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Synthesis of prolyl-hydroxyproline using prolyl aminopeptidase from *Streptomyces aureofaciens* TH-3

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ABSTRACT

Despite attention devoted to prolyl-hydroxyproline (Pro-Hyp) in nutraceuticals and pharmaceuticals, its enzymatic synthesis has not been achieved. This study investigated aminolysis reaction from proline esters or amide and hydroxyproline using prolyl aminopeptidases from *Streptomyces aureofaciens* TH-3 (PAP TH-3). The effects of pH, buffer concentration, reaction temperature, and the type and concentration of the acyl donor were examined for the aminolysis activity of PAP TH-3. The Pro-Hyp synthesis was conducted in alkaline conditions at low temperature. The type of acyl donor also affected the yield. Finally, optimum conditions were established as 5 μl of 1 M proline amide, 50 μl of 2 M hydroxyl-proline, 40 μl of 1200 mM boric buffer (pH 11), 5 μl of water containing 10 μg PAP TH-3, 4 °C and 3 h. Based on the acyl donor, 30% of the maximum yield was obtained.

1. Introduction

Prolyl hydroxyproline (Pro-Hyp) is known as a component of collagen [1–4]. Recently, Pro-Hyp has attracted much attention as a dipeptide that supports physiological effects of collagen hydrolysate because Pro-Hyp was identified as a major constituent of collagen peptides in human serum and plasma after oral ingestion of gelatin hydrolysate [1,2]. For example, Pro-Hyp exerts a stimulating effect on the growth and migration of mouse skin fibroblasts [3]. In addition, Pro-Hyp affects chondrocyte differentiation in an experiment under pathological conditions using mouse articular cartilage [4].

In general, collagen peptides are prepared through acidic or enzymatic hydrolysis of gelatin, a denatured collagen. This collagen preparation is a heterogeneous mixture of amino acids, dipeptides and oligopeptides. Therefore, purification step is necessary to supply Pro-Hyp from the collagen preparation at a high concentration. It is difficult and laborious to purify Pro-Hyp from collagen. For that reason, a convenient means to synthesize Pro-Hyp directly is needed.

Although chemical modes of dipeptide synthesis such as solid phase synthesis have been known [5,6], such methods require several steps, organic solvents, and restricted reaction conditions. In contrast, enzymatic methods of synthesis present the advantage of processing the specific synthetic reaction under mild conditions in an aqueous system. Particularly, prolyl aminopeptidase (PAP, EC: 3.4.11.5), an exopeptidase that catalyzes the hydrolysis of the N-terminus proline residue of peptides or proteins, is expected to be a good tool for Pro-Hyp synthesis; PAP mediated reaction is conducted in aqueous system and requires non-protected hydroxylproline as acyl acceptor. In thermolysin and trypsin, peptide production by condensation reaction has been reported as caused by a reverse reaction of hydrolysis; for subtilisin, peptide production by an aminolysis reaction has also been reported as caused by a nucleophilic attack of amine residue for water [7–10]. Nevertheless, these enzymes are endo-type, and the reactions require organic solvents for reaction media and N-terminal protections for acyl donors. In this regard, PAP has the potential to overcome these problems by catalyzing aminolysis using a non-protected amino acid as an acyl donor.

To date, in catalysis via wild type PAP mediated aminolysis, proline-containing dipeptides such as Pro-Phe, Pro-Tyr, and Pro-Trp are produced by PAP from *Bacillus brevis* [11]. Recently, diverse prolyl dipeptides were produced by PAP from *Streptomyces thermocyaneoviolaceus* using D-proline or L-proline benzyl ester as acyl donor and several amino acid esters as acyl acceptor [12]. Nevertheless, Pro-Hyp has never been synthesized. Previously, we reported that Ser144Cys variant of PAP from *Streptomyces thermo-*

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luteus subsp. *fuscus* strain NBRC14270 (PAP 14), which is improved its aminolysis activity by ser/cys engineering, produced many proline dipeptides, although Pro-Hyp did not [13]. In this study, we sought to synthesize Pro-Hyp using three PAP from *Streptomyces aureofaciens* TH-3 (PAP TH-3; accession no. AB248820), for which we have already reported the effects of salts on hydrolysis activity [14].

