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EVALUATION OF THE GROWTH PROMOTING POTENCY OF 6 DIFFERENT SYNTHETIC PEPTIDES USING RAMA-27 CELL-LINE

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ABSTRACT

Six versions of chemically synthesized peptides similar to milk peptides that have nutriceutical, therapeutic and cosmetic (anti-aging) properties were prepared by peptide synthesizer in the laboratory and subjected to bioassay method involved rat mammary gland cells (Rama-27) *in vitro*. Both molecular weights and sequences of these peptides were confirmed using Positive Ion Electroscopy Mass Spectrometry analysis by *Pepsyn*'s standard operating procedure for peptide synthesis. Cell culture techniques with doses ranging from (3.125ng/mL-450μg/mL) of these peptides dissolved in normal phosphate buffer saline (PBS) of *p*H 7.2 showed, alone, an insignificant effects as a growth promoting factor onto propagation of Rama-27 cells. Various doses of standard natural product (SNP), the extract of cow whey, alone, or mixed with equal volume of the synthesized peptides produced significantly higher growth promoting effects at doses 25-50 μg/mL. These results suggest that synthesized peptides are, alone, inactive and do lack the key co-factors as complementary elements to stimulate cell proliferation *in vitro*, unless mixed with other elements i.e. fetal calf serum (FCS) and SNP or alkaline buffer to activate or promote their growth activity characteristics. It is concluded that the composition of these peptides does still lack the natural key element to possess a full potency as a growth factor.

KEYWORDS: Growth promoting factors, Rama-27, Synthesized peptides, *in vitro*.

1.0 INTRODUCTION

The multiplication of many types of eukaryotic cells depends upon the presence of exogeneous polypeptide signaling molecules, growth factors (GF) which, after interacting with the cell, set in motion a chain of events leading to the onset of DNA synthesis and

mitosis. An explosion of discoveries in the field of cell culture and growth factors has already invaded many fields of biology, including direct clinical application as well as the most modern fundamental scientific problems in medicine. Generally, GFs are a large group of polypeptides, which induce cell multiplication both *in vitro* and *in vivo*[1,2]. They do not share any common structural features but could well be grouped into families of molecules with related sequences and structures[3]. All GFs exert their biological effects on cell multiplication at very low concentrations (typically 10^{-9} -10^{-11}M) through specific high affinity cell surface receptors. This is reflected by the fact that, with a few exception, they are typically present in natural sources at extremely low concentrations[4]. This action produces other intercellular signals which result in nutrient uptake, DNA synthesis and cell division, which eventually leads to tissue growth. The GFs have been found in a variety of body tissues and fluids, in both adults and embryos, and are believed to be released by most, if not all, cells in culture[5]. As such, GFs do not usually function in an endocrine manner but presumably diffuse over short distances through inter-cellular spaces, or act in an autocrine or paracrine fashion[4].

The growth factors, are also capable of stimulating cellular growth proliferation, healing, and cellular differentiation and usually are a protein or a steroid hormone[6]. Their ability to promote cell differentiation and maturation, varies pending on amino acids (AA) sequences and structures they have been grouped into five families i.e. transforming growth factors-β (TGF-β)[7]; epidermal growth factors (EGF); fibroblast growth factors (FGF); insulin-like growth factors (IGF) and platelet-derived growth factors (PDGF)[8]. Bovine milk and colostrums contain several growth factors, including, IGF-I, IGF-II and des(1-3)IGF-I[9,10].

The milk, in general, contains factors that stimulate cell growth in culture and the mammalian milk is unique in being a substance naturally designed to sustain the newborn i.e. rich in excellent source of nutrient mainly proteins which attracted the attention of scientists worldwide to support growth and development of babies. Beside, caseins which make up 76-86% of the total protein, the bovine milk contains whey proteins too[11,12].

