Purification and properties of tryptophan synthase from baker's yeast (*Saccharomyces cerevisiae*)

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Tryptophan synthase was purified from baker's yeast. The purified enzyme exhibited one band on polyacrylamide-gel electrophoresis, had no detectable *N*-terminal amino acid and *C*-terminal alanine. The amino acid composition was close to that predicted by recent studies on the DNA sequence of the structural gene for the enzyme. Kinetic parameters for the following three activities were measured: indole–serine condensation, indolylglycerol phosphate lyase and the overall reaction of serine with 1-(indol-3-yl)glycerol 3-phosphate. The K_m for indole was much lower than suggested by previous investigations, and the value of 11 μM was measured by a fluorimetric assay.

Tryptophan synthase catalyses the last stage in tryptophan biosynthesis:

The overall reaction may be considered as the sum of two parts:

In *Escherichia coli* the two partial reactions are controlled by two polypeptide species; in fungi one polypeptide species catalyses all the reactions (Yanofsky & Crawford, 1972; Miles, 1979). The fungal tryptophan synthase is thus a simple example of a multifunctional protein (Kirschner & Bisswanger, 1976).

Genetic studies with the yeast enzyme initially suggested that it was a dimer of one polypeptide species (Duntze & Manney, 1968). Attempts to purify the enzyme to homogeneity were unsuccessful (Katsunuma *et al.*, 1972; Wolf & Hoffmann, 1974), largely because of the extraordinary sensitivity towards proteolysis exhibited by the protein, and it is only comparatively recently that a single-component enzyme has been obtained. The available purification schemes require large-scale culture of yeast cells (Dettwiler & Kirschner, 1979) or appear to be suitable only for the purification of very small amounts of material (Bartholmes *et al.*, 1979). A major attraction of biochemical yeast research is that commercial supplies of cells are available on a large scale, and accordingly we have examined

purification from the traditional source, baker's yeast. The scheme presented provides a convenient source of stable homogeneous enzyme. Some of the structural properties are compared with those predicted by the DNA sequence of the structural gene for yeast tryptophan synthase (Zalkin & Yanofsky, 1982).

Experimental

Materials

Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Ultrogel AcA-34 was from LKB Instruments, South Croydon, Surrey, U.K. DEAE-cellulose (type DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Amino acid calibration standards, pyridine (Sequenal grade) and dimethylformamide (Sequenal grade) were from Pierce Chemical Co., Rockford, IL, U.S.A. Sodium tetrathionate was prepared by the method of Liu & Inglis (1972). Other standard chemicals were suitable grades from Sigma Chemical Co., Poole, Dorset, U.K., or BDH Chemicals, Poole, Dorset, U.K.

1-(Indol-3-yl)glycerol 3-phosphate

This substrate was prepared by enzymic synthesis in a solution containing 0.06 M-potassium phosphate buffer, pH 6.8, purified tryptophan synthase (0.15 units/ml), 6 mM-indole and 12 mM-DL-glyceraldehyde 3-phosphate (prepared from the diethylacetal by the method of Racker *et al.*, 1959). Reaction at 37°C was monitored by D-glyceraldehyde 3-phosphate-coupled NADH production in samples taken from the reaction mixture. The

product was worked up by charcoal column chromatography (Creighton & Yanofsky, 1970), rotary-evaporated to dryness and stored as a frozen aqueous solution at -40°C . It was assayed by periodate oxidation (Smith & Yanofsky, 1963) and enzymically by the indolylglycerol phosphate lyase procedure (see below). Only batches of substrate where the enzymically utilizable material was greater than 95% of the chemical titre were used in the experiments reported in the present paper.

Enzyme assays

Routine assay was by a colorimetric indole-disappearance assay similar to that described by Tsai & Susskind (1972). Samples (1–20 μl) of enzyme were added to 0.50 ml of solution containing 0.088 M- K_2HPO_4 , 0.038 M- KH_2PO_4 , 0.300 mM-indole, 40 mM-DL-serine, 0.2 mM-pyridoxal 5'-phosphate, 10 mM-EDTA and 0.8 mg of bovine serum albumin/ml at 37°C and pH (room temp.) 7.2. Reaction was continued for 5–60 min and stopped by addition of 3.0 ml of a solution of *p*-dimethylaminobenzaldehyde (6 g), 11.7 M-HCl (45 ml) and 100% ethanol (205 ml). Absorbance was measured at 570 nm and compared with that of an enzyme-free standard. For determination of the kinetic constants, bovine serum albumin and EDTA were omitted and measurements were made in 0.1 M-potassium phosphate buffer, pH 6.8, with other substrates as required.

