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INFLUENCE OF HEAT AND SODIUM DODECYL SULFATE ON THE ENDOPEPTIDASE I FROM *BACILLUS SPHAERICUS* 9602

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Endopeptidase I from *Bacillus sphaericus* is a stable enzyme which retains its activity at 37°C in the presence of sodium dodecyl sulfate. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed two forms of the enzyme : an active, fast-running form, for the enzyme preheated at 37°C and a denatured, slow-running form, for the enzyme preheated at 100°C. Such behavior is similar to that of the "heat-modifiable" outer membrane proteins from gram-negative bacteria. In the absence of sodium dodecyl sulfate, endopeptidase I aggregated in an enzymatically active dimer, with an apparent molecular weight of 90,000 daltons, which could be the native form of the enzyme.

Endopeptidase I has been characterized in *Bacillus sphaericus* NCTC 9602. It is a γ-D-glutamyl-(L)*meso*-diaminopimelate endopeptidase, active on the peptide sequence : L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelyl(L)-D-alanine of some bacterial peptidoglycans (1). The terminal D-alanine may be present or may be absent and the D-glutamyl residue can be replaced by a D-*iso*-glutaminyl one (2). The only strict substrate requirement for endopeptidase I is the presence of the *meso*-diaminopimelic acid residue with free ω-NH_2 and ω-COOH groups (2, 3). The accumulation of endopeptidase activity is closely related to sporulation (4-6). The enzyme exists in a particulate form in the integument fraction of the spores (6) and in an exocellular form in the culture media of sporulating *B. sphaericus* (7).

The present study describes the unusual properties exhibited by the exocellular endopeptidase I when examined by polyacrylamide gel electrophoresis either in the presence or in the absence of sodium dodecyl sulfate.

MATERIALS AND METHODS

Reagents. Acrylamide, N,N'-methylene-bis-acrylamide and N,N,N',N'-tetramethylethylene diamine were purchased from Merck. PAGE-Blue 83 was from BDH. All other reagents were from Sigma.

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Abbreviations used : SDS, sodium dodecyl sulfate ; *ms*-A_2pm, *meso*-diaminopimelic acid ; MurNAc, N-acetyl muramic acid ; Bis, N,N'-methylene-bis-acrylamide.

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Buffers. Buffer A : 0.02 M Tris-HCl at pH 8.0, plus 0.01 M $MgCl_2$. Buffer B : 0.025 M Tris-glycine buffer at pH 8.3.

Endopeptidase I. The exocellular endopeptidase I was obtained as described in (7) and purified (8). Its specific activity was 4 µmoles min^{-1} mg^{-1} with 0.4 mM MurNAc-L-Ala-γ-D-Glu-(L)*ms*-A_2pm(L)-D-(^{14}C)Ala as substrate.

Enzymatic assays. A 0.2 mM MurNAc-L-Ala-γ-D-Glu-(L)*ms*-A_2pm(L)-D-(^{14}C)Ala solution was incubated with endopeptidase I in buffer A at 37°C for 20 min (within the linear response). The percentage of released *ms*-A_2pm(L)-D-(^{14}C)Ala was estimated as described in (1).

Polyacrylamide gel electrophoreses. The slab-gel apparatus and the protein kits (HMW and LMW) were from Pharmacia.

The electrophoreses were carried out : (i) for 4 h at 10 V/cm in buffer B containing 0.1 % SDS, on a separating gel (80 x 120 x 0.7 mm) made at 10 % acrylamide and 0.27 % Bis according to Laemmli (9) ; (ii) at 20 V/cm and 4°C in buffer B, on a linear 4 to 30 % polyacrylamide gradient gel (Pharmacia, PAA 4/30, 75 x 75 x 2.7 mm).

For the localization of the proteins, the gel was immersed in 10 % sulfosalicylic acid for 30 min, stained with PAGE-Blue 83 and scanned in a Vernon apparatus.

For the research of the enzyme activity the gel was sliced into 3 mm-thick bands. Each band was eluted with 200 µl of buffer A containing 0.3 M NaCl and 0.02 % Brij 58. Aliquots of the extracts were dialyzed against buffer A and used for enzymatic assays (see above). Endopeptidase I activity was expressed as % *ms*-A_2pm(L)-D-(^{14}C)Ala released.

RESULTS

Enzymatic assays of endopeptidase I in the presence of sodium dodecyl sulfate.

Endopeptidase I activity was assayed, either in the absence or in the presence of 1 % SDS in buffer A at 37°C for 20 min, with MurNAc-L-Ala-γ-D-Glu-(L)*ms*-A_2pm(L)-D-(^{14}C)Ala as substrate. Hydrolysis of the substrate was respectively 42 % and 35 %, endopeptidase I is thus a stable enzyme which retains its activity at 37°C in the presence of SDS.

Gel electrophoresis under denaturing conditions.

