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Isolation and Characterisation of Latex Cyanogenic Glucosidase in Hevea brasiliensis

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Cyanogenic glucosidases play an important role in defence against pathogen attack in plants. Hevea brasiliensis *latex glucosidase was isolated from the latex B-serum by dialysis-induced protein precipitation and gel filtration. Following SDS-polyacrylamide gel electrophoresis carried out under reducing conditions, the protein was observed to be about 57 kDa. Partial amino acid sequences of the protein matched published sequences of several plant glucosidases, including those of cyanogenic glucosidases. Enzyme assays revealed that the protein possessed glucosidase activity, this being confirmed by isozyme staining of the protein after electrophoretic separation on native polyacrylamide gels. Enzyme activity was also observed when linamarin was used as the specific substrate, confirming that the protein was a cyanogenic glucosidase (linamarase) as suggested by the partial amino acid sequences. The latex cyanogenic glucosidase is a component of an allergenic protein complex, Hev b 4. Cloning and sequencing of the complete cDNA encoding the glucosidase is ongoing.*

Key words: latex B-serum; cyanogenic glucosidase; linamarase; gel filtration; RT-PCR; isolation; characterisation; *Hevea brasiliensis*; identification

Latex from the rubber tree (*Hevea brasiliensis*) is separated into the rubber cream, the clear aqueous phase (C-serum), and the sediment, which consists mainly of vacuole-like organelles called lutoids by high-speed centrifugation[1]. The fluid constituents of the latter, known as B-serum has drawn attention for many years, owing to its contents of hydrolytic enzymes[2] and pathogenesis-related proteins[3]. A number of these proteins were also pursued in latex allergy studies[4–6].

β-glucosidase is one the first hydrolases detected in the *H. brasiliensis* latex B-serum[2]. It is in the leaf tissue, however, that a *H. brasiliensis* β-glucosidase was first isolated. Besides β-mono-glucosides, this enzyme cleaves a wide range of indigenous heteroglycosides including coniferin and

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cyanoglucosides (linamarin and lataustralin) indicating a possible role in lignification and cyanogenesis Accordingly, it is given the name cyanogenic glycosidase (linamarase)[7] In cassava, hydrolysis of cyanoglucosides by linamarase produces cyanohydrins and glucose[8,9] Subsequent breakdown of cyanohydrins either spontaneous or by a α-hydroxynitrile lyase releases a ketone and hydrogen cyanide, which among others has a role in plant defense[10] Likewise, application of cyanide on *Microcyclus ulei*, a pathogen of the cyanogenic host *H brasiliensis* inhibited the fungal development and sporulation[11] Interestingly, at the fungal infection stage, very little hydrogen cyanide was detected from leaves of the resistant *H brasiliensis* clones compared to the susceptible clones[12] The present study is aimed at isolating, identifying and characterising a cyanogenic β-glucosidase in the latex B-serum, as well as cloning and sequencing of its transcript in *H brasiliensis* latex

EXPERIMENTAL

Preparation of B-serum

Latex B-serum was prepared based on a method previously described[13] Briefly, fresh latex from *H brasiliensis* clone RRIM 600 was collected into chilled containers and centrifuged at 43 000 g for one hour at 4°C The rubber cream and the C-serum were discarded while the bottom fraction was collected and re-suspended in 0 4 M mannitol to aid the removal of remnant C-serum while retaining the lutoids intact The cleansed bottom fraction was recovered after another centrifugation and subjected to alternate freezing and thawing (four times) to rupture the lutoids The fluid from the lutoids, the B-serum was recovered by centrifugation for subsequent analyses

Precipitation of B-Serum Proteins by Dialysis

Aliquots of 20 mL were dialysed using cellulose acetate tubings (molecular weight cut-off 2500 Da) against 5 litres of distilled water for 48 h at approximately 5°C The resulting protein precipitate was recovered by centrifugation at 20 000 g for 30 minutes The precipitate was re-dissolved in 20 mL of chilled 0 35 M NaCl

