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Full Length Research Paper

Degradation of pyrimidine ribonucleosides by extracts of *Aspergillus terreus*

Osama M. Abdel-Fatah*, Maysa A. Elsayed and Ali M. Elshafei

Department of Microbial Chemistry, National Research Centre, Dokki, Cairo, Egypt.

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Cell-free extracts of nitrate-grown mycelia of *Aspergillus terreus* could catalyze the hydrolytic deamination of cytidine to uridine and ammonia followed by the hydrolytic cleavage of the N-glycosidic bond of the produced uridine to the corresponding base (uracil) and ribose. The same extracts could not catalyze the hydrolytic deamination of cytosine. Addition of inorganic arsenate to the reaction mixture containing cytidine or uridine did not affect the amount of ribose liberated indicating the absence of pyrimidine ribonucleosides phosphorylase in the extracts. Cytidine deaminase showed an optimum activity at pH 7.0 and 60°C and stability to high degrees of temperature. Uridine hydrolase activity was optimized at pH 8.0 and 55°C. Incubation of the extracts at 55°C for 60 min showed no effect on uridine hydrolase activity whereas incubation of the extracts at 60 and 70°C for different interval times caused a gradual decrease in activity and the enzyme lost its activity completely by incubation at 80°C for 15 min. Dialyzing the extracts showed no effect on cytidine deaminase activity and a decrease in uridine hydrolase activity. Addition of EDTA at a concentration of 5 x 10^{-3} M and 10^{-2} M caused an inhibition to the two enzymes activities. The presence of $MgSO_4$ in the reaction mixture seems to activate greatly both enzymatic cytidine deamination (225 and 128% increases) and uridine hydrolysis (22 and 77% increases) at final concentrations of 5 x 10^{-3} M and 10^{-2} M respectively. However $HgCl_2$ and $CuSO_4$ were found to be potent inhibitors for both activities at the two concentrations.

Key words: Pyrimidine ribonucleosides, cytidine, uridine, cytidine deaminase, uridine hydrolase, *Aspergillus terreus*.

INTRODUCTION

Most of the studies concerning the cleavage of N-glycosidic bond of ribonucleosides in filamentous fungi have been reported mainly on purine ribonucleosides (Elzainy et al., 1978; Hassan et al., 1979; Elzainy et al., 1990; Abu-Shady et al., 1994; Elshafei et al., 1995; Abdel-Fatah et al., 2003). However the cleavage of N-glycosidic bond of pyrimidine ribonucleosides in filamentous fungi was very rare (Hassan et al., 1983; Allam et al., 1987). Cleavage of the amino group of cytidine was studied in extracts of *Penicillium citrinum* (Elzainy et al., 1990), *Aspergillus niger* (Ali, 1998) and *Aspergillus phoenicis* (Abdel-Fatah, 2005). A highly specific uridine hydrolase was firstly demonstrated from autolysates of baker's yeast (Carter, 1951). The presence of specific uridine hydrolase was also reported by Grishchknov et al., (1978) in extracts of *Corynebacterium glutamicus.* Uridine and cytidine hydrolase were also plant pathogenic bacterium namely, *Xanthomonas phaseoli* (Hochster and Nozzolillo, 1961). Uridine ribohydrolase from *Saccharomyces cereviciae* was identified cloned and characterized the corresponding URH1 gene and its physiological function was determined by the measurement of metabolic fluxes in several mutants im-paired in the pyrimidine salvage pathway (Kurtz et al., 2002). Pyrimidine nucleoside hydrolase from *Escherichia coli* showed high catalytic efficiency toward flourouridine which could be exploited for suicide gene therapy in cancer treatment (Giabbai and Degano, 2004). The availability of information about the degradation of pyrimidine ribonucleosides and their derivatives from different sources is the basis for studies on the use of these unique enzymes in biotechnology and in medical therapy (Johansson and Karlsson, 1997; Hocek et al., 2005). The aim of the present work was to investigate the presence of enzymatic cleavage of pyrimidine ribonucleosides in *A. terreus* and study the properties of the enzymes encountered in this degradation.

*Corresponding author: E - mail: abdelfatahom@yahoo.com.

Table 1. Degradation of pyrimidine ribonucleosides by extracts of *A. terreus.*

pH	Products (µmoles) formed from		
	Cytidine		Uridine
	Ammonia	Ribose	Ribose
5.0	2.00	0.0	0.0
7.0	2.27	1.4	1.8
9.0	2.12	0.9	1.6

Reaction mixture contained: cytidine or uridine, 5 µmoles Tris-acetate buffer pH as indicated, 50 µmoles; extracts protein, 3.96 mg; total volume, 1.0 ml; reaction temperature, 40°C; reaction time, 60 min.

