Journal of Biochemistry and Molecular Biology, Vol. 32, No. 6, November 1999, pp. 573–578

JOURNAL OF
Biochemistry
and
Molecular Biology

Properties of Trypsin-Mediated Activation of Aspartase from *Hafnia alvei*

Min-Sub Lee, Kyoung-Jae Choi, Si Joong Kwom, Insug Kang[2], Joohun Ha[2], Sung Soo Kim[2], Myung-Soo Han[1], and Moon-Young Yoon*

Department of Chemistry and Department of Life Science[1], Hanyang University, Seoul 133-791, Korea
[2] Department of Molecular Biology, College of Medicine, Kyung Hee University, Seoul 130-701, Korea

Received 5 July 1999, Accepted 17 August 1999

Treatment of *Hafnia alvei* aspartase with limited tryptic digestion resulted in a marked increase in enzymatic activity. The activation required a few minutes to attain maximum level and, thereafter, the activity gradually decreased to complete inactivation. The degree of cleavage associated with the activation was extremely small as judged by SDS-PAGE. Upon activation, the optimum pH and temperature were essentially unchanged. When trypsin-activated enzyme was denatured in 4 M guanidine-HCl followed by removal of the denaturant by dilution, the restoration of activity was similar (40%) to that of the native enzyme, indicating a degree of stability. The pK_a obtained on the acidic side and the pK_b obtained on the basic side of trypsin-activated aspartase were 6.6 and 8.6, respectively, the same as those of the native aspartase, indicating that aspartase may exist in a stable conformation after limited tryptic digestion. These results indicate that the activation of *H. alvei* may be mediated by a conformational change away from the active site of individual subunits.

Keywords: Aspartase, Trypsin activation, *Hafnia alvei*.

Introduction

Aspartase [L-aspartate ammonia-lyase (EC 4.3.1.1)] catalyzes the reversible conversion of L-aspartate to fumarate and NH_4^+. The enzyme has been regarded as a catabolic enzyme in both bacteria and plants. 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 from *E. coli* is a tetramer composed of four apparently identical subunits of molecular weight 48,000 (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 activity.

The pH dependence of the kinetic parameters has been studied in both the amination and deamination direction (Yoon and Cook, 1994; Kim *et al.*, 1995). The *V/K* for aspartase was bell-shaped with estimated pK values of 6.6 and 7.2. The maximum velocity for aspartate was also bell-shaped, giving pK values almost identical to that obtained from $V/K_{aspartate}$. These results indicate that two enzyme groups with pK values of 6.6 and 7.2 are necessary for binding of the substrate and/or catalysis. The *V/K* for fumarate was bell-shaped with pK values of 6.4 and 8.7. The maximum velocity for fumarate was also bell-shaped with pK values of 7.2 and 9.1. These results are consistent with two-enzyme groups that are necessary for catalysis. One residue that must be deprotonated has been identified, and another residue must be protonated for substrate binding. Both the general basic and acidic groups are in a protonation state opposite to that in which they started when aspartate was bound. A proton is abstracted from C-3 of the monoanionic form of L-aspartate by a general base, with a pK of 6.3–6.6 in the absence and presence of Mg^{2+}. A chemical modification study using diethylpyrocarbonate has predicted that a histidine residue is in the proton abstraction (Yoon and Cho, 1998; Shim *et al.*, 1999). Ammonia is then expelled with the assistance of a general acid group, giving NH_4^+ as the product (Yoon *et al.*, 1995).

* To whom correspondence should be addressed.
Tel: 82-2-2290-0946; Fax: 82-2-2299-0762
E-mail: myyoon@email.hanyang.ac.kr

Aspartase from *E. coli* was markedly activated by trypsin treatment without an appreciable alteration of its molecular weight (Mizuta and Tokushige, 1976; Yumoto *et al.*, 1980). Not only the V but also the $S_{0.5}$ value and Hill coefficient for aspartate considerably increased upon activation. The released peptide was separated by high voltage paper electrophoresis, and the amino acids composition and terminal residues were determined. The results showed that one or a few residues related from the COOH-terminal upon activation. Overall, it was concluded that the trypsin-activated aspartase is a more efficient catalyst than the native enzyme. In an effort to better elucidate structure-function relationships in the enzymatic reaction, we have tried to study the trypsin-mediated activation of aspartase from *Hafnia alvei*.

