Research Article

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**Implications of Stisa2 catalytic residue restoration through site directed mutagenesis**

Bölgeye yönlendirilmiş mutajenez ile Stisa2 katalitik amino asit restorasyonunun olası sonuçları

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Abstract

**Objective:** Restoration of catalytic activity of Isa2 from *Solanum tuberosum* (Stisa2) through restoration of conserved catalytic residues by site directed mutagenesis.

**Methods:** The six conserved amino acid residues absent in the Stisa2 gene were restored by mutation using the overlap extension PCR and the asymmetrical overlap extension PCR methods. Next, mutant Stisa2 with restored catalytic residues was expressed in *E. coli* Rosetta 2 under optimized conditions. Evaluation of debranching activity on starch, amylopectin and β-limit dextrin was carried out by measuring the amount of glucose equivalents released using the bicinchoninic acid assay.

**Results:** Both qualitative and quantitative analysis showed that the restoration of the conserved residues in the catalytic site did not restore starch debranching activity. Molecular modeling showed greater than expected distances between the catalytic triad in mutant Stisa2. These additional distances are likely to prevent hydrogen bonding which stabilizes the reaction intermediate, and are critical for catalytic activity.

**Conclusions:** These results suggest that during evolution, mutations in other highly conserved regions have caused significant changes to the structure and function of the catalytic network. Catalytically inactive Isa2, which is conserved in starch-producing plants, has evolved important non-catalytic roles such as in substrate binding and in regulating isoamylase activity.

**Keywords:** Isoamylase; Site directed mutagenesis; Catalytic site; Starch debranching; Isa2.

**Özet**

**Amaç:** Bölgeye yönlendirilmiş mutajenez ile *Solanum tuberosum* Isa2 (Stisa2) katalitik aktivitesinin, korunmuş katalitik amino asit restorasyonu ile geri kazanılması.

**Metod:** Stisa2 geninde bulunmayan 6 korunmuş amino asit, örtüşen uzatmalı PZR ve asimetrik örtüşen uzatmalı PZR yöntemleri ile gene eklendi. Mutasyana uğrattılarak katalitik bölgesi restore edilen Stisa2 geni optimize edilmiş şartlarda *E. coli* Rosetta 2 de ifade edildi. Bicinchoninik asit assayi kullanılarak; Stisa2 gen ürününün nişasta, amylopektin ve β-limit dextrin üzerindeki budayıcı aktivitesi glikoz türevlerinin salınım oranını ölçüllerdekilerin değerlendirildi.

**Bulgular:** Yapılan nicel ve/ve ya nitel çalışmalar katalitik bölgesinde korunmuş amino asitlerin restorasyonunun nişasta budayıcı aktivitesini restore etmeye yetmediğini gösterdi. Mutant Stisa2 ile yapılan moleküler modellle çalışmaları katalitik triad arasındaki uzaklıkların beklelenden çok daha uzun olduğunu gösterdi. Bu uzaklıklar muhtemelen ara ürün sabitlenmesinde ve dolayısı ile katalitik aktivitede önemli olan hidrojen bağının gerçekleştmesini önlemektedir.
Introduction

Isoamylases (EC 3.2.1.68) are essential enzymes in both the biosynthesis and degradation of starch. Isoamylases debranch glucan polymers by hydrolyzing the α-1,6 linkages which form the branch points in glucans. Three isoforms of isoamylase have been discovered and they are highly conserved in plants [1, 2]. These isoforms, Isa1, Isa2, and Isa3 are each believed to have different roles in starch metabolism, with Isa1 and Isa2 involved in starch biosynthesis, whereas Isa3 is involved in starch degradation plants [1, 2]. There are two Isa multimeric complexes involved in starch biosynthesis: an Isa1 homocomplex and an Isa1/Isa2 heterocomplex [3].

