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Primary structure, recombinant expression and homology modelling of human brain prolyl oligopeptidase, an important therapeutic target in the treatment of neuropsychiatric diseases‡

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INTRODUCTION

There is no doubt that Murray Goodman can be considered as a pioneer in the conformational analysis of proline-rich polypeptides. His decorated career was marked by seminal contributions to the synthesis and conformational studies of poly(L-prolyl-L-α-phenylglycyl-L-proline) [1], followed by theoretical calculations of poly[(*S*)-thiazolidine-4-carboxylic acid] [2], a 'thia-poly-L-proline', and important work in the field of collagen mimetics [3]. During a sabbatical year (1990–1991) spent by one of these authors, (E.G.) in San Diego as both a research associate at the Scripps Research Institute, La Jolla, CA, and a visiting professor in Murray's laboratory at the University of California, San Diego, he was able to appreciate first hand that his interest in Pro-rich peptides had not wavered. On the contrary, our two laboratories have since followed nearly parallel paths to develop novel Pro-based chemical systems, including Pro-based dendrimers [4–6]. Today, the key role of Pro-containing peptides in the central nervous system is well established and has triggered a surge of interest in enzymes, such as human brain prolyl oligopeptidase, that may regulate the biosynthesis and/or degradation of Pro-containing neuropeptides. E.G. is convinced that Murray would have been very happy to see the first ever detailed description of this important enzyme reported in this special issue.

Abbreviations: pNA, *para*-nitroaniline; POP, prolyl oligopeptidase; rmsd, root mean square deviation; z, benzyloxycarbonyl.

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Prolyl oligopeptidase (POP, EC 3.4.21.26) is a large (80 kDa) cytosolic serine protease characterized by oligopeptidase activity. Preferentially, POP hydrolyses proline-containing peptides at the carboxy end of Pro-residues [7]. Most peptide hormones and neuropeptides are processed and degraded by Pro-specific enzymes since classical peptidases cannot cleave at Pro residues [8]. Serum POP activity is increased in patients with bipolar affective disorder, schizophrenia [9], stress-induced anxiety [10] and post-traumatic stress disorder [11]. As the 3D-structure of porcine POP is known [12], this enzyme was used as a model for structure-based drug design projects. However, although POP from human lymphocytes has been cloned [13,14], the 3D-structure of the human brain enzyme is still elusive. Given that POP activity is increased in schizophrenia and in bipolar affective disorder, it was hypothesized that POP specific inhibitors would be useful for the treatment of these and related diseases. In fact, S-17092-1, a potent inhibitor of human POP, has entered clinical trials and could be used for the treatment of cognitive disorders [15]. There is an increasing number of reports on POP catalysis and new POP inhibitors. Nevertheless, many of these studies were performed using *Flavobacterium meningosepticum* [16–18] or porcine POP as models [19–21]. The inhibitors described in these papers were evaluated using the above model enzymes. In other studies protein extracts from several species were used as an enzyme source for the evaluation of POP inhibition, for example porcine brain [22] or human platelets [23]. However, in future studies aimed at obtaining an effective inhibitor of the human brain enzyme, it would be of interest to use the recombinant enzyme reported here.

This article describes the cDNA cloning of human brain POP and the expression, purification and characterization of the recombinant enzyme. Human and porcine brain POP were compared at different levels: enzymatic activity, primary structure and tertiary structure. A homology model of human POP is presented that could be used in future structure-based drug design approaches.

RESULTS

Expression and Purification of Human Brain POP

Human POP cDNA was generated by RT-PCR using human brain total RNA as a template and its sequence was deposited in the GenBank under the accession number AY660966. The protein sequence deduced from the isolated cDNA is shown in Figure 1. For expression in *E. coli*, the POP cDNA was subcloned into the vector pTrc99A. The resultant plasmid, named pTrc99A/HPO, was used for expression of recombinant human POP in *E. coli* BL21 cells. The recombinant enzyme was purified in two steps from an extract of *E. coli* soluble proteins. The first step consisted of an anion-exchange chromatography of the soluble extract using a HiTrap DEAE column (Figure 2A). Through this purification step, the main *E. coli* contaminant proteins were removed and the enzyme was concentrated in fractions 19–26. These fractions were applied to a size exclusion column and an isocratic elution was performed (Figure 2B). The yield obtained in the purification was 2 mg/l of culture. Then, the activity of the recombinant enzyme was determined in the presence of several POP inhibitors: Z-Pro-Pro-H [24], S-17092-1 [25], SUAM14746 [26] and Z-Pro-Pro-H dimethylacetal [27]. As expected, the recombinant human enzyme was inhibited by Z-Pro-Pro-H, S-17092-1, SUAM14746 and Z-Pro-Pro-H dimethylacetal and the corresponding IC_{50}s were calculated (Table 1).

