Recombinant Shrimp (*Litopenaeus vannamei*) Trypsinogen Production in *Pichia pastoris*

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Shrimp (Litopenaeus vannamei) trypsinogen has never been isolated from its natural source. To assess the production of L. vannamei trypsinogen, we engineered Pichia pastoris strains and evaluated two culture approaches with three induction culture media, to produce recombinant shrimp trypsinogen for the first time. The trypsinogen II cDNA was fused to the signal sequence of the Saccharomyces cerevisiae alpha mating factor, placed under the control of the P. pastoris AOX1 promoter, and integrated into the genome of P. pastoris host strain GS115. Using standard culture conditions for heterologous gene induction of a GS115 strain in shake flasks, recombinant shrimp trypsinogen was not detected by SDS-PAGE and Western blot analysis. Growth kinetics revealed a toxicity of recombinant shrimp trypsinogen or its activated form over the cell host. Thus, a different culture approach was tested for the induction step, involving the use of high cell density cultures, a higher frequency of methanol feeding (every 12 h), and a buffered minimal methanol medium supplemented with sorbitol or alanine; alanine supplemented medium was found to be more efficient. After 96 h of induction with alanine supplemented medium, a 29-kDa band from the cell-free culture medium was clearly observed by SDS-PAGE, and confirmed by Western blot to be shrimp trypsinogen, at a concentration of 14 μg/mL. Our results demonstrate that high density cell cultures with alanine in the induction medium allow the production of recombinant shrimp trypsinogen using the P. pastoris expression system, because of improved cell viability and greater stability of the recombinant trypsinogen. © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.,* 25: 1310–1316, 2009
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Introduction

Trypsin is a widely used serine protease with substrate specificity based on positively charged lysine or arginine side chains. In animals, this enzyme plays an important role in digestion, and in *Crustaceae,* for instance, is the most abundant digestive protease. Three major isoforms of this enzyme have been purified from the digestive glands of *Litopenaeus vannamei*,[1] and the molecular cloning and sequencing of cDNA encoding for two of the mature trypsin isoforms have been carried out.[2] Differing from trypsins from vertebrate sources, the three trypsin isoforms of *L. vannamei* have acidic isoelectric points, maximum activities in a broad range of temperatures (40–70°C),[1,3] with maxima activities between 50 and 55°C,[3] and have zymogens that do not have a specific recognition sequence for enteropeptidase.[2] Bovine trypsin is largely used in research laboratories and is the preferred trypsin for industrial protein processing or for proteomic studies. Shrimp trypsin, however, has a specific activity that is 30 times higher than bovine trypsin I, at 25°C and pH 7.5.[2] Because of its biochemical properties, shrimp trypsin is a candidate to be produced in a recombinant form. Because trypsin activity damages host cells,[4] a reasonable approach for its production is to initially produce the inactive precursor (trypsinogen) and then activate it to the active form (trypsin). Production of recombinant trypsinogen has been attempted mainly using *Escherichia coli* as a host, however, the low solubility of the produced trypsinogen, with formation of inclusion bodies, requires additional solubilization and refolding steps.[5,6]

The methylotrophic yeast *Pichia pastoris* has been developed as a host for the efficient production and secretion of foreign proteins.[7] Generally, this expression system uses the methanol-inducible alcohol oxidase 1 (*AOX1*) promoter for high cell density cultures with methanol-regulated expression cassettes.[8,9]

To assess the production of *L. vannamei* trypsinogen, we engineered *P. pastoris* strains and evaluated two culture approaches with three induction culture media to produce, for the first time, recombinant shrimp trypsinogen.

