Partial amino acid sequence and mRNA analysis of cytosolic pyridoxine- β -D-glucoside hydrolase from porcine intestinal mucosa: proposed derivation from the lactase–phlorizin hydrolase gene

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We have previously identified and purified a novel β -glucosidase, designated PNGH (pyridoxine-5'- β -D-glucoside hydrolase), from the cytosolic fraction of pig intestinal mucosal. PNGH catalyses the hydrolysis of PNG (pyridoxine-5'- β -D-glucoside), a plant derivative of vitamin B₆ that exhibits partial nutritional bioavailability in humans and animals. Preliminary amino acid sequence analysis indicated regions of close similarity of PNGH to the precursor form of LPH (lactase–phlorizin hydrolase), the β -glucosidase localized to the brush-border membrane. We report in the present study amino acid sequence data for PNGH and results of Northern blot analyses, upon which we propose a common genomic origin of PNGH and LPH. Internal Edman sequencing of the PNGH band isolated by SDS/PAGE yielded data for 16 peptides, averaging 10.8 amino acids in length. These peptides from PNGH (approx. 140 kDa) were highly similar to sequences existing over most of the length of the > 200 kDa precursor of rabbit LPH; however, we found no PNGH sequences that corresponded to approx. 350 amino acids between positions 463 and 812 of the LPH precursor, a region encoded by exon 7 of

the LPH precursor gene (amino acids 568–784), and no sequences that corresponded to regions near the N-terminus. MS analysis of tryptic peptides yielded 25 peptides, averaging 15 amino acids, with masses that matched segments of the rabbit LPH precursor. Northern blot analysis of pig and human small intestinal polyadenylated mRNA using a non-specific LPH cDNA probe showed an expected approx. 6 kb transcript of the LPH precursor, but also an approx. 4 kb transcript that was consistent with the size predicted from the PNGH protein mass. Using a probe specific to the region encoded by exon 7, hybridization occurred only with the 6 kb transcript. Based on these observations, we propose that both PNGH and LPH enzymes have the same genomic origin, but differ in transcriptional and, possibly, post-translational processing.

Key words: absorption, bioavailability, intestinal mucosa, lactase–phlorizin hydrolase (LPH), pyridoxine- β -D-glucoside hydrolase (PNGH), vitamin B₆.

INTRODUCTION

Pyridoxine-5'- β -D-glucoside (PNG) is a major form of vitamin B₆ in plant-derived foods. Dietary PNG exhibits approx. 25 % bioavailability, relative to pyridoxine, in rats [1,2] and approx. 50 % for humans [3,4]. Hydrolysis of the β -glycosidic bond of PNG to release free pyridoxine is a requisite step in the nutritional utilization of PNG in vitamin B₆ metabolism [1,5]. The rate and extent of PNG utilization are governed by the rate of hydrolysis of its glycosidic bond. Studies *in vivo* in rats and humans have shown that most of the hydrolysis of PNG occurs in the intestine [2,3].

Several β -glucosidases have been well characterized in mammalian cells. Lysosomal membrane bound acid β -glucosidase is responsible for the hydrolysis of the β -glycosidic linkage of glucocerebrosides [6]. Another membrane bound β -glucosidase in the small intestine is LPH (lactase–phlorizin hydrolase), a brush-border enzyme that catalyses the hydrolysis of dietary lactose and other β -glucosides [7]. Cytosolic broad specificity β -glucosidase catalyses the hydrolysis of various aryl β -D-glucosides [8]. We initially proposed that intestinal hydrolysis of PNG was catalysed by the broad specificity β -glucosidase [9], but found that PNG is neither a substrate nor an inhibitor of broad specificity β -glucosidase purified from pig intestine

[10,11]. These observations further led to the identification and purification of a distinct apparently cytosolic β -glucosidase capable of hydrolysing PNG, that was later designated PNGH (pyridoxine-5'- β -D-glucoside hydrolase) [11]. PNGH also was found to catalyse the hydrolysis of the β -glycosidic linkages of cellobiose and lactose, but not the α -glycosidic bonds of sucrose or maltose. We assumed that the activity of cytosolic PNG hydrolase is physiologically relevant in releasing pyridoxine from the fraction of dietary PNG taken up by enterocytes without prior hydrolysis. We have shown that purified LPH of the brush-border membrane also catalyses PNG hydrolysis [12]. This indicates that PNG is a secondary substrate for LPH and that either partial hydrolysis of dietary PNG in the mammalian small intestine can occur at the brush-border membrane prior to absorption or PNG can be absorbed in intact form and hydrolysed by cytosolic PNGH.

Although LPH also has the ability to catalyse the hydrolysis of a wide range of other substrates, including a range of glyceroceramides [13] and flavonoid glycosides [14], its primary function is to hydrolyse dietary lactose [15]. Since dietary lactose is not absorbed intact and is hydrolysed in the intestinal lumen, the lactase activity of cytosolic PNGH does not contribute to lactose digestion. In humans and most mammalian species, LPH is expressed as a single large precursor molecule termed pre-pro-LPH that undergoes post-translational modification via two

Abbreviations used: LPH, lactase–phlorizin hydrolase; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; PNG, pyridoxine- β -D-glucoside; PNGH, PNG hydrolase.

