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PURIFICATION OF PENICILLINASE (β-LACTAMASE) AND ACID PHOSPHATASE FROM *STAPHYLOCOCCUS AUREUS* IN ONE PROCEDURE

WERNER SCHAEG, RECEP BINGÖL AND HANS BLOBEL
Institut für Bakteriologie und Immunologie der Justus Liebig-Universität, Giessen (Germany)

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SUMMARY

Penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) and acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) from *Staphylococcus aureus* were purified in one procedure. The enzymes were selectively removed from the culture supernatant by adsorptions onto glass beads and cellulose phosphate. Subsequent elution and gel filtration on Sephadex G-100, superfine, allowed the separation of the two enzymes in one step. The enzymatic activities of penicillinase and acid phosphatase were not markedly inhibited by their respective antibodies.

INTRODUCTION

During our studies on the penicillin resistance and pathogenicity of *Staphylococcus aureus* a number of strains, predominantly from cattle, were investigated. Most of these strains were inducible penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) producers. One of them, however, yielded a constitutive mutant, strain 408, with a high capacity for penicillinase production. Isolation of this strain enabled us to prepare purified penicillinase and also acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2).

MATERIALS AND METHODS

Bacterial strains

The constitutive penicillinase-positive *Staphylococcus aureus* strain 408 was selected on CY-agar[1] from an originally inducible culture of bovine origin. The strain was not hemolytic on sheep blood agar and gave a type C reaction on crystal violet medium[2]. *S. aureus* strain 8325 (kindly supplied by Prof. Richmond, Bristol) was also a constitutive penicillinase producer and gave a type C reaction, but proved to be hemolytic (mainly δ-hemolytic) on sheep blood agar.

Cultivation

100 ml LO_3 medium[3] with 1 ml 50% glucose, but without sodium lactate, in 1-l

flasks were inoculated with 1 ml of culture which had been previously incubated at 37 °C for 9 h in the same medium. The inoculum was freshly derived from a penicillin blood agar slant culture. For enzyme production the staphylococci were cultured at 37 °C and 75 rev./min on a rotary shaker for 15 h.

Assay procedures

Penicillinase was routinely estimated by the photometric method[4]. For quantitative determinations we used automatic titration[4]. One unit penicillinase hydrolyzed 1 μmole penicillin G in 1 h at 25 °C[4]. For qualitative estimation of the acid phosphatase the reaction mixture consisted of 0.2 ml sample, 0.2 ml citrate buffer (0.1 M citric acid and 0.1 M $MgCl_2$ adjusted to pH 5 with KOH) and 0.2 ml of a saturated aqueous solution of phenolphthalein phosphate pyridinium salt (Fluka, Eschborn, Germany). The reaction mixture was incubated at 37 °C for 30 min. After addition of 2 ml of 1 M Na_2CO_3, the mixture was placed in a 2-cm cuvette and analyzed photometrically (Photometer Eppendorf, Netheler and Hinz, Hamburg) at 546 nm. Acid phosphatase was determined quantitatively according to the method of Barnes and Morris[5]. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) was estimated by the use of phenolphthalein phosphate and a 0.1 M Tris buffer (pH 9) containing 0.1 mole $MgCl_2$, as described above. Coagulase, fibrinolysin, nuclease, lipase and egg yolk factor were also determined[6]. Protein was estimated by the method of Lowry *et al.*[7], with bovine serum albumin as a standard.

DNA was prepared by the method of Schmidt and Thannhauser[8] and identified with deoxyribonuclease (Calbiochem., Lucerne, Switzerland).

