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EXPERIMENTAL ARTICLES

Enzyme Modification by Natural Chemical Chaperons of Microorganisms

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Abstract—We demonstrated for the first time that alkyl hydroxybenzenes (the d_1 microbial autoregulatory factors involved in stress responses of cells) are capable of stabilizing enzymes in aqueous media and increasing their catalytic activity. The stabilizing effect of a chemical analogue of alkyl hydroxybenzenes, C_7-AHB, was established in in vitro studies with enzymes of microbial origin: a protease produced by *Bacillus licheniformis*, cellulase produced by *Trichoderma viride*, and α-amylase produced by *Bacillus subtilis.* This effect manifested itself in considerable extension of the temperature and pH ranges of the enzymatic activity. The modulation of the catalytic activities of the stabilized enzymes depended on the C_7-AHB concentration and on the time of preincubation of the complexes obtained. We demonstrated that not only enzymes but also their polymeric substrates formed complexes with C_7-AHB and this significantly influenced the efficiency of hydrolytic reactions. We also conducted comparative studies on the efficiency of hydrolytic reactions in systems in which the structure of enzymes and/or substrates was modified with C_7-AHB.

Key words: enzyme stability, structural modification of biopolymers, chemical chaperons, alkyl hydroxybenzenes, stress.

The d_1 autoregulatory factors, which control the development of microbial cultures, accumulate in the cultivation medium. Once their concentration reaches the threshold level, the transition of the culture to the stationary growth phase occurs. A further increase in the d_1 concentration causes vegetative cells to lapse into an anabiotic state [1]. In chemical terms, the d_1 factors of some microorganisms belong to alkyl hydroxybenzenes (AHBs); a mixture of various AHB isomers and homologues occurs in developing cultures [2, 3]. The chemical properties of these autoregulators account for their capacity for noncovalent binding to biological molecules and supramolecular structures (membranes) due to the formation of hydrogen bonds and ion–ion and hydrophobic interactions. This results in changes in the functional activities of enzyme macromolecules [4–6] and biological membranes [7] and, at the cell level, in an increase in cell resistance to deleterious factors, including high temperatures, oxidative stress, etc. [8].

In order to elucidate the mechanisms involved in the development of anabiosis in dormant microbial forms, we conducted studies using hexylresorcinol (dissolved in ethanol) as a chemical analogue of d_1 factors. We showed that hexylresorcinol stabilizes the structure of enzyme macromolecules by forming complexes with them. This results in a decrease in the catalytic activity of enzymes in conjunction with an increase in their resistance to denaturing factors (photooxidation, temperature, and pH). From the data obtained, we inferred that alkyl hydroxybenzenes (the active components of d_1 factors) perform the functions of chemical chaperons. They can operate as natural modifiers of enzyme structures and contribute to the blockage of metabolic processes in hypometabolic and anabiotic cells by forming with enzymes thermostable complexes that possess low catalytic activity [4, 9]. Analogous functions are performed by long-chain 5-*n*-alkyl resorcinols, which are widespread components of plants and bacteria [5, 6, 10].

However, it has been recently suggested that some enzymes (hydrolases in particular) in dormant cells are characterized by a special conformation that ensures their high stability in conjunction with a high catalytic activity, which provides for intense hydrolysis of spore-specific structures in dormant cells upon initiation of their germination even though no de novo synthesis of enzymes occurs. Both this suggestion and our above statement are compatible with the theory of chemical enzymology that postulates that stabilization of the molecular structure of enzymes can cause either inhibition or stimulation of their catalytic activity [11].

The goal of this work was to investigate the in vitro effects of a chemical analogue of d_1 factors—an alkyl resorcinol, which, in contrast to hexylresorcinol, is characterized by low hydrophobicity—on the activity

and stability of microbial proteases, cellulases, and α-amylase.

