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PURIFICATION OF β-LACTAMASES ON QAE-SEPHADEX

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SUMMARY

The β-lactamase [penicillinase, or penicillin (cephalosporin) β-lactam amido hydrolase, EC 3.5.2.6] from *Klebsiella aerogenes* 1082E has been purified by chromatography on QAE-Sephadex at pH 7.5. The enzyme is adsorbed at this pH and eluted with increasing molarity of the buffer.

The β-lactamase from *Enterobacter cloacae* P99 has very different properties and is positively charged at pH 7.5. It has been purified by passage through QAE-Sephadex which binds contaminating protein but allows this β-lactamase to pass through unadsorbed.

Properties of the two enzymes, such as molecular weight, pH optimum, heat inactivation, substrate specificity and kinetic parameters (K_m and V), have been compared.

INTRODUCTION

The β-lactamases [penicillinase, or penicillin (cephalosporin) β-lactam amido-hydrolase, EC 3.5.2.6] from *Klebsiella aerogenes* 1082E and *Enterobacter cloacae* P99 are very different types of enzyme and the substrate specificities of partly purified preparations have been compared previously[1]. The *Klebsiella* strain was originally received from the Royal Hospital for Sick Children, Glasgow; its β-lactamase has been classified as type IVc[2] and, as far as we know, this type of Gram-negative β-lactamase has not been purified previously. The *E. cloacae* strain was first isolated at the Hospital for Sick Children, Toronto, Canada[3] and produces a β-lactamase classified as type Ia[2]; a very similar enzyme has been purified from *E. cloacae* 214 (ref. 4).

The preparations used in our previous study were shown to contain numerous protein components when examined by disc electrophoresis or immunoelectrophoresis[5] and attempts were made to purify the β-lactamases by chromatography on ion-exchange celluloses. Recoveries of enzyme activity were low until the combination of

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

QAE-Sephadex and TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid) buffer described in the present communication was tried. A good recovery of purified enzyme was achieved by the use of this combination. The method provides a convenient way of preparing purified samples of these two differently charged Gram-negative β-lactamases.

MATERIALS AND METHODS

Sephadex types G-50 and QAE A-50 were obtained from Pharmacia (Uppsala, Sweden). TES was obtained from B.D.H. Chemicals Ltd (Poole, England).

Growth of organisms

The medium and conditions for optimal production of the β-lactamases have been described previously[1].

Measurement of β-lactamase activity

Either the spectrophotometric (ultraviolet) method[6] or the iodometric method[7] was used. The amount of enzyme which hydrolyzed 1 μmole of cephaloridine/min at pH 7 and 37 °C was taken as 1 unit.

Enzyme stain

For detection of β-lactamase bands on disc electrophoresis either the cephaloridine–starch–iodide stain[5] or an indicator cephalosporin which changes colour when hydrolyzed by β-lactamase[8] was used.

Polyacrylamide disc electrophoresis

Gels were prepared as described by Tombs and Akroyd[9]. If the cephaloridine–starch–iodide stain was to be used, soluble starch (0.1%, w/v) was added to the gel components and the mixture was boiled for 0.5 min. 14.5 mM Tris–30 mM glycine buffer (pH 8.7) and a current of 4–5 mA/gel was used. Protein bands were located with Coomassie blue stain.

Molecular weight determination

Molecular weights were measured by the disc electrophoresis method of Weber and Osborn[10], except that the quoted gel buffer was diluted 20-fold.

Protein assay

Protein was measured by the method of Lowry *et al.*[11] using bovine serum albumin as the standard.

RESULTS

Purification of the β-lactamase from K. aerogenes 1082E

The enzyme from five 50-ml cultures of *K. aerogenes* 1082E was purified in each batch procedure. All steps were carried out at 4 °C. Samples were taken for β-lactamase and protein assay after each step. The purification is summarised in Table I.

