Biochimica et Biophysica Acta, 321 (1973) 369–381

BBA 67011

STUDIES ON ASPARTASE

I. PURIFICATION AND MOLECULAR PROPERTIES OF ASPARTASE FROM *ESCHERICHIA COLI*

SHINTARO SUZUKI, JUNKO YAMAGUCHI* AND MASANOBU TOKUSHIGE
Department of Chemistry, Faculty of Science, Kyoto University, Kyoto (Japan)

(Received March 23rd, 1973)

SUMMARY

Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) was highly purified from *Escherichia coli* W cells grown without aeration and its structural and enzymatic properties were studied. The purification procedures consisted of sonic extraction, streptomycin treatment, $(NH_4)_2SO_4$ fractionation, heat treatment, calcium phosphate gel treatment, and column chromatography on DEAE-Sephadex, hydroxylapatite, and Sepharose 6B. The purified enzyme preparations were homogeneous as judged by ultracentrifugation and polyacrylamide gel electrophoresis. The molecular weight of the native enzyme was determined to be 193 000 by sedimentation equilibrium analysis. The sedimentation coefficient was 9.4 S. The amino acid composition of the enzyme was determined and 4 tryptophan residues were found per 193 000 daltons of the enzyme. Studies on the subunit structure of the enzyme revealed the existence of a single component and its molecular weight was determined to be 48 500 ± 500 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. These results indicate that the native enzyme is composed of four subunits of identical molecular weight.

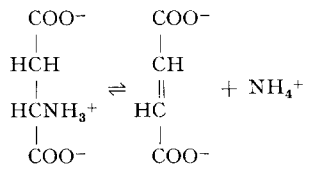
Among various structural analogs tested, only L-aspartate served as the substrate, as monitored by ammonia formation. The K_m value for L-aspartate was 1.0 mM and the optimum pH for the reaction was 8.7. The reaction was activated by divalent metal ions and K^+. The substrate saturation profile exhibited a cooperativity in the absence of the added metal ion or in the presence of K^+. In the reverse reaction, consumption of fumarate was observed in the presence of not only NH_4^+, but also NH_2OH. Mesaconate and maleate did not replace fumarate.

Abbreviations: MES, 2-(*N*-morpholino)-ethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TAPS, tris-(hydroxymethyl)-methylaminopropanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

* Present address: Kyoto Prefectural Institute of Environmental Disruption Research, Kyoto.

INTRODUCTION

Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and NH_4^+ as follows:



Its existence was first suggested by Harden in *Bacterium coli*[1], and the stoichiometry of the reaction was established by Quastel and Woolf[2]. Since then, the enzyme has been found in various bacteria[3,4], plants[4], and some animal tissues[5], and a number of studies have been published concerning molecular properties[6–9], reaction mechanism[10–15], and physiological roles[16,17]. Nevertheless, detailed information is not available about the molecular structure and properties mainly because stable and pure preparations of the enzyme have not been obtained till recently.

Present work was undertaken to elucidate structural and enzymatic properties of the enzyme. The purification and physical and chemical properties of highly purified preparations from *Escherichia coli* W cells are described in this communication.

MATERIALS AND METHODS

Sodium L-aspartate for growth medium was a product of Kyowa Hakko Kogyo Company (Tokyo). Polypeptone and yeast extract were obtained from Daigo Eiyo Kagaku Company (Osaka). 2-(*N*-morpholino)-ethanesulfonic acid (MES), *N*-2-hydroxylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), tris-(hydroxymethyl)-methylaminopropanesulfonic acid (TAPS), cyclohexylaminopropanesulfonic acid (CAPS), and Tris were from Sigma. DEAE-Sephadex A-50, Sephadex G-50, and Sepharose 6B were from Pharmacia. Ovalbumin, bovine serum albumin, cytochrome *c*, and chymotrypsinogen were obtained from Schwartz/Mann as a molecular weight standard kit. Yeast alcohol dehydrogenase was from Worthington. Dithiothreitol was from Sigma and 2-mercaptoethanol was from Nakarai Chemical Company (Kyoto). Hydroxylapatite was prepared according to the method described by Tiselius *et al.*[18]. Calcium phosphate gel was prepared by the method of Keilin and Hartree[19]. All other chemicals were of analytical grade.

Enzyme assay

The activity of aspartase was routinely determined spectrophotometrically by measuring the formation of fumarate following the increase in absorption at 240 nm. The standard assay system contained, in a total volume of 1.0 ml, 100 μmoles of sodium L-aspartate (pH 7.4), 2 μmoles of $MgCl_2$, 100 μmoles of Tris–HCl (pH 7.4), and the enzyme. The reaction was initiated by the addition of the enzyme. The molar extinction coefficient of fumarate, 2530 $mole^{-1} \cdot cm^2$ at 240 nm reported by Emery[15] was used. 1 unit of enzyme was defined as the amount producing 1 μmole of fumarate

per min under the standard assay conditions. Specific activity was expressed as units per mg of protein at 30 °C.

