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EXPERIMENTAL ARTICLES

Regulation of the Functional Activity of Lysozyme by Alkylhydroxybenzenes

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Abstract—In our study, we investigated the capacity of alkylhydroxybenzenes (AHB), which are microbial anabiosis autoinducers, for alteration of the enzymatic activity of the hen egg-white lysozyme, as well as the efficiency of hydrolysis of specific (peptidoglycan) and nonspecific (chitin) substrates catalyzed by lysozyme. AHB homologues (C7-AHB and C12-AHB), which differ in their hydrophobicity and effects in their interaction with lysozyme, were used as modifying agents. C7-AHB stimulated enzymatic activity within the whole range of concentrations used (10^{-7}–10^{-3} M). More hydrophobic C12-AHB exhibited this ability only at low concentrations and inhibited fermentative activity at high concentrations, acting as a mixed-type inhibitor. Both AHB homologues caused changes in the hydrophobicity of lysozyme molecules. An increase in the affinity level between the C7-AHB-modified enzyme and the nonspecific substrate (colloidal chitin or cell wall polymers of *Saccharomyces* sp.) was observed, which manifested itself in the enhancement of the hydrolysis rate by 200–500% (as compared to the native enzyme). A significant effect on the efficiency of the lysozyme-catalyzed modifications of the substrate (peptidoglycan, colloidal chitin) structure as a result of its complexation with AHB was demonstrated. A stabilizing effect of C7-AHB and C12-AHB was revealed, which ensured a high level of activity of the AHB-modified enzyme (as compared to the control) after heat treatment (functional stability), as well as at nonoptimal temperatures of catalysis (operational stability). The biological significance of lysozyme modification with AHB and the practical aspects of its application are discussed.

Key words: lysozyme, structural modification, alkylhydroxybenzenes, alkylresorcinols, chitin, stimulation and inhibition of catalytic activity, functional and operational stability.

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Enzymes are responsible for the development of all living organisms, as well as for their survival and interaction with the environment. In the course of evolution, enzyme systems with similar functional characteristics developed in living organisms at different organizational levels. Among these systems, there is a separate group of hydrolytic enzymes, lysozymes. According to the modern structural classification of proteins [1], this group includes seven families of molecules: C-type lysozymes, goose lysozyme, lysozymes of bacteriophages T4 and λ, bacterial muramidases, glycosidases and chitosanases, as well as two recently described groups of lysozyme-like proteins (COG3926/DUF847 and COG5526) [2]. Lysozymes of different origin exhibit a remarkable similarity in their secondary and tertiary structures, as well as in the structural features of their active centers [3]. The common property of lysozymes is their capacity for hydrolysis of the β-1,4-glycoside bonds between residues of *N*-acetylmuramic acid and *N*-acetyl-glucosamine in the peptidoglycan layer of bacterial cell walls. Some lysozymes with wider substrate specificity, e.g., glycosidases (EC 3.2.1.14) and chitosanases (EC 3.2.1.132), have the ability to hydrolyze the bonds between *N*-acetyl-glucosamine residues in chitin and chitosan molecules, respectively [4].

The biological functions of lysozymes are diverse. In bacteriophages, this enzyme enables viral nucleic acid to penetrate into the cells, as well as promotes the release of mature viral particles from infected host cells at the end of the lytic cycle [5]. Bacterial muramidases play an important role in the cell wall metabolism during cell growth and division; they are involved in the processes of macromolecule transport and interspecific competition [6, 7]. In vertebrates, lysozymes play a key role in the protection of host organisms against a wide spectrum of bacterial (and perhaps fungal) pathogens [8, 9]. Lysozymes are widely used in medicine for the

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treatment of various infections and in food industry to prevent bacterial contamination of foodstuffs [10].

Acquired resistance of microorganisms to lysozyme has therefore become an important problem [11]. In recent years, a great deal of attention has been devoted to the investigations aimed at studying lysozyme modifications affecting lysozyme properties while retaining its activity [12, 13].

This problem can be solved by the application of modifying agents influencing the enzyme activity and stability. Low-molecular-weight extracellular microbial metabolites acting as anabiosis autoinducers are possibly among the bioactive compounds that can control the lysozyme activity. In a number of bacterial and yeast species, these autoregulators belong to alkylhydroxybenzenes (AHB), in particular alkylresorcinols (AR); they induce the process of cell transition to the hypometabolic (anabiotic) state and fulfill this function via the interaction with a wide spectrum of cell biopolymers [14, 15]. The nonspecific effect of these autoregulators on enzymatic proteins is determined by the chemical structure of AHB and the type of their interaction with protein molecules [16, 17], which suggests that they may be active against lysozymes.

The purpose of this work was to study the effect of AHB (chemical analogues of microbial anabiosis autoinducers) on the activity, functional stability, and substrate specificity of lysozyme.

MATERIALS AND METHODS

In our studies, we used a commercial preparation of the hen egg-white lysozyme (EC 3.2.1.17, Sigma, United States) (M = 14.7 kDa).

The alkylresorcinol homologues C7-AHB and C12-AHB, which differ in the length of their alkyl chains and therefore in the hydrophobicity level, were used as chemical analogues of anabiosis factors. C7-AHB and C12-AHB were dissolved in PBS buffer or distilled water with or without 5% ethanol.

