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Detection and activity of plantaricin OL15 a bacteriocin produced by *Lactobacillus plantarum* OL15 isolated from Algerian fermented olives

By **Kacem Mourad*, Zadi-Karam Halima** and **Karam Nour-Eddine**

Laboratoire de Biologie des Microorganismes et Biotechnologie, Faculté des Sciences, Université d'Oran. B.P. 8053 SIDI El-BACHIR, 31026, Oran, Algérie.
E-mail: mourad.kacem1@caramail.com

RESUMEN

Detección y actividad de plantaricina OL15, una bacteriocina producida por *Lactobacillus plantarum* OL15 aislado de aceitunas fermentadas de Argelia.

Lactobacillus plantarum OL15, aislado de aceitunas verdes argelinas, produce un compuesto antimicrobiano en el medio de cultivo, observándose un efecto inhibidor frente a otras cepas de *Lactobacillus, Lactococcus* y *Propionibacterium*. Dicha actividad desaparece completa o parcialmente después del tratamiento con enzimas proteolíticas, es estable al pH en el rango de 3,0 a 8,0 y es, asimismo estable al calor incluso después de someterla a un proceso térmico de 121 ^{o}C durante 15 minutos. La bacteriocina pasa a través de membranas de celulosa de corte molecular de 1000.000 pero no a través de las de 10.000.

PALABRAS-CLAVE: Aceitunas de mesa - Bacterias del ácido láctico - Bacteriocina - Inhibición - Lactobacillus plantarum.

SUMMARY

Detection and activity of plantaricin OL15 a bacteriocin produced by *Lactobacillus plantarum* OL15 isolated from Algerian fermented olives.

Lactobacillus plantarum OL15 previously isolated from Algerian fermented green olives produces antimicrobial conpounds in its culture medium. Inhibitory action against other strains of *Lactobacillus, Lactococcus*, and *Propionibacterium* was observed. Activity was completely or partially inactivated by proteolytic enzymes, stable at pH values ranging from 3.0 to 8.0, and heat stable even after autoclaving at 121 ^{o}C for 15 min. The bacteriocin activity was able to pass through cellulose membranes with 100,000 but not through 10,000 molecular weight cut-off.

KEY-WORDS: Bacteriocin – Inhibition - Lactic acid bacteria - Lactobacillus plantarum – Table olives.

1. INTRODUCTION

It has long been agreed that the fermentation of olives is carried out by the naturally occurring strains of *L. plantarum* (Etchells *et al.*, 1966; Borbolla y Alcalá *et al.*, 1971 and Borcakli *et al.*, 1993). This bacterium has been extensively isolated from spontaneous processes of olive fermentations (Ruiz-Barba *et al.,* 1991; Lavermicocca *et al.*,1998 and Kacem *et al.,* 2004), and studied with the aim of using it in starter cultures for olive or other vegetable fermentations (Costilow and Fabian, 1953 and Leal-Sánchez *et al.,* 2002).

Some strains of *L. plantarum* isolated from fermented olives produce inhibitory substances, such as bacteriocins (Jiménez-Diáz *et al.,* 1993; Delgado *et al.,* 2001 and Maldonado *et al.,*, 2003). By definition, bacteriocins are biologically active proteins or protein complexes displaying a bactericidal mode of action towards Gram-positive bacteria and particularly towards closely related species (Tagg *et al.,* 1976 and Klaenhammer, 1988).

Research on bacteriocins from *L. plantarum* has expanded during the last decades. Plantaricin F (Fricourt *et al.*, 1994), plantaricin S and T (Jiménez-Diáz *et al.,* 1993), plantaricin C (Bruno-Bárcena *et al.,* 1998), and plantaricin NC8 (Maldonado *et al.*, 2003) have been detected, purified and characterized.

