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The patatin-like protein from the latex of *Hevea brasiliensis* (Hev b 7) is not a vacuolar protein

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Abstract

Upon centrifugation, rubber latex is divided into a layer of rubber particles, the cytosol, and the lutoid-body fraction, which is of vacuolar origin. One of the proteins isolated from the lutoid-body fraction is a protein with a molecular mass of 43 kDa, which has esterase activity on *p*-nitrophenylpalmitate and which shows significant sequence similarity with patatin, a vacuolar protein with esterase activity from potato (*Solanum tuberosum*). This protein is a major allergen in rubber latex products (Hev b 7) and can also be isolated from the cytosol fraction of rubber latex. The mature protein isolated from lutoid-bodies has no structural features expected for a vacuolar protein: the N-terminal methionine in the cDNA-derived sequence is cleaved off, the second residue is *N*-acetylated, and the C-terminal sequence is identical to that in the cDNA-derived sequence. Thus the patatin-like protein in *Hevea brasiliensis* is not a vacuolar protein, but may be associated with not yet characterized particles in the cytoplasm, which either sediment with lutoid-bodies or remain in the cytosol fraction, depending on the centrifugation conditions.

Keywords: *Hevea brasiliensis*; *Euphorbiaceae*; Rubber latex; Lutoid-bodies; Vacuoles; Patatin

1. Introduction

Latex of the rubber tree (*Hevea brasiliensis*) is the cytoplasm of specialized cells known as laticifers. The major components of latex are rubber particles, cytosol and organelles called lutoids, which are organelles of vacuolar origin. Upon centrifugation, rubber latex is divided into a layer of rubber particles, the cytosol, and the lutoid-body fraction (Moir, 1959). The latter fraction is composed mainly of lutoids, but may also contain other organelles. The major proteins in the lutoid-body fraction have been isolated and characterized (Subroto et al., 1996, 2001). Comparison of cDNA and mature sequences of these proteins show that after translation they are processed with removal of N-terminal signal and C-terminal putative vacuolar-targeting peptides (Subroto et al., 2001).

In a previous paper (Subroto et al., 1996) we described the isolation of a 43 kDa protein from the lutoid-body fraction, which had a blocked N-terminal sequence and from which a short CNBr-peptide could be isolated and sequenced that had significant similarity with a sequence in patatin, a major storage protein from potatoes (Rosahl et al., 1986). Independently from us Beezhold et al. (1996) have isolated a protein of similar size from rubber products, which causes rubber allergy, and also concluded that this protein is a homologue of potato patatin from sequence similarity of an unblocked N-terminal sequence and of a large internal CNBr-peptide, adjacent to the short one isolated by us. As potato patatin is a vacuolar protein with fatty acyl esterase and lipid acyl hydrolase activity (Racusen, 1984), and probably is part of a defence mechanism against pathogens, it was no surprise for us to find a homologue of this protein in the lutoid-body (vacuolar) fraction of rubber latex together with several pathogenesis related (PR) proteins.

However, later Yusof et al. (1998) described the isolation of an inhibitor of rubber biosynthesis also with a molecular mass of about 43 kDa, but from the cytosol (C-serum) of rubber latex. They isolated and sequenced several peptides of this protein, which has a blocked

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N-terminus, and also found significant homology with potato patatin. Finally, decisive evidence about the identity of the patatin homologue in rubber latex came from cDNA sequences of the rubber latex allergen Hev b7 determined by Sowka et al. (1998) and Kostyal et al. (1998), which confirmed the previous peptide sequences of 43 kDa proteins, obtained both from the lutoid-body fraction (Subroto et al., 1996), from rubber latex products (Beezhold et al., 1996), and from the cytosol fraction of latex (Yusof et al., 1998). However, the cDNA sequence lacks an N-terminal signal peptide sequence, which indicates that the protein is synthesized on free ribosomes and is expected to be present in the cytosol, in agreement with C-serum localization of the protein isolated by Yusof et al. (1998), but not with the vacuolar one described by us (Subroto et al., 1996).

