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A Stimulator Protein of Rubber Biosynthesis from Hevea brasiliensis *Latex*

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A rubber biosynthesis stimulator protein (RBSP) from the C-serum of Hevea brasiliensis *latex was purified and characterised. This proteinaceous factor stimulates the incorporation of isopentenyl diphosphate into rubber and thereby enhances the rate of rubber biosynthesis. The heat stable RBSP is shown to have a molecular weight of 13 068 Da by mass spectroscopy. Amino acid sequence of peptide fragments obtained from trypsin digestion revealed regions of sequence similar to eukaryotic Initiation Factor 5A (eIF-5A), present in alfalfa (*Medicago sativa*), tobacco (*Nicotiana tabacum *and* Nicotiana plumbaginifolia*), *Arabidopsis thaliana *and potato (*Solanum tuberosum*). Three* Hevea *eIF-5A clones were obtained from screening a latex cDNA library with a partial eIF-5A cDNA from tobacco. Small variations in their DNA sequences indicated that they represented three different isoforms.*

Hevea brasiliensis (Muell. Arg.) is presently the world's sole commercial source of natural rubber (*cis*-1,4-polyisoprene). Rubber is contained in the rubber particles found in the latex. In *Hevea*, latex is produced and stored in specialised cells called laticifers or latex vessels, located in the phloem in the bark of the tree[1]. These latex vessels are derived from the cambium and are arranged as concentric rings in the bark[1]. Between the vessels in each ring, there are anastomoses that allow withdrawl of latex from a large area of bark by means of a single tapping[1]. Rubber represents 30% to 50% by weight of the latex exuded by mature trees in regular tappings and constitutes more than 90% of the total latex solid. The yield of rubber that can be obtained from the rubber tree has always been an important factor to the growers. In the past, the yield of rubber has been increased by the combined application of selection and breeding, flow stimulation, crop extraction and agronomic practices. The yield will, no doubt, be increased further in the coming years but the stage will eventually be reached when the rate of formation of rubber within the tree becomes the limiting factor. At this point, any treatment that can be devised to increase the rate of rubber biosynthesis will prove most valuable. A thorough knowledge of all aspects related to the rubber biosynthesis is an essential prerequisite for progress in this direction.

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Rubber biosynthesis has been studied extensively especially in *Hevea brasiliensis*[2–5] and to a small extent in other natural rubber producing species such as *Parthenium argentatum* (guayule)[6,7] and *Ficus elastica*[8]. Rubber biosynthesis requires four distinct biochemical processes:

- The formation of isopentenyl diphosphate (IDP), the monomer which polymerises to produce *cis*-rubber;
- The initiation of rubber biosynthesis which refers to the production of dimethylallyl diphosphate (DMADP) followed by the production of other initiator molecules, such as geranyl diphosphate (GDP), farnesyl diphosphate (FDP) and geranyl geranyl diphosphate (GGDP);
- The propagation of rubber biosynthesis, achieved by the successive addition of IDP to initiator molecules to produce *cis*-rubber, and
- The termination of rubber biosynthesis which refers to the release of rubber from the enzyme rubber transferase.

Three mechanisms have been postulated for which rubber biosynthesis may have taken place *in vivo* and *in vitro*[9]. These mechanisms may operate alone, any two or three simultaneously. The mechanisms are:

- A chain extension step of a pre-existing rubber diphosphate chains by the addition of IDP. Although this step of rubber biosynthesis has not been proven to happen *in vitro*, it would be expected to happen, especially *in vivo*.
- *A de novo* synthesis of rubber molecules on washed rubber particles (WRP) with the IDP isomerase and *trans* prenyl transferase still present in the system. In this case, the initiator molecules, such as DMADP, GDP, FDP and GGDP can then be produced by the system. A new rubber chain is therefore made without added initiator molecules.
- *A de novo* synthesis of rubber molecules with added initiator molecules. In rubber biosynthesis, use of initiator molecules such as neryl diphosphate (NDP)/GDP, FDP and GGDP, has been shown *in vitro*[4–6,8].

By ultracentrifugation, fresh latex separates into four main phases, *i.e.* at the top, the rubber phase, below it, a thin yellowish layer containing the Frey-Wyssling complexes, followed by C-serum in the middle and at the base, the heavy bottom fraction[10]. Proteins extracted from the various fractions have been found to function in rubber biosynthesis[5,11–15]. Among the proteins studied thus far are the two rubber-particle bound proteins; the 14.6 kDa rubber elongation factor (REF)[11] and the 22.4 kDa small rubber particle protein (SRPP)[12]. In addition, two C-serum proteins, which were reported to have some role in rubber biosynthesis are the 43.7 kDa patatin-like protein, which is also a rubber biosynthesis inhibitor (RBI)[13] and the FDP synthase[5], a dimeric enzyme with a monomeric molecular mass of 38 kDa responsible for the production of an initiator molecule, FDP. A 45 kDa enzyme, 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR), from the bottom fraction, considered as the key rate-limiting enzyme in the rubber biosynthesis pathway has also been isolated and studied[14,15].

In 1987, Archer and Audley detected the presence of a proteinaceous factor in C-serum of centrifuged latex that seemed to stimulate rubber biosynthesis[4]. This stimulatory factor increased the rate of the *in vitro* uptake of IDP into rubber. In view of the potential of factors enhancing rubber biosynthesis, it is thus very

important to isolate the proteinaceous factor reported by Archer and Audley[4]. This paper describes the purification and characterisation of a stimulatory protein from the C-serum, which we have named as the 'rubber biosynthesis stimulator protein' or RBSP. Cloning of the corresponding *Hevea* cDNA clones from a previously constructed latex cDNA library is also presented.

