Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 49 (2006) 83–87
Protein Expression & Purification
www.elsevier.com/locate/yprep

Purification, characterization, and crystallization of human pyrroline-5-carboxylate reductase

Zhaohui Meng [a,b], Zhiyong Lou [a,b], Zhe Liu [a], Dong Hui [a], Mark Bartlam [a,b], Zihe Rao [a,b,*]

[a] *"Tsinghua-IBP Joint Research Group for Structural Biology," Tsinghua University, Beijing 100084, China*
[b] *National Laboratory of Biomacromolecules, Institute of Biophysics (IBP), Chinese Academy of Sciences, Beijing 100101, China*

Received 13 January 2006, and in revised form 10 February 2006
Available online 20 March 2006

Abstract

Pyrroline-5-carboxylate reductase (P5CR) catalyzes the reduction of Δ1-pyrroline-5-carboxylate (P5C) to proline with concomitant oxidation of NAD(P)H to $NAD(P)^+$. The enzymatic cycle between P5C and proline is very important in many physiological and pathological processes. Human P5CR was over-expressed in *Escherichia coli* and purified to homogeneity by chromatography. Enzymatic assays of the wild-type protein were carried out using 3,4-dehydro-L-proline as substrate and NAD^+ as cofactor. The homopolymer was characterized by cross-linking and size exclusion gel filtration chromatography. Human P5CR was crystallized by the hanging-drop vapor-diffusion method at 37 °C. Diffraction data were obtained to a resolution of 2.8 Å and were suitable for high resolution X-ray structure determination.

Keywords: Human P5CR; Homopolymer; Oxidative activity; Crystallography

The interconversion of Δ1-pyrroline-5-carboxylate (P5C)[1] and proline initiates a chain of cellular biochemical, energetic, and physiological processes through the transfer of oxidizing and reducing potential [1]. P5C, a physiological intermediate in the interconversions of proline, ornithine, glutamate, and arginine, can be transported into the cytosol as a source of oxidizing potential where its reduction by 1-pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2) to proline generates $NAD(P)^+$[2]. Proline can then be transported into mitochondria where its oxidation by proline dehydrogenase (POX, EC 1.5.99.8) to P5C transfers electrons into the mitochondrial electron transport with an intervening flavoprotein, thereby completing a P5C/proline redox cycle [2]. In addition to its well-established role as an osmoprotectant capable of withstanding stressful environments such as drought, excessive salinity, and low or high temperature in plants [3], proline can function as a non-enzymatic antioxidant required by microorganisms, animals, and plants to minimize damage caused by reactive oxygen species (ROS) [4]. Meanwhile, the conversion of proline to P5C by p53-induced POX can enhance generation of proline-dependent ROS [5], and leads to the p53-dependent initiation of apoptosis through a calcineurin-dependent pathway [6]. P5C could inhibit the proliferation and survival of ECV-304 and DECV cells, and induced apoptosis in both cell lines [7]. Abnormalities in the proline/P5C redox cycle have been associated with a number of mammalian diseases. For example, POX mutations were associated with hyperprolinemia in the schizophrenic patients [8]. The decreased P5C reductase activity may be implicated in retinal degeneration in mice [9].

Although several human P5CR isoforms have been deposited in GenBank and the existence of P5CR isoenzymes has recently been demonstrated in soybean and spinach leaves [10,11], only a single copy gene located to human chromosome band 17q25.3 has been identified to encode

* Corresponding author. Fax: +86 10 62773145.
E-mail address: raozh@xtal.tsinghua.edu.cn (Z. Rao).

[1] *Abbreviations used:* P5CR, pyrroline-5-carboxylate reductase; P5C, Δ1-pyrroline-5-carboxylate; ROS, reactive oxygen species; AS, ammonium sulfate; PCR, polymerase chain reaction; EGS, ethylene glycolbis.

