L-Asparaginase from *Proteus vulgaris*. Purification, Crystallization, and Enzymic Properties*

Tetsuya Tosa,† Ryujiro Sano, Kozo Yamamoto, Masatoshi Nakamura, and Ichiro Chibata

ABSTRACT: L-Asparaginase from *Proteus vulgaris* was purified by the following steps: cell lysis by lysozyme and toluene, pH treatment, ammonium sulfate fractionation, Sephadex G-100 gel filtration, DEAE-Sephadex chromatography, and crystallization by the addition of ammonium sulfate. This procedure yields the crystalline enzyme with a 30% recovery of the activity in crude extracts. The crystalline enzyme appears to be homogeneous, as judged by ultracentrifugation, disc electrophoresis, and isoelectric focusing with carrier ampholytes. Isoelectric point is 5.08. Specific activity of this crystalline enzyme is 300 IU/mg. The crystalline enzyme hydrolyzes L-asparagine, D-asparagine, L-glutamine, and some analogs of L-asparagine. Michaelis constants for L-asparagine, D-asparagine, and L-glutamine are 2.6×10^{-5}, 4.3×10^{-4} M, and 5×10^{-3} M, respectively. The pH optimum for L-asparagine hydrolysis is between pH 7 and 8. Although the enzyme is inactivated by heat, organic solvents, and chymotrypsin treatments, the presence of L-asparagine or its analogs protects the enzyme from the inactivation caused by these treatments. The enzyme activity is cleared from mouse and rabbit plasma with half-life values of 110 and 130 min, respectively.

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is an important enzyme as a drug for some tumors in man (Oettgen *et al.*, 1967; Hill *et al.*, 1969). A number of papers have been published on the production (Schwartz *et al.*, 1966; Cedar and Schwartz, 1968; Roberts *et al.*, 1968; Bilimoria, 1969; Robison and Berk, 1969), purification (Campbell *et al.*, 1967; Roberts *et al.*, 1968; Wagner *et al.*, 1969; Whelan and Wriston, 1969; Arens *et al.*, 1970; Ho *et al.*, 1970; Kristiansen *et al.*, 1970; Staerk *et al.*, 1970; Nakamura *et al.*, 1971), and enzymic properties (Campbell and Mashburn, 1969; Arens *et al.*, 1970; Cooney and Davis, 1970; Ho *et al.*, 1970; Lauinger and Ressler, 1970; Nakamura *et al.*, 1971) of the enzyme from *Escherichia coli*.

We previously observed that L-asparaginase from *Proteus vulgaris* also has an antitumor activity and that it differs immunologically from the *E. coli* enzyme. We also investigated the effects of cultural conditions of *P. vulgaris* on the enzyme production (Tosa *et al.*, 1971).

The present paper describes the purification of L-asparaginase from *P. vulgaris* and some enzymic properties of the crystalline enzyme.

Materials and Methods

Materials. L-Asparagine and L-aspartic acid were the products of Tanabe Seiyaku Co. Ltd., Osaka, Japan. *N*-α-Methyl-L-asparagine was prepared as described by Groot and Lichtenstein (1960), L-aspartic acid β-hydrazide was prepared as described by Roper and McIlwain (1948), and L-2-amino-2-carboxyethanesulfonamide was prepared as described by Heymann *et al.* (1959). Amino acid hydroxamates, D-aspartic acid, *N*-α-carbobenzoxy-L-asparagine, and α-chymotrypsin (42 U/mg) were purchased from Sigma Chemical Co., St. Louis, Mo. D-Asparagine, L-glutamine, and *S*-carbamyl-L-cysteine were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Sephadex G-100 and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. D-Glutamic acid, D-glutamine, and Nessler's reagent were obtained from Nakarai Chemicals, Co. Ltd., Kyoto, Japan. *n*-Butyramide and propionamide were obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. Lysozyme (55,700 U/mg) was obtained from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. All chemicals used in this study were either reagent or spectroscopic grade.

Organism. P. vulgaris OUT 8226 used in this study was obtained from the Faculty of Engineering, Osaka University, Osaka, Japan.

Culture of Bacteria. P. vulgaria was cultured under aerobic conditions at 30° for 18 hr in 100 l. of the medium containing 1% sodium fumarate and 5% corn steep liquor (pH 7.0). The cells were harvested by centrifugation. Approximately 2.1 kg of cells (wet weight) was obtained.