A variety of cell types including epithelial cells, normal and transformed fibroblast, smooth muscle cells and chondrocytes have been shown to proliferate in serum-free, milk-supplemented culture medium[13-15]. Such activity has been found in milk and human colostrums[16] and bovine sources[17]. A number of growth factors have been isolated from milk and purified to homogeneity in an attempt to characterize their structure and biological

function[18]. Recent intention of scientist directed towards synthesizing growth factors at laboratories mimicking the natural growth factors derived from organs due to their safety of any transmitted diseases. The biological activity of any given growth factor could be calculated by the dose-dependent proliferation of target cells *in vitro*.

The objectives of this study have been to: 1). Assess the natural activity of the chemically synthesized versions of peptides; 2). Whether or not they are active when treated with buffers within a certain *p*H range; 3). To what extend the mixtures of these peptides are more effective at growth promotion than any of them used individually and 4). Whether or not their potency increases in combination with standard natural product (SNP) which is derived from the cow-milk.

2.0. MATERIALS AND METHODS

2.1 Peptide synthesis

To synthesize a peptide with a given sequence of amino acids (AA) 20% of piperidine solution (an activating solution) was prepared by mixing 20 mL of piperidine with 80 mL of DMF (*N,N, Dimethylmorpholine; Perspective Biosystem, Warrington, UK*). 6% N-methyl morpholine (NMM) solution (4-Methylmorpholine-Fluka) was also prepared by mixing 6 mL of NMM with 100 mL of Dimethyl formamide (DMF). The columns were stirred first by spatula to remove Fmoc protecting group and the mixture then was poured into a funnel. The resin was washed 3 times with DMF by a glass pipette. The funnel from the bottom was closed by a stop lock and 10 mL of 20% piperidine solution was added on the resin. The solution was then filtered after 7 minutes using vacuum machine to activate the resin and to further remove the exes DMF. All the dried resin was transferred into a small beaker. A 10 mL of NMM solution was used to dissolve the given AA by gradually pouring it into the ampoule. The remaining Fmoc AA (9-Fluorenylmethyoxycarbonyl) and HOBt was mixed well and discarded into beaker to mix the resin.

2.2. Peptide Synthesizer

A computerized peptide Synthesizer System (*9050 Pepsynthesizer PerSeptive Biosystem-Bioresearch Product*) was used to prepare the peptides according to Pepsyn's standard procedure for peptide synthesis. The machine is designed for the scale of 0.2 mMol synthesis. Fmoc Leu-resin was used to start the synthesized after deprotection with piperidine/DMF, AA were added sequentially to the amino acid terminus of the grouping peptide. The amino

acid symbols, the structural compositions, and the molecular masses of these six peptides coded P1-P6 which were prepared by either peptide synthesizer or manually accordingly.

2.3. Culture of rat mammary fibroblastic cell-line

To provide a source of healthy responsive cells for the DNA synthesis assay tissue culture of rat mammary fibroblast cells, fast sticking fibroblastic cells derived from a single-cell-cloned cell line Rama-27 passage 31-38[3]. All samples were assayed simultaneously while an appropriate number of confluent cells were pooled out and was ensued an even distribution of cells before dispersing them into the assay plates while triplicate plates, including both negative and positive control i.e. PBS and 2% FCS respectively were used. The volume of both control and test samples were always the same as 50 μL.

2.4. Cell Line

The Rama-27, a rat mammary cell line derived from the fast sticking fraction of cells isolated from a normal mammary gland was defined as fibroblast on basis of its ability to differentiate to adipose phenotype[19]. The Rama-27 was cultured in Dublicco's MEM (Gibco) supplemented with 5% fetal calf serum (FCS), 50 ng/mL insulin and 50ng/mL hydrocortisone (Sigma). Rat mammary fibroblast were seeded into 9 cm diameter tissue culture Petri dish and grown in monolayer culture in the routine media (RM) at 37^{o}C in a humidified atmosphere of 10% CO_2:90% air. The strongly adherent Rama-27 cells were subcultured at ratio either 1:4 or 1:8. When the cell monolayer was 80% confluent, the cells were washed twice with PBS then incubated with 1 mL of 0.5% trypsin (w/v) in versene at 37^{o}C until the cell monolayer was dislodged from the culture dish. The dislodged or detached cells were re-suspended in 7 mL of the RM and the 1 mL of the re-suspended cells was placed in into 9 cm diameter tissue culture dishes with 9 mL of fresh RM.

