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General Procedures and Materials. Most general procedures and instrumentation associated with isolation and characterisation of LeuA and CbnB2 have been previously described.[11] Purified peptides were analyzed by the Alberta Peptide Institute (University of Alberta). The *N*-terminal amino acid sequences were obtained using Edman degradation on an automated gas phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A). All solvents for anhydrous reactions were dried according to Perrin *et al.*[20] Water was purified by a Milli-Q reagent water system (Millipore Corp., Milford, MA). Unless otherwise specified, solutions of NH_4Cl, $NaHCO_3$, KOH and NaOH refer to aqueous solution. Where possible, all reactions were monitored by thin layer chromatography on Merck 60 F-254 silica plates and were visualized using UV fluorescence or iodine staining. In solid phase reactions, the primary amino group was detected using a solution composed of 75 μL of each of the following three pre-prepared solutions: 5.0 g ninhydrin in 100 mL of 95% ethanol; 20.0 g phenol in 20 mL of ethanol; 2.0 mL of 0.66 mg/mL aqueous KCN was mixed with 98 mL of pyridine. A small amount of resin was mixed with the above solution and heated at 105 °C for 5 min. A brown or light yellow colour indicated no free amino groups. Flash chromatography was performed according to Still *et al.*[21] using Silicycle silica gel (Quebec City, PQ). High performance liquid chromatography (HPLC) was performed on a Beckman System Gold instrument equipped with a model 166 variable wavelength UV detector or Bio-Rad Value Chrom chromatography system equipped with a Bio-Rad Model 1305A UV monitor, with monitoring at 218 nm unless otherwise indicated. HPLC grade MeCN (190 nm cutoff) and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). All HPLC solvents were prepared fresh daily and filtered with a Millipore filtration system

under vacuum before use. 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids (both D- and L-isomers) and 2-(1*H*-benzotriazole-1-yl) 1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Wang resin (loading 0.95 mmol/g) was purchased from Chem-Impex. International, IL. 2-(1*H*-9-Azabenzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluoro-phosphate (HATU) was purchased from PerSeptive biosystems GmbH (Hamburg, Germany). Chemicals for peptide synthesis (dimethylformamide, dichloromethane, diisopropylcarbodiimide, dimethylsulfoxide, piperidine, trifluroacetic acid, hydroxybenzotriazole, acetonitrile, *N*-methylpyrrolidine, isopropyl alcohol, *N*-methyl morpholine) were obtained from commercial sources and were of the highest purity available. Routine mass spectra (MS) were recorded on Kratos AEI MS-50 for high resolution, electron impact ionization (EI), or MS-9 for fast atom bombardment (FAB) instruments, or HP1100 LC-MSD for electrospray mass spectra. Cleland matrix used in FAB refers to a 5:1 mixture of dithiothreitol and dithioerythritol. The mass spectra of the purified peptides were done by direct injection of their solutions (50% aqueous MeCN, 0.1% TFA) using a VG Quattro triple quadrupole instrument with an electrospray ionization source (Fisons, Manchester, England). Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM-300, Bruker WM-360, Bruker WH-400, Varian INOVA600 or Varian UNITY500 instruments.

Synthesis of *ent*-LeuA. *ent*-LeuA, the enantiomer of a naturally occuring L-LeuA (sequence shown in Figure 1), was manually synthesized by stepwise solid phase synthesis[13] using D-amino acids. The following side chain protection was used: Arg (Pmc); Asn (Trt); Cys (Trt); Glu (tBu);

