



Production of an antihypertensive peptide from milk by the brown rot fungus *Neolentinus lepideus*

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Abstract

Fermented milk has been shown to have beneficial effects on human health exerted through its bioactive peptides. The lactose-fermenting brown rot fungus *Neolentinus lepideus* has been shown to ferment milk; however, its ability to produce bioactive peptides has not been explored. This study, therefore, aimed to investigate the ability *N. lepideus* for the production of antihypertensive peptides in fermented milk and to characterize the peptides. Here we have used 9% skim milk as the substrate and fermented it with the mycelia of *N. lepideus*. The fermented milk exhibited higher angiotensin I-converting enzyme (ACE) inhibitory activity as compared to the commercially available sour milk containing Ile-Pro-Pro and Val-Pro-Pro. As a result of isolation and purification by reverse-phase high-performance liquid chromatography, followed by peptide sequencing and MS analysis, the dipeptide Tyr-Pro (YP) was identified as an active peptide. The concentration of YP in the fermented milk reached up to 450 µg/ml, which was > twofold higher than the IC₅₀ for ACE inhibition. Subsequently, the antihypertensive effect of YP was validated through intravenous injection of the peptide, which exhibited a significant lowering of blood pressure in a rat model of genetic hypertension. In addition, the transcriptomic analysis revealed the probable role of the carboxypeptidases for the increased yield of YP. These results together indicated that the fermentation with basidiomycete fungus could be a viable approach to produce bioactive peptides, and may, therefore, be applicable in the development of a cost-effective mass production system for functional peptides.

Keywords Angiotensin I-converting enzyme · ACE inhibition · Peptide · Milk fermentation · Brown rot fungus · Antihypertensive effect

Abbreviations

ACE	Angiotensin I-converting enzyme
RP-HPLC	Reverse-phase high-performance liquid chromatography
HPLC	High-performance liquid chromatography
ESI-MS	Electrospray ionization mass spectrometry
YP	Dipeptide Tyr-Pro
RAAS	Renin-angiotensin aldosterone system
TFA	Trifluoroacetic acid
IPP	Ile-Pro-Pro
VPP	Val-Pro-Pro

FPKM	Fragments per kilobase of transcript per million mapped reads
SHR	Spontaneously hypertensive rat
SHRSP	Stroke-prone spontaneously hypertensive rat
MAP	Mean arterial pressure

Introduction

Hypertension, also known as high blood pressure, is defined by systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 80 mmHg. It is associated with a high risk of serious diseases such as arteriosclerosis, cardiovascular disease, myocardial infarction, stroke, and renal failure [1]; it also raises the risk of developing cerebrovascular dementia [2]. Thus, hypertension, as a silent killer, is one of the most prevalent risk factors for premature death worldwide. According to a report from the World Health Organization, 9.4 million people die from complications of hypertension, such as myocardial infarction, cerebral stroke, and renal

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failure, every year [3]. Therefore, reducing the incidence of hypertension worldwide is imperative.

The renin–angiotensin–aldosterone system (RAAS) is one of the most important systems regulating blood pressure [4, 5], and several antihypertensive drugs have been developed to target the angiotensin I-converting enzyme (ACE, EC 3.4.15.1), a key enzyme in RAAS that catalyzes the conversion of angiotensin I to angiotensin II and causes blood vessel contraction [6, 7]. Although these synthetic inhibitors have shown an excellent antihypertensive effect, the use of such synthetic drugs to prevent or treat hypertension is not preferable owing to the related cost and side effects [8, 9].

In this context, several studies have been performed to identify nutrients with antihypertensive effects in regular foods [10], of which the studies evaluating the peptides with ACE inhibitory activity, have attracted much attention considering that they may provide a safe and inexpensive strategy to control blood pressure by inhibiting RAAS through regular meals [11]. Such studies attempted to produce functional peptides by *in vitro* enzymatic hydrolysis of, or fermentation of, protein-rich foods, such as fish, milk, peanuts, and soybean [12–27]. Although some of these studies have achieved commercial success by the development of foods with health-promoting benefits, these methods, in general, are limited in terms of cost-effectiveness, specifically, relatively low recovery of target peptides.

In our previous study, we had identified a basidiomycete fungus *Neolentinus lepideus* that harbored the unique ability to produce ethanol from whey or milk [28]. The study has also reported that milk protein was decomposed during the fermentation, besides the production of ethanol. We thus hypothesized that this basidiomycete fungus might be able to produce bioactive peptides from milk.

Therefore, the current study aimed to investigate the ability of *N. lepideus* to produce bioactive peptides in fermented milk and to evaluate the peptides quantitatively and qualitatively. In addition, we examined the effect of fermented milk on the ACE inhibitory activity to explore its possible physiological functions.

