



Bostrycin, a novel coupling agent for protein immobilization and prevention of biomaterial-centered infection produced by *Nigrospora* sp. No. 407

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ABSTRACT

Bostrycin, a red antibacterial agent with tetrahydroanthraquinone structure, has been isolated from *Nigrospora* sp. No. 407. This study investigated the potential antibacterial and multifunctional properties of matrixes through immobilization of bostrycin on their surface for immobilization of protein and prevention of bacterial growth. Bostrycin was immobilized on nonwoven polypropylene (PP) fabric by a technique using glutaraldehyde and polyethyleneimine for the activation of the surface. Glucose oxidase immobilized on bostrycin-treated nonwoven PP fabric showed high activity. The immobilization process improved thermal stability of the enzymes. During repeated assay for 30 cycles, the enzyme activity dropped to only 70% of the initial activity. Both bostrycin-treated nonwoven PP fabric sample and subsequently immobilized glucose oxidase sample on the surface also still exhibited a bacteriostatic effect. This is the first study to show that bostrycin is a promising coupling agent for surface modification on matrix and its potential applications in protein immobilization and biomaterial-centered infection.

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1. Introduction

Increasing knowledge and awareness among the people regarding disease transmission and cross-infections caused by microorganisms leads to thoughtful use of antimicrobial materials in many applications, such as the manufacturing of protective clothes for medical workers, underwear, sportswear, and others healthcare products [1]. However, prolonged use of these materials, usually referred to as biomaterials, has some drawbacks. One of the drawbacks is the possible occurrence of biomaterials-centered infections (BCI) [2]. In BCI, microorganisms closely associate with the surface of the biomaterials and form the so-called biofilms. A suitable approach to avoid the formation of such biofilms would be to covalently bind an antimicrobial agent to the surface of the biomaterials, while retaining its antimicrobial activity [3]. Some pretreatment is necessary to activate the biomaterial surface so that antimicrobial agents can then be introduced. Among the treatment methods, chemical activation is the most effective method to modify polymers, because it is a simple and an inexpensive method for the creation of a functional surface.

Nonwoven PP fabric is porous, has a rather larger surface area without dust particles, and can be easily processed; moreover, its

surface properties are readily modifiable. It can act as a good biomaterial because it can readily be conditioned to serve as an excellent dressing for wounds, as a biosensor, and as a clinical mask. In our previous study, nonwoven PP fabric was immersed in glutaraldehyde and PEI solutions, to achieve polymerization of $-NH_2$ groups on the nonwoven PP surface for further applications.

Bostrycin has a tetrahydroanthraquinone skeleton and was first isolated from *Bostryconema alpestre* [4]; subsequently, it was isolated from *Nigrospora oryzae* [5], *Arthrinium phaeospermum* [6], *Alternaria eichhorniae* [7], and *Aspergillus* sp. [8]. These studies described the antibacterial and phytotoxic activities of the drug. Recently, we conducted a screening program for antibiotic-producing microorganisms from soil, and purified and identified a bostrycin from *Nigrospora* sp. The red antibiotic bostrycin, which is a quinolone, consists of two active carbonyl groups and can act as a coupling agent in protein immobilization. When one of the carbonyl groups is immobilized on a support via covalent bonding, the other carbonyl group on the support could react with proteins. However, to date, no researcher has evaluated its potential as a coupling agent for prevention of biomaterial-centered infection and protein immobilization.

In our previous study, we established a method for covalent immobilization of enzymes on chitosan beads [9], and on nonwoven fabric pretreated with polyethyleneimine (PEI) and glutaraldehyde for preparation of biosensor. Thus, the aim of this study was to develop a method for immobilization of bostrycin

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as a coupling agent and such that it can be used for further applications on anti-microbial material, biosensor and wound dressing. For this purpose, we immobilized bostrycin on nonwoven fabric to develop biomaterial surfaces with anti-bacterial and anti-proliferative activities. The properties of an immobilized enzyme were evaluated and compared with those of the soluble enzyme.

