# Structural and Functional Relationship of the Catalytical Subunit of Recombinant Pyruvate Dehydrogenase Phosphatase (rPDPc): Limited Proteolysis

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# Pyruvate dehydrogenase phosphatase의 catalytical subunit의 구조와 활성에 대한 연구

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## 요    약

Pyruvate dehydrogenase phosphatase (PDP)와 kinase는 당대사시 해당과정에서의 대사 산물인 pyruvate 를 acetyl CoA로 만들어 구연산 회로로 진입시켜주는 효소인 pyruvate dehydrogenase complex (PDC)의 활성을 조절하는 중요한 효소이다. PDP의 catalytic subunit는 PDC의 dihydrolipoamide acetyltransferase (E2), PDP regulatory subunit (PDPr), 그리고 칼슘 결합 도메인 등으로 구성되어 있는 것으로 추측되어지 고 있다. 본 연구에서는 그 구조와 기능과의 상관관계를 알아보기 위해 PDPc를 *E. coli* JM101에서 발현 시켜 순수 정제 후 단백분해 효소를 이용한 제한적 가수분해 방법을 이용해 그 구조와 기능과의 상관관 계에 대해 연구하고자 하였다. 정제된 PDPc는 trypsin, chymotrypsin, Arg-C 그리고 elastase를 이용하여 30°C 그리고 pH 7.0에서 제한적으로 분해시켰으며 각 분해산물의 아미노 말단의 아미노산 배열을 분석 하였다. 그 결과 PDPc는 trypsin, chymotrypsin, elastase에 의해 N-terminal의 *50 kD*과 C-terminal의 *10 kD* 의 두 개의 분해산물을 만들었으며, Arg-C에 의해 *50 kD*의 분해산물은 약 *35 kD*와 *15 kD*으로 더 가수 분해가 되었다. 이러한 결과로 볼 때 PDPc는 앞에서 추측한데로 세 개의 주요한 기능적 도메인으로 이 루어져 있음을 알 수 있었다. 또한 C-terminal의 *10 kD*은 PDPc의 활성에는 영향을 주지 않는 것으로 밝 혀졌으나 다른 도메인의 기능은 더 연구가 되어져야 할 것으로 생각된다.

**Key words** : Limited proteolysis; Functional domain; Recombinant catalytic subunit of pyruvate dehydrogenase phosphatase (rPDPc); Inner lipoyl domain (L2); Dihydrolipoamide acetyltransferase (E2); Pyruvate dehydrogenase complex (PDC)

## INTRODUCTION

Pyruvate dehydrogenase phosphatase (PDP) is a

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mitochondrial protein serine/threonine phosphatase that catalyzes the dephosphorylation and concomitant reactivation of the pyruvate dehydrogenase component of the pyruvate dehydrogenase complex (PDC) (Linn *et al.*, 1969; Teague *et al.*, 1982). PDP consists of a $Mg^{2+}$-dependent and $Ca^{2+}$-stimulated catalytic

subunit (PDPc) of $Mr$ 52,600 (apparent $Mr$ approx. 60,000) and a FAD-containing regulatory subunit (PDPr) of $Mr$ 95,600 (Denton et al., 1972; Teague et al., 1982; Pratt et al., 1982; Yan et al., 1996). PDPc is a member of the protein phosphatase 2C family (Lawson et al., 1993). $Ca^{2+}$ mediates the translocation of PDP (or PDPc) to the 60-mer icosahedral dihydrolipoamide acetyltransferase (E2) component of the PDC in proximity to its substrate, the phosphorylated pyruvate dehydrogenase component (E1), thereby increasing the rate of dephosphorylation about 10-fold (Pettit et al., 1972; Teague et al., 1982; Chen et al., 1996). To date, PDPc has been suggested to have three major functional domains: E2-binding domain, PDPr-interaction domain, and calcium binding domain. However, it is not clear where the functional domains are in PDPc.

Limited proteolysis has been used to identify surface-exposed, flexible, or weakly structured areas of proteins (Neurath, 1980) and to assess protein-folding dynamics (Hubbard, 1998). The fundamental idea is that cleaving requires both the existence of an appropriate protease recognition sequence in the protein and the availability of that sequence for cleavage. The latter generally requires that the cleavage site exist at the center of a 4-10 residue segment in a flexible conformation that can bind and adapt to the protease active site (Hubbard, 1998). The observation of an especially rapid cleavage event in limited proteolysis means that sequence element cleaved is in an exposed conformation in the native state. Conditions for proteolysis are necessary to be adjusted to restrict proteolysis, if possible, to single cleavage event and the cleavage site is then identified by N-terminal analysis.

