Rapid Detection of *meso*-Diaminopimelic Acid in Lactic Acid Bacteria by Microwave Cell Wall Hydrolysis

Emanuele Marconi,* Elena Sorrentino, Liliana Mastrocola, and Raffaele Coppola

DISTAAM, Università degli Studi del Molise, Via De Sanctis, 86100 Campobasso, Italy

Two innovative microwave hydrolysis procedures for rapidly detecting *meso*-diaminopimelic acid in lactic acid bacteria have been developed. Whole-cell hydrolysis was performed both in liquid phase and vapor phase with 6 N HCl in sealed vessels using a microwave oven equipped with pressure and temperature probes. The presence or absence of *meso*-diaminopimelic acid determined by TLC, after the application of liquid- and vapor-phase microwave hydrolysis procedures, gave the same qualitative results as those obtained by traditional hydrolysis. These standardized microwave hydrolysis procedures permit a drastic reduction in hydrolysis time, from 16−20 h to less than 10 min and, consequently, in the total time of *meso*-diaminopimelic acid analysis (less than 90 min). Microwave hydrolysis in vapor phase is particularly convenient because, besides reducing hydrolysis time, it also eliminates the successive troublesome step of HCl removal.

Keywords: *meso-diaminopimelic acid; fast analysis; lactic acid bacteria; microwave cell wall hydrolysis; liquid and vapor-phase*

INTRODUCTION

A correct taxonomic classification of bacteria in foods is essential to attribute the actual contribution each species makes to the physical−chemical, organoleptic, nutritional, and functional characteristics of a food product. Likewise, their rapid detection is useful to monitor and/or correct the production processes of food products. This premise is particular relevant to lactic acid bacteria, which constitute the most important microbic group in the production of transformed foods and probiotic formulas (Hierro et al., 1999; Naidu et al., 1999; Goldin, 1998; Stiles and Holzapfel, 1997).

There are many procedures that enable a correct identification of lactic acid bacteria, and, for the most part, they make use of phenotypic and genotypic identification techniques (Kandler and Weiss, 1986; Hammes et al., 1992). Methods based on assessing the phenotype continue, for the moment, to be highly valid and, in many respects, irreplaceable. Many phenotypic features, particularly biochemical performances, however, vary a great deal, even in different strains of a single species. Their determination is often tedious and time-consuming and, for the above reasons, sometimes unsuccessful and/or misleading.

meso-Diaminopimelic acid (mDAP), an amino acid that forms the mureins of the bacteria cell wall but not the proteins is, instead, exclusive to certain species of lactic acid bacteria (Kandler and Weiss, 1986; Schillinger and Lucke, 1987; Hugas et al., 1993; Dellaglio et al., 1994) and of other bacterial groups, such as aerobic coryneforms (Bousfield et al., 1985), oral filamentous bacteria (Baboolal, 1969), and *Nocardia* and *Streptomyces* (Becker et al., 1964).

For this reason, mDAP detection was the first discriminating step in identifying homofermentative *Lactobacillus* and *Carnobacterium* spp. in meat in the simplified scheme proposed by Montel et al. (1991). In this scheme, *Carnobacterium* spp. could be clearly differentiated from *Lactobacillus* spp. as having *meso*-diaminopimelic-type peptidoglycan, producing only L-lactate and not growing on acetate agar.

The step that limits the qualitative determination of mDAP acid is the preparation of the whole-cell hydrolysate. The reference protocol, in fact, involves an acid hydrolysis in 6 N HCl of 16−20 h at 100−110 °C and thin layer or paper chromatography of about 1−3 h (Kandler and Weiss, 1986; Bousfield et al., 1985; Becker et al., 1964; Baboolal, 1969). Therefore, the use of microwave energy as a heat source was investigated to reduce the hydrolysis time.

