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Calf thymus ribonuclease H IIa activity lacks ribonuclease H specificity

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Summary. Less purified fractions of ribonuclease H IIa activity of calf thymus display divalent cation-dependent ribonuclease H activity and divalent cation-independent ribonuclease activity. Because the ratio of the two enzyme activities does not change during successive chromatographic procedures, we suggest that ribonuclease H IIa activity is indeed able to degrade both ssRNA and the RNA moiety of RNA·DNA-hybrids. Ribonuclease H IIa activity can therefore be differentiated from calf thymus ribonuclease H I and H IIb by its lack of ribonuclease H specificity. The native molecular mass of ribonuclease H IIa activity is between 23 and 28 kDa. Under denaturing conditions a 23 kDa-protein band copurifies with the enzyme activity suggesting that this enzyme is monomeric.
Key words. Ribonuclease H activity; ribonuclease activity; calf thymus.

Ribonucleases H are enzymes which specifically degrade the RNA moiety of RNA·DNA-hybrids [1, 2]. Calf thymus contains two enzymes with ribonuclease H specificity, named ribonuclease H I and H IIb, and another ribonuclease H activity, named ribonuclease H IIa activity [3, 4]. Ribonucleases H I and IIb have been purified to near homogeneity and polyclonal antibodies have been raised against these proteins [5–7]. All three enzymes could be differentiated by their physical properties as well as by serological analyses [3–8]. It has been previously shown that the ribonuclease H IIa fraction is specific in degrading the RNA moiety of RNA · DNA-hybrids, but not able to degrade the DNA part of such hybrids and single- or double-stranded DNA. On the other hand, this enzyme fraction degrades poly(rA) quite efficiently [3, 4]. Therefore the question remained whether ribonuclease H- and ribonuclease-activities are intrinsic properties of the enzyme IIa fraction or whether they can be separated from each other.

Materials and methods

Assay for ribonuclease H activity. Ribonuclease H activity determinations were carried out exactly as described elsewhere [1, 3, 7, 8]. The assay mixture contained in a final volume of 500 µl 50 mM Tris-HCl pH 7.8, 50 mM $(NH_4)_2SO_4$, 0.4 mM $MnCl_2$, 0.02% 2-mercaptoethanol, and 20 µl (^{3}H)RNA·DNA-hybrid (2000 cpm, corresponding to 60 pmol of ribonucleotides). 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 [9].

Purification of ribonuclease H IIa activity. Preparation of calf thymus crude extract (fraction 0) and separation of the different ribonuclease H activities were performed exactly as described elsewhere [3, 4, 7]. After separation from the other ribonuclease H activities (H I and H IIb) the ribonuclease H IIa activity fraction (fraction 2a) contained less than 0.1% of the protein of the calf thymus crude extract. The specific activity of fraction 2a amounted to 355 units/mg of protein. Fraction 2a was separated on an S-sepharose fast flow column using a linear salt gradient (0–1000 mM KCl in buffer B (30 mM Tris-HCl pH 7.8, 30% glycerol (mass/vol.), 2 mM EDTA, and 0.1% 2-mercaptoethanol)). Ribonuclease H IIa activity elutes from this column at 220 mM KCl (fraction 3a). The proteins were concentrated by chromatography on a CM-sepharose fast flow column and step elution with buffer B + 500 mM KCl. Fractions containing ribonuclease H IIa activity (fraction 4a) were separated on a Sephadex G 75 gel filtration column. Fractions containing enzyme activity (fraction 5a) were dialyzed against buffer B and applied to a blue sepharose CL-6B column. Proteins were eluted with a linear salt gradient (0–1000 mM KCl in buffer B). Ribonuclease H IIa activity

elutes at 200 mM KCl from this column. Fractions containing enzyme activity (fraction 6a) were dialyzed against buffer B.

One half of the dialyzed fraction 6a was chromatographed on a poly(rC)-agarose column and the proteins eluted with a linear salt gradient (0–1000 mM KCl in buffer B). Ribonuclease H IIa activity elutes at the beginning of the salt gradient. Activity-containing fractions were pooled (fraction $7a_1$). The specific activity of fraction $7a_1$ was determined to be 4420 units/mg. The other half of the dialyzed fraction 6a was applied onto a native DNA-cellulose column and the proteins were eluted from this matrix with a linear salt gradient (0–1000 mM KCl in buffer B). Ribonuclease H IIa activity elutes at 110 mM KCl from this column (fraction $7a_2$). The specific activity of fraction $7a_2$ was 5150 units/mg. Fractions $7a_1$ and $7a_2$ were stored at – 70 °C.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [10]. Proteins were visualized by Coomassie blue or silver staining [11, 12].

