폴리에톡시레이티드 아스코르빈산의 개발

송영숙, 정봉열, 조완구, 강세훈
(엘지화학 화장품연구소)

DEVELOPMENT OF POLYETHOXYLATED ASCORBIC ACID AS A WHITENING AGENT

Young-Sook Song, Bong-Yul Chung, Wan-Goo Cho and Seh-Hoon Kang
Cosmetics R&D Center, LG Chemical Ltd.

Abstract

A series of novel ascorbic acid derivatives, polyethoxylated ascorbic acid (PEAA) were synthesized by coupling ascorbic acid with polyethylene glycol (PEG) of two molecular weights (MW: 350 and 550) at the C-2 or C-3 hydroxyl group (2PEAA350, 3PEAA350, 2PEAA550, 3PEAA550) to increase the stability and retain the activity, as a skin whitening agent. Their stability, scavenging activity against free radical, inhibitory activity against tyrosinase and inhibitory activity of melanin synthesis in B16 melanoma cell of PEAAs were evaluated *in vitro* and compared with those of ascorbic acid and 3-O-ethyl ascorbic acid (3OEAA), a known stable vitamin C derivative.

Among PEAAs, 2PEAA350 and 2PEAA550 had high scavenging activity against free radical, inhibitory activity against tyrosinase and inhibitory activity of melanogenesis but low stability. 3PEAA350 had high stability and moderate scavenging activity against free radical, inhibitory activity against tyrosinase and inhibitory activity of melanogenesis. The stability, scavenging activity against free radical and inhibitory activity of melanogenesis of 3PEAA350 were higher than those of 3OEAA. The most stable 3PEAA350 among PEAAs was nontoxic in various toxicological tests. These results suggest that PEAA would be a good whitening agent for enhancing stability and bioavailability.

1. Introduction

Many Asian women want to have lighter skin and want to avoid hyperpigmentation, such as melasma or freckling. In order to meet this desire, many cosmetic companies have been researching the pigmentation process to develop whitening products. While skin thickness, hemoglobin and minor pigment like carotenoids affect skin color, the amount of melanin produced by the melanocytes

primarily determines skin color[1]. For this reason, research for the development of whitening products has focused on reducing melamin production in the melanocyte. Melanin formation is induced mainly by UV radiation and other stimuli such as toxic chemical agent[2]. UV radiation has been shown to generate active oxygen species (AOS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen or free radicals in living organisms[3]. AOS accelerate tyrosinase activity to increase dopa and dopaquinone generation from tyrosine in melanocyte[4] resulting in excess melanin formation[5,6]. Whitening products usually contain kojic acid, arbutin or ascorbic acid derivatives to decrease skin pigmentation. It is known that ascorbic acid (vitamin C) scavenges AOS and free radicals as a chain-breaking antioxidant[7,8] and inhibits melanin formation by reducing the intermediate dopaquinone and resulting eumelanin in melanogenesis[9]. These characteristic biological activities of vitamin C are derived from the enediol structure, which has a strong electron-donating ability.

A main problem that arose in using vitamin C in cosmetic formulation was its stability. The instability of vitamin C is difficult to apply it to cosmetic products. The well-known susceptibility of vitamin C to thermal and oxidative degradation have prompted a continuing search for vitamin C derivatives with increased stability and retained activity. To increase the stability and retain the activity, the chemical modification of hydroxyl group of vitamin C is of most interest, and several stable derivatives of vitamin C have been reported[10-14,21,22].

Based on this background, a series of novel ascorbic acid derivatives, polyethoxylated ascorbic acid (PEAAs, Figure1, Table1) were synthesized by coupling ascorbic acid with polyethylene glycol (PEG) with two molecular weights (MW of PEG: 350,550) at the C-2 or C-3 hydroxyl group (2PEAA350, 3PEAA350, 2PEAA550, 3PEAA550: for example, 2PEAA350 means PEAA with PEG of 350 molecular weight at C-2 hydroxyl group) with a view to simultaneously improving the thermal stability and preventing the diminution of activity. Stability and inhibitory activities against free-radicals, tyrosinase, melanin synthesis in B16 cells of PEAAs were evaluated and compared 3-O-ethyl ascorbic acid (3OEAA) and vitamin C. The most stable PEAA350 among PEAAs carried out various toxicological tests on animal to estimate its safety.

