Journal of Biochemistry and Molecular Biology, Vol. 31, No. 4, July 1998, pp. 345–349

JOURNAL OF
Biochemistry
and
Molecular Biology

Purification and Characterization of Aspartase from *Hafnia alvei*

Moon-Young Yoon*, Jae-Ho Park, Kyong-Jae Choi, Joung-Mok Kim, Yeon-Ok Kim, Jun-Bum Park and Jin-Burm Kyung#

Department of Chemistry, Hanyang University, Seoul 133-791, Korea

Received 2 March 1998
Revised 15 April 1998

Aspartase (EC 4.3.1.1) from *Hafnia alvei* was purified to homogeneity by a combination of DEAE-cellulose, Red A-agarose, and Sepharose 6B chromatography. The purified enzyme appeared homogeneous on denatured SDS-polyacrylamide gel electrophoresis. The purified enzyme was a tetrameric protein composed of identical subunits with a molecular weight of 55,000 daltons. The optimum pH for the enzymatic reaction was 8.5 and the optimum temperature for maximum activity was 45°C. The enzyme has an absolute requirement of divalent metal ions (Mg^{2+}, Mn^{2+}) at the alkaline pH. The enzyme, however, was inactivated in the presence of other divalent cations such as Zn^{2+}, Ca^{2+}. The helical content of the purified enzyme was estimated by CD spectropolarimetry to be 61%.

Keywords: Characterization, *Hafnia alvei* aspartase, Purification.

Introduction

L-aspartate ammonia-lyase [EC 4.3.1.1] (aspartase), an enzyme which catalyzes the reversible deamination of L-aspartate to yield fumarate, has been isolated from several sources including *E. coli* (Suzuki *et al.*, 1973), *Pseudomonas fluorescens* (Takagi *et al.*, 1984), and *Bacterium cadaveris* (Williams and Lartigue, 1967).

The enzyme has been regarded as a catabolic enzyme in both bacteria and plants, and the reaction is reversible and favors aspartate formation. The equilibrium constant for the aspartase reaction is 5×10^{-3} M (Bada and Miller, 1968). The aspartase is a tetramer composed of four apparently identical subunits (Williams and Lartigue, 1967). The enzyme was observed to have an absolute requirement for a divalent metal ion such as Mg^{2+} or Mn^{2+} at higher pH, with some indication that aspartase may possess activity in the absence of divalent metal ions at lower pH (Suzuki *et al.*, 1973). Other alkaline metals such as Be^{2+} and Ba^{2+} do not activate aspartase.

Because of our interest in the mechanism of these aspartate-utilizing enzymes, we have undertaken many studies (Yoon and Cook, 1994; Kim *et al.*, 1995; Shim *et al.*, 1997). Aspartase is commercially available, however, the purity is low and it is very expensive. Commercial aspartase contains fumarase which converts fumarate to malate. In order to perform a more efficient experiment, we have tried to purify and to characterize aspartase from *Hafnia alvei*. A single band by SDS-PAGE was obtained.

Materials and Methods

Materials *Hafnia alvei* (ATCC 9760) was purchased from ATCC (American Type Culture Collection). L-Aspartic acid, diethylaminoethyl(DEAE) cellulose, 4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES), potassium phosphate dibasic, dithiothreitol (DTT), Sepharose 6B, streptomycin sulfate, and ammonium sulfate were purchased from Sigma Chemical Co. (St. Louis, USA). Sodium dodecyl sulfate (SDS), ammonium persulfate, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylene diamine (TEMED) and acrylamide were purchased from Bio-Rad (Richmond, USA). Matrix Red A-agarose was purchased from Amicon (Beverly, USA). Nutrient broth and nutrient agar were purchased from Difco laboratories. All other chemicals were of pure or extra-pure grade commercially available.

