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PURIFICATION AND PROPERTIES OF ASPARAGINE SYNTHETASE FROM RAT LIVER

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Summary

Asparagine synthetase (L-aspartate:ammonia ligase (AMP-forming, EC 6.3.1.1) activity in rat liver increased when the animals were put on a low casein diet. The enzyme was purified about 280-fold from the supernatant of rat liver homogenate by a procedure comprising ammonium sulfate fractionation, DEAE-Sepharose column chromatography, and Sephadex G-100 gel filtration. The optimal pH of the enzyme was in the range 7.4—7.6 with glutamine as an amide donor. The molecular weight was estimated to be approximately 110 000 by gel filtration. Chloride ion was required for the enzyme activity. The apparent K_m values for L-aspartate, L-glutamine, ammonium chloride, ATP, and Cl^- were calculated to be 0.76, 4.3, 10, 0.14, and 1.7 mM, respectively. The activity was inhibited by L-asparagine, nucleoside triphosphates except ATP, and sulfhydryl reagents.

It has been observed that the properties of asparagine synthetase from rat liver are not so different from those of tumors such as Novikoff hepatoma and RADA 1.

Introduction

Biosynthesis and metabolism of asparagine have not been as fully clarified as glutamine, although asparagine is present in many animal and bacterial cells in the free state and as the constituent of proteins. Asparagine synthetase (L-aspartate: ammonia ligase (AMP-forming), EC 6.3.1.1) was first demonstrated by Ravel et al. [1] in *Lactobacillus arabinosus*, and similar enzymes have been studied in other bacteria [2,3]. These bacterial enzymes catalyze the conversion of L-aspartate to L-asparagine in the presence of ammonia (as an amide donor), ATP, and magnesium ion. In mammalian tissues, the biosynthesis of asparagine is catalyzed by glutamine-dependent asparagine synthetase, which

required the presence of L-glutamine or ammonium chloride at somewhat higher concentrations. The enzyme activity of tumors sensitive to the L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1), in contrast to those found to be resistant, have been shown to be either low or absent [4—7]. Asparaginase-resistant tumors such as Novikoff hepatoma [8] and mouse leukemia cell (RADA 1) [9] possess high asparagine synthetase activity, and have been used as sources for the purification and characterization of this enzyme. However, normal mammalian tissues have very low enzyme activity and little work has been published concerning this enzyme. The enzyme was partially purified about 15-fold from the tissues of guinea pig, which had a relatively high asparagine synthetase activity as compared with other animals [6,10,11].

Recently, we found that the asparagine synthetase activity of rat liver was increased when a diet containing 24% casein is changed for a low casein diet. In this paper, the purification and properties of the enzyme from these rat liver are presented and compared with those found in tumors.

Materials and Methods

Materials. L-[4-^{14}C]Aspartate and L-[U-^{14}C]aspartate were obtained from the Radiochemical Centre, Amersham, nucleotides were from Sigma Chemical Co., and carbamyl phosphate and *Escherichia coli* L-asparaginase (EC 3.5.1.1) (80 U/mg of protein) were from Boehringer mannheim. All reagents used were of analytical grade. Chows were supplied by Oriental Yeast Co.

Animals. Male Wistar strain rats weighing about 100 g were purchased. The rats were maintained on a diet containing 24% casein for approximately 1 month, followed by feeding on 15% casein diet for 5 days prior to killing. The body weight was 200—250 g at the time of use.

Enzyme assay. Asparagine synthetase activity was assayed based on measuring the radioactivity of L-[4-^{14}C]asparagine formed from L-[4-^{14}C]aspartate. The standard assay mixture contained 100 mM Tris/acetate, pH 7.8, 1.5 mM L-[4-^{14}C]aspartate (0.05 μCi), 20 mM L-glutamine, 10 mM magnesium acetate, 10 mM ATP, 20 mM KCl, and an aliquot of enzyme in a total volume of 0.5 ml. After incubation for 1 h at 37° C, the reaction was stopped by heating the mixture in boiling water for 5 min. The precipitate formed was removed by centrifugation, and an aliquot of supernatant was added on a column (0.5 × 5 cm) of Dowex 1 × 8 (200—400 mesh, formate type) equilibrated with 10 mM imidazol formate buffer, pH 7.0. L-[4-^{14}C]Asparagine formed from L-[4-^{14}C]-aspartate was separated from the latter by washing the column twice with 0.5 ml of the above buffer. The eluate was collected into directly scintillation vials, mixed with Bray's solution [12] and the radioactivity was measured by liquid scintillation counter.

