RESEARCH COMMUNICATION

A novel post-translational modification of the peptide antibiotic subtilin: isolation and characterization of a natural variant from *Bacillus subtilis* A.T.C.C. 6633

Weng C. CHAN,* Barrie W. BYCROFT,*‡ Mark L. LEYLAND,†§ Lu-Yun LIAN† and Gordon C. K. ROBERTS†

*Department of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, and †Biological NMR Centre and Department of Biochemistry, University of Leicester, Leicester LE1 9HN, U.K.

A variant of the peptide antibiotic subtilin has been isolated from *Bacillus subtilis* A.T.C.C. 6633, and its structure has been shown to be [N^{α}-succinyl-Trp^1]subtilin. The chemical structure of a fragment derived by tryptic hydrolysis of the variant is shown to be N^{α}-succinyl-Trp-Lys by ^{1}H and ^{13}C n.m.r., fast-atom-bombardment m.s. and total chemical synthesis. [N^{α}-Succinyl-Trp^1]-subtilin is produced later in the growth of the bacterium than is subtilin; reverse-phase h.p.l.c. analysis shows that after 24 h growth the ratio subtilin/[N^{α}-succinyl-Trp^1]subtilin is approx. 1:2. Although [N^{α}-succinyl-Trp^1]subtilin retains significant antibacterial activity, it is 10–20 times less active than subtilin.

INTRODUCTION

Subtilin [1] belongs to a unique group of post-translationally modified peptide antibiotics known as lantibiotics [2], which includes nisin, epidermin, gallidermin and pep-5. These antibiotics have been proposed to act by means of the voltage-dependent formation of pores in the cytoplasmic membrane [3]. They have in common the presence in their structure of one or more didehydroamino acid, *meso*-lanthionine and (2*S*,3*S*,6*R*)-3-methyl-lanthionine residues. The latter two residues introduce unique sulphide bridges in these peptide molecules, which thus contain a series of cyclic units.

Subtilin (Figure 1a) is structurally related to nisin [4], an important food preservative, in terms of both its chemical structure [1,5] and its conformation in aqueous solution [5,6]. The structural gene for the precursor of subtilin [7] encodes a 56-residue peptide, consisting of a 24-residue leader sequence followed by a 32-residue sequence which corresponds to that of mature subtilin except it contains serines, threonines and cysteines as precursors of the modified amino acid residues described above.

Hansen et al. [8] and we [5] have noted that, in the course of isolating subtilin from *Bacillus subtilis* A.T.C.C. 6633, two peptides with potent antibacterial activity were observed to be eluted close to one another on reverse-phase (RP-) h.p.l.c. The peptide with the shorter retention time (Hansen's subtilin A) has been unambiguously identified as subtilin [5]. We now report the determination of the structure of the second peptide, referred to as subtilin B, and show that it is subtilin which has been succinylated on its N-terminus.

EXPERIMENTAL

Isolation of subtilin B and its tryptic fragments

Subtilin B was isolated from a 48 h *B. subtilis* A.T.C.C. 6633 culture broth by the established protocol previously described in detail for the isolation of subtilin [5]. The final purification step was semi-preparative RP-h.p.l.c. on a Kromasil KR100-5C8 column (8.0 mm × 250 mm). The solvents used were: (A) aq. 0.06% trifluoroacetic acid (TFA), and (B) 0.06% TFA in aq. 90% acetonitrile. Elution was with a linear gradient of 40–46% B over 12 min, followed by isocratic 46% B for 4 min, at a flow rate of 2.60 ml/min. Analytical RP-h.p.l.c. was carried out on a Vydac 208TP5[C_8] column (4.6 mm × 150 mm) at a flow rate of 1.20 ml/min, with a linear gradient elution from 30% to 55% B in 16 min; subtilin and subtilin B were eluted at 10.95 and 11.74 min respectively. Antimicrobial activities, determined as minimum inhibitory concentrations, were estimated by an agar diffusion assay.

