Hyperdeproteinized Natural Rubber Prepared with Urea

Warunee Klinklai,[1] Takayuki Saito,[1] Seiichi Kawahara,[1] Kei Tashiro,[2] Yasuyuki Suzuki,[2] Jitladda Tangpakdee Sakdapipanich,[3,4] Yoshinobu Isono[1]

[1]*Department of Chemistry, Faculty of Engineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan*
[2]*Organic Material Department, Material Engineering Division II, Toyota Motor Corporation, Toyota, Aichi, 471-8572, Japan*
[3]*Department of Chemistry, Faculty of Science, Mahidol University, Rama VI, Bangkok 10400, Thailand*
[4]*Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus, Nakomprathom 73170, Thailand*

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ABSTRACT: Preparation of hyperdeproteinized natural rubber was made from fresh latex and preserved high-ammonia latex by treatment with urea in the presence of sodium dodecyl sulfate. Concentration of urea, temperature, and time for the incubation were investigated to remove the proteins effectively. Under the best conditions, the total nitrogen content and amount of allergenic proteins for the deproteinized rubbers were 0.005 wt % and 1.0 μg/ml, respectively, which were less than those of natural rubber deproteinized with proteolytic enzyme. The hyper-deproteinized natural rubber was proved through FT IR spectroscopy. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 93: 555–559, 2004

Key words: natural rubber; urea-deproteinization; protein; enzymes; latex allergy

INTRODUCTION

Removal of proteins from natural rubber (NR) may be essentially concerned with methods on how to control interactions between the rubber and proteins in the latex stage (i.e., chemical and physical interactions). The former is cleaved with proteolytic enzyme such as alkaline protease[1–2] and the latter is denatured with urea, which may change conformation of the proteins.[3,4] In the previous work,[1,5] the removal of proteins was mainly made in the latex stage by enzymatic deproteinization to remove proteins present on the surface of the rubber particle as a dispersoid. After the enzymatic deproteinization, the nitrogen content of NR was reduced to less than 0.02 wt %, which was about 1/20 of that of the untreated NR.[6–11] Despite the significant decrease in the nitrogen content, however, problems still exist, that is, both a long incubation time necessary for the enzymatic deproteinization (i.e., more than 24 h), and remaining proteins, peptides, or amino acid sequences that may result in intraoperative anaphylactic reactions of hypersensitive patients of allergy.[12–16] It is, thus, quite important to establish a novel procedure to remove the proteins from NR rapidly and efficiently.

The structure of NR has been proposed to consist of ω-terminal, two *trans*-1,4-isoprene units, long sequence of *cis*-1,4-isoprene units, and α-terminal, aligned in this order.[17–20] The ω-terminal was inferred to be a modified dimethylallyl group that can form hydrogen bonds between proteins, while the α-terminal was comprised of a phospholipid that may form chemical crosslinks with ionic linkages. According to the proposed structure of NR, it is expected that there is little possibility to form chemical linkages between NR and the proteins.

In previous work,[21–23] NR coagulated from fresh NR latex, just after tapping from *Hevea brasiliensis*, was found to be soluble in toluene, cyclohexane, and tetrahydrofuran. In contrast, the rubber from latex preserved in the presence of ammonia contained about 30–70% gel fraction, which was insoluble in the solvents. The formation of the insoluble fraction would be concerned with the interactions of rubber and proteins, because the gel fractions are reported to be soluble in the solvents after the enzymatic deproteinization.[24,25] If the interactions are physical but not chemical, it is possible to remove the proteins from the rubber after denaturation of the proteins with urea. In the present study, the removal of the proteins from fresh NR latex and preserved high-ammonia latex was investigated with urea in the presence of surfactant.

Correspondence to: S. Kawahara (kawahara@chem.nagaokaut.ac.jp).

