Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (11): 851-855, 2007
ISSN 1816-4935

Degradation of Pyrimidines by *Pseudomonas syringae*

Randi L. Gant, Mallory L. Hacker, Gail M. Meyer and Manuel F. Santiago
Department of Chemistry, University of Tennessee at Chattanooga,
Chattanooga, Tennessee 37403

Abstract: The focus of this project was to determine the method of catabolizing pyrimidines in cells of *Pseudomonas syringae* ATCC 12771. Enzyme assays of dihydropyrimidine dehydrogenase, dihydropyrimidinase and N-carbamoyl β-alanine amidohydrolase were performed under standard cell conditions and specific activities were determined for each enzyme with each auxotroph. Enzyme activities were determined for different nitrogen and carbon sources to observe the effects on their activity. It was demonstrated that glucose-ammonium sulfate and glucose-thymine grown cells showed repression of dihydropyrimidine dehydrogenase activity. It was observed that cells grown in both thymine and nitrogen-free medium showed repression of dihydropyrimidinase activity. It was also shown that succinate-uracil grown cells showed repression of the activity of the enzyme N-carbamoyl-β-alanine amidohydrolase. The primary reducing cofactor of dihydropyrimidine dehydrogenase was determined by comparative enzyme assays of varying substrates and cofactors. It was shown that the enzyme substrate uracil yielded the highest specific activity with each cofactor and the cofactor NADPH yielded the highest specific activity with each substrate. To verify the existence of NADPH in the cell, a pyridine nucleotide transhydrogenase enzyme assay was performed. The enzyme utilized NADPH in the cell extract to produce its product and it was then determined that the organism has NADPH available for use in its cell.

Key words: Pyrimidine, *Pseudomonas syringae*, dihydropyrimidine dehydrogenase, dihydropyrimidinase, N-carbamoyl β-alanine amidohydrolase, reductive pathway

INTRODUCTION

Pseudomonas syringae is an agriculturally important plant pathogen that is responsible for crop spoilage. The organism could be controlled with the use of pesticides that may be detrimental to the local ecological food chains and water cycles. It would be beneficial, then, to control the organism through bioregulation with an understanding of its metabolism. The method of catabolism of pyrimidines is one small component of wide-range metabolic activity that has not been identified in this organism. There are two known methods of catabolizing pyrimidines: the oxidative pathway and the reductive pathway (Vogels and van der Drift, 1976). Previously, it has been shown that other members of Pseudomonads such as, *P. lemmonieri*, *P. stutzeri* and *P. florescens* utilize the reductive pathway for catabolism of pyrimidines (Burnette *et al*., 2006; Santiago and West, 1999; Xu and West, 1992). The reductive pathway produces β-alanine from uracil in a three step process using three enzymes: dihydropyrimidine dehydrogenase (EC 1.3.1.2), dihydropyrimidinase (EC 3.5.2.2) and N-carbamoyl β-alanine amidohydrolase (EC 3.5.1.6). Upon the verification that *P. syringae* also utilizes this pathway; observation of this metabolic pathway's regulation with various nitrogen sources will provide assistance toward further research in bioregulation of the organism.

Corresponding Author: Dr. Manuel F. Santiago, Department of Chemistry, University of Tennessee at Chattanooga, Chattanooga, Tennessee 37403 Tel: 423-425-5364 Fax: 423-425-5234

This study investigated the effects on the regulation of the reductive pathway enzymes in cells of *P. syringae*. The results provide evidence that could be used to develop environmentally safe control agents to regulate the growth of this agricultural pathogen.

MATERIALS AND METHODS

This study was conducted at the University of Tennessee at Chattanooga from 2004 to 2006. The microorganism *Pseudomonas syringae* ATCC 12771 was used. It was grown in a modified Stanier minimal liquid medium that contained potassium monobasic (0.1% w/v), potassium dibasic (0.1% w/v), sodium chloride (0.1% w/v), sodium citrate (0.05% w/v), a carbon source of either glucose or succinate (0.4% w/v) and a nitrogen source (0.2% w/v) (Stanier, 1947). It was supplemented with iron chloride (0.02% w/v) and glutamic acid (10% w/v), which were found to be the optimal growth conditions (West, 2002). Three samples of organism in its nutrient solution were then shaken at 200 rotations per minute, incubated at 30°C and grown overnight. Two milliliters of these solutions were then re-inoculated into three fresh flasks containing 50 mL of liquid minimal medium to reach mid-exponential phase growth and the growth was measured spectrophotometrically at 600 nm.