The activity of the reverse reaction was determined spectrophotometrically by measuring the disappearance of fumarate following the decrease in absorbance at 270 nm*. The assay system contained, in a total volume of 1.0 ml, 2 μmoles of sodium fumarate, 100 μmoles of NH_4Cl, 2 μmoles of $MgCl_2$, 100 μmoles of Tris–HCl (pH 7.4), and the enzyme. The reaction was initiated by the addition of the enzyme. When necessary, the activity of deamination was assayed by the Nessler's reaction after performing the modified microdiffusion method of Brown *et al.*[20].

Polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was carried out according to the method of Davis[21]. The electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Laemmli[22].

Ultracentrifugal analysis

Ultracentrifugal experiments were performed at 20 °C in a Spinco Model E analytical ultracentrifuge. The samples were extensively dialyzed against 50 mM potassium phosphate (pH 6.8) containing 100 mM KCl and 5 mM 2-mercaptoethanol (KCl–potassium phosphate buffer). After removal of turbid materials by centrifugation, the samples were subjected to ultracentrifugal analyses.

The sedimentation velocity runs were carried out at 59 780 rev./min with schlieren optics. An AN-D rotor with single sector cells was used. Corrections were made on the observed sedimentation coefficients according to the method of Schachman[23].

Low speed sedimentation equilibrium runs were conducted according to Van Holde and Baldwin[24] with interference optics at 6995 rev./min. The experiment was performed simultaneously on five sample solutions by the use of an AN-G rotor. The molecular weight was obtained by the extrapolation of the apparent molecular weight to the zero protein concentration.

Amino acid analysis

Amino acid analysis was performed according to the method of Spackman *et al.*[25] with a Nihondenshi 6AH amino acid analyzer. Samples were extensively dialyzed against distilled water, lyophilized, and subjected to acid hydrolysis in 6 M HCl (constant boiling) in evacuated sealed tubes at 110 °C for 24, 48 and 72 h[26]. Cystine and cysteine were determined as cysteic acid after performic acid oxidation[27]. The tryptophan content was determined by the spectrophotometric method of Beaven and Holiday[28] based on the ultraviolet absorptions of tryptophan and tyrosine.

Other determinations

Protein was determined by the method of Lowry *et al.*[29]. All spectrophotometric determinations were carried out with a Hitachi 124 recording spectrophotometer equipped with a constant temperature cell housing.

* This wavelength was chosen in order to facilitate the employment of high concentrations of fumarate in the reaction mixture.

RESULTS

Purification of the enzyme

To obtain a good yield of the enzyme, an attempt was made to improve the culture conditions reported by other workers[6,30]. *E. coli* strain W was grown at 37 °C without aeration in 5 l of a medium containing 0.5% K_2HPO_4, 1% polypeptone, 1% yeast extract and 1% sodium L-aspartate (neutralized), in 5-l Erlenmeyer flasks. About 16 h later the cells were harvested by centrifugation when growth reached the stationary phase. The yield of wet packed cells was about 1.5 g per l of the medium. All subsequent procedures were carried out at 0–4 °C, unless otherwise specified.

Step 1. Sonic extraction. A total of 50 g (wet weight) of frozen cells was suspended in 150 ml of KCl–potassium phosphate buffer, and disrupted in 100-ml portions in an Ohtake sonic disintegrator (20 kHz) for 20 min. Cell debris was removed by centrifugation at 10 000 × *g* for 20 min.

Step 2. Streptomycin treatment. To the crude extract (188 ml) was added an equal volume of KCl–potassium phosphate buffer, and then 188 ml of 5% (w/v) streptomycin sulfate (pH 7.0) were slowly added with a constant stirring. After 1 h stirring, the precipitate was removed by centrifugation at 10 000 × *g* for 20 min. The enzyme protein in the supernatant solution (555 ml) was concentrated by adding 216 g of solid $(NH_4)_2SO_4$ (60% saturation). After 1 h of stirring, the protein was collected by centrifugation at 10 000 × *g* for 20 min.

Step 3. Heat treatment. The precipitate was dissolved in 250 ml of KCl–potassium phosphate buffer containing 10% glycerol as a stabilizer. The solution was divided into 50-ml portions and heated at 50 °C for 7 min with gentle shaking. After rapid cooling to 4 °C the precipitate formed was removed by centrifugation at 10 000 × *g* for 10 min.

Step 4. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ (209 g/l) was added with constant stirring to the supernatant solution from Step 3 to reach 35% saturation. After 1 h of stirring, the resulting precipitate was removed by centrifugation. Solid $(NH_4)_2SO_4$ (95 g/l) was then added to this solution to reach 50% saturation. After 1 h of stirring the precipitate was collected by centrifugation, and dissolved in a minimal volume of KCl–potassium phosphate buffer.