Materials and Methods

Chemical *Hafnia alvei* (ATCC 9760) was purchased from the ATCC (American Type Culture Collection, Rockville, USA). Bovine pancreatic trypsin (type I), soybean trypsin inhibitor (type I-S), L-aspartic acid, 2-(N-morpholino)ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), and 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) were purchased from Sigma Chemical Co. (St. Louis, USA). Sodium dodecyl sulfate (SDS), ammonium persulfate, N,N′-methylene-bis-acrylamide, acrylamide, and N,N,N′,N′-tetramethylene diamine (TEMED) were purchased from Bio-Rad (Richmond, USA). All other chemicals were pure or extra pure grade and commercially available.

Enzyme preparation Aspartase was purified from *Hafnia alvei* as described previously (Yoon *et al.*, 1998). Briefly, aspartase was obtained from a combination of DEAE-cellulose, Red A-agarose, and Sepharose 6B chromatography. The purified enzyme was divided into aliquots and stored at −70°C until use. The catalytic function of the enzyme remained stable for at least one month at 4°C without appreciable loss of enzymatic activity. Protein concentration was determined by the method of Bradford (1976).

Enzyme assay The activity of aspartase was determined at 25°C with a Hewlett Packard 8452 Diode-Array spectrophotometer equipped with a constant-temperature cell housing, by following the formation of fumarate a 240 nm (ε_{240} = 2,255 M^{-1} cm^{-1}) (Cook *et al.*, 1980). All reactions were carried out in a 1 ml cuvette with a 1 cm light path, which were incubated for at least 10 min in the cell compartment prior to initiation of the reaction by the addition of aspartase. The standard assay mixture contained 200 mM HEPES (pH 8.0) buffer, 10 mM aspartate, 20 mM Mg^{2+}, and the enzyme. The aspartate and Mg^{2+} concentrations were corrected for complexation with divalent metal using the following dissociation constant obtained at 0.1 mM ionic strength: Mg-aspartate, 4 mM (Dawson *et al.*, 1971).

SDS-polyacrylamide gel electrophoresis of trypsin-treated aspartase Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) using 12% acrylamide gels. The electrophoresis of the reaction mixture, 3 μg of aspartase and 0.4 μg of trypsin containing 100 mM HEPES (pH 8.0) buffer, was carried out at 30°C for 1, 2, 5, 40, 100, and 180 min. After the designated incubation times, 0.4 μg of trypsin inhibitor was added to the reaction mixture. Samples were incubated at 100°C for 5 min in a fourth part sample volume of a solution consisting of 12.5 mM Tris·HCl (pH 6.8), 0.4% SDS, 2.9 mM βME, 4% glycerol, and 0.02% bromophenol blue. The gel was stained with Coomassie Brilliant Blue G-250.

Circular dichroism (CD) spectroscopy CD spectra were obtained at 25°C on a Jovin-Yvan 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 at intervals of 1 nm with a protein concentration of 0.5 mg/ml. The sample buffer was 10 mM phosphate buffer, pH 7.2, and each spectrum was an average of three scans. The sample spectra were corrected by subtracting the appropriate buffer blanks. The unit of the molecular ellipsoid, $[\theta]$ is degree cm^2 per dmol. Values in degree were converted to rad to compare with the poly-L-lysine reference data in yielding the percentage of α-helix, β-sheet, and random coil.

pH studies Determination of V and V/K values for aspartate were carried out by varying the levels of aspartate at saturating concentrations of Mg^{2+}. All assays reflected the initial velocity conditions with less than 10% of the limiting reactant used over the time course of the reaction. Buffers used at 100 mM concentration were MES at pHs 5.5–6.5, HEPES at pHs 6.5–8.5, and CHES at pHs 8.5–9.5. All buffers were titrated to the appropriate pH level with KOH. The pH was measured before and after all reactions. Several of the assays were repeated at a given pH using different buffers to eliminate the possibility of activation by the buffers. At the pH extremes, the concentration of Mg^{2+} was doubled in separate assays to ensure saturation, but no significant rate change was detected. Data was analyzed according to the appropriate rate equation using the Fortran programs of Cleland (1979).

