Biological Activity of Oligomer Chitin Hydrolysate Produced Using Chitinase Enzymes from SSA2B4.1 (*Bacillus cereus* SW41) Isolate on Lymphocytes and Cancer Cell Lines

カニやエビなどの甲殻類の殻に含まれる成分には抗癌作用があるとされる。そうした水産資源の多いインドネシアで、抗癌剤開発の可能性を探った。

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Abstract

Local chitin waste from crab industries can be used as a source for production of oligomer which has important biological activity. The aim of this study was to evaluate the activities of oligomer produced by enzymatic hydrolysis upon proliferation of lymphocytes and cancer cells. The chitinase enzyme was obtained from thermophilic bacterium *Bacillus cereus* SW41 isolated from South Sulawesi. The reaction products were analyzed and fractionated using HPLC. Cytotoxic assay to determine the lethal concentration 50 (LC_{50}) used the BSLT method. The effect of oligomer hydrolysates on lymphocyte proliferative activity and inhibition of cancer cells was determined by MTT method. The oligomer hydrolysate processes with or without lyophilization at concentration of 62.5 and 125 µg/ml were able to increase lymphocytes proliferation (3-22%). Antiproliferation activity of oligomer chitin hydrolysate was detected in all tested cancer cell lines with the highest activity occurring in MT2 cell ranging from 17-48% followed by Raji cell (17-43%), and HeLa cell (5-33%). Based on these results, we conclude that oligomer chitin hydrolysate could be used as an anti-proliferation of cancer cell but a further study is needed to develop before commercial use.

Keywords oligomer chitin hydrolysate, cancer cell, lymphocyte

Introduction

The issue of food security and safety in Indonesia requires a solution and integrated effort in exploiting all of the potential existing local resources.

Invertebrate marine crustaceans such as shrimps and crab have hard shells that contain a compound known as chitin. Chitin in crustacean is of high content ranging from 20-60% depending on the species (Rochima et al., 2004).

Many studies have shown that kitooligomer compounds from chitin waste have the potential to be developed as an anti-cancer material. Therefore, this study is considered very important to carry out in efforts to increase the added value of chitin waste through the production of bioactive kitooligomer compounds that can be used as a functional food and nutraceutical immune system (Sanford, 2003).

Given this background, this study was conducted on the production of bioactive chitin oligomers by using chitinase enzyme produced by SSA2B4.1 iso-

lates (*Bacillus cereus* SW41) which had been previously characterized wholly, and a bioactivity test to observe the pharmacological activity of a compound. Further, this study was intended to produce the oligomer compounds of chitin which have the bioactive activity of cancer cell anti proliferation, thus making it possible to provide information on alternatives for increasing the value and usability of local marine waste into products of high economic value, and the information provided can be used for industrial development efforts, particularly food and marine-based nutraceutical products, which are likely to become export products.

Research Methodology

Time and Study Site

The study was conducted at the Laboratory of Microbiology and Biochemistry of the Research Center of Biological Sciences and Biotechnology of IPB and Tissue Culture Laboratory, Pathology Section, Department of Reproduction Clinics and Pathology (KRP) Faculty of Veterinary Medicine of IPB. The study was conducted from June - December 2011.

Study Materials

Chitin hydrolysate oligomers of 1% FBS for 6 hours and 1% EM for 12 hours. HeLa cervical cancer cells (ATCC CCL-2), lymphoma Raji cancer cell (ATCC CCL-86), and lymphoma cancer cells T (MT2) from the Stem Cell Cancer Institute of Jakarta. Other materials include among others the culture purposes such as Dulbecco's modified eagle's medium and 3-(4,5-dimethyl-2-thiazoly) -2.5-diphenyl-2H-tetrazolium bromide (MTT).

Research Method

This study consists of four main parts: 1) enzymatic production of bioactive oligomer compounds of chitin hydrolysate, 2) fractionation compounds of oligomers in hydrolysates resulting from enzymatic reaction, 3) cytotoxic testing of Lethal Concentration 50 (LC_{50}), 4) Testing of lymphocyte proliferation activity and inhibition activity of cancer cells.

