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STABILIZATION OF PHOSPHOLIPASE C FROM *Clostridium perfringens* BY CONJUGATION WITH A TRYPSIN INHIBITOR

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A new approach for the stabilization of phospholipase C by conjugation with a proteinase inhibitor is proposed. A heat-stable fraction obtained from a conjugate retained its initial activity for an hour at an incubation temperature of 50°C. An appropriate method of conjugation using an affinity adsorbent preventing the appearance of inactive forms of the conjugate has been developed.

The low stability of enzymes is the main disadvantage of their use in concrete processes. In particular, phospholipase C from *Clostridium perfringens*, especially a highly purified preparation, is extremely unstable and its activity falls both on storage and under conditions of use [1].

It is known that one of the main methods of stabilizing enzymes is the chemical modification of functional groups not involved in the active center. A special case of such modification is the immobilization of enzymes. A substantial contribution to the properties of the immobilized enzyme is also made by the surrounding groups of the support and the bonds formed by them.

There is extremely little information in the literature on the immobilization of phospholipase C [2], which is probably connected with the loss of activity by the enzyme in the immobilized state.

Our attempts to immobilize phospholipase C from *Clostridium perfringens* on a polyamide support were unsuccessful [1]. The enzyme immobilized by the formation of covalent bonds retained only 6-8% of its initial activity. The biospecific adsorption of phospholipase C on polycefamide (cephalin covalently bound to a polyamide through the amino groups with the aid of dialdehydes [3]) led to complete loss of activity. It is not excluded that in this case blocking of the active center by a ligand of the support (cephalin) took place.

Starting from the assumption that, after purification, phospholipase C loses the stabilizing action of a protein environment and has increased lability because of this, and also with the aim of protection from proteolysis, we decided to increase stability by conjugating the purified enzyme with a protein proteinase inhibitor.

The conjugation of phospholipase C with trypsin inhibitor was effected by covalent linkage through glutaraldehyde to the amino groups of the protein. To exclude the formation of nonspecific products and also to avoid partial inactivation of the already purified enzyme, conjugation was performed directly on the above-mentioned affinity sorbent. Phospholipase C was selectively adsorbed onto the affinity sorbent, and then the covalent binding of the enzyme with the trypsin inhibitor was conducted on the same sorbent, after which the finished conjugate was eluted:

$$PA-PEA + PhC \rightarrow PA-PEA-PhC$$

$$PA-PEA-PhC + GA + TI \rightarrow PA-PEA-PhC-GA-TI$$

$$PA-PEA-PhC-GA-TI \rightarrow PA-PEA + PhC-GA-TI,$$

where PA is polyamide, PEA phosphatidylethanolamine, PhC phospholipase C, GA glutaraldehyde, and TI trypsin inhibitor.

It can be seen from Fig. 1 that the ratios of phospholipase and trypsin-inhibiting activities in the various fractions of the eluted conjugate were different. The stabilities of the conjugate fractions also proved to be different. Thus, in the first combined fraction of the conjugate the thermal stability of the phospholipase had risen sharply in comparison with the native enzyme, as can be seen from Fig. 2. At 50°C the activity of the enzyme did not fall for 60 min, while in the first 15 minutes of incubation there was even some activation of the phospholipase.

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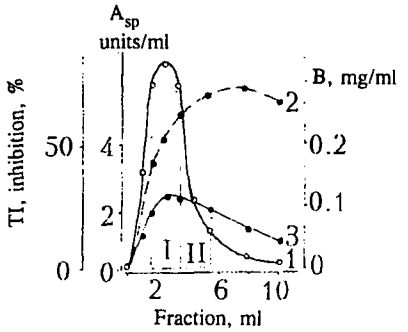

Fig. 1. Elution profile of a conjugate of phospholipase C with TI from polycefamide: *1*) phospholipase activity; *2*) trypsin-inhibiting activity; *3*) protein.

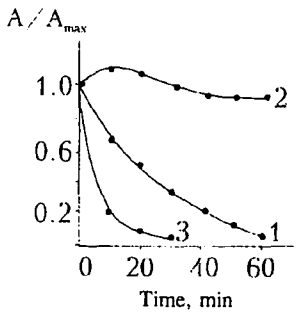

Fig. 2. Thermal stability of phospholipase C at 50°C: *1*) native enzyme; *2*) enzyme conjugated with TI, fraction I; *3*) enzyme conjugated with TI, fraction II.

Incubation of the second combined fraction of the conjugate at 50°C led to a rapid inactivation of the enzyme. In the latter variant, the thermal stability proved to be even lower than that of the native enzyme. The phenomenon observed is probably connected with a modification of the enzyme taking place on the formation of superfluous bonds with the inhibitor molecules, which could lead to a significant change in the conformational structure of the enzyme and to inactivation. Furthermore, the increase in the molecular mass of the conjugate due to an excess of inhibitor molecules may lead to thermal aggregation with loss of activity.

As the result of the conjugation of phospholipase C with trypsin inhibitor on polycefamide we obtained heat-stable and heat-labile fractions. The heat-stable fraction of the conjugate retained its activity at the initial level after incubation at 50°C for an hour although the native enzyme was practically inactivated in the same time and under the same conditions. As can be seen from Fig. 2, a considerable part of the total amount of active enzyme formed the heat-stable fraction that is of interest to us.

Thus, on the basis of the results of the investigations performed, it is possible to propose an unusual method of raising the thermal stability of phospholipase C by its conjugation with trypsin inhibitor. This simultaneously ensures antiprotease protection of the enzyme, which is extremely promising from the point of view of the practical use of phospholipase C.

EXPERIMENTAL

We used a partially purified fraction of isoenzyme 1 of phospholipase C (PhC) from *Clostridium perfringens*, strain BP6K N28, type A, obtained from the Scientific Research Institute of Vaccines and Sera (Moscow), and soybean trypsin inhibitor (Reanal).

Phospholipase activity was determined by potentiometric titration [4], the activity of the trypsin inhibitor was evaluated from the fall in the activity of trypsin determined by a modified Anson method [5], and protein was determined by the Lowry method and spectrophotometrically. An affinity sorbent on a polyamide base with immobilized phosphatidylethanolamine (polycefamide) was used.

For the adsorption of phospholipase C on polycefamide we incubated the polycefamide with a solution of the phospholipase C preparation in 0.1 M borate buffer, pH 8.0. The unbound protein was eliminated by washing the sorbent with the initial buffer, and then the calculated equimolar amounts of trypsin inhibitor and glutaraldehyde in the same buffer were added to the polycefamide–PhC. After incubation for 30 min, the sorbent was transferred to a column and washed with the initial buffer until the nonbound substances had been eliminated, and the conjugate was eluted with sodium deoxycholate under the conditions developed previously for the affinity chromatography of phospholipase C on polycefamide [1].

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