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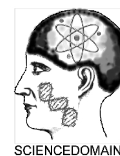

Mass Spectrometric Determination of the Primary Structure of β Amylase Isolated from Amylolytic Nigerian Maize Cultivar

O. A. Awoyinka[1*], O. Daini[2], K. Satisha[3] and Dipankar Chatterji[4]

[1]Department of Medical Biochemistry, College of Medicine, Ekiti State University Ado Ekiti, Nigeria.
[2]Kampala International University, Western Campus Ishaka Kampala, Uganda.
[3]School of Molecular and Biomedical Sciences, The University of Adelaide, Australia.
[4]Molecular Biophysics Unit, Indian Institute of Science, Bangalore, 56000-12, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author OA designed the protocol and wrote the first draft of the manuscript with authors OD and DC. Author KS managed the analyses of the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

This study was carried out after a five day germination period on TZEE*TZEE-W*DEMARSCUS*TZEE-W one of the most recommended high amylolytic Nigerian maize cultivar. Purification steps comprising of fractional precipitation by ammonium sulphate, gel filtration and anion exchange chromatography, was used respectively to purify β amylase (EC 3.2.1.2) from the malt. The sensitivity of the beta amylase indicated that it has serine at its active site. An apparent 60KDa monomeric protein was detected by one dimensional sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). Identity assigned to the purified protein by Matrix-assisted laser desorption ionization time –of- flight mass spectrometry (MALDI-ToF) reference to electronic protein data base is a 58542Da high putative beta amylase – Q9AV88-ORSA from *Oryza sativa*. Complete primary structure thereafter deduced with the aid of MS/MS MALDI- ToF showed a signature of a highly conserved ubiquitous not yet reported beta amylase. This study paved an insight to the gene encoding the β amylase in TZEE*TZEE-W*DEMARSCUS*TZEE-W and better understanding of the activity of the enzyme at molecular level.

**Corresponding author: Email: olayinka.awoyinka@eksu.edu.ng;*

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1. INTRODUCTION

TZEE* TZEE-W* DEMARSCUS*TZEE-W is a white dent maize cultivar developed at the International Institute for Tropical Agriculture (I.I.T.A.) Ibadan, Nigeria. It was found to possess high β amylase than other maize cultivars in Nigeria [1]. The profound importance and study of β amylase an enzyme generally believe to be most important of the diastatic power cannot be overemphasized [2].

β-amylase (1,4-α-glucan maltohydrase; E.C.3.2.1.2) catalyses the liberation of maltose from the non-reducing end of 1,4- α-D glucans. The enzyme is found in the embryo and the endosperm of most cereal seed. Unlike α-amylases, β amylase in most cereals is synthesized during seed development and deposited in the endosperm as a latent form [3]. However one of the main distinct features of β –amylase that separates it from α amylase is that it can only be found in bacteria and plants but not in animals hence it is the most important of the diastatic power [2].

Two distinct forms of β-amylase base on their expression patterns are generally known and use to describe its primary structure; one form being specific to the endosperm while the other has a tissue-ubiquitous pattern of expression [4]. To a certain extent β amylase primary structure has been well characterized from five major cereal crops: wheat, barley, rye, rice, and corn. β amylases from caryopses of barley [5], wheat and rye have been well described [6]. In this aforementioned *Triticeae* the β amylase is found to be endosperm-specific found to accumulate during caryopsis maturation and represents 1 to 2% of the total soluble protein fraction from mature seeds. The complete amino acid sequence of the barley endosperm-specific β-amylase and an incomplete sequence of that of rye were deduced from cDNA clones [7]. It revealed the presence of four (barley - β amylase) and three (rye β - amylase) short Gly-rich repeats close to the carboxyl terminus of the protein.

So far it has been suggested that rice and maize may not contain a typical endosperm specific β -amylase such as those found in wheat, rye, and barley but have just a single ubiquitously expressed form of β-amylase [8-10]. They are found not only to accumulate in maturing endosperm but also occur in other grain tissues such as pericarp and in vegetative organs such as leaves and roots. This ubiquitous type of β amylase is also known to lack the extensive Gly- rich repeats at the carboxyl terminus which is the common feature of endosperm-specific β -amylase [7,11].

