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Inhibitory effects of 4-chlorosalicylic acid on mushroom tyrosinase and its antimicrobial activities

Peng Han [a], Chao-Qi Chen [b], Chun-Le Zhang [a], Kang-Kang Song [a,c], Han-Tao Zhou [a,*], Qing-Xi Chen [a,c,*]

[a] *Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, China*
[b] *College of Environmental Sciences, Peking University, Peking 100871, China*
[c] *Key Laboratory for Chemical Biology of Fujian Province, Xiamen University, Xiamen 361005, China*

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Abstract

The inhibitory effects of 4-chlorosalicylic acid on the activity of mushroom tyrosinase have been investigated. The results showed that 4-chlorosalicylic acid could strongly inhibit both monophenolase activity and diphenolase activity. The IC_{50} values were estimated as 1.89 mM and 1.10 mM for monophenolase and diphenolase activities, respectively. For the monophenolase activity, 4-chlorosalicylic acid could not only lengthen the lag time, but also decrease the steady-state rate. For the diphenolase activity, kinetic analyses showed that the inhibition by 4-chlorosalicylic acid was reversible and its mechanism was mixed-II type, which is different from salicylic acid. The inhibition constants (K_I and K_{IS}) were determined to be 1.51 mM and 0.82 mM, respectively. Furthermore, the antibacterial activity against *Escherichia coli*, *Bacillus subtilis* and *Staphyloccocus aureus* and antifungal activity against *Aspergillus niger* and *Candida boidinii* were investigated. The results showed that 4-chlorosalicylic acid was the most effective against *E. coli* with the MIC of 250 μg/ml and with the MBC of 500 μg/ml.

Keywords: Mushroom tyrosinase; 4-Chlorosalicylic acid; Monophenolase activity; Diphenolase activity; Inhibition kinetics; Bacteriostatic activity; Antimycotic activity

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing multifunctional oxidase that is widely distributed in fungi, plants and animals (Whitaker, 1995). It catalyzes both the hydroxylation of monophenols and the oxidation of *o*-diphenol into *o*-quinones, which polymerize to form brown or black pigments (Martynez & Whitaker, 1995; Prota, 1988). The browning in vegetables and fruits which is caused by tyrosinase is an undesirable reaction. This unfavourable darkening from enzyme oxidation generally results in a less attractive appearance and a loss in nutritional quality. It becomes a major problem in the food industry. Therefore, tyrosinase inhibitors have been established as important constituents of depigmentation agents (Likhitwitayawuid & Sritularak, 2001) and have potential uses as food preservatives (Scotter & Castle, 2004). In addition, due to the decrease in melanization caused by the enzyme action in animals, tyrosinase inhibitors are supposed to have broad applications in medicinal and cosmetic whitening agents (Fenoll, Rodríguez-López, & García-Sevilla, 2001; Friedman, 1996).

Abbreviations: DMSO, dimethylsulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; Tyr, L-tyrosine; IC_{50}, the inhibitor concentrations leading to 50% activity lost; PDA, potato dextrose agar; CFU, colony forming unit; MIC, the minimum inhibitory concentration; MBC, the minimum bactericidal concentration; MFC, the minimum fungistatic concentration.

* Corresponding authors. Address: Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, China. Tel./fax: +86 592 2185487.
E-mail address: chenqx@xmu.edu.cn (Q.-X. Chen).

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Many efforts have been spent in the search for feasible and effective tyrosinase inhibitors. Although a large number of tyrosinase inhibitors have already been reported, some of their individual activities are not potent enough to be put to practical use. In the previous paper, some methyl or methoxyl or hydroxyl salicylic acid compounds were reported to inhibit the oxidation of L-DOPA, catalyzed by mushroom tyrosinase (Zhang, Chen, Song, & Xie, 2006). For the continuing investigation, 4-chlorosalicylic acid was found to have a more potent inhibitory effect on mushroom tyrosinase. The aim of this present work is, therefore, to carry out a kinetic study of the inhibition of the diphenolase and monophenolase activities of tyrosinase by 4-chlorosalicylic acid, to evaluate the kinetic parameters and inhibition constants characterizing the system and to investigate the inhibition mechanism. Furthermore, we have found that 4-chlorosalicylic acid has bacteriostatic and antifungal activity. All these data may provide a basis for developing novel tyrosinase inhibitors and new potent food preservatives or cosmetic additives.