Enzyme modification by AHB. Equal amounts of AHB and enzyme solutions in the concentrations twice as high as those required were mixed and preincubated at room temperature for 1 h. In the control systems, an equivalent amount of the solvent was used instead of the AHB solution.

Preparation and modification of AHB substrates. *Micrococcus luteus* ATCC 15307 cells were grown in a liquid nutrient medium [15] at 37°C for 24 h; *Saccharomyces cerevisiae* cells were grown in 2.5°B wort at 28°C for 12 h (exponential phase). The cells were harvested and resuspended in a phosphate buffer (pH 6.2) (*M. luteus*) or PBS buffer (pH 7.4) (yeast). The optical density (OD) attained was 0.5 (Specord, Germany, l = 1 cm, λ = 540 nm), which corresponded to the number of bacterial cells of ~2.8×10^7 CFU/ml and to the number of yeast cells of ~5×10^7 CFU/ml. To modify the polymers of the cell wall, bacterial suspensions were supplemented with various concentrations of AHB and incubated at 37°C for 1 h.

Peptidoglycan (PG) from the *M. luteus* cell wall was obtained by multi-stage extraction of biomass by successive treatment of disrupted cells with trypsin and acetone and resuspended in a phosphate buffer (pH 6.2) up to an OD = 0.5. To modify peptidoglycan, the obtained PG suspensions were supplemented with various concentrations of AHB and incubated at 37°C for 1 h.

Colloidal chitin was obtained by dissolving the crystalline chitin in 85% phosphoric acid; the sediment was then washed by decantation with distilled water to pH 5.8. The chitin content in the preparation was 20 mg/ml. To modify chitin, equal amounts of chitin and AHB solutions were mixed and incubated at 20°C for 1 h.

The modifying effect of AHB was assessed by the changes in the catalytic parameters of the reaction.

Determination of the hydrolytic activity of lysozyme. (1) In the case of *M. luteus* cells, the cell suspension (2.8 ml, in a spectrophotometer cuvette, 37°C) was supplemented with 0.2 ml of a lysozyme solution (2×10^{-5} M); the OD_{540} of the specimen was then monitored for 10 min with 10 s intervals. The kinetic parameters were calculated by the standard methods [18]. The lysis rate was expressed as the number of cells (calculated from OD) lysed in 1 ml of the reaction mixture per unit time (CFU/ml · s). (2) In the case of *S. cerevisiae* cells, the yeast suspension (2 ml) was supplemented with 0.5 ml of the lysozyme solution (1.3×10^{-4} M) and incubated at 37°C for 20 h. The samples were then centrifuged; the content of reducing sugars in the supernatant was determined with dinitrosalicylic acid (DSA) (the calibration curve for acetyl-glucosamine was used) [19]. (3) In the case of peptidoglycan, the PG suspension (2.9 ml, in a spectrophotometer cuvette) was supplemented with 0.1 ml of lysozyme (2×10^{-5} M); the OD values were then determined as described above. (4) In the case of colloidal chitin, 0.5 ml of colloidal chitin solution (20 mg/ml) was supplemented with 0.5 ml of the lysozyme solution (1.3×10^{-4} M) and incubated at 37°C or 55°C for 24 h. The sample was then centrifuged; the concentration of acetyl-glucosamine in the supernatant was then determined with DSA [19].

The lysozyme hydrophobicity was determined by HPLC [20].

The functional thermostability of lysozyme was determined from the levels of residual activity in the specimens after heating at 70°C and 80°C for 30, 60, and 90 min in a UV-10 ultrathermostat (Germany), using colloidal chitin as a substrate.

The operational thermostability of lysozyme was determined by conducting enzymatic reactions at various nonoptimal temperatures (20–63°C) for 24 h, using colloidal chitin as a substrate.

Table 1. Parameters of the lytic reaction in the "lysozyme–*M. luteus* cells" system in the presence of C7-AHB and C12-AHB (enzyme modification)

AHB	Reaction parameters (% of control)	Concentration of AHB used for enzyme modification, M					
		0 (control)	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
C7-AHB	V_{max}, CFU/ml · s	9961 ± 737 (**100**)	10883 ± 754 (**109**)	11990 ± 853 (**120**)	13005 ± 911* (**131**)	14388 ± 1223* (**144**)	15864 ± 1257** (**159**)
	Decrease in OD after 10 min, %****	19.56 ± 1.37 (**100**)	21.38 ± 1.50 (**109**)	23.55 ± 1.73 (**120**)	25.54 ± 1.77* (**131**)	28.26 ± 2.46* (**144**)	31.16 ± 2.47** (**159**)
C12-AHB	V_{max}, CFU/ml · s	10426 ± 771 (**100**)	10108 ± 687 (**97**)	8510 ± 685 (**82**)	7426 ± 590* (**71**)	6342 ± 503** (**61**)	2658 ± 275*** (**25**)
	Decrease in OD after 10 min, %****	20.48 ± 1.51 (**100**)	19.85 ± 1.35 (**97**)	16.71 ± 1.35 (**82**)	14.59 ± 1.16* (**71**)	12.46 ± 1.06** (**61**)	5.22 ± 0.52*** (**25**)

Notes: * $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ relative to control.
**** Initial OD = 0.5; number of cells = 2.8×10^7 CFU/ml.

