INTRACELLULAR SERINE PROTEASE FROM BACILLUS SUBTILIS. STRUCTURAL COMPARISON WITH EXTRACELLULAR SERINE PROTEASES- SUBTILISINS

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SUMMARY.Intracellular serine protease was isolated in a pure state from sporulating Bacillus subtilis A-50. The enzyme has the molecular weight about 30.000 daltons and pI 4.3, is completely inhibited with phenylmethylsulfonyl fluoride and EDTA, and possesses a rather low activity against protein substrates, but high specific activity with subtilisin chromogenic substrates. Amino acid composition shows higher level of Glx, Lys and Phe residues and lower content of Val residues in comparison with known subtilisins. N-terminal sequence of the enzyme, being two residues shorter from N-terminus than subtilisin BPN', is NH_2-Ser-Leu-Pro-Glu-Gly-Ile-X-Val-Ile-Lys-Ala-Pro--Glu-Leu-Gln-Ala-Gln-Gly-Phe-Lys-(residues identical with BPN' are underlined). This clearly indicates the presence of two homologous structural genes, for intra- and extracellular serine proteases, in B.subtilis genome.

Many of the proteins of the cells are eliminated or transformed by processes of protein turnover or limited proteolysis (1). Intracellular proteases are implicated as agents of these convertions, but little is known about their nature and functional role in bacteria. The role of proteases might be especially significant for sporeforming bacilli, as it has been well known that sporulation of B.subtilis coincides with an increased protein turnover, the modifications of certain "vegetative" proteins, the excretion of enzymes etc (1, 2). Intracellular protease(s) yet unknown may play a clue role in such sporulation-specific processes. Thus, the proteases appearing within the cells during the period of sporulation deserved careful examination. Up to now most studies of B.subtilis proteases have dealt

ABBREVIATIONS. ISP, intracellular serine protease; pCMB, p-chlormercurybenzoate; PMSF, phenylmethylsulfonyl fluoride; pNA, p-nitroanilide.

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with extracellular enzymes, especially with subtilisins (3), whose specific role in sporulation is still a matter of speculations, but yet not definitely clarified (4, 5). In contrast, intracellular proteases of B.subtilis as well as of the other bacteria are studied only scarcely. One of such enzymes, intracellular serine protease, was detected by Reysset and Millet(6) in sporulating B.subtilis 168. Any structural information about this protease is lacking.

We succeeded in isolation from sporulating B.subtilis A-50 of an intracellular protease belonging to serine enzymes (subtilisin group) as judged by substrate specificity, action of inhibitors and N-terminal sequence, but possessing a rather unusual amino acid composition and an acidic isoelectric point. Presumably, it is the same protease as one detected by Reysset and Millet (6).

METHODS. ISP activity was routinely measured with Z-L-Ala-L--Ala-L-Leu-pNA at 37°C for 10-30 min. The stock solution contained 0.5 mg/ml of the substrate in dimethylformamide. Incubation mixture contained 0.01-0.1 ml of enzyme solution, 50 mM Tris-1 mM $CaCl_2$, pH 8.5, to 1.25 ml, 0.25 ml of the substrate. Reaction was stopped with 0.5 ml of 2 M Na-citrate, pH 5.0, and p-nitroaniline released was estimated at 410 nm. Z-Gly-Gly-L-Leu-pNA and Z-Gly-Gly-L-Phe-pNA were also used as the substrates under the same conditions from the stock solutions of 1 mg/ml and 0.5 mg/ml, respectively. The molar absorbance of p-nitroaniline at 410 nm was equal to 8900 $M^{-1}cm^{-1}$.

SDS-acrylamide gel-electrophoresis was performed by the method of Weber and Osborn (7) using 10% gels. Prior to reduction and denaturation performed with 1% SDS-1% 2-mercaptoethanol in 10 mM Na-phosphate buffer, pH 7.1, at 100°C for 2-3 min, ISP was inhibited with 1 mM PMSF for 60 min at 20°C. For molecular weight determination myoglobin, chymotrypsinogen, pepsin, bovine serum albumin and ovalbumin were used as standards.

Acrylamide disc-gel electrophoresis was carried out at 4°C as described by Davis (8). R_f of protein bands was calculated against bromophenol blue.

Thin-layer gel-isoelectrofocusing was performed with an LKB Multiphor apparatus and Ampholine PAGplates (pH gradient 3.5-9.5) as described by manufacturer. Prior to focusing ISP was inhibited with 1 mM PMSF.

The protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry.

