Further Characterization of PL-1 Phage-associated N-Acetylmuramidase of Lactobacillus casei

By MIKIE HAYASHIDA,1* KENJI WATANABE,1 TSUYOSHI MURAMATSU2 AND MASA-AKI GOTO3
1 Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-01, Japan
2 Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Nagasaki 852, Japan
3 Research Institute of Life Science, Yukijirushi Co. Ltd, Tochigiken 329-05, Japan

(Received 14 November 1986)

The properties of an N-acetylmuramidase previously isolated from PL-1 phage lysates of Lactobacillus casei were studied in more detail. The enzyme differed from hen egg white lysozyme in its substrate specificity spectrum, in its physical and chemical nature and in other properties. The cell wall lytic action showed narrow specificity. The enzyme was most active against the phage's host. It was composed of a single polypeptide chain of M, about 37000 as estimated by gel filtration, SDS–PAGE and amino acid analysis. The circular dichroic spectrum showed that the peptide backbone of the enzyme molecule most probably had a mainly random structure. The protein was rich in neutral amino acids, and the enzyme had an isoelectric point of pH 5.0. The N-terminal sequence was determined up to 27 amino acid residues, showing alanine as the N-terminus; the C-terminus was a tyrosine residue.

INTRODUCTION

Studies with protoplasts of Lactobacillus spp. are greatly hampered, because little is known about lytic enzymes acting on these bacteria. Therefore, we attempted a search with PL-1 phage lysates for lytic enzymes acting on the cell walls of Lactobacillus casei, and found a lytic enzyme referred to as N-acetylmuramidase (Watanabe et al., 1984).

In this paper, we describe the further characterization of this enzyme and compare its features with those of hen egg white lysozyme.

METHODS

Phage and bacterial strains. Phage PL-1 and its host strain, L. casei ATCC 27092, were mostly used. Propagation of these organisms, their assay and other related methods were all described previously (Watanabe et al., 1970). Other bacterial strains used were Escherichia coli BH, L. casei YIT 9002, L. casei IAM 1045 and 'Lactobacillus arabinosus' (L. plantarum) 17-5 given to us by Dr T. Sakurai, L. casei Sm' obtained from Dr A. Murata, Bacillus subtilis K-49 and B. subtilis K-77 obtained from Dr J. Fukumoto, Bacillus megaterium ATCC 9885 and Micrococcus luteus (formerly M. lysodeikticus ATCC 4698) given to us by Dr L. J. Rode, and Staphylococcus aureus 6538-P, Streptococcus mutans HS-1, S. mutans BHT, S. mutans GS-5, S. mutans OMZ-176 and 'Streptococcus hemolyticus' (S. pyogenes) given to us by Dr Y. Hagihara. Cell walls and the peptidoglycan and polysaccharide fractions were prepared as described by Ishibashi et al. (1982).

Assay of enzyme activity. The enzyme activity was assayed by using the cell wall peptidoglycan of L. casei ATCC 27092 as a substrate. One unit of enzyme activity was defined as the amount of enzyme that reduced the OD0.600 of the incubation mixture by 0.001 min−1 during the initial period of linear decrease as previously described in detail (Watanabe et al., 1984). The enzyme activity was further tested against the other bacterial strains listed above, and against ethyleneglycol chitin by the method of Imoto & Yagishita (1971). Hen egg white lysozyme (Sigma) was always used for comparison.