Results

Effect of trypsin treatment on aspartase relative activity and its molecular size When aspartase was incubated with trypsin at pH 7.4 and 30°C, a marked increase in catalytic activity was observed. In 3 min, the activity was 1.5 times greater than that of the native enzyme, and thereafter, it gradually decreased to almost complete inactivation after 3 h (Fig. 1). Simultaneous inspection of the change in the molecular weight of subunits revealed that the maximally active enzyme had the same band as that of the native enzyme (55 kDa) and other faint bands (36 and 20 kDa) on electrophoresis in the presence of SDS. After prolonged exposure to trypsin for 3 h, the major band corresponding to the native subunits almost disappeared, but the faint band (20 kDa) remained on SDS-PAGE. This result indicates that the activated enzyme remained as a tetramer, and the molecular weight of the subunits did not change to an appreciable extent.

Time (min)

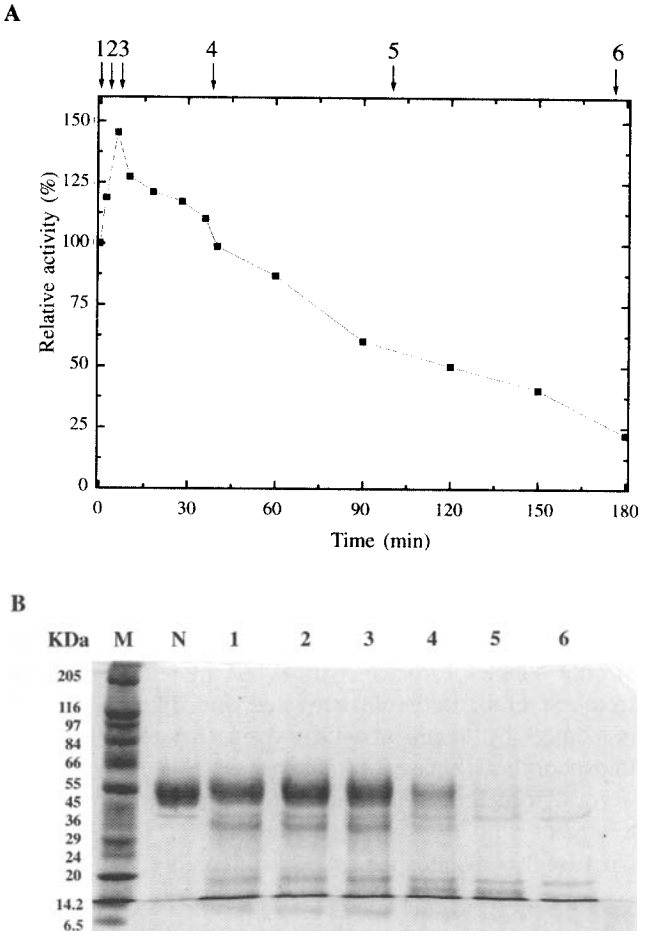

Fig. 1. Alteration of enzyme activity and molecular size of aspartase by trypsin treatment. A. Relative activity change of aspartase during trypsin treatment. The reaction was performed as described in Materials and Methods. B. SDS-PAGE of trypsin-treated aspartase. Lanes M and N are the marker and Non-trypsin treated enzyme (1.2 μg), respectively. The sample proteins were 3 μg (Lanes 1, 2, 3, 4, 5, and 6). Lane numbers of A correspond with lane numbers of B.

Optimum pH and temperature of trypsin-activated enzyme In order to examine whether the optimum temperature and pH have been changed by the limited trypsin treatment, temperature and pH vs. activity profiles were compared before and after trypsin treatment. The trypsin-activated and the native aspartase had the same optimum temperature at 45°C (data not shown). Optimum pH levels of the trypsin-activated and the native enzymes in the presence of metal were almost identical (pH 8.5); however, the activity of trypsin-activated aspartase was higher (1.5-fold) than that of the native aspartase. Optimum pH levels of the trypsin-activated and the native enzymes in the absence of metal were almost identical and broad (pHs 7.0–8.0). The trypsin-activated aspartase in the absence of metal showed similar activity to that of the native enzyme (Fig. 2).

