

## Removal of allergenic protein in natural rubber latex using protease from *Bacillus* sp.

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### Abstract

Natural rubber latex (NRL) contains more than 200 kinds of protein. Among these, 13 proteins have been regarded as an allergen that causes allergenicity to the user; especially healthcare workers. Thus, enzymatic degradation of these proteins using *Bacillus* sp. protease was introduced in this study instead of using chemical and physical method to reduce the allergenicity risk of NRL products. This study was aimed to produce protease powder from *Bacillus* sp. and determine minimum protease activity for NRL allergenic protein degradation. For the practical procedure, *Bacillus* sp. was cultured in Nutrient broth at 37 °C, on rotary shaker at 150 rpm for 24 h and subsequently centrifuged to remove cells. Crude protease was precipitated by ammonium sulfate (80% saturation) and dialyzed (12 kDa cut off). Powder protease was prepared by freeze drying and yellowish powder of protease from *Bacillus* sp. was subsequently obtained. The remaining protease activity was 120, 422.53Units/ g powder, while specific activity was 169.78 unit/mg proteins. Different amount of protease (0-500 unit) were added to fresh NRL serum and then incubated at 45°C for 6 hr. The remaining of allergen Hev b1 (rubber elongation factor; REF) was analyzed using western blot technique. REF could not be detected in NRL serum when more than 300 units of protease were added. Consequently, protease from *Bacillus* sp. could degrade REF in NRL.

**Keywords:** Protease, *Bacillus* sp., Natural rubber latex, Allergenic proteins, Rubber elongation factor

### 1. Introduction

Natural rubber latex (NRL) of Para rubber tree (*Hevea brasiliensis*) contains not only rubber particles in an aqueous dispersion, but other inorganic and organic matters are also found. Carbohydrates, lipids and proteins can be found at 1.5, 1.3 and 2.0% by wt., respectively (Perrella and Gaspari, 2002).

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In medical devices manufacturing, proteins seem to be the most considered compound. Fresh NRL can be separated into three phases by centrifugation. The upper phase is rubber particles, while the middle phase is aqueous that called serum phase (B fraction). The bottom phase is called as C fraction that contains lutoid and other intracellular particles (Yeang et al., 2002). Each phase contains unique proteins and 47% of total protein can be found in the serum phase (Beezhold et al., 1994). Among more than 240 kinds of protein that have been identified in NRL of Para rubber tree (Sussman et al., 2002a), 13 proteins have been recognized as allergenic protein by the International Union of Immunological Societies (IUIS) as they elicit an IgE response. Their names are systematically given from Hev b1 – Hev b13.

Rubber elongation factor (REF, Hev b1) was the first NRL allergen characterized at the molecular level and was suggested to be the major allergen in NRL and is the only allergen presenting in latex surgical glove (Palosuo, 1997). Hev b 1 facilitates the action of phenyltransferase enzyme to synthesize polyisoprene rubber chains (Hamilton, 2002). Sussman et al. (2002b) reported that Hev b1 reactivity was found to be presented in 81% of patients with spina bifida and latex allergy. Also only 50% of health care workers showed REF IgE reactivity. Therefore, REF - free NRL as premium grade raw material is required in medical and pharmaceutical devices manufacturing; i.e. condom, surgical gloves, feeding tube and cosmetic pore pack, etc.

Several reports have been introduced the methods to remove allergenic proteins in NRL; including chemical, physical and biological method. Typically some of the proteins are partially degraded by alkaline conditions that caused by ammonia (Perrella and Gaspari, 2002). Multi centrifugation, creaming, simple or ultrasonic leaching, chlorination and enzymatic method have been also applied to remove serum proteins (Pichayakorn et al., 2014). Since multi centrifugation of NRL can eliminate only serum proteins, the rubber particle bound proteins still remain, and alkaline treatment of NRL causes the change in the composition of NRL proteins that affect on the physical properties of latex, enzymatic method is then an alternative and interesting way to reduce allergenic proteins in NRL. Therefore enzymatic method using protease produced from *Bacillus* sp. to remove REF from NRL of Para rubber tree is introduced in this report. This *Bacillus* strain was isolated from fermented soybean food of Chiang Mai, Thailand and regarded as safe (Wongputtisin et al., 2012). The objective of this work was to investigate the REF removal efficiency of protease prepared from *Bacillus* sp.

## 2. Materials and Methods

### 2.1 Microorganism

*Bacillus* sp. was obtained from Agro-industrial Biotechnology Laboratory, Faculty of Science, Maejo University. It was isolated from fermented soybean food of Northern Thailand and maintained on Nutrient agar (NA).