Isolation of Latex Glucosidase

The solution (5 mL) of the re-dissolved proteins in sodium chloride was chromatographed on a column (77 cm x 1 6 cm) of Sephacryl S-200 (Amersham Biosciences) which had been equilibrated with 0 35 M NaCl Elution was carried out in the same solvent with the flow rate maintained at 20 mL per hour Sequential fractions of 5 mL were collected and the optical density of each fraction was measured at 280 nm Several such column chromatography runs were made to recover substantial quantities of the components eluted from the column for analyses

Concentration of the Eluted Components

The fractions under the major UV absorption peaks obtained from several runs were pooled and dialysed overnight against 3 litres of 1% glycine at approximately 5°C essentially to eliminate the salt while retaining the proteins in their soluble state The dialysed fractions were concentrated up to 10 x by partial freeze-drying

Glucosidase Activity

β-glucosidase activity was determined by measuring the change in absorbance at 400 nm resulting from the enzyme-catalyzed hydrolysis of the glucoside, *p*-nitrophenyl β,D-glucopyranoside. The assay mixture (60 μL) contained 8.3 mM of the glucoside in 10 mM citrate buffer, pH 5. The mixture was incubated at 30°C and the reaction stopped after 20 min by the addition of 100 μL 1 M sodium carbonate.

Linamarase Activity

Linamarase activity was measured by the release of HCN from the glucoside, linamarin (α-hydroxyisobutyronitrile β-D-glucopyranoside), that was used as the enzyme substrate. The reaction mixture (950 μL) containing 9.5 mM of the glucoside in 10 mM citrate buffer, pH 5, was placed in a vessel that had a central compartment holding 0.5 mL of picrate solution (0.5% picric acid in 2.5% sodium carbonate). The reaction mixture was incubated in a shaking water bath (30°C) for 90 min, at the end of which time HCN was released from the mixture by injecting 1 mL of 0.2 M NaOH through a parafilm seal that kept the reaction vessel air-tight. The injection puncture was immediately re-sealed. After a further 30-min incubation to trap HCN in the picrate solution, 0.4 mL of the picrate solution was pipetted into 0.6 mL water and the absorbance of the diluted solution read at 515 nm.

Native and SDS-Polyacrylamide Gel Electrophoresis

Native TBE polyacrylamide gel electrophoresis and isozyme staining was carried out according to Lebrun and Chevallier[14]. Analytical SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to standard procedures described elsewhere[15]. The gels were stained with Coomassie Brilliant Blue R250 (Sigma, USA) and molecular weight markers (14 kDa–97 kDa) were obtained from BioRad, USA. Preparative SDS-PAGE was performed to recover proteins at different molecular weights using the PrepCell (BioRad, USA) according to the instruction of the manufacturer.

Mass Spectrometry

Mass spectrometry of the peptides produced from tryptic digestion of each protein was undertaken using a Sciex API III triple quadrupole instrument fitted with a nano-electrospray source. Following desalting using a self-packed Poros R2 column, peptides were eluted directly into the nanospray needle in 2 mL of 50% methanol in 5% formic acid. Analysis of the peptides involved acquiring Q1 scans and precursor ion scans based on the immonium ion of Leucine/Isoleucine at m/z 86. Molecular ions relating to peptides were then selected for Tandem MS/MS to obtain fragment ions relating to the sequence of the peptide. All spectra were acquired in the positive ion mode.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and DNA Sequencing

Latex total RNA was isolated essentially according to the method of Prescott and Martin[16]. RT-PCR was performed using 5'/3' Rapid Amplification of cDNA Ends (RACE) kit (Roche Diagnostics, Germany) as described in the manufacturer's instruction manual. A Perkin-Elmer 9600 thermal cycler and an ABI377 Genetic Analyser (Applied

Biosystems, USA) were employed in sequencing the cDNA clones in *pGEM-T* vector (Promega, USA) using the T_7 and SP6 promoter primers according to the thermal cycling protocol of *Taq* Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA)

RESULTS AND DISCUSSION

Protein Isolation

On gel filtration by Sephacryl S-200, the reconstituted B-serum precipitate separated into four fractions, marked A–D (*Figure 1*) In SDS-PAGE run under non-reducing conditions, the major early eluting peak (described as fraction A) migrated predominantly as a 110 kDa protein When β-mercaptoethanol was included in the sample buffer, a broad band at *circa* 52 kDa–55 kDa and a distinct 57 kDa band appeared Preparative SDS-PAGE was employed to separate and recover the two proteins for amino acid sequencing Partial peptide sequences and database search revealed that the two proteins were dissimilar Analyses pertaining to the 57 kDa protein is presented here

