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NORMAL EXTRACELLULAR EXCRETION OF ACIDIC α-MANNOSIDASE ACTIVITY BY MANNOSIDOSIS FIBROBLAST CULTURES

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Summary

The effects of pH, metal ions, and heat were studied on α-mannosidase (EC 3.2.1.24) from normal and mannosidosis fibroblasts and in culture medium obtained from these. The results showed that enzymes secreted from mannosidosis fibroblasts behaved identically as the enzymes present in culture medium from normal fibroblasts. The implication of this finding is discussed.

Introduction

α-Mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) is a glycosidase involved in the degradation of mannose-containing substrates. The enzyme occurs in several different forms, some characterized by an acidic pH optimum and others characterized by more neutral, but different pH optima [1—4]. All the evidence available at present indicates that the acidic forms are inter-related and have a common genetic origin, but that these forms are genetically different from the neutral forms [5]. Subcellular centrifugation studies have shown that the acidic forms are lysosomal, whereas the neutral forms are either located in the Golgi membranes or cytosol [2]. The deficiency of the acidic forms has been attributed to a lysosomal storage disease, mannosidosis, affecting cattle [6] and man [1].

Recently, Beaudet and Nichols [7] showed that the residual acidic α-mannosidase activity from mannosidosis fibroblasts was considerably increased when a high substrate concentration was used. Moreover, the defective enzyme exhibited thermolability in contrast to normal acidic α-mannosidase [7]. We have shown earlier that the residual acidic activity from several tissues and fibroblasts from patients with mannosidosis was activated by high substrate concentration. Furthermore, a more pronounced activation could be achieved, even at low substrate concentrations, in the presence of Co^{2+} and Zn^{2+} [8]. These results indicate the presence of enzyme protein and suggest that the genetic

mutation has probably altered the metal-binding capacity of the enzyme.

The present communication deals with α-mannosidases from cultured fibroblasts and the extracellular excretion of these into the cell culture medium.

Experimental Procedure

Fibroblasts were cultured from skin biopsies obtained from normals and a patient with mannosidosis as described before [9]. The cells were harvested at the stage of confluent monolayer and the cell pellet obtained after centrifugation homogenized in 10 mM NaH_2PO_4/NaOH buffer, pH 6.0.

Extracellular excretion of α-mannosidase. At the stage of confluent monolayer, the cells were rinsed with physiological saline. New medium was preheated at 70° C for 2 h, cooled and added to the monolayer. Aliquots (1 ml) were removed for analysis immediately and after 4, 40 and 70 h.

Enzyme assay. For the analysis, 100 μl of the enzyme were incubated at 37° C for various times with 100 μl of a 2 mM solution of 4-methylumbelliferyl-α-D-mannopyranoside (Koch-Light Laboratories, Colnbrook, U.K.) in citrate/phosphate buffers (200 mM Na_2HPO_4 added to 100 mM citric acid). The rest of the procedure was as described before [8]. The incubation was repeated in the presence of 2.5 mM $ZnSO_4$ or $CoNO_3$.

Heat inactivation. Fibroblast homogenate or culture medium 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 pH 4.5 and 5.5, with and without metal ions.

Results

Effect of pH and metal ions

α-Mannosidase in normal fibroblasts had predominant activity at pH 4.5. A second activity, corresponding to 50—60% of the acidic activity, was found at pH 5.5. In mannosidosis fibroblasts, the acidic activity was drastically reduced, whereas no significant changes were observed in the level of enzymic activity at pH 5.5. A similar pH effect on the enzyme as the one seen in normal fibroblasts was also observed in the culture medium from both the normal and mannosidosis fibroblasts.

The activity of the enzyme from normal fibroblasts and culture medium from both the normal and mannosidosis fibroblasts was enhanced by 20—30% at pH 4.5 and 5.5 in the presence of Zn^{2+}. However, the same metal ion caused a 6—10-fold increase in enzymic activity at pH 4.5 and a 2—3-fold increase in activity at pH 5.5 when mannosidosis fibroblasts were used.

Co^{2+} inhibited the acidic α-mannosidase activity (by 30—40%) and activated the neutral enzyme activity (by 20—30%) from normal fibroblasts, normal culture medium, and medium from mannosidosis fibroblasts. However, it activated the α-mannosidase activity from mannosidosis fibroblasts significantly (6—10-fold at pH 4.5 and 2—3-fold at pH 5.5).