MATERIALS AND METHODS

Preparation of C-serum Solids

RBSP was purified from C-serum solids. C-serum solids were prepared from centrifuged latex as described in Yusof *et al.*[16] Latex was collected from *Hevea brasiliensis* RRIM 600 trees tapped $^1/_2$S d/2 (half spiral, every other day) in a flask chilled in melting ice. It was then centrifuged in a Sorvall RC-5C in SM-34 rotor for two h at 43 000 g (maximum) at 4°C. The latex separated into four main phases: the topmost is the light rubber phase followed by a thin yellowish layer containing the Frey-Wyssling complexes; the clear C-serum in the middle and the heavy yellowish lutoid fraction at the base[10]. 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 IDP. The activity is retained for many years on storage at –20°C.

Protein Quantification

During the purification of the RBSP, quantification of protein in samples was usually carried out. The quantification of protein was determined by the Lowry method as described in Yeang *et al.*[17] Bovine serum albumin was used as the protein standard.

Rubber Biosynthesis Assay

The rubber biosynthesis assay described by Yusof *et al.*[16] was used to detect the presence and to monitor the purification of the RBSP in C-serum solids. Initiator molecule of rubber biosynthesis, *e.g.* FDP, used in the routine assay was omitted (see for reference in *Table 1*). RBSP activity is always reported as % stimulation, with the RBSP replaced by buffer in the control (0% stimulation).

Polyacrylamide Gel Electrophoresis Analysis

To monitor the purification of RBSP, the fractions obtained from each column chromatography were submitted to polyacrylamide gel electrophoresis (PAGE). Proteins were separated by electrophoresis under denaturing conditions (sodium dodecyl sulphate-PAGE or SDS-PAGE)[18] with 4% stacking and 15% separating gel. The Coomasie Brilliant Blue R250 staining system was used to visualise the bands.

Molecular Weight Determination

An aliquot of the RBSP solution (20 μL) was injected onto a Poros R/H 320 μm (i.d.) 5 cm column equilibrated in aqueous 0.1% trifluoroacetic acid (TFA). The salts and buffers were removed by flowing an aqueous 0.1% TFA through the column at a flow rate of 40 μL per min. The protein was eluted by flowing 10% acetonitrile (MeCN) in 0.1% TFA through the column. The detection was carried out at 215 nm. 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.

N-Terminal Amino Acid Sequencing

An aliquot of the RBSP 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 Trypsin Enzymatic Digestion

An aliquot of 600 μL RBSP solution 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 NH_4HCO_3 (pH 8.3):MeCN. The enzyme trypsin (2 μg) was added to a 1 mL digestion buffer. This solution was allowed to flow through the column, which was then left overnight at room temperature, after which 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 per min beginning at 20% B (0.1% TFA/MeCN) and diluted with solvent A (0.1% TFA/H_2O) to 60% B in 40 min. Detection was at 215 nm. Peptide fragments were collected and sequenced.

cDNA Library Screening

Plaque lifts were prepared from 15 cm diameter plates containing clones from a λgt10 latex cDNA library[19] according to Sambrook *et al.*[20] A tobacco eIF-5A partial cDNA, NeIF-5A1[21], was DIG-labeled and hybridised with the plaque lift overnight at 40°C using DIG Easy Hyb buffer (Roche Molecular Biochemicals, Germany). Membrane washing and colorimetric detection using NBT/BCIP substrate solution were all performed according to manufacturer's instructions (Roche Molecular Biochemicals, Germany).

Analysis of *Hevea* eIF-5A cDNAs

Phage DNA isolation was performed using a commercial kit (Promega, USA). Restriction endonuclease digestion, subcloning of cDNAs and other DNA manipulations were carried out according to Sambrook *et al.*[20] The Centre for Gene Analysis and Technology (CGAT), National University of Malaysia (UKM), Bangi, performed DNA sequencing. Sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) algorithm against available databases (GenBank® Database at the NCBI). Nucleic acid and amino acid sequence alignments were carried out using DNASIS Version 2.5 software (Hitachi, USA).

RESULTS AND DISCUSSION

Effect of Initiator Molecules on the Activity of RBSP

The rubber biosynthesis assay[16] which was used to measure the uptake of IDP into rubber, has always included initiator molecule, *e.g.* FDP, as it has been shown to be important for further condensation of IDP into rubber molecules[4–8, 22–24]. However in the study of the RBSP, result shows that the stimulatory activity was only observed distinctly, 38% Activation

compared to 6%, in the absence of initiator molecules (*Table 1*). The differences in readings were calculated to be statistically significant (P = 0.0017). As a result of this observation, allylic initiator molecule was omitted in the incubation assay mixture throughout the RBSP study.

Purification of the RBPS

The RBSP is purified from C-serum solids and the protocol involved during the purification is summarised in *Table 2*. Purification was carried out at 4°C. About 1.5 g C-serum solids were reconstituted in 6 mL of 250 mM Tris-HCl, pH 8.0 plus 5 mM 2-mercaptoethanol to give a solution of 25% (w/v) concentration. The sample was loaded onto a previously equilibrated (with the same Tris-HCl buffer) Sephadex® G-150 (Pharmacia) column (80 cm × 2.6 cm) and the same buffer was used to elute the protein. *Figure 1* shows the elution profile of the RBSP from the Sephadex® G-150 column. RBSP-active fraction was eluted between 250 mL and 375 mL elution volume. The fractions were then pooled

TABLE 1. EFFECT OF INITIATOR MOLECULES (FDP) ON THE ACTIVITY OF RBSP

Samples	Incorporation (dpm)[a]	
	Without FDP	With FDP
Without RBSP	3128 ± 105	4205 ± 130
With RBSP	4326 ± 128	4486 ± 132
% Activation[b]	38	6