Study on the production of oligomer compounds of hydrolyzed chitin

The production of chitinase enzyme and manufacture of colloidal chitin used the method of Arnold and Solomon (1986), the production of enzymes FBS and EM adopted the method of Wahyuni (2010), the testing of chitinase enzyme activity and concentration of N-acetyl glucose-amine used the method of Ueda and Arai (1992), the measurement of protein content was based on the method of Bradford (1976) and the electrophoresis followed the method of Bollag and Edelstein (1991).

Fractionation of Oligomer Components of Chitin hydrolysate

Identification and fractionation by HPLC used carbohydrate column (waters) as the stationary phase, and the solvent is 60% acetonitrile in water as the moving phase. Detection is based on the retention time, with a UV detector of 440 model dual lambda, using an injection volume of sample as much as 20 µl and flow rate of 1 ml/min. As standard, an oligomer compound was used, a mixture of Seikagaku Japan with the monomer unit to hexamer at a concentration of 20 mg/ml, and chitosan as standard (Jeon and Kim, 2000).

Cytotoxic testing of Lethal Concentration 50 (LC_{50}) by the Brine Shrimp Lethality Test (BSLT)

BSLT is a test of toxicity on the larvae of *Artemia salina* L. It was carried out on a sample extract of chitin hydrolysate oligomers with a concentration of 0, 100, 125, 150, 200, and 250 (µg / ml). Data were then processed by a probit analysis to obtain LC50.

Testing of hydrolyzed chitin oligomer samples on lymphocytes

The test on lymphocytes is to see the ability of oligomer samples in enhancing the immune system. Suspension of lymphocyte cells (2 x 10^6 cells / ml) in complete medium (RPMI plus fetal bovine serum

10%, 100U/ml penicillin and 100 µg/ml streptomycin) was incubated for 72 hours with or without treatment of oligomer samples of chitin hydrolysate N-acetyl glucosamine, 1% FBS for 6 hours and 1% EM for 12 Hours (Zakaria, 1997). Sample concentration is the concentration of some dilution levels adjusted to the results of LC50 measurement. Cell viability was calculated with the help of trypan blue dye. Lymphocyte cell proliferation activity test was based on the method of 3- (4.5-dimethyl-2-thiazoly) -2.5-diphenyl-2H-tetrazolium bromide (MTT).

Testing of hydrolyzed chitin oligomer samples against cancer cells of HeLa, Raji and MT2

Testing on cancer cells was to see the inhibitory activity of oligomer against cancer cells. Cancer cells (1-2 x 106 cells/ml) in complete medium (DMEM plus 10% fetal bovine serum, 100U/ml penicillin and 100 µg/ml streptomycin) were cultured for 24 hours. After the cell density reached about 50%, the culture was further incubated for 48 hours with or without the treatment of oligomer samples. Sample concentration is the concentration of some dilution levels adjusted to the results of LC50 measurement. Testing of the inhibitory activity against cancer cells was based on ELISA reader at λ 595 nm by using the method 3- (4.5-dimethyl-2-thiazoly)-2.5-diphenyl-2H-tetrazolium bromide (MTT). (Kawaii, 1999).

Results and Discussion

Enzymatic Production of Oligomer Compounds

The testing of hydrolysis ability of some chitinase enzyme on 1% colloidal chitin resulted in some potential enzymes for use in producing the oligomer compounds. The activities of several enzymes are presented in Table 1. Based on these activities, some enzyme concentrations (0.005, 0.0085, and 0.10 units per milligram of chitin) were used to produce the oligomer compounds. The selected enzyme concentrations were based on estimates of the enzyme ability to produce reactions of oligomer compounds in a certain amount of units previously reported by Jeon and Kim (2000).

The results in Table 1 show that pure enzyme appears to have a good percentage of rendemen and specific activities – a low rendemen but with higher specific activities compared with other enzymes including AS because the magnitude of the specific activity is an indication of enzyme purity.

