INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 9, No. 6, p. 187-192, 2016

RESEARCH PAPER OPEN ACCESS

Antibacterial activity of *Trichosanthes cucumerina* seed lectin and study of its structural stability by fluorescence spectroscopy

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Key words: Fluorescence spectroscopy; bacterial agglutination; bacterial growth inhibition.

http://dx.doi.org/10.12692/ijb/9.6.187-192 Article published on December 14, 2016

Abstract

A *Trichosanthes cucumerina* seed lectin (TCSL) was purified previously that showed potent inhibitory effects against Ehrlich ascites carcinoma (EAC) cells *in vivo* in mice. In the present study, the lectin was treated with guanidine-HCl for 2 and 4h in the presence and absence of Ca^{2+} and changes in the tryptophan fluorescence shift were monitored by fluorescence spectroscopy. It was found that the lectin stability was increased in the presence of Ca^{2+}. Although the denaturant changed the environment of tryptophan residue, it did not affect the binding sites of TCSL as red blood cells became agglutinated after the treatment with EDTA. Besides the agglutination of three pathogenic bacterial species, the lectin also partially inhibited the growth of *Salmonella enteritidis* and *Staphylococcus aureus*.

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Introduction

Belonging to the family Cucurbitaceae, Snake gourd *(Trichosanthes cucumerina)* is found in the wild across much of South and Southeast Asia, including India, Bangladesh, Nepal, Pakistan, Sri Lanka, Indonesia, Malaysia, Myanmar (Burma), and southern China (Guangxi and Yunnan). It is an important summer vegetable in Bangladesh, but it may grow throughout the year except in extreme winter (Khatun *et al*., 2010).

Plant lectins are carbohydrate-binding proteins with different biological properties, such as agglutination, toxicity, anti-proliferation of cancer cells, anti-fungal and anti-bacterial activities (Sitohy *et al*., 2007; Tian *et al*., 2008; Liu *et al*., 2010; Kabir *et al*., 2011a; Kabir *et al*., 2011b; Kabir *et al*., 2013; Kabir and Reza 2014; Rafiq *et al*., 2013). Previously, a novel lectin was purified from Snake gourd seeds cultivated in Bangladesh with the molecular weight of 56±2 kD containing two sub units 34±1 and 22±1 (Kabir *et al*., 2012). This lectin was different from the one purified from the Indian Snake gourd seeds (Padma *et al*., 1999). The lectin showed broad range of pH optima with the heat stability up to 70°C. TCSL agglutinated Human blood (A, B, O & AB), mouse, chicken, cow and some pathogenic bacteria. It showed toxicity against brine shrimp nauplii. The lectin decreased the EAC cells growth 28% and 72% at a dose of 1 and 2 mg/ml concentrations, respectively. Moreover the lectin increased the blood parameters of the mice towards the normal level with a decrease of tumor burden and increased their life span. After observing this antitumor activity of TCSL, its structural stability, bacterial agglutination and antibacterial activity against the pathogenic bacteria was studied in this manuscript.

Material and methods

Purification of TCSL

Lectin was purified from Snake gourd seeds (Kabir *et al*., 2012) and the protein concentration was determined by Lowry's methods (Lowry *et al*., 1951) where BSA was used as the standard protein. Agglutination of red blood cells was studied according to the method described by Kabir *et al* (Kabir *et al*., 2012).

Bacterial Agglutination Activity

Listeria monocytogenes, *Salmonella enteritidis*, *Shigella flexneri*, *Staphylococcus aureus*, *Shigella boydii* and *Pseudomonas aeruginosa* were used to examine the bacterial agglutination activities of TCSL. The bacteria were grown at 37°C for 18 h in liquid nutrition medium and centrifuged at 1,027 g for 5 min and washed with 10 mM Tris-HCl buffer saline, pH 7.8. The bacteria were re-suspended in the same buffer with a turbidity of 2.3 at A_{630}. Then TCSL (0.8 mg/ml) was serially diluted with a hemagglutination buffer in the presence and absence of 0.8 mM of lactose and 50 µl of each bacterial suspension was mixed to a final volume of 100 µl in a 96-well microtiter plates. The plate was agitated for 2 min and was kept at room temperature for 60 min. Finally, light microscope was used to monitor the bacterial agglutinating activity.