This procedure has been successfully used for fast protein hydrolysis (4−30 min) in the liquid-phase determination of single amino acids, such as tryptophan (Carisano, 1993), lysine (Marconi et al., 1996), hydroxyproline (Kolar and Berg, 1990), and furosine, a nonnatural amino acid produced by the hydrolysis of the Amadori compound (Marconi et al., 1997; Acquistucci et al., 1996), as well as total amino acids by both vapor-phase hydrolysis (Gilman and Woodward, 1990; Woodward et al., 1990) and liquid-phase hydrolysis (Chen et al., 1987; Chiou and Wang, 1989; Carisano, 1992; Marconi et al., 1995). However, only a few reports have been published on the use of microwave energy for the acid hydrolysis of organic and biological samples such as starch (Khan et al., 1980) and dietary fiber polysaccharides (Li, 1998), besides proteins. The aim of this study was to set up fast liquid-phase and vapor-phase microwave irradiation procedures to prepare whole-cell hydrolysates for mDAP determination, to have a rapid specific intra-inter detection of lactic acid bacteria.

MATERIALS AND METHODS

Samples. The following types of strains from a DSM collection (Germany) were used: *Lactobacillus plantarum* 20174, *Lb. sakei* 20017, *Lb. paracasei* ssp. *paracasei* 5622, *Lb.*

* To whom correspondence should be addressed (telephone +39 0874 404616; fax +39 0874 404652; e-mail marconi@unimol.it).

10.1021/jf9907440 CCC: $19.00 © 2000 American Chemical Society
Published on Web 07/19/2000

mali 20444, *Lb. pentosus* 20314, *Lb. agilis* 20509, *Carnobacterium piscicola* 20730, and *Cb. divergens* 20623. *Lb. sakei* and *Lb. paracasei* were used as negative strains (control).

Lactobacilli were cultivated for 48 h in MRS broth (Unipath, Basingstoke, UK) at 30 °C (de Man et al., 1960), while carnobacteria were cultivated for 48 h in DMRS broth (Unipath), pH 8.5, at 30 °C (Hammes et al., 1992). Afterward, the cells were washed twice in physiological saline, collected by centrifugation, and stored at −20 °C when not for immediate usage.

Apparatus. A CEM Corporation (Matthews, NC) microwave hydrolysis system, model MDS 2000, maximum power 630 ± 50 W, magnetron frequency 2450 MHz, was used. This system was equipped with probes to detect and control the pressure and temperature inside the sealed vessels so as to regulate the hydrolysis conditions via magnetron power output control. The oven was equipped with a 12-positioned turntable (3 rpm) and a mode stirrer to prevent the uneven distribution of microwaves in the hydrolysis vessels.

The sealed vessels used in the liquid phase (Advanced Composite Vessel, ACV) consist of a PFA (perfluoroalkoxy) Teflon sample liner (volume = 50 mL) surrounded by an advanced composite sleeve. This apparatus is commonly and routinely used in food laboratories in sample preparations for Atomic Absorption Spectroscopy.

A protein hydrolysis accessory set (consisting of 45-mL vessels, a Teflon PFA, four-positioned turntable, and a vessel capping station) was used for microwave hydrolysis in the vapor phase. The vessels are sealed and unsealed using a capping station designed to quickly apply a reproducible torque to vessel caps for leak-free operations.

Traditional Whole-Cell Hydrolysis. About 50 mg wet weight of cells were put in a screw-cap Pyrex vial and left to hydrolyze with 1 mL of 6 N HCl at 100 °C overnight (Bousfield et al., 1985; Kandler and Weiss, 1986). The flask was then cooled, and the sample was evaporated to dryness under vacuum and redissolved in 0.5 mL of deionized water for TLC analysis. The hydrolysate may be stored at −18 °C until required.

Microwave Whole-Cell Hydrolysis. *Liquid Hydrolysis.* An aliquot of sample, corresponding to about 50 mg wet weight of cells, was placed in each of the four digestion vessels with 8 mL of 6 N HCl. The pressure and fiber optic probes were lined to the vessel with the triple ported cap.

The experimental conditions selected were the same as those standardized by Marconi et al. (1996) for lysine determination in food:

	first step	second step
power (% 630 W)	80	80
time (min)	1	5
temperature (°C)	100	155
max pressure (psi)	100	130

After the irradiation cycles, the vessels were cooled in an ice bath until the pressure was equal to atmospheric pressure (5 min). Then the samples were removed, evaporated to dryness under vacuum, and redissolved in 0.5 mL of deionized water for TLC analysis. The hydrolysate may be stored at −18 °C until required.

