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17 Study on Antibacterial Activity of Hydrogel from Irradiated Silk Protein

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ABSTRACT

Hydrogels for biomedical application were prepared from solution blends of 3% silk protein and 3%, 10% poly(vinyl alcohol) (PVA) and followed with irradiation. Mixture of hydrogels were gamma irradiated at 10, 20, 30, 40 and 50 kGy under N_2 atmosphere. To clarify anti-bacterial activity of hydrogels, modified of the Agar disk diffusion method and American Association of Textile Chemists and Colorists, AATCC Test Method 90-1977, were carried out. The four kinds of bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, were used. It was found that a 1:3 volume ratio of 3% silk protein and 3% PVA respectively, at 50 kGy irradiation, is suitable conditions for preparation hydrogels and trend to indicate the highest of an antibacterial activity against *E. coli*, *B. subtilis and S. aureus*. However the antibacterial activity of hydrogels against *S. epidermidis* was not clearly. These results are very useful to expand the application of hydrogel from irradiated silk protein to the medical products.

Keywords : Silk protein, Antibacterial activity, Hydrogel, Poly (vinyl alcohol)

1. Introduction

Silk fiber consists of two different types of protein, Fibroin and Sericin. Fibroin is a structural material and sericin is a covering material. A large amount of silk fibers have been wasted from hand and machine reeling in Thailand. It is reported about 200 tons per year as silk waste. The utilization of silk waste is interested to develop in the field of bio-materials. Products from silk have excellent physiological characters, it is expected to consider and develop the utilization as a new functional bio-materials [1].

Recently published papers reported gamma irradiation is the possible method for preparing hydrogel from poly (amino acid) such as poly (ε-lysine) [2] and poly (γ - glutamic acids) [3].

Hydrogels have been used widely for cosmetic and biomedical applications such a burns wound dressing, contacts lens, artificial skin and also in drug delivery system. Hydrogels for wound dressing have been produced by electron beam crosslinking on a mixture of natural and synthetic polymers, poly(vinyl pyrrolidone), polyethylene glycol and agar [4] and poly(vinyl alcohol) [5][6].

Poly(vinyl alcohol) is a nontoxic water soluble synthetic polymer, which is widely use in biochemical and biomedical applications [7]. PVA gels can be used in the medical field because they possess good biosuitability [8]. Hydrogels from solution blends of silk fibroin and PVA have been prepared by gamma irradiation under nitrogen [9]. However hydrogels were prepared for biomedical application, have to sterilize or prevent from many of microorganisms. These properties will be the most valuable hydrogel in the field of biomedical application. No reports have been found in the literature on an antibacterial activity of hydrogel preparing from irradiated silk protein. Therefore the aim of this research is to clarify an antibacterial activity of irradiated silk protein /PVA hydrogel.

2. Experimental

2.1 Materials

Raw silk waste fibers (STBII) was supplied from the SHINANO KENSHI (THAILAND) Co., LTD., Saraburi province, Thailand. The media to cultivate of bacteria were purchase from Difco Laboratories. Blank Paper disks (6 mm diameter) were purchase from Becton Dickinson and Company, USA (BBL^{TM}).

2.2 Preparation of silk protein solution

10 g of cleaned and crushed of silk waste fibers were dissolved in 90 ml $CaCl_2$ solution (73 g $CaCl_2$. $2H_2O$: 54ml H_2O : 47 g C_2H_5OH) at 90 °C for 30 min. The silk protein solution was dialyzed using seamless cellulose tube (molecular weight cut off 12,000-14,000 Da.) with de-ionized water at least for 3 days. Concentration of silk protein solution obtained after dialysis was 3w/v%. Dialysis process should be done just before mixing with PVA solution, to avoid silk protein crystallization.

2.3 Preparation of silk protein/PVA hydrogel

Two kinds of different concentrations of PVA, 3% and 10 w/v% were prepared by dissolving PVA with sterilize deionised water. Then 3% silk proteins were blended with 3% and 10% PVA (silk proteins : PVA, 1: 3). Subsequently the mixtures in sealed each glass tube, was filled with nitrogen gas and irradiation.