RESULTS

The effect of AHB on the functional activity of lysozyme against the specific substrate (PG of the cell wall of the *M. luteus* indicator culture) was determined from changes in the maximum lysis rate (V_{max}), which, according to the results of our preliminary experiments, was achieved after 250–300 s of contact, as well as from the percentage of decrease in the optical density of the bacterial suspension after 10 min of contact (Table 1).

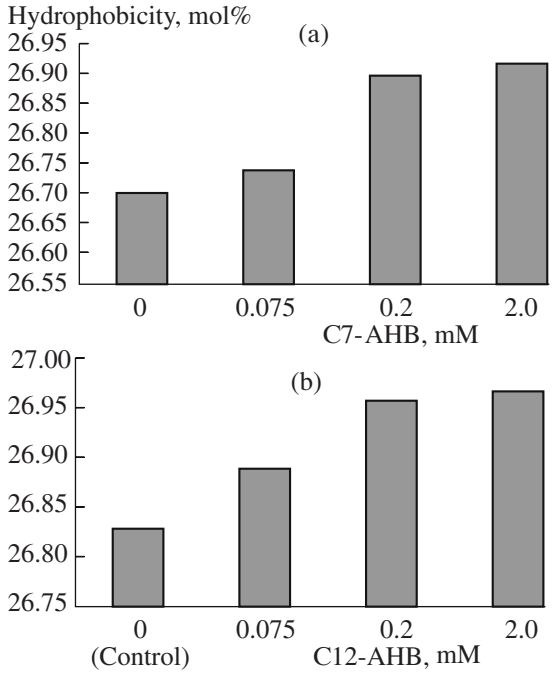

Fig. 1. Changes in the hydrophobicity of lysozyme modified with C7-AHB (a) and C12-AHB (b) in 5% ethanol.

Enhanced efficiency of the lytic reaction was the main effect of C7-AHB within the whole range of concentrations used. For instance, an increase in the C7-AHB concentration from 10^{-7} to 10^{-3} M resulted in the progressive increase of both V_{max} (from 10883 to 15864 CFU/ml · s) and the percentage of decrease in the optical density of the bacterial suspension (from 21.4 to 31.2%) (Table 1). C12-AHB exerted an opposite dose-dependent inhibitory effect on the lysozyme activity. When the maximum concentration used (10^{-3} M) was applied, the hydrolysis indices decreased to 2658 CFU/ml · s and 5.2%, respectively. These indices were reliably different ($P < 0.05$) from the control values when the AHB concentration was 10^{-6} M and higher, i.e., at the "enzyme : AHB" molar ratio of 1 : 1 and higher (Table 1).

The above-described changes in the activity of the AHB-modified lysozyme, which resulted in the changed kinetic parameters of the reaction, may be caused only by the conformational changes in the protein accompanied by the changes in the physicochemical characteristics of the enzyme. This effect has been demonstrated for other enzymes in [17], as well as in this work by the example of increasing hydrophobicity of the lysozyme modified with C7-AHB (Fig. 1a) and C12-AHB (Fig. 1b).

It may be suggested that the modifications of lysozyme by AHB, which are responsible for its changed activity and physicochemical properties, would affect the substrate specificity of the enzyme. Data on the activity of the hen egg-white lysozyme against chitin-like substrates are scarce [4]. Based on previous data, the enzyme : AHB molar ratio of 1 : 1 and higher was used to assess the activity of the modi-

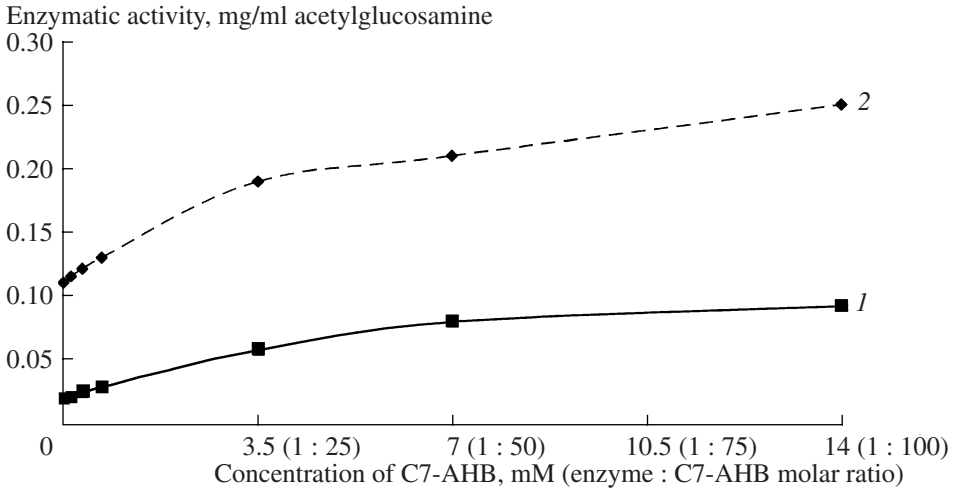

Fig. 2. Concentration dependence of the C7-AHB effect on the enzymatic activity of lysozyme during hydrolysis of colloidal chitin (enzyme modification). Temperatures of the enzymatic reaction: 37°C (*1*) and 55°C (*2*); time, 24 h.

