LIPASE AND TWEENASE ACTIVITIES OF Clostridium perfringens AND Cl. botulinum

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Enzymes of lipid metabolism were studied in strains of the gas gangrene organism Clostridium perfringens type A and of Cl. botulinum types A, B, and F, with different levels of toxigenicity. Lipase activity of the toxigenic strains of Cl. perfringens was found to be higher than that of strains with low toxigenicity, while this activity for Cl. botulinum was much higher than for Cl. perfringens. Cultural filtrates possessed much higher lipase activity than the bacterial cells. Neither the bacterial cells nor the cultural filtrates of Cl. perfringens and Cl. botulinum decompose tweens, but after a series of subcultures of these bacteria on media containing tweens an induced tweenase was clearly detectable. Accumulation of tweenases during growth of the bacterial cultures and the chief properties of the enzymes were studied.

The gas gangrene organism Clostridium perfringens, whose toxic complex consists of substances of enzymic nature, on entering a wound causes the breakdown of muscle and adipose tissues. However, although there have been many investigations of the proteolytic activity of the pathogenic clostridia, the investigation of their lipase and esterase activity has barely begun. For instance, an indirect conclusion regarding the high lipolytic activity of the pathogenic clostridia can be obtained by Nagler's diagnostic test, and in 1961 the presence of lipases in some clostridia was demonstrated [1]. The use of tweens for determination of lipolytic activity in this investigation, however, did not allow differentiation between lipase and esterase activity.

In the investigation described below the enzymes of lipid metabolism were studied in Cl. perfringens and Cl. botulinum in connection with the toxigenic function of bacterial cell.

EXPERIMENTAL METHOD

Highly toxigenic strains BP6K No. 28 and SR-12, the weakly toxigenic strains 2836, 2910, and No. 1, and the practically nontoxigenic strain A-27 of Cl. perfringens type A were used. The strains were kept on Tarozzi's medium and subcultured for growth on medium containing casein-mushroom hydrolysate with 0.5% glucose. Cultivation continued for 2 to 24 h at 37°C and samples were taken every 2 h. Since under these conditions it was impossible to detect tweenase activity, an attempt was made to induce the formation of this enzyme by subcultures on media containing tweens. Accordingly, 0.1% tween was added to the basic medium and after growth for 6 h the culture was transferred to fresh nutrient medium of the same composition for further growth, and so on. Tweenase with clearly defined activity appeared after the sixth subculture by the 4th hour of growth.

At the end of growth the bacterial mass was separated, washed twice with physiological saline, and a 10% bacterial suspension in physiological saline was made up. To determine lipase and tweenase activity

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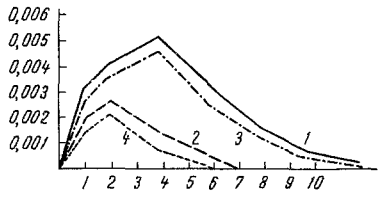

Fig. 1. Dynamics of lipase activity in toxin and bacterial cells of Cl. perfringens: 1) strain BP-6K (toxin); 2) BP-6K (cell); 3) 2836 (toxin); 4) 2836 (cell). Here and in Figs. 2 and 3: ordinate, content of oleic acid in mg/mg total nitrogen; abscissa, age of culture (in h).

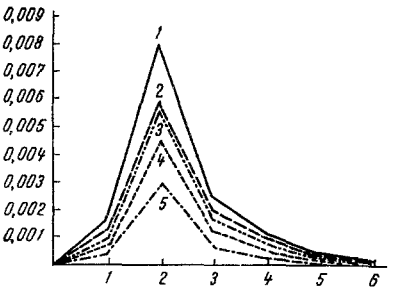

Fig. 2. Dynamics of lipase activity in toxin of Cl. botulinum: 1) strain B-175; 2) B-30; 3) A-98; 4) E-Nanaimo; 5) A-4/2.

the cell suspension and the supernatant of the culture (toxin) were used. Lipase activity was determined after incubation of the cell suspension or toxin for 1 h with olive oil at 45°C by Baker's method, and tweenase activity under the same conditions but with tweens as the substrates, using the same method to determine the fatty acids split off. Nitrogen in the samples was determined after mineralization with Nessler's reagent. The reaction mixture contained: 5 ml ammonia–ammonium buffer, pH 8.9, 2 ml 1.6% $CaCl_2$, 2 ml 2.4% solution of egg albumin, and 2 ml 1.6% solution of sodium oleate. The mixture was incubated with continuous stirring for 1 h at 37°C. A mixture with inactivated enzyme was used as the control. After the end of incubation 1 ml concentrated HCl and 20 ml benzene were added, the mixture was well stirred, then centrifuged for 15 min at 2500 rpm, 10 ml of the benzene phase was withdrawn and treated with 8 ml 5% copper acetate, and after stirring, photometry was carried out at 680 nm against a mixture of 10 ml benzene and 8 ml copper acetate. A calibration curve was plotted for oleic acid in benzene solution. The enzyme activity was expressed in milligrams of substrate decomposed per milligram total nitrogen of the enzyme preparation.

