FEBS Letters 453 (1999) 100–106 FEBS 22128

Introducing transglycosylation activity in a liquefying α-amylase

Gloria Saab-Rincón[1], Gabriel del-Río[1,2], Rosa I. Santamaría, Agustín López-Munguía, Xavier Soberón*

Instituto de Biotecnología, UNAM, Apartado Postal 510-3, Cuernavaca, Morelos 62271, Mexico

Received 8 March 1999; received in revised form 5 May 1999

Abstract By mutating Ala-289 by Phe or Tyr in the *Bacillus stearothermophilus* α-amylase, we induced this enzyme to perform alcoholytic reactions, a function not present in the wild-type enzyme. This residue was selected from homology analysis with neopullulanase, where the residue has been implicated in the control of transglycosylation [Kuriki et al. (1996) J. Biol. Chem. 271, 17321–17329]. We made some inferences about the importance of electrostatic and geometrical modifications in the active site environment of the amylase to explain the behavior of the modified enzyme.

Key words: α-Amylase; Transglycosylation; Hydrolysis; Site-directed mutagenesis; Reaction mechanism

1. Introduction

α-Amylases (α-1,4-glucan-4-glucanhydrolase, EC 3.2.1.1) are widely occurring monomeric enzymes that catalyze the hydrolysis of internal α-(1,4) glucosidic linkages in starch releasing α-anomeric products (therefore also designated as retaining) [1]. The α-amylase family, including cyclodextrin glycosyl transferases (CGTases) and neopullulanase, has a $(\beta/\alpha)_8$ barrel, also known as TIM barrel, that functions as the catalytic domain, both features shared by a large number of enzymes [3,4]. This domain harbors four highly conserved regions in the α-amylase family, in which two aspartic and one glutamic acid residues (i.e. Asp-206*, Asp-297* and Glu-230*, TAA numbering[3]) are involved in the acid-base catalytic mechanism of reaction [5,6]. A widely accepted classification of α-amylases groups them as saccharifying and liquefying depending on their mode of attack when reacting with starch: saccharifying amylases are α-amylases producing an increase in reducing power about twice that of the liquefying amylases [7]. Although all α-amylases act through a hydrolytic endo mechanism, saccharifying amylases catalyze α-1,4 transglycosylation in addition to the main reaction, the hydrolysis of α-1,4 glucosidic linkages [8]. Both types of α-amylases have been found in bacteria, but fungal amylases are mainly saccharifying [9].

It has been shown that many retaining glycosidases are able to transfer glycosyl residues to low molecular weight alcohols such as methanol, as well as to water, a property related to the transferase activity of the glycosidases. Furthermore, glycosidases such as β-galactosidase [10,11], α-glucosidase [12,13], β-xylosidase [14] and β-fructofuranosidase [15,16] have been studied in terms of their ability to perform alcoholysis reactions with high molecular weight insoluble alcohols. In the case of α-amylases few reports exist concerning alcoholysis reactions. We have recently reported a relationship between the ability to carry out alcoholysis and the classification of α-amylases as saccharifying or liquefying [32]. This is consistent with the fact that only amylases known as saccharifying have been successfully used in transferase reactions. Such is the case of α-amylase from *Aspergillus oryzae* in alcoholysis reactions with methanol, ethanol and *n*-butanol [17], in the presence of polyols [18] or the synthesis of oligosaccharides with α-amylase from *Thermoactinomyces vulgaris* R-47 [19]. However, little is known about which structural features of α-amylases are responsible for the transferase activity.

Kuriki and coworkers [2] have suggested that a series of three residues near the active site of the neopullulanase from *Bacillus stearothermophilus* are responsible for controlling the water activity in the active site. One of these residues, Tyr-377 (Tyr-252* in TAA numbering), was mutated to a polar, a charged and a non-polar amino acid residue. These mutations yielded a change in the transglycosylation/hydrolysis ratio consistent with non-polar mutations at this position favoring transglycosylation reactions. This was observed by the production of high molecular weight products after incubation with maltoheptaose.

If position 252* (TAA numbering, unless specified) is implicated in determining the transferase activity in the α-amylase family, mutating this residue in an α-amylase unable to carry out alcoholysis (a liquefying one) may introduce this

*Corresponding author. Fax: (52) (73) 17-2388.
E-mail: xavier@ibt.unam.mx

[1] These authors contributed equally to this work.
[2] Present address: Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037, USA.

Abbreviations: TAA, taka amylase or alpha-amylase from *Aspergillus oryzae*; ABST, α-amylase from *Bacillus stearothermophilus*; CGTase, cyclodextrin glycosyltransferase from *Bacillus circulans*; DNS, 3,5-dinitrosalicylic acid; MES, 2-(*N*-morpholino) ethanesulfonic acid; MOPS, 3′-(*N*-porpholino) propanesulfonic acid; G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; PCR, polymerase chain reaction; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PDB, Protein Databank

[3] Number residues followed by * indicate that taka amylase numbering is being used, otherwise the numbers correspond to ABST sequence unless specified.