Isa2, is a catalytically inactive isoform of isoamylase [1, 4]. However, despite being catalytically inactive, Isa2 still plays an important role in starch biosynthesis. In Arabidopsis, Isa2 null mutants, lacking the Isa heterocomplex, produced 80% less starch and accumulated water soluble polysaccharides instead [5]. Although Isa2 retains overall structural similarity with other members of the α-amylase superfamily, Isa2 from Solanum tuberosum (Stisa2) lacks six of eight conserved amino acid residues in its catalytic site [1]. Furthermore, the three carboxylic acid groups Asp-375, Glu-435 and Asp-510 which are essential for catalytic activity are substituted with Val, Asp, and Ser, respectively [1]. Despite lacking catalytic activity due to these changes, Stisa2 showed synergistic effects when combined with other isoamylase isoforms [1].

Site directed mutagenesis is a technique used to substitute, add, or delete specific bases in DNA [6]. Using this technique, DNA codons can be changed to encode for the desired amino acids. Restoration of catalytic residues through site directed mutagenesis has been reported to reactivate inactive enzymes such as azurocidin and carbonic anhydrase [7, 8].

PCR based methods are widely used in site directed mutagenesis as they are rapid, reliable and effective [9]. These PCR based methods involve the introduction of oligonucleotide primers containing one or more mismatched bases. One of these methods is the overlap extension PCR (OE-PCR) method [10]. The OE-PCR method involves two rounds of PCR. The primary PCR rounds generate two overlapping PCR fragments with the desired mutation in the overlapping region and the secondary PCR round joins the fragments via the overlapping regions to produce full length mutagenic DNA [11, 12].

A further modification of the OE-PCR method is the asymmetrical overlap extension PCR (AOE-PCR) method which uses asymmetrical primer concentrations [12]. In the AOE-PCR method, the primary rounds of PCR produce double stranded DNA, and also single stranded DNA when mutagenic primers are exhausted in later PCR cycles. The secondary round of PCR is replaced by a short incubation step where the single stranded primary DNA are joined into full length mutagenic DNA.

This work aims to restore catalytic activity to Stisa2 by restoring its catalytic residues to that of other catalytically active α-amylases. Restoring Stisa2 catalytic activity can facilitate the understanding of the role it plays especially related to substrate specificity and synergistic effects when combined with other isoamylase isoforms. To achieve this, the six conserved catalytic residues were restored through site directed mutagenesis of the corresponding codons.

Materials and methods

Genes and source materials

The Stisa2 gene (Accession AY132997) used in this work had been previously isolated and cloned into a pSTAG vector, where the Stisa2 gene has been fused to an S-tag coding sequence, facilitating the detection and purification of the S-tagged protein [1, 13].

Site directed mutagenesis

The codons encoding the six amino acids to be mutated were identified. Gene specific primers and mutagenic primers incorporating the desired mutations were then designed with the aid of PrimerX program (http://www.bioinformatics.org/primerx/index.htm). A list of primers used is shown in Table 1. Site directed mutagenesis was performed using the OE-PCR and AOE-PCR methods to mutate the codons (Table 2) [10–12]. After the mutagenesis,
the mutant PCR products were digested with Smal and Spe1 restriction enzymes and cloned back into pSTAG vector.

**OE-PCR method**

In the primary PCR reactions, the PCR mixtures (5 pmol gene specific primer, 5 pmol mutagenic primer, 5 nmol DNTP mix, 2 U Pfu DNA polymerase, 62.5 nmol MgCl2, and 2.5 pg plasmid DNA) were prepared in 25.0 μL reactions and were subjected to initial denaturation for 5 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature and 5 min at 72°C, followed by a final elongation of 72°C for 5 min. The products of the primary PCR reaction were gel purified. In the secondary PCR reaction, the PCR mixture (4 pmol gene specific primer, 4 pmol mutagenic primer, 8 nmol DNTP mix, 2 U Pfu DNA polymerase, 100.0 nmol MgCl2, and 25 pg DNA fragment A and 25 pg DNA fragment B) was prepared in a 40.0 μL reaction and subjected to initial denaturation for 5 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature and 5 min at 72°C, followed by a final elongation of 72°C for 5 min.

