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Cusativin, a new cytidine-specific ribonuclease accumulated in seeds of *Cucumis sativus* L.

M. Angeles Rojo[1], F. Javier Arias[1], Rosario Iglesias[1], J. Miguel Ferreras[1], Raquel Muñoz[1], Cristina Escarmís[2], Fernando Soriano[3], Juan López-Fando[4], Enrique Méndez[3], Tomás Girbés[1]

[1] Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Valladolid, E-47005 Valladolid, Spain
[2] Centro de Biología Molecular CSIC-UAM, Canto Blanco, E-28049 Madrid, Spain
[3] Servicio de Endocrinología, Hospital Ramón y Cajal, Carretera de Comenar Viejo km 9.1, E-28034 Madrid, Spain
[4] Departamento de Bioquímica y Biología Molecular, Universidad de Alcalá de Henares, E-28871 Alcalá de Henares, Spain

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Abstract. Dry seeds of *Cucumis sativus* L. were found to contain a heat-sensitive endoribonuclease of a novel type which we have named cusativin. It was purified to apparent electrophoretic homogeneity by chromatography through S-Sepharose Fast Flow, Sephadex G-75, CM-Sepharose, Superdex 75-FPLC (fast protein liquid chromatography) and Mono S-FPLC. It is a single unglycosylated polypeptide chain with an apparent molecular mass (M_r) of 22900. Polyclonal anti-cusativin antibodies raised in rabbits only reacted with melonin, the translation inhibitor from *Cucumis melo* L. Functional, Western blot and enzyme-linked immunosorbent assay (ELISA) analyses indicated that cusativin is present in the coat and cotyledons of dry seeds, but not in embryonic axes. Cusativin is accumulated in maturing seeds. By contrast, after seed germination there is degradation of the cusativin present in cotyledons but not that present in the seed coat. The preference of cusativin for polynucleotide cleavage was poly(C)$\gg$poly(A) acids, poly(U) and poly(G) being unaffected by cusativin. Under the denaturing conditions used for RNA sequencing, cusativin acted only on poly(C). Cusativin proved to be useful for RNA sequencing, in particular, complementing the data obtained with RNase CL3. Cusativin represents a new class of plant RNase and, as far as we are aware, is the first plant enzyme that shows cleavage specificity for cytidine under the denaturing conditions of RNA sequencing.

Key words: *Cucumis* – Cusativin – RNase – RNA degradation – Seed (germination maturation)

Introduction

Plants contain compounds, most of them unidentified, that strongly inhibit translation. Amongst them, RNases (Farkas 1982; Wilson 1982) and ribosome-inactivating proteins (RIPs; Stirpe et al. 1992; Citores et al. 1993) are proteins classified as the most active and best-identified compounds with translational inhibitory activity.

Ribonuclease activities and their changes have been studied in dry seeds in attempts to clarify their role during the early stages of germination. However, most such studies have been performed using rather crude or poorly purified preparations of these enzymes (Wilson 1982). Most known plant RNases have been isolated and purified from the endosperm and scutella of developing seeds (Wilson 1968, 1973; 1980), the leaves of several plants (Wyen et al. 1971; Lantero and Klosterman 1973; Jervis 1974; Chevrier and Sarhan 1980), cotyledons (Barker et al. 1974; Jacobsen 1980), and roots and seedlings (Kado 1968; Hirai and Asahi 1975; Kroeker et al. 1976; Beopoulos et al. 1978). Even though many plant seeds have been shown to contain RNase activities, only a few enzymes have been isolated from dry seeds (Wilson 1982). Most studies concerning RNase activities have been performed to correlate nucleolytic degradation of RNA with tissue development (Pietrzak et al. 1980).

Early reports indicated that the yellowing of cucumber cotyledons promoted increases in RNA degradation, presumably mediated by an RNase (Lewington et al. 1967). Furthermore, an RNase with an M_r of 12600 was isolated and partially characterized from etiolated seedlings (Kado 1968). In the present work we have isolated the protein-synthesis inhibitor from dry seeds of *C. sativus*, purified it to homogeneity and studied several structural and functional properties. This translational inhibitor is a powerful new cytidine-specific novel type of RNase with an M_r of 22900, and is present only in the seed coat and cotyledons. Furthermore, it was found to be much more active than RNase A and common RIPs in the inactivation of ribosomes from all sources. Cusativin is potentially useful for RNA-sequencing purposes.

Abbreviations: ELISA = enzyme-linked immunosorbent assay; M_r = molecular mass; RIP = ribosome-inactivating protein; rRNA = ribosomal ribonucleic acid

Correspondence to: T. Girbés; FAX: 34(83)423082

Materials and methods

Materials. All chemicals and biochemicals were obtained as previously described (Arias et al. 1992b). L-[^{3}H]Valine (sp. act. 1.22 TBq · mmol^{-1}) and L-[^{3}H]phenylalanine (sp. act. 4.11 TBq · mmol^{-1}) were purchased from Amersham Ibérica (Madrid, Spain). Alkaline phosphatase linked to polyclonal anti-rabbit antibodies, naphthol AS–TR phosphate and Fast Red were from Sigma (St. Louis, Mo. USA). Immobilon membranes were from Millipore (Madrid, Spain). XMO8 was purchased from Oncogene Science, Inc. Through ITISA (Madrid, Spain). Ribonuclease inhibitor from human placenta was purchased from Boehringer (Mannhein, Germany). One unit of each inhibitor was assumed to be as indicated by the suppliers. The RIPs were kindly provided by Prof. F. Stirpe (Dipartimento di Patologia Sperimentale, Università di Bologna, Italy). The plant seeds were obtained from commercial sources. *Cucumis sativus* L. seeds used for the isolation of the inhibitor were of the SMR–58 variety (FITO, Barcelona, Spain). Cleaning and germination of seeds was as described previously (Arias et al. 1992b). *Escherichia coli* MRE 600 was obtained from Prof. J.P. García-Ballesta (Centro de Biología Molecular, Madrid, Spain).

Isolation of the translational inhibitor. The procedure conducted at 10° C was as follows:

Step 1: Preparation of the crude protein extract. Dried seeds (500 g) were ground-up, and the resulting flour was extracted overnight, with vigorous stirring, in 4 l of 5 mM sodium phosphate (pH 7.2) containing 0.14 M NaCl. The lysate was filtered through two layers of cheesecloth. The resulting fluid was acidified with glacial acetic acid until pH 4. Precipitated solids were removed by centrifugation at 12400 · *g* for 45 min at 0° C and filtered through a pad of packed Sephadex G-25 (1 cm of bed and 5 cm inner diameter) equilibrated with 10 mM sodium acetate (pH 4.5).

Step 2: Cation-exchange chromatography on S-Sepharose Fast Flow. The clarified protein extract was applied to an S-Sepharose Fast Flow column (12 cm long, 2.6 cm i.d.) equilibrated with 10 mM sodium acetate (pH 4.5) at a flow rate of 100 ml · h^{-1}. The column was then washed with 5 mM sodium phosphate (pH 7) at the same rate and the resulting eluate was discarded. The protein retained by the column was then eluted with 5 mM sodium phosphate (pH 7) containing 1 M NaCl, at the same flow rate. All the protein eluted was pooled.

Step 3: Conventional size-exclusion chromatography. The protein from the previous step containing inhibitory activity was applied directly to a Sephadex G-75 column (95 cm long, 5 cm i.d.) previously equilibrated with 5 mM sodium phosphate (pH 7). The column was eluted with the same buffer at a flow rate of 275 ml · h^{-1}. The eluate was assayed for protein-synthesis inhibition and the fractions containing inhibitory activity (520 ml) were pooled.

