A Rapid Method for the Purification of Bovine Thrombin and the Inhibition of the Purified Enzyme with Phenylmethylsulfonyl Fluoride*

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ABSTRACT: A rapid method for obtaining bovine thrombin of high purity has been developed. Chromatography on Sulfoethyl-Sephadex C-50 was performed utilizing a stepwise elution pattern of sodium phosphate buffers (pH 6.5) at room temperature. Bovine thrombin, purified approximately 40-fold over the starting material, was obtained by elution with 0.25 M sodium phosphate (pH 6.5). This preparation was essentially homogeneous to gel filtration on G-100 Sephadex. The purified material is free of other coagulation factors and plasmin. The inhibition of this purified material with phenylmethylsulfonyl fluoride has been studied in some detail. The reaction appears to follow second-order kinetics with a rate constant of 8.9 M^{-1} sec^{-1} at pH 9.0. The pH dependence for this reaction shows an optimum between 8.5 and 9.0. The enzyme is protected from inhibition by either *p*-tosyl-L-arginine methyl ester or benzamidine.

Thrombin plays a pivotal role in blood coagulation. Not only does it catalyze the final reaction in coagulation, the formation of fibrin from fibrinogen, but also interacts with factor VIII (Rapaport *et al.*, 1963) as well as factor V (Coleman, 1969). In order to fully understand the nature of these interactions, a more complete understanding regarding the factors governing the catalytic activity and specificity of the enzyme is necessary. These studies will initially require a technique for the easy and rapid preparation of functionally homogeneous preparations of thrombin from readily available, relatively inexpensive commercial preparations.

The purification of bovine thrombin has been the subject of considerable investigation in the past. Rasmussen (1955) used Amberlite IRC-50 ion-exchange resin to obtain considerable purification of a commercial preparation. Baughman and Waugh (1967) have used a combination of a cation-exchange medium, either Amberlite CG-50 or cellulose phosphate, followed by the use of DEAE-cellulose to obtain material of high purity and enhanced stability. More recently Batt *et al.* (1970) used a combination of an acidic ion-exchange resin followed by a strong cation-exchange cellulose to purify prothrombin. Simultaneously, Rosenberg and Waugh (1970) were able to resolve commercial preparations of bovine thrombin into as many as six biologically active components by stepwise elution from cellulose phosphate.

The inhibition of thrombin by diisopropylphosphorofluoridate has been investigated by Gladner and Laki (1956). They observed a pH optimum for inhibition of approximately 8.0. The pH dependence curve for inhibition closely paralleled that for activity against fibrinogen. Subsequent work by these investigators (Gladner and Laki, 1958) revealed that the binding site for this reagent was the same as that in trypsin, that is, Gly-Asp-Ser-Gly. It has been shown (Seegers *et al.*, 1965) that phenylmethylsulfonyl fluoride, the trypsin and chymotrypsin inhibitor developed by Fahrney and Gold (1963), was a potent inhibitor of crude bovine thrombin. This observation was confirmed by Breckenridge and Ratnoff (1966). Subsequently, Lanchantin *et al.* (1967) reported that phenylmethylsulfonyl fluoride was considerably less effective than diisopropyl phosphorofluoridate in the inhibition of thrombin. However, their observations were based upon experiments utilizing functionally impure thrombin.

It is the purpose of this communication to present a facile method for the purification of commercially available bovine thrombin, partial characterization of the purified protein, and some studies on the reaction of this purified enzyme with phenylmethylsulfonyl fluoride.

Materials and Methods

Phenylmethylsulfonyl fluoride (PMSF)[1] and *p*-tosyl-L-arginine methyl ester were obtained from Calbiochem. Benzamidine hydrochloride was a product of Eastman Organic Chemicals. All other chemicals were of reagent grade.

SE-Sephadex C-25, SE-Sephadex C-50, and Sephadex G-100 were obtained from Pharmacia. The chromatographic apparatus was purchased from Glenco Scientific.

