Characterization of a soluble class I α-mannosidase in human serum

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Abstract Class I α-mannosidases are thought to exist exclusively as integral membrane proteins that play intracellulary an essential role in the *N*-glycan biosynthesis. Using [^{3}H]$Man_9GlcNAc_2$ as a substrate, we were able to identify a soluble α-mannosidase in human serum that trims the substrate $Man_9GlcNAc_2$ to $Man_{5-8}GlcNAc_2$ with $Man_6GlcNAc_2$ being the major product. This serum mannosidase is Ca^{2+}-dependent, sensitive to 1-deoxymannojirimycin but insensitive to the class II inhibitor swainsonine and, hence, belongs to class I mannosidases. The enzymatic properties of the serum class I mannosidase are similar to that of the membrane bound class I mannosidases Golgi-mannosidase IA and IB and Man_9-mannosidase.

Key words: Mannosidase; Glycosidase; *N*-glycan; HPAE chromatography; Demannosylation; Human serum protein

1. Introduction

Class I α-mannosidases specifically cleave α1,2-mannose linkages in a Ca^{2+}-dependent manner, but are inactive towards artificial substrates like aryl mannosides. These enzymes are type II integral membrane proteins that are specifically retained within the endoplasmic reticulum and the Golgi complex. Class I α-mannosidases play an essential role in *N*-glycan processing during the glycoprotein biosynthesis in mammalian cells removing up to four mannose residues from $Man_9GlcNAc_2$ to form the oligomannosidic glycans $Man_{8-5}GlcNAc_2$ (for review, [1,2,3]). The oligosaccharide $Man_5GlcNAc_2$ is the starting point for the synthesis of all hybrid and complex *N*-glycans found on mammalian glycoproteins.

Trimming of glycoprotein *N*-glycans of the oligomannosidic type, however, is not restricted to the biosynthesis but may also occur after the exit of glycoproteins from the secretory pathway and transport to the cell surface [4]. As has been shown in different cell types, cell surface glycoproteins undergo trimming of their oligomannosidic *N*-glycans by class I mannosidase(s) resulting in the conversion of $Man_{8-9}GlcNAc_2$ species to $Man_{5-7}GlcNAc_2$ with $Man_5GlcNAc_2$ being the major product. Demannosylation of cell surface glycoproteins has been shown to occur either at the cell surface or during endocytosis/recycling and is regarded as a mechanism to postbiosynthetically modify cell surface N-glycans of the oligomannosidic type [4].

In an attempt to identify mannosidases involved in the cell surface glycoprotein demannosylation, we have studied whether α1,2-mannosidase(s), assumed to exist exclusively as integral cellular membrane proteins, do also occur in a soluble form in human serum. This study was prompted by the observation that a diverse range of integral membrane proteins also exist as soluble forms in serum and other body fluids. These soluble forms are mostly derived from the membrane form by proteolysis [5].

In this study, we were able to identify a soluble serum α1,2-mannosidase with enzymatic properties similar to the membrane bound class I mannosidases Golgi-mannosidase IA and IB and Man_9-mannosidase. This soluble enzyme might be involved in the postbiosynthetic demannosylation of serum and cell surface proteins of endothelial and blood cells.

2. Materials and methods

2.1. Materials

Materials were obtained from the following sources. D-[2,6-^{3}H]-mannose (specific radioactivity 2 TBq/mmol) was from Amersham Buchler (Braunschweig, Germany). Dowex AG50W-X12 (hydrogen form) and Dowex AG3-X4 (free base form) were from Bio-Rad (München, Germany). Unless otherwise stated, all other chemicals and reagents were either from Sigma (Deisenhofen, Germany) or from Serva (Heidelberg, Germany). The oligosaccharides $Man_{5-9}GlcNAc_2$ and 1-deoxymannojirimycin (dMM) were from Oxford Glycosystems (Abingdon, UK).

2.2. Sample preparation

Venous blood was collected from healthy individuals and was allowed to clot at 37°C for 2 h. The serum was separated by centrifugation at $3000 \times g$ for 10 min.

