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Influence of antiviral factor on tobacco mosaic virus RNA biosynthesis in tobacco

M. ŠINDELÁŘOVÁ and L. ŠINDELÁŘ

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, CZ-16000 Prague 6, Czech Republic

Abstract

An antiviral factor (AVF) was separated by removing virus particles from extracts of tobacco mosaic virus (TMV) infected leaves using calcium phosphate gel and by column chromatography on DEAE cellulose. AVF was not found in the extracts from healthy plants. The AVF restricted the virus infectivity *"in vivo"* and significantly decreased the activity of key enzymes of metabolic pathways tending to the purine and pyrimidine nucleotides biosynthesis of viral-RNA (glucose-6-phosphate dehydrogenase, ribonucleases, phosphomonoesterase and phosphodiesterase). No inhibition of these enzymes was observed *"in vitro"* when the effect of different concentrations of AVF (0.25 - 250 μg cm^{-3}) was examined.

Additional key words: *Nicotiana tabacum*, AVF, protein, resistance, rRNA, virus RNA.

Introduction

Resistance can be defined as the ability of plants to prevent or restrict virus multiplication. We may distinguish between *passive preformed defences* developed in advance of infections, and *active defences* which generally develop after infection. The former type may include examples such as mechanical barriers, or lack of metabolites required for virus multiplication (Loebenstein *et al.* 1984).

Active host resistance generally develops after infection, and probably requires induction of transcription of a gene or genes in the host. This may lead to structural changes forming physical barriers to virus spread, or to functional changes that interfere with viral replication or translocation (Loebenstein and Stein 1985, Loebenstein and Gera 1990). The local lesion response is probably the most notable active resistance phenomenon, whereby after inoculation the virus invades and multiplies in several hundred cells but does not spread to other tissues.

Resistance is also induced in uninoculated parts of a hypersensitive host by inoculating other parts of the plant with necrotic local lesion-producing viruses (Loebenstein 1972), fungi (Kuč 1983) and bacteria (Jenns *et al.* 1979). Lesions developing after challenge inoculation of the resistant tissue are consistently smaller, and usually fewer in number, than those formed on previously uninoculated control plants. In tobacco plants with *N* gene, the induced resistance measured by reduction in number and size of lesions, was found to be closely correlated with the virus concentration determined by ELISA (Stein *et al.* 1985). This indicates that the virus replication, and not only the development of necrotic local lesions, was suppressed in the "resistant tissues". However, there have been other reports indicating that the virus multiplication is not suppressed in leaves with induced resistance (Fraser 1979, Coutts and Waigh 1983).

Together with the pathogenesis-related proteins (Van Loon and Van Strien 1999, Šindelářová and Šindelář 2001, Šindelářová *et al.* 2002) many natural inhibitors of virus infection are known to occur in plant tissues (Bawden 1954). Suggestions were made regarding the possible production of distinct inhibitors within the plant tissue as a result of virus infection (Loebenstein 1962).

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Abbreviations: AVF - antiviral factor; CPG - calcium phosphate gel; dpAVF - day post AVF application; dpTMV - day post TMV inoculation; G6P DH - glucose-6-phosphate dehydrogenase; PDE - phosphodiesterase; PME - phosphomonoesterase; RNase - ribonuclease; TMV - tobacco mosaic virus.

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Fax: (+420) 2 24310113; e-mail: sindelarova@ueb.cas.cz

The occurrence of an inhibitor was further demonstrated in virus-infected plants (Sela and Applebaum 1962, Antignus *et al.* 1975, Sela *et al.* 1978). This was discovered by removing virus particles from infected leaf extracts, using calcium phosphate gel, and comparing the antiviral activity of the extracts with that of extracts from healthy plants, treated similarly. The extracts from diseased plants contain a unique inhibitor named "antiviral-factor" (AVF). Loebenstein and Ross (1963), following a different procedure, arrived at similar results. Characteristic features of this type of inhibitors of virus replication were in detail studied by Sela *et al.* (1964, 1978), Antigus *et al.* (1975), Loebenstein and Gera (1990), Akad *et al.* (1999). Watanabe *et al.* (1997) isolated the "pokeweed antiviral protein" (PAP), which belongs to a group of ribosome-inactivating proteins that inactivate ribosomes by depurinating rRNA at a specific site. PAP inhibited protein synthesis in the virus-infected protoplasts and killed the cells, but it had no effect on the uninfected protoplasts. Proteins of this type has both cytostatic and cytotoxic activities as a competitive inhibitor of guanylate cyclase activity (Takemoto *et al.* 1983), inhibited the replication of several animal viruses, *e.g.* Polio, VSV, HSV, Sindbis and other (Andrei *et al.* 1985). The inhibitory activity against Ehrlich tumour was manifested by De Oliveira *et al.* (1993).