2. Materials and methods

2.1. Materials

The porous chitosan beads (Chitopearl BCW3007) were purchased from Fuji Spinning Co., Ltd., Tokyo, Japan. Nonwoven polypropylene (PP) fabric was obtained from Formosa Inc. Taiwan. Glucose oxidase was purchased from Sigma (*Aspergillus niger* type V) and all other chemicals were of reagent grade or higher purity.

2.2. Microorganism and culture conditions

A fungal strain, No. 407, was isolated from soil and identified as *Nigrospora* sp. by the CBS (Centraalbureau voor Schimmecultures, The Netherlands). This strain was not completely identical to a known *N. oryzae* strain, with only 95% rDNA sequence homology; thus, this strain was tentatively named *Nigrospora* sp. No. 407. The organism was maintained on YM agar slants (1% glucose, 0.5% peptone, 0.3% malt extract, and 0.3% yeast extract) at 30 °C. For antibiotic production, this strain was taken from a slant culture and inoculated in 50 ml of a medium containing 1.5% fructose and 1.5% malt extract in a 250 ml flask. Fermentation was carried out at 30 °C for 6 days on a rotary shaker at 150 rpm. The culture was filtered through 4 or 5 layers of muslin cloth, and the filtrate thus obtained was centrifuged at 12,000 × g at 4 °C for 20 min. The cell-free supernatant was considered the crude antibiotic, which was subsequently assayed.

2.3. Antimicrobial activity assay

The antimicrobial activity of the antibiotic was assessed by the agar-diffusion assay, according to a modified version of the method described by Barry [10]. Cultures of *Staphylococcus aureus* (OD₆₀₀ = 0.1) were mixed with 20 ml nutrient agar medium (Difco). After the nutrient agar had solidified, circular wells (diameter, 9 mm) were bored into the agar. Fifty microliters of the antibiotic sample (1–10 μg) was added to each well and areas of microbial growth inhibition were measured after incubation for 16 h at 35 °C. Zones of inhibited *S. aureus* growth were observed around the wells. The mean diameters of these zones were calculated, and the diameter of the well (9 mm) was subtracted. The antibacterial efficacy was determined by measuring the diameter of the zone of inhibition. The antibacterial activity of food poisoning bacterium *Clostridium botulinum* was assayed by the laboratories of National Defense Medical Center (Taipei).

The antibacterial effect of bostrycin against the nosocomial strains such as methicillin-resistant *S. aureus* (MRSA), *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* (VRE), and *Mycobacterium tuberculosis* were assayed that according to National Committee for Clinical Laboratory Standards guidelines, by the laboratories of Kaohsiung Veteran General Hospital and Institute of Preventive Medicine. Plastic microdilution trays contained the antimicrobial agent in a twofold serial dilution from 1:2 to 1:65,536 being tested. Inocula were prepared in broth from cultures grown on Columbia agar with 5% sheep blood. The final concentration was 1.5 × 10⁸ CFU/ml. All microdilution trays were incubated at 35 °C with 5% CO₂ for 24 h.

2.4. Recovery and purification

The culture fluid was extracted twice using chloroform (1000 ml each time), and the combined extract was concentrated to near dryness under reduced pressure. The residue obtained was then triturated with *n*-hexane, and a dark-red microcrystalline solid was obtained. Thereafter, the extract was placed on a silica gel 60 column (3.8 cm × 30 cm), after the column was packed with 100 g silica gel (80–100 mesh, Merck Co.) and washed with 500 ml of *n*-hexane. The column was developed in a batch-wise process using the following solvent sequence: 1000 ml of *n*-hexane-ethyl acetate (10:1); 1000 ml of *n*-hexane-ethyl acetate (10:2); and 1000 ml of *n*-hexane-ethyl acetate (10:3). Finally, the column was eluted with 1000 ml of chloroform-methanol solvent (6:4). A minor band was eluted first, after which the major red crystal pigments were filtered. The active fractions were collected, washed, dried, and analyzed for purity.

