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A Comparison of Protein Extraction Methods from Protease Treated Rubber Particles Suitable for 2-Dimensional Electrophoresis Study of Allergic Proteins

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Abstract

Some proteins in latex products may cause allergic reactions to human. This research was to investigate protein profiling in *Hevea brasiliensis* clone RRIM 600 rubber particles. Protease from *Bacillus subtilis* MR10 and non-protease treated rubber particles were prepared from seven-year old rubber trees to evaluate proteomics by using two-dimensional electrophoresis (2-DE) analysis in IPG pH 3 – 10, 7 cm and 12.50% gel conditions with silver staining. There were three protein extraction methods, first method (50 mM HEPES-Tris pH 7.60), second method (0.50 % SDS) and third method (0.10 % SDS and 50 mM Tris-HCl pH 6.80). Results showed that three extraction methods in non-protease treated rubber were estimated to 20 spots (22-175 kDa, pI 3-9), 40 spots (22-175 kDa, pI 4-10) and 200 spots (14-95 kDa, pI 3-9), respectively and protease treated rubber were estimated to 10 spots (42-175 kDa, pI 4-7), 30 spots (22-175 kDa, pI 4-7) and 160 spots (14-95 kDa, pI 3-10), respectively. Thus, protease treatment could decrease some proteins in rubber particle and third method was suitable protein extraction procedure because of highest protein expression. Protein identification in some spots on Coomassie brilliant blue R-250 staining of this extraction method with liquid chromatography-tandem mass spectrometry (LC-MS MS^{-1}) was also found four allergenic proteins (allergen Hev b 7, putative latex allergen Hev b 7.02, Hev 2.20 and small rubber particle protein Hev 3, respectively) on latex protein.

Keywords: protease; *Bacillus subtilis*; extraction solution; rubber; *Hevea brasiliensis*; 2-DE; LC-MS/MS

1. Introduction

Proteomics approaches in rubber particles to demonstrate the molecular mechanisms of allergic proteins to indicate new attempt for allergy elimination in latex products. Furthermore, protease derived bacteria, including *Bacillus subtilis* MR10, which are necessary for degradation of protein in natural rubber latex and have been implicated in quantity of allergic proteins [1]. Proteomics approach is applied to monitor protein pattern in between *Bacillus subtilis* MR10 and non-protease treated rubber particles and to identify allergic protein targets that perturbed to advantages of natural rubber products. This will be possible with a structural strategy in allergic protein on rubber particles by using suitable protein extraction methods. To manage with these conditions, we treated and compared three protein extraction methods reported in the literature to be successful with other recalcitrant tissue types: the extraction of HEPES-Tris pH 7.60 [2] and two modified extraction methods described for plant tissues [3 – 5], 0.50 % SDS solution and 0.10 % SDS with 50 mM Tris-HCl pH 6.80 solution. The virtues

andpitfalls of each of these approaches are determined using qualitative and quantitative gel electrophoresis methods including two-dimensional gel electrophoresis (2-DE). This experiment compared the proteins extracted by all three extraction methods.

2. Materials and Methods

Samples of rubber particle

Latex films were produced from natural rubber solution [1]. These latex films will be divided into two groups: 300 units in protease from *Bacillus subtilis* MR10 and non-protease treated rubber. Both treatments were incubated at 37 °C for 6 hours and then poured onto Petri dishes for incubation at a 70 °C to rubber sheet with a thin film. Then thin films were cut into small pieces with liquid nitrogen using a prechilled mortar and pestle for sample grinding and stored at –20 °C to enter the next protein extraction.

Protein Extraction

Prior to each type of extraction, 1g powder of thin films were transferred to a 15-mL tube containing the respective extraction solutions, and mixed as described below. A. First method (50 mM HEPES-Tris pH 7.60); Proteins were extracted in a buffer 7 M urea, 2 M thiourea, 10 mM *β*-mercaptoethanol, 60 mM DTT, 4% (w v^{-1}) CHAPS, 5 mM EDTA-Na2.2H2O, 1% NP-40 and 0.50 mM phenylmethanesulfonyl fluoride (PMSF) and mixed as described following Xiang and his group [2]. B. Second method (0.50% SDS); Powder of thin films was added directly to 0.50% SDS solution and mixed by inverting the tube 10 times. Tissue were incubated 1 hr. at 40 °C. Precipitated debris was centrifuged at 10,000 rpm for 5 min. Supernatant was collected into in 10 mL 12.50% TCA in ice-cold acetone of protein precipitation. Proteins were precipitated overnight for at least 12 hr. at 0 °C. Precipitated protein was centrifuged at 10,000 rpm for 10 min, washed three times in 10 mL in ice-cold acetone with vigorous disruption of the pellets with a glass rod between each wash, and air-dried. Pellets were dissolved on 0.10% SDS solution until used.C. Third method (0.10% SDS and 50 mM Tris-HCl pH 6.80); Powder of thin films was added directly to 0.50% SDS with 50 mM Tris-HCl pH 6.80 solution and mixed by blended tube for 5 min. Tissue were incubated 1 hr. at 0 °C and blended for 5 min again. Precipitated debris was centrifuged at 10,000 rpm for 5 min. Supernatant was collected into protein precipitation as described above for the TCA-acetone procedure.