2. Methods and Materials

Materials: PEAAs was synthesized by LG chemical Ltd. (Korea). Vitamin C was purchased from Fluka Chemie. AG. (Swiss). 3OEAA was purchased from Hypox Lab. (Japan). DMEM (Dulbecco's Modified Eagle's Medium), fetal bovine serum (FBS), streptomycin and penicillin were all purchased

from GibcoBRL (USA). Tyrosine, mushroom tyrosinase, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), sodium hydroxide, potassium phosphate, sodium chloride and sodium phosphate were obtained from Sigma Co. (USA). HPLC grade methanol, ethanol and acetonitril were all purchased from J.T.Baker Chemical Co. (USA).

Preparation of PEAA: The vitamin C derivatives, PEAAs could be synthesized by direct 3-O-alkylation reaction between ascorbic acid and the activated polyethylene glycol derivative. The starting materials for this synthesis, 1-iodopolyethyleneglycol monomethyl ether (MPEG-I) and 5,6-O-isopropyliden ascorbic acid were prepared by the previously known method[15].

At First, chlorination of polyethyleneglycol monomethyl ether (MPEG-OH) with thionyl chloride and triethylamin in toluene, followed by Finkelstein reaction with sodium iodide in acetone gave the MPEG-I in quantitative yield. In addition, 5,6-O-isopropyliden ascorbic acid was obtained in 81% yield by catalytic amount of acetyl chloride in acetone.

Finally, PEAAs were obtained as the pale yellow liquid by the regioselective 3-O-alkylation of 5,6-O-isopropyliden ascorbic acid with MPEG-I and subsequent deprotection of the corresponding 3-O-pegylated ascorbic acid. PEAAs were purified by column chromatography (SiO_2, mesh size 270-400, dichloromethane/methanol 15:1). The structure and purity of PEAAs were characterized by ^1H NMR, HPLC and FAB mass spectroscopy.

Free-radical scavenging activity: Free-radical scavenging activity was measured by the modified method of Fugita et al[16] using a moderately stable free radical, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) The sample solution was added to 0.1 ml of 0.1 mM DPPH methanol solution and keep at 37℃ for 30 minutes and 24 hours. The amount of free radicals in the mixture was measured by absorbance at 540 nm. The inhibition concentration 50 % on DPPH was calculated from the plot of the absorbance against concentration.

Inhibition of tyrosinase: Tyrosinase activity was measured as the procedure described by Vanni et al[17]. The reaction mixture contained 50mM sodium phosphate (pH 6.8), 0.3 ml of 1.5 mM L-tyrosine, and 2,000 U/ml mushroom tyrosinase in 50 mM sodium phosphate (pH 6.8). 0.2 ml of sample solution was added to reaction mixtures and was incubated at 37℃ for 30 minutes, and absorbances at 475 nm were measured by a spectrophotometer. The inhibitory activity of sample was expressed as concentration which inhibits 50% of enzyme activity (IC_{50}). The percent inhibition of tyrosinase

reaction was calculated as follows

$$\% \text{ inhibition} = \{(A-B)/A\} \times 100$$

A = Absorbance at 475 nm without test samples after incubation

B = Absorbance at 475 nm with test samples after incubation

Inhibition of melanin synthesis in B-16 melanoma cell: Inhibition of melanin synthesis in B-16 melanoma cell was measured by modified method of Maeda et al[18]. B16 mouse melanoma cells were cultured in DMEM supplemented with 10% fetal calf serum in humidified incubator at 37℃ under 5% CO_2. Cells were seeded into 60㎜ petridish at a density of 5×10^5 cells per dish. After cells were attached, medium was replaced with fresh medium containing various concentrations of samples. Then cells were cultured for 2 days and the medium was replaced with fresh medium with samples, further incubated for a day. Then cells were harvested with cell scrapper, counted with hemacytometer and collected by centrifugation. Inhibitory Activity of melanin formation is estimated by color of precipitation with eyes, using following scale; −: no effect, +: slightly effective, ++: moderately effective, +++: significantly effective.

Stability Stuides: To measure the temperature stabilities, 1% PEAAs, 3OEAA and vitamin C were prepared in aqueous solution. Samples were stored at 25℃and 40℃ up to 2 months. Periodically, 1 ml aliquots of each sample were pipetted out and diluted with distilled water. The amount of residual ascorbic acid derivatives was measured by HPLC.

