Mung bean nuclease: mode of action and specificity vs synthetic esters of 3'-nucleotides

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ABSTRACT

Mung bean nuclease hydrolyzes synthetic esters of 3'-nucleotides to nucleosides and phosphate esters; esters of 2'-nucleotides, and 2'→5' internucleotide linkages, are resistant. Esters of ribonucleotides are cleaved at 100-fold the rate for deoxyribonucleotides, the increased rate being due to presence of the 2'-hydroxyl and not to differences in conformation. Introduction of a 5'-substituent leads to a 3-fold increase in rate. The rates of hydrolysis vary up to 10-fold with the nature of the base, in the order adenine > hypoxanthine > uracil; and up to 6-fold with the nature of the ester radical. This form of cleavage of esters of 3'-nucleotides is also characteristic for nuclease-3'-nucleotidase activities from potato tubers and wheat, suggesting that one type of enzyme is responsible for all these activities.

INTRODUCTION

Mung bean type nuclease (EC 3.1.4.9), an endonuclease fairly widespread in plants, hydrolyzes ribo and deoxyribo polynucleotides with the liberation of nucleoside-5'-phosphates.¹⁻⁵ An additional property of this enzyme is its 3'-ω-monophosphatase activity, i.e. the ability to hydrolyze 3'-phosphates of nucleosides and terminal 3'-phosphates of oligonucleotides.⁶ Recently Nomura et al.⁷ and Sano et al.⁸ isolated from potato tubers a nuclease-3'-nucleotidase which hydrolyzed adenosine-3'-benzylphosphate to adenosine and benzylphosphate, and suggested that this represented a new class of enzymes. However, the isolation in this laboratory of a similar activity from wheat shoots, and the resemblances of all the foregoing activities, prompted us to examine the activity of mung bean nuclease against a variety of nucleotide esters.

MATERIALS AND METHODS

Highly purified mung bean nuclease⁹ was generously contributed by Dr. M. Laskowski, Sr. Poly 2'-O-methyluridylic acid (poly mU),¹⁰ was kindly prepared by Mr. J. Siedlecki and Dr. Barbara Żmudzka. Highly polymerized calf thymus DNA was obtained from Sigma (St. Louis, Mo.,

USA), CpC and ApA from Koch-Light (Colnbrook, England), *E. coli* alkaline phosphatase BAPC from Worthington (Freehold, N.J., USA), and thymidine-3'-(p-nitrophenylphosphate) from Rayle (Edmonton, Alta., Canada).

Cytidylyl-(2'→5')-cytidine was isolated by paper chromatography (with solvent B) from a limit digest of chemically synthesized oligo (C), containing a mixture of 2'→5' and 3'→5' internucleotide linkages,¹¹ incubated at pH 8 with an excess of pancreatic RNase and *E. coli* alkaline phosphatase. The remaining substrates were prepared as described in the references listed in Table I.

Chromatography (Table II) made use either of Whatman paper No. 1 or Eastman TLC 6065 cellulose sheets with the following solvent systems: (A) sat. ammonium sulphate - 1 M sodium acetate - isopropanol, 80:18:2 (v/v/v); (B) isopropanol - conc. NH_4OH - water, 7:1:2 (v/v/v).

The relative rates of hydrolysis of synthetic nucleotide esters were determined by incubation of 0.1 ml of a 5 mM substrate solution in 0.1 M acetate buffer pH 5.2 with the enzyme for 30 min at 37° or 50°C, following which 0.2 ml 0.2 M tris-acetate buffer pH 7.5 containing 1.5 units of alkaline phosphatase was added, and incubation continued for an additional 10 min at 37°C. Under these conditions the rate of hydrolysis was linear over a 30-min incubation period. Simultaneous controls were run with either nuclease or phosphatase omitted. The α -naphthol liberated from the naphthyl esters of nucleotides was assayed as elsewhere described;¹² the p-nitrophenol liberated from the p-nitrophenyl nucleotide esters was assayed according to Razzell & Khorana;¹³ for the methyl, phenyl and benzyl esters, the enzymatically liberated inorganic phosphate was determined according to Lowry & Lopez.¹⁴ The units of activity given in Table I were established with reference to the rate of hydrolysis of denatured DNA, estimated from formation of acid-soluble products, according to Johnson & Laskowski.¹⁵

The course of hydrolysis was also followed, in all instances, by TLC on Eastman 6065 cellulose sheets developed with Solvent A. For the α -naphthyl and p-nitrophenyl nucleotide esters the liberated α -naphthylphosphate and p-nitrophenylphosphate were identified by spraying the chromatograms with a solution of alkaline phosphatase in 0.1 M tris-HCl buffer pH 9 and subsequent visualization of liberated α -naphthol with Fast Red TR (1 mg/ml alkaline buffer) and liberated p-nitrophenol with 0.1 N NaOH.

