Biochem. J. (1979) **179**, 333–339
Printed in Great Britain

Intracellular Serine Proteinase of *Bacillus subtilis* Strain Marburg 168

COMPARISON WITH THE HOMOLOGOUS ENZYME FROM *BACILLUS SUBTILIS* STRAIN A-50

By ALEXANDER YA. STRONGIN, DMITRII I. GORODETSKY, IRINA A. KUZNETSOVA, VERA V. YANONIS, ZAKHAR T. ABRAMOV, LIDIYA P. BELYANOVA, LUDMILA A. BARATOVA and VALENTIN M. STEPANOV
Institute of Genetics and Selection of Industrial Micro-organisms, Dorojnaya, 8, 113545 Moscow, U.S.S.R.

(*Received* 10 *August* 1978)

Intracellular serine proteinase was isolated from sporulating cells of *Bacillus subtilis* Marburg 168 by gramicidin S–Sepharose 4B affinity chromatography. The enzymological characteristics, the amino acid composition and the 19 residues of the *N*-terminal sequence of the enzyme are reported. The isolated proteinase was closely related to, but not completely identical with, the intracellular serine proteinase of *B. subtilis* A-50. The divergence between these two intracellular enzymes was less than that between the corresponding extracellular serine proteinases (subtilisins) of types Carlsberg and BPN′, produced by these bacterial strains. This may be connected with the more strict selection constraints imposed on intracellular enzymes during evolution.

Intracellular serine proteinases have been identified in a number of bacteria, e.g. *Bacillus subtilis* A-50 (Stepanov *et al.*, 1977), *Bacillus thuringiensis* var. *berliner* (Lecadet *et al.*, 1977) *Bacillus cereus* T (Cheng & Aronson, 1977*a*,*b*) and *Escherichia coli* B (Pacaud *et al.*, 1976; Pacaud, 1976). These enzymes are presumed to fulfil important functions in intracellular protein catabolism and limited proteolysis. Because *B. subtilis* strain Marburg 168 is one of the most favoured bacterial species for extensive biochemical and genetic studies, we have undertaken a detailed characterization of its intracellular proteinases. Reysset & Millet (1972) and more recently Szulmajster & Keryer (1975) succeeded in the partial purification of intracellular serine proteinase from this strain and reported some properties of the enzyme. Nevertheless, the enzyme has not been obtained in a pure state in reasonable amount; it was not well characterized and structural data on this proteinase were completely lacking. We decided to extend our experience gathered in the course of the study of intracellular serine proteinase from *B. subtilis* strain A-50 to the investigation of the enzyme from the Marburg strain, applying the same approach for its isolation (Strongin *et al.*, 1978*a*). The comparison of intracellular proteinases of these two *B. subtilis* strains is of interest for the following reason. It is known that *B. subtilis* Marburg 168 produces an extracellular serine proteinase, subtilisin, that belongs to the Carlsberg type (Boyer & Carlton, 1968; Markland & Smith, 1971). On the other hand, *B. subtilis* A-50 was found to secrete subtilisin of another type, namely BPN′ (synonym Novo) (Strongin *et al.*, 1975). Subtilisins BPN′ and Carlsberg, though structurally homologous, nevertheless reveal striking divergence, their sequences differing in 83 residues from a total of 275 (Markland & Smith, 1971). It has been proposed previously that intracellular serine proteinase and subtilisin of *B. subtilis* A-50 arose from the same ancestral protein as a result of ancestral gene duplication (Strongin *et al.*, 1978*b*). Therefore direct comparison of corresponding intracellular enzymes synthesized by two *B. subtilis* strains, Marburg 168 and A-50, should reveal whether the extent of divergence characteristic for extracellular subtilisins is reproduced for intracellular serine proteinases.

Abbreviations used: Z, benzyloxycarbonyl; Nan, *p*-nitroanilide.