Enzyme assay All data were collected using the Hewlett Packard 8452 Diode-Array spectrophotometer and the Kontron UVIKON 860. The temperature was maintained at 25°C using a circulating water bath with the capacity to heat and cool the thermospacers in the cell compartment. All reactions were carried

* To whom correspondence should be addressed.
Tel: 82-2-290-0946; Fax: 82-2-290-1639
E-mail: myyoon@email.hanyang.ac.kr

Present address: Hanyang University, Ansan 425-791, Korea

out in a 1 ml cuvette with a 1 cm light pathlength. All cuvettes were incubated for at least 10 min in the water bath and 5 min in the cell compartment prior to reaction.

The reaction was started by the addition of aspartase. The aspartate concentration was corrected for complexation with divalent metal using the following dissociation constants obtained at 0.1 mM ionic strength; Mg-aspartate 4 mM (Dawson *et al.*, 1971). Aspartase has been assayed continuously using the absorbance of fumarate at 240 nm (ε_{240} = 2255 $M^{-1}cm^{-1}$) (Cook *et al.*, 1980). Standard assay mixture contained 100 mM HEPES (pH 8.0), 10 mM aspartate, and 20 mM Mg^{2+} based on the metal chelate correction. Initial velocity data were fitted by a least square method to the double reciprocal transformation of Eq. (1).

$$v = \frac{V_{max}[S]}{(K_m + [S])} \quad (1)$$

Protein concentration was estimated by the Bradford procedure with a bovine serum albumin standard (Bradford, 1976).

Enzyme purification All procedures were carried out at 4°C unless otherwise indicated. *Hafnia alvei* were grown at 30°C in a medium containing nutrient broth (0.5% bacto peptone, 0.3% bacto beef extract). A total of 30 g wet cell was immediately suspended in 100 ml cold standard buffer containing 100 mM potassium phosphate (pH 7.0), 1 mM DTT, and 1 mM EDTA (buffer A) and disrupted in an ultrasonic processor for 20 min. Assays of aspartase activity were conducted every 3 min during sonication. Sonication was terminated when no further increase was observed in the overall activity. The pellet was removed by a centrifuge at 7000 × *g* for 10 min. To the crude extract was added an equal volume of buffer A, and an equal volume (240 ml) of 2.2% (w/v) streptomycin sulfate (pH 7.0) was slowly added while stirring. After about 1 h of stirring, the precipitate was removed by a centrifuge at 7000 × *g* for 25 min. The supernatant solution was titrated to pH 5.7 with 200 mM acetic acid by continual stirring and heated at 50°C for 10 min with gentle shaking. After rapid cooling to 4°C, the precipitate formed was removed by a centrifuge at 10,000 × *g* for 30 min. Solid ammonium sulfate was added slowly to the stirred supernatant to achieve 40–70% saturation. The 40–70% ammonium sulfate precipitate was dissolved in a minimal volume of buffer and then dialyzed three times for 16 h against 20 vol of the same buffer.

DEAE- cellulose column (2 × 15 cm) was equilibrated with buffer A. The enzyme solution was applied to a column and then washed in a 10 bed vol with buffer A. Elution was carried out with a linear concentration gradient of phosphate buffer from 100 mM to 500 mM, pH 7.0. The active fractions were pooled and dialyzed twice for 16 h against 20 vol of 50 mM HEPES (pH 6.8) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 30 mM magnesium acetate (buffer B). The enzyme solution from the previous step was applied to a Red A-agarose column (2 × 15 cm) which had been equilibrated with buffer B. After washing the column with 10 bed vol of the same buffer, elution was carried out with the same buffer containing 10 mM L-aspartic acid. The active fractions were pooled and dialyzed for 20 h against 1 L of 100 mM HEPES (pH 6.8) with two changes. The dialyzed enzyme sample was applied to a column (3 × 90 cm) of Sepharose 6B equilibrated with 100 mM HEPES (pH 6.8). Elution was carried out with the same buffer at 0.4 ml/min. The active fractions were pooled and concentrated by ultrafiltration using a stirred cell (Amicon 8050) and 10% glycerol was added. The catalytic function of the enzyme remained stable for at least 1 month at 4°C without appreciable loss of enzymatic activity.