Crude bovine topical thrombin (bioactivated) was obtained from Parke-Davis in vials said to contain 10,000 NIH units (Bio 2076). Crude bovine fibrinogen was purchased from Nutritional Biochemical Corp. or Sigma Chemical Corp. In certain experiments, purified fibrinogen was prepared from the above crude material by the method of Laki (1951).

Chromatographic Fractionation. Crude bovine thrombin, 10,000 units, was placed in dialysis against 2 l. of the initial solvent, 0.025 M sodium phosphate (pH 6.5) for 8–12 hr under ambient conditions. The entire sample, 8–10 ml, was then placed on a 2 × 28 cm column of the indicated medium previously equilibrated with the initial solvent, under ambient conditions. Elution was performed at 60 ml/hr using a Milton Roy Minipump. During column development the absorbance at 254 mμ was continuously determined using an

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[1] The abbreviations are: PMSF, phenylmethylsulfonyl fluoride; Ts-L-ArgOMe, tosyl-L-arginine methyl ester; SE, sulfoethyl.

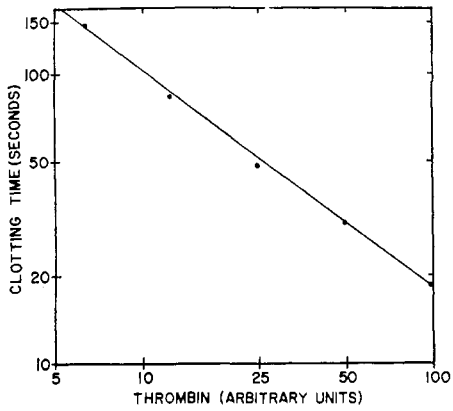

FIGURE 1: Thrombin standard curve. A solution of purified bovine thrombin was diluted 1:10, 1:20, 1:40, 1:80, and 1:160 in 0.06 M Tris-HCl (pH 7.5)–0.09 M NaCl. Portions (0.1 ml) of the above dilutions were added to 0.2 ml of crude fibrinogen (5 mg/ml) in 0.15 M NaCl. The clotting time was then determined at 37°.

ISCO UA-2 ultraviolet analyzer. Subsequently, protein concentration was estimated either by absorbance at 280 mμ or the ninhydrin reaction following alkaline hydrolysis (Fruchter and Crestfield, 1965) using the dimethyl sulfoxide ninhydrin reagent (Moore, 1968). After the void volume peak had been eluted (approximately 60 ml), the eluting solvent was changed to 0.1 M sodium phosphate (pH 6.5). This solvent was continued for 60 ml and then the solvent was changed to 0.25 M sodium phosphate (pH 6.5). This was continued until the elution of the active material. Thrombin activity was determined as described below. The fractions containing the greatest thrombin activity were combined and dialyzed against 2 l. of 0.01 M NaCl under ambient conditions. This material was then frozen in 5-ml portions for further use. For gel filtration studies, 20 ml of purified dialyzed thrombin containing approximately 4.5 mg of protein was lyophilized. This material was reconstituted with a minimal volume (*ca.* 1 ml) of distilled H_2O and subjected to gel filtration on a 1.5 × 58 cm of G-100 Sephadex. The solvent employed for these gel filtration studies was 0.1 M sodium phosphate (pH 6.5). Fractions (2.5 ml) were collected and assayed for biological activity as described below. Portions (100 μl) were taken and subjected to analysis for protein concentration as described below.

Measurement of Enzymatic Activity. Fibrinogen clotting activity was determined in the following manner. Fibrinogen (200 mg) was dissolved in 40 ml of 0.15 M NaCl. A portion of this solution (0.2 ml) was added to an uncoated glass clotting tube and maintained at 37° for 30 sec. The sample (0.1 ml) was then added and the clotting time determined. The clotting time, thus obtained, is converted into arbitrary units using a standard curve. The standard curve is obtained from determining the clotting time of various dilutions of the most active sample. The clotting times are plotted against the dilutions on double logarithm paper. A straight line is obtained as shown in Figure 1. The effect of various samples on the acceleration of the partial thromboplastin time in silicone tubes was assayed by the method of Lundblad and Davie (1964). Samples for the coagulation assays were diluted 1:10 in a 0.06 M Tris-HCl buffer (pH 7.5), 0.09 M with respect to sodium chloride.