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proteolytic steps and glycosylation to yield mature enzymically active LPH (approx. 160 kDa) that is directed to the brush-border membrane [7]. A large fragment (approx. 100 kDa, 849 amino acids, designated LPH α) that is released from the LPH precursor has no enzymic activity, but serves as a chaperone necessary for proper folding of mature LPH [16,17]. LPH is encoded by the LCT gene, which exists in the human genome as a single copy on chromosome 2 [18]. The LCT gene consists of a reading frame (17 exons) that is exclusively transcribed in small intestine to yield the approx. 6 kb mRNA, which encodes the LPH precursor (pre-pro-LPH) [18,19].

To determine more clearly the nature of PNGH and its relatedness to mammalian LPH, we modified the purification method described previously [10] to increase the yield of PNGH in a partially purified form suitable for determination of amino acid sequences of PNGH from SDS/PAGE gels. We report in the present study the results from internal peptide sequence analysis of PNGH and Northern blot analysis showing a novel mRNA consistent with that predicted for a PNGH transcript. On the basis of these data, we present a hypothesis regarding the relationship and proposed common genomic origin of cytosolic PNGH and brush-border LPH.

EXPERIMENTAL

Materials

PNG was biologically synthesized from pyridoxine and purified chromatographically as described previously [20]. DE-52 anion exchange cellulose was obtained from Whatman, Sephadex 300 gel filtration and Q Sepharose XL anion exchange matrices were from Pharmacia, and Macro Prep High S cation exchange matrix was from Bio-Rad. BenchMark prestained protein ladder for SDS/PAGE was from Gibco BRL. Human small intestine RNA was obtained from Ambion. All reagents employed were of analytical grade or better.

Enzyme purification

During the purification, all materials were kept on ice or at 4 °C. Pig jejunum was collected at the time of slaughter at the University of Florida Animal Science Department in a US Department of Agriculture-inspected facility. Longitudinally cut sections were washed with 0.9 % (w/v) NaCl and stored at -80 °C until use. Initial purification procedures were conducted as described by McMahon et al. [11], using approx. 300 g of frozen pig intestine thawed on ice and mucosa scraped from underlying tissue with a glass slide. The resulting approx. 100 g of mucosa was homogenized using a Polytron P10-35 device (Brinkmann Instruments) in 100 ml of buffer A (10 mM NaH₂PO₄, pH 7) containing 10 mM β -mercaptoethanol and 1 ml of protease inhibitor cocktail (Sigma P8340), and centrifuged at 200 000 *g* for 30 min. The homogenate was adjusted to pH 6 with acetic acid, and then centrifuged for 20 min at 20 000 *g*. Anion exchange chromatography of the supernatant was then conducted using a 4 cm $\times$ 30 cm DE-52 DEAE-cellulose column equilibrated in buffer B (10 mM NaH₂PO₄, pH 6). The column was washed with 1 litre of buffer B and protein was eluted with a 2 litre linear gradient of 0–0.4 M NaCl in buffer B. Fractions in this and subsequent chromatographic separations were monitored spectrophotometrically at A₂₈₀ for protein, and PNGH activity was assayed as described previously [11,20]. Fractions with the highest activity were pooled and concentrated in a stirred-cell ultrafiltration apparatus (Amicon Diaflo 10 PM30 membrane).

Gel filtration chromatography was performed on the resulting concentrate using a 2.6 cm $\times$ 60 cm Sephacryl 300 column equilibrated and eluted with buffer B containing 50 mM NaCl (60 ml/h). Fractions having the greatest PNGH activity were pooled and concentrated as described above. This concentrated PNGH pool was divided into two equal fractions and portions were applied separately to either a 1.6 cm $\times$ 10 cm Bio-Rad Macro-Prep High S cation exchange column or a 1.6 cm $\times$ 10 cm Q Sepharose XL anion exchange column as the next purification step. The columns were equilibrated in buffer B and the samples were eluted with a 0–0.5 M NaCl linear gradient at 60 ml/h for each column. Protein content was determined and fractions with enriched PNGH activity were pooled and concentrated. Enzyme purity was determined by SDS/PAGE according to Laemmli [21]. Fractions from different purification steps were loaded into an 8 % (w/v) total polyacrylamide mini-gel and stained with Simply Blue Safe Stain (Invitrogen).

PNGH activity assay, kinetics and protein determination

The PNGH activity was measured according to Nakano and Gregory [22]. Reaction mixtures were incubated at 37 °C for 1 h, followed by incubation in boiling water for 5 min to stop the reaction. The assay mixture contained 0.4 M NaH₂PO₄/1 mM PNG, pH 6. The concentration of pyridoxine released from PNG was measured using reversed-phase HPLC with fluorometric detection [20]. Kinetic constants ($V_{\max}$ and K_m) were calculated by non-linear regression (SigmaPlot Enzyme Kinetics Module, SPSS). PNG was used as the substrate from 0.2 to 10 mM. One unit of PNGH hydrolase activity was defined as the quantity of enzyme that catalysed the release of 1 nmol of pyridoxine from PNG per h. Protein concentration was determined according to Markwell et al. [23] using BSA as the standard.

