Effects of Asparaginase and Polyphenol Oxidase on Adhesive Characteristics of Microorganisms

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We studied the effects of polyphenol oxidase and asparaginase on microorganism adhesion to buccal epithelial cells. These enzymes reduced adhesion of pathogenic microorganisms (uropathogenic and *Escherichia coli*, *Salmonella enteritidis*, *Entamoeba spp.*, *Influenza virus*, *Candida albicans*, *Streptococcus spp.*) and had virtually no effect on adhesive characteristics of probiotic variants of *Escherichia coli* and *Lactobacillus fermentum.*

Key Words: *probiotic adhesion; L-asparaginase; polyphenol oxidase; adhesion inhibition*

Adhesion is a common property of microorganisms and an important component of colonization resistance. Adhesion can be regarded as the first stage in the development of various forms of symbiotic relationships [9]. Adhesion is determined by surface structures of bacterial cells recognizing specific receptors of target cells. Peptide sequences responsible for the adhesin-receptor binding are enriched with asparagine and tyrosine. Therefore, L-asparaginase (ASG) and polyphenol oxidase (PPO) were proposed as adhesion inhibitors [7]. These enzymes cleave tyrosine and asparagine residues, thus modifying the conformation of adhesion structures [4,5,7]. The inhibition of adhesion can prevent colonization of target cells by pathogenic microorganisms and the development of infection. On the other hand, adhesiveness is an important parameter of probiotics, as it is the first stage of colonization activity, ensuring fixation of microorganisms to the substrate and their subsequent multiplication and release of bioactive substances, suppressing the viability of other bacteria, including pathogens [2]. We compared the effects of ASG and PPO on adhesive activity of pathogenic and probiotic microorganisms.

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MATERIALS AND METHODS

Bacterial strains *Escherichia coli* M-17, producer of probiotic preparation Colibacterin, and M-17 fimH::ntp, recombinant variant of this strain with inactivated gene of type I pilus adhesin [8]; *Lactobacillus fermentum* 90-TS-4(21), a selection variant of producer of commercial probiotic Lactobacterin characterized by high adhesion to vaginal epithelial cells [7]; *Candida albicans*; *Sacharomyces cerevisiae*, a strain of commercial baking yeast, were used in the study.

Adhesion was studied on 18-h cultures of microorganisms grown at 37°C in Luria Bertani broth (*Escherichia coli* strains) and *Lactobacilli Man-Rogosa-Sharp* (strain *Lactobacillus fermentum*) under stationary conditions. The cells were washed (2×10 min at 1500 rpm) from the growth medium with 0.1 M buffered saline (PBS, pH 7.4) or 0.2 M phosphate buffer (PB, pH 7.4). Buccal epithelial cells (BEC) were obtained *ex temporo* from donors by delicate scraping from the buccal surface. BEC suspension in PSB was washed 4 times (5 min each time) and centrifuged at 1500 rpm, the optical density of suspension was brought to 0.4 (corresponding to a concentration of 10^6 cells/ml); the cells were counted in a Goryaev chamber. PPO and AGS (Sigma) were dissolved in 50 mM Tris-HCl to a concentration of 1 mg/ml (stock solutions). The enzymes were used in the following concentrations:

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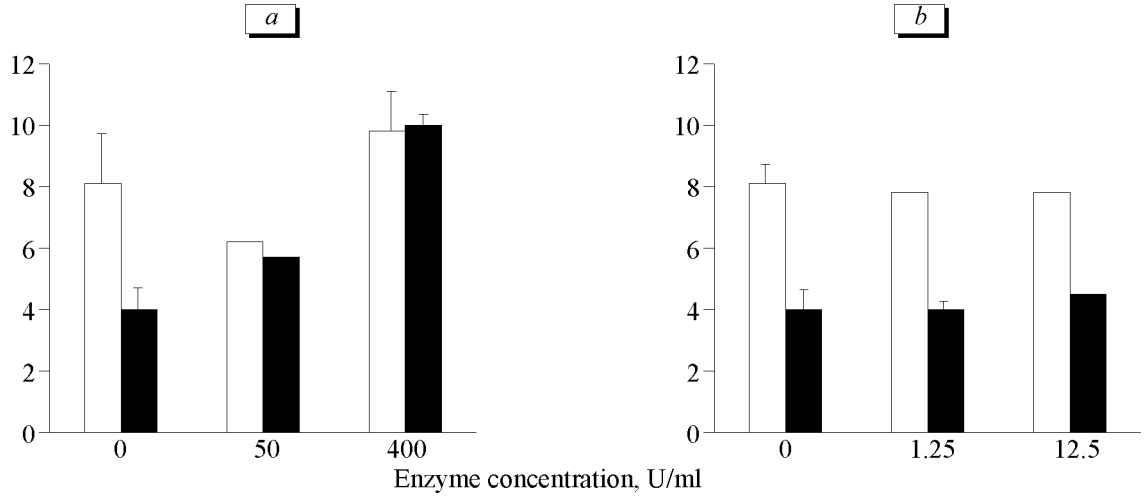

