ASSESSMENT OF ANTIMICROBIAL ACTIVITY FOR THE SMALLER CHAIN DIPEPTIDES AND TRIPEPTIDES

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Abstract: Disc diffusion method was employed to determine the antimicrobial effect of test compounds I, II and III (Trp-Phe-Asn, Gly-Pro, Gly-Asn) against eight microbial species viz., Staphylococcus aureus, Staphylococcus albus, Streptococcus fecalis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella aerogenes and Candida albicans. The disc was saturated with 100 μl of compound I and 200 μl of compound II and III, allowed to dry and introduced on the upper layer of seeded agar plate. The plates were incubated over night at 37° C. Microbial growth was determined by measuring the zonal inhibition diameters. Compound I showed maximum potency against gram positive S. aureus (28 mm) in comparison with standard Ciprofloxacin (40mm). Compound I was found to be highly sensitive against S. albus (20mm), S. fecalis (16 mm) and E. Coli (15 mm). Compound II shows good potency against S. aureus (13 mm) and S. albus (10mm). Compound III was found to be moderately sensitive against S. aureus (8mm) and Proteus vulgaris (10 mm). Among all the compounds tested, none of them was found to be effective against the fungi Candida albicans.

Key words: Antimicrobial activity, Dipeptides, Tripeptides

INTRODUCTION

Some biologically important peptides have only a few amino acid residues. Such peptides show large biological effects. This can readily be illustrated by the activity of the commercially synthesized dipeptide, l-aspartylphenylalanyl methyl ester [1]. This compound is an artificial sweetener better known as aspartame or nutra sweet. Among naturally occurring small peptides or hormones such as thyrotropin-releasing factor (three residues) which is formed in the hypothalamus and stimulates the release of thyrotropin, from the anterior pituitary gland. The peptide thyrotropin releasing factor (TRF) has the structure pyroglutamyl histidyl prolinamide [2]. A variety of peptides are involved in the mammalian oxygen-independent antimicrobial defense mechanism. Defensins are a family of small (29-35 amino acids) arginine and cysteine rich peptides that have been isolated from a variety of mammals, including rats, rabbits, and humans [3,4].

Defensins are one of the largest and most studied families of antimicrobial peptides. Most defensins function by penetrating the microbial cell membrane by way of electrical attraction, and once embedded, forming a pore in the membrane, which allows efflux. An imbalance of defensins in the skin may contribute to acne [5]. Several studies show that antimicrobial peptides are effective against ocular surface pathogens in vitro [6]. A variety of peptides have been tested, including defensins, magainins (antimicrobial peptides originally isolated from the African clawed frog) [7]) and cecropins. The
peptides might also be used as disinfectants in contact lens cleaning and storage solution. Cecropin D5C, synthetic insect antimicrobial peptide, has been shown to augment the antimicrobial activity of contact lens solutions against *P. aeruginosa* [8].

The increasing prevalence of multi drug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection fighting strategies and new effective therapeutic agents [9]. Therefore, the search for smaller chain peptide based drug analogues for the treatment of infectious diseases has become necessary.

**MATERIALS AND METHODS**

**Materials:** Both gram positive (*S. albus, S. aureus, S. fecalis*) and gram negative (*E. coli, P. aeruginosa, P. vulgaris, K. aerogenes*) bacteria and the fungi *Candida albicans* obtained from National Chemical Laboratory, Pune, were maintained on agar slants at 12-18°C. Prior to testing, they were grown in nutrient agar medium and incubated at 37°C for 48 hours followed by frequent sub culturing to fresh medium, and were used as test bacteria and fungi.

Standard antibiotics ciprofloxacin and clotrimazole were procured from Cadilla Pharmaceuticals, Gujarat and Glenmark Pharmaceuticals, Mumbai. Sterile discs (6mm) were procured from Hi-media Laboratories Pvt. Ltd., Mumbai. All the solvents and regents were of Anala R grade and are used without purification. Glassware is oven or flame dried prior to use. Synthesized peptides are purified by flash column chromatography using 230-400 mesh silica gel supplied by Acme chemicals limited, Mumbai, India. The Purified Peptides are designated as test compounds I, II and III respectively as Trp-Phe-Asn, Gly-Pro and Gly-Asn.

