ATYPICAL BEHAVIOUR OF RIBONUCLEASE SPL: DIFFERENT CONCENTRATIONS OF THE ENZYME GIVE DIFFERENT LIMIT DIGESTS

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1. Introduction

We have reported the isolation and characterisation of a new type of ribonuclease, RNase SPL, from bovine seminal plasma, which shows a RNase A-type activity on synthetic polyribonucleotides and on Mg^{2+}-containing heat-denatured or Mg^{2+}-depleted undenatured total *Escherichia coli* RNA, but which gives a limit digest containing only large ($\gtrsim M_r$ 10 000) fragments, with Mg^{2+}-containing undenatured (native) total *E. coli* RNA [1]. Here we show that one cannot compensate for a lower concentration of RNase SPL by increasing the reaction time within the limits studied, even though the enzyme continues to be fully active during this period. RNase SPL appears to be the first enzyme described to exhibit this phenomenon.

2. Materials and methods

2.1. *RNase SPL*

The enzyme was prepared as in [1,2]. Only preparations that were homogeneous [1] were used.

2.2. E. coli *RNA*

[^{3}H]Uridine-labelled Mg^{2+}-containing (native) total *E. coli* RNA was prepared either as in [1] or by the following method: The *E. coli* cells grown in the presence of the labelled uridine were suspended in 0.01 M Tris–HCl buffer (pH 7, containing 0.14 M NaCl, 0.001 M EDTA, 0.01 M $MgCl_2$, 10 μg polyvinyl sulphate/ml and 0.01% Bentonite). SDS was added to a final concentration of 1%, and the mixture shaken at room temperature for 10–15 min. RNA was extracted from the lysate as in [3].

2.3. *Nucleolytic activity*

RNase SPL was assayed for nucleolytic activity on the labelled *E. coli* RNA obtained as above, using paper-strip chromatography and polyacrylamide gel electrophoresis (PAGE) for determination of the acid-insoluble radioactivity, as in [1], except that a LKB slab-gel electrophoresis apparatus was used for PAGE. The radioactivity staying at the origin on paper-strip chromatography, or the combined radioactivity in the gel slices [1], was taken as a measure of the sum of radioactivity in the unhydrolysed RNA and the acid-insoluble products of hydrolysis. The difference between the initial radioactivity in RNA and the radioactivity staying at the origin on paper-strip chromatography, was taken to represent the acid-soluble products generated. The banding pattern on PAGE gave an indication of the extent of cleavage of the internucleotide linkages where the hydrolytic products were still large (acid-insoluble).

3. Results and discussion

We have shown [1] that incubation of Mg^{2+}-containing native total *E. coli* RNA (0.9–1.4 mg/ml) with 12–135 μg RNase SPL/ml for 5 min, gives a limit digest in which the fragments are usually 10 000–150 000 M_r; no acid-soluble material, or material which runs off the gel on PAGE of the digest, is formed. The molecular mass profile of the digest as assayed by its PAGE pattern, did not change beyond 12 μg RNase SPL/ml. In other words, increase of RNase SPL to >12 μg/ml (up to 135 μg/ml for 1.35 mg RNA/ml), did not lead to any further cleavage of the internucleotide linkages. (The above range of concentration of RNase SPL should be viewed in light of the fact that

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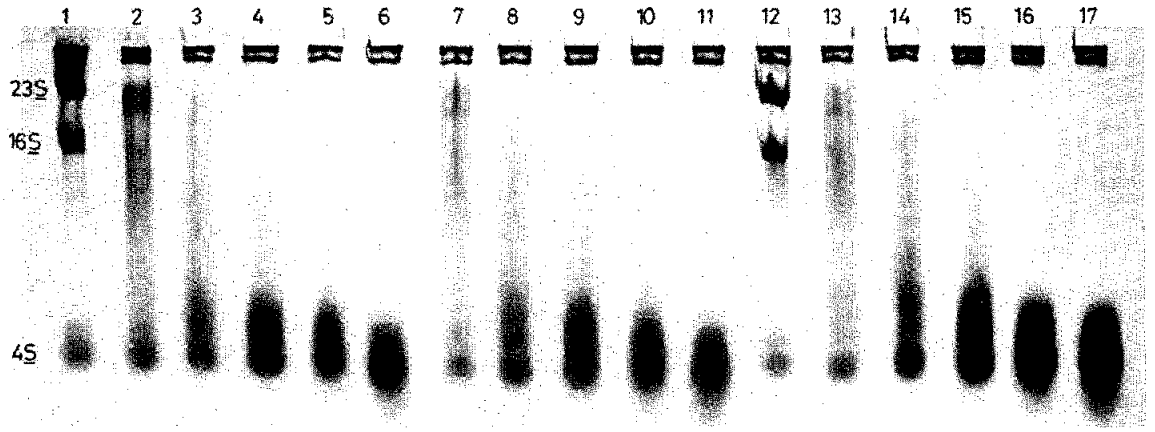

