Kasetsart J. (Nat. Sci.) 43 : 319 - 325 (2009)

Extraction and Characterization of Proteins from Skim Rubber

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ABSTRACT

To optimize the extraction of proteins from skim natural rubber (NR), solution type, number of extractions and sample size were examined. Two solutions, phosphate buffered saline (PBS) and sodium dodecyl sulfate (SDS), were used for protein extraction. The protein content was determined by Bradford assay. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to check the molecular weight of the extract. Analysis of protein purity by Fourier Transform Infrared (FTIR), spectrometer UV-Vis spectrometer and Carbon-Hydroger-Nitrogen (CHN) analyzer was also carried out on the extract from skim NR and compared with bovine serum albumin (BSA), a model protein.

The results revealed that all factors had significant effects on the levels of protein extracted. A high extractable protein (EP) content was obtained from the skim NR using a small amount of specimen in fresh SDS solution and repeating the extraction step three times (12 h/time). SDS-PAGE demonstrated that the EP had three subunit molecular weights of 66, 78 and 116 kDa. Protein impurities could be detected by the CHN method, infrared technique and UV absorbance analysis.

Key words: skim rubber, protein extraction

INTRODUCTION

Natural rubber (NR) has been available commercially for more than a century. Its latex-dipped products, particularly gloves, have been used preferentially as effective barriers to transmitted diseases. However, proteins remaining in the latex products are reported to cause a serious allergy in some people. Methods to reduce the allergenic proteins include: the use of low protein latex, leaching during processing, enzyme treatment, chlorination of the finished product, addition of fumed silica and an irradiation method. These methods are difficult to process and need a long incubation time for processing. A new technique for protein removal from natural rubber has been developed. It is a simple and rapid involving immersion of a functionalized support into natural rubber latex under optimum conditions. The proteins can be immobilized on the support surface via covalent linkage.

In general, bovine serum albumin (BSA) has been used as a model protein to investigate the behavior of proteins binding on functionalized supports (Ladeviére *et al*., 1999; Veron *et al*., 2001; Liu *et al*., 2005.). The binding of BSA molecules to solid supports involves mild reactions between the amino group of a protein and several groups of functionalized carriers. A study of the binding property of extractable proteins (EP) from NR on a support brings relevant information on the efficiency of protein immobilization and

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protein removal from NR. Therefore, it is necessary to extract protein from natural rubber.

Many studies showed that the water-soluble proteins in dry NR were extracted by eluting pieces of dry NR in PBS or SDS solutions. Alenius *et al*. (1994) extracted latex proteins overnight with PBS under continuous shaking conditions at room temperature. Hasma (1992) extracted protein from rubber particles by dispersing them in 2%SDS. It was found that an SDS solution could extract some membrane components amounting to about 0.3% of the rubber, which contained 10% nitrogen. Rogero *et al*. (2003), reported on the extraction of protein from an NR film with PBS solution (pH 7) in a shaking water bath at 37°C for 2 h.

Skim rubber was used in the current study because its protein content is higher than that in dry NR. The extraction of protein from a solid source often involves a compromise between recovery and purity. Optimization of extraction conditions should favor the release of the desired protein and leave difficult-to-remove contaminants behind. Several factors, such as solution type, the number of extractions and sample size have to be taken into consideration.

Thus, the objectives of this study were to investigate the optimal conditions for extraction of protein from skim NR and to analyze the characteristics of the protein obtained.

MATERIALS AND METHODS

Materials

All chemicals and solvents used were of analytical grade. Phosphate buffered saline (PBS) was purchased from Merck. Sodium dodecyl sulfate (SDS), urea and toluene were purchased from BDH. Bovine serum albumin (BSA) as a model protein, 2-mercaptoethanol and Bradford reagent were purchased from Sigma. Trichloroacetic acid (TCA) and Triton® X-100 were purchased from Fluka.

