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Novel activity of *Streptomyces* aminopeptidase P

Kun Wan[1,2], Misugi Uraji[1,3], Lingli Yang[1], Ryota Nakahigashi[4] and Tadashi Hatanaka[1*]

Abstract

Streptomyces aminopeptidase P enzymes are proline-specific peptidases that belong to the peptidase M24 family. To evaluate the activity of a commercial *Streptomyces* aminopeptidase P, named 'XPO DUET', we performed three experiments involving degradation of tryptic casein, production of free amino acids from casein hydrolysate, and hydrolysis of synthetic peptides. Using an ion-trap liquid chromatography–mass spectrometry (LC–MS) apparatus, we demonstrate that XPO DUET could degrade FFVAPFPEVFGK, an allergic and bitter peptide, VAPFPEVFGK, and PEVFGK from tryptic casein. All amino acids, except Ala, Asp, Glu, and Tyr, were released in an XPO DUET activity-dependent manner during the hydrolysis of casein hydrolysate. LC–MS analysis also revealed the ability of XPO DUET to completely hydrolyze Phe-Phe-Phe into free Phe. Thus, we confirm that XPO DUET possesses broader specificity than its known activity toward Xaa-Pro peptides. Because XPO DUET is a food-grade peptidase, it is useful in the bioprocessing of protein hydrolysates through its combination with other food-grade peptidases.

Keywords: Aminopeptidase P, *Streptomyces*, M24 family

Introduction

Aminopeptidases (APs) are enzymes that catalyze the cleavage of amino acid residues at the N-terminal position of peptides and proteins. Bacterial APs may be classified into one of the following two categories based on their substrate specificity: broad or narrow (Gonzales and Robert-Baudouy 1996). *Streptomyces* leucyl APs are well-known examples of broad-specificity APs (Ben-Meir et al. 1993; Arima et al. 2004) that prefer large hydrophobic amino acids at the P1 position of the substrate; however, these enzymes cannot release the N-terminal amino acids when the penultimate residue is Pro (Arima et al. 2006). In contrast, Pro-specific APs include narrow-specificity APs such as prolyl AP, an enzyme that catalyzes the hydrolysis of the N-terminal Pro residue of peptides and proteins. Another example includes aminopeptidase P (APP) that cleaves the N-terminal amino acid residue from a peptide carrying Pro as the penultimate residue. Thus, APPs have been categorized as narrow-specificity APs because of their specificity toward Xaa-Pro. To date, several commercial aminopeptidases are known by their trade names such as flavourzyme (Novo Nordisk), debitrase (Imperial Biotechnology Ltd.) coronase (Rohm GobH), and pronase (Calbiochem). However, these enzymes contained several peptidases as they were prepared by liquid culture or solid-state fermentation of microbes. In contrast, a commercial *Streptomyces* aminopeptidase P, named 'XPO DUET,' was intracellularly expressed using the APP gene (SCO1352) from *S. coelicolor* A3 (2) (http://www.sanger.ac.uk/resources/downloads/bacteria/streptomyces-coelicolor.html) and *S. lividans* as the host. Thus, XPO DUET is expected to be of high purity.

Among the 20 naturally occurring amino acids, Pro is unique owing to its unusual cyclic structure. This peculiarity deems Pro residues resistance to hydrolysis by exopeptidases. Peptides containing Pro residues often impart a bitter flavor to foods (Lemieux and Simard 1992). Until now, it was unknown that just one aminopeptidase could degrade various peptides to free amino acids completely.

*Correspondence: hatanaka@bio-ribs.com
[1] Okayama Prefectural Technology Center for Agriculture, Forestry, and Fisheries, Research Institute for Biological Sciences (RIBS), 7549-1 Kibichuo-cho, Kaga-gun, Okayama 716-1241, Japan
Full list of author information is available at the end of the article

Therefore, Pro-specific APs have applications in food industries by a combination of broad-specificity APs to degrade protein hydrolysates into free amino acids.

