PURIFICATION AND PROPERTIES OF UREIDOGLYCOLATASE FROM *CANDIDA UTILIS*

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ABSTRACT. — The specific activity of ureidoglycolatase in extracts of *Candida utilis* was increased about 5.4-fold by induction with uric acid and to a lesser extent with allantoin or allantoate as inducers. The enzyme was purified about 100-fold by ammonium sulfate fractionation and DEAE-cellulose chromatography. Maximum stability of the purified enzyme was near pH 7.7, the enzyme was inactivated when held at pH 7.0 and 60°C for 20 minutes and Q_{10} was 2.1. K_m was 0.5 m*M* when measured by the spectrophotometric assay method, glyoxylate was a competitive inhibitor, and allantoin and allantoate inhibited ureidoglycolatase. Mn^{++} (30-80μM) stimulated the activity of the yeast enzyme.

Valentine *et al.* (1962) demonstrated the presence of ureidoglycolatase in extracts of *Streptococcus allantoicus* and a *Pseudomonas* species when these bacteria were grown on allantoin. The enzyme from *S. allantoicus* was purified by Gaudy *et al.* (1965) and characterized by Gaudy and Wolfe (1965). Vogels (1963) studied ureidoglycolatase in *S. allantoicus* and in *Arthrobacter allantoicus* and Trijbels and Vogels (1966a,b) reported on the presence and some of the properties of this enzyme in *Pseudomonas* and *Penicillium* species; the microorganisms used for enzyme preparation were grown on allantoin in these reports. Domnas (1962) reported the presence of ureidoglycolatase in the yeasts *Candida utilis* and *Saccharomyces cerevisiae* grown on various nitrogen sources. Lee and Roush (1964) noted the presence of ureidoglycolatase in conjunction with allantoicase in these yeasts.

Choi *et al.*, (1966, 1968) studied the purification and properties of allantoicase in *C. utilis* and reported methods for the assay of yeast ureidoglycolatase. This paper is a report on the purification and properties of the ureidoglycolatase of *C. utilis* and is a continuation of our study of purine catabolism in yeasts (Lee and Roush, 1964; Choi *et al.*, 1966, 1968; Roush *et al.*, 1959). A preliminary report of this work has been presented (Choi and Roush, 1965).

MATERIALS AND METHODS

C. utilis (ATCC 9950) was grown as reported previously (Roush *et al.*, 1959). Sodium ureidoglycolate was prepared by the method of Valentine and Wolfe (1961). Tris buffers were adjusted to the desired pH with acetic acid; buffer molarities refer to the concentration of Tris. Other chemicals were from sources given in previous papers (Lee and Roush, 1964; Choi *et al.*, 1966, 1968).

The methods of enzyme induction and measurement of uric acid, al-

lantoin and allantoate uptake were reported previously (Choi *et al.*, 1968; Roush *et al.*, 1959). Enzyme extracts were obtained from *C. utilis* by shaking 10 g of induced yeast suspended in 30 ml of 0.1 *M* Tris acetate (pH 7.0) with glass beads (Lee and Roush, 1964) for 3.5 hours.

Ureidoglycolatase activities were measured either by the spectrophotometric method of Choi *et al.*, (1966), which depends upon the formation of glyoxylate phenylhydrazone, or by the microdiffusion assay of these authors, which depends upon the determination of urea with the aid of urease. Specific activities are given in units of μmoles/min/mg of protein.

Results

Induction and preparation of ureidoglycolatase

The results in Table I show that the specific activity of ureidoglycolatase in the yeast extracts was increased about 1.6-fold with allantoin and allantoate as inducers and about 5.4 fold with uric acid as the inducer, consequently, uric acid was used to induce ureidoglycolatase for the purpose of purification and characterization.

All steps of the purification process were carried out at 4°C and ammonium sulfate solutions were equilibrated for 30 minutes before centrifugation to remove precipitates. The crude enzyme extracts were concentrated by fractionation between 0.5 and 0.65 saturation with ammonium sulfate, the precipitate was dissolved in 0.1 *M* Tris acetate (pH 7.0) and the resulting solution was dialyzed against several changes of a large volume of 5 m*M* Tris acetate (pH 7.0) until the diffusate gave a negative Nessler's test for ammonium ion. This fractionation usually resulted in a several-fold increase in specific activity with a recovery of about 50% of the enzyme units.