Bacterial Growth Inhibition

In the presence and absence of different concentrations of TCSL (30 µg/ml - 240 µg/ml) in bacterial nutrient broth, the bacterial growth inhibition was studied according to the method previously described (Kabir *et al*., 2011). Three species of bacteria (*Pseudomonas aeruginosa, Salmonella enteritidis* and *Staphylococcus aureus*) were used for this study. The bacteria were grown overnight in the nutrient broth at 37°C and the absorbance was adjusted to 0.18-0.2 at A_{630} by adding the liquid nutrient medium. Then TCSL was serially diluted in the nutrient broth in a 96-well microtiter plate and 50 µl of each bacterial suspension was mixed to a final volume of 100 µl. Four wells without the lectin for each bacterium were used as control. The reading was taken at A_{630} after the plates were agitated for 8 h at 32°C by using temperature controlled titer plate shaker. According to the following formula the percentage of bacterial growth inhibition was determined:

% inhibition = {(Absorbance of control - Absorbance

of test) /Absorbance of control} × 100.

Fluorescence spectroscopy

The fluorescence measurement of TCSL (40 and 50 µg/ml) in tris buffer saline (TBS) has been performed at 30°C temperature by using a Shimadzu Spectrofluorometer RF-5301 PC. Fluorescence spectrum of 40 µg/ml TCSL was taken in the presence and absence of 1mM of $CaCl_2$. TCSL (50 µg/ml) was treated with 0.5 M of Guanidine-HCl for 2 h and 4 h in the presence of 1 mM of $CaCl_2$. All samples were placed in a 1×1×4.5 cm quartz cuvette, excited at 280 nm and the emission was recorded in the range of 300-400 nm Widths for the excitation and emission monochromators were maintained at 5 nm.

Results and discussion

Bacterial Agglutination Assay

Out of six, TCSL agglutinated three bacterial species including both gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Salmonella enteritidis* and *Shigella flexneri*) and the minimum agglutination concentration for each bacterial species was 50 µg/ml (as summarized in Table 1).

Table 1. Bacterial agglutination by TCSL**.**

Bacterium	TCSL Concentration (µg/ml)
Listeria monocytogenes	No agglutination (NA)
Salmonella enteritidis	50
Shigella flexneri	50
Staphylococcus aureus	50
Shigella boydii	NA
Pseudomonas aeruginosa	NA

In the previous study, it was observed that *Bacillus megaterium*, *Bacillus subtills*, *Salmonella* typhi, *Sarchina lutia*, *Shigella shiga* and *Shigella sonnei* were also agglutinated by this lectin in different concentrations and among those, *Shigella sonnei* was the most sensitive towards TCSL (Kabir *et al*., 2012). The lectin was unable to agglutinate *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Shigella boydii*. These results indicated that with a broad-spectrum antibiotic activity, TCSL can recognize the bacterial surface carbohydrate molecules on several species of bacteria and agglutinates those possibly due to the presence of cognate glycan antigens on their cell surface (Ghanekar *et al*., 1980).