Vapor-Phase Hydrolysis. Samples (about 10 mg wet weight of cells) were put into 2-mL vials and loaded in a five-positioned rack. Two drops of 6 N HCl were added to each sample/vial. Eight milliliters of 6 N HCl was added to both 45-mL vessels where the vial racks were then placed. As shown in Figure 1, the vials were placed in such a way that the acid could not directly come into contact with the sample. Both racks were placed inside the vessel body, and the vessel cap was sealed using the capping station. The vessels were placed on the protein hydrolysis turntable so that each vessel was connected by a 3-mm OD tube to the central manifold vessel in which 10 mL of 6 N HCl was added. Afterward, the turntable with the two vessels was placed on a drive lug inside the microwave oven, and the central manifold vessel was connected to the pressure sensing line.

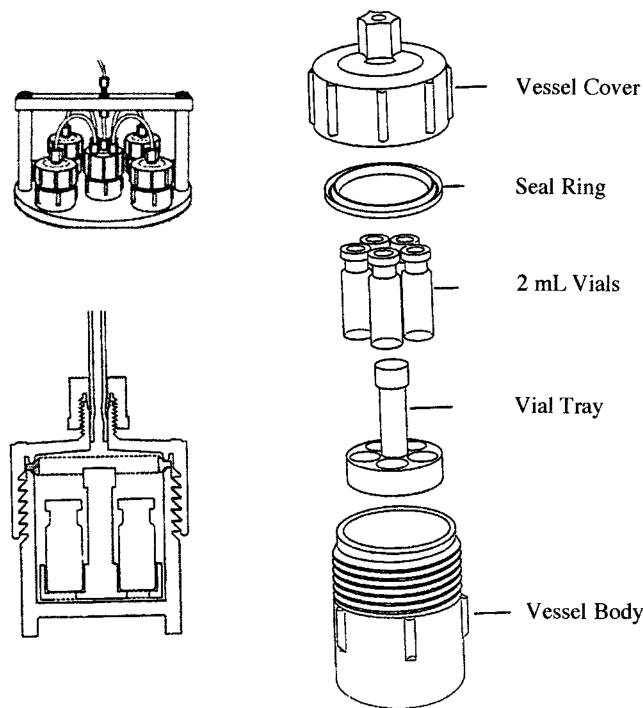

Figure 1. Vessel and hydrolysis accessory set used for vapor-phase microwave hydrolysis.

The experimental conditions selected, using two vessels [10 vials (5 + 5)], were as follows:

	first step	second step	third step
power (% 630 W)	80	80	80
time (min)	1	2	5
max pressure (psi)	50	60	70

After the irradiation cycles, the vessels were cooled in an ice bath until the pressure was equal to atmospheric pressure (5 min). Then the samples were directly redissolved in 0.1 mL of deionized water for TLC analysis; the HCl drops had evaporated during hydrolysis. The hydrolysate may be stored at −18 °C until required.

TLC Determination. The hydrolysates are obtained from wet cell materials, so the amount required for chromatography is a function of the moisture in the sample. Usually the amount of whole-cell hydrolysates required ranges from 0.5 to 2 μL.

A one-dimensional ascending chromatogram on cellulose aluminum sheets (20 × 20 cm) was developed in a chromatographic tank using the solvent system methanol/pyridine/10 M HCl/water (64:8:2:14, v/v) for 1 h at about 20 °C. After drying, the chromatogram was sprayed with ninhydrin/isobutanol (0.3% w/v) and then heated for 2 min at 100 °C. mDAP is well separated from other amino acids due to its very low R_f value. It also has a characteristic olive green color that turns yellow after several hours or days in the dark. The mDAP standard was purchased from Acros Organics, NJ. All the other chemicals were from Sigma Chemical Co. (St. Louis, MO). Analyses were carried out in triplicate as a minimum.