At mid-exponential phase, the cells were washed once with sterile sodium chloride (0.85%), once with sterile deionized water and collected by centrifugation at 7,719 x g for 20 min at 4°C. The resulting cell pellet was resuspended in 4 mL of dialysis buffer. This buffer contained 20 mM Tris-HCl buffer pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM β-mercaptoethanol (West *et al*., 1985). The cell extract was sonicated at maximum power in thirty-second intervals for 5 min per sample to disrupt the cell membrane and liberate the proteins from the cell. The cell extracts were then centrifuged 10,900 x g at 4°C for 30 min to remove the cell biomass from the solution. The resulting cell-free extract was then placed in Spectra/Por Regenerated Cellulose Membrane in a 300 mL solution of 20 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA and 1 mM 2-mercaptoethanol at 4°C and allowed to dialyze overnight.

The three enzymes of the reductive pathway were assayed to determine the specific activities. An average (standard deviation) of three independent cultures was determined. Dihydropyrimidine dehydrogenase was assayed in a reaction mixture containing 0.1 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADH or NADPH and cell-free extract (French *et al*., 1997). The mixture was incubated for 5 min at 30°C and then 0.2 mL of either uracil or thymine (5 mM concentration) was placed in the reaction mixture as starting material. The control mixture contained deionized water (0.1 mL). The conversion of NADPH to $NADP^+$ or NADH to NAD^+ was followed spectrophotometrically for 9.5 min at a wavelength of 340 nm. The specific activity of dihydropyrimidine dehydrogenase was reported in nanomoles of dihydrouracil formed/minute/milligram of protein using a molar absorptivity coefficient of $6.22\times10^3\ M^{-1}\ cm^{-1}$.

The second enzyme, dihydropyrimidinase, was assayed for activity colorimetrically. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.5), 0.01 M $MgCl_2$ and cell-free extract (Chu and West, 1990). Dihydrouracil (0.1 mM) was added as an enzyme substrate to start the reaction. The reaction was stopped by the addition of an acidic color mix reagent (1 mL) consisting of one part oxime and two parts antipyrine (West *et al*., 1982). The oxime was prepared by dissolving (5% v/v) of diacetylmonoxide in acetic acid (Prescott and Jones, 1969). The antipyrine was prepared by dissolving five grams of solid antipyrine (50% reagent grade) in 85% H_3PO_4. After termination of the reaction, the tubes were incubated at 70°C for 120 min. The absorbance was read at 466 nm and plotted against a standard curve of known concentrations versus absorbance of N-carbamoyl-β-alanine. The specific activity was reported in nanomoles of N-carbamoyl-β-alanine produced $minute^{-1}\ mg^{-1}$ of protein.

The determination of N-Carbamoyl-β-alanine amidohydrolase activity was conducted in two parts. The first reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.5), 0.01 $MgCl_2$ and cell-free

extract (Tamaki and Mizutani, 1987). Dihydrouracil (0.1 mM) was added as an enzyme substrate to start the reaction. The reaction was stopped by the addition of 0.1 mL of 50% (w/v) trichloroacetic acid. The reaction mixtures were then centrifuged at low speeds. The second reaction utilized the enzyme glutamate dehydrogenase and was followed spectrophotometrically at 365 nm. The ammonium ion concentration in the supernatant was determined in a reaction mix of 2 M $KHCO_3$, 0.5 M Tris-HCl buffer (pH 8.0), 0.1 M 2-ketoglutarate and 12 mM NADH (Xu and West, 1994). The NADH disappearance due to ammonium conversion by glutamate dehydrogenase was then measured spectrophotometrically after 60 min at 365 nm.

The initial enzyme of the reductive pathway requires a cofactor to reduce uracil to dihydrouracil. For this reason, the specific activity of pyridine nucleotide transhydrogenase (EC 1.6.1.1), a proton translocating enzyme for the reductive cofactor of dihydropyrimidine dehydrogenase, was determined to verify the presence of NADPH in cell extracts of the organism. The 1 mL reaction mixture contained 0.1 mM NADPH, 0.05 M Tris-HCl buffer (pH 7.5), the enzyme and cell-free extract (San Pietro *et al*., 1955). One milliliter of 0.1 mM of thionicotinamide adenine dinucleotide, or $tNAD^+$, was added to initiate the enzyme activity for the assay. The conversion of $tNAD^+$ to tNADH was followed at 400 nm and a molar absorptivity coefficient of 11.2×10^3 M^{-1} cm^{-1} was used in determining the activity.

Protein assays were performed using the Bradford method using lysozyme as a standard protein (Bradford, 1976).

