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Full Length Research Paper

Biodegradation of mononucleotides to nucleosides and free bases

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An intracellular phosphate, non repressible alkaline phosphate found in extracts of *Penicillium brevicompactum* NRC829 could catalyze dephosphorylation of ribonucleotides AMP, GMP, CMP, UMP and phenyl disodium orthophosphate (phph) optimally at pH 9.0 and 60°C. The extracts contain also hydrolytic deamination activities with adenosine, cytidine and cytosine out of the tested ribonucleotides, ribonucleosides and bases. While the optimum activity of deamination of adenosine or cytidine was achieved at pH 7.0 and 50°C. Neither cleavage of the N-glycosidic linkages of these nucleotides nor those of the corresponding nucleosides could be affected by the extracts. Heating the extracts at 80°C for 10 min, in absence of the substrate, inactivated the two enzymes. The extracts catalyzed hydrolytic cleavage of phosphate esters of different phosphorylated compounds with different rates. Hence, the enzyme appears to have abroad substrate specificity and the highest relative rate of hydrolysis was with UMP. No evidence for the involvement of specific nucleotidases in ribonucleotide dephosphorylation was recorded.

Key words: Ribonucleotides degradation, *Penicillium brevicompa*ctum alkaline phosphatase, aminohydrolase.

INTRODUCTION

Purine and pyrimidine nucleotides are major energy carriers, subunits of nucleic acids and precursors for the synthesis of nucleotide cofactors such as NAD and SAM. Despite the obvious importance of these molecules, we still have much to learn about how these nucleotides are synthesized and metabolize. (Barbara and Hiroshi, 2002). Nucleosides and free bases are commonly used for synthesis of various drugs, such as antitumor, antiviral and antibacterial drugs or immunomodulators. Usually they are obtained either by chemical or biological ways (Kruszewska et al., 2004). Moreover, of the research that has been done in this area relatively little. The catabolism of 5'-mononucleotides to the corresponding nucleosides and bases was demonstrated in bacteria (Barana, 1967; Schramm and Lazorik, 1975; Chmielowiec et al., 1996, 1999) The mode and the extent of ribonucleotides degradation by extracts of organisms belonging to the filamentous fungi has been reported for extracts of very few organisms, these organisms are *Penicillium chrysogenum* (Elzainy et al., 1979), *Aspergillus niger* strain (Elzainy et al., 1989), *A. niger* $NRRL_3$ (Ali and Elzainy , 2000) and *Penicillium politans* (Ali, 2006). Concerning the different ways by which fungal extracts could degrade the ribonuclotides, it appears that these ways include: (1) Hydrolytic cleavage of the phosphate-ester linkages, effected by extracts of the above mentioned fungi. (2) Hydrolytic cleavage of the N-glycosidic linkages of AMP and GMP catalyzed by extracts of *A. niger* (Masaaki and Tomoko 1978) and of *Neurospora crassa* (Trivedi and Mattoo, 1979). (3) Hydrolytic Cleavage of the amino-purine linkage of AMP, achieved by extracts of *A. oryzae* (Henderson and Paterson 1973).

The aim of the present study was to investigate the mode and the extent of degradation of these important bimolecules by extracts of a non previously studied organism which is *Penicillium brevicompactum*. Such study may add to the area of comparative biochemistry concerning this class of microorganisms.

