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BIOCATALYSIS

Changes in the catalytic properties and substrate specificity of *Bacillus* sp. US149 maltogenic amylase by mutagenesis of residue 46

Sameh Ben Mabrouk · Dorra Ayadi-Zouari · Hajer Ben Hlima · Samir Bejar

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Abstract Maltogenic amylase from *Bacillus* sp. US149 (MAUS149) is a cyclodextrin (CD)-degrading enzyme with a high preference for CDs over maltooligosaccharides. In this study, we investigated the roles of residue Asp46 in the specificity and catalytic properties of MAUS149 by using site-directed mutagenesis. Three mutated enzymes (D46V, D46G and D46N) were constructed and studied. The three mutants were found to be similar to the wild-type MAUS149 regarding thermoactivity, thermostability and pH profile. Nevertheless, the kinetic parameters for all the substrates of the mutant enzymes D46V and D46G were altered enormously as compared with those of the wild type. Indeed, the K_m values of MAUS149/D46G for all substrates were strongly increased. Nevertheless, the affinity and catalytic efficiency of MAUS149/D46V toward β-CD were increased fivefold as compared with those of MAUS149. Molecular modelling suggests that residue D46 forms a salt bridge with residue K282. This bond would maintain the arrangement of side chains of residues Y45 and W47 in a particular orientation that promotes access to the catalytic site and maintains the substrate therein. Hence, any replacement with uncharged amino acids influenced the flexibility of the gate wall at the substrate binding cleft resulting in changes in substrate selectivity.

Keywords Affinity · Catalytic efficiency · Kinetic parameters · Maltogenic amylase · Site-directed mutagenesis

S. Ben Mabrouk (✉) · D. Ayadi-Zouari · H. Ben Hlima · S. Bejar
Laboratoire de Métabolites Et de Biomolécules,
Centre de Biotechnologie de Sfax,
Université de Sfax, BP 1177, 3018 Sfax, Tunisia
e-mail: benm_sameh@yahoo.fr

Introduction

Maltogenic amylases (MAase, EC 3.2.1.133) are amylase-type enzymes in glycoside hydrolase family 13, along with neopullulanase (EC 3.2.1.135) and cyclomaltodextrinase (EC 3.2.1.54) [16]. The MAases exhibit broad specificity of hydrolysis activity on various carbohydrates such as cyclodextrins (CD), pullulan and starch, which distinguishes them from a typical α-amylase [21]. Unlike many typical α-amylases, the hydrolysis of starch results in maltose as major product. Furthermore, the hydrolysis of pullulan and CDs leads mainly to panose and maltose, respectively. MAases not only catalyse the cleavage of α-1,4 and α-1,6 glycosidic linkages, but also can transfer the products to the sugar moiety of various acceptor molecules [2, 5, 13, 17].

MAases exhibit 34–56 % sequence identity with each other and share four highly conserved regions (I, II, III and IV) in the primary amino acid sequences [16]. These regions contain the invariant catalytic residues Asp328, Glu357 and Asp424 (*Thermus* MAase numbering). In addition, Cha et al. in 1998 [4] and Duffner et al. in 2000 [7] reported that there are two additional conserved sequences, one between the third and fourth conserved regions and the second one is sited after the fourth conserved region. The role of these two sequences is not clear until now.

The aforementioned enzymes and other related ones display similar biochemical properties and share almost the same 3D structures [16, 20]. Thus, the structure of MAase of *Thermus* revealed that the enzyme was composed of three distinct domains [12], the N-domain (residues 1–124) composed of β-strands exclusively, the central $(\beta,\alpha)_8$ barrel domain, and the C-domain composed of eight β-strands (residues 505–588). The N-domain is distinctively separated from the central body, but it is involved in extensive

interaction with the $(\beta,\alpha)_8$ domain of the adjacent molecule, indicating a dimer formation of the enzyme. In fact, the N-terminal domain of one subunit covers part of the top of the $(\beta,\alpha)_8$ barrel of the other subunit, and shapes a narrow and deep active site groove. The width of the active site cleft of *Thermus* MAase appears to be optimal for binding CDs. As a result, *Thermus* maltogenic amylase with N-terminal deletion exhibited a significant decrease in β-CD hydrolysis [15]. Accordingly, the amino acid residues at the interdomain interface of MAase are likely to play a critical role in constituting the active and substrate binding sites, which allows efficient binding and catalysis of β-CD as compared with other substrates.

