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Rice Dehydrin K-Segments Have *in vitro* Antibacterial Activity

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Abstract—Dehydrins are groups of plant proteins that have been shown to response to various environmental stimuli such as dehydration, elevated salinity, and low temperature. However, their roles in plant defense against microbes have not been demonstrated. In an attempt to discover plant antimicrobial proteins, we have screened a rice cDNA library and isolated several cDNAs coding for dehydrins. Protein extracts from *Escherichia coli* expressing these cDNAs were tested for their activity against Gram-positive bacteria (*Bacillus pumilus*, *B. subtilis*, *Staphylococcus aureus*, and *Sarcina lutea*) and Gram-negative bacteria (*Escherichia coli* and *Xanthomonas oryzae* pv. *oryzae*). The results indicate that the crude protein extracts exhibited antibacterial activities against the Gram-positive bacteria. However, dehydrins purified by immunoaffinity chromatography were not active against the bacteria. To pinpoint the dehydrin peptides that were responsible for the bactericidal activity, we expressed DNA sequences coding for truncated dehydrins containing either K- or S-segment and found that K-segment peptides, and not S-segment, were responsible for the antibacterial activities against Gram-positive bacteria. Antibacterial assay with synthetic K-segments indicated that the peptides inhibited growth of *B. pumilus* with minimum inhibition concentration and minimum bactericidal concentration of 130 and 400 μg/ml, respectively.

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Key words: dehydrin, antimicrobial peptide, antibacterial activity, K-segment, rice

Proteins are known to play important roles in plant defense against microbial invasions, either directly as antimicrobials [1-3] or indirectly as signal mediators in defense pathways or plant–microbe interactions [4, 5]. Identification and characterization of the proteins with antimicrobial activities would provide insight on complex mechanisms of plant defense and offer opportunities for improving plant disease resistance. In our attempt to identify novel antimicrobial proteins, we have screened a rice cDNA library constructed and expressed in *E. coli* and isolated a number of cDNA clones that have anti-*E. coli* activity [6]. Unexpectedly, among them several clones have been found to contain cDNAs coding for dehydrins, a class of ubiquitous plant proteins well known for their response to abiotic stresses such as drought, salt, and cold stress [7-9]. However, their role in plant defense has not been clearly demonstrated. A recent study shows that expression level of a dehydrin (GenBank accession No. U60097) increased up to 20-fold in rice plant after inoculation of a bacterial pathogen *X. oryzae* pv. *oryzae* [10], indicating that dehydrin may be involved in plant–pathogen interactions.

Dehydrins share a number of structural features. One of the most notable features is the presence of a continuous run of five to nine serines (S-segment) followed by a cluster of charged residues. A second conserved feature is the presence of lysine-rich segment(s) (K-segment). Inhibition of *E. coli* cells was reported when two dehydrins ERD10 and LEA4-D113 from *Arabidopsis thaliana* were expressed in the bacterial cells, and deletion study showed that the lysine-rich K-segments are responsible for the *in vivo* inhibition [11]. Present study confirms that truncated rice dehydrin inhibited growth of *E. coli*. Furthermore, using synthetic K-segments, we have demonstrated that the K-segment peptides from a rice dehydrin have *in vitro* antibacterial activity, particularly against Gram-positive bacteria. These results indicate that dehydrins, as well as their derivatives, may play important roles in plant defense against pathogenic infection.

MATERIALS AND METHODS

Screening of antimicrobial genes. A rice cDNA library was screened for antimicrobial genes as described

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earlier [6]. The inserts of the isolated clones were sequenced and blasted against the public database for gene identification. Several clones coding for dehydrins were identified and further characterized for their antibacterial activities in the present study.

Antibacterial assay. Bacterial strains used in this study are Gram-positive *B. pumilus*, *B. subtilis*, *S. aureus*, *S. lutea*, and Gram-negative *X. oryzae* pv. *oryzae* and *E. coli*. The strains were obtained from China Veterinary Culture Collection Center, except *X. oryzae*, which is a laboratory strain. The bacteria, except *X. oryzae*, were cultured in LB medium at 37°C and used for antibacterial assay as described earlier [6]. *Xanthomonas oryzae* was cultured on PDA (potato dextrose agar) medium at 25°C.

