Latex clearing protein – an oxygenase cleaving poly(cis-1,4-isoprene) rubber at the cis-double bonds

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Abbreviations: ATR, attenuated total reflectance; BCS, bathocuproine sulfonate; FTIR, Fourier transform infrared spectroscopy; ICP-OES, inductively coupled plasma optical emission spectroscopy; IMAC, immobilized metal-affinity chromatography; Lcp, latex clearing protein; MALDI-TOF MS, matrix-assisted laser desorption/ionization–time-of-flight
mass spectrometry; NMR, nuclear magnetic resonance; OD, optical density; ppm, parts per million; RoxA, rubber oxygenase A; SEC, size-exclusion chromatography; SOD, superoxide dismutase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tat, twin arginine translocation.

ABSTRACT

*Gordonia polyisoprenivorans* strain VH2, a potent rubber-degrading actinomycete, harbors two latex clearing proteins (Lcp) which are known to be essential for the microbial degradation of rubber. However, biochemical information on the exact role of this protein in the degradation of polyisoprene was lacking, yet. In this study, the gene of Lcp1VH2 was heterologously expressed in strains of *Escherichia coli*, the corresponding protein was purified and its role in rubber degradation was examined by measurement of oxygen consumption as well as by chromatographic and spectroscopic methods. It turned out that active Lcp1VH2 is a monomer and responsible for the oxidative cleavage of poly(cis-1,4-isoprene) in synthetic as well as in natural rubber by the addition of oxygen (O2) to the cis-double bonds. The resulting oligomers possess repetitive isoprene units with aldehyde (CHO-CH2---) and ketone (---CH2-CO-CH3) functional groups at the termini. Two fractions with average isoprene contents of 18 and 10, respectively, were isolated, thus indicating an endocleavage mechanism. The activity of Lcp1VH2 was determined by applying a polarographic assay. Alkenes, acyclic terpenes or other rubber-like polymers such as poly(cis-1,4-butadiene) or poly(trans-1,4-isoprene) are not oxidatively cleaved by Lcp1VH2. The pH and temperature optima of the enzyme are at 7 and 30°C, respectively. Furthermore, it was demonstrated that active Lcp1VH2 is a Cu(II)-containing oxygenase that exhibits a conserved domain of unknown function which cannot be detected in any other hitherto characterized enzyme. The results presented here indicate that this domain might represent a new protein family of oxygenases.
INTRODUCTION

The high annual production and consumption rates of natural and synthetic poly(cis-1,4-isoprene) rubber and rubber containing-materials cause huge challenges in the disposal of this polymer. Most waste management strategies that are implemented at present, such as combustion or stockpiling in landfills, exhibit a serious hazard to the environment and to human health. Therefore, finding alternative processes for the recycling of polyisoprenoids is desirable (1). Processes involving complete or partial biodegradation could be an environment friendly opportunity for reutilization of the polymer. For such a process, enzymes capable for the initial cleavage of the very inert polymer would be of great interest.

Until now, only one enzyme responsible for a primary attack of poly(cis-1,4-isoprene) has been characterized in more detail. This rubber oxygenase A (RoxA) was first detected in Xanthomonas sp. strain 35Y that secretes this heme-dependent dioxygenase into the medium during growth on rubber (2, 3). Purified RoxA cleaves the double-bonds of rubber by incorporation of both oxygen groups into the polymer generating 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD) as main product (4, 5). Recently, RoxA was crystallized, structural and functional analyses were carried out and a first cleavage mechanism was proposed (6-9). However, RoxA could be detected only in a few Gram-negative bacteria (2, 10). In Gram-positive bacteria, especially in actinomycetes, in which the capability of rubber degradation seems to be quite widespread (11), no RoxA orthologs can be detected (10). In contrast, all rubber degrading actinomycetes seem to have at least one copy of a gene encoding a latex clearing protein (Lcp). This enzyme is considered to catalyze the initial oxidative cleavage of rubber within this group of Gram-positive bacteria (10, 12-16) and does not possess any similarity towards the amino acid sequence of RoxA. Lcp was first detected in Streptomyces sp. K30 by generating a chemically induced mutant that cannot form clear zones on latex-overlay plates. Complementation experiments using lcp restored this capability (16).
Homologs can also be detected in members of the non-clear-zone forming group of actinomycetes such as in strains of Nocardia and Gordonia (12, 13). Representatives of this group grow adhesively on the polymer and appear to be more effective in disintegration of the rubber than strains of the clear-zone forming group (17). Within the adhesively growing group G. polyisoprenivorans strain VH2 was selected as a model strain, \textit{inter alia}, due to its fast metabolism of poly(cis-1,4-isoprene). On the basis of the whole genome sequence together with practical experiments such as transposon mutagenesis and generation of deletion mutants a full rubber degradation pathway for strain VH2 could be predicted. Within the genome of this strain two Lcp-encoding genes were detected (14) and are thought to be responsible for the oxidative cleavage of the rubber polymer for several reasons: (i) when cells of strain VH2 grow on rubber, an increase of carbonyl functional groups as well as a decrease of the \textit{cis}-double bonds was observed (17); (ii) the deletion of both genes resulted in a non-rubber degrading phenotype, whereas single deletion mutants still grew on rubber (14); (iii) heterologous expression of \textit{lcp1}\textsubscript{VH2} in non-clear-zone forming \textit{Streptomyces} strain TK23, enabled this strain to form translucent halos and aldehydes on latex-overlay plates; (iv) incubation of poly(cis-1,4-isoprene) with this recombinant strain resulted in the generation of fragments of lower molecular weight, and (v) \textit{lcp1}\textsubscript{VH2} transcripts could only be detected by reverse transcription-PCR analysis when the cells were grown on rubber but not when grown on e. g. sodium acetate (13).

