1 Fernandes, J. F., Halsman, M., and Castellani, O., Nature *207* (1965) 1004.
2 Fernandes, J. F., Castellani, O., and Okumura, M., Rev. Inst. Med. Trop. Saõ Paulo *8* (1966) 151.
3 Fernandes, J. F., Halsman, M., and Castellani, O., Exp. Parasit. *18* (1966) 203.
4 Camargo, E. P., Rev. Inst. Med. Trop. Saõ Paulo *6* (1964) 93.
5 Bianco, C., Griffin, F. J. Jr, and Silverstein, S. C., J. exp. Med. *141* (1975) 1278.
6 Cohn, Z. A., and Benson, B., J. exp. Med. *121* (1965) 153.
7 Sobbel, H. M., Prog. nucleic Acid Res. molec. Biol. *13* (1973) 153.
8 Sobbel, H. M., Proc. natl Acad. Sci. *82* (1985) 5328.
9 Brascher, H. M., Vargens, J., Queiroz Cruz, M., and Oliveira-Lima, A., Mem. Inst. Oswaldo Cruz *83* (1988) 152.

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Class I and class II ribonuclease H activities in *Crithidia fasciculata* (Protozoa)

H. Vonwirth, J. Köck* and W. Büsen

*Lehrstuhl für Allgemeine Genetik, Biologisches Institut, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen, and * Max-Planck-Institut für Biologie, Correnstr. 38, D-7400 Tübingen (Federal Republic of Germany)*
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Summary. The protozoan *Crithidia fasciculata* contains two different ribonuclease H activities. These enzymes display similar physical and biochemical characteristics to their homologues in higher eukaryotes, for instance calf thymus class I and class II ribonuclease H. Class I ribonuclease H of lower and higher eukaryotes can be activated by Mg^{2+}- and Mn^{2+}-ions. However, the presence of Mn^{2+}-ions is inhibitory for the Mg^{2+}-dependent class II ribonuclease H activity of *Crithidia fasciculata* and calf thymus. The protozoan class I-homologue enzyme appears to be serologically related to the class I ribonuclease H of calf thymus.
Key words. Ribonuclease H activity; kinetoplastida; *Crithidia fasciculata.*

Ribonucleases H are enzymes which specifically degrade the RNA moiety of RNA DNA-hybrids [1, 2]. Higher eukaryotes contain two enzymes with ribonuclease H specificity, a class I and a class II ribonuclease H [3–6]. The classification of these enzyme activities, in particular those of calf thymus (here ribonuclease H I and H IIb correspond to class I and II ribonuclease H, respectively), is based on their physical and biochemical properties as well as on serological analyses [3–8]. Although ribonucleases H of yeast are well characterized, an unambiguous attribution of the distinct activities to class I and class II ribonucleases H, based on the criteria mentioned above, is not possible [9–14]. Extracts of macronuclei of the ciliate *Tetrahymena pyriformis* contain three ribonuclease H activities with biochemical characteristics similar to the class II ribonuclease H of higher eukaryotes [15].
Here we show that extracts of the protozoan *Crithidia fasciculata* display two distinct ribonuclease H activities, which can be classified as class I and class II ribonuclease H activity, as judged by physical and biochemical parameters. Moreover, the enzyme preparation of *C. fasciculata* containing the presumptive class I ribonuclease H activity is specifically recognized by an antibody directed against calf thymus ribonuclease H I.

Materials and methods

Preparation of C. fasciculata crude extract. C. fasciculata clone 1 was grown in 3.7% (w/v) brain heart infusion broth supplemented with 20 mg/l hemin as described [16]. Cells were harvested by centrifugation, washed and lysed in 50 vols of TGED [50 mM Tris/HCl pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25% (w/v) glycerol, 0.1 mM phenylmethylsulfonylfluorid] + 0.3 M $(NH_4)_2SO_4$ using a 'Stansted Cell Disrupter' [17]. The cell lysate was clarified by centrifugation and the sediment discarded. The crude extract contained 1.0 mg of protein/ml and 7.5 units/mg ribonuclease H activity (measured under Mg^+-conditions).
Separation of two ribonuclease H activities in C. fasciculata. C. fasciculata crude extract was absorbed to DEAE-cellulose, equilibrated in TGED and washed extensively. The DEAE-cellulose-binding ribonuclease H activity was eluted with TGED + 200 mM KCl. The DEAE-cellulose eluate contained one third and the flow-through two thirds of the remaining ribonuclease H activity. The yield of enzyme activity amounted to 77% compared with the crude extract. The DEAE-cellulose-binding and unbound ribonuclease H activities (1.0 mg of protein/ml each) displayed specific activities of 5.0 units/mg and 1.5 units/mg measured under Mg^{2+}-conditions, respectively. The ribonuclease H activity not bound to DEAE-cellulose bound to CM-Sephadex, and eluted from this column at around 0.2 M KCl.
Assay for ribonuclease H activity. Ribonuclease H activity determinations were carried out exactly as described elsewhere [1, 3, 5, 6]. The assay mixture contained, in a final volume of 500 µl, 30 mM Tris/HCl pH 7.8, 50 mM $(NH_4)_2SO_4$, 0.02% 2-Mercaptoethanol, 20 µl (^{3}H)RNA DNA-hybrid (2000 cpm, corresponding to 60 pMol of ribonucleotides), and either 10 mM $MgCl_2$ or 2 mM

