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TMV-particle borne enhancer of a tobacco RNA-replicase

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Abstract
According to separation studies it has been evident that a tobacco RNA-replicase after TMV-infection consists of two parts. The larger part is host-directed and will be combined with a virus borne small protein which considerably enhances the RNA-replicase activity and is therefore named replicase enhancer, Ree. This compound was found at HPLC-separations of TMV-coat proteins, and was detected using polymerase assay with a radioactive nucleotide involved. Molecular weight has been determined by mass spectrometry: with FT ICR MS to get the size – 6 023.3 - and with MALDI TOF MS to obtain a sequence of the 54 amino acids involved. Presumably Ree is fixed to the TMV-RNA at infection. The enzyme seems to produce minus-strands of the virus RNA.

Key Words: Enhancer, mass spectrometry, replicase, TMV, tobacco

INTRODUCTION

A soluble tobacco replicase (*i.e.* RNA dependent RNA polymerase) was obtained from fresh systemically TMV-infected tobacco leaves (TMV strain *"Flavum"; Nicotiana tabacum* L. var. Samsun) after a series of purification steps (Brishammar and Juntti, 1974). The molecular size of the enzyme was determined to be 130 000 using sedimentation analysis (Brishammar and Juntti, 1974) or by electrophoresis ((Romaine and Zaitlin, 1978; Ogawa *et al.,* 1991).It has been noted that the enzyme does not tolerate freezing which leads to a division of the enzyme into two parts. When preparations from frozen leaves are gel filtered over Sephadex G-75 a bigger part of the sample appears under the void peak and a smaller part after about two void volumes in the separation curve (Fig.1A) (Brishammar, 1976). The enzyme activity was followed by an assay where one of the four NTPs is radioactively marked (*i.e.*^{3}H-UTP) (Brishammar and Juntti, 1974) and expressed in cpm (*i.e.* counts per minute). The activity peak obtained has a very sharp and constricted profile. A division was also acquired when the purified replicase was gel filtered on Sephadex G-75 and the typical peak appeared (Fig.1B) (Brishammar, 1976). This division of the enzyme also brought about a drastic drop of the enzyme activity (from ~ 195 000 cpm to ~ 2 000 cpm). The second peak always had the same, limited enzymatic activity. The unity of these two enzymatic parts is surely dependent on hydrophobic interactions, since the enzyme does not stand low temperatures. The smaller part seems to be the replicase enhancer (Ree) inasmuch as its detachment from the bigger enzymatic part reduces the activity to only a few per cent of the normal activity levels. With material from uninfected tobacco leaves, this smaller component was absent but some replicase activity could be detected in the void volume at gel filtering over Sephadex G-75 (Brishammar, 1971**)**.

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MATERIAL AND METHODS

Enzyme purification

Enzyme purification of the prepared tobacco leaf extracts was performed by gel filtration on an 8% agarose gel (BioRad A-1.5) and then by affinity chromatography through Sepharose 2B coupled to yeast RNA (Brishammar and Juntti, 1974).

Enzyme detection

The enzymatic activity was determined according to Brishammar and Juntti (1974) by an assay where one of the four NTPs is radioactively marked (*i.e.*^{3}H-UTP) and expressed in cpm (*i.e.* counts per minute).

Mass Spectrometry 1

FT ICR MS – (*i.e.* Fourier Transform Ion Cyclotron Resonance Mass Spectrometry) is an electrospray ionization technique of mass spectrometry to produce ions (Bergquist *et al*., 2002). 1µL of the peak activity fraction (Fig. 3) was used as sample.

Mass Spectrometry 2

MALDI TOF MS (*i.e.* Matrix-Assisted Laser Desorption/Ionization–Time-Of-Flight Mass Spectrometry) was done according to Bergquist *et al*., 2002.

Chemicals and reagents

Acetonitrile (ACN, acetic acid (HAc), ammonium bicarbonate ($NH_4 HCO_3$) were obtained from Merck (Darmstadt, Germany). Iodoacetamide (IAA), urea and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) was obtained from GE Healthcare (Uppsala, Sweden). High

purity water was taken from a Milli-Q (Millipore, Bedford, MA, USA) purification system.