Results and discussion

Calf thymus ribonuclease H IIa activity is a minor activity and can first be detected after separation from the two main ribonuclease H activities, ribonuclease H I and H IIb [3, 4, 7, 8] (see fig. 1 of reference 7). Whereas for ribonuclease H I and H IIb ribonuclease H specificity could be clearly demonstrated even in less purified enzyme fractions, the question remained whether calf thymus ribonuclease H IIa activity displays ribonuclease H specificity or whether this enzyme displays ribonuclease H and ribonuclease activity. Figure 1 shows a comparison of the activities associated with the ribonuclease H IIa fraction 2a (see 'Materials and methods') with ribonuclease A. Ribonuclease H IIa activity, in the absence of divalent cations (x), is similar to ribonuclease A; under these conditions both enzyme activities degrade ssRNA but not the RNA moiety of RNA · DNA-hybrids (compare the top figures with the bottom ones (x)). However, in the presence of divalent cations (ribonuclease H IIa activity is preferentially activated by Mn^{2+}-ions as opposed to Mg^{2+}-ions) (o), fraction 2a degrades both substrates. It

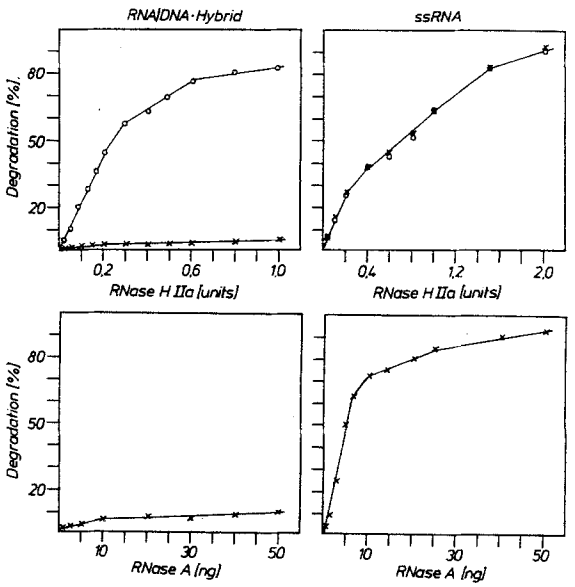

Figure 1. Comparison of the enzymatic properties of ribonuclease H IIa activity and ribonuclease A. Top: Ribonuclease H IIa activity (fraction 2a, see 'Materials and methods') was assayed for degradation of the RNA moiety of RNA · DNA-hybrids (left) or the degradation of ssRNA (right) both in the presence (o) and in the absence of 0.4 mM $MnCl_2$ (x). Bottom: Ribonuclease A was assayed under identical conditions for its ability to solubilize the RNA part of RNA · DNA-hybrids (left) or ssRNA (right) in the absence of $MnCl_2$ (in the presence of $MnCl_2$ ribonuclease A displays the same digestion pattern, not shown). 100% degradation corresponds to the release of 60 pmol ribonucleotides.

displays therefore ribonuclease H activity and ribonuclease activity.

To examine whether the two enzymatic activities can be separated from each other, we purified the ribonuclease H IIa activity by several chromatographic procedures as outlined in 'Materials and methods', analyzed the enzymatic properties for all six purification steps (3a to $7a_1$ and $7a_2$) and compared them with those found for fraction 2a (as outlined in fig. 1). As shown in the table, all fractions display divalent cation-dependent ribonuclease H activity and divalent cation-independent ribonuclease activity. Thus, even the most purified enzyme activity IIa displays both enzymatic activities, ribonuclease activity both in the presence and absence of divalent cations and ribonuclease H activity only in their presence

Ratio of ribonuclease/ribonuclease H activity during purification of ribonuclease H IIa activity

Fraction	Specific activity (units/mg)	% degradation of RNA · DNA-hybrid + $MnCl_2$	– $MnCl_2$	ssRNA + $MnCl_2$	– $MnCl_2$	ssRNA/ RNA·DNA-hybrid
2a	355	62.0	5.1	34.0	33.8	0.55
3a	507	67.5	4.9	33.0	33.2	0.49
4a	625	52.0	5.0	27.0	28.0	0.52
5a	1,600	81.0	6.1	44.0	43.8	0.54
6a	3,170	69.0	5.2	36.6	36.5	0.53
$7a_1$	4,420	71.0	5.5	37.0	36.0	0.52
$7a_2$	5,150	75.0	5.1	39.2	38.5	0.52

Activity determinations were carried out as described in 'Materials and methods' (100% degradation of RNA·DNA-hybrid or ssRNA corresponds to the release of 60 pmol of ribonucleotides). Fraction 2a is the first fraction of the purification free of the other main ribonuclease H activities (H I and H IIb). It is already a highly enriched protein fraction consisting of less than 0.1% of the original protein content of the crude extract.

