

MOLECULAR MODELING OF α -AMYLASE FROM GERMINATED SOYBEAN (*Glycine max*) AND ITS FUNCTIONAL DIVERSITY

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Abstract- Starch hydrolyzing α -amylase was purified from germinating soybean seeds. Amino acid sequence of soybean α -amylase (Accession No. Gm0237X0071) was taken from protein databases (http://soybeangenome.siu.edu/; http://soybase.org/; http:// soybeangenome.org/) and used for identification of full length gene with available clone data at NCBI (http://www.ncbi.nlm.nih.gov/). *Glycine max* strain Williams 82 clone GM_WBb0115J10 (AC235387.1) was used for similarity search and annotation of full-length gene. The present *in-silico* investigation deals with full length gene (TPA BK007878) identification; and *cis*-acting elements study; identified the important promoter's i.e. TATA, CAAT, GATABOX, DOFCOREZM, -300ELEMENT, WBOX, MYBST1, and EBOX for multifarious uses. A template structure (3AMK chain a) from *Oryza sativa* branching enzyme was selected for comparative modeling using an automated approach. Homology model was constructed using software DS Modeler and the quality of refined model was investigated using PDBSum, ERRAT and other bioinformatics softwares. The modeled structure showed acceptable Ramachandran statistics and remarkable active site residues. Structural analysis of the predicted model of α -amylase from soybean also gives an idea about potential sites inferring the region of catalytic active site responsible for inhibitory action; and opens the new opportunities for further investigations.

Keywords- a-Amylase, Glycine max, Homology modeling, Ramachandran plot, DS Modeler

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Introduction

α-Amylase (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1.1) categorized as family 13th of glycosyl hydrolase, functions the endohydrolysis of α,1→4 glycosidic linkages present in various polysaccharides [1-3]. The end product of its action constitutes glucose, maltose and oligosaccharides with varying length with an αconfiguration [4]. They are ubiquitous in nature occupy presence in all sections of life viz. plants, animals and microbes [5].

α-Amylases are composed of three structural domains. The largest is Domain A which forms a central eight-stranded (β/α)₈-barrel, to one end of which are located the active site residues [4, 6-7]. Domain B serves to form a calcium binding site against the wall of the (β/α)₈-barrel of Domain A. Domain B is probably responsible for the differences in substrate specificity and stability among the αamylases [8]. Domain C is made up of antiparallel β-structure and is only loosely associated with Domains A and B [9-10]. Residues equivalent to Asp-206, Asp-297, Glu-230 (catalytic site residues) and His-122, His-296 (part of substrate binding site) are conserved in all α-amylases [7,11-13]. Calcium ion, which is located at the interface between the A and B domains, is conserved in all αamylases with known three dimensional structures [14-17]. provides a valuable understanding of the function of molecular systems [18]. Protein structure determination is growing rapidly as can be seen from the large structural data available in Protein Data Bank (PDB) [19]. However, determination of the structure of protein complexes still remains a difficult task because of the experimental hurdles. Thus, computer algorithms and web server are used to predict the structure of the proteins that have not all been solved structurally. Homology modeling can be used to predict model structure of unknown protein (target) from its amino acid sequences based on the structure of related protein of known structure (template) [20-23]. Four steps are used to create a model (i) template selection (ii) alignment of template sequence with the target (iii) model construction (iv) evaluation of generated model [24].

Recently, Starch hydrolyzing α -amylase from germinated soybeans seeds (*Glycine max*) has been purified 400-fold to electrophoretic homogeneity with a final specific activity of 384 units/mg [25]. Peptide map obtained by MALDI along with its molecular mass information was used for the confirmation of the soybean α -amylase. In the present study, homology modeling of α -amylase from soybean was done using DSMODELER [26]. This software constructs three-dimensional structure of the protein to show the functional and binding site with their specific domains. Predicted model was evalu-

Knowledge of the three-dimensional structure of protein complexes

ated with the lowest value of PROCHECK statistics (http:// nihserver.mbi.ucla.edu/SAVES_3/saves.php) and quantitative measures of the predicted model building was carried out using Qmean (http://swissmodel.expasy.org/qmean/cgi/index.cgi) and Vadar (http://redpoll.pharmacy.ualberta.ca/vadar/) servers.

Methodology

Mass Spectrometry, Database Searching and Sequence Alignment

Soybean a-amylase was purified according to Kumari et al. [25] and analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS; Ultraflex III, Brüker-Daltonik, Bremen, Germany). Single band on SDS-PAGE was excised and was digested overnight with MALDI grade trypsin (Sigma). The digested mixture was analyzed by mass spectrometry to produce a peptide mass spectrum from which molecular masses of all of the proteolytic fragments can be read. The MASCOT software [27] was used to compare the peptide mass patterns obtained with those of all proteins from the theoretical soybean α -amylase proteome. The molecular weight search (MOWSE) scoring scheme [28] was used for unequivocal identification of proteins. Since a-amylase from soybean is already available in protein database (http://Glycine max genome.siu.edu/; http://soybase.org/); so the peptide masses as obtained with mass spectrometry was used for the confirmation of the protein.

Sequence of soybean α -amylase was aligned with the known sequence in database using the ClustalW [29]. These known sequences were taken from http://www.expasy.org/enzyme/3.2.1.1. It gives clear information about the best match for the given sequence and aligned them accordingly. There is no similar protein sequence available in NCBI public database but tblastn study gives an idea about the availability of α -amylase gene at genomic level so we did comparative study based on existing α -amylase sequence obtained from (http://www.matrixscience.com) with accession no. Gm0237X0071. It showed similarity with *Glycine max* strain Williams 82 clone GM_WBb0115J10 (AC235387.1). Full length gene prediction was done for obtained strain using Fgenesh (http:// sun1.softberry.com/berry.phtml) and -1000 upstream region was retrieved for promoter study using PLACE [30].

