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Production of functional recombinant bovine trypsin in transgenic rice cell suspension cultures

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ABSTRACT

A synthetic bovine trypsinogen (sbTrypsinogen) was synthesized on the basis of rice-optimized codon usage via an overlap PCR strategy, prior to being expressed under the control of the sucrose starvation-inducible rice α-amylase 3D (RAmy3D) promoter. Secretion of trypsin into the culture medium was achieved by using the existing signal peptide. The plant expression vector was introduced into rice calli (*Oryza sativa* L. cv. Dongjin), mediated by *Agrobacterium tumefaciens*. The integration of the sbTrypsinogen gene into the chromosome of the transgenic rice callus was verified via genomic DNA PCR amplification, and sbTrypsin expression in transgenic rice suspension cells was confirmed via Northern blot analysis. Western blot analysis detected glycosylated proteins in the culture medium, having masses from 24 to 26 kDa, following induction by sugar starvation. Proteolytic activity of the rice-derived trypsin was confirmed by gelatin zymogram, and was similar to that of the commercial bovine-produced trypsin. The yields of sbTrypsin that accumulated in the transgenic rice cell suspension medium were 15 mg/L at 5 days after sugar starvation.

Introduction

Trypsin is a serine protease that plays a key role in the activation of pancreatic enzymes involved in digestion. It is released from zymogen granules stored in pancreatic acinar cells, and becomes activated by enterokinase upon secretion. Enterokinase recognizes the $(Asp)_4$-Lys sequence in the propeptide, and cleaves after the lysine residue to release active trypsin [1]. Removal of the propeptide results in conformational changes that lead to the structural formation of an active site [2]. Trypsin cleaves the peptide bonds at the carboxyl-terminal end of lysine and arginine residues, and it can also activate its own zymogen. Once activated, trypsin goes onto activate other digestive proteases that have also been synthesized as zymogens, and it can also digest almost any protein that has basic amino acids as part of its sequence [3].

Trypsin is one of the most widely used, multi-purpose enzymes. Some examples of its use include serving as a detergent for animal cell cultures, having a role in the processing of leather, acting as a dietary supplement in the food industry, and being involved in substitutive therapies for patients with pancreatic diseases. Increasing quantities of highly pure trypsin are required to fulfill the needs of biopharmaceutical manufactures [4,5].

At the present time, bovine trypsin is produced by extraction from cow pancreas. However, the emergence of diseases, such as bovine spongiform encephalopathies, has raised concerns about the use of products with animal origin in industrial processes, especially in the pharmaceutical industry. Thus, there is an increased interest for recombinant trypsin to be produced in a number of systems, including mammalian cell culture [6–9], *Escherichia coli* [10–12], and yeast [13–15]. Despite the ability to express recombinant trypsin in many systems, these systems are not widely used for industrial-scale products.

Transgenic plants and plant cell cultures are now becoming focused as hosts for the production of valuable foreign proteins, as they offer a way to replace animal-derived proteins with a safe and economical alternative [16–19]. Recently, recombinant bovine trypsin was produced and used as a commercial protease from transgenic maize (*Zea mays*) seed [20]. However, the recovery and purification of recombinant proteins from plant biomass is an expensive and technically challenging business that may amount to 80–94% of the final product cost [21,22]. We explored the use of transgenic rice suspension cells to produce bovine trypsin. Bovine trypsin, fused with an existing signal peptide, could be expressed and secreted into the culture medium and then recovered and purified in the absence of large quantities of contamination proteins. The downstream processing of protein from the culture medium is a

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much simpler process, as well as less expensive in terms of product isolation and purification [23].

In the present study, a synthesized bovine trypsinogen (sbTrypsinogen) gene was modified on the basis of rice-optimized codon usage, and subsequently introduced into a transgenic rice suspension culture under the control of the rice amylase 3D promoter. The plant-derived bovine trypsin accumulation levels and functionality were analyzed from the transgenic rice culture medium.

Materials and methods

Trypsin vector construction

The trypsinogen gene from *Bos taurus* (GeneBank Accession No. D38507) harboring its own signal peptide, was synthesized on the basis of rice-optimized codon usage (http://www.kazusa.or.jp/codon) via an overlap PCR strategy (Fig. 1). The sbTrypsinogen gene was cloned into a pGEM T-easy vector (Promega, Madison, WI, USA), and its DNA sequence was verified by DNA sequence analysis. The resultant 747 bp PCR product harboring the gene expressing sbTrypsinogen was digested with *Xba*I and *Sac*I, and introduced into the same sites of the plant expression vector, pCAMBIA1300 [24], under the control of the RAmy3D promoter, with 3′UTR of the RAmy3D gene as the terminator. This plant expression vector was designated as pMYT111 and harbored the hygromycin phosphotransferase (*hpt*) gene as the selection marker for plant transformation (Fig. 2). The binary plant expression vector pMYT111 was transformed into *Agrobacterium tumefaciens* LBA4404 using the helper plasmid pRK2013 by the tri-parental mating method [25,26].