Hitherto except through the cDNA clones reported by Wang et al., [9] the primary structure of the ubiquitous β-amylase from maize is rarely known to be deduced from the purified enzyme and studied extensively at the molecular level. In the present study the amino acid sequence of β-amylase from the amylolytic Nigerian maize is found and compared to the existing sequence in the database. This effort would ultimately give an insight to the gene encoding the β amylase of the maize. This present work however is aim to proffer information on the possible catalytic mechanism of the β amylase. Against this backdrop the method of probability-based protein identification by searching sequence databases using mass spectrometry as described in the classic submission of Pappin-Darryl [12] was employed to achieve the aim of this research work.

2. MATERIALS AND METHODS

2.1 Enzyme Purification

Whole endosperms were collected from 5- day old seedlings for the purification of beta amylase by the modified method of Wang et al. [13]. The endosperms were homogenized in an extraction buffer [50mM Tris-HCl (pH6.8), 3mM NaCl, 4mM, $CaCl_2$, 0.2mM phenymethyl sulfonyl fluoride (PMSF), 1ppm leupeptin and 10mM β Mercaptoethanol] and filtered. The filtrate was centrifuged at 10,000 Xg for 10min supernatant collected further centrifuged at 28,000 Xg for 1hr and then fractionated with ammonium sulfate. Proteins precipitated from 2.4-3.2M ammonium sulfate were dissolved in extraction buffer and dialyzed against 50mM Tris-HCl (pH6.8) containing 4mM $CaCl_2$ and 10mM β Mercaptoethanol. The dialyzed sample was subjected to ion exchange chromatography with a DEAE cellulose column. Active fractions were set aside, electrophertically analyzed and then concentrated by ultrafiltration with a PM- 10 Centricon Macrosolute Concentrator. The concentrated enzyme preparation was made to bind to DEAE-Cellulose ion exchange column. After 3hrs on rotor spin the column was washed with 2 bed volumes of dialysis buffer containing 80mM NaCl before elution with a linear gradient from 100 to 500mM NaCl Fraction with amylolytic activity were then pooled together and then concentrated again by ultrafiltration with a PM- 10 Centricon Macrosolute Concentrator before homogeneity test.

2.2 Homogeneity Test

The homogeneity of the purified protein was tested by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) as described by Neurath and Hill [14]. The enzyme was dissociated by boiling for 10min in 6.25mM Tris-HCL buffer, pH 6.8, containing 40% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol. 30µL of the dissociated enzyme was loaded and electrophoresis was run at a constant 25mA for 2hrs. Separated protein bands were visualized by staining with silver stain adapted from Blum et al., [15] before in-gel trypsin digestion.

2.3 In-Gel Tryptic Digestion

Proteins were digested by the method of Hellman et al. [16]. This involves stepwise dehydration, rehydration and alkylation. Excised gel pieces were extensively washed with 25mM ammonium bicarbonate in 50% acetronitrile, and 50 to 100ng porcine trypsin (Promega, Charbonnières-les-Bains, France) was added to the dried gel pieces. The resulting peptides were extracted with 0.5% trifluoroacetic acid in 70% acetonitrile before loading onto the MALDI target.

2.4 Mass Spectrometry Analysis

ToF Spec 2E MALDI time –of- flight (TOF) instrument (Micromass, Manchester, UK) was used for the mass spectrometry (MS) study. Aliquots of 1µL from digest supernatant were mixed with acetate, 75% v/v methanol/0.1% v/v formic acid. This mixture was spotted onto a 100 well stainless steel MALDI plate and allowed to air dry with the SMA (N-succinimidyl-morpholine acetate) matrix before being placed in the MALDI instrument fitted with a nitrogen laser (337nm). Reflectron mass spectra were recorded by accumulating data from 20 laser shots. For PSD analysis (Post Source Decay) a two point calibration was carried out using trypsin autolysis products at m/z 850.687 and m/z1165 incipiently being the precursor

ion that was fragmented to generate full length structure of the analyzed protein sample from MS/MS search ion data at data base search engine (Bioinformatics). The search engine- Mascot used is the development of the MOWSE computer program. MS data are submitted in the form of peak lists of centroided mass values optionally with associated intensity values. In the case of MS/MS data, peak detection is also required in the chromatographic dimension so that multiple spectra from a single peptide are summed together to generate infallible amino acid sequence.

