Acarbose binding at the surface of *Saccharomycopsis fibuligera* glucoamylase suggests the presence of a raw starch-binding site

Juraj Gašperík, Eva Hostinová & Jozef Ševčík*

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, SK-84551 Bratislava, Slovakia; e-mail: jozef.sevcik@savba.sk

Abstract: There are two acarbose molecules in the structure of the complex of glucoamylase from *Saccharomycopsis fibuligera* HUT 7212 (Glu) with the inhibitor acarbose: one is bound to the active site, which is localized in the cavity of the $(\alpha/\alpha)_6$-barrel, the other one is on the surface of the enzyme molecule curved around Tyr464, which captures the inhibitor molecule as seen in the "sugar tongs" binding site of the barley α-amylase isozyme 1 complexed with a thiomaltooligosaccharide. The surface acarbose molecule suggests the presence of a raw starch-binding site. Based on the expected similarity of structures of glucoamylase Glu, which does not degrade raw starch, and the raw starch-degrading glucoamylase from *S. fibuligera* IFO 0111 (Glm), it is reasonable to expect the presence of a starch-binding site, at a structurally equivalent position, also on the surface of Glm. Verification of this hypothesis by preparation of mutants of glucoamylases Glu and Glm is presented.

Key words: glucoamylase, acarbose, X-ray structure, starch-binding site, sugar tongs.

Abbreviations: Glm, glucoamylase from *Saccharomycopsis fibuligera* IFO 0111; Glu, glucoamylase from *Saccharomycopsis fibuligera* HUT 7212.

Introduction

Glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3) is an inverting exo-glucan hydrolase that catalyzes the removal of α-D-glucose from the non-reducing ends of starch and other related poly- and oligosaccharides. This enzyme, which belongs to glycoside hydrolase family GH15 (Henrissat, 1991; Davies & Henrissat, 1995; Henrissat & Davies, 1997) and which is produced by many molds and yeasts, is also able to degrade α-1,6-glucosidic linkages but much less effectively. The primary industrial use of glucoamylase is in the production of glucose syrups, which in turn serve as a feedstock for biological fermentations in the production of ethanol or in the production of high fructose sweeteners.

Determination of the crystal structure of recombinant glucoamylase Glu from the yeast strain *Saccharomycopsis fibuligera* HUT 7212 at 1.8 Å resolution was reported a few years ago (Ševčík et al., 1998). The overall fold is an $(\alpha/\alpha)_6$-barrel and is closely similar to that of the catalytic domain of *Aspergillus awamori* glucoamylase (Aleshin et al., 1992). The active site is situated in the loop region pocket at the narrower end of the core of the $(\alpha/\alpha)_6$-barrel, as identified by the presence of a Tris molecule. The enzyme consists of 492 amino acid residues and has 14 α-helices, 12 of which form the barrel. The core at the center of the barrel is conical and is filled with hydrophobic side-chains. Both ends of the core are open to solvent.

In the structure of the glucoamylase-acarbose complex at 1.6 Å resolution, two acarbose molecules have been identified: one in the active site, whereas the other one at the surface of the enzyme molecule. The surface acarbose suggests the presence of a raw starch-binding site. Determination and description of the structure of the complex will be published elsewhere.

Two related glucoamylases, Glu (*S. fibuligera* HUT 7212) and Glm (*S. fibuligera* IFO 0111) are produced from the *GLU* (Itoh et al., 1987) and *GLM* (Hostinová et al., 2002; 2003) genes, respectively. Glu consists of 492 and Glm of 489 amino acid residues. Alignment of amino acid sequences shows a high degree of sequence identity (60%) and similarity (77%). The two enzymes differ in their properties, namely in the ability to digest raw starch. Glu is capable of raw starch adsorption, but not digestion. Glm adsorbs well to starch granules and is capable of raw starch digestion. The three-dimensional model of glucoamylase Glm (Hostinová et al., 2003), based on the structure of Glu (Ševčík et al., 1998), shows that the enzyme has a unique structure lacking a separate starch-binding domain typical for all

* Corresponding author

Table 1. Primers used for preparing the mutants of glucoamylases.