Sequence Analysis, Template Search and Model Generation

Sequence of α -amylase from soybean was aligned with diverse species using ClustalW and phylogenetic tree was constructed using UPGMA method [31,32]. The conserved motifs present in these sequences were analyzed using MEME (Multiple EM for Elicitation) [33]. Parameters have been set with number of different motifs: 30, minimum motif width: 100 and maximum motif width: 300, manually. The result obtained from PDB advance search showing their structural similarity with branching enzyme from *Oryza sativa* (PDB ID: 3AMK, chain a) at a resolution of 2.30 Å [34].The three dimensional structure of α -amylase from soybean has been predicted using DSMODELER [26].

Refinements and Evaluation

Energy minimization of the generated model was done using the Conjugate Gradient technique to remove the bad contacts between protein atoms. Model was evaluated according to the lowest energy value of the objective function and the backbone conformation of the model using DSMODELER [26].

Phi/Psi Ramachandran plot obtained with stereo chemical properties were assessed using PROCHECK server (http:// nihserver.mbi.ucla.edu/SAVES_3/saves.php) [35-36]. Structural comparison between template and target structure were calculated using PROSA server (https://prosa.services.came.sbg.ac.at/ prosa.php) and (ii) UCSF Chimera 1.5.1. UCSF Chimera 1.5.1. (http://www.cgl.ucsf.edu/chimera/) is a structural comparison matchmaker tool based on Needle-Wunsch Alignment algorithm BLOSUM -62 matrix [37]. QMEAN server (http://swissmodel.expasy.org/ amean/cai/index.cai) model quality estimation was used to analyzed QMEAN score / QMEANclust score, Residue error, Energy profiles and plot and Volume area dihedral angle for fractional accessible surface area, residue volume, 3D profile and stereo/packing quality index were done with VADAR (http://vadar.wishartlab.com/). QMEAN and VADAR were specially designed for quantitatively and qualitatively assessing protein structures determined by X-ray crystallography, NMR spectroscopy, 3D-threading or Homology modeling.

Active Site Prediction

Possible binding sites of the final obtained model were searched using Q-site Finder [38]. Q-site Finder determines the potential sites for ligand binding in docking calculations. Obtained binding sites were compared to the active site of the template for the determination of residues forming the binding pocket and to find their role in biological functions.

Results

Mass Spectrometry and Database Searching and Sequence Alignment

Peptide mass spectrum obtained from the digestion of purified sovbean a-amylase by trypsin provides fingerprint of great specificity. This peptide masses significantly revealed 9 tryptic peptides masses corresponding to a-amylase from soybean with accession no. Gm0237x00071 with 17% sequence coverage [25]. The obtained full length peptide sequence of soybean α -amylase was used for sequential and structural alignment. After sequence alignment using tblastn study it was observed that soybean α-amylase showed similarity with soybean (Glycine max) strain Williams 82 clone GM_WBb0115J10 (AC235387.1) and matches at position 104382 to 109759. Complete full length gene was fetched out and submitted to NCBI and obtained TPA Accession: TPA BK007878. Complete hypothetical mRNA showed the combination of gene with 14 CDS, TSS site at 921 position and Poly A site at 8824 position [Fig-1A]. cis-acting element study showed the gene containing regulatory promoters, as listed in [Table-1]. Seed germination specific promoter has been prominently identified in upstream region. Important promoters investigated were TATA, CAAT, GATABOX, DOFCOREZM, -300ELEMENT, WBOX, MYBST1 and EBOX. The length of a-amylase for soybean is 741 amino acids with a molecular mass of 84 kDa for (Accession No. Gm0237X0071) whereas. predicted a-amylase gene (i.e. TPA BK007878) containing 883 amino acids residues showed a molecular mass of 100 kDa. It was observed that 157 amino acids were missing in purified soybean aamylase sequence having an accession no. Gm0237X0071. The sequence was:

MAESLTIIVRSKQYLATQKPVNLALGYRNPHGYGFSFGSRRSI-HERVSSHFKGIAVMTDDKSTMSSTEEDLENIGIFHIDPSLKPYKDH FKYRLKRYVDQKKLIEEYEGGLEEFSQGYLKFGFNREEGGIVYCE WAPAAQEAQIIGDFNGWDGSNHQ

1	1 2	3	4 5		6 7		11 12	13	14
15	71	3000	4000	5000	600	0 7000	800)	9474
	CDSf	CI	DSI		il 📒	CDSo	PolA		TSS
	TSS	1571			-8.24				
1	CDSf	1651	-	1671	1.34	1651	-	1671	21
2	CDSi	1985	-	2101	14.35	1985	-	2101	117
3	CDSi	2590	-	2800	13.79	2590	-	2799	210
4	CDSi	3799	-	3868	16.08	3801	-	3866	66
5	CDSi	3949	-	4218	30.17	3950	-	4216	267
6	CDSi	4932	-	5838	79.75	4933	-	5838	906
7	CDSi	6000	-	6116	13.71	6000	-	6116	117
8	CDSi	6244	-	6306	13.38	6244	-	6306	63
9	CDSi	6493	-	6600	11.27	6493	-	6600	108
10	CDSi	7158	-	7259	19.49	7158	-	7259	102
11	CDSi	7562	-	7629	4.47	7562	-	7627	66
12	CDSi	7773	-	7854	17.74	7774	-	7854	81
13	CDSi	8374	-	8490	4.98	8374	-	8490	117
14	CDS1	8983	-	9381	45.96	8983	-	9381	399
	PolA	9474			1.06				





Fig. 1B- Dot plot analysis for soybean α-amylase and predicted amylase (Gm0237X0071, TPA BK007878). Arrow (a) indicates the missing fragment; arrow (b) indicates difference in residues

