



MOLECULAR WEIGHT DISTRIBUTION OF POLYISOPRENE FROM *LACTARIUS VOLEMUS*

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Key Word Index—*Lactarius volemus*; natural rubber *cis*-polyisoprene; polyprenol; M_r distribution.

Abstract—The *L. volemus* rubber showed a unimodal M_r distribution (MWD), centered around a M_r of 2.4×10^4 , with a shoulder in the high M_r region and two small peaks in the low M_r region. Each M_r fraction, except for the highest one, contained a dimethylallyl-*trans-trans* terminal, and poly-*cis* isoprene units with a hydroxyl or fatty-acid ester terminal group. The lowest M_r fraction showed a MWD corresponding to homologues of polyprenols with peaks from 17-mer to higher than 90-mer, having two centres around 21-mer and 32-mer by HPLC analysis. The distribution of chain-length between 35-mer and 90-mer in the next lowest M_r fraction, showed a 5 or 6 periodical repeating isoprene unit. It is postulated that the period observed by HPLC arises from the mechanism controlling M_r . © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

On the basis of ^1H NMR studies, polyprenols consisting of *cis*- and *trans*- isoprene units are classified into two groups, i.e. two-*trans*, poly-*cis* prenols and three-*trans*-, poly-*cis*-prenols [1]. ^{13}C NMR spectrometry has been used to determine the arrangement of *trans*- and *cis*-isoprene units along the chain [2]. The alignment of dimethylallyl-(*trans*)₂-*cis*- units was confirmed for ficaprenols [3] and that of dimethylallyl-(*trans*)₂-*cis*- units for polyprenols from *Ginkgo biloba* [4] and Pinaceae [5], and for dolichol from pig liver [6]. This ^{13}C NMR method has been applied to the structural characterization of rubber [2, 7–9]. Most natural rubbers, including that from *Hevea brasiliensis*, are composed of two- or three-*trans*-isoprene units and poly-*cis*-isoprene units, similar to polyprenols, but quite different from polyprenols in terms of molecular size [10]. Rubber from *Hevea brasiliensis* is a high M_r polymer with broad MWD [11–13]. The M_r of *Hevea* rubber isolated from freshly tapped latex of different clonal origins, has been shown to have either a distinctly bimodal distribution, or a unimodal distribution with a shoulder in the low M_r region [11]. The high and low M_r peaks usually centred around 10^6 and 10^5 , respectively.

Prenyltransferases catalyze the head-to-tail condensation between isopentenyl diphosphate and an

allylic diphosphate to produce prenyl diphosphates with various chain-lengths and geometric isomerisms. The mechanism controlling chain-length has been discussed for enzymes isolated from several bacteria [14–17]. Biochemical studies on rubber formation in *Hevea brasiliensis* latex have shown that rubber is synthesized on the surface of the rubber particles possibly by elongation of existing molecules, but also by the synthesis of new ones [18]. The problem of the origin of rubber molecules—other than from pre-existing particles, which must occur—has not been elucidated, though new work has thrown some light on this [19]. Recent *in vitro* studies have indicated a possible mechanism of M_r control in *Hevea* and other species [20].

We have analyzed the structure of rubber from *Lactarius* mushrooms in the hope of obtaining more information on the mechanism of rubber biosynthesis, and the control of M_r . ^{13}C NMR studies have shown that the fundamental structure of rubber from *Lactarius volemus* consists of a dimethylallyl group, two-*trans* isoprene units, a long sequence of *cis*-isoprene units, and a hydroxyl or fatty acid ester terminal group, aligned in that order [21–23].

The present paper reports on the M_r and MWD of rubber from *L. volemus*. This rubber is shown to be a high- M_r polyprenol with a chain-length between that of a long-chain polyprenol and *Hevea* rubber. The work provides new information on the mechanism controlling chain-length, and also clarifies the relationship between polyprenols and rubber.

