Purification and Characterization of a Novel Extracellular Tripeptidyl Peptidase from *Rhizopus oligosporus*

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Abstract

A novel extracellular tripeptidyl peptidase (TPP) was homogenously purified from the culture supernatant of Rhizopus oligosporus by sequential fast protein liquid chromatography. The purified enzyme was a 136.5 kDa dimer composed of identical subunits. The effects of inhibitors and metal ions indicated that TPP is a metallo- and serine protease. TPP was activated by divalent cations, such as Co2+ and Mn2+, and completely inhibited by Cu2+. Enzyme activity was optimal at pH 7.0 and 45 °C with a specific activity of 281.9 units/mg for the substrate Ala-Ala-Phe-pNA. The purified enzyme catalyzed cleavage of various synthetic tripeptides but not when proline occupied the P1 position. Purified TPP cleaved the pentapeptide Ala-Ala-Phe-Tyr-Tyr and tripeptide Ala-Ala-Phe, confirming the TPP activity of the enzyme.

Keywords:
Tripeptidyl peptidase; *Rhizopus oligosporus*; metalloprotease; serine protease

Introduction

Tripeptidyl peptidases (TPPs) cleave peptide bonds to liberate tripeptides sequentially from the free N terminal of polypeptides. TPPs are classified as TPP I (EC 3.4.14.9) and TPP II (EC 3.4.14.10) based on their catalytic mechanisms, molecular weights, and cellular localization.(1) TPP I is an acidic lysosomal protease that exhibits broad substrate specificity; its inhibitory profiles indicate that it may represent an unusual serine protease.(2-4) TPP II is a high-molecular-weight extralysosomal serine protease with a neutral pH optimum that exhibits both exo- and endoproteolytic activities.(5-8) In mammals, TPPs may play important roles in intracellular protein turnover,(1) antigen presentation,(9) peptide hormone and neuropeptide production,(10) bacteria-induced apoptosis,(11) and neurodegenerative disorders.(2)

In addition to the mammalian TPPs, a secreted tripeptidyl aminopeptidase (TAP) from Streptomyces lividans(12) and three secreted sedolisins (SedB, SedC, and SedD) from Aspergillus fumigatus(13) with TPP activity have been identified. Moreover, a protease classified as a prolyl tripeptidyl peptidase (PTP) specific for peptides with a proline residue in the P1 position has also been characterized.(14-17) TPPs in microorganisms may play substantial roles in provision of nutrients and may also contribute to pathogenesis.(13, 15, 16, 18) TPPs are used in industrial hydrolysis of protein sources(13) and in activating zymogens.(14, 17)

Rhizopus oligosporus, a filamentous fungus belonging to the class zygomycetes, is economically and medically important. It has been used for production of industrial enzymes,(19-21) treatment of wastewater,(22) and production of antibiotics that inhibit Gram-positive bacteria.(23) In the food and fermentation industries, R. oligosporus has been used in east and southeast Asia to produce soybean tempeh since ancient times, and there is increasing interest in this food worldwide(24) as a low-fat, high-protein food with high antioxidant activity.(25) Secretion of proteases from R. oligosporus appears to be a critical factor in the production of high-quality tempeh.(26) Furthermore, R. oligosporus has potential as a protease producer because it does not produce toxins.(27) The regulation and production of secretory acid proteases by R. oligosporus during fermentation have been extensively studied.(28, 29) However, the biochemical properties of this specific protease produced by this fungus have not been reported. Therefore, our objective was to purify and characterize the proteases secreted by R. oligosporus.

Materials and Methods

Materials

The chromogenic substrates listed in Table 4 were purchased from Bachem AG (Bubendorf, Switzerland). The peptides Ala-Ala-Phe-Tyr-Tyr (AAFYY) and Ala-Ala-Phe (AAF) were synthesized at Mission Biotech Co., Ltd. (Taipei, Taiwan) using a Symphony Multiple Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ). Pepstatin A, phenylmethylsulfonyl fluoride (PMSF), Pefabloc SC, E-64, N-ethylmaleimide, iodoacetic acid, β-mercaptoethanol, and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO), and Ala-Ala-Phe-chloromethyl ketone was obtained from New England Biolabs (Ipswich, MA). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich.

