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Short Communication

Discovery of Novel Flavonoids Fractions: Based Enzymes Inhibitors of Prolyl Oligopeptidase

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ABSTRACT

The following work describes the purification of recombinant expressed Prolyl Oligopeptidaseby GSH affinity capture beads *via* a GST-tag and protease cleavage site. The purification steps were monitored with Commission stained protein gel analysis. Therefore, an enzyme inhibition assay design for a well-established internally quenched fluorophore as substrate was established. Ethylacetate (flavonoid extract), hexane and dichloromethane plant extracts of different species of Allexis were analyzed for inhibition on POP.

Keywords: Prolyl oligopeptidase; Enzyme activity assay; Flavonoids extracts; Flavonoids inhibitors

INTRODUCTION

Proteases are the largest family of enzymes in the human organism. More than 550 genes 2% of the human genome encode proteolysis enzymes. Almost 600 of these enzymes, capable of cleaving proteins or peptides by hydrolyzing peptide bonds, have been identified so far. Based on their catalytic mechanism, proteases can be divided into five classes: Metallic, serine, cysteine, threonine and aspartic acid proteases. Additionally, they can be classified by their ability cleaving on a specific side or in the middle the targeted protein or peptide. The database MEROPS is the most comprehensive information resource for proteases, protease inhibitors and its classification. The database categorizes proteases and their protein/peptide-as well as small molecule inhibitors according to their name, an identification number and/or the source (microbes, plants, animals) [1].

Since the discovery of the first digestive enzymes pepsin by Schwann in 1836 and trypsin by Corvisart in 1856, proteases have been assigned too many other functions than protein digestion and intracellular turnover. Since then, proteases have been described to play a crucial role in physiological intra and extracellular processes such as cell-cycle progression, cell proliferation, cell death, DNA replication, tissue remodeling, homeostasis, wound healing and immune response [2]. It has been shown that dysregulated protease activity in such biological mechanisms is associated with serious illnesses, for instance coagulopathies, inflammation, infectious diseases, cancer and neurodegenerative diseases.

To identify and validate a specific protease as drug target, its complexity of its up and downstream effects, the mechanism of the protease activity (mechanism of action) and regulation and its underlying biochemistry including the Structure-Activity-Relationship (SAR) need to be understood before efficient agents with appropriate pharmacokinetic features can be designed. The starting point is the knowledge about these characteristics under normal physiological conditions to be able to investigate its changed properties in disease. A general approach to understand the biological role and the signaling pathway of a protease is the identification of its physiological substrate. To identify the substrate of an enzyme, different approaches were used in the past including a "bottom-up" strategy by searching for the protease of a known substrate, in silico biology, genetic approaches using knocking/knockout animal models or proteomics. But to see the whole picture of its physiological impact, it is not only important to see the immediate effect on the substrate, but also the following downstream effect. The entire enzyme pathway is tightly regulated at numerous levels consisting of the transcription, translation and post-translational modifications. In addition, most proteases are initially expressed as zymogen precursors that have to be activated by upstream proteolysis processing to control the spatial and temporal location of their activity.

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On the other side of the spectrum of enzyme dysregulation is excessive proteolysis. It has its origin in various endogenous and exogenous factors resulting in an unwanted activation of the enzyme caused by an increased activation or a loss of an endogenous key inhibitor. Genetic reasons such as mutations in genes encoding for enzymes can cause abnormally high concentrations (overexpression), but also point mutations leading to an enhanced specific activity due to a structural change in the active side of the enzyme can be the cause of increased levels of product formation. The major strategy and most widely explored approach to target overactive enzymes and its signaling pathway is the use of appropriate inhibitors. Already successfully targeted proteases by inhibitors in human disease therapy are for instance the renin and angiotensin converting enzyme.

RECOMBINANT PRODUCTION OF PROLYL OLIGOPEPTIDASE

Expression of POP in BL21 (DE3)

The cDNa clone of the human Prolyl Oligopeptidase (POP) was inserted into a commercially available pGEX-6P expression vector by customized service of Gene script Biotech (The Netherlands). The pGEX-6P1 vector is characterized by an ampicillin resistance gene, a sequence for an N-terminal GST-tag and a PreScission. The PreScission site consists of a recognition site and enables the specific cleavage between the Gln and Gly residues by the PreScission protease, a genetically engineered fusion protein from human rhinovirus 3C protease and Glutathione S-Transferees (GST).

