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Molecular characterization of a novel aspartyl aminopeptidase that contributes to the increase in glutamic acid content in chicken meat during cooking

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ABSTRACT

The enzyme involved in the increase in glutamic acid content in chicken meat during cooking was identified and characterized. Chicken homogenate produced significantly more free glutamic acid and exhibited higher glutamyl *p*-nitroanilide (Glu-*p*NA) hydrolyzing activity than beef when heat cooked. Amino acid sequencing revealed the presence of aspartyl aminopeptidase (DNPEP) in chicken meat. Using RT-PCR, *DNPEP* gene expression was detected in chicken breast and thigh muscles, liver, and small intestine, together with various other peptidase genes. Full-length DNPEP cDNA was cloned, and recombinant chicken DNPEP (cDNPEP) was expressed in *Escherichia coli*. cDNPEP showed five-fold higher activity against Glu-*p*NA than against aspartyl-*p*NA, which represents a different substrate specificity than observed for recombinant bovine DNPEP (bDNPEP). The Km values of both DNPEPs with Glu *p*-NA substrates indicated a higher affinity of cDNPEP for glutamyl residues. This unique substrate specificity of cDNPEP contributes to efficient glutamic acid production in chickens.

1. Introduction

Chicken is a relatively inexpensive meat that is healthy because of its high protein and low fat content; therefore, its consumption has tended to increase worldwide (Park et al., 2020). The world's consumption of poultry meat is the highest among meats, reaching 134 million tons in 2019 (Food and Agriculture Organization of the United Nations, 2020).

Meat products are obtained from animal muscle through a postmortem aging that is essential for the conversion of muscle into meat (England et al., 2013). Muscle metabolites including amino acids and sugars, which are the precursors of aroma compounds of meat, are altered according to the postmortem aging process (Muroya et al., 2020). The feeding is important factor that affects chicken meat taste. It has been reported that reduction in dietary lysine increases free glutamic acid in chicken breast muscle by promoting protein degradation (Watanabe et al., 2020). Two major aminopeptidases, H and C, have been reported in chicken (Nishimura et al., 1991, 1994), and bovine (Rhyu et al., 1992) muscles. During the postmortem aging of beef, the increase in free amino acid content is caused by the action of aminopeptidase C and H (Iida et al., 2016).

Depending on the cooking conditions, a variety of tastes and aromas are developed via chemical or enzymatic hydrolysis (Rotola-Pukkila et al., 2015). When using the same part and breed of chicken, roasting produces higher acidic amino acid content than stewing (Wang et al., 2018). Sous-vide cooking is a method in which raw food is cooked at a precisely controlled temperature for a specific duration of time (Baldwin, 2012). As raw food materials retain a variety of proteolytic enzyme activities, the enzymes are expected to function during cooking (Oosone et al., 2020). It has been reported that the optimized conditions for sous-vide cooking improve the sensory quality characteristics of chicken breast meat (Park et al., 2020). As glutamic acid is one of the major contributors to the brothy taste of chicken (Nishimura et al., 1988), it would be significant to identify and characterize the enzymes involved in the release of glutamic acid from meat during cooking and processing. An aminopeptidase with a preference for N-terminal glutamyl residues was purified from chicken meat and was considered to be a glutamyl aminopeptidase based on its typical substrate specificity (Maehashi et al., 2003). However, some of its enzymatic properties were not consistent with those of the glutamyl aminopeptidase present in chicken egg white (Petrovic & Vitale, 1990).

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With the advancement of the analysis of the chicken genome and transcriptome (International Chicken Genome Sequencing Consortium, 2004; Caldwell et al., 2004; Hubbard et al., 2005; Burt, 2005), chicken gene information is available on public databases. In this study, we identified the enzyme involved in the increase in glutamate in chicken meat as aspartyl aminopeptidase (DNPEP) using the chicken gene information. We report the expression profiles of DNPEP and other peptidase genes in chicken muscle, and the characterization of chicken DNPEP in comparison with bovine DNPEP.

2. Materials and methods

2.1. Materials

Chicken breast tender meat (from Kyushu, Japan) and bovine thigh meat (from Australia) for preparing meat homogenates were purchased from a grocery store in Tokyo, Japan. To extract total RNA, chicken (*Gallus gallus domesticus*, Red Corniche × White Rock crossbred, 60-day-old female) tissues were provided by Amatake Co. Ltd (Iwate, Japan). Thigh and breast tender skeletal muscles, liver, and intestine were excised from the whole body immediately after butchering and stored in RNA*later*® (Ambion, Inc., Austin, TX) until use. The PCR Ready First-Strand cDNA of bovine normal skeletal muscle (one donor, 2-year-old female) was purchased from BioChain Institute Inc. (Newark, CA). DEAE-Sepharose Fast Flow and Sephacryl S-300 gels were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). Toyopearl HW-65F was purchased from Tosoh Co. (Tokyo, Japan). L-Glutamyl-*p*-nitroanilide (Glu-*p*NA·H_2O, Peptide Institute, Osaka, Japan), L-aspartyl-*p*NA (Asp-*p*NA·HCl, Bachem Japan K. K., Tokyo, Japan), L-leucyl *p*-NA (Leu-*p*NA, Peptide Institute), L-prolyl-*p*NA (Pro-*p*NA, Watanabe Chemical Industries, Ltd., Hiroshima, Japan), L-phenylalanyl-*p*NA (Phe-*p*NA, Watanabe Chemical Industries, Ltd), L-glutamyl-L-glutamic acid (Glu-Glu, Peptide Institute), L-aspartyl-L-glutamic acid (Asp-Glu, Sigma-Aldrich Japan, Tokyo, Japan), and angiotensin Ⅱ (Fujifilm Wako Pure Chemical Co., Osaka, Japan) were used as substrates for DNPEP.