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Materials and Methods

Strains, plasmids, media composition, and chemicals

P. pastoris strain GS115 (*his4*) and *E. coli* strain TOP10F′ were purchased from Invitrogen (San Diego, CA). A Mut$^+$

P. pastoris recombinant strain, producer of human growth hormone (GS115-HGH22K) and previously constructed in our laboratory,[10] was used as a control strain in the growth kinetics assays. The plasmid pPIC9, from Invitrogen (GenBank No. Z46233), contained the promoter and terminator *AOX1* gene of *P. pastoris* and the α-mating factor prepro-secretion signal from *Saccharomyces cerevisiae,* and the HIS4 auxotrophic selection marker was used as a vector for expression of trypsinogen cDNA. The pBSTrp vector, harboring the full-length cDNA of the mature *L. vannamei* trypsinogen II (GenBank No. Y15040), was kindly provided by A. van Wormhoudt (Marine Biology Laboratory, URM-IFREMER-14-College de France, Concarneau Cedex, France).

Regeneration dextrose base (RDB), yeast extract-peptone-dextrose (YPD), buffered minimal glycerol (BMG), and buffered minimal methanol (BMM) media were prepared according to the manual of the *Pichia* Expression Kit (Invitrogen). BMM medium was also supplemented with 2% (w/v) D-sorbitol (BMMS) or with 0.8% (w/v) alanine (BMMA). In all induction media (BMM, BMMS, and BMMA), 0.75% (v/v) methanol was used instead of 0.5% (v/v) methanol, as was recommended by the Invitrogen protocols. Yeast nitrogen base, without amino acids, was purchased from Difco (Detroit, MI). All chemicals were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO) or from Productos Químicos Monterrey (Monterrey, N.L., Mexico).

Construction of P. pastoris *GS115-Tg strains*

The expression vector pPIC9 was cut with *Xho*I and *Avr*II restriction enzymes (New England Biolabs, Beverly, MA), to remove 23 nucleotides from the 3′ terminus of the alpha-factor signal sequence, including the sequence that encodes for the proteolytic processing site Leu-Glu-Lys-Arg and the Glu-Ala repeats. An oligonucleotide pair (forward 5′-GCTCTCGAGAAAAGAGCCCCCTCCAGGAAGCCC-3′ and reverse 5′-ACACCTAGGTTAAACAGCATTGGCCTT-3′) was designed to amplify with Pfu DNA polymerase (Stratagene, La Jolla, CA) the complete *L. vannamei* trypsinogen II cDNA by polymerase chain reaction (PCR) from the pBSTrp vector, with the simultaneous introduction of *Xho*I and *Avr*II restrictions sites at the 5′ and 3′ ends, respectively, of the amplified product, and the restoration of 11 nucleotides from the alpha-factor signal sequence. The PCR reaction was performed in a PCR Sprint thermal cycler (Hybaid, Middlesex, UK) in a 25-μL reaction volume containing 0.5 μM of each primer, 0.2 mM dNTP's each, 1X Pfu DNA polymerase buffer, 1.25 U Pfu DNA polymerase, and 50 ng of the plasmid. A 30-cycle amplification program was used: 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, with a first denaturation step of 94°C for 2 min and a final extension step of 72°C for 10 min. The amplified product was cut with *Xho*I and *Avr*II restriction enzymes and ligated to the vector pPIC9, previously digested with the same restriction enzymes, to produce the new expression vector pPIC9Tg. The correct construction of this vector was confirmed by PCR using the 5′ and 3′ *AOX1* primers from Invitrogen directed to the *AOX1* promoter and the transcription terminator, as previously described.[10] All DNA manipulations were performed according to standard methods.[11]

The *P. pastoris* host strain GS115 (*his4*), was transformed with *Sal*I-digested pPIC9Tg DNA by the spheroplasting method, as described.[12,13] The transformants were selected for their ability to grow on histidine-deficient medium (RDB-agar plates) at 30°C until colonies appeared (His^+ selection). Fourteen His^+ colonies were randomly selected (called GS115-Tg). The integration of the expression cassette into the genome of GS115-Tg strains was verified by PCR using the 5′ and 3′ *AOX1* primers, as previously described.[10]

