Antimicrobial and Anticancer Activity of a Novel Peptide (Musterolysin)
Extracted from Slurry of Mustard Oil Refinery Industry

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Abstract: Bacterial infections and cancer has been most prevalent diseases throughout the world. The discovery and development of new antibacterial and anticancer agents is critical. If such a valuable medicinal product is created from waste resources from the oil refinery business, academicians and industrialists will pay close attention. The sequential extraction process followed by RP-HPLC fractions was used to purify and identify a new peptide from mustard oil refinery sludge. The sequence of the peptide was determined as NH₂-KYQFFVP-COOH using MALDI TOF/TOF Mass spectrometry. The antibacterial activity of the peptide was determined against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, and Bacillus subtilis. The proapoptotic activity of the peptide was also determined against Hep2 (Human epidermoid cancer cell line). These findings suggest that readily available bio-waste might be a novel source of bioactive antibacterial and proapoptotic lipopeptides.

Keywords: Antibacterial activity, bioactive lipopeptide, mustard oil slurry, proapoptotic activity.
Introduction

Recently, the waste generation has grown fast in proportion to the world population, yet individuals in contemporary society have little propensity to regulate or recycle it. The exploitation of waste resources and energy-saving materials is critical, as is the creation of a suitable technology to recover valuable chemicals or materials from waste resources. The exploitation of waste materials and their conversion into valuable products has recently gained attention [1, 2]. As the human population grows, so does the need for consumer goods and consuming materials. The high production costs constrain the commercial apprehensions of consumer items or healthcare treatments. High manufacturing costs can often be minimized by utilizing innovative technology. The high production costs sometimes can be reduced using new technologies of novel and efficient multistep downstream process of the cheaper renewable substrates [3, 4].

The production of lipopeptides or biosurfactants and their application as healthcare products from waste resources are not commercialize yet. There are not available technologies to isolate and purify any product of medicinal value from oil refinery slurry [5, 6]. Only few reports are available for the proficient utilization of biomass resources in biorefineries [7, 8, 9]. Another sustainable technology to convert the biomass into other bio-based chemicals is trying to be implemented [10, 11]. A new technology have been developed to produce poly-(γ-glutamic acid) by Bacillus subtilis NX-2 using as substrate of rice straw utilization [12]. Bio-oil toxicity has been reduced by the microalgae Chlamydomonas reinhardtii in acetic acid rich pyrolytic bio-oil industry [13].

Several biosurfactant manufacturing strategies have been enhanced by recombinant and mutant hyper-producer microbial strains using less expensive renewable substrates. We devised a novel downstream process to purify a lipopeptide for medicinal applications such as proapoptotic and antibacterial properties.

Materials and methods:
Extraction and purification of peptides:

The whole solvent extraction technique is detailed in Figure 1 as a schematic figure, and the peptide yield was 0.0137%. The yellow seed variety (Vinay B-9) that is commonly accessible in West Bengal was primarily used in the mustard oil refinery industry. The lipopeptides were isolated using the method reported previously [14]. In brief, the extracted lipopeptides were dissolved in methanol containing 1% TFA (trifluoro acetic acid) and fractionated by reverse phase- HPLC (Agilent 1100...
series, CA, USA) with a ZORBAX 300-SB18 column (4.6 mm × 250 mm, particle size 5 μM), at a flow rate of 1 ml/min. The solvent system used was (A) 0.1% aqueous TFA and (B) acetonitrile containing 0.1% TFA following a gradient method and monitored at 215 nm (Figure 2a). Peaks were collected using a fraction collector (GILSON, France) and concentrated by speed vacuum, tested for their antimicrobial and anticancer activities. The concentrated fraction was re-chromatographed using 100% acetonitrile with a gradient of 0-10% for 30 min (Figure 2b).