Step 5. Calcium phosphate gel treatment. The solution of Step 4 (27 ml) was dialyzed overnight against 2 l of 100 mM potassium phosphate (pH 6.8) containing 5 mM 2-mercaptoethanol. The dialyzed enzyme solution was diluted to the protein concentration of 10 mg/ml with the same buffer. The gel was then slowly added to the solution to reach a gel–protein ratio of 2.0. The mixture was then stirred for 15 min, and the gel was removed by centrifugation. The enzyme in the supernatant solution (192 ml) was precipitated by adding 75 g of solid $(NH_4)_2SO_4$ (60% saturation). The precipitate was collected by centrifugation and dissolved in a minimal volume of KCl–potassium phosphate buffer.

Step 6. DEAE-Sephadex column chromatography. The enzyme solution from Step 5 (10.5 ml, 939 mg of protein) was dialyzed overnight against 2 l of 50 mM Tris–HCl (pH 7.0) containing 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 100 mM KCl and applied to a column of DEAE-Sephadex A-50 (3.0 cm × 40 cm), which had been equilibrated with the above buffer. After washing the column with 1 l of the same buffer, elution was carried out with a linear concentration gradient of $MgCl_2$

from 10 to 80 mM, 1 l of each solution. The main peak of enzyme activity was eluted at a $MgCl_2$ concentration of 35 mM. The active fractions (146 ml) were pooled and the protein components were precipitated by adding 57 g of solid $(NH_4)_2SO_4$ (60% saturation) in the presence of 1 mM dithiothreitol.

Step 7. Hydroxylapatite column chromatography. The precipitate from Step 6 was dissolved in a minimal volume of 10 mM potassium phosphate (pH 6.0) containing 5 mM 2-mercaptoethanol (7.4 ml, 141 mg of protein) and was dialyzed overnight against 2 l of the same buffer. Any insoluble material in the dialyzed enzyme solution was removed by centrifugation and the supernatant solution was applied to a hydroxylapatite column (1.6 cm × 30 cm), which had been equilibrated with the above buffer. After washing the column with 200 ml of the same buffer, elution was carried out with a linear concentration gradient of potassium phosphate from 10 (pH 6.0) to 200 mM (pH 6.8) containing 5 mM 2-mercaptoethanol, 200 ml of each solution. At this step most of the colored material was removed. The main peak of enzyme activity was eluted at a phosphate concentration of 170 mM. The active fractions (52 ml) were collected and the protein components were precipitated by adding 20 g of solid $(NH_4)_2SO_4$ (60% saturation) in the presence of 1 mM dithiothreitol.

Step 8. Sepharose 6B gel filtration. The precipitate from Step 7 was dissolved in a minimal volume of KCl–potassium phosphate buffer (3 ml, 62 mg of protein) and applied to a Sepharose 6B column (2.0 cm × 110 cm). Fractionation was carried out with KCl–potassium phosphate buffer. The flow rate was 4 ml/h. The active fractions with a constant specific activity (30 ml) were pooled and the protein component was precipitated by adding 11.5 g of solid $(NH_4)_2SO_4$ (60% saturation) in the presence of 1 mM dithiothreitol. The precipitate was dissolved in a minimal volume of KCl–potassium phosphate buffer containing 1 mM dithiothreitol. This enzyme solution was stored in an ice bath and used throughout all experiments, unless otherwise specified. A summary of typical purification procedures is shown in Table I.

Purity

The purified enzyme migrated as a monodisperse entity when subjected to sedimentation velocity experiments as shown in Fig. 1. On polyacrylamide gel electrophoresis, the protein migrated as a single band as shown in Fig. 2. When the

TABLE I

SUMMARY OF PURIFICATION

The purification procedures were repeated more than 10 times with satisfactory reproducibility, although the specific activity of the enzyme in the crude extracts varied by a factor of 0.5 depending upon the growth condition of the cells.

Step	*Total volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
1. Crude extract	188.0	7820	8 700	1.1	—
2. Streptomycin	37.0	4240	11 200	2.6	100
3. Heat treatment	252.0	3430	9 900	2.9	89
4. $(NH_4)_2SO_4$	27.0	1620	8 000	4.9	72
5. Calcium phosphate gel	10.5	939	7 350	7.8	66
6. DEAE-Sephadex	7.5	141	6 520	46.2	58
7. Hydroxylapatite	2.5	62.0	3 900	63.0	35
8. Sepharose 6B	1.6	49.0	3 360	68.5	30

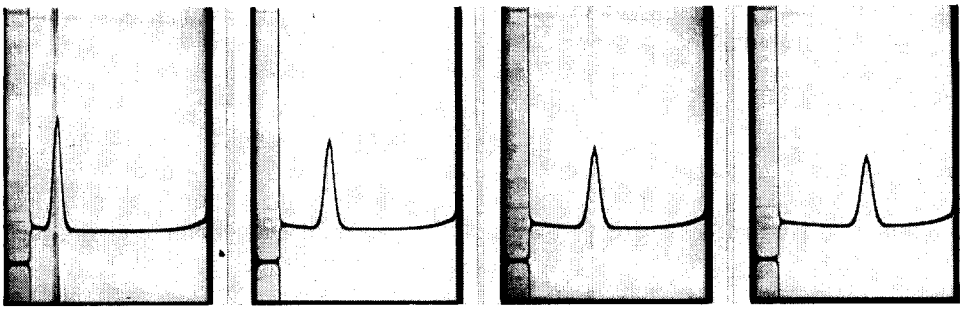


Fig. 1. Ultracentrifugal patterns of aspartase. The protein concentration was 0.8% in KCl-potassium phosphate buffer. The photographs were taken at 25, 37, 45, and 57 min, after reaching 59 780 rev./min at 20 °C. Sedimentation is from left to right.

