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Purification and Characterisation of an Inhibitor of Rubber Biosynthesis from C-serum of Hevea brasiliensis *Latex*

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A proteinaceous inhibitor of rubber biosynthesis was purified from the C-serum of Hevea brasiliensis *latex The protein inhibited the incorporation of isopentenyl diphosphate into rubber Purification was achieved by employing three column chromatography methods. Sephadex G-150® gel-filtration, DEAE-Cellulose® ion exchange chromatography and Phenyl Sepharose CL-4B® hydrophobic interaction chromatography The inhibitor makes up 0.3% of the total protein in the C-serum solids and was shown to have a molecular weight of 43 700 Da by mass spectrometry The protein was blocked at the N-terminal Amino acid sequence of peptide fragments obtained from CNBr, endoproteinase Lysine-C and trypsin digestions showed this protein to have regions of sequence similar to patatin, a protein that constitutes approximately 40% of the total protein present in mature potato tubers (*Solanum tuberosum*) Investigations showed that, like patatin, the inhibitor has lipolytic acyl hydrolase (LAH) activity Based on these observations, it is thought that the inhibitory effect is due to the destruction by LAH of the integrity of the rubber particle membrane in which the biosynthetic enzymes are thought to be embedded*

Rubber, or *cis*-1,4-polyisoprene, is formed in over 1800 species of plants distributed amongst 300 genera of seven families[1]. To date, rubber is produced commercially only from *Hevea brasiliensis* In *Hevea*, a rubber molecule is made up of 1500 to 60 000 isoprene monomers enzymatically linked in a head-to-tail configuration giving a product between 100 000 and 4 000 000 Da[2]. The presence of an inhibitor of rubber biosynthesis in C-serum of *Hevea brasiliensis* latex has been demonstrated by Archer and Audley[3]. The inhibitor was characterised to some extent but was not purified to homogeneity. Should this inhibitor have a physiological role, the understanding of its function could have important consequences for controlling and increasing rubber biosynthesis.

This paper describes the purification and characterisation of the inhibitor found in C-serum. A possible mechanism for the inhibition of rubber biosynthesis by the protein is discussed

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MATERIALS AND METHODS

Preparation of C-serum Solids

Latex was collected from *Hevea brasiliensis* RRIM 600 trees tapped ½S d/2 (half spiral, every other day) in a flask chilled in melting ice. It was then centrifuged in a SM-34 rotor in a Sorvall RC-5C for 2 h at 43 000 g (maximum) at 4°C. The latex separated into four main phases; the top-most is the light rubber phase followed by a thin yellowish layer that contains the Frey-Wyssling complexes. The clear C-serum equilibrates in the middle of the tubes, and the heavy and yellowish lutoid fraction at the base[4]. C-serum from all tubes was pooled and centrifuged again at the same speed for an hour to remove further rubber. The resulting, much clearer C-serum, which still contained small rubber particles, was then freeze-dried to give the yellowish C-serum solids (*ca.* 7 g per 100 ml). This material invariably contains small amounts of rubber particles and is highly active in synthesising rubber from isopentenyl diphosphate (IDP)[3]. The activity is retained for many years on storage at –20°C.

Quantitation of Protein

The quantitation of protein in samples was determined by the Lowry method as described in Yeang *et al.*[5] Bovine serum albumin was used as the protein standard.

Rubber Biosynthesis Assay

A rubber biosynthesis assay was developed by Yusof *et al.*[6,7] This was to detect the presence and to monitor the purification of the inhibitor of rubber biosynthesis in the reconstituted C-serum solids. Each 200 µl incubation mixture contained 50 µl of washed rubber particles (WRP), 0.29 nmol (28 750 dpm)[^{14}C]-isopentenyl diphosphate (IDP), 0.3 mM unlabelled IDP, 0.7 mM neryl diphosphate (NDP), 1% Tween-20®, 2 mM $MgSO_4$, 50 mM dithiothreitol (DTT), 120 µl inhibitor-containing fractions and 50 mM Tris-HCl at pH 7.5. Incubations were carried out for 3 h at 25°C in the wells of a microtitre plate. The reaction was stopped by the addition of 30 µl of 0.3 M EDTA and then 10 µl of 1% high ammonia latex (rubber content 1%) was added. Alcian Blue dye solution (50 µl of 1% solution) was then filtered through a Whatman gf/f glass fibre membrane on a Bio-Dot Apparatus® (Bio-Rad), followed by the incubated mixture. The filter membrane was disassembled from the apparatus, placed flat in a container, then heated at 110°C for 10 min, after which it was washed once in 20 ml of 1 M HCl for 15 min, followed by three times in 20 ml of 95% absolute ethanol for 10 min each wash. During each washing, the membrane was agitated on an orbital shaker. After drying at 110°C for about 5 min, the radioactive areas, as indicated by the dye, were cut out, placed in scintillation vials, scintillation fluid was added, and radioactivity determined. Inhibitor activity is reported as % incorporation, with the inhibitor replaced by buffer in the control (100% incorporation).

Polyacrylamide Gel Electrophoresis Analysis

Protein was separated by electrophoresis under native conditions [Native-polyacrylamide gel electrophoresis (Native-PAGE)] according to Ornstein[8] and Davis[9] and under denaturing conditions [sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)] according to Leammli[10]; both with 4% stacking and 15% separating gel. Native-PAGE

was run in the cold room (4°C) for 5 h at 20 mA. The Coomasie brilliant blue R250 staining system was used to visualise the bands.

