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Inhibition of Lytic Activity of *Escherichia coli* Toxin Hemolysin E against Human Red Blood Cells by a Leucine Zipper Peptide and Understanding the Underlying Mechanism†

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ABSTRACT: To investigate as to whether a peptide derived from hemolysin E (HlyE) can inhibit the cytotoxic activity of this protein or not, several peptides were examined for their efficacy to inhibit the lytic activity of the protein against human red blood cells (hRBCs). It was found that a wild-type peptide, H-205, derived from an amphipathic leucine zipper motif, located in the amino acid region 205−234, inhibited the lytic activity of hemolysin E against hRBCs. To understand the basis of this inhibition, several functional and structural studies were performed. Western blotting analysis indicated that the preincubation of HlyE with H-205 did not inhibit its binding to hRBC. The results indicated that H-205 but not its mutant inhibited the hemolysin E-induced depolarization of hRBCs. Flow cytometric studies with annexin V-FITC staining of hRBCs after incubation with either protein or protein/peptide complex suggested that H-205 prevented the hemolysin E-induced damage of the membrane organization of hRBCs. Tryptophan fluorescence and circular dichroism studies showed that H-205 induced a conformational change in HlyE, which was accompanied by the enhancement of an appreciable helical structure. Fluorescence studies with rhodamine-labeled peptides showed that H-205 reversibly self-assembled in aqueous environment, which raised a possibility that the H-205 peptide could interact with its counterpart in the protein and thus disturb the proper conformation of HlyE, resulting in the inhibition of its cytotoxic activity. The peptides derived from the homologous segments of other members of this toxin family may also act as inhibitors of the corresponding toxin.

Hemolysin E (HlyE)[1] is a 34 kDa protein-toxin that is believed to be a potential virulence factor, involved with the infections caused by pathogenic *Escherichia coli*. Bacteria expressing HlyE are able to lyse erythrocytes from several mammalian species including humans in both solid and liquid media (*1*, *2*). Macrophages grown in tissue culture media are also lysed by an *E. coli* strain expressing HlyE (*3*). Sequence comparisons show that the typhoid-causing bacterium *Salmonella typhi* and the dysentry causing organism *Shigella flexneri* have highly homologous proteins to HlyE, identified in *E. coli* (*4*). The expression of hemolysin E has also been observed in the clinical isolates of *E. coli* (*5*). The expression of the *hlyE* gene in the laboratory strain, *E. coli* K-12, is regulated by several proteins including H-NS, SlyA, MprA, HlyX, or a fumarate and nitrate reduction regulator (FNR) (*6*−*8*). Experiments in the lipid bilayer indicated that this toxin forms pores in the membrane (*3*).

The crystal structure of the water-soluble form of the toxin has been solved at high resolution. It shows that the toxin mainly consists of long helical structures (*4*). To date, no crystal structure of hemolysin E has been available in the presence of lipids. However, recent cryo-electron microscopic studies have revealed that the protein undergoes a significant structural change in the presence of lipids and forms oligomeric pores (*9*). Despite all these studies, very little is known about the hemolysin E segments or its structural units that contribute in the assembly and toxic activity of hemolysin E.

Recently, we have identified and characterized a leucine zipper-like motif (*10*), located very close to the β-tongue region of the protein. The synthetic segment (H-205) corresponding to this motif adopted a significant helical structure in the membrane-mimetic environment and also self-assembled in both zwitterionic and negatively charged lipid vesicles. Synthetic peptides derived from the heptad repeats have been reported to recognize its parent protein, disturb its proper assembly, and inhibit the activity. For example, synthetic peptides, derived from the heptad repeats of several viral fusion proteins such as HIV, human parainfluenza virus, sendai virus, etc. are known to inhibit the fusogenic activity of the corresponding virus (*11*−*13*). Therefore, we decided to investigate as to whether a synthetic peptide derived from the leucine zipper-like motif from *E. coli* toxin hemolysin E can inhibit the activity of the protein or not. Our results showed that only the wild-type peptide

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[1] Abbreviations: FITC, fluorescin isothiocyanate; GSH, glutathione; GST, glutathione *S*-transferase; HlyE, hemolysin E; hRBC, human red blood cell; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate buffered saline (pH 7.4); Rho, tetra-methylrhodamine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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H-205 but not its mutant or an extended peptide inhibited the lytic activity of hemolysin E against human red blood cells (hRBCs). The possible mechanism of inhibition of hemolysin E by the peptide H-205 and its probable implication in the toxic activity of the protein has been discussed.

MATERIALS AND METHODS

Hemolysin E Expression, Purification, and Antibody Production. Hemolysin E was expressed and purified as reported earlier (*14*). In brief, *E. coli* JM109 cells (a gift from Prof. Jefrey Green, University of Sheffield, U.K.) having a pGS-1111 plasmid, which contains the expression vector of GST-hemolysin E, were grown in LB broth for 3 h at 37 °C before the induction of GST-HlyE expression by the addition of isopropyl-1-thio-β-D-galctopyranoside (100 μg/mL). After a further 4 h of incubation at 37 °C, bacteria were collected by centrifugation at 7000 rpm at 4 °C. The pellet of the bacteria was suspended in 10 mM Tris-HCl, pH 8.0 containing 10 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride. The previous bacterial suspension was then sonicated for the disruption of the bacterial membrane followed by centrifugation at 12 000 rpm at 4 °C. The supernatant was taken and loaded on a GSH-Sepharose column equilibrated with 25 mM Tris-HCl, pH 6.8 containing 100 mM NaCl and 2.5 mM $CaCl_2$. After being washed with 10 volumes of the same buffer, HlyE was released by the thrombin treatment (5 units for 16 h at 25 °C). The protein concentration was estimated with the help of the Lowry et al. method (*15*), and HlyE-induced hemolysis of hRBCs was measured to check the activity of the protein.

To raise antibody against hemolysin E, rabbits were immunized by 120 μg of purified protein (protein was heated at 100 °C) emulsified in Freund's complete adjuvant subcutaneously (*14*). After 21 days of immunization, rabbits were given a booster dose of preheated purified protein emulsified in Freund's incomplete adjuvant. Rabbits were bled after 7 days of booster dosage, and the isolated serum was stored at −20 °C after adding 0.02% NaN_3. The antibody titer in the serum was analyzed by ELISA.

