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Antimicrobial activity of a protein obtained from fruiting body of *Lentinula edodes* against *Escherichia coli* and *Staphylococcus aureus*

Lilia Sánchez-Minutti[1,3], Maura Téllez-Téllez[4], Saul Tlecuitl-Beristain[3], Gerardo Santos-López[5], Ruben Díaz[2], Vijai Kumar Gupta[6], Gerardo Díaz-Godínez[2]*

[1]Postgraduate of Biological Sciences, Autonomous University of Tlaxcala, Tlaxcala, 90000, México
[2]Laboratory of Biotechnology, Research Center for Biological Sciences, Autonomous University of Tlaxcala, Tlaxcala, 90000, Mexico
[3]Biotechnology Laboratory, Polytechnical University of Tlaxcala, Tepeyanco, Tlaxcala, 90180, México
[4]Center for Biological Research, Autonomous University of State of Morelos, Morelos, 62209, México
[5]East Center for Biomedical Research, Mexican Social Security Institute, Puebla, 74360, México
[6]Molecular Glycobiotechnology Group, Discipline of Biochemistry, National University of Ireland Galway, Galway, 12074, Ireland
*Corresponding Author E-mail: diazgdo@hotmail.com

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Abstract

In thepresent study, a protein extracted from the fruiting body of *Lentinula edodes* showed growth inhibition of *Escherichia coli* and *Staphylococcus aureus*. An aqueous extract from the dry fruiting body for purification process was used. This process was carried out in three stages, isoelectric focusing, ion exchange, preparative electrophoresis. The antimicrobial activity was evaluated by diffusion disc technique. The aqueous extract reported a protein concentration of 57.4 mg l^{-1} and showed bacterial inhibition zone of 0.90 and 1.10 cm for *E. coli* and *S. aureus*, respectively; the isolated protein (9.69 mg l^{-1}) formed bacterial inhibition zone of 2.48 cm for *E. coliand* 2.68 cm for *S. aureus*. This protein with a purification factor of 16 showed molecular weight of 87.2 kDa and specific activity of 28 cm mg^{-1}. The isolated protein showed 2.8 and 2.4 times higher antimicrobial inhibition zone than aqueous extract for *E. coli* and *S. aureus* respectively, and their size was almost equal to that observed with 50 mg l^{-1} gentamicin.

Key words

Antimicrobial activity, *Escherichia coli, Lentinula edodes, Staphylococcus aureus*

Introduction

Bacterial infections have caused many epidemiological outbreaks around the world. Outbreaks associated with *Escherichia coli* and *Staphylococcus aureus* are very frequent, which are transmitted mainly by foods consumption but also there are nosocomial infections, which increase the problem (WHO and FAO, 2013; WHO, 2003). Despite the advances in medicine, occurrence of diseases caused by these pathogens has not reduced and antibiotic resistance could be a major threat to public health (Oldfield and Feng, 2014). Therefore, exists search for new antimicrobial compounds against those bacteria in mammals, insects, plants, bacteria, viruses and fungi (Tossi and Sandri, 2002). Some fungi like *Lentinula edodes, Pleurotus ostreatus, Fomes lignosus, Marasmius jodocodo, Pleurotus tuber-regium*, *Psathyrella atroumnonata, Polyporus giganeus, Termitomyces microcarpus, Termitomyces robustus, Pleurotus eryngii, Pleurotus sajor-cajuand Agaricus bisporus* produce molecules having antimicrobial activity (Gbolagade *et al*., 2007; Hearst *et al*., 2009; Akyuz *et al.*, 2010; Houshdar-Tehrani *et al.*, 2012). Among the

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molecules that act as an antimicrobial or antifungal include polysaccharides, peptides, thaumatin-like proteins, proteins inactivating ribosomes and ribonucleases (Ngai and Ng, 2004; Wang and Ng, 2004; Hu *et al.*, 2011). The mode of action of these molecules is usually associated with damage to the cell membrane (Hancock and Chapple, 1999).

