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Role of the phenylalanine 260 residue in defining product profile and alcoholytic activity of the α-amylase AmyA from *Thermotoga maritima*

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Abstract: Some α-amylases besides catalyzing the hydrolysis of α-1,4 glycosidic bonds in starch are also capable of carrying out some transglycosylation activity. The importance of aromatic residues near the catalytic site in determining the ratio of these two competing activities has been remarked in the past. In the present work we investigated the role of residue 260 in the product profile of the α-amylase AmyA from *Thermotoga maritima*. This phenylalanine residue, two positions after the glutamic acid/base catalyst was substituted by both tryptophan and glycine residues, showing opposite behaviors. The tryptophan mutant displayed a very similar product profile pattern to that of the wild-type enzyme; while the mutant Phe260Gly showed a higher transglycosylation/hydrolysis ratio. When the Phe260Trp mutation was constructed in the context of His222Gln, a mutant we have already reported with an increased transglycosylation/hydrolysis ratio and a higher alcoholysis activity, the resultant enzyme showed an apparent higher hydrolysis/transglycosylation ratio and a change to shorter products pattern than the single mutant enzyme, still maintaining the increased alcoholytic activity provided by the His222Gln mutation. The mutant Phe260Gly, on the other hand showed by itself a higher alcoholytic activity, similar to that of the His222Gln mutant.

Key words: alcoholysis; alkyl glycoside; α-amylase; site-directed mutagenesis; transglycosylation.

Abbreviations: AmyA, α-amylase from *Thermotoga maritima*; CGTase, cyclodextrin glucanotransferase; G1–G7, oligosaccharides from G1 (glucose) through G7 (maltoheptaose); GH, glycoside hydrolase; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

Introduction

α-Amylases (EC 3.2.1.1) belong to family 13 of glycoside hydrolases (GHs). These are retaining glycosidases whose main activity is to catalyze the hydrolysis of internal α-1,4-glycosidic bonds in starch through a double displacement mechanism and involving a covalent glycosyl-enzyme intermediate later deglycosylated by water (Mosi et al. 1997; Uitdehaag et al. 1999). However, some α-amylases display transglycosylation activity to some extent, an activity that can revert to elongated oligosaccharides the product of the reaction (glucose and maltose mainly). This transglycosylation activity results when another sugar, instead of water attacks the covalent intermediate glycosyl-enzyme at the acceptor binding site. The product profile reached at equilibrium is characteristic of each α-amylase, and according to this, they can be classified as saccharifying or liquefying enzymes. The firsts, yielding a more efficient hydrolysis of starch, from 50 to 60% while the seconds only about 30 to 40%, at a given produced reducing power (Vihinen & Mantsala 1989). Both types of amylases have been found in bacteria, but fungal amylases are mainly saccharifying (Fogarty 1983). The structural features responsible for these differences are not clearly identified yet. However, it has been suggested that combination of several factors, like the number of subsites in the active site (Robyt & French 1967; Brzozowski et al. 2000), affinity of each subsite (Allen & Thoma 1976; Kondo et al. 1980), and the presence of transglycosylation activity (Suganuma et al. 1996; del-Rio et al. 1997) contribute to the resulting product profile.

The industrial processing of starch requires the combined action of enzymes in order to reach a more efficient process. In an initial hydrolysis phase the use of liquefying (bacillar) thermostable α-amylase allows the fast viscosity reduction in the reactor at high temperature. In a second stage, the addition of saccharifying enzymes warrants a faster and more efficient hydrolysis process. However, the enzymes traditionally used at this stage, glucoamylase from *Aspergillus niger* and a compatible pullulanase that work under the same condi-

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tions, require a pH and temperature adjustment due to their lower thermal stability (Crabb & Mitchinson 1997; van der Maarel et al. 2002). The demand for enzymes that combine thermal stability and/or expanded reaction conditions to increase the efficiency of the process has been the focus of intense research in starch industry. The identification and characterization of GHs from thermophilic organisms has open a new era in the development of biocatalysts for this industry (Jorgensen et al. 1997; Liebl et al. 1997; Kim et al. 1999; Lim et al. 2003; Rey et al. 2003).

In 1997, Liebl et al. (1997) described an extracellular α-amylase (AmyA) found in the hyperthermophilic bacterium *Thermotoga maritima* MSB8 (DSM 3109) as a saccharifying amylase with an optimal temperature at 85 ℃, able to hydrolyze internal α-1,4-glycosidic bonds in various α-glucans such as starch, amylose, amylopectin and glycogen, yielding mainly glucose and maltose as final products. Although there is no crystal structure for this protein, the presence of the four highly conserved signatures among α-amylases (Friedberg 1983; Rogers 1985; Nakajima et al. 1986; Svensson 1988), has allowed the construction of a model (Damian-Almazo et al. 2008) based on homology to other α-amylases. These proteins are formed by three domains. Domain A, which contains the catalytic residues, is a $(\beta/\alpha)_8$ or TIM barrel domain interrupted between β-strand 3 and α-helix 3 by domain B. Domain B, less conserved among α-amylases (Jespersen et al. 1993; Janecek et al. 1997; Pujadas & Palau 2001), is difficult to model, especially because of its low sequence identity to any α-amylase whose structure has already been described. Finally, domain C at the C-terminal domain of the protein has a more conserved Greek-key motif and seems to be involved in substrate binding.