Nevertheless, biochemical information on how Lcp is enzymatically involved in the degradation of poly(cis-1,4-isoprene) and whether other enzymes are necessary for rubber disintegration by actinobacteria are missing. It was the aim of this study to unravel the reaction catalyzed by Lcp.
MATERIAL AND METHODS

Bacterial strains, media, growth conditions and oligonucleotides. Cells of *Escherichia coli* were cultivated aerobically at 37°C in Lysogeny Broth (LB) (18) whereas cells of *Gordonia polyisoprenivorans* strain VH2 (DSMZ 44266) were aerobically cultivated at 30°C in Standard-I (St-I) medium (Merck, Darmstadt, Germany). Liquid cultures were incubated in Erlenmeyer flasks on horizontal rotary shakers with an agitation of 100-150 rpm. Preparation of solid media was carried out by the addition of 1.5% (wt/vol) agar-agar. Antibiotics were added as described by Sambrook et al. (18).

Chemicals. Chemicals were purchased from Roth (Karlsruhe, Germany) and Sigma-Aldrich (Steinheim, Germany). Synthetic poly(cis-1,4-isoprene) (Roth, Karlsruhe, Germany) was purified and grained to a defined size of 63 - 500 µm as described elsewhere (14). Natural rubber (CAS-No. 9006-04-06) was received from Weber & Schäfer GmbH & Co. KG (Hamburg, Germany) as Latex milk (NEOTEX LATZ). The stabilizing ammonia was removed via centrifugation (5 min, 10,000 x g). The top layer was removed and used for further experiments.

Bioinformatic analyses. For prediction of cleavage sites TatP 1.0 (19) was applied using default parameters. Prediction and analyses of domains was carried out using the Pfam database (Pfam A and B) (20). Alignments were performed applying ClustalW with default parameters (21).

Isolation and modification of DNA. Total DNA of *G. polyisoprenivorans* strain VH2 was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The gene encoding Lcp1VH2 was amplified from total DNA of *G. polyisoprenivorans* strain VH2 using oligonucleotides lcp1_fw_oTAT_NdeI (5'-AAAACATATGCACCACCACCACCACCACCACAACTCGATGGGGTGGG-3') and
lcp_rv_Stop_BamHI (5’-AAAAGGATCCTCAGTTGTAGTTCGGGTTGTTGAAGTAGG-3’) synthesized by Eurofins MWG Operon (Ebersberg, Germany). Oligonucleotide lcp1_fw_oTAT_NdeI generates a hexahistidine tag at the N-terminus. Amplification was carried out using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The amplified DNA fragment was purified employing the peqGOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and cloned into pJET 1.2 Blunt Cloning Vector (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol, respectively. The construct was transferred into chemical competent cells (14) of E. coli Mach1™ T1R (Life Technologies, Carlsbad, CA, USA).

Plasmids were isolated from the cells growing on the medium containing an appropriate antibiotic using peqGOLD Plasmid Mini Kit (PEQLAB Biotechnologie, Erlangen, Germany). The correctness of the DNA fragment was verified by sequencing at Seqlab (Sequence Laboratories Göttingen, Göttingen, Germany) using pJET1.2 forward and reverse sequencing primer (Thermo Fisher Scientific, Waltham, MA, USA). The correct fragment was excised from pJET1.2::Hislcp1VH2 using the restriction endonucleases BamHI and NdeI (Thermo Fisher Scientific, Waltham, MA, USA) and was purified (peqGOLD Gel Extraction Kit; PEQLAB Biotechnologie, Erlangen, Germany) according to the manufacturer’s protocols. It then was ligated into the BamHI and NdeI-linearized expression vector pET23a(+) (Merck, Darmstadt, Germany), and the resulting plasmid was transformed into chemical competent cells (18) of E. coli Mach1™ T1R. Correctness of insertion and fragment was confirmed by plasmid isolation, subsequent restriction and sequencing analysis (using T7 promoter and terminator primer) as described above.

Purification of Lcp1_VH2. Overexpression of lcp1VH2 was carried out in E. coli C41(DE3) containing pET23a(+)::Hislcp1VH2 as described in the pET System Manual (Merck, Darmstadt, Germany) with the exception that cells were grown for 16 h at 20-25°C.
after induction at OD$_{600}$ = 0.4/0.5. They were harvested by centrifugation (4°C, 3500 x g) and stored at -20°C. Cell disruption was carried out after resuspension in 100 mM Tris-HCl buffer containing 40 mM imidazol and 500 mM NaCl (pH 7.4, 4°C) through a five-fold passage through a cooled French-press (Aminco, Silver Spring, MD, USA). The soluble fraction was obtained from the crude extract by 30 min centrifugation at 21,000 x g (4°C).