L. edodes is an edible mushroom that is used as traditional medicine, capable of inhibiting the growth of bacteria such as *E. coli, S. aureus, Micrococcus luteus, Bacillus cereus, Candida albicans, Pseudomonas* sp., among others (Kitzberger *et al*., 2007; Hearst *et al*., 2009; Casaril e*t al.,* 2011). This fungus has been reported to produce several compounds like a peptide called lentin capable of inhibiting *Physalosporapiricola, Botrytiscinerea and Mycosphaerella arachidicola; thaumatin-like proteinsthat inhibits Sacharomyces cerevisiae*, lentamicine that acts against *S. aureus*. Water-soluble lignin and lentinan obtained from mycelium of *L. edodes* have shown antiviral activity (Grenier *et al*., 2000; Ngai and Ng, 2003). Today, there is a growing interest in the search for molecules with potential therapeutic uses and *L. edodes* is an alternative. In view of the above, a protein extracted from the fruiting body of *L. edodes* was studied for its antimicrobial activity against *E. coli* and *S. aureus.*

Materials and Methods

Microorganisms: Fruiting body of *L. edodes* was used to extract the protein with antimicrobial activity.

Extraction process: Aqueous extract of fruiting body (previously dried in oven at 50 °C for 48 hr and pulverized) of *L. edodes* was obtained. Fungus powder and water in 1:3 (w/v) ratio was mixed using a stirrer plate at 4 °C for 24 hrs. Subsequently, the mixture was centrifuged at 15000 xg for 30 min and the supernatant and 1M acetate buffer pH 5.5 (1:10 v/v) were mixed.

Purification of protein with antimicrobial activity: Protein with antimicrobial activity was purified in three-step process. In the first step, an aqueous extract was fractionated by preparative isoelectric focusing technique (Rotofor, Bio-Rad) using a 60 ml chamber and ampholytes with pH ranging between 3-10 (Bio-Lyte 40%). In the second step, 5 ml of fraction number 3 (F3) from Rotofor with antimicrobial activity was eluted through an ion exchange column Econo-Pac High Q Anion Exchage Cartridge, Bio-Rad, coupled to a system FPLC (BioLogic System, BioRad), using a linear gradient 0-0.5 M NaCl in 25 mM Tris buffer pH 8.1. Fraction with antimicrobial activity (P1) obtained after separation by anion exchange was fractioned by preparative electrophoresis (Mini Prep Cell, Bio-Rad), using a 10 % acrylamide gel without SDS. In each purification step, the fractions were dialyzed with a 10 kDa membrane (Standard RC) and lyophilized. The molecular weight of the fractions obtained were determined by SDS-PAGE (Laemmli, 1970), gel staining was performed with silver nitrate. The protein concentration of aqueous extract and of each fraction was performed by the method of Bradford (1976). Statistical analysis with Statgraphics Plus version 5.1 program was conducted to determine significant differences between the individual means using ANOVA and Tukey's test (p<0.05).

Evaluation of antimicrobial activity: Aqueous extract and fractions obtained in each purification step were evaluated for antimicrobial activity by disc diffusion technique (Collin and Line, 2012), with few modifications described below. For this, an initial inoculum of pathogenic bacteria was adjusted to McFarland nephelometer turbidity standard No. 0.5 and was mixed with 20 ml of Muller-Hinton agar. The mixture was poured into Petri dishes of 100 x 15 mm. Four holes were made inside each Petri dish and 125 µl of each sample per well, were incubated for 18 hrs at 37 °C. Diameter of inhibition zone was measured with a Vernier Callifer. Gentamicin (50 mg l^{-1}) and water were used as positive and negative controls, respectively.

Results and Discussion

Aqueous extract of fruiting body of *L. edodes* with 57.4 mg l^{-1} protein showed bacterial inhibition zone of 0.90 and 1.10 cm for *E. coli* and *S. aureus* (Table 1). Several extracts obtained from basidiomycetes have been investigated for their antimicrobial activity, such as alcoholic extracts of fruiting bodies of *A. bisporus* and *P. eryngii*, showing antimicrobial inhibition zone of 0.75-0.85 mm diameter against *E. coli* and *S. aureus* (Akyuz *et al*., 2010), or ethanolic and cetonic extracts from *Ganoderma carnosum* and *Clitocybe geotropa* which formed inhibition zones >10 mm against *E. coli* and *S. aureus* (Yamac and Bilgili, 2006). It is important to mention that the polarity of solvent used in the extract is crucial to obtain a greater or lesser antimicrobial activity as it depends on the nature of the molecules obtained; for example, there is a report where protein extracts from *L. edodes* were prepared using a kit for protein extraction and found no zones of inhibition for Gram positive and Gram negative bacteria (Hearst *et al*., 2010), but there are also reports on antimicrobial activity of *L. edodes* extracts (Hearst *et al*., 2009). During the process of purification of aqueous extract, fraction F3 reported protein concentration of 17.2 mg l^{-1} and showed bacterial inhibition halos of 1.10 and 1.17 cm for *E. coli* and *S. aureus*, respectively. In this fraction at least six bands were observed on the SDS-PAGE with molecular weights ranging from 26 to 116 kDa (Fig. 1). The fraction obtained by anion exchange chromatography was called P1 and had a protein concentration of 10.08 mg l^{-1}, showed bacterial inhibition zone of 2.0 and 2.53 cm against *E. coli* and *S. aureus*. Three bands in P1 fraction observed in the SDS-PAGE had molecular

