BBA 66046

IODINE SENSITIVITY OF *BACILLUS CEREUS* PENICILLINASE

V. CSÁNYI, I. MILE, ILONA FERENCZ AND EVA SZABÓ
Institute of Medical Chemistry, University Medical School, Budapest (Hungary)

(Received October 3rd, 1969)

SUMMARY

Iodine sensitivity of *Bacillus cereus* penicillinase under various conditions (different iodine concentrations, pH, temperatures, *etc.*) was examined. The inactivation of the enzyme by iodine took place in two steps. During the first step about 30–35% of the enzyme was inactivated, and the rest of the enzyme activity disappeared under conditions favourable for the second step.

The conditions under which each of the steps of inactivation occurred were investigated.

INTRODUCTION

In the course of our studies on the regulation of penicillinase synthesis by *Bacillus cereus* 569 we made some observations suggesting that a certain role of the enzyme protein itself in the regulation of its own synthesis might not be ruled out[1,2]. It seemed to be of interest to start a closer study of the nature of penicillinase protein.

The behaviour of penicillinase in the presence of iodine was first examined by POLLOCK[3]. He found the exopenicillinase of *B. cereus* to be iodine-resistant at a given iodine concentration, unlike cell-bound penicillinase of the same species which showed a high sensitivity under similar conditions. Later CITRI and co-workers[4–6], while confirming these results, pointed out that under certain conditions the exopenicillinase may change its properties and become iodine-sensitive, like the cell-bound enzyme. Mild alkaline treatment, exposure to urea, and the presence of some competitive inhibitor of penicillinase were the most important circumstances in which the originally iodine-resistant exoenzyme could be transformed into an iodine-sensitive form. On the basis of these findings a transition between two conformations, different with respect to iodine sensitivity, was assumed.

Our recent experiments concerning the effects of different conditions (pH, temperature, iodine concentration, *etc.*) on iodine sensitivity have revealed some new facts influencing the evaluation of this property as a measure of conformational changes in exopenicillinase.

MATERIALS AND METHODS

Penicillinase

Exopenicillinase used in these experiments was prepared from the culture supernatant of the strain 569/H of *B. cereus* producing penicillinase constitutively. The cultures were grown as described previously[7]. At the end of the logarithmic phase the supernatant of the culture was stirred continuously for 1 h in the presence of Celite 535 (1 g per $2 \cdot 10^5$ units of penicillinase). The Celite was separated and washed in 0.01 M acetate buffer at pH 5.0 until the 280-mμ absorption of the washing solution disappeared. The adsorbent was then placed in a glass tube, and the penicillinase was eluted by a 50% saturated $(NH_4)_2SO_4$ solution at pH 8.0. Fractions containing more than $2 \cdot 10^4$ units per ml penicillinase were pooled. $(NH_4)_2SO_4$ was added to 80% saturation. The precipitate was dissolved in 0.01 M acetate buffer (pH 5.0), and a second chromatography on a Celite column was carried out. After dialysis of the eluate against 0.05 M phosphate buffer (pH 6.5), a highly purified enzyme preparation was obtained with a special activity of $2.12 \cdot 10^6$ units per mg N, similar to that reported by KOGUT *et al.*[8] for crystalline penicillinase of *B. cereus*. The penicillinase was kept at 0° after lyophilization.

Iodine treatment of penicillinase

The stock solution of iodine contained 0.1 M I_2 and 0.2 M KI. All the other concentrations used were prepared by dilution. The phosphate buffer was a mixture of KH_2PO_4 and Na_2HPO_4 solutions, each 0.05 M. The pH values were determined on a pH meter just before starting the measurement. Iodine treatment of the enzyme was carried out in small (6.0 ml) glass tubes and was interrupted by the addition of an equivalent amount of $Na_2S_2O_3$ solution. With temperatures higher than 0° the incubation mixtures (5 ml) were prewarmed to the appropriate temperatures for 5 min and the iodination was started by the addition of the enzyme in 0.1 ml solution. The control samples were passed through the same procedure without being treated by iodine.