In an infected host cell, virus RNA can be synthesised from the intermediates of reductive pentosephosphate pathway during photosynthesis or from the intermediates of oxidative pentosephosphate pathway active preferentially in the dark, or from the intermediates released from degraded host rRNA. These three metabolic pathways are involved in virus RNA biosynthesis, but their participation usually depends on the type of virus, host, and environmental conditions (Šindelářová *et al.* 1997).

This paper presents the results of the detailed study of changes in TMV content and in the key enzymes of host rRNA degradation (ribonucleases, phosphomonoesterases and phosphodiesterases - RNases, PME, PDE) and of oxidative pentosephosphate pathway (glucose-6-phosphate dehydrogenase - G6P DH) in locally-infected leaf tissue induced by AVF application.

Materials and methods

Preparation of crude AVF: Leaves of *Nicotiana tabacum* L. cv. Samsun NN, 2 d after inoculation with the common strain of tobacco mosaic virus (TMV), were harvested and ground in a Waring blendor with an equal amount of 10 mM phosphate buffer pH 7.5 (P-buffer). The sap obtained was squeezed through cheesecloth, centrifuged at 5 000 *g* for 10 min and mixed with about half its volume of calcium phosphate gel (CPG). This mixture was centrifuged at 5 000 *g* for 10 min; the supernatant was recentrifuged after being mixed again with half its volume of CPG. The clear, brown supernatant fluid was collected and tested for infectivity. The solutions were then dialyzed against P-buffer to remove compounds of low molecular mass and freeze-dried with a Lyovac GT2 lyophilizer (*Leybold-Heraeus*, Köln, Germany). A brown, solid material was obtained. This material had antiviral activity and was designated as "crude AVF". The "control material" was prepared by the same procedure from healthy *N. tabacum* L. cv. Samsun NN leaves. The dry materials were stored in sealed tubes at -20 °C.

Column chromatography of AVF: Column of DEAE-cellulose (*DEAE 52 Servacel*, *Serva*, Heidelberg, Germany) was used as ion exchanger. Crude AVF or control (50 mg) dissolved in 2 cm^3 of P-buffer were desalted by centrifugation through *Sephadex G-25 Fine* and applied to the top of the column with 35 cm^3 bed volume previously equilibrated with P-buffer. After washing with one bed volume of P-buffer, the AVF was eluted with 250 cm^3 of a linear gradient of NaCl between 0 and 1 M in P-buffer. Fractions of 10 cm^3 were collected, desalted by centrifugation through *Sephadex G-25 Fine* and assayed for proteins concentrations and antiviral activity by biological test. The active fractions eluted between 0.56 - 0.62 M NaCl were pooled, desalted and lyophilised (DEAE-AVF).

Biological test for AVF activity on TMV content: Purified TMV was prepared as described by Gooding and Hebert (1967). The same volumes of diluted TMV (10 μg TMV cm^{-3}) and crude AVF or control material (0.5 mg of lyophilised powder cm^{-3} distilled water) was used for the inoculation of *N. tabacum* L. cv. Xanthi-nc half-leaves (half-leaves method). Solution of TMV only (final concentration 5 μg TMV cm^{-3}) was applied as control on the opposite half-leaves. The fractions separated by DEAE-cellulose chromatography were directly tested for AVF activity in the same way with exception that the solution of crude AVF was replaced with the same volume of desalinated fraction. AVF activity was calculated as percentage protection on the basis of 0 % protection of control half-leaves inoculated with TMV only (Nitzany and Cohen 1960).

