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An Assessment of Stimulation of Hevea brasiliensis *Rubber Biosynthesis by eIF-5A Protein-enriched Bacterial Lysates*

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The effect of six unique isoforms of Hevea brasiliensis *eukaryotic translation initiation factor 5A (eIF-5A), previously reported as a rubber biosynthesis stimulator protein (RBSP) was evaluated using biosynthesis assays containing zone 2 rubber particles and radiolabelled isopentenyl diphosphate ([^{14}C]-IPP). Recombinant RBSP proteins were over-expressed as N-terminal glutathione-S-transferase (GST)-tagged proteins in a bacterial system. Crude extracts of six RBSP-GST isoforms were added to rubber biosynthesis assays in increasing volumes. Results showed that increases in the amount of each RBSP-GST isoform corresponded with increases in the level of [^{14}C]-IPP incorporation above that of the control which contained no recombinant protein. Statistical data analysis revealed that three RBSP isoforms increased [^{14}C]-IPP incorporation significantly compared to the GST control where the RBSP isoform with the highest stimulatory effect produced a 45% increase in IPP incorporation compared to GST.*

Key words: rubber biosynthesis; stimulator protein; eIF-5A; *cis*-polyisoprene; *Hevea brasiliensis*; glutathione-S-transferase; isopentenyl diphosphate

In *Hevea brasiliensis*, the bulk of rubber biosynthesis takes place in the cytoplasmic content of latex vessels. The mevalonate path way of isoprenoid biosynthesis has conventionally been studied in rubber biosynthesis[1,2]. More recently, there is evidence for enzymes of the non-mevalonate methylerythritol phosphate (MEP) pathway in latex[3] and thus, a possible alternative source of isopentenyl diphosphate (IPP) for *cis*-polyisoprene formation. Although *Hevea brasiliensis trans* and *cis*-prenyltransferase cDNAs have been identified[4–6], knowledge of rubber initiation and elongation by their enzymes is lacking. Besides the enzymes of intermediate pathway steps, other ancillary proteins contribute to rubber formation; these include the rubber elongation factor[7], the small rubber particle protein[8], a patatin-like inhibitor[9,10] and a rubber biosynthesis stimulator[10–12]. The rubber biosynthesis stimulator protein, RBSP, was previously purified from *Hevea brasiliensis* latex and identified as eIF-5A by amino acid sequencing[10,11]. The eIF-5A protein is involved in the formation of the first peptide bond during protein synthesis and is the only

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protein known to require post-translational modification of lysine at position *circa* 50 to hypusine for biological activity[13].

A large family of nine RBSP genes, representing seven unique isoforms of the protein was previously reported[12]. The occurrence of eIF-5A gene families in plants is not unusual; there are three genes in tobacco[14], five in potato[15] and four in tomato[16]. Besides protein synthesis, plant eIF-5A isoforms have been reported to be involved in other functions such as photosynthesis[14], organ senescence[16–18] and programmed cell death[19]. Therefore, the occurrence of variant RBSP isoforms infer diversification of enzyme function, in the case of *Hevea brasiliensis*, rubber biosynthesis. In this study, we show evidence for the stimulatory property of six unique isoforms on the incorporation of IPP by isolated rubber particles and suggest possible roles of RBSP in the rubber biosynthesis machinery.

MATERIALS AND METHODS

Expression Constructs and Recombinant RBSP Proteins

Six RBSP cDNAs (E11, E317, E318, E324, E328 and E332) subcloned into a GEX plasmid vector (Amersham Pharmacia Biotech)[12] were expressed in *Escherichia coli* BL21 cells using the glutathione-S-transferase (GST) Gene Fusion System (Amersham Pharmacia Biotech). Recombinant protein was not prepared using the seventh RBSP cDNA (E101) as it has a large 3' truncation[12]. Recombinant *E. coli* BL21 cells were cultured in LB media containing 100 µg/mL ampicillin at 37ºC with agitation. At mid-log phase, protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM. Subsequently, the culture was incubated at 37ºC with agitation for 5 h after which cells were harvested by centrifugation (5000 g, 10 min) and resuspended in PBS buffer at 50 µL volume per mL of culture. RBSP-GST fusion protein was harvested from cells by repeated freeze-thawing in liquid nitrogen. The broken cells were centrifuged (5000 g, 10 min) to obtain fusion protein-containing lysate in the supernatant fraction. RBSP-GST lysates were analysed by SDS-PAGE to verify recombinant protein expression. To determine that equivalent amount of RBSP-GST fusion proteins and GST tag protein was added to rubber biosynthesis assays, protein lysates that were diluted 30-50 times were standardised using Coomassie Blue-stained SDS-PAGE gels.