Table 1- List of identified regulatory elements

Promoter	Sequence	Function	PMID
GATBOX	GATA	Light regulation	2535536
CAATBOX 1	CAAT	Tissue specific promoter activity	2710102
DOFCOREZM	AAAG	DNA binding proteins	10074718
-300ELEMENT	TGHAAARK	Identification of an enhancer element for the endosperm-specific expression	2152160
WBOXHVISO1	TGACT	Sugar-responsive elements	12953112
MYBST1	GGATA	Transcriptional activator	7957104
EBOXBNNAPA	CANNTG	Light-responsive and tissue-specific	15821875
AAR1AT	NGATT	Response regulators operate as transcriptional activators.	11135105
TATABOX5	TTATT	cis elements and trans-acting factors	7630938
TBOXATGAPB	ACTTTG	Promoter analysis of the nuclear gene	11442054
NODCON2GM	CTCTT	Nodule specific genes from soybean.	11442054
PYRIMIDINE BOXOSRAMY1A	CCTTTT	Functional dissection of a sugar- repressed α -amylase gene RT (Ramy1A) promoter in rice embryos	9506846

There was also difference in fragment CILLHSSSHKTAVRCVIV in case of Gm0237X0071 [Fig-1B]. Based on sequence similarity we find 21 similar sequences from different species, retrieved sequences were used for multiple sequence alignment for checking the diversity among predicted α -amylase and other diverse species. By alignment study it was observed that there were unique consensus regions at various places as shown in [Fig-2]. Residues equivalent to Met-1; Val-28; Ile-89, Tyr-90; Glu-91, Ala-92, His-93; Val-94; Gly-95; Arg-107, Ala-110; Glu-132; Ser-138; Try-141; Val-143; Thr-144;

Phe-152; Asp-161; His-164; Gly-167; Val-170; Gly-207; Trp-212; Leu-228; Asn-231; Trp-235; Asp-242; Ser-251; Met-252; Lys-253; Try-254; Ala-279; Asn-287; Ala-301; Glu-302; Asp-303; Gly-318; Gly-320: Phe-321; Glu-361; Trp-462; Gly-524; Gly-564 and Pro-593 are conserved in soybean amylase (Gm0237X0071), whereas residue equivalent to Met-158; Val-185; Ile-246; Tyr-247; Glu-248; Ala-249; His-250; Val-251; Gly-252; Arg-264; Ala-267; Glu-289; Ser-295; Tyr-298; Val-300; Thr-301; Arg-309; Ser-310; Gly-311; Thr-312; Asp-32; His-324; Gly-327; Val-330; Gly-367; Trp-372; Leu-388; Asn-391; Trp-395; Asp-402; Ser-411; Met-412; Lys-413; Try-414; Ala-439; Asn-447; Ala-461; Glu-462; Asp-463; Gly-478; Gly-480; Phe-481; Glu-521; Trp-608; Gly-669; Gly-709 and Pro-738 are wellconserved in predicted soybean amylase gene (TPA BK007878). Two major clusters were obtained from phylogenic tree based on aamylase sequences from different species. Both the α-amylase (i.e. Gm0237X0071 and TPA BK007878) are closer to cluster A species i.e. Phaseolus vulgaris, Vigna radiata, 3AMK_chain A of Oryza sativa, respectively [Fig-3]. 11 unique motifs have been identified after MEME study [Fig-4A], [Fig-4B]. Motif diversity showed their evolutionary significance, which are inferred by phylogenetic study.