2.5. Immobilization of bostrycin onto nonwoven PP fabric and cross-linking with glucose oxidase

The technique of the nonwoven surface activation using glutaraldehyde and polyethyleneimine was carried out according to Garcia's method [11] with slight modification. The nonwoven PP fabric was cut into square-shaped samples of 1 cm² and immersed in 5 ml of a 10% solution of glutaraldehyde in water for 2 h at 90 °C with rotary agitation (150 rpm). Thereafter, the samples were thoroughly washed

with deionized water, immediately placed in 5 ml of a 5% PEI solution, and agitated at 40 °C for 2 h. They were washed again with distilled water, bostrycin was immobilized on nonwoven PP fabric via cross-linking, and this immobilization was performed in a bostrycin solution (10 mg/ml) at 4 °C for 12 h. The nonwoven samples were rinsed for further use. For enzyme immobilization, bostrycin-treated nonwoven PP fabric samples were immersed in 0.5 ml of a 1 mg/ml glucose oxidase (50 U/ml) solution in 50 mM Tris buffer (pH 7.8), for 16 h at 4 °C under mild agitation. Finally, the samples were washed with and stored in Tris buffer in a refrigerator until further use.

2.6. Antibacterial activity test

S. aureus was selected as the experimental bacterium for the antibacterial test. The antibacterial activities of the nonwoven samples were measured by a shake-flask test and assessed in terms of the ratio of bacteriostasis (*R*) to *S. aureus* [12]. *R* was calculated using the following equation:

$$R(\%) = 100 \times \frac{(A - B)}{B}$$

where *A* and *B* are the mean numbers of bacterial colonies on the nonwoven samples before and after the shake-flask test, respectively.

2.7. Enzyme activity assay

The enzymatic activity on the nonwoven PP fabric was estimated by a chromogenic method using peroxidase-4AA [13], and it was evaluated by measuring the increase in hydroperoxide concentration, caused by the nonwoven samples suspended in a well-agitated chromogenic solution. The sample was incubated at 30 °C in 1 ml of 50 mM Tris-HCl buffer (pH 7.8) containing 10 mM glucose, 2 U peroxidase, 0.1 mM 4-aminoantipyrine, and 1 mM phenol. The enzyme activity thus measured was compared with that of the soluble enzyme.

2.8. Protein assay

The protein concentration was determined by Bradford method [14], with bovine serum albumin as a protein standard. Bound protein was determined by the difference between the initial and residual protein concentration.

2.9. Characterizations and instrumentations

The chemical structure of the fabric samples was determined using the attenuated total internal reflectance Fourier transform infrared spectroscopy (ATR-FTIR-300E; Jasco, Japan). The surface morphologies of the nonwoven samples were observed using a Field-Emission Scanning Electron Microscope S-4800(II) (Hitachi Company, Japan), operating at a typical accelerating voltage of 10 kV. The samples were sputter-coated with gold for 60 s at 15 mA before observation.

3. Results

3.1. Characteristics and antimicrobial spectrum of bostrycin

The culture broth and chloroform extract of the strain PC-407 were red, and the purified crystal compound was a red pigment, whose structure was assigned from spectroscopic data and by comparison with the reported values for bostrycin [4,15–17]. The antibacterial activity of the purified bostrycin was evaluated in several microorganisms. Bostrycin showed a potent antibacterial activity against a wide range of Gram-positive bacteria, such as *Bacillus cereus*; however, it did not show any antibacterial activity against Gram-negative bacteria and also did not show antifungal activity against *Candida albican*. An interesting finding of our study was that the antibacterial effect of bostrycin was reinforced on the nosocomial strains such as methicillin-resistant *S. aureus* (MRSA), *S. pneumoniae*, vancomycin-resistant *Enterococcus* (VRE), *M. tuberculosis*, and the food poisoning bacterium *C. botulinum*, which are difficult to treat with commercial antibiotics (Table 1).