MATERIALS AND METHODS

Chemicals

AMP, GMP, CMP, UMP, adenosine, guanosine, cytidine, uridine,

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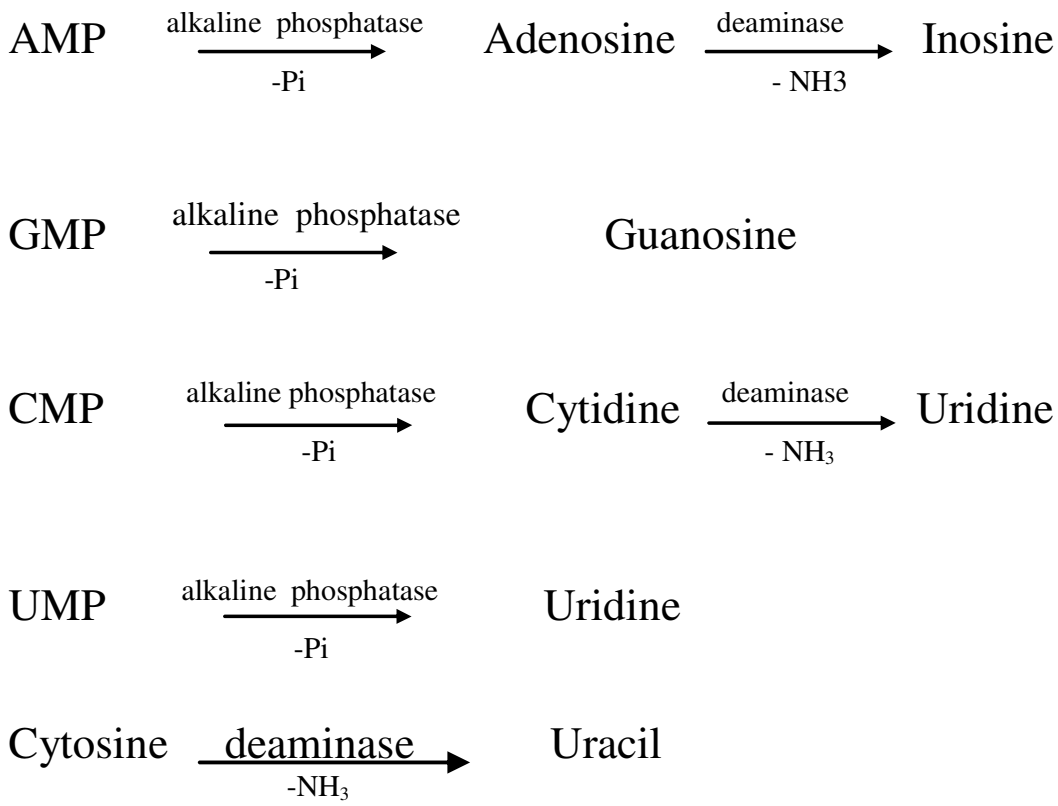

Figure 1. Ribonucleotides degradation.

adenine, guanine, cytosine and uracil are products of Sigma; phenyl disodium orthophosphate (phph) was a product of BDH Chemicals Ltd.

The organism

The *P. brevicompactum* strain was from the culture collection of the department of Microbial Chemistry, National Research Center, Cairo, Egypt.

Medium

P. brevicompactum NRC829 was grown and kept on slants of solid modified Czapek's Dox's medium containing (g/L tap water): glucose, 30; Na NO_3, 2.0; KH_2 PO_4, 1.0; Mg SO_4 7 H_2O, 0.5; KCl, 0.5 and agar 20.

METHODS

Preparation of *Penicillium brevi-compactum* extracts

The 4 days old mats, grown on liquid modified Czapek-Dox's medium at 28°C were harvested by filtration, washed thoroughly with distilled water, blotted dry with absorbent paper. The mats were then ground with cold washed sand in a chilled mortar and extracted with cold distilled water. The slurry so obtained was centrifuged at 1,522 x g for 10 min and the supernatant was used as the crude enzyme preparation.

Calorimetric determination

Assay of phosphatase: The enzyme was assayed as described by Heinonen and Lahti (1981) with some modification. Into test tubes containing 0.5 ml of samples, 4.0 ml of acetone- acid-molybdate (AAM) solution was added. The contents were mixed and 0.4 ml of 1 M citric acid was added into each tube. The yellow colour was measured at 420 nm.

Reducing compounds were determined (as ribose) by the method described by Ashwell (1957). Protein of the extracts was estimated by the method of Ohnistti and Barr (1978), with bovine serum albumin as standard. Ammonia is determined by direct nesslerization of ammonia released according to Borek et al. (2004).

Chromatographic analysis

Separation and identification of the ribonucleotides, the ribonucleosides and the bases were carried out by ascending paper chromatography technique using 3MM (46x57 cm) filter paper and two solvent systems. The solvent systems used for identification of the intermediates and products formed during AMP, GMP, CMP and UMP dephosphorylation by the phosphatase of the extracts were solvent I which consisted of n-butanol: acetone : acetic acid (glacial) : NH_3 (5%) : H_2O, 45: 15:10: 10: 20 and solvent II which consisted of n-butanol: acetic acid (glacial) : water : 70: 20 : 10 (Smith and Seakins, 1976). Appropriate control reaction mixtures where the enzyme source or the substrate was omitted were used as blanks through out the work.