The preservation of fermented olives could be improved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as a food additive. In olive fermentations, Ruiz Barba *et al.* (1994) inoculated an olive brine with a *L. plantarum* strain that was previously identified as a bacteriocin producer and they verified that this strain became readily dominant over wild bacteria. The same behaviour was not reproduced by the non-bacteriocin-producer mutant. Also Asehraou *et al.* (2002) described similar results in fermenting green olives that have been inoculated with *L. plantarum.*

In this study we report a bacteriocin (plantaricin OL15), produced by *L. plantarum* OL15 strain. This study also includes partial purification and preliminary characterization of the bacteriocin, determination of bacteriocin activity, and search for antimicrobial spectrum.

2. MATERIALS & METHODS

2.1. Bacterial cultures and media

The bacteriocin producer was *L. plantarum* OL15 and the indicator organism used in the bacteriocin assay was *Lactococcus lactis* B8. Both strains were isolated from fermented olives at the Biology of

Microorganisms and Biotechnology Laboratory, University of Oran, Algeria (Kacem *et al.* 2004).

The bacterial strains of different origin which were used as bacteriocin targets are listed in Table1. The stock cultures of lactic acid bacteria strains were maintained at -20°C in a solution of 10% sterile reconstituted nonfat skimed milk. Prior to use, the cultures were transferred twice in MRS broth (Merck Mikrobiologie) and incubated at 30°C for 16-18 h. All lactic acid bacterial strains used in this study were grown in MRS broth at 30°C for 24 h. *Propionibacterium* strains were grown aerobically in YELA agar (Malik *et al*., 1968) at 32°C for 48 h.

2.2. Preparation of crude extract samples (CE0)

L. plantarum OL15 was propagated in 500 ml MRS broth for 24 h at 30°C. For extraction of bacteriocin, the culture was centrifuged (10,000 g for 20 min. at 4°C). The supernatant was adjusted to pH 7.0 with 3M NaOH to exclude the antimicrobial effect of H^+, followed by filtration of the supernatant through a 0.22-μm pore size filter (Gelman Acrodisc 13, Pall Corp., Ann Arbor, USA). Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (C-100 bovine liver, Sigma). This solution was designated as the crude extract (CE0).

2.3. Determination of bacteriocin activity

The inhibitory activity of *L. plantarum* OL15 was screened by agar well diffusion assay (Schillinger and Lucke, 1989). Pre-poured MRS agar plates were overlaid with 7ml MRS soft agar containing 0.2 ml of indicator culture. In order to standarized the assay, the inoculum was approximately 10^6 indicator cfu ml^{-1}. Wells of 5 mm in diameter were cut into the agar plate using a cork borer, and aliquots of 50 μl from each bacteriocin solution samples were placed into the wells. The plates were incubated under conditions suitable for bacterial growth, and examined for the presence of 1.5 mm or larger clear zones of inhibition around the wells. Each assay was performed in duplicate.

2.4. Determination of bacteriocin titre

The titres of bacteriocin produced were quantified by the critical dilution method, as described by Schillinger and Lucke (1989). Two fold serial dilutions of concentrated or not CE0 were made in saline solution. Aliquots of 50 μl from each dilution were placed in wells in plates seeded with the indicator strain. These plates were incubated under conditions suitable for bacterial growth, and the diameters of the inhibition zones were measured. Each assay was performed in duplicate. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units per ml (AU/ml).

2.5. Partial purification of bacteriocin

*Ammonium sulphate precipitation***:**

CE0 was treated with solid ammonium sulphate (Mallinckrodth Chemical, Inc., Paris, KY, USA) to 0, 30, 35, 40, 45, 50, 55 and 60% saturation. The mixtures were stirred for 2 h at 4°C and later centrifuged at 14,000 g for 1 h at 4°C. The pellet was resuspended in 25 ml of 0.05 M potassium phosphate buffer pH 7.0. Dialysis was carried out against the same buffer for 18 h in spectrapor dialysis tubing (Specrapor, 1000 dalton MWco, Fisher Scientific Pittsburgh, PA USA). Assay of the bacteriocin activity was carried out and titer was determined. This product was named CE1.

