MANNOSIDOSIS: DETECTION OF THE DISEASE AND OF HETEROZYGOTES USING SERUM AND LEUCOCYTES.

Parvesh K. Masson and Arne Lundblad

Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

Seppo Autio

The Children's Hospital, University of Helsinki, Helsinki, Finland

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Summary: α-D-mannosidase activity in serum and leucocytes from normal individuals, patients with mannosidosis and their parents was measured at pH 4.4 and pH 6.0. When the results were expressed as total activity or specific activity at pH 4.4 in both tissues, or as a ratio of enzyme activity at the two pH conditions in serum, the disease could be diagnosed, but the heterozygotes could not be distinguished from the controls. However, all three groups could be recognised when acid α-mannosidase activity was related to total N-acetyl-β-D-glucosaminidase activity in serum and leucocytes. The distribution of α-mannosidases, as separated by ion-exchange chromatography was different in each tissue. The serum profile was unique, all components having substantial "neutral" activities which are unaltered in mannosidosis and carriers.

Introduction: Mannosidosis, a lysosomal storage disorder, attributed to a decreased activity of α-D-mannosidase (α-D-mannoside mannohydrolase, E.C. 3.2.1.24) was first described in 1967. (1) Carrol et al. demonstrated the presence of two "acidic" and one "neutral" forms of the enzyme in normal liver (2). Both the acidic forms were shown to be absent in livers of affected children. This defect is accompanied by an accumulation of mannose-rich material in certain tissues (3), and a urinary excretion of several mannose-rich oligosaccharides (4,5). The criteria mentioned above, in combination with clinical symptoms, can be used to diagnose the disease (6). No means to detect heterozygotes have yet been described.

Studies on α-mannosidase in serum and leucocytes from normal individuals, patients with mannosidosis and their parents show that these tissues can be used to detect both the disease and heterozygotes. Differences in ion-exchange chromatography profiles were observed in the two tissues, and the possible reasons for these are discussed.

Materials and Methods: Serum and leucocytes were collected from nine normal women, (five on oral contraceptives), ten normal men, three cases of

mannosidosis (cases which have been described before (7)), and their parents. Serum was also obtained from three new cases and their parents.

The leucocytes were prepared by mixing 5 ml of heparinised blood with an equal volume of 3% dextran (Pharmacia Fine Chemicals, Uppsala, Sweden). After 40 minutes at room temperature, the upper dextran layer was removed and centrifuged at 2,000 r.p.m. for ten minutes. Contaminating erythrocytes were lysed by osmotic shock with cold redistilled water for 90 seconds and 2.7% sodium chloride was then added to make the medium isotonic. The washed leucocytes, obtained after centrifugation were resuspended in 0.5 ml of water. α-mannosidase was released from the cells by rapid freezing and thawing, the process being repeated ten times. The supernatant obtained after centrifuging the samples as before was used for the analysis.

The pH dependance of α-mannosidase in serum was studied by diluting the samples elevenfold with phosphate-citrate buffers (McIlvaine), ranging in pH from 3.6 to 6.8. For the analysis, 0.2 ml were incubated at 37°C for 3 hours with 0.1 ml of the buffers and 0.1 ml of a 3.2 mM solution of 4 methyl-umbelliferyl α-D-mannopyranoside (Koch Light Laboratories, Colnbrook, Bucks. U.K.) in water.

For the leucocytes, the samples were diluted similarly in water. The reaction mixtures contained 0.1 ml of the enzyme solution, 0.2 ml of the same buffers as above, and 0.1 ml of the substrate solution. The incubation time was 1 hour.

The enzyme activities at the pH optima were determined in serum and leucocyte samples by diluting them elevenfold with buffers and incubating 0.2 ml with 0.1 ml of 1.6 mM buffered substrate, for two hours and half an hour respectively.

