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Pleiotropic enzyme activities of genetically engineered human prolidase

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Abstract: **AIM** To investigate whether the human prolidase possesses the G-type organophosphate hydrolyzing enzyme(Gase) activity besides its ability to catalyze the hydrolysis of the dipeptides bearing a proline residue at the C-terminus. **METHODS** Genetic engineering techniques were used in the cloning and expression of the recombinant human prolidase. Prolidase and Gase activities were assayed in the conventional ways. **RESULTS** The recombinant human prolidase expressed in COS-7 cells catalyzed the hydrolysis of organophosphorous compound soman as well as the hydrolysis of dipeptide Gly-Pro. Both activities were two-folds higher than that in the non-transformed COS-7 counterpart. Comparison between the two activities in COS-7 cells transfected with the recombinant vector containing the prolidase gene and the control cells showed parallel elevation with a constant ratio. **CONCLUSION** It is infered that the Gase and the prolidase are of the same enzyme, or at least belong to isozyme.

Key words: hydrolases, organophosphorous compounds; prolidase; liver; dipeptides; soman; human

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G-type organophosphate hydrolysing enzyme (Gase) is capable of hydrolyzing a wide variety of organophosphorous cholinesterase inhibitors, such as DFP, soman, sarin and tabun[1]. The wide phylogenetic distribution of Gase in prokaryotes and eukaryotes[2-6] suggests that the enzyme serve an important function, and it is of interest to note the potential use of Gase in decontamination and scavenging of these extremely toxic materials. The recombinant organophosphorous nerve agents degrading enzymes from *Alteromonas undina*[7] and *A. haloplanktis*[8] have been shown existing prolidase activity. The primary structure of the human prolidase has also been sequenced[9]. These experimental observations prompt us to clone and express the human prolidase gene, and try to find out whether the recombinant prolidase exhibits Gase activity as well.

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1 MATERIALS

Human normal liver tissue encompassing a hepatic angioma was dissected immediately after the surgical operation from a female adult. Stored at once in liquid nitrogen, and ready for use.

Restriction endonucleases, Klenow fragment, T4 DNA ligase and Taq plus Ⅱ DNA polymerase, PinPoint X_{a-1}-T and pGEM-7zf(+) plasmids, Wizards plus Minipreps DNA purification system, RNAgent Total RNA Isolation System, Reverse Transcription System, *E. coli* JM 109 were purchased from Promega; Dulbecco's modified Eagle's medium(DMEM) and lipofectin reagent from Gibco BRL; fetal calf serum from Tianjin Biochemical Products Co.; trypsin from Difco Co.; DNA rapid purification and recovery reagent kit from Tian Xiang Ren Co.; pSVL plasmid and COS-7 cells were kindly provided by Dr. LONG Jian-Yin and Institute of Basic Medicine respectively. All other reagents were analytically pure.

2 METHODS AND RESULTS

2.1 Reverse transcription

Total cellular RNA was isolated from the human liver tissue according to the protocol of the

kit. Synthesis of cDNA was carried out in the reaction buffer (mmol·L^{-1}: Tris-HCl 50, pH 8.3, KCl 75, $MgCl_2$ 3, dithiothreitol 10) using 2.5 μg of total cellular RNA (denatured at 65℃ for 10 min), 20 pmol of oligo$(dT)_{15}$ (prolidase mRNA-specific downstream antisense oligonucleotide primer), 10 U of human placenta ribonuclease inhibitor, 200 U of M-MLV reverse-transcriptase and 1 mmol·L^{-1} dNTP in a final volume of 20 μL. The mixture was incubated at 42℃ for 1 h, then inactivated the reverse transcriptase in boiling water for 5 min.