$MnCl_2$. One unit of ribonuclease H is that amount of enzyme that renders 100 pMol of ribonucleotides acid-soluble under optimal conditions in 10 min at 37 °C.

Protein determination. Protein determinations were carried out according to the method of Bradford [18].

SDS-Polyacrylamide gel electrophoresis and immunoblotting analysis. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [19]. Proteins were visualized by Coomassie blue or transferred to nitrocellulose and processed for immunoblotting analysis as described [5, 6].

Results and discussion

As outlined in the introduction, the published data on ribonuclease H in yeast and *Tetrahymena* do not allow a decision whether the occurrence of class I and class II ribonuclease H activities is typical for eukaryotes in general. Therefore, we became interested in ribonuclease H activities of another protozoan organism, *Crithidia fasciculata* (Kinetoplastida, Flagellata). During our studies we discovered that crude extracts of *C. fasciculata* contained two distinct ribonuclease H activities, which could be separated by DEAE-cellulose chromatography. One activity bound to this anion exchange matrix whereas the other was found in the flow-through fraction (see 'Materials and methods'). The DEAE-cellulose-binding and non-binding ribonuclease H activities displayed physical and biochemical parameters nearly identical with those of the class I and class II ribonucleases H of higher eukaryotes. The table shows a comparison of various characteristics of the calf thymus and the *C. fasciculata* enzymes. As can be seen, the distinct ribonuclease H activities of *C. fasciculata* are differentiated by these parameters. Moreover, the DEAE-binding and the DEAE non-binding activity display nearly the same characteristics as class I and class II ribonuclease H of calf thymus, respectively (in calf thymus, ribonuclease H I corresponds to class I ribonuclease H, and ribonuclease H IIb to the class II enzyme).

Physical and biochemical characteristics of class I and class II ribonuclease H activities of calf thymus and *C. fasciculata*

	Class I ribonuclease H activities of		Class II ribonuclease H activities of	
	calf thymus	*C. fasciculata*	calf thymus	*C. fasciculata*
DEAE-binding	+	+	−	−
CM-binding	−	−	+	+
Molecular mass[a]	67 kDa	67 kDa	45 kDa	45 kDa
Degradation of RNA DNA-hybrids [%]	100	100	100	100
Degradation of denatured hybrids [%]	4.9	5.1	5.1	5.0
Activity without divalent cations [%]	0	0	0	0
Activated by Mg^{2+}-ions	+ (10 mM)[b]	+ (10 mM)	+ (10 mM)	+ (8 mM)
Activated by Mn^{2+}-ions	+ (1 mM)[b]	+ (2 mM)	−	−
Inhibited by Mn^{2+}-ions	−	−	+	+
Sensitivity against N-ethyl-maleimide (2 mM)	no	weak	strong	strong
pH optimum	7.4	7.0–7.5	9.0–9.5	9.0

Activity determinations were carried out as described in 'Materials and methods' (100% degradation of RNA DNA-hybrid corresponds to the release of around 60 pmol of ribonucleotides).
[a] Molecular mass determinations were carried out by sucrose density centrifugation [5, 7]. Class I ribonuclease H activities sediment like bovine serum albumin (67 kDa) and class II ribonuclease H activities like ovalbumin (45 kDa). [b] Optimal concentrations are given in brackets.

