Cloning and characterization of human liver cytosolic β-glycosidase

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Cytosolic β-glucosidase (EC 3.2.1.21) from mammalian liver is a member of the family 1 glycoside hydrolases and is known for its ability to hydrolyse a range of β-D-glycosides, including β-D-glucoside and β-D-galactoside. We therefore refer to this enzyme as cytosolic β-glycosidase. We cloned the cDNA encoding the human cytosolic β-glycosidase by performing PCR on cDNA prepared from total human liver RNA. Specific primers were based on human expressed sequence tags found in the expressed sequence tag database. The cloned cDNA contained 1407 nt with an open reading frame encoding 469 amino acid residues. Amino acid sequence analysis indicates that human cytosolic β-glycosidase is most closely related to lactase phlorizin hydrolase and klotho protein. The enzyme was characterized by using cell lysates of COS-7 cells transfected with a eukaryotic expression vector containing the cDNA. The biochemical, kinetic and inhibition properties of the cloned enzyme were found to be identical with those reported for the enzyme purified from human liver.

Key words: β-galactosidase, β-glucosidase, glycoside, mammalian.

INTRODUCTION

In mammals, several β-glucosidases have been characterized. The best characterized are lysosomal β-glucosidase (also called acid β-glucosidase or glucocerebrosidase), lactase phlorizin hydrolase (LPH) and cytosolic (or broad-specificity) β-glucosidase [1]. These three enzymes are members of the family 1 glycoside hydrolases [2].

A distinguishing feature of cytosolic β-glucosidase is its broad substrate specificity. It has been shown to hydrolyse β-D-galactoside and β-D-glucoside substrates with comparable efficiencies [3]. We therefore refer to this enzyme here as cytosolic β-glycosidase. The metabolic role of cytosolic β-glycosidase is still unknown. It is also not known whether any specific disease is associated with a deficiency of this enzyme. It has been shown to hydrolyse several non-physiological glycosides. However, it does not hydrolyse any known physiological β-glycoside. The inability of cytosolic β-glycosidase to hydrolyse these endogenous substrates has led to the hypothesis that it is involved in the detoxification of xenobiotic (plant) glycosides [4]. This hypothesis is sustained by the fact that cytosolic β-glycosidase can efficiently hydrolyse several naturally occurring plant glycosides, including amygdalin and L-picein [5].

Cytosolic β-glycosidase is present in the cytosol of liver, spleen, kidney, small intestine and lymphocytes of mammals. It has a molecular mass of approx. 53 kDa, has a pH optimum of 5.5–6.0 and is not glycosylated [6]. It is inhibited by sodium taurocholate at very low concentrations [7,8]. In contrast with lysosomal β-glucosidase, cytosolic β-glycosidase is not inhibited by conduritol B epoxide [6,9]. It exhibits a high affinity for the β-D-glucoside and β-D-galactoside derivatives of 4-methylumbelliferone (4-MuGlc and 4-MuGal respectively) and *p*-nitrophenol [3].

Although human cytosolic β-glycosidase has also been purified and characterized to some extent, its cDNA sequence and primary structure are still unknown [1,6]. Until now the only cytosolic β-glycosidase cloned was the guinea-pig enzyme [10].

To clone the cDNA of human cytosolic β-glycosidase we searched the expressed sequence tag (EST) database to identify five human ESTs, which showed high similarity to the guinea pig cDNA for cytosolic β-glycosidase. One of these also showed high similarity to the human LPH. These ESTs were used to design primers for the cloning of human cytosolic β-glycosidase. We used these primers for a PCR on cDNA, which was prepared from total human liver RNA. After cloning of the primers, we sequenced the cDNA (GenBank® accession number AJ278964) and compared the predicted amino acid sequence with the sequences of several other family 1 glycoside hydrolases. We characterized the enzyme in terms of its molecular mass, pH optimum and activity towards several glycoside substrates to confirm that the cloned enzyme was indeed the human liver cytosolic β-glycosidase.