SDS polyacrylamide gel electrophoresis Nondenaturing polyacrylamide gel electrophoresis of native enzyme and the denaturing polyacrylamide gel was carried out according to the method of Laemmli (1970), and gels were stained with Coomassie Brilliant Blue G-250. Samples were incubated at 100°C for 5 min in 10 mM Tris-HCl (pH 8.0), 1% SDS, and 1% 2-mercaptoethanol to obtain complete denaturation of the enzyme. Myosin, galactosidase, phosphorylase, fructose-6-phosphate kinase, albumin, glutamic dehydrogenase, ovalbumin, and glyceraldehyde-3-phosphate dehydrogenase were used as marker proteins.

Metal effects All metals were corrected for the concentration of substrate chelate complexes according to Park *et al.* (1984) by using the following values for stability constants: Mg-aspartate, 4 mM; Mn-aspartate, 0.2 mM; Ca-aspartate, 25.1 mM; Zn-aspartate, 0.16 μM (Dawson *et al.*, 1971). The concentration of uncomplexed metal was maintained at 0.5 mM. Standard assay mixtures contained 100 mM HEPES (pH 8.0), 10 mM aspartate, and metals based on metal chelate correction.

pH profile and optimal temperature The dependence of aspartase activity on the pH was determined under standard conditions between pH 4.0–10.0. The buffers used were 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) in the pH range of 5.0–6.5, 100 mM MOPS in the pH range of 6.5–7.5, 100 mM 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid (TAPS) in the pH range of 7.5–9.0, and 100 mM 2-(N-cyclohexylamino) ethanesulfonic acid (CHES) in the pH range of 8.5-10.0. The pH values were adjusted at 25°C. The dependence of aspartase activity on temperature was studied under standard assay conditions of over 0–60°C.

Circular dichroism (CD) spectroscopy CD spectra were obtained at 25°C on a CD6 CD-ORD Spectropolarimeter. Samples were contained in a quartz cuvette able to hold a volume as great as 50 μl. All far UV spectra were scanned from 250 to 190 nm with a protein concentration of 0.5 mg/ml at intervals of 1 nm. The sample buffer was 10 mM phosphate buffer, pH 7.2. Each spectrum is an average of three scans. The sample spectra were corrected by substracting the appropriate buffer blanks. The unit of the molecular ellipticity, $[\theta]$ is degree cm^2 per dmol. The unit in degree was converted to rad to meet the condition of the reference data of poly-L-lysine. These data were compared to the circular dichroism of poly-L-lysine in different secondary structure conformations to yield the percentage of α-helix, β-sheet, and random coil.

Results and Discussion

The purification procedures for *Hafnia alvei* aspartase are summarized in Table 1. A homogeneous preparation of the aspartase obtained by combination of ammonium sulfate

Table 1. Stepwise purification of aspartase from *Hafnia alvei.*

Step	Protein (mg)[a]	Unit (U)[b]	Specific activity (U/mg)	Yield (%)	Purification fold
Crude	11,910	6,613	0.56	100	1
Low pH & Heat	9,698	6,206	0.63	93.84	1.44
$(NH_4)_2SO_4$	3,344	5,688	1.70	86.01	3.03
DEAE-cellulose	27	1,478	54.7	22.34	96.42
Red A-agarose	4.17	723	173.38	10.98	309.60
Sepharose 6B	1.79	424	236.8	6.4	422.15

[a]Total protein was determined by absorbance at 280nm ($A_{280}^{1mg/ml} = 1.0$). For the final step in the purification the total protein was determined by the biuret method.
[b]One unit of activity is defined as the amount of enzyme catalyzing the production of 1 μmol fumarate from L-aspartate per min at pH 8.5 at 30°C.

