Note

Inhibition of Sporulation of Genus *Bacillus* by Various Microbial Protease Inhibitors

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It has been suggested from various studies that a serine protease is required for sporulation in the genus *Bacillus*.^{1~3)} One approach to elucidate the requirement of a serine protease in the sporulation process has been to study the effect of serine protease inhibitors on the sporulation. Certain synthetic reagents such as phenylmethylsulfonylfluoride (PMSF)⁴⁾ and *m*-aminobenzenboronic acid,⁵⁾ which are known to inhibit serine proteases, have been shown to inhibit the sporulation. The mode of action of these inhibitors, however, is not well defined and their effect may not result from the inhibition of a protease. For example, the concentration of *m*-aminobenzenboronic acid required to inhibit the sporulation was well below that needed to inhibit proteases *in vitro*.⁵⁾ Therefore, an inhibitor with a more specific action seems to be desirable.

As previously reported, we have found that MAPIs (microbial alkaline proteinase inhibitors), serine protease inhibitors produced by *Sreptomyces nigrescens* WT-27, strongly inhibit the sporulation of *Bacillus subtilis* IFO 3027 without affecting the vegetative growth.⁶⁾

In this paper, we describe the effects of various microbial protease inhibitors on sporulation of the genus *Bacillus*.

B. subtilis IFO 3027, *B. subtilis* IFO 3009 and *B. thuringiensis* var. *thuringiensis* (obtained from Dr. Aizawa) were cultivated in the medium previously reported.⁶⁾ *B. subtilis* BR 16, *B. subtilis* BR 17 (obtained from Dr. Gallant), *B. megaterium* IAM 1030 and *B. thuringiensis* var. *aizawai* (obtained from Dr. Aizawa) were cultivated in a medium consisting of 0.8% meat extract, 0.8% peptone (polypepton, Daigo Eiyo Kagaku, Osaka), 0.1% glucose, 0.5% NaCl, 0.025% MgSO₄·7H₂O, 0.01% CaCl₂·2H₂O and 0.001% MnCl₂·4H₂O, pH 7.0. *B. cereus* IFO 3131 was cultivated in Golub's medium.⁷⁾ The cells were aerobically grown at 37°C in a L-shape test tube (containing 5 ml of medium) on a Monod-type shaker at 100 strokes/min. Inhibitors were added at the end of exponential growth.

The total viable cell titer was determined by plating the cells on nutrient agar plates and the spore titer was determined after heating the diluted cultures for 15 min at 70°C and then plating. Intracellular proteases were prepared and assayed by the methods previously reported.^{8,9)}

Microbial serine protease inhibitors used were MAPIs (inhibitors of microbial serine protease, chymotrypsin and papain),^{10,11)} chymostatin (inhibitor of chymotrypsin and papain),¹²⁾ antipain (inhibitor of trypsin and papain),¹²⁾ leupeptin (inhibitor of trypsin and papain)¹²⁾ and S-SI (inhibitor of microbial serine protease).¹³⁾ α -MAPI-O [*N*-(1-carboxy-2-phenylethyl)carbamoyl-L-arginyl-L-valyl-L-phenylalanine] and β -MAPI-OMe [*N*-(1-methoxycarbonyl-2-phenylethyl)carbamoyl-L-arginyl-L-valyl-D-phenylalaninal dimethyl acetal] were prepared in our laboratory (Murao *et al.*, unpublished results).

Table I summarizes the effects of the inhibitors on sporulation of *B. subtilis* IFO 3027 and their inhibitory activities against intracellular serine protease of this strain. The inhibitors were stable in the culture medium and practically no inactivation of the inhibitors was observed during the cultivation period. Low concentrations of MAPIs, chymostatin and β -MAPI-OMe strongly inhibited the sporulation and antipain exerted a similar effect at higher concentration. On the other hand, leupeptin, α -MAPI-O and S-SI did not inhibit the sporulation even at 100 μ g/ml. When added at the beginning of cultivation, MAPIs⁶⁾ or the other inhibitors showed no effect on vegetative growth. Intracellular serine proteases of the strain were strongly inhibited by MAPIs, chymostatin, antipain and β -MAPI-OMe at the concentration used in the *in vivo* experiment, whereas these enzymes were insensitive to leupeptin and α -MAPI-O. Accordingly, only inhibitors which are capable of inhibiting the protease inhibited the sporulation.