For amino acid analysis the samples were hydrolyzed in 5.7 M

HCL at 110°C in vacuo for 24 or 72 hours. The hydrolyzates were analysed on a Durrum D-500 analyser.

Automated sequential Edman degradation was performed on a Beckman 890 sequencer using modified protein program No.050972 with 0.2M Quadrol. At each cycle of degradation, ethylacetate and when necessary water layers were analysed by thin-layer and gas-chromatographies and amino acid analysis for the identification of phenylthiohydantoins or their respective trimethylsilylated derivatives.

B.subtilis, A-50 strain, a kind gift of Dr. L.Keay (New Enterprise Division, Monsanto Company, St.Louis, Missouri) was used. The 500-ml flasks containing rich nutrient broth were inoculated from an agar plate culture and incubated overnight at 35°C on a rotary shaker. This culture was used to inoculate 15 liters of Spizizen medium (9) in a fermenter. After 20 hours at 35°C when the culture was at stationary growth, the cells were harvested by centrifugation, washed with iced 50 mM Na-phosphate-100 mM NaCl, pH 6.5, and frozen until use.

For ISP isolation the cells, 100 g wet weight, were thawed, resuspended in 50 mM Tris-1 mM $CaCl_2$, pH 8.5, and sonicated with a MSE sonifier. All steps were performed at 4°C. The crude extract was prepared by centrifugation at 30.000xg for 60 min. To the crude extract streptomycin sulfate was added to the final concentration of 1 mg/ml, and the precipitated nucleic acids were removed by centrifugation. The supernatant was fractionated with solid ammonium sulfate. The protein fraction that precipitated between 0.55 and 0.8 ammonium sulfate saturation was dissolved in 50 mM Tris-1 mM $CaCl_2$, pH 8.5, and dialyzed against the same buffer overnight. Then it was applied to a DEAE-cellulose DE-52 Whatman column (2.5x15 cm) equilibrated with the buffer mentioned above, and the adsorbed proteins were eluted with a 500-ml linear gradient of 50 mM to 500 mM Tris-buffer, pH 8.5, containing 1 mM $CaCl_2$. The active fractions, eluted at 500 mM Tris, were pooled, concentrated by ultrafiltration in Amicon cells equipped with Diaflo UM-2 membranes, applied to a column (3x70 cm) of Ultragel AcA34 LKB and run in 50 mM Tris-1 mM $CaCl_2$, pH 8.5. The active fractions were pooled, ultrafiltrated, treated with 1 mM PMSF for 60 min at 20°C and subjected to disc-gel electrophoresis in 6x100 mm tubes using 10% acrylamide gels (8). After the completion of electrophoresis the gel slices containing ISP band were collected, homogenized, and protein was extracted overnight in 50 mM Tris-100 mM NaCl-1 mM $CaCl_2$, pH 8.5. Gel particles were removed on a glass No.4 filter and by centrifugation at 18.000xg. The supernatant was dialyzed against 1 mM $CaCl_2$ and freeze-dried. The obtained sample was denatured with phenol-water mixture (10:1 v/v), precipitated with aceton and ether and dried in vacuo. After such treatment the samples were subjected to amino acid analysis and automated Edman degradation.

For a series of experiments the native non-inhibited ISP samples after Ultragel step were separated by disc-gel electrophoresis, extracted from the gel slices as described above, but dialyzed against 50 mM Tris-1 mM $CaCl_2$, pH 8.5, and concentrated by ultrafiltration. After electrophoresis the specific activity of ISP decreased 2-3-fold.

TABLE 1. ISP purification from stationary grown B.subtilis A-50.

Step	Protein	Activity total	Activity specific
	mg	units	mmol×min^{-1}×mg^{-1} of p-nitroaniline released
Crude extract	not measured	135	not measured
Streptomycin sulfate	10.000	150	0.015
Ammonium sulfate	400	180	0.45
DEAE-cellulose DE-52	36	90	2.5
Ultragel AcA34	15	79	5.3
PMSF-treatment, electrophoresis	10	-	-

RESULTS & DISCUSSION. ISP was isolated from the stationary grown culture of B.subtilis as shown in Table 1. Exponentially grown cells contain no detectable ISP activity. When analyzed by disc-electrophoresis the obtained enzyme showed only one band with R_f 0.7 or 0.5 in 7.5% or 10% gels, respectively, after Coomassie staining. The purity was confirmed by SDS-electrophoresis and gel-isoelectrofocusing. The bands corresponded to the molecular weight 31.000±1000 and pI 4.3, respectively, were revealed. However, the molecular weight of ISP might be overestimated, possibly as a result of anomalies in SDS binding by rather acidic ISP.

