STUDIES ON STAPHYLOKINASE

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STAPHYLOKINASE is a protein found in the culture medium after growth of many strains of *Staphylococcus aureus* that converts plasminogen into the active proteolytic enzyme plasmin (Gerheim, 1948; Lack, 1948). Few reports have been published of the purification of staphylokinase. Davidson (1960) and Glanville (1963) precipitated protein containing staphylokinase from supernatant fluids of cultures by adjusting the pH to 3.3 with 10M-HCl. Glanville precipitated staphylokinase at 75 per cent. saturation with $(\text{NH}_4)_2\text{SO}_4$. The dissolved material was further purified by chromatography on carboxymethyl-cellulose (CM-cellulose) columns and one homogeneous component with staphylokinase activity was obtained. This preparation proved to contain no haemolysin, leucocidin, coagulase or hyaluronate lyase, and the staphylokinase was purified about 44 times. Immunodiffusion tests showed the presence of two or four antigens, depending upon how the material had been eluted from the CM-cellulose columns.

The fibrinolytic effect of staphylokinase was investigated by Lewis *et al.* (1964), who used enzyme that had been prepared by precipitation with ethanol (Lewis and Ferguson, 1951; Lewis *et al.*, 1964). The in-vivo tests were made with dogs, and this preparation of staphylokinase proved too toxic for adequate thrombolytic experiments. Whether the toxicity was dependent on the kinase itself or on some contaminating protein was not revealed.

Soru, Sternberg and Istrati (1959) purified staphylokinase by precipitation first with $(\text{NH}_4)_2\text{SO}_4$ and then with ethanol. The degree of purity of the kinase was controlled by electrophoresis, chromatography and immunodiffusion in gel, and these methods indicated homogeneity of the preparation. The amino-acid composition of staphylokinase was also investigated. Recently an article describing a purification of about 100 times was published by Lack and Glanville (1970); the yield was, however, only 15–20 per cent.

In the present investigation, staphylokinase has been purified by means of isoelectric focusing (Vesterberg *et al.*, 1967). Further characterisation of the kinase with respect to isoelectric point (*pI*), pH optimum for activity, influence of metal ions, and heat stability is reported, and some kinetic studies are also presented.

MATERIALS AND METHODS

Production of staphylokinase. *S. aureus*, strain V8, was grown and the culture supernatant was concentrated in principle as described earlier (Vesterberg *et al.*). Cultivation was made

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in a casein hydrolysate, yeast extract medium CCY_I at pH 7.5 and 37°C for 18 hr in a stirred aerated fermenter of 2.5-l working volume (Biotec FL103, Stockholm, Sweden).

Assay of staphylokinase. The method of casein hydrolysis according to Davidson, with the modifications described by Vesterberg *et al.*, was used. Thus, the staphylokinase solutions were incubated with plasminogen for 6 min. Casein was then added to the test-tubes and the incubation continued for 30 min. before the addition of perchloric acid was made. The solutions were then centrifuged, and the absorbancy at 280 nm (A_{280}) was measured on the clear supernatants. The staphylokinase activity is expressed as the increase in A_{280} per 30 min. The amount of enzyme that gives an increase in A_{280} per 30 min. of 1.0 is called one unit of enzyme.

FIG. 1.—Staphylokinase activity, expressed as increase of absorbancy at 280 nm (A_{280}) per 30 min., ○—○, after the first isoelectric separation in pH gradient ranging from pH 2 to 13; pH at +4°C, ●—●, 0.02 ml of the fractions were used in each assay.

Plasminogen (human, freeze-dried, grade A) was kindly provided by AB Kabi, Stockholm. The activity was expressed in caseinolytic units (c.u.) according to Sgouris *et al.* (1960).

Protein determination. Dialysed samples of the culture supernatant fluids and of concentrated solutions were analysed for protein by the biuret method and by the method of Lowry *et al.* (1951). Fractions from isoelectric columns were analysed by measuring the A_{280} after prolonged dialysis.

Spectrophotometric analyses were made in a Zeiss Spektral photometer model PMQII.

Measurements of pH were performed with a Radiometer pH-meter, model PHM 25 SE with a relative accuracy of ± 0.02 pH units.

Study of pH for optimal activity. Samples of the main components of staphylokinase A and C respectively (cf. fig. 2) were incubated with mixtures of plasminogen and the various buffer solutions indicated in fig. 5. Casein solution was added after 6 min. and the assay continued as indicated above.