Materials and methods

Fungal strain and culture conditions

The *N. lepideus* strain YP6096, used in this study, was isolated from fruiting bodies on decaying wood in Totтори Prefecture, Japan. The strain, YP6096, revealed the best casein degrading ability among the wild strains of *N. lepideus* collected by us (data not shown). The fungus was initially grown on an MYG agar plate (consisting of 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. Three 0.5 cm² pieces of

the mycelial mat were then inoculated in 500 ml Erlenmeyer flasks containing 50 ml of MYG liquid medium, and capped with silicone plugs (Shin-Etsu Polymer Co., Ltd., Tokyo, Japan) for mycelial growth; the flasks were incubated for 7 days at 28 °C without shaking. The mycelia were then inoculated into a 500 ml Erlenmeyer flask containing 50 ml of 9% (w/w) skim milk without any nutrient supplementation and sealed with a silicone rubber plug (AS ONE Co., Ltd., Osaka, Japan) for fermentation. The cultures were incubated at 28 °C without shaking. The fermented milk was then centrifuged for 15 min at 15,000 rpm at 4 °C in an Eppendorf bench-top centrifuge, and the supernatant was filtered through a 0.22-μm membrane filter (Millex-GP; Millipore Corp., Billerica, MA) to obtain a stock solution for further analysis.

Amino acid analysis

The free amino acid content in the fermented milk was measured by an automatic amino acid analyzer (JLC-500/V2; JEOL Ltd., Tokyo, Japan). The release rate of each amino acids in the supernatant of the fermented milk was calculated using the amino acid content in 9% skim milk as reported earlier [29], and the release rate was calculated as follows;

$$\text{Release rate (\%)} = (\text{Free amino acid content in the fermented milk} / \text{Amino acid content in 9\% skim milk}) \times 100.$$

Determination of ACE inhibitory activity

The ACE inhibitory activity of samples was determined using an ACE Kit-WST (Dojindo, Japan), according to manufacturer's instructions, modified based on the method described by Cushman and Cheung [30]. 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 *N. lepideus* fermented milk was measured by diluting the fermented milk 100-fold with sterile distilled water.

Comparison and evaluation of ACE inhibitory activities

We compared the ACE inhibitory activities of the *N. lepideus* fermented milk and the commercially available sour milk (Ameal S™; Calpis Co., Ltd., Tokyo, Japan) containing the potent ACE inhibitory peptides Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP), at a total concentration of 17 μg/ml. The fermented milk by *N. lepideus* and the sour milk were aseptically collected, centrifuged, and the supernatant was filtered in the same way as described earlier (in “Fungal strain and

[culture conditions](#)” section), which was subsequently diluted for comparative analysis of their ACE inhibitory activities.

Separation and purification of peptides released in fermented milk

At periodic intervals of 2 days starting from day 0 till day 8 followed by an interval of six and seven days till day 21, 1 ml of the sample was aseptically collected, centrifuged, and filtered in the same way as described earlier (in [“Fungal strain and culture conditions”](#) section). Then the filtrate was used for separation of the peptides by reverse-phase high-performance liquid chromatography (RP-HPLC) using an HPLC system (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a UV/VIS detector, SPD-20A, and reverse-phase column Cadenza CD-C18 (4.6 mm × 150 mm; Imtakt Corp., Kyoto, Japan). The analysis was performed 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 volume injected into the column was 10 µl, and the flow rate was 0.6 ml/min. The linear concentration gradient settings were as follows: 0–22% solvent B for 50 min. The UV absorbance of the eluent was monitored by recording the absorbance at 215 nm, and the fractions were collected for the estimation of ACE inhibitory activities. The fraction with the highest ACE inhibitory activity was then separated, concentrated, freeze-dried, and re-dissolved in 0.1% TFA. The purification was repeated thrice with the same elution conditions.

Identification of amino acid sequences of the purified peptide

Amino acid sequences of the purified peptide were determined by a protein sequencer PPSQ-31A (Shimadzu Co., Ltd., Kyoto, Japan).

Mass spectrometry analysis of the purified peptide

MS analysis of the active peptide was performed in a positive ion mode through electrospray ionization mass spectrometry (ESI–MS) using a mass spectrometer (Exactive™; Thermo Fisher Scientific, Waltham, MA, USA).

Synthesis and quantification of the purified peptide

The peptides IPP, VPP, VY, and GY, reported to have the ACE inhibitory activities, were chemically synthesized (BEX Co., Ltd. Japan) for analyzing the antihypertensive properties. In addition, we also synthesized the purified peptide based on its amino acid sequence for quantification and performed a comparative analysis to confirm its

properties. All the peptides were synthesized using a solid-phase method. The purified peptide was quantified using a standard curve developed by inferring to the area and concentration of the synthesized one by RP-HPLC.

The antihypertensive effect of YP on stroke-prone spontaneously hypertensive rats

The acute effects of YP and other peptides on blood pressure were studied in a rat model of genetic hypertension, the stroke-prone spontaneously hypertensive rat (SHRSP). Rats were housed under conditions of 12/12 h light/dark cycle, at 25 ± 1 °C, and 60% humidity. Male rats at 12 weeks of age were anesthetized by intraperitoneal injection of pentobarbital (50.0 mg/kg BW). The femoral vein was cannulated and connected to an auto-injector (ESP-32; Eicom, Kyoto, Japan) for continuous infusion of propofol to control anesthesia throughout the experiment (30.0 mg/kg/h). After that, the femoral artery was cannulated and connected to a pre-calibrated transducer (PowerLab 8/30; ADInstruments, NSW, Australia) to monitor intra-arterial blood pressure directly. The trachea was intubated for artificial respiration. Each peptide (1 mg/kg BW) was administered by a single intravenous injection through the indwelling venous catheter. Changes in blood pressure were monitored continuously, and the maximal change from baseline was considered for analysis.