The total N-acetyl-β-D-glucosaminidase (hexosaminidase) activity was analysed similarly, using 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch Light). The serum and leucocytes were incubated at 37°C for 40 and 20 minutes respectively. After the reaction was stopped, the fluorescence was measured as described for other glycosidases (8), using the Aminco-Bowmann spectrofluorimeter. One unit of enzyme activity is expressed as that amount which liberates one nM of product per minute at 37°C. Total protein was determined by the Folin method.

All the work concerning ion-exchange chromatography was performed at 4°C. For serum, 2.5 ml were dialysed overnight against one litre of the column buffer (10 mM sodium phosphate buffer pH 6.0) using Visking 8/32 inch dialysis tubing (Union Carbide, Illinois, USA). The dialysed sample was centrifuged at 2,000 r.p.m. for ten minutes and 2 ml were applied to a column (0.9 cm x 15 cm) packed with DEAE-cellulose (Whatman DE-52, W.R. Balston,

Maidstone, Kent, U.K.) pre-equilibrated with the column buffer. The same buffer was used to elute 4 ml fractions at a flow rate of 60 ml per hour for 40 minutes, when a linear sodium chloride gradient (0-0.15 M) in that buffer was applied. The supernatant from the leucocyte preparation was diluted with an equal volume of 20 mM sodium phosphate buffer, pH 6.0, and applied to a column of DE-52. The subsequent procedure was as outlined above.

The column fractions were analysed by incubating 0.5 ml of the sample with 0.2 ml of 1.6 mM substrate solution, made in McIlvaine buffers at pH 4.4 and 6.0.

The chloride concentration for the gradient was measured with a Philips conductivity meter, using a 1.5 ml electrode.

Results: Figure 1 shows the pH dependance of α-mannosidase in serum and

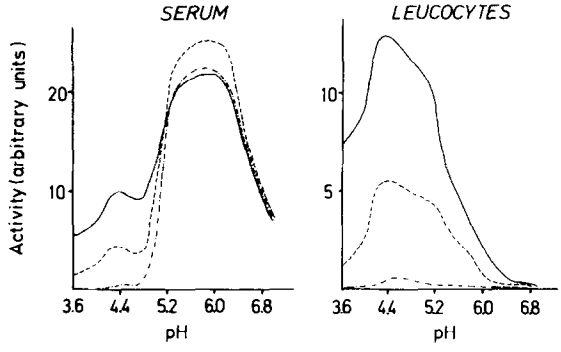

Figure 1: pH dependence of α-mannosidase in serum and leucocytes using phosphate-citrate buffers ranging from pH 3.6 to 6.8 (see text for methods used).

———— normal individual
---------- heterozygote
- ·-·-·-· mannosidosis

leucocytes from a normal subject, a patient with mannosidosis, and one of the parents. Two pH optima, one between pH 4.2 to 4.6 and another between pH 5.6 to 6.2 are observed in serum, the latter type being more predominant. In leucocyte, the main enzymes appear to be acidic with a pH optimum between pH 4.2 to 4.6. In mannosidosis, there is an almost complete loss of the acidic activity in both the tissues studied. In the parent the levels of the acidic enzymes appear to be intermediate between the normal levels and those

TABLE I

Ranges of α-mannosidase and hexosaminidase activities in serum and leucocytes.