Here we describe the results of more extensive studies on the patatin-like protein of 43 kDa, isolated from the lutoid-body fraction of latex of several rubber clones. The protein exhibits esterase activity and primary structure studies show no differences with the cDNA-derived sequences, except for absence of the N-terminal methionine and the presence of an *N*-acetyl group on the second residue. The protein is almost certainly not localized in lutoid bodies. However, it coprecipitates with these organelles under certain centrifugation conditions, which suggests some kind of association with not yet characterized particles in the cytoplasm.

2. Results

2.1. Isolation of the patatin-like protein (Hev b 7) from lutoid-body preparations

Proteins in an aqueous extract of lyophilized B-serum from clone PR 255 were precipitated by 100% saturation with ammonium sulfate, dissolved again in 0.1 M Tris buffer, pH 8.0, brought on a column of CM-cellulose and the unretarded peak was submitted to ion-exchange chromatography on DE-cellulose in the same buffer and gradient elution with NaCl (Fig. 1). The isolation procedure was followed by esterase activity measurements on *p*-nitrophenylpalmitate. A pure preparation of the patatin-like protein with a molecular mass of about 43 kDa (Fig. 2; lane A), and which shows esterase activity was obtained in a recovery of 80%. The amount of this protein was about 10 mg per gram lyophilized bottom fraction. Similar results were obtained starting with lyophilized B sera of rubber clones GT.1 and PR 261.

An unfolded form of the patatin-like protein with a molecular mass of 43 kDa could also be isotated from B-sera by reversed-phase HPLC with an elution position between those of hevamine and β-1,3-glucanase (see Fig. 5 in Subroto et al., 2001).

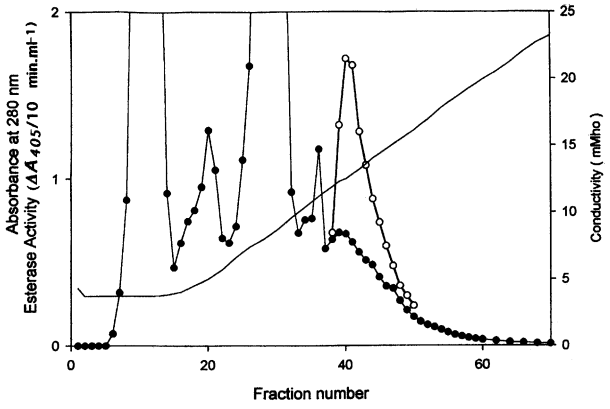

Fig. 1. Chromatography of acidic and neutral proteins from the lutoid-body fraction of 2.4 g lyophilized B-serum of latex of clone PR 255 of *Hevea brasiliensis*, on a DEAE-cellulose DE52 column (1.5×30 cm). Elution with a linear gradient of 0.0–0.5 M NaCl in 0.1 14 Tris–HCl buffer, pH 8.0. Fractions of 5 ml were collected. —•—, absorbance at 280 nm; —0—, esterase activity on *p*-dinitrophenylpalmitate; ——, conductivity.