Molecular Weight Determination

An aliquot of the sample solution (20 µl) was injected onto a *Poros R/H 320*® µm (internal diameter) 5 cm column equilibrated in aqueous 0.1% trifluoroacetic acid (TFA). The salts and buffers were removed by flowing aqueous 0.1% TFA through the column at a flow rate of 40 µl min^{-1}. The protein was eluted by flowing 10% acetonitrile (MeCN) in 0.1% TFA through the column. The detection was carried out at 215 nm 0.500AUFS. An aliquot (0.5 µl) of the collected protein material was spotted onto a stainless steel target pre-coated with α-cyano-4-hydroxy cinnamic acid and analysed by Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectrometry using a VG ToFSpec instrument fitted with a 337 nm nitrogen laser.

Amino Acid Sequence Determination

N-terminal amino acid sequence analysis. An aliquot of the sample solution (300 µl) was loaded onto a hydrophobic sequencing column. Salts and buffers were removed by washing with 2% TFA (2 ml). Three cycles of Edman sequencing chemistry were performed using a Hewlett Packard G1005A protein sequencer.

In-situ *cyanogen bromide digestion.* The sequencing column was removed from the sequencer and placed in the Hewlett Packard G1004B protein chemistry station where an automated cyanogen bromide (CNBr) digestion was performed using 0.25 M CNBr in 70% formic acid for an hour at 60°C. The column was then returned to the sequencer where twenty cycles of Edman sequencing chemistry were performed. A repeat of the CNBr digestion was carried out. An aliquot of the sample solution (600 µl) was loaded onto a pre-conditioned PCS hydrophobic column. All salts and buffers were removed by washing with 2 ml of 2% TFA. The column was placed *in the Hewlett Packard G1004B protein* chemistry station where an automated CNBr was performed using 0.25 M CNBr in 70% formic acid for an hour at 60°C. After that the PCS column was placed in line to a 2.1 mm Aquapore RP-300 C_{18}® reversed phase column *via* an adaptor. Peptide fragments were separated by gradient elution at a flow rate of 200 µl min^{-1} beginning at 20% B (0.1% TFA/MeCN), diluted with solvent A (0.1% TFA/H_2O) to 60% B in 40 min. Detection was at 215 nm 0.500AUFS. Peptide fragments were collected and analysed by MALDI and then sequenced.

In situ *trypsin enzymatic digestion.* An aliquot of 600 µl protein sample was loaded onto a pre-conditioned PCS hydrophobic column. All salts and buffers were removed by washing with 2 ml of 2% TFA. The column was then equilibrated in digestion buffer 1:4 (v/v) 50 mM ammonium bicarbonate (pH 8.3):acetonitrile. Trypsin (2 µg) was added to 1 ml digestion buffer. This solution was allowed to flow through the column, which was then left at room temperature overnight. After this time the PCS hydrophobic column was placed in line to a 2.1 mm Aquapore RP-300 C_{18}® reversed phase column *via* an adaptor. Peptide fragments were separated by gradient elution at a flow rate of 200 µl min^{-1} beginning at 20% B (0.1% TFA/MeCN) and diluted with solvent A (0.1% TFA/H_2O) to 60% of solvent B in 40 min. Detection was at 215 nm

0.300AUFS. Peptide fragments were collected and sequenced.

In situ *endoproteinase lysine-C enzymatic digestion.* An aliquot of 600 µl protein sample was loaded onto a pre-conditioned PCS hydrophobic column. All salts and buffers were removed by washing with 2 ml of 2% TFA. The column was then equilibrated in digestion buffer 1:4 (v/v) 50 mM ammonium bicarbonate (pH 8.3):acetonitrile. Endoproteinase Lysine-C (4 µg) was added to the digestion buffer (400 µl). This solution was allowed to flow through the column and the digest was then incubated at 37°C for 18 h. After this time the PCS column was placed in line to a 2.1 mm Aquapore RP-300 C_{18}® reversed phase column *via* an adaptor. Peptide fragments were separated by gradient elution at a flow rate of 200 µl min^{-1} beginning at 20% of solvent B (0.1% TFA/MeCN) and diluted with solvent A (0.1% TFA/H_2O) to 60% of solvent B in 40 min. Detection was at 215 nm 0.300AUFS. Peptide fragments were collected and sequenced.

Lipolytic Acyl Hydrolase Activity

Lipolytic acyl hydrolase (LAH) activity was assayed following the method of Racusen[11]. The assay was carried out with the purified inhibitor as the source of enzyme and *p*-nitrophenyl palmitate as the substrate. *P*-nitrophenyl palmitate stock solution was prepared by dissolving, with stirring, 28 mg in 100 ml of 1% (w/v) Triton 100-X® plus 1.7 ml of 1% sodium dodecyl sulphate in a boiling water-bath. The solution became quite turbid during heating, but cleared on cooling. The incubation mixture contained 1 ml of 0.1 M Tris-HCl, pH 8.2, 0.5 ml purified inhibitor and 1 ml *p*-nitrophenyl palmitate stock solution. The incubation mixture was incubated in a water-bath for 30 min at 37°C and the reaction stopped by the addition of 1 ml of 1 M NaOH. The product of the incubation, *p*-nitrophenol, was measured by reading the absorbance at 410 nm. The absolute amount of *p*-nitrophenol produced was calculated from a calibration graph constructed with known amounts of *p*-nitrophenol.

