ANALYTICAL BIOCHEMISTRY 118, 262-268 (1981)

Hemolytic Assay for Venom Phospholipase A₂^{1,2}

Carl-Wilhelm Vogel,^{*,3} Andreas Plückthun,^{†,4} Hans J. Müller-Eberhard,^{*,5} and Edward A. Dennis[†]

*Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037, and †Department of Chemistry, University of California at San Diego, La Jolla, California 92093

Received July 7, 1981

A rapid and sensitive spectrophotometric assay for venom phospholipase A_2 based on the hemolysis of guinea pig erythrocytes in the presence of decomplemented serum and cardiotoxin (direct lytic factor) is described. This assay is particularly useful for rapid multisample analyses, such as those used in monitoring chromatography fractions, and is specific for phospholipase A_2 in the presence of other potentially hemolytic venom components. The hemolytic mechanism is shown to be a combination of the action of lysophospholipids liberated from lipoproteins in the serum and the synergistic action of phospholipase A_2 and cardiotoxin on the erythrocyte membrane.

Assay procedures for phospholipase A_2 (EC 3.1.1.4) have been described (1-3), and the most commonly used procedures involve either the pH-stat or the thin-layer chromatographic separation of radiolabeled products. While each assay has some specific advantage and application, all are either laborious, nonspecific, or require specialized equipment. Thus, there is still a need for a specific and sensitive assay that is simple enough to allow rapid detection and quantification of phospholipase A_2 . We now wish to report a hemolytic assay for venom phospholipase A_2 that is especially useful when many samples have to be analyzed, as in the case of chromatography fractions.

EXPERIMENTAL PROCEDURE

Materials. Lyophilized cobra venom was obtained from the Miami Serpentarium (Naja naja naja, Lot No. NNP9STLZ) and from Sigma (Naja naja kaouthia, Lot No. 27C-0249). Phospholipase A₂ samples were obtained from Millipore (Crotalus adamanteus, Lot No. 59J402P), Calbiochem-Behring (bee venom, Lot No. 902118), and Sigma (porcine pancreas, Lot No. 128C-0252). Cardiotoxin (direct lytic factor⁶) (Naja naja, Lot No. 803031) was obtained from Calbiochem-Behring. Guinea pig serum was obtained from Pel-Freez Biologicals. Gelatin was purchased from Difco, bovine serum albumin and 5,5-diethylbarbituric acid from Sigma, DEAE- Sephacel from Pharmacia, and Triton X-100 from Rohm and Haas.

¹ Publication 2417 from the Research Institute of Scripps Clinic.

² This investigation was supported by U. S. Public Health Service Grants AI 07007, HL 16411, and GM 20501 and National Science Foundation Grant PCM 79-22839.

³ Recipient of a Fellowship from Studienstiftung des Deutschen Volkes and subsequently from Deutsche Forschungsgemeinschaft (No. Vo 303/1).

⁴ Recipient of a Fellowship from the Fulbright Commission and ITT and subsequently from Studienstiftung des Deutschen Volkes.

⁵ Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic.

Hemolytic assay for phospholipase A_2 . Into a small test tube were placed 20 μ l of

⁶ Cardiotoxin has also been referred to as "direct lytic factor" and by a variety of other names (4).



HEMOLYTIC ASSAY FOR PHOSPHOLIPASE A₂ 263

decomplemented serum reagent (DSR), 7 5 μ l of cardiotoxin (2.5 mg/ml) in isotonic veronal buffer (3.5 mm 5,5-diethylbarbituric acid, 143 mM NaCl, pH 7.4), 20 μ l of the phospholipase-containing sample, and last 20 μ l of a suspension of guinea pig erythrocytes (5 \times 10⁸ cells/ml). The test tubes were kept on ice. After vortexing, the samples were incubated in a water bath at 37°C for 10 min. (The incubation should be terminated sooner if inspection of the peak tubes reveals a high degree of hemolysis.) Then the rack was transferred into an ice bath, and 1 ml of cold veronal buffer was added to each tube. After vortexing, the tubes were centrifuged at 1000g for 2 min in a table-top centrifuge, and the supernatant was removed to measure the absorbance of the released hemoglobin spectrophotometrically at 412 nm. Control values without added enzyme were subtracted from the absorbance readings. Variations in the background hemolysis due to osmotic or pH differences of the samples are negligible if the erythrocyte suspension is added to the reaction mixture last. For quantitative determinations, standards of purified cobra venom phospholipase A_2 (*Naja naja naja*) of known concentration were analyzed simultaneously. The maximal amount of hemolysis possible in a sample (total hemolysis) was determined by substituting the DSR, cardiotoxin, and phospholipase sample with 45 μ l of distilled water, which completely lyses the cells instantaneously. Preparation of decomplemented serum reagent (DSR) (5). Guinea pig serum was incubated with an equal volume of cold 1 M KSCN for 16 to 18 h at 4°C. Then hydrazine was added to give a final concentration of 15 mm. The mixture was incubated at 37°C for 45 min and extensively dialyzed against veronal buffer containing 0.5 mM MgCl₂ and 0.15 mM CaCl_2 . This treatment results in a half-diluted serum lacking the functional