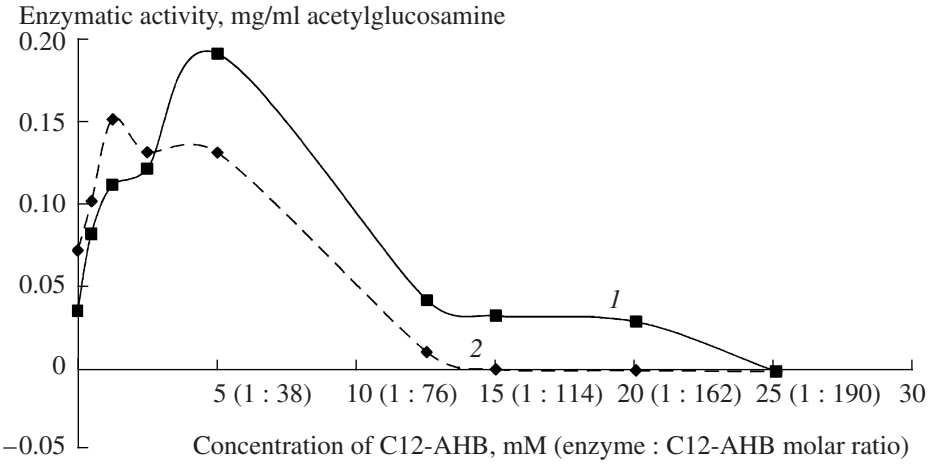

Fig. 3. Concentration dependence of the C12-AHB effects on the enzymatic activity of lysozyme during hydrolysis of colloidal chitin (enzyme modification). Temperatures of the enzymatic reaction: 37°C (*1*) and 55°C (*2*); time, 48 h.

fied enzyme against the nonspecific substrate (colloidal chitin). The effect that lysozyme modified with C7-AHB exerted on chitin (determined from acetyl-glucosamine accumulation) (Fig. 2), was much more pronounced than the effect exerted on *M. luteus* cells (Table 1). For instance, C7-AHB in millimolar concentrations enhanced the efficiency of the lytic reaction with the specific substrate by 60% of the control, whereas, in the experimental systems, the amount of product formed during hydrolysis of colloidal chitin was 200–500% larger than in the control experiment. The effect of C7-AHB probably increases the affinity of the enzyme to the nonspecific substrate. An increase in the reaction temperature up to 55°C resulted in a significant increase in the amount of the product; however, the effect of AHB became less pronounced (Fig. 2). It should be noted that we failed to decrease the enzymatic activity below the control level even at high C7-AHB concentrations (176 mM; the enzyme : AHB molar ratio of 1 : 1250).

Modification of lysozyme with another homologue, C12-AHB (in low concentrations, 0.1–12 mM), enhanced the efficiency of hydrolysis of the nonspecific substrate (Fig. 3). C12-AHB inhibited chitin hydrolysis at concentrations exceeding 12 mM. The differences in the effects on chitin of the lysozyme modified with C7-AHB and C12-AHB are due to the dose-dependency of the AHB–enzyme system and the temperature dependency of the reaction (Figs. 2, 3).

The revealed enhancement of the nonspecific activity of the lysozyme modified with C7-AHB was confirmed by the results of our experiments with *S. cerevi-*

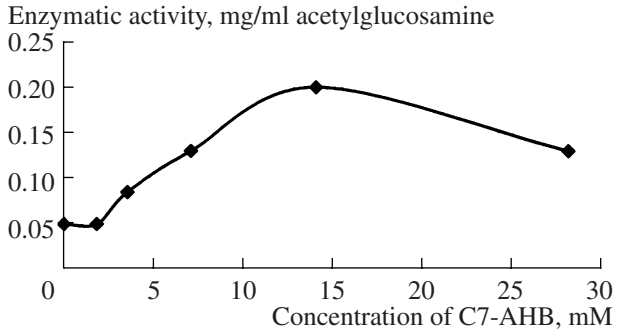

Fig. 4. Concentration dependence of the C7-AHB effects on the enzymatic activity of lysozyme against yeast cells (enzyme modification). Temperature of the enzymatic reaction, 37°C; time, 20 h.

siae cells used as a substrate (Fig. 4). The maximum yield of reducing sugars was attained at a C7-AHB concentration of 14 mM, which correlated with the results of our experiments with chitin (Fig. 3). Thus, the obtained results demonstrate the new effect of small molecules performing protein-modifying functions, i.e. to alter the substrate specificity of lysozyme by enhancing it against nonspecific substrates and thus to increase the rate of hydrolysis of nonspecific bonds.