To measure the UV stabilities of 1% PEAA and 3OEAA, 10 ml of aqueous solution (phosphate buffer, pH6) containing PEAAs or 3OEAA were divided into amount test tubes. UV was illuminated to the samples by artificial light generator (SUN TEST CPS, Heraeus Co.) for 4 hours. The amount of residual vitamin C derivatives was measured by HPLC.

High Performance Liquid Chromatography: The HPLC consisted of solvent delivery pump (Waters, 600 pump, Waters Co., MA, USA), C_{18} column (HP ODS Hypersil 5 ㎛, 4.6×200 mm, Hewlett Packerd, Germany), UV detector (waters, 486 UV detector) and data process system (Waters millennium). Vitamin C was analyzed with the mobile phase of distilled water in 0.05 % phosphoric acid. 3OEAA was analyzed with the mobile phase of 15 % acetonitril in 0.05 % phosphoric acid and at the flow rate of 1 ml/min. For 3PEAA350 and 3PEG550 analysis, the mobile phase was gradient

elution from 10 % Methanol in 0.05 % phosphoric acid to 50 % Methanol in 0.05 % phosphoric acid for 20 minutes and flow rate was 1 ml/min. The absorbence at 245nm was measured for the assay of vitamin C derivatives. The retention time was 3.5 min for vitamin C. The retention time was 4.5 min for 3OEAA. The retention time was from 8 minute to 18 minute for 3PEAA350. Temperature of the column was kept at 40℃.

Toxicity: We tested 3PEAA350, the most stable PEAA, on animals *in vivo* using toxicity tests based on the Organization for Economic Cooperation and Development's 1960 guidelines for testing of chemicals. To estimate the toxicity, acute oral toxicity, acute transdermal toxicity, primary skin irritation, ocular irritation, skin sensitization and human patch test were performed according to the method given in the reference[19].

3. Results and Discussion

Free-radical scavenging activity: Table 2 shows free radical-scavenging activity against DPPH. Free radical inhibition activity (IC_{50}) is defined as concentration of sample required for 50% of the free radicals to be inhibited; a lower IC_{50} means less is needed to counteract the same amount of free-radical activity. IC_{50} of vitamin C after 24 hours was similar to that after 30 minutes. But IC_{50} of PEAAs decreased after 24 hours. It indicates that scavenging reaction against free radical of vitamin C is completed in short time, but that of PEAA is retained after 24 hours. 2PEAA550 had the highest scavenging activity against free radical among PEAAs. The scavenging activity against free radicals of 3PEAA350 was higher than that of 3OEAA and was lower than vitamin C.

Inhibition of tyrosinase: Table 3 demonstrates inhibitory activity against tyrosinase. Melanogenesis, formation in melanocyte, is catalyzed by tyrosinase via the intermediate dopa and dopaquinone generation from tyrosine[4], and vitamin C inhibits melanogenesis by the reduction of dopaquinone to dopa[20]. The inhibitory activity of PEAAs, 3OEAA, and vitamin C on tyrosinase from mushrooms by using tyrosine *in vitro* was evaluated. IC_{50} is concentration of compound which inhibits 50% of enzyme activity. 2PEAA350 had the highest inhibition activity among PEAAs. The inhibitory activity of 3PEAA350 was similar to 3OEAA.

Inhibition of melanin synthesis in B-16 melanoma cell: Table 4 shows inhibition effect of compounds on melanin synthesis in B16 melanoma cells. The inhibitory effect of PEAAs and 3OEAA, vitamin C on melanin synthesis in B16 melanoma cells *in vitro* was evaluated; + represents slight

effect and ++ moderate effect of compounds on melanin synthesis. This result shows that inhibitory effect on melanogenesis was enhanced as molecular weight of PEG linked at PEAA increased. The inhibitory effect of 3PEAA350 on melanogenesis was higher than 3OEAA.