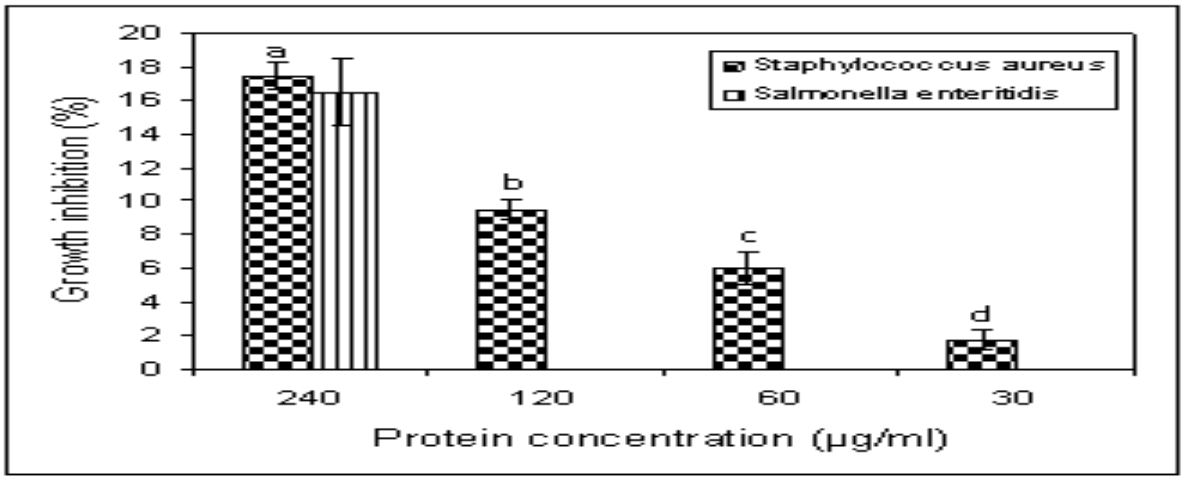

Fig. 1. Percentage of growth inhibition of *S. enteritidis* and *S. aureus* in presence of different concentrations of TCSL. (n = 3, mean ±S.D.). Values followed by different lowercase letters on top of column bar are significantly different at P<0.05.

Bacterial Growth Inhibition

TCSL partially inhibited the growth of *Salmonella enteritidis* and *Staphylococcus aureus* and did not show any inhibitory effect against *Pseudomonas aeruginosa*. Maximum growth inhibition was observed at a concentration of 240 µg/ml for both bacteria (Fig. 1). The inhibitory effect against *Staphylococcus aureus* was decreased gradually with the decrease of protein concentration, while the growth of *Salmonella enteritidis* was not affected at 120 µg/ml and in smaller protein concentrations.