MATERIALS AND METHODS

The following are the enzyme preparations of microbial origin used as models in our studies of the modification of enzyme structures by alkyl hydroxybenzenes: (i) neutral–alkaline protease from *Bacillus licheniformis* (produced by Ende Industrial Co, United States) with an activity of 440000 DAPU/g, a pH optimum of 7.0–8.0, and a temperature optimum of 60°C; (ii) cellulase from *Trichoderma viride* (produced by Vostok, Russia) with an activity of 2000 units/g, a pH optimum of 4.0, and a temperature optimum of 50°C; and (iii) a concentrated solution of α-amylase from *Bacillus subtilis* (produced by Vostok, Russia) with an activity of 2000 units/ml, a pH optimum of 6.0, and a temperature optimum of 60°C.

We used C_7-AHB, a short-chain amphiphilic alkyl resorcinol (pK 9) with a purification degree of 99.9% (a chemical analogue of d_1 factors of the bacteria of the genera *Pseudomonas* and *Azotobacter*), as a modifier of enzyme structures.

Determination of proteolytic activity (a modified Anson method). Hydrolysis was performed in a system containing 2% solution of substrate (casein as suggested by Hamersten) and the tested enzyme solution with an activity of 22 units/ml in 0.06 M phosphate buffer (pH 7.2). The C_7-AHB solution (350 mg of C_7-AHB per 50 ml of water) was diluted to yield the concentration to be tested. In the "no preincubation" system, the reaction mixture consisted of 2 ml of enzyme, 2 ml of substrate, and 1 ml of C_7-AHB (all reagents were kept in a thermostat at 40°C for 10 min prior to the assay). In the systems with preincubated AHB–enzyme or AHB–substrate complexes (termed hereinafter the "preincubation" systems), we added 1 ml C_7-AHB to 2 ml of enzyme/substrate, incubated the mixture at room temperature for 40 min and at 40°C for 10 min, and used it in the assay. If both the enzyme and the substrate were to be preincubated, we added 1 ml C_7-AHB to 4 ml of enzyme and 4 ml of substrate each and, following the procedures described above, combined 2.5 ml of the enzyme–C_7-AHB mixture with 2.5 ml of the substrate–C_7-AHB mixture to start the reaction. In the control systems, equivalent amounts of water were added instead of C_7-AHB; the enzyme was preincubated for the same time as in the experimental systems. Hydrolysis was carried out at 40°C for 20 min. The reaction was terminated by the addition of 4 ml of 5% trichloroacetic acid. The concentration of the low-molecular-weight protein substances that were not precipitated by trichloroacetic acid was determined from the change in the extinction ($\lambda = 280$ nm) of supernatants resulting from filtration of the reaction mixture (which was done at least twice). Because C_7-AHB per se absorbs light at 280 nm, we measured the control absorption value for each tested C_7-AHB concentration. We determined the true concentration of low-molecular-weight proteins by subtracting the control absorption value.

Determination of cellulolytic activity (based on determining reducing substances with 3,5-dinitrosalicylic reagent). Hydrolysis was carried out in a system containing 0.75% solution of substrate (the sodium salt of carboxymethylcellulose), the enzyme solution with an activity of 3 units/ml in 0.12 M citrate–phosphate buffer (pH 4.5), and C_7-AHB solution (350 mg of C_7-AHB per 50 ml of water). In the no preincubation system, the reaction mixture consisted of 2 ml of enzyme, 2 ml of substrate, and 1 ml of C_7-AHB (with the tested concentration). In the preincubation systems, the procedures carried out by us were analogous to those used to determine proteolytic activity. We added 1 ml of water instead of the C_7-AHB solution to control systems. Hydrolysis was carried out at 50°C for 2 h. The solution were cooled to room temperature after hydrolysis and, thereupon, an aliquot was sampled to determine the content of reducing substances (RSs) with 3,5-dinitrosalicylic reagent.

