Arch Pharm Res Vol 22, No 3, 232-236, 1999

Aloesin and Arbutin Inhibit Tyrosinase Activity in a Synergistic Manner via a Different Action Mechanism

Ying Hua Jin[1], Suk Jin Lee[1], Myung Hee Chung[2], Jeong Hill Park[1], Young In Park[3], Tae Hyeong Cho[4] and Seung Ki Lee[1]

[1]College of Pharmacy, [2]College of Medicine; Seoul National University, Seoul 151-742, Korea, [3]College of Natural Resources, Korea University, Seoul 136-701, Korea, [4]Namyang Aloe Bio-science Research Institute, 370-92, Yongjeong-Ri, Korea

(Received November 10, 1998)

In this study, we present evidence that cotreatment of aloesin and arbutin inhibits tyrosinase activity in a synergistic manner by acting through a different action mechanism. Aloesin or arbutin similarly inhibited enzyme activity of human- and mushroom-tyrosinases with an IC_{50} value of 0.1 or 0.04 mM, respectively. Lineweaver-Burk plots of the enzyme kinetics data showed that aloesin inhibited tyrosinase activity noncompetitively with a Ki value of 5.3 mM, whereas arbutin did it competitively (Maeda, 1996). We then examined whether cotreatment of these agents inhibits the tyrosinase activity in a synergistic manner. The results showed that 0.01 mM aloesin in the presence of 0.03 mM arbutin inhibited activity of mushroom by 80% of the control value and the reverse was also true. The inhibitory effects were calculated to be synergistic according to the Bürgi method. Taken together, we suggest that aloesin along with arbutin inhibits in synergy melanin production by combined mechanisms of noncompetitive and competitive inhibitions of tyrosinase activity.

Key words : Synergistic tyrosinase inhibitors, Aloesin, Arbutin

INTRODUCTION

Tyrosinase is a copper-containing mono-oxygenase (EC 1.14.18.1) that catalyzes the ortho-hydroxylation of monophenols and the oxidation of o-diphenols to o-quinones (Mason, 1965). The enzyme is responsible for the conversion of tyrosine into dopa (3,4-dihydroxy-phenyl-ananine) and in the synthesis of melanin (Pawelek 1976). Hyperpigmentation on human skin has been shown to be caused by over-production of dermal melanin pigment, which is synthesized in the melanocytes by the action of tyrosinase. Thus, tyrosinase has been extensively used as a target enzyme to search for depigmenting agents (Morrison, 1985; Robert, 1989; Shiroda, 1994; Maeda, 1991). A number of tyrosinase inhibitor have been isolated and characterized for the depigmenting action. For example, linoleic acid can efficiently inhibit tyrosinase activity but exhibits a cytotoxic effect (Meada, 1991). Hinokitiol, kojic acid, naturally occurring hydroquinones and catechols were also shown to inhibit the enzyme activity (Mishma, 1988; Smith, 1988). However, these compounds reveal not only weaker depigmenting effect than does arbutin, but also exhibit some types of adverse side effects (Meada, 1991). Others also reported that several vanillyl compounds, such as ferulic acid, curcumin, and capsaicin can inhibit tyrosinase activity (Shirota, 1994). Recently, aloesin, a chromone derivative isolated from fresh leaf of Aloe vera, and arbutin, a hydroquinone glycoside isolated from *gvae grsi* folium strongly inhibit tyrosinase activity (Yagi,1987). Albutin was reported to inhibit the enzyme activity competitively (Meada, 1996). Other reports, by contrast, have suggested that the inhibitory mechanism of alpha-arbutin may be mixed type inhibition, while that of beta-arbutin is non-competitive (Funayama, 1995). Here, we present evidence that aloesin inhibits mushroom-tyrosinase as well as human tyrosinase activity by non-competitive inhibition mechanism, whereas arbutin does it by competitive inhibition mechanism. On the basis of these results, we determined whether cotreatment of aloesin and arbutin can inhibit tyrosinase activity in a synergistic manner. The results showed that aloesin together with arbutin can synergistically inhibit tyrosinase activity as evaluated by Bürgi method (Okabe, 1976) and hence, the effective doses can be significantly reduced for the same inhibitory activity against tyrosinase.

Correspondence to: Seung Ki Lee, *College of Pharmacy*

MATERIALS AND METHOD

Materials

Aloesin (M.W: 394) was kindly provided by Namyang Aloe Co. Ltd. Mushroom-Tyrosinase, general biochemicals and chemicals were purchased from Sigma. L-[3,5-^{3}H]-Tyrosine was purchased from Amersham Corp. Calf serum, Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco BRL. sk-Mel I cells were obtained from the Seoul National University Cancer Center.