Influence of AVF on TMV multiplication and enzyme activities: Tobacco plants (*N. tabacum* cv. Xanthi-nc) hypersensitively reacting on TMV infection were used for the time response of TMV multiplication and enzyme activities to the DEAE-AVF application. The entire fully developed leaves from medium insertion were locally treated in the following ways: *1*) mock-inoculated with

distilled water (healthy control), *2*) inoculated with purified TMV diluted to 5 μg TMV cm^{-3} (TMV-inoculated), and *3*) TMV (final concentration 5 μg cm^{-3}) inoculated and DEAE-AVF (final concentration 0.25 mg cm^{-3}) treated (TMV+AVF).

Preparation of homogenates for the determinations of enzyme activities: Homogenates were prepared from samples by grinding in a mortar with fine silica sand, 10 % (m/m) insoluble polyvinylpyrrolidone and 20 mM Tris-HCl buffer pH 7.0 containing 1 mM EDTA, 2.5 mM $MgCl_2$, 0.5 mM PMSF, 1 mM benzamidine, 1 mM ε-aminocaproic acid and 30 mM 2-mercaptoethanol in a ratio of 1:5 (m/v). The resulting homogenate was squeezed through *Miracloth* and nylon sieve 100 mesh and centrifuged for 10 min at 20 000 *g*.

Preparation and storage of homogenates were carried out at 0 to 4 °C. Under these conditions the activity of the enzymes did not change for more than 5 h.

Determination of protein content and enzyme activities: Soluble protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

The ribonucleases (RNases) activity assay was a modified procedure of Šindelářová *et al.* (2000). The amount of degraded RNA in the supernatant was determined spectrophotometrically (*Helios* type, *Unicam*, Cambridge, UK) at 260 nm. One enzyme unit (U) was defined as the amount of enzyme causing an increase of 1.0 in the absorbance at 260 nm per hour.

G6P DH (EC 1.1.1.49) activity was also determined spectrophotometrically, NADPH generation was monitored at 340 nm according to Šindelář *et al.* (1999).

Phosphomonoesterase (PME, EC 3.1.3.2) and phosphodiesterase (PDE, EC 3.1.4.1) activities were assayed using *p*-nitrophenylphosphate or bis-*p*-nitrophenylphosphate as substrates at its pH optima (5.5, resp. 6.0) according to Chersi *et al.* (1966).

Enzyme activities were determined at their respective pH optima at 25 °C (with the exception 38 °C for ribonucleases).

Determination of TMV content: The TMV content was estimated on base of the number of necrotic lesions and using the quantitative DAS-ELISA (Clark and Adams 1977) with rabbit anti-TMV antibodies and alkaline phosphatase labelled antibodies raised against our isolate of TMV (common strain). Virus content was determined from the calibration curve of purified TMV using computer software described by Mančal (1987).

PAGE of proteins: Discontinuous nondenaturing polyacrylamide gel electrophoresis (PAGE) in a 1 mm thick 10 % resolving gel and 4 % stacking gel was performed to analyse acidic (Laemmli system) proteins (Hames and Rickwood 1990), using the *Mighty Small II* apparatus (*Hoefer Scientific Instruments*, San Francisco, USA). The amount of proteins loaded into a well corresponded to 15 μg of proteins. Gel was silver stained (Hames and Rickwood 1990).

Statistical treatment and chemicals: The results in tables are presented as arithmetical means ± standard deviation of mean of 3 - 7 measurements in four independent experiments. The *t* test was employed to characterise the differences.

Alkaline phosphatase was obtained from *Boehringer* (Heisenhofen, Germany), CPG and all other biochemicals were purchased from *Sigma Chemical Company* (St. Louis, USA).

Results and discussion

AVF isolation: Crude AVF at concentration of 250 μg cm^{-3} reduced the multiplication of TMV to 41.1 % as estimated on the basis of the number of necrotic lesions, whereas the control material applied at the same concentration did not change the TMV multiplication. Crude AVF alone was non-infectious. The fractions with AVF were eluted between 0.56 - 0.62 M NaCl (Fig. 1), which corresponds to findings of Sela *et al.* (1964). The fraction with the highest AVF content had more than 60 % protection activity. Isolated AVF was characterized by discontinuous nondenaturing polyacrylamide gel electrophoresis (PAGE - Laemmli system) as acidic protein and was represented by a single band (data not shown).