PII: S0014-5793(99)00671-7

property. We mutated the α-amylase from *B. stearothermophilus* (ABST) at residue Ala-289 (homologous position to Tyr-252 in TAA; 252* from now on) to Phe and to Tyr. We observed that both mutations produced an enzyme with alcoholytic capabilities, indicating that this position is implicated in the control of the transferase activity in the ABST. These mutants showed an altered pH profile, suggesting that an alteration of the electrostatic environment at the active site may be related to the transition of a hydrolytic amylase to a transferase amylase.

2. Materials and methods

2.1. Sequence alignment

A multiple sequence alignment including 49 sequences of α-amylases and 22 of cyclodextrin glycosyltransferases was obtained from the FSSP+HSSP database, and was kindly provided by Dr. Liisa Holm. This alignment is based on the three-dimensional structures of six α-amylases and three cyclodextrin glycosyltransferases, as previously described [20].

2.2. Site-directed mutagenesis and enzyme purification

The α-amylase gene of *B. stearothermophilus* ATCC 12980 (ABST) was isolated by PCR amplification from genomic DNA with the following oligonucleotides: 5′-CGG-GCG-AGA-TCT-AGA-AGG-AGT-TAA-ATA-TTA-TGC-TAA-CGT-TTC-ACC-GC-3′, and 5′-CCC-CGG-AAG-CTT-GGA-TGG-GCG-CCT-TGT-G-3′.

The strain was obtained from the American Type Culture Collection (ATCC) directly. The oligonucleotide sequences were based on the upstream (oligonucleotide 1) and downstream (oligonucleotide 2) regions of the ABST sequence previously reported [21]. The PCR product obtained was cloned into the plasmid pGBS18 [22] and sequenced. The amylase gene was expressed in *Escherichia coli* JM101.

Mutations at position 289 to Phe and Tyr were introduced by means of PCR, as previously described [23], using the oligonucleotides 5′-GTG-TAA-CGG-GAA-ATC-AAA-CAA-AAG-AC-3′, and 5′-GTG-TAA-CGG-GTA-ATC-AAA-CAA-AAG-AC-3′, respectively.

The wild-type and mutant enzymes were purified following a protocol previously described [24]. All proteins were purified to more than 98% homogeneity as determined by SDS-PAGE.

2.3. Other enzyme used

The glucoamylase from *Aspergillus niger*, used in the determination of methyl-glucoside, was obtained from Lakeside-Boehringer (USA), 99% purity.

2.4. Enzyme activity estimation and α-amylase product analysis

The depolymerization of starch (soluble starch from Sigma Aldrich, USA) was followed by the formation of reducing sugars by the method of 3,5-dinitrosalicylic acid [25]. A unit of enzyme activity is expressed as the number of μmol of glucose released per min by 1 mg of α-amylase. The reaction was carried out in 50 mM MOPS, 50 mM MES buffer at pH 5.8. All these reactions were carried out at 60°C for α-amylases, and 40°C for glucoamylase. For α-amylase reactions, 10 mM $CaCl_2$ was included in the reaction medium.

2.5. Alcoholysis of starch by α-amylases

Reactions of alcoholysis with starch and methanol were conducted at pH 6.0 (50 mM acetate buffer, 30 mM $CaCl_2$) and 60°C, using 3% starch and 40% methanol. The reactions were followed for 5 h, and samples were analyzed at 0, 0.5, 1, and 5 h of reaction. Methyl-glycosides were visualized with TLC, for which 10 cm × 10 cm × 200 μm Whatman Silica gel 60 plates were used, with butanol, ethanol and water (3:5:2) as solvent. The plates were developed by spraying α-naphthol and sulfuric acid in ethanol and heating at 100°C for 3 min. This method has a detection limit of 0.04 μg of methyl-glucoside. In order to quantify the degree of alcoholysis (since we do not have standards for the different methyl-glycosides), the reactions were stopped by addition of 2 M NaOH and exposed to glucoamylase action for 2 h to convert any methyl-saccharide to methyl-glucoside. Then, methyl-glucoside present in the reaction medium was quantified using a Waters 510 HPLC system with an automatic sampler model 717 Plus, a Waters column for carbohydrate analysis Nova-Pak aminated and an RI detector Waters model 410. A mixture of water: acetonitrile (70:30, v/v) was used as eluent, and methyl-glucoside (from Sigma, USA) was used as standard. Since the glucoamylase used had a slight contamination with α-amylase, the obtained values were corrected for the contribution of alcoholysis by the action of the amylase.

2.6. Transglycosylation of G7 by α-amylases

Transglycosylation reactions using 2 mM maltoheptaose were conducted with 0.04 units of activity at pH 5.8 and 60°C. The reaction was followed at 10 min, 3 and 6 h. Product analyses of α-amylase on maltoheptaose were visualized with TLC as described above with the proper standards.

2.7. Kinetic characterization

The initial velocity was plotted vs. starch concentration and Michaelis-Menten parameters K_m and k_{cat} were obtained for wild-type and mutant α-amylases from *B. stearothermophilus* by non-linear regression analysis using the program Kaleidagraph (Abelbeck software). The protein concentration in the solution was quantified using the Bradford method (Bio-Rad).