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Preparation of enzyme. PL-1 phage-induced lysates of *L. casei* ATCC 27092, which contained about $10^{10}$ p.f.u. ml$^{-1}$ of phage and 20–25 units ml$^{-1}$ of lytic enzyme, were centrifuged to remove cell debris and the supernatant was precipitated out with ammonium sulphate at 60% saturation. After storage overnight at 4 °C, the precipitate was collected by centrifugation, dissolved in 0.01 M-Tris/HCl buffer, pH 7.2, and dialysed against the same buffer at 4 °C for 24 h in a Visking cellulose bag to remove salt. The dialysate was then applied to a DEAE-Sephacel column (2.6 × 90 cm) previously equilibrated with 0.01 M-Tris/HCl buffer, pH 7.2. After washing with the same buffer, the column was eluted by a linear gradient from 0 to 0.4 M-NaCl in the same buffer at a flow rate of 20 ml h$^{-1}$ and 4 ml fractions were collected. Those containing the enzyme activity were pooled, concentrated, dialysed at 4 °C against the same buffer to remove the salt and subjected to rechromatography under the same conditions. The fractions containing enzyme activity were then applied to a Sephadex G-75 column (2.6 × 90 cm), and eluted with 0.01 M-Tris/HCl buffer, pH 7.2, at a flow rate of 10 ml h$^{-1}$. The active fractions were collected, concentrated and subjected to the second gel filtration under the same conditions to obtain a single protein fraction coinciding with enzyme activity. The resultant enzyme solution was pooled, dialysed against deionized water at 4 °C overnight, and then lyophilized.

**Determination of** $M_r$. PAGE was performed as described by Ui (1976) at pH 8.9 using a 7-5% (w/v) polyacrylamide gel at a constant current of 5 mA per tube for 1 h. SDS–PAGE was done by the method of Laemmli (1970) at pH 8.3 using a 0.1% SDS, 13% polyacrylamide gel at 40 V for 18 h. The $M_r$ of the enzyme was also determined by gel filtration as described by Whitaker (1963) on a column (2.6 × 90 cm) of Sephadex G-75 (Pharmacia).

**Determination of circular dichroic spectra.** Circular dichroism was recorded by a Jasco spectropolarimeter J-500A coupled to a data processor DP501N in a 1 mm quartz cell at about 20 °C in a nitrogen atmosphere. The measurement was made at a sensitivity of 0.002 degree cm$^{-1}$ and a time constant of 4. The intensity of circular dichroism was expressed as the mean residue ellipticity ($	heta$) in degrees cm$^2$ dmol$^{-1}$ by taking the residue weight of 110.4. The apparatus was standardized with ammonium d-camphor-10-sulphonate. All spectra are given as the signal average of four consecutive measurements and with appropriate base line subtraction.

**Determination of isoelectric point.** Analytical isoelectrofocusing was done as recommended in the LKB Chemical manual in 5% polyacrylamide gel containing 2.4% (w/v) carrier Ampholine (pH 3.5–9.5) (LKB-PAG) at 25 W for 2 h at 10 °C. Proteins were stained with 0.1% Coomassie brilliant blue R-250 (Sigma).

**UV spectrum measurement.** The ultraviolet absorption spectrum was measured in 0.01 M-Tris/HCl buffer, pH 7.2, with a Shimadzu UV-200 spectrophotometer.

**Amino acid analysis.** Analyses for amino acids and amino sugars were done with a Hitachi model 638 amino acid analyser. Purified enzymes (1.5 mg each) were hydrolysed with 0.5 ml 6 M-HCl at 110 °C in sealed ampoules for 24, 48 and 72 h. After removing HCl under reduced pressure, the residues were dissolved in 100 μl 0.01 M-HCl, and 25 μl of the solution were used for the analysis of amino acids. The amount of tryptophan was determined by the method of Liu & Chang (1971) and that of cysteine by the method of Moore (1963). Hen egg white lysozyme was used as a control to ensure the accuracy of the amino acid analysis. Cell wall peptidoglycans (5 mg) were hydrolysed with 0.5 ml 6 M-HCl at 100 °C for 16 h. After removing HCl, the residues were dissolved in the same volume of 0.01 M-HCl, and 2 μl of the solution was used for analyses of amino acids and amino sugars.