RESULTS AND DISCUSSION

Effect of Tween-20® and Initiator Molecules on the Inhibitory Activity

Results showed that the inhibitory effect was more pronounced if Tween-20® is added in the gel-filtration buffer. The inhibitory effect was even more distinct if Tween-20® was added to the rubber biosynthesis assay incubation mixture (see *Table 1*). The role of Tween-20® towards the inhibitory activity is still unclear but in all the detection, 1% Tween-20® was added. Results also showed that the presence of initiator molecules [any of these, neryl diphosphate (NDP), farnesyl diphosphate (FDP), geranyl diphosphate (GDP) or geranyl, geranyl diphosphate (GGDP)] is needed in the incubation mixture for the inhibitory effect to be distinctly observed, confirming the work of Archer and Audley[3] (see *Table 2*). This result shows that the inhibitor only blocks the condensation of IDP to the elongating rubber and not the initiation stage of rubber formation.

Purification of the Inhibitor

The purification of an inhibitor of rubber biosynthesis from C-serum is summarised in *Table 3*. Reconstituted C-serum solids (6 ml of

TABLE 1. EFFECT OF TWEEN-20® (1%) ON INHIBITORY ACTIVITY

	Incorporation (dpm)[a,b] (Control)[c]	Incorporation (dpm)[a,b] (Sample)
No Tween	3 898	2 973
With Tween	2 254	49

[a]Incorporation of [^{14}C]-IDP per ml of incubation mixture
[b]Values are average of duplicate determinations
[c]Incubations where samples (inhibitor) were replaced by buffer

TABLE 2. EFFECT OF INITIATOR MOLECULES, NDP, ON INHIBITORY ACTIVITY

	Incorporation (dpm)[a,b] (Control)[c]	Incorporation (dpm)[a,b] (Sample)
No Initiator molecules	3 128	2 354
With Initiator molecules	4 205	1 894

[a]Incorporation of [^{14}C]-IDP per ml of incubation mixture
[b]Values are average of duplicate determinations
[c]Incubations where samples (inhibitor) were replaced by buffer

25% w/v) was fractionated on a Sephadex G-150® (Pharmacia) column (81 cm × 2.6 cm) equilibrated in 250 mM Tris-HCl, pH 8.0 plus 5 mM 2-mercaptoethanol and 1% Tween-20®. The flow rate was 0.5 ml per min and 5 ml fractions were collected. Inhibitory activity eluted between 130 ml and 200 ml (*Figure 1*). These active fractions were pooled and then dialysed against two liters of 50 mM Tris-HCl, pH 8.0 plus 5 mM 2-mercaptoethanol at 4°C overnight. During dialysis a slight precipitate formed which was removed by centrifugation at 43 000 g for 30 min. The clarified solution was then loaded onto a DEAE-Cellulose® (Whatman) column (20 cm × 1.6 cm), equilibrated in 50 mM Tris-HCl, pH 8.0 plus 5 mM 2-mercaptoethanol at a flow rate of 0.5 ml per min. Once loaded, the column was further developed with another 300 ml of buffer to remove the unbound protein and then with a linear gradient (0 to 0.3 M NaCl in buffer) applied to the column over a volume of 500 ml. Inhibitory activity eluted between 0.15 and 0.2 M NaCl (*Figure 2*). The fractions with highest activity were pooled and dialysed against two liters of 50 mM Tris-HCl, pH 8.0 plus 5 mM 2-mercaptoethanol and 0.5 M NaCl. The sample was then loaded onto a column of Phenyl Sepharose CL-4B® (Pharmacia) (15 cm × 1.6 cm) equilibrated in the same buffer. The flow rate was 0.5 ml per min and 5 ml fractions were collected. Once loaded, a further 150 ml of buffer was run through the column to wash

TABLE 3. PURIFICATION OF INHIBITOR OF RUBBER BIOSYNTHESIS FROM C-SERUM OF *HEVEA BRASILIENSIS* LATEX

Purification	Protein (mg)	Yield (%)
1. C-serum (Freeze-dried, 1.5 g/6 ml, 250 mM Tris-HCl, pH 8.0 + 1% Tween-20® + 5 mM 2-mercaptoethanol)	257.46	100
2. Gel-filtration (*Sephadex G-150*, 250 mM Tris-HCl, pH 8.0 + 1% Tween-20® + 5 mM 2-mercaptoethanol)	60.55	23.35
3. Ion-exchange chromatography (DEAE-Cellulose, 0 to 0.3 M NaCl + 50 mM Tris-HCl, pH 8.0 + 5 mM 2-mercaptoethanol)	2.35	0.91
4. Hydrophobic interaction chromatography (Phenyl Sepharose CL-4B 50 mM Tris-HCl, pH 8.0 + 5 mM 2-mercaptoethanol)	0.77	0.30

the unbound proteins. The eluting buffer was then changed to 50 mM Tris-HCl, pH 8.0 plus 5 mM of 2-mercaptoethanol. The inhibitory activity eluted between 35 ml and 85 ml later (*Figure 3*). The purified inhibitor makes up 0.3% of the total protein of the C-serum solids.

