Identification and Characterization of Brush Border Membrane Bound Carboxypeptidase P from Rat Small Intestine

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= Abstract = Carboxypeptidase P in rat small intestine was identified and characterized using N-CBZ-Gly-Pro-Leu and N-CBZ-Pro-Ala as substrates, of which the hydrolysis was monitored by amino acid analyzer.

Specific carboxypeptidase P activites in bursh border membranes were increased 8 fold over that in the mucosal homogenate. Carboxypeptidase P had a pH optimum, 7.5 and activities were totally disappeared after incubation at 60°C for 20 min. Carboxypeptidase P activities were strongly inhibited by metalloenzyme inhibitors and reactivated by addition of Co^{++} to EDTA-treated brush border membranes. Molecular weight of carboxypeptidase was 259,000. Regional and cellular distribution of carboxypeptidase P suggested a physiologic role of this enzyme in protein digestion.

Key Words : *Carboxypeptidase P, Rat small intestine, Brush border membrane*

INTRODUCTION

Proline specific carboxypeptidases, prolylcarboxypeptidase (lysosomal carboxypeptidase C, angiotensinase C) (EC 3.4.12.4) and carboxypeptidase P (microsomal prolylcarboxypeptidase) (EC 3.4.12.-), release an amino acid from the C-terminus of a peptide provided that penultimate residue is proline.

Prolylcarboxypeptidase first detected in the kidney lysosomes is active in the acid pH-range and not stimulated by divalent cation (Yang *et al.* 1968; Yang *et al.*1970). This enzyme was subsequently found in a variety of tissues and purified from porcine and human kidney (Kakimoto *et al.* 1983; Odya *et al.*1978). In contrast, carboxypeptidase P was first detected and purified from porcine kidney microsomes which is active in neutral pH-range and activated by manganese ion (Dehm and Nordwig 1970). It was subsequently found that carboxypeptidase P was also localized in brush border membrane of porcine kidney (Kenny *et al.* 1977) and rabbit small intestine (Auricchio *et al.* 1978).

However the detailed information of kinetic properties and characteristics of this enzyme especially in intestinal brush border membrane is not available. In addition, the enzymatic monitoring of hydrolysis of the substrates used in these previous papers was found to have the problems.

In this paper, carboxypeptidase P is identified and characterized in brush border membrane of rat small intestine using N-CBZ-Gly-Pro-Leu and N-CBZ-Pro-Ala as substrates of which the hydrolysis is assayed by amino acid analyzer.

MATERIAL AND METHODS

1. Chemicals

N-CBZ-Gly-Pro-Leu was purchased from Penninsula (Belmont, CA). N-CBZ-Pro-Ala was obtained from Bachem Inc. (Torrance, CA). Transferrin, catalase, ferritin and various protease inhibitors were purchased from Sigma (St. Louis, CA). Brush border membrane aminopeptidase N and dipeptidyl aminopeptidase IV were purified from rat intestinal brush border membrane as described previously (Bella *et al.* 1982; Kim and Brophy 1975). All other chemicals were of reagent grade.

2. Isolation of the brush border membranes

Male Wistar rats (Simonsen Lab, Gilroy, CA)

weighing approximately 300g were maintained on a standard laboratory chow diet.

Overnight-fasted rats were killed by decapitation, and the small intestine was removed. The whole intestinal lumen was washed with 2mM Tris-Hcl-50 mM Mannitol buffer, pH 7.1 and mucosa was obtained by scraping with a glass slide. Brush border membranes were prepared from the mucosal scraping by the method described by Kessler et al.(1978).