3. RESULTS

Amylolytic activity was detected in the maize extract at every stage of the purification (Table 1). After final purification stage the electrophoretic assessment on the purified β amylase is shown in Fig. 1. The stretch-like single band may be as a result of the inability of β amylase to regain the random-coil configuration after denaturation with the sodium dodecyl salt. Similar observations were also made by Hayes and Mantle [17]. The sensitivity of the beta amylase indicated that it has serine at its active site (Table 2). These sensitivity tests are not exceptional to others β amylase found so far in other cereal plants [13,18,19].

Table 1. The Purification Profile of the β amylase

Steps	Protein (mg)	Activity (u)	Specific activity (u/mg)	Purification fold	Recovery
Crude extract	31.7	721.64	22.76	1.00	100
Differential centrifugation	8.20	410.05	50.01	2.19	57
NH4SO4 fractional precipitation	2.27	281.08	123.83	5.44	38.95
Gel filteration on Sephadex G-75	0.31	63.79	205.77	9.04	8.83
DEAE-Cellulose Chromatography	0.12	30.39	253.25	11.12	4.20

Table 2. Effect of some inhibitors on the purified beta amylase from TZEE*TZEE-W*DEMARSCUS*TZEE-W maize cultivar

Inhibitors 5mM, 15mM, 25mM	Beta amylase activity
Thiobabituric acid (TBA)	+++
Dithiothretol (DTT)	++
Iodoacetamide (IAM)	-
Mercaptoethanol (ME)	++
Phenylmethyl sulfonyl-fluoride (PMSF)	----
Ethylenediaminetetraacetic acid (EDTA)	***

*Key: + positive effect, - negative effect, * No effect*

A fine mass spectrum with less noise from MALDI indicating the peptide mass fingerprint results of the purified β amylase is shown in Fig. 2. In an exception of the trypsin autolysis a total number of 95 peaks were generated. From the spectra the peak with m/z 2581 was noted to be the highest. It has an area of 142, intensity of 63.74 and resolution of 6117 under the laser intensity of 4000a.u. shot on the matrix. However m/z 643.95 was the least peak

noted. It also has an area of 51, intensity 235.11 and resolution of 4567 under the same laser intensity shot.

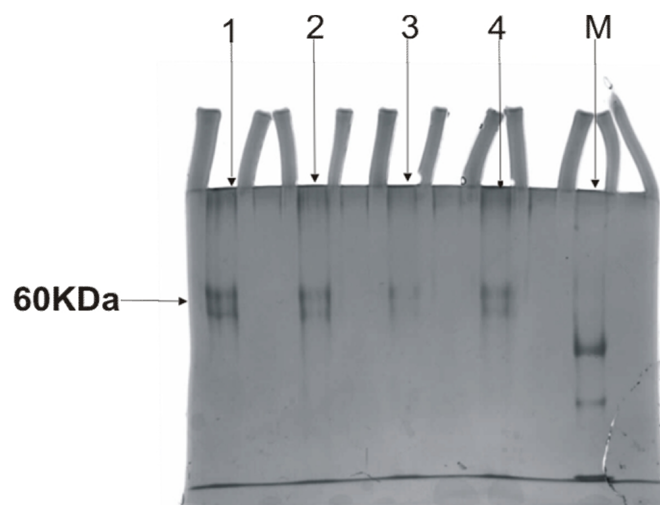

Fig. 1. Homogenity test of purified beta amylase from ion exchange chromatography fraction

Key: M Feremntas SM 0231 marker, 1234 different high βamylolytic fractions collected