In this report, I address that rPDPc is expressed in E. coli JM101 and subsequently purified to apparent homogeneity using the unique interaction between rPDPc and L2 and also I describe data to suggest the locations of the functional domains of rPDPc by lmited proteolysis and N-terminal sequence analysis.

## MATERIALS AND METHODS

### Materials

Escherichia coli strain BL21 (DE3) harboring a plasmid encoding L2 (amino acids 120-233 of E2) fused to GST was kindly provided by Drs Thomas E. Roche and Jason C. Baker (Kansas State University). Highly purified PDC was prepared from bovine kidney mitochondria (Damuni et al., 1984). GSH-Sepharose 4B was obtained from Pharmacia and [γ-$^{32}$P]ATP was from New England Nuclear. All proteases used in this study were obtained from Roche Applied Science (Indianopolis, USA) and the specificities of them were shown in table 1. Other reagents were of the highest purity available commercially.

### Expression of the Catalytic Subunit of Pyruvate Dehydrogenase Phosphatase (rPDPc)

The construct was transformed with p1922 plus pGroESL (pGroESL encodes E. coli chaperonin proteins groEL and groES). Fresh transformants were grown at 30°C to an $OD_{600}$ of 0.6-1.8. Expression was induced by addition of IPTG to a final concentration of 1.0 mM. Incubation was continued at 30°C for 5.5-6.5 h. Cells were centrifuged and used for the purification of rPDPc.

### Expression of the GST-Lipoyl domain of E2 of PDC and preparation of affinity matrix (GSH-Sepharose-GST-L2)

GST-L2 fusion protein was expressed in E. coli BL21 (DE3) and purified essentially as described (Denton et al., 1972; Lawson et al., 1993). Briefly, the fragment was subcloned into pGEX-2T and then this plasmid was transformed into E. coli strain BL21 (DE3). The E. coli BL21 (DE3) strain transformed with the pGEX-2T containing L2 (amino acids 120-233 of E2) construct was grown in 0.5% Yeast extract, 1% trypton, 1% NaCl, 0.1% glucose, 5 mM $Na_2HPO_4$, 100 (g/ml ampicillin, and 0.2 mM DL-α-lipoate. The culture was incubated at 37°C to an

$OD_{600}$ of 0.8-1.0. Expression was induced with IPTG to a final concentration of 0.5 mM, and growth was continued at 37°C for 5.5-6.5 h. Cells for L2-GST were suspended in 9 volume of ice-cold PBST buffer (20 mM sodium phosphate, pH 7.2, 0.15 M NaCl, 1.0% triton X-100) and disrupted by two passes with French press cell disrupter at a pressure of around 800 psi, and then it was centrifuged at 48,400 × g for 30 min. The extract was mixed with the GSH-Sepharose 4B that had been equilibrated with PBST buffer. The mixture was mildly shaken for 30 min at room temperature, and then it was loaded into an empty column. The column was washed with 10-column volume of PBST and then equilibrated with ice-cold buffer K (50 mM tris HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 10% glycerol, 0.5 mM dithiothreitol, 0.15 M NaCl, 1 mM benzamidine).

## Purification of rPDPc

PDPc was expressed and purified from *E. coli* by the procedure described (Choi *et al.*, 2000), with minor modification. Briefly, cells were suspended in 9 volume of ice-cold buffer K (50 mM tris HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 10% glycerol, 0.5 mM dithiothreitol, 0.15 M NaCl, and 1 mM benzamidine) and disrupted by one pass with French press cell disrupter at a pressure of around 1000 psi, and then it was centrifuged at 48,400 × g for 30 min. The extract was loaded on L2-GST*GSH-Sepharose 4B equilibrated with buffer K containing 1% triton X-100 and 1 M urea. It was washed extensively with buffer K containing 1% triton X-100 and 1 M urea and followed to wash with buffer K without 10% glycerol, and 0.15 M NaCl. And rPDPc was eluted with elution buffer (50 mM tris HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM benzamidine, and 1 mM EGTA).