Human and porcine POP were expressed following the same procedure. Nevertheless, the yield obtained for the former (2 mg/l) was five times lower than that obtained for the latter (10 mg/l), probably because of differences in stability of the two enzymes in *E. coli* cells.

Proteomic Characterization

The main band present in the SDS-PAGE was expected to correspond to human POP (Figure 2B). To confirm this hypothesis, it was analysed using a proteomic assay. The same protocol was applied to purified porcine POP. The percentages of sequence coverage

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Human    1 MLSLQYPDVYRDETAVQDYHGHKICDPYAWLEDPDSEQTKAFVEAQNKITVPFLEQCPIR
Pig      1 MLSFQYPDVYRDETAIQDYHGHKVCDPYAWLEDPDSEQTKAFVEAQNKITVPFLEQCPIR
           ***:**********:*******:************************************

Human   61 GLYKERMTELYDYPKYSCHFKKGKRYFYFYNTGLQNQRVLYVQDSLEGEARVFLDPNILS
Pig     61 GLYKERMTELYDYPKYSCHFKKGKRYFYFYNTGLQNQRVLYVQDSLEGEARVFLDPNILS
           ************************************************************

Human  121 DDGTVALRGYAFSEDGEYFAYGLSASGSDWVTIKFMKVDGAKELPDVLERVKFSCMAWTH
Pig    121 DDGTVALRGYAFSEDGEYFAYGLSASGSDWVTIKFMKVDGAKELPDVLERVKFSCMAWTH
           ************************************************************

Human  181 DGKGMFYNSYPQQDGKSDGTETSTNLHQKLYYHVLGTDQSEDILCAEFPDEPKWMGGAEL
Pig    181 DGKGMFYNAYPQQDGKSDGTETSTNLHQKLYYHVLGTDQSEDILCAEFPDEPKWMGGAEL
           ********:***************************************************

Human  241 SDDGRYVLLSIREGCDPVNRLWYCDLQQESSGIAGILKWVKLIDNFEGEYDYVTNEGTVF
Pig    241 SDDGRYVLLSIREGCDPVNRLWYCDLQQESNGITGILKWVKLIDNFEGEYDYVTNEGTVF
           ******************************.**:**********************

Human  301 TFKTNRQSPNYRVINIDFRDPEESKWKVLVPEHEKDVLEWIACVRSNFLVLCYLHDVKNI
Pig    301 TFKTNRHSPNYRLINIDFTDPEESKWKVLVPEHEKDVLEWVACVRSNFLVLCYLHDVKNT
           ******:*****:***** ********************:*****************:

Human  361 LQLHDLTTGALLKTFPLDVGSIVGYSGQKKDTEIFYQFTSFLSPGIIYHCDLTKEELEPR
Pig    361 LQLHDLATGALLKIFPLEVGSVVGYSGQKKDTEIFYQFTSFLSPGIIYHCDLTKEELEPR
           ******:****** ***:***:************************************

Human  421 VFREVTVKGIDASDYQTVQIFYPSKDGTKIPMFIVHKKGIKLDGSHPAFLYGYGGFNISI
Pig    421 VFREVTVKGIDASDYQTVQIFYPSKDGTKIPMFIVHKKGIKLDGSHPAFLYGYGGFNISI
           ************************************************************

Human  481 TPNYSVSRLIFVRHMGGILAVANIRGGGEYGETWHKGGILANKQNCFDDFQCAAEYLIKE
Pig    481 TPNYSVSRLIFVRHMGGVLAVANIRGGGEYGETWHKGGILANKQNCFDDFQCAAEYLIKE
           *****************:****************************************