fractionation, DEAE-cellulose, Red A-agarose, and Sepharose 6B showed a specific activity of 236 U/mg when aspartate was used as a substrate. While the first few steps in this purification scheme are somewhat time-consuming and result in only a three-fold overall purification of L-aspartase, the yield through the ammonium sulfate fractionation step has generally been about 86% or better. The low overall yield (22.3%) for the DEAE-cellulose purification step is primarily the result of activity loss during the dialysis following $(NH_4)_2SO_4$. For the Red A-agarose step in the purification, virtually all of the enzyme activity is bound to the dye during column loading. For the elution of L-aspartase, addition of 1 mM L-aspartate to the wash buffer was to remove greater than 90% of the bound enzyme (Fig. 1). Fumarase that catalyzes the reversible conversion of L-malate to fumarate and water are commonly copurified with aspartase. However, contaminating fumarase activity can be separated from aspartase activity by a Sepharose 6B column (Fig. 2). The procedure, as shown in Table 1, resulted in a 420-fold purification with a recovery yield of 6%. The specific activity of L-aspartase purified by this scheme is as high as or higher than has previously been reported (Karsten *et al.*, 1985).

The protein homogeniety of the purified enzyme was assessed by a single band migrating on 10% SDS polyacrylamide gel electrophoresis (Fig. 3). The native molecular size of the purified enzyme was estimated to be 198,000 daltons by pore gradient polyacrylamide gel electrophoresis (data not shown). The subunit size of the enzyme determined by SDS-PAGE was 55,000 daltons (Fig. 3), suggesting that the enzyme is composed of four identical subunits. The subunit size of the enzyme from other species such as *E. coli, Pseudomonas fluorescens,* and *Bacterium cadaveris* is also 50,000–56,000 daltons (Takagi *et al.*, 1984). The enzyme purified from *E. coli* has a molecular weight of 193,000 (Suzuki *et al.*, 1973). The molecular weight of *P. fluorescens* was 173,000 as determined by sedimentation equilibrium analysis (Takagi

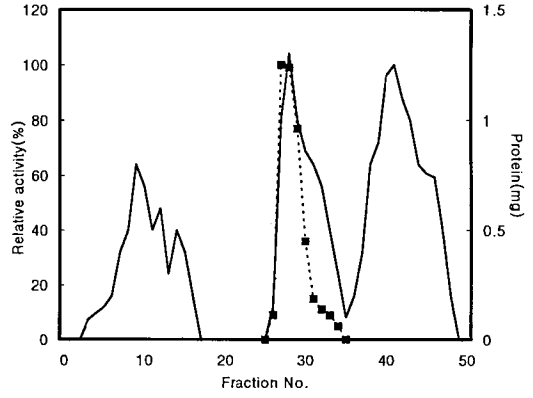

Fig. 1. Dye-ligand chromatography of aspartase on Red A-agarose. Concentrated fractions from the DEAE-cellulose step were applied to Red A-agarose column as described in Materials and Methods. The broken line (------) and the solid line (——) represent relative activity and protein amount (absorbance 280 nm), respectively.

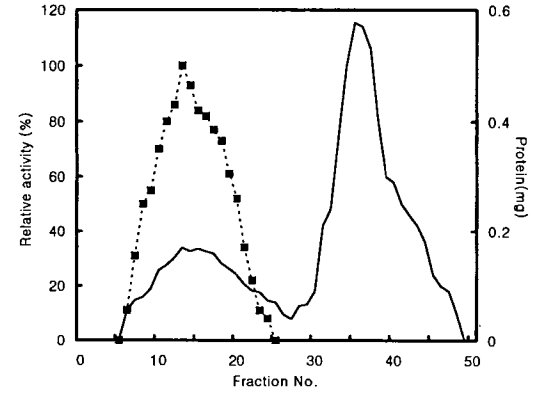

Fig. 2. Gel permeation chromatography of aspartase on Sepharose 6B. Concentrated fractions from the Red A-agarose step were applied to Sepharose 6B column as described in Materials and Methods. The broken (------) line and the solid (——) line represent relative activity and protein amount (absorbance 280 nm), respectively.