Tyrosinase assay

Tyrosinase activity was determined by Pomerantz method (Korner, 1977) with a slight modification. The reaction mixture contained 0.1 U/μl mushroom-tyrosinase, 0.1 M K_2HPO_4-KH_2PO_4 (pH6.8) buffer, 0.02 μCi/μl (6×10^{-5}M) L-[3,5-^{3}H]-tyrosine and indicated concentrations of aloesin, arbutin, or aloesin/arbutin. The reaction mixture was incubated at 37°C for 20 min, and then, 30 μl of activated charcoal (50mg/ml in 0.2N HCl) was added. This mixture was then mixed briefly with vortex mixer, centrifuged at 15,000 g for 15 min. 50 μl of the supernatant was mixed with 300 μl of activated Dowex 50 resin. After briefly centrifuging the mixture, radioactivity in the supernatant was counted with liquid scintillation counter. The synergistic inhibitory effect of aloesin and arbutin on tyrosinase activity was evaluated according to Bürgi method (Okabe, 1976). The synergistic inhibitory effect of aloesin together with arbutin on tyrosinase activity was calculated as followings: % activity=(A-C)/(B-C)×100, in that A and B denote enzyme activities in DPM in the presence and in the absence of inhibitors, respectively, and C denotes DPM without adding enzyme. When $m^xn > S$, M and N, inhibitors are evaluated to have synergistic effect on enzyme activity, in that m, n, and S denote % of control activities tested with inhibitors; M, N, and both M and N, respectively.

Cell cultures and purification of human melanoma tyrosinase

sk-Mel-1 cells were cultured at 37°C under humidified air with 5% CO_2 for 3 days in DMEM supplemented with 5% calf serum. Human tyrosinase was purified as previously described (Wittbjer, 1990) with a slight modification. Cultured cells were harvested, washed with 0.9% of NaCl, and then, homogenized in the same solution. After centrifugation at 40,000 g for 1 hour, the pellet was lysed in buffer containing 0.1 M Tris-HCl, pH 7.5, 2.5% Triton X-100, and dialysed in buffer solution containing 10 mM Tris-HCl, pH 7.5, 0.1 mM Triton X-100 for 24 hour. Proteins were then, precipitated with a gradient concentration of $(NH_4)_2SO_4$, and the fraction of proteins precipitated with 40~60% saturation of $(NH_4)_2SO_4$ was taken for the subsequent experiments. The pellet was dissolved and dialyzed in dialysis buffer. After dialysis, tyrosinase was purified by Con-A-Sepharose chromatography. The specific enzyme activity was 550 U/mg proteins.

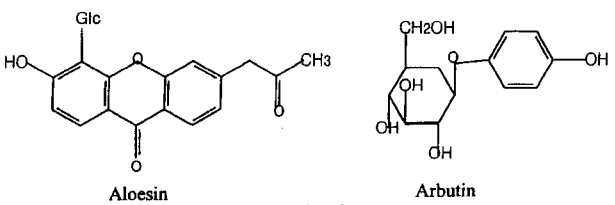

Fig. 1. Structures of aoesin and arbutin.

RESULTS AND DISCUSSION

Dose-dependent inhibition of mushroom- and human melanoma-tyrosinase activity by aloesin or arbutin

To examine whether aloesin or arbutin inhibits human melanoma-tyrosinase activity as does mushroom-tyrosinase activity, we determined the inhibitory effects of these agents on human tyrosinase activity in a dose dependent manner. The results showed that aloesin as well as arbutin strongly inhibited the enzyme activity of mushroom- and human melanoma-tyrosinase with a similar dose-dependency (Fig. 2A and B). The data showed that the IC_{50} values of aloesin and arbutin against human-tyrosinase activity were 0.1 and 0.04 mM, respectively

