Protein engineering of the pH dependence and substrate specificity of glucoamylase from Aspergillus awamori

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Protein engineering of the pH dependence and substrate specificity of glucoamylase from *Aspergillus awamori*

by

Tsuei-Yun Fang

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Major Professor: Clark F. Ford

Iowa State University

Ames, Iowa

1997
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This is to certify that the Doctoral dissertation of

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For the Major Program

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For the Graduate College
To my Parents

Mrs. Inn Lin & Mr. Chiao-Ya Fang
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ACKNOWLEDGMENTS

I would like to give my great appreciation to my major professor, Dr. Clark Ford for his guidance, support, advice, and understanding.

I would like to give my special thanks to Dr. Peter J. Reilly for his encouragement, wisdom, and his advice and help to this study.

I would like to sincerely thank Dr. Richard Honzatko for his valuable suggestions and help, and also my other committee members, Dr. Antony Pometto and Dr. Zivko Nikolov, for their time and encouragement.

I would like to thank my colleagues, Dr. Yuxing Li, Martin Allen, Kevin Liu, and Saber Khan, for their friendships and cooperation, and also Dr. Pedro M. Coutinho for his valuable suggestions and discussion. Thank all of the people that had ever helped me during my stay in Ames.

Finally, I would like to give my sincere appreciation to my family members, especially my parents, and Wen-Chi Tseng for their endless love and support.
GENERAL INTRODUCTION

Protein engineering holds promise for synthesis of new biocatalysts with predictable and useful properties. Strategies to achieve this goal include: (1) altering the amino acid residues in a catalytically active protein to obtain the desired catalytic properties; (2) adding enhancing catalytic or substrate-binding amino acid side chains to a catalytically inactive protein (or peptide) that binds a transition-state analogue (as in catalytic antibodies); and (3) making artificial structures that mimic a natural protein and adding catalytic residues to it (as in chymohelizyme) (Hahn et al., 1990; Corey et al., 1994).

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) (GA) is an enzyme that catalyzes the release of β-D-glucose by hydrolyzing α-1,4 and α-1,6-glucosidic linkages from the nonreducing ends of starch and related oligo- and polysaccharides. GA is widely used in industry to produce high-glucose syrups that are used as fermentation substrates to produce ethanol and other products or are converted to fructose sweeteners. The yield of glucose in starch processing, however, is limited to about 96% due to the synthesis of unwanted reversion products, especially isomaltose, resulting from glucose condensation. If the ability of the enzyme to synthesize isomaltose could be severely reduced, an increased D-glucose yield of up to 2% would be expected. In addition, the optimal pH of GA (4 - 4.5) is lower than other enzymes, such as α-amylase (5.5 - 6.5) and glucose isomerase (7 - 8), which are used in starch saccharification and high-fructose corn syrups production, respectively. Processing by GA must be done separately from that done by the other enzymes, and many inorganic salts produced from the neutralization of the digestion medium must be removed.
from the final product. If the optimal or sub-optimal pH of GA were close to the pH optima of α-amylase and glucose isomerase, the acidification and neutralization steps could be omitted. The concurrent use of GA and glucose isomerase would lower the glucose concentration, preventing the GA-catalyzed reverse reactions and accumulation of isomaltose.

Homology analysis and the understanding of the three-dimensional GA structure, combined with site-directed mutagenesis, has made alterations of GA properties to meet industrial requirements possible. By the analysis of the functions of mutated GAs, we can estimate the contribution of single amino acid residues to the catalytic properties of GA, and understand the relationships between GA structure and function in catalysis. These results could also aid in the design of a GA with industrially desirable properties.

This dissertation presents an investigation into the function and structure relationships of GA to obtain mutants with desired pH dependence and substrate specificity by altering specific amino acids in the active site of the enzyme.

The following section of this dissertation presents a literature review that provides a background to this study.

**Literature Review**

**Glucoamylase**

*Multiple forms of GA*

GAs, which are produced from yeasts, molds, and a few bacteria (Vihinen & Mantsala, 1989), have several forms that differ mainly in their molecular weight and capacity
to bind insoluble starch (Saha & Zeikus, 1989). Two major forms of *Aspergillus niger* GA are GAI and GAII. GAI contains 616 amino acid residues, and GAII, which is a digestion product from the proteolysis of GAI, contains a mixture of 512 and 514 amino acid residues (Svensson et al., 1982, 1986; Nunberg et al., 1984). The cloned GA genes of *Aspergillus awamori*, *Aspergillus niger*, and *Aspergillus awamori* var. kawachi code for full-length GA containing 615 or 616 amino acid residues and a signal peptide at the N-terminal, which is removed during the secretion of the enzyme (Svensson et al., 1983; Nunberg et al., 1984; Hayashi et al., 1989). The amino acid sequences of GAI from *Aspergillus awamori* and *Aspergillus niger* are identical (Svensson et al., 1983; Nunberg et al., 1984).

GAI has three domains: (1) a catalytic domain containing residues 1-467, which includes an O-glycosylated region from residues 441-467; (2) a starch-binding domain containing residues 509-606, which has the capacity to bind insoluble starch and enhances the hydrolysis of insoluble starch by the catalytic domain, and (3) a linker domain containing residues 468-508, which has many threonine and serine residues that are almost all O-linked to mannose (Coutinho & Reilly, 1994b) (Figure 1). The molecular weights of the polypeptide moiety (derived from the amino acid sequence) and the total GAI molecule are 65,424 and 82,000, respectively (Svensson et al., 1983).

*The three-dimensional structure of GA*

Crystallographic studies of GA have been done on a proteolytic fragment of GA from *Aspergillus awamori* var. X100, which consists of the first 470 residues (including the entire catalytic domain) formed by the natural action of fungal acid proteases (Aleshin et al., 1992, 1994b). The amino acid sequence of *Aspergillus awamori* var. X100 GA has about 95%
Figure 1. Possible structure of *Aspergillus niger* GA. The catalytic domain used was GA from *Aspergillus awamori* var. X100 (1gly), the linker was built from repetitions of the O-glycosylated belt around 1gly, and the starch-binding domain was domain E of cyclodextrin glucosyltransferase from *Bacillus circulans* no. 8 (1cgt) (Coutinho, 1996).
The catalytic domain of GA contains thirteen α-helices, twelve of which are arranged in pairs forming an α/α barrel (Figure 2). The cavity in the center of the barrel has highly conserved residues, which are involved in the active site. In addition, homology analysis of thirteen amino acid sequences of GAs shows five conserved regions in the catalytic domain (Coutinho & Reilly, 1994a). For the GA family these five conserved regions (S1 to S5) define the active site.

Crystallographic studies also have been applied to GA complexed with inhibitors and substrate analogs, including 1-deoxynojirimycin, acarbose and D-gluco-dihydroacarbose (Harris et al., 1993; Aleshin et al., 1994a; Stoffer et al., 1995). These structures are very similar to that of native enzyme. Crystal structures of GA complexed with the pseudotetrasaccharides acarbose and D-gluco-dihydroacarbose have shown that there are two different binding modes between pseudotetrasaccharides and GA at pH 6 and pH 4 (Aleshin et al., 1994a, 1996; Stoffer et al., 1995). The binding of the first two sugars residues of the pseudotetrasaccharides is the same, but there is extraordinary variation in the binding of the third and fourth sugars. Only one conformer (pH 6-type conformer) of pseudotetrasaccharide binding exists at pH 6, while two conformers (pH 4-type and pH 6-type conformers) of pseudotetrasaccharide binding appear at pH 4. At pH 4, the pH 4-type conformer has higher occupancy (0.53 in acarbose and 0.63 in D-gluco-dihydroacarbose) than the pH 6-type conformer (0.47 in acarbose and 0.37 in D-gluco-dihydroacarbose). Although subsite mapping indicates about six subsites for the active site of GA, the pH-dependent interaction
Figure 2. Cartoon display (RasMol Version 2.6) of the catalytic domain of GA from *Aspergillus awamori* var. *X100* (Aleshin et al., 1994b).
of acarbose and D-gluco-dihydroacarbose suggest that perhaps only the first two subsites are common to all maltooligosaccharides and their substrate analogs (Aleshin et al., 1996).

A solution structure of the starch-binding domain of GA from *Aspergillus niger* has been determined by heteronuclear multidimensional nuclear magnetic resonance spectroscopy and simulated annealing (Sorimachi et al., 1996). The solved structure shows a well defined β-sheet consisting of one parallel and six antiparallel pairs of β-strands which form an open-sided β-barrel (Figure 3). The structure has two binding sites for ligands at one end of the molecule on opposite faces.

**Catalytic properties of GA**

GA catalyzes the release of β-D-glucose from the nonreducing ends of starch and related oligo- and polysaccharides. Although GA hydrolyzes primarily the α-1,4-glucosidic linkage, it also hydrolyzes α-1,6-, α-1,3-, α-1,2- and α,β-1,1- glucosidic linkages slowly (Pazur & Kleppe, 1962; Meagher et al., 1989). GA also catalyzes the formation of glucose condensation products containing the glucosidic linkages described above, including maltose (4-O-α-D-glucopyranosyl-D-glucose), isomaltose (6-O-α-D-glucopyranosyl-D-glucose), isomaltotriose (6-O-α-D-glucopyranosyl-D-isomaltose), isomaltotetraose (6-O-α-D-glucopyranosyl-D-isomaltotriose), nigerose (3-O-D-glucopyranosyl-D-glucose), kojibiose (2-O-D-glucopyranosyl-D-glucose), panose (6-O-α-D-glucopyranosyl-D-maltose) and α,β-trehalose (α-D-glucopyranosyl β-D-glucopyranoside) (Nikolov et al., 1989). In general, the catalytic efficiency ($k_{cat}/K_m$) of *Aspergillus* GAs for cleaving α-1,6-linkages is about 500-fold less than
Figure 3. Cartoon display (RasMol Version 2.6) of the starch-binding domain of GA from *Aspergillus niger* (Sorimachi et al., 1996).
that for α-1,4-linkages (Hiromi et al., 1966a; Sierks et al., 1989). High activity for cleaving α-1,6-linkages is found in *Clostridium* sp. G005 GA and *Hormoconis resinae* GA P, with values of catalytic efficiency for isomaltose hydrolysis being 800 and 80 times, respectively, higher than in *A. niger* GA (Fagerström et al., 1990; Ohnishi et al., 1992). Conversely, very low activity for cleaving α-1,6-linkages compared to that of *A. niger* GA is found in *Clostridium thermosaccharolyticum* GA (Specka et al., 1991), in *H. resinae* GA S (Fagerström et al., 1990) and in *Thermomyces lanuginosus* GA (Basaveswara Rao et al., 1981).

Kinetic studies on the hydrolysis of different lengths of substrates by GA show that the catalytic efficiency increases with increasing substrate length up to six glucosyl units (Hiromi et al., 1973, 1983; Meagher et al., 1989; Sierks et al., 1989). Subsite mapping studies show that there are five to seven glucosyl binding subsites, and that the catalytic site is located between subsites 1 and 2 (Hiromi et al., 1973, 1983; Savel'ev & Firsov, 1982; Tanaka et al., 1983; Meagher et al., 1989).

Two essential carboxyl groups are involved in the catalysis of GA (Hiromi et al., 1966b). In *R. delemar*, p$K_1$ and p$K_2$ values for the free enzyme are 2.9 and 5.9, respectively, with both maltose and panose as substrate, whereas for the maltose-complexed form these values are 1.9 and 5.9, respectively, and for panose-complexed form are 1.9 and 6.4, respectively (Hiromi et al., 1966b). Kinetic results combined with the three-dimensional structures of GA suggest that Glu179 and Glu400 (in *Aspergillus awamori* or *Aspergillus niger*) are the catalytic acid and base, respectively (Sierks et al., 1990; Aleshin et al., 1992,
1994a; Harris et al., 1993; Frandsen et al., 1994). Crystallographic studies also suggest that a hydronium ion (carried by a water molecule) is located at the active site in place of the protonated Glu179 in the absence of substrate. Glu179 becomes protonated only in the presence of substrate (Aleshin et al., 1994a). Then Glu179 protonates the oxygen in the glycosidic linkage, acting as a general acid catalyst, and Glu400 activates water (Wat500) for nucleophilic attack at carbon C-1, acting as a general base catalyst (Frandsen et al., 1994).

Two types of reaction models have been proposed for the reaction mechanism of GA (Ohnishi et al., 1977, 1990; Tanaka et al., 1982; Hiromi et al., 1983; Olsen et al., 1992, 1993; Christensen et al., 1996). Model I is a three-step reaction mechanism proposed from presteady-state fast-flow kinetics. The substrate catalysis involving two intermediates, the initial association complex (ES) and the Michaelis complex (E*S, i.e. the most stable enzyme-substrate intermediate) (Ohnishi et al., 1977, 1990; Tanaka et al., 1982; Hiromi et al., 1983). The first step, a rapid bimolecular process, is followed by a slow unimolecular step.

\[
\begin{align*}
E + S & \xrightleftharpoons[k_1]{k_2} ES \xrightleftharpoons[k_3]{k_4} E*S \xrightleftharpoons[k_5]{k_6} E + P \\
E + S & \xrightleftharpoons[k_1]{k_2} ES \xrightleftharpoons[k_3]{k_4} E*S \xrightleftharpoons[k_5]{k_6} E + P \\
\end{align*}
\]  
(Model I)

There are two different explanations about the first two steps in Model I. Tanaka et al. (1982) proposed that the fast formation of an initial ES complex, in which the nonreducing end of the substrate does not occupy subsite 1, is followed by a slow unimolecular step involving the migration of the substrate to subsite 1 with a concurrent expulsion of water. Olsen et al. (1993) suggested an initial association of substrate at subsite 1 to form an ES complex followed by a conformational change to form the E*S complex.
However, crystallographic studies have not detected a conformational change in response to the binding of inhibitors.

Model II is a classical model of subsite theory (Hiromi et al., 1973) that has been widely used for calculation of subsite energy (Hiromi et al., 1983; Meagher et al., 1989; Sierks et al., 1989, 1993; Frandsen et al., 1995, 1996).

\[
\begin{align*}
K_{NP} & \quad K_P & \quad k_{int} \\
E S_{NP} & \rightleftharpoons E+S & \rightarrow ES_{P} & \rightarrow E + P & \text{ (Model II)}
\end{align*}
\]

Model II assumes the presence of an important nonproductive enzyme-substrate complex and also that the intrinsic rate constant \(k_{int}\) is independent of substrate length. \(K_{NP}\) and \(K_P\) represent the binding constants between enzyme and substrate to form a productive enzyme-substrate complex (ES\(_P\)) and a nonproductive enzyme-substrate complex (ES\(_{NP}\)). However, Christensen et al. (1996) reported that Model II is not valid based on the presteady and steady-state kinetic results on the interactions of wild-type and Glu180\(\rightarrow\)Gln and Asp176\(\rightarrow\)Asn mutants of \(A.\ niger\) GA1 with maltooligosaccharides.

**Mutations of glucoamylase**

Numerous mutations of GA (especially from *Aspergillus awamori* or *Aspergillus niger*) have been made. In general, there are two major purposes for making mutations of GA: 1) to understand the catalytic mechanism or structure and function relationships (Sierks et al., 1989, 1990, 1993; Bakir et al., 1993; Frandsen et al., 1994, 1995; Natarajan & Sierks, 1996); 2) to improve the catalytic properties, including substrate specificity, pH dependence and thermostability, for industrial application (Bakir et al., 1993; Chen et al., 1994a, 1994b, 1995, 1996; Fierobe et al., 1996). Mutations, including Trp120\(\rightarrow\)Phe, Leu, His and Tyr
(Sierks et al., 1989), Glu179→Gln (Sierks et al., 1990) and Asp (Bakir et al., 1993),
Glu180→Gln (Sierks et al., 1990), Asp55→Gly (Sierks et al., 1993), Trp178→Asp (Bakir et al., 1993), Tyr48→Trp (Frandsen et al., 1994), Glu400→Gln (Frandsen et al., 1994),
Arg305→Lys (Frandsen et al., 1995), Arg54→Lys and Leu (Frandsen et al., 1995),
Tyr317→Phe (Frandsen et al., 1995), and Asp309→Glu (Frandsen et al., 1995). seriously
decrease the catalytic efficiency ($k_{cat}/K_m$) of GA. The crystal structure of GA complexed with
acarbose has shown that all of these mutated residues, except Asp309 and Glu400, are
directly involved in enzyme-substrate interactions by hydrogen bonding or nonbonding
contacts (Aleshin et al., 1994a). Glu400 functions as the catalytic base and Asp309 is salt-
linked to Arg305. The loop region around Trp120 also has been mutated to investigate the
roles of these residues, with mutations including Arg122→Tyr, Pro123→Gly, Gln124→His,
Gly121→Thr and Arg125→Lys (Nagahara & Nishino, 1996). In addition, other mutations
have been made regarding substrate specificity (Sierks & Svensson, 1994; Fierobe et al.,
1996), thermostability (Chen et al., 1994a, 1994b, 1995, 1996; Fierobe et al., 1996), and pH
dependence (Bakir et al., 1993; Frandsen et al., 1994).

pH dependence

Factors involved in altering pH dependence of enzymes

The pH dependence of an enzyme is determined by the ionization of the catalytic
groups, the general catalytic base and catalytic acid. The ionization of the catalytic groups is
affected by factors involved in the microenvironment of the catalytic groups. These factors,
such as salt-bridges and hydrogen bonds between the catalytic group and other residues,
polarity, and charge distribution, can raise or lower the $pK_a$'s of the catalytic groups. Some enzymes have highly perturbed $pK_a$'s of catalytic groups (Schmidt & Westheimer, 1971; Fersht, 1972; Parsons & Raftery, 1972; Karplus et al., 1973; Johnson et al., 1981; Voet et al., 1981; Oda et al., 1994) which are unusually higher or lower than their usual range in proteins. If a carboxyl group is in a region of relatively low polarity or high negative charge, its $pK_a$ will be raised by destabilization of the anionic form (Parsons & Raftery, 1972; Aleshin et al., 1994a). The carboxylate ion can be stabilized by introducing a positive charge, especially by forming a salt-bridge with an ammonium ion, which lowers the $pK_a$ (Anderson et al., 1990). Conversely, if an amino group is buried in a nonpolar region, the $pK_a$ is lowered by inhibiting its protonation (destabilizing its protonated form) (Schmidt & Westheimer, 1971). An ammonium ion can be stabilized by a negative charge, especially by forming a salt-bridge with a carboxylate ion, which raises the $pK_a$ by inhibiting deprotonation (Fersht, 1972; Voet et al., 1981; Anderson et al., 1990).

In addition to the microenvironment of the catalytic groups, the surface charge of an enzyme may stabilize or destabilize buried or partially buried ionic catalytic groups. For example, a catalytic carboxylate ion in the active site can be stabilized with a positive surface charge (lowering the $pK_a$) and destabilized with a negative surface charge (raising the $pK_a$) by chemical modification (Spomer & Wootton, 1971; Valenzuela & Bender, 1971).

Based on the highly perturbed $pK_a$'s observed in natural and modified protein, the $pK_a$'s of catalytic groups can be theoretically altered by stabilizing or destabilizing the ionic forms ($CO_2^-$ or $NH_3^+$) of catalytic groups. Using site-directed mutagenesis is a better choice
than chemical modification, since it has the potential to change the microenvironment of catalytic groups without causing serious conformational disturbances.

**Protein engineering to modify the pH dependence of enzymes**

The three-dimensional structures of proteins provide detailed information about the microenvironment of the catalytic groups, and homology analysis of the amino acid sequences of enzymes with the same or similar functions also provide some important information about pH dependence. In addition, chemical modification of specific amino acid side chains can provide some valuable information, especially when the three-dimensional structure of the enzyme is not available. The pH dependence of many enzymes has been altered by single (or double) amino acid change using site-directed mutagenesis (Thomas et al., 1985; Russell & Fersht, 1987; Yamauchi et al., 1988; Ido et al., 1991; Martin & Hausinger, 1992; van Tilbeurgh et al., 1992; Myers et al., 1993; Pitts et al., 1993; Bastyns et al., 1996).