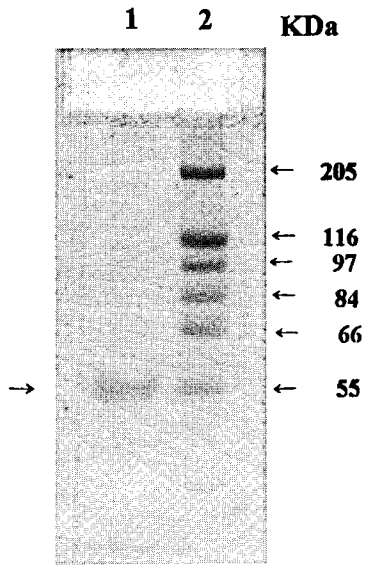

Fig. 3. SDS-polyacrylamide gel electrophoresis of aspartase isolated from *Hafnia alvei.* Lane 1, purified aspartase. Lane 2, molecular mass standards.

et al., 1984). In view of the native structure, the molecular weight of the *H. alvei* enzyme (M_r = 198,000) is slightly bigger than that of the other enzymes.

The optimum pH for the enzyme reaction was observed at pH 8.5 in the presence of 20 mM $MgCl_2$, while it shifts to about pH 7.0 in the absence of added metal ions (Fig. 4). The optimum pH for the enzyme stability was rather broad (pH 5.5–9). The optimum pH for catalysis was similar to the other enzymes (Suzuki *et al.*, 1973; Takagi *et al.*, 1984). On examining the heat stability of *Hafnia alvei* aspartase, the optimum temperature with a maximum activity was observed at 45°C (Fig. 5). This is consistent with the results obtained with other aspartases (Takagi *et al.*, 1984).

The enzyme shows an optimum pH at 8.5 and has an absolute requirement of Mg^{2+}. The dependence of the enzyme upon Mg^{2+} ion is shown in Fig. 6. No inhibition of activity was seen up to 50 mM $MgCl_2$. Some sources such as *E. coli* and *P. fluorescens* have shown that aspartase requires a metal ion in the same way for activity (Wilkinson and Williams, 1961; Rudolph and Fromm, 1971). The required divalent metal may be coordinated to only one of the carboxyl groups of aspartate (Nuiry *et al.*, 1984). Divalent metal ions such as Mg^{2+} and Mn^{2+} activate the enzyme at the alkaline pH. In the neutral and slightly acidic pH ranges, the divalent metal ions do not affect the activity to an appreciable extent. Enzyme activity was increased in the presence of Mg^{2+} and Mn^{2+} ions, but decreased by the addition of Zn^{2+} and Ca^{2+} ions (Table 2).

The CD spectrum of the purified enzyme is shown in Fig. 7. The purified enzyme exhibited negative cotton effects through 208 and 218 nm. From these values, the helical content of the purified enzyme was estimated to be

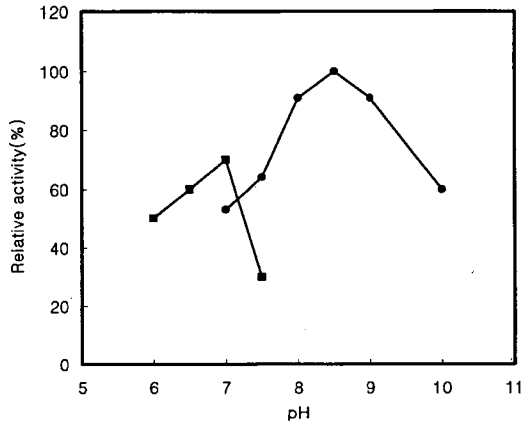

Fig. 4. Effect of pH on the purified enzyme. Maximal activity was shown as 100%. Aspartase activity was assayed as described in Materials and Methods. Maximal activity with Mg^{2+}(●). Maximal activity without Mg^{2+} (■).

