BBA 66517

THREONINE ALDOLASE FROM *CANDIDA HUMICOLA*

II. PURIFICATION, CRYSTALLIZATION AND PROPERTIES

HIDEHIKO KUMAGAI, TAKATOSHI NAGATE, HAJIME YOSHIDA AND HIDEAKI YAMADA

Research Institute for Food Science, Kyoto University, Uji 611, Kyoto (Japan)

(Received September 9th, 1971)

SUMMARY

A procedure is described for the preparation of crystalline threonine aldolase (L-threonine acetaldehyde-lyase, EC 4.1.2.5) from cells of *Candida humicola* grown in a synthetic medium containing L-threonine as the sole carbon source. The crystalline enzyme preparation appears homogeneous under ultracentrifugation and acrylamide gel electrophoresis. The molecular weight was determined as 277 000.

Pyridoxal phosphate was shown to be associated with the crystalline enzyme. The enzyme has two absorption maxima at 280 and 420 nm. Treatment with cysteine results in a decrease in absorption at 420 nm and a concomitant loss in enzyme activity. The treated, inactive enzyme preparation can be restored to full activity by the addition of pyridoxal phosphate. The amount of pyridoxal phosphate bound to the enzyme was determined by equilibrium dialysis as 6 moles per mole of enzyme.

INTRODUCTION

BRAUNSTEIN AND VILENKINA[1] reported the presence of an enzyme in various animal tissues, which forms glycine from serine, threonine and certain other α-amino β-hydroxy acids. The enzyme which catalyzes the cleavage of threonine into glycine and acetaldehyde was subsequently named threonine aldolase (L-threonine acetaldehyde-lyase, EC 4.1.2.5) by LIN AND GREENBERG[2]. It has been purified from rat liver[3] and sheep liver[4], and is characterized as a pyridoxal phosphate dependent enzyme.

Little is known about the threonine aldolase of microorganisms. DAINTY AND PEEL[5] demonstrated that the strict anaerobe *Clostridium pasteurianum* synthesizes glycine by way of threonine and that this pathway is catalyzed by threonine aldolase. Threonine aldolase from *Clostridium pasteurianum* has been partially purified and some of its properties have been investigated[6].

In a previous paper[7], we reported that threonine aldolase was formed in the cells of various bacteria and yeasts grown in media containing L-threonine as the sole

carbon source. It seems that the enzyme is adaptive in nature and is responsible for the growth of bacteria or yeasts on threonine as the carbon source. In this paper, we describe the purification and crystallization of threonine aldolase from *Candida humicola* grown in a threonine medium. Investigations of the physicochemical properties and cofactor requirements of the enzyme are also presented.

EXPERIMENTAL PROCEDURE

Materials. All chemicals used in this work were commercial products. L-Threonine was purchased from Tanabe Seiyaku Company, Ltd., Osaka. Pyridoxal phosphate was kindly provided by Dainippon Pharmaceutical Company, Ltd., Osaka. DEAE-Sephadex A-50 and Sephadex G-200 were purchased from Pharmacia. Hydroxylapatite was prepared according to the method of TISELIUS *et al.*[8].

Microorganism and medium. Candida humicola (Faculty of Agriculture, Kyoto University, AKU 4584) was used as the enzyme source. The basal medium for cultures consisted of 0.2% L-threonine, 0.1% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.1% $MgSO_4$ and 0.05% yeast extract in tap water. The pH of this medium was adjusted to 7.0.

Culture. C. humicola from a malt extract–agar slant was inoculated into a subculture (500 ml of the basal medium in a 2-l flask). Incubation was carried out at 30° for 24 h with reciprocal shaking. The subculture was, in turn, inoculated into a 30-l fermenter containing 20 l of basal medium. Incubation was carried out at 30° for 14 h with aeration (20 l per min). Cells were harvested with a continuous flow centrifuge, then washed with distilled water and suspended in 0.03 M potassium phosphate buffer, pH 6.4. Approx. 8 g of cells (wet weight) were obtained per l of medium.

Enzyme assay. Threonine aldolase activity was assayed by measuring the amount of acetaldehyde formed from L-threonine. The reaction mixture contained 50 μmoles of L-threonine, 0.2 μmole of pyridoxal phosphate, 400 μmoles of Tris–chloride buffer, pH 8.5, 400 μmoles of KCl and enzyme in a total volume of 4 ml. The reaction was carried out at 30° for 15 min and was stopped by the addition of 1 ml of 30% trichloroacetic acid. The amount of acetaldehyde formed was determined with the deproteinized filtrate according to the method of PAZ *et al.*[9]. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmole of acetaldehyde per min under the assay conditions described. Specific activity was expressed as units per mg of protein.