**MALDI TOF MS analysis**

The purified peptide was characterized using Matrix-assisted laser desorption ionization (MALDI) Time-of-Flight (ToF)/ToF Ultraflextreme mass spectrometry (BRUKER DALTONIK GmbH Life Sciences Fahrenheitstr. 4, D-28359 Bremen Germany). The peptide was re-suspended in methanol/TFA solution. Four microliter solution was mixed with 4 μL of matrix (CHCA, 10 mg/ml), 1.0 μL of this mixture solution was spotted onto the MALDI 100 well stainless-steel sample plate and allowed to air dry prior to the MALDI analysis [15]. The spectra were recorded in positive ion linear mode. To check the reproducibility of the spectrum, sample was separately spotted several times [16]. For peptide MS/MS sequencing, lipopeptide was incubated with 10% NaOH in methanol at room temperature for 16 h to cleave the lactone ring. The cleaved lipopeptide was lyophilized, again extracted with methanol and allowed for mass spectrometry analysis. The spectra were recorded in the post-source decay (PSD) ion mode as an average of 100 laser shots with a grid voltage of 75%. The reflector voltage was reduced in 25% steps and guide wire was reduced 0.02–0.01% with an extraction delay time of 100 ns [17].

**Antibacterial activity**

The lyophilized peptide fractions were dissolved in 0.1% aqueous TFA and test their antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis* (Table 1). Antimicrobial activity was tested in a microliter plate following CLSI guidelines (CLSI, 2010). MIC was determined at the lowest concentration of compound inhibiting the growth of test strain without showing any turbidity [18, 19].
**Pro-apoptotic activity**

Hep2 (*Human epidermoid cancer cells*) were obtained from the National Centre for Cell Sciences (Pune, India). The cells were cultured as monolayers in MEM (Minimum Essential Medium) with 10 % (v/v) FBS and antibiotics. The cells were incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂. Cells were plated and allowed to attach to the culture surface overnight and next day, the broth was aspirated off. Immediately, 200 µL of peptide-containing medium (with varying concentration) were added separately in triplicates. After 72 h incubation, MTT assay was performed using the protocol described earlier [20, 21, 22, 23].

**Cell cycle analysis**

After treatment, cells were harvested after washing with PBS(1X) and treated with 70 % ethanol for 45 min at 4°C in overnight for fixation. Cells were centrifuged and washed gently PBS buffer, incubated with propidium iodide solution (40 µg.ml⁻¹ PI in PBS) at 37 °C for 1 h. Apoptotic cells were determined by their hypochromic sub-diploid staining profiles. The percentage of cells in the different phases of cell cycle was calculated from the DNA histogram using Becton-Dickinson FACS Calibur flow cytometer and Cell Quest Pro software (Figure 4) [24].

**Results and discussions**

Both, cancer and microbial infections are the major cause of death throughout worldwide. Chemoprevention is the most promising approach aimed to reduce the rate of mortality [25]. Several chemotherapeutics molecules have been identified from natural source and synthetically derived, however most of them are not so effective neither cancer preventive nor anti-infictive. Therefore, search for new molecules with novel mechanism of actions to arrest the cell cycle, induce apoptosis and immunostimulation are more fascinating in current scenario.

Nowadays, the recycling of industrial waste and purification of bioactive molecules from agricultural residue is most challenging. The agro-industrial wastes are used as substrate in solid state fermentation for manufacturing of biofuels, enzymes, vitamins, antioxidants, animal feed, antibiotics, and other chemicals [26]. Here, the process was optimized for the production of a novel proapoptotic and antibacterial peptide from sewage of oil refinery industry, may be an effective measure for the formation of value-added health-care products.
The extraction and purification protocol was described in Figure 1. The purified peptide showed the antimicrobial activity against few tested pathogens and mostly active against Gram (+) ve bacteria (Table 1). The IC$_{50}$ value was calculated as 1.5 µM. The cell cycle analysis was performed at 1.5 µM and revealed that apoptotic cells number increased at M1 phase up to 48%. The peptide obtained by RP-HPLC chromatography used to determine molecular mass by MALDI-TOF analysis. The peptide showed a molecular mass of 1258.19 Da (+1) with a distinct difference of m/z 44 and sometimes m/z 42, suggesting the presence of aliphatic fatty chain. The MS/MS analysis revealed the lipopeptide sequence as KYQFFVP (Figure S1) and the sequence of fatty acid chain showed at Figure 3. The primary structure elucidation from the obtained mass spectrometry data is given in Figure 3d and named musterolysin.

