Rubber-Degradation Enzymes from a Bacterial Culture

AKIO TSUCHI and KIYOSHI TAKEDA
Fermentation Research Institute, Agency of Industrial Science and Technology,
Higashi, Tsukuba, Ibaragi 305, Japan

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Rubber-degrading activity was found in the extracellular culture medium of Xanthomonas sp. strain 35Y which was grown on natural rubber latex. Natural rubber in the latex state was degraded by the crude enzyme, and two fractions were separately observed by gel permeation chromatography of the reaction products. One fraction was of higher molecular weight (HMW) with a very wide MW distribution from 10^3 to 10^5, and the other fraction was of lower molecular weight (LMW) with a MW of a few hundred. 1H-nuclear magnetic resonance spectra of the partially purified fractions were those expected of cis-1,4-polyisoprene mixtures with the structure (CH2=CCH3)2CH=CH2 (HMW) and CH2=C(CH3)2-CH=CH2 (LMW), with average values of n of about 11 and 2 for HMW and LMW fractions, respectively. The LMW fraction consisted mostly of one component in gas-liquid chromatography as well as in gel permeation chromatography, and the main component was identified as 12-oxo-4,8-dimethyl trideca-4,8-diene-1-ol (acetonyl diprenyl acetaldehyde, A3P2A4) by 13C-nuclear magnetic resonance and gas chromatography-mass spectra. Not only the latices of natural and synthetic isoprene rubber, but also some kinds of low-MW polyisoprene compounds of cis-1,4 type, were degraded by the crude enzyme. The rubber-degrading reaction was found to be at least partly oxygenase catalyzed from the incorporation of 18O into A3P2A4 under an 18O2 atmosphere.

Spence and Van Niel observed in 1936 the formation of clear zones surrounding actinomycete colonies on a latex-agar plate, an indication of extracellular enzymatic decomposition of natural rubber latex (10). Since then, there have been a number of reports concerning microbial degradation of natural rubber (14). However, most of the studies were attempts to prevent microbial deterioration of rubber products, and no report has been available on the action of rubber-degrading enzymes.

We have previously reported the degradation of natural rubber in solid-state culture and the mechanism of rubber-glove degradation by an actinomycete, Nocardia sp. strain 835A (13). In this paper, we show the microbial degradation of rubber in the latex state and the chemical structure of isoprene oligomers produced by a crude enzyme of Xanthomonas sp. strain 35Y.

Actinomycetes have been considered the most important organisms in the degradation of natural rubber products (9). A bacterium, Xanthomonas sp. strain 35Y, however, was found to be a potent producer of rubber-degrading enzyme.

MATERIALS AND METHODS

Rubber latices and polyisoprene compounds. A commercial latex of natural rubber (Soctex-C; 60% rubber, dry weight) was obtained from Socin Co. Ltd. in Malaysia. Synthetic isoprene rubber latex (Maxprene IR-900; content of cis-1,4 structure was 78%, trans-1,4 content was 13%, and 3,4-content was 9% by 1H-nuclear magnetic resonance (NMR)) was from Seitetsu Chemical Industry Co. Ltd. Butadiene rubber latex (JSR 0700; vinyl content, 16 to 20%) was from Japan Synthetic Rubber Co. Ltd. Chlororubber rubber latex (LM-50) from Denki Chemical Industry Co. Ltd., and styrene butadiene rubber latex (Hyacar LX-110) from Nihon Zeon Co. Ltd. were also used. By the method of Spence and Van Niel (10), these latices were purified by dialysis against phosphate buffer (50 mM; pH 7.0) containing a surface-active agent (200 ppm [200 µg/ml]; Plysurf A210G: Daiichi Chemical Industry Co. Ltd.). A synthetic standard isoprene oligomer (PI 1000) (average molecular weight [MW], 940; cis-1,4 content, 67%; trans-1,4 content, 28%; and 3,4-content, 5% by 1H-NMR) was obtained from Polymer Laboratories Co. Ltd. Solanesol (all trans C45 isoprenoid alcohol; MW, 630), Ficaprenol (C45 to C65 prenol mixture; cis-1,4 content, 67%; MW, 830), and Dolichol (C90 to C105 dihydroprienol mixture; cis-1,4 content, 79%; MW, 1,300) were obtained from Sigma Chemical Co. Squalene was purchased from Tokyo Chemical Industry Co. Ltd. These low-MW polyisoprene compounds were treated by ultrasonication with an aqueous solution of the surface-active agent, and the emulsions formed were used as substrates for the enzyme reactions.

