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Interaction Proteome Analysis of Major Intracellular Serine Protease 1 in *Bacillus subtilis*

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Abstract Bacterial serine proteases, especially those from *Bacillus*, have been extensively studied. Intracellular serine protease 1 (Isp1) is responsible for most of the proteolytic activity in *B. subtilis*. To identify Isp1 substrates and study its physiological functions, a mutant of Isp1, which has lost the enzymatic activity, was constructed. Through a GST affinity chromatographic method, several *Bacillus* proteins that specifically interacted with S246A mutant Isp1 protein were isolated and then identified by MALDI-TOF analysis. ClpC and elongation factor Tu (EF-Tu) were among those proteins specifically bound to mutant Isp1. In addition, several proteins involved in stationary phase adaptive response (such as RNA polymerase sigma factor, spoIIIE) were also identified. These findings led us to suggest that the major function of this serine protease, whose expression is greatly increased during the stationary phase, is to mediate transition of the cell into the stationary phase in a proper and timely manner.

Key words: Isp1, interaction protein, MALDI-TOF, stationary phase

Four serine proteases (Isp1, Isp2, Isp3, and a membrane-bound protease) and a spore-germinating protease (Gpr) in *Bacillus subtilis* have been identified and studied as intracellular proteases [12, 16–18]. Among these intracellular proteases, Isp1 is the major protease, accounting for up to 80% of the intracellular azocollagen or azocasein hydrolytic activities, and has been well-characterized [3]. The activity of Isp1 begins to increase at the end of the exponential growth phase and reaches the maximum level during the initial stage of sporulation [15]. It was, therefore, originally suggested that Isp1 mediates the turnover of the bulk of cell proteins during sporulation and has an essential role in sporulation-associated processes. However, subsequent studies with the Isp1-deficient mutant and sporulation-defective mutant strains demonstrated that Isp1 is not required for the normal sporulation process [1]. Despite the above findings, the physiological substrates of Isp1 have not been clearly identified, and its regulatory process needs further studies to be clearly elucidated.

Bacillus subtilis gene encoding Isp1 was amplified from genomic DNA by PCR using a pair of primers covering the entire coding sequences (forward 5'-GC GGATCC ATG AAA AAG GGG ATC ATT CGC TTT C; reverse 5'-ACGC GTCGAC GTT TAT TCA ACA GTG AAA GGT TCT TC). The PCR-based site-directed mutagenesis of Ipsl (Ser246Ala) was performed by using following sets of primers; p1 primers (sense 5'-GC GG ATCC TTG AAA AAG GGG ATC ATT CGC TTT C; antisense 5'-CTG ACC GGC ACT GCA ATG GCT) and p2 primers (sense 5'-ACGC GTCGAC GTT TTA TTC AAC AGT GAA AGG TTC TTC; antisense 5'-AGC CAT TGC AGT GCC GGT CAG). The resulting PCR products were cloned into *E. coli* expression vector pGEX 6p-1. The mutation site of the *isp1* gene was verified by DNA sequencing.

The majority of Isp1 proteins were expressed as a soluble form, and had the expected molecular size of ~60 kDa (GST 26 kDa + Isp1 34 kDa) (data not shown). To purify GST-tagged Isp1 proteins, *E. coli* extracts containing soluble GST-tagged Isp1 proteins were adsorbed onto GSH-Sepharose beads, and the GST-tagged proteins were eluted by incubating the beads with 20 mM reduced glutathione. The purity of the eluted proteins was determined by SDS-PAGE analysis (data not shown).

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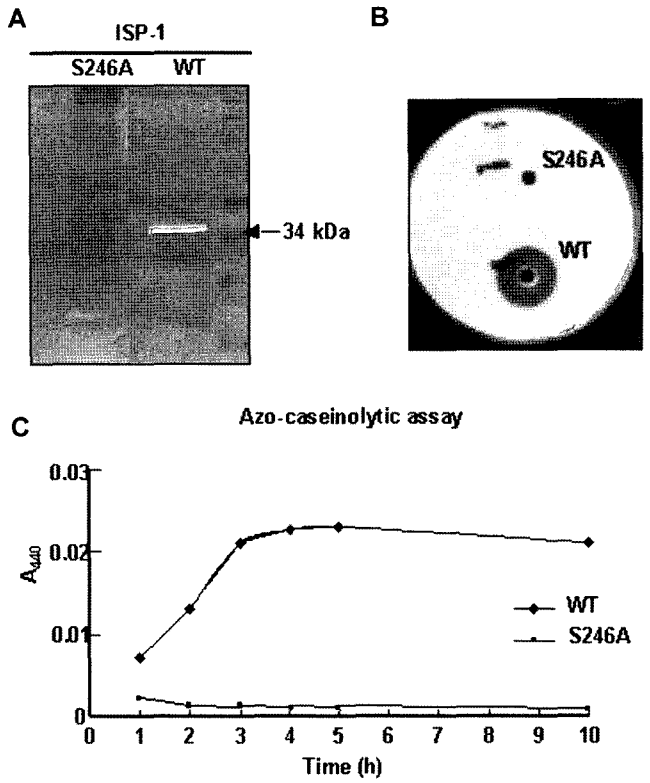

Fig. 1. Mutant Isp1 was enzymatically inactive.
Gelatin zymography (**A**), halo plate assay (**B**), and azo-caseinolytic assay (**C**) of purified GST-free Isp1 proteins showed that the wild-type enzyme was enzymatically active and the S246A mutant was inactive (50 μg of purified enzymes was used for the assays). WT, wild type.