The MAase from *Bacillus* sp. US149 (MAUS149) has been studied in our laboratory. The gene encoding MAUS149 (*amy*US149) was cloned, sequenced and overexpressed in *Escherichia coli* [3]. Furthermore, we have obtained a mutant named MA-A27 by error prone PCR. MA-A27 showed better thermostability at 50 and 55 °C as well as a higher affinity and catalytic efficiency (k_{cat}/K_m) toward β-CD in comparison with MAUS149. There are few data available comparing the kinetic parameters for maltogenic amylases; however, the K_m value of MA-A27 for β-CD was similar to that of *Thermus* maltogenic amylase [16]. The latter enzyme has 60 % similarity with MA-A27.

MA-A27 showed four substitutions at positions 46, 78, 145 and 548. Structural studies of that mutant showed that the change in the catalytic properties seems to be due to D46V mutation (submitted manuscript).

Here, we investigated the catalytic properties of three variant forms of MAUS149 modified at residue 46, namely D46V, D46G and D46N. Through kinetic parameters and substrates affinity studies of MAUS149 mutant enzymes, we determined the role of residue 46 in the function of MAUS149 during hydrolysis.

Materials and methods

Bacterial strains, plasmids and culture media

Escherichia coli DH5α (F-*sup*E44 Φ80 δ*lac* ZΔM15 Δ (lac$^-$ ZYA$^-$ argF) U169 end A1 recA1 hsdR17(r_k^-,m_k^+) deoR thi-1 λ$^-$ gyrA96 relA1) was used as a host strain in the present study. The pGEM-T Easy was used as a cloning vector, whereas pBMS2, pBMS19, pBMS20 and pBMS21 were the plasmids containing the genes encoding the wild-type MAUS149 and MAUS149/D46V, MAUS149/D46G and MAUS149/D46N mutant enzymes, respectively. *E. coli* strains harboring wild-type and mutated MAUS149 were grown in Luria-Bertani (LB) media. LB agar containing 1 % starch was used for the detection of colonies with MA activity. The media were supplemented, when necessary, with ampicillin (100 μg/ml) and isopropyl β-D-1-thiogalactopyranoside (IPTG; 16 μg/ml).

DNA manipulation and PCR

The preparation of plasmid DNA, digestion with restriction endonucleases, and separation of fragments by agarose gel electrophoresis were all performed as described by Sambrook et al. [22]. The PCRs were carried out with a Gene Amp PCR System 2700 thermocycler (Applied Biosystems). The amplification reaction mixtures (50 μl) contained 20 pg of each primer, 200 ng of DNA template, amplification buffer and 2 U of Pfu enzyme (*Pyrococcus furiosus* DNA polymerase). The cycling parameters were 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 60 s and 72 °C for 120 s. The PCR products were purified using an agarose gel extraction kit (Jena Bioscience).

Construction of maltogenic amylase mutant enzymes

The mutated enzymes (MAUS149/D46V, MAUS149/D46G and MAUS149/D46N) were generated using PCR-based site-directed mutagenesis [9]. Two non-mutagenic external primers, S237 (5′AGGTGCGGCCGCCACTGATT3′) and S238 (5′GGAGCATGCGTCTCTTGTCA3′), and two pairs of matching primers that harbour the appropriate single mutation were designed (Table 1). Using the pBMS2 plasmid as template, six PCR fragments (F1, F2, F3, F4, F5 and F6) were amplified with primer pairs (S237/RevD46V), (S238/Fwd D46V), (S237/Rev D46G), (S238/Fwd D46G), (S237/Rev D46N) and (S238/Fwd D46N), respectively. After the purification of these fragments, three other PCR reactions were performed using the external primer pair (S237/S238) and the appropriate mixture of the partial complementary fragments as template: The mixture (F1/F2) for the first reaction, the mixture (F3/F4) for the second one and the mixture (F5/F6) for the third one, leading to the mutated genes *amy*US149/D46V, *amy*US149/D46G and *amy*US149/D46N, respectively. These genes were cloned in the pGEM-T Easy

Table 1 Nucleotide sequences of primers used for site-directed mutagenesis and generated recombinant plasmids

Desired mutation	Forward primer sequence[a]	Plasmids
D46V	GGATCCGTAC**GTT**TGGACCAAGG	pBMS19
D46G	GGATCCGTAC**GGT**TGGACCAAGG	pBMS20
D46N	GGATCCGTAC**AAT**TGGACCAAGG	pBMS21

[a] Nucleotide sequences corresponding to the mutated amino acids are in bold

vector, thus generating the plasmids pBMS19, pBMS20 and pBMS21.