Since the study of the processes controlling inhibition of the enzyme activity under the effect of anti-lysozyme factors are of practical interest [11, 21], the kinetic parameters of hydrolysis of *M. luteus* cells by the lysozyme modified with C12-AHB, within a range of inhibitory concentrations from 10^{-7} to 10^{-3} M were studied in detail. Various concentrations of target cells were used in the experiment, from OD 0.7 (3.9×10^7 CFU/ml) to OD 0.1 (0.55×10^7 CFU/ml), in OD increments of 0.1, at a standard lysozyme concentration of 10^{-5} M. The obtained results were analyzed graphically using the Michaelis–Menten (Fig. 5) and Lineweaver–Burk equations to determine V_{max} and K_m (Fig. 5a). Low C12-AHB concentrations (10^{-7} M) resulted in an increase in K_m values (from 5.54 in the control to 7.18); however, V_{max} values remained unchanged (from 7.57×10^4 in the control to 7.46×10^4 CFU/ml · s). The increase in the C12-AHB concentrations up to 10^{-5} M and 10^{-3} M resulted in a considerable decrease in V_{max} values to 5.23×10^4 and 4.03×10^4 CFU/ml · s, respectively. Hence, the effect of C12-AHB on lysozyme is, according to existing concepts, typical of mixed-type inhibitors [18]. This conclusion was confirmed by the graphic analysis of the Michaelis–Menten equation with the S/V from S and V from the V/S coordinate axes (Figs. 5b and 5c). In particular, the plots of S/V against S (Fig. 5b) for the control measurement and C12-AHB concentration of 10^{-7} M were parallel, which is typical of competitive inhibition; however, as the C12-AHB concentration increased up to 10^{-5} M and 10^{-3} M, the curve shifted, which is typical of mixed-type inhibition. With the V from the V/S coordinate axes (Fig. 5c), the plot describing the lytic reaction in the presence of C12-AHB (10^{-7} M) was also typical of competitive inhibition, while with increasing C12-AHB concentrations it was typical of mixed-type inhibition.

The chemical structure of AHB determines their ability to interact with polymeric substrates and modify them, which should affect the lytic parameters of hydrolysis. Therefore, in the next series of experiments, the following substrates were modified with AHB: *M. luteus* cells, extracted peptidoglycan, and colloidal chitin. Taking into account that the C12-AHB homologue may induce the formation of resting cells, in our

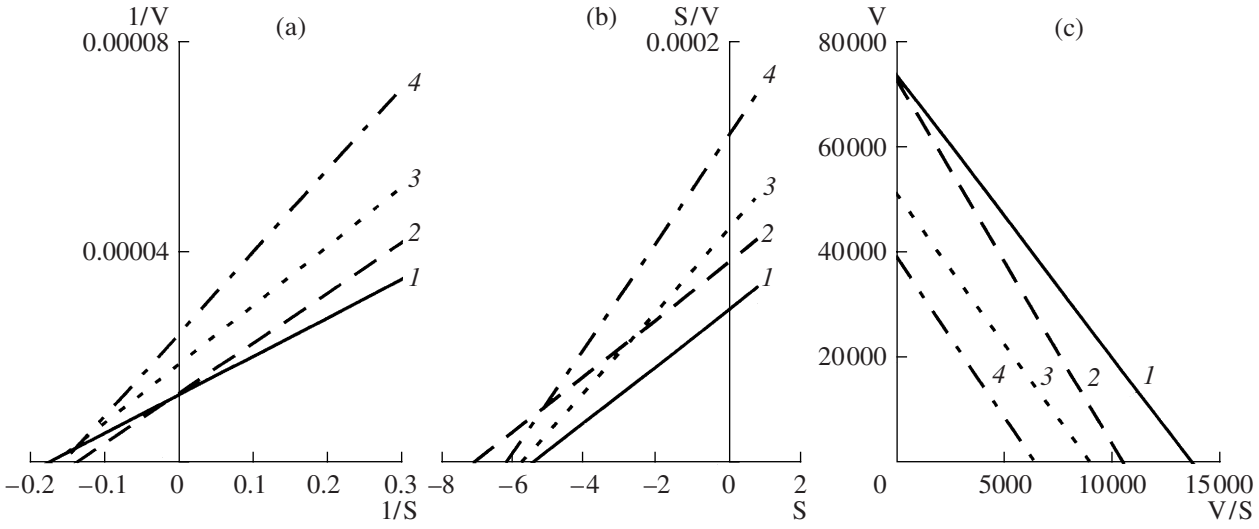

Fig. 5. Relationships between the substrate (*M. luteus* cells) concentration (S) and the rate (V) of substrate hydrolysis by lysozyme (10^{-5} M) modified with C12-AHB in the following concentrations: control (*1*); 10^{-7} M (*2*); 10^{-5} M (*3*); 10^{-3} M (*4*); (a) plot of 1/V against 1/S; (b) plot of S/V against S; (c) plot of V against V/S.

Table 2. Parameters of the lytic reaction in the "lysozyme–*M. luteus* cells" system in the presence of C7-AHB and C12-AHB (modification of the substrate (*M. luteus* cells)

AHB	Reaction parameters (% of control)	Concentration of AHB used for substrate modification, M					
		0 (control)	7×10^{-9}	7×10^{-8}	7×10^{-7}	7×10^{-6}	7×10^{-5}
C7-AHB	V_{max}, CFU/ml · s	6539 ± 318 (**100**)	6824 ± 344 (**104**)	7142 ± 351 (**109**)	7309± 358 (**112**)	7389± 361 (**113**)	7916± 387* (**121**)
	Decrease in OD after 10 min, %***	17.16± 0.83 (**100**)	17.70± 0.89 (**103**)	19.66± 0.97 (**115**)	20.15± 0.99 (**117**)	20.42± 1.00 (**119**)	21.66± 1.06* (**126**)
C12-AHB	V_{max}, CFU/ml · s	7175± 368 (**100**)	7402± 358 (**103**)	6963± 343 (**97**)	6149± 299 (**86**)	5600± 276* (**78**)	5427± 273* (**76**)
	Decrease in OD after 10 min, %***	16.89± 0.87 (**100**)	17.27± 0.83 (**102**)	15.39± 0.76 (**91**)	14.04± 0.68* (**83**)	12.52± 0.61** (**74**)	11.69± 0.59** (**69**)