HlyE Inhibition Assay in the Presence of the Peptides. To look into the peptide-induced inhibition of the toxin, the hemolytic activity of HlyE against the hRBCs was measured in the absence and presence of the specific peptides by a standard procedure (*3*, *16*, *17*). Briefly, fresh hRBCs that were collected in the presence of an anti-coagulant from a healthy volunteer were washed 3 times in PBS. Purified HlyE (~0.75 μM) was preincubated with an increasing concentration of H-205 or its analogue and other peptides derived from the protein for 10−15 min at room temperature and then incubated with the suspension of red blood cells (final density ~5×10^8 cells/mL, counted with the help of a LEICA DM 5000 microscope) for 2 h to detect the lysis of the RBC at 37 °C. The samples were then centrifuged for 10 min at 2000 rpm, and the release of hemoglobin was monitored by measuring the absorbance (A_{sample}) of the supernatant at 540 nm. For negative and positive controls, hRBC in PBS (A_{blank}) and hRBCs in 0.2% (final concentration v/v) Triton X-100 (A_{Triton}) were used, respectively. The percentage of hemolysis was calculated according to the following equation:

$$\text{percentage of hemolysis} = [(A_{sample} - A_{blank})/(A_{Triton} - A_{blank})]100$$

Moreover, H-205-induced inhibition of the activity of HlyE was studied at the specific hemolytic activity of the toxin. One hemolytic unit, the specific hemolytic activity of HlyE, is defined as the amount of protein required to cause 50% lysis of the hRBCs (~5×10^8 cells/mL) after an incubation of 2 h at 37 °C (*3*). The specific hemolytic activity of the toxin was determined by measuring its hemolytic activity with varying concentrations against the fixed number of hRBCs. Then, H-205-induced inhibition was further studied with the HlyE concentration that caused 50% lysis of hRBCs.

Analysis of Binding of Toxin to hRBCs after Preincubating with or without the Peptide by Western Blotting. The toxin was preincubated with or without H-205 at room temperature for 10 min followed by the further incubation of protein alone or the protein/peptide mixture with the hRBCs for another 10 min at 37 °C. Then, the cells were collected by centrifugation at 12 000 rpm for 15 min at room temperature. The pellet was then resuspended in 5× lysis buffer for electrophoresis and either heated for 10 min at 100 °C or kept for the same time at room temperature. For western blotting, protein samples with or without boiling were resolved on 12% SDS polyacrylamide gels and electroblotted onto a nitrocellulose membrane in Tris-glycine buffer at 50 V for 2 h. Blots were blocked with 5% milk in PBS overnight at 4 °C and then incubated with primary antibody (1:1000) for 1 h at 25 °C. After 5 washes with PBS containing 0.05% Tween-20 (PBS-T) were incubated with HRP conjugated secondary antibodies for another 1 h followed by washing with PBS-T. Blots were developed by a chemiluminescence substrate (ECL kit, Amersham, Pharmacia).

Tryptophan Fluorescence Studies of Hemolysin E. Tryptophan fluorescence of the protein was recorded in the absence and presence of H-205 and its mutant to detect a possible structural change in hemolysin E in the presence of the peptides. The tryptophan fluorescence of HlyE was recorded with excitation wavelength at 280 nm, emission range of 300−350 nm (*9*), and excitation and emission slits of 8 and 6 nm, respectively.

Fluorescence Studies with the Rho-Labeled Peptides. Since the fluorescence of rhodamine is sensitive to its self-association in aqueous environment, changes in the fluorescence of rhodamine-labeled peptides after the addition of unlabeled peptide was monitored to look into the nature of the self-association of H-205. When a peptide is aggregated in the aqueous environment, the fluorescence of its rhodamine-labeled version is quenched. However, an enhancement in fluorescence after the addition of unlabeled peptide to the aggregated rhodamine-labeled peptide occurs due to the reversible self-assembly of the unlabeled and labeled peptide molecules. Thus, the nature of self-association of a peptide can be determined by recording the fluorescence of a rhodamine-labeled peptide in the absence and presence of unlabeled peptide (*18*). These fluorescence experiments were performed in the time drive mode with the excitation and emission wavelengths of rhodamine set at 530 and 575 nm, respectively.

Assay of HlyE-Induced Membrane Depolarization of Human Red Blood Cells in the Absence and Presence of the Peptides. Hemolysin E-induced depolarization of the human

red blood cell membrane was determined by its efficacy to dissipate the membrane potential across the hRBC membrane (*19*−*22*). Fresh hRBCs were collected in the presence of an anti-coagulant from a healthy volunteer and washed 3 times with PBS and were incubated with a potential sensitive dye diS-C_3-5 for 1 h with a final cell density of 0.5×10^8 cells/mL. When the fluorescence level (with excitation and emission wavelengths set at 620 and 670 nm, respectively) became stable, a particular amount of hemolysin E that can cause the lysis of hRBCs was added, and the fluorescence of the dye was further recorded. A depolarization of the hRBC membrane was indicated by an increase in the fluorescence of the dye. To look into the effect of H-205 on the protein-induced depolarization of hRBCs, a fixed amount of hemolysin E (already used for the HlyE-induced depolarization of hRBCs) was incubated with a varying amount of H-205 or its analogue for 15 min. Then, instead of HlyE, the mixtures of HlyE and peptides with a fixed amount of the protein and varying amounts of H-205 or its mutant were added, and the fluorescence of the dye was recorded as before. Depolarization of the hRBC membrane as measured by the fluorescence recovery (F_t) is defined by the following equation:

$$F_t = [(I_t - I_0)/(I_f - I_0)]100\%$$

where I_f, the total fluorescence, was determined just after the addition of diS-C_3-5 to hRBCs; I_t, the observed fluorescence, was determined after the addition of the protein or protein/peptide mixtures to hRBCs that were already incubated for 1 h with diS-C_3-5 dye; and I_0, which is the steady fluorescence level, was determined after 1 h of incubation of hRBCs with the dye.