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Soyabean alphaamylase 25	VEPRPRHGDG	VWVDRIPAWI	EVATVOPTEP	AAPYDGVYND	PPLSERYOPE
GmAMY1predicted	VEFREREDO	VWVDRIPAWI	EVATVOPTEP	AAPYDGVYWD	PPLSERYOFK
3K1DAChainA	TEPEVHGADG	VVTDRADP-P	APGTEVPPOT	ASEVISSOYT	NGDDDHMAGR
Phaseolusvulgaris(kidneybean) Maluadomestica(Apple)	VEPEPEHGDG	VWVDRIPAWI	EYATVDPTEP	AAPYDGVYND	PPLSEPYOPK
Pisumsativum	VEPEPEHSDG	VWVDRIPAWI	EYATVDFTRF	AAPYDGVYWD	PPLSERYOFK
AAP72268Hordeumvulgare XP_002284841Vitisvinifera	VEFEFEGDG	VWVDRIPAWI	RYATADASAP	GAPYDGVHWD AAPYDGVYWD	PPPSERYOFK
NP_001105370Zeamays	VEPRFLHG-G	VWVDRIPALI AWUDRIPALI	RYATVDASEP DVATPDASEP	GAPYDGVHWD	PPASERYTER
XP_002439059Sorghumbicolor	VEFRFLHG-G	VWVDRIPAWI	RYATADASEP	GAPYDGVHWD	PPASERYTER
CAA54308Manihotesculenta ABN05321Populustrichocarpa	VEFRFKHGDG	VWVDBIPANI	ECATVDPASE	GAPYDGVYND GAPYDGVYND	PPPPERYOFN PPTSERYEFN
BAE96954Ipomoeabatatas	VEPEPEHNSG	VWIDEIPAWI	EXATVOPTEP	AAPYDGVYND	PPASERYDFK
ACM07441Nelumbonucifera	VEPEPEHGNG	VWVDEIPAWI	EYAVVDFTEF	AAPYDGVYWD	PPPSERTOPE
CAA70038Solanumtuberosum	VEPEPEHENG	VWVDBIPAWI	EVATADATEP	AAPYDGVYND	PPPSER YHPK
CAB40980Triticumaestivum	VEPRPHEGDG	LWVDEVPAWI	EYATPDASEP	GAPYDGVHWD	PPTGERYVFK
ABA43634Metroxylonsagu NP_001105370Zeamays.1	VEPEPLAG-G	VWVDRIPALI	EVATVOASEP	GAPYDGVHWD	PPASEEYTE
3	01	10000000		* *	
Soyabean_alphaamylase GmAMYlpredicted	YPRPPRPRPRP YPRPPRPRPRP	EIYEAHVGMS EIYEAHVGMS	SPEPEIN	SYREFADEIL SYREFADEIL	PRIBANNYNT
1M7XBChainB 3K1DAChainA	ALENPUNEAM	SIYEVHLGSW STYEVHLGSW	RPGL	SYRELADOLV	PYAR MGFTH
Phaseolusvulgaris(kidneybean) Malusdomestica(Apple)	YPRPPRPKAP YPRPPRPKAP	RIYEAHVGMS RIYEAHVGMS	SSEPRIN	SYREFADEIL SYREFADDVL	PRICAMPONT
Pigumgativum	HPRPPRPKPKAP	RTYEAHVGMS RTYRAHVGMS	SSEPRIN	SYREFADDVL	PRIBENDYNT
XP_002284841Vitisvinifera	PCPSKPNAP	RIYEAHVOMS	SSEPRIN	SYREFADDIL	PRINT
NP_0011053/02eamays NP_0010586290ryzasativa	HPRPPKPDAP	RIYEAHVGMS	GEEPEVS	TEREFADIVL	PRIRANNYNT
XP_002439059Sorghumbicolor CAA54308Manihotesculenta	HPRPSKPAAP YPRPPKPCAP	EIYEAHVGMS EIYEAHVGMS	SSEPRIN	TYREFADAVL	PRIEAMONYNT
ABN05321Populustrichocarpa BAE96954Ipomoeabatatas	YPRPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPP	EIYEAHVGMS EIYEAHVGMS	SSEPEVN	STREFADAVL STREFADDVL	PRIMENORYNT
XP_002510672Ricinuscommunis ACM07441Nelumbonucifera	APPPEPEPEPEP	EIYEAHVGMS	SSEPEVN	SYREFADDVL	PHICANSTANT
CAA70038Solanumtuberosum	YPRPPRPRAP	TYEAHVGHS	SSEPEVN	SYREFADDVL	PRIMANDIANT
CAB40980Triticumaestivum	HPRPREPDAP	RIYEAHVGMS	GERPEVS	TYREFADAVL	PRINADOWNT
NP_001105370Zeamays.1	HPRPSKPAAP	EIYEAHVGHS	GERPAVS	TYREFADNVL	PRIRANDINT
3	51 🖈	* * *	*	*	* * * *
Soyabean_alphaamylase GmAMYlpredicted	VOLMAVMERS	YYASPGYHVT	N - PFAVSSES	OT PEDLEYLI	DEARSLOLOV
1M7XBChainB 3K1DAChainA	VELLEVAEHP	DGS IG OPT	G-LYAPTEP	GTEDDPEYP1 GTPDDPEALV	DALHOAGLOV
Phaseolusvulgaris(kidneybean) Malusdomestica(Apple)	VOLMAGNERS	YYASPGYHVT	N-PYAVSSES	OT PEDLEVLI	DEAHSLGLOW
Pisumsativum	VOLMAVMERS	YYASPWYHVT	RPPFAVSSES	GSPEDLEYLI	DEARSLGLAN
XP_002284841Vitisvinifera	VOLMAVMEHS	YYASPGYHVT	N-PFAVSSES	OTPEDLEVLI	DEARSLOLEV
NP_001105370Zeamays NP_001058629Oryzasativa	VQLMAVMERS VQLMAIMERS	YYASPGYRVT YYASPGYRVT	N-PFAVSSES	GTPEDLKYLV GTPEDLKYLV	DEARSLGLEV
XP_002439059Sorghumbicolor CAA54308Manihotesculenta	VOLMAVMERS VOLMAVMERS	YYASPGYHVT YYGSPGYHVT	H - PFAVSSES H - PFAVSSES	GTPEDLEYLV GTPEDLEYLI	DEARSLGLEV
ABN05321Populustrichocarpa BAE969541pomocabatatas	VOLMAVIEHS	YYASPGYHVT YYASPGYHVT	N-PFAVSSES	GNPEDLEYLI GTPEDLEYLI	DEARSLGLEV
XP_002510672Ricinuscommunis	VOLMAVMERS	YYGSPGYHVT	N-PFAVSSES	OT PEDLEYLI	DEARSLOLEV
CAA70038Solanumtuberosum	VOLMAIMERS	YYGEPGYHVT	N-PFAVSSEY	GNPEDLEYLI	DEAHSLGLOV
CAB40980Triticumaestivum	VOLMAIMERS	YYASPGYHVT	N-PFAVSSES	OTPEDLEYLV	DEARSLOLEV