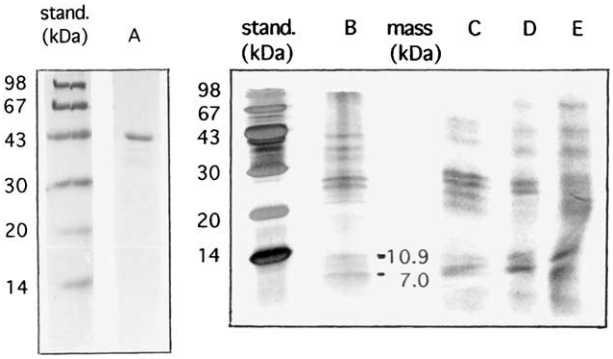

Fig. 2. SDS-PAGE on 15% gels, protein or peptide bands detected with Coomassie Brilliant Blue (lane A) or by silver staining (lanes B–E). (A) Purified patatin-like protein (43 kDa) from B-serum of clone PR 255. (B) CNBr digest of patatin-like protein from B-serum of clone PR 255. The positions of the N- and C-terminal peptides are indicated with masses as determined by mass spectrometry. (C) CNBr digest of patatin-like protein from C-serum of clone RRIM 600 (Hev b 7). (D) Patatin-like protein from B-serum of clone GT. 1 digested with CNBr in 70% formic acid. (E) Patatin-like protein from B-serum of clone GT. 1 digested with CNBr in 70% TFA.

SDS-PAGE patterns of lutoid-body preparations from several rubber clones obtained by centrifugation with the multi-speed attachment of an International PR-2 centrifuge (Bandung, Indonesia) were similar and showed bands with a molecular mass of 43 kDa (Subroto et al., 2001), irrespective of the isolation procedure of these preparations (B-sera prepared by repeated freezing and thawing of lutoid-body particles, followed by centrifugation, or direct lyophilization of these particles, followed by homogenization). However, freeze-dried bottom fractions of clone RRIM 600, obtained by ultracentrifigation of latex (kindly donated by Dr. H. Y. Yeang, Rubber Research Insitute of Malaysia), exhibited no or very weak protein bands with a molecular mass of 43 kDa (Lee et al., 1991; Subroto et al., 2001).

Efforts to isolate the protein from this material resulted in a more than 10 times smaller peak with esterase activity at the position where it was found in preparations from the other investigated rubber clones (not shown). In the tail fractions of the peak with esterase activity a protein was found with a mass of about 42 kDa, which stained intensely on SDS-PAGE gels. Initially we thought that this protein was a degradation product of the patatin-like protein. But studies of its antigenic properties and primary structure showed that it is unrelated to patatin and Hev b 7 (Dr. H.-Y. Yeang, personal communication). First we ascribed the high and low recoveries of the patatin-like protein from the investigated B-sera to differences between rubber clones, but it is more likely that the use of different centrifugation conditions to isolate lutoid-body fractions may explain the results.

2.2. Amino acid sequence studies

Studies on the amino acid sequence of the isolated patatin-like protein from lutoid-body fractions concentrated on finding identities and differences with the Hev b 7 sequences derived from cDNA studies (Sowka et al., 1998; Kostyal et al., 1998). These studies were performed on digests made by cleaving the protein at methionine residues with CNBr. The cDNA derived sequence of 388 residues has methionines at positions 1, 69, 80, 263 and 292, of which residue 263 is followed by a threonine residue with no cleavage by CNBr in 70% formic acid. This means that CNBr digestion of the cDNA derived sequence will yield an N-terminal peptide of 68 residues, a C-terminal one of 96 residues, the already demonstrated small peptide of eleven residues (Subroto et al., 1996), and peptides of 29 and 183 or 212 residues, respectively, depending on the cleavage conditions.

CNBr digests of several preparations of the protein showed reproducible SDS-PAGE patterns with a number of bands in the region of 20–30 kDa, and two bands with lower molecular masses in approximate agreement with the sizes of the N-terminal and C-terminal peptides expected from the cDNA sequence (Fig. 2; lane B). A preparation of the C-serum Hev b 7 protein (Yusof et al., 1998), kindly donated by Dr. H.-Y. Yeang (Rubber Research Institute of Malaysia), showed after CNBr digestion a similar pattern as the B-serum derived proteins (Fig. 2; lane C). Cleavage of the protein with CNBr in 70% TFA shifted several bands in the 20–30 kDa region to lower masses compared to the cleavage in 70% formic acid, confirming the presence of the Met–Thr bond (Fig. 2; lanes D and E).