Step 4: Cation-exchange chromatography on CM-Sepharose Fast Flow. The protein solution from the previous step was applied to a CM-Sepharose Fast Flow (12 cm long, 2.6 cm i.d.) column equilibrated with the same buffer as the Sephadex G-75 column. After washing with the same buffer the column was eluted at a flow rate of 420 ml · h^{-1} with the same buffer but now containing a gradient of 1.5 l of 0–400 mM NaCl. Fractions of 10 ml were collected. The eluate was then assayed for protein-synthesis inhibition and the fractions containing inhibitory activity were pooled.

Step 5: Size-exclusion FPLC on Superdex 75. The protein solution from the previous step was applied to a Superdex 75 column coupled to a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) preequilibrated with 5 mM sodium phosphate (pH 7) containing 400 mM NaCl. Elution was performed at a flow rate of 30 ml · h^{-1} with the same buffer and aliquots of 0.5 ml were taken. The fractions containing the second peak were pooled and analyzed by SDS–PAGE. The protein solution was dialyzed against 5 mM sodium phosphate (pH 7).

Step 6: Cation-exchange chromatography on Mono S. The protein solution from the previous step was applied to a Mono S column coupled to an FPLC system, previously equilibrated with 5 mM sodium phosphate (pH 7). Thereafter, the column was washed with the same buffer, this time containing 100 mM NaCl. Elution of the protein was achieved with a 100–300 mM NaCl gradient containing 5 mM sodium phosphate. The fractions containing the first peak eluted from the column were collected, dialyzed against water and finally freeze-dried and stored at −20° C.

Preparation of the cell-free translation systems, purified ribosomes and DNA. The cell-free translation systems were postmitochondrial supernatants (S–30) obtained in RNase-free conditions at 0–2° C. For the plant systems, essentially a previously described method was used (Arias et al. 1992a). The rat-liver cell-free extracts and the rabbit-reticulocyte lysates were obtained by standard procedures as described elsewhere (Pelham and Jackson 1976; Martin et al. 1979). Postmitochondrial rat-brain supernatant used to assay the effects of α-sarcin and cusativin on ribosomal RNA (rRNA) was prepared by centrifugation at 1700 · *g* of a rat-brain homogenate 1:2 (w/v) in 50 mM Hepes-KOH (pH 7.5), 140 mM K-acetate, 4 mM Mg-acetate and 2.5 mM dithiothreitol (Muñoz et al. 1990). The *E. coli* cell-free system (3000 · *g* supernatant, S 30) was obtained by grinding midlogarithmic-phase cell cultures with alumina using an extraction buffer that contained 10 mM Tris-HCl (pH 7.8), 10 mM Mg-acetate, 60 mM NH_4Cl and 10 mM 2-mercaptoethanol (Girbés et al. 1976). Rat-liver ribosomes were prepared and purified essentially as described elsewhere (Arias et al. 1992b). *Escherichia coli* MRE 600 ribosomes were obtained as previously described (Girbés et al. 1976). Deoxyribonucleic acid from *E. coli* MRE 600 was prepared according to a standard method (Sambrook et al. 1989).

Assays of cell-free protein synthesis. Protein synthesis encoded either by endogenous messengers or polyuridylic acid was assayed in several eukaryotic and prokaryotic cell-free systems under RNase-free and optimized conditions as indicated elsewhere (plant systems: Arias et al. 1992a, b, Rojo et al. 1993; rat liver: Girbés and Alonso 1986; rat brain: Muñoz et al. 1990; rabbit-reticulocyte lysates: Pelham and Jackson 1976; *E. coli* system: Girbés et al. 1976).

Tryptic digestion and peptide fractionation of cusativin. An aliquot of 182 µg of electrophoretically homogeneous cusativin was digested with 10 µg of trypsin in 0.3 ml of 0.2 M N-methyl-morpholine (pH 8.2) for 4 h at 37° C. The digest was resuspended in 0.35 ml of 0.1% trifluoroacetic acid and chromatographed by reverse-phase HPLC using a Nova Pak C18 column equilibrated with 0.1% trifluoroacetic acid. The column was eluted with a gradient of acetonitrile (0–40%) in 0.1% trifluoroacetic acid and operated at room temperature at a flow rate of 0.5 ml · min^{-1}. The peptide-containing peaks were lyophilized and stored for amino-acid sequencing.

Amino-acid analysis and sequence-analysis procedure. Amino-acid composition was analyzed as described (Arias et al. 1992b). Peptides were sequenced in the presence of polybrene, using a Knauer modular liquid-phase sequencer (model 810), equipped on line with a phenylthiohydantoin-amino acid analyzer as described by Limas et al. (1990).

Preparation of polyclonal anti-cusativin rabbit antibodies and Western blot analysis of cusativin and C. sativus extracts. Polyclonal anti-cusativin antibodies were raised in rabbits (Harlow and Lane 1988). The SDS–PAGE was carried out as described above, followed by electrophoretic blotting onto Immobilon membranes performed at 0.8 mA · cm^{-2} for 1 h with the Semi-phor system from Hoefer (San Francisco, Cal. USA). Immunodetection of cusativin was carried out following a general procedure (Harlow and Lane 1988), using polyclonal rabbit anti-cusativin as a first antibody and

alkaline-phosphatase-linked polyclonal anti-rabbit antibodies from Sigma. The colour reaction was performed with Naphtol AS–TR phosphate and Fast Red from Sigma and stopped with water.

Electrophoretic analysis of rRNA and DNA. Rabbit-reticulocyte lysates or postmitochondrial rat-brain supernatant were incubated with varying concentrations of cusativin at 37° C for 1 h and 15 min, respectively, in a reaction mixture of 50 µl of buffer that contained 20 mM Tris-HCl (pH 7.6), 125 mM KCl and 9 mM Mg-acetate. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA (pH 8.0) and 500 µl of 0.5% SDS containing 50 mM Tris-HCl (pH 7.6). The RNA was extracted by phenolization and ethanol-precipitation as described elsewhere (Sambrook et al. 1989). Electrophoresis of either rRNA or DNA was carried out in gels of either 2% agarose at 70 V for 2.5 h using a buffer containing 0.1 M 3-(N-morpholino)propanesulfonic acid (Mops) (pH 7), 40 mM Na-acetate and 5 mM EDTA (pH 8) (Sambrook et al. 1989), or 5% acrylamide at 21 mA for 50 min using a buffer containing 89 mM Tris-HCl (pH 8.3), 89 mM boric acid and 2.5 mM EDTA (Sallustio and Stanley 1990). The gels were photographed after staining for 20 min with 0.5 $\mu g \cdot ml^{-1}$ of ethidium bromide.

Generation of the RIP-diagnostic RNA fragment with single-chain RIP crotin 2,5'-end labelling and sequencing by the enzymatic method. The RIP-diagnostic RNA fragment was prepared from 200 µg of *E. coli* ribosomes which were treated with 0.6 µg of crotin 2 for 15 min at 37° C in a reaction mixture of 50 µl of buffer that contained 40 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl and 10 mM Mg-acetate. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA (pH 8.0) and 500 µl of 0.5% SDS containing 50 mM Tris-HCl (pH 7.6). Aniline treatment was carried out as reported elsewhere (Iglesias et al. 1993b). The rRNA fragment generated by the action of the RIP was isolated by electrophoresis in a 5% polyacrylamide gel (Iglesias et al. 1993b). The RNA fragment, accounting for roughly 1 µg of RNA, was extracted by immersion of the crushed gel pieces in 1 vol of TNE buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 5 mM EDTA) and 1 vol. phenol for 15 h at 4° C, and precipitated with 2 vol of ethanol at −90° C for 2 h. The RNA was 5'-dephosphorylated with alkaline phosphatase, deproteinized and 5'-phosphorylated with polynucleotide kinase, and sequenced by partial digestion with RNases as described elsewhere (Escarmis et al. 1992; Iglesias et al. 1993a).

