2. In several stages of the process the trimethylsilyl group has been used as temporary protection of the carboxyl of the amino component in the process of peptide synthesis.

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TRANSFERASE AND HYDROLASE ACTIVITIES OF PHOSPHOLIPASE D FROM THE ROOTS OF *Aconitum arcuatum*

Z. S. Evtushenko and T. V. Vlasenko

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The phospholipase D from *Aconitum arcuatum* has been studied. The optimum pH values have been determined: 6.3 for the hydrolase activity and 8.0 for the transferase activity. Calcium ions activate the phospholipase D: the hydrolase activity most strongly at 8 and 30 μmole and the transferase activity at 30 μmole. The hydrolase activity of the phospholipase D is not activated by low concentrations of sodium dodecyl sulfate, while the transferase activity is activated to a considerable degree. The results obtained are compared with those on the activation of cabbage phospholipase D.

Phospholipase D is widespread in higher plants [1-3]. The isolation and properties of this enzyme have been described in [4-7]. Its most interesting feature is its manifestation of two activities: hydrolase and transferase activities [8-11]. When phospholipase D was isolated from cabbage [5] and from peanuts [6], it was established that the ratio of hydrolase and transferase activities did not change during the purification process and the two activities require the same conditions for optimum catalysis. At the present time, it is assumed that both activities belong to a single enzyme [12], although Saito et al. [13] have shown that the maximum hydrolase and transferase activities of the phospholipase D from cabbage are shown at different optimum pH values and are activated by different concentrations of Ca^{2+}.

We have performed the partial purification of phospholipase D from the roots of *Aconitum arcuatum* Maxim., in which one of the highest hydrolase activities has previously been detected [2] and have described its properties. We have determined the transferase and hydrolase activities of the phospholipase D in the pH range of 5.0-9.0 and have studied the influence of various concentrations of Ca^{2+} and of sodium dodecyl sulfate on these activities. For comparison, we determined the two activities of cabbage phospholipase D under the same conditions. Figure 1A shows the dependence of the transferase and hydrolase activities of the aconitum and cabbage phospholipases D on the pH of the medium.

The hydrolase activities of the enzymes from the two sources are highest in the same pH region — 6.3 for the aconitum enzyme and 5.8 for the cabbage enzyme. It is known that the optimum hydrolase activities of preparations of phospholipase D from various sources are found at pH values of 5.5 to 6.0 [14, 16]. In our experiments, the transferase activities of the enzymes differed greatly according to their source: the preparation from cabbage possessed its maximum activity at pH 5.8 and that from the aconite at pH 8.0. In the paper of Yang et al. [4], the optimum pH was the same for the two activities of the cabbage phospholipase D, amounting to 5.5-6.0. According to the results of the investigations of Saito et al. [13], the optima of the two activities of the enzyme from the cabbage differed sharply: for the

Institute of Molecular Biology, Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostok. Far Eastern State University, Vladivostok.

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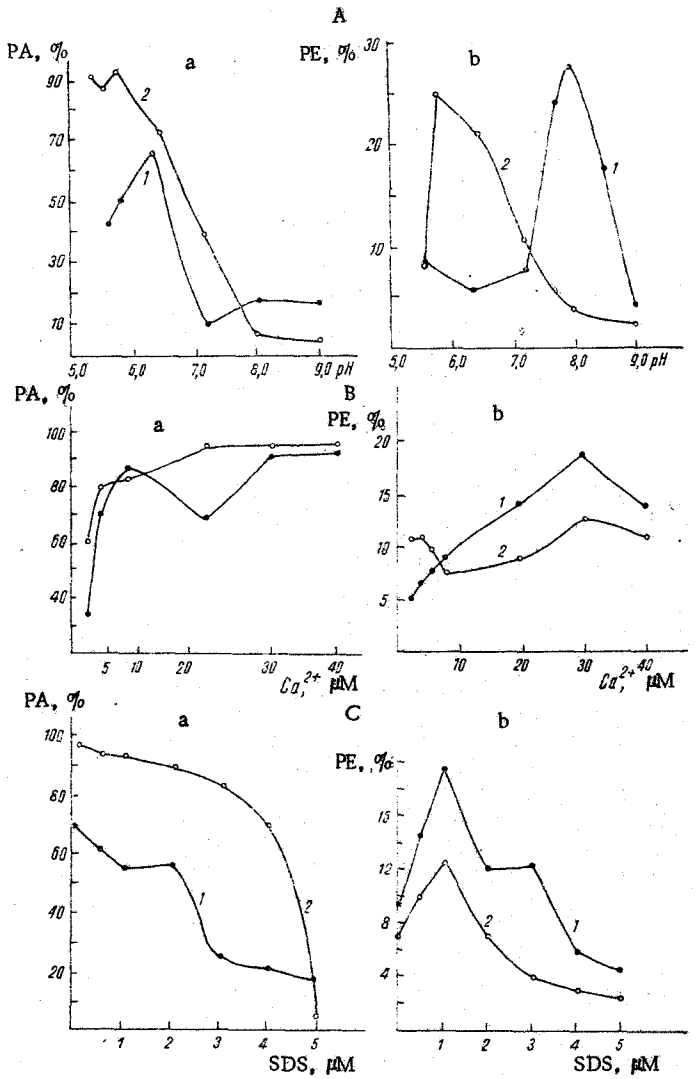

