Elsevier Editorial System(tm) for Journal of Biotechnology

Manuscript Draft

Manuscript Number:

Title: Production and characterization of recombinant human acid α-glucosidase in transgenic rice cell suspension culture

Article Type: Research Paper

Section/Category: Agro/Food Biotechnology

Keywords: Pompe disease, acid α-glucosidase (GAA), rice α-amylase 3D (RAmy3D) promoter, Agrobacterium tumefaciens · Transgenic rice cell suspension culture

Corresponding Author: Prof. Moon Sik Yang,

Corresponding Author's Institution:

First Author: Jae Wan Jung

Order of Authors: Jae Wan Jung; Nan Sun Kim; Seon Hui Jang; Moon Sik Yang

Abstract: Pompe disease is a fatal genetic muscle disorder caused by a deficiency of acid α-glucosidase (GAA), a glycogen-degrading lysosomal enzyme. In this study, the human GAA cDNA gene was synthesized from human placenta cells and cloned into a plant expression vector under the control of the rice α-amylase 3D (RAmy3D) promoter. The plant expression vector was introduced into rice calli (Oryza sativa L. cv. Dongjin) mediated by Agrobacterium tumefaciens. Genomic DNA PCR and Northern blot analysis were used to determine the integration and mRNA expression of the hGAA gene in the putative transgenic rice cells. SDS-PAGE and Western blot analysis showed that the glycosylated precursor recombinant hGAA had a molecular mass of 110 kDa due to the presence of seven N-glycosylation sites. The accumulation of hGAA protein in the culture medium was approximately 37 mg/L after 11 days of culturing in a sugar depletion medium. The His tagged-hGAA protein was purified using an Ni-NTA column and confirmed as the precursor form of hGAA without the signal peptide encoded by the cDNA on the N-terminal amino acid sequence. The acid alpha-glucosidase activity of hGAA produced in transgenic rice cells gave results similar to those of the enzyme produced by CHO cells.

Suggested Reviewers: Fengwu Bai
professor, Biotechnology, Shanghai Jiao Tong University
fwbai@sjtu.edu.cn
Professor Bai’s research interest is industrial biotechnology, with a focus on cost-effective production of biofuels and bioenergy from renewable biomass resources through biorefinery, which comprises the combination of engineering principles with biological fundamentals.

Nancy A. DaSilva
professor, Chemical Engineering & Materials Science, University of California

ndasilva@uci.edu
Nancy A. DaSilva's current research pursuits focus on cloned gene expression, gene amplification and integration, metabolic engineering, and protein secretion.

Pauline M. Doran
professor, Chemistry and Biotechnology, Swinburne University of Technology
pdoran@swin.edu.au
Professor Pauline M. Doran is a expert in tissue engineering,bioreactor engineering and nanobiotechnology.

Masaaki Terashima
professor, School of Human Sciences, Kobe College
terasima@mail.kobe-c.ac.jp
Professor Dr. Terashima's research area is molecular food functionality, Biochemical engineering and protein engineering.

Dear Editor,

December 10, 2015

I want to submit a manuscript entitled as **'Production and characterization of recombinant human acid α-glucosidase in transgenic rice cell suspension culture'** to be considered as a publication at *Journal of Biotechnology*. This manuscript was prepared from the work enhancing the production of recombinant human acid α-glucosidase in plant cell suspension culture.

This manuscript was sent to professional textcheck company and English in this manuscript was proofread by the specialist majoring related field of this study. The authors signify that the contents have not been published elsewhere and the paper is not being submitted elsewhere, and consent to the paper being submitted for consideration for publication.

Please inform the corresponding author of any additional requirements or information that may be needed. Thank you very much for your courtesy.

Sincerely yours,

Moon-Sik Yang
Professor
Department of Molecular Biology
Chonbuk National University
Jeonju 561-756, Republic of Korea
Tel: 82-63-270-3339, Fax: 82-63-270-4334,
E-mail: mskyang@Jbnu.ac.kr

Highlights

- We constructed transgenic rice cell suspension culture producing human GAA.
- The expression of rhGAA was determined by Northern and western blot analyses.
- The accumulated rhGAA protein in the culture medium was 37 mg/L.
- The rhGAA was purified using a Ni-NTA column.
- The acid alpha-glucosidase activity of hGAA produced in transgenic rice cells gave results similar to those of the enzyme produced by CHO cells.

Production and characterization of recombinant human acid α-glucosidase in transgenic rice cell suspension culture

Jae-Wan Jung [a,b], Nan-Sun Kim [a], Seon-hui Jang [a], Moon-Sik Yang [a,b,*]

[a]Department of Molecular Biology, Chonbuk National University, 664-14 Dukjindong, Jeonju, Jeollabuk-do 561-756, Republic of Korea

[b]Division of Bioactive Material Science, Chonbuk National University, 664-14 Dukjindong, Jeonju, Jeollabuk-do 561-756, Republic of Korea

[*]Corresponding author (Moon-Sik Yang)

Tel: 82-63-270-3339

Fax: 82-63-270-4334

E-mail: mskyang@jbnu.ac.kr

Jae-Wan Jung and Nan-Sun Kim contributed equally to this study

Abstract

Pompe disease is a fatal genetic muscle disorder caused by a deficiency of acid α-glucosidase (GAA), a glycogen-degrading lysosomal enzyme. In this study, the human GAA cDNA gene was synthesized from human placenta cells and cloned into a plant expression vector under the control of the rice α-amylase 3D (RAmy3D) promoter. The plant expression vector was introduced into rice calli (*Oryza sativa* L. cv. Dongjin) mediated by *Agrobacterium tumefaciens.* Genomic DNA PCR and Northern blot analysis were used to determine the integration and mRNA expression of the hGAA gene in the putative transgenic rice cells. SDS-PAGE and Western blot analysis showed that the glycosylated precursor recombinant hGAA had a molecular mass of 110 kDa due to the presence of seven N-glycosylation sites. The accumulation of hGAA protein in the culture medium was approximately 37 mg/L after 11 days of culturing in a sugar depletion medium. The His tagged-hGAA protein was purified using an Ni-NTA column and confirmed as the precursor form of hGAA without the signal peptide encoded by the cDNA on the N-terminal amino acid sequence. The acid alpha-glucosidase activity of hGAA produced in transgenic rice cells gave results similar to those of the enzyme produced by CHO cells.

Keywords Pompe disease, acid α-glucosidase (GAA), rice α-amylase 3D (RAmy3D) promoter, *Agrobacterium tumefaciens* · Transgenic rice cell suspension culture

1. Introduction

Acid alpha glucosidase (GAA) is an exo-1,4 and -1,6-α-glucosidase that hydrolyzes glycogen to glucose (Rosenfeld 1975). A deficiency of acid-alpha glucosidase causes Pompe disease (also known as glycogen storage disease type II, acid maltase deficiency or glycogenosis type II), which results in the accumulation of glycogen in lysosomes. Pompe disease is an autosomal recessive disorder that ranges from a fatal infantile form to a more slowly debilitating adult onset form, both of which exhibit various degrees of organ involvement and rates of progression to death. The incidence of Pompe disease is estimated to be 1:40,000 but may vary in different ethnic populations (Hirschorn and Reuser 2001; Raben et al., 2002).

Until recently there has been no specific treatment for Pompe disease other than palliative and supportive care. The discovery of the GAA gene led to rapid progress in understanding the biological mechanisms and properties of the GAA enzyme. As a result, enzyme replacement therapy (ERT) for Pompe disease was developed, and several sources of GAA have been investigated in the clinic for the treatment of patients with Pompe disease. Early attempts at ERT that utilized GAA purified from *Aspergillis niger* (Lauer et al., 1968) and human placenta (De Barsy et al., 1973) were unsuccessful, presumably due to low enzyme dosage, disease stage, and lack of correct post translational modification necessary for muscle targeting. Recombinant GAA from the milk of transgenic rabbits demonstrated that ERT could improve respiratory insufficiency and restore some muscle function in infants (Van den Hout et al., 2000; and Van den Hout et al., 2004). However, there is currently no single transgenic form of GAA useful for industrial-scale production. The later success of infantile trials using recombinant human GAA from Chinese hamster ovary (CHO) cells led to the development of a drug called alglucosidase alfa (Myozyme©), which received the US Food and Drug Administration (FDA) approval for the treatment of

infants and children with Pompe disease (McVie-Wylie et al., 2008). Nevertheless, there is a public concern regarding the high cost of the ERT enzymes derived from CHO cells approved for treating lysosomal storage disease (Gomord and Faye 2004; Grabowski et al., 2014).

Transgenic plants and plant cell cultures are gaining interest as hosts for the production of therapeutic proteins, as they offer a way to replace animal-derived proteins with a safe and economical alternative (Ma et al., 2003; Twyman et al., 2003; Schillberg et al., 2013; Lallemand et al., 2015). In addition, the entire manufacturing process is free from any animal-derived components, complementing the safety advantages associated with plant culture processing. Recently, the possibility of serious viral infection derived from animal cell cultures has become an important issue, as highlighted by an infection of calicivirus in CHO cells used in the production facility for Cerezyme ®, which is used in the treatment of Gaucher disease. Tailglucerase alfa (Elelyso®) is a biopharmaceutical drug developed by Protalix and Pfizer (Aviezer et al., 2009). The drug, a recombinant glucocerebrosidase used to treat Gaucher's disease, was the first plant-generated pharmaceutical to win approval by the U.S. Food and Drug Administration (FDA) (Grabowski et al., 2014). The manufacturers of two other Gaucher drugs—Genzyme in the US and Shire in Ireland—produce their therapeutic enzymes in mammalian cells. Structurally, Elelyso resembles Genzyme's Cerezyme, but is cheaper to produce because of the high maintenance required of animal cell culture. Further, the same viruses and pathogens that commonly contaminate mammalian cell culture stocks are not a threat to plant-cell cultures. Therefore, plant cell culture systems may be the most favorable means of producing high-priced, high-purity, specialty recombinant proteins (Aviezer et al., 2009).