RNA extraction, library preparation, and sequencing

Total RNA was extracted from freshly frozen mycelia using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The quality and concentration of RNA were validated using a BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan). Total RNA integrity was checked using the Agilent 2100 bioanalyzer system (Agilent Technologies, Palo Alto, CA, USA), and the samples with an RNA Integrity Number of ≥ 7.0 were used for paired-end sequencing. The mRNA sequencing was performed on the Illumina HiSeq platform. Library construction and sequencing were performed by Macrogen (Seoul, Korea). To obtain quantification scores between the two samples, fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated using Cufflinks, which correct for transcript length and the total number of mapped reads from the library to compensate for different read depths in different samples. Annotation of the raw data and differences in gene expression levels were analyzed by Maze Inc. (Tokyo, Japan).

Statistical analysis

Data were expressed as mean \pm standard deviation (SD) of 3–5 independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Student's *t* test for comparison of two means or Dunnett's post hoc test for multiple comparisons. The *p* value of <0.05 was considered significant.

Results

The amino acid content of milk fermented by *Neolentinus lepideus*

Analysis of the amino acid content of the supernatant of milk fermented by *N. lepideus* for 8 days revealed the release of 17 of the 20 amino acids suspected to be derived from casein (Table 1). The release rates of 7 of the 9 essential amino acids except for Met (13.25%) and His (40.02%) were $>50\%$, indicating that the fermented milk has a relatively high content of essential amino acids.

ACE inhibitory activity of the fermented milk

The ACE inhibitory activity of the *N. lepideus* fermented milk was observed to be increased daily, with approximately

70% or more on the fourth day of fermentation, and approximately 80% on the sixth day or later (Fig. 1).

Comparison of ACE inhibitory activity between the milk fermented by *N. lepideus* and the commercially available sour milk

The milk fermented by *N. lepideus* and sour milk containing the antihypertensive tri-peptides (IPP and VPP) was diluted 100-fold, 500-fold, and 1000-fold, each to prepare the test samples. The ACE inhibitory activity of the fermented milk was significantly higher (\sim twofold) than that of sour milk ($p < 0.05$, Student's *t* test) in all the three dilutions (Fig. 2).

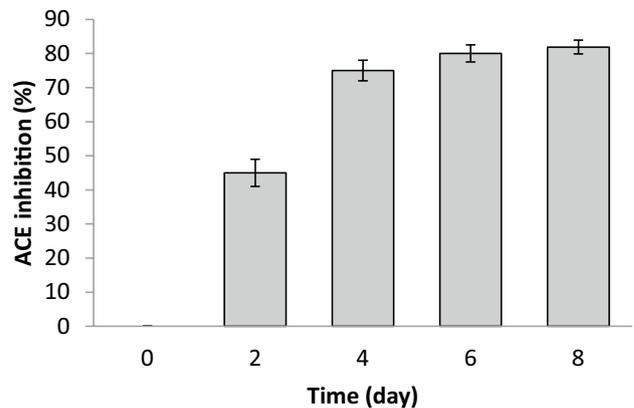


Fig. 1 The ACE inhibitory activity of the milk fermented by *N. lepideus* at different time intervals (days). All data are expressed as mean \pm SD, $n=5$

Table 1 The content of free amino acids released in fermented milk by *N. lepideus* and their release rate

Amino acid	Amount released into supernatant (mg)	Content of 9% skim milk per 50 ml (mg) ^a	Release rate (%) ^b
Ala	36.21 \pm 1.83	51.30	70.58 \pm 3.57
Gly	15.09 \pm 3.05	30.60	49.31 \pm 9.97
Ser	42.45 \pm 5.41	81.45	52.12 \pm 6.64
Thr	41.27 \pm 1.79	67.50	61.14 \pm 2.65
Met	4.95 \pm 2.86	37.35	13.25 \pm 7.66
Val	64.89 \pm 5.39	102.15	63.52 \pm 5.28
Leu	118.78 \pm 3.48	156.15	76.07 \pm 2.23
Ile	51.78 \pm 3.84	81.00	63.93 \pm 4.74
Tyr	35.97 \pm 1.51	72.45	49.65 \pm 2.08
Phe	53.16 \pm 1.50	76.50	69.49 \pm 1.96
Trp	15.67 \pm 2.35	24.30	64.49 \pm 9.67
Asp	23.97 \pm 4.36	120.60	19.88 \pm 3.62
Glu	83.06 \pm 7.91	331.65	25.04 \pm 2.39
Arg	36.49 \pm 1.34	54.00	67.57 \pm 2.48
Lys	68.16 \pm 5.58	130.50	52.23 \pm 4.28
His	19.27 \pm 1.64	48.15	40.02 \pm 3.41
Pro	45.01 \pm 4.47	149.85	30.04 \pm 2.98