Amino Acid Sequencing and Cloning of the Gene

A spectrum of molecular ions was obtained when the trypsin digested 57 kDa protein was scanned using a nano-electrospray tandem

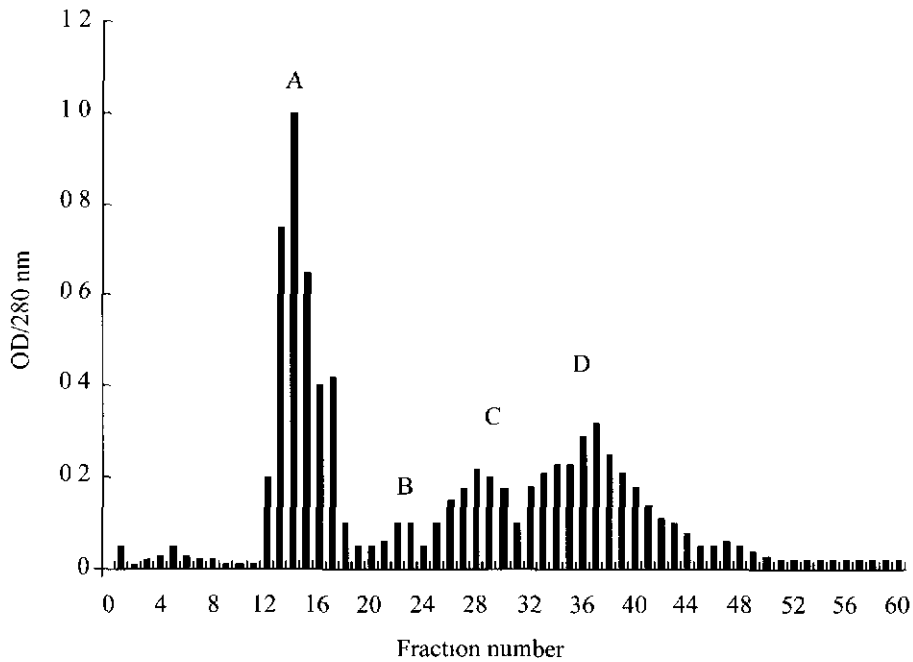

Figure 1 Sephacryl S-200 elution profile of the B-serum precipitate re-dissolved in 0 35 M NaCl The main fractions A – D are indicated

mass spectrometry (ESI-MS). A doubly charged precursor ion in the spectra at m/z 878.39 was chosen for peptide sequencing using MS/MS. This precursor which relates to a peptide with a molecular weight of 1745.76 kDa gave a complete series of y" ion fragments. BLASTP[17] search showed exact match of the sequence to a β-glucosidase/ linamarase from cassava (*Figure 2*), besides similarity to several other glucosidases of plant origin

Multiple nucleotide sequence alignment showed the presence of several highly conserved regions in plant β-glucosidases. RT-PCR was performed on latex total RNA using sense and antisense strand degenerated primers that were designed based on the conserved regions. A 435 bp fragment (GenBank accession number B1729486) with homology to glucosidases was obtained from the PCR (*Figure 3*). The 5'/3'RACE technique is presently being employed to generate the complete cDNA of *H. brasiliensis* latex glucosidase.

Glucosidase and Linamarase Activity

To determine if the isolated protein was a glucosidase, the major peak eluting from Sephacryl S200 was assayed for the ability to cleave *p*-nitrophenyl β-D-glucopyranoside, a glucoside. A latex glucan glucosidase (β-1, 3-glucanase), latex B-serum and latex C-serum were also tested at the same time. Enzyme activity was observed in test samples containing the putative glucosidase and the B-serum from which it originated. The glucanase, which also originated from latex B-serum but for which *p*-nitrophenyl β,D-glucopyranoside is not the preferred substrate, gave only a very low reading. Trace level of glucosidase activity was also seen in the latex C-serum. Since lutoids in the latex are fragile and are susceptible to rupture, contamination of the C-serum with small amounts of the lutoidic B-serum contents is normal. The slight presence of glucosidase activity in the latex C-serum is consistent with this occurrence (*Table 1*).