Table I. Induction of Ureidoglycolatase in *Candida utilis*. Each inducer was used in a concentration of 0.1 mg/ml. Ureidoglycolatase activities were measured at pH 7.0 by the spectrophotometric method.

Inducer	Induction Time	Protein in the Extract	Specific Activity
	(hours)	*(mg/ml)*	*(100·μmoles/min/mg of protein)*
None	0	6.6	0.35
Uric acid	5	7.4	1.2
Uric acid	7	8.0	1.7
Uric acid	10	8.0	1.9
Allantoin	1	9.9	0.23
Allantoin	3	5.6	0.57
Allantoin	5	6.6	0.48
Allantoate	1	5.9	0.45
Allantoate	3	6.9	0.54
Allantoate	5	7.6	0.54

Figure 1 shows the results of chromatography of ureidoglycolatase on DEAE-cellulose. In this experiment, the most active sample has a specific activity 96-fold greater than that of the enzyme placed on the column and the total recovery of enzyme units was 150%. The fractions eluted from DEAE-cellulose with the higher specific activities were used in studying the properties of the enzyme.

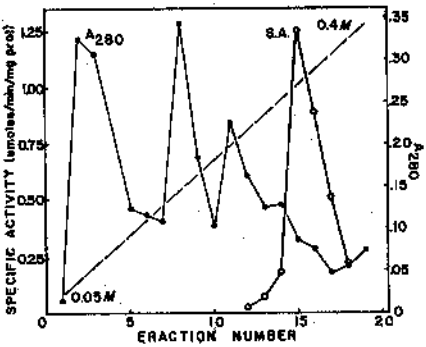

FIGURE 1. Chromatography of ureidoglycolatase on DEAE-cellulose. The DEAE-cellulose column (1.6 x 25 cm) was equilibrated with 0.05 *M* Tris acetate (pH 7.0) and ureidoglycolatase (34 mg of protein in 3.5 ml, specific activity of 0.035 unit) was added to the column. The column was eluded with a linear gradient of Tris acetate (pH 7.0) between 0.05 *M* and 0.4 *M* at the rate of 30 ml/hour and 7 ml fractions were collected.

Properties of ureidoglycolatase

Figure 2 shows that maximum stability of the purified enzyme was near pH 7.7 and Figure 3 shows that ureidoglycolatase was completely inactivated upon being held at 60°C for 20 minutes. Figure 4 is an Arrhenius plot of the effect of temperature on the reaction rate. An experimental activation energy of 12,000 cal/mole was calculated from the graph, Q_{10} between 11° and 21° was 2.1, and inactivation of the enzyme became evident at 40°C. A K_m value of 0.5 m*M* was obtained from Figure 5; however, K_m values estimated by studying the effect of substrate concentration with the microdiffusion assay were near 10 m*M* (see Figure 6, for example).

Uric acid (2 m*M*), urea (6.7 m*M*), L-arginine (3.3 m*M*) and ammonium sulfate (0.1 *M*) did not inhibit ureidoglycolatase in the indicated concentrations when the spectrophotometric assay system was used. Potassium allantoate (3.3 m*M*) inhibited 23% and allantoin (6.7 m*M*) inhibited 23% with this assay sys-

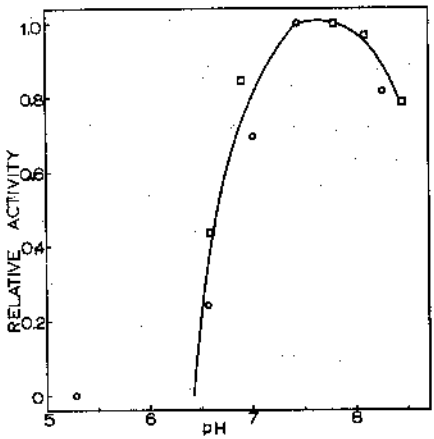

FIGURE 2. pH-stability curve for the ureidoglycolatase of *C. utilis*. Purified enzyme, eluted from a DEAE-cellulose column, was dialyzed for 4.5 hours against 2 changes of 5 m*M* Tris acetate (pH 7.0) and 0.5 ml portions of the dialyzed enzyme were mixed with 0.5 ml portions of Tris acetate buffers. These were placed in a 40°C water bath for 20 minutes, cooled in an ice bath, and 0.1 ml of each was assayed by the spectrophotometric method. The pH of each heated enzyme was measured with the glass electrode. The results of two experiments are combined in the figure.

tem. The results of glyoxylate inhibition experiments obtained by the microdiffusion assay system and plotted by the method of Dixon (1953) in Figure 6 show that glyoxylate was a competitive inhibitor with K_i = 4 m*M*.