Protein extraction and precipitation

The first factor investigated in this work was the solution type. Two different solutions were chosen for protein extraction: 0.01M PBS at pH 7 and 2% SDS. Dry skim NR (~ 5g) was cut into small pieces and immersed in 10 ml extract solution. After shaking at room temperature for 12 h, the mixture was filtered and then immediately subjected to protein precipitation.

In the study on the effect of sample size, the dry skim rubber was dissolved using toluene. The NR solution was poured onto the plate and dried at room temperature to obtain a rubber sheet with a thickness about 6 mm. The samples (~5g) were prepared using two different ratios of surface area to volume: a small sheet (0.5×0.5 cm^2) and a rubber disc (diameter~7cm). They were then extracted in the optimized solution using the selected extraction procedure.

In the protein precipitation step, 1 ml of the filtrate was treated with 3 ml of 2-mercaptoethanol (0.07% v/v) and trichloroacetic acid (TCA, 10 % w/v) in cooled acetone. The mixture was allowed to stand at -20°C for 24 h. The resulting precipitated proteins were collected by centrifugation at 10,000 rpm and 4°C for 10 min, and then were rinsed with acetone to remove the acid. Finally, the precipitated proteins were redissolved in lysis buffer solution and stored at -20°C.

The protein content of the skim rubber was given by Equation 1:

$$\text{Extractable protein, EP (mg/g)} = \frac{\text{Total content of protein (mg)}}{\text{Weight of skim rubber (g)}} \quad (1)$$

Characterization of extractable proteins

FTIR spectroscopy was used to analyze the composition of residual proteins after extraction and the structural characteristic of extracted proteins. The change in surface composition of rubber obtained from different treatments was studied by attenuated total

reflection FTIR (ATR-FTIR).

The nitrogen content in the amino group of extractable proteins was determined by CHN elemental analysis. The purity of protein in each sample was estimated quickly by a UV-VIS spectrophotometer. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the extractable proteins (Yunyongwattanakorn *et al*., 2008). Prior to fixing and staining with Coomassie brilliant blue, a 15% SDS–polyacrylamide gel was used for separation of proteins.

Determination of protein content by bradford micro-assay

To determine the protein content, Bradford micro-assay (Lowry *et al*., 1951) was utilized. After adding a Bradford reagent, the color of the protein solution was developed into violet. The absorbance of the solution was measured at 592 nm by UV-VIS spectrophotometer. The amount of protein was determined by comparison of the absorbance of the samples with a calibration curve of BSA.

RESULTS AND DISCUSSION

Selection of extract solution

The FTIR spectra of skim natural rubber before and after extraction for 12 h are shown in Figure 1. The principal peaks of proteins in skim NR were observed at 3,281, 1,656 and 1,539 cm^{-1}, corresponding to N-H stretching, C=O stretching (amide I) and N-H bending (amide II), respectively.

The efficiency of protein extraction with different solutions could not be directly investigated from a comparison of the extractable protein yield because the protein precipitate in PBS was not able to be collected. This may have been due to the extremely dilute concentration of EP in this solution. Thus, the effect of solution type on protein extraction was analyzed from the infrared results. After extraction, a sharp decay of the protein bands was observed in the SDS solution. It is believed that the SDS solution was more effective than the PBS solution for protein extraction.

To confirm this hypothesis, the removal of protein from rubber was determined by two