1046-5928/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.02.019

P5CR [12]. The alignment of the amino acid sequences of P5CR shows 26–42% identity with each other and a conserved GXGXXA/G sequence motif is present in the P5CR protein family [13]. Human P5CR extends by nearly 40 amino acids at the carboxyl terminal beyond other reductase sequences. Previous purification of P5CRs has revealed that the native enzyme is a polymer from different organisms [13–16]. P5CR from *Escherichia coli* is also involved in metabolism of some proline analogs [17]. The recent reports of the crystal structures of *Neisseria meningitides* MC58 and *Streptococcus pyogenes* have allowed a better understanding of the functional differences in this protein family on their structure determinants [18]. There is presently no known three-dimensional structure of human P5CR. In this study, we report the purification, characterization, and crystallization of human P5CR.

Materials and methods

Protein expression and purification

The full-length sequence (NCBI Accession No.: NM-006907) for human P5CR (33 kDa) was amplified from a human hepatoma cell line cDNA library using the polymerase chain reaction (PCR) method with primers:

PYCR1-F: 5′-GAAAGAATTCATGAGCGTGGGCT TCATCGGCGC-3′.
PYCR1-R: 5′-GAAACTCGAGTCAATCCTTGCCCG CTGG-3′.

The PCR products were restricted with *Eco*RI and *Xho*I, and ligated into *Eco*RI and *Xho*I restricted sites of the pET28a(+) vector (Novagen Inc.) with a 6× His tag at the N-terminus. A further transformation into *E. coli* DH5α competent cells was performed and positive clones with an insert of the right size were confirmed by DNA sequencing. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) and the transformants were selected on LB agar plates containing 50 μg/ml kanamycin. The cells were then cultured at 37 °C in LB medium containing 50 μg/ml kanamycin. When the culture density reached $A_{600} = 0.5$, the culture was induced with 0.25 mM IPTG or without IPTG and was grown for an additional 3 h before the cells were harvested.

Cell lysis and ammonium sulfate precipitation

The bacterial cell pellet was resuspended in lysis buffer (20 mM Tris–HCl, pH 8.4, 500 mM NaCl, and 10% glycerol) containing 1 mM EDTA, 1 mM dithiothreitol (DTT), and homogenized by sonication. The lysate was centrifuged at 15,000 rpm for 30 min to remove the cell debris. Next, ammonium sulfate (AS) was added with stirring to the solution at 4 °C. The precipitate formed between 0 and 20% AS was collected. The protein was suspended in storage buffer (20 mM Caps, pH 9.4, 0.5 M NaCl), and incubated with 1 mg/ml RNaseA (Sigma) and 15 U/ml DNaseI (Takara) at room temperature overnight.

Chromatographic purification

The supernatant was applied to an Ni^{2+}-chelating column (2 ml Ni–NTA agarose) and the contaminant protein was washed off with lysis buffer containing 5 mM imidazole. The target protein was eluted with lysis buffer containing 300 mM imidazole. The sample was concentrated with an Ultrafree 10,000 NMWL filter unit (Millipore) to less than 1 ml and injected into a Superdex-200 10/300 column (Pharmacia) in running buffer (storage buffer).

The sample eluted isocratically at 10–13 ml of running buffer volume was diluted to 100 mM NaCl in 20 mM Caps, pH 9.4, and applied through a 10 ml superloop (Pharmacia) onto a Resource Q 1 ml column (Pharmacia). The column was washed with 20 mM Caps, pH 9.4, before being eluted in a increasing salt gradient from 0 to 1 M NaCl in 20 mM Caps, pH 9.4.

P5CR activity assays

The 3,4-dehydro-L-proline dehydrogenase activity assays of P5CR were performed at room temperature using a sensitive spectrophotometric method [17]. In brief, after the enzyme was incubated at 37 °C for 10 min, the reaction was initialized by adding diluted P5CR (1 μl, 20 mg/ml) into 200 μl reaction buffer containing 300 mM Tris–HCl (pH 9.0, at which P5CR achieved maximized activity), NAD^+ (0.4 mM), varied concentrations of 3,4-dehydro-L-proline (0.02–1 mM). Using the mM extinction coefficient of NAD(P)H (6.22), initial rates of product formation were calculated by increase of absorbance at 340 nm/min from the first 60 s of a 5 min recording period. Reproducibility of all analyses was confirmed by taking each measurement at least twice with a fluctuation of less than 8%. A sample with both substrates except P5CR was served as a negative control.

