Biochimica et Biophysica Acta, 438 (1976) 231—238

BBA 67836

SOLID PHASE BOVINE THROMBIN

PREPARATION AND PROPERTIES

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(Received November 10th, 1975)

Summary

A new solid-phase thrombin (EC 3.4.21.5) was prepared through conjugation of the enzyme under mild conditions to a glass support bearing an active ester of *N*-hydroxysuccinimide. The immobilized enzyme retained 50 ± 10% of the specific esterase activity of the parent soluble enzyme. The K_m (apparent) for the esterase activity of the immobilized enzyme has a value of 5 mM, identical to the K_m value of the parent-soluble enzyme. Only 6 ± 1% of the specific proteolytic activity was retained and a higher K_m (apparent) value of 67 μM was obtained for the insoluble enzyme compared to K_m value of 12.5 μM for the parent soluble thrombin. Solid-phase thrombin prepared by the diazocoupling technique was previously reported to retain only 3% of the specific proteolytic activity. The observed loss of specific proteolytic activity can be attributed to steric interference, a change in charge characteristics, or both. Nevertheless, the present method of preparation has the advantages of rapidity and simplicity. It can be readily adapted to use for studying the fate of various complexes of fibrinogen, fibrin and their degradation products. It should also be useful for preparing radiolabeled autologous soluble fibrin for thrombus detection in patients undergoing active thrombosis.

Introduction

Various preparations of water-insoluble thrombin (EC 3.4.21.5) have been previously reported [1—3]. The methods so described are quite tedious and involve diazotization of the synthetic solid support, *p*-amino-DL-phenylalanine-L-leucine [1,2] or *m*-aminobenzyloxymethyl cellulose [3], followed by coupling of thrombin solution to the diazotized solid support. By employing commercially available solid supports bearing active ligands, the method of pre-

paring solid-phase thrombin can be simplified. The application of *N*-hydroxy-succinimide esters of agarose in immobilizing proteins has been extensively studied [4] and was found to be extremely useful due to the rapidity and simplicity of the procedure, and the extremely mild conditions under which a protein can be coupled to the solid phase. This paper describes the preparation of insoluble bovine thrombin employing a glass support bearing this type of reactive ligand. The kinetics of esterolytic and proteolytic activities of the insoluble enzyme, as well as the pH-dependence of the proteolytic activity, are compared with those of the parent-soluble enzyme. The stability of the solid-phase thrombin is also studied.

Materials and methods

Topical bovine thrombin (Parke-Davis, Detroit, Mich.) was employed for this study. The lyophilyzed powder was reconstituted in 0.05 M NaH_2PO_4 buffer, pH 7.0, and was further purified by ammonium sulfate precipitation at 0.55 g $(NH_4)_2SO_4$ per ml of thrombin solution. The enzyme precipitate was then redissolved in 0.15 M NaCl/0.05 M NaH_2PO_4 buffer, pH 7.0, and dialyzed against the same buffer to give a final protein concentration of 7 mg/ml [5]. The clotting activity assay by the method of Kline [5] gave values varying from 100—500 NIH units per ml of enzyme solution, depending on the batch of thrombin.

Preparation of insoluble thrombin. Corning glass support bearing aliphatic extension arm terminating in an active *N*-hydroxy-succinimide group (Pierce Chemical Co., Rockford, Ill.) was employed. Approx. 0.5—0.7 g of the activated support was added to 4 ml of the enzyme solution (7 mg/ml) and the mixture was gently agitated at 4° C for a period of 1—2 h. Unreacted protein was then removed, and the remaining active ester groups of the support were inactivated by addition of 4 ml of 1 M glycine, followed by gentle agitation for 15 min. The immobilized enzyme was then thoroughly washed with normal saline, until the addition of 0.2 ml of the washings to 0.8 ml of 0.2% fibrinogen solution did not result in clot formation, after 2 h incubation at room temperature. The immobilized enzyme was then resuspended in normal saline and stored at 4° C.

Determination of the protein content of immobilized thrombin. The protein content of the various insoluble thrombin preparations was determined from the amount of valine liberated on acid hydrolysis [7]. A sample of soluble thrombin solution of known concentration was used for comparison. After acid hydrolysis, the acid was removed by rotary evaporation in vacuo, and the residue dissolved in water. An aliquot was withdrawn and subjected to paper chromatography in the hyperphase of 4 : 5 : 1 mixture of *n*-butanol/H_2O/glacial acetic-acid. The paper was sprayed with Ninhydrin reagent; the valine spot was identified and quantitated by the technique of Kay and coworkers [8].