Protein determination. Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm. An E value of 3.98 for 10 mg/ml and for a 1-cm light path, which was used throughout, was obtained by absorbance and dry weight determinations.

Pyridoxal phosphate determination. Pyridoxal phosphate was determined with phenylhydrazine according to the method of WADA AND SNELL[10].

Spectrophotometric determinations. Spectrophotometric determinations were carried out with a Beckman model DB-G recording spectrophotometer.

Electrophoresis. Acrylamide gel electrophoresis was performed as described by DAVIS[11], except that Tris–glycine buffer, pH 8.3, containing $1 \cdot 10^{-2}$ M KCl, $1 \cdot 10^{-4}$ M mercaptoethanol and $1 \cdot 10^{-5}$ M pyridoxal phosphate was used. Stacking and running gels were polymerized in a Pyrex tube (5 mm × 65 mm). After the run, the gel was

stained with 1% naphthol blue-black, then destained electrophoretically and stored in 7% acetic acid.

Ultracentrifugal analysis. Sedimentation velocities were measured with a Spinco Model E ultracentrifuge operating at 59 780 rev./min. Diffusion measurements were made with the same apparatus operating at 12 590 rev./min with the boundary condition at the meniscus of the sector-shaped centrifuge cell. Molecular weight was calculated from sedimentation and diffusion coefficients, according to the equation of SVEDBERG AND PEDERSEN[12].

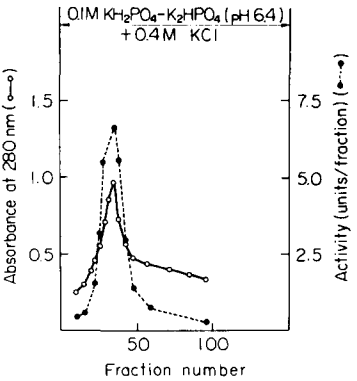
RESULTS

Purification procedure

All operations were carried out at 0–5°.

Step 1. Preparation of cell extract. The cell paste (300 g) was suspended in 0.03 M potassium phosphate buffer, pH 6.4, to give a suspension of about 1 g/5 ml. The suspension was divided into 300-ml portions and each portion was subjected to the action of a Kaijo denki ultrasonic oscillator at 20 kcycles/sec for 6 h. Cells and debris were removed by centrifugation at 20 000 × *g* for 20 min.

Step 2. DEAE-Sephadex column chromatography. The supernatant solution (1250 ml) was subjected to DEAE-Sephadex column chromatography. The adsorbent was packed in a column (6 cm × 70 cm) and equilibrated with 0.03 M potassium phosphate buffer, pH 6.4. The enzyme solution was passed through the column which was then washed with 4 l of 0.1 M potassium phosphate buffer, pH 6.4, containing 0.1 M KCl. The enzyme was subsequently eluted with 0.1 M potassium phosphate buffer, pH 6.4, containing 0.4 M KCl, $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA, at a flow rate of 1 ml per min in fractions of 20 ml. Elution of protein was followed



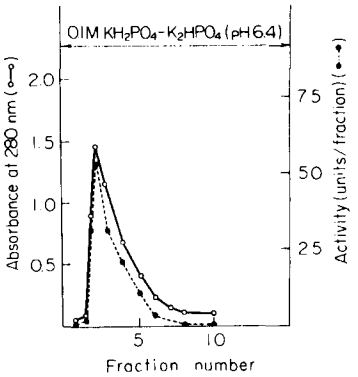


Fig. 1. Chromatography of crude threonine aldolase on DEAE-Sephadex A-50. Protein, 103.3 g, containing 593 units of activity, was applied to a column of DEAE-Sephadex A-50 (6 cm × 70 cm) equilibrated with 0.03 M potassium phosphate buffer, pH 6.4. Fractions of 20 ml were collected at a flow rate of 1 ml/min.

Fig. 2. Chromatography of partially purified threonine aldolase on hydroxylapatite. Protein, 1.17 g, containing 170 units of activity, was applied to a column of hydroxylapatite (3 cm × 5 cm) equilibrated with 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-5}$ M pyridoxal phosphate, $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA. Fractions of 12 ml were collected at a flow rate of 15 ml/h.

by measurement of absorbance at 280 nm as well as by determination of enzyme activity. Active fractions were combined to give 1.1 l which was concentrated by the addition of solid $(NH_4)_2SO_4$ to 0.50 saturation. The precipitate obtained by centrifugation (20 000 × *g*, for 20 min) was dissolved in 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate. It was then dialyzed for 15 h against 2 changes of 5 l of the same buffer. The elution pattern of the chromatographed enzyme is shown in Fig. 1.