Microorganism and culture. Bacterial strain 35Y, a potent producer of rubber-degrading enzyme, was selected from our culture collection and used throughout this study. The strain was a gram-negative rod (0.6 µm by 3 to 5 µm) and was motile with 1 (or 2) polar flagellum. It was a strict aerobic that produced insoluble yellow pigment in the cells. From these characteristics, strain 35Y was tentatively identified as a Xanthomonas sp. (7). A detailed description of the organism will be presented elsewhere. The strain was preserved on Nutrient Broth Agar (Difco Laboratories).

Preparation of the crude enzyme. The strain was inoculated into 10 ml of a mineral salt medium containing 5 mg (dry weight) of natural rubber latex and 0.2 mg of the surface-active agent in a test tube (inner diameter, 25 mm). The composition of the mineral salt medium has been reported previously (12). The culture tubes were incubated unshaken at 30°C but were shaken vigorously by hand a few times a day. After 5 days of cultivation, the culture was centrifuged at 104 rpm for 10 min, and the clear supernatant from the middle part of the centrifuge tube was collected with a syringe. The supernatant was then filtered through a 0.22-µm-pore-size filter (Millipex-GV; Millipore Corp.). This preparation contained 21 µg of protein per ml and was used as the crude enzyme. The preparation was stored at 5°C for at least

* Corresponding author.
a month without any loss of enzyme activity. Collodion Bag 12 (pore size, 8 nm; Sartorius Gmbh) was used for ultrafiltration of the crude enzyme.

The cells in the bottom of the centrifuge tube were washed with a phosphate buffer and suspended in 10 ml of the buffer. The cells were then disrupted by sonication twice for 30 s at 4°C. Cell debris was removed by centrifugation, and the supernatant (about 100 μg of protein per ml) was used as the cell extract. The protein content was determined by the modified method of Lowry et al. (3). The residual rubber in the upper part of the centrifuge tube was extracted with ether, dried in vacuo, and weighed.

**Conditions for the enzyme reaction.** For product analysis by gas-liquid chromatography (GLC) and carbonyl content determination, the following reaction conditions (condition 1 in Table 1) were used. The reaction mixture contained crude enzyme solution (0.4 ml), rubber latex (1.7 mg, dry weight), 40 μg of surface-active agent, and phosphate buffer (10 mM; pH 7.5) in a total volume of 2.0 ml. The mixture was incubated at 30°C for 16 h in a test tube (16.5 by 165 mm) with a screw cap and a Teflon liner. The tube was then cooled to −20°C; the mixture was acidified with 2 N HCl and extracted with ether. A portion of the extract was directly subjected to GLC or carbonyl content determination. No enzyme and boiled-enzyme controls were used in these experiments.

For separation analysis by gel permeation chromatography (GPC), crude enzyme solution (10 ml) and rubber latex (4.3 mg) were mixed in a test tube (25 mm in diameter) and incubated for 48 h at 30°C. The mixture was acidified with HCl and extracted with ether. The ether extract was dried in vacuo, dissolved in CHCl3, and subjected to GPC. Just prior to the injection, the CHCl3 solution was freed from any insoluble materials by passage through a 0.2-μm-pore-size Teflon filter (Millex-FGS; Millipore).

**Conditions of GPC.** GPC measurement was carried out at 40°C in CHCl3. The apparatus used was a model HLC-802UR (Toyo Soda Manufacturing Co.) equipped with both refractive index and UV (338 nm) detectors. TSK standard polystyrenes were used as reference materials for evaluation of the resolving power of the column system in GPC measurements. The following polystyrenes were obtained from Tosoh Co. Ltd.: F-288 (MW, 289 × 106), F-40 (MW, 42 × 106), F-1 (MW, 1.0 × 106), and A-2500 (MW, 2.800). Squalene (MW, 410) and pristane (MW, 268) were also used as MW standards.

