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LARGE SCALE PURIFICATION AND STRUCTURAL PROPERTIES OF YEAST ASPARTYL-tRNA SYNTHETASE

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Summary. A large scale purification procedure of baker's yeast aspartyl-tRNA synthetase is described which yields more than 200 mg pure protein starting from 30 Kg of wet commercial cells. The synthetase is an α_2 dimer of Mr = 125,000 ± 5,000 which can be crystallized (J. Mol. Biol. 138, 1980, 129-135). The enzyme has an elongated shape with a Stokes radius of 50 Å and a frictional ratio of 1.5 . The synthetase has a tendency to aggregate but methods are described where this effect is overcome.

In the field of tRNA and aminoacyl-tRNA synthetase a particular focus was given to the aspartic acid system from the yeast **Saccharomyces cerevisiae** after the successful crystallization of its components, $tRNA^{Asp}$ (1), aspartyl-tRNA synthetase (2) and the complex formed between one synthetase and two tRNA molecules (3,4) and the subsequent structural studies on this system (e.g. 5-10).

In this paper we describe the large scale purification procedure of yeast aspartyl-tRNA synthetase (EC 6.1.1.12) currently used in this laboratory, which yields enzyme preparations suitable for crystallization (2-4). Some structural properties including molecular weight, subunit structure and solubility characteristics of the enzyme are also described and discussed.

MATERIAL AND METHODS

Yeast and Miscellaneous Products. Fresh commercial baker's yeast was provided by the Société FALA (Strasbourg). Cells in the exponential growing phase were harvested over ice at the factory 5 hours before consumption of the growth medium. Cells were immediately centrifuged, washed and stored at -20°C (11). [^{14}C]-labeled aspartic acid was from the CEA (Saclay, France). Crude tRNA from brewer's yeast was from Boehringer Mannheim (Meylan, France);1-O-octyl-βD-glucopyranoside was from Serva (Heidelberg, FRG) and molecular weight marker proteins from Sigma (St Louis, USA) and Serva. Reference aminoacyl-tRNA synthetases, specific for arginine, leucine, phenylalanine and valine, were prepared at this Institute (11-14). The origin of chromatographic supports and of other chemicals was as indicated earlier (11,13).

General Purification Procedures and Handling of Proteins. The large scale purification procedure of yeast aspartyl-tRNA synthetase follows essentially the general methodology worked out for the purification of several yeast synthetases (13). 30 Kg of wet yeast cells are quickly ground in the presence of glass beads; the crude extract is centrifuged and the supernatant subjected

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to ammonium sulfate fractionation. Purification is pursued by filtration of the active fraction on Sephadex G200 followed by three successive chromatographies on DEAE-cellulose (DE 52, Whatman), hydroxylapatite (Bio Gel HTP, Biorad) and phosphocellulose (P11, Whatman). In recent preparations it was shown that the filtration step on Sephadex could be omitted. Purification was conducted in the presence of 0.1 mM DIFP, and chromatographic buffers contained 10% glycerol. Protein concentrations were measured spectrophotometrically at 280 nm; for pure aspartyl-tRNA synthetase $E^{280\ nm}_{mg.ml^{-1}cm^{-1}}$ = 0.60 ± 0.06 (4). Pure enzyme was stored at -20°C as a concentrated stock solution of about 20 $mg.ml^{-1}$ in 50 mM potassium phosphate buffer at pH 7.2 containing 5 mM 2-mercaptoethanol, 0.1 mM EDTA, and 50% glycerol. Optimal recovery of pure enzyme after dialysis is obtained when the dialysis is conducted in the presence of 0.1% (w/v) 1-O-octyl-βD-glucopyranoside in tubing previously treated with the detergent.

Aspartyl-tRNA Synthetase Assays. Along the purification, aspartyl-tRNA synthetase activity was measured by the rate of aspartyl-tRNA formation under the standard conditions described in (13). For specific activity measurements optimal aminoacylation conditions were used (4), except for the concentration of aspartic acid (0.1 mM) which was non-saturating.