The gel filtration pattern of the CNBr digest of the patatin-like protein from clone PR 255 on Sephacryl S200 in 0.1 M Tris buffer, pH 8.0 and 8 M urea, and SDS-PAGE patterns of selected fractions, are shown in Fig. 3. A peptide with a molecular mass of about 7.0 kDa (mass spectrometry) was obtained by reversed-phase HPLC of fractions 61–66. One of its tryptic subpeptides had a blocked N-terminal residue and yielded the sequence of residues 2–12 of the cDNA derived sequence of Hev b 7, with an additional *N*-acetyl group (Fig. 4). A peptide with a molecular mass of about 10.9 (mass spectrometry) was obtained by reversed-phase HPLC from fractions 54–58. Digestion by the glutamic acid specific protease from *Staphylococcus aureus* yielded a peptide with a sequence identical to that of the C-terminus of that derived from Hev b 7 cDNA (Fig. 4). Major peak fractions with the large centrally located CNBr peptide(s) were also digested with the glutamic acid specific protease and after reversed-phase HPLC two peptides with very similar sequences were obtained (Fig. 4), which could be assigned to an internal sequence in the cDNA derived sequences of isoforms S2 and D1 of Hev b 7 (Sowka et al., 1999).

3. Discussion

We repeated our previously (Subroto et al., 1996) described isolation of the patatin-like protein from the lutoid-body fraction (B serum) of rubber latex. Like patatin (Van Koningsveld et al., 2001) the protein is irreversibly precipitated at low pH values. Therefore, acidic conditions were avoided in the isolation procedure. It was essential that no polyphenoloxidase products were present in the starting material or are formed during the first steps of the isolation procedure, because they cannot be removed from the isolated protein, lower the specific enzymic activity and cause bands on SDS-PAGE gels in the 25 kDa region, which interfere with structural studies on CNBr digests. Therefore sodium dithionite was added not only during extraction of the freeze-dried lutoid body preparation, but also to the buffer used for ion exchange chromatography.

Structural studies of the protein aimed at finding identities and differences with amino acids derived from published cDNA sequences of Hev b 7 (Sowka et al., 1998; Kostyal et al., 1998). The blocked N-terminus of the protein can be accounted for by removal of the N-terminal methionine in the cDNA sequence and acetylation of the following alanine residue, which is a frequently observed posttranslational modification of cytoplasmic proteins. [The free N-terminal sequence reported in an earlier paper by Beezhold et al. (1996) may have resulted from proteolytic digestion. It is interesting that this sequence starts at almost the same position as processed potato patatin (Rosahl et al., 1986).]

The C-terminal amino acid sequence identified by us also shows that no vacuolar targeting signal is removed in contrast to the other investigated proteins from the lutoid-body fraction of latex of *Hevea brasiliensis* (Subroto et al., 2001).

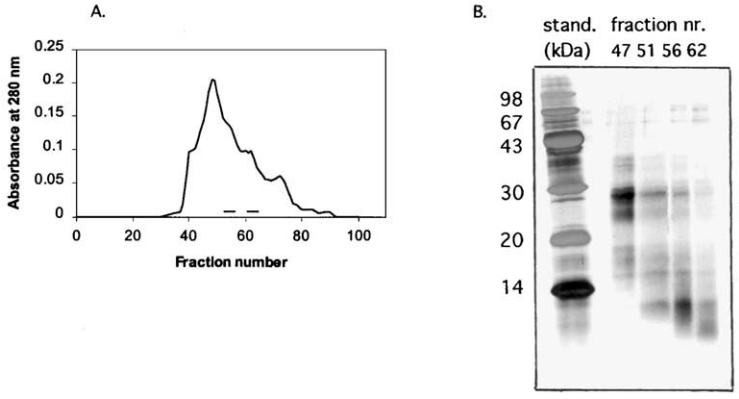

Fig. 3. A. Gel filtration of a CNBr digest of 3 mg of patatin-like protein from B-serum of clone PR 255 on a Sephacryl S-200 column (1.0×2×l40 cm). Etution with 0.1 M Tris–HCl buffer, pH 8.0 in 8 M urea. Fractions of 1 ml were collected. Fractions used for isolations of N-terminal and C-terminal peptides, respectively, are indicated with bars. B. SDS-PAGE patterns of selected fractions on a 15% gel. Detection of peptide bands by