For preparation of the tryptic fragments, 8.0 ml of a solution of subtilin B (12.2 mg) and tosylphenylalanylchloromethane ('TPCK')-treated trypsin (2 mg) in Tris/acetate buffer (30 mM sodium acetate, 5 mM Tris, 5 mM $CaCl_2$, pH 7.6) was incubated at 32 °C for 24–36 h. The mixture, after acidification and filter-sterilization, was then purified by semi-preparative RP-h.p.l.c. (Hypersil Pep 100-8C_{18}) to afford subtilin-(3–29)-peptide and N^{α}-succinyl-Trp-Lys. These peptides were characterized by amino acid analysis and fast-atom-bombardment (FAB-) or plasma-desorption mass spectroscopy (m.s.).

Chemical synthesis of N^{α}-succinyl-Trp-Lys

N^{α}-Succinyl-Trp-Lys was synthesized by continuous-flow solid-phase methodology on a Milligen PepSynthesizer 9050, with Fmoc-Lys(Boc)-PepSyn KA (0.1 mmol/g) as the derivatized resin support (Fmoc, fluoren-9-ylmethoxycarbonyl; Boc, t-butoxycarbonyl). N^{α}-Fmoc deprotection was accomplished with 20% piperidine in dimethylformamide (DMF). The acylation mixtures for the two steps in the synthesis were respectively Fmoc-Trp(Boc)-OH/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*-hydroxybenzotriazole/di-isopropylethylamine (DIPEA) (2:2:1:2) and succinic anhydride/DIPEA (2:1) (molar ratios), each in DMF at 4 equivalents excess. The assembled peptide-resin was then simul-

Abbreviations used: RP-h.p.l.c., reverse-phase h.p.l.c.; FAB-m.s., fast-atom-bombardment mass spectroscopy; TFA, trifluoroacetic acid.

‡ To whom correspondence should be addressed.

§ Present address: Department of Biochemistry, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.

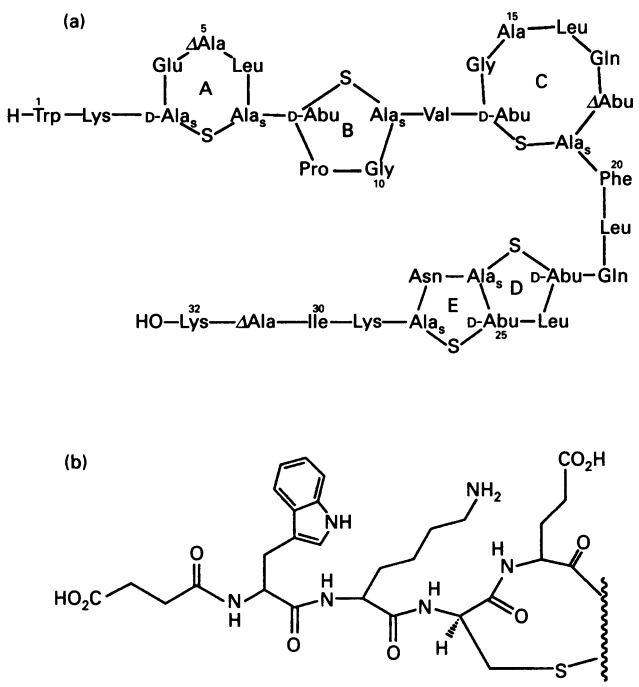

Figure 1 (a) Schematic chemical structure of subtilin and (b) chemical structure of the N-terminal region of [N^{α}-succinyl-Trp^1]subtilin

Abbreviations: ΔAbu, dehydrobutyrine; ΔAla, dehydroalanine; Ala_s, the alanine moiety of lanthionine or 3-methyl-lanthionine; D-Abu, 2-aminobutyric acid moiety of 3-methyl-lanthionine.

taneously deprotected and cleaved by treating with TFA/water/ethanedithiol (9.0 ml:5 ml:0.5 ml) at 28 °C for 1.5 h. After filtration, the filtrate was evaporated to dryness *in vacuo*, the residue triturated with ether (3 × 20 ml), dissolved in aq. 1% TFA (50 ml) and finally freeze-dried. This crude material (> 90% pure) was then purified by RP-h.p.l.c.