complement components C3, C4, and C5. The DSR was stored in aliquots at -70° C and can be kept for at least 1 year.

Preparation of guinea pig erythrocytes. Guinea pig erythrocytes were obtained by bleeding guinea pigs after cardiac puncture into an EDTA solution to prevent clotting. The cells were then washed three times with veronal buffer containing 0.5 mM MgCl₂, 0.15 mM CaCl₂, and 0.1% gelatin (w/v) and were adjusted spectrophotometrically to approximately 5×10^8 cells/ml, which corresponds to an absorbance of about 1.3 for 20 μ l of cells in 1 ml distilled water. The cell suspension was kept at 4°C and can be stored for several days. Other methods. The pH-stat assay for phospholipase A_2 was performed on egg phosphatidylcholine/Triton X-100 mixed micelles as described elsewhere (6). Phospholipase A₂ was purified from lyophilized cobra venom (Naja naja naja) as described previously (6,7). Lipoproteins (very low density, low density, and high density) and lipoprotein-depleted serum were prepared as described elsewhere (8).

RESULTS AND DISCUSSION

Standard hemolysis assay. The absorbance at 412 nm as a function of the amount of highly purified phospholipase A₂ from Naja naja naja venom is shown in Fig. 1. The curve is nonlinear and exhibits a sigmoidal shape characteristic of hemolytic assays. The data can also be plotted in linear form as shown in the insert. For multiple determinations of a sample (50 ng phospholipase A₂), the absorbance was 0.745 ± 0.016 (mean \pm standard deviation, N = 10), while the absorbance of the background control was 0.112 ± 0.010 (mean \pm standard deviation, N = 10). As little as 10-20 ng of enzyme can be easily detected. This sensitivity renders the hemolytic assay at least as sensitive as the pH-stat assay and the majority of other assays. Only assays involving the separation of radiolabeled products are

⁷ Abbreviation used: DSR, decomplemented serum reagent.

264

VOGEL ET AL.



FIG. 1. Dependence of the absorbance at 412 nm on the amount of phospholipase A_2 purified from cobra venom (*Naja naja naja*). Samples were incubated for 10 min under standard assay conditions, and the averages of duplicate determinations are shown. The insert shows a plot of the data as log $[A/(A_{x}-A)]$ versus log [phospholipase A_2], where A is the absorbance at 412 nm and A_{∞} is the absorbance at 412 nm for total hemolysis determined as described under Experimental Procedure.

more sensitive. Because of the reproducibility of this assay with the same batch of erythrocytes, single-sample analysis is sufficient for semiquantitative purposes, as in monitoring chromatography fractions.

The time course of hemolysis for a fixed amount of purified phospholipase A_2 is shown in Fig. 2. The optimum incubation time is dependent on the amount of phospholipase A_2 present in the samples, but the sensitivity of the assay and the length of the incubation time are limited by the continuously increasing background. Since in blanks about 15% of the red cells present in the assay are lysed after 20 min, longer incubation times are generally not useful. There is a small variation in standard curves obtained with different batches of DSR or fresh erythrocytes. If the erythrocyte suspension is aged for several days, the red cells become more susceptible to phospholipase A_2 . However, these cells can still be used for the assay. Consequently, the absorbance is only a semiquantitative unit for the phospholipase A₂ present in a sample, but the hemolytic test allows the quantitation of phospholipase A_2 in absolute units if standards of purified phospholipase A₂ are included. Guinea pig erythrocytes were employed because of their known susceptibility to lysis (9,10). In contrast, we found human erythrocytes resistant to lysis in this system. Application to venom chromatography. The elution profile of crude venom from Naja naja kaouthia after chromatography on DEAE-Sephacel is shown in Fig. 3. Because of its high activity, a very short in-





FIG. 2. Time course of hemolysis. Separate samples for each time point were incubated under standard assay conditions in the absence (O) and presence (\bullet) of 50 ng of purified cobra venom phospholipase A₂. The back-ground corrected time course (\blacksquare) is also shown.