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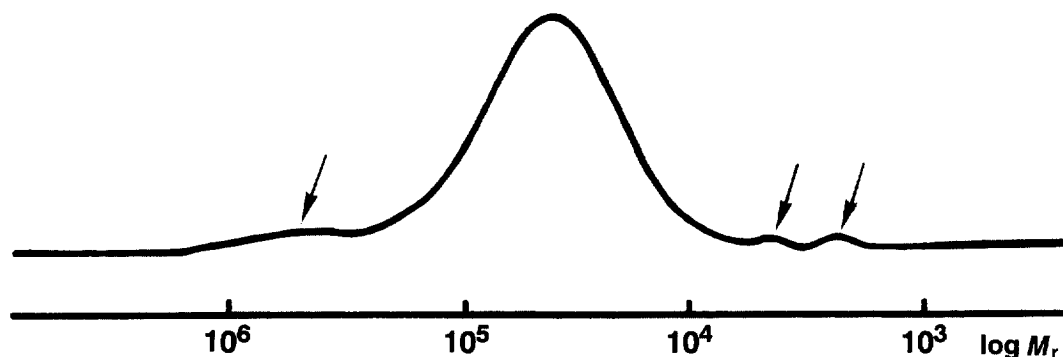


Fig. 1. MWD of rubber from *L. volemus* determined by GPC.

RESULTS AND DISCUSSION

The yield of rubber was 1.8–3.9%, based on the dry wt of the sporophores. As shown in Fig. 1, the rubber showed a unimodal distribution with a shoulder in the high M_r region and two small peaks in the low M_r region. The unimodal distribution, centred around 2.4×10^4 , was commonly observed for rubbers extracted from mature sporophores, and also for those extracted from young sporophores. The M_r was estimated to be $\bar{M}_n = 2.3 \times 10^4$ and $\bar{M}_w = 6.9 \times 10^4$ by GPC, using polystyrene standards. The degree of polymerization of 210 obtained by vapour pressure osmometry was in good agreement with the value of 260 obtained from ^1H NMR measurement, on the basis of the presence of two *trans*-isoprene units at the

initiating terminal. The two peaks, corresponding to degrees of polymerization of 53 and 27, based on polystyrene standards, were commonly observed for rubbers from sporophores. The above findings indicate that the *cis*-polyisoprene from the sporophores consists of at least four components.

The methyl-protons in polyisoprenols show ^1H NMR signals reflecting triad sequences of *trans*- and *cis*-isoprene units, including dimethylallyl (ω) and terminal *cis*-isoprene units with a hydroxyl group, i.e., an α -terminal group [24]. The ^1H NMR spectrum of *L. volemus* rubber showed splitting of the methyl-proton signals corresponding to the ω -(*trans*- configuration), ω -(*cis*- configuration), ω -*trans-trans*- and *trans-trans-cis*- sequences, as shown in Fig. 2. The methyl-proton signals in *cis-cis-cis*(α)-OH and in *cis*-