We have investigated *Streptomyces* APs for more than 10 years (Arima et al. 2004, 2006, 2008; Hatanaka et al. 2007, 2011; Uraji et al. 2007) and recently evaluated the synergistic effects of three *Streptomyces* APs on casein hydrolysis to degrade VLPVPQK and FFVAPFPEVFGK, which are known as allergenic and bitter peptides derived from tryptic casein (Chen et al. 2015; Weber et al. 2006; Nishiwaki et al. 2002). An APP from *Streptomyces costaricanus* TH-4 (Arima et al. 2008) was found to release the N-terminal Val from the sequence VLPVPQK (Wan et al. 2019). This result indicates that the APP from *Streptomyces* may exhibit a novel substrate specificity. In the present study, we confirm the activity of another APP from *S. coelicolor*.

Materials and methods

Materials

A protein assay kit and gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories Inc. (California, USA). Casein from bovine milk was obtained from Sigma-Aldrich (Tokyo, Japan), while synthetic peptides were supplied by Bachem AG (Bubendorf, Switzerland). Snake venom L-amino acid oxidase (LAAO) was procured from Sigma-Aldrich Japan, and horseradish peroxidase, from Wako Chemicals (Osaka, Japan). All other chemicals were commercial products of the highest grade.

Enzymes

A commercial *Streptomyces* APP, named 'XPO DUET', and a commercial endopeptidase (Bioprase SP) from *Bacillus* species (sp.) were obtained from Nagase ChemteX (Fukuchiyama, Japan). XPO DUET was intracellularly expressed using the *app* gene (SCO1352) from *S. coelicolor* A3 (2) (http://www.sanger.ac.uk/resources/downloads/bacteria/streptomyces-coelicolor.html) and *S. lividans* as the host. A commercial trypsin (isolated from pig liver), which also exhibited chymotrypsin activity, was obtained from Novozymes Japan (Chiba, Japan).

Partial purification of XPO DUET

Amorphous XPO DUET was dissolved in 25 mM Tris–HCl (pH 8.0) and dialyzed against the same buffer. The dialysate was loaded onto a Vivapure-Q spin column (Sartorius Japan, Tokyo, Japan), which was equilibrated with 25 mM Tris–HCl (pH 8.0). The bound protein was eluted with 25 mM Tris–HCl (pH 8.0) containing 0.5 M sodium chloride (NaCl). The eluate was dialyzed against 25 mM Tris–HCl (pH 8.0) and loaded onto a MonoQ HR5/5 column (GE Healthcare, Tokyo, Japan) pre-equilibrated with 25 mM Tris–HCl (pH 8.0). The active fraction was eluted with a linear gradient (0–0.5 M NaCl) using 20 bed volumes. The obtained fraction was dialyzed against 10 mM Tris–HCl (pH 8.0), and the resulting enzyme solution was used for analysis. The activity of the enzyme solution toward Ala-Pro-*p*NA (Bachem AG, Bubendorf, Switzerland) was determined by coupling with *Streptomyces* proline AP at 40 °C (Arima et al. 2008). The fold purification was 1.4, and its specific activity was 4.11 U/mg protein. The SDS-PAGE pattern of the purified XPO DUET is shown in Fig. 1.

Preparation of tryptic casein

The procedure was similar to a previously described method (Wan et al. 2019). A mixture of 20 mg trypsin (225 proteolytic units/mg), 2 g of casein, and 200 mL of 50 mM ammonium bicarbonate (NH_4HCO_3, pH 7.0) was incubated at 40 °C overnight on a rotary shaker at 750 rpm; the mixture was then incubated at 90 °C for 30 min without shaking to inactivate trypsin. After centrifugation, the supernatant (100 µL) was added to the enzyme solution (100 µL) and the reaction mixture was incubated at 40 °C for 1, 2, or 3 h with rotary shaking at 1500 rpm. The solution was then incubated at 95 °C for 5 min without shaking to inactivate the enzymes. All of these reactions were performed in triplicates. For peptide analyses, each reaction mixture (25 µL) was separated