MATERIALS AND METHODS

Materials and reagents

All materials were purchased from Life Technologies except as follows. *Pwo* polymerase, PCR buffer and dNTPs were obtained from Roche. The kits used for DNA isolation, purification and extraction from agarose gel were from Qiagen. Conduritol B epoxide and sodium taurocholate were purchased from Sigma. All materials needed for SDS/PAGE and Western blotting were obtained from Bio-Rad.

cDNA cloning

Total human liver RNA was used as the template for the synthesis of first-strand cDNA with the use of the Superscript

Abbreviations used: EST, expressed sequence tag; LPH, lactase phlorizin hydrolase; 4-MuGal, 4-methylumbelliferyl-β-D-galactopyranoside; 4-MuGlc, 4-methylumbelliferyl-β-D-glucopyranoside.

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Figure 1 Cloning strategy

The sizes and locations of ESTs and primers are shown to scale with reference to the human cytosolic β-glycosidase mRNA, depicted by the heavy black line. Primer P77 was based on EST AU076905, primers P79 and P80 on EST H60622. The ESTs AI796110, AI796175 and AI433547 were used to design primer P78.

P1 TAA TAC GAC TCA CTA TAG GG

P2 TAG AAG GCA CAG TCG AGG

P77 *GGC TAG* CCA TGG CTT TCC CTG CAG GAT TTG

P78 GGG *AAG CTT* CAG ATG TGC TTC AAG GCC ATT GTT

P79 GGC TTC CAG *AAT TCA* CTG AAG AAG AGA

P80 CTT CAG *TGA ATT* CTG GAA GCC TCG AT

Figure 2 Sequences of primers used for cloning and sequencing of the human cytosolic β-glycosidase

Primer P1 is the sense primer corresponding to the cytomegalovirus promoter on cloning vector pcDNA3.1(−)mycHisA. Primer P2 is the anti-sense primer corresponding to the bovine growth hormone poly(A) on pcDNA3.1(−)mycHisA. Primer P77 corresponds to the 5′ end of the mRNA and contains an additional *Nhe*I site. Primer P78 is the anti-sense primer corresponding to the 3′ end of the mRNA just upstream of the stop codon. It contains an additional *Hin*dIII site. Primers P79 and P80 both contain the *Eco*RI site present in the human cytosolic β-glycosidase cDNA sequence. The restriction sites are printed in italics.

first-strand synthesis system for reverse-transcriptase-mediated PCR. Priming was performed with oligo(dT) primers; reaction conditions were exactly as recommended by the manufacturer. All PCR primers were based on human ESTs found in the EST database at the National Centre for Biotechnology Information (Figure 1). The sequences of the primers are depicted in Figure 2. PCR was performed on first-strand cDNA with primers P77 and P78 for amplification of the complete cDNA. From the PCR product a 1470 bp DNA band was isolated by gel extraction. To increase the yield of the DNA fragment, PCR was performed on the purified PCR product with primers P77 and P80 for amplification of the 5′ part of the coding sequence or with primers P79 and P78 for amplification of the 3′ part.

Both PCR fragments were cloned into the expression vector pcDNA3.1(−)mychisA after digestion with restriction enzymes as follows. The PCR product of primers P77 and P80 was digested with *Nhe*I and *Eco*RI; the PCR fragment of primers P79 and P78 was digested with *Eco*RI and *Hin*dIII. Thus the enzyme was cloned carrying both a Myc tag for easy detection and a His_6 tag for purification at its C-terminus.

All sequencing was performed by BaseClear (Leiden, The Netherlands) with the dideoxy method on the cloned cDNA with primers P1, P2, P79 and P80 (Figure 2).

Enzyme expression

For expression, the expression vector containing the cDNA was transfected into COS-7 cells. The cells were transfected with LIPOFECTAMINE Plus reagent, by using the protocol provided by the manufacturer. Cells were grown for 48 h in Dulbecco's modified Eagle's medium containing 5% (v/v) fetal calf serum, 50 i.u./ml penicillin and 50 μg/ml streptomycin. After being harvested, the cells were taken up in 100 μl of PBS and lysed by freeze-thawing five times in liquid nitrogen.