Step 1. Ultrasonic disruption. A cell pad, obtained by centrifugation of the

TABLE I

PURIFICATION OF THE *K. aerogenes* β-LACTAMASE

Purification step	*% Yield activity*		*Volume (ml)*	*Total units*	*Total protein (mg)*	*Specific activity (units/mg protein)*
	Step	*Overall*				
1. Ultrasonic disruption	100	100	46	2576	639.4	4
2. High speed centrifugation	89.5	89.5	44	2305	480	4.8
3. Sephadex G-50 chromatography	91.3	81.8	146	2106	119.7	17.8
4. Concentration and equilibration	76	62.1	55	1600	92.4	17.4
5. QAE-Sephadex chromatography	68.2	42.4	840	1092	*	*
6. Final concentration step	55	23.3	46	600	3.7	163

* Protein value too low for accurate measurement.

culture at 5400 × *g* for 45 min, was washed in 50 ml 0.1 M phosphate buffer, pH 7.0 No activity was leached out. After resuspension in the phosphate buffer (45 ml) the cells were broken by ultrasound treatment for 3 min at 20 kHz with ice–water cooling (500-W generator, Measuring and Scientific Equipment Ltd, London).

Step 2. High speed centrifugation. Cell debris was removed by centrifugation at 105 000 × *g* for 90 min.

Step 3. Sephadex G-50 chromatography. The cell-free supernatant was layered onto a Sephadex G-50 column (25 cm × 6 cm) equilibrated in the phosphate buffer. Elution at 55 ml/h gave two protein peaks (Fig. 1). All the detectable β-lactamase activity was contained in the first peak.

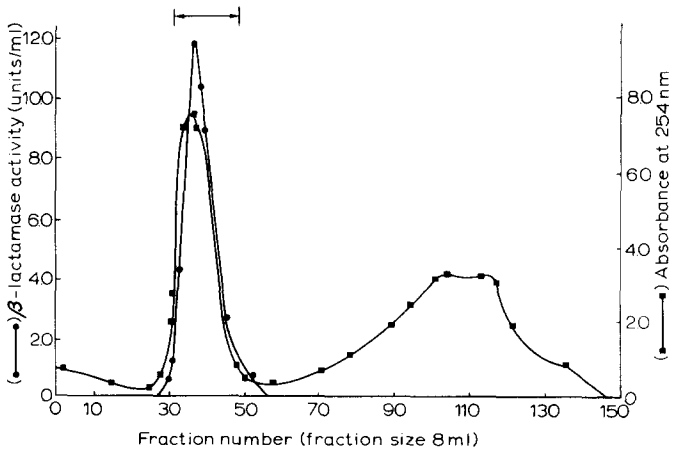

Fig. 1. Chromatography of the *K. aerogenes* β-lactamase on Sephadex G-50 (25 cm × 6 cm) equilibrated in 0.1 M phosphate buffer (pH 7.0). Elution at 55 ml/h gave two protein peaks. All the detectable β-lactamase activity was associated with the first peak. The fractions pooled are indicated.

Step 4. Concentration and equilibration. The active peak from Step 3 (146 ml) was dialysed overnight against demineralised water, concentrated to 53 ml by freeze-drying and then equilibrated against 0.025 M TES buffer (pH 7.5). Nearly a quarter of the activity was lost during this operation.

Step 5. QAE-Sephadex chromatography. Concentrated enzyme from Stage 4 was applied to a column of QAE-Sephadex (23 cm × 5.5 cm) equilibrated in the 0.025 M TES buffer and eluted at 55 ml/h. No β-lactamase activity was detected in the first 160 fractions (5.5 ml each). Very low levels of protein were eluted. Elution with 0.1 M TES buffer (pH 7.5) was started; Fractions 281–430 contained β-lactamase and were combined. Then 0.5 M NaCl was passed through the column and a second peak was obtained which contained most of the protein recovered from the column and slight β-lactamase activity. Further elution with 1 M NaCl removed a small amount of protein without detectable β-lactamase activity. The elution profile is shown in Fig. 2.