2.8. pH profiles

The activities of wild-type enzyme and A289F and A289Y mutants were determined using 10 mg/ml starch at 60°C and different pH values. The buffer used was a mixture of 50 mM MOPS, 50 mM MES, 10 mM $CaCl_2$ to have the whole range from 4.5 to 8.0 buffered. Triplicate determinations were performed. The amount of protein used varied according to the specific activity of each enzyme in order to have comparable activities in the plots. The values of activity in the plot correspond to the activity per tube of assay and are not normalized by the amount of enzyme used.

3. Results

3.1. Sequence- and structure-based analyses

In Fig. 1 the position equivalent to Tyr-252* (TAA) in a multiple sequence alignment of α-amylases and cyclodextrin glycosyltransferases (CGTases) is highlighted. It can be observed that depending on the nature of the enzyme a certain degree of correlation exists. Neopullulanase and CGTases, which are natural transferases, have Tyr or Phe residues in the homologous position, while bacterial α-amylases (usually liquefying amylases with no transferase activity) present small residues (Ala, Val, Ser), suggesting that this position could be important for transglycosylation. We infer that size and polarity of the side chain at this position may be the properties related to the transferase activity. It could then be predicted that TAA, as well as some plant and mammal α-amylases, that have Tyr or Phe residues at this position would be able to carry out transfer reactions. Indeed, this property has been reported for both TAA (fungal) [17] and human salivary α-amylases [26]. Hence, it would be expected that bacterial α-amylases, where a Tyr or Phe has not been observed at position 252*, would be unable to carry out transfer reactions.

In analyzing this alignment, we did not find the hydrophobicity pattern proposed by Kuriki and coworkers [2] to be related to the transferase activity. When superimposing the structures of α-amylases from a bacterium (*Bacillus licheniformis*) [27], a fungus (TAA) [28,29], and a CGTase (*Bacillus circulans*) [30,31], we found that the water accessibility to the active site is not altered whether a tyrosine (TAA), phenylalanine (CGTase), or a valine (bacillar amylase) residue is present at the entrance of the proposed pathway for water [2] at least in the ground state (Fig. 2A).

Based on these observations, we decided to test whether the presence of alternative side chains at residue 252* conferred

swiss	FSSP	no		A	T	T	F	D	F	T	T	K	G	I	L	N	V	A	
amya_aspor	2taa-A	1	247	D	G	V	L	N	Y	P	I	Y	Y	P	L	L	N	A	261
amy_bacli	1bpl-A	2	281	H	S	V	F	D	V	P	L	H	Y	Q	F	H	A	A	293
amy_bacam	1bpl-A	4	281	Q	S	V	F	D	V	P	L	H	F	N	L	Q	A	A	293
amt6_bacs7	1bpl-A	5	286	H	S	V	F	D	V	P	L	H	Y	N	L	Y	N	A	298
amy_bacst	1bpl-A	6	284	M	S	L	F	D	A	P	L	H	N	K	F	Y	T	A	296
amy2_salty	1bpl-A	9	285	T	M	L	F	D	A	P	L	Q	M	K	F	H	E	A	297
amy2_ecoli	1bpl-A	10	285	T	M	L	F	D	A	P	L	Q	M	K	F	H	E	A	297
cdgt_bacst	1cyg	1	274	M	S	L	L	D	F	R	F	G	Q	K	L	R	Q	V	288
amy_thetu	1cyg	2	279	M	S	L	L	D	F	R	F	S	Q	K	V	R	Q	V	293
amy_bacci	1cyg	3		M	S	L	L	D	F	R	F	S	Q	K	V	R	Q	V	
cdgu_bacci	1cyg	4	280	M	S	L	L	D	F	R	F	A	Q	K	V	R	Q	V	294
amyr_bacs8	1cyg	5	278	M	S	L	L	D	F	R	F	A	Q	K	V	R	Q	V	292
cdgt_bacsp	1cyg	6	278	M	S	L	L	D	F	R	F	A	Q	K	V	R	Q	V	292
cdgt_bacs0	1cyg	7	278	M	S	L	L	D	F	R	F	A	Q	K	A	R	Q	V	292
cdgt_bacci	1cyg	8	278	M	S	L	L	D	F	R	F	N	S	A	V	R	N	V	292
cdgt_bacoh	1cyg	9	271	M	S	L	L	D	F	Q	F	G	Q	T	I	R	D	V	285
cdgt_bacss	1cyg	10	278	M	S	L	L	D	F	R	F	N	S	A	V	R	N	V	292
cdgt_bacs3	1cyg	11	278	M	S	L	L	D	F	P	F	A	Q	K	A	R	Q	V	292
cdgt_bacli	1cyg	12	278	M	S	L	L	D	F	R	F	N	S	A	V	R	N	V	292
cdgt_bacs2	1cyg	13	271	M	S	L	L	D	F	Q	F	G	Q	T	I	R	N	V	285
cdg2_bacma	1cyg	14	279	M	H	L	L	D	F	A	F	A	Q	E	I	R	E	V	293
cdg1_bacma	1cyg	15	279	M	N	L	L	D	F	E	Y	A	Q	E	V	R	E	V	293
amym_bacst	1cyg	16	276	V	N	V	L	D	F	D	L	N	T	V	I	R	N	V	290
cdgt_klepn	1cyg	17	282	S	A	L	L	D	F	G	F	R	D	T	L	E	R	V	296
amyb_bacpo	1cyg	18	947	.	A	A	L	D	F	P	M	Y	Y	T	I	K	D	V	961
									d	252									