[a]Incorporation of [^{14}C]-IDP per mL of incubation mixture
[b]% Activation, assuming values without RBSP as no or 0% Activation

TABLE 2. PURIFICATION OF RBSP FROM C-SERUM OF *HEVEA BRASILIENSIS* LATEX

Steps	Purification
Step 1	C-serum solids, (freeze-dried,m 1.5g/6 ml, 250 mM Tris-HCl, pH 8.0 + 5 mM 2-mercaptoethanol)
Step 2	Gel-filtration, (Sephadex® G-150, 250 mM Tris-HCl, pH 8.0 + 5 mM 2-mercaptoethanol)
Step 3	Ion-exchange chromatography (DEAE-Cellulose®, 0 to 0.3 NaCl + 50 mM Tris-HCl, pH 8 + 5 mM 2-mercaptoethanol)
Step 4	hydrophobic interaction chromatography (Hexyl Agarose®, 50 mM Tris-HCl, pH 8 + 0.5 M NaCl + 5 mM 2-mercaptoethanol)
Step 5	Ion-exchange chromatography (CM-Cellulose®, 50 mM sodium acetate buffer, pH 5.0 + 5 mM 2-mercaptoethanol)

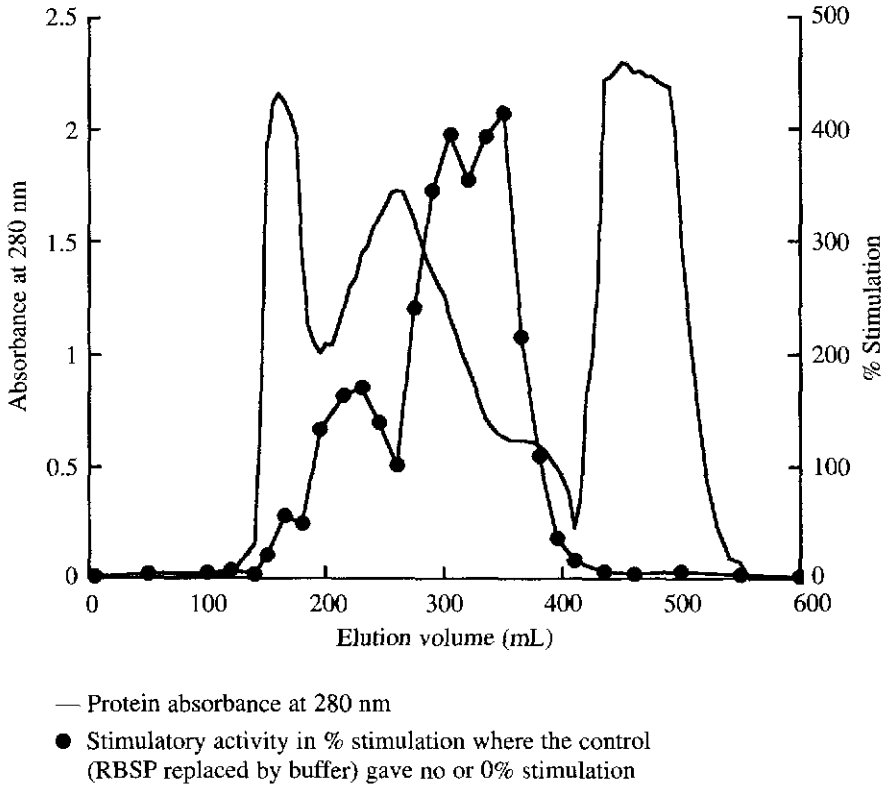

Figure 1. Elution profile of RBSP from Sephadex® G-150 gel column chromatography (80 × 2.6 cm). Elution buffer, 250 mM Tris-HCl pH 8.0 plus 5mM 2-mercaptoethanol. RBSP active fraction was eluted between 250 mL and 375 mL.

and dialysed against 50 mM Tris-HCl, pH 8.0 + 5 mM 2-mercaptoethanol at 4°C overnight and then loaded onto a previously equilibrated (with the same Tris-HCl buffer) DEAE-Cellulose® (Whatman) column (15 cm × 1.6 cm). The same buffer was used to elute the unbound proteins. When the absorbance of the fractions at 280 nm had returned to zero, bound protein was eluted with the same Tris-HCl buffer containing a linear NaCl gradient of 0 M to 0.3 M, over a volume of 500 mL. *Figure 2* shows the elution profile of the RBSP from the DEAE-Cellulose® column. RBSP-active fractions (eluted between 0.11 M to 0.17 M NaCl or between 440 mL to 530 mL) were pooled and dialysed against 50 mM Tris-HCl, pH 8.0 + 0.5 M NaCl + 5 mM 2-mercaptoethanol at 4°C overnight. The sample was then concentrated (five times) by ultrafiltration (Amicon, 3000 Da MWCO) and then loaded onto a previously equilibrated (with the same Tris-HCl buffer) Hexyl Agarose® (Pharmacia) column (10 cm × 1.6 cm). Similar buffer was used to elute the unbound protein. When the absorbance at 280 nm had returned to zero, the same Tris-HCl buffer without NaCl was run through to elute the bound proteins. Only traces of bound proteins were eluted. *Figure 3* shows the elution profile of RBSP from Hexyl Agarose®

