Processed products of the hevein precursor in the latex of the rubber tree (*Hevea brasiliensis*)

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Abstract The 20 kDa precursor of hevein and its C-terminal 14 kDa domain have been isolated. Sequence analysis of the C-terminal tryptic peptides of these proteins and comparison with the cDNA sequence indicate that they represent mature forms from which a C-terminal propeptide, possibly involved in vacuolar targeting, has been removed. The molar ratio of hevein to the C-terminal domain in the lutoid-body fraction of rubber latex is about 30:1. This indicates that not only the pre- and propeptides but also the 14 kDa domain are removed by proteolysis or other processes in the latex vessel after the processing of hevein has taken place.

Key words: Hevein; *Hevea brasiliensis*; Protein processing; Vacuolar targeting

1. Introduction

The first protein isolated from the lutoid-body fraction of rubber latex was hevein, a small cystine-rich protein [1] with a polypeptide chain length of 43 residues [2,3]. Broekaert et al. [4] have determined its cDNA sequence and found that it is synthesized as a precursor with a signal sequence of 17 residues, followed by the hevein domain of 43 residues and a C-terminal region of 144 residues. The C-terminal domain and the precursor consisting of the hevein and the C-terminal domain are also present in the lutoid-body fraction of latex [5]. N-Terminal sequence analysis of the C-terminal domain indicated that 6 amino acids between the hevein and the C-terminal domain are lost during processing of the precursor [5]. Analysis by ion-spray mass spectrometry of hevein showed that minor components with one and two additional C-terminal amino acids occur [6], indicating that processing probably occurs by cleavage at the N-terminus of the C-terminal domain, followed by removal of 4–6 residues from the C-terminus of hevein [6].

The hevein precursor belongs to the family of homologous 'pathogenesis-related' PR-4 plant proteins with a molecular mass of about 14 kDa and three conserved disulfide bridges [7]. This family includes both extracellular [8–10] and vacuolar [11] members. Some of them have additional N-terminal domains homologous with hevein [11,12]. Recently, a novel protein from tobacco leaves, designated CPB20, has been described, which is processed into the mature protein by the removal of an N-terminal signal peptide and a C-terminal propeptide most likely involved in the vacuolar targeting of the protein [11]. However, the hevein domain in this protein is not cleaved off in the mature form.

Here we report the isolation of the hevein precursor and its C-terminal domain and present evidence that they are the mature proteins from which a C-terminal propeptide has already been removed. The large molar excess of hevein in the lutoid-body fraction of rubber latex indicates that much proitein turnover occurs in the latex vessels of the rubber tree.

2. Materials and methods

Lyophilized B serum [13] from rubber latex of clone GT.1 of *Hevea brasiliensis* (collected at plantations in Western Java, Indonesia) was suspended at 4°C in water (4 mg/ml) to which 0.5 mg/ml sodium dithionite was added to inhibit polyphenol oxidases. After centrifugation the solution was 100% saturated with ammonium sulfate and centrifuged again. The precipitate was dissolved in a small volume of 0.2 M acetic acid and submitted to gel-filtration on a column of Sephadex G-25 (Fig. 1). The second peak contained hevein. The first peak was lyophilized and suspended in a small volume 0.04 M sodium borate buffer, pH 8.9, dialyzed overnight against the same buffer and centrifuged, and the solution was submitted to cation-exchange chromatography on a carboxymethylcellulose column CM32 with a gradient of 0.04 to 0.4 M borate buffer, pH 8.9 [14]. Fractions containing protein were pooled, dialyzed against water and lyophilized (Fig. 2). The second peak contained the hevein precursor. The first, unretarded peak contained predominantly the 14 kDa C-terminal fragment of the precursor. It was dissolved in 50 mM sodium acetate buffer, pH 4.0, and submitted to affinity chromatography on a chitin column [15]. The fractions containing proteins not bound were dialyzed again, dissolved in 0.01 M Tris-HCl buffer, pH 7.6, and submitted to anion-exchange chromatography on a Mono-Q column (0.5 × 5 cm) with a gradient of 0 to 0.75 M NaCl in the same buffer using a Pharmacia fast protein liquid chromatography (FPLC) system. The unretarded peak contained the 14 kDa C-terminal fragment of the hevein precursor. The hevein precursor and its C-terminal fragment were also isolated from rubber latex of clone PR-261 of *Hevea brasiliensis*; the salt-free protein peaks obtained after chromatography on CM-cellulose were submitted to reverse-phase HPLC on a nucleosil 10 C18 column (30 × 0.45 cm) with a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid for 60 min at a flow rate of 1 ml/min. The effluent was monitored at 214 nm, and the peaks were collected manually.

SDS-PAGE was performed on 15% gels [16]. Amino acid compositions of proteins were determined after acid hydrolysis in 6 M HCl at 110°C for 24 h by analysis with an LKB Alpha Plus amino acid analyzer. N-Terminal amino acid sequences of proteins and peptides were determined with Applied Biosystems Model 477A Protein Sequencers with on-line Model 120A PTH-Analyzers (Friedrich Miescher-Institut, Basel and Eurosequence BV, Groningen). Molecular masses of peptides were determined as described earlier [6].