2. Materials and methods

2.1. Reagents

Tyrosinase (EC 1.14.18.1) from mushroom was the product of Sigma Chemical Co (St. Louis, MO, USA). The specific activity of the enzyme was 6680 U/mg. 4-Chlorosalicylic acid, dimethylsulfoxide (DMSO), L-3,4-dihydroxyphenylalanine (L-DOPA) and L-tyrosine (Tyr) were obtained from Aldrich (St. Louis, MO, USA). All microorganisms were donated from Professor Zheng Z H (Institute of Microbiology and Marine Medicine of Xiamen University, China). All other reagents were local and of analytical grade. The water used was re-distilled and ion-free.

2.2. Enzyme assay

The enzyme activity assay was performed as reported by Chen et al. (2005). In this investigation, Tyr was used as the substrate for the monophenolase activity assay, and L-DOPA was used as a substrate for the diphenolase activity assay. The reaction media (3 ml) for activity assay contained 2.0 mM Tyr or 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). The final concentration of mushroom tyrosinase was 33.3 μg/ml for the monophenolase activity and 6.67 μg/ml for the *o*-diphenolase activity. The reaction was carried out at a constant temperature of 30 °C. 4-Chlorosalicylic acid was first dissolved in DMSO. The final concentration of DMSO in the test solution was 3.3%. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out (Chen, Liu, & Huang, 2003). The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). The inhibition type was assayed by the Lineweaver–Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor.

2.3. Antimicrobial assay

Antibacterial activities were determined in tryptone beef extract agar, at pH 7.2, with an inoculum of $1–2 \times 10^5$ cells/ml. Also, antimycotic activities of the yeast were determined using potato dextrose agar (PDA) medium, with an inoculum of $1–2 \times 10^5$ colony forming units (CFU)/ml. The antimicrobial activity of 4-chlorosalicylic acid was determined using the agar well diffusion method, following a published procedure with slight modification (Owais, Sharad, Shehbaz, & Saleemuddin, 2005; Perez, Pauli, & Bazerque, 1990). Briefly, culture medium was inoculated with the given microorganism by spreading the bacterial inoculum in the media. Wells (7 mm diameter) were punched in the agar and filled with 4-chlorosalicylic acid of different concentrations. Control wells, containing neat DMSO (negative control) and standard antibiotics, such as gentamycin sulfate (2000 U/ml) for the tested bacteria or K_2CrO_4 (1 mg/ml) against the tested fungi (positive control), were also run parallel in the same plate. Bacteria were incubated at 37 °C for 24 h, and fungi were incubated at 28 °C for 48 h. The antimicrobial activity was assessed by measuring the diameter of the zone of inhibition for the respective drug.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were tested by broth macrodilution methods (Kubo, Fujita, Kubo, Nihei, & Ogura, 2004). Briefly, serial 2-fold dilutions of the test compounds were prepared in DMSO, and 30 μl of each dilution was added to 3 ml of the above medium with the same inoculum of $1–2 \times 10^5$ cells/ml and under the same cultural condition. After the cultures were incubated at 37 °C for 24 h, MIC was determined as the lowest concentration of the test compound that demonstrated no visible growth. After the determination of the MIC, 100-fold dilutions with drug-free medium from each tube showing no turbidity were incubated at 37 °C for 48 h. The MBC was the lowest concentration of the test compound that showed no visible growth in the drug-free cultivation. For fungi, except the cultural condition, other processes were the same.

