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PRODUCTION AND IMMOBILIZATION OF PROTEASE INHIBITORS FROM *BCAILLUS SUBTILIS* (M24)-A POSSIBLE CANCER-FIGHTING AGENT AGAINST TUMOR PROLIFERATION IN HUMAN GUT

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ABSTRACT

Definite inhibition of proteases with their respective inhibitor is a special technique adopted for drug designing. PIs may also play their role as therapeutic agent for the treatment of cancer, cardiovascular and immunological diseases. Twenty-five bacterial strains were selected on the basis of their Protease Inhibitory activity, among them *Bcaillus subtilis* (M24) had highest protease inhibitory activity against trypsin. Then Protease Inhibitory activity was calculated before and after immobilization and there was a remarkable increase in Protease Inhibitory Activity against trypsin. Different Protease Inhibitory activities after immobilization against trypsin were 152 U/ml for Lewatite, 167U/ml for Dowex 80, 160U/ml for Dowex 66, 189 U/ml for Duolite, 175 U/ml for Amberlite, 207 Ulml for IR100, 157 U/ml for IR401. There were significant increase of protease inhibitory activity after immobilization, which were 9.4 fold increase for Lewatite, 10.8 fold for Dowex 80, 10 fold for Dowex 66, 12 fold for Duolite, 11 fold for Amberlite, 13 fold for IR100, 9.7 fold for IR401. Our final product, which is in the form of immobilized Protease Inhibitor of *Bacillus subtilis* are able to kill tumor protease and cancerous protein of human colon. Human colon has a natural habitat of *Bacillus subtilis*. The pro-biotic has ability to kill cancer protein and tumor protease due to the production of intracellular

Protease Inhibitor in human colon. *Bacillus subtilis* produces alkaline Protease Inhibitor has strong tendency to fight against human cancer.

Key words: *Bacillus subtilis*, Immobilization, Pro-biotic, Ion-exchange resins, Protease Inhibitor

INTRODUCTION

Protease inhibitors (PIs) are proteins naturally occurring in living organisms and able to inhibit and so, control the activity of proteases. They are ubiquitous proteins, occurring in animals, microorganisms and plants [1]. Different types of protease inhibitors inactivate the target protease in different human disease like high blood pressure, arthritis, pancreatitis, thrombosis, muscular dystrophy, cancer and AIDS [2]. Three generation of protease inhibitors (PIs) have been developed with the aim to improve the drug efficacy and/or properties such as better patient quality of live, increased survival, slowed diseases progression, decreased viral load, increased immunological response and decreased opportunist infections [3]. Alkaline Protease Inhibitors are extracted from *Bacillus subtilis,* a pro-biotic which has strong tendency to fight against cancer protein. *Bacillus subtilis* provides a pathogen free environment to our gut and stabilize the intestine of human being for its natural fighting against foreign microbes. *Bacillus subtilis* also act as antibiotic in treatment of cancer [4]. Initially pro-biotics are observed for the evaluation of gut microflora [5] and the findings proved that health-improving bacteria living in the gastrointestinal tract have invading pathogens and have strong ability to sustain activities of microbiota [6]. Furthermore, the probiotics-bacterial application in vivo described that current approach could boost innate and adaptive immunity [7]. The spores of *Bacillus subtilis* are able to produce intracellular Protease Inhibitor with increasing activity within the cell [8]. *Bacillus subtilis SK09,* a pro-biotic organism have isolated from dairy effluents with the ability of sporulation under stressful environment. The strain has the ability to fight against cancer as a result the strain has proved itself through MTT assay on Human Colon cancer cell line HT-29. Along this the strain has strongly positive result against in vitro cytotoxic activity [9]. The spores of *Bacillus subtilis* are used in oral bacteriotherapy during gastrointestinal disorders of human beings. *Bacillus* species are used as pro-biotic which is included in lactic acid producing bacteria [10]. The bacterium secretes protein directly to culture medium and does not produce endotoxins

[11]. It is responsible for the generation of antibiotics, protease and carbohydrases [12].

Anti-cancerous activities of various bacterial species especially from *Bacillus* are reported in several scientific reports. Different industries are trying to achieve some potential target drugs against cancer from different species of *Bacillus* [13]. *Bacillus subtilis* show different responses under different circumstances like antibiotic synthesis, extracellular enzyme synthesis, competency for taking DNA and mobility [14]. The strain FS05 of *Bacillus subtilis* have showed cytotoxic behavior against human cancerous cell line like HepG2 (hepatocellular carcinoma), HCT (colon carcinoma) and MCF-7 (breast carcinoma) through MTT assay [15]. *Bacillus sp.* strongly show its anti-fungal and anti-bacterial activities [16]. The bacterium suppress the tumor growth with the ability of cancer killing cells [17].