Expression and analysis of the recombinant shrimp trypsinogen

A randomly selected Mut^+ transformant strain (GS115-Tg2) was induced under low and high cell density conditions. For low cell density cultures, GS115-Tg2 was grown in 25 mL BMG medium at 30°C and 250 rpm until cultures reached an optical density at 600 nm (OD_{600}) of 2–6. Cells were harvested by centrifugation (1500 g, 5 min), used to inoculate 25 mL BMM medium to an initial OD_{600} of 1.4, and then incubated for 72 h at 30°C and 250 rpm to induce the expression of shrimp trypsinogen I cDNA. For the high cell density cultures, cells were grown in 100 mL of BMG medium at 30°C and 250 rpm for 12 h until cultures reached an OD_{600} of 6, harvested by centrifugation (1500 g, 5 min), and used to inoculate 20 mL of BMM, BMMS, or BMMA media at an initial OD_{600} of 30. Further incubation was carried out at 30°C for 96 h in continuous shaking at 250 rpm. In the induction step, methanol was added to a final concentration of 0.75% every 24 h or every 12 h, for the low or high cell density cultures, respectively.

Protein concentration was determined in the cell-free culture media by the Bradford protein assay, using bovine serum albumin as the standard. Cell-free culture media were concentrated 50-fold and diafiltrated by ultrafiltration using 10-kDa Amicon Ultra-4 centrifugal filter devices (Millipore, MA) and a 20 mM Tris-HCl, 20 mM $CaCl_2$ buffer (pH 7.6). Protein concentrates were analyzed by Coomassie blue-stained SDS-PAGE and Western blot. Shrimp trypsinogen concentration in the cell-free culture medium was estimated by densitometric analysis of the Coomassie blue-stained protein bands, using an EDAS 290 system and the Kodak Digital Science 1D program (Eastman Kodak Company, Rochester, NY). For Western blot analysis, a specific *L. vannamei* trypsinogen and trypsin polyclonal antibody, developed in rabbit using a keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide and purified by affinity chromatography using the synthetic peptide as ligand, was purchased from GenScript Corporation (Scotch Plains, NJ) and used as the primary antibody. Separated proteins in the SDS-polyacrylamide gels were transferred to Immobilon-NC transfer membranes (Millipore, Bedford, MA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA), blocked with 2% Tween 20 in PBS and incubated overnight with the primary antibody. Afterwards, the membrane was washed with PBS, incubated with a polyclonal goat anti-rabbit IgG-horseradish peroxidase conjugate as the secondary antibody (Sigma-Aldrich, St. Louis, MO), and stained with a 3,3′,5,5′-tetramethylbenzidine liquid substrate system for membranes (TMB, Sigma-Aldrich, St. Louis, MO).

Trypsin activity assays

Trypsin activity was tested in both non-concentrated and concentrated cell-free culture media measuring the amidase

activity on the synthetic substrate Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA) according to the Erlanger method.[14] Briefly, 40 μL of sample was added to 960 μL of freshly prepared substrate solution (4 mM BAPNA, 20 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.6, pre-incubated at 50°C), and the increase in absorbance at 405 nm was measured at 50°C for 3 min. The amount of the product generated was calculated using the absorption coefficient of p-nitroaniline (8270 M/cm). Trypsin activity was determined by the slope of linear regression of μmol p-nitroaniline vs. time in minutes. One trypsin unit was considered as the amount of trypsin that hydrolyzes 1 μmol BAPNA per min under the conditions described above. In addition, a preparation of a digestive gland extract (1.5 mg/mL) from *L. vannamei* in 20 mM Tris-HCl pH 7.6 obtained as described elsewhere[2] was used as a positive control for the trypsin assays. Trypsin (1 mg/mL in 1 mM HCl) from porcine pancreas (Sigma-Aldrich, St. Louis, MO) was also used as a positive control, with the incubation performed at 40°C instead of 50°C.