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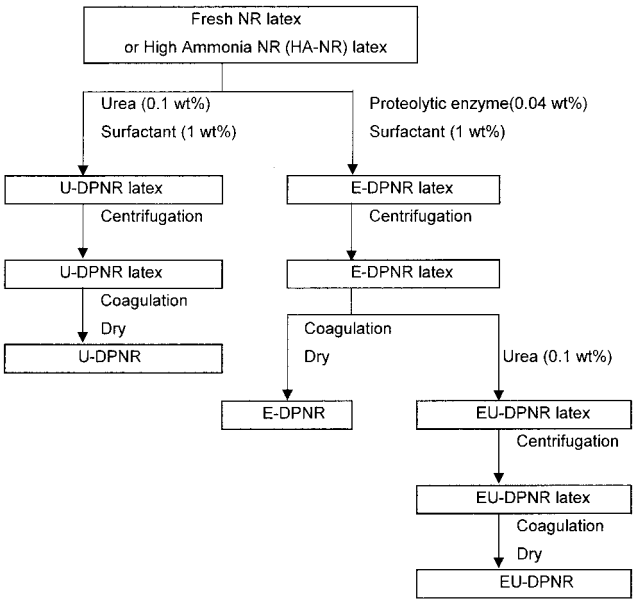

Figure 1 Schematic representation of experimental procedure.

EXPERIMENTAL

NR latexes used in this study were fresh NR latex, which was collected at a private plantation in Thailand just before deproteinization, and commercial high ammonia latex (HA-NR). The incubation of the latex was made with 0.1 wt % urea in the presence of 1 wt % sodium dodecyl sulfate (SDS, Kishida Co. Ltd., Japan) at 303, 333, or 363 K. The cream fraction was redispersed in 1 wt % SDS solution to make 30% dry rubber content (DRC) latex and was washed twice by centrifugation (fresh U-DPNR or U-DPNR). The NR latex was also deproteinized by incubation of the latex with 0.04 wt % proteolytic enzyme (Kao, KP-3939, Japan) and 1 wt % SDS for 12 h at 305 K followed by centrifugation.[25–26] The cream fraction was redispersed in 1 wt % SDS solution to make 30% DRC latex and was washed twice by centrifugation to prepare deproteinized NR (fresh E-DPNR or E-DPNR) latex.

The fresh E-DPNR or E-DPNR latex was, furthermore, deproteinized by incubation with 0.1 wt % urea at 303 K, followed by centrifugation. The cream fraction was redispersed in 1 wt % SDS solution to make 30% DRC latex and was washed twice by centrifugation (fresh EU-DPNR or EU-DPNR). The rubber was recovered by centrifugation followed by coagulation with methanol and dried under reduced pressure at ambient temperature until a definite weight was achieved. Schematic representation of the experimental procedure is shown in Figure 1.

Measurement of nitrogen content of the rubbers was made by Kjeldahl method as described in RRIM Test Method B7.[27]

The amount of allergenic proteins in NR samples was measured by an enzyme-linked immunosorbent assay (ELISA) of latex for antigenic proteins, that is, ELISA of latex for antigenic proteins (LEAP) method as described in ASTM D6499-00.[28] Films were prepared by pipeting the latex into a Petri dish. The film was allowed to dry overnight. Dried films were weighed, after which the samples were cut to allow buffer contact with all surfaces. Extractions were performed for 2 h with constant agitation at 298 ± 5 K in 100 mM phosphate-buffered saline (PBS) pH 7.4. The extraction ratio used (buffer/grams sample) was 5:1. The extracts were centrifuged to remove particulates and then processed to an ELISA inhibition assay. The resulting samples were assayed by using seven two-fold serial dilutions in duplicate. The data were expressed as antigenic proteins in μg/ml of sample.

The peptide linkages were characterized by Fourier transformation infrared (FTIR) spectroscopy, by using a JASCO FTIR 410 spectrometer with a resolution of 4 cm^{-1}.

TABLE I
Nitrogen Content and Incubation Time for HA-NR, Fresh NR, and Deproteinized NR

Specimens	Incubation time (min)	X (wt %)
HA-NR	0	0.300
E-DPNR[a]	720	0.017
U-DPNR[b]	60	0.020
EU-DPNR[c]	780	0.008
Fresh NR	0	0.450
Fresh E-DPNR	720	0.014
Fresh U-DPNR	60	0.004
Fresh EU-DPNR	780	0.005

[a] E-DPNR; enzymatically deproteinized HA-NR.
[b] U-DPNR: urea-treated HA-NR.
[c] EU-DPNR: urea-treated E-DPNR.

