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연어알중의 Carboxypeptidase B 분리정제 및 그 특성에 관한 연구

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Purification and Characterization of Carboxypeptidase B from Wild Salmon (*Salmo Salar*) Eggs

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요 약. 연어(*Salmo Salar*)알 중의 Carboxypeptidase B를 CM-셀룰로오스, 0.5 포화황산 암모늄, DEAE-셀룰로오스 및 세파덱스 G-75 젤로 정제하여 그 성질을 조사하였다. 이 효소의 최적 온도는 55°C였으며, 최적 pH는 4.0과 7.0이었고, pH 안정성은 2.0~3.0 및 5.5~7.0이었다. 히푸릴-L-아르기닌 기질에 대하여 글리실-L-아르기닌 부위를 절단하는 특이성을 보였고, 그 K_m 값은 0.21 mM이었다. Cu^{2+}와 Fe^{3+} 존재하에서는 효소의 활성도가 증가하였지만 Zn^{2+}의 경우에는 감소하였다. 특히 리신은 히푸릴-L-아르기닌 기질에 대하여 경쟁적 억제작용을 보였으며, K_i 값은 4.3mM이었다. 분자량은 36,400 돌톤이었고, 19종류의 아미노산으로 구성된 단위체이었다.

ABSTRACT. Carboxypeptidase B from *Salmo Salar* eggs was purified by CM-cellulose, 0.5 ammonium sulfate saturation, DEAE-cellulose, and Sephadex G-75 gel filtration and its enzymatic properties were investigated. Optimum temperature was 55°C, pH optima were 4.0 and 7.0 at 37°C, and the enzyme was stable at pH 2.0~3.0 and 5.5~7.0 for 1.5h. This enzyme showed substrate specificity hydrolyzing the peptide bond of glycyl-L-arginine. Its K_m value was 0.21mM, and the enzyme activity was stimulated by Cu^{2+} and Fe^{3+}, while inhibited by Zn^{2+}. The lysine was found to be competitive inhibitor and its K_i value was determined to be 4.3mM. Molecular weight of this enzyme was determined to be 36,400 daltons by SDS-PAGE and the enzyme was monomeric protein composed of 19 kinds of amino acid residues.

INTRODUCTION

A number of fishes and shellfishes are widely cultured in fish farms of all over the world. The aquaculture industries are now of greater importance as a production of protein-rich human foods. The aquaculture organisms, however, are suffering from various diseases caused by the culture such as parasitic, environmental, nutritional, and constitutional disease than are in natural conditions. Thus, it is quite natural that both pigment distributions and enzymatic pro-

perties, capable of influence on the sources of essential nutrients, would be abnormally changed in the fish with diseases. Despite of such physiological importance, comparative studies on the enzymes in fish[1] have seldom been investigated although the naturally occurring enzymes distributed in plants and mammalian cells[2,3] have been studied by various purification procedures. Trypsin like enzyme in Crayfish[4~8], however, has been studied, and amino acid compositions of carboxypeptidase A and B isolated from Dogfish[9,10] were reported. Pancreatic procarboxypeptidase B and carboxypeptidase B were purified from African Lungfish[11], and carboxypeptidase A and B purified from White Shrimp[12] had been found to be due to two different proteins. Carboxypeptidase B like enzyme from Starfish[13,14] was also purified and characterized. Inhibitors and activators of porcince pancreas were studied[15,16]. The identification of a glutamic acid at the active center of bovine carboxypeptidase B had been implicated[17] as a functional group by x-ray crystalography and affinity labeling.

However, enzymatic studies on Salmon's eggs have not ever been investigated. In a relation to importance of aquaculture development, fish pathology, and physiological functions, the carboxypeptidase B from wild salmon eggs was purified and characterized in this paper.