Polyacrylamide Gel Electrophoresis Analysis

The purification steps by column chromatography were usually followed by the analysis of protein on SDS- and Native-PAGE. Pooled inhibitor-active fractions from Phenyl Sepharose CL-4B column were concentrated three times by ultrafiltration. The sample was then submitted to 15% SDS-PAGE and stained with Coomasie brilliant blue dye. A band was observed at about 45 kDa as shown by *Figure 4(a)*. However, to confirm that this protein was truly causing the inhibitory effect, samples were run on a non-denaturing (Native) PAGE. A track, out of ten tracks, was Coomasie blue dye stained as shown by *Figure 4(b)*. The rest of the tracks (photograph not shown) were sliced every one centimeter from the top (altogether ten slices), macerated in 50 mM Tris-HCl, pH 8.0 and the supernatant assayed for the presence of inhibitor. Activity was only detected in the fractions corresponding to the band on the gel (see *Table 4*). Control refers to the gel section where no protein was run, only sample buffer.

Validation of Inhibition of Rubber Biosynthesis by Inhibitor by Gel-Filtration

To demonstrate that the inhibitor truly inhibits rubber formation, labelled rubber was purified differently, following the normal rubber biosynthesis incubation procedure. Instead of purifying the incubation mixture (5 × 200 µl) by glass membrane-filtration, the

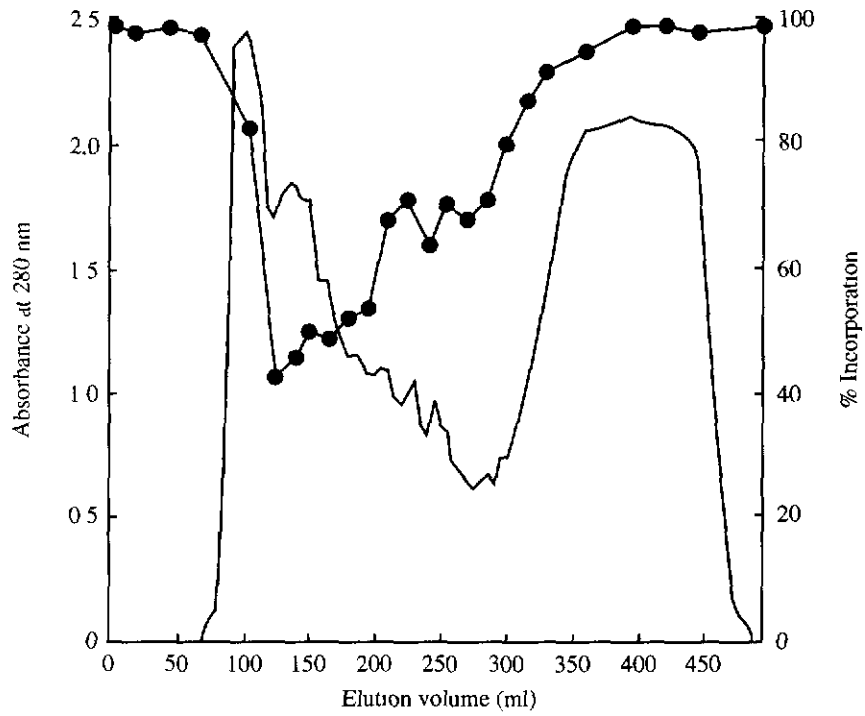

Figure 1 Elution profile of inhibitor of rubber biosynthesis from Sephadex G-150® gel column chromatography (80 × 2 6 cm) Elution buffer, 250 mM Tris-HCl, pH 8 0 plus 5 mM 2-mercaptoethanol and 1% Tween-20®.

incubated mixture was fractionated by gel-filtration on Sephadex G-150® column (50 cm × 1.6 cm). The sample was eluted with 250 mM Tris-HCl, pH 8.0. The eluant was collected in 1.5 ml fractions; scintillant was added and counted. The radioactivity readings of the eluted fractions in the absence and presence of inhibitor are shown in *Figure 5*. The presence of two peaks was as expected: Peak 1 was eluted in the void volume and presumably constituted of labelled rubber and Peak 2 constituted mainly of unreacted [^{14}C]-IDP. In the presence of the inhibitor, Peak 1 decreased and this reciprocated with an increase in Peak 2. This experiment confirms that the inhibitor protein does indeed inhibit rubber formation.

Molecular Weight Determination

The purified inhibitor protein, when chromatographed on Poros R/H® (320 μm i.d.), revealed one major peak at 215 nm