The rice α-amylase gene family is regulated by hormones in germinating seeds and metabolic repression in cultured cells. The expression of one member of this multigene

family, RAmy3D, is strongly controlled by sugar starvation (Simmons et al., 1991; Huang et al., 1993). Thus, the abundance of RAmy3D isozyme in cultured media indicates that the RAmy3D promoter and terminator together may act as a powerful system for recombinant protein production (Kim et al., 2011; Chung et al., 2014; Kim et al., 2014), and possess important advantages of plant cell cultures such as simplicity of use, safety of media, and ease of purification (Kim et al., 2011).

In this study, we used the RAmy3D rice α-amylase expression system to produce hGAA in transgenic rice cell suspension cultures as a low-cost, high-level expression system. In addition, we compared the characteristics of recombinant hGAA produced in animal cell culture (CHO-derived human GAA) with that produced using the RAmy3D system.

2. Materials and Methods

2.1. Cloning of human GAA gene and construction of plant expression vector

For the cloning of human GAA gene, total RNAs were prepared from human placenta (Hoefsloot et al., 1988) with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was conducted in order to amplify the hGAA cDNA (GenBank accession no. **NM_000152**) with the primers (Fig. 1A). With respect to cloning the 2.87 kb hGAA gene, three fragments surrounded by *Sal*I and *Sac*I restriction were generated: a 916 bp 5' fragment (hGAA Frag1with *Sal*I site) without signal peptide, an 867 bp middle fragment (hGAA Frag 2 with *Sal*I site and *Sac*I site), and a 1006 bp 3' fragment harboring a 6x His tag sequence to facilitate purification (hGAA Frag3 with *Sac*I site). The resulting hGAA Frag1 PCR product contained an open reading frame for hGAA lacking the signal peptide.

To fuse these fragments with the rice amylase 3D signal sequence, the rice amylase 3D signal sequence was amplified using the forward primer 3Dsp F *Bam*HI and reverse primer 3Dsp R (Fig. 1A). The hGAA Frag1 fused with the signal sequence of rice amylase 3D (3Dsp+hGAA Frag1), hGAA Frag 2, and hGAA frag 3 were then introduced into pGEM-T Easy vector (Promega, Madison, WI, USA) to generate the plasmids pMYD68, pMYD67, and pMYD80, respectively. The DNA sequence of each gene was confirmed by DNA sequence analysis.

In order to prepare the full length hGAA gene construct, the 3Dsp+hGAA Frag1 of pMYD68 was digested with *Spe*I and *Sal*I and subcloned into same site of pBlueScript (+) vector to generate pMYD74, while the hGAA Frag 2 of pMYD67 was digested with *Sac*I restriction enzyme and subcloned into same site of the pGEM-T Easy vector harboring the hGAA Frag3 to generate pMYD75 (hGAA Frag2+3). The correct orientation of the fragment was confirmed by enzyme digestion. The hGAA Frag2+3 of pMYD75 was then digested with *Sal*I and subcloned into same site of pMYD74 to generate pMYD76 (3Dsp+hGAA Frag1+2+3). To confirm the constructs, each hGAA fragment for cloning was electrophoresed on a 1.0% (w/v) agarose gel, visualized by staining with ethidium bromide, and observed under UV light (Fig 1B and C). The resulting DNA fragments representing full-length hGAA were digested with klenow-fragment blunt-ended *Nhe*I and *Xba*I and then introduced into klenow-fragment blunt-ended *Bam*HI and *Xba*I of pCAMBIA1300 (Hajdukiewicz et al., 1994) under the control of the Ramy3D promoter, with the 3'UTR of the RAmy3D gene used as the terminator. This plant expression vector was designated as pMYD84 and harbored the hygromycin phospho-transferase (*hpt*) gene as a selection marker for plant transformation (Fig. 2).

2.2. Rice transformation and PCR of genomic DNA

The binary plant expression vector pMYD84 was transformed into *Agrobacterium tumefaciens* LBA4404 using the helper plasmid pRK2013 by tri-parental mating method (Hoekema et al., 1983). Rice (*Oryza sativa* L. cv. Dongin) embryonic calli were prepared and infected with exponential-phase *Agrobacterium tumefaciens* LBA4404. Transformed calli were first selected by hygromycin B (50 mg/L) treatment and genomic DNA was analyzed by PCR to confirm insertion of the hGAA gene. Genomic DNA was isolated from the putative transgenic calli using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The hGAA gene was obtained by genomic DNA PCR analysis using primers: 3Dsp F *Bam*HI (5'- TTG GAT CCA TCA GTA GTG GTT AGC AG-3') and hGAA+6his R3 *Nhe*I (5'- GCT AGC CTA GTG ATG GTG AGT GTG ATG ACA CCA GCT GAC GAG AAA CTG-3'), which are specific for hGAA. Thermal cycling was performed for 30 cycles, consisting of 1 min at 94 °C 1 min at 55 °C, and 3 min at 72 °C. The PCR products were electrophoresed on a 1.0% (w/v) agarose gel, visualized by staining with ethidium bromide, and observed under UV light.

2.3. Establishment of rice cell suspension cultures

Transformed rice calli were propagated and cultured at 28 °C in darkness using a rotary shaker at a rotation speed of 110 rpm. To maintain the cell line, the cell suspension was cultured in 300-ml flasks using N6 medium containing 2 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.02 mg/l kinetin, and 3% sucrose (Chen et al., 1994). A 10-ml inoculum was transferred every 9 days for sub-culturing. In order to induce hGAA gene expression under the control of the Ramy3D promoter, N6 medium was removed from the cell suspension by aspiration, and the cells were transferred to fresh N6 (-S) medium (without sucrose) at a density of 10% (weight of wet cells /volume of medium). The culture medium supernatant of induced rice cells was collected by pouring the induced cell

suspension through 2-3 layers of Myracloth (Calbiochem, La Jolla, CA, USA). The total protein content of the media was collected by centrifugation at 15,000 X g at a temperature of 4 °C for 10 min in order to remove debris.

2.4. RNA isolation and Northern blot analysis

For Northern blot analysis, total RNA was isolated from suspension cells grown for 5 days in either N6 or N6 (S-) liquid media using the RNeasy plant total RNA extraction kit (Qiagen). RNA was electrophoretically separated on a formaldehyde-containing agarose gel (Sambrook and Russell 2001). The separated RNA was then transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech RPN82B, Piscataway, NJ, USA). This membrane was then hybridized with an α^{32}P-labeled hGAA probe using the Prime-a Gene labeling system (Promega U1100, Madison, WI, USA) at a temperature of 65 °C in a Hybridization Incubator (FINEPCR Combi-H, Seoul, Korea). The membrane was washed twice in 2XSSC (3 M NaCl and 0.3 M sodium citrate) and 0.1% SDS, and then washed twice more with 2XSSC and 1% SDS for 15 min each at 65 °C. The hybridized bands were detected via autoradiography on X-ray film (Fuji Photo Film Co. HR-G30, Tokyo, Japan).

2.5. SDS-PAGE and Western blot analysis

For Western blot analysis, 5 μg of protein from the culture medium were separated via 12% (w/v) SDS-PAGE (Laemmli 1970) and electroblotted onto a nitrocellulose membrane. The membrane was then incubated in blocking solution [5% (w/v) non-fat dried milk in TBST buffer (20 mM Tris-Cl, pH 7.5, 500 mM NaCl, and 0.05% Tween 20)], followed by rabbit polyclonal anti-hGAA antibody (Abcam, Cambridge, MA, USA) and then anti-rabbit IgG conjugated with alkaline phosphatase (Calbiochem, San Diego, CA, USA) as a

secondary antibody. The recombinant hGAA protein derived from CHO cell was used as positive control ((Genzyme, Cambridge, MA, USA)). Gels were stained with 0.25% Coomassie brilliant blue R-250 containing 45% methanol and 10% glacial acetic acid. Protein concentrations were determined using a protein assay reagent (Sigma, St. Louis, MO, USA) based on the Bradford method using bovine serum albumin (BSA) as a standard.

2.6. Direct enzyme-linked immunosorbent assay (direct *ELISA*)

For the detection and quantification of hGAA, direct ELISA was performed according to the manufacturers protocol (Abcam). Briefly, wells of microtiter plates (Nalgene NUNC International Corp., Rochester, NY, USA) were coated with 100 µl of 10 µg/ml standard hGAA (Genzyme) or recombinant hGAA produced from transgenic rice cells in 100 mM sodium bicarbonate buffer (pH 9.5) at 4 °C overnight. The following day, the coating solution was removed and the plate was washed with PBST (PBS buffer containing 0.05% Tween 20, pH 7.0). Plates were then incubated with 200 µl blocking buffer containing 0.1% skim milk and left at room temperature for 2 hours, followed by washing with PBST. Next, 100 µl per well of 2.5 µg/ml biotinylated rabbit anti-GAA polyclonal antibodies (Abcam) were added. After incubation at room temperature for 2 hours and subsequent washing with PBST, 100 µl of 1:1000 diluted avidin-horseradish peroxidase (Avidin-HRP, BD PharMingen Inc. San Diego, CA, USA) was added to each well. Samples were incubated at room temperature for 30 min, washed with PBST, and then each well was loaded with 100 µl of TMB substrate (BD PharMingen Inc.). The absorbance of the samples at 430 nm was recorded with a microplate reader.