Other procedures. Analysis of proteins by one dimensional SDS–PAGE was carried out on slabs (Hoefer Mighty-Small II system) using the discontinuous system of Laemmli (Laemmli 1970). The proteins used as standards were trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 29000), alcohol dehydrogenase (M_r 37000), glutamate dehydrogenase (M_r 54400), bovine serum albumin (M_r 68000). The presence of glycan chains in cusativin was studied using the Glycan Detection Kit from Boehringer (Mannheim, Germany) using transferrin (M_r 79500) as standard. Enzyme-linked immunosorbent assay (ELISA) was performed essentially using the method outlined in Pérez-Maceda et al. (1991) except that internal standards were included and the final concentration of protein was maintained constant at 200 ng per well (Metodiev and Demirevska-Kepova 1992). The RNase activity of cusativin on yeast RNA or polynucleotides was assessed at 37° C determining the cold-trichloroacetic acid-soluble material absorbing at 260 nm. For determination of the pH optimum, all buffers contained 0.01% gelatin and were as follows: Na-acetate for pH 3–6; Na-phosphate for pH 6.5–7.5; Tris-HCl for pH 8–9.5.

Results

Purification of protein-synthesis inhibitor. A salt-soluble protein extract from seeds strongly inhibited translation

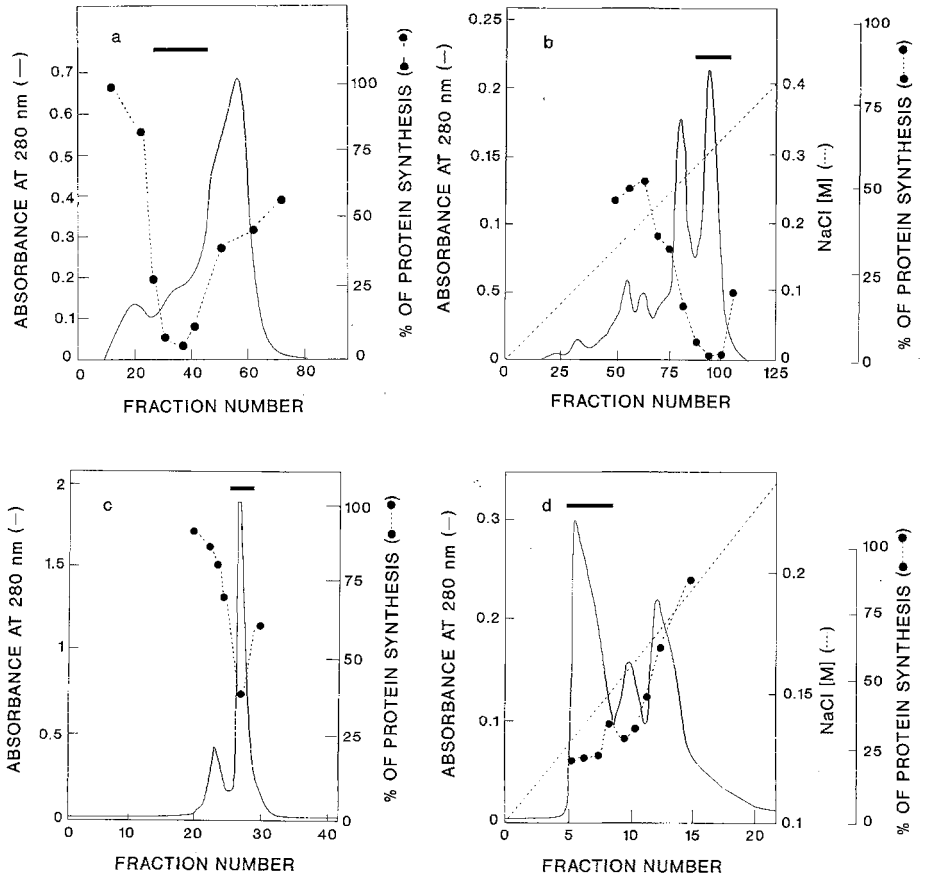


Fig. 1a–d. Purification of cusativin from dry seeds of *Cucumis sativus* L. by chromatography through Sephadex G–75 (**a**), CM-Sepharose FF (**b**), Superdex 75 (**c**), and Mono S (**d**). Fractions were assayed for inhibition of protein synthesis in rat-liver cell-free extracts at $\times 10^{-3}$ (**a, b**) and $\times 10^{-4}$ (**c, d**) dilutions. The fractions indicated by the *horizontal bar* were pooled and subjected to the next step

Table 1. Purification of cusativin from dry seeds of *Cucumis sativus*. The protein was purified as described in the text. Inhibition of protein synthesis was performed using a rat-liver cell-free system. One unit (U) is the amount of inhibitory protein required to reduce the 1-ml translation reaction mixture by 50%

Step	Vol. (ml)	Protein (mg)	IC_{50} ($ng \cdot ml^{-1}$)	Spec. act ($\times 10^{-6} U \cdot mg^{-1}$)	Total act. ($\times 10^{-6} U$)	Yield (%)
1. Acidified crude extract	3620	15928	4760	0.21	3346	(100)
2. S-Sepharose FF	429	1201	350	2.85	3431	102
3. Sephadex G 75	520	239.2	162	6.17	1477	31
4. CM-Sepharose FF	260	78.0	60	16.67	1300	39
5. Superdex 75	19	22.3	18	55.55	1239	24
6. Mono-S	16	4.8	14	71.43	340	10

by a rat-liver in-vitro model system. From this source we obtained a pure protein by using a purification scheme that involved several steps (Fig. 1). Throughout the different steps, purification of the protein – which we named cusativin – was followed by assays of protein-synthesis inhibition by rat-liver ribosomes directed by endogenous messengers. The isolation procedure is summarized in Table 1. The most effective step in terms of the concentration of the inhibitory activity was the S-Sepharose Fast Flow chromatography. As shown below, the preparation was electrophoretically homogeneous. With the current procedure the final preparation had a 340-fold enrichment in inhibitory activity over the crude extracts, with an overall recovery of 10%.

Relative molecular mass and glycosylation state. Both the purity and the molecular size of the inhibitory protein were assessed by 15% SDS–PAGE, either in the absence or the presence of 2-mercaptoethanol (Fig. 2). Despite the presence of the reducing agent, under denaturing conditions the protein moved as a single sharp peak with an apparent M_r of 22900. Gel filtration on Sephadex G–75 gave an apparent M_r of around 25000. Additionally, we studied the glycosylation state of cusativin by electrophoresis, blotting onto Immobilon membranes, and immunological detection. Our results indicated that the protein was apparently not glycosylated (Fig. 2).