Stability studies: Table 5, 6 and Figure 2, 3 demonstrate the stabilities of PEAAs, 3OEAA and vitamin C in aqueous solution during 2 months storage at 25℃ and 40℃. This results show that the stability of vitamin C derivatives mainly depended on pH and temperature. The stability of vitamin C derivatives decreased as pH and temperature increased. Especially vitamin C and vitamin C derivatives was unstable at pH6 and pH7. The stability of 3PEAA350 was higher than 3PEAA550 and 2PEAA was less stable than 3PEAA (data is not shown). The stability of 3PEAA350 was higher than that of 3OEAA below pH5.

Table 7 and Figure 4 shows stabilities of 3PEAA350, 3PEAA550 and 3OEAA in aqueous solution (phosphate buffer, pH6) under UV treatment during 4 hours. 3PEAA350 was more stable than 3PEAA550 and 3OEAA under UV.

This results show that 3PEAA350 was the most stable among PEAAs and was more stable than 3OEAA. We carried out various toxicological tests of most stable 3PEAA350 among PEAAs.

Toxicity: To estimate the safety of 3PEAA350, various safety tests were carried out.
Acute oral toxicity: When 10,000, 5,000, 2,500 mg of 3PEAA350 was administered orally per kg of healthy Sprague-Dawley rats and New Zealand white rabbits, neither observable symptom nor death was observed.
Acute transdermal toxicity: When 10,000, 5,000, 2,500, 1,250, 625 mg/kg of 3PEAA350 was applied to the skin of selected healthy Sprague-Dawley rats and New Zealand white rabbits, no observable change was observed. Autopsies revealed no visual pathological symptoms.
Dermal primary irritation in rabbits: Neither observable any general symptoms nor weight change was observed. Nor did we see any erythema or formation of scale or edema at the application site. The P.I.I. (Primary Irritation Index by Draize) value was zero. Thus, there is no skin irritancy associated with 3PEAA350.
Ocular irritation in rabbits: Neither general symptoms nor weight change was observed. There was no observable turbidity of corne, abnormality of the iris, redness of the conjunctiva, edema or secretion. We concluded that 3PEAA350 does not induce eye irritancy in the New Zealand white rabbits.

Skin sensitization in guinea pig: According to Magnusson and Kligmam's evaluating standard, hypersensitivity score was zero and hypersensitivity induction rate was 0 %. Thus, 3PEAA350 caused no sensitized hypersensitivity in guinea pigs.

Human patch test: Draize score was zero. Therefore, the emulsion containing 5 % 3PEAA350 was nontoxic in various toxicological tests proving that it can be safely introduced to the skin care formulations.

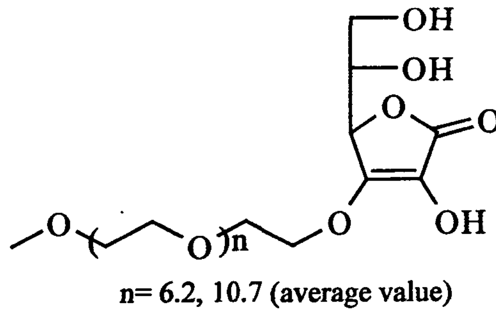
4. Conclusion

Among PEAAs, 2PEAA350 and 2PEAA550 had high scavenging activity against free radical, inhibitory activity against tyrosinase and inhibitory activity of melanogenesis but low stability. 3PEAA350 had high stability and moderate scavenging activity against free radical, inhibitory activity against tyrosinase and inhibitory activity of melanogenesis. The stability, scavenging activity against free radical and inhibitory activity of melanogenesis of 3PEAA350 were higher than those of 3OEAA. The most stable 3PEAA350 among PEAAs was nontoxic in various toxicological tests. These results suggest that PEAA would be a good whitening agent for enhancing stability and bioavailability.

5. References

1. P.R. Gordon et al, *J Invest Dermatol*, 92, 565 (1989).
2. M. Kubo and H. Matsuda, *Fragrance J*, 8, 48-55 (1995).
3. R.A. Clark et al, *J Biol Chem*, 262, 4065-4072 (1987).
4. G. Imokawa and Y.Mishima, *Canser Res*, 42 (1994).
5. Y. Tomita et al, *J InvestDermatol*, 82, 573 (1984).
6. Y. Tomita, *J Act Oxyg Free Rad*, 3, 284 (1992).
7. A.A. Frimer and P.G.Sharon, *J Org Chem*, 60, 2796 (1995).
8. B.Frei et al, *Proc Natl Acad Sci U.S.A.*, 85, 9748 (1988).
9. M. Tanaka et al, *Biochem Biophys Acta*, 1078, 127 (1983).
10. Y. Nihro et al, *J Chem Pharm Bull*, 39, 1731 (1991).
11. S.F. Quadri et al, *Carbohydr Res*, 29, 259 (1973).
12. K kato et al, *J Med Chem*, 31, 793 (1988).
13. M.E. Jung and T. J. Shaw, *J Am Chem Soc*, 102, 6304 (1980).
14. K. Wimalasena and M.P.D. Mahindaratne, *J Org Chem*, 59, 3427 (1994).

