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Production of the Antihypertensive Peptide Tyr-Pro from Milk Using the White-Rot Fungus *Peniophora* sp. in Submerged Fermentation and a Jar Fermentor

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Abstract: In order to evaluate the blood pressure-lowering peptide Tyr-Pro (YP) derived from casein, we wanted to develop an efficient fermentation method. Therefore, we chose to use a jar fermentor for this purpose. Strains with an excellent antihypertensive peptide-releasing ability from casein were selected from basidiomycete fungi that grow well in milk under shaking conditions accompanied by physical stimulation. Among them, the white-rot fungus *Peniophora* sp., which is suited for growth only in cow's milk or low-fat milk under vigorous shaking conditions, was found to release peptides and amino acids from milk. When comparing the growth in cow's milk and low-fat milk, there was no particular difference in the growth of mycelia between the two, but this fungus tended to preferentially consume lactose under low-fat conditions. The fermented milk exhibited good production of the target peptide YP. The expression of many genes encoding proteolytic enzymes, such as aminopeptidases and carboxypeptidases, was observed during the milk fermentation. Furthermore, this fungus showed good growth in a jar fermentor culture using only cow's milk or low-fat milk, which enabled the efficient production of YP and ACE-inhibitory activity. At this time, it was more effective to use cow's milk than low-fat milk. These results suggest that *Peniophora* sp. could be potentially useful in the production of the functional YP peptide from milk.

Keywords: antihypertensive peptide; milk; submerged fermentation; jar fermentor; white-rot fungus; *Peniophora* sp.



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1. Introduction

Over the years, the number of patients with lifestyle-related diseases, such as hypertension, has been increasing, as has the number of health-conscious consumers, resulting in a shift in the focus of the food industry to functional foods that contain bioactive peptides. Most of these peptides are prepared by digesting proteins that are derived from milk, soybeans, or seafood with defined enzyme preparations [1]. Importantly, oligopeptides with high in vitro activity are decomposed by digestive enzymes in the body when orally administered; therefore, the expected effect may not be obtained in vivo unless the core sequence is maintained. In contrast, dipeptides and tripeptides are not readily degraded by digestive enzymes and heat and are therefore more stable and easily absorbed by the body through the intestinal tract.

Enzymatic hydrolysis is a peptide production method that can be carried out using a protease preparation; however, this method does not efficiently yield the target sequence alone, and it is possible to expose hydrophobic amino acids inside the protein via excessive decomposition. Moreover, it is difficult to control the type and combination of enzymes and the treatment time, which can result in the release of bitter peptides. In addition, the production of casein-derived functional peptides by lactic acid bacteria was commonly used for fermentation [2], and various angiotensin-converting enzyme (ACE, EC 3.4.15.1)-

inhibitory peptides, including Val-Pro-Pro and Ile-Pro-Pro, were shown to be produced by this process [3]. However, the production of the target peptide cannot be increased as expected due to feedback inhibition to peptidases [4,5]. The amount of peptides that can be produced using fermentation with lactic acid bacteria is limited. In contrast, chemical syntheses via *in silico* predictions were widely used to design and verify new peptides based on databases and reports of physiologically active peptides found using enzymatic hydrolysis and fermentation [6]. Furthermore, unlike the above-mentioned enzymatic hydrolysis of substrates, enzymatic synthesis methods using aminoacyltransferases or L-amino acid ligases are expected to be applied to peptide production [7,8].

Although there are many reports on the identification of ACE-inhibitory peptides from fruiting bodies and the mycelial extracts of basidiomycetes [9–18], the content of each peptide in them was negligible. In our previous study, we found that the brown-rot fungus *Neolentinus lepideus* possesses a unique ability to produce ethanol from lactose and whey or milk [19]. The fermented milk showed ACE-inhibitory activity during the milk fermentation process and significant activity compared with other foods marketed for specified health uses. The main component of the active peptide derived from *N. lepideus* fermented milk is Tyr-Pro (YP), which may originate from seven positions in bovine caseins (α_{s1} -CN, f 146–147; α_{s1} -CN, f 159–160; β -CN, f 60–61; β -CN, f 114–115; β -CN, f 180–181; κ -CN, f 35–36; κ -CN, f 58–59). The fungus exhibits a YP production capacity that was over 40 times higher than *Lactobacillus helveticus*, as reported by Yamamoto et al. [20]. Notably, intravenous administration of YP to stroke-prone spontaneously hypertensive rats (SHRSP) showed an effective antihypertensive action. For the functional evaluation of YP, a detailed study was conducted to establish a method for efficient YP production utilizing the fermentation ability of basidiomycetes [21].