In addition, the whole small intestine was divided into six segments of equal length (18 cm). Brush border membranes were obtained from each segment by the same procedure. A gradient of intestinal cells from villus tips to crypt was obtained by serial incubation of excised rat proximal intestine in EDTA-phosphate-buffered saline according to the method of Breimer et al. (1981). Twelve incubations were necessary to obtain the last crypt cell fraction. Cells from the 12 incubations were pooled into five representative fractions as follows; 1. villus tip, 2. middle villus, 3. villus base, 4. intermediate or mixed portion, 5. crypt zone. Each fraction contained approximately 25%, 25%, 25%, 15% and 10% of the isolated cells respectively, as determined by total protein content. The final pellet of each cell fraction was homogenized and brush border membranes of villus and crypt cell were purified from the pooled fraction by the procedure of Kessler et al. (1978).

Brush border membranes were also obtained from the mucosa of Thiry-Vella loops 72 hrs after operation, as previously described (Curtis et al. 1978).

3. Enzyme assay

Enzyme preparation was incubated with 1mM of substrate in 0.05 M Tris-Hcl buffer, pH 7.0 in a total volume of 0.25 ml. N-CBZ-Gly-Pro-Leu and N-CBZ-Pro-Ala were used as the substrates.

After incubation for 60 min at 36°C, the reaction was stopped by the addition of 0.25 ml of 6% sulfosalicylic acid.

The released hydrolytic products were assayed using Beckmann amino acid analyzer.

Aminopeptidase N and sucrase were assayed as described previously (Bella et al. 1982; Dahlqvist 1968). Protein was measured by the modified method of Lowry et al. (1951).

4. Determination of pH optimum, thermostability and Km value

Optimum pH of carboxypeptidase P was determined using 0.05 M Tris-Hcl buffer in the range of pH 6.0 to 9.0.

Heat stability was checked by means of 20 min. preincubation of the enzyme preparation in the range of 40°C to 70°C. For determination of Km value and Vmax the concentrations of N-CBZ-Gly-Pro-Leu were changed from 31.3 μM to 1 mM.

5. Determination of the effect of protease inhibitors and metal ions

The effect of protease inhibitors on carboxypeptidase P was studied by preincubation of enzyme preparation with inhibitors at room temperature for 30 min.

One mM EDTA-pretreated brush boder membranes, of which carboxypeptidase P activities was inhibited by less than 4% of original activities, were preincubated with various concentrations of divalent cations and then reacted with substrates. Reactivation of carboxypeptidase P by divalent cations was compared with orginal activities.

6. Determination of molecular weight

Solubilization of brush border membranes was done as follows. One % (v/v) of Triton X-100 was added to brush border membranes incubated on ice for 1 hr and then sonicated for 10 seconds. This preparation was centrifuged at 27,000 g for 30 min and then supernatant was used for determination of molecular weight of rat small intestinal carboxypeptidase P.

Molecular weight of carboxypeptidase was estimated by disc gel electrophoresis described by Hedrick et al. (15).

Standard proteins such as transferrin, catalase and ferritin were separated in 5, 7 and 9% gels and stained with Coomasie blue.

At the same time electrophoresis of solubilized brush border membranes was performed in 5, 7 and 9% gels and then gels were sliced into 3 mm pieces. Each pieces were soaked overnight at 4°C in 0.5 ml distilled water. Appropriate aliquots were taken for determination for carboxypeptidase P activities.

From the slope-molecular weight relation curve of standard protein, molecular weight of carboxypeptidase P was calculated.

RESULTS

1. Isolation of brush border membranes (Table 1)

The purity of brush border membranes was determined by assaying sucrase and aminopeptidase N of which activities were all increased 14-15 fold

Table 1. Enrichment of carboxypeptidase P in rat small intestinal brush -border membranes

| Substrate | Specific enzyme activities (n mol/min/mg protein) | | Enrichment |
	Homgenate	BBM	
N-CBZ-Gly-Pro-Leu	5.43±0.88	43.16±4.75	7.9
N-CBZ-Pro-Ala	1.30±0.19	10.01±1.21	7.7

• Mean±S.D are given (n=4)
• Specific enzyme activities of cytosol fraction cleaving N-CBZ-Gly-Pro-Leu was 1.22 n mol/min/mg protein
• Marker enzyme (sucrase and aminopeptidase N) showed mean enrichment of 14-15 over that in the homogenate.