Notes: * $P < 0.05$.
** $P < 0.01$.
*** Initial OD = 0.5; number of cells = 2.8×10^7 CFU/ml.

experiments with *M. luteus* it was used in low concentrations (no more than 7×10^{-5} M) (Table 2). The C7-AHB concentrations ranging from 7×10^{-9} to 7×10^{-5} M enhanced the efficiency of the lytic reaction by increasing V_{max} values and the percentage of the OD decrease: the peak lysis rate increased from 6539 (control) to 7916 CFU/ml · s, whereas the percentage of decrease in the optical density of the bacterial suspension increased from 17.2 to 21.7 % (7×10^{-5} M). The use of C12-AHB in similar experiments has had the opposite effect: V_{max} decreased from 7175 (control) to 5427 CFU/ml · s, while the percentage of the OD decrease reduced from 17 to 12% (7×10^{-5} M). The effect of modification of *M. luteus* cells with C7-AHB and C12-AHB was less pronounced than the effect of enzyme modification with the same AHB homologues.

Similar results were obtained when modifying the peptidoglycan extracted from *M. luteus* cells (Table 3) and colloidal chitin (Fig. 6) with AHB. It is notable that modification of the extracted peptidoglycan with both C7-AHB and C12-AHB had a more pronounced effect on the efficiency of hydrolysis (Table 3) than modification of peptidoglycan in the cell walls of intact *M. luteus* cells (Table 2). With the application of C7-AHB in concentrations up to 14 mM, the hydrolysis efficiency increased by less than 23%; however, it increased significantly (up to 230% of the control) with further increase in the C7-AHB concentration up to 70 mM (Fig. 6). Modification of the substrate probably results in its partial reorganization and increases the number of accessible reaction sites. Changes in the hydration characteristics of the molecules may cause an increase in the enzyme–substrate affinity.

Modification of the enzyme structure affecting its functional activity should affect the resistance of the protein molecule to denaturing factors, i.e., the preservation of its catalytic function under these conditions. In the next series of experiments, the functional stability of the lysozyme modified with C7-AHB (14 mM) (on average, causes a twofold increase in the catalysis efficiency under standard conditions) and C12-AHB (5 mM) (increases the catalysis efficiency by 80% on average under standard conditions) were studied. It was established that, after heating (30, 60, and 90 min at 70°C (C7-AHB and C12-AHB) or 80°C (C7-AHB)), the enzyme modified with C7-AHB and C12-AHB exhibited higher residual activity than the native enzyme (Fig. 7). For instance, incubation of native lysozyme at 70°C and 80°C for 90 min resulted in a decrease in its activity by 44 and 91%, respectively, while in the case of the enzyme modification with C7-AHB, its activity decreased by 24 and 76%, respectively (Figs. 7a and 7b). The lysozyme modified with C12-AHB exhibited even higher resistance to thermal denaturation (70°C) (Fig. 7c) and higher residual activity (up to 40%) than the native one (1%).

Another indication of the stability of the modified enzyme is its high operational stability, which was assessed by high hydrolytic activity at nonoptimal temperatures (Fig. 8): lysozyme modified with C7-AHB

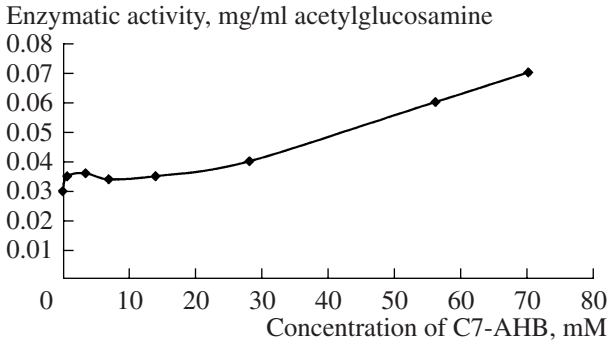

Fig. 6. Concentration dependence of the C7-AHB effects on the efficiency of hydrolysis of colloidal chitin by lysozyme (substrate modification). Temperature of the enzymatic reaction, 37°C; time, 24 h.

Table 3. Parameters of the lytic reaction in the "lysozyme–peptidoglycan isolated from the *M. luteus* cell wall" system in the presence of C7-AHB and C12-AHB (modification of the substrate (peptidoglycan)

AHB	Reaction parameters (% of control)	Concentration of AHB used for substrate modification, M					
		(control)	7×10^{-9}	7×10^{-8}	7×10^{-7}	7×10^{-6}	7×10^{-5}
C7-AHB	V_{max}, CFU/ml · s	23585 ± 1650 (**100**)	23914 ± 1889 (**101**)	24874 ± 1617 (**105**)	26850 ± 1853 (**114**)	28319 ± 2067 (**120**)	29468 ± 2240 (**125**)
	Decrease in OD after 10 min, %****	11.16 ± 0.67 (**100**)	13.68 ± 0.68* (**123**)	15.5 ± 0.78** (**139**)	15.8 ± 0.92*** (**142**)	16.28 ± 0.85*** (**146**)	18.25 ± 0.91*** (**163**)
C12-AHB	V_{max}, CFU/ml · s	27116 ± 2137 (**100**)	15525 ± 1102*** (**57**)	11252 ± 833*** (**41**)	9164 ± 614*** (**34**)	9682 ± 581** (**36**)	5771 ± 433*** (**21**)
	Decrease in OD after 10 min, %****	10.31 ± 0.62 (**100**)	7.23 ± 0.36* (**70**)	7.21 ± 0.36** (**70**)	7.02 ± 0.35** (**68**)	7.11 ± 0.61** (**69**)	6.42 ± 0.32*** (**62**)

Notes: * $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ relative to control.
**** Initial OD = 0.5; number of cells = 2.8×10^7 CFU/ml.