Accordingly, in this study, we aimed to elucidate the characteristics of a white-rot fungal strain. Our results showed that this strain grew well on milk only, showed excellent casein degradation ability, and was suitable for the efficient production of functional peptides, such as YP.

2. Materials and Methods

2.1. Fungal Strains and Culture Conditions

In this study, we used strains that were collected from Japanese forests and those owned by the Fungus/Mushroom Resource and Research Center of Tottori University (TUFC cultures), except for *Peniophora* sp. YM5314 [22]. Each fungus was maintained on MYG agar plates containing 10 g/L malt extract, 4 g/L yeast extract, 4 g/L glucose, and 15 g/L agar powder at 28 °C for 7 days. For screening, three 0.5 cm² pieces of the mycelial mat were inoculated into a 500 mL Erlenmeyer flask containing 50 mL of 9% (*w/w*) skim milk without any nutrient supplementation and pH adjustment. For the culture test, three 0.5 cm² pieces of the mycelial mat were inoculated into a 500 mL Erlenmeyer flask containing 200 mL cow's milk (lactose: 4.8%, protein: 3.3%, and fat: 3.8%; Daisen Dairy Agricultural Cooperative, Tottori, Japan) or low-fat milk (lactose: 5.1%, protein: 3.6%, and fat: 1.4%; Daisen Dairy Agricultural Cooperative) without any nutrient supplementation or pH adjustment. The flask was capped with a sponge plug (SILICOSEN; Shin-Etsu Polymer Co., Ltd., Tokyo, Japan). The submerged cultures were incubated at 30 °C on a rotary shaker at 200 rpm. In the case of the reciprocal shaking culture, a 500 mL Sakaguchi flask was used instead of the 500 mL Erlenmeyer flask. In cultures that were carried out in a jar fermentor, the preculture described above with an inoculum amount of 6% (*v/v*) was then transferred into an 8 L stirred jar fermentor (EYELA, Tokyo, Japan) with a working volume of 3 L milk at 30 °C without adjusting the pH, an agitation speed of 250 rpm, and an aeration rate of 1.0 L/min.

2.2. Analysis of YP and Aromatic Amino Acids in Fermented Milk

At periodic intervals during the milk fermentation, samples were collected, centrifuged for 10 min at 15,000× *g* in an Eppendorf benchtop centrifuge, and then filtered

through a 0.22 µm membrane filter (Millex-GP; Merck Millipore, Darmstadt, Germany). The lactose concentrations in the culture filtrates were determined using a high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Kyoto, Japan) that was equipped with a refractive index detector (RID-10A) and a Shodex KS-801 column (8.0 mm × 300 mm; Showa Denko Co., Ltd., Tokyo, Japan) and operated at 80 °C with distilled water as the mobile phase at a flow rate of 0.6 mL/min. The concentrations of YP and aromatic amino acids (tyrosine, phenylalanine, and tryptophan) were assessed using an HPLC (Shimadzu Corp.) that was equipped with an ultraviolet (UV)/visible detector (SPD-20A) and a reversed-phase (RP) column (Cadenza CL-C18; 3.0 mm × 150 mm; Imtakt Corp., Kyoto, Japan) and operated at 28 °C with deionized water containing 0.1% (v/v) trifluoroacetic acid (TFA) and acetonitrile containing 0.1% (v/v) TFA as solvents A and B, respectively. The C18 column was conditioned with solvent A. The sample (10 µL volume) was injected into the column, where the flow rate was 0.6 mL/min. The linear concentration gradient settings were as follows: 100% solvent A for 5 min and 0–20% solvent B for 100 min. The UV absorbance of the eluent was monitored by measuring the absorbance at 215 nm. The YP in the fermented milk was identified via agreement with the retention time of the synthesized YP (BEX Co., Ltd., Tokyo, Japan) and the result of the peptide sequence based on the purified peak fraction. Quantification was performed based on the calibration curve created from the RP-HPLC area and the concentrations of the synthesized YP and standard aromatic amino acids.