Another approach used to test β-glucosidase activity in the active fraction was isozyme staining. Besides the isolated protein fraction, rubber particle protein, latex B-serum and C-serum were also run on the native TBE polyacrylamide gel. When tested with β-naphtyl-β,D-glucopyranoside staining protocol, a distinct red band appeared at the top end of the separating gel in the lanes containing the active fraction and B-serum, indicating β-glucosidase activity. The latex C-serum gave a faint band but β-glucosidase activity was absent in a preparation of rubber particle proteins (*Figure 4*). When

Query: Partial peptide of the 57 kDa *Hevea brasiliensis* latex B-serum protein
Subject: Cassava β-glucosidase (EC 3.2.1.21) gi|112564|pir||S23940
Length = 531

```
Query:     1  ILDGSNGDVAVDFYNR 16
              ILDGSNGDVAVDFYNR
Subject:  74  ILDGSNGDVAVDFYNR 89
```

Figure 2. BLASTP match of Hevea brasiliensis *latex cyanogenic glucosidase partial peptide to a cassava β-glucosidase.*

```
1   TAATGTCCAT TTCATGGTCT AGAATAGTAC CTAGTGGAAG GGTATGGGAA
51  GGAGTGAATG AGCAAGGAAT TGACTTTTAC AACAATGTTA TCAACGAAAT
101 TATAGCAAAT GATATGAAGC CTTTTGTAAC TATGTTCCAT TGGACTACTC
151 CTCGGGCGCT AGAGGATAAG TATGGTGGCT TTTTAAGTCC CNATATTGTG
201 AATGATTTTC GAGACTATGC GGATCTTCTT TTTGAAAAAT TTGGTAATCG
251 AGTGAAGCAT TGGATGACTT TAAATGAACC ATGGACTGTT GCTGGATTTG
301 GCTATGATGA TGGCATCCAT GCCCCTGGAA GATGCTCACC TTGGGTGAAT
351 TATCGATGCC CGGCTGGAAA CTCATCCACA GAACCTTATA TAGTTGCCCA
401 TAATTTACTC CTTACTCATG CTACCCCTGT TCACC
```

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>gi|112564|pir||S23940 beta-glucosidase (EC 3.2.1.21) -
cassava gi|249262|gb|AAB22162.1|     linamarase    [Manihot
esculenta] Length = 531
```

```
Query: 3   MSISWSRIVPSGRVWEGVNEQGIDFYNNVINEIIANDMKPFVTMFHWTTPRALEDKYGGF 182
            MSISWSR++PSGR  EGVNE+GI FYN+VINEII+N ++PFVT+FHW TP+AL+DKYGGF
Sbjct: 107 MSISWSRVIPSGRRREGVNEEGIQFYNDVINEIISNGLEPFVTIFHWDTPQALQDKYGGF 166

Query: 183 LSPXIVNDFRDYADLLFEKFGNRVKHWMTLNEPWTVAGFGYDDGIHAPGRCSPWVNYRCP 362
            LS  IV D+  YADLLFE+FG+RVK WMT NEP    GF +DDG+ APGRCS WVN +C
Sbjct: 167 LSRDIVYDYLQYADLLFERFGDRVKPWMTFNEPSAYVGFAHDDGVFAPGRCSSWVNRQCL 226

