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SYNTHESIS AND CHARACTERIZATION OF SOME PEPTIDES HAVING SURFACE ACTIVITY USING POLYETHYLENE GLYCOL

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ABSTRACT

In order to obtain peptide based amphoteric surfactants with antimicrobial activity, six polyethylene glycol bounded peptide chains were synthesized using the liquid phase method. The investigated chains contain aliphatic and aromatic amino acids in different sequences. Polyethylene glycol monomethyl ether was empolyed as soluble polymeric support; dicyclohexyl carbodiimide was used as the coupling reagent while the acid labile tert. butyloxy carbonyl group was applied in N-protection. The synthesized peptide chains was found to have promising surface properties beside their antimicrobial activity.

INTRODUCTION

Amphoteric surfactants, with and without antimicrobial activities, containing pure amino acid and/or protein hydrolysates have been studied [1,2]. A series of long chain N^{α}- acyle amino acid derivatives was synthesized and investigated as soluble surfactants[3]. N^{α}-laurolyl arginine and arginine dipeptide were found to exhibit good surfactant properties and antimicrobial activity [4].

This stimulates many studies aim to investigate the surfactant properties of amino acids and thier derivatives. N-(2-hydroxyethyl) -N- (2-hydroxy alkyl) -β-alanine and their oxyethylated derviatives were studied in blends with convention anionic surfactants. It was indicated that these systems have excellent detergency and other active surface properties[5,6].

It has also been established that derivatives of amino acids containing the 2-perfluoroalkyl-2-hydroxyethyl group considered a good amphoteric surfactants with different application[7]. Amphoteric surfactants containing amino acids and their derivatives found to have wide applications [8] as shampoos, cosmetics, emulsion paints, textile industry, corrosion inhibition, industrial cleaning and many others.

One of the most important features of these surfactants is that they are effective over wide pH range, in their cationic form in acidic solution and in their anionic form in alkaline solution, except at the isoelectric points [9]. Furthermore, these compounds are less toxic to higher animals and less irritating to human skin because they have structures similar to natural amino acids [10,11].

The present work deals with synthesis of some model peptide chains bounded to polyethylene glycol according to lpps (liquid phase peptide synthesis) [12] in order to evaluate their surface active properties. The bacteriostatic activities of the investigated chains were also studied.

RESULTS AND DISCUSSION

Synthesis of investigated peptides :

In an attempt to investigate the surface properties of polymer bounded peptides which possess antimicrobial activity, the following peptide chains were synthesized using lpps .

I. H- Ala-Tyr- $(Gly)_2$- MPEG

II. Boc-Gly-Ala-Tyr-Gly-MPEG

III. Boc- $(Ala)_2$- $(Gly)_2$ - MPEG

IV. Boc $(Tyr)_2$- $(Gly)_2$ - MPEG .

V. Boc Ala-$(Tyr)_2$- Gly- MPEG

VI. Boc Tyr - Ala - $(Gly)_2$ - MPEG

These peptides are covalently bounded to monofunctional ethylene glycol of molecular weight 3000 which exerts a strong solublizing effect on the attached peptide chains in all solvents. The physicochemical properties of PEG and permits its penetration through the cell membrane, so antimicrobial measurements could be carried out on PEG- bounded peptides [13]. It is also essential to notice that the polymer has nG influence on the conformational behaviour of the peptide chains bounded to it [14]. Peptide chains under investigation were synthesized using liquid phase method which primets direct application of analytical control and continuous monitoring of coupling and deprotection reactions during the synthesis cycle [15].

The acid labile t.butyl oxycarbonyl group (Boc) was employed as an N-terminal protecting group. The first amino acid (BOC Gly) was covalently bounded to PEG chain with molecular weight 3000. Coupling reactions were carried out via symmetrical anhydride separately prepared by the reaction of two moles of Boc amino acid with one mole of dicyclohexyl carbodiimide (DCCI) and were monitored by Kaiser test[16].