2.4 Irradiation

Irradiation was carried out at room temperature using Co-60 Gamma cell-220 irradiator with a dose rate of 100Gy/min. The doses of irradiation were conducted 10, 20, 30, 40 and 50 kGy.

2.5 Culture Medium

AATCC Broth contains of Peptone (Bacto-peptone) 10g, Beef extract 5g, sodium chloride 5g, and distilled water 1000ml. And AATCC agar, add 1.5% agar. Boil to dissolve ingredients. Adjust to pH 6.8-7.0, Dispense in 150ml amounts in Duran bottles and sterilized at 121 ° C (15 psi) , 15 min.

2.6 Test Bacteria

Pure cultures of bacteria were cultivated and obtained from Bamrajnaradul Hospital, Nonthaburi province, Thailand. Four kinds of organisms such *as Escherichia coli* (gram-negative), *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (gram-positive) were carried out.

2.7 Hydrogels preparation to determine the antibacterial activity

The specimen of hydrogels in each glass tubes was removed by breaking tubes. Then hydrogels was cut into a circular piece by a sharp cutter using aseptic technique. Each pieces of hydrogel was placed on and pressed down lightly to a heavily seeded bacteria.

2.8 Antibacterial activity testing

The procedure was performed as the modified methods of Agar disk diffusion method [10] and AATCC Test method 90-1977 [11]. The procedures are as following :

1. Twenty-four-hour AATCC broth cultures of four test bacteria, were conducted.

2. Count a number of each test bacteria (direct count with counting chamber and measure turbidity of OD 660 nm) and dilute become to 1.0×10^6 cells/ml.
3. Melt 150 ml of AATCC Agar in a Duran bottle, cool at 45 °C, inoculate with 1.0×10^6 cells/ml of a tested bacterium.
4. Pour the test organisms with medium into a 100 mm diameter Petri-dish. Allow 25 minutes lapse of time before placed on the tested specimen
5. Gently press down the tested hydrogel (d) and an untreated control (PVA solution (a), non-irradiated silk/PVA mixture (b) and silk protein solution (c) using a blank paper disk) into intimate contact with the seeded agar Petri-dish, using sterile forceps. Set tests in duplicate.
6. Incubated the Petri-dish for 24-48 hours at 37 °C
7. Observed and measured clear zone around the hydrogel specimens through the bottom of the plate. Calculated the width of the clear zone as follows:

$$W = \frac{T - D}{2}$$

Where:

W : width of clear zone in millimeter (mm.)

T : total diameter of test specimens and clear zone

D : diameter of test specimens

8. A clear zone of no growth surrounding the specimen, indicates an antibacterial activity of hydrogel specimens.

3. Results and Discussion

3.1 To select a suitable concentration of silk protein and PVA for preparation hydrogels on antibacterial activity testing

The 3% silk protein blended with 3% PVA and 10% PVA, and followed with γ – irradiation, were carried out. The appearances of hydrogels which contained 3% silk protein /10% PVA at a ratio of 1:3 respectively, were brittle and easy to break. Furthermore the resulting hydrogel contained many bubbles which come from gases evolved during irradiation. Thus it is very difficult to cut into a circular shape for an antibacterial activity testing. And it was reported that hydrogel derived from 3% silk

protein /3% PVA concentration , showed higher water absorption than 10% PVA (Fig. 1)[9]. Absorbs fluid is one required properties of hydrogel for wound dressing [12]. Based on these reasons, 3% PVA concentration was selected to prepare hydrogel on antibacterial activity testing.