The ability of fractions to hydrolyze Ts-L-ArgOMe was determined after the spectrophotometric method of Hummel (1959). The assays were performed at pH 8.0 (0.05 M Tris-

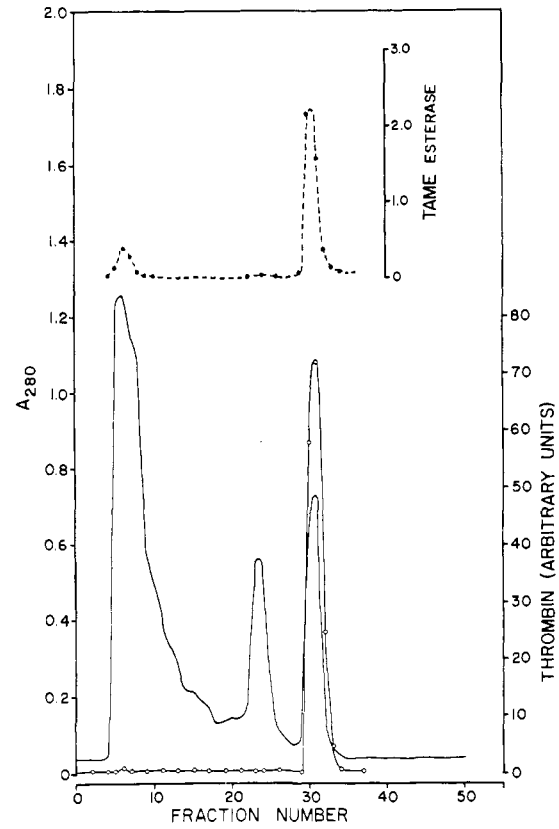

FIGURE 2: Chromatographic purification of crude bovine thrombin on SE-Sephadex C-50. Bovine thrombin (10,000 NIH units) was dialyzed against the initial solvent, 0.025 M sodium phosphate (pH 6.5), and then applied to column which had previously been equilibrated with the initial solvent. The column was developed with initial solvent for 60 ml, 0.1 M sodium phosphate (pH 6.5) for another 60 ml, and finally with 0.25 M sodium phosphate (pH 6.5). Assays for biological activity are performed as described in the text. The solid line indicates absorption at 280 mμ, the dashed line shows the activity in the hydrolysis of Ts-L-ArgOMe, and the open circles show the fibrinogen clotting activity.

HCl) in the absence of calcium ions. The results are expressed as ΔA_{247}/min per ml at 23°.

Inhibitor Studies. A 0.1-ml portion of a solution of PMSF in methyl alcohol was added at zero time to a reaction mixture containing 0.6 ml purified thrombin (containing 0.09 mg of protein) and 0.3 ml of the indicated buffer solution. Portions of 0.1 ml were removed at the indicated times, diluted 1:10 in the Tris–sodium chloride buffer described above, and assayed for their ability to clot fibrinogen, also as described above. The concentration of PMSF is indicated in the individual experiments. All reactions were performed at 23°. Benzamidine, Ts-L-ArgOMe, and Ts-L-Arg were dissolved in the indicated buffer prior to use.