All purification steps were carried out at 4°C. The soluble fraction was loaded (0.5 ml/min) onto a Ni-NTA Sepharose column (HisTrap™ FF, 1 ml; GE Healthcare Lifescience, Uppsala, Sweden) that was equilibrated according to the manufacturer’s instructions. Binding conditions were 100 mM Tris-HCl buffer containing 40 mM imidazol and 500 mM NaCl (pH 7.4). For purification, the following program was chosen: flow-rate was 1ml/min; the first washing step was carried out for 20 min with 40 mM imidazole, and the second washing step was done for 10 min with 60 mM imidazole in 100 mM Tris-HCl buffer containing 500 mM NaCl (pH 7.4). Elution was carried out for 10 min at a concentration of 500 mM imidazol in the latter buffer. The eluted fractions were pooled and then concentrated to approximately 1 ml using Vivaspin ultrafiltration spin columns (10 MWCO PES) by centrifugation (3,500 x g). Since the protein was not totally pure after immobilized metal-affinity chromatography (IMAC), a further purification step was performed via size-exclusion chromatography (SEC) using a Superdex 200 HP 16/600 column (GE Healthcare Lifescience, Uppsala, Sweden). This step also served for molecular mass detection of Lcp1VH2. Calibration was carried out applying the high-molecular-mass gel filtration calibration kit (GE Healthcare Lifescience, Uppsala, Sweden), according to the manufacturer’s protocol. The concentrated eluate was loaded onto the column that was equilibrated with 20 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.4). A flow rate of 1 ml/min was applied. Fractions were determined by monitoring the absorbance at 280 nm. The fractions were pooled, concentrated, and the buffer was changed to Bis-Tris buffer (200 mM, pH 7.0) by centrifugation (3,500 x g) using Vivaspin ultrafiltration spin columns (10 MWCO PES).
Protein concentrations were determined according to Bradford (22). Proteins were separated, and purity was examined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 23). Protein verification and control of purity was executed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) at the Institut für Mikrobiologie, Ernst-Moritz-Arndt Universität, Greifswald, Germany.

**Oxygen consumption assay.** Oxygen consumption was determined using the module-based microrespiration system and MicOx software of Unisense (Aarhus, Denmark). Bis-Tris buffer (200 mM, pH 7.0), 5 µg/ml purified protein and 20 µl/ml of a 20% latex emulsion were mixed in Unisense micro-respiration chambers (1 ml). Consumption of oxygen was determined at 23°C and a stirring rate of 200 rpm, using a standard glass sensor (OX-MR, Unisense, Aarhus, Denmark) that was pre-polarized and calibrated according to the manufacturer’s protocol. This assay was also applied for determination of the temperature optimum which was performed within a heating cabinet at temperatures mentioned in the text. Determination of the pH optimum was achieved using the buffers mentioned in the text. Furthermore, the substrate range of Lcp1VH2 was also tested via oxygen consumption using substrates other than latex (as specified in the text). For that 1% (vol/vol) of each compound was added to the assay. In order to exclude inhibitory effects of the corresponding compound 20 µl/ml of 20% latex emulsion was additionally added, and the oxygen consumption was compared to that of the assays with latex emulsion only. The oxygen consumption assay was also used by default to check the activity of the purified protein as well as for the chelating experiments.

**Isolation of degradation products and spectral analyses.** For spectral analyses 50 µl/ml of a 20% latex (vol/vol) emulsion were mixed with 15 µg/ml of purified protein (negative control: heat inactivated enzyme; 98°C, 30 min) in Bis-Tris buffer (200 mM, pH 7.0) and inoculated in test tubes for 20 h at 30°C on a rotary shaker at 150 rpm (sample
volume 1 ml). Furthermore, 15 mg/ml granules of synthetic rubber (in 200 mM Bis-Tris buffer, pH 7.0) were inoculated in test tubes for five days with a daily addition of 10 µg/ml of purified protein (negative control: heat inactivated enzyme). The test tubes containing synthetic rubber were autoclaved before adding the enzyme for the first time. The mixtures were extracted using diethyl ether. The resulting fractions were dried using anhydrous MgSO$_4$, filtered and concentrated in a rotary evaporator at 40°C under reduced pressure. The degradation products were purified using chromatography on a silica gel 60 (0.040-0.063 mm; 230-400 mesh) column using dichloromethane as an eluent. Analysis of the fractions (each ca. 10 ml) with a TLC plate using dichloromethane as an eluent was performed on pre-coated plastic sheets with detection by UV (254 nm) and by coloration with cerium molybdenum solution [phosphomolybdic acid (25 g), Ce(SO$_4$)$_2$·$x$H$_2$O (10 g), concentrated H$_2$SO$_4$ (60 mL), H$_2$O (940 mL)]. Two product containing fractions (fraction 1 and fraction 2) were collected, and the solvent was removed in a rotary evaporator at 40°C under reduced pressure.