In order to establish an identity for the result derived from the mass spectrum the raw data was fed into the computer system for mascot software analysis. The result generated is World Wide Web facilitated. The peptide mass fingerprint on the purified β amylase (Fig. 2) derived from MALDI indicated that the amino acid sequence of β amylase of maize is homologous to the β amylase from *Oryza sativa* (Fig. 3). Except at position number 23 the skeletal backbone in the primary structure of the purified β amylase shows the same sequence with Q9AV88_ORYSA from the first amino acid at the N-terminal end to amino acid at number 42. The C terminal end lacks the 39 glycine-rich tails noted for the β amylase endosperm-specific [18,20] hence indicating that the purified β amylase is ubiquitously expressed (that is non- endospermic).

The probability Based Mowse Score shown in Fig. 4 summarized the basis of the acceptability of the search engine database which was in agreement with the ideology of Pappin Darryl and his colleagues [12]. A very high score of 139% was recorded with beta amylase signature where protein scores greater than 66% is known to be significant. In order to generate the complete primary structure of the protein, isolation and subsequent ion fragmentation was carried out on Post Source Decay (PSD). The peaks with mass per charge (m/z) values of 850.687 and 1165 were effortlessly isolated from the spectrum. Hence they were regarded as the precursor ion and both fragmented. The raw data subsequently collated was fed into the matrix science software on the computer system to match its database. Fig. 5 showed the complete structure deduced for our protein sample after the raw data from MS/MS fragment ion search was fed into a computer system. It should be noted that when this was generated there was no signature assigned for this type of β amylase thus clearly indicating that it has not yet been reported prior to this study.

Thereafter the complete sequence was BLAST run in a dedicated protein structure software-Ex Pas Y. The list of matching sequences showed P55005 AMYB_MAIZE cultivar to be

topmost hit with a 662 score, sharing 80% positive identity even at the conserved regions (Fig. 6). The entire amino acid sequence of the β amylase in P55005 AMYB_MAIZE cultivar was found to be 488 showing to be 17 amino acid less to β amylase from TZEE*TZEE-W*DEMARSCUS*TZEE-W. However the alignment is shown to be scattered between the amino acid number 43 and amino acid number 496 (Fig. 6).