Crosslinking with EGS

The P5CR protein was diluted to 10 mg/ml in 20 mM Hepes, pH 8.9, 100 mM NaCl. Ethylene glycolbis (EGS) was dissolved in DMSO to 1 mM and then added to 10 μl of protein sample with a final concentration ranging from 2 to 400 μM. After the mixture was incubated at room temperature for 10 min, the reaction was quenched for 5 min by adding 1 M Tris–HCl (pH 7.5) to a final concentration of 50 mM. After loading the sample containing 2 μl SDS load buffer, it was run on an 8% SDS–polyacrylamide gel in SDS electrophoresis buffer.

Crystallization and X-ray data collection

The purified human P5CR was concentrated to 15 mg/ml in 20 mM Caps, pH 9.4, 0.5 M NaCl, and 1 mM DTT.

The protein concentration was confirmed spectrophotometrically by the Bradford method. Crystallization was performed by the hanging-drop vapor-diffusion method at 16 and 37 °C. In preliminary assays, the Hampton Research Crystal Screen I and Crystal Screen II reagent kits were used to screen crystallization conditions. One microliter of protein solution was mixed with 1 μl reservoir solution containing 0.8–1 M sodium acetate, 30–40 mM imidazole (pH 6.5), 50–60 mM Tris–HCl (pH 7.5), and the mixture was equilibrated against 400 ml reservoir solution at 16 and 37 °C. The best crystals were obtained in a 37 °C incubator by mixing 1 μl protein with 1 μl reservoir solution and 1 μl 100% paraffin oil or 1 μl protein with 1 μl reservoir solution containing 0.1–0.2% (w/v) agarose.

Initial cube-shaped crystals appeared after 1–3 days. Crystals were immersed in cryoprotectant for 1 min, mounted into a nylon cryo loop, and then flash-cooled in a stream of nitrogen gas cooled to 100 K. The cryoprotectant was prepared by adding 20% glycerol to the mother-liquor reservoir. Preliminary diffraction data sets were collected at room temperature in-house using a Rigaku MM007rotating-anode CuKα X-ray generator operating at 40 kV and 20 mA ($\lambda = 1.5418$ Å) with a RAIXS IV++ image plate detector. The beam was focused using Osmic mirrors. All intensity data were indexed, integrated, and scaled using the HKL2000 programs DENZO and SCALEPACK [19].

Results and discussion

Human P5CR is expressed as a soluble protein

Human P5CR was over-expressed in *E. coli* as a fusion protein with a 6× His and T7-tag at its N-terminus under T7 promoter with or without IPTG induction. After being induced for 3 h, the expressed P5CR comprised ~40% of the total protein and most of the expressed P5CR was soluble. High expression of human P5CR does not affect the cell growth. Soluble P5CR in the crude lysate was used for further purification, and P5CR was fractionated from the crude lysate by 20% AS precipitation. The P5CR recovered was associated with small amounts of nucleic acid, and could be digested by incubation with RNaseA and DNaseI. Sequential systematic purification of P5CR using chromatographic methods, including affinity, size exclusion, and anionic exchange, turned out to be efficient and convenient. The various purification steps are summarized in Table 1. Human P5CR readily precipitated at low pH (<8.0), low temperature (<4 °C) and low salt concentration (<0.3 M NaCl), so the purification temperature was optimized at 16 °C.

Human P5CR forms a stable homopolymer and oligomer

Affinity-purified protein showed a big peak at a very high molecular weight (>660 kDa) and a small peak at a high molecular weight (~370 kDa, about 10 times that of P5CR) from size exclusion chromatography (Fig. 1A). Both of them were target proteins as determined by SDS–PAGE