Column system I was designed to provide a resolving power high enough to cope with the high-MW region; accordingly, four columns (TSK-gels G-5000H, G-4000H, G-3000H, and G-2000H) were used at a flow rate of 0.95 ml/min and a pressure of 53 kg/cm². Each column was 60 cm long with an internal diameter of 7.5 mm. Although system I had a high resolving power in the region of MW higher than 106, the separations in the MW range from 102 to 106 were not satisfactory.

For separation analysis of low-MW oligomers, column system II (TSK-gels G-6000H, 30 cm; G-2000H, 60 cm [two columns]; and G-1000H, 60 cm) was used at a flow rate of 1.1 ml/min and a pressure of 73 kg/cm².

**Determination of carbonyl content.** The content of carbonyl compounds in the reaction mixture was determined as 2,4-dinitrophenyl hydrazones by the procedure of Katz and Keeny (6). The A338 of the hexane solution of 2,4-dinitrophenyl hydrazones was determined, and the concentration was calculated from a molar absorptivity of 21,500.

**Conditions of GLC.** GLC was performed with a Shimazu GC-5A equipped with a flame ionization detector. A glass column (3 mm by 2 m) packed with Tenax GC was used. N2 gas was used as the carrier gas at a flow rate of 60 ml/min. Oven temperature was 130 to 310°C (5°C/min). Retinal, tetradecyl aldehyde, citronellal, and isovaleraldehyde were used as standard reference materials.

**Isolation of the reaction products.** To prepare a relatively large amount of the reaction products, natural rubber latex (230 mg) was mixed with 220 ml of the crude-enzyme solution and incubated for 48 h at 30°C. The ether extract (221 mg) was dissolved in a small volume of CH2Cl2 and subjected to thin-layer chromatography (Kieselgel 60F254, 2-mm thickness; Merck & Co., Inc.). After the plate was developed two times with the solvent (benzene-acetone [20:1, vol/vol]), two distinct spots of the products, fraction R (Rf, 0.7 to 1.0; 130 mg) and fraction L (Rf, 0.46; 39 mg) could be obtained and were located on the chromatogram with UV light. Fraction L was further purified by another preparative thin-layer chromatography with the same solvent to remove a small amount of high-MW component (32 mg recovered).

**Spectral analysis.** Nuclear magnetic resonance spectra in CDC13 were recorded with a JEOL JNM-GX 270 FT-NMR spectrometer with tetramethylsilane as the internal standard. 1H-NMR spectra were obtained at 270 MHz, and 13C-NMR spectra were obtained at 68 MHz. The distortionless enhancement by polarization transfer sequence was used to separate a 13C spectrum into CH2, CH3, and CH subspectra (1). Neryl acetone (Fluka AG) and dolichol (from porcine liver; Sigma) were used as the standards for peak assignment.

Gas chromatography (GC)-mass spectra were obtained with a Hitachi M-2000 instrument equipped with an M-0201 data processing unit. Helium was used as a carrier gas, and the electron impact masses were measured at a 20-eV ionization potential. A glass column (1 m) packed with Tenax-GC was used with the following temperature program: isothermal for 5 min at 150°C, followed by increasing the temperature at a rate of 10°C/min to 340°C.

**Reaction with 18O2.** After complete removal of air by vacuum pump, the crude enzyme (2.0 ml) and the latex (1.7 mg) were mixed in a Thunberg tube. The tube was filled with 20% 18O2 in N2. The reaction mixture was incubated for 1 h at 30°C and extracted with ether. A portion of the extract was subjected to GC-mass spectrometry. 18O (97 to 98 atom%) was obtained from Cambridge Isotope Laboratories.

