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Starch-binding domain shuffling in Aspergillus niger glucoamylase

Abstract
Aspergillus niger glucoamylase (GA) consists mainly of two forms, GAI [from the N-terminus, catalytic domain + linker + starch-binding domain (SBD)] and GAII (catalytic domain + linker). These domains were shuffled to make RGAI (SBD + linker + catalytic domain), RGAIΔL (SBD + catalytic domain) and RGAII (linker + catalytic domain), with domains defined by function rather than by tertiary structure. In addition, *Paenibacillus macerans* cyclomaltodextrin glucanotransferase SBD replaced the closely related *A.niger* GA SBD to give GAE. Soluble starch hydrolysis rates decreased as RGAII ≈ GAII ≈ GAI > RGAIΔL ≈ RGAI ≈ GAE. Insoluble starch hydrolysis rates were GAI > RGAIΔL > RGAI >> GAE ≈ RGAII > GAII, while insoluble starch-binding capacities were GAI > RGAI > RGAIΔL > RGAII > GAII > GAE. These results indicate that: (i) moving the SBD to the N-terminus or replacing the native SBD somewhat affects soluble starch hydrolysis; (ii) SBD location significantly affects insoluble starch binding and hydrolysis; (iii) insoluble starch hydrolysis is imperfectly correlated with its binding by the SBD; and (iv) placing the *P.macerans*cyclomaltodextrin glucanotransferase SBD at the end of a linker, instead of closely associated with the rest of the enzyme, severely reduces its ability to bind and hydrolyze insoluble starch.

Keywords
Aspergillus niger, cyclomaltodextrin glucanotransferase, glucoamylase, Paenibacillus macerans, shuffing, starch-binding domain

Disciplines
Biochemical and Biomolecular Engineering | Biological Engineering | Chemical Engineering | Food Chemistry | Food Science | Human and Clinical Nutrition

Comments
This is a post-print of an article from *Protein Engineering Design & Selection*, 16, no. 7 (2003): 521–529, doi: 10.1093/protein/gzg066.

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Starch-Binding Domain Shuffling in *Aspergillus niger* Glucoamylase

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Aspergillus niger glucoamylase consists mainly of two forms, GAI (from the *N*-terminus, catalytic domain + linker + starch-binding domain (SBD)) and GAII (catalytic domain + linker). These domains were shuffled to make RGAI (SBD + linker + catalytic domain), RGAIΔL (SBD + catalytic domain), and RGAII (linker + catalytic domain), with domains defined by function rather than by tertiary structure. In addition, *Paenibacillus macerans* cyclomaltodextrin glucanotransferase SBD replaced the closely related *A. niger* glucoamylase SBD to give GAE. Soluble starch hydrolysis rates decreased as RGAII ≈ GAII ≈ GAI > RGAIΔL ≈ RGAI ≈ GAE. Insoluble starch hydrolysis rates were GAI > RGAIΔL > RGAI >> GAE ≈ RGAII > GAII, while insoluble starch-binding capacities were GAI > RGAI > RGAIΔL > RGAII > GAII > GAE. These results indicate that: 1) moving the SBD to the *N*-terminus or replacing the native SBD somewhat affects soluble starch hydrolysis; 2) SBD location significantly affects insoluble starch binding and hydrolysis; 3) insoluble starch hydrolysis is imperfectly correlated with its binding by the SBD; and 4) placing the *P. macerans* cyclomaltodextrin glucanotransferase SBD at the end of a linker, instead of closely associated with the rest of the enzyme, severely reduces its ability to bind and hydrolyze insoluble starch.

Keywords: *Aspergillus niger*/cyclomaltodextrin glucanotransferase/glucoamylase/*Paenibacillus macerans*/shuffling/starch-binding domain

Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3, GA) is an exo-hydrolase that catalyzes the release of β-D-glucose by hydrolyzing α-1,4- and α-1,6-glycosidic linkages at the non-reducing ends of starch and related oligo- and polysaccharide chains. The most studied GAs are from *Aspergillus awamori* and *Aspergillus niger*, which are identical (Svensson *et al*., 1983; Nunberg *et al*., 1984) and henceforth will be called *A. niger* GA, since the first species has recently been consolidated into the second. There are two major forms of this enzyme. GAI has three regions: (1) a catalytic domain containing residues 1–470, which includes an *O*-glycosylated region from residues 441 to 470; (2) a linker containing residues 471–508, with mannosyl residues *O*-linked to many serine and threonine residues both here and in the earlier thirty residues (Gunnarsson *et al.*, 1984), and (3) a starch-binding domain (SBD) containing residues 509–616, which can bind insoluble starch. GAII, a proteolysis product from GAI lacking its SBD, has 512 or 514 residues (Svensson *et al*., 1982, 1986; Nunberg *et al*., 1984). GAI and GAII can hydrolyze soluble substrates equally well (Meagher and Reilly, 1989; Meagher *et al.*, 1989). GAI hydrolyzes insoluble starch in addition to soluble starch, but GAII, lacking an SBD, is much less able to do this (Svensson *et al*., 1982).

All GAs have fairly homologous catalytic domains (Coutinho and Reilly, 1997). Their tertiary structures are similar, consisting of an $(\alpha,\alpha)_6$ barrel (Aleshin *et al.*, 1992) with a peripheral thirteenth α-helix present in fungal forms. The active site is located in a well (Aleshin *et al.*, 1992). GAs from filamentous fungi have linkers of varying primary sequences, decreasing in length from the roughly forty residues found in GAs from most *Aspergillus* species to approximately twenty residues in *Corticium rolfsii* GA (Coutinho and Reilly, 1997).

SBDs are found in carbohydrate-binding module Families 20, 21, 25, and 26 (Coutinho and Henrissat, 1999a,b). Family 20 members include all SBDs located at the *C*-termini of GAs, along with SBDs from cyclomaltodextrin glucanotransferases (EC 2.4.1.19, CGTases, Domain E), α-amylases (EC 3.2.1.1), β-amylases (EC 3.2.1.2), maltotetraose-forming exo-amylases (EC

3.2.1.60), maltogenic α-amylases (EC 3.2.1.133), and other hydrolases. GA and CGTase SBDs are quite homologous (Figure 1), with those from α-amylases intermediate to them, suggesting that perhaps they could be interchanged.

At present, tertiary structures are available for nine Family 20 SBDs (Coutinho and Henrissat, 1999a). These structures have no α-helices, containing only β-strands and loops (Figure 2).

Rhizopus oryzae and *Arxula adeninivorans* GAs have *N*-terminal Family 21 SBDs. There are no available tertiary structures of these SBDs.

The functional domain boundaries of *A. niger* GA are somewhat different than those suggested by its tertiary structure. For instance, the catalytic domain requires a portion of its glycosylated region to achieve full secretion and thermostability. In a *C*-terminal truncation study of GAI, yeast expressing *A. niger* GA residues 1–460 had a little activity on a starch-clearing plate but no measurable secreted GA activity. Forms with residues 1–482 and 1–496 were fully active but were slightly lower in high-temperature thermostability than wild-type GAI and GAII (Evans *et al.*, 1990). Furthermore, when residues 466–512, 485–512, and 466–483 were deleted, the GA resulting from the first deletion was almost undetectible, while the GAs resulting from the second and third were expressed extracellularly to about 60% and 20% the activities of wild-type GAI and GAII, which were essentially equal (Libby *et al.*, 1994). Activities of these latter two forms on soluble starch were about the same as those of GAI and GAII, while those on insoluble starch were similar to GAI. However, their thermostabilities were somewhat lower.