Enzyme Reactions. Unless otherwise noted, L-asparaginase activity was determined under the standard conditions previously described (Tosa *et al.*, 1971). For the studies on hydrolytic rate of D-asparagine, L-glutamine, D-glutamine, *N*-α-methyl-L-asparagine, *N*-α-carbobenzoxy-L-asparagine, L-aspartic acid β-hydrazide, *S*-carbamyl-L-cysteine, 2-amino-2-carboxyethanesulfonamide, *n*-butyramide, propionamide, and amino acid hydroxamates, the enzyme reaction was carried out under standard conditions substituting these compounds for L-asparagine. Liberated products and remaining substrates were determined as follows. Ammonia was determined by Nesslerization combining with Conway's microdiffusion and L-aspartic acid was determined manometrically using L-aspartic acid β-decarboxylase from *Pseudomonas dacunhae* (Chibata *et al.*, 1965). Amino acid hydroxamates were determined by the method of Lipmann and Tuttle (1945).

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as a standard. After the crystalline enzyme was obtained, the extinction coefficient ($E^{1\%}_{1cm}$ 6.6 at 280 mμ) was used to determine protein concentration. The extinction coefficient was obtained as follows. Crystalline enzyme, dried over phosphoric anhydride, was dissolved in 0.05 M sodium phosphate buffer (pH 7.0), and the optical measurement was made with Hitachi EPS-2T recording spectrophotometer.

* From the Department of Applied Biochemistry, Chemical Research Laboratory, Tanabe Seiyaku Co., Ltd., Kashima-cho Higashiyodogawa-ku, Osaka, Japan. *Received September 14, 1971.*

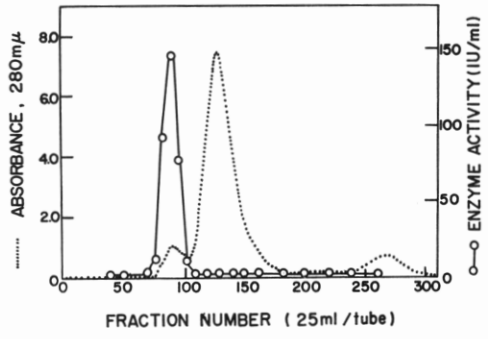

FIGURE 1: Sephadex G-100 gel filtration of L-asparaginase from ammonium sulfate fraction (50–90% saturation). Conditions are given in the text.

Enzyme Unit. One enzyme unit (IU) is defined as that amount of enzyme which hydrolyzes 1 μmole of substrate/min under the conditions of the assay. Specific activity is expressed as units per milligram of protein.

Ultracentrifugal Analysis. A Beckman-Spinco Model E analytical ultracentrifuge was used and sedimentation pattern was followed with schlieren optics.

Disc Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Davis (1964), but sample gel was omitted. The enzyme (50 μg) was placed on the top of spacer gel in 1 M sucrose. Electrophoresis was conducted at room temperature at 4 mA/tube for 80 min. The gels were stained with 1% Amido-Schwarz in 7% acetic acid.

Isoelectric Focusing. Isoelectric focusing was carried out by the method of Vesterberg and Svensson (1966). The carrier ampholytes (LKB-Produkter AB) used in this study were selected to give a gradient of pH 3–10, and the density gradient was made up with sucrose. Enzyme (5 mg) was applied and focused for 64 hr at 4° with a final potential of 900 V.

Clearance of L-Asparaginase from Plasma. Male mice (dd strain, 25 g) and male rabbits (2 kg) were used in this experiment. The animals were injected intravenously with L-asparaginase (300 IU/kg of body weight) dissolved in a physiological saline solution. They were bled into heparinized tubes from the carotid artery (mice) or from auricular vein (rabbits) at the indicated intervals after a single injection of the enzyme. The plasma was collected by centrifugation, and L-asparaginase activity in it was determined immediately.