Materials and Methods

Growth conditions

B. subtilis Marburg 168 (*Trp*$^-$) was grown at 32°C in a 100-litre fermenter containing 75 litres of modified Spizizen medium [$(NH_4)_2SO_4$, 0.5g; K_2HPO_4, 14g; KH_2PO_4, 6.0g; sodium citrate, 3.0g; $MgSO_4,7H_2O$, 0.2g; Hottinger meat broth (Experimental Moscow Meat Factory, Moscow, U.S.S.R.), 130ml; glucose, 5g; and DL-tryptophan, 10mg/litre, pH7.1–7.2]. Growth was followed by the determination of A_{650}. Sporulating cells at the

t_2–t_5 of the sporulation were harvested, washed with ice-cold 50mM-sodium phosphate buffer, pH6.5, containing 100mM-NaCl, and kept frozen at −20°C until use.

Enzyme assay

Enzyme activity was usually measured at 40°C with Z-L-Ala-L-Ala-L-Leu-Nan as substrate in 50mM-Tris/HCl buffer, pH8.5, containing 1mM-$CaCl_2$. Incubation mixture contained 0.005–0.1ml of enzyme solution, buffer up to 1.25ml and 0.25ml of the substrate dissolved in dimethylformamide at a concentration of 0.5mg/ml. After 5–15min incubation the reaction was stopped with 0.5ml of 2M-sodium citrate buffer, pH5.0. *p*-Nitroaniline released was assayed at 410nm (Strongin *et al.*, 1978*a*). To compare the specificity of the proteinases, a series of other chromogenic substrates synthesized in our laboratory [Z-Gly-Gly-L-Phe-Nan, Z-Gly-Gly-L-Leu-Nan, Z-Gly-L-Pro-L-Leu-Nan, Z-L-Ala-L-Ala-L-Phe-Nan and Z-L-Ala-L-Pro-L-Leu-Nan (Lyublinskaya *et al.*, 1977)] was used under the same set of conditions. One unit of activity is the amount of the enzyme that liberated 1μmol of *p*-nitroaniline from Z-L-Ala-L-Ala-L-Leu-Nan under the standard conditions/min. The molar absorption coefficient of *p*-nitroaniline at 410nm was $8900 M^{-1} \cdot cm^{-1}$. Protein content was measured at 280nm (Strongin *et al.*, 1978*a*).

Electrophoretic methods

Polyacrylamide-gel electrophoresis was performed in 10% acrylamide gel (acrylamide/*NN'*-methylenebisacrylamide ratio 19:1, w/w) as described by Davis (1964), sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described by Weber & Osborn (1969) and thin-layer gel isoelectrofocusing according to the manufacturer's instructions on Ampholine PAG-plates 'pH 3.5–9.5' (LKB, Bromma, Sweden). The references for molecular-weight estimation were myoglobin (mol.wt. 17800), chymotrypsinogen (25000), ovalbumin (45000) and bovine serum albumin (67000) from Serva, Heidelberg, Germany, pig pepsin (35000) isolated in our laboratory and subtilisin of BPN' type (28000) (trade name 'Nagarse'; Nagase, Nagoya, Japan).

Gel filtration

The molecular weight of the native proteinase was estimated by gel filtration through a Sephadex G-100 column (1.5cm × 100cm) as described by Andrews (1964). The proteinase peak was located by its activity. The column was run in 50mM-Tris/HCl buffer, pH8.5, containing 1mM-$CaCl_2$ and 100mM-NaCl, flow rate 10ml/h, and the fractions (2ml) were collected and analysed.

Amino acid analysis

For amino acid analysis, proteinase samples were hydrolysed for 24h with 5.7M-HCl *in vacuo*, and the resulting hydrolysates were analysed with a Durrum D500 amino acid analyser. Intracellular serine proteinases of *B. subtilis* Marburg 168 and A-50 were treated and analysed in parallel for more reliable comparison.

N-Terminal sequence determination

Sequence analysis with a Beckman 890 Sequenator and identification of the phenylthiohydantoins or their respective trimethylsilyl derivatives by g.l.c., t.l.c. and amino acid analysis after back hydrolysis were carried out exactly as reported by Strongin *et al.* (1978*a*). Before the sequence analysis, proteinase samples were treated with 1mM-phenylmethanesulphonyl fluoride for 60min at 20°C, dialysed against water, freeze-dried, denatured for 15min with phenol/water (10:1, v/v) at 20°C, precipitated and dried with ice-cold acetone and ether as described by Strongin *et al.* (1978*a*).

