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INHIBITION OF PROPHAGE INDUCTION BY SYNTHETIC PENTAPEPTIDE

Somaya E. Abdel Rahman* and Gamal El - daidamony +

** Department of Chemistry and + Department of Botany*
Faculty of Science, University of Zagazig, Egypt.

ABSTRACT

Six peptide chains different in length and sequences were synthesized by continuous flow solid phase method. Their effects on prophage induction were tested. It was found out that a pentapeptide at concentration of 25 µg/ml inhibited the spontaneous induction of prophage Lambda in *E. coli* strain W 3110 (λ) and (ϕ)RL_{ZLS} in *R. Leguminosarum* (lysogenic local isolate) by (99.5%) and (98.5%), respectively. This concentration was also able to inhibit the replication of the developed phages in the indicator strains. it is suggested that the peptide may block the receptor sites of the induced phages or inhibit Rec A Protease.

INTRODUCTION

In the last two decades, synthetic peptide have been proved to be useful in wide areas of current research. They have widely been used for structural elucidation of many recently isolated natural products having a peptide structure such as hormones, neuropeptides and antibiotics [1].

Another interesting application of synthetic peptides sequences was reported by Duita et al [2]; Cohen et al [3]. and Abdel Rahman et al [4]. They found that some new synthetic peptide sequences corresponding to RR_2 subunit of herpsvirus ribonucleotide reductase behave as specific inhibitors of the enzyme. They speculated that synthetic peptides could have potential application for antiviral chemotherapy.

The above results encouraged us to investigate the effect of some synthetic peptides on the prophage induction from lysogenic bacteria. Since compounds capable of mutagenic, carcinogenic and teratogenic effects are also capable of prophage induction from lysogenic bacteria[5].

Induction of prophage is usually associated with damage to DNA.

This induces a set of cellular response that have been termed SOS responses [6] result in formation of SOS signal [7].

Lysogenic induction assays were used in order to evaluate genotoxic and mutagenic properties of these compounds. Nguyen et al [8]; Brito et al [9],da Fonseca et al. [10] and Heinemann [11] recommended prophage induction system as a useful detection for compounds which potentially hazard environments.

On the other hand, substances are able to inhibit the phrophage (S); lysogenic and mutagensis induction such as caffeine [12] and Cobaltous chloride [13] gained great importance.

This paper describes the inhibitory effect of a synthetic penta peptide on the replication of λ and ϕ RL_{ZLS} phages in the indicator strains.

MATERIAL AND METHODS

HPLC measurements:

HPLC conditions: Column nucleosil 120 C_{18}, 25 cm x 4.5mm. Solvent system A (0.1% TFA in H_2O), B (O.1% TFA in acetonitrile) gradient = 0 -50%B in 30 min. Detection at 220 and 254 nm.

Amino acid analysis:

An amount of 0.1(mmol) of the peptide was hydrolysed by 3 - 4 ml 6 N HCl in an air evacuated and sealed tube for 24 h at 110°C. Then the solvent was removed in vacuam to give a residue which was washed several times with water, dried and analyzed. Amino acid analyzer is a Biotronik system LC 6000 E with intergrator system 1.

Ion spray mass spectrometry :

Ion spray mass spectroscopy was measured using Sciex spectrometer, Tubingen University, Germany.

Peptide synthesis :

Peptide synthesis was carried out on a PS - PEG graft copolymer, kindly presented by Dr. W. Rapp (Tentagel, TM, Rapp Polymer, Tubingen). Fmoc amino acid were purchased from Novabiochem. Tert. butyl group was used to protect Tyr. and Asp. side chains. All solvents and bulk chemicals were reagent grade. DMF was MCB spectroquality stored over 4A° molecular sieves. PS - PEG graft copolymer was coupled with the acid labile anchor group, 4-hydroxymethy 3 methoxy phenoxy acetic acid through the DIC/ HOBT activation method [14].

The first amino acid was coupled to the polymer using 6 g polymer anchor (1.26 meq.) dissolved in 50ml DMF/ CH_2 CI_2 (1: 1) added to a mixture of 1.6g DIC (12.6 m mol), Fmoc amino acid (12.6 mmol) 1.7 g

HOBT (1.26 mMol) and 0.06g DMAP (0.5 m Mol) dissolved in 10 ml DMF/CH_2Cl_2 (1:1).The mixture was shaked for 24 h at RT, filtered, washed several times by DMF, CH_2Cl_2, MeOH, DMF, CH_2Cl_2 and dried under high vacuum.

The column of Milligen peptide synthetizer was packed with the polymer bounded to the first amino acid and the continous flow synthesis was carried out according to the method described by Abdel-Rahman et al (4). Purity of the synthetic peptide chains was checked by ion spray mass spectroscopy and amino acid analysis after HPLC separation .