Analyses. The formation of L-asparagine in the reaction mixture was confirmed by analyses as described below. The eluate from the column was concentrated and chromatographed using Whatman filter paper NO 2 with following solvent system: phenol/water (4 : 1, by vol.) and 2-propanol/formic acid/water (20 : 1 : 5, by vol.). The spots (visualized by spraying with 0.2% ninhydrin solution and heating) were cut out into vials, Bray's solution added and the radioactivity measured. The radioactivity was coincident with the spot of

L-asparagine. When crude extract of rat liver (as enzyme source) and L-[U-^{14}C]-aspartate were used, a substantial radioactive substance was present in a region distinct from that of L-asparagine. This substance, the amount of which was 2.5 times as high as that of L-asparagine, coincided with L-alanine on chromatogram and seems to be produced with a release of CO_2 at the site of C_4 of L-aspartate. Therefore L-[4-^{14}C]aspartate was used and, with a crude extract, asparagine formed in another assay mixture was degraded to aspartate throughly by adding *E. coli* L-asparaginase. The radioactivity in this fraction was subtracted from that of the fraction without added L-asparaginase. Synthesis of L-asparagine with the purified enzyme preparation was further confirmed by amino acid analysis.

Results

Effect of dietary casein content on the asparagine synthetase activity of rat liver. The activity of asparagine synthetase in rat liver increased as shown in Table I when a diet containing 24% casein was changed to one containing 15% casein.

Subcellular distribution of asparagine synthetase activity in rat liver. As shown in Table II, most of the enzyme activity measured by the use of L-glutamine as an amide donor was localized in the 105 000 × *g* supernatant fraction. The distribution of the ammonia-dependent enzyme activity was the same as that of the glutamine-dependent enzyme.

Purification of the enzyme. All procedures were carried out at 0—4°C.

Step 1. Preparation of crude extract: The rats were killed by a blow on the head and the livers were perfused thoroughly with cold saline and rapidly excised. Livers were immediately homogenized with 6 vols. of 0.05 M Tris · HCl buffer, pH 7.8, containing 0.25 M sucrose in Potter-Elvejhem Teflon/glass homogenizer. The homogenate was centrifuged at 20 000 × *g* for 30 min and the precipitate was removed.

Step 2. Ammonium sulfate fractionation: Powdered ammonium sulfate was added to the supernatant to give 35% saturation. After 20 min stirring, the precipitate formed was removed by centrifugation at 10 000 × *g* for 15 min. Additional ammonium sulfate was added to the supernatant to give 50% saturation

TABLE I

EFFECT OF DIETARY CASEIN CONTENT ON THE ASPARAGINE SYNTHETASE ACTIVITY OF RAT LIVER

Male Wistar rats (body wt. 130—170 g) maintained on a 24% casein diet were put on a 15% casein diet for 5 days, and the asparagine synthetase activity of liver extracts as described in the purification procedure was determined by the standard assay method. Results are given as mean values ± S.D. for the numbers of rats indicated in parentheses.

Diet	Asparagine synthetase activity (nmol/h per mg protein)
24% casein	0.60 ± 0.36 (10)
15% casein	7.9 ± 0.94 (7)

TABLE II

SUBCELLULAR DISTRIBUTION OF ASPARAGINE SYNTHETASE IN RAT LIVER

Rat liver was homogenized with 4 vols. of 3 mM Tris · HCl buffer, pH 7.8, containing 0.25 M sucrose. The homogenate was fractionated by a sequential centrifugation method as follows: 550 × *g*, 10 min; 10 000 × *g*, 15 min; 17 500 × *g*, 15 min; and 105 000 × *g*, 60 min. The sediments were suspended in a small volume of the above buffer and the activity of each fraction was measured by the standard assay method. Protein content was determined by the method of Lowry et al. [13] with bovine serum albumin as a standard.