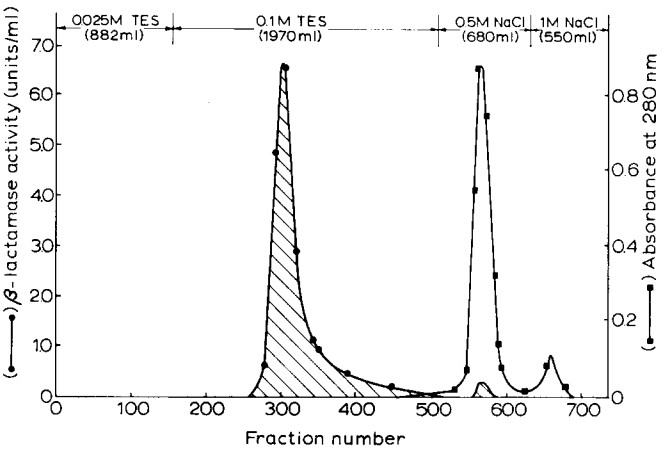

Fig. 2. Chromatography of the *K. aerogenes* enzyme on QAE-Sephadex. The conditions are described in Results.

Step 6. Final concentration. The active fractions from the QAE-Sephadex column were concentrated and dialysed by ultrafiltration using a UM-10 membrane (Amicon Ltd, High Wycombe, England) at 15 lbs/inch^2 N_2 with minimal stirring. Considerable losses of β-lactamase activity occurred during the process but the purified enzyme has been stored at −20 °C for at least a year without appreciable loss of activity.

Purification of the β-lactamase from E. cloacae P99

The β-lactamase from five 50-ml cultures of *E. cloacae* was purified by essentially the same method as that used for the purification of the β-lactamase from *K. aerogenes*. The purification is summarised in Table II. Some β-lactamase activity was detected in the culture supernatant, but only intracellular enzyme was purified. The elution profile after Sephadex G-50 chromatography was very similar to that ob-

TABLE II

PURIFICATION OF THE *E. cloacae* β-LACTAMASE

Purification step	*% Yield activity*		*Volume (ml)*	*Total units*	*Total protein (mg)*	*Specific activity (units/mg protein)*
	Step	*Overall*				
1. Ultrasonic disruption	100	100	35	1390	420	3.3
2. High speed centrifugation	98	98	34	1360	280	4.6
3. Sephadex G-50 chromatography	98	96.3	160	1340	120	11.2
4. Concentration and equilibration	52.0	50.0	48	696	105.6	6.6
5. QAE-Sephadex chromatography	96.8	49.1	232	673	*	*
6. Final concentration step	97.4	47.4	71	646	4.97	134.3

* Protein value too low for accurate measurement.

tained with the *K. aerogenes* enzyme and the concentration and equilibration (Step 4) again caused considerable loss of activity.

The main difference in the purification of the two enzymes occurred at Step 5. The *K. aerogenes* β-lactamase was negatively charged in 0.025 M TES buffer (pH 7.5) and was adsorbed on the QAE-Sephadex column, whereas the *E. cloacae* enzyme was positively charged at this pH and passed straight through the column. Contaminating proteins in the partially purified *E. cloacae* enzyme preparation from Step 4 were adsorbed on to the column until they were eluted successively with 0.1 M TES, 0.5 M NaCl and finally 2 M NaCl. The elution profile is shown in Fig. 3. The purified β-lactamase has been stored at −20 °C for at least a year without appreciable loss of activity.

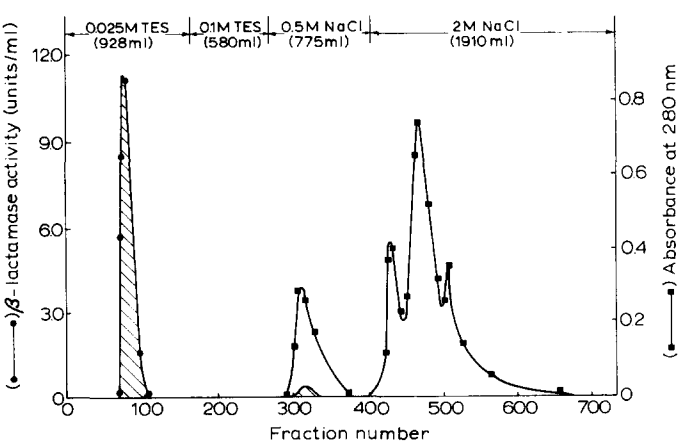

Fig. 3. Chromatography of the *E. cloacae* enzyme on QAE-Sephadex. The conditions are described in Results.