Protein identification and amino acid sequence analysis

Analyses for determination of PNGH amino acid sequence information were performed at the Emory University Microchemical Facility and at the University of Florida Protein Chemistry Core. At the Emory facility, the SDS/PAGE-purified protein was in-gel digested using 500 ng of sequencing grade trypsin (Promega) for 15 h at 37 °C [24]. For tryptic peptide mass matching, the extracted peptides (25 % of total material) were micro-desalted using a C18ZipTip (Millipore, Bedford, MA, U.S.A.) and analysed by delayed-extraction reflectron MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS (ReflexIII mass spectrometer; Bruker Daltonics, Billerica, MA, U.S.A.), as described by Hubalek et al. [25]. The relationship of PNGH to known proteins was evaluated initially by matching mono-isotopic masses of tryptic peptides against the all Taxa proteome using the ProFound algorithm (http://129.85.19.192/profound_bin/WebProFound.exe). The remaining 75 % of the extracted peptides was separated by capillary reversed-phase HPLC [25], and the eluate with an A₂₁₀ was manually collected for sequence analysis. N-terminal sequencing of peptides was done by automated Edman degradation (Procise-cLC model protein sequencer; Applied Biosystems, Foster City, CA, U.S.A.) [26]. In order to increase sequence coverage of PNGH, the aliquots of HPLC fractions of the digest were also analysed by MALDI-TOF/TOF MS/MS using a model 4700 Proteomics Analyzer (Applied Biosystems). For each fraction, an MS spectrum was initially collected, and up to ten precursor molecular ions observed in each fraction were selected for subsequent MS/MS analysis. Using the manufacturer's GPS Explorer 2.0 software, the MS

and MS/MS data were submitted to a MASCOT search engine (<http://www.matrixscience.com/>) for positive identification. The NCBI non-redundant database and the Mammalia taxonomy were used for these and all other searches.

In analyses conducted at the University of Florida, SDS/PAGE-purified protein was enzymically cleaved by endoproteinase LysC (Promega) or trypsin, fragments were separated by small-bore reversed-phase HPLC on a Vydac C18 column, and collected peptides subjected to internal fragment N-terminal sequence analysis by standard Edman degradation chemistry (Procise 494 HT protein sequencer; Applied Biosystems).

Northern blot analysis

Total RNA from pig small intestinal mucosa was extracted using Trizol reagent (Life Technologies), followed by oligo (dT) affinity isolation of the polyadenylated RNA fraction (PolyA-Purist; Ambion). Pig small intestine cDNA clone c8a10 [27] inserted into the Bluescript plasmid vector (Stratagene) was used as the probe. Standard methods were used for plasmid amplification, isolation and restriction enzyme digestion [28]. Maxiscript In Vitro Transcription kit and [α - 32 P]dCTP were used to label the single-stranded probe. This cDNA has been reported to comprise 243 bases (GenBank accession number, F14711); however, our sequencing of the amplified plasmid revealed an insert of 2190 bases with 86–87% similarity to the range of nucleotides 795–2562 of the human LPH sequence and 763–2533 of the rabbit sequence (BLAST E values $\leq 10^{-55}$). This range spanned sequences encoded by all or part of exons 3–8 of the LPH gene [18].

We prepared a second probe with specificity for a sequence corresponding to exon 7 of the human LPH/LCT gene, a segment that we hypothesized, on the basis of amino acid sequence, was missing from the PNGH mRNA sequence. In this procedure, total pig jejunum DNA was isolated using Trizol reagent, and LPH exon 7 was amplified by PCR using following the set of primers: sense, gactggcagaacccctgtct; and antisense, ggcaccacacgaatccaagag. The conditions of the PCR were: 65 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min, repeated for 25 cycles. The product (approx. 400 bases) was isolated after agarose gel electrophoresis (Invitrogen E-Gel). Bio-Rad Freeze 'N Squeeze DNA gel extraction kit was used to extract the product, and a portion was sequenced by the University of Florida DNA Sequencing Core Laboratory. This analysis yielded the following sequence: acccctgtct ccagagaggg ctgaggacct gagagcctct gaggcctct tgcactcat gctgggtcgg ttgcacacc ccgtaattgt gtagggagat taccagcca cctgaggaa ccagatcca catagtaaca gacagtctc ccatcctgtg gctcaccatc cgggagttac agaggcagag aagcagctcc tgaaaggctc tgcgtgatttt ctggtctgtc gcattacacc tcccgcctca tcagaacgc ccacaaaac acctgcaccc ctagctatga taccattgga aggtctctcc caacacgtga accatgtgtg gccccagacc tcatccttt gg (352 bases). This matched reported sequences from human and rabbit LPH exon 7 with 96% and 85% similarity respectively. The amplified LPH exon 7 probe was radiolabelled using [α - 32 P]dCTP and Maxiscript In Vitro Transcription kits (Ambion), and probed against the blot obtained with freshly extracted pig small intestinal mucosa mRNA after Trizol reagent followed by oligo (dT) affinity isolation (Ambion PolyA-Purist).