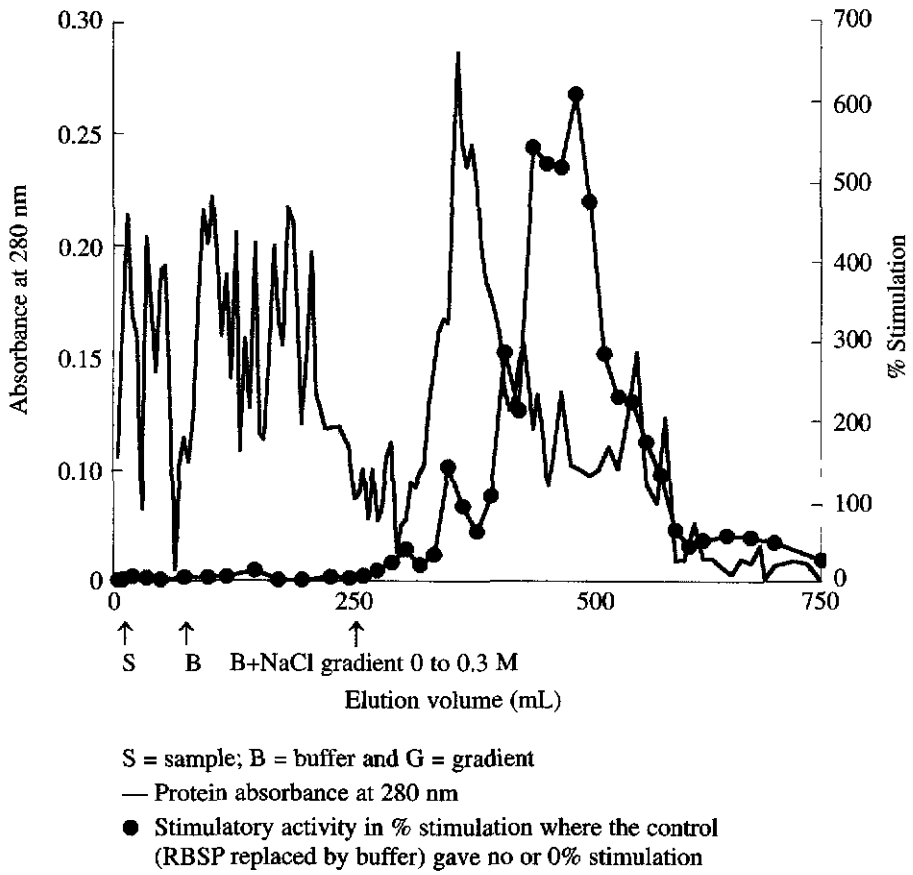

Figure 2. Elution profile of RBSP from DEAE-Cellulose® ion-exchange column chromatography (20 cm × 1.6 cm). The unbound proteins were eluted with 250 mL 50 mM Tris-HCl pH 8.0 plus 5 mM 2-mercaptoethanol and the bound proteins were eluted with a linear NaCl gradient of 0 M to 0.3 M (250 mL each) in the same buffer (between 250 mL and 750 mL elution). RBSP active fraction was eluted between 0.11 M to 0.17 M NaCl gradient or 440 mL and 530 mL.

column. RBSP-active fractions were eluted between 20 mL and 55 mL in the unbound protein fractions. The fractions were pooled and dialysed against 50 mM sodium acetate buffer, pH 5.0 + 5 mM 2-mercaptoethanol at 4°C overnight. On dialysis, a formation of precipitate was observed. The precipitate was removed by centrifugation at 43 000 g at 4°C for 30 min. The clear supernatant was concentrated (five times) by ultrafiltration (3000 MWCO) and loaded onto a previously equilibrated (with the same acetate buffer) CM-Cellulose® (Whatman) column (10 cm × 1.6 cm). When the absorbance at 280 nm had returned to zero, the unbound protein was eluted with the same buffer but this time containing 0.5 M NaCl. Very small traces of bound proteins were eluted. *Figure 4* shows the elution profile of the RBSP from CM-