Strains, Media, and Culture Conditions

Rhizopus microsporus var. oligosporus BCRC31750 (R. oligosporus) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and grown in DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$) under aerobic conditions at 30 °C for 36 h on an orbital shaker at 150 revolutions per minute (rpm). TPP medium (1% glucose, 11.2 mM KH_2PO_4, 7 mM KCl, 2.1 mM $MgSO_4 \cdot 7H_2O$, 0.04 mM $FeCl_2 \cdot 4H_2O$, 0.04 mM $CoCl_2 \cdot 6H_2O$, and 0.07 mM $ZnCl_2$ at pH 7.0) supplemented with different nitrogen sources, i.e., 2% casein, skim milk, beef extract, bovine serum albumin (BSA), Soytone, or NH_4Cl, was used to induce TPP activity.

Purification of Proteases

R. oligosporus (10^7 spores) was cultured in 400 mL of TPP medium containing 2% Soytone as the sole nitrogen source at 30 °C for 3 days. Cells were removed by centrifugation (8000g at 4 °C for 30 min), and the supernatant was collected for enzyme purification. All purification steps were performed using an AKTA purifier 10 system (GE Healthcare Biosciences, Uppsala, Sweden) at 4 °C.

The culture supernatant was concentrated by ultrafiltration using an Amicon Ultra-15 10K Centrifugal Filter Device (10 kDa cutoff; Millipore, Bedford, MA) and dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0). The dialyzed supernatant was loaded onto a HiTrap Q Sepharose Fast Flow column (GE Healthcare Biosciences), previously equilibrated with 50 mM sodium phosphate buffer (equilibration buffer; pH 7.0). TPP was eluted at a flow rate of 0.2 mL/min using the equilibration buffer with a NaCl stepwise gradient of 0–55 mM (10 mL), 55 mM (12 mL), 55–155 mM (10 mL), 155 mM (12 mL), and 155 mM–1.0 M (5 mL). TPP activity in the eluted fractions was measured using Ala-Ala-Phe-p-nitroanilide (Ala-Ala-Phe-pNA) as the substrate. TPP-containing fractions were pooled and

dialyzed against 50 mM sodium phosphate buffer (pH 6.5) in an Amicon Centrifugal Filter Device.

The pooled fractions from the HiTrap Q Sepharose Fast Flow column were further purified on a MonoQ HR 5/5 high-performance column (GE Healthcare Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 6.5). TPP was eluted using a NaCl gradient of 0–120 mM (5 mL), 120–270 mM (15 mL), and 270 mM–1.0 M (3 mL) in 50 mM sodium phosphate buffer (pH 6.5) at a flow rate of 0.15 mL/min. The active fractions were pooled, concentrated, and dialyzed against 50 mM sodium phosphate buffer (pH 6.5). The pooled sample was then subjected to gel filtration through a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 6.5). Elution of TPP was performed using a 50 mM sodium phosphate buffer (pH 6.5) at a flow rate of 0.4 mL/min. Fractions with the highest TPP activity were pooled, concentrated by ultrafiltration, and then stored at −20 °C.

Electrophoresis and Protein Determination

The proteins obtained from each chromatography step were separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to standard protocols.(30) After electrophoresis, the gel was stained with 0.1% silver nitrate. Broad-range protein markers were used to estimate protein size (6.5–200 kDa; Bio-Rad Laboratories, Richmond, CA). Protein concentrations were quantified using a Bio-Rad Protein Assay Kit with BSA as the standard.

Estimation of the Molecular Mass

The molecular mass of the purified enzyme was estimated by both size-exclusion chromatography using a Superdex 200 column and SDS–PAGE. The purified enzyme and a solution of standard molecular-weight markers, such as β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa), were loaded separately onto a Superdex 200 column, using 50 mM sodium phosphate (pH 6.5) as the mobile phase at a flow rate of 0.4 mL/min. Blue Dextran (2000 kDa) was used to determine the void volume. Protein bands on the polyacrylamide gels were analyzed by densitometry, and protein sizes were estimated using Kodak 1D Image Analysis Software (Windows, version 2.0.3; Eastman Kodak, Rochester, NY).