The N-protecting group (Boc) was cleaved by dissolving the polymer peptide in the mixture of TFA/CH_2Cl_2 (Trifluoro acetic acid/ dichloro methane) (1:1) with stirring for half an hour at room temperature. Quantitative cleavage of Boc group was tested by ninhydrin reaction. The crude polymer bounded chains were dissolved in distilled water and lyophilized. Part of the products was tested by thin layer chromatography and amino acid analysis of peptide synthesized chains.

Results of amino acid analysis (A.A.A.) and thin layer chromatography :

Peptide I : H- Ala -Tyr $(Gly)_2$ - MPEG

R_f = 0 (butanol : acetic acid : water 3 : 1:1)

A.A. A.	Ala	Tyr	Gly
Calculated	1	1	2
Found	1	0.8	2

Peptide II: Boc- Gly - Ala -Tyr-Gly - MPEG

R_f = 0 (butanol : acetic acid : water 3 :1:1)

A.A. A.	Ala	Tyr	Gly
Calculated	1	1	2
Found	0.98	0.86	2

Peptide III: Boc$(Ala)_2$ $(Gly)_2$ - MPEG

R_f = 0 (butanol : acetic acid : water 3 :1:1)

A.A. A.	Ala	Gly
Calculated	2	2
Found	1.92	2

Peptide IV: Boc$(Tyr)_2$ $(Gly)_2$ - MPEG

R_f = 0 (butanol : acetic acid : water 3 :1:1)

A.A. A.	Tyr	Gly
Calculated	2	2
Found	1.68	2

Peptide V : Boc- Ala - $(Tyr)_2$- Gly -MPEG

R_f = 0 (butanol : acetic acid : water 3 :1:1)

A.A. A.	Ala	Tyr	Gly
Calculated	1	2	1
Found	1	1.88	1

Peptide VI: Boc-Tyr-Ala-$(Gly)_2$ -MPEG

R_f = 0 (butanol : acetic acid : water 3 :1:1)

A.A. A.	Tyr	Ala	Gly
Calculated	1	1	2
Found	1	0.91	2

Antimicrobial activity :

The antimicrobial activity of the six synthesized chains was assayed as described by **Molinero et al.** [17] against Gram positive (+ve), Gram negative (-ve) organisms and one yeast. Results are summerized in table (1). The investigated peptide chains (II and VI) showed high activity against *Candida albicans* compared to other chains.

Peptide chain (I) showed activity against *Pseudamons aeruginosa* (Gram-ve) and *Bacillus subtilis* (Gram +ve). Peptide chain (I and III) inhibited the growth of *Pseudamons aeruginosa*. Peptides (I and V) have activity against *Bacillus subtilis*.

All tested chains showed no activity aganist *E.Coli* or *Staphylococcus aureus*.

Table (1) : Antimicrobial activity.

Samples / Organism	I	II	III	IV	V	VI
Bacillus subtilis	+ve	-ve	-ve	-ve	+ve	-ve
Staphylococcus aureus	-ve	-ve	-ve	-ve	-ve	-ve
Escherichia Coli	-ve	-ve	-ve	-ve	-ve	-ve
Pseudomonas aeruginosa	+ve	-ve	+ve	-ve	-ve	-ve
Condia albicans	+ve	++ve	+ve	+ve	+ve	+++ve

Physicochemical and surface active properties measurements:

A large number of protein based surfactants drived from protein hydrolysates (Elastin, Keratin, Collagen....) and fatty acids have been reported as amphoteric surfactants [18, 19]. Although these products generally can be considered as non sensitizing and non irritating, it should be remembered that they may offer an excellent growth medium for biological organism if they are not preperly protected.

The present work represents new synthesized polymer bounded peptide chains with antimicrobial activity and promising surface properties suitable for wide area of applications. The surface tension of the under investigated chains were measured at room temperature by Du Nouy method [20]. Results illustrated in table (2) showed that the tested chains produced a reduction in surface tension ranged from 32.3 to 37.5 dynes/cm. Chain V found to be the most effective one while chain III was the least active in this respect. This may be attributed to the abscence of Tyr residue with its phenolic moiety in the sequence of chain III.