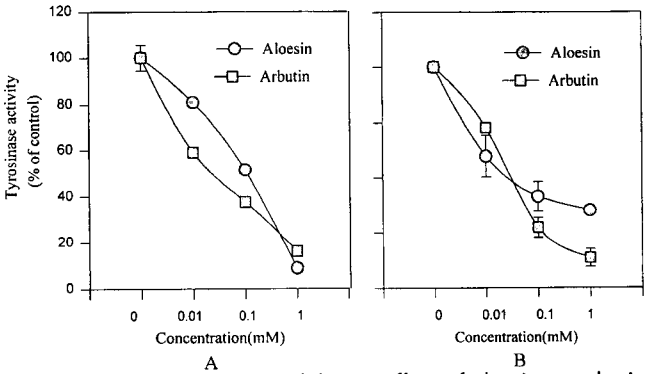

Fig. 2. A: Dose-dependent inhibitory effect of aloesin or arbutin on tyrosinase activity. Tyrosinase activity was determined by Pomerantz method as described in materials and methods. A: Inhibitory effects on mushroom tyrosinase activity. IC_{50} values were calculated to be 0.1 and 0.04 mM for aloesin and arbutin, respecely. B: Inhibitory effect on human melanoma sk-Mel-1 tyrosinase activity. Sk-Mel-1 tyrosinase was partially purified from cultured sk-Mel-1 cells as described in Materials and Methods. Specific activity of tyrosinase was 550 U/mg proteins. IC_{50} values were calculated to be approximately 0.1 and 0.04 mM for aloesin and arbutin, respectively. Each value represents the means of triplicate determinations±SE.

(Fig. 2B), whose values were similar to those against mushroom-tyrosinase activity (Fig. 2A). The results suggested that aloesin and arbutin can similarly inhibit enzyme activity of both human melanoma- and mushroom-tyrosinases.

Kinetic analysis of the inhibitory effect of aloesin on tyrosinase activity

In the earlier reports, it has been suggested that arbutin inhibits mushroom-tyrosinase activity competitively with a Ki value of 2.01×10^{-3} M (Maeda, 1996). Here, we examined whether aloesin can inhibit mushroom-tyrosinase activity with a similar inhibition mechanism. As shown in Fig. 3, Lineweaver-Burk plots showed that aloesin at 4×10^{-4} M and 8×10^{-4} M could inhibit in non-competitive manner the tyrosinase activity with a Ki value of 5.25×10^{-3} M. Under the same experimental conditions, we also confirmed that arbutin can competitively inhibit mushroom-tyrosinase activity with a similar Ki value as previously reported (data not shown). We also examined whether aloesin or arbutin can inhibit human-tyrosinase activity with the same action mechanisms of these compounds shown against mushroom-tyrosinase activity. The results showed that aloesin and arbutin inhibit the human tyrosinase activity with similar Ki values and the same inhibition mechanisms (data not shown). The data suggested that aloesin and arbutin can similarly inhibit the enzyme activity of both mushroom- and human-tyrosinases with the same action mechanisms and potency.

Cotreatment of aloesin and arbutin inhibits tyrosinase activity in a synergistic manner

Since aloesin and arbutin inhibit tyrosinase activity with a different action mechanism, we tested whether cotreatment of these compounds can synergistically inhibit the enzyme activity by combined inhibitory action mechanisms. For this, we determined the enzyme activity in the presence of a fixed concentration of arbutin, 0.03 mM with varying concentration of aloesin, or vice versa (Table I, Fig. 4A and 4B). The results showed that aloesin could synergistically inhibit in the presence of 0.03 mM both mushroom- and human-tyrosinase activity (data not shown for human tyrosinase activity) (Fig. 4A). The data showed that 0.01 mM aloesin

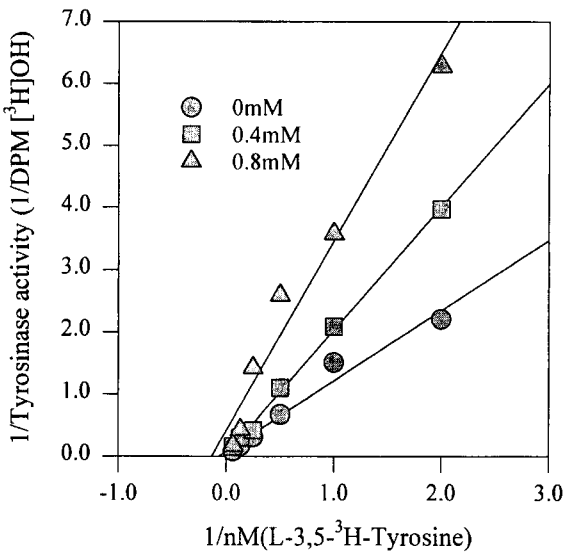

Fig. 3. A double-reciprocal plot of mushroom tyrosinase kinetics in the absence or presence of aloesin, 0.4 or 0.8 mM. The values represent incremental [^{3}H]OH. Each value represents the means of triplicate determinations±SE. Lineweaver-Burk Plots indicate that aloesin inhibits tyrosinase activity noncompetitively with Ki value of 5.25×10^{-4} M.