Table I

Mung bean nuclease activity towards synthetic esters of nucleotides. Figures in brackets are references to preparative procedures for the substrates.

S u b s t r a t e	$\mu\text{moles} \times 10^2$ substrate hydrolyzed/ unit of enzyme/30 min	
	37°C	50°C
Adenylyl-(3' $\rightarrow$ 5')-adenosine	198.4	-
Adenosine-3'-(α -naphthylphosphate) (19)	460.0	-
Inosine-3'-(α -naphthylphosphate) (20)	62.4	-
Uridine-3'-(α -naphthylphosphate) (20)	44.0	90.00
Uridine-2'-(α -naphthylphosphate) (21)	-	<0.40
5'-O-benzyluridine-3'-(α -naphthylphosphate) (22)	190.8	-
5'-O-ethyluridine-3'-(α -naphthylphosphate) (22)	129.6	-
5'-O-ethyl-2'-O-tetrahydropyranyloridine-3'-(α -naphthylphosphate) (22)	-	1.44
Uridine-3'-methylphosphate (23)	117.6	-
Uridine-3'-phenylphosphate (24)	18.4	-
Cytidine-3'-benzylphosphate (25)	322.8	-
Thymidine-3'-(α -naphthylphosphate) (12)	-	0.84
5'-O-acetylthymidine-3'-(α -naphthylphosphate) (12)	-	2.44
5'-O-tetrahydropyranyloridine-3'-(α -naphthylphosphate) (12)	-	2.44
Thymidine-3'-(p-nitrophenylphosphate)	-	1.44
5'-O-acetylthymidine-3'-(p-nitrophenylphosphate) (12)	-	3.88
5'-O-tetrahydropyranyloridine-3'-(p-nitrophenylphosphate) (12)	-	3.88

Table II

TLC and paper chromatography (ascending) of relevant compounds.

Compound	Eastman TLC 6065		Whatman No.1
	Solvent A	Solvent B	Solvent B
Adenylyl-(3' $\rightarrow$ 5')-adenosine	0.03		
Adenosine-3'-(α -naphthylphosphate)	.00		
Inosine-3'-(α -naphthylphosphate)	.04		
Uridine-3'-(α -naphthylphosphate)	.08		
Uridine-2'-(α -naphthylphosphate)	.08		
5'-O-benzyluridine-3'-(α -naphthylphosphate)	.00		
5'-O-ethyluridine-3'-(α -naphthylphosphate)	.01		
5'-O-ethyl-2'-O-tetrahydropyranyluridine-3'-(α -naphthylphosphate)	.00		
Uridine-3'-methylphosphate	.62		
Uridine-3'-phenylphosphate	.34		
Cytidine-3'-benzylphosphate	.30		
Thymidine-3'-(α -naphthylphosphate)	.06		
5'-O-acetylthymidine-3'-(α -naphthylphosphate)	.01		
5'-O-tetrahydropyranylthymidine-3'-(α -naphthylphosphate)	.01		
Thymidine-3'-(p-nitrophenylphosphate)	.04		
5'-O-acetylthymidine-3'-(p-nitrophenylphosphate)	.04		
5'-O-tetrahydropyranylthymidine-3'-(p-nitrophenylphosphate)	.01		
Adenosine	.14		
Inosine	.48		
Uridine	.68		
5'-O-benzyluridine	.17		
5'-O-ethyluridine	.49		
Cytidine	.68	0.52	0.6
Thymidine	.48		
5'-O-acetylthymidine	.25		
5'-O-tetrahydropyranylthymidine	.13		
α -naphthylphosphate	.42		
p-nitrophenylphosphate	.61		
Poly (U)	.00		
Poly (mU)	.00		
UMP	.76		
2'-O-methyl-UMP	.63		
CMP		.08	.17
Cytidylyl-(2' $\rightarrow$ 5')-cytidine		.23	.31
Oligo (C)		.00	.00