$^1$H and $^{13}$C spectra of the extracts and isolated products were recorded at room temperature in CDCl$_3$ on a spectrometer at 600 and 151 MHz respectively. The chemical shifts are given in ppm relative to internal standard TMS ($^1$H: $\delta$ [Si(CH$_3$)$_4$] = 0.00 ppm) or relative to the resonance of the solvent ($^{13}$C: $\delta$ [CDCl$_3$] = 77.0 ppm). $^1$H and $^{13}$C signals were assigned by means of C, H, COSY and HSQC spectroscopy. Infrared spectra were recorded on a FTIR-Perkin Elmer (SpectrumOne; Perkin Elmer, Waltham, MA, USA). The samples were directly measured on an ATR-sample head. The positions of the absorption bands are given in cm$^{-1}$. FTIR spectrum is given for the second fraction with an average n of 10 for the FTIR (film) with 2961, 2918, 2853, 1722, 1449, 1376, 1082, 837 cm$^{-1}$. Furthermore, nuclear magnetic resonance spectroscopy data ($^1$H and $^{13}$C) for both fractions are:
Fraction 1: $^1$H NMR (600 MHz, CDCl$_3$): $\delta = 1.68$ (s, 53.3 H, 4-Me, 8-Me), 2.01 – 2.09 (m, 67.6 H, 6-H, 7-H), 2.13 (s, 3 H, 12-H), 2.26 (td, $^3$J$_{10,11} = 7.7$ Hz, $^3$J$_{10,9} = 7.4$ Hz, 2 H, 10-H), 2.35 (t, $^2$J$_{3,2} = 7.8$ Hz, 2 H, 3-H), 2.44 (t, $^3$J$_{11,10} = 7.7$ Hz, 2 H, 11-H), 2.49 (td, $^3$J$_{2,3} = 7.8$ Hz, $^3$J$_{2,1} = 1.6$ Hz, 2 H, 2-H), 5.08 (t, $^3$J$_{9,10} = 7.4$ Hz, 1 H, 9-H), 5.11 – 5.18 (m, 17.0 H, 5-H), 9.78 (t, $^3$J$_{1,2} = 1.6$ Hz, 1 H, 1-H) ppm.

Fraction 1: $^{13}$C NMR (151 MHz, CDCl$_3$): $\delta = 22.3$ (C-10), 23.1 (4-Me), 23.4 (8-Me), 24.3 (C-3), 26.4 (C-6), 29.7 (C-12), 32.2 (C-7), 42.4 (C-2), 44.0 (C-11), 123.4 (C-9), 125.0 (C-5), 135.2 (C-8), 202.1 (C-1), 208.7 (C=O) ppm.

Fraction 2: $^1$H NMR (600 MHz, CDCl$_3$): $\delta = 1.68$ (s, 29.7 H, 4-Me, 8-Me), 2.01 – 2.09 (m, 35.4 H, 6-H, 7-H), 2.13 (s, 3 H, 12-H), 2.26 (td, $^3$J$_{10,11} = 7.7$ Hz, $^3$J$_{10,9} = 7.4$ Hz, 2 H, 10-H), 2.35 (t, $^2$J$_{3,2} = 7.8$ Hz, 2 H, 3-H), 2.44 (t, $^3$J$_{11,10} = 7.7$ Hz, 2 H, 11-H), 2.49 (td, $^3$J$_{2,3} = 7.8$ Hz, $^3$J$_{2,1} = 1.6$ Hz, 2 H, 2-H), 5.08 (t, $^3$J$_{9,10} = 7.4$ Hz, 1 H, 9-H), 5.11 – 5.18 (m, 9.0 H, 5-H), 9.78 (t, $^3$J$_{1,2} = 1.6$ Hz, 1 H, 1-H) ppm.

Fraction 2: $^{13}$C NMR (151 MHz, CDCl$_3$): $\delta = 22.3$ (C-10), 23.1 (4-Me), 23.4 (8-Me), 24.3 (C-3), 26.4 (C-6), 29.7 (C-12), 32.2 (C-7), 42.4 (C-2), 44.0 (C-11), 123.4 (C-9), 125.0 (C-5), 135.2 (C-8), 202.1 (C-1), 208.7 (C=O) ppm.

Possible degradation of poly(trans-1,4-isoprene) and poly(cis-1,4-butadiene) using purified Lcp1VH2 was also determined by using $^1$H NMR analysis. For this 1.5% (w/v) of each polymer was incubated in in 200 mM Bis-Tris buffer, pH 7.0 in test tubes for five days with a daily addition of 10 µg/ml of purified protein (negative control: without enzyme) as also described for rubber. The test tubes were autoclaved before starting the test. The mixtures
were extracted, dried, filtered and concentrated as described for rubber (above). $^1$H spectra of
the extracts were recorded at room temperature in CDCl$_3$ on a spectrometer at 600 MHz.

Identification and characterization of cofactors in purified Lcp1$_{VH2}$. Metal content
analyses using inductively coupled plasma optical emission spectroscopy (ICP-OES) were
carried out at Chemical Analysis Lab/Center for Applied Isotope Studies at the University of
Georgia, USA.

The oxidation state of the transition metal within the active enzyme, was determined
applying a spectroscopic assay with bathocuproine sulfonate (BCS) which specifically
chelates Cu(I) (24). Samples contained 50 µM Lcp1$_{VH2}$ in 20 mM sodium phosphate buffer
containing 150 mM NaCl (pH 7.4) and 1% (w/v) sodium dodecyl sulfate (SDS) within a
quartz cuvette. BCS (final concentration of 500 µM) was added to form yellow Cu(I)(BCS)$_2$
complexes with Cu(I) that were detected spectroscopically at approx. 480 nm. Reduction of
Cu(II) was performed by addition of Na-ascorbate (final concentration of 500 µM). To chelate
divalent ions, ethylenediaminetetraacetic acid (EDTA) was added to a concentration of
20 mM. UV/visible spectra were recorded on a Jasco V-550 UV/VIS Spectrophotometer
(JASCO Corporation, Tokyo, Japan) or a Shimadzu UV-2600 UV/VIS Spectrophotometer
(Shimadzu, Kyoto, Japan).