Cloning and modifications of dehydrin. To express full-length dehydrin, a cDNA coding for a SK-3 type dehydrin (clone RR46, GenBank accession No. EF444534) was cloned into *E. coli* expression vector pET28a as described earlier [12]. To construct truncated versions of the dehydrin containing either S-segment or K-segment, two dehydrin variants were made by PCR using two sets of primers. The variant containing S-segment (S-dehydrin) was amplified using forward primer 5′-GAATTCCATATGGAGGATGAGAGGAACACGG and reverse primer 5′-CGGGATCCTTAGCCGTTGTCATCGATCACC. This amplification generated a 329-bp DNA fragment whose coding region spans nucleotide 1-309 of the dehydrin, with a stop codon after glycine at a.a. 102. The DNA coding for the variant containing K-segment (K-dehydrin) was generated by PCR using forward primer 5′-GGAATTCCATATGGAGGTGATCGACGACAAC and reverse primer 5′-CGGGATCCTTAAGCGCTGCTCTTGTGC, which produced a DNA fragment of 612-bp coding for C-terminal half of the dehydrin (a.a. 96-291). Restriction sites *Nde*I and *Bam*HI were introduced as underlined in the primer sequences. The PCR products were digested with *Nde*I and *Bam*HI and ligated to pET28a that had been linearized by *Nde*I and *Bam*HI digestion. This generated two expression vectors with the mutant dehydrins fused to C terminal of thrombin cleavage site in pET28a. As such, the variant proteins contain a 6×His tag plus a thrombin cleavage site followed by histidine and methionine prior to the truncated native dehydrins.

Extraction of dehydrins expressed in *E. coli*. *Escherichia coli* cells (strain BL21(DE3) pLysS) carrying expression vectors for the dehydrin and its truncated variants were inoculated into LB medium with 50 mg/liter kanamycin. Isopropyl β-D-1-thiogalactopyranoside (ITPG) was added into the culture at final concentration of 1 mM when the optical density of the culture reached 0.6-0.8, and the cells were cultured for three more hours. Fifty-milliliter cell suspension samples were then collected by centrifugation, and the pellets were rinsed three times, each with 15 ml of sterile distilled water, by repeating the centrifugation steps. The washed cells were resuspended in 5 ml PBS buffer and sonicated on ice for 10 min with 5 sec on/off scheme using a JY92-II N disintegrator (Ningbo Scientz Biotechnology Co., Ltd.). The sonicated cells were spun for 10 min at 12,000 rpm at 4°C, and the supernatants were saved and used as crude protein extracts for antibacterial study. Dehydrin was further purified from the extracts under native condition using a Ni-NTA column according to the manufacturer's instruction (Qiagen, USA).

Determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). Three lysine-rich peptide fragments designated as K1, K2, and K3 following the S-segment in the SK3-type dehydrin were synthesized chemically using solid phase synthesis technology (HD Biosciences, China). They were used in antibacterial assay as described in the previous section, as well as MIC and MBC determination against *B. pumilus* as described [13]. All experiments were repeated three times.

RESULTS AND DISCUSSION

Dehydrins expressed *in vivo* inhibit *E. coli* growth. In an effort to isolate cDNA sequences with antibacterial activity, we screened a rice cDNA library constructed with *E. coli* plasmid pBluescript. The screening resulted in isolation of bacterial colonies whose cells were impaired due to expression of the cloned genes, which were visualized through a colony staining method [6]. Analysis of the cDNA sequences from the colonies isolated from the screen revealed unexpectedly that several clones contain cDNAs encoding for dehydrins, as well as