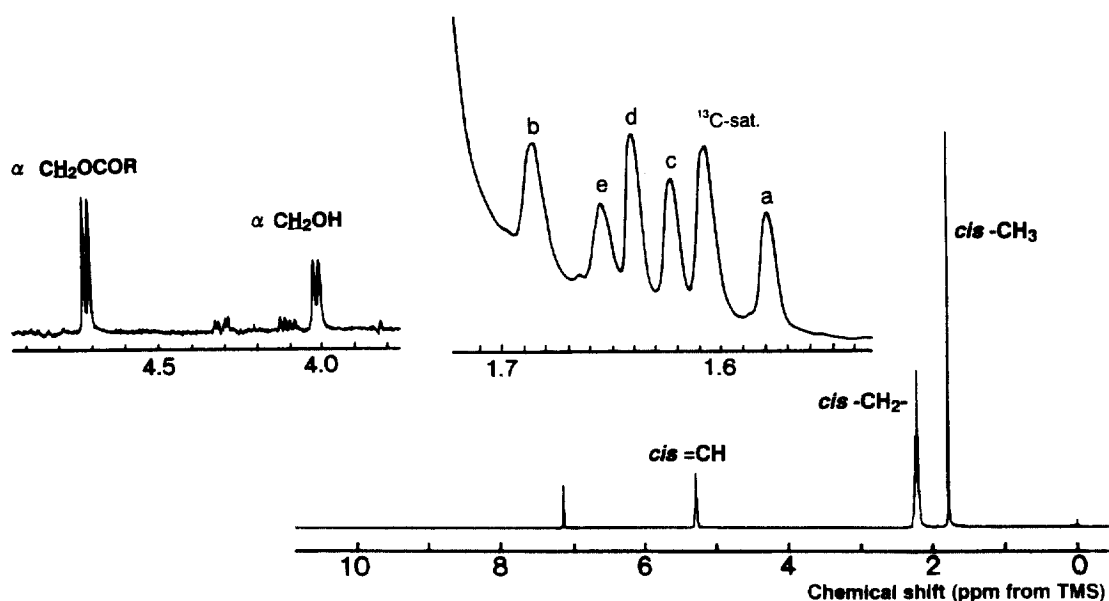


Fig. 2. ^1H NMR spectrum of *L. volemus* rubber.

a, $\omega(t)$; b, $\omega(c)$; c, $\omega-t-t$; d, $t-t-c$; e, α ; ^{13}C -sat, ^{13}C satellite signal of $c-c-c$.

Table 1. Number of terminal groups and *trans*-units in fractionated *L. volemus* rubber

Relative intensity		Methyl-proton signal*						
Fraction	DPh [†]	ω (t)	ω -t-t	t-t-c	ω (c)	α -ester	α -OH	α (total)
1	1300	1.0	1.4	2.4	1.1	0.38	0.21	0.59
2	680	1.0	1.2	1.2	1.1	0.49	0.32	0.81
3	330	1.0	1.0	1.1	1.1	0.58	0.29	0.87
4	250	1.0	1.1	1.2	1.0	0.59	0.33	0.92
5	130	1.0	1.2	1.1	1.1	0.54	0.36	0.90

* the methyl-proton signals in the dimethylallyl group in the *cis*- and *trans*- configurations are designated as ω (c) and ω (t), respectively, and those of *trans*- units in the ω -*trans-trans*- and *trans-trans-cis*- sequences are abbreviated as ω -t-t and t-t-c, respectively. The relative intensity of each signal is shown against the signal intensity of ω (t).

[†] Degrees of polymerization.

cis-cis(α)-OCOR were not separated. The hydroxyl and fatty acid ester terminal groups can be differentiated by the characteristic methylene-proton signals of CH₂OH and CH₂OCOR at δ 4.055 and 4.722, respectively. The numbers of the terminal groups and *trans*-isoprene units were determined on the basis of the relative intensity of these signals.

The rubber was separated into five fractions using solvents. As listed in Table 1, the relative intensities of both terminal groups, *trans*- units and *cis*- units, were determined for each fraction, assuming that one dimethylallyl terminal group was present per molecule. Each fraction, except that of the highest *M_r* (fr. 1), showed relative intensities of the methyl-proton signals in the ω -*trans-trans*- and *trans-trans-cis*-sequence of 1.0–1.2 and 1.1–1.2, respectively, and that of the hydroxyl terminal group plus the fatty acid ester terminal group, was close to unity. This indicated that each fraction, except for fr. 1, contained ω -*trans-trans*-terminal and poly-*cis*- isoprene units, together with hydroxyl or fatty acid ester terminal groups. On the other hand, a significant difference in the number of terminal groups and *trans*- units was observed for fr. 1 compared with the other fractions; the relative intensities of methyl-proton signals in the ω -*trans-trans*- and *trans-trans-cis*- sequences was 1.4 and 2.4, respectively, and the total number of hydroxyl and fatty acid ester terminal groups was smaller than that observed for the other fractions.