Northern blots were performed following the manufacturer's instructions using the NorthernMax kit (Ambion), with 2 μ g of each mRNA sample (human or pig small intestine mucosa) separated on a denaturing 1% (w/v) agarose gel. Each gel was stained with ethidium bromide and photographed on an UV transilluminator. The mRNA was capillary transferred for 2–4 h in 20 $\times$ saline sodium citrate to a positively charged nylon membrane (BrightStar-Plus membrane, Ambion) by downward

flow to reduce transfer time and increase efficiency [29], then fixed by UV cross-linking. Millenium Markers (Ambion) were used to provide accurate size determination of single-stranded RNA. Membranes were incubated overnight with radiolabelled probe and washed with a high stringency solution (0.1 $\times$ saline sodium citrate/0.1% SDS) at 45 °C and exposed to X-ray film (X-OMAT, Eastman Kodak). Human jejunal mRNA was obtained commercially (Ambion).

RESULTS AND DISCUSSION

Purification and properties of PNGH

The present study reports a partial purification procedure for PNGH, which is a modification of our previous method [11], to increase yield for sequence analysis. The profiles of four chromatographic steps are shown (Figure 1), and a summary of the purification of PNGH is presented (Table 1). The variations on the previous procedure mainly involved the use of conventional chromatographic columns, rather than lower capacity HPLC-based separations used previously [11]. The initial DE-52 anion exchange column, as used previously, achieved the largest quantitative separation of PNGH from other proteins (9-fold). Gel filtration chromatography on Sephacryl S-300 yielded another approx. 3-fold further purification. We then evaluated cation exchange chromatography on a Bio-Rad Macro-Prep High S column and anion exchange chromatography on Macro-Prep HS as the next and final step of this partial purification. In this comparison, the cation exchange chromatography was superior, with approx. 3.6-fold further purification. In addition, in cation exchange chromatography PNGH was eluted in the void volume, which contributed to speed and convenience, because many other proteins were retained. This result suggests that anion exchange chromatography using the Q Sepharose XL, and possibly gel filtration on Sephacryl 300, could be eliminated from the purification scheme to reduce time and effort in obtaining a sufficiently purified enzyme fraction for amino acid sequence analysis.

Partially purified cytosolic PNGH exhibited Michaelis–Menten kinetics with PNG as the substrate. The $V_{\max}$ and K_m values were 11.8 nmol/h per mg of protein and 0.71 mM respectively, which were consistent with values reported by McMahon et al. [11].

Electrophoresis and molecular mass

SDS/PAGE indicated that PNGH had been partially purified (Figure 2). We estimate that the final PNGH preparation in this purification was 30–40% pure on the basis of the relative intensity of Coomassie Blue G-250 staining. Using 8% (w/v) total polyacrylamide gel; concentration in SDS/PAGE analysis, the PNGH band was well resolved from other proteins and well suited for excision and sequencing (Figure 2). The apparent molecular mass of PNGH under these denaturing conditions was 140 kDa, which is in agreement with the molecular mass of 140 kDa determined by SDS/PAGE analysis of PNGH purified by previous methods [11]. McMahon et al. [11] also observed an apparent molecular mass of approx. 160 kDa by gel filtration chromatography under non-denaturing conditions, which suggests that PNGH exists as a monomer under physiological conditions.

Amino acid sequence analysis

Internal sequence analysis of PNGH by Edman degradation yielded data for 16 peptides with an average of 10.8 amino acids

Figure 1 Chromatographic purification of pyridoxine- β -D-glucoside hydrolase

(A) DE-52 DEAE-cellulose anion exchange column. A_{280} (●) was determined for each fraction of 8 ml/tube. Elution was accomplished using a 0–0.4 M NaCl gradient in 10 mM sodium phosphate buffer, pH 6. Fractions were collected throughout the NaCl gradient only. Fractions 94–124 with enriched PNGH activity (○) (relative activity) were pooled and concentrated. (B) Sephacryl 300 gel filtration chromatography. Concentrated fractions from the DE-52 column were applied to a Sephacryl 300 column. A_{280} was determined for each fraction of 5 ml/tube. Fractions 27–37 with enriched PNGH activity were pooled and concentrated. (C) Pharmacia Q Sepharose XL anion exchange chromatography. Half of the concentrated fractions of Sephacryl 300 column were applied to a Q Sepharose XL anion exchange column. A_{280} was determined for each fraction of 4 ml/tube. Elution was performed with a 0–0.5 M NaCl gradient in 10 mM sodium phosphate buffer, pH 6. Fractions 41–49 with enriched PNGH activity were pooled, concentrated and stored at 4 °C. (D) Bio-Rad Macro-Prep High S cation exchange chromatography. Half of the concentrated fractions from the Sephacryl 300 column were applied to a Macro-Prep High S cation exchange column. A_{280} was determined for each fraction of 4 ml/tube. A 0–0.5 M NaCl gradient in 10 mM sodium phosphate buffer, pH 6, was used during elution. Fraction 4 with enriched PNGH activity was concentrated and stored at 4 °C.