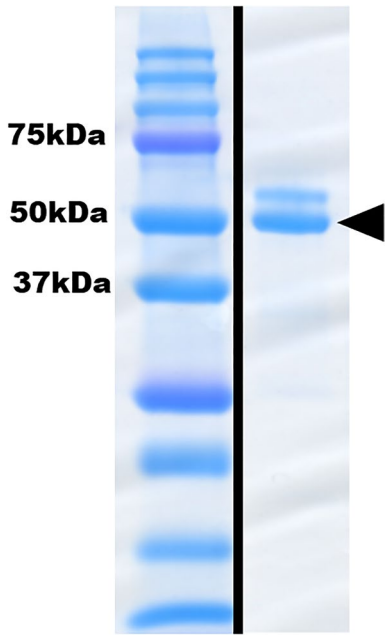

Fig. 1 SDS-PAGE of the purified XPO DUET. The left lane includes the molecular weight marker, while the right lane includes 10 µg of the purified enzyme

using high-performance liquid chromatography (HPLC; LC-20AD, Shimadzu) with a TSK gel ODS-120H column (3 μm, 2.0 mm × 15 cm; TOSOH) maintained at 50 °C. Following separation, the obtained reaction solutions were analyzed using an ion-trap mass spectrometry (MS) apparatus (Amazon SL; Bruker) in positive mode. The flow rate of the mobile phase comprising (A) ultrapure water with 0.1% (v/v) formic acid and (B) acetonitrile with 0.1% formic acid (v/v) was 0.25 mL/min. The gradient profile was as follows: 0–5 min, 10% B; 5–15 min, 10–70% B; 15–15.1 min, 70–95% B; 15.1–20 min, 95% B; 20–20.1 min, 95–10% B; 20.1–25 min, 10% B. In MS analyses, data were recorded in the 100–800 m/z range. The MS/MS data for the reaction solution in which casein was digested with trypsin alone were recorded in the 350–1400 m/z range and submitted to the "Mascot server" protein identification/characterization search engine (Matrix Science) to confirm the identity of the peptides produced. According to this search, the specific fragmentation of m/z 390.8 ± 0.1 (charge: +2; retention time: 9.8 min) and m/z 692.9 ± 0.1 (charge: +2; retention time: 13.0 min) represented the oligopeptides VLPVPQK and FFVAPFPEVFGK, respectively. The specific fragmentation of m/z 545.8 ± 0.1 (charge: +2; retention time: 11.8 min) and m/z 328.9 ± 0.1 (charge: +2; retention time: 9.6 min) from the MS data represented the oligopeptides VAPFPEVFGK and PEVFGK, respectively. The amount of the oligopeptide in each reaction solution was determined from the residue intensity for the corresponding m/z of the oligopeptides identified in the MS analysis.

Preparation of casein hydrolysate and analysis of free amino acids

The procedure was similar to a previously described method (Wan et al. 2019). In brief, a mixture of 33 μL of Bioprase SP (100 proteolytic units/mg, 10 mg/mL) and 10 mL of 0.6% (w/v) aqueous dispersion of casein (40 mM monosodium phosphate [NaH_2PO_4], pH 7.5) was prepared and incubated at 40 °C overnight and then at 80 °C for 30 min to inactivate Bioprase SP. After centrifugation, 250 μL of the resulting supernatant was added to 100 μL of the enzyme solution (20 mM NaH_2PO_4, pH 7.5) or 100 μL of 20 mM NaH_2PO_4 (pH 7.5) buffer. The reaction solution was incubated at 40 °C for 6 h with rotary shaking at 1400 rpm and then at 80 °C for 30 min to inactivate XPO DUET. Both reactions were performed in triplicates. For the analysis of amino acids from casein hydrolysate, a 100 μL aliquot of each of the triplicate reaction solutions was acidulated with 0.2 M HCl and analyzed with gas chromatography (GC-2025; Shimadzu) using an EZ: faast™ kit (Phenomenex). This analysis was performed as described by Wan et al. (2016). The degree of hydrolysis was determined and compared with the total number of free amino acids obtained following treatment with 6 N HCl at 100 °C for 22 h. As Arg and Cys could not be detected using the EZ: faast™ kit, the level of Arg was analyzed using LC–MS and a Discovery HS F5 column (3 μm, 2.1 mm × 15 cm; SPELCO) maintained at 40 °C. The separated reaction solutions were analyzed using an ion-trap MS apparatus (Amazon SL; BRUKER) in the positive mode. The flow rate of the mobile phase comprising (A) ultrapure water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid (v/v) was 0.25 mL/min. The gradient profile was as follows: 0–2 min, 0% B; 2.1–5 min, 0–25% B; 5.1–11.0 min, 25–35% B; 11.1–15 min, 95% B; 15.1–20 min, 95% B; 20.1–25 min, 0% B. A total of 3 μL of each sample was injected into the apparatus.