**RESULTS**

**Time course of microbial growth and enzyme activity.** Strain 35Y grew well on natural rubber latex as the sole source of carbon and energy. The typical time course is shown in Fig. 1. The total protein in the culture medium, which was used to express cell growth, attained its maximum on days 5 to 7, and the weight loss of rubber was about 60% on day 7. Enzyme activity in the extracellular culture medium reached a maximum on day 5 and then decreased. Activity in the cell extract was at an extremely low level compared with that in the culture filtrate.

Enzyme activity was destroyed by heating the crude enzyme at 100°C for 10 min. Furthermore, the activity was concentrated two or three times by means of ultrafiltration, and the filtrate contained no activity after passing through the membrane.

**Substrate specificities of the crude enzyme.** The latices of natural and synthetic isoprene rubber were degraded by the
FIG. 1. Time course of the degradation of natural rubber latex by strain 35Y. Symbols: ○···○, rubber recovered (milligrams per 10 ml); ○—○, cell growth (milligrams of protein per 10 ml); •—•, protein in the culture filtrate (milligrams per 10 ml); •—○, enzyme activity in the culture filtrate (micromoles per 16 h); ○—○, enzyme activity in cell extract (micromoles per 16 h).

crude enzyme, and a substantial amount of carbonyl compounds was formed (Table 1). The GPC analysis of the hydrazones suggested that more than 95% (in molar concentration) of the carbonyl compounds formed from natural rubber corresponded to one main component with a MW of a few hundred. The enzyme activity shown in Fig. 1 was expressed as the amount of carbonyl compounds (in micromoles) produced by the action of the crude enzyme under condition 1. No reaction was observed, however, on the latices of the other three kinds of synthetic rubber.

Among the low-MW polyisoprene compounds, dolichol, which has a relatively high content of cis-1,4 linkage, was rapidly degraded. Although the action of the enzyme upon ficaprenol and PI 1000 was slow, substantial amounts of carbonyl compounds were formed with a prolonged incubation period and a larger amount of the enzyme (condition 2 in Table 1). Even in reaction condition 2, no enzymatic action was detected upon trans-1,4-type isoprene oligomers like solanesol and squalene.

TABLE 1. Action of the crude enzyme on various kinds of substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Carboxyl compound produced (μmol) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Condition 1*</td>
</tr>
<tr>
<td>Natural rubber latex</td>
<td>1.4</td>
</tr>
<tr>
<td>Isoprene rubber latex</td>
<td>0.96</td>
</tr>
<tr>
<td>Butadiene rubber latex</td>
<td>ND*</td>
</tr>
<tr>
<td>Chloroprene rubber latex</td>
<td>ND</td>
</tr>
<tr>
<td>SBR latex</td>
<td>ND</td>
</tr>
<tr>
<td>PI 1000</td>
<td>0.08</td>
</tr>
<tr>
<td>Dolichol</td>
<td>0.68</td>
</tr>
<tr>
<td>Ficaprenol</td>
<td>0.12</td>
</tr>
<tr>
<td>Solanesol</td>
<td>ND</td>
</tr>
<tr>
<td>Squalene</td>
<td>ND</td>
</tr>
</tbody>
</table>

* 1.7 mg of substrate, 0.4 ml of crude enzyme, and 16 h of reaction.
* 0.85 mg of substrate, 2.0 ml of crude enzyme, and 48 h of reaction.
* 0.5 mg of substrate was used.
* ND, Not determined.

FIG. 2. GPC of the degradation products (column system I). ———, Boiled-enzyme control (or enzyme-less control); ———, degradation products; ····, standard reference materials.

GPC analysis of the degradation products. The elution profiles of the degradation products formed by the action of the crude enzyme on natural rubber are shown in Fig. 2 and 3. A very high-MW polymer of the original rubber (peak 1 of the boiled-enzyme control in Fig. 2) was almost completely degraded by the enzyme reaction. Without enzymatic digestion, 10 to 20% of the latex rubber was left unextracted by ether and was recovered as an insoluble rubber gel. No such insoluble fraction was obtained, however, after the enzyme reaction. The results indicated that the insoluble gel fraction in natural rubber was degraded and became soluble in ether by the action of the crude enzyme.