The optimal functional binding domains of fusions of the *A. niger* GA SBD to β-galactosidase expressed in *Escherichia coli* also contain some of the glycosylated linker region. The SBD possessed the greatest binding capacity when the last eleven amino acid residues of the glycosylated region were included. A further addition of fourteen residues or the removal of sixteen residues causes decreased binding capacity (Chen *et al.*, 1991). This suggests that a 119-residue SBD is a better functional domain than the 108-residue SBD defined by its tertiary structure. Therefore, the boundaries of the functional domains used in this research are residues 1–482 for the catalytic domain and residues 497–616 for the SBD. The role of the remaining fourteen-

residue glycosylated region (residues 483–496) is unclear except to separate the catalytic and starch-binding domains.

Given that different GAs have SBDs at either their *C*- and *N*-termini, the possibility exists that *A. niger* GA may function with its SBD at its *N*-terminus. In addition, considering that many GAs and CGTases have similar *C*-terminal SBDs, it is possible that substitution of a CGTase SBD for the *A. niger* GA SBD may yield a functional enzyme. The goals of this project therefore were to determine whether *A. niger* GA with its SBD at its *N*-terminus rather than its *C*-terminus and whether *A. niger* GA with a *C*-terminal substitution of the *Paenibacillus* (formerly *Bacillus*) *macerans* CGTase SBD can still hydrolyze soluble starch and bind and hydrolyze insoluble starch. Although there is no known tertiary structure for the latter, its primary sequence is so similar to the SBD of *Bacillus circulans* CGTase (Figure 1) that its tertiary structure should be almost identical to that of the latter shown in Figure 2.

To achieve these goals, genetic engineering was used to reverse the domain order of GAI and GAII to give RGAI and RGAII, respectively. In addition, a reduced-linker version of RGAI (RGAIΔL) was made. Finally, a GAI with a CGTase SBD (GAE) was constructed. All four rearranged GAs and the wild-type forms, GAI and GAII, were expressed in *S. cerevisiae* and purified. The abilities of the purified enzymes to bind insoluble starch and to hydrolyze soluble and insoluble starch were then compared.

Materials and Methods

Plasmids, strains, and media

Construction and sequencing of GA variants was carried out in plasmid pBS$^+$ from Stratagene. Plasmid pDS4, expressing a truncated GA designated as GACDO (Suominen *et al.*, 1993), and the yeast expression vector YEpPM18 (Cole *et al.*, 1988), containing the GAI cDNA from *A. awamori*, a gift from Cetus, were sources of GA coding and expression vector sequences. The plasmid pRE513 (Evans *et al.*, 1990), was used to express GAII. The plasmid pLCGT1 (Lee and

Tao, 1994), containing the CGTase cDNA from *P. macerans*, was obtained from Dr. Zivko Nikolov.

All cloning was done in *E. coli* TG1 [*supE, hsdδ5, thi*δ(*lac-proAB*)*F'*(*traD36, proAB*$^+$*, lacI*^{q}*, lacZ, δM15*)]. GAs were expressed by *S. cerevisiae* strain C468 (α*, leu2-3, leu2-112, his3-11, his3-15, mal*$^-$) (Innis *et al.*, 1985), also from Cetus.

E. coli strains were grown in LB + Amp medium (10 g/L Difco Bacto-tryptone, 5 g/L Difco Bacto-yeast extract, 10 g/L NaCl, pH adjusted to 7.5 with NaOH, with or without 1.5% Difco agar, and 60 mg/L ampicillin). Yeast strains were grown in SD + His medium (1.7 g/L Difco yeast nitrogen base without amino acids, 5 g/L ammonium sulfate, 2% glucose, 100 mg/L L-histidine, with or without 1.5% Difco agar).

Reagents, enzymes, and oligonucleotides

Reagents were from Sigma or Fisher. T4 DNA ligase, restriction enzymes, and buffers were purchased from Boehringer Mannheim Biochemicals, Promega Biotech, Stratagene, or New England Biolabs. HK™ phosphatase from Epicentre Technologies was used for dephosphorylation. Acarbose was donated by Miles Laboratories. Maltose, glucose oxidase, and peroxidase were from Sigma. The Iowa State Nucleic Acid Facility supplied the oligonucleotides used for adaptors and sequencing primers.

Construction of plasmids

A cloning scheme for the three domain-reversed GAs consisting of eleven plasmids was necessary to achieve the desired constructs. It was designed to take advantage of existing restriction sites in the GA gene. Adaptors were synthesized to code for residues 483–496 and 606–616. They were also used to add restriction sites for subsequent plasmid construction. Unique restriction sites to be added were chosen for compatibility so that one type of sticky end could be reannealed to another. The first eight plasmids were constructed in the pBS$^+$ cloning vector. The last three were constructed in the expression vector portion of YEpPM18 while maintaining the

pre-pro leader sequence cleavage sites and the terminator.

Constructs were produced in four stages. The first of these was to join adaptors to GA gene regions to obtain plasmids pCG1 and pCG2. The next stage involved combining coding sequences from pCG1 and pCG2 with another adaptor, resulting in pCG3, the progenitor to pRGAI. Deletions were then made to produce pCG4 and pCG5, the progenitors of pRGAIΔL and pRGAII, respectively. In the final stage, the coding sequences were completed by adding the *C*-terminal half of the catalytic domain to pCG3, pCG4, and pCG5 and placing the coding sequences in the expression vector. The specific details follow, and are also shown on Figure 3.

pCG1. This plasmid, coding for most of the SBD, was produced by a directional three-part ligation. The synthesized *N*-terminal *Eco*RI/*Nhe*I adaptor (Adoptor 1) (Figure 4) contained an internal *Bss*HII site. The *Nhe*I/*Sal*I portion of the SBD was derived from YEpPM18. The adaptor and insert were annealed to the pBS^+ vector at the *Eco*RI and *Sal*I sites. The *Bss*HII site was used in the final three constructs.

pCG2. This was constructed to place the linker upstream of the *N*-terminal portion of the catalytic domain. This involved ligation of a *Xba*I/(*Bss*HII)-synthesized linker (Adoptor 2) (Figure 4) and a YEpPM18-derived coding sequence, *Bss*HII to *Pst*I, for the *N*-terminal half of the catalytic domain with the pBS^+ vector. The cohesive end (*Bss*HII) of Adaptor 2 was coded to leave no *Bss*HII site after ligation.

pCG2A. This plasmid was the end-product of a spontaneous deletion in pCG2. Inverted repeat sequences of *Bam*HI, *Xba*I, and *Bam*HI at the vector/adaptor joint were most likely responsible for a homologous recombination event.

pCG2B. This was constructed to replace the deleted section. pCG2A was restricted with *Eco*RI and *Spe*I and Adaptor 3 (Figure 4) was inserted. pCG2B then contained a synthetic glycosylated linker region surrounded by engineered *Xba*I and *Spe*I sites.

pCG2C. A 5.5-kb *Eco*RI fragment derived from YEpPM18 was cloned into *Eco*RI-restricted and dephosphorylated pCG2B. This step was necessary to achieve complete restriction of the plasmid with *Eco*RI and *Xba*I for pCG3 construction.