Based on the generated model, we have constructed some mutants with the aim of increasing the alcoholytic activity already present in the wild-type enzyme and associated to transglycosylation. Mutations have been directed towards residues forming the subsites +1 and +2 (acceptor binding site) according to the modeled structure. Mutants at His222 (subsite +1) gave place to more alcoholytic enzymes (Damian-Almazo et al. 2008). Crystallographic studies showed the importance of aromatic residues in the formation of binding subsites (Brzozowski & Davies 1997) and for this reason, they have been target for mutagenic studies intended to modify the transglycosylation/hydrolysis ratio of GHs (Matsui et al. 1994; Kuriki et al. 1996; Saab-Rincon et al. 1999; van der Veen et al. 2001; Rivera et al. 2003). The comparison among glycoside hydrolases through multiple sequence alignment has also made possible the identification of residues involved in transglycosylation activity. In this context, the residue Phe259 in cyclodextrin glucanotransferases (CGTases) from *Bacillus circulans* has been identified as a switch between tranglycosylation and hydrolysis reaction (van der Veen et al. 2001). CGTases are natural transferases that promote the formation of cyclodextrins by transferring the covalently bound glycosyl end of a short maltooligosaccharide chain to its non-reducing end instead of to a water molecule. Although there are several structural differences between CGTases and α-amylases – CGTases have two more domains than α-amylases, as well as some very conserved signatures – it is interesting to observe that AmyA, in contrast to previously sequenced α-amylases (Janecek et al. 1995), contains the equivalent Phe259 residue, part of the highly conserved dipeptide Phe259–Trp260 among CGTases.

In the present study we investigated the effect of mutating Phe260 (AmyA numbering) in the wild-type enzyme and in the H222Q mutant context. A comparison of product profile and activities was carried out.

Material and methods

Site-directed mutagenesis and gene cloning

Site-directed mutagenesis was carried out in the AmyA gene from *Thermotoga maritima* by the megaprimer method (Sarkar & Sommer 1990). The used template was the AmyA gene without signal peptide-coding region cloned into pET28a(+) as reported previously (Damian-Almazo et al. 2008) in order to obtain a higher yield in protein production and to avoid contamination of unprocessed peptide. The DNA fragments carrying the mutations at position Phe260 to glycine and tryptophan were amplified using the oligonucleotides 5'-CCGCTACCCACCTCTCCCAC-3', 5'-CCGCTCCACACCTCTCCCAC-3', respectively (mutation sites underlined), in combination with the oligo 5'-TTGCTGAGCCGAAGGCATATGTGCTTTCAAACGT CTATGAGTCAATCC-3' that anneals at the 5' termini of the gene. The megaprimers obtained were purified and further extended in a second round of PCR with oligo 5'-CCGCAAGCTTTTTGAAAATGTACGCTTTC-3' that anneals at the 3' end to complete the genes. Double mutant H222Q-F260W was constructed using as template the AmyA H222Q mutant gene constructed previously (Damian-Almazo et al. 2008). The resulting mutated genes as well as the wild-type and mutant H222Q were cloned in the pET28a(+) expression vector; transformation, sequencing and enzyme purification were made as reported previously (Damian-Almazo et al. 2008).

Specific activity determination

Enzyme activities of wild-type AmyA and mutants were estimated by measuring initial velocities of formation of reducing sugars released upon starch hydrolysis by the 3,5-dinitrosalicylic acid method as reported previously (Rivera et al. 2003). The reaction was carried out in 1 mL of 10 mg/mL soluble starch (Sigma-Aldrich) dissolved in 50 mM Tris-bis-HCl, 150 mM NaCl, 2 mM $CaCl_2$ buffer pH 7 at 85 ℃, employing approximately 4 U/mL of activity in each enzyme assay.

Product profile

Starch hydrolysis reactions carried out by 16 U/mL of wild-type AmyA and mutants were conducted at 85 ℃ in a Thermomixer Compact (Eppendorf) in 1 mL of 50 mM Tris-bis-HCl, 150 mM NaCl, 2 mM $CaCl_2$ buffer pH 7 with 3% of soluble starch as substrate. Reactions were incubated for 42 hours to obtain the products concentration at equilibrium. Samples were analyzed by thin layer chromatography (TLC) as previously reported (Saab-Rincon et al. 1999) and quantified by high performance liquid chromatography

(HPLC) in a Waters-Millipore 510 HPLC system with a refractive index detector (Waters model 410) equipped with an automatic sampler (model 717 Plus) and a C18 column (4.6 × 250 mm) using water as mobile phase at a flow rate of 0.7 mL/min and a Nova-Pak aminated column (4.6 × 250 mm) using acetonitrile and water as mobile phase (68:32) at a flow rate of 1 mL/min. The peak areas were measured and compared against those of a standard curve containing known amounts of oligosaccharides from glucose (G1) to maltoheptaose (G7) (Sigma-Aldrich) as described previously (Damian-Almazo et al. 2008). In order to increase resolution among small oligosaccharides, a digestion sample from the wild-type protein was run in the aminated column using acetonitrile:water (80:20) at a flow rate of 1 mL/min.