Thomas et al. (1985) studied electrostatic effects on the pH dependence of subtilisin (a serine protease) by site-directed mutagenesis. Mutant Asp99→Ser was constructed. Asp99 is not conserved in highly homologous subtilisins, and is replaced by a Ser or Thr in subtilisins from other organisms. The three-dimensional structure showed that Asp99 is about 14-15 Å from the imidazole of His64, the catalytic base. Removing the negative charge of Asp99 was designed to destabilize the low-pH, positively-charged form of His64 and therefore lower its pKₐ, assuming that electrostatic interactions would be effective at the long distance of 14-15 Å. At ionic strength 0.1, mutant Asp99→Ser decreased the pKₐ values by 0.29 ± 0.04 units and decreased the catalytic efficiency by 20%. The existence of
electrostatic interactions was shown indirectly based on the effect of ionic strength. High concentrations of ions are able to mask electrostatic interactions. At ionic strength 1.0, the electrostatic interactions were masked as expected, since the mutation Asp99→Ser had no effect on the catalytic properties of the enzyme. This study showed that the modification of a single charge in active site of an enzyme can have a significant effect on the pH dependence of the catalytic reaction. Such effects should be larger if the mutated residue is closer to catalytic groups of an enzyme.

Ido et. al. (1991) investigated the importance of hydrogen bonding in the pH dependence of aspartic proteases. Human immunodeficiency virus type 1 (HIV-1) protease optimally catalyzes in the pH range of 4 - 6, however, nearly all of the other eukaryotic aspartic proteases catalyze best in the pH range of 2 - 4. Compared to the other eukaryotic aspartic proteases, HIV-1 protease lacks a hydrogen bond between residue 28 and the carboxyl group of active-site Asp25. Mutation Ala28→Ser was constructed to introduce a hydrogen bond between residue 28 and Asp25. The high pKₐ values of the free enzyme are 6.8 and 5.6, and the corresponding values of the enzyme-substrate complex are 6.9 and 6.0 for the wild-type and mutant enzymes, respectively. The low pKₐ values of the free enzyme are 3.3 and 3.4, and the corresponding values of the enzyme-substrate complex are 5.1 and 4.3 for the wild-type and mutant enzymes, respectively. The lowered pKₐ values in mutant HIV-1 protease indicated that the hydroxyl group of Ser28 formed a new hydrogen bond to Asp25.
Bakir et al. (1993) tried to alter the pH dependence of glucoamylase by introducing mutations, including Asp176→Glu, Leu177→Asp, Trp178→Asp, Glu179→Asp, Glu180→Asp, Val181→Asp, Asn182→Asp and two insertion mutations, in the region near or at Glu179, the catalytic acid. In spite of the increased total negative charge in most cases, the pH dependence of the active mutant GAs was very similar to wild-type GA. In another study, mutations Gly183→Lys and Ser184→His broadened the optimal pH range for activity by replacing amino acid residues with those containing positive charge (Sierks & Svensson, 1994).

Substrate specificity

Factors involved in altering substrate specificity of an enzyme

The substrate specificity of an enzyme is determined by its ability to form a stable enzyme-ligand complex in both the ground state and the transition state. The stability of the enzyme-ligand complex is affected by steric constraints, hydrogen bonding, van der Waal's and electrostatic forces, and hydrophobic contacts (Fersht, 1985). If a mutation can change one or more factors involved in the stabilization of enzyme-ligand complex, the substrate specificity may be changed.

Protein engineering the substrate specificity of enzymes

The rational redesign of an enzyme's active site requires extensive knowledge of the catalytic mechanism and the enzyme structure. The three-dimensional structures of the complexes of an enzyme (or functionally related enzyme) with inhibitor or pseudosubstrate can provide valuable information in determining the key interactions that might affect
substrate specificity. Homology analysis of amino acid sequences of enzymes with the same or similar functions also provide important information about substrate specificity and other properties of an enzyme.

Based on the three-dimensional structure of the enzyme or related enzyme and homology analysis, substrate specificities of a large number of enzymes have been altered by redesigning their substrate binding pockets by site-directed mutagenesis to change steric constraints and/or hydrogen bonding (Wilkinson et al., 1984; Craik et al., 1985; Bone et al., 1989; Meng et al., 1991; Rheinnecker et al., 1994), electrostatic interactions (Wells et al., 1987; Dean & Koshland, 1990; Sullivan et al., 1991), or hydrophobic interactions (Estell et al., 1986) with the substrate.

Meng et al. (1991) switched the substrate preference of thermophilic xylose isomerase from D-xylose to D-glucose (D-glucose has an additional —CH₂OH compared to D-xylose) by reducing steric constraints and enhancing the hydrogen-bonding capacity of the active site for bound glucose. Based on the known crystal structure of *Arthrobacter* xylose isomerase and homology analysis, Trp139, Thr141, Val186, and Glu232 of thermophilic xylose isomerase were chosen for mutagenesis based on potential steric hindrance for effective binding of D-glucose. Mutation Trp139→Phe reduced the $K_m$ and enhanced the $k_{cat}$ of the mutant thermophilic enzyme toward xylose. Mutant Val186→Thr, enhancing the hydrogen bonding capacity with glucose, also enhanced the catalytic efficiency toward glucose. Double mutants Trp139→Phe/Val186→Thr and Trp139→Phe/Val186→Ser had a higher
catalytic efficiency than wild-type enzyme, and also had 1.5 and 3 times higher catalytic efficiency for D-glucose than for D-xylose, respectively.

Craik et al. (1985) replaced the glycine residues with alanine at positions 216 and 226 in the binding cavity of rat trypsin. In comparison to the three-dimensional structure of bovine trypsin, which has 74% identical primary structure with rat trypsin, the glycine residues at positions 216 and 226 are conserved and seemed to permit the entry of large amino acid side chains of peptide substrate into the hydrophobic pocket. Computer graphic analysis using bovine trypsin structures complexed with pancreatic trypsin inhibitor (with Lys15 of the inhibitor in the substrate binding pocket) or benzamidine (an arginine analogue) as a model, suggested these replacements would differentially affect arginine and lysine substrate binding of the enzyme. Two single mutants and a double mutant were constructed. In spite of the reduced catalytic rate in these mutant enzymes, they did show enhanced substrate specificity toward arginine or lysine substrates relative to the native enzyme.

Subtilisin BPN' is a serine-class endoprotease secreted by Bacillus amylo liquefaciens. It has a broad peptidase specificity, mainly by interactions between its two binding pockets, $S_1$ and $S_4$, and the corresponding amino side-chains, $P_1$ and $P_4$, of the substrate. The three-dimensional structures of Subtilisin BPN' have been solved since 1969. Estell et al. (1986) replaced the conserved Gly166, located at the bottom of the substrate binding cleft ($S_1$ pocket), by 12 nonionic amino acids to probe steric and hydrophobic effects on the substrate specificity of subtilisin by cassette mutagenesis. Mutant enzymes were analyzed by using $P_1$ substrates of varying size and hydrophobicity. Resultant mutant enzymes had large changes in substrate specificity caused by the combined effect of steric hindrance and enhanced
hydrophobic interactions. In general, the catalytic efficiency toward small hydrophobic substrates was increased up to 16 times by hydrophobic substitutions at position 166. Wells et al. (1987) protein-engineered electrostatic interactions between charged substrates and complementary charged amino acids of subtilisin BPN', resulting in an increased catalytic efficiency toward complementary charged substrates (up to 1900 times) and a decreased catalytic efficiency toward similarly charged substrates. Sixteen mutants were constructed by applying single or double mutations at position 156 and 166, which are located in the substrate binding pocket S_1, since side chains from either residue 156 or 166 have the potential to form an ion pair with a complementary charge at the P_1 position of the substrate. Rheinnecker et al. (1993) tried to improve the substrate specificity of subtilisin BPN' toward substrates with large hydrophobic P_4 side chains. Ile107, which is at the bottom of the S_4 binding pocket, was replaced by Val, Ala and Gly to increase the size of the S_4 binding pocket, and Tyr104 was replaced by Phe to remove the hydrogen bond between Tyr104 and Ser130 at the entrance of the S_4 binding pocket. All mutant subtilisins, except Ile107→Val, had increased specificity for residues with large side chains at P_4 compared with wild-type enzyme. The catalytic efficiency of mutant Ile107→Gly was increased up to 214-fold for Phe compared with Ala at the P_4 position of the substrate, and the activity of Ile107→Gly was comparable to that of the wild-type enzyme. Again, Rheinnecker et al. (1994) tried to further improve the substrate specificity of subtilisin BPN' toward substrates with large hydrophobic P_4 side chains by increasing the size of the S_4 binding pocket. Single and double mutations were made at positions 104, 107 and 126. Leu126 was replaced by valine, alanine and
glycine; however, only the Leu126→Ala subtilisin showed a significantly improved specificity profile. Double mutants Ile107→Gly/Leu126→Val, Ile107→Gly/Leu126→Ala and Ile107→Gly/Tyr104→Ala did not show additive effects of the single amino acids on the kinetic parameters of the enzyme. Mutant Ile107→Gly/Leu126→Val subtilisin showed the largest improvement in P4 substrate specificity, with a catalytic efficiency increased 340-fold for leucine compared to wild-type enzyme.

Sierks and Svensson (1994) tried to improve the substrate specificity of GA by single amino acid replacement. Based on the sequence similarities between starch-degrading enzymes, mutations Ser119→Tyr, Gly183→Lys and Ser184→His were designed to enhance selection for α-1,4- over α-1,6-linked substrates in order to suppress formation of α-1,6-linked glucose condensation products. These three mutants all slightly increased the activity toward α-1,4-linked maltose. The selectivity of these three mutant GAs, determined from the ratio of catalytic efficiency for hydrolysis of maltose to that of isomaltose, was enhanced 2.3 to 3.5-fold. However, a later study (Svensson et al., 1995) showed Ser119→Tyr has an unusually high capacity to produce branched oligosaccharides under conditions similar to industrial saccharification.

**Research Objectives**

There are four objectives in this research:

1) Alter the pH dependence of GA to obtain a higher optimal pH.

2) Investigate the contribution of single amino acid residues on the pH dependence of GA.
3) Alter the substrate specificity of GA to reduce the formation of isomaltose from glucose condensation reaction.

4) Investigate the contribution of single amino acid residues on the substrate specificity of GA.

The first and second objectives are the subject of the first manuscript. The third and fourth objectives are the subjects of both the second and third manuscript.

Dissertation Organization

This dissertation contains a general introduction, general conclusions and recommendations, general references, and three manuscripts, which are written in a format for publication in Protein Engineering (Oxford University Press). The general introduction is provided as a more detailed background to this research program. The general conclusions and recommendations summarize the results of this research program and provide some suggestions resulting from this research program. The general references contain the references cited in the general introduction and are listed at the end of this dissertation. In addition, each manuscript has an individual reference list.
PROTEIN ENGINEERING OF GLUCOAMYLASE FROM ASPERGILLUS AWAMORI TO INCREASE ITS OPTIMAL pH

A paper to be submitted to Protein Engineering
Tsuei-Yun Fang and Clark Ford

Abstract

To increase the optimal pH of glucoamylase, site-directed mutagenesis was used to construct five amino acid replacement mutations at residue Ser411. Although Ser411 is not a conserved residue, it hydrogen bonds to the general catalytic base Glu400. The mutations were designed to destabilize the carboxylate ion form of Glu400 by removing or weakening the hydrogen bond between Ser411 and Glu400 and thereby raising its pK. The additional effects of polarity and both positive and negative charges were also studied by the substitution of alanine, histidine and aspartate, respectively. Compared to wild-type glucoamylase, Ser411→Gly glucoamylase was highly active with a slightly increased $K_m$ for the hydrolysis of isomaltose, maltose and maltoheptaose at pH 4.4. Ser411→Ala glucoamylase maintained 65 - 74% of wild-type catalytic efficiency ($k_{cat}/K_m$) with a slightly decreased $k_{cat}$ and a slightly increased $K_m$. The Ser411→Cys enzyme maintained 54 - 73% of wild-type catalytic efficiency with a decrease in both the $k_{cat}$ and $K_m$. Ser411→His and Ser411→Asp glucoamylases had only about 6 - 12% of wild-type catalytic efficiency with a seriously decreased $k_{cat}$ and an increased $K_m$. All five mutations increased the optimal pH in the enzyme-substrate complex, mainly by raising p$K_1$ values. Ser411→Ala is the best
performing pH mutant of glucoamylase isolated to date. The Ser411→Ala mutation increased the optimal pH by 0.84 units while maintaining a high level of catalytic activity and catalytic efficiency. These results suggest that the hydrogen bond between Glu400 and Ser411 plays an important role in increasing the optimal pH of glucoamylase by affecting the ionization of Glu400. The positive and negative charges that were introduced at position 411, however, had more effects on the ionization of Glu179, the catalytic acid. The hydrolysis of 28% DE 10 maltodextrin also showed that Ser411→Ala glucoamylase has the potential to be used at higher pHs in starch processing.

Key words: glucoamylase/optimal pH/protein engineering/site-directed mutagenesis/hydrogen bond/catalytic base/catalytic acid/ionization

Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3, GA) is an enzyme that catalyzes the release of β-D-glucose by hydrolyzing α-1,4- and α-1,6-glucosidic linkages from the nonreducing ends of starch and related oligo- and polysaccharides. GAs from Aspergillus awamori and Aspergillus niger, which are encoded by an identical gene (Svensson et al., 1983; Nunberg et al., 1984), are used in industry to produce high-glucose syrups from dextrin already liquefied by α-amylase. Glucose syrups are isomerized by glucose isomerase to produce fructose sweeteners. The optimal pH of GA (4 - 4.5) is lower than that of α-amylase (5.5 - 6.5) and glucose isomerase (7 - 8). GA hydrolysis must be done separately from the other enzymatic reactions, and many inorganic salts produced from the neutralization of the digestion medium must be removed from the final product. An
increased optimal pH for glucoamylase would be industrially desirable so that it could be used in process conditions more similar to that of other enzymatic steps in starch processing. Furthermore, the isomerization reaction is performed in a high-glucose medium at basic pHs that cause glycosylation and finally inactivation of the glucose isomerase (Pickersgill & Goodenough, 1991). In addition, glucose and fructose are not stable at these pHs. Some research has been done to decrease the optimal pH of glucose isomerase (van Tilbeurgh et al., 1992; Siddiqui et al., 1993). Since a significant change, such as 3 pH units, in the optimal pH of an enzyme is still very difficult to achieve, an effort to shift the optimal pHs of both GA and glucose isomerase would more likely be successful in bringing their optimal pHs close to each other. If the optimal pHs of GA and glucose isomerase were close to that of α-amylase (pH 6.0 - 6.5), the acidification and neutralization steps could be omitted. The concurrent use of GA and glucose isomerase in a reactor would have the additional benefit of lowering the glucose concentration, thus preventing GA reversion reactions and accumulation of isomaltose.

pH dependence of an enzyme is determined by the ionization of the catalytic groups, which is affected by various interactions involved in the microenvironments of the catalytic groups. Two carboxyl groups are involved in the catalytic mechanism of GA (Hiromi et al., 1966), Glu179 and Glu400 (Aspergillus awamori and Aspergillus niger numbering) (Sierks et al., 1990; Harris et al., 1993; Frandsen et al., 1994). Glu179, the catalytic acid, protonates the oxygen in the glucosidic linkage. Glu400, the catalytic base, activates water (Wat 500) for nucleophilic attack at carbon C-1. Our strategy for increasing optimal pH of GA, based on homology among related GAs and the three-dimensional structure of GA, was to change...
the polarity, charge distribution and hydrogen bonding in the microenvironment of Glu400 by using site-directed mutagenesis. According to the three-dimensional structure of GA (Aleshin et al., 1992, 1994a, 1994b, 1996; Harris et al., 1993; Stoffer et al., 1995). Ser411 hydrogen bonds to the catalytic base Glu400. Position 411 is either Ser or Gly in the known GA sequences (Coutinho & Reilly, 1994). The stereo view of the environment around Glu400 of GA from Aspergillus awamori var. X100 containing acarbose (an inhibitor of GA) in the active site is shown in Figure 1. Substitution of glycine at position 411 was designed to remove the hydrogen bond between Glu400 and Ser411. In this study, mutation Ser411→Gly was constructed to remove the hydrogen bond between S411 and Glu400, which would destabilize the carboxylate ion of Glu400, thereby raising the pK of Glu400 (pK\(_i\)). Mutations Ser411→His, Ser411→Asp, Ser411→Cys and Ser411→Ala were constructed to further investigate the effects of charge distribution, hydrogen bonding and polarity on the pH dependence of GA.

**Materials and Methods**

**Materials**

The yeast strain *Saccharomyces cerevisiae* C468 (α leu2-3 leu2-112, his3-15, mal³) and the yeast expression vector YEPM18 (containing the wild-type GAI cDNA from Aspergillus awamori) were gifts from Cetus Corporation (Emeryville, CA). Acarbose was a gift from Miles Laboratories (Elkhart, IN). Maltose, maltoheptaose, glucose oxidase and peroxidase were from Sigma (St. Louis, MO). Isomaltose was from TCI America (Portland, OR). DE (dextrose equivalent) 10 maltodextrin (Maltrin® M100) was from Grain
Figure 5. Stereo view of the environment of Glu400 (the catalytic base) of GA from *Aspergillus awamori* var. X100 (Aleshin et al., 1994a) with acarbose (bold line) in the active site. Dashed lines represent hydrogen bonds.
Processing Corporation (Muscatine, IA). Restriction enzymes, T4 DNA ligase and pGEM-7Z(+), an *Escherichia coli* phagemid vector, were from Promega (Madison, WI).

**Construction of mutant GA genes**

The GA gene was mutated by site-directed mutagenesis according to the protocols of the Muta-Gene phagemid *in vitro* mutagenesis kit (from Bio-Rad, Hercules, CA) based on the method described by Kunkel et al. (1987). pGEM-GA, an *E. coli* phagemid vector containing the wild-type GA cDNA, was transformed into *E. coli* CJ236 (*dua*, *ung*) to produce uracil-containing single-stranded DNA to be used as the template in site-directed mutagenesis.

pGEM7-GA was constructed by inserting a small *Xho* I-*EcoR* I fragment, which contains the GAI cDNA, of YEpm18 into pGEM-7Z(+). Mutation-containing oligonucleotide primers were synthesized in the Nucleic Acid Facility of Iowa State University, including 5'-GGC GAG CAG CTT GGA GCA CGC GAC CTG AC-3' (Ser411→Gly), 5'-GGC GAG CAG CTT GAT GCA CGC GAC CTG AC-3' (Ser411→Asp), 5'-GGC GAG CAG CTT CAT GCA CGC GAC CTG AC-3' (Ser411→His), 5'-GGC GAG CAG CTT TGT GCA CGC GAC CTG AC-3' (Ser411→Cys), and 5'-GGC GAG CAG CTT GCA GCA CGC GAC CTG AC-3' (Ser411→Ala). The nucleotides for designed GA mutations are shown in bold. The silent mutations, decreasing the melting temperature of primer hairpins, are underlined. All designed mutations were verified by DNA sequencing. The mutated GA cDNAs were subcloned into YEpm18, the yeast expression vector, and transformed into *S. cerevisiae* C468 as previously described (Chen et al., 1994).
Production and purification of GA

Wild-type, Ser411→Gly, Ser411→Ala, Ser411→Asp, Ser411→Cys and Ser411→His GAs were produced by growing YEpPM18-transformed *S. cerevisiae* C468 at 30°C for 5 days with shaking (170 rpm) in 2-L flasks each containing 1 L SD-His broth (Innis et al., 1985). The culture supernatants containing secreted GA were concentrated via ultrafiltration and diafiltered with 0.5 M NaCl-0.1 M NaOAc buffer at pH 4.3 and purified by acarbose-Sepharose affinity chromatography (Chen et al., 1994).

Protein concentration measurement

The concentration of GA was measured by the Pierce bicinchoninic acid protein assay with bovine serum albumin as a standard (Smith et al., 1985).

Enzyme kinetics

Initial rates of hydrolysis of isomaltose, maltose and maltoheptaose were determined at 45°C in 0.05 M NaOAc buffer at pH 4.4 by using ten to twelve substrate concentrations ranging from 0.1*Km* to 10*Km*. Samples were taken at five different time points and reactions were quenched by adding 0.4 volumes of 4 M Tris-HCl buffer, pH 7.0. The release of glucose was measured by the glucose oxidase method (Rabbo & Terkildsen, 1960). *kcat* and *Km* were calculated by fitting initial rates, *v*, as a function of substrate concentration, [S], to the Michaelis-Menten equation using Enzfitter software (Elsevier-BIOSOFT, Amsterdam, The Netherlands). Experiments were duplicated and the average initial rates were used. The change of transition-state binding energy (∆(∆G)) for substrate hydrolysis caused by the mutation, which was used to estimate the binding strength of the substrate in the transition-
state complex, was calculated by the equation \( \Delta(\Delta G) = -RT\ln[(k_{\text{cat}}/K_m)_{\text{mut}}/(k_{\text{cat}}/K_m)_{\text{wt}}] \), where the subscripts \( \text{mut} \) and \( \text{wt} \) refer to mutant and wild-type enzymes, respectively (Wilkinson et al., 1983).