		SERUM				LEUCOCYTES			
		Man 4.4[1]		Man 6.0[1] / Man 4.4	Hex 4.4[2] / Man 4.4	Man 4.4		Man 6.0 / Man 4.4	Hex 4.4 / Man 4.4
		Activity nM/ml/min	Specific activity mU			Activity nM/ml/min	Specific activity U		
Controls	n								
Females (O.C.)[3]	5	5.3-6.5	53-75	1.4-1.9	12.6-18.0	28-95	34-73	0.16-0.24	5.9-9.2
Females	4	4.9-7.7	51-82	1.2-1.9	10.7-18.3	54-142	42-121	0.18-0.24	5.7-9.4
Males	10	4.5-8.0	51-85	1.4-1.9	10.0-19.2	30-92	58-141	0.18-0.24	5.8-9.2
Heterozygotes									
Female (S.S.)	1	6.8	67	2.0	33.6	33	54	0.25	14.8
Male (T.S.)	1	1.8	17	6.0	36.5	34	36	0.18	14.2
Female (R.K.)	1	2.0	23	4.6	26.0	28	42	0.20	15.3
Male (J.K.)	1	1.9	19	5.2	48.5	28	45	0.21	15.2
Female (P.N.)	1	6.0	66	1.3	24.4	-	-	-	-
Male (O.N.)	1	2.1	23	3.5	37.4	-	-	-	-
Mannosidosis									
Males	3	0.4-0.5	4-5	23.2-24.3	185-340	3.3-8.0	7-8	2.37-2.54	67.0-75.2
Males	3	0.3-0.4	3-5	17.0-21.4	255-620	-	-	-	-

(1) Man 4.4 = α-D-mannosidase activity at pH 4.4
Man 6.0 = α-D-mannosidase activity at pH 6.0

(2) Hex 4.4 = Total N-acetyl-β-D-glucosaminidase activity at pH 4.4

(3) O.C. = Females on oral contraceptives

observed in mannosidosis. However, the activity of α-mannosidase is unaltered between pH 5.2 and 6.8 in all serum samples examined.

Table 1 shows the results obtained from the enzyme analysis in serum and leucocyte preparations, expressed as total and specific activity of α-mannosidase at pH 4.4, the ratio of the enzyme activity at pH 6.0 to that at pH 4.4, and the ratio of hexosaminidase to α-mannosidase, both at pH 4.4.

The first three sets of results enable diagnosis of mannosidosis but do not allow a clear differentiation between the controls and the known heterozygotes. However, there is no overlap between any of the categories when the results are expressed as a ratio of the activity of hexosaminidase at pH 4.4 to that of acid α-mannosidase in either serum or leucocytes.

The separation of α-mannosidases from serum and leucocytes by ion-exchange chromatography shows that the distribution of enzyme components in each is different (figure 2). The adsorbed acid activity in each case may consist of at least two forms in different proportions. The profiles also

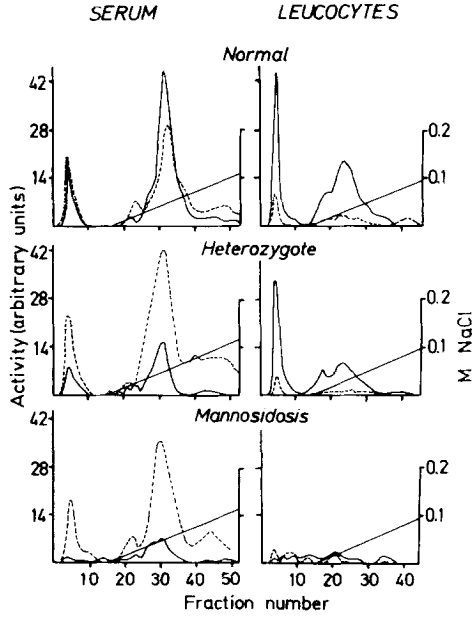

Figure 2: DEAE-cellulose column chromatography at pH 6.0, of 2 ml dialysed serum and 1 ml leucocyte supernatant on normal, heterozygote and mannosidosis samples.

———— α-mannosidase activity at pH 4.4
---------- α-mannosidase activity at pH 6.0
The sodium chloride gradient is indicated by a straight line.

vary from that described in human liver (2), where form A is unadsorbed and forms B and C are eluted by an increasing salt concentration gradient. Form C is the most active component at pH 6.0. In serum, all the peaks eluted have activities at pH 6.0 comparable with, or higher than those at pH 4.4. The most interesting feature in serum is that the "neutral-type" of activities remain essentially unaltered in all cases, including serum from mannosidosis and carriers where the "acidic-type" of activities are either very much reduced or intermediate. However, the DE-52 pattern seen in the case of one of the mothers (S.S.) of the affected boys resembled that found in controls at both the pH conditions used.