Determination of amylolytic activity (based on determining reducing substances with 3,5-dinitrosalicylic reagent). Hydrolysis was performed in a system containing 1% substrate (starch) solution, the enzyme solution with an activity of 0.1 units/ml in phosphate buffer (pH 6.0), and C_7-AHB solution (420 mg of C_7-AHB per 50 ml of water). In the no preincubation system, the reaction mixture consisted of 2 ml of substrate, 0.5 ml of enzyme, and 0.5 ml of C_7-AHB solution (with the tested concentration). The preincubation systems were analogous to those used for determining proteolytic activity. We added 0.5 ml of water instead of the C_7-AHB solution to control systems. Hydrolysis was carried out at 30°C for 10 min. The reaction was terminated by the addition of 3 ml of 1.0 N HCl. The RS concentration in the hydrolysate obtained was determined using the color reaction with 3,5-dinitrosalicylic reagent.

Because 3,5-dinitrosalicylic reagent interacts with starch to form a colored compound in the presence of C_7-AHB, we measured its background concentration for each C_7-AHB concentration. The true RS concentration was calculated by deducting the background concentration.

The C_7-AHB effect on the temperature range of catalytic activity was investigated in the no preincubation system. Hydrolysis was carried out within the 20–90°C and 5–90°C temperature ranges with proteases and cellulases, respectively.

Changes in the catalytic pH range of cellulases in the presence of C_7-AHB were determined using systems with various pH values ranging from 2.5 to 8.0.

Three independent series of experiments were conducted; five repeats of each experiment were done. We present the mean values of the data obtained. The statistical treatment of the data was carried out using stan-

dard mathematical methods, including the calculation of the mean-square deviation within the data array and Student's test ($P < 0.05$).

RESULTS

Since the stabilization of an enzyme is based on the modification of its structure upon complex formation with a ligand, the efficiency of this process is expected to depend on the concentration of the ligand involved (C_7-AHB) and the time of its incubation with the enzyme [11]. In the first series of studies, therefore, we investigated the influence of the stabilizing factor C_7-AHB on the proteolytic, cellulolytic, and amylolytic activities of hydrolases within a wide concentration range (0.4–11.27 mM C_7-AHB). It was added to the reaction mixture (i) by simultaneously mixing the enzyme, substrate, and stabilizing factor solution, i.e., in the no preincubation system, and (ii) by supplementing the substrate solution with enzyme that was prestabilized with C_7-AHB for 40 min (the preincubation system). The preincubation system enabled us to monitor the time course of the direct C_7-AHB–enzyme interaction and to take into account the gradual structural maturation of the enzyme due to the establishment of an equilibrium in the reaction of enzyme–C_7-AHB complex formation [9]. In the no preincubation system, C_7-AHB could form complexes with the polymer substrate, apart from the enzyme protein. The enzyme without the factor was assumed to have 100% activity (the control level).

Within the whole tested concentration range, C_7-AHB increased the catalytic activity of the hydrolases used by us (Figs. 1a–1c). All the enzymes displayed nonlinear activity changes depending on the C_7-AHB concentration both in the no preincubation and preincubation systems. With supraoptimal C_7-AHB concentrations added, we observed a decrease in enzyme (especially protease and α-amylase) activities. This is characteristic of the effects of chemical chaperons and, in all likelihood, reflects a modification of the protein structure that assumes a nonoptimum conformation in catalytic terms.

Using the preincubation system with proteases (Fig. 1a, curve *1*), we detected two activity maxima, at C_7-AHB concentrations of 3.22 and 8.05 mM (200% and 260% of control activity, respectively). Since AHB only acts on the enzyme during the preincubation step whose time is constant, the first activity maximum could be due to a modification of the enzyme structure that increases its catalytic activity; hence, the optimum C_7-AHB concentration for modifying the enzyme molecule is 3.22 mM. The second activity maximum, corresponding to high AHB concentrations, implies that excess AHB molecules modify, apart from the enzyme per se (during the preincubation step), the polymer substrate (casein). This inference is indirectly supported by the results of experiments in which we found only one clear-cut peak of proteolytic (Fig. 1a, curve *2*) and amylolytic (Fig. 1c, curve *2*) activities. In this no preincubation system, we administered C_7-AHB simultaneously with the enzyme and the substrate, which implies that C_7-AHB interacts to an equal extent with the polymer substrate and the enzyme macromolecule.