Proteinase purification

Intracellular serine proteinase was isolated from 400g of the frozen cell paste of *B. subtilis* Marburg 168 sporulating cells. A combination of previously reported procedures was used: cell disruption, nucleic acid precipitation, fractionation with $(NH_4)_2SO_4$ and DEAE-cellulose DE-52 chromatography were done as described by Stepanov *et al.* (1977), followed then by gramicidin S–Sepharose 4B affinity chromatography performed exactly as described by Strongin *et al.* (1978*a*). Neither column flow rates, dimensions, protein load, nor pH and ionic strength of buffer used were changed. Affinity adsorbent was prepared by coupling twice-crystallized commercial gramicidin S with CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and contained about 1μmol of ligand/ml of packed wet gel as judged by amino acid analysis (Strongin *et al.*, 1978*a*).

Results and Discussion

Isolation of the enzyme

As judged on an activity basis, the content of the enzyme in *B. subtilis* Marburg 168 sporulating cells was 20–25-fold less than that of intracellular proteinase in *B. subtilis* A-50 cells, complicating the enzyme preparation. Nevertheless, the isolation procedure previously described for *B. subtilis* A-50 proteinase (Stepanov *et al.*, 1977; Strongin *et al.*,

Table 1. *Purification of intracellular proteinase from sporulating cells of B. subtilis strain Marburg* 168
The enzyme purification started with 400g wet wt. of *B. subtilis* cells. The buffers used contained 1mM-Ca^{2+}. All purification steps were performed at 4°C. Activity was measured with Z-L-Ala-L-Ala-L-Leu-Nan as substrate. Protein is based on A_{280} measurements. Abbreviation: n.d., not determined.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Crude cell extract (30000*g* supernatant)	n.d.	210	n.d.	100	n.d.
Streptomycin sulphate	140000	160	0.00115	76.5	1
$(NH_4)_2SO_4$ (55–85% saturation)	24000	140	0.00585	66.5	5
DEAE-cellulose DE-52	1600	125	0.078	59.5	68
Two cycles of gramicidin S–Sepharose 4B affinity chromatography	16	60	3.75	28.5	3250

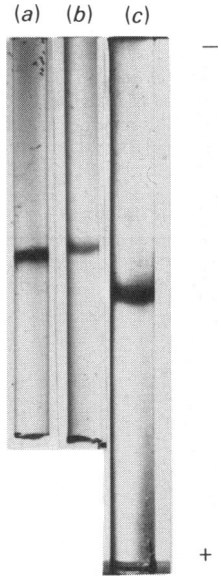

Fig. 1. *Polyacrylamide-gel electrophoresis (a, b) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (c) of intracellular serine proteinases from B. subtilis Marburg* 168 *and A*-50
(*a*) Proteinase of *B. subtilis* Marburg 168, (*b*) proteinase of *B. subtilis* A-50, (*c*) proteinase of *B. subtilis* Marburg 168 plus proteinase of *B. subtilis* A-50. About 20μg of protein was applied on each gel. Migration was from top to bottom. Protein was detected by staining with Coomassie Blue.

1978*a*) was also successfully used for the enzyme after minor modifications, the two reported procedures being combined. The enzyme purification included cell disruption with a MSE sonifier, nucleic acid precipitation with 1% (w/v) streptomycin sulphate, fractionation of the supernatant with solid $(NH_4)_2SO_4$ (55–85% of saturation), followed by DEAE-cellulose DE-52 column chromatography and two cycles of gramicidin S–Sepharose 4B affinity chromatography. This resulted in about 3000-fold purification with 30% recovery of the enzyme (Table 1). The product was essentially homogeneous, as shown by polyacrylamide-gel electrophoresis, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel isoelectrofocusing.

Electrophoretic properties and the molecular weight

The electrophoretic mobility of the enzyme was slightly higher at non-denaturing conditions than that of *B. subtilis* A-50 proteinase (Fig. 1), whereas in the presence of sodium dodecyl sulphate and 2-mercaptoethanol these enzymes migrated as a single band with mol.wt. 31000 ± 1000. The pI of the enzyme determined by gel isoelectrofocusing was 4.9 ± 0.1 (compared with the pI 4.3 for *B. subtilis* A-50 proteinase). When the proteinases from *B. subtilis* A-50 and Marburg 168 strains were gel-filtrated through Sephadex G-100, both the enzymes showed a mol.wt. of 54000–56000, corresponding to polypeptide-chain dimers (cf. Pacaud *et al.*, 1976).