Trypsinogen transcript detection

Reverse transcriptase polymerase chain reaction (RT-PCR) assays were carried out to detect trypsinogen transcripts. Total RNA was isolated from GS115-Tg2 cells grown under induction conditions using Tri-Reagent (Molecular Research Center, Cincinnati, OH), following the manufacturer's recommendations. The isolated RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI). Primary shrimp trypsinogen cDNA was synthesized by reverse transcription of the RNA using 2.3 μM oligo$(dT)_{23}$ primer (Sigma-Aldrich, St. Louis, MO) and 400 U M-MLV reverse transcriptase (Promega, Madison, WI) in a final reaction volume of 30 μL, according to the manufacturer's recommendations. The cDNA was amplified by PCR, as described above for the construction of the expression vector pPIC9Tg using Taq DNA polymerase (Promega, Madison, WI).

Growth kinetics

Growth kinetics in BMG and three induction media (BMM, BMMS, BMMA) were determined for the GS115-Tg2 strain. For the BMG growth kinetics, YPD cultures were used for inoculation of 25 mL BMG medium at an initial OD_{600} of 0.1, and cells were grown at 30°C and 250 rpm for 12 h. OD_{600} was measured every 2 h. For growth kinetics in the induction media, cells from BMG cultures (OD_{600} of 1.0–1.4) were harvested by centrifugation (1500 g, 5 min) and used to inoculate the induction media (BMM, BMMS, or BMMA) at an initial OD_{600} of 1.0–1.4. Each culture was grown at 30°C and 250 rpm for 12 h. Samples were taken every 2 h to measure OD_{600} and determine the colony-forming units (CFU) per mL in YPD-agar plates after incubation at 30°C for 24–48 h. Specific growth rates were determined by the slope of the log-linear regression of OD_{600} or CFU/mL vs. time, and were statistically compared between groups using an analysis of variance (ANOVA) and Tukey's multiple comparisons, with a significance level of $P < 0.05$.

Ultrastructural analysis of methanol-induced cells

Methanol-induced cells harvested by centrifugation at different induction times from low cell density cultures were washed with water, fixed in 1.5% $KMnO_4$ for 45 min at room temperature, stained with 1% uranil acetate, dehydrated with increasing concentrations of ethanol, and embedded in Epon 812. Ultrathin sections were prepared and examined with a ZEISS-EM109 transmission electron microscope.

Table 1. Final OD_{600}, pH, and Protein Concentration in the Cell-Free Culture Medium for the Four Culture Assays with the GS115-Tg2 Strain

Culture Medium	OD_{600}	pH	Protein Concentration (μg/mL)
BMM[1]	13	2.7	11.7
BMM[2]	68	2.8	23.1
BMMS[2]	89	2.5	38.5
BMMA[2]	82	6.9	93.0

[1] Low cell density assay.
[2] High cell density assay.

Results

Construction of the expression vector pPIC9Tg

The expression vector pPIC9Tg was constructed harboring *L. vannamei* trypsinogen II cDNA sequence in-frame with the *S. cerevisiae* alpha-factor signal and between the promoter and transcriptional terminator of the *AOX1* gene. We engineered the DNA sequences to produce shrimp trypsinogen II as a mature polypeptide of 251 amino acids in accordance with the sequence of GenBank No. CAA75310 described by Klein et al.[2] and the signal peptide cleavage site prediction by the SignalP 3.0 Server.[15] A PCR analysis of the recombinant plasmid, using *AOX1* primers, showed a 1225-bp product that corresponded to the 756-bp of the trypsinogen II cDNA with the termination codon, and the 469-bp of the vector pPIC9, confirming the pPIC9Tg construction.

P. pastoris *transformation and DNA analysis*

Transformation of the *P. pastoris* GS115 strain with *Sal*I-digested pPIC9Tg gave about 500 *His*$^+$ transformants. With the *Sal*I-linearized vector, a single-crossover type integration event was expected to take place into the chromosomal *his4* locus, and *His*$^+$ Mut$^+$ (methanol utilization positive) phenotype transformants were expected to be generated keeping the *AOX1* gene intact and functional.