Human  541 GYTSPKRLTINGGSNGGLLVAACANQRPDLFGCVIAQVGVMDMLKFHKYTIGHAWTTDYG
Pig    541 GYTSPKRLTINGGSNGGLLVATCANQRPDLFGCVIAQVGVMDMLKFHKYTIGHAWTTDYG
           **********************:************************************

Human  601 CSDSKQHFEWLVKYSPLHNVKLPEADDIQYPSMLLLTADHDDRVVPLHSLKFIATLQYIV
Pig    601 CSDSKQHFEWLIKYSPLHNVKLPEADDIQYPSMLLLTADHDDRVVPLHSLKFIATLQYIV
           ***********:************************************************

Human  661 GRSRKQSNPLLIHVDTKAGHGAGKPTAKVIEEVSDMFAFIARCLNVDWIP
Pig    661 GRSRKQNNPLLIHVDTKAGHGAGKPTAKVIEEVSDMFAFIARCLNIDWIP
           ******.*****************************************:****
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Figure 1 Protein sequence alignment of human and porcine brain POP (Swiss-Prot accession number P23687). Asterisks and periods indicate conserved and semi-conserved residues, respectively. Underlined residues show the sequence coverage obtained by the proteomic analysis. The MASCOT scores obtained for the identification were 344 and 252 for human and porcine POP, respectively.

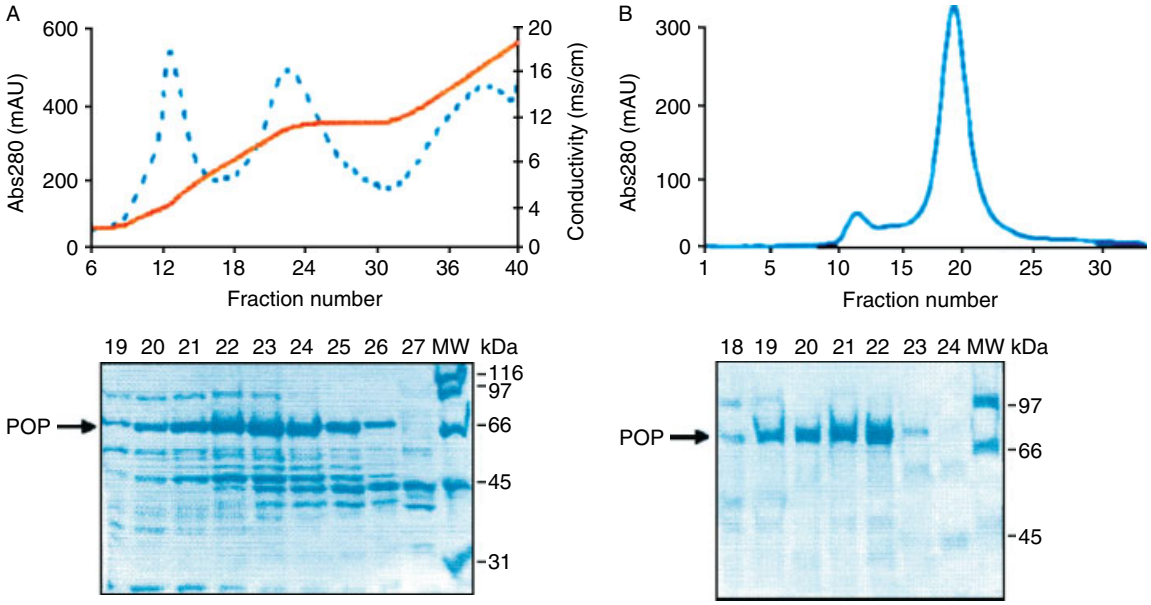

Figure 2 Purification of human brain POP produced in *E. coli*. A: First purification step of the recombinant enzyme by anion-exchange chromatography with HiTrapDEAE. A soluble extract of *E. coli* protein was loaded on a HiTrapDEAE column and elution was performed with a NaCl gradient. The chromatogram of the elution is shown on the top of the panel. The dashed blue line corresponds to absorbance (280 nm, mAU) and the red line to conductivity (mS/cm). A SDS-PAGE gel of fractions 19–27 is shown below the chromatogram. B: Second purification step by size-exclusion chromatography with Superdex 200. The chromatogram is shown on the top of the panel. The blue line shows absorbance (280 nm, mAU). Below the chromatogram, an SDS-PAGE gel of fractions 18–23 is shown. In A and B, 5 µl of the indicated fractions on the top of each SDS-PAGE were loaded on a 10% SDS-PAGE. After running, the gels were stained with Coomassie G-250. The arrow indicates the running position of recombinant POP. MW are molecular weight markers. The molecular masses of MW are indicated on the right of each panel.