The specific enzyme activities of carboxypeptidase P cleaving both substrates were enriched 8 times in the brush border membranes of small intestine.

Carboxypeptidase P activities in cytosol fractions were only 1/4 of those in mucosal homogenate.

In addition the mucosal homogenate and the brush border membranes obtained from Thiry-Vella 100 ps showed similar level of carboxypeptidase P activities in normal small intestine (specific enzyme activities in homogenate and brush border membranes; 6.4 and 43.7 n mol/min/mg protein, respectively).

2. Determination of pH optimum, thermostability and Km vallue

Carboxypeptidase P in brush border membranes showed a pH optimum of 7.0 (Fig. 1)

Carboxypeptidase P was a relatively heat labile enzymes of which activities was totally disappeared after incubation at 60°C for 20 min (Fig. 2). Using N-CBZ-Gly-Pro-Leu as substrate the Km value and Vmax were estimated as 633 um and 83 n mole/min/mg protein, respectively (Fig. 3).

3. The effect of protease inhibitors and metal ions

Carboxypeptidase P activities were strongly inhibited by metal chelating agents and thiol reagents (Table 2).

Other protease inhibitors had no effect on carboxypeptidase P activities in brush border membranes.

As showin Fig. 4, 1 mM EDTA-treated brush border membranes of which carboxypeptidase P activities were less than 4% of original activities,
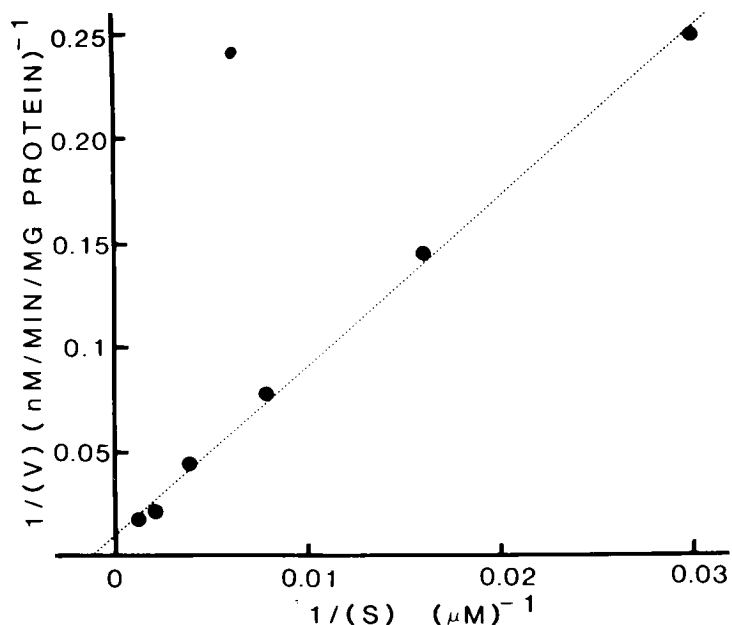

Fig. 1. Double reciprocal plots of carboxypeptidase P in brush border membranes. Brush border membranes were incubated with N-CBZ-Gly-Pro-Leu as described in Methods.
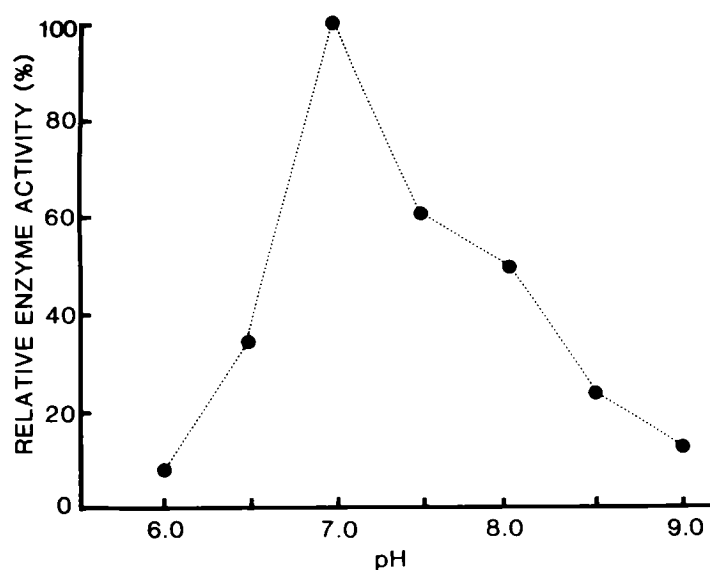