2.7. Recombinant hGAA purification

The rhGAA secreted in rice cell suspension culture was purified using a Ni-NTA column (Invitrogen) following the manufacturer's instructions. The total medium proteins were collected by centrifugation at 15,000 X g at a temperature of 4 °C for 10 min in order to remove the debris. Samples were loaded onto 1-ml Ni-NTA columns (Invitrogen), which were then washed with 10 ml of 1XPBS pH 6.5 buffer to remove unbound protein. Finally, His-tagged recombinant proteins were eluted with 1XPBS pH 4.0 buffer (Flanagan and Forstner 1978; Fuller et al. 1995). The purified recombinant proteins were quantified by Bradford protein assay (Bio-Rad, Hercules, CA, USA) and identified by separate SDS-PAGE and Western blot analysis using a rabbit anti-hGAA antibody (Abcam) and mouse anti-His antibody (Qiagen).

2.8. N-terminal amino acid sequence analysis

Five µg of purified hGAA were applied to 10% SDS-PAGE under reducing conditions and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Protein bands were visualized with 0.02% Coomassie Blue R-250. Fifteen cycles of automated Edman chemistry were performed on protein bands excised from PVDF electroblots, and amino-terminal sequencing was performed using an ABI Procise N-terminal sequencer (Moreland et al., 2005).

2.9. Acid α-glucosidase enzyme activity

GAA activity was measured by determining the rate of GAA-catalyzed hydrolysis of the synthetic substrate *p*-nitrophenyl-$_{D}$-α-glucopyranoside (*p*-NPG) (Santa Cruz Biotechnologies, Dallas, TX, USA) in 50 mM sodium acetate and 0.1% BSA at pH 4.3 as previously described by McVie-Wylie et al., (2008). In this assay, the substrate *p*-NPG is hydrolyzed to liberate *p*-nitrophenol (*p*-NP), which can be quantified by its absorbance at

400 nm. Briefly, 10 μg of purified rice-derived hGAA and 10 μg of CHO cell-derived hGAA (Genzyme) as a standard were prepared and 2-ml of assay buffer (50 mM sodium acetate in 0.1% BSA with 0.01M *p*-NPG, pH 4.3) was added to each sample. For temperature equilibration and enzyme activation, the solution was incubated for 5 min at 37 °C. The release of *p*-NA was continuously monitored at 400 nm in a spectrophotometer (Shimadzu Corp., Tokyo, Japan) for 15-120 min. One unit of GAA was defined as that amount of activity that resulted in the hydrolysis of 1 μmol of substrate per minute at 37 °C under the assay conditions.

3. Results

3.1. Generation of the plant expression vector and rice transformation

To obtain high level expression of recombinant human acid α-glucosidase in transgenic rice cell suspension culture, cDNAs encoding hGAA with the signal sequence of the rice amylase 3D gene were introduced into a plant expression vector under the control of the rice amylase 3D promoter, which is induced by sucrose starvation (Fig. 2). Constructs were transformed into rice embryogenic calli using *Agrobacterium*-mediated transformation (Hoekema et al., 1983). Putative transgenic calli were selected on medium containing hygromycin B and the integration of the hGAA gene was confirmed by PCR analysis of genomic DNA using gene specific primers (Fig. 3A). Six callus lines were selected for further analysis and cultured as suspension cells.

3.2. Northern Blot Analysis

Northern blot analysis was conducted in order to confirm hGAA gene expression in transgenic cell suspension cultures after the induction of gene expression by sucrose starvation. Positive signals were detected in 6 transgenic suspension cell lines (Fig. 3B

and C), while no signal was detected in non-transgenic calli. The expression levels of hGAA varied significantly most likely due to position effects and copy number of the target gene into the host genome (Peach and Velten 1991; Streatfield 2007). Six transgenic rice cell lines in which hGAA mRNA was expressed were selected for the establishment of suspension cell cultures.

3.3. Production and quantitative assay of hGAA in transgenic rice suspension culture

The production of rhGAA protein in culture medium during sucrose depletion was identified via SDS-PAGE, Western blot analysis, and ELISA. The secreted proteins that accumulated in the culture medium of transgenic rice cell suspensions after sucrose starvation were separated via SDS-PAGE and analyzed by Western blot (Fig. 4A and B). There was a strong band at 44-46 kDa (indicated by asterisks) in all lanes except for the PC lanes, which corresponded to rice α-amylase induced by sugar starvation conditions (Chen et al., 1994). High expressing of recombinant hGAA was observed in all culture conditions (indicated by arrowheads), except for the PC and NC lanes (Fig. 4A). Western blot analysis revealed that the rhGAA was expressed in all transgenic cell lines but not in the non-transformed cell line. Under reducing conditions, immunoblotting reactions with anti-human GAA indicated various levels of expression of recombinant hGAA expression in the selected cell lines. hGAA in culture medium was isolated predominantly as the glycosylated 110 kDa precursor due to the presence of seven N-glycosylation sites on hGAA (Moreland et al., 2005). In these experiments, CHO-derived hGAA as a 110 kDa precursor form and was used as a positive control. Our results indicated that the highest production of recombinant hGAA from the culture medium both mRNA transcript level and protein accumulation level was observed in the D84-04 line, which was selected for further analysis. The molecular mass of α-glucosidase from rice cell culture was 110 kDa,

which was similar to the mass of the acid α-glucosidase precursor produced in genetically engineered CHO cells, consistent with the fact that both enzyme species contain uncleaved N-and C-terminal pro-peptides (Fuller et al., 1995; van Hove et al., 1996).

In order to determine the maximum production phase of recombinant hGAA in the high expression acid α-glucosidase cell line (D84-04), we conducted a time series experiment, in which the quantities of recombinant GAA that accumulated in the suspension culture medium were determined by direct ELISA. The amount of hGAA increased in a time-dependent manner, reaching a maximum level of 37 mg/L after culture for eleven days in sugar starved culture medium (Fig. 5).

3.4. Purification and characterization of hGAA

To purify the hGAA in transgenic rice cell suspension culture, culture medium was harvested after growth in sugar starvation conditions for eleven days. The culture medium was filtered through a 0.22-μm membrane and purified using a Ni-NTA column. His-tag binding of the recombinant hGAA from transgenic rice cell culture medium was confirmed through interaction with a Ni-NTA column and bound protein eluted with 1X PBS with pH 4.0 buffer (Fuller et al., 1995). The purified protein was then analyzed by SDS-PAGE (Fig. 6A) and Western blot analysis (Fig. 6 B and C). Specifically, the purified hGAA protein was detected as a 110 kDa precursor form under reducing conditions on 10% SDS-PAGE and Western blot analysis using both anti-hGAA antibody and anti-His antibody.

To structurally identify the recombinant GAA, N-terminal amino acid sequencing was performed using the Ni-NTA column purified enzyme. The N-terminal amino acid sequence was H-Q-Q-G-A, which conformed to the one predicted by the GAA amino acid starting at residue 56 from the first methionine. Thus, the recombinant hGAA enzyme was

secreted without signal peptide and was isolated as the GAA precursor form, suggesting that the processing sites recognized in recombinant hGAA from CHO cell and the natural hGAA enzyme from urine or placenta are similar (Oude et al., 1984; Hoefsloot et al., 1988, Wisselaar et al., 1993)

3.5. Activity of recombinant hGAA

A total of 10 μg of the purified precursor form of hGAA from transgenic rice cell suspension cultures was assayed for acid α-glucosidase activity by measuring the rate of GAA-catalyzed hydrolysis of a synthetic substrate, *p*-nitrophenyl-$_{D}$-α-glucopyranoside (*p*-NPG). The activity of the rice-derived precursor form of GAA was similar to that of 10 μg of the CHO-derived precursor form of GAA (Myozyme©, Genzyme) at 3.2 and 3.3 U/mg of protein, respectively, and GAA activity gradually increased during prolonged incubation (Fig.7A and B).

4. Discussion

Pompe disease, a lysosomal storage disorder caused by a deficiency of the gene encoding acid α-glucosidase (GAA), is currently treated by enzyme replacement therapy (ERT) using recombinant GAA. Large amounts of safe human acid α-glucosidase are required for therapeutic trials in patients with Pompe disease. The currently reported transgenic rabbit production line supplies ~10 g of rhGAA per animal per year. At a dose of 10 to 40 mg/kg per patient per week, the production of rhGAA in rabbit milk is feasible in the initial phase of product development, but falls short of meeting the global demand of Pompe disease patients (Van den Hout et al., 2004). Genetically modified CHO cells represent another source of hGAA, which was developed as a drug called alglucosidase alfa, and although it can be used as an alternative, it has capacity, cost, and safety

limitations (Fuller et al., 2013). Even with extremely high dosages of the drug (20-40 mg/kg body weight, significantly higher than other LSDs), patients with the childhood and adult forms of the disease experience limited clinical benefit (Angelini and Semplicini 2012; Lim et al., 2014). Patients must be injected with 20 mg of GAA every 2 weeks, and at a cost of approximately 500,000 USD per year for the life of the adult patient, GAA is one of the most expensive therapeutic enzymes in use (Fuller et al., 2013). Thus, timely investments need to be made towards developing alternative production platforms.