2.2 PCR amplification

One tenth of the total cDNA product and 40 pmol of each primer (P1 + P2 or P3 + P4) (Tab 1) were added to 50 μL of reaction buffer(mmol·L^{-1}: Tris-HCl 10, pH 8.3, KCl 25, $MgCl_2$ 1.5 and 200 μmol·L^{-1} dNTPs), layered with 50 μL of liquid paraffin, and incubated at 95℃ for an initial 5-min denaturation. The PCR amplification was started by adding 2.5 U of Taq DNA polymerase, carrying out 30 PCR-amplification cycles (1 min at 94℃, 1 min at 55℃ and 1.5 min at 72℃ in tandem), and then a final elongation at 72℃ for 7 min. The RT-PCR products were isolated by agarose gel electrophoresis and extracted by glassmilk.

Tab 1. Oligonucleotide primers for PCR amplification of human prolidase cDNA

Primer orientation	Primer sequence	Location
P1(sense)	5′-AACATGGCAGCGGCA-ACCGGACCCTCGTTTTGG-3′	3 – 30
P2(antisense)	3′-CGTTCAAGTGACGTC-TGGT-5′	887 – 905
P3(sense)	5′-CTCCTTTGACGGCAT-CAGC-3′	483 – 501
P4(antisense)	3′-GGTTCATCTCGGTCG-GTCTTTACCTAGGCGC-5′	1475 – 1505

The full length of the prolidase gene was amplified in two steps. At first, a 0.9 kb fragment was obtained using primers P1(sense) and P2(antisense), and a 1.0 kb fragment using primers P3 (sense) and P4(antisense). Then the 0.9 and 1.0 kb fragments were used as the templates for amplification of the 1.5 kb full length human prolidase gene. The prolidase gene was then constructed into the PinPiont$^{TM}X_{a-1}$-T vector for identification of the correctness of the whole nucleotide sequence.

2.3 Construction of eukaryotic expression plasmid of human liver prolidase

The recombinant PinPoint^{TM} X_{a-1} plasmid bearing human prolidase gene was inserted into plasmid pGEM-7zf(+) (3.0 kb), and then into pSVL plasmid(4.9 kb) to construct the expression plasmid pSVL-P(Fig 1). The correctness of the recombinant plasmid was verified by restriction mapping.

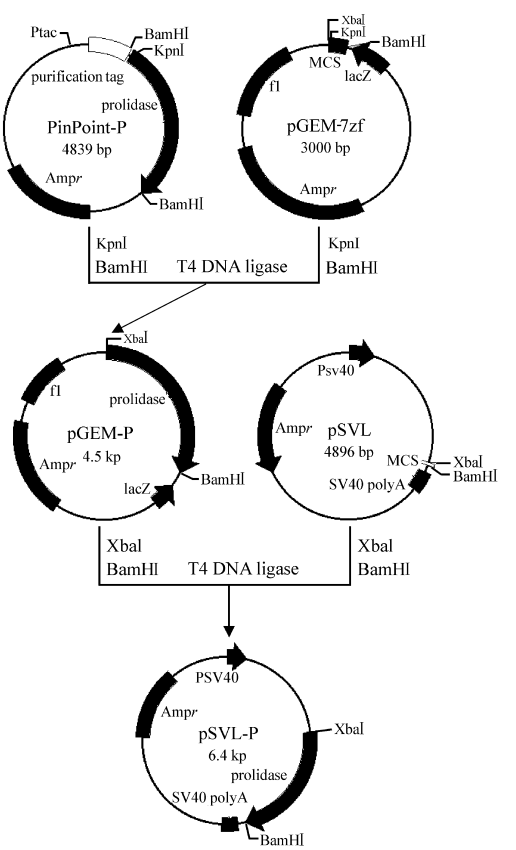

Fig 1. Construction of recombinant plasmid pSVL-P

2.4 Transient expression of recombinant human liver prolidase gene in COS-7 cells

COS-7 cells (5×10^5) grown in DMEM containing 10% calf serum, 100 kU·L^{-1} peni-

cillin, 100 kU·L^{-1} streptomycin at 37℃ in 5% CO_2 incubator were washed with DMEM (serum-free).