We have found that manganous and zinc ions in the concentration range from 30 to 80 μM stimulate the activity of the purified ureidoglycolatase. The rate was occasionally doubled with manganous ion and smaller effects were observed with zinc ion. We have been unable to demonstrate an absolute requirement for a divalent cation; attempts to obtain an inactive enzyme which could be reactivated with manganous or zinc ions were unsuccessful.

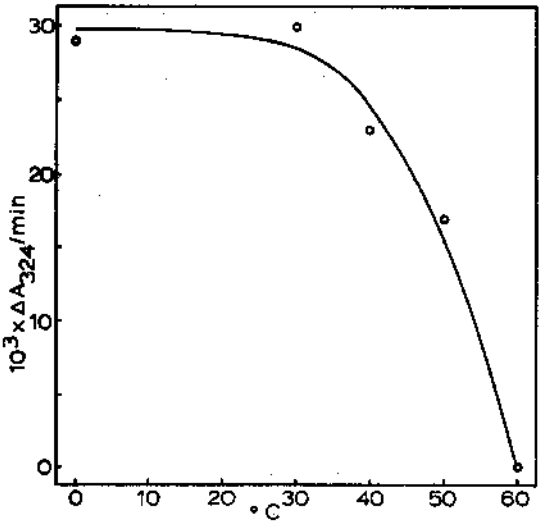

FIGURE 3. Thermal inactivation of ureidoglycolatase. Purified enzyme was held at the indicated temperature for 20 minutes, placed in an ice bath and assayed for residual ureidoglycolatase activity by the spectrophotometric method; 13 μg of protein was present in the assay solution.

DISCUSSION

The results of various workers indicate that ureidoglycolatase is required for the metabolism of allantoin or allantoate as carbon or nitrogen sources and that the enzyme is inducible in microorganisms. Gaudy and Wolfe (1965) reported that ureidoglycolatase was present in allantoin-grown but not in glucose-grown cells. Domnas (1962) found ureidoglycolatase in *C. utilis* grown with ammonium sulfate as the nitrogen source and the specific activity of ureidoglycolatase in extracts was increased 21-fold by growth on allantoin; growth conditions were not

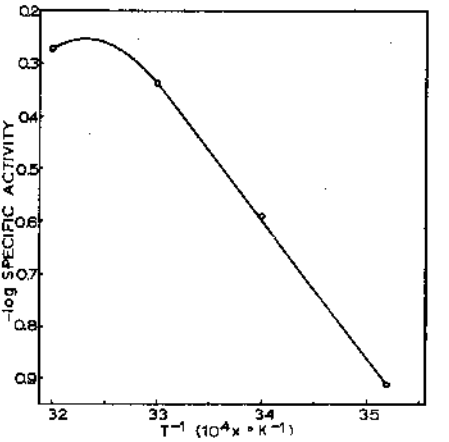

FIGURE 4. Variation of ureidoglycolatase activity with temperature. Purified enzyme was assayed by the spectrophotometric method over the indicated temperature range. Water from a constant temperature bath was circulated through the thermospacers of the spectrophotometer and each reaction solution was equilibrated to constant temperature before starting the reaction. The temperature was measured at the end of the reaction by placing a thermometer previously equilibrated at the temperature of the bath in the cuvette. The logarithm of the measured specific activity is plotted against the reciprocal of the absolute temperature.

defined for these experiments. Our results are in qualitative agreement but we find much lower specific activities and only a slight increase in specific activity upon incubation in allantoin as the sole nitrogen source under our conditions. Uric acid was the best inducer, possibly because it accumulates in the vacuole in crystalline form (Roush, 1961) and provides a constant source of purine catabolites for induction over a prolonged period of time. The results of Choi *et al.* (1968) taken in conjunction with the present results support the conclusion of Lee and Roush (1964) that allantoicase was induced in *C. utilis* by purine metabolites and was not present in detectable amounts in *C. utilis* grown on ammonium sulfate; ureidoglycolatase is present in non-induced cells and did not prevent the detection of allantoicase in the assay procedure of Lee and Roush (1964).