enzyme activity was examined in the sliced gels by the Nessler's reaction, its location exactly coincided with the protein band. In sedimentation equilibrium experiments, logarithms of the fringe displacement *versus* r^2 were linear. All these results indicated that the purified enzyme preparations were homogeneous.

Molecular weight and subunit structure

The weight average molecular weight of the enzyme was determined by sedimentation equilibrium experiments as described in Materials and Methods. A typical

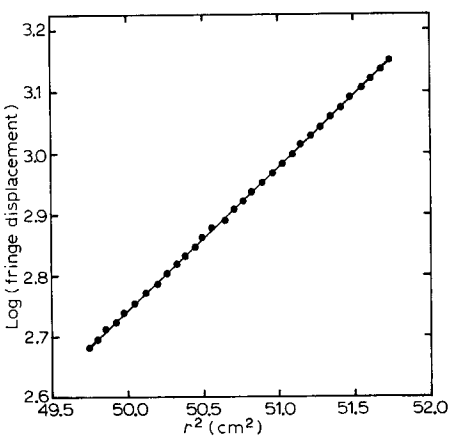


Fig. 2. Polyacrylamide gel electrophoresis of aspartase. Approximately 30 μg of purified enzyme were applied to the column, and electrophoresed at a constant current of 3 mA. The direction of migration was toward the anode (down). Protein was stained with Amido Schwartz.

Fig. 3. Determination of molecular weight of aspartase. Centrifugation was performed at 6995 rev./min for 21 h in a Spinco Model E ultracentrifuge. The sample solution contained 1.2 mg/ml of the enzyme and KCl–potassium phosphate buffer. The ordinate shows the logarithm of the fringe displacement (c), and the abscissa, r^2, where r is the distance from the center of rotation to the point at which the fringe displacement was measured.

example of the results is shown in Fig. 3. Assuming the partial specific volume to be 0.734 as calculated from the amino acid composition according to the method of Cohn *et al.*[31], the mol. wt was determined to be 193 000. The molecular weight of the subunits was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Laemmli[22]. Gel concentrations of 8 and 10% were used. A single band was observed upon staining with 0.1% Coomassie brilliant

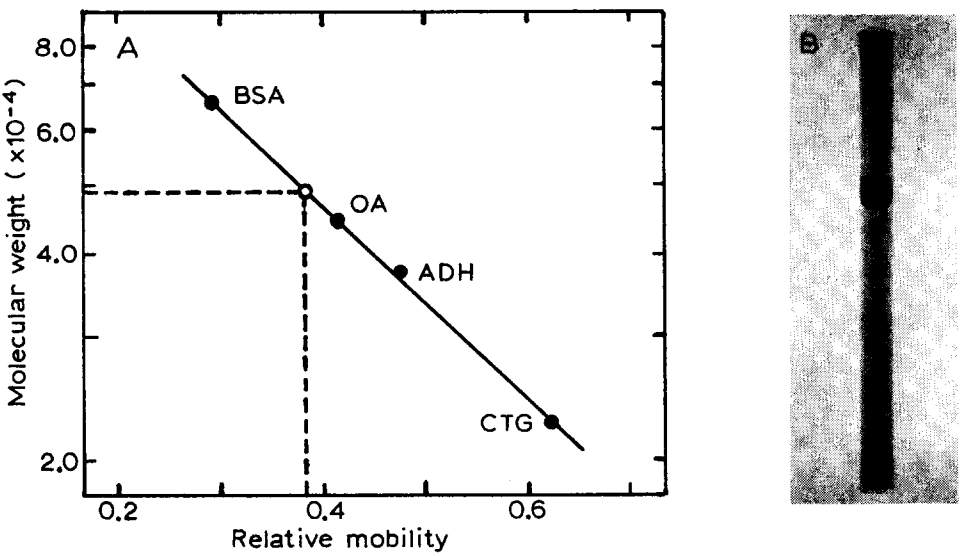


Fig. 4. Determination of molecular weight of aspartase by sodium dodecyl sulfate disc electrophoresis. Standard proteins (10 μg each protein) and aspartase (10 μg protein) were heated at 100 °C for 1.5 min in the presence of 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8, and 0.001% bromophenol blue and were subjected to electrophoresis at 3 mA per tube as described by Laemmli[22]. The proteins were stained with 0.1% Coomassie brilliant blue. (A) Molecular weight determination. Mobilities of standard proteins were plotted against log molecular weights. The standard proteins used were: BSA, bovine serum albumin (M_r, 67 000); OA, ovalbumin (M_r, 45 000); ADH, yeast alcohol dehydrogenase (M_r, 37 000); CTG, chymotrypsinogen (M_r, 25 700). (B) Electrophoretic pattern of aspartase.

blue as shown in Fig. 4B. This band corresponded to a mol. wt of 48 500 ± 500 on a scale calibrated with the standard proteins as shown in Fig. 4A. These results indicate that the native enzyme is composed of four subunits of identical molecular weight.

