

**ANTIMICROBIAL ACTIVITY OF EXTRA CELULAR PROTEIN
DERIVED FROM SIX ISOLATED THERMOPHILIC BACTERIA**

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Abstract

Antibiotics are metabolic by-products of complex biosynthetic pathways in microorganisms, some of which are proteins or peptides, known for their bactericidal and/or fungicidal effect on microorganisms. This Antimicrobial Peptides (AMP) produced by certain bacteria are toxic for other bacteria or for fungi but not for mammalia. The aim of this study was to investigate the antimicrobial activity of protein derived from six isolated thermophilic bacteria (113a, 94b, 153, 104c, 83, 110a). Bacteria was fermented in NB medium with 1% glucose in 55°C for 24 hr. Protein was isolated from cell-free extract by salting out method with 60% ammonium sulphate followed by dialysis. Antimicrobial activity was tested by growing several pathogenic microbes (*Eschericia coli*, *Staphylococcus aureus*, and *Candida albican*) in NA and PDA medium and then layering the papper disk with protein extract onto the top of those pathogenic microbes. Protein which exhibits antimicrobial activity was shown by the presence of a clear zone around the papper disk. The result of this study showed that almost protein derived from six isolated thermophilic bacteria demonstrate antimicrobial activity to *Eschericia coli*, *Staphylococcus aureus*, and *Candida albican*, except the ones isolated from thermophilic bacteria no 104c and 83, which have no antimicrobial effect to *Eschericia coli*.

Key words: *thermophilic, bacteria, antimicrobial peptide*

INTRODUCTION

Microorganisms develop various ways to increase their defense against drugs, causing what is called as drug resistance. They can transfer their genes horizontally (plasmid, transposon and bacteriophages), incorporate the host foreign DNA to their chromosome (in bacteria) and cause a mutation on different bacterial locus (Alam M. J. at al., 2013). There is a growing incidence of cell resistance against antibiotics, antiviral and antifungi drugs. Indeed, some therapeutic drugs have shown an inadequacy to cope with medical problems related to this cell resistance. Making human body more susceptible towards mutant pathogens that no drugs can treat, drug resistance has become a crucial problem around the world (Strobel G and Daisy B, 2003).

Nevertheles, some natural products demonstrate great potencies to be developed as therapeutic agents to overcome these resistant problems (Fawzy G.A et al., 2011; Strobel G, et al., 2003). A large number of drugs have been developed in medicinal practice from natural products. Natural resources, such as plants, microorganisms, vertebrates and invertebrates, are valuable source of bioactive compounds (Amador et al. 2003). One of which is bacteria, the inexhaustible source of chemical compounds, produce a wide variety of biologically active

secondary metabolites. For example is marine bacteria, from which a large number of bioactive compounds are developed and nowadays they become essential target for biotechnology industry. The bioactive metabolites may be produced as a response to ecological pressures, i.e. competition, predation deterrence, and reproduction (Gómez et al., 2010).

The success and discovery of penicillin has opened such opportunities to utilize structurally diverse bioactive agents derived from various microorganisms as antibiotics. Thereafter, we have seen the discovery of other antibiotics synthesized from microbial metabolites such as erythromycin, streptomycin, amphotericin and polyketides (Phonnok S, et al., 2010). The antimicrobial peptides, produced by many kinds of microorganism like bacteria, are involved in innate immunity, representing host defence effector molecules. However, there should be a great awareness that there are principal differences between microbial and mammalian cells that may represent distinct targets for antimicrobial peptides.

Moreover, the development of antifungal peptides from bacteria as therapeutic agent also provides a great challenge. Hospital reports anywhere has stated that the fourth most common cause of nosocomial infection is now the major human fungal pathogen *Candida albicans*. Mortality rates associated with systemic fungal infection are close to 50 %, with rates reaching 100 % for some fungal pathogens in the developing world. In clinical practice, the current applied antifungal treatments seems fail because several reasons such as enormous toxic side effects, poor efficacy, and drug resistance. Until now, most research only focuses on the activity of antimicrobial peptides against bacteria. Although some study demonstrated that several antimicrobial peptides specifically target fungal cells and are not active against bacteria. Others with broader specificity often have different mechanisms of action against bacteria and fungi. Therefore, understanding the mechanism of anti fungal actions to investigate the potential use of innate immunity peptides as novel therapeutics against fungi is of great interest for human (van der Weerden et al., 2013).