3. Results

3.1. Effect of 4-chlorosalicylic acid on the diphenolase activity of mushroom tyrosinase

The progress curve of the oxidation reaction of L-DOPA by mushroom tyrosinase was a line passing through the origin, which indicated that the formation of product was in proportion to the reaction time. The value of the slope of the line indicated the diphenolase activity. Using 4-chlorosalicylic acid (see Fig. 1 for structure) as inhibitor, the inhibitory concentration-effect on the diphenolase activity

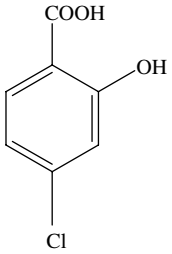

Fig. 1. Chemical structure of 4-chlorosalicylic acid.

of mushroom tyrosinase was assayed. The diphenolase activity decreased with increasing inhibitor concentrations. When the concentration of inhibitor reached 2.0 mM, enzyme activity was inhibited by 65% (Fig. 2). The IC_{50} value of 4-chlorosalicylic acid was determined to be 1.05 mM. The data are summarised in Table 1.

3.2. The inhibitory mechanism of 4-chlorosalicylic acid on the diphenolase activity of mushroom tyrosinase

The inhibition mechanism, by 4-chlorosalicylic acid, on the enzyme for the oxidation of L-DOPA was first studied.

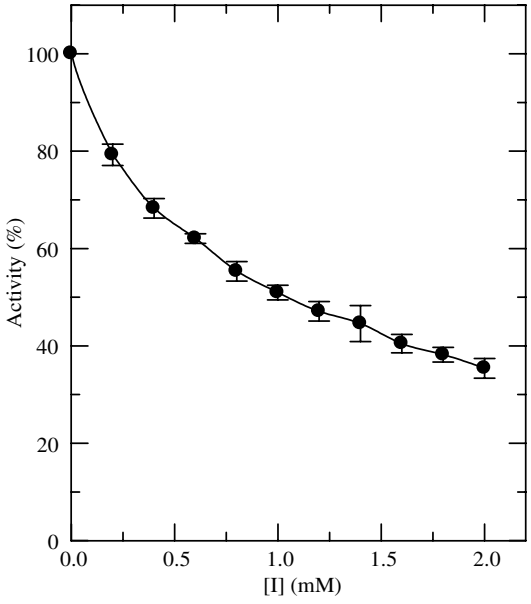

Fig. 2. Effect of 4-chlorosalicylic acid on the diphenolase activity of mushroom tyrosinase.

Table 1
Kinetic constants and inhibition constants of 4-chlorosalicylic acid on mushroom tyrosinase

	Constants
IC_{50}	
Monophenolase	1.89 mM
Diphenolase	1.05 mM
Inhibition	Reversible
Inhibition type	Mixed-II
K_I	1.51 mM
K_{IS}	0.82 mM

The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of 4-chlorosalicylic acid gave a family of straight lines, which all passed through the origin (Fig. 3). Increasing the inhibitor concentration resulted in a descending of the slope of the line, indicating that the inhibition by 4-chlorosalicylic acid of the diphenolase was reversible. The presence of 4-chlorosalicylic acid did not bring down the amount of the efficient enzyme, but just resulted in the inhibition of enzyme activity.

3.3. Determination of the inhibition type of 4-chlorosalicylic acid on the activity of mushroom tyrosinase

The inhibitory kinetics of mushroom tyrosinase by 4-chlorosalicylic acid are illustrated in Fig. 4. Under the experimental conditions employed, the oxidation reaction of L-DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. Double-reciprocal plots yielded a family of straight lines intersected at the 3rd quadrant. The values both of K_m and V_m decreased with increasing inhibitor concentration. Thus, 4-chlorosalicylic acid was a competitive-uncompetitive mixed-II type inhibitor. The uncompetitive effect was stronger than the competitive effect, indicating that 4-chlorosalicylic acid inhibited the free enzyme more weakly than the enzyme-substrate complex. The equilibrium constants for inhibitor binding with the free enzyme and the enzyme-substrate complex, K_I and K_{IS}, were obtained from the double-reciprocal plots of the slope and the vertical intercept versus the concentration of 4-chlorosalicylic acid, respectively. The values of K_I and K_{IS} were determined to be 1.51 mM and 0.82 mM, respectively. The results are summarized in Table 1.

