

## 혈액 검체에서 여러 가지 단백분해효소 억제제에 대한 부갑상선 호르몬의 분해 효과 비교

김영식 · 권희정 · 이혜경

가톨릭대학교 의과대학 진단검사의학교실

### The Comparison of Parathyroid Hormone Degradation Effect by Various Protease Inhibitors in Blood Specimen

Yeong Sic Kim, M.D., Hi Jeong Kwon, M.D., and Hae Kyung Lee, M.D.

Department of Laboratory Medicine, College of Medicine, The Catholic University of Korea, Seoul, Korea

**Background :** The objective of this study was to evaluate the role of proteases on the degradation of parathyroid hormone (PTH) in blood samples.

**Methods :** Protease inhibitors with specificity against serine proteases (aprotinin), cysteine proteases (E-64), serine and cysteine proteases (leupeptin), metalloproteases (EDTA), or a protease inhibitor cocktail with a broad spectrum of inhibitory activity were added to blood samples. After storage at room temperature (0-48 hr), PTH levels were measured.

**Results :** PTH levels in samples with the protease inhibitor cocktail did not change significantly after 48 hr of storage at room temperature, but the average PTH levels decreased by 40.7% and 20.1%, in samples stored at room temperature and stored at 4°C without protease inhibitors, respectively. PTH levels in samples with leupeptin were stable for up to 24 hr. After 48 hr, the mean PTH levels decreased by 17.1%, 16.0%, 26.2%, and 32.1%, with 500 KIU/mL aprotinin, 100 μmol/L leupeptin, 10 μmol/L E-64, and 10 μmol/L EDTA, respectively, in the samples stored at room temperature.

**Conclusions :** The decrease in PTH levels in blood samples seemed to be due to the degradation of PTH by proteases. Various proteases, including especially serine proteases, would act together to degrade PTH in blood specimen. The PTH degradation may be inhibited in blood specimen with protease inhibitor cocktail. (*Korean J Lab Med 2009;29:104-9*)

**Key Words :** Metal ion; Serine proteases; Parathyroid hormone

## INTRODUCTION

Parathyroid hormone (PTH) is released from the parathy-

roid glands and regulates bone and mineral metabolism. It is a single-chain 84-amino acid polypeptide with a molecular weight of 9,425 Daltons. It is synthesized and secreted by the parathyroid glands, and rapidly cleared from plasma (half-life <5 min). PTH is metabolized to C-terminal fragments by the liver and kidneys, and cleared by the kidney, either as intact PTH or as C-terminal fragments [1]. The circulating C-terminal fragments result from peripheral cleavage of PTH between residues 33 and 34 or between

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Corresponding author : Hae Kyung Lee, M.D.

Department of Laboratory Medicine, The Catholic University of Korea, Uijeongbu St. Mary's Hospital, 65-1 Kumoh-dong, Uijeongbu 480-130, Korea  
Tel : +82-31-820-3159, Fax : +82-31-847-6266  
E-mail : hkl@catholic.ac.kr

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residues 36 and 37. Kupffer cells, but not hepatocytes, were found to be responsible for cleavage at these sites [2].

PTH concentrations in serum specimens are reduced after incubating at room temperature for more than a few hours [3–5], probably due to proteolytic cleavage by proteases. Proteases generally have 5 groups (serine, cysteine, aspartic, metallo, and threonine). Some proteases of these groups, including metalloproteases, need metal ions as cofactors in the enzymatic reactions, and so these proteases are called metal ion-dependent proteases. For metalloproteases, they contain one or two metal ions (in most cases zinc but also cobalt, nickel or manganese) that are usually bounded by three amino acids in the catalytic nucleophile [6]. There are a number of proteases in blood, including serine proteases (thrombin, protein C, factor VII, IX, X, and XII, plasmin, tissue plasminogen activator, urokinase plasminogen activator), cysteine proteases (cathepsins), metalloproteases (gelatinase A, matrixins), aspartic proteases, and threonine proteases [6–8]. Blood also contains endogenous inhibitors of many of these proteases.

PTH is shown to be stable at room temperature for approximately 2 days in plasma containing ethylene diamine tetra-acetic acid (EDTA), at a clinically used concentrations in blood specimen [5, 9]. However, since the concentration of EDTA used in the blood collection is about 5,000  $\mu\text{M}$ , a concentration sufficient to remove almost all metal ions in blood samples, including zinc, calcium, and copper, it is impossible to distinguish whether the effect of EDTA is inhibition of proteolysis by metalloproteases or metal ion-dependent proteases [10, 11].

To date, it remains unclear which class or classes of proteases are involved in degrading PTH in serum and plasma specimens. The objective of this study was to evaluate the role of proteases on the degradation of PTH in blood samples.