The lowest *M_r* fraction was further separated by GPC (fr. 5a, fr. 5b and fr. 5c). The detailed chain-length distribution was analyzed by reversed-phase HPLC using gradient elution. Fig. 3 shows the distribution of homologues of polyprenol from polyprenol-17 to higher than -80, in the lowest-*M_r* fraction (fr. 5a). Two families were clearly observed in the chromatogram, centred around 21-mer and 32-mer. These families correspond to the two peaks in the low-*M_r* region in the GPC curve in Fig. 1. The chain-length was determined by calibration with polyprenols isolated from the needles of *Pinus thunbergii*. These compounds consist of two-*trans*- and poly-*cis*- isoprene units, as in the case of *L. volemus* rubber. In the

two families in Fig. 3, the lower-*M_r* one was attended by tiny shoulder-peaks, with a slightly delayed elution volume for each peak. On the other hand, the higher-*M_r* family was accompanied by a series of much larger shoulder-peaks corresponding to one-half–one-third of the main peaks in intensity. Each shoulder peak disappeared on saponification, showing that they were due to polymers esterified with fatty acids at the terminal groups. It is interesting that the two families were found to have a different ratio of hydroxyl and fatty acid ester groups. In the high-*M_r* region (40–80-mer), there were many families showing a periodical repetition of *M_r*.

The next lowest *M_r*-fraction (fr. 5b) had peaks corresponding to polyprenols-25 to -90, with a definite repeating unit (Fig. 4). Fourier transformation showed that the distribution of chain-length had a period of 5 or 6 isoprene units. It was demonstrated that this distribution did not result from the conditions used for HPLC; i.e., a simple monotonous chain-length distribution was observed for low-*M_r* synthetic *cis*-polyisoprene (Fig. 5).

These findings clearly indicate that the rubber from sporophores comprises at least four components: two-types of polyprenols with *M_r* centred around 21-mer (a) and 32-mer (b); and rubbers with *M_r* centred around 350-mer (c) and longer than 1000-mer (d).

This is the first time that a periodical fluctuation in chain-length-distribution has been observed for poly-prenols/rubber. It is postulated that the period of 5 or 6 isoprene units arises as a result of the mechanism controlling the *M_r*.

The number of *trans*-isoprene units in fr. 1 was estimated to be 3.8 on the assumption of one dimethylallyl group per molecule. In the ¹³C NMR spectrum of this fraction, no carbon signals in a *trans-trans-trans*-sequence were detected (data not shown). This indicates selective modification of the dimethylallyl group. Such a change has been inferred for *L. volemus* rubber during the storage of the sporophores [23, 25]. Thus, it is likely that one of the *trans*-isoprene units seen in fr. 1 is derived from the dimethylallyl group. In confirmation, the number of