N-Terminal Amino Acid Sequencing

The purified enzyme on the polyacrylamide gel was electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF, Millipore) in 3-(cyclohexylamino)-1-propanesulfonic acid buffer at pH 11.0 and stained with Coomassie Blue R-250. The stained protein band was excised from the membrane and subjected to N-terminal amino acid sequencing. The N-terminal sequence was determined by the Edman degradation method using an ABI Procise 494 protein

sequencer (Applied Biosystems, Foster City, CA).

Enzyme Activity Assay

The activity of purified TPP was determined spectrophotometrically as described by Exterkate.(31) The reaction mixture, containing 100 mM sodium phosphate buffer (pH 7.0), 1 mM $CoCl_2$, 1 mM Ala-Ala-Phe-pNA as the substrate, and the purified enzyme (20 ng), was incubated at 45 °C for 30 min. The reaction was terminated by the addition of acetic acid to a final concentration of 30%, and the absorbance at 405 nm was measured using a Hitachi U3000 spectrophotometer (Hitachi, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μM pNA per minute at 45 °C, with pNA as the standard.

Enzyme Characterization

The optimal temperature for TPP was determined at temperatures ranging from 15 to 65 °C for 30 min in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM $CoCl_2$ and 1 mM Ala-Ala-Phe-pNA as the substrate. The optimal pH for TPP was determined by measuring its activity at 45 °C for 30 min over a pH range of 4.0–9.0 using the following buffers: 100 mM glycine-HCl (pH 4–6), sodium phosphate (pH 6–8), potassium phosphate (pH 6–8), and Tris-HCl (pH 7–9).

To determine the effects of protease inhibitors and reducing agents on TPP activity, the purified enzyme (20 ng) was incubated in 100 mM sodium phosphate (pH 7.0) with each of the chemical agents at 30 °C for 30 min, followed by measurement of its activity under the standard assay conditions described above. To investigate the effects of metal ions on TPP activity, the purified enzyme was incubated at 30 °C for 30 min in 100 mM sodium phosphate buffer (pH 7.0) with 10 mM ethylenediaminetetraacetic acid (EDTA) or 5 mM dipicolinic acid. After the chelating agent was removed by centrifugal ultrafiltration (Amicon Ultra-15 10K Centrifugal Filter Device), the divalent metal salt solution was added and incubation was continued for an additional 30 min before the enzyme assay. TPP activity was assayed using the standard procedure in the absence of metal ions and chelating agents. To avoid interference by the anions, all metal ions were added as chlorides (Table 3). Substrate specificity was examined using a range of synthetic tripeptidyl-pNAs, dipeptidyl-pNAs, and monopeptidyl-pNAs at a final concentration of 1 mM (Table 4) under the standard assay conditions.

TPP Activity of the Enzyme

The TPP activity was investigated using the AAFYY synthetic pentapeptide. The peptide was dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM $CoCl_2$ to a final concentration of 1 mM, and purified TPP (20 ng) was added and incubated at 45 °C for 30 min. The reaction was terminated by the addition of acetic acid to a final concentration of 30%, and the samples were then analyzed by

high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). The HPLC/MS/MS system consisted of two PerkinElmer Series 200 LC micro pumps, a Series 200 Autosampler (PerkinElmer Co., Waltham, MA), and an AB Sciex API 2000 triple quadrupole mass spectrometer with a TurboIonSpray probe (Applied Biosystems). The data were processed using AB Sciex software (Analyst, version 1.3.2; Applied Biosystems).

HPLC analysis was conducted using a reversed-phase Polaris C18-A column (2 mm inner diameter × 50 mm; particle size, 3 μm; Varian, Inc., Palo Alto, CA). The mobile phases were (A) 0.1% formic acid and (B) acetonitrile, and the flow rate was 0.1 mL/min. The linear gradient was initiated with 10% B and was increased to 95% B within 10 min. For MS/MS detection, the TurboIonSpray, orifice voltages, temperature, collision energy, and entrance potential were set to 5500 V, 60 V, 200 °C, 16 eV, and −9.5 V, respectively. The collision gas (nitrogen) was maintained at a pressure of 2.3 × 10–5 Torr. The positive-ion mode and multiple-reaction monitoring (MRM) mode were used to detect AAFYY (m/z 634.3–453.2) and AAF (m/z 308.2–166.1), respectively. Total analysis time was 10 min for each sample.