The mutated genes were analysed by restriction enzyme assays, and the presence of the desired mutations and the absence of other undesirable changes were verified by DNA sequencing using an automated DNA sequencer (Applied Biosystems).

Preparation of crude extracts and enzyme purification

The recombinant *E. coli* strains were grown in LB medium, containing ampicillin (100 μg/ml) and IPTG (16 μg/ml). The cells were harvested by centrifugation at 6,000 $\times g$ for 10 min, and the pellets were crushed with alumina at 4 °C for 30 min in the presence of 100 mM PMSF (phenylmethanesulfonyl fluoride) prepared in isopropyl alcohol. The mixture was suspended in 50 mM sodium phosphate buffer pH 6.5 and the cell debris was removed by centrifugation at 30,000 $\times g$ for 30 min. The supernatant constituted the enzyme crude extract. The purification of MAUS149 and its mutants was achieved using a protocol previously described by the authors [3] wherein ammonium sulfate precipitation and fast-performance liquid chromatography (FPLC) were employed.

Enzyme assays

The activity of MAUS149 was determined by measuring the amount of reducing sugars released after incubation with starch. A 200-μl aliquot of the purified enzyme was mixed with 300 μl of 50 mM sodium phosphate buffer pH 6.5 and 500 μl of 1 % (w/v) starch from potato, and the samples were incubated for 10 min at 37 °C. The amount of released reducing sugars was determined by the dinitrosalicylic acid method [18]. One unit of maltogenic amylase was defined as the amount of enzyme that released reducing sugars equivalent to 1 μmol of glucose per min under the specified assay conditions.

Kinetic parameters determination and characterization of the hydrolysis products

The hydrolysis of β-CD, starch and pullulan was performed in 50 mM sodium phosphate buffer pH 6.5 at 37 °C. The substrate concentrations used ranged from 1 to 7.5 g/l for starch and from 0.5 to 4 g/l for β-CD and pullulan. The generated reducing sugars were determined at regular time intervals and expressed in equivalent micromoles of glucose per millilitre of purified enzyme used for the reaction. The V_{max} and K_m values were calculated from a Lineweaver–Burk plot using the hyper-32 program.

Characterization of the hydrolysis products of starch was performed as previously described [3]. Wild-type and mutated MAUS149 were added to starch solutions separately and the reaction mixtures were incubated at 40 °C for 24 h. Resulting products were analysed by HPLC (high-performance liquid chromatography) using a Eurokat Ag 10 μm column (KNAUER).

Bioinformatic analysis

ClustalW multiple sequence alignment of various maltogenic amylases was performed using Bioedit (version 7.0.4.1) (http://www.mbio.ncsu.edu/). The sequences included for alignment were retrieved from GenBank. The homology models of the wild-type MAUS149 and derived mutants were generated using Swiss-Model [1, 8, 23] and edited using the PyMol editor [6]. The template used was 1GVI, i.e. the crystal structure of *Thermus* maltogenic amylase in a complex with β-CD.

Results and discussion

Design of mutations and purification of mutant enzymes

MAUS149, as with other MAases, can hydrolyse starch, pullulan and β-CD with better affinity toward the last substrate [3]. The enzyme has an *exo*-acting mode on starch, giving maltose as a unique end product. In order to enhance MAUS149 performance, a mutated enzyme (MA-A27) was obtained by error prone PCR (submitted manuscript). The comparison of kinetic parameters of MA-A27 with those of MAUS149 indicates that the affinity toward β-CD increased fivefold, whereas that of the starch and pullulan decreased. The results also show that the catalytic efficiency of MA-A27 toward β-CD was improved as compared with that of MAUS149.

The gene *amy*MA-A27 was sequenced and compared with the wild *amy*US149. The alignment of two protein sequences shows four substitutions: D46V, P78L, V145A, and K548E (submitted manuscript). The study of the location and nature of these substitutions has shown that the change of catalytic characteristics of MA-A27 could mainly be the effect of mutation at position 46. To test this hypothesis and provide an insight into structure–function relationships of MAUS149, we attempted site-directed mutagenesis of D46. Hence, three mutants enzyme were designed: MAUS149/D46V, MAUS149/D46G and MAUS149/D46N as described in the "Materials and methods" section.

The wild-type and mutant enzymes were produced and purified as previously described [3]. The mutant proteins had molecular weights that were similar to that of the wild-type protein, 67.5 kDa.