Critical micelle concentration (CMC) values of the synthesized peptides were determined by applying the electrical conductivity methods. The values of (CMC) measurements which, ranged from $6.2x10^{-2}$ to $3.2x10^{-5}$ m mole/l (Fig I, II) indicated that the tested compounds posses good surface properties.

The performance of the tested substances were given in terms of foaming and wetting power, emulsion, cloud point and stability to acid.

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Table (2) : Surface properties of the synthesized poly peptides .

Compound	Surface tension dynes/ cm 0.1%	Interfacial tension dynes / cm 0.1	emulsion stability sec.	Cloud point °C	Foam height mm 0.1	Wetting time sec.	Stability to hydrolysis min: sec	CMC
I	34.8	7.3	215	86	66	130	22: 26	$5.7x10^{-4}$
II	32.9	6.9	340	87	78	106	18 :23	$1.1x10^{-4}$
III	37.5	8.9	198	94	63	137	30 : 46	$3.2x10^{-5}$
IV	33.6	7.4	302	76	102	112	15 : 36	$1.3x\ 10^{-2}$
V	32.3	6.5	282	73	112	114	16 : 02	$6.2x10^{-2}$
VI	34.7	7.7	217	80	76	120	32 : 13	$6.0x10^{-4}$

Result in table (2) showed that the investigated polymer bounded peptide chains are low to moderate foamers. These low foaming effects may be attributed to the presence of many hydrophilic groups which cause a considerable increase in the area per molecule and produce less cohesive forces at the surface.

Low foaming power is the characterstic property of nonionic surfactants [21] which premits some recent applications for these in dyeing auxiliaries textile industry. Examination of wetting properties of the peptide chains showed that they posses a potent wetting inducing efficiency. The dilute solutions of all peptide chains could wet the cotton skins in periods ranging from 106-137 seconds (Table 2). In this respect, these compounds may be potentially useful in variety of applications where wetting is desired e.g dyeing processings, paintings, cosmetics and many other operations [22].

The cloud points, of individual chain are listed in (Table 2). The results indicated that the values of cloud point increase by increasing the number of hydrophilic groups and decreases by the presence of aromatic rings. So compounds (IV and V) showed low cloud points since each of them contains two Tyr residues.

Stability to acid hydrolysis was also tested (Table 2) and the chains showed good stability towards acid hydrolysis.

Emulsifying stability of the peptide chains was estimated according to **Fukmoto et al.** [23]. Results indicated the all six chains exhibit to adequate emulsification stability, specially compounds (II, IV and V). Member of this series could produces oil/water emulsion of considerable stability. This shows that the peptide chains under investigation could be useful in textile processing and dye bathes. Biodegradability is also tested using die-away[24] test in river water. All compounds showed high rate of degradation reach 97% during 6 days (table 3).

The above mentioned results of physicochemical and surface active properties indicated that polyethylene glycol bounded peptides may be potentially useful as antimicrobial surface active agent posses pronounced surface activities. In order to obtain amphoteric surfactants of peptide nature which present better antimicrobial activities and surface properties of applications research will continue to study influence of the sequence chain length and the terminal amino acids on the surfactant and antimicrobial properties of this type of compounds.

Table (3) : Biodegradability (%) of the prepared poly peptides.