Enzyme activity assay

All determinations were performed at 37 °C. The standard reaction contained 1.0 mM 4-MuGlc and 4-MuGal (Fluka) and 5 μl of cell lysate, in a final volume of 100 μl. For determination of the pH optimum of cytosolic β-glycosidase, a series of 0.2 M sodium citrate/0.1% BSA buffers for pH 4.5, 5.0, 5.5, 6.0 and 6.5, and 0.2 M sodium phosphate/0.1% BSA buffers for pH 6.0, 6.5, 7.0 and 7.5 were used. The assay for the determination of K_m was performed with a 0.2 M sodium acetate/0.1% BSA buffer, pH 5.5. The reaction was stopped after 1 h of incubation, by adding half of the reaction volume to 1 ml of 0.1 M glycine/NaOH buffer, pH 10.6. Substrate conversion was measured in a fluorescence spectrometer (PerkinElmer) at an excitation wavelength of 360 nm and an emission wavelength of 470 nm. One unit of enzyme activity is defined as 1 nmol of product formed/h at 37 °C under the defined assay conditions.

SDS/PAGE and Western blotting

Cell lysates from the transfection were analysed by SDS/PAGE as described by Laemmli [11]. After electrophoresis, proteins were transferred to a PVDF membrane [12]. Proteins were detected with anti-Myc antibody 9E10 [13] and horseradish-peroxidase-conjugated rabbit anti-mouse antibody in Tris-buffered saline [10 mM Tris/HCl (pH 7.5)/150 mM NaCl] with 0.1% Tween (Dako). The blots were developed by chemi-luminescent detection with Lumi-Light Plus (Boehringer Mannheim).

FPLC

Cell lysate from the transfection (50 μl) was loaded on a HiLoad™ 16/60 Superdex 200 column and eluted with PBS. Fractions of 2 ml were collected and β-D-galactosidase activity was assayed as

described above. The column was calibrated with reference proteins: IgM, IgG, BSA and cytochrome *c*.

RESULTS AND DISCUSSION

cDNA cloning

Cloning of the human cytosolic β-glycosidase was performed with specific primers based on the sequences of human ESTs showing high similarity to the guinea-pig cytosolic β-glycosidase sequence (U50545) and the human LPH gene. The cDNA obtained by PCR was cloned into the expression vector pcDNA3.1(−)mycHisA. After cloning, the cDNA sequence of several different independent clones was determined (Figure 3).