Fraction	Specific activity (nmol/h per mg)	Percentage
Sediments:		
550 × *g*	0.03	0.4
10 000 × *g*	0.02	0.1
17 500 × *g*	0.5	0.9
105 000 × *g*	0.1	0
Supernatant	10.7	98

and the mixture was stirred for 20 min. The precipitate was collected by centrifugation and dissolved in 0.02 M Tris · HCl buffer, pH 7.8, containing 1 mM EDTA and 0.5 mM 2-mercaptoethanol (TEM buffer). Ammonium sulfate in this solution was removed by passing through a Sephadex G-50 column (4 × 40 cm) equilibrated and eluted with the TEM buffer.

Step 3. DEAE-Sepharose column chromatography: The desalted solution was applied to a DEAE-Sepharose column (3 × 24 cm) equilibrated with TEM buffer. The column was washed with the same buffer and the enzyme was eluted with linear gradient of NaCl (0—0.35 M) in TEM buffer. Fractions of 10 ml were collected at 24 ml/h and the elution pattern is presented in Fig. 1. Active fractions were pooled and concentrated with ammonium sulfate (50% saturation).

Step 4. Sephadex G-100 gel filtration: The precipitate in Step 3 was dissolved in 4 ml of TEM buffer containing 2 mM L-aspartate, 2 mM L-asparagine, 2 mM ATP, 11 mM magnesium acetate, and 30 mM KCl (supplemented TEM

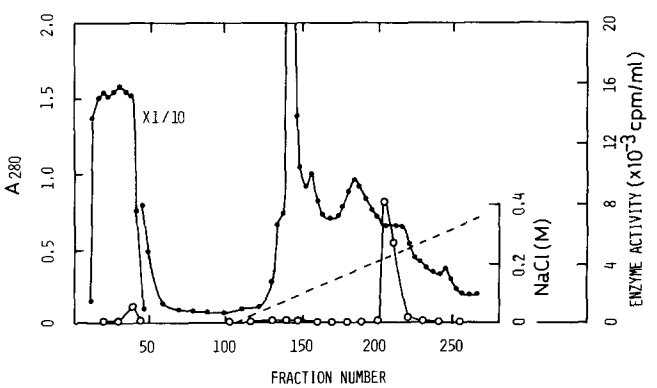


Fig. 1. Elution pattern of asparagine synthetase on DEAE-Sepharose column chromatography. (●), absorbance at 280 nm; (○), asparagine synthetase activity; (- - - - -), NaCl concentration.

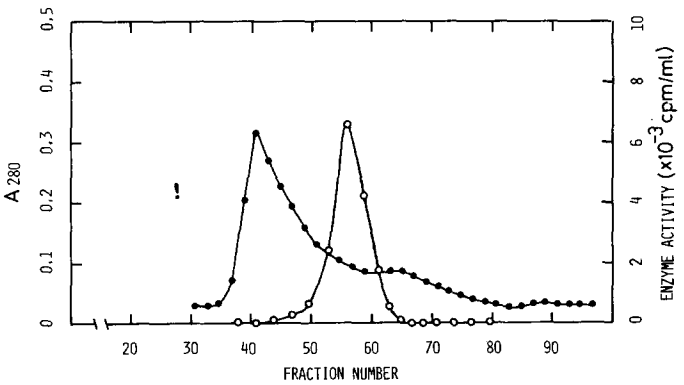


Fig. 2. Gel filtration of asparagine synthetase on Sephadex G-100. (•), absorbance at 280 nm; (○), asparagine synthetase activity.

buffer). The solution was layered on a Sephadex G-100 column (2.6 × 90 cm) equilibrated and eluted with supplemented TEM buffer. Fractions of 4 ml were collected at 17 ml/h and the elution pattern is shown in Fig. 2. Fractions with enzymatic activity were combined and used as purified asparagine synthetase throughout this work, unless otherwise stated. This preparation could be stored at −20° C for several months without loss of activity. Species included in the preparation for the stabilization of the enzyme were removed by Centriflo CF 25 membrane (Amicon Co.) just before use.

