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Computer-Aided Analysis of Spatial Structure of Some Hydrolytic Enzymes

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Abstract—Using the MolScript version 2.1 computer program for protein molecule modeling and X-ray structure analysis data the spatial structures of several hydrolytic enzymes have been compared. These include glucoamylase from *Aspergillus awamori* and *Saccharomycopsis fibuligera* and lipases from *Rhizopus japonicus*. Results on homology of amino acid sequences and topology of secondary structure elements were obtained. 3D models of these enzymes with positioning of functionally important groups in the active site cavity were built.

Key words: enzyme, amino acid sequence, secondary structure, spatial model, topology, computer-aided analysis

Increased interest in hydrolases is due to their wide use as effective biocatalysts in medicine, food, and light industries [1, 2]. Studies of physicochemical, kinetic, and thermodynamic parameters and features of enzyme substrate interactions during catalysis by hydrolytic enzymes has significantly changed and improved old technologies and resulted in development of new technologies.

Studies of mechanisms of enzymatic catalysis by classic methods (e.g., IR- and UV-spectroscopy, enzymatic methods, ion-exchange and gel chromatography) emphasized effects of various physicochemical factors on the conformation of *Aspergillus awamori* glucoamylase and *Rhizopus japonicus* lipase [1-4].

Currently developed software programs for computer modeling of protein molecules based on crystallography data provide three-dimensional (3D) images of research objects. This helps in better understanding of experimental data on structural–functional properties of enzymes and therefore recognition of additional aspects of molecular mechanisms responsible for enzymatic conversion of a substrate.

In this study, we have analyzed hierarchic levels of hydrolytic enzyme structure based on protein X-ray structure analysis using the MolScript version 2.1 program for modeling of spatial organization of protein molecules and experimental data.

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MATERIALS AND METHODS

Aspergillus awamori G-20X glucoamylase (Ladyzhinskii Factory for Enzyme Preparations) and *Rhizopus japonicus* 1403 lipase isolated at the Department of Biochemistry and Microbiology, Voronezh State Technological Academy, were used in experiments. Glucoamylase (α-1,4:1,6-glucan-4,6-glucohydrolase, EC 3.2.1.3) can hydrolyze both α-1,4- and α-1,6-glycoside bonds in a starch molecule. Lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3) catalyzes cleavage of primary ether bonds formed by glycerol and fatty acid. The hydrolases were purified by ion exchange and gel chromatography. Homogeneity of the resulting enzyme preparations was confirmed by electrophoresis in polyacrylamide gel. Catalytic activity of glucoamylase was evaluated by glucose oxidase methods using kits for assay of glucose concentration in biological fluids (Olvex Diagnosticum, Russia) and starch as substrate. Lipase activity was assayed spectrophotometrically using rhodamine 6G and tributyrin as substrate [5]. Protein content in the enzyme preparations was determined by the Lowry method [6]. Infrared absorbance spectra of protein molecules were registered using a Specord M-80 spectrophotometer (Germany). Supramolecular organization levels of these hydrolases were studied using $3.5 \cdot 10^{-5}$ M sodium dodecyl sulfate followed by subsequent chromatography on Sephadex G-200. Sulfhydryl groups were assayed by the

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method of Thannhauser et al. [7]. The content of disulfide bonds in hydrolase molecules was calculated by the method based on the increase in optical density during nitrothiobenzoate formation. UV-irradiation of intact enzyme solutions and those treated with methylene blue as a photosensitizer was carried out in a thermostatted cuvette at 20-22°C under constant stirring using a DRT-400 mercury-quartz lamp through a 240-390 nm bandpass UVS-1 filter at a distance of 10 cm and intensity of 0.15 kJ/m^2 per min.

Computer modeling of biopolymers based on results of X-ray structure analysis was carried out using the MolScript program version 2.1 [8].

