Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 109–112

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Cleavage of L-leucine-containing dipeptides by *Clostridium butyricum*

Nasser Khelifa[1,*], Mohabeddine Brik[2], Annie-Claude Tessedre[1], Hugues De Rocquigny[3], Bernard-Pierre Roques[3], Jacques Courtieu[2], and Alain Rimbault[1]

[1]Laboratoire de Microbiologie, UMA, Faculté des Sciences Pharmaceutiques et Biologiques de Paris, Université René Descartes, 4 avenue de l'Observatoire, F-75270 Paris Cedex 06, France; [2]Laboratoire de Chimie Organique Structurale, CNRS URA 1384, Faculté des Sciences d'Orsay, Université Paris XI, 91405 Orsay, France and [3]Laboratoire de Chimie Organique, Faculté des Sciences Pharmaceutiques et Biologiques de Paris, 4 avenue de l'Observatoire, Paris, France

Received 19 October 1998; accepted 19 November 1998

Abstract: The ability of *Clostridium butyricum* cultures to hydrolyze three L-leucine-containing dipeptides (Leu-Leu, Leu-Gly and Gly-Leu) in a synthetic minimal medium is demonstrated by using gas chromatography coupled with mass spectrometry. The ^{13}C nuclear magnetic resonance and a labeled dipeptide L-[1-^{13}C]Leu-Gly were used to confirm this activity.

Keywords: peptides, bacteria, NMR, enzyme, labelling, *Clostridium butyricum*, 2-hydroxy acid, gas chromatography-mass spectrometry

Introduction: The genus *Clostridium* is one of the most diversified genera[1,2] and even if this genus is subject to restructuring, the type species remains *Clostridium butyricum*[3]. In this genus, many studies have been focused on proteinase and peptidase activities, mainly for botulism and tetanus neurotoxins[4], peptidoglycan-degrading peptidases, collagenase and gelatinases[5]. To our knowledge, no studies have been devoted to peptidases of *C. butyricum*. Strains of this species were implicated in the etiology of neonatal necrotizing enterocolitis[6] and their role in this pathology is still a matter of debate[7]. Since aminopeptidases and proteinases of bacterial origin have been implicated in the mechanism of several human pathogeneses in associated conditions and evoked as an important virulence factor[8-11], there is a need to investigate the oligopeptide metabolism by *C. butyricum* which is included with the saccharolytic, non proteolytic clostridia.

The aim of this paper was to study the metabolism by *C. butyricum* of three L-leucine-containing dipeptides (Leu-Leu, Leu-Gly and Gly-Leu). The hydrolysis of the peptides was confirmed by the detection of 2-hydroxy-4-methylpentanoic acid using gas chromatography coupled with mass spectrometry (GC-MS). This metabolite has been reported earlier as a bioconversion-product of L-leucine in *C. butyricum* cultures in a basal mineral glucose (BMG) medium[12]. The ^{13}C NMR and a labeled dipeptide L-[1-^{13}C]Leu-Gly were used to confirm this activity. *C. butyricum* strain used throughout this study is the type strain MGPP 26^T (VPI 3266^T), stored at -80°C in brain-heart medium with 15% (v/v) glycerol as cryoprotective agent. The synthetic medium BMG, used in this study, could be supplemented with either L-Leu-L-Leu, L-Leu-Gly, Gly-L-Leu or L-[1-^{13}C]Leu-Gly[13] added as a 0.22-μm membrane-filtered solution (final concentration, 5 mM).

0960-894X/99/$ - see front matter
PII: S0960-894X(98)00694-5

Strains were grown using the Hungate method under an anaerobic atmosphere (nitrogen: carbon dioxide, 80:20, v/v) at 37°. For a given series of experiments, 3 precultures were performed in the same conditions.

Results and discussion. Acid-extractable metabolites, detected by GC-MS in *C. butyricum* 24h-cultures after growth in BMG medium and analyzed as their *tert*-butyldimethylsilyl (TBDMS) derivatives[14], were formic, acetic, butyric and lactic acids (figure 1A). When BMG medium was supplemented with Leu-Gly, Leu-Leu or Gly-Leu, an additional peak clearly appears at a retention time of 32.0 min (figure 1B). The comparison of retention time and mass spectra with authentic standards indicated that this peak corresponded to the di-TBDMS derivative of 2-hydroxy-4-methylpentanoic acid. The mass spectrum corresponding to this peak shows the molecular weight at m/z 360 and characteristic fragments at *m/z* 303 $[M-57]^+$, *m/z* 345 $[M-15]^+$ and *m/z* 275 [M–57–28] (figure 1C).