Preparation of Zone 2 Rubber Particles (Z2RP)

Latex was collected from field-grown *Hevea brasiliensis* trees (clone RRIM 600) into chilled containers for preparation of Z2RP. The latex was centrifuged in 50 mL tubes at 44 000 g in a Sorvall RC 5C high-speed centrifuge for 2 h to obtain three main fractions: the upper-most rubber cream layer, the middle aqueous C-serum fraction and the 'bottom fraction'[20]. The rubber cream layer was punctured with a spatula to allow the C-serum to drain out by tilting the tube. The entire cream layer was scooped out intact and Z2RP located at the base of the rubber cream (about 2 mm thick) was collected by skimming with a spatula. About 1 g of Z2RP was suspended in 14 mL 50 mM Tris-HCl pH 7.5 buffer. Before use, this Z2RP mixture was filtered through 4M Whatman paper to remove any coagulated rubber.

Rubber Biosynthesis Assays

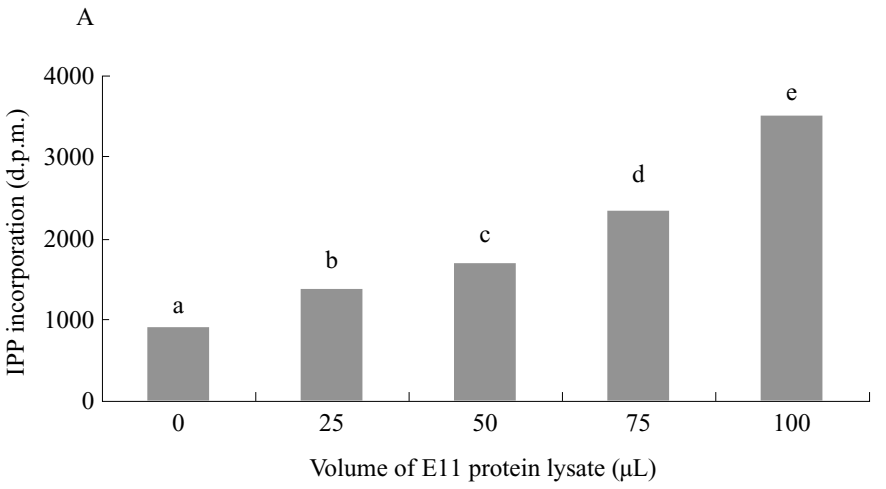
Rubber biosynthesis assays containing washed rubber particles[21] were performed

with several changes. Washed rubber particles used in the original assay were replaced with Z2RP. Each 200 µL incubation mixture contained 50 µL of Z2RP mixture, 0.29 nmole [^{14}C]-IPP (1.92 GBq/mol), 0.3 mM unlabelled IPP, 2 mM $MgSO_4$, 50 mM DTT and 50 mM Tris-HCl at pH 7.5. No initiator molecules of rubber biosynthesis were included in the assays. Incubations were carried out for three hours at 37ºC instead of at 25ºC in the original assay. Each RBST-GST protein isoform was added in volumes of 25, 50, 75 and 100 µL to assay mixtures and each assay was performed in triplicate. Analysis of variance of [^{14}C]-IPP incorporation data was performed using the Statgraphics software (Statistical Graphics Corp., Maryland, USA).

RESULTS AND DISCUSSION

Six RBSP cDNAs were expressed in *E. coli* as N-terminal GST-tagged proteins of approximately 45 kDa[12] and the crude proteins obtained from the cell lysates were used in biosynthesis assays. The 29 kDa GST tag protein was similarly expressed for use in the control assay. Two sets of experiments using different batches of Z2RP preparations were performed to determine the effect of different amount of protein lysate on IPP incorporation. Our results showed that increasing the amount of each RBSP-GST lysate resulted in increases in the level of IPP incorporation above that of the control which contained no lysate protein. The statistical significance of these results was assessed using Duncan's Multiple Range Test which accounted for effects of experimental replicates (*Figure 1A–E*). The enhancement of IPP incorporation was statistically significant at $p = 0.05$ in each case with the exception of RBSP-GST isoform, E317. This isoform could not be included in the statistical analysis as the amount of recombinant protein produced was only sufficient for one experiment. However, the average of incorporation measurements done in triplicate showed that although E317 increased IPP incorporation, it was not the best stimulator among the six candidates (data not shown).