PCR analysis of the DNA, isolated from several *P. pastoris* GS115-Tg strains (*His*$^+$ transformants), showed two bands: one of 2107 bp and another of 1225 bp that were derived from the *AOX1 locus* and from the trypsinogen expression cassette. These results indicate that the *AOX1* structural gene was intact, and that the expression cassette was integrated into the *P. pastoris* genome, thus confirming the Mut$^+$ phenotype.

Heterologous gene expression and protein analysis

Table 1 shows the final OD_{600}, pH, and protein concentration in the cell-free culture medium for the four culture assays with the GS115-Tg2 strain. Despite grown in BMMS and BMMA using high cell density conditions was almost identical, only in BMMA medium the pH was maintained between 6 and 7. Furthermore, cultures grown in the BMMA showed the highest protein concentration in the cell-free culture medium.

Although a clear increase in absorbance was observed in the trypsin activity assays with shrimp digestive gland extract (0.32 U/mL) and porcine trypsin (0.40 U/mL), trypsin

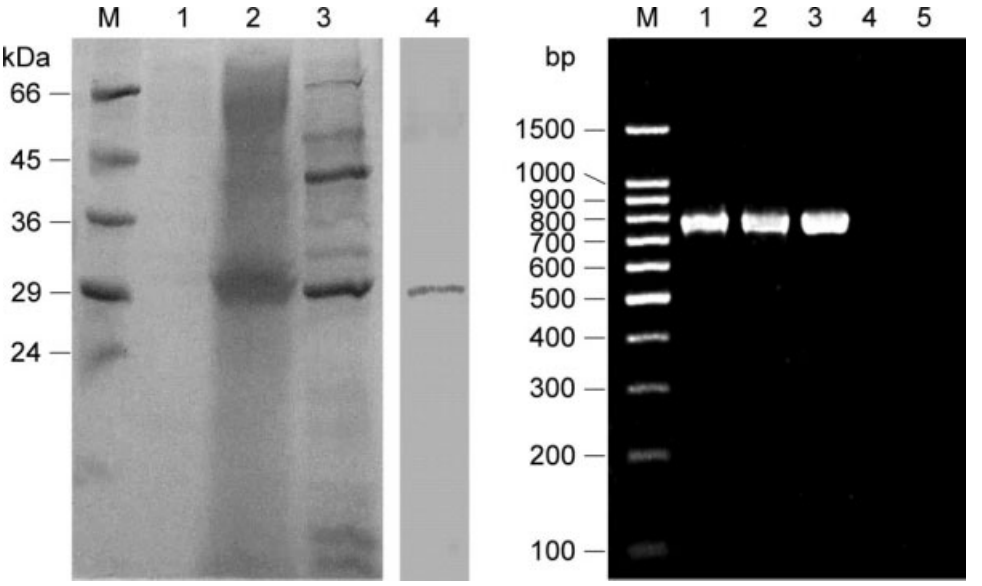

Figure 1. Left, SDS-polyacrylamide gel and Western blot analysis of concentrated cell-free culture medium from 96 h induction of GS155-Tg2 strain using high cell density and methanol feeding every 12 h. Lane M, molecular weight marker; Lane 1, BMM culture medium; Lane 2, BMMS culture medium; Lane 3, BMMA culture medium; Lane 4, Western blot for the sample of Lane 3. Right, agarose gel of RT-PCR assays. Lane M, molecular size marker; Lane 1, BMM culture medium; Lane 2, BMMS culture medium; Lane 3, BMMA culture medium; Lane 4, negative control of reverse transcriptase step (without reverse transcriptase); Lane 5, negative control of PCR (without cDNA).

activity was not detected in either the non-concentrated or the concentrated cell-free culture media.

Cell-free culture medium from BMM cultures of the GS115-Tg2 strain at low and high cell density conditions were negative for the trypsinogen theoretical band of 26.7 kDa by SDS-PAGE and Western blot analysis. Nevertheless, a 29-kDa band was observed in the SDS-polyacrylamide gels from the cell-free culture media of BMMS and BMMA cultures, using high cell-density conditions (Figure 1, left). In addition, in both cultures, the 29-kDa band positively reacted with the specific *L. vannamei* trypsinogen and trypsin antibody in the Western blot analysis (Figure 1, left). In the BMMA culture, recombinant shrimp trypsinogen was 17% (14 μg/mL) of the protein concentration in the cell-free culture medium. RT-PCR assays for induced cells from all culture conditions tested were positive for the expected 782-bp trypsinogen transcript band (Figure 1, right).