Fig.1. Characterisation by PAGE on a 3% gel, of ^{3}H-labelled Mg^{2+}-containing native total *E. coli* RNA treated with RNase SPL for different periods of time. The labelled RNA (200 μg; 6 × 10^5 cpm) was treated for 5,20 and 30 min with various concentrations of the nuclease in a total volume of 0.1 ml. An aliquot of the reaction mixture was removed at each time point, the reaction stopped, and the resulting solution electrophoresed as in section 2: (1–6) 0,1,2,4,8 and 16 μg RNase SPL/ml, respectively, incubation time 5 min; (7–11)1,2,4,8 and 16 μg RNase SPL/ml, respectively, incubation time 20 min; (12–17) 0,1,2,4,8 and 16 μg RNase SPL/ml respectively, incubation time 30 min.

0.09 μg of the nuclease/ml hydrolysed quantitatively poly(U) (230 nmol U/ml) in 5 min, and 4 μg/ml rendered 90% of Mg^{2+}-free (or depleted) total *E. coli* RNA (300 μg/ml) acid-soluble in 5 min [1].)

In the case of most enzymes described so far which do not lose activity during the incubation period, it appears possible to compensate for a lower enzyme concentration by an increase in the time of reaction, over a fairly wide concentration range. We, therefore, thought that it should be possible to use concentrations of RNase SPL <16 μg/ml (for, say 2 mg Mg^{2+}-containing RNA/ml) to obtain the limit digest if the time of incubation was increased. It was, however, found (fig.1) that although the extent of cleavage of the Mg^{2+}-containing native total RNA (2 mg/ml) with 1,2 and 4 μg RNase SPL/ml in 5 min, was less than in the limit digest obtained with a nuclease concentration of 8–16 μg/ml, a 6-fold increase in the time of incubation, using a lower concentration (1,2 or 4 μg/ml) of the enzyme, did not lead to any further cleavage, as adjudged by the profile of the degradation products on PAGE. Thus the extent of degradation of the RNA by 1 μg RNase SPL/ml was the same at 20 and 30 min as at 5 min (compare channels 2,7,13 in fig.1); the same was true for 2 (compare channels 3,8,14 in fig.1) or 4 μg (compare channels 4,9,15 in fig.1) of RNase SPL/ml, even though the degradation of RNA at 5 min by 2 μg enzyme was more than by 1 μg enzyme (compare channels 2,3 in fig.1), and that by 4 μg enzyme more than that by 2 μg enzyme (compare channels 3,4 in fig.1). In other words, each of the lower concentrations (1,2 and 4 μg/ml) of the nuclease, gave a 'limit digest' characteristic of the concentration, in 5 min, the average size of the cleav-

Table 1
Comparison of the nucleolytic activity of RNase SPL on ^{3}H-labelled Mg^{2+}-containing native total *E. coli* RNA incubated with the nuclease for 5 and 30 min

RNase SPL (μg/ml)	Acid-insoluble radioactivity (% control)	
	Incubation time (min)	
	5	30
0	100[a]	100[a]
4	92	95
8	88	95
16	92	90
20	88	90

[a] 6 × 10^5 cpm

The labelled *E. coli* RNA was isolated and the assay for nucleolytic activity carried out, as in section 2. The reaction mixture (0.1 ml) contained 200 μg (6 × 10^5 cpm) of the labelled RNA and the stated concentration of RNase SPL. After hydrolysis for the specified period, the acid-insoluble radioactivity was determined by paper-strip chromatography of the hydrolysate. The values given for acid-soluble radioactivity are % of the control which had no RNase SPL but was otherwise treated exactly as above