Glucoamylase	Primer[a]
GLU gene: H447A, D450A	forward 5'-GCAAGTCATTTTGGATGCTATTAATGCTGATGGCTCCTTGAATGAAC-3' reverse 5'-GTTCATTCAAGGAGCCATCAGCATTAATAGCATCCAAAATGACTTGC-3'
GLM gene: H444A, D447A	forward 5'-GAAAGTTCTCCTTGACGCTATTGATGCTAATGGCCAACTCACCGA-3' reverse 5'-CTCGGTGAGTTGGCCATTAGCATCAATAGCGTCAAGGAGAACTTTC-3'

[a] The mutated codons are underlined. All mutations in the *GLU* and *GLM* sequences were verified by DNA sequencing.

known raw starch-degrading amylases. It results, that in Glm, the raw starch affinity site is an integral part of the single-domain enzyme. From this point of view, Glm represents a new type of raw starch-degrading enzymes. High structural similarity between Glu and Glm made it possible to identify five amino acid residues in Glu (Arg15, His447, Asp450, Thr462, Tyr464) as well as in Glm (Arg15, His443, Asp447, Thr459, Phe461), which might constitute the raw starch-binding site located in identical positions in both enzymes. It is important to note the difference in amino acids: at the position structurally equivalent to Tyr464 in Glu, there is Phe461 in Glm. The main goal of the present article was to verify the hypothesis on the presence of raw-starch-binding sites at the surface of Glu and Glm by preparation of mutants of both glucoamylases.

Material and methods

In vitro mutagenesis
Site-directed mutagenesis of *GLU* and *GLM* genes was performed using the QuickChange^{TM} site-directed mutagenesis kit (Stratagene). The primers used are described in Table 1.

Preparation of enzymes
The recombinant glycosylated enzymes Glu and Glm were prepared from the wild type and mutant *GLU* and *GLM* genes in *Saccharomyces cerevisiae* AH 22, as described by GAŠPERÍK & HOSTINOVÁ (1993) and HOSTINOVÁ et al. (2003). Expression of the *GLU* and *GLM* genes was achieved from vectors pVT100L-Glu and pVT100L-Glm in *S. cerevisiae* AH 22. Yeast transformants were grown in YPD medium for 48 h. Proteins, which showed electrophoretic homogeneity, were obtained from extracellular media after ultrafiltration through Amicon PM-30 membrane, molecular sieving chromatography on Superose 12 P and ion exchange chromatography on FQ (both from Pharmacia). The mutant glucoamylases were prepared in the same way.

Electrophoretic methods
Polyacrylamide electrophoresis was performed on 10% gels under denatured and native conditions, respectively. The positions of glucoamylases after SDS/PAGE were detected with Coommassie Brilliant Blue R-250 staining.

Two modifications of native electrophoresis were used:

(1) Soluble starch was applied onto the gel after electrophoresis: the starch was diffused into the gel after electrophoretic separation by immersing it into the solution of 1% soluble starch in Na acetate buffer, pH 5.6, with several buffer exchanges to reach the pH 5.6. After 60 min incubation of the gel at room temperature it was shortly washed with water and stained with an iodine solution (0.3% I_2 w/v in 3% KI w/v).

(2) Soluble starch was co-polymerized into the gel before electrophoresis: suspension of 37.5 mg of soluble starch in 1.25 mL of 1.5 M Tris.HCl buffer, pH 8.8, and 2 mL of water was boiled for 5 min and after cooling to room temperature 1.65 mL of an acrylamide solution (30%), 60 μL of 10% ammonium persulfate solution and 2.5 μL TEMED were added. After electrophoretic separation the pH of the gel was changed by immersing it into 0.1 M Na acetate buffer, pH 5.6, and incubated for 60 min at room temperature. The positions of glucoamylases were identified by staining with an iodine solution.

Enzyme activity
Estimation of enzyme activity on soluble and raw starch was described by HOSTINOVÁ et al. (2003).