Isoelectric focusing and separation of proteins. This method was used to separate staphylokinase from other extracellular proteins produced by *S. aureus* as described earlier (Vesterberg *et al.*). However, in this study Ampholine pH 5–7 (LKB-Produkter, S-161 25 Bromma 1, Sweden) was used to obtain a higher resolution.

Dialysis. The enzyme solutions were dialysed against 0.05M-phosphate buffer, pH 7.0. Before isoelectric focusing the samples were dialysed against 1 per cent. (w/v) glycine.

Chemicals used, unless otherwise stated, were of reagent grade. The metal salts were from Merck, Darmstadt, Germany.

FIG. 2.—Staphylokinase activity (increase in A₂₈₀ per 30 min.), ○—○, after a second isoelectric focusing (see text) in a pH gradient mainly ranging from pH 5 to 8; pH at +4°C, ●—●. 0.01 ml of the fractions were used in each assay. Protein content (A₂₈₀ nm) ★—★.

RESULTS

Isoelectric focusing of staphylokinase

The concentrated and dialysed culture supernatant originating from 2.5 l was applied to a column for isoelectric focusing (LKB-Produkter) of 110-ml capacity (Vesterberg *et al.*). After the first separation in a pH gradient ranging from pH 2 to 10, one broad peak of staphylokinase activity was obtained (fig. 1) with a maximum at pH 6.5. The active fractions were pooled and then applied to a new column. This was prepared with ampholytes giving a narrower pH gradient mainly ranging from pH 5 to 8. The staphylokinase was now found in three peaks with *pI* at pH 5.8 (A), 6.2 (B) and 6.8 (C) (fig. 2). The two most acidic components were not quite separated. The reproducibility of *pI* in different experiments with material from four different cultivations was

very good, but the relative amounts of staphylokinase in different peaks varied, e.g., in some experiments peak A contained twice as much activity as peak C.

Degree of purification of staphylokinase

The staphylokinase activity per ml of crude supernatant was 3.5 units (mean value of several cultivations). The protein concentration of this material was about 0.1 mg per ml. This gives an approximate specific staphylokinase activity of 35 units per mg of protein. Purified component C had a specific activity of about 1.9 units per μg of protein. Staphylokinase was thus purified about 55 times. The specific activity of component A was somewhat less. For component B no value can be given because of the very low protein concentration and low activity. In most runs the protein concentration of fractions from the isoelectric column was estimated by absorbancy measurements at 280 nm, where $A_{280} = 1.00$ is assumed to correspond to 1 mg of protein per ml. The carrier ampholytes interfere with the Lowry method, but after long dialysis of the fractions (36 hr) this method gives almost the same result as A_{280} measurement, except in the acidic region of the gradient, where A_{280} gives a higher value partly due to oxidation products formed at the anode. The three components of purified staphylokinase were free from alpha haemolysin (Wadström, 1968), leucocidin, lipase, phosphatase and protease (Vesterberg *et al.*). DNA-ase activity was also studied (Wadström, 1967) and only staphylokinase B contained a minor part of this activity.

Properties of staphylokinase preparations

Stability. When the fractions containing staphylokinase, sucrose and Ampholine were stored at -20°C for 6 mth only small losses of activity were observed. However, component B was less stable than the other components. Repeated freezings and thawings of components A and C did not seem to have any effect on their activity. Fractions A, B and C and unpurified staphylokinase could be heated to 90°C for 10 min. without any detectable loss of activity. However, later experiments have shown that when staphylokinase is further purified by means of ion-exchange chromatography it is much more labile (Eriksson, Möllby and Vesterberg, to be published). When electrofocusing was applied to the heated culture supernatant no significant changes in the isoelectric point of staphylokinase were observed.

Recovery of activity after isoelectric focusing. The total staphylokinase activity increased about 100 per cent. after the first isoelectric separation. This was confirmed in several experiments. As a rule, no significant loss of activity was observed during the second separation. To find out whether the increase in activity was due to the presence of some inhibiting factor in the culture supernatant that was removed during electrofocusing, concentrated culture supernatant was heated to 96°C for 10 min. Portions of this material were mixed with purified staphylokinase (components A and C separately). The mixtures were kept at 37°C for 15 min. before assay. Untreated concentrated supernatant was also mixed with purified staphylokinase in the same way and

assayed. Controls without any additions were also included. Neither heated nor unheated supernatant had any effect on the activity of purified staphylokinase. Thus, this experiment gave no explanation for the gain of activity during isoelectric focusing.