cubation time was employed, although the samples could have been further diluted instead. Every other fraction was assayed for phospholipase A₂ activity using both the hemolytic assay and the pH-stat technique. Both methods gave an identical pattern of phospholipase A_2 distribution and relative activities in all fractions, indicating similar specificity for both assays. The shapes of the activity peaks obtained with both assays need not be identical since the hemolytic assay is not linear with phospholipase concentration (see Fig. 1), whereas the pH-stat assay is. While the testing of column fractions took about 6 h using the pH-stat technique, the hemolytic analysis was performed in less than 1 h. Application to phospholipase A_2 from other sources. The assay system was worked out with highly purified phospholipase A₂ from Naja naja naja venom. To demonstrate the applicability of the hemolytic assay to phospholipase A_2 from different sources, phospholipases A₂ from Crotalus adamanteus venom, bee venom, and porcine pancreas were also tested. The phospholipase A₂ from Crotalus adamanteus reportedly does

not hydrolyze phospholipids in intact erythrocytes (11), and an especially interesting difference from cobra venom is the absence of any cardiotoxinlike activity in the venom of Crotalus adamanteus (4,12). Bee venom and mammalian pancreas were chosen because they represent major sources of phospholipase A_2 other than snake venoms (2). All three enzymes could be detected with the hemolytic assay. The sensitivity was comparable to that found for the Naja naja naja enzyme. In addition, the ratios of hemolytic activities of all four enzymes were similar to the ratios of esterolytic activities in the pH-stat assay. Therefore, the hemolytic assay appears to be applicable in general to phospholipases A_2 secreted by exocrine glands. For any particular enzyme, however, additional testing would be necessary. Intracellular phospholipases A₂ from mammalian tissues as well as from serum exhibit very low activities (2,13) and can only be detected with the radioactive assay. Specificity of the assay. Phospholipase A₂ was discovered in 1903 by its ability to induce lysis of erythrocytes (14), and hemolysis was subsequently developed as an assay



FIG. 3. Chromatography of crude cobra venom (*Naja naja kaouthia*) on DEAE-Sephacel. Lyophilized venom (50 mg) was dissolved in 1 ml of 10 mM Tris-HCl buffer, pH 7.8. Insoluble material was removed by centrifugation. The supernatant was applied to a DEAE-Sephacel column (0.9×15 cm), equilibrated with the same buffer at 4°C. Protein was eluted with 70 ml of the same buffer, followed by a linear NaCl gradient in the same buffer (400 ml, 0-0.4 M NaCl). Fractions (2 ml each) were collected and analyzed for protein by absorbance at 280 nm (\longrightarrow) and phospholipase activity by both the hemolysis (1:5 dilution, 0.5-min incubation) (\bullet) and the pH-stat (\triangle) assay. The conductivity is indicated (--).

principle for phospholipase A_2 (15–18). However, hemolytic assays for phospholipase A₂ were only rarely used. Some are very laborious (15,17), but the major disadvantage of previous assays is the intrinsic lack of specificity of hemolysis. Therefore, all of these assays, including those that are rapid and, in performance, most comparable to the present assay, have usually been employed only as a probe for the lytic activity of the enzyme under study or even for its absence (15-20). Lysis of erythrocytes can be achieved by lysophospholipids generated from exogenous phospholipids by phospholipase A₂, by the direct action of phospholipase A₂ on the phospholipids of the erythrocyte membrane (10,21), by cardiotoxin and similar basic peptides like melittin (22-24), by the synergistic action of phospholipase A_2 and cardiotoxin (22,25,26), and by complement activators such as cobra venom factor (27-29). Since these additional lytic agents are present in snake and bee venoms and are extremely difficult to separate from phospholipase A_2 (18,30,31), they can interfere with hemolytic determinations of phospholipase A₂. Only in recent years have the actions of these agents become well enough understood to make it possible to take the presence of these lytic agents into consideration and to develop a hemolytic assay specific for phospholipase A_2 . In the assay system employed herein, hemolysis due to the presence of cardiotoxin or other lytic peptides from animal venoms in the sample does not interfere with the phospholipase A_2 determinations because the assay system contains cardiotoxin in a large excess. Thus, hemolysis becomes a function of phospholipase A₂ concentration only. Any complement-induced hemolysis in the presence of activators of the complement system, including the specific anticomplementary proteins present in snake venoms, is excluded because DSR, a serum lacking a functional complement system, is used.