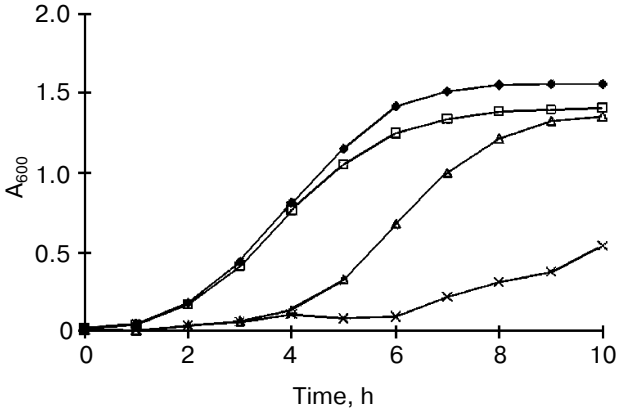

Fig. 1. Effect of overexpression of dehydrin protein on *E. coli* growth. Solid and open squares indicate the growth kinetics of *E. coli* cultures harboring empty vector pBluescript, without (solid square) and with (open square) addition of IPTG in the culture medium. Open triangle and star indicate the growth kinetics of *E. coli* cultures harboring vector pBluescript with full-length dehydrin clone RR46, without (open triangle) and with (star) addition of IPTG in the culture medium.

other well known antimicrobial genes [6]. When comparing the growth of *E. coli* cells harboring these genes under induction and non-induction conditions, we found that the growth of the *E. coli* cells (strain SOLR) was reduced as a result of the induced cDNA expression by IPTG, as shown in Fig. 1 with dehydrin clone RR46.

Dehydrins exist widely in plants, belonging to the LEA D-II family of late embryogenesis abundant (LEA) proteins [14]. They have been demonstrated to play protective roles in plant response to abiotic stresses such as cold and drought [15, 16]. However, their roles in plant defense against pathogens remain unclear. Recently, there is a report showing that dehydrins from *Arabidopsis thaliana* inhibited the growth of *E. coli* cells [11]. To analyze the antimicrobial activity of the dehydrins from rice, the clone RR46 (GenBank accession No. EF444534), which codes for an SK3-type dehydrin, was further investigated in the present study.

Dehydrins inhibit bacterial growth *in vitro*. We noted that the inhibition of *E. coli* growth, either observed in our experiments or reported otherwise, occurred when the dehydrins were synthesized within the bacterial cells. It was therefore interesting to see if the proteins would inhibit bacterial growth when applied from outside cells, like other antimicrobials. To investigate this activity, we expressed the full-length cDNA coding for the SK3-type dehydrin using an overexpression vector pET28a behind the His-tag. The recombinant plasmid was introduced into *E. coli* strain BL21(DE3)pLys. Expression of the recombinant plasmid was induced by IPTG, and recombinant dehydrin was synthesized in the strain [12]. The crude proteins from the *E. coli* cells were extracted after sonication, and used to test their activity against a panel of bacteria. As shown in Fig. 2, inhibition zones were visible around disks loaded with cell extracts from IPTG-induced *E. coli* cells on bacterial lawns from Gram-positive bacteria *B. pumilus*, *B. subtilis*, *S. aureus*, and *S. lutea*, but not from Gram-negative bacteria *X. oryzae* and *E. coli*. No inhibition zone was seen in samples loaded with extracts from non-induced cells or cells containing empty