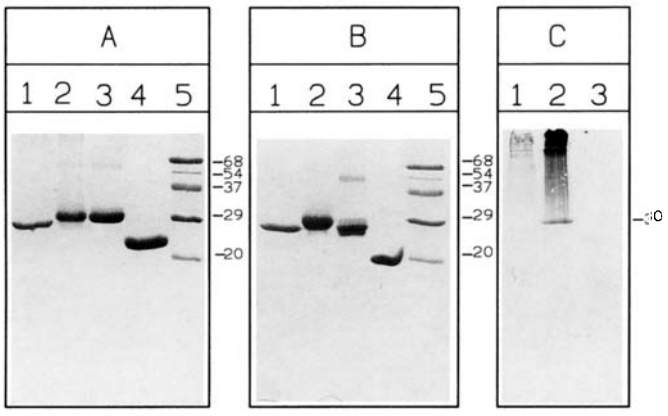


Fig. 2A–C. Polyacrylamide gel electrophoresis of cusativin and other protein-synthesis inhibitors. The SDS–PAGE, run in the presence (**A**, **C**) or the absence (**B**) of 2-mercaptoethanol, was conducted in 15% gels at 20 mA for 60 min. Thereafter, two gels (**A**, **B**) were stained for visual assessment and the third gel (**C**) was blotted onto an Immobilon membrane and treated for glycan detection. Standards were those indicated in *Materials and methods*. *Lanes 1A* and *1B*, petroglaucin 2; *lanes 2A* and *2B*, saporin 5; *lanes 1C*, *2C* and *3C*, petroglaucin 2, dianthin-32 and cusativin, respectively. The M, values of standards (*lanes 5A* and *5B*) are marked in kDa

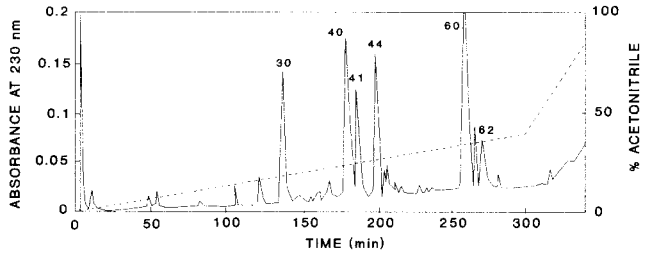
Amino-acid composition, tryptic peptides and sequence of some peptides. Table 2 shows the amino-acid composition in terms of the approximate number of residues. Attempts to obtain the amino-terminal amino-acid sequence were unsuccessful, most probably because the amino-terminal amino-acid had been blocked. Unblocking with trifluoroacetic acid (Wellner et al. 1990) also proved unsuccessful. Thus, in order to obtain details of the internal sequence, tryptic peptides were obtained by HPLC (Fig. 3a). The six major tryptic peptides (Nos. 30, 40, 41, 44, 60, and 62) were sequenced (Fig. 3b). No sequence homology was found between these sequences and those reported for several RIPs which, even considering the large variability among RIPs, suggests a different mode of action for cusativin. By contrast, pep-

Table 2. Amino-acid composition of cusativin. Composition is expressed in rounded-off numbers of residues per mole of protein based on an M_r of 22900 (parentheses)

Amino acid	Number of residues
Cys[a]	–
Asp	22.6 (23)
Thr	13.1 (13)
Ser	9.5 (10)
Glu	17.8 (18)
Pro	13.8 (14)
Gly	16.6 (17)
Ala	11.9 (12)
Val	13.8 (14)
Met	3.5 (4)
Ile	9.9 (10)
Leu	13.4 (13)
Tyr	3.5 (4)
Phe	15.0 (15)
Lys	14.6 (15)
His	6.3(9 6)
Arg	10.7 (11)
Trp[a]	–

[a] Trp and Cys were not determined

a) Tryptic peptides



b) N-terminal aminoacid sequence of tryptic peptides

(30)- Y F Q T A I N M R
(40)- P P X G H E X N K
(41)- S F T I H G L W P N K
(44)- I A H L E N D L N V V W P N V V T G N N K
(60)- Y V G R
(62)- A S N G Q V L L T E I V M X F D D D X X T L

Fig. 3. Fractionation by HPLC (**a**) and amino-acid sequence (**b**) of tryptic peptides of cusativin from *Cucumis sativus* seeds. Peptides were chromatographed on a reversed-phase C18 column and eluted with a gradient of acetonitrile as indicated. Only peptides indicated by numbers were sequenced

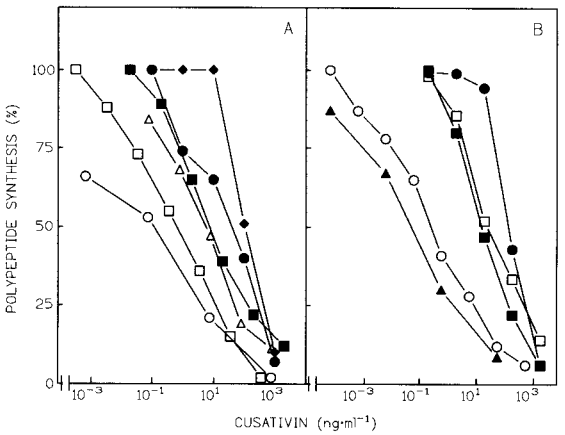


Fig. 4A, B. Effect of cusativin on protein synthesis by several cell-free translation systems. **A:** △, ribosomes plus S 100 rat liver directed by poly(U); ○, S 30 rat liver directed by poly(U); ●, S 30 rat liver directed by endogenous messengers; □, rabbit-reticulocyte lysates; ■, S 30 rat brain. For comparative purposes RNase A was also assayed in the S 30 from rat liver (◆). **B**: ○, S 30 from *C. sativus* directed by poly(U); ●, S 30 from *C. sativus;* ▲, *E. coli* ribosomes plus S 100 directed by poly(U); □, S 30 from wheat germ; ■, S 30 from *Vicia sativa*

tide 40 is identical to the tryptic peptide numbered 18 recently described in melonin (Rojo et al. 1992), a protein translational inhibitor isolated from the dry seeds of *Cucumis melo* L. (Ferreras et al. 1989). In addition, peptide 41 shared 91% of the identity of another tryptic peptide of melonin (Rojo et al. 1992). A computer-aided search in protein-sequence data banks indicated that tryptic peptide 41 of cusativin shares 82% sequence homology with *Petunia inflata* S 3 protein between amino acids 290 and 300 (genEMBL accession number Em-Pl:Pis3alle), 73% with *Petunia inflata* RNase (rn 2) between amino acids 185 and 195 (genEMBL accession number Em-Pl:Pirnx2a) and 73% with *Rhizopus niveus* RNase between amino acids 224 and 234 (Horiuchi et al. 1988).

Translational inhibitory activity. The effects of the protein inhibitor on protein synthesis were studied using ten translation systems from mammals, plants and bacteria. As shown in Fig. 4, the protein inhibitor acted strongly on all the in-vitro translation systems. It is noteworthy that the lowest inhibitor concentration required to inhibit the poly(U)-encoded rat liver system by 50% was 0.1 ng · ml^{-1}, which classifies this inhibitor as one of the strongest translational inhibitors hitherto found, comparable only to certain RIPs acting on rabbit reticulocyte lysates (Stirpe et al. 1992). Strikingly, all the IC_{50} values fell within the 0.1–80 ng · ml^{-1} range with the notable exception of the S 30 system from *C. sativus,* which displayed a lower sensitivity (i.e. an IC_{50} of 270 ng · ml^{-1}), thus indicating that it acts catalytically. In the case of the *C. sativus* cell-free system, which was the least sensitive, the protein-to-ribosomes ratio was $5 \cdot 10^{-2}$ and for rat liver directed by poly(U), which was the most sensitive, it was $1 \cdot 10^{-5}$. For comparative purposes, we studied the effects of RNase A on translation by rat-liver ribosomes programmed with poly(U) (Fig. 4). Under our standard conditions, this enzyme had no effect on translation by the rat-liver S 30 directed by poly(U) at concentrations lower than 10 ng · ml^{-1} and had an IC_{50} of 110 ng · ml^{-1}; this is nearly three orders of magnitude the value obtained with cusativin and purified rat-liver ribosomes directed by poly(U).