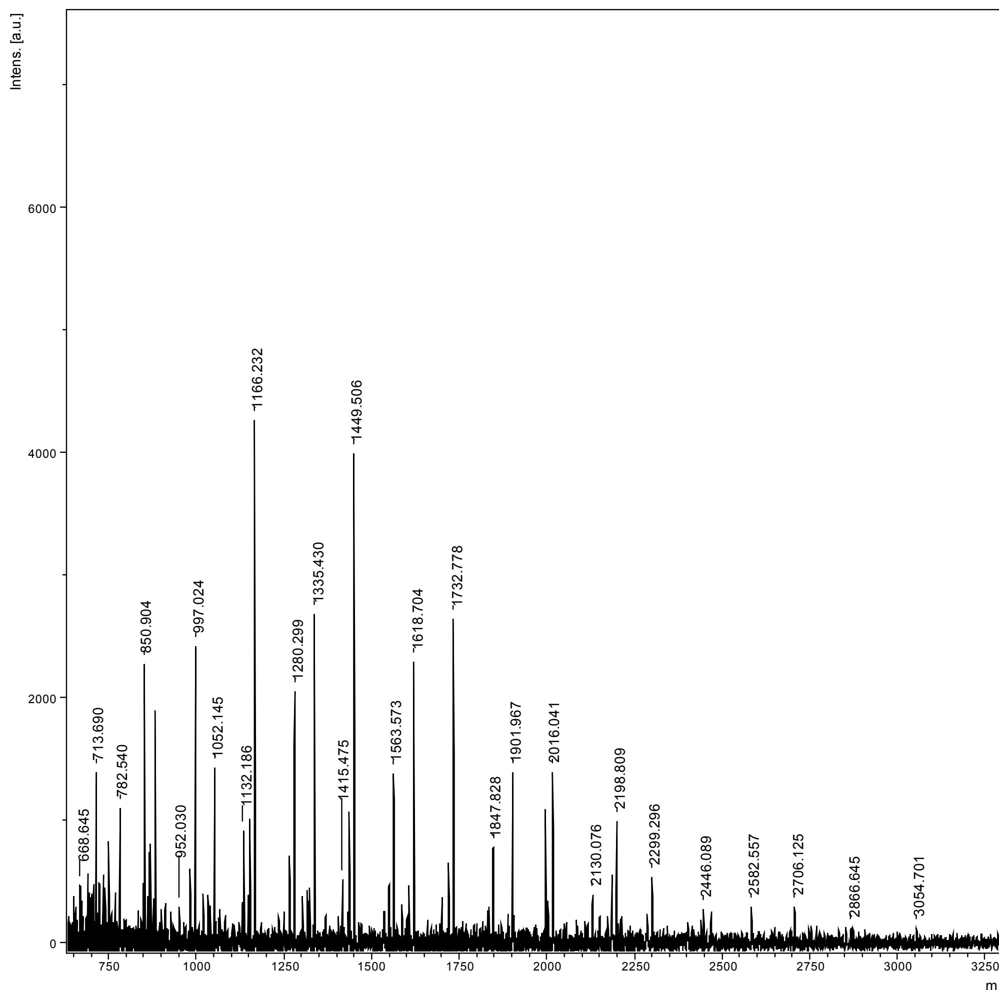

Fig. 2. MALDI-TOF-MS of Peptide Finger Print Mass (PFPM) of purified beta amylase from TZEE*TZEE-W*DEMARSCUS*TZEE-W

In order to get more information on the β amylase isolated from TZEE*TZEE-W*DEMARSCUS*TZEE-W. The method of Pujadas et al., [21] was adopted to align the complete sequence of the β amylase showing the conserved regions and notable amino acid sequence around this region (Fig. 7). It was also known that there was strict alignment at the eight conserved regions noted. This conserved region span through the length of the protein structure from amino acid 51 to the amino acid 428.

1 **MALNLAQSAA AAACFATAGD AR**R**AASVVAM PSSSSSATTS LR**MKRQAACE
51 PVACRAVARH VAAAAASSRR **NGVPVFVMMP LDTVSK**CGSA LNRRKAVAAS
101 LAALKSAGVE GIMVDVWWGI VESEGPGR**YN FDGYVELMEM AR**KTGLK**VQA**
151 **VMSFHQCGGN VGDSVNIPLP RWVVEEMEK**D NDLAYTDQWG RR**NFEYISLG**
201 **CDAMPVFK**GR **TPVECYTDFM R**AFRDHFASF LGDTIVEIQV GMGPAGELRY
251 PSYPESNGTW RFPGIGAFQC NDRYMRSSLK AAAEARGKPE WGHGGPTDAG
301 GYNNWPEDTV FFRGDCGGWS TEYGEFFLSW YSQMLLEHGE RVLSGATSVF
351 GDGAGAKISV KVAGIHWHYG TRSHAPELTA GYYNTRHRDG YLPIARMLAR
401 **HGAVLNFTCV EMR**DHEQPQE AQCMPEALVR QVAAAARAAG VGLAGENALP
451 RYDGTAHDQV VAAAADRAAE DR**MVAFTYLR MGPDLFHPDN WR**RFVAFVRR
501 **MSESGSPR**EA AESAAHGVAQ ATGSLVHEAA VALRS

Fig. 3. Mascot database result assigning Putative amylase (Amylase, putative).- Oryza sativa (japonica cultivar-group). Match to: Q9AV88_ORYSA Score: 139 Expect: 3.1e-09
Nominal mass (M_r): 58542; Calculated pI value: 6.74; Matched peptides shown in Bold Red