MATERIALS AND METHODS

Materials. The mature eggs of wild salmon (*Salmo Salar*) caught in the east sea during the first week of December in 1983 were examined. Reagents such as DEAE-cellulose, Sephadex G-75, bovine hemoglobin(BH), hippuryl-L-arginine(H-arg), hippuryl-L-phenylalanine (H-phe), bovine albumin(BA), egg albumin(EA), pepsin(Pep), trypsin(Try), diazoacetyl-DL-norleucine(DAN), and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane(EPNP) were purchased from Sigma Co. LTD. N,N,N′,N′-tetramethylethylene diamine(TEMED), N,N′-methylene-bisacrylamide(MBAA), and acrylamide(AA) were purchased from Wako Co. LTD. MBAA and AA were repurified by the method of Loening[18] before use. All other chemicals were of analytical grade.

Isolation and Purification of Carboxypeptidase B. Mature eggs(300g) were homogenized for 3 minutes using a Waring blender with 600m*l* of 0.05M phosphate buffer(pH 7.0) and allowed to stand for overnight at 4°C. According to the method of Zagalsky[19] with slight modifications, the homogenate was centrifuged at 3,000rpm for 30 minutes and the yellow lipids floating at the surface were discarded. The supernatant was then stirred for 15 minutes with CM-cellulose powder, filtrated, and the solution was precipitated at 0.5 ammonium sulfate saturation. This was centrifuged, and then the supernatant and fats floating at the surface were discarded. The precipitates were dialysed against 0.05*M* phosphate buffer(pH 7.0) in cold room, followed by centrifugation at 18,000 × g for 20 minutes. After freeze concentration, the enzyme preparation was applied onto a column (2.7× 40cm) of DEAE-cellulose which was previously equilibrated with 0.05*M* phosphate buffer (pH 7.0) and eluted by stepwise gradient with 0.1 *M*, 0.3*M*, 0.5*M*, and 0.7*M* phosphate buffer (pH 7.0) at the flow rate of 0.5m*l*/min respectively. The fractions eluted with 0.1*M* phosphate buffer (pH 7.0) were concentrated up to 20m*l* using freeze-dryer. This was then purified by gel filtration using Sephadex G-75 gel columns. All operations were performed at 4°C.

Enzyme Assay. The active fractions (A and B) toward the BH substrate were assayed according to the method of Oda[20~22]. One unit of enzyme activity was defined as the change of

1μg tyrosine per minute at 37°C. The active fraction B was assayed using H-arg as substrate by the modified method of Decker[23]. One unit hydrolyzes 1μmole of H-arg per minute at pH 7.0 and 37°C under the following specified conditions. Assay mixture contained 0.2m*l* of 10mM H-arg in 0.01*M* McIlvaine buffer (pH 7.0), 0.5m*l* of the same buffer, and 0.1m*l* of enzyme solution. After incubating for 20 minutes at 37°C, the enzyme reaction was terminated by heating in boiling water bath for 3 minutes. After centrifugation and filtration (millex-filter, 0.45μm), the rate of reaction was determined by an increase in absorbancy at 254nm resulting from hydrolysis of substrate. The amount of total hippuric acid formed was determined from standard curve. The protein content was determined according to the method of Lowry *et al*[24].

Disc-Polyacrylamide Gel (PAG) and SDS-PAG Electrophoresis. Disc-PAG electrophoresis was performed on 7.5% gels by the method of Cooper.[25] For estimation of molecular weight, SDS-gel electrophoresis was performed on 11% gels using current density 8mA per gel tube for 2.5hr at pH 8.3 by the method of Lugtenberg[26]. SDS-PAG electrophoresis system contained 0.2% SDS, 11% running gel, and 3% stacking gel, Scanning of the electrophoresis patterns was performed with densitometer (Toyo model DMU-33C) at 620nm, slit, 0.2×2mm, and intensity, 3.0.

Km and Ki Values. The Km of the enzyme for H-arg was estimated by Lineweaver-Burk double reciprocal plot. To determine the inhibition constant (Ki) of the enzyme for lysine, Dixon plot was done.

Amino acid Analysis. Amino acid was analyzed on a Beckman model 116 amino acid analyzer under the same analytical conditions[21,22]. 0.2mg (4.6m*l*) of the enzyme was hydrolyzed with 6*N* HCl at 110°C for 20h in vacuo. Tryptophan was determined according to the method of Goodwin[27] and cysteine was determined as cysteic acid after oxidation with performic acid using the method of Moore[28].