Results and Discussion

Production of R. oligosporus TPP

TPP production from R. oligosporus reached a maximum of 1.0 unit/mL after 48 and 72 h of incubation when the cells were cultivated in medium containing skim milk or Soytone as the sole nitrogen source, respectively (Figure 1). When the cells were cultured in TPP medium containing casein, BSA, or beef extract as the sole nitrogen source, the maximum activity of TPP was 0.5, 0.6, and 0.2 unit/mL, respectively. No detectable activity was found when NH4Cl was used as the sole nitrogen source. TPP enzyme activity was induced by organic nitrogen sources, which may indicate that this enzyme plays a role in the provision of nutrients. Proteolytic enzymes are secreted by a variety of fungi and likely supply nitrogen for cell survival when inorganic nitrogen in the environment is depleted.(28, 32) The extracellular carboxyl proteinase of R. oligosporus is also suppressed when amino acids are used as a nitrogen source, while the enzyme activity is enhanced when the medium contains protein as the sole nitrogen source.(28)

Purification of *R. oligosporus* TPP

The concentrated supernatant of R. oligosporus BCRC31750 exhibiting TPP activity (18.5 units/mg) was subjected to chromatography using a HiTrap Q Sepharose Fast Flow column and eluted with a stepwise NaCl gradient. The chromatographic profile is shown in Figure 2A. The column yielded four peaks, of which that eluting at 155 mM NaCl was active. The specific activity of the collected active fractions was 24.2 units/mg, with a yield of 22.3%. The active fractions were fractionated on a MonoQ HR 5/5 column. All of the proteins present in fractions 25–31 were active (Figure 2B), and the target enzyme, with specific activity of 29.5 units/mg and a yield of 7.1%, was obtained. All active fractions were further subjected to chromatography on a HiLoad 16/60 Superdex 200 prep-grade column, which resulted in elution of five protein peaks, among which only the third peak showed proteolytic activity (Figure 2C). A summary of the purification steps for TPP produced by R. oligosporus is shown in Table 1. The enzyme was purified 15.4-fold from the culture supernatant, with a yield of 0.9% and a specific activity of 285.4 units/mg.

Low purification yields were also observed with TPP II purified from rat liver and from Arabidopsis thaliana, with 2 and 5% yields, respectively.(7, 8) The yields of the enzyme purified from Prevotella nigrescens and Streptomyces mobaraensis were also low, 8.3 and 4%, respectively.(15, 17) In our previous work, ammonium sulfate precipitation was used to concentrate TPP, which resulted in almost the complete loss of TPP activity. Whether the low yield of the R. oligosporus enzyme reflects greater initial heterogeneity or greater instability of the enzyme remains to be determined.

Purity and Molecular Mass

The purity of the enzyme was confirmed by SDS–PAGE after silver staining (Figure 3). The purified protein migrated as a single band on the denaturing gel with an apparent molecular mass of 70 kDa (lane 4 of Figure 3). The molecular mass of the native enzyme determined by gel filtration chromatography was 136.5 kDa, indicating that purified TPP consisted of two subunits with similar molecular masses.

PTP from Porphyromonas gingivalis is a homodimeric cell surface-associated serine protease with two 77 kDa subunits,(33) while the enzyme from P. nigrescens is a 56 kDa intracellular protease.(15) TAPs from S. lividans and S. mobaraensis have molecular masses of 55 and 53 kDa, respectively.(12, 17)A. fumigatus was reported to secrete the proteases SedB (60–90 kDa), SedC (65 kDa), and SedD (70–100 kDa), which possess TPP activity.(13) The molecular mass of rat spleen TPP I was estimated to be 47 kDa by SDS–PAGE and 64 kDa by gel filtration chromatography.(3) TPP II from rat liver, Drosophila melanogaster, and A. thaliana is composed of a single 135–150 kDa subunit that forms an oligomeric complex with a native molecular mass of ≥1000 kDa.(5-8) Unlike the known TPPs from prokaryotic and eukaryotic organisms, R. oligosporus TPP represents a novel homodimeric extracellular TPP.