Hydrolysis of synthetic peptides

The enzyme reaction was performed at 40 °C at a final concentration of 0.8 mM peptides in 0.1 M Tris–HCl (pH 7.0) for 30 min. The reaction was terminated with heat treatment (95 °C for 5 min). All reactions were performed in triplicates. The specific activity of the purified XPO DUET toward the peptides was determined using the horseradish peroxidase and 4-aminoantipyrine–phenol method coupled with the reaction of LAAO (Arima et al. 2006).

Time course of hydrolysis toward Phe-Phe-Phe

The enzyme reaction was performed at 40 °C at a final Phe-Phe-Phe concentration of 0.8 mM in 0.1 M Tris–HCl (pH 7.0) for 1, 2, and 3 h. The reaction was terminated with heat treatment (95 °C for 5 min). All reactions were performed in triplicates. The reaction mixture was tenfold diluted with ultrapure water containing 0.1% formic acid. Each diluted sample (2 μL) was subjected to HPLC (LC-20AD; Shimadzu) analysis using a Triart C18 column (1.9 μm, 3.5 mm × 7.5 cm; YMC) maintained at 40 °C. The separated reaction solutions were analyzed using an ion-trap MS apparatus (Amazon SL; BRUKER) in positive mode. The mobile phase consisted of (A) ultrapure water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid at a flow rate of 0.3 mL/min. The gradient profile was as follows: 0–2 min, 10% B; 2–10 min, 10–90% B; 10.1–15 min, 10% B. The standard curves of Phe, Phe-Phe, and Phe-Phe-Phe were obtained from the quantification of peak intensity at concentrations from 0 to 100 μM.

Results

Degradation of tryptic casein by XPO DUET

VLPVPQK is known as an allergenic and bitter peptide. It was retained even after 3 h of treatment with XPO DUET