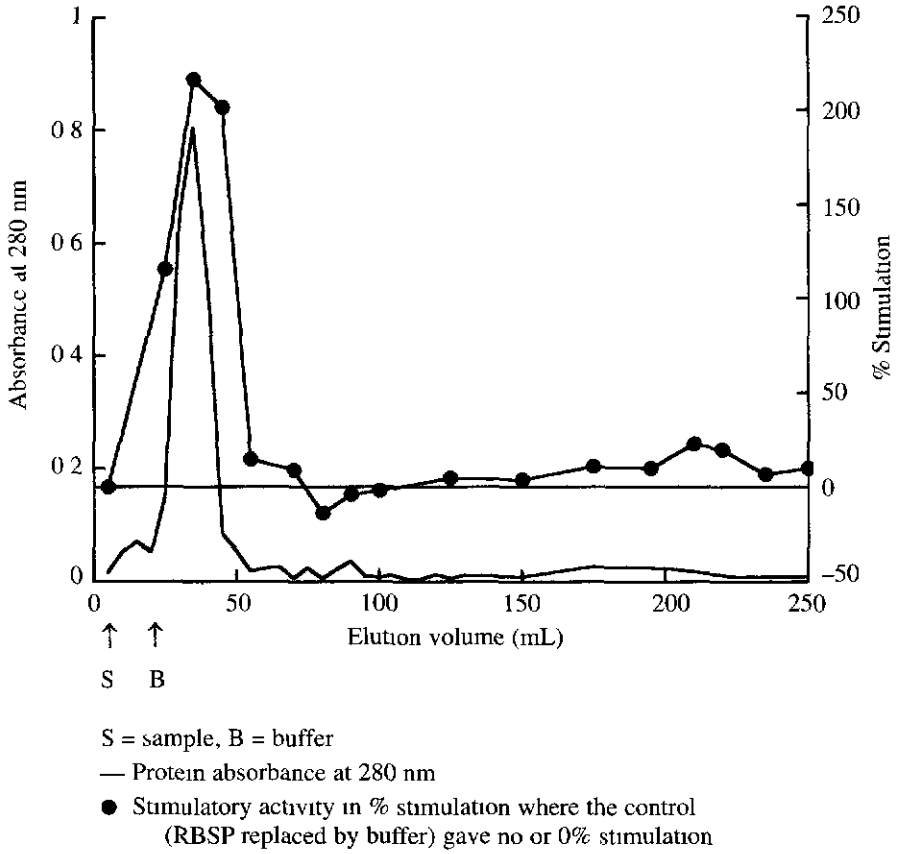

Figure 3 Elution profile of RBSP from Hexyl Agarose® hydrophobic interaction column chromatography (10 cm × 1 6 cm) The unbound proteins were eluted with 250 mL of 50 mM tris-HCl pH 8 0 plus 5 mM 2 mercaptoethanol plus 0 5 M NaCl RBSP active fraction was eluted between 20 mL and 55 mL in the unbound protein fraction

Cellulose® column RBSP activity was detected only in the fractions of the unbound protein, eluting between 45 mL and 70 mL elution volume The RBSP active fraction was pooled, concentrated and then dialysed against 50 mM Tris-HCl, pH 8 0 at 4°C for overnight At the end of the purification steps, only about 0 07% protein was recovered Assay shows the purified RBSP increased the uptake of labelled IDP into rubber (up to 240% increase) and the relationship between the uptake and the sample size is linear over the range tested (*Figure 5*)

Polyacrylamide Gel Electrophoresis Analysis

The purified RBSP obtained from CM-Cellulose® ion-exchange chromatography

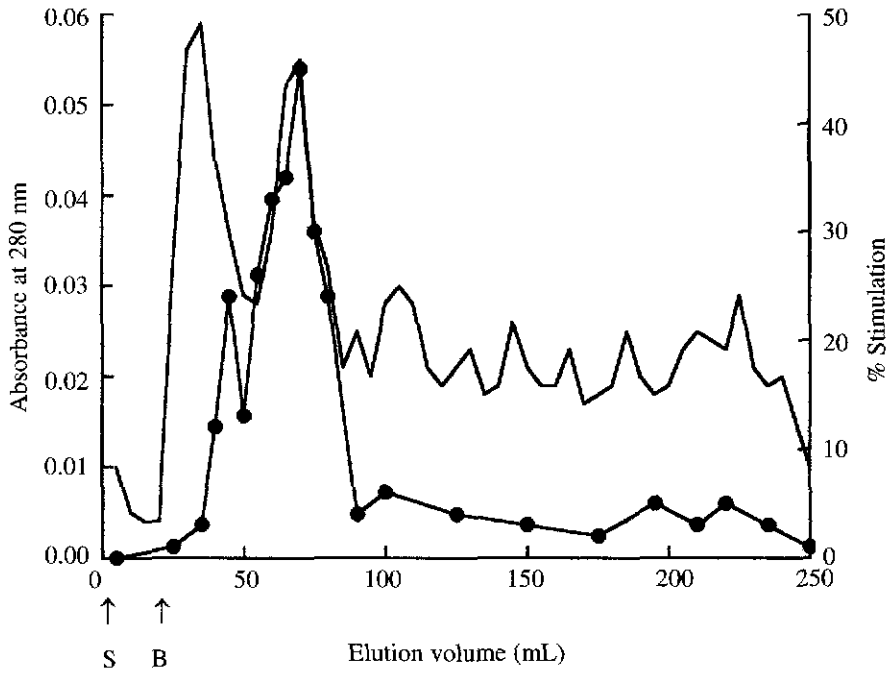

Figure 4. Elution profile of RBSP from CM-Cellulose® ion-exchange column chromatography (10 cm × 1.6 cm). The unbound proteins were eluted with 250 mL of 50 mM Tris-HCl pH 8.0 plus 5 mM 2-mercaptoethanol. RBSP active fraction was eluted between 45 mL and 70 mL of the unbound protein fraction.

showed a single strong band at about 18 kDa on 15% SDS-PAGE (*Figure 6*).

Molecular Weight Determination and Amino Acid Sequencing

By mass spectroscopy, the molecular weight of the RBSP was found to be 13 068 Da. This result is quite different from the value found by SDS-PAGE. At this moment, the reason for the observed differences in molecular weight is still unclear.

The protein was blocked at the N-terminal. *In situ* enzymatic trypsin digestion gave a few fragments of which the two largest ones were sequenced. The amino acid sequence data

obtained was compared against a protein sequence database [GenBank® Database at the National Center for Biotechnology Information (NCBI), U. S. A.] using the BLAST algorithm. The search revealed that the sequences have regions similar to protein eukaryotic Initiation Factor 5A (eIF-5A), found in alfalfa (*Medicago sativa*), tobacco (*Nicotiana tabacum*

TABLE 3. AMINO ACID SEQUENCE DATA OF PEPTIDES DERIVED FROM THE RBSP, COMPARED WITH THE SEQUENCE OF eIF-5A FROM ALFALFA (*MEDICAGO SATIVA*)

Peptides	Amino acid sequences	Similarity
RBSP Tryptic Peptide 1	Q D E H E H F E S K	60%
Alfalfa eIF-5A	S D E E H Q F E S K	
RBSP Tryptic Peptide 2	T Y P Q Q A G T I R	100%
Alfalfa eIF-5A	T Y P Q Q A G T I R	