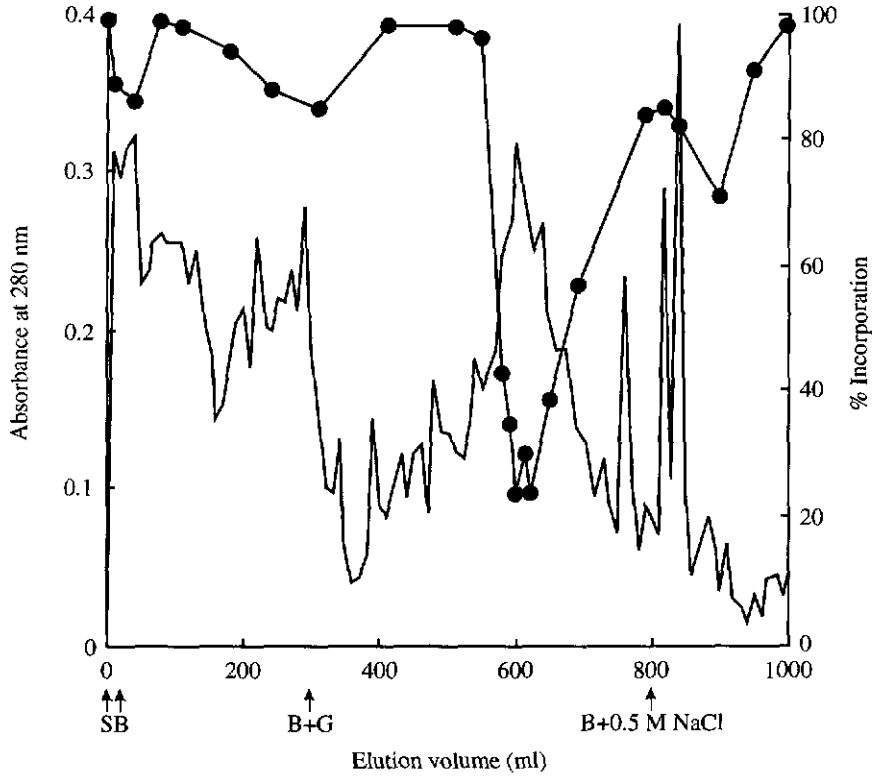

Figure 2. Elution profile of inhibitor of rubber biosynthesis from DEAE-Cellulose ion exchange column chromatography (20 cm x 1.6 cm). Elution buffer, 50 mM Tris-HCl, pH 8.0 plus 2-mercaptoethanol.

0.500AUFS. Eluant of the peak was collected and submitted to mass spectrometry. The MALDI mass spectrum for the peak (*Figure 6*) shows ions at 43.7 kDa, 21.8 kDa, 14.5 kDa and 10.9 kDa. These can be assigned as +1, +2, +3 and +4 molecular ions, all relating to a species with molecular weight of 43.7 kDa. The peaks for the ions were broad suggesting microheterogeneity due to post translational modifications.

Amino Acid Sequencing

There was no sequence data obtained when protein was submitted to N-terminal sequencing, suggesting that the protein be blocked at the N-terminal. To prove that this was really so, and not because of an absence of protein material in the experiment, *in situ* digestion of protein was then carried out

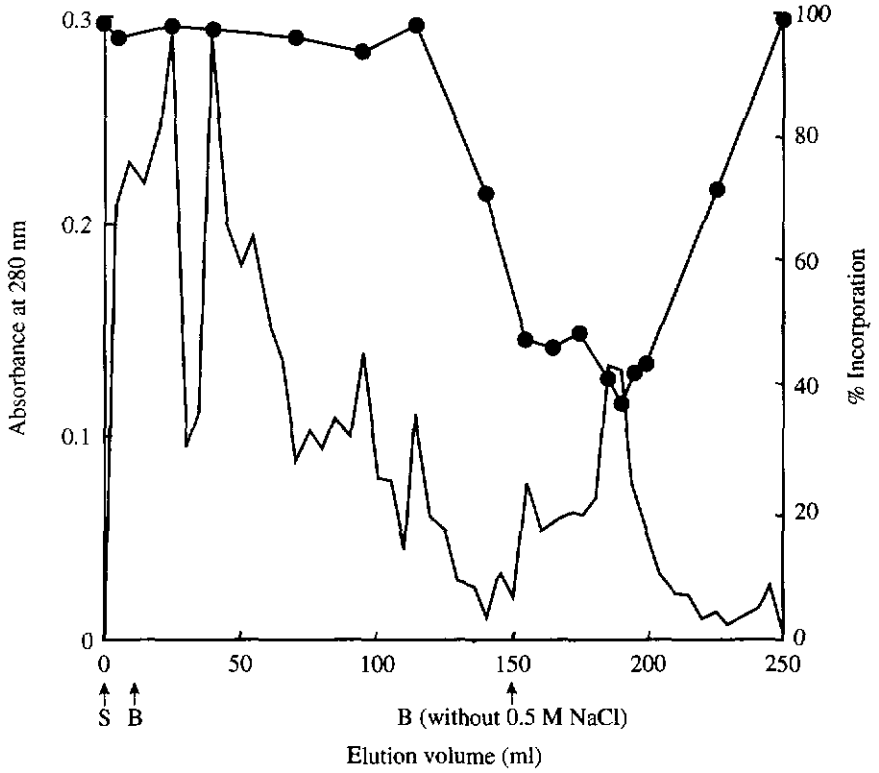

Figure 3. Elution profile of inhibitor of rubber biosynthesis from Phenyl Sepharose CL-4B hydrophobic interaction column chromatography (15 cm x 1.6 cm). Elution buffer, 50 mM Tris-HCl, pH 8.0 plus 0.5 M NaCl and 2-mercaptoethanol.