ABA43634Metroxylonsagu NP_001105370Zeamays.1	VOLMAINERS VOLMAVNERS	YYASPGYHVT YYASPGYHVT	N-PFAVSSES	GTPEDLKILV	DEARSLOLEV
40	01			*	\$
Soyabean_alphaamylase GmAMY1predicted	LMDV IHSHAS	NN TOG NGP	DVGOTSODSY	PHTGDRGYHN	LWDSRLPNYA
1M7XBChainB	I LOWVPGHPP	TDDFAL	AEFDOTNLYE	HSDPREGYHO	DWNTLIYNYG
Phaseolusvulgaris(kidneybean)	LMDVIHSHAS	NNITDGLNGF	DVGOTSODSY	PHTGDEGYHE	LWDSELFNYA
Malusdomestica(Apple)	LMDVIHSHAS	NN ITDGLNGF	EVGOSSOESY	PHTGDRGYHK	LNDSRLPNYA
AAP72268Hordeumvulgare	LMDVVHSHAS	SNMTDGLNGY	DUGONTOESY	FHTGERGYHK	LWDGRLPNYA
XP_002284841Vitisvinifera NP 001105370Zeamays	LMDVVHSHAS	NIVTOGLNGP	DVGOSSODSY DVGOSTOESY	PHIGDEGYNE	LNDSKLPNYA LNDSKLPNYA
NP_0010586290ryzasativa	LMDVVHSHAS	NIVTDGLNGY	DVGONTHESY	PHTGDRGYHK	LUDGRLPNYA
CAA54308Manihotesculenta	LMDVVHSHAS	NNITDGLNGP	DVGOSTODEY	PHTGDEGYH	LWDSRLPNYA
ABN05321Populustrichocarpa BAE96954Ipomocabatatas	LMDVVHSHAS	NEVTOGLAGP	DIGOGAOESY DAGOGSODEY	PHTGDRGYHN	LADSRLPNYA VWDSRLPNYA
XP_002510672Ricinuscommunis	LMDVVHSHAS	NNVTDGLNGP	DVGOSSODSY	PHTADRGYHK	LNDGRLPNYA
ACM07441Nelumbonucifera CAA70038Solanumtuberosum	LVDVVHSHAS	NIV TOGLAGP	DIGOGSOESY	PHAGERGYHK	LUDSRLPNFA
AAT76445Vignaradiata	LMDVIHSHAS	NHITDGLNGP	DVGOTSODSY	PHAGERGYHK	LWDSRLPNYA
ABA43634Metroxylonsagu	LMDVVHSHAS	NIVTOGLNGP	DUGOSTODSY	PHTGERGYHK	LNDSRLPNYA
NP_001105370Zeamays	LMDVVHSHAS	NNVTDGLNGY	BUGOSTQEST	PHAGDRGYHK	LUDSELENYA
4 Soyabean_alphaamylase	NHEVLEPLLS	NLEWNLEEPE	POGPEPOGVT	SMLYHHHHGIN	IAPTODYNEY
GmAMY1predicted 1M7XBChainB	REVIEFLUS	NALYWIERP	PDGPBPDGVT IDALEVDAVA	SMLYHHHGIN SMIYEDYSE	- REGENIPHE
3K1DAChainA Phaneolunyulgarin (kidneybern)	BPEVENFLVA	NALYWLOBPH	TOGLEVDAVA	SMLYLDYSR-	- PEOGNTPNV
Malusdomestica (Apple)	NWEVLEFLLS	NLEWWLEEPE	PDGFRFDGVT	SMLYHHHGIN	MAPSODYHEY
AAP72268Hordeumvulgare	NWEVLEFLLS	NLEYWHEEFM	PDGPRPDGVT	SMLYNHHGIN	MSPSGDYREY
AF_002284841Vitisvinifera		COLUMN TWO IS NOT	A REAL PROPERTY OF	BALL I HHHGYN	HIP IGN INEY
NP_001105370Zeamays	NWEVLEFLLS	NLEYWLDEPM	PDGPRPDGVT	SMLYHHHGIN	ACK TON LOB L
NP_001105370Zeamays NP_0010586290ryzasativa XP_002439059Sorghumbicolor	NWEVLEFLLS NWEVLEFLLS NWEVLEFLLS	NLEYWLDEPM NLEYWLDEPM NLEYWLDEPM	PDGPRPDGVT PDGPRPDGVT	SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN	KGPTGN KEY
NP_001105370Zeamays NP_0010586290ryzasativa XP_002439059Sorghumbicolor CAA54308Manihotesculenta ABN05321Populustrichocarpa	NWEVLEFLLS NWEVLEFLLS NWEVLEFLLS NWEVLEFLLS NWEVLEFLLS	NLEYWLDEPM NLEYWDDEPM NLEYWLDEPM NLEYWLDEPM NLEWWLEEYE	PDGPRPDGVT PDGPRPDGVT PDGPRPDGVT PDGPRPDGVT PDGPRPDGVT	SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN	NGPTGNY KEY NGPTGNY KEY NGPTGNY QEY MAPTGDYNEY MAPTGDYNEY
NP_0011053702eamays NP_0010564290ryzasativa XP_00243905980rghumbicolor CAA54308Manihotesculenta ABN05321Populustrichocarpa BAE969541pomoeabatatas VD_0025104729bicturecommunic	NHEVLEFLLS NHEVLEFLLS NHEVLEFLLS NHEVLEFLLS NHEVLEFLLS NHEVLEFLLS NHEVLEFLLS	NLEYNLDEP NLEYNDEP NLEYNDEP NLEYNLDEP NLEWNLEEP NLEWNLEEP	PDGPRPD3/7 PDGPRPD3/7 PDGPRPD3/7 PDGPRPD3/7 PDGPRPD3/7 PDGPRPD3/7 PDGPRPD3/7	SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN SMLYHHHGIN	KGPTGNYEEY VGPTGNYEEY MAPTGDYNEY MAPTGDYNEY LTPTGDYNEY
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Soyabean_alphaamylase Gm&MTlpredicted MILDCAuA Phaseclusvujgaris(kidneybean) Maluadomestics(Apple) Picummativum AAP72264841Vitisvinifera NP_0010561290Tytasativa XP_002349580Tytasativa XP_002390580Tytasativa XP_002390580Tytasativa XP_002390580Tytasativa XP_002390580Tytasativa XP_00230057281Cinuscommunis XP_00250057281Cinuscommunis ACM07441Melumbonucifera CAM76445Vignaradiata AT76445Vignaradiata CAM40980Trificumasetivum ABA43634Metroxylonaagu		DIJITG PEOJ P DIJITG PEOJ P VILSDETAR VILSDETAR VILSDETAR VILSDETAR VILSDETAR DIJITG PEOJ P DIJITG			