Determination of K_m for indole in the indole-serine condensation reaction required measurement of indole at micromolar concentration. The following fluorescence assay is suitable. Enzyme solution was added to make up a final volume of 1 ml containing 0.1 M-potassium phosphate buffer, pH 6.8, 10 mM-L-serine and 0.2 mM-pyridoxal 5'-phosphate at 37°C . After incubation, the reaction was stopped by rapid mixing with 2 ml of ethyl acetate, which served to remove indole completely. The phases were separated by brief centrifugation. Termination of the reaction was complete, because the indole concentration in the organic phase remained constant. Samples of the ethyl acetate phase were assayed at 30°C by using a Perkin-Elmer MPF-44B fluorimeter, with excitation at 280 nm and emission at 310 nm, both slits being set at 10 nm, together with extracts of solutions containing no indole and no enzyme. The fluorimetric signal was directly proportional to indole concentration in the range 0.5–15 μM . Pyridoxal 5'-phosphate and tryptophan did not interfere with this assay at the concentrations used.

Indolylglycerol phosphate lyase and the overall reaction involving tryptophan production from indolylglycerol phosphate and L-serine were measured by an assay coupled to D-glyceraldehyde 3-phosphate production (Creighton, 1970).

The 1 ml final volume contained 0.1 M-Tris/HCl buffer, pH 7.8, 12 mM- Na_2HAsO_4 , 0.9 mM- NAD^+ , 1 mM-dithiothreitol and 0.44 unit of glyceraldehyde 3-phosphate dehydrogenase. Activity at 37°C was measured by continuous measurement of A_{340} by using a Unicam SP.8-200 spectrometer.

For each activity, 1 unit of enzyme activity turns over 1 μmol of substrate or product in 1 min at 37°C .

Protein determination

The method of Lowry *et al.* (1951) was used, with bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

The system of Laemmli (1970), containing sodium dodecyl sulphate, was used with rod gels.

End-group determinations

Active enzyme solution was dialysed at 2°C for 3 days against deionized water and freeze-dried. The dried protein was dissolved in hot 0.02 M- NaHCO_3 /1% (w/v) sodium dodecyl sulphate/1% (v/v) 2-mercaptoethanol, precipitated with acetone and dried. The denatured washed protein was dissolved at 13 mg/ml in 0.2 M- NaHCO_3 /1% (w/v) sodium dodecyl sulphate for end-group assays.

C-Terminal investigation was conducted by digestion of protein solution (5 μl) with carboxypeptidase A (di-isopropyl phosphorofluoridate-treated; Sigma) solution (1 μl) in 0.2 M- NaHCO_3 . Reaction was stopped by addition of dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride in acetone (2.6 mg/ml; 5 μl), and dansyl-amino acids were identified by t.l.c. Control samples without exopeptidase and without sample protein were also taken through the procedure.

N-Terminal analysis of dansyl-amino acid residues was conducted after reaction in the dimethylformamide/*N*-ethylmorpholine system (Gray, 1972).

The validity of the procedures used was checked by using haemoglobin and lysozyme as control proteins for the C-terminal assay and epidermolytic toxins (Bailey *et al.*, 1980) as controls for the N-terminal assay.

Amino acid analysis

Dialysed freeze-dried samples of tryptophan synthase were hydrolysed *in vacuo* with 4 M-methanesulphonic acid containing 0.2% 3-(2-amino-ethyl)indole at 115°C (Simpson *et al.*, 1976). The tetrathionylated hydrolysates were analysed on a JEOL 5-AH Amino Acid Analyser by using an accelerated two-column procedure (Spackman *et al.*, 1958).

Results

Purification of the enzyme

Lysate. Dried active baker's yeast (700g) was added with stirring to a solution of EDTA (1.2g) in 0.38M-NH₃ solution (1500ml) at room temperature. Addition and suspension was complete after about 30–45 min, and the homogeneous suspension was then stirred for a further 1h before being left overnight at room temperature.

The lysate was cooled with stirring to 2°C, and saturated (at 2°C) (NH₄)₂SO₄ was added to give 35% final saturation. After 1h the suspension was centrifuged (12000g for 40min) to obtain the crude extract.

