Transaminase and D-Amino Acid Oxidase of *Trigonopsis variabilis*

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SUMMARY

The yeast *Trigonopsis variabilis* can utilize many single amino acids as sole source of nitrogen. Of the amino acids utilized, the D-isomers are oxidatively deaminated, whilst the L-isomers are transaminated with either α -ketoglutarate or pyruvate. The D-amino acid oxidase activity of this organism, when grown on DL-methionine as sole source of nitrogen, is the highest yet recorded, and may be recommended for the preparation of keto acids.

INTRODUCTION

It was shown in a preceding paper (SentheShanmuganathan & Nickerson, 1962*a*) that *Trigonopsis variabilis* can utilize a variety of single amino acids as sole source of nitrogen. The growth obtained with D- and L- methionine indicated that both the rate of growth and the total yield of organism with the D-isomer were almost twice that obtained with the L-amino acid. To elucidate the mechanism whereby amino acids are metabolized by this yeast, cell-free extracts were prepared from organisms grown with methionine or $(\text{NH}_4)_2\text{SO}_4$ as sole source of nitrogen. The data presented in this paper reveal the presence in this organism of extremely powerful D-amino acid oxidase and transaminase activities.

METHODS

Microbiological methods. *Trigonopsis variabilis* Schachner (CBS 1040) was used throughout this work. The culture medium and conditions of growth were as previously described (SentheShanmuganathan & Nickerson, 1962*a*).

Cell-free extracts. Cell-free preparations of the yeast were obtained from organisms grown in the medium containing salts, trace elements, glucose, biotin and thiamine. The nitrogen sources were DL-methionine or $(\text{NH}_4)_2\text{SO}_4$ at 25 mg. N/100 ml. The organisms were harvested by centrifugation, washed twice with distilled water, and suspended in 0.1M-phosphate buffer (pH 8.0) at a concentration equivalent to 1.5 g. dry wt. organism/20 ml. buffer. This suspension was subjected to sonic oscillation for 1 hr. in a Raytheon 50 W., 9 kc. magnetostriction oscillator at a plate voltage of 130 V. and an output voltage of 150 V. The suspension was then centrifuged for 15 min. at 19,600 g in the high speed angle-head of an International

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refrigerated centrifuge. The clear supernatant fluid was dialysed against 0.01M-KCl at 2° for 16–18 hr. with continuous stirring. The non-dialysable portion contained no debris and was used as the source of enzymes. The extracts from organisms grown with methionine or $(\text{NH}_4)_2\text{SO}_4$ as sole source of nitrogen will be referred to as extracts M and A, respectively. These were stored at -20° until required.

Reagents. The amino acids used were described in Sentheshanmuganathan & Nickerson (1962*a*); 2-ketoglutarate and pyruvate were obtained from the California Corporation for Biochemical Research (Los Angeles 63, California, U.S.A.) and Schwarz BioResearch, Inc. (Mount Vernon, N.Y.), respectively. Solutions of the amino acids were made in 0.05M-phosphate buffer (pH 8.0) whilst those of the keto acids were prepared in 1% (w/v) Na_2CO_3 solution, adjusted to pH 8.0. Pyridoxal phosphate was purchased from the California Corporation for Biochemical Research.

Estimation of protein. The protein content of the preparations was determined by a turbidimetric method of Sentheshanmuganathan (1960).

Assay of transaminase activity. The formation of glutamate or alanine from the amino acid and 2-ketoglutarate or pyruvate was taken as an index of transaminase activity. All incubations were made in duplicate in Thunberg tubes at 37° , gas phase N_2 (unless stated otherwise). Except where indicated, the complete system contained in a total volume of 3.0 ml.: pyridoxal phosphate (0.04 μmole), enzyme solution (0.1 ml.), and 2-keto acid (20 μmole) in 0.067M-phosphate buffer (pH 8.0). The mixture was incubated for 10 min. before the addition of the amino acid (10 μmole L- or 20 μmole DL-amino acid). Two control systems were used, one without the amino acid and the other without the keto acid. At the end of the incubation period, 6.0 ml. ethanol were added and, after heating in a boiling water bath for 15 min., the denatured protein was removed by centrifugation. Equal volumes of the supernatant fluid were placed on paper chromatograms. The procedure of Woiwod (1949) was adopted for the separation of amino acids. After drying the papers, the positions of the amino acids were located by spraying with 0.1% (w/v) ninhydrin in acetone (Dent, 1947).