15. S. Zalipsky et al, *Eur Polym J,* 19, 1177-1183 (1983).
16. Y. Fugita et al, *Yakugaku Zasshi,* 108(5), 1968-1979 (1986).
17. A. Vanni et al, *Annali di Chimica,* 80, 35-60 (1990).
18. K. Maeda and M. Fukuda, *J Soc Cosmet Chem*, 42, 361 (1991).
19. D.S. Seo et al, *Cosmetics & Toiletries,* 112, 83-90 (1997).
20. Y. Ishida, *Fragrance J,* 28 (1983).
21. K. Morisaki and S. Ozaki, Bull Chem Soc Jpn, 69, 725-734 (1996)
22. Y.S. Song et al, *Cosmetics &Toiletries*, 114, 53-58 (1999).



3-O-[2-[ω -methoxypoly-(oxy-1,2-ethanediyl)] ethyl] ascorbic acid

Fig 1. Chemical Structure of PEAAs.

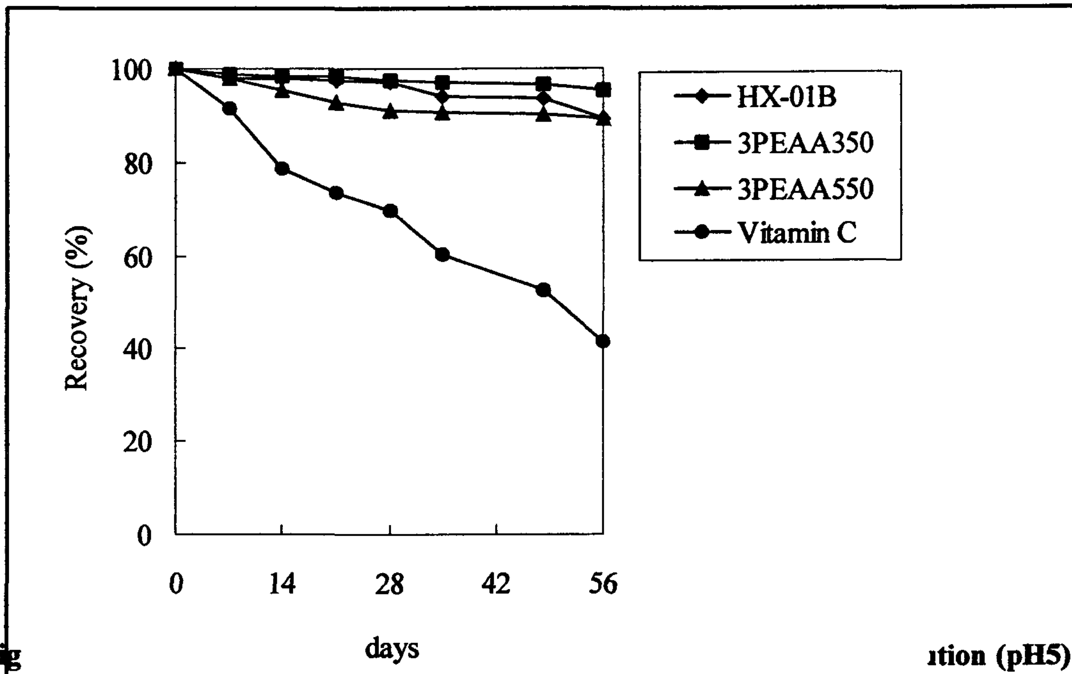


Fig ... ıtion (pH5) stored at 25℃.