Protein Digestion

The sample was reconstituted in a volume of 100µL denaturation buffer (8 M urea, 400 mM NH_4HCO_3), followed by addition of 10µl DTT (45 mM) and incubation at 55ºC for 15min for protein reduction. For alkylation a volume of 10µL IAA (100mM) was added followed by incubation at RT in darkness. 25 µg sequence grade trypsin from bovine pancreas (Roche, Basel, Switzerland) were reconstituted in 250µL ddH_2O to give a final concentration of100 ng/µL. A volume of 20µL Trypsin solution (2µg, 1:25 w: w) was added to the protein solution and incubation at 37ºC overnight. The samples were desalted on ZipTip® C_{18} columns (Millipore, Bedford, MA, USA), according to a protocol described in detail elsewhere (Bergquist *et al*., 2002).

MALDI TOF MS

An aliquot of 1µL was spotted on a MALDI ground steel target and allowed to dry down. 1µL of 4-hydroxy-alpha cyanocinnamic – matrix solution (10 mg/mL, 30% CAN, 0.1 % TFA) was added onto of the dried sample spots. Mass data was acquired with Ultraflex II MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) in reflector positive mode. A mass range of 700-4000 Da was analyzed with a sum of 1000/shots/spot. The laser frequency was set to 100 Hz using a large laser focus. MALDI-TOF/TOF tandem MS analysis was performed in LIFT mode by software-controlled data acquisition. Here, 500 shots were acquired in PARENT mode at normal laser energy level but enabled precursor ion selection resulting in intact precursor ion spectra. A one point calibration was performed before switching to FRAGMENT mode. Here 2000 shots/spot with 35% increased laser energy were acquired to give the fragmentation spectra. Post LIFT motherion suppression was applied to deflect the precursor and elevate fragment ion intensity.

Data Processing and Protein identification

Peptide monoisotopic signals were analyzed using the SNAP algorithm, implemented in the Flex Analysis (v. 3.0) software (Bruker Daltonics). The spectra were calibrated externally using calibrants-spots that have been prepared adjacent to the sample spots containing a calibration mix with the following monoisotopic signals: Angiosin-II, [M+H]+, 1046.54180; Angiosin-I, [M+H]+, 1296.68480, Substance P (M+H)+, 1347.73540, Bombesin (M+H)+, 1619.82230, ACTH clip (1-17), [M+H]+, 2093.08620; ACTH clip (18-39), [M+H]+,2465.19830; Somatostatin, [M+H]+, 3147.47100. For protein identification, MS and MS/MS data were run submitted to the BioTools software (v.3.1 SR2, Bruker Daltonics). MS data were submitted to peptide mass fingerprint search using the Mascot search engine version 2.2 (Matrix Science, London, UK). The following specified parameters were applied for database-search: database (Swiss Prot v. 51.6); taxonomy (virus); proteolytic enzyme (Trypsin); peptide mass tolerance (±100 ppm); peptide charge state (1+); instrument (MALDI TOF)); max missed cleavage (1); fixed modifications (carbamidomethyl) and variable modifications (oxidation Met). Tandem MS (MS/MS) data were searched by Mascot MS/MS ion search using the same settings as described for PMF search with a set fragment mass tolerance of ± 0.8 Da. False positive identification was estimated by searching every dataset against a decoy database using the corresponding feature implemented in the Mascot software.

RESULTS

The smaller part, which provides a limited amount of polymerase activity, can be isolated from frozen TMV-infected tobacco leaves (Fig.1A) (Brishammar, 1970) and thus must tolerate temperatures below zero. To obtain the intact holoenzyme of this replicase, fresh infected leaf material is required for the purification procedure (Brishammar and Juntti, 1974). In both cases the separation conditions will have to be perfectly balanced. At chromatography *e.g*. the material of the end plates shall not be too hydrophilic or too hydrophobic.

The RNA-replicase enhancer, Ree, was surprisingly detected among the coat proteins of the TMV-particle after removal of the nucleic acid by an acid procedure according to Fraenkel-Conrat(1957) or by an alkali treatment (Onda*et al*.,1970). HPLC on inert protein columns made it possible to separate Ree from the viral capsomers and it was detected byreplicase assay and then the typical peak of enzyme activity was obtained (Figs. 2 and 3).