Results

Chromatographic Fractionation. The chromatographic studies were initiated to determine whether a suitable medium could be found to replace the IRC-50 system developed by Rasmussen (1955). Initial studies showed that SE-Sephadex C-25 gave resolution comparable to that found for IRC-50 ion-exchange resin. Subsequent experiments demonstrated that SE-Sephadex C-50 had a greater capacity to bind thrombin and showed increased resolution in the stepwise elution system described above. A typical chromatogram is shown in

TABLE I: Purification of Bovine Thrombin.[a]

Sample	Thrombin (Units/ml)	Vol (ml)	Total Units	Protein (A_{570}/ml)[b]	-Fold Purifcn
Starting material	100	4.5	450	113.6	1
Peak 3	17.5	20	350	0.45	44

[a] Solutions of crude bovine thrombin and thrombin purified by SE-Sephadex chromatography were assayed as described against fibrinogen. Aliquots of these solutions were analyzed for protein using the ninhydrin reaction after alkaline hydrolysis.

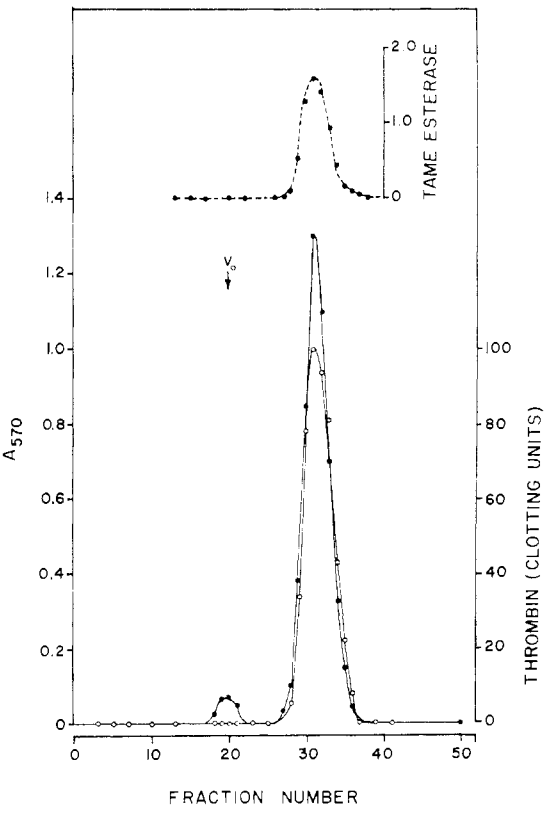

FIGURE 3: Gel filtration of purified bovine thrombin. Bovine thrombin, purified by chromatography on SE-Sephadex as described under Figure 2, was lyophilized after dialysis against 0.05 M NaCl. A portion of this lyophilized material (taken up in distilled H_2O to a volume of 1.5 ml) containing approximately 5 mg of protein was applied to 1.5 × 95 cm column of G-100 Sephadex previously equilibrated with 0.1 M sodium phosphate (pH 6.5). Effluent fractions (2.5 ml) were collected and assayed for esterase (-------) and clotting activity (○—○) as described under Figure 2. Protein (●—●) was determined by the ninhydrin reaction following alkaline hydrolysis.

Figure 2. A considerable amount of material is eluted in the void volume of the column with the initial solvent, 0.025 M sodium phosphate (pH 6.5). This material contains an insignificant amount of fibrinogen-clotting activity. It does, however, show considerable activity in accelerating the partial thromboplastin time of normal plasma in siliconized tubes. A 1:10 dilution of material from the center of this peak clotted normal plasma as described above in 6–10 sec. This peak also showed activity in the hydrolysis of Ts-L-ArgOMe. The second peak, that eluted by 0.1 M sodium phosphate (pH 6.5), contains no demonstrable fibrinogen-clotting activity. It does, however, show some activity in accelerating the clotting of normal plasma. A 1:10 dilution gives a clotting time of 60–70 sec.

The third peak, that eluted by 0.25 M sodium phosphate (pH 6.5) contains thrombin. This peak contains 60–80% of the fibrinogen-clotting activity applied to the column and is approximately 40-fold purified compared to the starting material (Table I). The peak also has considerable activity in the hydrolysis of Ts-L-ArgOMe. A clot formed by this thrombin preparation did not dissolve in 36 hr at 37° suggesting the absence of plasmin. Furthermore, the esterase activity was not inhibited by soybean inhibitor lending additional support for the functional homogeneity of this preparation. The distribution patterns were of the types described by Mann and Batt (1969) and varied from preparation to preparation (K. G. Mann, 1970, personal communication). The factors responsible for the formation of a particular distribution pattern are not clear at this time. For future studies, the most active thrombin fractions were combined and dialyzed against 200 volumes of 0.05 M NaCl at room temperature and stored in suitable portions at −20°.