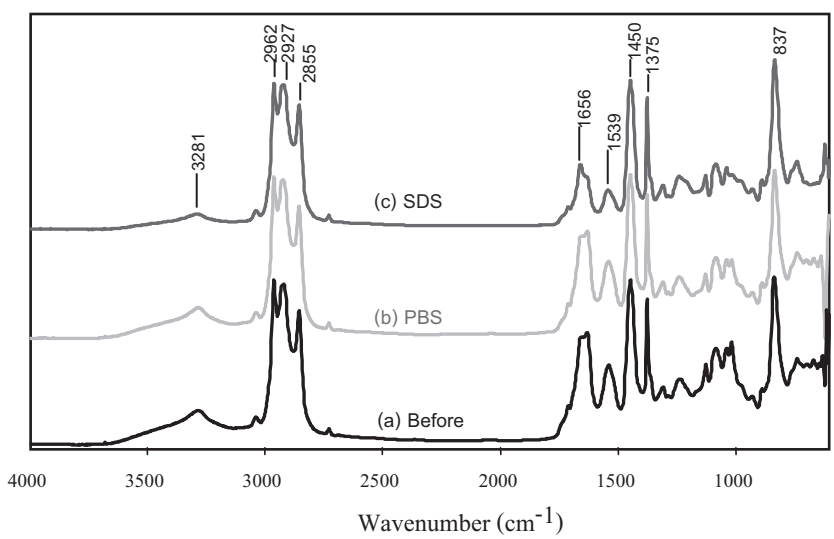

Figure 1 FTIR spectra of skim natural rubber: (a) before; and (b) after extraction with PBS; and (c) and after extraction with SDS.

modes of infrared analysis, i.e., ATR and transmittance. The difference between the ATR and transmittance modes is based upon the depth of IR beam penetrating into the sample. The results obtained were represented in terms of the relative intensity of N-H bending of the amide group at 1,539 cm^{-1} to C-H bending of isoprene at 1,375 cm^{-1}.

In the case of transmission (Figure 2a), the protein in polymer bulk decreased after extraction in both solutions and the extracted NR using SDS showed a slightly lower residual protein level in the sample than that obtained using PBS. In the case of ATR, Figure 2b clearly shows that the SDS solution was very highly efficient at protein removal from the rubber surface. Hasma (1992) reported that the SDS was an effective detergent in solubilizing rubber particle-bound proteins. Therefore, an SDS solution was selected to remove the tightly associated proteins from the dry skim rubber in the following experiments.

Effect of multiple extractions

By repeating the extraction with fresh SDS solution, protein was continuously solubilized. Therefore, the protein content was expected to be higher than that obtained using multiple extractions. In this work, the skim NR was cut into small pieces and the extraction with SDS was repeated three times.

Figure 3 shows that the extractable protein content from the skim NR continued to increase as the extraction process was repeated. Three extractions yielded the maximum protein content. A similar result was observed in the extraction of oil from soft oilseeds by Vâronique and James (2002), who reported that multiple extractions (2×30 min) had a greater recovery than a single extraction for the same amount of time (60 min).

Effect of sample size

In general, contact between the surface of a sample and the extract solvent is necessary for protein removal. Protein on the rubber surface is easily removed with extract solution. To evaluate the effect of sample size on protein removal from

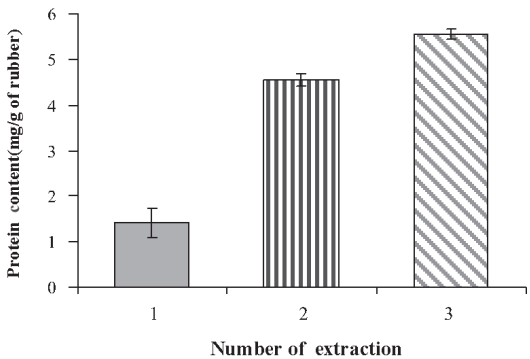

Figure 3 Protein content from extraction with SDS using multiple extractions.