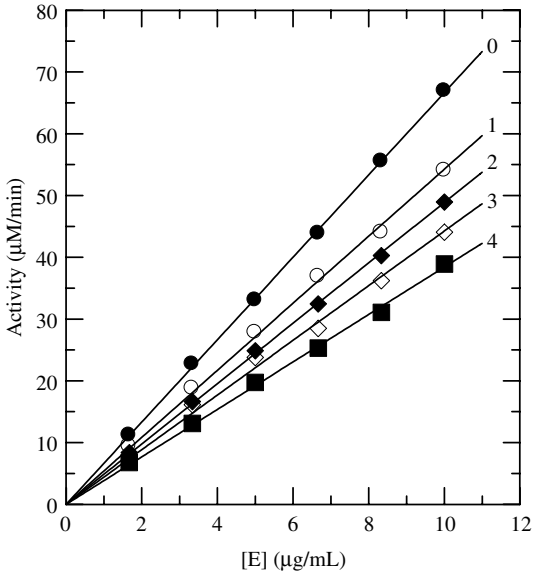

Fig. 3. Determination of the inhibitory mechanism of 4-chlorosalicylic acid on mushroom tyrosinase. The concentrations of 4-chlorosalicylic acid for curves 0–4 were 0, 0.25, 0.5, 0.75 and 1.0 mM, respectively.

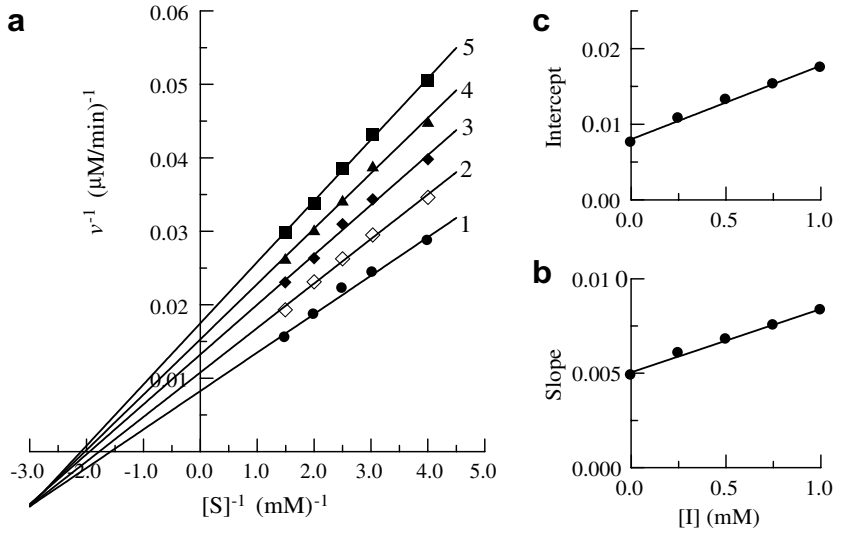

Fig. 4. Determination of the inhibitory type and inhibition constants of 4-chlorosalicylic acid on mushroom tyrosinase. (a) Lineweaver–Burk plots for inhibition of 4-chlorosalicylic acid on mushroom tyrosinase. The concentrations of 4-chlorosalicylic acid for curves 1–5 were 0, 0.25, 0.50, 0.75 and 1.0 mM, respectively. (b) and (c) represent the plots of slope and intercept versus the concentration of 4-chlorosalicylic acid for determining the inhibition constants K_I and K_{IS}, respectively.

