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α-Amylase from mung beans (*Vigna radiata*) – Correlation of biochemical properties and tertiary structure by homology modelling

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Abstract

α-Amylase from germinated mung beans (*Vigna radiata*) has been purified 600-fold to electrophoretic homogeneity and a final specific activity of 437 U/mg. SDS–PAGE of the final preparation revealed a single protein band of 46 kDa. The optimum pH was 5.6. The energy of activation was determined to be 7.03 kcal/mol in the temperature range 15–55 °C. K_m for starch was 1.6 mg/mL in 50 mM sodium acetate buffer, pH 5.5. Thermal inactivation studies at 70 °C showed first-order kinetics with rate constant (k) equal to 0.005 min^{-1}. Mung bean α-amylase showed high specificity for its primary substrate starch. Addition of EDTA (10 mM) caused irreversible loss of activity. Mung bean α-amylase is inhibited in a non-competitive manner by heavy metal ions, for example, mercury with a K_i of 110 μM. Homology modelling studies with mung bean α-amylase using barley α-amylases Amy 1 and Amy 2 as templates showed a very similar structure as expected from the high sequence identity. The model showed that α-amylase from mung beans has no sugar-binding site, instead it has a methionine. Furthermore, instead of two trptophans, it has Val^{277} and Lys^{278}, which are the conserved residues, important for proper folding and conformational stability.

Keywords: α-Amylase; *Vigna radiata*; Enzyme purification; Characterization; Homology modelling

1. Introduction

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are widely distributed in plants, mammalian tissues and micro-organisms. They catalyze the hydrolysis of α-1 → 4 glucosidic linkages of polysaccharides such as starch, glycogen, or their degradation products. They play a vital role in degradation of starch in germinating seeds. Seed germination is triggered by an increase in temperature and humidity. As a consequence the embryo synthesizes the phytohormone gibbrellic acid, which in turn induces *de novo* synthesis of α-amylase and an array of other hydrolases (Jones and Jacobsen, 1991). The energy for proper growth of plant is provided by the progressive release of sugars from the stored starch. The widespread occurrence of α-amylases in various organisms and the consumption of their substrates for food reserves and energy sources have led to intense interest in their biomedical properties and to major biotechnological applications in industry (Van der Maarel et al., 2002; Bertoldo et al., 1999). Also their value in specific industrial processes depends critically on their pH and temperature optima, which vary depending on the origin of the organisms (Linden et al., 2003).

Abbreviations: VrAMY, *Vigna radiata* α-amylase; AMY 1, barley α-amylase 1; AMY 2, barley α-amylase 2; STI, soybean trypsin inhibitor; β-CD, β-Cyclodextrin; PPA, porcine pancreatic amylase.

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In general α-amylases are classified based on their sequence into family-13 of glycoside hydrolases (Henrissat, 1991; CAZY server, www.cazy.org), with related enzymes classified in families 70 and 77. Many X-ray crystal structures have been reported in case of α-amylase such as *Aspergillus oryzae* TAKA α-amylase (Matsuura et al., 1984; Swift et al., 1991), porcine pancreas PPA (Qian et al., 1993), *Aspergillus niger* α-amylase (Boel et al., 1990; Brady et al., 1991), barley α-amylase isozyme 2 (AMY 2), (Kadziola et al., 1994), barley α-amylase isozyme 1 (Robert et al., 2003), and from *Alteromonas haloplanctis* (Aghajari et al., 1998). The structures consist of single polypeptide chains folded into three domains (A, B and C). The catalytic domain A consists of $(\beta/\alpha)_8$-barrel. Domains B and C are located roughly at opposite sides of this TIM-barrel. Domain B is probably responsible for the differences in substrate specificity and stability among the α-amylases (Svensson, 1994). Domain C constitutes the C-terminal part of the sequence and contains a Greek key motif and its functional role is yet to be established (Neilsen et al., 2004). With nearly all α-amylases, X-ray structures contain at least one conserved calcium ion, which is located at the interface between the A and B domains (Boel et al., 1990; Machius et al., 1995; Robert et al., 2002). One or more additional calcium ions have been found in several structures. It has been suggested that the role of the calcium ions is mainly structural (Gilles et al., 1996) and the conserved regions are involved in the architecture of the Ca^{2+} binding site and of the active site. The essential role of calcium ion has been explained by the observation that its ligands belong to domains A and B, the active site cleft is stabilized by inducing an ionic bridge between domain A and B (Buisson et al., 1987). A hyperthermophilic α-amylase with a novel (Ca, Zn) two metal centres has also been reported and it does not require the addition of metal ions for its full activity (Linden et al., 2003).

In the present study, we report purification of α-amylase from mung beans (*Vigna radiata*), its biochemical characterization and computer modelling studies using AMY 1 and AMY 2, as templates.