MATERIALS AND METHODS

Microorganism

Aspergillus terreus NRRL 265 was obtained from the culture collection of the Northern Utilization Research and Development Division, United States, Department of Agriculture, Peoria, Illinois, U.S.A.

Media: Aspergillus terreus was cultivated and kept on slants of modified solid Czapek-Dox medium (Difco Manual, 1972). The composition of this medium is as follows (g/l): D-glucose, 20; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.5; KCl, 0.5 and agar 20. The liquid medium was sterilized by autoclaving under 1.5 atmosphere for 20 min. In case of solid media, the sterilization time was prolonged for 30 min under the same pressure.

Cultivation of organism

Conidia were scraped from mycelia, which were grown on slants for 7 days at 30°C and suspended by hand shaking, in sterile distilled water. Two ml aliquots of this suspension were used to inoculate, under aseptic conditions, 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium. The inoculated flasks were incubated statically at 30°C for 4 days.

Preparation of cell-free extracts

After four days incubation period the mycelia were collected and harvested by filtration, then washed thoroughly with cold distilled water and finally blotted dry with absorbent paper. The blotted-dry mycelia were ground with approximately twice its weight of washed cold sand in a cold mortar and extracted with 0.1 M Tris-HCl pH 8.0. The slurry so obtained was centrifuged at 5500 rpm for 5 min. The supernatant was used as the crude enzyme preparation.

Dialysis of the extracts

Dialysis of the extracts was made against 200 fold its volume of cold 0.01 M Tris-HCl pH 8.0 for a period of 24 h at 4°C using dialysis bags (cellulose tubing 21mm dia., Sigma Diagnostic. St Louis. MO 631 78 USA).

Colorimetric determination

Determination of ribose was made by the method described by Ashwell, (1957). Ammonia was determined by Nessler's reagent described by Schramm and Lazorik, (1975). Protein was determined by the method of Lowry et al., (1951).

Enzyme assay

Cytidine deaminase activity was assayed by measuring the appearance of ammonia when the enzyme preparation was incubited with cytidine. This was accompanied by chromatographic identification of the formed products. Uridine hydrolase activity was determined by measuring the reducing sugar as ribose formed from the pyrimidine ribonucleosides, as previously described by Elzainy et al., (1990). This was accompanied by chromatographic identification of the base. One unit of enzyme is defined as the amount that formed one micromole of ribose or ammonia under the standard assay conditions. Specific activities are expressed as units/ml/mg protein. The estimation was repeated for three times and the data given represents the mean value of the three repetitions.

Identification of ribose: Ribose was identified using the ascending paper chromatographic (Whatman N°.1) technique according to the method of Smith and Seakins, (1976). Two solvent systems were used; solvent 1 consists of n-propanol -ethyl acetate - water (70:10:20) and solvent 2 consists of isopropanol - water (160: 40). The developed brown spots of the identified and authentic ribose were located by using aniline oxalate reagent.

Identification of the pyrimidine ribonucleosides and their bases

Chromatographic identification of the pyrimidine ribonucleosides and their bases was made using chromatographic Whatman N°3 MM filter paper and two solvent systems. Solvent 1 consists of n-butanol - glacial acetic acid - water (120:30:50) and solvent 2 consists of n-butanol - formic acid - water (154:20:26) (Smith and Seakins, 1976). The spots were located with an ultraviolet lamp.

RESULTS

Enzymatic degradation of pyrimidine ribonucleosides by extracts of *A. terreus*

An experiment was made to test the ability of cell-free extracts of nitrate-grown mycelia of *A. terreus* to catalyze the degradation of the pyrimidine ribonucleosides namely, cytidine and uridine. Each substrate was added to the reaction mixture containing Tris-acetate buffer at pH 5.0, 7.0 and 9.0. The results obtained indicate that ammonia was detected in the reaction mixtures containing cytidine adjusted at pH 5.0, 7.0 and 9.0 whereas ribose was detected in the reaction mixtures containing cytidine or uridine adjusted at pH 7.0 and 9.0 with different proportions. The results cited in Table 1 indicated that ex-tracts of *A. terreus* could catalyze the deaminating and hydrolytic degradation of cytidine and uridine respectively.