## Assay of rPDPc

Measurements of rPDPc activity were based on the measurement of the initial rate of release of [$^{32}$P]

phosphate from the bovine $^{32}$P-labeled pyruvate dehydrogenase complex (Damuni *et al.*, 1984). One unit is defined as the amount of phosphate that releases 1 nmole [$^{32}$P] phosphate/min.

## SDS-PAGE and Immunoblotting

Proteins were separated by SDS-PAGE on 12.5% gels and transferred electrophoretically to Immobilon -P membrane (Millipore). Blots were probed with a 1 : 4000 dilution of rabbit anti-rPDPc IgG followed by detection with 1 : 4000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase as described by the supplier (Bio-Rad). Rabbit antibodies to rPDPc was purified on protein A-agarose (Sigma) to obtain the IgG fraction.

## N-terminal amino acid sequence

After electrophoresis, the polyacrylamide gel was blotted onto PVDF membrane (Millipore). Subsequently, N-terminal Edman degradation was performed according to the manufacturer's instruction using an Applied Biosystem 477A protein sequencer.

## Proteolysis with trypsin and chymotrypsin

Purified rPDPc was incubated with trypsin at an enzyme/trypsin ratio of 1/350 (w/w) in 50 mM tris-HCl, pH 7.5, 0.1 M NaCl, 2 mM DTT, and 20% glycerol (buffer A). Reactions were carried out at 30°C and for various times and terminated by heating at 100°C for 3 min in electrophoresis 3X sample buffer unless otherwise indicated. To measure the remained activity, they were terminated by a mixture of 1 mM PMSF and 1 mM benzamidine. The reaction products were separated by SDS-PAGE and either examined after staining with Coomassive Blue R-250 or transferred to Immobilin-P membranes for N-terminal sequencing.

## Proteolysis with Arg-C

Purified rPDPc was treated at an Arg-C/rPDPc ration of 1/5 (w/w) in buffer A. Reactions were per-

formed at 30°C, terminated and the products were
analyzed as described above.

## Proteolysis with elastase

Purified rPDPc was treated at an elastase/rPDPc
ratio of 1/200 (w/w) in buffer A. Reactions were
performed at 30°C, terminated and the products were
analyzed as described above. To investigate effects
of calcium ion, magnesium ion, and manganese ion
on the proteolytic reaction, reactions were performed
in buffer A without 20% glycerol.

## Other methods

Protein was determined based on Bradford meth-
ods (Bradford, 1976), using bovine serum albumin as
the standard.

## RESULTS AND DISCUSSION

A biochemical technique that has been used in
structural analysis of globular proteins is limited pro-
teolysis (Neurath, 1980; Hubbard, 1998). Protease
binding sites generally require the availability of up
to 10 amino acid residues in an accessible, flexible
conformation (Hubbard, 1998). If a globular protein
undergoes a single proteolytic cleavage event at a
particular site under controlled, partial proteolysis
conditions, it can be concluded that this site must be
in an exposed, flexible, or weakly structured portion
of the protein in the native state (Neurath, 1980). The
method is particularly effective in dissecting multi-
domain structure within protein (Neurath, 1980).
PDPc has been suggested to have three major puta-
tive functional domains of E2-binding domain,
PDPr-binding domain, and $Ca^{2+}$-binding motif. I
address here by using this specific technique that
rPDPc has structurally three major domains.

## Expression and Purification of PDPc

I transformed PDPc DNA construct into E. coli
JM101 competent cells and selected four clones.
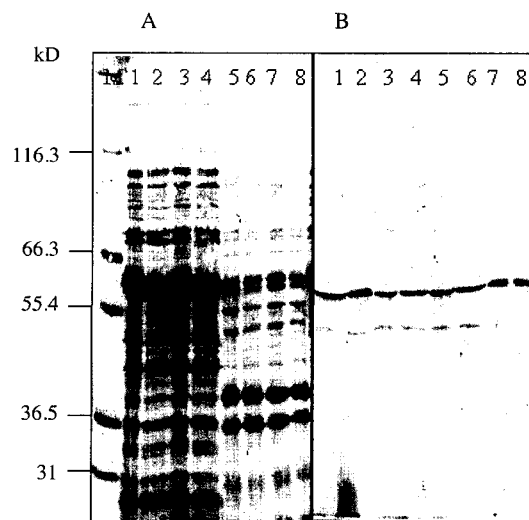