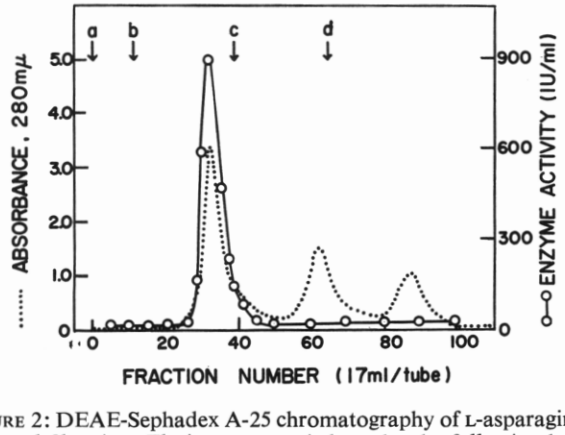

FIGURE 2: DEAE-Sephadex A-25 chromatography of L-asparaginase from gel filtration. Elution was carried out by the following buffer changes: (a) 0.01 M phosphate, (b) 0.01 M phosphate + 0.05 M NaCl (c) 0.01 M phosphate + 0.2 M NaCl, (d) 0.01 M phosphate + 1 M NaCl. Other conditions are given in the text.

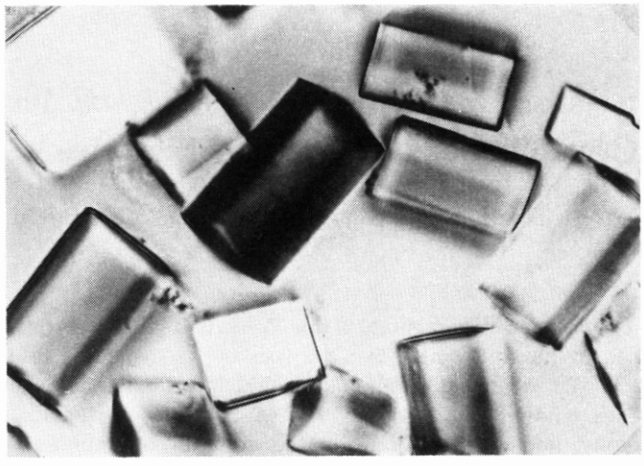

FIGURE 3: Crystals of L-asparaginase from *P. vulgaris* (×600).

Results

Purification Procedure of L-Asparaginase. All of steps except step I were carried out at around 5°.

STEP I. Cells harvested from 100 l. of medium were suspended in 5 l. of 0.05 M phosphate buffer (pH 8.0). To this suspension 1.05 g of lysozyme and 50 ml of toluene were added, and the mixture was incubated for 24 hr at 30° with gentle stirring. After the addition of an equal volume of water, cell debris was removed by centrifugation, and crude extract was obtained.

STEP II. The crude extract was adjusted the pH to 4.5 with the dropwise addition of 50% aqueous acetic acid, and allowed to stand for 2 hr. The precipitate was discarded after centrifugation.

STEP III. The supernatant was brought to 50% saturation with ammonium sulfate and the precipitate was removed by centrifugation. To the supernatant ammonium sulfate was added to 90% saturation. The resulting precipitate was collected by centrifugation, and dissolved in 100 ml of water. The solution was dialyzed over night against 0.01 M phosphate buffer containing 0.05 M sodium chloride (pH 6.8). Any precipitate which was formed during dialysis was removed by centrifugation.

STEP IV. The supernatant was applied to a Sephadex G-100 column (10 × 80 cm) equilibrated with 0.01 M phosphate

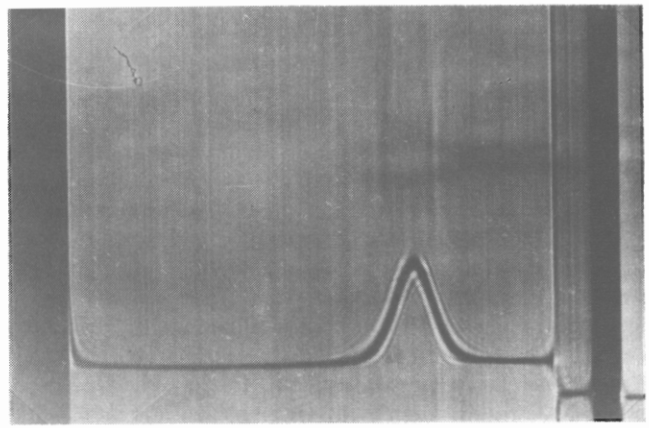

FIGURE 4: Schlieren pattern of crystalline L-asparaginase in 0.1 M sodium acetate buffer (pH 6.8). Picture was taken 37 min after reaching a speed of 52,640 rpm. Protein concentration was 0.6%. Temperature was 23.3°.

TABLE I: Purification of L-Asparaginase from *P. vulgaris*.