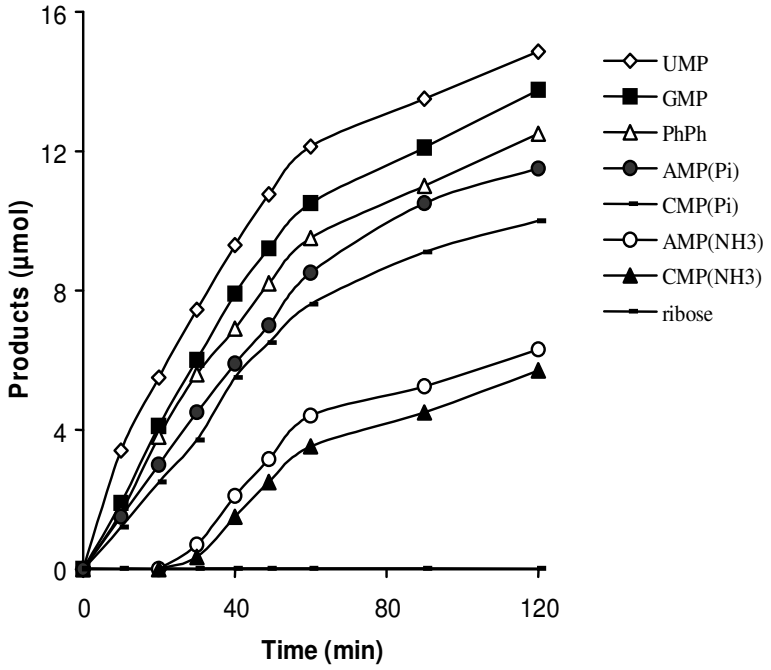

Figure 2. Degradation of nucleotides by cell-free extracts of *P. brevi-compactum* Reaction mixture contained: substrate,15 µmoles; buffer,100 µmoles (Tris-acetate, pH 9); protein of the extracts, 2.7mg; total volume,1ml; temperature,40°C and incubation time, as indicated.

RESULTS

Mode of degradation of mononucleotides by cell-free extracts of *P. brevicompactum*

Reaction mixtures containing AMP, GMP, CMP, UMP and Ph. Ph as substrates were incubated for 120 min at 40°C at different incubation periods and after different time intervals aliquots were removed for phosphate and ammonia determination. Figure 2 indicated that the four nucleotides were dephosphorylated at nearly equal rates by dephosphorylating enzyme. Ribose could not be detected at different time intervals in any of the reaction mixtures suggesting the absence of nucleoside hydrolase activity at this condition. As seen also in Figure 2 ammonia was formed in the reaction mixtures containing either AMP or CMP and the rate of phosphate liberation from AMP or CMP was higher than the ammonia formation. It suggested that dephosphorylation of AMP and CMP to adenosine and cytidine respectively occurs before the cleavage the nucleosides to ammonia and the corresponding deaminated nucleosides (inosine and uridine) by the nucleoside deaminase. The affinity of the phosphatase for the different ribonucleotides was in the order of magnitude UMP > GMP > AMP > CMP and revealed changes in the dephosphorylating activities with the four ribonuclotides similar to the changes recorded with phenyl phosphate. These findings indicate that the non specific alkaline phosphatase of the extracts (which catalyzed the hydrolysis of phenyl phosphate) was the only dephosphorylating enzyme that catalyzed the hydrolytic dephosphorylation of the four ribonucleotides, as, if certain specific nucleotidases (which are known to dephosphorylate only nucleotides) were participating in the catalytic process, the variations in the activities of the extracts with the four ribonucleotides will not have been so similar to the variations obtained with phenyl phosphate.