Transgenic plants and plant cell cultures for the production of human biopharmaceutical proteins has been under evaluation in recent years (Hellwig et al., 2004; Beutler 2006; Aviezer et al., 2009; He et al., 2012). Plant cell cultures offer several advantages over both field-grown transgenic plants and mammalian cell cultures. They are cost-effective, do not involve use of mammalian-derived components in the manufacturing process, have high batch reproducibility, allow precise control over the growth process, and enable compliance with current Good Manufacturing Procedures (Hellwig et al., 2004). However, the relatively low productivity of foreign proteins of these systems has been considered to be one of the limiting factors. To increase the protein yields, we chose the rice alpha-amylase 3D (RAmy3D) promoter system in this study, which is induced by sugar starvation in rice cell suspension culture. Alpha-amylase3/RAmy3D promoter is one of the most widely used metabolite-regulated promoters and is highly expressed during sugar starvation (Whitaker and Sonnet 1989). In a rice suspension cell system expressing recombinant human granulocyte-macrophage colony stimulating factor (hGM-CSF) using RAmy3D promoter, protein expression level was improved by 1,000-fold as compared to the 35S CaMV promoter expression system in a transgenic tobacco cell suspension culture system (Shin et al., 2003)

For that reason, the RAmy3D promoter system has been used for the production of

recombinant proteins such as hGM-CSF (Shin et al., 2003), human growth hormone (Kim et al., 2008), human VEGF165 (Chung et al., 2014), FimA monoclonal antibody (Kim et al., 2014) and bovine trypsin (Kim et al., 2011).

In our initial attempt to express and produce acid α-glucosidase in the transgenic rice cell culture system, we used a human GAA cDNA expressed from a rice amylase 3D promoter, which is activated by sugar starvation (Fig. 2). Expression of biologically active hGAA in rice cell cultures resulted in a 110 kDa precursor form due to N-glycosylation (Fig. 4). Sequence analysis indicated that the N-terminal amino acid of purified 110 kDa precursor hGAA from rice cell culture medium was located at histidine 56. In the case of the precursor forms of CHO-GAA and rabbit milk-GAA (tgGAA), the N-termini are located at pyroglutamic acid residue 57 and aspartic acid 67, respectively (McVie-Wylie et al., 2008). Thus, heterogeneity at the N-terminus of other GAA preparations expressed in either CHO cells or purified from human urine and placenta does not appear to impact enzyme function (Wisselaar et al., 1993; Fuller et al., 1995; Bijvoet et al., 1998). Most importantly, the enzymatic properties of purified rice-derived hGAA were similar to those of alglucosidase alfa. Moreover, maximum amount of hGAA reached in transgenic rice suspension culture with the Ramy3D system was 37 mg/L at day 11 after sugar depletion.

In order for GAA to be effective in ERT, terminal mannose residues must be present on the glycan chains to permit binding to mannose receptors of cardiomyocytes and skeletal muscle cells, and subsequent internalization (Bijvoet et al., 1998). Oligosaccharide analysis revealed that the seven N-linked glycosylation sites of CHO-GAA (alglucosidase alfa) and tgGAA were comprised of a mixture of oligomannose, phosphorylated oligomannose and complex oligosaccharide structures. Also, they developed HP-GAA produced in CHO cells which involves sequential in *in vitro* deglycosylation using enzymatic engineering to exposure high levels of terminal mannose

residues. Higher levels of GAA activity in GAA knockout mice was observed in the hearts of the mice treated with HP-GAA, although similar levels of enzyme activity were detected in CHO-GAA (McVie-Wylie et al., 2008). In our study, analysis of N-glycosylation patterns of rice-derived GAA revealed the complex and hybrid N-glycan structures without high mannose residues (data was not shown). Recently, several strategies for therapeutic approach in plant-derived protein have been developed to reduce or eliminate plant-specific N-glycan maturation, including ER retention, targeting the protein of interest to protein storage vacuoles via a pathway that by passes the Golgi complex, or by suppressing the gene that specific plant glycans using RNAi technology (Ko et al., 2003; Shaaltiel et al., 2007; Shin et al., 2011). Another approach is to use the plant mutant, which lacks GnTI activity due to mutation in the gene encoding GnTI (von Schaewen et al., 1993; Strasser et al., 2005; He et al., 2012). GnTI is the first enzyme in the pathway of hybrid and complex *N*-glycan biosynthesis. He et al., reported that the production of active human lysosomal acid β-glucosidase using seeds of the *Arabidopsis thaliana complex-glycan-deficient* (*cg1*) mutant, which are deficient in the activity of *N*-acetylglucosaminyl transferase I (*gntI*) for therapeutic efficacy (He et al., 2012). It will be studying in the future to develop the rice GnTI mutant for production of hGAA with high mannose terminal residues for both activity and targeting to the effected cells.

5. Conclusion

In this study, we showed that biologically active, soluble hGAA can be produced at high yields in transgenic rice cells. As far as we know, this is the first report on the expression of hGAA in transgenic plant cell cultures. Our data suggest that the production of recombinant hGAA with the rice α-amylase RAmy3D expression system may be a good alternative to production by mammalian cells because of lower costs and similar

GAA activity.

Author contribution statement

JW Jung carried out the experiments, participated in vector development, interpretation of results and manuscript preparation. NS Kim was involved in devising the work, interpretation of results and writing the manuscript. SH Jang participated in vector development and provided technical assistance in the experiments. MS Yang was involved in devising the study, data analysis and preparation of the manuscript. All authors read and approved the manuscript.

Acknowledgements

This research was supported by the Advanced Production Technology Development Program, Ministry for Food Agriculture, Forestry and Fisheries (312037-05); a National Research Foundation of Korea Grant funded by the Korea Government (NRF-20151A1A3A04001542); and partly by the Joint Degree and Research Center Program of Korea Research Council of Fundamental Science and Technology, Republic of Korea. Jae-Wan Jung was supported by the BK21 plus program in the Department of Bioactive Material Sciences, Chonbuk National University, Republic of Korea.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Angelini, C., Semplicini, C., 2012. Enzyme replacement therapy for Pompe disease. Curr. Neurol. Neurosci. Rep. 12, 70-75.

451 Aviezer, D., Brill-almon, E., Shaaltiel, Y., Hashmueli, S., Bartfeld, D., Mizrachi, S.,
452 Liberman, Y., Freeman, A., Zimran, A., Galun, E., 2009. A plant-derived recombinant
453 human glucocerebrosidase enzyme-A preclinical and phase I investigation. PLoS One
454 4, e4792.
455 Bijvoet, A.G., Kroos, M.A., Pieper, F.R., Van Der Vliet, M., De Boer, H.A., Van
456 DerPloeg, A.T., Verbeet, M.P., Reuser, A.J., 1998. Recombinant human acid alpha-
457 glucosidase: high level production in mouse milk, biochemical characteristics,
458 correction of enzyme deficiency in GSDII KO mice. Hum Mol Genet 7, 1815-1824.
459 Beutler, E., 2006. Lysosomal storage disease: natural history and ethical and economic
460 aspects. Mol Genet Metab 88: 208-215.
461 Chen, M.H., Liu, L.F., Chen, Y.R., Wu, H.K., Yu, S.M., 1994. Expression of α-amylases,
462 carbohydrate metabolism, and autophagy in cultured rice cells is coordinately
463 regulated by sugar nutrient. The Plant Journal 6, 625-636.
464 Chung, N.D., Kim, N.S., Giap, D.V., Jang, S.H., Oh, S.M., Jang, S.H., Kim, T.G., Jang,
465 Y.S., Yang, M.S. 2014. Production of functional human vascular endothelial growth
466 factor $_{165}$ in transgenic rice cell suspension cultures. Enzyme Microb Technol 63,58-
467 63.
468 De Barsy, T., Jacquemin, P., Van Hoof, F., Hers, H.G., 1973. Enzyme replacement in
469 Pompe disease: an attempt with purified human acid alpha-glucosidase, Birth Defects
470 Orig Artic Ser 9,184-190.
471 Flanagan, P.R., Forstner, G.G., 1978. Purification of rat intestinal maltase/glucoamylase
472 and its anomalous dissociation either by heat or by low PH. Biochem J 173,553-563.
473 Fuller, M., Van Der Ploeg, A., Reuser, A.J., Anson, D.S., Hopwood, J.J., 1995. Isolation
474 and characterization of recombinant, precursor form of lysosomal acid alpha-
475 glucosidase. Eur J Biochem 234, 903-909.