RESULTS

Purification of Lcp1$_{VH2}$. Although functional expression of lcp1$_{VH2}$ in an lcp-double-
deletion mutant of G. polyisoprenivorans strain VH2 (14) resulted in the complementation of
the rubber-degrading activity, a purification of the enzyme was not successful from this
mutant. Furthermore, the protein of heterologously expressed lcp1$_{VH2}$ was not processed and
secreted in E. coli, although analysis of the protein sequence of Lcp1$_{VH2}$ (GenBank accession
no. YP_005285193) revealed the occurrence of a twin-arginine signal peptide cleavage site
between amino acid no. 36 and 37 (…WAPA-NSIP…). Instead, it remained in the cells mainly as inclusion bodies. Additionally, Lcp1\textsubscript{VH2} could not be purified by IMAC when a Hexahistidine-tag was adhered C-terminally under non-denaturing conditions; a purification was only possible under denaturing conditions (not shown).

For these reasons we replaced the predicted TAT-cleavage site by an N-terminal Hexahistidine-tag. The resulting protein (377 amino acids) exhibited a theoretical molecular mass of 42.59 kDa. The overproduced protein was purified to electrophoretic homogeneity (Fig. 1) in two subsequent steps: (i) via immobilized metal-affinity chromatography and (ii) by size-exclusion chromatography. The latter chromatographic step revealed an apparent molecular mass of 38.5 kDa (± 1.5 kDa) for the modified Lcp1\textsubscript{VH2} which correlates with a monomeric protein. The average yield of purified Lcp1\textsubscript{VH2} from 1000 ml of culture volume was approx. 1.5 mg.

**Oxygen consumption assay.** Since it was anticipated that Lcp is an oxygenase, measurements of oxygen consumption by Lcp1\textsubscript{VH2} were carried out with the active enzyme as well as the heat inactivated protein (98°C, 30 min) in presence of latex in an enzyme assay as described in the Materials and Methods section (Fig. 2). The assay clearly showed that oxygen consumption of Lcp1\textsubscript{VH2} is latex dependent and does not occur in the absence of the polyisoprene substrate. In order to demonstrate that the consumption of oxygen is due to the active enzyme and not to the other components of the applied assay, negative controls were carried out. The assay was for example performed with heat inactivated protein, with only buffer or with buffer together with latex but without enzyme. Since all these controls were negative the oxygen consumption can be attributed to the enzymes activity. Furthermore, a clearing of the samples with active enzyme was observed in these assays whereas the controls (use of denatured enzyme or latex only) remained milky. This clearly demonstrated that active Lcp1\textsubscript{VH2} consumes oxygen to change the structure of molecules which are present in latex.
Evaluation of the oxygen consumption within the first 10 min (without lag phase) revealed a specific activity of Lcp1VH2 of approx. 1.3 µmol/min per mg of purified protein.

**Analysis of degradation products via spectral analyses.** After incubation of latex with active or heat inactivated enzyme the resulting degradation products were extracted, dried, filtered and concentrated as described in the Materials and Methods section. Thereafter, they were analyzed by FTIR and NMR spectroscopy. Furthermore, the degradation products were purified using preparative column chromatography with silica gel. The products were eluted with dichloromethane, and two fractions were collected. The product fractions were also analyzed by means of FTIR and NMR spectroscopy.

Latex treated with active or heat inactivated Lcp1VH2 gave FTIR spectra (Fig. 3) that were identical to that of poly(cis-1,4-isoprene) in literature (25, 26). This includes the assignment of the C= C double bonds as well as of the aliphatic CHx groups confirming that the extracts consisted of natural rubber from latex milk. However, the analysis of the product fractions, which could be isolated when the rubber was incubated with active Lcp1VH2, showed FTIR spectral bands at 1722 cm⁻¹ that clearly indicated the presence of carbonyl functional groups. Latex samples of the negative control incubated with denatured enzyme did not exhibit these groups. This is in good agreement with the analyses of microbiologically deteriorated rubber analyzed via infrared spectroscopy in the past (17, 27) and indicate a degradation mechanism involving the addition of oxygen to the polyisoprene molecules.