Fig. 1. Dependence of the hydrolase (a) and transferase (b) activities of phospholipases D on the pH (A), on the concentration of calcium (B), and on the concentration of sodium dodecyl sulfate (C): 1) phospholipase D from *A. arcuatum*; 2) phospholipase D from cabbage; PA — phosphatidic acid; PE — phosphatidylethanolamine; SDS — sodium dodecyl sulfate.

hydrolase activity pH 5.6 and for the transferase activity pH 9.0. In our work, the optima of the hydrolase and transferase activities of the phospholipase D from the cabbage proved to be the same and the optima of the activities of the enzyme from the aconitum differed greatly: 6.3 for the hydrolase and 8.0 for the transferase activities.

Our results on the preparation from the cabbage agree with the generally accepted opinion that the two activities require the same conditions for optimum catalysis, but the results that we obtained with the preparation from aconitum and the facts reported by Saito et al. [13] throw doubt on the conclusion that the conditions for the manifestation of the hydrolase and transferase activities of phospholipases D from different sources are identical. The isolation of the enzyme from other sources and the examination of the optimal conditions for the two activities will permit this question to be answered. The influences of the concentration of Ca^{2+} on the two activities of the preparation from aconitum and cabbage are shown in Fig. 1B. The influences of Ca^{2+} on the activity of the enzymes from the two sources differ. The hydrolase activity of the aconitum phospholipase D rises sharply with the addition of small amounts of Ca^{2+}, gives a considerable jump at 20 μM and issues onto a plateau at 30 and 40

μM Ca^{2+} in the incubation medium. The enzyme from the cabbage is also activated by Ca^{2+}, reaching a maximum and issuing onto a plateau at 20 μM. The transferase activity of the aconitum phospholipase D increases only slightly at low concentrations of Ca^{2+}, reaching a maximum at 30 μM while the same activity of the cabbage enzyme scarcely changes on the addition of Ca^{2+}, giving a slight rise in the rate of the reaction at 4 and 30 μM.

The results that we have obtained on the influences of Ca^{2+} on the two activities of the enzymes of the cabbage and the aconitum show different relationships of the phospholipases to the action of an activator and are not in harmony with the results of Yang et al. [4], indicating the same influence of Ca^{2+} on the two activities. Stanecev et al., [17] in the preparation of a cardiolipid from a phosphatidylglycerol by the transferase reaction of cabbage phospholipase D reported an inhibiting action of Ca^{2+} on this reaction. Saito et al. [13] give different optimum concentrations of Ca^{2+} for the two activities: 4 μM for the transferase activity with a subsequent jump in the rate of the reaction, and 28 μM for the hydrolase activity with issuance onto a plateau.

A widely used activator of phospholipase D is sodium dodecyl sulfate [15, 18]. Figure 1C gives the curves of the dependence of the enzyme activities of the phospholipase D preparations on the concentration of sodium dodecyl sulfate. In our experiments, the hydrolase activities of the phospholipases D from aconitum and cabbage decreased with the addition of 1-5 μM of the detergent. The transferase activity of the enzyme increased on the addition of 1 μM of sodium dodecyl sulfate and fell with a further increase in the concentration of detergent. This applies to the enzymes from the aconitum and from the cabbage. The absence of an activation of the hydrolase activity by low concentrations of sodium dodecyl sulfate in our experiments can obviously be explained by the state of the substrate: a high degree of dispersity of [^{14}C]lecithin obtained after considerable sonication treatment of the substrate.

