Purification and properties of staphylocoagulase

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PURIFICATION AND PROPERTIES OF STAPHYLOCOAGULASE

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SUMMARY

Staphylocoagulase, an exoprotein of coagulase-positive *Staphylococci*, has been purified to a state in which only trace amounts of contaminating proteins are detectable. Aspartic acid was found as a single N-terminal amino acid in this preparation. The molecular weight is 61 000; the isoelectric point lies at pH 4.53. The amino acid composition was determined.

INTRODUCTION

Staphylocoagulase is a protein secreted by certain strains of *Staphylococcus aureus*. Upon the interaction of this protein with prothrombin, a thrombin-like activity arises. Several authors have reported on the purification of staphylocoagulase [1–9] but none of them describes a procedure yielding a product of sufficient purity to permit the type of experiments intended to investigate the mode of interaction of staphylocoagulase and prothrombin [13].

MATERIALS AND METHODS

Unless stated otherwise, all chemicals were obtained from Merck and were of analytic grade. Brain–heart infusion was obtained from Difco. Sephadex G-100 and DEAE-Sephadex A-100 were from Pharmacia Fine Chemicals. Polyacrylamide gels for chromatography were obtained from Biorad. The DEAE-cellulose used for step 2 was Batch D 8382 from Sigma. For the final chromatography, microgranular DEAE cellulose DE 32 from Whatman was used; for ultrafiltration, a Diaflow apparatus with membrane XM-50. Rabbit plasma was obtained from healthy rabbits by cardiac puncture. The blood was collected in 1/10 volume 0.01 M sodium oxalate and was centrifuged twice (10 min at 4000 $\times$ g and 20 min at 20 000 $\times$ g) to obtain platelet-poor plasma. Bovine fibrinogen was a freeze-dried preparation obtained from Behringwerke A.G.

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Protein determination

Protein was determined according to Lowry et al. [10] with crystalline bovine serum albumin as standard. For rapid protein measurement, the biuret method [11] was used. E_{280} was measured with column fractions.

Lipid analysis

Phospholipids were extracted from the samples according to Bligh and Dyer [12]. The chloroform phase was dried under an air stream and digested with conc. H_2SO_4 and 72% perchloric acid. The phosphorus content was then measured according to Chen et al. [14]. Total phospholipids were calculated from the phosphorus content, as indicated by Wagener [15]. Total lipids were extracted from the samples by the procedure of Bligh and Dyer [12] and determined by a gravimetric procedure [16].

Amino acid analysis was carried out on a Beckman type Unichrom amino acid analyser, using a Beckman PA 27 column and the Beckman calibration mixture no. 312220. The procedure of ref. 17 was followed. Losses during hydrolysis were compensated for by extrapolation to zero hydrolysis time.

Determination of N-terminal amino acids

The N-terminal amino acids of the purified protein were determined by the dansyl chloride method of Gray [18] and the dansyl amino acids were identified on 5 × 5 cm polyamide sheets by using the system of Woods and Wang [19] with the modification described by Croft [20].

For the estimation of molecular weights, sodium dodecylsulphate–polyacrylamide gel electrophoresis was performed according to Weber and Osborn [21]. The samples (volume 0.05–0.10 ml) were mixed with tracking dye (5 μl 0.05% (w/v) Bromphenol Blue) after which sodium dodecylsulphate was added (10% w/v, 5 μl). When necessary, β-mercaptoethanol (5 μl) was added and the mixture was then heated (100 °C, 2 min) before electrophoresis. The gels (8% w/v acrylamide and 0.27 %w/v methylene bisacrylamide) were polymerized and run at a constant current of 8 mA/gel until the tracking dye had moved at least 5 cm. The gels were stained in Coomassie Brilliant Blue and destained by soaking in 7.5% (v/v) acetic acid–5% (v/v) methanol.

Analytical polyacrylamide gel electrophoresis was carried out in an analogous way with omission of the sodium dodecylsulphate.

Gel filtration

Molecular weight determinations were performed with Biogel P-100 according to Whitaker in a 1.5 × 100 cm glass column [22]. The flow rate was kept constant at 12 ml/h by pumping. Fractions (3 ml) were collected and the absorbance was read at 280 nm.