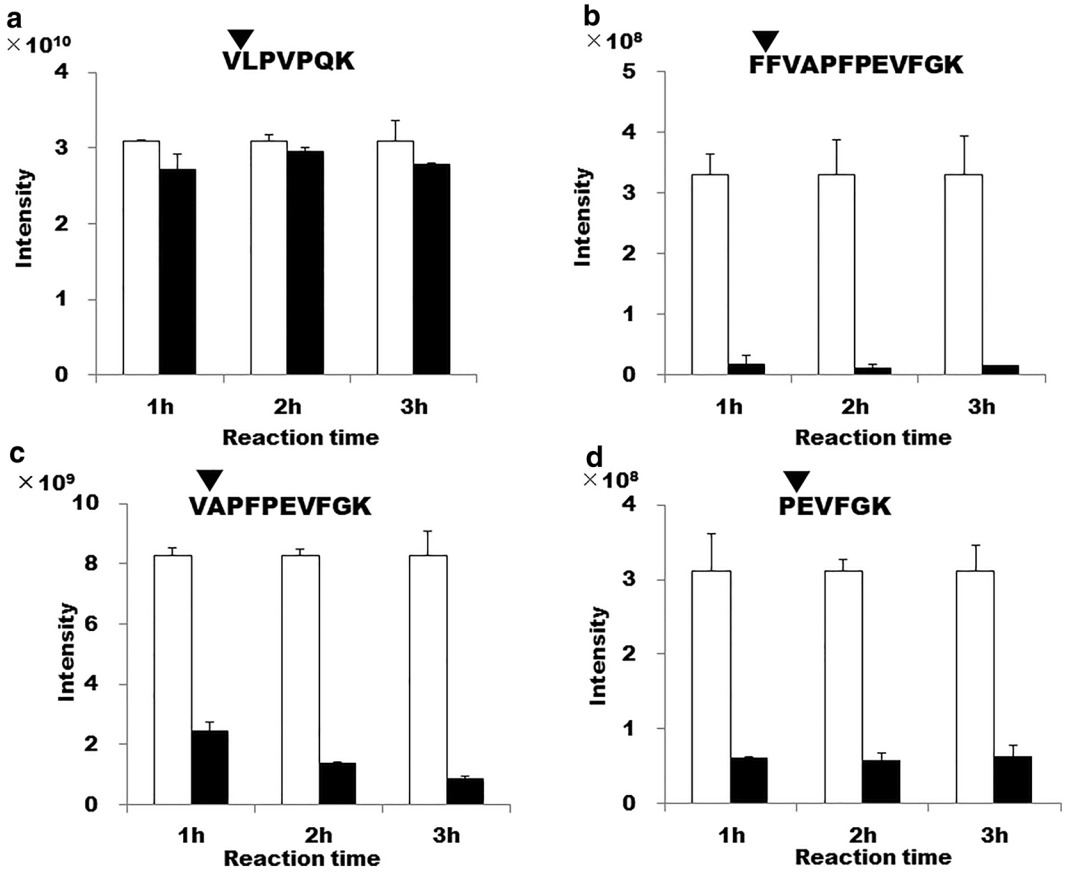

Fig. 2 Evaluation of XPO DUET during the degradation of tryptic casein. **a** The contents of VLPVPQK, as determined by LC–MS analysis. **b** The contents of FFVAPFPEVFGK, as determined by LC–MS analysis. **c** The contents of VAPFPEVFGK, as analyzed with LC–MS. **d** The contents of PEVFGK, as determined with LC–MS analysis. The white and black columns indicate tryptic digestion and treatment with XPO DUET, respectively

(Fig. 2a), indicating that XPO DUET could not release the N-terminal Val residue from VLPVPQK.

After 1 h of treatment with XPO DUET, FFVAPFPEVFGK was scarcely detected with LC–MS (Fig. 2b). Thus, the enzyme could easily release the N-terminal Phe residue from FFVAPFPEVFGK.

For tryptic casein, VAPFPEVFGK was detected with LC–MS and LC–MS/MS analyses and its peak intensity was tenfold higher than that of FFVAPFPEVFGK. This result was attributed to the chymotrypsin activity of trypsin. After 1 h of treatment with XPO DUET, VAPFPEVFGK was scarcely detected with LC–MS (Fig. 2c), indicating that the enzyme could hydrolyze the N-terminal Val residue of VAPFPEVFGK but not of VLPVPQK.

Similar to VAPFPEVFGK, PEVFGK was also produced owing to the chymotrypsin activity of trypsin. This oligopeptide could only be distinguished after 1 h of treatment with XPO DUET. Thus, XPO DUET could also release the N-terminal Pro residue of PEVFGK (Fig. 2d).

Thus, XPO DUET as one of the APPs of *Streptomyces* showed broader specificity than its known activity toward Xaa-Pro peptides, which preferred Met-Pro peptides (Arima et al. 2008; Nandan and Nampoothiri 2014).

Profile of free amino acids from casein hydrolysate by XPO DUET

Figure 3 shows the comparison of the free amino acids produced from casein peptides after treatment with XPO DUET. As Bioprase SP is an endopeptidase that rarely exhibits exo-peptidase activity (Hatanaka et al. 2012), the free amino acid content of the control samples was sparse. Among the 19 amino acids, Gln level was the highest after treatment with XPO DUET (Fig. 3a). All amino acids except Ala, Asp, Glu, and Tyr were produced in an XPO DUET activity-dependent manner. However, the acidic amino acids Asp and Glu could not be released upon enzyme treatment. This result is consistent with the XPO DUET-mediated digestion of tryptic casein and explains the release of Phe, Val, and Pro upon treatment with XPO DUET.

Figure 3b indicates the free amino acid profile of casein hydrolysate after treatment with XPO DUET. Among all