RESULTS AND DISCUSSION

The microwave system permits the use of both systems (liquid and vapor phase) and different operational conditions, as reported in Materials and Methods. The presence or absence of mDAP in the various lactic acid bacteria, obtained after liquid microwave and traditional hydrolysis, is reported in Figure 2 and Table 1. The microwave procedure gives the same qualitative results as the traditional procedures: as expected, mDAP was found in *Lb. plantarum*, *Lb. mali*, *Lb. pentosus*, *Lb. agilis*, *Cb. piscicola*, and *Cb. divergens*,

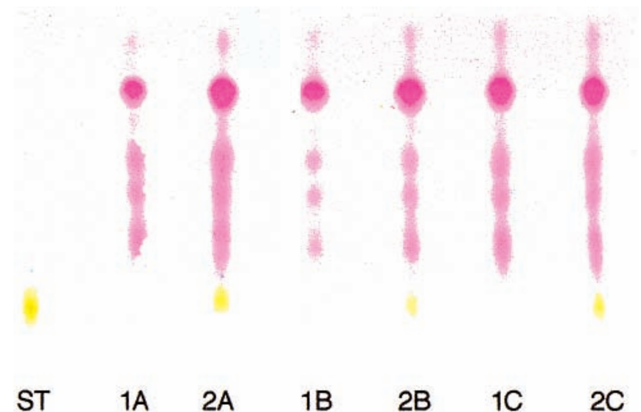

Figure 2. Detection of mDAP in *Lactobacillus sakei* (1) and *Lb. plantarum* (2) by traditional hydrolysis (A) and microwave hydrolysis in liquid phase (B) and vapor phase (C); ST= mDAP standard.

Table 1. Presence (+) or Absence (−) of mDAP in Different Lactic Acid Bacteria Determined by Microwave and Traditional Cell Wall Hydrolysis

species	mDAP[a]		
	microwave hydrolysis liquid-phase	microwave hydrolysis vapor-phase	traditional hydrolysis
Lactobacillus plantarum	+ + +	+ + +	+ + +
Lb. mali	+ + +	+ + +	+ + +
Lb. pentosus	+ + +	+ + +	+ + +
Lb. agilis	+ + +	+ + +	+ + +
Carnobacterium piscicola	+ + +	+ + +	+ + +
Cb. divergens	+ + +	+ + +	+ + +
Lb. sakei	− − −	− − −	− − −
Lb. paracasei ssp. *paracasei*	− − −	− − −	− − −
Lb. sakei + mDAP standard	+ + +	+ + +	+ + +
Lb. paracasei + mDAP standard	+ + +	+ + +	+ + +

[a] Each analysis was carried out in triplicate.

while it was not found in *Lb. sakei*, *Lb. paracasei* ssp. *paracasei*, since their cell walls did not contain mDAP.

The repeatability of the liquid-phase microwave procedure, evaluated on six determinations per sample (analyzed in duplicate in three separate hydrolysis sets) was very good because the presence or absence of mDAP was always confirmed.

A typical temperature/pressure diagram of whole-cell hydrolysates is given in Figure 3. Pressure and temperature control in situ is essential for the safety, reproducibility, and normability of the system.

Therefore, the microwave hydrolysis system in liquid phase can provide a valid support for the analysis of mDAP with regard to the rapid identification of lactic acid bacteria, since it significantly reduces hydrolysis time from 16−20 h to <10 min. To improve the microwave procedure, by increasing the sample number per cycle and avoiding the tedious HCl evaporation phase after hydrolysis, a microwave procedure in vapor phase was standardized. The presence or absence of mDAP in various lactic acid bacteria obtained after vapor-phase microwave hydrolysis and traditional hydrolysis is reported in Figure 2 and Table 1. The same qualitative results (presence or absence of mDAP) as traditional hydrolysis and liquid-phase microwave hydrolysis were obtained.

To test the possibility of material exchange between vials placed in the same vessel, which would result in incorrect positive (+) results, repeated tests were made,

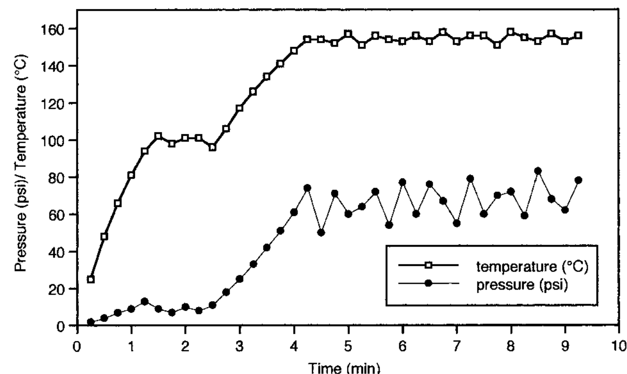

Figure 3. Typical temperature/pressure diagram of whole-cell microwave hydrolysis in liquid phase.