X-Ray structure analysis data were obtained from The Research Collaboratory for Structural Bioinformatics (RCSB) available at http://www.RCSB.org/pdb for the following proteins: *A. awamori* glucoamylase (3GLY; pid: g576132; gi: 576132), *Saccharomycopsis fibuligera* glucoamylase (1AYX; pid: g3212473; gi: 3212473), *R. niveus* lipase (1LGY; pid: g1942798; gi: 1942798). Primary structures of the enzymes studied were compared using the programs Gene Bee and Gene Doc available at http://www.genebee.msu.ru/genebee.html and http://www.psc.edu/biomed/genedoc/, respectively.

RESULTS AND DISCUSSION

Analysis of primary structures of glucoamylases from micromycetes of the *Aspergillus* genus (*A. niger*, *A. awamori* X100, *A. awamori* var. *kawachi*, *A. shirousami*, *A. oryzae*) using the Gene Bee program revealed high homology of their amino acid sequences (86.1%). Their signal peptides contain 24 amino acid residues; Ala and Thr are N-terminal residues, whereas Trp and Arg are C-terminal residues. The signal peptide of *A. oryzae* glucoamylase contains two additional valine residues, Val2 and Val20. Table 1 summarizes data on the longest, absolutely identical for these enzymes fragments of amino acid sequences, their positions in primary structures and the presence of functionally important residues. Conservative regions of most glucoamylases contain Asp, Glu, and Trp; carboxyl groups of Asp and Glu residues are involved in cleavage of glycoside bonds of starch molecules, whereas Trp is important for substrate binding [3].

Comparison of primary structures of *Aspergillus* species glucoamylases revealed (Table 2) that the frequency of amino acid residue substitutions is characterized by high variability. This analysis of amino acid sequences also revealed amino acid positions insignificant for manifestations of structural–functional manifestations of these enzymes; these positions are characterized by high frequency of amino acid substitutions. Evidently, high frequency of substitutions of amino acids with similar properties (e.g., Val-Ile, Ser-Thr, Met-Leu) insignificantly influences physicochemical properties of glucoamylases. Mutations resulting in substitutions Ala-Ser, Ala-Thr, Ile-Ser, Phe-Thr, Ser-Glu are not numerous and they insignificantly influence hydrophobicity of protein molecules. The predominance of positions with low frequency of amino acid substitutions suggests evolutionary relation of these species.

Table 2 shows that the primary structure of the glucoamylase subunit from *A. oryzae* contains additional residue Gly3, but it lacks three rather extended fragments and Ser584. This polypeptide chain is significantly shorter and contains only 586 amino acid residues.

Loss of 13 Thr residues and five Ser residues in the glucoamylase molecule from *A. oryzae* may suggest

Table 1. Conservative regions of primary structures of glucoamylases from *Aspergillus* species

Polypeptide chain fragment	Position of the fragment in the primary structure	Presence of functionally important residue
PDYFYTWTRDSG	46-57[1,2] 47-58[3]	D55[1,2] D56[3]
GLGEPKFNVDETA	103-115[1,3] 102-114[2]	– –
WGRPQRDGPALRATAMI	120-136[1,3] 119-135[2]	W120[1,3] W119[2]
VRNDLSYVAQYW	159-170[1,3] 158-169[2]	– –
PLWEEV	176-181[1,3] 175-180[2]	– –
GYLQSFWTGS	222-231[1,3] 221-230[2]	– –
LGSIHTFDP	250-258[1,3] 249-257[2]	– –
AVAVGRYPED	300-309[1,3] 299-308[2]	– –
TLAAAEQLYDALYQWDK	321-337[1,3] 320-336[2]	– –
SARDLTWSYAALLTANNRRN	411-430[1] 410-429[2]	– –
VGSISQLG	534-541[1] 533-540[2] 505-512[3]	– – –
WESDPNR	590-596[1] 589-595[2] 560-566[3]	– – –

[1] *A. niger.*
[2] *A. awamori* X100, *A. awamori* var. *kawachi*, *A. shirousami.*
[3] *A. oryzae.*

Table 2. Structural variability of amino acid sequences of glucoamylase subunits from different *Aspergillus* species

