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EXPERIMENTAL ARTICLES

Production of Cell-Wall-Bound Proteinases in *Bifidobacterium adolescentis* 94-BIM

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Abstract—*Bifidobacterium adolescentis* 94-BIM was found to produce cell-wall-bound proteolytic enzymes active at acidic, neutral, and alkaline pH values. The solubilization of proteinases with 0.5% Triton X-100 substantially improved the yield of the enzymes. The most active accumulation of cell-bound proteinases was observed in the third hour of cultivation at rates of 156.7, 179.5, and 111.1 U/(mg h), measured at pH 2.5, 7.0, and 9.0, respectively. It is suggested that the cell-wall-bound proteinases of *B. adolescentis* 94-BIM are the precursors of the enzymes secreted into the medium.

Key words: bifidobacteria, proteolytic activity, solubilization of proteinases, cell wall associated proteinases

The literature on the production of proteolytic enzymes by bifidobacteria is very scarce. The available information concerns primarily the proteolytic activity of some representatives of the genus *Bifidobacterium* [1–3]. This problem is not only of scientific interest, but also of applied interest, since starter cultures for milk fermentation must possess high proteolytic activity [4–5].

During growth in complex media, *Bifidobacterium adolescentis* 94-BIM secretes extracellular proteolytic enzymes active at acidic, neutral, and alkaline pH values [6]. Further studies showed that washed bifidobacterial cells also possess proteolytic activity, suggesting that proteolytic enzymes are bound to the cell surface.

The present work was undertaken to study the production of cell-wall-bound proteinases by bifidobacteria and the effect of surfactants on their solubilization.

MATERIALS AND METHODS

Experiments were performed with *Bifidobacterium adolescentis* 94-BIM deposited as *Bifidobacterium adolescentis* BIM B87 from the Collection of Nonpathogenic Microorganisms of the Institute of Microbiology, Belorussian Academy of Sciences. The bifidobacterium was cultivated in a modified corn–lactose medium [7] supplemented with casein hydrolysate.

Proteolytic activity was measured using the modified Anson's method [8] with a 2% casein solution in 0.1 M universal buffer with pH 2.5, 7.0, and 9.0. The reaction of casein proteolysis was allowed to proceed for 20 min, after which the residual casein was precipitated with trichloroacetic acid, and then the optical density of the supernatant containing casein peptides that do not precipitate with trichloroacetic acid was measured. One unit of proteolytic activity (U) was defined as the amount of enzyme necessary to produce an increase in the optical density of 0.0005 OD units of the supernatant. Enzyme activity was expressed in U/ml-medium or in U/mg-biomass (to characterize the productivity of enzyme synthesis by bifidobacteria).The proteolytic activity of washed bifidobacterial cells was assessed as follows. First, bifidobacterial cells were precipitated by centrifugation from a specific volume of culture and then washed with 0.01 M phosphate buffer (pH 6.9) until the proteolytic activity after washing reached zero. The cells were then resuspended in 0.1 M universal buffer one-tenth the volume of the culture sample that was taken for centrifugation. The proteolytic activity of the cells was measure and expressed in U/ml-culture.

To study the dynamics of growth and proteolytic activity of bifidobacteria, the growth medium was inoculated at 5 vol % with an18-h *B. adolescentis* culture that was preliminarily subcultured in the same medium three times. The culture thus obtained was then incubated for three days at 37°C with a regular (3-h interval) sampling of cells for analysis. To solubilize cell-wall-bound proteinases (hereafter called, CW proteinases), the washed cells were incubated and regularly stirred, for 24-and 48-h at 4°C in a 0.01 M phosphate buffer (pH 6.9) containing different concentra-

Table 1. Proteolytic activity of washed *B adolescentis* 94-BIM cells

Cultivation time, h	Proteolytic activity, U/ml culture		
	pH 2.5	pH 7.0	pH 9.0
24	78	45	42
48	56	32	28
72	40	26	15

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Table 2. Solubilization of the cell-wall-bound proteinases of *B. adolescentis* 94-BIM with various detergents*

