

Molecular Cloning and Characterization of the Phenylalanine Aminomutase Gene From *Taxus baccata* L.

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ABSTRACT

Background: Taxol is one of the most important anti-cancer drugs, which is obtained from yew trees (*Taxus* sp.). T+he first step in side chain assembly of taxol is catalyzed by phenylalanine aminomutase, which converts α -phenylalanine to β -phenylalanine.

Objectives: In this study, for the first time, we report on the cloning, preliminary expression and characterization of a full-length gene and cDNA encoding phenylalanine aminomutase from *Taxus baccata* L.

Materials and Methods: Comparison of the full-length gene with other ones identified from the *Taxus* species showed high similarity, particularly with *Taxus x media*.

Results: The results showed that the expression level of this gene in *Taxus baccata* is very low and therefore this enzymatic step could be a rate limiting step in the taxol biosynthesis pathway. Successful amplification of the cDNA was only obtained from RNA samples isolated from methyl jasmonate elicited suspension cells of *Taxus baccata*. The cloned cDNA contained a 2064 bp open reading frame encoding a protein composed of 687 amino acids. Sequence comparison analysis revealed that the gene is very similar (98 - 99 %) with respect to the nucleotide and amino acid sequences in different *Taxus* species and also share the signature active site motif (175ASG177).

Conclusions: The predicted structure of TbPAM was analyzed using bioinformatic tools. The results indicated that the protein has similar overall folding to tyrosine aminomutase.

Keywords: Molecular Cloning; Phenylalanine Aminomutase; Taxol; Taxus baccata L.

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Implication for health policy/practice/research/medical education: This article has implication on researchers and scientific collaborators.

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1. Background

Taxus species are known to produce a wide range of natural diterpenoids known as taxoids (taxanes), with approximately 350 identified forms. One of the bestknown taxoids, taxol, has been well proven to be a potent anticancer drug with powerful effects against a range of cancers. The production of taxol from the original plant source is very limited due to the low abundance and slow growth of Taxus trees and the low concentration of taxol in the trees (1). On the other hand, demand for the drug has increased considerably due to the expansion of clinical trials and treatments (2). Therefore, it seems that the only way to increase the yields of taxol and other clinically important taxoids by genotypes, is to study and manipulate the biochemical and enzymatic reactions in the taxoid pathway (3). This is a precondition for the eventual over expression of the slow steps and/or silencing of the subsidiary steps of the pathway (3). Generally, the taxol molecule consists of two components (Figure 1): the taxane ring moiety (10-deacetyl baccatin III) derived from the terpenoid pathway and C-13 side chain derived from the phenylpropanoid pathway (4). Since, the taxane ring moiety is much more readily available

than taxol itself (4), the low content of taxol may be related to its side chain assembly. The taxol biosynthesis pathway contains about 20 distinct enzymatic steps (2). Tremendous progress has been made in the cloning and functional expression of the genes involved in the pathway. The first step in the side chain assembly of taxol is catalyzed by phenylalanine aminomutase (PAM) which converts α -phenylalanine to β -phenylalanine. The activity of the enzyme was demonstrated in the soluble fraction of crude cell-free extracts of T. brevifolia (4). Feeding studies showed that β-phenylalanine is incorporated into both phenylisoserine and benzoate moieties of the side chain (4). Thus, PAM may limit the rate of taxol production at the commercial scale and could be an important target for genetic engineering in yew and its derived cell cultures to increase the taxol productivity. On the other hand, characterization of the enzyme is essential to understand the nature of taxol side chain biosynthesis (5). PAM gene from Taxus cuspidata and Taxus chinensis has been cloned and functionally expressed in Escherichia coli (4, 5). Until now, there is no report on the cloning of PAM gene from *T. baccata*. This species contains comparatively lower content of taxol and higher content of baccatin III in its needles than the other Taxus species (6).



a) biosynthesis of taxane ring moiety from the terpenoid pathway, b) conversion of α -phenylalanine to β -phenylalanine by phenylalanine aminomutase (PAM), c) attachment of C-13 side chain to the Baccatin III and biosynthesis of Taxol.

2. Objectives

In this paper, we described the cloning and characterization of the PAM gene from *T. baccata* (TbPAM) as an initial step to investigate the physiological role of the gene in this species.

3. Materials and Methods

3.1. Materials

General molecular biology reagents were obtained from Fermentas (Germany), Merck (Germany) or Sigma (St. Louis, MO), unless otherwise indicated. Enzymes for PCR and RT-PCR were purchased from Roche (Roche Applied Science, Germany). Cloning of the full-length gene and cDNA were carried out using pTZ57R/T vector (InsTAclone[™] PCR Cloning Kit) from Fermentas (Germany). Plant growth regulators, nutrients and other plant tissue culture materials were purchased from Duchefa (Biochemie B.V., Netherlands).