Glucoamylase producer	Positions of amino acid residues characterized by high frequency of substitutions	Inserts	Deletions
A. niger	L60, S73, N80, A84, V88, I91, T150, E219, F232, S240, A246, A261,T310, C320, V343, D357, D375, M398, Y402, E408, Q409, A435, S436, V445, T455, P481, S486, A495, S503, D560, I582, T606	–	–
A. awamori X100	I60, D73, H80, S84, I88, V91, A150 , Q219, Y232, S240, T246, G261, S310, C320, I343, G357, S375, L398, F402, D408, E409, P435, S436, V445, I455, P481, S486, A495, S503, D560, I582, T606	–	A102*
A. awamori var. *kawachi*	I60, S73, N80, A84, V88, I91, T150, E219, F232, R240, A246, A261, T310, S320, V343, D357, D375, M398, Y402, E408, Q409, A345, S436, W445, I455, T481, G486, T495, T503, N560, I582, E606	–	A102*
A. shirousami	L60, D73, H80, S84, Q88, V91, A150, Q219,Y232, S240, T246, G261, S310, C320, I343, G357, S375, L398, D402, E408, L409, S435, W436, P445, S455, T481, G486, T495, T503, N560, V582, E606	–	A102*
A. oryzae	M60, D73, E80, S84, I88, I91, I150, Q219, Y232, G240, T246, T261, S310, T320, I343, A357, S375, M398, Y402, S408, Q409, A435, P436, I445, S455, N560, V582, V606	G3	VATGGTTTTATPPTG (471-484)*, SVTSSTSKTTATASKT (487-501)*, A102*, S584*

* Asterisks indicate that numeration of lost fragments is carried out using the conservative amino acid sequence of glucoamylase subunit from *A. niger*.

reduced glycosylation of this enzyme because these residues are involved into attachment of carbohydrate components to the polypeptide chain.

Analysis of available data revealed that the deletions involve only a O-glycosylation domain of the protein globule and they do not influence the functional properties of this enzyme. Amino acid sequences of glucoamylase subunits from *A. awamori* X100, *A. awamori* var. *kawachi*, *A. shirousami*, and *A. oryzae* are characterized by a gap at Ala102 in the catalytic domain. Deletion of Ala102 is not essential for manifestation of catalytic activity and polypeptide chains of these molecules consist of 615 residues.

Comparison of amino acid sequences of glucoamylases from *A. awamori* and *S. fibuligera* revealed their rather low homology (39.2%, Fig. 1). The primary structure of glucoamylase from yeast *S. fibuligera* contains 492 residues; it lacks Cys [9]. *Aspergillus awamori* glucoamylase contains nine SH-groups; eight groups form four disulfide bonds and one SH remains free.