Fig. 2. pH profile of carboxypeptidase P in brush border membranes.

restored 94% of its original activities by addition of 4 mM of Co^{++} and 17% of its original activities by addition of 4 mM of Zn^{++}.

But other divalent cation such as Ca, Mg, Mn, Hg didn't reactivate the carboxypeptidase P activities.

4. Molecular weight of carboxypeptidase P

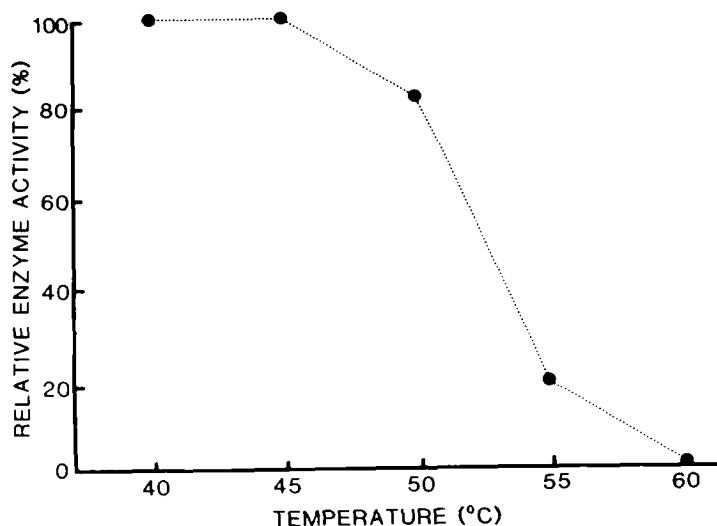As shown in Fig. 5, the slope of transferrin, catalase and ferritin was 0.17, 0.24 and 0.36, respecti-

Fig. 3. Thermostability of carboxypeptidase P in brush border membranes. Heat stability was checked by means of 20 min range of 40°C to 70°C.

Table 2. Effect of protease inhibitors on carboxypeptidase P

Inhibitors	Final conc.	% inhibiton
None		0
Serine enzyme inhibitor		
Phenylmethylsulfonyl floride	2mM	0
Cysteine enzyme inhibitor		
Iodoacetate	1mM	0
Metaloenzyme inhibitors		
EDTA	1mM	96.7
EGTA	1mM	94.1
1-10 Phenanthroline	1mM	97.1
Dithiothreitol	1mM	94.5
3-Phenylpropionic acid	2mM	0
Catopril*	1mM	0
Phosphoramidone**	30mM	0

* Inhibitor of dipeptidyl peptidase
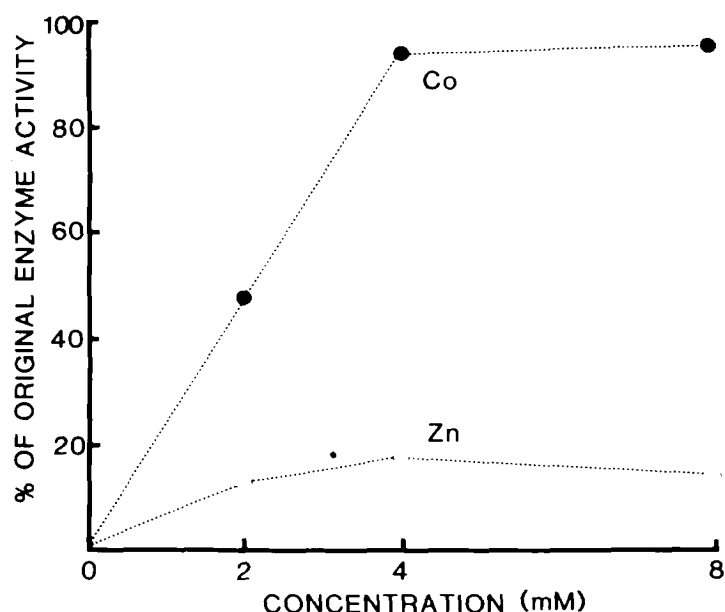** Inhibitor of neutral endopeptidase

