BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 560–564 (1997)
ARTICLE NO. RC977344

The Role of Chondroitin Sulfate Chains of Urinary Trypsin Inhibitor in Inhibition of LPS-Induced Increase of Cytosolic Free Ca^{2+} in HL60 Cells and HUVEC Cells

Naohiro Kanayama, Kayoko Maehara, Masako Suzuki,* Yutaka Fujise,** and Toshihiko Terao
*Department of Obstetrics and Gynecology, **Department of Chemistry, and *Equipment Center, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan*

Received August 7, 1997

Preincubation of HL60 cells and HUVEC cells with urinary trypsin inhibitor (UTI) inhibited increase of cytosolic free Ca^{2+} induced by LPS. In contrast, an increase of cytosolic free Ca^{2+} induced by LPS was not inhibited by deglycosylated UTI, UTI treated with monoclonal antibody of chondroitin sulfate. $^{45}Ca^{2+}$ binding showed that UTI binds $^{45}Ca^{2+}$ dose-dependently. Scatchard plot analysis showed that UTI has two binding sites for Ca^{2+}, a high affinity binding site (Kd=15 μM) and a low affinity site (Kd=150 μM), and that UTI has more than 70 Ca^{2+} binding sites per molecule. The Ca^{2+} binding capacity of deglycosylated UTI and UTI treated with monoclonal antibody of chondroitin sulfate was markedly depressed. Furthermore, UTI forms multi-polymers in the presence of Ca^{2+} as demonstrated by gel filtration and agarose gel electrophoresis. These results suggest that UTI is a physiological Ca^{2+} chelator on the cells and that the action is due to chondroitin sulfate chains of UTI.

Urinary trypsin inhibitor (UTI) is a Kunitz-type preotease inhibitor which exists in plasma and urine. It is identical to HI-30 (1) or urinary trypsin inhibitor (UTI) and is a heat-and acid-stable glycoprotein (2-3). Most of the UTI in plasma exists as a part of inter-α trypsin inhibitor (ITI) (4). Molecular biology has revealed that ITI is composed of three genetically different peptides: a light chain and two heavy chains (5). The three peptides are covalantly cross-linked by a unique linkage through chondoroitin sulfate chains (6). UTI contains two tandem repeats of Kunitz-type domains. UTI circulates in the blood stream as a form of ITI in normal physiological conditions, and it is released in pathological conditions such as thrombus or inflammation (7). UTI is released from ITI by a neutrophil elastase-like enzyme on the cell surface after ITI binding to hyaluronic acid (8-9). A high concentration of ITI is present in normal human serum, but it plays only a 5% role in trypsin inhibitory activity in serum (10). Other strong trypsin inhibitors such as α_1 antitrypsin and α_2 macroglobulin are present in the blood. ITI or UTI is thought to have another physiological role in addition to protease inhibition in the blood. Some current studies suggest UTI may be a modulator of cell growth (11); however, the precise functions of UTI converted from ITI remains unknown.

Previous work from this laboratory has shown that UTI inhibited smooth muscle contraction by inhibition of calcium influx (12) and that UTI acts on down-regulation of interleukin-8 gene expression induced by LPS (lipopolisaccharide) via inhibition of an increase of cytosolic free calcium (13).

To clarify the mechanism of inhibition of Ca^{2+} influx by UTI in cells, we focused on the binding affinity between UTI and Ca^{2+}.

Abbreviations used in this paper: UTI, urinary trypsin inhibitor; ITI, inter-a trypsin inhibitor; Ch0S, chondroitin 0-sulfate; Ch4S, chondroitin 4-sulfate.

MATERIALS AND METHODS

Materials

UTI was purified from human urine according to methods described by Proksch (Proksch et al., 1972). Highly purified UTI (specific activity, 2330 units/mg soluble protein, with a molecular mass of 67000 K), which migrated as a single band on SDS-PAGE and on Sephadex gel chromatography, was kindly provided by Mochida Pharmaceutical Co., Tokyo, Japan. ITI was purchased from BioPur AG (Basel, Switzerland). Chondroitin 0-sulfate (Ch0S) and chondroitin 4-sulfate (Ch4S) monoclonal antibodies were purchased from Seikagaku Co., Tokyo, Japan. $^{45}Ca^{2+}$ was obtained from Amasham, USA.

A partially deglycosylated UTI was obtained as follows: UTI (100 μM) was incubated with chondroitinase ACII (10 μM for 1 h at 37°C; Sigma, USA). UTI treated with chondroitinase ACII was purified by reversephase HPLC.

0006-291X/97 $25.00

Methods

HL60 cells (11) and HUVEC (human umbilical cord vein endothelial cell) cells (12) were prepared as described previously. HUVEC cells were cultured on a thin cover glass in a CO_2 incubator. Serum-free RPMI 1640 medium (Nissui, Japan) was used for HL60 cells and HUVEC cells. HL60 cells and HUVEC cells were washed with PBS, and incubated in 1 ml of serum-free medium for 30 min in a CO_2 incubator before experiments.