Growth kinetics

Under non-induction conditions (BMG medium), no significant differences in specific growth rate were observed between the GS115-Tg2 (0.324/h) and the control strain (0.310/h).

Figure 2 shows GS115-Tg2 growth kinetics in the three induction media (BMM, BMMS, and BMMA) compared with the control strain in BMM medium, measured by OD_{600} (left) and CFU/mL (right). Although the OD_{600} for the BMM cultures of the control strain increased exponentially during the 12-h assay, the OD_{600} for the GS115-Tg2 strain grown in the same conditions did not increase during the two first hours of culture, and then increased exponentially from 2 to 6 h of culture (specific growth rate of 0.091/h) before reaching a plateau after 6 h of culture. These findings were not observed for the GS115-Tg2 kinetics carried out in BMMS and BMMA media, where the OD_{600} increased exponentially during the whole assay. Nevertheless, significant differences were seen in specific growth rates between the GS115-Tg2 strain in BMMS (0.243/h) and in BMMA (0.084/h) media, compared with specific growth rate for the control strain in the BMM medium (0.137/h).

Although no significant differences were found among the specific growth rates for the control strain in the BMM medium determined by OD_{600} and CFU/mL, both specific growth rate determinations were statistically different for the GS115-Tg2 strain (using CFU/mL: BMM −0.02/h, BMMS 0.191/h, and BMMA 0.062/h). It is noteworthy that the CFU/mL for the BMM cultures of the GS115-Tg2 strain decreased slightly during the 12-h assay, instead of the expected increase, as observed for the GS115-Tg2 cultures in BMMS and BMMA media, and for the control strain culture in BMM medium.

Ultrastructural analysis of methanol-induced cells

The ultrastructural analysis of methanol-induced GS115-Tg2 cells showed cellular damage, cellular disorganization, and a decrease in peroxisome number in an induction time-dependent manner, whereas intact cells and typical clusters of large peroxisomes were observed in the methanol-induced control strain (data not shown).

Discussion

The *P. pastoris* expression system is well known for its ability to synthesize biologically active proteins and to secrete these into the culture medium. With this expression system, we engineered, for the first time, a *P. pastoris* strain to produce and secrete recombinant shrimp trypsinogen II. The integration of the shrimp trypsinogen II cDNA into the *P. pastoris* genome and Mut^+ phenotype were confirmed by PCR analysis. Although we did not include the Glu-Ala