Rice transformation and detection of the sbTrypsinogen gene in transgenic calli

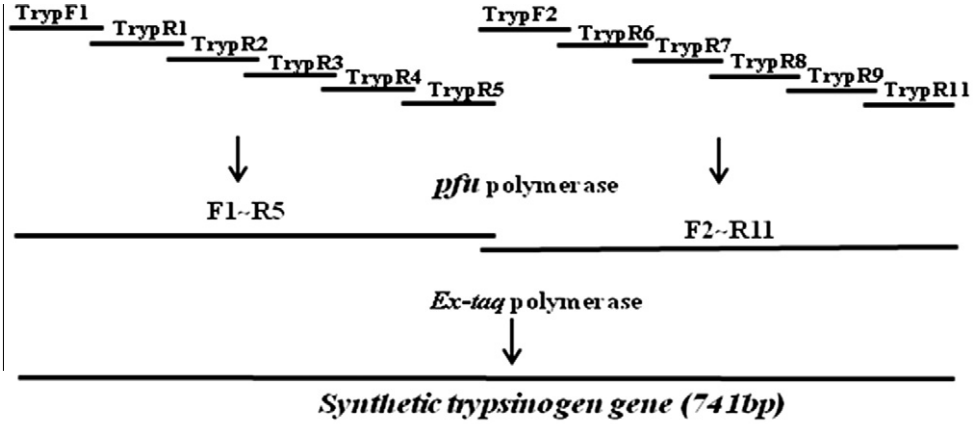
Rice (*Oryza sativa* L. cv. Dongin) embryonic calli were infected with exponential-phase *A. tumefaciens* LBA4404 containing the synthetic trypsinogen gene, as described by Hiei et al. [27]. Transformed calli were first selected by hygromycin B (50 mg/L) treatment and analyzed by genomic DNA PCR to check the insertion of the sbTrypsinogen gene. Genomic DNA was isolated from the putative transgenic calli using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). PCR analysis was performed using the primers 5′-TCT AGA GAT TAT GAA GAC CTT CAT CTT CCT C-3′, and 5′-GAG CTC TCA GTT GGA GGC GAT GGT CTG-3′, which are specific for sbTrypsinogen. Thermal cycling was performed for 30 cycles, consisting of 1 min at 94 °C, 1 min at 55 °C, and 40 s 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.

Propagation and induction of rice cell culture

Transformed rice calli were propagated and cultured at 28 °C in darkness, using a rotary shaker with 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 [28]. A 10-ml inoculum was transferred every 9 days for sub-culturing. In order to induce sbTrypsin gene expression under the control of the RAmy3D promoter, the N6 medium was removed from the cell suspension by aspiration, and the cells were transferred to fresh N6 (–S) medium (without sucrose) at 10% (weight of wet cells/volume of medium) density. The culture supernatant from the culture medium of induced rice cells was collected by pouring induced cell suspension through 2–3 layers of Myracloth (Calbiochem, La Jolla, CA, USA). The total medium proteins were collected by centrifugation at 18,000*g* with at a temperature of 4 °C for 10 min, in order to remove the debris.

Northern blot analysis

For Northern blot analysis, total RNA was isolated from suspension cells grown during 5 days in N6 and N6 (S–) liquid media using



Tryp-F1:5'-ATG AAG ACC TTC ATC TTC CTC GCC CTC CTC GGC GCT GCT TGG CCT TCC CAG TGG ACG ACG ACG ACA AGA TCG TGG GAG G-3'
Tryp-R1:5'-AAG TGG TAT CCG GAG TTG AGG GAC ACC TGG TAT GGG ACG GTG TTT GCT CCG CAG GTG TAT CCT CCC ACG ATC TTG TCG TC-3
Tryp-R2:5'-GAC TTG TAG CAG TGT GCT GCG GAC ACC ACC CAC TGG CTG TTG ATG AGG GAT CCT CCG CAG AAG TGG TAT CCG GAG TTG AG-3'
Tryp-R3:5'-AAC TGC TCG TTT CCC TCC ACG ACG TTG ATG TTG TCC TCT CCC AGG CGC ACC TGG ATT CCG GAC TTG TAG CAG TGT GCT GC-3'
Tryp-R4:5'-TCG TTG TTG AGG GTG TTG GAG TTG TAG CTT GGG TGG ACG ATG CTC TTG GAG GCG CTG ATG AAC TGC TCG TTT CCC TCC AC-3'
Tryp-R5:5'-GAG ATG GAT GCC ACC CTG CTG TTG AGG CTT GCT GCG GAC TTC AGC TTG ATG AGC ATG ATG TCG TTG TTG AGG GTG TTG GA-3'
Tryp-F2:5'-AGC AGG GTG GCA TCC ATC TCC CTG CCG ACC TCC TGC GCT TCC GCT GGC ACC CAG TGC CTC ATC TCC GGA TGG GGC AAC AC-3'
Tryp-R6:5'-GAG AGG ATT GGG GCC TTC AGG CAC TTC AGG ACG TCC GGG TAG CTG GTG CCG CTG CTC TTG GTG TTG CCC CAT CCG GAG AT-3'
Tryp-R7:5'-CCA GCG CAG AAC ATG TTG CTG GTG ATC TGT CCT GGG TAG GCG CTC TTG CAG GAG CTG TCG GAG AGG ATT GGG GCC TTC AG-3'
Tryp-R8:5'-CTG CAG ACC ACC GGT CCT CCG GAG TCG CCC TGG CAG GAG TCC TTT CCT CCC TCC AGG TAG CCA GCG CAG AAC ATG TTG CT-3'
Tryp-R9:5'-CCC GGC TTG TTC TTC TGG GCG CAG CCA GAT CCC CAG GAG ACG ATG CCC TGG AGC TTG CCG CTG CAG ACC ACC GGT CCT CC-3'
Tryp-R11:5'-TCA GTT GGA GGC GAT GGT CTG CTT GAT CCA GCT CAC GTA GTT GCA GAC CTT GGT GTA CAC TCC CGG CTT GTT CTT CTG GGC-3'

Fig. 1. Overlap PCR strategy for the synthesis of sbTrypsinogen. Primers are used with PCR to get the sbTrypsinogen gene.