Assay of penicillinase

After the iodine treatment the glass tubes containing the enzyme were placed in a prewarmed solution consisting of 10 ml 0.5% gelatin, 20 ml 0.05 M phosphate buffer (pH 6.5) and 0.06 g penicillin G. They were then incubated for 5 min at 30°. Enzymic reaction was stopped by the addition of 10 ml 0.5 M sodium tungstate in 1.0 M acetate buffer (pH 4.0). Penicilloic acid was measured iodimetrically and the results were expressed in units as described previously[9].

RESULTS

Since the iodine sensitivity of penicillinase was first described, a wide variety of different conditions (pH, temperature, iodine concentration, presence or absence of gelatin, *etc.*) has been employed in the iodine treatment. But the effects of these conditions on the iodination process have not been investigated. According to our experiments, these conditions have a great influence on the iodine sensitivity of penicillinase.

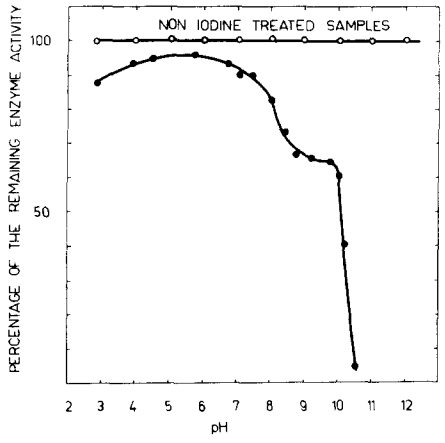


Fig. 1. Effect of pH on the inactivation of penicillinase by iodine. The reaction mixtures consisted of 300 units of penicillinase in 5 ml 0.05 M phosphate buffer at pH values indicated in the figure and 1 mM iodine. After 1 min of incubation at 0° the iodine was reduced by adding $Na_2S_2O_3$. The remaining enzyme activities of the respective mixtures were determined as described in MATERIALS AND METHODS. The residual activity is expressed as the percentage of activity of the samples treated in the same way, except that iodine was omitted.

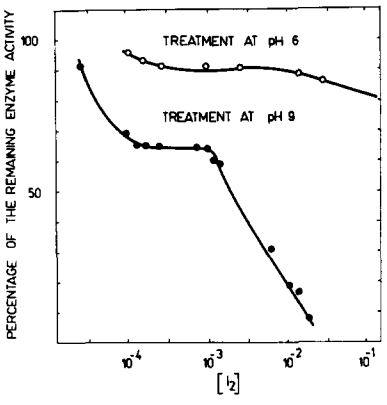
Effect of pH

For iodine treatment 0.001 M concentration of iodine, 0° and 1 min incubation time were chosen as standard conditions, similarly to those used by CITRI and co-workers[4–6]. The procedure was carried out in 5 ml 0.05 M phosphate buffer at pH values as given in Fig. 1. The same buffer was used in the whole pH range investigated, although the capacity of the buffer varied at different pH values. Nevertheless, working with the same buffer had a certain advantage, eliminating the different effects of different ions on the iodine sensitivity of exopenicillinase, not discussed here. After treatment the residual activity was measured. In the absence of iodine a considerable resistance of the enzyme towards alkaline pH was observed. Penicillinase samples may be incubated for more than 30 min at pH 10–11 and at 0° without any loss in activity. (The same results were obtained using other buffers, for example 0.1 M borate.)

When the pH was shifted from low towards higher regions, the inactivation process of penicillinase by iodine seemed to be divided into two major steps. At pH values between 2 and 7 only little of the penicillinase was inactivated during the treatment, the bulk of the enzyme remaining iodine resistant. When the pH was shifted towards alkaline values, the inactivation of the enzyme became more pronounced, though a wide plateau was observed between pH 8.5 and 10.0. The inactivation that had taken place before the plateau was reached was identified as a first step in the inactivation process. The second step of inactivation took place above pH 10 very rapidly. In the following experiments pH 6 and 9, *i.e.*, the values at the middle of the plateaus observed, were chosen as standard conditions for examining the influence of other factors.