Measurement of cytosolic free Ca^{2+}. Digital imaging microscopy was carried out as described previously with some modifications (13). Briefly, cells were first incubated with 4 μg/ml fura-2 AM (Kumamoto, Japan) for 1 h. Before the examination, the cells were washed with PBS, pH 7.3, and serum-free RPMI 1640 medium was added for 1 h at 37 °C. Fura-2 loaded cells on a thin cover glass were placed on a microscope stage warmed to 37 °C. Cells in discs were preincubated with or without UTI (1 μM) for 10 min and then 0.1 mg/ml LPS was added. Cytosolic free calcium ($[Ca^{2+}]_i$) was measured every 30 s for 390 s. The microscopic system consisted of an inverted microscope (Nikon TMD-EFQ, Japan) with a 100 × UV objective (Olympus, Japan). The cells were excited by ultraviolet light at 340 nm and 380 nm. Sequential images were collected through a single broad band pass filter (500 nm, band width 20 nm) at intervals of 30 s. The light source, a DC-stabilized Xenon lamp, was fitted with a computer-assisted device to alternate filters for the two wave lengths. Video images were acquired with a silicon-intensified target camera (2400-08, Hamamatsu Photonics K.K., Japan). The out-put was digitized with a color image analyzer ARGUS 100 (Hamamatsu Photonics K.K., Japan). The images were integrated to improve the S/N ratio and calculated as 340/380 nm ratio images.

To determine the $[Ca^{2+}]_i$, we made a calibration curve in our optical set up according to a previous report (14). $[Ca^{2+}]_i$ in all experiments was calculated from the fluorescence ratio values using the calibration curve. In all experiments, $[Ca^{2+}]_i$ was measured at the single cell level and means ± SD for 10 cells in repeated experiments (> 6 times) are presented in graphs. Statistical analysis were performed by Student's t test.

$^{45}Ca^{2+}$ binding study. The binding of Ca^{2+} ions to 1 μM of UTI was investigated by the Millipore filtration method using $^{45}CaCl_2$ (15). The filtration apparatus consisted of a stainless steel injection chamber, a filter holder introduced from the bottom of the housing with a piston, and four adjustable pipettes used to inject various solution into the filter. The pipettes were connected with thin nozzles converging a few millimeters above the filter. The filters were 0.45 μm Millipore (USA), and the radioactivity was counted with a Aloka LSC-3100 (Japan). The standard buffer used in this study was Tris buffer containing 0.125 M NaCl, pH 7.0. The different concentrations of UTI (0-10 μM) were incubated with different concentrations of $^{45}CaCl_2$ (0.1-500 μM) for 30 min at 37°C. To determine the specificity the binding of $^{45}Ca^{2+}$ to UTI, excess amount of cold $CaCl_2$ (100 μM) was also added to each sample before incubation of UTI and $^{45}Ca^{2+}$. After the incubation was completed by washing with the buffer, the radioactivity bound to the proteins on the filter was calculated after subtracting the contribution of the buffer trapped in the filter. We also measured 100 μM $^{45}Ca^{2+}$ binding to 1 μM UTI at a different pH. The $^{45}Ca^{2+}$ binding study was also performed using 1 μM deglycosylated UTI, ITI and UTI treated with 10 μM of chondroitin 0-sulfate and chondroitin 4-sulfate monoclonal antibodies. Scatchard analysis of UTI and Ca^{2+} was performed.

Gel filtration. Ten mg UTI or 2 mg ITI in 1 ml of Tris buffered saline (0.125 M NaCl), pH 7.0 was incubated with or without 100 mM $CaCl_2$. The aliquots were applie d to Sepharose CL4B gel filtration (2×100 cm). The running buffer was Tris buffered saline or Tris buffer containing 1.25 M NaCl solution. Three mls of each fraction were collected, frozen, and dried.

Agarose gel electrophoresis. To examine the electrophoresis pattern of UTI after gel filtration, 2% agarose gels were used. The samples from filtration (approximately 50 μg of UTI) were applied to the gel, and electrophoresis was performed at 50 V for 20 minutes at room temperature.

TABLE 1

Levels of $[Ca^{2+}]_i$ in Neutrophils and HUVEC Cells on Various Treatments

	Neutrophils nM (mean ± SD)	HUVECs nM (mean ± SD)
LPS	420 ± 60	324 ± 62
UTI + LPS	105 ± 27**	91 ± 32**
Deglycosylated-UTI + LPS	246 ± 75*	241 ± 45
UTI with MoAb Ch0S + LPS	350 ± 55	288 ± 49
UTI with MoAb Ch4S + LPS	390 ± 70	319 ± 54

Note. The cytosolic free Ca^{2+} concentration was measured 60 s after stimulation. Values are means ± SD for 5 replicate experiments. *P < 0.05. **P < 0.01.