**pH dependence of glucoamylase activity**

The pH dependence of glucoamylase activity was measured at 45°C at 16 different pH values, ranging from 2.2 to 7.0, using 0.025 M citrate-phosphate buffer (McIlvane, 1921) with maltose or maltoheptaose as substrate. The ionic strength of the citrate-phosphate buffer was maintained at 0.1 by adding KCl. The pK values of free enzyme and enzyme-substrate complex were measured at substrate concentrations (i) smaller than 0.2\( K_m \), so that the initial rate \( v \) was proportional to \( k_{\text{cat}}/K_m \), and (ii) higher than 10\( K_m \), so that the initial rate \( v \) was proportional to \( k_{\text{cat}} \) (Sierks & Svensson, 1994; Whitaker, 1994). Experiments were duplicated and the average initial rates were used. The pK values of two catalytic groups of free enzyme and enzyme-substrate complex were calculated by fitting the initial rates as a function of pH values to the equation \( \log Y = \log [C/(1+H/K_1+K_2/H)] \) by using the software of Enzfitter. \( Y \) is the observed value of the parameter of interest (i.e. \( k_{\text{cat}}/K_m \) or \( k_{\text{cat}} \)) measured at different pH values, \( C \) is the pH-independent value of \( Y \) (i.e. the maximal value of \( k_{\text{cat}}/K_m \) or \( k_{\text{cat}} \) ), \( H \) is the concentration of hydrogen ion, and \( K_1 \) and \( K_2 \) are dissociation constants of catalytic groups of the enzyme. When the values of apparent pK\(_1\) and pK\(_2\) were separated by less than 3 pH units, the pK values were adjusted by equations

\[
(H^+) + (H^+)_2 = K_1 + 4(H^+)_{\text{opt}} \quad \text{and} \quad (H^+)_{\text{opt}} = \sqrt{K_1 K_2} \quad (\text{Whitaker, 1994}).
\]

The concentration of hydrogen ion at the optimal pH, \( (H^+)_{\text{opt}} \), was calculated from pH\(_{\text{opt}}\) which is equal to the
average of apparent p$K_1$ and p$K_2$. \((H^+)_1\) and \((H^+)_2\) (apparent $K_1$ and $K_2$) correspond to the concentrations of hydrogen ion when the pH values are equal to apparent p$K_1$ and p$K_2$, respectively.

**Hydrolysis of DE 10 maltodextrin**

Hydrolysis was performed at 35°C by using 28% (w/v) DE 10 maltodextrin in 0.025 M citrate-phosphate buffer (McIlvane, 1921) at different pH values. Sodium azide (0.02%) was used to inhibit microbial growth in the reaction mixtures. The enzyme concentration was 2.64 μM for wild-type and mutant GAs. Samples were taken at various times (from 0.5 to 120 h) and the reactions were stopped by adding samples to the same volume of 1 M Tris-HCl buffer at pH 7.0, since Tris is a known inhibitor of GA (Clarke & Svensson, 1984). The production of glucose was determined by the glucose oxidase method (Rabbo & Terkildsen, 1960). Experimental data were fitted to the equation $c = At/(1+Bt)$, where $c$ is the product concentration, $t$ is time, $A$ (the initial rate) and $B$ are obtained from the nonlinear regression.

**Results**

**Enzyme kinetics**

The kinetic parameters, $k_{cat}$ and $K_m$, for the hydrolysis of α-1,4-linked maltose and maltoheptaose and α-1,6-linked isomaltose at 45°C and pH 4.4 are given in Table 1. Mutant Ser411→Gly GA was highly active compared to wild-type GA, with increased $k_{cat}$ and $K_m$ values of 13 - 30% and 11 - 59%, respectively, on the substrates tested. The catalytic efficiencies ($k_{cat}/K_m$) were 71 - 116% that of wild-type GA. Mutant Ser411→Ala GA maintained 65 - 74% of wild-type GA catalytic efficiency with a slightly decreased $k_{cat}$ and a
Table 1. Kinetic parameters of wild-type and mutant GAs for hydrolysis of isomaltose, maltose and maltoheptaose

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild-type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ser411→Gly</td>
</tr>
<tr>
<td>Isomaltose (iG₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(k_{cat}(s^{-1}))</td>
<td>0.72 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93 ± 0.06</td>
</tr>
<tr>
<td>(K_m(mM))</td>
<td>23.5 ± 0.6</td>
<td>26.2 ± 2.7</td>
</tr>
<tr>
<td>(k_{cat}/K_m(s^{-1}mM^{-1}))</td>
<td>0.031 ± 0.001</td>
<td>0.036 ± 0.004</td>
</tr>
<tr>
<td>(\Delta(\Delta G)'/(kJ mol^{-1}))</td>
<td>-</td>
<td>-0.39</td>
</tr>
<tr>
<td>Maltose (G₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(k_{cat}(s^{-1}))</td>
<td>20.4 ± 0.2</td>
<td>23.0 ± 0.4</td>
</tr>
<tr>
<td>(K_m(mM))</td>
<td>1.01 ± 0.03</td>
<td>1.59 ± 0.08</td>
</tr>
<tr>
<td>(k_{cat}/K_m(s^{-1}mM^{-1}))</td>
<td>20.3 ± 0.6</td>
<td>14.5 ± 0.6</td>
</tr>
<tr>
<td>(\Delta(\Delta G)'/(kJ mol^{-1}))</td>
<td>-</td>
<td>0.89</td>
</tr>
<tr>
<td>Maltoheptaose (G₇)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(k_{cat}(s^{-1}))</td>
<td>72.3 ± 0.9</td>
<td>84.0 ± 2.5</td>
</tr>
<tr>
<td>(K_m(mM))</td>
<td>0.083 ± 0.004</td>
<td>0.132 ± 0.012</td>
</tr>
<tr>
<td>(k_{cat}/K_m(s^{-1}mM^{-1}))</td>
<td>870 ± 35</td>
<td>634 ± 41</td>
</tr>
<tr>
<td>(\Delta(\Delta G)'/(kJ mol^{-1}))</td>
<td>-</td>
<td>0.84</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined at 45°C in 0.05 M NaOAc buffer, pH 4.4.

<sup>b</sup>Standard error.

<sup>c</sup>Changes of transition-state energy \(\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]\).

<sup>d</sup>Not determined.
slightly increased $K_m$. Mutant Ser411→Cys GA maintained 54 - 73% of wild-type GA catalytic efficiency with a decrease in both the $k_{cat}$ and $K_m$ values. Since mutant Ser411→His and Ser411→Asp GAs had only about 6 - 12% of wild-type catalytic efficiency resulting from a seriously decreased $k_{cat}$ and an increased $K_m$, their kinetic parameters for the hydrolysis of isomaltose were not determined. Only mutant Ser411→His and Ser411→Asp GAs had large increases (5.5 to 7.5 kJ/mol) in the transition-state energy, $\Delta(\Delta G)$, for the hydrolysis of maltose and maltoheptaose. The large increases of transition-state energy indicated that the introduction of histidine or aspartic acid into position 411 substantially destabilized the binding between GA and substrate in the transition state.

**pH dependence of GA activity**

The kinetic parameters, $k_{cat}/K_m$ and $k_{cat}$, of the hydrolysis of maltose by wild-type and mutant GAs at different pH values were calculated from initial rates obtained at low (smaller than 0.2$K_m$) and high (higher than 10$K_m$) concentrations of maltose. The effects of pH on the $k_{cat}/K_m$ and $k_{cat}$ of maltose hydrolysis (Figures 2 and 3) were used to determine the pK values (Table 2) of both the free enzymes and the enzyme-substrate complexes. Although wild-type GA had a higher catalytic efficiency ($k_{cat}/K_m$) than all of the mutant GAs at all of the pH values tested (Figure 2), mutant Ser411→Gly and Ser411→Ala GAs had higher $k_{cat}$ values than that of wild-type GA at some pH values (Figure 3). The uncomplexed and maltose-complexed Ser411→His and Ser411→Asp GAs showed more narrow bell-shaped curves than that of wild-type GA. The effects of pH on the hydrolysis of maltoheptaose by wild-type, Ser411→Gly and Ser411→Ala GAs were measured (Figure 4) to further investigate the change of pK values and optimal pH of enzyme-substrate complexes using a long-length
Figure 2. pH-activity dependence for uncomplexed wild-type and mutant GAs. Maltose concentrations were 0.2, 0.2, 0.2, 0.1, 0.5 and 0.5 mM for wild-type (O), Ser411→Gly (●), Ser411→Ala (♦), Ser411→Cys (▼), Ser411→His (□) and Ser411→Asp (▲) GAs, respectively.
Figure 3. pH-activity dependence for maltose-complexed wild-type and mutant GAs. Maltose concentration was 40 mM for wild-type (○), Ser411→Gly (●), Ser411→Ala (◆), Ser411→Cys (▽), Ser411→His (□) and Ser411→Asp (▲) GAs.
Figure 4. pH-activity dependence for maltoheptaose-complexed wild-type (○), Ser411→Gly (●) and Ser411→Ala (◆) GAs. Maltoheptaose concentration was 2 mM.
Table 2. pK and optimal pH values of wild-type and mutant GAs for hydrolysis of maltose and maltoheptaose at 45°C

<table>
<thead>
<tr>
<th></th>
<th>Free enzyme (uncomplexed)</th>
<th>Enzyme-substrate complex (maltose-complexed)</th>
<th>Enzyme-substrate complex (maltoheptaose-complexed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK₁  pK₂  pH_{opt}</td>
<td>pK₁  pK₂  pH_{opt}</td>
<td>pK₁  pK₂  pH_{opt}</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.77  5.80  4.29</td>
<td>2.11  5.85  3.98</td>
<td>2.60  6.78  4.69</td>
</tr>
<tr>
<td>Ser411→Gly</td>
<td>3.01  5.57  4.29</td>
<td>2.68  5.81  4.24</td>
<td>3.22  6.73  4.98</td>
</tr>
<tr>
<td>Ser411→Ala</td>
<td>3.11  5.86  4.49</td>
<td>3.32  6.32  4.82</td>
<td>3.91  7.18  5.54</td>
</tr>
<tr>
<td>Ser411→Cys</td>
<td>3.26  5.86  4.56</td>
<td>3.32  6.38  4.85</td>
<td>ND  ND  ND</td>
</tr>
<tr>
<td>Ser411→His</td>
<td>3.10  5.01  4.05</td>
<td>3.58  4.69  4.13</td>
<td>ND  ND  ND</td>
</tr>
<tr>
<td>Ser411→Asp</td>
<td>3.13  5.72  4.42</td>
<td>3.34  5.53  4.44</td>
<td>ND  ND  ND</td>
</tr>
</tbody>
</table>

*Not determined.
substrate (Table 2). Surprisingly, not only Ser411→Gly but also Ser411→Ala GA were highly active compared to wild-type GA at optimal pH. Wild-type GA pK₁ values (ionization of the catalytic base) were 2.77, 2.11, and 2.6 for the free enzyme, the maltose-complexed form, and the maltoheptaose-complexed form, respectively. The pK₂ values (ionization of the catalytic acid) of wild-type were 5.80, 5.85, and 6.78 for the free enzyme, the maltose-complexed form, and the maltoheptaose-complexed form, respectively (Hiromi et al., 1966; Bakir et al., 1993; Sierks & Svensson, 1994). Compared to wild-type GA, the Ser411→Gly mutation increased the pK₁ of both the maltose-complexed form and the maltoheptaose-complexed form by ~0.6 units, whereas the Ser411→Gly mutation had no effect on the pK₂ of either enzyme-substrate complexes and only had a minor effect on the pK₁ and pK₂ of the free enzyme. The combined effect of the Ser411→Gly mutation on pK₁ and pK₂ was an increased optimal pH of both the maltose-complexed form and the maltoheptaose-complexed form by ~0.3 units. The Ser411→Gly mutation, however, had no effect on the optimal pH of the free enzyme. The Ser411→Ala and Ser411→Cys mutations had very similar effects on the pH dependence of maltose hydrolysis, increasing the pK₁ of the free enzyme and the maltose-complexed form by 0.3 - 0.5 and 1.21 units, respectively. Surprisingly, the Ser411→Ala and Ser411→Cys mutations also increased the pK₂ of the maltose-complexed form by ~0.5 units. In addition, the Ser411→Ala mutation increased the pK₁ and pK₂ of the maltoheptaose-complexed form by 1.31 and 0.4 units, respectively. The Ser411→His mutation increased the pK₁ of the free enzyme and the maltose-complexed form by 0.33 and 1.47 units, respectively; however, it decreased the pK₂ of the free enzyme and the maltose-
complexed form by 0.79 and 1.16 units, respectively. The Ser41→Asp mutation increased the pK\textsubscript{1} of the free enzyme and the maltose-complexed form by 0.36 and 1.23 units, respectively, while the Ser41→Asp mutation also decreased the pK\textsubscript{2} of the maltose-complexed form by 0.32 units. For wild-type, Ser41→Gly, and Ser41→Ala GAs, the values of pK\textsubscript{1}, pK\textsubscript{2}, and pH\textsubscript{opt} for the maltoheptaose-complexed forms were higher than those of the corresponding maltose-complexed forms by ~0.5, ~0.9 and ~0.7 units, respectively. For Ser41→Gly and Ser41→Ala GAs, the increases in optimal pH (compared to that of wild-type GA) obtained using the long-length substrate (maltoheptaose) were almost the same as that obtained using the short-length substrate (maltose).

All five mutants at position 411 showed a shift of 0.15 to 0.87 units in the optimal pH of the enzyme-substrate complex compared to wild-type GA, mainly due to increased pK\textsubscript{1} values. Compared with other mutant GAs, Ser41→Ala GA was the best performing. This mutation increased the optimal pH by 0.84 units while also maintaining a high level of both catalytic activity (k\textsubscript{cat}) and catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}).

**Hydrolysis of DE 10 maltodextrin**

The hydrolysis of 28% (w/v) DE 10 maltodextrin was used to study the pH dependence of GA activity at a high concentration of a long-length substrate. DE 10 maltodextrin is a mixture of maltodextrins with an average (and major) degree of polymerization of 10. The production of glucose by wild-type and Ser41→Ala GAs during the hydrolysis of DE 10 maltodextrin at eleven different pH values was determined and used to calculate the initial rates of glucose production at different pH values (Figure 5). The production of glucose increased following a hyperbolic curve. Ser41→Ala GA had higher
Figure 5. Initial rates of glucose production by wild-type (●) and Ser411→Ala (■) GAs during DE 10 maltodextrin hydrolysis at different pH values. Hydrolysis was performed at 35°C and 28% (w/v) DE 10 maltodextrin with 0.02% sodium azide in 25 mM citrate-phosphate buffer at different pHs for 5 days.
initial rates of glucose production than wild-type GA when the pH values were above 6.6 (Figure 5).

Discussion

Ser411 was chosen as a target for site-directed mutagenesis because it hydrogen bonds to Glu400, the catalytic base, and Ser411 is replaced by Gly in several other related GAs (Aleshin et al., 1992, 1994a; Coutinho & Reilly, 1994). Substitutions of glycine and alanine at position 411 were designed to remove the hydrogen bond between Glu400 and Ser411 (Figure 1), which was expected to destabilize the carboxylate ion form of Glu400 and thus raise its pK. The Ser411→Gly mutation did result in an increase of the pK1 of the enzyme-substrate complex, which either used maltose or maltoheptaose as substrate, by ~0.6 units (Table 2). The alanine substitution also should decrease the polarity in the microenvironment around Glu400, which would raise the pK of Glu400. Substitution of cysteine at position 411 was designed to weaken or remove the hydrogen bond between Glu400 and Ser411. The very similar effects on pH dependence of Ser411→Ala and Ser411→Cys GAs suggest that the substitution of cysteine at position 411 did in fact remove the hydrogen bond between Glu400 and Ser411. Since loss of the hydrogen bond accounted for the change in pH dependence of both Ser411→Ala and Ser411→Cys GAs, the decreased polarity in the microenvironment around Glu400 may have had little effect.

The removal of the hydrogen bond between Glu400 and Ser411 increased the pK1 of the enzyme-substrate complexes by only ~0.6 units for Ser411→Gly GA, compared to ~1.2 units for Ser411→Ala and Ser411→Cys GAs. One explanation for the smaller increase in
the $pK_1$ of Ser411$\rightarrow$Gly GA is the possible presence of a water molecule in the space
between Gly411 and Glu400, which may form a hydrogen bond that compensates for the loss
of the hydrogen bond between Glu400 and Ser411 (Figure 6).

Substitution of histidine at position 411 was designed to remove the hydrogen bond
and also introduce a positive charge at position 411. The Ser411$\rightarrow$His mutation not only
increased the $pK_1$ of the free enzyme and enzyme-substrate complex but also decreased the
$pK_2$ of the free enzyme and enzyme-substrate complex. The contribution of the introduced
positive charge may be estimated from the comparison of Ser411$\rightarrow$His and Ser411$\rightarrow$Ala
GAs. The Ser411$\rightarrow$His mutation increased the $pK_1$ of the maltose-complexed form by only
0.26 units relative to that of Ser411$\rightarrow$Ala GA, while it reduced the $pK_2$ of the free enzyme
and maltose-complexed form by 0.85 and 1.63 units relative to that of Ser411$\rightarrow$Ala GA.
Since the distance between atom OE1 of Glu179 and atom OG of Ser411 is 10.9 Å (Aleshin
et al., 1994a) and the side-chain of histidine is bigger than that of serine, the distance between
atom OE1 of Glu179 and the imidazole of His411 should be much closer. The decreased $pK_2$
values of the free enzyme and maltose-complexed form in Ser411$\rightarrow$His GA indicate that the
positive charge of histidine stabilized the negatively charged form of Glu179 and then
lowered the $pK_2$ values. The electrostatic interaction between two ions can exist at a distance
as far as 14-15 Å and still affect the pH dependence of an enzyme (Thomas et al., 1985).

Substitution of aspartate at position 411 was designed to remove the hydrogen bond
and also introduce a negative charge at position 411. The Ser411$\rightarrow$Asp mutation increased
the $pK_1$ of the free enzyme and the maltose-complexed form, and decreased the $pK_2$ of the
Figure 6. (A) Hydrogen bonding network in wild-type GA.
(B) The putative hydrogen bonding network in Ser411→Gly GA.
maltose-complexed form. Since the Ser411→Asp mutation produced similar increases in the pK1 as that of the Ser411→Ala mutation, the negative charge introduced by the Ser411→Asp mutation apparently did not contribute to the pK1 of the free enzyme and enzyme-substrate complex. However, the Ser411→Asp mutation did decrease the pK2 of the free enzyme and the maltose-complexed form by 0.14 and 0.79 units, respectively, relative to that of Ser411→Ala GA. In light of the large decrease in the pK2 of the maltose-complexed form compared to that of the free enzyme of Ser411→Asp GA and the expected increase in the pK values by introducing a negative charge, the lowered pK2 values may alternatively be accounted for by the effect of increased size of the amino acid side chain on the maltose binding instead of electrostatic interaction between Asp411 and Glu179.

In wild-type GA, the pK1 for the maltose-complexed form was lower than that of the free enzyme form by 0.66 units, indicating that the ionization of the carboxyl group of Glu400 was enhanced by the binding of maltose (Hiromi et al., 1966). Interestingly, the Ser411→Gly mutation decreased this difference to 0.33 units, whereas in the other four mutants, the pK1 values of the maltose-complexed form were higher than that of the free enzyme by 0.06 - 0.48 units. These results indicate that the mutation at position 411 weakened or even reversed the effect of maltose binding on the ionization of Glu400.

In general, the mutations at position 411 affected the pK values and optimal pHs of the enzyme-substrate complex more than that of the free enzyme. This may be explained from the three-dimensional structures of GA complexed with the inhibitor acarbose (Aleshin et al., 1994a, 1996) which showed that the 6-OH of the first sugar residue of acarbose
hydrogen bonds to Wat500 (Figure 1), and Wat500 is involved in the hydrogen bonding network in the active site (Figure 6A). The hydrogen bonding network around position 411 might be affected more in enzyme-substrate complex than in the free enzyme.