ferentiate between phospholipase A_2 and A_1 (1). Phospholipase A_1 has been detected predominantly in microorganisms and, intracellularly, in mammalian tissues. These enzymes exhibit low activities as compared to the venom phospholipase A_2 and are difficult to isolate and purify (2,13). While we cannot rule out that phospholipase A_1 might cause hemolysis in the present assay, the presence of phospholipase A_1 in animal venoms has not been reported, and its absence in the secreted products of exocrine glands is generally accepted (2,4,32). Therefore, the hemolytic test can be considered specific for phospholipase A₂ with respect to all venom components. Mechanism of lysis. To elucidate the mechanism of lysis in the present hemolytic test system, we carried out additional experiments. Omitting cardiotoxin from the test system abrogates phospholipase-induced hemolysis, indicating that the lysis of erythrocytes is not accomplished by the action of phospholipase alone on membrane phospholipids or lipoprotein phospholipids. When DSR was substituted with veronal buffer (containing $MgCl_2$, $CaCl_2$, and gelatin as described), hemolysis was still observed in the presence of phospholipase A_2 . This test system was less sensitive, however, and exhibited a rather sudden increase of hemolysis with increasing phospholipase A₂ concentration. These differences showed that hemolysis in our test is not only a result of the synergistic action of phospholipase and cardiotoxin on the erythrocytes but that an additional factor present in DSR is also required. This factor is lipoprotein, because DSR could be substituted by a solution of lipoproteins of physiological concentration and composition, resulting in a normal test sensitivity and dependence of hemolysis on phospholipase A_2 concentration. These results taken together indicate that the mechanism of lysis in the assay described is a combination of (a) lysophospholipids liberated from lipoproteins by the phospholipase A₂ and (b) the synergistic action of phos-

The majority of assays for phospholipase A (including the pH-stat assay) cannot dif-

pholipase A₂ and cardiotoxin on the erythrocyte membrane. This is consistent with the finding that hemolysis was inhibited when, instead of the normal 0.1 mM CaCl₂, a higher concentration (10 mm) was present, a concentration that is optimal for the action of phospholipase A_2 (33,34) but known to inhibit the synergistic action of phospholipase A_2 and cardiotoxin (25). The presence of 10 mm EDTA in the diluting buffer after completion of the incubation had no effect on the degree of lysis, regardless of whether the high or low concentration of CaCl₂ was used. This indicates that Ca²⁺ did not inhibit the rupture of erythrocytes in our system, whereas this was found for the action of phospholipase A_2 alone (33). Because albumin is present in DSR and is known to influence the action of phospholipases toward lipoproteins (35) and erythrocytes (33,36), we checked its effect on the hemolysis in our test system. While substitution of DSR with veronal buffer still resulted in some hemolysis (see above), an albumin solution (2 mg/ml in veronal buffer)or lipoprotein-depleted serum totally suppressed red cell lysis. Although it is known that albumin enhances hemolysis caused by phospholipase A₂ alone, probably due to liberating sequestered lysophospholipids and fatty acids from the erythrocyte membrane (33,36), our results demonstrate that the synergistic action of phospholipase and cardiotoxin is inhibited by albumin. This inhibitory effect is somehow compensated for by lipoprotein since hemolysis does occur with DSR, which contains albumin in half-physiological concentration. Inhibition of venominduced hemolysis by plasma (37) and albumin (38) has been reported, but the functions of albumin in our system seem to be complex and remain unsolved. Albumin might prevent the cardiotoxin from binding to the erythrocyte surface, either by a direct interaction or by coating the surface itself, since it has been observed that preincubation with albumin can protect the red cell against hemolysis caused by the action of phospholipase A_2 alone (33). In the presence of lipoprotein, the reaction products (fatty acids and lysophospholipids) appear to be generated immediately and these products are known to bind to albumin (35). It is therefore possible that albumin, when saturated with these reaction products, loses its capacity to interact with either the red cell membrane or the cardiotoxin. Consequently, both cardiotoxin and lipoproteins appear to be needed for lysis in the presence of albumin.

ACKNOWLEDGMENT

The authors are indebted to Dr. D. B. Weinstein, Division of Metabolic Disease, Department of Medicine, University of California at San Diego, for a gift of isolated lipoproteins and lipoprotein-depleted serum.