3.4. Effect of 4-chlorosalicylic acid on the monophenolase activity of mushroom tyrosinase

The effects of 4-chlorosalicylic acid, at different concentrations, on the oxidation of L-tyrosine by the enzyme were assayed. The kinetic course of the oxidation of the substrate in the presence of 4-chlorosalicylic acid is shown in Fig. 5. A marked lag period, characteristic of monophenolase activity, was observed simultaneously with the appearance of the first stable product, dopachrome (Fig. 5) (Fenoll et al., 2001). The system reached a constant rate after the lag period, which was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa. After the reaction system reached the steady state, the curve of product increased linearly with increasing reaction time; the slope of the line denoted the steady-state rate. In the presence of different concentrations of 4-chlorosalicylic acid, the lag time and the steady-state rate were determined and the results are showed in Fig. 6. The lag time was prolonged to a great extent with increasing inhibitor concentration; meanwhile, the steady state rate decreased distinctly. When the concentration of 4-chlorosalicylic acid increased from 0 to 2.0 mM, the remaining activity of the monophenolase was determined to decrease, from 23.5 to 10.6 μM/min, indicating that the inhibitory effect of 4-chlorosalicylic acid on monophenolase was concentration-dependent. The IC_{50} value for the monophenolase activity inhibited by 4-chlorosalicylic acid was 1.87 mM.

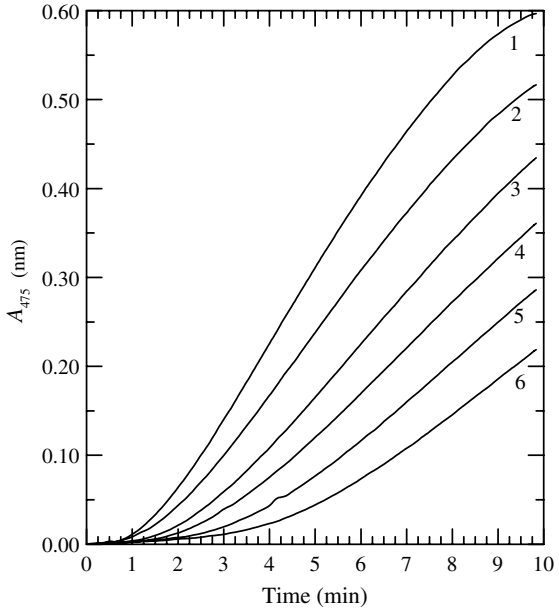

Fig. 5. Progress curves for the inhibition of monophenolase of mushroom tyrosine by 4-chlorosalicylic acid. The concentrations of 4-chlorosalicylic acid for curves 1–6 were 0, 0.4, 0.8, 1.2, 1.6 and 2.0 mM, respectively. The concentrations of the enzyme and the substrate (Tyr) were 6.67 μg/ml and 0.5 mM.

3.5. The antimicrobial activity of 4-chlorosalicylic acid

The antimicrobial activities of 4-chlorosalicylic acid on *Escherichia coli*, *Bacillus subtilis*, *Staphyloccocus aureus*, *Aspergillus niger* and *Candida boidinii* were first studied. The results are showed in Fig. 7 and Table 2. In the test, the bacteriostatic activities and antimycotic activities were assayed, taking 2000 U/ml gentamycin sulfate and 1 mg/ml K_2CrO_4 as controls, respectively. It was found that 4-chlorosalicylic acid could inhibit the proliferation of these five different kinds of microbials to different extents, but less than the corresponding control. DMSO had no obvious effect on the proliferation of these five different kinds of microbial.

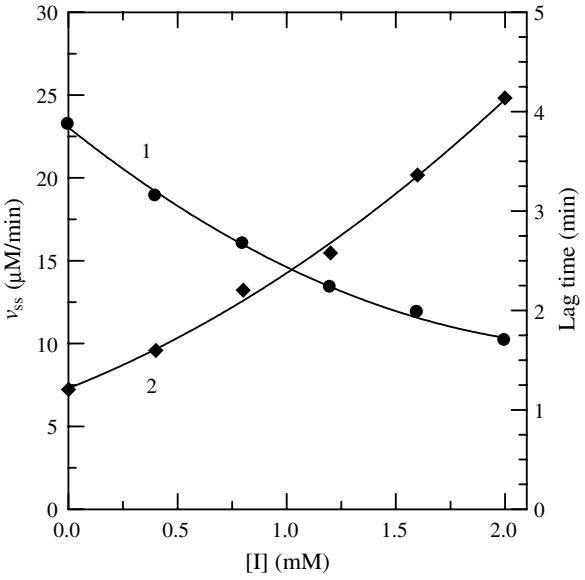

Fig. 6. Effect of 4-chlorosalicylic acid on the steady-state rate of monophenolase activity (1) and the lag period of mushroom tyrosinase (2) for the oxidation of L-tyrosine, using 0.5 mM of L-tyrosine as substrate.