### 2.2 Natural rubber latex

NRL sample was collected from Para rubber trees (*Hevea brasiliensis*) of the cultivar RRIM 600 that kindly obtained from rubber plantation farmer in Phrao District, Chiang Mai. NRL was added with sodium sulfite and ampicillin to the final concentration at 0.05% (w/v) and 10 µg/ml; respectively, and placed in an ice box during transportation to preserve the stability of NRL.

### 2.3 Preparation of NRL serum

Fresh latex was centrifuged at 13,000 rpm (13,413 g) (Hettich zentrifuge; Universal 320 R) for 30 min and at 4 °C. After centrifugation, three phases of NRL were obtained. NRL serum (water phase) was taken using pasteur pipette. Serum was subsequently clarified again by centrifugation with the same condition. Clear serum was stored and preserved in the freezer at -20 °C.

### 2.4 Preparation of crude protease

Inoculum of *Bacillus* sp. was prepared in 5 ml of Nutrient broth (NB) for 24 hr with aeration by shaking at 150 rpm. Inoculum was transferred to 100 µl of sterile NB in 250 ml Erlenmeyer flask. It was cultured at 37 °C, on rotary shaker at 150 rpm for 24 hrs, after that the culture broth was collected by centrifugation at (8000 rpm, 30 min and 4 °C). Salting out technique using ammonium sulfate  $(\text{NH}_4)_2\text{SO}_4$  at 80% saturation was applied to culture broth. The proteins were allowed to precipitate at 4 °C overnight with agitation. The precipitate was collected by nitrocellulose membrane filtration under low temperature. The pellet was dissolved in minimum volume of 0.05 M phosphate buffer pH 7.0 and subsequently dialyzed against 0.05 M phosphate buffer pH 7.0 at 4 °C using regenerated cellulose dialysis membrane with 12 kDa molecular mass cut off. Finally, crude protease was formulated by freeze dryer (FTS Systems Dura-Dry MP) and stored at -20 °C. Protease activity was determined according to the method of Wongputtsin et al. (2012). One unit of protease was defined as amount of enzyme that releases 1 µg of tyrosine per min.

## 2.5 Removal of allergenic proteins in natural rubber latex using protease from *Bacillus* sp.

Ten ml of NRL serum was mixed with various concentrations of crude protease (0-500 Unit) and stand at 45 °C for 6 hrs. The remaining of REF in serums was analyzed using dot blot technique based on the immunological assay.

## 2.6 Analysis of REF protein

Two µl of serum sample was dotted on PVDF membrane that was previously soaked in methanol for 5 min and then in transfer buffer for 15 min before used. After drying, dotted membrane was incubated in 4% (w/v) non-fat milk in TBS buffer for 1 hr at room temperature. Then the membrane was incubated with mouse anti-latex REF monoclonal antibody (1:10000 dilutions in 4% non-fat milk) (clone 07-001; Pierce®) for 2 hr. After that, the reacted membrane was washed 3 times (15 min each) with 0.01% tween 20 in TBS buffer (TBST) and 5 min with TBS buffer. Then membrane was further incubated with horseradish peroxidase-labelled goat anti-mouse IgG (1:10000 dilutions in 4% non-fat milk) (Pierce®) for 1 hr and washed 3 times (15 min each) with TBST and TBS buffer for 5 min. Finally, REF band was visualized by the reaction with 3 ml of HRP substrate (Luminate™ Crescendo Western HRP substrate; Merck Millipore) for 5 min and subsequently expose on X-ray film (KODAX™).

## 3. Results and Discussion

### 3.1 Crude protease preparation

After culturing of *Bacillus* sp. in NB at 37 °C for 24 hrs, protease activity was determined. The results are shown in Table 1. Also crude protease powder was prepared and its activity was determined as shown in Table 1. It was found that 159,040 unit of protease could be produced in 1 L of NB; however, the recovery yield of protease activity was only 49.8% after protein precipitation step but the purity of protease was obviously increased. Moreover, after crude protease was freeze dried, recovery yield decreased.