MATERIALS AND METHODS

Microorganism

Different bacterial strains was isolated from soil, air and water near protein decomposing bodies**.** Among 75, isolated bacterial strains, 24 were short-listed for the screening of Protease inhibitory activity. The strain having highest protease inhibitory activity was selected for further experimentation.

Identification

For the identification of bacterial strains, isolated sample was send to First BASE Laboratories Sdn Bhd (604944-X) Malaysia. It was proved from the result of DNA sequencing that *Bacillus subtilis* was studied pro-biotic of our research.

Production of Protease Inhibitor: Inoculum was prepared by adding loop full of bacteria in prepared autoclaved flask with 0.8% of nutrient broth. Then flask was kept in rotatory shaker at 37 ° C for 24 hours within a speed of 200rev./min. Sub-merged fermentation technique was used for the production of enzyme. Media was consisting of 2% of soybean meal, 1.5% of glucose, 2% of peptone, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 and 0.5% sodium carbonate For this purpose, 0.5ml of inoculum was poured in well-prepared and autoclaved media flask. The flask kept in rotatory shaker for 48hrs at 37 ° C within a speed of 200rev./min

Protease Inhibitory activity

Protease Inhibitory activity was calculated with little amendments by Kunitz (1947) methods [18]. 1 ml of Trypsin was mixed with 1 ml of Protease Inhibitor for 15 min with optimum temperature of 37°C. Then 2ml of casein (1%) was transferred in

above mentioned test tube for 30 min. For the termination of reaction 2.5ml 0f TCA (0.44M) was added in given test tube. In this method, the TCA soluble fractions formed by action of trypsin on the protein substrate hammerstein casein was measured by the change in absorbance at 280 nm. The residual caseinolytic activity of the trypsin in the presence of inhibitor, at 37 ° C, was used as a measure of inhibitory activity. Appropriate blanks for enzyme, inhibitor, and substrate was included in the assay along with the test. The mixture was further processed for 15 min in centrifugation machine with speed of 1000rpm. Then absorbance of supernatant was taken at 280 nm.

Immobilization on ion exchange resins

Physical adsorption is a method, which is adopted for immobilization. For this purpose 5ml of Protease Inhibitor was taken in test tube and 0.6 gram of different ion exchange resin was added in same test tube and kept it in shaking water bath for 60 min at 37 ° C within the speed of 100 rpm. After one hour sample was centrifuged for 15min at the speed of 6000 rpm [19]

Enhancement in Protease Inhibitory activity after immobilization

Immobilized ion exchange resins was used to calculate the activity of Protease Inhibitor. There was remarkable increase in the activity of Protease Inhibitor as previously calculated through Kunitz method [18].

Proposed application of novel drug against cancer

As already mentioned above that *Bacillus subtilis*, which is a pro-biotic, has strong tendency to fight against cancer. The bacteria, is immobilized on ion exchange resins and stored its activity in the form of spores. Now this immobilized probiotic is ready to use as a cancer-fighting agent. Human gut is a natural habitat of *Bacillus subtilis* and have ability to eliminate any pathogenic activity in its surrounding. As it is known that Alkaline Protease Inhibitor is able to degrade cancer protein especially trypsin protease and our novel bacterial Alkaline Protease Inhibitor after immobilization can perform the same function.

RESULTS

It was calculated from Figure 1, that's even different ion exchange resins (from S1 to S7) used for Immobilization. Lewatite, Dowex 80, Dowex 66, Duolite, Amberlite, 1R 100, IR401 were denoted with S1, S2, S3, S4, S5, S6 and S7 respectively. Protease Inhibitory Activity of Bacterial Strain M24 against Trypsin (Alkaline Protease) was

calculated after Immobilization. Immobilized sample of PIs showed enhanced activity as compare to partially purified sample of same strain. IR 100 showed highest (13) fold of purification as compare to Lewatite which showed least (9.4) fold of purification. There were significant increase of protease inhibitory activity after immobilization, which were 9.4 fold increase for Lewatite, 10.8 fold for Dowex 80, 10 fold for Dowex 66, 12 fold for Duolite, 11fold for Amberlite, 13 fold for IR100, 9.7 fold for IR401.