Bacterial strains:

Escherichia coli strains, a lysogen strain ic W3110 tna A_2 trp E_5 (λ)and its sensitive one were provided by Prof. Dr. El. Wafai, Nahed (Bot. Dept., Fac. Agric., Zag. University.). She obtained it from Dr. C. Yanofdky, Dept. of Biological Sciences Stanford, University of California, USA.

Rhizobium leguminosarum a lysogeic strain was isolated after exposure the sensitive strain of R. leguminosarum S (local strain) to phage RLz 13 which isolated by El-Didamony (15).

Growth conditions:

The growth media used for Propagation of E. *coli* strains have been previously described by El-Wafai and Shawky (16). Rhizobium leguminosarum strains were cultivated on yeast mannital broth or solid media(17).

Prophage induction:

A loop of each E. coli W 3110 (λ) and *R. leguminosarum* lysogeny strains was inoculated in 50 ml of their respective liquid medium and incubated for 24 h, at 25 °C. After the incubation period, 0.1ml of cultures (8.0X 10^9 CFU ml and 12.0 X 10^9 CFU ml)was added into 50 ml fresh liquid medium, supplemented with the required concentration of synthesized peptide and mitomycin C, then the cultures were incubated for 24 h. Five ml was filtered through0.45 µm Millipore filter desk membrane. The phage was assayed by overlay method (18) and the phage forming units (PFU/ml)were calculated.

Single plaque from each untreated indicator strain was picked up and suspended on fresh medium. The titer of each suspention was determined.

The inhibitory concentration of peptide was added to 5 ml of suitable phage dilution and to the same volume of indicator strain then incubated for 10 min at 28°C. The titer of phage was determined.

Electron microscope of phage isolated from R. leguminosarum lysogeny strain:

The overnight culture of lysogeny strain was filtered and plaque assayed. Single plaque was isolated and propagated with R.leguminosarum S. (sensitive strain).

The lysate was filtered concentrated by centrifugation for 3 h at 20,000 rpm at 4°C, the pellet was resuspended in small volume of phosphte buffer (PH 7.2) and viewed under EM after negative staining with 1% aqueous uranyl acetate (PH 6.4) (15)

RESULTS AND DISCUSSION

Synthesis of peptide chains under investigation:

To investigate the effect of synthesized peptides on prophage induction in lysogenic bacteria, the following chains were synthesized using the continuous flow technique, H-Val-Val- Asn-Asp- Leu OH (1), H-Val- Ala- Asn- Asp- Leu OH (2), H- Tyr- Val- Asn- Asp Leu- OH (3), H- Tyr- Val- Ala- Asn- Anp- Leu OH (4) and H-Val-Ala-Asn-Asp-Leu-Val-Asn-Asp-Leu OH(6).

Synthesis was carried out on polystyrene polyethylene glycol graft copolymer which is recommended to be used as C-terminal protecting group for rapid peptide synthesis (1).

This polymer shows pressure stability, similar swelling behaviour in different solvents and chemical stability beside its spherical form and equal bead size. All these properties are prequisited for continuous flow method.

An essential advantage of the continuous flow method is the reduction of use of expensive coupling components and the solvents.

Fmoc group was utilized as N-terminal protecting group. This enables UV monitoring of the coupling and deprotection reactions at inlet and outlet of the columm (19).Coupling was carried out using HOBT/ DIC reagents [20,21]. Synthesis was carried out at 50 °C which reduced the time of standard cycle by Ca. 29 %. Cleavage of the polymeric support and side chain protecting groups was carried out stimultaneously using a mixture of TFA/ ethane dithiol (4:1). Free peptide was precipitated using ice cold ether, the precipitate was dissolved in distilled water and lyophlized before it has been subjected to HPLC purification.

The HPLC-pure peptide was characterized by ion spray mass spectroscopy (Fig. 1) and correct amino acid analysis (Fig.1) shows the ion spray mass of peptide chain (1) after purification by HPLC.

Prophage induction

The relative capabilities of six, HPLC pure peptide chains to induce Lambda and ϕ RL 7LS (Fig.) bacteriophage formations in lysogenic strains were tested and compared to that of mitomycin C.