**Antimicrobial Screening** [10,11]: The testing of antimicrobial activity of test compounds were carried out *in vitro* by Kirby-Bauer disc diffusion technique [12]. The disc-diffusion method is highly effective for rapidly growing microorganisms and the activities of the test compounds are expressed by measuring the diameter of the zone of inhibition [13]. The formation of inhibition zones represents the dynamic interaction between antibiotic diffusion and bacterial growth [14]. Generally, the more susceptible the organism, the bigger the zone of inhibition. The method is essentially a qualitative or semi quantitative test, indicating sensitivity or resistance of microorganisms to the test materials as well as bacteriostatic or bactericidal activity of a compound [15].

The antimicrobial activity of peptide based test compounds I, II and III were determined against three Gram-positive (*S. aureus, S. albus and S. fecalis*) and four Gram-negative (*E. coli, P. vulgaris, P. aeruginosa and K. aerogenes*) bacteria along with the fungi *Candida albicans*. Mueller Hinton Agar No.2 was used as an assay medium. Inoculum size was maintained as 1X10⁸ cells/ml. The media and the test bacterial cultures were poured in to petri dishes (Hi-media). The test strain (200 ml) was inoculated in to the media when the temperature reached 40-42°C. The test compounds I, II, and III (100ml, 200ml and 200ml) were impregnated in to sterile discs (6mm) and then allowed to dry. The disc was then introduced in to medium with the bacteria. The plates were incubated overnight at 37°C. Microbial growth was determined by measuring the diameter of zone of inhibition in millimeters. For each bacterial strain, controls were maintained where pure solvents were used instead of the compounds [16].

The control zones were subtracted from the test zones and the resulting zonal inhibition diameter in millimeters is made and its size was compared to that contained in a standardized chart [17]. Based on this comparison, the test organism was determined to be resistant, intermediate, or susceptible to the antibiotic. The diameters of the zones of inhibition produced by the compounds were also compared with the standard antibiotic (ciprofloxacin 5mg/disc). The experiments were performed three times to minimize error. Similarly, for antifungal screening, sabouraud dextrose agar was used as an assay medium. Clotrimazole (5mg/disc) was used as the standard for antifungal susceptibility testing. The Procedure adopted was similar to antibacterial susceptibility testing except that, the plates were incubated at 28°C for 48 hours and the zone of inhibition was measured [18].

**RESULTS AND DISCUSSION**

The antimicrobial activity of test compounds I (Trp-Phe-Asn), II (Gly-Pro) and III (Gly-Asn) against the different Gram negative bacteria is demonstrated in figures A to D. *In vitro* antimicrobial activity of
Fig. A: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds I (Trp-Phe-Asn), II (Gly-Pro) and III (Gly-Asn ) against *E. coli*. Tripeptide (Trp-Phe-Asn) is highly sensitive among the tested compounds with half their diameter of zone of inhibition (± 15mm) in comparision to the standard ciprofloxacin (± 30 mm). CE and CC in all figures represent the Solvent control ethanol, and Solvent control chloroform respectively.

Fig. B: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds stated above against *P. aeruginosa*. Both the test compounds I & II are moderately sensitive with their respective zones of inhibition (± 9mm; ± 8mm) against the opportunistic pathogen *P. aeruginosa*.

Fig. C: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds stated above against *P. vulgaris*. Tripeptide (Trp-Phe-Asn) and the Dipeptide (Gly-Asn) lead compounds exhibits moderate sensitivity against *P. vulgaris* with similar zonal inhibition diameter (± 10 mm).