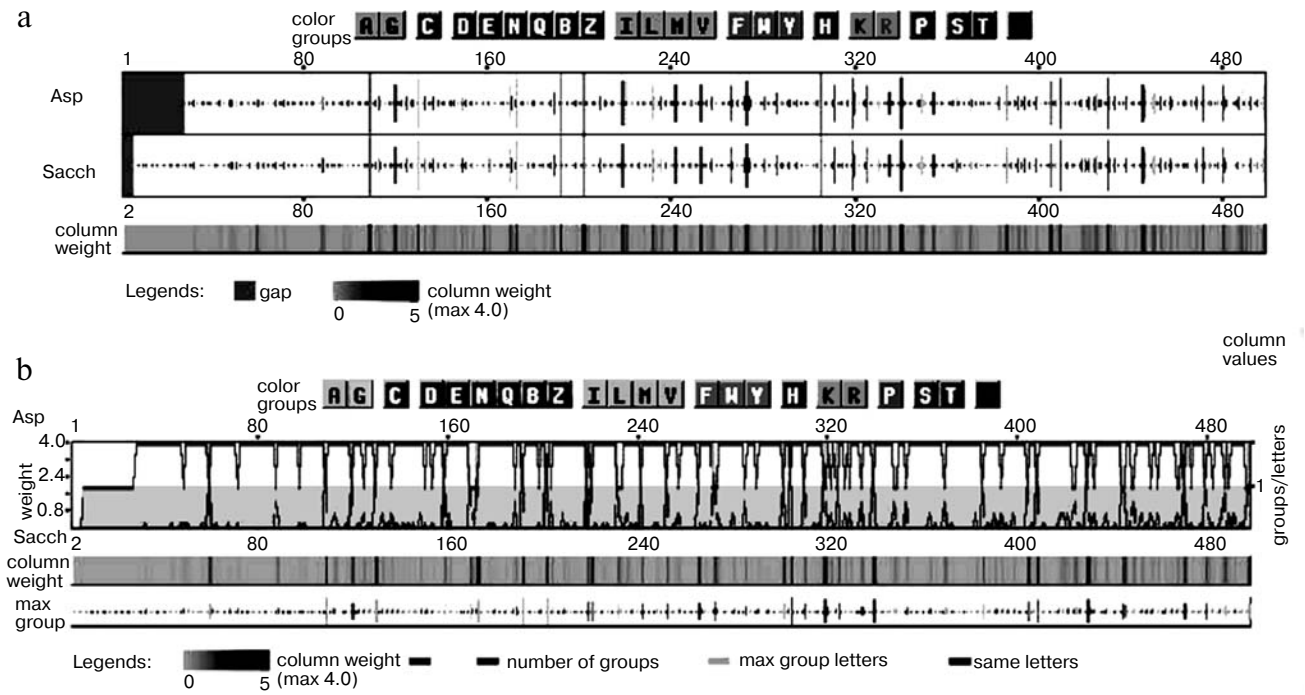
Using X-ray structure analysis data obtained by Aleshin et al. [10] and the MolScript program, it was found that one subunit of *A. awamori* glucoamylase is characterized by a tightly packed core of 13 helices, 11 β-sheets, and 19 unstructured sites. The glucoamylase molecule from *S. fibuligera* contains 13 helices, 13 β-sheets, and 23 amorphous sites [11, 12]. Figure 2 shows the folding mode of the studied enzymes (from N- to C-terminus), which clearly demonstrates secondary structure order in protein globules.

Information on such elements of secondary structures as β- and γ-turns, β-bulge, etc. is available at the Web site of the Department of Biochemistry and Molecular Biology, University College London (http://www.biochem.ucl.ac.uk/bsm/pdbsum/1lgy/main.html).

Results of computer modeling studies, IR-spectrophotometry, and literature data [9, 10, 13] indicate the order of glucoamylase globules that can be referred to α-helical proteins (43-46% of amino acid residues are in α-helices and ~5% represent β-sheets).

Formation of ordered elements of secondary structures involves more than 60% of all amino acid residues of glucoamylases from *A. awamori* and *S. fibuligera*. However, α-helices and β-sheets do not tend to position at particular places of the tertiary structure (inside the globule or on its surface, within N- or C-terminal regions). Topology of α-helices, β-sheets, and unstructured sites in molecules of these proteins suggests their evolutionary relationship.

Studies of structural–functional properties of enzymes are often focused on analysis of their tertiary structure because this level of protein molecule organization is mainly responsible for catalytic functions of these macromolecules.



c

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USR1:_   2/3     TLDSWLSNEATVARTAILNNIG--------
ADGAWVSGADSGIVVASPSTDNPDYFYTWT
USR2:_   17/18  DLETFLDKQKEVSLYYLLQNIAYPEGQFNNG--------
VPGTVIASPSTSNPDYYYQWT

USR1:_   54/55  RDSGLVIKTLVDLFRNG—DTDLLSTIEHYISSQAIIQGVSNPSGDLSS---
GLGEPKF
USR2:_   69/70  DSAFLTVLSELEDNNFNTTLAKAVEYYINTSYNLQRTSNPSGSFDDENHKGLGEPKF

USR1:_  109/111  NVDETAYTGSVGRPQRDGPALRATAMIGFGQWLLD—NGYT-----------
AATEIVWP
USR2:_129/130  NTDGSAYTGAWGRPQNDGPALRAYAISRYLNDVNSLNEGKLVLTDSGDINFSSTEDIYKN

