β-LACTAMASE ASSAY USING 2,2′-BICINCHONINATE AT NEUTRAL pH AND ROOM TEMPERATURE

Sir:

The iodometric procedure[1] has been very commonly used for estimation of β-lactamase activity. In modifications which increase assay sensitivity[2,3] the iodine may affect the enzyme while cellular amylases may give distorted results due to hydrolysis of starch in the mixture. β-Lactamases have also been estimated from the reduction of Cu(II) by penicilloates with neocuproine as the detecting agent[4]. Investigations into the action of copper ion on penicillins[5,6] in this laboratory have led to the use of 2,2′-bicinchoninate, a reagent used for assay of proteins[7], for estimating β-lactamase activity. Details are reported.

Color reagent consisted of 1.5% sodium bicinchoninate and 0.75% sodium potassium tartrate in 0.05 M phosphate buffer pH 7. Solution containing β-lactamase (0.4 ml) was mixed with 1.6 ml of 6.25 mM penicillin and after incubation at 37°C with shaking 0.1 ml samples were added to 0.1 ml ice cold 2.5% TCA. This was immediately followed by addition of 2.7 ml of color reagent and tubes were allowed to equilibrate to room temperature (20~25°C). The color reaction was initiated by addition of 0.1 ml of 20 mM $CuSO_4 \cdot 5H_2O$ and terminated after 10 minutes by addition of 0.1 ml 2×10^2 mM EDTA in buffer. Readings were made at 562 nm after centrifugation where required.

Labpenase (β-lactamase from Commonwealth Serum Laboratories, Melbourne) was diluted 1/1,000 for use. After 1 hour incubation under the conditions described above a specific activity of 8.3 mmol of benzylpenicilloate (BPOA)/μg protein was noted. The rate of formation was linear with time and it was also proportional to enzyme concentration at lower dilutions. Organisms containing β-lactamase were grown in nutrient broth with shaking and after 8 hours methicillin was added to a concentration of 1.2×10^{-3} mM[2]. Cells were harvested after 24 hours, washed with buffer, concentrated 10-fold and enzyme activity measured as described above after 1/2, 1, 2, 3, 4, 5 hours incubation. The supernatant fluid of the growth broth was used without further treatment and samples tested for BPOA formation after 0.33, 0.67, 1, 1.5, 2.25 hours incubation.

Fig. 1. Reaction of bicinchoninate with varying concentrations of BPOA (●) in presence of sodium potassium tartrate as described in text (○) in absence of sodium potassium tartrate.

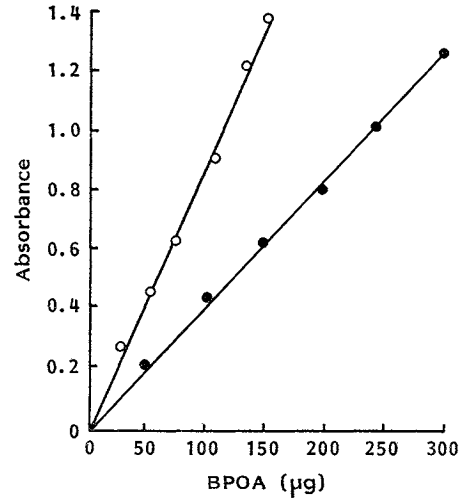
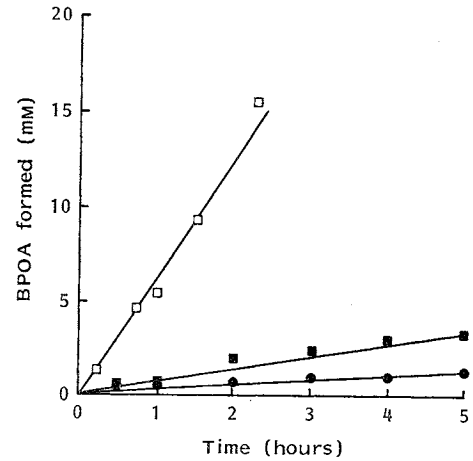


Fig. 2. Estimation of β-lactamase activity using bicinchoninate assay.

Cells of *Escherichia coli* (●) and *Bacillus subtilis* (■) and supernatant fluid of the growth broth of *B. subtilis* (□) were used.



Sodium potassium tartrate depressed colour of the BPOA standard curve (Fig. 1) but was used routinely to obviate potential for precipitates. The blank was lower than that obtained with neocuproine and low enzyme activity was more readily detected by a change in colour of the test. Intracellular and extracellular activity in

culture broth (Fig. 2) could be measured as well as purified enzyme activity. Immediate neutralization by dilution eliminated any effect of TCA on penicillins and other potential reducing agents in the culture fluid and cells also showed minimal interference. The open β-lactam ring forms of phenethicillin, methicillin, ampicillin, 6-aminopenicillanic acid and cephalothin all reduced Cu(II) and could thus be used as β-lactamase substrates with this reagent. Potential modifications are now being examined to increase sensitivity without the heating step or higher pH which is used for proteins[7] and carbohydrates[8].

The overall mechanism has proved difficult to determine. At equilibrium, in the presence of excess copper, 4 mol of Cu(I) are formed per mol of BPOA added. Extrapolation of reduction rates to zero time gives an initial 2:1 stoichiometry. Penicilloates and penicic acid show an absorbance peak with copper in the wavelength range 320~400 nm and thiol groups can be detected with ELLMAN's reagent[9]. The most likely mechanism is multistage and involves an initial electron transfer from Cu(II) to open the thiazolidine ring between S-1 and C-2 followed by further copper catalysed degradation. This might include the formation of a disulfide dimer or a Cu(I) complex analogous to mercuric chloride induced penamaldates as well as the formation of penicillamine and penaldate derivatives.

W. L. BAKER

Chemistry Department,
Swinburne Institute of Technology,
John Street,
Hawthorn, Victoria 3122,
Australia

(Received October 24, 1988)

References

1) PERRET, C. J.: Iodometric assay of penicillinase. Nature 174: 1012~1013, 1954
2) NOVICK, R. P.: Microiodometric assay of penicillinase. Biochem. J. 83: 236~240, 1962
3) SYKES, R. B. & K. NORDSTRÖM: Microiodometric determination of β-lactamase activity. Antimicrob. Agents Chemother. 1: 94~99, 1972
4) COHENFORD, M. A.; J. ABRAHAM & A. E. MEDEIROS: A colorimetric procedure for measuring β-lactamase activity. Anal. Biochem. 168: 252~258, 1988
5) BAKER, W. L.: A note on the detection of penicillin acylase in *Escherichia coli* by the reaction of ampicillin with biuret reagent. J. Appl. Bacteriol. 49: 225~229, 1980
6) BAKER, W. L. & B. D. BLAKE: Estimation of penicillin concentrations and penicillin metabolising enzyme activities by the LOWRY reaction. J. Antibiotics 33: 1386~1387, 1980
7) SMITH, P. K.; R. I. KROHN, G. T. HERMANSON, A. K. MALLIA, F. H. GARTNER, M. D. PROVENZANO, E. K. FUJIMOTO, N. M. GOEKE, B. J. OLSEN & D. C. KLENK: Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76~85, 1985
8) MCFEETERS, R. F.: A manual method for reducing sugar determinations with 2,2'-bicinchoninate reagent. Anal. Biochem. 103: 302~306, 1980
9) ELLMAN, G. L.: Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70~77, 1959