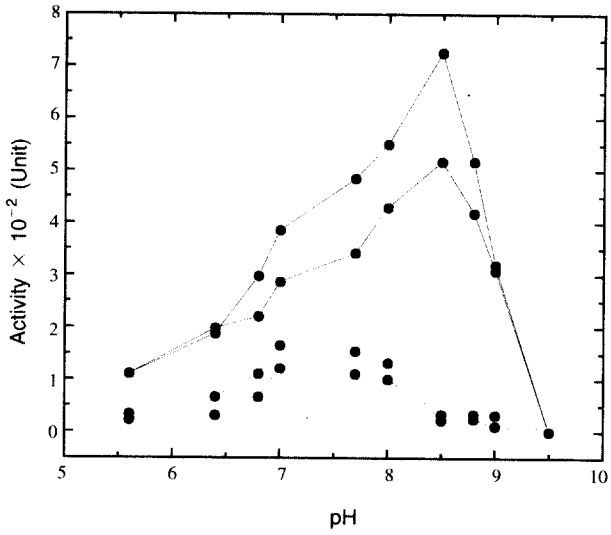

Fig. 2. pH vs. activity profile of trypsin-activated aspartase (●) and native aspartase (○). Initial velocity of the trypsin-activated and the native enzyme reactions were determined at 30°C using a reaction mixture which contained 100 mM HEPES buffer (pH 8.0), 10 mM aspartate, the enzyme, and 20 mM $MgSO_4$ (——) or in its absence (----) in a total volume of 1.0 ml. The pHs were maintained using the following buffers: MES, 5.5–6.5; HEPES, 6.5–8.5; CHES, 8.5–9.5.

Reversible denaturation of trypsin-activated enzyme From the previous studies (Park, 1997), it was revealed that *Hafnia alvei* aspartase denatured in 4 M guanidine(HCl maintained its activity and quaternary structure upon dilution. We also investigated the stability of the trypsin-activated and the native aspartase. The enzyme activity was assayed at designated time intervals using small aliquots of renatured enzyme solution. The untreated enzyme was also examined for reversible denaturation under the same conditions. The original activities of trypsin-activated and native aspartase were restored to their 40% and 50% activities after 20 min, respectively (Fig. 3).

pH Study of trypsin-activated aspartase At various pHs, the maximum velocities and K_m values were obtained to determine the pH dependence of V and V/K of the trypsin-activated aspartase. The K_m for aspartase was near 5.4 ± 0.1 mM. On the other hand, the V_{max} was 0.11 s^{-1}. We calculated pK_a and pK_b values of the trypsin-activated enzyme by processing the data into a computer program developed by Clelend (1979). In the presence of Mg^{2+}, the maximum velocity decreased from constant values at high pH to another constant value at low pH, giving a pK of 7.6. The pK, obtained as V becomes constant at low pH, was 6.5 (Yoon *et al.*, 1995). The V/K profile for aspartate had a maximum value at pH 7.9 and decreased on either side of this maximum. pH vs. Log V and Log V/K profiles of trypsin-activated aspartase were very similar to those of

the native aspartase. The pK_a obtained on the acidic side and the pK_b obtained on the basic side of trypsin-activated aspartase were 6.6 ± 0.2 and 8.6 ± 0.1, respectively (Fig. 4).