2. Results and discussion

2.1. Purification and biochemical properties

The specific activity of α-amylase from VrAmy increased to a maximum in 24 h following imbibition. Thereafter, α-amylase specific activity showed a marked decline (results not shown). As outlined in Table 1, α-amylase from 24 h germinated mung beans was purified 599-fold to a final specific activity of 437 U/mg (Fig. 1) and an overall yield of 9%. Koshiba and Minamikawa (1981) purified α-amylase from *V. mungo* with specific activity of 203 U/mg. Mar et al. (2003) recently purified two isoforms (VAAmy1 and VAAmy2) from Azuki beans (*V. angularis*) with specific activities of 75 and 63 U/mg, respectively. Ziegler (1988) reported α-amylase from mature pea leaves using affinity chromatography with a yield about 20%; another report from shoots and cotyledons of pea seedlings showed fold purification of 6700 and 850, respectively (Beers and Duke, 1990). Greenwood et al. (1965) reported α-amylase from broad beans (*Vicia faba*) with specific activity of 360 U/mg. In the β-cyclodextrin Sepharose column chromatography of kidney beans (*Phaseolus vulgaris*) cotyledons, a small peak of starch hydrolysing activity was first eluted by a lower concentration of β-cyclodextrin, while most of the enzyme activity was more tightly bound to the column (Mori et al., 1995). According to Mori et al. (1995) the two isoforms may be common in leguminous plant α-amylase.

2.2. MALDI-TOF mass spectrometry and electrophoresis

MALDI-TOF analysis of the trypsin digested single band of molecular mass 46 kDa (from SDS–PAGE) revealed a set of peptides, which matched with mass data of α-amylase from *Vigna angularis* from the available database with 98% sequence homology with *V. radiata* (Fig. 2) (Koizuka et al., 1990; Mar et al., 2003).

Coomassie Blue/silver staining showed a single band corresponding to 46 kDa on polyacrylamide gel electrophoresis. The identification of this band as α-amylase was further confirmed by immunoblotting with antibodies against barley Amy2 and activity staining (Fig. 3).

2.3. Kinetic studies

2.3.1. Effect of pH on activity

The effect of pH on purified α-amylase was examined in the pH range 3.6–9.0. The α-amylase from mung beans showed maximal activity at pH 5.6 in 0.1 M sodium acetate buffer (Fig. 4). Koshiba and Minamikawa (1981) reported pH optima at pH 6.0 from *V. mungo* α-amylase; Mar et al. (2003) reported optimum pH from Azuki beans at pH 5.3 and 5.2 for VAAmy1 and VAAmy2 isoforms, respectively;

Table 1
Purification of α-amylase from germinated mung beans (300 g)

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)[a]	Yield (%)
Crude extract	11,000	15,000	0.73	–	–
$(NH_4)_2SO_4$ (30–50%)	9900	1620	6.1	8.4	90
Affinity chromatography	2000	6.4	312	427	18
Superdex 75	1000	1.6	437	599	9

[a] Fold purification calculated with respect to the specific activity of the crude extract.

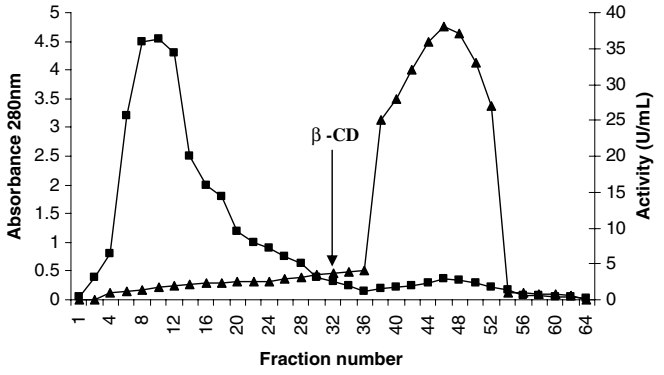

Fig. 1. Affinity chromatography of α-amylase on β-cyclodextrin Sepharose 6B column. The enzyme after 30–50% ammonium sulphate precipitation was loaded onto the column (40 mL) pre-equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 5 mM $CaCl_2$. After washing, elution was started with 10 mg/mL β-cyclodextrin in the same buffer as is indicated by an arrow (β-CD). Eluate was collected in 3-mL fractions and assayed for enzyme activity (▲) and $A_{280\,nm}$ (■).

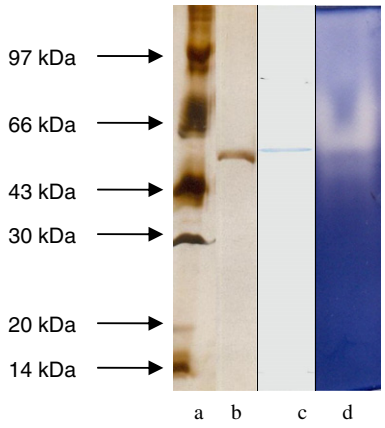

Fig. 3. Molecular weight markers in the lanes were (a) Phosphorylase b (97 kDa), BSA (68 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (30 kDa), STI (20 kDa), Lysozyme (14 kDa), Silver stained gel (lane b), immunoblotting (lane c) and activity staining (lane d) of α-amylase from *V. radiata*.