Sedimentation velocity coefficient

The sedimentation velocity coefficient was determined as described by Schachman[23]. Protein concentration was varied from 3 to 20 mg/ml. The results are shown in Fig. 5. The sedimentation coefficient in water at 20 °C extrapolated to zero protein concentration was estimated to be 9.4 S.

Amino acid composition

The amino acid composition of the enzyme is shown in Table II. Analyses were performed at three time intervals, and the average of the determined values was used. The number of tryptophan residues estimated by the spectrophotometric method was 4 moles per mole of protein.

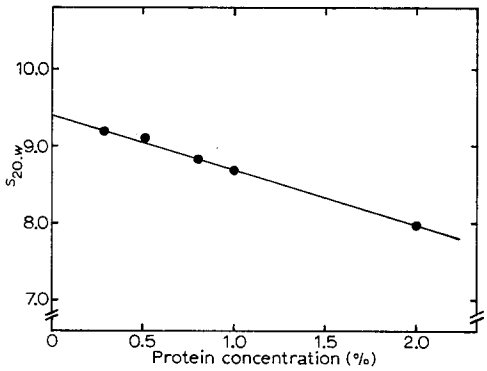


Fig. 5. Sedimentation coefficients of aspartase as a function of protein concentration. Ultracentrifugations were performed at 59 780 rev./min at 20 °C. The sample solutions contained designated amounts of the enzyme in KCl–potassium phosphate buffer. Sedimentation coefficients were corrected to water at 20 °C.

TABLE II

AMINO ACID COMPOSITION OF ASPARTASE

Analysis was performed on each hydrolyzate as described under Materials and Methods. The results are expressed as residues per molecule, assuming a mol. wt of 193 000.

Amino acid	*Hydrolysis (residues/molecule)*			*Calculated*
	24 h	*48 h*	*72 h*	
Lysine	91	94	100	95
Histidine	32	29	29	30
Arginine	68	64	65	66
Aspartic acid	207	221	203	210
Threonine	86	82	82	86*
Serine	71	63	59	73*
Glutamic acid	236	237	249	241
Proline	72	75	80	76
Glycine	125	133	131	130
Alanine	155	155	162	157
Valine	147	155	163	155
Methionine	51	50	46	49
Isoleucine	113	112	117	114
Leucine	157	156	163	159
Tyrosine	56	57	59	57
Phenylalanine	52	48	51	50
Half-cystine	38	38	—	38
Tryptophan	—	—	—	4**

* These values were extrapolated to zero time hydrolysis.
** This value was calculated from ultraviolet absorption.

Absorption spectrum

The absorption spectrum of the enzyme was determined in 50 mM potassium phosphate (pH 6.8). A maximum and a minimum absorption were observed at 278 and 253 nm, respectively. There was no absorption detected in the visible region. The ratio of the absorption of the enzyme at 280 nm to that at 260 nm was determined to be 2.0. This value appeared to be somewhat higher than those of many other proteins, and suggests the virtual absence of intrinsic nucleotide or nucleic acid.

Stability

When the enzyme was stored at 4 °C in a dilute buffer at a neutral pH, the activity was almost completely lost in 2 weeks. However, in the presence of various salts, thiol compounds, or glycerol, the enzyme was stabilized to a considerable extent and the loss of the activity was almost undetectable over 2 weeks at 4 °C. The enzyme was also stable upon storage at −20 °C. These results are shown in Table III.

TABLE III

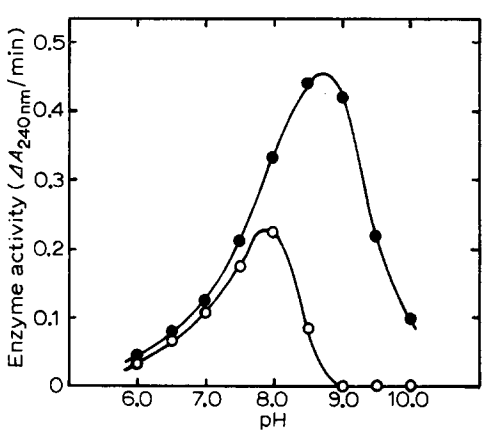
EFFECTS OF VARIOUS COMPOUNDS ON STORAGE OF ASPARTASE

The enzyme solution used was desalted by gel filtration on a small column of Sephadex G-50. The sample solution contained 25 μmoles of potassium phosphate (pH 6.8), 42 μg of enzyme, and indicated compounds in a total volume of 1.0 ml, and stored at 4 °C or −20 °C for 14 or 30 days. The residual activity was assayed by using each aliquot in the standard assay system.