Fig. 4. Reactivation of carboxypeptidase P by divalent cations. Carboxypeptidase-P activities in EDTA-treated brush border membranes were less than 4% of originial activities. EDTA-treated brush border membranes were incubated with various concentrations of several divalent cations.

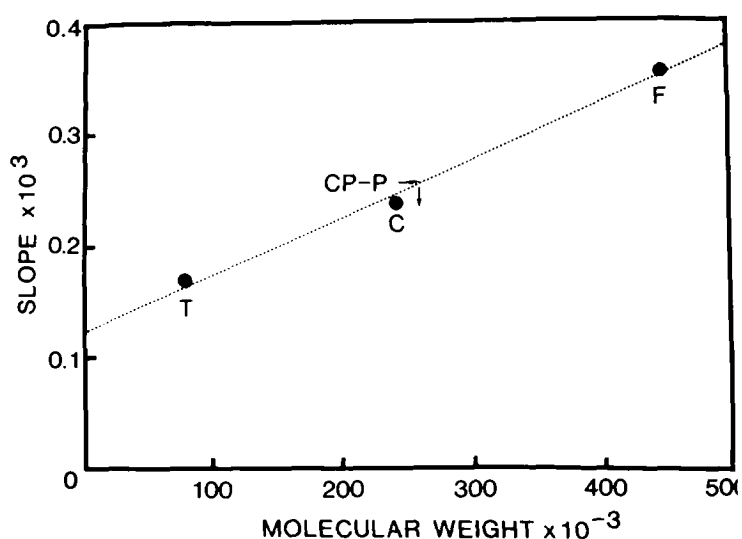　　Ca, Mg, Mn and Hg did'nt reactivate carboxypeptidase P activities.

Fig. 5. Determination of molecular weight of carboxypeptidase P by disc gel electrophoresis. Standard proteins, transferrin (T), catalase (C), and ferritin (F) were elecrophoresed in 5, 7 and 9% gels and stained with Coomassle blue, and slope-molecular weight relation curve was acquired. Supernatant of 1% Triton-solubiluzed brush border membranes was also electrophoresed in 5, 7 and 9% gels and carboxypeptidase P activities were assayed in each gel slice (3 mm).

viely. And the slope of carboxypeptidase P was 0.259. Molecular weight of carboxypeptidase P was estimated to be 259,000.