3.2 To clarify an antibacterial activity of irradiated silk protein/ PVA hydrogels

Four kinds of bacteria, such as *E .coli, B. subtilis, S. aureus* and *S. epidermidis*, were examined. Clear zone of growth inhibition adjacent to hydrogel indicates antibacterial activity which was compared with silk protein/PVA without irradiation (b) as a control. An antibacterial activity of hydrogels at 20 (Fig.2) and 50 kGy (Fig. 3) against *E. coli*, were clearly observed, whereas low activity was observed in PVA (a), non-irradiated silk protein/PVA solution (b) and no activity on silk protein solution (c). Similar phenomenon of hydrogels (d) were also observed against *B. subtilis* at 20 kGy (Fig. 4) and at 50 kGy (Fig. 5) and *S. aureus* at 20 kGy (Fig. 6) and at 50 kGy (Fig. 7). In addition, no activity of untreated control (a, b, c) against *B. subtilis* and *S. aureus* were found. These results suggest that *E. coli* is highly sensitive to PVA and silk protein solution. On the other hand a clear zone of growth inhibition of hydrogels against *S. epidermidis* at 20 kGy (Fig. 8) and at 50 kGy (Fig. 9), was not clearly observed. This indicated that prepared hydrogel trend to lack of that activity against *S. epidermidis*.

3.3 To study the effect of irradiation doses on an antibacterial activity of hydrogel

The appearance of silk protein/PVA at 10 kGy, hasn't been a hydrogel, it is still liquid solution. From this experience, we used the samples which were irradiated at 20, 30, 40 and 50 kGy for an antibacterial activity testing. An antibacterial activity was determined by the presence of growth inhibition (clear) zones surrounding the various disks and hydrogel used. Width of the diameters of such zones can be measured with a fine metric ruler. An antibacterial activity of hydrogel against four bacteria at various doses was shown in Fig. 10. It can be seen that an antibacterial activity of hydrogel against *E. coli* (gram negative) was increased gradually with increasing doses of irradiation especially at 50 kGy, can be clearly observed. Similar trend of this activity of hydrogel against *B. subtilis* and *S. aureus* (gram positive) (Fig. 10) were

found whereas that of activity against *S. epidermidis* was not clearly observed. It can be seen that the maximum of such activity of hydrogel was found at 50 kGy [Fig. 10]. It mentioned that irradiation degraded silk protein to produce lower molecular weight products [9][13]. Base on these reports, silk fiber changed to low molecular weight fragments by irradiation. Therefore it assumed that increased inhibitory effect of hydrogel on each bacteria was higher than that of non-irradiated one. This similar behavior was found in irradiated chitosan has an antibacterial activity than that of non-irradiated chitosan [14]. Similar effect of molecular weight dependent antimicrobial activity by chitosan, was found [15][16]. The results can be explained on the higher irradiation doses degraded higher silk proteins. No significant different in the width of clear zone of growth inhibition for three kinds of bacteria (*E. coli, B. subtilis* and *S. aureus*) were observed. On the contrary, it is a difference of *S. epidermidis*. It was supposed that an antibacterial susceptibility of hydrogel depended upon species of microorganisms. However the hydrogel seem to be the highest antibacterial susceptibility to *E. coli*. As the previous investigators mentioned that the size of growth inhibition zones can be affected by several factors, including (1) the culture medium used; (2) incubation conditions; (3) the rate of diffusion of the solution or antibiotic; (4) the concentrations of the antibiotics used; and the antibiotic sensitivity of the organism being tested[10][17]. These conditions are very important role to determine an antibacterial activity. However, irradiation doses are trend to indicate the effective to hydrogel on an antibacterial activity.