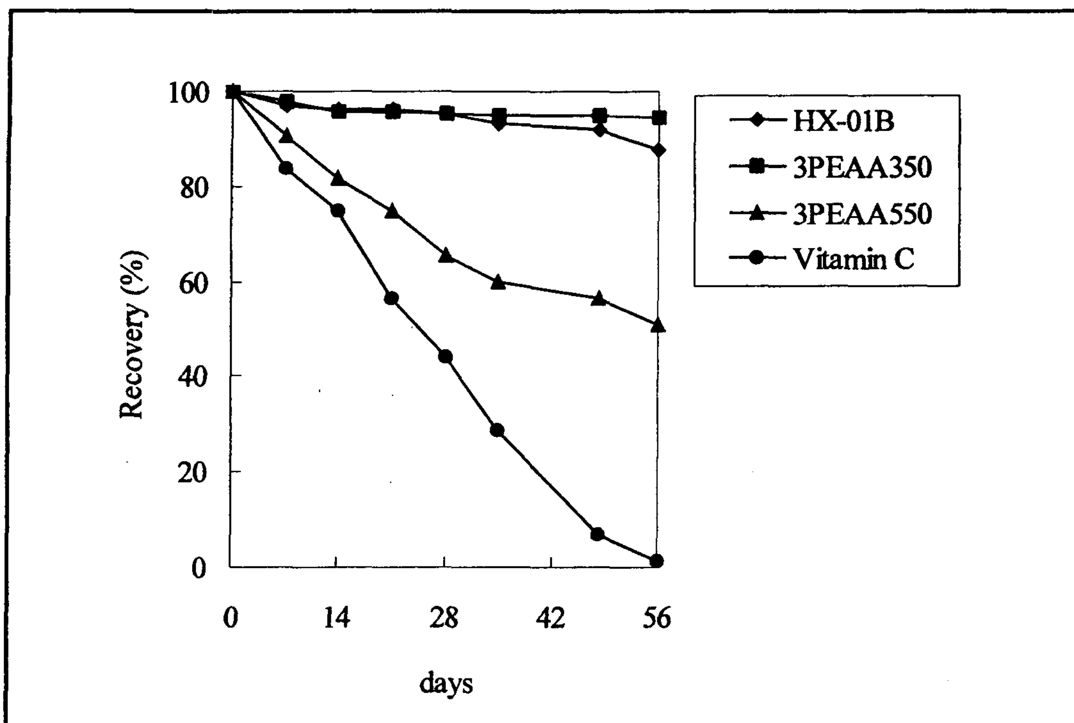


Fig 3. Recovery percent of PEAAs, 3OEAA and vitamin C in aqueous solution (pH5) stored at 40℃.