RESULTS AND DISCUSSION

Purification of Carboxypeptidase B. As mentioned in experimental part, the result of the DEAE-cellulose column chromatography was shown in *Fig.* 1. The fractions eluted with 0.1*M* phosphate buffer (pH 7.0) showed a strong enzyme activity toward the BH. Therefore, these fractions were collected and concentrated to 20m*l*, and then placed on a Sephadex G-75 column (2.0×60cm) which was previousely equilibrated with 0.01*M* McIlvaine buffer (pH 7.0). The column was then eluted with the same buffer at slow flow rate (0.1m*l*/min). The fraction was separated into two different

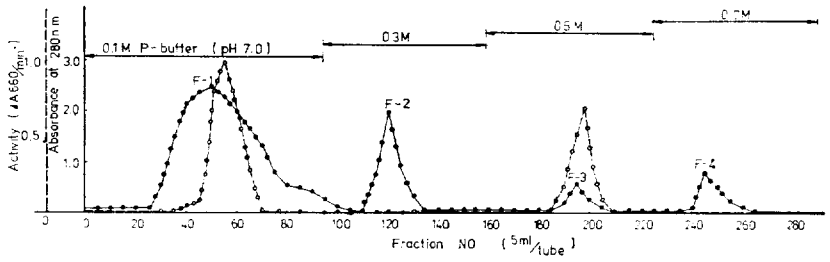

Fig. 1. Elution profile and localization of enzyme activity from DEAE-cellulose column chromatography. The column (2.7×40cm) was equilibrated with 0.05*M* phosphate buffer (pH 7.0) and eluted by stepwise gradient with 0.1*M*, 0.3*M*, 0.5*M*, and 0.7*M* same buffer, respectively. ●; absorbance at 280nm. ○; activity by Folin's method (Oda, 1974 and Min *et al.*, 1983).

forms of enzymic active fractions (A and B) toward the BH as shown in *Fig.* 2. Fraction B hydrolyzed H-arg but not H-phe, while fraction A hydrolyzed only the H-phe. Fraction B was collected, and freeze dried up to 10*ml*, and then rechromatographed on a longer Sephadex G-75 column (1.0×120cm) for further purification as shown in *Fig.* 3. The results of purification and specific activity from each step were summarized in *Table* 1. Compared to the initial step, the purification of carboxypeptidase B was 320 fold, and specific activity was 83 units/mg protein.

Disc-and SDS-PAG Electrophoresis. As shown in *Fig.* 4, disc-PAG electrophoresis pattern(A) of this enzyme appeared to be single component on 7.5% polyacrylamide gel electrophoresis. SDS-PAGE pattern(B) obtained after treatment with 2-ME showed that the enzyme was composed of monomeric protein. Molecular weights of carboxypeptidase B previousely determined by SDS-PAGE from Dogfish[10], African Lungfish[11], White Shrimp[12], Porcine[30], Bovine[31], and Rat[32] were 35,000~37,000, 34,000, 31,000, 34,300, 34,000, and 35,000, respectively, and procarboxypeptidase B from Bovine[31] and Rat[32] were reported being 57,000 and 55,000 respectively. Molecular weight of this enzyme was found to be 36,400 daltons and this was very close to that of Dogfish carboxypeptidase B as

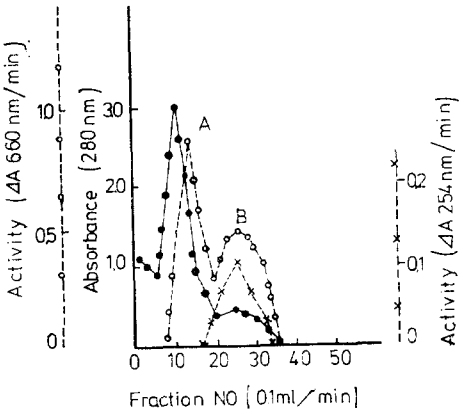

Fig. 2. Elution profile and localization of carboxypeptidase activity after gel filtration with Sephadex G-75 gel. The column (2.0×60cm) was equilibrated with 0.01*M* Mcllvaine buffer(pH 7.0) and eluted with the same fuffer. ●; protein absorbance at 280nm. ○; activity by Folin's method (Min *et al.*, 1983). ×; carboxypeptidase B activity toward the H-arg.