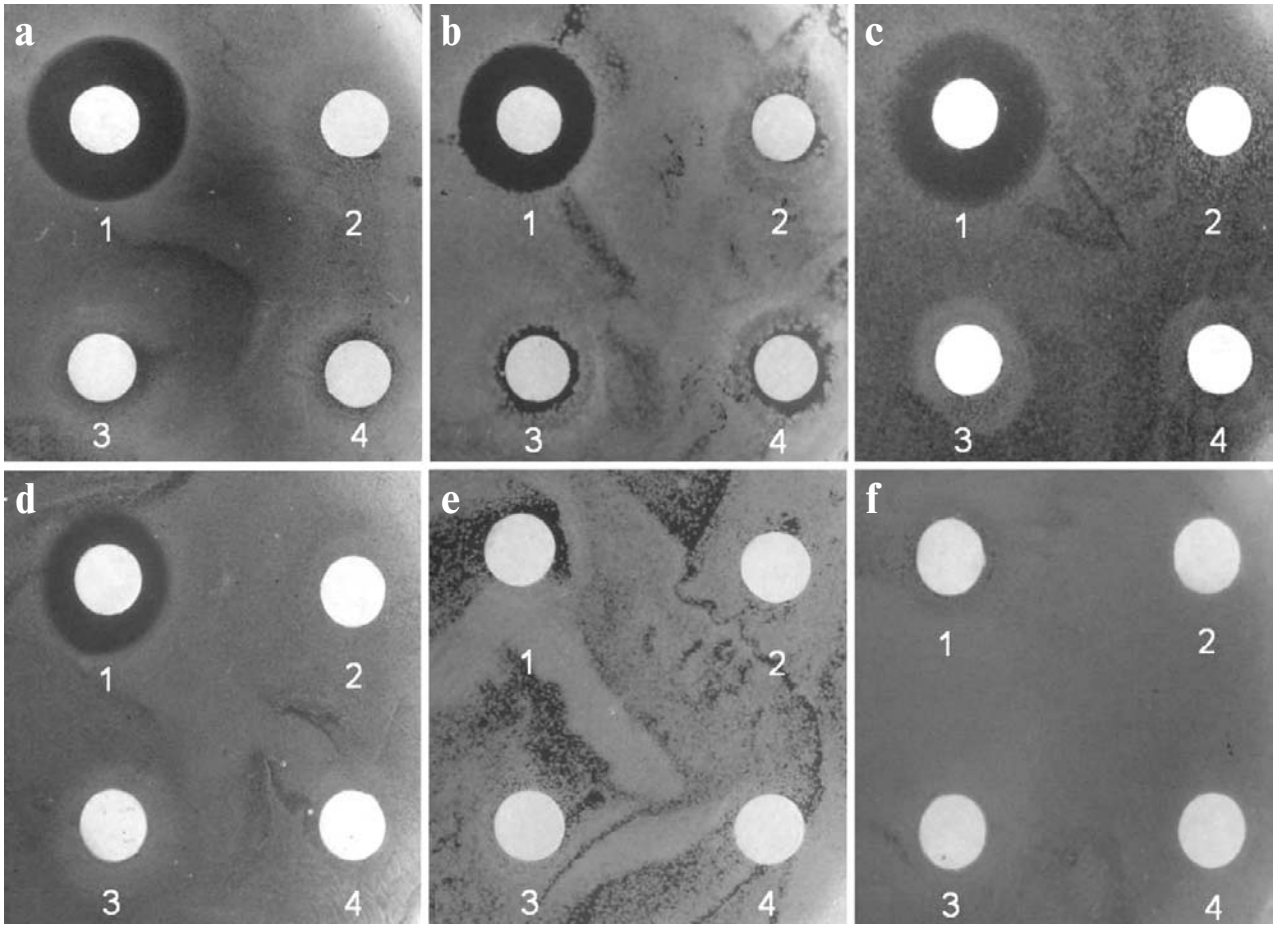

Fig. 2. Disc agar diffusion assay of cell lysates from dehydrin-expressing *E. coli* against Gram-positive and negative bacteria. Bacteria *B. pumilus* (a), *B. subtilis* (b), *S. lutea* (c), *S. aureus* (d), *X. oryzae* (e), and *E. coli* (f) were seeded on LB agar plates and cultured with paper discs loaded with 20 μl of the lysates from IPTG induced (samples 1 and 2) and non-induced (samples 3 and 4) pET28-dehydrin (samples 1 and 3) and empty vector culture (samples 2 and 4). The photos were taken after 12-24 h growing of the culture at 37°C.

vector. The results clearly indicated that the protein extracts containing the rice dehydrin could exert their antibacterial activity against Gram-positive bacteria from outside the bacterial cells, similar to common antibiotics.