## MATERIALS AND METHODS

### 1. Blood collection and PTH measurement

Blood samples were collected from volunteer patients (8

male, 2 female) and dispensed (30 mL/tube) into each of 7 plain glass tubes (Sinyoungscience Co., Seoul, Korea). Tubes 1–5 contained: 1) aprotinin (inhibitor of serine proteases); 2) E-64 (inhibitor of cysteine proteases); 3) leupeptin (inhibitor of serine and cysteine proteases); 4) EDTA (inhibitor of metalloproteases); 5) protease inhibitor cocktail (inhibitor of serine, cysteine and aspartic acid proteases, and metalloproteases). Tubes 6–7 contained no protease inhibitors. Blood samples were allowed to clot by standing for 30 min at room temperature. After centrifugation at  $2,000\times g$  for 10 min, the serum or plasma was transferred to another plain glass tube. One of the two remaining serum sample without protease inhibitors was stored at  $4^{\circ}\text{C}$  for 48 hr; the other was stored at room temperature for 48 hr with samples containing protease inhibitors.

Four kinds of protease inhibitors and one kind of inhibitor cocktail used in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The final concentrations of aprotinin, E-64, leupeptin, and EDTA in blood samples were 500 KIU/mL, 10  $\mu\text{M}$ , 100  $\mu\text{M}$ , and 10  $\mu\text{M}$ , respectively. The protease inhibitor cocktail is a mixture of water-soluble protease inhibitors with a broad specificity against serine proteases, cysteine proteases, aspartic acid proteases, and metalloproteases. The protease inhibitor cocktail was used at a concentration recommended by the manufacturer (0.3  $\mu\text{M}$  aprotinin, 130  $\mu\text{M}$  bestatin, 1,000  $\mu\text{M}$  EDTA, 14  $\mu\text{M}$  E-64, 1  $\mu\text{M}$  leupeptin, and 2,000  $\mu\text{M}$  4-(2-Aminoethyl) benzenesulfonyl fluoride.

PTH concentrations were measured on 0, 12, 24, and 48 hr after centrifugation using IMMULITE 2000 Intact PTH (DPC, Los Angeles, CA, USA). The assay kit uses a noncompetitive method that requires two antibodies capable of simultaneously binding PTH. The antibodies immobilized to the solid phase are specific for the C-terminal fragment (residues 44–84), but the enzyme-labeled antibody recognizes only the N-terminal fragment (residues 1–34). Accordingly, the assay is able to recognize only intact PTH and very large PTH fragments that are nearly so long as intact PTH. Informed consent was obtained from each patient, and the institutional review board approved the use of clinical samples in this study.

## 2. Statistics

A Wilcoxon signed-rank test was used to compare initial PTH levels to those after 48 hr of storage and was also used to compare the PTH levels between different storage conditions. A  $P$  value  $<0.05$  was considered statistically significant. Statistical analyses were performed using the SPSS program (Version 11.5, SPSS Inc., Chicago, IL, USA).

## RESULTS

EDTA of  $10 \mu\text{M}$  did not prevent clot formation, nor did any of the other protease inhibitors, with the exception of leupeptin.

At zero time, PTH levels in all samples with the exception

of leupeptin showed almost the same as each other. The mean concentration of PTH in samples with leupeptin was 20.1% higher than that in samples without protease inhibitors. This difference was considered to be caused by the effect of leupeptin on the measurement process of the PTH assay kit (Table 1, Fig. 1).

All the samples except the samples with protease inhibitor cocktail showed significant reductions in PTH level during 48 hr storage ( $P<0.05$ ). After 48 hr, the mean PTH levels in samples stored at room temperature and stored at  $4^\circ\text{C}$  without protease inhibitors decreased by 40.7% and 20.1%, respectively. In the samples stored at room temperature with protease inhibitors, the mean PTH levels with aprotinin, leupeptin, E-64, and EDTA decreased by 17.1%, 16.0%, 26.2%, and 32.1%, respectively. Among samples with different stor-