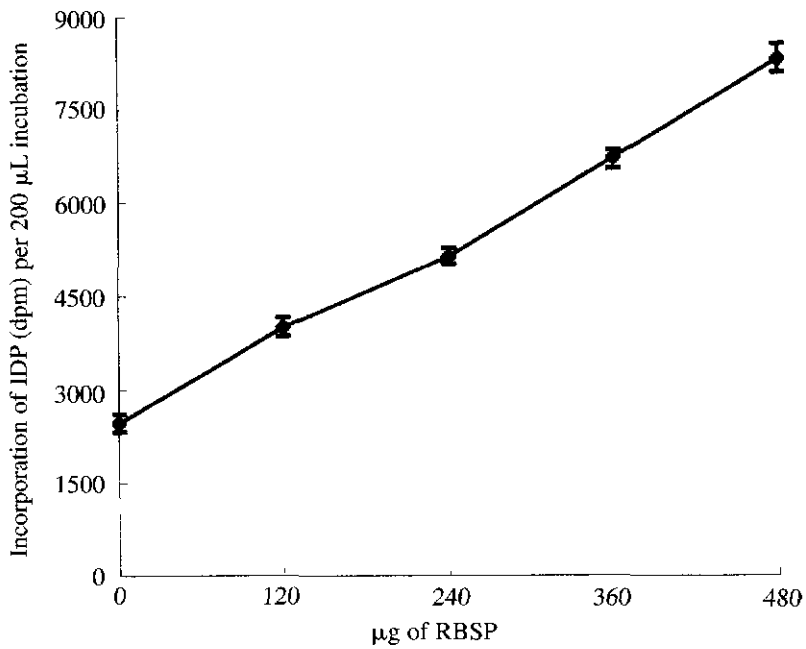

Figure 5. Incorporation of labelled IDP with increasing amount of RBSP in the incubation mixture containing 50 µL WRP suspension, 0.29 nmole (28 750 dpm) [^{14}C]-IDP, 0.3 mM unlabelled IDP, 2 mM $MgSO_4$, mM DTT, 50 mM Tris-HCl, pH 7.5.

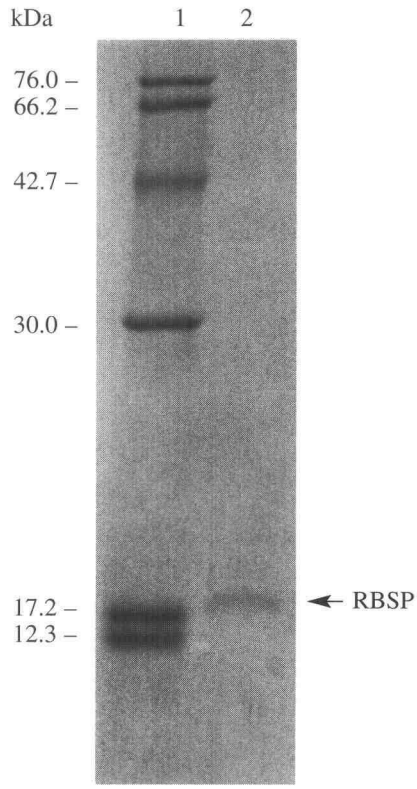

Figure 6. Analysis of the purified RBSP by 15% SDS-Polyacrylamide Gel Electrophoresis; lane 1: molecular weight markers; lane 2: purified RBSP.

and *Nicotiana plumbaginifolia*), *Arabidopsis thaliana* and potato (*Solanum tuberosum*). Both the sequences are located very near towards the N-terminal of eIF-5A sequence. *Table 3* shows the degree of similarity between RBSP tryptic peptide sequences with eIF-5A from alfalfa.

Validation of the Activity of RBSP by Sephadex® G-150 Gel-filtration

To validate the observed increase of radioactive measurement in the presence of RBSP is due to the increased uptake of labelled IDP into rubber, an assay was conducted but instead of purifying the product *via* membrane-filtration, it was filtered through a Sephadex® G-150 column (50 cm × 1.6 cm). A buffer containing 50 mM Tris-HCl, pH 8.0 was used to elute the sample. *Figure 7* shows the elution profile of labelled product from the column in the absence and in the presence of the RBSP. The profile shows the elution of two labelled populations. Peak 1 was eluted at the void volume and presumably constituted of the labelled rubber particles and Peak 2 constituted of mainly unreacted [^{14}C]-IDP. In the presence of the RBSP, Peak 1 increased and this reciprocated with a decrease in Peak 2. This shows that there was an increase in the incorporation of the [^{14}C]-IDP into rubber and at the same time more of [^{14}C]-IDP was used. These findings strongly indicate that the RBSP does indeed stimulate rubber synthesis, validating results obtained by membrane-filtration.

Purification of Labelled Rubber by Bio-Beads® S-X1 Gel-filtration

To eliminate the possibility of any binding of [^{14}C]-IDP to protein on the rubber particles or elsewhere, which might behave as ^{14}C-rubber, assay in the presence of the RBSP was carried out up to the filtration of product through the glass fibre membrane. The product on the membrane instead of being prepared for scintillation counting was dissolved in toluene overnight, and then filtered through a previously equilibrated (with toluene) column of Bio-Beads® S-X1 (50 cm × 1.6 cm), following the method by Audley and Archer[22]. The elution profile demonstrated by *Figure 8* shows that the labelled population was eluted at the void volume where rubber would be expected to elute. Since it was the only labelled population eluted, this confirms that all the unreacted [^{14}C]-