chemically (CNBr) and enzymatically (trypsin and endoproteinase Lysine-C). The HPLC separations of all digest gave ranges of fragments but only major fragments were subsequently sequenced: Peaks 2 and 3 from CNBr digest, Peaks 13 and 14 from tryptic digest and finally, Peaks 19 and 21 from endoproteinase Lysine-C digest. The summary of the amino acid sequences derived from all fragments is presented in *Table 5*. Only primary calls are reported here. All of these sequences were compared against a protein sequence database (GenBank® Database) using the Basic Local Alignment Search Tool (BLAST) algorithm programme. Result shows that three of the sequences do have regions of sequence similar to patatin, a storage protein in mature potato, *Solanum tuberosum* (*Table 6*). During

TABLE 4. INCORPORATION OF [^{14}C]-IDP INTO RUBBER IN THE PRESENCE OF ELUANT OBTAINED FROM NATIVE-PAGE GEL

Gel slices	Incorporation[a] (dpm) (Control)	Incorporation[a] (dpm) (with inhibitor)
1	557	537
2	537	207
3	567	547
4	540	555
5	550	542
6	545	549
7	559	543
8	541	542
9	549	538
10	539	555

[a]Values are average of duplicate determinations.

TABLE 5. SUMMARY OF AMINO ACID SEQUENCES DERIVED FROM THE PURIFIED INHIBITOR OF RUBBER BIOSYNTHESIS

CNBr digest:	
Peak 2	V D F H L X C L F F X T D X T D A Y X R
Peak 3	A Q F P D I D D F Y L T N A
Trypsin digest:	
Peak 13	Y N A D M I Y D G G G I R G I I X G I I
Peak 14	D N Y D P I H S I G P I Y D G X Y L R
Endoproteinase Lysine-C digest:	
Peak 19	Y N A D M T Y N A D M Y N G A L Y G I X
Peak 21	E I T V L S I D G G G I X G I I X G I I

X = residue not determined.

the digestion, trypsin should cleave behind lysine and arginine and endoproteinase Lysine-C behind lysine, which means those identical peptides, may be expected in digests of protein with these two enzymes. In comparing sequences from peptides obtained from the two enzymes, it is rather certain that Peak 13 of the tryptic digest could have been a mixture of Peak 19 (N-terminus) and Peak 21 (C-terminus) of the endoproteinase lysine-C digest. A very unusual repetitive amino acid sequence, Y N A D M occurs in Peak 19. At this point,

TABLE 6. COMPARISON OF AMINO ACID SEQUENCE DATA FOR PEPTIDES DERIVED FROM THE PURIFIED INHIBITOR OF RUBBER BIOSYNTHESIS WITH SEQUENCES IN PATATIN[14]

Peptides	Amino acid sequences	Similarity
Endo Lys-C	E I T V L S I D G G G I X G I I X G I I	75%
Patatin	M V T V L S I D G G G I K G I I P A I I	
Tryptic	D N Y D P I H S I G P I Y D G X Y L R	38%
Patatin	I F N Y S G S I L G P M Y D G K Y L L	
CNBr	A Q F P D I D D F Y L T N A	35%
Patatin	A A A K D I V P F Y F E R G	

X = residue not determined.

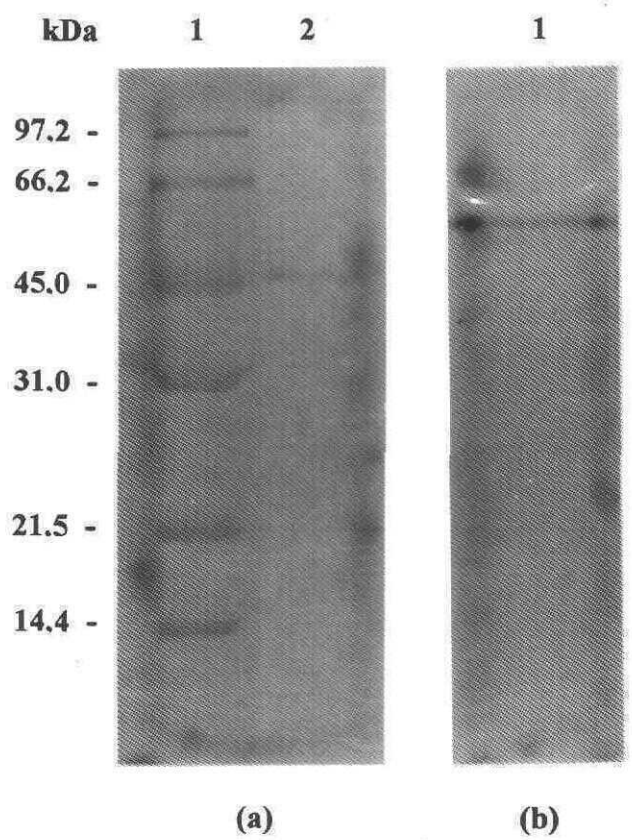

Figure 4. Analysis of the purified inhibitor of rubber biosynthesis by Polyacrylamide Gel Electrophoresis; (a) Purified inhibitor analysed on 15% SDS-PAGE, lane 1, molecular weight markers and lane 2, inhibitor, (b) Purified inhibitor analysed on 15% Native-PAGE on lane 1.