	Molecular mass found	Molecular mass expected	Sequence evidence
2 12 Acetyl A T G S T T L T Q G K	1105	1105	mass spectrometry (MSMS)
70 80 (M) L T A P N E D K K P "homoserine"			sequencer (Subroto et al., 1996)
203 210 220 224 (E) L I D G G V A A A N P T L L A L T H I R N E (isoform S2)	2260	2259	mass spectrometry (MSMS)
203 210 220 224 (E) L I D G G V A A T N P T L L A L T H I R N E (isoform D1)	2289	2288	mass spectrometry (MSMS)
382 388 (E) R K L R Q L K	942	940	sequencer

Fig. 4. Primary structures of investigated peptides of patatin-like protein isolated from the lutoid-body fraction of latex of clone PR 255 and sequence or molecular mass comparison with identical peptide sequences of the cDNA-derived sequences of Hev b 7 from clone RRIM 600.

Sowka et al. (1998) also made a construct of the cDNA sequence of Hev b7 with an N-terminal leader peptide and expressed it in the yeast *Pichia pastoris,* which resulted in an extracellular glycosylated protein of about 47 kDa with esterase activity. They also demonstrated that natural Hev b 7 isolated from rubber latex C-serum does not contain carbohydrate, although the sequence contains two *N*-glycosylation sites. These observations confirm that the protein is not synthesized on endoplasmatic reticulum associated ribosomes. The molecular mass of the protein isolated by us (43 kDA; Fig. 2A) is similar to those of the non-glycosylated preparations or the protein as isolated by Sowka et al. (1998) and Yusof et al. (1998).

Other homologous sequences of patatin-like proteins in the database are those of tobacco (*Nicotiana tabacum,* U68484) and *Arabidopsis thaliana* (AY128840). The sequence of potato patatin can be divided in three parts:

1. An N-terminal signal peptide sequence of about 20 residues, also present in tobacco, but not in *Hevea* Hev b7 and also not in the *Arabidopsis* sequence. This indicates that the latter two proteins are synthesized on free ribosomes.
2. The larger part of the sequence of more than 300 residues, located at the C-terminus. Identities with the homologous sequences of tobacco, *Arabidopsis* and *Hevea* in this part of the molecule are about 60, 40 and 35%, respectively.
3. The N-terminal sequence of about 70 residues of mature patatin. Sequence identities with tobacco and *Hevea* Hev b7 are higher than in the C-terminal fragment (73% and 61%, respectively). But this sequence fragment is missing in *Arabidopsis,* except for a short stretch of 13 residues with no or very little identity with the other three. It may be that this sequence constitutes a separate domain in patatin and the patatin-like proteins from tobacco and *Hevea,* but which is lacking in *Arabidopsis.* No other proteins with sequence similarity with this N-terminal fragment of patatin could be found in the database.

The Hev b 7 sequences determined by Sowka et al. (1998) and Kostyal et al. (1998) differ at seven amino