Two-Dimensional Gel Electrophoresis

Each extraction types were used for quantitative analysis by modified Lowry's method [6], and were used 2-D gel for spot-picking. A total of 300 *μ*g protein was loaded onto immobilized pH gradient (IPG) strips (pH 3–10 nonlinear, 7 cm; GE Healthcare) during an overnight passive rehydration of the strips, according to the manufacturer's specifications. The preparative gels were staining in both Coomassie blue R-250 and silver staining. Silver staining was operated as described following Xiang and his group [2]. In Coomassie blue R-250 staining, gels were fixed in a solution of 50% methanol and 10% acetic acid for 1 h, stained for 15 min in conventional Coomassie blue R-250 solution, and developed in de-staining solution (25% methanol and 7% acetic acid) for overnight prior to scanning.

Gel Analysis

Gels were scanned on the Image Scaner UTA-1120 (Amersham Biosciences) according to the manufacturer's specifications and Coomassie blue R-250 stained gels were visualized with the 632.80-nm helium-neon laser with no emission filter. 2-D gel images were analyzed using Progenesis same spots, v. 3.1 (Nonlinear Dynamics, Newcastle Upon Tyne, UK).

Mass Spectrometry and Protein Identification

Approximately 22 protein spots were picked manually from only preparative gels in Coomassie blue R-250 staining using a 3-mm picking tips of micropipette. Three subsets of spots were selected: if they were unique to a particular extraction; if they were differentially extracted; or if they were not differentially extracted. The gel plugs were investigated protein analysis and peptide mass profiling using liquid chromatography tandem mass spectrometry (LC-MS MS^{-1}) in National Center for Genetic Engineering and Biotechnology, Thailand. Mass spectrometry data were automatically registered, analyzed, and searched by using National Center for Biotechnology Information public protein databases (NCBI databases). MASCOT (Matrix Science Ltd., London, UK) search engines were used for peptide mapping. Data identifications were registered when search results of protein score greater than 82 were significant ($p < 0.05$).

3. Results and Discussion

Quantitative Differences

Determination of protein quantitation from three rubber extraction methods was shown in Table 1. The results showed that the extraction of 0.10% SDS and 50 mM Tris-HCl pH 6.80 had higher protein content than either of the two methods and an addition of protease would have the potential to significantly decrease the protein content over the rubber. It is possible that protease can help eliminate in some proteins.

Table 1 The concentration of the protein solution (Mean ± SD) from protease (from Bacillus subtilis MR10) and non-protease treated rubber particles in three methods was investigated by modified Lowry's method with the BSA standard curve and the absorbance at a wavelength of 480 nm.

Protein extraction methods	Treated rubber particles	The concentration of the protein solution ($\mu g\ \mu l^{-1}$)
1	non-protease	8.59 ± 0.16^{a}
	protease	4.16 ± 0.07^{a} *
2	non-protease	10.40 ± 0.12^{b}
	protease	4.57 ± 0.13^{a} *
3	non-protease	15.32 ± 0.50^{c}
	protease	13.71 ± 0.07^{b} *

^{abc} significantly different between methods (three samples/method) at the same condition and * significantly different from non-protease.