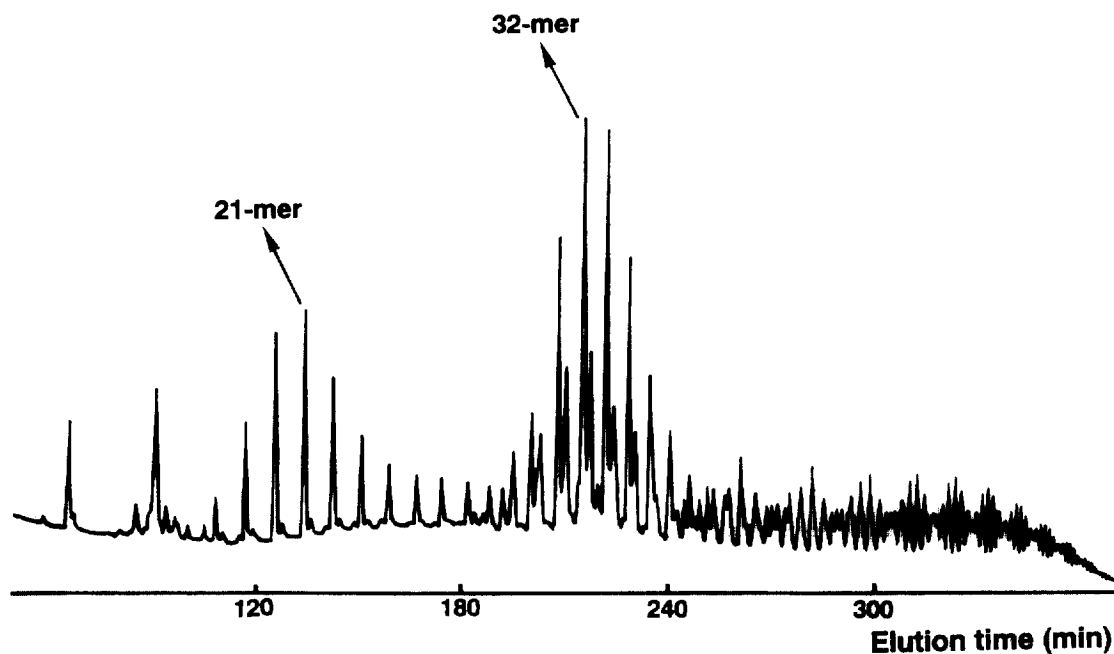


Fig. 3. HPLC of the lowest M_n -fraction (fr. 5a) of rubber from *L. volemus*.

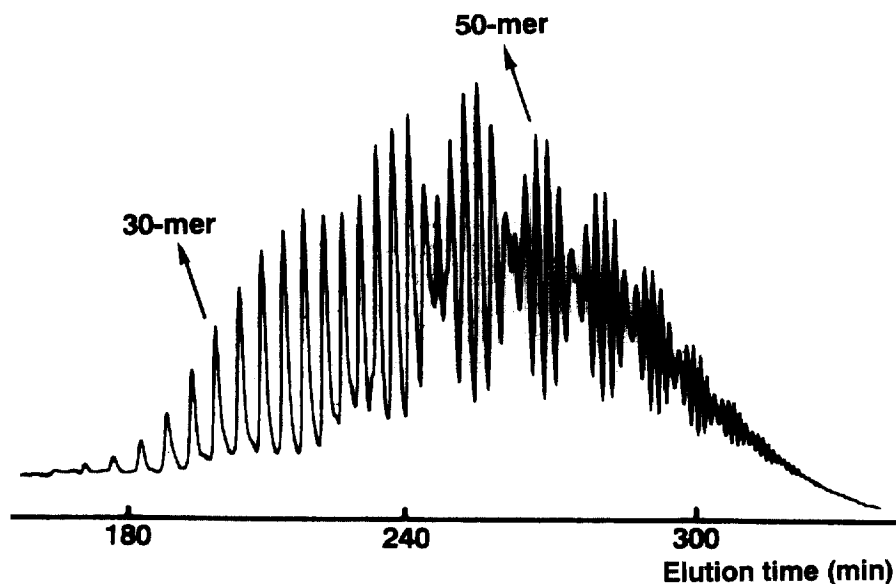


Fig. 4. HPLC of the second-lowest- M_n fraction (fr. 5b) of rubber from *L. volemus*.

methyl protons was in the order; *trans-trans-cis* > ω -*trans-trans* > ω . The high- M_n rubber might be modified at the chain-end during storage in the sporophores of after polymerization.