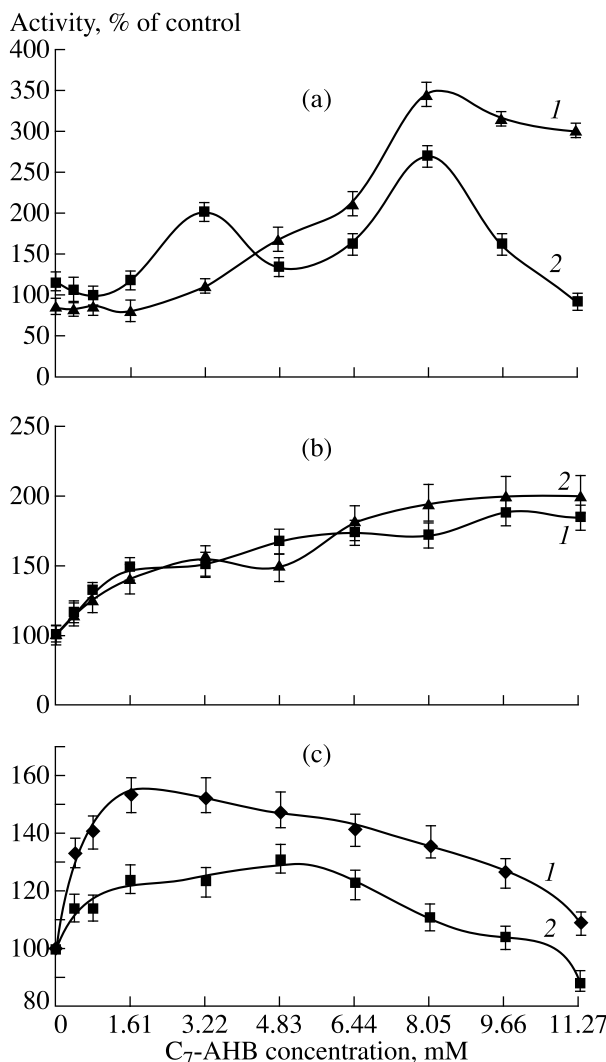

Fig. 1. C_7-AHB effects on (a) proteolytic, (b) cellulolytic, and (c) amylolytic activities in the systems (*1*) with enzyme preincubation with C_7-AHB and (*2*) without preincubation.

The concentration ranges of the RSs formed in the preincubation and the no preincubation system overlapped in our studies with C_7-AHB–stabilized cellulases (Fig. 1b). Presumably, the C_7-AHB modification of cellulases, in contrast to proteases, requires no enzyme maturation time. Alternatively, the efficiency of cellulose hydrolysis depends, in addition to the changes in enzyme conformation, on the modification of the substrate structure.

A comparative analysis of changes in the catalytic activities of the three AHB-stabilized hydrolases revealed that the preincubation system was character-