476 Fuller, D.D., Eimallah, M.K., Smith, B.K., Corti, M., Lawson, L.A., Falk, D.J., Byrne,
477 B.J., 2013. The respiratory neuromuscular system in Pompe disease. Respir Physiol
478 Neurobiol 189, 241-249.
479 Gomord, L., Faye, L., 2004. Posttranslational modification of therapeutic proteins in
480 plants. Curr Opin Plant Biol 7,171-181.
481 Grabowski, G.A., Golembo, M., Shaaltiel, Y., 2014. Tailglucerase alfa: An enzyme
482 replacement therapy using plant cell expression technology. Molecular Genetics and
483 Metabolism 112, 1-8.
484 Hajdukiewicz, P., Svab, Z., Maliga, P., 1994. The small versatile pPZP family of
485 *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25, 989-994.
486 He, X., Galpin, J.D., Tropak, M.B., Mahuran, D., Haselhorst, T., Von Itzstein, M.,
487 Kolarich, D., Packer, N.H., Miao, Y., Jiang, L., Grabowski, G.A., Clarke, L.A.,
488 Kermode, A.R., 2012. Production of active human glucocerebrosidase in seeds of
489 *Arabidopsis thaliana* complex-glycan-deficient (*cgl)* plants. Glycobiology 22, 492-503.
490 Hellwig, S., Drossard, J., Twyman, R.M., Fischer, R., 2004. Plant cell cultures for the
491 production of recombinant proteins. Nat Biotech 22, 1415-1422.
492 Hirschhorn, R., Reuser, A.J., 2001. Glycogen storage disease type II: acid α-glucosidase
493 (acid maltase) deficiency, In: Scriver CR (Ed.), The metabolic and molecular basis of
494 inherited disease, pp. 3389-3420.
495 Hoefsloot, L.H., Hoogeveen-Westerveld, M., Kroos, M.A., Van Beeumen, J., Reuser,
496 A.J.J., Oostra, B.A., 1988. Primary structure and processing of lysosomal alpha-
497 glucosidase; homology with the intestinal sucrose-isomaltase complex. The EMBO J 7,
498 1697-1704.
499 Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., Schieroort, R.A., 1983. A binary plant vector
500 strategy based on separation of vir and T-region of Agrobacterium tumefaciens Ti-

plasmid. Nature 303, 179-180.

Huang, N., Chandler, J., Thomas, B., Koizumi, N., Rodriguez, R., 1993. Metabolic regulation of α-amylase gene expression in transgenic cell cultures of rice (*Oryza sativa* L.). Plant Mol Biol 23,737-747.

Kim, B.G., Kim, S.H., Kim, N.S., Huy, N.X., Choi, Y.S., Lee, J.Y., Jang, Y.S., Yang, M.S., Kim, T.G., 2014. Production of monoclonal antibody against FimA protein from *Porphyromonas gingivalis* in rice cell suspension culture. Plant cell Tiss Organ Cult 118, 293-304.

Kim, N.S., Yu, H.Y., Chung, N.D., Shin, Y.J., Kwon, T.H., Yang, M.S., 2011. Production of functional recombinant bovine trypsin in transgenic rice cell suspension cultures. Protein Expr Purif 76,121-126.

Kim, T.G., Back, M.Y., Lee, E.K., Yang, M.S., 2008. Expression of human growth hormone in transgenic rice cell suspension culture. Plant Cell Rep 27, 885-891.

Ko, K., Tekoah, Y., Rudd, P.M. Harvey, D.J., Dwek, R.A., Spitsin, S., Hanlon, C.A., Ruppercht, C., Dietzschold, B., Golovkin, M., Koprowski, H., 2003. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci USA 100, 8013-8018.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680-685.

Lallemand, J., Bouche, F., Desiron, C., Stautemas, J., De Lemos Esteves, F., Perilleux, C., Tocquin, P., 2015. Extracellular peptidase hunting for improvement of protein production in plant cells and roots. Front Plant Sci 6, 1-10.

Lauer, R.M., Mascarinas, T., Racela, A.S., Diehl, A.M., Brown, B.I., 1968. Administration of a mixture of fungal glucosidases to a patient with type II glycogenosis (Pompe's disease). Pediatrics 42, 672-676.

526 Lim, J.A., Li, L., Raben, N., 2014. Pompe disease: from pathophysiology to therapy and
527 back again. Front Aging Neurosci 6, 1-14.
528 Ma, J.K., Drake, P.M., Christou, P., 2003. The production of recombinant pharmaceutical
529 proteins in plants. Nature Reviews Genetics 4, 794-805.
530 McVie-Wylie, A.J., Lee, K.L., Jin, Q.H.X., Gotschall, H.D.R., Thurberg, B.L., Rogers, C.,
531 Raben, N., O'Callaghan, M., Canfield, W., Andrews, L., McPherson, J.M., Mattaliano,
532 R.J., 2008. Biochemical and pharmacological characterization of different recombinant
533 acid α-glucosidase preparations evaluated for the treatment for the treatment of pompe
534 disease. Mol Genet Metab 94, 448-455.
535 Moreland, R.J., Jin, X., Zhang, X.K., Decker, R.W., Albee, K.L., Lee, K.L., Cauthron,
536 R.D., Brewer, K., Edmunds, T., Canfield, W.M., 2005. Lysosomal acid alpha-
537 glucosidase consists of four different peptides processed from a single chain precursor.
538 J Biol Chem 280, 6780-6791.
539 Oude Elferink, R.P.J., Brouwer-Kelder, E.M., Surya, I., Strijland, A., Kroos, M., Reuser,
540 A.J.J., Tager, J.M., 1984. Isolation and characterization of a precursor form of
541 lysosomal α-glucosidase from human urine. Eur J Biochem 139, 489-495.
542 Peach, C., Velten, J., 1991. Transgene expression variability position effect of CAT and
543 GUS repoter genes driven by linked divergent T-DNA promoter. Plant Mol Biol 17,
544 49-60.
545 Raben, N., Plotz, P., Byrene, B.J., 2002. Acid alpha-glucosidase deficiency (glycogenosis
546 type II, Pompe disease). Curr Mol Med 2, 145-166.
547 Rosenfeld, E.L., 1975. Alpha-glucosidases (gamma-amylases) in human and animal
548 organisms. Pathol Biol 23, 71-84.
549 Sambrook, J., Russell, D.W., 2001. Molecular cloning: A laboratory manual, 3rd edition.
550 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

551 Schillberg, S., Raven, N., Fischer, R., Twyman, R., Sciermeyer, A., 2013. Molecular
552 farming of pharmaceutical proteins using plant suspension cell and tissue cultures.
553 Curr Pharm Des 19, 5531-5542.
554 Shaaltiel, Y., Bartfeld, D., Hashmueli, S., Baum, G., Brill-Almon, E., Galili, G., Dym, O.,
555 Boldin-Adamsky, S.A., Silman, I., Sussman, J.L., Futerman, A.H., Aviezer, D., 2007.
556 Production of glucocerebrosidase with terminal mannose glycans for enzyme
557 replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol J
558 5, 579-590.
559 Shin, Y.J., Hong, S.Y., Kwon, T.H., Yang, M.S., 2003. High Level of expression of
560 recombinant human granulocyte-macrophage colony stimulating factor in transgenic
561 rice cell suspension culture. Biotechnol Bioeng 82, 778-783.
562 Shin, Y.J., Chong, Y.J., Yang, M.S., Kwon, T.H., 2011. Production of recombinant human
563 graulocyte macrophage-colony stimulating factor in rice cell suspension culture with a
564 human-like N-glycan structure. Plant Biotechnol J 9,1109-1119.
565 Simmons, C.R., Huang, N., Cao, Y., Rodriguez, R.L., 1991. Synthesis and secretion of α-
566 amylase by rice callus: Evidence for differential gene expression. Biotechnol Bioeng 38,
567 545-551.
568 Strasser, R., Stadlmann, J., Svoboda, B., Altmann, F., Glossl, J., Mach, L., 2005.
569 Molecular basis of *N*-acetylglucosaminyltransferase I deficiency in *Arabidopsis*
570 *thaliana* plants lacking complex N-glycans. Biochem J 387,385-391.
571 Streatfield, S.J., 2007. Approaches to achieve high-level heterologous protein production
572 in plants. Plant Biotechnol J 5, 495-510.
573 Twyman, R.M., Stoger, E., Schillberg, S., Christou, P., Fischer, R., 2003. Molecular
574 farming in plants: host systems and expression technology. Trends Biochnol 21, 570-
575 578.

Van den Hout, H., Reuser, A.J., Vulto, A.G., Loonen, M.C., Cromme-Dijkhuis, A., Van Der Ploeg, A.T., 2000. Recombinant human alpha-glucosidase from rabbit milk in pompe patients. Lancet 356, 397-398.

Van den Hout, J.M.P., Kamphoven, J.H.J., Winkel, L.P.F., Arts, W.F.M., De Klerk, J.B.C., Loonen, M.C.B., Vulto, A.G., Cromme-Dijkhuis, A., Weisglas-Kuperus, N., Hop, W., Van Hirtum, H., Van Diggelen, O.P., Boer, M., Kroos, M.A., Van Doorn, P.A., Van der Voort, E., Sibbles, B., Van Corven, E.J.J.M., Brakenhoff, J.P.J., Van Hove, J., Smeitink, J.A.M., De Jong, G., Reuser, A.J.J., Van der Ploeg, A.T., 2004. Long-term intravenous treatment of Pompe disease with recombinant human alpha-glucosidase from milk. Pediatrics 113, e448-e457.

Van Hove, J.L., Yang, H.W., Wu, J.Y., Brady, R.O., Chen, Y.T., 1996. High-level production of recombinant human lysosomal acid alpha-glucosidase in chinese hamster ovary cells which targets to heart muscle and corrects glycogen accumulation in fibroblasts from patients with Pompe disease. Proc Natl Acad Sci USA 93, 65-70.

Whitaker, J.R., Sonnet, P.E., 1989. Biocatalysis in agricultural biotechnology. In: Simmons CR, Rodriguez RL (ed) High-level synthesis and secretion of a-amylase from rice callus. ACS press, Washington, pp. 202-214.

