SUBSTRATE SPECIFICITY OF RAPE POLLEN 3'-NUCLEOTIDASE *(Brassica napus)****

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3'-Nucleotidase isolated from rape pollen (*Brassica napus*) is a metalloenzyme with pH-optimum of 8·0–8·5 by which 3'-ribonucleotides are degraded in the ratio G ~ U ≫ A = C. The enzyme requires the presence of a free 2'-hydroxylic function in the *ribo* configuration and *cis*-situated with respect to the 3'-phosphomonoester bond. The 5'-nucleotides as well as the 3'- and 5'-nucleotides of the 2'-deoxyribo series are resistant towards this degradation. The enzyme is classified as 3'-D-ribonucleotide phosphohydrolase (EC 3·1·3·6).

In preceding papers[1,2] of this series, isolation of two nucleolytically active protein fractions from the extract of bee-gathered rape pollen (*Brassica napus*) has been reported. Fraction 1 was found to contain as main components nonspecific ribonuclease, nonspecific phosphodiesterase ("ribonuclease-hydrolase"), and nonspecific decyclizing phosphodiesterase. These three enzymes were characterised in detail and their structural requirements towards the substrate molecule were determined. In the present work, fraction 2 has been examined.

As indicated by preliminary results, fraction 2 contains as the only enzymatic activity towards nucleic acids the activity of a phosphomonoesterase dephosphorylating exlusively the 3'-nucleotides, namely, 3'-nucleotidase. The content of 3'-nucleotidase is not surprising since enzymes of this type frequently occur in plant material such as ryegrass seeds[3], wheat germs[4], and mung bean sprouts[5]. These enzyme preparations have not been so far obtained free of further nucleolytic activities or characterized by data on their mechanism of action and particularly on their structural requirements towards substrates.

Since uridine 2'(3')-phosphate is well degraded by the rape pollen 3'-nucleotidase, the basic data such as pH optimum and effect of ions on the enzymatic activity were

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determined with the use of uridine 3′-phosphate as model substrate. Fig. 1 shows dependence of the initial hydrolysis rate of Up on the pH value of the medium. The pH optimum of this reaction lies in the region of pH 8·0 – 8·5; the present enzyme is thus an alkaline 3′-nucleotidase analogous to the enzyme from ryegrass[3] and mung bean sprouts[5] (the wheat germ 3′-nucleotidase is an acidic phosphatase[4]). The pH dependence of the Up degradation is very steep both in acidic and alkaline region (at pH 5, the degradation does not practically take place). A similar observation has been recorded in the case of the mung bean 3′-nucleotidase which is irreversibly inactivated at pH 5.

The requirement of the enzyme on the presence of metal ions was unequivocally inferred from investigations on the effect of ions in the uridine 3′-phosphate degradation with the rape pollen enzyme: the enzyme activity is markedly lowered by the

TABLE I

Effect of Metal Ions Upon the Rate of Pollen 3′-Nucleotidase Degradation of Uridine 3′-Phosphate

Compound	Concentration	v_0[a]
$ZnSO_4$	10^{-3}	1·05
$MgSO_4$	10^{-2}	1·21
EDTA	10^{-2}	0·61

[a] Initial velocity of U 3′-p degradation referred to standard conditions.

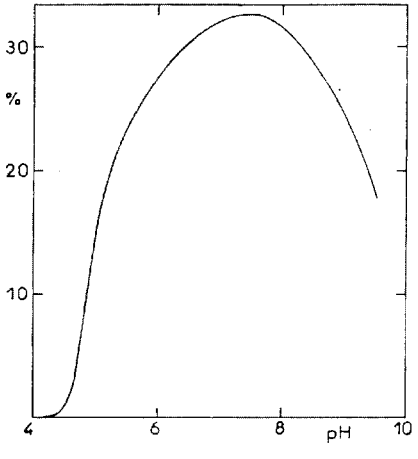


FIG. 1

pH-Profile of Uridine 3′-Phosphate Degradation by Rape Pollen 3′-Nucleotidase

presence of EDTA but is stimulated by zinc ions and particularly by magnesium ions (Table I). The 3′-nucleotidase may thus be considered as a metalloprotein but not as a zinc metalloprotein like the above mentioned 3′-nucleotidases from other sources[7].