Compound		*Days*	*Temperature (°C)*	*Residual activity (%)*
None		14	4	trace
None		30	−20	96
NaCl	(100 mM)	14	4	52
KCl	(100 mM)	14	4	34
$(NH_4)_2SO_4$	(100 mM)	14	4	100
$MgCl_2$	(50 mM)	14	4	57
Dithiothreitol	(1 mM)	14	4	100
2-Mercaptoethanol	(5 mM)	14	4	77
Glycerol	(10%)	14	4	92

Kinetic properties

The K_m value for L-aspartate was 1.0 mM and the optimum pH for the activity was 8.7. When the pH–rate profile was determined in the absence of the added metal



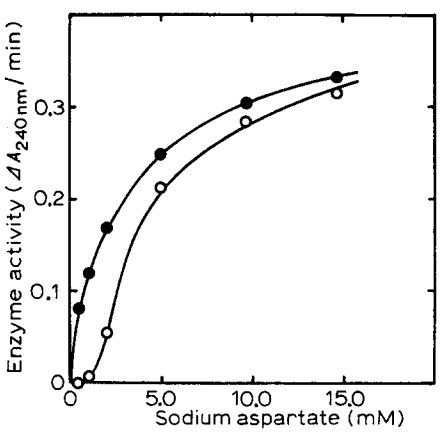


Fig. 6. pH–rate profile of aspartase. The reaction mixture contained 100 μmoles of potassium aspartate, (pH was adjusted to indicated values), 100 μmoles of the designated buffer, 0.75 μg of the enzyme, and 2 μmoles of $MgCl_2$ (●—●) or in its absence (○—○) in a total volume of 1.0 ml MES–KOH (pH 6.0–7.0), HEPES–KOH (pH 7.0–8.0), TAPS–KOH (pH 8.0–9.0), CAPS–KOH (pH 9.5–10.0).

Fig. 7. Effect of $MgCl_2$ on substrate saturation profile. The reaction mixture contained, in a total volume of 1.0 ml, 100 μmoles of Tris–HCl (pH 7.4), 2 μmoles of $MgCl_2$ (●—●) or in its absence (○—○), varied concentrations of sodium aspartate (pH 7.4), and 2.5 μg of the enzyme.

ion, the optimum pH shifted to 7.7 as shown in Fig. 6. Although aspartase has been recognized as a divalent metal-requiring enzyme[7], the metal–activity relationship appeared to be rather complex. The effect of addition of the metal ion was not obvious at pH 7.0, or at high concentrations of the substrate. At high pH values, however, there was little enzyme activity in the absence of the metal ion as shown in the same figure. When sodium aspartate solution was subjected to dithizone–chloroform extraction, an appreciable amount of the metal ions was detected. Nevertheless, extensive treatment of the substrate with dithizone or desalting of the enzyme solution by a Sephadex G-50 column did not result in abolition of the activity in the absence of added metal ions.

When low concentrations of the substrate were employed, the activating effect of divalent metal ions was observed even at pH 7.4 as shown in Fig. 7. For example, the activity in the presence of 2 mM $MgCl_2$ was 8 times higher than that in its absence at the substrate concentration of 1 mM. The activating effect of Mg^{2+} was plotted as a function of the metal concentration as shown in Fig. 8, and the activation constant was calculated to be 0.15 mM. Besides Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, and Cd^{2+} were also effective under the above conditions.

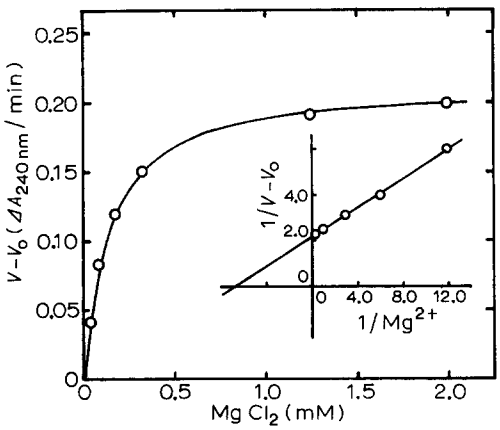


Fig. 8. Effect of $MgCl_2$ concentration on aspartase activity. The reaction mixture contained, in a total volume of 1.0 ml, 100 μmoles of Tris–HCl (pH 7.4), varied concentrations of $MgCl_2$, 1 μmole of sodium aspartate, and 7.7 μg of the enzyme. V and V_0 are the rates of the reaction in the presence and absence of $MgCl_2$, respectively.