Alcoholysis reactions

16 U/mL of each enzyme were incubated at 85 ℃ in a Thermomixer Compact (Eppendorf) with a suspension of 6% starch – and either 20% methanol in 50 mM Tris-bis-HCl, 150 mM NaCl, 2 mM $CaCl_2$ buffer pH 7 or a solution containing 8% of butanol saturated with the same buffer. After 42 hours of incubation, reaction products were visualized by TLC and determination of the methanolysis and butanolysis products was carried out in a reversed-phase HPLC equipped with a C18 column and a Nova-Pak aminated column, respectively, under conditions described above. Quantification of methyl and butyl glycosides was made by the hydrolysis of the total products by *Aspergillus niger* glucoamylase (43 U/mL) (Sigma-Aldrich) to transform all the alkyl glycosides in alkyl glucoside and glucose; the peak areas of the hydrolyzed products were compared against those of a standard curve containing known amounts of methyl glucoside and butyl glucoside (Sigma-Aldrich). Alcoholysis yields were determined as reported previously (Damian-Almazo et al. 2008).

Results

Specific activity

The specific activity of the wild-type AmyA purified in our hands in 50 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$ buffer pH 7 at 85 ℃ was of 1200 U/mg of protein. This value is in the same order of magnitude but lower than previously reported by Liebl et al. (1997). The fact that we used a slightly higher temperature (85 ℃, instead of 80 ℃) may account for the difference. The mutant F260W showed about half the specific activity of the wild-type enzyme (550 U/mg of protein) and the double mutant H222Q/F260W showed an intermediate value of 730 U/mg of protein. Mutant H222Q had a specific activity very close to that of the wild-type enzyme (1050 U/mg); while mutant F260G had about one third (300 U/mg). We can conclude from these results that none of the mutations had a significant effect on the activity of the enzyme.

Product profile

Figure 1 shows the product profile of the soluble starch digestion obtained for each of the variants. The wild-type is a saccharifying enzyme that yields mainly glucose and maltose. However, the presence of a transglycosylation activity different from α-1,4 gives place to the accumulation of some products different from G1–G7 according to their retention factor by TLC (Fig. 1a) and their elution time by reversed phase HPLC (Fig. 1b). Although the mutants present practically the same product profile as the wild-type enzyme, subtle differences are visible by TLC. H222Q, F260G and H222Q/F260W double mutant show a light spot with a mobility close to G5. When these digestions are analyzed by HPLC, there is a small peak that has an elution time slightly longer than G4 when the products are run in an aminated column (Fig. 1c), and closer to the G5 peak when run in the C18 column (Fig. 1b). To judge by its abundance in the samples this peak must correspond to the spot observed in H222Q, F260G and H222Q/F260W double mutant around G5 by TLC. The fact that its elution pattern, when methods based on different physico-chemical properties are used, is different, suggests that this compound is not G5. A similar observation can be done for the strong spot, observed for all samples, close to the spot corresponding to G4 but with a lower mobility (Fig. 1a). When the samples are separated by HPLC (Figs 1b,c) the peak that should correspond to this spot in TLC (judged by is abundance) leaves right after the G3 peak in both columns. If G3 or some compound with similar mobility is present in small amounts as might be suggested by TLC, these bands may not be resolved by HPLC under these conditions. In fact, some of the samples, like wild-type (WT), F260W and the F260W-H222Q double mutant seem to show a small shoulder right before this peak by reversed-phase HPLC (Fig. 1b) that could correspond to the spot near G3 observed by TLC. To verify if these peaks are indeed different from the oligosaccharides expected, the digestion carried out by the wild-type enzyme was run under different conditions to increase the resolution. As can be observed in Figure 1d, a peak near the maltose peak, but with longer retention time is present. Maltotriose seems to be absent in the sample, while other species are observed with longer retention times. These results suggest that the main and probably the only product of hydrolysis observed for these enzymes is glucose, and the rest of products accumulated are indeed products of transglycosylation different from α-1,4. This point is under investigation and will be further discussed in the next section. Another difference worth to highlight is the small but clear difference in the G1/G2 ratio obtained, i.e., what could be defined as saccharification power. Samples were incubated for long time to assure that equilibrium was reached. Although, stability of the enzymes under these conditions has been tested in the past only for the wild-type and H222Q mutant (Damian-Almazo et al. 2008), the rather similar equilibrium pattern obtained for the F260W mutant suggests that the mutant was active under these conditions. Mutants H222Q and F260G leave small but higher amounts of oligosaccharides at the expense of G1, while the wild-type and F260W mutant present higher proportion of G1 (Table 1). The difference can be due to a higher hydrolysis/transglycosylation ratio for these two enzymes. Interestingly, the double mutant H222Q/F260W reverses