Quantitative estimation of transaminase activity. The transamination between aspartic acid and 2-ketoglutarate was studied by measuring the change in absorption at 280 $m\mu$ in the Beckmann spectrophotometer (Cohen, 1955).

Assay of D-amino acid oxidase activity. The extent of amino acid oxidase activity was estimated by measurement of the amount of O_2 absorbed by the substrate (Krebs, 1951). The uptake of O_2 was followed manometrically in Warburg manometers by conventional techniques. Unless stated otherwise, all manometric experiments were carried out at 30° , in 0.05M-phosphate buffer (pH 8.0) with air as gas phase. The centre well contained 0.2 ml. of 10% (w/v) NaOH to trap any CO_2 produced. Reactions were initiated by the addition of the substrate from the side arm.

Growth measurements. Growth was measured in terms of dry weight, as described in Sentheshanmuganathan & Nickerson (1962*a*).

RESULTS

The presence of transaminase activity in extracts prepared from organisms grown in methionine or in $(\text{NH}_4)_2\text{SO}_4$ as sole source of nitrogen, was tested with a variety of single amino acids as amino-group donors, and 2-ketoglutarate or pyruvate as amino-group acceptors. These estimations were carried out qualitatively by paper chromatography. Except with aspartic acid, no quantitative estimations were conducted. The results (Table 1) indicate that of the fourteen amino acids tested all, with the exception of proline, transaminated with 2-ketoglutarate in the presence of extracts A and M. In nine cases transaminase activity (glutamate formation) was observed with the DL form of the amino acid. In the other five cases the L form was active and it seemed likely that the L form of the DL mixtures might be the active component. This was tested and shown to be true with methionine since L-methionine but not D-methionine gave glutamate. When 2-ketoglutarate was replaced by pyruvate, only six of the fourteen acids (namely, α -aminobutyric acid, serine, threonine, tryptophan, tyrosine, valine) transaminated in the presence of either extract.

Table 1. *Transaminase activity in cell-free extracts of Trigonopsis variabilis prepared from organisms grown in methionine (M) or $(\text{NH}_4)_2\text{SO}_4$ (A) as sole source of nitrogen*

The complete system contained in a total volume of 3.0 ml.: pyridoxal phosphate (0.04 μ mole), enzyme solution (0.1 ml.), and oxo acid (20 μ mole) in 0.067 M-phosphate buffer (pH 8.0). The mixture was pre-incubated for 10 min. before the amino acid was added (10 μ mole L or 20 μ mole DL). Gas phase N_2 ; temp. 37°; incubation period, 60 min. Extract M from organism grown with methionine, extract A from organism grown with $(\text{NH}_4)_2\text{SO}_4$.

Amino acid	Glutamate formation		Alanine formation	
	Ext. M	Ext. A	Ext. M	Ext. A
None	—	—	—	—
DL- α -Alanine	+	+	—	—
DL- α -Aminobutyric acid	+	+	+	+
DL-Aspartic acid	+	+	—	—
L-Cysteine	+	+	—	—
L-Histidine	+	+	—	—
DL-Isoleucine	+	+	—	—
L-Leucine	+	+	—	—
DL-Methionine	+	+	—	—
L-Proline	—	—	—	—
DL-Serine	+	+	+	+
DL-Threonine	+	+	+	+
L-Tryptophan	+	+	+	+
DL-Tyrosine	+	+	+	+
DL-Valine	+	+	+	+

Transamination between aspartic acid and 2-ketoglutarate

To a quartz optical cell of the Beckmann spectrophotometer were added 0.1 ml. cell-free extract, 1.0 ml. 0.05 M-phosphate buffer (pH 8.0), DL-aspartic acid (20 μ mole) and pyridoxal phosphate (0.04 μ mole). After mixing, 2-ketoglutarate (20 μ mole) was added and the change in absorption at 280 m μ was measured. Two controls were run simultaneously, one without the keto and the other without the amino acid. The results of such an experiment are shown in Fig. 1, where it is seen that both extracts A and M catalyse the transfer of amino group from aspartic acid

to 2-ketoglutarate. The rate of transamination with extract M was higher than that observed with extract A (0.060 μ mole/min. as compared to 0.045 μ mole/min.). When extract M was used, one of the control optical cells (which contained only aspartic acid) produced 0.01 μ mole oxaloacetic acid/min. Such behaviour was not observed with extract A, implying that the extract M possessed a very slight amino acid oxidase activity.