pCG3. The *Eco*RI/*Sal*I SBD coding region of pCG1 and a *Sal*I/*Xba*I-synthesized adaptor (Adoptor 4) (Figure 4) were annealed to *Eco*RI- and *Xba*I-restricted pCG2C. This clone yielded a completed SBD upstream of the *N*-terminal half of the catalytic domain with the linker region between them. This is the parent of pRGAI.

pCG4. The progenitor of pRGAIΔL was constructed by restricting pCG3 with *Xba*I and *Spe*I to remove the linker region. The sticky ends were reannealed, destroying both restriction sites.

pCG5. The progenitor of pRGAII was constructed by restricting pCG3 with *Nhe*I and *Xba*I to remove the SBD. The *Nhe*I and *Xba*I sites were destroyed upon ligation of their cohesive ends.

pRGAI, pRGAIΔL, and *pRGAII*. The final expression vectors for the domain-reordered GAs were all constructed by three-part directional cloning. Sequences upstream of the catalytic domain *Pst*I site were joined to the *C*-terminal half of the catalytic domain and placed in the expression vector. This was accomplished by annealing the *Bss*HII/*Pst*I fragments from pCG3, pCG4, and pCG5 to the *Pst*I/*Hin*dIII fragment of pDS4 and the *Bss*HII/*Hin*dIII vector portion of YEpPM18.

GAE. The fusion gene GAE was constructed by using a fragment containing the GAI cDNA, cleaving it to obtain the GA gene holding residues 1–514, and ligating it to that part of the CGTase gene containing SBD residues 579–687. Specifically, the fusion gene of GAE was constructed by using modified pGEM-7Z(+) (the *Bst*XI site had been destroyed) as a cloning vector. The small *Xho*I-*Eco*RI fragment, which contained the GAI cDNA, of YEpPM18 was inserted into the modified pGEM-7Z(+) to construct pGEM7m-GA. The big *Bst*XI/*Eco*RI fragment containing the GA gene from amino acid residues 1–514 of pGEM7m-GA, the small *Hin*dIII/*Eco*RI fragement (containing the CGT gene from amino acid residues 579–687) of pLCGT1, and a single-strand adaptor (5'-AGCTGGCG-3') were ligated to construct pGEM7m-GAE. Then the small *Xho*I/*Hin*dIII fragment of pGEMm-GAE, containing the fusion gene of GAE, was ligated to the big *Xho*I/*Hin*dIII fragment of YEpPM18 to reconstruct a yeast expression vector YEpPM-GAE.

Qiagen columns from Diagen were used for purification of plasmid DNA for fragment prep-

aration. Electrophoresed DNA fragments were extracted from agarose gels with the GeneClean product from Bio 101, Inc. Other cloning work was done using standard molecular biology protocols (Sambrook *et al.*, 1989).

Sequencing

Adaptors were verified by sequencing through the regions containing them. All complete gene constructs were verified by restriction analysis and DNA sequencing, the latter being done by the Iowa State Nucleic Acid Facility. The secreted sequences of the four constructed GAs are as follows:

RGAI: Ala-(497–616)-Ser-Arg-(483–496)-Thr-Ser-Ile-Glu-Gly-Arg-(1–484)-Met-Ala-Tyr

RGAIΔL: Ala-(497–616)-Ser-Ser-Ile-Glu-Gly-Arg-(1–484)-Met-Ala-Tyr

RGAII: Ala-Ala-Arg-(483-496)-Thr-Ser-Ile-Glu-Gly-Arg-(1–484)-Met-Ala-Tyr

GAE: (1–514)-(CGTase 579–687)

Therefore, using domains defined by function, RGAI consists of the SBD followed by the linker and the catalytic domain, while RGAIΔL is the SBD followed by the catalytic domain, RGAII is the linker followed by the catalytic domain, and GAE is GAII followed by the CGTase SBD. In terms of tertiary structure, RGAI consists of the last part of the linker followed by the SBD, then two short adaptors around the middle of the linker, and finally the catalytic domain with the first part of the linker. RGAIΔL is the same less one of the adaptors and the middle part of the linker. RGAII is the middle of the linker, followed by an adaptor, the catalytic domain, and the first part of the linker. GAE is GAII followed by domain E of CGTase.

Transformation of yeast

S. cerevisiae strain C468 was transformed with the constructed expression plasmids pRGAI, pRGAIΔL, and pRGAII, as well as with YEpPM18 (wild-type GAI), pRE513 (wild-type GAII), and pAC1 (negative control) by the lithium acetate method (Ito *et al.*, 1983). It was transformed with YEpPM-GAE by electroporation. Cells containing expression plasmids were then selected

by leucine prototrophy, which was conferred by the expression vector.

Starch-clearing plate assay

A starch-clearing plate assay verified GA activity in the resulting yeast strains. Aliquots of 5 mL of selective yeast medium (SD + His minimal medium) were inoculated with the appropriate yeast strain and grown at 30°C with shaking. At the end of the exponential phase, when OD_{600} was about 0.5, equivalent numbers of cells were plated on SD + His medium containing 1% (w/v) soluble starch. Plates were incubated at 30°C for 5 d and then at 50°C for 5 h. Plates were stained for 1 min with iodine vapors. Clear halos result around colonies producing active GA.

GA production and purification

GAs were secreted from the yeast strains in shake-flask fermentations. Six liters of selective yeast medium containing 2% glucose were inoculated with the appropriate yeast strain and shaken at 30°C and 160 rpm for 5 d. Cells were removed from the culture by centrifugation. Medium containing GA was concentrated approximately 20-fold in an Amicon ultrafiltration system. Concentrate was then diafiltered with three times its volume of wash/diafiltration buffer (0.1 M NaOAc, pH 4.3/1.5 M NaCl) and then reconcentrated.

GA in the diafiltered concentrate (approximately 120 mL) was purified by acarbose affinity chromatography (Chen *et al.*, 1994). The loaded column was washed with wash/diafiltration buffer and eluted with 1.7 M Tris-HCl, pH 7.6. Column loading, washing, and elution were monitored with a UV detector. Eluted GA was dialyzed extensively with water and the purified GA was lyophilized for storage. The entire harvesting, purification, and lyophilization process was completed in under 32 h, largely at 4°C.

SDS-PAGE of GA

GAs were electrophoresed under standard SDS-PAGE conditions on a Bio-Rad 4–15% Tris-HCl/2.6% crosslinker linear gradient gel to determine purity and apparent molecular mass. Each

GA-containing lane was loaded with 7 μg of protein sample, electrophoresed under the suggested conditions, and stained with Coomassie Blue.

GA concentration

Concentrations of rehydrated GAs were determined by one of two methods. GAs used for SDS-PAGE and soluble and insoluble starch hydrolysis assays were quantified with the Pierce biocinchoninic acid kit. GA concentrations in solutions used in the insoluble starch-binding assays were determined with the Bio-Rad Bradford assay kit. Bovine serum albumin was the reference standard in both methods.

Soluble starch hydrolysis

Soluble starch substrate was prepared daily by boiling Fisher soluble starch (1.8% w/v) in 50 mM NaOAc, pH 4.4, optimal for GA activity. For each reaction, 1.0 mL of starch substrate was equilibrated in a 35°C water bath for 5 min, a sufficiently low temperature that no enzyme activity was lost during the assay. Assays were initiated with 0.2–2 μg of enzyme in a total volume of 200 μL. At times ranging from 5 to 90 min, 100-μL samples were removed and quenched with 40 μL of 4 M Tris-HCl, pH 7.0.

