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The Thermostability of Penicillinase

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SUMMARY: Extracellular penicillinase produced by the growth of five different strains of the genus *Bacillus* in casein hydrolysate is rapidly inactivated at 100°, but can be strikingly protected against such inactivation by addition of 1 % gelatin or high molecular weight substances present in tryptic digest and meat infusion broth.

Since Abraham & Chain (1940) first described penicillinase there have been conflicting reports on the thermostability of the enzyme. Most workers (see Florey, Chain, Heatley, Jennings, Sanders, Abraham & Florey, 1949) have reported rapid destruction on heating above 50° in aqueous solution at neutral pH. Duthie (1944), however, showed that penicillinase in the filtrate of a broth culture of *Bacillus subtilis* was unaffected by heating at 100° for 20 min. at pH 7·0, and Smith & Smith (1945) reported that penicillinase from a strain of paracolon bacillus was stable at 100° for 30 min. when dry, but was completely inactivated in aqueous solution. In view of these reports it was decided to test a number of penicillinase preparations from different bacterial cultures of the genus *Bacillus* to discover whether their thermostability was determined by the strain of organism used.

METHODS

Media and materials. Casein hydrolysate medium (CH) was made from acid hydrolysed vitamin-free casein (Allen and Hanbury's Ltd.) 0·8 % (w/v) supplemented with 8×10^{-5} M-L-tryptophan, 2×10^{-4} M-DL-cystine, $1 \cdot 7 \times 10^{-3}$ M-MgSO₄ and 0·02 M-phosphate buffer, pH 7·0.

Infusion broth was prepared as described by Wright (1933), but using only a quarter of the amount of water, to give a concentrated medium. Tryptic digest broth was prepared as described by Hartley (1922).

The penicillin used was sodium benzylpenicillin from Glaxo Laboratories Ltd. The gelatin solutions were prepared from 'Difco' standardized gelatin.

Five commercial penicillinase preparations (A–D and F) and the strains used in their production, with the exception of strain F, were obtained. These commercial strains were called (A) *B. licheniformis*, (B) *B. cereus*, (C) *B. subtilis* and (D) *B. subtilis* and were used for the laboratory penicillinase preparations together with a penicillinase-producing strain of *B. cereus* (NRRL 569 designated here as E).

Enzyme production. Washed cell suspensions were prepared from CCY medium (Gladstone & Fildes, 1940) (organisms A–D) or tryptic digest broth (organism E) after incubation for 18–24 hr. at 35° on a bacteriological shaker. The dry weights were determined by opacity measurements on the 'Spekker' absorptiometer by reference to a standard curve of the relation between

opacity and dry weight. Cell suspensions at an initial concentration of 1 mg./ml. were incubated in 50 ml. CH medium or tryptic digest broth in a 250 ml. conical flask and penicillin (1–10 units/ml.) was added to increase the yield of enzyme. These cultures were shaken for 3–3½ hr. at 35° before the cells were centrifuged off. The supernatant fluids (having penicillinase activities from 200 to 1000 μ l. CO₂/hr./ml.) were stored at 2°.

Gelatinase activity. Strains were tested for gelatinase activity by incubating 'stab' cultures in broth containing 15 % (w/v) gelatin for 3 weeks at 18°; a positive result was recorded if any liquefaction had occurred. The culture supernatant fluids were similarly tested, after sterilization by filtration, by incubating 0.5 ml. on the surface of 2 ml. of the gelatin-broth in a test-tube at 18° for 5 days.

Penicillinase assay. The enzyme was assayed manometrically at pH 7.0 and 30° by the method of Henry & Housewright (1947), and the penicillinase activity expressed in μ l. CO₂ liberated/hr./ml. of medium. No corrections have been made for the small amounts of CO₂ retention in the different media, which will cause a slight error in the enzyme activity values. The flask constants did not vary enough for such retention to affect comparative results.