EXPERIMENTAL

Materials

Sporophores of *Lactarius volemus* were collected in Fukushima, Tochigi and Tokyo, Japan. Ficaprenol

was extracted from silkworm faeces with Me_2CO followed by separation by florisil CC [3]. Polyprenol was obtained from leaves of *Ginkgo biloba* [4]. Synthetic liquid rubber was supplied by Kuraray Co. and was used without purification. $\bar{M}_n$ and $\bar{M}_w$ were estimated to be 1.01×10^4 and 4.9×10^3 , respectively, by GPC measurement using polystyrene standards. GPC columns packed with styrene-divinylbenzene gel were products of TOHCO Co. Ltd (TSK-Gel). ODS-silica gel was purchased from Sakata, Japan. All other chemicals were of analytical grade.

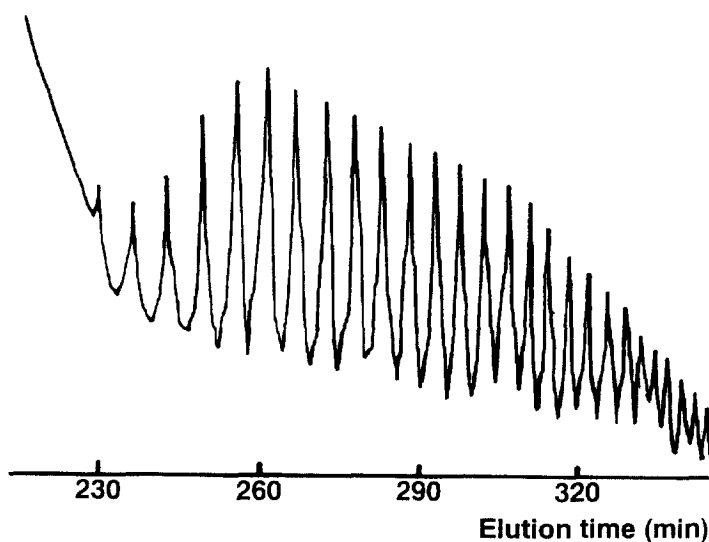


Fig. 5. HPLC of the low- M_n synthetic *cis*-polyisoprene.

Method

The fresh sporophores were cut into small pieces and extracted with EtOH followed by toluene. The toluene extract was concentrated by rotary evaporation and the rubber was precipitated by pouring the toluene soln into MeOH. Fractionation by M_r was performed on a 1% w/v toluene soln in the usual way [26] to give five fractions (fr. 1–5). That of the lowest M_r (fr. 5) was further fractionated using a GPC column (500×21.2 mm) having an exclusion limit of 3×10^4 . Saponification was carried out in a 1% w/v toluene soln (30 ml) of rubber with 10 ml of 10% KOH and 10 ml of 5% methanolic pyrogallol for 1 h at 90°. The hydrolysate was extracted with Et₂O–hexane (1:1) and the extract washed with water.

The polyprenol mixtures from silkworm faeces and from leaves of *G. biloba* were separated into 7–14-mers and 14–22-mer of polyprenol, respectively, by RP-HPLC using an ODS-silica gel column.

The M_r and MWD of rubbers were determined by means of GPC using two columns in series packed with styrene-divinylbenzene gel having an exclusion limit of 8.0×10^6 and 1.7×10^5 . Measurements were made using CHCl₃ as eluent. Commercial standard polystyrenes were used to calibrate the elution.

The distribution of chain-length in the low M_r fraction of rubber from *L. volemus* was determined by RP-HPLC using an ODS silica-gel column. HPLC measurements were performed as described by Swiezewska *et al.* [27]. A dual pump apparatus, gradient programmer and UV detector set at 210 nm were used. The gradient used was MeOH–*iso*PrOH–H₂O–hexane changing from a volume ratio of 12:8:1:0 to 12:23:1:34.

The M_n of the rubber was determined by means of a Hitachi 117 vapour pressure osmometer. ¹H NMR measurements were performed on deuterated benzene solns of rubbers, with TMS as int. standard, using

a JEOL GX-400 spectrometer at 50°, and a pulse repetition time of 5 s for a 45° pulse.

