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NEUTRAL α-D-MANNOSIDASE ACTIVITY IN HUMAN GRANULOCYTES

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Several forms of α-D-mannosidases, playing different functional roles in the processing and degradation of mannose-containing glycoproteins, exist in human and animal cells [6, 15]. In human blood cells two forms of α-mannosidases have been found: acid and neutral [11, 12]. Since the structure of the glycoproteins is modified in various forms of myelo- and lymphoproliferative diseases [9], the study of α-mannosidases, as the chief enzymes involved in degradation of mannose-containing N-bound glycoproteins, is of considerable interest. It was shown previously that in chronic myeloid leukemia (CML) activity of acid α-mannosidase is increased in the myeloid cells, whereas in chronic lymphatic leukemia activity of this enzyme in the lymphoid cells is sharply depressed [2, 4, 5, 10, 13]. Changes in activity of neutral α-mannosidase in these forms of diseases have not previously been studied. It was difficult to determine activity of neutral α-mannosidase because of, first, the great lability of this form of the enzyme [14] and, second, the presence of an acid form, interfering with determination of the neutral form. The study of the distribution of activities of acid and neutral α-mannosidases in healthy human blood cells showed that only the neutral enzyme is present in platelets, whereas both acid and neutral forms are present in erythrocytes and lymphocytes; only the acid form of α-mannosidase was found in granulocytes [11].

The aim of this investigation was to develop a method of determination of neutral α-mannosidase in blood cells and to study the presence of neutral α-mannosidase in mature and immature granulocytes in normal individuals and patients with CML.

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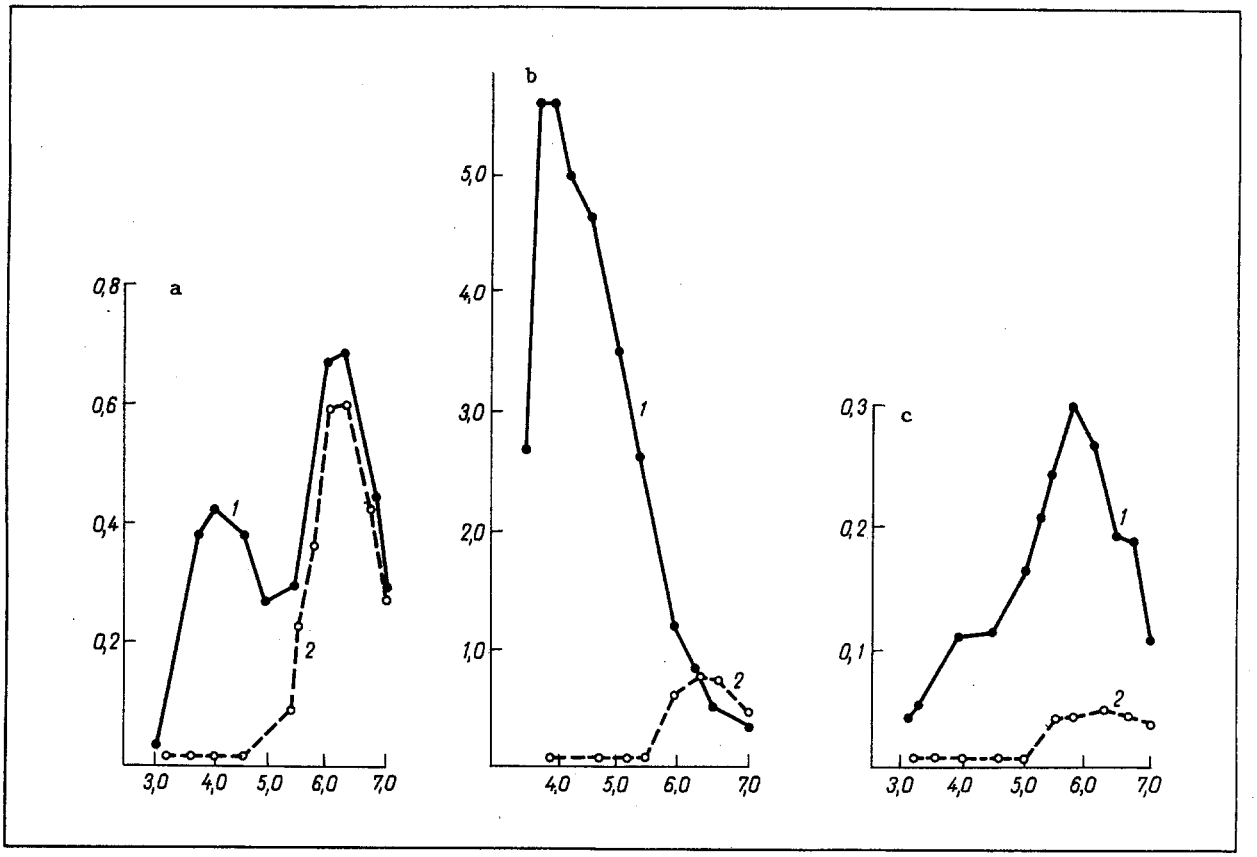
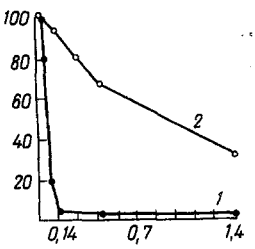


