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Purification, characterization and the use of recombinant prolyl oligopeptidase from *Myxococcus xanthus* for gluten hydrolysis

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ABSTRACT

Prolyl oligopeptidase (POP, EC 3.4.21.26) is a cytosolic serine protease that hydrolyses proline containing small peptides. The members of prolyl oligopeptidase family play important roles in many physiological processes such as neurodegenerative diseases, maturation and degradation of peptide hormones. Thus the enzyme has been purified and characterized from various sources to elucidate the potential use as therapeutics. In this study recombinant *Myxococcus xanthus* prolyl oligopeptidase expressed in *E. coli* was purified 60.3 fold, using metal-chelate affinity and gel permeation chromatography. The recombinant enzyme had a monomeric molecular weight of 70 kDa. Isoelectric point of the enzyme was found to be approximately 6.3 by two-dimensional polyacrylamide gel electrophoresis. The optimum pH and temperature was estimated as 7.5 and 37 °C, respectively. The purified enzyme was stable in a pH range of 6.0–8.5 and thermally stable up to 37 °C. The K_m and V_{max} values were 0.2 mM and 3.42 μmol/min/mg. The proteolytic activity was inhibited by active-site inhibitors of serine protease, Z-Pro-Prolinal, PMSF, and metal ions, Cd^{2+}, and Hg^{2+}. Furthermore, the hydrolysis efficiency of the recombinant prolyl oligopeptidase was investigated with wheat gluten.

1. Introduction

Prolyl oligopeptidase (POP; E.C.3.4.21.26) is an enzyme that is among serine protease family, with an approximate molecular weight of 80 kDa [1]. POP family includes prolyl oligopeptidase, dipeptidyl peptidase IV (E.C.3.4.14.5), oligopeptidase B (3.4.21.83) and acylaminoacyl peptidase (3.4.19.1) enzymes. This enzyme family differs from trypsin-subtilisin-like serine proteases with their catalytic triad region arrangement and specificities for small peptide substrates. POP hydrolyses peptides consisting of less than 30 residues from carboxy terminal of proline. The role of POP enzyme in hydrolysing bioactive peptides such as thyrotropin releasing hormone, substance P, arginine-vasopressin, neurotensin, angiotensin is known [2,3].

Prolyl oligopeptidase was discovered by the release of leucyl-glycinamide peptide via hydrolysis of oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2) from C-terminus (COO^-) in human uterus homogenate [4,5]. Prolyl oligopeptidase enzyme is widespread such as bacteria, plant, arceae and human [6–14]. Only in yeasts and actinomycetes, prolyl oligopeptidase activity has not been observed [1,15,16]. Although membrane bound form of prolyl oligopeptidase enzyme has been characterized, it is generally reported to be cytosolic [17,18].

Recent studies showed that POP enzyme has an important role in the treatment of diseases such as Alzheimer's, amnesia, depression, cancer and celiac disease. Discovering the potential role of POP in pathophysiology of some diseases has led to many researches for the development and identification of POP inhibitors [2,19–21]. POP enzyme thought to be participating in cheese ripening acceleration and used for production of recombinant functional peptides, gluten degradation for celiac disease cases and antibody directed enzyme prodrug therapy [22].

Expression of prolyl oligopeptidase from *Sphingomonas capsulate*, *Flavobacterium meningosepticum* and *Myxococcus xanthus* have been reported (9, 14, 23). However, functional characterization of recombinant Prolyl oligopeptidase activity have not been fully investigated up to now. Therefore, in this study recombinant production of *Myxococcus xanthus* prolyl oligopeptidase enzyme in *E. coli* and purification of the enzyme with Nickel-Chelate Affinity and gel filtration chromatography were carried out. Characterization of the purified enzyme included explorations of pH and temperature optimum, isoelectric point, molecular weight, substrate

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specificity, kinetic parameters, effects of metal ions, effects of various chemicals and inhibitors; pH, thermal, and storage stabilities.

2. Materials and methods

2.1. Cloning of POP gene

A strain of *Myxococcus xanthus ATCC 25232* was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). The bacteria was grown and maintained on liquid CTT medium (1% Casitone, 10 mM Tris-HCl [pH 8.0], 8 mM MgSO4, 10 mM KPO4 [pH 7.4]). The culture was incubated at 30 >°C for 6 days on an orbital shaker (225 rpm). Genomic DNA from *Myxococcus xanthus* ATCC *25232* extraction was carried with the Invitrogen genomic DNA kit as described (Invitrogen, France). Forward and reverse primers were used based on the *POP* gene sequence of *Myxococcus xanthus* according to the method of Shan et al., 2004 [23]. The cycling conditions were as follows: an initial denaturation of 95 °C for 5 min; 30 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s; and a final extension of 72 °C for 5 min. The PCR product and pET21a (+) were digested with *Nde*I and *Hin*dIII restriction endonucleases. PCR products were separated on 1% agarose gels, extracted and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and then ligated together by T4DNA ligase. Competent *Escherichia coli* cells were transformed with the ligation mixture to obtain the required recombinants.