Effect of heating

The activity of acidic α-mannosidase from normal fibroblasts was unaffected

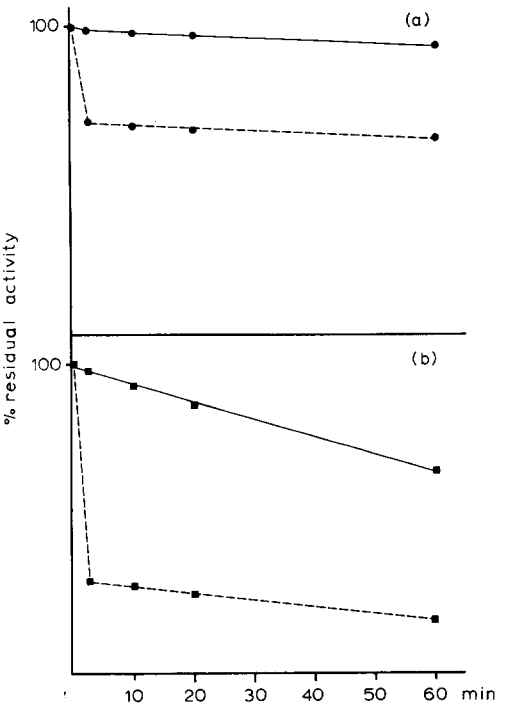

Fig. 1. Effect of heat on α-mannosidase activity from fibroblasts. Fibroblast homogenates were pre-heated at 60°C for timed intervals and enzyme activity analysed as outlined in Experimental Procedure using 3 h incubation time. The results are expressed as percent residual activity. (a) Normal fibroblasts (●). (b) Mannosidosis fibroblasts (■). α-Mannosidase activity at pH 4.5 (———) and pH 5.5 (- - - - -).

by heating and approx. 30% of the neutral activity was lost. In comparison, 35% of the residual acidic activity and 70—80% of the neutral activity from mannosidosis fibroblasts was lost upon heating (Fig. 1). The enzymes from mannosidosis fibroblasts were partially stabilized by 10 mM substrate or 2.5 mM Co^{2+}.

The effects of heat on the α-mannosidase activity from the culture media from either normal or mannosidosis fibroblasts were identical and resembled the effect observed in the case of normal fibroblasts.

Extracellular excretion of α-mannosidases from fibroblasts to culture medium

Heat treatment of the medium prior to addition to the cell culture monolayer inactivated most of the neutral α-mannosidase, but did not affect acidic α-mannosidase to any great extent. These endogeneous activities were therefore subtracted from the total activities obtained. The levels of excreted enzymes at pH 4.5 and 5.5 increased with time (Table I). Furthermore, the levels of the excreted activities at the two pH values were almost identical. As far as excreted enzymes were concerned, the mannosidosis medium could not be differentiated from the normal medium, whatever the pH of analysis. This was also confirmed when the excretion of α-mannosidases from mannosidosis fibroblasts to the culture medium was studied by omitting foetal calf serum from the medium broth.

TABLE I

EXTRACELLULAR EXCRETION OF α-MANNOSIDASE

Pre-heated culture medium containing 18—24 nmol/h per ml α-mannosidase activity at pH 4.5 and 1.5—2.1 nmol/h per ml activity at pH 5.5 was added to a monolayer of fibroblasts. Aliquots were withdrawn immediately and at the times given and analysed for enzyme activity at the two pH values specified. The results are given after correction for the endogeneous activity and represent a range of enzyme activity for six normal fibroblasts and fibroblast from a patient with mannosidosis.

Time (h)	Enzyme activity (nmol/h per ml medium)			
	pH 4.5		pH 5.5	
	Normal	Mannosidosis	Normal	Mannosidosis
0	0	0	0	0
4	1.7— 5.7	4.0	1.7— 4.0	3.2
44	11.4—17.6	15.5	11.4—15.0	12.4
70	15.0—21.0	18.3	12.0—19.0	14.8

Discussion

The occurrence of mannosidosis has been attributed to a deficiency of acidic α-mannosidase activity, whilst the neutral type of activity is unaffected [1]. Recent results have indicated that the residual acidic α-mannosidase activity present in several tissues from mannosidosis patients can be activated significantly either by high substrate concentration or by metal ions [7,8]. These findings demonstrate the presence of defective enzyme protein molecules, which may be produced in the same quantities as the normal enzyme. Since the defective acidic enzyme can be partially stabilized and activated by high substrate concentration, it is possible that the enzyme may be partially functional in the mannosidosis patients. This may explain the finding that free mannose is also found in increased quantities in those tissues from mannosidosis which are characterized by an accumulation of mannose-containing oligosaccharides [10]. Secondly, the pathologic course of the disease is less severe than that observed in GM_1-gangliosidosis and Tay-Sachs disease.

Both normal and mannosidosis fibroblasts excreted acidic and neutral α-mannosidases in approximately the same quantity and proportions. The effect of pH, metal ions, and heat were also identical. It is not clear what caused this apparent normalization of the enzymes secreted from mannosidosis fibroblasts. Since amniotic fluid cells have identical enzyme distribution as fibroblasts [11], they may also secrete apparently "normal" acidic α-mannosidase into the amniotic fluid. Hence results of enzymes in amniotic fluid for prenatal diagnosis of mannosidosis must be cautiously interpreted. The intracellular level of acidic α-mannosidase in mannosidosis fibroblasts was always very low despite the increase of activity in extracellular fluid, suggesting that sequestration of the normalized enzyme, as suggested for several glycosidases [12], did not occur. It is probable that the inherent defect in the acidic α-mannosidase molecule (despite apparent normalization) prevents its reabsorption by mannosidosis fibroblasts in a similar manner as the glycosidases in I-cell disease [12].

Preliminary studies by us indicate that the neutral activity in fibroblasts con-

sists of both the Golgi-type of α-mannosidase (form G) and α-mannosidase C [2,4], whereas the medium contains both α-mannosidase C and serum type (form S) of enzyme [4]. Thus the possibility that the different forms of neutral activity are related to the Golgi-form of enzyme cannot be excluded.

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