Stability of insoluble thrombin. The insoluble thrombin was kept as a suspension in normal saline at 4° C. An aliquot of the solid material was taken at weekly intervals and assayed for its proteolytic activity by the method described below. The results are expressed as a percent of the initial activity.

Proteolytic activity. The proteolytic acitivity of insoluble thrombin and its parent-soluble thrombin was determined by the spectrofluorimetric measure-

ment of fibrinopeptides released after a certain period of incubation of the enzyme with a human fibrinogen solution of known concentration at pH 7.4. This method is based on the principle that the fibrinopeptides released contain C-terminal arginyl groups [9] which form fluorophor in strong alkaline ninhydrin solution [10]. The fibrinopeptides released were separated from residual protein by precipitating the protein with 10% trichloracetic acid, and the pH of the supernatant was readjusted to basic by addition of saturated KOH solution prior to its reaction with ninhydrin reagent [11]. A standard curve was obtained with purified human fibrinopeptide A solution the concentration of which was determined by our modification (S.S.L. Harwig and L.A. Sherman, unpublished data) of the method of Moore and Stein [13]. The proteolytic activity of insoluble thrombin and its parent-soluble thrombin was then expressed as μmol of fibrinopeptides released/min.

Esterolytic activity. Tos-Arg-OMe (α-*N*-toluene-*p*-sulfonyl-L-arginine methyl ester) esterase activity was determined by the pH-stat technique [14]. All measurements were carried out in 0.004 M NaH_2PO_4, pH 7.0, at 25° C, with 0.05 M NaOH as the titrant. A pH-meter connected to a strip chart recorder was employed. Results are reported as μmol of sodium hydroxide per min required to maintain constant pH and are therefore equivalent to μmol of Tos-Arg-OMe hydrolyzed per min.

pH-dependence curve of proteolytic enzymatic activity of insoluble thombin and its parent-soluble thrombin. Topical bovine thrombin was further purified by cation-exchange chromatography employing Bio-Rex 70 carboxylate resins [15]. The fractions with the highest clotting activity [5] were pooled. Solid-phase thrombin was prepared from the purified thrombin solution by the method described in a previous section. The pH-dependence curve of its proteolytic enzymatic activity was then determined by incubating the enzyme with solutions of human fibrinogen at various pH values ranging from 5.5—9.6, followed by the fluorimetric assay of the fibrinopeptides released.

Results

Although solid clot formation was not observed when the insoluble thrombin preparation was added to fibrinogen solution at room temperature, the insoluble enzyme did exhibit clotting activity. This was indicated by aggregation of the thrombin particles as a result of fibrin adherence to the particles after 5 min incubation. When radio-iodinated human fibrinogen was incubated with insoluble thrombin, more than 80% of the radioactivity was found to adhere to the solid thrombin particles.

Shown in Fig. 1 is the comparison of proteolytic activity of 25 μl of a typical insoluble-thrombin preparation with 5 μl of its corresponding parent-soluble thrombin solution containing 0.4 NIH clotting unit. From these data and the protein content assay, the specific proteolytic activity of the insoluble-thrombin preparation was found to be 6.0 ± 1.0% of its parent-soluble thrombin solution. The Lineweaver-Burk plot shown in Fig. 2 over a substrate concentration range of $4 \cdot 10^{-6}$—$15 \cdot 10^{-6}$ M gave a K_m (apparent) value of 67 μM for insoluble thrombin and 12.5 μM for soluble thrombin.

Fig. 3 shows the comparison of Tos-Arg-OMe esterase activity of 20 μl of a

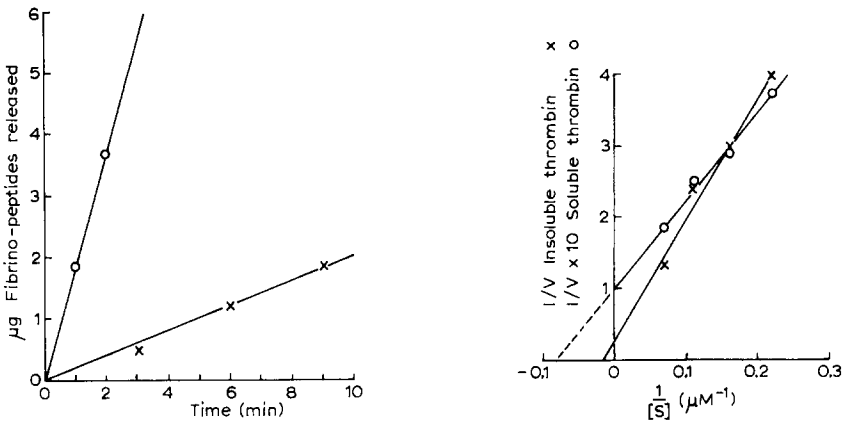

Fig. 1. Proteolytic activity of thrombin preparations. To 3 mg of human fibrinogen in 1 ml 0.1 M NaH_2PO_4/0.005 M EDTA, pH 7.4, is added: X, 25 µl insoluble thrombin, ○, 5 µl parent soluble thrombin. The reaction is stopped at various time intervals and assayed for fibrinopeptide concentration.