3.2. Bostrycin immobilization on nonwoven PP fabric

Bostrycin consists of two carbonyl functional groups and can act as a bifunctional cross-linking agent. Thus, the carbonyl groups can react with the amine groups present in activated nonwoven fabric. The process for the preparation of bostrycin immobilization on nonwoven PP fabric is shown in Fig. 1. The carbonyl-terminated

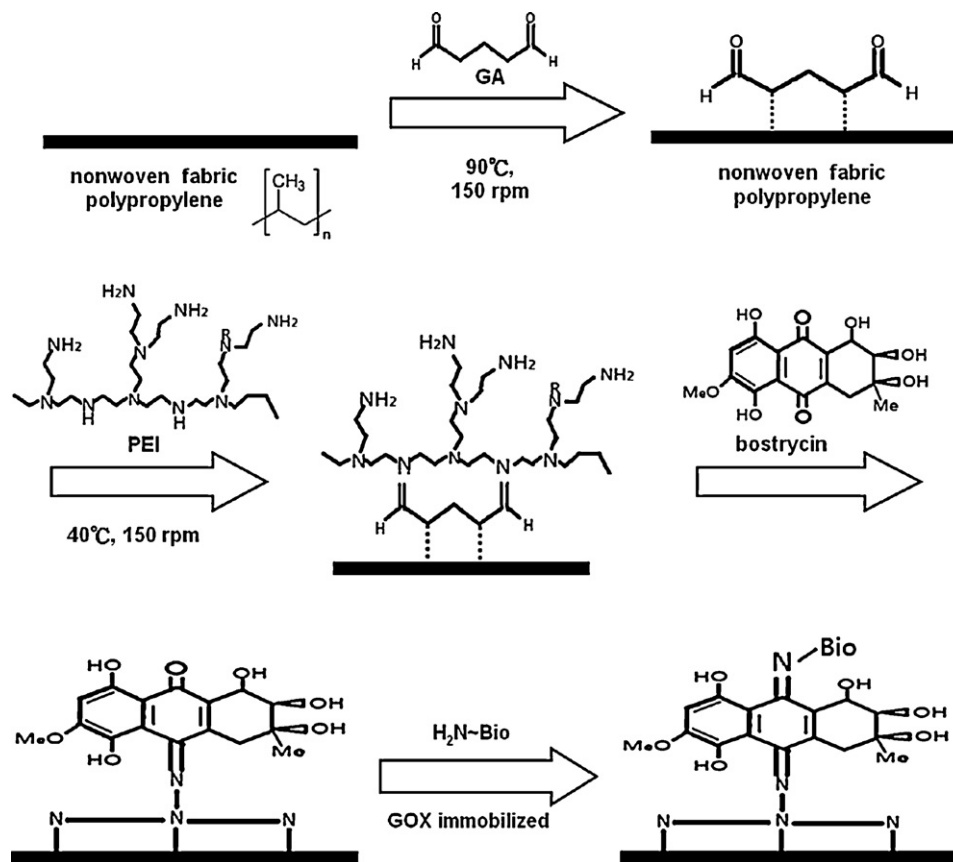


Fig. 1. Schematic diagram for the immobilization of bostrycin and enzyme on nonwoven fabric. The nonwoven PP fabric was immersed in a 10% glutaraldehyde (GA) solution at 90°C with 150 rpm rotary agitation. The sample was placed in a 5% PEI solution, and agitated at 40°C with 150 rpm. The GA-PEI linkage was formed. Bostrycin was then immobilized on nonwoven PP fabric via cross-linking between carbonyl group of bostrycin and amino group of PEI. The enzyme (e.g. glucose oxidase, GOX) was then immobilized through another carbonyl group of bostrycin and amino group of the enzyme. The dotted line indicates the Van der Waals force between nonwoven PP fabric and glutaraldehyde. $\text{NH}_2\text{-Bio}$ represents for the amino group of enzyme.

nonwoven PP fabric was obtained by glutaraldehyde treatment under the activation of high temperature, which is possible to physically adsorb around nonwoven PP fabric by Van der Waals forces interaction. Subsequently, the peripheral carbonyl groups onto nonwoven PP fabric were reacted with PEI. The as-formed NH_2 -terminated nonwoven PP fabric should be used to interact with the carbonyl groups on the bostrycin and the bioconjugates were constructed. The influence of bostrycin concentration was investigated and showed that there was no difference when the concentration was above 1%. All the immobilized bostrycins were prepared with an initial bostrycin of 1% in the present study, and the amount of bostrycin retained after immobilization was about 40% (data not shown). Bostrycin deposition on the nonwoven PP fabric was evident from a change in color of the surface to strong

red, as showed in Fig. 2. The color intensity of the coating depends on the amount of bostrycin deposited on the surface.