USR1:_   157/159  LVRNDLSYVSQYWNQTGYDLWEEVNGSSFFTIAVQHRALVEGSAFATAVGSS—CSWCDS
USR2:_   189/190  IIKPDLEY
VIFYWDSTGFDLWEENQGRHFFTSLVQQKALAYAVDIAKSFDDGDFANTLSS

USR1:_   215/217  QAPQILCYLQ----SFWTG—SYILANFDS----SRSGKDTNTLLGSIHTF--------DP
USR2:_   249/250
TASTLESYLSGSDGGFVNTDVNHIVEANPDLLQQNSRQGLDSATYIGPLLTHDIGPLLTHDIGESSST-

USR1:_   258/260  EAGCDDSTFQPCSPRALANHKEVVDSFRSIYTLNDGLSDSEAVAVGRYPEDSYY-----N

USR2:_   308/309--------PFDVDNEYVLQSYYLLLEDNKDRYSVNSAYS—ADAAIGRYPEDVYNGDGSSE

USR1:_   313/315  GNPWFLCTLAAAEQLYDALYQWD-KQGSLEITDVSLDFFKALYSGAA----------TGT

USR2:_   359/360  GNPWFLATAYAAQVPYKLAYDAKSASNDITINKINYDFFNKYIVDLSTINSAYQSSDSVT

USR1:_   362/364  YSSSSSTYSSIVSAVKTFADGFVSIVETHAASNGSLSEQFDKSDGDELSARDLTWSYAAL

USR2:_   419/420  KSGSDEFNTNLVTFDDSFLQVILDHINDDGSLNEQLNRYTGYSTGAYSLTWSSGAL

USR1:_   422/424  LTANNRRNS
USR2:_   479/480  LEAIRLRNK
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Fig. 1. Comparison of amino acid sequences of glucoamylases from *A. awamori* (*1*) and *S. fibuligera* (*2*). a, b) Graphic analysis of amino acid positioning in glucoamylases from *A. awamori* and *S. fibuligera*. c) Structural comparison of polypeptide chains of glucoamylases from *A. awamori* (usr1) and *S. fibuligera* (usr2).