2.3. RNA Extraction, Library Preparation, and Sequencing

Transcriptome analysis was performed to select for the expression of a specific proteolytic enzyme during fermentation or YP production using RNA expressed in the cultured mycelium on days 3 and 7 of the culture. Total RNAs were extracted from freshly frozen mycelia on days 3 and 7 of the culture using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and concentration of the RNA were validated using a BioSpec-nano spectrophotometer (Shimadzu). The integrity of the total RNA was checked using an Agilent 2100 bioanalyzer system (Agilent Technologies, Palo Alto, CA, USA). Only samples with high-quality RNA (RNA integrity number ≥ 7.0) were used for paired-end sequencing. mRNA sequencing was performed on an Illumina HiSeq platform. Library construction and sequencing were performed by Macrogen (Seoul, Korea). To obtain the quantification scores between two samples, fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated using Cufflinks, which corrects for transcript length and the total number of mapped reads from the library to compensate for different read depths for various samples. Annotation of the raw data and differences in gene expression levels were analyzed by Maze Inc. (Tokyo, Japan).

2.4. Assay of Angiotensin-I-Converting Enzyme (ACE)-Inhibitory Activity

The ACE-inhibitory activity of samples fermented in a jar fermenter was assayed using an ACE Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions, which were modified based on the method described by Cushman and Cheung [23]. The absorbance of the released 3-hydroxybutyric acid in the reaction mixtures at 450 nm was recorded using a microplate reader (Multiskan™ GO; Thermo Fisher Scientific, Waltham, MA, USA). The ACE-inhibitory activity of each sample during *Peniophora* sp. fermentation was measured by diluting the fermented milk 500-fold with sterile distilled water. The percent inhibition of ACE activity was calculated according to the following equation: the inhibitory activity (%) = [(control abs) – (sample abs – sample blank abs)] / (control abs – control blank abs) × 100, where control refers to the activity of the enzyme with added distilled water instead of the sample solution and blank refers to the activity without the enzyme. The values presented in the paper are the means of triplicate analyses.

3. Results and Discussion

3.1. Screening of a Basidiomycete Fungus Suitable for YP Production

To efficiently produce functional peptides with mushrooms, we screened for a strain that, unlike *N. lepideus*, showed good growth in milk, even under shaking culture conditions, and had excellent casein-degrading ability. First, of the laboratory-preserved strains and TUF C strains, those that were able to grow in the MYG medium under shaking culture conditions were selected. Next, 24 strains in which growth was observed even when skim milk was used as a raw material were further selected, and the dipeptide YP concentrations in these fermented milk samples were evaluated using RP-HPLC (Figure 1). High YP production was observed for *Peniophora* sp. YM5314. The amino acid analysis showed that free amino acids equivalent to those of *N. lepideus*, such as glutamic acid, aromatic amino acids, and branched-chain amino acids, were present (data not shown). In general, most basidiomycete fungi showed optimal growth at approximately 24 °C and poor growth at approximately 30 °C; however, the optimum growth temperature for strain YM5314 was approximately 30 °C. In addition, this strain was characterized by an extremely fast growth rate compared to other basidiomycete fungi. In a previous report, this fungus was found to exhibit excellent ethanol production capacity not only from sugars, such as glucose, cellobiose, maltose, and starch, but also from kitchen waste [22]. These results suggest that the white-rot fungus *Peniophora* sp. YM5314 strain could assimilate lactose and showed effective protein-degrading ability, even when only milk was used as a growth medium.