The expression plasmid was transfected into an *E. coli* expression cells (BL21 D3 strain), plated on LB agar plate for selection of AmpR clones and three clones were used for DNA purification and DNA sequencing by LGC Genomics (Berlin). Until usage the cells were stored in a glycerol stock at -80°C. In the course of this work, two batches of recombinant Prolyl Oligopeptidasewere prepared, which had the quality as well as quantity for characterization and application in the inhibition assays. These two batches will be referred to as batch A and batch B in the further [2].

100 µg/ml AMP was added to avoid unspecific bacterial growth. For the protein expression a pre-culture was prepared from an initial inoculum of the glycerol stock of sequenced clones. A pipette tip was scratched into the glycerol stock and was added into a 15 ml Falcon Tube with 5 ml LB Media. The Falcon Tube was put into an incubator with a loose cap for 5-6 hours at 37°C and 120 rpm. Afterwards, 2-3 ml of the pre-culture was transferred in a 200 ml laboratory flask (scale up) with 50 ml fresh LB Media inclusive AMP. The flask was covered with aluminum foil and incubated at 37°C, 120 rpm overnight. The next day, 15-20 ml of the second pre-culture were transferred to 200-300 ml fresh LB media inclusive AMP in a 1 L flask. During the growth process optical density was continuously measured with a UV/Visible spectrophotometer. At this point, all cells should be in the same cell division stage [2,3]. Thereafter, 0.3 mm IPTG was added to Batch A and 0.5 mm IPTG to Batch B to induce the protein expression of POP. As a negative control of protein expression, no IPTG was added to 100 ml of the bacterial culture. For protein expression the flasks were shaken on an orbital shaker (GFL 3020, Germany) at room temperature (~150 rpm) until an OD of 1.4-1.5. Expression at lower temperatures is intended to prevent excessive formation of inclusion bodies. For cell harvest, the bacteria culture was centrifuged with an Avanti JXN-26 (Beckman Coulter) for 15 minutes (15000 rpm) at 4°C. The supernatant was discarded and the cell pellet was shock frozen with liquid nitrogen and stored at -80°C until further use.

Purification of POP *via* GST-affinity capture beads

The modifications of the protein, the GST-tag as well as the PreScission cleavage site, were used for the further protein purification. To maintain the bioactivity of the enzyme, all working steps of the protein purification were performed on ice and all equipment was set to 4°C. During the purification process, aliquots of 200 µL were taken and stored at -20°C until further analyzes. All used reagents for the protein purification and equipment. Sodium phosphate buffers were adjusted to pH 8.0 with HNa_2O_4P and NaOH with a pH meter and stored at 4°C until use.

Cell wall dissociation

For cell wall dissociation the cell pellet was suspended with 30 ml Lysis Buffer by up and down pipetting. After that, it was transferred to 50 ml Falcon tubes and treated with three freeze-and-thaw-cycles with liquid nitrogen and a water bath (37°C). Subsequently, the suspension was processed with an Ultra sonic stab connected with a power supplier for seven cycles. Then the samples were centrifuged for 30 min with a Sigma laboratory centrifuge 1-14 K (15000 rpm) to separate the soluble proteins from membrane proteins. The obtained supernatant was used for further purification. The remaining pellet was used to dissolve insoluble membrane proteins.

Sepharose bead reactivation

The GSH-loaded Sepharose b (SERVA) was stored in at 4°C in 20%. To reuse the beads, the following reactivation protocol was applied. According to the manufacturers manual (SERVA) 5 mg of GST-tagged protein can be expected from 1 L bacterial culture. Therefore, it was assumed that 1200 ml bacterial culture produced approximately 6 mg GST-tagged POP. With a binding capacity of 8 mg protein/ml settled GSH beads, there was sufficient binding capacity for 32 mg protein to ensure that all GST-tagged proteins were captured [4].