placing 3 samples with mDAP (*Lb. plantarum, Lb. mali, Cb. piscicola*) and 2 samples without mDAP (*Lb. sakei*, *Lb. paracasei* ssp. *paracasei*) in the same vessel. The repeatability of the vapor-phase microwave procedure, evaluated on six determinations per sample (analyzed in duplicate in three separate hydrolysis sets) was very good because the qualitative results (presence or absence of mDAP) were always confirmed.

Vapor-phase microwave hydrolysis improves mDAP analysis because, besides reducing hydrolysis time, it enables the analysis of a large number of samples per cycle (up to 10 with two vessels and up to 20 with four vessels) and eliminates the successive troubling step of HCl removal.

To further assess the reliability of the microwave procedures, mDAP standard was added to mDAP negative (−) samples (*Lb. sakei* and *Lb. paracasei),* and they underwent vapor- and liquid-phase microwave hydrolysis. After hydrolysis was performed, both samples were mDAP positive (+) (Table 1), proving that the microwave irradiation procedures, standardized in this work, are appropriate for the qualitative detection of mDAP in lactic acid bacteria.

CONCLUSION

The microwave irradiation system drastically reduces the preparation time for whole-cell hydrolysates, from 16−20 to <10 min, enabling rapid mDAP detection in lactic acid bacteria (less than 90 min). This result enables a rapid screening of lactic acid bacteria. Therefore, this test would be particularly suitable as a preliminary step in the procedure for the classification of lactic acid bacteria strains. Moreover, the apparatus used in this work is usually used in food laboratories for preparing samples for atomic absorption analysis; therefore, its utilization can be easily extended to mDAP analysis.

LITERATURE CITED

Acquistucci, R.; Panfili, G.; Marconi, E. Application of the microwave hydrolysis to furosine determination in cereal and dairy foods. *J. Agric. Food Chem.* **1996**, *44*, 3855−3857.

Baboolal, R. Cell wall analysis of oral filamentous bacteria. *J. Gen. Microbiol.* **1969**, *58*, 217−226.

Becker, B.; Lechevalier, M. P.; Gordon, R. E.; Lechevalier, H. A. Rapid differentiation between *Norcadia* and *Streptomyces* by paper chromatography of whole-cell hydrolyzates. *Appl. Microbiol.* **1964**, *12*, 421−423.

Bousfield, I. J.; Keddie, R. M.; Dando, T. R.; Shaw, S. Simple rapid methods of cell wall analysis as an aid in the identification of aerobic coryneform bacteria. In *Chemical*

Methods in Bacterial Systematics; Goodfellow, M., Minnikin, D. E. Eds.; Academic Press: London, 1985; pp 222–234.

Carisano, A. Digestione rapida delle proteine in matrici alimentari, con forno a microonde. I. Determinazione degli amminoacidi con esclusione di triptofano, cistina e metionina. (Quick protein digestion in food matrices with a microwave system. I. Amino acids determination excepted tryptophan, cystine and methionine). *Ind. Alim.* **1992**, *31*, 891–895.

Carisano, A. Digestione delle proteine in matrici alimentari, con forno a microonde. II Determinazione del triptofano. (Protein digestion in food matrices with a microwave system. II Determination of tryptophan). *Ind. Alim.* **1993**, *32*, 346–349.

Chen, S. T.; Chiou, S. H.; Chu, Y. H.; Wang, K. T. Rapid hydrolysis of proteins and peptides by means of microwave technology and its application to amino acid analysis. *Int. J. Pept. Protein Res.* **1987**, *30*, 572–576.

Chiou, S. H.; Wang, K. T. Peptide and protein hydrolysis by microwave irradiation. *J. Chromatogr.* **1989**, *491*, 424–431.