Properties of the β-lactamases

The purity of the final products was examined by polyacrylamide gel electrophoresis. As shown in Fig. 4, a protein band which coincided with the β-lactamase activity was obtained with each of the purified enzymes. The *K. aerogenes* β-lactamase moved towards the anode during electrophoresis (mobility 0.57 compared to the bromophenol blue marker at pH 8.7); the *E. cloacae* β-lactamase remained at the cathodic end of the gel (mobility 0.16).

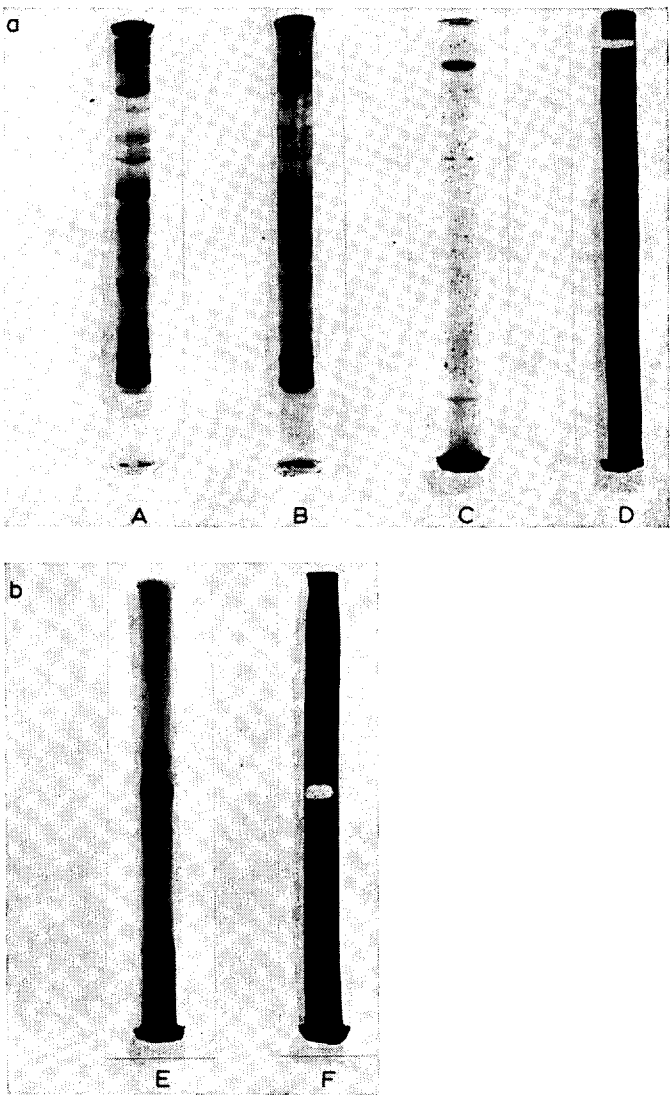

Fig. 4. Polyacrylamide disc electrophoresis of β-lactamase preparations at various stages of purification. (a) *E. cloacae* β-lactamase. A, cell-free supernatant after Step 2; B, after chromatography on Sephadex G-50, Step 3; C, purified enzyme after Step 6; D, enzyme stain (cephaloridine–starch–iodide) of C. (b) *K. aerogenes* β-lactamase. E, purified enzyme after Step 6; F, enzyme stain of E. Protein was located by staining with Coomassie blue in A, B, C and E.

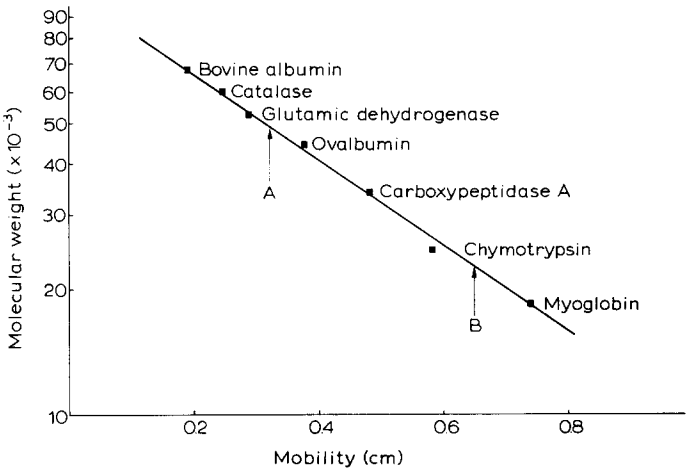

Fig. 5. Estimation of the molecular weights of the purified β-lactamases by disc electrophoresis. Arrows refer to *E. cloacae* β-lactamase (A) and *K. aerogenes* β-lactamase (B).