Fig. 1. Effects of polyphenol oxidase (*a*) and L-asparaginase (*b*) on adhesion of *E. coli* M-17 strains (light bars) and M-17 fimH::ntp (dark bars) to buccal epithelial cells. Here and in Fig. 2: Ordinate: number of bacteria per epithelial cell.

ASG, 10 and 100 mg/ml (1.25 and 12.5 U/ml); PPO, 25 and 200 mg/ml (50 and 400 U/ml). All experiments with ASG were carried out with PSB and experiments with PPO using PB, because PPO activity decreases in the presence of Cl^- [4]. The enzyme was added to the microorganism suspension (optical density 1 at λ=540 nm; corresponds to a concentration of 10^9 CFU/ml) to attain the studied concentration and incubated for 1 h at 37°C with shaking. After incubation the bacterial suspension was washed twice in PSB (2×10 min, centrifugation at 1500 rpm). BEC suspension was added (1:3) to bacterial suspension and incubated for 1 h at 37°C with shaking. After incubation BEC were washed with PSB (5×10 min at 800 rpm). Smears similar to blood smears were fixed in 96% ethanol and stained with gentian violet or after Romanowskii—Giemza. At least 50 cells in 5 visual fields were counted. Adhesiveness was determined as the mean number of microorganisms per BEC.

Alternatively, BEC (but not microorganisms) were treated with the enzymes (BEC suspension before ASG or PPO addition was prepared in PSB or PB, respectively) [3,6].

Yeast suspension (10 mg/ml) washed twice in PSB with 0.1% BSA was used in the yeast aggregation test. The experiment was carried out in U-shaped bottom immunological plates preincubated with 40 µl 0.1 BSA in PSB for 40 min for saturation of nonspecific binding; 40 µl *Escherichia coli* M-17 culture prepared as described above was added and titered to 1:250 dilution. Type I pili aggregate yeast by the mannose-sensitive type, that is, the reaction is inhibited by mannose and its derivatives, and therefore a parallel test with 1% α-methyl-D-mannopyranoside was carried out in order to prove the presence of type I pili. All stages of the experiment were carried out on the cold. Aggregation was evaluated visually [3].

The data were processed statistically using parametrical methods with estimation of the mean variation value. The presented data are the means of at least 3 independent experiments in 3 repetitions.

RESULTS

Effects of PPO and ASG on adhesive activity of pathogenic microorganisms were extensively studied in the laboratory of one of the authors (R. J. Doyle). Their effects were best studied on types I and P pili of pathogenic *E. coli* on a model of uroepithelial cells. In order to prove that adhesion is caused by one of these pili, a control with inhibitors (α-methyl-D-mannopyranoside for type I pili and globoside for P fimbria) was performed in each experimental series. The decrease in erythrocyte agglutination titers

TABLE 1. Effects of ASG and PPO on Adhesion of *Candida albicans* to BEC (*M*±*m*)

Control	PPO, U/ml			ASG, U/ml	
	70	140	210	20	60
9.0±3.5	6.0±1.5	4.5±1.5	8.5±3.5	7.0±2.0	4.5±1.5

TABLE 2. Effects of ASG and PPO on Aggregation of Baking Yeast by *Escherichia coli* M-17