Effect of some metal ions, ethylenediaminetetra-acetate (EDTA) and cysteine. When the metal salts or the other agents listed in the table were added simultaneously with the casein solution the effect observed was the influence on the caseinolytic activity of plasmin (the table, "inhibition of ... plasmin").

TABLE
Inhibition of staphylokinase activity by metal ions and by cysteine

Substance added	Percentage inhibition, by the substance added, of		
	the total activity* of component		plasmin†
	A (pI: 5.8)	C (pI: 6.8)	
H ₂ O	0	0	0
0.02M-NaCl	0	0	0
0.02M-CaCl ₂	88	88	66
0.02M-MgSO ₄	5	2	0
0.02M-MnSO ₄	94	93	96
0.02M-CuSO ₄	100	100	100
0.01M-Pb(Ac) ₂	60	70	45
0.01M-ZnSO ₄	88	98	91
0.02M-AgNO ₃	100	100	100
0.02M-cysteine	24	30	46
0.02M-CoCl ₂	97	96	97

* Substance added simultaneously with staphylokinase.

† Substance added simultaneously with casein solution.

For experimental details see text.

On the other hand, when the metal-salt solution was added simultaneously with staphylokinase the effect could be caused by its influence (1) on the conversion of plasminogen to plasmin promoted by staphylokinase, (2) on the caseinolytic activity of plasmin, or (3) on both (the table, "inhibition of ... total activity"). Many of the metal salts studied had an inhibitory effect on plasmin as well as on the total activity. In no case was it possible to see an isolated effect on staphylokinase, when allowance is made for a certain degree of uncertainty in the activity determination. None of the agents had an enhancing effect on the activity. The influence of different concentrations of lead acetate on the total reaction when staphylokinase component C was used is shown in fig. 3. The same type of curve was obtained with component A. The sodium salt of EDTA in concentrations higher than 0.01M had an inhibitory effect on the staphylokinase activity in unpurified material. Lower concentrations of EDTA had no effect.

pH for optimal activity. The pH-optima of the two main components

(A and C) were investigated. The optimal pH lies between pH 7.5 and 8.5 (fig. 4). There was no significant difference between the two components. The peak at pH 7.4 (fig. 4) could be the optimal pH of plasmin. Work is in progress to verify this hypothesis.

FIG. 3.—The effect of lead acetate ($Pb(Ac)_2$) on total reaction (i.e., salt added before activation). Staphylokinase activity (increase in A_{280} per 30 min.), $\circ$ — $\circ$. The figures at the abscissa indicate the final concentration of $Pb(Ac)_2$. 0.05 ml of staphylokinase C is here used as activating agent (see text).

FIG. 4.— pH optimum of staphylokinase component C (pI : 6.8). 0.05 ml of the staphylokinase solution was used for each assay. The pH values given are those in the reaction mixture before the addition of casein. Staphylokinase activity (increase in A_{280} per 30 min.) when the following buffers were used: acetic acid-sodium acetate (pH 4.2–6.9) $\bullet$ — $\bullet$; potassium dihydrogen phosphate-disodium hydrogen phosphate (pH 6.8–7.7) $\circ$ — $\circ$; tris (hydroxymethyl) aminomethane hydrochloric acid-tris (hydroxymethyl) aminomethane (pH 7.6–9.2) $\square$ — $\square$; and glycine-sodium glycinate (pH 8.7–9.4) $\triangle$ — $\triangle$. All buffers had an ionic strength of 0.1.

Kinetics. Fig. 5 shows the kinetics of activation of plasminogen by purified staphylokinase. Activation of plasminogen occurs in a few seconds. The kinetics of the reaction with components A and C were almost identical. Under

FIG. 5.—Activation of plasminogen by purified staphylokinase (component A). 0.02 ml of staphylokinase solution was incubated for various times with 8 caseinolytic units (c.u.) of human plasminogen, after which casein was added. Staphylokinase activity (increase in A_{280} per min.), $\bigcirc$ — $\bigcirc$. The $A_{280}^{1\%1\text{cm}}$ of the staphylokinase solution used was 0.09.