First ammonium sulphate pellet. The crude extract was adjusted to pH4.7 (apparent; glass electrode) at 2°C with 4M-acetic acid 35%-saturated with (NH₄)₂SO₄. The suspension was stirred for 2h and left overnight on ice, then centrifuged as previously.

Heat supernatant. The pellet from the previous stage was dissolved in 400ml of solution M (0.13M-K₂HPO₄, 0.056M-KH₂PO₄, 0.4mM-pyridoxal 5'-phosphate, 20mM-DL-serine, 10mM-EDTA), adjusted to 0.1mM-phenylmethane-sulphonyl fluoride immediately before use. Protamine sulphate (0.7g) dispersed in solution M (25ml) was added with stirring for 45min. The mixture was re-centrifuged and the supernatant, after further adjustment to 0.1mM-phenylmethane-

sulphonyl fluoride, was heated rapidly to 54°C and held at that temperature for 4min. The supernatant was recovered after centrifugation and adjusted at 2°C to 35% saturation with (NH₄)₂SO₄ at pH4.7 as previously. After 30min the pellet was collected by centrifugation, and dissolved in a minimal volume of solution M (50–100ml).

DEAE-cellulose chromatography. The solution from the previous step was dialysed overnight against 15mM-potassium phosphate buffer, pH6.8, to a final conductivity of less than 3mS, clarified by centrifugation and loaded on to a DEAE-cellulose column (3cm × 30cm) previously equilibrated with the same buffer. After washing with the equilibration buffer, the enzyme was eluted on a linear 0–0.4M-KCl gradient formed from 600ml volumes of equilibration buffer, at a flow rate of about 200ml/h. Active fractions were eluted near the beginning of the gradient (Fig. 1), and were pooled and precipitated by 35% saturation with (NH₄)₂SO₄ at pH4.7 as previously. The enzyme was taken up in solution M.

Hydroxyapatite chromatography. The active DEAE-cellulose chromatography fraction was dialysed against 10mM-potassium phosphate buffer, pH6.8, containing 0.01mM-pyridoxal 5'-phosphate, 0.5mM-DL-serine, loaded on to a column (1.5cm × 15cm) of hydroxyapatite and eluted at 2°C with a gradient formed from 120ml of 10–200mM-potassium phosphate buffer, pH6.8, containing 0.1mM-pyridoxal 5'-phosphate and 5mM-DL-serine.

Fig. 1. DEAE-cellulose chromatography of tryptophan synthase from baker's yeast

For experimental details see the text. Fractions (14ml) were assayed for tryptophan synthase activity (●), absorbance at 280nm (○) and conductivity (▲), and pooled as indicated by the bar.

Fig. 2. *Hydroxyapatite chromatography of tryptophan synthase from baker's yeast*

For experimental details see the text. Fractions (3.5 ml) were assayed for tryptophan synthase activity (●), absorbance at 280 nm (○) and conductivity (▲), and pooled as indicated by the bar.

Table 1. *Purification of yeast tryptophan synthase*

For experimental details see the text. Enzyme activities at the first three stages are approximate because of non-linear kinetics. The stability values given are not for the tabulated purification but are averaged from other data.

Fraction	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Enzyme stability $t_{\frac{1}{2}}$ (days)
Lysate	1990	280	0.0012	5
Lysate + $(\text{NH}_4)_2\text{SO}_4$	3110	400	0.0013	12
Crude extract	1770	170	0.0020	11
$(\text{NH}_4)_2\text{SO}_4$ -fractionation pellet	490	181	0.0074	>30
Post-heat-treatment pellet	140	165	0.0143	>30
DEAE-cellulose chromatography pellet	12	84	0.143	—
Hydroxyapatite chromatography pellet	3	51	1.00	—
Ultrogel chromatography pellet	2	31	1.69	—

The active pool (Fig. 2) was adjusted to 35% saturation of $(\text{NH}_4)_2\text{SO}_4$ and pH 4.7 as previously, centrifuged and resuspended in solution M without DL-serine.

Ultrogel chromatography. The active solution from the previous stage (2–3 ml) was run on an Ultrogel AcA-34 column (2.6 cm $\times$ 30 cm) in solution M without serine at a flow rate of about 20 ml/h. The active material in the symmetrical peak (at 96–106 ml) was pooled and precipitated by 35% saturation with $(\text{NH}_4)_2\text{SO}_4$ as before.

Omission of pyridoxal 5'-phosphate from all solutions used after the DEAE-cellulose chromatography stage gave an apoenzyme preparation, as was the case in the example detailed in Table 1.