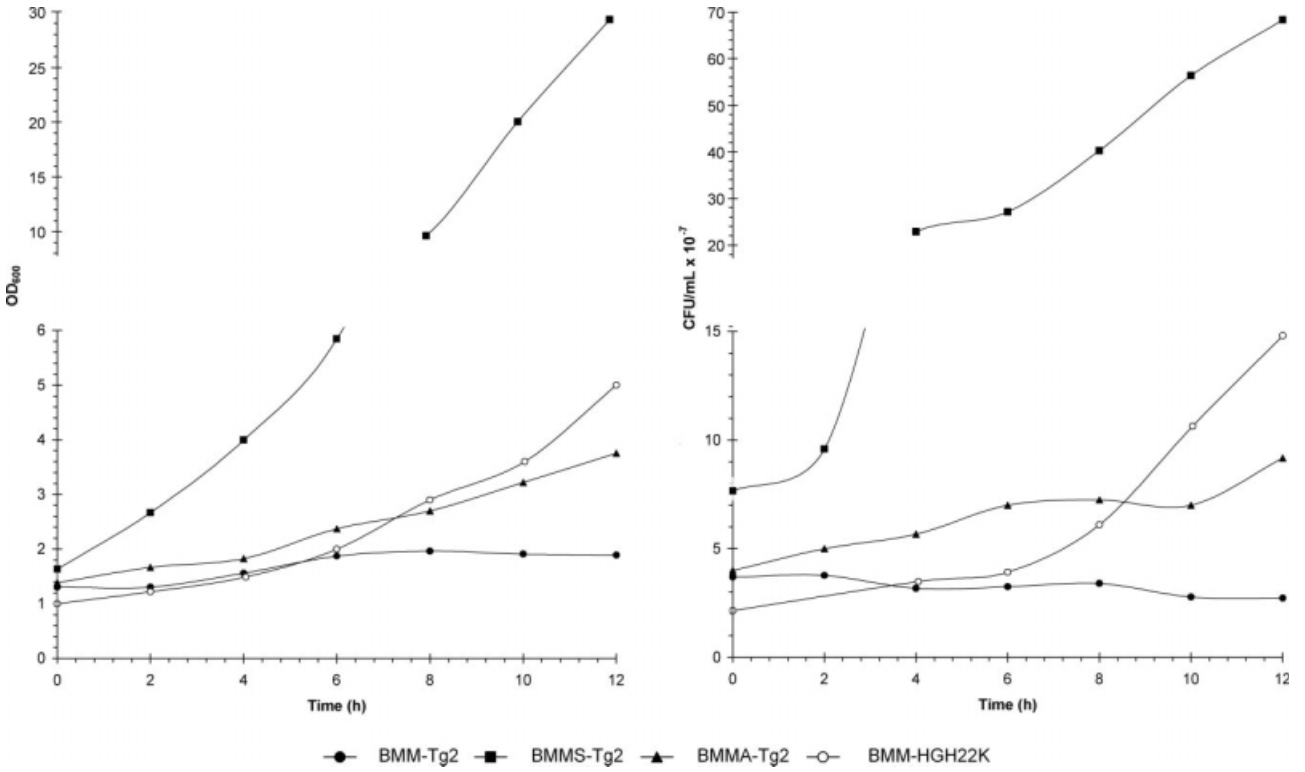

Figure 2. GS115-Tg2 growth kinetics in the three induction media (BMM, BMMS, and BMMA) compared with the control strain (GS115-HGH22K) in BMM medium, measured by OD_{600} (left) and CFU/mL (right). Points represent the mean of three assays (coefficient of variation less than 5%) from three independent kinetics experiments.

repeats after the cleavage site of the alpha-factor signal for peptide release in our heterologous gene construction, the GS115-Tg strains that were constructed in this work produce and secrete recombinant shrimp trypsinogen II into the culture medium. We previously used this type of construction in *P. pastoris* for the production and secretion of 22 kDa[10] and 20 kDa (unpublished results) human growth hormone, a bacterial phytase[16] and a laccase from *Trametes* (unpublished results). In addition, other authors have successfully used this construction,[17] and an inefficient removal of the Glu-Ala repeats from a secreted recombinant protein produced in *P. pastoris* was described.[18-20]

The transcription process of the shrimp trypsinogen II cDNA expression cassette was demonstrated by RT-PCR assays. The expected band for shrimp trypsinogen II, however, was not detected by SDS-PAGE of the cell-free culture medium from the BMM cultures at either low or high cell-density conditions. The BMM culture at the low cell density as used here, is the recommended procedure for producing a recombinant protein in shake-flasks using the *P. pastoris* expression system,[12] and we have successfully used to produce human growth hormone.[10] Growth kinetics for the BMM medium clearly showed a toxicity of the recombinant shrimp trypsinogen, or its activated form, over the cell host. For this reason, we tested a different culture approach for the induction step that involved the use of a high density cells, a higher frequency of methanol feeding (every 12 h), and a buffered minimal methanol medium supplemented with a carbon source other than methanol (sorbitol or alanine) that would not repress the methanol metabolic pathway enzyme synthesis.[21] This approach increased the concentration of the heterologous protein in the cell-free culture medium, since a new 29-kDa band was observed in the SDS-polyacrylamide gels, with alanine supplementation being the most effective. Because the 29-kDa band is close to the molecular weight of shrimp trypsinogen, positively reacted with the specific antibody, and we did not detect any trypsin activity in the cell-free culture media, we concluded that the expression product was recombinant shrimp trypsinogen and not trypsin.