2.2. Partial purification of Glu-pNA hydrolyzing enzyme from meat

Glu-*p*NA hydrolyzing enzyme was partially purified by the method described previously (Maehashi et al., 2003) with some modifications. Chicken breast meat (2 kg) was homogenized with 3 volumes of 10 mM Tris-HCl containing 1 mM $CaCl_2$ buffer (pH 7.5), and 6,340 ml of supernatant was recovered by centrifugation (11,400 × *g*, 30 min, 4 °C). Subsequently, 40% saturated ammonium sulfate fractionation was performed and the precipitate was collected by centrifugation. The precipitate was dissolved, dialyzed against the same buffer, and heat treated (60 °C, 20 min), and the insoluble matter was removed by centrifugation. The supernatant was separated on a DEAE-Sepharose CL-6B column (φ2.5 × 15 cm). The peak fraction with Glu-*p*NA hydrolysis obtained by a gradient elution with NaCl 0–0.1 M in the same buffer was concentrated, followed by separation on a Toyopearl HW65F column (φ1.5 × 95 cm).

2.3. Protein sequencing

To determine the partial amino acid sequences of sample proteins, in-gel digestion was performed according to the method of Cleveland et al. (1977) using *Staphylococcus aureus* V8 protease (Fujifilm Wako Pure Chemical Industries, Ltd.) as described previously (Maehashi et al., 2007). After electroblotting onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA), the separated band of a proteolytic fragment was excised from the membrane and subjected to amino (N)-terminal sequence (30 cycles) analysis using a gas-phase protein sequencer (PPSQ-21; Shimadzu, Kyoto, Japan). By using the first 20 amino acid sequences data were analyzed using NCBI's protein BLAST program.

2.4. Reverse transcription–polymerase chain reaction (RT − PCR)

For the preparation of cDNAs for chicken muscles, liver, and intestine, total RNA was extracted from the tissues using the RNeasy mini kit (QIAGEN KK, Tokyo, Japan), according to the manufacturer's instruction. Single-stranded cDNA synthesis was performed using a SuperScript III First-Strand Synthesis SuperMix kit and an oligo-dT (20) primer (Invitrogen Co., Carlsbad, CA, USA).

RT-PCR was conducted as described previously (Maehashi et al., 2012). For the tissue expression profiling of various peptidase mRNAs, the primer sets listed in supplementary Table S1 were designed based on the DNA sequences listed in supplementary Table S2. The primers and cDNAs of chicken thigh and breast tender muscles, small intestine, and liver were used for PCR amplification. PCR was performed using Ex Taq DNA polymerase (Takara Bio Inc. Shiga, Japan) and the following condition: 94 °C for 45 s, followed by 30 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min.

For the expression profiling of the DNPEP mRNA in chicken tissues, the primer set composed of DNPEP-10F/DNPEP-11R and chicken breast tender muscle, liver, and intestine cDNAs were used for RT-PCR analysis. PCR amplification was performed using Ex Taq DNA polymerase (Takara Bio Inc.) and the following conditions: 94 °C for 45 s, followed by 30 cycles at 94 °C for 45 s, 51–56 °C (annealing temperature) for 45 s, and 72 °C for 1 min. The annealing temperature depended on the primer set, as indicated in supplementary Table S1. To calibrate the cDNA concentration among samples, the β-actin gene was amplified as a housekeeping gene using the primer set of bact-11F/bact-12R or bact-11F/bact-14R, as determined using the same program. Amplicon sizes were confirmed by 1% agarose gel electrophoresis with ethidium bromide staining, followed by the sequencing of the amplicons.

2.5. Cloning of the chicken and bovine DNPEP cDNAs

The amplified chicken liver cDNA and purchased bovine skeletal muscle cDNA were used for subsequent PCR amplifications with Platinum *Pfx* DNA polymerase (Thermo Fisher Scientific K. K. Tokyo, Japan) and the following conditions: 94 °C for 2 min; followed by 27 cycles at 94 °C for 45 s, 60 °C for 45 s, and 68 °C for 2 min; and a final cycle of 68 °C for 10 min. The primers used for the amplification of the chicken and bovine *DNPEP* genes are listed in supplementary Table S3. The amplicons were cloned into pCR-blunt II-TOPO (Thermo Fisher Scientific K. K.) and then sequenced.

2.6. DNA sequencing

The nucleotide sequences of the DNAs cloned into pCR-blunt II-TOPO were analyzed by Macrogen Japan Co. (Tokyo, Japan). The T7 promoter primer and M13 reverse primer (Invitrogen Co.) were used as sequencing primers. The resultant nucleotide sequence data were assembled using the ATGC program (Genetyx Corp., Tokyo, Japan) and analyzed using NCBI's nucleotide BLAST program.

2.7. Expression and purification of recombinant DNPEP

The cloned chicken and bovine *DNPEP* genes were subcloned into the pET15b vector (Sigma-Aldrich Japan) using the *Nde* I and *Bam*H I sites and *Nde* I and *Xho* I sites, respectively, and subsequently introduced into *Escherichia coli* BL21(DE3)pLysS (Sigma-Aldrich Japan). The *E. coli* transformant was cultured to express recombinant DNPEP using the Overnight Expression Autoinduction System I® (Sigma-Aldrich Japan) at 37 °C for 17 h. The cells harvested by centrifugation were frozen overnight, thawed, and then disrupted by sonication. The

cell lysate was subjected to a Protino Ni-IDA resin (Macherey-Nagel GmbH, Düren, Germany) and DNPEP was eluted by an imidazole solution at pH8.0.

2.8. Western blot analysis

A purified recombinant DNPEP sample was diluted to 0.44, 0.55, 0.73, 1.1, and 2.2 μg on a protein basis, and a partially purified chicken meat sample was diluted to 19 μg on a protein basis. Subsequently, these samples were loaded and separated on SDS − PAGE and the bands were electroblotted onto a polyvinylidene fluoride (PVDF) membrane. Protein concentration was determined using the bicinchoninic acid (BCA) method using a Pierce® BCA protein assay kit (Thermo Scientific K. K), according to the manufacturer's instructions. A primary antibody against chicken DNPEP (anti-DNPEP antibody) obtained from rabbits immunized with a synthesized peptide with the sequence LYDNEEVGSESAQGA was purchased from Sigma-Aldrich Japan. A Vectastain ABC-AP Rabbit IgG kit (Vector Laboratories, Ltd., Burlingame, CA, USA) was used as a secondary antibody, and an alkaline phosphatase substrate kit (IV BCIP/NBT; Vector Laboratories, Ltd.) was used to detect the secondary antibody.