Fig. 2. Calculation of K_m for soluble and insoluble thrombin where [S] is fibrinogen concentration and v is the rate of fibrinopeptide release.

typical insoluble-thrombin preparation with 20 µl of its corresponding parent-thrombin solution. The specific Tos-Arg-OMe esterase activity of the insoluble-thrombin preparation was found to be 50 ± 10% of its parent-soluble thrombin solution. Identical values of 5 mM were obtained for K_m (apparent) of insoluble thrombin and K_m of soluble thrombin from the Lineweaver-Burk plot over a substrate concentration range of $2 \cdot 10^{-3}$—$10 \cdot 10^{-3}$ M as shown in Fig. 4.

The stability of insoluble thrombin stored at 4°C as a suspension in normal

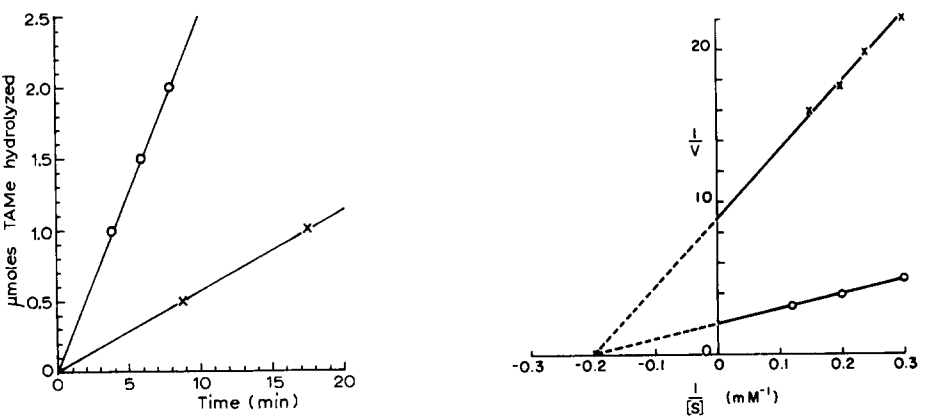

Fig. 3. Esterolytic activity of thrombin preparations. To 6 ml of a 5 mM TAMe (Tos-Arg-OMe) solution in 0.15 M NaCl/0.004 M NaH_2PO_4, pH 7.0, is added: X, 20 µl insoluble thrombin; ○, 20 µl parent-soluble thrombin.

Fig. 4. Calculation of K_m for soluble and insoluble thrombin where [S] is the concentration of TAMe (Tos-Arg-OMe) and v represents micromoles of TAMe hydrolysed per min. X, insoluble thrombin; ○, soluble thrombin.

TABLE I

STABILITY OF INSOLUBLE THROMBIN

The insoluble thrombin was kept at 4°C as a suspension in normal saline; aliquots were drawn at weekly intervals for its proteolytic activity assay. The activity is expressed as percent of the initial activity.

Day	Activity (%)
0	100
7	90
14	95
21	95

saline is shown in Table I. The insoluble thrombin maintained its full proteolytic activity for a period of at least 3 weeks when stored under the specified conditions.

The pH-dependence of proteolytic activity is shown in Fig. 5. The optimum pH for proteolytic activity of insoluble thrombin is near 7.4, while the optimum pH for the parent purified bovine thrombin is near 9.0.

Discussion

The insoluble-thrombin preparation described here is extremely simple and rapid as compared to the method previously described [1—3]. The preparation also exhibits excellent stability over a period of at least three weeks.

Previous reports [2,3] have compared the kinetic data of Tos-Arg-OMe esterase activity of insoluble thrombin prepared through diazocoupling reaction with their respective parent-soluble thrombin. No kinetic data on its proteolytic or clotting activity was provided. The K_m value for Tos-Arg-OMe esterase activity determined by the pH-stat technique was found by Hussain and Newcomb [2] to be 3.22 mM at pH 7.0 and by Owen and Wagner [3] to be 3.70 mM at pH 7.5, for their respective insoluble-thrombin preparations and parent-

