Germination-specific cortex-lytic enzymes from *Clostridium perfringens* S40 spores: time of synthesis, precursor structure and regulation of enzymatic activity

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Abstract

Germination-specific enzymes, an amidase and a muramidase, of *Clostridium perfringens* S40 were synthesized at the time of forespore formation during sporulation. The amidase had a unique precursor structure consisting of four domains: the N-terminal pre-sequence, the N-terminal pro-sequence, mature enzyme and the C-terminal pro-sequence. The N-terminal pre-sequence and the C-terminal pro-sequence were sequentially processed at the time of development of phase-bright spores, and the resulting inactive pro-enzyme was activated by cleavage of the N-terminal pro-sequence with a specific protease during germination. A possible mechanism for the regulation of activity of muramidase, which is produced as a mature form and does not need processing for activation, is presented.

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1. Introduction

Bacterial spore germination is defined as a series of interrelated degradation events, triggered by specific germinants, which causes the irreversible loss of dormant spore characteristics [1,2]. Once triggered, germination proceeds in the absence of both germinant and germinant-stimulated metabolism. This indicates that germination is a process controlled by sequential activation, or allosteric conformational alteration, of a set of germination-related enzymes pre-existing in the dormant spore. One of the key enzymes involved in the germination process is spore cortex-lytic enzyme, which degrades the spore cortex peptidoglycan implicated in the maintenance of spore dormancy.

An amidase and a muramidase are involved in germination of *Clostridium perfringens* S40 spores as major germination-specific cortex-lytic enzymes [3–5]. The amidase is produced as a precursor with at least N-terminal pre- and pro-sequences, exists as an inactive pro-form with the N-terminal pro-sequence in dormant spores, and the pro-sequence is...
removed to release active mature enzyme [4]. On the other hand, the muramidase is synthesized in a mature form and does not need proteolytic activation [5]. However, little is known about the details of the expression of germination-specific enzymes, the processing of the precursor, the regulation of activity and the construction of the germination apparatus. As a first step towards the elucidation of these problems, the present study was undertaken to clarify when spore-lytic enzymes of *C. perfringens* are synthesized and how their activities are controlled.

2. Materials and methods

2.1. Preparation of vegetative cells, sporulating cells, dormant spores, decoated spores and germinated spores

*C. perfringens* S40 was grown and sporulated as described previously [3]. Vegetative cells, sporulating cells and dormant spores were harvested, washed with distilled water and stored at 4°C. Decoated spores, which are a substrate of the germination-specific amidase, were prepared as described previously [3]. Dormant spores were germinated at 32°C for 2 h by incubating with 0.15 M KCl, 50 mM potassium phosphate, pH 7.0, containing 10 mM L-alanine and 2 mM inosine, in the presence of CO₂. Germinated spores were separated from germination exudate by centrifugation (6000×g for 5 min at 4°C), washed with distilled water and stored at 4°C.

2.2. Purification of mature enzymes and preparation of antisera

A 31-kDa mature amidase (C₃₁) and a 38-kDa mature muramidase (M₃₈) were purified from germination exudate [3,5]. Anti-C₃₁ antiserum and anti-M₃₈ antiserum were prepared as described previously [4,5].

2.3. SDS-PAGE of extracts from vegetative cells, sporulating cells and dormant spores

Vegetative cells, sporulating cells and dormant spores were boiled for 5 min in 10 mM sodium phosphate, pH 7.0, containing 1% SDS and 0.1% 2-mercaptoethanol and centrifuged (6000×g for 5 min at 4°C). The supernatants were subjected to SDS-PAGE (0.1% SDS and 12.5% polyacrylamide gel) according to the method of Laemmli [6]. The gels were immunoblotted as described previously [4,5].

2.4. Preparation of a 35-kDa inactive precursor of amidase and its activation by germination-specific protease

A 35-kDa inactive precursor of amidase (C₃₅) was extracted by treating dormant spores (1 g packed weight) with 5 ml of 0.1 M potassium borate, pH 10.0, containing 50 mM dithiothreitol at 32°C for 1 h [4]. The extract was dialyzed against 50 mM potassium phosphate, pH 7.0. Germination-specific protease (GSP) which activates C₃₅ was extracted from fully germinated spores (0.5 g packed weight) with 2 ml of 0.25 M KCl-50 mM potassium phosphate, pH 7.0, containing 0.2% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) at 30°C for 2 h. The extract was used as GSP solution without further purification. The GSP solution (20 μl) was added to 400 μl of the C₃₅ solution and the mixture was reacted at 32°C. At appropriate time intervals, an aliquot (10 μl) was mixed with 130 μl of decoated spore suspensions in a cell of 1 mm light path. Initial OD₆₀₀ was 0.2 and the decrease in OD₆₀₀ was monitored at 32°C as a measure of amidase activity [3]. Furthermore, after the reaction was stopped by boiling for 5 min, an aliquot (20 μl) was analyzed by immunoblotting to detect the appearance of mature enzyme.