A residual amount of bacterial host proteins is present in fusion protein-containing lysates as detectable by SDS-PAGE analysis[12]. The GST tag protein which is not expected to stimulate IPP extension, produced increasing IPP incorporation levels with more amount of GST protein (*Figure 1F*) and this indicates that data in *Figures 1A–E* include some background stimulatory activity contributed by bacterial proteins. This is most likely attributed to the bacterial *cis*-prenyltransferase, undecaprenyl diphosphate synthase, an enzyme which functions in the synthesis of cell wall polysaccharide components[22]. In our study, bacterial proteins had not been removed from lysates prior to assays for the sake of convenience in handling the concurrent expression of a panel of RBSP-GST proteins. To verify that stimulation by RBSP isoforms is not artefactual, our results were analysed by comparing the stimulatory effect of individual isoforms relative to GST and to one another by assessment of the same sets of experiments using Duncan's Multiple Range Test. Having removed variation due to effects of protein amount and experimental replicates, three out of five RBSP-GST isoforms (E324, E328 and E11) were found to perform significantly better than the GST control (*Figure 2*). Therefore, it can be concluded that in spite of the presence of background bacterial proteins, RBSP isoforms which produced statistically significant increases in IPP incorporation were identified. The better performance of the E324, E328 and E11 isoforms is not easily explained by sequence differences between them due to the highly identical protein alignment of RBSP isoforms[12]. Among the three, RBSP-GST



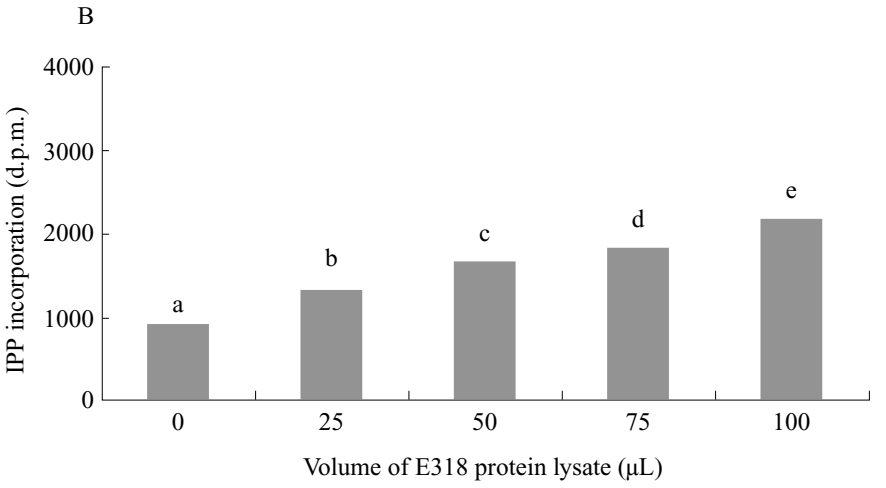
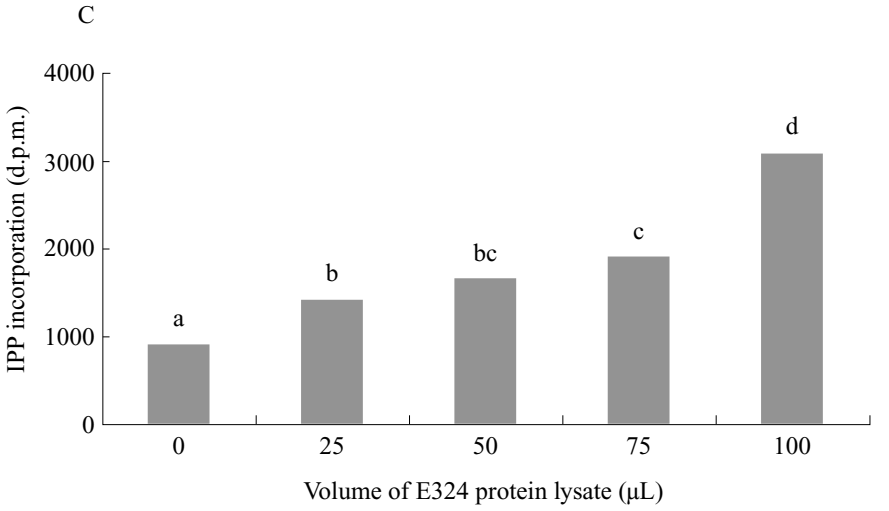


Figure 1. Analysis of variance of effect of RBSP-GST protein amount on IPP incorporation. Rubber biosynthesis assays comparing the effect of volume of RBSP-GST protein lysates (E11, E318, E324, E328, E332) and GST tag protein with the control (no added protein). The alphabets a–e denote categorical statistical significance at p=0.05 according to Duncan's Multiple Range Test.