Compound	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
I	47	54	66	82	91	98	-
II	45	56	65	72	83	91	79
III	52	57	72	79	83	97	-
IV	34	50	61	68	75	89	97
V	38	44	58	62	72	85	96
VI	39	53	61	70	78	88	90

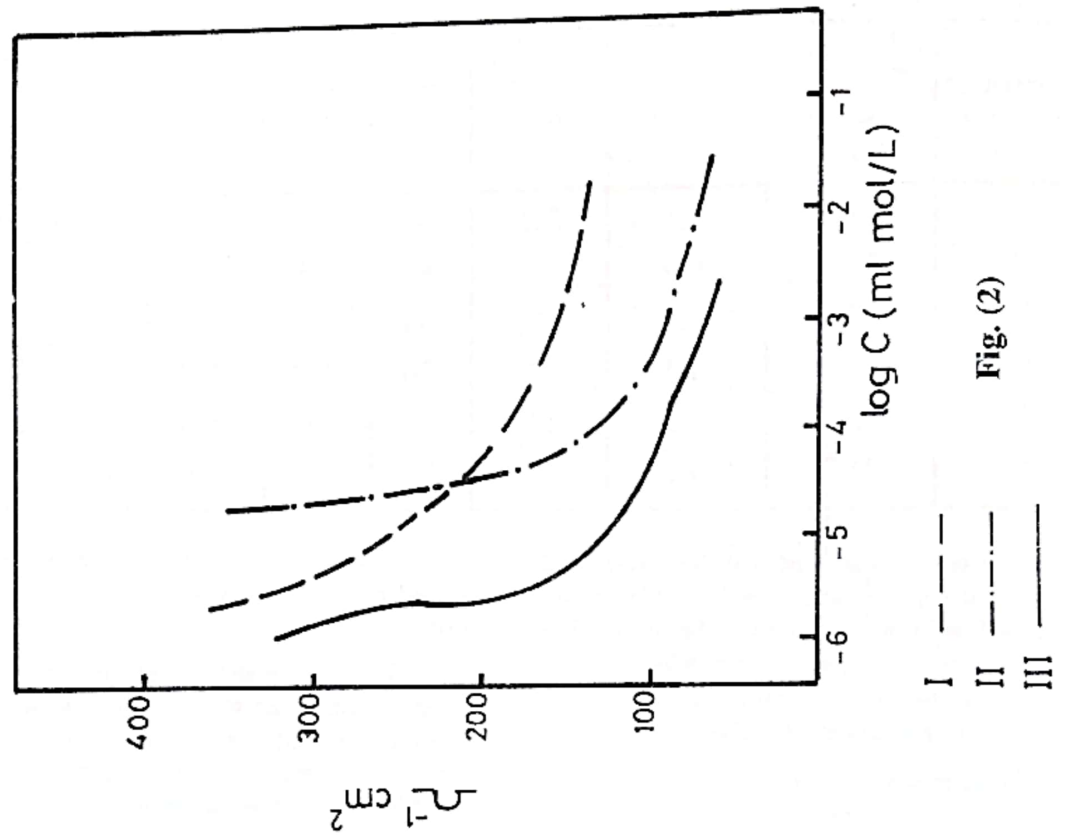
400
300
200
100
-6
-5
-4
-3
-2
-1
log C (ml mol/L)
I
II
III

Fig. (2)