Data cited in Figure 2 shows that there is a gradual increase in the amounts of ammonia formed up to 30 minutes of incubation, after that there is a steady state occurred in which the increase in incubation time does not reflect noticeable increase in the amounts of ammonia

pH optimum

To determine precisely the optimum pH value(s) at which optimum dephosphorylation of four ribonucleotides and ph.ph and optimum deamination of AMP or CMP by cell-free extracts of *P. brevicompactm* , occurred, reaction mixtures were made each contained the same amount of protein, the same amount of ribonucleotides or ph.ph and the same amount of buffer. Tris-acetate buffer were adjusted at pH (3.0 - 9.0) and carbonate- bicarbonate

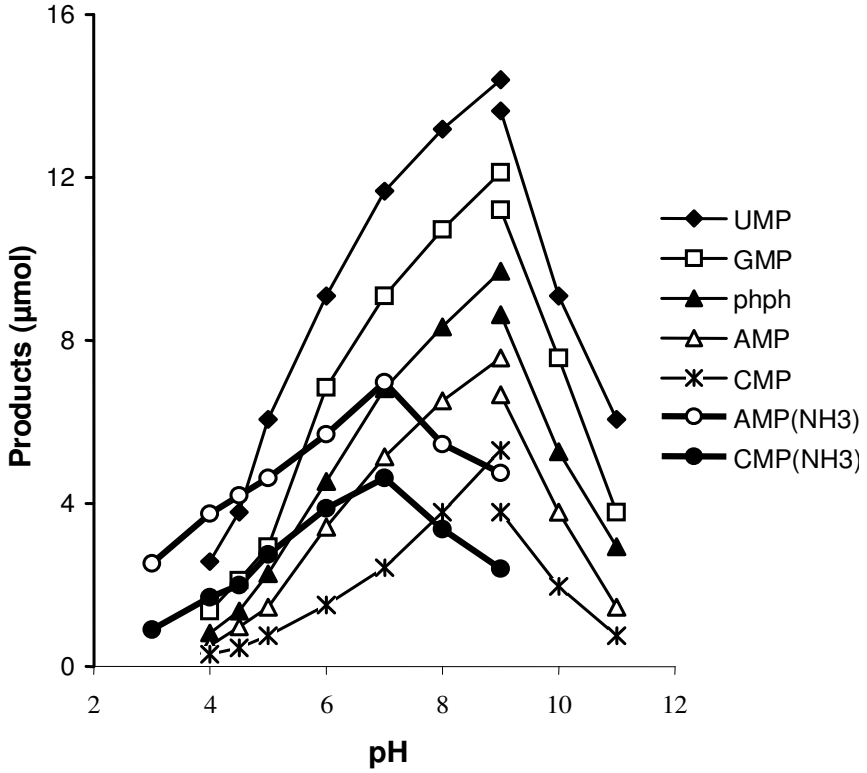

Figure 3. pH-dependence of phosphatase and deaminase activities. Reaction mixture contained: substrate,15 µmoles; buffer,100 µmoles (Tris-acetate, pH 4 - 9 and carbonate- bicarbonate, pH 9- 11); protein of the extracts,2.7 mg; total volume,1ml; temperature,40°C and incubation time,30 min.

buffer pH (9-11). The reaction mixtures were then incubated at 40°C for 30 min after which determination of phosphate and ammonia was determined in all of them. Data obtained recorded in Figure 3 which shows that phosphate was liberated in all the reaction mixtures and its liberation was optimum at pH 9.0 the same figure also shows that the pH-activity profiles obtained with the four substrates were similar to the analogous profile recorded with the synthetic substrate ph.

Nature of buffer

Testing the phosphates and deaminase activities in some buffer systems including, Tris-acetate, Tris-HCl and Carbonate-bicarbonate revealed data in Figure 4 indicating that Tris-acetate buffer was more suitable than the other two buffers in both activities of phosphatase and deaminase.

Temperature dependence of ribonucleotides phosphhydrolase and aminohydrolase

Reaction mixtures containing four ribonucleotides and ph.ph as substrates were incubated at different degrees of temperature ranged from 30 - 90°C for 30 min. Results obtained in Figure 5 indicate that optimum temperature of phosphatase for all substrates was obtained at 60°C while the optimum temperature of aminohydrolase of adenosine or cytidine was 50°C and the amount of ammonia released from adenosine is higher than that of cytidine.