This component evidently exists in extremely low numbers since no peak could be seen by UV-absorbancy at 280 nm and probably there is only one molecule of Ree per virus particle. It has not been noticed by crystallography, electron microscopy and evidently not either with the use of antibodies. It has never been possible to scale up the purification of Ree since this compound only tolerates distinctly selected conditions of separation media. A variety of separation conditions have been tested while the enzymatic activity fails to come off. Therefore ion exchangers and reversed phase columns were not possible to use because quite inert column materials are required.

To obtain a characterization of Ree it was necessary to perform analysis by mass spectrometry. Unfortunately the distinct HPLC separation on the Waters column (Fig. 2) required Tris-buffer, which disturbs the MS-measurements. Therefore, another type of protein column, SUPELCO SigmaCrom GFC-100 protein Column was used, which made it possible to perform a separation with a low ion-strength buffer and at a higher pH (Fig. 3). With the use of mass spectrometry method with very low detection limit – FT ICR MS – (*i.e*. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry) – it has been possible to gain a determination of the molecular size of Ree with very high accuracy. A signal indicating a value of 6 023.3 was obtained. The same fraction was also run in equipment for MALDI TOF MS (*i.e.* Matrix-Assisted Laser Desorption/Ionization–Time-Of-Flight Mass Spectrometry) to obtain a sequence of the 54 amino acids involved:

SDNFTDNAKTIIVQLKEAVEINCTRPNNNTRKSIPIGPGRAFYTTGDII GDIRQ

Except from the capsomers, no other proteins than Ree could be detected.Ree exhibits characteristics which keeps it apart from the coat protein. Any enzymatic activity could not be detected in the sample added to the HPLC-column. It has been discussed if the soluble cell replicase in non-infected tobacco leaves is responsible for the viral RNA-production after stimulation from the virus (Ikegami and Fraenkel-Conrat, 1978; Fraeenkel-Conrat, 1979; De Zoeten, 1995).With the knowledge of the existence of the smaller active, viral

component (here called Ree), which is answering to the replicase assay, it might be probable. However, it should be stressed that a restoration of the high replicase activity from the intact holoenzyme of this tobacco RNA-replicase so far has not been achieved *in vitro* by fusion of the bigger and smaller components of the enzyme.

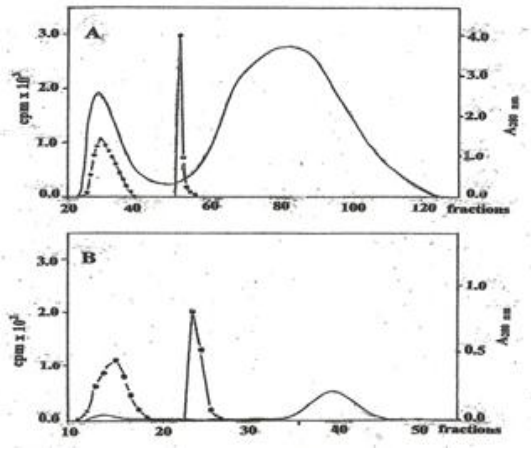

Fig 1. Gel filtration on Sephadex G-75; buffer 50 mM Tris-HCl pH 7.2 + 15 mM β-mercaptoethanol; Continuous line: UV-absorbancy at 280 nm; - o - : replicase activity: expressed as cpm. A. Sample: Extract from frozen, TMV-infected leaves; Column: 3.2 x 16.5 cm; B. Sample: Purified replicase; Column 2 x 15.2 cm

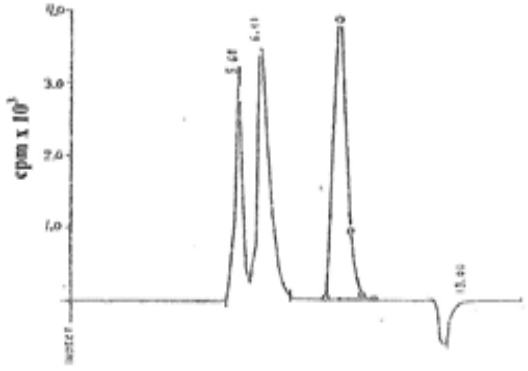

Fig 2. HPLC-separation on Waters Protein Column I-125; Scan Wawelenghth 280 nm; buffer: 10 mM Tris-HCl, pH 7.6 Fractions: 0.5 ml; Flow rate: 1 ml/min; Registration rate: 1 cm/ml; - o - : replicase activity; expressed as cpm: Sample: 15 µl from TMV-protein preparation (corresponding 120 µg protein)