Results and discussion

In the Glu-A structure two acarbose molecules have been localized: one in the active site and the other one at the surface of the enzyme molecule (Fig. 1). The active site acarbose fits tightly into the pocket where

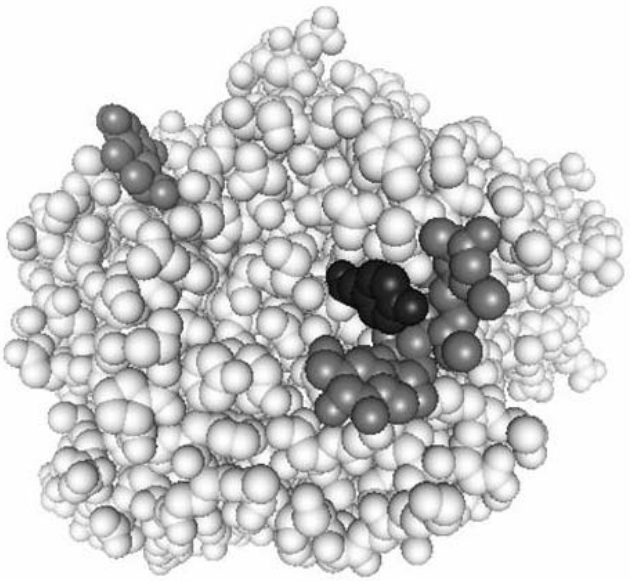

Fig. 1. Space-filling model of the Glu-A complex with two acarbose molecules in dark-grey; Tyr464 is black. Active site acarbose (left) is buried in the void of the barrel.

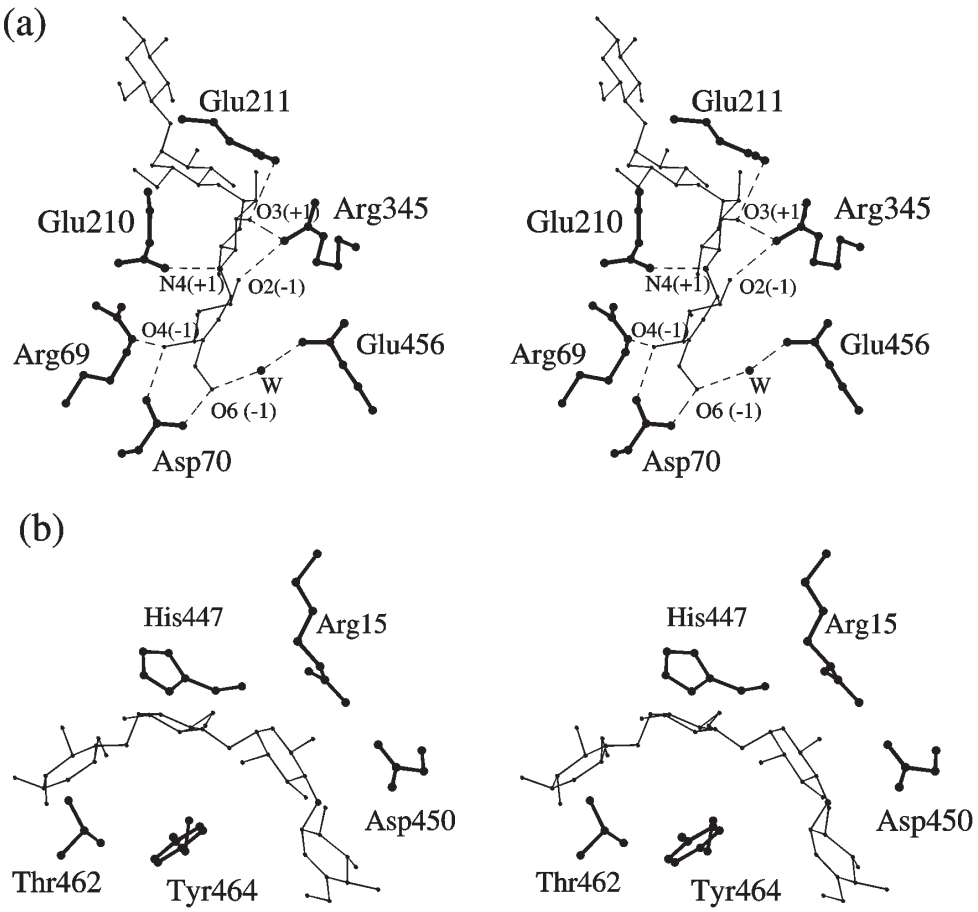

Fig. 2. (a) Hydrogen-bonds formed by the acarbose molecule with the active site residues. The catalytic residues are Glu210 and Glu456. (b) Surface acarbose curved around Tyr464 and the interacting partners Arg15, His447, Asp450, and Thr462.

it forms several hydrogen bonds with the enzyme depicting the active site residues (Fig. 2a). The mode of acarbose binding to the active site readily explains the exoglucanase activity of glucoamylases.