When the labeled dipeptide L-[1-^{13}C]Leu-Gly was used to supplement the medium, the molecular mass of the corresponding metabolite was found at *m/z* 361 since the mass spectrum shows fragments containing the ^{13}C at *m/z* 304 $[M-57]^+$ and 346 $[M-15]^+$ and fragment at *m/z* 275 $[M-57-29]^+$ with loss of a carbonyl fragment ^{13}CO (figure 1D). The molecular weight, the retention time and the mass spectrum were compatible with the di-TBDMS derivative of a 2-hydroxy-4-methyl[1-^{13}C]pentanoic acid.

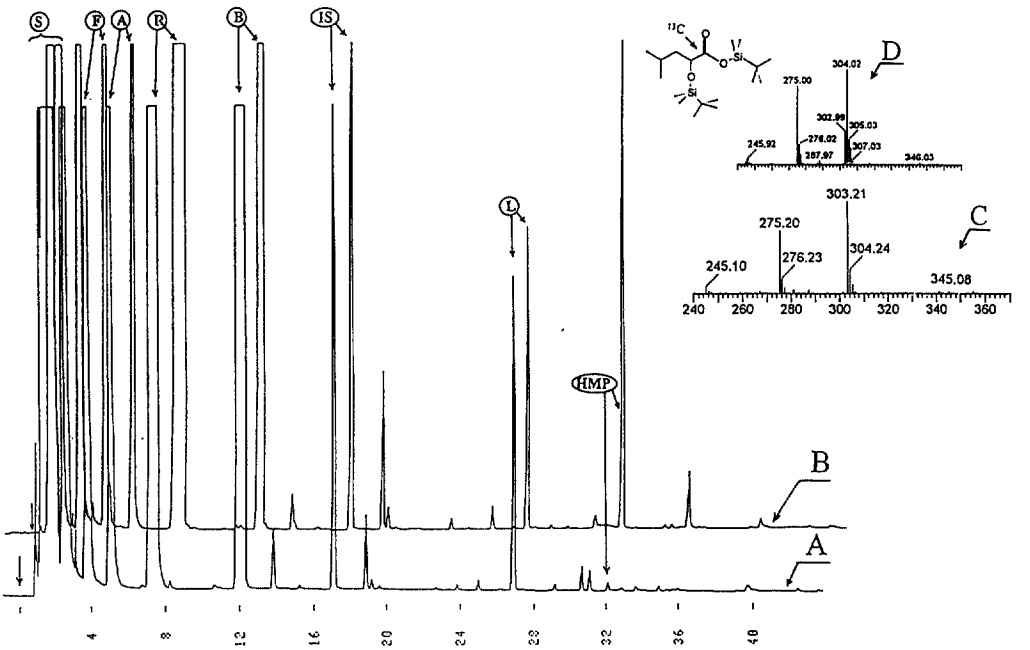

Figure 1: GC profiles obtained for *C. butyricum* grown (A) in BMG medium and (B) in BMG + L-Leu-Gly (5 mM). For identification: S, solvent (acetonitrile); R, silylating agent; IS, internal standard (2-methylpentanoic acid). Mono-TBDMS derivatives of formic acid (F); acetic acid (A); butyric acid (B). Di-TBDMS derivatives of lactic acid (L), 2-hydroxy-4-methylpentanoic acid (HMP). Mass spectra of di-TBDMS derivatives of (C) 2-hydroxy-4-methylpentanoic acid (MW: 360) and (D) 2-hydroxy-4-methyl[1-^{13}C]pentanoic acid (MW: 361).

The ^{13}C NMR spectrum[15] of a culture of *C. butyricum* in BMG medium supplemented with the ^{13}C-labelled dipeptide at the beginning of the incubation shows a signal at $\delta = 170$ ppm corresponding to the C_1 leucyl group in L-[1-^{13}C]Leu-Gly [spectrum (a), figure 2]. After 24 h, a signal appears at $\delta = 176$ ppm corresponding to L-[1-^{13}C]leucine [spectrum (b), figure 2]. After extraction with *tert*-butylmethyl ether (TBME), 2-hydroxy-4-methyl[1-^{13}C]pentanoic acid was characterized by a signal at $\delta = 179$ ppm [spectrum (c), figure 2].