**AOE-PCR method**

In the primary PCR reactions, the PCR mixtures (5 pmol gene specific primer, 1.25 pmol mutagenic primer, 5 nmol DNTP mix, 2 U Pfu DNA polymerase, 62.5 nmol MgCl2, and 2.5 pg plasmid DNA) were prepared in 25.0 μL reactions and were subjected to initial denaturation for 5 min at 95°C, followed by 55 cycles of 30 s at 94°C, 30 s...
Hasnain Hussain and Nikson Fatt Ming Chong: Implications of Stisa2 catalytic residue restoration at annealing temperature and 5 min at 72°C, followed by a final elongation of 72°C for 5 min. If single bands were obtained, gel purification was unnecessary. The products of the primary PCR reaction were then mixed together and incubated using a PCR machine at 55°C for 1 min, 72°C for 5 min, 55°C for 1 min and 72°C for 5 min.

**Heterologous expression and partial purification of mutant Stisa2**

The pSTAG vector carrying the mutant Stisa2 gene was transformed into *E. coli* Rosetta 2 using the heat shock method. Protein expression was carried out under optimized conditions (enriched LB media, incubation temperature of 20°C, shaking at 250 rpm, and an incubation time of 12–16 h). The cells were harvested by centrifugation at 4°C and the cell pellet was frozen. To release soluble proteins, the frozen cell pellet was resuspended in lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10% ethylene glycol, 0.5 mM PMSF) and lysed by glass bead vortexing for 2 min (1 g glass beads/mL cell suspension). The soluble S-tagged mutant Stisa2 proteins were partially purified using S-protein agarose beads (Novagen) according to the manufacturer’s recommendation.

**In silico characterization**

Three dimensional structures of mutant Stisa2 and other debranching enzymes were modeled using Phyre2 server ([http://www.sbg.bio.ic.ac.uk/phyre2/ html/page.cgi?id=index](http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index)) and viewed using Rastop Molecular Visualization Software [15]. The catalytic site of debranching enzymes were superimposed using Swiss-PB viewer ([http://www.expasy.org/spdbv](http://www.expasy.org/spdbv)) [16].

**Results**

**Site directed mutagenesis**

A total of six mutagenesis experiments, corresponding to the six codons encoding the six conserved residues lacking in Stisa2 were performed. The first five were performed using the AOE-PCR method while the sixth was performed using the OE-PCR method after the AOE-PCR method failed to give satisfactory results. Example results are shown in Figure 1. DNA sequencing of the mutant gene showed that the desired mutations were successfully made, with no random mutations occurring elsewhere in the gene.

**Expression and partial purification of mutant Stisa2**

Mutant Stisa2 was successfully expressed in soluble form. In Figure 2, unpurified mutant Stisa2 was observed on the western blot as a purple band of about 75 kDa. Partially purified Stisa2 was observed as a purple band of about 80 kDa. Additional bands of low molecular weight were observed and are likely to arise from binding of other *E. coli* cellular proteins to S-protein agarose.

**Evaluation of starch debranching activity**

Evaluation of debranching activity on starch, amyllopectin and β-limit dextrin was carried out by measuring the amount of glucose equivalents released using the bicinchoninic acid assay. Briefly, mutant Stisa2 bound to S-protein agarose was incubated with glucan substrate (5 mg/mL) in MES buffer (50 mM MES, pH 6.0) on a rotating platform at 30°C for 3 h. The bicinchoninic acid assay was carried out as described, but with slight modifications where the incubation time and temperature was reduced to 65°C for 30 min [1].

Debranching activity was also measured qualitatively though zymogram analysis of native PAGE gels. The Ornstein-Davis discontinuous native PAGE system was used as described previously [14]. Soluble glucan substrate (amyllopectin, β-limit dextrin, or starch) was incorporated into the separating gel at a 0.3% final concentration. Samples were prepared by mixing ice cold soluble cell extracts with 5x sample buffer in a 4 : 1 ratio. For each lane, 10 μL sample was loaded and the gel was run at a constant 90 V for 2–2.5 h. The running chamber was kept on ice during the run. After the run, the gels were incubated overnight at room temperature in MES buffer stained with Lugol’s iodine the following day.