Binding of the acarbose molecule to the surface of the enzyme suggests the presence of a starch-binding site. It is a unique property of glucoamylases that consist of only a catalytic domain. It is reasonable to assume that this site substitutes for a separate starch-binding domain, which is typical for other amylolytic enzymes. The acarbose is localized in a crevice between Asp450, Arg15, His447, Thr462 on one side and Tyr464 on the other (Fig. 2b). It is bent around the Tyr464 side-chain in a form of a semicircle and captures the inhibitor molecule as seen in the "sugar tongs" binding site in barley α-amylase isozyme 1 complexed with a thiomaltooligosaccharide (Robert et al., 2002; 2003). A similar situation was found also in the structure of the amylomaltase-acarbose complex (Sträter et al., 2002) in which the acarbose molecule winds around Tyr54. In the complex of glucoamylase Glu with acarbose, there are two direct H-bonds and three H-bonds mediated through water molecules between acarbose and the enzyme. The plane of the guanido group of Arg15 is parallel with the second sugar ring within the acarbose molecule forming a stacking interaction.

To verify the hypothesis that the site on Glu surface interacting with acarbose represents a starch-binding site, double mutants – H447A, D450A in Glu and H444A, D447A in Glm – were prepared and tested for enzyme activity and starch adsorption. It was assumed that double mutants would have more pronounced effect than a single mutant. Biochemical analysis showed that towards soluble starch both double mutants retained specific activities identical with the wild type enzymes. Degradation of raw starch by the Glm mutant decreased to 12% in comparison to that by the wild type enzyme (not shown). Starch adsorption of the enzymes under different electrophoretic conditions is documented in Figures 3 and 4.

Electrophoretic mobility of the wild type Glu and its H447A, D450A double mutant under denaturing conditions of SDS-PAGE is shown in Figure 3. The mobility of both enzymes is identical. Under native conditions, where proteins are separated in the gel not containing starch, the mobility of wild type enzyme and the mutant is also identical. Electrophoretic separation of enzymes in the gel co-polymerized with starch showed,

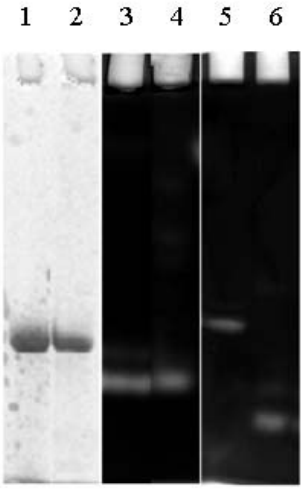

Fig. 3. PAGE of glucoamylase Glu. Denatured conditions – SDS: Lane 1, Glu wild type; Lane 2, Glu H447A, D450A. Native conditions – soluble starch diffused into the gel after electrophoresis: Lane 3, Glu wild type; Lane 4, Glu H447A, D450A. Soluble starch present in the gel during electrophoresis: Lane 5, Glu wild type; Lane 6, Glu H447A, D450A.

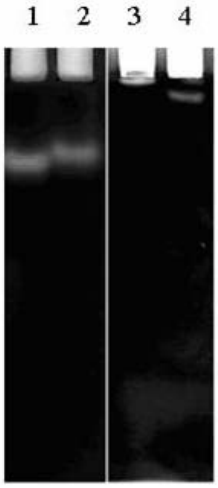

Fig. 4. PAGE of glucoamylase Glm under native conditions. Starch diffused into the gel after electrophoresis: Lane 1, Glm wild type; Lane 2, Glm H443A, D447A. Starch present in the gel during electrophoresis: Lane 3, Glm wild type; Lane 4, Glm H443A, D447A.

however, significant differences in mobility of wild type and mutant enzymes that are caused by different affinities of the enzyme toward starch.