3.1.1. Plant Material, Media and Culture Conditions

Young leaves and stems were collected from adult *Taxus* baccata trees at the flower garden of Isfahan in Isfahan, Iran. Callus was obtained from the leaf explants in B5 medium containing 2 mg.L⁻¹ NAA and 0.2 mg.L⁻¹ kinetin in dark condition as previously described (7). In order to prepare cell suspension culture of *Taxus*, three grams of two-month old callus were added to 50 mL of the same liquid B5 medium in a 250 mL flask. The flask was incubated for 21 days in darkness at 25°C, shaking at 110 rpm. The culture was elicited at day 14 by methyl jasmonate with a final concentration of 100 μ M. The elicited cells were separated from the remaining medium at day 21 by centrifugation at 5000 rpm for 10 min and used for RNA isolation procedure.

3.2. DNA and RNA Isolation

Total RNA was extracted from the elicited cell suspension using a special method for Taxus species (3). Briefly, the cells were ground to a fine powder in liquid nitrogen and added to 10 mL of preheated extraction buffer containing a high concentration of β -mercaptoethanol (4%) and PVP (4%). The homogenate was then extracted with chloroform: isoamylalcohol (24:1 v/v), 3 times. The upper phase was carefully mixed with a 0.5 volume of 3 M sodium acetate (pH 4.8), incubated at -20 °C for 30 min and centrifuged. The supernatant was mixed gently with 1/4 volume of 10 M LiCl and placed at 4°C overnight. The RNA was pelleted by centrifugation, dissolved in 100 µL DEPCtreated water, and then treated with RNase-free DNase I (Fermentas). The homogenate was extracted twice with chloroform: isoamylalcohol and the RNA was pelleted using ice-cold absolute ethanol, and finally dissolved in DEPC treated water. Genomic DNA was extracted using a CTAB (hexadecyltrimethylammoniumbromide)-based method (3) and used for cloning of the full length PAM gene. The quality and concentration of RNA and DNA samples were examined by agarose gel electrophoresis and spectrophotometer (ND-1000, USA) analysis.

3.3. PCR and RT-PCR

The PCR was done in a total volume of 25 µL containing 2 mM MgCl2, 10 pmol of each primer of PAM (Forward primer: 5⁻TTTTGAATTCATGGGGTTTGCCGTGGAATC-3⁻; reverse primer: 5⁻-TTTTGGTACCCTAGACGCCGTTGGCGCA-3⁻), 200 uM of each dNTPs, 1 unit Tag DNA polymerase (Roche Applied Science, Germany), and 100 ng of genomic DNA in 1x PCR buffer. The PCR amplifications were carried out using a MyCycleTM Thermal cycler (Bio-Rad, USA). Each PCR was initiated by a preheating step for 3 min at 94°C followed by 30 cycles consisting of a denaturation step (30 sec at 94°C), primer-annealing step (30 sec at 61°C), and elongation step (3 min at 72°C). The final elongation step was extended for 8 min. The first-strand cDNA synthesis was done using AMV reverse transcriptase with Oligo-(dT)17 primer and approximately 2 µg of total RNA according to the manufacturer's instructions (Roche Applied Science, Germany). The second strand amplification was carried outusing high fidelity PCR system (Roche Applied Science, Germany) and the PCR conditions similar to that explained before. The PCR products were then analyzed by gel electrophoresis.

3.4. Cloning of Full-Length cDNA and Genomic Gene

The PCR product of appropriate size was gel purified and extracted carefully using a DNA Extraction Kit (Fermentas). The purified fragment was then cloned into the pTZ57R/T vector according to the manufacturer's instructions (InsTAclone[™] PCR Cloning Kit, Fermentas). The vectors were introduced into *E. coli* XL1-Blue cells by electroporation with a Gene Pulser XcellTM apparatus (Bio-Rad, USA) and the cells were cultured overnight at 37°C in Luria Bertani (LB) medium containing 50 µg.mL⁻¹ ampicillin. The positive clones were selected based on lacZ gene marker and finally sent for sequencing to the Macrogene Company (Seoul, Korea).