Tryptic peptide No.30 (YFQTAINMR) retained a substantial degree of activity as an inhibitor of rat-liver cell-free protein synthesis (IC_{50} of 8 μg · ml^{-1}), although this was lower than that exerted by cusativin. When the concentration of ribosomes in the cell-free extracts is taken into account, this result points to a stoichiometric mode of action of the peptide. Nonetheless, this concern awaits further molecular explanation.

The high activity of the *C. sativus* cell-free translation system led us to suspect that, if indeed present in embryonic axes, cusativin is either inactive or is blocked by an endogenous inhibitor of cusativin. To test this hypothesis, we compared the potential inhibitory effects of salt-soluble protein extracts obtained from either dry seeds or 4 to 5-d-old embryonic axes of *C. sativus* on the cell-free translation systems from rat liver and *C. sativus* embryonic axes. The crude salt-soluble protein extract from dry seeds strongly inhibited translation by both systems with nearly the same efficiency (i.e. IC_{50} of around 10 ng · ml^{-1} in both cases; data not shown). By contrast, the salt-soluble protein extract from embryonic axes was much less inhibitory, acting only at concentrations of around 1 mg · ml^{-1}; this suggests a rather non-specific effect (data not shown). Such results lead us to speculate that the protein inhibitor would be present in an active form in the dry seeds but not in the embryonic axes; alternatively, cusativin might be absent from the embryonic axes.

Western blot analysis of cusativin distribution and accumulation during maturation and germination of seeds of C. sativus. To test the distribution of cusativin and whether the active form of cusativin is confined to dry seeds or not, we analyzed the cross-reactive process immunologically using polyclonal anti-cusativin antibodies raised in rabbits and developed an ELISA procedure to measure the concentration of cusativin. As outlined in Table 3, the protein was present only in the dry-seed extracts (0.25% of total protein in the dry seeds), or fractions derived thereof. In seeds, the protein was concentrated in the coat, where it accounted for 0.17% of the total protein content. Cusativin was also located in the cotyledons.

We next investigated whether cusativin is synthesized as a large precursor and whether the protein inhibitor shares immunoreactive epitopes with other strong protein inhibitors present in plant seeds, suchs as RIPs. Western blot analysis of purified cusativin and protein from some of the steps of the isolation procedure of cusativin from dry seeds, from a salt-soluble extract of embryonic axes, from a 3000 · *g* translation supernatant (S 30) prepared from embryonic axes, and from germinating seeds is shown in Fig. 5. Cusativin reacted very well, as expected from the activity of anti-cusativin antibodies. The salt-soluble protein extract prepared from dry seeds reacted just as well, as also expected. Analysis of the protein bound to S-Sepharose FF at low salt concentrations and eluted at 1 M NaCl clearly showed that cusativin may have two reactive proteins that could be two isoforms or glycoforms. Both protein bands were observed when the basic proteins were concentrated by S-Sepharose FF. Neither the crude extract from embryonic axes nor the S 30 contained reactive material, thus suggesting that both cusativin and its putative precursors are absent from 4- to 5-d-old embryonic axes. On the other hand, when the seeds were imbibed for 2 d with tap water, cusativin activity partly disappeared. This indicated that some kind of imbibition-triggered proteolytic process must lead to the elimination of most of the cusativin in the germinated seeds.

We further investigated the appearance of cusativin in maturing and germinated seeds. As shown in Fig. 6, Western blot analysis of cusativin with anti-cusativin antibodies as a first antibody indicated that a reactive

Table 3. Enzyme-linked immunosorbent assay of cusativin from various parts of *C. sativus* plant

ELISA source	Total protein (mg · g^{-1})
Seeds	2.47 ± 0.45
S–30	0.25 ± 0.13
Embryonic axes	0.25 ± 0.20
Coat	1.73 ± 0.20
Cotyledons (4 d of imbibition)	0.74 ± 0.11

Values are means of six determinations ± SEM

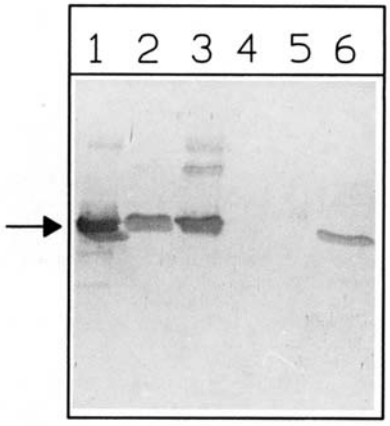


Fig. 5. Western blot analysis of cusativin from *Cucumis sativus*. Cusativin and protein extracts from several stages of purification were electrophoresed and blotted onto an Immobilon membrane. Thereafter, the material reactive to anti-cusativin antibodies was developed as indicated in *Materials and methods*. *Lanes: 1,* cusativin (3 µg); *2,* protein from crude seed extract (20 µg); *3,* protein eluted from S-Sepharose FF (20 µg); *4,* protein from crude extract of embryonic axis (17 µg); *5,* protein from S 30 extract (100 µg); *6,* protein from crude seed extract after 2 d of imbibition with tap water (18 µg)

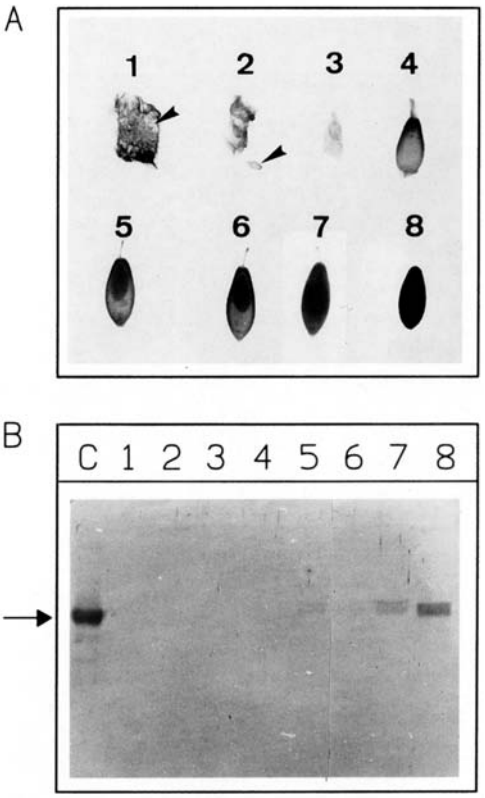


Fig. 6A, B. Detection of cusativin at several stages of seed maturation by immunoblotting. **A** Seeds of *C. sativus* at several arbitrary stages of maturation were used. The average large diameters of seeds at the different stages of maturation were: *stage 1,* 1.17 ± 0.15 mm; *stage 2,* 2.17 ± 0.17 mm; *stage 3,* 6.17 ± 0.33 mm; *stage 4,* 8.83 ± 0.33 mm; *stage 5,* 10.00 ± 0.29 mm; *stage 6,* 10.83 ± 0.17 mm; *stage 7,* 10.83 ± 0.17 mm; *stage 8,* 9.60 ± 0.10 mm. The *arrowheads* indicate seed stages 1 and 2 of maturation. **B** Crude protein extracts from seeds at each stage of maturation were prepared as indicated in *Materials and methods*. Western blots of these protein samples were carried out. Protein contents per lane were: 1.7 µg in *lane C* and 26.4 µg in all other lanes. *Lane C,* cusativin; the numbers of the other lanes correspond to the stages of maturation