Fig. 1. α-D-mannosidase activity as a function of pH in lysates of different types of cells in the presence of DTH and $MnCl_2$: a) mononuclears, b) granulocytes, c) platelets. 1) Without addition of swainsonin, 2) in presence of 0.14 μM sainsonin. Abscissa, pH values; ordinate, activity (in units/mg protein).

EXPERIMENTAL METHOD

Blood was taken from healthy blood donors and patients with CML, with the aid of 6% EDTA as anticoagulant. The leukocytes were sedimented as described previously [3] and fractionated in a Verografin–Ficoll density gradient [1] into fractions of granulocytes and mononuclears (lymphocytes + monocytes). The cells were washed off with 0.9% NaCl, counted, and suspended in the same solution in a concentration of $20 \cdot 10^6$-$30 \cdot 10^6$ cells/ml. To stabilize the neutral α-mannosidase, the cell suspension was treated with $MnCl_2$ and dithiothreitol (DTH) to a final concentration of 1 and 2 mM respectively, after which the cell suspension was frozen at –18°C. Before activity was determined the cells were thawed and disintegrated in a homogenizer with Teflon pestle, the homogenate was centrifuged for 10 min at 24,000*g*, and the supernatant was used as the enzyme preparation. After determination of neutral α-mannosidase activity the incubation mixture contained (in a volume of 50:1): 10 μl of 5 mM 4-methylumbelliferyl-α-D-mannopyranoside as substrate, 20 μl of 0.1 M citrate-phosphate buffer, pH 6.2, and 10 μl water (or swainsonin solution in a concentration of 2.5 μg/ml). The samples were incubated at 37°C for 10-120 min depending on enzyme activity. The reaction was stopped by the addition of 4 ml 0.1 M Na_2CO_3 and fluorescence was measured on a BIAN 130 fluorometer (λ_{ex} = 365 nm, λ_{em} = 436 nm). The unit of enzyme activity was taken to be the quantity of the enzyme catalyzing removal of 1 nmole of 1-methylumbelliferone in 1 min. Specific activity was calculated in units/10^8 cells or /mg protein. Protein was determined by Lowry's method. Acid α-mannosidase either was inhibited with swainsonin or was removed by adsorption on concanavalin A-sepharose (con A-seph). In the latter case, the centrifuge tubes contained 100 μl of 0.02 M phosphate buffer, 0.5 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, together with 100 μl of a suspension of con A-seph and 400 μl of the enzyme preparation. The mixture was incubated for 1 h at room temperature with continuous shaking, and centrifuged for 10 min at 17,000*g*. The supernatant contained neutral α-mannosidase.



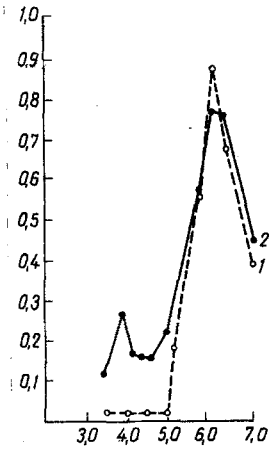


Fig. 2 Fig. 3

Fig. 2. Effect of swainsonin on neutral and acid α-D-mannosidase activity in lysates of human mononuclears. 1) Acid α-D-mannosidase, 2) neutral α-D-mannosidase. Abscissa, concentration of swainsonin (in μM), ordinate, residual activity (in percent).

Fig. 3. Dependence of α-D-mannosidase activity on pH in lysates of granulocytes in the presence of swainsonin and after treatment with con A-seph. 1) In presence of 0.14 μM swainsonin, 2) after treatment with con A-seph. Abscissa, pH; ordinate, activity (in units/mg protein).