Influence of AVF on TMV content and activities of enzymes *"in vivo"*: The number of necrotic lesions on

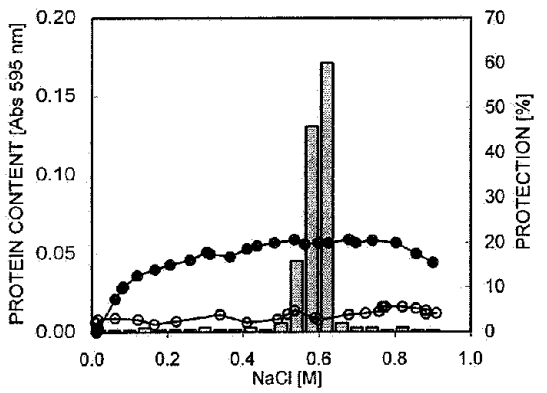

Fig. 1. The elution profiles of the proteins of crude AVF (*closed circles*) and of control material (*open circles*) on DEAE cellulose, and the percentage of protection value (*columns*) of fractions.

the TMV locally infected leaves treated with DEAE-AVF was decreased to 14.9 % when compared with the application of TMV alone. The necrotic lesions were firstly apparent on the 3rd dpTMV. However, the lower protection of DEAE-AVF (only 11 % on the 2nd dpTMV) was found if the quantitative DAS-ELISA method was used for a determination of TMV (Fig. 2).

The DEAE-AVF+TMV application initiated a decrease in soluble protein content (to 82 - 85 % of healthy non-treated control - Table 1, Fig. 2), but on the 5th dpTMV the protein content was rebalanced.

The TMV inoculation induced an intensive increase in the activity of the glucose-6-phosphate dehydrogenase (G6P DH) up to 209 % of healthy mock-inoculated control in the tobacco cv. Xanthi-nc on the 5th dpTMV. The DEAE-AVF treatment likewise decreased the activity of this enzyme to 154 % soon after the application (Table 1, Fig. 2).

Activities of RNases (pH optimum 5.5) were also increased in the locally-infected leaves with TMV (up to 156 % of healthy control on the 5th dpTMV). The application of DEAE-AVF decreased the activity of this enzyme (to 121 %) as well as the activity of G6P DH for the entire period (Table 1, Fig. 2).

The trends of the PME and PDE activities appear similar to that of RNases, but the DEAE-AVF restricted their activities later, on the 3rd day for PDE and on the 4th day for PME (Table 1, Fig. 2) while the G6P DH and RNases activities were lowered as early as on the first day.

Influence of AVF on TMV content and enzyme activities "*in vitro*": The direct effect of the AVF on activities of enzyme involved in the TMV-RNA biosynthesis was studied "*in vitro*" by measuring the activities with addition of the DEAE-AVF (concentrations from 0.25 - 250 μg cm^{-3}) into spectrophotometric cuvette. No effect on the enzyme activities was found with the exception of 250 μg cm^{-3} (concentration used in mechanical inoculation of experimental plants) that weakly ($0.01 \leq P < 0.05$) inhibited the PME and PDE activity to 85.4 %, resp. 88.6 % (Table 2). Therefore, the decrease in studied enzymes induced by AVF "*in vivo*" is not likely caused by the direct action of AVF.

The present study upgrades the characteristics of antiviral factors (inactivation of ribosomes by depurinating rRNA at a specific site, inhibition of protein synthesis in the virus-infected protoplasts, cytostatic and cytotoxic activities, competitive inhibition of guanylate cyclase activity, inhibition of the replication of several animal viruses) with the additional feature: to decrease the activity of the key enzymes of metabolic pathways tending to the purine and pyrimidine nucleotides biosynthesis of viral nucleic acid (glucose-6-phosphate dehydrogenase, ribonucleases, phosphomonoesterase and

