Hydrolysis of Tosylarginine Methyl Ester by Russell's Viper Venom

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Many preparations of proteolytic enzymes have been found to accelerate the clotting of plasma. In partially purified systems some of these preparations have been reported to be capable of aiding in the conversion of prothrombin to thrombin; others have been shown to coagulate fibrinogen. One property that these enzyme preparations have in common is the ability to hydrolyze benzoyl- or tosylarginine esters and presumably certain arginyl peptide bonds. Among the enzymes included in this group are: trypsin (1, 2, 3), papain (4, 5), ficin (6, 7), B. jararaca venom (8), and thrombin (9).

In this paper, the ability of another clot-accelerating material, Russell's viper venom (RVV), to hydrolyze tosylarginine methyl ester (TAME) is reported. Some of the properties of the TAME splitting factor in RVV are also reported. The action of RVV on TAME and in accelerating the coagulation of plasma is compared with the action of trypsin.

Materials and Methods

Materials. — (1) p-Toluenesulfonyl-L-arginine methyl ester hydrochloride was obtained from H. M. Chemical Co. Ltd. It was made up fresh for each experiment by dissolving the powder in distilled water at 28° C just before use.

(2) Russell's viper venom was "Stypven" from the Burroughs Wellcome and Co. supplied in 0.5 mg lyophilized lots. It was prepared for use by diluting to the desired protein concentration with isotonic saline.

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(3) Trypsin was a crystalline preparation, salt-free and lyophilized from Mann Research Laboratories. Solutions of the desired protein concentration were made by adding 0.001 N HCl.

(4) Canine plasma was used for all clotting experiments. It was prepared by drawing 9 ml of blood from the external jugular vein of a mongrel dog into 1 ml of 3.2% citrate. The blood was centrifuged for 10 minutes and the plasma was drawn off.

(5) Formaldehyde (NF) was a 37% solution adjusted to pH 8.0—8.5 with 1 N NaOH.

(6) Tris(hydroxymethyl)aminomethane was Reagent Grade from Fisher Scientific Company. The buffer was prepared by addition of distilled water and 0.1 N HCl to the desired pH.

(7) Phenol red was used as a 0.01% solution in absolute ethyl alcohol.

(8) Cephalin used was a crude dog brain cephalin obtained by a previously described method (10). A 5 mg % suspension was prepared in isotonic saline.

(9) Calcium-imidazole-saline was prepared according to a method described earlier (10).

Methods. — TAME Esterase Assay: The test was a modification of that described by Troll and Sherry (9) in which the carboxyl group liberated by the split at the ester link is titrated. The test consisted of the addition of the following to a 12 × 120 mm test tube: a) 1.0 ml of isotonic saline; b) 0.5 ml of 0.375 Tris buffer pH 9; c) 0.5 ml of TAME, 0.1 M except in experiments for Figure 2; d) 0.5 ml of the enzyme preparation to be tested. The incubations of this test system were carried out in a 37° C water bath. Five-tenths ml of the final incubation mixture was added to 0.5 ml of formaldehyde in a 5 ml pH cup. The mixture was titrated with 0.05 N NaOH from a syringe type micro-burette with 0.02 ml phenol red indicator. Careful manual mixing was used. The control for spontaneous TAME hydrolysis was incubation of the complete test system except for enzyme, with the addition of the enzyme at the time of titration. The control was subtracted from the test titration to obtain the value for enzymatic hydrolysis.

Plasma Clotting Test: The test was run at 28° C. The following materials were added to a 10 × 75 mm test tube: a) 0.1 ml of calcium-imidazole-saline solution; b) 0.1 ml of a 5 mg % cephalin suspension; c) 0.1 ml of the enzyme preparation to be tested; d) 0.1 ml of citrated plasma. The reaction was timed from the addition of the plasma; the end point was the first appearance of a clot. Neither RVV nor trypsin had any ability to clot fibrinogen.

Experimental

TAME Hydrolysis by RVV. Fig. 1 shows the hydrolysis of TAME by RVV as a function of the incubation time. The final concentration of RVV in this experiment was 0.1 mg per ml. With this concentration of enzyme and over the period of time studied, the reaction is apparently zero order.

Use of Silicone-Treated Glass. It has been demonstrated that thrombin in weak concentrations is significantly adsorbed into glass surfaces (11). Early in our work we found that glass adsorption also occurred with RVV in the range of concentrations with which we were working. This effect is illustrated in Table 1. In this experiment the final concentration of RVV was 0.10 mg per ml. Because of this effect silicone-treated glass was used for preparing enzyme solutions and for the TAME assay, except where noted.

The effect of pH on the TAME hydrolysis by RVV was studied and the optimum was pH 8.7—9.0. Fig. 2 illustrates the experiments run to determine the Michaelis constant (K_m) for RVV. The rate hydrolysis of TAME by RVV

Table 1: Comparison of TAME esterase activity of RVV in glass and silicone-treated tubes.

Hydrolysis Conditions	TAME Hydrolyzed in 120 Min. per cent	TAME Hydrolyzed in 240 Min. per cent
Glass tubes	9	18
Silicone-treated tubes	17	26

Conditions as in Fig. 1, except glass or silicone-treated tubes used as indicated.