Trichloroacetic acid (TC) precipitation:

Five percent (5%) equivalent of TC was added to 25 ml of CE1 to precipitate target fraction. The mixture was centrifuged at 12.000 g for 10 min. after which the supernatant was decanted. The resulting pellet was dissolved in potassium phosphate buffer, obtaining CE2.

Ultrafiltration:

Several aliquots of 1ml CE2 were ultrafiltered through various filtron membranes (Filtron Technology Corp; Northborough, Mass), of 1,000,000, 100,000, 10,000 and 1,000-molecular exclusion sizes. Bacteriocin activity was determined in retained and eluted fractions. For the quantification of protein concentration a modification of the Bradford method has been used, according to manufacturer's instructions for micro-assay procedure (Bio-rad Protein Assay, Bio-Rad Laboratories, Hercules-CA, USA).

2.6. Preliminary characterization of the bacteriocin and search for antimicrobial spectrum

The concentrated CE2 was subjected to different treatments. For all tests agar well diffusion method was used to check the remaining activity of the inhibitory agent. In all assays, untreated CE2 samples were tested simultaneously to serve as controls. Each assay was performed in duplicate.

CE2 was adjusted pH 7.0 and treated with trypsin (EC3.4.21.4), α-chymotrypsin (EC3.4.21.1), pronase E (Type XXV) or catalase (EC1.11.1.6). All enzymes

were obtained from Sigma (Chemical Company, St. Louis, Mo) except trypsin, which was from Serva (Heidelberg, Germany). Enzymes were dissolved in phosphate buffer (0.2 mol l^{-1}, pH 6) and mixed with CE2 (1:1) to a final concentration of 1mg/ml. Controls included sterile MRS broth, a 1:1 mixture of CFS with buffer but without enzyme, and buffer with only the enzyme.

To assess the effect of pH, CE2 was adjusted to pH values from 1-12 with 4M HCl or 4M NaOH.

Heat stability of the bacteriocin was assessed by heating the CE2 at 80°C and 100°C in a water bath. Samples were withdrawn at different time intervals and the antimicrobial activity was tested. Heat resistance was also checked after autoclaving the CE2 at 121°C for 15 minutes. The CE2 was also treated with Tween 20, SDS and Triton X-100 at a final concentration of 1% (w/v). Sample (200 µl of CE0 or CE2) stability at 37°C, 4°C and at –20°C was also evaluated in the same way for sterile CE0 or CE2.

Finally, inhibitory activity of CE2 was tested against lactococci, lactobacilli (in MRS agar at 30 °C for 18 h) and propionibacteria (in YELA agar at 32°C for 48 h) by the well diffusion method (Table 1).

3. RESULTS & DISCUSSION

During the study (Kacem *et al.*, 2004) on lactic acid bacteria from naturally fermented olives, 11 strains of *L. plantarum* were screened for antimicrobial activity against target microorganisms by well diffusion method similar to that described by Barefoot and Klaenhammer (1983). Finally, the supernatant from one strain of *L. plantarum* OL15 proved its ability to inhibit several strains of *Lactococcus, Lactobacillus* and *Propionibacterium.* Respectively, *L. plantarum* OL15 and *L. lactis* BO8 strain were then selected as an antibacterial agent-producing and indicator strains.

The antibacterial activity of the CE0 was estimated directly from the first dilution where inhibition of *L. lactis* BO8 (indicator strain) was not observed. The titer was found to be 1600 AU/ml.