Detergent concentration, %		Proteolytic activity, U/ml culture		
		pH 2.5	pH 7.0	pH 9.0
Tween 20,	0.1%	112	188	200
	0.5%	149	132	215
	1.0%	166	194	249
Tween 40,	0.1%	157	227	195
	0.5%	194	276	218
	1.0%	245	259	246
Tween 60,	0.1%	148	188	194
	0.5%	162	205	216
	1.0%	177	185	182
Tween 80,	0.1%	163	234	154
	0.5%	198	249	186
	1.0%	175	196	180
Triton X-45,	0.1%	124	131	142
	0.5%	108	158	118
	1.0%	142	210	189
Triton X-100,	0.1%	158	200	287
	0.5%	234	266	318
	1.0%	218	244	290
Triton X-305,	0.1%	142	178	226
	0.5%	186	242	244
	1.0%	179	147	215
0.1 M universal buffer				
	pH 2.5	72	80	80
	pH 7.0	114	146	132
	pH 9.5	122	176	164

* Cells from a 24-h bifidobacterial culture were treated with detergent solutions for 48 h.

Table 3. Effect of surfactants (1 vol %) on the activity of the extracellular proteinases of *B. adolescentis* 94-BIM

Detergent	Proteolytic activity, U/ml culture		
	pH 2.5	pH 7.0	pH 9.0
Control (without detergent)	460	408	346
+ Tween 20	437	412	374
+ Tween 40	206	219	135
+ Tween 60	423	389	354
+ Tween 80	450	418	312
+ Triton X-45	438	374	327
+ Triton X-100	470	395	368
+ Triton X-305	404	380	338

tions (0.1, 0.5, and 1.0%) of various surfactants (Tween 20, 40, 60, and 80; and Triton X-45, X-100, and X-35). After 24- and 48-h time periods, the cells were removed by centrifugation, and the proteolytic activity of the supernatant was measured.

In the control experiments, the direct effect of surfactants on the proteinases was assessed. In these experiments, 1 vol % of each of the detergents studied was added to the supernatant, which was obtained by removing cells from a 24-h bifidobacterial culture. These mixtures were kept at 4°C for 24 h and then assayed for proteolytic activity. For comparison, the proteolytic activity of the culture was determined by adding 1 vol % of 0.01 M phosphate buffer instead of detergents.

The specific growth rate of bifidobacteria, μ, has been estimated and described elsewhere [6].

Intensity of accumulation of the proteinases was determined by measuring the specific rate of enzyme synthesis (ε), calculated by the formula $\varepsilon = dE/dtx$, where x is the biomass expressed in mg/ml, and dE is the increase in enzyme activity (U/ml) over the time period dt [9].

All experiments and measurements were performed three more times.

RESULTS AND DISCUSSION

The experiments showed that *B. adolescentis* 94-BIM cells washed with 0.01 M phosphate buffer showed proteolytic activity that decreased with the amount of cultivation time (Table 1).

Since this proteolytic activity might be due to CW-bound proteinases, we studied the efficiency of solubilization of these enzymes by various surfactants (for their relevant properties, see the handbook [10]). The data obtained in experiments with 48-h incubation with surfactants are summarized in Table 2. It is shown that 0.5% Triton X-100 solubilized the maximum amount of cell-bound enzymes to the medium. Solubilization was most efficient at high pH values. Similar results were obtained for bifidobacteria incubated with detergents for 24-h (data not shown), but the degree of solubilization of proteinases in this case was, as a rule, much lower than after the 48-h incubation period.

Control experiments checking the probability of inactivation of proteolytic enzymes by detergents showed that they did not affect proteolytic activity at 1 vol % concentration except for Tween 40, which inhibited proteolytic activity by approximately two times (Table 3). These results allowed us to use detergents for the isolation and further study of the CW-bound proteinases of *B. adolescentis* 94-BIM.