Wisselaar, H.A., Kroos, M.A., Hermans, M.M., Van Beeumen, J., Reuser, A.J., 1993. Structural and functional changes of lysosomal acid alpha-glucosidase during intracellular transport and maturation. J Biol Chem 268, 2223-2231.

601 Figure legends

602

603 Fig. 1. Strategy for cloning the hGAA gene. Primer sequence for PCR use to amplify the
604 hGAA gene (A); RT-PCR for synthesis of hGAA gene from human placenta cDNA (B);
605 Each PCR product was analyzed on agarose gels (C). Lane M, 1 kb plus 100 bp DNA size
606 marker (ELPIS, DaeJeon, Korea); Lane 3Dsp, signal peptide of the rice amylase 3D gene;
607 Lane hGAA Frag1, 5' fragment of 916 bp of hGAA; Lane 3Dsp+hGAA Frag1, hGAA
608 fragment 1 fused with 3Dsp; Lane hGAA Frag 2, a 867 bp of middle fragment of hGAA;
609 Lane hGAA Frag 3, a 1006 bp 3' fragment of hGAA harboring 6 His tag sequence; Lane
610 hGAA Frag 2+3, ligated with fragment 2 and 3 of hGAA; Lane 3Dsp+hGAA Frag 1+2+3,
611 the full of hGAA gene with 3Dsp, respectively.

612

613 Fig. 2. The plant expression vector, pMD84. The human acid alpha-glucosidase (hGAA)
614 gene, harboring the signal peptide of rice amylase 3D gene and own pro-peptides, is
615 located between the rice amylase 3D promoter (Ramy3D-p.) and the 3'untranslated region
616 (3'UTR). Transferred DNA (T-DNA) of the final plasmid is shown. RB, T-DNA right
617 border; 3'UTR, 3' untranslated region of the rice α-amylase 3D gene; 35S-p., CaMV35S
618 promoter; HPT, hygromycin phosphotransferase (Hyg^R); 35S polyA, terminator of 35S
619 gene; LB, T-DNA left border.

620

621 Fig. 3. Detection of the hGAA gene in transgenic callus via PCR. (A) Lane M, 1 kb plus
622 100 bp DNA size marker (ELPIS); Lane PC, pMYD84 plasmid as a positive PCR control;
623 Lane NC, non-transgenic callus genomic DNA as a negative control; Lanes 1-6 indicate
624 the PCR products obtained from genomic DNA of transgenic callus. Northern blot
625 analysis used to determine expression of the hGAA mRNA in a transgenic rice cell

suspension culture (B). Lane NC, total RNA extraction of non-transformed rice cell as a negative control; Lanes 1-6 are the total RNA extracts of transgenic rice suspension cells. Loading standards are indicated by ethidium bromide-stained rRNA (C).

Fig. 4. SDS-PAGE and Western blot analysis of hGAA in transgenic rice cell suspension culture medium. SDS-PAGE stained with Coomassie blue (A); Western blot analysis (B) using anti-hGAA antibodies to detect the target protein. Lane M, pre-stained molecular weight standard (PageRuler ™ Protein Ladder,); Lane PC, 200 ng of CHO-derived rhGAA; Lane NC, culture medium from non-transgenic cell line; Lanes 1-6, transgenic cell lines. Arrowheads indicates precursor forms of secreted rhGAA; Asterisks indicate rice α-amylase.

Fig. 5. Time course study of recombinant hGAA production in transgenic rice culture medium by direct ELISA. The culture medium was used to measure hGAA production from day 1 to 17 after induction by sugar starvation. Error bars indicate the standard error obtained from triplicate cultures.

Fig. 6. Analysis of purified rhGAA in transgenic rice suspension cell culture medium. SDS-PAGE (A) and Western blot analysis (B and C) were conducted to evaluate rhGAA purification using an Ni-NTA column. PC, 200 ng of wheat germ-derived rhGAA using an anti-hGAA antibody (B) and our laboratory protein with a His tag using an anti-His antibody (C); NC, culture medium from non-transgenic suspension cells; C, culture medium after induction by sugar starvation; FT, flow-through from His-tag affinity column; W, wash from His-tag affinity column; E1 and E2, eluted fractions from His-tag affinity column. Arrowheads indicate precursor forms of secreted rhGAA; Asterisks indicate rice α-amylase, respectively.

Fig. 7. Representative curve of the acid alpha-glucosidase activity as a function of incubation time (A) and specific GAA activity (B). GAA activity of ten µg of purified rice-derived hGAA and 10 µg of CHO-derived hGAA were measured with *p*-nitrophenyl-$_D$-α-glucopyranoside (*p*-NPG) synthetic substrate. The release of *p*-NPG was continuously monitored at 400 nm in a spectrophotometer for 15-200 min. One unit of GAA was defined as that amount of activity that resulted in the hydrolysis of 1 µmol of substrate per minute at 37 °C under the assay conditions. Error bars indicate the standard error of triplicate cultures.

Figure 1.
Click here to download high resolution image

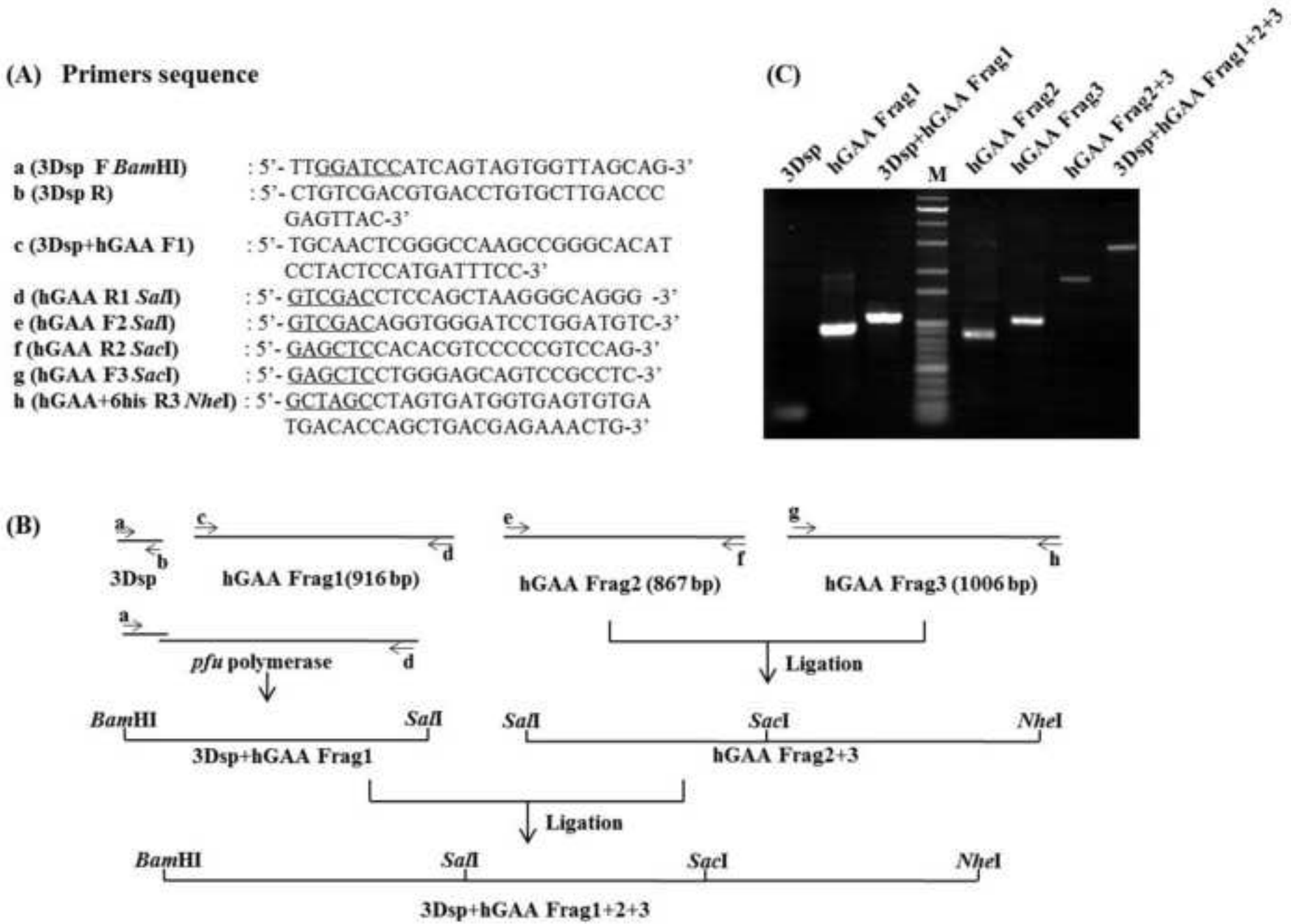

Figure 2.

