ANTIMICROBIAL PEPTIDES EXTRACTED FROM THE AUTOLYSATE OF *Saccharomyces cerevisiae* AS POWERFUL CANDIDATES FOR FOOD PRESERVATION

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RESUMO - Peptídeos antimicrobianos são capazes de inibir o crescimento bacteriano, evitando ou minimizando a ameaça de resistência, sendo encontrados em matrizes alimentares, como o fermento biológico, um produto de baixo custo e GRAS (Geralmente Reconhecido Como Seguro). Um extrato rico em peptídeos foi produzido por autólise induzida de *Saccharomyces cerevisiae*, obtida do fermento biológico. O extrato, enriquecido em peptídeos <10 kDa por ultrafiltração, inibiu o crescimento de *Acinetobacter* genomospécies 3, *Pseudomonas aeruginosa*, *Escherichia coli*, *Listeria innocua*, *Staphylococcus aureus* e *Staphylococcus saprophyticus* coagulase-negativa, com IC_{50} de 0,79 a 0,10 mg/mL. O fracionamento do extrato filtrado por gel filtração originou frações com potencial superior ou similar a antibióticos convencionais. F1 e F5 apresentaram atividade promissora contra *E. coli*, com IC_{50} de 6,57 µg/ml (F1), 8,80 µg/ml (F5) e 31.08 µg/ml (F6) enquanto para *S. aureus*, F5 apresentou potencial com IC_{50} de 16,17 µg/ml. Estudos adicionais estão em andamento para testar novas espécies bacterianas.

PALAVRAS-CHAVE: antimicrobianos; autólise; peptídeos; bactérias alimentares.

ABSTRACT – Antimicrobial peptides are capable of inhibiting bacteria growth while avoiding or minimizing resistance threat, being found in food matrices such as baker's yeast, a low cost, and GRAS (Generally Recognized As Safe) product. A peptide-rich extract was produced through heat-induced autolysis of *Saccharomyces cerevisiae* from baker's yeast. The extract was enriched in <10 kDa peptides by ultrafiltration and inhibited the growth of *Acinetobacter* genomospecies 3, *Pseudomonas aeruginosa*, *Escherichia coli*, *Listeria innocua*, *Staphylococcus aureus* and coagulase-negative *Staphylococcus saprophyticus* with IC_{50} from 0.79 to 0.10 mg/mL. The fractionation of the filtered extract by gel filtration released fractions with superior or similar antimicrobial activities compared to conventional antibiotics. F1, F5 and F6 exhibited promising activity against *E. coli*, with IC_{50} of 6.57 µg/ml and 8.80 µg/ml and 31.08 µg/ml, respectively, while for *S. aureus*, only F5 exhibited potential with IC_{50} of 16.17 µg/ml. Further studies are in progress to test other bacterial species.

KEYWORDS: antimicrobials; autolysis; peptides; foodborne bacteria.

1. INTRODUCTION

Food preservation aims to prevent or decrease deterioration speed of natural products caused by microorganisms or enzymes. Deterioration decreases the nutritional value and food quality, thus affecting color, flavor and texture, impairing consumption. The presence of some microorganisms species such as *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella*, *Shigella*, *Pseudomonas aeruginosa*, among others, is directly linked to food deterioration or food poisoning, attributing undesirable qualities to food products, including gas production, toxic substances release and greenish coloring. The consumption of food contaminated with pathogenic microorganisms may lead to food poisoning, which cause adverse effects in healthy individuals according to Rahman (2007), Amit et al. (2017), Odeyemi et al. (2020).

The use of preservation methods, along with sanitary actions, is essential to guarantee food integrity, preventing deteriorating or contamination. Different methods including drying, thermal processing, freezing, various packaging methods, irradiation, high pressure and the addition of chemical preservatives have been applied to produce safe food according to Rahman (2007), Rai et al. (2016), Amit et al (2017), Mukhopadhyay et al.(2017), Sharif et al. (2017)

Currently, there is an increasing demand for alternatives preservatives from natural matrices, especially bioactive peptides with antimicrobial activity, which in addition to avoid food poisoning, would still add nutritional value to food products, improving shelf life and microbiological safety, while maintaining organoleptic characteristics, and reducing losses. An alternative and low-cost method for food preservation would be the use of antimicrobial peptides (AMPs) extracted from GRAS (generally recognized as safe) sources, such as baker's yeast, which consists of *Saccharomyces cereviseae* biomass, millennially used by various cultures throughout history for bakery and beverage production according to Del Nobile et al. (2012), Carocho et al (2015).

Based on the aforementioned, this study aims to produce and partially purify new AMP candidates, extracted from the autolysate of *Saccharomyces cerevisiae* obtained from baker's yeast, with potential for application in food preservation, conferring microbiological safety, while maintaining physicochemical characteristics and improving shelf time of perishable food products.