**Fig. 1.** Expression and purification of rPDPc from E. coli
JM101. Cell extracts from four different clones were
electrophoresed on 12.5% SDS-PAGE and blotted
on Immobilin-P membrane (Millipore). Blots were
probed with a 1 : 4000 dilution of rabbit anti-rPDPc
IgG followed by detection with 1 : 4000 dilution of
goat anti-rabbit IgG conjugated to alkaline phos-
phatase. A, SDS-PAGE; B, Immunoblotting analy-
sis (lane 1-4, soluble fractions; lane 5-8, insoluble
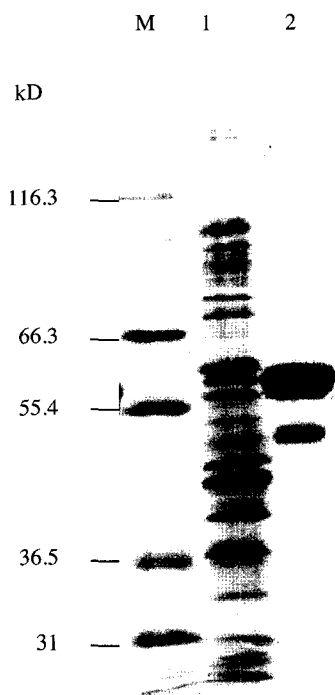cell pellets).

PDPc was expressed well as shown in fig. 1. For this
study, I used clone number 2. And PDPc was puri-
fied to near homogeneity with the yield of approx.
79% and the specific activity was approx. 1,150
nmole/min/mg protein (Fig. 2). The gel pattern is qui-
te similar with the data by Choi et al. (Choi et al.,
2000).

## Proteolysis with Chymotrypsin and Trypsin

To investigate the functional domains of PDPc, the
limited proteolysis was done with various conditions.
Fig. 3 shows proteolysis by time for the generation of
domain fragments from PDPc at pH 7.5 and 30°C.
The PDPc was digested by chymotrypsin and trypsin
and as the results (Fig. 3A and 3B), a big fragment of
Mr 50 kD and a small peptide, Mr 10 kD (data not
shown for Mr 10 kD) were prepared with chymotry-

psin and trypsin. To determine the cleavage sites, the digested 50 kD fragment were separated by SDS–PAGE, blotted to PVDF membranes, and the N-terminal sequence was determined by Edman auto-degradation as in method section. It shows that the cleavage is between Arg-394 and Arg-395 in the N-terminal of PDPc (Table 1). In order to measure the change of activities by proteolysis, I measured

PDPc activities with the same batch of samples. Fig. 3E shows that the proteolysis of PDPc with chymotrypsin did not decrase any activity. This strongly suggests that the small N-terminal fragment is not necessary for the activity of PDPc. However, as expected, the proteolysis with trypsin proteolysis caused PDPc to be inactive over time because the major fragment was further proteolysed by time in Fig. 3B. Together, the results show that the 10 kD N-terminal peptide is not necessary for the activity.
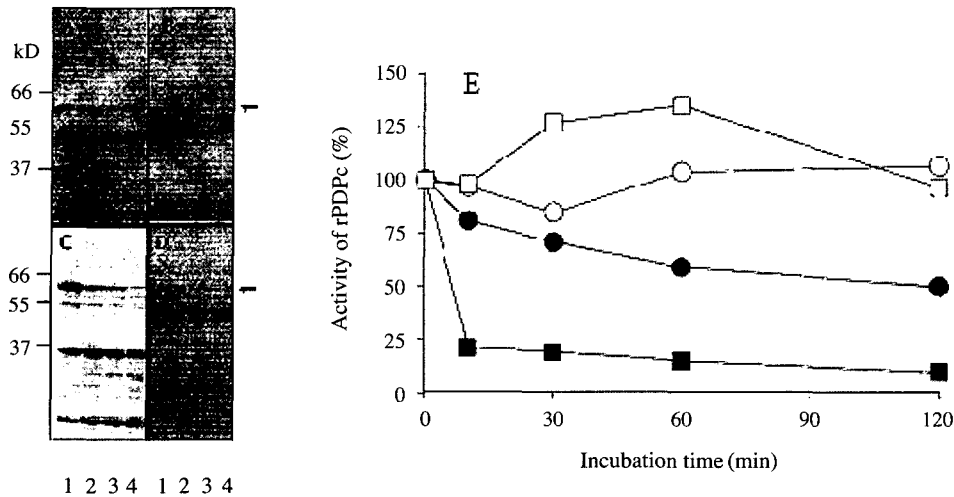
## Arg-C