Isoelectric focusing

Isoelectric focusing was carried out in a sucrose gradient in 1% w/v Ampholine buffers in the pH range of 3–6 as described by Haglund [23]. To prevent precipitation at the isoelectric point of the protein, focusing was carried out in the presence of 7 mM urea [24] and BRIJ-35 (polyoxyethylene dodecylether) a non-ionic detergent [25, 26].

Determination of staphylocoagulase

Essentially, the method of Soulier was followed [7]. A glass tube in a water bath was provided with: 0.1 ml rabbit plasma diluted 1:20, 0.1 ml bovine fibrinogen 4 mg/ml, 0.2 ml veronal acetate buffer (pH 7.35) and a 0.1-ml sample containing staphylocoagulase.

At the addition of the sample, a stopwatch was started. The moment of coagulation was assessed by hand with a 5 × 30 mm stainless steel hook, the stopwatch being stopped at that moment. When the activity was to be assessed in slices of a polyacrylamide gel, each 2-mm slice was fragmented in 0.2 ml buffer after which 0.2 ml of a mixture of equal parts of diluted rabbit plasma and bovine fibrinogen solution were added. One crude preparation of staphylocoagulase (obtained after the acid precipitation step to be described below) was considered to contain 100 arbitrary units per ml.

Coagulation times obtained with dilutions of this preparation were used to construct a reference curve. This curve was a hyperbola, since the plot of coagulation times against the inverse of the concentration of staphylocoagulase was a straight line. The standard preparation was frozen at −70 °C in small portions and remained stable for more than two years.

This approach is preferable to basing the definition of a unit on a coagulation time [7] because variations in rabbit plasma or fibrinogen will not influence the unit defined.

PROCEDURE

A strain of *Staphylococcus aureus* (originally strain 104 of Tager [7]) kindly provided by Professor Soulier was stored at −70 °C in broth. Every two months a fresh broth was inoculated and stored after 24 h of culture growth. For mass culture, the broth was used to inoculate 1% glucose–blood agar. After 24 h at 37 °C three tubes of blood agar were mixed into 100 ml brain–heart infusion (Difco). This infusion was kept at 37 °C for 48–72 h in 1-litre Roux flasks, after which the material from 30 flasks was pooled and centrifuged for 30 min at 7000 × *g*. The supernatant was then filtered through a G-5 glass filter.

The material was brought to pH 4.5 with 3 M HCl and left for 18 h at 0 °C. The precipitate was collected by centrifugation (20 min at 5000 × *g*, 4 °C) and washed twice in 1/10 vol. 0.15 M sodium acetate (pH 3.8) before being dissolved in 1/20 vol. 0.05 M Tris–HCl (pH 7.35) containing 0.10 M NaCl. Then one half volume of DEAE-cellulose slurry (75 mg/ml in 0.015 M Tris–HCl, pH 7.35) was added. After stirring for 10 min at room temperature, the cellulose was separated on a G-2 sintered glass filter and washed repeatedly with 20 ml of Tris–HCl 0.05 M (pH 7.35) containing 0.15 M NaCl. Washing was stopped when coagulation times longer than 60 s were found in the eluting fluid (after 9–12 washings). The pooled washing fluids were concentrated by ultrafiltration using a Diaflow apparatus with a XM-50 membrane.

A 100-ml aliquot of the preparation (0 °C) was added slowly to a mixture of 100 ml ethanol and 100 ml diethylether at −10 °C. After 30 min of incubation 2.5 ml water was added. The resulting ethanol–water layer (about 240 ml) was separated from the ether layer and 160 ml of a saturated solution of ammonium sulphate was added to it. This again separated into two phases. The lower one contained the staphylocoagulase in the form of a fine precipitate which was collected by centrifugation (10