Figure 1 shows the dynamics of production of CW-bound proteinases in *B. adolescentis* 94-BIM culture. It can be seen that proteolytic activity rose throughout the exponential phase, somewhat decreased in the stationary phase, and fell in the phase of culture death. The

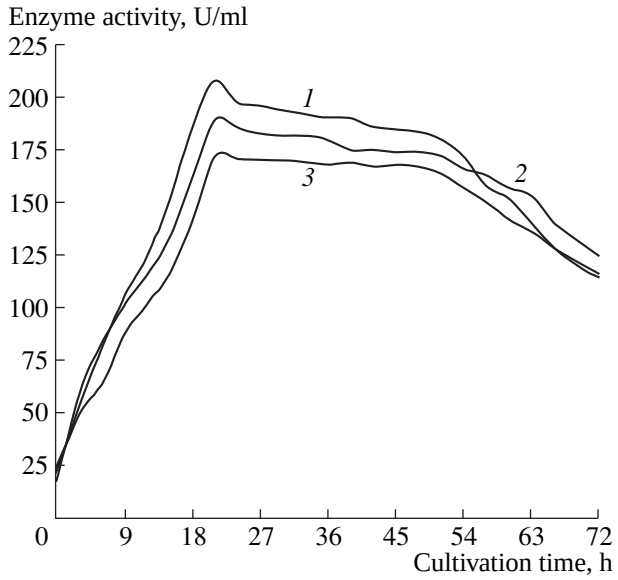

Fig. 1. Dynamics of (*1*) acidic, (*2*) neutral, and (*3*) alkaline cell-wall-bound proteinases during the course of the growth of *B. adolescentis* 94-BIM.

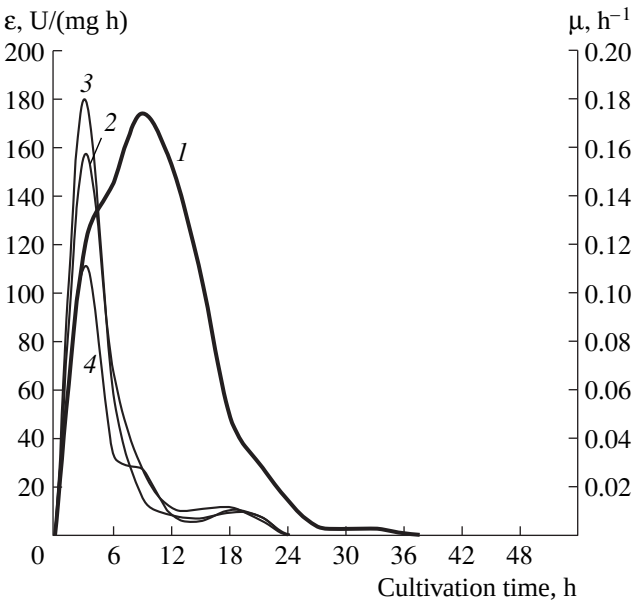

Fig. 2. Specific rates of (*1*) *B. adolescentis* 94-BIM growth (μ) and synthesis of cell-wall-bound proteinases (ε) active at pH (*2*) 2.5, (*3*) 7.0, and (*4*) 9.0.

specific rate of synthesis of CW proteinases in the *B. adolescentis* 94-BIM culture reached a maximum by the third hour of growth, when ε comprised 179.48, 156.75, and 111.1 U/(mg h) at pH 2.5, 7.0, and 9.0, respectively (Fig. 2). As shown previously, the maximum activity of extracellular proteinases is observed by the sixth hour of growth [6]. It can be suggested that the CW-bound proteinases synthesized by *B. adolescentis* 94-BIM are the precursors of extracellular proteolytic enzymes.

According to data available in the literature, these characteristics of the production of proteolytic enzymes an typical of related groups of microorganisms (lactobacilli and lactococci), which, like bifidobacteria, are components of natural intestinal microflora [11, 12]. The maximum activity of CW-bound proteinases was observed in the exponential and early stationary phases of growth of these microorganisms. For instance, the maximum activity of cell-bound proteinases in the *Lactococcus lactis* Wg2 culture was observed at $\mu = 0.23$ h^{-1} [13], while at $\mu = 0.12$ h in the *B. adolescentis* 94-BIM culture.

It should be noted that the intracellular, cell-bound, and extracellular proteinases of lactic acid bacteria are well-studied. Unlike the proteinases of bifidobacteria, the proteinases of lactobacilli and lactococci are mainly associated with the cell surface [14].