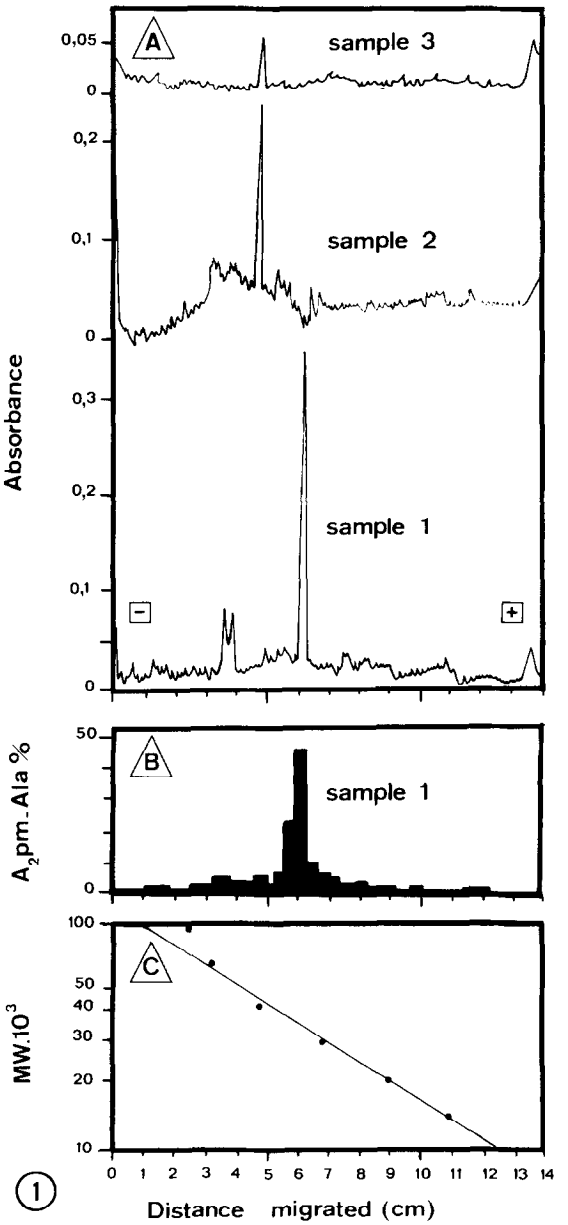
Pretreated samples of endopeptidase I were analyzed with low molecular weight proteins as standards. The results are presented in Fig. 1.

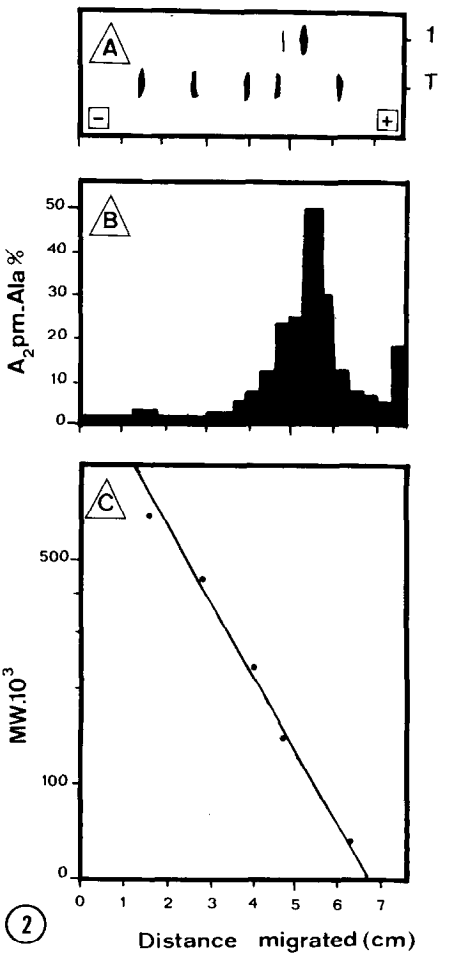
When pre-incubated at 37°C for 20 min in the presence of 1 % SDS, endopeptidase I showed one major band with a molecular weight of 35,000 daltons (Fig. 1A, sample 1) and retained its activity (Fig. 1B, sample 1).

When pre-incubated at 100°C in the presence of 1 % SDS and 5 % 2-mercaptoethanol (Fig. 1A, sample 2) one could observe an alteration in the mobility of the enzymatic protein from a faster (35,000 daltons) to a slower-running form (45,000 daltons). This latter form is likely to be completely denaturated since it migrates in the same position as does the sample that was pre-treated with 8 M urea (Fig. 1A, sample 3).

Gel electrophoresis under non-denaturing conditions.

Endopeptidase I, in the absence of SDS, was loaded on a linear 4 to 30 % poly acrylamide gradient slab gel. Such a gel fractionates proteins over an approximate molecular weight range of 50,000-2,000,000 daltons. If electrophoresis is carried out for sufficient time (16 h at 20 V/cm), the proteins reach a point where the pore size prohibits their further migration. The results are shown in Fig. 2.