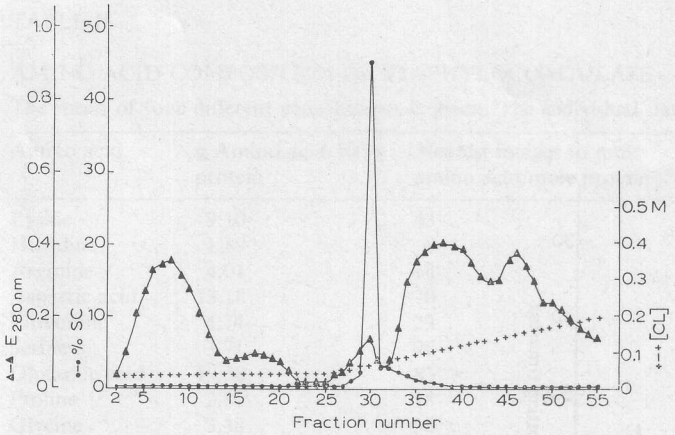

Fig. 1. DEAE-cellulose chromatography of staphylocoagulase. ▲—▲, adsorbance at 280 nm; ●—●, activity of staphylocoagulase in % (SC scale); +++, concentration of chloride ions (CL scale).

min at −10 °C, 2000 × *g*) and suspended in 100 ml *n*-pentane at −10 °C, again centrifuged and freeze-dried. The dry powder was repeatedly extracted by 20 min of stirring at room temperature with 20 ml Tris buffer (pH 7.35) 0.15 M NaCl, until the coagulation time of the supernatant exceeded 60 s (2–4 times). Lipid extraction with alcohol–ether and pentane in this system was absolutely necessary for the following chromatography to be successful. Before extraction the material contained 43 μg phospholipids and 628 μg triglyceride per mg protein. After extraction no phospholipid and less than 20 μg triglyceride was detected. The preparation was diluted five times with a 0.02 M borate buffer (pH 5.5) and applied to a 2.5 × 10 cm column of DEAE-cellulose equilibrated with the same buffer containing 0.03 M NaCl. When 280 nm-adsorbing material no longer eluted. a linear gradient of 0.03–0.25 M NaCl in the same buffer was applied (2 × 100 ml). The staphylocoagulase elutes as a sharp peak shortly afterwards, at about 0.07 M NaCl (Fig. 1). The course of the procedure is summarized in Table I.

Fig. 2 shows a scan of a polyacrylamide-electrophoresis pattern of the final product. In a parallel unstained run the activity was measured. The maximal activity

TABLE I

PURIFICATION OF STAPHYLOCOAGULASE

Values represent the means of 10 purification procedures. The highest specific activity observed was 41 800 units/mg, the lowest 35 200.

Procedure	Vol. (ml)	Activity (units/ml)	Protein (mg/ml)	Spec. Act. (units/mg)	Yield (%)	Purification (×)
Filtered broth	20 000	17	15.4	1.1	100	1
Isoelectric ppt	1 000	110	8.7	12.6	32	11
DEAE-cellulose adsorbance	4 000	24.5	0.650	37.7	29	34
Ultrafiltration	200	479	0.742	661	20	600
Lipid extraction	120	532	0.325	1 640	18	1 490
Chromatography	20	2360	0.060	39 300	14	35 700

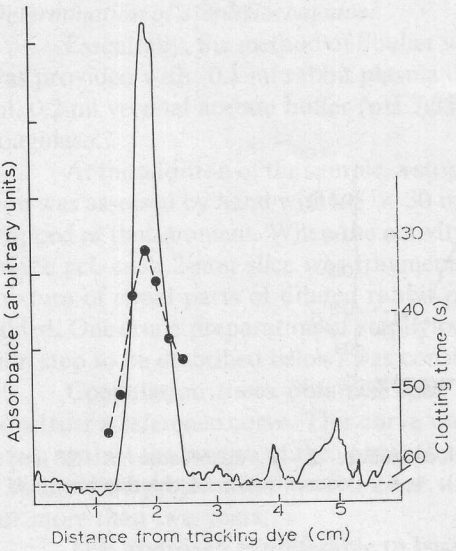

Fig. 2. Absorbance scanning of the polyacrylamide-gel electrophoresis of staphylocoagulase. ———, Absorbance trace; - - - - - -, baseline; ●---●, coagulation times obtained with fragmented 2-mm segments of an unstained gel prepared under the same conditions as the stained gel used for scanning.

coincided with the protein band. Faint contaminating bands amounting to 16% of the area under the curve and without activity were observed. Also on sodium dodecylsulphate–polyacrylamide electrophoresis of the reduced or unreduced sample only very faint contaminating bands could be observed.