Analytical Techniques. The antibodies against aspartyl-tRNA synthetase were prepared from rabbits and immunotitrations of the enzyme in the crude extract or after phosphocellulose chromatography were conducted using established procedures (15). Polyacrylamide gel electrophoresis of native enzyme and molecular weight determinations were as described in (16,17); SDS polyacrylamide gel electrophoresis was according to Weber and Osborn (18) or Laemmli (19). Sedimentation velocity measurements were performed as described in (20). Molecular weight determination by sucrose gradient centrifugation was according to Martin and Ames (21); 5 to 20% sucrose gradients (11.2 ml) in 50 mM Hepes (Na) pH 7.2, 50 mM KCl, 5 mM 2-mercaptoethanol and 0.2 mM DTE were loaded with 150 µl medium containing a mixture of synthetase (80 µg) and of one marker protein (1mg hemoglobin or 0.3 mg alcohol dehydrogenase or 0.15 mg catalase) and run at 40,000 rpm (4°C) for 16 hours in a Beckman SW41 rotor. Fractions of 300 µl were collected and protein localized by spectrophotometry (hemoglobin) or by established activity assays. Gel filtration was conducted on a Sephadex G200 column (1cm x 56 cm) equilibrated at 4°C in 20 mM potassium phosphate buffer at pH 7.5 containing 100 mM KCl, 0.5 mM 2-mercaptoethanol and 0.2 mM DTE. The flow rate was 27 $ml.h^{-1}$ and 0.9 ml fractions were collected. Samples of 100 µl containing the appropriate amounts of proteins were filtered and detected in the eluate using conventional methods. The Stokes radius of aspartyl-tRNA synthetase was determined by the method of Ackers (22). The partial specific volume and the average hydrophobicity of the enzyme were calculated from the aminoacid composition (9) according to Schachman (23) and Bigelow and Channon (24) respectively.

RESULTS AND DISCUSSION

A typical large scale purification of yeast aspartyl-tRNA synthetase is summarized in Table I. Starting from 30 Kg of yeast cells it was possible to obtain in this particular purification 220 mg of pure enzyme after the last step on a column of phosphocellulose. In the case shown on Figure 1, the enzyme is eluted as one regular peak; in some experiments however the elution profiles were irregular and even could be split in several peaks, all exhibiting aspartyl-tRNA synthetase activity and apparently similar catalytic and structural properties (not shown). This behaviour might be related to a

Table I: Large scale purification of aspartyl-tRNA synthetase from commercial baker's yeast in the exponential growing phase.

Fraction	Total proteins	Specific activity *	Purification ratio	Yield
	(mg)	(Units.mg^{-1})		(per cent)
Crude extract	7,800,000	0.14	1	100
Sephadex G 200	88,300	6	42	48
DEAE-cellulose	3,900	116	840	42
Hydroxylapatite	320	550	3,900	16
Phosphocellulose	220	800	5,800	16

The enzymatic activity unit is that amount of enzyme which catalyzes the incorporation of 1 nmol aspartic acid to $tRNA^{Asp}$ in 1 min under the conditions (0. 1 mM aspartic acid) described in (4).