Strain	Agglutination titer						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128
E. coli M-17							
native	++++	++++	+++	++	++	+	—
ASG							
1.25 U	++++	++++	+++	++	+	—	—
12.5 U	++++	++++	+++	++	+	—	—
PPO							
50 U	++++	++++	+++	++	+	—	—
400 U	++++	+++	++	+	—	—	—
1% α-methyl-D-manno-pyranoside treatment	+	—	—	—	—	—	—
E. coli M-17 fimH::ntp	—	—	—	—	—	—	—
Lactobacillus fermentum 90-TS-4(21)	—	—	—	—	—	—	—

Note. "++++" pronounced aggregation, "+++" clear-cut aggregation, "++" poor aggregation, "+" very poor aggregation, "-" no aggregation.

in the presence of these enzymes was demonstrated for clinical strains of *Salmonella enteritidis*, *Entamoeba spp.*, and influenza virus. In the latter case treatment with PPO or ASG in a concentration of 70 U/ml reduced agglutination titer to 64 *vs.* 256 in the control. The inhibition of adhesion to BEC was demonstrated for cocci and *Candida albicans.* A 50% inhibition of *Candida albicans* adhesion was attained with 140 U/ml of PPO and 60 U/ml of ASG (Table 1).

It was found that the enzymes had no effects on adhesive activity of normal microflora (Figs. 1 and 2). In some cases adhesion slightly increased, for example after treatment of *E. coli* M-17 fimH::ntp strain expressing no pili (Fig. 1). The mechanism of this phenomenon is not quite clear; presumably the enzymes act as liases "loosening" the cell surface and exposing the structures for additional fixation of the bacterium to receptors. Enzyme treatment of BEC did not appreciably inhibit adhesion: the changes in adhesion were similar to those after enzyme treatment of the microorganisms.

Evaluation of the effects of PPO and ASG on adhesive activity of bacteria in the yeast aggregation test showed a negligible decrease of agglutination titers of *E. coli* M-17 production strain. *Lactobacillus fermentum* 90-TS-4(21) and *E. coli* M-17 fimH::ntp strains did not aggregate yeast (Table 2).

Hence, our experiments showed that both ASG and PPO blocked adhesion of mainly pathogenic microorganisms, while normal microflora was virtually insensitive to these enzymes. These results

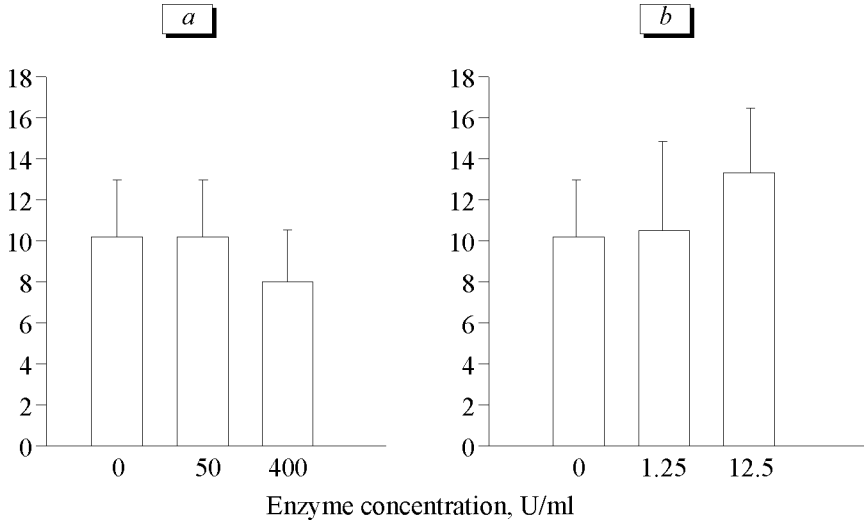

Fig. 2. Effects of polyphenol oxidase (*a*) and L-asparaginase (*b*) on adhesion of *Lactobacillus fermentum* 90-TS-4(21) to buccal epithelial cells.

open new prospects in the use of these enzymes together with probiotics for prevention and/or treatment of infections caused by microorganisms sensitive to the studied enzymes. It is important that *Candida albicans*, an agent of highly prevalent vulvovaginal candidosis, is sensitive to the enzymes, while *Lactobacillus fermentum* 90-TS-4(21), perspective for the creation of a vaginal probiotic, is insensitive to them. The fact prompts combined use of this probiotic and enzyme for the treatment of this condition.

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