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Catalytic site of human protein-glucosylgalactosylhydroxylysine glucosidase: Three crucial carboxyl residues were determined by cloning and site-directed mutagenesis☆

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ABSTRACT

Protein-glucosylgalactosylhydroxylysine glucosidase (PGGHG; EC3.2.1.107) cleaves glucose from disaccharide unit (Glc-α1,2-Gal) linked to hydroxylysine residues of collagen. In the present paper we first show that PGGHG is the product of *ATHL1* gene as follows. (1) PGGHG was purified from chick embryos and digested with trypsin. LC-MS/MS analysis suggested the tryptic-peptides were from the *ATHL1* gene product. (2) Chick embryo *ATHL1* cDNA was cloned to a cloning and expression vector and two plasmid clones with different *ATHL1* CDS insert were obtained. (3) Each plasmid DNA was transformed into *Escherichia coli* cells for expression and two isoforms of chicken PGGHG were obtained. (4) Both isoforms effectively released glucose from type IV collagen. Next, we searched for carboxyl residues crucial for catalytic activity as follows; human *ATHL1* cDNA was cloned into a cloning and expression vector and 18 mutants were obtained by site-directed mutagenesis for 15 carboxyl residues conserved in *ATHL1* of jawed vertebrates. The expression analysis indicated that substitutions of Asp301, Glu430 and Glu574 with sterically conservative (D301N, E430Q, E574Q) or functionally conservative (D301E, E430D, E574D) residues led to the complete elimination of enzyme activity. These findings lead us to the conclusion that PGGHG is encoded by *ATHL1* and three carboxyl residues (corresponding to Asp301, Glu430 and Glu574 of human PGGHG) might be involved in the catalytic site of PGGHG.

1. Introduction

PGGHG is the enzyme that specifically hydrolyzes glucose from disaccharide unit linked to hydroxylysine residues of collagen and collagen-like proteins [1]. PGGHG is highly specific to Glc-Gal-Hyl, and requires free, positively charged ε-amino group of hydroxylysine [1–4]. Although more than three decades have passed since PGGHG was purified from chick embryos [2], rat kidneys [3] and rat spleens [4], the structural information has not been available. Recently, the significance of disaccharide unit attached to collagen-like domain of some proteins was suggested; for instance, it is reported that the disaccharide unit is essential for the oligomeric structure of adiponectin and the removal of the sugar chain affects the function of the protein [5]. The cloning of PGGHG will be necessary to understand the structure and the biological function(s) of PGGHG and to produce recombinant PGGHG that will be a useful tool to remove glucose residue from collagen-like proteins. In addition, the cloning and site-directed mutagenesis will make it possible to resolve the question why jawed-vertebrates have PGGHG activity but jawless-vertebrates have not. In this study we substituted carboxylic amino acids of human PGGHG by site-directed mutagenesis and determined three carboxyl residues that were supposed to constitute the catalytic site, since it is generally accepted that in most glycoside hydrolases only aspartate and/or glutamate residues has been found to perform catalysis [6,7].

Abbreviations: PGGHG, protein-glucosylgalactosylhydroxylysine glucosidase; Glc-Gal-Hyl, 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosylhydroxylysine; GH, glycoside hydrolase.

☆ The nucleotide sequence data have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC011569 for transcript variant 1 and LC011570 for transcript variant 2.

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2. Materials and methods

2.1. Materials

Trehalose, *o*-dianisidine, glucose oxidase, horseradish peroxidase, and silver staining kit for MS were purchased from Wako Chemicals (Osaka, Japan). Type IV collagen from human placenta was obtained from Sigma–Aldrich (St. Louis, MO). Trizol™ Reagent, GeneRacer™ Kit, Ni-NTA Agarose and ChargeSwitch-Pro Miniprep Kit were obtained from Invitrogen/Thermo Fisher Scientific (Waltham, Mass). Expresso™ T7 SUMO Cloning and Expression System was from Lucigen (Middleton, WI). In-Fusion cloning kit was from Clontech/Takara. PrimeSTAR HS DNA Polymerase and PrimeScript II RTase were from TaKaRa Bio Corp (Shiga, Japan). PCR clean-up gel extraction system was from Macherey–Nagel GmbH & Co. KG. (Bethlehem, PA). Polyacrylamide gel was from ATTO Corp. (Tokyo, Japan). Glc-Gal-Hyl and acetylated Glc-Gal-Hyl were prepared according to the method of Spiro [8]. Primers were synthesized by Invitrogen. All procedures in chicken embryos were approved by the Institutional Animal Use and Care Committee of Kitasato University Laboratory Animal Center and performed according to the National Guide for the Care and Use of Laboratory Animals.

2.2. LC-mass spectrometry

PGGHG was purified from 100 g of 14-day chick embryos as described previously [2]. After SDS-polyacrylamide gel electrophoresis, PGGHG was in-gel digested by trypsin. The digested peptides were applied to Thermo Scientific LTQ Orbitrap XL Mass Spectrometer equipped with Shiseido Nanospace SI2 HPLC system (Capicell Pak HPLC column 2.0 × 50 mm (3 μm)). The obtained fragment ion mass data were analyzed with MS/MS Ions Search software in MASCOT using International Protein Index database (IPI_chicken).