The ATR-FTIR spectra, as shown in Fig. 3, are used to evaluate the chemical structure of nonwoven PP fabric. The structure of the modified monomer can be verified according to the presence of some unique functional group shown in the ATR-FTIR spectra. Compared to the ATR-FTIR spectra of the original nonwoven fabric (Fig. 3A), peaks of 3200 cm^{-1} and $1620\text{--}1660\text{ cm}^{-1}$ can be ascribed to the -NH_2 group and Schiff base group ($\text{C}=\text{N}$ group), respectively (Fig. 3B). After the addition of bostrycin, the peaks of 1569 cm^{-1} is the $\text{C}=\text{C}$ of aromatic ring on bostrycin (Fig. 3C), this proves that a new Schiff base was formed between $\text{-C}=\text{O}$ on bostrycin and -NH_2 on PEI. Therefore, the introduction of the $\text{C}=\text{O}$ groups on bostrycin can provide a surface for binding of proteins by cross-linkage.

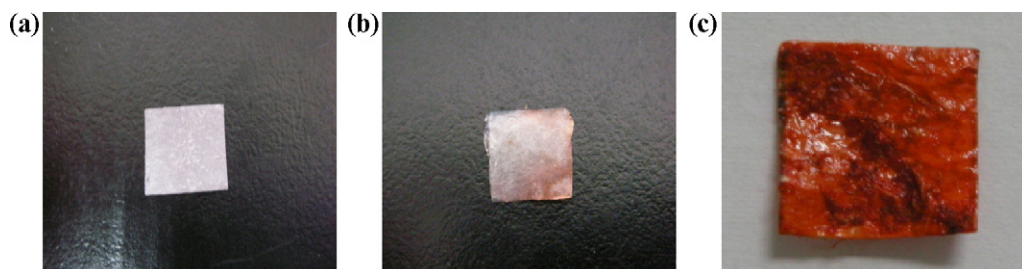


Fig. 2. Visual comparison of (a) glutaraldehyde-treated, (b) PEI-treated, and (c) bostrycin-treated nonwoven PP fabric samples.

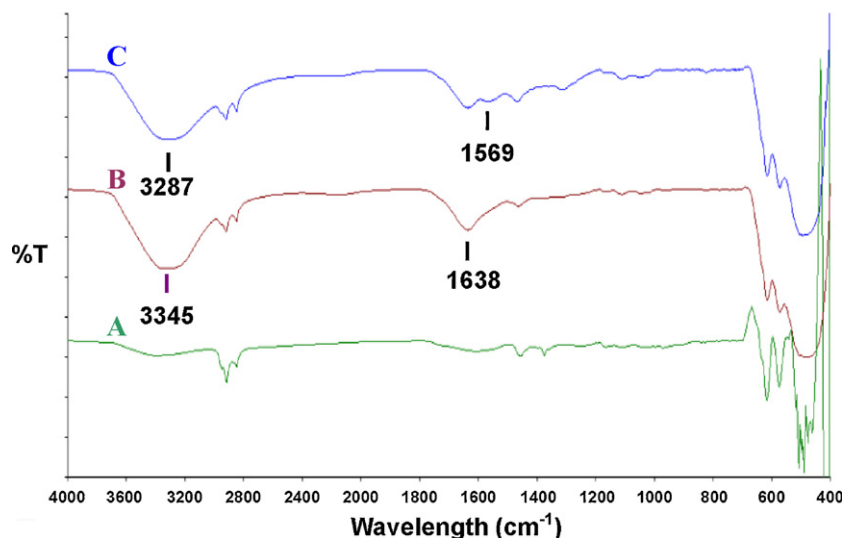


Fig. 3. ATR-FTIR spectra of nonwoven PP fabric: (A) original, (B) PEI-treated, and (C) bostrycin-treated.