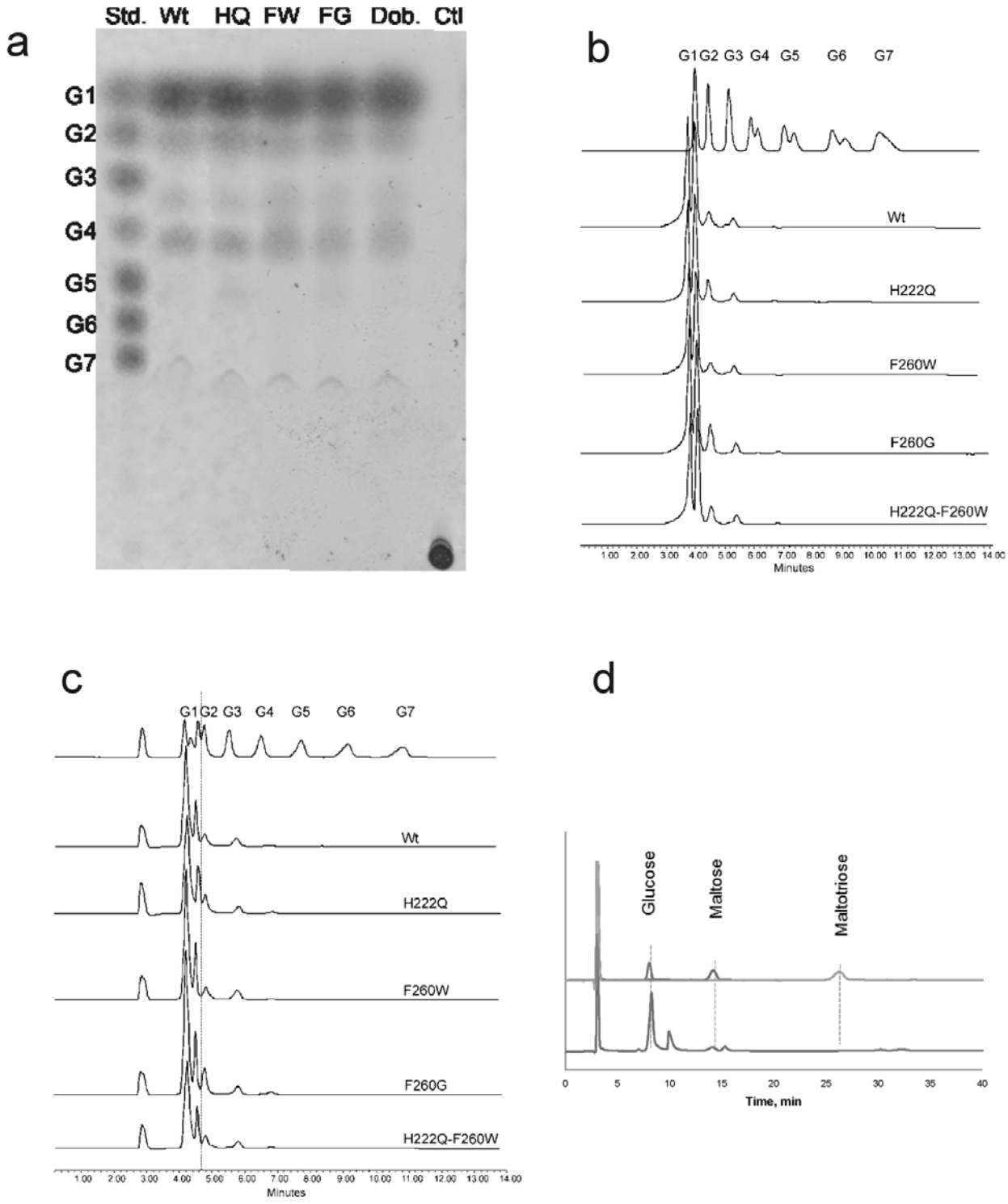

Fig. 1. (a) TLC product profile of hydrolysis reaction of a 30 mg/mL starch solutions with 16 U/mL of the wild-type (Wt) and the H222Q (HQ), F260W (FW), F260G (FG) and H222Q-F260W (Dob.) α-amylases mutants from *T. maritima* after 42 hours of reaction at 85 °C; as control (Ctl) a suspension of starch 30 mg/mL incubated without enzyme was used and a mixure of 5 mg/mL of glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7) was used as standard (Std.). Products profile of the hydrolysis reactions carried out by the enzymes were compared against standard curves from glucose (G1) to maltoheptaose (G7) by HPLC equipped (b) with a C18 column, (c) and (d) with a Nova-Pak aminated column, using a mixture of acetonitrile:water 68:32, and 80:20, respectively. In (c), hydrolysis products obtained with the different variants are shown as indicated; in (d), upper chromatogram are oligosaccharide standards (G1 to G3) and lower chromatogram is the product profile obtained with the wild-type enzyme.

the more transglycosylic pattern of the single mutation H222Q, eliminating or reducing the presence of longer oligosaccharides.

Alcoholysis reactions

Being alcoholysis a special case of transfer reaction, we decided to test the alcoholytic behavior of the mutants.

Table 1. Quantification of starch hydrolysis products by the different mutants.

Enzyme	G1 (mg/mL)	G2 (mg/mL)	G3 (mg/mL)	G1/G2	G1/(G1+G2+G3)
Wild-type	18.6	4.7	0.4	3.95	0.78
H222Q	18.1	5.3	2.1	3.41	0.71
F260W	18.8	4.2	0.5	4.48	0.80
F260G	20.1	7.2	2.8	2.79	0.67
H222Q-F260W	19.9	5.0	0.3	3.98	0.79

Table 2. Glucose and butyl glucoside quantified after butanolysis reactions carried out by the wild-type and mutant variants of AmyA.

Enzyme	Before glucoamylase treatment		After glucoamylase treatment		
	Glucose (mg/mL)	Butyl glucoside (mg/mL)	Glucose (mg/mL)	Butyl glucoside (mg/mL)	Increase of butyl glucoside[a] (%)
Wild-type	7.5	4.0	43.7	4.6	15.2
H222Q	2.3	4.4	29.6	5.8	31.8
F260W	10.1	3.2	24.2	3.8	18.8
H222Q-F260W	12.6	5.0	27.6	5.7	13.1

[a] Increase of glucose and alkyl glucoside due to the hydrolysis of longer butyl glycoside products upon glucoamylase treatment.