In contrast to low molecular weight inhibitors (MAPI *etc.*), S-SI which has an inhibitory activity against extra- and intracellular (cytoplasmic) enzymes, showed no effect on the sporulation. This seemed to be due to a permeability problem since this inhibitor is a protein of a molecular weight of 23,000. Indeed, only extracellular protease activity was decreased (less than 12% of the control culture), when the cells were cultivated with S-SI (90 μ g/ml). Thus, the inhibition of extracellular protease was insufficient to inhibit the sporulation.

From these results, it is reasonable to consider that the inhibition of the sporulation by MAPIs and other inhibitors is due to inhibition of intracellular serine protease(s).

Next we examined the influence of α -MAPI on sporulation of various species of the genus *Bacillus*. As shown in Table II, the inhibitory effect of α -MAPI was observed in most strains so far tested.

Based on the facts mentioned above, it can be considered that an intracellular chymotrypsin-like protease¹⁴⁾ which is widely distributed in the genus *Bacillus* plays an important role in sporulation.

TABLE I. EFFECT OF VARIOUS PROTEASE INHIBITORS ON SPORULATION OF *B. subtilis* IFO 3027

The strain was cultivated in the presence of the indicated concentrations of the inhibitors at 37°C for 24 hr, then cell titers were determined.

Inhibitor	Concentration ($\mu\text{g/ml}$)	Viable cells ($\times 10^8/\text{ml}$)	Heat-resistant spores ($\times 10^8/\text{ml}$)	Inhibitory activity toward protease (%) ^a	
				Membrane bound ^b	Cytoplasmic ^b
α -MAPI	25	13	0.6	82	79
	10	14	0.8		
	5	14	3.5		
β -MAPI	25	15	0.6	80	76
	5	16	7.3		
γ -MAPI	25	11	0.6	62	68
	5	11	6.4		
Chymostatin	25	10	0.5	64	82
	15	13	1.1		
	5	11	2.3		
Antipain	100	7.3	0.1	20	65
	50	16	3.1		
	20	15	10		
Leupeptin	100	13	12	0	0
S-SI	100	21	16	0	51
α -MAPI-O	50	19	11	0	3
β -MAPI-OMe	20	19	2.0	60	51
Control,	—	18	14	—	—

^a Results are given as percent inhibition. Inhibitory activity of the inhibitor was assayed as follows: enzyme solution (0.25 ml) was preincubated with the inhibitor (0.25 ml, 20 $\mu\text{g/ml}$) at 37°C for 10 min, then the residual activity was measured with casein (1.5 ml, pH 9.5) as substrate.

^b The cells were harvested at T₈₋₉ and disrupted with lysozyme. Crude membrane and cytoplasmic fractions were separated by centrifugation at 10,000 *g* for 20 min. Membrane bound enzyme was extracted from the pellet, and purified by phenyl-Sepharose CL-4B column chromatography and Sephacryl S-300 gel filtration. The supernatant was used as the cytoplasmic fraction without further purification.

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TABLE II. EFFECT OF α -MAPI ON SPORULATION OF VARIOUS SPECIES OF GENUS *Bacillus*

Strain	α -MAPI ^a	Viable cells ^b ($\times 10^8$ /ml)	Heat-resistant ^b spores ($\times 10^8$ /ml)
<i>B. subtilis</i>			
IFO 3027	+	10	0.1
	—	17	14
IFO 3009	+	2.2	0.6
	—	3.6	3.4
BR-16	+	10	0.1
	—	15	9.5
BR-17	+	8.6	0.5
	—	17	13
<i>B. cereus</i>			
IFO 3131	+	1.1	0.1
	—	1.1	0.3
<i>B. megaterium</i>			
IAM 1030	+	1.8	0.1
	—	13	5.4
<i>B. thuringiensis</i> var. <i>aizawai</i>	+	3.8	0.2
	—	12	12
<i>B. thuringiensis</i> var. <i>thuringiensis</i>	+	6.1	0
	—	12	11

^a +, with 20 μ g/ml α -MAPI; —, without α -MAPI.^b After 24 hr cultivation.