2.5. Thawing cells

Frozen cells were thawed quickly in a water bath at 37^{o}C and the cells were then transferred to a 30 mL plastic universal to which 10 mL of DMEM, supplemented with 20% FCS (v/v) was added slowly. The cell suspension was then centrifuged at 100 rpm for 5 minutes. The cell supernatant was discarded and the cell pellet was re-suspended to three 9 cm diameter Petri dishes and RM was added to give a final volume of 15 mL.

2.6. Trypsinisation procedure

The medium is aspirated from the cells which were washed twice with PB. One mL trypsin-EDTA solution was added and incubated for 5-6 minutes at 37^{o}C to ensure that all the cells are dislodged. If necessary, the plate was carefully knocked to further dislodge any stuck cells.

2.7. Passaging Cells and the passaging procedure

The original cell culture of Rama-27 used was from passage 31st as the younger generations look healthier with higher tendency to grow steadily. The incubator was always kept clean and sterile to avoid any source of contamination and the temperature was always kept on 37^{o}C and the inside moist all the time. The cells were passaged when they reached about 80% confluence (2x weekly) to allow for variable rates of growth while the cells were passaged at 2 concentrations (1:4 and 1:8) according to Bazzaz, *et al*,[1] methodology. Freshly prepared PBS solution with *p*H 7.2 was prepared and adjusted prior each experiment. A 100 mL of glycine/NaOH buffer was prepared and the *p*H 7.2 was adjusted.

2.8 Growth promoting activity assay

In the day-1 the original culture of cells of an appropriate number of 30,000-40,000 cells/mL counted by Coulter Electronic particle counter following trypsinising the 80% confluent cells with 1 mL Trypsin, suspended in 10 mL RM. Only 500 μL of cell suspension (at a density 1-1.5x10^{-4} cells/cm diameter well in 0.5 mL RM) were pipette into each well of he assay plate (15,000-20,000 cells/well) followed by incubating them for 24 hrs at 37^{o}C in moisturized oven. Wells were washed twice with 500 μL of PBS in day 2 and were replaced with 500 μL of step down media (SDM) and incubated for 24 hrs at 37^{o}C. After 24 hrs, 50 μL of serum (FCS) for a positive control and SDM or synthesized peptides were added to the cells. Test samples were similarly added to the wells and incubated for 18 hrs at 37^{o}C. In day-4 micropipettes of 20 μL of 40 μCi/mL [^{3}H]-thymidine was added to each well and the plated were then incubated for 1 hr at 37^{o}C. After 1 hr the wells were then washed twice with PBS followed by adding 500 μL of ice-cold of 5% TCA (w/v) to precipitate macromolecules. The cells left in TCA for at least 30 minutes at 4^{o}C. The TCA was then replaced with another 500 μL fresh TCA and removed immediately. The TCA was then removed and the cells wrer washed twice with ice-cold 95% ethanol at 4^{o}C. After drying the ethanol on the bench for 30 minutes, 0.5 mL of 0.2M NaOH was added to each well which were left at 37^{o}C for 1 hour to allow solubilisation. One mL of the ULTIMA Sciencentillant fluid was added into each vial

and then 0.3 mL of the solubilised macromolecules was added to Optimize Scintillation fluid in a scintillation vial. To homogenize the suspension the caps were inserted firmly and shaken several times. Radioactivity was determined by counting for 10 minutes in a Packard TRI Carb 1900TR Scintillation counter. Each experimental value was derived from triplicate wells.

2.9. Recording and expressing the results

The growth activity was recorded as the mean count/minute (cpm) value and the standard deviation was calculated automatically via the computer software linked to the system. Radioactivity was determined by counting for 10 minutes in a Packard TRI Carb 1900TR, mean negative (SDM) and positive control values (5% FCS) were recorded and saved automatically in computer. The calculations are based on:

Fold activity= Mean test cpm/mean -tive control……..(1).