Glucose concentrations were determined by the Sigma glucose oxidase/peroxidase/*o*-dianisidine assay kit. Activities were determined from slopes of glucose concentration *vs.* time plots. Specific activities were then calculated on IU/mg and k_{cat} bases, where 1 IU is defined as the enzyme necessary to produce 1 μmol/min glucose at 35°C in a 1.5% soluble starch reaction mixture. Protein molecular masses were calculated from the number of amino acid residues.

Insoluble starch hydrolysis

Insoluble starch substrate was prepared by suspending 1.8% (w/v) Sigma corn starch in 50 mM NaOAc, pH 4.4, at 35°C. Reactions were initiated with 4 μg GA solution plus reaction buffer in 200 μL. Reaction mixture samples (150 μL) were taken at 10-min intervals for 1 h and were

quenched with 60 µL of 4 M Tris-Cl, pH 7.0, before being microfuged to pellet the unreacted starch. A 140-µL portion of each microfuged sample was removed and analyzed for glucose content. Activity of GA on insoluble starch was determined from the plot of glucose released over time and recorded on IU/mg and k_{cat} bases as described above.

Insoluble starch binding

Insoluble starch substrate was prepared by washing Sigma corn starch twice with water. The water was drawn off by suction and the starch was air-dried for several days, with occasional repowdering with a mortar and pestle. A 0.2-g/mL stock mixture of the washed starch in 50 mM NaOAc, pH 4.4, was made fresh for each set of assays and chilled on ice. Chilled GA and buffer were added to aliquots of the stock mixture, resulting in 0–30 µg of GA in a 0.1 g/mL starch mixture. Reaction tubes were shaken at 5°C for 30 min. The starch was pelleted by centrifugation and the supernatant was assayed for mass of unbound GA. Equilibrium binding constants (K_{ad}) were calculated from the linear slopes of plots of nmol bound GA/g starch *vs.* nmol unbound GA/L solution.

Results and Discussion

Starch-clearing plate assay

Soluble starch hydrolysis was initially characterized by starch-clearing plate assays (Figure 5). The negative control did not show GA activity. GAI, RGAI, and RGAIΔL produced similarly-sized cleared zones. The cleared zones from GAII and RGAII were larger than those from the other GAs, perhaps enhanced by differences in diffusion due to enzyme size. GAE gave a smaller cleared zone.

SDS-PAGE

Purified GAs were subjected to SDS-PAGE (Figure 6). Apparent molecular masses for GAI, RGAI, and RGAIΔL are 110 kDa. GAII has an apparent molecular mass of about 90 kDa, with

that of RGAII being a little lower. GAE had two bands of somewhat greater than 110 kDa and about 90 kDa, suggesting that in some molecules the SBD was cleaved during processing. These molecular masses are much higher than the 81.7 and 69.2 kDa found by MALDI-TOF for glycosylated GAI and GAII expressed by *S. cerevisiae* C468 containing plasmid YEpPM18 (Khan *et al.*, 2000). GAI and GAII produced from pGAC9, a vector with one-fifth the expression ability of the YEpPM18-based vector, gave apparent molecular masses of 97 and 87 kDa, respectively, measured by SDS-PAGE (Innis *et al.*, 1985; Evans *et al.*, 1990). This suggests that SDS-PAGE yields erroneously high molecular masses for GA forms. YEpPM18 produces GAI of higher molecular mass than pGAC9 (H.-M. Chen, personal communication), probably caused by more glycosylation (Innis *et al.*, 1985).

Soluble starch hydrolysis

GA specific activities on soluble starch substrate are compared in Table I. GAII and RGAII have similar activities based on protein mass, somewhat higher than that of GAI. RGAI, RGAIΔL, and GAE have similar activities, slightly less than that of GAI. These results generally agree with the results from the starch-clearing assay. When specific activities are converted to k_{cat} values, differences between enzyme forms become smaller, these values ranging from 13.6 to 22.7 s^{-1}.

Great variations in GA domain architecture do not affect specific activity on soluble starch greatly. The GA catalytic domain can hydrolyze soluble starch with an SBD at its *C*-terminus 20 to 25% faster than when these structures are at its *N*-terminus. RGAII activity is equivalent to GAII activity. A possible reason for RGAI and RGAIΔL being slightly less active than GAI is that an *N*-terminal SBD may physically inhibit soluble starch hydrolysis. The similarities in k_{cat} values of GAI and GAII agree with an earlier study (Meagher and Reilly, 1989).

Insoluble starch hydrolysis

GAI, RGAI, and RGAIΔL have high specific activities on insoluble starch (Table I). GAII, RGAII, and GAE specific activities are much lower. GAs lacking SBDs have 15% or less the

activity on insoluble starch as those containing SBDs, as earlier shown (Svensson *et al.*, 1982). GAE activity on insoluble starch is much closer to activities of GAII and RGAII, which lack SBDs, than it is to those GAs with SBDs. The already-mentioned loss of an SBD in some GAE molecules (Figure 4) may explain some but not all of its low activity. The very low k_{cat} values of three GA forms indicate that activity on any soluble starch associated with the insoluble starch substrate was close to negligible.

It is apparent that the GA domains can cooperate to hydrolyze insoluble starch whether the SBD is *N*- or *C*-terminal to the catalytic domain; however, the former is less effective than the latter. The positioning of the SBD relative to the catalytic domain affects insoluble starch hydrolysis much more than it affects soluble starch hydrolysis. GAI has 45 and 80% greater activity than RGAIΔL and RGAI, respectively, on insoluble starch, whereas the differences for soluble starch hydrolysis are 20 and 25%. Presence of the whole linker in RGAI significantly inhibits insoluble starch hydrolysis, demonstrated by RGAIΔL possessing 25% more activity than RGAI.

Insoluble starch binding

GAI has the greatest ability to bind insoluble starch, while RGAI and RGAIΔL bind insoluble starch less strongly. GAE, GAII, and RGAII have very little binding ability (Figure 7, Table I).

The SBD functions with residues at its *C*-terminus, as demonstrated by the relatively high K_{ad} values of RGAI and RGAIΔL, 60% and 25% that of GAI, respectively. The position of domains relative to one another may be responsible for these K_{ad} differences. This is especially true for RGAI, since it contains the same sequences that GAI does. Possibly the three-dimensional configuration of RGAI interferes with access of starch to the SBD.

The very low ability of GAE to bind insoluble starch, 3% that of GAI, appears to be the main reason for its low activity on this substrate. The CGTase SBD (Domain E) is not separate from the rest of the enzyme in its native state, and it is quite possible that its structure is so severely modified when detached from the other CGTase domains and attached to GA through a linker that its binding ability is diminished. In addition, as mentioned earlier, not all GA molecules

retain their fused CGTase SBDs after processing. These factors appear to overcome the fact that GA and CGTase SBDs in their native states have very similar primary and tertiary structures (Figures 1 and 2).