The divalent cation dependence and sensitivity of the protozoan ribonuclease H activities can be taken as evidence that both activities correspond to their homologues in higher eukaryotes. Whereas the DEAE-binding ribonculease H activity is active in the presence of Mn^{2+}-ions as well as Mg^{2+}-ions (fig. 1 a), the DEAE non-binding ribonuclease H activity shows a strict dependence on the presence of Mg^{2+}-ions. As shown in figure 1 b, Mn^{2+}-ions are not only unable to activate the DEAE non-binding ribonuclease H activity, but also strongly inhibit the Mg^{2+}-dependent activity. This sensitivity is not only characteristic for the *C. fasciculata* enzyme but also for the class II ribonuclease H of higher eukaryotes (shown for calf thymus ribonuclease H IIb in fig. 1 B). The class I enzymes of calf thymus and *C. fasciculata* do not display this type of sensitivity (fig. 1 A and a). Thus, the Mn^{2+}-sensitivity of the Mg^{2+}-dependent class II ribonuclease H activity seems to be an important, new feature to distinguish between eukaryotic ribonuclease H of class I and of class II. If the Mn^{2+}-sensitivity of the class II enzyme can be confirmed as a general feature of eukaryotic class II ribonuclease H, then this sensitivity should be helpful in in vitro systems for transcription and replication for finding indications, or for excluding the possible participation of class II ribonuclease H.

As a further step, we wanted to see whether polyclonal antibodies against calf thymus ribonuclease H I and H IIb, respectively, cross-reacted with proteins in the corresponding *C. fasciculata* fractions containing ribonuclease H activities. Figure 2 shows that the protein fraction displaying class I ribonuclease H activity is recognized by the anti-calf thymus ribonuclease H I-serum (A), but not by the control serum (C)[7]. The cross-reacting 64 kDa-protein band can be followed in association with the Mn^{2+}-activatable ribonuclease H activity on phosphocellulose chromatography (not shown). Therefore, this protein band may correspond to the native class I ribonuclease H in *C. fasciculata*. The anti-calf thymus ribonuclease H IIb-serum [5], however, does not recognize a cross-reacting protein in *C. fasciculata* fractions containing class II ribonuclease H activity (not shown). Interestingly, this antibody cross-reacts specifically with another, DEAE-binding protein band, which seems to be associat-