The purified material may be stored as a frozen solution at -20°C ($t_{\frac{1}{2}} > 0.5$ year) or as a suspension in 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ at 2°C ($t_{\frac{1}{2}} > 2$ years, more than 40% residual activity after storage for 3 years).

Homogeneity and integrity of the purified enzyme

The final preparation (Fig. 3) showed essentially one band, of M_r 74 000 $\pm$ 4000, on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. No dansylated *N*-terminal amino acids were found in 4 h hydrolysates of dansyl chloride-treated enzyme solution, suggesting that a blocked *N*-terminus was present. Digestion of tryptophan synthase with carboxypeptidase A (enzyme/substrate ratio 1:80,

w/w) yielded unchanging amounts of alanine and a trace of valine after 1 h, 2 h and 18 h digestion. Release of alanine was observed when digestion was performed at 20°C for 5, 15 and 30 min. This evidence indicates that yeast tryptophan synthase has a C-terminal alanine residue.

The amino acid composition of the enzyme preparation is shown in Table 2, with for com-

parison that predicted from the DNA sequence of the structural gene and the single-hydrolysate protein composition found by Zalkin & Yanofsky (1982). Fourteen of the DNA-predicted values lie within, or very close to, the 95% confidence interval about the experimental values. Arginine, half-cystine, threonine and glycine are not in exact agreement.

Table 2. *Amino acid composition of yeast tryptophan synthase*

The experimental values were from four analyses of 22 h hydrolysates and two analyses after 49 h hydrolysis. Preferred values [$\pm$ S.E.M. or S.E.E. (standard error of the estimate)] in the third column are averages of all values except for the following: those for serine and threonine were extrapolated to zero time, assuming first-order decay; those for valine and isoleucine are 49 h values; that for half-cystine is from 22 h values. If half-cystine values are extrapolated to zero time a value of 8.6 ± 0.5 is obtained. All data are given in mol of residue/76 544 g.

Amino acid residue	Amino acid composition (mol of residue/mol)				
	Experimental values			Zalkin & Yanofsky (1982) values	
	22 h hydrolysate	49 h hydrolysate	Best value	From DNA sequence	From protein analysis
Trp	5.05	4.94	5.0 ± 0.2	5	—
Lys	46.1	43.7	45.1 ± 0.8	44	42
His	18.8	18.4	18.6 ± 0.2	19	19
Arg	31.9	30.7	31.4 ± 0.5	28	28
CyS	7.9	7.1	7.9 ± 0.3	7	8
Asx	69.0	70.3	69.5 ± 0.9	66	67
Thr	41.8	40.9	42.5 ± 1.0	46	44
Ser	41.5	37.7	44.8 ± 1.2	43	39
Glx	70.2	70.6	70.4 ± 0.9	69	70
Pro	31.8	30.4	31.3 ± 0.6	31	31
Gly	61.3	62.4	61.7 ± 0.4	66	68
Ala	60.3	61.0	60.6 ± 1.2	64	64
Val	51.9	59.9	59.9 ± 2.9	61	58
Met	10.6	10.4	10.5 ± 0.1	11	11
Ile	32.3	34.5	34.5 ± 0.9	34	33
Leu	58.8	58.9	58.8 ± 0.6	62	61
Tyr	19.3	19.8	19.5 ± 0.2	19	15
Phe	30.6	31.8	31.0 ± 0.5	32	28

Table 3. *Kinetic constants of yeast tryptophan synthase*

The K_m values are apparent Michaelis constants at the given fixed substrate concentration. The confidence interval for K_m is a 95% zone as calculated by the non-parametric procedure (Porter & Trager, 1977). V_{max} values are relative to that for indole-serine condensation.

Reaction	Concn. of fixed substrate (mM)			Variable substrate	K_m (confidence interval) (μ M)	Relative V_{max}
	L-Serine	Pyridoxal 5'-phosphate	Indole			
Indolylglycerol phosphate lyase	10	0.02	0	1-(Indol-3-yl)glycerol 3-phosphate	32 (26–37)	0.33
Indolylglycerol phosphate lyase	0	0	0	1-(Indol-3-yl)glycerol 3-phosphate	810 (220–940)	0.04
Indole-serine condensation	10	0.2	—	Indole	11 (8–12)	—
Indole-serine condensation	—	0.4	0.2	L-Serine	2300 (400–3400)	1
Indole-serine condensation	10	—	0.15	Pyridoxal 5'-phosphate	3100 (2900–3300)	—

Fig. 3. Polyacrylamide-gel electrophoresis of purified yeast tryptophan synthase

The relative molecular mass of the tryptophan synthase band (mobility 0.236) was measured by calibration of the system with the following standard proteins (M_r , mobility): β -galactosidase (135 000, 0.144); urease (95 000, 0.183); *Neurospora crassa* tryptophan synthase (76 000, 0.224); bovine serum albumin (69 000, 0.262); glyceraldehyde 3-phosphate dehydrogenase (36 000, 0.463); lactate dehydrogenase (35 000, 0.545); haemoglobin (15 500, 0.907).