Two other studies of CGTase SBD fused to other proteins also bear light on this difference. Dalmia *et al.* (1995) fused *P. macerans* CGTase SBD or the *A. niger* GA SBD to the *C*-terminus of β-galactosidase, finding that both constructs followed Langmuir adsorption isotherms, the former binding half as much insoluble corn starch but about the same amount of crosslinked amylose as the latter at high enzyme concentrations. Furthermore, the GA SBD-β-galactosidase fusion protein has the same K_{ad} as the CGTase SBD-β-galactosidase protein on insoluble corn starch, but double its value on crosslinked amylose. They explained these results as being caused by the CGTase SBD being potentially more susceptible to unfolding and proteolysis, countered in the case of amylose by the greater evolutionary advances of the CGTase SBD toward small-molecule binding.

In a second study, Ohdan *et al.* (2000) fused a *Bacillus* sp. CGTase SBD, with and without the immediately preceding Domain D, to the *C*-terminus of a *Bacillus subtilis* α-amylase not previously possessing an SBD. The fused protein lacking Domain D hydrolyzed soluble corn starch at the same rate as the parent α-amylase, while that with Domain D was only one-eighth as active, apparently because of structural distortion caused by the extra domain. Both fusion proteins had several times the activity of α-amylase on insoluble corn starch, the form with Domain D being more active, perhaps because of its role as a linker. No comparison with other SBDs was made.

In all three cases when CGTase SBDs were fused to other proteins, to GA in this study and to β-galactosidase and α-amylase earlier, diminished binding and/or activity on either soluble or insoluble starch was noted. These effects appear to be caused by enhanced susceptibility to proteolysis and to changes in tertiary structure.

Comparison of insoluble starch hydrolysis and binding data

Consideration of soluble and insoluble starch hydrolysis as well as insoluble starch binding provides insight into the nature of the cooperative interaction between binding and hydrolysis. When the three data sets for GAI, RGAI, and RGAIΔL are considered simultaneously, it appears that the relative positioning of the binding domain to the catalytic domain is an important factor influencing insoluble starch hydrolysis. The lower ability of RGAI and RGAIΔL than of GAI to hydrolyze insoluble starch is not mainly the result of one catalytic domain having greater activity than the other, since RGAI and RGAIΔL both hydrolyze soluble substrate at 80% the rate of GAI. Nor does their difference in insoluble starch hydrolysis rate appear to be completely correlated with starch-binding ability; in fact, RGAI binds starch over twice as well as RGAIΔL, yet it hydrolyzes insoluble starch less effectively than RGAIΔL, perhaps because the orientation of its SBD places the insoluble substrate in a less optimal position for hydrolysis than does the orientation of the RGAIΔL SBD, where no linker is present. Three-dimensional enzyme/substrate studies would be needed to substantiate this hypothesis.

Furthermore, it may be noted that rates by which GAII, RGAII, and GAE hydrolyze insoluble starch are not well correlated with K_{ad} beyond the fact that they all bind and hydrolyze insoluble starch poorly compared to GAI, RGAI, and RGAIΔL.

Conclusions

This study has produced significant information regarding GA structure and function. It is now clear that the *A. niger* GA catalytic domain can hydrolyze soluble starch with an SBD attached to its *N*-terminus and that the SBD can bind insoluble starch with a catalytic domain attached to its *C*-terminus. The results also demonstrate that the catalytic and starch-binding domains can cooperate to hydrolyze insoluble starch when their order is reversed from that of wild-type *A. niger* GA. In addition, insoluble starch binding does not solely correlate with insoluble starch hydrolysis, as evidenced by the results from GAI, RGAI, and RGAIΔL on one hand and from GAII, RGAII, and GAE, on the other hand.

Acknowledgments

The authors thank James Meade, Zivko Nikolov, and Jayarama Shetty for their generous gifts of the *A. niger* GA gene, the *P. macerans* CGTase gene, and acarbose, respectively. They also thank Alain Laederach for his help with Figure 2. This project was funded by the U.S. Department of Agriculture through its National Research Initiative Competitive Grants Program, by the U.S. Department of Energy through the Consortium for Plant Biotechnology Research, Inc., and by Genencor International, Inc.

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Table I. Specific activities and binding constants of wild-type, domain-reordered, and SBD-substituted GAs on soluble and insoluble starch.

GA form	Protein molecular mass (Da)	Specific activity				K_{ad} (L/g)
		Soluble starch		Insoluble starch		
		IU/mg	k_{cat} (s^{-1})	IU/mg	k_{cat} (s^{-1})	
GAI	65,790	17.0 ± 1.7 [a]	18.6 ± 1.9 [a]	5.98 ± 0.47 [a]	6.55 ± 0.52 [a]	0.051 ± 0.002 [a]
GAII	50,474	24.8 ± 3.1	20.9 ± 2.6	0.27 ± 0.04	0.22 ± 0.03	0.0023 ± 0.0002
RGAI	67,257	13.4 ± 3.0	15.0 ± 3.3	3.24 ± 0.15	3.63 ± 0.17	0.030 ± 0.004
RGAIΔL	65,734	14.3 ± 1.5	15.7 ± 1.6	4.14 ± 0.18	4.54 ± 0.20	0.013 ± 0.0003
RGAII	54,253	25.1 ± 2.5	22.7 ± 2.3	0.62 ± 0.03	0.56 ± 0.03	0.0053 ± 0.0003
GAE	66,283	12.3 ± 1.6	13.6 ± 1.7	0.68 ± 0.08	0.75 ± 0.09	0.0017 ± 0.0001

[a]Standard error.

Figure captions

Fig. 1. Multiple sequence alignment of GA and CGTase SBDs, arranged manually. SWISS-PROT designations unless noted. **Ax**: *A. niger X*-100 (Q12537); **As**: *Aspergillus shirousami* (P22832); **Ak**: *Aspergillus kawachi* (P23176); **An**: *A. niger* (P04064); **Te**: *Taleromyces emersonii* (GenBank AJ304803); **Ao**: *Aspergillus oryzae* (P36914); **Nc**: *Neurospora crassa* (P14804); **Hg**: *Humicola grisea* (Q12623); **Hr**: *Hormoconis resinae* (Q03045); **Cr**: *Corticium rolfsii* (Q12596); **Le**: *Lentinula edodes* (GenBank AF220541); **Bc**: *Bacillus circulans* (P43379); **Bl**: *Bacillus licheniformis* (P14014); **Pm**: *P. macerans* (P04830); **Tt**: *Thermoanaerobacter thermosulfurogenes* (P26827); **Bo**: *Bacillus obensis* (P27036); **Bb**: *Brevibacillus brevis* (O30565); **Gs**: *G. stearothermophilus* (P31797); **Kp**: *Klebsiella pneumoniae* (P08704). *: residues totally conserved. ●: residues semi-conserved (all residues of similar character less at most one outlier).

Fig. 2. Tertiary structures of the SBDs of (a) *A. niger* GA (Protein Data Bank 1AC0). (b) *B. circulans* 251 CGTase (1CDG); (b) Prepared with MOLMOL (Koradi *et al.*, 1996).

Fig. 3. Complete cloning scheme. B: *Bam*HI; Bs: *Bss*HII; E: *Eco*RI; H: *Hin*dIII; N: *Nhe*I; P: *Pst*I; Sa: *Sal*I; Sp: *Spe*I; X: *Xba*I. ( ): nonfunctional site when ligated. Catalytic domain: ▬▬ ; linker: ''''''''''''' ; SBD: ⊏------⊐ ; *Eco*RI–*Eco*RI spacer: ∿∿∿∿ .