Step 3. Hydroxylapatite column chromatography. The dialyzed enzyme solution (17.4 ml) was subjected to hydroxylapatite column chromatography. Hydroxylapatite was packed in a column (3 cm × 5 cm) and equilibrated with 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate. The enzyme solution was placed on the column, which was then washed with 350 ml of 0.1 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate. The buffer was allowed to flow at a rate of 15 ml per h and 12-ml fractions were collected. The elution pattern of the enzyme is shown in Fig. 2. Active fractions were combined to give 65 ml which was concentrated by the addition of solid $(NH_4)_2SO_4$ to 0.60 saturation. The precipitate was collected by centrifugation at 20 000 × *g* for 15 min, then was dissolved in a minimal amount of 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate.

Step 4. Sephadex G-200 gel filtration. The enzyme solution (3.8 ml) was subjected to Sephadex G-200 gel filtration. Sephadex G-200 was packed in a column (2 cm × 100 cm) and equilibrated with 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate. The enzyme solution was then introduced into the column and the buffer was allowed to flow at a rate of 20 ml per h. 10-ml fractions were collected. The elution pattern of the enzyme is shown in Fig. 3. Filtration yielded a single, symmetrical protein peak with which enzyme activity was entirely associated. Active fractions containing

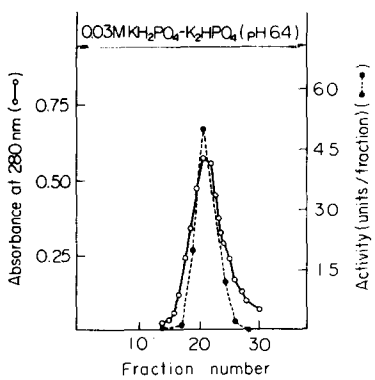


Fig. 3. Filtration of purified threonine aldolase through Sephadex G-200. Protein, 109 mg, containing 156 units of activity, was applied to a column of Sephadex G-200 (2 cm × 100 cm) equilibrated with 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-5}$ M pyridoxal phosphate, $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA. Fractions of 10 ml were collected at a flow rate of 20 ml/h.