We previously demonstrated that AmyA was able of transferring the covalently bound saccharide to alcohols, and that mutant H222Q had an increased alcoholytic activity (Damian-Almazo et al. 2008). Figure 2 shows the chromatograms of the methanolysis (Fig. 2a) and butanolysis (Fig. 2b) reactions. The only methanolysis product that can be resolved from the rest of products is the methyl glucoside, therefore the clear differences are the higher methyl glucoside/glucose ratio in the H222Q mutant relative to the rest of variants and that the mutant F260W has not only a lower alcoholysis/hydrolysis ratio, but it shows a more saccharifying behavior, as can be observed by the larger peak of glucose obtained (Fig. 2a).

The products of butanolysis are better resolved by HPLC as can be observed in Figure 2b. The presence of longer products may reflect either the lower stability of the enzymes to butanol or an increase in the transglycosylation activity in the presence of butanol. As with respect to the product profile of the reaction, the pattern observed is very similar to methanolysis, being the H222Q the most alcoholytic variant followed by the H222Q/F260W double mutant, with a more saccharifying behavior giving the highest amount of glucose (see Table 2). The effect of this mutation in the context of the H222Q mutant does not change the alcoholytic activity of the single mutant, but changes the final product profile, so that the double mutant produces mainly butyl glucoside, reducing the amount of longer butyl glucosides. This is also reflected by the lower increase of butyl glucoside in all the variants containing the F260W mutation upon digestion of the final alcoholysis product with glucoamylase (see Table 2). Note in the chromatogram of the H222Q variant, the presence of three peaks before the glucose peak, presumably, butyl glucoside, butyl maltoside and butyl matotrioside (Fig. 2b). In contrast, the rest of variants show only the first two peaks, and the second in con-

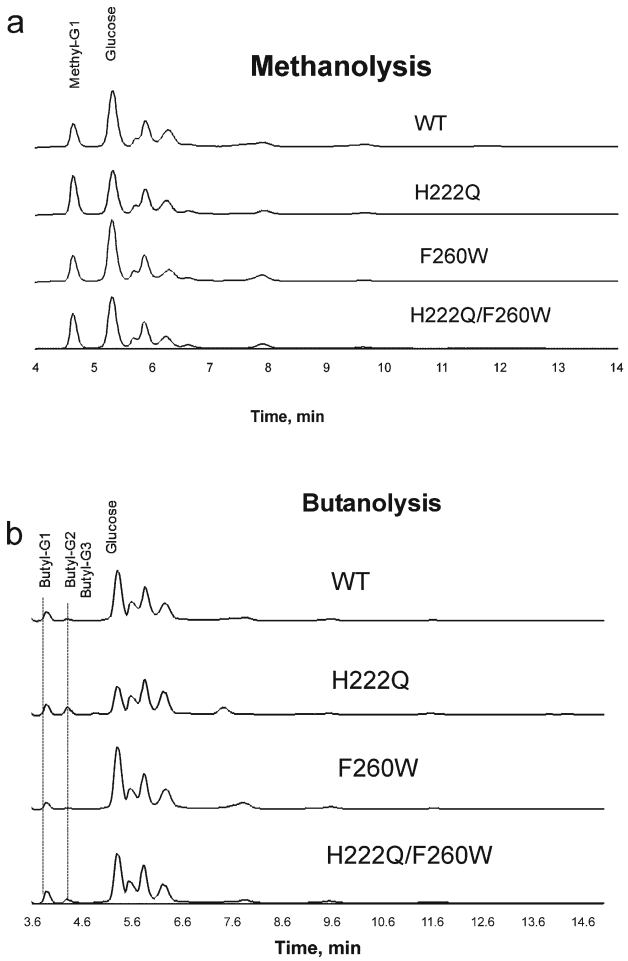

Fig. 2. HPLC profile products of alcoholysis reaction obtained from (a) 6% starch – 20% methanol reaction medium and (b) from 6% starch – 8% butanol solution with 16 U/mL of wild-type (WT) and mutants (H222Q, F260W, H222Q/F260W) after 42 hours of incubation at 85 °C before glucoamylase treatment. Analysis was carried out by HPLC equipped with a Nova-Pak aminated column. The main products, methyl glucoside, butyl glycosides and glucose are indicated.