A summary of the purification of the enzyme is presented in Table III.

Estimation of molecular weight. The molecular weight of the enzyme was estimated to be 110 000 by gel filtration on Sephadex G-100.

Effect of pH on the enzyme activity. The enzyme activity was measured at various pH in 0.1 M Tris/acetate buffer. As shown in Fig. 3, optimal activity was observed in the range pH 7.4—7.6 when glutamine was used as an amide donor. While the activity increased sharply as pH was raised from 5 to 9 when ammonium chloride was used. This supports the proposal that active species for the amide donor is ammonia rather than ammonium ion [8,10].

Stability of the enzyme. The enzyme was most stable around pH 8 at 4° C,

TABLE III

SUMMARY OF PURIFICATION OF ASPARAGINE SYNTHETASE FROM RAT LIVER

The purification was carried out as described in the text, starting from 150 g of rat liver.

Step	Protein (mg)	Activity (nmol/h)	Specific activity (nmol/h per mg)	Yield (%)	Purification (-fold)
Crude extract	13 320	33 300	2.5	100	1
Ammonium sulfate	3 633	19 618	5.4	59	2.2
DEAE-Sepharose	34	4 885	142	14.7	57
Sephadex G-100	2.9	2 036	702	6.1	281

TABLE IV

EFFECT OF SUBSTRATES AND PRODUCTS ON THE STABILIZATION OF ASPARAGINE SYNTHETASE

In this experiment the enzyme preparation at Step 3 in the purification was used. The preparation was dissolved in TEM buffer in the text and passed through a Sephadex G-50 column (1.8 × 14 cm) to remove ammonium sulfate. L-Asp, $Mg(CH_3COO)_2$, ATP, L-Asn, and L-Glu were added to the enzyme solution, and samples were assayed by the standard method before and after incubation of the solutions at 50°C for 20 min.

Addition	Enzyme activity remaining (%)
2 mM L-Asp	0
2 mM ATP	5
11 mM Mg^{2+}	10
2 mM L-Asn	2
2 mM L-Asp and 11 mM Mg^{2+}	7
2 mM ATP and 11 mM Mg^{2+}	36
2 mM ATP and 2 mM L-Asp	0
2 mM ATP, 2 mM L-Asp, and 11 mM Mg^{2+}	89
2 mM ATP, 2 mM L-Asp, 2 mM L-Asn, and 11 mM Mg^{2+}	100
2 mM ATP, 2 mM L-Asp, 2 mM L-Glu, and 11 mM Mg^{2+}	86
None	4

but the activity decreased rapidly even at pH 8 when the enzyme was allowed to stand at room temperature. The effect of substrates and products on stabilization for the enzyme is shown in Table IV. L-Asparagine, ATP, Mg^{2+}, and L-aspartate had no effect individually on a protection of the enzyme from heat inactivation, but the enzyme was protected significantly by a combination of L-aspartate, ATP, and Mg^{2+}.

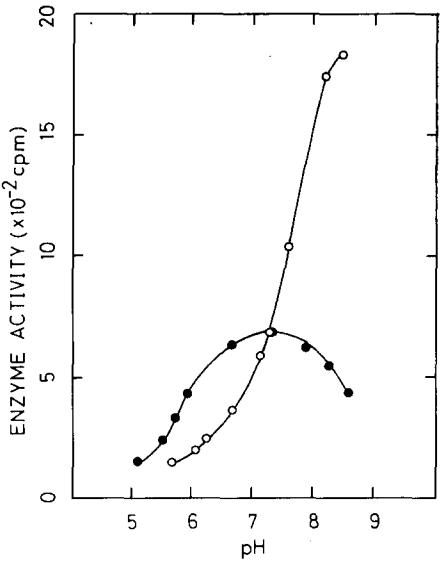
Glutaminase activity of the enzyme. The enzyme preparation exhibited the activity to hydrolyze L-glutamine in the medium lacking an amide group acceptor, as observed in other glutamine-utilizing enzymes [14—16]. As indicated in Table V, the amount of L-glutamate formed in the complete assay system was higher than that of L-asparagine. The amount of L-glutamate produced in the

TABLE V

GLUTAMINASE ACTIVITY OF ASPARAGINE SYNTHETASE

The reaction mixture contained the component similar to the standard assay mixture of asparagine synthetase in a final volume of 1.0 ml. After incubating for 2 h at 37°C, the reaction was stopped by heating in boiling water for 5 min. The formation of L-Glu and L-Asn was determined by the method of Bernt and Bergmeyer [17]. The values indicated for L-Glu were corrected by subtracting blank value which was exhibited in the absence of the enzyme.

