STUDY OF THE *N*-ACETYLMURAMYL-L-ALANINE AMIDASE ACTIVITY IN *ESCHERICHIA COLI*

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1. Introduction

N-Acetylmuramyl-L-alanine amidases hydrolyze in the bacterial cell wall peptidoglycan the *N*-acetylmuramyl-L-alanine amide bond linking the glycan and peptide moieties of this heteropolymer. In some cases, these amidases are produced as extracellular enzymes which can be purified and used as valuable analytical tools for the specific degradation of cell wall peptidoglycans of other bacteria [1–4]. They have also been characterized as autolysins capable of digesting the cell walls to which they are more or less fixed [5–10]. Aside from their probable fortuitous function as autolysins, little is known about their true physiological role. In the case of *Bacillus subtilis* 168, the amidase which is the major autolysin [5], is fixed to the cell wall teichoic acid [11] and is perhaps someway involved with transformation competence [12, 13].

Escherichia coli contains at least five different enzymes which can degrade specifically the cell wall peptidoglycan into smaller fragments [14]. However, only two of them, carboxypeptidases I and II have been studied to some extent [15]. Pelzer detected in *E. coli* B a high level of amidase activity associated with the cell wall [16]. Schwarz and Weidel [17, 18] showed that this activity was partly responsible for the degradation of the peptidoglycan and of its precursors when growing cells of *E. coli* B are treated with penicillin.

In the present paper, general properties of the *N*-acetylmuramyl-L-alanine amidase activity associated with the cell wall fraction of *E. coli* B are described. Our study was greatly facilitated by the use of radioactive substrates prepared from precursors of cell wall peptidoglycans.

2. Materials and methods

E. coli B was grown in minimum medium M 9 according to Pelzer [16]. Cells were collected at the outset of the exponential phase, washed once with cold water, suspended in 0.02 M K Na phosphate buffer, pH 6.6 (1.5 ml/g wet cells) and broken by sonication (Sonicator 150 T.S. Ultrasons, 74 Annemasse, France) in the presence of deoxyribonuclease and ribonuclease (both crystalline preparations from Koch-Light Lab., Colnbrook Bucks, England). The resulting suspension was centrifuged for one hour at 43,000 *g*. The pellet was washed three times with the same buffer containing 10^{-3} M EDTA. The final pellet was resuspended in buffer (1 ml/g wet cells) and dialyzed overnight against the same buffer without EDTA. The dialyzed suspension was stable at least three months at −20° and was used as the enzyme. The protein content [19] was about 30 mg/ml. A particulate membrane fraction sedimenting between 15,000 *g* and 100,000 *g* was also prepared.

2.1. *Preparation of substrates*

The following procedures were used for the preparation of substrates. (A) gel filtration on Sephadex G 25; (B) chromatography on Whatman 3 MM filter paper in ethanol, 1 M ammonium acetate pH 7 (5:2) for 48 hr; (C) same procedure for 96 hr; (D) rheoelectrophoresis on Whatman 3 MM filter paper in 0.1 N formic acid for 45 min at 25 V/cm.

UDP-*N*-acetylmuramyl-L-ala-γ-D-glu accumulates in *Staphylococcus aureus* H when cells are incubated in the medium described by Park and Chatterjee [20] from which lysine and vancomycin are omitted. The compound was isolated from the trichloroacetic acid

(TCA) extract by procedure A and purified by chromatography on Dowex AG 1X2 (200–400 mesh) according to Park and Chatterjee [20].

UDP-*N*-acetylmuramyl-L-ala-γ-D-glu-(L)-meso-DAP was prepared according to Izaki et al. [21] from *Bacillus cereus*. The compound was purified from the TCA extract by procedures A, C and A.

UDP-*N*-acetylmuramyl-L-ala-γ-D-glu-(L)-^{3}H-meso-DAP was prepared by adding ^{3}H-meso-DAP to UDP-*N*-acetylmuramyl-L-ala-D-glu according to Ito et al. [22] with the meso-DAP adding enzyme isolated from *Corynebacterium xerosis* ATCC 7711. The compound was isolated from the reaction mixture by procedures A,B and A.

UDP-*N*-acetylmuramyl-L-ala-γ-D-glu-(L)-meso-DAP-^{14}C-D-ala-^{14}C-D-ala was prepared from UDP-*N*-acetylmuramyl-L-ala-γ-D-glu-(L)-meso-DAP according to Izaki et al. [21]. The compound was isolated from the reaction mixture by procedures A, C and D.

The different *N*-acetylmuramyl peptides were obtained from the corresponding UDP derivatives by mild acid hydrolysis with 0.05 N HCl for 5 min at 100°. The compounds were purified by procedure D.

The disaccharide tripeptide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-ala-γ-D-glu-(L)-meso-DAP and the disaccharide tetrapeptide β-1,4-*N*-acetylglucosaminyl-*N*-acetymuramyl-L-ala-γ-D-glu-(L)-meso-DAP-(L)-D-ala were isolated from the cell wall of *E. coli* B as previously described [23].

The composition of the different compounds was verified by determining the amino acid (Technicon analyser), the phosphorus [24] and the *N*-acetylhexosamine [25] contents.