RESULTS AND DISCUSSION

Enzyme specific activity was detected for each of the three enzymes of the reductive pathway in cells of *P. syringae*. It can therefore be concluded that this organism does utilize the reductive pathway. Dihydropyrimidine dehydrogenase exhibits the highest specific activity in cells of *P. syringae* grown in medium contain glucose as the carbon source and uracil as the nitrogen source (Table 1). These results are similar to the investigations conducted with *Pseudomonas stutzeri* (Xu and West, 1992) and *Pseudomonas lemonnieri* (Burnette *et al*., 2006). This could be expected considering that these organisms are in the same ribonucleic acid (RNA) homology group (Palleroni, 1984). In addition, the nutrient source could be derepressing the expression of this enzyme. Dihydropyrimidine dehydrogenase exhibited the lowest specific activity when cells were grown in liquid medium containing glucose as the carbon source and thymine or ammonium as the nitrogen sources (Table 1). This is similar to *P. lemonnieri* where thymine and dihydrothymine demonstrated the lowest activity (Burnette *et al*., 2006). The low specific activity of this enzyme could be due to the repressive effect caused by thymine.

It was previously demonstrated that the dehydrogenase enzyme in cells of *P. chlororaphis* required a cofactor (West, 1991). The initial enzyme in and present study showed activity when either

Table 1: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source and a nitrogen source

Nitrogen source	Dihydropyrimidine dehydrogenase specific activity*	Dihydropyrimidinase specific activity *	N-carbamoylBeta-alanine amidohydrolase specific activity*
No nitrogen	0.42±0.21	0.009±0.0004	52.10±3.30
Uracil	3.54±0.38	66.100±1.75	9.01±0.65
Thymine	0.08±0.02	0.011±0.002	4.80±0.80
Dihydrouracil	1.42±0.30	140.500±11.9	71.30±1.18
Dihydrothymine	0.74±0.33	142.900±0.45	29.10±3.69
Beta-alanine	0.50±0.05	128.800±9.20	27.80±3.20
Ammonium sulfate	0.05±0.01	23.190±2.10	28.30±11.9

*The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nmoles min^{-1} mg^{-1} of protein at 30°C. Each value is the mean of the independent values obtained from three samples (Mean±SD)

NADH or NADPH served as the enzyme cofactor (Table 2). Since dehydrogenase activity was observed with the phosphorylated nicotinamide, pyridine nucleotide transhydrogenase a proton-transferring enzyme was assayed. The proton transferring enzyme (±SD) activity was observed (9.30±1.32 nmol min^{-1} mL^{-1} of protein) when glucose and uracil served as the carbon and nitrogen sources, respectively. It was demonstrated in West's (1991) study that NADH stimulated higher activity as a cofactor than was observed using NADPH. It also showed that the activity of the initial enzyme when NADPH served as the cofactor was due to the expression of the enzyme pyridine nucleotide transhydrogenase.

In the dihydropyrimidinase reaction, the highest specific activity is observed when the organism is grown on succinate and dihydrothymine as the carbon and nitrogen sources, respectively (Table 3). It is also interesting to note that the specific activities of the second enzyme were relatively higher compared to the first and third enzymes regardless of the carbon source (Table 1 and 3). This can also been seen in studies conducted on *P. aeruginosa*, *P. lemonnieri* and *P. fluorescens* (Kim and West, 1991; Burnette *et al*., 2006; Santiago and West, 1999). Dihydropyrimidinase is also referred to as hydantoinase and is capable of hydrolyzing hydantoins. This is relevant because this molecule can be used to synthesize D-amino acids and could be important commercially (Morin *et al*., 1990).

In the N-carbamoyl β-alanine amidohydrolase reaction, the highest enzyme specific activity was observed when glucose served as the carbon source and dihydrouracil served as the nitrogen source (Table 1). Compared to succinate, the specific activity of the enzyme was consistently higher when the carbon source was glucose (Table 1 and 3). Xu and West (1992) previously demonstrated in cells from *P. stutzeri* that the specific activity was highest when succinate served as the carbon source. This shows a difference between microorganisms assigned to the same RNA homology group and how the carbon sources regulate the enzyme specific activity.

It can be concluded that the enzymes of the reductive pyrimidine pathway in the cells of *P. syringae* were expressed. In addition, this investigation showed that the pathway can be regulated through the use of various carbon and nitrogen sources in the growth medium. As a pathogen of various agricultural products, the regulation of the growth of this organism is critical. This study demonstrates that this could be accomplished through the use of bio-medications.