FIG. 6.—Effect of concentration of staphylokinase on rate of hydrolysis of casein (increase in A_{280} per 30 min.). Different amounts of purified staphylokinase (component A) incubated with 8 c.u. of human plasminogen for 6 min. The $A_{280}^{1\%1\text{cm}}$ of the staphylokinase solution used was 0.09.

the test conditions the rate of hydrolysis of casein was proportional to the staphylokinase concentration up to an increase in A_{280} per 30 min. = 1.00 (fig. 6). This was true both for purified and unpurified staphylokinase.

DISCUSSION

Glanville (1963) found staphylokinase activity in a single component after ion-exchange chromatography on CM-cellulose columns, but the enzyme seemed to be inhomogeneous on immunodiffusion. This was probably due to the presence of some contaminating protein. The work of Soru *et al.* (1959) seemed to indicate that staphylokinase was a homogeneous substance.

The present investigation showed that staphylokinase can be separated into three components by means of isoelectric focusing. All three components were identified in repeated experiments over a narrow *pH* range (5–7), but the relative amounts in the peaks varied from experiment to experiment. Further investigations will be made in order to find out the cause of the heterogeneity. Recent experiments indicate that staphylokinase can be separated into three components by means of disk electrophoresis and that these show identity in gel immunodiffusion (Arvidson *et al.*, to be published). The increase in activity after isoelectric focusing seems to suggest that there are inhibiting factors in the culture medium that are removed during this process.

It is difficult to estimate the degree of purification of staphylokinase, partly because of the small amount of protein in the fractions after isoelectric focusing, and partly because different methods for protein analysis were used at different stages in the purification. The protein concentration in the dialysed culture supernatant is about 0.1 mg per ml according to the Lowry method (Lowry *et al.*, 1951). A_{280} measurement on the same material gives a value about 100 times higher. The biuret method was used on concentrated culture supernatants, but this method was too insensitive for use in the later stages of purification. The carrier ampholytes interfered with the Lowry method for protein analysis, so extensive dialysis of the fractions from the isoelectric focusing columns had to be made. The protein concentration could then be estimated by the Lowry method. The accuracy and reproducibility of these tests was not satisfactory because of the low protein content in the fractions after the second electrofocusing. More accurate values will be reported when more staphylokinase is available. Efforts have therefore later been made to increase the production of staphylokinase and to use bigger culture volumes (Arvidson *et al.*, to be published).

The study of the optimal *pH* for staphylokinase activity and the influence of metal ions is rather complicated since, in fact, two reactions are studied in the assay: firstly, the activation of plasminogen to plasmin by staphylokinase, and, secondly, the digestion of casein by plasmin. By adding metal salt simultaneously with the casein it was possible to observe the influence of this on the caseinolytic activity (the table), but it was not possible to isolate the influence of metal salts on the activation of plasminogen to plasmin by staphylokinase.

Moreover, the *pH* is changed by adding different buffers to a solution containing plasminogen and staphylokinase. When the buffered solution of casein

(the substrate of plasmin) is added, the *pH* ought to be reset to 7.4, which is the *pH* used in all other assays. If plasmin has a different *pH*-optimum from staphylokinase, this might cause the appearance in the diagram of an extra peak of activity. One of the peaks in fig. 4 might therefore be due to the *pH* optimum for plasmin activity. This possibility calls for further investigation. We have not been able to find a record of the *pH*-optimum of human plasmin (caseinolytic activity) in the literature.

Work is in progress to purify staphylokinase further by chromatography on Sephadex gels and by ion chromatography combined with isoelectric focusing (Eriksson, Möllby and Vesterberg, to be published).

SUMMARY

Staphylokinase in the supernatant of cultures of *Staphylococcus aureus* was concentrated and then purified twice successively by isoelectric focusing. The total activity of staphylokinase was increased about 100 per cent. after the first isoelectric focusing. The cause of this was investigated. It was also shown by means of this separation technique that staphylokinase is a heterogenous protein, with isoelectric points at *pH* 5.8 (component A), 6.2 (component B) and 6.8 (component C). The degree of purification was calculated.

Some properties of the components were studied. The effects of the following metal ions: Ca^{++} , Mg^{++} , Mn^{++} , Cu^{++} , Pb^{++} , Zn^{++} , Ag^{+} and Co^{++} ; and of EDTA and cysteine, were examined. No significant differences in these respects between the three components were observed. The *pH* for optimal activity also seemed to be the same for the main components.

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