Table 1 : Antimicrobial activity of aqueous extract (AE) and each fraction obtained through purification process

Purification step	Protein (mgl^{-1}±SE)	Inhibition zone (cm±SE)		Specific activity (cm mg^{-1} proteína±SE)		Purification factor (Media±SE)	
		E. coli	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
AE	57.40±0.06	0.90±0.10[b]	1.10±0.10[a]	0.02	0.02	1	1
F3	17.20±2.74	1.10±0.00[b]	1.17±0.06[a]	0.06	0.07	4.0	3.5
P1	10.08±0.60	2.00±0.00[b]	2.53±0.04[a]	0.20	0.25	12.6	13.1
P2	9.69±1.62	2.48±0.03[a]	2.68±0.04[a]	0.26	0.28	16.3	14.2

Values are mean SE (n=3). Different superscript letters in the same row indicate significant difference between the means ($p<0.05$). Gentamicin was used as positive control (Inhibition zone for *E. coli* and *S. aureus* were 3.2±0.00 and 2.8±0.02 cm, respectively) and water was used as negative control without inhibition zones in both bacteria

weight of 87.2, 32.2 and 26.2 kDa. Finally, in the third stage of extraction, a single peak (P2) was obtained with a protein concentration of 9.69 mg l^{-1}showed bacterial inhibition zone of 2.48 and 2.68 cm against *E. coli* and *S. aureus*, respectively. This fraction showed a single band of 87.2 kDa. Fig. 2 shows the bacterial inhibition zones in Petri dishes.

Recently, antimicrobial activity of 91 isolates of endophytic fungi from ethnomedicinal plant *Melastoma malabathricum* has been reported. Fungi classified into 18 genera and 24 isolates showed antimicrobial activity against one or more pathogen (Gram negative bacteria: *Pseudomonas aeruginosa* and *E. coli* and the Gram positive bacterium *S. aureus*). *Diaporthe phaseolorum* var. *meridionalis* inhibited all the bacterial strains (Mishra *et al.*, 2016). On the other hand, compounds with antimicrobial activity against drug-resistant strains of human pathogens were studied in *Neurospora crassa* isolated from leaves of *Rhizophora mucronata*. Fungal extracts showed antimicrobial activity against *E. coli, P. aeruginosa*, *B. subtilus* and several other bacterial strains. The GC MS analysis reported 9-Octadecenoic acid as an active compound (Joel and Bhimba, 2013). Cyclosporine A in the marine fungus *Microdochium nivale* associated with *Porteresia coarctata*. The antifungal properties of Cyclosporine A against Aspergillus *niger, A. fresenii, A. japonicus, Cryptococcus neoformans, Candida sp., Trichophyton mentagrophytes, T. violaceum, T. tonsurans, Microsporiumgypsum* and *Fusarium sp.* was assessed by Bhosale *et al*. (2011).

Although several studies have evaluated the antimicrobial and antifungal activity of extracts obtained from basidiomycetes, few studies have purified the molecules responsible for this antimicrobial activity. Houshdar-Tehrani (2012) purified 22.5 kDa protein from *A. bisporus*, which showed antibacterial properties against *E. coli, S. aureus* and other bacterial strains. Similarly, a protein isolated from *Clitocybe sinopica* showed positive antimicrobial activity against *Agrobacterium rhizogenes, Agrobacterium tumefaciens, Agrobacterium vitis, Xanthomonas oryzae* and *Xanthomonas malvacearum* and negative effect against *E. coli* and *S. aureus* (Zheng *et al.*, 2010), respectively. In a few studies, however the antifungal activity of a purified proteins did not show antimicrobial activity, as reported for purified peptidic compound from *Agrocybe cylindracea* (Ngai *et al.*, 2005).