ISP appeared stable in 50 mM Tris-1 mM Ca^{++}, pH 8.5, at 4°C. Without Ca^{++} the enzyme activity was lost rapidly and irreversibly at 20°C. 1 mM PMSF, the inhibitor of serine proteases, inhibited ISP completely. pCMB treatment led to a weak inhibition of ISP - 20% of activity was lost. EDTA or EGTA completely and irreversibly inhibited ISP. The enzyme activity could not be restored by addition of divalent ions - Ca^{++}, Zn^{++}, Co^{++}, Mn^{++}, Mg^{++}. The main properties of ISP are given in Table 2.

ISP cleaved subtilisin chromogenic substrates, pNA of benzyloxycarbonyl tripeptides, Z-L-Ala-L-Ala-L-Leu-pNA being the most suitable. On the contrary, when hemoglobin or bovine serum albu-

TABLE 2. The main properties of ISP.

Isoelectric point	4.3	pH optimum	7-10
Molecular weight	31.000±1000	pH stability	6-7
PMSF, EGTA, EDTA	100% inhibition	Thermal stability up to	60°C
pCMB	20% inhibition	Thermal optimum	40°C

Table 3. Activity of ISP and subtilisin Novo "Nagarse".

Substrates	Specific activity ISP[x]	Subtilisin Novo
Z-L-Ala-L-Ala-L-Leu-pNA	5.3 (100%)	0.45 (100%)
Z-Gly-Gly-L-Phe-pNA	0.13 (2.5%)	0.031 (7%)
Z-Gly-Gly-L-Leu-pNA	0.21 (4%)	0.036 (8%)

[x]ISP activity was measured after Ultragel step.

min was used as substrate, a low activity was detected. K_m, the Michaelis constant, of ISP and subtilisin Novo "Nagarse" calculated from the Lineweaver-Burk plots were (pH 8.5, 30°C): with Z-L-Ala-L-Ala-L-Leu-pNA 6.5×10^{-4} and 3×10^{-4} M, respectively, and with Z-Gly-Gly-L-Leu-pNA 4×10^{-3} and 1×10^{-3} M, respectively.

ISP and subtilisin Novo "Nagarse" were treated with CNBr in trifluoroacetic acid, and the peptides resulted were studied electrophoretically in 6M urea-gel. Electrophoretic pattern of CNBr-peptides of ISP (5 bands) and subtilisin (4 bands) differed markedly, indicating the differences in the number and location of Met residues and/or peptide composition.

The amino acid composition of ISP is given in Table 4. Comparison with the composition of known subtilisins reveals significant differences, especially clear for Glx, Val, Lys and Phe.

Nevertheless, N-terminal sequence determination performed

TABLE 4. Amino acid composition of ISP and subtilisins A-50, BPN' (Novo), 221, Carlsberg, Amylosaccharitícus and Pfizer.

Residues	A-50 (10)	BPN' (11)	221 (12)	Carls-berg (11)	Amylosaccha-riticus (11)	Pfizer (13)	ISP (our data)
Lys	11	11	6	9	8	11	20
His	6	6	8	5	6	4	6
Arg	2	2	8	4	4	4	6
Asx	28	28	29	28	25	28	36
Thr	13	13	18	19	17	17	13
Ser	35	37	23	32	41	31	25
Glx	15	15	16	12	15	15	33
Pro	14	14	16	9	13	14	13
Gly	32	33	39	35	33	29	36
Ala	38	37	45	41	35	32	32
Val	30	30	27	31	25	22	19
Met	-	5	4	5	4	5	5-6
Ile	13	13	9	10	16	9	13
Leu	15	15	22	16	15	13	25
Tyr	9	10	9	13	12	9	7
Phe	3	3	2	4	4	4	7
Trp	-	3	3	1	3	-	-
Total	264	275	283	274	275	247	296

TABLE 5. N-terminal sequence of ISP and subtilisins BPN', Carlsberg and Amylosaccharitícus (11). Subtilisin A-50 has the same N-terminal sequence as BPN'.

