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STABILIZATION OF RIBONUCLEASE B ACTIVITY BY CONCENTRATED XYLOSE SOLUTIONS

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SUMMARY: Ribonuclease B has become a paradigm as a simple example of an *N*-linked glycoprotein. We have found that certain affinity-purified preparations of this enzyme demonstrated a pronounced tendency to lose activity if stored as dilute aqueous solutions. Such inactivation is accelerated by the presence of NaCl, but can be counteracted by inclusion of high (1 mol/l) concentrations of xylose. Enzyme activity cannot be restored by addition of xylose after storage of the enzyme. In marked contrast to α-methyl-mannoside, xylose does not prevent ribonuclease B from binding to concanavalin A and so may be used to stabilize the enzyme during purification by lectin affinity chromatography.

Bovine pancreatic ribonuclease B (RNAase B; ribonucleate 3'-pyrimidino-oligonucleotide hydrolase; EC 3.1.27.5) is a well-characterized glycoform of RNAase A (1), a digestive nuclease which cleaves RNA by a transphosphorylation mechanism (recently reviewed in 2). RNAase B possesses a single, *N*-linked oligomannose chain, the predominant form of which is Manα1,3[Manα1,3(Manα 1,6)Manα1,6]Manβ1,4GlcNAcβ1,4GlcNAc-Asn (3-5). Because of its simple sugar structure, the enzyme has seen widespread use as a standard whole glycoprotein substrate to determine the efficacy of deglycosylation by endo-β-*N*-acetyl-

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Abbreviations: Con A, concanavalin A; Endo, endo-β-*N*-acetylglucosaminidase [EC 3.2.1.96]; GlcNAc, *N*-acetyl-*D*-glucosamine; Man, mannose; PNGase, peptide-N^4-(*N*-acetyl-β-*D*-glucosaminyl) asparagine amidase [EC 3.5.1.52]; RNAase B, ribonucleate 3'-pyrimidino-oligonucleotide hydrolase [EC 3.1.27.5]; TBS, Tris-buffered saline.

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glucosaminidase type (EC 3.2.1.96) *and peptide-N^4-(N-acetyl-β-D-glucosaminyl)* asparagine amidase type (EC 3.5.1.52) enzymes (reviewed in 6).

We have used RNAase B to measure endoglycosidase activity in a novel assay method which employs the nuclease as a general endoglycosidase substrate (7). Intact RNAase B is removed by treatment with concanavalin A (Con A)-agarose and the ribonuclease activity of the deglycosylated product is used to assay endoglycosidase activity. We have found that certain batches of affinity-purified RNAase B lose activity rapidly after preparation; such lability is an infrequent and unpredictable occurrence which cannot be attributed to the mode of preparation or storage, but can be overcome by incorporating the sugar, xylose, at high concentrations (1.0 mol/l) in reaction mixtures.

MATERIALS AND METHODS

Materials: *D*+ xylose, methyl-α-*D*-mannopyranoside, Con A-agarose (type III-ASCL), RNA (calf type IV and yeast type X) and RNAase B (type III-B) were all from Sigma (Poole, Dorset, England).

Buffers: Tris-buffered saline (TBS; Tris, 0.05 mol/l; NaCl, 0.5 mol/l; $MgCl_2$, 1 mmol/l; $CaCl_2$, 1 mmol/l; pH 8.1); HEPES buffer (0.05 mol/l, pH 8.0).

Preparation of RNAase B: Commercial RNAase B was further purified to homogeneity by affinity chromatography on Con A-agarose (8). RNAase B concentration was measured using an extinction coefficient of 0.63 ($\varepsilon^{1\ mg/ml}$ at 280 nm).

Ribonuclease assay: RNAase B activity was determined by a modification of the method of Corbishley *et al.* (9) in which the working concentration of RNA was increased to 5 mg/ml. Results were expressed as ribonucleotide yield (absorbance at 260 nm).

RNAase B-Con A-agarose binding reaction: 200 µl of RNAase B (10 µmol) were mixed with 200 µl of Con A-agarose (5mg Con A per ml gel) in a 1 ml microfuge tube on a Spiramix rolling device (Denley, Sussex, England). This was performed at 4°C for 4 h. Con A-agarose was made up in HEPES buffer. Each tube was then centrifuged at 10,000 x *g* for 15 min. and 120 µl of the supernate removed and analyzed for RNAase B activity.

RESULTS AND DISCUSSION

Activity variation in RNAase B. In general, we have found RNAase B to be a stable protein, with little propensity to lose activity during short-term (up to 24 h) storage at 4°C (10). However, certain batches of RNAase B display a depressed specific activity and inherent instability when stored in water or neutral pH buffer; debility is evident after in-house purification on Con A-agarose and is not attributable to batch variations which may have been introduced by the manufacturer (Fig. 1, lot C).