Table 2 Kinetic parameters of MAUS149 wild-type and mutated enzymes

	K_m (g l^{-1})			k_{cat}/K_m (l g^{-1} s^{-1})		
	Starch	Pullulan	β-CD	Starch	Pullulan	β-CD
MAUS149	12.00 ± 1.5	15.25 ± 2	1.75 ± 0.3	18.00 ± 1.5	40.00 ± 2.0	180.00 ± 10
D46V	16.75 ± 1.5	19.00 ± 2	0.20 ± 0.15	13.00 ± 2.0	32.00 ± 3.5	410.00 ± 8.0
D46G	17.50 ± 00	20.40 ± 1	3.50 ± 1.5	12.5 ± 1.0	29.70 ± 2.5	100.60 ± 5.0
D46N	13.00 ± 0.5	14.50 ± 00	1.5 ± 0.3	16.5 ± 2.5	39.50 ± 2.0	176.80 ± 4.0
MA-A27	17.00 ± 1.5	19.50 ± 1	0.30 ± 0.08	13.0 ± 1.0	30.0 ± 2.6	400 ± 15.0

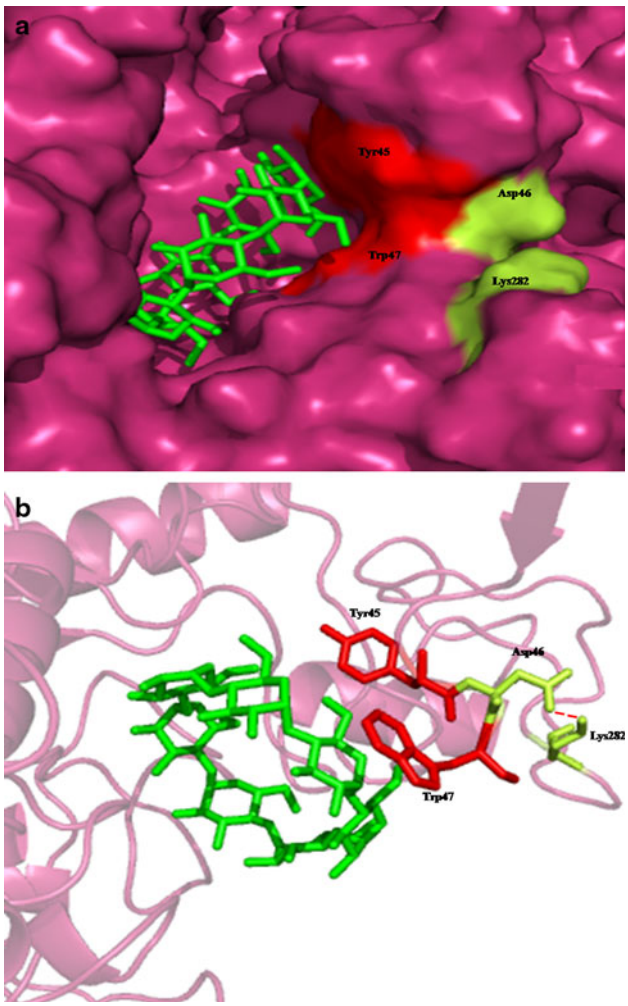

Fig. 1 Model of the monomeric structure of MAUS149. Residues Tyr45, Asp46 and Trp47 are shown as *sticks*. A molecule of cyclodextrin was docked to indicate the location of the active site. **a** Surface representation. **b** A close-up view showing the position and the interaction of residues Asp46 and Lys282. Salt bridge interaction is indicated as *dashed lines*

Physicochemical properties of mutated enzymes

Thermostability and thermoactivity of the purified mutants were determined and compared with those of the wild-type enzyme (data not shown). No distinct differences in the two aforementioned parameters were observed between the four enzymes. In fact, all enzymes showed maximum activities at 40 °C and had a half-life of about 15 min at 55 °C. Furthermore, no significant differences were observed in optimum pH between all enzymes and maximum activities occurred at pH 6.5.