Assay condition	Product formed	
	L-Glu (nmol)	L-Asn (nmol)
Complete	70	38
Minus L-Asp	66	



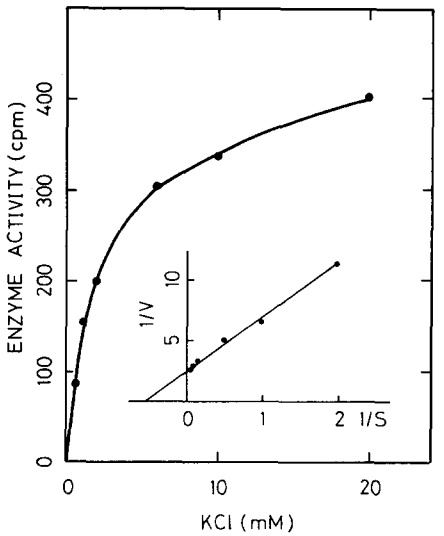


Fig. 3. Effect of pH on the activity of asparagine synthetase from rat liver. Reaction was carried out in the 0.1 M Tris/acetate buffer at various pH. (●), glutamine-dependent enzyme activity in the standard assay medium; (○), ammonia-dependent enzyme activity in the medium containing ammonium chloride in place of glutamine and KCl. The procedure of assay was modified as follows. After stopping the reaction, the reaction mixture was diluted 2-fold with water. 0.4 ml of this solution was removed and adjusted to pH 7.8 by adding 0.1 ml of 0.5 M Tris/acetate buffer of appropriate pH prior to applying on Dowex column.

Fig. 4. Response curve and double reciprocal plot of asparagine synthetase activity against concentration of KCl. The activity was measured in the standard assay condition.

TABLE VI

EFFECT OF VARIOUS COMPOUNDS ON PURIFIED ASPARAGINE SYNTHETASE FROM RAT LIVER

The activity was measured in the standard assay medium containing various compounds at each concentration. In the examination of sulfhydryl reagents the enzyme was preincubated at 37°C for 5 min and remaining activity was measured. The concentration of sulfhydryl reagent in preincubating medium are shown. The activity was expressed as a percentage of that in the control experiment. Abbreviation: PCMB, *p*-chloromercuribenzoate.

Compound	Concentration (mM)	Relative activity (%)	Compound	Concentration (mM)	Relative activity (%)
L-Asn	10	15	Carbamyl phosphate	10	54
L-Glu	10	98	PP_i	10	43
Gly	10	98	PCMB	5	0
L-Ala	10	98		1	11
L-Ser	10	101	*N*-Ethyl-maleimide	5	0
D-Asp	10	78		1	16
GTP	10	31	Iodoacet-amide	5	11
ADP	10	59		1	35
AMP	10	74			
GMP	10	108			
IMP	10	106	None		100

assay system lacking L-aspartate was nearly equivalent to that of L-glutamate formed in the standard complete assay system. Therefore, an excess formation of L-glutamate in the reaction seems to be due to asparagine synthetase itself rather than a contaminant glutaminase, as explained by Meister [18].

Requirement of chloride ion and K_m values for various compounds. Chloride ion was required for the optimal enzyme activity in rat liver asparagine synthetase as in the case of RADA 1 enzyme [9] when glutamine was used as an amide donor. A response curve and double reciprocal plot for various chloride ion concentrations are shown in Fig. 4. The apparent K_m value for Cl^- was calculated to be 1.7 mM. The K_m values for L-aspartate, L-glutamine, ammonium chloride, and ATP in the standard assay condition were found to be 0.76, 4.3, 10, and 0.14 mM, respectively.