Chromatographic identification of the products

Ribose was chromatographically identified in the reaction mixture containing cytidine or uridine adjusted at pH 8.0. The developed brown spots of the identified and authentic ribose had the same R_f values of 0.51 and 0.72 in solvent 1 and 2 respectively (materials and methods). Uri-

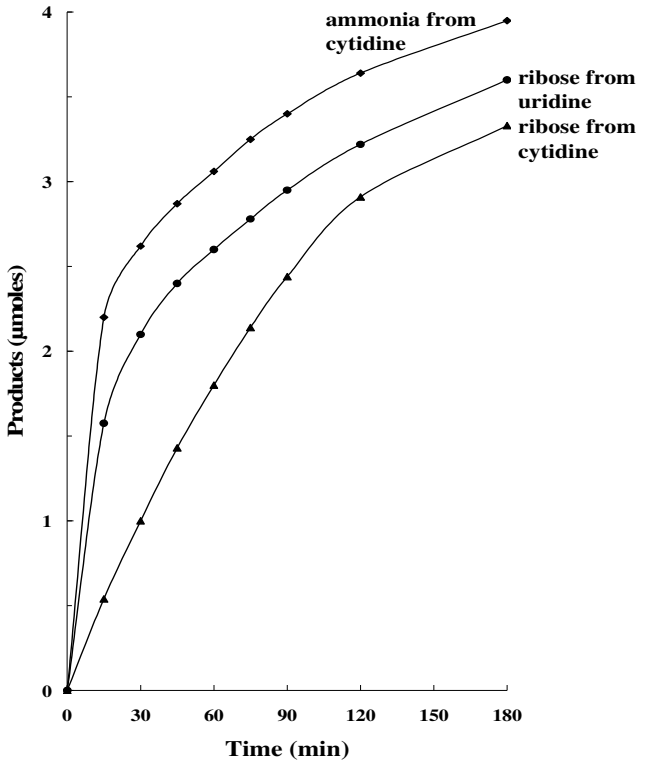


Figure 1. Degradation of cytidine and uridine as a function of time. Reaction mixture contained: Cytidine or uridine, 5 µmoles; Tris-HCl buffer pH 8.0, 50 µmoles; extract protein, 3.96 mg; total volume, 1.0 ml; temperature, 55°C; and reaction time as indicated.

dine and uracil were detected in the reaction mixture containing cytidine and uracil in the reaction mixture containing uridine. Cytosine could not be detected in the reaction mixture containing cytidine indicating that cytidine was degraded to uridine due to the action of cytidine deaminase. N-glycosidic bond of the formed uridine was then cleaved to produce uracil and ribose. The R_f values of the identified and authentic uridine were identical and were found to be 0.38 in solvent 1 and 0.25 in solvent 2. The expected base, uracil had R_f coincide with the authentic sample (R_f 0.49 in solvent 1 and 0.4 in solvent 2) (not shown).

Degradation of cytidine and uridine as a function of time

Figure 1 shows that the amount of ammonia formed in the reaction mixture containing cytidine was higher than the amount of the formed ribose. This result suggests that the degradation process starts by the deamination of cytidine producing ammonia and uridine which in turn could be cleaved by a hydrolase to produce uracil and ribose. The previously mentioned assumption was confirmed by the formation of considerable amounts of ribose from a reaction mixture containing uridine as a substrate (Figure1) .

Inability to detect pyrimidine ribonucleoside phosphorylase

Investigating this point was achieved through comparing the activities of the extracts in the presence and absence of inorganic arsenate with the two substrates cytidine and uridine. Results obtained show that the amount of ribose in presence of arsenate equals to that formed in the absence of arsenate indicating that phosphorolytic activity could not be detected in the extracts under these experimental conditions (not shown).

Substrate specificity of cytidine deaminase of *A. terreus*

This experiment was carried out to investigate substrate specificity of the enzyme that catalyzes the hydrolytic deamination of cytidine. Equimolar amount of a specific substrate namely cytidine, cytosine, adenosine, adenine, guanosine and guanine was incubated with Tris-HCl buffer at pH 8.0 at 60°C for 120 min after which ammonia was determined in all reaction mixtures. Results obtained indicate that from the six tested substrates, only cytidine was degraded by hydrolytic deamination, as 3.64 µ moles of ammonia was formed from 5 µmoles of cytidine indicating its specificity for this substrate (not shown).

pH value dependence of pyrimidine ribonucleosides degradation

Figure 2 shows the effect of pH values of the reaction mixtures on ammonia formation due to the action of cytidine deaminase. Optimum pH value for ammonia formation from cytidine occurred at pH 7.0, while as pH 8.0 was the optimum value for ribose formation from uridine and cytidine by cell-free extracts of *A. terreus* (Figure 3).