2.9. Assay of aminopeptidase activity

The determination of the aminopeptidase activity is based on the hydrolysis of the substrate, Glu-*p*NA, to *p*-nitroaniline and L-glutamic acid by DNPEP. The reaction was carried out at 50 °C within 30 min. The initial increase rate of the intensity of the color of *p*-nitroaniline was determined by measuring OD at 405 nm using a U-2910 spectrophotometer (Hitachi High-Tech Co., Tokyo, Japan). Aminopeptidase activity was expressed as units per mg of protein. One unit of enzyme activity is defined as the amount of 1 μmol of *p*-nitroanilide/min from the substrate. Total protein was determined using the BCA method.

2.10. Enzyme characterization

DNPEP activity was measured against several aminoacyl-*p*NA derivatives (Glu-, Asp-, Leu-, Arg-, and Pro-*p*NA), dipeptides (Glu-Glu and Asp-Glu), and angiotensin II, as substrates (2 mM), in 10 mM Tris-HCl buffer (pH 7.5), as a standard assay medium. The L-glutamic acid produced from dipeptide substrates was determined using the L-glutamate assay kit II (Yamasa Co., Chiba, Japan), whereas the L-aspartic acid produced from angiotensin II was determined by HPLC using an L-7000 series system with a ninhydrin reaction unit (Hitachi Hi-Tech Co., Tokyo, Japan). For the assessment of Glu-Glu hydrolyzing activity, half of the amount of glutamic acid produced was used for activity calculation. Enzyme activities are defined as the amount of 1 μmol of glutamate or aspartate per min from dipeptides or angiotensin II, respectively. The effect of potential inhibitors, activators, or metal salts was tested by incubating under the standard assay condition using Glu-*p*NA as the substrate.

The Michaelis-Menten constant (Km) for the hydrolysis of Glu-*p*NA and Asp-*p*NA was determined from Lineweaver − Burk plots with 10 mM Tris-HCl buffer (pH 7.5) at 50 °C.

The optimum temperature was measured using various temperatures (30–70 °C) in 10 mM Tris-HCl buffer (pH 7.5). The thermal stability of the purified recombinant DNPEP was determined by incubation of the enzyme in the same buffer at 30–90 °C for 20 min. Aliquots of the solution were collected to measure the remaining activity using a standard assay. Activity was expressed as a percentage against the activity recorded before incubation. The optimum pH was measured at 50 °C in reaction mixtures containing 100 mM GTA buffer system (3,3-dimethylglutaric acid, 2-amino-2-methyl-1,3-propanediol, and tris (hydroxymethyl) aminomethane) at pH 5.0–9.0. Relative activity was expressed as a percentage against the maximum activity.

2.11. Determination of glutamic acid content in meat homogenate

Chicken breast tender meat and beef thigh were finely chopped, and an equal volume of water was added; the meats were then homogenized using a blender. These homogenates were incubated with or without 1% (w/w) of the meat improver "Miola" (Ohtsuka Chemical Industrial Co. Ltd., Saitama, Japan) at 55 °C for 16 h, followed by boiling for 15 min; the glutamic acid content in the supernatant obtained by centrifugation was then measured using the L-glutamate assay kit II (Yamasa, Co.), according to the manufacturer's manual.

3. Results and discussion

3.1. Glutamic acid release in chicken and beef homogenates during incubation

The amount of glutamic acid in chicken and beef homogenates after incubation at 55 °C for 16 h is shown in Fig. 1Aa. The free glutamic acid concentration increased in both beef and chicken homogenates, from 0.14 to 0.23 mg/mL in beef homogenate and from 0.12 to 0.36 mg/mL in chicken homogenate. After the addition of the meat improver, glutamic acid in the beef homogenate increased from 0.11 to 0.53 mg/mL, while in the chicken homogenate it increased more significantly, from 0.13 to 3.11 mg/mL, as shown in Fig. 1Ab. Glutamic acid is released from muscle proteins by the action of peptidases on peptides produced by the activity of proteases. The difference in the increases in chicken and beef in Fig. 1Aa was considered to be due to the difference in protease or peptidase activity. On the other hand, as the shown in Fig. 1Ab, the addition of the meat improver as a protease agent caused a remarkably high increase in glutamic acid in chicken, which suggests the presence of a peptidase that can highly release glutamic acid in chicken meat. In our previous study, we presumed that bromelain efficiently produced glutamyl peptides from chicken proteins (Maehashi et al., 1999). Koide et al. (2010) reported that treatment of chicken meat with bromelain enhanced the free glutamic acid content. The main component of the meat improver used in this study was papain, which is the same cysteine protease as bromelain. Therefore, if papain acts similarly to bromelain, it is possible that the aminopeptidase with specificity toward glutamyl residues in chicken efficiently acts on the glutamyl peptides produced by papain in the meat improver.

The glutamic-acid-releasing activity of the extracts prepared from chicken and beef using the respective buffers at pH 5 to pH 9 was measured. The stability of the glutamic-acid-releasing enzyme is affected by the pH value at the preparation of the meat extract; moreover, meat chicken extract exhibited a significantly higher glutamic acid-releasing activity compared with meat beef extract at any pH. In particular, in meat chicken extract, this activity was highest at pH 7–7.5. (Fig. 1B). Nishimura et al. (1990) also showed that the aminopeptidase activity of chicken muscle extract against glutamyl substrate was much higher than that of bovine muscle extract. The pH values of meat chicken and meat beef extracts are around 6 (Kadıoğlu et al., 2019) and 5 to near 6 (Feng et al., 2020), respectively. At pH 6, meat chicken extract showed much higher glutamic acid-releasing activity than meat beef extract. Blanchard & Mantle (1996) reported that the highest levels of activity for proteases and aminopeptidases were observed in chicken thigh and breast muscles compared with those of lamb, pig, and rabbit muscles. At pH 5, glutamic acid-releasing activity was not detected in meat beef extract, but was detected in meat chicken extract, suggesting that the glutamic-acid-releasing enzyme in meat chicken extract could act to release glutamic acid, even under general cooking conditions.