**Determination of N-terminal amino acid sequence.** The N-terminal sequence of the enzyme was determined by an automated version of the stepwise Edman degradation method (Edman & Henschen, 1975; Hunkapiller et al., 1983; Hunkapiller & Hood, 1983) using an Applied Biosystems 470A gas-phase sequencer, which was coupled with HPLC identification of the resulting phenylthiohydantoin derivatives of amino acids. The N-terminal amino acid residue was also determined by both the method of Gray (1972) and the method described by Ikenaka (1976), and the C-terminal residue was determined by the procedure described by Akabori et al. (1956).

**RESULTS**

**Purification of enzyme**

An *N*-acetylmuramidase, present in PL-1 phage lysates of the host cells, *L. casei* ATCC 27092, was purified by using ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sephacel (Fig. 1) and gel filtration on Sephadex G-75 (Fig. 2). The result of a typical purification procedure at each step in terms of the yield and specific activity of the enzyme is summarized in Table 1. The specific activity of the final preparation was about 400-fold that of the phage lysate. The purified enzyme preparation showed a single band both on non-dissociating PAGE and on SDS–PAGE (Fig. 3). After PAGE of the native protein, the gel was sliced and the lytic enzyme activity of each slice was measured. The activity was detected only in the slice where the protein was found. This indicates that the enzyme is electrophoretically
Fig. 1. Second ion-exchange chromatography of PL-1 enzyme on DEAE-Sephacel. PL-1 enzyme adsorbed to a column of DEAE-Sephacel was eluted by a linear gradient from 0 to 0.4 M-NaCl in 0.01 M-Tris/HCl buffer, pH 7.2, at 20 ml h⁻¹. 4 ml fractions were collected. Lytic enzyme activity (●) and protein content (○) were measured. Fractions 55–65 were pooled. ---, NaCl concentration.

Fig. 2. Second gel chromatography of PL-1 enzyme on Sephadex G-75. PL-1 enzyme applied to a column of Sephadex G-75 was eluted with 0.01 M-Tris/HCl buffer, pH 7.2, at a flow rate of 10 ml h⁻¹; 4 ml fractions were collected. Lytic enzyme activity (●) and protein content (○) were measured. Fractions 30–40 were pooled.

Table 1. Purification of PL-1 enzyme

The details of the purification procedures and the assay for the determination of lytic activity are described in Methods. The total volume before and after the purification was 1000 and 40 ml, respectively.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total lytic activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units mg⁻¹)</th>
<th>Purification (±fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage lysates</td>
<td>21000</td>
<td>4900</td>
<td>4.3</td>
<td>1.0</td>
</tr>
<tr>
<td>0–60% (NH₄)₂SO₄</td>
<td>19600</td>
<td>810</td>
<td>24</td>
<td>5.6</td>
</tr>
<tr>
<td>fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephacel 1st</td>
<td>11900</td>
<td>150</td>
<td>79</td>
<td>18</td>
</tr>
<tr>
<td>DEAE-Sephacel 2nd</td>
<td>9400</td>
<td>35</td>
<td>269</td>
<td>62</td>
</tr>
<tr>
<td>Sephadex G-75 1st</td>
<td>7400</td>
<td>13</td>
<td>569</td>
<td>132</td>
</tr>
<tr>
<td>Sephadex G-75 2nd</td>
<td>6600</td>
<td>3.9</td>
<td>1690</td>
<td>394</td>
</tr>
</tbody>
</table>
The purified enzyme is referred to as PL-1 enzyme throughout the rest of the paper.