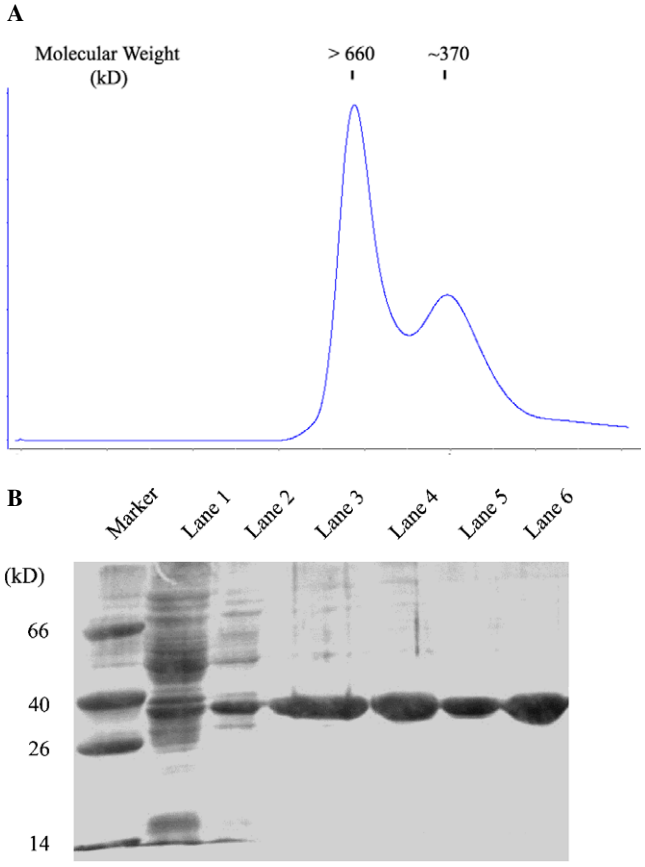

Fig. 1. (A and B) Human P5CR appears to form a decamer and homopolymer, as determined by size exclusion chromatography and SDS–PAGE. Marker, molecular mass markers. Lane 1, before induction. Lane 2, after induction. Lane 3, purified by Superdex-200. Lane 4, purified by Resource Q. Lanes 5 and 6, peak 1 (>660 kDa) and peak 2 (~370 kDa) from size exclusion chromatography.

Table 1
Purification summary for human P5CR

Purification step	Volume (ml)	Protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Yield (%)
Soluble cell fraction	80	15	1920	1.6	100
Ammonium sulfate	4.0	9	744	21	38
Ni–NTA agarose	60	0.5	420	14	22
Superdex 200	5.0	5	187	32	9.7
Resource Q	0.5	20	275	14	7.1

[a] Units are defined as the amount of enzyme activity which will catalyze the transformation of 1 μM 3,4-dehydro-L-proline of the substrate per minute under standard conditions.

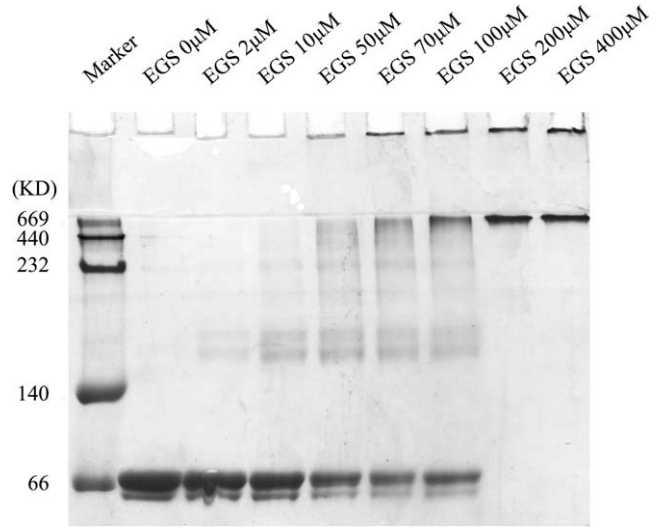

Fig. 2. Cross-linking gel of P5CR. The dimer (~66 kDa) and homopolymers (⩾660 kDa) were detected. Molecular weight marker is labeled at the side of each lane. EGS: ethylene glycolbis.