Adsorptions to glass beads

After centrifugation at 24 000 × *g*, 2 l of the culture supernatant were applied to a column of 150 cm × 4 cm. The column contained 400 g glass beads of 0.1 mm diameter, which had been suspended in distilled water and allowed to settle by gravity. After the supernatant had passed the column, 5 g cellulose phosphate (Whatman P-1) were added. The mixture was stirred magnetically for 1 h at room temperature. During this time 200 ml of distilled water were applied to the column to wash off nonadsorbed culture constituents from the glass beads. At the moment when the water level reached the surface of the glass beads, 50% satd $(NH_4)_2SO_4$ (pH 7.8) was applied to the column. After the remaining 80–85 ml distilled water had passed through the column—also noticeable by the appearance of a "schlieren" in the elution fluid—20 × 5-ml fractions were collected. The penicillinase activity of all fractions was estimated photometrically[4]. All fractions reducing by 0.3 the absorbance at 578 nm of the iodine–starch solution within 15 s were pooled. The fractions with lower penicillinase activity were saved for the first step in the elution of the cellulose phosphate.

Preparation of cellulose phosphate

The cellulose phosphate was collected on a porous porcelain candle by suction, after it had been used in two of the above-mentioned parallel adsorptions. It was washed with 100 ml distilled water. The water was removed and the adsorbed enzymes were eluted from the cellulose phosphate in a stepwise manner by 5 times 30 ml 50% satd $(NH_4)_2SO_4$ (pH 7.8).

Pressure filtration

All eluates from the glass beads and the cellulose phosphate were pooled and 600–700 ml were concentrated on a cellulose nitrate filter (type 12136, diameter of 25 mm, Membranfiltergesellschaft, Göttingen) at 11 atm N_2.

Sephadex chromatography

The concentrated enzyme preparation, containing 130–150 mg protein, was dissolved in 10 ml 50% satd $(NH_4)_2SO_4$ (pH 7.8) and clarified by centrifugation at 24 000 × *g* at 4 °C. Initially, the enzyme preparations were applied to a column (5 cm × 60 cm) filled with Sephadex G-100 (Pharmacia, Uppsala, Sweden). The flow rate was 17 ml/h at 4 °C. The Sephadex G-100 had been equilibrated with 50% satd $(NH_4)_2SO_4$ (pH 7.8). Finally, we used a column (5 cm × 72 cm) with 100 g Sephadex G-100, superfine, at 4 °C. The Sephadex G-100, superfine, had also been equilibrated with 50% satd $(NH_4)_2SO_4$ (pH 7.8). The speed of flow was adjusted to 9 ml/h with a peristaltic pump (LKB-Produkter, Stockholm, Sweden). Fractions of 12 ml were collected.

Polyacrylamide electrophoresis

The enzymes were characterized by polyacrylamide electrophoresis and amidoblack was used for staining[9].

Isoelectric focusing

20 mg of protein were applied to an electrofocusing column of 110-ml capacity, equipped with double cooling jackets (LKB-Produkter). The carrier ampholytes (LKB-Produkter) ranged from pH 3–10 and from 7–10. The density gradient was adjusted essentially by the method of Delmotte[10], except that the saccharose concentration in the dense column solution was 26 g instead of 27 g, and in the light column solutions I and II it was 1.5 g instead of 0.7 g and 0.5 g instead of 0.2 g. During the first 6 h of focusing the potential was 400 V and during the remaining 42 h 600 V.

Antisera

For the production of antibodies, respectively, 2 rabbits of about 2.5 kg were injected with purified penicillinase or purified acid phosphatase. Each rabbit received, as a first intramuscular injection into 4 different sites, 10 mg protein in 1 ml 0.14 M NaCl mixed with 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). 3 weeks later 10 mg protein of the purified enzyme preparations, either penicillinase or acid phosphatase in 1 ml 0.14 M NaCl, were injected intramuscularly into 2 different sites of each rabbit. The specific penicillinase activity was originally 35 000 units/mg protein and that of acid phosphatase 70 000 units/mg protein. There was, however, a considerable loss of enzyme activity during dialysis against 0.14 M NaCl. 10 days after the second injection the rabbits were exsanguinated. The antisera were stored at −18 °C. Precipitating antibodies could be demonstrated in the capillary ring test[11].