Molecular weights of the enzymes, determined by disc electrophoresis, were 23 000 ± 4000 for the *K. aerogenes* β-lactamase and 49 000 ± 3000 for the *E. cloacae* β-lactamase (Fig. 5).

A comparison of the rates of hydrolysis of cephaloridine solutions (50 μg/ml) at various pH values by the two enzymes was made using the ultraviolet assay at 37 °C. The cephaloridine solutions were made up in 0.1 M phosphate buffers at pH values from 4.6 to 9.0. The averages of triplicate determinations of activity at each

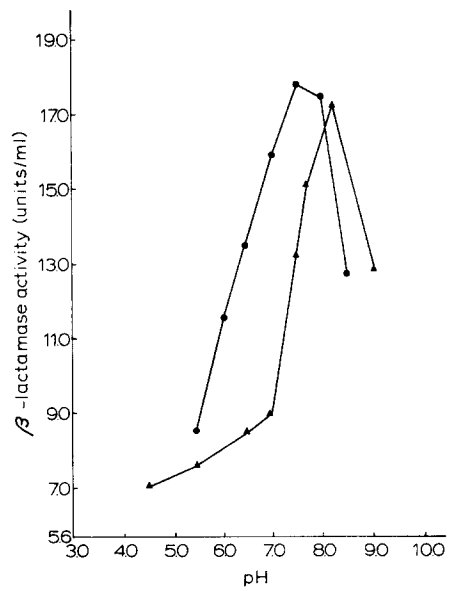

Fig. 6. pH–activity curves of the purified β-lactamases. ●—●, *K. aerogenes* enzyme; ▲—▲, *E. cloacae* enzyme. Ultraviolet assay at 37 °C in 0.1 M phosphate buffers.

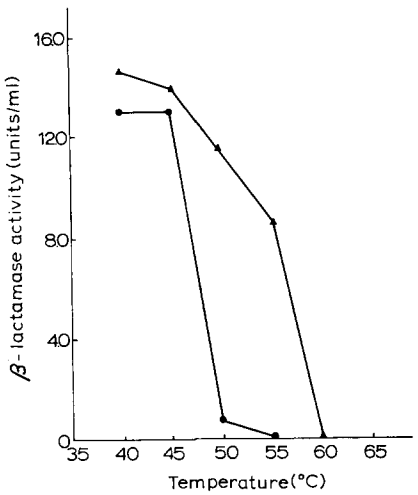

Fig. 7. The effect of temperature on β-lactamase activity. The purified enzymes were heated at various temperatures for 30 min before β-lactamase activity was measured (ultraviolet assay at 37 °C). ●—●, *K. aerogenes* enzyme; ▲—▲, *E. cloacae* enzyme.

pH were plotted against pH as shown in Fig. 6. The optimum pH for the *K. aerogenes* enzyme was 7.5; for the *E. cloacae* enzyme it was pH 8.2.

The heat stability of each enzyme was examined by measuring the activity of enzyme solutions heated in a water bath at 40, 45, 50, 55 and 60 °C for 30 min and then assayed at 37 °C. As shown in Fig. 7, the activity of the *K. aerogenes* enzyme fell sharply between 45 and 50 °C. The *E. cloacae* enzyme had lost only 20% of its initial activity after 30 min at 50 °C but activity fell rapidly on heating at higher temperatures.

The substrate profiles of the two purified enzymes were determined using the iodometric assay and are shown in Table III. Cephalosporins 5/1 and 291/1 are 3-

TABLE III

COMPARISON OF THE SUBSTRATE SPECIFICITIES AND KINETIC PARAMETERS OF THE *K. aerogenes* AND *E. cloacae* β-LACTAMASES

Substrate (5 mM) was incubated with enzyme at 30 °C for 5 min before the amount of substrate hydrolysed was measured by the iodometric assay. The relative rates of hydrolysis shown in the table are expressed as a percentage of the value for cephaloridine hydrolysis. K_m and V values were obtained from ultraviolet assay results with cephaloridine as substrate.