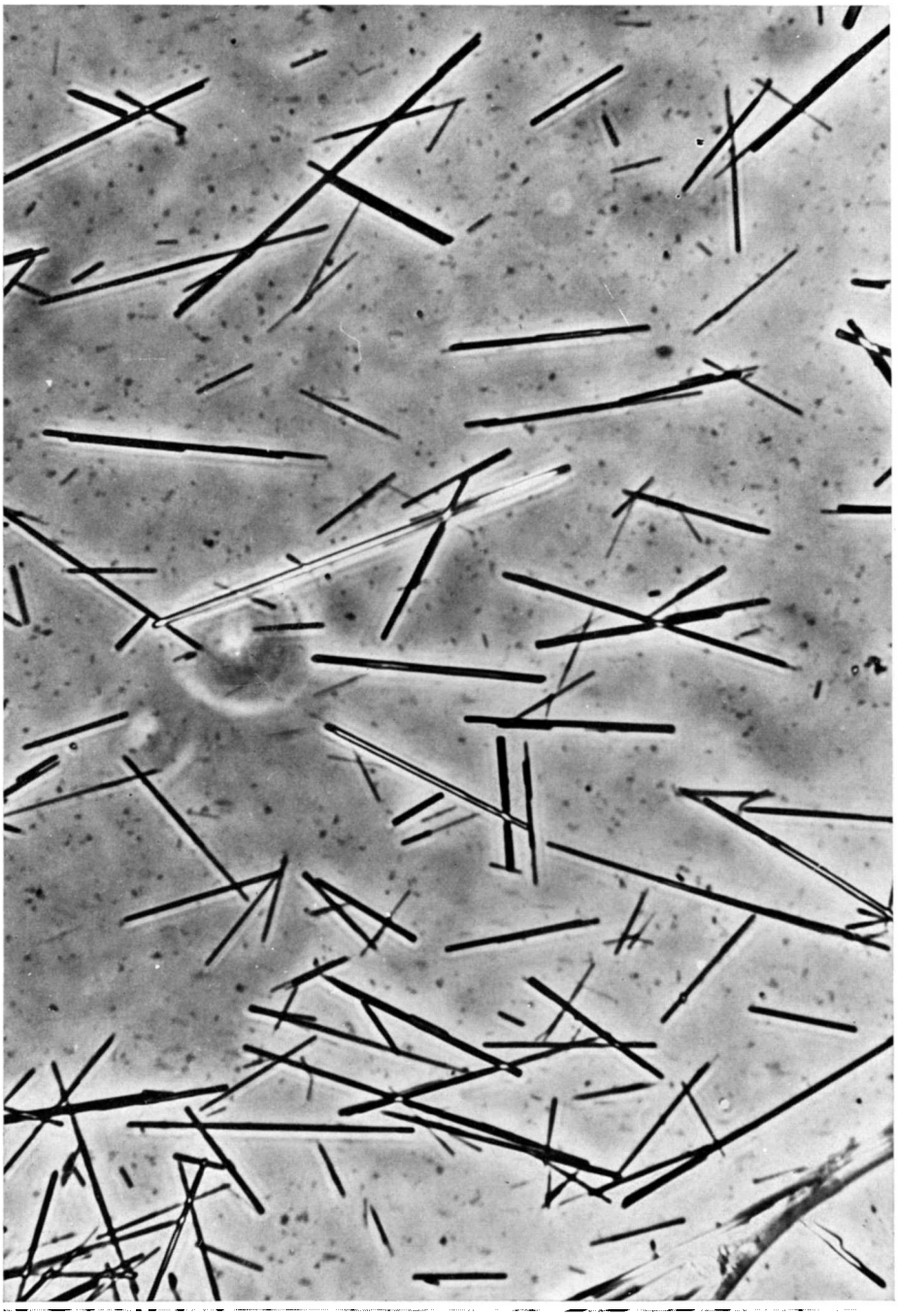


Fig. 4. Photomicrograph of crystalline threonine aldolase. Magnified 300-fold.

TABLE I

PURIFICATION OF THREONINE ALDOLASE FROM *Candida humicola*

Step	*Fraction*	*Total protein (mg)*	*Total units*	*Specific activity*
1	Cell extract	103 300	593	0.00573
2	DEAE-Sephadex	1 170	170	0.145
3	Hydroxylapatite	109	156	1.43
4	Sephadex G-200	58.8	150	2.56
5	Crystals (I)	47.5	130	2.75
	Crystals (II)	30.9	90	2.91

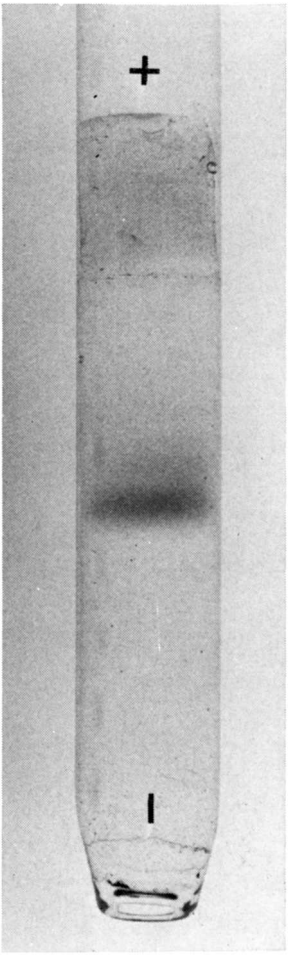


Fig. 5. Acrylamide gel electrophoresis of threonine aldolase. Recrystallized enzyme, 13 μg, was applied to the stacking gel and subjected to the electrophoresis at a current of 3.0 mA for 6 h in Tris–glycine buffer, pH 8.3, containing $1 \cdot 10^{-2}$ M KCl, $1 \cdot 10^{-5}$ M pyridoxal phosphate and $1 \cdot 10^{-4}$ M mercaptoethanol. The direction of migration is from the cathode to the anode.

enzyme of specific activities greater than 2.0 were combined to give 90 ml. This was concentrated by the addition of solid $(NH_4)_2SO_4$ to 0.60 saturation. The precipitate was collected by centrifugation at 20 000 × *g* for 20 min, then was dissolved in a minimal amount of 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate.

Step 5. Crystallization. Finely powdered $(NH_4)_2SO_4$ was cautiously added to the purified enzyme solution (3.0 ml) until faint turbidity appeared, then the mixture was placed in an ice bath. Crystallization began about 2 h later and was virtually complete within 3 days. A photomicrograph of the crystalline enzyme, which appears as fine needles yellow in color, is shown in Fig. 4. For recrystallization, the crystals were dissolved in a minimal volume of 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate. Solid $(NH_4)_2SO_4$ was added as described above. Approx. 510-fold purification was achieved with an over-all yield of 15%. The typical purification procedure is summarized in Table I.