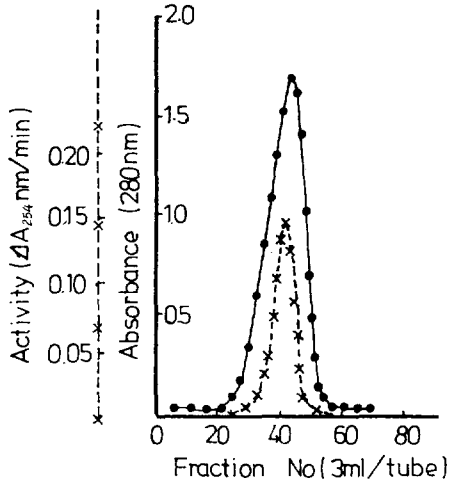

Fig. 3. Sephadex G-75 rechromatography of carboxypepitdase B. The column (1.0×120cm) was equilibrated with 0.01*M* Mcllvaine buffer (pH 7.0), and eluted with the same buffer. ●; protein absorbance at 280nm. ×; carboxypeptidase B activity toward the H-arg.

Table 1. Purification of carboxypeptidase B from *Salmo Salar* eggs

Procedure	Volume (*ml*)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Relative purity
Cell homogenate	1,360	84.6	0.26	100	1
0.5 $(NH_4)_2SO_4$ saturation	477	60.5	2.8	71	10.4
DEAE-cellulose chromatography	72	30.1	20	35.5	76.9
Sephadex G-75 chromatography	40	9.0	51	10.6	196.1
Sephadex G-75 rechromatography	20	4.83	83.3	5.7	320.3

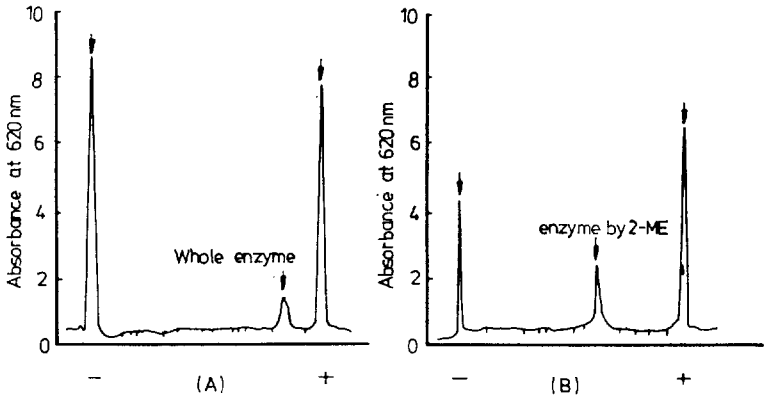

Fig. 4. Disc- and SDS-PAG electrophoresis patterns of carboxypeptidase B scanned by densitometer. (A); Disc-PAGE. (B); SDS-PAGE, slit, 0.2×2mm intensity, 3.0.

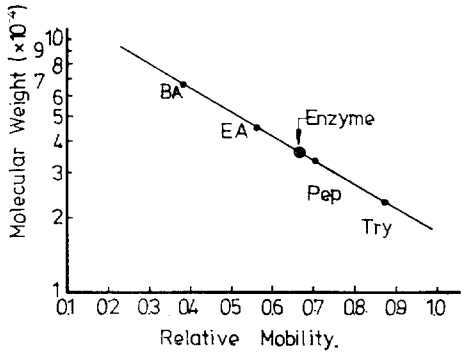

Fig. 5. Molecular weight of carboxypeptidase B determined by SDS-PAG electrophoresis. Marker proteins were bovine albumin (mol. wt. 66,000), egg albumin (mol. wt. 45,000), pepsin(mol. wt. 34,700), and trypsin (mol. wt. 24,000), respectively. Relative mobilities were 0.38, 0.56, 0.66 (carboxypeptidase B), 0.71, and 0.87, respectively. Electrophoresis was performed at pH 8.3 using current density 8mA/gel, 140 volts for 2.5hr.