To monitor the reactions of some prepared enzymes with various parameters of enzyme concentration values, substrate concentration and incubation time of enzyme and substrates, as an initial stage a measurement of the N acetyl glucosamine concentration that could predict the formation rate of oligomer compounds in various reactions was carried out. The various production patterns of N acetyl glucosamine are presented in the graph below.

Table 1. Activities of Some Enzymes.

Types of enzymes	Activities (U/mL)	Protein (mg/ml)	Specific activities (U/mg)	Rendemen
Cell-free filtrate (FBS)	0,056	0,203	0,279	1,011
Hot cell-free filtrate 60°C, 20 minutes (FBSp)	0,050	0,175	0,286	0,893
Enzymes with lyophilized ammonium sulfate (AS)	0,068	0,108	0,629	1,213
Purified enzymes (EM) (UV method)	0,096	0,043	2,215	1,713
Purified enzymes (EM) (Bradford method)	0,096	0,022	4,363	1,714

Figure 1 shows differences in the production of N acetyl glucosamine from various enzyme preparations with the same enzyme concentration (0.0085 units per milligram chitin) and the same substrate concentration (1%). More monomer chitin of N acetyl glucosamine will be produced on the enzyme preparations of greater units per milligram of chitin (enzyme concentration) than those of smaller units per milligram of chitin in the same incubation time. The production of chitin oligomers is through an optimized production of specific size/type of chitin oligomers based on the kinetics study of enzyme-substrate reaction. This study includes that of enzyme concentration of 0.0085 U / mg of chitin and substrate concentration of 1% for the optimal production of chitin oligomers.

From the graph above of N acetyl glucosamine production, it can be seen that higher substrate concentrations (within certain limits) will produce higher amount of N acetyl glucosamine with faster incubation times than a smaller concentration of substrate.

For the production of oligomers derived from the purified enzyme, the enzyme was purified by using cell-free filtrate that had previously been treated with saturated ammonium sulfate of 30%, and column chromatography of HIC (Hydrophobic Interaction Chromatography) using a matrix butyl separose as the stationary phase and ammonium sulfate buffer as the mobile phase.

By detection of enzyme purity, the fraction of the highest activity was taken and measured as the basis to be used in the reaction production of oligomer compounds with the targeted enzyme concentration of 0.0085 units per milligram chitin.

Hydrolysate Fractionation of Oligomer Compounds

Oligomer compounds resulting from various reactions of enzymes and substrates were monitored by analyzing the composition and concentration of the compounds in the hydrolysate oligomers from mono to hexamer by using the chromatographic techniques HPLC. Calculation of the concentration of oligomer compounds on each hydrolysate after being analyzed with HPLC is presented in Figure 2.

Resulted composition analysis of the oligomer compounds from some hydrolysates in Figure 2 shows that only the purified enzyme hydrolysate (12 hours) has a composition of monomer to pentamer, while FBS (6 hours) has the composition of only the monomer to tetramer but with the highest level of tetramer, thus it is concluded that the hydrolysate of enzymatic reaction used should contain a tetramer (FBS of 6 hours) and pentamer (EM of 12 hours) which would be further confirmed by a further test of bioactivity. The different composition and concentrations of oligomer compounds can answer the different responses of various hydrolysates bioassay on the proliferation testing of lymphocyte cell culture and cancer cells.

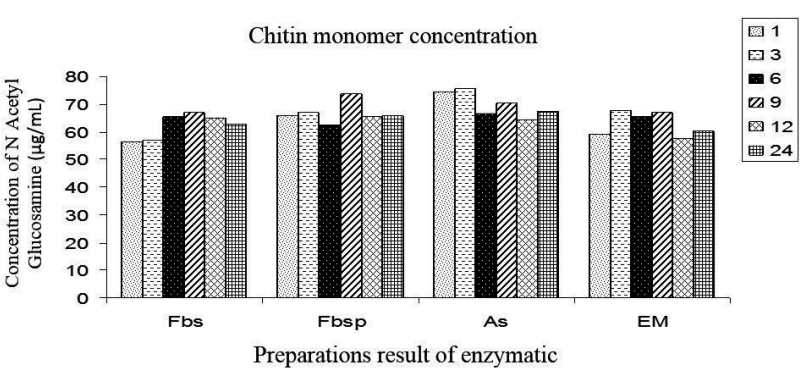

Fig. 1. Concentration of N Acetyl Glucosamine in Various Enzymatic Hydrolisate.