In summary, the hydrogen bond between Glu400 and Ser411 plays an important role in the pH dependence of GA. All five mutants at position 411 successfully increased the optimal pH of their respective enzyme-substrate complexes from 0.15 to 0.87 units, mainly due to increased pK values. Ser411→Ala is the best pH performing mutant of GA isolated to date, increasing the optimal pH by 0.84 units while maintaining a high level of catalytic activity and catalytic efficiency. The hydrolysis of 28% DE 10 maltodextrin also showed that Ser411→Ala GA has the potential to be used at higher pHs in starch saccharification.

Acknowledgments

This project was supported by the Consortium for Plant Biotechnology Research and Genencor International, Inc. (Palo Alto, CA). We thank Dr. James Meade for the gift of the wild-type GA gene and plasmid, Dr. Pedro M. Coutinho for the fruitful suggestions and discussion, and Drs. Wen-Chy Chu, Harold Hills and Gary Polking of the Iowa State University Nucleic Acid Facility for help with DNA sequencing and oligonucleotide synthesis.

References


PROTEIN ENGINEERING THE SUBSTRATE SPECIFICITY OF GLUCOAMYLASE FROM ASPERGILLUS AWAMORI TO REDUCE ISOMALTOSE FORMATION FROM THE GLUCOSE CONDENSATION REACTION

A paper to be submitted to *Protein Engineering*

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**Abstract**

Site-directed mutagenesis was used to construct glucoamylase variants designed to reduce the isomaltose formation from the glucose condensation reaction and thus increase the glucose yield in starch processing. In order to maintain wild-type glucoamylase ability to digest α-1,4 linked substrates, six mutations were designed to produce minor changes in the active site. Mutations were based on both homology analysis of amino acid sequences of GAs and the three-dimensional structure of glucoamylase at positions that are not totally conserved or unchangeable. Compared to wild-type glucoamylase, Tyr175→Phe, Arg241→Lys and Ser411→Gly glucoamylases were highly active for the hydrolysis of isomaltose and maltooligodextrins of DP 2 - 7 at pH 4.4. Tyr116→Trp and Ser411→Ala glucoamylases had moderately decreased activity, while Tyr48Phe49→Trp glucoamylase had severely decreased activity. The ratio of the initial rate of isomaltose production (from glucose condensation reactions) to that of glucose production (from the hydrolysis of DE 10 maltodextrin) was used to represent the ability of glucoamylase to synthesize isomaltose at a normalized level of DE 10 maltodextrin hydrolytic activity. Mutant Tyr175→Phe,
Ser411→Ala and Ser411→Gly glucoamylases had decreased ratios of the initial rate of isomaltose production to that of glucose production by 15, 32 and 58 at 35°C, respectively, and by 24, 60 and 62 at 55°C, respectively, compared to that of wild-type glucoamylase. Arg241→Lys glucoamylase had a very similar ratio as that of wild-type glucoamylase at both 35 and 55°C. Tyr116→Trp glucoamylase increased this ratio by 26 and 55% at 35 and 55°C, respectively. Our hypothesis that decreasing the ability of glucoamylase to synthesize isomaltose (via α-1,6 activity) would result in increased glucose yield has been supported in this study. Mutations Tyr175→Phe, Ser411→Ala and Ser411→Gly have great potential for increasing the glucose yield in starch processing.

Key words: glucoamylase/substrate specificity/protein engineering/site-directed mutagenesis/isomaltose/glucose yield/glucose condensation reaction/reversion product

Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) (GA) is an enzyme that catalyzes the release of β-D-glucose from the nonreducing ends of starch and related oligo- and polysaccharides. Although GA hydrolyzes primarily the α-1,4-glucosidic linkage, it also hydrolyzes α,β-1,1-, α-1,2-, α-1,3- and α-1,6-glucosidic linkages slowly (Pazur & Kleppe, 1962; Meagher et al., 1989). GA is widely used in industry to produce high-glucose syrups which are used as fermentation substrates to produce ethanol and other products or are converted to fructose sweeteners. The yield of glucose in starch processing, however, is limited by the unwanted synthesis of reversion products containing the above glucosidic linkages. These reversion products, resulting from glucose condensation, include α, β-
trehalose, kojibiose, nigerose, maltose, isomaltose, panose, isomaltotriose, and isomaltotetraose (Nikolov et al., 1989). The production of reversion products, especially isomaltose, limits the yield of D-glucose to about 96%. If the substrate specificity of GA could be engineered in order to decrease the ability of the enzyme to synthesize isomaltose via α-1,6-glucosidic linkages while at the same time maintaining wild-type levels of α-1,4 hydrolytic activity, an increased D-glucose yield of up to 2% would be expected.

The substrate specificity of an enzyme is determined by its ability to form a stable complex with a ligand in both the ground state and the transition state. The stability of the enzyme-ligand complex is affected by steric constraints, hydrogen bonding, van der Waal's and electrostatic forces, and hydrophobic contacts (Fersht, 1985). Kinetic studies have indicated that there are five to seven glucosyl binding subsites in GA, and the catalytic site is located between subsites 1 and 2 (Hiromi et al., 1973; Hiromi et al., 1983; Tanaka et al., 1983; Meagher et al., 1989). The catalytic domain of GA contains thirteen α-helices, twelve of which are arranged in pairs forming an α/α barrel (Aleshin et al., 1992). The cavity in the center of the barrel is defined by five regions of highly conserved residues, which form the active site. Homology analysis (Coutinho & Reilly, 1994) and understanding of the three-dimensional structures of GA complexed with inhibitors (Harris et al., 1993; Aleshin et al., 1994a, 1994b, 1996; Stoffer et al., 1995) make alternation of substrate specificity of GA meeting industrial requirements possible using site-directed mutagenesis.

In this study, site-directed mutagenesis was used to construct six mutations in the active site designed to reduce isomaltose formation due to glucose condensation in order to
increase the glucose yield of starch processing. The six mutations were designed to have decreased ability to synthesize isomaltose (an α-1,6 linked product) while maintaining wild-type ability to digest α-1,4 linked substrates. All mutations were made at positions that are not completely conserved based on homology analysis of GA amino acid sequences (Coutinho & Reilly, 1994). The stereo view of the GA active site in its complex with acarbose (a pseudotetrasaccharide inhibitor) (Aleshin et al., 1994a) is given in Figure 1. The reasons why each mutation was made are as follows. 1) Replacement of Tyr48 and Phe49 by Trp: Most GAs have Tyr and Phe (or Tyr) corresponding to positions 48 and 49 of Aspergillus awamori GA. Neurospora crassa GA, however, has only one Trp in the position of residues 48 and 49. These positions are involved in the first conserved region of the active site. Tyr48 hydrogen bonds to Glu400, the catalytic base, and makes a nonbonded contact to acarbose (Figure 1). 2) Tyr116→Trp: The three-dimensional structure of GA complex with acarbose has shown that the 6-OH of the second sugar of acarbose extends into a void which is large enough to accommodate an additional sugar residue (Aleshin et al., 1994a) (Figure 1). This void has been suggested to be the location of the α-1,6-branch during starch hydrolysis (Honzatko, 1995). The amino acid side-chains of Tyr116 are at the edge of the void. This residue is replaced by Phe in several other GAs. Changing Tyr116 to Trp was designed to limit the size of this void and also introduce more hydrophobic interactions, thus limiting α-1,6 activity. 3) Tyr175→Phe: Tyr175 is within the third conserved region. The nearest distance between Tyr175 and the fourth sugar residue of inhibitor D-gluco-dihydroacarbose is 4.06 Å (Stoffer et al., 1995). Tyr175 is replaced by Phe or Gln in several
Figure 1. Stereo view of the active site of GA from *Aspergillus awamori* var. X100 (Aleshin et al., 1994a) in its complex with acarbose (bold line). Dashed lines represent hydrogen bonds.
other GAs. Changing Tyr175 to Phe was designed to increase the hydrophobic interactions between enzyme and substrate. 4) Arg241→Lys: This position is not in the five conserved regions of the active site. This residue, however, hydrogen bonds to Glu180 and Tyr306, which are both highly conserved residues in the active site. In addition, Glu180 hydrogen bonds to the second sugar residue of acarbose (Figure 1). The GAs from *Rhizopus oryzae* and *Clostridium* sp. *G005* have Lys in the position corresponding to residue 241. The Arg241→Lys mutation was designed to alter the binding between Glu180 and the second sugar residue of the substrate. 5) Ser411→Gly and Ser411→Ala: Ser411 hydrogen bonds to the catalytic base Glu400 (Figure 1), and is replaced by Gly in some GAs. Although mutations Ser411→Gly and Ser411→Ala were originally designed to remove the hydrogen bond between Ser411 and Glu400 in order to raise the optimal pH of GA (Fang & Ford, 1997), these mutations were also tested for their effects on substrate specificity.

**Materials and Methods**

**Materials**

The yeast strain *Saccharomyces cerevisiae* C468 (α leu2-3 leu2-112, his3-15, mal') and the yeast expression vector YEpm18 (containing the wild-type GAI cDNA from *A. awamori*) were gifts from Cetus Corporation (Emeryville, CA). Acarbose was a gift from Miles Laboratories (Elkhart, IN). Maltose, α,β-trehalose, nigerose, maltotriose, panose, isomaltotriose, isomaltotetraose, maltoheptaose, glucose oxidase and peroxidase were from Sigma (St. Louis, MO). Kojibiose was from Koch-Light Laboratories Ltd. (Colnbrook Bucks, England). Isomaltose, maltotetraose, maltopentaose and maltohexaose were from TCI
America (Portland, OR). DE (dextrose equivalent) 10 maltodextrin (Maltrin® M100) was from Grain Processing Corporation (Muscatine, IA). Restriction enzymes, T4 DNA ligase and pGEM-7Z(+), an *Escherichia coli* phagemid vector, were from Promega (Madison, WI). High performance thin layer chromatography (HPTLC) plates (LHPK silica gel 60 Å, 20 x 10 cm) were obtained from Whatman (Hillsboro, OR).

**Construction of mutant GA genes**

The GA gene was mutated by site-directed mutagenesis according to the protocols of the Muta-Gene phagemid *in vitro* mutagenesis kit (from Bio-Rad, Hercules, CA) based on the method described by Kunkel et al. (1987). pGEM-GA, an *E. coli* phagemid vector containing the wild-type GA cDNA, was transformed into *E. coli* CJ236 (*dut*, *ung*) to produce uracil-containing single-stranded DNA to be used as the template in site-directed mutagenesis. pGEM7-GA was constructed by inserting a small *Xho* I-*EcoR*I fragment, which contained the GAI cDNA, of YEPM18 into pGEM-7Z(+). Mutation-containing oligonucleotide primers were synthesized in the Nucleic Acid Facility of Iowa State University, including 5'-GAT AAC CCG GAC TGG TAC ACC TGG ACT C-3' (Tyr48Phe49→Trp), 5'-GAT GAG ACT GCC TGG ACT GGT TCT TGG G-3' (Tyr116 to Trp), 5'-AAC CAG ACA GGA TTT GAT CTA TGG GAA GAA-3' (Tyr175→Phe), 5'-TTC GAT AGC AGC AAA TCC GGC AAG GAC-3' (Arg241→Lys), 5'-GGC GAG CAG CTT GGA GCA CGC GAC CTG AC-3' (Ser411→Gly), 5'-GGC GAG CAG CTT GCA GCA CGC GAC CTG AC-3' (Ser411→Ala). The nucleotides for designed GA mutations are shown in bold. The silent mutations, decreasing the melting temperature of primer hairpins, are underlined. All designed
mutations were verified by DNA sequencing. The mutated GA cDNAs were subcloned into the yeast expression vector YEpPM18, and transformed into *S. cerevisiae* C468 as previously described (Chen et al., 1994).

**Production and purification of GA**

Wild-type and mutant GAs were produced by growing YEpPM18-transformed *S. cerevisiae* C468 at 30°C for 5 days with shaking (170 rpm) in 2-L flasks each containing 1 L SD-His broth (Innis et al., 1985). The culture supernatants containing secreted GA were concentrated and diafiltrated with 0.5 M NaCl-0.1 M NaOAc buffer at pH 4.3, and purified by acarbose-Sepharose affinity chromatography (Chen et al., 1994).

**Protein concentration measurement**

The concentration of GA was measured by the Pierce bicinchoninic acid protein assay with bovine serum albumin as a standard (Smith et al., 1985).

**Enzyme kinetics**

Initial rates of hydrolysis of isomaltose and linear maltooligodextrins (G$_2$ - G$_7$) were determined at 45°C in 0.05 M NaOAc buffer at pH 4.4 by using ten to twelve substrate concentrations ranging from 0.1$K_m$ to 10$K_m$. Samples were taken at five different time points and reactions were quenched by adding 0.4 volumes of 4 M Tris-HCl buffer, pH 7.0. The release of glucose was measured by the glucose oxidase method (Rabbo & Terkildsen, 1960). $k_{cat}$ and $K_m$ were calculated by fitting initial rates, $v$, as a function of substrate concentration, [S], to the Michaelis-Menten equation using Enzfitter software (Elsevier-BIOSOFT, Amsterdam, The Netherlands). Experiments were duplicated and the average initial rates were used. The change of transition-state binding energy ($\Delta (\Delta G)$) for substrate hydrolysis
caused by the mutation, which was used to estimate the binding strength of the substrate in the transition-state complex, was calculated by the equation $\Delta(\Delta G) = -RT \ln\left(\frac{(k_{cat}/K_m)_{mut}}{(k_{cat}/K_m)_{wt}}\right)$, where the subscripts $mut$ and $wt$ refer to mutant and wild-type enzymes, respectively (Wilkinson et al., 1983).

**Hydrolysis of DE 10 maltodextrin**

Hydrolysis was performed at 35 and 55°C with 28% (w/v) DE 10 maltodextrin as substrate in 0.05 M NaOAc buffer at pH 4.4 with the addition of 0.02% sodium azide, used to inhibit microbial growth in the reaction mixtures. The enzyme concentration was 2.64 μM for both wild-type and mutant GAs. Samples were taken at various times (from 0.5 to 288 h) and the reactions were stopped by adding samples to the same volume of 1 M Tris-HCl buffer at pH 7.0, since Tris is a known inhibitor of GA (Clarke & Svensson, 1984). The production of glucose was determined by the glucose oxidase method (Rabbo & Terkildsen, 1960). Initial rates of glucose production were determined by fitting the experimental data to the equation $c = At/(1+Bt)$, where $c$ is the product concentration, $t$ is time, and $A$ (the initial rate) and $B$ are obtained from the nonlinear regression. At 55°C, only the time points before 70 h were used for the calculations, since the glucose concentration by that time had already declined for wild-type GA.

**Glucose condensation reactions**

Glucose condensation reactions were performed at 35 and 55°C with 30% (w/v) D-glucose as substrate in 0.05 M NaOAc buffer at pH 4.4 for 12 days with the addition of 0.02% sodium azide. The enzyme concentration was 2.64 μM for both wild-type and mutant
GAs. Samples were taken at various times and the reactions were stopped by adding samples to the same volume of 1 M Tris-HCl buffer at pH 7.0. High performance thin layer chromatography (HPTLC) and imaging densitometry were used to determine the production of isomaltose by a method modified from that described by Robyt and Mukerjea (1994). One microliter of variously diluted samples and six different concentrations of standard (containing glucose, maltose and isomaltose) were applied to the HPTLC plates. The developing solvent system contained acetonitrile, ethyl acetate, 1-propanol and water in the volume proportions of 85:20:50:40. Only one ascent was used to develop the carbohydrate separation on HPTLC plates. After development, the plates were air-dried, dipped into an EtOH solution containing 0.3% (w/v) α-naphthol and 5% (v/v) H₂SO₄, air-dried again, and incubated ~10 min at 120°C to visualize the carbohydrates. Densities of the isomaltose spots on HPTLC plates were quantified by imaging densitometry (Bio-Rad, Model GS-670), using Molecular Analyst software (Bio-Rad). The experimental data were fitted to the equation \( c = \frac{At}{(1 + Br)} \), described above for the hydrolysis of DE 10 maltodextrin, to obtain the initial rates of isomaltose production.

**Results**

**Enzyme kinetics**

Kinetic parameters (\( k_{cat} \) and \( K_m \)) for the hydrolysis of \( \alpha-1,6 \)-linked isomaltose and \( \alpha-1,4 \)-linked maltooligodextrins (DP 2-7) at 45°C and pH 4.4 are given in Table 1. Mutant Tyr48Phe49→Trp lowered the catalytic efficiencies (\( k_{cat}/K_m \)) for hydrolysis of maltose and maltoheptaose by 850-2100 fold. Since the activity of mutant Tyr48Phe49→Trp was so low,
Table 1. Kinetic parameters of wild-type and mutant GAs for hydrolysis of isomaltose and maltooligodextrins of DP 2 - 7

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>(k_{\text{cat}}/K_m) (G)</th>
<th>(k_{\text{cat}}/K_m) (IG)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(s')</td>
<td>(mM)</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
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<td></td>
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<tr>
<td>(k_m) (s')</td>
<td>0.72 ± 0.01b</td>
<td>20.4 ± 0.2</td>
<td>48.2 ± 0.7</td>
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<tr>
<td>(K_m) (mM)</td>
<td>23.5 ± 0.6</td>
<td>1.01 ± 0.03</td>
<td>0.25 ± 0.014</td>
</tr>
<tr>
<td>(k_{\text{cat}}/K_m) (s(^{-1})mM(^{-1}))</td>
<td>0.031 ± 0.001</td>
<td>20.3 ± 0.55</td>
<td>196 ± 9</td>
</tr>
<tr>
<td>Tyr48Phe49→Trp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(k_m) (s')</td>
<td>ND</td>
<td>0.236 ± 0.016</td>
<td></td>
</tr>
<tr>
<td>(K_m) (mM)</td>
<td>ND</td>
<td>9.9 ± 1.8</td>
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<tr>
<td>(k_{\text{cat}}/K_m) (s(^{-1})mM(^{-1}))</td>
<td>0.024 ± 0.003</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td>(\Delta(\Delta G)^c) (kJ mol(^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr116→Trp</td>
<td></td>
<td></td>
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<tr>
<td>(k_m) (s')</td>
<td>0.69 ± 0.02</td>
<td>11.7 ± 0.2</td>
<td>19.4 ± 0.3</td>
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<tr>
<td>(K_m) (mM)</td>
<td>28.8 ± 2.5</td>
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<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>(k_{\text{cat}}/K_m) (s(^{-1})mM(^{-1}))</td>
<td>0.024 ± 0.001</td>
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<td>98 ± 6</td>
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<tr>
<td>(\Delta(\Delta G)^c) (kJ mol(^{-1}))</td>
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<td>1.84</td>
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<td>Tyr175→Phe</td>
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<td>(k_m) (s')</td>
<td>1.02 ± 0.05</td>
<td>21.2 ± 0.2</td>
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<tr>
<td>(K_m) (mM)</td>
<td>40.1 ± 4.3</td>
<td>1.13 ± 0.04</td>
<td>0.29 ± 0.02</td>
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<tr>
<td>(k_{\text{cat}}/K_m) (s(^{-1})mM(^{-1}))</td>
<td>0.025 ± 0.002</td>
<td>18.8 ± 0.5</td>
<td>136 ± 6</td>
</tr>
<tr>
<td>(\Delta(\Delta G)^c) (kJ mol(^{-1}))</td>
<td>0.55</td>
<td>0.20</td>
<td>0.97</td>
</tr>
</tbody>
</table>

\(^a\)Determined at 45°C in 0.05 M NaOAc buffer, pH 4.4.

\(^b\)Standard error.

\(^c\)Changes of transition-state energy \(\Delta(\Delta G)=-RT \ln[(k_{\text{cat}}/K_m)_{mut}/(k_{\text{cat}}/K_m)_{wt}]\).