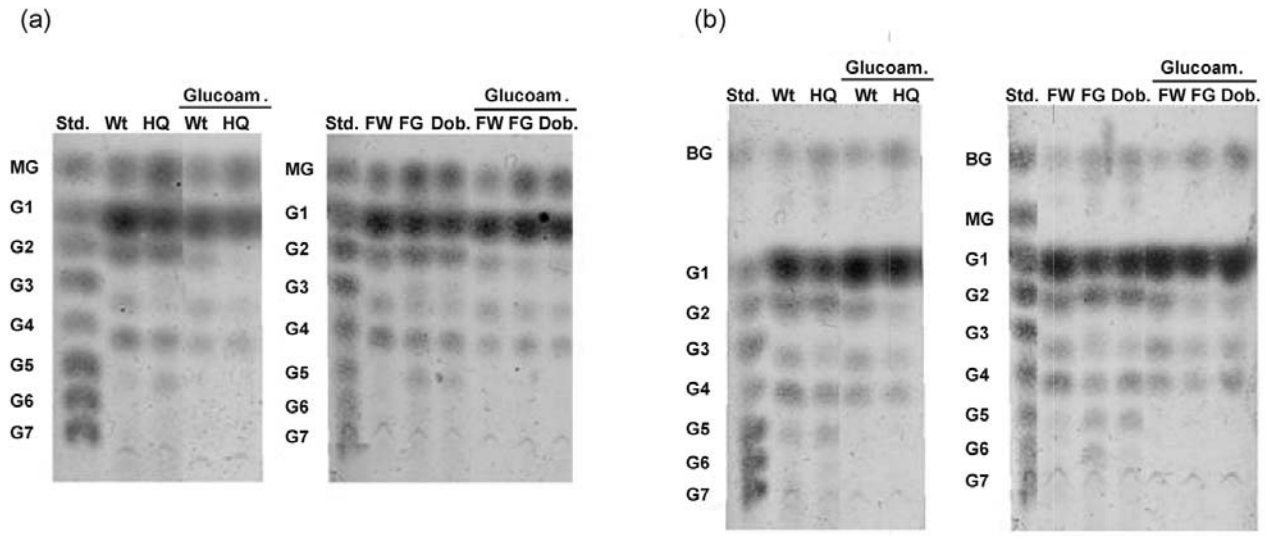

Fig. 3. TLC product profile of alcoholysis reaction obtained from (a) 6% starch – 20% methanol reaction medium and (b) from 6% starch – 8% of butanol solution after 42 hours of incubation at 85 °C with 16 U/mL of wild-type (Wt) and H222Q (HQ), F260W (FW), F260G (FG) and double mutant H222Q-F260W (Dob.) mutant enzymes before and after *A. niger* glucoamylase (Glucoam.) treatment. Methyl glucoside (MG), butyl glucoside (BG), glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7) were used as standards.

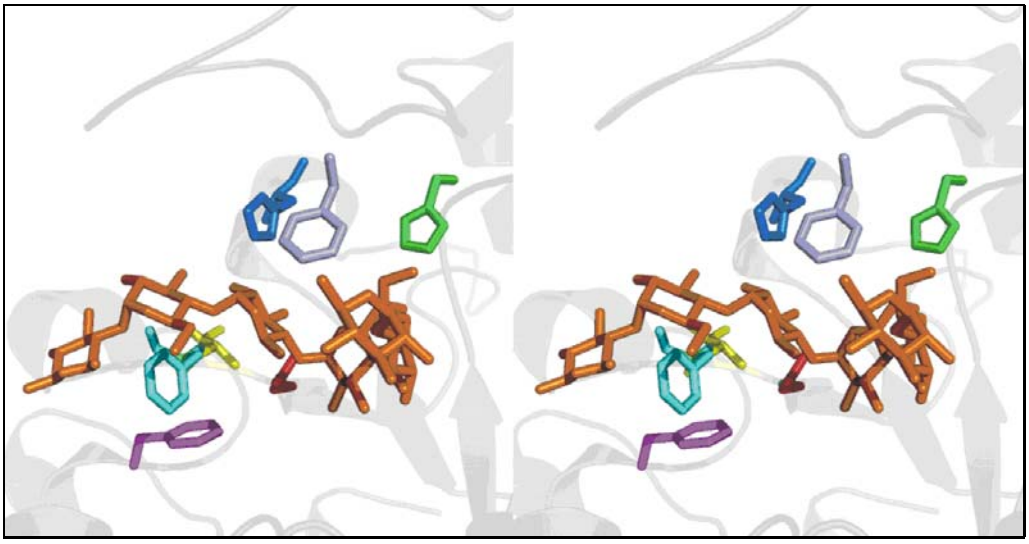

Fig. 4. Stereoscopic view of the homology model obtained for AmyA active site. The acarbose inhibitor (orange) is located between domains A and B, and is surrounded by various residues located within 4 Å, among them the catalytic residue Glu258 (red). Some of the residues involved in substrate binding are indicated by stick format; among them the amino acids subjected to site-directed mutagenesis, His222 (blue navy), and Phe260 (cyan). Also shown are Val259 (yellow), Phe277 (purple), and His128 (green).

siderably lower amount than obtained with the H222Q mutant. The same results are visualized by TLC. Figure 3a shows the methanolysis reaction and Figure 3b shows the butanolysis one. These figures also show the product profile after treatment of the alcoholysis reactions with glucoamylase. It is noteworthy that some of the longer products observed are not digested by glucoamylase, in agreement with the previous suggestion that these are the result of transglycosylation reactions different from α-1,4. Thus, in the butanolysis with F260W mutant, the presumably G2 spot seems to have lower mobility than the corresponding spot observed for the F260G and H222Q/F260W double mutant reaction products. It becomes apparent that this is a different species since the spot remains after the glucoamylase treatment. In contrast, the equivalent spot obtained with the H222Q variant disappears, and only traces are observed after the glucoamylase digestion for the F260G and H222Q/F260W mutants.