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8	51				
Soyabean_alphaamylase GmAMYlpredicted JMCYDChainP	ESOEDDDNNS	LVGVEETSAA LVGVEETSAA	ADVARIPDES ADVARIPDES	ASTESEDIKL ASTESEDIKL	DGVRETLAA DGVRETLAA
3K1DaChaina					
Phaseolusvulgaris(kidnevbean)	ENCEGSNDSL	VGLEDTFAAA	DVARIPDESA	SIESEYSNNL	DOVKETSTS
Malusdomestica(Apple)	ESEEADAEET	LIBEEVGVGO	ENFEEOTGPI	NEDNAVGPRA	OESDOGSSS
Pisumsativum	ERCEESNNEN	LGSVEETFAA	ADTDVARIPD	VSMESEDSNL	DRIEDNSED
AAP72268Hordeumvulgare	EKAEKPKDGG	AAFWGELLED	TSMLKLLASK	DATDGEALSG	SEKVSTGDG
XP_002284841Vitisvinifera	ESLEESDDDH	NSTGANATLV	ADVVAEOESL	BEPASYKDHE	FEPRLIEGS
NP_001105370Zeamayo	EAGAGRELHA	KABTGETSPA	ESIDVKASRA	SSKEDKEATA	GGERGHEFA
NP_001058629Oryzasativa	EDREELRRGG	AVASGRIVTE	YIDVEATSGE	TISG	GWKGSEKDD
XP_002439059Sorghumbicolor			STATISTICS.	D DOUBLE	
CAA54308Maninotesculenta	EREGNHASSD	IGAANETLITD	TARLGDFEGT	NBISPAD	AVAROEDLA
ABN05321Fopulustrichocarpa	ESEESHDUDD	DACETETICT	DVIPEOBDVE	OPI DTENI OP	PHLVDGDGD
XP 002510672Ricinuscommunis	ESOESNINGIN	PGARNETSTA	DEVPELEDTE	UGMECTLAAD	TGARBODLE
ACM07441Nelumbonucifera	ESPODITYRE	ESPODSDAGD	KLDLPEENSG	DELVGGDEAA	VANVVASGE
CAA70038Solanumtuberogum	ERMSETEDYO	TDICSELLPT	ANTEESDEKL	KDSLSTNISN	IDERMSETE
AAT76445Vignaradiata					
CAB40980Triticumaestivum	EKAEKPEDEG	AASWGKAAPG	YIDVEATRVK	DAADGEATSG	SKKASTGGD
ABA43634Metroxylonsagu					
NP_001105370Zeamays	EAGAGRELHA	KAETGETSPA	ESIDVKASRA	SSKEDKEATA	GGKKGWKFA
	001				
Sovabean alphaamvlase	DVARTPDESA	PLESEDSNLD	VVERPLAAAN	AEVTEISGEL	VSVETEGIN
GmAMY1predicted	DVARIPDESA	PLESEDSNLD	VVNEPLAAAN	AEVTKI SGEL	VSVETEGIN
1M7XBChainB					
3K1DAChainA					
Phaseolusvulgaris(kidneybean)	QISVESEVIN	LDKVGIVA			
Malusdomestica(Apple)	THE R OWNER WHEN THE	NUMBER OF STREET			
Pisumsativum	ADAGITEARE	EVVGDN			
VD 002204041Vitigurinifera	UPDUUPPATD	SPURDING			
NP_001105370Zeamaya	OPSDODT				
NP 001058629Orvzagativa	GERGMEFVER	SSDEDCE			
XP_002439059Sorghumbicolor					
CAA54308Manihotesculenta	AQPSLIADDI	ATKANTETEE	IREETSDOK-		
ABN05321Populustrichocarpa	DGEWVMVVDL	ABQ			
BAE96954Ipomoeabatatas	- NSDVDGLQD	LPAMGNSDVD	DLQELGTVAN	SDVGQSKVQD	LEENPPELD
XP_002510672Ricinuscommunis	VVNQTLASDA	ATMLEVPKEP	ADDEDTEEPT	VSVKKGNSKA	ORT
Charlenge and a second and a se	VOTBICCELL	DTANI PRODP	BUGDVGLDD-	CHITDOPIDATO	UPPPPPPPT PT
AAT76445Vignaradiata	TATA TOOPTOD	FIRMIDDOVD	BURNOLO1ME	DATEVITO	APPENDED
CAB40980Triticumaestivum	SKEGINFVFG	SPDKDNK			
ABA43634Metroxylonsagu					
NP_001105370Zeamays.1	QPSDQDTK				
1	951		-		
GmaMylanedicted	DELEBITIVAA	OUDODINODE	DPDAAM		
1M7XDChainB	LEUGOA LYNN	PYROPAARD	COLUMN		
3K1DAChainA					
Phaseolusvulgaris(kidnevbean)					
Malusdomestica(Apple)					
Pisumsativum					
AAP72268Hordeumvulgare					
XP_002284841Vitisvinifera					
NP_001105370Zeamays					
NP_0010586290ryzasativa					
XP_002439059Sorghumbicolor					
AA54308Maninotesculenta					
BAE96954Inomocabatatas	PRDD				
XP 002510672Ricinuscomunia	No. of Concession, Name				
ACM07441Nelumbonucifera					
ACM07441Nelumbonucifera CAA70038Solanumtuberosum	SPSVSIISDV	VPAEWDDSDA	NVWGED		
ACM07441Nelumbonucifera CAA70038Solanumtuberosum AAT76445Vignaradiata	SPSVSIISDV	VPAEWDDSDA	NVNGED		
ACM07441Nelumbonucifera CAA70038Solanumtuberosum AAT76445Vignaradiata CAB40980Triticumaestivum	SPSVSIISDV	VPAEWDDSDA	MWGED		
ACM07441Nelumbonucifera CAA70038Solanumtubercaum AAT76445Vignaradiata CAB40980Triticumaestivum ABA43634Metroxylonsagu	SPSV811SDV	VPAEWODSDA	MAGED		