2. MATERIAL AND METHODS

2.1 Organisms

The dried baker's yeast from fleischmann brand was acquired in the local trade of Rio de Janeiro (Latitude 22° 54' 13 S, Longitude 43° 12' 35 O). For antimicrobial activity, the following microorganisms were used: *Acinetobacter genomospecies* 3 (ATCC 17922), *coagulase-negative Staphylococcus saprophyticus* (KT955005), *Listeria innocua* (ATCC 33090) *Staphylococcus aureus* (ATCC 14458), *Pseudomonas fluorescens* (ATCC 13525), *Escherichia coli* (DH5alfa), kindly provided by the INCQS cell bank of the Oswaldo Cruz Institute.

2.2 Preparation Of Peptidic Extract

The autolysate was prepared according to Freitas et al. (2019) with modifications. Distilled water (50 mL) and 10.5 g dry baker's yeast were homogenized and the pH adjusted to 6.0. The homogenate was incubated in a water bath at 50°C for 24 h to induce autolysis and proteolysis by endogenous enzymes. After centrifugation, the cell-free supernatant was reincubated in water bath at 90°C for 10 min to inactivate proteases and then filtered through a 0.22 µm pore membrane.

The obtained autolysate was ultrafiltered in a 10 kDa-cutoff membrane to obtain an extract rich in <10 kDa peptides , that was termed the filtered extract.

2.3 Determination Of Protein/Peptide Content

The protein and peptide content of the autolysate and filtered extracts was determined using BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) and performed according to the manufacturer's recommendations.

2.4 Evaluation Of Proteins And Peptides Size Distribution Profile

The protein distribution profile of the autolysate and filtered extracts was evaluated by 16% tricine-SDS-PAGE according to Schägger (2006). The ultra-low range molecular weight marker (Invitrogen, Missouri, USA) was used as the molecular mass pattern.

To characterize the size distribution, samples of the autolysate and filtered extracts were fractionated by high-efficiency liquid chromography (HPLC) LC-20A (Shimadzu, Kyoto, JPN) in a prosec 300S 300 x 7.5 mm GPC/SEC gel filtration column (Agilent Technologies, CA, USA), coupled to a photodiode array detector (PDA) model SPD-M30A (Shimadzu Corp.). The chromatographic column was equilibrated with 0.15 M NaCl in 0.05M Na_2HPO_4, pH 7 at a flow rate of 1 mL/min.

2.5 Fractionation Of The Filtered Extract

The filtered extract (<10kDa) was fractionated in a gel filtration column (Superdex-75/10 300GL) using the Fast-Performance Liquid Chromatography (FPLC) AKTA purifier 10 system. The column was previously equilibrated with 0.05 M sodium phosphate buffer at pH 7.0 in a constant flow rate of 0.5 mL/min. An aliquote (1 mL) of the filtered extract (15.39 mg/mL) was loaded and the column was washed with the equilibration buffer. Absorbances were monitored at 280 and 215 nm and fractions of 1 mL were collected.

2.6 Evaluation Of Antimicrobial Activity

The antimicrobial potential of the extracts, antibiotics (cephalexin, chloramphenicol and vancomycin) and fractions collected by gel filtration (section 2.5) were evaluated by the microdilution method according to Wei et al. (2016), using microorganisms of clinical and food importance in accordance with international standards established by the Clinical & Laboratory Standards Institute (CLSI).