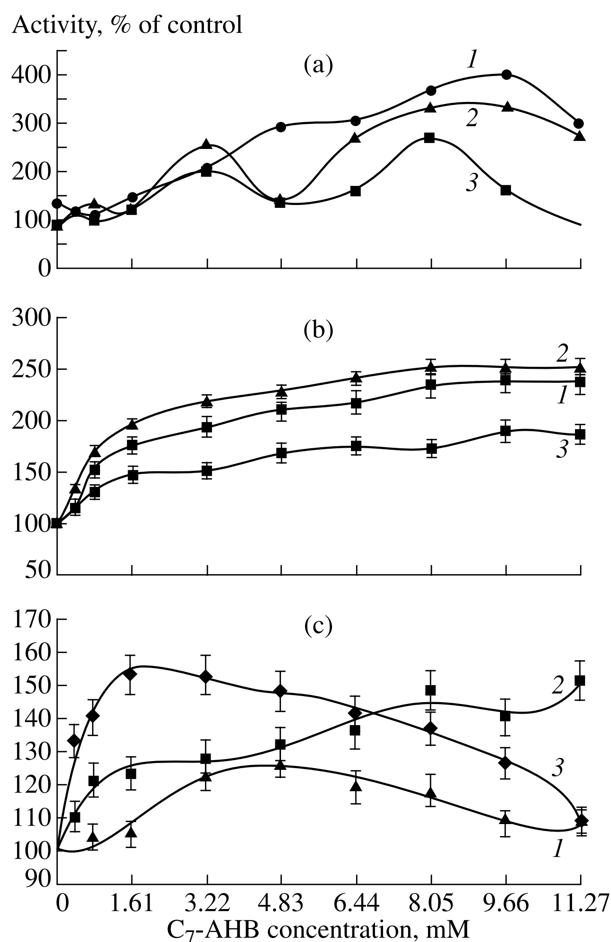

Fig. 2. Changes in (a) proteolytic, (b) cellulolytic, and (c) amylolytic activities after the preincubation with C_7-AHB of (*3*) the enzyme, (*2*) the substrate, and (*1*) both the substrate and the enzyme.

ized by a more significant stimulatory effect at low AHB concentrations. At high AHB concentrations, conversely, the system with direct addition of AHB to the incubation mixture (containing the enzyme and the substrate) was more efficient. Possibly, this was due to the ability of AHB to form complexes not only with enzyme macromolecules but also with polymeric substrates (casein, starch, and carboxymethylcellulose).

In order to test the above suggestion, we separately preincubated both reaction partners, the enzyme and the substrate, with AHB (Fig. 2) in the subsequent series of studies. Separate preincubation of the reaction mixture components produced different effects with each of the three catalytic reactions. Proteolytic activity (Fig. 2a) displayed an oscillatory pattern of AHB concentration dependence in all tested systems, suggesting that several biopolymer conformers may form upon the formation by AHB of complexes with the enzyme and with casein. The maximum proteolytic activity attained by modifying the substrate (at a C_7-AHB concentration of 9.6 mM) and the enzyme (at a C_7-AHB concentration of 8.05 mM) was 330 and 260%, respectively. The strongest stimulatory effect (up to 350–400% of the control level) occurred in a system combining preincubated enzyme–AHB and substrate–AHB complexes.

The system in which AHB-premodified starch was hydrolyzed by amylase (Fig. 2c) demonstrated the same increase in enzyme activity (150%) as the system with the AHB-modified enzyme (150%); however, the systems with AHB-premodified substrate and with AHB-premodified enzyme differed in the AHB concentration required for the maximum effect (11.27 and 1.61 mM, respectively). Simultaneous treatment of the enzyme and the substrate resulted in a lower product yield in the hydrolytic system (in contrast to the proteolytic system). Apparently, the modified enzyme was characterized by a suboptimal affinity for the modified substrate in the hydrolytic system.

In our studies with cellulases (Fig. 2b), substrate modification produced a more significant effect on catalytic activity than did enzyme modification: e.g., the difference between the activity increase caused by substrate modification and enzyme modification was 70% at a C_7-AHB concentration of 8.05 mM. If the enzyme and the substrate were treated separately, the reducing sugar yield was higher than that obtained upon modifying only the enzyme and only the substrate by 37 and 18% (mean values), respectively.

Accordingly, an individual approach to structural modification of each enzyme and respective polymer substrate is prerequisite for increasing the efficiency of hydrolytic reactions. However, our studies also revealed general regularities concerning the effect of AHB as a structural modifier of biopolymers: (i) the extent of the C_7-AHB effect both on the enzyme and the polymer substrate is concentration-dependent and (ii) exceeding the threshold concentration of the ligand results in decreasing the efficiency of the hydrolysis reaction, which is due either to noncompetitive inhibition of the enzyme involved or to a decrease in the enzyme–substrate affinity.