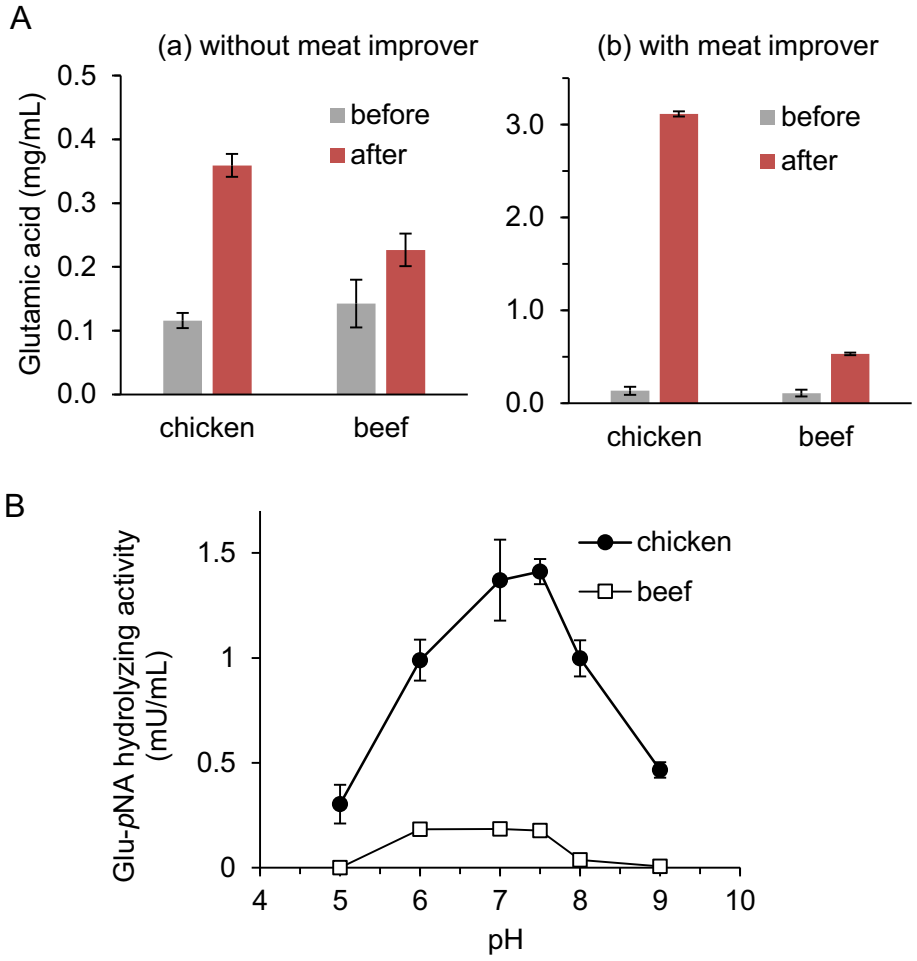

Fig. 1. Glutamic acid release in chicken and beef homogenates. (A) Glutamic acid content in chicken and beef extracts before and after incubation without (a) or with 1% of meat improver (b). (B) Glutamic-acid-releasing activity of chicken and beef homogenates prepared using buffers with different pH values. The Glutamyl-*p*-nitroanilide (Glu-*p*NA) hydrolyzing activities of chicken and beef extracts were measured and expressed as mU per ml of meat extract. Chicken breast tender meat and bovine thigh meat were used in both of (A) and (B).

In the investigation of several breeds of chicken and beef for their Glu-*p*NA hydrolyzing activities, we found that chickens tended to present higher enzymatic activity than beef, although differences in individual and geographic location may have an effect (data not shown).

3.2. Identification of the glutamic-acid-releasing enzyme in chicken meat

The glutamic acid-releasing (Glu-*p*NA hydrolyzing) enzyme was partially purified from chicken breast meat according to a previous report (Maehashi et al., 2003). As the result of purification from 2 kg of chicken breast tender meat, the fraction with 0.0767 U/mg of specific activity was obtained (supplementary Table S4). The fraction was then examined by SDS − PAGE (Fig. 2A). Although a large number of bands were still observed in the fraction, the 55 kDa band expected to be the target protein based on the results of a previous report was excised from the gel and subjected to in-gel digestion with protease V8. As shown in Fig. 2B, some fragments were obtained on the blotted PDVF membrane and subjected to amino acid sequence analysis. As a result, a sequence of 20 amino acid residues (LCLADTQPATLGGAFDEFIF) was identified.

We elucidated the partial amino acid sequence of the 55-kDa protein detected as a candidate respomsible enzyme for the release of glutamic acid in chicken meat. As a great amount of chicken gene information currently exists in the public database, a sequence matching the protein was found by the BLAST search and identified as aspartyl aminopeptidase (DNPEP, EC 3.4.11.21).

DNPEP is a widely distributed cytosolic enzyme with preference for N-terminal aspartyl and glutamyl residues (Wilk et al., 1998). Although there is no report of an avian source, origins including mammals (Chaikuad et al., 2012) and microbes (Gao et al., 2018) have been characterized. DNPEP belongs to the poorly understood M18 metallopeptidase family (Chaikuad et al., 2012). Although it has been implicated in the metabolism of angiotensin peptides (Chen et al., 2012), its biological and pathological roles remain poorly studied (Chen et al., 2014). Recently it was found that DNPEP is a novel biomarker of aggressive chronic lymphocytic leukemia (Kakodkar et al., 2020).

3.3. Expression of DNPEP and various peptidase genes in chicken muscle

A vast number of reports have shown that aminopeptidase proteins are expressed in avian skeletal muscles; however, no report has shown the expression of the respective genes. As DNPEP and glutamyl aminopeptidase (ENPEP) are probably involved in the release of glutamate, the expression distribution of the *DNPEP* and *ENPEP* genes was examined in chicken tissues. As shown in Fig. 3A, the *DNPEP* gene was expressed in chicken breast muscle, small intestine, and liver. Several studies have addressed *DNPEP* gene expression: the rabbit *DNPEP* gene is highly expressed in the testis and is also expressed in the small intes-