Properties of the enzyme

Homogeneity. The specific activity of enzyme achieved after the second crystallization was not altered with further recrystallization. The recrystallized enzyme preparation gave a single band on acrylamide gel electrophoresis in Tris–glycine buffer, pH 8.3, containing $1 \cdot 10^{-2}$ M KCl, $1 \cdot 10^{-4}$ M mercaptoethanol and $1 \cdot 10^{-5}$ M pyridoxal phosphate (Fig. 5). Recrystallized enzyme sedimented as a single symmetrical peak in the ultracentrifuge in 0.03 M potassium phosphate buffer, pH 6.4,

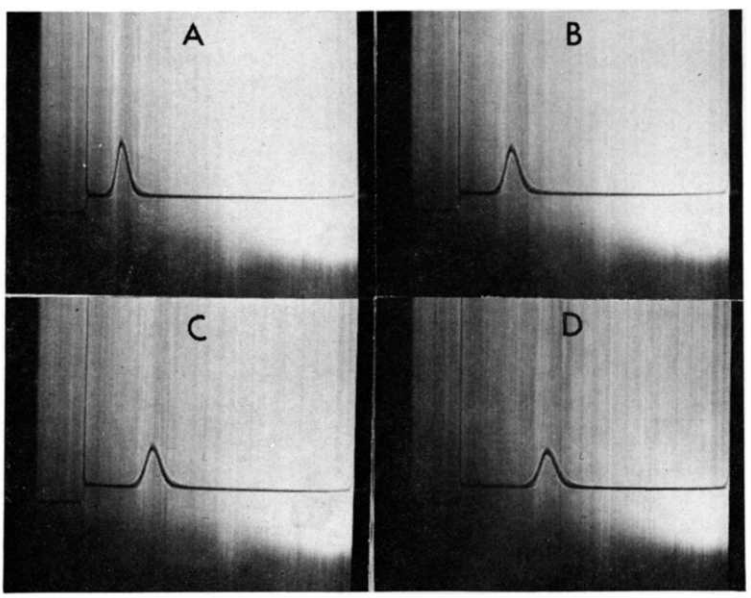


Fig. 6. Sedimentation patterns of threonine aldolase. Recrystallized enzyme was used at a concentration of 0.64% in 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-5}$ M pyridoxal phosphate, $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA. Photographs were taken 18 (A), 24 (B), 30 (C) and 36 (D) min after reaching 59 780 rev./min. The sedimentation is left to right.

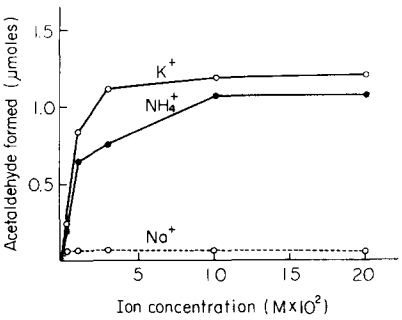


Fig. 7. Effect of cation concentration on activity of threonine aldolase. Recrystallized enzyme was freed from alkali metal ions by filtration through a Sephadex G-25 column (1 cm × 10 cm) equilibrated with 0.02 M Tris–chloride buffer, pH 6.8. The reactions were carried out at 30° for 15 min in reaction mixtures containing 34.3 μg of the enzyme, 50 μmoles of L-threonine, 0.2 μmole of pyridoxal phosphate, 400 μmoles of Tris–chloride buffer, pH 8.5, and variable amounts of KCl, NH_4Cl or NaCl as indicated, in a total volume of 4 ml.

containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate (Fig. 6).

Molecular weight. Extrapolation of the data from 4 ultracentrifuge runs to zero protein concentration gave an $s^0_{20,w}$ of $11.60 \cdot 10^{-13}$ cm/sec. A diffusion coefficient, $D_{20,w}$ of $4.06 \cdot 10^{-7}$ cm²/sec was determined for a 6.4 mg/ml solution of protein. Assuming a partial specific volume of 0.75, a value of 277 000 was calculated for the molecular weight of the enzyme.