RESULTS AND DISCUSSION

Deproteinization of NR with urea

Total nitrogen content, X, of both untreated and deproteinized rubbers, is shown in Table I. The total nitrogen content of HA-NR was reduced to 0.017 wt % after enzymatic deproteinization (E-DPNR), as reported in the previous study.[26–27] On the other hand, it was reduced to 0.020 wt % after the treatment with urea, being similar to the nitrogen content of E-DPNR. This implies that most proteins present in NR are attached to the rubber with weak attractive forces. To remove further the proteins, the treatment with urea was carried out after the enzymatic deproteinization of HA-NR latex. The nitrogen content of the resulting rubber, EU-DPNR, was 0.008 wt %, less than that of E-DPNR and U-DPNR. This suggests that the most of proteins are removed by denaturation with urea,

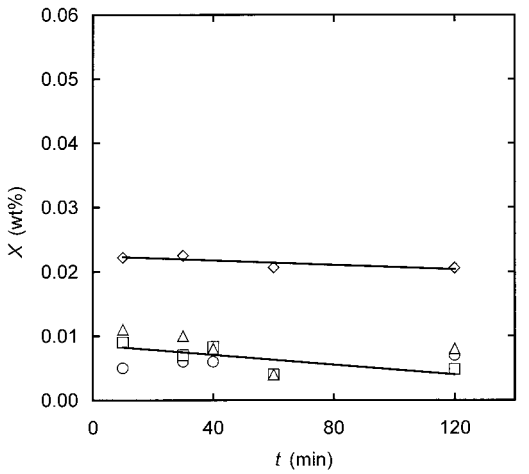

Figure 2 Nitrogen content of HA-NR at 303 K (◇), and fresh U-DPNR at 303 K (○), 333 K (□), and 363 K (△) versus time for incubation.

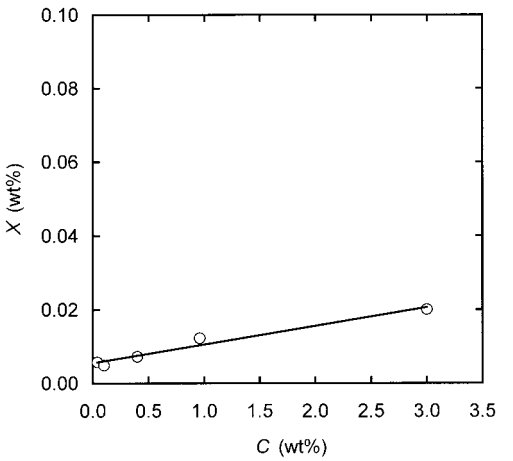

Figure 3 Nitrogen content of fresh U-DPNR versus urea concentration.

whereas the residue must be removed with proteolytic enzyme in conjunction with urea.

To assure the difference in the role between the proteolytic enzyme and urea, the treatment of fresh NR latex was made as well as HA-NR latex. The total nitrogen content of both untreated and deproteinized rubbers, which were prepared from fresh NR latex, is also shown in Table I. The total nitrogen content of fresh NR was reduced to 0.014 wt % after enzymatic deproteinization (fresh E-DPNR), and 0.005 wt % after enzymatic deproteinization followed by the treatment with urea (fresh EU-DPNR), as in the case of HA-NR. On the other hand, after treatment with urea (fresh U-DPNR), the total nitrogen content of fresh NR was 0.004 wt %, being the least among the deproteinized rubbers. This may be explained in that most proteins present in fresh NR are attached to the rubber with weak attractive forces, which are able to be disturbed with urea. Thus, it is possible to expect that almost all proteins present in fresh NR are removed rapidly from the rubber by urea treatment.

A plot of total nitrogen content versus time, *t*, required for the deproteinization of HA-NR and fresh NR with urea at 303 K is shown in Figure 2. The nitrogen content of HA-NR and fresh NR decreased suddenly to 0.022 and 0.005 wt % after 10 min, respectively, after adding urea. The difference in the nitrogen content between HA-NR and fresh NR may be attributed to the amount of the proteins that are weakly attracted to the rubber, as mentioned above. It is quite important to note that the nitrogen content of fresh NR decreases to and reaches a definite value of 0.004 wt % within 10 min, expressing an advantage of urea compared to proteolytic enzyme in view of the rapid, efficient deproteinization. The dependence of the total nitrogen content on temperature is also shown for fresh NR in Figure 2. The nitrogen content decreased to 0.004 wt % within 10 min at all temperatures, ranging from 303 to 363 K. This may be in part due to the ability of urea to form hydrogen bonds with the proteins and detach themselves from the rubber particles with urea, based upon the previous work.[4] Consequently, urea is proved to be more effective to remove the proteins from fresh NR, rather than the proteolytic enzyme.