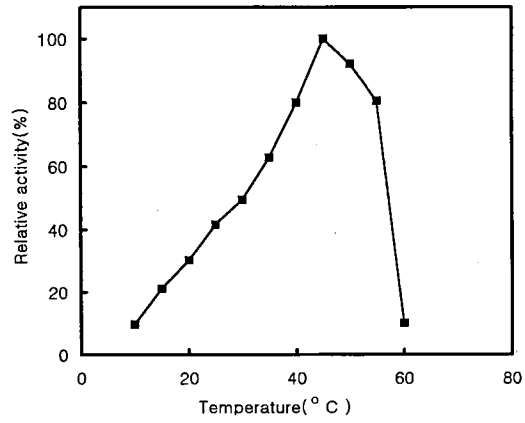

Fig. 5. Optimal temperature on the purified enzyme. The enzyme was incubated at various temperatures. Aspartase activity was assayed as described in Materials and Methods.

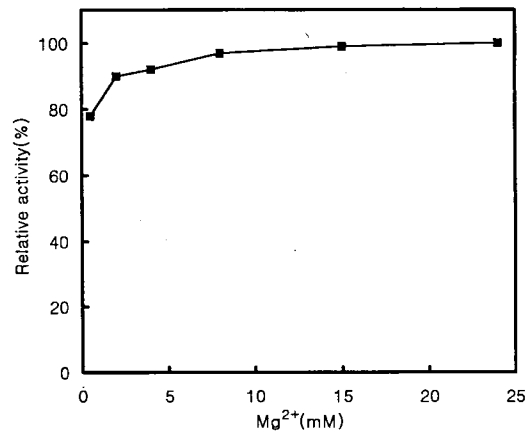

Fig. 6. Activation of aspartase by Mg^{2+}. The activities were measured in the presence of various concentrations of $MgCl_2$ as a control under the standard assay conditions.

Table 2. Effects of divalent cations on aspartase activity[a].

Divalent Cations	Relative Enzyme Activity (%)
Mg^{2+} (5 mM)	90
Mg^{2+} (10 mM)	100
Mn^{2+} (1 mM)	90
Mn^{2+} (3 mM)	100
Ca^{2+} (2 mM)	100
Ca^{2+} (10 mM)	82
Zn^{2+} (0.2 μM)	100
Zn^{2+} (0.5 μM)	60

[a]The activities were measured with the various divalent cations under the standard assay conditions.

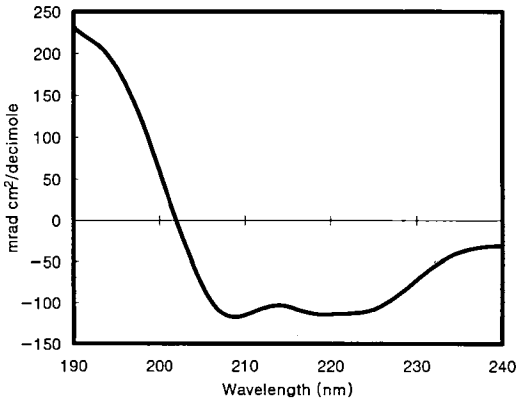

Fig. 7. CD spectra of *Hafnia alvei* aspartase. Assay conditions are described in Materials and Methods.

at least 61%, taking that of poly-L-lysine as a standard (100%). The *E. coli.* enzyme exhibited negative cotton effects through 210 and 224 nm. The helical content of the *E. coli* enzyme was about 70% (Yumoto *et al.*, 1980). The spectrum for the two enzymes between *H. alvei* and *E. coli.* exhibited no significant differences. However, the helical content of the *H. alvei* enzyme is about 9% less than that of the *E. coli* enzyme.