Protocols often used for purification of proteins with antimicrobial, antifungal or antiviral activity includes ion exchange chromatography, affinity and gel filtration. This purification protocol was used for purifying a 28 kDa peptide called alveolarin from the fungus *Polyporus alveolaris* showed antimicrobial activity against *B. cinerea, Fusarium oxysporum, M. arachidicola* and *P. piricola* (Wang *et al*., 2004). In an other study a 14.4 kDa peptide called Eryngin isolated from *P. eryngii* showed antifungal activity against *F. oxysporum* and *M. arachidicola* (Wang and Ng, 2004). Similarly, a 7 kDa peptide isolated from *P. ostreatus* showed antifungal activity against *F. oxysporum, M. arachidicola* and *P. piricola* (Chu *et al.*, 2005), whereas apurified protein from *Tricholoma giganteum* showed positive antimicrobial activity against *F. oxysporum, M. arachidicola* and *P. piricola* (Guo *et al*., 2005), respectively.

A purified peptide of 27.5 kDa called lentin extracted from *L. edodes* showed positive antimicrobial activity against *P. piricola, B. cinerea* and *M. arachidicola* (Ngai and Ng, 2003). Besides this, reports on the purification process of protein with antimicrobial activity from *L. edodes* is meagre. In addition, the purification protocol used in the present research has not been reported before, where the preparative techniques of isoelectric focusing and electrophoresis including anion exchange were used.

The protein reported in the present study, showed 2.8 and 2.4 times higher antimicrobial inhibition zone than aqueous extract for *E. coli* and *S. aureus* respectively, and

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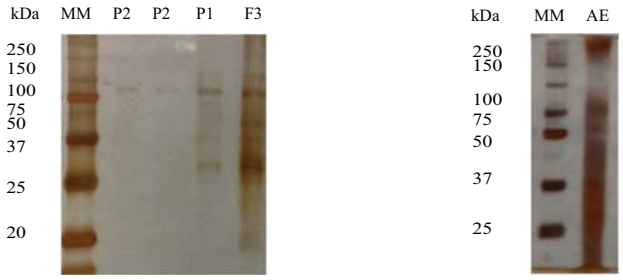

Fig. 1: SDS-PAGE of aqueous extract (AE) and each fraction with antimicrobial activity obtained through purification process

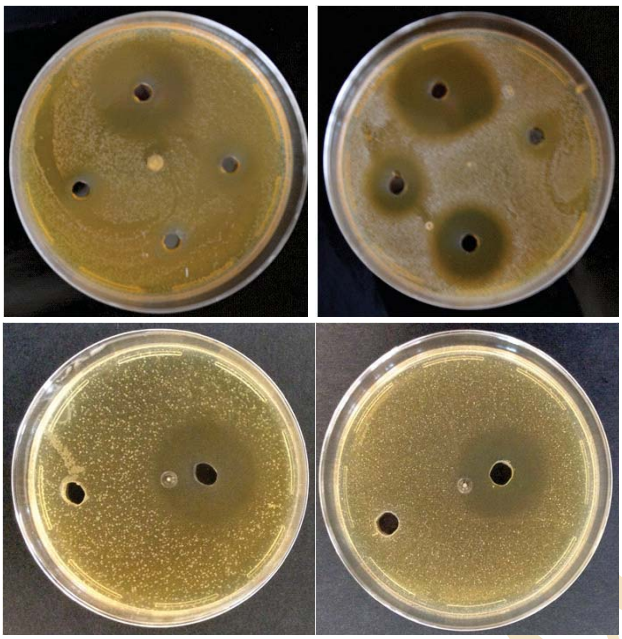

Fig. 2: Inhibition zones for *E. coli* (left up) and *S. aureus* (right up) of aqueous extract (AE) and each fraction with antimicrobial activity obtained through purification process. Controls for *E. coli* (left down) and *S. aureus* (right down); negative (left hole) and positive (right hole)

their size were almost than those observed with 50 mg l^{-1} of gentamicin. The specific activity was increased 13-16 times with purification process for both bacteria (Table 1). In general, the antimicrobial activity was slightly greater for *S. aureus* than for *E. coli*. The size of antimicrobial inhibition zones of aqueous extract and all fractions including the pure protein was higher than those reported in above studies.

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