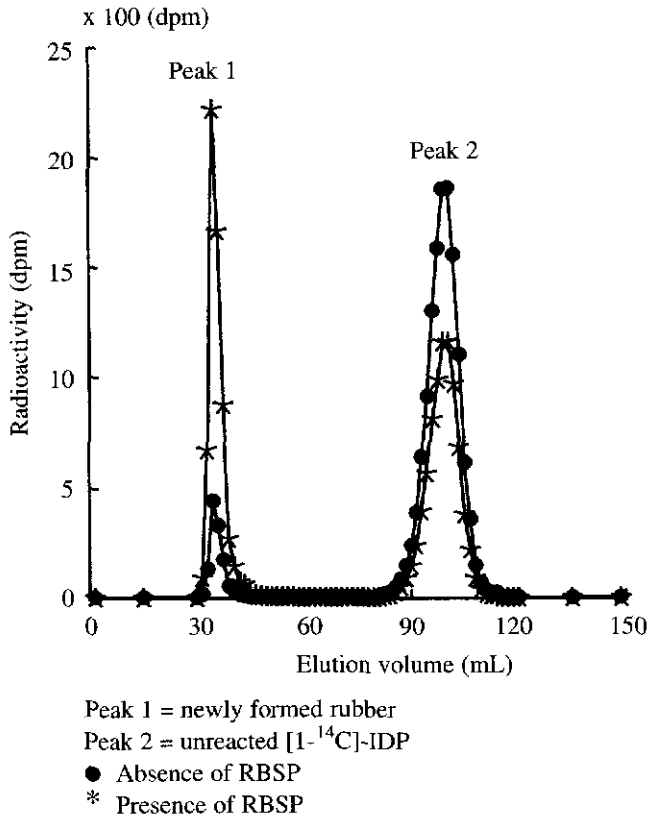

Figure 7. The effect of RBSP on rubber biosynthesis, demonstrated by gel-filtration on Sephadex® G-150 gel column (50 cm × 1.6 cm). 5 × 200 μL incubated product was loaded onto the gel and then eluted with 250 mM Tris-HCl, pH 8.0 plus 5 mM 2-mercaptoethanol. The figure shows the elution profile of labelled products [radioactivity counts (dpm)] from the column in the absence and presence of the RBSP.

TABLE 4. THE INCORPORATION OF [^{14}C]-IDP (DPM) INTO RUBBER OBTAINED BY TWO DIFFERENT METHODS OF PURIFICATION OF LABELLED RUBBER

Samples	Incorporation (dpm)
Membrane-filtered (no RBSP)	1252 ± 75
Membrane-filtered (with RBSP)	6153 ± 115
Membrane-filtered (with RBSP)/toluene/Bio-Beads® S-X1	5875 ± 123

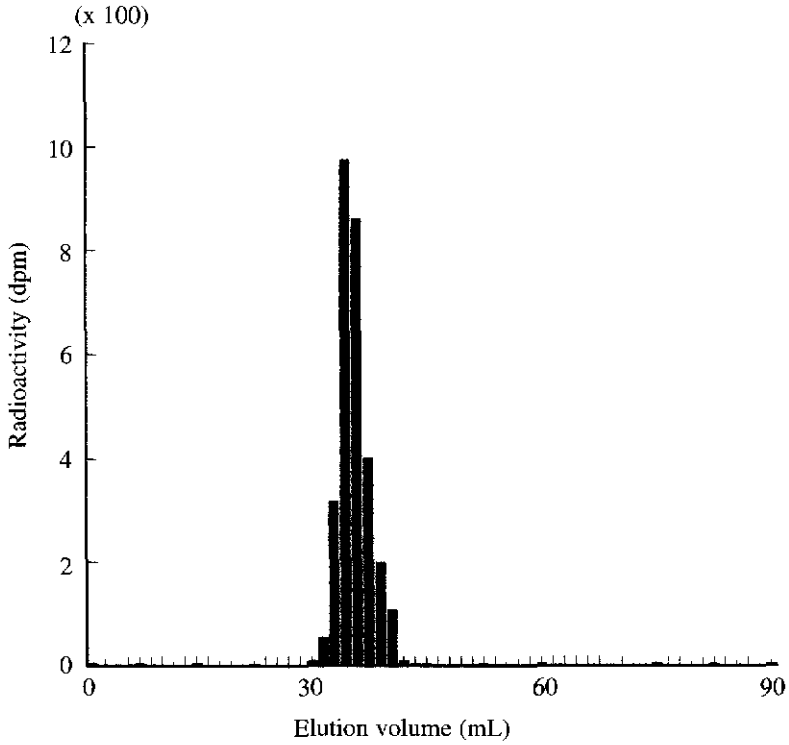

Figure 8. A gel-filtration chromatographic profile of 14C-rubber in toluene on a column (50 cm × 1.6 cm) of Bio-Beads® S-X1 (exclusion limit, 14 000 Da). A total of five purified products (originally from 5 × 200 µL incubation mixture), prepared as described in the text, were used in the chromatography.

IDP has been eliminated during membrane washing. The counts obtained in the void volume were quantitatively compared with the ones obtained by the routine assay (*Table 4*). This experiment confirms that the increase in counts in the presence of the RBSP is due to the increase of [^{14}C]-IDP incorporation into rubber. The possibility of the presence of protein-[^{14}C]-IDP complexes on the rubber particles, if any, which would have contributed to the increase in counts, was dismissed as the toluene purification step would have got rid off any non-rubber from the rubber particles, leaving only the newly formed ^{14}C-rubber. The fact that there was only one population of labelled eluant shows that no protein-[^{14}C]-IDP complexes were formed and being trapped on the rubber particles during the normal rubber biosynthesis assay steps, because if there was any, there would be another labelled population eluted, besides the one attributed to the [^{14}C]-IDP-rubber complexes.

Heat stability

The purified RBSP was heated at 100°C for 30 min and the stimulatory activity was assayed. Result (*Table 5*) shows that the RBSP

was heat-stable as it retained 98.5% of its original capability of stimulating rubber biosynthesis.