^{1}H and ^{13}C n.m.r. spectroscopy

N.m.r. measurements were carried out on a Bruker AMX 600 spectrometer operating at 600.14 MHz for ^{1}H, and 150.91 MHz for ^{13}C experiments. Samples contained 2 mM subtilin or subtilin B in 85% ^1H_2O/15% ^2H_2O (pH 2.50), or 5–10 mM N^{α}-succinyl-Trp-Lys in [$^2H_6$]dimethyl sulphoxide. Two-dimensional ^{1}H-n.m.r. experiments were carried out as described in detail previously [5,6,9]. All data were processed in the phase-sensitive mode, by using as window functions either a Lorentzian–Gaussian transform or a sine-bell squared function with a $\pi/6$ phase shift. ^{13}C spectra were accumulated under conditions of noise-modulated broad-band ^{1}H decoupling with a recycle time of 3 s. The ^{13}C chemical shifts were measured relative to dioxan, but are expressed relative to tetramethylsilane (TMS), taking the shift of dioxan relative to TMS as 67.4 p.p.m.

RESULTS AND DISCUSSION

Isolation and initial characterization of subtilin B

Butanol extraction of culture broths of *B. subtilis* A.T.C.C. 6633 grown at 37 °C for 24–48 h yielded crude materials with potent antimicrobial activities against a number of Gram-positive test organisms (e.g. *Micrococcus luteus* NDCO8166 and *Lactococcus lactis* MG1614). However, RP-h.p.l.c. analysis revealed that the activity resided predominantly in two closely-eluting components, which were subsequently purified to homogeneity by semi-preparative RP-h.p.l.c. The earlier-eluted component is the expected peptide antibiotic subtilin, as shown by n.m.r. and mass spectroscopy [5]. Amino acid analysis of the second component reveals that it has the same amino acid composition as subtilin, as previously reported by Hansen et al. [8]. This second component, a natural variant of subtilin, is designated subtilin B.

Subtilin B has potent antibacterial activity, although this is significantly less than that of subtilin. The minimum inhibitory concentrations of subtilin and subtilin B are respectively 0.05 and 0.34 μg/ml against *M. luteus*, and 1.1 and 21.3 μg/ml against *L. lactis* MG1614.

Determination of the molecular mass of subtilin B by FAB-m.s. gave a value (MH^+ 3420.9) indicating a mass of 99.0 units greater than that of subtilin (MH^+ 3321.9). However, from the amino acid analysis, this mass difference is clearly not due to an extra valine (requires 99.1) or proline (requires 97.1) residue. In addition, in contrast with our experience with subtilin, attempted sequencing of subtilin B by Edman degradation was unsuccessful. This suggests that in subtilin B the α-amino group of Trp^1 residue is blocked by a chemical moiety of ~ 99 mass units, which is not an α-amino acid.

^{1}H n.m.r. analysis

A sample of subtilin B was analysed by ^{1}H n.m.r. spectroscopy. The spectrum of the peptide in aqueous solution was assigned by using a range of two-dimensional n.m.r. experiments, as described in detail for nisin [6,9]. Briefly, resonances were first assigned to types of amino acids from the patterns of scalar connectivities revealed in the homonuclear Hartmaan–Hahn (HOHAHA) spectra. By analogy with subtilin, the ABMX and the unique A_3MPX spin systems of the Ala_s and Abu (2-aminobutyrate) moieties respectively were readily identified, and assignment of the Trp spin system was aided by the long-range indole C^2H–$C_{\beta}H_2$ scalar connectivity. Also, the presence of dehydroamino acids (ΔAla and ΔAbu), not detectable by amino acid analysis, is confirmed by their unique chemical shifts (e.g. $C_{\beta}H$ δ 5.5–6.9 p.p.m.) and spin systems. Assignment to specific residues in the sequence was then based on the observation of nuclear Overhauser enhancement spectroscopy (NOESY) cross-peaks between the $N_{\alpha}H$ of one residue and the $C_{\alpha}H$, $C_{\beta}H$ or $N_{\alpha}H$ of the preceding residue in the sequence. Some of the measured ^{1}H chemical shifts of subtilin B are compared with those of subtilin in Table 1.