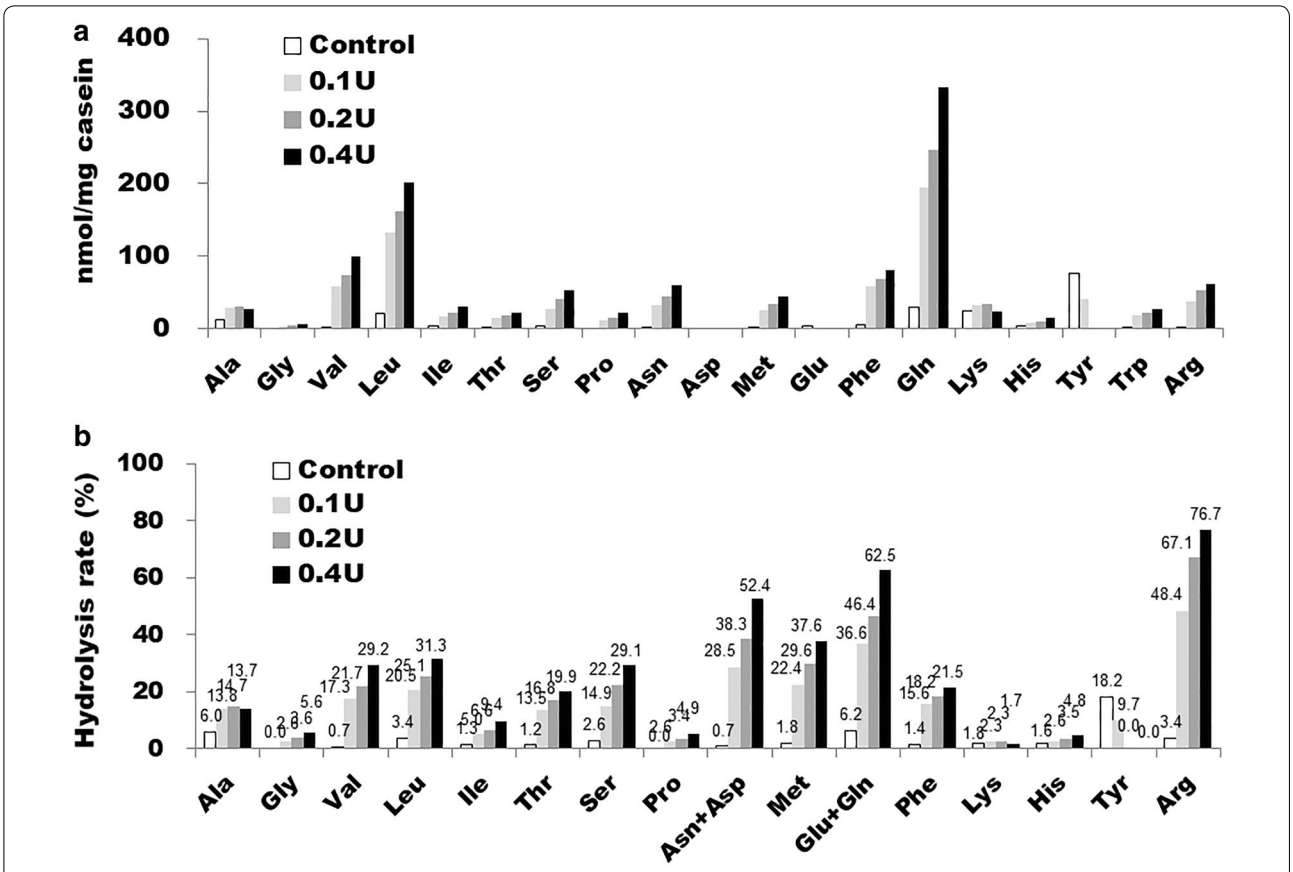

Fig. 3 **a** Free amino acid profiles of casein hydrolysates. The contents of 19 amino acids in the control (without XPO DUET treatment [white bar]) and after treatment with 0.1 U of XPO DUET (light gray bar), 0.2 U of XPO DUET (dark gray bar), and 0.4 U of XPO DUET (black bar). **b** Degree of hydrolysis (%) of casein hydrolysate in the control (without XPO DUET treatment [white bar]) and after treatment with 0.1 U of XPO DUET (light gray bar), 0.2 U of XPO DUET (dark gray bar), and 0.4 U of XPO DUET (black bar). The degree of hydrolysis is presented as the relative content of free amino acids present after treatment with 6 N HCl at 110 °C for 15 h

amino acids, Arg content was the highest (48–78%) after the hydrolysis with the enzyme. Casein from bovine milk comprises αs1, α-s2, β, and κ caseins (Wal 1998). There are 21 Arg residues in four casein proteins; however, the blockage of Arg-Pro exists only in one of the 21 residues. These results suggest that the enzyme possesses broader specificity than its known activity toward Xaa-Pro peptides.