The objective of this study was to investigate the antibacterial and antifungal activities of the protein extracellular extracts derived from six isolated thermophilic bacteria of Gendol river after Merapi eruption in 2010.

RESEARCH METHOD

This study was designed as an exploration experiment. The objects were thermophilic bacteria isolated from water and mud of Gendol river, Merapi volcano. We synthesized protein from cell-free extracts of these bacteria and use it to test its effects against some pathogens which were *E coli*, *Staphylococcus aureus* and *Candida albicans*. We employed materials and tools as follow: isolated thermophilic bacteria, isolated pathogenic bacteria and fungi, microbial test disk blank, medium NB, NA, PDA, PD Broth, aquades, polyachrylamid, SDS, protein marker, ammonium sulfat, glucose 1%, LAF, Flash culture bottle, conicle tubes, pipet, ose, petri dish, drygalski, micropipet, blue tip, tissue, erlenmeyer tubes, incubator, analytic balancer, sentrifuge, electrophoresis tank, dialysis pocket, spectrofotometer, and refrigerator.

Antibiotic production

Preparation

The inoculum was grown on a 100 ml of Nutrien Broth medium. Briefly, the medium was prepared in a 250 ml of erlenmeyer tube and autoclaved at 121°C and 15 psi pressure for 15 mins. The thermophilic bacteria was grown on a slope nutritious agar and incubated at a temperature of 55°C for 24 hours. Reisolation was performed to create a new culture, using a sterile ose to incubate again at the same temperature for another 24 jam. Then, it was moved to NB medium by using 1 ose.

Batch fermentation

A 1% glucose in 1,5 L of aquadest was added to NB media, creating a production medium then

was sterilized in a 250 ml Erlenmeyer. The 10% inokulum was put onto the medium and incubated again at 55°C for another 24 hours. Then, the sample was removed into a 15 mL of conicle tube and centrifuged for 30 minutes at 3000 rpm to get a cell-free supernatant.

Isolating and Purifying Protein

The protein from the supernatant was collected by using a 60% of ammonium sulfat, followed by dialysis to get rid of the remnant ammonium sulfat. The protein profile was then checked with SDS PAGE.

The Antimicrobial Test

1. Antifungal effect

A series of dilution was performed on the protein to get concentration of 100%, 80%, 60%, 40% and 20%. Afterward, a 10 µl sample was dipped onto a 5 mm paper disk. Meanwhile, the sterile 20 mL of PDA was kept hardened to be inoculated with a 100 µl of *Candida albicans* and put aside for 30-60 minutes. Afterwards, the previous paper disk with protein was layered and we waited for another 24 hours at a temperature of 37°C to measure the barrier zone.

2. Antibacterial effect

The same preparation as above, only this time the protein in paper disk was tested on a 20 mL of sterile NA inoculated with 100 µl of *E coli* and *Staphylococcus aureus*. The same measurement as above was also employed.

RESULTS AND DISCUSSION

The results showed that each protein demonstrates different effects against the three tested pathogens as seen in Table 1. These data showed that protein derived from each isolate have various anti bacterial and anti fungal actions. However, the protein derived from isolate number D83 dan D104c showed no anti bacterial effect against *E coli*, while protein from other isolate demonstrated antimicrobial activity with different clear zone result. The result of antimicrobial activity derived from 6 isolated thermophilic bacteria was presented in these figures number 1 to 6 below.

Table 1. Antimicrobial effect of extracellular protein derived from the 6 isolated thermophilic bacteria

Isolate Number	Protein concentration (µg/µl)	<i>Staphylococcus aureus</i>		<i>Eschericia coli</i>		<i>Candida albican</i>	
		Average clear zone (mm)	Average inhibition zone (mm)	Average clear zone (mm)	Average inhibition zone (mm)	Average clear zone (mm)	Average inhibition zone (mm)
113a	0,94	7,2±0,2	2,2	6,2±0,1	1,2	8,4±0,6	3,4
	0,75	7,1±0,4	2,1	6,5±0,2	1,5	8,3±0,3	3,3
	0,56	7,0±0,4	2,0	6,3±0,2	1,3	8,2±0,5	3,2
	0,38	6,6±0,1	1,6	6,2±0,1	1,2	7,8±0,1	2,8
	0,19	6,5±0,2	1,5			7,1±0,1	2,1
94b	0,85	7,2±0,2	2,2	8,0±0,2	3,0	8,8±0,6	3,8
	0,68	6,9±0,1	1,9			8,7±0,1	3,7
	0,51	7,0±0,2	2,0			7,7±0,1	2,7
	0,34	6,7±0,3	1,7			7,0±0,1	2,0
	0,17	6,5±0,1	1,5			7,0±0,3	2,0

