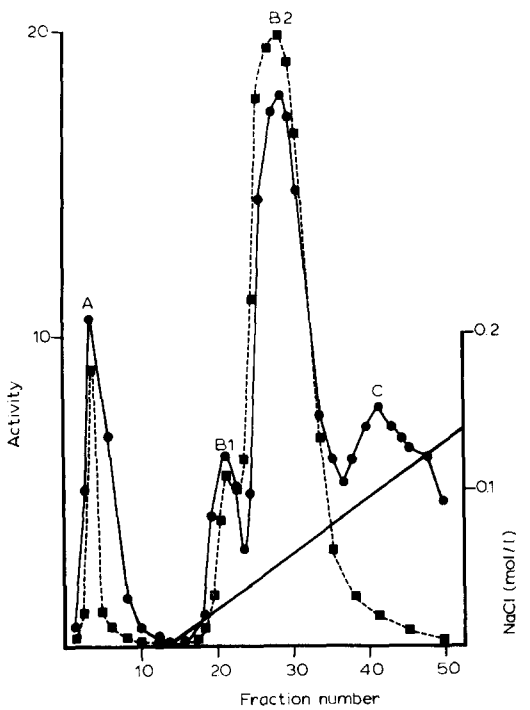

Fig. 1. Anion-exchange chromatography of serum α-mannosidases. 1 ml of serum, dialysed against 10 mM NaH_2PO_4/NaOH buffer, pH 6.0, was applied to a column (0.9 × 15 cm) of DEAE-cellulose, pre-equilibrated with the same buffer. Fractions (4 ml) were eluted at a flow rate of 60 ml/h, initially using the same buffer and afterwards with a salt gradient (0—150 mM NaCl) in buffer. The enzyme activity was analysed by incubating 200 µl sample with 200 µl of a 2 mM solution of the substrate dissolved in citrate/phosphate buffer, and was expressed as nmol of 4-methylumbelliferone released/200 µl/4 h. α-Mannosidase activity at pH 4.5 (■------■) and at pH 5.5 (●———●). The NaCl gradient is indicated by a straight line.

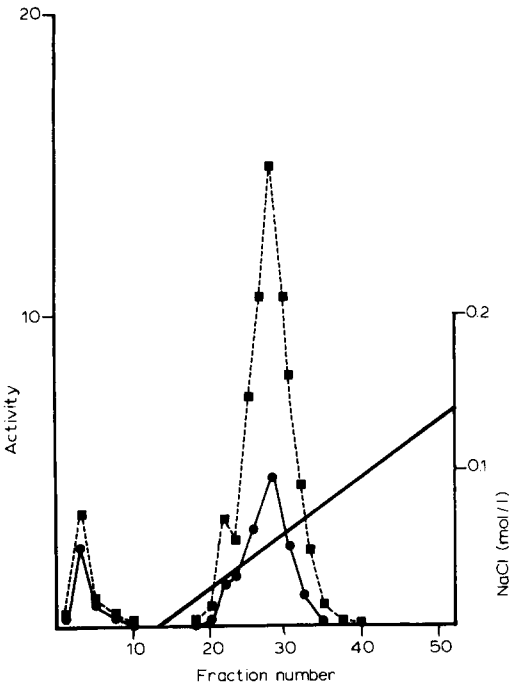

Fig. 2. Anion-exchange chromatography of serum α-mannosidases after heat-treatment. 1 ml of serum, which was heated as described in Experimental Procedure, was dialysed before chromatography as outlined in Fig. 1. All the other details are as given in Fig. 1.