His (Trt); Lys (Boc); Ser (tBu); Thr (tBu); Trp (Boc); Tyr (tBu). The synthesis started with coupling of Fmoc-tryptophan (Boc) to 0.3 mmoles of Wang resin using 1,3-dicyclohexylcarbodiimide (DCC) as the activating agent in the presence of a catalytic amount of 4-*N,N*-dimethylaminopyridine (DMAP) in DMF. Following this, each amino acid was assembled in turn by cycles of deprotection, activation and coupling. Each step was followed by extensive washing sequentially with DMF, dichloromethane, isopropanol, dichloromethane and DMF. Freshly prepared 20% piperidine in DMF was used for the deprotection of Fmoc groups. Couplings were carried out in DMF by activating with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-methyl morpholine using a four-fold excess of the Fmoc protected amino acid between 1 to 2 h at room temperature. Coupling yields were quantitated by ninhydrin assay for residual free amine.[22] If the Kaiser test gave a positive result after double coupling, either more coupling under the same conditions or capping with acetic anhydride was performed. For difficult residues (Cys and most of the residues after position 20), elevated temperatures of up to 50 °C were used, and 2-(1*H*-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was used as the coupling reagent instead of HBTU. A test cleavage was performed after each five residues were coupled and the desired product was given each time as was confirmed by electrospray mass spectrometry. Cleavage from the resin was done in a mixture of 90% TFA, 5% thioanisole, 3% DTT and 2% anisole for 2 h at room temperature with mechanical shaking. One third of the resin was removed after the first 20 amino acids had been attached.

After the chain assembly was complete, the crude peptide was cleaved from the resin and deprotected under the same conditions used for test cleavages. Following purification on a preparative RP-HPLC with a C_{18} column (21 x 250 mm, Zorbax 300 SB column; flow rate of 8

mL/min; monitored at 210 nm) using a gradient from 25 to 50% MeCN in 0.1% aqueous TFA over a period of 90 min. The fractions showing the desired mass (calcd mass 3932.33, reduced form) were pooled and lyophilized. The lyophilized peptide was further purified to homogeneity by RP-HPLC with a semi-preparative VYDAC C_8 column (10 x 250 mm, 10-μm particle size, 300-Å pore size; flow rate 2.5 mL/min) using a gradient from 20 to 40% MeCN in 0.1% aqueous TFA over a period of 50 min. The purified sample was then concentrated and lyophilized.

The lyophilized peptide was redissolved in 20 mM of ammonium bicarbonate solution (pH 8.1) with a peptide concentration of 0.5 mg/mL. The solution was stirred overnight under oxygen at room temperature. The oxidization state of the two cysteine residues was monitored by iodoacetamide reaction. After disulfide bond formation was complete, the solution was diluted with water and lyophilized. The oxidized pure peptide was stored at -20 °C or dissolved in water containing 0.1% TFA to make a 2 mg/mL solution for biological tests.

The structure of the purified *ent*-LeuA was characterized by automatic amino acid sequencing, mass spectrometry, co-injection on analytical HPLC with an authentic L-LeuA sample and circular dichroism (CD) spectroscopy (see figures at end of this section). The *N*-terminal amino acid sequences of the purified peptide were obtained using Edman degradation on an automated gas phase sequencer (Applied Biosystems, model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems, model 120A). The mass spectra of the purified peptides were obtained by direct injection of their solutions (50% aqueous MeCN, 0.1% TFA) using a HP1100 LC-MSD instrument. In order to confirm the stereochemistry of the peptide back bone and to obtain conformational information of *ent*-LeuA, CD spectra were obtained on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) at 25 °C. The CD spectra were averages of four scans, collected at 0.1 nm intervals between

190 and 250 nm with a LeuA concentration of 0.1 mM. Spectra were recorded in: 90% trifluoroethanol (TFE) in 0.1% aqueous TFA; aqueous dodecylphosphocholine (DPC) with 0.1% TFA (1:40 ratio of LeuA:DPC); and 0.1% aqueous TFA. For L-LeuA, the CD spectra were collected under identical conditions as those of *ent*-LeuA.