3.5. Preliminary expression and Analysis of Recombinant Protein

The recombinant plasmid containing PAM cDNA was cotransformated into *E. coli* BL21 (iDE3) cells (Merck Biosciences, Schwalbach, Germany) and cultivated at 30°C overnight in 5 ml LB medium containing 100 μ g.mL⁻¹of ampicillin. The overnight culture was then inoculated into 500 mL of the same medium and cultivated at 30°C. The culture was grown at 30°C to A600= 0.5 – 0.6, and then induced by addition of 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) to express the PAM gene. The cells were grown for another 4 h at 30°C and finally the presence of the respective protein was detected using SDS-PAGE (12% Laemmli gels). Protein concentrations were determined on the basis of the calculated extinction coefficient at 280 nm: 51185 M-1 cm⁻¹.

3.6. Bioinformatics Analysis

BLAST was used to investigate for homology in the Gen-Bank database and the Gene Runner program was used to deduce the amino acid sequence of the gene. Multiple sequence alignments were performed using the DNASTAR Lasergene core suit software. Analysis and characterization of the protein were performed using protparam belonging to the Swiss Institute of Bioinformatics [http:// www.expasy.ch/tools/protparam.html/]. Secondary structure of TbPAM was analyzed by the Swiss-pdb viewer tool (version 4.01). Tertiary structure of TbPAM was predicted by using phyre webserver (Version 0.2) and Swiss Model 8.05 [http://www.expasy.org/swissmodel/].

4. Results

4.1. DNA and RNA Isolation

Isolating high-quality samples of nucleic acid is a critical step in many molecular biology experiments especially in cDNA synthesis of low expressed genes or rare mRNAs. Degradation and contamination of the RNA samples during the isolation process may cause the failure of the following experiments. Different tissues of Taxus contain high levels of secondary metabolites especially phenolic compounds that are rapidly oxidized and vigorously bond nucleic acids during isolation and finally result in brown and insoluble nucleic acid pellets. The presence of such powerful oxidizing agents in the samples may result in decreased purity and productivity of the final extracted nucleic acids. Agarose gel electrophoresis of nucleic acids also showed good quality of DNA and RNA samples with no considerable polysaccharide, polyphenol and protein contaminations (Figure 2). The results indicate that high quality DNA and RNA samples with appropriate yields can be obtained using the protocol.

4.2. Cloning and Sequence Analysis of the Full-Length Gene

For identification and amplification of the full length of thePAM gene from *Taxus baccata*, forward and reverse primers were designed based on highly conserved 5' and 3' terminal sequences of ORF regions of the PAM genes in other *Taxus* species. Digestion sites of *EcoRI* (under lined sequence) and *KpnI* (bold sequence) restriction enzymes were also added to the ends of primers for cloning the gene in the vector (pASK-IBA43plus) for subsequent experiments. In order to improve the digestion efficiency, four thymine nucleotides were also added to the 5'-ends of primers. As a result of these nonspecific nucleotides

in primers, the annealing temperature was reduced considerably. PCR reaction using the primers and *Taxus* baccata genomic DNA (as template) resulted in the specific amplification of a single fragment of about 2250 bp (Figure 2) that was cloned into the vector and sequenced subsequently. Investigating the sequence of the cloned fragment in Gene Bank showed high similarity to the sequences of PAM genes identified in other Taxus species. Moreover, specific amplification of the fragment in the PCR reaction confirmed the assumption that the 3'- and 5'ends of PAM gene from Taxus baccataare similar to those reported previously from other Taxus species. The cloned full-length of PAM gene from Taxusbaccata was 2233 bp in lengh (designated as TbPAM, Genbank accession number: GU214709) and contained an exon within the gene from nucleotide 1092 to 1260. Searches against Gen Bank using the BLAST algorithm revealed that the similarity between TbPAM and the gene obtained from *Taxus* x media is more than that from Taxus canadensis and Taxus chinensis with respect to the sequence, length and exon site. Since Taxus x media originated from the hybridization of T. baccata and T. cuspidate (8), such a result was predictable.



A, Taxus baccata Genomic DNA (1: Marker and 2, 3: Isolated DNA); B, RNA isolated from Taxus baccata (1: 1 µg total RNA and 2: Marker); C, PCR product (1: Marker and 2: Full length of Phenylalanine aminomutase gene (2233 bp)); D, RT-PCR product (1: Marker and 2: Phenylalanine aminomutase cDNA (2064 bp)).