Heat inactivation kinetics

To test the stability of the ribonucleotides phosphorhydrolase and aminohydrolase enzymes as a function of exposure to 50 - 80°C in absence of the substrate, aliquots of the exposed crude enzyme preparations were withdrawn at different time intervals, cooled then incubated with substrates and assayed for phosphatase and deaminase activities. Results obtained indicated that complete stability of the enzymes activities when exposed at 50 and 60°C for 10 min. However data obtained from exposure of crude extracts to 70°C were represented in Table 1 demonstrates a difference in stability of the two activities. Where the deaminase is more stable than the phosphatase and complete inhibition occurred at 80°C after 10 min for two enzymes

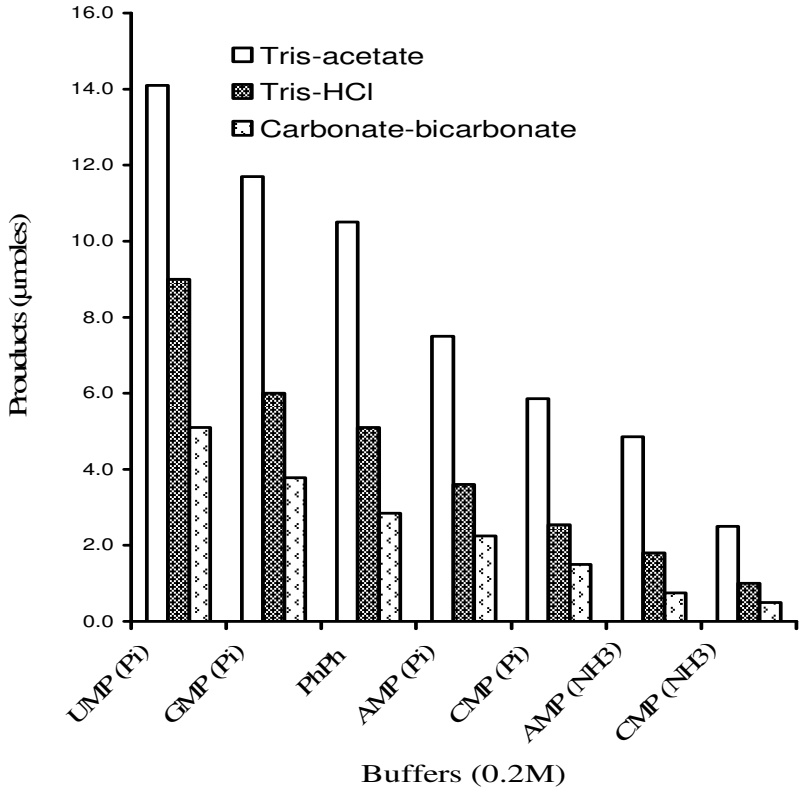

Figure 4. Effect of different buffers on the phosphatases and the aminohydrolase. Reaction mixture contained: substrate,15 µmoles; different buffer (pH),100 µmoles; protein of the extracts,2.7 mg; total volume,1ml; temperature,40°C and incubation time, 30 min.

activities.

Extents of degradation of ribonucleotides by *P. brevicompactm* extracts

Results of Table 2 show the extents of degradation of the four ribonucleotides under the optimum conditions of pH and temperature. From this table, it appears that the Pi that only resides from UMP and GMP was released by the end of the incubation period out of the tested ribonucleotides. This amount of Pi seems to be liberated directly from the nucleotide molecule and not from an intermediate in the way of its degradation such as ribose-5-phosphate or a deaminated nucleotide. This suggestion was based on the absence of reducing compounds and also the absence of equimolar amounts of NH_3 from all the reaction mixtures. However, an amount of NH_3 represented about 56 and 21% was detected of that which originally resides in the amount of the AMP and CMP respectively used at the end of the incubation period. From this last result, it was suggested that NH_3 was liberated from the adenosine and cytidine formed from AMP and CMP respectively by the action of the phosphatase of the extracts. Chromatographic analysis of all the reaction mixtures at the end of the incubation period did not reveal appearance of any base.