\(^d\)Not determined.
Table 1 (cont’d).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (G_4)</th>
<th>$k_{cat}/K_m$ (iG_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg241→Lys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>1.34 ± 0.08</td>
<td>20.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>39.3 ± 5.8</td>
<td>2.27 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (s⁻¹mM⁻¹)</td>
<td>0.034 ± 0.003</td>
<td>8.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>$\Delta(\Delta G)$ (kJ mol⁻¹)</td>
<td>-0.28</td>
<td>2.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser411→Ala</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>0.63 ± 0.02</td>
<td>18.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>27.9 ± 2.9</td>
<td>1.26 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (s⁻¹mM⁻¹)</td>
<td>0.022 ± 0.002</td>
<td>15.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>$\Delta(\Delta G)$ (kJ mol⁻¹)</td>
<td>0.84</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser411→Gly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>0.93 ± 0.06</td>
<td>23.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>26.2 ± 2.7</td>
<td>1.59 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (s⁻¹mM⁻¹)</td>
<td>0.036 ± 0.004</td>
<td>14.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>$\Delta(\Delta G)$ (kJ mol⁻¹)</td>
<td>-0.39</td>
<td>0.89</td>
<td></td>
</tr>
</tbody>
</table>

* Determined at 45°C in 0.05 M NaOAc buffer, pH 4.4.
* Standard error.
* Changes of transition-state energy $\Delta(\Delta G) = -RT \ln(\frac{k_{cat}/K_m}{m} / \frac{k_{cat}/K_m}{r})$.
* Not determined.
only the hydrolysis of maltose and maltoheptaose were tested. Mutant Tyr116→Trp had moderately decreased $k_{cat}$ values. The $K_m$ values were slightly increased except for the hydrolysis of maltose and maltotriose. In addition, for hydrolysis of maltotriose and maltotetraose, $K_m$ values were not twofold different as in wild-type GA but had almost the same values. The catalytic efficiencies were 44 - 77% that of wild-type GA depending on the substrate tested. Mutant Tyr175→Phe GA, on the other hand, was highly active. The $k_{cat}$ and $K_m$ values were 83 - 141% and 106 - 171%, respectively, that of wild-type GA for the different substrates tested, and the catalytic efficiencies were 69 - 102% that of wild-type GA. Mutant Arg241→Lys GA was also highly active, having $k_{cat}$ and $K_m$ values 97 - 185% and 167 - 404%, respectively, that of wild-type GA, with catalytic efficiencies 28 - 110% that of wild-type GA. Mutant Ser411→Ala GA had moderately decreased activity, with the $k_{cat}$, $K_m$, and catalytic efficiency values 74 - 93%, 107 - 191%, and 48 - 74%, respectively, that of wild-type GA. Mutant Ser411→Gly GA, by contrast, was highly active, with the $k_{cat}$ and $K_m$ values 93 - 129% and 83 - 203%, respectively, that of wild-type GA, and with catalytic efficiencies 55 - 122% that of wild-type GA.

Transition-state energy was used to estimate the binding strength of the enzyme-substrate-complex in the transition state. The change of transition-state energy, $\Delta(\Delta G)$, associated with loss of a hydrogen bond between an uncharged group of substrate and an uncharged group of enzyme is about 2.1 - 6.3 kJ mol$^{-1}$. The change of transition-state energy for loss of a hydrogen bond between an uncharged group on the substrate and a charged group on the enzyme is about ~14.6 - 18.8 kJ mol$^{-1}$ (Fersht et al., 1985). The large change of
transition-state energy, $\Delta(\Delta G)$, for the hydrolysis of maltose and maltoheptaose by Tyr48Phe49$\rightarrow$Trp (17.8 and 20.3 kJ mol$^{-1}$) indicated loss of a charged hydrogen bond between GA and the substrate in the transition state. Other mutant GAs showed small $\Delta(\Delta G)$ values (from -0.52 - 3.36 kJ mol$^{-1}$), indicating these mutants only had a minor effect on substrate binding in the transition state.

Mutant Tyr175$\rightarrow$Phe GA increased the catalytic efficiency ratio of $\alpha$-1,4 activity (maltose as substrate) to $\alpha$-1,6 activity (isomaltose as substrate) by about 15% compared to that of wild-type GA. Therefore, the selectivity for hydrolysis of maltose ($\alpha$-1,4 linked) over isomaltose ($\alpha$-1,6 linked) was slightly increased, while maintaining almost the same hydrolytic activity as wild-type GA for maltose. Ser411$\rightarrow$Ala GA had a similar catalytic efficiency ratio as wild-type GA; however, Tyr116$\rightarrow$Trp, Arg241$\rightarrow$Lys and Ser411$\rightarrow$Gly GAs had decreased catalytic efficiency ratios of $\alpha$-1,4 activity (maltose as substrate) to $\alpha$-1,6 activity (isomaltose as substrate) compared to that of wild-type GA.

**Hydrolysis of DE 10 maltodextrin**

The hydrolysis of DE 10 maltodextrin was used to study the production of glucose by wild-type and mutant GAs at high substrate concentrations in order to simulate industrial conditions (Figure 2). DE 10 maltodextrin is a mixture of maltodextrins with an average (and major) degree of polymerization of 10. The initial rates of glucose production are given in Table 2.

At 35°C, the glucose yield from the hydrolysis of DE 10 maltodextrin was still apparently increasing over the 280-hour course of the experiment for all of the GAs tested.
**Figure 2.** (a) Production of glucose by wild-type and mutant GAs during hydrolysis of DE 10 maltodextrin at 35°C. This experiment was performed at 28% (w/v) DE 10 maltodextrin and 0.02% sodium azide in 0.05 M NaOAc buffer at pH 4.4 for 12 days. (○) wild-type, (■) Tyr116→Trp, (▲) Tyr175→Phe, (▼) Arg241→Lys, (◆) Ser411→Ala, (●) Ser411→Gly GAs.
Figure 2 (cont’d). (b) Production of glucose by wild-type and mutant GAs during hydrolysis of DE 10 maltodextrin at 55°C. This experiment was performed at 28% (w/v) DE 10 maltodextrin and 0.02% sodium azide in 0.05 M NaOAc buffer at pH 4.4 for 12 days. (●) wild-type, (■) Tyr116→Trp, (▲) Tyr175→Phe, (▼) Arg241→Lys, (◆) Ser411→Ala, (○) Ser411→Gly GAs.
Table 2. Initial rates of glucose and isomaltose production in the hydrolysis of DE 10 maltodextrin and glucose condensation reactions, respectively, at 35 and 55°C, and their ratios by wild-type and mutant GAs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Product</th>
<th>Glucose&lt;sup&gt;a&lt;/sup&gt; (G&lt;sub&gt;1&lt;/sub&gt;) x 10&lt;sup&gt;3&lt;/sup&gt; (mol/h*mol GA)</th>
<th>Isomaltose&lt;sup&gt;b&lt;/sup&gt; (iG&lt;sub&gt;2&lt;/sub&gt;) (mol/h*mol GA)</th>
<th>Ratio x 10&lt;sup&gt;3&lt;/sup&gt; (iG&lt;sub&gt;2&lt;/sub&gt;/G&lt;sub&gt;1&lt;/sub&gt;)</th>
<th>Relative ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>35°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td>125 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>59.2 ± 2.8</td>
<td>0.47</td>
<td>1</td>
</tr>
<tr>
<td>Tyr116→Trp</td>
<td></td>
<td>90.6 ± 3.6</td>
<td>54.0 ± 2.7</td>
<td>0.60</td>
<td>1.26</td>
</tr>
<tr>
<td>Tyr175→Phe</td>
<td></td>
<td>127 ± 9.7</td>
<td>51.3 ± 1.9</td>
<td>0.40</td>
<td>0.85</td>
</tr>
<tr>
<td>Arg241→Lys</td>
<td></td>
<td>147 ± 8.6</td>
<td>69.5 ± 2.7</td>
<td>0.47</td>
<td>1</td>
</tr>
<tr>
<td>Ser411→Ala</td>
<td></td>
<td>32.9 ± 2.3</td>
<td>10.5 ± 1.0</td>
<td>0.32</td>
<td>0.68</td>
</tr>
<tr>
<td>Ser411→Gly</td>
<td></td>
<td>108 ± 7</td>
<td>21.8 ± 3.1</td>
<td>0.20</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>55°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td>815 ± 67</td>
<td>432 ± 17</td>
<td>0.53</td>
<td>1</td>
</tr>
<tr>
<td>Tyr116→Trp</td>
<td></td>
<td>457 ± 29</td>
<td>376 ± 18</td>
<td>0.82</td>
<td>1.55</td>
</tr>
<tr>
<td>Tyr175→Phe</td>
<td></td>
<td>762 ± 32</td>
<td>309 ± 12</td>
<td>0.41</td>
<td>0.76</td>
</tr>
<tr>
<td>Arg241→Lys</td>
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<td>643 ± 36</td>
<td>349 ± 13</td>
<td>0.54</td>
<td>1.02</td>
</tr>
<tr>
<td>Ser411→Ala</td>
<td></td>
<td>261 ± 15</td>
<td>55.7 ± 2.3</td>
<td>0.21</td>
<td>0.40</td>
</tr>
<tr>
<td>Ser411→Gly</td>
<td></td>
<td>554 ± 27</td>
<td>113 ± 8</td>
<td>0.20</td>
<td>0.38</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined from the hydrolysis of 28% (w/v) maltodextrin 10 in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide.

<sup>b</sup>Determined from the glucose condensation reactions of 30% D-glucose in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide.

<sup>c</sup>Standard error.
The initial rates of glucose production for wild-type and Tyr175→Phe GAs were very similar. Arg241→Lys GA had slightly increased initial rates of glucose production compared to that of wild-type GA, whereas Tyr116→Trp and Ser411→Gly GAs had slightly decreased initial rates of glucose production compared to that of wild-type GA. Ser411→Ala GA had an unexpectedly low initial rate for the glucose production compared to that of wild-type GA.

At 55°C, all of the GAs, except Ser411→Ala GA, reached their highest glucose yield very fast (Figure 2b). The glucose yield of Ser411→Ala GA, on the other hand, slowly increased for a very long period of time. The initial rates of glucose production for wild-type and Tyr175→Phe GAs were still very similar; however, Arg241→Lys GA had a slightly decreased initial rate of glucose production compared to that of wild-type GA. Tyr116→Trp, Ser411→Ala and Ser411→Gly GAs had strongly decreased initial rates of glucose production compared to that of wild-type GA. The initial rates of glucose production at 55°C were 4 to 8 times higher than those at 35°C. Tyr116→Trp, Tyr175→Phe, Arg241→Lys, Ser411→Ala and Ser411→Gly GAs had increased initial rates of glucose production ~5, ~6, ~4, ~8 and ~5 times, respectively, from 35 to 55°C, while wild-type GA had a sevenfold increased initial rate of glucose production from 35 to 55°C.

**Glucose condensation reactions**

Glucose condensation reactions were used to study the ability of wild-type and mutant GAs to synthesize isomaltose at high glucose concentrations. The same concentrations of GAs (2.64 µM) were used as in the hydrolysis of DE 10 maltodextrin. Isomaltose production
was determined by HPTLC and imaging densitometry. The HPTLC separation of isomaltose from the other glucose condensation products and glucose is given in Figure 3. Figure 3 shows that isomaltose was well separated from glucose, maltose, α, β-trehalose, kojibiose, nigerose, isomaltose, panose, isomaltotriose and isomaltotetraose. The production of isomaltose by wild-type and mutant GAs during glucose condensation is given in Figure 4.

At 35°C, mutants Tyr116→Trp, Tyr175→Phe, Ser411→Ala and Ser411→Gly GAs produced less isomaltose than wild-type GA, whereas Arg241→Lys GA produced more isomaltose than wild-type GA (Figure 4a). This same relative relationship between wild-type and mutant GAs was also seen for the initial rates of isomaltose production (Table 2). In addition, isomaltose production for wild-type and mutant GAs increased almost linearly over the 280-hour course of the experiment at 35°C.

At 55°C, the initial rates of isomaltose production for Ser411→Ala and Ser411→Gly were much lower than wild-type GA (Figure 4b). Tyr116→Trp, Tyr175→Phe and Arg241→Lys GAs had a slightly decreased initial rate of isomaltose production, however, Tyr116→Trp GA had a much lower isomaltose production as reaction time increased compared to wild-type, Tyr175→Phe and Arg241→Lys GAs. Therefore, Tyr116→Trp GA might have decayed during this experiment. The initial rates of isomaltose production at 55°C were five to seven times greater than those at 35°C. Tyr116→Trp, Tyr175→Phe, Arg241→Lys, Ser411→Ala and Ser411→Gly GAs had ~7, ~6, ~5, ~5 and ~5 times increased initial rates of isomaltose production, respectively, from 35 to 55°C, while wild-type GA had a sevenfold increased initial rate of isomaltose production from 35 to 55°C.
Figure 3. HPTLC separation of isomaltose from the other glucose condensation products and glucose.

1~6: six different concentrations of standard containing glucose, maltose and isomaltose (0.125, 0.25, 0.5, 0.75, 1, 1.25 mg/ml for each sugar). 7~12: diluted samples (12X) of 96 h in glucose condensation reactions at 55°C; 7, wild-type GA; 8, Tyr116→Trp GA; 9, Tyr175→Phe GA; 10, Arg241→Lys GA; 11, Ser411→Ala GA; 12, Ser411→Gly GA. 13~18: glucose condensation products; 13, α,β-trehalose; 14, nigerose; 15, panose; 16, isomaltotriose; 17, isomaltotetraose; 18, kojibiose.
Figure 4. (a) Production of isomaltose by wild-type and mutant GAs during glucose condensation at 35°C. This experiment was performed at 30% (w/v) D-glucose in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide for 12 days. (○) wild-type, (■) Tyr116→Trp, (▲) Tyr175→Phe, (▼) Arg241→Lys, (●) Ser411→Ala, (♦) Ser411→Gly GAs.
Figure 4 (cont'd). (b) Production of isomaltose by wild-type and mutant GAs during glucose condensation at 55°C. This experiment was performed at 30% (w/v) D-glucose in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide for 12 days. (○) wild-type, (■) Tyr116→Trp, (▲) Tyr175→Phe, (▼) Arg241→Lys, (♦) Ser411→Ala, (●) Ser411→Gly GAs.
Selectivity of GA for the synthesis of α-1,6 linked products versus the hydrolysis of α-1,4 linked substrates

The ratio of the initial rate of isomaltose production (from glucose condensation reactions) to that of glucose production (from the hydrolysis of DE 10 maltodextrin) was calculated to evaluate selectivity for the synthesis of α-1,6 linked products versus the hydrolysis of α-1,4 linked substrates. This ratio represents the ability of GA to synthesize isomaltose at a normalized level of DE 10 maltodextrin hydrolytic activity.

Mutant Tyr175→Phe, Ser411→Ala and Ser411→Gly GAs had a decreased ratio of the initial rate of isomaltose production to that of glucose production by 15, 32 and 58% at 35°C, respectively, and a decreased ratio by 24, 60 and 62% at 55°C, respectively, compared to wild-type GA (Table 2). Mutant Tyr116→Trp GA had an increased ratio by 26 and 55% at 35 and 55°C, respectively, compared to wild-type GA. Arg241→Lys GA had a very similar ratio to that of wild-type GA at both 35 and 55°C. Since the increase of initial rates of glucose and isomaltose production due to raising the temperature were not always proportional, the ratio of the initial rate of isomaltose production to that of glucose production is apparently temperature dependent.

We have hypothesized that selectively decreasing the ability of GA to synthesize isomaltose (via α-1,6 activity) would increase the glucose yield. Since the hydrolysis of DE 10 maltodextrin and the glucose condensation reactions were performed in the same buffer system using the same amount of GA, these reactions were compared to test our hypothesis. Since the hydrolysis of DE 10 maltodextrin at 35°C did not reach completion, we used only
the data obtained at 55°C. Instead of using glucose yield at an arbitrary single time point, the average glucose yield (after reaching 90%) was used in order to distinguish differences in yield within 5%. The average glucose yield was calculated using 13 to 17 time points for each GA. In addition, the average of glucose yields from top four contiguous points was also calculated and defined as peak glucose yield. Except for Tyr116→Trp GA, average glucose yields (or peak glucose yields) and initial rates of isomaltose production had a trend of linear relationship as shown in Figure 5. This result supports our hypothesis. Since Tyr116→Trp GA might have decayed during glucose condensation reactions at 55°C (Figure 4b), it was not surprising that this mutant GA did not follow the linear relationship shown by the wild-type and other mutant GAs. Since mutant Tyr175→Phe, Ser411→Ala and Ser411→Gly GAs had substantially decreased ratios of initial rates of isomaltose production to that of glucose production, these mutant GAs have great potential for increasing the glucose yield in starch processing.

Discussion

Although many mutations have been made in GA, this study is a first attempt to investigate the effects of mutations on substrate specificity using high concentrations of glucose and maltodextrin as substrates (conditions similar to that of industrial saccharification). Although altered substrate specificity of GA has been previously reported, only the hydrolysis of maltooligodextrins and isomaltooligodextrins were studied (Sierks et al., 1990, 1993; Sierks & Svensson, 1994; Fierobe et al., 1996). Glucose condensation reactions catalyzed by mutant GAs have rarely been studied. Sierks and Svensson (1994)
Figure 5. (a) The relationship between average glucose yields (after reaching 90%) and initial rates of isomaltose production in the hydrolysis of DE 10 maltodextrin and glucose condensation reactions, respectively, at 55°C. The hydrolysis of DE 10 maltodextrin 10 and glucose condensation were performed at 28% (w/v) DE 10 maltodextrin and 30% (w/v) D-glucose, respectively, in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide for 12 days.
Figure 5 (cont'd). (b) The relationship between peak glucose yields (average of glucose yields from top four contiguous points) and initial rates of isomaltose production in the hydrolysis of DE 10 maltodextrin and glucose condensation reactions, respectively, at 55°C. The hydrolysis of DE 10 maltodextrin and glucose condensation were performed at 28% (w/v) DE 10 maltodextrin and 30% (w/v) D-glucose, respectively, in 0.05 M NaOAc at pH 4.4 with 0.02% sodium azide for 12 days.
suggested that a mutant GA with enhanced selection for $\alpha$-1,4- over $\alpha$-1,6-linked substrates would decrease the production of $\alpha$-1,6-linked condensation products, and finally increase the glucose yield. This hypothesis, however, has not previously been tested. In this study, all mutant GAs increased the average glucose yield (or peak glucose yield) during DE 10 maltodextrin hydrolysis, and only Tyr175$\rightarrow$Phe showed slightly increased selection for $\alpha$-1,4- over $\alpha$-1,6-linked substrates.

Since we have been attempting to isolate mutants that ultimately could be used in the industrial saccharification to increase glucose yield, we did not want to lower $\alpha$-1,4 hydrolytic activity too much. $\alpha$-1,4 and $\alpha$-1,6 activity share the same cleavage site (between subsite 1 and subsite 2). It is therefore difficult to only change one catalytic activity (for $\alpha$-1,6 linkages) without affecting the other (for $\alpha$-1,4 linkages). In this study most of the mutations were designed to make minor changes in the active site, yet we anticipated that these minor changes might be large enough to decrease the enzyme ability to synthesize isomaltose.

*Mutant Tyr48Phe49$\rightarrow$Trp GA.* Unexpectedly, this mutant seriously decreased the catalytic efficiency. Although Tyr48Phe49$\rightarrow$Trp GA had only 1 - 3% of wild-type GA activity, this value is very similar to Tyr48$\rightarrow$Trp GA studied by Frandsen et al. (1994). Tyr48Phe49$\rightarrow$Trp GA, however, increased $K_m$ values ($\sim$10 and $\sim$60 times that of wild-type GA) much more than Tyr48$\rightarrow$Trp GA ($\sim$1.5 to 3.5 times that of wild-type GA). Therefore, the additional deletion of Phe49 apparently affects only substrate binding.
Mutant Tyr116→Trp GA. In spite of the introduction of a larger side-chain and increased hydrophobic interactions, this mutation had only a minor effect on the kinetic parameters for hydrolysis of α-1,4- and α-1,6-linked substrates (Table 1). During glucose condensation reactions at 55°C, Tyr116→Trp GA only had a slightly decreased initial rate of isomaltose production; however, this mutant GA had a much lower isomaltose production as reaction time increased compared to wild-type GA. Therefore, Tyr116→Trp GA might have decayed during this experiment. The thermostabilities of Tyr116→Trp and wild-type GAs have been studied at 70°C. Inactivation constants (kd) of Tyr116→Trp and wild-type GAs were 0.00556 and 0.00169 s⁻¹, respectively. Inactivation constants showed that Tyr116→Trp GA was ~3.3 times less stable than wild-type GA.

Mutant Tyr175→Phe GA. The kcat and Km values for the hydrolysis of maltooligodextrins were very similar to that of wild-type GA (Table 1). However, the kcat and Km values for isomaltose hydrolysis increased 41% and 71%, respectively. Therefore, the increased hydrophobic interaction near subsite 4 resulting from changing Tyr175 to Phe may affect the catalytic activity and substrate binding at subsites 1 and 2. The resultant minor change at subsites 1 and 2 produced a slightly increased average (and peak) glucose yield during DE 10 maltodextrin hydrolysis at 55°C (Figure 5) and decreased the ratios of initial rates of isomaltose production to that of glucose production in the glucose condensation reactions at 35 and 55°C.