4.3. Cloning and Sequence Analysis of the TbPAM cDNA

Isolating RNA samples from the native tissues and also non-elicited cell suspensions of *Taxus baccata* to use in RT-PCR reactions for synthesis of PAMcDNA, didn't result in any amplified product and all of the experiments failed. This may be related to the low expression level of the gene in *Taxus baccata* L. Moreover, the lower productivity of taxol in Taxus baccata compared to the other taxus species may also be related to the low expression of the PAMgene in this species and perhaps PAM would be a rate limiting enzyme in taxol biosynthesis pathway. However, more studies on the taxol biosynthesis pathway and the functions of the enzyme in the pathway could be useful in this context. Several studies have previously emphasized on the elicitor effects of methyl jasmonate on taxol synthesis from culture suspension of Taxus cells (9). Wang et al. (9) presumed that methyl jasmonate may activate the biosynthesis pathway from baccatin III to taxol and inhibit biosynthesis from baccatin III to cephalomannine. Based on this assumption, it seemed that methyl jasmonate is a suitable choice to induce the expression of PAM gene and consequently to increase the probability of gene amplification. Therefore, we isolated RNA samples from *T. baccata* cell suspensions, which were induced to result maximal taxol synthesis with 100 µM methyl jasmonate at day 14 of culture. RT-PCR reactions by using these RNA samples resulted in amplification of a single fragment with lengh of about 2050 bp (Figure 2). Sequencing result of the fragment (*Figure* 3) coincided with the sequences from ORF region of PAMcDNAs identified

previously from other Taxus species (AY582743, AY724735). The cloned cDNA of TbPAM (Genbank accession number: GU214708) was 2064 bp in length. Nucleotide BLAST of the sequence from the NCBI website indicated that the gene shows only very minor sequence differences (almost as silent mutation) with the other known sequences of Taxus PAM (99% similarity with Taxus chinensis and Taxus x mediaand 98% with Taxus canadensis). Since all Taxus species are known to be very closely related and are considered to represent geographic variants of the basic species, T. baccata, such a result had been anticipated. The protein contained 687 amino acids (Genbank accession number: ADA57703) with a molecular weight of about 75.25 kDa and an isoelectric point of 5.7. As also showen by Figure 4, amino acid sequence of TbPAM had high similarity (98%) with the other known Taxus Phenylalanine aminomutases (AAU01184, AAU01183, and AAU01185). The minor differences are likely attributable to the species differences or to allelic variations. The secondary structure of TbPAM was analyzed by Swiss-pdb viewer tool (version 4.01) and the results showed that the putative TbPAM peptide contained 51% alpha helix, 7% beta sheet, and 42% random coil.

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V	A	A	Ц	A	К	K	н	D	V	ĸ	V	A	-	E	A	E	Q	C	К	A	K	V
GΑ	AGA	CGG	CGC	CGA	CAT	ATA	CGG	CGT	CAC	CAC	GGG	CTT	CGG	cgo	:GTG	CTC	GAG	CCG	GAG	GAC	CAA	CCA
Ε	D	G	A	D	Ι	Y	G	V	Т	T	G	F	G	A	С	S	S	R	R	Τ	N	Q
СТ	GCT	CGC	GGG	GGT	GTT	TAC	TAA	AGG	ATC	cad	ттc	СТС	CGT	CGA	CGA	GCT	ccc	CGC	GAC	CGC	CAC	ccg
T.	т.	A	G	v	F		R	G	C	A	S	s	v	D	E	т.	P	A	Ψ	A	Ψ	R
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ΤA	TGG	ATG	TTC	CGG	CAT	CCG	GTG	GGA	GGI	CAT	GGA	AGC	GCT	GGA	AAA	GCT	TCT	CAA	CAG	CAA	TGT	CTC
Y	G	С	S	G	I	R	W	E	V	Μ	E	A	L	E	K	L	L	Ν	S	N	V	S
ΤC	GGG	AGA	CCT	CAT	ççç	GCT	CGC	CTA	CAT	TGC	AGG	GCT	CCI	GAT	'CGG	GAA	.GCC	TAG	CGT	AAT	CGC	TCG
S	G	D	L	I	P	L	A	Y	Ι	A	G	L	L	Ι	G	K	Ρ	S	V	Ι	A	R
GC	GTT	GAG	CAG	GGT	GGG	GCT	GCG	GCC	ATT	CAA	GCT	CCA	GGC	CAA	AGA	AGG	GCT	GGC	GCT	CGT	CAA	CGG
A	Г	s	R.	v	G	L	R	P	F	K	L	Q	A	K	Ε	G	L	A	L	V	N	G
AT	GTA	CGA	ago	CAA	TGT	TCT	GTT	GCT	GCT	CGT	CGA	AAC	GCT	TTG	CGG	AAT	GTT	CTG	CGA	GGT	GAT	CTT
Μ	Y	D	A	Ν	V	L	L	L	L	V	Ε	Т	L	С	G	Μ	F	С	Ε	V	I	F
ЪЪ	AGT	GAN	GCC	GCA	ccc	GGG	CCA	GAT	CGA	ATC	GGC	GGA	GCT	GCT	CGA	GTG	GCT	GCT	GCG	GTC	GAG	ccc
K	V	ĸ	P	Н	P	G	Q	I	Е	S	A	Ε	L	L	Е	W	L	L	R	s	S	P
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GA	TAA	GCT T	GAA	.GAA v	ACC	GAA	ACA	ADD.	TCG P	UTA V	.T.G.C.	TCT	GAG D	G.L.C	.GAG o	rCCC D	GCA	GTG. W	ст. т	200	.TCC	TCT
D	r.	Ц	n	n	Ľ	r.	Q	D	к	T	A	Ц	к	Ð	D	Ľ	Q	vv	Ц	A	Ľ	Ц
GΑ	GAC	GGA	GGT	CAA	TTC	CGC	CAA	TGA	TAA	CCC	CAT	CAT	TGA	CCA	CGC	CAA	TGA	CAG	GGC	TCT	CCA	TGG
Ε	Т	Ε	V	Ν	S	A	Ν	D	Ν	P	Ι	Ι	D	Η	A	N	D	R	A	L	Н	G
AT	GGA	CTA	CGT	GCG	CAT	CGC	AGT	CGC	CGG	GCT	GGG	GAA	ACT	CTT	GTT	CGC	TCA	.GTT	CAC	GGA	GCT	GAT
Μ	D	Y	V	R	Ι	A	V	A	G	L	G	K	L	L	F	A	Q	F	Т	Ε	L	Μ
СТ	CTC	сст	GGG	GCC	GGA	сст	GAG	CGT	GGA	CTA	CGG	сст	CAA	GGG	GCT	CGA	CAT	CGC	CAT	GGC	CGC	CTA
L	s	L	G	P	D	L	S	V	D	Y	G	L	K	G	L	D	I	A	М	A	A	Y
AC	CAC	ACA	CGT	TCA	.CAG	CGC	GGA	ACA	GCA	CAA	CCA	GGA	CAT	CAA	CTC	TCT	GGC	GCT	CAT	CTC	CGC	CCG
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Figure 3. The Full-Length cDNA Sequence and Deduced Amino Acid Sequence of Taxus baccata Phenyl Alanine Aminomutase