(7 mM) within a broad temperature range (35–57°C) exhibited similar (or higher) activity as the native lysozyme modified at the optimal temperature (53°C).

DISCUSSION

The obtained results indicate that AHB, which are microbial anabiosis autoinducers, are able to modify the functional activity of the hen egg-white lysozyme, as well as to regulate the efficiency of hydrolysis of specific (peptidoglycan) and nonspecific (chitin) substrates. The direction of the observed effects was determined by the chemical properties of AHB. For instance, C7-AHB enhanced the enzymatic activity within the whole range of concentrations, whereas C12-AHB displayed this ability only at low concentrations; high C12-AHB concentrations inhibited the catalytic activity.

The results of our study of the inhibitory effect of C12-AHB on lysozyme suggested its action as a mixed-type inhibitor: the effect of low C12-AHB concentrations is typical of competitive inhibition (an increase in K_m without a change in V_{max} was observed), whereas the effect of high C12-AHB concentrations is typical of mixed-type inhibition, accompanied by changes in the V_{max}.

Hence, the principal role of the chemical structure of the modifying agent responsible for modification of the enzymatic activity is always modified by the agent's concentration, i.e., by the enzyme : AHB molar ratio. The obtained results correlate well with the data on the effect exerted by AHB on other mono-subunit enzymes (trypsin, α- and β-amylase, ribonuclease, etc.) and confirm the functional role of AHB in modification of the activity of enzymatic proteins nonspecifically to their structure [15–17]. This trait, as well as the chemical composition of AHB, confirms that they perform the functions of chemical chaperons which modify proteins by means of weak physicochemical interactions [16].

There are several descriptions of the modifications that alter the lysozyme structure by incorporating additional amino acids or a hydrophobic pentapeptide. These modifications stimulated the functional activity of the modified enzyme in respect to its bactericidal effects on *Escherichia coli* cells (without changing the level of the peptide hydrolyzing activity) and caused a decrease in the valinomycin-induced transmembrane electrochemical potential [12]. Lysozyme acquired similar properties after gradient thermal denaturation (pH 7.0) [12] and the thermal assembly of the dimeric molecule [13]. The described modifications enhanced the lysozyme hydrophobicity; the molecules therefore acquired the ability to disrupt the outer membrane of gram-negative bacteria and gained access to the peptidoglycan layer of the cell wall [22, 23].

Our experiments indicated that the interaction of the hen egg-white lysozyme with both C7-AHB and C12-AHB resulted in an increase in the lysozyme hydropho-

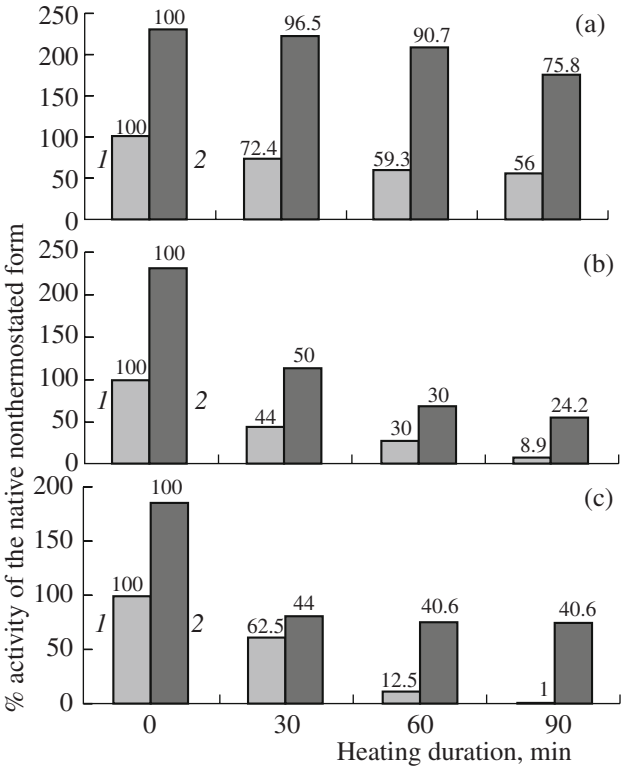

Fig. 7. Functional thermostability of modified lysozyme (colloidal chitin was used as a hydrolyzed substrate): C7-AHB (14 mM, water solution) (a, b); C12-AHB (5 mM, 5% ethanol) (c). Heating temperature: 70°C (a, c), 80°C (b). Designations: native enzyme (*1*); modified enzyme (*2*).