Effect of iodine concentration

In the following experiments we studied the course of inactivation during



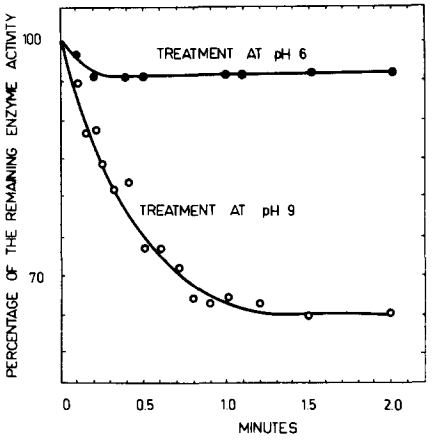


Fig. 2. Effect of iodine concentration. The reaction mixtures consisted of 300 units of penicillinase in 5 ml 0.05 M phosphate at pH 6 and 9 respectively, and various amounts of iodine. After incubation for 1 min at 0° the residual activities of the mixtures were determined and expressed as the percentage of activity of the untreated samples.

Fig. 3. Time-course of inactivation. The reaction mixture consisted of 300 units of penicillinase per ml in 0.05 M phosphate at pH 6 and 9, respectively, and 1 mM iodine. After treatment at 0° for various times, samples were removed and the remaining enzyme activity was determined and expressed as the percentage of the untreated samples.

iodine treatment carried out at different iodine concentrations. The samples were treated with iodine at pH 6 and 9, all the other invariable conditions being the same as above. The results are shown in Fig. 2.

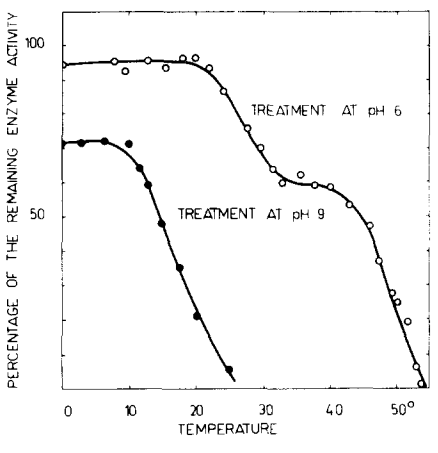
Some penicillinase was inactivated by low (0.5 mM) iodine concentrations, but the extent of inactivation remained at almost the same level until a 0.1 M concentration of iodine was reached at pH 6. In samples treated at pH 9 the amount of the inactivated enzyme was considerably higher than at pH 6, and the curve of inactivation showed a plateau between 0.5 and 2 mM iodine concentrations.

Time-course of inactivation by iodine

Fig. 3 shows the kinetics of inactivation of the iodine-treated enzyme at pH 6 and 9. The iodine concentration was 0.001 M. The time necessary for the partial inactivation at both pH values was less than 1 min.

Effect of temperature

In the following experiments we examined the influence of temperature on iodine treatment. The standard conditions were: 0.001 M iodine concentration, 1 min incubation time and 5 ml 0.05 M phosphate buffer at pH 6 and 9. The variation of temperature during iodine treatment and the extent of inactivation are shown in Fig. 4. These curves again reflect clearly the stepwise nature of penicillinase inactivation by iodine, showing two plateaus, when iodine treatment was carried out at pH 6. Along the first plateau (0–20°) only a slight amount (5%), while between 20–30° some 35% of the enzyme initially present became inactivated. The second plateau of 30–45° precedes the inactivation of the remaining penicillinase. Only one plateau was observed when iodine treatment was carried out at pH 9.



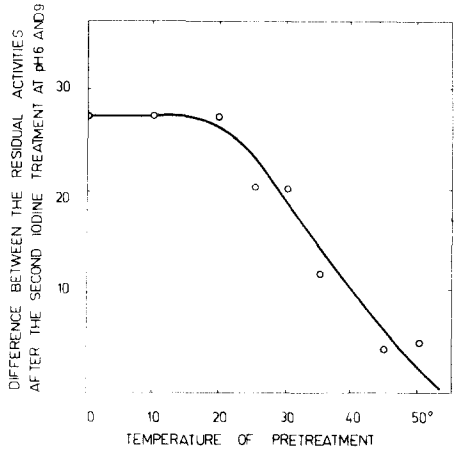


Fig. 4. Effect of temperature. The reaction mixtures consisted of 300 units of penicillinase added to 5 ml prewarmed 0.05 M phosphate at pH 6 and 9, and 1 mM iodine. The samples were treated at various temperatures for 1 min. The residual activities of the mixtures were determined and expressed as the percentage of the activity of samples treated in the same way, except that iodine was omitted.