Discussion

In the present study we investigated the role of the Phe260 in defining the product profile of AmyA. This residue located two positions after the acid/base Glu258 is in the loop following β-strand 4 and is part of the third highly conserved region in α-amylases (Friedberg 1983; Rogers 1985; Nakajima et al. 1986; Svensson

Enzyme	Region I		Region II		Region III		Region IV			
			*		*			*		
T. maritima	KVIMDLVINHT	129	DGFRIDAAKHI	223	VGEVFSGN	263	FNFALM	280	FLENHDL 311	
A. oryzae	YLMVDVVANHM	123	DGLRIDTVKHV	211	IGEVLDGD	235	LNYPIY	255	FVENHDN 298	
A. niger	YLMVDVVPDHM	123	DGLRIDSVLEV	211	VGEIDNGN	235	LNYPIY	255	FIENHDN 298	
H. sapiens sal	RIYVDAVINHM	102	AGFRIDASKHM	202	YQEVIDLG	238	TEFKYG	259	FVDNHDN 301	
H. sapiens pan	RIYVDAVINHM	102	AGFRLDASKHM	202	YQEVIDLG	238	TEFKYG	259	FVDNHDN 301	
Pig Panc	RIYVDAVINHM	102	AGFRIDASKHM	202	FQEVIDLG	238	TEFKYG	259	FVDNHDN 301	
Tenebrio	RIYVDAVINHM	100	AGFRVDAAKHM	190	YQEVIDLG	227	LEFQFG	248	FVDNHDN 288	α-Amylases
Alteromonas	DIYVDTLINHM	90	KGFRFDASKHV	179	FQEVIDQG	205	TEFKYS	226	FVDNHDN 265	
Barley	KAIADIVINHR	93	DGWRFDFAKGY	184	VAEIWTSL	209	FDFTTK	249	FVDNHDT 286	
B. lichen	NVYGDVVINHK	106	DGFRLDAVKHI	236	VAEYWQND	266	FDVPLH	289	FVDNHDT 329	
B. amylo	QVYGDVVLNHK	104	DGFRIDAAKHI	236	VAEYWQNN	266	FDVPLH	289	FVDNHDT 329	
B. stearo	QVYADVVFDHK	107	DGFRLDAVKHI	239	VGEYWSYD	269	FDAPLH	292	FVDNHDT 332	
B. sub	KVIVDAVINHT	103	DGFRFDAAKHI	180	YGEILQDS	213	TASNYG	232	WVESHDT 270	
B. circ1	KVIIDFAPNHT	141	DGIRMDAVKHM	234	FGEWFLGV	262	LDFRFA	286	FIDNHDM 329	
B. circ2	KIVIDFAPNHT	141	DGIRVDAVKHM	234	FGEWFLGS	262	LDFRFN	286	FIDNHDM 329	
B. specie	KVIIDFAPNHT	141	DGIRVDAVKNM	234	FGEWFLGV	262	LDFRFA	286	FIDNHDM 329	CGTases
B. stearo	KVIIDFAPNHT	137	DGIRMDAVKHM	230	FGEWFLSE	257	LDFRFG	281	FIDNHDM 325	
Termo sulfu	KVIIDFAPNHT	142	DGIRLDAVKHM	235	FGEWFLGT	263	LDFRFS	283	FIDNHDM 330	
Thermus sp	RVMLDAVFNHC	248	DGWRLDVANEI	233	LGEIWHDA	362	MNYPLA	380	LLGSHDT 422	
B. lichen	KIMLDAVFNHI	252	DGWRLDVANEV	334	LGEIWHQA	363	MNYPFT	381	LLDSHDT 426	Maltogenic
B. stearo	RVMLDAVFNHS	248	DGWRLDVANEV	333	LGEIWHDA	361	MNYPFT	380	LLGSHDT 425	

Fig. 5. Multiple sequence/structure alignment around the four highly conserved regions observed in members of the glycoside hydrolase family 13. The catalytic residues conserved in all the sequences are marked with asterisks. Aromatic residues involved in transglycosylation activity and the residues that structurally interact with them are highlighted in grey; His222 is underlined. α-Amylases: T. maritima, *Thermotoga maritima* (GenBank accession number: CAA72194); A. oryzae, *Aspergillus oryzae* (PDB code: 2TAA); A. niger, *Aspergillus niger* (PDB: 2AAA); H. sapiens sal, *Homo sapiens*, saliva (PDB: 1JXK); H. sapiens pan, *Homo sapiens*, pancreas (PDB: 1HNY); Pig panc, *Sus scrofa*, pancreas (PDB: 1HX0); Tenebrio, *Tenebrio molitor* (PDB: 1JAE); Alteromonas, *Pseudoalteromonas haloplanktis* (PDB: 1G94); Barley, *Hordeun vulgare* (PDB: 1AMY); B. lichen, *Bacillus licheniformis* (PDB: 1VJS); B. amylo, *Bacillus amyloliquefaciens*, chimera (PDB: 1E43); B. stearo, *Bacillus stearothermophilus* (PDB: 1HVX); B. sub, *Bacillus subtilis* 2633 (PDB: 1BAG). Cyclodextrin glucanotransferases (CGTases): B. circ1, *Bacillus circulans* 251 (PDB: 1CDG); B. circ2, *Bacillus circulans* 8 (PDB: 1CGT); B. specie, *Bacillus* sp. 1011 (PDB: 1D7F); B. stearo, *Bacillus stearothermophilus* (PDB: 1CYG); Termo sulfu, *Thermoanaerobacter thermosulfurogenes* (PDB: 1A46). Maltogenic amylases (Maltogenic): Thermus sp, *Thermus* sp. IM6501 (PDB: 1SMA); B. lichen, *Bacillus licheniformis* (GenBank: CAA47612); B. stearo, *Bacillus stearothermopilus* (GenBank: AAC46346).