Effect of various compounds on the enzyme activity. Inhibitory effect of amino acids, nucleotides, and other various compounds on the asparagine synthetase activity was examined (Table VI). L-Asparagine inhibited the enzyme activity about 85%, but L-glutamate did not affect the activity. Other amino acids had no effect, and D-aspartate had a small effect on the enzyme activity. Nucleoside triphosphates such as GTP, ITP, CTP, and UTP inhibited the activity considerably (44—70%) and nucleoside diphosphates of ADP and GDP to a lesser extent. Little effect was shown by GMP, IMP, CMP, and UMP. Inhibition by AMP, which has been reported to be a reaction product [1—3,8,9], was less than with ADP. Carbamyl phosphate, an inhibitor of glutamine synthetase of rat liver [19] and *E. coli* [20], inhibited the asparagine synthetase activity, as did sulfhydryl reagents.

Discussion

Asparagine synthetases in bacteria and L-asparaginase-resistant tumors have been purified and characterized. But in normal mammalian tissues the enzyme activity was very low and little work has been done on this enzyme. When rats were fed on a low protein diet, asparagine synthetase activity in the liver increased markedly. In this experiment, asparagine synthetase was purified about 280-fold from these rat livers by a procedure consisting of ammonium sulfate fractionation, DEAE-Sepharose column chromatography, and gel filtration. Chromatography on DEAE-Sepharose was the most efficient purification procedure.

The purified enzyme showed the apparent K_m values for L-aspartate, ammonium chloride, ATP, and Cl^- similar to that in RADA 1 enzyme [9], but the value for L-glutamine was calculated at 4.3 mM. This is about 4 times as high as that of the enzyme from Novikoff hepatoma [8] and RADA 1 [9] (for L-glutamine 1 and 0.9 mM, respectively). This high K_m value for L-glutamine may explain the observed low asparagine synthetase activity in rat liver. The molecular weights of asparagine synthetase from *E. coli* and RADA 1 have been estimated to be 82 000 and 105 000, respectively. With purified rat liver enzyme, the molecular weight was calculated to be 110 000. The thermal stability of the enzyme at 50° C was similar to that from *E. coli* [21].

It has been reported that glutamine amidotransferase such as anthranilate synthetase [14], carbamyl phosphate synthetase [15], and formylglycinamide

ribonucleotide amidotransferase [16] have glutaminase activity in the absence of the substance which accepts the amide nitrogen of glutamine. However, these reactions proceed more slowly than in the complete system. In the reaction of RADA 1 asparagine synthetase, Horowitz and Meister [9] have reported that the formation of L-glutamate was higher than that of L-asparagine, and even in the medium lacking L-aspartate the amount of L-glutamate formed was equivalent to that in the complete assay system. Meister [18] proposed the possibility that the above phenomenon observed with RADA 1 asparagine synthetase may be a reflection of the neoplastic process as follows: this enzyme was produced by tumor cells in amounts much greater than produced by normal tissues. The augmentation of asparagine synthetase by tumor cell is in some way related to the synthesis of an "imperfect" enzyme which does not utilize glutamine efficienctly. It is conceivable that the glutaminase activity exhibited by the asparagine synthetase may serve a useful function in the metabolism of the tumor cell. However, a similar phenomenon was observed in purified rat liver asparagine synthetase. Therefore, it seems probable that the above property exists only in asparagine synthetase and is not characteristic of the enzyme from tumors only.

Horowitz and Meister [9] showed that glutamine-dependent asparagine synthetase activity from RADA 1 required chloride ion. This property seems to be unique in the glutamine amidotransferase enzymes, and it was found that asparagine synthetase from rat liver had the same property.

From these data in this experiment, it appears that the properties of asparagine synthetase from rat liver are similar to those of Novikoff hepatoma and RADA 1.

When crude extracts of rat liver were used as an enzyme source, the formation of L-alanine was observed in the reaction mixture. It might be possible that the enzymatic 4-decarboxylation mechanism of L-aspartate exists in the crude rat liver extract.

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