Percentage activity= (Mean test cpm/mean +tive control cpm) x 100…… (2)

2.10. Determination of cell numbers

The Coulter Electronic particle counter was switched on for 10 minutes counting and the probe was thoroughly washed with isoton-II which also measured the background. The value of the latter was then recorded. A cell suspension (0.5 mL), obtained by the above procedure was mixed with 9.5 mL of isoton-II. Three counts were performed upon each suspension of cells and the mean was used (minus the background count value) to calculate the number of cells in the original suspension.

2.11. Mass Spectrometry

Micromass Quattro Mass Spectrometry which operates on positive ion Electrospray has been used to measure the molecular weight (m.wt.) of the synthetic peptide. The mobile phase used was 50% acetonitrie contained 0.01% formic acid with a flow rate of 10 uL/min and nitrogen at 100psi was used. A small amount of each peptide sample was dissolved in a 20 µL aliquot of the mobile phase. The solution was then injected via rheodyne injector into the MS source i.e. 50^{o}C temperature, 3.5 kV capillary voltage, 5V skimmer offset, cone 81V, 4.6 ion energy and mass range scanned m/z 100-2000Da for 5 second. The mass was calibrated using Horse Heart Myoglobin and the mass spectra were produced by MCA acquisition of 20 scans.

3.0. RESULTS

3.1 The Mass Recovery and Spectrum analysis

The chemical structure of each synthetic peptide appears as (NH2-) molecule in the left side while (-COOH) on the right side e.g. (NH2-L-Y-R-V-Y-P-I-V-K-COOH) and so on with others. All the AA sequence is the means of defining proteins or peptides. All of the required AA sequences of the synthetic peptide and the peaks of the de-protected Fmoc groups from each AA were observed indicating that the synthesis of the peptides was successful. The 9050 peptide synthesizer was designed for the scale of 0.2 mmol synthesis. Because the five peptides were produced consecutively with one column packing of the resin, the theoretical yield of each individual peptide should be approximately 0.04 mmol. The recovery of the peptide synthesis is between 74.5-166.2%. The control P1 is the reverse of P2 has been produced as separate batch of resin (Table-1). All the synthetic peptides were examined by *Mass Spectrometry* and the size observed for the nested series of the six peptides were obtained as expected. The positive Electrospray Mass Spectrometry analysis confirmed the molecular masses of the synthetic peptides, indicating the successful synthesis of these peptides.

*3.2 The growth promoting effects of the synthesized peptides of Rama-27 cell-line at neutral and alkaline p*H

All the five synthetic peptides, with nested series of sequences, and the control peptide, were separately reconstituted in PBS with various concentration ranging between 0.312 ng/mL and 450 μg/μL as a final solution. At neutral or at *p*H 7.2 all the six peptides, alone, exhibited none or an insignificant promotion of DNA synthesis of Rama-27 in comparison with the positive control (5% FCS) ranged from 10.5%-16.0% (Table. 2). With alkaline PBS (*p*H 10-12.99), a shorter chain peptide, a longer chain peptide P5, was chosen as examples to assess the effects of the increase in the *p*H on the growth activity (Table-3). The cpm steadily showed an increase in the *p*Hs starting from *p*H 9.0-12.0 but declined by 12.8. However, a relatively higher cpm in the final solution was noticed at *p*H 8.0 while alkaline glycine/NaOH buffer (*p*H 10), used instead of PBS for peptide P5 and P6 did not stimulate the cell growth.

(Table-1): Mass recovery details of the 6 synthetic peptides, molecular weight of homologus synthetic peptide in (Dalton), theoretical amount in mg, the obtained amount in mg and yields as percentages. Note that P1 is used as control while P2 is the reverse of P1 in AA sequencing. Note both have the same molecular weights. Each letter in capital refers to an amino acid (AA). The twenty AA are: (L), Leucine $C_6H_{11}NO$; (Y), Tyrosine $C_9H_9NO_2$; (R), Arginine $C_6H_{12}N_4O$; Valine C_5H_9NO; (P), Proline C_5H_7NO;

Isoleucine $C_6H_{11}NO$; Lysine $C_6H_{12}N_2O$; (T), Threonine $C_4H_7NO_2$; (A), Alanine C_3H_5NO; (M), Methionine C_5H_9NOS; (W), Tryptophan $C_{11}H_{10}N_2O$; (Q), Glutamine $C_5H_8N_2O_2$; (H), Histidine $C_6H_7N_3O$; (S), Serine $C_3H_5NO_2$; (F), Phenylalanine C_9H_9NO; (G), Glycine C_2H_3NO; (E), Glutamic acid $C_5H_7NO_3$; (C), Cystine C_3H_5NOS; (D), Aspartic acid $C_4H_5NO_3$ and (N), Asparagine $C_4H_6N_2O_2$. (Da), Dalton; and (mg) Milligram.