Assay of Hemolysin E-Induced Damage of hRBC Membrane Organization in the Absence and Presence of Peptides as Probed by Annexin V-FITC Staining. Alteration in the morphology or organization of lipid bilayers of hRBCs is often probed by annexin V-FITC staining (*23*). Since hemolysin E lyses hRBCs, the effect of the peptide on the activity of hemolysin E was detected by annexin V-FITC staining of the cells in the presence of calcium chloride in PBS after the treatment of the protein or protein/peptide mixture with varying peptide concentrations. After the addition of annexin V-FITC to the hRBCs that have already been treated with protein or protein/peptide mixture, damage of the cells was analyzed by using a Becton Dickinson FACSCalibur flow cytometer and CellQuest Pro software. The exitation and emission wavelengths of annexin V-FITC were set at 488 and 530 nm, respectively.

RESULTS

Purification of Hemolysin E. The overexpression and purification of hemolysin E from the fusion protein GST-HlyE was achieved as described earlier (*14*). The upper panel A of Figure 1 shows the overexpression of *gst-hlyE* in *E. coli* JM109 cells after induction by IPTG for 4 h. The lower panel B shows the protein band corresponding to the molecular weight marker (lane 1) and GST-HlyE (lane 2); GST-HlyE after thrombin cleavage (lane 3); and the purified HlyE band after GST-HlyE cleavage by thrombin and passing through GSH-Sepharose column (lane 4).

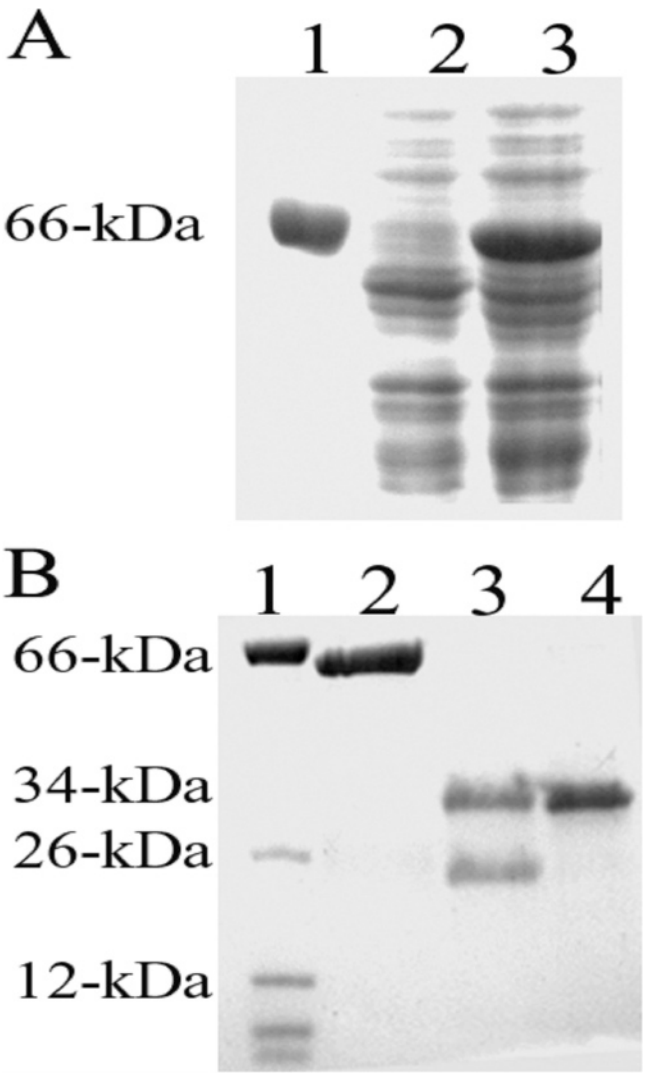

FIGURE 1: Overexpression and purification of hemolysin E from the fusion protein GST-HlyE. Panel A shows the overexpression of GST-HlyE in *E. coli* JM109 cells after induction by IPTG for 3 h. Lane 1, 66 kDa marker; lane 2, before induction; and lane 3, after induction of GST-HlyE expression by IPTG. Panel B: lane 1, the bands corresponding to molecular weight markers; lane 2, GST-HlyE; lane 3,GST-HlyE after thrombin cleavage; and lane 4, purified HlyE band after GST-HlyE cleavage by thrombin and passing through GSH-Sepharose column.

Only H-205 But Not Its Mutant Inhibits the Lytic Activity of Hemolysin E against Human Red Blood Cells. To investigate as to whether H-205, a peptide derived from an amphipathic leucine zipper motif of HlyE, can inhibit the activity of the protein or not, the lytic activity of HlyE against the hRBCs (*16*) was determined in the absence and presence of either varying concentrations of H-205 or its mutant Mu1-H-205. Figure 2A clearly indicates that H-205 inhibited the lytic activity of hemolysin E against human red blood cells in a dose-dependent manner (columns a−d) as the percentage of hemolysis of hRBCs by the toxin decreased progressively with an increase in peptide concentration. However, Mu1-H-205, which has the same amino acid composition as H-205 but four amino acids interchanged in their positions (Table 1), was negligibly active in inhibiting the HlyE-induced hemolysis of hRBCs (column e in Figure 2). It is to be mentioned that H-205 exhibited its inhibition to the toxin only when it was first incubated with the protein before the