As far as the Glm is concerned, the electrophoretic mobility of the wild type and H444A, D447A double mutant under native conditions is presented in Figure 4. Both enzymes move differently in the gel. While the wild type enzyme moves in the gel not containing starch faster than the double mutant, in the gel containing incorporated starch the wild type enzyme shows impaired mobility and stronger affinity to starch in comparison to the double mutant.

Electrophoretic separation of the wild type Glu and Glm on native gels containing co-polymerized starch clearly documents that Glm, capable of raw starch degradation, has significantly higher affinity to starch than Glu, which does not degrade raw starch. Further, these results demonstrate that mutation of amino acid residues, which are involved in binding of acarbose onto the surface, caused reduction of enzyme adsorption onto starch, thus proving that these amino acids are involved in starch-binding site in spite of different key residues – Tyr464 in Glu versus Phe461 in Glm.

Acknowledgements

This work was supported by Howard Hughes Medical Institute grant No. 75195-574601 and the grants No. 1/0101/03 and 2/1010/96 awarded by the Slovak Grant Agency VEGA.

References

ALESHIN, A.E., GOLUBEV, A., FIRSOV, L.M. & HONZATKO, R.B. 1992. Crystal structure of glucoamylase from *Aspergillus awamori* var. X100 to 2.2-Å resolution. J. Biol. Chem. **267:** 19291–19298.

GAŠPERÍK, J. & HOSTINOVÁ, E. 1993. Glucoamylases encoded by variant *Saccharomycopsis fibuligera* genes – structure and properties. Curr. Microbiol. **27:** 11–14.

HENRISSAT, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **280:** 309–316.

DAVIES, G. & HENRISSAT, B. 1995. Structures and mechanisms of glycosyl hydrolases. Structure **3:** 853–859.

HENRISSAT, B. & DAVIES, G. 1997. Structural and sequence-based classification of glycoside hydrolases. Curr. Opin. Struct. Biol. **7:** 637–644.

HOSTINOVÁ, E. 2002. Amylolytic enzymes produced by the yeast *Saccharomycopsis fibuligera*. Biologia, Bratislava **57 (Suppl. 11):** 247–251.

HOSTINOVÁ, E., SOLOVICOVÁ, A., DVORSKÝ, R. & GAŠPERÍK, J. 2003. Molecular cloning and 3D structure prediction of the first raw-starch-degrading glucoamylase without a separate starch-binding domain. Arch. Biochem. Biophys. **411:** 189–195.

ITOH, T., OHTSUKI, L., YAMASHITA, I. & FUKUI, S. 1987. Nucleotide sequence of the glucoamylase gene GLU1 in the yeast *Saccharomycopsis fibuligera*. J. Bacteriol. **169:** 4171–4176.

ROBERT, X., HASER, R., SVENSSON, B. & AGHAJARI, N. 2002. Comparison of crystal structures of barley α-amylase 1 and 2: implications for isozyme differences in stability and activity. Biologia, Bratislava **57 (Suppl. 11):** 59–70.

ROBERT, X., HASER, R., GOTTSCHALK, T.E., RATAJCZAK, F., DRIGUEZ, H., SVENSSON, B. & AGHAJARI, N. 2003. The structure of barley α-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding: a pair of sugar tongs. Structure **11:** 973–984.

ŠEVČÍK, J., SOLOVICOVÁ, A., HOSTINOVÁ, E., GAŠPERÍK, J., WILSON, K.S. & DAUTER, Z. 1998. Structure of glucoamylase from *Saccharomycopsis fibuligera* at 1.7 Å resolution. Acta Crystallogr. **D54:** 854–866.

STRÄTER, N., PRZYLAS, I., SAENGER, W., TERADA, Y., FUJII, K. & TAKAHA, T. 2002. Structural basis of the synthesis of large cycloamylose by amylomaltase. Biologia, Bratislava **57 (Suppl. 11):** 93–99.

Received March 03, 2005
Accepted May 05, 2005