Disulfide bridges of proteins isolated from rubber latex of clone PR-261 were cleaved by reduction with tributylphosphine followed by S-pyridylethylation with 4-vinylpyridine [17]. The reduced proteins were digested with trypsin (treated with L-1-tosylamide-2-phenylalanylchloromethane, TPCK) in 0.2 M ammonium bicarbonate at 37°C for 4 h at substrate to enzyme ratios of 100:1 (by mass). Tryptic peptides were isolated by reverse-phase HPLC as described above.

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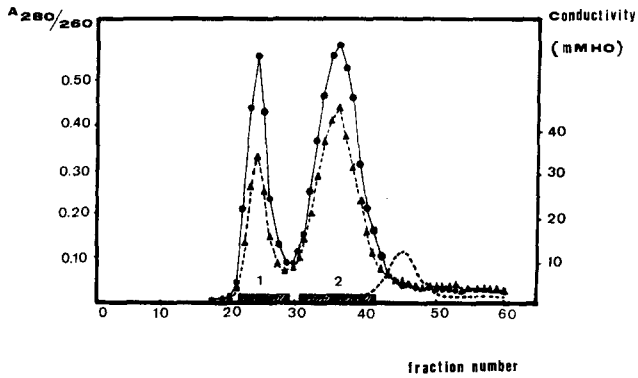

Fig. 1. Gel-filtration of a 100% saturated ammonium sulfate precipitate of 100 mg lyophilized B serum of rubber clone GT.1 on a Sephadex G-25 column (3 × 50 cm). Elution with 0.2 M acetic acid. Fractions of 5.0 ml were collected. ●, A_{280}; ▲, A_{260}; - - -, conductivity. The fractions were pooled as indicated by bars.

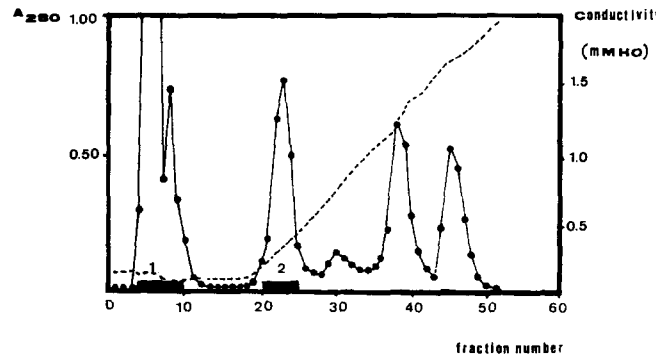

Fig. 2. Chromatography of the first Sephadex G-25 peak (Fig. 1) of 1 g lyophilized B serum of clone GT.1 on a carboxymethylcellulose CM32 column (1.5 × 20 cm). Elution with a linear gradient of 0.04–0.4 M borate buffer, pH 8.9. Fractions of 2 ml were collected and pooled as indicated by bars. ●, A_{280}; - - -, conductivity.

3. Results and discussion

The second peak from the CM-cellulose column had a molecular mass of about 20 kDa and was identified by amino acid analysis and N-terminal sequence analysis (16 residues) as the hevein precursor with a hevein domain at its N-terminus [5]. The unretarded peak had a molecular mass of 14 kDa and was identified by amino acid analysis and sequence analysis (17 residues) as the processed C-terminal part of the hevein precursor.

The two proteins were submitted to tryptic digestion after reduction of their disulfide bridges and pyridylethylation. Peptides were isolated by reverse-phase HPLC and N-terminal amino acid sequences were determined by manual Edman degradation [6]. The C-terminal peptide of the 14 kDa protein was identified by comparing its N-terminal sequence with the translated cDNA sequence of the 14 kDa protein. This peptide was submitted to automatic Edman degradation and was found to have the following sequence:

Gly-His-Leu-Thr-Val-Asn-Tyr-Gln-Phe-Val-Asn-Cys-Gly-Asp-Ser

No heterogeneities were observed in the peptide sequence and the molecular mass of the peptide found by ion-spray mass spectrometry was 1758, in exact agreement with the sequence, confirming that serine is its C-terminal residue. It is identical to residues 159–173 corresponding to the cDNA sequence of the hevein precursor [4], except for the two underlined residues, which are Ile and Asp, respectively, in the latter. We do not know the cause of these differences. The published cDNA sequence may not represent the major component of the hevein precursor, or there may be differences between rubber tree cultivars. A partially sequenced cDNA clone also has these two replacements but, in addition, Glu instead of Gln at position 166 and no basic residue at position 158 (W.J. Broekaert, personal communication), so it does not represent our peptide sequence either. An identical C-terminal tryptic peptide was isolated from the 20 kDa hevein precursor (according to elution position on HPLC, automatic sequence degradation, and molecular mass).