In leucocytes, the contribution to activity at pH 6.0 due to a "neutral" form is very small. In mannosidosis there are traces of all the acidic activities left, and in the heterozygotes there seem to be a general decrease of all the components.

Discussion: The results show that the diagnosis of mannosidosis and of heterozygotes is possible with serum and leucocytes. The total and specific activities of α-mannosidase at pH 4.4 are very low in serum and leucocytes from patients with mannosidosis. In serum from all carriers except two, the same values were intermediate between those in controls and disease. In the exceptional cases, the total and specific activities of α-mannosidase at pH 4.4 were within the normal limits, as was the distribution of the enzymes as seen by ion-exchange chromatography. The reasons for these discrepancies are not understood but the finding demonstrates the need to relate the activity of acid α-mannosidase to a different lysosomal hydrolase, to avoid misdiagnosis.

The total serum hexosaminidase level appears to vary slightly in females on oral contraceptives, and is increased substantially in pregnancy and diabetes, while one of the components within this system, denoted as N-acetyl-β-glucosaminidase A, decreases in all these conditions (9). However, both the total and hexosaminidase A activities are relatively unaffected in leucocytes from these cases. Hence the ratio of total hexosaminidase to α-mannosidase activity at pH 4.4 provides a satisfactory alternative to differentiate between controls, heterozygotes and mannosidosis in leucocytes, where wider variations in either total or specific activities may theoretically be expected due to variations in leucocyte numbers taken for analysis or due to the failure to remove contaminating proteins such as haemoglobin during the preparation. In another disease, fucosidosis, such an approach of relating α-L-fucosidase to α-D-mannosidase in white blood cells has been used to detect fucosidosis and carriers (10).

As the results show, the ratio of hexosaminidase to α-mannosidase at pH 4.4 in serum also allowed the separation of the three categories without any overlap. Since serum is easier to obtain and handle, it could be used initially, followed by leucocyte studies on borderline-cases, which may include pregnant women and diabetics. Recently, the presence of acidic α-mannosidase has been shown in uncultered amniotic fluid cells (11). This means that prenatal diagnosis of mannosidosis is also possible.

The results also showed that neither α-mannosidase activity in serum seemed to be affected in women on oral contraceptives.

Despite the wide variation of acid α-mannosidase in serum, the enzyme appeared to vary much less at pH 6.0, the activity ranging from 6.7 to 13.8 nM/ml/min. in the 35 serum samples studied. The fact that this level does not vary so much in any case examined, when measured over the pH range 5.2 to 6.8 explains why earlier analysis at pH 5.5 did not enable the diagnosis of mannosidosis (12,6,7). This also explains the reason for the different ratios of the pH 6.0 to pH 4.4 activities in the controls, heterozygotes and disease. Leucocytes, on the other hand, do not have any "neutral" component comparable to the form C in liver. This could account for the observation that the pH 6.0 to pH 4.4 ratio remains constant at around 0.2 in all cases.

In ion-exchange chromatography of serum, all the peaks had substantial activity at pH 6.0. These are essentially unchanged in mannosidosis or carriers when the acidic activity in the same fractions is either deficient or decreased.

This can be explained if one assumes that α-mannosidases, once released from the lysosomes reorganize, but retain the same net charge. The defective enzyme molecules that most likely are present in heterozygotes and mannosidosis would then undergo similar reorganization.

Alternatively, serum may have characteristic "neutral" enzymes under an independent genetic control, and different from all other tissue α-mannosidases. The most predominant form of the enzyme in sheep serum has been shown to have a pH optimum intermediate between that of the acidic and the "neutral" forms. (B.G. Winchester, personal communication). An identical situation may prevail in human beings, and the residual enzyme activity in mannosidosis may be due to this component.

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