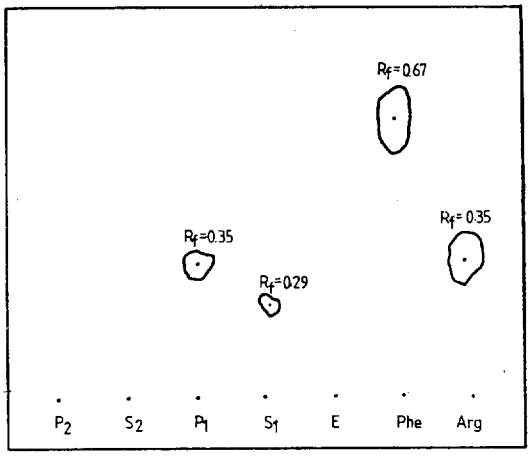

Fig. 6. Paper chromatogram of substrate specificity. Solvent; *n*-BuOH/HAc/H_2O (12 : 3 : 5, v/v/v) Coloring reagent; 0.2% ninhydrin in acetone. P_2 : hydrolysate of H-phe. P_1 : hydrolysate of H-arg. S_2 : H-phe. S_1 : H-arg. E : enzyme. Phe : phenylalanine. Arg : arginine.

shown in *Fig.* 5.

Substrate Specificity. The enzyme-catalyzed reaction for H-arg as substrate was carried out for 20 minutes at 37°C and optimum pH, and the reaction was terminated by heating in water bath (100°C) for 3 minutes. The reaction mixture was then concentrated in lyophilyzer, filtrated, and then paper chromatographed with *n*-BuOH/HAc/H_2O(12 : 3 : 5, v/v) as a developing solvent. As shown in *Fig.* 6, the enzyme showed substrate specificity hydrolyzing the peptide bond of glycyl-L-arginine of substrate.

Optimum pH and Stability. To estimate the optimal pH, the enzyme reaction was carried out under the various pH for 20 minutes at 37°C in the solution of H-arg(10m*M*) dissolved in

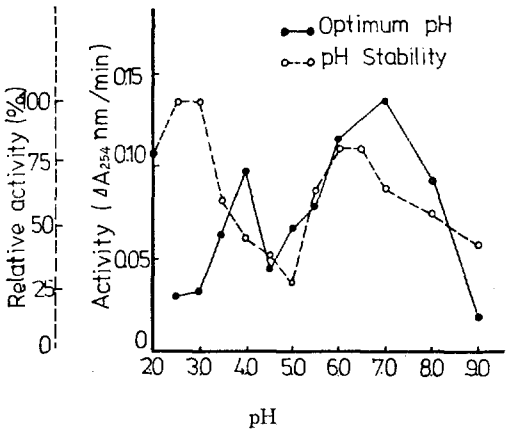

Fig. 7. pH optima and stability of carboxypeptidase B. pH 2.0~pH 3.0; 0.01*M* Sørensen buffer. pH 3.0-pH 7.5; 0.01*M* Mcllvaine buffer. pH 8.0-pH 9.0; 0.01*M* Tris-HCl buffer.

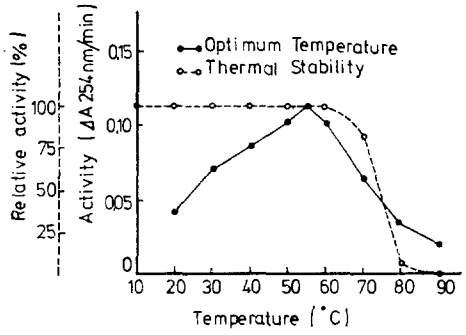

Fig. 8. Optimum temperature and thermal stability of carboxypeptidase B.