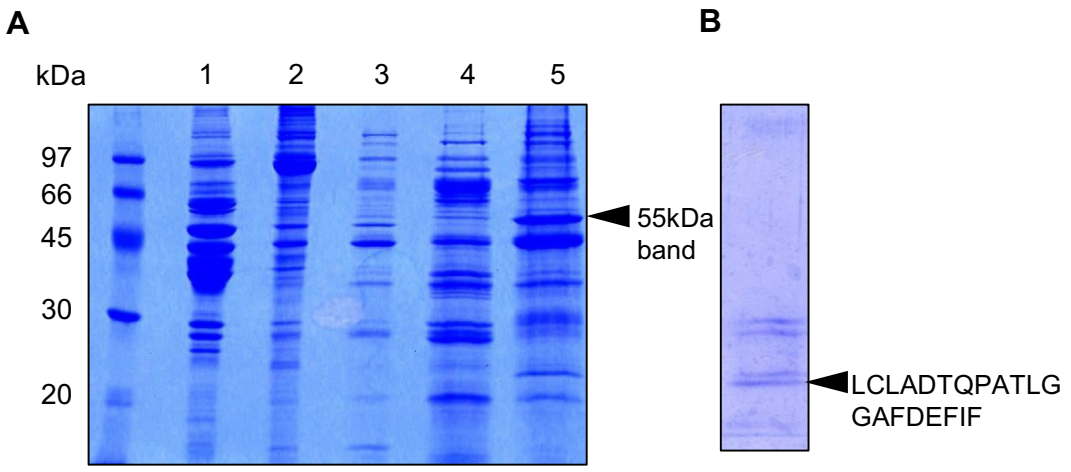

Fig. 2. Partial purification of the Glu-*p*NA hydrolyzing enzyme from chicken breast tender meat. (A) SDS − PAGE of the purification steps. Lane 1, crude extract; lane 2, 40% ammonium sulfate saturation; lane 3, 60 ˚C treatment; lane 4, DEAE-Sepharose; and lane 5, Toyopearl HW65F. The 55 kDa band indicated in lane 5 was cut out from the gel and subjected to in-gel digestion. (B) PVDF membrane with Coomassie brilliant blue (CBB) staining of SDS − PAGE of the V8 protease-digested fragments from the 55 kDa band. The band indicated by the arrow was subjected to N-terminal amino acid sequencing and determined as shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

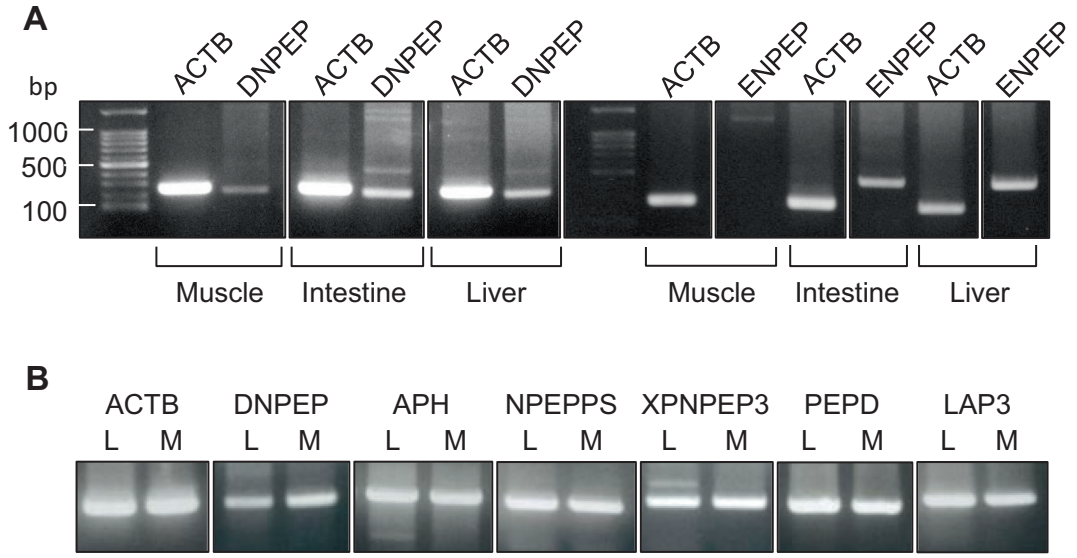

Fig. 3. Expression of various aminopeptidase genes in chicken tissues. (A) Tissue distribution of the expression of the aspartyl aminopeptidase (*DNPEP*) and glutamyl aminopeptidase (*ENPEP*) genes by RT − PCR in chicken breast tender muscle, small intestine, and liver. The amplified products for *DNPEP* and *ENPEP* were run on two separate gels. (B) Expression profiles of the aminopeptidase genes in chicken tissues, as assessed by RT − PCR. The amplified products of each set of L and M were run on separate gels. The template cDNAs were from liver (L) and thigh muscle (M). The primers used are listed in supplementary Table S2. ACTB, β-actin; APH, aminopeptidase H; ENPEP, glutamyl aminopeptidase; XPNPEP3, X-prolyl aminopeptidase 3; PEPD, peptidase D; NPEPPS, aminopeptidase puromycin sensitive; LAP3, leucine aminopeptidase 3. The GenBank accession numbers of these genes are indicated in supplementary Table S1.

tine and liver (Wilk et al., 1998), whereas the bovine *DNPEP* gene is highly expressed in the whole eye and the neural retina (Chen et al., 2012).

Conversely, *ENPEP* was clearly expressed in the liver and small intestine, but not in breast muscle. ENPEP (EC 3.4.11.7) has been extensively studied and characterized as being specific to acidic amino acid residues and as preferring glutamyl residues (Holmes et al., 2017). ENPEP exists as a membrane-bound enzyme in mammalian kidney and intestinal villi and its role in blood pressure regulation has been well studied, as it decomposes angiotensin II in the renin − angiotensin system (Holmes et al., 2017). Recently, it was proposed as one of the candidate SARS-CoV-2 receptors (Qi et al., 2020). Although the substrate specificity of the aminopeptidase purified from chicken meat by Maehashi et al. (2003) was consistent with that of ENPEP, it was found that the *ENPEP* gene was not expressed in chicken muscles (Fig. 3A), which implies that ENPEP does not exist in chicken meat and the enzyme reported by Maehashi et al. (2003) was not ENPEP.