High molecular weight proteins were used as standards (Fig. 2A, lane T and Fig. 2C). Endopeptidase I showed two bands (Fig. 2A, lane 1) : a major one at a molecular weight 90,000 daltons and a minor one at a molecular weight 135,000 daltons. The enzymatic activity covered a broad area from one band to another with a maximum at 90,000 daltons (Fig. 2B). According to the hydrophobic nature of the enzyme (8), one can assume that two and, to a lesser extent, three monomeric units of the molecule have undergone association in the absence of detergent without loss of the activity. If the monomer were still present it might not have been detected, since it would have eventually migrated out of the gel. To elucidate this, another electrophoresis of endopeptidase I was carried out for a limited time (2.5 h at 20 V/cm), so that bromophenol blue was not excluded from the gel. High (669,000 to 67,000 daltons) and low (67,000 to 14,400 daltons) molecular weight proteins were used as standards. The activity of endopeptidase I was solely located in the upper part of the gel, area of migration of the high molecular weight proteins (data not shown) ; therefore endopeptidase I was not present in the monomeric form and its self-association was complete in the absence of SDS.

DISCUSSION

The self-association which is characteristic of endopeptidase I *in vitro* may also be true *in vivo* and it is concluded that this enzyme is an enzymatically

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Figure 1 : Sodium dodecyl sulfate electrophoresis of endopeptidase I on a 10 % polyacrylamide gel (see Material and Methods).
Prior to the electrophoresis, samples of endopeptidase I (5 µg) in buffer A were :
1 - heated at 37°C for 20 min in 1 % SDS.
2 - heated at 100°C for 5 min in 1 % SDS and 5 % 2-mercaptoethanol.
3 - heated at 100°C for 10 min in 2 % SDS, 5 % 2-mercaptoethanol and 8 M urea.
A - The gel was stained with PAGE-Blue 83 and scanned in a Vernon apparatus.
B - Endopeptidase I activity (see Material and Methods).
C - Plots of log molecular weight versus distance migrated of reference proteins : phosphorylase B (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), α-lactalbumin (14,400).

Figure 2 : Electrophoresis of endopeptidase I on a linear 4 to 30 % polyacrylamide gradient gel.
Sample 1 : endopeptidase I (5 µg).
Sample T : reference proteins.
A - Staining with PAGE-Blue 83.
B - Endopeptidase I activity.
C - Plots of log molecular weight versus distance migrated of reference proteins : thyroglobulin (668,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), albumin (67,000).

active dimer of about 90,000 daltons apparent molecular weight. The dimer dissociates into a monomer of about 45,000 daltons apparent molecular weight in strongly denaturing conditions as in the presence at 100°C of SDSor 8 M urea. Nevertheless endopeptidase I is a stable enzyme and in less drastic denaturing conditions, as in the presence of SDS at 37°C, it dissociates into an active monomer. One can observe a difference in the electrophoretic mobility of the active and the denaturated monomers, the active monomer has a greater mobility than that of the denaturated one

The behavior of endopeptidase I is then analogous to that of the "heat-modifiable" outer membrane protein (Omp A) of many gram-negative bacteria which probably means that it has a highly ordered secondary structure (for recent reviews, see references 10 and 11). The Omp A protein was initially characterized in *Escherichia coli* as a major outer membrane polypeptide that exhibited anomalous heat-modifiable migration in SDS polyacrylamide gels (12, 13). Its mobility decreases when the membrane is dissolved into SDS solutions above 50°C (12, 14-16). Reithmeier and Bragg suggested a more unfolded structure in the heat-modified form (17). Nakamura and Mizushima showed that the native protein has a high β-structure content and that this conformation was stable into SDS solution but was destroyed upon heating above 50°C (18).

Similar heat-modifiable proteins have also been described in *Pseudomonas aeruginosa* (19), *Neisseria gonorrhoeae* (20), *Neisseria meningitidis* (21) and *Salmonella typhimurium* (22).

To our knowledge no "heat-modifiable" protein from gram-positive bacteria has yet been described. However two forms (fast and slow-running forms) of the DD-carboxypeptidase from *Streptomyces albus* G were noted in SDS gel electrophoresis, without explanation at that time (23). Recently the amino acid sequence of the enzyme was established leading to the unequivocal molecular weight of 22,076 daltons (slow-running form) (24). The presence of the fast-running form in SDS gel electrophoresis was then explained by an incomplete unfolding of the protein in insufficient denaturing conditions (B. Joris, personal communication).

The anomalous behavior of those "heat-modifiable" proteins on SDS gels demonstrates that one must be careful before to assign them a molecular weight on the basis of their electrophoretic mobilities.

At last, another interesting point of comparison between outer membrane proteins of gram-negative bacteria and endopeptidase I is that they are peptidoglycan-associated proteins. Cross-linking experiments have shown a direct cross-linking of the Omp A protein of *E. coli* to peptidoglycan in whole cells as well as in the enveloppe fraction (25-27), and endopeptidase I was shown to be located in the forespore and spore integuments of *B. sphaericus* (6). This and the above results should be a starting point for further investigations concerning the secondary structure, assembly, function and regulation of endopeptidase I.

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