PROPERTIES OF STAPHYLOCOAGULASE

Molecular weight

Gel filtration on P-100 acrylamide gel columns with the use of yeast alcohol dehydrogenase (mol. wt; 126 000), bovine serum albumin (mol. wt: 70 000), ovalbumin (mol. wt: 43 000), lactic dehydrogenase (mol. wt: 36 000), and chymotrypsinogen (mol. wt: 25 700) as reference molecules gave a molecular weight of 62 000 $\pm$ 3000 as the mean of ten separate estimations, calculated according to Andrews [27]. The molecular weight as estimated with the aid of sodium dodecylsulphate electrophoresis was 60 000 $\pm$ 2300 (15 estimations); with both methods we obtained a calculated molecular weight of 61 000 $\pm$ 2300 (see ref. 13, Fig. 5). Reduction does not change the behaviour in sodium dodecylsulphate–polyacrylamide electrophoresis.

Isoelectric points

Upon isoelectric focusing the staphylocoagulase activity eluted in a single peak in fractions with a pH of 4.51–4.55.

Chemical composition

The mean amino acid composition of four different preparations is shown in Table II. The variation between the four figures for each amino acid was less than

TABLE II

AMINO ACID COMPOSITION OF STAPHYLOCOAGULASE

The mean of four different preparations is given. The individual data differed less than 11%.

Amino acid	g Amino acid/100 g protein	Nearest integer to mole amino acid/mole protein
Lysine	9.10	43
Histidine	1.89	8
Arginine	4.01	16
Aspartic acid	13.18	70
Threonine	4.74	29
Serine	3.71	26
Glutamic acid	17.58	83
Proline	2.82	18
Glycine	3.38	36
Alanine	5.09	44
Cystine (half)	1.64	10
Valine	6.96	43
Methionine	1.51	7
Isoleucine	6.42	35
Leucine	7.70	42
Tyrosine	3.99	15
Phenylalanine	4.53	19
Tryptophan*	0.60	2

* Determined according to Scoffone et al. [28].

9%. Carbohydrate staining of the polyacrylamide gels of the purified product was negative. A 0.2-ml aliquot containing 100 μg protein was dansylated and hydrolysed as indicated in ref. 18. The maximal yield of dansylated N-terminal amino acid is $\approx$ 1.5 nmoles. Upon polyamide-sheet chromatography, apart from the usual ε-Dns–lysine, *O*-Dns–tyrosine, Dns–NH_2 and Dns–OH, only Dns–aspartic acid could be identified. This was verified by co-chromatography of Dns–aspartic acid or Dns–glutamic acid. No parasitic spots were observed. Co-chromatography of 0.1 nmole of various Dns–amino acids gave readily discernable spots. As this concentration is $< 7\%$ of the maximal yield of the N-terminal of the main protein it was concluded that no contaminants were present in a concentration of more than 5–10% unless they had N-terminal amino acids that form labile Dns compounds (proline, tryptophan). When 3–6 times the normal amount of the hydrolysate was applied to the sheets hardly discernable parasitic spots were observed.

DISCUSSION

Staphylocoagulase is a single-chain protein with a molecular weight of 61 000 $\pm$ 2300 as determined by sodium dodecylsulphate-gel electrophoresis and gel filtration. The degree of purification obtained by our method compares favourably with that obtained by other authors as far as can be judged from their data. Siwecka and Jeljaszewicz [6] reported obtaining a 1355-fold purification at a 1.3% yield with the method of Duthie and Haughton [2] and a 5000-fold purification at a 77% yield with the method of Zolli and San Clemente [9]. This is the highest purification we found to

have been reported. Our method produces staphylocoagulase that routinely has a specific activity which is more than seven times higher. This may be due either to a difference in the starting material. resulting from a different strain of *Staphylococcus*, or to a better purification method. From the N-terminal amino acid analysis we concluded that individual contaminating proteins were present in a concentration of less than $\approx 7\%$. In the polyacrylamide electrophoresis pattern (Fig. 2) three small accessory peaks could be seen. They contributed 16% to the total area between the curve and the baseline in Fig. 2. Comparable results were obtained in sodium dodecylsulphate–polyacrylamide electrophoresis. As the scanning tends to underestimate high density bands we estimate the amount of contaminating proteins to be $< 15\%$. We were not able to raise antibodies against our preparation. When antibodies were raised against coagulase-thrombin one precipitation line was seen between this antibody and our preparation (see ref. 13).

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