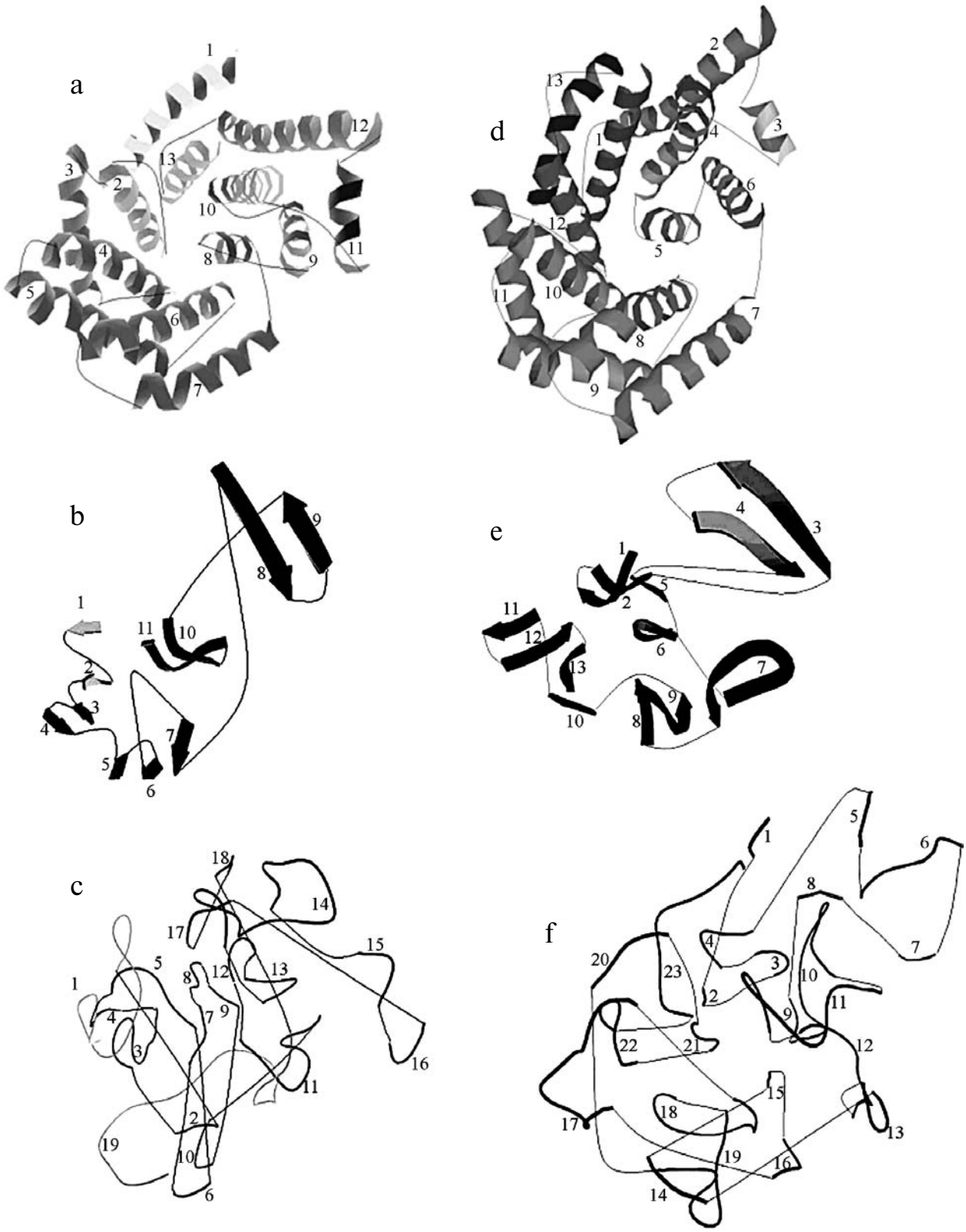

Fig. 2. Topology of secondary structural elements of glucoamylases from *A. awamori* (a, b, c) and *S. fibuligera* (d, e, f). a, d) Helical sites; b, e) β-sheets; c, f) unstructured sites.

Table 3. UV-induced changes in catalytic activity of *A. awamori* glucoamylase (GA)

Specific activity, U/mg	Sample	Irradiation dose, kJ/m^2			
		1.5	3.0	4.5	6.0
	Native GA	9.5 ± 0.74	6.25 ± 0.69	3.41 ± 0.36	1.96 ± 0.19
	GA + methylene blue	8.33 ± 0.42	4.83 ± 0.34	2.15 ± 0.32	
	Native GA*	10.25 ± 0.86			
	GA* + methylene blue (reaction in the darkness)	10.0 ± 0.78			

* Glucoamylase preparations were not subjected to UV-modification.

Table 4. UV-induced changes in catalytic activity of *S. fibuligera* glucoamylase (GA)

Specific activity, U/mg	Sample	Irradiation dose, kJ/m^2			
		1.5	3.0	4.5	6.0
	Native GA	13.94 ± 0.92	10.4 ± 0.73	6.5 ± 0.38	3.9 ± 0.32
	GA + methylene blue	9.8 ± 0.43	5.75 ± 0.36	2.56 ± 0.26	
	Native GA*	15.7 ± 0.98			
	GA* + methylene blue (reaction in the darkness)	15.3 ± 0.94			

* Glucoamylase preparations were not subjected to UV-modification.

Identification of functional groups of hydrolase active sites traditionally employs the following methods: 1) determination of pK values for ionizing groups of enzymes and enzyme–substrate complexes; 2) photooxidation in the presence of thiazole dye, methylene blue; 3) chemical modification of functional groups of the active site involved in substrate binding and catalysis.

Photooxidation in the presence of methylene blue identifies the presence of amino acid residues containing aromatic or sulfur-containing side chains in the active site. We found that the increase in UV-irradiation dose within 1.5-6.0 kJ/m^2 was accompanied by a decrease in catalytic activities of glucoamylases from *A. awamori* and *S. fibuligera* and methylene blue potentiated this effect (Tables 3 and 4).