**Lytic action spectrum of PL-1 enzyme**

Since the phage enzyme, like hen egg white lysozyme, was an N-acetylmuramidase (peptidoglycan N-acetylmuramoylhydrolase; EC 3.2.1.17), the lytic action spectra of the two enzymes were compared (Fig. 4). The phage enzyme rapidly dissolved the peptidoglycan from *L. casei* ATCC 27092, while at the same concentration, hen egg white lysozyme dissolved it slowly and only partially. However, the enzyme had little effect either on cells of *M. luteus* or on ethyleneglycol chitin, which are commonly used for the assay of hen egg white lysozyme. The enzyme was essentially inactive on the cell walls of *L. casei* YIT 9002, *L. casei* Sm', 'L. arabinosus' (*L. plantarum*) 17-5, *S. mutans* HS-1, *S. mutans* BHT, *S. mutans* GS-5, *S. mutans* OMZ-176, 'S. hemolyticus' (*S. pyogenes*), *S. aureus* 6538-P, *M. luteus*, *B. megaterium* ATCC 9885 and *E. coli* BH, although it dissolved those of *L. casei* IAM 1045, *B. subtilis* K-49 and *B. subtilis* K-77 only slightly under the same conditions. Thus, the lytic action of the phage enzyme was distinguishable from that of hen egg white lysozyme.

**Physical and chemical properties of PL-1 enzyme**

The maximum absorption of this enzyme was at 280 nm, and the absorption coefficient of a 1·0% (w/w) solution at 280 nm ($A_{280}^{1%}$) was 4·6. The $M_r$ of the enzyme was estimated to be about 37000 from an SDS–PAGE pattern of the enzyme and six standard proteins (Fig. 3). This value was the same as that estimated by a Sephadex G-75 gel filtration method, described above. The enzyme appears to consist of a single polypeptide chain. It had an isoelectric point of pH 5·0 as determined by electrofocusing (Fig. 5).
**Fig. 4.** Comparison of the lytic activities of PL-1 enzyme and hen egg white lysozyme. The cell wall peptidoglycan of *L. casei* ATCC 27092 (a) or the cell walls of *M. luteus* (b) (each OD$_{600}$ about 0.6) were incubated with PL-1 enzyme (●) or hen egg white lysozyme (○) (each 50 µg ml$^{-1}$) in 0.01 M-Tris/HCl buffer, pH 7.2, at 37 °C, and at intervals the OD$_{600}$ was measured.

**Fig. 5.** Isoelectric focusing of PL-1 enzyme and marker proteins on LKB-PAG. PL-1 enzyme (●) and marker proteins (each 55 µg) (○) were applied to a LKB-PAG (pH 3.5-9.5) and subjected to electrofocusing at 25 W for 2 h at 10 °C.

**Fig. 6.** Circular dichroic spectra of PL-1 enzyme. Native (-----) and denatured (----) PL-1 enzymes (0.24 and 0.31 mg ml$^{-1}$, respectively, in 10 mM-phosphate buffer, pH 7.0) were measured in a 1 mm cell at 20 °C. See text for details.

Circular dichroism is one of the most useful tools for studying the conformation of the polypeptide backbone. The spectrum in the far-ultraviolet region is a measure of α-helix, β-sheet and random coil structure, as is widely shown in the literature. Therefore, the circular dichroic spectra of both the native and denatured preparations of the enzyme were measured in the range 200–280 nm (Fig. 6). The denatured enzyme was prepared by dissolving the native enzyme in 10 mM-phosphate buffer containing 6 M-guanidine.HCl, pH 5.5, at about 20 °C for 2 h. A negative band was observed at around 200 nm with a shoulder at 233 nm for the native enzyme, while for the denatured one a positive peak at 221 nm appeared, making the trough at 233 nm more distinct.
Table 2. Amino acid composition of PL-1 enzyme

Values are presented as moles per mole of enzyme ($M_\text{r} = 37000$). They are averages of analyses of 24, 48 and 72 h hydrolysates, except for threonine, serine and methionine, which were obtained by linear extrapolation to 0 h of hydrolysis, and for isoleucine and valine, which were obtained with 48 h hydrolysates. Tryptophan was determined by hydrolysis with p-toluenesulphonic acid. Half-cystine was determined by performic acid oxidation.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>PL-1 enzyme</th>
<th>Hen egg white lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp + Asn</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Thr</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Ser</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Pro</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Gly</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Ala</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Val</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Met</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>335</td>
<td>129</td>
</tr>
</tbody>
</table>