5. Regional and cellular distribution of brush

border membrane carboxypeptidase P

　　Enzyme activities in brush border membranes from different region along the longitudinal axis o' rat small intestine are shown in Fig. 6.
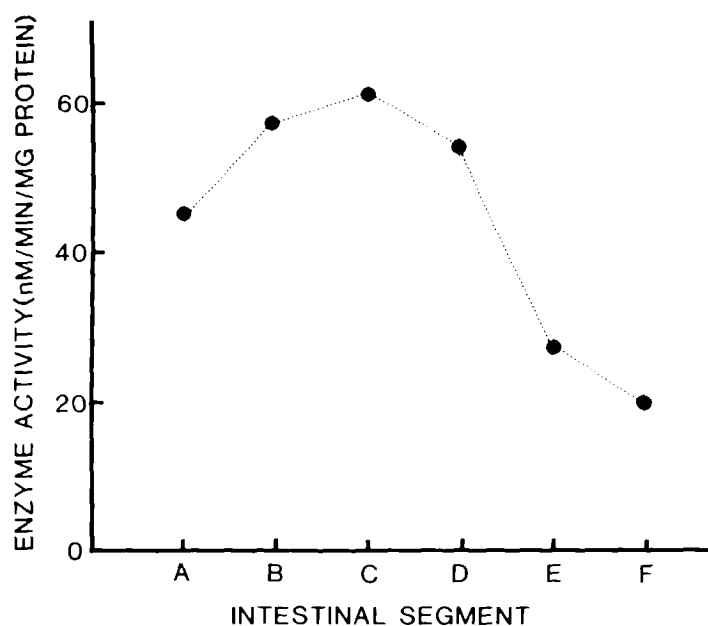
Fig. 6. Brush border membrane carboxypeptidase P activities along the longitudinal axis of small intestine. Excised whole small intestine was divided into six equal segments (from A, proximal, to F, distal 18 cm each). Data were expressed as n mole/min/mg protein and are the mean value ± SEM of 4 experiments.
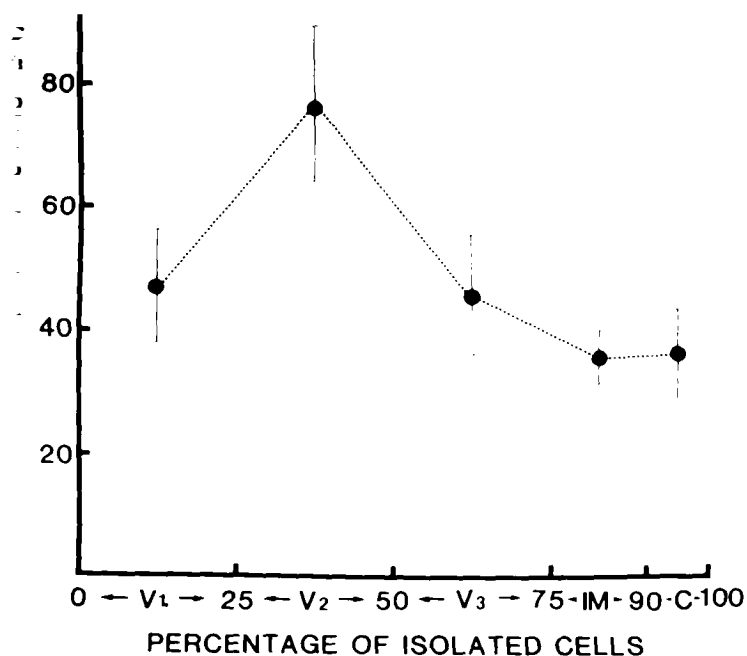

ig. 7. Brush border membranes carboxypeptidase P activities along the villous-crypt axis.
Cells were pooled into five segments.
1. V_1 = villus tip 2. V_2 = middle villus,
3. V_3 = villus base 4. IM = intermediate
5. C = crypt zone, containing 25%, 25%, 25%, 15% and 10% of the isolated cells respectively as determined by protein. Data were expressed as n mole/min/mg protein and are the mean value ± S.E.M. of 4 experiments.

Carboxypeptidase activities were maximal in the middle region of small intestine with lowest value found in the distal segment. Fig. 7 shows the distribution of carboxypeptidase P along the villous-crypt axis.

The enzyme activities in villous cell were around 2 times higher than that in crypt cell.

DISCUSSION

Enrichment of carboxypeptidase P in the rat intestinal brush border membranes is remarkably lower than that of marker enzymes, sucrase and aminopeptidase N. By contrast, carboxypeptidase P identified from kidney brush border memranes was unexpectedly highly enriched, that is, enrichment of carboxypeptidase was two times higher than that of marker enzymes (Kenny et al. 1977). In addition carboxypeptidase P in rabbit small intestine was enriched as high as marker enzymes (Auricchio et al. 1978). But in previous two papers, hydrolytic products were monitored enzymatically by L-amino acid oxidase which is different from our method using amino acid analyzer.