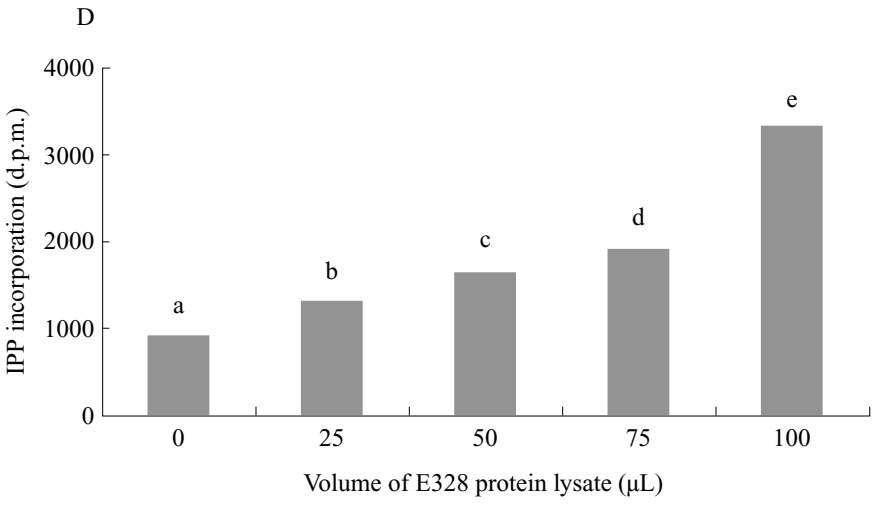
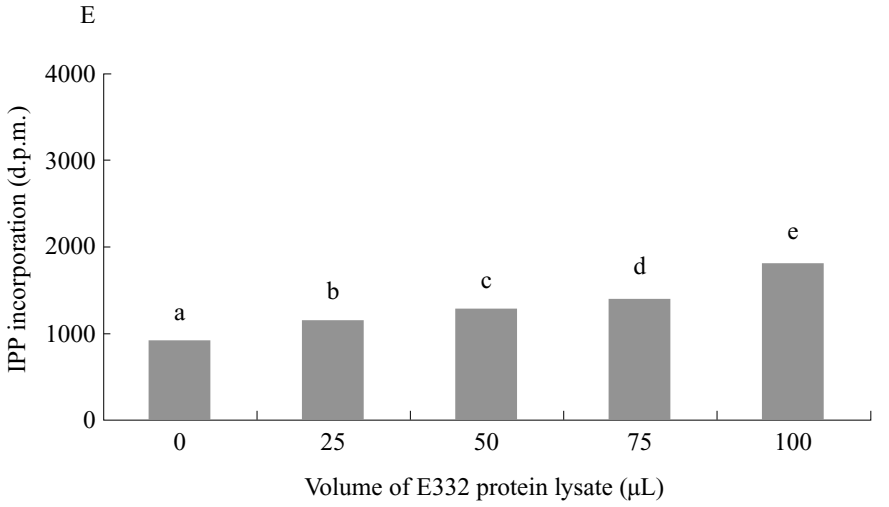


Figure 1. (cont.) Analysis of variance of effect of RBSP-GST protein amount on IPP incorporation. Rubber biosynthesis assays comparing the effect of volume of RBSP-GST protein lysates (E11, E318, E324, E328, E332) and GST tag protein with the control (no added protein). The alphabets a–e denote categorical statistical significance at p=0.05 according to Duncan's Multiple Range Test.