Next, RT − PCR was performed to assess the gene expression corresponding to some peptidases available in the gene database. The gene expression of five aminopeptidases other than DNPEP in chicken thigh muscle and liver is shown in Fig. 3B. All genes tested were apparently expressed in the liver. It was confirmed that many peptidase genes were also expressed in muscle. Of these aminopeptidases, only APH (similar to bleomycin hydrolase) has been characterized. APH has been reported to contribute greatly to amino acid release in chicken muscle, because it also has endopeptidase activity (Rhyu et al., 1992). APH releases Met, Leu, and Lys residues, whereas its

activity against Glu residues is about halved. NPEPPS is probably identical to aminopeptidase C (APC), which has been purified from chicken muscle and characterized by Nishimura et al. (1991), because the predicted molecular weight and puromycin sensitivity of NPEPPS (Yamamoto et al., 2000) are in good agreement with those of APC. Chicken APC releases Lys, Ala, and Leu residues, but almost does not release Glu (Nishimura et al., 1991). Chicken PEPD and XPNPEP2 are orthologs of human prolidase (Wilk et al., 2017) and human aminopeptidase P (Bazan et al., 1994), respectively. The activity of LAP3, an ortholog of human leucine aminopeptidase 3 (EC3.4.11.1), in chicken muscle has been determined in relation to the process of meat tenderization by Blanchard and Mantle (1996); however, chicken LAP3 has not been molecularly characterized.

This study confirmed that many peptidases, including DNPEP, exist in chicken meat at the gene level. Nishimura (1998) reported that, during the postmortem aging process, muscle proteins are broken down into peptides via the action of proteases, such as cathepsins and calpains, and aminopeptidases, such as aminopeptidase C, aminopeptidase H, and aminopeptidase P, which act to release amino acids. Chicken aminopeptidase P has not been characterized; however, Nishimura (1998) described it as being similar to rat brain aminopeptidase P, an enzyme that cleaves any terminal amino acid from the Xaa-Pro-sequence (Harbeck & Mentlein, 1991). We detected the expression of the *PEPD* and *XNPEP3* genes in chicken muscle; however, it is unknown whether they have the same properties as aminopeptidase P, as reported by Nishimura (1998). In this study, it was confirmed that various peptidases, including DNPEP, in addition to aminopeptidase H, are present in chicken meat. It is expected that various enzymes act not only in the postmortem process, but also during cooking or processing to increase flavor through proteolysis and amino acid production. Therefore, it is necessary to characterize the enzymes expressed in chicken muscle in future studies.

3.4. Molecular cloning and amino acid sequence of chicken DNPEP

DNPEP was identified as the glutamic-acid-releasing enzyme in chicken meat; however, DNPEP is generally known as an enzyme that releases aspartic acid preferentially (Chaikuad et al. 2012). To resolve this contradiction, we examined the enzymatic properties of DNPEP. To this end, molecular cloning of the chicken and bovine DNPEPs was conducted to characterize chicken DNPEP in comparison with bovine DNPEP. As the result of PCR amplification, the full-length *cDNPEP* gene was obtained from chicken liver cDNA. However, the full-length *cDNPEP* gene could not be obtained from chicken thigh and breast muscles. Conversely, a full-length *bDNPEP* gene was amplified from bovine muscle cDNA. The nucleotide sequences of the obtained *cDNPEP* and *bDNPEP* genes were identical to NM_001012919 and NM_001045952, respectively.

The amino acid sequences deduced from the chicken and bovine *DNPEP* genes were aligned with the sequence of porcine DNPEP (Supplemental Fig. S1). The co-catalytic zinc atom binding sites His90 and His438 postulated in human DNPEP (Wilk et al., 2002) were conserved among the three DNPEPs. The metal binding sites His90, Asp263, Glu300, Asp344, and His438 (Chen et al, 2012) were all conserved among the three DNPEPs. Only cDNPEP had a 3-amino-acid insertion at position 199–201 and a 1-amino-acid deletion between residues 283 and 284.

Recently, it was predicted that chicken (Red Jungle Fowl) DNPEP has two isoforms, X1 (XM_025152198) and X2 (XM_025152199), as assessed by automated computational analysis. Both have an extra 125 amino acids at N-terminal, and X2 lacks 27 amino acids between residues 273 and 301. Moreover, the theoretical molecular weights of X1 and X2 are 64 and 61 kDa, respectively. Conversely, the DNPEP found in chicken meat exhibited a molecular weight of ~55 kDa, whereas the theoretical molecular weight of the protein encoded by the *DNPEP* gene cloned in this study was 52 kDa. A high level of genetic divergence between Red Jungle Fowl and commercial chickens has been reported (Tadano et al., 2014). Therefore, it was suggested that our sequence of DNPEP of broiler is identical to that of NM_001012919 of Leghorn, but different from those of X1 and X2 of Red Jungle Fowl.

3.5. Characterization of recombinant chicken DNPEP

The chicken *DNPEP* gene amplified from liver was cloned and expressed in *E. coli* as 6 × Histidine-tagged *DNPEP* (*cDNPEP*). The bovine *DNPEP* gene amplified from muscle cDNA was cloned to obtain recombinant *DNPEP* (*bDNPEP*), similar to *cDNPEP*. *E. coli* transformants carrying the *cDNPEP* and *bDNPEP* genes were cultured, and the expressed cDNPEP and bDNPEP proteins were purified from each cell extract on an affinity column to a single band on SDS − PAGE (Fig. 4A).

Western blot analysis using an anti-DNPEP antibody confirmed the identity and immune reactivity of the recombinant cDNPEP, as shown in Fig. 4B. Chicken tender meat extract was fractionated by 40%-saturated ammonium sulfate precipitation and DEAE-Sepharose CL-6B column chromatography. The resulting fraction, which exhibited enzymatic activity, was then run on SDS − PAGE and detected with Coomassie brilliant blue (CBB) and western blotting using an anti-DNPEP antibody. As a result, it was also confirmed that cDNPEP exists in chicken meat, indicating that recombinant DNPEP was slightly larger than native DNPEP because of the 6x His-tag of recombinant DNPEP. In addition, it was confirmed that this enzyme was not identical to the DNPEP X1 isoform, because the band did not correspond to the 64 kDa of X1, although it had an epitope region to the anti-DNPEP antibody, as shown in Supplementary Fig. S1; rather, it ran ~ 52 kDa on western blot analysis. However, the presence of X1 and X2 in chicken meat should be clarified in future studies. Purified bDNPEP also reacted with the anti-DNPEP antibody, similar to cDNPEP on western blotting (Supplementary Fig. S2). Beef was separated in a similar manner to that used for chicken, to attempt to detect DNPEP by western blot analysis; however, the enzyme activity was not specified in any fractions of the beef extract (data not shown) and DNPEP was hardly detected in the fraction of beef obtained by the same procedure as for chicken (Supplementary Fig. S2).