0.01*M* Mcllvaine buffer. The enzyme solutions for the test of pH stability were preincubated at each pH for 90 minutes before the addition of substrate, followed by the remaining activities were assayed. The results showed double pH optima toward the H-arg at pH 4.0 and pH 7.0, but did not show single optimum pH as shown in *Fig.* 7. The dual optimum characteristics was similar to that of β-glucosidase from Rat Uterus[29] except acidic and basic conditions, and also similar to pH profile for the carboxypeptidase B from Starfish[14] which showed an activity maximum in the region pH 7.0~7.5

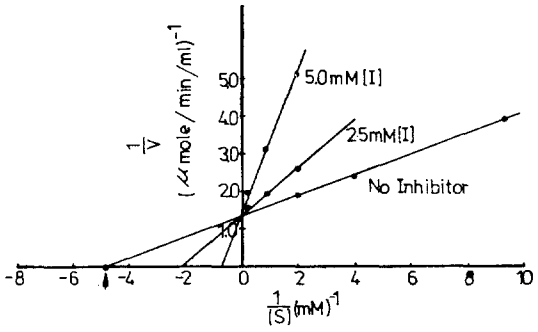

Fig. 9. Lineweaver-Burk double reciprocal plots for inhibition of the enzyme hydrolysis by lysine. The slopes for the lines increase as the inhibitor concentration increases from 2.5m*M* to 5.0m*M*, respectively.

Table 2. Effect of various reagents on carboxypeptidase B activity

Compound	Concentration (m*M*)	Relative activity (%)
None		100
$FeCl_3$	10	156
	1	125
$CuCl_2$	10	142
	1	109
$MgCl_2$	10	100
	1	100
$CaCl_2$	10	100
	1	100
$CoCl_2$	10	100
	1	100
$ZnCl_2$	10	92
	1	97
Lysine	10	86
Cysteine	10	100
DAN	1	unusual
EPNP	1	unusual

and a second peak at high pH. These were consistent with the facts that the pH optima of Shrimp[12] carboxypeptidase B were at the pH range of 4.35~8.80 and exhibited maximum activity at pH 6.0~8.0. It seemed to be quite stable over the pH range of 5.5 to 7.0 at least for 90 minutes at 37°C, but completely inacti-

vated at pH 5.0 and over pH 9.0 respectively.

Optimum temperature and Thermal Stability. All the enzyme reactions were carried out at optimum pH and various temperatures for 20 minutes. For the test of thermal stability of the enzyme, the reaction mixture was preincubated at each temperature for 30 minutes and the remaining activities were assayed at 37°C. The optimum tem perature for the enzyme reaction was 55°C, and the enzyme was stable at below 60°C, but lost its activity rapidly at 80°C as shown in *Fig.* 8. These facts were in full agreement with carboxypeptidase B from Starfish[13,14].

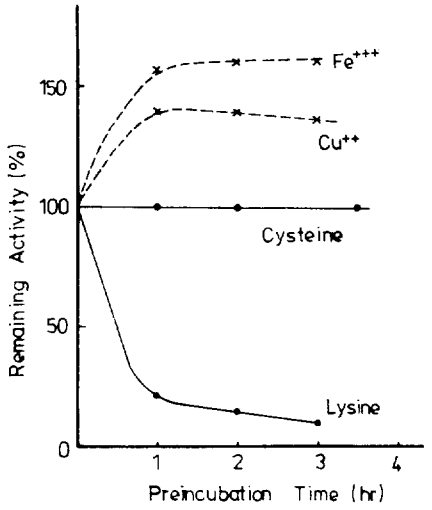

Fig. 10. Effect of modifiers on the rate of hydrolysis of carboxypeptidase B. Concentrations of modifiers were 10m*M* each.

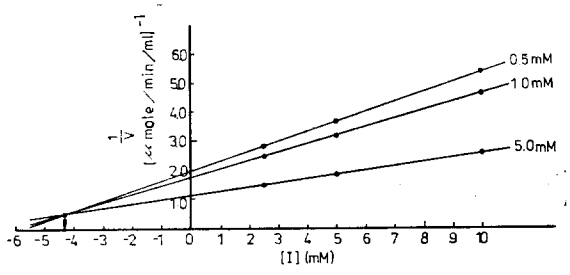

Fig. 11. Dixon plot for the inhibition of the enzyme hydrolysis by lysine. The slopes for the lines decrease as the substrate concentration increases from 0.5m*M*, to 1m*M*, and 5m*M*, respectively.