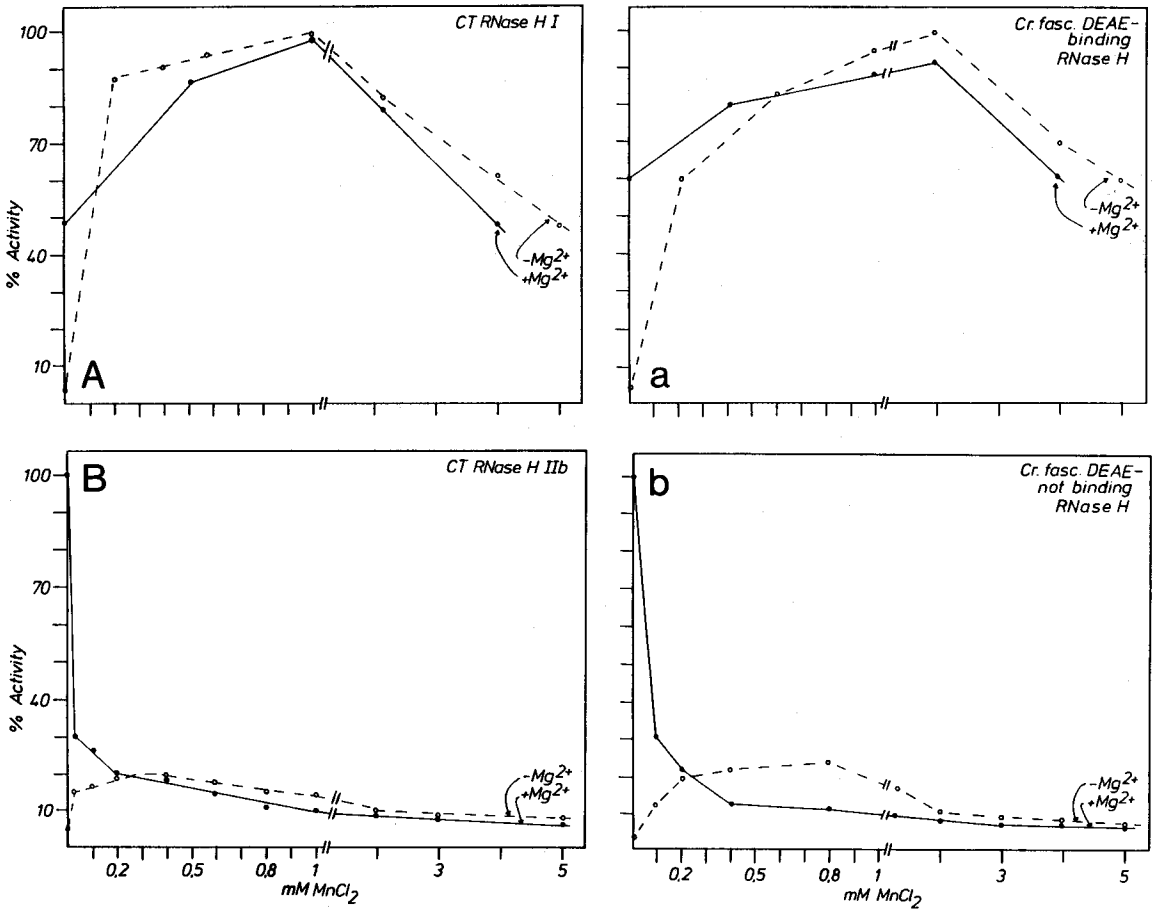

Figure 1. Effect of Mn^{2+}-ions on the two Mg^{2+}-dependent ribonuclease H activities of calf thymus and *C. fasciculata*. The figure shows the activities of calf thymus ribonuclease H I (*A*), *C. fasciculata* DEAE-binding ribonuclease H activity (*a*), calf thymus ribonuclease H IIb (*B*), and *C. fasciculata* DEAE non-binding ribonuclease H activity (*b*) in the presence of $MnCl_2$ alone (○) or in the presence of 10 mM $MgCl_2$ *and* $MnCl_2$ (●). (100% activity corresponds to the release of 30–45 pmol of ribonucleotides; –○–○–○– ribonuclease H activity in the presence of increasing concentrations of $MnCl_2$; –●–●–●– ribonuclease H activity in the presence of 10 mM $MgCl_2$ and increasing concentrations of $MnCl_2$.)

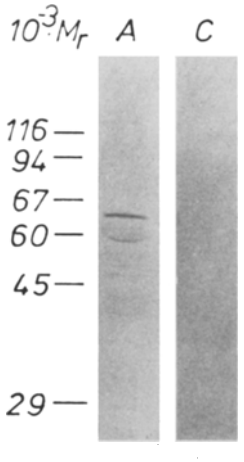

Figure 2. Immunoblot analysis of *C. fasciculata* DEAE-cellulose-binding proteins. *C. fasciculata* DEAE-cellulose-eluate (100 µg of protein) was separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose and processed for immunoblotting as described[5,6]. (*A*, 1:1000 dilution of anti-calf thymus ribonuclease H I-serum; *C*, 1:500 dilution of control serum)[7].

ed with RNA polymerase I in this protozoan system (Köck et al., manuscript in preparation).

Only two other, lower eukaryotic systems have been characterized for their ribonuclease H activities: yeast and *Tetrahymena pyriformis*. Yeast ribonuclease H(70) might correspond to our class I ribonuclease H, and yeast ribonucleases H(55) and H(42) to our class II ribonuclease H. The DEAE-binding behavior and the reported molecular masses are in favor of this interpretation, but the divalent cation requirements and N-ethyl-maleimide-sensitivities argue against this notion[12–14]. In macronuclear extracts of *T. pyriformis*, only DEAE non-binding ribonuclease H activities have been characterized. They most probably correspond to our class II ribonuclease H[15].

Although the exact biological functions of the class I and class II ribonucleases H are not known, indications favor a role for the class I enzyme during replication and for the class II enzyme during transcription[2,6]. The fact that

both classes of ribonuclease H occur in metazoa and in kinetoplastida suggests that their occurrence is a general feature of eukaryotes. The kinetoplastids diverged very early in evolution form the other eukaryotes[20]; this can be taken as an indication that the class I and class II ribonuclease H appeared very early in eukaryotic evolution.