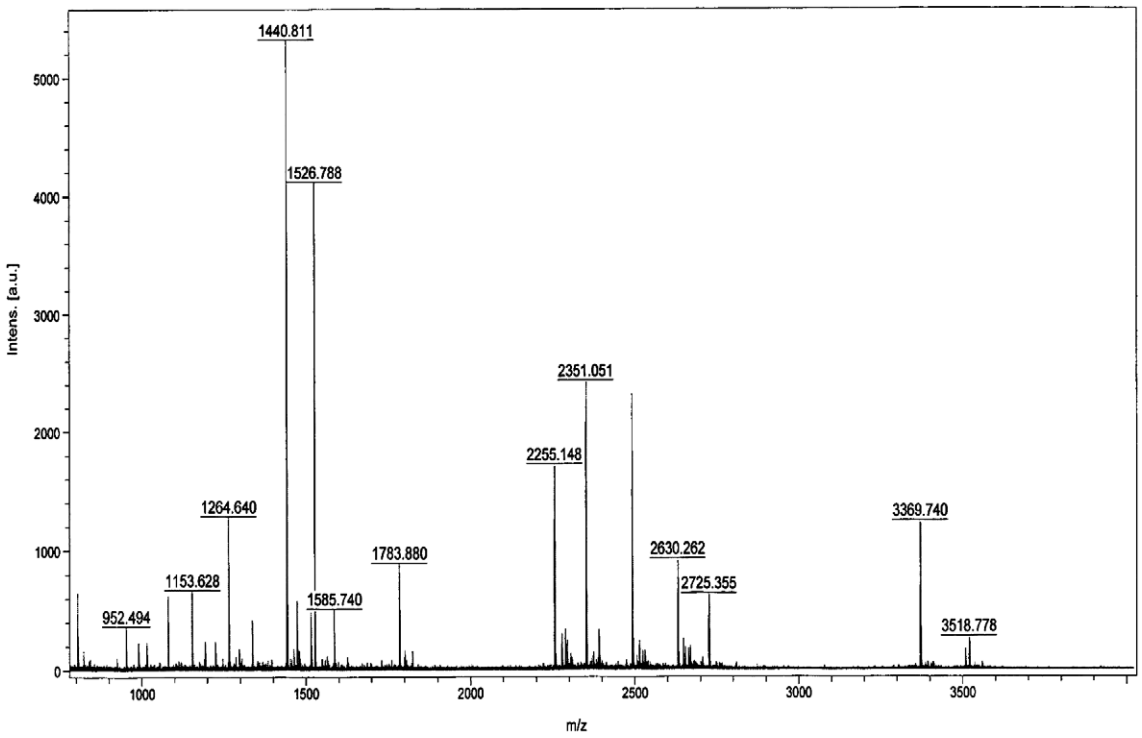

Fig. 2. MALDI-TOF spectra of tryptic peptides of α-amylase from *Vigna radiata*. Single band from SDS–PAGE was subjected to trypsin digestion and later analysed by MALDI-TOF.

Ziegler (1988) reported from mature pea leaves α-amylase pH maximum at 6.0; broad beans pH optimum was found to be 5.6 (Greenwood et al., 1965).

2.3.2. Effect of temperature on activity

The effect of temperature was studied by keeping the enzyme at different temperature ranging from 15 to 55 °C for 10 min in a water bath. The value of activation energy (E_a) was determined to be 7.03 kcal/mol for mung bean α-amylase. E_a for *V. mungo* α-amylase was determined to be 10.8 kcal/mol (Koshiba and Minamikawa, 1981). In *Vigna radiata* the highest activity was observed at 65 °C. The highest activity was observed at 70 °C for both the isoforms of Azuki beans α-amylase in 10 min reactions (Mar et al., 2003).

2.3.3. Effect of EDTA

EDTA had irreversible effect on activity of mung bean α-amylase. Enzyme was dialyzed against buffer containing 10 mM EDTA for 4 h, followed by the activity assay. α-Amylase activity was completely lost. This enzyme was

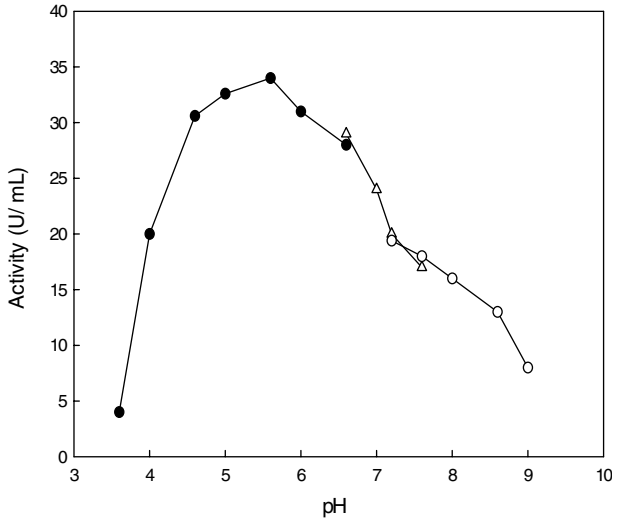

Fig. 4. Effect of pH on α-amylase activity. Buffer used were: 0.1 M sodium acetate buffer (●), 0.1 M potassium phosphate buffer (△), and 0.1 M Tris–HCl buffer (○).

dialyzed overnight against buffer containing 50 mM $CaCl_2$. However, α-amylase activity was not restored by addition of calcium.