Action of inhibitors

The enzyme and *B. subtilis* A-50 proteinase undoubtedly are serine proteinases, being completely inactivated with 1mM-phenylmethanesulphonyl fluoride and 1mM-di-isopropyl phosphorofluoridate in 50mM-Tris/HCl buffer, pH8.5, containing 1mM-$CaCl_2$, at 20°C in at least 30min. No enzyme activity was restored after the dialysis. Moreover, a specific feature presumably characteristic for all *Bacilli* proteinases (Lecadet *et al.*, 1977), namely complete inhibition by 2mM-EGTA and 2mM-EDTA as a consequence of strict Ca^{2+}-dependence, was also found for both the *B. subtilis* enzymes. Intracellular serine proteinase of *Bacillus licheniformis* recently isolated in our laboratory was

Table 2. *Relative rates of chromogenic-substrate hydrolysis*

Chromogenic peptide substrates were synthesized as described by Lyublinskaya *et al.* (1977). Specific activity of *B. subtilis* A-50 proteinase against Z-L-Ala-L-Ala-L-Leu-Nan was taken as 100%; it was 19 μmol of *p*-nitroaniline released/min per mg of protein under the standard conditions (see the Materials and Methods section). The conversion of the substrates did not exceed 5–10% under the conditions of the assay.

Substrate	Subtilisin BPN′	Intracellular proteinase from *B. subtilis* A-50	Intracellular proteinase from *B. subtilis* Marburg 168	Ratio A-50/168
Z-L-Ala-L-Ala-L-Leu-Nan	8	100	11	9:1
Z-L-Ala-L-Pro-L-Leu-Nan	0.72	12.5	5.5	2:1
Z-L-Ala-L-Ala-L-Phe-Nan	0.23	25	5	5:1
Z-Gly-Gly-L-Leu-Nan	0.5	2.5	0.85	3:1
Z-Gly-Gly-L-Phe-Nan	0.47	2.5	0.55	5:1
Z-Gly-L-Pro-L-Leu-Nan	0.45	3.8	2.6	1.5:1

Table 3. *Amino acid composition of intra- and extra-cellular serine proteinases of B. subtilis A-50 and Marburg 168*

Amino acid composition of subtilisins BPN′ and Carlsberg are taken from Kurihara *et al.* (1972). Subtilisin 168 is the extracellular serine proteinase isolated from *B. subtilis* Marburg 168 (indole$^-$) (Boyer & Carlton, 1968). Amino acid composition of *B. subtilis* A-50 proteinase is taken from Stepanov *et al.* (1977). One Cys residue, at least, was found in *B. subtilis* A-50 proteinase (Strongin *et al.*, 1978*a*). Abbreviation: n.d., not determined.

Residues	Intracellular proteinases from *B. subtilis* A-50	Intracellular proteinases from *B. subtilis* Marburg 168	Extracellular proteinases: Subtilisin BPN′	Subtilisin Carlsberg	Subtilisin 168
Lys	20	17	11	9	9
His	6	10	6	5	5
Arg	6	6	2	4	5
Asx	36	36	26	28	28
Thr	13	13	13	19	17
Ser	25	23	37	32	33
Glx	33	38	15	12	13
Pro	13	13	14	9	11
Gly	36	40	33	35	32
Ala	32	26	37	41	30
Val	19	19	30	31	22
Met	5	5*	5	5	4
Ile	13	13	13	10	14
Leu	25	22	15	16	16
Tyr	7	7	10	13	12
Phe	7	8	3	4	5
Trp	n.d.	n.d.	3	1	n.d.
Total	296	296	275	274	256

* Not determined; assumed to be 5 by analogy with *B. subtilis* A-50 proteinase.

also strictly Ca^{2+}-dependent and had a mol.wt. of 54000–56000 under non-denaturing conditions (Strongin *et al.*, 1979).

The peptide antibiotic gramicidin S, produced by *Bacillus brevis*, in a concentration of 0.1 mM completely inhibits the enzyme, consistent with its application as a ligand for affinity chromatography. On the other hand, the enzyme is resistant to bacitracin, a peptide antibiotic from *B. licheniformis*, and to natural trypsin inhibitor from soya bean.