The substrate specificity of the enzyme appeared to be quite strict and the following structural analogs (40 mM) of L-aspartate did not serve as the substrate, when the activity for deamination was examined by the use of the Nessler's reaction; D-aspartate, DL-α-methylaspartate, DL-β-methylaspartate, DL-*threo*-β-hydroxy-aspartate, DL-*erythro*-β-hydroxyaspartate, L-cysteate, L-α-aminobutyrate, L-asparagine, L-alanine, and L-glutamate. D-Aspartate acted as a competitive inhibitor and its K_i value was 10 mM. The substrate specificity of the reverse reaction was examined. As shown in Table IV, consumption of fumarate was observed in the presence of not only NH_4^+, but also NH_2OH. Since the apparent K_m values for NH_4^+ and NH_2OH were determined to be 20 and 5 mM, respectively, under the experimental conditions, it is unlikely that NH_4^+ in NH_2OH preparations served as the substrate. Methylamine did not replace NH_4^+. Likewise, mesaconate and maleate did not replace fumarate.

TABLE IV

SUBSTRATE SPECIFICITY FOR REVERSE REACTION OF ASPARTASE

Assay conditions are described in Materials and Methods. The amount of protein used was 21 μg.

Substrates		*Enzyme activity* ($\Delta A_{270\ nm}$/*min*)
2 mM	*100 mM*	
Sodium fumarate	NH_4Cl	0.410
Sodium fumarate	NH_2OH	0.480
Sodium fumarate	NH_2CH_3	0
Sodium mesaconate	NH_4Cl	0
Sodium maleate	NH_4Cl	0*

* Due to the lower molar extinction coefficient, the activity was determined at 260 nm.

Activators and inhibitors

In the presence of K^+, the enzyme exhibited complex kinetics. At low substrate concentrations, K^+ exhibited an inhibitory effect, whereas the activity was rather enhanced at high substrate concentrations. In addition, the substrate saturation profile exhibited a cooperative nature. These results are shown in Fig. 9. NH_4^+ and Li^+ also exhibited similar activating effects, although to lesser extents. In contrast, Na^+ showed a slight inhibition.

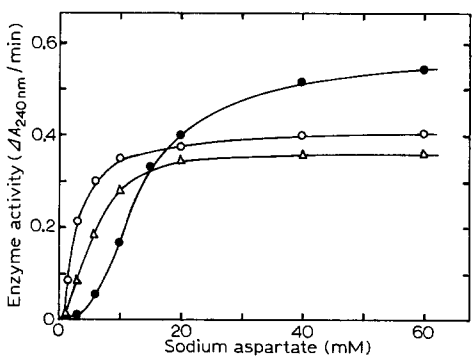


Fig. 9. Effect of KCl and NaCl on substrate saturation profile. The reaction mixture contained, in a total volume of 1.0 ml, 100 μmoles of Tris–HCl (pH 8.6), 2 μmoles of $MgCl_2$, varied concentration of sodium aspartate (pH 8.6), 0.87 μg of the enzyme, and 100 μmoles of the designated salt or in its absence. ○—○, no salt; ●—●, + 100 mM KCl; △—△, + 100 mM NaCl.

The enzyme was completely inhibited by EDTA or thiol reagents such as *p*-hydroxymercuribenzoate or DTNB. NH_2OH competitively inhibited the deamination of L-aspartate with a K_i value of 10 mM, while it served as a substrate for the backward reaction as already reported by Emery[15]. $NaBH_4$ and carbonyl reagents exhibited no appreciable effect.

DISCUSSION

Aspartase purified from *E. coli* W cells grown without aeration appeared to be homogeneous as judged by sedimentation analysis and disc electrophoresis. The

native enzyme has a mol. wt of 193 000 as estimated by sedimentation equilibrium analysis. The enzyme is composed of four subunits of seemingly identical mol. wt (48 500) as estimated by sodium dodecyl sulfate disc electrophoresis. Rudolph and Fromm[9] recently obtained a homogeneous aspartase preparation from *E. coli* B cells and concluded that their enzyme has four subunits, too, although the molecular weight of their enzyme appeared to be a little smaller than the enzyme from the W strain.

Gale[30] demonstrated the presence of two distinct molecular species of aspartase in *E. coli* cells. An attempt to fractionate the enzyme by the use of $(NH_4)_2SO_4$ fractionation, sucrose density gradient centrifugation, and starch gel electrophoresis of the sonic extracts prepared from aerobically and anaerobically grown *E. coli* cells was without success and indicated the presence of a single species of the enzyme[32].

Unlike histidine ammonia lyase[33] and phenylalanine ammonia lyase[34], the *E. coli* aspartase was not inhibited by carbonyl reagents in agreement with the results of Williams and Dougherty[13] and Rudolph and Fromm[9].

The requirement of the enzyme for divalent metal ions appeared to be rather complex. When the metal ion was omitted from the standard assay system, almost complete activity was still observed. However, when the pH was set above 8.0 or a lower substrate concentration was employed, the activating effect of added metal ion was observed. Under more alkaline conditions (pH $>$ 9.0), the enzyme exhibited an absolute requirement for the metal ions regardless of the substrate concentrations.