153	0,67	7,2±0,3	2,2	6,9±0,1	1,9	9,9±0,1	4,9
	0,54	6,6±0,4	1,6	7,4±0,1	2,4	9,2±0,5	4,2
	0,4	7,4±0,6	2,4	7,9±0,1	2,9	8,5±0,3	3,5
	0,27	7,1±0,1	2,1	9,7±0,3	4,7	8,2±0,2	3,2
	0,13	6,6±0,1	1,6	8,0±0,3	3,0	8,0±0,6	3,0
104c	0,33	7,3±0,1	2,3			8,2±0,2	3,2
	0,26	7,3±0,1	2,3			8,1±0,4	3,1
	0,19	7,4±0,1	2,4			7,6±0,3	2,6
	0,13	7,2±0,3	2,2			7,1±0,1	2,1
	0,07	7,0±0,2	2,0			6,4±0,1	1,4
83	0,63	6,3±0,2	1,3			8,7±0,3	3,7
	0,5	7,5±0,1	2,5			7,4±0,3	2,4
	0,38	6,7±0,3	1,7			7,2±0,7	2,2
	0,25	6,6±0,1	1,6			6,9±0,1	1,9
	0,13	6,4±0,2	1,4			6,7±0,1	1,7
110a	0,77	8,9±0,5	3,9	8,2±0,4	3,2	7,8±0,4	2,8
	0,62	8,9±0,5	3,9			8,1±0,3	3,1
	0,46	8,8±0,5	3,8			7,6±0,1	2,6
	0,3	8,6±0,1	3,6			7,7±0,3	2,7
	0,15	8,3±0,4	3,3			7,4±0,2	2,4

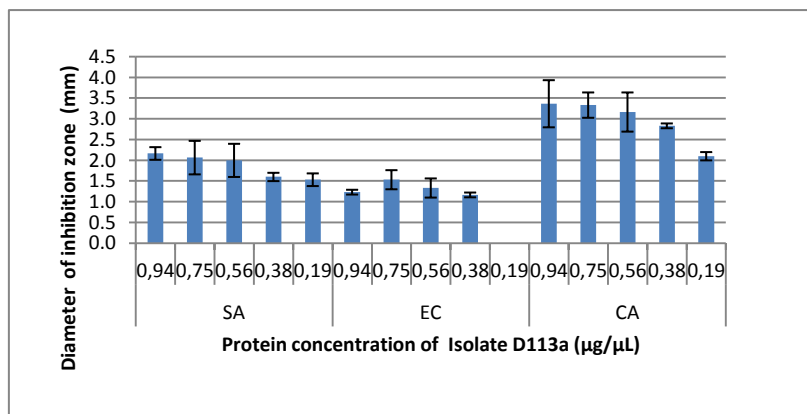


Figure. 1. The diameter of inhibition zone demonstrated by protein from isolate number D113a

The graph number 1 shows that protein derived from isolate number D113a possesses a minimum inhibitory concentration (MIC) against *S aureus* and *C albican* of 0,19 µg/µl respectively and against *E coli* of 0,38 µg/µl. Whereas, figure number 2 demonstrated that protein fermented from isolate number D94b has a minimum inhibitory concentration (MIC) against *S aureus* and *C albican* of 0,17 µg/µl respectively and against *E coli* of 0,85 µg/µl.