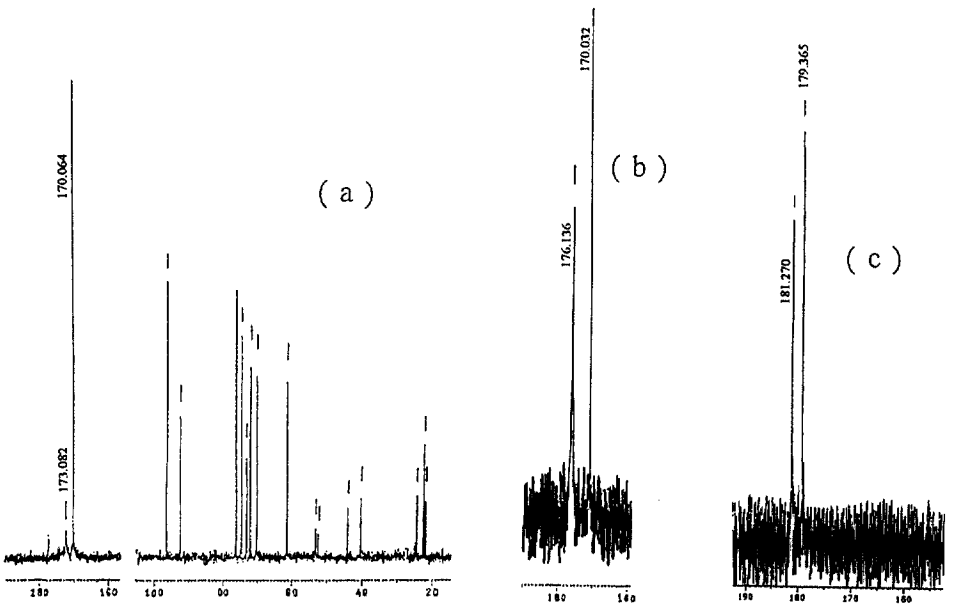

Figure 2: ^{13}C NMR spectra of medium from *Clostridium butyricum* in BMG + L-[1-^{13}C]Leu-Gly medium incubated for 6 h (a), 24 h (b) and 24 h (c) followed by an extraction with TBME.

The proportion of di-TBDMS derivative of 2-hydroxy-4-methylpentanoic acid in the extracts of *C. butyricum* is similar even if the media were supplemented with Leu-Gly or Gly-Leu (~25.2 $\mu mol.mol^{-1}$). This derivative has doubled in proportion when the medium was supplemented with Leu-Leu (~49.6 $\mu mol.mol^{-1}$).

This 2-hydroxy-4-methylpentanoic acid detected after culture in medium supplemented with a L-leucine-containing dipeptide may originate from the L-leucyl moiety. The detection of L-[1-^{13}C]leucine by ^{13}C NMR and 2-hydroxy-4-methyl[1-^{13}C]pentanoic acid by GC-MS and ^{13}C NMR in cultures of *C. butyricum* in BMG medium supplemented with L-[1-^{13}C]Leu-Gly confirms, unambiguously, the implication of this strain in the hydrolysis of this peptide.

Many bacterial peptidases have been investigated, principally in lactic bacteria from genera *Lactococcus* and *Lactobacillus*, in *Capnocytophaga gingivalis* and various anaerobic bacteria - e.g. *Prevotella ruminicola*, *Peptostreptococcus micros*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Treponema denticola*[8,9,16,17]. Two distinct intracellular peptidases were purified in *Lactobacillus sake* L110: one of them was responsible of the hydrolysis of Leu-Gly dipeptide[16]. In clostridia, *C. histolyticum* produces a clostridiopeptidase and an

extracellular cysteine peptidase, clostripain[18]. *C. bifermentans*, *C. sordellii*, *C. difficile*, and *C. glycolicum* exhibit a leucine aminopeptidase activity, a protease that removes Leu from the NH_2-terminal position of peptide substrates. Proline aminopeptidase activity was found in the cell-free extract of *C. bifermentans*, *C. difficile*, *C. glycolicum*, *C. sordellii* and *C. sporogenes*, but was not present in *C. butyricum*[19,20].