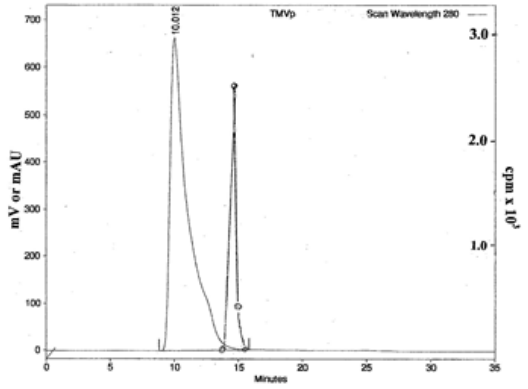

Fig 3. HPLC-separation on SUPELCO SigmaCrom GFC-100 protein Column; buffer: 0.1mM NH_4HCO_3, pH7.8; Continuous line: UV-absorbancy at 280 nm; - o- : replicase activity expressed as cpm

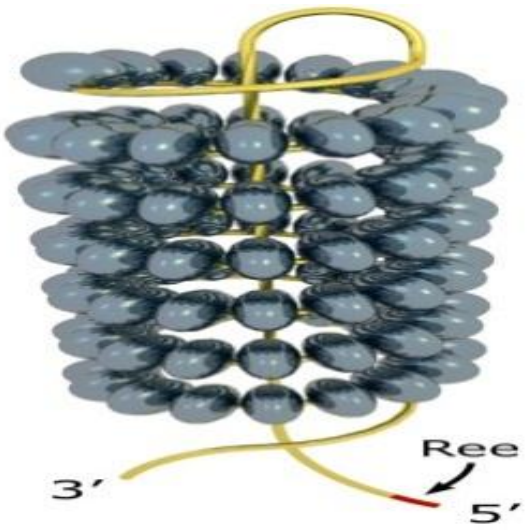

Fig 4. Sketch of the TMV-particle with the RNA (in red) inside and with Ree (in blue) fixed to its 5´end. (N.B. TMV has a length of 300 nm and a diameter of 18 nm)

DISCUSSION

Ree is presumably fixed to the 5´-end of the RNA, inside the intact TMV-particle and hidden at antibody production towards TMV (Fig. 4). An attachment of Ree to the viral RNA is probably a requisite for a fast production of the minus viral RNA-strands which will be used as templates when the TMV-RNA-dependent RNA-polymerase (Mas and Beachy, 1999) makes the complementary plus-strands.

Infectivity with viral nucleic acid was reported (Fraenkel-Conrat*et al*., 1957) but the amount of RNA required to cause infection was very big and it is likely that Ree anyhow could have been attached to any of the RNA-strands.

Some researchers have earlier maintained that the RNA-dependent RNA-polymerase in non-infected tobacco-leaves could be responsible for the production of any type of viral RNA. That idea has been criticized since the activity of this enzyme is very low. However, when the small,- enhancing protein,-Ree, from the virus-particle, combines with this cellular enzyme the RNA-synthesis significantly increases. There is a problem that the amount of Ree is extremely low and has been difficult to detect. Anyhow, it is quite probable that a component like Ree might be found in many other plant viruses and also in viruses attacking animals and man.

A number of functions are ascribed to the capsid proteins (Callaway *et al*., 2001)but some of them may be preserved by Ree, which of course has not been detected among them before.

The tobacco RNA-replicase produces the minus-strand of TMV-RNA. The main part of this enzyme is host-directed, but its activity is increased by a TMV-particle borne enhancer with a particular amino acid sequence. Data from “Network ProteinSequence Analysis” reveal that this amino acid sequence has been detected in at least 26 different proteins, discovered in eucaryotic cells, in bacteria and in viruses. Therefore it is quite probable that its outermost origin is from the host cell and its code is from there and not from the viral nucleic acid.

In Fig. 2 Ree distinctly lies apart from the coat proteins and this may indicate an adhesion to the virus RNA. Its presumed fixation to the 5´-end of the viral plus-strand is anything Ree shares with VPg

- the primer protein at RNA-dependent RNA polymerization by picorna-viruses (Flint *et al*., 2012).

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