We also used a broth dilution method to test the antimicrobial activities of 4-chlorosalicylic acid against *E. coli*, *B. subtilis*, *S. aureus*, *A. niger* and *C. boidinii*. The results obtained are listed in Table 3. 4-Chlorosalicylic acid was effective against the five kinds of representative microbes. The antimicrobial activity against *E. coli* was the most effective with the MIC of 250 μg/ml and with the MBC of 500 μg/ml. For the two kinds of fungi, the values of MIC and MBC were 500 μg/ml.

4. Discussion

Tyrosinase is a multifunctional oxidase with both activities of monophenolase and diphenolase. The effects of 4-chlorosalicylic acid on the diphenolase activity and the monophenolase activity of tyrosinase were studied. The results showed that 4-chlorosalicylic acid could inhibit both the diphenolase activity and the monophenolase activity of mushroom tyrosinase. 4-Chlorosalicylic acid was a reversible competitive-uncompetitive mixed-II type inhibitor like 4-methylsalicylic acid. As shown in Table 1, the IC_{50} of 4-chlorosalicylic acid for the diphenolase activity was obviously different form other salicylic acid family compounds. The inhibitory potency of 4-chlorosalicylic acid on diphenolase activity was greater than that of the salicylic acid derivatives (Zhang et al., 2006). It may be induced by the effect of the chlorine group at *para*-position of the benzene ring in salicylic acid compounds. The chlorine group is an electron-donating and hydrophobic group. According to the literature reported, the electron-donating group can strengthen the inhibitory effect on tyrosinase (Huang et al., 2006). From the result, it can be supposed that the combination of the substrate with the enzyme molecule will induce a new hydrophobic pocket in the enzyme-substrate complex, and the *para*-position hydrophobic group can just be inserted into the pocket. Thus, 4-chlorosalicylic acid and 4-methylsalicylic acid showed the same inhibitory effects on the diphenolase activity of tyrosinase. The polarity of the chlorine group was weaker than that of the methyl group, which made the inhibitory capacity of 4-chlorosalicylic acid more potent than that of 4-methylsalicylic acid. From Table 1, the inhibitory constants were determined and compared. The results showed that the value of K_I was about twice higher than that of K_{IS}, indicating that the attachment of inhibitor to enzyme-substrate complexes was stronger than to the free enzyme.

In the process of catalysis, tyrosinase has three existing forms: E_{met}, E_{oxy} and E_{deoxy} (Espín, Varón, & Fenoll, 2000). 4-Chlorosalicylic acid has a carboxyl group adjacent to a hydroxy group, which can chelate copper at the active