^aCalculated based on previously reported data [29]

^b(Free amount/original amount) \times 100

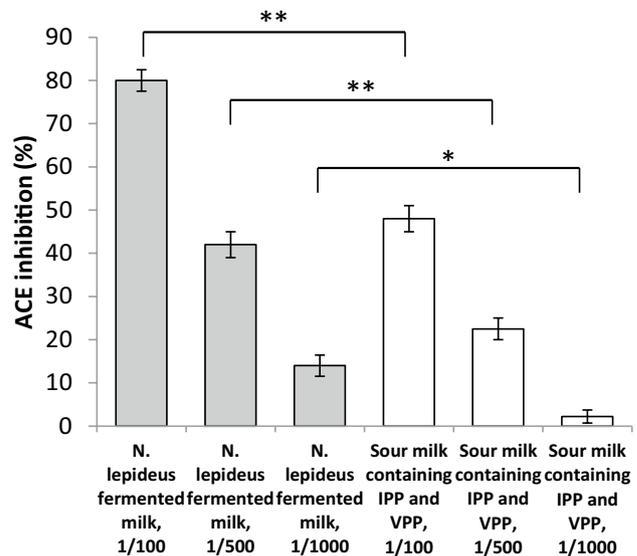


Fig. 2 Comparison of the ACE inhibitory activity of the *N. lepideus* fermented milk and the commercially available sour milk. All data are expressed as mean \pm SD, $n=3$. * $p < 0.05$, ** $p < 0.01$

Isolation and purification of the fraction with maximum ACE inhibitory activity

Next, we separated the peptides by RP-HPLC. We focused on peaks that increased remarkably following the progression of fermentation, and the ACE inhibitory activity was measured after fractionation. Among 12 fractions collected, the fraction 9 displayed the highest ACE inhibitory activity (Fig. 3) and purified (Fig. 4a) for further analysis.

Identification of the peptide from fraction 9

The amino acid sequencing of the purified peptide in the fraction 9 (Fig. 4a) identified the dipeptide Tyr-Pro (YP) and the retention time of YP (31 min) in fermented milk was confirmed to be consistent with that of synthetic YP, by RP-HPLC analysis (Fig. 4a, b). Further, the ESI-MS

analysis showed the signal m/z 279.1337 $[M+H]^+$, corresponding to one proton added to the molecular weight of 278.31 of YP (Fig. 5). Taken together, these results confirmed that the main component showing ACE inhibitory activity in the *N. lepideus*-fermented milk was YP.

The yield of YP by *N. lepideus* over time

Fermentation was performed using *N. lepideus* in a liquid containing 9% skim milk, and the sample was collected at different time intervals up to 21 days to measure the yield of YP, using RP-HPLC. The results are shown in Fig. 6. The concentration of YP in samples collected on day 2 was approximately 100 $\mu\text{g/ml}$, which reached a maximum of 450 $\mu\text{g/ml}$ on day 21.

Fig. 3 The RP-HPLC chromatogram of the peptides present in the *N. lepideus* fermented milk (a), and the ACE inhibitory activities of the identified fractions 1–12 (b). All data are expressed as mean \pm SD, $n = 3$