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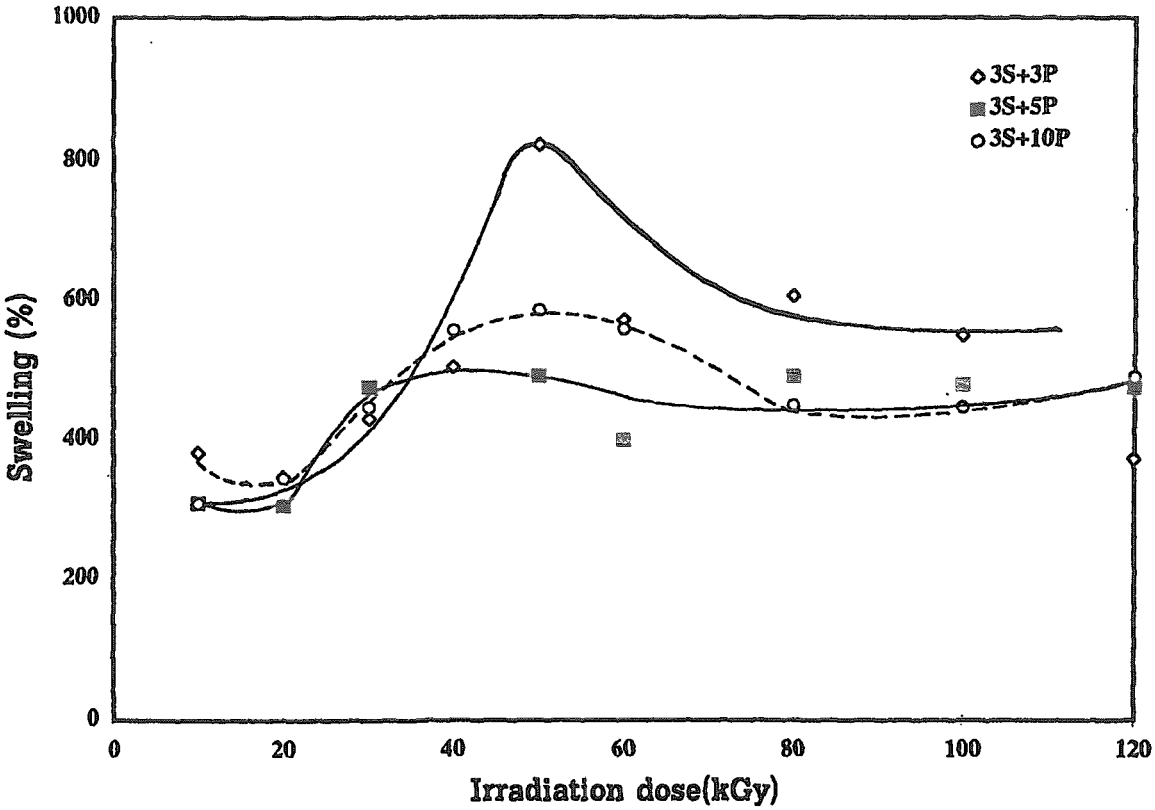

Fig. 1 Change in the swelling of silk fibroin/PVA hydrogels as a function of irradiation dose

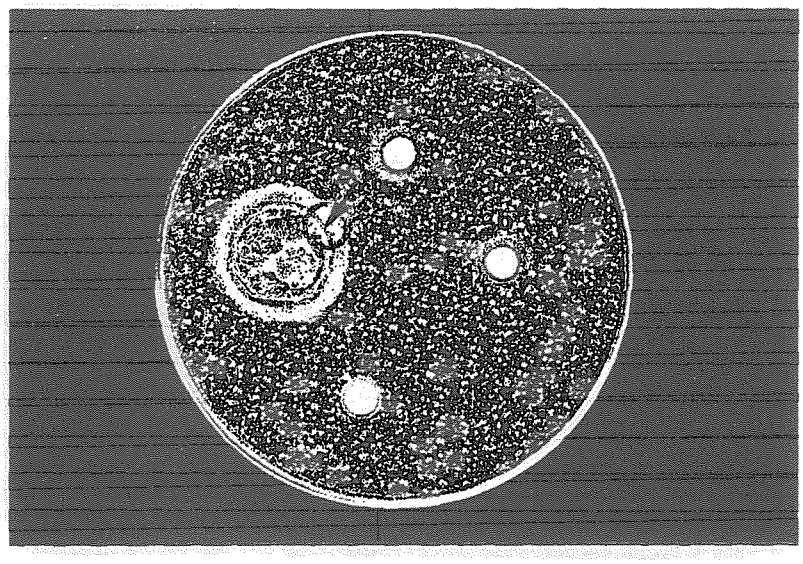

Fig. 2 Clear zone of growth inhibition of *E.coli* at 20 kGy
a : PVA
b : silk protein / PVA
c : silk protein solution after dialysis
d : hydrogel (irradiated silk protein / PVA)