VrAMy α-amylase is similar to other leguminous α-amylases w.r.t. optimum pH, K_m, activation energy and molecular weight; however, differences were observed w.r.t. EDTA inhibition and its reversibility. Pea α-amylase activity was completely inhibited by 5 mM EDTA (Ziegler, 1988), whereas *V. mungo* (Koshiba and Minamikawa, 1981), VrAMY and *V. angularis* α-amylase activity was completely inhibited by 10 mM EDTA (Mar et al., 2003). Interestingly, *V. mungo* α-amylase activity was partially restored by the addition of $CaCl_2$, but that of pea, *V. angularis* and VrAMY inhibitions were irreversible.

2.3.4. Effect of substrate concentration

When the substrate concentration was varied from 0.5 to 10 mg/mL, the K_m value for starch was found to be 1.6 mg/mL for α-amylase from mung beans. For *V. mungo* α-*amylase,* K_m was reported to be 1.1 mg/mL (Koshiba and Minamikawa, 1981), for mature pea leaves α-amylase it was observed to be 1 mg/mL (Ziegler, 1988).

2.3.5. Substrate specificity

Substrate specificity was measured for amylopectin, maltose, dextrin III, dextrin IV, β-cyclodextrin and pullulan (Table 2). VrAmy hydrolyzed amylopectin and other substrates at a rate much lower than that of starch whereas, mature pea leaves α-amylase degraded amylose most readily and branched starch preparations from higher plant material were also effectively hydrolyzed (Ziegler, 1988; Masuda et al., 1987).

2.3.6. Thermal inactivation

Thermal inactivation of VrAmy has been studied at 75 °C. The thermal inactivation followed first-order kinetics with $t_{1/2}$ equal to 150 min and rate constant (k) of thermal inactivation was 0.0046 min^{-1}.

Table 2
Substrate specificity α-amylase from mung beans, showing starch as the best substrate for VrAMY

Substrate	Activity (%)
Starch	100
Amylopectin	53.8
Maltose	18
DextrinIII	17
DextrinIV	14
β-Cyclodextrin	6.3
Pullulan	5.0

2.3.7. Inhibition studies

Some of the heavy metal ions tested on α-amylase activity were found to be inhibiting the enzyme. Mercury was observed to be potent inhibitor with a K_i of 110 μM (results not shown) and mode of inhibition was non competitive. Zinc inhibited only in millimolar range and is probably not physiologically significant in case of VrAMY. Heavy metal ions have been found in some cases to selectively inhibit plant amylases (Irshad et al., 1981). Hg^{2+}, Pb^{2+} and Cu^{2+} have been reported to inhibit microbial amylases (Lin et al., 1998). The effect of Zn^{2+} varied between amylases. For instance, it had a potent inhibitory effect on *Schwanniomyces alluvius* (Moranelli et al., 1987).

2.3.8. Modelling studies

Since the yield after final purification (approx. 10%, see Table 1) was too low to take up crystallographic studies, homology-modelling using the automated Swiss-Model server was undertaken to decipher the structure of α-amylase from *V. radiata*. The two known structures of plant amylases, barley AMY 1 and AMY 2 were chosen as templates. The sequence identity between target and templates is over 65%, and the number of amino acids in AMY 1, AMY 2 and VrAMY are 404, 402 and 402, respectively. As there are no long loop insertions in VrAMY as compared to the template structures, this is a simple homology-modelling problem that can be tackled by an automated server with no manual adjustments to the alignments. The Ramachandran Z-score for the model is −1.132. The score is within the expected range. The score expresses how well the backbone conformations of all residues are corresponding to the known allowed areas in the Ramachandran plot.

As to be expected, the model is very similar to the structures of the templates. The conserved active site residues in *V. radiata* α-amylase are Asp^{182}, Glu^{207} and Asp^{290} (Asp^{180}, Glu^{205}, Asp^{291} in AMY 1 and Asp^{179}, Glu^{204} and Asp^{289} in AMY2). Site directed mutagenesis showed that the mutants $Asp^{180} \rightarrow Asn^{180}$, $Glu^{205} \rightarrow Gln^{205}$, and $Asp^{291} \rightarrow Asn^{291}$ (on AMY 1) were catalytically inactive, strongly suggesting that these homologous residues are crucial for catalysis (Søgaard et al., 1993; Terashima and Katoh, 1996).