Although cell viability in the induction step was improved when either sorbitol or alanine was added to the BMM medium, a comparison of the growth kinetics of GS115-Tg2 in the induction media to the control strain, as well as a comparison of kinetics parameters determined by OD_{600} and UFC/mL, clearly shows that a toxicity over the host cells was still present. Methanol feeding every 12 h has been used for foldon production in *P. pastoris,*[22] sorbitol has also been used successfully for the growth of peroxisome-biogenesis-defective mutants of *P. pastoris,*[23] and alanine-supplemented induction medium has been described for controlling the pH in recombinant *P. pastoris* cultures.[24] GS115-Tg2 strain cultures in BMM, without alanine supplementation, reached a pH below 3.0, whereas with alanine added to the culture medium, a slight pH increase was observed (final pH 6.9). Because rapid, irreversible enzyme inactivation at pH below 6 has been described for shrimp trypsins from *L. setiferus*[25] and from *L. vannamei,*[1] the improved production of recombinant shrimp trypsinogen with added alanine might be explained by an improvement in recombinant trypsinogen stability. Therefore, two factors could have important roles in the production of recombinant shrimp trypsinogen in BMMA: cell toxicity and trypsinogen stability.

From a sequence comparison of 70 trypsinogen activation peptides from different species, an efficient inhibition of the

trypsinogen autoactivation process was suggested when a tetra-aspartate sequence is present in the activation peptide preceding the Lys-Ile scissile peptide bond,[26] which is cleaved in the first step of the activation process. Similar conclusions have been made[27] after observing a rapid autoactivation process of a mutated human cationic trypsinogen, where the tetra-aspartate motif was replaced with tetra-alanine, in comparison with the wild-type tetra-aspartate human cationic trypsinogen. Because shrimp trypsinogen has no aspartate in the activation peptide, the recombinant shrimp trypsinogen produced was likely partially autoactivated to release active trypsin in the induction step, with this protease causing the cell toxicity. Furthermore, because we did not detect any trypsin activity in the cell-free culture media, intracellular trypsinogen activation was likely taking place, which caused the cell damage observed in the ultrastructural analysis of the methanol-induced GS115-Tg2 strain.

A toxicity to *P. pastoris* cells has also been reported for wild-type bovine trypsinogen production.[28] Therefore, a single mutation in the activation site destroying the Lys-Ile scissile peptide bond was necessary for the accumulation of this trypsinogen in the culture medium.[28] Despite this mutation approach, trypsinogen levels in the culture medium were similar to our findings. In addition, a decrease in viability below 70% in the induction step has been described during the production and secretion of human trypsinogen in *P. pastoris*.[29]

Similar to our results with the BMM or BMMS media, a cold-adapted fish trypsinogen was produced in *P. pastoris* and the recombinant protein was only detected by Western blot.[30] Using the BMMA medium, however, we improved the production levels of shrimp trypsinogen so that it was clearly detected by SDS-polyacrylamide gels and Western blot analysis. Because high cell densities of *P. pastoris* can be reached in a simple bioreactor, which simultaneously provides a good control of pH, a 10 to 100-fold increased shrimp trypsinogen production can be expected.

Because shrimp trypsinogen does not have a specific recognition sequence for enteropeptidase[2] as does the activation peptide in vertebrate trypsinogens, shrimp trypsinogen must therefore have a different activation mechanism, being important autoactivation and trypsin inhibition control. With a sufficient source of shrimp trypsinogen, functionality studies could be carried out. Furthermore, since trypsin is frequently used in proteomics, the availability of a recombinant version would be beneficial as it would be free of other proteases such as chymotrypsin.

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