Sequence of Pi and NH_3 release during AMP and CMP degradation

In order to determine the sequence of release of Pi and NH_3 during AMP and CMP degradation, the two reaction mixtures containing AMP and the other containing CMP as substrate were calorimetrically and chromatogram-phically analyzed at different time intervals over a period of 2 h. Data recorded and presented in Table 3 and 4 shows that Pi liberation preceded NH_3 release during AMP and CMP degradation. This suggests that AMP and CMP were degraded to inosine and uridine respectively, via the intermediate formation of adenosine and cytidine. Data in Table 3 show chromatographic appearance of adenosine prior to inosine, and IMP was not detected throw out the incubation period, also data presented in Table 4 are in accordance with this suggestion as they show chromatographic appearance of cytidine prior to uridine during the degradation process and that UMP was

Table 1. Heat inactivation kinetics of phosphohydrolase and aminohydrolase.

Substrate	Remaining activity %					
	Phosphatase exposed for 60 min at			Deaminase exposed for 60 min at		
	60°C	70°C	80°C	60°C	70°C	80°C
UMP	100	68.3	0.0	100	--	--
GMP	100	55	0.0	100	--	--
Phph	100	45	0.0	100	--	--
AMP	100	38	0.0	100	70	0.0
CMP	100	31.5	0.0	100	39.4	0.0

Reaction mixture contain; substrate, 7.5 µmoles; Tris-acetate buffer (pH 9) 50 µmoles; protein of the extracts, 2.7 mg; total volume, 0.5 ml; temperature, 40°C and incubation time, 60 min.

Table 2. Extents of ribonucleotides degradation by extracts of P. *brevi-compactum.*

Substrate	Products (µmoles)		
	Pi	Ribose or ribose-5- phosphate	NH_3
UMP	45	0.0	0.0
GMP	41.6	0.0	0.00
CMP	34.8	0.0	7.5
AMP	30.3	0.0	17.0
Adenosine	---	0.0	33
Guanosine	---	0.0	0.0
Cytidine	---	0.0	15
Uridine	---	0.0	---

Reaction mixture contained: substrate, 45 µmoles; Tris-acetat buffer (pH 7),300 µmoles; protein of the extracts, 2.7 mg; total volume, 3ml; temperature,40°C and incubation time, 120 min.

not detected throw out the incubation period.

Substrate specificity of the deamidase(s)

Data of Table 5 show that the amidohydrolase(s) exhibited activities only with adenosine, cytidine and cytosine out of the tested compounds such as nucleotide, nucleosides and bases at the same experimental conditions.

From these data ribonucleotides degradation in this organism are suggested to proceed as in Figure 1.

Absence of evidence for involvement of specific nucleotidases in Pi liberation from the four ribonucleotides

Data presented in Figures (2 and 3) show that change in the pH and in the temperature of the reaction mixtures, revealed changes in the dephosphorylating activities with the four ribonuclotides similar to the changes recorded with phenyl phosphate. These findings indicate that the non specific alkaline phosphates of the extracts (which catalyzed the hydrolysis of phenyl phosphate) was the only dephosphorylating enzyme that catalyzed the hydrolytic dephosphorylation of the four ribonucleotides, as, if certain specific nucleotidases (which are known to dephosphorylase only nucleotides) were participating in the catalytic process, the variations in the activities of the extracts with the four ribonucleotides will not have been so similar to the variations obtained with phenyl phosphate.

Inability of the extracts to catalyze cleavage of the N-glycosidic linkages of either the four nucleotides or their corresponding nucleosides

Data of Table 1 show that the extracts could not catalyze cleavage of the N-glycosidic linkages of the four nucleotides, as neither reducing compounds (ribose or ribose 5-phosphate) nor bases could be detected in any of the reaction mixtures. In addition, neither hydrolytic nor

Table 3. Chromatographic identifications of the intermediates and products formed during AMP degradation.

Compounds identified in the reaction mixture	R_f values of the compounds identified					
	At					
	Zero time		30 min		120 min	
	SI	SII	SI	SII	SI	SII
AMP	0.27	0.09	0.27	0.09	-	-
Adenosine	-	-	0.48	0.4	-	-
Inosine	-	-	0.41	0.3	0.41	0.3
IMP	-	-	-	-	-	-

Reaction mixture contained : substrate, 15 µmoles; protein, 2.7 mg; buffer, Tris-acetate pH9, 100 µmoles; vol. ,1ml. Time, as indicated; temp., 40°C.