Increase in pH of the reaction medium was accompanied by increase in inactivation rate constant (k_f) for most yeast glucoamylases (Table 5). Lack of a plateau on the plots of pH dependence of k_f value (within pH range 6.0-7.0) suggests a passive role of a His residue in the active site of glucoamylase to the catalytic act. It is possible that glucoamylase photoinactivation may be attributed to damage to a Trp indole ring involving singlet oxygen.

Study of the dependence of catalytic activity of glucoamylases from *A. awamori* and *S. fibuligera* on substrate concentration at various pH values followed by graphic treatment of the experimental data plotted as depen-

Table 5. pH-dependence of the rate constant of glucoamylase photoinactivation

pH	k_f	
	A. awamori	*S. fibuligera*
4.7	0.45 ± 0.02	0.55 ± 0.07
6.5	0.82 ± 0.06	2.76 ± 0.16
7.0	1.22 ± 0.09	4.07 ± 0.21
7.6	2.84 ± 0.18	5.45 ± 0.29

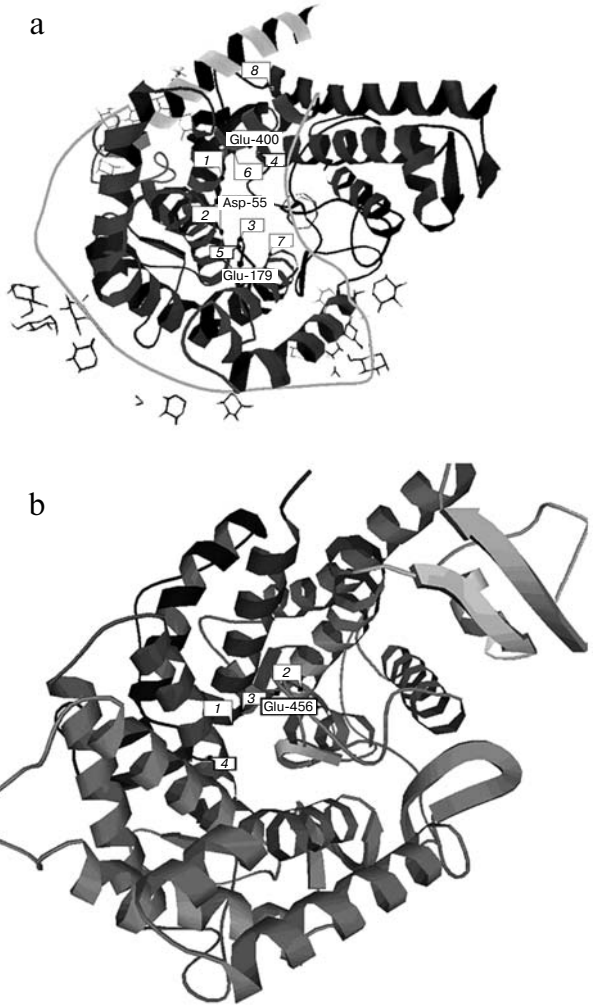

Fig. 3. Spatial location of the active site of glucoamylases from *A. awamori* (a) and *S. fibuligera* (b) and its microenvironment. a: *1*) Leu58; *2*) Leu130; *3*) Leu177; *4*) Leu319; *5*) Trp178; *6*) Trp417; *7*) Phe187; *8*) Trp120. b: *1*) Gly467; *2*) Gly353; *3*) Ala468; *4*) Tyr63.

dences of $\log V_{max}$ versus pH and $\log(V_{max}/K_m)$ versus pH revealed that ionization constants pK_a of functionally essential active side groups correspond to pK_a of Asp and Glu carboxyl groups (2.7-2.8).

Using the MolScript program and X-ray structure analysis data [10] we modeled spatial structure of *A. awamori* X100 glucoamylase (Fig. 3).

Figure 3a shows that the active site of this enzyme is located in a cavity (~1.5 nm) restricted by the following amino acid residues: Leu58, Leu130, Leu177, Leu319, Trp178, Trp417, Phe187, and Trp120. Asp55 and Glu179 are positioned at one side of glucoamylase active site, and Glu400 is at the other side; carboxyl groups of these residues are involved in hydrolysis of α-1,4-glycoside bonds in starch molecules.