80	
157 GTDTMGVTTGFGATSHR RTS QG VELOKE LI RFLNAGVFGS-CEGNV LPEATTRAAMLVRTNTLPQG	cal z
155 GTDTYGVTTGFGATSHR RTNQGAELQKE LI RFLNAGVLGK-CPENV - LSEDTTRAAM LVRTNTLLQG	pal Pm
133 GADIY GVTTGFGACS SR RTNO LSELQES LI RCLLAGV FTKGCASSV DELPATATRSAML LRLNSFT YG	pam ch
115 GADIYGVTTGFGACS SR RTNQ LSELQES LI RCLLAGVFTKGCASSVDELPATVTRSAML LRLNSFT YG	pam xm
61 NIPIYSVTTGYGEMIYMQVDKSKEVELQTNLVRSHSAGVGPL F AEDEARAIVA ARLNTLAKG	TAM
76 GADIYSVTTGFGACS SR RTN Q LSELQESL I RCILAGVFTKGCASSVDELPATATRSAML LRLNSFT YG	pam tb
175-177	
222 YSGI RWAL LET IEKLLNAGIT PK LPLRGT ITASGDLVPL SY I AGLLTGRPNSKVRTR DG TEM SG LEALKQ	pal g
220 Y SG I R W D I LET V EK LLN A G LT P K L P L R G T I T A S G D L V P L S Y I A G L T G R PN S R V R S R D G I EM S G A E A L K K	pal Pm
201 CSGI RWEVMEALEKLLNSNVSPKVPLRGSVSASGDLI PLAY I AGLL IGKPSVIAR IGDDVEVPAPEA LSR	pam ch
183 CSGI RWEVMEALEKLLNSNVSPKVPLRGSVSASODLI PLAY I AGLL IGKPSVIAR IGDDVEVPAPEALSR	pam xm
123 H SAVR P IIL ERLAQYLNEG ITPA IPE IGS LGX DLAPLSHV ASTLIGEGYVLR DGRPVETAQVLAE	TAM
144 CSGI RWEVMEALEKLLNSNVSPKVPLRGSVSASODL IPLAY I AGLL IGKPSVIAR IGDDVEVPAPE ALSR	pam tb
214 231	
292 VGLEKPFELQPKEGLAIVNGTSVGAALASIVCFDANVLAVLSEVMSAMFCEVMNGKPE-FTDPLTHRLKH	palg
290 V G LEKP FELQ PK EG LAIV NGTS V GAALAS I V CFDANVLA L LS EV I SAM FCEVM NG KPE-FTDPLT H KLKH	pal Pm
271 VGLR-PFKLQAKEGLALVNGTSFAT AVASTVMYDANVLL L LVE TL CGM FCEV I FGREE-FAHPLIHKVKP	pam ch
253 V G L R - P F K L Q A K E G L A L V N G T S F A T A L A S T V M Y D A N V L L L V E T L C G M F C E V I F G R E E - F A H P L I H K V K P	pam xm
188 RGI E-PLEL RFKEGLAL INGTSGMTGLGSLV VGR ALEQAQQAE IV TA LLIE AV RGSTSPFLAEGHDI ARP	TAM
214 VGLR-PFKLQAKEGLALVNGTSFA TALASTVMYDANVLLLLVETLCGMFCEVIFGREE-FAHPLIHKVKP	pam tb
321-325	
361 H P G Q M E A A A I M E Y V L D G S S Y M K Q A A K LQ E L N P LQ K P K Q	palg
359 H P G Q M E A A A I M E Y V L D G S ···· S Y M K H A A K LH EMN P L Q K P K Q ···· · P R Y A L R T SPQ W L G P Q 4 P E I I R S	pal Pm
339 H P G Q T ESA E LL E W L L R S S ···· P F Q E L S R E Y Y S I D K L K K P K Q ······ C R Y A L R S S P Q W L A P L V Q T I R D	pam ch
321 H P G Q I ESA E LL E W L L R S S ···· P F Q E L S R E Y Y S I D K L K K P K Q ······ G R Y A L R S S PQ W L A P L V Q T I R D	pam xm
257 HEGQ I DTAANMRA LMRGSGLTVEHAD LRRELQKDKEAGKDVQRSEIVLQ KAYSLRAIPQ VVGAVRDTLYH	TAM
282 HPGQI E SAELLEWLLRSS PFQELSREYYSIDKLKKPKQ	pam tb
371	
420 ATH MIEREINS V NDN PV IDVSRDKALHGGNFQGT PI GVSM DNLRLSIAAIGKLMFAQFSELV NDVV NGGL	pal g
418 ATH MIEREINS VNDN PV IDV ARDKALH GGN FQGT PI GVSM DNLRLS IS AIGKLM FA DFSELV NDY NGGL	pal Pm
398 ATT TV ET EV N SAN DN PI ID HAN DRALH GAN FQ G SAV G FY M DY VRIAV AG LG KLL FAQ ET ELMI EY Y/S NG L	pam ch
380 ATT TVETEVNSANDNPI IDHANDRALHGANFQG SAVGPYM DYVRIAVAGCORLL FAQFTELMI EYYS NGL	pam xm
827 ARHKLRIELNSANDNPLFFEGRE - IFHGANFHGQPIAFAMDFVTIALIOLGVLAERQINRVLNRHLSYGL	TAM
341 ATT TV ETEVNSANDNHI I DHANDRALH GANEQGSAV GFYM DY VRI AV AGLG KLL FAQ FTELMI EY YSNGL	pamtb
427	
490 PSNLSGG PNPS LDYGLKG	pal g
ASS PSNLSGGPNPS LDTGLKG	pal Pm
	pam ch
	pam xm
350 PETRO SC - DPULAS GRAG	LAM
ALL PONIS LOPDESV DTOLKG	pam to