Substrate specificity

The enzyme is capable of cleaving chromogenic substrates of subtilisin, namely *p*-nitroanilides of benzyloxycarbonyl-tripeptides. The specific activity of the enzyme against a series of these substrates

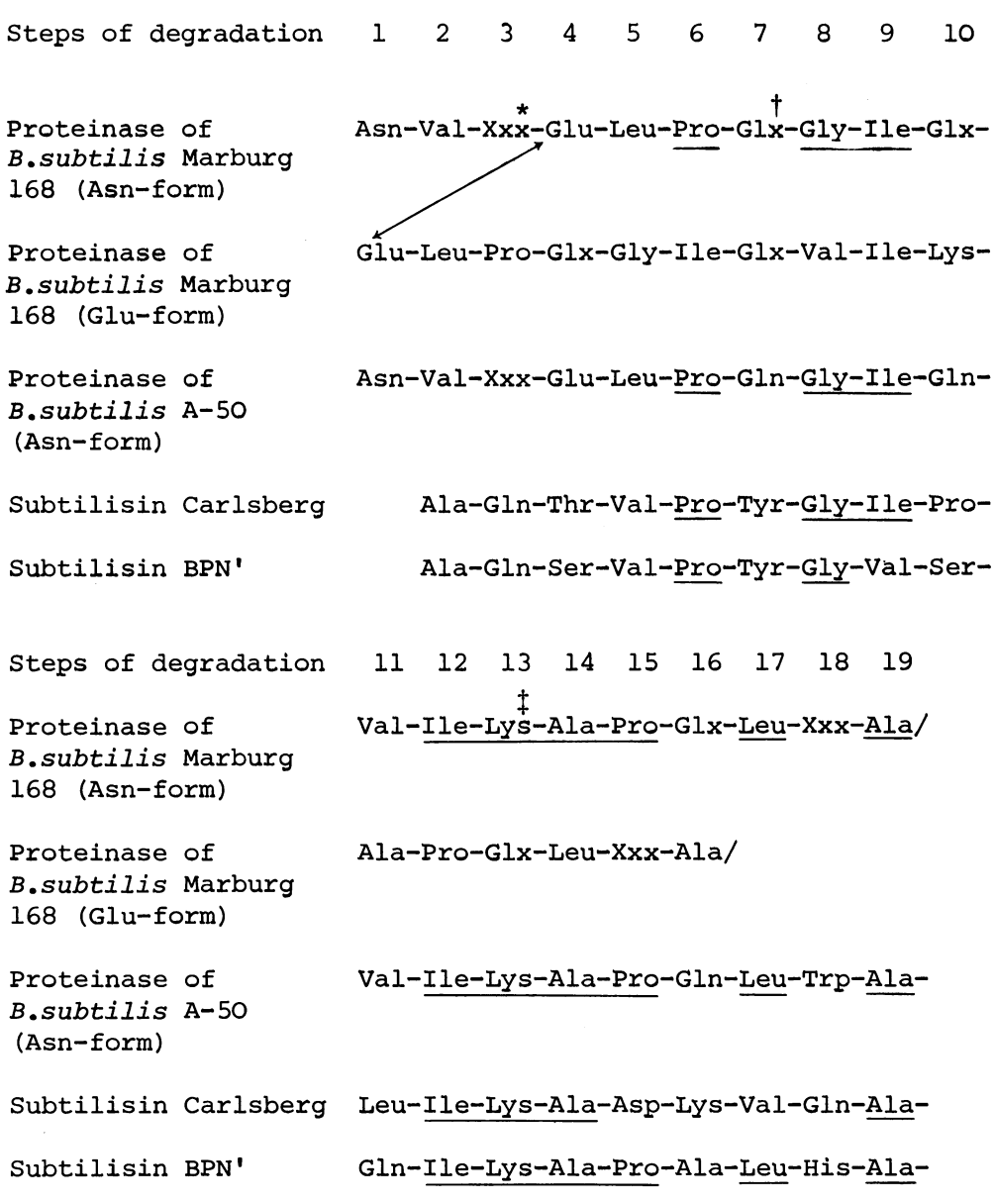

Fig. 2. *N-Terminal sequence of intracellular serine proteinases and extracellular subtilisins*
N-Terminal sequences of subtilisins are taken from Kurihara *et al.* (1972). Subtilisin A-50 from *B. subtilis* A-50 has the same *N*-terminal sequence as subtilisin BPN′ (Strongin *et al.*, 1978*a*). *N*-Terminal sequence of *B. subtilis* A-50 proteinase is taken from Strongin *et al.* (1978*a*). Identical residue positions in intracellular proteinases and any of the subtilisins are underlined. Xxx, unidentified residue.

* This is apparently Thr.
† A prominent amount of Val appeared at this step, but was not taken into consideration when constructing the sequence.
‡ Lys was revealed only by amino acid analysis.

was always higher than that of a secretory subtilisin of BPN′ type ('Nagarse'), but lower in comparison with *B. subtilis* A-50 proteinase. The most suitable substrate for all these enzymes was Z-L-Ala-L-Ala-L-Leu-Nan. The relative rates of substrate hydrolysis indicated that some difference between *B. subtilis*

A-50 and Marburg 168 intracellular enzymes should be awaited (Table 2).

The proteolytic activity of the enzyme against standard protein substrates such as azocasein, casein, haemoglobin and bovine serum albumin was negligible, being 30–100-fold less as compared with secretory subtilisins. Moreover, the enzyme shows extremely low activity, if any, against its presumed natural substrates, the mixture of *B. subtilis* intracellular proteins, although the latter was effectively cleaved by secretory subtilisin of BPN′ type (Strongin *et al.*, 1978*c*). Presumably, the enzyme cannot be considered as a proteinase with general breakdown functions.

Amino acid composition