Effect of temperature

Maximal deaminating and hydrolytic activities occurred at 60 and 55°C respectively (Figures 4 and 5). This can be explained by stability of cytidine deaminase and uridine hydrolase towards high degrees of temperature.

Thermal stability behaviour

Cell-free extracts of *A. terreus* was subjected to different degrees of temperature to test the stability of cytidine deaminase and uridine hydrolase against high degrees of temperature. Results cited in Figures 6 and 7 show that cytidine deaminase has a thermal stability higher than uridine hydrolase where cytidine deaminase was still active at high temperatures (33% of activity remained when the extracts was

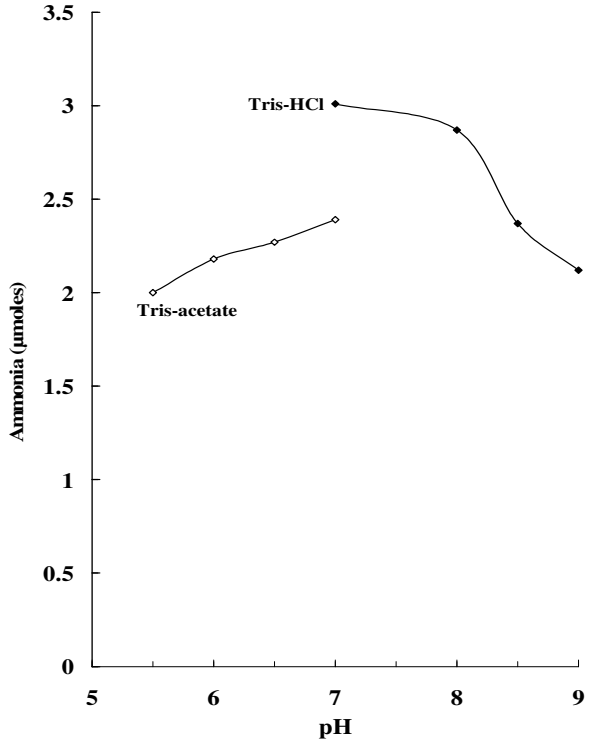


Figure 2. Effect of pH on cytidine deaminase activity. Reaction mixture contained: Cytidine, 5 µmoles; (•) Tris-acetate or (◦) Tris-HCl buffer pH as indicated, 50 µmoles; extract protein, 3.52 mg; total volume, 1.0 ml; temperature, 60°C and reaction time, 60 min.

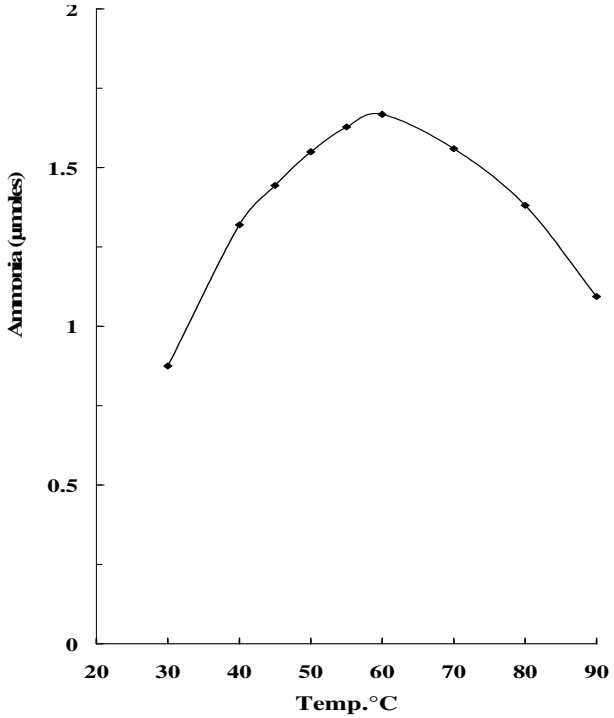


Figure 4. Effect of temperature on cytidine deaminase activity. Reaction mixture contained: Cytidine, 5 µmoles; Tris-HCl buffer pH 7.0, 50 µmoles; extract protein, 3.52 mg; total volume, 1.0; temperature, as indicated and reaction time, 60 min.