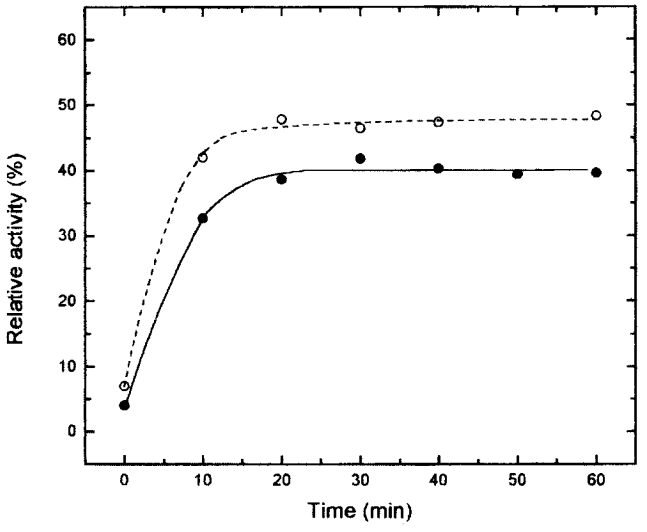

Fig. 3. Reactivation of denatured aspartase in guanidine·HCl. Aspartase (17.9 μg) was incubated with 1 μg of trypsin at 30°C. After 3 min, excess trypsin inhibitor was added. From this 145%, the activated enzyme portion was transferred into test tubes containing 50 μl of guanidine·HCl. After a 30 min incubation at 30°C, the reaction mixture was diluted 21-fold with a buffer containing 50 mM potassium phosphate buffer, pH 6.8, 100 mM KCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA at 30°C. The restored activity was assayed at the designated time internals [native enzyme (○), trypsin-activated enzyme (●)].

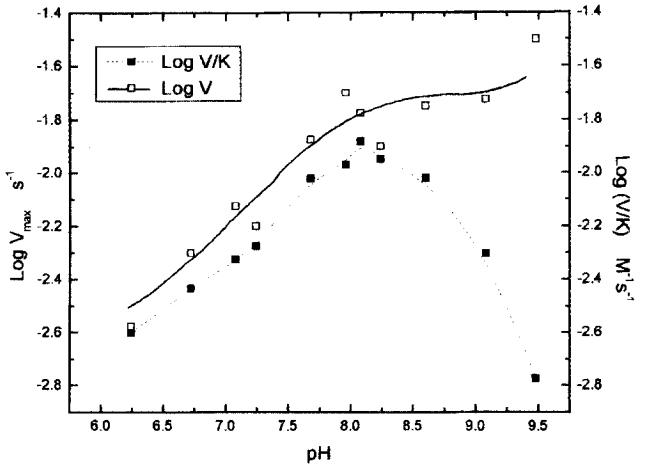

Fig. 4. pH dependence of *V* and *V/K* of the deamination reaction of the trypsin-activated aspartase. The pHs were maintained using the following buffers: MES, 5.5–6.5; HEPES, 6.5–8.5; CHES, 8.5–9.5. The data was fit to Log (Y) = Log [c/(1 + H/K_a + K_b/H)] to yield pK_a and pK_b.

Circular dichroism spectra of trypsin-activated enzyme The CD spectrum of the trypsin-activated aspartase is shown in Fig. 5. The trypsin-activated aspartase exhibited negative cotton effects from 208 through 222 nm. From these values, the helical content of the trypsin-activated enzyme was estimated to be at least 71% (Table 1), taking that of poly-L-lysine as a standard (100%). The native enzyme exhibited negative cotton effects from 208 to 218 nm. The helical content of the native enzyme was about 61% (Yoon *et al.*, 1998). The percentage (71%) of helical structure of the trypsin-activated aspartase was increased, while the percentage (18%) of its sheet structure was decreased.

Discussions

Limited proteolysis has been widely employed as a useful technique for investigation of the structure-function relationship in various enzymes (Hayakawa *et al.*, 1973; Kuczenski, 1973; Yumoto *et al.*, 1980). Molecular mechanisms of activated enzymes by limited proteolysis are not simple. Tyrosine hydroxylase of tetrameric form from rat brain is 2-fold activated and dissociated into monomers by treatment with trypsin (Kuczenski, 1973). Phosphorylase kinase of rabbit muscle is activated by several-fold and not dissociated into monomers by treatment with trypsin (Hayakawa *et al.*, 1973). When fructose-1,6-bisphosphatase is activated by a limited