3.3. Measurement of antibacterial activity

The antibacterial activities of the nonwoven PP fabric treated with bostrycin were determined by using the shake-flask test. The test was performed on untreated and bostrycin-treated nonwoven fabric by incubation of the fabric in an oven at 35 °C for 16 h. The ratio of bacteriostasis (*R*) to *S. aureus* was up to 90.1%, which indicates that a better antibacterial effect was observed on the nonwoven fabric with immobilized bostrycin. As shown in Fig. 4, a clear bacteria-free test solution was observed, confirming bostrycin-induced inhibition of bacterial growth. No bacterial cells were present in the clear supernatant of the liquid culture. For further investigation, the surface of the nonwoven samples that were subjected to the above treatment was analyzed using SEM. Fig. 5 shows a SEM image of a surface of a bostrycin-treated nonwoven sample. *S. aureus* cells were locally attached to the surface, as observed in the image. The red-antibiotic, bostrycin, bound to the surface of the nonwoven sample was found to maintain its inhibitory effect on cell growth.

3.4. Enzyme immobilization onto bostrycin-treated nonwoven PP fabric

Bostrycin was able to act as a bifunctional cross-linking agent, and this is supported by the following observations. Various

Table 1
Antimicrobial spectrum of bostrycin from *Nigrospora* sp. strain PC-407.

Microorganisms	Antimicrobial activity	
	Inhibition zone	MIC test
<i>Staphylococcus aureus</i>	10 mm	–
<i>Clostridium botulinum</i>	9 mm	–
Methicillin-resistant	–	1:512
<i>Staphylococcus aureus</i> (MRSA)	–	1:512
<i>Streptococcus pneumoniae</i>	–	1:256
Vancomycin-resistant	–	1:512
<i>Enterococcus</i> (VRE)	–	1:512
<i>Mycobacterium tuberculosis</i>	–	–

quantities of purified glucose oxidase solutions were shaken with 1 cm² nonwoven samples as described above. The catalytic activity of immobilized glucose oxidase was 8 U/cm² on the nonwoven sample surface, and the activity retained after immobilization was about 16%. However, no bacterial cells were found in the clear supernatant of the liquid culture after overnight incubation; this finding confirmed the bacterial growth inhibition effect observed on the bostrycin-treated nonwoven PP fabric surface on which glucose oxidase was immobilized (Fig. 6). Various proteins, such as D-amino acid oxidase, lipase, peroxidase-conjugated antibody and collagen were tested for this immobilized procedure, and obtained the similar effects of immobilization (data not shown).

3.5. Thermal and operational stability of the immobilized enzyme

The thermal stabilities of free and immobilized enzymes were studied by incubating the enzyme solutions at different temperature for 1 h, and then, determining their residual activities at 30 °C. Both enzymes were found to be stable at temperatures lower than 40 °C. At higher temperatures (50–70 °C), however, the



Fig. 4. Antibacterial activity of nonwoven PP fabric samples. Left: untreated nonwoven sample; right: bostrycin-treated nonwoven sample. Comparison with the untreated nonwoven sample, a clear bacteria-free solution was observed in bostrycin-treated nonwoven sample, indicating the bostrycin inhibited the growth of bacteria.