Table I. The synergistic effect of aloesin and arbutin on tyrosinase activity

Treatment	Dose (mM)	Inhibition rate (%)	Incidence (%)	Calculation for potentiation
Aloesin	0.00	0.0±5.7	100 ±5.7	
	0.01	19.0±1.0	81.0±1.0	
	0.03	23.0±1.8	77.0±1.8	
	0.10	48.5±13.4	51.5±1.4	
	1.00	91.2±1.0	8.8±1.0	
Arbutin	0.00	0.0±7.9	100 ±7.9	
	0.01	40.9±1.9	59.1±0.9	
	0.03	54.2±0.9	45.8±0.9	
	0.10	62.4±0.3	37.6±0.3	
	1.00	83.9±1.0	16.1±1.0	
Aloesin+Arbutin	0.03+0.01	79.4±2.2	20.6±2.2	77.0×59.1=45.5>20.6
	0.03+0.03	89.8±0.7	10.2±0.7	77.0×45.8=35.3>10.2
	0.03+0.10	90.8±0.3	9.2±0.3	77.0×37.6=29.0> 9.2
Arbutin+Aloesin	0.03+0.01	76.4±3.8	23.6±3.8	45.8×81.0=37.1>23.6
	0.03+0.03	89.8±0.7	10.2±0.7	45.8×77.0=35.3>10.2
	0.03+0.10	95.5±0.4	4.5±0.4	45.8×51.5=23.6> 4.5

*Enzyme assay was carried out under the conditions given in materials and methods. The values are means±SE of three measurements. The synergistic effects were evaluated according to Bürgi method.

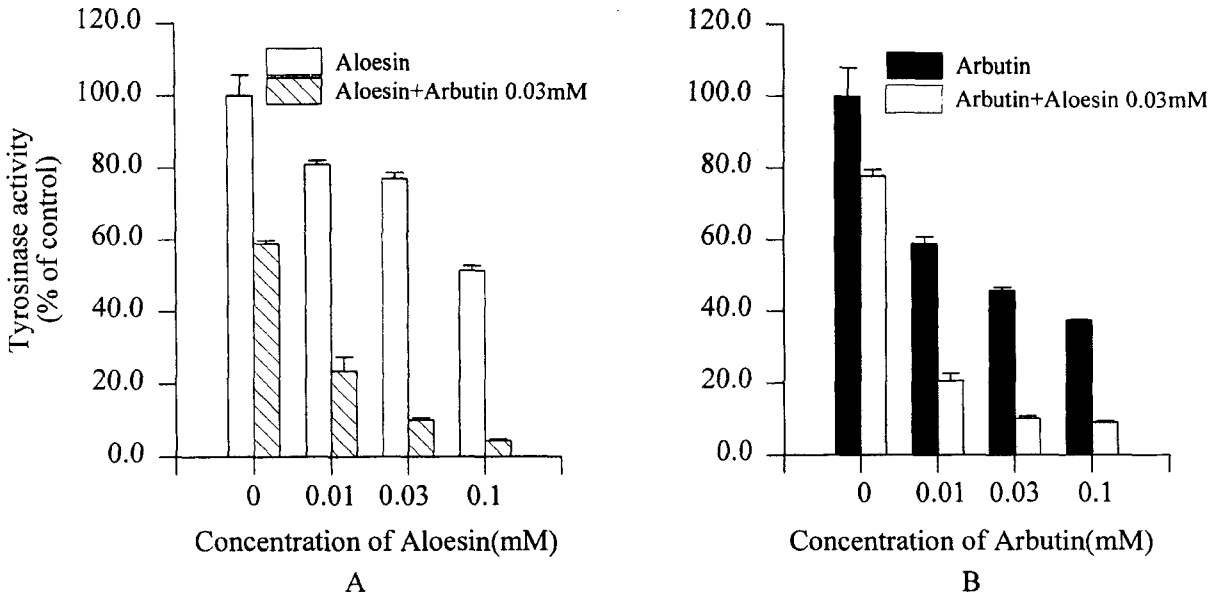

Fig. 4. The synergistic inhibitory effect of aloesin and arbutin on tyrosinase activity. Assays were carried out with mushroom tyrosinase. A: Dose-dependent effect of aloesin on tyrosinase activity in the presence of 0.03 mM arbutin. B: Dose-dependent effect of arbutin on tyrosinase activity in the presence of 0.03 mM aloesin. Each value represents the means of triplicate determinations ± SE. The synergistic effects were evaluated according to Bürgi method.