Immunelectrophoresis

Electrophoretic separation was conducted in 1% agarose gel (Schuchard, München) and 0.05 M acetate (pH 5) at 10 mA per slide (LKB-Produkter) in the range of

250–300 V for 2 h at 4 °C. After electrophoresis the respective antisera were applied. For identification of the precipitin lines, parallel electrophorograms were overlaid with media specific for penicillinase or acid phosphatase. The medium for penicillinase consisted of 1% agarose (Schuchard) with the iodine starch solution[4]. After solidification the indicator medium was flooded with a solution of 1% penicillin G in distilled water. The penicillinase reaction became visible after 5–10 min at room temperature. The medium for acid phosphatase activity contained 1% agarose and $1 \cdot 10^{-3}$ mole *p*-nitrophenyl phosphate per l, 0.1 M citric acid and 0.1 M $MgCl_2$ adjusted to pH 5 by addition of KOH. After solidification 1 M Na_2CO_3 was spread over the medium and 20–60 min later a positive reaction became visible by yellow spots.

Neutralization test

About 30 units penicillinase or 60 units acid phosphatase in 0.1 ml 0.14 M NaCl were incubated for 1 h at 37 °C with 10, 50 and 100 μl of their specific antisera. The enzyme activities were determined quantitatively. In a further test 0.5 ml of the antisera was incubated for 14 h at room temperature with 2 mg protein of the respective purified enzyme preparations in 0.5 ml 0.14 M NaCl. The precipitate was collected at 9000 × *g* and washed twice in 0.14 M NaCl at 9000 × *g*. The precipitate was dissolved in 5 ml 6 M urea, and the solution dialyzed against 0.14 M NaCl for 20 h at 4 °C. The newly formed precipitate was collected at 9000 × *g*, suspended in 0.1 ml 0.14 M NaCl and tested for enzyme activity as mentioned above.

RESULTS

Purification of the enzymes

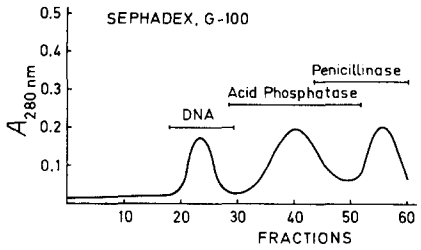
The primary goal of these studies was to isolate penicillinase from *S. aureus*. After incubation of the staphylococci in LO_3 medium for 14 h at 37 °C on a rotary shaker maximum concentrations of penicillinase were reached. At that time the culture medium supernatant usually had specific activities of approx. 1–2 units penicillinase and about 2 units acid phosphatase (Table I). Most of the penicillinase and acid phosphatase were removed from the culture supernatant by selective adsorption to glass beads and cellulose phosphate and could subsequently be eluted by 50% satd $(NH_4)_2SO_4$ (pH 7.8). The pooled penicillinase eluates had a specific activity of 11–13 units penicillinase and 23–25 units acid phosphatase. The enzymes were concentrated by pressure filtration.

TABLE I

ISOLATION OF PENICILLINASE AND ACID PHOSPHATASE FROM *Staphylococcus aureus* STRAINS 408 AND 8325

Activities are expressed as (*a*) μmoles penicillin G/h per μg protein and (*b*) μmoles *p*-nitrophenol/min per μg protein from glass beads and cellulose phosphate.

	(a) Penicillinase		*(b) Phosphatase*	
	408	*8325*	*408*	*8325*
Culture supernatant	1.8	0.9	2.1	1.8
Pooled eluate	11	13	23	25
Sephadex G-100, superfine	35	37	72	68



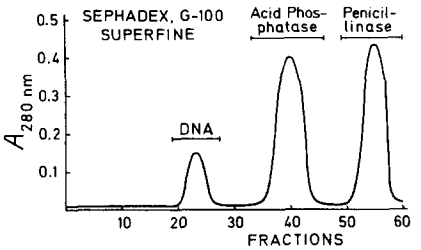


Fig. 1. Filtration on Sephadex G-100 of the crude enzyme preparation which had been previously concentrated by pressure filtration.