Syntheses of Peptide Fragments of LeuA and CbnB2. Methodology similar to that employed to synthesize and analyze *ent*-LeuA was used to prepare LeuA(18-32), LeuA(18–37), N–Ac-LeuA(18-37) and CbnB2(1-22). The peptides were purified to >95% homogeneity by reverse phase HPLC using an acetonitrile-H_2O gradient (0.05% TFA) using a Beckman System Gold Nouveau instrument. The molecular weights of the peptides were determined by electrospray mass spectrometry (Fisons VG Trio 2000 ESMS). The molecular weight of LeuA(18-32) was 1571.78 +/- 0.37 (calculated mass 1571.76 Da) and that of LeuA(18–37) was 2133.1 +/- 0.67 (calculated mass 2133.39 Da). The molecular weight of N–Ac-LeuA(18-37) was 2174.69 +/-0.50 (calculated mass 2175.43 Da). The molecular weight of the reduced CbnB2(1-22) was 2346.21 +/- 2.63 (calcd mass 2349.6).

The reduced CbnB2(1-22) was dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.0 and stirred under an atmosphere of oxygen overnight. The oxidized disulfide product was easily separated from the starting material by RP-HPLC with a C_8 VYDAC column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; Scientific Products and Equipment (Concord, ON); flow rate 1 mL/min) using a gradient from 12 to 44% MeCN in 0.1% aqueous TFA over a period of 30 min. The retention time of the reduced form was 15.2 min and that of oxidized form was 16.8 min. About one third of starting material was oxidized according the peak integration on HPLC. Samples of both oxidized and reduced forms of the 22-mer peptide were reacted with

iodoacetamide under the same conditions as those described for CbnB2 modification.[11] Mass spectrometric and HPLC analyses confirmed that, as expected, only the reduced peptide was modified.

Antimicrobial tests, bacterial strains and culture media. The antimicrobial activity of the peptide or peptide mixtures was determined by spot-on-lawn test.[23] All cultures were grown in APT broth (Difco Laboratories Inc., Detroit, MI). Agar was also obtained from Difco. Thus, plates containing approximately 20 mL of APT agar were overlaid with 10 mL of soft APT agar (0.75% agar) inoculated with a 16 h culture of various indicator strains (1% inoculum). The following cultures were screened: *C. divergens* LV13, *C. piscicola* N5, *C. piscicola* LV17A, *C. piscicola* LV17B (Bac^+ and Bac^-), *Enterococcus faecium* BFE 900, *Leuconostoc gelidum* UAL187, *L. gelidum* UAL187.13, *Leuconostoc mesenteroides* 23386, *Lactobacillus sakei* 20017 and *Listeria monocytogenes* LI0502. The purified peptide was dissolved in water containing 0.1% TFA to give a concentration of 1.0 or 2.0 mg/mL. To test for an agonistic and/or antagonistic effect of L-LeuA and *ent*-LeuA, solutions of the mixture of the two isomers were prepared with different molar ratios of *ent*-LeuA to L-LeuA ranging from 0.1 to 100 (solution A, 0.004 : 0.04 μg/μL; solution B, 0.04 : 0.04 μg/μL; solution C, 0.4 : 0.04 μg/μL; solution D, 0.4 : 0.004 μg/μL). Pure peptides with the same concentration were used as controls and tested in the same way. Serial two-fold dilutions of these stock solutions were performed in water containing 0.1% TFA, and up to 10 μL of each concentrations were spotted onto the bacterial lawn. The plates were kept at 25 °C for approximately 16 h before the presence of inhibition zones was recorded. The results were expressed in arbitrary units (AU) of bacteriocin (one AU is the minimal amount of peptide required to produce a visible clearing on the lawn of the indicator

strain). Inhibition was recorded as a positive if a distinct clearing was observed. No inhibition of the indicator strain was detected when 0.1% TFA alone was used in the assay.