The stabilization of an enzyme resulting from its structural modification manifests itself in a change in its functional activity and, which is still more important, in an increase in the stability of the enzyme molecule to denaturing factors, i.e., in the retention of the enzyme function despite these factors. The stabilizing effect of C_7-AHB was determined on the basis of a comparative assay of enzyme activities. In these studies, hydrolysis was performed within a wide range of temperature and pH values. In order to evaluate the thermostability of proteases (Fig. 3), the enzymes were stabilized with C_7-AHB at concentrations of 3.22 and 8.05 mM, which caused optimum stimulation of enzyme activity based on the results of the preceding studies (Fig. 1a). The C_7-AHB solution was added to the medium in combination with the enzyme and the substrate solutions. We established that the temperature optimum (60°C) of the catalytic reaction did not change

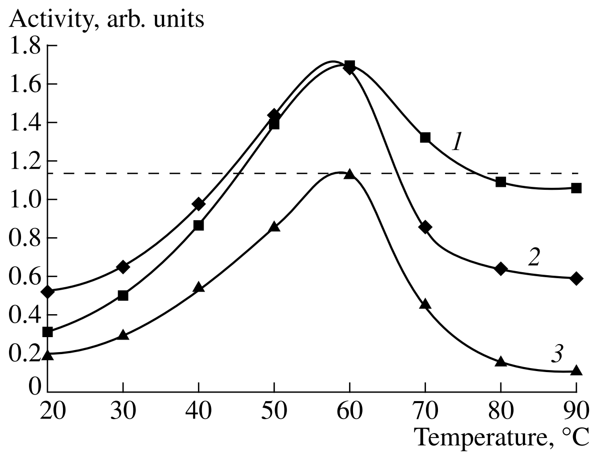

Fig. 3. Effects of C_7-AHB concentrations of (*1*) 8.05, (*2*) 3.22, and (*3*) 0 mM (control system) on proteolytic activity at various temperature values. The dashed line shows the activity maximum in the control system.

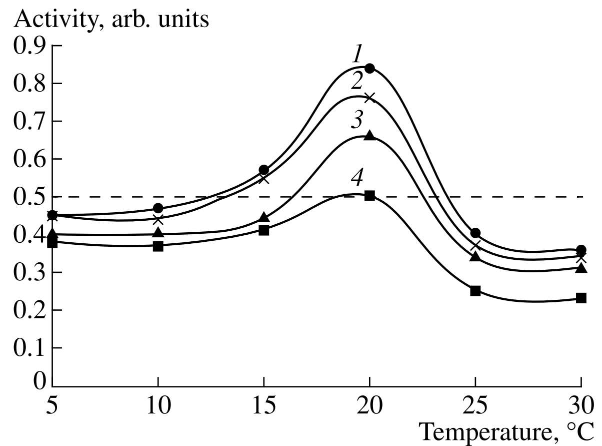

Fig. 4. Effects of C_7-AHB concentrations of (*1*) 9.66, (*2*) 6.44, (*3*) 1.61, and (*4*) 0 mM (control system) on cellulolytic activity at various temperature values. The dashed line shows the activity maximum in the control system.

upon stabilizing the proteases with C_7-AHB. The C_7-AHB effect manifested itself in a considerably enlarged temperature range (40–80°C) within which the reaction rate was higher than (or at least the same as) with the unstabilized enzyme used at optimum temperature (the dashed line in the figure).

In studies on the thermal and pH stability of cellulases, we added C_7-AHB to the reaction mixture at concentrations of 1.61, 6.44, and 9.66 mM, in accordance with the data on the concentration dependence of the AHB effects (Fig. 1b). Based on the results obtained (Fig. 4), the addition of C_7-AHB results in a considerably enlarged range of suitable temperatures (20–65°C), retaining the temperature optimum of 50°C. The stabilization of the cellulase influenced its catalytic activity in a similar fashion (Fig. 5). The activity level characteristic of the optimum pH value (4.0) in the control system without C_7-AHB was also attained at pH values of 2.5 and 8.0 if the cellulase was stabilized with C_7-AHB at concentrations of 6.44 and 9.66 mM, respectively.