Soluble proteins in the Lysis Buffer were added to the reactivated GSH beads in the 15 ml Falcon Tube. The Falcon tubes were shaken on a Tube Rotating Shaker for 21 h at 4°C to allow binding of the GST-tagged proteins on the beads [3]. On the next day, the tubes were centrifuged for 3 min (3500 rpm) with the Misgauge 1.0 R. A 200 µL aliquot was taken from the supernatant for further analysis and the rest of the supernatant was discarded.

Following washing steps were carried out with each falcon tube to wash off unspecific bound compounds. The beads were mixed with the buffer, well mixed and centrifugation afterwards. Between each step 200 µL aliquots were taken and stores at -20°C for further analysis. The remaining supernatant was discarded.

The theoretical mechanism of the PreScission protease consists of the cleavage between the Gln and Gly residues of the recognition sequence of the PreScission site and its ability to bind to the immobilized glutathione beads *via* its GST protein. PreScission protease was added to the GSH beads with the expected protein in a ratio of 1:20. Additionally, 1 ml of PreScission cleavage Buffer was added to allow movement within the falcon tube. Furthermore, 50 µL of fresh agarose was added assuming that all binding capacity could be already occupied. 50 µL of fresh agarose beads were washed with ddH_2O twice centrifuged in between and mixed with 150 µL PreScission cleavage buffers. 50 µL of the suspension was added to each falcon tube with the sample. The tubes were shaken at 4°C on the Tube Rotating Shaker to allow cleavage of the protein and binding of the PreScission protease overnight [5].

Elution of the product prolyl oligopeptidase

The product recovery was conducted by transferring the slurry agarose beads with a cut pipette tip into Pierce Spin Columns. The soluble product was spanned down for 30-45 seconds and was collected in 1.5 ml vials. A new vial was placed under the spin columns. The falcon tubes were washed with 1 ml PreScission cleavage Buffer and transferred to the Spin Columns as well. The agarose beads in the spin columns were rinsed with 1 ml PreScission cleavage buffer to collect any remaining product. The elution product (POP) was stored at -80°C.

Elution of the PreScission protease and the GST-tag

The agarose beads in the Pierce Spin Columns were transferred back into the 15 ml Falcon tubes with a cut pipette tip with ddH_2O. The water was discarded after centrifugation. 7.5 ml of GSH Elution Buffer was freshly prepared and 2.5 ml were added to each centrifugation tube and incubated on a vertical shaking plate for 2 h at room temperature [6]. Afterwards, an aliquot of the supernatant was taken for further analysis and the remaining buffer was discarded. The agarose beads were washed with water and stored in 20% EtOH at 4°C.

Release of insoluble proteins of the membrane fraction

After the separation of the soluble proteins from the cell pellet, the pellet was centrifuged with 0.5 ml Lysis Buffer for 30 minutes with the Sigma laboratory centrifuge 1-14 K (13000 rpm). The supernatant was discarded; the pellet was mixed and shaken with 200 µL Urea Buffer for 4 h on the Thermomixer (37°C) until the pellet had dissolved [3]. The samples were centrifuged again for 30 minutes (13000 rpm). Next, 200 µL of the resulting supernatant were taken out as insoluble membrane proteins (Aliquot: Membrane fraction).

BICINCHONINIC ACID ASSAY

Protein concentrations were measured from all taken aliquots with a PierceTM BCA Protein Assay Kit (Thermo scientific) according to the manufactures manual. A standard curve was generated with bovine standard albumin in a concentration range of 25-2000 µg/ml solved in Lysis Buffer without additives. The aliquots of the soluble proteins, the empty supernatant and the membrane fraction were diluted 1:5 and the GSH-Eluate 1:10 before measuring its protein concentration. Absorption was measured at 562 nm in a 96 well plate with a Synergy™ H4 Hybrid Multi-Mode Micro plate Reader (Biotech Instruments) [5,7]. Measured absorption values were blanked against Lysis Buffer without additives, based on the incompatibility of DTT and the assay components. Calculated protein concentrations were used to normalize the aliquots to 2 µg/µL for the following protein gel analysis. The protein concentration was determined in two replicates for each batch before it was used for the enzyme assays [7,8].

Protein gel analysis and commission staining

Protein gel analysis was performed to follow up the purification steps of the isolation of POP. All used reagents for the protein gel analysis; prepared buffers and the composition of the protein and used equipment. For the purification steps of Batch A (induced with 0.3 mm IPTG), a 8% SDS gel was prepared, whereas a 8% and a 10% SDS protein gel was used for Batch B (0.5 mm IPTG) [9].