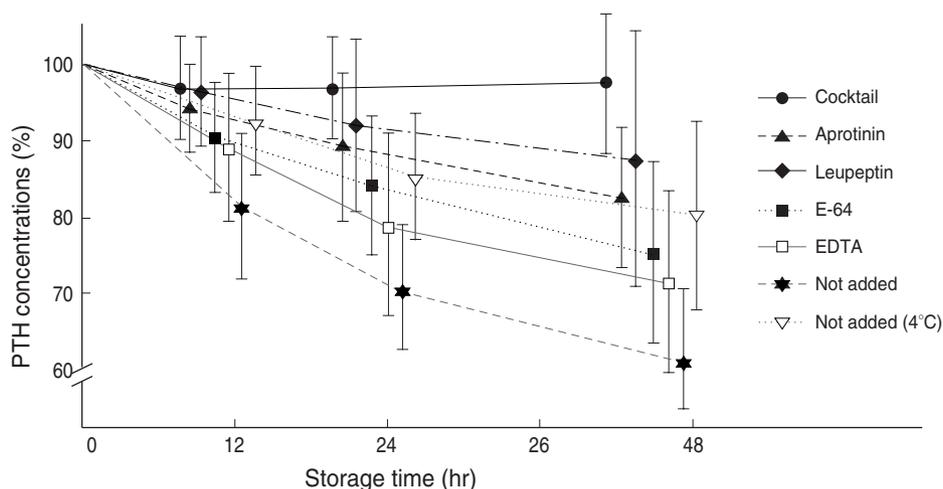


Fig. 1. Time-dependent changes of the PTH levels in samples with different kinds of protease inhibitors. Data points are the mean  $\pm$  SD (error bars) of PTH levels. Six groups of samples were stored at room temperature and the other group was stored at  $4^\circ\text{C}$ . Abbreviation: PTH, parathyroid hormone.

Table 1. Changes of PTH levels in blood specimen according to different storage conditions

Storage condition	PTH levels (pg/mL)*				$P^\dagger$
	Zero time	After 12 hr	After 24 hr	After 48 hr	
Storage at room temperature					
Without protease inhibitors	$35.4 \pm 13.1$	$28.1 \pm 8.4$	$23.8 \pm 4$	$21.0 \pm 6.2$	0.005
With protease inhibitors					
Inhibitor cocktail	$34.7 \pm 12.4$	$33.6 \pm 12.7$	$33.14 \pm 11.9$	$33.3 \pm 11.5$	0.139
Aprotinin, 500 KIU/mL	$35.6 \pm 12.6$	$33.5 \pm 11.8$	$31.8 \pm 12.7$	$29.5 \pm 11.0$	0.005
Leupeptin, 100 $\mu\text{mol/L}$	$45.0 \pm 9.0$	$43.6 \pm 15.9$	$41.9 \pm 15.7$	$37.8 \pm 14.2$	0.028
E-64, 10 $\mu\text{mol/L}$	$34.3 \pm 12.2$	$30.6 \pm 10.9$	$28.7 \pm 11.3$	$25.3 \pm 9.9$	0.005
EDTA, 10 $\mu\text{mol/L}$	$34.9 \pm 13.5$	$30.1 \pm 9.8$	$26.4 \pm 9.5$	$23.7 \pm 8.3$	0.008
Storage at $4^\circ\text{C}$					
Without protease inhibitors	$35.9 \pm 14.2$	$33.6 \pm 13.7$	$30.6 \pm 12.4$	$28.7 \pm 11.1$	0.009

\*, PTH levels are given as mean  $\pm$  SD;  $^\dagger$ , Comparison between zero time PTH levels and those after 48 hr was performed using the Wilcoxon signed-rank test.

Abbreviations: PTH, parathyroid hormone; EDTA, ethylene diamine tetra-acetic acid.

age conditions, samples stored at room temperature without protease inhibitors showed the largest reduction in PTH level after 48 hr. The PTH levels after 48 hr in samples with protease inhibitor cocktail, aprotinin, leupeptin, and E-64 were higher than those in samples stored at room temperature without protease inhibitors ( $P < 0.05$ ).

PTH levels in samples with the protease inhibitor cocktail did not change significantly during 48 hr storage at room temperature ( $P > 0.05$ ). After 12 hr, there were significant reductions in PTH level in all samples except samples with protease inhibitor cocktail and leupeptin ( $P < 0.05$ ). After 12 hr, the mean PTH levels in samples stored at room temperature and stored at 4°C without protease inhibitors decreased by 17.8% and 6.4%, respectively. The mean PTH levels in samples with aprotinin, E-64, EDTA decreased by 5.9%, 10.8%, and 13.8%, respectively. PTH levels in samples with leupeptin were stable for up to 24 hr at room temperature.

## DISCUSSION

Consistent with previous reports, we found that PTH levels in serum samples without protease inhibitors significantly decreased during storage at room temperature. Among the protease inhibitors tested in this study, only the protease inhibitor cocktail, consisting of 0.3  $\mu\text{M}$  aprotinin, 130  $\mu\text{M}$  bestatin, 1,000  $\mu\text{M}$  EDTA, 14  $\mu\text{M}$  E-64, 1  $\mu\text{M}$  leupeptin, and 2,000  $\mu\text{M}$  4-(2-Aminoethyl) benzenesulfonyl fluoride, was able to completely prevent PTH degradation in samples stored at room temperature for 48 hr. Leupeptin, a serine and cysteine protease inhibitor, could prevent PTH degradation for 24 hr, and aprotinin, a serine protease inhibitor, could partially protect PTH degradation for 24 hr. These findings indicate that PTH degradation in blood specimen is protease-dependent, and various proteases, including especially serine proteases, act together to degrade PTH in blood specimen. These results are consistent with those of Anderson et al., who reported that aprotinin significantly reduced the in vitro PTH degradation in plain tubes from 24.7% to 9.6% at 24 hr [12].