As shown in Table 1, the inhibitory action of CE2 was tested against a total of 18 microorganisms represented by 6 strains of natural competitors of *L. plantarum*, 6 strains of *L. lactis*, 2 strains of *Enterococcus* sp. and 4 strains of *P. freudenriechii shermanii*. The CE2 inhibited by some strains of the

Table 1
Bacterial strains used as indicators for inhibition studies

Origin	Target strain	Inhibitory action of CE2
Fermented olives (Kacem *et al.*, 2004)	*L. lactis* B08	+
	L. lactis B018	+
	L. lactis B025	+
	Enterococcus sp. 0L32	-
	Enterococcus sp. 0L98	-
	L. plantarum OL16	-
	L. plantarum OL23	+
Cow's milk (Kacem *et al.*, 2003)	*L. lactis* LVA1	-
	L. lactis LVA10	-
	L. lactis LVA18	-
Sheep's milk (Kacem *et al.,* 2003)	*L. plantarum* BA1	+
	L. plantarum BA2	+
	L. plantarum BA3	+
	L. plantarum BA16	+
ATCC	*P. freudenriechii shermanii* 9615	+
	P. freudenriechii shermanii 9619	+
	P. freudenriechii shermanii 1367	+
	P. freudenriechii shermanii 8262	+

ATCC: American Type Culture Collection.
Symbols:+: inhibition -: inhibition not detected.
CE2 is the concentrated crude extract (trichloroacetic acid precipitation) obtained from a *L. plantarum* OL15 culture. It was tested against microorganisms by the well diffusion method.

Table 2
Concentration and partial purification of bacteriocin produced by *L. plantarum* OL15

Purification Stages	Volume (ml)	Activity (AU/ml)[1]	Total activity[2]	Total protein (µg/ml)[3]	Specific activity[4] (AU/µg)	Purification factor[5]
Crude extract samples (CE0)	500	400	200000	210	1.9	1
Ammonium sulphat precipitation (CE1)	15	800	12000	140	5.7	3
Tricholoroacetic precipitation (CE2)	2	1600	3200	38	42.1	7.4
Ultrafiltration (1,000,000 Kda cut-off)	5	800	4000	3	267	6.3

[1]Antimicrobial activity of the bacteriocin solution against *L. lactis* B08 (indicator bacteria) (AU/ml).
[2] Multiplication of total volume (ml) by activity (AU/ml).
[3]Determined by the Bradford method
[4]Activity (AU/ml) (column 2) divided by the protein concentration (µg/ml) (column 4)
[5]Fold increase in the initial specific activity, when compared to previous step.

Table 3
Ultrafiltration study of bacteriocin produced by *L. plantarum* OL15

Membrane molecular weight cut-off	AU (% Initial bacteriocin activity)[1]	
	Retentate (%)	Eluted fraction (%)
1,000,000	200 (12.5)	800 (50.0)
100,000	800 (50.0)	400 (25.0)
10,000	1600 (100.0)	0 (0.0)

[1]Initial bacteriocin activity was 1600 AU/ml

genus *Lactococcus* (*L. lactis*, B018 and B025), *Lactobacillus* (*L. plantarum* OL23, BA1, BA2, BA3 and BA16), and *Propionibacterium* (*P. freudenriechii shermanii* 9615, 9619, 1367 and 8262). On the other hand, the CE2 was not active against strains such as *L. lactis* (LVA1, LVA10 and LVA18) *Enterococcus* sp. (0L32 and 0L98) and *L. plantarum* OL16.

Partial purification steps of the bacteriocin are summarized in Tables 2 and 3. During the purification procedure, each step resulted in a considerable loss of protein concentration while specific activity increases. The optimal bacteriocin recovery was achieved by including ammonium sulphate precipitation and trichloroacetic acid precipitation. The bacteriocin was able to pass through cellulose membranes with 100,000-molecular weight cut-off , but not through 10,000-molecular weight cut-off. However, partial loss of bacteriocin activity was observed during ultrafiltration (Table 3).

The effects of enzymes, heat, storage time, pH, and surfactants on EC2 (1600 AU/ml) activity were determined using *L. lactis* BO8 as the indicator organism.