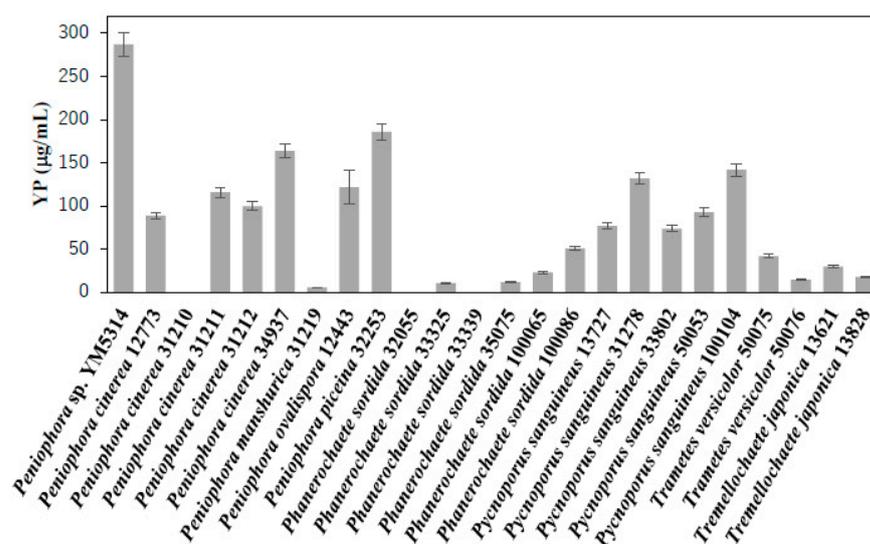


Figure 1. Screening of basidiomycete fungi capable of growing in milk and producing dipeptide YP. All experiments were performed in triplicate, and the error bars represent means \pm standard deviations.

3.2. Effects of Shaking Conditions on YP Production

In the above screening, the *Peniophora* sp. YM5314 strain showed the best growth and YP-producing ability; thus, we subjected this strain to further examination. In a rotary shaking culture, the mycelium grows as if it were attached to the centrally collected fat. In a reciprocal shaking culture, the mycelium grows in a shattered state because the culture solution is vigorously stirred. Figure 2 shows the YP and lactose concentrations in two types of media (low-fat milk and cow's milk) and with different shaking methods (rotary or reciprocal), as well as the changes in aromatic amino acid concentrations as an indicator of casein decomposition over time. Although the types of milk used in Figures 1 and 2 were different, the productivity of YP was higher when the medium volume was 200 mL than when it was 50 mL at the time of screening under the conditions of a rotary shaking culture. This might have been because the depth of the liquid in the Erlenmeyer flask when milk was added affected the growth, including the proteolysis by the mycelium.

Therefore, it was inferred that this fungus may be suitable for deep cultures. Both low-fat milk and cow's milk showed a YP concentration of approximately 520 $\mu\text{g}/\text{mL}$ on day 16 of the cultures, and no significant differences were observed between the two in terms of YP production in the rotary shaking culture; however, in the former, there was a tendency for the YP concentrations to decrease after the peak of YP production. Low-fat milk consumed more lactose than cow's milk (Figure 2a,b,e,f). Additionally, the free aromatic amino acid concentration tended to be higher in low-fat milk (Figure 2c,d,g,h). Because the fat content of cow's milk was higher than that of low-fat milk, this fungus also utilized fat, and lactose may be preferentially utilized in low-fat milk (Figure 2a,b,e,f). No significant differences were observed regarding mycelium growth, regardless of the amount of milk fat. When comparing the different shaking methods, both low-fat milk and cow's milk showed higher YP and free aromatic amino acid concentrations in the rotary shaking culture than in the reciprocal shaking culture (Figure 2a–h). This may have been because the latter shaking method caused more stress, forcing the mycelium apart via physical stimulation. In all cases, no significant decrease in pH or lactic acid production was observed (Figure 2i). From these results, this strain was suggested to have good growth and protein degradability in milk, regardless of the milk fat content. Among the conditions tested, we found that rotary shaking culture in cow's milk was most suitable for YP production accompanying casein decomposition.

