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Hydrolysis potential of recombinant human skin and kidney prolidase against diisopropylfluorophosphate and sarin by *in vitro* analysis

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ABSTRACT

Human prolidase (PROL), which has structural homology to bacterial organophosphate acid anhydrolase that hydrolyze organophosphates and nerve agents has been proposed recently as a potential catalytic bioscavenger. To develop PROL as a catalytic bioscavenger, we evaluated the *in vitro* hydrolysis efficiency of purified recombinant human PROL against organophosphates and nerve agents. Human liver PROL was purified by chromatographic procedures, whereas recombinant human skin and kidney PROL was expressed in *Trichoplusia ni* larvae, affinity purified and analyzed by gel electrophoresis. The catalytic efficiency of PROL against diisopropylfluorophosphate (DFP) and nerve agents was evaluated by acetylcholinesterase back-titration assay. Partially purified human liver PROL hydrolyzed DFP and various nerve agents, which was abolished by specific PROL inhibitor showing the specificity of hydrolysis. Both the recombinant human skin and kidney PROL expressed in *T. ni* larvae showed ~99% purity and efficiently hydrolyzed DFP and sarin. In contrast to human liver PROL, both skin and kidney PROL showed significantly low hydrolyzing potential against nerve agents soman, tabun and VX. In conclusion, compared to human liver PROL, recombinant human skin and kidney PROL hydrolyze only DFP and sarin showing the substrate specificity of PROL from various tissue sources.

1. Introduction

Rapid development of efficient catalytic bioscavengers of human origin to combat potential exposure to organophosphates (OPs) and chemical warfare nerve agents (CWNAs) are of high demand. An emerging class of such enzyme is human prolidases (PROL, EC 3.4.13.9). Human PROL exhibits ~28% amino acid identity with that of organophosphate degrading enzyme, *Alteromonas* organophosphate acid anhydrolase (OPAA) (Cheng et al., 1997; Endo et al., 1989a,b). The OPAA from *Alteromonas undina* and *Alteromonas haloplanktis* also displays PROL activity indicating that PROL is an enzyme that has similarity to OPAA and may possibly hydrolyze OPs and CWNAs (Cheng et al., 1993, 1996, 1997, 1998, 1999). Exploring the catalytic activity of crude preparation of human liver PROL expressed in yeast against CWNA substrate showed hydrolysis of soman suggesting that human PROL is a kind of OPAA (Wang et al., 2004). Therefore, studies on the expression, purification and evaluation of the catalytic activity of human PROL have considerable prospects to develop it eventually as a human catalytic bioscavenger for protection against OPs/CWNAs.

Prolidases are classes of enzymes that catalyze the cleavage of the peptide bond within a dipeptide containing a prolyl residue at the carboxy terminus (X-Pro), where X is any amino acid. OPAA has a broad spectrum of substrate specificity which can hydrolyze P–F and P–O bonds of various OP compounds. The PROL gene has been cloned from a variety of sources including human liver (Endo et al., 1989b; Ishii et al., 1996). In humans, PROL has been reported to exhibit multiple variants with differences in catalytic activity (Endo et al., 1989b; Lewis and Harris, 1969; Tanoue et al., 1991). The human kidney and skin PROL have approximately 98% sequence similarity to human liver PROL (GenBank Accession

Abbreviations: PROL, prolidase; OPs, organophosphates; OPAA, organophosphate acid anhydrolase; CWNAs, chemical warfare nerve agents; PON1, paraoxonase 1; DFP, diisopropylfluorophosphate; AChE, acetylcholinesterase; ATCh, acetylthiocholine; DTNB, 5-5′-dithio-*bis*(2-nitrobenzoic acid); SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DEAE, diethyl amino ethyl; Cbz-Pro, *N*-Benzyl oxycarbonyl L-proline; Gly-Pro, glycyl proline; BCA, bicinchoninic acid; GB, sarin; GD, soman; GA, tabun.

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Numbers BC028295 – kidney, BC015027 – skin and J04605 – liver). The amino acid differences in kidney PROL includes L66R, S183C, G221E, SV283CF, R294A, L311R, D324V, ID329MH, L435F and I457T compared to liver PROL. The human skin PROL shows all the same amino acid differences as that of kidney PROL except L435F compared to liver PROL. Thus, human skin PROL is more similar to kidney PROL but lacks only L435F (Strausberg et al., 2002). Very little information is available on the catalytic bioscavenger potential of PROL isolated from different human tissues against OPs and CWNAs, except human liver PROL (diTargiani et al., 2010; Wang et al., 2006) . Because of the human origin, PROL can overcome the incompatibility of bacterial OPAA for human use as a catalytic bioscavenger for protection against OPs and nerve agents.

We expressed human skin and kidney PROL in *T. ni* larvae followed by purification using affinity chromatography as described earlier (Valiyaveettil et al., 2010). We also prepared partially purified human liver PROL by using conventional chromatography procedures as reported earlier (Endo et al., 1987; Nakayama et al., 2003). Purified recombinant human skin and kidney PROL and partially purified human liver PROL were analyzed for the catalytic efficiency by using the recently established AChE back-titration assay which requires small amount of enzymes for the complete hydrolysis of substrates to draw more accurate results (Valiyaveettil et al., 2010, 2011) .