Bacteriocin activity was also measured with a 96-well microtiter plate assay system. L-LeuA solution at twofold dilutions, various amounts of the 15-mer peptide fragment or the 20-mer peptide fragment, and 2 μL of 16 h culture (indicator strain, *C. divergens* LV13 or *Lactobacillus sakei* 20017) were added to each microtiter plate well containing 200 μL of APT broth. The microtiter plate cultures were incubated for 16 h at 25 °C, after which the growth of the indicator strain was measured spectrophotometrically at 650 nm with a microplate reader (Molecular Devices Inc., Softmax 2.3.2). The culture absorbance was then plotted against the peptide concentration and MIC was read from the graph directly. The MIC was defined as the bacteriocin concentration that inhibited the growth of the indicator strain by 50% (50% of the absorbance of the control culture without bacteriocin at 650 nm). All of the tests were repeated twice or more.

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(22) Sarin, V. K.; Kent, S. B. H.; Ram, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147-157.

(23) Ahn, C.; Stiles, M. E. *Appl. Environ. Microbiol.* **1990**, *56*, 2503-2510.

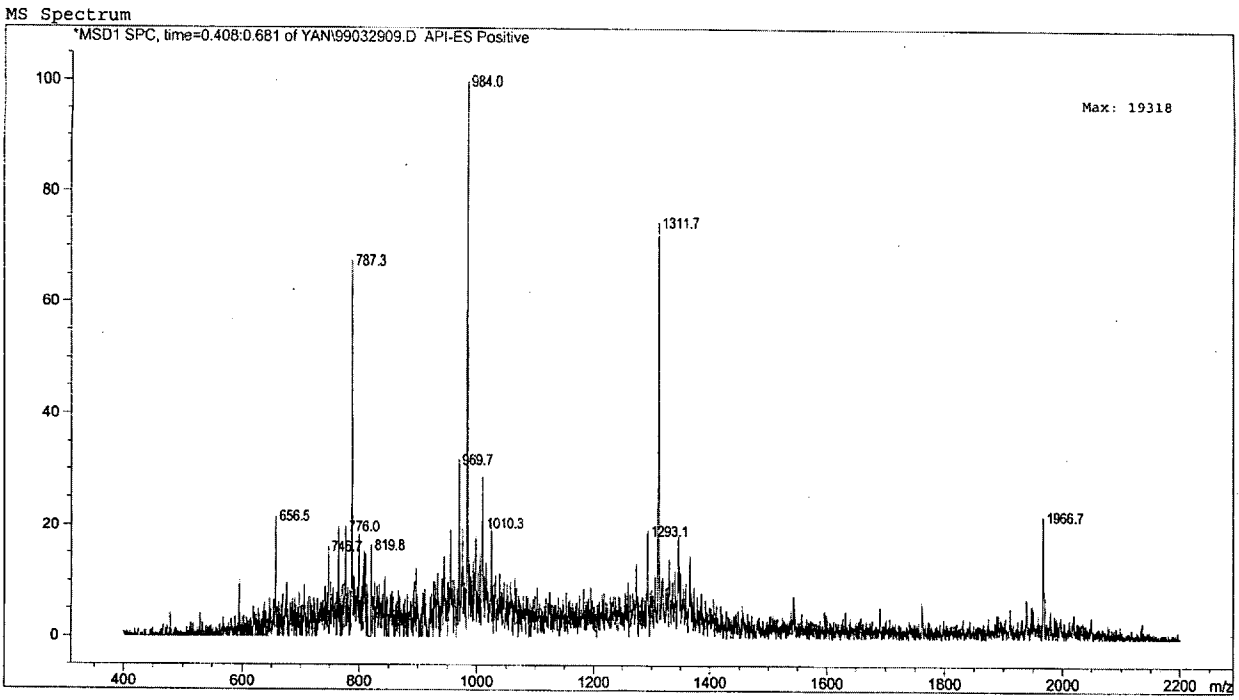

Figure 2. Electrospray mass spectrum of the synthetic *ent*-LeuA

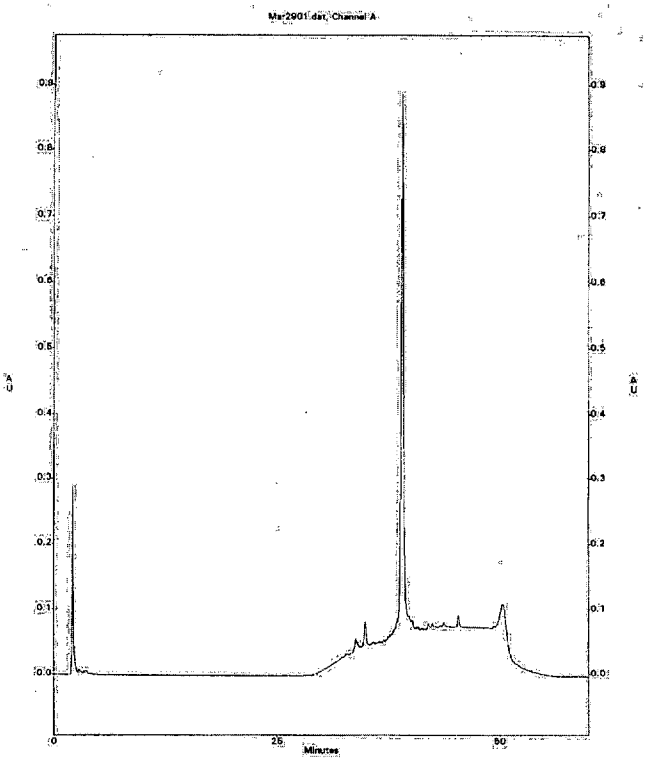

Figure 3a. HPLC trace of the purified *ent*-LeuA

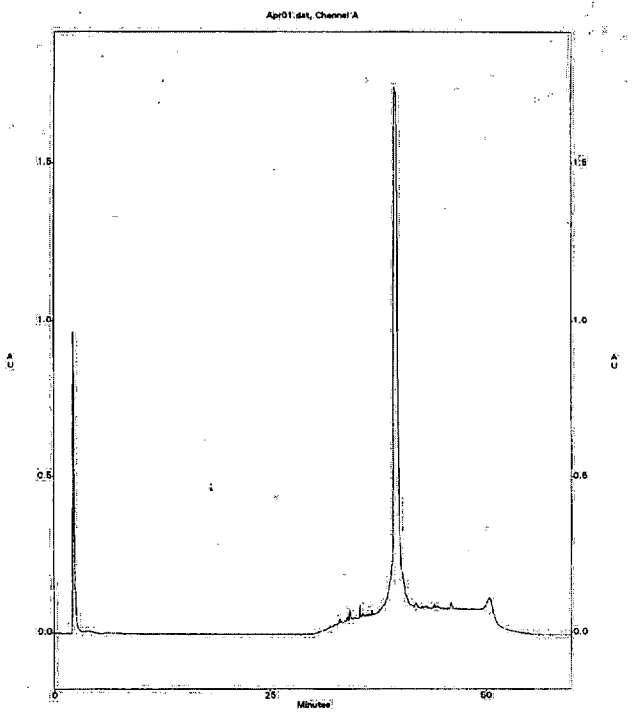

Figure 3b. HPLC trace of a 1:1 mixture of L-LeuA and *ent*-leuA