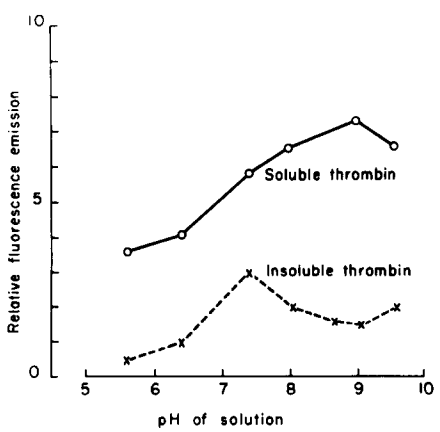

Fig. 5. Proteolytic activity of purified bovine thrombin and insoluble bovine thrombin at various pH values. The fibrinogen concentration for these assays was kept constant.

soluble thrombin solutions. The K_m (apparent) of our insoluble bovine thrombin preparation for its Tos-Arg-OMe esterase activity is identical to the K_m of its parent-soluble enzyme, 5 mM at pH 7.0. The K_m value for Tos-Arg-OMe esterase activity of soluble thrombin measured spectrophotometrically according to the method of Hummel [16] was reported to be 0.24 mM [17] and 0.297 mM [18].

Very few kinetic studies have been performed on the proteolytic or clotting activity of soluble thrombin. The K_m value for clotting activity of human thrombin has been reported to be 0.438 μM by clotting-time measurement [19]. The K_m value for proteolytic activity of bovine thrombin when bovine fibrinogen was used as substrate was found to be 5 μM [17]. In the latter study, the fibrinopeptides released were allowed to undergo alkaline hydrolysis followed by reaction with ninhydrin [20]. Our fluorimetric technique yielded a value of 12.5 μM for the proteolytic activity of soluble bovine thrombin, using human fibrinogen as substrate, and a relatively higher K_m (apparent) value was obtained for insoluble bovine thrombin described here.

The insoluble thrombin preparation retained an average of 50% of its specific Tos-Arg-OMe esterase activity. However, only an average of 6% of the specific proteolytic activity was retained. This is similar to the finding of Alexander and Engel [1] who observed an average yield of only 3% for specific clotting activity of their insoluble bovine thrombin prepared by the diazocoupling reaction. The much lower yield for specific clotting or proteolytic activity of insoluble thrombin can possibly be explained by the fact that fibrinogen is a much larger substrate than Tos-Arg-OMe; therefore steric interference may play an important role during the interaction of insoluble thrombin with fibrinogen.

An alternative explanation is based on the fact a greater K_m (apparent) value was obtained with insoluble thrombin for its proteolytic activity, suggesting a decreased substrate affinity secondary to altered charge characteristics of the insoluble enzyme. It was previously suggested [21] that during the interaction of thrombin with fibrinogen the fibrinopeptides, due to their polyanionic nature, are bound to positively charged sites on the surface of the enzyme. This brings the fibrinogen molecule into a position to allow the nucleophilic attack of the activity serine center of the enzyme on the arginylglycine peptide bond of the fibrinogen molecule. Acetylation of thrombin resulted in reduction of clotting activity [22]. A subsequent study by Walz and coworkers [23] suggested that the critical site in the case of fibrinogen is to the right of arginine and not only to the left where it was formerly thought to be. In our case, since some of the amino groups of the thrombin molecule have already participated in the coupling reaction to the solid support, forming a strong amide linkage, these are no longer available for binding the anionic sites of the fibrinogen molecule to allow enzyme-substrate interaction. A greater loss in specific proteolytic activity of insoluble thrombin is therefore to be expected. The slight shift we observed in the pH-dependence curve of its proteolytic activity also tends to support this idea. However, even in the case of Alexander's preparation [1], where none of the amino groups of the thrombin molecule participated in the diazo coupling reaction, only 3% specific clotting activity was retained. Thus, it is possible that either the steric interference or the charge characteristic change, or both, could explain the decrease in proteolytic activity

in the insoluble thrombin. However, a more definitive conclusion must await further investigation.

The solid-phase thrombin described here is easy to prepare, exhibits good stability, and retains reasonable enzyme activity. This immobilized enzyme may be applicable to certain biomedical studies. For example, the solid-phase thrombin is ideally suited to the preparation of soluble fibrin for current investigations of fibrinogen/fibrin/fibrin-degradation product complexes [24—27]. This is important because a variety of experimental and clinical studies have focused on soluble fibrin as an important stage in the patho-physiology of intravascular coagulation [28—30]. Furthermore, the potential use of radioiodinated soluble fibrin for thrombus localization [31,32] in patients undergoing active thrombosis would require the immobilized thrombin to achieve an injectable soluble fibrin solution, devoid of any residue of thrombin or thrombin inhibitor.

Acknowledgements

This work was supported by a S.C.O.R. Thrombosis Center Grant No. 1 P17 HL 14147-04.

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