Different concentrations of peptide as well as mitomycin C ranged from 5 to 45 µg/ml were prepared

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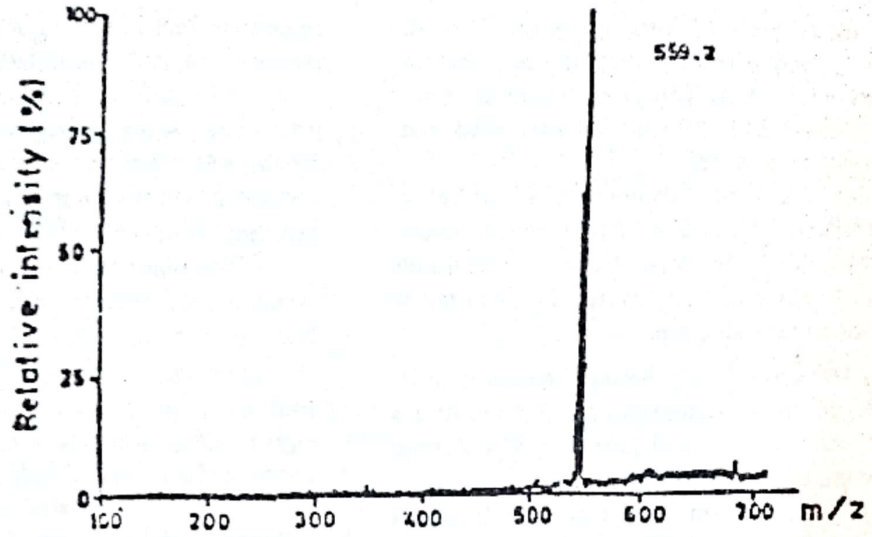
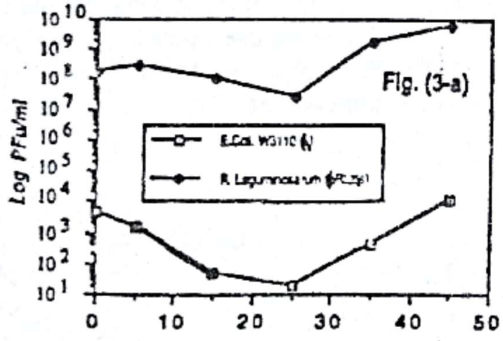
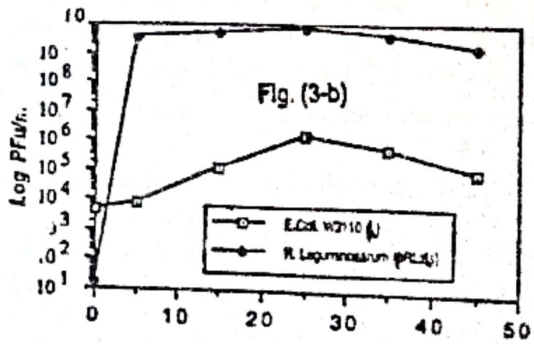


Fig. 1: Ion spray mass spectrum of peptide (1) after HPLC purification

Fig 2: Electron micrograph for bacteriophage ϕ RL_{ZLS} negatively stained with 1% aqueous - uranyl acetate showed hexagonal headd and very short tail . Mg . x = 200,000



Peptide (1) concentration µg/ ml



Mitomycin C concentration µg/ ml

Fig 3: Prophage induction from lysogenic strainas of *E.coli* W 3110 λ and *Rhizobium leguminosarum* (ϕ RL_{ZLS}) by different concentrations of synthesized peptide (1) and mitomycin C.

and sterilized by filteration, then, fifty ml of fresh medium supplemented with the required concentration were inoculated with young lysogenic bacteroa. After incubation period of 24h, five ml were filtered and plaque formation was assayed.

Peptide chain (1) showed a remarkable inhibition of prophage induction of both strains under investigation, while the other peptide chains (2-6) found to have no significant effect on neither Lambda nor ϕ RL_{ZLS} bacteriophages induction.

Figure (3 a) shows the prophage induction in E. coli W 3110 (λ) and R. leguminosarum lysogenic strains when different concentrations of peptide (1) as well as mitomycin C were used.

It is evidence from the presented data that addition of peptide (1) to both strains resulted in a significant inhibition of prophage induction and an increase in the concentration of peptide, incrreased the inhibition rate.

The greatest extent of inhibition rate (99.5%) in case of Lambda bacteriophage and (98.5%) in ϕ RL_{ZLS} is achieved at concentration of 25 µg/ml at higher concentrations > 25 µg/ml. The addition of the peptide (1) caused an increase in the prophage (s) induction of both strains.

Figure (3b) showed also that mitomycin C induced Lambda and ϕ RL_{ZLS} bacterphage formation in the range of used concentrations . Increment in phage induction was more significant increase in case of ϕ phage compared with ϕ RL_{ZLS}

These results indicated that peptide (1) is able to inhibit the spontaneous induction of prophage in E. coli W 3110 (λ) and *R. leguminosarum* lysogenic cells. We propose that, peptide (1) may deactivate Rec A protease which cleaves (λ) and ϕ RL_{ZLS} repressors. These repressors are responsible for maintaining the lysogenic state (2,4, 22- 24).

In order to elucidate the mechanism of inhibition the prophage induction caused by the synthesized peptide; the phages (λ and /or ϕ RL_{ZLS}) isolated from single plaque were incubated with peptide (1) at concentration of 25 ug/ml for 10 min at 28°C in phosphate buffer (pH 7.2). Then, 0.1 ml of this mixture was poured with 0.1ml of indicator bacterial and 3 ml molten soft agar into plates.