Steps	Vol (ml)	Total Protein (mg)	Sp Act. (IU/mg)	Total Act. (IU)	Yield (%)
I. Crude extract	10,500	198,200	1.1	218,000	100
II. pH 4.5 supernatant	9,300	94,200	1.9	179,000	82
III. Ammonium sulfate (50–90%)	420	10,200	13.2	135,000	62
IV. Sephadex G-100	750	660	182	120,000	55
V. DEAE-Sephadex	136	279	280	78,000	36
VI. Crystals	10	217	300	65,000	30

buffer containing 0.05 M sodium chloride (pH 6.8). The enzyme was eluted with the same buffer. The flow rate was approximately 150 ml/hr. The elution pattern is shown in Figure 1.

STEP V. The active fractions in step IV were collected and saturated with ammonium sulfate. The resulting precipitate was collected by centrifugation, dissolved in 30 ml of 0.01 M phosphate buffer (pH 6.8), and dialyzed against the same buffer over night. The enzyme solution was applied to a DEAE-Sephadex A-25 column (2.5 × 90 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the same buffer, the enzyme was eluted stepwise with the buffer supplemented with sodium chloride. The flow rate was approximately 60 ml/hr. The enzyme activity was found only in the fraction eluted with 0.01 M phosphate buffer supplemented with 0.05 M sodium chloride (pH 6.8). The detailed elution pattern is shown in Figure 2. The active fractions were collected, and concentrated by the addition of ammonium sulfate to 90% saturation. The precipitated protein was collected by centrifugation and dissolved in 0.01 M phosphate buffer (pH 6.8) at the concentration of 2%.

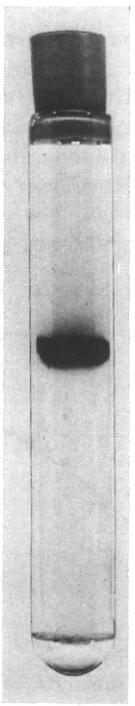

FIGURE 5: Disc electrophoresis of crystalline L-asparaginase. The direction of migration is from the cathod (top of photo) to the anode. Other conditions are given in the text.

STEP VI. To the enzyme solution solid ammonium sulfate was added until a slight turbidity was observed. The faint insoluble material was discarded by centrifugation. From the supernatant the enzyme crystallized by standing over night. The crystals took the form as shown in Figure 3. The procedure described here usually yielded the crystalline enzyme about 300-fold purification with a recovery of 30% of the enzyme activity in crude extract. The specific activity of the crystalline enzyme was 300 IU/mg of protein and was not enhanced by recrystallization. A summary of the purification is presented in Table I.

Purity of Crystalline Enzyme. The purity of crystalline enzyme was investigated as follows.

ULTRACENTRIFUGAL ANALYSIS. Figure 4 shows the sedimentation pattern of crystalline enzyme. The crystalline L-asparaginase appeared to be homogeneous in this pattern.

DISC GEL ELECTROPHORESIS. Disc gel electrophoresis of crystalline enzyme yielded only one protein band on the gel, as shown in Figure 5.

ISOELECTRIC FOCUSING. On the basis of isoelectric focusing with carrier ampholytes, this crystalline L-asparaginase appeared to be a homogeneous substance having a pI of 5.08 (Figure 6).

Stability of L-Asparaginase. As a suspension in 3.5 M ammonium sulfate the crystalline enzyme was stable for one year at 5° without decrease of enzyme activity. In 0.05 M borate buffer (pH 8.4), the enzyme at the concentration of 0.01% may be stored at 37° for 24 hr, at 5° for 1 week, and at −20° for 1 month without considerable loss of activity.

Substrate Specificity. The hydrolysing activities of L-asparaginase toward various L-asparagine analogs are shown in Table II. The rate of hydrolysis of L-glutamine is only 2%

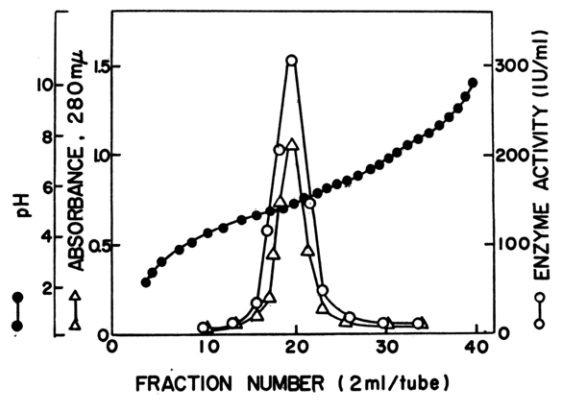

FIGURE 6: Isoelectric focusing of crystalline L-asparaginase. Conditions are given in the text.