Kinetic Parameters. The activity of enzyme as a function of substrate concentration was assayed spectrophotometrically in 0.01*M* McIlvaine buffer (pH 7.0). The double reciprocal plot between substrate concentration and the rate of reaction was show in *Fig.* 9. The K_m and V_{max} values for purified carboxypeptidase B, when H-arg was used as a substrate, was 0.21 m*M* and 1.40μmole/min/m*l* respectively. These values were very close to those of carboxypeptidase B from White Shrimp[12], which was 0.2m*M*, but the Kms of procarboxypeptidase B and carboxypeptidase B from Lungfish[11] were 0.62m*M* and 0.32m*M* respectively when synthesized benzoylglycyl-L-arginine was used as substrate.

This enzyme was inactivated by Zn^{2+} whereas the reaction was stimulated by addition of Cu^{2+} and Fe^{3+} respectively, but showed no effect on the activity by Mg^{2+}, Ca^{2+}, and Co^{2+} as shown in *Table* 2. The enzyme was strongly inhibited

Table 3. Amino acid content of carboxypeptidase B

Amino acid	Component (mg/100m*l*)
Lysine	0.53
Histidine	0.16
Arginine	0.33
Aspartic acid	0.60
Threonine	0.30
Serine	0.27
Glutamic acid	0.75
Proline	0.33
Glycine	0.25
Alanine	0.58
Valine	0.47
Methionine	0.10
Isoleucine	0.37
Leucine	0.64
Tyrosine	0.27
Phenylalanine	0.29
Cysteine	0.20
Tryptophan	0.23
Total	6.67

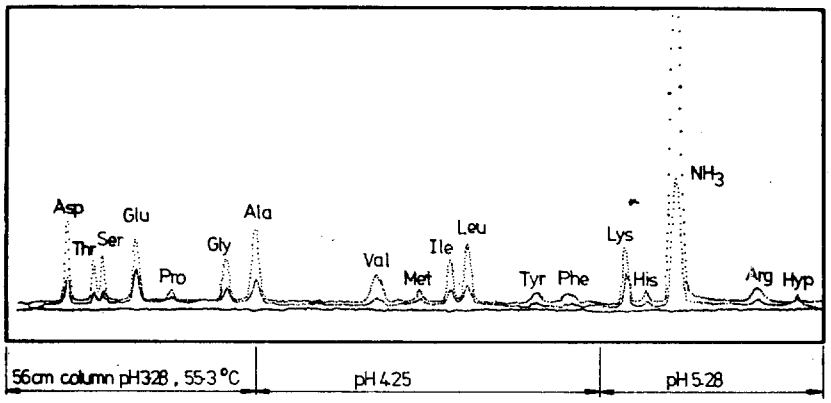

Fig. 12. Amino acid profile of carboxypeptidase B.

by lysine (10m*M*) in one hour but the inhibition gradually decreased thereafter one hour as shown in *Fig.* 10. However, the enzyme was not inhibited by cysteine. Inhibition constant(K_i) of this enzyme for lysine was 4.3m*M* by Dixon plot as shown in *Fig.* 11. The lysine was found to act as a fully competitive inhibitor.

Amino Acid Composition. The amino acid contents were shown in *Table* 3 and *Fig.* 12. Of particular interest was the presence of hydroxyproline like residue. The amounts of hydrophobic amino acid residues and small amino acid residues were high (30% and 27.4%), but those of the hydrogen bonding groups, playing a significant role in the protein structure, were low (18.8%). The helix breaking amino acids were present in low amounts (23.2%), while amino acids (Ileu, Val, Thr, and Glu) which occur in frequency in β-structures of marine products[19] were most abundant (30.3%). This enzyme had high contents of leucine, the main stabilizing influence on the conformation of protein in the inner sections of α-helices. The contents of leucine and glutamine were 22.3% of total amino acid residues. The amino acid composition was similar to thase of carboxypeptidase B from Crayfish[7], and its structure probably may have random coil and β-formation in terms of the α-helix contents(%) of carboxypeptidases.