Fig. 1. Multiple sequence alignment of α-amylases. Regions of homology around Tyr252* positions in taka amylase are shown for representative α-amylases and CGTases. The Swiss-Prot name for each protein sequence is indicated, as well as the numbering of each sequence. Tyr-252 is shown in bold.

on bacterial α-amylases the ability to carry out transfer reactions in the context of ABST.

3.2. Transfer reactions by wild-type and mutant α-amylases

We conducted two types of experiments to determine the ability of our mutants to carry out transglycosylation reactions: alcoholysis with methanol and starch, and transglycosylation using G7 as substrate. It has already been observed that TAA and other glycosylases are able to carry out alcoholysis [17]. If an α-amylase is capable of transferring starch chains to methanol, this would be an indication that the enzyme is adapted also to carry out transference reactions besides hydrolysis. Thus, based on the capability of α-amylases to carry out alcoholysis reactions in methanol, we measured directly transferase activity through the formation of methylmaltosides [32]. A second approach was to use maltoheptaose as a substrate, and look for higher molecular weight oligosaccharide formation by TLC. The presence of G8 or longer oligosaccharides would indicate the ability of the enzyme to catalyze transglycosylation reactions.

The resulting products of alcoholysis reactions using the wild-type and mutant enzymes are shown in Fig. 3 and Table 1. It can be observed that both Ala289Tyr[4] and Ala289Phe mutants transfer oligosaccharides from starch to methanol, in agreement with the idea that this position is involved in controlling the transferase activity [2]. It is noteworthy that in Fig. 3 no spot corresponding to methyl maltoside or meth-

[4] The mutant proteins are named using the three letter amino acid code with the first three letters corresponding to the wild-type residue, the number indicating the position of the residue, and the second three letters corresponding to the mutation.

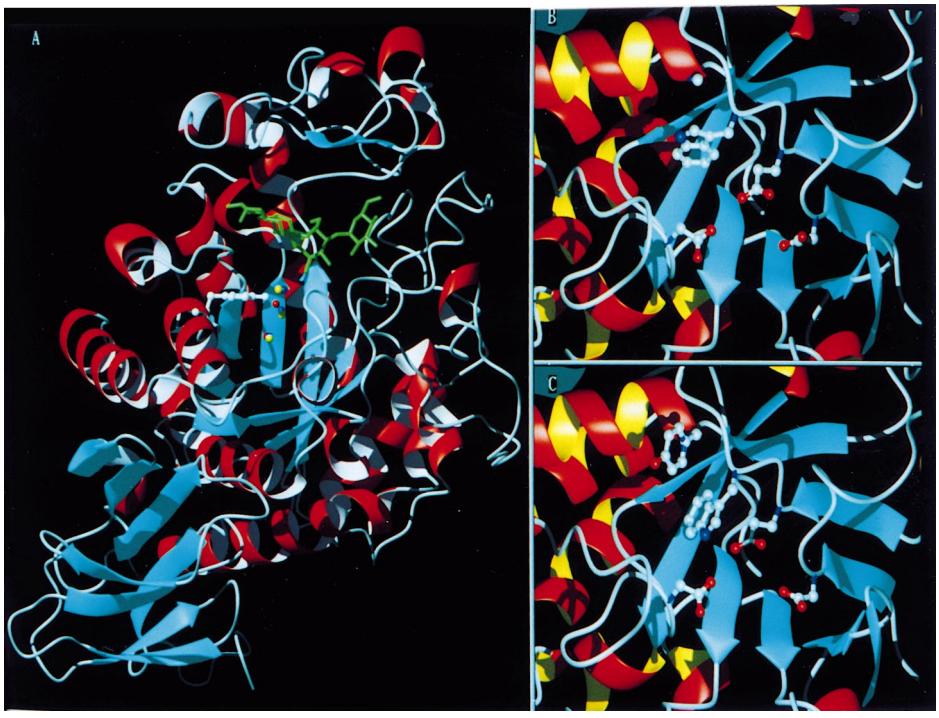

Fig. 2. A: Structural superposition of α-amylases and CGTases at the Tyr-252* position. Ribbon representation of taka amylase with residue Tyr-252 (white) and the inhibitor acarbose (green) in neon format. The proposed path of water, represented as balls, is shown for taka amylase (PDB name 6taa, yellow), the α-amylase from *B. licheniformis* (PDB name 1vjs, red) and CGTase from *B. circulans* (PDB name 1cxe, blue). B and C: Molecular graphic representation of close-up of models of α-amylases from *B. stearothermophilus* based on the structure of α-amylase from *B. licheniformis* (PDB name 1vjs). B: Wild-type enzyme. C: Ala289Tyr mutant, where residue alanine 289 was replaced by tyrosine in the sequence before running the program Modeller rel. 4 [34,35]. Residues 289, Asp-234, Glu-264, and Asp-331 and Trp-266 are indicated by ball and stick format. Figure prepared with the program MOLMOL [41] and rendered with POV-Ray program 'trademark'.