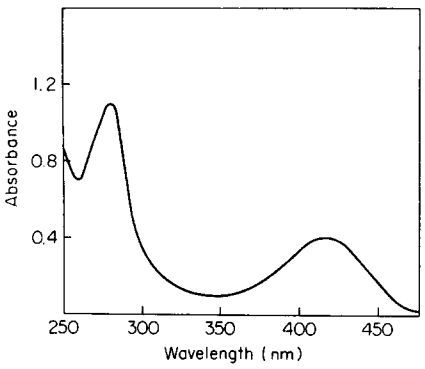
Cofactor requirements

Cation requirement. Several pyridoxal phosphate enzymes require K^+ or NH_4^+ for their activity[13–15]. Recrystallized threonine aldolase showed a similar requirement for K^+ or NH_4^+ for maximal activity. Na^+, Ca^{2+}, Ba^{2+} or Mg^{2+} did not activate but, instead, inhibited activation by K^+ or NH_4^+. Effects of cation concentrations on enzyme activity are shown in Fig. 7.

Pyridoxal phosphate requirement

Enzyme spectra. The recrystallized enzyme solution showed an absorption peak at 420 nm besides at 280 nm (Fig. 8). Extinction coefficient of the enzyme at 280 nm was determined by weighing desiccated samples of the purified enzyme on a microbalance. A 1% solution of enzyme in 0.03 M potassium phosphate buffer, pH 6.4, gave an absorbance of 3.98 in a standard 1-cm cuvette*. This value was used for quantitative measurement of pure threonine aldolase. The 410–420-nm peak is characteristic of enzymes containing pyridoxal phosphate. L-Cysteine inhibited enzyme activity and caused the disappearance of the 420-nm peak with the concomitant appearance of a peak at 330 nm (Fig. 9). The new absorption at 330 nm disappeared on subsequent dialysis. This indicates that L-cysteine resolved the enzyme by

* The E value of 3.98 is rather lower than those found for most proteins. The absorbance and dry weight determinations were made repeatedly with a variety of the purified enzyme preparations prepared from the yeast cells of different batches. The determinations on the different samples agreed within 1.0%.



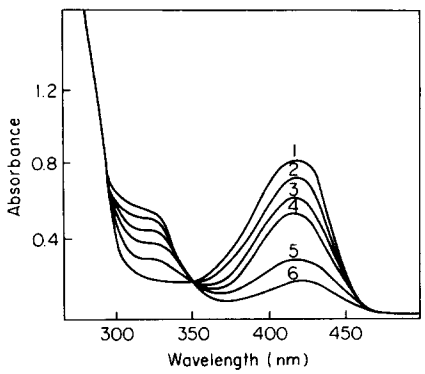


Fig. 8. Absorption spectrum of threonine aldolase. Recrystallized enzyme was used at a concentration of 0.28% in 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA.

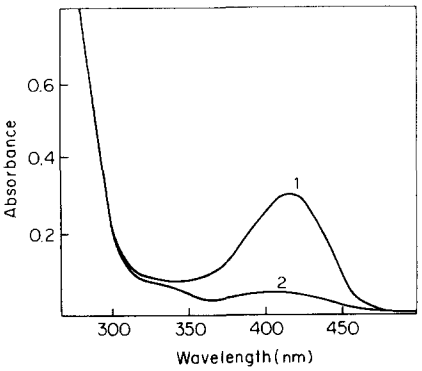
Fig. 9. Variation in absorption spectrum of threonine aldolase after addition of cysteine. Curve 1, absorption spectrum of threonine aldolase at a concentration of 0.56% in 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA. Curve 2– 6,the absorption spectra of the enzyme after addition of neutral cysteine. Curve 2, 0.6 μmole; Curve 3, 1.2 μmoles; Curve 4, 5.4 μmoles; Curve 5, 9.4 μmoles; Curve 6, 13.6 μmoles. Each curve was taken 15 min after the addition of cysteine.

combining with the enzyme bound pyridoxal phosphate (420-nm peak) to form the more stable thiazolidine compound (330-nm peak)[16,17].

Resolution of enzyme. Resolution of the enzyme was carried out by incubating it with L-cysteine, essentially as described by SCHIRCH AND MASON[18]. Thus, recrystallized enzyme (5.40 mg) was diluted with an equal volume of 0.1 M L-cysteine, pH 6.4, and was held at 5° for 5 min. The enzyme protein was then precipitated by the addition of cold saturated $(NH_4)_2SO_4$ solution, pH 6.4, to 0.60 saturation. The precipitate was collected by centrifugation at 20 000 $\times$ g for 20 min and dissolved in a small amount of 0.1 M L-cysteine, pH 6.4. The enzyme solution was dialyzed for 12 h at 4° against 100 ml of 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $5 \cdot 10^{-2}$ M L-cysteine. Then it was dialyzed against 3 changes of 500 ml of 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA. Fig. 10 shows the spectrum of the enzyme before and after resolution. The 330-nm peak, which appeared on the addition of L-cysteine, disappeared during dialysis, indicating that pyridoxal phosphate was completely removed from the enzyme protein. The enzyme thus treated, had less than 5% of its original activity in the absence of added pyridoxal phosphate and retained more than 95% of its activity in the presence of excess pyridoxal phosphate.