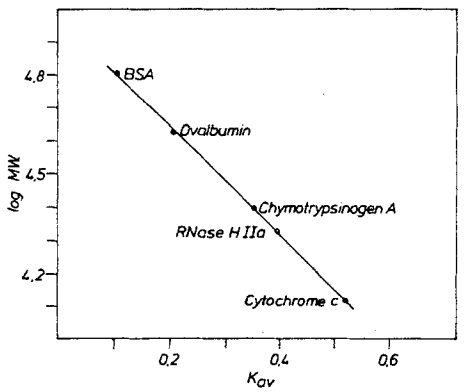

Figure 2. Native molecular mass determination of ribonuclease H IIa activity. Gel filtration was carried out on a Sephadex G 75 column equilibrated in buffer B + 500 mM KCl. Fraction 4a (8 ml) was loaded onto the column, 1-ml fractions were collected and the ribonuclease H activity determined. The calibration plot shows the position of ribonuclease H IIa activity relative to the position of the indicated marker proteins.

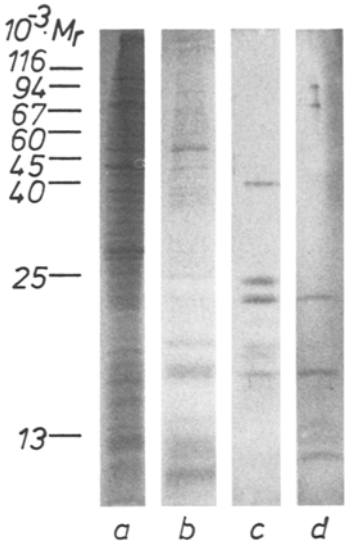

Figure 3. SDS-polyacrylamide gel electrophoresis of various purified fractions of ribonuclease H IIa activity. Proteins were separated on a 15% polyacrylamide gel and visualized by Coomassie blue (lanes a and b) or processed for silver staining (lanes c and d). Lane a: 100 µg of fraction 0 of the purification; lane b: 50 µg of fraction 2a of the purification; lane c: 3 µg of fraction $7a_1$ of the purification; lane d: 3 µg of fraction $7a_2$ of the purification (see 'Materials and methods').

($Mn^{2+} > Mg^{2+}$). Moreover, the ratio between the ribonuclease activity and the ribonuclease H activity does not change during the purification (see table). We interpret this to mean that ribonuclease H IIa activity is most probably able to degrade the RNA moiety of RNA · DNA-hybrids as well as ssRNA. Therefore, even the most purified ribonuclease H IIa activity does not display ribonuclease H specificity; it can be clearly differentiated from ribonuclease H I and H IIb enzymes which show ribonuclease H specificity.

We propose that enzymes with ribonuclease H specificity should be called ribonuclease H, and those which do not show specificity in degrading only the RNA moiety of RNA · DNA-hybrids ribonuclease H activity.

Gel filtration experiments were performed to determine the native molecular mass of the protein with ribonuclease H IIa activity. Figure 2 shows that the enzyme elutes from a sephadex G 75 column as a protein with à molecular mass of around 23 kDa. Sucrose gradient centrifugation experiments yield a value of around 28 kDa[4].

Figure 3 shows SDS-polyacrylamide gel electrophoreses of aliquots of fractions 0, 2a, $7a_1$ and $7a_2$ of the purification. As one can see, distinct polypeptides have been purified from the crude extract. Fraction $7a_2$ (lane d) shows only three prominent protein bands with molecular masses of 23, 16, and 11.5 kDa, respectively. We presume that the 23 kDa-polypeptide is the most likely candidate for the ribonuclease H IIa activity protein because this protein band is the protein band with the highest molecular mass common to purified enzyme fractions prepared by different chromatographic procedures (compare fraction $7a_1$ and $7a_2$ (fig. 3, lanes c and d)). Thus, molecular mass determinations indicate that ribonuclease H IIa activity is most probably a monomeric protein with an apparent native molecular mass of 23 kDa.

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