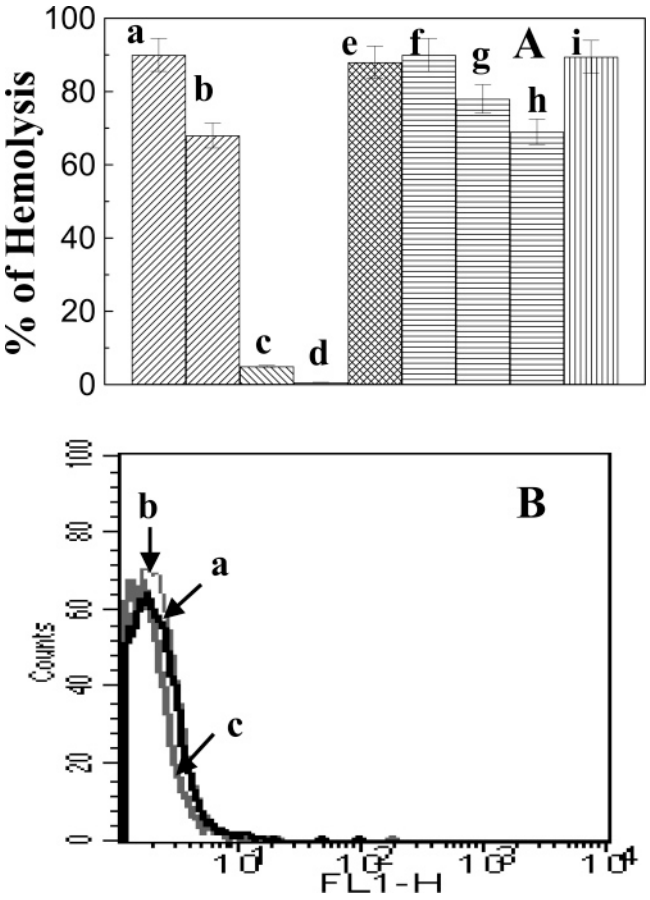

FIGURE 2: Inhibition of hemolysin E-induced lysis of hRBCs by H-205 and study of binding of the peptides to hRBCs by flow cytometry. Panel A shows the hemolytic activity of hemolysin E, preincubated without or with H-205/Mu1-H-205 against hRBCs. Plots of percentage of hRBCs lysis induced by 0.75 μM HlyE in the absence of any peptide (a) and in the presence of varying concentrations of H-205, namely, b−d represent 4.2, 8.4, and 12.6 μM peptide, respectively. Plot e depicts the percentage of hRBCs lysis induced by 0.75 μM HlyE, with preincubation of 14.6 μM, of Mu1-H-205 peptide. Plots f−h represent the percentage of hemolysis induced by the same concentration of HlyE (0.75 μM) when H-205 with varying concentrations was incubated with hRBCs first and then the protein was added. The concentrations of H-205 in columns f−h were the same as in b−d, respectively. Plot i shows the percentage of hemolysis induced by HlyE (0.75 μM) when Mu1-H-205 (12.6 μM) was preincubated with hRBCs and then the protein was added. Panel B shows the binding of H-205 or Mu1-H-205 to hRBCs by flow cytometry employing their NBD-labeled analogues. (a) hRBCs without any peptide; (b) 12 μM NBD−-H-205 in hRBCs; and (c) 12 μM NBD−-Mu1-H-205 in hRBCs. A total of 10 000 events were counted for each of these experiments.

addition of hRBCs. When H-205 was added to the protein that was already incubated with the hRBCs, no inhibition of the protein's hemolytic activity was observed. The results probably suggest that once the hemolysin E molecules are already bound to the target hRBCs, the peptide could not inhibit the protein's toxic activity. Furthermore, it was investigated as to whether the H-205 peptide and the toxin compete for the same binding sites onto the hRBC or not. For this purpose, H-205 or its mutant was incubated with hRBC first, and then hemolysin E was added, and the toxin-induced lysis of human red blood cells was measured in the usual procedure. As shown in the Figure 2A (columns f−h), only a marginal inhibition of the hemolytic activity of hemolysin E was observed at higher peptide concentrations, which was much less as compared to that when the same amount of peptide was incubated to the protein prior to the addition of hRBCs. However, in this experiment, when H-205 was replaced by its mutant, a negligible inhibition of hemolytic activity of HlyE was observed (Figure 2A, column i), indicating that whatever small inhibition of hemolysin E was observed in the presence of preincubated H-205 and human red blood cells was sequence specific.

The binding of H-205 to the hRBCs was explored by flow cytometric experiments by employing its NBD-labeled version. Figure 2B clearly indicates that H-205 did not bind to the hRBCs. Therefore, a partial inhibition of HlyE's hemolytic activity when H-205 was incubated with hRBCs prior to the addition of HlyE could be due to the weak interaction of the peptide molecules present in the hRBC suspension with the protein toxin.

The specific hemolytic activity of hemolysin E was determined as described in the Materials and Methods, which came at a 0.23 μM protein concentration. Considering the molecular weight of HlyE as 33757 kDa, one hemolytic unit for the protein corresponded to 7.76 μg. Thus, the specific hemolytic activity of HlyE was ~129 hemolytic units/mg against human red blood cells, which is ~65% of the reported value of the specific activity of the protein (~200 hemolytic units/mg) against the horse red blood cells (*3*). The value is consistent with the report that HlyE is ~70% active against the hRBCs as compared to that of horse blood cells (*24*). H-205-induced inhibition of the hemolytic activity of the toxin was measured at the specific activity of the protein (0.23 μM). It was observed that ~4.5 μM H-205 totally inhibited the hemolytic activity of the toxin at this concentration (Supporting Information Figure 1).

Moreover, three more peptides, namely, H-88, H-167, and H-130 derived from the amino acid regions, 88−120, 130−157, and 167−197, were also tested for their efficacy to inhibit the hemolytic activity of hemolysin E. However, all three peptides did not exhibit any appreciable inhibition toward the toxicity of HlyE (data not shown) in the concentration range where the peptide H-205 showed its activity against the protein. Interestingly, an extended version of H-205 (H-198; a.a. 198−234) (*25*) with the addition of seven hydrophobic amino acids at the N-terminus also did not inhibit the cytotoxic activity of hemolysin E in the same concentration range.