FIG. 3. GPC of the degradation products (column system II). (a) ———, Degradation products; (b) purified fractions: ———, fraction M; ····, fraction L; (c) ····, standard reference materials.
A relatively higher-molecular-weight (HMW) fraction (peak 2 in Fig. 2) and a lower-molecular-weight (LMW) fraction (peak 3 in Fig. 2) in the degradation products were separated by column system I. Although the HMW fraction had a very wide MW distribution (from $10^1$ to $10^5$), peak 3 was narrow (Fig. 2). The resolving power of system II was very high in the MW region below $10^2$, and peaks of squalene and pristane were well separated (Fig. 3c). Even in column system II, only one single and sharp peak was observed in the region shown in Fig. 3a, which indicates that the LMW fraction consists mostly of one component with a MW of a few hundred. Because of the insufficient resolving power of system II in the high-MW region, the HMW fraction was also condensed in a narrow peak 2 (Fig. 3a). A ghost peak was often observed near the exclusion limit (MW, $10^2$) of system II, probably because of the high viscosity of the rubber solution.

The elution profiles of the purified products prepared by thin-layer chromatography are shown in Fig. 3b. From a comparison between Fig. 3a and b, it was revealed that fraction M corresponded well to the HMW fraction and fraction L corresponded well to the LMW fraction. It must be noted that fraction M contains no detectable amount of the LMW fraction, and fraction L contains no detectable amount of the HMW fraction.

**GLC analysis.** In the gas-liquid chromatogram of the reaction products from natural rubber, one large peak was observed at the retention time of 51 min (peak D in Fig. 4). All of the small peaks except peak B also appeared in the chromatogram of the solvent; they were considered background peaks, probably due to the column packing. The peak area of peak B (retention time, 43 min) was less than 1% of that of peak D. Neither peak D nor peak B was observed in the chromatogram with no enzyme control. When fraction L was analyzed by GLC, only peak D and background peaks were observed; peak B was not detected.

**$^1$H-NMR spectra of the purified fractions.** The positions of the bands and their relative areas of $^1$H-NMR spectra are summarized in Table 2. The spectra and the details of the peak assignments were very similar to those reported in our earlier report (13). From the comparison with the spectra of standard compounds (neryl acetone and dolichol) and from the spin-decoupling experiments, the spectra of the purified fractions were considered to be those expected of cis-1,4-polyisoprene mixtures with the structure OHC-CH$_2$=CH-CH$_2$-C(-OH)-CH$_2$ with average values of $n$ of about 113 for fraction M and 2 for fraction L.

**$^{13}$C-NMR spectra of the purified fractions.** There were 15 peaks in the spectrum of fraction L. The assignments of these peaks were estimated by comparison with those in the spectra of standard compounds, and distortionless enhancement by polarization transfer experiments were used for distinguishing methylene and methyl carbons.

Five strong bands, corresponding to the repeated structure of the polymer backbone, and eight small bands were observed in the $^{13}$C spectrum of fraction M. The results corresponded well with those of $^1$H-NMR spectra (Table 3).

By means of distortionless enhancement by polarization transfer sequence, an error in the peak assignment of the $^{13}$C spectra was found in our earlier report (13). The band at 24.3 ppm (previously assigned as $\delta$-methyl carbon) was reassigned as $\beta$-methylene carbon, and the $\delta$-methyl carbon band (23.1 ppm) was considered to be buried within the strong band at 23 ppm of the main chain E-methylene carbon (Table 3, footnote b).

**GC-mass spectra of the degradation products.** Mass spectrum of the main component (peak D) of the LMW fraction showed a peak of parental molecular ion at $m/e$ 236 and other fragments at $m/e$ 228 (M-H$_2$O), 192 (M-44), 125 (M-111), 111 (M-125), 107 (125-H$_2$O), 93 (111-H$_2$O), 81 (125-44), and 43 (CH$_3$C=O). The spectrum confirmed that fraction L is 12-oxo-4,8-dimethyl trideca-4,8-diene-1-al (acetyl diprenyl acetoldehyde, A$_1$P$_2$A$_2$).