The mechanisms by which *C. butyricum* hydrolyses those dipeptides and the role of dipeptides as nutrients in this species are not known and must be explored. Our observation demonstrates that *C. butyricum* is able to cleave dipeptides, opening new aspects in the study of *C. butyricum* metabolism.

References

1 Andreesen, J.R.; Bahl, H.; Gottschalk, G. In *Clostridia*; Minton, N.P.; Clarke, D.J., eds.; Plenum Press: New York, **1989**, pp. 27-62.
2 Hippe, H.; Andreesen, J.R.; Gottschalk. G. In *The Prokaryotes*; Balows, A.; Trüper, H.G.; Dworkin, M.; Harder, W.; Schleifer K.H. eds., Springer-Verlag Inc.: New York, 1992; pp. 1800-1866.
3 Collins, M.D.; Lawson, P.A.; Willems, A.; Cordoba, J.J.; Fernandez-Garayzabal, J.; Garcia, P.; Cai, J.; Hippe, H.; Farrow. J.A.E. *Int. J. Syst. Bacteriol.* **1994**, 44, 812-826.
4 Rossetto, O.; Deloye, F.; Poulain, B.; Pellizari, R.; Schiavo, G.; Montecucco, C. *J. Physiol.* **1995**, 89, 43-50.
5 Morihara, K.; Oda K. In: *Microbial degradation of natural products*; Winkelmann, G. (ed.); VCH: Weinheim, **1992**, pp. 294-364.
6 Stark, P.L.; Lee, A. *J. Pediatr.* **1982**, 100(3), 362-365.
7 Kliegman, R.M.; Walker, W.A.; Yolken, R.H. *Pediatr. Res.* **1993**, 34, 701-708
8 Spratt, D.A.; Greenman, J.; Schaffer, A.G. *Microbiology* **1995**, 141, 3087-3093.
9 Beighton, D.; Homer, KA; de Graaff, J. *Arch. Oral Biol.* **1997**, 42(12), 827-834.
10 Lantz, M.S. *J. Periodont Res.* **1997**, 32, 126-132.
11 Johansson, A; Kalfas, S. *Eur. J. Oral Sci.* **1998**, 106(4), 863-871.
12 Butel, M.J.; Rimbault, A.; Khelifa, N.; Campion, G.; Szylit, O.; Rocchiccioli, F. *FEMS Microbiol. Lett.* **1995**, 132, 171-176.
13 L-[1-^{13}C]Leu-Gly was prepared as follows; L-[1-^{13}C]Leucine, protected on the amino position under a N-benzyloxycarbonyl derivative, was coupled to glycine protected on the carbon position as *O-tert*-butylglycine derivative. The grouping guards are eliminated by the acid to give peptide The dipeptide was obtained by action of trifluoroacetic acid.
14 Khelifa, N.; Butel, M.J.; Rimbault, A. *Bioorg. & Med. Chem. Lett.* **1998**, 8(23), 3429-3434.
15 For ^{13}C-NMR, samples (5 ml) were centrifuged at 3000 rpm for 20 min. Each supernatant was membrane-filtered (0.22-μm), acidified to pH<2, then freeze-dried. The residues, dissolved in 1 ml of D_2O, were transferred to NMR tubes. Acid-extractable metabolites were characterized after filtration, acidification, extraction with TBME, evaporation under dry nitrogen then dissolution in 1 ml of a D_2O and transfer into an NMR tube. NMR spectra were recorded with a Bruker AM 250 spectrometer operating at 62 MHz for ^{13}C using a 200 ppm spectral width. Spectra were obtained after 35000 scans and chemical shifts were calibrated in respect to 1,4-dioxane peak at $\delta = 67{,}4$ ppm for ^{13}C.
16 Montel, M.-C.; Seronie, M.-P.; Talon, R.; Hebraud, M. *Appl. Environ. Microbiol.* **1995**, 61, 837-839.
17 Wallace, R.J., Mckain, N.; Broderick, G.A.; Rode, L.M.; Walker, N.D.; Newbold, C.J.; Kopecny, J. *Anaerobe* **1997**, 3, 35-42.
18 Gilles, A.-M.; Lecroisey, A.; Keil, B. *Eur. J. Biochem.* **1984**, 145, 469-476.
19 Fedorko, D.P.; Williams., E.C. *J. Clin. Microbiol.* **1977**, 35, 1258-1259.
20 Garcia, A., Garcia, T.; Perez. J.L. *J. Clin. Microbiol.* **1997**, 35, 3007.