Furthermore, the formation of aldehydes as well as ketones during the degradation of rubber was approved by ¹H-NMR spectroscopy of the two product fractions (Fig. 4; for full ¹H-NMR spectrum of the second fraction in comparison with the negative control see Fig. S1). The triplet at 9.78 ppm, which was not detected in the negative control, clearly shows the appearance of aldehyde groups during the degradation process and therefore confirmed the findings made in FTIR spectroscopic analysis. Furthermore, an occurrence of a singlet at 2.13
ppm, which represents the protons of the methyl ketone, appeared only in samples that were treated with active Lcp1VH2 but not with denatured protein. The signals, which appeared in the sample with active enzyme between 2.26 ppm and 2.49 ppm, are due to the formation of terminal alkyl chains during the Lcp1VH2-mediated degradation process. The position of the multiplet signals at 5.13 ppm, 2.04 ppm as well as the singlet signal at 1.68 ppm were detected in both samples and are to be assigned to the poly(cis-1,4-isoprene) structure as presented at position 5 (olefinic protons), 6 and 7 (methylene protons), 4 and 8 (methyl protons), respectively, in Fig. 4. All these spectra are in agreement with spectra that were published for intact poly(cis-1,4-isoprene) as well as for the biodegradation products, like those isolated from a culture of Nocardia sp. strain 835A growing on rubber as sole source of carbon and energy (3, 28-31). Furthermore, the occurrence of these products was confirmed by $^{13}$C-NMR spectroscopy, and the data also corresponded with spectra obtained for biodegraded rubber particles in the past (3, 29). The two isolated fractions differed from each other by the average chain length n, which could be estimated using the $^1$H-NMR integrals by dividing the signal integrals by the number of protons of the corresponding groups (3). Herein an average chain length of n = 17.9 for the first fraction and n = 10.0 for the second fraction could be calculated (for detailed calculations see Table S1). All experiments were also carried out with synthetic poly(cis-1,4-isoprene) and lead to identical results (not shown).

**Biochemical characterization of Lcp1VH2.** The temperature and pH optima of purified Lcp1VH2 were elucidated using different buffers and the oxygen consumption assay described above (Fig. 5). The temperature optimum was at around 30°C, that of the pH was around 7. The activity of Lcp1VH2 at a pH lower than 5 could not be determined since the rubber of latex coagulates at low pH values. Furthermore, the substance range of the enzyme was evaluated and it clearly appeared that alkanes and also alkenes were not oxidatively cleaved by Lcp1VH2 as revealed by the oxygen consumption assay. This assay also revealed that acyclic terpenes such as squalene or squalane and also unsaturated fatty acids, such as
palmitoleic acid or linoleic acid were also not cleaved. Furthermore, enzyme assays employing active Lcp1VH2 and subsequent NMR analyses as described in the materials and methods section were applied. These analyses clearly revealed that the enzyme does not cleave the double-bonds of polymers having a similar structure like poly(cis-1,4-isoprene) such as poly(trans-1,4-isoprene) and poly(cis-1,4-butadiene) since the spectra of polymers treated with active Lcp1VH2 exhibited no differences to that of the negative controls (in absence of the enzyme) (data not shown).

Lcp1VH2 possesses no domain of which a function is known and also shows not any similarity to an already characterized protein. Therefore, a distinct prediction of a putatively utilized transition metal cannot be done in silico. However, purified and concentrated Lcp1VH2 shows an orange color with an UV/visible spectrum shown in Fig. 6. This spectrum shows a broad band around 420 nm, also typical for e.g. copper-dependent “white” laccases (32, 33) and indicating a putatively bound transition factor. Therefore, the influence of different chelating agents on the enzyme activity was tested. EDTA and tiron did not significantly inhibit the activity of the enzyme even after an incubation time of 30 min but 1,10-phenanthroline as well as 2,2-bipyridyl inhibited the activity at high concentrations (Table 1). The latter compounds are not only known to chelate Fe(II) but also to form complexes with e.g. Cu(II) (34, 35). Therefore, ethylxanthate, highly specific for copper at low concentrations (36), was also applied. The latter clearly showed significant inhibition of the reaction at low concentrations of even 0.2 mM.

In order to detect whether other metals could also be involved in the enzymatic activity, a metal content analysis of purified Lcp1VH2 using inductively coupled plasma optical emission spectroscopy (ICP-OES) was performed. This analysis clearly showed that the only metal present in the purified and active Lcp1VH2 is copper. As also described for other recombinant copper-dependent enzymes that were purified from cells of *E. coli* (37), the
active Lcp1VH2 only had a sub-stoichiometric content of copper. In order to specify the oxidation state of the transition metal within the active enzyme, a spectroscopic assay applying bathocuproine sulfonate (BCS), specifically chelating Cu(I) (24), was employed (Fig. 6): When BCS was added to the enzyme, denatured with SDS, an increase at approx. 480 nm (associated with formation of the Cu(I)(BCS)₂ complex) indicated the presence of Cu(I). However, reduction of possibly contained Cu(II) to Cu(I) applying ascorbate resulted in a further increase of the absorbance - thus indicating that Cu(II) is also present. Since Cu(I) could also evolve by intrinsic reduction of Cu(II) e.g. by cysteine, tyrosine or tryptophan (38) within this environment, the assay was performed with addition of EDTA before the enzyme was denatured with SDS. After subsequently adding BCS, the absence of Cu(I)(BCS)₂ complexes clearly indicated that there was no Cu(I) within the sample. Therefore, it was concluded that the purified and active Lcp1VH2 contains Cu(II).