Concentration of urea for deproteinization

It is quite important to determine the amount of urea necessary to remove the proteins from NR. Figure 3 shows the relationship between the total nitrogen content of fresh NR latex versus concentration of urea after treatment with urea. The nitrogen content was dependent upon the concentration of urea and was found to be the lowest (i.e., 0.005 wt %, at urea concentration of 0.1 wt %). The higher nitrogen content of 0.007 wt % at 0.05 wt % urea may be due to a lesser amount of urea that interacts with the proteins present in the rubber. In contrast, the higher nitrogen content at higher concentration of urea may be expected to be due to the residual urea that interacts with the rubber, because the nitrogen content increased linearly as the concentration of urea increased.

Figure 4(A) shows FTIR spectra for fresh U-DPNR, in which the peak at 3320 cm^{-1} is identified to mono- or dipeptides, as reported in the previous study.[25,29] In the spectrum of Figure 4(A)a–d, a small peak was observed at 3450 cm^{-1} in addition to the peak at 3320 cm^{-1}, the intensity of which increased as the concentration of urea increased. A mixture of synthetic *cis*-1,4-polyisoprene with urea showed a peak at 3450 cm^{-1} characteristic of urea[30] in addition to the peak at 3320 cm^{-1}, which is identified to an antioxidant as a mixture,[31] as shown in Figure 4(B). Thus, we estimated the concentration of residual urea present in

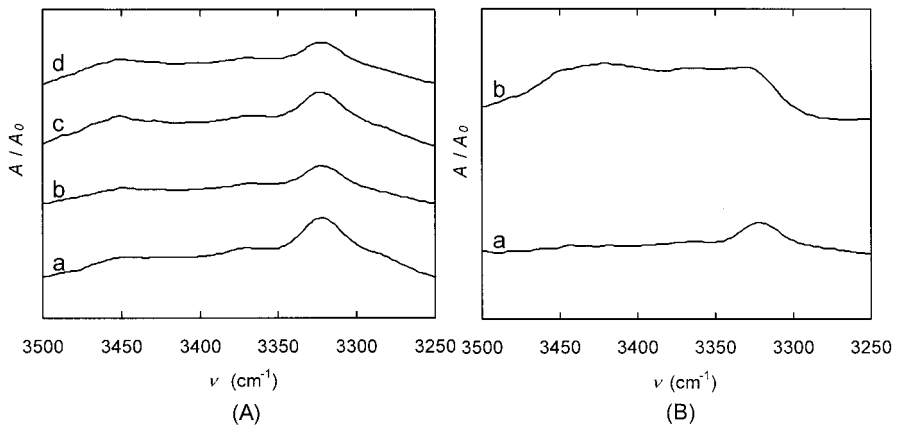

Figure 4 (A) FTIR spectrums of fresh U-DPNR at various urea concentrations: (a) 0.05 wt % urea, (b) 0.1 wt % urea, (c) 0.5 wt % urea, and (d) 1.0 wt % urea; (B) FTIR spectrums of synthetic *cis*-1,4-polyisoprene and its mixture (a) synthetic *cis*-1,4-polyisoprene, and (b) synthetic *cis*-1,4-polyisoprene mixed with urea.

fresh U-DPNR from the intensity of the peak at 3450 cm^{-1}, using a calibration curve made with the mixture of synthetic *cis*-1,4-polyisoprene with urea. A plot of the concentration of the residual urea versus urea concentration is shown in Figure 5. The residual urea increased as the urea concentration increased. Furthermore, the nitrogen content estimated from the concentration of the residual urea was similar to that shown in Figure 3. Thus, the increase in nitrogen content of the deproteinized rubber was proved to be due to not only the mono- or dipeptides but also the residual urea. In the present study, a suitable condition of urea for the deproteinization was determined to be 0.1 wt %.

Amount of allergenic proteins

The ELISA is known to be a method to detect allergenic proteins present in NR (i.e., rubber elongation factor, small rubber particle protein, acidic latex protein, and mature hevein), in which their molecular weight is about 14, 22, 17, and 4.7 KDa, respectively.[32] We, thus, measured the deproteinized rubbers through LEAP. The amount of allergenic proteins for E-DPNR, EU-DPNR, fresh E-DPNR, and fresh U-DPNR is tabulated in Table II. The amount of allergenic proteins for EU-DPNR was as low as 25% of E-DPNR. The lesser amount of allergenic proteins for EU-DPNR was consistent with the lesser nitrogen content shown in Table I, suggesting that whole proteins were removed by enzymatic deproteinization of HA-NR latex followed by the treatment with urea. This demonstrates that the EU-DPNR is a hyperdeproteinized NR that ever appeared. On the other hand, the amount of allergenic proteins in fresh E-DPNR was in a similar level to that of fresh U-DPNR and EU-DPNR. This may be attributed to the weak attractive forces between the fresh NR and proteins, as mentioned above. This indicates that the hyperdeproteinized NR can be prepared from not only HA-NR but also fresh NR.