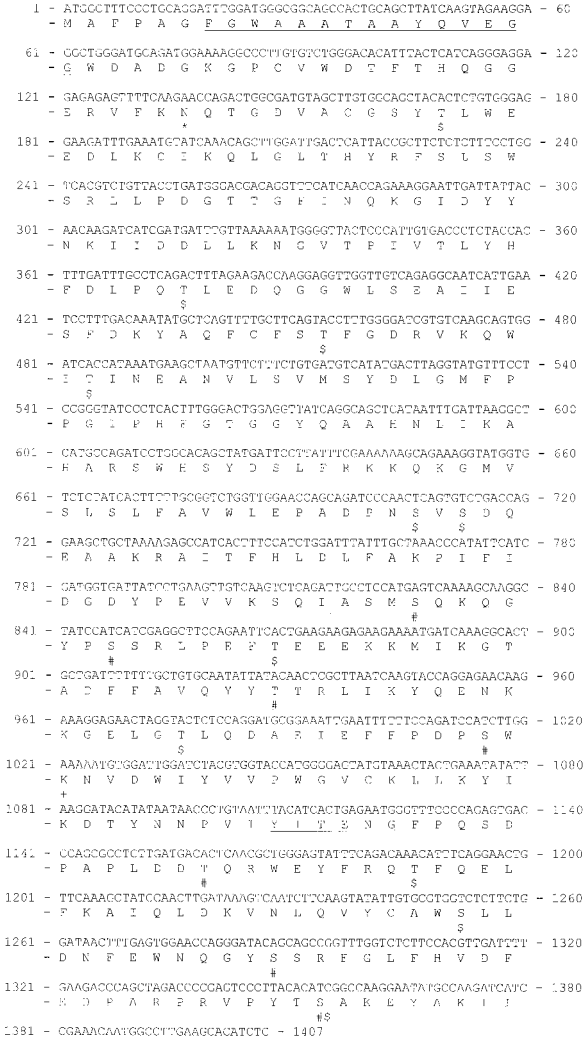

Figure 3 cDNA and amino acid sequences of the human cytosolic β-glycosidase

The underlined amino acid sequence represents the glycoside hydrolases family 1 N-terminal consensus sequence. The doubly underlined sequence represents the highly conserved YITENG motif containing the nucleophile (Glu residue), which is involved in the formation of a glycosyl-enzyme intermediate and occurs in all family 1 glycoside hydrolases [18]. The characters under the sequence correspond to the following: *, consensus N-glycosylation site; #, consensus protein kinase C phosphorylation site; $, consensus casein kinase II phosphorylation site; +, consensus tyrosine kinase phosphorylation site.

Sequence analysis

The cDNA encoding human cytosolic β-glycosidase is 1407 bp long. Translation of the cDNA sequence into an amino acid sequence predicted a molecular mass of 53 kDa. The sequence contains potential sites for post-translational glycosylation and phosphorylation (Figure 3). As expected from the guinea-pig counterpart, sequence similarity places this enzyme with the family 1 glycoside hydrolases [2]. Guinea-pig cytosolic β-glycosidase showed high similarity with the human enzyme (91 % similarity). The amino acid sequences of four family 1 glycoside hydrolases were compared and aligned with that of the human cytosolic β-glycosidase. The functional domains III (64 % similarity) and IV (63 % similarity) of human LPH and human klotho protein (57 % similarity) exhibited comparable similarities to human cytosolic β-glycosidase. LPH is an intestinal glycoprotein, hydrolysing dietary lactose and phlorizin. Deficiency of this enzyme causes lactose intolerance [14]. The klotho protein is a family 1 glycoside hydrolase associated with aging and diseases related to aging, including arteriosclerosis and osteoporosis [15,16].

The cDNA sequence encoding human cytosolic β-glycosidase is identical with that of the klotho-related protein, called cytosolic β-glucosidase-like protein1 ('cBGL1') [17].

We wished to confirm by biochemical characterization that the cloned cDNA sequence did indeed encode human cytosolic β-glycosidase. The biochemical properties of the recombinant human cytosolic β-glycosidase were compared with the properties previously reported for the cytosolic β-glycosidase purified from human liver.

Biochemical characterization

The apparent molecular mass of human cytosolic β-glycosidase in cell lysates from transfected cells was determined by SDS/PAGE and Western blotting and by size-exclusion chromatography as described in the Materials and methods section. For easy detection and purification we cloned the cytosolic β-glycosidase with a Myc tag and a His_6 tag at its C-terminus. Although these tags facilitate both processes, we are aware that these tags can influence the activity of the enzyme. We therefore also cloned the enzyme without these tags, by adding a stop codon to the 3′ primer immediately upstream of the cDNA sequence (results not shown). The enzyme containing the Myc and His tags exhibited an enzyme activity comparable to that of the enzyme without the tags (results not shown).

The apparent mass of the Myc-tagged enzyme as determined by Western blot was approx. 53 kDa, as expected [6] (results not shown). This was confirmed by size-exclusion chromatography (results not shown). The enzymic activity was present in the fraction containing proteins with a molecular mass of approx. 52 kDa, confirming that human cytosolic β-glycosidase is active as a monomer.