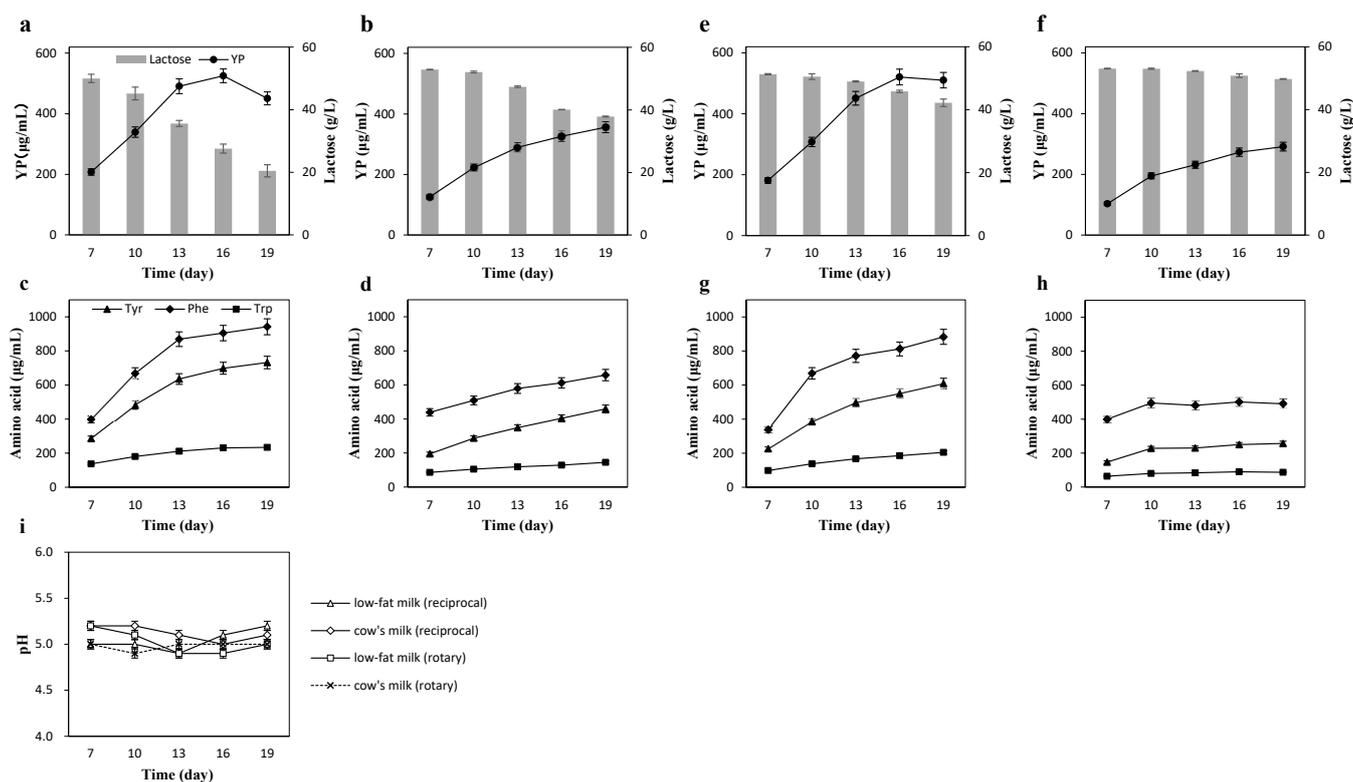


Figure 2. Time course of YP production by *Peniophora* sp. YM5314 in shaken flasks. (a) Lactose consumption and YP production with rotary shaking in low-fat milk. (b) Lactose consumption and YP production with reciprocal shaking in low-fat milk. (c) Release of aromatic amino acids with rotary shaking in low-fat milk. (d) Release of aromatic amino acids with reciprocal shaking in low-fat milk. (e) Lactose consumption and YP production with rotary shaking in cow's milk. (f) Lactose consumption and YP production with reciprocal shaking in cow's milk. (g) Release of aromatic amino acids with rotary shaking in cow's milk. (h) Release of aromatic amino acids with reciprocal shaking in cow's milk. (i) Changes in pH in each fermentation experiment. All experiments were performed in triplicate, and the error bars represent means \pm standard deviations.