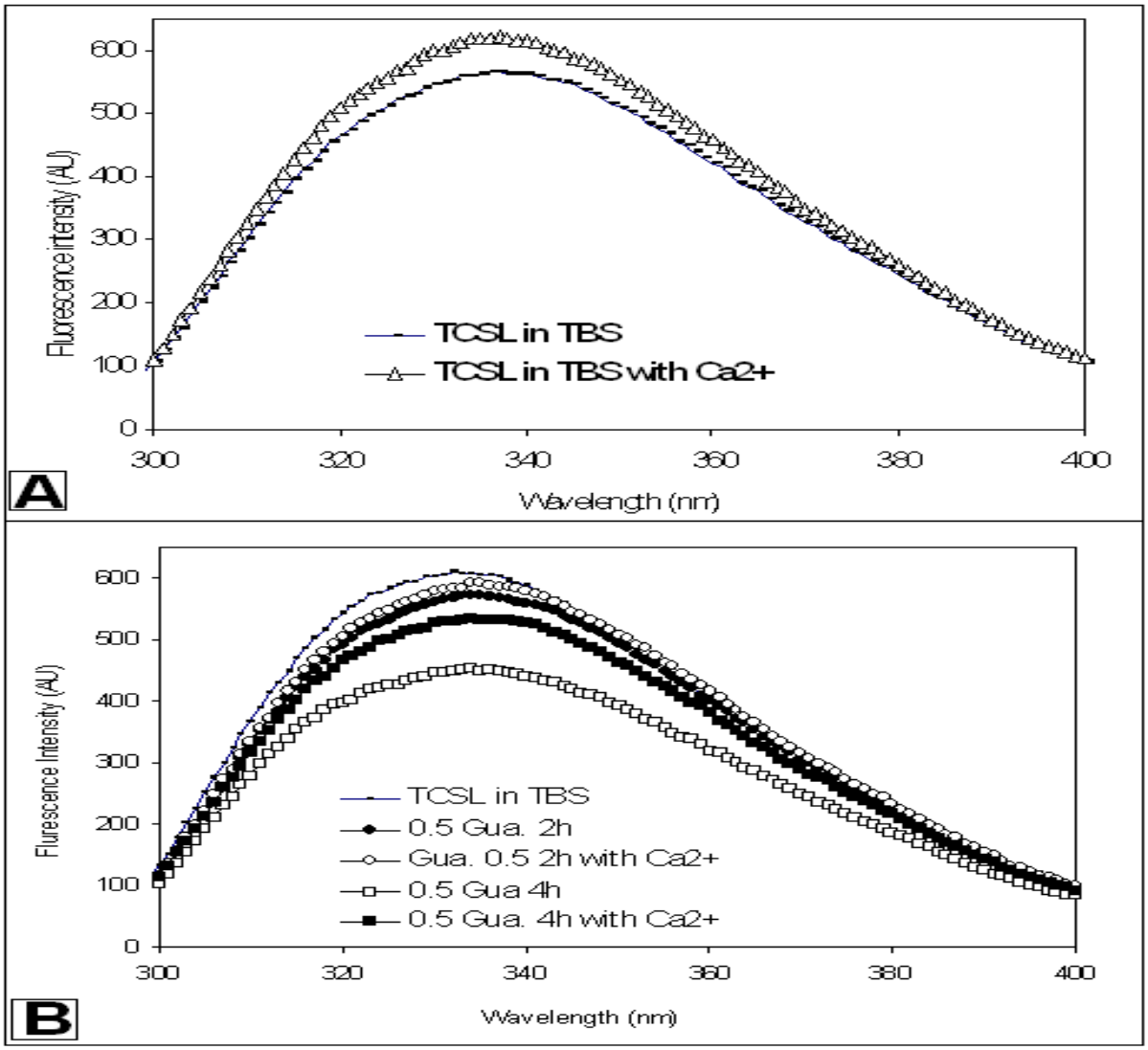

Fig. 2. Fluorescence spectra of TCSL under different conditions. (A) Fluorescence emission spectra of 40 µg/ml TCSL in TBS (-), TBS containing 0.5 mM $CaCl_2$ (-Δ-). (B) Fluorescence emission spectra of 50 µg/ml TCSL (-). TCSL incubated in 0.5 M guanidine-HCL for 2 h with Ca^{2+} (-◦-) and without Ca^{2+} (-•-) and 4 h with Ca^{2+} (-▪-) and without Ca^{2+} (-▫-).

Two distinct levels of antibacterial activity were demonstrated: a low percentage of inhibition, possibly suggesting bacteriostatic activity and a higher activity that may be bactericidal (Hubert *et al.*, 1996). In fact, observations under the microscope revealed that some bacteria were deformed or showed decreased mobility during the antibacterial assay when compared to the control (Tunkijjanukij & Olafsen, 1998). TCSL showed this type of low percentage of inhibition towards *Staphylococcus aureus* and *Salmonella enteritidis* whereas high antibacterial activity was exhibited by *Holothuria scabra* lectin (Gowda *et al.*, 2008) inhibiting the complete growth of both gram-positive bacteria

(*Staphylococcus sp*) and gram negative bacteria (*Shigella sp*, *E. coli*, *Proteus sp* and *Serratia sp*).

Fluorescence Measurement of TCSL at different conditions

Structural changes of TCSL were measured upon binding with Ca^{2+}, by measuring fluorescence emission spectra with excitation at 280 nm as shown in Fig. 2A. The fluorescence spectrum of TCSL reached to the peak at 330 nm, mainly from tryptophan residues in the protein molecule. Ca^{2+} stabilization of TCSL was examined by incubating the lectin solution with 0.5M of guanidine-HCl in the presence and absence of 1 mM $CaCl_2$ during a shorter time period (2 h) and a longer time period (4 h) at 30°C as shown in the figure. When TCSL was incubated with 0.5M of guanidine-HCl for 2 h, a drop in the spectrum was found but when the lectin became treated with 0.5 M of guanidine-HCl for 4 h, a large drop in the spectrum was observed. On the other hand, when the lectin was incubated in 0.5 M of guanidine-HCl for 2 h in the presence of Ca^{2+}, the peak of the spectrum reached higher than that of the peak obtained in the absence of Ca^{2+}. A significant increase in the peak level was observed when TCSL was treated with Ca^{2+} for 4 h (Fig. 2B).

In the previous study, it was found that the hemagglutination activity of TCSL was not affected by the pH values from 3 to 12 and also by the divalent cations, guanidine-HCl, urea and EDTA (Kabir *et al*., 2012).

In the present study, the fluorescence intensity of TCSL at 330 nm became remarkably increased in the presence of Ca^{2+} salt. When TCSL in TBS was incubated with 0.5 M guanidine-HCl for 2 h and 4 h in the presence and absence of 1 mM $CaCl_2$ a remarkable change was observed. This result indicated that the change in environment of tryptophan residue(s) does not affect the binding sites. From this result it can be concluded that guanidine-HCl induced a structural change that did not affect the lectin binding sites. On the other hand, Ca^{2+} induced a conformational change in the TCSL molecule that stabilized the lectin structure.

Conclusion

TCSL inhibited the growth of a number of pathogenic bacteria. Though the denaturant changed the environment around its tryptophan residues, binding sites of TCSL did not become affected. The lectin stability increased in the presence of Ca^{2+} salt that would be very important for the formulation of the lectin as an antibacterial agent.

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