**Debranching activity on amylopectin, starch and beta limit dextrin of mutant Stisa2 was compared to native Stisa2, native Stisa1, and the negative control which was**
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The soluble extract of *E. coli* expressing an empty pSTAG vector. The results of the quantitative BCA assay are shown in Figure 3 while the results of the zymogram analysis is shown in Figure 4. Both qualitative and quantitative results showed that mutant Stisa2 did not have debranching activity. The negative control showed no activity for the BCA assay, indicating that the *E. coli* proteins which co-purified during S-protein agarose purification did not affect the debranching activity.

**In silico characterization**

Three dimensional molecular modeling (Figure 5) showed that the molecular structure of the mutant Stisa2 was unchanged, conforming to the characteristic (αβ)₈ structure as previously reported [1]. However, closer examination of the catalytic network of mutant Stisa2 showed that spatial differences between the catalytic residues of mutant Stisa2 were greater than that of other active debranching enzymes (Figure 6).

The catalytic triad in the α-amylase family are the β₄-aspartic acid (catalytic nucleophile), β₅-glutamic acid (proton donor) and β₇-aspartic acid (transition-state stabilizer) [17]. The distance between the β₄-aspartic acid and β₇-aspartic acid in the other debranching enzymes ranged from 8.06–9.14 Å, while that of the mutant Stisa2 was 11.96 Å, much greater than expected. The distance between the β₅-glutamic acid and β₇-aspartic acid in the other active debranching enzymes ranged from 6.38–7.22 Å, while that of the mutant Stisa2 was 8.95 Å, also much further than expected.

From Figure 6, it can be seen that the β₄-aspartic acid side chain in mutant Stisa2 (Asp-509) is angled away from the catalytic cleft instead of towards the substrate. Similarly, the β₇-aspartic acid (Asp-618-transition-state stabilizer) in mutant Stisa2 is angled away, instead of towards the glucan substrate/catalytic cleft. As seen in Figure 7,
Discussion

Inactivity of mutant Stisa2

Restoration of the catalytic residues did not restore starch debranching activity of the mutant Stisa2. Examination of the superimposed catalytic sites of debranching enzymes shows that despite being restored, the catalytic site of mutant Stisa2 still differs from that of the other debranching enzymes.

Restoration of the catalytic residue of Stisa2 did not restore starch debranching activity. Examination of the superimposed catalytic sites of debranching enzymes yielded some clues for this inactivity. Firstly, the larger than expected distance between the catalytic residues could explain the inactivity of mutant Stisa2 as it may influence binding of the substrate and formation of reaction intermediates. Side chain residues interact with each other, and with the catalytic network yielded some clues for this inactivity. Firstly, the larger than expected distance between the catalytic residues could explain the inactivity of mutant Stisa2 as it may influence binding of the substrate and formation of reaction intermediates. Side chain residues interact with each other, and with the
substrate through extensive hydrogen bonding interactions [18, 19]. Increased hydrogen bonding in the active site has been reported to increase pH and temperature stability of $\alpha$-amylases [20]. For hydrogen bonding to occur, a distance of 2.2–3.5 Å between donor and acceptor are usually observed [21]. Thus, the larger distance observed in the catalytic network of mutant Stisa2 may restrict hydrogen bonding which is essential for the enzymatic reaction.

Besides that, changes in side-chain angles, especially that of the $\beta_4$-aspartic acid and the $\beta_7$-aspartic acid could possibly affect the catalytic network. In the $\alpha$-amylase retaining hydrolysis mechanism, the $\beta_7$-aspartic acid, the transition-state stabilizer is suggested to tightly bind the substrate in cooperation with the other residues by forming hydrogen bonds with the glucose units [22]. In mutant Stisa2, this $\beta_7$-aspartic acid (Asp-618) is angled away, instead of towards the glucan substrate/catalytic cleft like for the other catalytic members of the $\alpha$-amylase superfamily.