Fbs: cell-free filtrate,
Fbsp: hot cell-free filtrate,
AS: enzyme resulting from a concentrated ammonium sulfate,
EM: purified enzyme
1,3,6,9,12,24 : incubation time (in hours) of enzymes and substrates in oligomer production

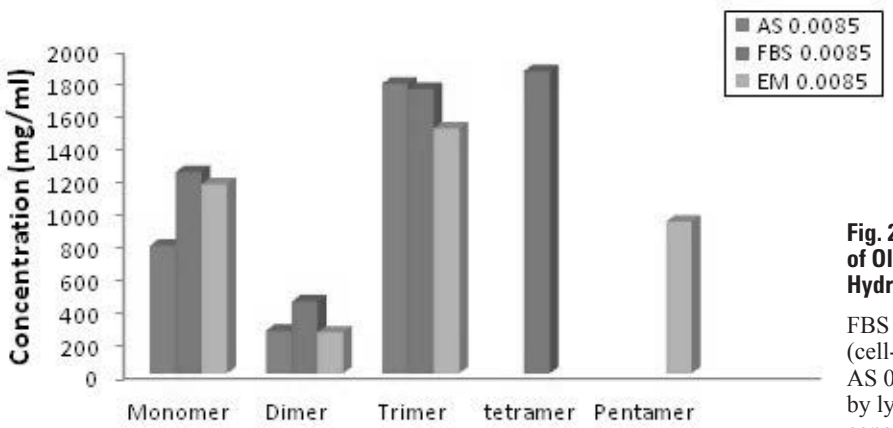

Fig. 2. Composition and Concentrations of Oligomer Compounds in Various Hydrolizates.

FBS 0.0085 6j = Result of enzyme reaction (cell-free filtrate) at a concentration of 0.0085
AS 0.0085 12j = Result of enzyme reaction by lyophilization of ammonium sulfate at a concentration of 0.0085
EM 0.0085 12j = Result of purified enzyme reaction at a concentration of 0.0085

Table 2. Resulted Data of BSLT for Hydrolysate Extract of Chitin Oligomers.

Types of extracts	Concentration (µg/ml)	Log concentration	Mortality (%)	Probit (y)	LC50 (µg/ml)
N-acetyl glucosamine	100	2	25	4.33	153
	125	2.09	38.9	4.72	
	150	2.18	52.6	5.05	
	200	2.3	63.2	5.33	
	250	2.4	76.5	5.71	
FBS 1% 6J	100	2	5.4	3.36	199
	125	2.09	12.9	3.87	
	150	2.18	26.9	4.39	
	200	2.3	47.8	4.95	
	250	2.4	71.4	5.55	
EM 1% 12J	100	2	41.2	4.77	107
	125	2.09	70	5.52	
	150	2.18	88	6.18	
	200	2.3	96.9	6.88	
	250	2.4	100	8.09	

Toxicity Test with BSLT Methods

BSLT test is used as an initial test to determine the activity of a substance or compound contained in an extract or a purified isolates.

The data shown in Table 2 is the mortality data by a probit analysis to get the value of LC_{50} (lethal concentration of 50%). The data showed LC_{50} extracts of chitin oligomers produced from each of calculated amounts: 153 µg/ml, 199 µg/ml and 107 µg/ml. This value indicates that the chitin oligomer extract is included in the toxic category because of LC50 <1000 µg/ml, which has potential bioactivity (Meyer et al, 1982).