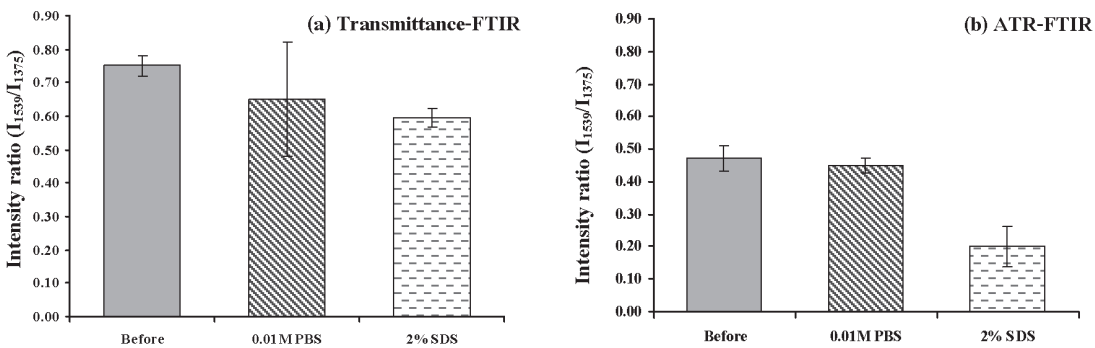

Figure 2 Effect of extract solvent on intensity ratio of protein in skim NR: (a) transmittance mode and (b) ATR mode.

skim NR, two samples with different surface area to volume ratios were extracted by SDS solution. In this study, a rubber sheet with a thickness about 6 mm was obtained by dissolving skim NR in toluene. The samples were prepared into a small sheet (0.5 × 0.5 cm^2) and a rubber disc with a diameter of 7 cm. Total extractable protein was based on the sum of three SDS extractions (12 h/time) at room temperature. Data are shown in Table 1.

The results show that the EP content from small rubber sheets was five times higher than from a NR disc, which indicated that the protein content obtained by extraction was higher from a smaller sample having a higher surface area; the increased surface exposure to the extract solvent was believed to improve extraction.

From all the results, it can be concluded that using a small specimen in fresh SDS solution and repeating the extraction three times produced a high extractable protein content.

Characterization of extractable protein

The inclusion of contaminants in the protein can be investigated by infrared spectroscopy, SDS-PAGE (Wilson, 1973) and UV absorbance analysis (Panov *et al*., 1977).

Molecular weight of extractable protein

Protein was removed from skim NR by extraction with 2% SDS, precipitated with TCA and then analyzed by SDS-PAGE. From SDS-PAGE, the extractable proteins were separated and identified based on their primary molecular weight, as shown in Figure 4. It was found that the extractable proteins presented three bands, with molecular weight of 66, 78 and 116 kDa.

Composition and structure of extractable protein

After protein extraction, the extract solution was precipitated with TCA and immediately freeze-dried. Finally, a brownish powder was obtained and the composition of the freeze-dried protein from the extract was demonstrated by elemental analysis. The weight percentages of C, H and N of the extracted protein and BSA are compared in Table 2.

In general, the raw protein content is obtained by multiplying the nitrogen content by known matrix-specific protein factors (Vemuri, 2005). The results indicated that the nitrogen content of EP was lower than that of the BSA standard. This seemed to imply that with CHN analysis, the protein content of BSA was higher than that of EP. However, it is possible that the

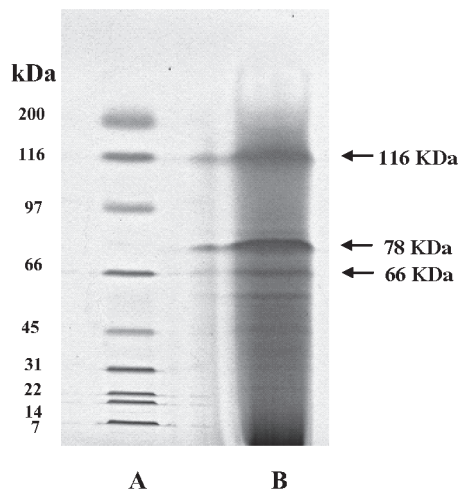

Figure 4 SDS-PAGE of (A) standard proteins; and (B) extractable proteins. (5 μg of BSA proteins in Lane A and 10 μg of extractable proteins in Lane B)

Table 1 Effect of skim NR size on efficiency of protein extraction.