Fig. 2- Sequence alignment of soybean α-amylase with predicted amylase and with 21 diverse amylases from different species. Asterisk denotes the consensuses amino acid residue



Fig. 3- Phylogenic tree based on α-amylase sequences from different species.

Sequence	E-value	Block Diagram
Soyabean_alphaamylase	0	
GmAMY1predicted	0	
1M7XBChainB	0	
3K1DAChainA	0	
Phaseolusvulgaris(kidneybean)	0	
Malusdomestica(Apple)	0	
Pisumsativum	0	
AAP72268Hordeumvulgare	0	
XP_002284841Vitisvinifera	0	
NP_001105370Zeamays	0	
NP_001058629Oryzasativa	0	
XP_002439059Sorghumbicolor	0	
CAA54308Manihotesculenta	0	
ABN05321Populustrichocarpa	0	
BAE969541pomoeabatatas	0	
XP_002510672Ricinuscommunis	0	
ACM07441Nelumbonucifera	0	
CAA70038Solanumtuberosum	0	
AAT76445Vignaradiata	0	
CAB40980Triticumaestivum	0	
ABA43634Metroxylonsagu	0	
NP_001105370Zeamays	0	
		0 10d 20d 30d 40d 50d 60d 70d 80

Fig. 4a- Schematic distribution of respective conserved motifs; identified by means of MEME software

	Motif	Sequences
Motif 1	1	NNINTVQLMAVME SYYASFGY VTNFFAVSSRSGT EDLKILIDKA SLGLQVLMDVV S ASMIVTDGLNGFDVGQSTQESIF TGDRGY KLMDSRLF
Motif 2	2	KIKNDEENSNKEISNSLTNRRITEKCIAVAES DQAIVODKTIAFLINDKENVSONSCLQDAS TIERGIALQKHI FITNALGGEGVINFNONEFGHEN
Motif 3	3	EVA AAQEAQIIGDENGNNGSN QNEKNQEGVWSIKI DODON AI HISRVKEREK GDGVWVDRI ANIKVATVD TKEAA VDGV ND TI SERVQEK
Motif 4	4	NLLDEKFSFLASTKQIVSSTNEEDKVIVFERGDLVFVFNF ENTVEGVKVCCDL CKVRVALDSDAMEFGGUCRVGDVDUFTS EGI CV ETNFINR
Motif 5	5	YCCFCC RRSICORKVKSFKIVAVNTDDKSTNTTTEEDMENIGILSID KLE FKD FR RNKRVLDOKKLIEK EGGLEE FAKGVLKFOFNREEGGIV
Motif 6	6	QOFT LELL INE FOCSUGYQ TOYYA TRRFGTRODFRVFIDAA QAGIOVILDWY OFF KODWALGEFOGTNLVE SD KEGEQQDWGTVIFDFOR
Motif 7	7	NNSLVOVEETSAAADVAKI DESASTESEDIKLDOVKETLAAADVAKI DESA LESEDSNLDVVKE LAAANAEVTKISGELVSVETEGINLDKLEETI
Motif 8	8	W NGIQRLVRDINDIVRC KANNELDFD EGFEWIDVDDKERNVLIFMRROKEONELICVFNFT V RRDVRFGINQ GKWREILNTDAMIV GSGIGNGG
Motif 9	9	VTOVRFAVNA NAKRVSLIGOFNONDERE HELEK SEINELFN OF COOLIKFENIGADENLEDKAD FAFETONE OTASRICOL EKNEQDENEK
Motif 10	10	NQDGQWDICI DT GNNIE DEKLKORFR GDGNWDERN EWEK QTDD TEFA VDIEEND KLKER QFKI R Q KV RIEERDKENKOF RI
Motif 11	11	EN K VA RI ELGINGG E CO-TTREFFON A EIKANNGKORQLMQIME GYFAVFG VONFOTRLORTGE CE KTLIDKTRFL TRGENDL

Fig. 4b-Multilevel consensus sequences for the MEME defined motifs; among different species

Homology Modeling of α -Amylase from Soybean

To the best of our knowledge three dimensional structure for α -amylase from soybean is not known till date. The 3D structure of soybean α -amylase can be used to predict its molecular functions, active sites, and its interactions with suitable inhibitors. Three models were built by DSMODELER based on CHARMm force field using Conjugate Gradient (CONJ) [26] method that exhibits better convergence than the steepest descent method. Three distinct domains organization is fairly-well conserved in the generated model [Fig-5]. The predicted model of α -amylase from soybean consists of 14 α -helices and 17 β -sheets [Fig-6].







Fig. 6- Secondary structure prediction for generated model

Validation of the Predicted Structure

The stereo chemical quality of the predicted structure was assessed after energy minimization. Energy was-12828.653 after energy minimization. The total numbers of residues in selected model were observed as: 94.4% residues in most favored region, 4.7% in additional allowed region, 0.9% in generously allowed region and no residues in disallowed region in Ramachandran plot statistics [Fig-7A] [Table-2]; which indicates that generated model quality is good. QMEAN score / QMEANclust score of the whole model reflecting the predicted model reliability ranging from 0 to 1. In this predicted model QMEAN score 0.716 with global scores estimated absolute quality Z-score: -0.52 result show that model is reliable. Fractional accessible surface area volumes of all residues close to 1.0±0.1, Statistics of hydrogen bonds of predicted model show equal to expected mean hbond distance score 2.2 sd=0.4, mean hbond energy observed -1.7 sd=1.0 (74% residues) closest to expected -2.0 sd=0.8 (expected 75% residues). Dihedral Angles were observed closest to expected, Total Accessible Surface Area score 27365.1 Angs. with expected score 22651.6 Angs., Total volume (packing)