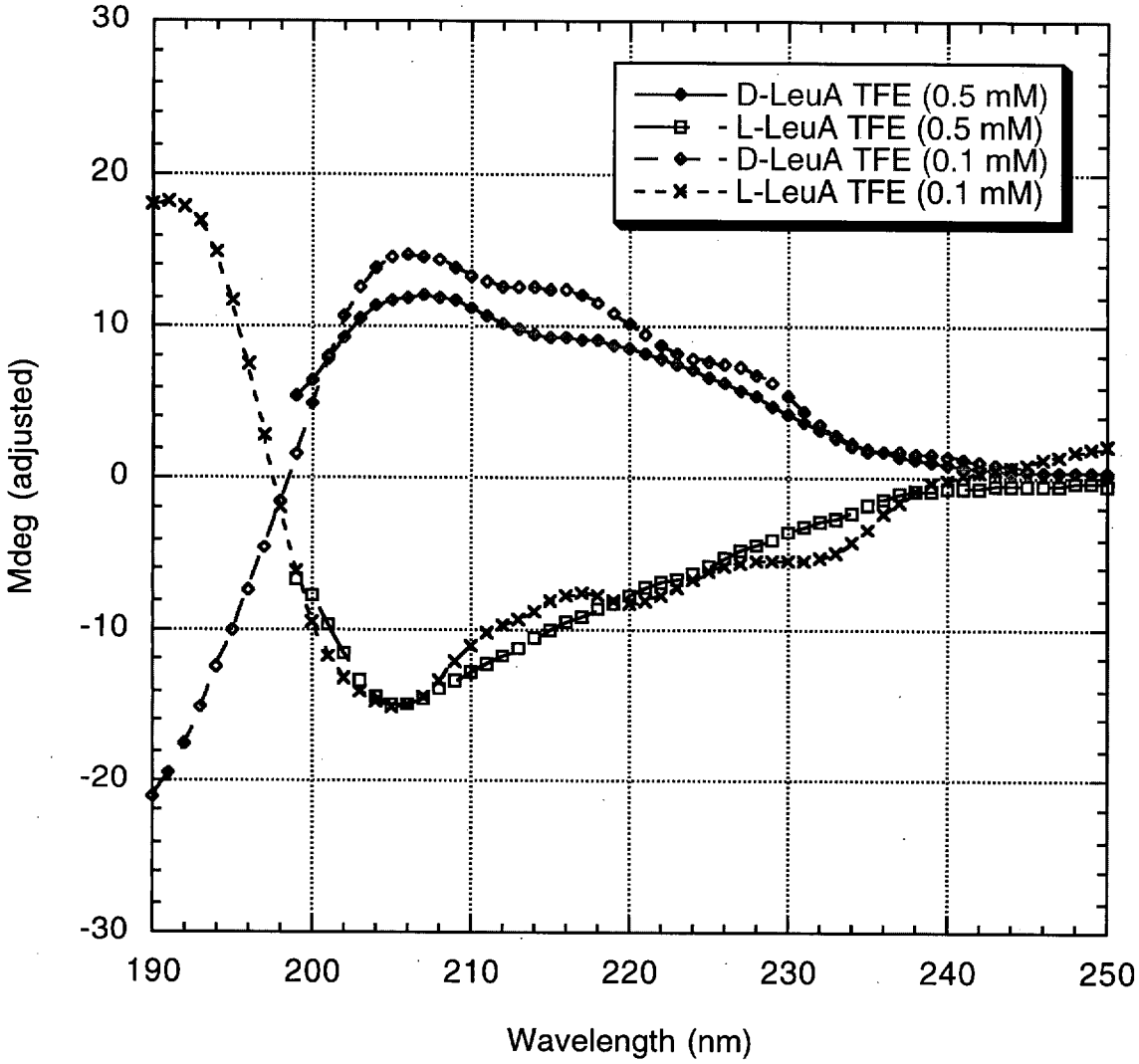

Figure 4. CD Spectra of *ent*-LeuA (D-LeuA) and L-LeuA in aqueous TFE (0.5 mM)

Table 1. Agonistic/Antagonistic Tests with *Carnobacterium divergens* LV13

Dilution	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
ent- : L-LeuA (A) 0.004 : 0.04 (μg/μl)	+	+	+	+	+	+	+	+	+/-	-
ent- : L-LeuA (B) 0.04 : 0.04 (μg/μl)	+	+	+	+	+	+	+	+	+/-	-
ent- : L-LeuA (C) 0.4 : 0.04 (μg/μl)	+	+	+	+	+	+	+	+	+/-	-
ent- : L-LeuA (D) 0.4 : 0.004 (μg/μl)	+	+	+	+	+/-	-	-	-	-	-
L-LeuA (0.04 μg/μl)	+	+	+	+	+	+	+	+	+/-	-
L-LeuA (0.004 μg/μl)	+	+	+	+	+/-	-	-	-	-	-
ent-LeuA (0.4 μg/μl)	-	-	-	-	-	-	-	-	-	-
ent-LeuA (0.04 μg/μl)	-	-	-	-	-	-	-	-	-	-
ent-LeuA (0.004 μg/μl)	-	-	-	-	-	-	-	-	-	-