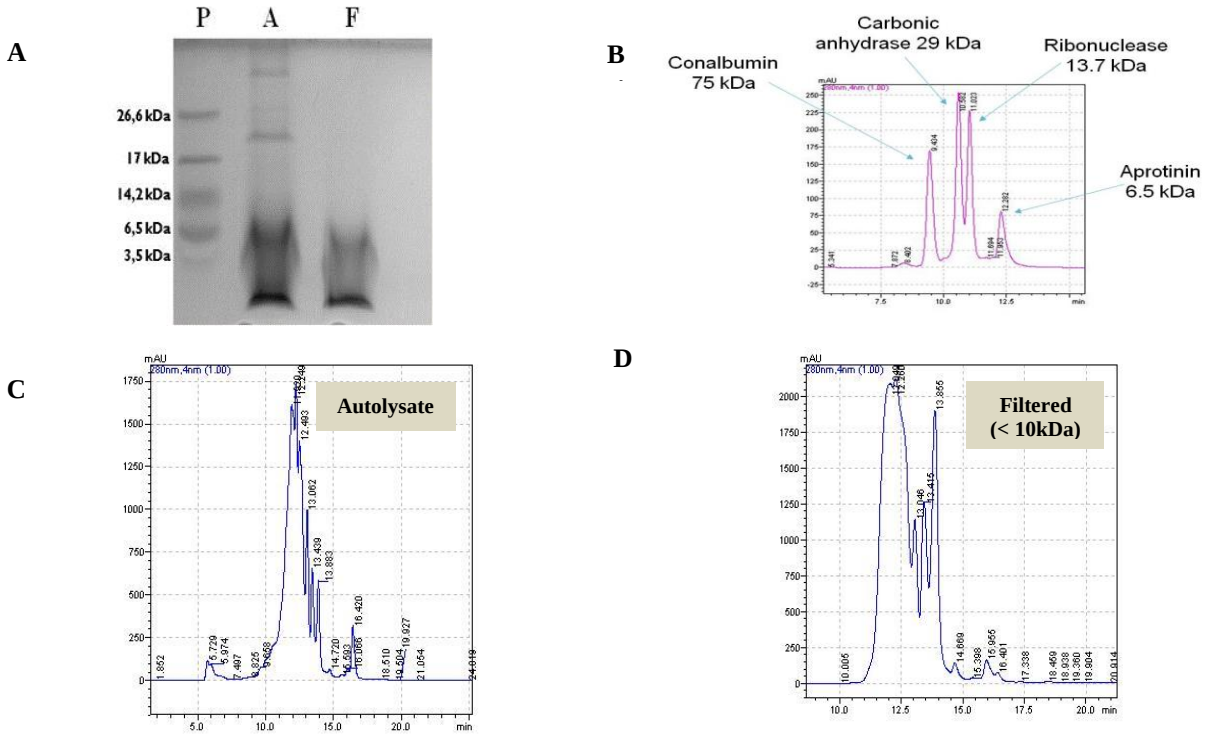
The inoculums were prepared in Mueller Hinton Broth (MHB) medium (2.0 g/L meat extract; 17.5 casein hydrolysate; 1.5 g/L starch) (KASVI, ITA) and incubated at 37° C for 18h. A bacterial suspension containing 10^8 cells was prepared according to the McFarland 0.5 scale followed by 10 × dilution in MHB medium for utilization in antimicrobial activity assays. The samples (100 µL) were serially diluted (1/2) in 100 µL MHB medium followed by the addition of 100 µL bacteria suspension. The microplates were incubated at 37 ºC for 18 h, under constant agitation. For cell viability assay, 30 µL of 2% resazurin was added to each well and incubated at 37 ºC during additional 2 h for resazurin metabolization. After incubation, the Absorbance was determined in a 2030 Multilable Reader VICTOR™ X4 (PerkinElmer, MA, USA) at 570 nm and 600 nm.

3. RESULTS

3.1 Proteins/Peptides Distribution Profile Of The Extracts And Their Antimicrobial Potential

Evaluation of the autolysate and filtered extracts by 16% tricine-SDS-PAGE and HPLC revealed they are mostly composed of low molecular mass proteins (< 10 kDa), as shown in Figure 1. Besides low molecular mass proteins (< 10kDa), the autolysate contains proteins of molecular masses as high as 17 and >26.6 kDa (Figure 1A, lane A, and Figure 1C) that after ultrafiltration were eliminated, giving rise to a filtered extract enriched in peptides with molecular masses ranging from ~10 kDa to < 3.5kDa as shown in Figure 1A, lane F, and Figure 1D.

Figure 1- Protein/peptidic distribution profile of baker's yeast extracts. (A) 16% tricine-SDS-PAGE. lane P - ultra-low range molecular weight markers; lane A - autolysate extract, lane F - filtered extract. (B) Pattern proteins fractionated in HPLC with ProSec300S gel filtration column. (C) Autolysate and (D) filtered extracts fractionated by the same methodology.



Both extracts, autolysate and filtered, were able to inhibit bacteria growth by at least 55% and a maximum of 100%. *A.* genomospecies 3 growth was completely inhibited by the two extracts, especially the filtered extract that was able to cause cell death since its MBC (minimal bactericidal concentration) and MIC (minimum inhibitory concentration) coincided at 0.97 mg/mL. On the other hand, the autolysate at 1.97 mg/mL inhibited 100% of *A.* genomospecies 3 growth ,but was not able to cause bacterial death as indicated by its MBC at Table 1. For other bacteria, the inhibition was partial, and it was not possible to determine the MIC or MBC with the maximum concentrations used in the assay, as indicated in the MBC column shown in table 1. For this reason, the concentration capable of inhibiting 50% growth (IC_{50}) was determined for each bacteria considering both extracts. Except for *E. coli* and *P. aeruginosa*, the filtered extract showed superior efficiency, since it produced lower IC_{50} values against *A. genomospecies* 3, *S. aureus*, coagulase-negative *S. saprophyticus* and *L. innocua*. No tendency in sensitivity of Gram positive or Gram-negative bacteria to peptide fractions was observed.