Synthetic Peptides	Molecular weight (Da)	Theoretical amount (mg)	Obtained amount (mg)	Yield (%)
LYRVYPIVK (P1) [control]	1150	230	141	61.3
KVIPYVRYL (P2)	1150	46	50	108.7
TKVIPYVRYL (P3)	1252	50	54	108
KTKVIPYVRYL (P4)	1380	55	41	74.5
AMKPWIQPKTKVIPYVRYL (P5)	2272	91	74	82.3
PQYLKTVYQKAMKPWIQPKT KVIPYVRYL (P6)	3847	154	254	166.2

(Table-2): The relative mean growth activity of Rama-27 in PBS at *p*H 7.2 of the 6 peptides. Concentrations given are those before addition to the cells. (±sd), standard deviation; negative (-ve), SDM and (+ve), fetal calf serum FCS. Note the peak has been at positive control in comparison with other doses.

Peptides	P1	P2	P3	P4	P5	P6
-ve Control	784±66	728±94	868±203	335±42	342±44	329±85
+ve control	6937±234	5732±980	6232±992	7234±911	1421±86	1420±92
100 ng/mL	730±35	674±136	809+251	377±153	406±13	311±10
50 ng/mL	1123±381	807±102	1029±31	380±143	393±20	371±27
25 ng/mL	748±187	660±71	991±81	469±64	424±84	477±39
12.5 ng/mL	885±21	610±31	1071±130	413±47	388±11	411±24
6.25 ng/mL	930±171	821±221	794±111	441±121	343±22	380±17
3.125 ng/mL	837±78	663±112	944±56	424±42	289±12	276±14

(Table-3): The relative mean growth activity of Rama-27 in PBS at alkaline *p*H (10.0-12.8) of the peptide 5 and folds of (–ve SDM). Concentrations given are those before addition to the cells. (±sd), standard deviation; negative (-ve), SDM and (+ve), fetal calf serum FCS. Note the remarkable increase in the cpm at *p*H 12.0 in comparison with other *p*Hs.

Sample lables	*p*H 10.0	*p*H 12.0	*p*H 12.8
-ve Control & Folds of –ve (SDM)	917±57 1.0	648±175 1.0	815±13 1.0
+ve control & Folds of –ve (SDM)	7235±368 7.9	6880±859 10.6	6718±339 8.2
100 ng/mL &	853±167	3995±481	2541±152

Folds of –ve (SDM)	0.9	6.2	3.1
50 ng/mL & Folds of –ve (SDM)	691±123 0.8	3871±175 6.0	2918±126 3.6
25 ng/mL & Folds of –ve (SDM)	747±70 0.8	3821±254 5.9	2959±126 3.6
12.5 ng/mL & Folds of –ve (SDM)	804±33 0.9	3847±333 5.9	2400±241 2.9
6.25 ng/mL & Folds of –ve (SDM)	940±277 1.0	3921±495 6.1	2070±379 2.5
3.125 ng/mL & Folds of –ve (SDM)	839+12 0.9	3860+157 6.0	2100±252 2.6

3.3 Effects of fetal calf serum (FCS) on cell growth

A 5% fetal calf serum (FCS) was added to the SDM used as a positive control in the almost whole project. The positive control was chosen because in a preliminary experiment, it showed a maximum increase in DNA synthesis relative to that of the negative control (SDM) only. The mean cpm of the 10% FCS showed less than that of 5% FCS whereas lower doses showed a steady increase depending upon the amount of FCS in the media (Table-4).