Click here to download high resolution image

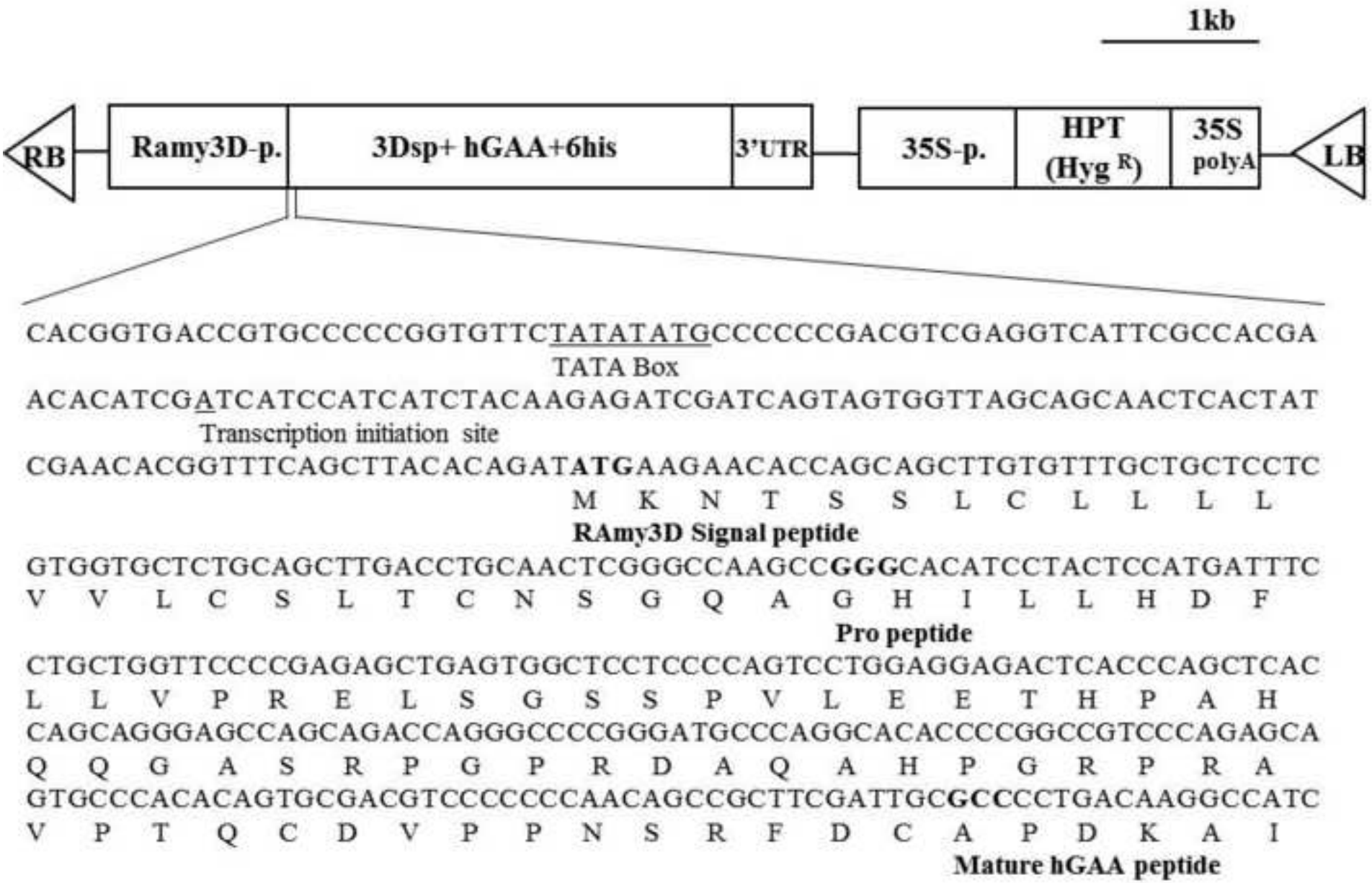

Figure 3.
Click here to download high resolution image

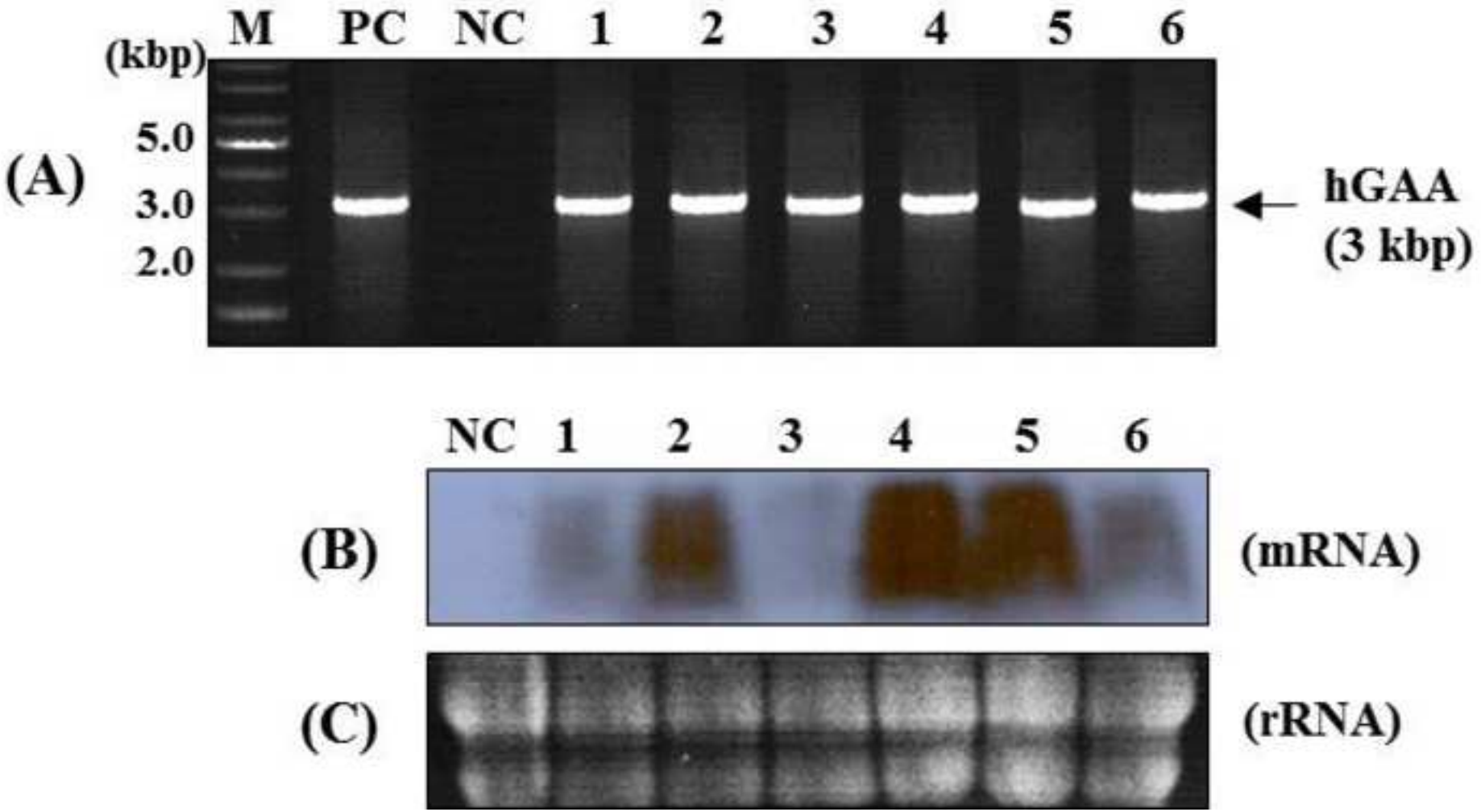

Figure 4.