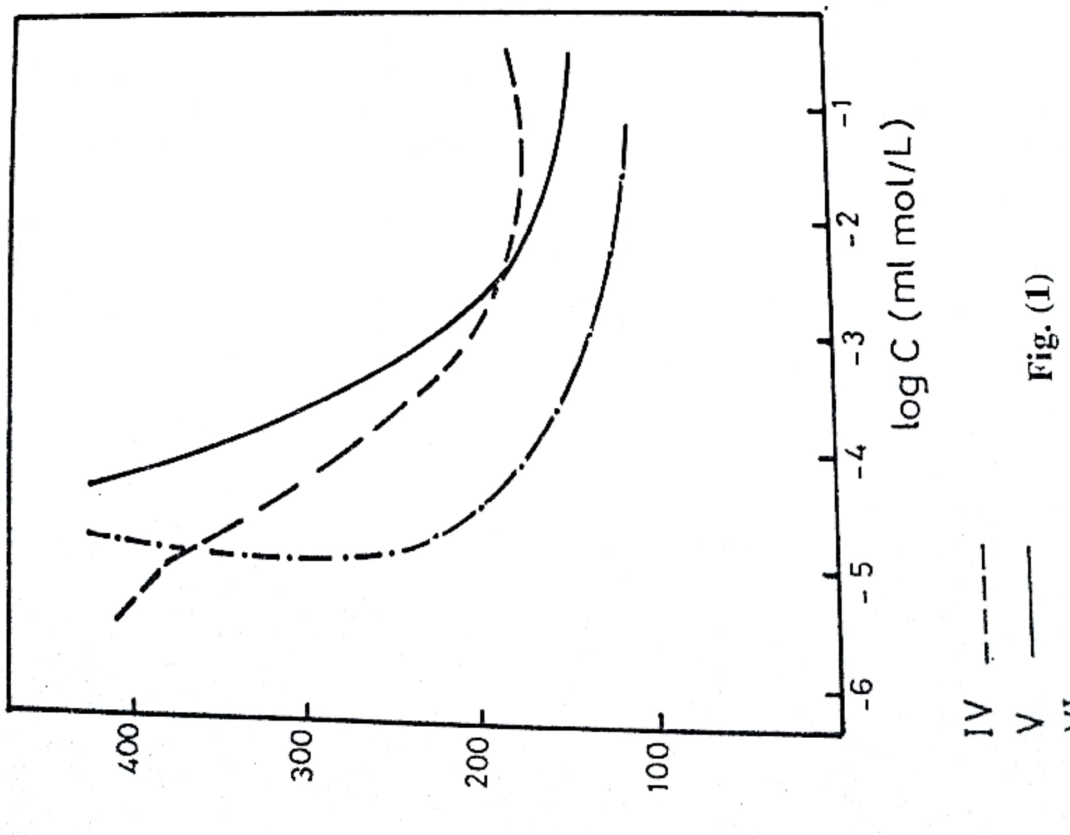
400
300
200
100
-6
-5
-4
-3
-2
-1
log C (ml mol/L)
IV
V
VI

Fig. (1)

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EXPERIMENTAL

1. Synthesis of peptide chains :

Peptide chains under investigation were covalently bounded to polyethylene glycol (MPEG) of molecular weight 3000. All the compounds were chromatographically and analytically pure. Analytical controls were carried out after each step of the synthesis. Purity was indicated by amino acid analysis and thin layer chromatography. All the amino acids were Boc-Protected at the α-amino group using Schnable method [25].

The Boc group was removed by treatment of the PEG-peptide for 30 min with mixture of trifluoro acetic acid and dichloromethane (1:1) using 10 ml of the deprotecting agent per 1g PEG-pepetide. The volume of the solution was then reduced by flash evaporation to an oil and the PEG-peptide was precipitated by the addition of anhydrous ether under vigorous stirring. The mixture was stirred another 15-30 min at 0°C, the precipitate was filtered, washed with ether, and dried under vacuum.

The coupling reactions were carried out via the *in situ* symmetrical anhydride method applying excess anhydride component. To this end, the Boc-Protected amino acid derivative was dissolved in a minimum amount of dichloromethane and the solution was cooled to 0°C. 0.48 equivalent of dicyclohexyl -carbodiimide (DCCI) in a 2M stock solution of dichloromethane was added, and the mixture was allowed to stand 30 min at 0°C. The precipitated dicyclohexylurea was removed by filtering the anhydride solution directly into a flask containing the deprotected amino component in dichloromethane. The extent of coupling was monitored first by qualitative fluorescamin test on thin layer plates. Quantitative ninhydrin and kaiser tests were carried out after isolation of the protected PEG-Peptide by precipitation. The coupling yield was not lower than 99.5%.