Table 1 The IC_{50} values of the Recombinant Human and Porcine Brain POP Enzymes Calculated with Distinct Inhibitors

Inhibitor	IC_{50} (nM)	IC_{50} (nM)
	Human brain POP	Porcine brain POP
Z-Pro-Pro-H	2.3 ± 1.8	1.7 ± 1.3
S17092	1.3 ± 0.6	1.4 ± 0.4
SUAM14746	55.3 ± 16	61.3 ± 10.3
Z-Pro-Pro-H dimethylacetal	64.5 ± 20.5	68.5 ± 50.2

To measure the IC_{50} values, Z-Gly-Pro-pNA was used as a substrate. The reaction was performed by mixing a range of concentrations of inhibitors with the enzyme (0.5 ng/µl) and pre-incubating them for 15 min at 30 °C. The substrate was then added to the mixture and the reaction was allowed to proceed for 1 h at 30 °C. Finally, the amount of pNA produced was measured by reading the absorbance at 405 nm. The data were obtained from three independent experiments. No statistically significant differences were observed.

obtained by the proteomic analysis were 68% and 28% for the human and porcine enzyme, respectively (Figure 1). Using mass spectrometry, it was possible to distinguish unequivocally the two protein sequences. The good coverage in the human sequence allowed the identification of the amino acid differences between human and porcine sequences expected in accordance with the cDNA sequence. Moreover, we also identified the only two amino acid changes between human brain POP and human lymphocytes POP (Swiss-Prot accession number P48147). The good score obtained in the identification opens up avenues to perform the proteomic characterization of the human native brain enzyme in a range of physiological situations.

Comparison of Primary Structure and Enzymatic Activity between Human and Porcine POP

The alignment between the protein sequences of human and porcine POP (Figure 1) showed that the primary structures of the two proteins were very similar. These two POP enzymes shared an identity of 97% and a similarity of 98%. The main amino acid differences between them corresponded to conserved changes (Figure 1). Moreover, the residues involved in the catalysis (S554, H680 and D641) were also conserved. As porcine POP has been widely used as a model of the POP family of enzymes, the activities of the two enzymes were compared. The IC_{50} values of the four POP inhibitors with the two recombinant enzymes were calculated using Z-Gly-Pro-pNA as a substrate. No statistically significant differences between the IC_{50}

values obtained with human and porcine enzymes were observed (Table 1).

3D-Structure: A Homology Model of Human Brain POP

A homology model of the human POP was obtained based on the x-ray diffraction structure of porcine POP as a template (PDB accession number 1QFS) [12]. The 3D-structure of the modelled human enzyme is shown in Figure 3 (coordinates of this structure are available on request from the authors). The presence of the two domains, catalytic and structural, is clearly visible. The catalytic domain (top of panel A, Figure 3) corresponds to a peptidase domain with an α/β hydrolase fold. The structural domain (bottom of panel A, Figure 3) is based mainly on β-sheets, which form a 7-fold repeat of four-stranded antiparallel sheets that are twisted and generate a central tunnel (Figures 3A and 3B). The entrance to the cavity inside the structural domain, is clearly visible (Figure 3B). The backbones of the human and porcine structures were superimposed and a low rmsd was found (0.17 Å).

DISCUSSION

In this study the cDNA of human brain POP was isolated and expressed in *E. coli*. To our knowledge, this is the first report of the expression and characterization of this enzyme. It was demonstrated that the isolated cDNA codes for a protein with POP activity and the protein was purified for use in enzymatic studies. In addition, human and porcine POP were compared at three levels: enzymatic activity, primary structure and tertiary structure. No significant differences were found between the IC_{50}s for the two enzymes. This result can be explained by the high identity (97%) and similarity (98%) between the two sequences [28]. On the basis of our findings, the results described in the literature for porcine POP can also be taken into account when working with the human enzyme. However, in future studies that aim at obtaining an effective inhibitor of the human brain enzyme, it would be of interest to use the recombinant enzyme reported here.