4.4. Predicted Structure and Active Site of TbPAM

Similarity searches using the BLAST algorithm indicated that the amino acid sequence of TbPAM is most similar (up to 98%) to phenylalanine ammonia-lyase (PAL). Among the other aminomutases, Tyrosine Aminomutase (TAM) showed most similarity (70%) to TbPAM with respect to the amino acid sequence. Based on PAL-derived TAL activity in monocots and fungi (10) and also high structure similarity between phenylalanine and tyrosine, it seems that the binding mechanism and mode of action could be similar in PAM and TAM. Based on this assumption and the scientific literature on the crystal structure of TAM (11), probable protein structure of TbPAM was predicted by using the phyre2 server from the Centre for Bioinformatics Imperial College, London (Figure 5). The predicted structure of TbPAM adopts a predominantly α-helical fold that is linked together by β -sheets. Such a structure could be essential for flexibility and plasticity of the enzyme and seems to be a functional requirement for substrate binding and catalysis. Data indicated that TbPAM has the same overall protein fold as tyrosine aminomutase (Sg-TAM) that was reported by Montavon and coworkers (11). Moreover, superposition of the theoretical structure of TbPAM with TAM and PAL indicated significant homology where TbPAM contains a 4-methylidene-imidazole-5-one (MIO) cofactor. The MIO moiety formed in the active site of the enzyme by spontaneous (autocatalytic) cyclization and dehydration of an internal 175Ala-Ser-Gly177 tripeptide motif was similar to TAM and histidine/phenylalanine ammonia lyase (PAL/HAL) family (*Figure 4*). These similarities suggest a common mechanism in the vicinal interchange catalyzed by PAM and TAM, and also in the elimination of ammonia catalyzed by the related lyases. Furthermore, the amino acids involved in active site of SgTAM exactly conserved in TbPAM.