When we compare the level of the enzyme activities in mucosal homogenate and brush border membranes using both methods simultaneously, the values in homogenate monitored enzymatically is 1/3 of that monitored by amino acid analyzer. But the enzyme activities of brush border membranes were shown to have no difference between two methods.

Kenny et al. (1977) asserted that unexpectedly high enrichment of carboxypeptidase P in kidney brush border membranes comparing with marker enzymes could be explained by the presence of inhibitors in homogenate which is not present in brush border membranes.

According to our observation inhibitors claimed to be present only in mucosal homogenate might be amino acids, of which concentration in homogenate were remarkably higher than that in brush border membranes. When hydrolysis of substrate was monitored enzymatically by L-amino acid oxidase, high level of various amino acids in homogenate mask the detection of released L-amino acids from substrate by enzymatic reaction. By our experiment, enrichment of carboxypeptidase P in brush border membranes is 8 using amino acid analyzer, but it is 17-22 using L-amino acid oxidase which is unexpectedly high like Kenny's observation. Our results strongly indicated that

such a high enrichment was resulted not from inhibitors of homogenate but from inappropriate assay method.

Now we have no data to answer the question why enrichment of carboxypeptidase P is around half of that of marker enzymes even though the enzyme activities in cytosolar fraction were remarkably lower than that in mucosal homogenate.

Lower recovery of carboxypeptidase P in brush border membranes comparing with marker enzymes suggests that carboxypeptidase P is destroyed significantly during preparation of brush border membranes.

Comparing with other brush border membrane peptidases such as aminopeptidase N and DAP IV (Bella *et al.* 1982), carboxypeptidase P is a relatively heat labile enzyme, of which activities disappeared after incubation at 60°C for 20 min.

Optimum pH of carboxypeptidase P is 7.0, a little bit lower than that in kidney microsome or rabbit small intestine. This small difference can be explained by different assay method.

Metalloenzyme inhibitors such as EDTA, EGTA and 1-10 phenathroline strongly inhibited carboxypeptidase P activities indicating carboxypeptidase P is a metalloenzyme. In addition, this is supported by our observation that addition of Co^{++} to EDTA-treated brush border membranes reactivated the carboxypeptidase P activities by 94% of original activities. Other protease inhibitors of various carboxypeptidase didn't inhibit the carboxypeptidase P of rat small intestine. These findings are in contrast with those by Aurichio *et al.* (1978) that carboxypeptidase P in rabbit small intestine was strongly inhibited by 3-phenylpropionate.

The molecular weight of carboxypeptidase P is 259,000 as estimated by disc gel electrophoresis which is similar to carboxypeptidase P in kidney microsome (Dehm and Nordwig. 1970).

Regional and cellular distribution of carboxypeptidase P in rat small intestine, being maximal in the upper middle region where maximal absorption of the nutrient are considered to occur, and higher in villus than in crypt, suggests that carboxypeptidase P might play a physiologic role in mucosal digestion of dietary protein and polypeptide.

Due to unique structure of proline, peptide bonds involving proline residues are often resistant to the action of peptidase, even to peptidases of broad specificity. To initiate or complete the degradation of peptide or protein involving proline residue, a series of peptidases which can uniquely recognize the pyrrolidine ring of proline are needed.

These specific proline peptidases are even further restricted in the action in that the proline residue must exist in a particular position in the peptide substrate before hydrolysis will take place.

When N-CBZ-Gly-Pro-Leu or N-CBZ-Pro-Ala not hydrolyzed by brush border membranes aminopeptidase N and DAP IV was incubated with intestinal brush membranes, only Leu or Ala was released respectively without release of Pro.

For breakdown of the collagen endowed with an unusually high proportion of X-Pro-Y sequences, this carboxypeptidase P might be involved.