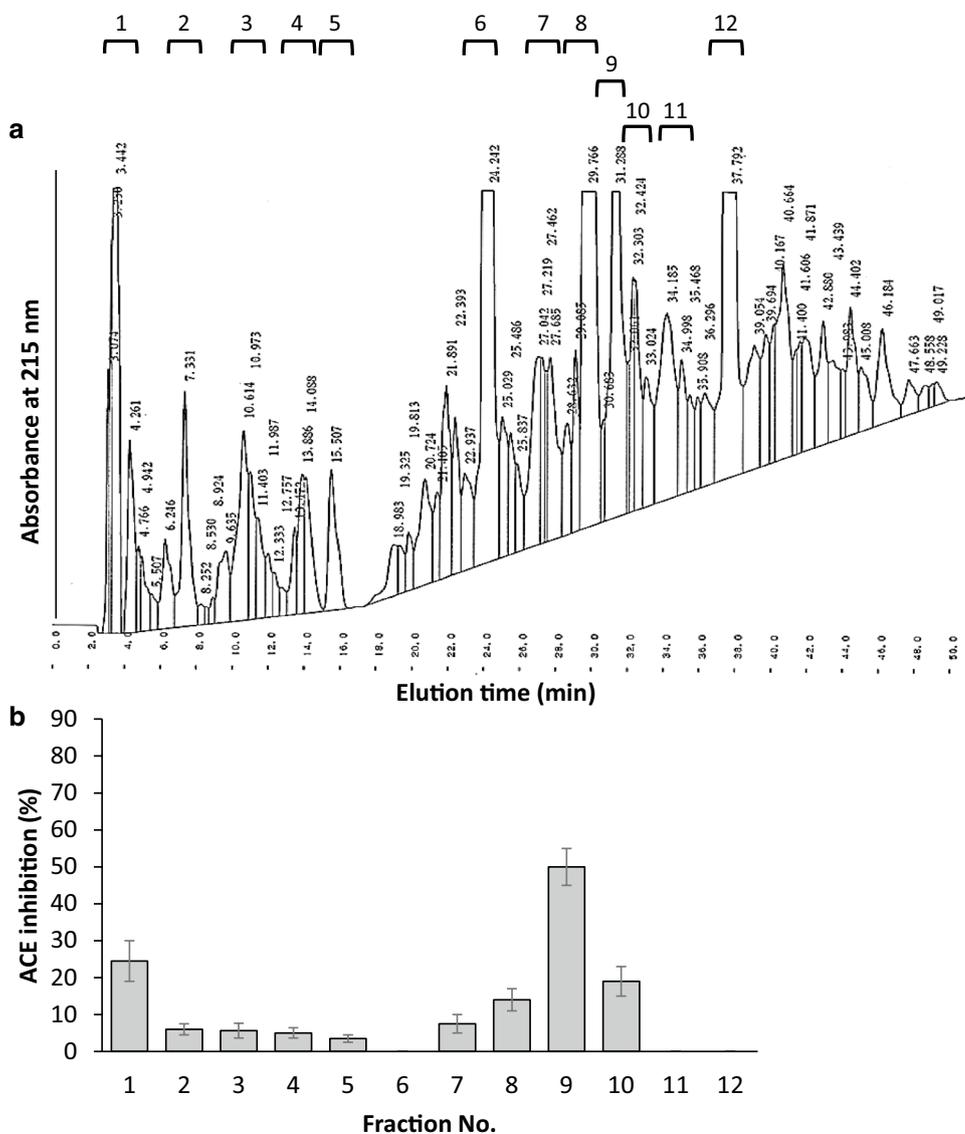
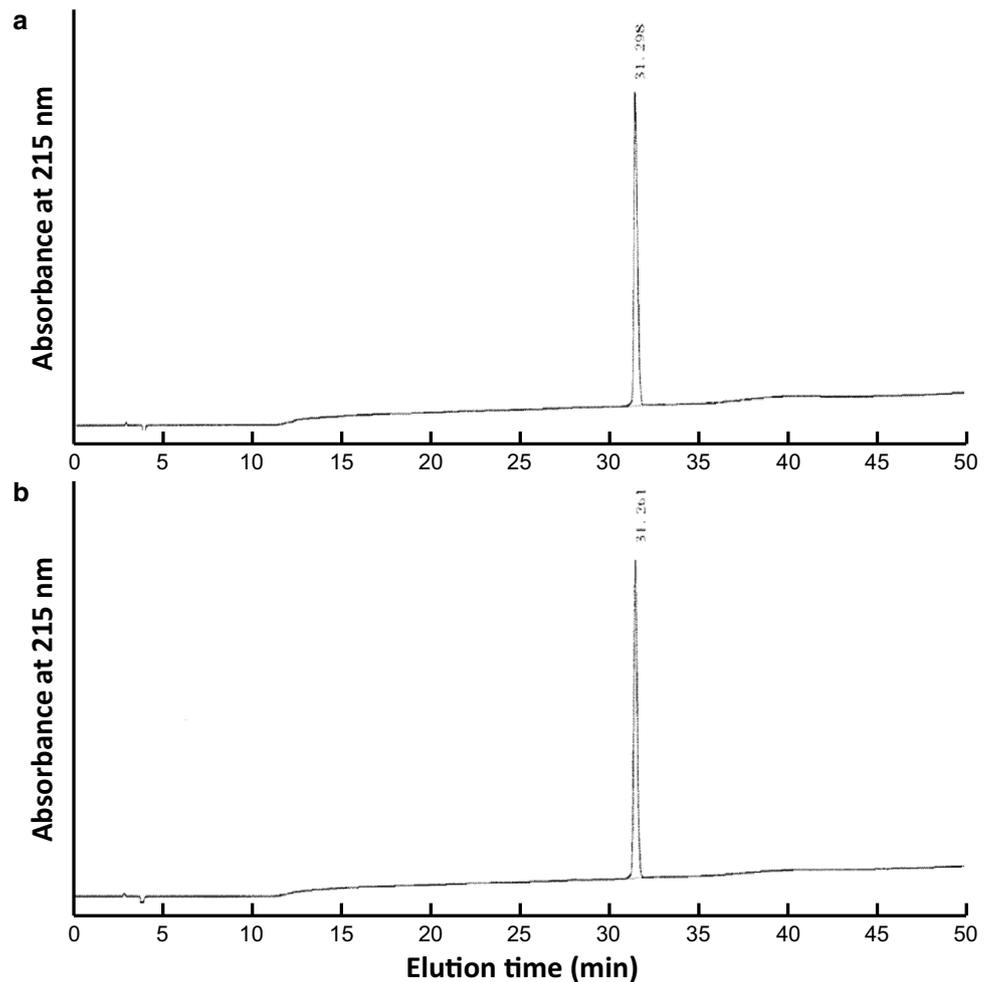