To further confirm that the observed antibacterial activity was caused by the dehydrin, not other cellular components in the *E. coli* cell extracts, the recombinant protein in the *E. coli* cell lysate was purified using immunoaffinity chromatography and then used in antibacterial assay. Unexpectedly, when the purified protein was used to assay its activity against the same group of bacteria, no antibacterial activity was found (data not shown). These results prompted us to speculate that the previously observed antibacterial activity in *E. coli* lysates (as shown in Fig. 2) might have resulted from degraded dehydrin protein fragments, rather than the intact dehydrin itself. Furthermore, we did not observe reduced growth of *E. coli* strain BL21(DE3)pLys that was used to overexpress the protein. Since this protein expression strain is deficient in both *lon* and *omp*T proteases [17], proteins expressed in the strain are subjected to less protease activity. It is likely that there is more intact dehydrin when expressed in the strain, and thereby it is less or not toxic to the host cells.

K-Segment is involved in antibacterial activity. Dehydrins have highly conserved domains including the lysine-rich segments (the K-segment) and serine-rich S-segment (the S-segment). A common feature in the dehydrins we isolated due to their antimicrobial activity is that they have one or more lysine-rich 11-19-amino acid seg-

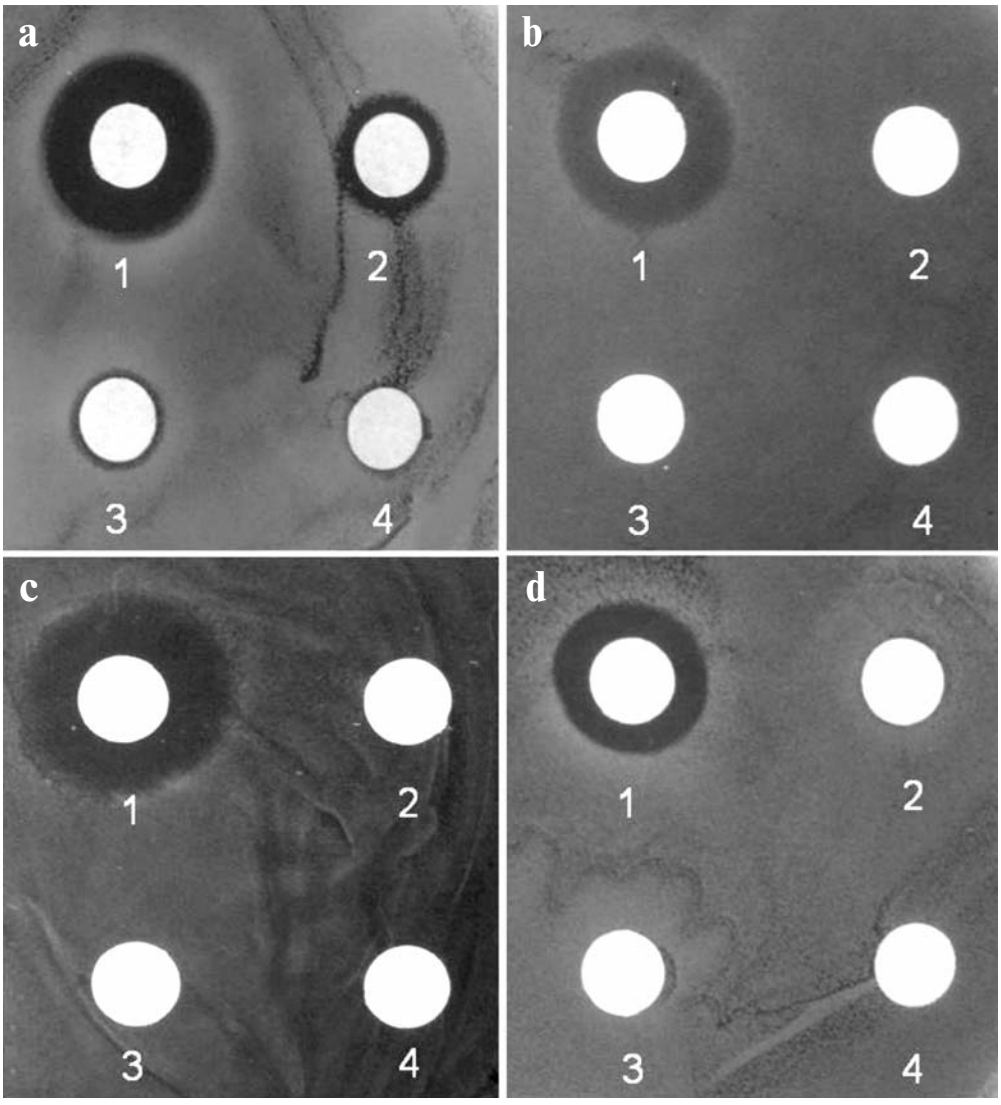

Fig. 3. Disc agar diffusion assay of cell lysates from *E. coli* expressing truncated K-dehydrin against Gram-positive bacteria. Bacteria *B. pumilus* (a), *B. subtilis* (b), *S. lutea* (c), and *S. aureus* (d) were seeded on LB agar plates and cultured with paper discs loaded with 20 μl of the lysates from IPTG induced (samples 1 and 2) and non-induced (samples 3 and 4) pET28-truncated dehydrin (samples 1 and 3) and empty vector culture (samples 2 and 4). The photos were taken after 12-24 h culture growth at 37°C.

ments [6]. In addition, two of the clones also carry typical serine-rich S-segments commonly found in dehydrins. To pinpoint the domains related to the antibacterial activity, we constructed two truncated dehydrins carrying either S- or K-segments in pET28a (S- and K-dehydrins) and expressed them in *E. coli* strain BL21 (DE3)pLys. Antibacterial activity was determined