*Bacillus* sp. is not only the source of protease, but the other interesting industrial enzymes can be produced from this bacterium; for example phytase, cellulase and hemicellulase (Wongputtisin et al., 2012). Normally, the optimal pH for enzymes from *Bacillus* sp. is neutral or weak alkalinity that is similar to pH of NRL, therefore it was expected that our protease might exhibit its hydrolytic activity in NRL, so that complete allergenic proteins degradation could be obtained. Comparing to the production of protease from *Bacillus* F603.1 in Nutrient broth (Chomsri,

2001), our *Bacillus* sp. produced higher protease activity but the recovery yield was lower. There were many factors that effected to our recovery yield. Environmental and technical factors might be the main effects; but higher purity of protease was obtained. There were many factors that affected to our recovery yield. Environmental and technical culture broth as used in this study may not be practical for industrial use. Therefore, low cost culture medium should be investigated for this purpose. There are many reports that had studied the production of protease from *Bacillus* sp. in low cost medium; such as soybean meal (Wongputtisin, 2008), rice bran (Naidu and Devi, 2005), molasses (Hezayen et al., 2009) and potato peel (Mukherjee et al., 2008).

**Table1.** Recovery yield of *Bacillus* sp. protease in each preparation step.

Preparation step	Protease activity (Unit)	Protein (mg)	Specific activity (Unit/mg protein)	Recovery Yield (%)
Crude protease	159,040.00	15,221.93	10.44	100.00
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation + Dialysis	79,200.00	136.80	578.94	49.80
Freeze drying	16,422.00	96.66	169.78	10.32

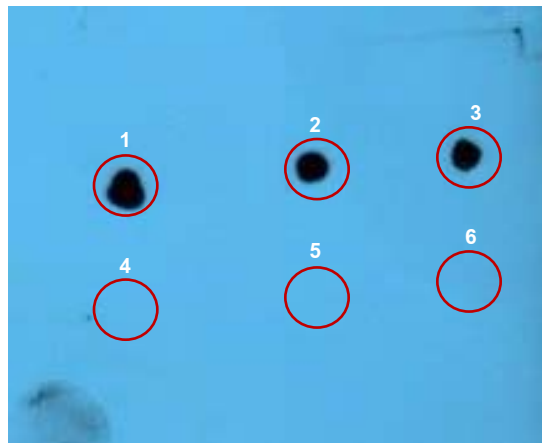
### 3.2 Removal of allergenic proteins in natural rubber latex using protease from *Bacillus* sp.

The ability of protease powder prepared from *Bacillus* sp. in REF removal from NRL was investigated. As it was tested at various levels from 0-500 unit/ 100 ml NRL, the results showed that REF; one of high risk allergenic protein found in NRL, could be degraded by *Bacillus* sp. protease. However, it was also found that protease at lower than 300 unit/100 ml was low efficiency to eliminate REF as shown in Figure 1.

Enzymatic degradation of proteins in NRL has been reported by many researchers (Perrella and Gaspari, 2002; Klinklai et al., 2003; Rattanasom et al., 2005). The treated NRL has been called as deproteinized natural rubber latex (DNRL). Most of reports had mentioned on the total protein content in rubber after enzyme treatment. Although the total protein content of rubber was decreased, but no evidence had proven that individual allergenic protein had been degraded. SDS-polyacrylamide gel electrophoresis with Coomassie blue staining was a technique that was used by Perrella and Gaspari (2002) for analysis of protease-treated NRL proteins. However, the detection sensitivity of this technique is not too high. Thus higher detection sensitivity and

specificity technique; i.e. immunological assay using anti-REF IgG, was applied and reported in this study.

REF is a major and high risk allergen from NRL and mostly found in rubber particles (Yeang et al., 2002). Proteolytic degradation of REF by protease enzyme may be more practical process comparing to the other methods because of its specificity to the target proteins. We have expected that NRL treated by our protease can be a premium grade raw material for the production of rubber products that contact to human skin; especially medical and pharmaceutical devices.



**Figure 1.** The appearance of rubber elongation factor (REF) after treated by *Bacillus* sp. protease that visualized by immunological assay

Note: Dot 1 is NRL serum without protease addition

Dot 2 is NRL serum added with 100 unit protease

Dot 3 is NRL serum added with 200 unit protease

Dot 4 is NRL serum added with 300 unit protease

Dot 5 is NRL serum added with 400 unit protease

Dot 6 is NRL serum added with 500 unit protease

#### 4. Conclusions

From all of our results, it might be concluded that crude protease powder could be prepared from *Bacillus* sp. with recovery yield approximately 10%. Crude protease was able to remove an allergenic rubber elongation factor (REF, Hev b1) from NRL. The enzymatic treated NRL; so called REF free NRL is very interesting and expected to be used as premium raw material for application in the production of many medical pharmaceutical devices.

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