Fig. 4 RP-HPLC chromatogram of the purified fraction (fraction 9) with maximum ACE inhibitory activity (a) and the synthesized dipeptide, YP (b)



Intravenous injection of YP to SHRSP

Antihypertensive effects of YP and other peptides with ACE inhibitory activity, chemically synthesized as acetate salts, were investigated in vivo. As shown in Fig. 7, YP and IPP had a strong antihypertensive effect while VPP, VY, and GY were relatively weak. Blood pressure returned to the initial level after about 1 hour of injection. In Dunnett's test, YP was significantly different from VPP, VY, GY ($p < 0.001$). However, no significant difference was observed between YP and IPP ($p > 0.05$).

Transcriptome analysis

Transcriptome analysis was performed to identify the proteolytic enzymes involved in YP production that is expressed during the fermentation process. A total of 22 proteolytic enzymes, including endopeptidases, aminopeptidases, carboxypeptidases, dipeptidases, dipeptidyl peptidases, and tripeptidyl peptidases were identified to be expressed with

at least 10-times higher FPKM after 2 days of fermentation than at the beginning of fermentation (Table 2).

Discussion

Among the various kinds of mushrooms, the brown rot fungus, *N. lepideus*, is not only capable of assimilating carbon sources in woods but can also ferment dairy products, despite not requiring such materials as nutrients for their survival [28]. This fungus could not only grow in milk, but also decomposed casein, a major component of milk proteins. In this study, we newly discovered that, in addition to amino acids, this fungus produced physiologically active peptides during the fermentation of milk.

Seventeen amino acids were found abundantly in the fungus-fermented milk (Table 1). In particular, arginine and tryptophan, which are not so rich in lactic acid bacteria fermented milk, constitute a unique content of this fungus-fermented milk. Various beneficial effects of the amino acids in the body, such as reducing anxiety and promoting

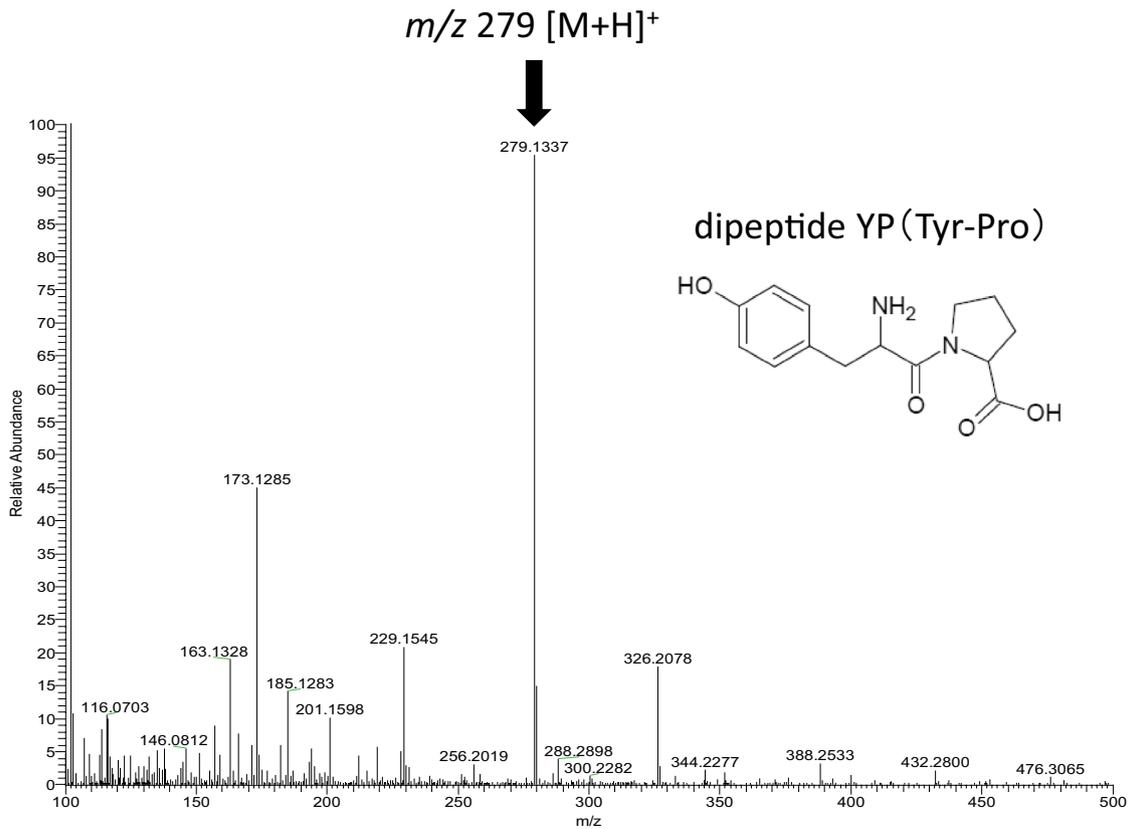


Fig. 5 The spectra obtained through ESI-MS of YP (demarcated by the black arrow) obtained from the *N. lepideus* fermented milk