Fig. 5. Effect of pretreatment. Samples of enzyme, 1500 units each, were pretreated with 1 mM iodine at different temperatures in 3 ml 0.05 M phosphate for 1 min at pH 6. After the residual activity had been measured a second iodination was carried out with a 1-ml part of the samples with 1 mM iodine at 0° and at both pH 6 and 9. All the samples were supplemented to 5 ml with 0.05 M phosphate at the appropriate pH value. The residual activities after the second iodine treatment are expressed as percentages of the original activity. The values obtained at pH 6 were subtracted from the values obtained at pH 9, and plotted against the temperature of the last iodine treatment.

The form of the curve obtained under the conditions of this experiment at pH 6 closely resembles that of Fig. 1 representing the effects of pH on the iodine treatment. In both experiments the heights of the second plateaus are some 65% of the remaining enzyme activity. The question arises, whether the reaction of the first major step in the inactivation process taking place at 0° and pH 9 is the same as the reaction occurring between 30 and 45° at pH 6. In order to obtain an answer to this question the following experiment was carried out. Samples of enzyme were treated with 0.01 M iodine at different temperatures for 1 min at pH 6, as described above. After this pretreatment a second iodination was carried out with each sample at both pH 6 and 9. The extent of inactivation of an untreated enzyme at pH 6 and 9 shows some 30% difference, in agreement with the results shown in Fig. 1. If the reactions of inactivation taking place during iodination of the pretreated enzyme at pH 6 but at relatively high temperature are the same as those occurring at 0° and at pH 9, the difference between the extents of the second inactivation at pH 6 and pH 9 must decrease with the rise of temperature during pretreatment. The results presented in Fig. 5 clearly show that the identity of the two reactions may be assumed.

Effect of protein concentration

All the measurements mentioned above were performed using highly diluted samples of the enzyme (1–10 μg/ml). It seemed to be of interest to establish whether

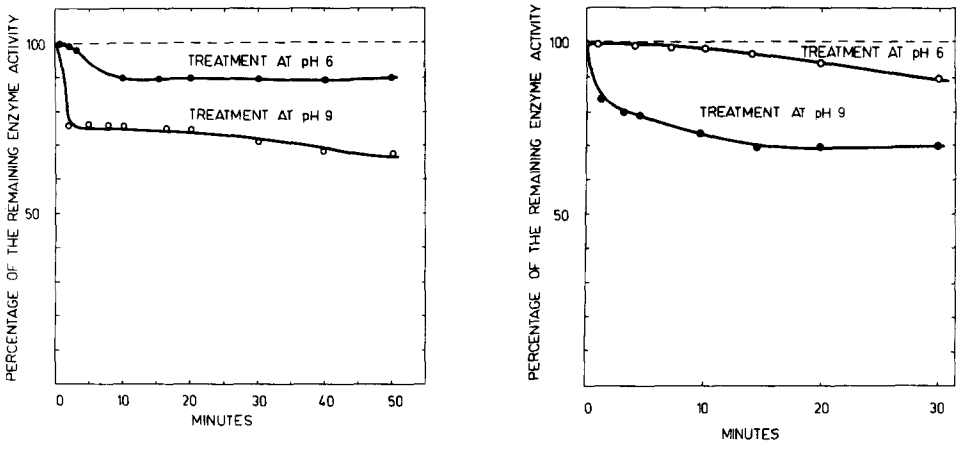


Fig. 6. Effect of protein concentration. The reaction mixture consisted of 0.75 mg penicillinase in 1 ml 0.05 M phosphate at pH 6 and 9, and 1 mM iodine. After treatment for various times at 0° the residual activities of the mixture were determined at an appropriate dilution and expressed as percentage of the activity of the untreated samples.

Fig. 7. Effect of the presence of gelatin. The reaction mixture consisted of 300 units of penicillinase in 5 ml 0.05 M phosphate containing 0.5% gelatin at pH 6 and 9, and 1 mM iodine. After treatment at 0° for various times the residual activity of the mixture was determined and expressed as percentage of the activity of the untreated samples.

iodine sensitivity was affected by the concentration of the enzyme. The experiments were carried out in the presence of 0.75 mg of penicillinase per ml, all the other conditions being the same as for the measurement of the time-course of inactivation of the diluted enzyme. The only exception was the volume of the incubation mixture which was only 1 ml in the iodine treatment. The remaining enzyme activity was measured after dilution. The results are shown in Fig. 6. No differences were observed in the extent of inactivation during iodine treatment even at the high enzyme concentration. Only little of the enzyme was inactivated at pH 6, and some 30% was inactivated at pH 9.