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1 Hausen, P., and Stein, H., Eur. J. Biochem. *14* (1970) 278.
2 Crouch, R. J., and Dirksen, M.-L., Cold Spring Harb. Monogr. *14* (1982) 211.
3 Büsen, W., and Hausen, P., Eur. J. Biochem. *52* (1975) 17.
4 Büsen, W., Dissertation, University of Tübingen, Fachbereich Biologie 1976.
5 Vonwirth, H., Frank, P., and Büsen, W., Eur. J. Biochem. *184* (1989) 321.
6 Vonwirth, H., Dissertation, University of Tübingen, Fachbereich Biologie 1990.
7 Büsen, W., J. biol. Chem. *255* (1980) 9434.
8 Büsen, W., in: Biological Implications of Protein-Nucleic Acid Interactions, pp. 571–584. Ed. J. Angustyuiak. Elsevier/North-Holland, Amsterdam 1980.
9 Wyers, F., Sentenac, A., and Fromagoet, P., Eur. J. Biochem. *69* (1976) 377.
10 Wyers, F., Huet, J., Sentenac, A., and Fromagoet, P., Eur. J. Biochem. *69* (1976) 385.
11 Iborra, F., Huet, J., Breant, B., Sentenac, A., and Fromagoet, P., J. biol. Chem. *254* (1979) 10920.
12 Karwan, R., Blutsch, H., and Wintersberger, U., Biochemistry *22* (1983) 5500.
13 Karwan, R., and Wintersberger, U., J. biol. Chem. *263* (1988) 14970.
14 Wintersberger, U., Kühne, C., and Karwan, R., Biochim. biophys. Acta *951* (1988) 322.
15 Tashiro, F., and Mita, T., Eur. J. Biochem. *65* (1976) 123.
16 Evers, R., Hammer, A., Köck, J., Jess, W., Borst, P., Mémet, S., and Cornelissen, A. W. C. A., Cell *56* (1989) 585.
17 Kooter, J. M., and Borst, P., Nucl. Acids Res. *12* (1984) 9457.
18 Bradford, M. M., Analyt. Biochem. *72* (1976) 248.
19 Laemmli, U. K., Nature *227* (1970) 680.
20 Gunderson, J. H., McCutchan, T. F., and Sogin, M. L., J. Protozool. *33* (1986) 525.

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Mitogenic activity of selenoorganic compounds in human peripheral blood leukocytes

J. A. Czyrski and A. D. Inglot*

Laboratory of Virology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Czerska 12, 53–114 Wroclaw (Poland)
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Summary. A variety of organoselenium compounds were originally described as antiinflammatory, antioxidant or glutathione-peroxidase-like agents, and as inhibitors of prostaglandin and leukotriene synthesis. Recently, the compounds have also been found to be inducers of interferon gamma and tumor necrosis factor in human peripheral blood leukocytes (PBL). We evaluated the effects of bis [2-(N-phenylcarboxamido)phenyl] diselenide and Ebselen®; 2-phenyl-1,2-benzisoselenazol-3(2H)one, on the incorporation of tritiated thymidine into the DNA of PBL cultured in vitro. Both compounds were mitogenic and this effect was correlated with the expression of interleukin 2 receptor in T-lymphocytes. Therefore, we suggest that the selenoorganic compounds may induce mitogenic cytokines.
Key words. Selenoorganic compounds; Ebselen®; human leukocytes; mitogenic activity; IL-2 receptor expression.

The organoselenium compounds have many different biological activities in vitro and in vivo. Originally they were described as glutathione-peroxidase-like agents, antioxidants and inhibitors of prostaglandins and leukotrienes[1–5]. Ebselen®; 2-phenyl-1,2-benzisoselenazol-3(2H)one is a potential antiinflammatory drug of a new generation. The toxicity of Ebselen in mice, rats, pigs and man is low because in this structure Se is not bioavailable[4,5].

Recent studies conducted in our laboratory have provided evidence that the selenoorganic compounds are inducers of cytokines including interferon gamma (IFN-γ) and tumor necrosis factor (TNF) in human peripheral blood leukocytes (PBL). Therefore, the compounds may be also regarded as biological response modifiers[6].

Here, we describe the activation of human lymphoid cells to proliferate brought about by Ebselen and bis [2-(N-phenyl-carboxamido)phenyl] diselenide (diselenide 1). This activity was correlated with the expression of receptors for interleukin-2 (IL-2R).

Materials and methods

The selenoorganic compounds were synthesized at the Institute of Organic and Physical Chemistry, Technical University, Wrocław[7,8]. The stock solutions (20 mg/ml) of the compounds were prepared in dimethyl sulfoxide. The compounds were chemically pure and they were free of lipopolysaccharides.

The buffy coats of leukocytes were obtained from the Wrocław Regional Blood Transfusion Center. The erythrocytes from the suspension of leukocytes were removed be repeated lysis with 0.83% ammonium chloride according to Cantell et al.[9]. The separated PBL were