Table 4. Chromatographic identification of the intermediates and products formed during CMP degradation.

Compounds identified in the reaction mixture	R_f valus of the compounds identified					
	At					
	Zero time		30min		120 min	
	SI	SII	SI	SII	SI	SII
CMP	0.24	0.11	0.24	0.11	-	-
Cytidine	-	-	0.5	0.32	-	-
Uridine	-	-	0.55	0.4	0.55	0.4
UMP	-	-	-	-	-	-

Reaction mixture contained : substrate, 15 µmoles; protein, 2.7 mg; buffer, Tris-acetate pH 9, 100 µmoles; vol. 1 ml. Time, as indicated; temp., 40°C.

Table 5. Substrate specificity of the deaminase.

Compounds	Ammonia Formed (µ moles)
AMP	3.6
GMP	0.0
CMP	1.25
Adenosine	7.0
Guanosine	0.0
Cytidine	2.5
Adenine	0.0
Guanine	0.0
Cytosine	0.88

Reaction mixture contained (in 1 ml vol): substrate, 15 µmol; extract protein, 2.7 mg; buffer, Tris- acetate pH7, 100 µmol; temperature,40°C and time, 30 min.

phosphorylytic cleavage of N-glycosidic linkages of the corresponding purine and pyrimidine ribonucleosides could be effected by the same extracts over a pH range from 4 - 11. This conclusion was based on inability to detect either ribose or any bases when each of the four ribonucleosides (cytidine, uridine, adenosine and guanosine) was incubated with the extracts in presence and absence of Pi or arsenate at a concentration that represented (on molar bases) about four times that of the substrate.

DISCUSSION

From the findings recorded during the present investigation and those previously demonstrated

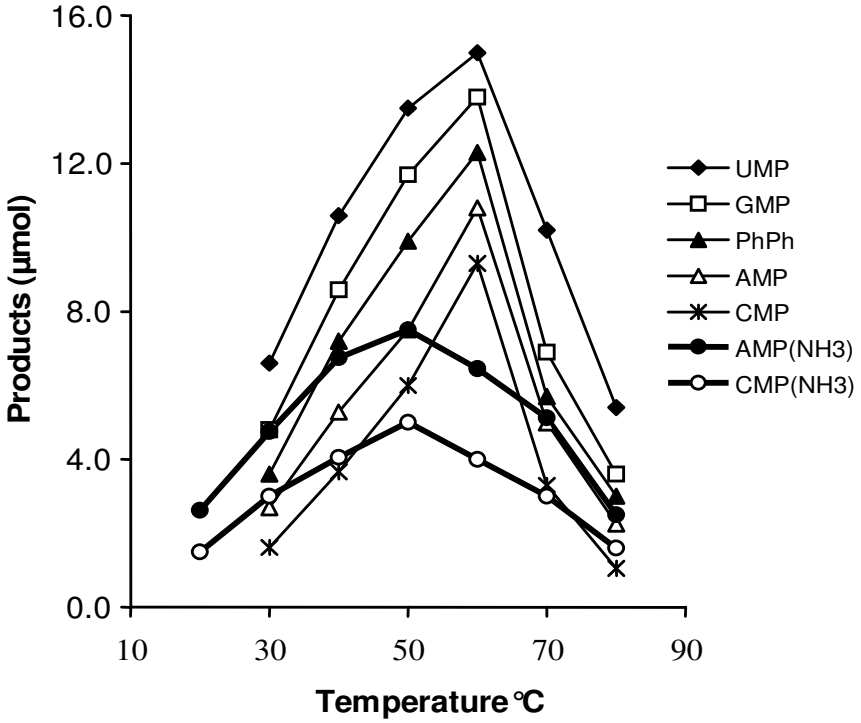

Figure 5. Effect of temperature on dephosphorylation and deamination of ribonucleotides Reaction mixture contained: substrate, 15 µmoles; Tris- acetate buffer (pH 9),100 µmoles; protein of the extracts, 2.7 mg; total volume, 1ml; temperature, as indicated and incubation time, 30 min.