N-Terminal Amino Acid Sequence of TPP

The N-terminal amino acid sequence of purified TPP was identified as "S-K-I-Q-V-K-Y-A-T-T-P-K-M-S-T". Protein homology searches revealed no significant similarity with published TPP amino acid sequences in the National Center for Biotechnology Information (NCBI) GenBank database. The highest similarity was observed to Polysphondylium pallidum aminopeptidase N (GenBank EFA77142.1) spanning amino acid residues 297–307, indicating that the purified enzyme possesses aminopeptidase activity. For protein identification, the N-terminal sequence was also searched for within the genome database for Rhizopus oryzae RA 99-880 (http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/Blast.html); however, no detectable similarity was observed.

Effects of the Temperature and pH on TPP Activity

The optimum temperature for TPP activity was determined at pH 7.0 using the substrate Ala-Ala-Phe-pNA; TPP was found to be active between 35 and 55 °C, with maximum activity at 45 °C (Figure 4A). The effect of pH on TPP activity was studied using Ala-Ala-Phe-pNA as the substrate at 45 °C. TPP was found to be active between pH 6.0 and 8.0, with maximum activity at pH 7.0 in sodium phosphate buffer (Figure 4B). The relative activities at pH 6.0 and 8.0 in sodium phosphate buffer were about

63 and 39%, respectively, of that at pH 7.0. Only about 10% of the optimum activity was observed at pH 5.5 and 8.5. These results are similar to those for PTP from P. gingivalis(16) and TAP from S. mobaraensis,(17) which had an optimum pH of 7.0–7.5. An optimum alkaline pH (7.5–8.5) was reported for TAP from S. lividans,(12) while an optimum acidic pH (5.0–6.0) was observed for SedB, SedC, and SedD from A. fumigatus.(13) Mammalian TPP II has an optimum pH of 7.5,(6, 8) while an acidic environment is preferable for TPP I.(3, 34)

Effects of Enzyme Inhibitors and Metal Ions on TPP Activity

To determine the nature of the peptidase, the effects of various inhibitors on enzyme activity were examined (Table 2). TPP was inhibited by the serine protease inhibitors PMSF (1 mM) and Pefabloc SC (1 mM), with 60 and 30% inhibition, respectively. A tripeptidyl chloromethyl ketone analogue, Ala-Ala-Phe-chloromethyl ketone, was also inhibitory. The aspartyl protease inhibitor pepstatin A had no effect on enzyme activity. E-64 (1 mM) inhibited activity slightly, while other cysteine protease inhibitors (N-ethylmaleimide and iodoacetic acid) had no effect on enzyme activity. The cysteine protease activator agents dithiothreitol (1 mM) and β-mercaptoethanol (1 mM) were also ineffective.

These inhibition results are similar to those for TPP from P. nigrescens. Enzyme activity was inhibited by diisopropylfluorophosphate (DIFP) by 60% at 1 mM and 70% at 10 mM and was reduced to about 40% in the presence of Pefabloc SC (1 mM).(15) PTP from P. gingivalis was strongly inhibited by the serine protease inhibitors DIFP and Pefabloc SC, while PMSF had no effect.(16) Low effectiveness of PMSF and differing degrees of inhibition have also been observed for dipeptidyl peptidases II from various organisms and tissues.(35) Variations in inhibition profiles for different serine protease families remain poorly understood. Our results indicate that purified TPP was inhibited, if not strongly, by serine protease inhibitors and may be classified as a serine protease.

The effects of chelating agents and various metal ions on the activity of TPP were examined at pH 7.0 and 45 °C with Ala-Ala-Phe-pNA (Table 3). TPP was strongly inhibited by chelating agents, such as 10 mM EDTA (90%) and 5 mM dipicolinic acid (97%), suggesting that it is a metalloenzyme. After EDTA was removed by ultrafiltration, the enzyme activity could be strongly reactivated and enhanced to 233 and 269.3% of the original activity by 1 mM Mn^{2+} or Co^{2+}, respectively. The addition of 1 mM Mg^{2+}, Ni^{2+}, or Ca^{2+} caused slight recovery of TPP activity to about 19.1, 52.9, and 66.1%, respectively, whereas the addition of 1 mM Zn^{2+} or Cu^{2+} inhibited the activity. Similarly, upon treatment of the enzyme with dipicolinic acid, 288.7, 312.3, and 101% of the original activity could be reactivated by 1 mM

Mn^{2+}, Co^{2+}, or Ni^{2+}, respectively. The addition of 1 mM Zn^{2+}, Mg^{2+}, or Ca^{2+} increased TPP activity to about 7.2, 26.2, and 77.9%, respectively, whereas the addition of 1 mM Cu^{2+} inhibited residual enzyme activity. When TPP was not treated with chelating agents, 1 mM Co^{2+} or Mn^{2+} stimulated activity by 294.5 and 162.8%, respectively. Zn^{2+} reduced TPP activity by up to 50%. Moreover, 0.1 mM $CuCl_2$ inhibited 88.2% of the original activity and completely inhibited activity at 1 mM (data not shown).