Hydrolysis of synthetic peptides by XPO DUET

Table 1 shows the specific activities of XPO DUET toward the peptides. Among the peptides, Met-Pro-Gly was deemed as the best substrate. In addition, XPO DUET also showed activity toward Phe-Phe and Phe-Phe-Phe (Fig. 4).

Figure 4b shows the time course of Phe-Phe-Phe hydrolysis. The level of Phe-Phe-Phe decreased and that of Phe increased with time. After 3 h of treatment, the degree of hydrolysis for Phe-Phe-Phe was ca. 40%. On the other hand, the content of Phe-Phe was the highest after 2 h of treatment. Phe-Phe content slightly decreased after 3 h as compared to that after 2 h. This

Table 1 Hydrolysis of synthetic peptides by XPO DUET

Substrate	Specific activity (μmol/min/mg protein)	Relative activity (%)
Met-Pro-Gly	0.782 ± 0.03	100.0
Phe-Phe-Phe	0.122 ± 0.02	15.6
Phe-Phe	0.178 ± 0.01	22.8
Lys-Lys-Lys	0.000	0
Ala-Ala-Ala	0.000	0
Ala-Ala-Ala-Ala	0.000	0
Arg-Gly-Asp-Ser	0.0001 ± 0.0002	0.01

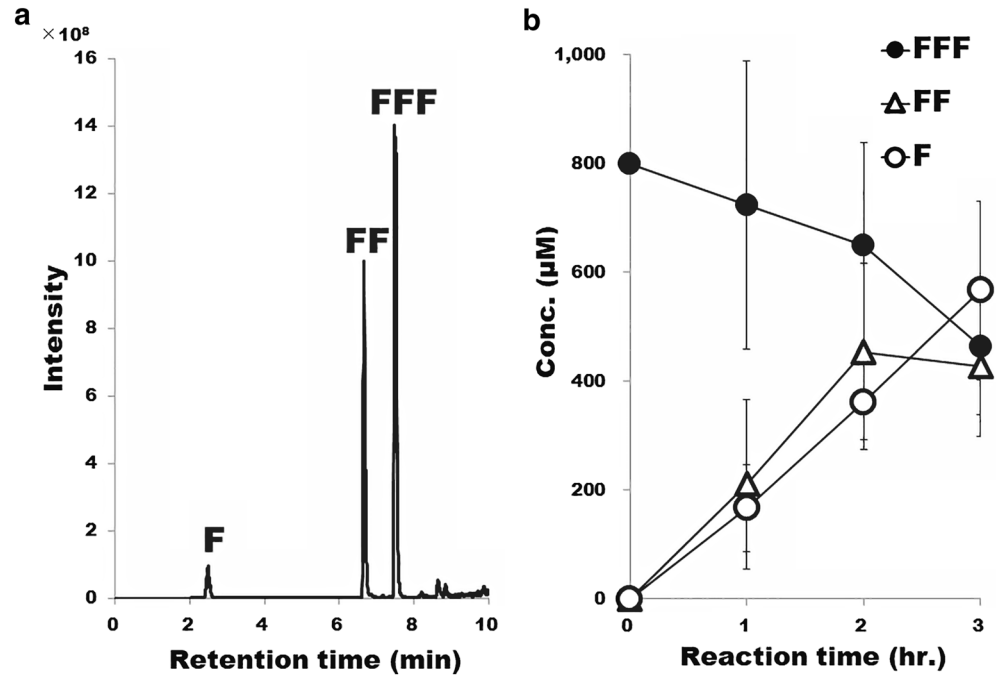

Fig. 4 Evaluation of the hydrolysis of Phe-Phe-Phe by XPO DUET. **a** Pattern of the base peak chromatogram of the standards. **b** Time course of hydrolysis of Phe-Phe-Phe by XPO DUET

result indicates that the resultant Phe-Phe was also hydrolyzed into free Phe by XPO DUET.

The above results suggest that XPO DUET could completely hydrolyze Phe-Phe-Phe into free Phe.