Because of the limited amount of enzyme at our disposal, hydrolysis of the poorer substrates was followed at 50°C. The rates at this temperature were considered to be slightly more than 2-fold those at 37°C, since this was the increase in rate observed between 37° and 50° with either DNA or uridine-3'-(α -naphthylphosphate) as substrates, in fair agreement with the results of Johnson & Laskowski¹⁶ for DNA. Furthermore the pH optimum for activity vs uridine-3'-(α -naphthylphosphate) was found to be identical with that reported for activity against macromolecular substrates.¹

The susceptibilities to mung bean nuclease of poly (U), poly (mU), CpC and cytidyl-(2'→5')-cytidine were examined only qualitatively, by incubation of 5 mM solutions with enzyme in 0.1 M acetate buffer pH 5.2 at 50°C and following product formation by TLC on Eastman 6065 sheets with solvent A for the polynucleotides and solvent B for the dinucleoside monophosphates.

RESULTS AND DISCUSSION

The following facts testify to hydrolysis by mung bean nuclease of esters of 3'-nucleotides to nucleosides and phosphate esters (Scheme 1): (a) incubation of a nucleotide ester with the enzyme did not liberate the ester substituent; (b) incubation with the enzyme and an excess

Scheme 1

of phosphatase led to the appearance of inorganic phosphate and the ester substituent; (c) in the case of the α -naphthyl and p-nitrophenyl esters, chromatographic methods demonstrated directly the liberation by mung bean nuclease of the nucleosides and phosphate esters.

The rates of hydrolysis of various esters of 3'-nucleotides are dependent on the nature of the heterocyclic base (Table I), e.g. adenosine-3'-(α -naphthylphosphate) is hydrolyzed 7x faster than inosine-3'-(α -naphthylphosphate) and 10x faster than uridine-3'-(α -naphthylphosphate).

From Table I it will be noted that the enzyme exhibits a pronounced preference for esters of 3'-ribonucleotides as compared to 3'-deoxyribonucleotides, for uridine-3'-(α -naphthylphosphate) is hydrolyzed 100x faster than thymidine-3'-(α -naphthylphosphate). Since replacement of uracil by thymine apparently does not markedly affect the rate of hydrolysis of internucleotide linkages,^{1,6} this demonstrates a preference of the enzyme for the ribose ring, or for a free 2'-OH group. The second possibility was examined by comparing the rates of hydrolysis of 5'-O-ethyluridine-3'-(α -naphthylphosphate) and 5'-O-ethyl-2'-O-tetrahydropyranyluridine-3'-(α -naphthylphosphate); the latter, in fact, required 180x more enzyme for comparable rates of hydrolysis (Table I); paper chromatography showed that poly (mU) required $\sim$ 200x more enzyme for hydrolysis at a rate comparable with that for poly (U). These differences cannot be ascribed to differences in conformation, since it has been shown in the case of monomers that O'-alkylation of the ribose ring of nucleosides only minimally affects its conformation,¹⁷ while at 37°C the conformation of poly (mU) is essentially the same as that of poly (U).¹⁸

An additional point of interest in Table I is that a 5'-substituent (ethyl, benzyl, acetyl, tetrahydropyranyl) leads to a 3 to 4-fold increase in rate of hydrolysis of esters of 3'-ribo or deoxyribonucleotides. By contrast, 3'-UMP and 5'-O-benzyl-3'-UMP were both hydrolyzed at comparable rates, suggesting that the influence of a 5'-substituent is less pronounced in the case of the 3'- ω -phosphatase activity of the enzyme.

The nature of the ester substituent also influences the rate of hydrolysis, e.g. uridine-3'-methylphosphate is hydrolyzed 6x faster than uridine-3'-phenylphosphate and 2.6x faster than uridine-3'-(α -

naphthylphosphate).

It is of interest to compare the relative rates of hydrolysis when the ester substituent is replaced by a nucleoside to give a "natural" substrate (Scheme 1, R = nucleoside). This has been done for adenosine-3'-(α -naphthylphosphate) and adenylyl-(3' $\rightarrow$ 5')-adenosine, i.e. ApA. From Table I it will be seen that the synthetic substrate is hydrolyzed more than 2-fold faster than the "natural" one.