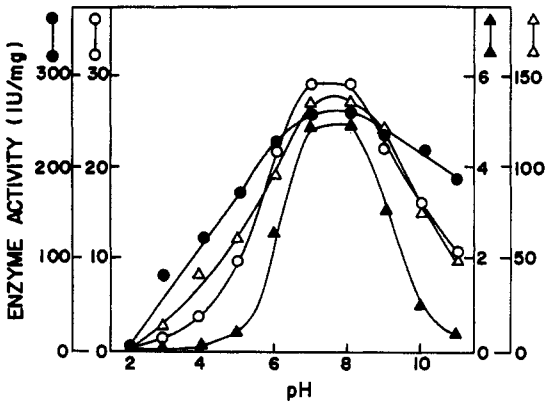

FIGURE 7: Effect of pH on the hydrolysis of L-asparagine (●), D-asparagine (○), L-glutamine (▲), and L-aspartic acid β-hydroxamate (△). The enzyme assay was carried out under standard conditions except for the substrates and buffers employed. The following buffers were used: pH 2–4, sodium acetate–hydrochloric acid buffer; pH 4–6, sodium acetate–acetic acid buffer; pH 6–8, sodium phosphate dibasic–potassium phosphate monobasic buffer; pH 8–11 sodium carbonate–boric acid buffer.

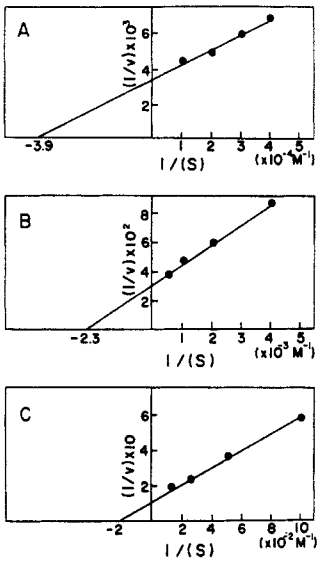

FIGURE 8: Lineweaver–Burk plots for the L-asparaginase reaction. (A) L-Asparagine, (B) D-asparagine, and (C) L-glutamine. In the cases of L-asparagine and D-asparagine the following assay method was employed. The enzyme reaction was started by the addition of 1 ml of preincubated enzyme solution into 4 ml of prewarmed substrate solution, and the mixture was incubated for 10 min. The reaction was stopped by the addition of 1 ml of Nessler's reagent and measured the optical density at 420 mμ after 30 min. In the case of L-glutamine, its hydrolytic velocity was measured by the standard assay method. The reaction velocity (v) is expressed as micromoles of liberated ammonia per minute per milligram of enzyme. Michaelis constant (k_m) was determined from Lineweaver–Burk plots.

of that of L-asparagine. The enzyme hydrolyzed some L-asparagine derivatives such as *N*-α-methyl-L-asparagine, L-aspartic acid β-hydroxamate, and L-aspartic acid-β-hydrazide but at a slower rate than L-asparagine. D isomers of asparagine, aspartic acid β-hydroxamate and glutamine were also hydrolyzed about 3–10% of the rate for corresponding L isomers.

Effect of pH. The pH dependence of the rates of hydrolysis of L-asparagine, D-asparagine, L-glutamine, and L-aspartic acid β-hydroxamate is shown in Figure 7. The optimum pH range for these substrates is 7.0–8.0.

TABLE II: Substrate Specificity of L-Asparaginase.[a]

Compounds	Enzyme Act. IU/mg[b]	Rel Rate of Hydrolysis
L-Asparagine	300	100
D-Asparagine	29	10
L-Glutamine	6	2
D-Glutamine	0.2	0.1
N-α-Methyl-L-asparagine	77	26
N-α-Carbobenzoxy-L-asparagine	0	0
L-Aspartic acid β-hydroxamate[c]	178	59
D-Aspartic acid β-hydroxamate[c]	18	6
L-Aspartic acid β-hydrazide[d]	6	2
L-Glutamic acid γ-hydroxamate[c]	3	1
L-Isoleucine α-hydroxamate[c]	0	0
L-Valine α-hydroxamate[c]	0	0
2-Amino-2-carboxyethanesulfonamide	0	0
S-Carbamyl-L-cysteine	0	0
n-Butyramide	0	0
Propionamide	0	0

[a] Conditions are given in the text. [b] Compounds hydrolyzed in micromoles per minute per milligram of protein. [c] After enzyme reaction, the remaining compound was estimated, and hydrolytic velocity was calculated by the decrease of original amounts. [d] L-Aspartic acid formation was determined.