TABLE 7. COMPARISON OF AMINO ACID SEQUENCE OF PATATIN[14], THE INHIBITOR, BEEZHOLD AND SUSSMAN'S 46 AND 110 KDA PROTEINS[12] AND SUBROTO *ET AL.*'S 45 KDA PROTEIN[13]

Peptides	Sequence
Patatin	K L E E M V T V L S I D G G G I K G I I P A I I L
B&S P46	L T Q G K K I T V L S I D X G
B&S P110	L T Q G K K L T V F S I D X X
Inh Pk 21 Lys-C	E I T V L S I D G G G I X G I I X G I I
Inh Pk 13 Try	Y N A D M I Y D G G G I R G I I X G I I
Inh Pk 19 Lys-C	Y N A D M T Y N A D M Y N G A L Y G I X
Patatin	I F N Y S G S I L G P M Y D G K Y L L
Inh Pk 14 Try	D N Y D P I H S I G P I Y D G X Y L R
Patatin	M I T T P N E N N R P F A A A K D I V P F Y F E R G P H I F
Subroto CNBr	L T A P N E D K K P M
B&S P46 CNBr	Y Q A K D E K D F Y L E N C P K I F
Inh Pk 3 CNBr	A Q F P D I D D F Y L T N A

X = residue not determined
Inh = inhibitor
B&S = Beezhold and Sussman

this occurrence cannot be confirmed as real or artifact. However, no such repetitive sequence exists in the amino acid sequence of patatin from potato.

Other authors have also reported the existence of patatin-like protein in *Hevea*[12,13]. Beezhold and Sussman[12] identified two latex allergens with molecular weights of 46 kDa and 110 kDa, respectively. Both of the proteins were reported to have the same N-terminal sequence and the sequences were also found to have some similarity to patatin from potato, *Solanum tuberosum*. These proteins are not blocked at the N-terminal, contrary to the inhibitor. Subroto *et al.*[13] later reported an existence of a latex protein with a molecular weight of 43 kDa, which was also having some similarity to patatin. The source of the protein was from the lutoid-body fraction of the *Hevea* latex or the bottom fraction. The protein was, however, blocked at the N-terminus. An internal CNBr peptide (total of 29 residues) was reported to have 60% similarity to sequences of patatin. *Table 7* demonstrated the comparison of amino acid sequences of patatin (*Solanum tuberosum*[14]) and the various reported patatin-like latex proteins; the inhibitor, the proteins reported by Beezhold and Sussman[12] and the protein reported by Subroto *et al.*[13] Although these patatin-like proteins were all derived from *Hevea*, none of the amino acid sequences matches patatin or each other completely. This suggests that there may be several forms of patatin-like protein in *Hevea brasiliensis*.

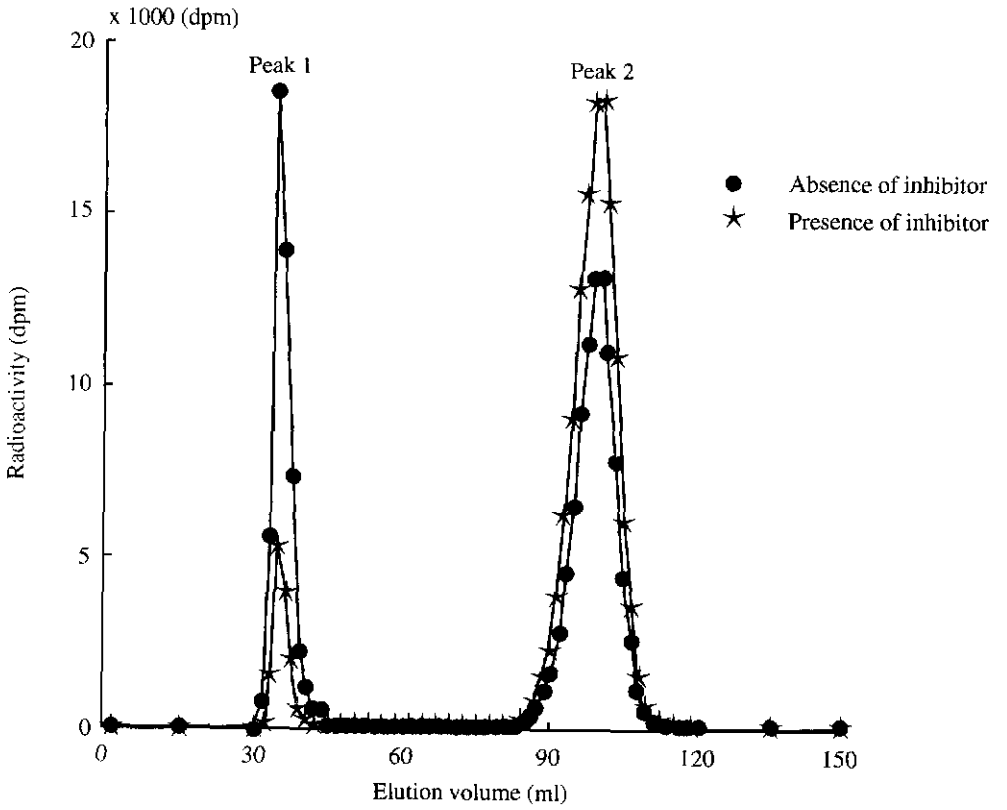

Figure 5. The effect of inhibitor protein on rubber biosynthesis demonstrated by gel-filtration on Sephadex G-150® column (50 × 1.6 cm). The elution profile of labelled products [Radioactivity (dpm)] from the column is shown. Peak 1 = newly formed rubber, Peak 2 = unreacted [1-^{14}C]-IDP.