2.2. Expression and purification of recombinant POP gene

Expression was carried out in *E. coli* BL21(DE3) strain. Cultures transformed with recombinant plasmid were grown in Luria – Bertani medium containing 50 μg/mL chloramphenicol and 150 μg/mL ampicillin. Cultures were induced with 0.3 mM isopropyl thio-b-D-galactoside (IPTG). Before induction, cultures were grown at 37 °C to an A_{600} of 0.6–0.8. The induced cultures were centrifuged at 4.000 rpm for 30 min at 4 °C. The cell pellets were suspended in extraction buffer (50 mM sodium-phosphate buffer, pH 7.4 containing 500 mM NaCl and 5 mM imidazole) per 1 g cell pellet and disrupted by sonication. The lysate was centrifuged at 13,000 rpm at 4 °C for 30 min. The soluble protein was filtered through a 0.45-μm syringe filter (Millex, Millipore), and loaded onto the ÄKTA FPLC system (ÄKTA Pharmacia GE FPLC) equipped with a HisTrap HP column (5 mL, GE Healthcare), with Ni^{2+} ions immobilized on Sepharose. The purification procedure was carried out under increasing gradient of elution buffer (phosphate buffer, 50 mM, pH 7.8; NaCl, 500 mM; imidazole, 250 mM) at the flow rate of 2.5 mL/min. The protein was eluted with a linear gradient of imidazole (0 to 250 mM) in the buffer. Fractions were immediately analyzed for the prolyl oligopeptidase activity. Gel filtration was performed on an Ultragel AcA 44 column (1.8 × 30 cm) attached to a Biorad MPLC system. Runs were performed at a flow rate of 1.0 mL/min and protein elution was monitored at a wavelength of 280 nm. The column was calibrated with bovine serum albumin (66 kDa), peroxidase (40 kDa), proteinase K (28 kDa) and lysozyme (14 kDa). Protein concentration was determined by the Bradford method [24], using bovine serum albumin (BSA, Sigma®) as standard.

The purity of the enzyme was assessed by SDS-PAGE with 4% concentrating gel and 12% separating gel using a Mini PROTEAN II gel system (Bio-Rad Laboratories), according to the method of Laemmli [25]. The isoelectric point of the enzyme was determined using ampholytes with pH 3.0–10.0 (Ampholyte; Bio-Rad) in a 2D-PAGE system (Bio-Rad). A set of standards with range of isoelectric points of 3–10 (Amersham) was used.

2.3. Biochemical characterization

2.3.1. Substrate specificity and kinetic analysis

Prolyl oligopeptidase activity was determined using synthetic and natural substrates. The mixture (0.2 mL) contained 10 μL enzyme solution, 2 mM Suc-Ala-Pro-pNA (Bachem AG, Bubendorf, Switzerland) as chromogenic substrate and 100 mM Tris-HCl buffer (pH 7.4). After incubation for 5 min at 37 °C, the amount of liberated *p*-nitroaniline (pNA) was calculated by spectrophotometric absorption at 405 nm. One unit of prolyl oligopeptidase activity (U) was expressed as micromoles of substrate hydrolyzed per minute by the enzyme [25]. The POP activity was also assayed on other chromogenic substrates (Leu-pNA, Pro-pNA, Glu-pNA, Glu-Ala-pNA, H-Ala-Pro-pNA, H-Arg-Pro-pNA, H-Val-Ala-pNA, H-Gly-Pro-pNA, Suc-Ala-Pro-pNA, Z-Gly-Pro-pNA, and Suc-Gly-Pro-pNA). Activity of recombinant POP preparate from *Myxococcus xanthus* tested against natural substrates IGF-1 (H-Gly-Pro-Glu-OH) and Substance P (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) by RP-HPLC analysis. Kinetic parameters estimated by the use of Suc-Ala-Ala-Pro-pNA as substrate (0.05–2 mM). Assays carried out under standard conditions (37 °C, 5 min) and replicated 3 times. GraphPrism 6 used for evaluation of Km, V_{max}, and K_{cat}.

2.3.2. Effect of temperature and pH on activity and stability of recombinant POP

The optimal pH was determined by measuring the activity between pH 6.0 and 10.0 (100 mM) using sodium-phosphate (pH 6.0–7.5), Tris-HCl (pH 6.0–7.5) and sodium-borate buffer (pH 9.0–10.0) at 37 °C. The optimum temperature for enzyme activity was determined by performing the enzymatic reaction in temperatures ranging from 4 °C to 80 °C. The pH stability was studied by pre-incubating the enzyme for 30 min at 37 °C at different pH values ranging from 3.0 to 10.0 using buffered substrate. In addition to that, thermostability studies of the enzyme investigated by preincubating the enzyme solution at different temperatures ranging from 4 to 55 °C for 30 min at constant pH 7.4. Finally, the storage stability of the enzyme was tested at 4 °C.

2.3.3. Effect of various chemicals, inhibitors and metal ions on recombinant POP

In order to determine the effects of metal ions, chemical reagents and classical protease inhibitors on the enzyme activity with inhibitors were pre-incubated at 37 °C for 30 min. The relative activity, defined as the activity value relative to the activity of control (without any effector), was also estimated. IC_{50} values were only determined for POP inhibitors by nonlinear regression analysis from the residual activity versus inhibitor concentrations curve.

2.4. Hydrolysis of gluten with recombinant POP

The gluten 3% (w/v) was suspended in 0.1 M sodium-phosphate (pH 7.4) and heated at 90 °C for 10 min. Recombinant POP was added at an enzyme-to-substrate ratio of 4.6 U/mg and hydrolysis was carried out at 37 °C for 8 h with constant agitation. Reaction was terminated for the samples that were taken from the media at defined time intervals by heating in 90 °C water bath for 15 min. The hydrolysates were centrifuged at 9000 rpm for 15 min to remove the unhydrolyzed residue, followed by lyophilisation and storage at −20 °C until further use. Free amino groups were measured using TNBS and the degree of hydrolysis (DH) was calculated as:

$$DH(\%) = [(h - h_0)/h_{tot}] \times 100$$

where, h_{tot} is the total number of peptide bonds in the protein