3.3. Transcriptome Analysis

Table 1 shows 33 proteases that were likely to be involved in the YP release from casein in the white-rot fungus *Peniophora* sp. YM5314 based on marked increases in the FPKM values during the fermentation process in cow's milk via rotary shaking. The changes in the expression of genes encoding enzymes involved in proteolysis in *Peniophora* sp. YM5314 during milk fermentation were similar to those in the brown-rot fungus *N. lepideus* [21]. Notably, AAP1-alanine/arginine aminopeptidase; cytosol aminopeptidase; peptidases M14, M17, M20, M28, M48, M50B, and S41; tripeptidyl peptidase A; and carboxypeptidase Y were detected in *Peniophora* sp. YM5314. In addition, Xaa-Pro dipeptidase (EC 3.4.13.9), which cleaves the bond between the tyrosine and proline of dipeptide YP, was also expressed in *Peniophora* sp.; however, its expression level tended to be lower than that in *N. lepideus*. These enzymes were expected to be specifically related to efficient YP accumulation in *Peniophora* sp. YM5314. The imbalance between the release of YP from casein and the degradation of YP affected the concentration of accumulated YP rather than the ability to produce high concentrations of YP. Therefore, in the future, when selecting a fungus that produces even higher YP, it may be effective to select a strain that has low Xaa-Pro cleavage activity or low expression levels.

Table 1. Putative proteolytic enzymes upregulated during the fermentation of cow's milk by *Peniophora* sp. YM5314.

Contig Length (bp)	Putative Proteolytic Enzymes	FPKM	
		3 Days	7 Days
2288	Acid protease	0	3.62
2737	Metalloprotease	0	0.11
227	Zn-dependent exopeptidase	0	1.08
2037	Aspartic endopeptidase Pep2	1.04	5.43
373	Aspartic peptidase A1	0	1.7
701	Aspartyl aminopeptidase	0	2.77
3133	AAP1-alanine/arginine aminopeptidase	0.48	1.88
250	Cytosol aminopeptidase	0	0.95
1425	Leucine aminopeptidase	18.74	31.5
2068	Methionine aminopeptidase	0	9.69
483	Peptidase M14	1.5	2.2
324	Peptidase M17	0.48	2.44
2194	Peptidase M20	0	0.05
952	Peptidase M24A	0	4.43
241	Peptidase M28	0	0.5
1294	Peptidase M36	0	0.45
2337	Peptidase M48	0	4.01
702	Peptidase M50B	3.54	16.83
1910	Peptidase S10	20.17	30.78
371	Peptidase S28	0	3.56
776	Peptidase S41	0	0.78
236	Xaa-Pro aminopeptidase	0	2.05
5975	Xaa-Pro dipeptidase	0.94	2.64
1261	Proline iminopeptidase	2.02	7.18
213	Proline-specific peptidase	0	1.76
1370	Dipeptidyl aminopeptidase BIII	14.02	31.89
701	Tripeptidyl peptidase A	0	1.95
224	Tripeptidyl peptidase sed2	0	1.1
2178	Tripeptidyl peptidase sed3	0	1.59
530	Carboxypeptidase S	0	2.58
1828	Carboxypeptidase Y	0	3.78
2724	Serine carboxypeptidase	0	6.43
516	Family S53 protease	0	1.84