Mutant Arg241→Lys GA. As seen in Figure 1, Arg241 hydrogen bonds to Glu180 and Tyr306. The replacement of Arg by Lys should theoretically retain the hydrogen bonding
network among Arg241, Tyr306 and Glu180, although it was anticipated that the strength of
the hydrogen bonds might be altered, thus altering hydrogen bonding between the substrate
and Glu180. Although the $k_{cat}$ values for the hydrolysis of maltooligodextrins were very
similar to that of wild-type GA (Table 1), the $K_m$ values increased ~2 - 4 times. The $k_{cat}$ and
$K_m$ values for the hydrolysis of isomaltose also increased 85 and 67%, respectively, compared
to that of wild-type GA. In spite of the changes in $k_{cat}$ and $K_m$ values, this mutation affected
both the hydrolysis of DE 10 maltodextrin and glucose condensation similarly. Arg241→Lys
GA, therefore, had very similar ratios of initial rates of isomaltose production to that of
glucose production at both 35 and 55°C compared to wild-type GA.

**Mutant Ser411→Ala and Ser411→Gly GAs.** In general, for the hydrolysis of both
isomaltose and maltooligodextrins, Ser411→Ala GA had moderately decreased $k_{cat}$ values
(74 -93% that of wild-type GA), whereas Ser411→Gly GA had slightly increased $k_{cat}$ values
(103 - 129% that of wild-type GA) except for the hydrolysis of maltotetraose (93% that of
wild-type GA). Both mutants caused only minor changes on the $K_m$ values (Table 1).
Although Ser411→Ala and Ser411→Gly GAs only had minor effects on the kinetic
parameters for the hydrolysis of both α-1,4- and α-1,6-linked substrates, Ser411→Ala and
Ser411→Gly GAs had increased average glucose yields in the hydrolysis of DE 10
maltodextrin at 55°C and decreased ratios of the initial rates of isomaltose production to that
of glucose production in the glucose condensation reactions at 35 and 55°C. An unexpected
loss of hydrolytic activity at high substrate concentration (28%) for both Ser411→Ala and
Ser411→Gly GAs was observed. During DE 10 maltodextrin hydrolysis, the initial rates of
glucose production for Ser411→Ala GA were only 26 and 32% that of wild-type GA at 35 and 55°C, respectively, and for Ser411→Gly GA they were 87 and 68% that of wild-type GA at 35 and 55°C, respectively. As seen in Figure 1, atom OG of Ser411 hydrogen bonds to atom OE1 of Glu400 (the catalytic base), atom OE2 of Glu400 hydrogen bonds to both the catalytic water (Wat500) and atom OH of Tyr48, and Wat500 also hydrogen bonds to atom O6A of the inhibitor acarbose. Ser411→Ala and Ser411→Gly GAs were designed to remove the hydrogen bond between Ser411 and Glu400. In our previous study (Fang & Ford, 1997) we showed that Ser411→Ala and Ser411→Gly GAs had increased pKᵢ values for the enzyme-substrate complex due to loss of a hydrogen bond between residue 411 and Glu400. In mutant Ser411→Gly GA, we have proposed the presence of a water molecule in the space between Gly411 and Glu400 to form a hydrogen bond that compensates for the loss of the hydrogen bond between Glu400 and Ser411. When the substrate concentration is very high, the substrate competes with the enzyme to form hydrogen bonds with water. Since the removal of a hydrogen bond between residue 411 and Glu400 decreased the binding strength of the catalytic water, at high substrate concentrations mutant Ser411→Ala and Ser411→Gly GAs might require more time for a new catalytic water to be properly positioned for each hydrolytic reaction, therefore reducing the rate of hydrolysis. Since Ser411→Gly GA was less affected by high concentrations of substrate than Ser411→Ala GA, it is possible that the proposed water molecule between Gly411 and Glu400 that hydrogen bonds to Glu400 may help to keep the catalytic water in its proper position.
In summary, based on the homology analysis of amino acid sequences of GAs and the three-dimensional structures of GAs with inhibitors, the designed minor changes in active site that we made were successful in altering substrate specificity and reducing isomaltose production during glucose condensation. Mutant Tyr175→Phe, Ser411→Ala and Ser411→Gly GAs decreased the ratios of the initial rates of isomaltose production to that of glucose production. These mutant GAs have great potential for increasing the glucose yield in starch processing.

Acknowledgments

This project was supported by the Consortium for Plant Biotechnology Research and Genencor International, Inc. (Palo Alto, CA). We thank Dr. James Meade for the gift of wild-type GA gene and plasmid, Dr. Richard B. Honzatko for the fruitful suggestions and discussion, and Drs. John F. Robyt and Motomitsu Kitaoka for help with isomaltose quantitation by thin-layer chromatography.

References


PROTEIN ENGINEERING THE SUBSTRATE SPECIFICITY OF GLUCOAMYLASE FROM ASPERGILLUS AWAMORI BY ALTERING THE HYDROGEN BONDING

A paper to be submitted to *Protein Engineering*

Tsuei-Yun Fang, Richard B. Honzatko, Peter J. Reilly and Clark Ford

**Abstract**

Site-directed mutagenesis was used to construct mutant glucoamylase (GA) with altered hydrogen bonding in order to change the substrate specificity of the enzyme. Four single mutations (Ser119→Glu, Ser119→Gly, Ser119→Trp and Gly121→Ala) and a double mutation (Gly121→Ala/Ser411→Gly) have been constructed. Previously, mutation Ser411→Gly had been found to decrease the selectivity of GA toward the formation of isomaltose. Compared to wild-type GA, mutant Ser119→Glu, Ser119→Gly and Ser119→Trp GAs were highly active in hydrolysis of isomaltose and maltooligodextrins of DP 2 - 7 at pH 4.4. Mutant Gly121→Ala and Gly121→Ala/Ser411→Gly GAs had moderately decreased activity. Mutant Gly121→Ala and Gly121→Ala/Ser411→Gly glucoamylases had an approximately twofold increased ratio of catalytic efficiency of α-1,4 activity (maltose as substrate) to α-1,6 activity (isomaltose as substrate) compared to that of wild-type GA, resulting in an approximately twofold increase in selectivity for hydrolysis of maltose (α-1,4 linked) over isomaltose (α-1,6 linked). The ratio of the initial rate of isomaltose production (from glucose condensation reactions) to that of glucose production...
(from the hydrolysis of DE 10 maltodextrin) was used to indicate the ability of GA to synthesize isomaltose at a normalized level of DE 10 maltodextrin hydrolytic activity. At 35°C, mutant Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs showed a decrease in this ratio of 14, 32, 42 and 68%, respectively, compared to that of wild-type GA, while mutant Ser119→Glu GA had a very similar ratio to that of wild-type GA. At 55°C, the double mutant Gly121→Ala/Ser411→Gly GA showed a decrease in this ratio of 56% compared to that of wild-type GA, whereas Ser119→Gly, Ser119→Trp and Gly121→Ala GAs had very similar ratios to that of wild-type GA. Ser119→Glu GA had an increased ratio 22% higher than that of wild-type GA. Although the effect of mutations Gly121→Ala and Ser411→Gly on the ratios of isomaltose production to that of glucose production was not additive, the double mutant Gly121→Ala/Ser411→Gly GA had the lowest ratio of isomaltose production to that of glucose production at 35°C. In summary, we have successfully changed the substrate specificity of GA by altering the hydrogen bonding between enzyme and substrate in the enzyme-substrate complex, resulting in reduced isomaltose formation during glucose condensation reactions.

Key words: glucoamylase/substrate specificity/protein engineering/site-directed mutagenesis/isomaltose/glucose yield/glucose condensation reaction/reversion product

Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) (GA) is an enzyme that catalyzes the release of β-D-glucose from the nonreducing ends of starch and related oligo- and polysaccharides. Although GA hydrolyzes primarily α-1,4-glucosidic linkages, it also
hydrolyzes $\alpha,\beta-1,1$, $\alpha-1,2$, $\alpha-1,3$ and $\alpha-1,6$-glucosidic linkages slowly (Pazur & Kleppe, 1962; Meagher et al., 1989). GA is widely used in industry to produce high-glucose syrups. At a high concentration of glucose, however, reversion products, especially isomaltose (containing an $\alpha-1,6$-glucosidic linkage), produced from glucose condensation limit the D-glucose yield to about 96%. If the substrate specificity of GA could be engineered to selectively decrease the ability of the enzyme to synthesize isomaltose via $\alpha-1,6$-glucosidic linkages, an increased D-glucose yield would be expected.

Kinetic studies have indicated that there are five to seven glucosyl binding subsites, and the catalytic site is located between subsites 1 and 2 (Hiromi et al., 1973, 1983; Tanaka et al., 1983; Meagher et al., 1989). The solved three-dimensional structure of the catalytic domain of GA from *Aspergillus awamori* var. X100, which has about 95% homology with the corresponding regions of GAs from *A. awamori* and *A. niger* (Coutinho & Reilly, 1994), contains thirteen $\alpha$-helices, twelve of which are arranged in pairs forming an $\alpha/\alpha$ barrel (Aleshin et al., 1992, 1994). The active site is located in the cavity of the barrel center. In addition, homology analysis of thirteen amino acid sequences of GAs showed that five conserved regions define the active site (Coutinho & Reilly, 1994). The mechanism of GA catalysis involves two carboxyl groups (Hiromi et al., 1966), Glu179 and Glu400 (in *A. awamori* or *A. niger* GAs) (Sierks et al., 1990; Harris et al., 1993; Frandsen et al., 1994). Glu179 protonates the oxygen in the glycosidic linkage, acting as a general acid catalyst, and Glu400 activates water (Wat500) for nucleophilic attack at carbon C-1, acting as a general base catalyst (Frandsen et al., 1994). The crystal structures of GA complexed with the
pseudotetrasaccharides (acarbose and D-glucodihydroacarbose), showed that there are two different binding conformers, pH 4-type and pH 6-type, for pseudotetrasaccharides at pH 4 (Stoffier et al., 1995; Aleshin et al., 1996). Binding of the first two sugar residues of the pseudotetrasaccharides is the same, but there is an extraordinary variation in binding of the third and fourth sugar residues of the pseudotetrasaccharides. The stereo view of the active site of GA complexed with D-glucodihydroacarbose (Stoffier et al., 1995) is given in Figure 1.

The substrate specificity of an enzyme is determined by its ability to form a stable complex with a ligand in both the ground state and the transition state. The stability of the enzyme-ligand complex is affected by steric constraints, hydrogen bonding, van der Waal's and electrostatic forces, and hydrophobic contacts (Fersht, 1985). In this study, site-directed mutagenesis was used to construct mutations at residues 119 and 121 to alter the hydrogen bonding between enzyme and substrate. Atom OG of Ser119 hydrogen bonds to the 3-OH of the fourth sugar residue of pseudotetrasaccharides only in the pH 6-type conformer, whereas the amide nitrogen of Gly121 hydrogen bonds to the 6-OH of the third sugar residue in both pH 4-type and pH 6-type conformers (Figure 1). Since we have been attempting to isolate mutations with potential for industrial application, the mutations in this study were designed to change substrate specificity (decrease α-1,6 condensation reactions) while maintaining wild-type ability to hydrolyze α-1,4 linked substrates. Ser119 is not conserved and is replaced by Ala, Pro and Glu in other GAs. Mutation Ser119→Glu was designed to strengthen the hydrogen bond between the enzyme and the fourth sugar residue of the
Figure 1. Stereo view of the active site of GA from Aspergillus awamori var. X100 (Stoffer et al., 1995) in its complex with D-glucodihydroacarbose (bold line). Dashed lines represent hydrogen bonds.
substrate to stabilize the pH 6-type conformer, and to bring a negative charge near subsite 4 in order to increase electrostatic interactions in the active site. Mutation Ser119→Gly was designed to remove the same hydrogen bond in order to destabilize the pH 6-type conformer. Mutation Ser119→Trp was designed to remove the same hydrogen bond and to increase the hydrophobic interactions between the enzyme and the pH 6-type conformer. Gly121 is highly conserved in all GA sequences except in Clostridium sp. G005 GA, which has high α-1,6 activity and in which Gly is replaced by Thr. Since the φ and ψ angles of Gly121 would allow an alanine in this position without causing a conformation distortion, the Gly121→Ala mutation was designed to introduce a β-carbon at position 121 to displace the 6-OH group of the third sugar residue from its hydrogen bonding position. In addition, the double mutation Gly121→Ala/Ser411→Gly was designed to investigate additivity of the two substrate specificity mutations. The Ser411→Gly mutation was previously shown to reduce the ratio of initial rates of isomaltose production (from glucose condensation reactions) to that of glucose production (from the hydrolysis of DE 10 maltodextrin) (Fang et al., 1997).

Materials and Methods

Materials

The yeast strain Saccharomyces cerevisiae C468 (α leu2-3 leu2-112, his3-15, mal') and the yeast expression vector YEpPM18 (containing the wild-type GAl cDNA from A. awamori) were gifts from Cetus Corporation (Emeryville, CA). Acarbose was a gift from Miles Laboratories (Elkhart, IN). Maltose, maltotriose, maltoheptaose, glucose oxidase and peroxidase were from Sigma (St. Louis, MO). Isomaltose, maltotetraose, maltopentaose and
maltohexaose were from TCI America (Portland, OR). DE (dextrose equivalent) 10 maltodextrin (Maltrin® M100 Maltodextrin) was from Grain Processing Corporation (Muscatine, IA). Restriction enzymes, T4 DNA ligase and pGEM-7Z(+), an *Escherichia coli* phagemid vector, were from Promega (Madison, WI). High performance thin layer chromatography (HPTLC) plates (LHPK silica gel 60 Å, 20 x 10 cm) were obtained from Whatman (Hillsboro, OR).

**Construction of mutant GA genes**

The GA gene was mutated by site-directed mutagenesis according to the protocols of the Muta-Gene phagemid *in vitro* mutagenesis kit (from Bio-Rad, Hercules, CA) (Fang & Ford, 1997) or according to the protocols of the Altered Sites II *in vitro* Mutagenesis Systems (from Promega). An *Xba I*-*Hind III* fragment (containing the wild-type GA cDNA) of pGEM-GA (Fang & Ford, 1997) was inserted into the pALTER-1 vector (from Promega) to construct a GA cDNA-containing vector, pALTER-1-GA, to be used as the double-stranded DNA template in the Altered Sites II *in vitro* mutagenesis systems. Mutation-containing oligonucleotide primers were synthesized at the Nucleic Acid Facility of Iowa State university, including 5'-GCC TAC ACT GGT GAA TGG GGA CGG CC-3' (Ser119→Glu), 5'-GCC TAC ACT GGT **GGA** TGG GGA CGG CC-3' (Ser119→Gly), 5'-CC TAC ACT GGT **TGG** TGG GGA CGG CC-3' (Ser119→Trp), and 5'-ACT GGT **TCT** TGG **GCT** CGG CCG CAG C-3' (G121→Ala). The nucleotides for designed GA mutations are shown in bold. The double mutation Gly121→Ala/Ser411→Gly was constructed by ligating an *Xho I*-Pst I fragment carrying the Gly121→Ala mutation and a Pst I-BamH I fragment carrying the
Ser411→Gly mutation (Fang & Ford, 1997) to the Xho I-BamH I fragment of YEpPM18 to reconstruct the yeast expression vector YEpPM18. For single mutations, the mutated GA cDNAs were subcloned into YEpPM18 as previously described (Chen et al., 1994). All designed mutations were verified by DNA sequencing of the entire subcloned fragment at the Nucleic Acid Facility of Iowa State University. The mutated YEpPM18 was transformed into S. cerevisiae C468 by electroporation.

Production and purification of GA

Wild-type and mutant GAs were produced by growing YEpPM18-transformed S. cerevisiae C468 at 30°C for 5 days with shaking (170 rpm) in 2-L flasks each containing 1 L of SD-His broth (Innis et al., 1985). The culture supernatants, containing secreted GA, were concentrated and diafiltrated in 0.5 M NaCl-0.1 M NaOAc buffer at pH 4.3, and purified by acarbose-Sepharose affinity chromatography (Chen et al., 1994).

Protein concentration measurement

The concentration of GA was measured by the Pierce bicinchoninic acid protein assay with bovine serum albumin as a standard (Smith et al., 1985).

Enzyme kinetics

Initial rates of hydrolysis of isomaltose and linear maltooligodextrins (G2 - G7) were determined at 45°C in 0.05 M NaOAc buffer at pH 4.4, using 10 to 12 substrate concentrations ranging from 0.1K_m to 10K_m. Samples were taken at five different time points and reactions were quenched by adding 0.4 volumes of 4 M Tris-HCl buffer, pH 7.0. The release of glucose was measured by the glucose oxidase method (Rabbo & Terkildsen, 1960). k_cat and K_m were calculated by fitting initial rates, v, as a function of substrate concentration,
[S], to the Michaelis-Menten equation using Enzfit software (Elsevier-BIOSOFT, Amsterdam, The Netherlands). Experiments were duplicated and the average initial rates were used. The change of transition-state binding energy ($\Delta(\Delta G)$) for substrate hydrolysis caused by the mutation, which was used to estimate the binding strength of the substrate in the transition-state complex, was calculated by the equation

$$
\Delta(\Delta G) = -RT \ln \left( \frac{(k_{cat}/K_m)_{mut}}{(k_{cat}/K_m)_{wt}} \right),
$$

where the subscripts \textit{mut} and \textit{wt} refer to mutant and wild-type enzymes, respectively (Wilkinson et al., 1983).

**Specific activity assays**

Specific activity assays were performed at 35, 45 and 55°C with 3.33% maltose in 0.05 M sodium NaOAc buffer at pH 4.4. Reactions were stopped at six different time points and the release of glucose was measured as described above for the enzyme kinetic studies. Experiments were duplicated and the average values were used. One unit (IU) is defined as the amount of enzyme required to produce 1 μmol glucose per minute under the conditions of the assay.

**Hydrolysis of DE 10 maltodextrin**

Hydrolysis of 28% (w/v) DE 10 maltodextrin was performed at 35 and 55°C in 0.05 M NaOAc buffer at pH 4.4 with the addition of 0.02% sodium azide to inhibit microbial growth in the reaction mixtures. The enzyme concentration was 2.64 μM for both wild-type and mutant GAs. Samples were taken at various times (from 0.5 to 288 h) and the reactions were stopped by adding samples to the same volume of 1 M Tris-HCl buffer at pH 7.0, since Tris is a known inhibitor of GA (Clarke & Svensson, 1984). The production of glucose was
determined by the glucose oxidase method (Rabbo & Terkildsen, 1960). Initial rates of glucose production were determined by fitting the experimental data to the equation \( c = \frac{At}{(1+Br)} \), where \( c \) is the product concentration, \( t \) is time, and \( A \) (the initial rate) and \( B \) are obtained from the nonlinear regression. At 55°C, only the time points before 70 h were used for the calculations, since the glucose concentration by that time had already declined for wild-type GA.

**Glucose condensation reactions**

Glucose condensation reactions were performed at 35 and 55°C with 30% (w/v) D-glucose in 0.05 M NaOAc buffer at pH 4.4 for 12 days. Sodium azide (0.02%) was used to inhibit microbial growth in the reaction mixtures. The enzyme concentration was 2.64 μM for both wild-type and mutant GAs. Samples were taken at various times and the reactions were stopped by adding samples to the same volume of 1 M Tris-HCl buffer at pH 7.0. High performance thin layer chromatography (HPTLC) and imaging densitometry were used to determine the production of isomaltose by a method modified from the one described by Robyt and Mucerjea (1994). One microliter of variously diluted samples and six different concentrations of standard (containing glucose, maltose and isomaltose) were applied to the HPTLC plates. The developing solvent system contained acetonitrile, ethyl acetate, 1-propanol and water in the volume proportions of 85:20:50:40. Only one ascent was used to develop the carbohydrate separation on HPTLC plates. After development, the plates were air-dried, dipped into an EtOH solution containing 0.3% (w/v) α-naphthol and 5% (v/v) \( \text{H}_2\text{SO}_4 \), air-dried again, and incubated ~10 min at 120°C to visualize the carbohydrates. Densities of the isomaltose spots on HPTLC plates were quantified by imaging densitometry.
(Bio-Rad, Model GS-670), using Molecular Analyst software (Bio-Rad). The experimental data were fitted to the equation $c = At/(1+Bt)$, described above for the hydrolysis of DE 10 maltodextrin, to obtain the initial rates of isomaltose production.