Bovine thrombin, thus prepared, was subjected to further analysis on other separatory media. Figure 3 shows the results from gel filtration of this purified material after lyophilization on Sephadex G-100. A small amount of ninhydrin-positive material (less than 5% of the starting material) was found in the void volume of the columns. This material did not show any activity in clotting fibrinogen or in the hydrolysis of Ts-L-ArgOMe. This material may contain inactive aggregates of thrombin described by Baughman and Waugh (1967). The fibrinogen-clotting and esterase activity were found in the major protein peak which is retained within the column. No attempt was made to calibrate the gel filtration column but the elution position of this material is consistent with a globular macromolecule of mol wt 30,000–40,000. In this procedure more than 95% of the protein was recovered and approximately 65–70% of the fibrinogen-clotting activity. The loss in activity can be accounted for by stability problems as described below. We have not observed any specific interaction of our thrombin preparations with the Sephadex matrix as described by Berg *et al.* (1966) under our conditions of gel filtration.

Stability of the Purified Enzyme. The stability of the purified enzyme was examined in a number of different buffer systems. The results of a typical experiment are shown in Figure 4. The proteinase activity (fibrinogen clotting) of this preparation is somewhat unstable under ambient conditions at pH 7.5 while the esterase activity remains unchanged over the same period. The fibrinogen-clotting activity is considerably more stable at 4°. The results of the inhibition studies described below have been corrected for loss of activity during the experimental time period.

Inhibition with Phenylmethylsulfonyl Fluoride. In these experiments the PMSF was always present initially in a large molar excess (500–1000). Thus, pseudo-first-order kinetics were observed. At concentrations of PMSF of 2×10^{-3} M the reaction follows strict first-order kinetics over the course of the reaction. However, at a lower concentration (1×10^{-3} M) at high pH (9.0) the reaction velocity is observed to decrease as the reaction progresses. Presumably, this is

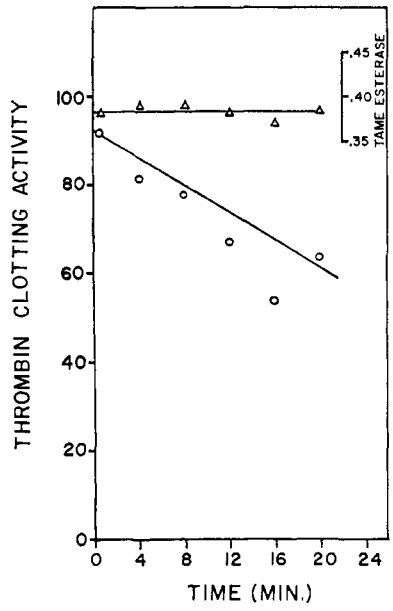

FIGURE 4: The stability of purified thrombin. The reactions were performed at pH 7.5 at 23°. Esterase (△—△) assays and fibrinogen-clotting (○—○) assays were performed as described under Figure 1.

TABLE II: Rate Constants for the Inhibition of Thrombin by PMSF.[a]

PMSF (M)	pH	First-Order Rate Constant (sec^{-1})	Second-Order Rate Constant (M^{-1} sec^{-1})
1×10^{-3}	8.0	6.1×10^{-3}	6.1
2×10^{-3}	8.0	11.0×10^{-3}	5.5
1×10^{-3}	9.0	8.6×10^{-3}	8.6
2×10^{-3}	9.0	17.9×10^{-3}	8.9

[a] The observed first-order rate constants were calculated from data obtained from inhibition reactions at the indicated pH and PMSF concentrations. The inhibition reactions were performed at 23° and aliquots assayed against fibrinogen at 37°. The second-order rate constants were obtained by dividing the observed first-order rate constant by the PMSF concentration of the reaction. Each first-order rate constant is the average results of at least five separate experiments.

due to the base-catalyzed hydrolysis of sulfonyl fluorides. Preincubation of PMSF with the pH 9.0 buffer for increasing periods of time prior to the addition of thrombin results in decreasing rates of inhibition. The data reported here were calculated from early reaction rates.