The success in purification of ureidoglycolatase by chromatography on DEAE-cellulose depended upon the strong adsorption of the enzyme by DEAE-cellulose in 0.05 *M* Tris buffer; much of the protein and nucleic acid are not adsorbed strongly under these conditions and appear in the eluate at lower ionic strengths than provided by the 0.3 *M* Tris buffer required for elution of the ureidoglycolatase. The high recovery of activity may be due to the removal of inhibitors during chromatography. Possible inhibitors include divalent heavy metal ions (Gaudy and Wolfe, 1965) or proteins and nucleic acids. The latter classes of compounds have structural similarities to the substrate.

Our K_m value of 0.5 m*M*, obtained by the spectrophotometric assay, is much smaller than the value of 92 m*M* reported by Trijbels and Vogels (1966a) for the enzyme from *Pseudomonas acidovorans* or the value of 33 m*M* reported by Gaudy and

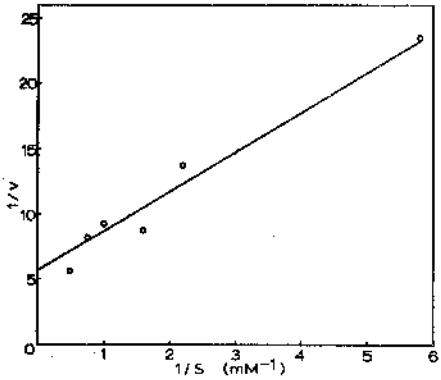

FIGURE 5. The effect of substrate concentration on ureidoglycolatase activity. Purified enzyme (specific activity = 0.52 unit) was assayed by the spectrophotometric method. The units of v are μmoles/min/3 ml and (0.1) $(1/v)$ is the ordinate in the reciprocal plot. The line was drawn by the method of least squares.

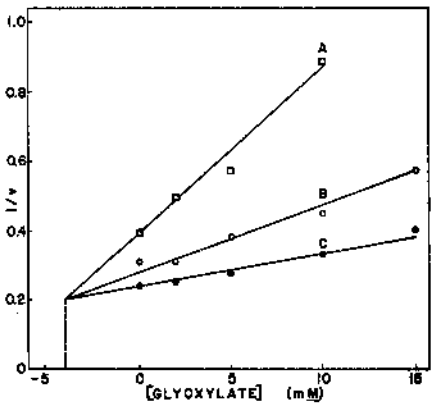

FIGURE 6. The inhibition of ureidoglycolatase by sodium glyoxylate. The activities were measured at pH 7.0 and 30°C for 30 minutes by the microdiffusion assay in a volume of 2 ml containing 69 μg of purified ureidoglycolatase and 0.8 mg of urease. Ureidoglycolate concentrations:
A, 5.25 m*M*; B, 9.50 m*M*; C, 15.90 m*M*.

Wolfe (1965) for the enzyme from *S. allantoicus*. The K_m value of 10 m*M* which we estimated from microdiffusion assay data is more nearly of the order of magnitude of the values given by the other workers. The difference between the K_m values obtained by the two assay systems might be due to phenylhydrazine acting as a direct acceptor of the glyoxylate from an enzyme-substrate complex in the spectrophotometric assay system.

Glyoxylate was a competitive inhibitor of yeast ureidoglycolatase which indicates that the glyoxylate moiety of ureidoglycolate may be involved in the formation of the enzyme-substrate complex. Urea was not inhibitory, in agreement with the results of Gaudy and Wolfe (1965), but these authors found that 1 m*M* glyoxylate did not inhibit their enzyme, in contrast to our results with the yeast enzyme.

Gaudy *et al.* (1965) recovered 174% of their enzyme activity in a purification step which employed 20 m*M* Mn^{++} and Gaudy and Wolfe (1965) found that 1 m*M* Mn^{++} stimulated the activity of their enzyme to 131% of the control value. Vogels (1961) and Trijbels and Vogels (1966a) have reported stimulations of ureidoglycolatase activity by 0.16 m*M* and by 0.77 m*M* Mn^{++} respectively. We have found maximum stimulation of the yeast ureidoglycolatase by 60 μM Mn^{++} and no effect or inhibition at higher concentrations. More recent results obtained in this laboratory (N. Spalek and A.H. Roush, unpublished observations) show that Mn^{++} in concentrations of 1 m*M* or higher catalyzes the cleavage of ureidoglycolate in the spectrophotometric assay system and therefore we conclude that caution must be exercised in attributing stimulation of ureidoglycolatase activity by higher concentrations of Mn^{++} to an effect on the enzyme. Some of the observed results may be due to direct Mn^{++} catalysis of ureidoglycolate cleavage.

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