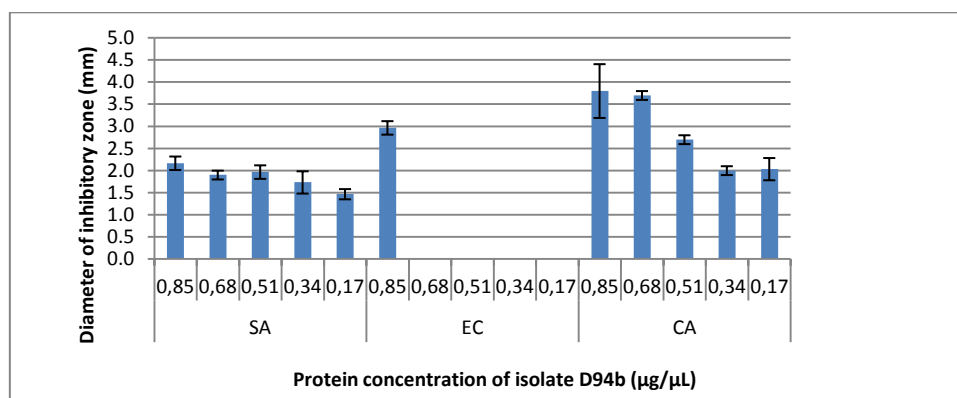


Figure. 2. The diameter of inhibition zone demonstrated by protein from isolate number D94b

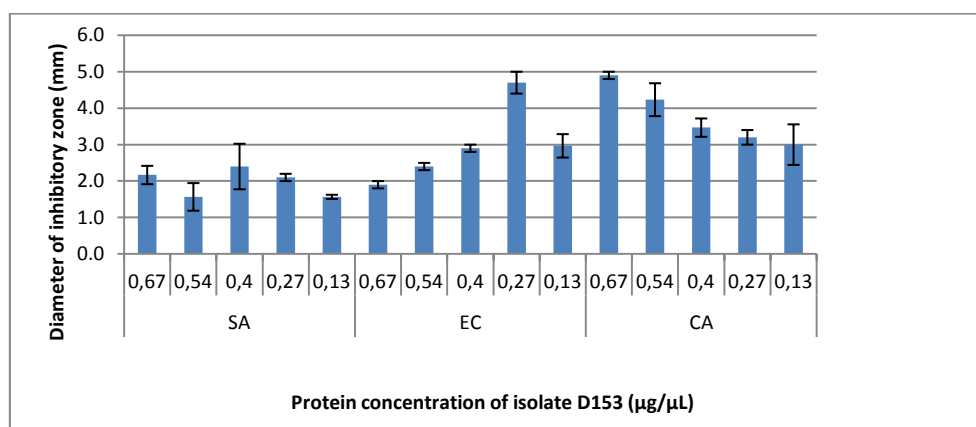


Figure. 3. The diameter of inhibition zone demonstrated by protein from isolate number D153

The above figure demonstrates that the protein isolate number D153 has a minimum inhibitory concentration (MIC) against *S aureus*, *E coli* dan *C albican* of 0,13 µg/µl respectively. Meanwhile, the figure below number 4 shows that protein isolate number D104c has a minimum inhibitory concentration (MIC) against *S aureus* dan *C albican* of 0,07 µg/µl respectively but not against *E coli* .

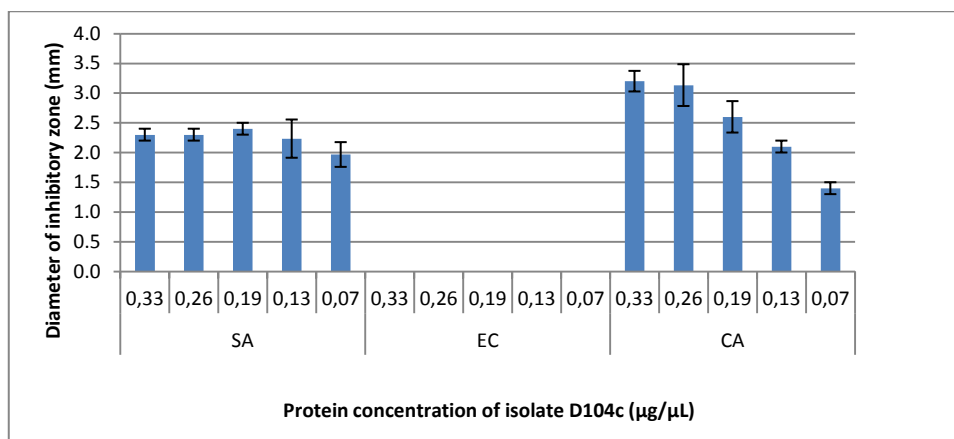


Figure.4. The diameter of inhibition zone demonstrated by protein from isolate number D104c