Kinetic parameters and hydrolysis products

The kinetic parameters of the hydrolysis of β-CD, pullulan and starch by wild-type MAUS149, MAUS149/D46V, MAUS149/D46G and MAUS149/D46N were determined and are summarized in Table 2. The wild-type and the mutant enzymes have the highest affinity and catalytic efficiency toward β-CD when compared with the other substrates, as do many other maltogenic amylases [4, 10, 11, 14, 20]. Nevertheless, some dissimilarity between these enzymes was observed. Indeed, the kinetic parameters of MAUS149/D46N for all substrates were quite similar to those of wild-type MAUS149 (Table 2). However, the replacement of Asp46 by Gly or Val affected the kinetic parameters, especially the binding affinity for β-CD. Accordingly, the K_m value of MAUS149/D46V was 11 % of that of the wild type, leading to an increase of the affinity and the catalytic efficiency by a factor of about 5 and 3, respectively (Table 2). Regarding the MAUS149/D46G mutant, the affinity and the catalytic efficiency for all substrates decreased enormously. Hence, the K_m values of MAUS149/D46G mutant for starch, pullulan and β-CD increased about 150, 135 and 200 %, respectively, as compared with those of the wild-type MAUS149. These results showed that residue 46 plays an important role in substrate binding and catalysis.

On the other hand, maltogenic amylase was known to produce maltose as a unique product from starch. Thus, observed kinetic parameters changes between the studied mutants prompted us to analyse the products of starch hydrolysis. This analysis indicated that mutation at position 46 has no influence on the starch breakdown products. In fact, maltose was the unique product generated by the action of all the mutant enzymes on starch.

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                 10         20         30         40         50
MAUS149   VFKEAIDHRP KGAYAYAFDK ETLHIKLRAK KNDLKTVTLI GGDPYDWTKE
ThMA      MRKEAIHHRS TDNFAYAYDS ETLHLRLQTK KNDVDHVELL FGDPYEWHDG
BSMA      MFKEAIYHRP KDNFAYAYDE QTLHIRLRTK KNDVEHVRLI YGDPYEWENG
Cdase1    MIKEAIFHKS DVPYAYPLNE NQLKIVLRTA VFDVDRVYVL YKDRYDWLG-
NEOP1     MRKEAIYHRP ADNFAYAYDS ETLHLRLRTK KDDIDRVELL HGDPYDWQNG
CDase2    MLKEAIYHRP KNNYAYAYSK DTLHIRLRTK KNDLTQVELL YADPYNWNED
NEOP2     MRKEAIYHRP ADNFAYAYDS ETLHLRLRTK KDDIDRVELL HGDPYDWQNG
Cdase 3   MRKEAIHHRS TDHFAYAYDS ETLHLRLQTK KHDVDHVELL FGDPYEWHDG
                260        270        280        290        300
MAUS149   YFEPFQDVLE KGEKSAYKDW FHLREFPVVA EPKPNYDTFA FTPM-MPKLN
ThMA      EFAPFQDVLK NGAASRYKDW FHIREFPLQT EPRPNYDTFA FVPH-MPKLN
BSMA      EFPPFQDVLK YGENSKYKHW FHIREFPLQT VPRPNYDTFA FTPN-MPKLN
Cdase1    DFFAFQDVIK NGKKSKYWDW FNIYEWPIKT HGKPSYEAFA DTVWRMPKLM
NEOP1     EFAPFQDVWK NGESSKYKDW FHIHEFPLQT ESRPNYDTFA FVPQ-MPKLN
CDase2    YFEAFQDVLK HQEQSKYKDW FHIRDFPVTP GPKPNYDTFG FVEY-MPKLN
NEOP2     EFAPFQDVWK NGESSKYKDW FHIHEFPLQT EPRPNYDTFR FVPQ-MPKLN
Cdase 3   EFAPFQDVLK NGAASRYKDW FHIREFPLQT EPRPNYDTFA FVPQ-MPK
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Fig. 2 Amino acid sequence alignment of the regions surrounding Asp46 and Lys282 of maltogenic amylases from different species. *Boldface letters* indicate residues Tyr45 and Trp47. ThMA, maltogenic amylase from *Thermus* sp. IM6501 (accession no. AF060204.1); *BSMA* maltogenic amylase from *Bacillus stearothermophilus* (accession no. U50744.1); *Cdase1* cyclodextrinase from *Clostridium thermohydrosulfuricum* 39E (accession no. P29964.1); *NEOP1* neopullulanase from *B. stearothermophilus* (accession no. AAK15003.1); *Cdase2* cyclodextrinase from *Bacillus* sp. A2-5a (accession no. BAA31576.1); *NEOP2* neopullulanase from *Geobacillus stearothermophilus* (accession no. P38940.1); *Cdase 3* cyclodextrinase from *G. stearothermophilus* (accession no. BAB63955.1)

Structural interpretation

In the absence of a 3D structure, the model structure generated using known structures of homologous enzymes is helpful in understanding the roles of the various mutations in improving the characteristics of enzymes. We therefore tried to dissect out the possible consequences of the three individual mutations at position 46 in MAUS149 enzyme, using the homology-modelled structure along with the known structure of *Thermus* maltogenic amylase [12].