DISCUSSION

Stabilization of enzyme proteins and modulation of their activities are essential mechanisms involved in the regulation of cell metabolism associated with the growth of microbial cultures and their stress responses to various deleterious factors [8]. Apart from the expression and functional role of stress proteins that operate as molecular chaperons (stabilizing the native structure of enzymes by "complementarily" binding to them [12]), much attention has been recently given to low-molecular-weight metabolites that serve as chemical chaperons [13–15]. In contrast to molecular chaperons, chemical chaperons function as ligands that nonspecifically interact with biopolymer molecules, change their conformation and, increase the resistance to denaturing factors. However, model studies of this type of interactions of enzyme proteins and small-size molecules revealed that enzyme stabilization in the resulting complexes is frequently accompanied by a decrease in enzyme activity [16]. As mentioned above, long-chain alkyl hydroxybenzenes such as C_{12}-AHB and 5-*n*-alkyl(C_{19}–C_{25}) resorcinols produce a protein structure–stabilizing effect in conjunction with an inhibition of its biological activities [4–6, 9, 10].

In our studies, we used an AHB homologue that had a shorter alkyl radical and, therefore, was less hydrophobic than the AHB mentioned above. An analysis of the data obtained revealed that the effect of C_7-AHB, like that of other chemical chaperons, is concentration-dependent. The nonlinear pattern of the concentration dependence is due to the formation of several new conformers that exhibit higher catalytic activity than the native enzyme. The data on the enlargement of the temperature and pH ranges of cellulase, amylase, and protease activities indicate that complex formation with C_7-AHB results in an increase in the stability of the enzyme proteins involved.

The results obtained are consistent with our earlier data on the stabilization of the enzyme systems of yeast cells [17]. They confirm the idea that AHB produces nonspecific effects on various enzymes. There is no contradiction between our findings and data on the effects of structurally different chemical chaperons, e.g., the osmoprotectants glycine-betaine and ectoine [18, 19].

In contrast to data on a *decrease* in functional activity associated with the stabilization of protein globules (documented in scientific works and patent bulletins), we revealed that an increase in the stability of enzyme proteins was accompanied by *stimulation* of proteolytic, amylolytic, and cellulolytic activities. A paral-

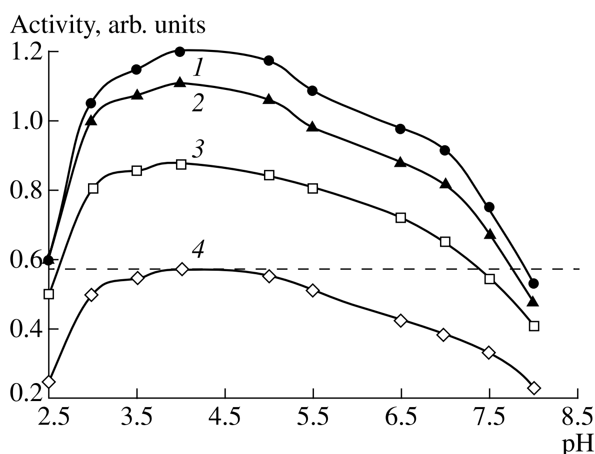

Fig. 5. Effects of C_7-AHB concentrations of (*1*) 9.66, (*2*) 6.44, (*3*) 1.61, and (*4*) 0 mM (control system) on cellulolytic activity at various pH values. The dashed line shows the activity maximum in the control system.