H-205 Does Not Inhibit the Binding of Hemolysin E to Human Red Blood Cells. To understand the mechanism of H-205-induced inhibition of the hemolytic activity of HlyE, it was addressed as to whether the peptide inhibited the binding of the toxin onto the hRBCs. Protein, preincubated with or without H-205, was added to hRBCs followed by centrifugation and lysis of the cells. Western blotting experiments with the antibody raised against HlyE were performed after the lysates were run in the SDS-PAGE with or without boiling in the presence of the sample buffer as mentioned in the Materials and Methods. The pattern and positions of the protein bands in both the conditions were similar, and therefore, a gel picture with the boiling condition has been presented in Figure 3. However, the gel picture with a non-boiling condition can be found in the Supporting Information Figure 2. HlyE in PBS and the lysates of only human red blood cells were used as positive and negative controls. Hemolysin E was nicely detected by the antibody

Table 1: Designations and Sequences of the Peptides in This Study[a]

peptide designation	amino acid sequences derived from hemolysin EX = H or rhodamine
H-205	X-NH-GKL**I**PELKNK**L**KSVQNF**F**TTLSNT**V**KQANK-CONH$_2$
Mu1-H-205	X-NH-G**L**L**S**PELKNK**K**K **I**VQNF**F**TTLSNT**V**KQANK-CONH$_2$
H-167	NH$_2$-QSQVDKIRKEAYAGAAAGVVAGPFGLIISYSIA-CONH$_2$
H-88	NH$_2$-GVATQLLAAYILLFDEYNEKKASAQKDILIKVL-CONH$_2$
H-130	NH$_2$-AQKSLLVSSQSFNNASGKLLALDSQLTN-CONH$_2$

[a] Heptadic amino acids are in bold in H-205 and Mu1-H-205, and mutated amino acids are in bold and underlined.

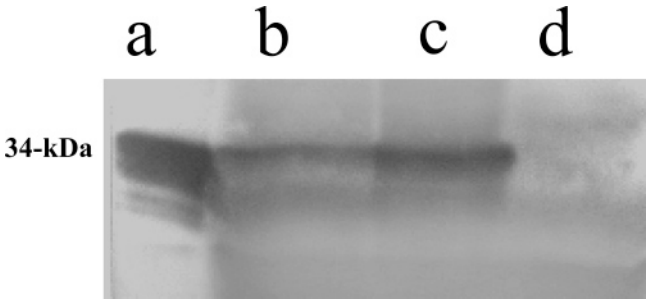

FIGURE 3: Detection of binding of HlyE to hRBCs after its preincubation with the H-205 peptide by Western blotting. (a) 0.40 μM HlyE in PBS; (b) 0.40 μM HlyE with hRBCs; (c) 0.40 μM HlyE, preincubated with 8.4 μM H-205 in hRBCs; and (d) hRBCs only.

raised against it (Figure 3, lane a). Hemolysin E was detected onto the hRBCs as expected when the toxin was incubated with the cells (Figure 3, lane b). Interestingly, HlyE was also appreciably bound to the hRBCs as is evident from the corresponding protein band (Figure 3, lane c) when the toxin interacted with H-205 first before the addition of hRBCs. Thus, the data indicated that the H-205 peptide did not inhibit the binding of the toxin to the human red blood cells.

H-205 Inhibits the Hemolysin E-Induced Depolarization of the Human Red Blood Cell Membrane. To understand the molecular basis of inhibition of the cytotoxic activity of hemolysin E, the toxin-induced depolarization of the hRBC membrane was measured in the absence and presence of H-205 and its mutant. Figure 4A depicts the experimental profiles of hemolysin E-induced hRBC membrane depolarization in the absence and presence of H-205, as is evident from the profiles that hemolysin E depolarized the hRBC membrane readily, indicating its ability to permeabilize the hRBC membrane. However, when instead of only HlyE, the preincubated mixtures of HlyE and increasing amounts of H-205 were added, a gradual decrease in the HlyE-induced hRBC membrane depolarization was observed (profiles a−c in Figure 4A). Thus, the results indicated that hemolysin E lost its membrane permeability toward human red blood cells after incubation with H-205. The dose response activity of H-205 and a complete decrease in HlyE-induced hRBC membrane depolarization (Figure 4B) at a certain concentration of H-205 are consistent with the inhibition of the hemolytic activity of the protein in the presence of the peptide. Interestingly, the mutant peptide (Mu1-H-205) did not show any significant effect on the hemolysin E-induced hRBC membrane depolarization (profile d of Figure 4A) even at higher concentrations.

H-205 Blocks the Hemolysin E-Induced Damage of the Membrane Organization of Human Red Blood Cell. To further look into how H-205 inhibits the lytic activity of hemolysin E against human red blood cells, annexin V-FITC staining of human red blood cells after the treatment

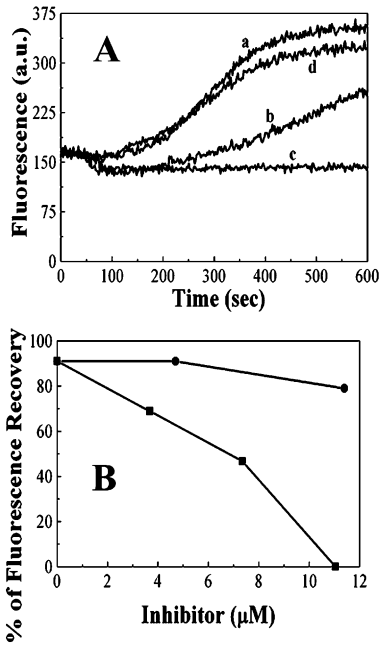

FIGURE 4: H-205 inhibits the hemolysin E-induced depolarization of human red blood cell membrane. (A) Representative profiles of hRBCs depolarization induced by 0.75 μM HlyE in the absence of any peptide (a), in the presence of 3.67 μM H-205 (b), in the presence of 7.35 μM H-205 (c), and in the presence of 11.4 μM mutant peptide (d). Panel B shows the plots of percentage of fluorescence recovery induced by hemolysin E (0.75 μM) as a result of hRBCs membrane depolarization in the presence of H-205 (squares) and its mutant scrambled peptide Mu1-H-205 (circles).

of either hemolysin E or the mixture of hemolysin E and peptides was carried out. Hemolysin E altered the membrane organization of hRBCs as is evident by the significant staining of the cells after hemolysin E treatment (Figure 5B). However, when the cells were treated with a mixture of hemolysin E and H-205 (Figure 5C), an appreciable decrease in annexin V-FITC staining of hRBCs was observed. Thus, the results probably indicated that H-205 blocked the HlyE-induced damage or alteration of membrane organization of hRBCs. However, an insignificant reduction in annexin V-FITC staining of hRBCs was observed when H-205 was replaced by its mutant, Mu1-H-205 (Figure 5D).