yl-glucoside is observed for the wild-type enzyme (detection limit 40 μg of methyl-glucoside). However, after the action of glucoamylase in the alcoholysis products of the wild-type enzyme, some methyl-glucoside is obtained due to the action of some contaminating α-amylase in the Sigma glucoamylase used. It can also be observed in Fig. 3 that both mutants produce glucose and maltose in larger amounts than the wild-type amylase. Finally, the results suggest that Ala289Tyr mutant is a more efficient transferase than Ala289Phe.

The hydrolysis products obtained from maltoheptaose using the wild-type and mutant enzymes are shown in Fig. 4. At short reaction times (10 min) transglycosylation products (oligosaccharides of molecular weight higher than the substrate) are observed for the mutants in higher amounts than for the wild-type enzyme. It is noteworthy that the substrate (lane 11) had some contamination with higher molecular weight oligosaccharides. Some differences in the cleavage pattern are also observed. The mutants show higher proportions of G1, G3, and G4 than the wild-type enzyme. These results are consistent with the idea that the inclusion of a transglycosylation activity would increase the efficiency of starch degradation to smaller maltosides [20].

We also determined the k_{cat} and K_m of the wild-type and mutant enzymes for starch. There were only moderate modifications in the K_m values for the mutants (see Table 1). In contrast the k_{cat} values were significantly decreased.

3.3. How does position 289 in B. stearothermophilus α-amylase promote a transferase activity?

From the sequence analysis (Fig. 1) we observed that size and polarity at position 252* are properties that may constitute an alternative to the hydrophobicity pattern observed by Kuriki and coworkers [2] to explain the behavior of the mutant at this position. Factors other than hydrophobicity, such

Table 1
Characterization of WT and mutant α-amylases

Enzyme	k_{cat}^{a} (μmol/min mg)	K_m^{b} (mg starch/ml)	Alcoholysis[c] (mg/ml MG)	Units used per assay[d]
WT	18 680 (1280)	3.4 (0.5)	0.46 (0.034)	173
A289F	5 230 (346)	2.0 (0.3)	0.52 (0.025)	172
A289Y	1 190 (81)	1.9 (0.4)	1.23 (0.028)	76

Errors are shown as the standard deviations and are in parentheses.
[a] k_{cat} = μmol dextrose equivalents per min per mg of protein.
[b] K_m = substrate (starch) dissociation constant.
[c] Alcoholysis = mg/ml of methyl-glucoside obtained by the action of glucoamylase after 5 h of alcoholysis reaction. The methyl-glucoside obtained for the wild-type enzyme is due to a contaminating α-amylase in the glucoamylase, since we did not observe any alcoholysis product for wild-type enzyme by TLC.
[d] Units of activities = amount of enzyme to produce 1 DE/min.

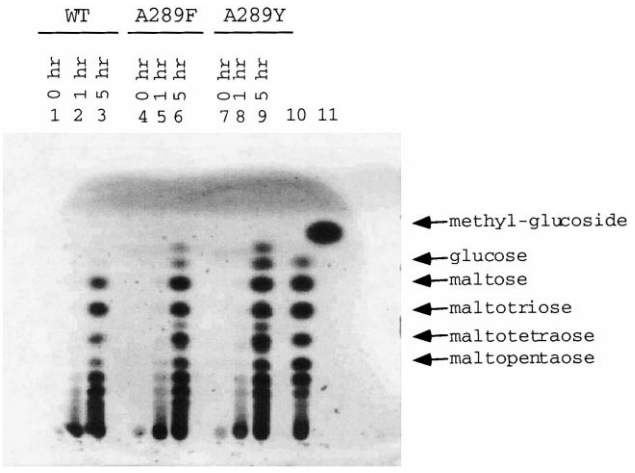

Fig. 3. Alcoholysis of starch by the wild-type α-amylase of *B. stearothermophilus* and its (Ala-252*) Ala-289 mutants. TLC plate showing the processing of 3% starch in 40% methanol, by wild-type (lanes 1–3), (Ala252*Phe) Ala289Phe mutant (lanes 4–6) and (Ala252*Tyr) Ala289Tyr mutants (lanes 7–9). The reaction times for each enzyme are 0, 1 and 5 h. Lanes 10 and 11 are the molecular markers: a mixture of oligosaccharides from glucose to maltohexose and methyl-glucoside, respectively.

as the geometry and electrostatic environment of the active site, also noted by Kuriki and coworkers [2], may be important. In this regard, the size of the residue at this position might enable it to reach and interact with some other residues at the active site, while the polarity can affect the ionization state of the catalytic residues. We explore these possibilities below, concluding that both effects play a role in changing the transglycosylation/hydrolysis ratio observed.