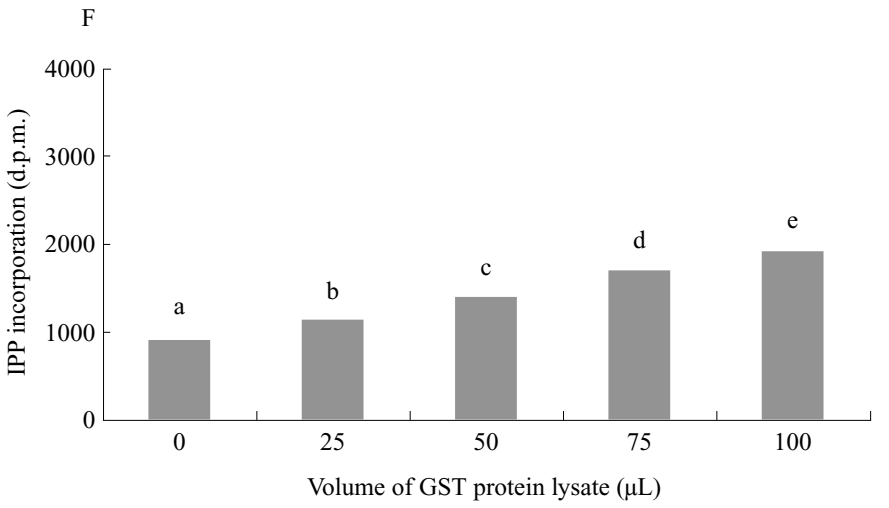


Figure 1. (cont.) Analysis of variance of effect of RBSP-GST protein amount on IPP incorporation. Rubber biosynthesis assays comparing the effect of volume of RBSP-GST protein lysates (E11, E318, E324, E328, E332) and GST tag protein with the control (no added protein). The alphabets a–e denote categorical statistical significance at p=0.05 according to Duncan's Multiple Range Test.

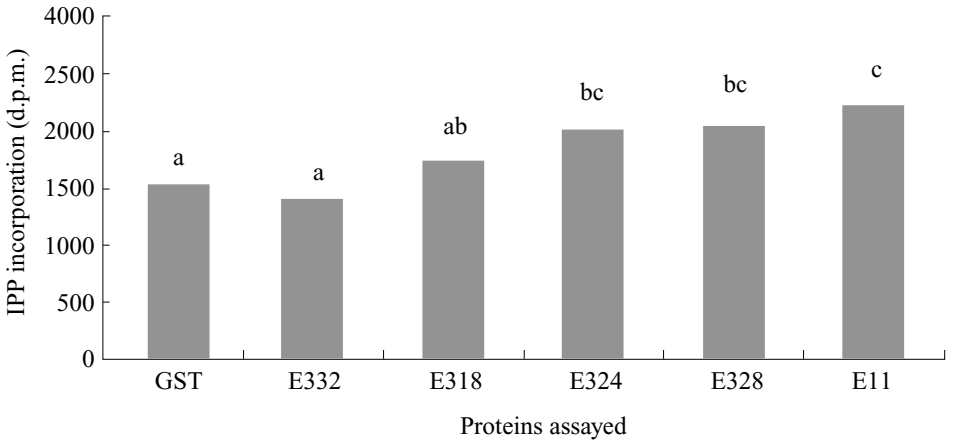


Figure 2. Analysis of variance of effect of RBSP-GST isoforms on IPP incorporation. Rubber biosynthesis assays comparing the effect of each RBSP-GST isoform (E11, E318, E324, E328, E332) with the control GST tag protein. The alphabets a–c denote categorical statistical significance at p=0.05 according to Duncan's Multiple Range Test.

isoform E11 was the best stimulator, producing a 45% increase in IPP incorporation of that of the GST control.

Incorporation measurements alone based on the performance of individual isoforms compared to the GST control is not sufficient to delineate a *bona fide* subset of isoforms which stimulate biosynthesis. This is because the physiological disposition of isolated rubber particles is likely to vary from batch to batch and can thus affect the absolute incorporation values. Thus, the main contribution from our assays is the identification of isoform E11 as the best RBSP. The preparation of recombinant E11 protein free of bacterial proteins is important in future experiments so as to establish a more realistic magnitude of stimulation of IPP incorporation. The removal of the GST tag from the fusion protein will also be necessary for quantitative comparison of the stimulatory properties of both recombinant and native RBSP.

CONCLUSIONS

This study provides evidence for differential stimulatory effect of RBSP isoforms on rubber biosynthesis based on *in vitro* rubber biosynthesis assays. Previously, it was established that native RBSP acts at the initiation stage of rubber formation[11]. Although this study does not identify the isoform corresponding to the native protein, the results showed that more than one RBSP isoform is likely to be involved in stimulating rubber biosynthesis *in vivo*. In addition, this study reinforces the participation of other proteins, including RBSP, acting in concert with prenyltransferases. Concerning the role played by RBSP, several postulations are conceivable. It is plausible that RBSP could mimic the bond-forming function of eIF-5A during protein synthesis[13], in this case, by biochemically stimulating the condensation of IPP units into initiator molecules. Alternatively, RBSP could play a regulatory role

by interacting with the patatin-like inhibitor protein[9] as part of a mechanism of controlling rubber biosynthesis in latex vessels.

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