Figure 5. Predicted Structure of Phenylalanine Aminomutase from Taxus baccata L.

5. Discussion

Study and manipulation of the enzymes involved in metabolic pathways are considered as one of the most efficient methods to improve productivity and also to alter pathways for production of novel compounds. Study of substrate specificity and enzymatic chemistry is an essential step towards this aim. Aminomutases that are classified as isomerases, catalyze the vicinal interchange of an amino group and hydrogen either via radical-dependent homolytic or ion-dependent heterolytic mechanisms. Taxus baccata PAM (TbPAM) catalyzes the conversion of α -phenylalanine to β -phenylalanine; the first committed step in the biosynthesis of N-benzoyl phenylisoserinoyl side-chain of the anticancer drug,taxol. PAM revealed a multifunctional activity which catalyzes the isomerization of $2S-\alpha$ -phenylalanine to $3R-\beta$ -phenylalanine with retention of configuration at C3, reversely the conversion of both R- and S-β-phenylalanine to the same 2S-αphenylalanine, and also the α,β -elimination of ammonia from the α -isomer to form trans-cinnamic acid (4). Recent mechanistic studies on the aminomutases (PAM and TAM) revealed that 4-hydroxycinnamic acid (in the case of TAM) and cinnamic acid (in the case of PAM) are intermediates in the aminomutase reaction that can be released from the active site of the enzyme. These observations indicate that TAM and PAM might exhibit ammonia lyase activity and suggest the possibility of using the second half of the aminomutase reaction to synthesize β-amino acids from cinnamates (12). Comparison of the amino acid sequence of TbPAM with PAL and TAM indicate that the enzyme also contain a signature Ala-Ser-Gly motif which rearranges auto-catalytically to the MIO moiety in the active site of the enzyme. Unlike the aminomutases that require external cofactors, PAM activity depends on a MIO-derived cofactor. Based on crystallographic experiments on the MIO-dependent tyrosine aminomutase, Christanson et al. (13) suggested that MIO reacts with the amino group of the substrate during the initial deamination step of the aminomutase reaction. It seems that the α -amine of the substrate (α -phenylalanine) adds into the electrophilic moiety (MIO) via a conjugate addition that facilitates deprotonation of the β-hydrogen. In this condition, the carbon-nitrogen bond is broken and the MIO-NH2 bond and α , β -unsaturated carboxylic acid are formed. Then the MIO-bound amine adds on at the β -position while these intermediates retain in the active site of the enzyme. Finally, the product (β -phenylalanine) is ultimately released and the MIO group regenerated. Walker et al. (4) revealed that potassium cyanide and sodium borohydride both eliminate the function of the MIO in PAM similar to ammonia lyases. The result suggests that the MIO moiety is essential for the activity of PAM similar to TAM and PAL. Comparison of PAM from T. baccata to those previously acquired from other Taxus species revealed that these enzymes are very identical (99%) at the amino acid level and they share, as expected,

the signature active site motif 175Ala-Ser-Gly177. This may be related to the essential role of the enzyme in the taxol biosynthesis pathway. Similarity searches using the amino acid sequence of TbPAM indicated that the enzyme is most similar (up to 98%) to phenylalanine ammonia-lyase (PAL) that catalyzes the first committed step in the phenylpropanoid biosynthetic pathway. PAM and PAL share several enzymatic properties specially the lack of cofactor requirements and monomeric molecular weight (14) that suggest a common mechanism in the action of these enzymes. The higher Km for PAM (1100 µM) than PAL (29-260 μ M) indicates that if the phenylpropanoid and the taxoid pathways were in direct competition for substrate then the phenylpropanoid pathway would be favored (14). More importantly, the high Km for PAM suggests that PAM may be a rate-limiting enzyme in the biosynthesis of taxol (5). Based on the points mentioned above and the relative abundance of some precursors such as baccatin III compared totaxol in the Taxus tissues, it seems that PAM catalyzes a rate limiting enzymatic step in taxol biosynthesis pathway and is considered as an important target for genetic engineering in yew. Over expressing the enzyme or genetic engineering to increase the affinity of the enzyme to the substrate in Taxus, may improve the biosynthesis of taxol. On the other hand, competition between PAM and PAL for α-phenylalanine may be an important factor that decreases the efficiency of PAM.