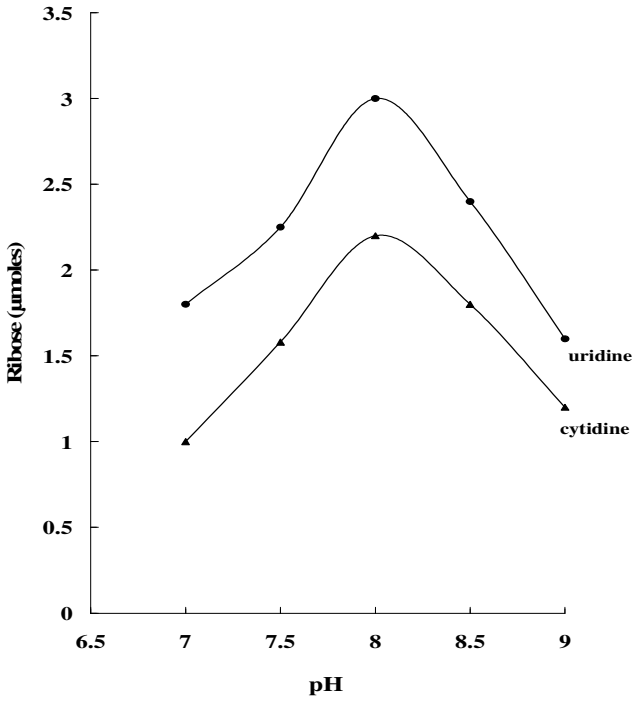


Figure 3: Ribose formation from cytidine and uridine as a function of pH value. Reaction mixture contained: Cytidine or uridine, 5 µmoles; Tris-HCl buffer pH as indicated, 50 µmoles; extract protein, 3.96 mg; total volume, 1.0 ml; temperature, 55°C; and reaction time, 60 min.

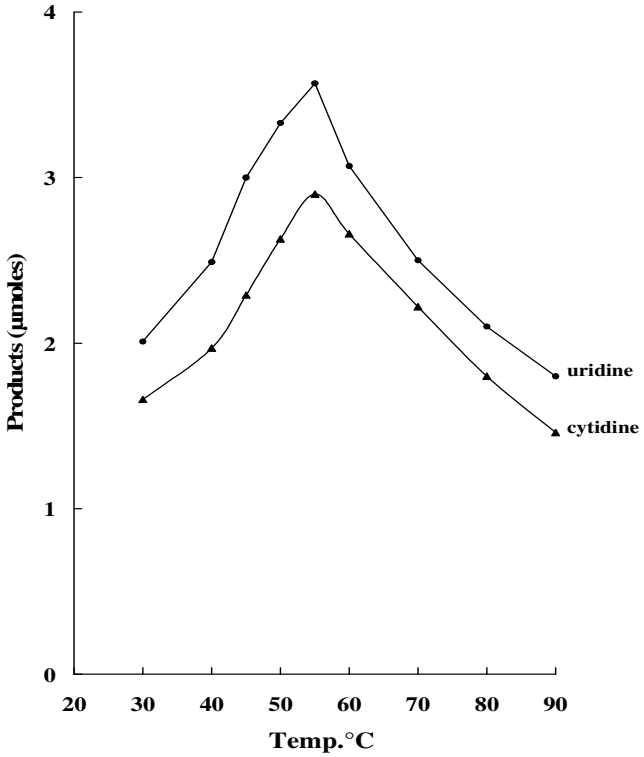


Figure 5. Effect of temperature on the formation of ribose from cytidine and uridine. Reaction mixture contained: Cytidine or uridine, 5 µmoles; Tris-HCl buffer pH as indicated, 50 µmoles; extract protein, 3.96 mg; total volume, 1.0 ml; temperature, 55°C; and reaction time, 60 min.