Heat stability test. A 250 ml. flask with a stoppered side arm was connected to a water cooled reflux condenser and placed in a boiling water-bath. Through the side arm, so made that a pipette passing through it could reach the base of the flask, approximately 8 ml. of enzyme solution were added rapidly. Samples of 2 ml. were removed at intervals and immediately placed in test-tubes, precooled in a beaker of ice, and kept cold until assayed.

RESULTS

The thermostability of the five commercial preparations was examined; three (A–C) were found to be fairly stable at 100°, whereas the other two (D, F) were rapidly inactivated (see Table 1).

Further studies were made of the thermostability of penicillinase from four of the organisms concerned (A–D) and also organism (E).

Gelatin. Enzyme produced in CH medium was found to be rapidly inactivated at 100° irrespective of the bacterial strain used or stability of the commercial preparation; but this inactivation was markedly reduced if 1 % (w/v) gelatin was added to the enzyme preparation before heat treatment. Enzyme produced in a 'complex' medium, e.g. tryptic digest broth, was inactivated only slightly at 100° and to about the same extent as CH preparations containing 1 % gelatin (Table 1 and Fig. 1). The concentration of gelatin which gave maximum protection was found to be 1 % when a range of concentrations from 0.01 to 2.5 % (w/v) was tested (Table 2). A twofold difference in concentration of enzyme (from growth of E in CH) did not affect the extent of protection afforded by 1 % gelatin.

The addition of 1 and 2.5 % gelatin to the commercial preparation (F) had no protective effect on the enzyme which was still completely inactivated after 2 min. at 100°.

Table 1. *Thermostability of penicillinase preparations*

Organism	Growth medium	Commercial preparation		Laboratory preparation			Time of heating (min.)
		Activity (μ l. CO ₂ /hr./ml.)	Residual activity (%)	Casein hydrolysate -gelatin (%)	Casein hydrolysate +gelatin* (%)	Hartley broth -gelatin (%)	
A. <i>B. licheniformis</i>	Papain digest 1:3 distilled water + 0.5% glucose	1080	—	38	92	—	2
			—	38	88	—	5
			46	—	—	—	15
			20	—	—	—	60
B. <i>B. cereus</i>	Difco yeast beef broth	4500	—	19	84	87	2
			—	16	80	83	5
			80	—	—	87	15
			27	—	—	—	60
C. <i>B. subtilis</i>	Tryptic digest broth + 1% glucose	600	90	< 0.1	75	—	2
			85	< 0.1	70	—	5
			73	—	—	—	15
			37	—	—	—	60
D. <i>B. subtilis</i>	Lemco broth	700	4	22	79	84	2
			< 0.1	13	73	74	5
			< 0.1	—	—	65	15
			< 0.1	—	—	—	60
E. <i>B. cereus</i> † (NRRL 569)	—	—	—	19	48	53	2
			—	14	42	47	5
			—	—	—	43	15
			—	—	—	—	60
F. Unknown†	Unknown	340	< 0.1	—	—	—	2
			< 0.1	—	—	—	5

Residual activity figures represent percentage of original activity remaining after heating at 100° for periods stated.

* 1% gelatin added to enzyme preparation.

† Positive gelatinase activity.

Fig. 1. Protective effect of 1% gelatin against inactivation of penicillinase at 100°. Enzyme was the supernatant fluid from a culture of organism B in casein hydrolysate.

Concentrated infusion broth. The addition of concentrated infusion broth to an equal volume of the enzyme preparation had the same protective effect as 1 % gelatin. Concentrated broth (25 ml.), when dialysed against glass-distilled water (4.5 l.) at 2°, suffered no loss in protective ability (Table 2).