Table 2: Dihydropyrimidine Dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source and either NADH or NADPH as a reducing cofactor

Cofactor	Enzyme substrate	Specific activity
NADH	Uracil	3.54±0.38
NADH	Thymine	0.70±0.12
NADPH	Uracil	0.48±0.05
NADPH	Thymine	0.49±0.10

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nmol min^{-1} mg^{-1} of protein at 30°C. Each value is the mean of the independent values obtained from three samples (Mean±SD)

Table 3: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source and a nitrogen source

Nitrogen source	Dihydropyrimidine dehydrogenase specific activity*	Dihydropyrimidinase specific activity *	N-carbamoylBeta-alanine amidohydrolase specific activity*
No nitrogen	0.15±0.09	0.001±0.0001	1.20±0.30
Uracil	0.33±0.002	166.500±17.7	1.04±0.23
Thymine	0.66±0.26	0.030±0.008	4.10±0.02
Dihydrouracil	0.31±0.02	95.300±2.30	7.45±1.55
Dihydrothymine	0.66±0.02	327.600±29.2	11.10±3.79
Beta-alanine	0.24±0.01	195.800±39.9	9.08±0.40
Ammonium sulfate	0.32±0.05	165.100±20.1	7.71±0.41

*The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as $nmole^{-1}$ min^{-1} mg^{-1} of protein at 30°C. Each value is the mean of the independent values obtained from three samples (Mean±SD)

ACKNOWLEDGMENTS

Gratitude for the funding for this research is given to the University of Tennessee Provost Student Research Grant and Grote Fund. The research was performed during the Undergraduate Research Program at the University of Tennessee at Chattanooga Chemistry Department.

REFERENCES

Bradford, M.M., 1976. A rapid and sensitive method for the quantitzation of microgram quantities of protein utilizing the principle of dye-binding. Anal. Biochem., 72: 248-254.

Burnette, E.B., R. Gant, G.M. Meyer and M.F. Santiago, 2006. Regulation of pyrimidine catabolism in *Pseudomonas lemonnieri* ATCC 12983. J. Undergraduate Chem. Res., 5: 9-13.

Chu, C.P. and T.P. West, 1990. Pyrimidine biosynthetic pathway of *Pseudomonas fluorescens.* J. Gen. Microbiol., 136: 875-880.

French, C.E., B. Boonstra, K.A.J. Bufton and N.C. Bruce, 1997. Cloning, sequence and properties of the soluble pyridine nucleotide transhydrogenase of *Pseudomonas fluorescens.* J. Bacteriol., 79: 2765-2765.

Kim, S. and T.P. West, 1991. Pyrimidine catabolism in *Pseudomonas aeruginosa.* FEMS Microbiol. Lett., 77: 175-180.

Morin, A., D. Leblanc, A. Paleczek, W. Hummel and M.R. Kula, 1990. Comparison of seven microbial D-hydantoinases. J. Biotechnol., 16: 37-48.

Palleroni, U.J., 1984. Bergey's Manual of Systemic Bacteriology. Garrity, G.M. and D.R. Boone, (Eds.), 1984, Springer, 1: 162.

Prescott, L.M. and M.E. Jones, 1969. Modified methods for the determination of carbamyl aspartate. Anal. Biochem., 32: 408-419.

San Pietro, A., N.O. Kaplan and S.P. Colowick, 1955. Pyridine nucleotide transhydrogenase. VI. Mechanism and stereospecificity of the reaction in *Pseudomonas fluorescens.* J. Biol. Chem., 212: 941-952.

Santiago, M.F. and T.P. West, 1999. Effect of nitrogen source on pyrimidine catabolism by *Pseudomonas fluorescens.* Microbiol. Res., 154: 221-224.

Stanier, R.Y., 1947. Simultaneous adaptation: A new technique for the study of metabolic pathways. J. Bacteriol., 54: 339-348.

Tamaki, N. and N. Mizutani, 1987. Purification and properties of β-ureidopropionase from the rat liver. Eur. J. Biochem., 169: 21-26.

Vogels, G.D. and C. van der Drift, 1976. Degradation of purines and pyrimidines by microorganisms. Bacteriol. Rev., 40: 403-468.

West, T.P., M.S. Shanley and G.A. O'Donovan, 1982. Improved colorimetric procedure for quantitating N-carbamoyl-β-alanine with minimum dihydrouracil interference. Anal. Biochem., 122: 345-347.

West, T.P., T.W. Traut, M.S. Shanley and G.A. O'Donovan, 1985. A *Salmonella typhimurium* strain defective in uracil catabolism and β-alanine synthesis. J. Gen. Microbiol., 131: 1083-1090.

West, T.P., 1991. Isolation and characterization of dihydropyrimidine dehydrogenase mutant of *Pseudomonas chlororaphis.* Arch. Microbiol., 156: 513-516.

West, T.P., 2002. Control of pyrimidine synthesis in *Pseudomonas fragi.* Lett. Applied Microbiol., 35: 380-384.

Xu, G. and T.P. West, 1992. Reductive catabolism of pyrimidine bases by *Pseudomonas stutzeri.* J. Gen. Microbiol., 138: 2459-2463.

Xu, G. and T.P. West, 1994. Characterization of dihydropyrimidinase from *Pseudomonas stutzeri.* Arch Microbiol., 161: 70-74.