Table 3
Segments of VrAMY with homologous segments of AMY 1, AMY 2 and TAKA

VrAMY	Trp41-Pro44	Gly53-Tyr54	Asp90-His95	Arg180-Lys185	Gly206-Trp209	Phe245-Phe247	Asn288-Asp290
AMY 1	Trp39-Pro42	Gly51-Tyr52	Asp88-His93	Arg178-Lys183	Ala 204-Trp207	Phe246-Phe248	Asn289-Asp291
AMY 2	Trp38-Pro41	Gly50-Tyr51	Asp87-His92	Arg177-Lys182	Ala203-Trp206	Phe244-Phe246	Asn287-Asp289
TAKA	Trp61-Pro64	Gly81-Tyr82	Asp117-His122	Arg204-Lys209	Gly229-Leu232	Leu250-Tyr252	Asn295-Asp297

Sequence alignment between PPA and TAKA α-amylases have also revealed these highly conserved residues viz., TAKA-Asp^{206}, PPA-Asp^{197}; TAKA-Asp^{297}, PPA-Asp^{300}; TAKA-Glu^{230}, PPA-Glu^{233} (Svensson, 1988).

Table 3 shows the homologous segments among AMY 1, AMY 2 and TAKA amylase found by superimposing these segments (Svensson, 1988) and it was compared with VrAMY sequence, which also revealed the similar homology. According to MacGregor (1988), the conservation of these four amino acids (His^{122}, Asp^{206}, Glu^{230}, Asp^{297} of *A. oryzae*) and a few semi-conserved amino acids indicated strong similarities throughout the α-amylase family at the catalytic site and subsites F and G. This is quite reasonable since all catalyze the same hydrolytic reaction. Also the conservation of His^{95} of VrAMY explains the fact that histidine is required for activity of the enzymes from plants

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TARGET    4         PALLFQG FNWESSKKG- GWYNSLKNSI PDLANAGITH VWLPPPSQSV
1ht6A     1         HQVLFQG FNWESWKQSG GWYNMMMGKV DDIAAAGVTH VWLPPPSHSV
1amy      1          QVLFQG FNWESWKHNG GWYNFLMGKV DDIAAAGITH VWLPPASQSV
                      .**** ***** * .  **** .. .  *.* **.** *****.*.**

TARGET    50    SPEGYLPGRL YDLDASRYGS KNELKSLIAA FHEKGIKCLA DIVINHRTAE
1ht6A     48    SNEGYMPGRL YDIDASKYGN AAELKSLIGA LHGKGVQAIA DIVINHRCAD
1amy      47    AEQGYMPGRL YDLDASKYGN KAQLKSLIGA LHGKGVKAIA DIVINHRTAE
                . .**.**** **.***.**.    .*****.* .* **.  .* ******* *.

TARGET    100   RKDGRGIYCI FEGGTPDSRL DWGPSFICRD DTAYSDGTGN NDSGEGYDAA
1ht6A     98    YKDSRGIYCI FEGGTSDGRL DWGPHMICRD DTKYSDGTAN LDTGADFAAA
1amy      97    HKDGRGIYCI FEGGTPDARL DWGPHMICRD DRPYADGTGN PDTGADFGAA
                 **.****** *****.*.** ****  **** *  *.***.*  *.*  . **

TARGET    150   PDIDHLNPQV QRELSEWMNW LKTEIGFDGW RFDFVKGYAP SISKIYMEQT
1ht6A     148   PDIDHLNDRV QRELKEWLLW LKSDLGFDAW RLDFARGYSP EMAKVYIDGT
1amy      147   PDIDHLNLRV QKELVEWLNW LKADIGFDGW RFDFAKGYSA DVAKIYIDRS
                ******* .* *.** **. * **...***.* *.** .**..  ..*.*.. .

TARGET    200   RPDFAVGEKW DAISYGQDGK PNYNQDSHRG ALVNWVESAG ---GAITAFD
1ht6A     198   SPSLAVAEVW DNMATGGDGK PNYDQDAHRQ NLVNWVDKVG GAASAGMVFD
1amy      197   EPSFAVAEIW TSLAYGGDGK PNLNQDQHRQ ELVNWVDKVG GK-GPATTFD
                 * .**.* *    .. * *** ** .** **   *****.  *     ..   **

TARGET    247   FTTKGILQAA VQGELWRLID PNGKPPGMIG VKPENAVTFI DNHDTGSTQR
1ht6A     248   FTTKGILNAA VEGELWRLID PQGKAPGVMG WWPAKAVTFV DNHDTGSTQA
1amy      246   FTTKGILNVA VEGELWRLRG TDGKAPGMIG WWPAKAVTFV DNHDTGSTQH
                ******* * *.******     **.**..*   * .****. *********