**Results**

**Enzyme kinetics**

Kinetic parameters ($k_{\text{cat}}$ and $K_m$) for the hydrolysis of $\alpha$-1,6-linked isomaltose and $\alpha$-1,4-linked maltooligodextrins (DP 2 - 7) at 45°C and pH 4.4 are given in Table 1.

Mutant Ser119→Glu GA was highly active, with $k_{\text{cat}}$ and $K_m$ values 81 - 131% and 74 - 148%, respectively, that of wild-type GA for the different substrates tested. $k_{\text{cat}}$ and $K_m$ values for the hydrolysis of DP 2 - 6 maltooligodextrins were very similar to that of wild-type GA, whereas the $k_{\text{cat}}$ values for the hydrolysis of isomaltose and maltoheptaose were larger than that of wild-type GA. The catalytic efficiencies ($k_{\text{cat}}/K_m$) were 66 - 121% that of wild-type GA.

Mutant Ser119→Gly GA was also highly active, with $k_{\text{cat}}$ and $K_m$ values 69 - 172% and 52 - 143%, respectively, that of wild-type GA. $k_{\text{cat}}$ values increased for hydrolysis of maltooligodextrins of DP 4 - 7, whereas they decreased for hydrolysis of isomaltose, maltose and maltotriose. The $K_m$ values decreased only for hydrolysis of isomaltose and maltose. The catalytic efficiencies of Ser119→Gly GA were 77 - 139% that of wild-type GA.

Mutant Ser119→Trp GA, again, was highly active, with $k_{\text{cat}}$ and $K_m$ values 74 - 131% and 139 - 301%, respectively, that of wild-type GA. $k_{\text{cat}}$ values increased only for the hydrolysis of isomaltose and maltoheptaose. Although catalytic efficiency was 94% that of
Table 1. Kinetic parameters of wild-type and mutant GAs for hydrolysis of isomaltose and maltooligodextrins of DP 2 - 7

| Enzyme | Substrate | \( k_{cat}/K_m \) (G|G|) | \( k_{cat}/K_m \) (IG|G|) |
|--------|-----------|----------------|-----------------|
| Wild-type\(^{e}\) | Isomaltose (IG|G|) | 656 | 422 |
| | Maltose (G|G|) | 435 |
| | Maltotriose (G|G|) | 459 |
| | Maltotetraose (G|G|) | | |
| | Maltopentaose (G|G|) | | |
| | Maltohexaose (G|G|) | | |
| | Maltoheptaose (G|G|) | | |
| Ser119\({\rightarrow}\)Glu | \( k_{cat} \) (s\(^{-1}\)) | 0.72 ± 0.01\(^{b}\) | 20.4 ± 0.2 | 48.2 ± 0.7 | 64.5 ± 2.9 | 71.8 ± 1.9 | 73.7 ± 2.1 | 72.3 ± 0.9 |
| | \( K_m \) (mM) | 23.5 ± 0.6 | 1.01 ± 0.03 | 0.25 ± 0.014 | 0.111 ± 0.017 | 0.110 ± 0.010 | 0.107 ± 0.010 | 0.083 ± 0.004 |
| | \( k_{cat}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) | 0.031 ± 0.001 | 20.3 ± 0.55 | 196 ± 9 | 582 ± 65 | 654 ± 43 | 685 ± 47 | 870 ± 35 |
| Ser119\({\rightarrow}\)Gly | \( k_{cat} \) (s\(^{-1}\)) | 0.53 ± 0.01 | 14.1 ± 0.4 | 38.4 ± 0.9 | 72.8 ± 2.2 | 75.7 ± 1.6 | 81.1 ± 2.4 | 124 ± 4 |
| | \( K_m \) (mM) | 12.2 ± 1.2 | 0.75 ± 0.09 | 0.26 ± 0.03 | 0.159 ± 0.015 | 0.111 ± 0.008 | 0.124 ± 0.012 | 0.112 ± 0.013 |
| | \( k_{cat}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) | 0.043 ± 0.004 | 18.7 ± 1.8 | 151 ± 12 | 458 ± 30 | 680 ± 39 | 652 ± 50 | 1107 ± 97 |
| | \( \Delta(\Delta G)^{c} \) (kJ mol\(^{-1}\)) | -0.89 | 0.21 | 0.69 | 0.63 | -0.10 | 0.13 | -0.64 |
| Ser119\({\rightarrow}\)Trp | \( k_{cat} \) (s\(^{-1}\)) | 0.94 ± 0.03 | 19.0 ± 0.7 | 45.0 ± 0.7 | 47.7 ± 2.3 | 59.8 ± 1.2 | 66.0 ± 3.3 | 88.0 ± 1.6 |
| | \( K_m \) (mM) | 32.7 ± 2.4 | 1.43 ± 0.16 | 0.48 ± 0.02 | 0.27 ± 0.03 | 0.23 ± 0.01 | 0.28 ± 0.03 | 0.25 ± 0.01 |
| | \( k_{cat}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) | 0.029 ± 0.001 | 13.3 ± 1.1 | 94.6 ± 3.4 | 180 ± 14 | 263 ± 10 | 235 ± 18 | 346 ± 10 |
| | \( \Delta(\Delta G)^{c} \) (kJ mol\(^{-1}\)) | 0.16 | 1.11 | 1.93 | 3.10 | 2.41 | 2.83 | 2.44 |

\(^{a}\)Determined at 45°C in 0.05 M NaOAc buffer, pH 4.4.

\(^{b}\)Standard error.

\(^{c}\)Changes of transition-state energy \( \Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_mut/(k_{cat}/K_m)_{wt}] \).

\(^{d}\)Not determined.

\(^{e}\)Data from Fang et al. (1997).
Table 1. Kinetic parameters of wild-type and mutant GAs for hydrolysis of isomaltose and maltooligodextrins of DP 2 - 7

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Isomaltose (G2)</th>
<th>Maltose (G2)</th>
<th>Maltotriose (G3)</th>
<th>Maltotetraose (G4)</th>
<th>Maltopentaose (G5)</th>
<th>Maltohexaose (G6)</th>
<th>Maltoheptaose (G7)</th>
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<td>Wild-type&lt;sup&gt;4&lt;/sup&gt;</td>
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</tr>
<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>0.72 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.4 ± 0.2</td>
<td>48.2 ± 0.7</td>
<td>64.5 ± 2.9</td>
<td>71.8 ± 1.9</td>
<td>73.7 ± 2.1</td>
<td>72.3 ± 0.9</td>
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<tr>
<td>$K_m$ (mM)</td>
<td>23.5 ± 0.6</td>
<td>1.01 ± 0.03</td>
<td>0.25 ± 0.014</td>
<td>0.111 ± 0.017</td>
<td>0.110 ± 0.010</td>
<td>0.107 ± 0.010</td>
<td>0.083 ± 0.004</td>
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<tr>
<td>$k_{cat}/K_m$ (s⁻¹mM⁻¹)</td>
<td>0.031 ± 0.001</td>
<td>20.3 ± 0.55</td>
<td>196 ± 9</td>
<td>582 ± 65</td>
<td>654 ± 43</td>
<td>685 ± 47</td>
<td>870 ± 35</td>
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<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>0.91 ± 0.04</td>
<td>16.5 ± 0.47</td>
<td>41.4 ± 0.5</td>
<td>63.0 ± 1.4</td>
<td>67.6 ± 0.7</td>
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<td>$K_m$ (mM)</td>
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<td>0.32 ± 0.01</td>
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<td>0.079 ± 0.004</td>
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<td>$k_{cat}/K_m$ (s⁻¹mM⁻¹)</td>
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<td>13.5 ± 1.0</td>
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<td>385 ± 19</td>
<td>691 ± 23</td>
<td>832 ± 36</td>
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<td>$\Delta(\Delta G)^{c}$ (kJ mol⁻¹)</td>
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<td>1.07</td>
<td>1.11</td>
<td>1.09</td>
<td>-0.14</td>
<td>-0.51</td>
<td>-0.04</td>
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<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>0.53 ± 0.01</td>
<td>14.1 ± 0.4</td>
<td>38.4 ± 0.9</td>
<td>72.8 ± 2.2</td>
<td>75.7 ± 1.6</td>
<td>81.1 ± 2.4</td>
<td>124 ± 4</td>
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<tr>
<td>$K_m$ (mM)</td>
<td>12.2 ± 1.2</td>
<td>0.75 ± 0.09</td>
<td>0.26 ± 0.03</td>
<td>0.159 ± 0.015</td>
<td>0.111 ± 0.008</td>
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<tr>
<td>$k_{cat}/K_m$ (s⁻¹mM⁻¹)</td>
<td>0.043 ± 0.004</td>
<td>18.7 ± 1.8</td>
<td>151 ± 12</td>
<td>458 ± 30</td>
<td>680 ± 39</td>
<td>652 ± 50</td>
<td>1107 ± 97</td>
</tr>
<tr>
<td>$\Delta(\Delta G)^{c}$ (kJ mol⁻¹)</td>
<td>-0.89</td>
<td>0.21</td>
<td>0.69</td>
<td>0.63</td>
<td>-0.10</td>
<td>0.13</td>
<td>-0.64</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>0.94 ± 0.03</td>
<td>19.0 ± 0.7</td>
<td>45.0 ± 0.7</td>
<td>47.7 ± 2.3</td>
<td>59.8 ± 1.2</td>
<td>66.0 ± 3.3</td>
<td>88.0 ± 1.6</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>32.7 ± 2.4</td>
<td>1.43 ± 0.16</td>
<td>0.48 ± 0.02</td>
<td>0.27 ± 0.03</td>
<td>0.23 ± 0.01</td>
<td>0.28 ± 0.03</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (s⁻¹mM⁻¹)</td>
<td>0.029 ± 0.001</td>
<td>13.3 ± 1.1</td>
<td>94.6 ± 3.4</td>
<td>180 ± 14</td>
<td>263 ± 10</td>
<td>235 ± 18</td>
<td>346 ± 10</td>
</tr>
<tr>
<td>$\Delta(\Delta G)^{c}$ (kJ mol⁻¹)</td>
<td>0.16</td>
<td>1.11</td>
<td>1.93</td>
<td>3.10</td>
<td>2.41</td>
<td>2.83</td>
<td>2.44</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined at 45°C in 0.05 M NaOAc buffer, pH 4.4.

<sup>b</sup>Standard error.

<sup>c</sup>Changes of transition-state energy $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.

<sup>d</sup>Not determined.

<sup>e</sup>Data from Fang et al. (1997).
wild-type GA for the hydrolysis of isomaltose, catalytic efficiencies were only 31 - 66% that of wild-type GA for the hydrolysis of maltooligodextrins of DP 2 - 7.

Mutant Gly121→Ala GA had moderately decreased $k_{\text{cat}}$ values (68 - 97% that of wild-type GA), but a large change in the $K_m$ values. The $K_m$ values for hydrolysis of maltooligodextrins of increasing length up to maltoheptaose did not decrease as in wild-type GA but maintained a nearly constant level. The catalytic efficiency was 74 and 137% that of wild-type GA for the hydrolysis of isomaltose and maltose; however, the catalytic efficiencies were only 8 - 30% that of wild-type GA for the hydrolysis of maltooligodextrins of DP 3 - 7.

The double mutant Gly121→Ala/Ser411→Gly GA also had moderately decreased $k_{\text{cat}}$ values (65 - 92% that of wild-type GA). The effects of mutations Gly121→Ala and Ser411→Gly on the kinetic parameters were fairly additive only for the $k_{\text{cat}}$ values of maltotriose, maltotetraose and maltoheptaose hydrolysis. The $K_m$ values of the double mutant GA were more similar to that of Gly121→Ala than Ser411→Gly GA. The $K_m$ values of the double mutant GA still increased 1.5 - 2.6-fold compared to that of Gly121→Ala GA. The catalytic efficiency was 39 and 88% that of wild-type GA for the hydrolysis of isomaltose and maltose, however, catalytic efficiencies were only 5 - 15% that of wild-type GA for DP 3 - 7 maltooligodextrin hydrolysis.

Transition-state energy is a useful estimating for the binding strength of the enzyme-substrate complex. The change of transition-state energy, $\Delta(\Delta G)$, associated with loss of a hydrogen bond between an uncharged group of substrate and an uncharged group of enzyme
is about 2.1 - 6.3 kJ mol⁻¹. The change of transition-state energy for loss of a hydrogen bond between an uncharged group on the substrate and a charged group on the enzyme is about ~14.6 - 18.8 kJ mol⁻¹ (Fersht et al., 1985). For DP 4 - 7 maltooligodextrin hydrolysis by mutant Ser119→Trp GA, the Δ(ΔG) values (2.41 - 3.1 kJ mol⁻¹) indicated that mutation Ser119→Trp GA caused loss of a hydrogen bond between an uncharged group on GA and the substrate, probably at the fourth subsite. For hydrolysis of DP 3 - 7 maltooligodextrins, the Δ(ΔG) values for mutant Gly121→Ala (3.28 - 6.53 kJ mol⁻¹) and Gly121→Ala/Ser411→Gly (5 - 8.08 kJ mol⁻¹) GAs also indicated that mutation Gly121→Ala caused loss of a hydrogen bond between an uncharged group on GA and the substrate, probably at the third subsite.

The small Δ(ΔG) values (-0.64 - 1.11 kJ mol⁻¹) for mutants Ser119→Glu and Ser119→Gly GAs indicated that these two mutants had only minor effects on substrate binding in the transition state.

Mutations Gly121→Ala and Gly121→Ala/Ser411→Gly increased the ratio of catalytic efficiency of α-1,4 activity (maltose as substrate) to α-1,6 activity (isomaltose as substrate) about twofold compared to that of wild-type GA. Therefore, the selectivity for hydrolysis of maltose (α-1,4 linked) over isomaltose (α-1,6 linked) was increased about twofold, while α-1,4 hydrolytic activity remained at 87% the wild-type GA level using maltose as a substrate. Mutations Ser119→Glu, Ser119→Gly and Ser119→Trp had decreased ratios of catalytic efficiency for α-1,4 activity (maltose as substrate) to α-1,6 activity (isomaltose as substrate) compared to that of wild-type GA.
Specific activity of GA at different temperatures

Specific activity of GA at different temperatures (Table 2) was used to determine the effect of temperature on the hydrolysis of maltose by wild-type and mutant GAs. The specific activities of wild-type and mutant GAs at 55°C increased 3.7- to 4.2-fold compared to that at 35°C. The specific activities of Ser119→Glu, Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs were 79 - 86%, 67 - 69%, 93 - 104%, 77 - 87% and 76 - 84% that of wild-type GA at corresponding temperatures, respectively. This result showed that the relative specific activity differences between wild-type and mutant GAs were almost unaffected by reaction temperature.

Hydrolysis of DE 10 maltodextrin

Hydrolysis of DE 10 maltodextrin was used to study the production of glucose by wild-type and mutant GAs at high substrate concentrations in order to simulate industrial saccharification (Figure 2). DE 10 maltodextrin is a mixture of maltodextrins with an average degree of polymerization of 10. The initial rates of glucose production are given in Table 3.

At 35°C, the glucose yield from the hydrolysis of DE 10 maltodextrin was still apparently increasing for all of the GAs tested (Figure 2a). The initial rates of glucose production for wild-type and Ser119→Gly GAs were very similar. Ser119→Trp GA had slightly increased initial rates of glucose production compared to that of wild-type GA, whereas mutant Ser119→Glu, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs had slightly decreased initial rates of glucose production compared to that of wild-type GA (Table
Table 2. Specific activity (IU/mg enzyme) of wild-type and mutant GAs determined from the hydrolysis of maltose at different temperatures

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35°C</td>
</tr>
<tr>
<td>Wild-type</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Ser119→Glu</td>
<td>6.8 ± 0.1</td>
</tr>
<tr>
<td>Ser119→Gly</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Ser119→Trp</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>Gly121→Ala</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Gly121→Ala/Ser411→Gly</td>
<td>5.9 ± 0.2</td>
</tr>
</tbody>
</table>

* Determined from the hydrolysis of 3.33% (w/v) maltose in 0.05 M NaOAc buffer at pH 4.4.
* Standard error.

3). For wild-type, Ser119→Glu, Ser119→Gly and Ser119→Trp GAs, the increase of glucose yield leveled off to a very slow increase at yields over about 90% (Figure 2a). The change in the the slope, however, appeared at a yield of 75 - 80% for Gly121→Ala and the double mutant Gly121→Ala/Ser411→Gly GAs. For Gly121→Ala and Gly121→Ala/Ser411→Gly GAs, two higher enzyme concentrations were also used for DE 10 maltodextrin hydrolysis (Figure 2b and 2c). For mutant Gly121→Ala GA, the initial rates of glucose production were 66 and 79.2 g/l/h at enzyme concentrations of 3.30 and 3.96 μM, respectively. For the double mutant Gly121→Ala/Ser411→Gly GA, the initial rates of glucose production were 62.3 and 74.3 g/l/h using enzyme concentrations of 3.30 and 3.96 μM, respectively. Although the
Figure 2. (a) Production of glucose by wild-type and mutant GAs during hydrolysis of DE 10 maltodextrin at 35°C. This experiment was performed at 28% (w/v) DE 10 maltodextrin and 0.02% sodium azide in 0.05 M NaOAc buffer at pH 4.4 for 12 days. The enzyme concentration was 2.64 μM. (•) wild-type, (□) Ser119→Glu, (▲) Ser119→Gly, (▼) Ser119→Trp, (◆) Gly121→Ala, (●) Gly121→Ala/Ser411→Gly GAs.
Figure 2 (cont'd). (b) Production of glucose by wild-type and different concentrations of Gly121→Ala GAs during hydrolysis of DE 10 maltodextrin at 35°C. This experiment was performed at 28% (w/v) DE 10 maltodextrin and 0.02% sodium azide in 0.05 M NaOAc buffer at pH 4.4 for 12 days. (●) wild-type GA, 2.64 μM; (◇) Gly121→Ala GA, 2.64 μM; (△) Gly121→Ala GA, 3.30 μM; (▽) Gly121→Ala GA, 3.96 μM.
Figure 2 (cont’d). (c) Production of glucose by wild-type and different concentrations of Gly121→Ala/Ser411→Gly GAs during hydrolysis of DE 10 maltodextrin at 35°C. This experiment was performed at 28% (w/v) DE 10 maltodextrin and 0.02% sodium azide in 0.05 M NaOAc buffer at pH 4.4 for 12 days. (●) wild-type GA, 2.64 μM; (◆) Gly121→Ala/Ser411→Gly GA, 2.64 μM; (○) Gly121→Ala/Ser411→Gly GA, 3.30 μM; (◇) Gly121→Ala/Ser411→Gly GA, 3.96 μM.
Figure 2 (cont'd). (d) Production of glucose by wild-type and mutant GAs in the hydrolysis of DE 10 maltodextrin at 55°C. This experiment was performed at 28% (w/v) DE 10 maltodextrin and 0.02% sodium azide in 0.05 M NaOAc buffer at pH 4.4 for 12 days. The enzyme concentration was 2.64 μM. (●) wild-type, (■) Ser119→Glu, (▲) Ser119→Gly, (▼) Ser119→Trp, (♦) Gly121→Ala, (●) Gly121→Ala/Ser411→Gly GAs.
Table 3. Initial rates of glucose and isomaltose production in the hydrolysis of DE 10 maltodextrin and glucose condensation reactions, respectively, at 35 and 55°C, and their ratios by wild-type and mutant GAs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glucose $^a$ (G$_1$) x $10^3$ (mol/h*mol GA)</th>
<th>Isomaltose $^b$ (iG$_2$) (mol/h*mol GA)</th>
<th>Ratio x $10^3$ (iG$_2$/G$_1$)</th>
<th>Relative ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>35°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type$^d$</td>
<td>125 ± 7</td>
<td>59.2 ± 2.8</td>
<td>0.47</td>
<td>1</td>
</tr>
<tr>
<td>Ser119$^e$→Glu</td>
<td>103 ± 7</td>
<td>51.3 ± 1.6</td>
<td>0.50</td>
<td>1.05</td>
</tr>
<tr>
<td>Ser119$^e$→Gly</td>
<td>134 ± 8</td>
<td>54.4 ± 1.7</td>
<td>0.41</td>
<td>0.86</td>
</tr>
<tr>
<td>Ser119$^e$→Trp</td>
<td>143 ± 7</td>
<td>46.4 ± 1.9</td>
<td>0.32</td>
<td>0.68</td>
</tr>
<tr>
<td>Gly121$^e$→Ala</td>
<td>108 ± 8</td>
<td>29.8 ± 1.8</td>
<td>0.28</td>
<td>0.58</td>
</tr>
<tr>
<td>Gly121$^e$→Ala/Ser411$^e$→Gly</td>
<td>102 ± 7</td>
<td>15.5 ± 0.8</td>
<td>0.15</td>
<td>0.32</td>
</tr>
<tr>
<td>Ser411$^e$→Gly$^d$</td>
<td>108 ± 7</td>
<td>21.8 ± 3.1</td>
<td>0.20</td>
<td>0.42</td>
</tr>
<tr>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type$^d$</td>
<td>815 ± 67</td>
<td>432 ± 17</td>
<td>0.53</td>
<td>1</td>
</tr>
<tr>
<td>Ser119$^e$→Glu</td>
<td>687 ± 57</td>
<td>445 ± 24</td>
<td>0.65</td>
<td>1.22</td>
</tr>
<tr>
<td>Ser119$^e$→Gly</td>
<td>735 ± 36</td>
<td>366 ± 12</td>
<td>0.50</td>
<td>0.94</td>
</tr>
<tr>
<td>Ser119$^e$→Trp</td>
<td>693 ± 46</td>
<td>372 ± 14</td>
<td>0.54</td>
<td>1.01</td>
</tr>
<tr>
<td>Gly121$^e$→Ala</td>
<td>434 ± 34</td>
<td>225 ± 6</td>
<td>0.52</td>
<td>0.98</td>
</tr>
<tr>
<td>Gly121$^e$→Ala/Ser411$^e$→Gly</td>
<td>398 ± 21</td>
<td>93.1 ± 2.2</td>
<td>0.23</td>
<td>0.44</td>
</tr>
<tr>
<td>Ser411$^e$→Gly$^d$</td>
<td>554 ± 27</td>
<td>113 ± 8</td>
<td>0.20</td>
<td>0.38</td>
</tr>
</tbody>
</table>

$^a$ Determined from the hydrolysis of 28% (w/v) DE 10 maltodextrin in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide.