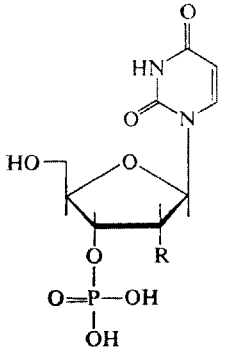
The substrate specificity of 3′-nucleotidase present in the EIII protein fraction of rape pollen has been examined at the uridine 3′-phosphate pH-optimum with the use of numerous nucleotide derivatives modified on the heterocyclic as well as sugar moieties. The corresponding data are summarised in Table II. All the four naturally

TABLE II
Nucleotide Degradation by Rape Pollen 3′-Nucleotidase

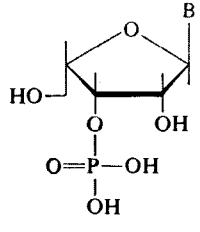
Formula	Compound	Degradation, %
I	Uridine 5′-phosphate	0
II	Cytidine 5′-phosphate	0
III	Adenosine 5′-phosphate	0
IV	Guanosine 5′-phosphate	0
V	Uridine 2′(3′)-phosphate	60
VI	Uridine 3′-phosphate	72
VII	Uridine 2′-phosphate	0
VIII	Cytidine 3′-phosphate	25
IX	Adenosine 2′(3′)-phosphate	9
X	Adenosine 3′-phosphate	20
XI	Guanosine 3′-phosphate	80
XII	2′-Deoxyuridine 3′-phosphate[9]	0
XIII	2′-Deoxyadenosine 3′-phosphate[10]	0
XIV	2′-Deoxyguanosine 3′-phosphate[11]	0
XV	2′-Deoxythymidine 5′-phosphate	0
XVI	2′-Azido-2′-deoxyuridine 3′-phosphate[12]	0
XVII	2′-Amino-2′-deoxyuridine 3′-phosphate[12]	0
XVIII	2′-O-Tetrahydropyranylguanosine 3′-phosphate[13]	0
XIX	1-(β-D-Arabinofuranosyl)cytosine 3′-phosphate[14]	0
XX	9-(α-L-Lyxofuranosyl)adenine 3′-phosphate[15]	10
XXI	1-(α-L-Lyxofuranosyl)uracil 3′-phosphate[15]	15
XXII	9-(β-D-Ribopyranosyl)adenine 3′-phosphate[16]	0
XXIII	L-Cytidine 2′(3′)-phosphate[17]	0
XXIV	L-Adenosine 2′(3′)-phosphate[17]	

occurring 5′-ribonucleotides *I*–*IV* are not degraded by the present enzyme regardless the prolonged incubation time or the use of an excess of the enzyme. On the other hand, the corresponding 2′(3′)- and 3′-nucleotides *V*, *VI*, *VIII*–*XI* are degraded by the enzyme in the order G ~ U ≫ A = C (this order is different from that reported[5] in the case of the bean 3′-nucleotidase, A > G > C). In the interaction of the substrate with rape pollen 3′-nucleotidase, the —NH—CO— grouping characteristic of uridine and guanosine is thus more favourable than the —N=C—NH_2 system of cytosine and adenine.

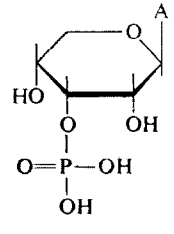
Uridine 2′-phosphate (*VII*) is resistant towards the present enzyme preparation. The enzyme is thus strongly specific to the geometry of the phosphomonoester bond on the *cis*-diol system of the ribonucleoside and may be therefore considered to be a true 3′-nucleotidase. The inhibitory effect of isomeric 2′-nucleotides on the degradation rate of 3′-isomers has not been directly examined but nevertheless it may be inferred from comparison of the data obtained with the 2′(3′)-nucleotides *V* and *IX* with those of pure 3′-isomers *VI*, *X*, and *XI* that the 2′-nucleotides are marked inhibitors.



VI, R = OH; *XVII*, R = NH_2;
XII, R = H
XVI, R = N_3



XX, B = adenin-9-yl
XXI, B = uracil-1-yl



XXII, A = adenin-9-yl

No degradation has been observed in the group of the corresponding 2′-deoxyribonucleoside 3′-phosphates *XII*–*XIV* regardless the prolonged incubation time. In analogy to the *ribo* derivatives, the 5′-nucleotide *XV* cannot be degraded. A similar resistance of 2′-deoxyribonucleotides has also been reported in the case of the ryegrass 3′-nucleotidase[7]. Modification of the 2′-hydroxylic function of the substrate is of essential importance since substitution by the tetrahydropyranyl group (compound *XVIII*), replacement by the azide (*XVI*) or amino (*XVII*) groups or con-

figurational change (compound *XIX* of the *arabino* series) result in a complete resistance towards the enzyme. The configurational change at position 4' of the sugar moiety is not as important. In spite of the *cis* orientation of the 4'-hydroxymethyl group in *ribo* compounds with respect to the 3'-phosphomonoester, the corresponding α-L-lyxofuranosyl derivatives *XX* and *XXI* are enzyme substrates. The extent of the degradation is of course lower than in the case of the *ribo* series derivatives.