H-205 Interacts with Hemolysin E as Evidenced by Tryptophan Fluorescence Studies of the Protein. To understand the structural changes in hemolysin E following its interaction with H-205, the tryptophan fluorescence of the protein was recorded in the absence and presence of the peptides. The tryptophan fluorescence studies were convenient for looking into the interaction of the peptide and the protein due to the fact that although hemolysin E contains two tryptophan residues, according to the crystal structure, they are located close to each other (*4*), and the peptide H-205 does not possess any tryptophan residue. Figure 6A

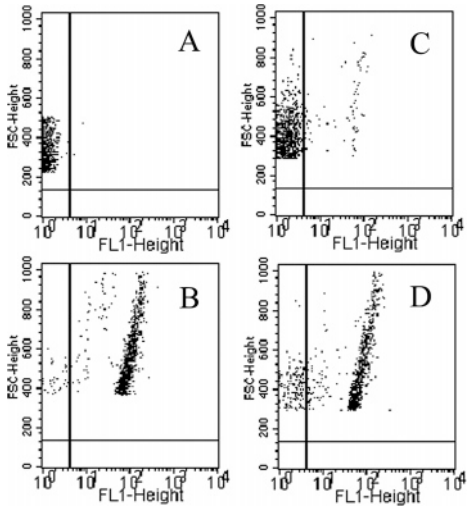

FIGURE 5: H-205 blocks the hemolysin E-induced damage of membrane organization of human red blood cells. The dot plot of the annexin V-FITC stained hRBCs in the absence of hemolysin E or any peptide (A), in the presence of 0.75 μM HlyE (B), in the presence of preincubated 0.75 μM HlyE with 8.82 μM H-205 (C), and in the presence of preincubated 0.75 μM HlyE with 11.4 μM Mu1-H-205 peptide (D). A total of 10 000 events were counted for each experiment.

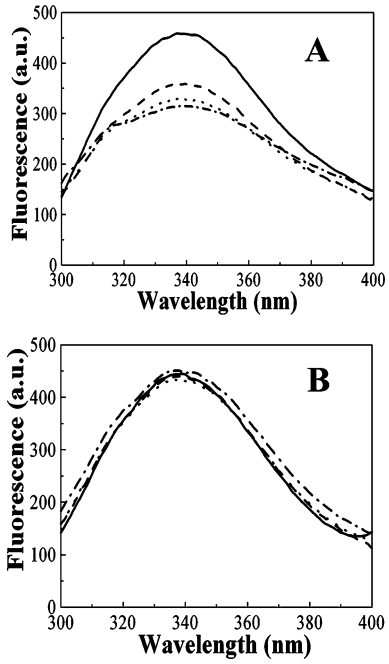

FIGURE 6: Tryptophan fluorescence spectra of HlyE in the absence and presence of H-205 or Mu1-H-205 peptides. (A) Fluorescence spectra of 0.75 μM HlyE with no peptide (solid lines), 3.67 μM H-205 (dashed lines), 7.35 μM H-205 (dotted lines), and 8.82 μM H-205 (dashed dotted lines). (B) Line symbols, HlyE protein, and peptide concentrations were the same as in panel A; however, peptide H-205 was replaced by Mu1-H-205.

depicts the fluorescence spectra of the protein in the absence and presence of varying amounts of H-205. Hemolysin E exhibited tryptophan emission maxima ~337 nm. However, with an increasing concentration of H-205, a progressive quenching of tryptophan fluorescence was observed. This decrease in fluorescence could be due to the change in the environment of the tryptophan residues, which results from a conformational change of HlyE following its interaction with the peptide H-205. However, in the presence of Mu1-H-205 (Figure 6B), no appreciable change in the tryptophan fluorescence of HlyE was observed.

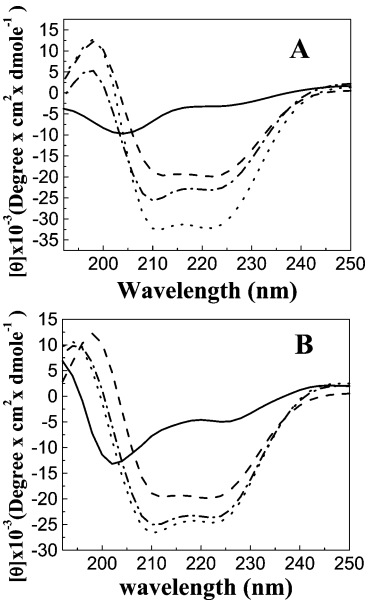

FIGURE 7: Detection of H-205-induced change in the secondary structure of HlyE by recording the circular dichroism spectra. (A) Plots of mean residue ellipticity values of H-205 and HlyE (each) separately and in a mixture. Solid line, 8.82 μM H-205; dashed line, 1.1 μM HlyE; dashed dotted line, algebraic sum of both H-205 and HlyE; and dotted line, experimental profile when H-205 and HlyE were mixed together. (B) Solid line, 8.82 μM Mu1-H-205 peptide; dashed line, 1.1 μM HlyE; dashed dotted line, algebraic sum of both; and dotted line, experimental profile of the mixture of Mu1-H-205 and HlyE.

H-205-Induced Change in the Secondary Structure of Hemolysin E. To further look into the structural changes in hemolysin E in the presence of H-205, circular dichroism spectra of the protein and peptide alone and their mixture were recorded. The peptide did not exhibit an appreciable secondary structure in aqueous environment as also reported earlier (*10*). However, hemolysin E exhibited a significant helical structure as evidenced by the characteristic CD spectra. Interestingly, when hemolysin E was mixed with H-205 and then CD spectra were recorded, a significant enhancement in the helical structure (Figure 7A) was observed, as the mean residue ellipticity value of the mixture of protein and peptide (−32 197) was appreciably higher than the corresponding algebraic sum (−23 113) of their individual CD spectra. This enhancement of a negative molar ellipticity value corresponded to a 22.7% increase in helix content, taking 100% helicity as a −40 000 mean residue ellipticity value (*26*). However, when H-205 was replaced by the mutant peptide, Mu1-H-205, the enhancement of the helical structure of the protein and peptide mixture was much less (Figure 7B). Thus, the results clearly indicated that only the wild-type H-205 but not its mutant-induced significant secondary structural changes in hemolysin E.