2.3. Preparation of [^{3}H]mannose-labelled oligosaccharides

HepG2 cells were metabolically labelled with [2,6-^{3}H]mannose in the presence of 1 mM dMM. Cells were then surface-labelled with NHS-SS-biotin and biotinylated proteins were isolated from detergent extracts by affinity chromatography on streptavidin agarose [4]. Oligosaccharides were released by PNGase F from heat-denatured and trypsin-treated glycoproteins and were separated by HPAE chromatography as described [4]. Fractions containing $Man_9GlcNAc_2$ or $Man_8GlcNAc_2$, respectively, were pooled and passed through a column containing AG3-X4 in the bottom layer and AG50W-X12 in the top layer. After washing with two bed volumes of water, the filtrates were dried by evaporation.

2.4. HPAE separation of oligosaccharides

Oligosaccharides were separated using a Dionex (Sunyvale, CA, USA) DX-300 system and a CarboPac PA-100 (4×250 mm) in series with a CarboPac PA-100 guard column as described [6]. Columns were calibrated with authentic oligosaccharides $Man_{5-9}GlcNAc_2$.

2.5. [^{3}H]$Man_9GlcNAc_2$-mannosidase assay

[^{3}H]$Man_9GlcNAc_2$-cleaving mannosidase activity was assayed in

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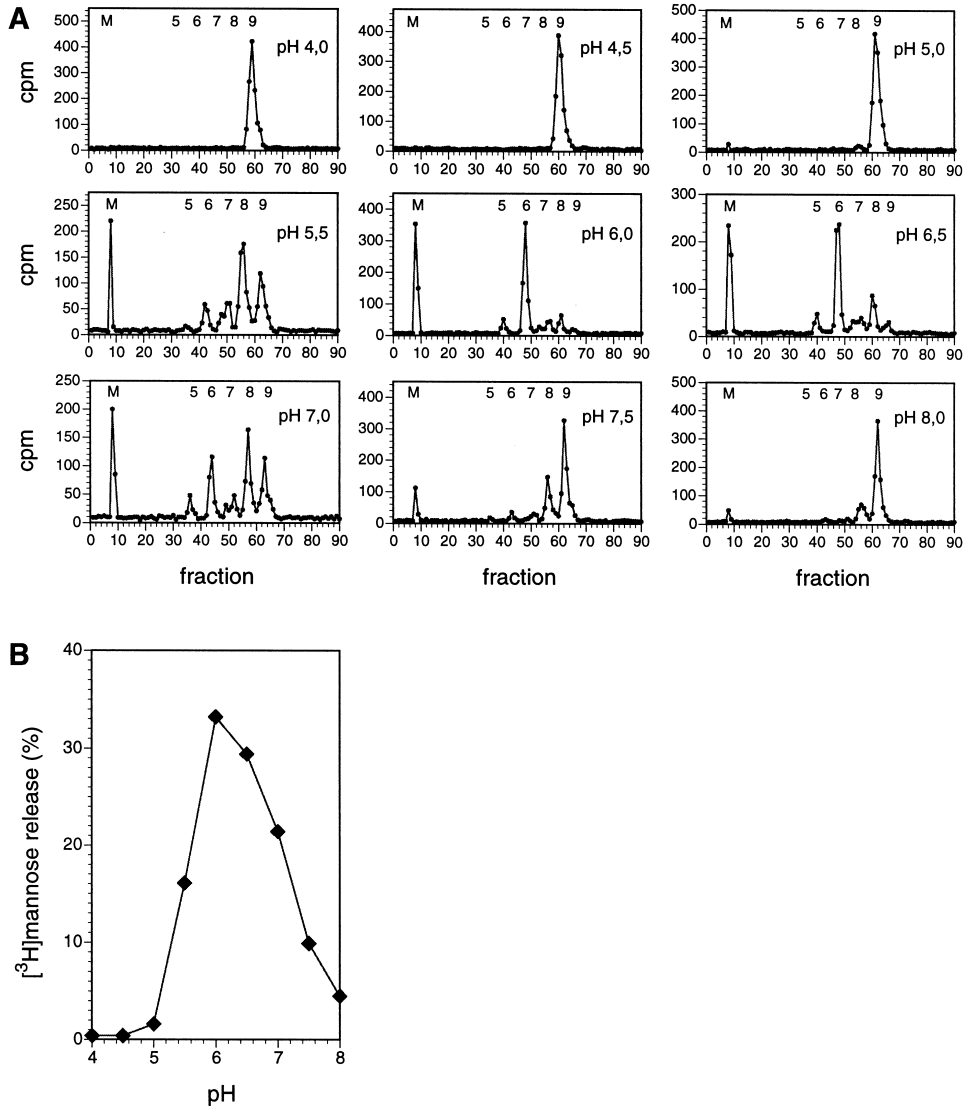