E-64, a cysteine protease inhibitor, and EDTA, a metal-

loprotease inhibitor, were ineffective to protect PTH degradation. Our data are a contrast to previous reports on the effects of EDTA [5, 9, 12, 13]. Teal et al. reported that PTH remained stable in EDTA-plasma for up to 48 hr at room temperature, and Evans et al. provided data showing that PTH remained stable in EDTA-plasma for 36 hr at 30°C [5, 9]. These differences likely reflect due to differences in EDTA concentrations used. In the current study, EDTA was used at 10  $\mu\text{M}$ , a concentration recommended by the manufacturer to effectively inhibit metalloproteases. However, the previous studies used EDTA at concentrations of about 5,000  $\mu\text{M}$  to obtain plasma, that is higher enough to remove almost all metal ions in blood samples, including zinc, calcium, and copper [10, 11]. EDTA displays a high binding constant for zinc and a weak one for calcium and magnesium (about 1.5 times and 2 times, respectively) [11]. So, at the low concentration of EDTA (10  $\mu\text{M}$ ), EDTA binds zinc more easily than calcium or magnesium. Because the overwhelming majority of the metalloproteases are zinc enzymes, lower concentration of EDTA can inhibit many of metalloproteases. EDTA of 10  $\mu\text{M}$  as a zinc-chelating agent could not prevent blood coagulation. Furthermore, the protease inhibitor cocktail containing 1,000  $\mu\text{M}$  EDTA could not also prevent blood coagulation. Taken together these findings, cysteine protease and metalloprotease inhibitors have less effect on PTH degradation than other protease inhibitors, and EDTA would play a role for the inhibitor of metal ion-dependent proteases, but not metalloproteases, in PTH degradation.

Leupeptin is also known to have inhibitory activity toward proteases involved in blood coagulation and fibrinolysis [14, 15]. In the present study, leupeptin of 100  $\mu\text{M}$  could prevent blood coagulation, but the protease inhibitor containing 1  $\mu\text{M}$  leupeptin could not prevent coagulation. This finding seemed to be due to the difference in concentration of leupeptin.

Although PTH level was more stable in serum specimen stored at 4°C compared to serum specimen at room temperature, the mean PTH reductions after 12 hr, 24 hr, and 48 hr storage at 4°C were 6.4%, 14.8%, and 20.1%, respectively. Therefore, although serum specimen is stored at 4°C, careful attention to the interpretation of PTH levels mea-

sured after 12 hr of storage would be given. PTH is unstable in serum specimen even stored at 4°C. Thus, adding protease inhibitors to serum appears to be a good method to prevent the degradation of PTH for some days.

In conclusion, PTH degradation in blood specimen would be protease-dependent and various proteases, including especially serine proteases, would act together to degrade PTH in blood specimen.

## 요 약

**배경** : 혈액 검체에서 부갑상선 호르몬(PTH, parathyroid hormone)의 분해 과정에 단백질 분해효소의 역할을 평가하고자 하였다.

**방법** : 단백질 분해효소 억제제인 aprotinin (inhibitor of serine proteases)과 E-64 (inhibitor of cysteine proteases), leupeptin (inhibitor of serine and cysteine proteases), EDTA (inhibitor of metalloproteases), 광범위한 억제 작용을 보이는 protease inhibitor cocktail을 혈액 검체에 각각 혼합하였다. 실온에서 48시간 보관하면서 PTH 농도를 측정하였다.

**결과** : 48시간 실온 보관 후 protease inhibitor cocktail이 함유된 검체의 PTH 농도는 48시간 동안 유의하게 변하지 않았지만 단백질 분해효소 억제제가 함유되지 않은 실온 보관 검체와 냉장 보관 검체의 평균 PTH 농도는 각각 40.7%와 20.1% 감소하였다. Leupeptin이 함유된 검체의 PTH 농도는 24시간까지 안정적이었다. 실온 보관 48시간 후 평균 PTH 농도는 500 KIU/mL aprotinin과 100 µmol/L leupeptin, 10 µmol/L E-64, and 10 µmol/L EDTA이 함유된 검체에서 각각 17.1%와 16.0%, 26.2%, 32.1% 감소하였다.

**결론** : 혈액 검체내에서 PTH 농도 감소는 단백질 분해효소에 의한 파괴 때문으로 여겨진다. 주로 serine protease를 포함하여 다양한 단백질 분해효소들이 혈액 검체 내 PTH 분해에 함께 관여하는 것으로 판단되며 protease inhibitor cocktail을 사용하면 PTH 분해를 억제할 수 있을 것으로 판단된다.

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