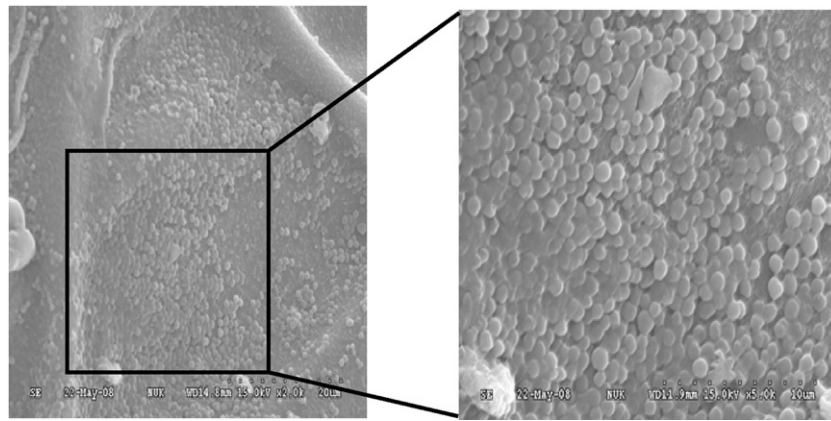


Fig. 5. SEM images of bostrycin-treated nonwoven PP fabric surface shows *Staphylococcus aureus* at low magnification (1000 \times), and inset at high magnification (5000 \times).

immobilized enzyme showed better stability (Fig. 7A). The operational stability of the immobilized enzyme was studied by continuous application of an activity assay. Even after 30 cycles of continuous operation, the immobilized enzyme retained approximately 70% of its original activity (Fig. 7B).

4. Discussion

This is the first study to show that bostrycin, owing to its tetrahydroanthraquinone structure, is a promising coupling agent for protein immobilization. The tetrahydroanthraquinone skeleton of bostrycin creates a matrix having antibacterial activity and protein immobilization property. Tetrahydroanthraquinone derivatives related to bostrycin were produced by fungi such as *A. eichhorniae* [7], *Alternaria porri* [18], *Alternaria solani* [19–21], *Auxarthron umbrinum* [22], *Chrysosporium queenslandicum* [2,23], *Dactylaria lutea* [24,25], *Dermocybe* sp. [26], *Phomopsis juniperovora* [27], *Pleospora* sp. [28], and *Stemphylium botryosum* [29]. The antimicrobial, antiprotozoal, phytotoxic, and cytotoxic activities of these compounds have been described in the papers cited above. Therefore, the quinone structure is necessary for their bioactivities.

Immobilization of bostrycin on the surface of biomaterials has the potential to overcome BCI. Covalent binding of an antimicrobial agent to the surface of a biomaterial is one of the most popular

methods of protein immobilization. Glutaraldehyde is often used as a coupling reagent in the support; however, it is a toxic substance. The other well-known coupling agents such as *N*-succinimidyl 6-maleimidocaproate (EMCS) and *N*-hydroxy-succinimide (NHS) were recently reported to bring about protein immobilization by formation of a C=N bond via a coupling reaction [30,31]. Kang et al. [32] employed tyrosinase to oxidize the tyrosyl residues in

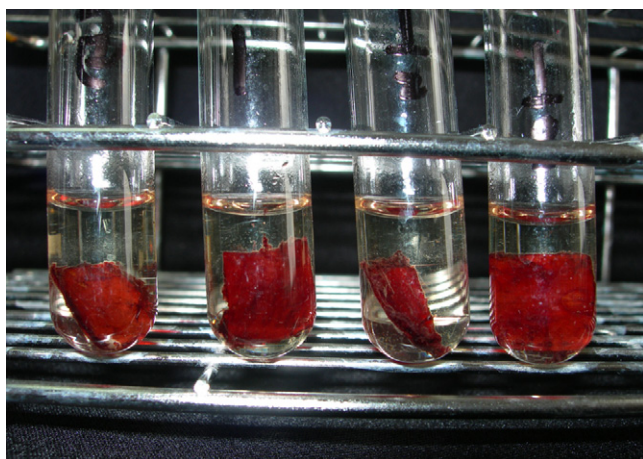


Fig. 6. Antibacterial activity of bostrycin-treated nonwoven PP fabric samples with immobilized glucose oxidase. No bacterial cells were found in the supernatant of the liquid culture after overnight incubation. It indicated that the glucose oxidase immobilized bostrycin-treated nonwoven PP fabric still maintained the antibacterial activity. Sample in each tube represents the result from four different batches of treatment.