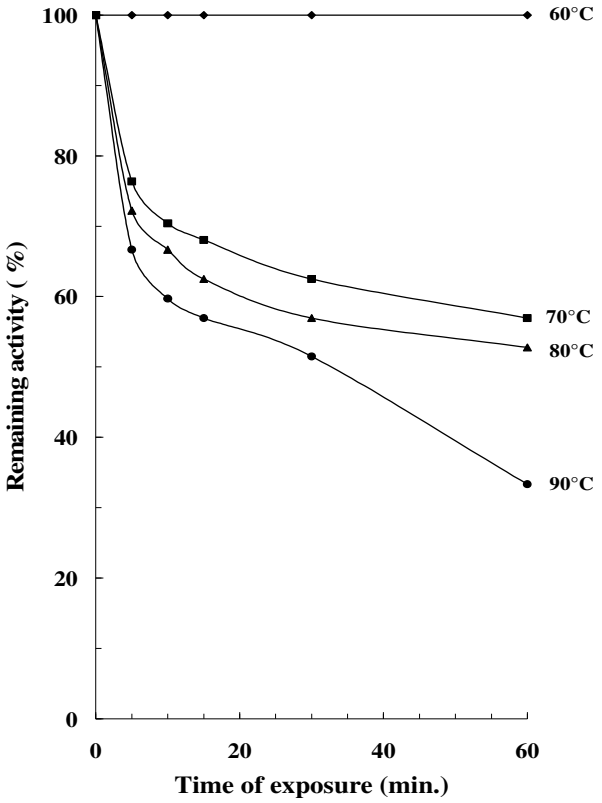


Figure 6. Heat inactivation kinetics of cytidine deaminase. Reaction mixture contained: Cytidine, 5 µmoles; Tris-HCl buffer pH 8.0, 50 µmoles; extract protein, 3.03 mg; total volume, 1.0 ml; reaction temperature, 60°C; time of exposure, as indicated and reaction time, 60 min.

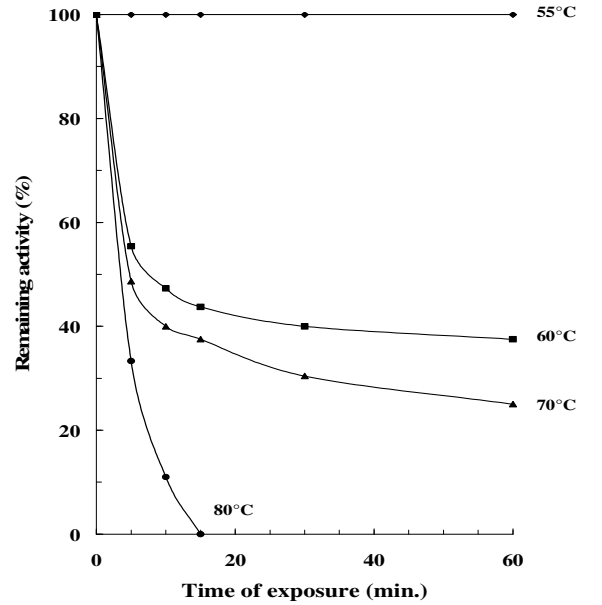


Figure 7. Heat sensitivity of uridine hydrolase. Reaction mixture contained: uridine, 5 µmoles; Tris-HCl buffer pH 8.0, 50 µmoles; extracts protein, 3.03 mg; total volume, 1.0 ml; reaction temperature, 55°C; time of exposure, as indicated and reaction time, 60 min.

Table 2. Effect of different buffer systems on pyrimidine ribonucleosides degradation

Type of buffer (pH 8.0)	Products (µmoles) formed from		
	Cytidine		Uridine
	Ammonia	Ribose	Ribose
Tris-HCl	4.9	3.8	4.8
Tris-acetate	4.0	1.6	1.8
Boric-Borax	2.9	1.4	2.4
Phosphate	4.5	3.6	3.8

Reaction mixture contained: cytidine or uridine, 5 µmoles; different buffers as indicated at pH 8.0, 50 µmoles; extract protein, 4.08 mg; total volume, 1.0 ml; temperature, 55°C and reaction time, 60 min.

exposed to 90°C for 60 min), while uridine hydrolase lost its activity completely when the crude extracts were exposed to 80°C for 15 min.

Best buffer system

Four reaction mixtures were made containing the same amount of substrate (cytidine or uridine) and received equimolar amount of Tris-HCl, Tris-acetate, Boric-Borax and phosphate buffer (0.05 M) at pH 8.0. Results obtained in Table 2 indicate that the activity of each of the two enzymes in Tris-HCl buffer is the highest when compared with the analogous activities obtained from the other three tested buffers.