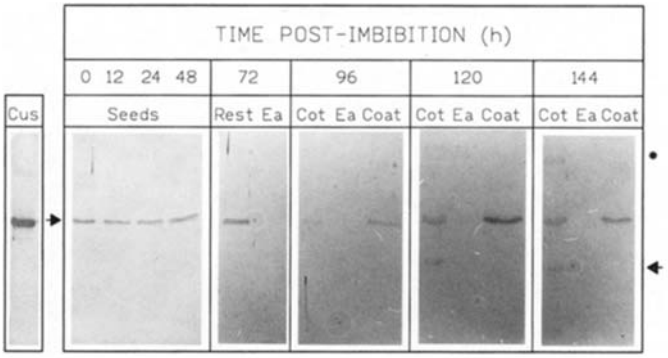


Fig. 7. Detection of cusativin after imbibition and during the germination of *C. sativus* seeds. Seeds of *C. sativus* were imbibed in sterile tap water in darkness for the times indicated. Crude protein extracts, and the corresponding. Western blots, were obtained as indicated under *Materials and methods*. The amounts of protein per lane were 1.7 µg for cusativin (*Cus*) and 26.4 µg for all other lanes. *Rest* refers to the rest of the seed after removal of the embryonic axe; *Ea*, embryonic axis; *Coat*, seed coat; *Cot*, cotyledons. The *arrow on the left* indicates the position of cusativin. The *arrow on the right* indicates the position of a band appearing at M_r 16500. The *asterisk* indicates the position of the precursors

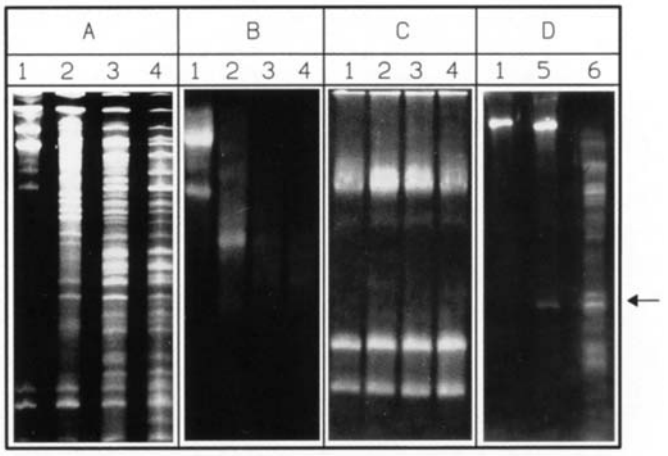


Fig. 8A–D. Effects of cusativin from *C. sativus* seeds on RNA and DNA electrophoretic profiles. Cusativin was incubated either with rabbit-reticulocyte lysates (**A, B**), rat-brain cell-free extract (**D**) or *E. coli* DNA (**C**). Thereafter, the rRNA (**A, B, D**) and DNA (**C**) were extracted and subjected to PAGE (**A, D**) or to 2% agarose submerged gel electrophoresis (**B, C**). *Lanes: 1*, control; *2*, plus 10 ng cusativin; *3*, plus 100 ng cusativin; *4*, plus 600 ng cusativin; *5*, plus 0.2 µg α-sarcin; *6*, plus 0.2 µg cusativin. The *arrow* on the right indicates the α-sarcin fragment

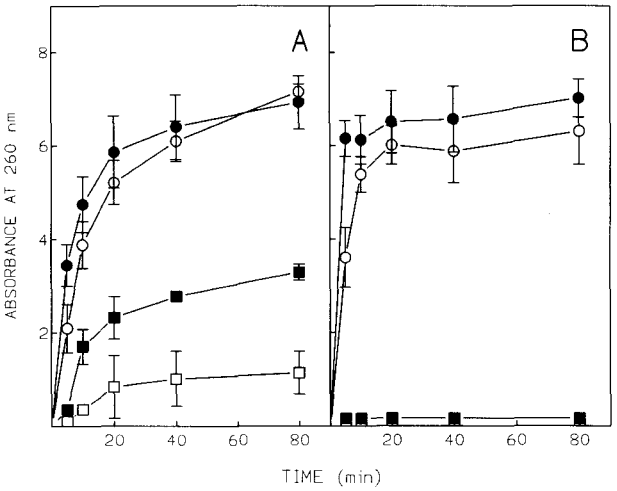


Fig. 9A, B. Effects of cusativin from *C. sativus* seeds on the degradation of polynucleotides. The effect of cusativin on polynucleotides was determined by the release of acid-soluble UV-absorbing material either in control (**A**) or RNA sequencing conditions (**B**). ○, ●, poly(C); □, ■, poly(A); ●, ■, 600 ng · ml^{-1} cusativin; ○, □, 100 ng · ml^{-1} cusativin. Neither poly(U) nor poly(G) were affected at all by cusativin

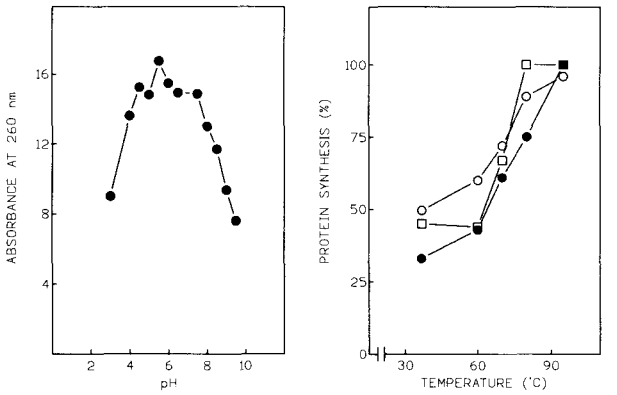


Fig. 10. Optimum of pH and thermal stability of cusativin from *C. sativus* seeds. To find the pH optimum for nucleolytic cleavage, crude yeast RNA was incubated with cusativin (100 µg · ml^{-1}) at 37° C for 15 min. Thereafter, acid-soluble UV-absorbing material was measured. Thermal stability was studied as follows: cusativin (134 ng · ml^{-1}) was incubated in 40 µl of H_2O for 20 min at the indicated temperature either in the absence (●), or the presence of 10% (v/v) glycerol (○), or 250 mM trehalose (□). Thereafter, the protein (26.8 ng · ml^{-1}) was assayed for inhibition of protein synthesis in a rat-liver cell-free system

material with an M_r of 22900 appeared in the arbitrary seed stage 5 (Fig. 6). A maximal accumulation of cusativin was evident in the last steps of seed maturation. The appearance of material reactive with anti-cusativin antibodies was concurrent with the appearance of a strong inhibitory activity of protein synthesis in crude extracts obtained from the different seed growth stages defined in Fig. 6 (data not shown).

As seedling growth progressed, the cusativin present in the seed coat remained intact (Fig. 7). Conversely, the cusativin present in cotyledons seemed to be degraded under the same conditions; thus, a band of low-molecular-weight reactive material appears.

At 120 h two bands of apparent M_r of 44000 and 40000 appeared that persisted at 140 h. These proteins may be either precursors of cusativin or immunologically related material that only appear in cotyledons. The fact that these organs produce and degrade cusativin points to their very active metabolism after germination.