Three-dimensional models of *A. awamori* glucoamylase confirm and supplement results of biochemical studies; they also improve evaluation (and interpretation) of available experimental data. It is also interesting to compare spatial structures of enzymes from various sources for elucidation of common and different features in their tertiary structures. So we obtained an image of the 3D structure of the yeast glucoamylase molecule and compared it with 3D structure of *A. awamori* glucoamylase.

Figure 3b shows that the active site of *S. fibuligera* glucoamylase contains Glu456; it is analogous to Glu400 in *A. awamori* glucoamylase. The microenvironment of the active site of this enzyme includes Gly467, Gly353, Ala468, and Tyr63.

Study of spatial models of subunits of glucoamylase molecules from *A. awamori* and *S. fibuligera* revealed the following similarities in the tertiary structure of these enzymes: 1) tight package of hydrophobic core; 2) active site location in the active site cavity (cleft) and presence of water in it; 3) involvement of carboxyl groups of Glu into catalysis. Such results suggest similarity in catalytic mechanism of starch hydrolysis by these enzymes.

Study of supramolecular organization of glucoamylases revealed that the molecules of these enzymes are homodimers constituted by two identical subunits of 53.6 kD.

Comparative analysis of primary structures of lipases from the Mucoraceae family (*Rhizomucor miehei*, *Rhizopus niveus*, *Rhizopus arrhizus*, *Rhizopus oryzae*) revealed that these enzymes share 61.7% homology. The amino acid residues of the catalytic triad Ser-His-Asp are located in conservative sites of polypeptide chains of these enzymes. Six Cys residues involved in formation of disulfide bonds are located at fixed positions and in lipases from the *Rhizopus* genus they are localized in homologous fragments of polypeptide chains [14].

Computer aided study of lipase structure using the MolScript version 2.1 program revealed that each enzyme subunit is characterized by the presence of seven α-helices, nine β-sheets, and 15 unstructured sites (Fig. 4). This enzyme is an α/β-protein: ~28% of residues are involved in formation of α-helices and 24% are in β-sheets.

Graphic treatment of the experimental data on the dependence of catalytic activity of *R. japonicus* lipase on substrate concentrations plotted as dependences of $\log V_{max}$ versus pH and $\log(V_{max}/K_m)$ versus pH revealed ionization constants pK_a of functionally essential active site groups ($pK_{a1} = 6.5$, $pK_{a2} = 7.5$).

Lipase photooxidation revealed that the increase of UV-irradiation dose within the range 1.5-6.0 kJ/m^2 is accompanied by partial enzyme inactivation potentiated by methylene blue. With increase in pH within the pH range 6.0-7.0 the curve k_f (pH) reaches a plateau; this suggests UV-induced breakage of the imidazole ring of a His residue.

Analysis of experimental data on chemical inactivation of lipase by phenylmethylsulfonyl fluoride and study

Fig. 4. Spatial structure of *R. japonicus* lipase molecule: a) general view; b) helical sites; c) β-sheets; d) unstructured sites.

of stoichiometry of inhibition suggest the presence of one hydroxyl group of a Ser residue in the active site.

Study of the tertiary structure of *R. japonicus* lipase by gel chromatography revealed that the enzyme represents a complex oligomeric protein that consists of two symmetrical catalytically active subunits with molecular masses of 46 kD.

The tendency of lipases to dimerization has been recently demonstrated by Berryman et al. [15], Ruiz et al. [16], Pernas et al. [17], and Palomo et al. [18]. Lipases from *Candida rugosa*, *Penicillium candidum*, *Mucor miehei*, and *Humicola lanuginosa* form bimolecular structures characterized by higher catalytic activity and thermo- and pH-stability.

Thus, computer-aided modeling of structure of complex oligomeric enzymes in combination with IR spectrophotometry, enzymatic methods of analysis, and ion-exchange and gel chromatography provide more cor-

rect interpretation of results of studies of structural–functional properties of hydrolases. Combined use of these methods promotes better characterization of molecular mechanisms underlying reactions of polysaccharide and lipid hydrolysis.

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