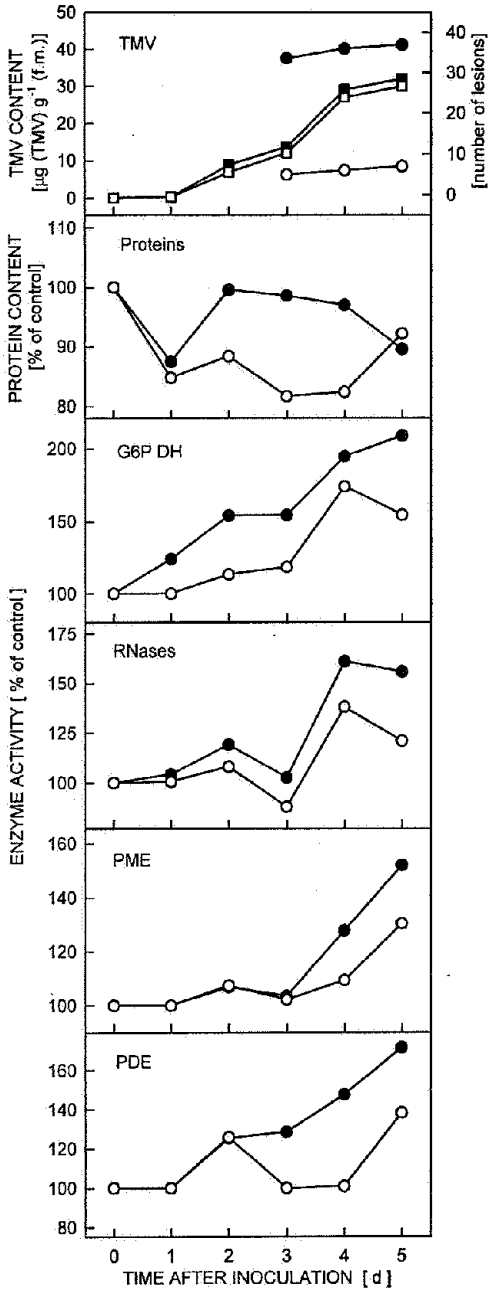

Fig. 2. The TMV content and infectivity (TMV), the protein content (proteins), the glucose-6-phosphate dehydrogenase (G6P DH), the ribonucleases (RNases, pH optimum 5.5), the phosphomonoesterase (PME) and the phosphodiesterase (PDE) activities in the locally-infected leaves of *N. tabacum* L. cv. Xanthi-nc. The TMV infectivity is given as the number of local necrotic lesions (*closed circles* - TMV infected leaves, *open circles* - TMV infected DEAE-AVF treated leaves). The TMV content estimated by ELISA method (*closed squares* - TMV infected leaves, *open squares* - TMV infected DEAE-AVF treated leaves) is given in μg(TMV) g^{-1}(f.m.). The protein content and the enzyme activities are expressed in % of the healthy DEAE-AVF untreated control (*closed circles* - TMV infected leaves, *open circles* - TMV infected DEAE-AVF treated leaves). The first lesions appeared on the 3rd dpTMV.

phosphodiesterase). This could be one of the phenomena which takes part in the inhibition of replication of plant and animal viruses, cytostatic and cytotoxic activities observed by the authors mentioned above.

Potential analogy was suggested between AVF and interferon produced in infected animal tissues (Isaacs and Lindenmann 1957). Interferon is induced by virus infection, has antiviral activity, and is non-specific as regard the inducing virus (Sela *et al.* 1964). Recently, Edelbaum *et al.* (1990) showed the relation of AVF and human interferon by specific reaction of polyclonal antibodies raised against human β-interferon with two plant antiviral proteins.

Conclusion: AVF probably reduces "*in vivo*" the TMV infectivity and its content by the decrease of the activity of the key enzymes of host rRNA degradation (RNases, PME and PDE) and of oxidative pentosephosphate pathway (G6P DH), but not by the direct AVF inhibition of these enzymes.

Table 1. Protein content [mg g^{-1}(f.m.)], activities of glucose-6-phosphate dehydrogenase (G6P DH) [nmol g^{-1}(f.m.) s^{-1}], ribonucleases (RNases of pH optima 5.5) [U g^{-1} (f.m.)], phosphomonoesterases (PME) and phosphodiesterases (PDE) [μmol g^{-1} (f.m.) s^{-1}] in crude tissue homogenate from healthy (H), TMV infected (TMV) and TMV infected DEAE-AVF treated (TMV+AVF) tobacco leaves. Statistical evaluation was done between the samples: "TMV infected" and "TMV infected AVF treated". * - the difference is statistically significant at $0.01 \leq P < 0.05$; ** - at $0.001 \leq P < 0.01$; and *** - at $P \leq 0.001$ (data without symbols are not statistically significant - NS).