In conclusion, the strain *B. adolescentis* 94-BIM was found to produce CW-bound proteolytic enzymes active at acidic, neutral, and alkaline pH values in addition to the proteolytic enzymes secreted into the cultivation medium. The solubilization of cell-bound proteinases with surfactants substantially increases the yield of extracellular enzymes. The results obtained in this present study contribute to our knowledge of the proteolytic systems of microorganisms, particularly the cell-wall-bound proteinases of bifidobacteria.

REFERENCES

1. Gudkov, A.V., Ervol'der, T.M., Sviridenko, G.M., and Gudkova, M.Ya., Biological Activity of Bifidobacteria in Milk, *Molochn. Prom–st.*, 1984, no. 1, pp. 21–23.
2. Etsuo, M., Shuichi, K., Fudji, T., Hidemasa, M., and Kunio, Y., Exopeptidase Profiles of Bifidobacteria, *J. Nutr. Sci. Vitaminol.*, 1985, vol. 31, no. 6, pp. 599–606.
3. Abu-Taraboush, H.M., Al-Dagal, M.M., and Al-Royli, M.A., Growth, Viability, and Proteolytic Activity of Bifidobacteria in Whole Camel Milk, *J. Dairy Sci.*, 1998, vol. 81, no. 2, pp. 354–361.
4. Androsova, N.L., Firsov, V.K., and Baryshnikova, B.P., Quality of Starter Cultures as a Factor of Optimization of Technological Processes in the Children's Food Industry, *Mater. mezhd. konf. "Natsional'naya politika v oblasti zdorovogo pitaniya v RB"* (Proc. Intl. Conf. on Governmental Politics toward Healthy Nutrition in the Republic of Belarus), Minsk, 1997, p. 226.
5. Visser, S., Proteolytic Enzymes and Their Relationship to Cheese Ripening and Flavor: An Overview, *J. Dairy Sci.*, 1993, vol. 76, no. 1, pp. 329–350.
6. Samartsev, A.A., Astapovich, N.I., and Novik, G.I., Growth and Production of Extracellular Proteinases by *Bifidobacterium adolescentis* 94-BIM, *Mikrobiologiya*, 1997, vol. 66, no. 5, pp. 635–639.

7. Sundukova, M.B., Khor'kova, E.A., Semenikhina, V.F., Ganina, V.P., Khaleneva, M.P., Pospelova, V.V., and Rakhimova, M.G., Growth Medium for the Isolation and Cultivation of Bifidobacteria, USSR Inventor's Certificate no. 37994941/30-13, *Byull. Izobret.*, no. 43, p. 104.
8. Anson, M.Z., The Estimation of Pepsin, Trypsin, Papain and Cathepsin with Hemoglobin, *J. Gen. Physiol.*, 1938, vol. 22, no. 1, pp. 79–83.
9. Terui, J., Shinmyo, A., and Okazaki, M.P., *Rein Hardsbunner Symposium der Sektion Microbiologie der Biologischen Gellschaft der DDR*, Berlin, Academie-Verlag, 1981, no. 3, pp. 273–279.
10. Dawson, R.M.C., Elliott, D.C., Elliott, W.H., and Jones, K.M., *Data for Biochemical Research*, Oxford: Clarendon, 1986, 3rd ed. Translated under the title *Spravochnik biokhimika*, Moscow: Mir, 1991.
11. Nissen-Meyer, J. and Sletten, K., Purification and Characterization of the Free Form of the Lactococcal Extracellular Proteinase and Its Autoproteolytic Cleavage Products, *J. Gen. Microbiol.*, 1991, vol. 137, pp. 1611–1618.
12. Pritchard, G.G. and Coolbear, T., The Physiology and Biochemistry of the Proteolytic System in Lactic Acid Bacteria, *Fed. Eur. Microbiol. Soc. Microbiol. Rev.*, 1993, vol. 12, pp. 179–206.
13. Laan, H., Bolhuis, H., Polman, B., Abee, T., and Konings, W.N., Regulation of Proteinase Synthesis in *Lactococcus lactis*, *Acta Biotechnol.*, 1993, vol. 13, no. 1, pp. 95–101.
14. Gilbert, C., Blanc, B., Frot-Contaz, J., Portalier, R., and Atlan, D., Comparison of Cell Surface Proteinase Activities within the *Lactobacillus* Genus, *J. Dairy Res.*, 1997, vol. 64, no. 4, pp. 561–571.