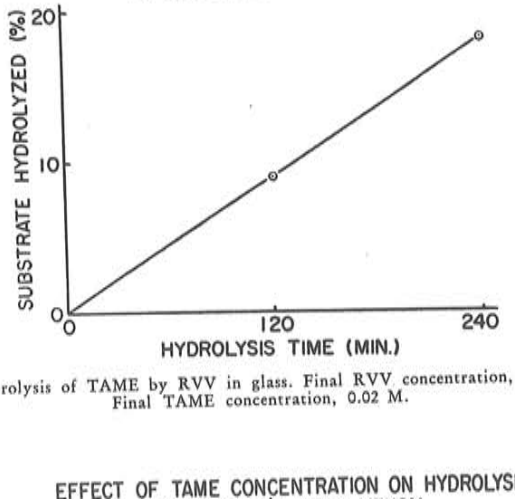

Fig. 1: Hydrolysis of TAME by RVV in glass. Final RVV concentration, 0.1 mg per ml. Final TAME concentration, 0.02 M.

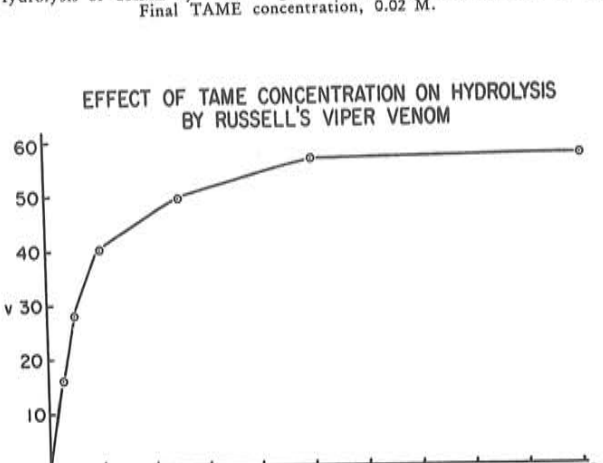

Fig. 2: Effect of TAME concentration on hydrolysis by Russell's viper venom. RVV concentration, 0.167 mg per ml; hydrolysis time, 60 min.; v, relative velocity of hydrolysis in arbitrary units; V_{max}, 0.62 μM per min. per mg RVV.

is plotted as a function of the substrate concentration. The K_m calculated from these data was 1.05×10^{-2} M. Maximum velocity, V_{max}, was calculated to be 0.62 μM per min. per mg RVV (12).

Comparison of TAME Esterase and Clot-Accelerating Activity of RVV and Trypsin. Table 2 is a comparison of the clot-accelerating and esterase activities of a crystalline trypsin preparation and the crude RVV preparation. From this table it can be seen that the crude RVV has considerably more clot-accelerating activity per μg than the highly purified trypsin, although the RVV has much less activity in hydrolyzing TAME.

Table 2: Comparison of clot-acceleration and TAME esterase activity of trypsin and RVV.

Initial Trypsin Concentration μg per ml	Clotting Time sec	TAME Hydrolyzed in 60 Min. per cent
100	15.3	100
10	24.0	100
1	32.2	52
0.1	45.5	16
Control	48.0	—

Initial RVV Concentration μg per ml	Clotting Time sec	TAME Hydrolyzed in 60 Min. per cent
1000	—	22
100	13.2	20
10	13.7	13
1	18.4	6
0.1	27.6	—
0.01	35.8	—
Control	42.3	—

Discussion

RVV is a frequently used reagent in a variety of blood clotting tests. It does not clot fibrinogen or citrated plasma, but has a pronounced acceleratory effect on recalcified clotting tests in the presence of a source of "cephalin". Its action in clotting tests most closely resembles that of trypsin. The ability of RVV to split TAME and presumably certain arginyl peptide bonds suggests a correlation between this activity and its clot-accelerating action.

Crude RVV has considerably more clot-accelerating activity per μg than highly purified trypsin. An explanation of this difference lies either in the relative specifities of the two preparations or the presence in the RVV of other active enzymes.

Summary

Russell's viper venom (RVV) is capable of hydrolyzing TAME slowly. It is readily adsorbed by glass from weak solutions. The pH optimum for the hydrolysis was 8.7 to 9.0. Under the conditions specified, K_m was found to be 1.05×10^{-2} M and V_{max} was 0.62 μM per min. per mg RVV. On a weight basis, RVV has a greater clot-accelerating activity than trypsin but less TAME hydrolyzing activity. It is suggested that there is a correlation between the clot-accelerating action of RVV and the hydrolysis of specific arginyl peptide bonds.

Résumé

Le venin du vipère Russell (RVV) est capable d'hydrolyser lentement le TAME. L'agent actif en solution diluée est aisément adsorbé au verre. La valeur optimale du pH pour l'hydrolyse est de 8.7 à 9.0. Dans les conditions expérimentales spécifiés, Km est 1.05×10^{-2} M et V max 0.62 μM par minute par mg de RVV. A poids égale, le RVV a des propriétés accélérant la coagulation plus accentuées que la trypsine mais est moins actif dans l'hydrolyse du TAME. Une correlation entre l'action accélératrice du RVV sur la coagulation et l'hydrolyse de liaisons spécifiques d'arginyl peptides est suggérée.

Zusammenfassung

Russell-Vipern-Gift vermag Tosylargininmethylester langsam zu hydrolisieren. Es wird aus verdünnten Lösungen leicht an Glas adsorbiert. Das pH-Optimum der Hydrolyse liegt zwischen 8,7 und 9,0. Unter diesen Bedingungen beträgt K_m $1{,}05 \times 10^{-2}$ M und V_{max} 0,62 μM/Min./mg Russell Vipern Gift. Dieses hat eine stärkere gerinnungsbeschleunigende und eine geringere TAME hydrolysierende Wirkung als Trypsin bezogen auf gleiche Gewichtsmengen. Es wird angenommen, daß eine Beziehung zwischen der gerinnungsbeschleunigenden Wirkung von Russell-Vipern-Gift und der Hydrolyse spezifischer Arginyl-Peptid-Bindungen besteht.

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