When quantitative coupling was achieved, the N-Boc-Protected PEG-peptide was precipitated by adding ether to the coupling mixture to obtain partially crystalline product which could be readily filtered off and which resulted in a fine powder after drying. The phenolic OH group of Boc-Tyr was protected with benzyl gruop. At the end of the synthesis; if was removed by catalytic hydrogenation, which was carried out in the least amount of methanol on a Pd catalyst (1g per 3g PEG-peptide). The mixture was subjected to continuous stream of hydrogen while stirring for 12h. The pure product was obtained by filteration of the solution and addition of ether while stirring at 0°C, the precipitate was filtered, washed with dry ether and dried under vacuum to yield pure peptide as indicated by thin layer chromatography, HPLC measurements and amino acid analysis.

2. Surface active properties :

2.1 Surface and interfacial tension :

Method for evaluation of surface active properties are described in Du-Nouuyìs interficial tensiometer (Kruss, type 4851) was used taking distilled water at 25°C as 73.1 dyne/cm and the interficial tension between medicinal paraffin oil and distilled water as 56.2 dyne/cm.

2.2. Wetting properties :

Wetting power of the tested peptides were determined by measuring the sinking time in seconds of a grey cotton skin in the surfactant solutions (0.5% by weight) at 28°C.

2.3. Foaming properties :

Foaming properties were tested according to Ross- Millîs method [21].

2.4. Emulsion stability :

The emulsion was prepared by adding 10ml of a 20 mmol aqueous solution of the surfactants to 5ml toluene at 40°C. The emulsifying property was determined by the time it took for an aqueous volume separating from the emulsion layer to reach 9 ml. counting from the moment of the cession of shaking[23].

2.5. Cloud point :

The cloud point of the individual chains were determined as reported by **Cohen et al** [26].

2.6. Stability to hydrolysis :

A mixture of 10 ml of the 10 m mol surfactants and 10ml of 2N sulphuric acid was placed in a thermostat 40°C. The time takes for a sample solution to be clouded as the result of hydrolysis shows the stability of the surfactant to hydrolysis [27]

2.7. Biodegradability :

Samples taken daily or more frequently were filter through Wattmann filter paper no I before measuring the surface tension. Surface tension measurements were made periodically (each day) on each sample during degradation test. Biodegradation percent (D) for each sample was calculated using the following relation.

$$D = \frac{\gamma_t - \gamma_0}{\gamma_{bt} - \gamma_0} \times 100$$

Where

γ_t = surface tension at time t

γ_0 = surface tension at zero time

γ_{bt} = surface tension of blank experiment at time t (without samples).

2.8. Critical micelle concentration (CMC) :

The (CMC) values for the synthesized peptide chains were determined by electrical conductivity method [28].

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تخليق وخواص بعض الببتيدات ذات النشاط السطحي باستخدام البولي ايثيلين جليكول

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في هذا البحث تم تحضير عدد ستة ببتيدات كمواد ذات نشاط سطحي امفوتيرية لها نشاط ضد الميكروبات.
وقد تم تحضير هذه المركبات باستخدام طريقة الطور السائل وهذه المركبات تحتوى سلاسلها الكربونية على احماض امينية اليفاتية وأروماتية بتتابع مختلف . وقد استخدم البولى ايثيلين جليكول احادي الميثيل اثير كشق منشط للاذابة فى جزئى البوليمر وكذلك الداى سيكلو هكسيل كاربو داى إميد كعامل الازدواج واستخدام رباعى البيوتيل أوكسى كاربونيل كحماية لذرة النيتروجين .
وقد تم تحليل المركبات بواسطة الكروماتوجرافيا وكذلك تحليل الأحماض الأمينية ودرس النشاط ضد البكتريا موجبة الجرام وسالبة الجرام وأظهرت بعض المركبات نشاط ضد هذه البكتريا.
كذلك درست الخواص الفيزيقوكيمائية والسطحية لهذه المركبات وكذلك تم تعيين التركيز الحرج للمسيلات وأظهرت هذه المركبات تحللا بيولوجيا مما يفيد البيئة ويمنع التلوث.