H-205 Molecules Self-Assemble Reversibly in an Aqueous Environment. To understand the nature of self-association of H-205 peptide molecules, the fluorescence of its rhodamine-labeled version was recorded in the presence of unlabeled wild-type and mutant peptide and proteinase-k in PBS as reported earlier (*18*). Figure 8A shows the changes in fluorescence of rhodamine-labeled H-205 in the presence of proteinase-k, unlabeled H-205, and the mutant Mu1-H-205.

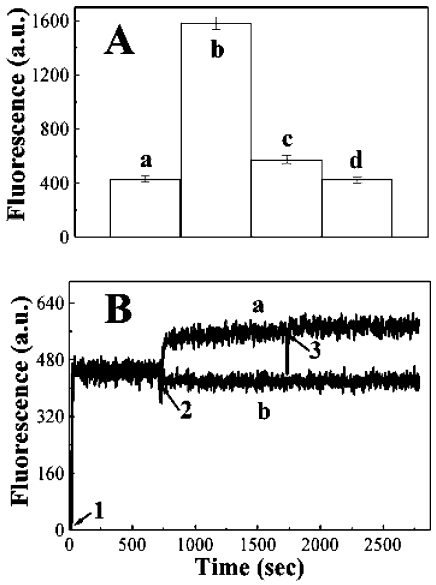

FIGURE 8: Detection of sequence specific reversible self-association of H-205 peptide molecules by recording the fluorescence of Rho-H-205 in the presence of proteinase-k and unlabeled H-205 and Mu1-H-205 in PBS. Panel A shows the bar diagram of the fluorescence level of Rho-H-205 in aqueous environment alone (a), in the presence of proteinase-k (b), in the presence of unlabeled H-205 (c), and in the presence of unlabeled Mu1-H-205 (d). Concentration of Rho-H-205 was 5.25 μM and proteinase-k 10 μg/mL. (B) Profiles showing the changes in fluorescence of Rho-H-205 with respect to time after the addition of unlabeled H-205 and Mu1-H-205. Profile a: 5.25 μM Rho-H-205 was added at time point 1, and and 5.25 and 10.5 μM unlabeled H-205 were added at time points 2 and 3, respectively. Profile b: 10.5 μM unlabeled Mu1-H-205 was added to Rho-H-205 at time point 2.

The fluorescence of Rho-H-205 ($\sim$5.25 μM) increased significantly in the presence of proteinase-k, indicating that at this peptide concentration, H-205 was aggregated in an aqueous environment. As proteinase-k cleaves the peptide molecules nonspecifically, the aggregated peptide molecules become dissociated from each other, and the rhodamine fluorescence level of Rho-H-205 increases. Also, an appreciable increase in fluorescence was observed when unlabeled H-205 was added to Rho-H-205 (Figure 8B, profile a), indicating a reversible nature of self-association of the H-205 peptide molecules. The addition of unlabeled H-205 probably resulted in the dissociation of Rho-H-205 molecules from the aggregate and an association of unlabeled and Rho-H-205 molecules, and thus, a dequenching of rhodamine fluorescence occurred. However, the addition of unlabeled Mu1-H-205 to Rho-H-205 did not result in any significant increase in fluorescence, indicating a sequence specific self-association of H-205 molecules.

DISCUSSION

Results described here indicated that H-205, a peptide derived from the amphipathic leucine zipper motif located near the β-tongue region of *E. coli* toxin hemolysin E, inhibits the lytic activity of the protein against hRBCs (Figure 2 and Supporting Information Figure 1). To the best of our knowledge, the presented data for the first time showed the inhibition of the lytic activity of hemolysin E by a peptide derived from the protein. A peptide derived from the other regions of HlyE, namely, amino acid 88−120, 130−157, and 167−197, did not show an appreciable inhibition toward the toxin in the concentration range where H-205 inhibited the activity of HlyE against hRBCs, and therefore, further studies on their interactions with the protein were not performed.

H-205 specifically inhibited the hemolytic activity of the toxin when it interacted with hemolysin E before incubation with the hRBCs. When the toxin was added to a mixture of H-205 and hRBCs, the activity of the toxin was inhibited only partly at a higher peptide concentration (Figure 2). The first possible reason for this small inhibition could be that due to the binding of H-205 onto the hRBCs, the binding of toxin onto the these cells is disturbed. However, the flow cytometric studies clearly indicated that the H-205 peptide does not bind to the human red blood cells (Figure 2), ruling out any possibility of competition between the peptide and the protein for their binding onto the hRBCs. However, despite the fact that the peptide is free in the suspension of human red blood cells, it inhibited the hemolytic activity of the toxin only partly as compared to when the toxin was incubated with the same concentration of the peptide and then hRBC was added. Most likely, the toxin interacts with hRBCs with a higher affinity and/or faster kinetics than to H-205, and hence, HlyE escapes the peptide-induced inhibition of its hemolytic activity.

The presence of HlyE on the human red blood cells when HlyE was preincubated with or without H-205 was studied by Western blotting experiments (Figure 3 and Supporting Information Figure 2). The results clearly indicated that even after interacting with H-205, HlyE was appreciably bound to the human red blood cells. In other words, the inhibition of hemolytic activity of HlyE by H-205 was not associated with the inhibition of binding of the protein to the hRBCs.

Membrane depolarization studies indicated that hemolysin E induced permeation in human red blood cells (Figure 4), while annexin V-FITC staining suggested that it altered the membrane organization of hRBC also (Figure 5). Although HlyE is a pore-forming toxin with a hemolytic activity, the studies depicted here provide evidence for the first time that the toxin induced the depolarization of human red blood cells and also damages in its membrane organization. Interestingly, hemolysin E lost its ability to depolarize and damage the organization of the hRBC membrane after its preincubation with H-205 but not with its mutant. Thus, although preincubation with H-205 did not inhibit the binding of the toxin onto the human red blood cells, it probably caused some structural change in the protein, which resulted in a loss of its hemolytic activity.