Day p.i.		Proteins	G6P DH	RNases	PME	PDE
0	H	4.11 ± 0.08	0.69 ± 0.01	87.09 ± 1.31	39.12 ± 1.26	6.83 ± 0.08
1	H	5.16 ± 0.09	1.21 ± 0.02	90.20 ± 1.26	40.66 ± 1.21	8.03 ± 0.13
	TMV	4.52 ± 0.06	1.50 ± 0.06	93.93 ± 1.28	40.30 ± 1.19	7.21 ± 0.13
	TMV+AVF	4.37 ± 0.05	1.16 ± 0.02*	90.51 ± 1.21	40.13 ± 1.18	7.80 ± 0.14
2	H	9.38 ± 0.12	1.18 ± 0.06	89.58 ± 1.28	41.37 ± 1.19	7.12 ± 0.14
	TMV	9.34 ± 0.11	1.83 ± 0.08	106.38 ± 1.32	44.22 ± 1.24	8.93 ± 0.16
	TMV+AVF	8.29 ± 0.10**	1.35 ± 0.06***	132.50 ± 1.51*	40.48 ± 1.18	8.55 ± 0.15
3	H	7.58 ± 0.08	0.85 ± 0.02	130.64 ± 1.53	34.61 ± 1.23	5.64 ± 0.05
	TMV	7.47 ± 0.08	1.32 ± 0.05	133.75 ± 1.67	35.86 ± 1.28	7.25 ± 0.14
	TMV+AVF	6.20 ± 0.06***	1.01 ± 0.01***	115.08 ± 1.36**	31.95 ± 1.22	5.55 ± 0.04***
4	H	9.27 ± 0.12	0.78 ± 0.02	138.10 ± 1.41	39.06 ± 1.23	6.95 ± 0.06
	TMV	6.50 ± 0.08	1.52 ± 0.04	222.39 ± 2.44	35.80 ± 1.31	6.80 ± 0.04
	TMV+AVF	7.66 ± 0.12***	1.36 ± 0.03**	190.67 ± 2.38**	38.82 ± 1.28***	7.03 ± 0.14***
5	H	8.18 ± 0.16	0.99 ± 0.02	146.19 ± 1.41	28.09 ± 1.22	4.90 ± 0.02
	TMV	7.36 ± 0.07	2.07 ± 0.05	227.68 ± 2.48	42.73 ± 1.35	8.42 ± 0.13
	TMV+AVF	7.55 ± 0.12	1.53 ± 0.06***	176.67 ± 2.44***	36.63 ± 1.31***	6.78 ± 0.08***

Table 2. Influence of the DEAE-AVF on the "*in vitro*" activity of control enzymes of metabolic pathways for TMV-RNA biosynthesis. The values in parentheses express the percentage of the healthy DEAE-AVF untreated control. * - the difference is statistically significant at $0.01 \leq P < 0.05$ (data without symbols are not statistically significant - NS).

Enzyme activity	DEAE-AVF concentration [μg cm^{-3}]				
	0	0.25	2.5	25	250
G6P DH	0.71 ± 0.17	0.70 ± 0.17	0.74 ± 0.19	0.69 ± 0.14	0.68 ± 0.12
[nmol g^{-1}(f.m.) s^{-1}]	(100.0)	(98.9)	(104.6)	(97.6)	(95.8)
PME	41.26 ± 0.91	42.17 ± 0.96	39.61 ± 0.79	39.98 ± 0.82	35.24 ± 0.74
[μmol g^{-1}(f.m.) s^{-1}]	(100.0)	(102.2)	(99.0)	(96.9)	(85.4*)
PDE	7.06 ± 0.11	6.73 ± 0.09	6.72 ± 0.10	6.49 ± 0.08	6.26 ± 0.06
[μmol g^{-1}(f.m.) s^{-1}]	(100.0)	(95.3)	(95.2)	(91.9)	(88.6*)
RNases pH 5.5	95.14 ± 1.16	95.81 ± 1.18	94.57 ± 1.19	94.76 ± 1.15	86.10 ± 1.12
[U g^{-1}(f.m.)]	(100.0)	(100.7)	(99.4)	(99.6)	(90.5)

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