As no tertiary structure of human POP is available, a homology model of the enzyme was obtained based on the experimental structure of porcine POP [12] and on the sequence of human POP established in the present

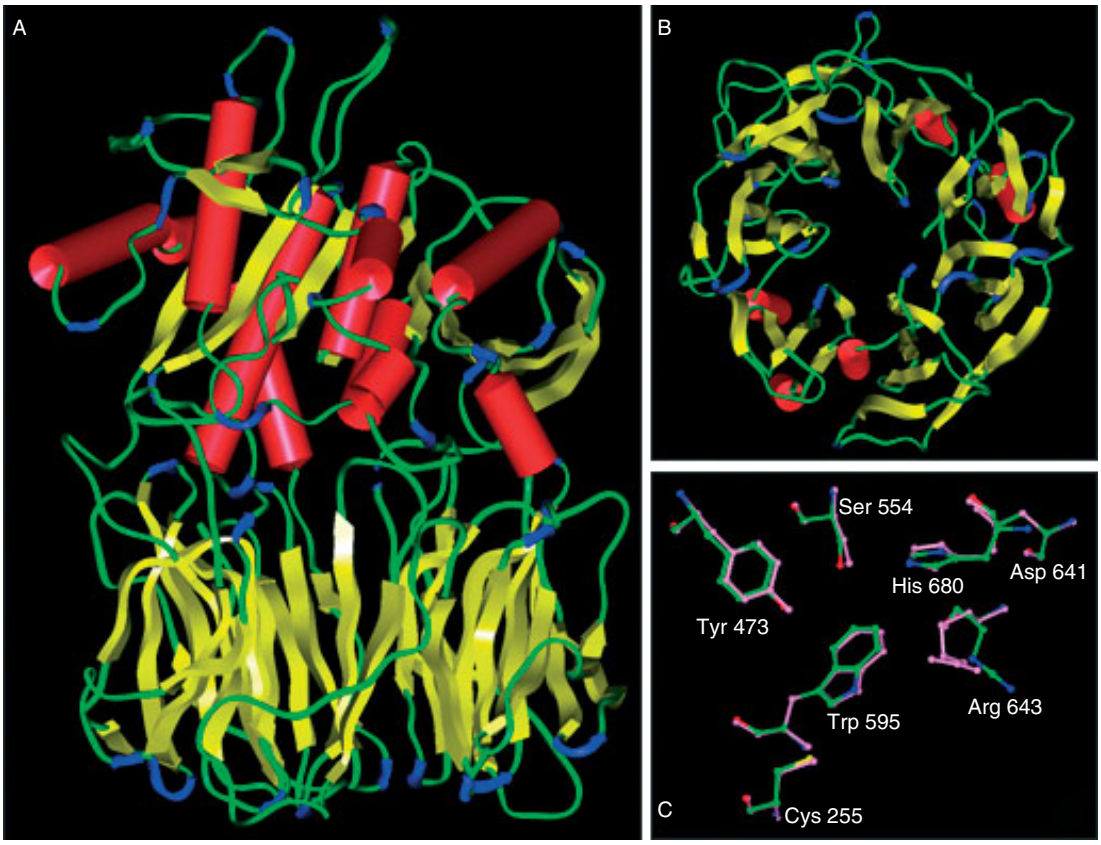

Figure 3 Homology model of human brain POP. A: Human brain POP 3D-structure obtained by molecular modelling. B: POP structural domain represented perpendicularly to the picture in B. The entrance to the central cavity and the 7-fold repeat of the β-sheets are shown. In A and B, the α-helices are represented in red, in blue the turns, in yellow the β sheets and in green the random-coil segments. C: Superimposition, in a ball and stick representation, of the residues which form the active site of the molecule, following ref. [12].

study. When the human POP modelled structure was compared with the porcine POP x-ray structure, a high similarity was observed. Nevertheless, although the backbones of the residues that belong to the catalytic centre of the two proteins can be superimposed, there is a slight difference in the disposition of the Arg 643 residue (Figure 3C).

The homology model of human brain POP reported here could be applied to structure-based drug design approaches and thereby could contribute to improving the treatment of serious psychiatric diseases such as schizophrenia and bipolar affective disorder.

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