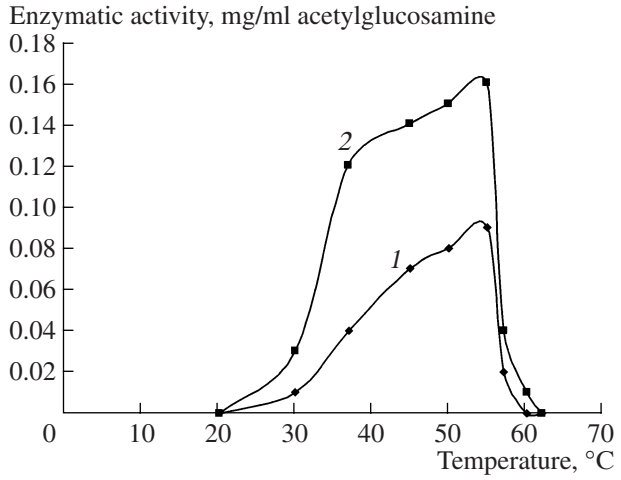

Fig. 8. Operational thermostability of lysozyme modified with C7-AHB (7 mM). Substrate (colloidal chitin): native enzyme (*1*); modified enzyme (*2*).

bicity (Fig. 1), which can be attributed to a certain loosening of the protein molecule [24] resulting in the exposure of intramolecular hydrophobic groups, e.g., thiol ones. Changes in the AHB-modified lysozyme hydrophobicity (Fig. 1) resulted in alteration of its catalytic activity (Tables 1–3), similar to the above-described variants of enzyme modification.

It should be noted that changes in the hydrolytic and antimicrobial activities of lysozyme do not always correlate with each other. For example, interactions between lysozyme and polymers, dextrans, or organic acids (caffeic or cinnamic) resulted in a decrease in the enzymatic activity against *M. luteus* (up to 62%); however, in this case, a dose-dependent increase in the enzymatic activity against *Staphylococcus aureus* and *E. coli* was observed in both model and food systems (for instance, they reduced the number of *E. coli* cells by three orders of magnitude in cheese curds after a 40 day storage [25, 26]).

Importantly, native conformational transitions of lysozyme at physiological temperatures may be biologically appropriate events switching the antimicrobial specificity of lysozyme against food pathogens and opportunistic microflora [22].

Even though the spectrum of modifications resulting in the production of lysozymes active against a wide variety of gram-positive and gram-negative bacteria is broad enough, lysozymes active against fungal or yeast cells are very rare. Some works describe an enhanced lysozyme activity against yeast cells after their pretreatment with antimycotic substances (nystatin, amphotericin B) as an example of the enzyme–antibiotic synergism [27].

In our studies, we observed that intact yeast cells were sensitive to the AHB-modified lysozyme (Fig. 4), possibly due to some changes in the enzyme–substrate affinity.

This conclusion was confirmed by the results of a series of experiments demonstrating a significant increase in the affinity of the AHB-modified enzyme to a nonspecific substrate, chitin. The rate and the degree of hydrolysis of the specific substrate (peptidoglycan incorporated into the cell walls of *M. luteus* cells or extracted from them) by modified lysozyme increased by 60% (as compared to the native enzyme) (Tables 1 and 2), whereas the amount of product formed during hydrolysis of the nonspecific substrate (colloidal chitin or polymers from yeast cell walls) was 200–500% larger (Fig. 2). The detected increase in the affinity level of modified lysozyme may be a useful tool for practical diagnostics, since the peptidoglycan contained in the infecting agent induces lysozyme biosynthesis in the host organism; this biosynthesis can be detected in the reaction of lysozyme with a nonspecific substrate (chitin). An increase in the reaction sensitivity will permit diagnostics at an earlier stage. This goal could be also attained (though less effectively) by modifying the substrate (colloidal chitin) (Fig. 6).

Finally, another effect of lysozyme modification with AHB is that it alters the lysozyme stability, as was confirmed by the findings that the enzyme activity did not change after thermal denaturation (functional sta-

bility) (Fig. 7) and that the temperature range of active catalysis was enlarged (operational stability) (Fig. 8).

The regulation of lysozyme activity and stability may be of interest for the theory of bacterial persistence (especially in the case of respiratory infections) and for the application of highly active and stable lysozymes in medical practice [28]. Modification of lysozyme in order to enhance its activity and stability could be used for production of synergetic prebiotics, the application of which is a new trend in the production of synbiotic nutrients [29].

As for the biomedical aspect of our results, we think that alkylhydroxybenzenes of the C12-AHB-type can be classified as metabolites of a bacterial origin, as the so-called antilysozymes or antilysozyme factors which have a pronounced inhibiting effect on the functional activity of lysozyme [30].

AHB have some specific features that distinguish them from other antilysozymes. (1) Unlike the relatively large protein molecules exhibiting antilysozyme properties [21], they belong to the group of small regulatory molecules which presumably were the first molecules that acquired this ability; they are relatively evolutionarily conservative and, therefore, widespread. (2) Unlike the metabolites exhibiting "specific" inhibitory activity, AHB are polyfunctional in respect to various enzyme and nonenzyme proteins [15–17]. (3) Finally, unlike antilysozymes whose key function is to protect microorganisms against exogenous lysozyme produced in human and animal bodies by their antiinfective systems, the initial biological function of AHB is dormancy induction in bacterial cells. To suppress autolysis, this process requires inactivation of endogenous lysozyme-like enzymes. It should be noted that the nonspecific effect of AHB does not rule out its possible role in the activity regulation of various lysozymes (in addition to the hen egg-white lysozyme) in the case of such biological phenomena as phage resistance, microbial antagonism, and infection persistence in the host organism.

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