The amino acid composition of the enzyme (Table 2) was characterized by a relatively high content of leucine, lysine and alanine, and by a lack of tryptophan. The N-terminal amino acid was alanine and the C-terminal one, tyrosine. From amino acid analysis, a minimal $M_\text{r}$ of 37 200 was calculated according to Delaage (1968). This number coincides well with that determined by both the SDS–PAGE and gel-filtration methods. The N-terminal sequence was determined by an Edman degradation of up to 27 amino acid residues as follows: Ala-Tyr-Hyp-Ile-Asn-Lys-Glu-Phe-Ala-Leu-Gly-Ala-Asn-Glu-Gly-X-Lys-Gln-Val-Ala-Asn-X-Leu-Tyr-Ile-Ile-Leu-. This sequence is also different from that of hen egg white lysozyme.

**DISCUSSION**

Many phage-associated lytic enzymes have been reported and characterized as muramidase, glucosaminidase, amidase and endopeptidase (Rogers, 1979; Tsuru, 1983). We have found an N-acetylmuramidase, which can hydrolyse the glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan of *L. casei* ATCC 27092, in PL-1 phage-induced lysates of this bacterium. As described in a previous paper (Watanabe et al., 1984), this enzyme differs from hen egg white lysozyme in several properties such as optimum lytic conditions, temperature, pH stability and sensitivity to metal ions and enzyme inhibitors. In this paper, we have compared other properties of the phage enzyme with those of hen egg white lysozyme, such as isoelectric point, circular dichroic spectrum, amino acid composition, N- and C-terminal amino acids and N-terminal amino acid sequence. The lytic action of this enzyme is quite different from that of hen egg white lysozyme, and has a high specificity for the PL-1 phage's host.

The absorption coefficient ($A_{1\text{cm}}^{1\text{cm}}$) at 280 nm of the PL-1 enzyme is about one-sixth that of hen egg white lysozyme, suggesting a difference in the amino acid composition. Actually this enzyme has no tryptophan and few tyrosine residues, but many leucine, lysine and alanine residues. The N-terminal amino acid sequence, up to 27 amino acid residues, indicated no homology between the two enzymes.

As for the circular dichroic spectra, the negative bands near 206 and 222 nm, which are characteristic of the $\alpha$-helix form of a peptide backbone, were scarcely detectable. The appearance of the trough at 233 nm could not be explained with respect to the local conformation of the protein molecule or amino acid side chains. Random structure was possibly dominant in the main chain of this enzyme, since the circular dichroic spectrum resembled that of phosvitin, which is known to have a disordered structure (Timasheff et al., 1967). Even in this respect, the enzyme was different from hen egg white lysozyme, which contained about 43%
α-helix form (Mulveny et al., 1974). However, the present circular dichroic spectrum did not exclude the existence of a small amount of ordered structure in the native enzyme, as compared with the denatured enzyme.

Since the isoelectric point of this enzyme was much lower than that of hen egg white lysozyme, it seemed that the difference in electric charge between these enzymes may be reflected in their different lytic activity profiles with various peptidoglycans. Thus, this enzyme is considered to be quite different from hen egg white lysozyme both genetically and in its evolution.

The applicability of the PL-1 muramidase for the production of protoplasts of some strains of L. casei, which are resistant to the action of hen egg white lysozyme, commonly used for that purpose, is now under investigation.

The authors wish to thank Dr T. Sakurai of Yakult Central Institute, Dr Y. Hagiwara of Fukuoka Dental College, Dr J. Fukumoto of Osaka Municipal University, Dr A. Murata of Saga University and Dr L. J. Rode of University of Texas for their generous supply of bacterial strains as shown in the text. We also thank the following students of Fukuoka University for their technical assistance in parts of the experiments: T. Nishimura, R. Arinori, H. Kawasaki, S. Koga, R. Yahiro and Y. Iwakuma.

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