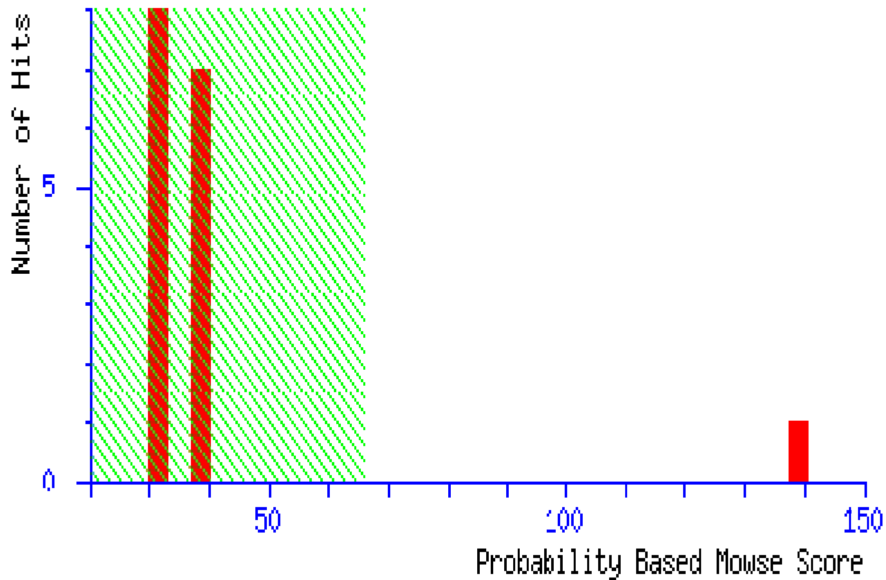

FIG. 4. Probability based mowse score
Protein scores greater than 66 are significant (p<0.05)

1 MALNLAQSAA AAACFATAGD ARDAASVVAM PSSSSSATTS LRKKLTEAGA
51 DGVMVDVWWG LVEGKEIGVY NGVPVFVMMP LDTVSKLKLQ AIMSCHQCGG
101 NVGDNVNIPI PQWVRDVGKS NPDIFYTNYN FDGYVELMEM ARDDQPLVQA
151 VMSFHQCGGN VGDSVNIPLP RWVVEEMEKI EVGLGPAGEM RYPSYPQSQG
201 WVFPGVGEFI TPVECYTDFM RAVAEEAGHP EWDLLDEDAG GYNDTPEKTQ
251 FFADNGTYQT DKGKFFLTWY SNKLIKHGDK ILOEANKVFL GCKVQLAIKV
301 SGIHWWYNVP NHAAELTAGY YNLDDRDGYR TIAHMLTRHR ASMNFTCAEM
351 RDSEQSSEAK SAPEELVQQV LSAGWREGLN LACENALNYR DATAYNTILR
401 HGAVLNFTCV EMREHKLHGF TYLRVSDELF QEQNYTTFKT RVRRMHANLD
451 YNPNVDPVAP LERSKAEIMV AFTYLRMGPD LFHPDNWRKD DTDLPVMSES
501 GSPRS

Fig. 5. Complete Amino Acid Sequence as Deduced from the MS/MS ion fragment data

Query: 43-102

KKLTEAGADGVMVDVWWGLVEGKEIGVYNGVPVFVMMPLDTVSKLKLQAIMSCHQCGGNV
KKLTEAGADGVM+DVWWGLVEGKE GVY+ + L + LKLQAIMSCHQCGGNV
Sbjct: 38 KKLTEAGADGVMIDVWWGLVEGKEPGVYDWSAYRQVFKLVQEAGLKLQAIMSCHQCGGNV 97

Query: 103 GDNVNIPIPQWVRDVGKSNPDIFYTNYNFDGYVELMEMARDDQPLVQ---AVMSFHQCGG 159
GD VNIPIPQWVRDVGKSNPDIFYTN + +E + + DDQPL A+ +
Sbjct: 98 GDVVNIPIPQWVRDVGKSNPDIFYTNRSGLTNIEYLTLGVDDQPLFHGRTAIQLYADYMK 157