Discussion

In this study, we conducted three types of experiments, including degradation of tryptic casein, hydrolysis of casein hydrolysate, and hydrolysis of synthetic peptides. From the obtained results, we demonstrate that the commercial *Streptomyces* APP, XPO DUET, possessed broader specificity than its known activity toward Xaa-Pro peptides.

Streptomyces APPs are zinc-containing peptidases belonging to the peptidase M24 family [https://www.ebi.ac.uk/merops/] (Lowther and Matthews 2002; Nandan and Nampoothiri 2017). Until now, two types of *Streptomyces* APPs from *S. lividans* (slAPP I and slAPP II) have been reported as aminopeptidases P, which is specific toward Xaa-Pro peptides (Arima et al. 2008; Butler et al. 1993, 1994). XPO DUET is similar to slAPP II, which forms a dimer (Arima et al. 2008) and shows 99% sequence identity to XPO DUET in the primary structure, while slAPP I and the APP from *S. costaricanus* TH-4 (TH-4 APP) form tetramers. XPO DUET and TH-4 APP show 65% identity in their primary structures. Consistent with the activity of XPO DUET, TH-4 APP could degrade VAPFPEVFGK (Wan et al. 2019).

Thus, *Streptomyces* APPs possess broader specificity than their known activities toward Xaa-Pro peptides. Further, these enzymes may have their own substrate specificities. TH-4 APP, but not XPO DUET, can degrade VLPVPQK. Arima et al. (2008) have reported that TH-4APP possessed higher and broader activity toward Xaa-Pro derivatives than slAPP II. We hypothesize that their different quaternary structures led to their differing hydrolysis activity toward VLPVPQK.

From the result of Fig. 3, XPO DUET prefers peptides possessing Arg, Gln, or Asn at the N-terminal position. The highest hydrolysis rate of Arg from casein hydrolysate (Fig. 3) for XPO DUET indicates its activity toward the N-terminal Arg except for Arg-Gly-Asp-Ser (Table 1). Further studies are warranted to confirm the kind of Arg-terminal sequence amenable to degradation by this enzyme. XPO DUET could release N-terminus Pro from PEVFGK (Fig. 2d); however, the released Pro was minorly involved in the degradation of tryptic casein by XPO DUET (Fig. 3). We have already reported that it was important to note the significance of the penultimate position in addition to the N-terminus for defining the specificities of M1 family aminopeptidase and M28 family aminopeptidase (Arima et al. 2006; Hatanaka

et al. 2007). Thus, the specificity of XPO DUET may be affected by the penultimate position of substrates.

Enzymatic hydrolysis of food proteins is widely used to increase the value of the final product by improving their nutritional characteristics, imparting functional properties, and removing inhibitory peptides (Clemente 2001). Some studies have reported the synergistic effects of the combination of a broadly specific AP and a proline-specific AP in protein hydrolysis (Byun et al. 2001; Stressler et al. 2013).

Conclusions

We have shown that XPO DUE is able to release N-terminal amino acids from FFVAPFPEVFGK, VAPFPEVFGK, PEVFGK, and FFF, which were not Xaa-Pro peptides. Thus, this is the first report to describe the broader specificity of XPO DUET, a *Streptomyces* APP from *S. coelicolor*, than its known activity toward Xaa-Pro peptides. XPO DUET is commercially produced as a food-grade peptidase and is expected to be applicable in food protein hydrolysis.

Abbreviations

AP: Aminopeptidase; HPLC: High-performance liquid chromatography; GC: Gas chromatography.

Acknowledgements

Not applicable.

Authors' contributions

KW, MU, LY, RN, and TH conceived the project. KW and TH designed the experiments and modified the manuscript. KW carried out all experiments. MU and LY gave advice to KW regarding his experiment and modified the manuscript. KW and TH wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Okayama Prefectural Technology Center for Agriculture, Forestry, and Fisheries, Research Institute for Biological Sciences (RIBS), 7549-1 Kibichuo-cho, Kaga-gun, Okayama 716-1241, Japan. [2] College of Animal Science, Southwest University, Rongchang, Chongqing 402460, China. [3] Graduate School of Science, Technology and Innovation, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan. [4] Faculty of Agriculture, Nagase ChemteX Corporation, 1-52 Osadano-cho, Fukuchiyama, Kyoto 620-0853, Japan.

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