lel increase in stability and activity, which is due to conformational changes in the enzyme protein, was also demonstrated in the α-chymotrypsin model system [11]. However, in this case the enzyme was solubilized in inverted AOT micelles in octane, i.e., in an organic phase. From the circular dichroism spectra obtained, the conclusion was drawn that α-chymotrypsin molecules increase their "rigidity" (spiralization degree) if placed in inverted micelles whose size is close to that of an α-chymotrypsin molecule. The increase in the enzyme activity in this system was partially due to the effects of the microenvironment of the enzyme molecule in the inverted micelle [11]. In our work, we demonstrated for the first time that short-chain phenolic lipids (AHB) enable us to stabilize enzymes in conjunction with a stimulation of their activities in aqueous media. We believe that this is a consequence of conformational changes in proteins that are mainly due to the formation of intermolecular hydrogen bonds involving hydroxy groups of the aromatic nucleus of AHB.

Importantly, enzyme stabilization by AHB did not change the temperature or pH optima of the catalytic reactions. If conformational changes influence the pK values of an enzyme, they affect the ionogenic groups involved in the formation of the active center [11]. Protein structure stabilization by alkyl hydroxybenzenes does not cause such effects because the pH optimum does not change despite the formation of new conformers. Presumably, the increase in enzyme activity upon complex formation with C_7-AHB is linked to conformational changes in the protein molecule involved that increase the accessibility of its catalytic center and binding sites.

Nonspecific complex formation of structurally different enzymes with AHB (based on physicochemical interactions) implies that AHB can also nonspecifically interact with polymer substrates. Studies conducted by us revealed that conformational changes not only in enzymes but also in polymer substrates contribute to the resulting increase in the efficiency of catalytic reactions. Substrate modification probably causes an increase in enzyme–substrate affinity. This seems to be due to partial unfolding of the substrate that increases the number of accessible sites. Substrate modification produced the most significant effect in the cellulose hydrolysis reaction. Based on the data reported in the literature, the capacity of cellulases to adhere to a substrate is as important for the efficiency of hydrolysis reactions as their catalytic activity per se. Compelling evidence was presented that the degrees of adhesion of cellulolytic enzymes from various microorganisms to cellulose differ by two or three orders of magnitude. This is correlated with the degree of hydrolysis of crystalline cellulose [20]. Bearing in mind these data, we suggest that the modification of the polymer structure of a substrate by AHB promotes enzyme adhesion to its surface, resulting in an increase in enzyme–substrate affinity.

Hence, we established that C_7-AHB, a chemical analogue of the d_1 autoregulatory factors of microorganisms characterized by low hydrophobicity, is capable of posttranslational modification of the structure of enzyme proteins (proteases, amylases, and cellulases) that results in an increase in their resistance to denaturing factors (temperature and pH) in conjunction with a stimulation of catalytic activities. In terms of catalysis efficiency, the structural modification of polymer substrates by C_7-AHB is at least as important as the modification of the enzyme per se. Substrate modification can produce a more significant stimulatory effect on the intensity of the catalytic reaction than enzyme modification. The fact that enzyme macromolecules are stabilized by small-size molecules operating as anabiosis autoinducers is of considerable interest in relation to the mechanisms stabilizing dormant forms and contributing to stress responses of microorganisms and cells at higher organizational levels (fibroblasts, melanocytes, etc.) [8]. The biological importance of the interactions of enzymes and anabiosis inducers is also due to the fact that this dynamic system is constantly involved in the development of microbial cultures [1, 3]. Changes in the properties of enzymes forming complexes with alkyl hydroxybenzenes (d_1 factors), the lability of the complexes per se, and the accessibility of AHB-modified cell polymers as substrates are conditional on the qualitative composition of AHB, among other factors. In our publications [4, 9], we have recently investigated two AHB homologues that differed in their hydrophobicity degree. We have shown that one of them stimulates and the other inhibits enzyme activities. In order to understand more fully the mechanisms of AHB effects on biomacromolecules, it is necessary to conduct further studies concerning the physicochemical properties of conformers that form under the influence of AHB.

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