concerning extents and pathways of ribonucleotides degradation by the extracts of organisms belonging to the filamentous fungi, it can be stated that, alkaline phosphatase of *P. brevi-compactum* (used in the present work) resembles the two alkaline isozymes of *A terrecola* (Ali, 2003), acid phosphatase of *P. politans* (Ali, 2006), and acid phosphatases of the two strains of *A. niger* and *A. niger* $NRRL_3$ studied by Elzainy et al., 1989 and Ali and Elzainy, 2000 in that all of these enzymes could catalyze Pi liberation from the ribonucleotides over a wide range of pH values and this catalytic process was effected by phosphate non-repressible phosphatase. The four extracts could not catalyze cleavage of the N-glycosidic linkages of either ribonucleotides or the two pyrimidine ribonucleosides. All previous extracts and *Arabidopsis thaliana* protein (Vincenzetti et al., 1999) could hydrolytically deaminate cytidine out of the tested amino bases, their ribonucleosides and ribonucleotides except the extracts of *P. brevi-compactum* which can deaminate adenosine and cytosine moreover cytidine. *P. brevi-compactum* phosphatase resembles *Penicillium chrysogenum* phosphatase (Elzainy et al., 1979) and *P. politans* phosphatase (Ali, 2006) in having one phosphatase only, however the enzyme in the two first organisms are alkaline phosphatases but the enzyme in the third organism was active in acidic medium, and they were differ from the two *A. niger* strains and *A. terricola* which contained two phosphatases. The hydrolysis of nucleotides to nucleosides and free bases by environmental bacteria–*Stenotrophomonas (Xanthomonas) maltophilia* were described by Chmielowiec et al., 1996, 1999), they were able to obtain all nucleosides and guanine and thymine from nucleotides.

The suggested specificity of the *P. brevi-compactum* phosphatase towards the four ribonucleotides was based on the given lines of evidence which all indicate that one enzyme was involved complete conversion of each of the four ribomomonucleotide to the corresponding nucleoside plus inorganic phosphates. These lines of evidence included: similar responses of the four activities to changes in the conditions of the reactions such as pH, nature of the buffer, temperature further more, the activity with the four substrates was with almost constant ratio. All these criteria are in favor of classification of the dephosphorylating enzyme as non specific alkaline phosphatase.

Also *P. brevi-compactum* deaminase was similar to *P. politans* which could deaminate cytidine and adenosine, the cytidine and adenosine deaminase properties of *P. politans* were studied by Elshafei et al. (2005 a + b) and purified adenosine deaminase at the same organism was reported by Elshafei et al. (2007). Where as *Streptomyces hygroscopicus* NRRL B-1476 contain cytidine deaminase (Ali and Mohamed, 2008). No evidence could

be obtained demonstrating participation of specific nucleotidases in dephosphorylation of ribonucleotides by the three extracts, but differ from of *A. niger* phosphatase which studied by Elzainy et al. (1989) proved to contain a purine nucleoside hydrolase which was active with adenosine and guanosine while, extracts of *P. chrysogenu*m were suggested to contain both purine and pyrimidine nucleosides hydrolases (Allam et al., 1987). But extracts of *P. brevi-compactum, A. niger* $NRRL_3$ and *P. politans* strains were devoid of this activity. Inorganic phosphate liberation by extracts of *P. brevi-compactum* was optimum at pH9*, P. politans* phosphatase (Ali, 2006) was optimum at pH6 while the analogous catalytic activity of extracts of the two other strains *A. niger* $NRRL_3$ (Ali and Elzainy 2000) and *A. niger* studied by Elzainy et al. (1989) was optimum at pH 2 and pH 4 respectively.

Conclusion

The present study describes the properties of enzymes of the catabolic pathways of some purine and pyrimidine nucleotides, nucleosides and bases in *P. brevi-compactum* NRC829 which contains two enzymes, a phosphate- non repressible alkaline phosphates and aminohydrolase. The presence of the enzymes involved in the salvage pathway implies the presence of their substrates, nucleosides. Furthermore, these nucleosides might provide substrates not only for this pathway but also for the enzymes involved in their “catabolism”. In this case the catabolism of nucleosides might represent a possible source of carbon and nitrogen.

ACKNOWLEDGMENT

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