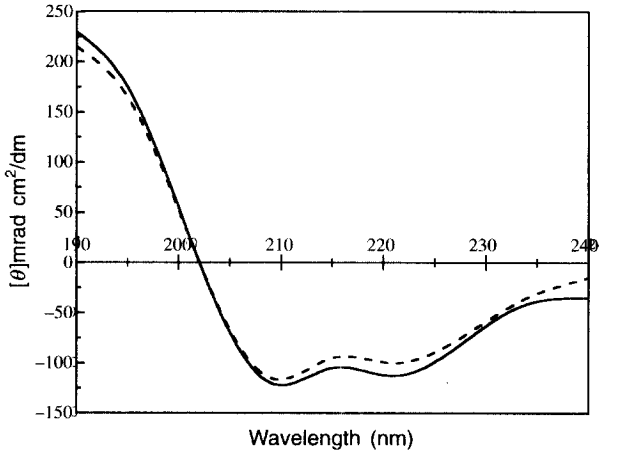

Fig. 5. Circular dichroism (CD) spectra of the trypsin-activated (----) and the native (——) aspartase. CD spectra were obtained as described in Materials and Methods.

Table 1. Percentage of different secondary structure conformations of the trypsin-activated and the native aspartase.

Aspartase	Helix	Sheet	Coil
Native	61%	24%	15%
Trypsin-activated	71%	18%	11%

proteolysis, the optimum pH for the activity is altered to a marked extent, and an apparent activation is attained by this pH shift (Traniello *et al.*, 1972). Thus, a variety of features are known for protease-activation.

Hafnia alvei aspartase was activated 1.5-fold by limited treatment with trypsin, while aspartase purified from *E. coli* was activated 3.3-fold under similar conditions (Mizuta and Tokushige, 1976). The activation of the two different bacterial species by limited treatment with trypsin showed 2-time differences. Limited proteolysis at the activation site is assumed to induce to the conformation change. This is an indication that the conformational change difference is attributed to the different amino acid compositions of the two species. The degree of peptide cleavage associated with the activation seems to be extremely small as judged from the fact that no appreciable change in the molecular weight of the enzyme subunits is detected by SDS-PAGE. Upon activation, the optimum pH and the optimum temperature were essentially unchanged.

Aspartase, denatured in 4 M guanidine·HCl, was renatured *in vitro* by simple dilution with a concentration restoration of the 50% activity (Park, 1997), i.e. aspartase is reversibly denatured. After denaturation of the trypsin-activated enzyme in guanidine·HCl, the restoration of the activity occurred at a similar ratio (40%) to the native enzyme activity. This is an indication that the stability degree of the trypsin-activated enzyme and the native enzyme are in similar states. The CD spectra for the enzymes from *H. alvei* and *E. coli* exhibited no significant differences. However, the helical content of the *H. alvei* enzyme was about 9% less than those of the *E. coli* enzyme (*vide ante*). The helical content of the *E. coli* enzyme was 70%. However, the helical content of trypsin-activated aspartase from *E. coli* was 65% (Yumoto *et al.*, 1980). The helical content of the trypsin-activated enzyme from *E. coli* appears to be 5% less than that of the native enzyme. Interestingly, the helical content in trypsin-activated aspartase from *H. alvei* was rather increased (9%) as compared to that of the native enzyme. These results indicate that the activated enzyme species has a somewhat tightened conformation than that of the native enzyme.

Aspartase obtained from limited tryptic digestion exhibited dramatically altered kinetic properties when compared to the native enzyme. The native enzyme exhibited apparent K_m for aspartate near 1.3 mM, whereas, aspartase by limited tryptic digestion exhibited apparent K_m for aspartate near 5.4 mM, which is an increase of 4-fold. The trypsin-activated aspartase exhibited a V_{max} near 0.11 s^{-1} which was increased to 0.02 s^{-1} by limited tryptic digestion. The maximum velocity and $V/K_{aspartate}$ profile by limited tryptic digestion are similar to those of the native enzyme within error. Generally, the change of V_{max} and $V/K_{aspartate}$ induces a change in the ionization of the enzyme. This result suggests that aspartase by limited tryptic digestion may exist in a conformationally stable but relatively active state.