Fig. 2. Filtration on Sephadex G-100, superfine, of the crude enzyme preparation which had been previously concentrated by pressure filtration.

Initially, the enzyme preparations were applied to Sephadex G-100. This revealed 3 peaks. One contained mainly DNA, and the other 2 both penicillinase and acid phosphatase. Finally, penicillinase could not be separated from acid phosphatase (Fig. 1). However, Sephadex G-100, superfine, yielded 3 distinctly separate peaks. The 2 major peaks contained either penicillinase or acid phosphatase, the minor peak mainly DNA (Fig. 2). The relative enzyme activities corresponded with the absorbance at 280 nm. The separation of the 2 enzymes was complete. The 2 separated enzymes had no activities for coagulase, fibrinolysin, hemolysin, nuclease, lipase, alkaline phosphatase and egg yolk factor. The purification factor for penicillinase was 16–38; that for acid phosphatase approx. 35 (Table I).

Electrophoretic characterizations

Polyacrylamide electrophoresis revealed 1 identical band when purified penicillinase and acid phosphatase were applied separately or together (Fig. 3). This could not be changed by variations in the buffer within the acid range and by various polyacrylamide concentrations.

Isoelectric focusing of penicillinase and acid phosphatase was conducted be-

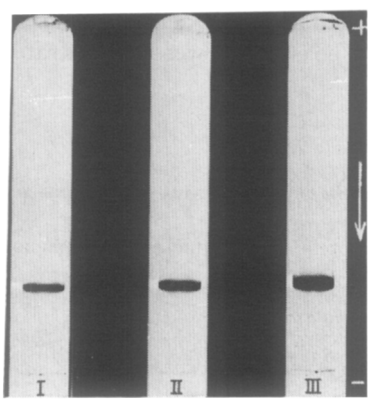


Fig. 3. Acrylamide electrophoresis of 50 μg penicillinase (I), 50 μg acid phosphatase (II), and a mixture of 50 μg penicillinase and 50 μg acid phosphatase (III). The enzymes had been previously purified by filtration on Sephadex G-100, superfine.

TABLE II

ISOELECTRIC POINTS OF PENICILLINASE AND ACID PHOSPHATASE FROM STAPHYLOCOCCAL STRAINS 408 AND 8323

The isoelectric points were determined by isoelectric focusing with deviations in pH of ±0.1.

Enzymes	*Components*	*From strains*	
		408	*8325*
Penicillinase	Major	9.25	9.55
	Minor	8.38	8.45
Acid phosphatase	Major	9.58	9.47
	Minor	8.40	8.42

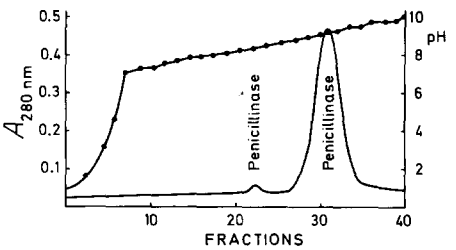


Fig. 4. Isoelectric focusing of penicillinase, previously purified on Sephadex G-100, superfine, in ampholine buffer in the range of pH 7–10. Penicillinase was in Fractions 20–38 with a maximum in Fraction 31.

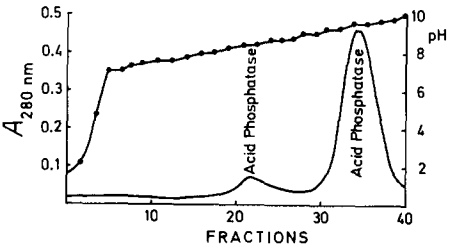


Fig. 5. Isoelectric focusing of acid phosphatase previously purified on Sephadex G-100, superfine, in ampholine buffer in the range of pH 7–10. Acid phosphatase was in Fractions 18–40 with a maximum in Fraction 34.