As far as the geometry of the active site is concerned, it has recently been observed that the homologous position of Tyr-252* in the α-amylase from barley can be involved in residue-residue interactions important for the orientation of the catalytic residue [33]. These interactions include residues that are not conserved among α-amylases, for instance Trp-266 (ABST numbering) (data not shown). In an attempt to understand the molecular effect of this mutation on the α-amylase

Table 2
pK_a values of WT and mutants

	WT[a]	A289F[a]	A289Y[b]
pK_a1	4.2 (0.09)	4.2 (0.14)	3.9 (0.05)
pK_a2	7.3 (0.08)	7.2 (0.11)	7.6 (0.36)
pK_a3	N/A	N/A	6.8 (0.24)

Errors are shown as one standard deviation and are in parentheses.
[a]Calculated from fitting the data to two ionizable forms.
[b]Calculated from fitting the data to three ionizable groups.

from *B. stearothermophilus* we built models for both the wild-type and Ala289Tyr mutant using the program Modeller, rel. 4 [34,35] and the structure of the α-amylase from *B. licheniformis* as a template [27]. These enzymes share 62% identity, providing a more reasonable model than that based on the structure of TAA whose identity is only 25% [36]. In Fig. 2B,C the interactions between Ala-289, Trp-266 and Glu-264 (252*, 230* and 232*, respectively) are indicated. It can be observed that placing a ring at position 252* would produce an interaction of this position with Trp-266 (equivalent to 206 in barley amylase), and indirectly with the catalytic Glu residue (230*). Hence, as proposed for the barley amylase [37], this network of interactions may have an effect on the geometry of the active site that can influence either the activation of the nucleophilic acceptor, or the effective concentration of less polar acceptors at the active site resulting in an increase of the transglycosylation/hydrolysis ratio.

The widely accepted mechanism for retaining α-amylases consists of a double displacement reaction involving a glycosyl-enzyme intermediate [5,38]. This intermediate can be nucleophilically attacked either by a water molecule (hydrolysis), by another glycosyl group (transglycosylation) or by an alcohol (alcoholysis). It is not clear what factors favor one reaction over the other. However, a change in the hydrophobicity and/or geometry of the active site can increase the effective concentration of less polar acceptors (like glycosyl groups or alcohols), by increasing the affinity of the acceptor site towards these groups, thus favoring these reactions. Additional electrostatic effects can also be involved in determining the

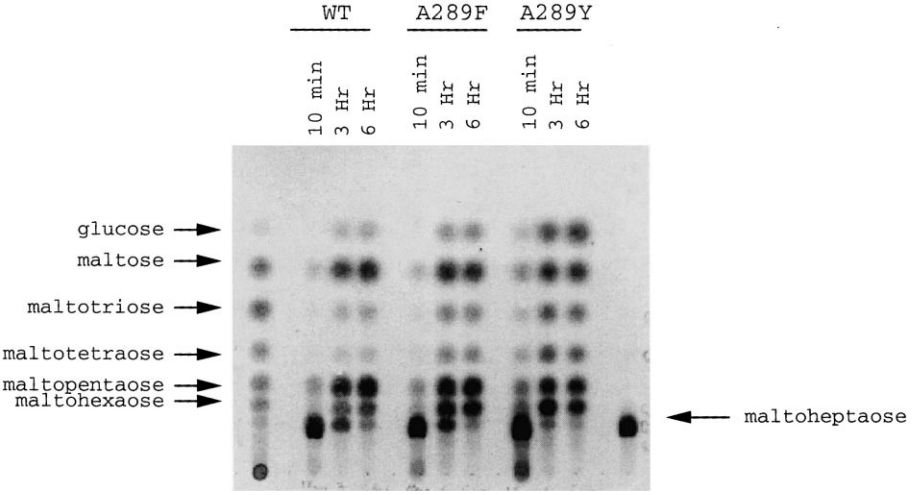

Fig. 4. Transglycosylation of G7 by wild-type α-amylase from *B. stearothermophilus* and mutants. The TLC plate shows the results of the action of 0.04 units of activity of each enzyme on a 2 mM solution of maltoheptaose at pH 6.0 and 60°C. Lanes 2–4, wild-type; lanes 5–8, Ala289Phe mutant; lanes 9–11, Ala289Tyr mutant at 10 min, 3 and 6 h, respectively; lane 1, mixture of oligosaccharides from G1 to G6; lane 12, 2 mM maltoheptaose.