Activity of Oligomer Compounds against Lymphocyte Cell Proliferation of Spleen

Enzymatic hydrolysates containing a mixture of enzymes and oligomer compounds used to test the proliferation of lymphocyte cells and cancer cells are FBS and EM with a concentration of 0.0085 units /mg of chitin. In the preliminary study on the enzyme produced, hydrolysates were at an early stage screened at several levels of dilution to see the proliferative activity of lymphocyte cells. The resulted screening showed that hydrolysate with a chitin concentration of 125 µg/ml solution turned out to have shown a quite good proliferative activity

of lymphocyte cells compared to the use of hydrolysate with lower concentration (Wahyuni, 2010). This is consistent with the study by Agustine (2005), with a positive effect on lymphocyte proliferation in vitro at the chitin oligomer concentration of 125 µg/ml. The concentration was then adjusted to the results of LC_{50} measured at a concentration of 125 µg/ml.

One of the parameters to see the immunomodulatory activity of a component is the ability to stimulate the proliferation of lymphocyte cells. The lymphocyte cell proliferation is the process of maturation and multiplication of cells through cell division or mitosis. The proliferating activity of lymphocytes cells T and B can be measured with the stimulation index (SI). Mitogen was used to trigger a non-specific proliferation of lymphocyte cells, in which mitogen lipopolysaccharide (LPS) and Concavalin A (Con A) are used as controls for the stimulation of B cells and T cells.

The resulted observation indicates that the oligomer samples of hydrolyzed chitin have immunomodulatory properties that could stimulate lymphocyte cells. The increased stimulation index of chitin oligomer sample was 3-22%. The oligomer samples of FBS 1% in 6 (six) hours lyophilized at the sample concentration of 125 µg/ml had the highest lymphocyte proliferation of 121.51% or a stimulation index score (SI) of 1.22 (increase of 22%), almost equivalent to mitogen LPS with the SI of 1.28 (28% increase). This result implies that there was an increase in the number of cells up to 1.22 times from the initial cell count of 1 x 10^6 cells/ml. The increased ability of lymphocytes to proliferate or establish clones showed that lymphocyte cells have the ability of immunologic respond or levels of immunity.

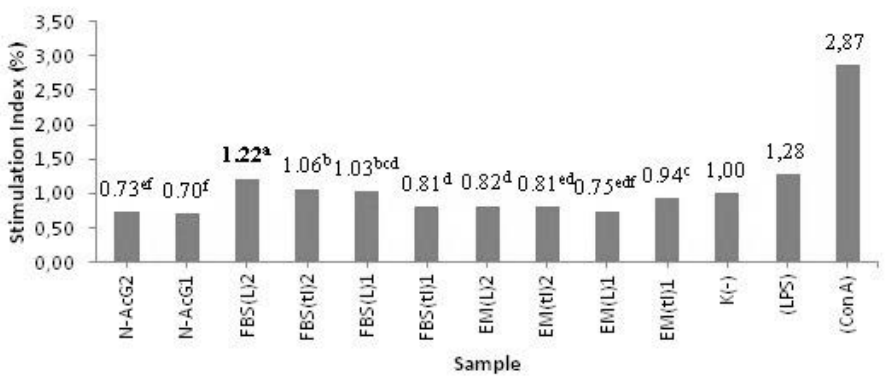

Fig. 3. Stimulation Index of Mice Lymphocyte Proliferation *In Vitro*.

N-AcG2: N acetyl glucosamine at a concentration of 125 µg/ml
N-AcG1: N acetyl glucosamine at a concentration of 62.5 µg/ml
FBS *(l)* 2: FBS 1% lyophilization at a concentration of 125 µg/ml
FBS *(tl)* 2: 1% FBS without lyophilization at a concentration of 125 µg/ml
FBS *(l)* 1: FBS 1% lyophilization at a concentration of 62.5 µg/ml
FBS *(tl)* 1: FBS 1% without lyophilization at a concentration of 62.5 µg/ml
EM *(l)* 2: EM 1% lyophilization at a concentration of 125 µg/ml
EM *(tl)* 2: EM 1% without lyophilization at a concentration of 125 µg/ml
EM *(l)* 1: EM 1% lyophilization at a concentration of 62.5 µg/ml
EM *(tl)* 1: EM 1% without lyophilization at a concentration of 62.5 µg/ml
K (-): negative control
LPS: Positive control with mitogen LPS
Con A: Positive control with mitogen Concanavalin A
Numbers followed by the same letters show no significant difference at the level of 5%