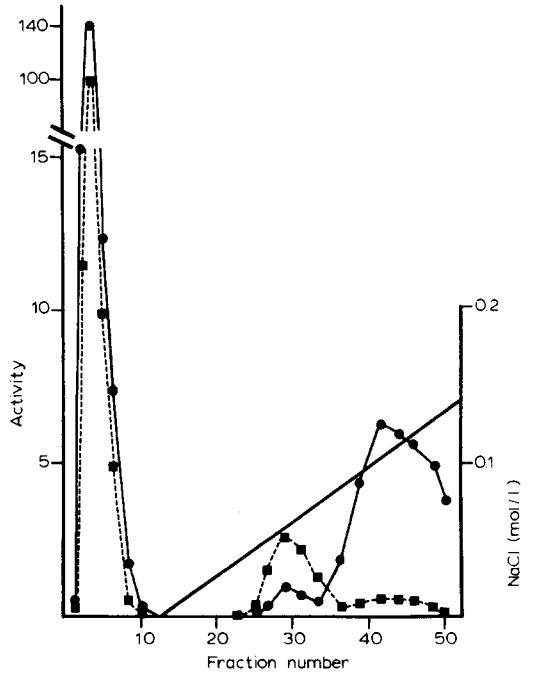

Fig. 3. Anion-exchange chromatography of serum α-mannosidases after neuraminidase treatment. Serum was treated with neuraminidase from *Clostridium perfringens* type VI (1) before chromatography as outlined in Fig. 1. Other details are as given in Fig. 1.

TABLE I

CHROMATOGRAPHY OF SERUM AND LIVER ON CONCANAVALIN-A SEPHAROSE

	Serum		Liver	
	Fraction 1	Fraction 2	Fraction 1	Fraction 2
1. Acidic activity *				
(a) Initial	5	75	15	65
(b) After heating	3	70	12	55
(c) 200 mM methyl-α-D-mannopyranoside	3	35	12	30
2. Neutral activity *				
(a) Initial	20	60	70	10
(b) After heating	5	15	17	8
(c) 200 mM methyl-α-D-mannopyranoside	20	55	60	9

* All the results are expressed as % α-mannosidase activity. For serum the pH of analyses were 4.5 and 5.5, and for liver, the pH values were 4.0 and 6.0.

ty at pH 5.5 than at pH 4.5, and the activities of the enzyme at the two pH values were approximately equal in peak B (Fig. 1). In contrast, the neutral activity in all the peaks was substantially reduced after the heat treatment (Fig. 2). A 6—10-fold increase in α-mannosidase activity at pH 5.5 was observed in all peaks from control serum when 2.5 mM Co^{2+} was included in the assay mixture. On the other hand, very little activation was observed in the peaks from the heat treated serum.

The activities of peaks B_1 and B_2 at both pH values disappeared after ion-exchange chromatography of serum treated with neuraminidase. However, the recoverable activity was accountable by an increase in activity of the unadsorbed peak. Peak C, on the other hand, was unaffected by neuraminidase treatment (Fig. 3).

After gel-filtration on Sephadex G-200, the activities of α-mannosidase at pH 4.5 and 5.5 from serum eluted as a single peak directly after Dextran Blue. This was in contrast with the elution of the two types of activities in human liver, where neutral activity eluted slightly before acidic activity.

Table I summarizes the distribution of the acidic and neutral activities in human serum and liver after chromatography on concanavalin-A Sepharose, together with the effects of heat and methyl-α-D-mannopyranoside on these activities. The acidic activity in each instance was stable to heating, but the breakdown of methylumbelliferyl-α-D-mannopyranoside was inhibited by methyl-α-D-mannopyranoside, whereas the neutral activity was heat-labile, but unaffected by methyl-α-D-mannopyranoside. In addition, the neutral activity from Fraction 1 from serum or liver was eluted as α-mannosidase C after chromatography on DEAE-cellulose. The effect of methyl-α-D-mannopyranoside was also studied on the acidic and neutral activities in peaks A, B_1, and B_2, and confirmed the results obtained with the concanavalin-A Sepharose fractions.

Discussion

Mannosidosis can be explained by the deficiency of two types of acidic α-mannosidases, called A and B, which have immunologic identity [8] and which

react non-specifically towards natural substrates [9], and probably only differ in neuraminic acid content [1], indicating that the two acidic enzymes have a common genetic origin. In addition to these enzymes, there are α-mannosidases with pH optima closer to neutral. Of these, the tissue neutral enzyme has a pH optimum between 6.0 and 6.5, and is unaffected in the disease [6]. This enzyme, called α-mannosidase C, has a molecular weight which is greater than the acidic enzymes, does not cross-react with antibodies raised for the acidic enzymes [8] and is unaffected by neuraminidase [1], suggesting its genetic independence. Characterization of this enzyme is hampered by the fact that it exhibits lability even at 4°C in those tissues where it dominates [1].