Table 2. *Effect of added substances on heat stability of penicillinase from organism B grown in casein hydrolysate*

Exp.	Added substance	Percentage original activity remaining after treatment at 100° for	
		2 min.	5 min
I	Nil	7	4
	Infusion broth	71	69
	Infusion broth after dialysis for 24 hr.	71	—
II	Nil	19	16
	0.01 % gelatin	42	39
	0.05 % gelatin	55	56
	0.1 % gelatin	65	66
	1.0 % gelatin	84	80
	2.5 % gelatin	84	76
III	Nil	11	17
	5×10^{-3} M penicillin	56	50
	5×10^{-2} M penicillin	76	76
	5×10^{-3} M penicilloic acid	28	25
	5×10^{-2} M penicilloic acid	38	46

Penicillin, added to the enzyme solution cooled to 0° immediately before heating at 100°, protected the enzyme to some extent, whereas an equivalent concentration of the product of enzymic hydrolysis (penicilloic acid) had only a slight effect on the thermostability (Table 2). The penicilloic acid was prepared by incubating the penicillin and enzyme together at 30° before treatment. The greater part of the enzyme prepared as described in 'Methods' was formed adaptively, following induction by addition of the substrate. This preparation was compared with that of 'basal' enzyme produced without added penicillin, but no difference in thermostability could be detected (Manson & Pollock, to be published).

Miscellaneous. 8-Hydroxyquinoline at the concentration (8.3×10^{-4} M) used for inhibiting adaptation had no effect on thermostability, but another chelating agent ethylenediamine tetra-acetic acid ('Versene', Bersworth Chemical Co., Framingham, Mass.) in high concentrations (0.005 and 0.5 M) and gum acacia 1 % (w/v) each had slight protective properties.

DISCUSSION

The results show that the penicillinase in these preparations is rapidly inactivated in aqueous solution at 100°, but can be strikingly protected by the addition of gelatin or some high molecular weight compounds present in complex media. A similar observation with pectic enzymes has been made by

Matus (1948), who found that gelatin and other compounds gave some protection against heat inactivation up to 60°. This effect was shown by penicillinase from four of the five strains tested and to a lesser extent by that from the fifth strain (E). It thus appears that the heat stability of penicillinase in crude preparations depends on the other constituents present during heat treatment, rather than on the bacterial source. The decreased protective action of gelatin on the penicillinase from strain E and its inability to protect enzyme in commercial preparation F, may be partly due to destruction of the gelatin by the gelatinase known to be present in both these cases.

It is possible that the protective effect of gelatin is due to its combination with the enzyme to form a heat stable complex. This is indeed suggested by the time curves for penicillinase inactivation shown in Tables 1 and 2, where it can be seen that little further enzyme was destroyed by prolonging heat treatment from 2 to 5 min. The immediate loss in 2 min. might thus be due to inactivation of the labile unassociated enzyme and the slow subsequent loss to that of the fraction stabilized by combination with gelatin or other protecting substance. The stabilizing action of a substrate on its enzyme is well known, and the protective effect of penicillin against destruction of penicillinase at 100° is almost certainly due to the formation of a relatively stable enzyme-substrate complex.

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REFERENCES

- ABRAHAM, E. P. & CHAIN, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature, Lond.* **146**, 837.
- DUTHIE, E. S. (1944). The production of penicillinase by organisms of the subtilis group. *Brit. J. exp. Path.* **25**, 96.
- FLOREY, H. W., CHAIN, E., HEATLEY, N. G., JENNINGS, M. A., SANDERS, A. G., ABRAHAM, E. P. & FLOREY, M. E. (1949). *Antibiotics*, vol. II, ch. 33, London: Oxford University Press.
- GLADSTONE, G. P. & FILDES, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.
- HARTLEY, P. (1922). The value of Douglas's medium for the production of diphtheria toxin. *J. Path. Bact.* **27**, 479.
- HENRY, R. J. & HOUSEWRIGHT, R. D. (1947). Studies on penicillinase. II. Manometric method of assaying penicillinase and penicillin, kinetics of the penicillin-penicillinase reaction and the effects of inhibitors on penicillinase. *J. biol. Chem.* **167**, 559.
- MATUS, J. (1948). Untersuchungen über die Aktivität der Pektinase. *Ber. schweiz. bot. Ges.* **58**, 319.
- SMITH, W. & SMITH, M. M. (1945). Production of sterile and stable penicillinase. *Lancet*, i, 809.
- WRIGHT, H. D. (1933). The importance of adequate reduction of peptone in the preparation of media for the pneumococcus and other organisms. *J. Path. Bact.* **37**, 257.

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