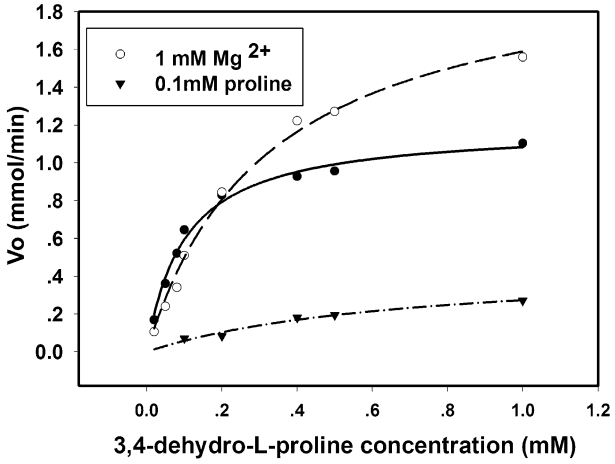

Fig. 3. Initial velocity (V_0) versus substrate concentration ([S]) plot for the 3,4-dehydro-L-proline dehydrogenase activities of P5CR. The activities were first measured in reaction buffers described under Materials and methods (solid circle), and then in the same buffers containing 1 mM magnesium ion (hollow circle) or 0.1 mM proline (solid triangle).

Coomassie blue staining (Fig. 1B). Only the 370 kDa fraction protein was chosen to be further purified with anionic exchange. P5CR eluted as a single peak with a high A_{280}/A_{260} ratio of 1.8 at about 300 mM salt concentration and its purity was confirmed by SDS–PAGE (Fig. 1B). The 370 kDa P5CR protein was subsequently used for crystallization and enzyme activity assays. The cross-linking behavior of P5CR further supports the assembly mode of the supercomplex. The homopolymer was also detected in cross-linking experiments (Fig. 2). These results suggest that a decamer may be the essential form existing in solution. Based on this evidence, human P5CR could further aggregate into polydecamers or homopolymers.

Human P5CR implicates in dehydrogenation of 3,4-dehydro-L-proline

A functional characterization of the enzyme was performed by dehydrogenation of the 3,4-dehydro-L-proline. The stability studies showed that the enzyme still had activity after incubation at 37 °C for 3 h, with irreversible loss of activity at 90 °C for 5 min. Thus, human P5CR was very stable in the Caps buffer, pH 9.4. The enzyme was stabilized for up to 6 months at −80 °C in storage buffer. 3,4-Dehydro-L-proline, which has a double bond between C3 and C4 of proline, can be dehydrogenated by human P5CR with apparent $K_m = 0.141 \pm 0.012$ mM, $K_{cat} = 10\ s^{-1}$. The NAD^+-dependent activity of human P5CR was almost fully inhibited by 0.1 mM proline; interestingly, 1 mM Mg^{2+}stimulated the activity up to 60% (Fig. 3).

Crystallization and preliminary crystallographic characterization

Besides the requirement of an accurate chromatographic purification to avoid minor contamination that could interfere with the crystallization of the enzyme, the growth of well-ordered crystals of human P5CR suitable for X-ray diffraction analysis was found to be critically dependent on temperature (Fig. 4). As a rule, we found the bigger the crystal size, the better the quality of diffraction.

To avoid autoaggregation, crystallization experiments were started just after the incubation of P5CR and reservoir solution at 37 °C for 20 min. Large crystals were obtained using agarose to slow the growth. After growing at 37 °C for 7 days, the crystals achieved a final size of 550 × 80 × 50 μM. A set of data were subsequently collected from this crystal, which belonged to space group C2 with

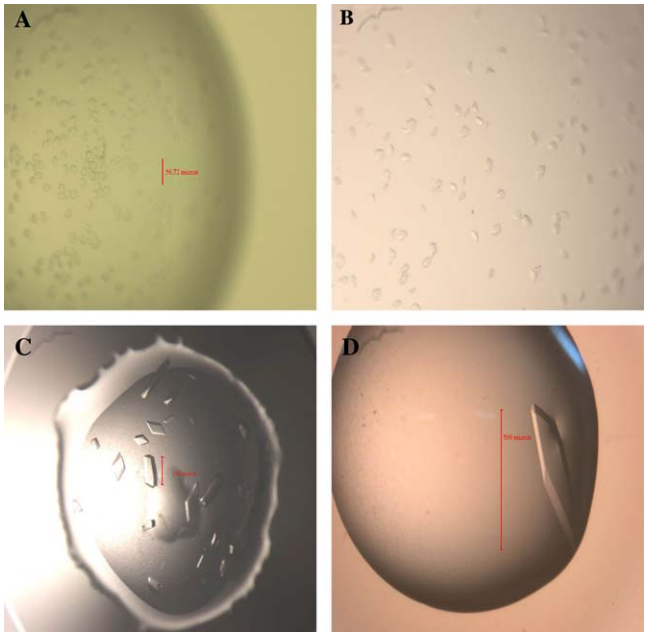

Fig. 4. Crystals from different conditions. (A) 16 °C; (B) room temperature; (C) covered with 1 μl of 100% paraffin at 37 °C; (D) containing 0.1% agarose at 37 °C.