The neutral activity in serum exhibits a pH optimum between pH 5.2 and 6.0 and is relatively stable at 4°C even upon prolonged storage compared with the neutral activity from some tissues. However, this enzyme is labile if heated at 60°C. Upon ion-exchange chromatography, most of the neutral activity co-elutes with the acidic fractions A, B_1 and B_2, and shows a similar change in elution after neuraminidase treatment. In addition to this, most of the neutral activity in serum also co-elutes with the acidic activity on Sephadex G-200 and concanavalin-A Sepharose. Thus the chromatographic properties of the major serum neutral enzyme are identical to acidic activity. However, this activity can be differentiated from the α-mannosidase C, both by chromatography on DEAE-cellulose or on concanavalin-A Sepharose. The significant differences between the serum neutral activity and acidic activity in the various fractions

TABLE II

SOME CHARACTERISTIC PROPERTIES OF α-MANNOSIDASE FORMS

	Acidic forms (pH optimum = 3.8—4.5)	Neutral form (pH optimum = 6.0—6.5)	Intermediate forms (pH optimum = 5.2—6.0)		
			Form S	Form I	Form G
Chromatography on DEAE-cellulose	Resolved as A, B_1 and B_2	Resolved as C	Co-elutes with A, B_1 and B_2	Co-elutes with C	Not studied
Chromatography on concanavalin-A Sepharose	Adsorbed	Not adsorbed	Adsorbed	Not adsorbed	Not studied
Effect on neuraminidase	Give one precursor form	Unaffected	Gives one precursor form	Unaffected	Not studied
Effect of heat (60°C)	10—20% loss of activity	60—80% loss of activity	60—80% loss of activity	Unaffected	Lability dependent upon state of solubilization
Effect of Co^{2+}	10—30% loss of activity	2—3-fold increase in activity	6—10-fold increase in activity	6—10-fold increase in activity	Unaffected
Effect of methyl-α-mannopyranoside	40—50% loss of activity	Unaffected	Unaffected	Unaffected	Unaffected

where these two co-elute are the effect of heating and methyl-α-D-mannopyranoside. Whereas the acidic activity is stable to heating, it demonstrates a decrease of breakdown of methylumbelliferyl-α-D-mannopyranoside in the presence of methyl-α-D-mannopyranoside. In contrast, the neutral activity is labile to heating but unaffected by methyl-α-D-mannopyranoside. Thus it appears that the bulk of the serum neutral activity is different from the tissue type neutral activity (α-mannosidase C) and represents a new type of α-mannosidase. About 20% of the serum neutral activity can be attributed to α-mannosidase C. The structural difference between the serum-type neutral enzyme and the acidic activity was recently shown by Phillips et al. [10].

The existence of this neutral α-mannosidase activity in serum explains the earlier finding that the neutral activity in the peaks of α-mannosidase obtained by ion-exchange chromatography of serum was unaffected in patients with mannosidosis, despite the fact that acidic activity in the same fractions was very much reduced [7].

In human liver, α-mannosidase activity at pH 5.5, which is heat-stable and activated by Co^{2+}, has been shown to co-elute with α-mannosidase C as a minor component [11]. The serum neutral activity, although activated by Co^{2+}, is different from this because it elutes differently and shows heat lability. The effect of heat and metal ions can also be considered in the case of the Golgi membrane α-mannosidase. This enzyme is insensitive to Co^{2+} or Zn^{2+} and shows different heat labilities depending on whether it is solubilized from the Golgi membrane or not. The Golgi membrane enzyme, like the serum neutral α-mannosidase, is not affected by methyl-α-D-mannopyranoside. Some of the properties which can be used to distinguish between the various forms of α-mannosidase are summarized in Table II. In order to avoid confusion, we suggest that the serum type neutral enzyme should be designated α-mannosidase S, the tissue neutral α-mannosidase, which is either a contaminant or a minor constituent of α-mannosidase C [10,11], should be called α-mannosidase I and that the Golgi membrane enzyme should be called α-mannosidase G. Whether these forms are related to each other or not, or what possible function they have, must await their purification and characterization.

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