REFERENCES

1. Feeney, J. and Hemming, F. W. *Anal. Biochem.*, 1967, **20**, 1.
2. Tanaka, Y., in *NMR and Macromolecules*, ACS Symposium Ser. 247, ed. J. C. Randall. American Chemical Society, Washington D.C., 1984, p. 233.
3. Tanaka, Y. and Takagi, M., *Biochem. J.*, 1979, **183**, 163.
4. Iбата, K., Mizuno, M., Takigawa, T. and Tanaka, Y., *Biochem. J.*, 1983, **213**, 305.
5. Iбата, K., Kageyu, A., Takigawa, T., Okada, M., Nishida, T., Mizuno, M. and Tanaka, Y., *Phytochemistry*, 1983, **23**, 2517.
6. Tanaka, Y., Sato, H., Kageyu, A. and Tomita, T., *Biochem. J.*, 1987, **243**, 481.
7. Tanaka, Y., Sato, H. and Kageyu, A., *Rubber Chem. Technol.*, 1983, **56**, 299.
8. Tanaka, Y., *Proc. Int. Rubb. Conf., Kuala Lumpur*, 1985, **2**, 73.
9. Tanaka, Y., *J. Appl. Polym. Sci. Appl. Polym. Symp.*, 1989, **44**, 1.
10. Skoczylas, E., Swiezewska, E., Chojnacki, T. and Tanaka, Y., *Plant Physiol. Biochem.*, 1994, **32**, 825.
11. Subramaniam, A., *Rubb. Chem. Technol.*, 1972, **45**, 346.
12. Subramaniam, A., *Rubb. Res. Inst. Malaysia Technol. Bull.*, 1980, No. 4.
13. Subramaniam, A., *Proc. Int. Rubb. Conf., Kuala Lumpur*, 1975, **4**, 3.
14. Chen, A., Poulter, C. D., *J. Biol. Chem.*, 1993, **268**, 11002.
15. Fujii, H., Koyama, T., Ogura, K., *J. Biol. Chem.*, 1982, **257**, 14610.

16. Ohnuma, S., Koyama, T., Ogura, K., *J. Biol. Chem.*, 1991, **266**, 23706.
17. Ishii, K., Sagami, H., Ogura, K., *Biochem. J.*, 1986, **233**, 73.
18. Audley, B. G., Archer, B. L., *Natural Rubber Science and Technology*, ed. A. D. Roberts. Oxford Univ. Press, Oxford, New York, 1988.
19. Tangpakdee, J., Tanaka, Y., Ogura, K., Koyama, T., Wititsuwannakul, R. and Wititsuwannakul, D., *Phytochemistry*, 1997, **45**, 269.
20. Cornish, K., Siler, D. J., Castillon, J. and Chapman, M. H., *Int. Symposium on Isoprenoid Biochemistry*, Zao, Japan, 1996, p. 19.
21. Tanaka, Y., Kawahara, S., Eng, A. H., Shiba, K. and Ohya, N., *Phytochemistry*, 1995, **39**, 779.
22. Tanaka, Y., Mori, M. and Takei, A. *J. Appl. Polym. Sci. Appl. Polym. Symp.*, 1992, **50**, 43.
23. Tanaka, Y., Kawahara, S., Eng, A. H., Takei, A. and Ohya, N., *Acta Biochim. Polon.*, 1994, **41**, 303.
24. Tanaka, Y. and Hirasawa, H. *Chem. Phys. Lipids*, 1989, **51**, 183.
25. Tanaka, Y., Mori, M., Ute, K. and Hatada, K., *Rubber Chem. Technol.*, 1990, **63**, 1.
26. Berfield, D. R., Chew, L. C. and Gan, S. N., *Polymer*, 1976, **17**, 713.
27. Swieczewska, E., Chojnacki, T., Jankowski, W. J., Singh, A. K. and Olsson, J., *Biochem. Cell Biol.*, 1992, **70**, 448.