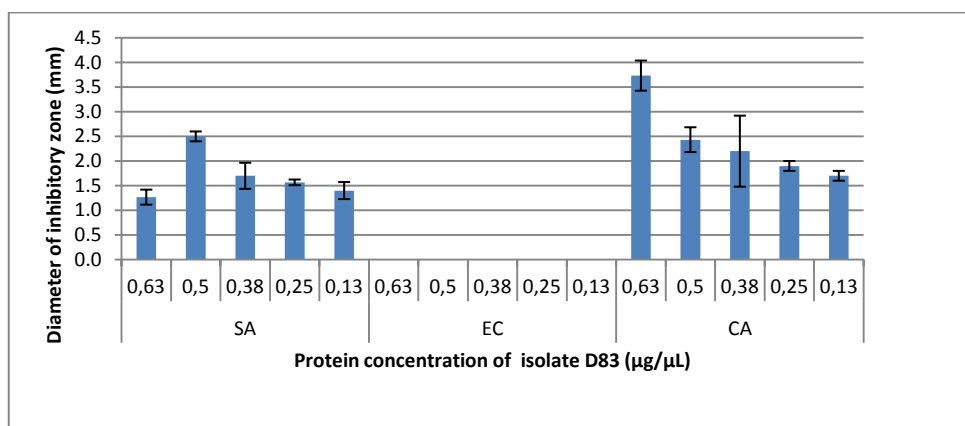


Figure.5. The diameter of inhibition zone demonstrated by protein from isolate number D83.

The figure 5 above displays the activity of protein isolate number D83 against *S aureus* and *C albican*, showing a minimum inhibitory concentration (MIC) of 0,13 µg/µl respectively but did not have potency as an antibacterial agent against *E coli*. This figure 6 below shows that protein isolate number D110a has a minimum inhibitory concentration (MIC) against *S aureus* and *C albican* of 0,15 µg/µl respectively and against *E coli* of 0,77 µg/µl.

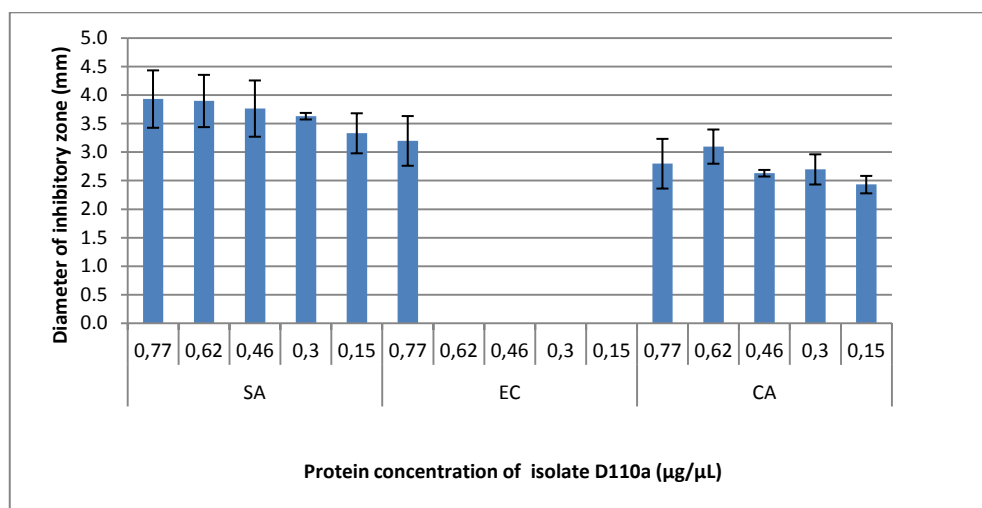


Figure.6. The diameter of inhibition zone demonstrated by protein from isolate number D110a.

The protein was coagulated with ammonium sulfate and purified with dialysis before they were tested with SDS page.