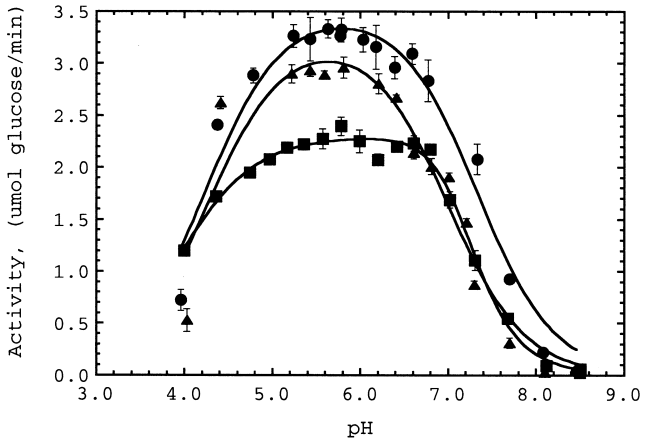

Fig. 5. pH profile of the activity of the wild-type α-amylase from *B. stearothermophilus* and mutants. Wild-type (filled circles), (Ala252*-Phe) Ala289Phe (filled triangles), and (Ala252*Tyr) Ala289Tyr (filled squares). The points are the average of three independent determinations and the bars represent the standard deviation of each determination. The lines represent the fit to a model of three ionizable groups for the (Ala252*Tyr) Ala289Tyr mutant, and to two ionizable groups for the wild-type and (Ala252*Phe) Ala289Phe mutant.

transglycosylation/hydrolysis ratio by modifying the ionic state of the general acid/base of the reaction (Glu-230*).

Since transglycosylation occurs when a non-polar and large amino acid residue (Tyr, Phe) is at position 252*, we decided to test whether such side chains specifically affect the electrostatic environment of the catalytic site. To accomplish this we determined the pH profile of activity of the mutants and the wild-type enzymes (Fig. 5). Although the experimental data obtained for the three enzymes fit very well to a kinetic model that assumes three ionization groups in the active site [39], only the data of the Ala252*Tyr mutant allows unequivocally the assignment of three pK_a values (see Table 2). Three ionizing groups may also exist for the wild-type and Ala252*Phe enzymes, but probably two of the pK_a values are too close to be distinguishable. In the case of the Ala252*Tyr mutant two of the pK_a values are shifted to lower values, increasing the separation among them and as a result, the precision in their determination.

Hence, the Ala289Tyr mutant alters substantially the pH profile, and interestingly, this is the mutant that showed more transferase activity (see Fig. 3). These results can explain the increased transferase activity of the Ala252*Tyr mutant as follows: during the second part of the reaction mechanism, the intermediate is nucleophilically attacked by a water molecule which is activated (deprotonated) by the carboxylate group of Glu-230*. If in the Ala252*Tyr mutant, Glu-230* has a lower pK_a, its ability to activate the hydrolytic water will be decreased. This, together with a higher effective concentration of other acceptor groups (glycosyl or alcohols), would result in an overall reduced catalytic activity but an increased transferase activity relative to hydrolysis, as is indeed observed.

From our results we can conclude that both the hydrophobic nature of the residue at position 252* and the electrostatic effects are important for the transglycosylation reaction. In agreement with these results, a Ser residue at position 252* in neopullulanase did not significantly reduce the transferase activity [2], while an Ala in the ABST does.

4. Discussion and conclusions

It was shown that mutations at the equivalent position to 252* induce transfer reactions in the liquefying α-amylase from *B. stearothermophilus*. This result indicates that the observed variation at this position in saccharifying and liquefying amylases correlates with the transferase activity. In this regard, it is interesting to note that our mutants are more saccharifying enzymes, since they produce more glucose and maltose than the wild-type enzyme.

Our results lend support to the hypothesis that not only hydrophobicity at position 252* is an important factor for transglycosylation reactions to occur, but also electrostatic interactions that may affect the geometry of side chains in the active site.

These observations suggest that the transferase activity can be further increased in the ABST with no drastic modification in the architecture or chemical nature of the catalytic residues, like in the case of subtilisin [40]. We are currently exploring the limits of the transferase activity attainable by α-amylases.

Acknowledgements: We are thankful to Dr. Lorenzo Segovia and Gabriel Moreno for computational assistance, to Dr. Lisa Ellerby for critical review of the manuscript and to Fernando González, Filiberto Sánchez, Leticia Olvera, Eugenio López and Paul Gaytán for technical support. This project was funded by DGAPA Research Grant IN212696 from the Universidad Nacional Autónoma de México.