Our results are an indication of different characteristics of the hydrolase and transferase activities of the phospholipases D from cabbage and aconite. These results do not show that the two activities are catalyzed by different enzyme proteins or complexes; this conclusion can be drawn only if individual proteins are isolated.

EXPERIMENTAL

As the substrate we used 1-acyl-2-[^{14}C]oleoylglycerophosphocholine obtained biosynthetically by the method of Robertson and Lands [19]. The labeled lecithin was purified on a column of silica gel and the [^{14}C]lecithin (5 mg/ml, $3.15 \cdot 10^5$ counts/min per mole of lipid) was dispersed in the appropriate buffer on a UZDN-1 ultrasonic generator at 44 kHz for 10 min. Phospholipase D was isolated from the roots of *A. arcuatum* collected in the flowering period in September. It was purified by the method of M. M. Rakhimov et al. [15]. The dried powder was stored at −20°C. A sample of the powder (30 mg/ml) was dissolved in 0.05 M sodium acetate buffer, pH 5.6, the undissolved matter being eliminated by centrifugation (6000 × g, 15 min). The supernatant was used as the solution of phospholipase D for the experiment. The enzyme from the cabbage was obtained by a similar method.

Determination of Enzymatic Activity. In the determination of hydrolase activity, the incubation mixture contained 20 μl of a dispersion of [^{14}C]lecithin in the appropriate buffer at a given pH, 20 μl of the enzyme solution, 40 μl of 0.02 M $CaCl_2$, and 20 μl of diethyl ether. In the determination of the transferase activity of phospholipase D, 1.5% of ethanolamine (on the total volume of the mixture) was added to the incubation mixture. The time of incubation was 30 min in the determination of the hydrolase activity and 15 min in the determination of the transferase activity. Enzymatic hydrolysis was carried out with constant shaking on a 326 M shaker at room temperature. In the investigation of the influence of sodium dodecyl sulfate, the diethyl ether was replaced by sodium dodecyl sulfate in amounts of 1, 2, 3, 4, and 5 μmole in a constant volume of 20 μl. In the investigation of the influence of calcium ions, 2, 4, 8, 20, 30, and 40 μmole of calcium in a constant volume of 40 μl was added to the reaction mixture. The optimum pH was determined in 0.05 M sodium acetate buffer, pH 5.4-5.8, in 0.05 M phosphate buffer, pH 6.5-7.2, and in 0.05 M Tris-HCl buffer, pH 8.0 and 9.0.

The hydrolase activities of the phospholipases D were determined from the amount of [^{14}C]phosphatidic acid liberated in per cent of the total radioactivity of the [^{14}C]lecithin added to the incubation mixture. The reaction was stopped by the addition of 0.05 ml of

chloroform–methanol (2:1), and after vigorous shaking the mixture was centrifuged at 5000 rpm for 5 min. The lower layer was taken off and the lipids were separated off by microthin-layer chromatography with the deposition of 10 μl of solution at each point. The chloroform–methanol–28% aqueous ammonia (65:35:5) system was used for the separations. The lipids were detected in iodine vapor. The zones of the silica gel containing the phosphatidic acid, lecithin, and phosphatidylethanolamine were transferred to bottles and the radioactivities were measured on an Intertechnique SL-30 scintillation spectrometer with 1.0 ml of scintillation solution. The reagents Tris and sodium dodecyl sulfate from Reanal were used, the others being of kh.ch. ["chemically pure"] or ch.d.a. ["pure for analysis"] grades.

CONCLUSION

The dependences of the transferase and hydrolase activities of the phospholipase D from the root of *Aconitum arcuatum* on the pH and the concentrations of calcium and of sodium dodecyl sulfate have been studied. Different optimum pH values and also different effects of Ca^{2+} and of sodium dodecyl sulfate on the activities of the phospholipase D have been shown. The hydrolase and transferase activities of the aconitum phospholipase D have been shown to have different characteristics.

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