Additional resonances, beyond those required to account for the structure of subtilin, were observed at δ 2.61 and 2.53, with coupling patterns suggestive of a CH_2–CH_2 fragment; these presumably arise from the proposed 'blocking' group on the N-terminus. The observation of an $N_{\alpha}H$ resonance for Trp^1 at δ 8.17, characteristic of an amide NH, is consistent with the N-terminal substituent being an acyl moiety. It is apparent from Table 1 that the ^{1}H chemical shifts of the residues in the N-terminal region of the molecule, from Trp^1 to Leu^6, differ between subtilin B and subtilin. The connectivity in this region of subtilin B is, however, firmly established by the n.m.r. experiments, and it is clear that the only structural difference between subtilin B and subtilin is the acyl substituent on the N-terminus in subtilin B.

Nature of the N^{α}-acyl substituent

In order to identify the N^{α}-acyl substituent, subtilin B was treated with trypsin to give two fragments which were purified by RP-h.p.l.c. One of these was identical with a fragment produced by

Table 1 ^{1}H-n.m.r. (600 MHz) chemical shifts of subtilin (2 mM) and subtilin B (2.5 mM) in aqueous solution (pH 2.5; 85% ^1H_2O/15% ^2H_2O) at 303 K

	δ (p.p.m.)					
	Subtilin			Subtilin B		
	N*H*	$C_\alpha H$	$C_\beta H$	N*H*	$C_\alpha H$	$C_\beta H$
Succinyl	–	–	–	–	2.61	2.53
Trp[1]		4.48	3.50, 3.55	8.17	4.71	3.30, 3.45
Lys[2]	8.87	4.37	1.86, 1.92	8.46	4.15	1.68, 1.79
D-Ala_s[3]	8.69	4.86	3.11, 3.32	8.17	4.68	3.21 3.29
Glu[4]	7.98	4.15	1.58, 1.90	8.15	4.59	2.06, 2.40
ΔAla[5]	9.71	–	5.50, 5.66	10.04	–	5.34, 5.60
Leu[6]	8.85	4.47	1.78	8.09	4.41	1.56
Ala_s[7]	8.29	4.58	3.08, 3.18	8.36	4.53	3.08, 3.20
D-Abu[8]	8.82	5.15	3.49	8.76	5.11	
Pro[9]	–	4.48	2.00, 2.50	–	4.45	2.02, 2.55
Gly[10]	8.77	3.68, 4.42	–	8.70	3.48, 4.48	–
Ala_s[11]	8.03	4.08	3.09, 3.72	7.83	4.00	3.05, 3.67
Val[12]	8.41	4.26	2.12	8.40	4.23	2.10
D-Abu[13]	8.35	4.69	3.65	8.35	4.67	3.65
Gly[14]	8.39	4.11, 4.28	–	8.36	4.07, 4.29	–
Ala[15]	8.61	4.20	1.48	8.61	4.18	1.47
Leu[16]	8.53	4.31	1.83	8.54	4.31	1.82
Gln[17]	7.85	4.51	2.26	7.82	4.50	2.25
Δ^2Abu[18]	8.74	–	6.91	8.69	–	6.91
Ala_s[19]	7.60	4.48	2.94, 3.00	7.57	4.46	2.92, 3.00
Phe[20]	8.10	4.68	3.15, 3.22	8.10	4.67	3.16, 3.22
Leu[21]	7.97	4.40	1.68	7.96	4.40	1.67
Gln[22]	8.25	4.36	2.12, 2.20	8.24	4.36	2.11, 2.18
D-Abu[23]	8.68	5.00	3.64	8.66	5.00	3.64
Leu[24]	8.02	4.72	1.96	8.01	4.70	1.95
D-Abu[25]	9.34	4.94	3.60	9.33	4.93	3.58
Ala_2[26]	7.88	4.00	2.84, 3.78	7.87	4.00	2.83, 3.76
Asn[27]	8.72	5.01	2.76, 2.97	8.70	5.01	2.76, 2.97
Ala_s[28]	7.74	4.32	2.81, 3.70	7.73	4.32	2.79, 3.70
Lys[29]	8.52	4.45	1.82, 1.91	8.51	4.45	1.81, 1.91
Ile[30]	8.24	4.30	1.98	8.23	4.30	1.98
ΔAla[31]	9.67	–	5.79, 5.82	9.67	–	5.78, 5.82
Lys[32]	8.35	4.47	1.88, 2.02	8.33	4.46	1.88, 2.00