The activation of aspartase by limited tryptic digestion is qualitatively similar for the *H. alvei* (this study) and *E. coli* enzymes. The activation for both enzyme exhibits 1.5–3.3 fold increases in rate. Both activated enzymes also remained as tetramers and the molecular weight of the subunits did not change to an appreciable extent. The optimum pH and the optimum temperature are essentially similar. When the trypsin-activated enzyme for *H. alvei* was denatured in 4 M guanidine·HCl, followed by removal of the denaturant by dilution, the enzyme activity is restored to its 50% activity, while the enzyme activity in the case of *E. coli* restored to its 150% activity. This is an indication that the *E. coli* enzyme by limited tryptic digestion is a rather stable molecule. However, the native aspartase restored to 40% for *H. alvei* and 70% for *E. coli*. Even more striking were the differences in the CD spectra of trypsin-activated enzyme. The helical structure of trypsin-activated aspartase from *H. alvei* was increased from 61% to 71%, while that of *E. coli* decreased from 70% to 65%, an indication that *H. alvei* aspartase has a somewhat tighter conformation than *E. coli* aspartase. On the basis of these observations, we would suggest that the activation of aspartase from *H. alvei* may be mediated by a conformational change away from an active site of the individual subunits. Further investigation is needed for elucidation of the molecular mechanism of the trypsin-mediated activation of *H. alvei* aspartase.

Acknowledgments This work was supported in part by research grants from Hanyang University (1999).

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.

Cleland, W. W. (1979) Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **63**, 103–138.

Cook, P. F., Blanchard, J. S. and Clelend, W. W. (1980) 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., Eillot, W. H. and Jones K. M. (1971) Data for Biochemical Research, p. 430, Oxford University Press, London, England.

Hayakawa, T., Perkins, J. P. and Krebs, E. G. (1973) Studies on the subunits structure of rabbit skeletal muscle phosphorylase kinase. *Biochemistry* **12**, 574–580.

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

Kuczenski, R. (1973) Rat brain tyrosine hydroxylase. *J. Biol. Chem.* **248**, 2261–2265.

Laemmli, U. K. (1970) Cleavage of structural protein during the

assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Mizuta, K. and Tokushige, M. (1976) Alteration of enzymatic properties upon trypsin-mediated activation. *Biochim. Biophys. Acta* **452**, 253–261.

Park, J. B. (1997) Studies of denaturation and reactivation of aspartase from *H. alvei*. Master Thesis, Hanyang University, Korea.

Shim, J. H., Kim, H. J. and Yoon, M. Y. (1999) Diethylpyrocarbonate inactivation of aspartase from *Hafnia alvei*. *J. Biochem. Mol. Biol.* **32**, 326–330.

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

Traniello, S., Melloni, E., Pontremoli, S., Sia, C. L. and Horecker, B. L. (1972) Rabbit liver fructose-1,6-diphosphatase properties of the native enzyme and their modification by subtilisin. *Arch. Biochem. Biophys.* **149**, 222–231.

Williams, V. R. and Lartigue, D.J. (1967) Quaternary structure and certain allosteric properties of aspartase. *J. Biol. Chem.* **242**, 2973–2987.

Yoon, M. Y. and Cho, C. G. (1998) Affirmation of a general base of *Hafnia alvei* aspartase by organic solvent perturbation method. *Bull. Korean Chem. Soc.* **19**, 31–33.

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

Yoon, M. Y., Thayer-Cook, K. A., Berdis, A. J., Karsten, W. E., Schnackerz, K. D. and Cook, P. F. (1995) Acid-base chemical mechanism of aspartase from *Hafnia alvei*. *Arch. of Biochem. Biophys.* **320**, 115–122.

Yoon, M. Y., Park, J. H., Choi, K. J., Kim, J. M., Kim, Y. O., Park, J. B. and Kyung, J. B. (1998) Purification of aspartase from *Hafnia alvei*. *J. Biochem. Mol. Biol.* **31**, 345–349.

Yumoto, N., Tokushige, M. and Hayashi, R. (1980) Typsin mediated activation releasing carboxy terminal peptides. *Biochem. Biophysic. Acta* **616**, 319–328.