References

[1] Sinnott, M.L. (1990) Chem. Rev. 90, 1171–1202.
[2] Kuriki, T., Kaneko, H., Yanase, M., Takata, H., Shimada, J., Handa, S., Takada, T., Umeyama, H. and Okada, S. (1996) J. Biol. Chem. 271, 17321–17329.
[3] Farber, G.K. and Petsko, G.A. (1990) Trends Biochem. Sci. 15, 228–234.
[4] Reardon, D. and Farber, G.K. (1995) FASEB J. 9, 497–503.
[5] McCarter, J.D. and Withers, S.G. (1994) Curr. Opin. Struct. Biol. 4, 885–892.
[6] Davies, G. and Henrissat, B. (1995) Structure 3, 853–859.
[7] Fukumoto, J. (1963) J. Ferm. Technol. 41, 427.
[8] Hehre, E.J., Genghof, D.S. and Okada, G. (1971) Arch. Biochem. Biophys. 142, 382–393.
[9] Fogarty, W.M. (1983) in: Microbial Enzymes and Biotechnology (Fogarty, W.M., Ed.), pp. 1–92, Applied Science Publishers, New York.
[10] Svensson, B. (1994) Plant Mol. Biol. 25, 141–157.
[11] Moreno, A., Salgado, L., Vazquez-Duhalt, R. and López-Munguía, A. (1998) J. Mol. Catal. B Enzym. (in press).
[12] Vulfson, E.N., Patel, R., Beecher, J.E., Andrews, A.T. and Law, B.A. (1990) Enzyme Microb. Technol. 12, 950–954.
[13] Chahid, Z., Montet, D., Pina, M. and Graille, J. (1992) Biotechnol. Lett. 14, 281–284.
[14] Shinoyama, H., Kamiyama, Y. and Yasui, T. (1988) Agric. Biol. Chem. 52, 2197–2202.
[15] Straathof, A.J.J., Vrijenhoef, J.P., Sprangers, E.P.A.T., Bekkum, H.v. and Kieboom, A.P.G. (1988) J. Carbohydr. Chem. 7, 223–238.
[16] Rodríguez, M., Gómez, A., González, F., Bárzana, E. and López-Munguía, A. (1996) Appl. Biochem. Biotechnol. 59, 167–175.
[17] Matsubara, S. (1961) J. Biochem. 49, 226–231.
[18] Graber, M. and Combes, D. (1990) Biotechnol. Bioeng. 36, 12–18.
[19] Tonozuka, T., Sakai, H., Ohta, T. and Sakano, Y. (1994) Carbohydr. Res. 261, 157–162.
[20] del-Río, G., Morett, E. and Soberon, X. (1997) FEBS Lett. 416, 221–224.
[21] Suominen, I., Karp, M., Lautamo, J., Knowles, J.K.C. and Montsaelae, P. (1987) in: Extracellular Enzymes of Microorganisms

(Chaloupka, J. and Krumphanzl, V., Eds.), Plenum Press, New York.
[22] Spratt, B.G., Hedge, P.J., Heesen, S., Edelman, A. and Boome Smith, J.K. (1986) Gene 41, 337–342.
[23] Merino, E., Osuna, J., Bolivar, F. and Soberon, X. (1992) BioTechniques 12, 508–510.
[24] Vihinen, M., Ollikka, P., Niskanen, J., Meyer, P., Suominen, I., Karp, M., Holm, L., Knowles, J. and Mantsala, P. (1990) J. Biochem. (Tokyo) 107, 267–272.
[25] Summer, J.B. and Howell, S.F. (1935) J. Biol. Chem. 108, 51–54.
[26] Omichi, K. and Ikenaka, T. (1983) J. Biochem. (Tokyo) 94, 1797–1802.
[27] Machius, M., Wiegand, G. and Huber, R. (1995) J. Mol. Biol. 246, 545–559.
[28] Brady, R.L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E.J. and Dodson, G.G. (1991) Acta Crystallogr. B, 527–535.
[29] Brzozowski, A.M. and Davies, G.J. (1997) Biochemistry 36, 10837–10845.
[30] Knegtel, R.M., Strokopytov, B., Penninga, D., Faber, O.G., Rozeboom, H.J., Kalk, K.H., Dijkhuizen, L. and Dijkstra, B.W. (1995) J. Biol. Chem. 270, 29256–29264.
[31] Strokopytov, B., Penninga, D., Rozeboom, H.J., Kalk, K.H., Dijkhuizen, L. and Dijkstra, B.W. (1995) Biochemistry 34, 2234–2240.
[32] Santamaría, R.I., Rodríguez, M.E., Del Río, G., Saab, G., Soberón, X. and López-Munguía, A. (1999) FEBS Lett. 452, 346–350.
[33] Fujimoto, Z., Takase, K., Doui, N., Momma, M., Matsumoto, T. and Mizuno, H. (1998) J. Mol. Biol. 277, 393–407.
[34] Sali, A., Potterton, L., Yuan, F., van, V.H. and Karplus, M. (1995) Proteins Struct. Funct. Genet. 23, 318–326.
[35] Sanchez, R. and Sali, A. (1997) Proteins Struct. Funct. Genet. 25, 50–58.
[36] Holm, L., Koivula, A.K., Lehtovaara, P.M., Hemminki, A. and Knowles, J.K. (1990) Protein Eng. 3, 181–191.
[37] Kadziola, A., Sógaard, M., Svensson, B. and Haser, R. (1998) J. Mol. Biol. 278, 205–217.
[38] Koshland, D.E.J. (1953) Biol. Rev. 28, 416–436.
[39] Ishikawa, K., Matsui, I., Honda, K. and Nakatani, H. (1990) Biochemistry 29, 7119–7123.
[40] Abrahmsen, L., Tom, J., Burnier, J., Utcher, K.A., Kossiakoff, A. and Wells, J.A. (1991) Biochemistry 30, 4151–4159.
[41] Koradi, R., Billeter, M. and Wüthrich, K. (1996) J. Mol. Graphics 14, 51–55.