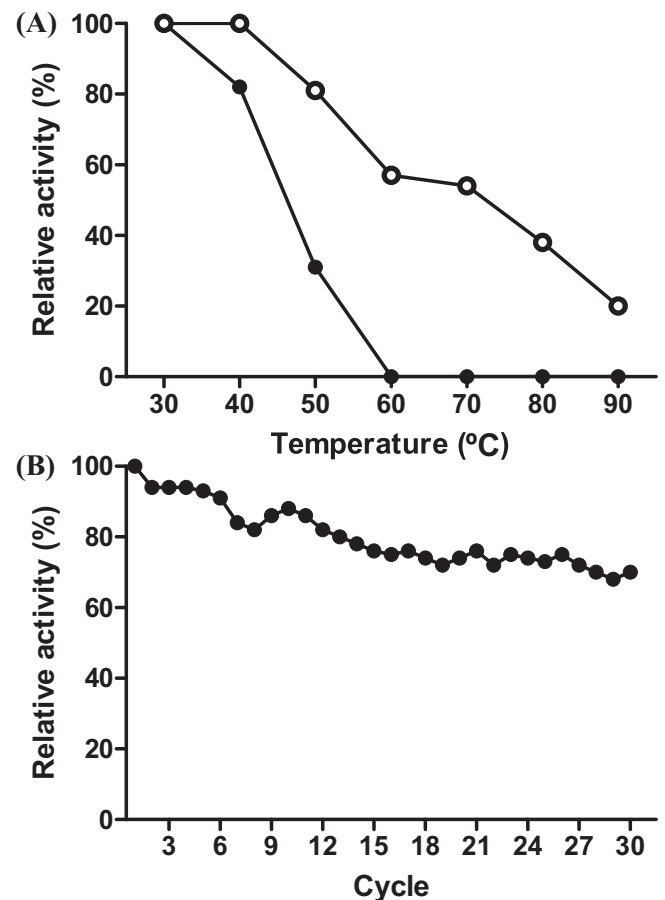


Fig. 7. (A) Temperature stabilities of free glucose oxidase (●) and glucose oxidase immobilized on bostrycin-treated nonwoven PP fabric (○). The relative activity of enzyme at each temperature is presented as percentage of the original activity. (B) Stability of glucose oxidase immobilized on bostrycin-treated nonwoven PP fabric samples after 30 cycles of continuous operation. The stability of the immobilized enzyme was measured by enzyme activity assay and presented as percentage of the original activity.

silk fibroin with oxygen, resulting in the production of *o*-quinone residues. Subsequently, the inter- or intra-molecular crosslinks are formed by reaction with amino groups through nonenzymatic process (Maillard and Michael addition reaction, respectively). Bostrycin also contains a functional group (C=O) in a ring-like structure, similar to that of coupling reagents, and it is expected to be a novel coupling agent for protein immobilization via Schiff base formation.

Bostrycin shows antibacterial activity only against Gram-positive bacteria but not against Gram-negative bacteria. The major difference between the 2 types of bacteria is their cell envelope. The cell wall of the Gram-positive bacteria contains a single, thick homogeneous layer of peptidoglycan outside the plasma membrane. In contrast, the Gram-negative bacteria are quite complex. It has a thin peptidoglycan layer covered by an outer membrane. In Gram-positive bacteria, bostrycin directly acts on the nascent penta-peptide of the peptidoglycan and blocks cell wall biosynthesis. However, bostrycin does not act on Gram-negative bacteria, because the outer envelope of the Gram-negative bacteria is a membrane and not a peptidoglycan.

The present study indicates that bostrycin is a promising coupling agent that can be used to immobilize proteins on a matrix by chemical activation. The use of bostrycin could cause the matrix to exhibit protein immobilization and antibacterial properties. It might replace the use of glutaraldehyde in the biomaterials owing to its well-known toxic properties. Further, the bostrycin-treated matrix developed in this study proved to be a rapid and convenient approach to surface modification and immobilization for prevention of biomaterial-centered infection. It might be applied to many products such as wound dressing, high-efficiency particulate air (HEPA) filter, and biosensors. In summary, this immobilized bostrycin preparation could be exploited for several biomedical and public health purposes.

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