The larger than average distance between the catalytic residues and the different side-chain angle are likely to be caused by different side chain conformations, rather than distance of the polypeptide backbone. Side chain conformations are not freely distributed, but tend to favor low energy states, although there are deviations, where the adopted side chain conformation is not energetically or geometrically favorable [23–25]. Some side chain conformations are flexible, while some are held in place by hydrogen bonding [26, 27]. There exists a “conformational selection” where the substrate prefers to bind a subset of enzyme conformations [28]. It is possible that the side chain conformations in the mutant Stisa2 do not belong to the preferred subset of conformations, thus leading to the inactivity of mutant Stisa2.

**Stisa2 catalytic residues and role in catalytic process**

In isoamylases, hydrolysis of the $\alpha$-1,6-linkages occurs via a retaining mechanism which is shown in Figure 8 [29]. In the first step, when the glucan substrate binds, its glucosyl ring adopts a half-chair conformation, with its C2-OH and C3-OH groups hydrogen bonded to the transition-state stabilizer [30]. In mutant Stisa2, the transition-state stabilizer, Asp-618 is likely to be too far from the substrate for hydrogen bonding to occur since a distance of 2.2–3.5 Å is required for hydrogen bonding to occur [21].

In the second step, the glycosidic oxygen is protonated by the proton donor ($\beta_5$-glutamic acid), and at the same time, the C1 carbon of the glucan substrate is attacked by the carboxylate anion of the catalytic nucleophile ($\beta_4$-aspartic acid) [30]. This leads to the formation of a glucosyl-enzyme intermediate [30]. In mutant Stisa2, the distance between the $\beta_4$-aspartic acid (Asp-509) and the $\beta_5$-glutamic acid (Glu-546), which was 5.7 Å, was in range of the other debranching enzymes and conforms with the average distance between catalytic nucleophile and proton donor of ~ 5.5 Å in retaining enzymes [29].

The glucosyl-enzyme intermediate is stabilized by hydrogen bonding with three conserved residues: the transition-state stabilizer, an arginine and a histidine [30]. In mutant Stisa2, both the transition-state stabilizer (Asp-618) and the conserved arginine (Arg-507) are further away than expected, and they are also angled wrongly. Thus, they are unlikely to stabilize the high energy half-chair conformation of the glucosyl-enzyme intermediate, thus, the energy-barrier cannot be overcome.

In the final step of the catalytic mechanism, the glucosyl-enzyme intermediate is attacked by a water molecule at the C1 carbon. This nucleophilic attack produces a product which retains the same stereochemistry as the substrate [29].

Therefore, based on the isoamylase catalytic mechanism, it is likely that the distances between the catalytic
residues are too large, thus preventing hydrogen bonding which stabilize the glucosyl-enzyme intermediate. Without the hydrogen bonding which stabilize the glucosyl-enzyme intermediate, the energy barrier cannot be overcome, thus reducing catalytic rates.

**Implications of catalytically inactive mutant Stisa2**

Stisa2 has remained catalytically inactive despite the restoration of the catalytic residues. This implies that the
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loss of Stisa2’s catalytic activity during evolution cannot be attributed to the substitutions of the catalytic residues alone. Although Stisa2 still maintains structural similarity with other α-amylases, Stisa2 has undergone more changes during evolution than just the substitutions of the catalytic site [1]. There is a possibility of mutations in other highly conserved regions which have caused significant changes to the structure and function of the catalytic network. In other mutational studies, relatively small changes were enough to restore long lost enzymatic activity if the substrate-binding and catalytic network have been well conserved [7, 31]. Restoration of catalytic residues have been reported to reactivate inactive enzymes such as carbonic anhydrase and azurocidin [7, 8]. In the case of Stisa2, catalytic activity was not restored because the geometry of the catalytic network appears to have been significantly altered, as shown by the in silico analysis and/or because of alteration of the substrate binding where the substrate is not positioned correctly in the catalytic site, making conditions unfavourable for catalysis. Furthermore, this supports the idea that the loss of catalytic activity is not a recent evolution for Stisa2 [32].