Substrate	*K. aerogenes*	*E. cloacae*
Cephaloridine	100	100
Cephalothin	80	25
Cephalosporin C	20	80
Cephalexin	10	15
5/1	5	0
291/1	1.5	0
Benzyl penicillin	200	1.5
Ampicillin	185	0
Methicillin	10	0
Cloxacillin	15	0
K_m (μM)	173	258
V (μmoles/min per mg protein)	187	313

acetoxymethyl-7β-(2′,6′-dimethoxybenzamido)-ceph-3-em-4-carboxylic acid and 3-acetoxymethyl-7-β-(3-(*o*-chlorophenyl)-5-methylisoxazole-4-carboxamido)-ceph-3-em-4-carboxylic acid, respectively.

Kinetic parameters for the enzymes were obtained from Lineweaver–Burk plots. Cephaloridine was selected as the substrate and the ultraviolet assay was used. Values for K_m and V are given in Table III.

DISCUSSION

Comparison of the properties of the two β-lactamases emphasised the differences between them. A study of the substrate specificities confirmed that the *K. aerogenes* β-lactamase had a very wide range of activity, although it was predominantly a penicillinase, while the *E. cloacae* β-lactamase was a cephalosporinase and was relatively inactive against penicillins. The *E. cloacae* enzyme had a molecular weight more than twice that of the *K. aerogenes* enzyme and the charge difference between the two enzymes was illustrated by their behaviour on ion-exchange chromatography and on electrophoresis. Small differences were detected in pH optimum and heat stability.

Partly purified preparations corresponding to the product obtained after step 3 in the present process had been used in earlier studies with these enzymes[1]. Comparison of the substrate specificities of the purified enzymes (Table III) with those of the partly purified enzymes[1] showed that the substrate profile was not altered to a significant extent by purification. This indicated that the type of β-lactamase activity had not changed during purification and there was no evidence for the presence of more than one β-lactamase in each strain. However, in recent experiments with heavily loaded polyacrylamide gels (200 μg protein), reproducible patterns were obtained in which some minor β-lactamase bands were detected in addition to the major β-lactamase band.

Hennessey and Richmond[4] showed that the inducible β-lactamase from *E. cloacae* 214 had very similar properties to the *E. cloacae* P99 enzyme; the two enzymes also gave a reaction of identity against P99 antiserum[5]. However, the molecular weight of the P99 enzyme (49 000) was much higher than that obtained for the 214 enzyme (14 000 (ref. 4)), Recent studies using an isoelectric focusing technique (Matthew, M., Boulton, M. G. and Ross, G. W., unpublished) have shown that both β-lactamases give a similar pattern of enzyme bands, but that the major bands focus at different pH values; work is in progress to check the molecular weights of these major bands.

Essentially the same method was used to purify the β-lactamases from *K. aerogenes* and *E. cloacae* despite their very different properties. The method was originally developed for purification of the *K. aerogenes* β-lactamase which had an isoelectric point of pH 6.5 and was adsorbed by QAE-Sephadex equilibrated in 0.025 M TES buffer (pH 7.5). Approximately 70% of the β-lactamase activity was recovered from the column; the active fraction contained only 4% of the protein loaded on to the column.

The β-lactamase from *E. cloacae* P99 had an isoelectric point of pH 7.9 and was not adsorbed on QAE-Sephadex equilibrated in 0.025 M TES buffer at pH 7.5. The contaminating proteins were strongly adsorbed under these conditions and nearly all the β-lactamase activity (97%) was recovered; the active fraction contained only

5% of the protein loaded on to the column. This high recovery of activity from the column was probably achieved because the labile enzyme passed through the column and so was less likely to become denatured or otherwise lost during ion exchange.

Other methods of concentration were examined in attempts to reduce loss of activity during concentration and dialysis (Steps 4 and 5), but ultrafiltration remained the method of choice. Freeze-drying was also used successfully, particularly when subsequent removal of inorganic ions was not required. Losses during dialysis could be reduced by addition of 0.1% gelatin or 5 mM dithiothreitol, but this was not done during the purification described above.

A good recovery of highly purified samples of both β-lactamases was obtainable by this method.

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