(Table-4): The relative growth activity of cpm of Rama-27 using various doses of fetal calf serum (FCS) added to the SDM. (±sd) mean standard deviation.

Doses	Mean cpm & sd	Folds (times –ve)
Negative control	700±81	1.0
0.625% FCS	616±54	0.9
125% FCS	747±123	1.1
2.5% FCS	1633±206	2.3
5% FCS	2870±340	4.1
10% FCS	2628±188	3.8

3.4 Effects of standard natural product (SNP) on cell growth

The standard natural product (SNP) alone was tested, at different *p*Hs of both PBS and glycine/NaOH buffers and together with different doses of the 6 peptides, to assess its effects on the growth activity of Rama-27 cell line. Using doses ranged 6.25-200 μg/mL alone, SNP, exerted a remarkable growth promoting activity on Rama-27 cells at doss 25-50 μg/mL and 100μg/mL a neutral double concentration of PBS at *p*H 7.2 with a peak of 240% at doses 50 and 100 μg/mL. However, the highest dose of the SNP (200 μg/mL) showed a down

regulation of cells (Table-4). The best dose (100 μg/mL) showed was then chosen to assess its effects on all the six peptides, produced higher cpm for P2 and P4 (Table-5). Simultaneously, a new batch of 100 μg/mL of P1-P6 was ran without SNP confirmed the very low or an insignificant growth activity of the synthetic 9 folds of the peptide alone. The 25 μg/mL dose yielded a reasonable growth activity of cpm too. Therefore, 25 and 50 μg/mL were chosen to evaluate further two volume mixture proportions of these six synthetic peptides with. Equal volume mixture of SNP and synthetic peptides showed higher cpm with mixed SNP+peptide at dose (SNP:100 μg/mL>50 μg/mL>25 μg/m) in comparison with SNP alone with almost all the peptides, respectively. Mixing an equal volume of both P5 and P6 at higher concentration 800 μg/mL of each showed an increase in the mean cpm (109% of the positive control and 3.9 folds of the negative control). Using glycine/NaOH (*p*H: 10.0 as a buffer instead of PBS produced no stimulation of growth for two short peptide (P1and P3) and longer peptide (P5-P6) with doses ranging from 10-40 μg/mL as indicated in table-5. A double concentration PBS ranged 7.37-12.0 again produced relatively higher cpm of Rama-27 cells and showed up to 49% increase in comparison with the positive control (Table-6 and 7).

(Table-5): the relative growth activity of Rama-27 of SNP doses (3.125-200 μg/mL) of the 6 synthetic peptide in double concentration of PBS at pH 7.2.

Sample labels	Mean cpm+sd	Folds of -ve (SDM's)	Percentage of -ve
SDM	479±64	1.0	100
200 μg/mL SNP	174±46	0.4	36.3
100 μg/mL SNP	1148±65	2.4	239.6
50 μg/mL SNP	1148±61	2.4	239.6
25 μg/mL SNP	962±80	2.0	200.8
12.5 μg/mL SNP	746±34	1.6	155.7
6.25 μg/mL SNP	698±31	1.5	145
3.125 μg/mL SNP	448±13	0.9	93.5

(Table-6): The relative growth activity of Rama-27 of 100 μg/mL of each of the 6 synthetic peptides (P1-P6) at double concentration PBS pH7.2 plus 100 μg/mL of SNP.

Sample labels	Mean cpm+sd	Folds of -ve (SDM's)	Percentage of -ve
SDM	1176±88	1.0	79.6
FCS	1478+32	1.3	100.0
100 μg/mL SNP+100 µg/mL P1	790±24	0.7	53.5
100 μg/mL SNP+100 µg/mL P2	1458±125	1.2	98.7
100 μg/mL SNP+100 µg/mL P3	991±46	0.8	67.0
100 μg/mL SNP+100 µg/mL P4	1626±410	1.4	110.1

100 µg/mL SNP+100 µg/mL P5	620±255	0.5	42.0
100 µg/mL SNP+100 µg/mL P6	476±189	0.4	32.2

(Table-7): The relative growth activity of Rama-27 of P3 synthetic peptide tested in series pHs (7.37-12.0) in double concentration of PBS.