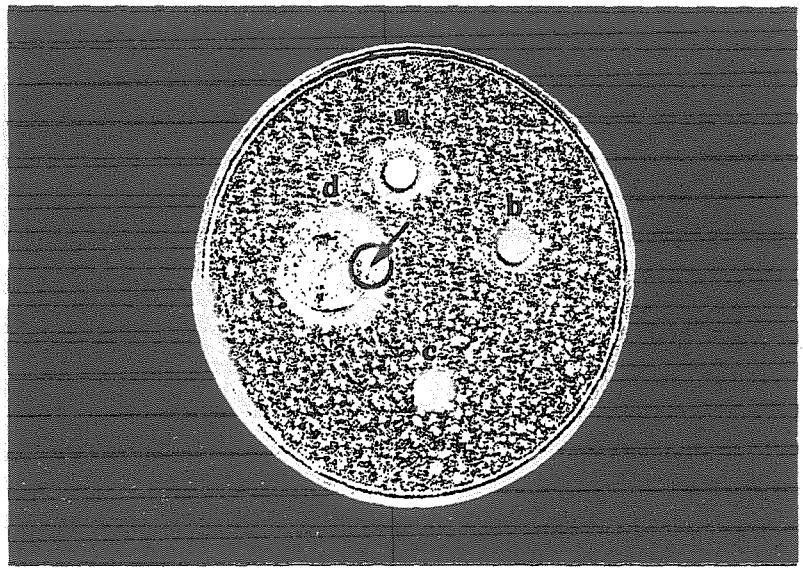

Fig. 3 Clear zone of growth inhibition of *E.coli* at 50 kGy
a : PVA
b : silk protein / PVA
c : silk protein solution after dialysis
d : hydrogel (irradiated silk protein / PVA)

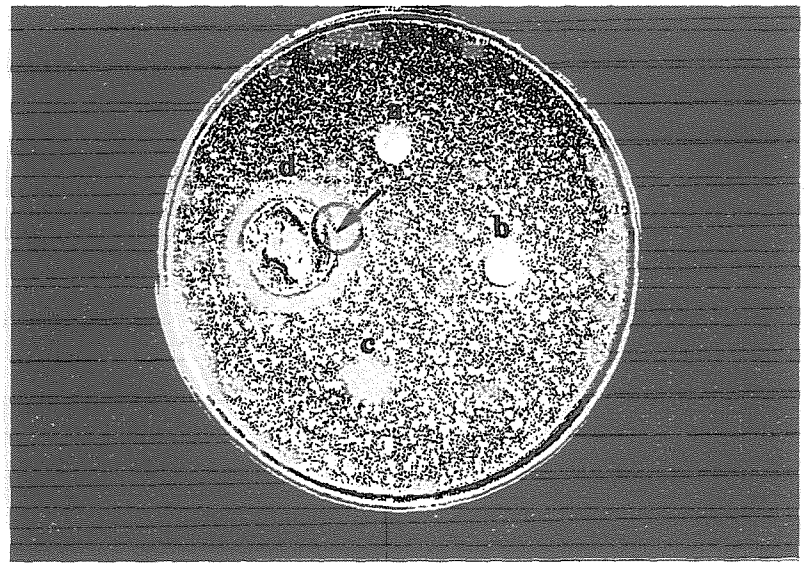

Fig. 4 Clear zone of growth inhibition of *Bacillus subtilis* at 20 kGy

a : PVA

b : silk protein / PVA

c : silk protein solution after dialysis

d : hydrogel (irradiated silk protein / PVA)