EXPERIMENTAL RESULTS

On determination of dependence of mannosidase activity on pH in healthy human granulocytes and mononuclears without the addition of stabilizing agents, only acid α-mannosidase with pH-optimum at about 4.0 was found. Absence of the neutral form could be explained by inactivation of the neutral enzyme during disintegration of the cells. After addition of the stabilizing agents, the curve showing α-mannosidase activity of mononuclears as a function of pH had two distinct peaks at pH 4.0 and 6.2, indicating the presence of two forms of mannosidases, namely acid and neutral (Fig. 1a). It is interesting to note that in preparations of mononuclears obtained from patients with mannosidosis (a hereditary defect of acid α-mannosidase) activity of neutral α-mannosidase was detected if no stabilizing agents were added, even after keeping for a long time (over 6 months). This suggests increased stability of neutral α-mannosidase in mannosidosis. In granulocytes, however, no peak of neutral activity was found even after addition of stabilizing agents (Fig. 1b). This could be explained by the presence of high acid α-mannosidase activity, interfering with the detection of neutral α-mannosidase. To suppress or remove the acid α-mannosidase, we used two approaches: inhibition by swainsonin and removal of acid α-mannosidase by affinity sorption on con A-seph. We know that the plant alkaloid swainsonin is an inhibitor of acid α-mannosidase, but it also weakly inhibits neutral cytosol mannosidase [8]. Under our conditions, with swainsonin in a concentration of 0.14 μM, activity of acid α-mannosidase in mononuclears was inhibited by 95%, whereas activity of neutral α-mannosidase was virtually completely preserved (Fig. 2). In the presence of swainsonin, a peak of neutral activity, not previously detected because of high acid α-mannosidase activity, was discovered in preparations of granulocytes from healthy blood donors (Fig. 1b).

It is interesting to note that platelet neutral α-mannosidase was inhibited by swainsonin in the same concentration by 70-80% (Fig. 1c), suggesting that platelets contain a neutral mannosidase which differs in its properties from the neutral mannosidase of lymphocytes and granulocytes. In order to obtain further proof of the presence of neutral α-mannosidase in granulocytes, activity of this enzyme was determined after removal of the acid α-mannosidase by sorption on con A-seph [7]. In granulocytes, just as in all other cell fractions, the acid α-mannosidase was adsorbed virtually completely on con A-seph, whereas

TABLE 1. Neutral α-D-Mannosidase Activity in Mononuclears and Granulocytes from Healthy Blood Donors and Patients with Mannosidosis and CML

Parameter	Mature granulocytes			Immature granulocytes, CML, n = 1	Mononuclears, normal, n = 6
	normal, n = 6	mannosidosis, n = 3	CML, n = 1		
Activity, units/mg protein	0,78 (0,28—1,75)	0,20 (0,10—0,31)	0,23	2,42	0,64 (0,3—1,0)
Activity, units/10^8 cells	3,6 (0,5—6,2)	1,9 (1,0—3,6)	1,8	17,4	17,7 (4,2—47,5)

Legend. n) Number of cases; limits of variations shown between parentheses.

neutral α-mannosidase remained unbound, and its activity was determined in the supernatant after removal of the con A-seph (Fig. 3).

It can be concluded from these results that granulocytes, like other blood cells, contain neutral α-mannosidase. To determine activity of neutral α-mannosidase quantitatively we used the two methods described above. As Table 1 shows, specific activity of neutral α-mannosidase, calculated per milligram protein in granulocytes and mononuclears from healthy blood donors was about equal, but if calculated per cell, it was significantly higher in the mononuclears than in the granulocytes. Accordingly the question arose, whether activity of neurtal α-mannosidase discovered in granulocytes is the result of their contamination by mononuclears in the process of fractionation of the cells. This question could be answered by using granulocytes obtained from a patient with CML, in whom lymphocytes and monocytes in the blood accounted for not more than 3% of the total number of leukocytes. Contamination of the granulocyte fraction by mononuclears in this case was minimal and could not have introduced an error into the calculation of activity. As Table 1 shows, neutral α-mannosidase activity in granulocytes obtained from the blood of patients with mannosidosis, and also in the fraction of mature granulocytes from a patient with CML, corresponded to the activity of this enzyme in healthy human granulocytes. Meanwhile, activity of neutral α-mannosidase in immature granulocytes of a patient with CML was more than three times higher than the average activity of this enzyme in healthy human granulocytes and more than 10 times higher than its activity in the same patient's granulocytes. This showed that neutral α-mannosidase is present in both immature and mature myeloid cells, and its activity in immature cells, moreover, is significantly higher. Since acid α-mannosidase activity in these cells also is significantly higher than in mature granulocytes, it can be tentatively suggested that the general process of metabolism of mannose-containing compounds in immature, undifferentiated myeloid cells is more active than in mature granulocytes.

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