DISCUSSION

Until now several studies on the participation of Lcp in the degradation of poly(cis-1,4-isoprene) have been carried out (12, 13, 16), and the essentiality of Lcp for the degradation process was unequivocally demonstrated recently (14). However, concrete statements on how Lcp is involved in the degradation of rubber and if other coenzymes or cofactors are essential for the initial step of rubber disintegration by actinobacteria could not be made until now. In this study, we could clearly show that Lcp1VH2 is responsible for this initial oxidative step by demonstrating the reaction in vitro employing the purified enzyme. Lcp1VH2 cleaves rubber at the cis-double bonds of the polymer by incorporation of oxygen what leads to the formation of oligomers containing terminal carbonyl functional groups. Furthermore, this process relies on an endocleavage mechanism resulting in degradation products of different molecular weights.
The degradation products possess the structures shown in Fig. 4 which are in good agreement with the degradation products generated by actinomycetes growing on natural and synthetic as well as vulcanized rubber. However, the molecular weights of the fractions always differ from strain to strain (2 - 190 isoprene units) (3, 12, 13, 29). Since Lcp is present in all rubber degrading actinomycetes (10, 12-16), Lcp’s from different strains obviously degrade the polymer to cleavage products of different average chain length by endocleavage. However, it should be pointed out that all publications mentioned above depict a completely different experimental setup since they were carried out with living cells and never with the purified enzyme. Therefore, an additional modification of the resulting degradation products or the rapid consumption of lower molecular weight products by the cells is conceivable. Furthermore, other factors such as the length of the incubation period or the ratio of enzyme to substrate in these experiments might also influence the average length of the identified products.

For purified RoxA of *Xanthomonas* sp. strain 35Y, the only rubber cleaving oxygenase that was investigated in more detail up to now, the formation of products varying in size and consisting of repetitive isoprene units with carbonyl functional groups at the termini was also described. This enzyme also cleaves rubber at the double bonds upon the incorporation of oxygen outside the cell and without the utilization of cosubstrates such as NAD(P) (4, 5, 29). For RoxA a degradation mechanism involving an oxidative cleavage of the polyisoprene molecule via the generation of an allylic hydroperoxide and a subsequent formation of a substrate-dioxetane-intermediate was proposed (5, 9). Since Lcp also cleaves rubber by adding oxygen to the double bonds of the polymer outside the cell (13, 14, 16, 17, 39) and since *in vitro* experiments with Lcp1VH2 could be also performed without additional cosubstrates, parallels within the degradation mechanism are possible.
However, the amino acid sequences of latex clearing proteins exhibit no similarities towards that of RoxA, and the characterization of Lcp1_{VH2} clearly revealed great differences between Lcp and RoxA: (i) The main average cleavage products of Lcp1_{VH2} contain five and nine times more isoprene units than RoxA, (ii) Lcp amino acid sequences contain a domain of unknown function (DUF2236; Pfam accession no. PF09995) whereas RoxA sequences possess conserved sequences that belong to the family of di-heme cytochrome c peroxidases (Pfam accession no. PF03150), (iii) Lcp1_{VH2} is in contrast to the heme-containing RoxA a Cu(II)-dependent oxygenase, (iv) BLAST analyses reveal that Lcp is highly distributed whereas RoxA is only detectable in very few Gram-negative bacteria (4, 10) (v) and a comparison of the evaluated specific activities indicates that Lcp1_{VH2} has a higher specific activity (1.3 µmol/min per mg) than RoxA of *Xanthomonas* sp. strain 35Y (0.3 µmol/min per mg) (5). These activities are, however, comparable with other double bond-cleaving oxygenases such as benzene or toluene dioxygenases (activity between 0.045 and 2.58 µmol/min per mg) (40, 41), but are clearly lower than e.g. the secreted, copper-dependent quercetin 2,3-dioxygenases (up to 177 µmol/min per mg) (42).

Due to (i) the inhibition of Lcp1_{VH2} activity by the utilized chelating agents with a simultaneous absence of other metals than copper, (ii) the results of the applied bathocuproine sulfonate-based spectroscopic assays, and (iii) since it is known from the literature that hexa-histidine tagged, purified copper-binding enzymes do not contain significant higher amounts of copper than the same proteins that were purified applying streptavidin tags or of which the hexa-histidine tag was removed (43, 44) it can be concluded that the purified Lcp1_{VH2} possess Cu(II) as transition factor. The divalent copper ions are known to preferably complex with amino acid residues of histidine, serine, threonine, or tyrosine (45). Furthermore, copper sites can be assigned into three groups due to their spectroscopic capabilities. Since the enzyme shows no strong absorption band at 600 nm (typical for type 1 copper centers) or 300 nm (typical for type 3 copper centers) (46, 47) but shows a UV/visible spectrum that is similar to...
that of a non-typical, single copper containing laccase-related enzyme (33), it can be speculated that the Cu(II) center within Lcp1VH2 is of type 2. These centers are mainly found in oxygenases, Cu/Zn superoxide dismutases and copper oxidases that depict no blue color and most commonly ligate Cu(II) by histidine (45, 48). A comparison of more than fifty Lcp-similar sequences taken from publicly accessible databases revealed that the amino acid sequences of Lcp are highly conserved, especially in the area presented in Fig. 7. Within this region all Lcp-sequences of adhesive growing strains but also of clear-zone forming bacteria show three highly conserved histidine residues: H195 and H200, H228 (in Lcp1VH2). It might therefore be possible that these three histidine residues play a substantial role in the coordination of the copper ion as also described for other copper-containing oxygenases, such as the quercetin 2,3-dioxygenase mentioned above (42). However, this assumption has to be verified by further investigations in the future.