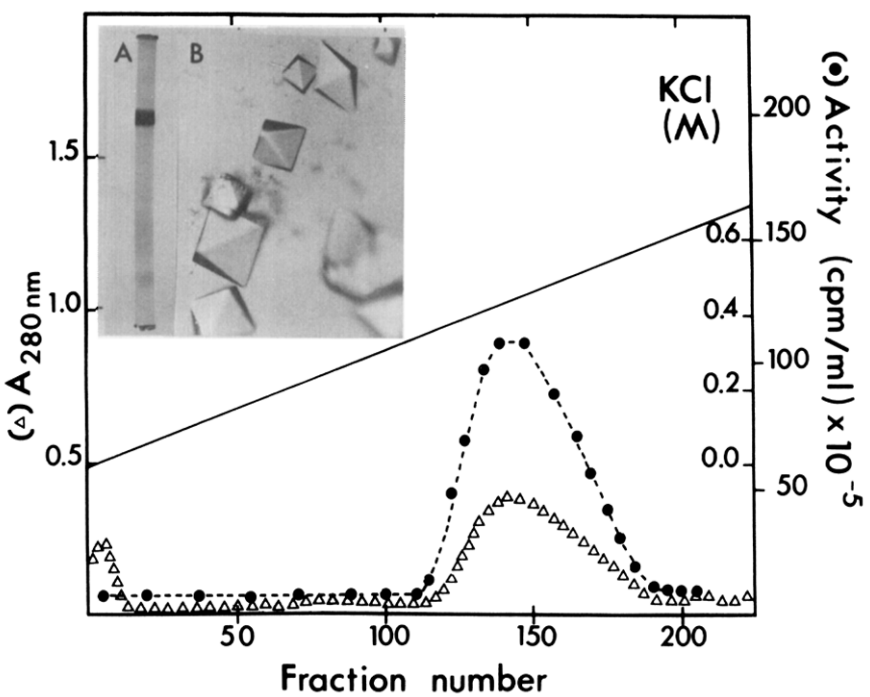

Fig.1 : Elution of aspartyl-tRNA synthetase from phosphocellulose. The pooled aspartyl-tRNA synthetase fractions from the hydroxylapatite column were extensively dialyzed (11-13) and applied on a phosphocellulose column (5 cm x 32 cm). Elution was obtained with a linear gradient from 0 to 0.9 M KCl in the buffer described in (11-13) (the total volume of the gradient was 4 litres). (△) Absorbance at 280 nm; (●) aspartyl-tRNA synthetase activity. Inset photographs :(A) SDS-polyacrylamide gel electrophoresis (18) of 15 µg of pure enzyme,(B) crystals of pure aspartyl-tRNA synthetase shaped as tetragonal bipyramids (2).

microheterogeneity of the enzyme which will be described elsewhere. A similar effect was observed with aspartyl-tRNA synthetase from porcine thyroid which is eluted as four distinct forms from phosphocellulose columns (25, 26).

The pure yeast aspartyl-tRNA synthetase is obtained with a yield of 16% and exhibits typically a specific activity of 600 to 800 $U.mg^{-1}$. This apparently low value is due to the fact that usual aminoacylation assays are done for practical reasons in the presence of a non-saturating amount of aspartic acid ($10^{-4}M$) because of the weak affinity of the aminoacid for the synthetase ($K_m = 2.10^{-3}M$). The specific activity is raised up to 12,000 $U.mg^{-1}$ when corrected for a saturating aspartic acid concentration; this value is among the highest found for yeast aminoacyl-tRNA synthetases.

After phosphocellulose chromatography the enzyme appears homogeneous on SDS-polyacrylamide gels according to Weber and Osborn (18) (see Figure 1). As judged after immunotitration all purified synthetase molecules appear to be active. Furthermore the enzyme can be crystallized in the presence of ammonium sulfate, either in its free state (Fig. 1 and ref. 2) or complexed with tRNA (3,4).

Some structural parameters of yeast aspartyl-tRNA synthetase have been determined and are summarized in Table II. The molecular weight of the native enzyme was estimated by five different methods : sedimentation equilibrium, sucrose gradient centrifugation, Sephadex G200 filtration, polyacrylamide gel electrophoresis (Fig. 2) and neutron scattering (26). As can be seen on Table II the values range around 120,000 except for the enzyme eluted from Sephadex G200 which presents an apparent molecular weight of 205,000. This high value cannot solely be accounted for by the hydration of the protein (22); more likely it reflects an elongated shape of aspartyl-tRNA synthetase, a structural property also supported by the large Stokes radius (50 Å) of the protein and the frictional ratio of 1.5, as well as by crystallographic evidences (2) and neutron small-angle scattering measurements (27). Other aminoacyl-tRNA synthetases present similar hydrodynamic features; this is the case of yeast phenylalanyl- and valyl-tRNA synthetases. Bean chloroplastic leucyl-tRNA synthetase (14) and yeast arginyl-tRNA synthetase, however, behave more like globular proteins. It might be noticed that sucrose gradient centrifugation yields the lowest values of the molecular weight; this results from a denser particle as compared to the reference proteins.

Under denaturing conditions on SDS-polyacrylamide gels the enzyme migrates as a particle of about 63,000 daltons molecular weight (Fig. 3), indicating a dimeric structure for aspartyl-tRNA synthetase. Since the two subunits of the enzyme are related by a two-fold crystallographic symmetry axis (2) it can be concluded that the oligomeric structure is of the α_2 type. This can be correlated with binding studies showing that two tRNA molecules

Table II: Physical parameters of yeast aspartyl-tRNA synthetase.

Parameter	Method	Value
Molecular weight [a]		
Native	Sedimentation equilibrium	116,000
	Sucrose gradient centrifugation [b]	89,000 - 106,000
	Polyacrylamide gel electrophoresis	122,000
	Neutron scattering	116,000 [d]
	Gel filtration	205,000
Subunit	SDS-gel electrophoresis [c]	63,000 [c]
Extinction coefficient ($E^{280\ nm}_{mg.ml^{-1}.cm^{-1}}$)	Aminoacid composition, dry weight and colorimetric titration	0.60 ± 0.06 [d]
Partial specific volume ($\bar{v}$)	Aminoacid composition	0.73 $cm^3.g^{-1}$
Stokes radius (R_S)	Gel filtration	50 Å
Frictional ratio (f/f_0)	Calculated from R_S	1.50
Average hydrophobicity ($H\phi_{ave}$)	Aminoacid composition	983 $cal.mol^{-1}$

[a] Molecular weights are mean values ; the maximum standard deviation (± 10%) were found for sucrose gradient centrifugation and gel filtration; for the other methods the error was less than 5%; [b] The molecular weights were 89,000; 99,000 and 106,000 with catalase, alcohol dehydrogenase and hemoglobin as reference proteins ; [c] This value is an average number obtained on two types of SDS-gels (18,19), for details see the text ; [d] These values have been published elsewhere (2,4). The aminoacid composition was taken from (9).