Table 2. Agonistic/Antagonistic Tests on *Lactobacillus sakei* 20017

Dilution	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
ent- : L-LeuA (A) 0.004 : 0.04 (μg/μl)	+	+	+	+	+	+	+	+	+	+*
ent- : L-LeuA (B) 0.04 : 0.04 (μg/μl)	+	+	+	+	+	+	+	+	+	+*
ent- : L-LeuA (C) 0.4 : 0.04 (μg/μl)	+	+	+	+	+	+	+	+	+	-
ent- : L-LeuA (D) 0.4 : 0.004 (μg/μl)	+	+	+	+	-	-	-	-	-	-
L-LeuA (0.04 μg/μl)	+	+	+	+	+	+	+	+	-	-
L-LeuA (0.004 μg/μl)	+	+	+	-	-	-	-	-	-	-
ent-LeuA (0.4 μg/μl)	-	-	-	-	-	-	-	-	-	-
ent-LeuA (0.04 μg/μl)	-	-	-	-	-	-	-	-	-	-
ent-LeuA (0.004 μg/μl)	-	-	-	-	-	-	-	-	-	-

* Further dilutions all showed negative activity.

Table 3. Induction of Bacteriocin Production of *Carnobacterium piscicola* LV17B with L-LeuA[a]

Dilution of supernatant	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.01 x $1/2^0$ (μg/μl)[b]	+	+	+	+	+	-	-	-	-	-
0.01 x $1/2^1$ (μg/μl)	+	+	+	+	+/-	-	-	-	-	-
0.01 x $1/2^2$ (μg/μl)	+	+	+	+/-	-	-	-	-	-	-
0.01 x $1/2^3$ (μg/μl)	+	+	+/-	-	-	-	-	-	-	-
0.01 x $1/2^4$ (μg/μl)	+	+/-	-	-	-	-	-	-	-	-
0.01 x $1/2^5$ (μg/μl)	+	-	-	-	-	-	-	-	-	-
0.01 x $1/2^6$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^7$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^8$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^9$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^{10}$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
L-LeuA, 0.01 (μg/μl)[c]	+	+	+	+	+	+	+/-	-	-	-
Control (+)[d]	+	n/d[e]	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Control (-)[d]	-	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d

a. *C. divergens* LV13 was the indicator. The plates were read after incubation at 25°C for 16 h.
b. The concentration of L-LeuA in the culture medium.
c. Test of background activity due to the added L-LeuA in the medium.
d. Positive control: 1% supernatant of Bac+ culture, which induces bacteriocin production. Negative control was the direct subculture of Bac- culture.
e. n/d: not determined

Table 4. Induction of Bacteriocin Production of *C. piscicola* LV17B with *ent*-LeuA[a]

Dilution of supernatant	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.01 x $1/2^0$ (μg/μl)[b]	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^1$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^2$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^3$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^4$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^5$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^6$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^7$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^8$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^9$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
0.01 x $1/2^{10}$ (μg/μl)	-	-	-	-	-	-	-	-	-	-
D-LeuA, 0.01 (μg/μl)[c]	-	-	-	-	-	-	-	-	-	-
Control (+)[d]	+	n/d[e]	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Control (-)[d]	-	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d

a. *C. divergens* LV13 was the indicator. The plates were read after incubation at 25 °C for 16 h.
b. The concentration of L-LeuA in the culture medium.
c. Test of background activity due to the added L-LeuA in the medium.
d. Positive control: 1% supernatant of Bac+ culture, which induces bacteriocin production. Negative control was the direct subculture of Bac- culture.
n/d: not determined