The enzymatic properties of cDNPEP were investigated via a comparison with those of bDNPEP. The optimum temperature for the Glu-*p*NA hydrolyzing activity was around 50 °C for bDNPEP and 60 °C for cDNPEP (Fig. 4C). The activity of bDNPEP decreased from 50 °C, whereas that of cDNPEP was stable up to 50 °C (Fig. 4D). The optimum pH of the Glu-*p*NA hydrolyzing activity was pH 7.5 for both cDNPEP and bDNPEP, and almost no activity was observed at pH 5. The activity of bDNPEP was remarkably reduced at pH 6, whereas that of cDNPEP remained at 40% at pH 6 (Fig. 4E). The relationship between pH and cDNPEP activity was consistent with Fig. 1B, which shows the relationship between the pH of chicken homogenate and glutamate-releasing activity.

The substrate specificity of cDNPEP and bDNPEP was examined as shown in Table 1. cDNPEP showed 5-fold higher activity against Glu-*p*NA than against Asp-*p*NA. Conversely, bDNPEP was also specific to the acidic amino acid *p*NA, but its activity against Asp-*p*NA was higher than that against Glu-*p*NA. cDNPEP also showed higher hydrolytic activity against Glu-Glu than did bDNPEP, whereas cDNPEP had lower hydrolytic activity against Asp-Glu and angiotensin II compared with bDNPEP. The Km values also showed that cDNPEP had a remarkably higher affinity for Glu-*p*NA than for Asp-*p*NA, which was different from that observed for bDNPEP, demonstrating that cDNPEP has unique substrate specificity.

This substrate specificity of cDNPEP was typical of ENPEP, and was consistent with that of the enzyme purified from chicken meat by Maehashi et al. (2003). The N-terminal acidic amino acid-specific aminopeptidase from soybean cotyledons (Asano et al. 2010) also

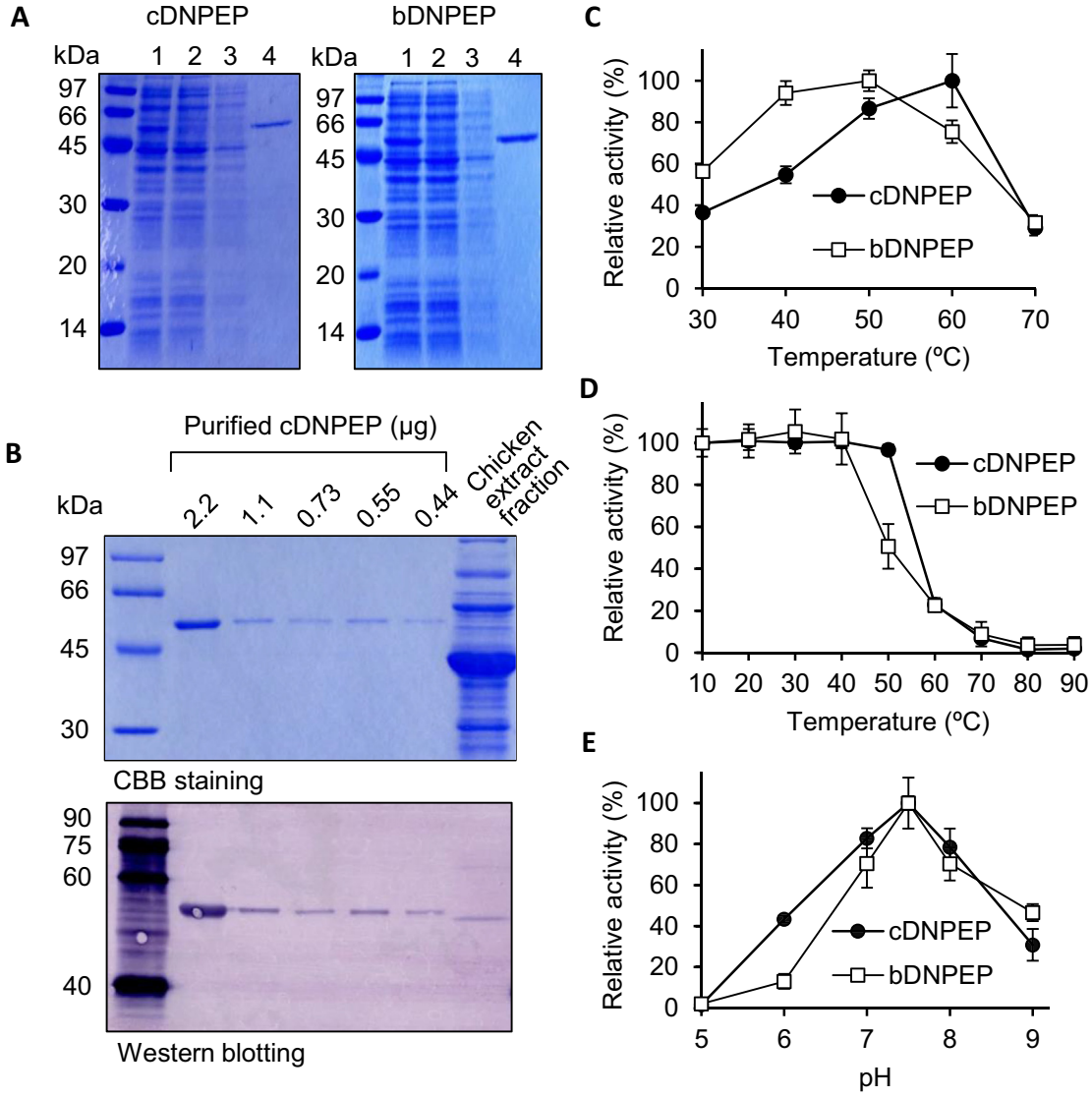

Fig. 4. Characteristics of recombinant chicken and bovine aspartyl aminopeptidases. (A) SDS − PAGE of each purification step of chicken aspartyl aminopeptidase (cDNPEP) and bovine DNPEP (bDNPEP) expressed in *E. coli* on the Ni-affinity column. Lane 1: crude extract; lane 2: flow-through fraction; lane 3: wash fraction; and lane 4: elute fraction. (B) Western blot analysis of recombinant cDNPEP and chicken meat extract. Purified cDNPEP (0.44–2.2 μg of protein) and fractionized chicken meat extract (19 μg of protein) were loaded onto SDS − PAGE for CBB staining and western blot analysis using an anti-DNPEP antibody. (C) Optimal reaction temperature at pH 7.5. (D) Thermal stability after incubation for 30 min at pH 7.5. (E) pH dependency at 50 °C. The enzymatic activities of cDNPEP and bDNPEP were assayed using Glu-*p*NA as the substrate. Values are the mean ± SE (n = 3).