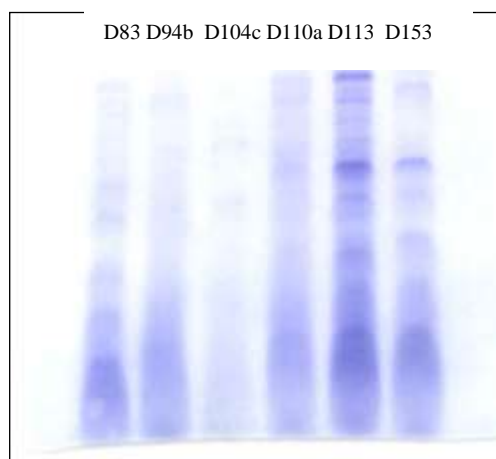


Figure 7. The results of SDS PAGE test on the protein derived from the 6 isolates

Discussion

The results showed different antimicrobial effect of each protein isolate against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albican*. Protein isolated from six thermophilic bacteria have antimicrobial activity to *Escherichia coli*, *Staphylococcus aureus*, and *Candida albican*, except the isolates number 104c and 83, which have no antimicrobial activity against *Escherichia coli*. The antimicrobial capability of these extracellular protein is due to the presence of Antimicrobial Peptides (AMP) produced by a fermentation process of each isolates in NB medium with 1% glucose. Several studies had demonstrated that AMP, either in the form of synthetic or natural, is capable of improving the immunity system and therefore is considered as a potent antibiotic. The later effect is caused by a strong electrostatic bond between the negative charge of bacteria and the positive charge of AMP. When a critical concentration of

AMPs accumulate on the microbial surface, intercolation and assembly of their hydrophobic faces within the cytoplasmic membrane leads to formation of ion channels or aqueous pores, leading to microbial death through hypoosmotic lysis (Gutsmann *et al.*, 2001; Oren *et al.*, 1999). In bacteria, the principal site of AMP action is the cytoplasmic membrane, therefore the result is a disruption to the bacterial cell membrane.

Almost AMP can kill either negative or positive gram Gram-positive bacteria lack an outer membrane, but possess a thick cell wall composed of heavily crosslinked polymers of teichoic or lipoteichoic acids and peptidoglycan. Gram-negative bacteria do not produce teichoic acids, but have a multilayered surface structure including a peptidoglycan matrix in the periplasmic space beneath an outer membrane. This outer membrane contains the bound complex of lipid A, core polysaccharide and specific side-chain (O) polysaccharides known as lipopolysaccharide (LPS) (Nizet N, 2011). These antimicrobial effects are due to the ability to neutralize the lipopolysaccharide (LPS) therefore the extracellular protein ability against negative or protein gram bacteria was distinctive (Hoskin, D. W. and Ramamoorthy, A., 2008). Furthermore, some bactericidal peptides have the cancer and anti viral activity due to their effects in improving the adaptive immune system

On the other hand, some studies have shown that some peptides can cause cell death without significant membrane perturbation. Thus, it is proposed that CAMPs can be divided into two functional groups: membrane-disruptive and membrane-nondisruptive peptides (Powers & Hancock, 2003). However, this grouping is not completely clear, since peptides that attack membranes of some species may be membrane-nondisruptive in other species. Furthermore, many peptides have a multifunctional role, affecting both cell membranes as well as internal targets. Whatever the actual target of peptides, the ability to interact with lipid bilayers is crucial for their action. Whether the final target of a peptide is the cell membrane or some intracellular component, it has to find its way through the cell envelope (Yount *et al.*, 2006).

The mechanism of action of antifungal peptides involves membrane permeabilization/translocation, inhibition of cellular processes and formation of reactive oxygen species (ROS) in the fungal species. AMPs have been known to translocate across the cell membrane and exert antimicrobial action by affecting macromolecular processes such as protein, nucleic acid and cell wall synthesis and enzyme activities. The formation of ROS has been suggested to play a pivotal role in the fungicidal activity of most of the antifungal peptides. (Pushpanathan M, et al., 2013).

CONCLUSION AND SUGGESTION

It can be concluded that almost protein derived from six isolated thermophilic bacteria demonstrate antimicrobial activity to *Escherichia coli*, *Staphylococcus aureus*, and *Candida albican*, except the ones isolated from thermophilic bacteria no 104c and 83, which have no antimicrobial effect to *Escherichia coli*. Further studies are needed to explore the working mechanisms of these AMPs we found and to investigate the other potential effects.

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