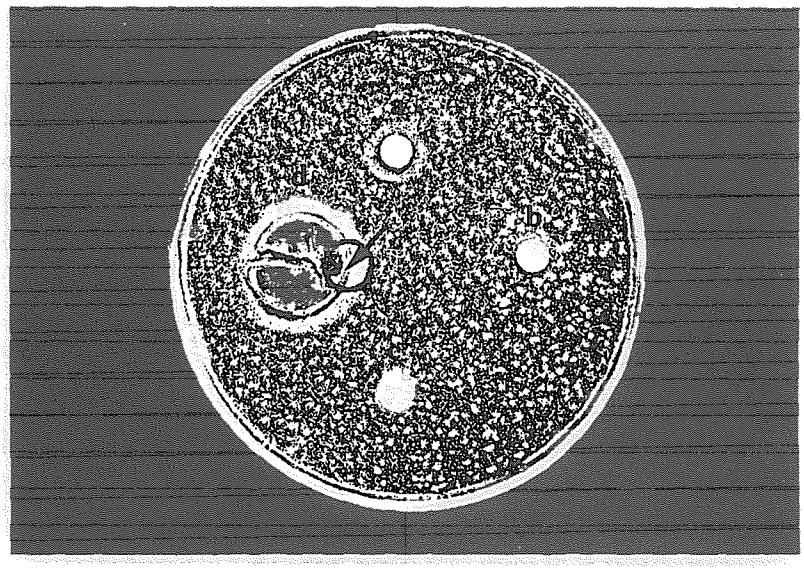

Fig. 5 Clear zone of growth inhibition of *Bacillus subtilis* at 50 kGy

a : PVA

b : silk protein / PVA

c : silk protein solution after dialysis

d : hydrogel (irradiated silk protein / PVA)

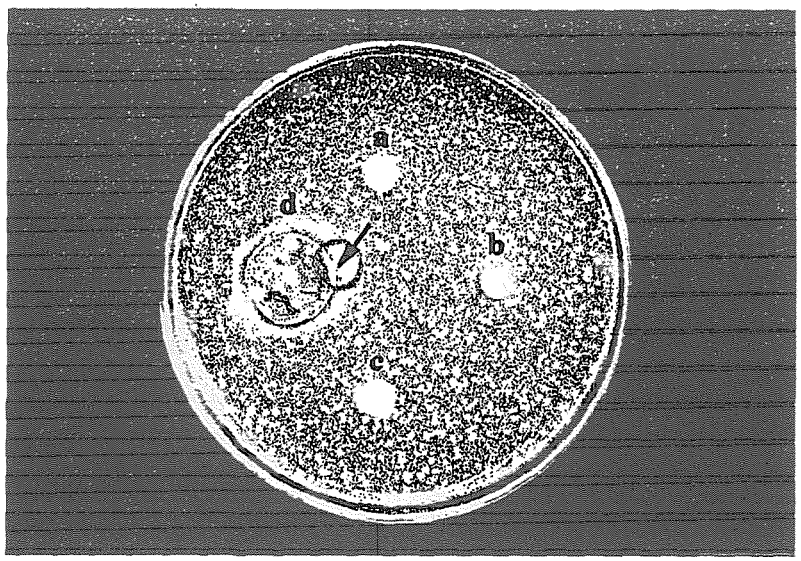

Fig. 6 Clear zone of growth inhibition of *S. aureus* at 20 kGy

a : PVA

b : silk protein / PVA

c : silk protein solution after dialysis

d : hydrogel (irradiated silk protein / PVA)