2.5. Mass spectroscopy

Electrospray ionization mass spectroscopy was performed with a VG Platform II-LC mass spectrometer (Micromass) at a flow rate of 10 ml min⁻¹, employing a capillary voltage of 4 kV and nitrogen as a drying gas. The sample was dissolved in 1% formic acid/50% acetonitrile and the mobile phase was 50% acetonitrile. Horse heart myoglobin was used to calibrate the quadrupole. Masslynx (Micromass) was used for the calculation of the molecular mass.
Fig. 1. Synthesis of germination-specific, spore cortex-lytic enzymes and processing during sporulation. A, B: Immunoblots with anti-C\textsubscript{31} antiserum and anti-M\textsubscript{38} antiserum, respectively, of extracts from sporulating cells. Vegetative cells grown in TG medium were further cultured in DS medium for sporulation. At various times after the cells were resuspended to DS medium, cells were harvested, solubilized and analyzed by immunoblotting, as described in Section 2. Germination exudate was similarly analyzed. Approximately 100 mg of protein was loaded on the gel. C\textsubscript{50}, C\textsubscript{38}, C\textsubscript{35} and C\textsubscript{31} in A and M\textsubscript{38} in B indicate migration positions of various forms of amidase and muramidase, respectively. Sporulation times of samples in lanes 1-7 were, T\textsubscript{0} (vegetative cells), t\textsubscript{3}, t\textsubscript{4}, t\textsubscript{6}, T\textsubscript{12} and t\textsubscript{24} (dormant spores), respectively. Germination exudate was also electrophoresed (lane 8) for comparison. C: Microscopic observation of sporulating cells. Sporulating cells were observed by phase-contrast microscopy (Olympus BH-2). Sporulation times of cells shown in pictures 1-6 were t\textsubscript{0} (vegetative cells), t\textsubscript{3}, t\textsubscript{4}, t\textsubscript{6}, t\textsubscript{8}, and t\textsubscript{24} (dormant spores), respectively. All pictures are shown at the same magnification. Bar, 5 \(\mu\)m.
2.6. N-terminal amino acid analysis

A 31-kDa peptide produced from GSP-treated C<sub>35</sub> was separated by SDS-PAGE and transferred to ProBlott membrane (Applied Biosystems). Sequencing of N-terminal amino acids of the peptide was performed on a protein sequencer (model 477A/120A, Applied Biosystems), according to the method of Matsudaira [7].

3. Results

3.1. Timing of the synthesis of germination-specific cortex-lytic enzymes

Previous work indicated that a germination-specific amidase of C. perfringens S40 is synthesized in the form of a 50-kDa precursor termed C<sub>50</sub> (a 438 amino acid residues long precursor with N-terminal 114-residue pre- and 35-residue pro-sequences) [4]. Furthermore, after the processing of the pre-region, the enzyme was thought to accumulate in dormant spores as a 35-kDa protein with the N-terminal pro-sequence termed C<sub>35</sub>, which converts to a mature enzyme, C<sub>31</sub>, during germination. As shown in Fig. 1A, immunoblotting analysis of extracts prepared throughout vegetative growth and sporulation revealed that the synthesis of C<sub>50</sub> began 3 h after resuspension of vegetative cells in sporulation medium (T<sub>3</sub>). The results showed that C<sub>35</sub> is produced through an intermediate of 38 kDa (C<sub>38</sub>).

It was suggested [5] that a germination-specific muramidase is produced as mature enzyme M<sub>38</sub> with an apparent molecular mass of 38 kDa, as determined by SDS-PAGE. M<sub>38</sub> was synthesized at T<sub>3</sub> (Fig. 1B), and no cleavage was found during sporulation and germination as indicated previously [5].

3.2. Precursor structure of germination-specific amidase and its processing

As shown in Fig. 1A, the conversion from C<sub>50</sub> to C<sub>35</sub> during sporulation occurred through an inter-
mediate, C_{38}. A likely possibility for this is the presence of a cleavable C-terminal extended sequence with a molecular mass of about 3 kDa, in addition to the N-terminal pre-sequence which is cleaved during sporulation. Comparison of the amino acid sequence of C_{50} with the precise size of the mature enzyme, C_{31}, might indicate whether the enzyme liberates such a C-terminal sequence or not. On this basis, the molecular mass of purified C_{31} was measured by electrospray ionization mass spectroscopy (Fig. 2). The multi-charged molecular ion analysis revealed one major C-terminal extended sequence of C_{50} which was cleaved during sporulation. Comparison of the amino acid sequence of C_{50} with the precise size of the mature enzyme, C_{31}, might indicate whether the enzyme liberates such a C-terminal sequence or not. On this basis, the molecular mass of purified C_{31} was measured by electrospray ionization mass spectroscopy (Fig. 2). The multi-charged molecular ion analysis revealed one major peak whose molecular size was determined to be 30,239.0 ± 4.9 Da (average of 17 peaks of multi-charged ion). The molecular size was in good agreement with the expected molecular mass, 30,236 Da, based on the known N-terminus of C_{31} and the hypothetical C-terminus, Arg-413 of C_{50} [4]. Since C_{50} is composed of 438 amino acid residues [4], it is indicated that C_{50} has a C-terminal extended sequence consisting of probably a 25 amino acid residues long peptide.