TARGET    297   LWPFPSDKVM QGYAYILTHP GTPSIFYDHF FDWGLKEQIA KLSSIRLRNG
1ht6A     298   MWPFPSDKVM QGYAYILTHP GIPCIFYDHF FNWGFKDQIA ALVAIRKRNG
1amy      296   MWPFPSDRVM QGYAYILTHP GTPCIFYDHF FDWGLKEEID RLVSVRTRHG
                .******.** ********** * * ****** *.**.*..*   * ..* *.*

TARGET    347   INEKSSVKIM ASEGDLYVAK IDNKIMVKIG PKMDLGNLIP SNLHVATSGQ
1ht6A     348   ITATSALKIL MHEGDAYVAE IDGKVVVKIG SRYDVGAVIP AGFVTSAHGN
1amy      346   IHNESKLQII EADADLYLAE IDGKVIVKLG PRYDVGNLIP GGFKVAAHGN
                *   * . *.   ..* *.*  ** *..**.* .. *.* .** . .  .. *

TARGET    397   DYAVWE
1ht6A     398   DYAVWEK
1amy      396   DYAVWEKI-
                ******
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Fig. 5. Sequence alignment of the VrAMY (target; Seq. Acc. No. 1803517 A) with AMY 1 (1ht6A; Seq. Acc. No. AAA 32927) and AMY 2 (1amy; Seq. Acc. No. AAA 32925).

Table 4
α-Amylase from different plant source showing the presence and absence of the tyrptophan residues

Plant source	Accession number	Amino acid identity (%)	Residues
V. radiata	1803517A	100	VK
Vigna mungo	CAA37217	98	VK
Vigna angularis	BAC76729	98	VK
Phaseolus vulgaris	BAA33879	94	VK
Ipomoea nil	BAC02435	72	IS
Arabidopsis thaliana	NP_564977	68	IM
Malus domestica	AY939870	68	VK
Hordeum vulgare (Amy 1)	P00693	70	WW
Hordeum vulgare (Amy 2)	P04063	73	WW

(Greenwood and Milne, 1968a), animals and bacteria (Dua and Kochhar, 1985) as well as *A. oryzae* (Kita et al., 1982).

A sequence alignment between VrAMY and the two templates is shown in (Fig. 5) in which clearly Amy2 residues of Tyr^{51}, His^{92}, Tyr^{104} and His^{288} are conserved and also present in VrAMY. Mar et al. (2003) have also shown multiple alignments of α-amylases from Azuki bean (*V. angularis*), *V. mungo*, VrAMY, *Phaseolus vulgaris* and AMY 2. The residues Tyr^{51}, His^{92} and His^{288} were predicted to associate with substrate binding in subsite-1, and Tyr^{104} in subsite-6.

Crystallographic and mutagenesis studies with Amy 1 and Amy 2 have revealed that two consecutive tryptophan residues, Trp^{278} and Trp^{279} in AMY 1 (homologous to Trp^{276} and Trp^{277} in AMY 2) are involved in carbohydrate ligand-binding, in particular important for adsorption onto granular starch (Søgaard et al., 1993; Kadziola et al., 1998; Robert et al., 2005). Table 4 shows comparative studies of α-amylase from different sources, showing the presence and absence of tryptophans on the above-mentioned positions. This site is also known as starch granule binding surface site (Maeda et al., 1978; Gibson and Svensson, 1987; Søgaard et al., 1993; Wolfgang and Sauter, 1996). An interesting fact is that Trp^{276} is conserved in all reported cereal α-amylases (Søgaard et al., 1993) and its equivalent is not found in α-amylases from microbes, animals or other plants, including VrAMY. Instead, Val^{277} and Lys^{278} residues were present in VrAMY (Fig. 6.).

Another sugar binding site present in AMY 1, the so-called sugar tongs binding site in domain C, is not present in VrAMY, as compared to AMY 1; Tyr^{380}, involved at the sugar tongs site, is present only in AMY 1 and in AMY 2, Tyr^{378}; instead VrAMY has a Met^{379}. Thus, VrAMY is expected to have a reduced affinity for poly and oligosaccharides compared to AMY 1 and AMY 2.

3. Materials and methods

3.1. Chemicals and plant material

Epoxy-activated Sepharose-6B and Superdex 75 were purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. Amylopectin, maltose, dextrin III, dextrin IV, pullulan, β-cyclodextrin, BSA, TBS-T [25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% (v/v) Tween 20], Nitro blue tetrazolium (NBT), bromo-chloro-indoryl phosphate (BCIP) were purchased from Sigma Chemical Co., St. Loius, MO, USA. Soluble starch and all other chemicals were from E. Merck or BDH Chemicals. All solutions were prepared in Milli Q (Millipore, Bedford, MA, USA) water. Primary antibody (rabbit anti-Amy2) was a generous gift from Dr. B. Svensson, Denmark, secondary antibody (Goat anti Rabbit Immunoglobulins) was purchased from DAKO A/S, Denmark.