Table 1- Inhibitory potential of autolysate and filtered extracts against bacteria of clinical and food importance.

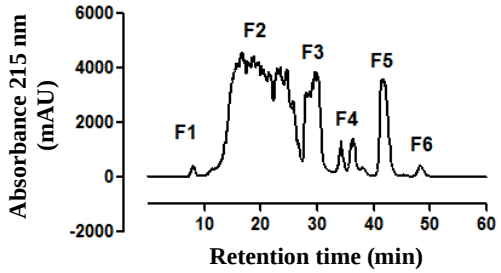
Bacteria	Gram	Autolysed			Filtered <10kDa		
		IC_{50} (mg/mL)	MCB (mg/mL)	Maximum inhibition %	IC_{50} (mg/mL)	MCB (mg/mL)	maximum inhibition %
Acinetobacter genomospecies 3 ATCC17922	-	0.61	>1.97	100	0.45	0.969	100
Escherichia. Coli ATCCDH5 alfa	-	0.54	>2.17	75	0.79	>2.03	82
Pseudomonas aeruginosa ATCC13525	-	0.2	>1.64	95	0.29	>1.54	85
Staphylococcus aureus ATCC 14458	+	0.69	>1.40	66	0.4	>1.34	81
Staphylococcus saprophyticus coagulase-negativa KT955005	+	0.19	>1.64	66	0.1	>1.54	73
Listeria innocua ATCC33090	+	1.81	>1.64	67	0.74	>1.54	55

3.2 Fractionation Of The Filtered Extract And Antimicrobial Activity Of The Obtained Fractions

The filtered extract was fractionated in a gel filtration column (Superdex-75/10 300GL) giving rise to six fractions with peaks that, in some cases, were not very well defined as shown in Figure 2A and with concentrations ranging from 63.07 to 1,080 µ g/mL as indicated in Figure 2B.

Figure 2- Fractionation of the filtered extract (< 10 kDa). (A) An aliquot of 1 mL of the filtered extract was loaded into a gel filtration column (Superdex-75/10 300GL) and the absorbance was monitored at 280 and 215 nm As shown in the chromatogram. (B) Table with retention times and concentration of each collected fraction.

A



B

Fractions	Retention time (min)	Dosage (µg/ml)
F1	7-9	77.23
F2	11-26	1080
F3	29-31	395.97
F4	34-38	174.41
F5	41-45	63.07
F6	48-50	77.52

The fractions obtained by gel filtration exhibited antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus.* Fractions F1 and F5 stood out with IC_{50} of 6.57 µg/ml and 8.80 µg/ml against *Escherichia coli*, respectively, while for *Staphylococcus aureus*, only F5 presented promising potential with IC_{50} of 16.17 µg/ml as shown in Table 2. In comparison to conventionally used antibiotics, fractions 1, 5 and 6 tested against *E. coli* presented a superior or similar potential than that of cephalexin (IC_{50} = 8.16 µg/mL) and vancomycin (IC_{50} = 33.51 µg/mL). Regarding *S. aureus,* all IC_{50} values were inferior when compared to chloramphenicol (IC_{50} = 4.67 µg/mL). However, fraction 5 stood out for presenting a potential close to that of cephalexin (IC_{50} = 11.69 µg/mL) as shown in the Table 3.

Table 2- Inhibitory potential of the 6 fractions obtained from gel filtration against bacteria of food importance.

Fractions	IC50 µg/ml	
	Escherichia coli	*Staphylococcus aureus*
F1	6.57	NI
F2	306.7	224.3
F3	604.4	NI
F4	796	NI
F5	8.8	16.17
F6	31.08	61.73

*NI – No inhibition

Table 3 - Inhibitory potential of conventional antibiotics against bacteria of food importance.

Antibiotics	IC50 µg/ml	
	Escherichia coli	*Staphylococcus aureus*
Vancomicina	33.51	ND*
Cefalexina	8.16	11.69
Cloranfenicol	ND*	4.67

ND – Not determined

4. CONCLUSIONS

The heat-induced autolysis of *Saccharomyces cerevisiae* originated an AMP-rich extract with potential for application in food preservation. The extract effectiveness is intensified when autolysate is subjected to ultrafiltration followed by gel filtration, giving rise to < 10 kDa peptide-rich fractions with superior or similar antimicrobial activity compared to conventional antibiotics. Additional experiments are in progress to test other bacterial species, assess the toxicity of obtained fractions and identify the peptides responsible for antimicrobial activity.

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