Query: 160 NVGDSVNIPLPRWVVEEMEKIEVGLGPAGEMRYPSYPQSQGWVFPGVGEFITPVECYTDF 219
+ +++ L VV + IEVGLGPAGEMRYPSYPQSQGWVFPGVGEFI CY +
Sbjct: 158 SFRENMADFLDAGVVVD---IEVGLGPAGEMRYPSYPQSQGWVFPGVGEFI----CYDKY 210

Query: 220 M----RAVAEEAGHPEWDLLDEDAGGYNDTPEKTQFFADNGTYQTDKGKFFLTWYSNKLI 275
+ +A AEEAGHPEWDLLD DAG YNDTPEKTQFFADNGTYQTDKGKFFLTWYSNKLI
Sbjct: 211 LQADFKAAAEEAGHPEWDLLD-DAGTYNDTPEKTQFFADNGTYQTDKGKFFLTWYSNKLI 269

Query: 276 KHGDKILOEANKVFLGCKVQLAIKVSGIHWWYNVPNHAAELTAGYYNLDDRDGYRTIAHM 335
KHGDKIL EANKVFLGCKVQLAIKVSGIHWWYNVPNHAAELTAGYYNLDDRDGYRTIAHM
Sbjct: 270 KHGDKILDEANKVFLGCKVQLAIKVSGIHWWYNVPNHAAELTAGYYNLDDRDGYRTIAHM 329

Query: 336 LTRHRASMNFTCAEMRDSEQSSEAKSAPEELVQQVLSAGWREGLNLACENALNYRDATAY 395
LTRHRASMNFTCAEMRDSEQSSEAKSAPEELVQQVLSAGWREGLNLACENALN DATAY
Sbjct: 330 LTRHRASMNFTCAEMRDSEQSSEAKSAPEELVQQVLSAGWREGLNLACENALNRYDATAY 389

Query: 396 NTILRHGAVLNFTCVEMREHKLHGFTYLRVSDELFQEQNYTTFKTRVRRMHANLDYNPNV 455
NTILR+ EHKLHGFTYLRVSDELFQEQNYTTFKT VRRMHANLDYNPNV
Sbjct: 390 NTILRNARPQGINKNGPPEHKLHGFTYLRVSDELFQEQNYTTFKTFVRRMHANLDYNPNV 449

Query: 456 DPVAPLERSKAEIMVAFTYLRMGPDLFHPDNWRKDDTDLPV 496
DPVAPLERSKAEI + L + P + K DTDLPV
Sbjct: 450 DPVAPLERSKAEIPIE-EILEVAQPKLEPFPFDK-DTDLPV 488

Fig. 6. Result of most hit match on query sequence with other maize. Beta-amylase
(EC 3.2.1.2) (1,4-alpha-d-glucan maltohydrolase)

51 DGVMVDVWWG LVEGKEIGVY NGVPVFVMMP LDTVSKLKLQ AIMSCHQCGG

101 NVGDNVNIPI PQWVRDVGKS NPDIFYTNYN FDGYVELMEM ARDDQPLVQA

151 VMSFHQCGGN VGDSVNIPLP RWVVEEMEKI EVGLGPAGEM RYPSYPQSQG

201 WVFPGVGEFI TPVECYTDFM RAVAEEAGHP EWDLLDEDAG GYNDTPEKTQ

251 FFADNGTYQT DKGKFFLTWY SNKLIKHGDK ILOEANKVFL GCKVQLAIKV

301 SGIHWWYNVP NHAAELTAGY YNLDDRDGYR TIAHMLTRHR ASMNFTCAEM

351 RDSEQSSEAK SAPEELVQQV LSAGWREGLN LACENALNYR DATAYNTILR

401 HGAVLNFTCV EMREHKLHGF TYLRVSDELF QEQNYTTFKT RVRRMHANLD

Fig. 7. Sequence of the purified β amylase from high amylolytic nigerian maize cultivar showing the conserved region
Key: Green font is for the conventional conserved region of beta amylase signature
*Black font for the sequence found exclusively for this maize- TZEE*TZEE-W*DEMARSCUS*TZEE-W*

4. DISCUSSION

Except at position number 23 the skeletal backbone in the primary structure of the purified β amylase shows the same sequence with Q9AV88_ORYSA (Fig. 3) from the first amino acid at the N-terminal end to amino acid at number 42. Though other match sequences discovered appear to be scattered on the fabric of the protein structured at amino acid positions 71-76, 128-142, 148-179, 211-221, 401-413, 469-488, 497-504. The inferred conserved regions (Fig. 7) corroborate the work of Pujadas et al., [21] in which they submitted that maize has a closer relationship with rice than any other cereal on the phylogenetic dendogram.