3.4. YP Production and ACE-Inhibitory Activity in a Jar Fermentor

When the jar culture was performed using low-fat milk as the main medium, the mycelial growth was good, and YP and aromatic amino acid production levels were equivalent to those at the flask level (Figure 3a,b). The low-fat milk cultures consumed abundant amounts of lactose, and changes in pH were also observed, suggesting that metabolism was activated (Figure 3c). Next, the jar culture using cow's milk was performed. The results showed that the mycelial growth was also good and that the YP production was higher than that in low-fat milk culture. Additionally, on day 8 of the 3.2 L culture (Figure 3e), the same level of YP production (520 $\mu\text{g}/\text{mL}$) was observed as on day 16 of the flask culture performed using 200 mL of the same milk. These findings suggested that the culture period could be shortened and scaled up. The amount of phenylalanine released was slightly higher than that of low-fat milk; however, no significant differences were observed in the amounts of tyrosine and tryptophan between the cow's milk culture and the low-fat milk culture (Figure 3f). No particular changes in pH were observed, and unlike the case with low-fat milk, an almost constant value was observed (Figure 3g).

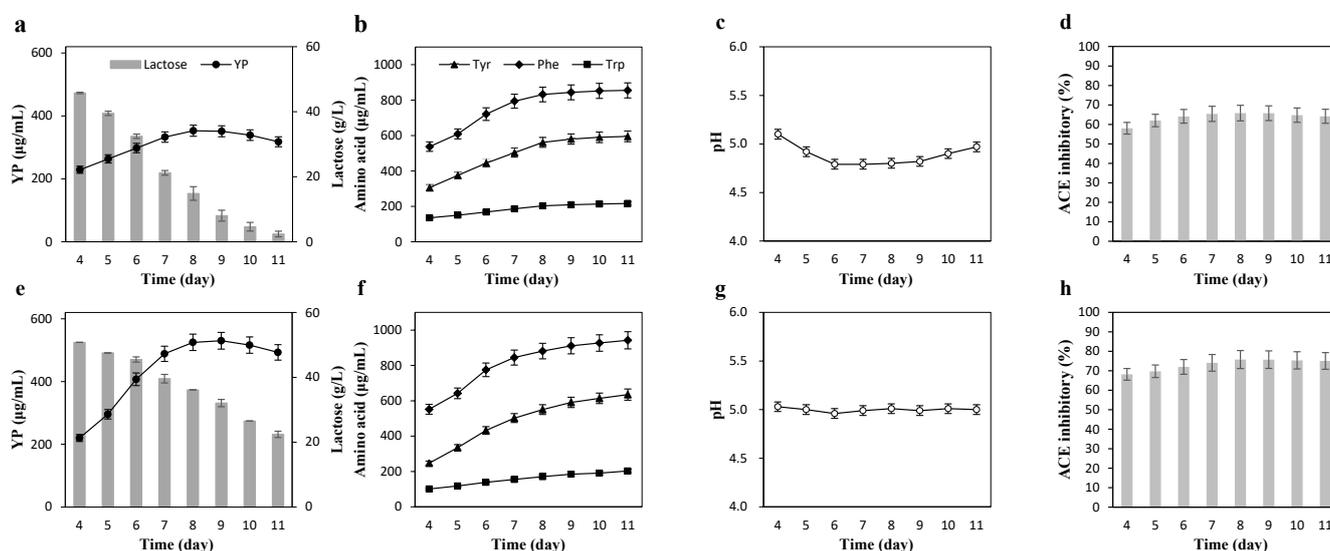


Figure 3. Time course of YP production by *Peniophora* sp. YM5314 in a jar fermentor. (a) Lactose consumption and YP production in low-fat milk. (b) Release of aromatic amino acids in low-fat milk. (c) Changes in pH in low-fat milk. (d) ACE-inhibitory activity in low-fat milk. (e) Lactose consumption and YP production in cow's milk. (f) Release of aromatic amino acids in cow's milk. (g) Changes in pH in cow's milk. (h) ACE-inhibitory activity in cow's milk. All experiments were performed in triplicate, and the error bars represent means \pm standard deviations.