Stabilization of RNAase B. Xylose increased the activity of anomalous RNAase B preparations and rendered them more stable to overnight storage (Table

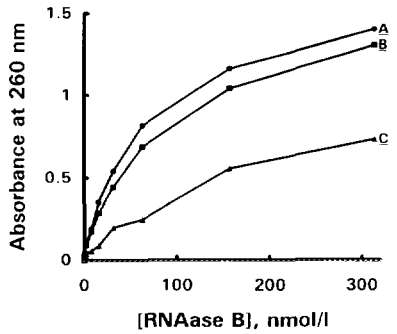

Fig. 1. Specific activity variation in RNAase B prepared by Con A-agarose chromatography. RNAase B protein concentrations were standardized by extinction coefficient and the RNA substrate hydrolysis rate compared. Affinity-purified RNAase B from the following batches of the Sigma type III-B preparation were used: A, lot 17F-8170 (June 1990); B, lot 96F-8190 (September 1990); C, lot 96F-8190 (January 1991). The dates of preparation by affinity chromatography are given in parentheses. RNAase B and Con A-agarose were made up in TBS. The average of duplicate determinations minus the blank is shown.

1). NaCl, usually incorporated into buffers to prevent non-specific binding reactions, appeared to have a deleterious effect on RNAase B activity; this could only be partially countered by sugar presence.

Effect of xylose. RNAase B activity increased markedly when higher xylose concentrations were used (Table 2). A sugar concentration of 0.5-1.0 mol/l was optimal for this purpose. There was an activity loss after 4 hours in the absence of xylose which was not reversed by xylose addition.

Table 1. Effect of diluent composition on RNAase B activity

Diluent	Absorbance at 260 nm ± S.D. [n = 2]	
	0 h	21 h
Water	0.114 ± 0.02	0.014 ± 0.00
NaCl	0.090 ± 0.01	0.017 ± 0.00
Xylose	0.473 ± 0.01	0.419 ± 0.01
Xylose-NaCl	0.369 ± 0.00	0.116 ± 0.00

RNAase B (in water), mixed with the diluents shown, was assayed for activity at the indicated time intervals. The 21-h incubation was performed at 4°C. The final concentration of components in the pre-assay mixtures was as follows: RNAase B, 5 nmol/l; NaCl, 0.02 mol/l; Xylose, 0.2 mol/l.

Table 2. Effect of xylose concentration on RNAase B activity

[Xylose], (mol/l)	Absorbance at 260 nm ± S.D. [*n* = 2]
0.01	0.026 ± 0.001
0.05	0.043 ± 0.001
0.10	0.033 ± 0.002
0.20	0.097 ± 0.004
0.50	0.350 ± 0.004
1.00	0.373 ± 0.028
0.20[a]	0.023 ± 0.001
1.00[a]	0.055 ± 0.001

RNAase B (5 nmol/l final) was mixed with xylose to yield the sugar concentrations shown. After incubation for 4 h at 4°C, the samples were assayed for ribonuclease activity. Both RNAase B and xylose were in HEPES buffer.

[a] RNAase B (10 nmol/l) was maintained at 4°C for 4 h in HEPES buffer. Xylose was then added to achieve the sugar concentrations indicated and the solutions were assayed for ribonuclease activity.

Effect of sugar on Con A-RNAase B reaction. Xylose did not interfere with RNAase B-Con A binding (Table 3) and so may be used to stabilize the enzyme in the presence of this lectin. This contrasts with methyl-α-*D*-mannopyranoside, which appeared to have a similar stabilizing influence on RNAase B, but totally abolished the affinity of the latter for Con A.

Concluding comments. Since 'unstable' RNAase B retains Con A binding (Table 3), loss of activity cannot be attributed to glycan removal during purification or storage. The presence of the same amino acid composition and sequence in both RNAase A and B (11) permits a consideration of stability data for the naturally-occurring aglycone. Classical protein unfolding studies with RNAase A (12) have contributed to a common perception of this nuclease as a somewhat 'indestructible' enzyme. However, pronounced variations in enzymatic activity due to unknown factors have been widely reported (13, 14) and much argument surrounds the precise stimulatory/inhibitory effects of salts (13, 15-19) and metal ions (20-24) on the activity of pancreatic ribonuclease.

The findings presented in this communication indicate that the instability problem was associated with certain batches of RNAase B prepared by Con A-

Table 3. Comparison of effect of xylose and methyl-α-*D*-mannopyranoside on the RNAase B-Con A reaction

Diluent	Absorbance at 260 nm ± S.D. [*n* = 2]	
	+ Con A	- Con A[a]
HEPES	0.052 ± 0.002	0.016 ± 0.001
methyl-α-*D*-mannopyranoside	1.485 ± 0.003	1.221 ± 0.087
Xylose	0.084 ± 0.006	1.128 ± 0.141

RNAase B in the diluents indicated was incubated with Con A-agarose as detailed under 'Materials and Methods'. Final concentrations during the lectin mixing step were RNAase B, 25 nmol/l; HEPES, 0.05 mol/l; sugars, 0.2 mol/l.
[a] Denotes absence of lectin (HEPES buffer only).

agarose chromatography. NaCl accelerates the inactivation of such anomalous batches and should therefore be excluded from buffers. Xylose stabilizes the activity of RNAase B. Evidence for RNAase A stabilization by other sugars (25), sugar alcohols (26) and polysaccharides (27) has been previously documented. The universality of the phenomenon has been the subject of some debate, with some sugars seemingly better than others (28). Work is underway to test the effect of other sugars on RNAase B stability.

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