It was observed from figure 2, that ion exchange resins have different effect after immobilization on same Protease Inhibitors against Trypsin. Different Protease Inhibitory activities after immobilization against trypsin were 152 U/ml for Lewatite, 167U/ml for Dowex 80, 160U/ml for Dowex 66, 189 U/ml for Duolite, 175 U/ml for Amberlite, 207 Ulml for IR100, 157 U/ml for IR401.

The results obtained from Figure 3 showed that Effects of Protease Treatment on Protease Inhibitory Activity was calculated for strain M24 against Trypsin (Alkaline Protease) through standard assay mentioned before. Protease Inhibitory Activity was checked for five different protease concentration (0.2%, 0.4%, 0.6%, 0.8%, 1%). Strain M24 showed highest (16.7U/ml) Inhibitory Activity (U/ml) at 0.2% Protease concentration against Trypsin and least (6.3U/ml) value at 1% Protease concentration.

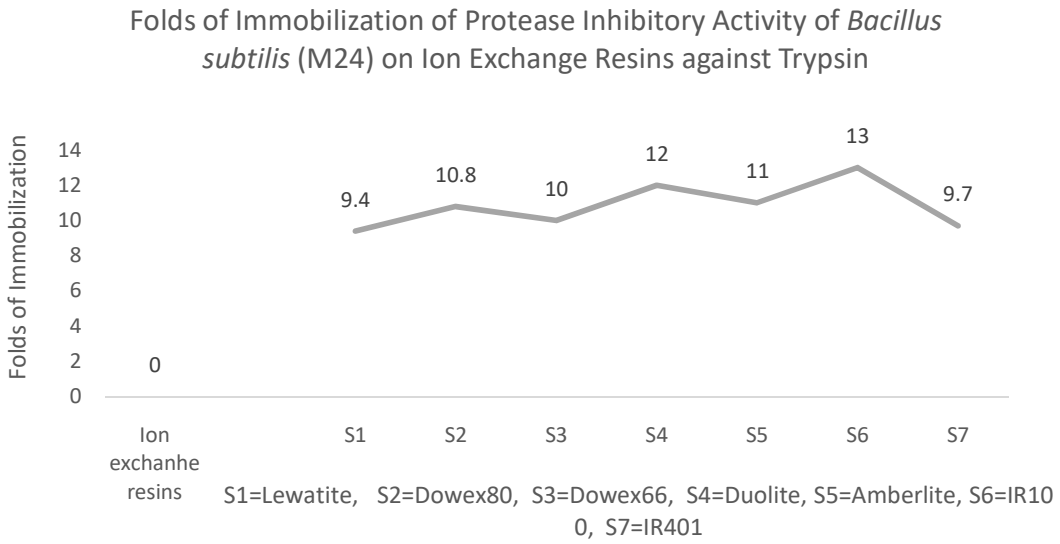

Figure 1

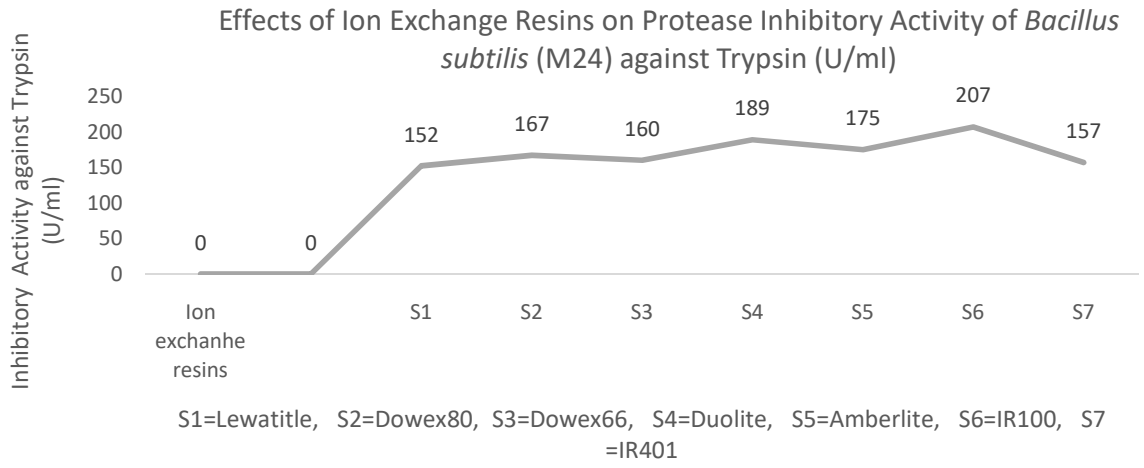

Figure 2

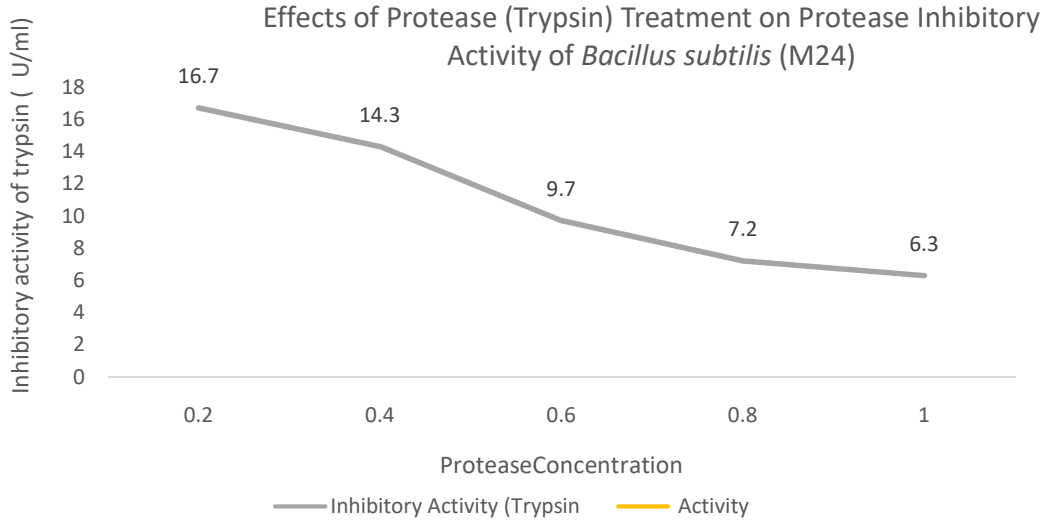

Figure 3

DISCUSSION

It was reported from Vilmaminoska *et al.,* [20] that activity of enzyme was increased up to 3.3 folds for Amberlite IRC-50. Finding observed from Abdle- Naby, *et al.,* [21] showed that immobilized protease from *Bacillus mycoides* had proteolytic activity 28.09U/ml on Amberlite IR-120. Research conducted by Ben A. C. *et al.,* [22] described that Amberlite IRC 50 had Inhibitory Activity 42.3U/ml, Amberlite CG-120 (NA) type III had Inhibitory Activity 31.2 U/ml and Amberlite CG-4B (OH) type I had Inhibitory Activity 28.4 U/ml. Research published by Liu L et al., [23] have clearly matched with our discussion that *Bacillus*