Amino acid oxidase activity

The amino acid oxidase activity in extracts A and M was studied by measuring the O₂ uptake by the substrate amino acid. All experiments were carried out with the DL-isomers. Of the nineteen amino acids tested (Table 2), O₂ uptake was observed with fifteen of them, but the rates of O₂ absorption differed appreciably. With extract M, homocysteine was found to be the best substrate (415 μ l. O₂/hr./mg. protein). When extract A was used, homocysteine was again found to be the best (136 μ l. O₂/hr./mg. protein). With most of the amino acids studied, the rate of O₂ uptake in the presence of extract M exceeded (by more than three times) the rate observed with extract A.

Table 2. D-Amino acid oxidase activity in cell-free extracts of *Trigonopsis variabilis* prepared from organisms grown in methionine (M) or (NH₄)₂SO₄ (A) as sole source of nitrogen

The complete system contained in a total volume of 3.0 ml.: cell-free extract (0.3 ml.); amino acid (80 μ mole); and 0.05 M-phosphate buffer (pH 8.0; 1.5 ml.); 10% (w/v) NaOH (0.2 ml.) in the centre well. The enzyme preparation was added from the side arm when equilibrium was attained. Temp. 30°, incubation period, 60 min.

Amino acid	μ l. O ₂ uptake/hr./mg. protein		
	Extract M	Extract A	<i>Penicillium chrysogenum</i> (Krebs, 1951)
None	0	0	—
DL-Homocysteine	415	136	—
DL-Methionine	800	107	188
DL-nor-Valine	205	38	—
DL- α -Aminobutyric acid	201	55	184
DL-Valine	200	37	112
DL-Phenylalanine	172	19	126
DL- α -Alanine	153	31	146
DL-Leucine	151	30	208
DL-Tryptophan	122	6	38
DL-nor-Leucine	120	30	128
DL-Isoleucine	105	22	64
DL-Homocystine	104	28	—
DL-Tyrosine	86	19	—
DL-Serine	48	6	0
DL-Lysine	35	3	26
DL-Threonine	14	4	14
DL-Aspartic acid	8	1	0
DL-Ornithine	7	0	—
DL- α -Amino isobutyric acid	5	0	—

To learn whether the D, L, or both isomers were deaminated, four amino acids were tested in their L form (leucine, histidine, valine, phenylalanine). There was no uptake of O₂ with any of these amino acids in the presence of extracts A or M, indi-

cating that the amino acid oxidase was specific for the D-isomer only. For comparison, the D-amino acid oxidase activity obtained with *Penicillium chrysogenum* Q 176 (Krebs, 1951, based on data of Emerson, Puziss & Knight, 1950) is also given in Table 2. The results obtained with *Trigonopsis variabilis* run almost parallel to those obtained with *Penicillium chrysogenum*. This is the first time the presence of D-amino acid oxidase activity has been demonstrated in a yeast. The organisms grown with methionine as sole source of nitrogen are the richest source of amino acid oxidase found to date. *T. variabilis* may therefore be recommended as a potential source of D-amino acid oxidase for the preparation of keto acids.

Fig. 1

Fig. 2

Fig. 1. Transamination between 2-ketoglutarate and aspartic acid by cell-free extracts of *Trigonopsis variabilis* grown with DL-methionine or $(\text{NH}_4)_2\text{SO}_4$ as sole sources of nitrogen. The quartz cell of the Beckman spectrophotometer contained in a total volume of 3.0 ml.: extract (0.1 ml.), phosphate buffer (0.05 M; pH 8.0; 1.0 ml.), DL-aspartic acid (20 μmole); pyridoxal phosphate (0.04 μmole); 2-ketoglutarate (20 μmole). O—O Complete system containing extracts of methionine grown cells (M); ×—× complete system (M), less 2-ketoglutarate; Δ—Δ complete system containing extracts of $(\text{NH}_4)_2\text{SO}_4$ grown cells (A); □—□ complete system (A), less 2-ketoglutarate.

Fig. 2. D-Amino acid oxidase activity of cell-free extracts of cells of *Trigonopsis variabilis* grown with DL-methionine or $(\text{NH}_4)_2\text{SO}_4$ as sole source of nitrogen. O_2 uptake measured in Warburg manometers. The complete system contained, in a total volume of 3.0 ml.: cell-free extract, 0.3 ml. = 1.5 mg. protein; 0.05 M-phosphate buffer (pH 8.0; 1.5 ml.); 10% (w/v) NaOH (0.2 ml.) in the centre well. Reaction was initiated by the addition of the amino acid (10 μmole) from the side arm; temp. 30°. ×—× Extract M, DL-methionine; ●—● extract M, DL-alanine; Δ—Δ extract A, DL-methionine; □—□ extract A, DL-alanine; □····□ extracts A or M.