(7) as well as two of the small ligands (to be published), can interact with the synthetase. It must however be noted that under certain gel conditions (19) the enzyme migrates as a doublet with electrophoretic bands of apparent molecular weight of 62,000 and 64,000 daltons contrarily to other conditions (18) under which it migrates as a unique band of 63,000 daltons. This versatile electrophoretic behaviour of aspartyl-tRNA synthetase likely is due to structural microheterogeneities in the protein; it will be described in details in a forthcoming paper.

Summarizing the previous data we propose for the dimeric yeast aspartyl-tRNA synthetase a Mr value of 125,000 ± 5,000 (because the enzyme migrates just after yeast valyl-tRNA synthetase (Mr = 130,000) on non- denaturing gels and like the α subunit of yeast phenylalanyl-tRNA synthetase (Mr = 63,000) on SDS-polyacrylamide gels).

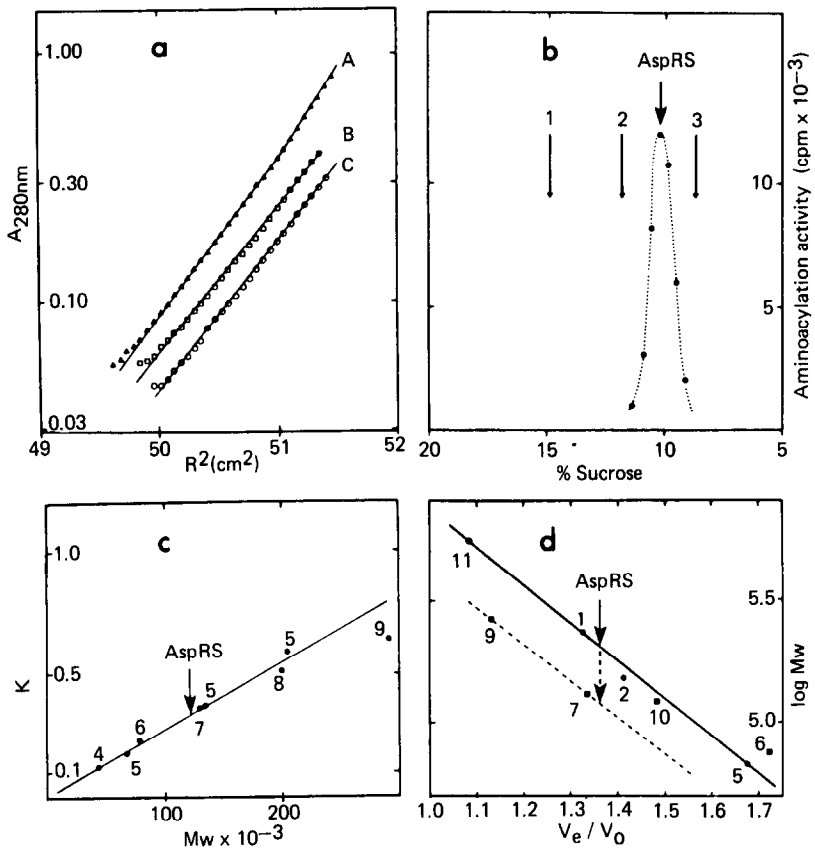

Fig.2 : Molecular weight determination of native aspartyl-tRNA synthetase. **(a)** Sedimentation equilibrium (the three runs represented correspond to loading concentrations of 0.18 (A), 0.14 (B) and 0.04 (C) mg.ml^{-1} and were done at 20°C). **(b)** Sucrose gradient centrifugation. The arrows show the positions of the reference proteins on the gradient. **(c)** Polyacrylamide gel electrophoresis (the reference curve and the K value for aspartyl-tRNA synthetase were obtained using 5, 6, 7, 8, 9 and 10% polyacrylamide gels). **(d)** Filtration on Sephadex G200; V_e is the effluent peak volume and V_o the void volume of the column (20.4 ml), the total volume of the column is 44 ml. The full straight line is the calibration curve obtained with globular proteins(•);V_e/V_o values for aminoacyl-tRNA synthetases of known molecular weight are indicated (■) (the doted line is a calibration curve obtained with phenylalanyl- and valyl-tRNA synthethases).In (b), (c) and (d) the marker molecules with their molecular weights were :1, catalase (240,000);2,alcohol dehydrogenase (150,000); 3,hemoglobin (67,000); 4, ovalbumin (44,000); 5, bovine serum albumin,monomer (67,000), dimer and trimer;6, yeast arginyl-tRNA synthetase(74,000);7, yeast valyl-tRNA synthetase (130,000);8, fumarase (200,000) ;9, yeast phenylalanyl-tRNA synthetase (276,000); 10, bean chloroplastic leucyl-tRNA synthetase (122,000) and 11, ferritin (550,000).

Yeast aspartyl-tRNA synthetase possesses some special aggregation properties. When dialyzed in aqueous buffers in the absence of glycerol part of the enzyme precipitates or is adsorbed on the dialysis tubing. This effect is reduced when ammonium sulfate (40% of saturation) or/and the non-ionic detergent octylglucoside are added in the medium. Interestingly the presence of ammonium sulfate (7) or of the detergent does not significantly modify the apparent tRNA aminoacylation properties of the enzyme. Furthermore when the