REFERENCES

Auricchio S, Greco L, de Vizia B, Buoncore V. Dipeptidyl-aminopeptidase and carboxypeptidase activities of the brush border of rabbit small intestine. Gastroenterology 1978, 75:1073-1079

Bella AM Jr, Erickson RH, Kim YS. Ret intestinal brush border membranes dipeptidyl-aminopeptidase IV; Kinetic properties and substrate specificities of purified enzyme. Arch. Biochem. Biophys. 1982, 218:156-162

Breimer, ME, Hansson, GO, Karlsson KA, Lefflir H. Glycosphingolipids and the differentiation of intestinal epithelium. Exp. Cell. Res. 1981, 135:1-13

Curtis KS, Gaines HD, Kim YS. Protein digestion and absorption in rats with pancreatic duct occlusion. Gastroenterology 1978, 74:1271-1276

Dahlqvist A.M Method of assay of intestinal disacharidases. Annal Biochem. 19678, 27:99-107

Dehm P, Nordwig A. The clearage of prolyl peptides by kidney peptidases. Isolation of a microsomal carboxypeptidases from swine kidney. Eur. J. Biochem. 1970, 17:372-377

Hedrick JL, Smith AJ. Size and charge isomer separation and estimation of molecular weights of protein by disc gel electrophoresis. Arch. Biochem. Biophys. 1968, 126:155-162

Kakimoto T, Oshima G, Yeh HSJ, Erdos EG. Purification of lysosomal prolylcarboxypeptidase angiotensinase C. Biochem. Biophys. Acta 1973, 302:178-182

Kenny AJ, Booth AG, Macnair RDC. Peptidase of the kidney microvillus membrane. Acta Biol. Med. Germ. 1977, 36:1575-1585

Kessler M, Acuto O, Storelli C, Murer H, Muller M, Semenza G. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Biochim. Biophys Acta 1978, 506:136-154

Kim YS, Brophy DJ. Rat intestinal brush border membrane peptidases solubilization 1. purification and physicochemical properties of two different forms of the enzyme. J. Biol. Chem. 1975, 251:3199-3205

Lowry OH, Rosebrough NJ, Farr AI, Rondall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951, 193:265-275

Odya CE, Marinkovic DV, Hammon KJ, Stewart JD , Erdos EG. Purification and properties of prolylcarboxy-peptidase (Angiotensinase C) from human kidney. J. Biol. Chem, 1978, 253:5927-5931

Yang HYT, Erdos EG, Chiang TS. New enzymatic route for the inactivation of angiotensin. Nature 1968, 218:1224-1226

Yang HYJ, Erdos EG, Chiang TS, Jenssen TA, Rodgers JG. The effects of p-cl-phenylalanine on the content and cellular distribution of 5-HT in the rat pineal gland; combined biochemical and effect on microscopic analysis. Biochem. Pharmacol. 1970, 19:1210-1211

= 국문초록 =

쥐 소장점막의 Carboxypeptidase P

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송 인 성

단백분해효소인 carboxypeptidase P의 존재를 쥐 소장점막 미소 융모막에서 확인하고 그 효소적 특징을 조사하였다.

효소활성도는 N-CBZ-Gly-Pro-Leu와 N-CBZ-Pro-Ala를 기질로 하여, 분해산물은 단백산 분석기를 이용, 측정하였다.

Carboxypeptidase P 활성도는 점막균등액에 비해 미소융모막에 8배 증가되어 있었고, 효소의 적정 pH는 7.5이었으며, 60°C에서 20분간 전처치로 효소활성도가 없어짐을 알 수 있었다.

효소활성도는 금속효소 억제인자에 의해 강력히 억제되었으며, 억제물질인 EDTA로 전처치된 미소 융모막에 Co^{++}의 첨가로 효소가 재활성화되었다.

전기영동법에 의해 측정된 carboxypeptidase P의 분자량은 259,000이었다.

효소의 소장에서의 지역적 분포나 세포내 분포상태는 단백분해흡수에 있어서 carboxypeptidase P의 생리적 기능을 시사하고 있었다.