The proteolysis was shown in Fig. 3C. rPDPc was fragmented by Arg-C to two major fragments of N-terminal 35 kD and 15 kD with several minor peptides with low activities (Fig. 3E). This result indicates that there is a cleavage site between two functional domains of N-terminal and C-terminal end and both ends are necessary for the activity of PDPc. However, the proteolysis of PDPc with Arg-C was inhibited by 2 mM calcium (Fig. 5C, lane 8), which suggest that the proteolysed site was protected by the conformational change of PDPc by calcium. The cleavage is much likely to prevent the conformational change of two ends and thus it did have very low activities. In another side, it is suggested that calcium-binding site was disappeared by the proteolysis.
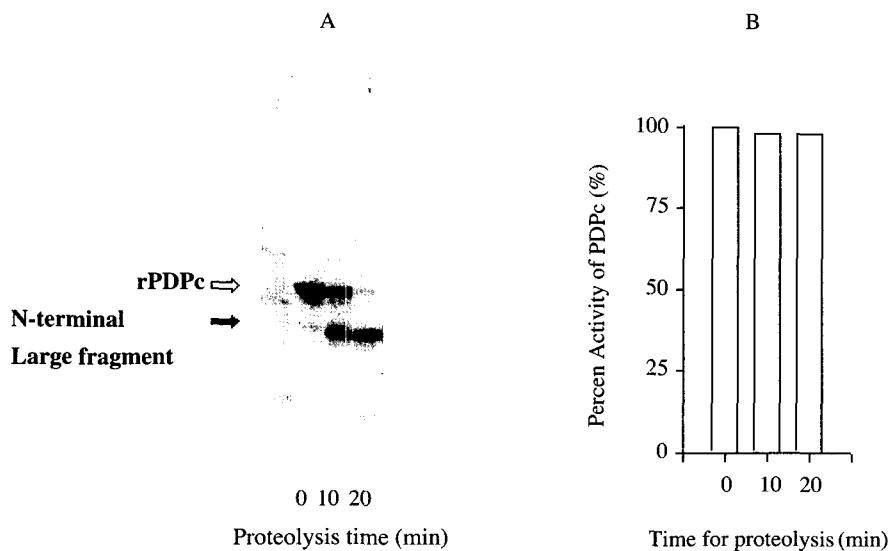
## Elastase

The proteolysis of PDPc with elastase is shown in Fig. 3D. PDPc was proteolyed to form two major peptides of approx. 50 kD and approx. 10 kD without any significant activity decrease (Fig. 3E). To further probe whether the N-terminal end itself has the intact activity, C-terminal end of rPDPc was removed



M    1    2

kD

116.3

66.3

55.4

36.5

31

Fig. 2. SDS–PAGE analysis of purified rPDPc. Purified rPDPc (6.7 μg) was electrophoresed on 12.5% SDS–polyacrylamide gel and was stained with coomassie brilliant blue G. lane M, Mr marker; lane 1, soluble cell extract; lane 2, purified rPDPc).

Table 1. Proteases for limited proteolysis and specific cleavage sites

| Protease | Classification | Specificity | M.W.(kD) | Optimal pH | Inhibitors |
|----------|----------------|-------------|----------|------------|------------|
| Chymotrypsin | Serine protease | W, L, M, A, D, E | 25 | 7.0–9.0 | PMSF, TPCK |
| Trypsin | Serine protease | K, R | 23.5 | 8.0 | PMSF, TLCK |
| Elastase | Serine protease | Neutral amino acid | 25.9 | 8.5 | PMSF, DFP |
| Arg-C | Serine protease | R | 30 | 8.0–8.5 | DFP, TLCK |

1 2 3 4   1 2 3 4

**Fig. 3.** Proteolysis of rPDPc by chymotrypsin (A), trypsin (B), Arg-C (C), and elastase (D) and their activities (E). rPDPc was proteolysed at 30°C and pH 7.5 for 10, 30, 60 and 120 min by the proteases and the activity was assayed by the measurement of the initial rate of release of [$^{32}$P] phosphate from the bovine $^{32}$P-labeled pyruvate dehydrogenase complex. O , chymotrypsin; ● , trypsin, ■ , Arg-C; □ , elastase.