Fig. 4. Oligonucleotide sequences of adoptors synthesized to 1) provide *N*-terminal restriction sites for the SBD; 2) provide *N*-terminal restriction sites for the *N*-terminal half of the catalytic domain; 3) replace the deleted part of pCG2; 4) complete the *C*-terminal portion of the SBD.

Fig. 5. Starch-clearing plates showing activities of different GAs on soluble starch.

Fig. 6. SDS-PAGE gels of different GAs.

Fig. 7. Insoluble starch binding to GAI (○), GAII (●), RGAI (▽), RGA1ΔL (▼), RGAII (□), GAE (■).

Figure 1

```
                    . .  *    . *  *  . . * .  *. *    .            . .   .
Ax GA   508 CTT PTA  VAV TFDL TATTTYGENIYLVGSISQLGDWETSDG IALSADKYTSSNPLWYVTV
As GA   508 CTT PTA  VAV TFDL TATTTYGENIYLVGSISQLGDWETSDG IALSADKYTSSNPPWYVTV
Ak GA   508 CTT PTA  VAV TFDL TATTTYGENIYLVGSISQLGDWETSDG IALSADKYTSSNPLWYVTV
An GA   509 CTT PTA  VAV TFDL TATTTYGENIYLVGSISQLGDWETSDG IALSADKYTSSDPLWYVTV
Te GA   485 CTT PTS  VAV TFDE IVSTSYGETIYLAGSIPELGNWSTASA IPLRADAYTNSNPLWYVTV
Ao GA   481 CQV PTT  VSV TFAV KATTVYGESIKIVGSISQLGSWNPSSA TALNADSYTTDNPLWTGTI
Nc GA   485 CAA DHE  VLV TFNE KVTTSYGQTVKVVGSIARLGNWAPASG LTLSAKQYSSSNPLWSTTI
Hg GA   478 CAD ASE  VYV TFNE RVSTAWGETIKVVGNVPALGNWDTSKA VTLSASGYKSSNPLWSTTI
Hr GA   476 CQ       VSI TFNI NATTYYGENLYVIGNSSDLGAWNIADA YPLSASAYTQDRPLWSAAI
Cr GA   451 PGGSSGS  VEV TFDV YATTVYGQNIYITGDVSELGNWTPANG VALSSANY    PTWSATI
Le GA   442 CGGGPVAQAVSV TFNV DASTLEGQNVYLTGAVDALEDWSTDNA ILLSSANY    PTWSVTV
Bc CGT  579 FEVLSGDQ VSV RFVVNNATTALGQNVYLTGSVSELGNWDPAKA IGPMYNQVVYQYPNWYYDV
Bl CGT  578 FTILSGDQ VSV RFVINNATTALGENIYLTGNVSELGNWTTGAASIGPAFNQVIHAYPTWYYDV
Pm CGT  580 FNVLTGDQ VTV RFLVNQANTNYGTNVYLVGNAAELGSWDPNKA IGPMYNQVIAKYPSWYYDV
Tt CGT  576 INILTGNQ ICV RFVVNNASTVYGENVYLTGNVAELGNWDTSKA IGPMFNQVVYQYPTWYYDV