	1				5				10		
BPN'	Ala-	Gln-	Ser-	Val-	Pro-	Tyr-	Gly-	Val-	Ser-	Gln-	Ile-
Carlsberg	Ala-	Gln-	Thr-	Val-	Pro-	Tyr-	Gly-	Ile-	Pro-	Leu-	Ile-
Amylosaccha-riticus	Ala-	Gln-	Ser-	Val-	Pro-	Tyr-	Gly-	Ile-	Ser-	Gln-	Ile-
ISP			Ser[a]	Leu-	Pro-	Glu-	Gly-	Ile-	X	-Val-	Ile-

	15						20				
BPN'	Lys-	Ala-	Pro-	Ala-	Leu-	His-	Ser-	Gln-	Gly-	Tyr-	Thr-
Carlsberg	Lys-	Ala-	Asp-	Lys-	Val-	Gln-	Ala-	Gln-	Gly-	Phe-	Lys-
Amylosaccha-riticus	Lys-	Ala-	Pro-	Ala-	Leu-	His-	Ser-	Gln-	Gly-	Tyr-	Thr-
ISP	Lys-	Ala-	Pro-	Glu-	Leu-	Gln-	Ala-	Gln-	Gly-	Phe-	Lys-

[a]The first step for ISP (the third one upon subtilisin numbering) was lost due to sequencer failure and, therefore, identified by the dansylation technique. X - unidentified residue.

with automated Edman degradation revealed a rather close relationship of ISP with subtilisins (Table 5). N-terminal sequence of ISP is two residues shorter than that of subtilisins. The essential substitutions were found for two positions: No.6 and No.15 (subtilisin numbering). The other substitutions detected are conservative, and the total number of substitutions are 9, 8 and 7 in comparison with subtilisins BPN', Amylosaccharitics and Carlsberg, respectively. In the region analyzed BPN' differs from Carlsberg with 10 residue substitutions. Earlier it has been shown that subtilisin from B.subtilis A-50 has practically the same amino acid composition as BPN' (Novo)(10). N-terminal sequence assay revealed no difference between subtilisin A-50 and BPN' throughout 15 determined N-terminal residues. Hence, N-terminal sequence of serine proteases from the same strain A-50, ISP and extracellular subtilisin A-50, is clearly homologous. This shows that there are two homologous structural genes for serine proteases in the genome of the same B.subtilis strain. It seems likely that ISP and subtilisin evolved from the same ancestor as a result of gene duplication. Upon our knowledge it is the first gene duplication in bacterial genome confirmed by structural data, except ribosomal proteins. It is of interest that the mechanism of secretion is well suited for subtilisin, but not ISP. Nevertheless, upon special conditions ISP might be found as extracellular enzyme.

Assuming the presence of two homologous structural genes for proteases in B.subtilis genome a rather significant divergence of various subtilisins (11) might be explained not by the point mutations solely, but also upon the basis of their recombination and more rapid evolution of one duplicated gene, which might be under relaxed control (14).

Nothing is known about the true substrates for ISP within a cell. However, a rather restricted substrate specificity of ISP especially with protein substrates, may lead to assumption that ISP might be considered as the "modifying" enzyme, rather than "degrading" one.

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REFERENCES

1. Pine, M.J.(1972) Ann.Rev.Microbiol., 26, 103-126.
2. Bott, K.F.(1976) in Second International Symposium on the Genetics of Industrial Microorganisms (Macdonald, K.D., ed.) pp.419-436, Academic Press, London-N.Y.-San-Francisco.
3. Markland, F.S., and Smith, E.L.(1971) The Enzymes, 3, 564-608.
4. Millet, J., Larribe, M., and Aubert, J.-P.(1976) Biochimie, 58, 109-117.
5. Piggot, P.J., and Coote, J.G.(1976) Bacteriol.Reviews, 40, 908-962.
6. Reysset, G., and Millet, J.(1972) Biochem.Biophys. Res. Commun., 49, 328-334.
7. Weber, K., and Osborn, M.(1969) J.Biol.Chem., 244, 4406-4412.
8. Davis, B.J.(1964) Ann. N.Y. Acad.Sci., 121, 404-427.
9. Spizizen, J.(1958) Proc.Natl.Acad.Sci., 44, 1072-1078.
10. Strongin, A.Ya., Abramov, Z.T., Levin, E.D., and Stepanov, V.M.(1975) Bioorganicheskaya Khimiya, 1, 1441-1448.
11. Kurihara, M., Markland, F.S., and Smith, E.L.(1972) J.Biol. Chem., 247, 5619-5631.
12. Nakamura, K., Matsushima, A., and Horikoshi, K.(1973) Agr. Biol.Chem., 37, 1261-1267.
13. Munnelly, K.P., and Kapoor, A.(1976) Int.J.Peptide and Protein Res., 8, 141-153.
14. de Haën, C., Neurath, H., and Teller, D.C.(1975) J.Mol.Biol., 92, 225-259.