2. Materials and methods

2.1. Materials

L-Proline benzyl ester hydroxyl chloride (Pro-OBzl), L-proline methyl ester hydroxyl chloride (Pro-OMe), L-proline amide (Pro-NH_2 (HCl–)), L-proline amide hydroxyl chloride (Pro-NH_2), L-hydroxyl proline (Hyp-H) and Pro-Hyp were purchased, respectively, from Bachem (Bubendorf, Switzerland), Aldrich Chemical Co. Inc. (St. Louis, MO, USA), Sigma Chemical Corp. (St. Louis, MO, USA), Sigma Chemical Corp. and Wako Pure Chemical Industries Ltd. (Osaka, Japan). We obtained L-Proline *p*-nitroanilide (Pro-*p*NA) from Sigma Chemical Corp. Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All solvents and other chemicals used for this study were of analytical grade.

2.2. Cloning of the PAP gene, expression of PAP in Escherichia coli and protein purification

The *pap th-3* gene has already cloned and the enzyme with C-terminus His-tag has expressed using pET-22b (+) expression vector as previously described [14]. In this study, PAP TH-3 with N-terminus His-tag was expressed using pET-28a (+) expression vector (Novagen Inc., Darmstadt, Germany). The *pap th-3* gene was PCR-amplified using GXL DNA polymerase (Prime STAR®; Takara Bio Inc., Shiga, Japan), a set of a sense primer containing the *Nde*I site (CATATG TCCACCGTCAGCCGGCTG; the start codon is underlined) and an anti-sense primer containing the *Hind*III site at the downstream of the stop codon of genomic DNA (AAGCTTACACCTCGCCGTGGGCCAT; the stop codon is underlined). The amplified DNA fragment was cloned and sequenced using a PCR cloning kit (Zero Blunt II TOPO; Life Technologies Inc., Carlsbad, CA, USA). The PAP coding region was excised by *Nde*I and *Hind*III, and inserted into the pET-28a (+) expression vector.

The resulting plasmid was transformed into *E. coli* BL21-Gold (DE3) (Agilent Technologies Inc., Santa Clara, CA, USA) using GenePulseXcell™ (Bio-Rad Laboratories Inc., CA, USA). Transformants were inoculated into 50 ml of Overnight Express™ Instant TB Medium using an overnight express system 1 (Novagen Inc.). After cultivation at 30 °C for 24 h with 180 rpm of shaking level, cells were collected by centrifugation and disrupted with sonication (Elestin NP035SP; Nepa Gene Co. Ltd., Chiba, Japan) at 1 min intervals for 5 min to a total time of 45 min at maximum output. The obtained cell free extract was purified using cobalt affinity resin (Talon; Clontech Japan) according to the manufacturer's instructions. The purified proteins were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under a reducing condition with Coomassie Blue staining (Fig. S1 in supplemental materials). The protein concentration was measured using a protein assay reagent (Bio-Rad Laboratories Inc.) according to the manufacturer's protocol.

2.3. Assay of PAP hydrolytic activity

Continuous spectrophotometric assay for PAP TH-3 was conducted using Pro-pNA. First, 20 μl of enzyme solution was added to the mixture of 380 μl of water, 320 μl of 200 mM Tris buffer (pH 7.5) and 80 μl of Pro-pNA/DMSO at 37 °C. The increased absorption at 405 nm attributable to the release of pNA per minute was monitored using a spectrophotometer (U2800; Hitachi Ltd., Tokyo, Japan). The initial velocity was determined from the linear portion of the optical density profile ($\varepsilon_{405\,nm}$ = 10,600 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 μmol of pNA per minute under the assay conditions.

2.4. Biochemical characterization of PAP

The optimum pH was determined using phosphate buffer (pH 6.0–8.0) and Tris buffer (pH 7.5–9.0) with 80 mM final concentration. The optimum temperature was determined at 30.0–70.0 °C. Thermal stability was determined as residual activity, which was relative to that stored at 4 °C, by 30 min incubation with optimum pH at 30.0–70.0 °C. The pH stability was determined as residual activity, which was relative to that pH which showed highest activity, by 21 h incubation at 30 °C.