Bo CGT  568 FEVLTGDQ VSI RFAVNNATTSLGTNLYMVGNVNELGNWDPDQA IGPMFNQVMYQYPTWYYDI
Bb CGT  566 FEVLSGNQ VSV RFAVNNATTNSGTNVYIVGNVSELGNWDPNKA IGPMFNQVMYKYPTWYYDI
Gs CGT  572 FEVLTNDQ VSV RFVVNNATTNLGQNIYIVGNVYELGNWDTSKA IGPMFNQVVYSYPTWYIDV
Kp CGT  521 SDDAENPT VQSINFTCNNGYTISGQSVYIIGNIPQLGGWDLTKA VKISPTQYPQWSASLELP

          .      . ....        . ..    .                          .
Ax GA   TLPA GESFEYKFIRVES DDSVEWES DPNREYTVPQ     ACGESTATVTDTWR           615
As GA   TLPA GESFEYKFIRVES DDSVEWES DPNREYTVPQ     ACGESTATVTDTWR           615
Ak GA   TLPA GESFEYKFIRVES DDSVEWES DPNREYTVPQ     ACGESTATVTDTWR           615
An GA   TLPA GESFEYKFIRIES DDSVEWES DPNREYTVPQ     ACGTSTATVTDTWR           616
Te GA   NLPP GTSFEYKFFKNQT DGTIVWED DPNRSYTVPA     YCGQTTAILDDSWQ           592
Ao GA   NLPA GQSFEYKFIRVQ  NGAVTWES DPNRKYTVPS     TCGVKSAVQSDVWR           587
Nc GA   ALPQ GTSFKYKYVVVNS DGSVKWEN DPDRSYAVGT     DC ASTATLDDTWR           591
Hg GA   PIKATGSAVQYKYIKVGT NGKITWES DPNRSITLQTASSAGKCAAQ  TVNDSWR           589
Hr GA   PLNA GEVISYQYVRQEDCDQPYIYET  VNRTLTVP      ACGGAAVTTDDAWMGPVGSSGNC  587
Cr GA   ALPA DTTIQYKYVNID  GSTVIWEDAISNREITTPA        SGTYTEKDTWDES         554
Le GA   DLPG STDVQYKYIKKDG SGTVTWES DPNMEITTPA       NGTYAT NDTWR           545
Bc CGT  SVPA GKTIEFKFLKKQ  GSTVTWEG GSNHTFTAP  SS    G  TATINVNWQP          686
Bl CGT  SVPA GKQLEFKFFKKN  GATITWEG GSNHTFTTP  TS    G  TATVTINWQ           685
Pm CGT  SVPA GTKLDFKFIKKG  GGTVTWEG GGNHTYTTP  ASGV  G  TVTV  DWQN          687
Tt CGT  SVPA GTTIQFKFIKKN  GNTITWEG GSNHTYTVP  SSST  G  TVIV  NWQQ          683
Bo CGT  SVPA EENLEYKFIKKDS SGNVVWES GNNHTYTTP  ATGT  D  TVLV  DWQ           675
Bb CGT  SVPA GKNLEYKYIKKDH NGNVTWQS GNNRTYTSP  ATGT  D  TVIS  NW           672
Gs CGT  SVPE GKTIEFKFIKKDS QGNVTWES GSNHVYTTP  TNTT  G  KIIV  DWQN          680