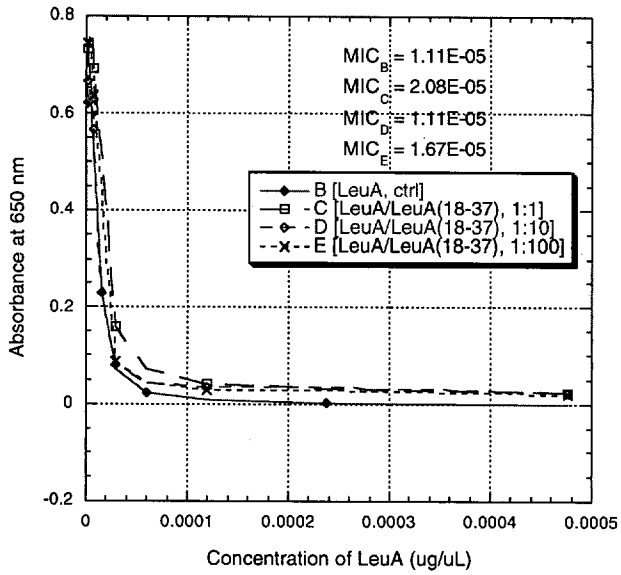

Figure 5. Inhibition Studies of L-LeuA(18-37) towards L-LeuA

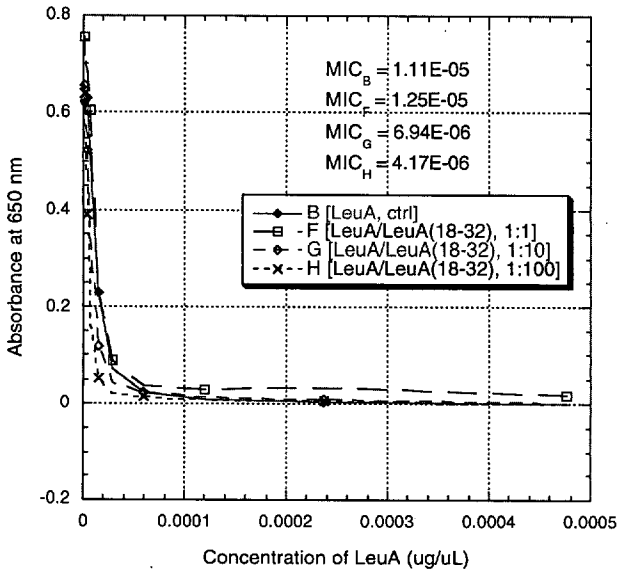

Figure 6. Inhibition Studies of L-LeuA(18-32) towards L-LeuA

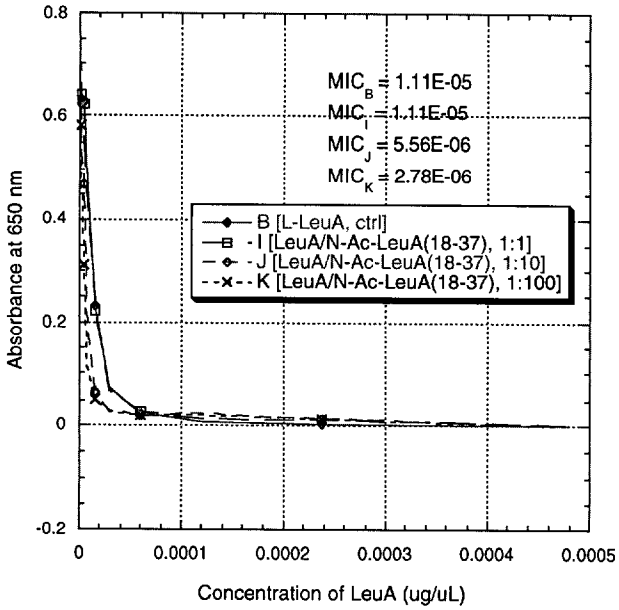

Figure 7. Inhibition Studies of N-Ac-L-LeuA(18-37) towards L-LeuA