On the other hand, each indicator strain (about 8..0 X 10^9 CFU/ml) was incubated with the same concentration of peptide for the same period, then 0.1 ml of the mixture was added to 3ml of molten soft agar previously inoculated with 0.1 ml phage suspension and poured into agar plates. Therefore, plates were incubated over night at 28°C.

Results in Table (1) showed that, addition of peptide (1) to the isolated phage λ and ϕ RL_{ZLS} inhibited its virulencey and vegetative replication on its respective indicator host. Meanwhile, such addition to the indicator strain stimulated the phage replication.

The ability of the peptide to inhibit the phage activation toward its respective indicator bacteria may be attributed to the interference of the peptide with the specific receptor sites present on the phage tail results in blocking its activity (25,26).

The observed activity of the peptide towards the receptor sites present on the cell wall of the indicator bacteria may support this interpretation. Our result are in agreement with the result of Rastogi et al., (12) who found that, caffeine at concentration of 0.6 mg/ml significantly inhibited the spontaneous induction of prophage Lambda in *E.coli* strain Gy 5022.

It was suggested that Caffeine represses the induction of prophage Lambda by inhibiting the excision of prophage Lambda DNA from *E. coli* genome DNA. Both Caffeine and peptide (1) have shown inhibitory effect on spontaneous induction of prophage Lambda in *E. coli* but the concentration (ug/ml) required for maximum effect in case of Caffeine is 240 times more than that of peptide (1).

Here we report that a synthetic peptide has inhibitory effects on prophage induction.

The effects of the peptide on induction of prophage from lysogeny strains and it's interference with receptor sites of the isolated phages are complex, so further studies are needed to explain it.

Abbreviations:

CFU, Colony formating unit; DIC, N, N Diisopropyl carbodiimide; DMAP, Dimethyl amino -4- pyridine; DMF, Dimethyl formamide; HOBT, N-Hydroxybenzotriazole; PFU, plaque formating unit; PS-PEG, Polystyrene - polyethylene glycol copolymer; SPPS, Solid phase method of peptide synthesis; TFA, Triflouroacetic acid.

Table (1): Plaques formation units of λ and ϕ $RI-_{ZLS}$ Phages and their infectivity of the indicator strains after incubation with 25 ug/ml of peptide No 1 at 28°C for 10 min.

Treatments	PFU / ml	
	λ	ϕ RL_{ZLS}
Control	6.2×10^3	17.9×19^7
Phage (s) + peptide (1)	8.2×10^2	5.0×10^6
Indicator strain (s) + Peptide (1)	4.8×10^5	13.0×10^8

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الأثر المثبط لبعض الببتيدات الخماسية المخلقة كيميائياً على حث تحرر الفيروسات الخام

سيمة الشيخ عبدالرحمن - جمال الديدامونى أحمد*
قسم الكيمياء - و *قسم النبات
كلية العلوم- جامعة الزقازيق - مصر

فى هذا البحث تم إختبار مقدرة ستة أنواع من الببتيدات خماسية الأحماض الأمينية والمخلقة كيميائياً بطريقة الوسط الصلب على تحرر الفيروس الخام البروفاج. وقد تم تنقية كل السلاسل بعد فصل البولمر وإنتهاء التحضير بواسطة الـ HPLC وتم تعريفها وإثبات تركيبها بواسطة تحليل الأحماض الأمينية وكذلك مطياف الكتلة الأيونى. كما تم دراسة تأثير هذه الببتيدات على تحرر الفيروس الخام من أحد سلالات بكتريا القولون [E. coli w3110(λ)] وكذا سلالة بكتريا ريزوبيوم لجيوكانوزام القول وكلاهما سلالة ليجوسينين.

ودلت النتائج على أن الببتيد رقم (١) له مقدرة فائقة على تثبيط تحرر الفيروس الخام بنسبة تصل إلى (٩٨-٩٩٫٥٪) تركيز ٢٥ ميكروجرام لكل ملليلتر. وقد يرجع هذا التأثير لقدرة الببتيد على التفاعل مع مستقبلات ذنب الفيروس (الفاج) وبذلك لا يتم التعارف بين هذه المستقبلات ونظيراتها التى توجد على الجدار الخلوى للعائل (البكتريا).

ولما كان لبعض المركبات تأثير مسرطن للخلية وذلك بأحداث تشوهات فى الحامض النووى بها ويستدل على ذلك بواسطة قدرة هذه المركبات على إحداث تحرر للفيروسات الخام من البكتريا فإن أهمية هذا الببتيد ترجع إلى كونه مثبط لتحرر الفيروس الخام وبالتالى فهناك إمكانية لإستخدامه فى تجنب آثار المواد المسرطة.