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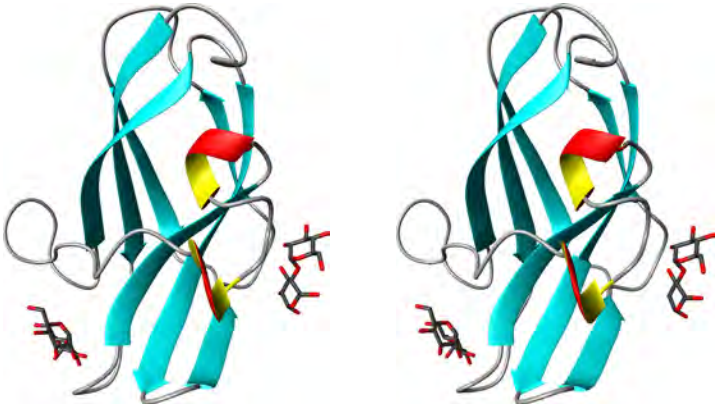
Figure 2

a



b

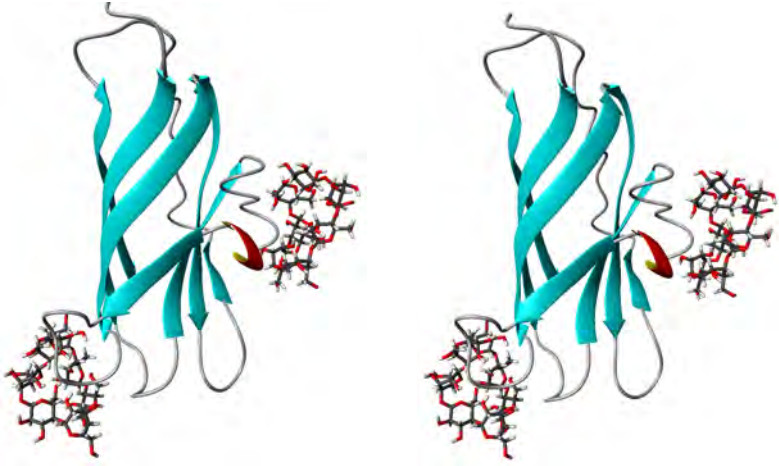
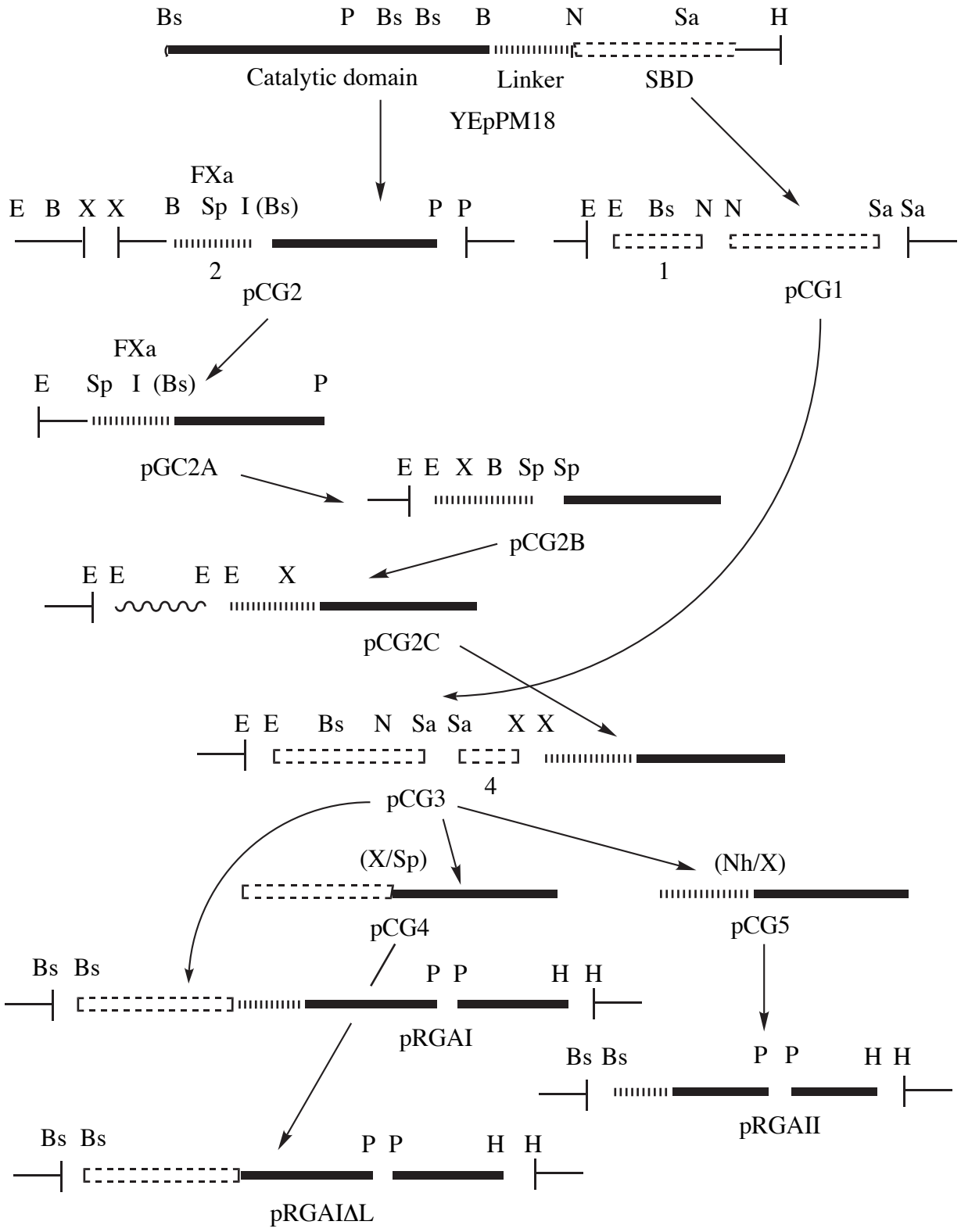


Figure 3


Bs P Bs Bs B N Sa H
Catalytic domain
Linker
SBD
YEpPM18
FXa
E B X X B Sp I (Bs) P P
2
pCG2
E E Bs N N Sa Sa
1
pCG1
FXa
E Sp I (Bs) P
pGC2A
E E X B Sp Sp
pCG2B
E E E E X
pCG2C
E E Bs N Sa Sa X X
4
pCG3
(X/Sp)
(Nh/X)
pCG4
pCG5
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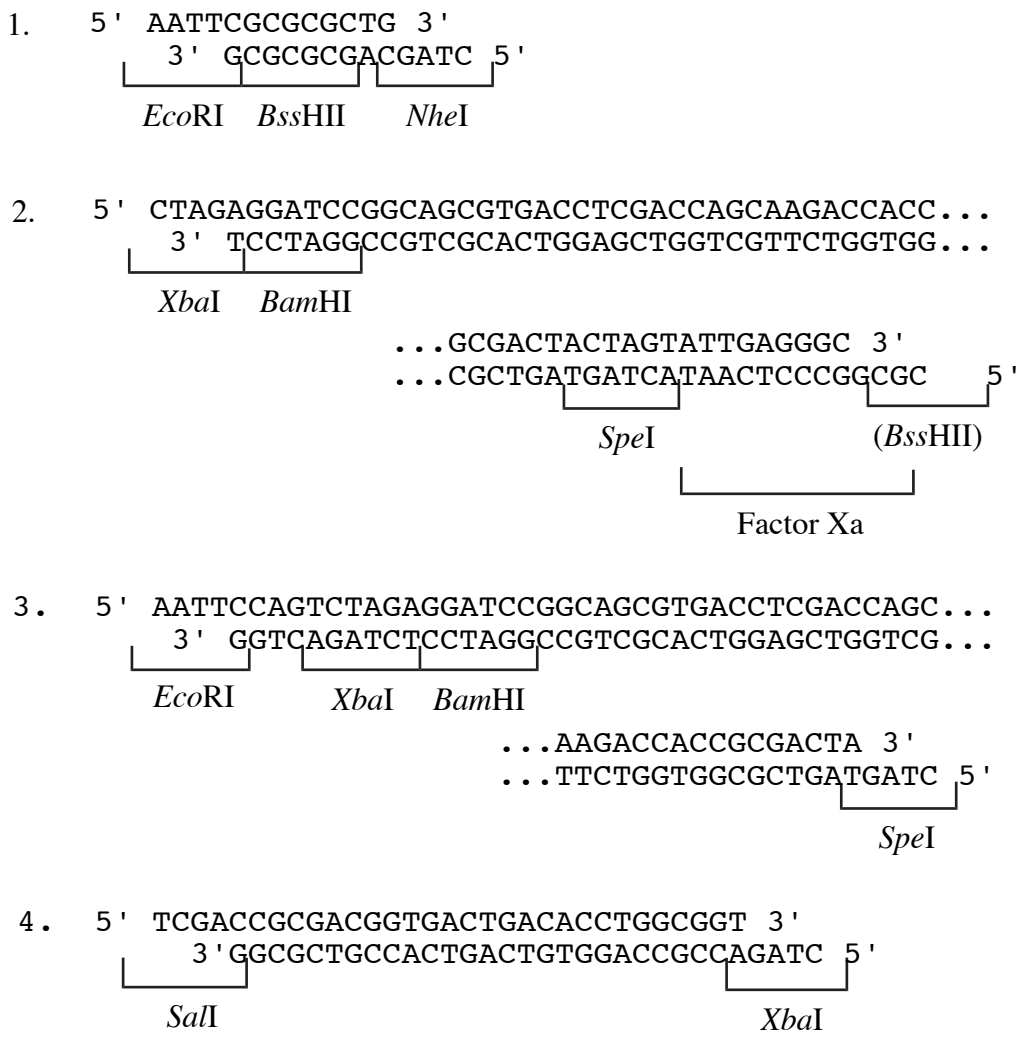
pRGAI
Bs Bs P P H H
pRGAII
Bs Bs P P H H
pRGAIΔL

Figure 4


1.
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3' GCGCGCGACGATC 5'
EcoRI
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NheI
2.
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3' TCCTAGGCCGTCGCACTGGAGCTGGTCGTTCTGGTGG...
XbaI
BamHI
...GCGACTACTAGTATTGAGGGC 3'
...CGCTGATGATCATAACTCCCGGCGC 5'
SpeI
(BssHII)
Factor Xa
3.
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3' GGTCAGATCTCCTAGGCCGTCGCACTGGAGCTGGTCG...
EcoRI
XbaI
BamHI
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SpeI
4.
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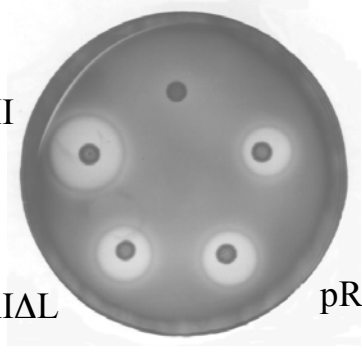
SalI
XbaI

Figure 5



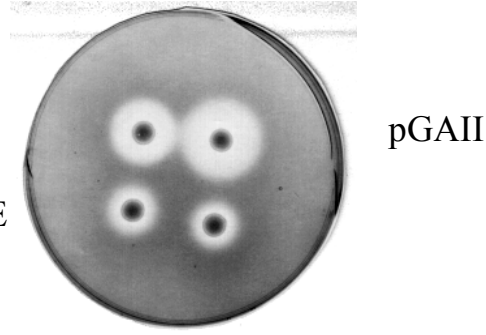
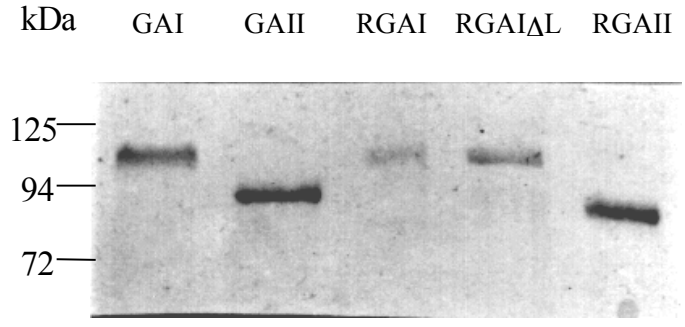


Figure 6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GAII
RGAI
RGAIΔL
RGAII
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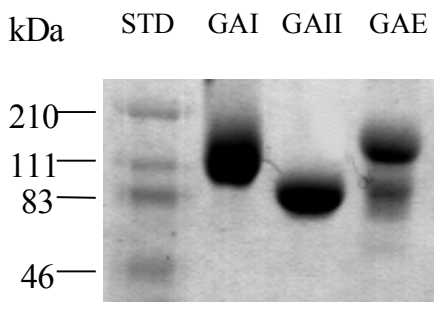
94
72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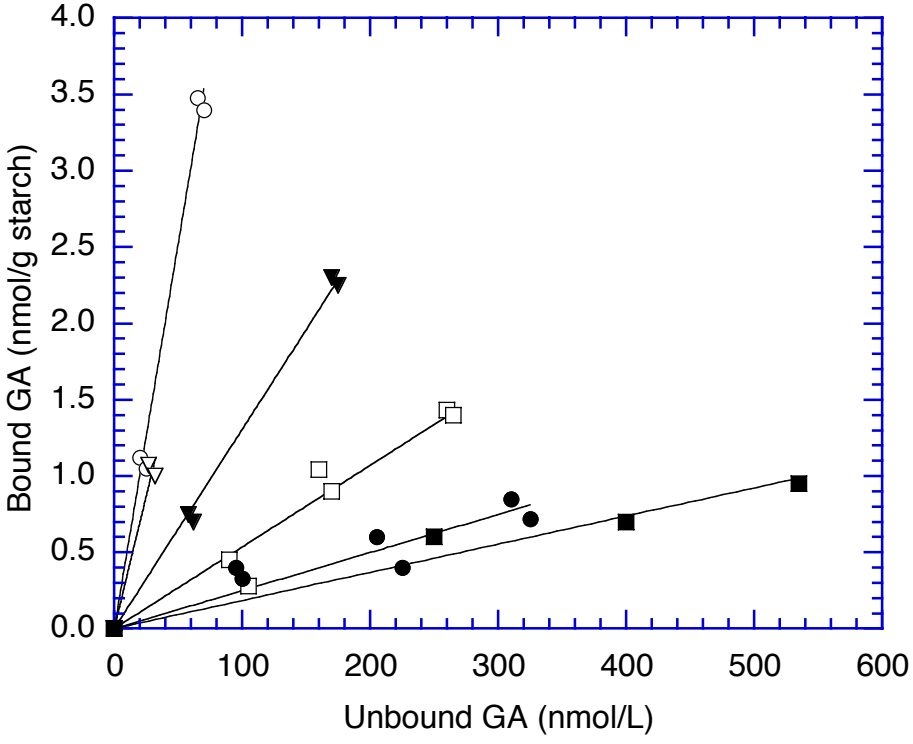
STD
GAI
GAII
GAE
210
111
83
46

Figure 7


Bound GA (nmol/g starch)
Unbound GA (nmol/L)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
100
200
300
400
500
600