Nevertheless, catalytically inactive Isa2 has been conserved and plays important roles in substrate binding and in regulating isoamylase activity.

Roles of Stisa2 in starch biosynthesis

It has been postulated that the original function of isoamylase was in degradation of glucans [33]. However, gene duplication events allowed evolution of multiple isoforms which now have roles in glucan synthesis and degradation [33]. Similar to the case of Ammodytin L, where evolutionary specialization caused loss of catalytic activity but increase in myotoxicity, Isa2 appears to have undergone evolutionary specialization [7]. Isa2 has lost catalytic activity, but has gained other specialized functions. Non-catalytic Isa2 has been conserved in all chloroplast-containing species examined to date, suggesting a functional selective advantage [32]. Indeed, plants lacking Isa2 such as in Arabidopsis mutant lines have abnormal starch biosynthesis [5].

Isa2 has been suggested to regulate the activity of the Isa1 subunit in the Isa1/Isa2 heterocomplex [1]. In developing rice seeds, a transcription factor, OsbZIP58, which is a key transcriptional regulator controlling starch synthesis in the endosperm, binds to the promoter of Isa2 and regulates its expression [34]. In vivo experiments showed that OsbZIP58 binds to the promoter of Isa2, but not Isa1 [34]. This suggests that the availability of the Isa heterocomplex is dependent on the expression of Isa2, which in turn is regulated by OsbZIP58. This is especially critical in dicots which require the Isa heterocomplex for normal starch biosynthesis, whereas in monocots such as maize and rice, the Isa1 homocomplex can compensate for the absence of the heterocomplex and have near-normal starch biosynthesis [32, 35, 36].

The catalytic properties of the Isa heterocomplex has also been reported to vary by the stoichiometry of Isa1 and Isa2, where the heterocomplex had increased affinity for highly branched polyglucans such as phytylgycogen [1, 36] Isa2 has also been reported to have greater affinity to Isa1, than for Isa1 to itself, where in rice leaves, only the Isa heterocomplex exists [36]. Furthermore, Isa2 has been reported to contribute towards increased thermostability of the Isa heterocomplex, and increased hydrophobicity [3, 36]. The Isa heterocomplex has also been reported bind more easily to glucan granules and to repress granule initiation in maize [35, 36]. The different properties of the Isa heterocomplex and the Isa1 homocomplex means that each complex plays a distinct role, which may or may not be compensated by the other complex. It is still unclear
why different plant species and even different types of tissue in the same plant (e.g. leaves, tuber, or endosperm) require a different complex, although it has been suggested that substrate properties and availability, as well as physiological and environmental conditions are among the factors why these two complexes with different catalytic properties exist [35, 36].

**Conclusions**

With the goal of restoring starch debranching activity to catalytically inactive Stisa2, the catalytic residues were restored through site directed mutagenesis of the Stisa2 gene. However, experimental results indicated that the mutant Stisa2 remained inactive. In silico analysis of the catalytic network showed spatial differences in the catalytic network of Stisa2 when compared to other catalytically active debranching enzymes. The larger than expected distances between catalytic residues are likely to prevent hydrogen bonding which stabilize the glucosyl-enzyme intermediate, thus preventing restoration of debranching activity in the mutant Stisa2. This indicates that during evolutionary specialization, significant changes to the structure and function of the catalytic network have occurred. Although catalytically inactive, Isaa2 is conserved in starch-producing plants as it has evolved important non-catalytic roles such as in substrate binding and in regulating isoamylase activity.

**Figure 8:** The retaining catalytic mechanism of isoamylases based on Davies and Henrissat (1995) [29]. (A) shows two glucose residues in a glucan substrate linked via α-1,6-linkage. In (B), the glycosidic oxygen is protonated by the proton donor (X), while at the same time, the C1 carbon is attacked by the catalytic nucleophile (Y), which results in the glycosyl-enzyme intermediate shown in (C). In (D), the glycosyl-enzyme intermediate is attacked by a water molecule at the C1 carbon, which results in the hydrolysed products shown in (E).
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