The inhibitory factor produced by *L. plantarum* OL15 was neither hydrogen peroxide nor organic acid. The inhibitory activity was not affected by catalase and was retained in neutralized EC0. Activity was completely inactivated by α-chymotrypsin but partially inactivated after treatment with trypsin and pronase. The loss of the antimicrobial activity after treatment with enzymes indicated the sensitivity of the active compounds secreted by *L. plantarum* OL15 strains which reflect the proteinaceous nature of the inhibitory agent.

The inhibitory activity of this proteinaceous substance (s) was stable when exposed to 100^{o}C and even retained complete activity after autoclaving at 121^{o}C for 15 min.

The inhibitory activity of CE0 was stable at pH 3 to 8 but was completely inactive after exposure to pH 9.

Detergents seem to have a marked effect on the bioactive substance. The antagonistic activity of CE0

was greatly reduced when treated with SDS whereas Triton X-100 and Tween 20 completely inhibited the activity.

The effect of time and temperature of storage on bacteriocin activity was also (studied, examined, determined etc.) . It was observed that the bacteriocin produced by *L. plantarum* OL15 maintained full stability after storage for 60 days at –20°C; partial stability after storage for 120 days at 4°C, while no activity was detected after storage for 80 to 120 days at 37°C.

The antagonistic effect produced by *L. plantarum* OL15 against *L. lactis* BO8 was completely or partially inactivated when preparations were treated with proteolytic enzymes, suggesting that the substance (s) is proteinaceous, or at least has a proteinaceous activator. From these findings, and in accordance with the guidelines outlined by Tagg *et al.* (1976), the proteinaceous antibacterial substance can be attributed to a bacteriocin.

As we have reported in the introduction of this paper, several bacteriocin-producing strains of *L. plantarum* have been isolated from olives (Jiménez-Diáz *et al*., 1993; Ruiz Barba *et al.,* 1994; Leal-Sánchez *et al.,* 1998; Delgado *et al.,* 2001).

L. plantarum OL15, previously isolated from algerian fermented green olives (Kacem *et al.,* 2004) also produces a proteinaceous substance which can be regarded as a bacteriocin according to the standard criteria. This bacteriocin, named «plantaricin OL15», demonstrated an antibacterial effect on bacteria tested for sensivity.

This proteinaceous substance was heat resistance and even retained complete activity after autoclaving for 15 min, stabe to pH, has the ability to precipitate by «salting-out» and to retain membranes by ultrafiltration (10,000-molecular weight cut-off). The inhibition action over some other bacteria was also examined. We registered the inibition of some strains from two lactic acid bacteria genera (*Lactococcus* and *lactobacillus*) and several strains of the same subspecies of *Propionibacterium* genus.

In this respect, the substance produced by *L. plantarum* OL15 strain exhibited properties similar to those of bacteriocins of several other lactic acid bacteria (Nettles & Barefoot, 1993; Jack *et al.,* 1995), including strains isolated from fermenting olives such as *L. plantarum* LB17.2b (Delgado *et al.*, 2001) and *L. plantarum* NC8 (Maldonado *et al.*, 2003). These authors have shown that *L. plantarum* NC8 produced plantaricin NC8 (PLNC8) which was heat resistant and of a proteinaceous nature. Maldonado *et al.* (2002) reported that bacteriocin producer strains are commonly distributed among wild-type of *L. plantarum* strains. These authors have shown that among 68 independent isolates from different olive fermentations in southern Spain, 15 of them were shown to produce bacteriocins that were active against other lactic acid bacteria, as well as spoilage and pathogenic bacteria.

4. CONCLUSION

L. plantarum OL15 and its bacteriocin (plantaricin OL15), can have positive impacts on the fermentation of olives produced in Algeria as well as other vegetable products, with the aim of improving the hygiene and safety of the food products so produced. Further experiments concerning molecular identification (PCR analysis) of the bacterium as well as its bacteriocin are in progress.

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