Effect of additives

Addition of ethylenediamine-tetraacetate (EDTA), a metal chelating agent, at a concentration of 5 x 10^{-2} and 10^{-2} M caused about 50 and 43% inhibition on cytidine deaminase activity. The same concentrations caused about 54 and 74% inhibition on uridine hydrolase activity (Table 3). The previously mentioned results can be interpreted by the fact that cytidine deaminase and uridine hydrolase may require some metal cation(s) or activator(s) in the enzyme catalysis process. Addition of iodoacetate or 2-mercaptoethanol at a concentration of 5 x 10^{-3} and 10^{-2} M had no effect on cytidine deaminase and uridine hydrolase activities. The previous findings indicate that sulfhydryl group has no role in deaminating and hydrolytic catalysis process. These results were supported by the addition of reduced glutathione at the same concentrations which had no effect on the activity of the two enzymes under study (Table 3).

Effect of dialyzing the extracts

To study the effect of dialyzing cell-free extracts of *A. terreus* on the formation of ammonia and ribose from cytidine and uridine respectively, dialyzed extracts were tested and the obtained activity was compared with the analogous activity of nondialyzed extracts. The data presented in Table 5 show that the dialyzing process has no

Table 3. Effect of the addition of some compounds on the activity of cytidine and uridine-degrading enzymes.

Additive compounds	Concentration (M)	Enzyme activity (%)	
		Cytidine deaminase	Uridine hydrolase
---	---	100	100
Iodoacetate	5×10^{-3}	100	96
	10^{-2}	100	94
Mercaptoethanol	5×10^{-3}	100	97
	10^{-2}	100	95
Reduced glutathione	5×10^{-3}	100	100
	10^{-2}	100	100
EDTA	5×10^{-3}	50	46
	10^{-2}	57	26

Reaction mixture contained: cytidine or uridine, 5 µmoles; Tris-HCl buffer pH 8.0, 50 µmoles; extract protein, 2.62 mg; total volume, 1.0 ml; reaction temperature, 55°C and reaction time, 60 min

Table 4. Effect of dialysis process on the activity of cytidine and uridine- degrading enzymes.

Type of extract	Cytidine deaminase		Uridine hydrolase	
	Ammonia (µmoles)	Sp. activity	Ribose (µmoles)	Sp. activity
Crude	3.40	1.14	3.0	1.01
Dialyzed	2.74	1.2	0.6	0.26

Reaction mixture contained: cytidine or uridine, 5 µmoles; Tris-HCl buffer pH 8.0, 50 µmoles; extract protein, 2.97 mg; dialyzed extract protein, 2.25; total volume, 1.0 ml; reaction temperature, 55°C; reaction time, 60 min.

no effect on cytidine deaminase activity while as, uridine hydrolase lost about 75% of its activity due to the dialyzing process. The decrease in the enzymatic activity in case of dialyzed extracts can be explained by the release of certain dialyzable cofactors or activators that are present in the non-dialyzed extracts and required for the catalysis process (Table 4).

Effect of addition of some metal salts

From the previous experiment it was shown that dialyzing the extracts caused a decrease in the activity of uridine hydrolase. This finding may indicate the importance of certain dialyzable cofactors or activators that are present in the non-dialyzed extracts and required for the catalysis process. The presence of $MgSO_4$ in the reaction mixture seems to activate greatly both enzymatic cytidine deamination (225 and 128% increases) and uridine hydrolysis (22 and 77% increases) at a final concentration of 5×10^{-3} M and 10^{-2} M respectively. However $HgCl_2$ and $CuSO_4$ were found to be potent inhibitors for both activities at the two concentrations. $CuSO_4$ was also found to inhibit completely uridine hydrolase and partially cytidine deaminase (32 and 42% decreases in activity at both concentrations respectively) (Table 5).

Effect of freezing and thawing

The enzyme preparation was assayed for activity in the usual manner, stored in the refrigerator at about -5°C, left for 24 h, after which it was thawed and an aliquot was withdrawn for assay of activity under the same experimental conditions. This process was repeated every 24 h for 3 days. Results of the analysis of products (ammonia and ribose) cited in Table 6 show that the amounts of products remain more or less the same after 72 h (3 cycles) indicating the stability of cytidine deaminase and uridine hydrolase towards this treatment.