Mung beans (*V. radiata*) were procured from local market. Seeds were imbibed for 4 h in water at 30 °C and germinated for 30 °C in dark by spreading the imbibed seeds over moist filter paper on a moist sand bed. For enzyme purification, 24-h germinated seeds (containing radical and cotyledons) were used.

3.2. Enzyme and protein assays

The activity was measured by the method of Fuwa (1954). The reaction mixture, containing 0.5 mL of 1% starch, 0.3 mL of 0.1 mol/L sodium acetate buffer (pH 5.5) and 0.1 mL of Milli Q water, was incubated at 55 °C for 10 min for equilibration. The reaction was started by the addition of 0.1 mL of enzyme solution and allowed to proceed for 5 min. The reaction was stopped by the addition of 0.5 mL of 1 mol/L HCl and cooled rapidly to room temperature. A 0.2 mL of volume of this reaction mixture was diluted with distilled water to 15 mL including 0.1 mL of 1 mol/L HCl and 0.1 mL iodine reagent (0.2% iodine in 2.0% KI). A blank was prepared by adding the enzyme solution after the reaction has been stopped by the addition of HCl. The absorbance was measured at 610 nm.

One unit of α-amylase was defined as the amount of enzyme, which caused a decrease of absorbance by 0.05 in starch iodine colour assay under the assay condition. Starch and sodium acetate buffer was first incubated at 55 °C.

For checking substrate specificity using starch, amylopectin, maltose, dextrin III, dextrin IV, β-cyclodextrin and pullulan, DNS method (Bernfeld, 1955) was used, as they do not form a complex with iodine. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar/min.

Protein was determined by the method of Bradford (1976) with crystalline bovine serum albumin as the standard protein.

3.3. Enzyme purification

All steps were performed at 0–5 °C unless stated otherwise. Germinated mung beans (300 g) were homogenized using Waring blender in extraction buffer (50 mM sodium acetate, pH 5.5, containing 5 mM $CaCl_2$ and 1 mM DTT), filtered through double layers of cheesecloth, and

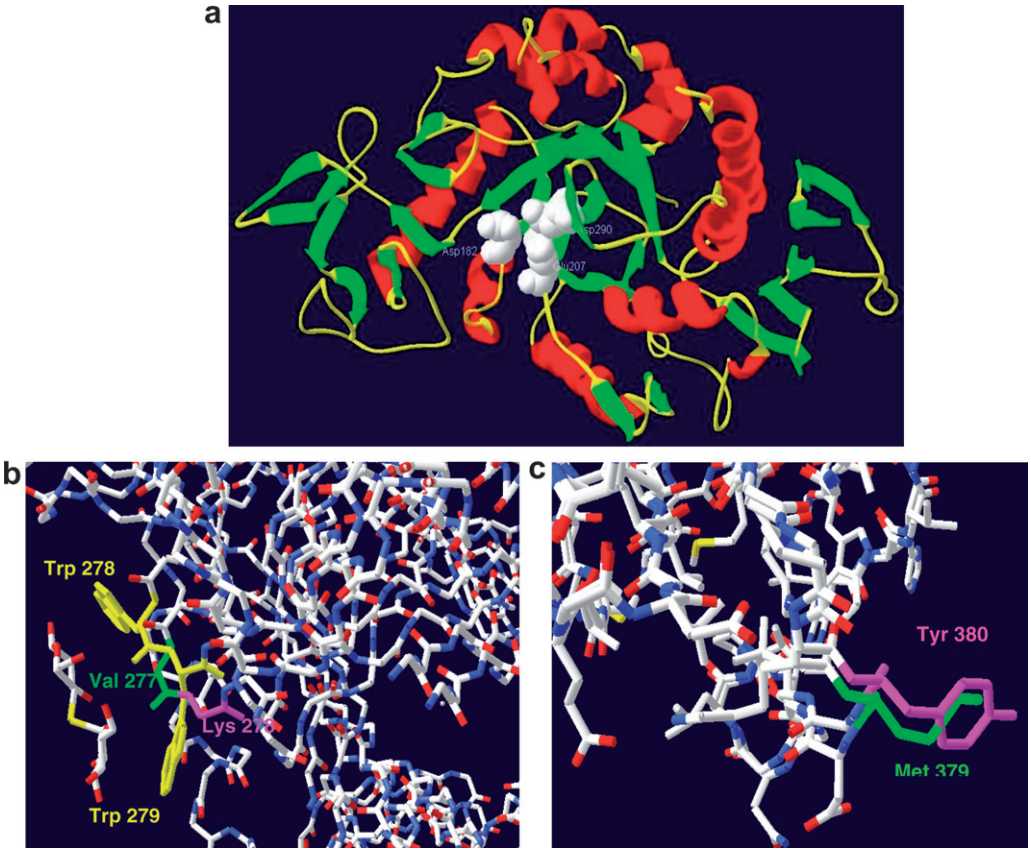

Fig. 6. Homology model of α-amylase from *V. radiata*. (a) Overall view showing the conserved active site residues, (b) comparison with the structure of AMY 1 at the starch granule binding site (AMY 1, Trp^{278} and Trp^{279}; VrAmy, Val^{277} and Lys^{278}) and (c) sugar tongs binding site (AMY 1,Tyr^{380}; VrAmy, Met^{379}).

stirred for 30 min and then centrifuged at 10,000 rpm (21,000*g*) for 25 min.