The affinity of the resolved enzyme for pyridoxal phosphate was determined using the method of LINEWEAVER AND BURK[19]. Fig. 11 is a double reciprocal plot of the threonine aldolase reaction rate as a function of pyridoxal phosphate concentration. The K_m of the enzyme for pyridoxal phosphate, as determined from this plot, is $2.5 \cdot 10^{-7}$ M.

Amount of pyridoxal phosphate bound to enzyme. The amount of pyridoxal phosphate bound to the enzyme was determined after dialysis of the enzyme, 5.8 mg of protein, against 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-5}$ M pyridoxal phosphate. After 24 h,



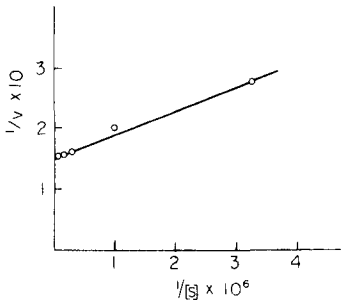


Fig. 10. Absorption spectra of threonine aldolase before and after resolution. Curve 1, absorption spectrum of threonine aldolase, 2.38 mg/ml. Curve 2, spectrum of resolved threonine aldolase, 2.40 mg/ml. Buffer was 0.03 M potassium phosphate buffer, pH 6.4, containing $1 \cdot 10^{-3}$ M mercaptoethanol and $1 \cdot 10^{-3}$ M EDTA. The resolved enzyme was prepared by the use of cysteine as described in the text.

Fig. 11. Effect of pyridoxal phosphate concentration on activity of resolved threonine aldolase. The resolved enzyme was prepared by the use of cysteine as described in the text. The reactions were carried out at 30° for 15 min in reaction mixtures containing 22.6 μg of the resolved enzyme, 50 μmoles of L-threonine, 400 μmoles of Tris-chloride buffer, pH 8.5, 400 μmoles of KCl and variable amounts of pyridoxal phosphate as indicated, in a total volume of 4 ml. The resolved enzyme was preincubated for 10 min (essentially the same enzyme activity was obtained when the resolved enzyme was preincubated with pyridoxal phosphate for longer periods) with pyridoxal phosphate before the addition of substrate. Velocity (v) was expressed as μmoles of acetaldehyde formed per min and pyridoxal phosphate concentration ([S]) as moles/l.

when control experiments without protein showed complete equilibration, the concentration of pyridoxal phosphate inside and outside the dialysis bag was determined by the method of WADA AND SNELL[10]. An excess concentration of pyridoxal phosphate was found within the dialysis bag, corresponding to the binding of 1 mole of pyridoxal phosphate by 46 000 g of protein.

DISCUSSION

A survey of threonine aldolase in microorganisms has been made by DAINTY with a variety of bacteria[25]. He found that the enzyme is present in anaerobic species, *Clostridium pasteurianum*, *Selenomonas ruminatium* and various Caecal anaerobes. DAINTY AND PEEL[5] indicated that the enzyme is constitutive on *C. pasteurianum* and that it may be involved in the biosynthesis of glycine from glucose via threonine. Threonine aldolase of *C. pasteurianum* was purified about 200-fold by DAINTY[6], by $(NH_4)_2SO_4$ fractionation, acid and calcium phosphate gel treatments and DEAE-cellulose chromatography. Pyridoxal phosphate was shown to be associated with the purified enzyme.

The occurrence of threonine aldolase activity in bacteria and yeasts was recently re-investigated in our laboratory[7] with strains contained in the type culture collection (AKU) preserved in the Faculty of Agriculture, Kyoto University, Kyoto. Activity was found in cells of various yeasts, mainly those belonging to Candida. Preliminary experiments on enzyme formation showed that threonine aldolase was formed when these yeasts were grown in media containing L-threonine. Maximal formation of the

enzyme was observed during the early logarithmic phase of growth; thereafter the enzyme gradually disappeared. The enzyme seems to be adaptive in nature and is responsible for cell growth on a medium with L-threonine as the sole carbon source. *Candida humicola*, grown under these conditions, produced markedly high enzyme activity and was selected as a likely source of enzyme for the present investigation.