Characteristic of sample	Protein content (mg/g rubber)
Small sheet (0.5 × 0.5 cm^2)	11.16 ± 2.27
Rubber disc (diameter ~7 cm)	2.27 ± 0.16

precipitated sample from the extract solution of skim NR consisted of protein and non-protein components. Another reason to explain this result is the difference in amino acid type between BSA and extractable proteins. BSA has a high content of lysine and arginine amino acids with $-NH_3^+$ groups in their side chains, leading to high nitrogen levels (Peters, 1985; Carter *et al*., 1994; Coradin *et al*., 2003). Because of this result, the structural characteristic of the extracted sample was investigated. The extract would be expected to contain readily extractable protein.

The structures of freeze-dried protein and BSA standard protein were analyzed using FTIR. From Figure 5, although there were impurity peaks at 1,732 and 1,626 cm^{-1} due to interference by non-protein substances, the spectrum of extractable protein was similar to that of BSA.

It can be seen clearly (Figure 6) that the UV spectra of BSA consisted of a single maximum at wavelength 276 nm, due to light absorption by amino acids (tryptophan, tyrosine and phenylalanine), while the peak of EP shifted to the left with a maximum at 265 nm. It is suggested that any non-protein components that absorb ultraviolet light may interfere with the protein.

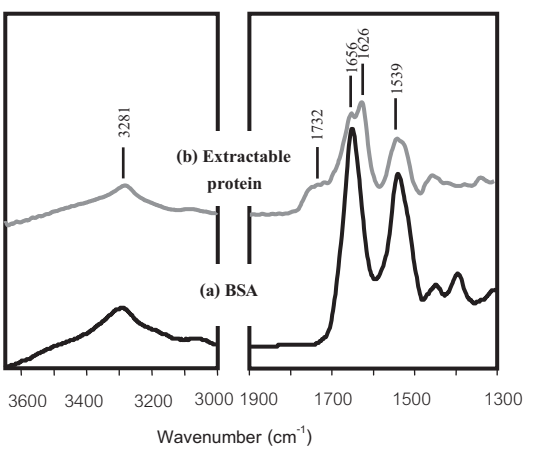

Figure 5 FTIR spectra of: (a) BSA standard protein; and (b) extractable protein.

CONCLUSION

Several factors were investigated to determine their effects on the extraction of proteins from NR and to optimize the extraction protocol. Protein extraction using a small specimen in fresh SDS solution and repeating the extraction three times (12 h/time) gave a high extractable protein content.

Furthermore, these studies provided basic information on the characteristics of extractable proteins. SDS-PAGE indicated that the extractable proteins had three subunit molecular weights of 66, 78 and 116 kDa. Protein impurities could be detected by the CHN method, infrared technique and UV absorbance analysis. However, all results confirmed that the sample obtained from SDS-extraction was composed of readily

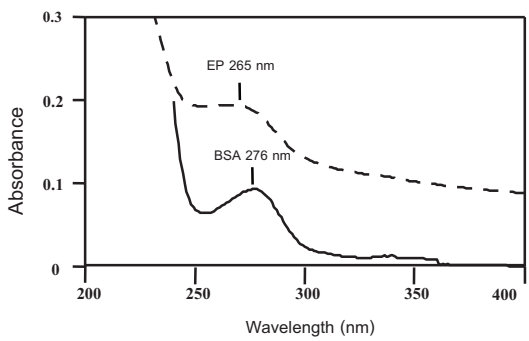

Figure 6 UV absorbance spectra for BSA and extractable protein.

Table 2 CHN content of extractable protein and BSA standard protein.

Sample	%		
	C	H	N
BSA	45.39	7.55	13.51
Extractable protein	46.84	8.05	2.75

extractable proteins, which have an NH_2 group to link to a support. It was also found that the extractable proteins could be redissolved in lysis buffer solution. This may be useful in the design of experiments for protein-binding study on functionalized support.

ACKNOWLEDGEMENT

The authors wish to thank Associate Professor Jitladda T. Sakdapipanich, Faculty of Science, Mahidol University for kind permission to use the UV-Vis spectrophotometer and the SDS-PAGE apparatus and for her valuable discussions.

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