Table 1
Substrate specificity of recombinant chicken and bovine aspartyl aminopeptidases.

Substrate	cDNPEP		bDNPEP	
	Relative activity* (%)	Km (mM)	Relative activity* (%)	Km (mM)
Glu-*p*NA	100	0.137	85.2	0.314
Asp-*p*NA	21.1	0.767	100	0.389
Leu-*p*NA	0.3		4.8	
Phe-*p*NA	5.7		5.5	
Pro-*p*NA	4.2		7.4	
Glu-Glu	100		54.5	
Asp-Glu	37.9		65.2	
Angiotensin II	57.6		210.3	

cDNPEP = chicken aspartyl aminopeptidase
bDNPEP = bovine aspartyl aminopeptidase
* The relative activities of cDNPEP and bDNPEP toward aminoacyl-*p*NAs were presented as a percentage against the activities of cDNPEP toward Glu- *p*NA and bDNPEP toward Asp- *p*NA, respectively. The relative activities toward peptides were presented as a percentage against the activity of cDNPEP toward Glu-Glu.

showed higher activity on the Glu substrate than on the Asp substrate, but no report has been found regarding other sources. This unique substrate specificity of cDNPEP was thought to contribute to the release of a large amount of glutamic acid detected in chicken meat during processing and cooking.

The effects of inhibitors and metals on the Glu-*p*NA hydrolyzing activity of cDNPEP and bDNPEP are shown in Table 2. The activities of both cDNPEP and bDNPEP were inhibited by 10 mM monoiodoacetic acid, 0.1–1 mM 2-ME, 0.1–1 mM DTT, 20 mM EDTA, 0.4 mM PCMB, and 0.4 mM $ZnCl_2$. These results are almost consistent with the results obtained for the chicken enzyme, as reported by Maehashi et al. (2003), and for bDNPEP, as reported by Chen et al. (2012), respectively. However, 10 mM cysteine inhibited cDNPEP but activated bDNPEP. In addition, cDNPEP was activated by $MnCl_2$ and bDNPEP was activated by $CoCl_2$. In contrast, Chen et al. (2012) described the activation of bDNPEP by Mn^{2+}. It is known that mammalian DNPEP activity is enhanced by Mn (II) and inhibited by zinc and metal chelators (Chen et al., 2012).

4. Conclusion

In this study, we identified a glutamic-acid-releasing enzyme in chicken meat as being DNPEP. It was found that the *DNPEP* gene was expressed in breast and thigh muscles, in addition to the liver and small intestine of chicken. Furthermore, it was found that various peptidase genes were expressed together with *DNPEP* in chicken muscle. cDNPEP showed high specificity for N-terminal glutamic acid over aspartic acid residues, which was a substrate specificity that was different from that of bDNPEP and other origins. Based on the fact that none of the aminopeptidases are reportedly highly active against glutamyl residues, with the exception of DNPEP found in the chicken muscle, it was concluded that DNPEP mainly contributes to the high glutamic acid release from chicken meat that occurs during cooking and processing. As the presence of some uncharacterized peptidases was detected in muscle, it was considered that DNPEP releases a large amount of glutamic acid by cooperating with these peptidases, thus assisting the release of all amino acids in chicken. It is necessary to characterize these peptidases for a full understanding of the mechanism of amino acid increase in chicken meat in the future. The findings of this study may lead to the development of novel processing or cooking techniques that use enzymes to improve the palatability of chicken meat further.

Table 2
Effects of potential inhibitors and metals on the activities of recombinant chicken and bovine aspartyl aminopeptidases.

Chemicals	(mM)	Relative activity (%)*	
		cDNPEP	bDNPEP
None		100	100
Cysteine	0.1	120 ± 11.8	95 ± 4.3
	1	111 ± 1.9	66 ± 0.6
	10	55 ± 2.6	160 ± 8.5
Monoiode acetate	0.1	183 ± 15.6	97 ± 9.1
	1	124 ± 19.7	95 ± 11.4
	10	8.7 ± 3.8	13 ± 3.7
2-Mercaptoethanol	0.1	76 ± 5.4	71 ± 3.5
	1	59 ± 7.4	55 ± 0.4
DTT	0.1	66 ± 6.4	34 ± 3.1
	1	38 ± 9.5	34 ± 1.9
EDTA	5	98 ± 2.8	117 ± 6.0
	20	23 ± 3.5	33 ± 2.9
Bacitracine	0.02	115 ± 4.0	119 ± 11.1
	0.2	109 ± 2.2	102 ± 9.7
p-Chloromercuribenzoate	0.04	13 ± 4.1	131 ± 18.6
	0.4	11 ± 1.6	6.7 ± 3.3
o-Phenanthroline	2.4	81 ± 10.9	153 ± 1.6
$ZnCl_2$	0.4	43 ± 2.6	25 ± 4.8
$MnCl_2$	0.4	128 ± 0.7	124 ± 3.8
	4	196 ± 8.4	119 ± 6.7
$CoCl_2$	0.4	121 ± 7.3	289 ± 5.6
	1	118 ± 9.9	562 ± 70.0
$CaCl_2$	40	129 ± 13.2	171 ± 3.6

cDNPEP = chicken aspartyl aminopeptidase.
dDNPEP = bovine aspartyl aminopeptidase.
*The values are means ± SE (n = 3). Relative activity was expressed as a percentage against the control.

CRediT authorship contribution statement

Hitomi Yuhara: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Akira Ohtani:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Mami Matano:** Methodology, Validation, Formal analysis, Investigation, Data curation. **Yutaka Kashiwagi:** Writing - review & editing. **Kenji Maehashi:** Conceptualization, Methodology, Writing - original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2021.100015.

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