for the two dehydrin variants using crude and purified S- and K-dehydrins. The results indicated that the crude, but not the purified K-dehydrin showed antibacterial activity against Gram-positive bacteria *B. pumilus*, *B. subtilis*, *S. aureus*, and *S. lutea* (Fig. 3), but not Gram-negative bacteria *X. oryzae* and *E. coli* (data not shown). On the other hand, either crude or purified S-dehydrin was found to be inactive against these bacteria (data not shown). These results, along with our earlier antibacterial experiment data with purified full-length dehydrin, suggested that it was the peptides derived from the dehydrin, not the intact dehydrin, that caused inhibition of bacterial growth, and the active peptides are likely within the K-dehydrin sequences.

Synthetic K-segments are active against bacteria. In clone RR46 there are three lysine-rich fragments following the S-segment, which could be potentially responsible for the antimicrobial activity. We synthesized chemically the three peptides as K1 (KKKKGLKEKIKEKLPGHK), K2 (KKGFLDKIKEKLPGGHKK), and K3 (KEKKGILGKIMEKLPG) to investigate their antibacterial activity. In agar diffusion assays, peptide K1 and K2 produced clear inhibition zones on the lawns of Gram-positive bacteria *B. pumilus*, *B. subtilis*, and *S. aureus*, but not *S. lutea* (Fig. 4), while up to 1 mM K3 did not inhibit the bacterial growth in the agar diffusion assay (data not shown). The MIC and MBC for peptide K1 against *B. pumilus* were 130 and 400 μg/ml, respectively. The data confirm that the K-segments are at least partially responsible for the bactericidal activities observed in the dehydrins. The K-segments are highly lysine-rich motifs, particularly for K1 and K2 (lysine contents are 50 and 40%, respectively) with strong positive net charge of 7.1 and 5.1 at pH 7, respectively. On the other hand, peptide K3 is neutral in charge. The "dense alignment surface" (DAS) method [18] indicates that the K1 peptide has transmembrane regions between two glutamic acids at amino acid position 8 and 12. This property could lead to interaction of the peptide with bacterial cell membranes, as observed with other antimicrobial peptides [19], and be attributed to observed antimicrobial activity. Furthermore, sequence comparison of the K-segment with known antimicrobial peptides indicates that it contains a hexa-