2.5. Pro-Hyp synthesis mediated by PAPs

Typical reaction conditions were the following: 5 μl of 1 M Pro-NH_2/DMSO as the acyl donor, 50 μl of 2 M Hyp-H as the acyl acceptor, 40 μl of 400 mM borate buffer (pH 11.0), and 5 μl of enzyme solution containing 10 μg of PAP (720 mU), at 900 rpm of shaking level, for 3 h at 4 °C. The reaction was terminated by addition of 200 μl of methanol. After centrifugation at 10,000 rpm, 2.5 μl of 33-fold diluted solution of supernatant was applied to liquid chromatography–mass spectrometry (LS–MS) analysis. All experiments were repeated at least three times and are shown as mean ± standard deviation.

Table 1
Biochemical properties of PAP TH-3.

Property	PAP TH-3
Molecular mass of protomer[a]	42 kDa
Quaternary structure[a]	Hexamer
Optimum pH	7.5
Optimum temperature	57 °C
pH Stability	6.5–9.0
Thermal stability	59 °C
Inhibitor[a]	p-(Chloromecurio)benzoic acid, pefablock SC, phenylmethylsulfonyl fluoride

[a] Data were obtained from previous study [13].

2.6. LC–MS MS analysis

An ultra-performance liquid chromatography (UPLC) system (ACQUITY; Waters Corp., Milford, MA, USA) equipped with a TUV detector and binary solvent manager, and an API 2000 LC–MS MS System (AB SCIEX, Foster City, CA, USA) were used. The column used for this study was an XBridge™ amide column (3.5 μm, 2.1 mm × 50 mm; Waters Corp.). The mobile phase was (A) water with 0.1% HCOOH and (B) CH_3CN with 0.1% HCOOH. The gradient profile was as follows: 0–2 min, 2% A; 2–13.5 min, 2–30% A. The flow rate was 0.3 ml/min. To determine the reaction products including Pro-Hyp and substrate, corresponding peak areas of the extracted ion chromatogram were calculated according to a device system of API 2000 LC–MS MS, i.e., multi reaction monitoring (MRM) mode. The specific fragmentation patterns of *m/z* 206.2/91.0, *m/z* 114.7/70, *m/z* 229.0/131.9, and *m/z* 132.1/86.0 were used, respectively for detection of Pro-OBzl, Pro-NH_2, Pro-Hyp, and Hyp-H. The amount of each compound was calculated using the corresponding calibration curve. The yield of Pro-Hyp was based on the acyl donor.

3. Results

3.1. Biochemical properties and kinetic characterization of PAP TH-3

Biochemical properties of PAP TH-3 are presented in Table 1. On thermal stability, PAP TH-3 with N-terminus His-tag has 10 °C superior to that of PAP TH-3 with C-terminus His-tag [14]. The recombinant PAP TH-3 followed the Michaelis–Menten kinetics with respect to hydrolysis of Pro-pNA as a substrate, and data calculated form Lineweaver–Burk plot are presented in Table 2.

3.2. Pro-Hyp synthesis using PAP TH-3

Using LC–MS MS analysis, it was elucidated that Pro-Hyp was synthesized with PAP TH-3 in the reaction conditions of 5 μl of 1 M Pro-NH_2/DMSO, 50 μl of 2 M Hyp-H, 40 μl of 400 mM borate buffer (pH 11.0), 5 μl of enzyme solution containing 10 μg of PAP, at 900 rpm, for 3 h at 4 °C (Fig. 1). The retention times of Pro-NH_2, Pro-Hyp, and Hyp-H were, respectively, 5.9, 8.1, and 9.7 min. In the reaction condition without PAP TH-3, Pro-Hyp was not detected (data not shown).

Table 2
Kinetic parameters of PAP TH-3.

Parameters	PAP TH-3
Specific activity (U/mg)	66.0 ± 0.9
V_{max} (μmol/min/mg)	88.6 ± 3.9
K_m (mM)	1.8 ± 0.1
k_{cat} (s^{-1})	40.8 ± 1.0
k_{cat}/K_m (s^{-1}/mM)	73.6 ± 3.3