Extraction and isolation

Dried and powdered root of each Allexis specie (300 g) were extracted with MeOH (0.5 L) at room temperature and evaporated under vacuum to yield a crude extract. 50 g of these extracts were dissolved in MeOH-H_2O (8:2) and partitioned with n-hexane, dichloromethane and ethyl acetate (3 × 50 ml).

Protein gels of the isolation of prolyl oligopeptidase

Protein concentrations of taken aliquots were measured with a BCA Assay as described in chapter 2.4.1. The samples were normalized to a protein concentration of 2 µg/µL by diluting them with 12 µL 5 x LD and ddH_2O to a total volume of 60 µL. 20 µL of this dilution were loaded onto the gel, so each lane contain 40 µg protein. Exceptions were samples with too low protein concentrations to reach 2 µg/µL or concentrations that were no longer measurable [10].

Expressed GST-tagged Prolyl Oligopeptidasewere expected at 106.6 kDa, consisting of POP with 81.6 kDa and the GST-tag with 25 kDa. The soluble proteins (lane 2) showed a signal at the height of approximately 105 kDa. Based on a signal of the same size in lane 3 (empty supernatant) it may be possible, that not all of the enzyme could bind to the agarose beads. Interestingly, the same signal appears at lane 4 consisting of the soluble proteins of a non-induced bacterial culture. Therefore, another, more likely, reason of the signal in lane 3 and 4 at the level of the GST-tagged POP could be another expressed protein in *E. coli* cells with the same molecular weight or electrophoretic

migration [10]. The lanes of the washing significant signal loss indicating that all unspecific proteins should now be washed off the agarose beads. Lane 7 represent the elution of beads bound GST-fusion proteins using PreScission Protease and 8 the elution control through rinsing the beads with 2 ml PreScission cleavage buffer. Additionally, the glutathione transferees with a migration at corresponding to about 25 kDa and a very slight signal at 60-70 kDa of the cleaved PreScission protease are visible. The signal at cleaved POP (~80 kDa) could be resulted from unspecific bound POP released through a pH change from 7.5 (PreScission cleavage buffer) to 8.0 (GSH elution buffer). The membrane fraction (soluble proteins after treating the remaining cell pellet with 8 M urea) showed an intense signal at the height of GST-tagged POP suggesting the formation of inaccessible inclusion bodies.

The purity of POP compared within the two manufacturers demonstrated small differences of the binding efficiency of the PreScission protease. The elution from SERVA beads yielded a slightly purer product than the elution of Micherey-Nagel. No difference was visible in in lane 14 to 15. Both lanes show signals at the height of POP, PreScission protease and GSH. Hence, elution efficiency was independent by the bead manufacturer and future optimization steps could be performed in other directions.

Optimized enzyme concentration

First activity experiments were performed with 88.5 ng POP per well and showed a concentration-dependent cleavage of Z-Gly-Pro-AMC by POP, but impractical signal response in the initial phase. All substrate concentrations resulted into the same slope of the initial linear phase, so that a calculation of the enzyme activity was not possible. The substrate was dissolved in 100% DMSO and diluted in Tris-HCl (pH 7.6) with 1 mm EDTA. To observe the influence of enzyme concentrations, it was reduced to 50 mg, 25 mg and 10 mg per well in the following three adjacent follow-up experiments [11].

These experiments were only performed once, to gain principal information on how much enzyme to use for further experiments. Slopes with 60 µM substrate showed the weakest linearity in all experiments. Based on the better separation of the observed concentration-response curves and the limited amount of recombinant POP, 25 ng was chosen for further experiments. Additional limitations are the different concentrations of solvent per substrate concentration, which were investigated in the following experiments.

Optimized solvent of the substrate Z-Gly-Pro-AMC

Previously, bovine POP was reported to have strong independence on the organic solvents in the assay which is needed to prepare stock solution of the substrate (REF). Hence, we intended to optimize the 'solvent' as an assay parameter to gain optimal conditions for dose-response and enzyme activity over assay time. Four different solvents were investigated in four different concentrations [12]. To investigate the effect of the solvent concentration on the enzyme activity, and to eliminate possible adverse impact, four different solvents were tested in four final concentrations. Studied solvents were Methanol, 1,4-Dioxane, Isopropanol and DMSO in 2, 4, 5 and 10% per well.