Table 2 ^{1}H n.m.r. (600 MHz) and ^{13}C n.m.r. (150 MHz) chemical shifts of N^α-succinyl-Trp-Lys (5 mM) in [$^2H_6$]dimethyl sulphoxide at 296 K

	δ (p.p.m.)
Succinyl	2.34 ($C_\alpha H$), 2.17, 2.28 ($C_\beta H$); 177.8 (C_αO), 30.0, 31.3 (C_αH, C_βH), 174.2 (C_βOOH)
Trp[1]	8.15 ($N_\alpha H$), 4.46 ($C_\alpha H$), 2.91, 3.18 ($C_\beta H$), indole: 10.78 (N*H*), 7.13 (C^2H), 7.54 (C^5H), 6.96 (C^6H), 7.04 (C^7H), 7.30 (C^8H); 176.3 (C_αO), 55.5 ($C_\alpha H$), 27.7 (C_βH), indole: 125.5 (C_2H), 109.9 (C^3), 127.9 (C^4), 120.3 (C^5H), 122.8 (C^6H), 119.4 (C^7H), 112.8 (C^8H), 137.2 (C^9)
Lys[2]	7.95 ($N_\alpha H$), 4.05 ($C_\alpha H$), 1.65 ($C_\beta H$), 1.33 ($C_\gamma H$), 1.52 ($C_\delta H$), 2.74 (C_ϵ); 175.42 (C_αO), 53.6 (C_αH), 30.7 (C_β), 22.5 (C_γH), 26.9 (C_δH), 40.0 (C_ϵH).

trypsin treatment of subtilin, which has been shown [10] by amino acid analysis and plasma-desorption m.s. (MH^+ found 2696.7, requires 2697.19) to be subtilin-(3–29-peptide). The structure of the second fragment isolated from subtilin B was shown to be N^α-succinyl-Trp-Lys, by ^{1}H and ^{13}C n.m.r. (Table 2), FAB-m.s. (MH^+ found 434, requires 433.21) and total chemical synthesis. The ^{1}H n.m.r. spectrum (Figure 2) and RP-h.p.l.c. profile of the synthetic peptide were found to be identical with those of the tryptic-derived N^α-succinyl-Trp-Lys, thus indirectly confirming the chemical structure of subtilin B (Figure 1b). The succinyl moiety also accounts favourably for the observed mass difference between subtilin B and subtilin determined by FAB-m.s.

The possibility that the N^α-acyl substituent might be a succinamyl [2-(aminocarbonyl)propionyl] group was also investigated, but the h.p.l.c. behaviour of synthetic N^α-succinamyl-Trp-Lys was observed to be quite different from that of the tryptic fragment of subtilin B.

Hansen et al. [8] have reported the isolation of a variant of subtilin from both *B. subtilus* A.T.C.C. 6633 and *B. subtilis* LH45