Gel Analysis of Protein in 2-DE Gel

The results of silver staining were presented on spot higher than Coomassie brilliant blue R-250 staining (data not shown). The protein expression profiles of protease and non-protease treated rubber with first method were examined in Fig. 1(A) and Fig. 1(B). Protein was found to estimate 20 spots (22-175 kDa, pI 3-9), in non-protease treated rubber and 10 spots (42-175 kDa, pI 4-7) in protease treated rubber, however, these gels were found unidentifiable smear in the middle of the gel. We could speculated that this method had some problems with protein extraction. The protein expression profiles of second method (Fig. 1(C) and Fig. 1(D)) can be demonstrated appearance of their non-protease and protease treated rubber particles, 40 spots (22-175 kDa, pI 4-10) and 30 spots (22-175 kDa, pI 4-7), respectively. Data revealed that this protein extraction method has more effective process than first method in which the extraction solution is used in the detergent SDS, which can be accelerated the performance of this material by heating at 40 °C to produce high expression [4]. The results

demonstrated that the third method (Fig. 1(E) and Fig. 1(F)) showed highest protein expression in non-protease and protease treated rubber particles, 200 spots (14-95 kDa, pI 3-9 and 160 spots (14-95 kDa, pI 3-10), respectively. This extraction method of 0.50% SDS with 50 mM Tris-HCl pH 6.8 could be pulled out many proteins more than both first and second methods. In addition, the amount of protein expressed in the gels was also corresponding to protein quantitation from this rubber extraction method (Table 1). The reduced protein was also appeared after protease treatment as low the number and intensity of the protein spots in all methods. The consequences of these results can be analyzed that the addition of protease to the second extract method has eliminated some proteins, but not all. The analysis in each spots of protein by using 2-DE technique will need to select a spot that is free and clear in protein expression to identify the type of protein associated with allergic reaction to do next step. Thus, the third method was the best procedure suited for the proteomic study in rubber particles by using 2-DE. To determine in the protein identification by using LC-MS MS^{-1}, some selected proteins (22 protein spots) were extracted from preparative gels stained with Coomassie brilliant blue R-250 (Fig. 1(G)) compared to silver stained gel (Fig. 1(E)) in third extraction method. The third method stained with Coomassie brilliant blue R-250 showed that it had 53 spots (11-95 kDa, pI 3-10) less than the protein gels stained with silver in the same conditions. However, protein gels stained with silver had even more difficult to analyze the process of MS that act to identify certain proteins. A summary of selected abundant proteins of allergic proteins extracted was demonstrated in Table 2. The MS analysis confirmed that non-protease and protease treated rubber particles still have some allergenic proteins (allergen Hev b 7, putative latex allergen Hev b 7.02, Hev 2.20 and small rubber particle protein Hev 3, respectively) on latex protein. In addition, some proteins were identified to be the rubber particles in well-known rubber biosynthesis-related protein (data not shown) corresponding to previous research [7].