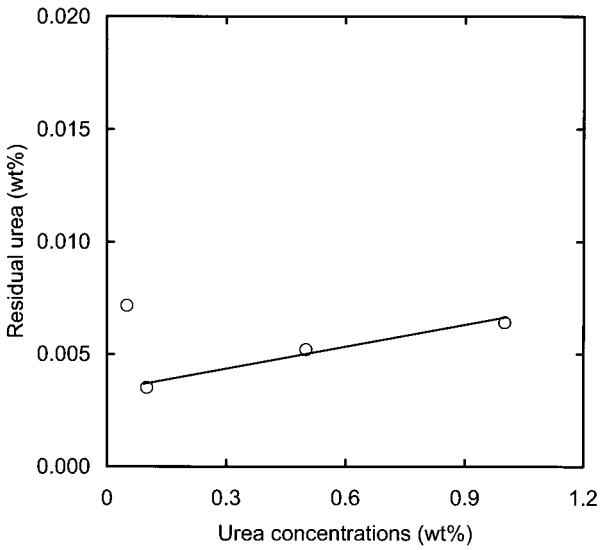

Figure 5 Concentration of residual urea in fresh U-DPNR versus urea concentration.

Figure 6 shows the NH stretching region of FTIR spectra of fresh NR, fresh E-DPNR, fresh U-DPNR, and EU-DPNR. Fresh NR showed a clear peak at 3280 cm^{-1}, characteristic of proteins or peptides. After the deproteinization of fresh NR latex with the proteolytic enzyme, the peak disappeared and a new peak ap-

TABLE II
Amount of Allergenic Proteins for E-DPNR, EU-DPNR, Fresh E-DPNR, and Fresh U-DPNR

Specimens	C (μg/ml)
E-DPNR	3.2
EU-DPNR	0.8
Fresh E-DPNR	1.5
Fresh U-DPNR	1.0

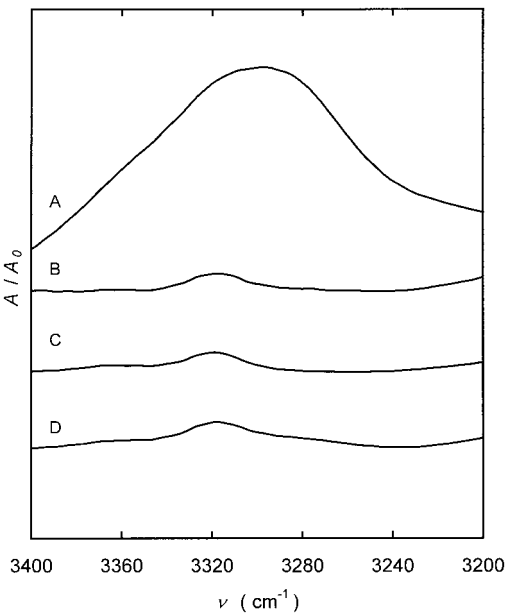

Figure 6 FTIR spectrums of (A) fresh NR, (B) fresh E-DPNR, (C) fresh U-DPNR, and (D) EU-DPNR.

peared at 3320 cm^{-1}, which was identified to mono- or dipeptides, as reported in the previous study.[25] On the other hand, fresh U-DPNR showed the peak at 3320 cm^{-1} and no peak at 3280 cm^{-1}. The relative intensity of the peak at 3320 cm^{-1} peak in fresh E-DPNR and fresh U-DPNR was quite similar to that of EU-DPNR. This is supporting evidence that the EU-DPNR and fresh E-DPNR and fresh U-DPNR are the hyperdeproteinized NR.

CONCLUSION

Hyperpurified NR was prepared by incubation of HA-NR latex with a proteolytic enzyme followed by treatment with urea and by incubation of fresh NR with a urea. The total nitrogen content and amount of allergenic proteins, determined by means of Kjeldahl and LEAP methods, were about 0.005 wt % and 1.0 μg/ml, respectively, for the hyperdeproteinized NR. It is concluded that the hyperdeproteinized NR is almost free from the proteins.

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