The amino acid content, chemical structure, folding, and self-assembly of antimicrobial peptides all influence their antimicrobial activity [27]. Instead of relying on their individual amino acid components or three-dimensional designs, some antimicrobial peptides rely on their interfacial action [28]. Such peptides interact physically and chemically with membrane triggers to destabilize and make the membrane more permeable, which results in cell death. They also create pores in the membrane, which might result in a cell dying quickly. Due to the existence of a long hydrophobic chain in this instance, the peptide is more hydrophobic, which may have fast attachments to the membrane and may follow the same mode of action. As like as antimicrobial peptides, anticancer peptides are also target membrane alterations of cancer cells, which is crucial for a cell to become malignant. When peptides disrupt the membrane of a cancer cell, it inhibits functions such as motility, tissue invasion, and metastasis.

Thus, the novel bioactive molecule from cheaper renewable substrates and their efficient application may sustain the commercial apprehension from the high production costs. In this way, the waste of oil refinery slurry can be valorized. By applying the value adding opportunities this peptide may be used in relation to their biological applications such as antibiotic, antifungal, and antitumor agent. In addition to that it may also be considered as insecticide, antiviral, immunomodulators, and enzyme inhibitors after proper trial.

Conclusions

To explore the utilization of waste material and to search for bioactive healthcare molecules, a novel peptide was identified with the sequence of NH$_2$-KYQFFVP-COOH from oil refinery slurry. The
peptide showed significant antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *B. subtilis*. It also showed good proapoptotic efficacy against Hep2 cancer cells. The purified peptide, musterolysin may be the future chemotherapeutic drug for antimicrobial and anticancer therapy.

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**References:**


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FIGURE CAPTIONS

**Figure 1.** Extraction and purification of lipopeptides from the waste slurry of mustard oil refinery industry. Schematic representation of extraction procedure.

**Figure 2.** HPLC separation of lipopeptide through 300SB-C18 column (Fig. 2a). Re-chromatograph of active fraction through different gradient procedure (Fig. 2b).

**Figure 3.** MALDI TOF/TOF MS analysis of the lipopeptide. The molecular mass was determined of the whole peptide (Fig. 3a), MS/MS analysis of the lipid part of the peptide (Fig. 3b); amino acid sequence was determined from MS/MS analysis (Fig. 3c) and structure determination from MS/MS analysis (Fig. 3d).

**Figure 4.** Determination of cell viability against human breast cancer cell line, Hep2 (Human epidermoid cancer cells) (Fig. 4a). Cell cycle analysis using flow cytometer of Hep2 (Human epidermoid cancer cells) (Fig. 4b), and treated with peptide (Fig. 3d). Cells were stained with propidium iodide.

**Table 1.** Determination of MIC values against different bacterial strains (Table 1).
Figure 1

**Schematic diagram of extraction and purification of peptide:**

1. Mustard oil refinery slurry (20g) → Add n-hexane (40 mL) and vortexed → Add ACN containing 0.1% TFA (20 mL), vortexed and centrifuged (5000 g at 4°C for 30 min)
2. HCl phase separated and neutralized with 3(N) NaOH adding dropwise, white ppt appeared, centrifuged (5000 g at 4°C for 30 min), taken the ppt.
3. ACN phase separated and mixed with 3(N) HCl (equal volume), vortexed and centrifuged (5000 g at 4°C for 30 min)
4. Ppt. dissolved into ACN containing 0.1% TFA soln (1 mL), Filtered through 0.22 µM filter. → Inject into HPLC, separated with zorbax 300SB C-18 column, solvent used 0.1%TFA in ACN and 0.1%TFA in water, monitored at 215 nm.
   → Fractions were collected and lyophilized.
Figure 2

a)

b)
Figure 3
Figure 4

(a) (b) (c)

Table 1:

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC Value (µM/mL)</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td>0.75</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1.5</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.75</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>0.375</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>0.375</td>
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