Assuming that enzyme activity corresponds to the amount of fluorescence signal generated by the enzyme able to cleave the substrate, 2% isopropanol is the optimal solvent for measuring POP activity out of all screend variations. A decreasing activity with increasing concentration was observed for isopropanol, 1,4-Dioxane and DMSO. In contrat, the fluorescence signal in response to methanol was similar for all concentrations, but overall an activity loss of approximately 50% compared to 2% isopropanol was noticeable. 1,4-Dioxane showed the most adverse impact on the activity of POP in all concentrations.

OPTIMIZED BUFFER CONDITIONS

Response-dependent curves were created by incubating 25 ng POP with 50 µM substrate and each buffer for 4 hours. Buffer systems in course of the optimization were 100 mm Tris-HCl with 1 mm EDTA (pH 7 and pH 7.6), 100 mm HEPES with 1 mm EDTA (pH 7) and 100 mm phosphate buffer with 1 mm EDTA (pH 7). For data evaluation, the final responses and the velocity of the linear phase of two experiments were compared [13].

Considering the velocity of the enzyme using different buffer systems, Tris-HCl (pH 7.6) showed the best results, but was excluded due to the final response compared with HEPES and phosphate buffer. Phosphate and HEPES buffer showed similar results in the final fluorescence signals, but unspecific signal fluctuations in the background with phosphate buffer were observed [13].

NaCl to adversely effected on the activity of POP with increasing salt load, whereas DTT affected POP in an non-linear relationship for the limited testing range by reducing its activity from zero to 0.5 mm DTT and then increasing the activity to 5 mm DTT.

FINAL KINETIC ENZYME ACTIVITY ASSAY

The enzymatic activity of POP was determined kinetically in a black 96-well plate. Final test conditions for the kinetic measurement were 100 mm HEPES buffer with 1 mm EDTA and 5 mm DTT adjusted to pH 7. The solvent of choice for the substrate stock solution was 40% isopropanol leading to a final concentration of 2%. The initial velocity in the linear range from eleven substrate concentrations was used to calculate the velocity in µmol/min. Following, concentration-dependent curves were generated by co-incubating distinct substrate concentrations with 25 ng of POP. The data are represented as the mean ± SD of three independent measurements. The values were blanked by subtraction with the control consisting of buffer and substrate for each time point [14].

Substrate concentrations higher than 150 µM had adverse effects on the response. A possible cause of these observed signal fluctuations could be issues with solubility of the substrate. Prior attempts at solubility in the preparation of a stock solution have already shown difficulties. Therefore, only those response

curves up to 150 µM were used for the further determination of the enzyme activity. The endpoint fluorescence signals were used to generate a calibration curve to calculate the velocity of POP in the initial phase substrate processed per minute'. The maximum velocity (V_{max}) of the enzyme and the Michaelis constant (km) were determined with Graph Pad Prism for both graphs, resulting in slight different values [12]. The Lineweaver-Burk plot is only displayed for visualization, because it does not obey the assumption of linear regression due to a distortion of the experimental error caused by the double-reciprocal transformation of the data points. Hence, the intercepts are not suitable to obtain accurate values for V_{max} and km. The more accurate way to get the values for V_{max} and km is the nonlinear regression, as used in the Michealis-Menten diagram. Thus, a V_{max} of 0.0001473 µmol/min and a km of 42.35 µM were used for the calculation of the enzyme activity and the assay design for the inhibition tests, respectively. The final enzyme activity was calculated from V_{max} [15].

CONCLUSION

Five different flavonoids enriched plant extracts and hexane plant extracts from Allexis species and CH_2Cl_2 fraction of two species of these plants were screened for their inhibition activity against POP. Substrate concentration was set to the calculated km of 42.35 µM. Four concentrations (c (µg/ml)=400, 200, 100, 50) were tested and compared to 1 µM synthetic KYP-2047 as positive control. The enzyme activity without inhibition (100%) was used for the calculation of the inhibition. This review allows us to conclude that Allexis species can be used for the treatment of many diseases due by the production of human POP in the organism.

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