2.3. Molecular cloning and expression of chicken PGGHP

Total RNA was prepared from a 14-day chick embryo using Trizol reagent. First strand cDNA was synthesized from the total RNA using oligo-dT primer and PrimeScript II RTase. To construct pT7-His-SUMO chicken *ATHL1* variant 1 a DNA fragment corresponding to CDS having 18-bp overhang ends complementary to the vector was amplified from chicken first strand cDNA by PCR using primers F3 (5′-CGCGAACAGATTGGAGGTGGAAGCATGAGAGGTTCG-3′) and R3 (5′-GTGGCGGCCGCTCTATTATCAAAGTCCTTCGCT-3′). The PCR was purified by gel electrophoresis and cloned into a linear cloning and expression vector, pETite N-His SUMO Kan Vector, according to the manufacturer's instruction. pT7-His-SUMO chicken *ATHL1* variant 2 was constructed by the same way as variant 1 using primers F4 (5′-CGCGAACAGATTGGAGGTGGATCCCTGGGAGGAA-GATGG-3′) and R3.

pT7-His-SUMO chicken *ATHL1*variant 1 and variant 2 were transformed into HI-Control BL21 (DE3) cells (Lucigen) according to the manufacturer's instruction. The transformed cells were cultured in LB medium containing kanamycin (0.1 mg/ml) for 6 h at 37° and subsequently expression was induced with 1.0 mM IPTG for 16 h at 25°. Cells were collected by centrifugation, suspended in 2 ml of 150 mM NaCl-50 mM Tris–HCl (pH 8.0), treated with lysozyme (1 mg/ml) for 30 min on ice, and finally sonicated 10 pulses of 10 s each allowing 2 min for the sample to cool between pulses using Tomy Seiko UR-200P sonicator on ice. The cell lysate was centrifuged to collect the supernatant, which was applied to a column packed with 2-ml aliquots of Ni-NTA agarose resin. The column was washed with 10 ml of 20 mM imidazole-500 mM NaCl-50 mM phosphate (pH8.2), and finally the His-tagged protein was eluted with 250 mM imidazole-500 mM NaCl-50 mM phosphate (pH8.0). Purified His-SUMO-tagged ATHL1 was treated with SUMO-protease (Lucigen) to remove His-SUMO tag according to the manufacturer's instruction. SDS-PAGE was performed using 5–20% gradient gel to confirm purity of chicken PGGHP. After electrophoresis proteins were detected using silver staining kit.

2.4. Substrate specificity

The glucosidase assay for substrate specificity of chicken PGGHG was performed in 50 μl of 100 mM acetate buffer (pH5.3), containing 50 nmol of substrate (Glc-Gal-Hyl, acetylated Glc-Gal-Hyl, or trehalose) and 0.25 μg of the recombinant enzyme. In the case of placental type IV collagen, 250 μg of the substrate and 1.5 μg of the recombinant enzyme were contained in 50-μl enzyme reaction media. The enzyme blank, containing the same amounts of buffer and enzyme but water instead of substrate, was treated identically. After incubation at 37 °C, the reaction was stopped by heating for 3 min in boiling water. The released glucose was assayed by glucose oxidase-peroxidase methods [9] with a modification as follows; 100-μl aliquots of glucose assay reagent (200 mM phosphate buffer (pH6.8), 0.02% glucose oxidase, 0.01% horseradish peroxidase, and 0.02% *o*-dianisidine) was added to the samples and incubated at 37 °C for 30 min. Glucose assay was stopped by adding 650 μl of 400 mM HCl and the optical density at 420 nm was determined against an enzyme blank.

2.5. Molecular cloning and site-directed mutagenesis of human PGGHG

Total RNA was prepared from white blood cells of the author (HH) using Trizol reagent. The informed consent was obtained for experimentation. First strand cDNA was synthesized from the total RNA using GeneRacer Oligo dT Primer (5′-GCTGTCAACGA-TACGCTACGTAACGGCATGACAGTG$(T)_{24}$-3′) and GeneRacer Kit. Human *ATHL1* mRNA sequence data (**GenBank Accession Number**: NM_025092) was used to amplify human *ATHL1* CDS by PCR. The first PCR was performed using the first strand cDNA and primer pair, Hm Primer 1 (5′-ATGGAGGACGCCGGCGAGGA-3′) and GeneRacer 3′Nested Primer (5′-CGCTACGTAACGGCATGACAGTG-3′). Human *ATHL1* cDNA insert having 18-bp overhang ends complementary to the vector was synthesized by the second PCR using the first PCR as a template and primers SUMO-Hm Forward (5′-CGCGAACAGATTGGAGGTGAGGACGCCGGCGAGGA-3′) and SUMO-Hm Reverse (5′-GTGGCGGCCGCTCTATTATTATTCA-GAGGCAGGGTCCACA-3′). The amplified DNA fragment was purified by agarose gel electrophoresis and cloned into linear pETite N-His SUMO Kan Vector according to the manufacture's instruction. pT7-His-SUMO human *ATHL1* was purified using Charge Switch-Pro Miniprep Kit and the sequence analysis was performed to confirm the junctions of the insert with the vector as well as the predicted coding sequence. Site-directed mutagenesis was performed using In-Fusion methods according to the manufacturer's instruction using mutated primers and pT7-His-SUMO human *ATHL1* as a template. For example, pT7-His-SUMO human *ATHL1* (E167Q) was prepared as follows. PCR 1 was performed using a forward primer (5′-ATTCAAGCTGATCAGACCCCTGAA-3‘) and a reverse primer (5’-CTgAGGGGTGAGGGTGTGG-3′). PCR 2 was performed using a forward primer (5′-ACCCTCACCCCTcAG-CAGCCCGGGGGGCCA-3′) and a reverse primer (5′-CTGATCAGCTTGAATTTCAATACC-3′) (15-bp overhang ends complementary to each other were underlined). The products of PCR 1 and PCR 2 were purified by gel electrophoresis, treated with In-fusion enzyme and used for transformation of DH5α cells. Plasmid DNA was purified from transformed DH5α cells using