Fig. 1. Influence of the pH on the [^{3}H]$Man_9GlcNAc_2$-mannosidase activity present in human serum. (A) Serum samples were assayed for [^{3}H]$Man_9GlcNAc_2$ activity at the indicated pH. After incubation, oligosaccharides and the released [^{3}H]mannose were analyzed by HPAE chromatography. M, mannose; 9, $Man_9GlcNAc_2$; 8, $Man_8GlcNAc_2$; 7, $Man_7GlcNAc_2$; 6, $Man_6GlcNAc_2$; 5, $Man_5GlcNAc_2$. (B) pH optimum of the soluble α-mannosidase present in human serum.

human serum by incubating 100 μl of freshly prepared serum with radiolabelled substrate (–1500 cpm) and 200 μl of the reaction buffer: sodium acetate (0.5 M, pH 4.0, 4.5, 5.0, 5.5) or sodium cacodylate (0.5 M, pH 6.0; 0.2 M, pH 6.5, 7.0, 7.5, 8.0) for 8 h at 37°C. Immediately after incubation, oligosaccharides and the released [^{3}H]mannose were purified by mixed bed ion exchange chromatography and analyzed by HPAE chromatography. Inhibition studies were carried out by preincubating the samples in the presence of 1 mM dMM, 1 mM swainsonine or 20 mM EDTA for 30 min at 37°C. After preincubation, the samples were assayed for [^{3}H]$Man_9GlcNAc_2$-mannosidase activity at pH 6.5 for 4 h at 37°C in the presence of the inhibitor. To examine the Ca^{2+}-dependence, 30 mM $CaCl_2$ was added after the incubation with EDTA.

3. Results and discussion

3.1. Identification of a soluble α-mannosidase in human serum

To identify soluble class I mannosidases in human serum, freshly prepared serum samples were assayed for the [^{3}H]$Man_9GlcNAc_2$-cleaving activity. The substrate [^{3}H]$Man_9GlcNAc_2$ was prepared from HepG2 cells after metabolic labelling with [^{3}H]mannose as described in section 2. The mannosidase activity was assayed at various pH values ranging from pH 4.0 to 8.0. Separation of the reaction products by HPAE chromatography clearly showed a mannosidase activity cleaving $Man_9GlcNAc_2$ to $Man_{5-8}GlcNAc_2$ and free mannose in a pH-dependent manner (Fig. 1A). The soluble α-mannosidase in serum showed an optimal activity at pH 6.0 where $Man_6GlcNAc_2$ was the major product. 30–64% of the enzyme remained active at pH 7.0–7.5, whereas only a very low activity was detected at pH 4.0–5.0 (Fig. 1B).

3.2. Kinetics of the soluble serum α-mannosidase

As a measure for the substrate specificity, the time course of $Man_9GlcNAc_2$ hydrolysis and formation of the reaction products by serum α-mannosidase was determined quantitatively at pH 6.5. In these experiments, oligomannosidic *N*-glycans and released [^{3}H]mannose were analyzed by HPAE chromatography after different times of incubation (Fig. 2). Human serum mannosidase readily cleaved three of the four α1,2-