REFERENCES

- 1. Grossman, S., Oestreicher, G., and Singer, T. P. (1974) Meth. Biochem. Anal. 22, 177-204.
- 2. Brockerhoff, H., and Jensen, R. G. (1974) Lipolytic Enzymes, Academic Press, New York.
- 3. van den Bosch, H., and Aarsman, A. J. (1979) Agents and Actions 9, 382-389.
- Condrea, E. (1979) in Handbook of Experimental Pharmacology, Vol. 52, Snake Venoms (Lee, C. Y., ed.), pp. 448–479, Springer-Verlag, Berlin.
- Cooper, N. R., and Müller-Eberhard, H. J. (1970) J. Exp. Med. 132, 775-793.
- Deems, R. A., and Dennis, E. A. (1981) in Methods in Enzymology (Lowenstein, J. M., ed.), Vol. 71, 703-710.
- Darke, P. L., Jarvis, A. A., Deems, R. A., and Dennis, E. A. (1980) *Biochim. Biophys. Acta* 626, 154-161.
- 8. Weinstein, D. B., Carew, T. E., and Steinberg, D. (1976) *Biochim. Biophys. Acta* 424, 404-421.
- 9. Condrea, E., Mammon, Z., Aloof, S., and DeVries, A. (1964) *Biochim. Biophys. Acta* 84, 365-375.
- Martin, J. K., Luthra, M. G., Wells, M. A., Watts, R. P., and Hanahan, D. J. (1975) *Biochemistry* 14, 5400-5408.
- Zwaal, R. F. A., Roelofsen, B., Comfurius, P., and van Deenen, L. L. M. (1975) *Biochim. Biophys.* Acta 406, 83-96.
- Karlsson, E. (1979) in Handbook of Experimental Pharmacology, Vol. 52, Snake Venoms (Lee, C. Y., ed.), pp. 159-212, Springer-Verlag, Berlin.

- 13. van den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191-246.
- 14. Kyes, P. (1903) Berl. Klin. Wochenschr. 40, 956-959.
- 15. Neumann, W., and Habermann, E. (1954) Hoppe-Seyler's Z. Physiol. Chem. 296, 166-179.
- 16. Boman, H. G., and Kaletta, U. (1957) Biochim. Biophys. Acta 24, 619-631.
- 17. Bernard, M. D., and Denis, F. (1969) C. R. Soc. Biol. 163, 1926-1928.
- 18. Lachmann, P. J., Halbwachs, L., Gewurz, A., and Gewurz, H. (1976) *Immunology* 31, 961-968.
- 19. Wahlström, A. (1971) Toxicon 9, 45-56.
- Branganca, B. M., Sambray, Y. M., and Ghadially, R. C. (1969) *Toxicon* 7, 151–157.
- 21. Lankisch, P. G., and Vogt, W. (1971) Biochim. Biophys. Acta 270, 241-247.

- Pickering, R. J., Wolfson, M. R., Good, R. A., and Gewurz, H. (1969) Proc. Nat. Acad. Sci. USA 62, 521-527.
- 28. Ballow, M., and Cochrane, C. G. (1969) J. Immunol. 103, 944-952.
- 29. Minta, J. O., and Man, D. (1980) Immunology 39, 503-509.
- 30. Visser, L., and Louw, A. I. (1977) Biochim. Biophys. Acta 491, 349-353.
- 31. Mollay, C., Kriel, G., and Berger, H. (1976) Biochim. Biophys. Acta 426, 317-324.
- 32. Iwanaga, S., and Suzuki, T. (1979) in Handbook of Experimental Pharmacology, Vol. 52, Snake Venoms (Lee, C. Y., ed.), pp. 101–158, Springer-Verlag, Berlin.
- 33. Gul, S., and Smith, A. D. (1974) Biochim. Biophys. Acta 367, 271-281.

- 22. Condrea, E., DeVries, A., and Mager, J. (1964) Biochim. Biophys. Acta 84, 60-73.
- 23. Louw, A. I., and Visser, L. (1977) Biochim. Biophys. Acta 498, 143-153.
- 24. Habermann, E. (1972) Science 177, 314-322.
- Louw, A. I., and Visser, L. (1978) Biochim. Biophys. Acta 512, 163-171.
- 26. Yukelson, L. Y., Sadykov, E., Sakhibov, D. N., and Sorokin, V. M. (1975) *Biokhimiya* 40, 698-702.
- 34. Salach, J. I., Seng, R., Tisdale, H., and Singer, T. P. (1971) J. Biol. Chem. 246, 340-347.
- 35. Aggerbeck, L. P., Kezdy, F. J., and Scanu, A. M. (1976) J. Biol. Chem. 250, 3823-3830.
- 36. Drainas, D., and Lawrence, A. J. (1980) FEBS Lett. 114, 93-97.
- 37. Ibrahm, S. A., and Thompson, R. H. S. (1965) Biochim. Biophys. Acta 99, 331-341.
- 38. Kurup, P. A. (1965) Naturwissenschaften 52, 478.

ž.

922