Click here to download high resolution image

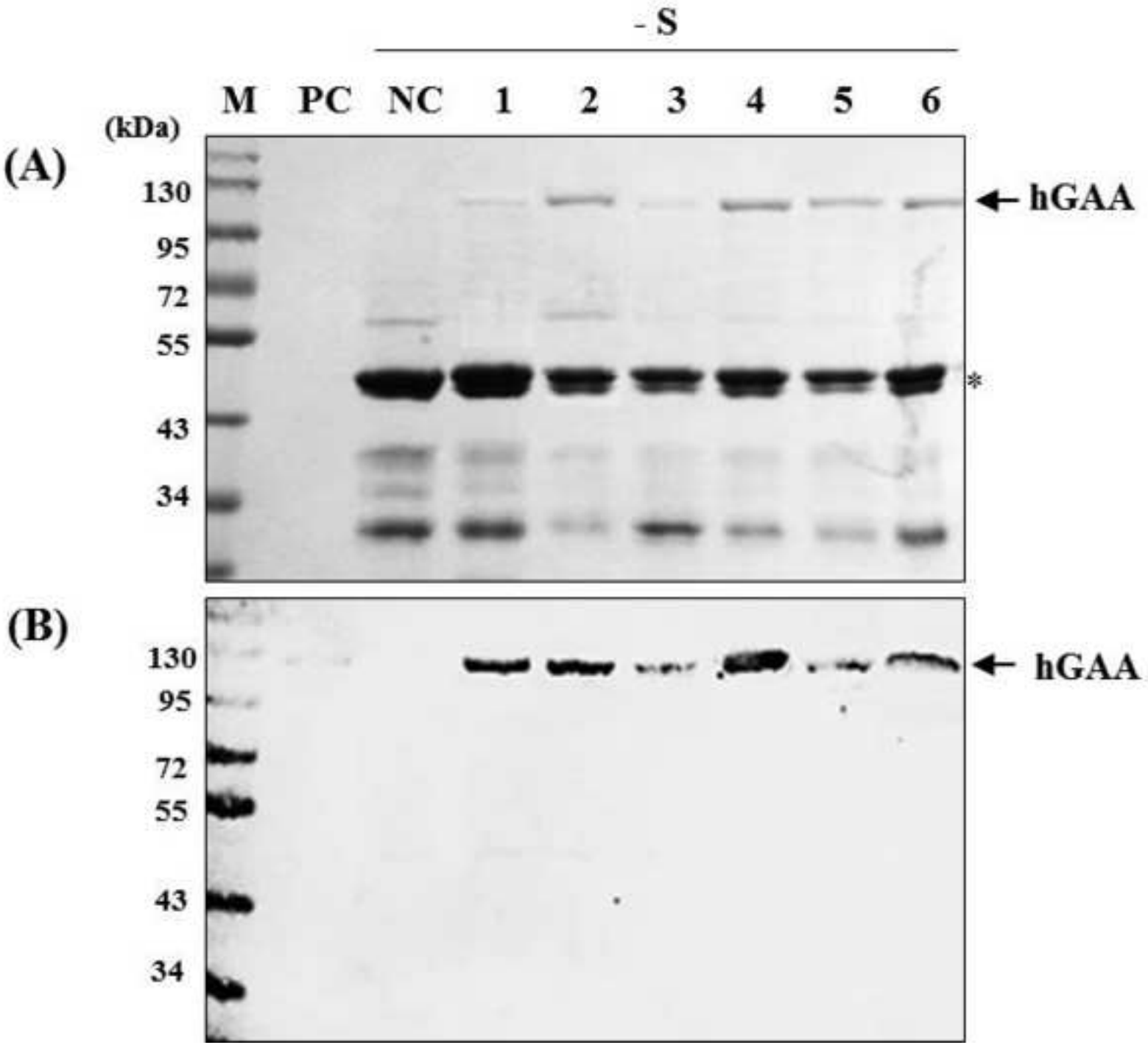

Figure 5.

Click here to download high resolution image

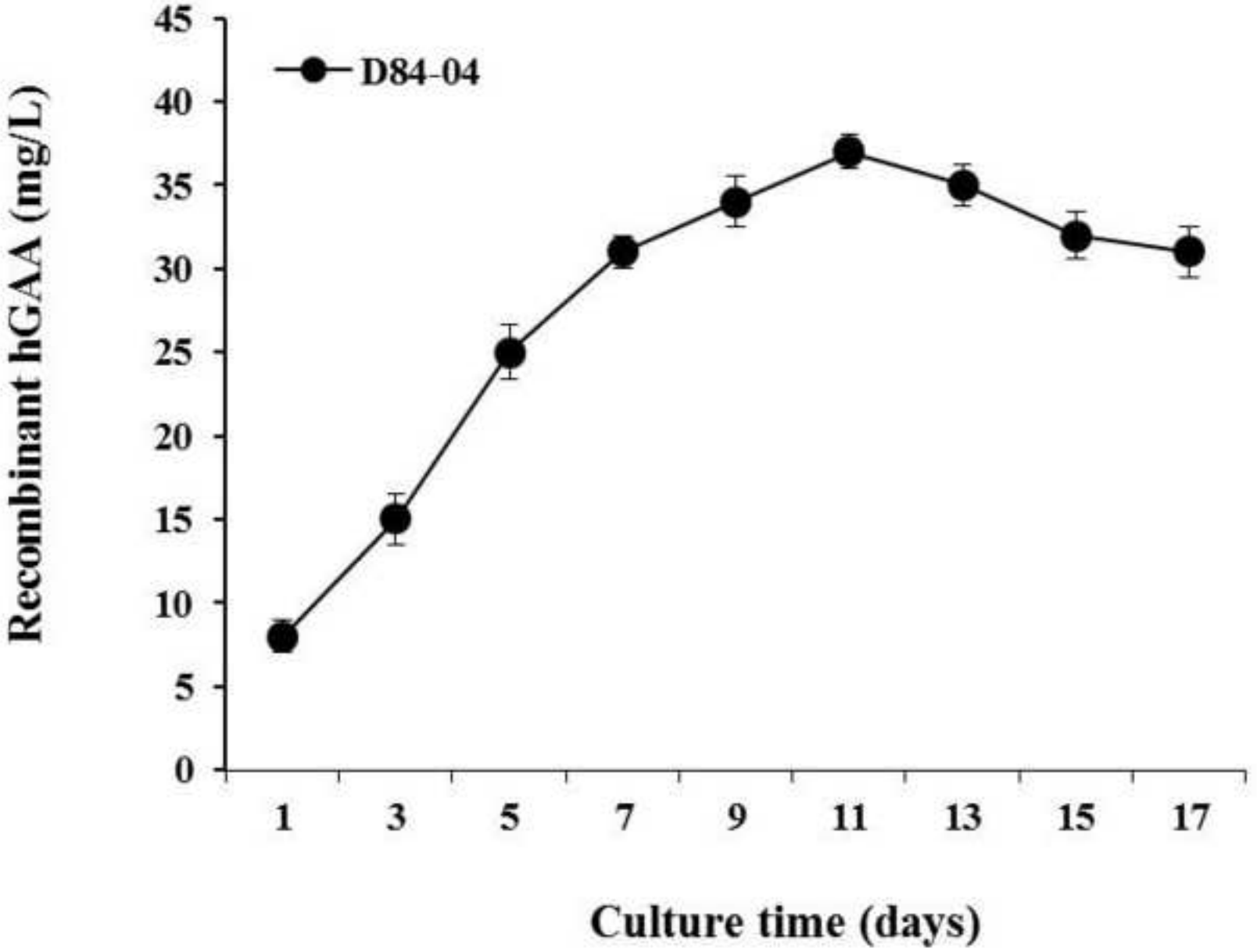

Figure 6.

Click here to download high resolution image

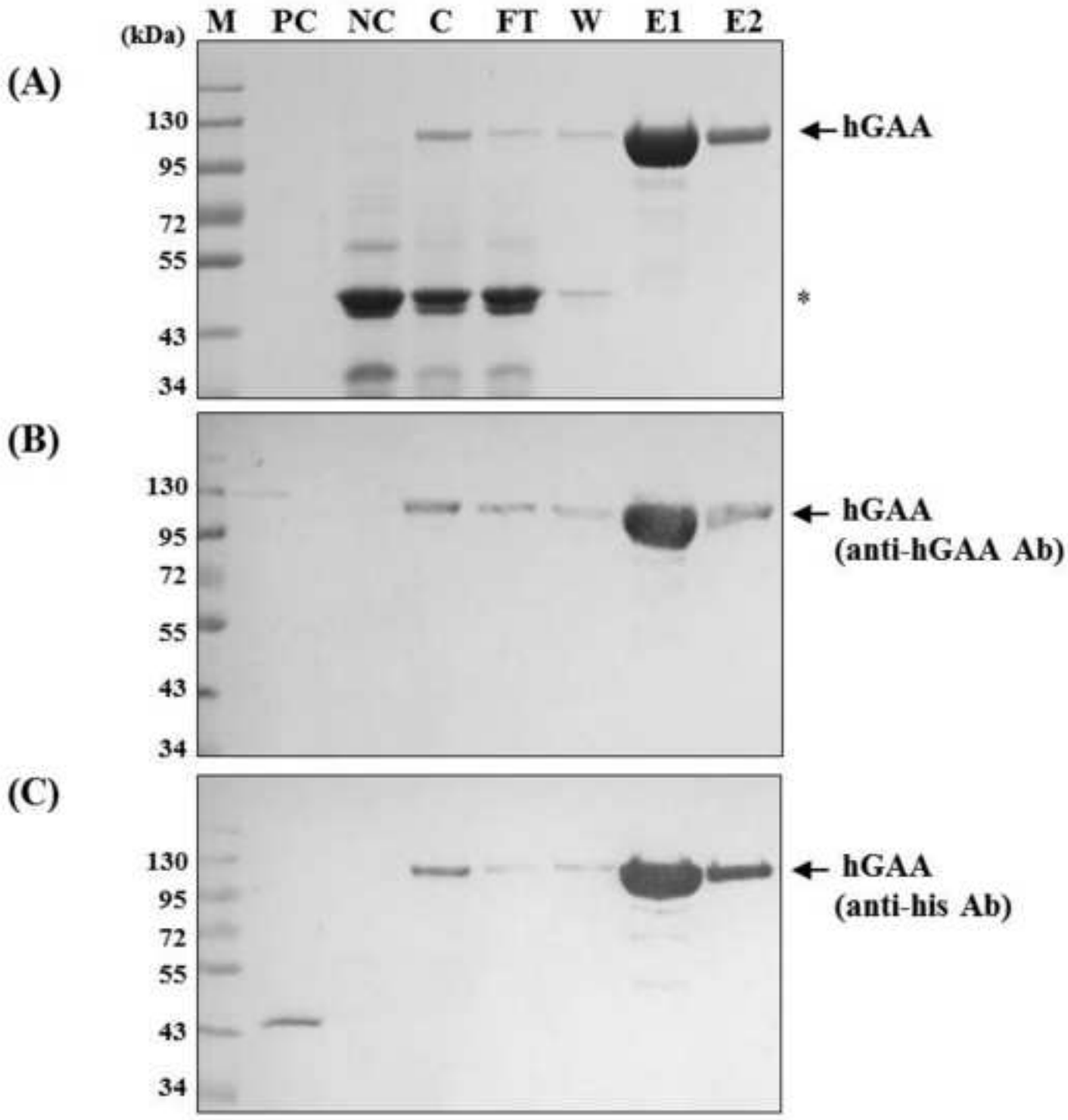

Figure 7.
Click here to download high resolution image