Desalting of the enzyme with a Sephadex column and treatment of the substrate with dithizone did not abolish the activity even in the absence of the added metal ions. In addition, our preliminary experiments of metal analyses by ESR and atomic absorption spectroscopy indicated that the purified enzyme preparations did not contain appreciable amounts of Mn^{2+} and Zn^{2+}. Nevertheless, a possibility that the enzyme contains intrinsic metal ions still can not be excluded in consideration of the inhibitory effect of EDTA on the enzyme.

Williams and Lartigue[6] and Rudolph and Fromm[9] independently observed pH-dependent cooperative kinetics. In addition, the present investigation revealed that the effect of K^+ was also of a cooperative nature, although elucidation of the mechanism needs further analysis.

ACKNOWLEDGMENTS

The authors wish to express their sincere gratitude to Dr H. Katsuki of this Department for continuous encouragement and valuable discussions during the present investigation. The authors are grateful to Dr M. Irie, Faculty of Pharmaceutical Sciences, Dr M. Yanagida of this Faculty, Dr M. Nozaki of Medical School, and Mr H. Takeuchi of this Faculty for the helps with amino acid analysis, sodium dodecyl sulfate disc electrophoresis, atomic absorption analysis and ESR analysis, respectively. Thanks are also due to the Kyowa Hakko Kogyo Co. for making the cell culture facilities available in the early part of this work. This work was supported in part by grants from the Ministry of Education of Japan.

REFERENCES

1 Harden, A. (1901) *J. Chem. Soc.* 79, 610–628
2 Quastel, J. H. and Woolf, B. (1926) *Biochem. J.* 20, 545–555
3 Cook, R. P. and Woolf, B. (1926) *Biochem. J.* 22, 474–481
4 Virtanen, A. I. and Tarnanen, J. (1932) *Biochem. Z.* 250, 193–211
5 Kurata, Y. (1962) *Exp. Cell Res.* 28, 424–429
6 Williams, V. R. and Lartigue, D. J. (1967) *J. Biol. Chem.* 242, 2973–2978
7 Depue, R. H. and Moat, A. G. (1961) *J. Bacteriol.* 82, 383–386
8 Haight, R. D. and Morita, R. Y. (1962) *J. Bacteriol.* 83, 112–120
9 Rudolph, F. B. and Fromm, H. J. (1971) *Arch. Biochem. Biophys.* 147, 92–98
10 Englard, S. (1958) *J. Biol. Chem.* 233, 1003–1009
11 Krasna, A. I. (1958) *J. Biol. Chem.* 233, 1010–1013
12 Gawron, O. and Fondy, T. P. (1959) *J. Am. Chem. Soc.* 81, 6333–6334
13 Williams, V. R. and Dougherty, T. B. (1970) *Fed. Proc.* (Abstr.) 29, 462
14 Dougherty, T. B., Williams, V. R. and Younathan, E. S. (1971) *Fed. Proc.* (Abstr.) 30, 1239
15 Emery, T. F. (1963) *Biochemistry* 2, 1041–1045
16 Halpern, Y. S. and Umbarger, H. E. (1959) *J. Bacteriol.* 80, 285–288
17 Liess, K., Mecke, D. and Holzer, H. (1966) *Biochem. Z.* 346, 244–251
18 Tiselius, A., Hjertén, S. and Jevin, O. (1956) *Arch. Biochem. Biophys.* 65, 132–155
19 Keilin, D. and Hartree, E. F. (1938) *Proc. Roy Soc. London, Ser. B* 124, 397–405
20 Brown, R. H., Duda, G. D., Korkes, S. and Handler, P. (1957) *Arch. Biochem. Biophys.* 66, 301–309
21 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427
22 Laemmli, U. K. (1970) *Nature* 227, 680–685
23 Schachman, H. K. (1957) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds), Vol. 4, pp. 32–103, Academic Press, New York
24 Van Holde, K. E. and Baldwin, R. L. (1958) *J. Phys. Chem.* 62, 734–743
25 Spackman, D. H., Stein, W. H. and Moore, S. (1958) *Anal. Chem.* 30, 1190–1206
26 Hirs, C. H. W., Stein, W. H. and Moore, S. (1954) *J. Biol. Chem.* 211, 941–950
27 Moore, S. (1963) *J. Biol. Chem.* 238, 235–237
28 Beaven, G. H. and Holiday, E. R. (1952) *Adv. Protein Chem.* 7, 319–386
29 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
30 Gale, E. F. (1938) *Biochem. J.* 32, 1583–1599
31 Cohn, E. J., McMeekin, T. L., Edsall, J. T. and Blanchard, M. H. (1943) *J. Am. Chem. Soc.* 56, 784–794
32 Yamaguchi, J. (1971) Thesis for the degree of Master of Science, Graduate School, Kyoto University
33 Smith, T. A., Cordelle, F. H. and Abeles, R. H. (1967) *Arch. Biochem. Biophys.* 120, 724–725
34 Havir, E. A. and Hanson, K. R. (1968) *Biochemistry* 7, 1904–1914