2.2 *Enzymatic assay*

A typical assay mixture containing in a total volume of 50 μl, 12.5 μmoles of tris-HCl buffer, pH 8, 30 nM of substrate and 50 μl of the enzyme preparation, was incubated 15 min at 30°.

In the case of radioactive substrates, the reaction mixture was spotted on Whatman 3 MM filter paper and the products of the reaction were separated by procedure D. Peaks were detected by scanning the paper strips on a 4π Tracerlab scanner. The radioactive peaks were cut out and counted in a Packard Tri-Card liquid scintillation spectrometer with the solvent system of Kaufman et al. [26]: 2 ml of water and 13 ml of a solution containing in 1 l of dioxane, 6 g of PPO (2,5-diphenyloxazole), 300 mg of POPOP (*p*-bis-2-(5-phenyloxazoyl)-1-benzene) and 100 g of naphthalene.

With non radioactive substrates the enzymatic reaction was followed by determining the *N*-terminal groups of alanine liberated according to the method of Ghuysen et al. [25].

3. Results

When the enzymatic reaction is performed with compound I, (table 1) only one radioactive peak other than the substrate peak could be detected by rheoelectrophoresis. The product isolated in this way contained Ala, Glu and DAP in the ratio 1:1:1, but no muramic acid. The amount of *N*-terminal groups of L-alanine liberated by the enzymatic reaction was equivalent to that of substrate hydrolyzed, determined by the radioactivity measurements. Furthermore the product of the reaction and the tripeptide L-ala-γ-D-glu-(L)-meso-DAP isolated either from *Escherichia coli* B or *Bacillus megaterium* KM [23] had the same chromatographic and electrophoretic properties when compared under the conditions previously described [23].

The amidase activity was located in the 43,000 *g* pellet as found by Pelzer [15] and none could be detected in the supernatant. The time course of the reaction (fig. 1) is essentially linear for the first 30 min. The pH optimum is at about 8.3 (fig. 2) and the temperature optimum at 30° (fig. 3). The initial velocity is proportional to the concentration of enzyme (fig. 4). When the initial velocity was plotted against the substrate concentration (fig. 5), it appeared that the enzymatic reaction was inhibited by excess substrate.

Among the other substrates tested (table 1), compound II was as good a substrate as compound I. Under the conditions used for the enzymatic assay and for the determination of the *N*-terminal groups of alanine liberated, no activity could be detected either with compound III or IV and only a very low level of activity was found with compound V. Several compounds (table 2) were tested for a possible inhibitory effect. The tripeptide L-ala-γ-D-glu-(L)-meso-DAP inhibits the enzymatic reaction to a certain extent and this is in agreement with the inhibition by an excess of substrate. A much lower extent of inhibition was found with meso-DAP.

Table 1
Compounds tested as substrates for the amidase.

No.	Compound	Specific activity[a]
I	MurNAc[b]-L-Ala-γ-D-Glu-(L)-^{3}H-meso-DAP	16
II	MurNAc-L-Ala-γ-D-Glu-(L)-meso-DAP-^{14}C-D-Ala-^{14}C-D-Ala	18
III	MurNAc-L-Ala-D-Glu	none
IV	disacch[c]-L-Ala-γ-D-Glu-(L)-meso-DAP	none
V	discch-L-Ala-γ-D-Glu-(L)-meso-DAP-(L)-D-Ala	low

[a] Expressed in nanomoles of substrates hydrolyzed per mg of enzyme protein, per hour under the conditions described in materials and methods.
[b] MurNAc : *N*-acetylmuramic acid
[c] Disacch : β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuranic acid.

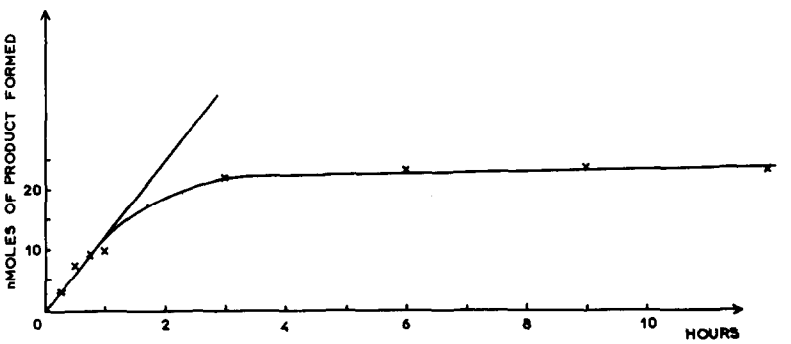

Fig. 1. Time course of the enzymatic reaction with compound I.

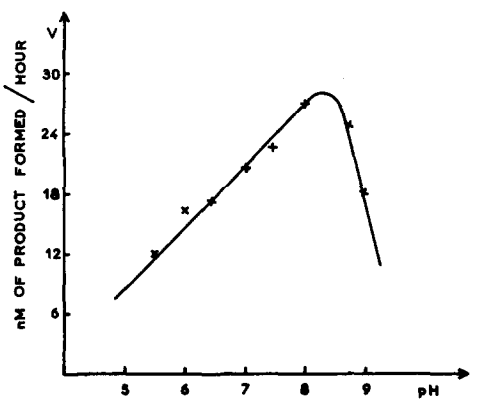

Fig. 2. Effect of pH on the intial velocity of the enzymatic reaction with compound I. Buffer tris-maleate, 0.25 M final concentration.