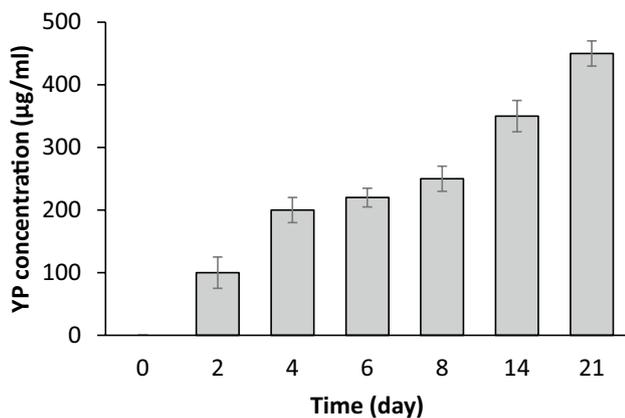


Fig. 6 The yield of YP at different time intervals (days) of the fermentation of the milk with *N. lepideus*. Data are mean ± SD, $n=3$

recovery of muscle damage after exercise [31], the fungus-fermented milk could be potentially valuable as a health-promoting food.

In addition to the production of amino acids, the release of peptides with ACE inhibitory activity was remarkable in the fermented milk. As fermentation progressed, ACE inhibitory activity of the milk increased over time (Fig. 1); the

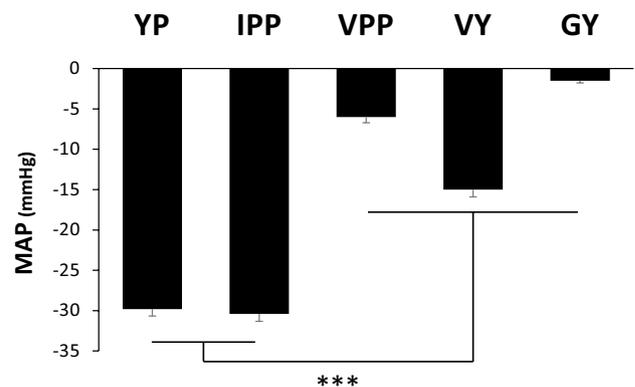


Fig. 7 The antihypertensive effect of the YP, comparative analysis as obtained by the intravenous injection of YP and other ACE inhibitory peptides to SHRSP. All data are expressed as mean ± SD, $n=4-5$. *** $p < 0.001$

fungus-fermented milk showed a more potent ACE inhibitory effect than lactic acid bacteria-fermented milk (Fig. 2).

Among the various ACE inhibitory peptides found in lactic acid bacteria-fermented milk [20–23], IPP and VPP have been reported to have a strong antihypertensive effect [15–18]. In this report, the fungus-fermented milk was

Table 2 The putative proteolytic enzymes up-regulated during fermentation of milk by *N. lepidus*

Contig length (bp)	Proteolytic enzymes	FPKM	
		0 day	2 days
1951	Acid protease	32.78	450.82
2857	Metalloprotease	0	4.1
5020	Zn-dependent exopeptidase	0	0.15
937	Aspartic-type endopeptidase (CtsD)	0	3.83
283	Aspartic peptidase A1	0	2.46
3262	Aminopeptidase C	1.01	10.42
2084	Aminopeptidase P, Xaa-Pro aminopeptidase	0.01	1.48
1730	Peptidase M22	0	1.84
2033	Peptidase M24	0	1.62
2641	Peptidase family M28	0	1.94
1492	Leucine aminopeptidase 1	0	1.88
3261	Methionine aminopeptidase type 1	0.04	1.82
1529	Proline iminopeptidase, prolyl aminopeptidase	0.16	4.36
430	Prolidase pepP, Xaa-Pro dipeptidase	0	8.13
785	Xaa-Pro dipeptidyl aminopeptidase B (DapB)	0	2.19
3209	Dipeptidyl peptidase III	0.44	4.65
343	Tripeptidyl-peptidase sed3	0	1.1
3476	Carboxypeptidase S	0	2.78
2013	Carboxypeptidase Y	0	3.99
1610	Serine carboxypeptidase S10	0.02	1.4
2093	Serine carboxypeptidase S28	0	4.78
4016	Family S53 protease	3.24	33.43

clearly shown to have a stronger ACE inhibitory activity than the commercially available sour milk rich in IPP and VPP, although the fungus-fermented milk did not contain these tripeptides (data not shown). We identified that the peptide playing a central role in the fungus-fermented milk was a dipeptide, YP. As shown in Table 3, many different

peptides with the YP sequence were reported to be produced in fermentation [19, 32–38]; however, *L. helveticus* was reported to be the only one that could release YP from casein per se [19].