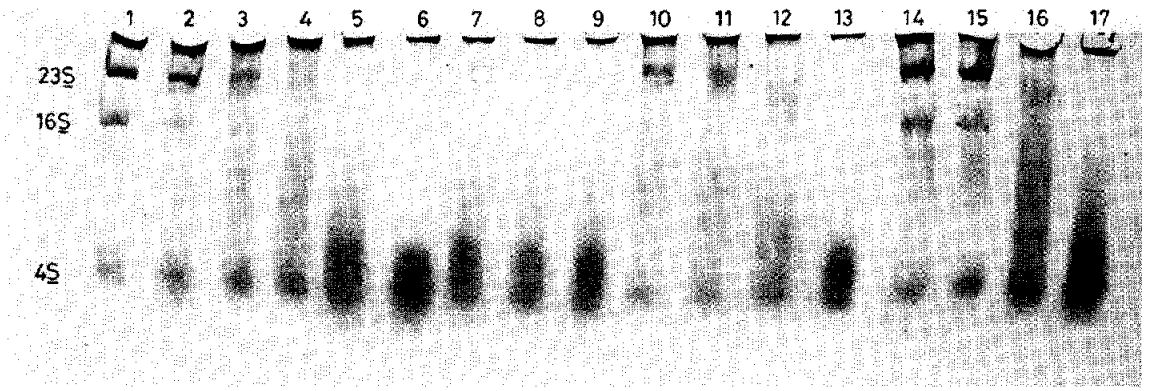

Fig.2. Effect of addition of more substrate or more RNase SPL, after treatment of ^{3}H-labelled Mg^{2+}-containing native total *E. coli* RNA with RNase SPL for 10 min. The labelled RNA (40 μg) was treated for 10 min with the nuclease in a total volume of 0.025 ml as in section 2; more nuclease (0.05 0.2 μg in 2.5 μl water) or more of the labelled RNA (20 μg in 2.5 μl 0.5 mM $MgCl_2$) was added at 10 min and the reaction continued for another 10 min. The reaction was stopped and the resulting solution electrophoresed as in fig.1. (1) RNA was incubated without the nuclease for 20 min; (2–6) RNA (40 μg) was treated with 1,2,4,8 or 16 μg RNase SPL/ml, respectively, for 10 min; more *E. coli* RNA (40 μg) was then added and the samples incubated for another 10 min; (7–9) RNA (40 μg) was treated with 1,2 or 4 μg RNase SPL/ml, respectively, for 10 min; more RNase SPL was then added to make the final concentration of the enzyme as 9,10 or 12 μg/ml, respectively, and the samples incubated for another 10 min; (10–13) RNA (40 μg) was treated with 1,2,4 or 8 μg RNase SPL/ml respectively, for 20 min; (14,15) RNA was treated with 0.8 and 0.6 μg RNase SPL/ml respectively, for 20 min; (16,17) RNA (40 μg) was treated with 2 and 4 μg RNase SPL/ml, respectively, for 10 min; more RNase SPL was then added to make the final concentration of the enzyme as 4 and 8 μg/ml, respectively, and the samples incubated for another 10 min. Channels (14,15) not discussed in the text, show that below 1 μg RNase/ml, much of the 23 S and 16 S rRNAs remained intact.

age products in the digest decreasing as the enzyme concentration increased, reaching the final limit value at 8–16 μg RNase SPL/ml. Table 1 shows that in an experiment such as fig.1, no acid-soluble radioactivity was released by the enzyme in any of the cases.

Fig.2 shows that the enzyme (RNase SPL) was fully active up to at least 20 min at all the above-mentioned enzyme concentrations; addition of more substrate to the limit digest obtained at 10 min with various concentrations of the enzyme, resulted in rapid cleavage of the newly-added substrate to the same extent as was obtained initially (compare channels 2 with 10,3 with 11,4 with 12,5 with 13 in fig.2). Again, all the cleavage occurred within the first 5 min.

Fig.2 also shows that if, at the end of the first 10-min incubation of the substrate with various concentrations of the enzyme as in fig.1, more enzyme was added, the substrate was cleaved further to the extent to which it would have been cleaved if the additional amount of the enzyme was present right from the beginning (compare, in fig.2, channel 12 with 16; 13 with 17; 7–9 with 5,6 and 13, noting that for 7–9 the final enzyme concentration was 9–10 μg/ml, whereas for 5 and 13 it was 8 μg/ml and for 6,16 μg/ml). Thus, the extent of limit cleavage of the Mg^{2+}-containing RNA obtained with 1 μg of the nuclease added at zero time and 3 μg of the enzyme then added at 10 min, was the same as obtained with 4 μg of the enzyme added at zero time.

The exact reason for the above-mentioned atypical

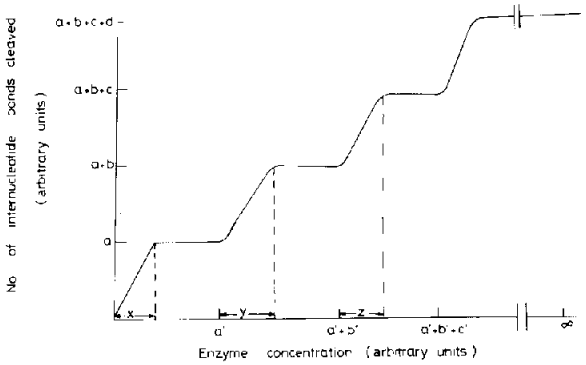

Fig.3. Postulated enzyme concentration *vs* enzyme activity curve for RNase SPL: x/a', $y/(a' + b')$ and $z/(a' + b' + c')$ may have values different from those illustrated here; $a + b + c + d$ represents the limit digest obtained with high concentrations of the enzyme. The number of 'steps' may actually be larger or smaller than those shown here.

behaviour of RNase SPL is not understood. One possibility is that RNase SPL may bind to different sites in naturally occurring RNAs (which are known to be highly 'structured' in the presence of Mg^{2+}, in the native state) with widely varying affinities. In such a case, the relationship illustrated in fig.3 between concentration of the enzyme and the number of internucleotide linkages broken at a given time, could hold true. Such a relationship would explain the observations reported here.

The ability of RNase SPL to give a whole series of 'limit digests' which increase in complexity (in terms of the number of cleavage products) with an increase in the concentration of the enzyme, in what appears to be a step-wise manner, may be useful in generating fragments for sequence analysis.

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