Fig. 1C shows phase-contrast microscopic pictures of sporulating cells at t_0, t_3, t_4, t_6, t_8 and t_24. C_{50} was expressed at the time of forespore formation and the C_{50}-to-C_{38} and C_{38}-to-C_{35} conversions were observed with the development of phase-bright spores.

We looked for a protease which cleaves the N-terminal pro-sequence to activate C_{35}. Such protease activity was detected in the extract from germinated spores with CHAPS, but not from vegetative cells, sporulating cells and dormant spores. As shown in Fig. 3, treatment of C_{35} with the protease generated spore-lytic activity, in parallel with the appearance of a 31-kDa peptide. Protein sequence analysis of the 31-kDa product provided the N-terminal sequence of VLPEPVPEYIVVHNG (16 residues), which is identical to that of C_{31}, unequivocally indicating the processing of the N-terminal pro-sequence of C_{35}. Various proteases tested (trypsin, lysyl endopeptidase, subtilisin Carlsberg, proteinase K, thermolysin) could not activate C_{35}.

4. Discussion

The results show that the synthesis of both spore cortex lytic enzymes, an amidase and a muramidase, of C. perfringens S40 takes place at the time of fore-
spore formation in sporulation, corresponding to stage III of sporulation of *Bacillus subtilis* [8]. Many germination-related genes of *B. subtilis*, *gerA*, *gerB*, *gerD* and *gerK*, are expressed at stage III of sporulation under control of σ^G^, a sporulation-specific sigma factor [9]. The sigma factor responsible for the synthesis of *C. perfringens* cortex-lytic enzymes is not known at present.

A new finding in this study is that a germination-specific amidase of *C. perfringens* S40 is produced in a form with a C-terminal extension which consists of probably 25 amino acid residues and is cleaved during sporulation, in addition to the known N-terminal pre- and pro-sequences. During the development of phase-bright spores which corresponds to stages IV–VI in *B. subtilis* sporulation [8], C₅₀ was converted to C₈₈, then to C₃₅, by a sequential removal of the N-terminal pre- and the C-terminal pro-sequence, and C₃₅ was processed to release an active enzyme C₃₃ during germination. A protease which converted C₃₅ to C₃₃ has been extracted from germinated spores and its characteristics will be published elsewhere.

Although activation of cortex-lytic enzyme by processing during germination has also been observed for the germination-specific amidase of *Bacillus megaterium* KM [10], the precursor structure in this organism has not been clarified. The precursor structure of the *C. perfringens* amidase differs in at least the lack of a signal sequence from those of aqualysin I from *Thermus aquaticus* [11] and minor extracellular protease from *B. subtilis* [12], which are proteases of bacterial origin synthesized in a form consisting of a four-domain structure: the signal sequence, the N-terminal pro-sequence, mature enzyme, and the C-terminal pro-sequence. The N-terminal pro-sequence of *C. perfringens* amidase might directly contribute to the regulation of cortex-lytic activity. On the other hand, it seems that the N-terminal pre- and C-terminal pro-regions of the amidase which are processed during sporulation are involved in the translocation of the enzyme to the final destination in the dormant spore. In contrast to the amidase, no modification in the primary structure of *C. perfringens* muramidase occurred during sporulation and germination. Both amidase and muramidase are known to be closely localized on the exterior of cortex layer in the dormant spore [13]. The mechanism(s) by which enzymes having different structural characteristics are sited outside the cortex layer is unknown.

The results presented here show that, in cortex hydrolysis of *C. perfringens*, the germination-specific muramidase does not need activation during germination, while proteolytic cleavage is a crucial event for activation of the germination-specific amidase. It should be emphasized that the muramidase lyses only cortical fragments [5], in contrast to the amidase which attacks only intact spore cortex [3]. This implies that the muramidase might hydrolyze cortex partially degraded with the amidase. Therefore, it is most likely that the in vivo activity of *C. perfringens* muramidase is tightly regulated by its requirement for disrupted unstressed cortex as substrate. On the other hand, germination-specific amidases of *Bacillus cereus* and *B. subtilis*, which attack only intact spore cortex, exist in a mature form in dormant spores [14,15]. This suggests that the expression of the activity of *B. subtilis* and *B. cereus* amidases is regulated by a mechanism which is different from those proposed for *C. perfringens* amidase and muramidase. Thus, it appears that there is diversity among germination-specific cortex-lytic enzymes with respect to the regulation of activity and expression.

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**References**