To follow the rate of oxidation of the amino acids, O_2 consumption was estimated manometrically at 10-min. intervals. For both D- and L-amino acids the rate of O_2 uptake with extract M was more than three times that obtained in the presence of extract A (Fig. 2). In 30 min. the substrates were completely deaminated by extract M, but incubation for 60 min. with extract A was required to achieve complete deamination of methionine and about one-third of the alanine. From

these results, and those in Table 2, it may be concluded that incubation of *Trigonopsis variabilis* with an amino acid as nitrogen source, can result in stimulation of deaminase activity by more than threefold.

To establish that the end product of the reaction between an amino acid and oxygen in the presence of the extracts was the keto acid corresponding to the amino acid (reaction 1):

the product was isolated as the 2:4-dinitrophenyl hydrazone.

As a typical example, reaction (1) was examined in the case of methionine. When O_2 uptake ceased in the reaction flask, protein was precipitated with trichloroacetic acid (10 %, w/v, final concentration) and removed by centrifugation. The supernatant fluid was then extracted with ether and the ether extract dried with anhydrous Na_2SO_4 . On removal of the ether, a thick syrupy liquid remained. This was treated with 5 ml. N-HCl and shaken with 10 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in N-HCl . After standing at 2° for 12 hr., the precipitated mass was filtered, washed with N-HCl and distilled water until the washings were colourless. The crude yellow product was recrystallized three times from ethanol + water until a constant melting point was obtained. The recrystallized product melted at 150° . The keto acid, α -keto- γ -methiolbutyric acid, corresponding to methionine, was also prepared according to the method described by Meister (1952) by using acetone powders of hog kidney cortex as source of D-amino acid oxidase. The 2:4-dinitrophenylhydrazone was also prepared from this keto acid as described above. The melting point was found to be 150° (Meister, 1957, m.p. 150°). A mixed melting determination also gave a value of 150° .

Table 3. *Growth of Trigonopsis variabilis under anaerobic conditions with $(\text{NH}_4)_2\text{SO}_4$ or D-, L-, or DL-methionine as sole source of nitrogen*

After inoculation, the flasks were flushed with N_2 , stoppered with rubber stoppers carrying Bunsen valves, and incubated at 28° with continuous agitation for 72 hr.; medium at pH 6.0.

Nitrogen source	Growth after 72 hr. of incubation (mg. dry wt./l.)
None	5
$(\text{NH}_4)_2\text{SO}_4$	372
DL-Methionine	145
L-Methionine	135
D-Methionine	13

From these findings it is apparent that *Trigonopsis variabilis* metabolizes amino acids by transamination or by deamination. The D-isomer is deaminated oxidatively (reaction 2), whilst the L-amino acid is transaminated with either 2-ketoglutarate or pyruvate (reaction 3):

If only reactions 2 and 3 are available to the organism, then this yeast should be able to utilize only the L-isomer, and not the D-amino acid, in growth under anaerobic conditions. To test this supposition, $(\text{NH}_4)_2\text{SO}_4$ and D-, L-, or DL-methionine was used as sole nitrogen source in the medium previously described. After inoculation, the flasks were flushed with N_2 , and incubated at 28° . At the end of 72 hr. of incubation, growth was measured in terms of dry weight of organism. In Table 3 it may be seen that with L- or DL-methionine growth was very much greater than that obtained with the D-isomer.

DISCUSSION

D-Amino acid oxidase activity has been demonstrated in cell-free preparations obtained from a number of filamentous fungi (including *Neurospora crassa*, *N. sitophila*, *Aspergillus niger*, and several species of *Penicillium*; see Krebs, 1951) and from a few bacteria. The enzyme has been found in liver and kidney of all vertebrates in which it has been sought. In view of the very high activity of this enzyme in our preparations from *Trigonopsis variabilis*, it is somewhat remarkable that this is, apparently, the first report on the presence of D-amino acid oxidase in a yeast. Since the recognition of the existence of separate enzyme systems for the oxidation of L- and of D-amino acids (Krebs, 1935) it is also somewhat remarkable that 'no satisfactory answer can be given to the question of the physiological significance of D-amino acid oxidases' (Krebs, 1951). The statement, apparently, holds good today. However, as Krebs pointed out in his review, D-amino acid oxidase is a useful chemical tool for the determination of the presence of D-amino acids, and for the preparation of α -keto acids from several of the α -amino acids. In *T. variabilis* there is provided an organism, easily grown, that is a rich source of D-amino acid oxidase.

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