The effects of the enzyme dilution may be prevented by adding gelatin to the enzyme solution. This method was widely used by CITRI and co-workers[4-6]. Therefore, an examination of the effect of gelatin on the inactivation caused by iodine seemed to be necessary. We reinvestigated the time-course of the inactivation of the diluted enzyme in the presence of 0.5% gelatin. The results are shown in Fig. 7. In the presence of gelatin the inactivation of the diluted enzyme usually proceeded in the same way as with concentrated enzyme samples in the absence of gelatin (Fig. 6). The only difference we observed was that the rate of inactivation was considerably slower in the presence of gelatin, and the curves of inactivation were not so unequivocal. There seemed to be no advantage in using gelatin during measurements of iodine sensitivity, especially in short-time experiments.

DISCUSSION

The sensitivity of *B. cereus* exopenicillinase to iodine is known to be dependent on the conditions of the iodine treatment. CITRI and co-workers[4,5] have found exo-

penicillinase generally iodine resistant, but they also reported circumstances (urea treatment, adsorption on a glass surface, presence of competitive inhibitors, *etc.*) in which the resistance disappeared and the enzyme was inactivated in the presence of iodine. They assumed that a transition might occur between two conformations different with respect to iodine sensitivity.

In analysing the effects of the different conditions during the iodine treatment of penicillinase we concluded that the inactivation of the enzyme by iodine took place in two major steps depending on circumstances. Low temperature (0–10°) at pH 9 or higher temperature (30–45°) at pH 6 were favourable conditions for the first step of inactivation. The second step leading to the disappearance of the enzyme activity took place if the pH was above 10 at 0°, or if the temperature was over 45° at pH 6. The height of the inactivation plateaus observed in different circumstances seemed to be nearly constant. Some 30% of the enzyme activity was inactivated during the first step of inactivation, and some 70% disappeared during the second step.

There are several possible explanations for this phenomenon. The stepwise character of the penicillinase inactivation by iodine may be the consequence of the presence of penicillinase conformations differing in iodine sensitivity, as originally supposed by CITRI *et al.*[5], and the steps in the inactivation may reflect the transition of an iodine-resistant conformation to an iodine-sensitive one. But we cannot exclude some other possibilities, for example that the inactivation of each molecule of penicillinase takes place in two steps, *i.e.*, there are two reacting groups on the enzyme moiety that differ in iodine sensitivity, and only the iodination of both groups results in the total inactivation of the enzyme. Another possibility is that there are two different kinds of penicillinase responsible for different reactivity to iodine. The latter possibility is supported by the findings of J. IMSANDE (personal communication) who found three different fractions of exopenicillinase with respect to electrophoretic mobility. This finding was confirmed by our preliminary results, but the examination of these fractions on iodine sensitivity has not been completed. So the question remains open.

REFERENCES

1 V. CSÁNYI, G. JACOBI AND F. B. STRAUB, *Biochim. Biophys. Acta*, 145 (1967) 470.
2 V. CSÁNYI, *The Regulation of Penicillinase Synthesis in B. cereus*, Thesis, Budapest, 1964, p. 102.
3 M. R. POLLOCK, *J. Gen. Microbiol.*, 15 (1956) 154.
4 N. CITRI AND N. GARBER, *Biochim. Biophys. Acta*, 30 (1958) 664.
5 N. CITRI, N. GARBER AND M. SELA, *J. Biol. Chem.*, 235 (1960) 3454.
6 N. GARBER AND N. CITRI, *Biochim. Biophys. Acta*, 62 (1962) 385.
7 V. CSÁNYI, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 1 (1966) 149.
8 M. KOGUT, M. R. POLLOCK AND E. J. TRIDGELL, *Biochem. J.*, 62 (1965) 391.
9 V. CSÁNYI, *Acta Physiol. Acad. Sci. Hung.*, 18 (1961) 261.