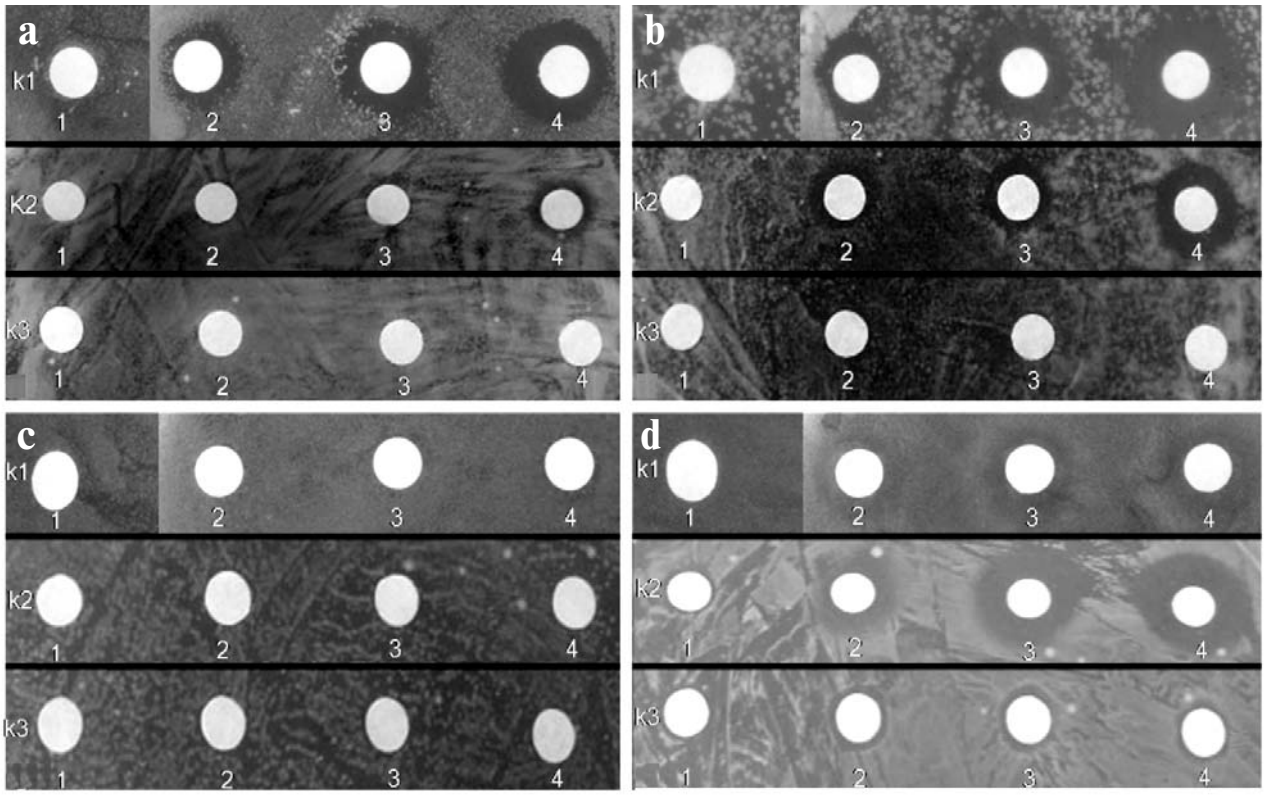

Fig. 4. Disc agar diffusion assay of synthetic K-segment peptides (K1, K2, and K3) against Gram-positive bacteria. Bacteria *B. pumilus* (a), *B. subtilis* (b), *S. lutea* (c), and *S. aureus* (d) were seeded on LB agar plates and cultured with paper discs loaded with 5 μl solution of the peptides at 0, 0.25, 0.5, and 1 mM (samples 1-4, respectively). The photos were taken after 12-24 h culture growth at 37°C.

peptide sequence EKIKEK that also exists in the 22-a.a. antimicrobial peptide Caerin 3.3 from green tree frog [20], although the significance of the 6-a.a. fragment with regard to its bactericidal activity is not clear.

Living organisms, including plants, produce a number of antimicrobial peptides, sometime in response to microbe invasion [21]. These peptides are released from precursor proteins after posttranslational proteolytic cleavage [22]. In our study, we show that short synthetic peptides from the dehydrin have *in vitro* antibacterial activity. However, further investigation is needed to see if the peptides or their analogs are produced *in planta* from dehydrins as precursors and their role in microbial defense.

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