Table 1 Purification summary of PNGH from pig intestinal mucosa

Supernatant refers to the starting material after centrifugation at 20 000 *g*.

Fraction	Total volume (ml)	Total protein (mg)	Total units*	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Supernatant	92	1571	11 588	7.4	100	1
DE-52 DEAE-cellulose column	24	102	6605	64.5	57	9
Sephacryl 300 column†	10	21	3989	190	34	26
Q Sepharose XL anion exchange column	4	3	1389	413	12	56
Macro-Prep High S cation exchange column	2	1	811	688	7	93

* Units are expressed as nmol of pyridoxine released per h.

† Concentrated PNGH pool was divided into two equal fractions and applied separately to either Bio-Rad Macro-Prep High S cation exchange column or Q Sepharose XL anion exchange column as the next purification step.

each. Using BLASTP analyses, these peptides were found to exhibit at least 98% similarity to the sequence of rabbit LPH precursor (pre-pro-LPH) (Figure 3) and aligned over almost the entire length of pre-pro-LPH. However, the large difference in molecular mass between pre-pro-LPH (approx. 220 kDa) and PNGH (approx. 140 kDa) indicates that, in spite of the high degree of sequence similarity, PNGH is not identical with the LPH precursor. MS analysis of the masses of tryptic peptides complemented the amino acid sequencing and provided extended coverage of the full PNGH sequence (Figure 3). The MALDI-TOF MS analysis detected 25 peptides from tryptic hydrolysis of

PNGH whose masses matched segments of pre-pro-LPH. Similar to the results from Edman degradation studies, peptides identified by MS analysis covered most of the range of pre-pro-LPH. Further analysis by MALDI-TOF/TOF MS/MS confirmed the identities for all or part of 9 of these PNGH tryptic peptides.

BLASTP analysis of our entire set of pig PNGH amino acid sequences obtained by Edman and/or MS against sequences in the mammalian database indicated close matches to full-length sequences of rabbit and human pre-pro-LPH (E-values, 4×10^{-60} and 2×10^{-48} respectively). It should be noted that rabbit and human pre-pro-LPH exhibit close similarity, as indicated by

Figure 2 SDS/PAGE analysis

Electrophoresis was performed using 8% (w/v) total polyacrylamide gel; with the exception of lane A each lane contains 8 μ g of protein. Lane A, prestained protein ladder with molecular mass in kDa (Gibco BRL); lane B, 20 000 g centrifugation concentrated fraction; lane C, DE-52 anion exchange concentrated fraction; lane D, Sephacryl 300 gel filtration concentrated fraction; lane E, Q Sepharose XL anion exchange concentrated fraction; lane F, Macro-Prep High S cation exchange concentrated fraction. Protein band at 140 kDa corresponds to PNGH.

81% identity and BLAST E-value $< 10^{-180}$. As pig PNGH exhibited closer similarity to rabbit, rather than human, pre-pro-LPH sequence, we elected to show the comparison of pig PNGH with rabbit pre-pro-LPH in Figure 3.

Data from Edman degradation and/or MS revealed a large segment of the pre-pro-LPH sequence in which no alignment with PNGH peptides occurred, which ranged from positions 464 and 782 of pre-pro-LPH (Figure 3). In this regard, the peptide identified by MALDI-TOF MS as AIKEDSV DVR (positions 783–792) could not be confirmed by MS/MS and, thus, may be regarded as ambiguous. For this reason, the region lacking similarity may extend to approx. position 812 of the pre-pro-LPH sequence.

The segment of pre-pro-LPH encoded by exon 7 of the LPH/LCT gene [18] corresponds to amino acids 573–788 for human pre-pro-LPH. Assuming similar intron–exon structure of the pig LPH/LCT gene, the segment for which no correspondence exists between pre-pro-LPH and PNGH would include the region encoded by exon 7. The total molecular mass of these segments of pre-pro-LPH which did not align with PNGH peptide sequences was approx. 36–39 kDa. The absence of matching sequences in this region cannot be attributed to a lack of tryptic cleavage sites, since lysine and arginine residues are dispersed throughout. A possible explanation of the lack of sequence alignment in these sequencing analyses could be the large size, hydrophobic nature and neutral charge of these fragments which might hinder efficient extraction and removal from the SDS/PAGE gel. We examined the exon 7 region of pre-pro-LPH and found that many of the predicted tryptic peptides would be of polar character. Thus at least some of these would be available for recovery during liquid chromatography and detectable in Edman sequencing.