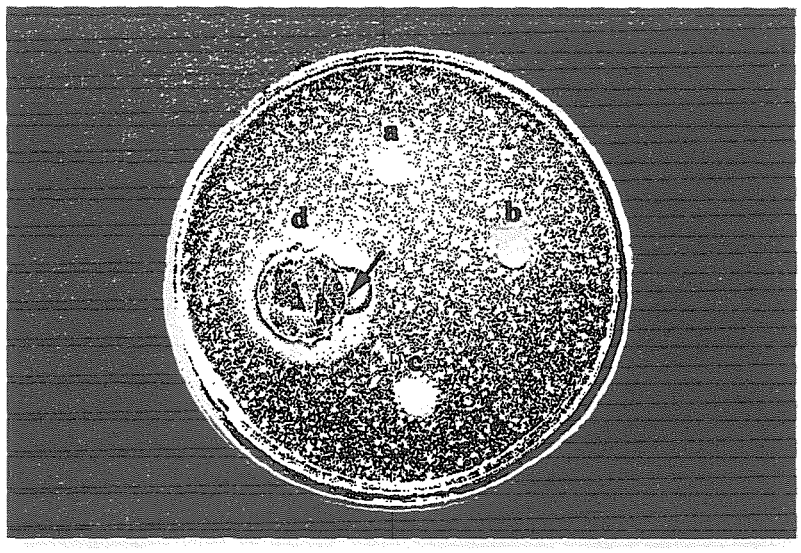

Fig. 7 Clear zone of growth inhibition of *S. aureus* at 50 kGy

a : PVA

b : silk protein / PVA

c : silk protein solution after dialysis

d : hydrogel (irradiated silk protein / PVA)

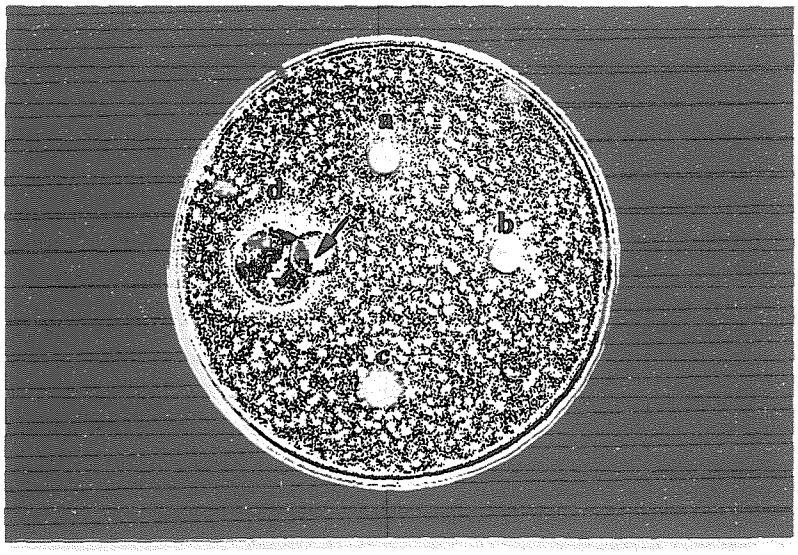

Fig. 8 Clear zone of growth inhibition of *S.epidermidis* at 20 kGy
a : PVA
b : silk protein / PVA
c : silk protein solution after dialysis
d : hydrogel (irradiated silk protein / PVA)

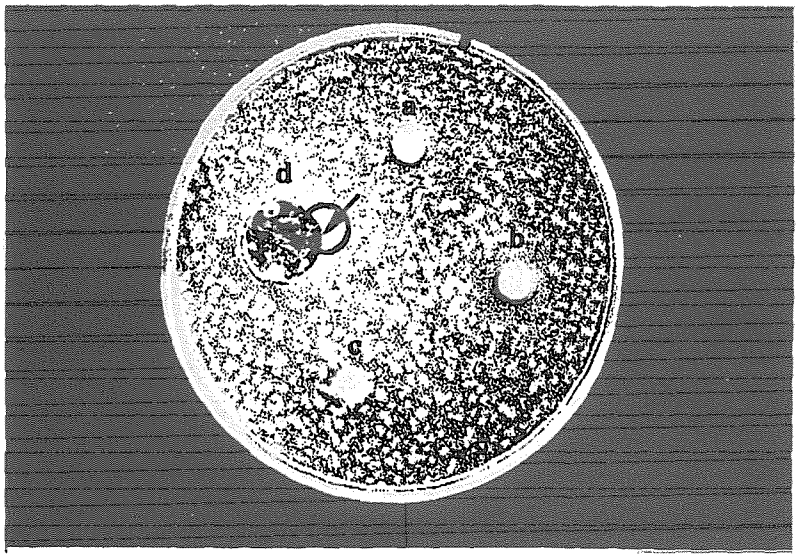

Fig. 9 Clear zone of growth inhibition of *S.epidermidis* at 50 kGy
a : PVA
b : silk protein / PVA
c : silk protein solution after dialysis
d : hydrogel (irradiated silk protein / PVA)