Threonine aldolase from *C. humicola* was purified about 450- to 470-fold in this investigation, using column chromatographies with DEAE-Sephadex, hydroxylapatite and Sephadex G-200. Crystals were obtained from solutions of the purified enzyme by the addition of $(NH_4)_2SO_4$. This crystalline preparation was apparently homogeneous based on ultracentrifugation and acrylamide gel electrophoresis studies.

Pyridoxal phosphate was shown to be associated with the crystalline threonine aldolase of *C. humicola*. The enzyme exhibited two absorption maxima at 278 and at 410–420 nm. The 410–420-nm absorption is characteristic of enzymes containing pyridoxal phosphate as a hydrogen-bonded Schiff base and suggests that the cofactor is associated with the enzyme by way of an azomethine bond; probably involving the ε-amino group of lysine. Adding cysteine to enzyme of *C. humicola* resulted in a decrease in the 420-nm peak and the appearance of a new peak at 330 nm. Loss of enzyme activity was closely related with these spectral changes. The compound absorbing at 330 nm, probably the thiazolidine of pyridoxal phosphate, was removed by dialysis. The inactive dialyzed enzyme preparation was restored to full activity with its original spectral properties, by the addition of pyridoxal phosphate. These observations suggest that pyridoxal phosphate is, in fact, a catalytic cofactor for the cleavage of threonine into glycine and acetaldehyde. This does not, however, rule out the possibility that pyridoxal phosphate is also involved in maintaining the structural integrity of the enzyme.

The amount of pyridoxal phosphate bound to the crystalline threonine aldolase of *C. humicola* was determined by equilibrium dialysis as 1 mole per 46 000 g of enzyme. This value corresponds to 6 moles of pyridoxal phosphate per mole of enzyme. If pyridoxal phosphate is, in fact, a component of the active site of the enzyme, it seems likely that the threonine aldolase of *C. humicola* would have a structure containing 6 active sites. This possibility is currently being investigated.

ACKNOWLEDGMENT

We wish to thank Prof. Y. Morita and Prof. K. Ogata, Kyoto University, Kyoto, for their interest and advice during the course of this work.

REFERENCES

1 A. E. Braunstein and G. Y. Vilenkina, *Dokl. Akad. Nauk. U.S.S.R.*, 66 (1949) 1243.
2 S. C. Lin and D. M. Greenberg, *J. Gen. Physiol.*, 38 (1954) 181.
3 L. I. Malkin and D. M. Greenberg, *Biochim. Biophys. Acta*, 85 (1964) 117.
4 M. A. Karasek and D. M. Greenberg, *J. Biol. Chem.*, 227 (1957) 191.
5 R. H. Dainty and J. L. Peel, *Biochem. J.*, 117 (1970) 573.
6 R. H. Dainty, *Biochem. J.*, 117 (1970) 585.
7 H. Yamada, H. Kumagai, T. Nagate and H. Yoshida, *Agr. Biol. Chem.*, 35 (1971) 1340.
8 A. Tiselius, S. Hjerten and O. Levin, *Arch. Biochem. Biophys.*, 65 (1956) 132.
9 M. A. Paz, O. O. Blumenfeld, M. Rojkind, E. Henson, C. Furfine and P. M. Gallop, *Arch. Biochem. Biophys.*, 109 (1965) 548.

10 H. WADA AND E. E. SNELL, *J. Biol. Chem.*, 236 (1961) 2089.
11 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
12 T. SVEDBERG AND K. O. PEDERSEN, *The Ultracentrifuge*, Oxford University Press, New York, 1940.
13 W. A. NEWTON AND E. E. SNELL, *Proc. Natl. Acad. Sci. U.S.*, 51 (1964) 382.
14 Y. MORINO AND E. E. SNELL, *J. Biol. Chem.*, 242 (1967) 2800.
15 H. KUMAGAI, H. YAMADA, H. MATSUI, H. OHKISHI AND K. OGATA, *J. Biol. Chem.*, 245 (1970) 1773.
16 M. V. BUELL AND R. E. HANSEN, *J. Am. Chem. Soc.*, 82 (1960) 6042.
17 Y. MATSUO, *J. Am. Chem. Soc.*, 79 (1957) 2011.
18 L. SCHIRCH AND M. MASON, *J. Biol. Chem.*, 237 (1962) 2578.
19 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.