Based on the above observations, we propose that the segment of pre-pro-LPH encoded by exon 7 is absent from PNGH. The 'statistics' of this analysis provide support this hypothesis. As summarized in Table 2, we evaluated the amino acid sequences of peptides that were identified by Edman degradation or matched by MS or MS/MS for the three 'segments' of the PNGH protein, the N-terminus, exon 7 and the C-terminus. At present, only one 'exon 7 peptide', AIREDSVDVR, was matched by MS, but this match could not be confirmed by the MS/MS analysis as the peptide molecular ion yielded a very poor MS/MS spectrum. No peptide from exon 7 could be found by Edman sequencing.

The pattern of alignment between PNGH and pre-pro-LPH eliminates the possibility that PNGH is the approx. 100 kDa, 849-amino-acid fragment (LPH α) that is released during post-

Table 2 Summary of amino acid sequencing results for PNGH by Edman degradation and MS procedures for three segments of the corresponding rabbit LPH precursor sequence

Values in parentheses indicate the percentage sequence coverage (%).

Identification method	PNGH residues		
	1–462	463–812 (exon 7)	813–1920
Edman sequencing of purified peptides	42 residues (9.09)	not found	131 residues (11.8)
MS or MS/MS matching of peptide fractions	115 residues (24.8)	10 residues (2.87)	257 residues (23.2)

translational processing. Additionally, the alignment pattern shows that PNGH is not equivalent to mature LPH. Mature LPH (molecular mass approx. 160 kDa) originates from amino acids 867–1926 of the lactase precursor, and we have obtained sequence data for PNGH peptides that align with regions of pre-pro-LPH that do not correspond to mature LPH. This eliminates the possibility that PNGH is an artifact arising from contamination of the cytosol with LPH from the brush-border membrane or that PNGH is a soluble form of LPH.

Segments of predicted cDNA sequences corresponding to PNGH amino acid sequences were subjected to BLAST search in the human genome (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html>) and were found to match only the LPH/LCT gene located on chromosome 2. In view of this observation and the apparent lack of PNGH amino acid sequences that corresponded to the range encoded by exon 7 of the LPH/LCT gene (pre-pro-LPH amino acids approx. 570–784), we hypothesize that PNGH is a product of alternative splicing during transcription of the LPH/LCT gene. This proposed mechanism appears to be the most tenable explanation of the overall amino acid sequence alignments and the differences in molecular mass of PNGH and pre-pro-LPH. The existence of an enzymically inactive splice variant of human β -galactosidase has been reported [30,31], which provides precedence for alternative splicing in the mammalian glycohydrolase family.

Northern blot analysis

Based on the molecular masses of the proteins (pre-pro-LPH approx. 220 kDa; PNGH approx. 140 kDa), the respective mRNAs are predicted to be approx. 6300 and approx. 4000 nucleotides. Northern blot analysis of pig intestinal mucosa, using the 2190 nucleotide probe derived from cDNA clone c8a10 [27] as the probe, revealed an expected transcript of 6 kb mRNA for the LPH precursor (i.e. pre-pro-LPH). However, after 12 h of exposure to X-ray film, a second, less intense transcript of approx. 4 kb appeared and became more evident after 24 h of exposure (Figure 4A). On the basis of the size of the 4 kb band, its lower abundance and hybridization with the LPH probe, we believe that this is strong, but not absolutely conclusive, evidence for the existence of a specific mRNA encoding PNGH, either as an alternative splice variant derived from the LPH gene or the transcript of a closely related gene. Similar results were obtained in Northern blot analysis of human intestinal mRNA, where after 24 h of film exposure transcripts of 6 kb and 4 kb were observed (Figure 4B).

To determine whether the 4 kb band may be an artifact caused by fragmentation of pre-pro-LPH mRNA during analysis, both transcripts were excised and extracted separately from agarose gel and then purified with the EZNA Poly-Gel RNA extraction kit (Omega Bio-Tek) using the manufacturer's protocol. Northern

Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	1	MELFWSIVFT VLLSFSCRGS DWESDSNFIS AAGPLTTDLL LSLQYPQGNQ TSDFAAGGKD
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	61	LYVCSQPLPA FLPEYFSSLR ASEITHYKVF LSWAQLLPAG HSGDPDGNV RCYRQLLEAL ASEITHYK CYRQLLEAL
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	121	RAAQLQPMVV LHHQHLPASS ALRSVAFADL FAEYATFAFH AFGDLVGVL TFSLEAAIR FAEYATFA ¹ R
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	181	ELPQPESRAS RLQLLTAHR KAYEIHQKY AAQGGKVSVV LQAEIESELL LESSTSALAK KAYEIHQK ² ELPQQDSRAW RLQLLTAHR KAYEIHQK
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	241	DSIDFLSLDL SYECQSEMSL PEKLSKLQTI EPKVKVFIFT LRLQDCPSSR KSPASLLFSF LQTI EPKVKVFIFT LKLQDCPSSR K
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	301	IEAINKQVL TLGFDVNAFL NCSSTSCKSI SCFLTDSLAL QTDHERAARN SAPVSTYQRV AARN SAPVSTYQ ² AARN SAPASAYQRV
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	361	WEMFAHQPR ERDAFLQDTF PQGFLWGVST GAFNVEGGWA EGGRGPSVWD QFGHLKAAQG WEMFAHQPR
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	421	QATPEVASDS YYKASDVAL LRGLRAQVYK FSISWSRIFP MGRGSSPSPQ GVAYYNKLID FSISWSRIFP MG ² GLRAQVYK FSISWSRIFP MGR
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	481	SLLDSHIEPM ATLFHWDLPQ ALQDEGGWQN ESVVDAFVDY AAFCSFAFN RVKLVWTFHE
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	541	PWVMSYAGYG TGQHAPGISD PGIASFQVAH LVLKAHARTW HHYNSHHRPQ QQGRVGIVLN
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	601	SDWAEPLSPE RPEDLAASER FLHFMWGFA HPFIVDGDYP ATMKAQIQOR NEQCPSPVAQ
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	661	LPEFTDTEKQ LLKGSADFLG LSHYTSRLIS KAPEDSCIPS YDTIGGFSQH TDPAWPQTSS
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	721	PWIRVVPWGI RRLQFVSLE YTKGKVIYL AGNGMPIGES ENLLSDSLRV DYFNQYINEV
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	781	LKAIKEDSVD VRSYIARSIM DGFEGPAGYS QRFGLYHVN F NESSKPTPR KSAFLTSII FGLYHVN N ¹ AIREDSVD VR
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	841	EKNGFLTAV KQPLPPNSAH LPSKTRASAL PSEVPSKAKV VWEKFSNQTK FERDLFYHGT DLFYHGT NGFLTAV K TRASAL PSEVPSKAKV VWEKFSNQTK FERDLFYHGT
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	901	FRDDFLWGS SSAYQIEGAW DADGKGPSIW DNFTHTPGNG VTDNSTGDIA CDSYNQLDAD FR ² FR
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	961	LNVLRAKVK AYRFSLSWSR IFPTGTNSSI NSHGVDIYNR LIDGLLASDI FPMVTLFHWL FSLWSR ² FSLWSR
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	1021	LPQALQDIGG WENPSLIDLF DSYADYCFQT FGDRVKFWIT FNEPTIYSWW SYGSGTFPPN F DSYADYCFQ
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	1081	VNDPGWAPYR ISHALIKAHA RVYHTYDEKY RQSQNGVISL SLVAQWAEKP SPDVLRDVEA Y RQSQNGVISL SLVAQWA ² AHA RVYHTYDEK VISL SL
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	1141	ADRMQFTLG WYAHPIFKTG DYPDAMKWKV GNRSELQHLA TSRLPSFTEE EKSYIRGTAD LPSFTEE EKSYIR ² MQFTLG WYAHPIFKTG DYPDAMK SELQHLA TSR
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	1201	VFCLNTYSSK IVQHKTPALN PPSYEDDQEL ABEEDTSWPT TAMNRAASFG MRLLNWIKE
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	1261	↓ EYGDIPYIT ENGVGLTNPR LEDIDRIFY KTYINEALKA YRLDGVNLRG YFAWSLMDNF YGDIP ¹ TYINEALK
Rabbit prepro-LPH: Pig PNGH-Edman: Pig PNGH-MS:	1321	EWLQGYTIKF GLYHVDENV NRPRTARISA SYTTELITNN GMPLPSEDEF VYGQFPEGV F GLYHVDENV N ¹