A                      B



rPDPc ⟹

N-terminal   ➡

Large fragment

0 10 20

Proteolysis time (min)            Time for proteolysis (min)

**Fig. 4.** The N-terminal large fragment without C-terminal end has the inact activity. To prepare the N-terminal large fragment, rPDPc was incubated with elastase at an elastase/protein ratio of 1/200 (w/w) at 30°C and the activity was measured by the procedure in method section.

by elastase under the tight-controlled condition. The activity of the N-terminal end was exactly same to inact rPDPc (Fig. 4). The N-terminal end was further proteolysed (Fig. 5B) in buffer A without 20% glycerol with decreasing activities by time (Fig. 5A). However, proteolysis of the N-terminal end was

**Table 2.** N-terminal sequence of proteolysed PDPc

| # of major fragment | Chymotrypsin 2 | Trypsin 2 | Arg-C 4 | Elastase 2 | Apparent $M$r (kD) |
|---|---|---|---|---|---|
| cPDPc | $^{1}$ASTPQKF | | | | 60 |
| Peptide I | $^{1}$ASTPQKF | $^{1}$ASTPQKF | $^{1}$ASTPQKF | $^{1}$ASTPQKF | 50 |
| Peptide II | | | | $^{1}$ASTPQKF | 35 |
| Peptide III | | | | $^{264}$KSVVKQD | 15 |
| Peptide IV | $^{395}$RAKMSSV | $^{391}$LTERRAK | $^{395}$RAKMSSV | $^{391}$LTERRAK | 10 |

The purified rPDPc was electrophoresed on 12.5% SDS-polyacrylamide gel, and after blotting on Immobilin-P membrane, the protein band was excised for protein sequencing. N-terminal amino acid sequences of proteins were obtained using an Applied Biosystem Automatic amino acid Sequencer. Amino acid residues are shown in standard single alphabet representation.
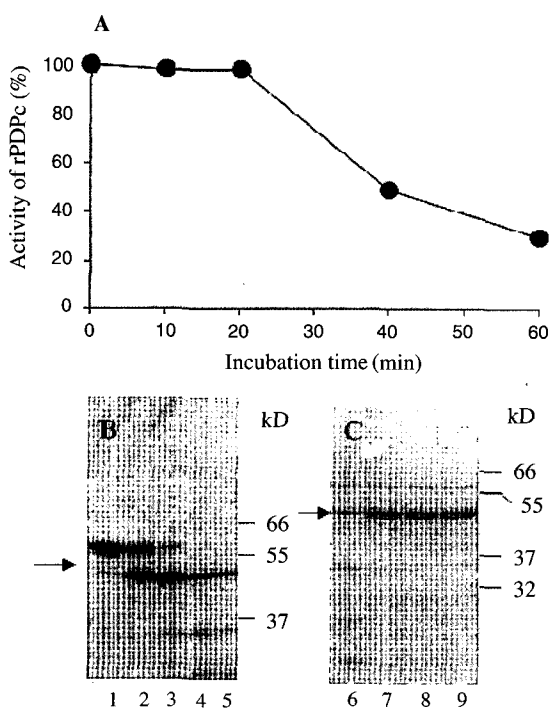


Fig. 5. $Ca^{2+}$ prevents the further proteolysis. rPDPc was proteolysed by elastase in 50 mM tris-HCl, pH 7.5, 0.1 M NaCl, and 2 mM DTT without 20% glycerol for 0, 10, 20, 40, and 60 min (lane 1-5) and the rPDPc activities were assayed with the same samples. A, rPDPc activity; B, SDS-PAGE gel analysis by elastase without 2 mM CaCl₂; C, proteolysis by elastase for 60 min without 2 mM CaCl₂ (lane 6); Proteolysis with 2 mM CaCl₂ for 60 min (lane 7, by elastase; lane 8, by Arg-C; lane 9, by Chymotrypsin). Arrow represents 50 kD N-terminal end.

mational change of the second proteolytic region between N-terminal and C-terminal ends.

Taken together, PDPc consists of three major structural/functional domains and C-terminal end (peptide IV in table 2) is not necessary for the activity but, peptide II and peptide III are critical for the activity. Also, the calcium binding domain seems to be located in the border of between peptide II and peptide III in N-terminal large fragment (Table 2).

## ACKNOWLEDGEMENTS

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inhibited by 2 mM CaCl₂ like those of Arg-C (Fig. 5C). This indicates that calcium caused the confor-

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