C-Terminal propeptides which are necessary for vacuolar targeting, and which are cleaved off during post-translational processing, have been identified in several proteins [17,18]. It can be concluded from the sequence of its C-terminal tryptic peptide that the hevein precursor may also have a C-terminal vacuolar targeting signal, which is cleaved off in the mature protein. The cleavage position is identical to that in the tobacco CPB20 protein [11], although the residues between which the cleavages occur are different (Fig. 3). Fig. 3 shows some general features and C-terminal sequences of PR-4 proteins. All mature vacuolar or extracellular PR-4 proteins have no or few residues C-terminal to the last half-cystine residue involved in one of the three disulfide bridges in the 14 kDa subunit [7]. Therefore, Ponstein et al. [11] already suggested that the potato Win1 protein probably is an extracellular PR-4 protein with an

PROTEIN	Localisation of the protein	Hevein domain	C-terminal sequence
HEVEIN PRECURSOR (cDNA)		present	C G D S F N - P L F S - V M K S S V I N *
HEVEIN PRECURSOR (processed)	vacuoles	cleaved off	C G D S
TOBACCO CBP20 (cDNA)		present	C G D N M N V - L L S P V D K E *
TOBACCO CBP20 (processed)	vacuoles	uncleaved	C G D N
TOBACCO PR4a (cDNA)	extracellular	absent	C N *
TOMATO PR2 (cDNA)	extracellular	absent	C *
POTATO Win1 (DNA)	unknown	present	C G D N *
POTATO Win2 (DNA)	unknown	present	C G D N V N V P L L S V V D K E *
BARWIN (protein)	extracellular	unknown	C R D

Fig. 3. Comparison of several features of PR-4 proteins including prohevein, the intracellular 20 kDa protein CPB20 from tobacco [11], the extracellular proteins PR4a from tobacco [8,9] and PR2 from tomato [8], the wound-induced proteins from potato [12], and the extracellular PR-4 type protein from barley grain barwin [10]. Gaps introduced to optimize the alignment of the C-terminal sequences of the proteins are indicated by hyphens. *Stop codon in cDNA or DNA sequence.

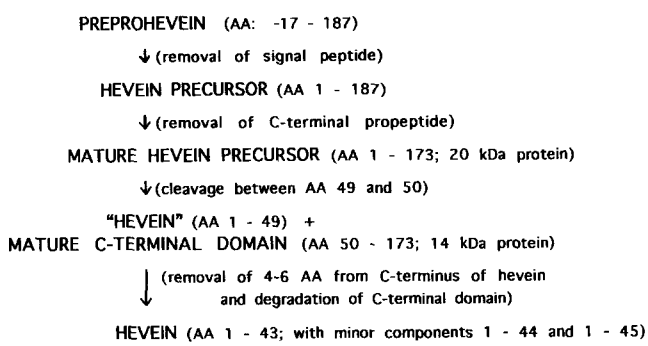

Fig. 4. Scheme with the hypothetical pathway of hevein processing, derived from the cDNA sequence [4], and structural studies ([5,6]; this paper). Mature hevein precursor and the C-terminal domain are major proteins in the lutoid-body fraction of rubber latex and occur in a molar ratio of about 1:30 relative to hevein.

N-terminal hevein domain while the potato Win2 protein may be a vacuolarly located one with a cleaved-off C-terminal targeting signal.

The unretarded peak 1 from the CM-cellulose chromatography contains a small amount of a protein with an apparent molecular mass slightly larger than that of the major 20 kDa hevein precursor. This protein was purified by reverse-phase HPLC and was found to have the N-terminal hevein sequence. As there is only one methionine residue in the published cDNA sequence of the hevein precursor, located near its C-terminus (Fig. 3), we submitted this slightly larger 20 kDa protein to CNBr digestion followed by automatic sequencing of the digest. However, no additional N-terminal sequence could be identified. Therefore, we could not decide if this protein is the hevein precursor with uncleaved C-terminal targeting sequence or a processed variant with slightly higher apparent molecular mass.

Rubber latex contains 5–10 g/l protein, about one third of which occurs in the lutoid-body fraction [20,21]. More than half of the latter is hevein (1–2 g/l latex). Protein recoveries indicate that the 14 kDa protein and the 20 kDa precursor of hevein occur in the lutoid-body fraction in about equal quantities and in a molar ratio to hevein of about 1:30. This means not only that the precursor of hevein is processed at several positions, with removal of the signal peptide, the C-terminal targeting peptide and 4–6 residues between the hevein and 14 kDa domains (Fig. 4), but also that most of the latter domain disappears from the lutoid-body fraction by further proteolysis or other processes. A moderately productive rubber tree exudes about 100 ml latex during one tapping, which is completely regenerated within 3 days [20]. During this period there is a net synthesis of about 0.5–1 g protein, including 0.1–0.2 g hevein. However, for the production of this quantity of hevein, there has to be an additional turnover of 0.5–1 g of its precursor, which indicates much higher levels of total protein synthesis.

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