together with 0.03 mM arbutin inhibited the enzyme activity by 86% as compared with the control value. The inhibitory effect of aloesin at the same concentration was 4.5-fold lower than that of cotreatment of aloesin together with 0.03 mM arbutin. The reversed experiments also showed the same results (Fig. 4B). To evaluate whether the cotreatments may result in synergistic inhibitory effects on tyrosinase activity, we calculated the potentiation values according to the Bürgi method. As shown in Table I, the mxn values, potentiation values were larger than those of the incidence values in all the concentrations of inhibitors as indicated. Thus, it is evident that aloesin and arbutin act through different inhibitory mechanisms and hence, synergistically inhibit the enzyme activity by binding to different acting sites. In the earlier reports, it has been suggested that arbutin inhibits tyrosinase activity by a mixed type- or competitive-inhibition (Funayama, 1995). However, our data clearly demonstrated that arbutin inhibits the enzyme activity by competitive inhibition mechanism. The notion is supported by the results that aloesin together with arbutin synergistically inhibits the enzyme activity. Taken these data together, it is evident that aloesin and arbutin, which are world-widely used as whitening agents in cosmetics, efficiently inhibit both human and mushroom-tyrosinase activity via acting through non-competitive and competitive inhibition mechanisms, respectively. In addition, cotreatment of these agents clearly showed a synergistic inhibitory effect on the enzyme activity of human-and mushroom-tyrosinases. Taken together, we suggest that aloesin along with arbutin inhibit tyrosinase activity in a synergistic manner. Thus, it is advantageous to use aloesin and arbutin as a mixture for depigmenting effect, as the cotreatment can cut down the effective doses of these agents for the same inhibitory effect on tyrosinase activity and can reduce potentially adverse side-effect.

ACKNOWLEDGEMENTS

This work was supported by research grants from Namyang Aloe Co. Ltd. and from the Ministry of Korean Science and Technology (1997).

REFERENCES CITED

Funayama, M., Arakawa, H., Yamamoto, R., Nishino, T., Shin, T. and Murao, S., Effects of alpha-and beta-arbutin on activity of tyrosinases mushroom and mouse melanoma (1995) *Biosci Biotechnol Biochem.*, 59(1), 143-144 (1995)

Lerch, K., Monophenol monooxygenase from Neurospora crassa. *Methods Enzymol.*, 142, 165-169 (1987).

Maeda, K. and Fukuda, M., *In vitro* effectiveness of several whitening cosmetic components in human melanocytes *J. Soc. Chem.*, 42, 361-368 (1991).

Maeda, K., Fukuda, M., Arbutin: mechanism of its depigmenting action in human melanocyte culture. *J Pharmacol Exp Ther.*, 276(2), 765-769 (1996).

Mason, H. S., *Annu. Rev. Bichem.*, 34, 595-634 (1965).

Mishima, Y., Hatta, S., Ohyama, Y. and Inazu, M. Induction of melanogenesis suppression: cellular pharma-

cology and mode of differential action *Pigment Cell Res.*, 1(6), 367-374 (1988).

Morrison, M. E., Yagi, M. J., Cohen G., *In vitro* studies of 2,4-dihydroxyphenylalanine, a prodrug targeted against malignant melanoma cells. *Proc Natl Acad Sci USA*, 82(9), 2960-4 (1985)

Okabe, S., Takeuchi, R., Urushidani, T. and Murata, T., Effects of aluminum hydroxide- magnesium oxide(AH MO), Dicyclomine HCL and AH-MO+Dicyclomine HCL on acute gastric ulcers in experimental animals *Applied Pharmacology (Japan)*, 12(5), 759-764 (1976).

Pawelek, J. M., Factors regulating growth and pigmentation of melanoma cells. *J Invest Dermatol.*, 66(4), 201-209 Review (1976).

Pomerantz, S. H., Separation, Purification, and Properties of Two Tyrosinases from Hamster Melanoma *J. Biol. Chem.*, 238, 2351-2357 (1963).

Robert, C., Hider and Lerch, K., The inhibition of tyrosinase by pyridinones *Biochem. J.*, 257, 289-290 (1989).

Shirota, S., Miyazaki, K., Aiyama, R., Ichioka, M. and Yokokura, Tyrosinase inhibitors from crude drugs. *Biol Pharm Bull.*, 17(2), 266-9 (1994).

Smith, C. J., OHare, K. B., Allen, J. C., Selective cytotoxicity of hydroquinone for melanocyte-derived cells is mediated by tyrosinase activity but independent of melanin content *Pigment. Cell Res.*, 1(6), 386-389 (1988).

Wittbjer, A., Odh, G., Rosengren, A. M., Rosengren, E. and Rorsman, H., Isolation of soluble tyrosinase from human melanoma cells *Acta. Derm. Venereol.*, 70(4), 291-294 (1990).

Yagi, A., Kanbara, T. and Morinove, N., Inhibition of mushroom-tyrosinase by Aloe extract. *Planta Medica.*, 515-517 (1987).