Lipolytic Acyl Hydrolase Activity

Lipolytic acyl hydrolase (LAH) activity assay of the purified inhibitor was measured according to the conditions given in Materials and Methods. The results of the analysis showed that the inhibitor has LAH activity, with a specific activity of 0.6 μmoles of *p*-nitrophenyl palmitate hydrolysed per milligram of protein per min. LAH activity was not observed if the inhibitor was inactivated by boiling for 5 min or if the incubation was stopped immediately, *i.e.*, zero time incubation.

CONCLUSION

A proteinaceous inhibitor of rubber biosynthesis was purified from the C-serum and partially characterised. The inhibitor was shown by mass spectroscopy to have a molecular weight of

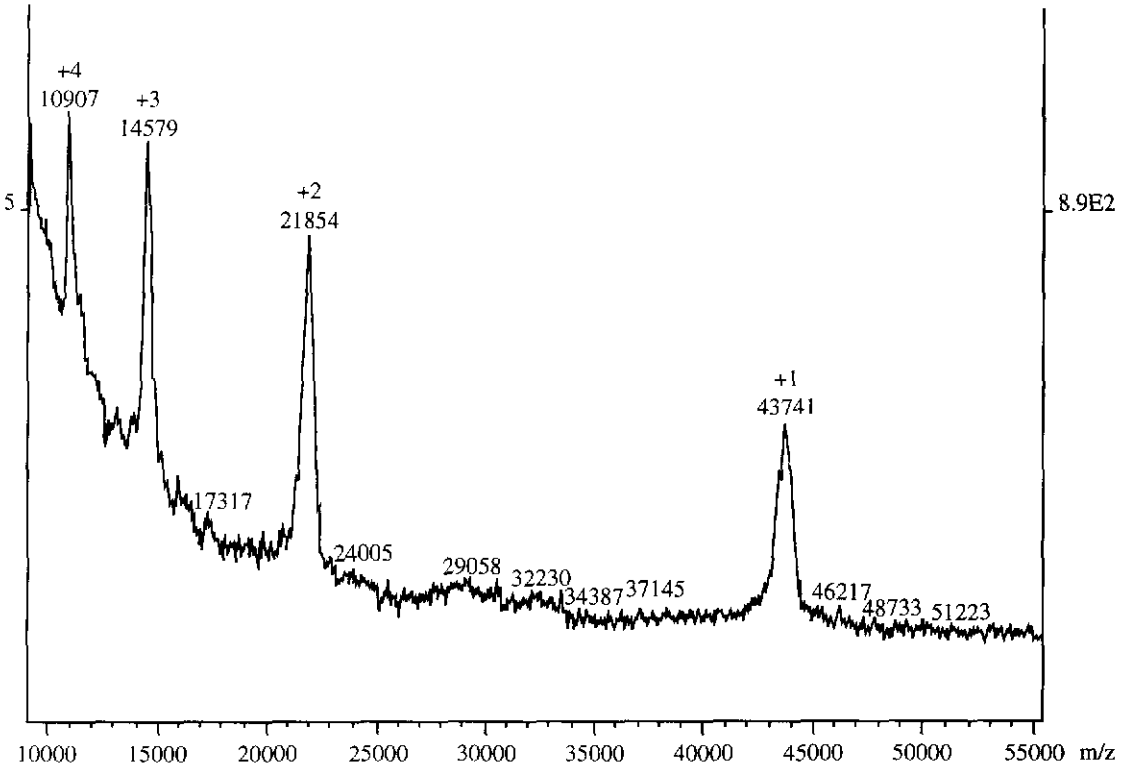

Figure 6. Matrix Assisted Laser Desorption Ionisation mass spectrum of inhibitor of rubber biosynthesis.

43 700 Da. The protein was submitted to amino acid sequencing. Edman degradation *via* the N-terminal was not successful which indicates that the terminal is blocked. The protein was chemically (CNBr digestion) and enzymatically (trypsin and endoproteinase Lysine-C digestions) fragmented. The fragments when sequenced, showed strong similarity to patatin[14] (*Table 6*), a protein which constitutes approximately 40% of the total protein present in mature potato tubers (*Solanum tuberosum*)[15]. Patatin in potato may have a dual role as a somatic storage protein and as an enzyme involved in host resistance.

It is known that patatin has LAH activity[11]; an activity which is thought to be involved in the protection of tubers against pathogens. The inhibitor from *Hevea* was also demonstrated to have LAH activity with a specific activity of 0.6 μmoles of *p*-nitrophenyl palmitate hydrolysed per milligram of protein per min.

For comparison, the specific activity of LAH determined by Galliard and Dennis[16] in three different potato varieties, using the same substrate, were 0.012, 3.3 and 4.4 μmoles of *p*-nitrophenyl palmitate hydrolysed per milligram of protein per min. Based on the observation, it is thought that the inhibitory effect may be due to the destruction by LAH of the integrity of the rubber particle membranes[17–19] in which the biosynthesis enzymes are thought to be embedded[20,21]. This event would lead to the cessation of IDP condensation onto the elongation rubber molecules, thus inhibiting rubber biosynthesis. The polar lipid backbone that makes up part of the rubber particle membrane[17–19] is the most likely target of the LAH activity.

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