1988). Figure 4 shows a generated model of the active site constellation of this protein (Damian-Almazo et al. 2008). In most of saccharifying α-amylases the position equivalent to Phe260 is occupied by an aliphatic amino acid, while in bacillary α-amylases (liquefying) by tryptophan, as can be observed in the sequence alignment (Fig. 5). Interestingly, CGTases have the dipeptide Trp-Phe highly conserved signature next to the catalytic glutamate residue (Janecek et al. 1995). The role of the phenylalanine residue in cyclization has been demonstrated by site-directed mutagenesis (van der Veen et al. 2001). We have previously mutated the Val259 residue to tryptophan to make a CGTase-like signature, however, the resultant mutant had a considerably reduced activity and stability (Damian-Almazo et al. 2008), so it was not further characterized.

In the present work we mutated Phe260 to tryptophan, this would turn into a maltogenic amylase-like signature. The resultant enzyme increased the production of G1, yielding a more saccharifying enzyme. In the context of the mutant H222Q, a more alcoholytic and apparently more transglycosylic enzyme, this mutation changed the product profile to get higher amounts of the glucosyl derivatives. Interestingly, the F260G mutation had the opposite effect, an increase in transglycosylation products was observed, as well as in alkyl glucosides when tested for alcoholysis. Phe260 is part of the subsite +2. When the equivalent position was mutated in CGTases, the resultant enzymes became more hydrolytic and less transglycosylic, apparently by reducing the hydrophobicity of the active site (van der Veen et al. 2001). Our results suggest that AmyA presents not only α-1,4 transglycosylation, but also other kinds of transglycosylation, probably α-1,3 and α-1,6 that give rise to products non-digested by glucoamylase. The nature of these products is under investigation. We found that mutations at positions 222 and 260 alter this/these activity(ies). This phenomenon is not exclusive of AmyA; it has been detected that *Thermus* maltogenic amylase also possesses these activities and they are considerably reduced when Glu332 (equivalent to His222 in AmyA) is replaced with histidine or glutamine (Kim et al. 2000). The interpretation to these results was that upon mutation of Glu322 to glutamine, the enzyme increased the hydrolysis/transglycosylation ratio for α-1,3 and α-1,6 bonds, so that the corresponding transglycosylation products are not accumulated at equilibrium. These results are in good agreement with the results obtained with the H222Q AmyA mutant, for which a null or considerably reduced accumulation of some transglycosylation products is observed after alcoholysis and treatment with glucoamylase. Interestingly, the mutation F260G had the same effect as H222Q mutant. In contrast, F260W mutant seems to favor the accumulation of other transglycosylation products at the same time than being more saccharifying when acting upon α-1,4 bonds.

We have proposed in the past that the presence of

transglycosylation activity in α-amylases favors a more saccharifying pattern (Saab-Rincon et al. 1999). In fact, the final product profile depends on the number of subsites of the enzyme (Robyt & French 1967; Brzozowski et al. 2000) and their relative affinities for substrate (Allen & Thoma 1976; Kondo et al. 1980). However, we have observed that in those cases where the affinity of subsites favor the production of medium-size oligosaccharide, a change in transglyosylation/hydrolysis ratio is usually associated to a change in the product profile reached at equilibrium towards a more saccharifying pattern (Saab-Rincon et al. 1999). This makes sense if we consider that transglycosylation activity allows the recycle of products that other way would not be cleaved anymore, regenerating better substrates for the enzyme that can be processed further until accumulating only small-size oligosaccharides that are not bound anymore by the enzyme.

Generally transglycosylation reactions catalyzed by α-amylases are unfavorable under normal conditions. To be observed, they require high concentration of substrates and a decreased water activity. In fact, these two activities can be competing all the time and the speed of one relative to the other determines the products observed at equilibrium. By the end of the reaction, when small oligosaccharides, that are not good substrates for hydrolysis reactions, are the predominant species, transglycosylation reactions start being important. AmyA has an important transglycosylic activity, however its α-1,4 hydrolytic activity seems to be higher, and then an important accumulation of transglycosylation products is not observed. On the other hand, the other transglycosylic activities present in the enzyme seem to be faster than their hydrolysis, giving rise to the accumulation of the resultant transglycosylation products. These activities can be modulated by mutating residues His222 to glutamine and Phe260 to glycine. Kim et al. (2000) proposed that a charged residue at position 322 in *Thermus* maltogenic amylase allows the positioning and orientation of the acceptor saccharide for transglycosylation. Substitution of the charged residue thus decreases the speed of trasglycosylation reactions. The behavior observed for F260G mutation of the AmyA can be explained using the same arguments. Aromatic residues interact favorably with the sugar moieties through stacking interactions. The replacement of phenylalanine by glycine may remove the necessary interactions for positioning and orienting a sugar acceptor at subsite +2, reducing the transglycosylation reaction rates as it has also been observed for CGTase (van der Veen et al. 2001), at least with acceptors larger than G2. The opposite effect, observed for the F260W mutant, strongly supports our hypothesis. But it is also possible that the introduction of a bulkier side-chain at subsite +2 restricts the entrance not only of acceptor saccharides during the second part of the reaction, but also of the initial substrate, i.e., subsite +2 may have a lessened affinity for the substrate, and this may be the reason of a higher glucose yield for the variants containing this mutation.

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