$^b$ Determined from the glucose condensation reactions of 30% D-glucose in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide.

$^c$ Standard error.

$^d$ Data from Fang et al. (1997).
initial rates of glucose production at enzyme concentrations of 3.30 and 3.96 μM for Gly121→Ala and Gly121→Ala/Ser411→Gly GAs were higher than that of wild-type GA at the enzyme concentration of 2.64 μM (59.2 g/l/h), the change in slope of glucose yield still appeared at a lower yield for Gly121→Ala and Gly121→Ala/Ser411→Gly GAs, compared to that of wild-type GA.

At 55°C, wild-type, Ser119→Glu, Ser119→Gly and Ser119→Trp GAs reached a high glucose yield very quickly, whereas the glucose yield of Gly121→Ala and the double mutant Gly121→Ala/Ser411→Gly GAs slowly increased for a long period of time (Figure 2d). Surprisingly, all mutant GAs had decreased initial rates of glucose production compared to that of wild-type GA (Table 3). The initial rates of glucose production for wild-type and Ser119→Glu GAs at 55°C increased about sevenfold compared to those at 35°C, whereas the initial rates of glucose production for the other mutant GAs at 55°C increased only about four- to sixfold compared to those at 35°C.

The initial rates of glucose production for Ser119→Glu, Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs were 83, 107, 115, 86 and 82%, respectively, that of wild-type GA at 35°C, whereas they were 84, 90, 85, 53 and 49%, respectively, that of wild-type GA at 55°C. These results showed that the relative relationships between wild-type and mutant GAs (except mutant Ser119→Glu GA) for the initial rate of glucose production were substantially affected by reaction temperature.
Glucose condensation reactions

Glucose condensation reactions were used to study the ability of wild-type and mutant GAs to synthesize isomaltose at high concentrations of glucose (Figure 3). The same concentrations of GAs (2.64 μM) were used as in the hydrolysis of DE 10 maltodextrin. The initial rates of isomaltose production are given in Table 3.

At 35°C, all mutant GAs produced less isomaltose than wild-type GA, and the initial rates of isomaltose production also showed the same relative relationship between wild-type and mutant GAs (Figure 3a and Table 3). Isomaltose production for wild-type and mutant GAs increased almost linearly over the 280-hour course of the experiment at 35°C.

At 55°C, all mutant GAs except Ser119→Glu GA produced less isomaltose than wild-type GA. The isomaltose production for Gly121→Ala/Ser411→Gly GA was much lower than wild-type GA and almost linear as at 35°C. The initial rates of isomaltose production at 55°C increased about six- to ninefold compared to those at 35°C. The initial rates of isomaltose production for all mutant GAs except Ser119→Glu GA at 55°C were also lower than that of wild-type GA. Ser119→Glu GA had a very similar initial rate of isomaltose production at 55°C compared to that of wild-type GA, since the initial rates of isomaltose production increased about nine- and sevenfold, respectively, for Ser119→Glu and wild-type GAs from 35 to 55°C.

The initial rate of the isomaltose production for Ser119→Glu, Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs were 87, 92, 78, 50 and 26%, respectively, that of wild-type GA at 35°C, whereas they were 103, 85, 86, 52 and 22%,
Figure 3. (a) Production of isomaltose by wild-type and mutant GAs in glucose condensation reactions at 35°C. This experiment was performed at 30% (w/v) D-glucose in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide for 12 days. (●) wild-type, (■) Ser119→Glu, (▲) Ser119→Gly, (▼) Ser119→Trp, (♦) Gly121→Ala, (◆) Gly121→Ala/Ser411→Gly GAs.
Figure 3 (cont'd). (b) Production of isomaltose by wild-type and mutant GAs in glucose condensation reactions at 55°C. This experiment was performed at 30% (w/v) D-glucose in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide for 12 days. (●) wild-type, (■) Ser119→Glu, (▲) Ser119→Gly, (▼) Ser119→Trp, (♦) Gly121→Ala, (●) Gly121→Ala/Ser411→Gly GAs.
respectively, that of wild-type GA at 55°C. These results showed that the relative relationships between wild-type and mutant GAs (except mutant Ser119→Glu GA) for the initial rate of isomaltose production were fairly unaffected by reaction temperature.

**Selectivity of GA for the synthesis of α-1,6 linked products versus the hydrolysis of α-1,4 linked substrates**

The ratio of the initial rate of isomaltose production (from glucose condensation reactions) to that of glucose production (from the hydrolysis of DE 10 maltodextrin) was used to indicate the selectivity for synthesis of α-1,6 linked products versus the hydrolysis of α-1,4 linked substrates. This ratio represents the ability of GA to synthesize isomaltose at a normalized level of DE 10 maltodextrin hydrolytic activity.

Mutant Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs had decreased ratios of the initial rate of isomaltose production to that of glucose production by 14, 32, 42 and 68% at 35°C, respectively, compared to that of wild-type GA (Table 3). Mutant Ser119→Glu GA had a very similar ratio to that of wild-type GA at 35°C, while it had an increased ratio at 55°C. Unexpectedly, mutant Ser119→Gly, Ser119→Trp and Gly121→Ala GAs had very similar ratios to that of wild-type GA at 55°C. The double mutant Gly121→Ala/Ser411→Gly GA maintained a decreased ratio of the initial rate of isomaltose production to that of glucose production by 56% at 55°C compared to that of wild-type GA. As shown previously (Fang et al., 1997), the ratio of the initial rate of isomaltose production to that of glucose production was temperature dependent, since the
increase of initial rates of glucose and isomaltose production due to the increased temperature were not always proportional.

Since the hydrolysis of DE 10 maltodextrin and the glucose condensation reaction were performed in the same buffer system using same amount of GA, these reactions were compared to show the relationship between isomaltose production and glucose yield. Since the hydrolysis of DE 10 maltodextrin at 35°C did not reach completion, we used only the data obtained at 55°C. Both average glucose yields (after reaching 90%) and peak glucose yields (average of glucose yields from the top four contiguous points) versus initial rates of isomaltose production had a linear relationship as shown in Figure 4. These results support the hypothesis that decreasing the ability of GA to synthesize isomaltose results in an increase in glucose yield as shown previously (Fang et al., 1997).

**Discussion**

Residues 119 and 121 were chosen for site-directed mutagenesis in order to study the effect of hydrogen bonding between enzyme and substrate on substrate specificity. In this study, substrate specificity was evaluated not only from the kinetic parameters but also from the production of isomaltose and glucose using high concentrations of glucose and DE 10 maltodextrin as substrates, respectively.

Mutation Ser119→Glu was designed to strengthen the hydrogen bond between residue 119 and the fourth sugar residue of the substrate in order to stabilize the pH 6-type substrate conformer and to bring a negative charge near subsite 4. The kinetic parameters of Ser119→Glu GA showed that this mutation only slightly alters the active site. The catalytic
Figure 4. (a) The relationship between average glucose yields (after reaching 90%) and initial rates of isomaltose production in the hydrolysis of DE 10 maltodextrin and glucose condensation reactions, respectively, at 55°C. The hydrolysis of DE 10 maltodextrin and glucose condensation reactions were performed at 28% (w/v) DE 10 maltodextrin and 30% (w/v) D-glucose, respectively, in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide for 12 days.
Figure 4 (cont'd). (b) The relationship between peak glucose yields (average of glucose yields from top four contiguous points) and initial rates of isomaltose production in the hydrolysis of DE 10 maltodextrin and glucose condensation reactions, respectively, at 55°C. The hydrolysis of DE 10 maltodextrin and glucose condensation reactions were performed at 28% (w/v) DE 10 maltodextrin and 30% (w/v) D-glucose, respectively, in 0.05 M NaOAc buffer at pH 4.4 with 0.02% sodium azide for 12 days.
efficiency on short-length substrates, however, was affected more than for long-length substrates. This was possibly due to increased electrostatic interactions from the introduced negative charge near subsite 4 affecting hydrolysis of short-length substrates.

Mutation Ser119→Gly was designed to remove the hydrogen bond between residue 119 and the fourth sugar residue of the substrate in order to destabilize the pH 6-type conformer. Observed changes in transition-state energy ($\Delta(\Delta G)$), however, did not show loss of a hydrogen bond. Fersht et al. (1985) reported that deletion of a hydrogen bond in the enzyme-substrate complex may not cause overall loss of absolute binding strength of a hydrogen bond. In certain cases, loss of a hydrogen bond may cause no loss of binding energy, such as a deletion of a side-chain on an enzyme allowing the access of water into the enzyme-substrate complex. Mutation Ser119→Gly removed the side-chain of Ser119, potentially allowing a water molecule to enter the space between residue 119 and the substrate in the enzyme-substrate complex. Since the water molecule could hydrogen-bond to the substrate, this would explain why mutation Ser119→Gly caused only a minor change in the transition-state energy. The kinetic parameters of Ser119→Gly GA showed that this mutant increased $k_{cat}$ values for the hydrolysis of long-length substrates tested (DP 4-7), whereas it decreased the $k_{cat}$ values for the hydrolysis of short-length substrates tested (DP 2 and 3), compared to that of wild-type GA. $K_m$ values decreased only for the hydrolysis of isomaltose and maltose. The decreased $k_{cat}$ and $K_m$ values for the hydrolysis of isomaltose and maltose compared to that of wild-type GA indicated that the Ser119→Gly mutation affected subsites 1 and 2. Since mutation Ser119→Gly altered the flexibility of the peptide
bond near Trp120, it may affect the side-chain position of Trp120. Trp120 is an important residue, as shown by three-dimensional structures of GA complexed with pseudotetrasaccharide (Stoffel et al., 1995; Aleshin et al., 1996). Atom NE1 of Trp120 hydrogen bonds to atom OE2 of Glu179, the catalytic acid, and atoms CE3, CZ3 and CD2 of Trp120 make nonbonded contacts with the atom O5 of the third sugar residue of pseudotetrasaccharide inhibitors (Figure 1). Sierks et al. (1989) reported that mutation of Trp120 to His, Leu, Phe and Tyr decreased the $k_{\text{cat}}$ and $K_m$ values for the hydrolysis of maltose, and they also suggested that Trp120 is important for stabilization of the transition-state enzyme-substrate complex in subsites 1 and 2.

Mutation Ser119→Trp was designed to remove the same hydrogen bond and to increase hydrophobic interactions between the enzyme and the pH 6-type conformer. The changes of transition-state energy, $\Delta(\Delta G)$, indicated loss of a hydrogen bond between residue 119 and the fourth sugar residue of the substrate. The Ser119→Trp mutation also caused some minor changes in the hydrolysis of maltose and isomaltose. Increased hydrophobic interactions near subsite 4 might have affected subsites 1 and 2, as shown by our previous study on mutant Tyr175→Phe (Fang et al., 1997).

Mutation Gly121→Ala was designed to introduce a β-carbon at position 121 to displace the 6-OH group of the third sugar residue from its hydrogen bonding position. The changes of transition-state energy, $\Delta(\Delta G)$, indicated that the hydrogen bond between residue 121 and the third sugar residue of the substrate had been removed. The loss of the hydrogen bond is likely responsible for the increased $K_m$ values for DP 3 - 7 maltooligodextrin.
hydrolysis. The Gly121→Ala mutation also affected the hydrolysis of isomaltose and maltose. Since mutation Gly121→Ala also affected the flexibility of the peptide bond near Trp120, it might also have affected the side-chain position of Trp120, and therefore subsites 1 and 2 as in mutant Ser119→Gly GA.

The double mutation Gly121→Ala/Ser411→Gly was designed to investigate additivity of these substrate specificity mutations. The effects of mutations Gly121→Ala and Ser411→Gly on the kinetic parameters appeared to be additive only at certain $k_{cat}$ values. Therefore, the introduced mutation Gly121→Ala at subsite 3 and mutation Ser411→Gly near subsite 1 are not totally independent of each other. Although the effect of mutations Gly121→Ala and Ser411→Gly on the ratios of isomaltose production to that of glucose production was not additive, the double mutant GA had the lowest ratio of isomaltose production to that of glucose production at 35°C.

In spite of minor changes at subsites 1 and 2 caused by the mutations at subsite 3 or near subsite 4, mutant GAs (except Ser119→Glu GA) showed decreased initial rates of isomaltose production (in glucose condensation reactions) and had increased average (or peak) glucose yields (in the hydrolysis of DE 10 maltodextrin) at 55°C, compared with that of wild-type GA (Figure 4).

During hydrolysis of 28% DE 10 maltodextrin at 35°C, mutant Gly121→Ala and Gly121→Ala/Ser411→Gly GAs produced a slowly increasing glucose yield that leveled off at a lower level than that of wild-type GA (Figure 2a), even when higher enzyme concentrations of these two mutant GAs were used (Figure 2b and 2c). Since these two
mutants had large $K_m$ value increases for DP 3 - 7 maltooligodextrin hydrolysis compared to that of wild-type GA (Table 1), hydrolysis by these mutants became much slower than that of wild-type GA when the substrate concentration decreased due to the hydrolysis reaction. A similar but less pronounced trend was also observed for the hydrolysis of DE 10 maltodextrin by these mutants at 55°C.

In the production of isomaltose and glucose using high concentrations of glucose and DE 10 maltodextrin as substrates, respectively, mutant Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs had a decreased ratio of the initial rate of isomaltose production to that of glucose production compared to that of wild-type GA at 35°C (Table 3). Mutant Ser119→Glu GA had a very similar ratio to that of wild-type GA at 35°C. However, the relative ratios (the ratio of initial rate of isomaltose production to glucose production of each mutant GA relative to that of wild-type GA at the corresponding temperature) of all five mutant GAs (Ser119→Glu, Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly) at 55°C were higher than that at 35°C (Table 3). The increased relative ratios at 55°C were caused by either increased isomaltose production or decreased glucose production relative to that of wild-type GA. The relative relationships between wild-type and mutant GAs (except mutant Ser119→Glu GA) of the initial rate of glucose production were affected by reaction temperature, while the relative relationships of the initial rate of isomaltose production were almost totally unaffected by reaction temperature. The initial rate of glucose production of Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs were 107, 115, 86 and 82% that of wild-
type GA at 35°C, respectively, whereas they were 90, 85, 53 and 49% that of wild-type GA at 55°C, respectively. Specific activity assays at different temperatures, however, showed that the relative relationships between wild-type and mutant GAs on specific activity (determined from the hydrolysis of maltose) were almost completely unaffected by reaction temperature (Table 2). We suggest that the removal of the hydrogen bond between the mutated enzyme and the substrate in Ser119-Gly, Ser119-Trp, Gly121-Ala and Gly121-Ala/Ser411-Gly GAs was responsible for the loss of hydrolytic activity on long-length substrates at 55°C, and therefore the relative ratios at 55°C were higher than that at 35°C.

In summary, the substrate specificity of GA has been changed by altering hydrogen bonding between enzyme and substrate in the enzyme-substrate complex. Mutant Ser119-Gly, Ser119-Trp, Gly121-Ala and Gly121-Ala/Ser411-Gly GAs showed decreased ratios of initial rates of isomaltose production to that of glucose production compared to that of wild-type GA at 35°C. Removal of the hydrogen bond, however, lowered the hydrolytic activity (compared to that of wild-type GA) on long-length substrates at 55°C. Therefore, Ser119-Gly, Ser119-Trp and Gly121-Ala GAs had very similar ratios of initial rates of isomaltose production to that of glucose production, compared to that of wild-type GA, at 55°C. Only the double mutant Gly121-Ala/Ser411-Gly GA still showed decreased ratios of initial rates of isomaltose production to that of glucose production, compared to that of wild-type GA, at 55°C.
Acknowledgments

This project was supported by the Consortium for Plant Biotechnology Research and Genencor International, Inc. (Palo Alto, CA). We thank Dr. James Meade for the gift of wild-type GA gene and plasmid, and Drs. John F. Robyt and Motomitsu Kitaoka for help with isomaltose quantitative determination by thin-layer chromatography.

References


GENERAL CONCLUSIONS AND RECOMMENDATIONS

General Conclusions

The hydrogen bond between Glu400 and Ser411 plays an important role in the pH dependence of GA. Five mutations, Ser411→Cys, Ser411→Ala, Ser411→His, Ser411→Asp and Ser411→Gly, at residue 411 removed the hydrogen bond between Glu400 and Ser411 and increased the optimal pHs of their enzyme-substrate complexes from 0.15 to 0.87 units. Ser411→Ala is the best performing pH mutant of GA isolated to date. Ser411→Ala GA increased the optimal pH by 0.84 units while maintaining a high level of catalytic activity and catalytic efficiency. The hydrolysis of DE 10 maltodextrin also showed that Ser411→Ala GA has the potential to be used at higher pH in starch saccharification.

The minor changes designed in the active site successfully altered the substrate specificity of GA to reduce isomaltose formation from glucose condensation. Mutant Tyr175→Phe, Ser411→Ala and Ser411→Gly GAs had decreased ratios of initial rates of isomaltose production to that of glucose production compared to wild-type GA at both 35 and 55°C. These mutant GAs have great potential for increasing the glucose yield in starch processing.

Alterating the hydrogen bonding between enzyme and substrate in the enzyme-substrate complex also produced changes in the substrate specificity of GA. Mutant Ser119→Gly, Ser119→Trp, Gly121→Ala and Gly121→Ala/Ser411→Gly GAs had a decreased ratio of initial rates of isomaltose production to that of glucose production compared to wild-type GA.
at 35°C. Removal of the hydrogen bond, however, lowered the hydrolytic activity (compared to that of wild-type GA) on long-length substrates at 55°C. Mutant Ser119→Gly.

Ser119→Trp and Gly121→Ala GAs showed a very similar ratio to that of wild-type GA at 55°C, and only the double mutant Gly121→Ala/Ser411→Gly GA had a decreased ratio. compared to that of wild-type GA, at 55°C. Since the saccharification of starch processing is performed at 55 - 65°C, the loss of hydrolytic activity on long-length substrates at 55°C by removal of the hydrogen bond between substrate and GA suggested that this type of mutation had limited industrial potential. Although the effect of mutations Gly121→Ala and Ser411→Gly on the ratios of isomaltose production to that of glucose production was not additive, the double mutant Gly121→Ala/Ser411→Gly GA had the lowest ratio of isomaltose production to that of glucose production at 35°C, suggesting that it is possible to obtain superior GA variants by combining single mutations.

**General Recommendations**

1) Design mutations with increased hydrophobic interactions in the active site to improve substrate specificity.

2) Design mutations with additional hydrogen bonding between enzyme and substrate to improve substrate specificity.

3) Combine single mutations that have improved substrate specificity.
GENERAL REFERENCES


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