The enzyme exhibits no activity against esters of 2'-nucleotides, testified to by the resistance of uridine-2'-(α -naphthylphosphate) to a 200-fold excess of enzyme required to cleave the analogous ester of 3'-UMP. A 2' $\rightarrow$ 5' internucleotide linkage is also resistant, since cytidylyl-(2' $\rightarrow$ 5')-cytidine was unaffected by a 40-fold excess of enzyme which hydrolyzed CpC.

From the foregoing results it is clear that not only the nuclease-3'-nucleotidase from potato tubers,⁸ but also mung bean nuclease, belong to a class of enzymes which hydrolyzes esters of 3'-nucleotides to nucleosides and phosphate esters (Scheme 1). Our own data with a partially purified nuclease-3'-nucleotidase from wheat⁴ indicate that the latter is also a member of this class. The remaining specificity requirements of these three enzymes are also similar,^{1,4,7,8} with perhaps a more marked preference for single-stranded polymucleotides in the case of the enzyme from mung bean.¹⁶ The potato enzyme also exhibits a somewhat different pH optimum.⁷ Basically the differences between the three enzymes are not very marked; it is rather the similarities which point to one type of activity widely distributed in the plant kingdom.

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REFERENCES

- 1 Sung, S.C. and Laskowski, M., Sr. (1962) J. Biol. Chem. 237, 506-511
- 2 Tone, H. and Osaki, A. (1968) Enzymologia 34, 101-128

- 3 Wilson, C.M. (1968) *Plant Physiol.* 43, 1339-1346
- 4 Hanson, D.M. and Fairley, J.L. (1969) *J.Biol.Chem.* 244, 2440-2449
- 5 Wyen, H.V., Erdel, S. and Parkas, G.L. (1971) *Biochim.Biophys.Acta* 232, 472-483
- 6 Mikulski, A.J. and Laskowski, M., Sr. (1970) *J.Biol.Chem.* 245, 5026-5031
- 7 Nomura, A., Suno, M. and Mizuno, Y. (1971) *J.Biochem.* 70, 993-1001
- 8 Suno, M., Nomura, A. and Mizuno, Y. (1973) *J.Biochem.* 73, 1291-1297
- 9 Ardelt, W. and Laskowski, M., Sr. (1971) *Biochem.Biophys.Res.Comm.* 44, 1205-1211
- 10 Żmudzka, B. and Shugar, D. (1971) *Acta Biochim.Polon.* 18, 321-337
- 11 Michelson, A.M. (1959) *J.Chem.Soc.* 3655-3669
- 12 Sierakowska, H. and Shugar, D. (1971) *Acta Biochim.Polon.* 18, 143-152
- 13 Razzell, W.E. and Khorana, H.G. (1961) *J.Biol.Chem.* 236, 1144-1149
- 14 Lowry, O.H. and Lopez, J.A. (1946) *J.Biol.Chem.* 162, 421-426
- 15 Johnson, P.H. and Laskowski, M., Sr. (1968) *J.Biol.Chem.* 243, 3421-3424
- 16 Johnson, P.H. and Laskowski, M., Sr. (1970) *J.Biol.Chem.* 245, 891-898
- 17 Remin, M. and Shugar, D. (1973) *J.Am.Chem.Soc.* 95, 8146-8156
- 18 Rabczenko, A. and Shugar, D. (1971) *Acta Biochim.Polon.* 18, 387-402
- 19 Kole, R., Sierakowska, H. and Shugar, D. (1972) *Biochim.Biophys. Acta* 289, 323-330
- 20 Kole, R. and Sierakowska, H. (1971) *Acta Biochim.Polon.* 18, 187-197
- 21 Zan-Kowalczevska, M., Sierakowska, H. and Shugar, D. (1966) *Acta Biochim.Polon.* 13, 237-250
- 22 Kole, R., Sierakowska, H. and Shugar, D. (1971) *Biochem.Biophys. Res.Comm.* 44, 1482-1487
- 23 Heppel, L.A. and Whitfield, P.R. (1955) *Biochem.J.* 60, 1-7
- 24 Zan-Kowalczevska, M., Bardoń, A., Sierakowska, H. and Shugar, D. (1974) *Biochim.Biophys.Acta* 341, 138-156
- 25 Tener, G.M. and Khorana, H.G. (1955) *J.Am.Chem.Soc.* 77, 5349-5351