As it may be inferred from activity comparison of the four naturally occurring 3'-ribonucleotides *VI*, *VIII*, *X*, and *XI*, the enzyme obviously interacts with the heterocyclic base of the substrate. This interaction does not consist in bond formation or recognition of the base of the substrate by the enzyme but its existence is corroborated by resistance of the L-*ribo* enantiomers *XXIII* and *XXIV* of the naturally occurring 3'-nucleotides. The enzyme-substrate interaction must therefore include the chiral portion of the molecule[8]. In addition to the interaction of the active centre region with the substrate by means of the phosphomonoester group and apparently, also the 2'-hydroxylic function, an interaction with the heterocyclic base of the substrate is therefore most likely involved. Furthermore, the resistance of adenosine 3'-phosphate isomer, namely, 9-(β-D-ribopyranosyl)adenine 3'-phosphate (*XXII*) may be ascribed to rigorous steric requirements of the enzyme with respect to the substrate molecule, particularly on the mutual orientation and distance of the heterocyclic base, the hydroxylic function, and the phosphomonoester group for the purposes of an ES-complex formation. In the molecule of compound *XXII*, the three factors are properly orientated but the original distance of the base and the phosphate or the bond angles between the particular functional groups changed because of the replacement of the naturally occurring five-membered furanose ring by the six-membered pyranose ring; for this reason, the necessary binding and non-binding interactions cannot assert themselves and the probability of the ES-complex formation is considerably lowered. Worth of mention is the rigorous requirement of the enzyme on the presence of a free 2'-hydroxylic function in the *ribo* configuration; this requirement is identical with those of the ribonucleases. 3'-Nucleotidase does not require the presence of this group for the formation of a stable intermediate of cyclic phosphodiester type but it cannot be excluded that the 2'-hydroxylic function of 3'-ribonucleotides takes part either directly in the catalytic process or in the formation of the activated intermediate.

In conclusion, the above rape pollen 3'-nucleotidase may be classified as 3'-D-ribonucleotide phosphohydrolase (EC 3.1.3.6).

EXPERIMENTAL

Paper chromatography was performed by the descending technique on paper Whatman No 3 MM in the solvent system S_1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2). Spectrophotometrical measurements were carried out on the Spectromom 203 apparatus at 260 nm.

Materials

Uridine 3′-phosphate, cytidine 3′-phosphate, and guanosine 3′-phosphate were prepared by the pancreatic ribonuclease or ribonuclease T1 degradation of the corresponding 2′,3′-cyclic phosphates. Uridine 2′-phosphate was obtained by the rape pollen cyclic phosphodiesterase degradation[2] of uridine 2′,3′-cyclic phosphate. Ribonucleoside 5′-phosphates, 2′(3′)-phosphates, and dTMP were preparations of Calbiochem, Los Angeles. Adenosine 3′-phosphate was preparation of Boehringer, Mannheim. The remaining test substances were synthesized by procedures given in Table II.

Degradation of Nucleotides by the EIII Protein Fraction

The incubation mixture (50 µl) contained 1·5 µmol of the substrate (sodium or ammonium salt) and 200 µg of the EIII protein fraction in 0·05M-Tris-HCl buffer solution (pH 7·3). After 3 h of incubation at 37°C, the mixture was analysed by chromatography in the solvent system S_1. The extent of the degradation was determined spectrophotometrically by the method mentioned above. For the results see Table II.

Determination of the pH-Optimum of 3′-Nucleotidase from the EIII Rape Pollen Protein Fraction

The incubation mixture (100 µl) contained 3 µmol of uridine 3′-phosphate lithium salt and 200 µg of the EIII protein fraction in 0·1M-Tris-HCl or 0·1M sodium acetate buffer solution (intervals, pH 0·5). After 1 h of incubation at 37°C, the mixture was analysed in the solvent system S_1. The extent of the degradation was determined spectrophotometrically from chromatographic eluates.

Effect of Metal Ions and EDTA on the EIII Protein Fraction Degradation of Uridine 3′-Phosphate

The effect was determined using 3 µmol of uridine 3′-phosphate lithium salt and 200 µg of the EIII protein fraction in 100 µl of 0·1M-Tris-HCl buffer solution (pH 7·5). After 1 h of incubation at 37°C, the mixture was analysed analogously to the pH-optimum determination. For the results see Table I.

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