Fig. D: The disk represents the antibacterial activity with their zone of inhibition of the three test compounds stated above against *K. aerogenes*. Test compound II does not possess significant activity against this bacteria. Compound I & III are resistant to the same organism (± 2mm; ± 4mm). Only solvent controls are effective against these species.

CE and CC in all figures represent the solvent control ethanol, and solvent control chloroform respectively.
smaller chain dipeptides and tripeptides against eight clinically important microbial strains are also presented in table 1. Trp-Phe-Asn shows maximum potency against gram positive Staph. aureus with a zonal diameter 28 mm (standard 40 mm). The above test compound is also highly effective against S. albus (20 mm), S. fecalis (16 mm) and E. coli (15 mm). Test compound I shows moderate potency against gram negative P. aeruginosa (9mm) and P. vulgaris (10 mm) bacteria (Table 1).

Test compound II, Gly-Pro, showed good potency against the gram positive S. aureus (13mm) and S. albus (16 mm). However, it was found to be moderately sensitive against gram negative P. vulgaris (10 mm) bacteria. For details see table 1.

As evident from table 1, the test compound III, Gly-Asn, was completely devoid of activity against S. albus, S. fecalis and E. coli. A negligible activity was of this compound was recorded against gram negative P. aeruginosa (2 mm) and K. aerogenes (4 mm). All the test compounds (I, II and III) were ineffective against the fungi Candida albicans.

Among the peptides tested, tripeptide compound I (Trp-Phe-Asn) was found to be highly effective against gram positive organisms tested. As far as the gram negative organisms are concerned, only the test compound I exhibits maximum potency against E. coli (± 15mm) and test compound II (Gly-pro) shows moderately equipotent action against Proteus vulgaris and Pseudomonas aeruginosa (± 8 mm each). It was concluded that the compounds doesn’t possess antifungal activity at all. Despite the significant diversity of different antimicrobial peptides, it is generally accepted that the cationic peptides exert their antimicrobial activity just by having interaction electrostatically with negative charged compounds, particularly phospholipids, of microbial cell membranes, leading to increased permeability of the cell membrane and death. The relative selectivity of the peptides for microorganisms versus eukaryotic cells has been attributed to the low membrane potential, fewer negatively charged phospholipids, and high cholesterol levels of the latter [19,20].

The carpet model has been used to describe the mechanism of action of many antimicrobial peptides, including human b-defensins [21] and human cathelicidin [22]. In this model, the microbial membrane is first coated in a “carpet” of peptide. When the point of saturation is reached, the membrane collapses, creating “worm holes”, leading to lysis of the organism.

Moreover, the histatins are believed to reach their intracellular target, the mitochondria, by using an aggregate channel model [23]. Here the peptides cluster in to unstructured aggregates within the membrane, which causes the transient formation of channels through the membrane. These channels allow for the leakage of ions, and additionally, allow the peptides to cross the membrane and reach intracellular targets, where they exert their killing effect.

The a-defensins are thought to produce antimicrobial action by using barrel-stave model [24]. In this model, the peptide monomers associate with the membrane
and then assemble together to form a stable transmembrane pore. The pore size increases as more peptide monomers are added, and leakage of intracellular contents through the pores eventually leads to cell death.

By utilizing one of the approaches outlined above, antimicrobial peptides are able to kill gram negative and positive bacteria and some fungi. From the above observations and findings, it is believed that our test compounds (I & II) exert their antimicrobial effect similar to β-defensins mode of action as they possess few peptide linkages (one or two) only. With the emergence of new infectious diseases, as well as the appearance of microbial strains resistant to existing antibiotics, there is an enormous challenge to researchers to develop new methods for treating both existing infectious diseases and those emerging as new health threats [25]. Keeping this view in mind, we have assessed the biofriendly smaller chain peptides as antimicrobial compounds to surmount the problem of resistance of existing antibiotics. Since the test compounds I (Trp-Phe-Asn) and II (Gly-Pro) possess antimicrobial property against clinically important pathogens, they can be used as potent therapeutic agents after further investigations. The work in this respect is in progress.

REFERENCES