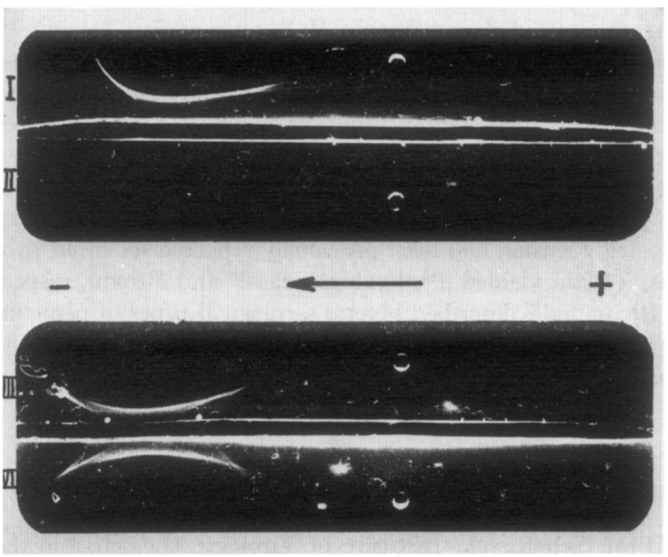


Fig. 6. Immunoelectrophorograms of acid phosphatase from *S. aureus*, strain 408 (I) with the homologous antiserum and the "DNA fraction" (II), and of penicillinase from *S. aureus*, strain 408 (III) with the homologous antiserum and penicillinase from *S. aureus*, strain 8325 (IV). All preparations used as antigens had been previosuly purified on Sephadex G-100, superfine.

tween pH 3–10. Both enzymes had isoelectric points above pH 9. Focusing between pH 7–10 revealed that the major activities of both enzymes were above pH 9 and minor activities below pH 9 (Table II, figs 4, 5).

Immunelectrophoresis of penicillinases from strains 408 and 8325 as well as acid phosphatase gave similar, if not identical precipitin lines (Fig. 6) which could be identified by the respective indicator media in an overlay-procedure. There were no serological cross-reactions between penicillinase and acid phosphatase.

Neutralization

Neither the penicillinase nor the acid phosphatase activities could be significantly inhibited by their respective antisera.

DISCUSSION

Glass beads[4] and cellulose phosphate[12] selectively adsorbed not only penicillinase, but also acid phosphatase of *S. aureus*. The enzymes could be eluted with good yields by 50% satd $(NH_4)_2SO_4$ (pH 7.8). There was no significant loss of enzyme activities in the eluent. Further purification of penicillinase on Sephadex G-100, superfine, proved to be more efficient than the multi-step procedure of Richmond[13]. Our method led to a complete separation of penicillinase and acid phosphatase. The relatively high isoelectric point of penicillinase could possibly be explained by its considerable content of lysin[13]. Because of their high isoelectric point penicillinase and acid phosphatase migrated only in acid buffers, unfortunately with identical R_F values. Electrophoretic separation of the enzymes was not achieved by variations in the buffer and in polyacrylamide concentrations. Thus, electrophoretic analyses did not yield a criterion for the degree of purity. The enzymes did not migrate in alkaline buffers. This had been observed already for acid phosphatase by Malveaux and San Clemente[14]. It was important, however, that in alkaline buffers neither penicillinase nor acid phosphatase yielded protein contaminations. Isoelectric focusing of penicillinase and acid phosphatase revealed 2 components. This could possibly be explained by some degree of enzyme denaturation at the low ionic strength, indicated also by a considerable loss in enzyme activity. Therefore, it appeared unlikely that the smaller second peak in the focusing diagram was an "isoenzyme". More likely, it was an artifact. Splitting into several peaks during isoelectric focusing had been previously reported for other substances of *S. aureus*. Thus, α-toxin yielded 4[15], hyaluronidase[16] and δ-toxin, respectively, 2 "isoenzymes"[15]. Richmond[17] described several serological types of penicillinases of *S. aureus* on the basis of specific serological inhibition of enzyme activity. The penicillinase and acid phosphatase isolated by us were not inhibited by specific antisera.

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