Dellaglio, F.; De Roissart, H.; Torriani, S.; Curk, M. C.; Janssens, D. General characteristics of lactic acid bacteria. In *Bacteries Lactiques*; de Roissart, H., Luquet, F. M., Eds.; Lorica: Uriage, France, 1994; pp 25–116.

de Man, J. C., Rogosa, M., Sharpe, M. E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **1960**, *23*, 130–135.

Gilman, L. B.; Woodward, C. An evaluation of microwave heating for vapor phase hydrolysis of proteins. Comparison to vapor phase hydrolysis for 24 hours. In *Current Research in Protein Chemistry*; Gilman, L. B., Ed.; Academic Press: New York, 1990; pp 23–27.

Goldin, B. R. Health benefits of probiotics. *Br. J. Nutr.* **1998,** *80*, S203–207.

Hammes, W. P.; Weiss, N.; Holzapfel, W. The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes*, 2nd ed.; Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K. H., Eds.; Springer-Verlag Inc.: New York, 1992; Vol. II., pp 1534–1594.

Hierro, E.; de la Hoz, L.; Ordonez, J. A. Contribution of the microbial and meat endogenous enzymes to the free amino acid and amine contents of dry fermented sausages. *J. Agric. Food Chem.* **1999**, *47*, 1156–1161.

Hugas, M.; Garriga, M.; Aymerich, T.; Monfort, J. M. Biochemical characterization of lactobacilli from dry fermented sausages. *Int. J. Food Microbiol.* **1993**, *18*, 107–113.

Kandler, O.; Weiss, N. Regular, nonsporing gram-positive rods. In *Bergey's Manual of Systematic Bacteriology*; Sneath, P. H. A., Mair, N. S., Sharpe, M. E., Holtz, J. G., Eds.; Williams and Wilkins: Baltimore, 1986; pp 1208–1234.

Khan, A. R.; Robinson, R. J.; Johnson, J. A. Starch hydrolysis by acid and microwave energy. *J. Food Sci.* **1980**, *45*, 1449–1451.

Kolar, K.; Berg, H. Microwave hydrolysis for rapid determination of hydroxyproline in meat and meat products. Swedish Meat Research Institute Publication, **1990**, KK/Ba K 0924a, 13–21.

Li, B. W. Comparison of microwave oven and convection oven for acid hydrolysis of dietary fiber polysaccharides. *J. AOAC Int.* **1998**, *81*, 1277–1280.

Marconi, E.; Panfili, G.; Bruschi, L.; Vivanti, V.; Pizzoferrato, L. Comparative study on microwave and conventional methods for protein hydrolysis in food. *Amino Acids* **1995**, *8*, 201–208.

Marconi, E.; Panfili, G.; Messia, M. C.; Cubadda, R.; Compagnone, D.; Palleschi, G. Fast analysis of lysine in food using protein microwave hydrolysis and an electrochemical biosensor. *Anal. Lett.* **1996**, *29*, 1125–1137.

Marconi, E.; Panfili, G.; Acquistucci, R. Microwave hydrolysis for the rapid analysis of furosine in foods. *Ital. J. Food Sci.* **1997**, *9,* 47–55.

Montel, M. C.; Talon, R.; Fournaud, J.; Champomier, M. C. A simplified key for identifying homofermentative *Lactobacillus* and *Carnobacterium* spp. from meat. *J. Appl. Bacteriol.* **1991**, *70*, 469–472.

Naidu, A. S.; Bidlack, W. R.; Clemens, R. A. Probiotic spectra of lactic acid bacteria (LAB). *Crit. Rev. Food Sci. Nutr.* **1999**, *39*, 13–126.

Schillinger, U.; Lucke, F. K. Identification of lactobacilli from meat and meat products. *Food Microbiol.* **1987**, *4*, 199–208.

Stiles, M. E.; Holzapfel, W. H. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.* **1997,** *29*, 1–29.

Woodward, C.; Gilman, L. B.; Engelhart, W. G. An evaluation of microwave heating for vapor-phase hydrolysis of proteins. *Int. Lab.* **1990**, *20*, 40–46.

Received for review July 7, 1999. Revised manuscript received May 10, 2000. Accepted May 24, 2000.

JF9907440