3.3.1. Ammonium sulphate fractionation

Protein precipitating in the range 30–50% (saturation) ammonium sulphate were collected by centrifugation, dissolved in extraction buffer and dialyzed against the same buffer with four buffer changes (500 mL each) to remove ammonium sulphate.

3.3.2. Epoxy-activated Sepharose 6B affinity chromatography

The dialyzed solution was applied to a column of Sepharose 6B equilibrated with the acetate buffer. Column was washed with extraction buffer and was then eluted with 200 mL of buffer containing a linear β-cyclodextrin gradient from 0 to 15 mg/mL (Silvanovich and Hill, 1976). The active fractions (5 mL each) were pooled and concentrated with Amicon 3 K (Millipore, Bedford, MA, USA).

3.3.3. Superdex 75 gel filtration chromatography

The concentrated enzyme was loaded on to an equilibrated gel filtration column, Superdex 75. The eluate was collected in 3 mL fractions and the active fractions were pooled and concentrated as above and stored at −20 °C.

3.4. Electrophoresis and immunoblotting

Native and SDS–PAGE, subunit M_r estimations and immunoblotting were performed using the Bio-Rad mini-gel apparatus according to the method by Laemmli (1970). SDS-gels (12%) were stained both by silver staining and Coomassie Brilliant Blue. To detect α-amylase activity following Native-PAGE (8%), gel was incubated at 30 °C for 15 min in 0.1 M sodium acetate buffer, pH 5.4, containing 1% soluble starch, then immersed in 0.01 NI_2-KI solution for development of α-amylase band. For Western blotting protein was separated on SDS–PAGE (12%) and transferred to nitrocellulose membrane, using an immunoblotter. Membrane was then incubated in TBS-T/1% BSA for 1 h, and then washed with TBS-T buffer three times. The membrane was then sequentially incubated with primary antibody (1:3000) in TBS-T for 1 h and then with secondary antibody (1:2000) for 1 h. Membrane was then stained with BCIP + NBT until blue colour appeared.

3.5. Kinetic studies

For all kinetic studies, enzyme obtained after gel-filtration on Superdex 75 was used. Effect of pH was checked using the following buffers like, 0.1 M sodium acetate buffer

(pH 3.6–5.6), 0.1 M phosphate buffer (pH 6.0–7.6), 0.1 M Tris–HCl buffer (pH 7.2–9.0). Enzyme activity was assayed as described above except that the starch solution was in the appropriate buffers. The activation energy (E_a) of the mung bean α-amylase was determined by an Arrhenius plot in the temperature range 15–55 °C. For thermal inactivation, the enzyme was maintained at the specified temperature ±1 °C in a water bath (Multitemp Bath, LKB, Sweden). Small aliquots were withdrawn at different time intervals; chilled immediately and tested for enzyme activity at 37 °C, using routine assay.

Substrate specificity was checked with starch and six other substrates. All parameters are the mean of triplicates determinations from three independent preparations of the purified enzyme and are reproducible to within ±10% SE of the mean value.

3.6. MALDI-TOF mass spectrometry

Identification and determination of molecular masses was performed by matrix assisted laser desorption mass spectrometry (Bruker-Daltonik, Bremen, Germany). In order to establish the identity of each fragment in-gel tryptic digest was employed. Single band of VrAMY were excised from the SDS–PAGE gel, washed and incubated with MALDI grade trypsin, digested overnight and analysed. The peptides obtained were matched with the sequence available in the database.

3.7. Modelling studies

Modelling studies were performed using Swiss Model server (http://swissmodel.expasy.org/) (Schwede et al., 2003). The sequence used was taken from α-amylase from mung beans (Koizuka et al., 1990), accession number 1803517A. The chosen templates were the only α-amylases from plant origin for which X-ray structure information is available, isozyme 1 and isozyme 2 of α-amylase from barley, sharing 70% and 73% sequence identity with the *V. radiata* α-amylase. The highest resolution structures available at the time were chosen as templates corresponding to pdb codes 1ht6 for AMY 1 (1.5 Å resolution) (Robert et al., 2003) and 1 amy for AMY 2 (2.8 Å resolution) (Kadziola et al., 1994). A higher resolution structure for AMY 2 is available in the PDB, but since it is in complex with a proteinaceous inhibitor, this structure was not chosen as template. SWISS-MODEL was used in first approach mode using default parameters. Structures were visualized using Swiss-PDBViewer (Guex and Peitsch, 1997).

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