Kinetic Constants for Several Substrates. The effect of substrate concentration on the enzyme activity is shown in Figure 8. K_m values of 2.6×10^{-3} M for L-asparagine, 4.3×10^{-4} M for D-asparagine, and 5×10^{-3} M for L-glutamine were obtained.

Effect of Temperature on the Reaction Rate. Figure 9 shows the rate of hydrolysis for L-asparagine as a function of temperature. The highest reaction rate was observed at 57°. The apparent activation energies were calculated to be 6630 cal/mole below 35° and 2470 cal/mole above 35°.

Protective Effect of L-Asparagine and Its Analogs on L-Asparaginase from Inactivation. The enzyme activity was decreased by heat, organic solvents, and protease treatments,

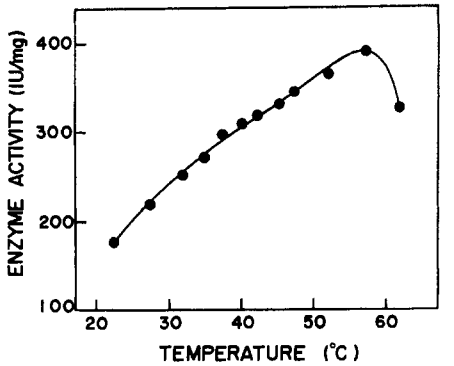

FIGURE 9: Effect of temperature on the hydrolysis of L-asparagine. The enzyme assay was carried out under the standard conditions modified by varying the temperature.

TABLE III: Effect of L-Asparagine and L-Aspartic Acid on L-Asparaginase Inactivation.[a]

Treatments	Remaining Activity (%) Addition of: NaCl	L-Asn	L-Asp
None	100	100	100
Heat (°C) 65	58	62	68
70	44	51	54
Organic solvents (%)			
Acetone 33	64	100	100
50	44	100	67
Ethanol 20	85	100	100
33	10	92	100
Chymotrypsin (min) 5	48	71	72
20	3	31	29

[a] Crystalline L-asparaginase was dissolved in 0.1 M borate buffer (pH 8.4) at the concentration of 0.05% and this enzyme solution was treated as follows. In the case of thermal inactivation, 4 ml of 0.0125 M additional compound solution (sodium chloride, L-asparagine, or sodium L-aspartate) was added to 1 ml of enzyme solution and the mixtures were heated in a water bath for 30 min. In the case of organic solvent treatment, 4 ml of 0.125 M additional compound solution containing prescribed acetone or ethanol was added to 1 ml of enzyme solution. The mixtures were allowed to stand at 25° for 1 hr, and then diluted with borate buffer. In the case of chymotrypsin digestion, 4 ml of 0.125 M additional compound solution equilibrated at 37° containing 25 μg of chymotrypsin was added to 1 ml of enzyme solution and the mixtures were incubated at 37° for specified period. After these treatments, remaining enzyme activity was immediately determined by the standard assay system.

TABLE IV: Effect of L-Asparagine and Its Analogs on the Inactivation of L-Asparaginase by Organic Solvents.[a]

Compounds	Remaining Activity (%) Solvents: Acetone	Ethanol
None	11	4
L-Asparagine	100	95
D-Asparagine	100	100
L-Aspartic acid	100	82
L-Glutamine	100	68
D-Glutamine	100	51
L-Glutamic acid	100	5
L-Aspartic acid β-hydroxamate	100	100
L-Aspartic acid β-hydrazide	100	100
S-Carbamyl-L-cysteine	3	2
L-Valine	18	3

[a] Crystalline L-asparaginase was dissolved in 0.05 M borate buffer (pH 8.4) at the concentration of 0.1% and this enzyme solution was treated as follows. To 1 ml of enzyme solution, 100 μmoles of compounds was added. Then 1 ml of acetone or ethanol was added and the mixtures were allowed to stand at 5° for 16 hr. The mixtures were diluted with borate buffer (0.05 M, pH 8.4) and remaining enzyme activity was determined by the standard system.

but the addition of L-asparagine or L-aspartic acid protected the enzyme from these inactivating treatments, especially from organic solvent treatments (Table III). Other substrates or products of L-asparaginase also effective for protection of the enzyme against inactivation by organic solvents (Table IV).