The pH dependence for the inhibition reaction is shown in Figure 5. As can be observed, the reaction approaches maximum velocity in the range of pH 8.5–9.0. The observed pseudo-first-order rate constants at several pH values are shown in Table II. The table also shows the calculated second-order rate constants. These were obtained, as by Fahrney and Gold (1963), by dividing the pseudo-first-order constants by the PMSF concentration.

The time course for the inhibition reaction at pH 8.0 with a PMSF concentration of 1 mM is shown in Figure 6. It can be observed that the proteinase (fibrinogen clotting) activity and esterase activity are inhibited at an identical rate. This supports the contention that the same catalytic site of the thrombin molecule is responsible for proteolysis and esterolysis.

The inhibition by PMSF is affected by the presence of substrate. Ts-L-ArgOMe, at a concentration of 1 mM, caused a 70% decrease in the rate of inhibition. Benzamidine, a competitive inhibitor of thrombin, at a concentration of 1 mM caused a 75% decrease in the rate of inhibition. Ts-L-Arg had no effect on the inhibition at concentrations as high as 3 mM. These compounds, at the concentrations employed, had no inhibitory effect on the assay system.

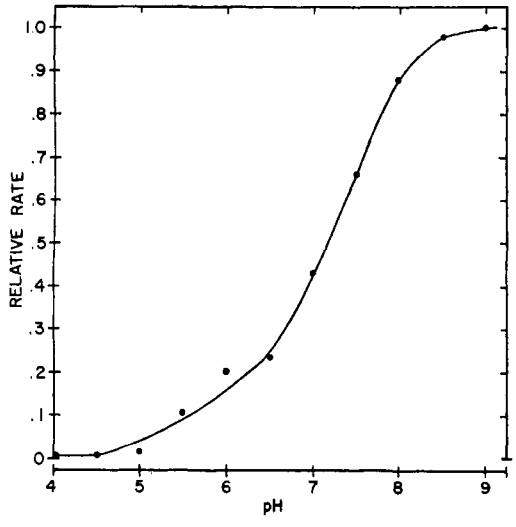

FIGURE 5: The pH dependence of the inhibition of purified bovine thrombin by phenylmethylsulfonyl fluoride. Phenylmethylsulfonyl fluoride (1.7 mg/ml in methyl alcohol), 0.1 ml, was added to 0.6 ml thrombin (0.09 mg/ml) and 0.3 ml of 0.02 M triethanolamine–0.02 M imidazole–0.02 M acetate at the indicated pH. Assays against fibrinogen were performed on 1:10 dilutions of the reaction mixture as described under Figure 1. The inhibition reactions were maintained at 23°. Data were calculated from initial reaction rates.

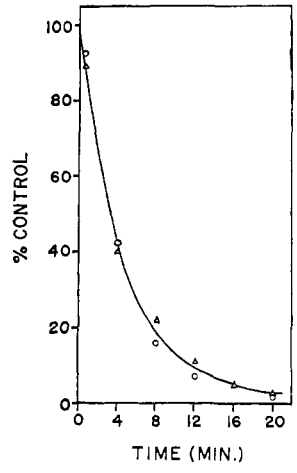

FIGURE 6: The time course for the reaction of PMSF with thrombin. The reactions were performed at pH 8.0 as described under Figure 5. Assays for fibrinogen clotting activity were performed as described under Figure 1 (○—○). Portions (100 μl) were removed for assay against Ts-L-ArgOMe at the indicated times (△—△).