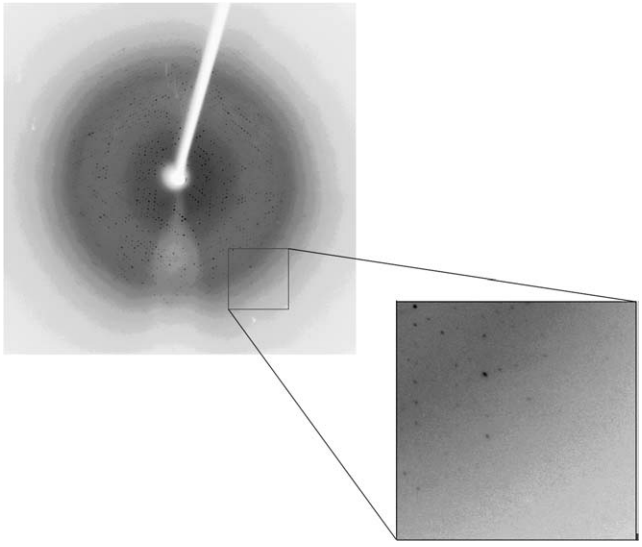

Fig. 5. A typical X-ray diffraction pattern collected from a human P5CR crystal. The diffraction image was collected on a MAR research image-plate detector. The detector edge corresponds to 2.4 Å resolution. The exposure time was 300 s, the crystal-to-detector distance was 150 mM, and the oscillation range per frame was 1°.

Table 2
Data collection statistics

Detector	R-AXIS IV++
Space group	C2
Unit cell parameters (Å, °)	$a = 207.6$, $b = 123.7$, $c = 120.6$, $\beta = 121.7$
Wavelength (Å)	1.5418
Resolution (Å)	50.0–2.8 (2.9–2.8)
Total observations	302,670
Unique observations	63,449
$\langle I/\sigma \rangle$	8.2 (2.1)
Completeness (%)	99.3 (97.8)
R_{merge} (%)	14.8 (53.0)

Numbers in parentheses correspond to the highest resolution shell.

unit-cell parameters $a = 207.6$ Å, $b = 123.7$ Å, $c = 120.6$ Å, and $\beta = 121.7°$ (Fig. 5). Scaling and merging of the crystallographic data resulted in an overall R_{merge} of 9.9% and an R_{merge} in the highest resolution shell (2.8–2.9 Å) of 53.0%. There are five molecules in one asymmetric unit, as defined by a self-rotation search, with an estimated solvent content of 65.6%. Data collection statistics for crystals grown at 37 °C are summarized in Table 2. Preparation of selenomethionyl P5CR derivative protein and heavy-atom soaking derivatives are currently underway to determine the structure of human P5CR.

Acknowledgments

This work was supported by Project "973" (Grant No. G1999075602), Project "863" (Grant No. 2002BA711A12), and the NSFC (Grant No. 30221003).