Sample labels	Mean cpm+sd	Folds of -ve (SDM's)	Percentage of +ve control (FCS's)
SDM	470±80	1.0	23.2
10% FCS	2030±212	4.3	100.0
***p*H 7.37**	342±76	0.7	16.8
***p*H 8.0**	986±116	2.1	48.6
***p*H 9.0**	434±62	0.9	21.4
***p*H 10.0**	500±26	1.1	24.6
***p*H11.0**	833±134	1.8	41.0
***p*H 12.0**	1086±89	2.3	53.5

4.0 DISCUSSION

Several growth factors i.e. insulin-like growth factor (IGF) and epidermal growth factor (EGF) are present in the mammalian milk i.e. human, mouse, rat and swine[12, 20-22]. Their physiological mechanism of action typically depends upon acting as signalling molecules between cells e.g. cytokines and hormones that bind to specific receptors on the surface of their target cells[9]. Bovine milk contains a major growth-promoting activity for a rat mammary fibroblast cell-line (Rama-27) which is different than other growth factors[12]. The EGF concentration in human milk has significantly been correlated to the growth promoting activity measured by bioassay techniques[23]. Due to fear of transmission of some unexpected germs e.g. virus from their natural source to human beings attention has been given to synthesize peptides at laboratories on the industrial scale to mimic the action of these natural growth hormones[3].

In all the six peptides were synthesized (both manually and mechanically) and their growth stimulating potency assessed, amino acid components, molecular weights detected by Mass Spectrometry technique had confirmed the reliability and the successfulness of the preparation procedures. However, the growth promoting bioassays showed that alone these synthetic peptides cannot stimulate cell proliferation of Rama-27 cells and, alone, they are inactive or have an insignificant growth promoting factor effects in PBS at neutral *p*H (7.20). There also was no growth promoting activity of these peptides when an alkaline/NaOH buffer replaced the neutral PBS. Moreover, at acidic *p*H (3.0-7.0) the peptides had shown an insignificant increase in cpm (Smith and co-workers, unpublished work). Therefore no more

bioassays were further carried out with these buffers but rather neutral and alkaline PBS were tested. Generally, the cpm of Rama-27 being measured for all six peptides in alkaline buffer, were higher than at neutral *p*H. This could either be attributed to the promoting effects of the alkaline solutions originated from –OH ions, or to a new configuration of the peptides within the alkaline solution, but unlikely to be due to the peptides themselves.

The P5, was chosen, particularly, due to its longer sequence showed higher cpm in the final solution at *p*H 12.0 and significantly decreased at *p*H12.8. This may indicate either to down regulation of cell growth at *p*H 12.8, to the decrease in tolerance or perhaps to the death of a proportion of cell growth at higher *p*Hs.

The remarkable increase in the cpm at low doses of EGF confirms its potency, as naturally stimulating growth factor extracted from mouse sub-maxillary gland as a natural-non-synthesized product which is in agreement some similar works[20,24-27].

All the doses of the Standard Natural Product (SNP) of the bovine milk used have proved high potency as a natural stimulating growth factor, each on its own. This is in agreement with the finding of Cox and Burk[7]. The NSP has also proved its high necessity as a co-factor to initiate stimulation in cell growth when mixed up with synthetic peptide. It is recommended that an equal volume of the SNP, as little as 25 ug/mL mixed with any synthetic peptide could activate them as growth promoting factors. The highest cpm produced by 5% FCS has been recommended as an optimal dose to be adopted in similar bioassays. The above three agents EGF, SNP and alkaline *p*H value, plus fetal calf serum, could well be catalytic agents to improve the credibility of the chemically synthesized peptide at industrial scale.

5.0 CONCLUSION

All the six synthetic peptides, regardless of dose, are inactive in their own and cannot stimulate the growth of Rama-27 and each of them needs a complementary catalyst to turn them on as an active factor. Further studies are recommended to optimize the potency of the synthetic peptides to promote their usability upto industrial scale as nutria-ceutical therapeutic and cosmetic (anti-aging) agents.

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