The generated models showed that residue 46 is located at the edge of the catalytic cavity between residues Y45 and W47 (Fig. 1). These two amino acids are key residues for accessing and maintaining the substrate in the catalytic site [10, 14, 16, 19]. According to the MAUS149 model, residue D46 could form a salt bridge with residue K282 of the other molecule (Fig. 1b). This bond would maintain the arrangement of residues Y45 and W47 side chains in a very particular orientation that promotes not only the access of the substrate but also its residence in the catalytic cavity. In addition, the inspection of many maltogenic amylase sequences showed the presence of an acidic residue (Asp or Glu) at position 46 and a basic residue (Lys or Arg) at position 282 (Fig. 2). This could explain the importance of the linkage formed between the amino acids at positions 46 and 282 in the hydrolytic activity of these enzymes. As a result, any substitution in the amino acid at position 46 would modify the present bond and might disrupt the interactions necessary for substrate binding and catalytic steps. Accordingly, the substitution D46V could eliminate this binding and confer more flexibility to the sides chains of residues 45 and 47 (Fig. 3). In fact, from the structural modelling analysis it cannot be unequivocally demonstrated that V46 significantly affects the binding cavity dimensions, but it is clear that this mutation leads to a change in the side chain orientations of 45 and 47 residues. Therefore, it seems that this cavity could more easily accommodate the cyclic substrates without affecting the affinity for linear substrates. As a consequence, the affinity

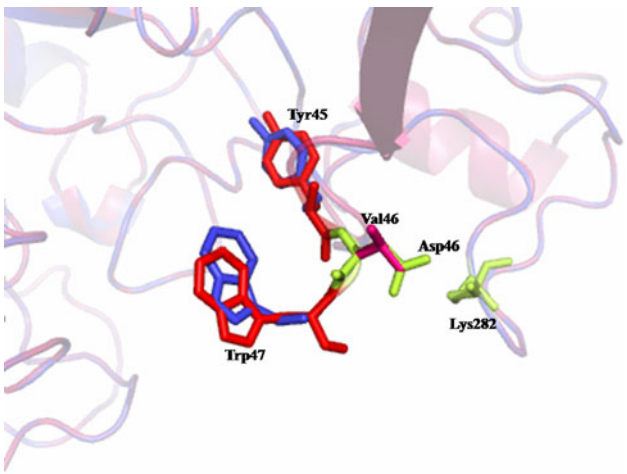

Fig. 3 Superposition of residues Tyr45 and Trp47 from MAUS149 and those from MAUS149/D46V. Asp46, Val46 and Lys282 residues are shown as *sticks*

of MAUS149/D46V toward β-CD would increase in comparison with that of MAUS149. On the other hand and according to our model, the substitution of Asp46 in MAUS149 with Asn, as observed in CDase of *Bacillus* sp. A2-5a (accession BAA31576.1), would allow the side chain oxygen atom of Asn to form H-bonds with the NH1 atom of Lys282, which seems to mimic the salt bridge model and could maintain the active site structure as in the wild-type model. This would explain the unchanged kinetic parameters of the mutant D46N when compared with the wild-type MAUS149.

Concerning MAUS149/D46G, the replacement Asp/Gly appears to completely eliminate the bond between residues at positions 46 and 282, which increases the degree of freedom, thus leading to the destabilization of the side chains of Y45 and W47 residues. This could account for the immense decrease of the affinity parameter of MAUS149/D46G. Hence, any replacement of residue 46 with uncharged amino acids may have influenced the shape and flexibility of the gate wall at the substrate binding cleft, which resulted in high substrate selectivity. Thus, the polarity of the amino acid at position 46 was an important factor for substrate binding.

Conclusion

The role of residue 46 in the MAUS149 maltogenic amylase substrate binding was investigated through site-directed mutagenesis and analysis of the catalytic performance of the variant enzymes. The results obtained in this study suggest that the Asp46 residue plays an important role in substrate recognition and hydrolysis. These results provide useful information for rational protein engineering of the GH-13 enzyme. The investigation of the catalytic performance of mutant enzymes with various amino acids at position 282, and other positions near the catalytic cavity, will be among the perspectives of a further study.

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