score observed 93847.6 Angs. with expected 92249.8 Angs., Stereo/Packing, 3D quality index results shows that less than 1% of error residues in predicted model. [Fig-7B], [Fig-7C]. Over all quality score was 82.866 for predicted models. PROSA score for Template and Target were -10.48 and -10.89, respectively [Fig-8A]; whereas root mean square deviation of 0.160Å between 651 atom pairs was observed UCSF Chimera 1.5.1. [Fig-8B]. Structural comparison between template and target structures show less deviation at atomic level. Validated and refined α -amylase model from soybean was successfully submitted to Protein Model Database (http://mi.caspur.it/PMDB/) with PMDBID PM0078685.



Fig. 7A- Ramachandran plot, based on most favoured, allowed generous and disallowed residues. Quantitative measures of the model protein

S. No.	Description	% Value
1	Residues in most favoured regions	94.40
2	Residues in additional allowed regions	4.70
3	Residues in generously allowed regions	0.90
4	Residues in disallowed regions	0.00

Comparison with non-redundant set of PDB structures



Fig. 7B- QMEAN score and Global scores estimated absolute quality Z-score



Fig. 7C- Volume area dihedral angle reporter results from VADAR.



Fig. 8A- Superimposition of modeled structures of α-amylases from soybean with the template 1M7X using the combinatorial extension (CE) method (Template structure in red colour and modeled structure in green colour).



Fig. 8B-Superimposition of modeled structures of α -amylases from Glycine max with the templet 1M7X using UCSF Chimera 1.5.1.

Active Site Identification

Active site was calculated by using active site finder tool of Qsitefinder. A total of 10 active sites have been predicted [Fig-9] and after sequence comparison it has been determined that showed that the best possible binding sites Gm0237X0071 were-His- 91,Val -92, Gly-93, Met-94, Ser-95, Ser-96, Phe-97, Glu-98, Asn-102, Ser-103, Tyr-104, Phe-107, Tyr-134, Ser-148, Arg-150, Ser-151, Gly-152, Lys-468, where as in case of TPA BK007878 structure; the best possible sites showing the possible binding regions were Phe114, Tyr-118, Glu-134, Trp-135, Ala-136, Phe-189, Arg-199, Thr-208, Met-288, Val-300, Thr-301, Asn-302, Phe-303, Phe-304, Ala-305, Val-334, Ile-335, His-336, His-338, Ser-340, Asp-345, Gly-346, Asn-348, GLY349, Phe-350, Asp-351, His-369, Lys-370, Phe-377, Val-384, Phe-387, Leu-388, Leu-389, Asn-391, Leu-392 and Tyr-442. With comparison with template protein structure (3AMK.pdb) Lys-101, Lys-234, Arg-237, Lys-322, Ser-325, His-369, Leu-371, Trp-372, Ser-411, His-417, Phe-423, Tyr-430, Val-464, Ser-465, Lys-498, Asn-499, Lys-500, Asp-502, Pro-561, Lys-628, Asn-631, Ala-632, Pro-679 and Arg-748 residues were found to be best active binding sites.



Fig. 9- Active site identification in predicted structure

Discussion

Predicted gene of α-amylase contains 13 introns and 14 exons. Cisacting element study was carried out by retrieving promoter region and number of regulatory elements were observed closely related with 3 main physiological phenomenon i.e. endosperm specificity, stress response and hormone response. Earlier studies revealed that despite considerable structural diversity, all a-amylase from different sources shared a similar topology and fold. a-Amylases from soybean have similar domain topology and conserved regions as predicted by sequence alignment and homology modeling. Homology modeling can be used to generate 3D model of unknown protein from its homologous protein. Predicted 3D structure of the protein gives important information which is based on the quality of the model generated. This can be useful for drug discovery processes i.e. for the selection of target protein for which drug has to develop, for designing mutants and to identify active and binding site residues [39-40]. a-Amylase from soybean showed higher similarity with branching enzyme from Oryza sativa (PDB ID: 3AMK, chain a). The template and the target have 76% identity with 89% positive identity with 0 E-value and score was 1169.07bits (3023). The template structure consists of three major domains, an NH₂terminal seven-stranded β -sandwich domain, a COOH-terminal domain, and a central (β/α) barrel domain containing the enzyme active site. The central domain is similar to that of all the other amylase family enzymes [34].

The structurally conserved regions were determined by multiple sequence alignment, based on the Needleman and Wunsch Algorithm [37]. Homology modeling provides high quality structure alignment for structure prediction. Phi and Psi torsion angles of predicted models were checked by PROCHECK. The RMSD of the equivalent C_{α} atoms of the modeled structure was 0.161 Å. In Ramachandran plot analysis, no residues were found in disallowed region. The Ramachandran plot for the model showed 100% of the residues in

the allowed regions and contains non-glycine and non-proline residues. Structural alignment was done using Swiss-PDB viewer (http://spdbv.vital-it.ch/) to calculate the structure deviation between template (3AMK) and selected model of α -amylase from soybean. Active site prediction showed that residues Lys-101, Lys-234, Arg-237, Lys-322, Ser-325, His-369, Leu-371, Trp-372, Ser-411, His-417, Phe-423, Tyr-430, Val-464, Ser-465, Lys-498, Asn-499, Lys-500, Asp-502, Pro-561, Lys-628, Asn-631, Ala-632, Pro-679 and Arg-748 were well-conserved and containing active binding site for TPABK007878. This model can be helpful for generating hypotheses and to explore and design new potent inhibitors of soybean α -amylase.

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