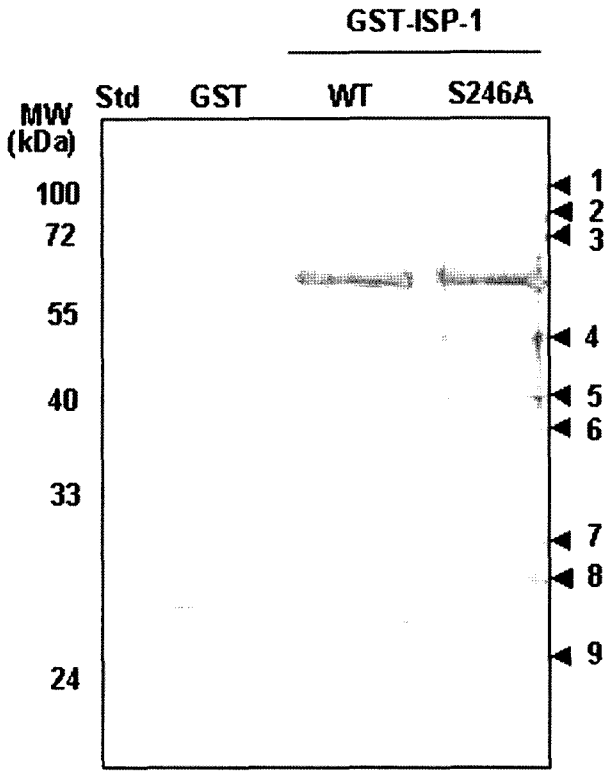

Fig. 2. Isolation of Isp1 interacting proteins.
Bacillus cell extract (50 mg of proteins) obtained from the KN2 strain (Δ*ispA* mutant) were incubated with GST-tagged proteins bound to GSH-Sepharose beads (GST, GST-Isp1 WT, and GST-Isp1 S246A mutant) for 3 h at 4°C. After the incubation, the beads were washed and the associated proteins were analyzed by 12% SDS-PAGE. Proteins specifically interacting with mutant GST-Isp1 were subsequently identified by MALDI-TOF mass spectrometry. Std, molecular weight standards; GST, GST only; WT, wild-type; S246A, Ser246Ala mutant of Isp1.

To confirm the proteolytic activity of purified Isp1 proteins, a zymography assay was performed [5]. The zymogram gel showed a single activity band at the expected molecular size of 34 kDa. On the contrary, mutant Isp1 was enzymatically inactive and showed no activity band in the zymogram (Fig. 1A). The halo plate assay of purified Isp1 also confirmed the result from the zymography assay [4] (Fig. 1B). In parallel, the caseinolytic activity of GST-free Isp1 proteins was measured by monitoring changes of absorption at 440 nm using azocasein as a substrate (Fig. 1C).

To elucidate the potential substrates of Isp1, *Bacillus* extracts (50 mg) obtained from the KN2 strain (*isp1* deletion mutant) were incubated with the GSH-Sepharose beads carrying GST-tagged Isp1 proteins (wild-type or S246A mutant) for 3 h at 4°C. After repeated washes, the bead-associated *Bacillus* proteins were separated by 12% SDS-PAGE. A number of proteins were found to specifically interact with S246A mutant Isp1 (Fig. 2), and subsequently, nine of them were identified by MALDI-TOF mass analysis [7]. Interestingly, many of these proteins identified in this study were the same as those proteins that have been reported to be specifically digested upon *in vitro* Isp1 treatment of KN2 extract [9].

Interestingly, some of the Isp1 interacting proteins identified in this study (Table 1), putative physiological substrates of Isp1, have been reported to have regulatory function(s) during the stationary phase and also to be involved in the stationary phase adaptive response. For example, transcriptional regulatory protein LevR and trehalose operon transcriptional repressor TreR are required for RNA synthesis. LevR is a positive transcriptional regulator of the levanase operon and contains a sigma-54 interaction ATP binding domain [10]. The expression of this operon is induced by fructose and is subjected to catabolite repression. Trehalose is a nonreducing disaccharide composed of α-glycoside-linked glucose moieties. This sugar can be used as a sole carbon and energy source. The trehalose operon is subject to regulation by induction, mediated by the repressor TreR [2]. Presumably, Isp1-mediated degradation of LevR and TreR leads to a decrease in the expression of levanase and constitutive expression of the trehalose operon, respectively. Moreover, RNA polymerase Sigma-F factor and SpoIIIE are involved in sporulation [14]. SpoIIIE is required for chromosome partitioning through the septum into the

Table 1. Isp1 interacting proteins.