Although the amino acid composition of the enzyme was clearly not identical with that of *B. subtilis* A-50 intracellular proteinase, it shared with the latter many common features. These included the high content of Glx, Lys, Leu and Phe, and the lower values of Ser, Val and Ala, compared with the composition of secretory subtilisins BPN′ and Carlsberg produced by these bacterial strains (Table 3). The subtilisin isolated by Boyer & Carlton (1968) from *B. subtilis* Marburg 168 indole$^-$-derivative also possesses the lower content of Val and Ala, though it is still clearly of the Carlsberg type (Table 3).

N-Terminal sequence

The *N*-terminal sequence of the enzyme was traced through 19 residues by automated Edman degradation (Fig. 2). The scarcity of the material available for sequence analysis (about 40nmol) did not permit more extended data, and the identification of certain residues (those marked Xxx) remained dubious.

Two polypeptide chains differing by the *N*-terminal extension were revealed in the enzyme during sequence determination. The main one started with Asn and the minor one with Glu and was three residues shorter at the *N*-terminus. These chains were not separable electrophoretically owing to the negligible difference in their molecular weights and the absence of charge difference. The presence of two polypeptide chains in the proteinase preparation complicated the sequence analysis. Thus the third residue of the main chain (apparently Thr) was not identified clearly, interfering with Pro, which appeared at the third step of degradation from the minor chain (Fig. 2). The same phenomenon of protein 'ragged ends' was observed for *B. subtilis* A-50 proteinase (Strongin *et al.*, 1978*a*). These enzyme forms appeared likely to result from limited proteolytic cleavage at two sites of an unknown proteinase precursor. They may also arise from the action of aminopeptidase(s) on the parent form of the enzyme with Met at the *N*-terminus. In the latter case it would be hard to explain the existence of only two forms without the intermediate products, e.g. Val-Xxx-Glu-enzyme. Nevertheless, 'nicking' of the enzyme at the *N*-terminus during isolation cannot be excluded also. The ratio Asn-form/Glu-form was about 1 : 5 for *B. subtilis* A-50 proteinase, as estimated by the yields of the corresponding residues at the appropriate steps of the Edman degradation (Strongin *et al.*, 1978*a*); in contrast, the ratio Asn-form/Glu-form was near 1.5 : 1 for the enzyme from *B. subtilis* Marburg 168.