subtilis is available commercially for the production of enzyme and antibiotics. It is also proved through different scientific researches that scientist are trying to improve the efficacy of probiotic by changing their culture through different methods. Observation reported by Vilmaminoska *et al.,* [20] concluded that the Lipase enzyme was immobilized on Dowex 2X8 with activity 39.4 U/ml. Yang D, and Rhee J. S. [24] showed that Ionic binding was proved as better source of immobilization with higher Inhibitory Activity and increased mechanical stability. For Amberlite IR-4B activity was 402 U/g of support while for Amberlite IRC-50 activity was 600 U/g of support.

It was concluded through discussion from Takayuki I., *et al.,* [25] that the growth of human myeloid leukemia cell showed degenerated trend while using HIV-1 Protease Inhibitors. In his research he concluded that PIs like Ritonavir, Indinavir and squinavir were used as inhibitory agent against growth of DU145 and PC-3 androgen-independent prostate cancer. Data published by Bredy J, in 2005 described about probiotic that *Bacillus strain* have great significant role in human gut because of the high metabolic activities [26]. There are 66 antibiotic are reported from *Bacillus subtilis*. *Bacillus* antibiotic are differs in structure and spectrum, most of them are peptides [27]. Research from Hosoi T *et al*., in 2000 reported that Enzymes of *Bacillis* improve the normal flora of gut [28].

CONCLUSION

Protease Inhibitory activity was calculated before and after immobilization and there was a remarkable increase in Protease Inhibitory Activity against trypsin (protease present in cancer). The novel Pro-biotic bacterial strains were immobilized and their activity was retained in the form of spores on pharmaceutically active grades of ion-exchange resins. Our final product, which is in the form of immobilized protease inhibitor of *Bacillus subtilis* are able to kill tumor protease (trypsin) and cancerous protein of human colon. Human colon is a natural habitat for *Bacillus subtilis,* which has strong tendency to suppress tumor proliferation in human gut.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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