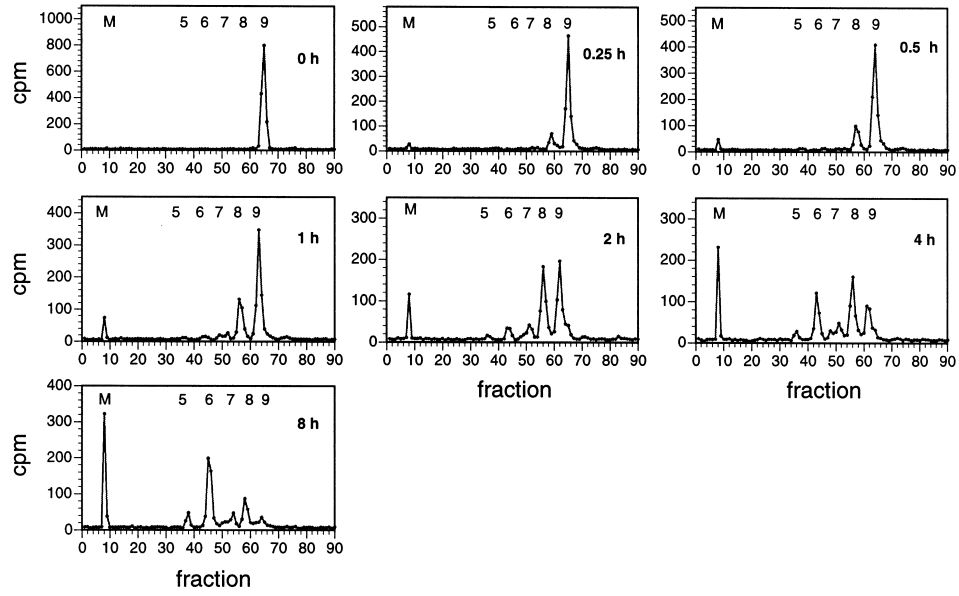

Fig. 2. HPLC fractionation of the oligosaccharides generated from $Man_9GlcNAc_2$ by the soluble serum α-mannosidase after various times of incubation. M and 5-9 indicate where free mannose and standard $Man_{5-9}GlcNAc_2$ oligosaccharides fractionate on HPLC.

mannosidic linkages in the substrate $Man_9GlcNAc_2$ to form $Man_6GlcNAc_2$ but displayed only a marginal activity towards the major product $Man_6GlcNAc_2$. The oligosaccharide $Man_6GlcNAc_2$ was also shown to accumulate as a reaction product when class I mannosidases, Man_9-mannosidase and Golgi-mannosidases IA and IB were incubated with the substrate $Man_9GlcNAc_2$ [7–10]. Therefore, it is likely that the serum mannosidase detected in the present study is related to these class I mannosidases.

Data from HPAE fractionations were quantitated by totaling the radioactivity in each peak and by correcting for the number of mannose residues, assuming that the specific activity in all mannose residues is the same. In Fig. 3A, the radioactivity of each oligosaccharide species, expressed as percentage of the total oligosaccharides recovered, is plotted versus the time of incubation. The α-mannosidase activity was quantitated by determining the radioactivity of the released [^{3}H]mannose, expressed as percentage of the total radioactivity recovered (Fig. 3B). With the assumption of first order kinetics, the release of [^{3}H]mannose followed the equation $D_t = D_{t\to\infty} \times (1-e^{-kt})$ where D_t is the extent of [^{3}H]mannose at the time t = x. The constant k can be determined from this equation as the negative slope of a plot of $\ln(D_{t\to\infty}-D_t)$ over the time (Fig. 3C). The half-time of the [^{3}H]mannose release (4.2 h) was calculated according to the equation $t_{1/2} = \ln 2/k$. Using the data of the hydrolysis of [^{3}H]$Man_9GlcNAc_2$ (Fig.