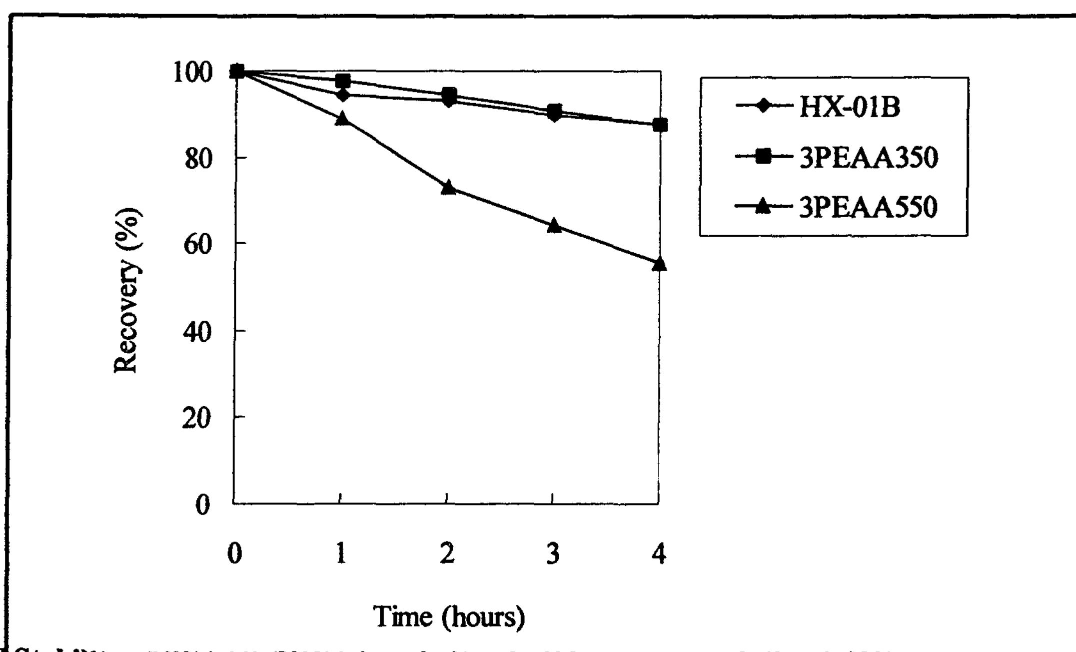


Fig 4. UV Stability of PEAAS, 3OEAA and vitamin C in aqueous solution (pH6).

TABLE 1.
Summary of a series of PEAAs .

compounds	n	MW of PEG	total molecular weight	location linked
2PEAA350	6.2	350	504	C-2
3PEAA350	6.2	350	504	C-3
2PEAA550	10.7	550	725	C-2
3PEAA550	10.7	550	725	C-3
3OEAA			208	C-3
Vitamin C			176	

TABLE 2.

Free radical scavenging activity against DPPH for 30min and 24 hours.

IC_{50} is the concentration at which 50% of free radicals are scavenged/inhibited.

	($IC_{50}/10^{-5}M$)	
compounds	30 min	24 hr
2PEAA350	7.9	6.0
3PEAA350	8.0	6.0
2PEAA550	4.1	3.4
3PEAA550	27.6	3.4
3OEAA	10.0	10.0
Vitamin C	3.4	3.4

TABLE 3.

Inhibitory activity against tyrosinase-catalyzed oxidation of tyrosine.

IC_{50} is concentration of compound which inhibits 50% of enzyme activity.

compounds	**($IC50/10^{-3}$ M)**
2PEAA350	2
3PEAA350	10
2PEAA550	7
3PEAA550	14
3OEAA	10
Vitamin C	0.06~0.1

TABLE 4.

Inhibitory activity of melanin synthesis in B-16 melanoma cell.

This is concentration of compounds that has inhibitory effect on melanin synthesis; + slightly effective, ++ moderately effective.

($10^{-3}M$)

compounds	+	++
2PEAA350	4	10
3PEAA350	4	10
2PEAA550	3	7
3PEAA550	3	7
3OEAA	20	ppt
Vitamin C	1	3

TABLE 5.

Stability of PEAAs, 3OEAA and vitamin C in aqueous solution stored at 25℃.

(Recovery %)

compounds	pH3		pH4		pH5		pH6		pH7	
	1mon.	2mon.	1mon.	2mon.	1mon.	2mon.	1mon.	2mon.	1mon.	2mon.
3PEAA350	98.9	96.1	97.6	96.1	97.5	95.5	96.9	93.5	73.2	59.1
3PEAA550	91.9	89.1	90.6	85.5	90.9	89.3	73.2	59.1	2.9	0.0
3-OEAA	98.0	90.5	97.5	89.8	96.8	89.2	96.1	8.9	85.3	71.2
Vitamin C	72.4	46.8	-	-	69.5	41.5	53.0	27.5	0.0	0.0

TABLE 6.

Stability of PEAAs, 3OEAA and vitamin C in aqueous solution stored at 40℃.

(Recovery %)

compounds	pH3		pH4		pH5		pH6		pH7	
	1mon.	2mon.	1mon.	2mon.	1mon.	2mon.	1mon.	2mon.	1mon.	2mon.
3PEAA350	98.5	97.3	95.6	94.5	95.1	94.3	86.2	83.1	38.1	32.4
3PEAA550	77.0	68.3	75.1	69.3	65.2	51.0	28.1	8.4	0.0	0.0
3-OEAA	97.4	88.0	5.3	87.3	95.1	87.4	94.7	86.9	53.5	39.2
Vitamin C	50.6	6.1	-	-	44.2	1.2	10.2	0.0	0.0	0.0

TABLE 7.
UV Stability of PEAAs, 3OEAA and vitamin C in aqueous solution (pH6).

			(Recovery %)		
compounds	0	1 hour	2 hours	3 hours	4 hours
3PEAA350	100	97.5	94.5	91.0	87.5
3PEAA550	100	89.1	73.1	64.2	55.3
3OEAA	100	94.4	93.3	90.0	87.8