Human prolidase expression plasmid pSVL-P (8 μg DNA) in DMEM and lipofectin reagent (20 μg) in DMEM were mixed, added to the washed COS-7 cells in 2.4 mL of DMEM and incubated for 10 h. COS-7 cells were then transferred into DMEM containing 10 % calf serum and cultivated successfully for 54 h. Finally, the cells were harvested and washed with Na/K phosphate buffer (67 mmol·L^{-1}, pH 7.2). COS-7 cells transformed with pSVL plasmid without prolidase gene was performed in the same way as a control.

The harvested COS-7 cells were subjected to three cycles of freezing (in liquid nitrogen) and thawing. Supernatant of the cell lysate was collected and kept frozen at −20℃ ready for the enzyme assay.

2.5 Assay of prolidase and Gase activities

Prolidase activity was determined colorimetrically[10] using Gly-Pro dipeptide as the substrate. Proline was used for the calibration curve. The supernatant of COS-7 cell lysate was added to 100 mL of Gly-Pro in 0.3 mol·L^{-1} Tris-HCl (pH 7.8) containing 3 mmol·L^{-1} $MnCl_2$, incubated at 37℃ for 30 min, terminated with 300 μL glacial acetic acid and developed with 500 μL 3% ninhydrin (dissolved in 70℃ heated glacial acetic acid and phosphoric acid, 3∶2, *V*/*V*) at 100℃ for 10 min. The absorbance was read at 492 nm. The Gase activity was assayed by the colorimetric method[11] using soman as substrate in presence of Mn^{2+} ion.

The enzyme activity assays showed that the recombinant plasmid SVL-P (6.4 kb) expressed well in COS-7 cells. The recombinant human prolidase exhibited not only prolidase activity but also Gase activity. Both activities were two-folds higher than that in their counterpart controls. The ratio of the two enzyme activities of the recombinant enzyme and the control were nearly equal, and the elevating multiple of enzyme activities *vs* that of the control were identical (Tab 2). It implies that the two kinds of enzymatic activities seemingly come from a single enzyme.

Tab 2. Gase and prolidase activities in the supernatant of COS-7 cell lysate

Sample	Enzyme activity/μmol·min^{-1}·g^{-1} protein		Ratio of Gase and prolidase
	Gase	Prolidase	
Supernatant of cell lysate	1008(3)	72(3)	14
Control	332(1)	26(1)	13

The numbers in brackets are the multiple compared with control.

3 DISCUSSION

Organophosphate degrading enzymes from two kinds of bacteria, *A. undina*[8] and *A. haloplanktis*[9], have been documented to manifest prolidase activity. In this paper, we showed that the recombinant human prolidase exhibits Gase activity. The likelihood of the ratio of Gase activity to prolidase activity, the elevation multiple of each of these activities in the transformed *vs* non-transformed COS-7 cells, and the similarity in biochemical properties of Gase and prolidase[1,11-13] suggests that the Gase and prolidase are of the same enzyme, or at least belong to isozyme.

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人的基因工程氨酰基脯氨酸二肽酶的多效酶活性

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摘要：目的　研究人氨酰基脯氨酸二肽酶除催化水解 C 端为脯氨酸残基的二肽外，是否还有 G 类有机磷化合物水解酶(G 酶)活性。**方法**　用基因工程技术克隆及表达人的重组氨酰基脯氨酸二肽酶。氨酰基脯氨酸二肽酶及 G 酶活性用常规方法测定。**结果**　COS-7 细胞表达的人氨酰基脯氨酸二肽酶催化有机磷化合物梭曼的水解，也水解二肽化合物 Gly-Pro。两种活性比未转染的 COS-7 细胞高 2 倍。比较转染了带有氨酰基脯氨酸二肽酶基因的重组载体的 COS-7 细胞和对照组细胞中的两种酶活性，可以看到有平行的升高趋势及恒定的酶活性比值。**结论**　G 酶和氨酰基脯氨酸二肽酶为同一个酶，或至少属于同工酶。

关键词： 水解酶类，有机磷化合物；氨酰基脯氨酸二肽酶；肝；二肽类；梭曼；人

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