Activity of Oligomer Compounds in Proliferation Inhibition of Several Cancer Cell Lines

The data in Table 3 shows the anti-proliferation of some cancer cells in the oligomer samples of chitin hydrolysates. The inhibitory activity in the oligomer samples of chitin hydrolysate in cancer cells shows significant differences: inhibiting the proliferation of HeLa cancer cell (5-33%), Raji cancer cells (17-43%), and MT2 cancer cells (17-48%). The largest inhibitory activity by oligomer samples of chitin hydrolysate was on MT2 cancer cells, which were derived from the human cell culture T isolated from the stem cells of blood lymphocytes and sub-cultured from the cells of adult patients suffering from T-cell leukemia. The inhibition of cancer cells MT2 (48%) was found in the samples of N-acetyl glucosamine (62.5µg/ml). This correlates with the chitin structure with the monomer N-acetyl glucosamine. Chitin and its derivatives such as chitin oligomers have the ability to inhibit and form cationic (polyelectrolyte), which is predicted to be caused by the bio-functionality of amine group in its structure.

The test results indicated that the use of oligomer compound samples had inhibitory activity against the proliferation of cancer cells. Enzymatic hydrolysate containing monomer unit of chitin oligomers would better inhibit the proliferation of epithelial cells of HeLa type and the suspension cells of MT2 type than those of tetramer and pentamer units of chitin. Each type of cancer cells have different active sides, so the sample or compound that can inhibit or kill cancer cells also differs (Primadona et al, 2006). Cancer cells in a proliferative cycle of cells are sensitive to the effects of cytotoxic compounds.

Conclusions

1. Oligomer compounds can affect the proliferation activity of lymphocyte cells, with an SI increase of 3-22%.
2. The inhibitory activity by the oligomer compounds against cancer cell proliferation in the enzymatic hydrolysate is greater on the suspension type cells (Raji and MT2) rather than the type of one-layer cells (HeLa).

Table 3. Testing of Inhibited Proliferation in Several Cancer Cell Lines.

Samples	Concentration (µg/ml)	Proliferation Inhibition Index (%)		
		HeLa	Raji	MT2
N-Acetyl Glucosamine	125	-11,4bc	32.1a	35.4abcd
N-Acetyl Glucosamine	62,5	**33,2d**	22.9a	**47.9d**
FBS 1% of 6 hours *(l)*	125	16,1cd	**42.7ab**	27.5abc
FBS 1% of 6 hours *(wl)*	125	5,5bcd	35.8a	32.5abcd
FBS 1% of 6 hours *(l)*	62,5	6,7bcd	35.5b	22.5bcd
FBS 1% of 6 hours *(wl)*	62,5	-3,1bc	17.0a	43.3cd
EM 1% of 12 hours *(l)*	125	-2,9bc	29.6a	45.4cd
EM 1% of 12 hours *(wl)*	125	-41,3a	20.2a	23.8ab
EM 1% of 12 hours *(l)*	62,5	-18,5ab	24.8a	43.3cd
EM 1% of 12 hours *(wl)*	62,5	9,4bcd	19.3a	17.9a
Positive controls	5	78	94.5	89.6

Notes: *(wl)*: without lyophilization or without a concentration process
(l): with lyophilization or concentration process
Numbers followed by the same letters show no significant difference at the level of 5%

Suggestions

1. It is necessary to do a study on the mechanisms of lymphocyte cell proliferation and anti-cancer at the cellular and molecular levels such as receptors on cells associated with oligomer compounds.
2. A further study is required to identify and clarify the mechanisms of cancer cell apoptosis and cell membrane damage as a result of oligomer compound treatment.
3. A further study in vivo is also needed in terms of anti-cancer activity in experimental animals.

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