Enzymic properties

The purified enzyme catalysed the characteristic three reactions (Table 3). The kinetic constants were derived from initial-rate studies at concentration ranges of at least 0.5–3 times K_m . All substrates obeyed Michaelis–Menten kinetics except for indole at high substrate concentration, where substrate inhibition was detectable above 0.5 mM.

Indole–serine condensation was undetectable in the absence of pyridoxal 5'-phosphate.

Discussion

Initial attempts to purify the enzyme by conventional procedures confirmed the extreme lability of crude extracts of yeast tryptophan synthase

(Manney, 1968; Katsunuma *et al.*, 1972). The procedure developed depends crucially on extraction conditions that restrain proteinase activity: the ammoniacal lysis procedure (de la Morena *et al.*, 1968; Scopes, 1971) followed by neutralization at high ionic strength with the conjugate acid. Observation of the stability of the enzyme at these stages showed that inactivating processes are quite slow during the initial extraction and clarification (Table 1), but the concentrated enzyme solutions used after the first ammonium sulphate fractionation were entirely stable. The ammonium sulphate fractionation procedure (cf. Manney, 1968) served to concentrate the protein and also amplified the purification achieved at the initial chromatography stages.

The final preparation was essentially homogeneous on gel electrophoresis, and this conclusion is supported by end-group and compositional analysis, although the final specific activity was 1.9-fold lower than that detailed by Dettwiler & Kirschner (1979) and very much less than the value claimed by Bartholmes *et al.* (1979). Each of the three groups has obtained a different specific activity for homogeneous enzyme, and this is a remaining source of concern.

The C-terminal amino acid and the overall composition indicate that an enzyme similar to that predicted by DNA-sequencing studies of the structural gene has been obtained. The size and C-terminus of yeast tryptophan synthase are as predicted, but the amino acid analysis shows possible differences. Of these the value for arginine is most likely to represent a real difference, but the overall accuracy of amino acid analytical techniques is insufficient to be confident that a difference of 12%, in one value out of 18, is significant. The composition of our material is also close to that of the Kirschner sample analysed by Zalkin & Yanofsky (1982). Their single-point analysis is inadequate for tryptophan, serine and possibly tyrosine; phenylalanine is the only other possible difference. Notably, the two protein analyses suggest that 8 rather than 7 half-cystine residues/molecule are present in the mature protein, and the 11 methionine residues/molecule found agree with the DNA sequence of the structural gene only if the initiator methionine residue is retained. These differences are unexpected, in that sulphur amino acids are likely to be underestimated because of their lability. Our selected half-cystine analytical value is conservative, for there is evidence of hydrolytic loss (Table 2).

The purified enzyme catalyses the characteristic three reactions at relative V_{max} values indistinguishable from those of wild-type crude extracts (Duntze & Manney, 1968). By comparison the *Neurospora* enzyme has a lower kinetic capacity for

1-(indol-3-yl)glycerol 3-phosphate lysis in the absence of serine (DeMoss, 1962). A notable feature of the K_m values presented here is that our value for indole ($11\mu\text{M}$) is very much lower than previously reported values: 70 and $160\mu\text{M}$ (Bartholmes *et al.*, 1979; Wolf & Hoffmann, 1974) for the yeast enzyme, and 70 and $140\mu\text{M}$ (DeMoss, 1962; Matchett, 1972) for the *N. crassa* enzyme. Exact determination of the value of the apparent K_m for indole required the fluorescence assay, but the conventional colorimetric assay was sensitive enough to confirm that the K_m for indole is less than $50\mu\text{M}$. This finding may prompt some re-appraisal of the 'channelled-intermediate' conception (Matchett, 1974) of the operation of fungal tryptophan synthase, which requires that the concentration of free indole should be insufficient to account for the rate of indole-serine condensation (Kirschner & Bisswanger, 1976).

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