Immunoreactive properties of anti-cusativin antibodies. The anti-cusativin antibodies reacted, on immunodif-

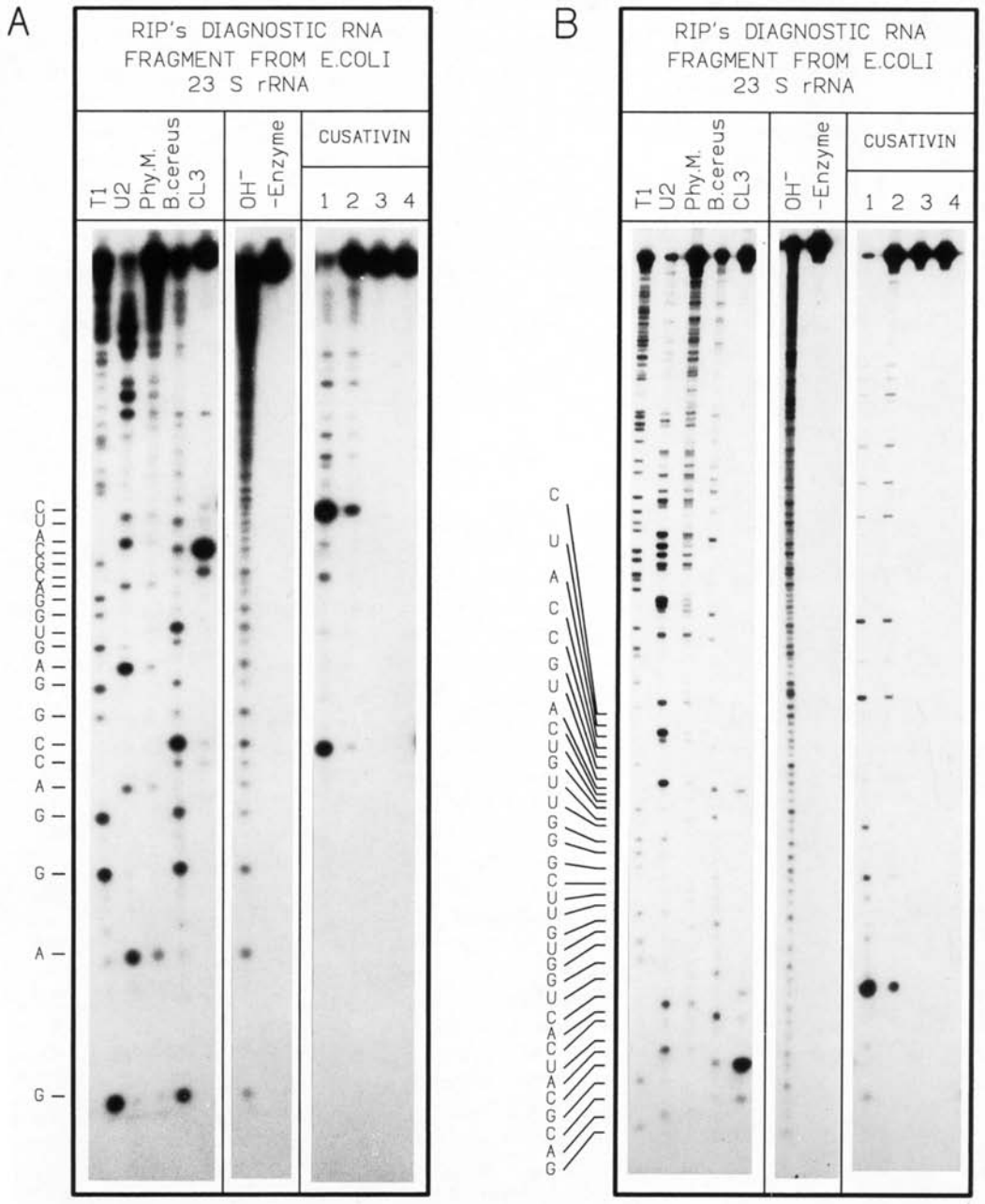


Fig. 11 A, B. Specificity of cusativin using the RIP-diagnostic fragment from *E. coli* 23 S rRNA released by crotin 2 after acid-aniline treatment. Partial digestions of the 5′-end-labelled Endo's fragment from *E. coli* 23 S rRNA were conducted as described elsewhere (Iglesias et al. 1993b). *Lanes* indicate, from left to right, treatment with RNase T1 (*G*), RNase U2 (*A*), RNase Physarum M (*A/U*), RNase from *Bacillus cereus* (*C/U*), RNase CL3 (*C*), alkaline buffer, a sample without enzyme, and cusativin at 17 (*1* and *3*) and 1.7 ng·ml^{-1} (*2* and *4*) and 50° C and either with 7 M urea (*1* and *2*) or in the absence of urea (*3* and *4*). Electrophoresis was performed in gels with either 20% (**A**) or 8% (**B**) polyacrylamide

fusion Ouchterlony plates, with purified cusativin and almost with the same efficiency with melonin (data not shown). By contrast, no cross-reactivity was detected between these antibodies and other proteins such as the plant protein-translation inhibitors N-glycosidase RIPs and RNase A (data not shown).

Action on nucleic acids and polynucleotides. To determine whether cusativin might behave as an RIP or as an RNase, we isolated the rRNA from cusativin-treated ribosomes and subjected it to electrophoresis either without further treatment, to assess direct nucleolytic attack, or with treatment with acid aniline in an attempt to detect the release of the RIP-diagnostic fragment (Endo and Tsurugi 1987; Endo et al. 1987; data not shown). As shown in Fig. 8, electrophoretic analysis of the rRNA extracted from rabbit reticulocyte-lysate ribosomes treated with a low concentration of cusativin revealed a large number of rRNA fragments. This appears to be a consequence of the action of cusativin.

The effects of cusativin on rRNA were seen to be dose-dependent and were clearly appreciable in both polyacrylamide and agarose gels even at doses as low as 10 ng · ml^{-1} (Fig. 8A, B). A direct nucleolytic effect of cusativin was also observed with ribosomes from other mammals. As shown in Fig. 8D, treatment of rat-brain ribosomes with cusativin also generated a large number of oligonucleotides, including one that appears to be the RIP-diagnostic RNA fragment or, at least, a fragment with nearly the same length. However, α-sarcin, restrictocin and mitogillin, highly specific RNases which break the *E. coli* 23 S rRNA at A_{2661} and the rat-liver 28 S

rRNA at A_{4234} (Fando et al. 1985), only generated the RIP–RNA fragment. The collection of discrete fragments of rRNA generated by the action of cusativin on intact ribosomes suggested that cleaved sites would be more exposed to cusativin on the ribosomal surface than uncleaved ones.

Next, we studied whether cusativin might also be able to perform DNA degradation. At a rather high concentration, the protein was unable to promote changes in *E. coli* DNA size as detectable by electrophoresis in 2% agarose submerged gels (Fig. 8C). This clearly indicated that cusativin promoted endonucleolytic cleavages only on RNA.

We further investigated the action of cusativin on polyribonucleotides. As illustrated in Fig. 9A, cusativin promoted an effective degradation of polycytidylic and polyadenylic acids although the former was degraded with higher efficiency than the latter. By contrast, neither polyuridylic nor polyguanylic acids were attacked at all, even at 600 ng · ml^{-1} of cusativin (data not shown). Protein RNasin inhibitors such as RNase inhibitor from human placenta and XMEO8 strongly inhibited the action of cusativin; 16 units · ml^{-1} of either XMEO8 or RNase from human placenta fully abolished the nucleolytic activity of a solution of 16 ng · ml^{-1} of cusativin on 0.5 mg · ml^{-1} of poly(C). This indicates that cusativin is highly sensitive to such protein inhibitors. By contrast, neither EDTA nor EGTA at 3 mM affected the action of cusativin at all.