EXPERIMENTAL RESULTS

The lipase activity was found to be stronger in the toxin than in the cell suspension, and it was much higher in filtrates from cultures of toxigenic strains than in those from strains of low toxigenicity. Lipase activity began to appear in the filtrate during the first 2 h of growth of the cultures of both strongly and weakly toxigenic strains (Fig. 1). By these times the lipase activity in the filtrates and cell suspensions was about equal, but later the activity in the filtrate increased until 4 h, while in the bacterial cells at this time it fell considerably, to reach 20% of its initial activity after 6–8 h. Lipase activity also fell in the filtrates, but the rate of decrease was much less: 50% of the activity still remained after 6 h. Lipase activity in the toxin remained at this level, and then fell slightly until 18–20 h of growth of the culture; it was accordingly postulated that lipase is synthesized in the cell most intensively during the first hours of growth of the culture, but the rate of synthesis then falls off although the enzyme continues to leave the cell until the end of incubation. Activity of the enzyme decomposing tweens was detected in the bacterial cells and filtrates only after repeated subculture of the Cl. perfringens strains on media containing tweens. For instance, tweenases capable of hydrolyzing tween-40 and tween-65 appeared at the sixth subculture of the strains regardless of their toxigenicity after incubation for 4 h, and they remained until the eighth subculture, after which the tweenase activity declined. The total tweenase activity was higher in weakly toxigenic strains. Cultivation of the bacteria on media with tweens 20, 60, 80, and 85 did not induce tweenase formation.

Investigation of the properties of the lipase showed that the enzyme activity reached its maximum at pH 8.2 and 9.6, whereas tweenase activity was maximal at pH 8.2 and 9.2. The presence of two peaks of activity thus suggests the heterogeneity of these enzymes in Cl. perfringens. The temperature optimum for lipases was 45°C and for tweenases 40°C. At 65°C both enzymes completely lost their activity. Lipase was activated by ascorbic acid and cysteine, indicating that the enzyme contains SH-groups, and also by ions of the metals Fe, Mn, Co, Ca, Mg, and Na. Low concentrations of Hg ions activate while high concentrations inhibit lipase activity.

The toxigenic strains A-98, B-175, B-30/I, and F 470 and the weakly toxigenic strains A-4/2 and E-Nanaimo of Cl. botulinum were studied. The strains were kept on Tarozzi's medium and subcultured on casein-mushroom medium. The culture was grown for 6 days and enzyme activity determined every day in the cells and toxin as described above. Tweenase activity likewise could not be detected in Cl. botulinum and an attempt was made to induce tweenase formation by the method described above, but the subcultures were made every 2 days. An enzyme decomposing only tween-20 was discovered at the fifth subculture,

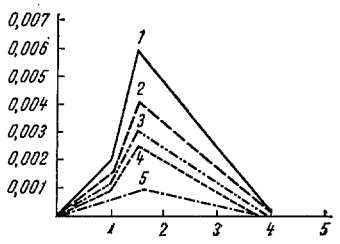

Fig. 3. Dynamics of lipase activity in bacterial cells of Cl. botulinum. Legend as in Fig. 2.

and its activity continued until the seventh subculture, after which it began to decline. The experiments showed that lipase activity began to appear on the 1st day of growth of the culture in both the cells and the toxin (Figs. 2 and 3), and reached a maximum in the 2nd day of growth. On the 3rd day the lipase activity began to decrease, and by the 6th day it had fallen to 10–15% of the maximum. Lipase activity was weaker in strains of low toxigenicity than in the more highly toxigenic strains, and it was stronger in the toxin than in the bacterial cells. Lipase activity of Cl. botulinum was maximal at pH 9.3 and 10.3. The optimal temperature was 45°C. The lipase was activated by ascorbic acid and by Mn, Mg, and Zn ions and it was inactivated by Fe and Hg ions. Co, Ca, and Na ions had no appreciable action.

The results suggest that the lipase synthesized in the bacterial cells is rapidly liberated from them and acts on the medium in accordance with the needs of the cell. In pathogenic strains of Cl. perfringens the high lipase activity could be one of the biochemical characteristics enabling the microorganism to exist in the lipid-rich tissues of the host. The presence of lipolytic enzymes in Cl. botulinum, which is not an organism of wound infections, nevertheless corresponds completely to the conditions of its existence as they have evolved, and to the production of toxin on meat, fish, and vegetable products rich in lipids.

LITERATURE CITED

1. C. Gonzalez and G. Sierra, Nature, 189, 601 (1961).