Clearance of L-Asparaginase from Plasma. The rate of disappearance of L-asparaginase activity from mouse and rabbit plasma was determined after a single intravenous injection of the enzyme. The decrease in enzyme activity followed first-order kinetics (Figure 10). Half-life values of the enzyme in mouse and rabbit plasma were 110 and 130 min, respectively.

Discussion

L-Asparaginase has been purified about 300-fold and crystallized with a recovery of 30% from the crude extract of *P. vulgaris*. The crystalline enzyme is homogeneous by the criteria of ultracentrifugation, disc electrophoresis, and isoelectric focusing, and has a specific activity of 300 IU per mg of protein. On the other hand, a number of reports (Roberts *et al.*, 1968; Whelan and Wriston, 1969; Arens *et al.*, 1970; Nakamura *et al.*, 1971) have been published on the specific activity of homogeneous L-asparaginase from *E. coli*, and values of 280–620 IU/mg of protein have been reported. These discrepancies may have resulted from the different methods employed for protein determination. In fact, Arens *et al.* (1970) reported that none of five pure *E. coli* L-asparaginase preparations from different laboratories had a specific activity higher than 280 IU/mg of protein. Therefore, *P. vulgaris* L-asparaginase may be considered to have the almost same specific activity as *E. coli* L-asparaginase.

A number of papers have been published on the substrate specificity of *E. coli* L-asparaginase. Cooney and Handschumacher (1970) summarized these results and noted that the enzyme can hydrolyze the C–N bonds such as amide, hydroxamate, hydrazide, diazoketone, and nitril of L-asparagine and its analogs. *P. vulgaris* L-asparaginase has a similarly broad substrate specificity (Table II).

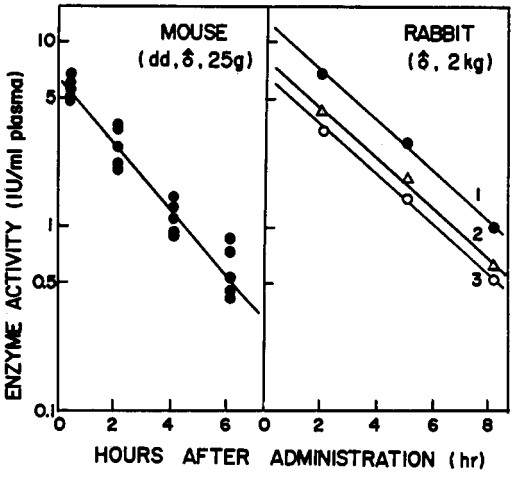

FIGURE 10: Disappearance of L-asparaginase from normal mouse and rabbit plasma. Conditions are given in the text.

The Michaelis constant of *P. vulgaris* L-asparaginase for L-asparagine is 2.6×10^{-5} M. That of *E. coli* enzyme has been reported to be 1.25×10^{-5} M (Broome, 1968), 1.15×10^{-5} M (Ho *et al.*, 1970), and 1.1×10^{-5} M (Lauinger and Ressler, 1970). No significant difference is found between the two enzymes.

It is well known that enzyme is, in general, stabilized by the presence of substrate or substrate analogs. In the case of *P. vulgaris* L-asparaginase, enzyme activity is protected by the presence of substrate or product from the inactivation by heat, organic solvents, and chymotrypsin. L-Valine or *S*-carbamyl-L-cysteine, which is not a substrate for L-asparaginase, showed no protective effect. These results suggest that conformational changes in the protein structure of L-asparaginase occur in the presence of substrates or products.

Boyse *et al.* (1967), Broome (1968), Campbell and Mashburn (1969) reported that the half-life of *E. coli* L-asparaginase in normal mouse plasma was about 2.5, 2.6, and 3 hr, respectively. *P. vulgaris* L-asparaginase showed almost the same half-life value as the *E. coli* enzyme (Figure 10).

From the results above mentioned, *P. vulgaris* L-asparaginase seems to have a character quite similar to that of the *E. coli* enzyme. The two enzymes differ, however, with respect to immunochemical properties (Tosa *et al.*, 1971). Some physicochemical properties and subunit structure of the *P. vulgaris* L-asparaginase will be reported elsewhere.

Acknowledgments

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