Figure 3 For legend see facing page

Rabbit prepro-LPH:	1381	WSTSTAAPQI EGAWRADGKG LGIWDTFHT RLKIENDDIA DVACDSYHKI SEDVVALQNL
Pig PNGH-Edman:		WRADGKG LSIWDTFHT PLKI ^{1,2}
Pig PNGH-MS:		I SEDVVALQNL
Rabbit prepro-LPH:	1441	AVTHYRFSIS WSRILPDGTT NYINEAGLNY YVRLIDALLA ANIKPQVTMY HFDLPQALQG
Pig PNGH-Edman:		FSIS WSR ²
Pig PNGH-MS:		AVTHYRFSIS WSR
Rabbit prepro-LPH:	1501	VGGWENETIV QRFKEYADVL FQRLGDKVKF WITLNEFFVY ATHGYGTGLY APGLYFRPQT
Pig PNGH-Edman:		FREYADVL FQR ²
Pig PNGH-MS:		EYADVL FQRLGDK
Rabbit prepro-LPH:	1561	APTIVGHNLI KAHAEAWHLY NDVYRASQGG VISITISSDW AEPDPSNQE DVEAAKRYVQ
Pig PNGH-Edman:		
Pig PNGH-MS:		AHAEAWHLY NDVYRASQGG VISITISSDW AEPR
Rabbit prepro-LPH:	1621	FMGGWFAHPI FKNQDYNEVM KTQIRERSLA AGLNESRLPE FTESEKRRIN GTYDFGPNH
Pig PNGH-Edman:		
Pig PNGH-MS:		NGDYHEVM K ERSLS AGLNESRLPE FTESEKR
Rabbit prepro-LPH:	1681	YTTVLAYNPN YPSIMSTVDA DRGVASIVDR SWPGSGSYWL KMTPTGFRRI LNWIKKEYNM
Pig PNGH-Edman:		GVASITDR SWPDGSGFWL K ¹
Pig PNGH-MS:		GVASITDR SWPDGSGFWL KMTPTG FR
Rabbit prepro-LPH:	1741	PPIYVTENG V SHRGDSYLD TTRIYYLRSY INEALKAVQQ DKVDLRGYTV WTIMDNFENY
Pig PNGH-Edman:		
Pig PNGH-MS:		SY INEALKAVQQ DKVDLR
Rabbit prepro-LPH:	1801	TGFSODKFLGH FVNYSDPFLP RIPRESAKFY ASIVRCNGFP DPAGPHPC LQPEDTPTM
Pig PNGH-Edman:		FY ASIVR ²
Pig PNGH-MS:		FGLH FVNYSDPFLP R ESAKFY ASIVR
Rabbit prepro-LPH:	1861	SPVSEQEVQF LGLSLGSTE ETALYVLFSL MLLGVCGLAF LSYALCKSSK QRKLSQQEL
Pig PNGH-Edman:		
Pig PNGH-MS:		LSQEEL
Rabbit prepro-LPH:	1921	SPVSSF
Pig PNGH-Edman:		
Pig PNGH-MS:		SPVSSF

Figure 3 Alignment of the deduced amino acid sequences of pig intestinal mucosa PNGH with those from rabbit LPH precursor [19]

Identical residues are in bold type. Light shading represents the LPH α fragment [17]. The mature LPH is marked by the dark-shaded area [13]. The active sites are indicated by arrows; lactase activity is localized to glutamine 1273 and that of phlorizin hydrolase to glutamine 1749 [32–34]. Amino acids encoded by exon 7 of LPH/LCT gene are in italic type. Sequences obtained from University of Florida and Emory University are annotated with ⁽¹⁾ and ⁽²⁾ respectively. Sequence data for pig PNGH were obtained using Edman degradation and MS techniques, as indicated. In rows labelled Pig PNGH-MS, results were obtained by mass matching of PNGH tryptic peptides by using MALDI-TOF MS. Underlining of MS data indicates confirmation by MALDI-TOF/TOF MS/MS.

Figure 4 Northern blot analysis

Analyses were conducted as described in the text. (A–C and E) used a 2190 nucleotide probe derived using cDNA clone c8a10 and (D) used the exon 7 probe. (A) Northern analysis showing expected transcript of 6 kb for LPH at 6 h of film exposure (lane 1) and, as exposure time increases to 12 h (lane 2) and (lane 3) to 24 h, appearance of an approx. 4 kb transcript. (B) Human jejunum mRNA after 24 h of exposure showing two distinct transcripts of 6 kb and 4 kb. (C) Gel-extracted pig jejunum 6 kb and 4 kb mRNA transcripts analysed by Northern blotting, after 24 h of exposure individual transcripts appeared on Northern blot analysis. (D) Pig jejunal mRNA analysed using exon 7 probe showing expected transcript of 6 kb for LPH at 24 h (lane 1) and 48 h (lane 2) of film exposure and lack of a 4 kb transcript. (E) Northern blots of pig jejunal mRNA conducted parallel to those in (D), except using the probe derived from cDNA clone c8a10, showing two distinct transcripts of 6 kb and 4 kb after 24 h and 48 h of exposure (lanes 1 and 2 respectively).

blot analysis on both transcripts (Figure 4C) supported the conclusion that the 4 kb transcript is a distinct and unique mRNA, and not an artifact from the degradation of the 6 kb transcript.

Further Northern blot analyses of pig jejunal mRNA were conducted to compare results obtained using an exon 7-specific probe, which was compared with the less restrictive 2190 nucleotide probe derived from cDNA c8a10. In this analysis, the exon 7 probe hybridized only with the 6 kb LPH transcript and did not yield a 4 kb band (Figure 4D). As a control, the cDNA c8a10 probe again showed both transcripts (Figure 4E). These results are consistent with observations from amino acid sequencing and confirm the lack of sequence derived from LPH/LCT exon 7 in the 4 kb band that we presumptively identify as the PNGH mRNA.

In summary, we have shown by analysis of amino acid sequences that PNGH is closely related to the LPH precursor and that PNGH is not a form of LPH. Detection of an mRNA transcript of a size consistent with that predicted for PNGH and which hybridizes with an LPH probe, but not with a specific exon 7 probe, supports the hypothesis that cytosolic PNGH is a product of alternative splicing of the LPH/LCT gene.

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