acid positions (isoforms S1 and D1). Recently the two groups sequenced 32 other cDNA clones of Hev b 7 (Sowka et al., 1999), of which 17 corresponded to isoform S1 (Sowka et al., 1998), and eight and two to two new isoforms with four and eight amino acid differences, respectively (S2 and D2). No isoform corresponding to D1 (Kostyal et al., 1998) was identified in this study. All cDNA sequence studies were performed on latex originating from rubber clone RRIM 600. Recombinant Heb b 7 isoform S1 and S2 had similar specific esterase activities on *p*-nitrophenylpalmitate expressed as ΔA_{400} of about 10/10 min×(mg protein)$^{-1}$ (Sowka et al., 1998, 1999), while that of recombinant isoform D2 was 10 times lower. Specific activities of two natural patatin isoforms from *S. tuberosum* expressed in the same units were found to be 1.5 and 0.25 (Hoefgen and Willmitzer, 1990). The specific activity of the protein isolated from the lutoid-body fraction of rubber clone PR 255 as shown in Fig. 1A was about 2 expressed in these units, which is in the same order of magnitude as observed for recombinant preparations of the Hev b 7 protein (Sowka et al., 1998).

There are five Cys residues in the sequence of Hev b 7. Only one of these is also present in the homologous sequences from potato and tobacco (Sowka et al., 1998). No X-ray structure of potato patatin has yet been determined and it is not known if the Cys residues in this dimeric protein are present with free sulfhydryl groups or form one or more intra- or intersubunit disulfide bridges. Likewise we do not know anything about the presence of disulfide bridges in the patatin-like protein in rubber latex. Four Cys residues occur in the centrally located CNBr fragment of 212 residues and may have free sulfhydryl groups or be involved in disulfide bonds. However, the C-terminal CNBr peptide could be isolated in good recovery without prior reduction of the protein. This indicates that this Cys may have a free sulfhydryl group, and it may be that this residue is capable of forming intersubunit disulfide bridges, resulting in a Hev b 7 derivative with molecular mass of about 80–90 kDa (Beezhold et al., 1996).

Two peptides with very similar sequences were obtained from a digest of the large central CNBr peptide of the patatin-like protein with the glutamic acid specific protease from *S. aureus,* one in agreement with the cDNA sequence of isoform S2 and the other with that of D1 (Sowka et al., 1999). It should be mentioned here that in the present study we were not able to isolate the short CNBr peptide isolated and sequenced before, which originated from clone GT.1 (Subroto et al., 1996). In isoform S2 the C-terminal Met of this peptide is replaced by Ile, explaining that this peptide cannot be formed from this isoform. But the peptide should have been present in the digest of isoform D1.

Although the patatin-like protein with a mass of 43 kDa (Hev b 7) from rubber latex could be isolated from lutoid-body preparations, the molecular features indicate that the protein is not synthesized on ribosomes associated with the endoplasmic reticulum. This is in agreement with the fact that the protein could also be isolated from the cytosol fraction (C-serum). Probably different centrifugation conditions used to isolate cytosol and lutoid-bodies from fresh latex may explain observed differences. However, the fact that the protein is also found in lutoid-body preparations indicates that it may be associated with not yet characterized particles localized in the cytoplasm.

4. Experimental

4.1. Isolation of the patatin-like protein

Freeze-dried B-sera of clones GT.1, PR 261 and PR 255 of *H. brasiliensis* (obtained from lutoid-body fractions of latex by centrifugation in the multi-speed attachment of an International PR-2 centrifuge, followed by repeated freezing and thawing of these fractions, and centrifugation and lyophilization of the supernatants) were kindly supplied by Dr. Toto Subroto, Laboratorium Biokimia, Universitas Padjadjaran in Bandung (Indonesia).

Lyophilized B-serum was suspended and lyophilized bottom-fraction was homogenized (Ultra Turrax, Janke & Kunkel, Staufen, F.R.G.) at 4 °C in 200 ml water to which 0.5 g l^{-1} Na dithionite was added to inhibit polyphenol oxidases. After centrifugation, the solutions were 100% saturated with ammonium sulfate and centrifuged again. The precipitates were dissolved in small volumes of 0.1 M Tris buffer, pH 8.0, to which also 0.5 g l^{-1} Na dithionite was added, and after centrifugation submitted to cation exchange chromatography on a carboxymethylcellulose CM32 column, with a gradient of 0–0.1 M NaCl in the same buffer to isolate the basic proteins hevamine, a thaumatin-like protein and β-1,3-glucanase (Subroto et al., 1996, 2001). Unretarded material was submitted to anion-exchange chromatography on a DEAE-cellulose column, with a gradient of 0–0.5 M NaCl in 0.1 M Tris buffer, pH 8.0, to which also 0.5 g l^{-1} Na dithionite was added, which yielded pure protein with a molecular mass of 43 kDa. Denatured protein could also be isolated directly from extracts of lyophilized B-serum by reversed-phase HPLC (see below).