Discussion

The chromatographic fractionation described here is a relatively simple method for obtaining bovine thrombin of high purity. It is a rapid method, taking approximately 4 hr from sample application to combination of the active fractions. The thrombin activity is well resolved from other coagulation factors present in the crude starting material. For instance, activated factor X (activated Stuart factor) which is present in the crude commercial preparations (Kerwin and Milstone, 1967; Yin and Wessler, 1968) appears to be in the void volume fraction. The coagulant material in the second peak has not yet been identified.

The purified material shows considerable homogeneity in gel filtration and the elution position is consistent with a globular macromolecule having a molecular weight of 35,000 ± 5000. Heterogeneity of the active material similar to that obtained by Mann and Batt (1969) and Rosenberg and Waugh (1970) has been observed with chromatography on anion-exchange media (R. L. Lundblad, 1970, unpublished observations). Such heterogeneity is commonly found with proteolytic enzymes (Stein, 1970). It is most probable that, in order to obtain a single, homogeneous thrombin molecule, one will have to activate highly purified prothrombin under carefully controlled conditions. This was found to be the case with pepsin (Rajagopalan *et al.*, 1966). Such heterogeneity of a functionally homogeneous preparation has not been shown to significantly inhibit studies on the catalytic activity of a proteolytic enzyme (Delpierre and Fruton, 1966).

The differential stability of the proteinase and esterase activities of purified bovine thrombin is somewhat puzzling. Such differential stability has been observed by Lanchantin *et al.* (1967) during prolonged citrate activation of their prothrombin preparations. Since thrombin is a highly specific proteolytic enzyme, the nature of the substrate binding site must be exceedingly complex. Thus, it is possible to have distortion of the enzyme molecule which would destroy the binding site for proteolysis without significantly affecting the catalytic process for the less specific esterase substrate. Indeed, differential stability of proteinase and esterase activity has been observed by Sokolovsky and Riordan (1969) on reaction of tetranitromethane with crude bovine thrombin. In the above instance, the tetranitromethane is modifying tyrosyl residues in the binding site, thus causing a more rapid loss of proteinase activity as compared to esterase activity.

The interaction of PMSF with thrombin has been studied by Lanchantin *et al.* (1967). These workers presented evidence indicating the rate of inhibition of thrombin with PMSF was considerably slower than with diisopropyl phosphorofluoridate. This opinion is not supported by the present data. It should be pointed out that the preparations of thrombin and reaction conditions utilized by Lanchantin *et al.* (1967) were considerably different from those employed in the present study.

The reaction of thrombin with phenylmethylsulfonyl fluoride shows a pH dependence very similar to that observed by Gladner and Laki (1956) for the inhibition of thrombin by diisopropyl phosphorofluoridate. Presumably, the phenylmethylsulfonyl fluoride reacts with the same serine residue in the thrombin that reacts with diisopropyl phosphorofluoridate (Gladner *et al.*, 1958; Gladner and Laki, 1958). In α-chymotrypsin the inhibition with phenylmethylsulfonyl fluoride is associated with the sulfonation of a specific serine residue (Gold, 1965).

The protective effect of a substrate, *p*-Ts-L-ArgOMe methyl ester, and the competitive inhibitor, benzamidine, suggests that the reaction with phenylmethylsulfonyl fluoride occurs at or near the catalytically active site in the thrombin molecule. The failure of *p*-Ts-L-Arg to have any similar effect on the inhibition reaction is difficult to explain. The presence of acidic groups at the substrate binding site on the thrombin molecule may prevent significant interaction with the free acid form of Ts-L-Arg. Efforts are underway to elucidate the amino acid modified and the nature of the substrate binding on the thrombin molecule.

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Dissociation of Yeast Enolase into Active Monomers*

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ABSTRACT: Yeast enolase exists as a monomer at concentrations below 0.7 μg/ml, temperatures above 40°, and at pH 7.4, even in the presence of Mg^{2+} and substrate, both of which favor dimer formation. Monomers formed under these conditions, in contrast to those obtained in the presence of high concentrations of Br^- or Cl^-, are fully active. Standard thermodynamic parameters estimated for the dissociation process are $\Delta H^\circ = 80$ kcal and $\Delta S^\circ = 221$ eu.