Cloning of RBSP cDNAs

Since the peptide sequence of RBSP matched with other plant eIF-5A, a cDNA for tabacco eIF-5A (a kind gift from Prof. C. Kuhlemeier, University of Berne, Switzerland) was used as a probe to isolate cDNAs encoding the stimulator protein. Three phage clones were isolated after screening approximately 2×10^4 plaque forming units of a previously constructed λgt10 latex cDNA library. Phage DNA was isolated from these clones after which restriction with *Eco* RI revealed cDNA inserts of approximately 600, 400 and 350 bp (E11, E101 and E21 respectively). Subsequently, these cDNAs were subcloned into the pBluescript plasmid vector and sequenced (sequences described in Chow, K. S. *et al.*, manuscript in preparation). A comparison of their sequences with DNA databases showed that all three clones encode partial eIF-5A cDNAs, and are highly homologous with other plant eIF-5A. In addition, small variations in DNA sequence between the three cDNAs indicated that they are derived from three isoforms. Different isoforms of eIF-5A has been reported in other plant species: three in tobacco[21] and five in potato[25].

Table 6 shows the alignment of the translation frames of E11 and E101 in relation to the known tryptic peptide sequences of the RBSP. Both *E11* and *E101* are identical in amino acid sequence within the region, which corresponds with the tryptic peptides. The first amino acid of E101, which is missing in E11,

TABLE 5. EFFECT OF HEATING ON RBSP

Incubation mixture contains	Incorporation (dpm)	%
No RBSP	1423 ± 89	–
RBSP — unheated	4215 ± 118	100.0[a]
RBSP — heated	4153 ± 120	98.5

[a]The incorporation of [^{14}C]-IDP into rubber in the presence of unheated RBSP is assumed as 100%

TABLE 6. AMINO ACID ALIGNMENT OF E11, E101 AND TRYPTIC PEPTIDES

E101:	MSDEEHHFESKADAGASKTYPQQAGTIR . . .
E11:	SDEEHHFESKADAGASKTYPQQAGTIR . . .
RBSP Tryptic peptide 1:	QDEHEHFESK
RBSP Tryptic peptide 2:	TYPQQAGTIR

is the initiating methionine based on their alignment with other plant eIF-5A (data not shown). The third clone, E21, could not be included in the alignment as it has a large 5′ end cDNA truncation. Although the exact sequence of RBSP Tryptic Peptide 2 was found in both E101 and E11, however, the match with RBSP Tryptic Peptide 1 was not precise. Two explanations for this are either the peptide sequence was inaccurate or an exact match with both tryptic peptide sequences is found within the 5′ end of the E21 cDNA.

CONCLUSION

In this study, we have established a purification method for RBSP followed by biochemical characterisation of this protein. The stimulatory property of RBSP was demonstrated by rubber biosynthesis assays, where the incorporation of IDP onto rubber particles was enhanced in the presence of RBSP. This is the first time a protein, capable of stimulating IDP incorporation *in vitro*, has been purified to homogeneity from the C-serum of *Hevea brasiliensis* following the detection of a C-serum stimulatory factor by Archer and Audley[4]. It is possible that RBSP is the same proteinaceous factor reported by these workers. Nonetheless, the presence of additional stimulatory proteins in the C-serum cannot be ruled out.

The heat stable RBSP, with a molecular weight of 13 068 Da by mass spectroscopy, was identified as eIF-5A based on similarity of its partial peptide sequences to eIF-5A proteins from several plants[21,25]. In eukaryotes, eIF-5A is a small and abundant protein that is highly conserved[26,27] and is suggested to be involved in promoting the formation of the first peptide bond in a protein biosynthesis[28–30]. Unlike eIF-5A, RBSP is comparatively less abundant in latex, as our data shows that only about 0.07% total RBSP was recovered from the C-serum solids. We have not tested RBSP for the presence of eIF-5A activity. In any event, even if RBSP functions in protein synthesis, it would not explain its stimulatory property as shown by the increased rate of IDP incorporation into rubber in the rubber biosynthesis assay. EIF-5A is also the only known cellular eukaryotic protein to have the unique amino acid, hypusine [N-ε-(4-amino-2-hydroxybutyl)lysine], which is a post-translationally modified lysine, formed by the addition of a butylamino group (from spermidine)[31].

The rubber biosynthesis assay used in this study was previously described by Yusof *et al.*[16] and contains the initiator molecule, FDP, as one of the basic components. We found that RBSP produced a higher level of IDP incorporation in the assay mixture in the absence of FDP compared to when FDP was routinely included in the basic assay. This would be the expected result if RBSP acted as an initiator molecule. This result also indicates that RBSP did not have any role in the elongation of rubber molecules. Therefore, as a first observation of the mechanism of action of RBSP, it may be postulated that RBSP stimulates the initiation of rubber biosynthesis, *i.e.* the isomerisation of IDP to DMADP and/or the synthesis of any of the initiator molecules such as GDP, FDP or GGDP, and not in the subsequent addition of IDP to produce *cis*-rubber in the elongation phase.

Due to the significance of a protein with stimulatory function in rubber biosynthesis, we have also substantiated our assay results by confirming that the increased radioactive measurement in the presence of RBSP is due to increase in the amount of incorporated labelled IDP onto rubber and not due to accumulation of artefactual protein-^{14}C-IDP complexes. Evidence supporting this was provided by

chromatographic separation of assay products by Sephadex® G-150 gel-filtration and the purification of labelled rubber by Bio-Beads® S-X1 gel-filtration.

In addition, we extended our study of RBSP by isolating cDNAs for this protein. As a result, three isoforms of *Hevea* eIF-5A were isolated; two of which partially match the available amino acid sequence of the RBSP. The occurrence of multiple forms of *Hevea* eIF-5A, though consistent with similar reports in other plant eIF-5As[25,26], raises the question of whether the stimulatory effect is a result of one, or more than one isoform. This will be investigated further in future.

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