DISCUSSION

Studies cited in this work revealed that extracts of *A. terreus* catalyzed the hydrolytic deamination of cytidine to uridine and ammonia and could not catalyze the deamination of cytosine .The formed uridine or uridine itself as a substrate could be cleaved to produced uracil and ribose. Cytidine deaminase of *A. terreus* reached its maximum activity in Tris-HCl buffer at pH 7.0 which resembles cytidine deaminase of *A. niger* (Ali, 1998) and differ from cytidine deaminase of *A. phoenicis* in having its maximum activity in citrate buffer at pH 6.0 (Abdel-Fatah, 2005). Cytidine deaminase of *A. terreus* showed a relatively high optimum temperature (60°C) and thermal stability behaviour compared to that obtained with cytidine deaminase of *P. citrinum* and *A. niger* at 50°C (Allam et al., 1991 and Ali, 1998 respectively) and *A. phoenicis* at 45°C (Abdel-Fatah, 2005). Uridine hydrolase of *A. terreus* showed an activity at a rather extreme pH value (8.0) compared to that reported with purine ribonu-

Table 5. Effect of addition of some metal salts on the activity of cytidine and uridine-degrading enzymes.

Metal salts	Concentration (M)	Relative activity (%)	
		Cytidine deaminase	Uridine hydrolase
---	---	100	100
$CuSO_4$	5×10^{-3}	0.0	0.0
	10^{-2}	0.0	0.0
$CaCl_2$	5×10^{-3}	100	178
	10^{-2}	100	122
KCl	5×10^{-3}	118	0.0
	10^{-2}	106	0.0
$HgCl_2$	5×10^{-3}	0.0	0.0
	10^{-2}	0.0	0.0
$CoSO_4$	5×10^{-3}	68	0.0
	10^{-2}	58	0.0
$MgSO_4$	5×10^{-3}	325	122
	10^{-2}	228	177
$ZnSO_4$	5×10^{-3}	100	0.0
	10^{-2}	100	0.0
$FeSO_4$	5×10^{-3}	100	0.0
	10^{-2}	100	0.0
$MnCl_2$	5×10^{-3}	100	55
	10^{-2}	100	0.0
NaCl	5×10^{-3}	113	200
	10^{-2}	124	150

Reaction mixture contained: cytidine or uridine, 5 µmoles; Tris-HCl buffer pH 8.0, 50 µmoles; dialyzed extract protein, 2.25 mg; total volume, 1.0 ml; reaction temperature, 55°C and reaction time, 60 min.

Table 6. Effect of freezing and thawing on the activity of cytidine and uridine- degrading enzymes.

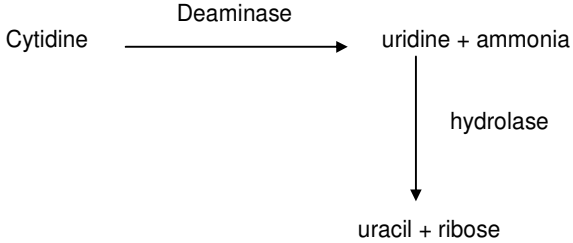
Time of storage at -5°C	Relative activity (%)	
(hr)	Cytidine deaminase	Uridine hydrolase
--	100	100
24	100	100
48	98	97
72	97	97

Reaction mixture contained: cytidine or uridine, 5 µmoles; Tris-HCl buffer pH 8.0, 50 µmoles; extracts protein, 3.96 mg; total volume, 1.0 ml; reaction temperature, 55°C and reaction time, 60 min.

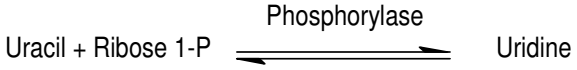
cleoside hydrolase of *P. citrinum* and *A. terricola* at pH 4.0 (Elzainy et al., 1990; Abu-Shady et al., 1994 respectively) and *A. phoenicis* at pH 3.5 (Abdel-Fatah et al., 2003). On the other hand the cleavage of N-glycosidic bond of guanosine, inosine, adenosine, cytidine anduridine by extracts of *Fusarium moniliforme* occurred at pH 6.0 (Allam et al., 1987). Results obtained in this work indicate the absence of pyrimidine ribonucleosides phosphorylase while as phosphorolytic cleavage of the pyrimidine ribonucleosides by extracts of *P. oxalicum* occurred at pH 6.2 (Hassan et al., 1983). In conclusion the cleavage of cytidine and uridine by extracts of *A. terreus* revealed the following facts:

1. Presence of a constitutive cytidine deaminase which is responsible for the deamination of cytidine to uridine and ammonia.
2. Presence of a constitutive uridine hydrolase which catalyzes the cleavage of uridine to uracil and ribose.

The way through which cytidine and uridine were degraded can be summarized as follows:



The cessation of further breakdown of uracil is not unexpected, as the denovosynthesis of this molecule requires a lot of energy. So it is much logical for the cell not to degrade this preformed molecule. Furthermore what has been actually found in some biological systems is that the cell invented very short pathway for its reutilization as:



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