2. Materials and methods

2.1. Materials

Diethyl amino ethyl (DEAE)-Sepharose, glycyl-proline (Gly-Pro), *N*-benzyl oxycarbonyl L-proline (Cbz-Pro), AChE (Torpedo Californica), acetylthiocholine (ATCh), 5-5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), and diisopropylfluorophosphate (DFP) were purchased from Sigma (St. Louis, MO). SDS–PAGE running buffer, 4–20% gradient Tris–glycine gel, protein molecular weight marker, and SDS–PAGE transfer buffer were purchased from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) assay kit for protein estimation was purchased from Pierce (Rockford, IL). Diluted chemical warfare nerve agents were obtained from United States Army Medical Research Institute of Defense, Aberdeen Proving Ground, MD.

2.2. Purification of human liver prolidase

Prolidase was isolated from human liver (National Disease Research Interchange, Philadelphia, PA) by the method of Endo et al. with modifications (Endo et al., 1987; Nakayama et al., 2003). Briefly, 50 g of tissue was homogenized with 500 ml of 50 mM Tris–HCl buffer, pH 7.4 containing 1 mM $MnCl_2$ and centrifuged at 500×*g* for 10 min followed by 10,000×*g* for 15 min at 4 °C. The supernatant was fractionated by ammonium sulfate (40–70%) precipitation. The protein precipitate was dissolved in a mixture of 10 mM Tris–HCl containing 1 mM $MnCl_2$ and 2 mM sodium phosphate buffer, pH 7.4 and loaded onto a DEAE-Sepharose column pre-equilibrated with 10 mM Tris–HCl/1 mM $MnCl_2$/2 mM sodium phosphate buffer, pH 7.4. The column was washed extensively with 50 mM Tris–HCl buffer/1 mM $MnCl_2$, pH 7.4, followed by elution of PROL enzyme with a linear gradient of 50–350 mM Tris–HCl/1 mM $MnCl_2$ buffer, pH 7.4. Fractions of 5 ml were collected and analyzed for PROL activity by using Gly-Pro substrate as described earlier (Park et al., 2004). Aliquots of PROL fractions were incubated with 1 mM Gly-Pro substrate followed by measuring the absorbance difference at 222 nm by using a spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA). The high PROL activity containing fractions were pooled and dialyzed against 10 mM Tris–HCl/1 mM $MnCl_2$/2 mM sodium phosphate buffer, pH 7.4. The resulting preparation was applied to a second DEAE column pre-equilibrated with 10 mM Tris–HCl/1 mM $MnCl_2$/2 mM sodium phosphate buffer, pH 7.4, followed by washing and elution as described above. The protein content of the PROL enzyme fractions were determined by BCA assay. The specificity of PROL enzyme activity in the human liver enzyme preparations were confirmed by pre-incubation with 5 nM of Cbz-Pro as described earlier (Lupi et al., 2005).

2.3. Expression and purification of human skin and kidney prolidase

Human skin and kidney PROL was expressed in *T. ni* larvae (cabbage looper-worm) in collaboration with Chesapeake PERL (Savage, MD). Briefly, the larvae was infected orally using viral inoculums (baculovirus expression vector containing human skin or kidney PROL cDNA) impregnated diet and incubated at controlled conditions for 96 h. The harvested larvae were stored under frozen conditions until the recovery of PROL enzyme. The recombinant protein was extracted and purified from the infected larvae by using classical protein purification technology including, ion-exchange and size-exclusion chromatography, ultra-filtration and finally by his-tagged affinity column (the baculovirus expression system has 6 histidine residues to facilitate this purification procedure) as described earlier (Otto et al., 2010; Valiyaveettil et al., 2010). The purified recombinant PROL showed >99% purity was stored under −80 °C until use.

2.4. SDS–PAGE

The purity of recombinant PROL expressed in *T. ni* larvae was analyzed by SDS–PAGE using XCell Sure Lock Mini-Cell electrophoresis system from Invitrogen (Carlsbad, CA). Briefly, ~2 μg of recombinant protein was mixed with SDS–PAGE sample buffer containing reducing agent and incubated at 100 °C for 5 min. The protein samples were separated on 4–20% gradient Tris–glycine gels at a constant voltage of 100 V, stained with Coomassie Blue and photographed with AlphaImager (Cell Biosciences, Santa Clara, CA) (Laemmli, 1970).

2.5. Kinetic assay

Human recombinant skin and kidney PROL (1 μg) were incubated with varying concentrations of Gly-Pro substrate (0.25–8 mM) in 10 mM Tris–HCl, pH 8.0, containing 1 mM $MnCl_2$ followed by measuring the absorbance difference at 222 nm using a spectrophotometer as described earlier (Park et al., 2004). The rate of enzyme activity was calculated from the absorbance difference per minute using the molar extinction coefficient of the peptide bond (904 M^{-1} cm^{-1}). By using GraFit5 Version 5.0.13 software (Erithacus Software Ltd., Surrey, UK), the kinetic parameters (K_M and V_{max}) were determined. k_{cat} values were expressed as the ratio of V_{max} over moles of enzyme used in the reaction system.

2.6. AChE back-titration assay

To analyze the hydrolyzing potential of purified recombinant human skin and kidney PROL against OPs and nerve agents, an *in vitro* back-titration of AChE Ellman assay was employed as reported previously (Ellman et al., 1961; Valiyaveettil et al., 2010; Wille et al., 2010). This methodology helps to evaluate complete hydrolysis of substrates with relatively lower concentration of enzymes. To determine the optimum concentration of OPs and nerve agents required for ~95% inhibition of AChE activity, different concentrations of OPs or nerve agents were incubated with 5 ng of AChE for 15 min at room temperature followed by incubation with