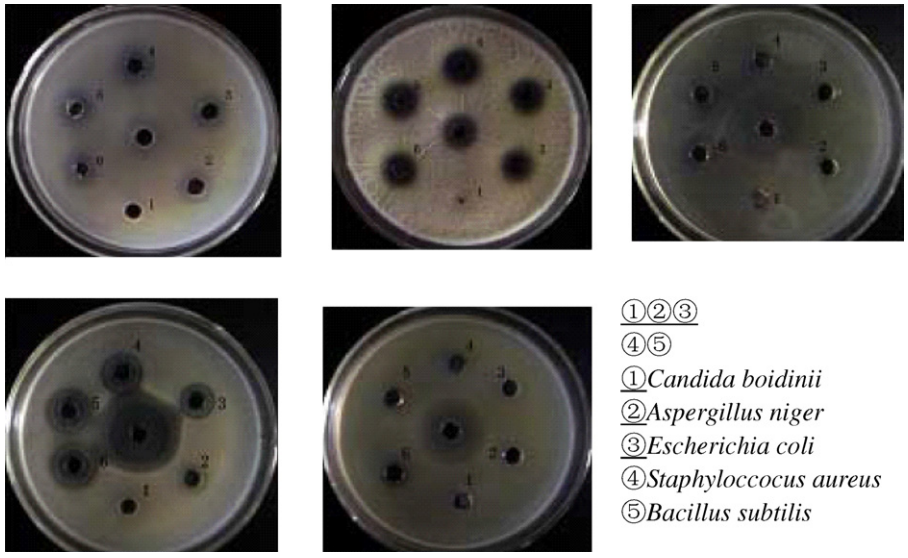

Fig. 7. The antimicrobial activity of 4-chlorosalicylic acid at different concentrations. The concentrations of 4-chlorosalicylic acid for dishes 1–5 were 5, 7.5, 10, 15 and 20 mg/ml, respectively.

Table 2
The antimicrobial activity of 4-chlorosalicylic acid

Concentration (mg/ml)	*Escherichia coli*	*Bacillus subtilis*	*Staphyloccocus aureus*	*Candida boidinii*	*Aspergillus niger*
a	+++	++	+++		
b				++	++
c	±	−	±	−	−
20	++	++	++	++	++
15	++	++	++	++	+
10	++	+	++	+	+
7.5	±	+	++	+	+
5	±	+	+	±	+

a, Positive control with 2000 U/ml of Gentamycin sulfate for bacterium.
b, Positive control with 1.0 mg/ml of K_2CrO_4 for fungi.
c, Negative control with DMSO.
+++, antimicrobial zone is above 12 mm in diameter.
++, antimicrobial zone is between 10 mm and 12 mm.
+, antimicrobial zone is less than 10 mm.
±, antimicrobial zone is faint.
−, no inhibition.

Table 3
Antimicrobial activity (μg/ml) of 4-chlorosalicylic acid

Microbe	MIC	MBC/MFC
E. coli	250	500
B. subtilis	500	750
S. aureus	500	750
C. boidinii	500	500
A. niger	500	500

site of enzyme (Kubo & Kinst-Hori, 1998). The carboxyl group forms a Schiff base with the primary amino group around the active site of the enzyme, and the chlorine group can insert a new hydrophobic pocket into the enzyme-substrate complex. According to these interactions of inhibitor with the enzyme, 4-chlorosalicylic acid was a mixed-type inhibitor. It could not only combine with the free enzyme but also with the enzyme-substrate complex. The K_I value of 4-chlorosalicylic acid was lower than those of other salicylic acid compounds reported. The tight binding of the competitive inhibitors to the active site of the enzyme may be responsible for the low K_I values.

The monophenolase activity of mushroom tyrosinase was assayed using L-tyrosine as a substrate. The lag time could be estimated by extrapolation of the linear portion of the product accumulation curve to the *X*-axis. As shown in Fig. 6, 4-chlorosalicylic acid could decrease the steady state rate of the monophenolase activity and prolong the lag time. 4-Chlorosalicylic acid was obviously an inhibitor of monophenolase.

Salicylic acid has antimicrobial activity. *E. coli*, *B. subtilis*, *S. aureus*, *A. niger* and *C. boidinii* were the microbials resulting in the greatest decay of cosmetics. 4-Chlorosalicylic acid could inhibit the proliferation of these five different kinds of microbial to different extents. The result showed that the antimicrobial activity of 4-chlorosalicylic acid was broad-spectrum, and could inhibit the Gram-positive (G^+) and Gram-negative (G^-) bacteria and fungi. The antimicrobial activity of salicylic acid on *E. coli* was the most effective. In addition, 4-chlorosalicylic acid could inhibit the proliferation of two kinds of fungi. So, it may become a new potent preservative in the food industry or a skin-whitening agent with antimicrobial activity in cosmetics.

Acknowledgements

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