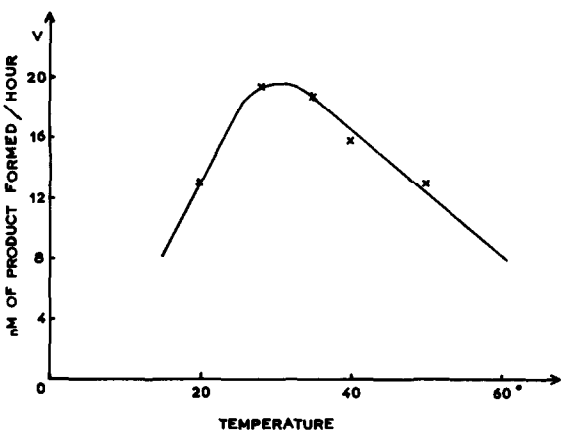

Fig. 3. Effect of temperature on the initial velocity of the enzymatic reaction with compound I.

Table 2
Compounds tested for their possible inhibitory effect on the enzymatic reaction.

Compound (M)	Inhibition[a]
meso-DAP (10^{-3})	0
(10^{-2})	22
N-Acetylmuramic acid[b] (8×10^{-4})	0
(8×10^{-3})	0
L-Ala-D-Glu[c] (7×10^{-4})	0
(7×10^{-3})	0
L-Ala-γ-D-Glu-(L)-meso-DAP[d] (8×10^{-4})	19
(8×10^{-3})	67

[a] Expressed as the percentage of decrease in specific activity when the enzymatic reaction is performed with *N*-acetylmuramyl-L-Ala-γ-D-Glu-(L)-^{3}H-meso-DAP under the conditions described in materials and methods.
[b] Purchased from Mann Research Lab., New York, N.Y., USA.
[c] Prepared from BOC-L-alanyl-D-glutamyl-γ-benzyl ester synthesized in the laboratory by F. Lerique.
[d] Prepared as described previously [23].

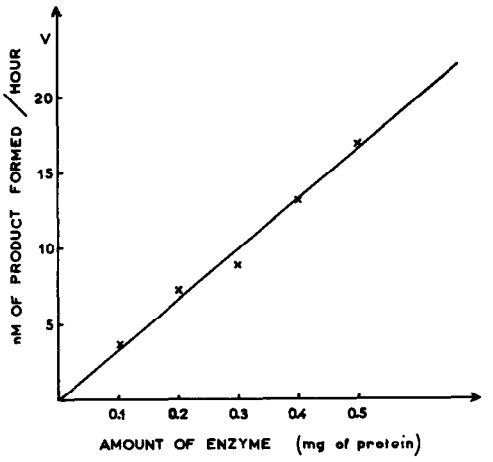

Fig. 4. Effect of the enzyme concentration on the initial velocity of the reaction compound I.

When the enzyme preparation was separated into a cell wall fraction sedimenting at 15,000 *g* and a membrane fraction sedimenting between 15,000 *g* and 100,000 *g*, amidase activity was found in both fractions.

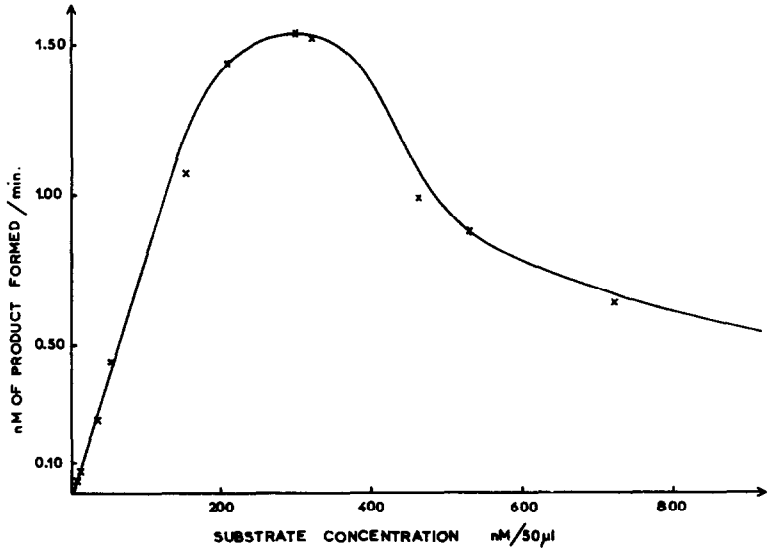

Fig. 5. Effect of the concentration of substrate (compound I) on the initial velocity of the enzymatic reaction.

4. Conclusion

With the use of readily available radioactive substrates such as compounds I and II, it is now possible to examine conveniently other properties of this amidase: activity in the membrane fraction, in mutants and under different growth conditions. These problems are now under investigation and their study may help to determine the function of the admidase activity during cell growth and division.

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