The amino acid sequences of both Lcp genes of *G. polyisoprenivorans* strain VH2 are very similar and show over 60% of identity. Both possess like all Lcp sequences a twin-arginine signal peptide and the DUF2236 domain. Since a deletion of only one *lcp* within the genome of strain VH2 did not result in a mutant that lost its ability to grow on rubber as sole source of carbon and energy whereas a double deletion mutant exhibited a total loss of this ability (14), it is likely that Lcp2VH2 also cleaves rubber by incorporation of oxygen in a similar manner.

The conserved region presented in Fig. 7 is located within the conserved domain of unknown function that is detectable in all Lcp sequences. In Lcp1VH2 this domain covers more than 50% of the amino acid sequence. However, not all DUF2236-domain containing proteins can be annotated as Lcp by similarity but the domain can be detected in 541 species of Eubacteria (mainly Actinobacteria and Proteobacteria) and 113 species of Eukaryota (mainly Ascomycota) (Pfam version 27.0; 20). All these phyla are known for their high degradative
capabilities, in particular for the degradation of hardly degradable polymers. Since lcp1VH2 encodes for an oxygenase, this domain might be detectable in a new family of oxygenases. However, this statement requires further experiments in the future.

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Table 1. Inhibitory compounds of Lcp1VH2.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Concentration [mM]</th>
<th>Relative activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard assay without added compound</td>
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<td>100</td>
</tr>
<tr>
<td><strong>Chelators</strong></td>
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</tr>
<tr>
<td>Ethylenediaminetetraacetic acid</td>
<td>10</td>
<td>108</td>
</tr>
<tr>
<td>Tiron</td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
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<td>53</td>
</tr>
<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>2,2-Bipyridyl</td>
<td>10</td>
<td>65</td>
</tr>
<tr>
<td>Ethylxanthate</td>
<td>0.2</td>
<td>70</td>
</tr>
<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td><strong>Alkylation reagent</strong></td>
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<tr>
<td>N-Ethylmaleimid</td>
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<td>101</td>
</tr>
<tr>
<td></td>
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<td>70</td>
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<tr>
<td><strong>Reducing agent</strong></td>
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</tr>
<tr>
<td>2-Mercaptoethanol</td>
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<td>17</td>
</tr>
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</table>
LEGENDS TO FIGURES

FIG. 1. Purification of Lcp1VH2 applying an immobilized metal-affinity chromatography step (lane 3) and subsequent size-exclusion chromatography (lane 4) as analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10 µg of protein were applied in lane 3 and 4). In lane 1 and 2: 40 µg of protein of soluble fraction of the control (E. coli C41(DE3) containing pET23a(+) (lane 1) as well as of E. coli C41(DE3) containing pET23a(+):his/lcp1VH2 (lane 2); both induced with isopropyl β-D-1-thiogalactopyranoside at an OD600 of 0.4. Marker proteins (M).

FIG. 2. Measurements of Lcp1VH2–driven oxygen consumption. Analyses were carried out in presence of 20 µl/ml latex suspension and 5 µg/ml of Lcp1VH2 in Bis-Tris buffer (200 mM, pH 7.0). All components for the negative controls were added as indicated in the box with the concentrations mentioned above. All measurements were carried out at 23°C at a stirring rate of 200 rpm. Lcp1VH2 was inactivated by heat.

FIG. 3. FTIR absorbance spectrum of the second product fraction after incubation of latex milk with active Lcp1VH2 in comparison to the spectrum of latex of the negative control. From a latex emulsion containing 20% (vol/vol) latex, 50 µl/ml were mixed with 15 µg/ml of purified protein (negative control: heat inactivated enzyme) in Bis-Tris buffer (200 mM, pH 7.0) and incubated for 20 h at 30°C on a rotary shaker (150 rpm).
FIG. 4. ¹H-NMR spectrum of the second fraction. For clearance parts of the spectrum have been omitted. Symbols: s, singlet; d, doublet; t, triplet; td, triplet of doublet; m, multiplet; Me, methyl.

FIG. 5. Temperature (A) and pH optima (B) of purified Lcp1 VH2. 20 µl/ml latex suspension was incubated with 5 µg/ml of purified Lcp1 VH2 and oxygen consumption was measured. For determination of the temperature optimum Bis-Tris buffer (200 mM, pH 7.0) was chosen. For evaluation of the pH optimum of the enzyme Bis-Tris ▲, PIPES ● and Tris ■ (each 200 mM) was applied with corresponding pH values at 23°C.

FIG. 6. UV/visible spectrum of purified Lcp1 VH2 (A) and results of spectroscopic assay for determination of the oxidation state of copper within Lcp1 VH2 applying bathocuproine sulfonate (BCS; B). A and B: buffer of all samples was 20 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.4). B: All samples contained 50 µM Lcp1 VH2 and 1% (w/v) SDS. The black solid line of B represents the absorption of all components including 20 mM EDTA, but excluding BCS. Light grey and broken line: sample contained 500 µM BCS. Dark grey and broken line: Sample contained 500 µM BCS and 500 µM ascorbate. Light grey and solid line: sample contained 20 mM EDTA and 500 µM BCS.

FIG. 7. Alignment of the most conserved segment within Lcp sequences of actinomycetes showing adhesive growth on rubber (strains of Gordonia and Nocardia) as well as of strains forming clear-zones on latex overlay plates (strains of Streptomyces). Highly conserved histidine amino acids are marked with arrows.