Fluorescence and circular dichroism studies indicated significant conformational and secondary structural changes in hemolysin E only in the presence of H-205 but not the mutant Mu1-H-205 (Figures 6 and 7). Tryptophan fluorescence studies indicated a specific direct interaction between the peptide molecules and the hemolysin E toxin. The decrease in the fluorescence of HlyE indicated a change in local environment of the tryptophan residues, which probably occurred due to the conformational change in the toxin induced by the binding of H-205 onto it as evidenced by CD spectra.

An extended H-205 peptide (H-198) after the addition of seven amino acids at its N-terminus (*25*) did not show any inhibition toward HlyE's hemolytic activity. Presumably,

inhibition of hemolysin E's toxicity by a peptide depends on its ability to bind to the protein, resulting in an impaired membrane perturbing activity of the toxin. It could be possible that the addition of a hydrophobic stretch to the H-205 peptide (*25*) makes the peptide more aggregated in an aqueous environment, which consequently disturbs the binding of the peptide to the protein (indicated by the CD and fluorescence studies, data not shown), and hence, no H-198-induced inhibition of HlyE's toxic activity was observed.

Recent studies (*9*) indicated that lipids induce structural changes in HlyE. The results described here suggested that the wild-type H-205 peptide inhibited the cytotoxic activity of HlyE before the toxin was incubated with hRBCs. It is most likely that structural changes take place in hemolysin E following its interaction with hRBCs, which is essential for the cytotoxic activity of the protein. However, when H-205 interacts with hemolysin E and induces structural changes in it, probably it prevents the structural change that is supposed to occur in the protein in the presence of hRBCs, and thus, HlyE loses its hemolytic activity toward the hRBCs. On the other hand, when the protein is allowed to interact with hRBCs first before the addition of peptide, structural changes occur in the protein, which the peptide H-205 cannot disturb anymore and probably therefore the peptide does not show any inhibition to the hemolytic activity of HlyE. The H-205-induced inhibition of the lytic activity of hemolysin E showed a remarkable similarity with the inhibition of the fusogenic activity of the sendai virus by a peptide derived from the viral fusion protein (*18*). The activity of virions was inhibited only when they were treated with the peptide first before incubation with the target hRBCs, which is similar to the inhibition of the hemolytic activity of hemolysin E by H-205. Also, this peptide does not inhibit the binding of virions to human red blood cells but probably disturbs the structural changes in the fusion protein, which occurs during or after the binding of virions to target cells (*18*).

That H-205 inhibited the activity of hemolysin E implies that it interacted with an important structural and/or functional element in hemolysin E that participates in the lytic activity of HlyE toward hRBCs. Tryptophan fluorescence and circular dichroism studies suggested that H-205 interacted directly with the toxin hemolysin E. Furthermore, fluorescence studies with a rhodamine-labeled peptide indicated that H-205 could self-assemble reversibly with a sequence specificity (Figure 8). This observation led us to speculate that the synthetic peptide H-205 may recognize and interact with its counterpart (amino acids 205−234) in the whole protein while inhibiting the lytic activity of the protein. This could further be implicated to an important role of the amino acid region 205−234 containing the leucine zipper motif in the assembly of hemolysin E, responsible for its toxicity as was proposed in our earlier studies involving the phospholipid membrane interaction of the synthetic segment derived this region (*10*). Another possibility could be that the H-205 peptide interacts with the H-130 segment (amino acids 130−157) of the toxin to exhibit its inhibition as the two synthetic segments can interact with each other in aqueous environments (*10*). The other speculation one could think of is that the H-205-induced enhancement of the helicity of the toxin could be associated with a structural change in the non-helical regions of the toxin such as the amino acid region 180−207, which contains the β-tongue region, an important region implicated in the toxicity of HlyE.

It should to be mentioned that H-205 does not self-assemble up to ~0.6 μM (*10*) peptide concentration; however, it has been observed that at a relatively higher peptide concentration (~1.1 μM), it begins to self-associate (Supporting Information Figure 3). Interestingly, H-205 showed its inhibition to the hemolytic activity of HlyE only at and above ~3.0 μM peptide concentration.

Altogether, the present study reports the inhibition of the cytotoxic activity of hemolysin E by a leucine zipper peptide and also shows the studies that indicated a possible mechanism of inhibition. Disturbing directly the membrane-damaging activity of hemolysin E by its own leucine zipper peptide is an interesting finding and could be used as an approach for designing an inhibitor of this class of protein toxin. With the results of inhibition of viral fusion protein by heptad repeats already there in the literature, the present results strengthen the emerging trend that synthetic peptides, derived from the heptad repeats, could be employed as the potential lead molecules to design inhibitors of a diverse class of proteins having this structural element.

CONCLUSION

A peptide H-205, derived from an amphipathic leucine zipper motif located in the amino acid region 205−234 of hemolysin E interacting with the protein toxin, induced structural changes in it, which resulted in the inhibition of the lytic activity of the protein against human red blood cells. This inhibition of hemolytic activity of HlyE is probably associated with the loss of HlyE's ability to perturb the membrane organization or to induce permeation in human red blood cells in the presence of H-205. However, the H-205 peptide did not inhibit the binding of the toxin onto the human red blood cells. Along with other options, the reversible self-association of Rho-H-205 in an aqueous environment raised a possibility that H-205 may interact with its counterpart (amino acids 205−234) in the whole protein to exhibit its inhibition.

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SUPPORTING INFORMATION AVAILABLE

Figure 1: Inhibition of specific hemolytic activity of hemolysin E by H-205. Figure 2: Detection of binding of HlyE to hRBCs after its preincubation with the H-205 peptide by Western blotting when the samples were loaded in the gel without boiling. Figure 3: Determination of aggregation of peptide H-205 in PBS by plotting the concentration dependence fluorescence vs Rho-labeled peptide. This mate-

rial is available free of charge via the Internet at http://pubs.acs.org.

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