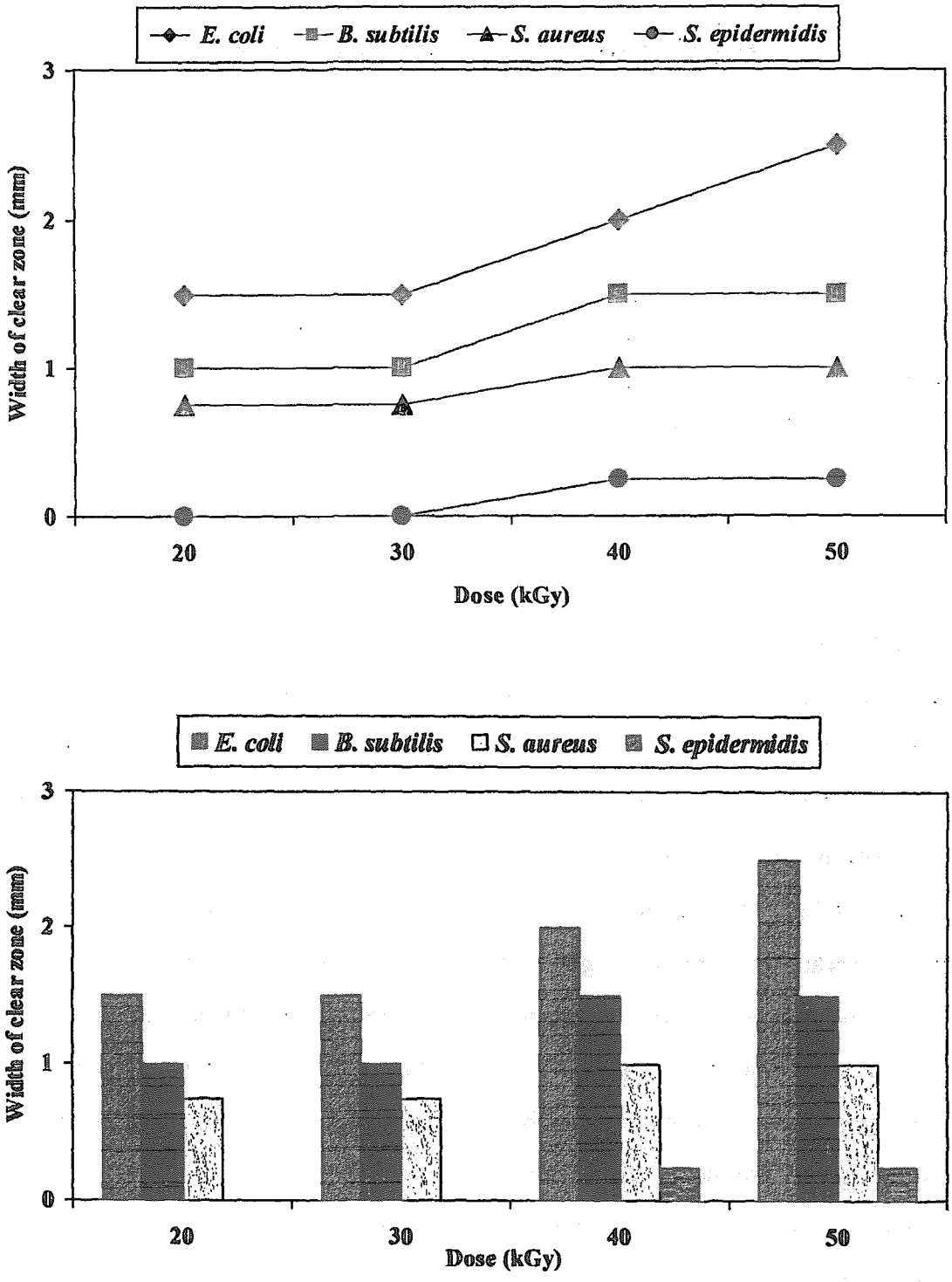

Fig. 10 Width of clear zone of growth inhibition of four bacteria by hydrogel at various doses