Mass spectrum of the component corresponding to peak B in GLC was also obtained by GC-mass spectroscopy (Fig. 5b). The spectrum showed peaks at $m/e$ 150 (M-H$_2$O), 124 (M-44), 107 (125-H$_2$O), 81 (125-44), and 43 (CH$_3$C=O). Although a peak of parental molecular ion at $m/e$ 168 (C$_{11}$H$_{20}$O$_2$) was not detected, the spectrum might be considered as that of 8-oxo-4-methyl-4-none-1-al (A$_1$P$_2$A$_2$). Further analysis by GC-mass spectroscopy revealed that none of the related compounds like acetyl acetoldehyde (re-vulcanization aldheyde, A$_2$P$_2$A$_2$) or acetyl tripropenyl acetoldehyde (A$_3$P$_2$A$_2$) were present in the degradation products.

The spectra of all other small background peaks appearing in GLC were quite different (all synchronized fragments at $m/e$ 221, 281, 355, 489, etc.) and may have been due to the column packing.
TABLE 3. $^{13}$C-NMR chemical shifts and signal assignment for the purified products

<table>
<thead>
<tr>
<th>Fraction L</th>
<th>$\delta_C$ (ppm)</th>
<th>Assignment</th>
<th>$\delta_C$ (ppm)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>208.7</td>
<td>C-12</td>
<td></td>
<td>208.7</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>202.2</td>
<td>C-8</td>
<td></td>
<td>135.2</td>
<td>C$_A$</td>
</tr>
<tr>
<td>136.1 (q)</td>
<td>C-4</td>
<td></td>
<td>125.1</td>
<td>C$_B$</td>
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<tr>
<td>126.9 (CH)</td>
<td>C-5</td>
<td></td>
<td>126.5</td>
<td>C$_A$</td>
</tr>
<tr>
<td>123.7 (CH)</td>
<td>C-9</td>
<td></td>
<td>123.4</td>
<td>C$_Y$</td>
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<tr>
<td>43.8 (CH$_2$)</td>
<td>C-6</td>
<td></td>
<td>44.0</td>
<td>C$_n$</td>
</tr>
<tr>
<td>42.3 (CH$_2$)</td>
<td>C-2</td>
<td></td>
<td>42.4</td>
<td>C$_n$</td>
</tr>
<tr>
<td>31.8 (CH$_2$)</td>
<td>C-7</td>
<td></td>
<td>32.3</td>
<td>C$_C$</td>
</tr>
<tr>
<td>29.9 (CH$_3$)</td>
<td>C-13</td>
<td></td>
<td>29.7</td>
<td>C$_n$</td>
</tr>
<tr>
<td>26.3 (CH$_2$)</td>
<td>C-8</td>
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<td>C$_D$</td>
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<tr>
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<td>C-10</td>
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<td>C$_B$</td>
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<tr>
<td>23.4 (CH$_3$)</td>
<td>C-15</td>
<td></td>
<td>23.4</td>
<td>C$_E$</td>
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<td>23.1 (CH$_3$)</td>
<td>C-14</td>
<td></td>
<td>22.3</td>
<td>C$_B$</td>
</tr>
</tbody>
</table>

* The numbers are standard carbon designations of the formula: 12-oxo-8,8-dimethyltrideca-4,8-diene-1-ol.

$^{19}$O$_2$ experiment. Under an $^{19}$O$_2$ atmosphere, formed AP$_2$A contained $^{18}$O$_2$-AP$_2$A (M$^+$ 236), $^{18}$O$_2$-AP$_2$A (M$^+$ 238), and $^{18}$O$_2$-AP$_2$A (M$^+$ 240) in a ratio of 18:77:5, whereas a ratio of 98:2:0 was obtained under air. When the reaction was performed in an atmosphere of nitrogen, no detectable amount of AP$_2$A was formed after 16 h of incubation.