As the IC₅₀ of YP for ACE inhibition was reported to be 720 μM [19], which is equivalent to 200 μg/ml, the fermented milk must contain such a high concentration of YP to elicit ACE inhibition. In the present study, we observed that the concentration of YP in milk gradually increased during the fermentation process, and finally reached as high as 450 μg/ml (Fig. 6), twice as high as that required to achieve the IC₅₀, which confirmed the ACE inhibitory activity of YP. The YP content in 9% skim milk has been estimated to be about 640 μg/ml, whereas it was 450 μg/ml (70.3%) in *N. lepidus* fermented milk, which indicated a comparatively high efficiency of the fungus to release most of the YP residues from skim milk. This is the advantage of fermentation using *N. lepidus* since lactic acid bacteria produced YP at a maximum concentration of 10 μg/ml [19]. This difference might be due to the poor sensitivity of fungi to acidification of the medium during their growth. In contrast, lactic acid bacteria are known to stop their growth and peptide production in the acidified medium due to the accumulation of lactic acid and feedback inhibition of peptidase by branched-chain amino acids [39, 40]. Considering the high ACE inhibitory activity of the fungus-fermented milk, we may, in the future, need to examine the possibility of co-production of other peptides with potent ACE inhibitory activity.

An antihypertensive effect of YP was confirmed in vivo using a rat model of genetic hypertension. Although the ACE inhibitory activity of YP in vitro, was comparatively lower than other di- and tripeptides with antihypertensive effects (IC₅₀ was 720 μM, 5 μM, 9 μM, 22 μM, and 210 μM for YP, IPP, VPP, VY, and GY, respectively) [14, 19, 41], YP showed the largest reduction of blood pressure; however, was comparable to the antihypertensive effect of IPP (Fig. 7). This discrepancy between the ACE inhibitory activity in vitro and antihypertensive effect in vivo implied that

Table 3 List of ACE inhibitory peptides with YP sequence isolated from various materials and their IC₅₀ values

Sequence	Preparation	IC ₅₀ (μM)	References
YP	Fermentation (lactic acid bacteria)	720	[19]
IYPRY	Sake lees	4.1	[32]
YPPFGPI	Brie cheese	–	[33]
YPPFGPIP	Cheddar cheese	–	[34]
YPPFGPIP	Gouda cheese	14.8	[35]
MPFPKYPVQF		–	
AVPYPQR	Fermentation (lactic acid bacteria)	274	[36]
DAYPSGAW		98	
LAYFYF		65	
VYP	Enzymatic	288	[37]
VYPPFG		221	
YPPFGPI	Enzymatic	500	[38]

the antihypertensive effect of the peptides, observed in vivo, may not be via inhibition of ACE. This is consistent with the result of the oral administration test of YP to SHR by Yamamoto et al. [19]. Additional studies would be required to understand the antihypertensive mechanisms of YP in vivo.

Many genes encoding putative proteolytic enzymes, active in casein decomposition, were expressed and up-regulated even in an early stage of fermentation with *N. lepidus* (Table 2). In addition to endopeptidase, this fungus seemed to have a balanced expression of aminopeptidase, XPDAP, and carboxypeptidase, indicating that YP might be specifically produced. Previously, it was reported that the exopeptidase treatment of whey protein had a positive effect on its ACE inhibitory activity and antihypertensive action [42]. In particular, the transcriptomic data showing higher FPKM for carboxypeptidases indicated that *N. lepidus* expressed the gene(s) encoding carboxypeptidase, which is not found in lactic acid bacteria [43–45] could be associated with higher ACE inhibitory activity and higher YP production (30–40 times) than lactic acid bacteria. Furthermore, many genes, found in *N. lepidus*, had a low homology (around 40%) with *Aspergillus* fungi (to be reported elsewhere), thus suggesting a possibility that *N. lepidus* could be unique in terms of YP production, even amongst fungi. Through transcriptome analysis, we further found that prolidase, an enzyme degrading the bond between Y and P, was expressed in *N. lepidus*. This suggested that we could recover more YP through ‘knockout’ of the prolidase gene in *N. lepidus*. This possibility would be worth exploring in a future study.

Conclusion

This is the first report that identifies the brown rot fungus, *N. lepidus*, as a promising microorganism for the production of the antihypertensive peptide, YP, also having ACE inhibitory activity from milk. However, further studies are warranted to verify the antihypertensive effect of fermented milk, containing YP, on SHR or SHRSP, and to improve the productivity of peptides by this fungus. Production of peptides from milk by the naturally occurring basidiomycete fungus would be preferable not only for peptide production, but also for its cost-effectiveness, and lower environmental footprint.

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Author contributions KO conceptualized and designed the study, performed the experiments, drafted the initial manuscript, reviewed the manuscript, and revised the manuscript. SK and MT performed the

experiments and analyzed the data. TM contributed to the amino acid analysis, peptide sequencing, and MS analysis. HMZ performed the animal experiments. TN designed animal experiments and reviewed the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirements The procedures of animal experiments were reviewed and approved by the local committee for animal research at Shimane University. The animal experiments were performed in accordance with relevant guidelines and regulations.

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