NO	#AC	Description	MW(kDa)/*pI*[a]	Match[b]	Coverage[c]
	RNA synthesis				
1	P23914	Transcription regulatory protein LevR	106/5.9	4	4
8	P39796	Trehalose operon transcriptional repressor TreR	27/6.73	6	14
	Protein biosynthesis				
5	P33166	Elongation factor Tu	43/4.92	5	23
	Adaptation to atypical conditions				
2	P37571	Negative regulator of genetic competence ClpC	90/5.8	4	6
	Sporulation				
3	P21458	Similar to DNA translocase stage III sporulation protein	87/6.72	4	13
7	P07860	RNA polymerase sigma-F factor	29/5.25	5	26
	Phage-related function				
4	P54331	Phage-like element PBSX protein XkdK	50/4.74	4	19
	Unknown				
6	P42422	Hypothetical sensor-like histidine kinase YxdK	37/6.22	6	25
9	O34392	Hypothetical protein YtrE	25/5.99	4	17

[a]Theoretical value using ExPASY tool.
[b]Number of peptide sequence matches.
[c]Percent coverage obtained for the sequence of the identified protein.

forespore compartment during the spore formation. During the sporulation process, SpoIIIE could function as a DNA pump that actively moves one of the replicated pair of chromosomes into the prespore. The first transcription factor to be activated during the sporulation is the prespore-specific Sigma-F factor, which is responsible for the activation of the first mother cell-specific transcription factor and, through a cascade of events, of the later sporulation-specific transcription factors in both compartments [11]. The digestion of spoIIIE and Sigma-F factor by Isp1, therefore, may be crucial for sustaining the stationary phase by blocking the entry into sporulation.

More interesting candidates are those involved in stationary phase adaptive response (ClpC) and in protein synthesis (EF-Tu). ClpC is a negative regulator of genetic competence, and an ATP-dependent Hsp100/Clp protein is involved in general stress survival and the overall proteolysis of misfolded proteins [6, 13]. A complex of ClpC with the protease ClpP and the adaptor protein MecA controls competence development by regulating proteolysis of the transcription factor ComK, which is required for the transcription of genes encoding DNA transport proteins. It has been suggested that binding of ClpC to MecA targets ComK for degradation by the ClpP-ClpC complex. The small regulatory protein ComS, which is induced by a quorum-sensing pathway, triggers the release of ComK from the ClpC-MecA complex and leads to the stabilization of ComK. Competence is an important physiological state during the stationary phase, distinct from sporulation and vegetative growth, that enables cells to bind and internalize transforming DNA. Possibly, upon entry into the stationary phase, *Bacillus* begins to express Isp1, which first mediates the rapid breakdown of ClpC and the stabilization of ComK, and then induces genes required for competence. EF-Tu is a protein ubiquitously present in all kingdoms [8]. It plays a central role in protein biosynthesis, where it serves in the GTP-bound form for the transport of aminoacyl-tRNA to the A-site of the mRNA-programmed ribosome. Protein synthesis at the stationary phase is usually limited, because of depletion of nutrients. To save energy by blocking unnecessary protein synthesis and eventually to adapt to harsh conditions, it might be necessary to rapidly degrade EF-Tu by Isp1. Finally, it is plausible that other proteins (XkdK for Phage-like element PbsX protein and two proteins with unknown function) are also involved in stationary phase physiology. Further studies are needed to clarify their exact physiological functions and roles in stationary phase transition.

Isp1 is a major intracellular serine protease of *Bacillus subtilis*, whose exact functions still remain unknown, and whose physiological substrates are yet to be determined. To identify Isp1 substrates and study its physiological functions, a mutant form of Isp1, which was enzymatically inactive but still capable of binding substrates, was constructed and the *Bacillus* proteins that specifically interacted with this mutant Isp1 were isolated from an Isp1-deficient mutant

strain (KN2). Finally, several of such Isp1 interacting proteins were identified by MALDI-TOF mass spectrometry.

The Isp1 interacting proteins seem to be involved in various cellular functions, such as protein biosynthesis, genetic competence, RNA synthesis, and sporulation. Interestingly, a number of Isp1 interacting proteins are related to stationary phase adaptation, suggesting that a major function of this serine protease, whose expression is greatly increased during the stationary phase, is to mediate *Bacillus* cells transition into the stationary phase in a proper and timely manner. More studies will be required to further elucidate the precise functions of Isp1 during the stationary phase as well as its involvement in the regulation of competence.

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