Aspartases have been purified to homogeneity from *E. coli* (Suzuki *et al.*, 1973), *P. fluorescens* (Takagi *et al.*, 1984), and *H. alvei.* The interesting difference between *P. fluorescens* and *H. alvei* is that the former precipitates between 0–35% saturation of $(NH_4)_2SO_4$, while the latter precipitates at 40–70% saturation. It seems to be rather unusual that the *P. fluorescens* enzyme with its molecular weight around 170,000 precipitates at such low salt concentrations. The purified preparation obtained in the present investigation was homogeneous as judged by gel electrophoresis and appeared to be pure enough for mechanism study. The purified enzyme was stable over a few weeks at $-20^{\circ}C$ and stable over 3 months at $-70^{\circ}C$.

Acknowledgements This work is supported in part by a University research grant from Hanyang University (1998).

References

Bada, J. L. and Miller, S. L. (1968) Equilibrium constant for the reversible deamination of aspartic acid. *Biochemistry* **7**, 3403–3408.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Cook, P. F., Blanchard, J. S. and Cleland, W. W. (1981) Secondary deuterium and nitrogen-15 isotope effects in enzyme-catalyzed reaction. Chemical mechanism of liver alcohol dehydrogenase. *Biochemistry* **20**, 1817–1825.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M. (1971) *Data for Biochemical Research*, p430, Oxford Press, London, England.

Karsten, W. E., Hunsley, J. R. and Viola, R. E. (1985) Purification of aspartase and aspartokinase-homoserine dehydrogenase I from *Escherichia coli* by dye-ligand chromatography. *Anal. Biochem.* **147**, 336–341.

Kim, S.K., Choi, J. H. and Yoon, M. Y. (1995) The kinetic parameters of *Hafnia alvei* from pH study. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **28**, 204–209.

Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Nuiry, I. I., Hermes, J. D., Weiss, P. M., Chen, C. Y. and Cook, P. F. (1984) Kinetic mechanism and location of rate-determining steps for aspartase from *Hafnia alvei. Biochemistry* **23**, 5168–5175.

Park, S. H., Kiick, D. M., Harris, B. G. and Cook, P. F. (1984) Kinetic mechanism in the direction of oxidative decarboxylation for NAD-malic enzyme from *Ascaris suum. Biochemistry* **23**, 5446–5453.

Rudulph, F. B. and Fromm, H. J. (1971) The purification and properties of aspartase from *Escherichia coli. Arch. Biochem. Biophys.* **147**, 92–98.

Shim, J. B., Kim, J. S. and Yoon, M. Y. (1997) Chemical modification of cysteine residues in *Hafnia alvei* aspartase by NEM and DTNB. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **30**, 113–118.

Suzuki, S., Yamaguchi, J. and Tokushige, M. (1973) Studies on aspartase I: Purification and molecular properties of aspartase from *Escherichia coli. Biochim. Biophys. Acta* **321**, 369–381.

Takagi, J., Fukunaga, R., Tokushige, M. and Katsuki, H. (1984) Purification, crystallization, and molecular properties of aspartase from *Pseudomonas fluorescens. J. Biochem. (Tokyo)* **96**, 545–552.

Williams, V. R. and Lartigue, D. J. (1967) Quaternary structure and certain allosteric properties of aspartase. *J. Biol. Chem.* **242**, 2973–2978.

Wilkinson, J. S. and Williams, V. R. (1961) Partial purification of bacterial aspartase by starch electrophoresis. *Arch. Biochem. Biophys.* 93, 80–84.

Yoon, M. Y. and Cook, P. F. (1994) pH Studies in the direction of deamination of aspartase from *Hafnia alvei. J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **27**, 1–5.

Yumoto, N., Tokushige, M. and Hayashi, R. (1980) Studies on aspartase VI: Trypsin-mediated activation releasing carboxy-terminal peptides. *Biochim. Biophys. Acta* **616**, 319–328.