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Authors' Contribution

Abolghasem Abbasi Kajani: experimental procedures, preparing the manuscript, Mohammad Reza Mofid: bioinformatics analysis, the manuscript preparation, Khalil Alami Saeid: experimental procedures.

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همسانهسازی مولکولی و توصیف ژن فنیل آلانین آمینوموتاز از گیاه سرخدار گونه Taxus baccata L.

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صه مقاله	اطلاعات مقاله
دمه: ت اکسول یکی از مهمترین داروهای ضد سرطان است که از درخت سرخدار (جنسTaxus) به دست میآید. اولین مرحله در واکنش کار نیم مان سال کار اورا سرار آناد ذنا آلاد می سارخدا آلاد مار سرکتر می از آن منا آلاد مار سرکتر کار اورا می د	نوع مقاله مقاله پژوهشی
حیل ریچره جایی تا تسول سامل بیدیل الفا ^س قیل ا نین به بنا – قیل ا نین است که به وسیله اتریم قیل ا نین امیوموتاز را برای اولین بار از خدار گونه Laxus baccata گزارش کردیم. اد و روش ها: ابتدا نمونههای DNA و RNA از سلولهای تحریکشده گونه Taxus baccata L استخراج شد. سپس به کمک زگرهای اختصاصی و با استفاده از روشهای PCR و RT-PCR، به ترتیب توالی کامل و cDNAی ژن فنیل آلانین آمینوموتاز تکثیر و پلاسمید PTZ57RT همسانهسازی شد. از پلاسمیدهای نوترکیب برای توالییایی و بیان مقدماتی ژن استفاده گردید. در نهایت تولی ژن و	تاریخچه مقاله اهت تاریخ دریافت: ۲۱ مرداد ۱۳۹۱ سر تاریخ تجدید نظر: ۱۰ دی ۱۳۹۱ موا تاریخ پذیرش: ۱۴ اسفند ۱۳۹۱ ا
فتار احتمالی آنزیم PAM با استفاده از نرم افزارهای مختلف مورد بررسی قرار گرفت. نته ها : تکثیر موفقیت آمیز cDNA نها با استفاده از نمونه RNA، استخراج شده از سلول های کشت سوسپانسیون تحریک شده با متیل سمونات به دست آمد. cDNA ی همسانه سازی شده شامل یک چارچوب خواندن باز ۲۰۶۴ نوکلئوتیدی بود که پروتئینی متشکل از ۶۸۷ اسید نه را رمز می کرد. بررسی مقایسهای توالی این ژن بین گونههای مختلف <i>Taxus</i> نشان داد که از نظر توالی نوکلئوتیدی و اسید آمینهای به دار را مرز می کرد. بررسی مقایسهای توالی این ژن بین گونههای مختلف <i>Taxus</i> نشان داد که از نظر توالی نوکلئوتیدی و اسید آمینهای بسیار را ۹۸ – ۹۹ ٪) هستند و موتیف مشخصه جایگاه فعال (ASG177175) را نیز در بر دارند. مقایسه توالی ژن کامل با نسخههای دیگر ژن در گونههای دیگر سرخدار شناسایی شده بود، مشابهت بسیار زیاد را به ویژه با گونه یا گونه Taxus تعلی توالی نوکلئوتیدی ا تو و نتیجه گیری : نتایج نشان داد که سطح بیان این ژن در گونه و Taxus <i>baccata</i> بسیار پایین است و بابراین این مرحله آنزیمی تواند مرحله محدود کنده سرعت در مسیر بیوستنژی تاکسول باشد. ساختار آنزیم Tby با استفاده از ایزارهای بیوانفورماتیکی پیش بینی روسی شد. نتایج نشان داد که سطح بیان این ژن در گونه Pay الار Taxus baccata بسیار پایین است و بنابراین این مرحله آنزیمی روسی شد. نتایج نشان داد که سطح بیان این ژن در کونه Tay محمدو تا Tay می بایونه و اندام از بازارهای بیوانفورماتیکی پیش بینی	سا- کلمات کلیدی: فنیل اَلانین آمینو موتاز جاد مش می همسانه سازی مولکولی و ب

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