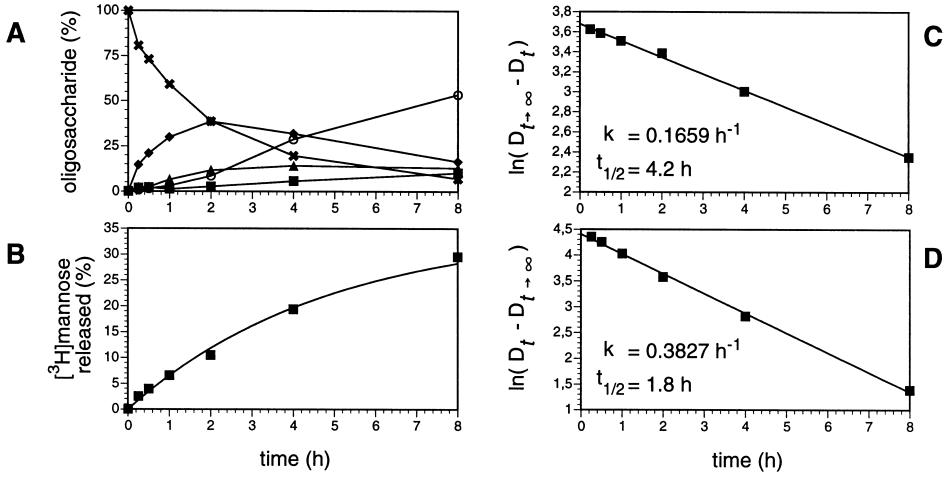

Fig. 3. Kinetic analysis of [^{3}H]$Man_9GlcNAc_2$ hydrolysis by the soluble α-mannosidase from human serum. (A) The rate of appearance of the oligosaccharide species generated from [^{3}H]$Man_9GlcNAc_2$ by the serum α-mannosidase. Chromatographic data from the experiment in Fig. 2 were quantitated by totaling the radioactivity in each peak and correcting for the number of mannose residues in that species. The amount of each oligosaccharide species is expressed as percentage of the total oligosaccharides recovered and plotted versus the time. (×) $Man_9GlcNAc_2$; (♦) $Man_8GlcNAc_2$; (▲) $Man_7GlcNAc_2$; (○) $Man_6GlcNAc_2$; (■) $Man_5GlcNAc_2$. (B) The percentage of [^{3}H]mannose released at each time point was determined from the radioactivity eluting in the position of free mannose on HPLC (see Fig. 2). (C) Plot of $\ln(D_{t\to\infty}-D_t)$ from the data displayed in B versus the time. A linear regression leads to $k = 0.1659$ h^{-1} ($R^2 = 0.999$). (D) Plot of $\ln(D_t-D_{t\to\infty})$ from the hydrolysis of $Man_9GlcNAc_2$ (data displayed in A) versus the time. A linear regression leads to $k = 0.3827$ h^{-1} ($R^2 = 0.998$).

Table 1
Enzymatic properties of the α-mannosidase from human serum

Effectors	Relative activity (%)
None	100
1 mM dMM	0.1
1 mM Swainsonin	108
20 mM EDTA	0.1
20 mM EDTA, 30 mM $CaCl_2$	119

3A), a half-time of 1.8 h could be calculated for this process (Fig. 3D). The hydrolysis of $Man_9GlcNAc_2$ by the serum α-mannosidase occurs distinctly faster as compared to the half-life of the majority of serum glycoproteins (for review, [11]) as well as of cell surface glycoproteins (for review, [12]), indicating that oligomannosidic *N*-glycans on these glycoproteins (for review, [13]) could undergo postbiosynthetic demannosylation by this enzyme.

3.3. Enzymatic properties of the serum α-mannosidase.

The [^{3}H]$Man_9GlcNAc_2$-mannosidase activity present in human serum could be completely blocked by the class I mannosidase inhibitor dMM, but was insensitive to the class II inhibitor swainsonine (Table 1). From these results, it can be concluded that human serum $Man_9GlcNAc_2$-mannosidase is a class I mannosidase. So far, serum mannosidases have been detected solely by the use of the artificial substrates 4-nitrophenyl-α-D-mannopyranoside or methyumbelliferyl-α-D-mannopyranoside [14,15]. Since only class II mannosidases are active towards aryl mannosides, the serum mannosidases described so far [14,15] have to be regarded as class II mannosidases. Hence, the present data provide the first evidence for the existence of a soluble class I mannosidase in serum. Moreover, the data support the conclusion that human serum does not contain dMM-resistant, swainsonine-sensitive class II mannosidases capable of cleaving $Man_9GlcNAc_2$ under standard assay conditions.

The soluble α1,2-mannosidase present in human serum was strongly inhibited by EDTA. The full enzyme activity was recovered when the incubation mixtures were supplemented with an excess of Ca^{2+} ions (Table 1). These data provide further evidence that the soluble mannosidase belongs to the class I mannosidases that contain an EF-hand Ca^{2+} binding consensus sequence and require Ca^{2+} for activity [3].

The soluble serum α1,2-mannosidase exhibits similar enzymatic properties as the Man_9-mannosidase [7,8,16] and Golgi-mannosidases IA and IB [9,10,17,18]. Studies are under way to examine whether serum mannosidase represents a proteolytically-cleaved form or a secreted splice variant of one of these membrane bound enzymes or may reflect a protein encoded by a gene, unknown so far.

The physiological role of serum class I mannosidase is unknown. The enzyme could be involved in demannosylation of serum glycoproteins or plasma membrane glycoproteins on endothelial cells or blood cells. Alternatively, serum class I mannosidase could exhibit lectin-like properties and could play a role in the recognition and binding of glycoconjugates containing oligomannosidic oligosaccharides as has been shown for mouse sperm surface α-mannosidase presumably involved in the sperm-egg binding [19].

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