As shown in Fig. 9B, under conditions of RNA sequencing (i.e. at 50° C and in the presence of 7 M urea; Donis-Keller 1980), cusativin catalyzes cleavage of only polycytidylic acid, over a broad concentration of enzyme tested.

Effects of temperature and pH on cusativin activity. In order to gain further insights into the characterization of cusativin, we undertook a series of experiments aimed at investigating the thermal and chemical sensitivity and the optimum pH. As illustrated in Fig. 10, incubation of cusativin at temperatures higher than 60° C led to its inactivation, which was completed in the 80–95° C range. Neither trehalose nor glycerol were able to prevent the thermally-induced deactivation of cusativin. The optimum pH for the action of cusativin on purified yeast RNA, measured as the release of trichloroacetic-acid-soluble material, was broad and was found to lie between 5 and 7 (Fig. 10).

Ribonuclease sequencing. To define in molecular terms the site at which cusativin cleaves rRNA, we sequenced the already-known RIP-diagnostic RNA fragment, labelled at the 5'-end, by limited digestion using the sequence RNase procedure (Escarmís et al. 1992), and compared this with the pattern of cleavage promoted by cusativin. As shown in Fig. 11, using low concentrations of cusativin and under denaturing conditions, most phosphodiester bonds containing cytidylic acid (e.g. CpG) were hydrolyzed. In the first 40 nt of the RIP-diagnostic RNA fragment from *E. coli,* cytidylic acid occurs at positions 2666, 2667, 2676, 2678, 2681, 2683, 2691 and 2699. Cusativin hydrolyzed cytidylic residues at positions 2666, 2667, 2676, 2678, 2683 and 2691. Positions 2666 and 2678 were poorly hydrolyzed as compared with the CL3 chicken-liver enzyme (Levy and Karpetsky 1980). By contrast, positions 2667, 2681 and 2691, poorly hydrolyzed by CL3, were readily hydrolized by cusativin. In comparison with the other RNases it has been found that, using the RIP-diagnostic RNA fragment as substrate, cusativin cleaves CpG, CpA and CpU bonds with great efficiency. By contrast, UpG bonds were inefficiently hydrolyzed and CpC were not hydrolyzed. Only at high concentration did cusativin also weakly cleave GpU and ApU bonds.

Discussion

The results presented in this work demonstrate the existence of a very active RNase in the dry seeds of *C. sativus* L. – designated cusativin by us – that acted on cell-free translation systems from a variety of sources with slightly different efficiencies. An exception was the *C. sativus* cell-free translation system which was more resistant to cusativin that the other translation systems.

The properties of cusativin allow us to classify it among the RNases on the basis of the following: (i) cusativin catalyzes endonucleolytic cleavage of rRNA in purified ribosomes, of isolated rRNA, of a crude RNA mixture from yeast, of polycytidylic, and of polyadenylic acid; (ii) under sequencing conditions, cusativin catalyzes an almost specific nucleolytic cleavage of cytidylic-acid-containing bonds; (iii) cusativin was completely inactive against DNA even at very high concentrations; (iv) the enzyme has an apparent M_r of 22900 and is thermolabile. This clearly differentiates if from the rather unspecific M_r-12600 thermostable RNase isolated from etiolated cucumber seedlings many years ago (Kado 1968). In view of its nucleotide-specific attack, especially under RNA-sequencing conditions (Donis-Keller 1980), cusativin could be considered as a new class of RNase.

Since at low concentrations cusativin cleaves cytidylic acid-containing bonds, except CpC, it must be useful for RNA sequencing. Cusativin would be complementary to CL3 in RNA sequencing, since CL3 does not cleave some cytidine-containing bonds. However, since the RNA used in this work is small, further studies are required to better define the specificity of cusativin before its use in current RNA sequencing.

It is noteworthy that cusativin retained almost all its hydrolytic activity on CpN bonds (where N is any nucleotide) even under denaturing conditions. The ApN bonds, which are sensitive under native conditions, were unrecognized under denaturing conditions, unlike the CpN bonds. This points to a strong specificity in the interaction between the active site of the protein and cytidine-containing bonds.

Concerning the sensitivity of cusativin towards current RNase inhibitors, our results indicate that the enzyme does not require divalent cations. Its sensitivity to protein inhibitors opens the possibility of some kind

of strong, perhaps stoichiometric, interaction between cusativin and the inhibitor. This, however, requires further research.

From an immunological point of view, melonin, the protein-translation inhibitor isolated from *Cucumis melo* L. (Ferreras et al. 1989; Rojo et al. 1992), is more closely related to cusativin than to RIPs. Anti-cusativin antibodies did not react with other protein-translation inhibitors such as RIPs (data not shown), or RNase A, whereas they did react with melonin. This suggests that melonin might also be an RNase, in contrast to previous reports (Ferreras et al. 1989).

It has been reported that up to 100 nucleolytic cleavages in rRNA do not impair the translational activity of ribosomes (Fong et al. 1991). By contrast, a single RIP-promoted depurination in a highly conserved loop of the largest rRNA of mammalian and bacterial ribosomes leads to irreversible inactivation of translation (Stirpe et al. 1992). In *E. coli* it has been shown that this loop is protected by elongation factor G from attack by dimethyl sulphate (Moazed et al. 1988) and by the type-1 RIP crotin 2 (Iglesias et al. 1993b). Therefore, this rRNA loop must play a key role in polypeptide chain elongation. Cusativin cleaves mammalian rRNA into a series of fragments of very different sizes, including one fragment with the same electrophoretic mobility as the RNA fragment (Fig. 8), which is released by the action of α-sarcin on the phosphodiesther A_{4325} of the 28–S rRNA of mammalian ribosomes (Fando et al. 1985). The conserved rRNA loop, whose cleavage leads to excision of Endo's fragment (Fong et al. 1991; Stirpe et al. 1992), is exposed to the medium and is therefore accessible to low-M_r proteins such as cusativin, α-sarcin (Jiménez and Vázquez 1985, and data in this work) and RIPs (Stirpe et al. 1992). However, the fact that not all the cell-free translation systems assayed in this work displayed the same sensitivity to cusativin would indicate that the conserved loop is not equally accessible in all ribosomes. The pinpoints the differences among ribosomes, specially those belonging to different kingdoms.

The biological role played by cusativin in maturing *C. sativus* seeds is speculative at present. It is possible that the accumulation of cusativin in seeds might be related to the cessation of transcriptional and translational activities preceding desiccation and dormancy of the seeds. This activity of cusativin could be related to seed-coat formation through tissue death and subsequent hardening of the coat (there is an increase in the opaqueness of maturing seeds in seed stage 4, Fig. 6). The absence of cusativin in actively elongating embryonic axes, which require high rates of both transcription and translation, and in mature plants is consistent with this hypothesis. Assessing the hypothesis will require determining the localization of cusativin.

Cusativin in the seed coat may constitute a new class of plant defense proteins (i.e. against fungi and RNA-viruses), a role that has also been suggested for three proteins of barley seeds – an RIP, a chitinase and a glucanase (Leah et al. 1991) – and perhaps from attack by insects. Further work will address the potential correlation existing between the appearance of cusativin and the pattern of seed RNAs and the potential antipathogenic activity of the enzyme.

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