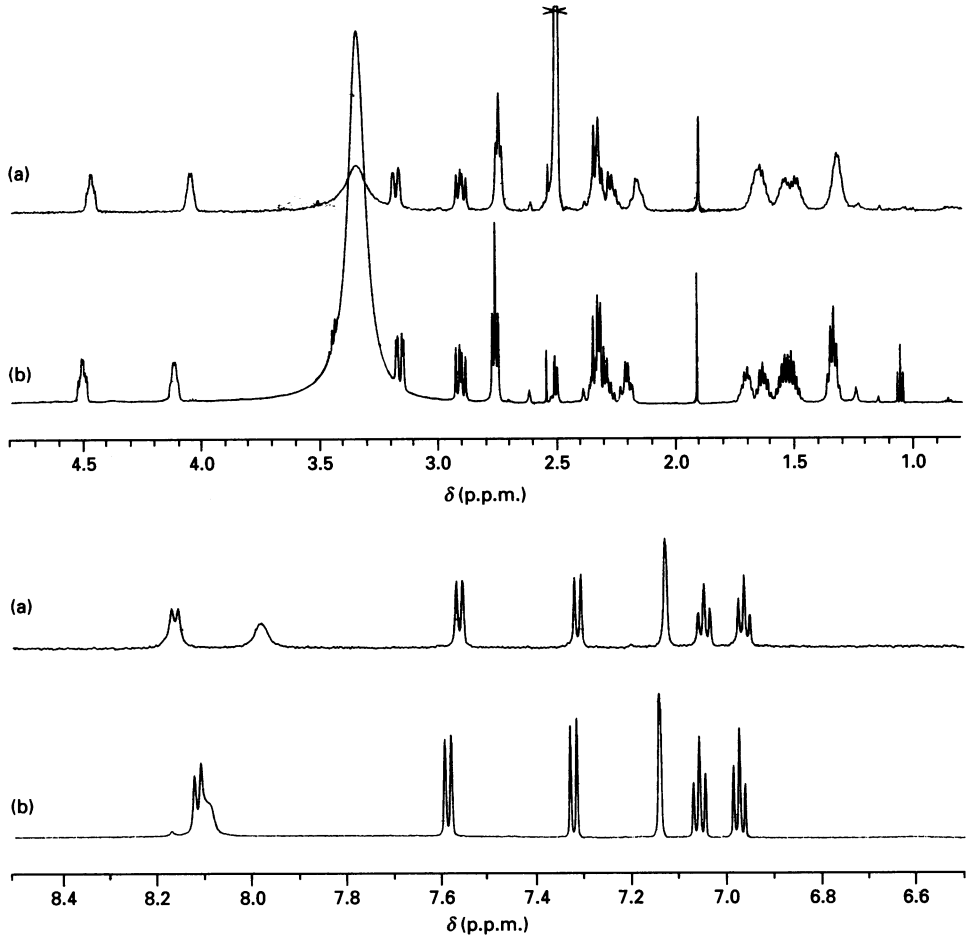

Figure 2 Regions of the ^1H n.m.r. (600 MHz) spectra of (a) tryptic-derived and (b) synthetic N^{α}-succinyl-Trp-Lys in [$^2H_6$]dimethyl sulphoxide at 296 K

which had the same amino acid composition as subtilin, and the same activity as an inhibitor of outgrowing bacterial spores; they speculated that the two molecules might differ only in the chirality at C_{α} of Ala_s^3. It is clear from the data in the present paper that the subtilin variant which we have isolated does not have the structure proposed for subtilin B by Hansen et al. [8]. However, it is not yet entirely clear whether the variants isolated by our two groups are the same molecule. In particular, Hansen et al. [8] report that their subtilin B could, in contrast with our observations, be sequenced by Edman degradation as far as ΔAla^5, giving the same sequence as subtilin itself. This would appear to indicate that, as isolated, their subtilin B does not have a substituent on the terminal α-amino group. It is nevertheless interesting to note that the chemical shifts for ΔAla^5 in (N^{α}-succinyl-Trp^1)subtilin are nearly identical with those reported by Hansen et al. for their subtilin variant (δ 5.34, 5.60, cf. 5.30, 5.56 p.p.m. [8], corrected by positioning $\Delta Abu^{18}C_{\beta}H$ at δ 6.91). It remains to be established whether there are in fact two different structural variants of subtilin; to avoid confusing nomenclature, we propose that chemically explicit names such as (N^{α}-succinyl-Trp^1)subtilin should be used in preference to, for example, subtilin B.

A number of naturally occurring variants in the family of lantibiotics have been reported, including (Leu^6)epidermin [11] and (Asn^{27})nisin [12], but these have all involved point mutations in the structural genes encoding the pre-lantibiotic peptides. The case of (N^{α}-succinyl-Trp^1)subtilin appears to be the first example

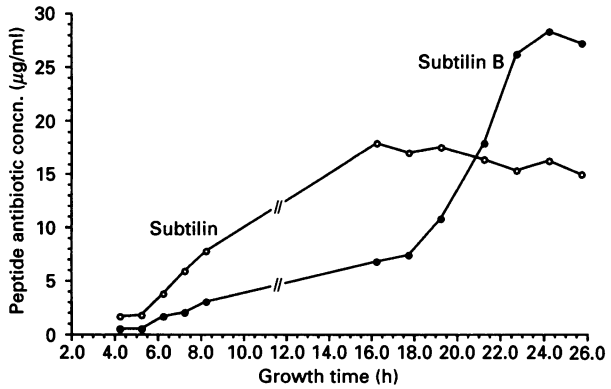

Figure 3 Production of subtilins by *B. subtilis* A.T.C.C. 6633 in culture broth (50 ml) at 37 °C