As shown for the flask culture, the decrease in YP after day 10 may have been due to Xaa-Pro dipeptidase, which cleaves the peptide bond between tyrosine and proline. In the transcriptome analysis described above, the expression of this enzyme tended to increase as the fermentation progressed. Despite the good mycelial growth in cow's milk, the consumption of lactose was lower than that in the low-fat milk culture. Since a small amount of glycerol, which is thought to be derived from milk fat, was detected in fermented milk, it is considered that this fungus preferentially used milk fat over lactose in cow's milk. There are several reports on lipolytic enzymes in basidiomycetes [24–31]. Although there are no reports of lipases derived from the genus *Peniophora*, transcriptome analysis during the fermentation process of cow's milk by this fungus also confirmed that the lipase gene was remarkably increased based on the FPKM value (data not shown). In contrast, for low-fat milk, which showed better lactose consumption, the accumulation of YP may have decreased because of the enhanced decomposition of YP (Figure 3a,e). That is, the presence of milk fat may have suppressed the consumption of lactose and the degradation of YP. These results were similar to the results of the flask culture, as described

above (Figure 2a,b,e,f). To increase the productivity of YP, it is necessary to promote proteolysis, accompanied by the growth of mycelium. Nonetheless, suppressing the activity of the enzymes involved in YP degradation is also important. A possible solution is gene knockout; for example, the gene encoding Xaa-Pro dipeptidase in *Peniophora* sp. can be knocked out using CRISPR/Cas9. Moreover, transcriptome analysis showed that the expression of lipase tended to increase during the fermentation process (data not shown). Thus, further examinations are required to analyze the free fatty acids in fermented milk and to investigate the relationships of milk fat with lipolytic activity, lactose consumption, and protein degradation.

The measurement results of the ACE-inhibitory activity of the sample that was diluted 500-fold are shown in Figure 3d,h. The YP production was higher in cow's milk, as described previously herein, but the difference in ACE-inhibitory activity between the two was approximately 14%, which was not as remarkable as the difference in the amount of YP released. This suggests that other peptides with ACE-inhibitory activity were produced in addition to YP. It is highly possible that many dipeptides other than YP have an Xaa-Pro sequence. Supporting this, Phe-Pro (FP) and Val-Pro (VP) were detected in the fermented milk. Similar to YP, these dipeptides were reported to show ACE-inhibitory activity [32]. We plan to isolate ACE-inhibitory peptides with an Xaa-Pro sequence in fermented milk and investigate the blood pressure-lowering effect of milk fermented by the *Peniophora* sp. YM5314 strain.

There were many studies on the production of biomass, enzymes, and secondary metabolites by fungi using jar fermentors. Most of them are for yeasts and molds, while there are few studies on basidiomycetes [33–36]. Moreover, no reports have described milk fermentation by basidiomycetes and the production of casein-derived peptides using jar fermentors. The results of this study exhibit a possibility for the efficient production of functional peptides using a basidiomycete. Additionally, the culture period may be further shortened via analysis of the dissolved oxygen content, culture temperatures, and optimal starter conditions.

YP is an important constituent peptide of endogenous opioid peptides, hormones, and neuropeptides with analgesic activity. However, the detailed functions of YP have not been fully elucidated. Dipeptide YP has an important sequence that is found within the endogenous opioid peptide, which plays important roles in biological regulation [37,38]. Recently, YP was shown to penetrate the blood–brain barrier and accumulate around various regions of the brain, including the hippocampus, hypothalamus, and cerebellum [39]. As described above, dipeptide YP, which has a simple sequence, has attracted much attention as a bioactive, functional peptide in the living body. As an alternative to conventional methods, fermentation with mushrooms was suggested as a technique for the specific production of YP. We expect that more efficient fermentation production will be possible via further optimization of the growth conditions.

4. Conclusions

This is the first report to show the ability of the white-rot fungus *Peniophora* sp. to grow well in milk without additional nutrients and efficiently release the target antihypertensive peptide YP from milk in a jar fermentor. This study revealed a new way to produce YP from milk that harnesses the potential of this white-rot fungus. Further investigations to optimize the fermentation conditions (e.g., temperature, pH, aeration, agitation speed, and nutrient contents) are expected to improve the peptide productivity.

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