4.2. Analytical methods and protein primary structure studies

4.2.1. Esterase activity

The substrate solution was prepared by dissolving 33 mg *p*-nitrophenylpalmitate (Sigma), 1 g Triton X-100 and 170 μl 10% SDS with some heating in 100 ml water.

The assay mixture contained 2 ml substrate solution, 1 ml 0.1 M Tris buffer, pH 8.0, and 20–200 μl of the esterase solution under investigation. The assay was performed at 37 °C with measurement of the absorbance at 405 nm. Linear increases of absorbance were observed. Enzyme quantities were expressed as ΔA_{405} per 10 min and per ml esterase solution.

Protein concentrations were determined as absorbancies at 280 nm. The tryptophan and tyrosine contents of the patatin-like protein (Hev b 7) allow to use these absorbancies as approximate protein concentrations in mg ml^{-1}.

SDS-PAGE of proteins and large peptides was performed on 15% gels (Laemmli, 1970). Reversed-phase HPLC separations of protein or peptide mixtures were performed on a nucleosil 10 C18 column (30×0.45 cm), with a gradient of 0–70% acetonitrile in 0.1% CH_3COOH for 60 min, at a flow rate of 1 ml min^{-1}. The effluents were monitored at 214 nm, and the peaks were collected manually. N-terminal amino acid sequences of proteins and peptides were determined with an Applied Biosystems Model 477 A protein sequencer with an on-line Model 140A PTH analyser. Masses of peptides were determined by MALDI-TOF mass spectrometry and primary structures by mass spectrometiy of the peptide, followed by determination of masses after fragmentation (MSMS).

Protein samples were cleaved with CNBr (1.5 mg mg $protein^{-1}$) in 70% formic acid or 70% trifluoroacetic acid (0.4 ml mg $protein^{-1}$) at room temperature under nitrogen for 24 h. After digestion, the cleavage mixtures were lyophilized several times from water and investigated by SDS-PAGE in the presence or absence of β-mercaptoethanol.

A CNBr digest of 3 mg patatin-like protein from rubber clone PR 255 was dissolved in a small volume 0.1 M Tris buffer, pH 8.0 in 8 M urea and submitted to gel filtration on a column of Sephacryl S200 (Fig. 3). Fractions were investigated by SDS-PAGE and reversed phase HPLC. The N-terminal CNBr peptide of the protein of about 6 kDa in fractions 61–66 was isolated by preparative HPLC and lyophilized (about 30 μg), dissolved in 0.5 ml 0.2 M ammonium bicarbonate and digested with 0.6 μg trypsin (treated with L-tosylamide-2-phenylalanyl-chloromethane, TPCK) at 37 °C for 4 h. Subpeptides were isolated by reversed-phase HPLC. In a similar way the C-terminal CNBr peptide of about 10 kDa was isolated from fractions 54–58 by reversed-phase HPLC and digested with the glutamic acid specific protease S from *S. aureus* at 37 °C during 20 h and subpeptides were isolated. Part of the fractions (45–53) with the large, centrally located peptides of the protein in 8 M urea (about 1 ml) was slowly added to 3 ml Glu specific protease (50 μg in 0.2 M ammonium bicarbonate), digested overnight at 37 °C. Several subpeptides were isolated by reversed-phase HPLC and submitted to further analysis.

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