of a variant arising due to post-translational modification. It is interesting that, as shown in Figure 3, the production of (N^{α}-succinyl-Trp^1)subtilin by *B. subtilis* A.T.C.C. 6633 follows a different time course from that of subtilin, the ratio of N^{α}-succinyl-Trp^1)subtilin to subtilin increasing quite markedly after prolonged incubation of the culture. However, the total quantity of peptide antibiotics produced, i.e. subtilin plus (N^{α}-succinyl-Trp^1)subtilin, appears to be linear with time from 7 to 24 h growth. The physiological significance of the production of the N-terminally modified peptide antibiotic is not yet clear. It is

conceivable that it is related to 'self-protection' by the producing organism, since (N^{α}-succinyl-Trp^{1})subtilin has appreciably lower (although still considerable) antibacterial activity. More detailed studies of the mechanism of action of both the native and the modified subtilin will be required before the function of this post-translational modification can be properly understood.

N^{α}-Acetylation of proteins is a common post-translational process in both eukaryotes and prokaryotes. The biological significance of this modification is still an open question, but it has been proposed that it confers resistance to proteolysis, particularly for intracellular proteins. However, it is worth noting that N^{α}-acetylation of peptides can have a profound affect on their biological activities, e.g. N^{α}-acetyl-β-endorphin lacks analgesic activity [13]. Examples of other N-terminal post-translational modifications include myristoylation and glucuronylation. We believe that (N^{α}-succinyl-Trp^{1})subtilin is the first example of post-translational modification of peptides or proteins by N^{α}-succinylation.

This work is supported by grants from S.E.R.C. and A.F.R.C., U.K. FAB-m.s. was carried out at the Centre for Mass Spectroscopy, UMIST, Manchester.

Received 18 December 1992/19 January 1993; accepted 19 January 1993

REFERENCES

1 Gross, E., Kiltz, H. H. and Nebelin, E. (1973) Hoppe-Seyler's Z. Physiol. Chem. **354**, 810–812
2 Jung, G. (1991) Angew. Chem. Int. Ed. Engl. **30**, 1051–1068
3 Kordel, M., Benz, R. and Sahl, H.-G. (1988) J. Bacteriol. **170**, 84–88
4 Hurst, A. (1981) Adv. Appl. Microbiol. **27**, 85–123
5 Chan, W. C., Bycroft, B. W., Leyland, M. L., Lian, L.-Y., Yang, J. C. and Roberts, G. C. K. (1992) FEBS Lett. **300**, 56–62
6 Lian, L.-Y., Chan, W. C., Morley, S. D., Roberts, G. C. K., Bycroft, B. W. and Jackson, D. E. (1992) Biochem. J. **283**, 413–420
7 Banerjee, S. and Hansen, J. N. (1988) J. Biol. Chem. **263**, 9508–9514
8 Hansen, J. N., Chung, Y. J., Liu, W. and Steen, M. T. (1991) in Nisin and Novel Lantibiotics (Jung, G. and Sahl, H.-G., eds.), pp. 287–302, ESCOM, Leiden
9 Chan, W. C., Lian, L.-Y., Bycroft, B. W. and Roberts, G. C. K. (1989) J. Chem. Soc. Perkin Trans. I 2359–2367
10 Chan, W. C., Roberts, G. C. K. and Bycroft, B. W. (1993) in Peptides 1992 Proceedings of the 22nd European Peptide Symposium (Schneider, C. H. and Giralt, E., eds.), ESCOM, Leiden, in the press
11 Kellner, R., Jung, G., Horner, T., Zahner, H., Schnell, N., Entian, K.-D. and Gotz, F. (1988) Eur. J. Biochem. **177**, 53–59
12 Mulders, J. W. M., Boerringter, I. J., Rollema, H. S., Siezen, R. J. and de Vos, W. M. (1991) Eur. J. Biochem. **201**, 581–584
13 Smyth, D. G., Massey, D. E., Zakarian, S. and Finnie, M. D. A. (1979) Nature (London) **279**, 252–254