On the basis of the above results, the peptidase purified from R. oligosporus was identified as a metallo-dependent TPP, which has not previously been reported in eukaryotes. The enzyme activity of TAP from S. mobaraensis was found to be enhanced by Ca^{2+} and reduced by up to half in the presence of chelating agents.(17) However, in contrast to TPP, TAP from S. mobaraensis is a proline-specific tripeptidyl and tetrapeptidyl peptidase.(14, 17) Dipeptidyl peptidase III, belonging to the serine protease family, is also a metalloprotease harboring a conserved HEXXXH motif as a Zn-binding site, which provides a rationale for substrate recognition.(36) The role of metal ions in proteolytic activity of TPP from R. oligosporus remains to be determined, including the exact function of metal ions in catalysis and the enzyme proteolytic mechanism.

Proteolytic Activity of TPP

Specificities of purified TPP for various chromogenic substrates are summarized in Table 4. The enzyme most actively cleaved Ala-Ala-Phe-pNA, and hydrolysis of Ala-Ala-Ala-pNA was also efficient (about 60% of the relative activity toward Ala-Ala-Phe-pNA). About 10% relative activity was detected for Phe-Pro-Ala-pNA and Pro-Leu-Gly-pNA. No activity toward Ala-Phe-Pro-pNA was observed in the purified enzyme. Hydrolysis of dipeptidyl-pNA, monopeptidyl-pNA, and N-terminally blocked tripeptidyl-pNA substrates was also not detected. These results indicated that purified TPP had no endoproteolytic activity and catalyzed most tripeptidyl-pNA substrates, except when proline occupied the P1 position. Moreover, these data also indicated that purified TPP was free of any contamination with aminopeptidase, dipeptidyl peptidase, and endopeptidase activities.

Substrate specificity of R. oligosporus TPP was similar to that reported for mammalian TPP I and TPP II, which show the highest activity toward Ala-Ala-Phe-pNA.(3, 8) SedB, SedC, and SedD from A. fumigatus also efficiently catalyze Phe-Pro-Ala-pNA and Ala-Ala-Phe-pNA.(13) The TPPs described above show no catalytic activity toward the P1-Pro tripeptide substrates. In contrast, PTPs isolated from S. mobaraensis,(14, 17)P. nigrescens,(15) and P. gingivalis(16) specifically hydrolyze tetra-, tri-, and/or dipeptide substrates with a P1-proline

residue.

To further demonstrate the proteolytic activity of TPP, the synthetic peptide AAFYY was used as the substrate and the resulting reaction products were analyzed by HPLC/MS/MS. Using the full-scan mode for MS analysis, the most abundant [M + H]+ ions for AAFYY and AAF standards were m/z 634.3 and 308.2, respectively; these were selected as the precursor ions for MS/MS analysis. Figure 5 shows the product ion spectra for AAFYY and AAF obtained by infusion in the positive-ion mode. The primary product ions for AAFYY were m/z 290.2, 345.2, and 453.2 (Figure 5A). The primary product ions for AAF were m/z 143.1, 166.1, and 237.1 (Figure 5B). The MS/MS transitions m/z 634.3–453.2 and m/z 308.2–166.1 were then selected for AAFYY and AAF analysis, respectively, using MS/MS in the MRM mode. To determine AAFYY and AAF using HPLC/MS/MS analysis, the standard peptides (10 μM each) were separated by a Polaris C18-A column and subjected to MS/MS. AAFYY and AAF were eluted at retention times of 6.65 and 5.60 min, respectively, in the MRM chromatograms (panels C and D of Figure 5). When purified TPP was incubated with the peptide AAFYY, a mass peak consistent with the degradation product AAF was detected, confirming the TPP activity of the enzyme (Figure 5E).

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