Human cytosolic β-glycosidase exhibited high β-galactosidase and β-glucosidase activity over a broad pH range (5–7) when assayed with 4-MuGal and 4-MuGlc (Figure 4). The β-glucosidase activity of human cytosolic β-glycosidase is less dependent on the pH than the β-galactosidase activity. As has been reported previously [6], the enzyme shows higher β-galactosidase activity than β-glucosidase activity. In fact, a distinguishing feature of cytosolic β-glycosidase purified from human liver has been its broad substrate specificity. The enzyme hydrolyses not only β-D-galactosides and β-D-glucosides but also β-D-fucosides, β-D-xylosides and α-L-arabinosides.

Substrate saturation curves were constructed for the cytosolic β-glycosidase with 4-MuGal and 4-MuGlc. The β-glucosidase

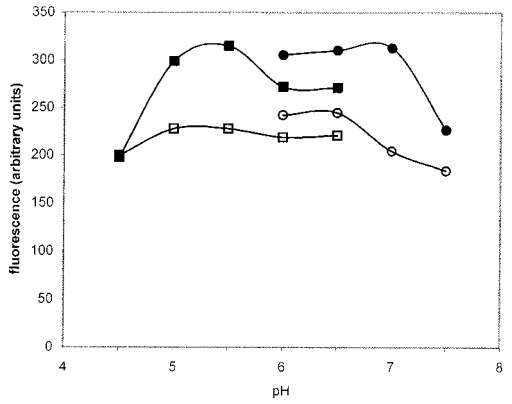

Figure 4 Effect of pH on the activity of human cytosolic β-glycosidase

Comparison of β-D-galactosidase (●, ■) and β-D-glucosidase (○, □) activity of human cytosolic β-glycosidase in lysate of transfected COS-7 cells as a function of pH with 0.2 M sodium citrate (□, ■) and 0.2 M sodium phosphate (○, ●) buffers. The pH optimum of human cytosolic β-glycosidase is broad, ranging from 5 to 7.

and β-galactosidase activities of cytosolic β-glycosidase in cell lysate of transfected COS-7 cells were assayed at pH 5.5. The activities were measured over a 500-fold range of concentrations. The curves fitted Michaelis–Menten kinetics in both cases (results not shown). The K_m values of the cloned enzyme for 4-MuGlc and 4-MuGal were 0.04 and 0.05 mM respectively, similar values to those reported earlier [6].

For further confirmation of the identity of the enzyme, we compared the effect of two inhibitors on the β-glucosidase and β-galactosidase activities of human cytosolic β-glycosidase. Conduritol B epoxide is a potent inhibitor of lysosomal β-glucosidase but does not affect the activity of the cytosolic β-glycosidase [9]. In contrast, sodium taurocholate is an inhibitor of the cytosolic enzyme and activates lysosomal β-glucosidase [7]. We assayed the cloned enzyme in the presence of either of these inhibitors. The presence of conduritol B epoxide (6.2 mM) did not decrease the β-glucosidase and β-galactosidase activities of the cloned enzyme, whereas sodium taurocholate (13.0 mM) had an inhibitory effect on both activities of cytosolic β-glycosidase. These results confirm that the cloned enzyme is indeed cytosolic β-glycosidase.

In summary, characterization of the enzyme cloned in this study confirms that we have cloned the human cytosolic β-glycosidase. It has a broad pH optimum (5–7) and a molecular mass of approx. 53 kDa, and it is active as a monomer. The enzyme kinetics displayed by this enzyme is similar to that reported with enzyme purified from human liver, as are the effects of inhibitors on enzyme activity. Moreover, sequencing of the cloned cDNA revealed a high similarity to guinea-pig cytosolic β-glycosidase.

Received 20 September 2000/14 March 2001; accepted 30 March 2001

The cDNA sequence of cytosolic β-glycosidase reported here should facilitate further studies to unravel the metabolic function of the enzyme, for example by the development of a knock-out strain of mice.

We gratefully acknowledge the gift of the total human liver RNA from D. A. M. Heideman.

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