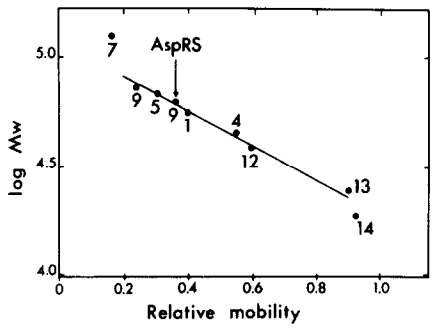

Fig.3 : Molecular weight determination of SDS denatured aspartyl-tRNA synthetase by disc gel electrophoresis. The reference curve was obtained with 8 proteins of known molecular weight treated before electrophoresis similarly to aspartyl-tRNA synthetase : 2 min incubation at 100°C in a total volume of 30 µl containing 0.5 - 1.0 µg protein, 1% SDS and 1% 2-mercaptoethanol. Marker proteins were : 12, aldolase (4 x 40,000); 13, chymotrypsinogen A (25,000); 14, trypsin inhibitor (21,000) and proteins listed in Figure 2 (proteins 1 and 9 are tetramers α_4 and $\alpha_2\beta_2$ respectively). The average position of aspartyl-tRNA synthetase on the reference curve is indicated by an arrow; when the protein migrates as a doublet the two bands migrate at each side of the α subunit of phenylalanyl-tRNA synthetase.

enzyme (at 1 to 5 $mg.ml^{-1}$) is mixed under low ionic strength conditions with small amounts of tRNA ([tRNA]/[enzyme]< 0.2) the protein precipitates, an effect reversed after addition of more tRNA. All these properties would indicate an hydrophobic character of aspartyl-tRNA synthetase. The average hydrophobicity of the enzyme (Table II), however, is not particularly high when compared to other proteins (24) and does not significantly deviate from values one can calculate for other aminoacyl-tRNA synthetases from their aminoacid composition. A likely explanation is that hydrophobicity is confered by well defined structural domains at the surface of the protein. The presence of such domains can be correlated with the ability of aminoacyl-tRNA synthetases to form multienzymatic aggregates in the presence of tRNA (7, 28) and in the case of eukaryotic enzymes, to form high molecular weight complexes containing a variable number of aminoacyl-tRNA synthetase species (reviewed in 25).

Partial enrichments and purification procedures of aspartyl-tRNA synthetases from different sources, including yeast, as well as determination of some structural and functional parameters of these enzymes have been reported in literature (25, 26, 29-37). When determined, the molecular weights of these synthetases were around 120,000 daltons, (33, 34, 36, 37) in agreement with the value presented in this work (Mr = 125,000 ± 5,000). Dimeric α_2 structures were described for aspartyl-tRNA synthetase from porcine thyroid (26), from **N.crassa** (32) and from **E.coli** (37). Interestingly enough and resembling properties of yeast aspartyl-tRNA synthetase is the multiband pattern of the porcine enzyme on SDS-polyacrylamide gels and the allotropism

of this protein (25, 26) suggesting the existence of similar structural properties among eukaryotic aspartyl-tRNA synthetases.

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