Query: 363 AGNSSTEPYIVAHNLLLTHATPVH 434
            AG+S+TEPYIVAHNLLL+HA  VH
Sbjct: 227 AGDSATEPYIVAHNLLLSHAAAVH 250
```

Figure 3. A 435 bp reverse transcription coupled PCR product (GenBank accession number B1729486) of Hevea brasiliensis *latex β-glucosidase and its BLASTX match to a cassava linamarase.*

β-naphtyl-β,D-galactopyranoside was substituted as the substrate, a similar zymogram was observed although at lower intensity (results not shown). These results confirmed the isolated protein to be a glucosidase, similar to the leaf glycosidase, which also showed weak galactosidase activity in the enzyme assay (about 1.6% that of the glucosidase activity)[7].

The cassava glucosidase that shared the same partial amino acid sequence with the latex glucosidase was also a linamarase. To investigate if the *H. brasiliensis* latex protein fraction has cyanogenic glucosidase activity, linamarase assay was carried out on four samples derived from latex. These were the protein fraction, latex β-1,3-glucanase, latex B-serum and latex C-serum. The protein fraction hydrolysed linamarin, thus confirming that it contained a cyanogenic glucosidase (linamarase) as suggested by the partial amino acid sequences. As might be expected,

TABLE 1. β-GLUCOSIDASE ACTIVITY IN LATEX FRACTIONS

Test sample	Absorbance $_{515\ nm}$
Latex β-glucosidase (110 μg / mL)	0.970
Latex β-1,3-glucanase (100 μg / mL)	0.049
Latex B-serum (Diluted 1/10)	0.505
Latex C-serum (Diluted 1/10)	0.050

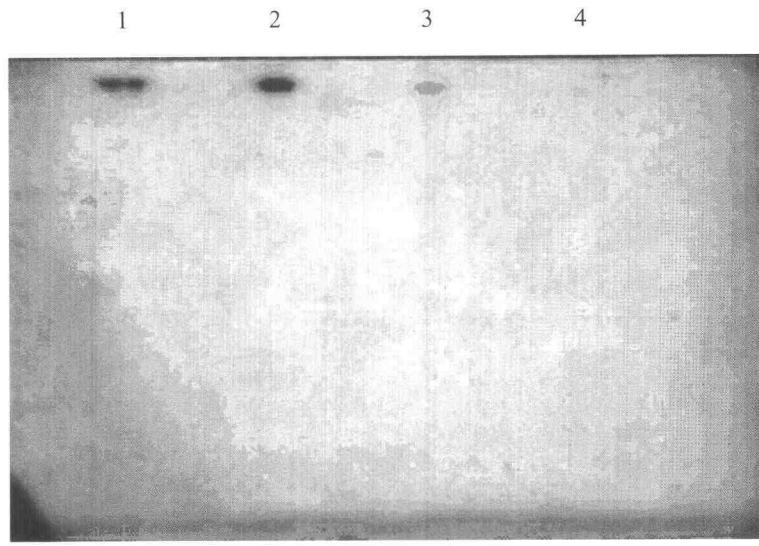

Figure 4. β-glucosidase activity (band) in various latex fractions discerned by isozyme staining of a 7% TBE native polyacrylamide gel. Lane 1: Major protein peak eluted from Sephacryl S-200 column; lane 2: Latex B-serum; lane 3: Latex C-serum; and lane 4: Rubber particle proteins.

linamarase activity was also observed in the B-serum from where the latex glucosidase was isolated. The C-serum showed trace linamarase activity arising from contamination by lutoidic B-serum, as mentioned above. On the other hand, latex β-1,3-glucanase did not show linamarase activity (*Table 2*). The containment of *H. brasiliensis* latex linamarase in the lutoids was comparable to the case of cassava latex linamarase where the enzyme was also found in vesicles in the latex[18]. Again, the characteristics of H. brasiliensis latex glucosidase/linamarase are in agreement with those reported for the leaf glycosidase/linamarase[7].

Besides its relevance in plant defence and pathogenicity, the *H. brasiliensis* latex cyanogenic glucosidase characterised in this investigation is also important in the study of latex allergy. This protein is a component

TABLE 2. LINAMARASE ACTIVITY IN LATEX FRACTIONS

Test sample	Absorbance $_{515\ nm}$
Latex β-glucosidase (86 µg / mL)	0.202
Latex β-1,3-glucanase (86 µg / mL)	0.001
Latex B-serum (Undiluted)	0.505
Latex C-serum (Undiluted)	0 050

of Hev b 4, a major latex allergen. Hev b 4 has also been identified as a part of the microhelices, a large lutoidic protein complex visible under the electron microscope. Immunoassays performed on Hev b 4 showed binding to IgE antibody from blood serum of latex-allergic patients[5]. Its allergenicity has also been confirmed by skin prick tests on latex-allergic patients[19].

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