It is clear that the *N*-terminal sequences of *B. subtilis* A-50 and Marburg 168 proteinases are very similar, being at the same time related to that of secretory subtilisins (Fig. 2). All the 17 residues of the enzyme *N*-terminal sequence that had been identified unambiguously coincided with those in the corresponding position of *B. subtilis* A-50 proteinase, which confirms the close structural relationship between these two enzymes. Clearly, one ought not to conclude from these data that the proteinases of the two *B. subtilis* strains compared are identical throughout the whole length of their polypeptide chains.

It appears that the amino acid sequences of intracellular serine proteinases are not so divergent as those of extracellular serine proteinases produced by *B. subtilis* A-50 and Marburg 168, that is, respectively, subtilisin BPN' and subtilisin Carlsberg. The latter proteinases differ by eight amino acid substitutions in the *N*-terminal stretch of 19 residues (Markland & Smith, 1971). The difference between intracellular proteinases might be compared with that between the closely related subtilisin BPN′ and subtilisin from *Bacillus amylosacchariticus*, possessing a single amino acid substitution within the same stretch of *N*-terminal sequence (Kurihara *et al.*, 1972).

Thus the difference in the primary structure between intracellular serine proteinases of two *B. subtilis* strains is substantially less than that between corresponding extracellular serine proteinases. A rationale for this might be seen in more stringent selection constraints imposed on the intracellular enzymes during evolution.

References

Andrews, P. (1964) *Biochem. J.* **91**, 222–233

Boyer, H. W. & Carlton, B. C. (1968) *Arch. Biochem. Biophys.* **128**, 442–455

Cheng, Y. E. & Aronson, A. I. (1977*a*) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1254–1258

Cheng, Y. E. & Aronson, A. I. (1977*b*) *Arch. Microbiol.* **115**, 61–66

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Kurihara, M., Markland, F. S. & Smith, E. L. (1972) *J. Biol. Chem.* **247**, 5619–5631
Lecadet, M.-M., Lescourret, M. & Klier, A. (1977) *Eur. J. Biochem.* **79**, 329–338
Lyublinskaya, L. A., Yakusheva, L. D. & Stepanov, V. M. (1977) *Bioorg. Khim.* **3**, 273–279 (in Russian)
Markland, F. S. & Smith, E. L. (1971) *Enzymes, 3rd Ed.* **3**, 561–608
Pacaud, M. (1976) *Eur. J. Biochem.* **64**, 199–204
Pacaud, M., Sibilli, L. & Le Bras, G. (1976) *Eur. J. Biochem.* **69**, 141–151
Reysset, G. & Millet, J. (1972) *Biochem. Biophys. Res. Commun.* **49**, 328–334
Stepanov, V. M., Strongin, A. Ya., Izotova, L. S., Abramov, Z. T., Ermakova, L. M., Baratova, L. A., Belyanova, L. P. & Lyublinskaya, L. A. (1977) *Biochem. Biophys. Res. Commun.* **77**, 298–305
Strongin, A. Ya., Abramov, Z. T., Levin, E. D. & Stepanov, V. M. (1975) *Bioorg. Khim.* **1**, 1441–1448 (in Russian)
Strongin, A. Ya., Izotova, L. S., Abramov, Z. T., Gorodetsky, D. I., Ermakova, L. M., Baratova, L. A., Belyanova, L. P. & Stepanov, V. M. (1978*a*) *J. Bacteriol.* **133**, 1401–1411
Strongin, A. Ya., Izotova, L. S., Abramov, Z. T., Gorodetsky, D. I. & Stepanov, V. M. (1978*b*) *Mol. Gen. Genet.* **159**, 337–339
Strongin, A. Ya., Izorova, L. S., Abramov, Z. T., Ermakova, L. M., Gorodetsky, D. I. & Stepanov, V. M. (1978*c*) *Arch. Microbiol.* **119**, 287–293
Strongin, A. Ya., Abramov, Z. T., Yaroslavtseva, N. G., Baratova, L. A., Shaginyan, K. A., Belyanova, L. P. & Stepanov, V. M. (1979) *J. Bacteriol.* in the press
Szulmajster, J. & Keryer, E. (1975) in *Spores VI* (Gerhardt, P., Sadoff, H. L. & Costilow, R. W., eds.), pp. 271–278, American Society for Microbiology, Washington, D.C.
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412