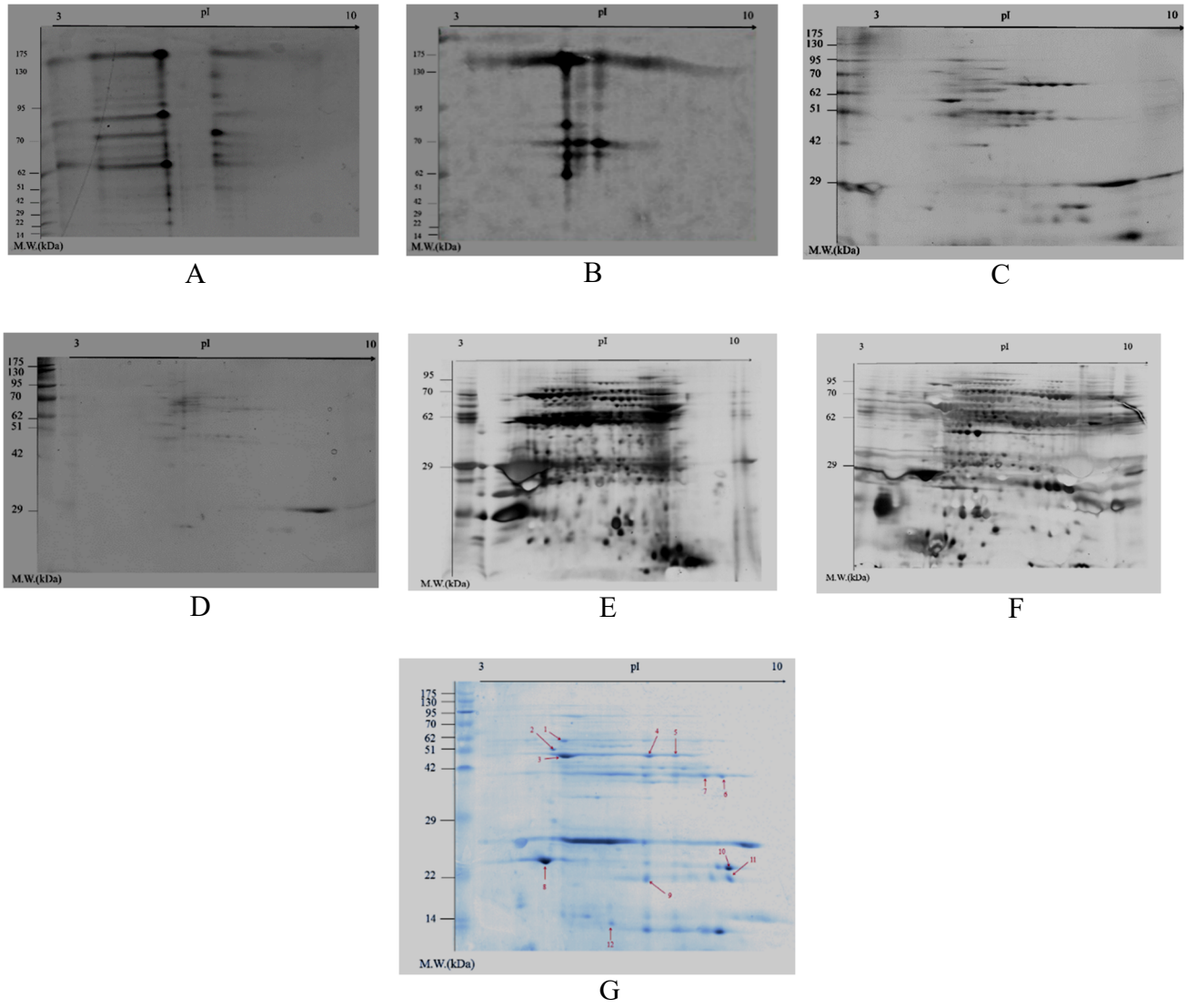

Fig. 1 The expression of proteins in protease from Bacillus subtilis MR10 and non-protease treated rubber particles from three extraction methods by 2-DE analysis of IPG pH 3-10, 7 cm and 12.50% gel conditions with silver staining; A and B on first method (50 mM HEPES-Tris pH 7.60), C and D on second method (0.50% SDS), E and F on third method (0.10% SDS and 50 mM Tris-HCl pH 6.80), respectively, and with Coomassie brilliant blue R-250 staining; G on third method and at 300 µg protein.

Table 2 Some Mascot databases of MS/MS score identified with ≥ 95% confidence protein within individual protein spot on 2-DE analysis of non-protease treated rubber particles (Fig. 1(E)) in the IPG strip pH 3-10 NL, 7 cm, 12.50% gel staining techniques by Coomassie brilliant blue R-250 at 300 μg of protein

Spot number	Accession	M.W.	pI	Types	Score
2	tr|Q9SEM0|Q9SEM0_HEVBR	42.82	5	Latex protein allergen Hev b 7 OS=Hevea brasiliensis PE=2 SV=1	28.30
3	tr|Q9SEM0|Q9SEM0_HEVBR	42.80	5	Latex protein allergen Hev b 7 OS=Hevea brasiliensis PE=2 SV=1	9,377.70
4	tr|Q706V4|Q706V4_HEVBR+	42.70	5.1	Putative latex allergen hev b 7.02 (Fragment) OS=Hevea brasiliensis PE=2 SV=1	4,870.80
5	tr|Q706V4|Q706V4_HEVBR	42.70	5.1	Putative latex allergen hev b 7.02 (Fragment) OS	3,744.90
9	tr|Q6JYQ7|Q6JYQ7_HEVBR	21.85	5.1	Hevea brasiliensis PE=2 SV=1	2,563.40

4. Conclusion

This research was determined three protein extractions on *Bacillus subtilis* MR10 and non-protease treated rubber particles using qualitative and quantitative gel electrophoresis methods including two-dimensional gel electrophoresis (2-DE). Taken together, there are two points highlighted by these data. First, for suitable extraction method, the extraction with 0.10% SDS and 50 mM Tris-HCl pH 6.80 was suitable protein extraction procedure rather than extraction with 50 mM HEPES-Tris pH 7.60 or only 0.50 % SDS. Secondly, protease treatment can decrease some proteins in rubber particle and it is possible that some remained proteins have involved in allergic reaction.

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6. References

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