REFERENCES

1. T. Sakai and H. Kawatsu, *Bull. Japan. Soc. Sic. Fish.* **49**, 683 (1983).
2. M. T. Everitt and H. Neurath, *Febs lett.*, **110**, 292 (1980).
3. C. Quinto, M. Quiroga, W. F. Swain, W. C. Nikovits, Jr, D. N. Standring, R. L. Pictet, P. Valenzuela, and W. J. Rutter, *Proc. Natl. Acad. Sci. U. S. A.*, **79**, 31(1982).
4. R. Zwilling, G. Pfleiderer, H. M. Sonneborn, V. Kraft, and I. Stucky, *Comp. Biochem. Physiol.*, **28**, 1275(1969).
5. R. Zwilling, H. Neurath, *Methods Enzymol.* **80**, 633 (1981).
6. R. Zwilling, F. Jakob, H. Bauer, H. Neurath, and D. L. Enfield, *Eur. J. Biochem.* **94**, 223 (1979).
7. K. Titani, L. H. Ericsson, S. Kumar, F. Jakob, H. Neurath and R. Zwilling, *Biochem.* **23**, 1245 (1984).
8. K. Titani, T. Sasagawa, R. G. Woodbury, L. H. Ericsson, H. Dörsam, M. Kraemer, H. Ne urath, and R. Zwilling, *Biochem.* **22**, 1459(1983).
9. A. G. Lacko and H. Neurath, *Biochem.* **9**, 4680 (1970).
10. J. W. Prahl, H. Neurath, *Biochem.* **5**, 4137 (1966).
11. G. R. Reek and H. Neaurath, *Biochem.* **11**, 3947 (1972).
12. B. J. Gates and J. Travis, *Biochem.* **12**, 1867 (1973).

13. Z. Camacho, J. R. Brown, and G. B. Kitto, *J. Biol. Chem.*, **245**, 3964 (1970).
14. R. E. Ferrell, Z. Camacho, G. B. Kitto, *Biochim. Biophys. Acta*, **386**, 260 (1975).
15. N. Zisapel, N. Kurn-Abramowitz and M. Sokolovsky, *Eur. J. Biochem.*, **35**, 507 (1973).
16. N. Zisapel, N. Kurn-Abamowitz and M. Sokolovsky, *Eur. J. Biochem.*, **35**, 512 (1973).
17. M. T. Kimmel and T. H. Plummer, Jr, *J. Biol. Chem.*, **247**, 7864 (1972).
18. U. E. Loening, *Biochem. J.* **102**, 251 (1967).
19. P. F. Zagalsky, H. J. Ceccaldi, and R. Daumas, *Comp. Biochem. Physiol.*, **34**, 579 (1970).
20. K. Oda and S. Murao, *Agr. Biol. Chem.*, **38**, 2435 (1974).
21. T. J. Min, S. I. Hong and J. W. Kim, *Kor. Bicchem. J.* **16**, 42(1983).
22. T. J. Min, K. S. Chung and J. W. Kim, *ibid.*, **16**, 205 (1983).
23. L. A. Decker, "Worthington Enzyme Manual," Freehold, New Jersey, U. S. A., 1977.
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 263 (1951).
25. T. G. Cooper, "Tools of Biochemistry," John Wiley & Sons, Inc. 1977.
26. B. Lugtenberg, J. Meijers, R. Peters, P. V. A. Hoek, and L. V. Alphen, *Febs letters*, **58**, 254 (1975).
27. T. W. Goodwin and R. A. Morton, *Biochem. J.* **40**, 628 (1946).
28. S. Moore, *J. Biol. Chem.* **238**, 235 (1963).
29. H. W. Kim and C. H. Yang, *Kor. Biochem. J.*, **16**, 111 (1983).
30. J. E. Folk, K. A. Piez, W. R. Carroll, and J. A. Gladner, *J. Biol. Chem.* **235**, 2272(1960).
31. E. Winstersberger, D. J. Cox, and Neurath, *Biochem.* **1**, 1069 (1962).
32. T. G. Sanders, Ph. D. Thesis, University of Illinois (1970).