The active site of the purified β amylase (catalytic and substrate binding site) could be found in this conserved regions. Four out of the eight sites detected as the conserved region could be regarded as the catalytic site. This is due to the presence of the serine residue in the amino acid sequence between positions 185- 195, 301-305, 351-353, 381-387 and 421 to 428 (Fig. 7). This indicates that the purified enzyme is serine protein and affirming the result in respect of sensitivity to PMSF (Table 2) well, the other part of the conserved region could be regarded as the substrate binding site. The presence of amino acid Cysteine found only in other part of the other conserved regions (amino acid positions-95-105, 344- 353 and 381-387) not noted as the catalytic site is another clear confirmation of the result from the sensitivity test with an alkylating agent- Iodoacetamide. Even though sulfyhydryl group (SH) is found close to the active site it may not play a major role in the catalytic action because of positive effect on the activity of the purified β amylase in the presence of a stronger alkylating agent such as Thiobabituric acid and disulfide reducing agents -Dithiothretol (Table 2). Though there has been presumptive report on the catalytic role of this SH group in β amylase [13]. However the exact site of this SH group is a revelation from this primary structure study which to the best of our knowledge has not been affirmatively reported in any study. Also critical look into the primary structure (Fig. 7) showed that there is only one Cysteine amino acid found close, but outside the conserved region at position 292. It is sufficient to say that since cysteine will be needed to form two sulfyhydryl bridges [22]. The cysteine at position 292 will “complement” the other three Cysteine in the conserved region to form two sulfyhydryl bridges that will in turn orient the protein structure to form the active site cleft of this purified β amylase. Though all these observations are non -experimental qualifiers they contribute a lot to the understanding of how this maize cultivar- TZEE* TZEE-W* DEMARSCUS*TZEE-W orientate to form the catalytic domain thereby accounting for the marked difference noted in the amylolytic activity in our previous work [23].

Further to the understanding of the possible mechanism of the catalytic action of the purified β amylase from maize cultivar- TZEE* TZEE-W* DEMARSCUS*TZEE-W the submission of Totsuka and Fukazawa [24] can be applied. They made likely proposition on the action mechanism of β amylase on their work with β amylase isolated from Soybean. Their X-ray crystallographic findings suggest that the β amylase catalytic action in Soybean involves the interaction of three essential amino acid residues (ASP101, GLU 186 and GLU 380) in concert with Leu 383 and assumed an indispensable role for Asp101. It is interesting to note that all these amino acids are found in these same positions in TZEE* TZEE-W* DEMARSCUS*TZEE-W except at sequence 383 where it is substituted for Cytosine and at sequence number186 it is substituted for amino acid Glycine. Hence the presence of Asp 101, Asp 380, Cys 383 and Gly 186 to the functional analysis in maize cultivar- TZEE* TZEE-W* DEMARSCUS*TZEE-W cannot be overlooked.

Moreover a point of note in this amino acid sequence is at C terminal end which indicates that the purified β amylase is ubiquitously expressed. It is has been established that the endosperm-specific β amylase enzyme is characterized by a glycine-rich region at the carboxyl terminus end that is subject to extensive post-translation modification [20]. This further support our submission that the β amylase in this work is monomeric from the result of SDS –PAGE (Fig. 1) giving no clue for the possibility of post translational modification.

5. CONCLUSION

In this work we have been able characterized at the molecular level β amylase purified from the most recommended high amylolytic Nigerian maize cultivar and comparing it with that of barley thus setting the pace for a genetic manipulation study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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