References

[1] J.M. Phang, The regulatory functions of proline and pyrroline-5-carboxylic acid, Curr. Top. Cell. Regul. 25 (1985) 91–132.
[2] C.H. Hagedorn, J.M. Phang, Catalytic transfer of hydride ions from NADPH to oxygen by the interconversions of proline and Δ1-pyrroline-5-carboxylate, Arch. Biochem. Biophys. 248 (1986) 166–174.
[3] T. Nanjo, M. Kobayashi, Y. Yoshiba, Y. Kakubari, K. Yamaguchi-Shinozaki, K. Shinozaki, Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*, FEBS Lett. 461 (1999) 205–210.
[4] C. Chen, M.B Dickman, Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*, Proc. Natl. Acad. Sci. USA 102 (2005) 3459–3464.
[5] S.P. Donald, X.Y. Sun, C.A. Hu, J. Yu, J.M. Mei, D. Valle, J.M. Phang, Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species, Cancer Res. 61 (2001) 1810–1815.
[6] A. Rivera, S.A. Maxwell, The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway, J. Biol. Chem. 280 (2005) 9346–9354.
[7] S.A. Maxwell, G.E. Davis, Differential gene expression in p53-mediated apoptosis-resistant vs. apoptosis-sensitive tumor cell lines, Proc. Natl. Acad. Sci. USA 97 (2000) 13009–13014.
[8] H. Jacquet, C. Demily, E. Houy, B. Hecketsweiler, J. Bou, G. Raux, J. Lerond, G. Allio, S. Haouzir, A. Tillaux, C. Bellegou, G. Fouldrin, P. Delamillieure, J.F. Menard, S. Dollfus, T. D'Amato, M. Petit, F. Thibaut, T. Frebourg, D. Campion, Hyperprolinemia is a risk factor for schizoaffective disorder, Mol. Psychiatr. 10 (2005) 479–485.
[9] T. Matsuzawa, Disease of the ornithine–proline pathway: delta 1-pyrroline-5-carboxylate reductase deficiency in the retina of retinal degeneration mice, Metab. Pediatr. Syst.Ophthalmol. 6 (1982) 123–128.
[10] O.P. Chilson, A.E. Kelly-Chilson, N.R. Siegel, Pyrroline-5-carboxylate reductase in soybean nodules: isolation/partial primary structure/evidence for isozymes, Arch. Biochem. Biophys. 288 (1991) 350–357.
[11] M. Murahama, T. Yoshida, F. Hayashi, T. Ichino, Y. Sanada, K. Wada1, Purification and characterization of Δ1-pyrroline-5-carboxylate reductase isoenzymes, indicating differential distribution in spinach (*Spinacia oleracea* L.) leaves, Plant Cell Physiol. 42 (2001) 742–750.
[12] K.M. Dougherty, S.Q. Marjorie, C. Brandrissq, D. Valle, Cloning human pyrroline-5-carboxylate reductase cDNA by complementation in *Saccharomyces cerevisiae*, J. Biol. Chem. 267 (1992) 871–875.
[13] S.R. Misener, V.K. Walker, Complementation cloning and characterization of the pyrroline 5-carboxylate reductase gene from *Drosophila melanogaster*, Biochem. Genet. 39 (2001) 15–31.
[14] M.C. Brandriss, D.A. Falvey, Proline biosynthesis in *Saccharomyces cerevisiae*: analysis of the PRO3 gene, which encodes delta 1-pyrroline-5-carboxylate reductase, J. Bacteriol. 174 (1992) 3782–3788.
[15] T. Shiono, P.F. Kador, J.J. Kinoshita, Purification and characterization of rat lens pyrroline-5-carboxylate reductase, Biochim. Biophys. Acta 881 (1986) 72–78.
[16] M.J. Merrill, G.C. Yeh, J.M. Phang, Purified human erythrocyte pyrroline-5-carboxylate reductase. Preferential oxidation of NADPH, J. Biol. Chem. 264 (1989) 9352–9358.
[17] C.E. Deutch, J.L. Klarstrom, C.L. Link, D.L. Ricciardi, Oxidation of L-thiazolidine-4-carboxylate by delta1-pyrroline-5-carboxylate reductase in *Escherichia coli*, Curr. Microbiol. 42 (2001) 442–446.
[18] B. Nocek, C. Chang, H. Li, L. Lezondra, D. Holzle, F. Collart, A. Joachimiak, Crystal structures of delta1-pyrroline-5-carboxylate reductase from human pathogens *Neisseria meningitides* and *Streptococcus pyogenes*, J. Mol. Biol. 354 (2005) 91–106.
[19] Z. Otwinowski, W. Minor, Processing of X-ray diffraction data collected in oscillation mode, in: C.W. Carter Jr., R.M. Sweet (Eds.), Macromolecular Crystallography, Part A, Academic Press, New York, 1997, pp. 307–326.