**DISCUSSION**

We have previously reported that isoprenoid oligomers were accumulated during rubber-glove degradation by an actinomycete, Nocardia sp. strain 835A (13). The chemical structures of the two fractions of oligomers were shown to fit the formula OHC-CH$_2$-(CH$_2$-C(CH$_3$)$_n$=CH-CH$_2$)$_n$=CH-CH$_2$-CH=$($=O$)$-CH$_2$, where the values $n$ were about 114 (acetonyl polyisoprene acetoaldehyde, AP$_{114}$A$_2$) and 19 (AP$_{19}$A$_2$). Another series of isoprenoid oligomers were shown in this report to be produced from natural rubber latex by the action of a bacterium, Xanthomonas sp. strain 35Y. The chemical structures were also of the same formula as those described above, and the average values of $n$ were 113 (AP$_{113}$A$_2$) and 2 (AP$_2$A$_2$) for the two fractions of oligomers. The proposed chemical structures of these oligomers suggested that the polymeric chains in rubber were cleaved at the double bond shown by a wavy line in the formula shown in Fig. 6. It was of great interest that these two entirely different organisms degraded natural rubber in exactly the same way via chain scissions.

Rubber-degrading activity was destroyed by heating the culture filtrate at 100°C for 10 min, and the activity was concentrated by ultrafiltration. These results suggest that the cleavage reaction was enzyme catalyzed. It was supposed that strain 35Y secreted rubber-degrading enzyme into the extracellular culture medium and that AP$_2$A formed by the enzyme action was used as a carbon substrate for growth.

As the degradation products by the crude enzyme(s) were separated into two fractions by GPC (and by thin-layer chromatography), the enzyme reaction could be regarded as a two-step reaction. In the first step, the original polymer of natural rubber with a very high MW of over 10$^6$ was degraded and converted to the polymers with medium MW (the HMW fraction or fraction M). The wide MW distribution of the fraction suggested the random scissions of the
original polymer in endwise form. In the second step, the HMW fraction was further degraded to form mainly 12-oxo-4,8-dimethyl trideca-4,8-diene-1-al (acetonyl diprenyl acetaldehyde, A₁P₂A₃; MW, 236). Although the formation of a very small amount of 8-oxo-4-methyl-4-nonene-1-al (A₁PA₁) was indicated by GC-mass spectrometry, other oligomers corresponding to A₁A₃ or A₁P₂A₃ and A₁P₂A₄ were not detected in the degradation products with GPC or GC-mass spectrometry analysis. The high product specificity for the formation of AP₂A indicated that the double bonds in the HMW fraction were not cleaved at random but in a somewhat regular manner. Whether these apparent two-step reactions were catalyzed by two or more enzymes in the culture filtrate or by a single enzyme will be a subject of further study.

The formation of AP₂A was found to be at least partly oxygenase catalyzed from the incorporation of 18O into AP₂A under an 18O2 atmosphere. The incorporation of one atom of 18O into AP₂A was 77% and that of two atoms of 18O was only 4% at a neutral pH and with a 1-h incubation period. These relatively lower values were probably due to the rapid exchange of oxygen atoms in the carbonyl group (especially in the aldehyde group) and water (2). In fact, further lower incorporation of 18O was observed with a prolonged incubation period or at an acidic pH.

The conversion of β-carotene to retinal in rat liver was reported as a dioxygenase reaction (8). An oxygenase in a Microcystis sp., which cleaves β-carotene to form β-cyclocitrinal and crocetinal, was also reported (5). Squalene was shown to be cleaved to geranylacetone partly by an oxygenase in the cell-free system of an Arthrobacter sp. (4). In these cases, trans-1,4-type double bonds in low-MW isoprene oligomers were attacked and the enzymes involved were considered to be present only in the cells.

The extracellular crude enzyme from strain 35Y has a high substrate specificity to cis-1,4-polysoprene compounds and attacks high-MW polymers rather than low-MW oligomers. In recent years, an extracellular lignin-degrading enzyme from white rot fungus was reported to be a H₂O₂-dependent oxygenase (11). Natural rubber may also be degraded by oxygenase reactions of microorganisms, but the depolymerization mechanisms of high-MW polymers and the properties of enzymes involved require further study.

**LITERATURE CITED**