The glycolytic enzyme enolase, isolated from yeast cells, is reportedly a dimeric molecule (Brewer and Weber, 1968). Dissociation into monomers has been achieved in the presence of 1 M Br^- or Cl^- with a concommitant loss of activity (Gawronski and Westhead, 1969). Thus, it could be assumed that the dimeric structure is essential for the proper functioning of the enzyme. An alternative possibility is that this observed inactivation is the consequence of the high salt concentrations used to effect dissociation and not a result of the dissociation process *per se*. If dissociation could be brought about under very mild conditions, the question whether or not the monomers are active could be resolved. One such mild procedure for dissociation is the lowering of the total protein concentration. However, molecular weight determinations in very dilute solutions are difficult to perform with most physical techniques. Of these, Sephadex chromatography provides the most effective approach, since elution volumes, reflecting molecular weight, can be detected at extremely low enzyme concentrations by activity measurements. By this procedure, evidence indicating that the monomers are active has been obtained and is presented here along with some observations concerning this monomer–dimer equilibrium.

Methods and Materials

Yeast enolase and its substrate, the sodium salt of 2-phosphoglycerate, were purchased from the Sigma Chemical Co., lots 27B7290 and 118B1410, respectively. Purified enzyme preparations were obtained by ion-exchange chromatography according to Rosenberg and Lumry (1964).[1] All other chemicals used were of reagent grade.

All column chromatography experiments described herein were performed on a 2 × 50 cm column packed with Sephadex G-75 gel. Two reservoirs, one for sample, one for elution solvent, were connected to the column through a three-way valve. Both column and reservoir system were enclosed in a continuous-jacketing device through which water was circulated from a constant-temperature water bath. The column was operated under a hydrostatic pressure of approximately 80 cm. resulting in a flow rate that varied with temperature from 60 to 125 ml per hr.

The column was calibrated using the zonal analysis technique. Samples (2.5 mg) of various marker molecules in 1-ml volumes were applied to the column. The effluents were monitored at 280 nm by means of a Beckman Model DBG spectrophotometer equipped with a 0.3-ml flow cell of 1.0-cm path length and the absorbance recorded on a Beckman 10-in. recorder. The elution volumes reported are those corresponding to maximal absorptions in the effluents. Calibrations with BSA,[2] ovalbumin, chymotrypsinogen, and Blue Dextran were performed at both 25 and 45°.

To relate elution volumes to distinct protein concentrations, the experiments with yeast enolase were performed by frontal analysis or large volume chromatography technique. For the column described above, a 50-ml sample application was sufficient to produce a plateau region in the elution pattern. The protein concentration in this region is identical with that of the sample applied to the column. In theory, the elution volume is the centroid point of the boundary. For symmetrical or nearly symmetrical boundaries as observed with enolase, this point can be approximated as the half-height of the plateau. Thus, all elution volumes reported here refer to observed half-heights.

Elution volumes of monomeric and dimeric enolase in Tris-HCl buffer (pH 7.4) were determined by frontal analysis and verified by the zonal technique. In both cases, monomers were obtained in the presence of 1 M KBr. The column was equilibrated with the buffer also 1 M in Br^-. For the frontal analysis determinations, the protein concentration applied to the column was 50 μg/ml. For zonal experiments, the 1.0-ml sample used contained 2.5 mg of enolase. Effluents were monitored at 280 nm. Dimeric elution volumes were similarly obtained in the absence of bromide.

The effect of dilution on the elution volume of yeast enolase was determined at 25°. Solutions (50 ml) of enzyme were prepared at various dilute concentrations in the assay buffer,

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[1] The specific activity of the preparation was within 5% of that cited by Malmstrom (1957).

[2] Abbreviation used is: BSA, bovine serum albumin.