RESULTS

Effects of UTI on Cytosolic Ca^{2+}

Unstimulated HL60 cells and HUVEC cells with or without UTI showed $[Ca^{2+}]_i$ values of 88-110 nM and 78-94 nM, respectively. LPS increased cytosolic Ca^{2+} time-dependently, the peak of concentrations was at about during 60-90 s (data not shown). Table 1 shows results on $[Ca^{2+}]_i$ at 60 s. Preincubation with 1 μM UTI completely inhibited the increase in cytosolic Ca^{2+} induced by LPS, but deglycosylated UTI and UTI treated with monoclonal antibody of chondroitin sulfate chains patially inhibit the increase of cytosolic Ca^{2+} induced by LPS.

$^{45}Ca^{2+}$ Binding Study

The radioactivity was measured and compared with controls (blanks without membranes). The binding of Ca^{2+} ions to different concentrations of UTI was shown in Figure 1. UTI bound $^{45}Ca^{2+}$UTI-dose dependent manner. The Ca^{2+} binding capacities of UTI are shown in Figure 2. Figure 2a shows a saturation curve for binding of Ca^{2+} to 1 μM of UTI. UTI bound $^{45}Ca^{2+}$ in a dose-dependent manner up to more than 70 times higher of the UTI concentrations. Scatchard plot analysis showed that UTI has two binding sites for Ca^{2+}, a high affinity binding site (Kd=15μM) and a low affinity one (Kd=150 μM) (Figure 2b). Binding of Ca^{2+} to UTI at saturation was approximately 70 μM $^{45}Ca^{2+}$/1μM UTI. On the other hand, UTI treated with excess amount of chondroitin 0-sulfate (Ch0S) and chondroitin 4-sulfate (Ch4S) monoclonal antibodies, UTI treated with chondroitinase ACII and ITI (inter-α trypsin inhibitor) bound $^{45}Ca^{2+}$ much less effectively than UTI

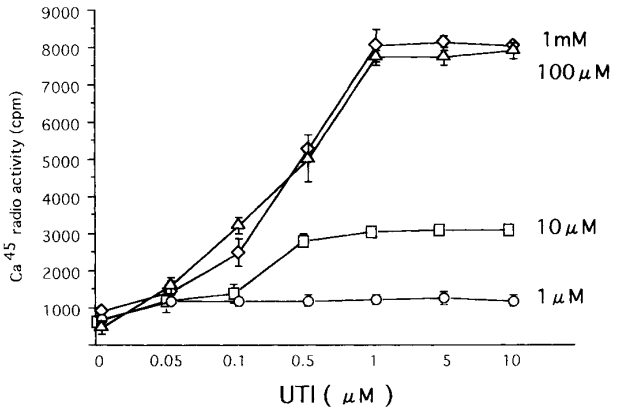

FIG. 1. The binding of $^{45}Ca^{2+}$ ions and UTI. UTI bound $^{45}Ca^{2+}$ in a UTI-dose-dependent manner. More than 100 μM $^{45}Ca^{2+}$ shows the similar curves indicating that UTI binding capacity to artificial membranes is saturated at 1 μM.

(Figure 3). $^{45}Ca^{2+}$ binding to UTI was only slightly increased in response to elevated pH (data not shown).

Gel Filtration

As shown by gel filtration, the peak of UTI treated with Ca^{2+} (100 mM) appeared in the early number of fraction tubes suggesting that UTI was a multimer (Figure 4). In the presence of high concentrations of NaCl, UTI treated with Ca^{2+} still formed a multimer. On the other hand, ITI treated with Ca^{2+} did not form a multimer regardless of the presence of sodium ions. Agarose gel electrophoresis showed that the movement of multimeric UTI was delayed compared to free UTI (Figure 4).

DISCUSSION

UTI inhibited increase of $[Ca^{2+}]_i$ induced by LPS in HL60 cells and HUVEC cells. However, deglycosylated UTI, UTI treated with chondroitin sulfate monoclonal antibody supressed increase of $[Ca^{2+}]_i$ induced by LPS slightly. These suggest that chondroitin sulfate part of UTI is involved in regulation of intracellular calcium. Our $^{45}Ca^{2+}$ binding study clearly showed that UTI can bind more than 70 calcium ions per molecule and that this binding is dependent upon chondroitin sulfate chains of UTI. $^{45}Ca^{2+}$ binding to UTI treated with chondroitin sulfate monoclonal antibodies was depressed, and $^{45}Ca^{2+}$ binding to deglycosylated UTI was also inhibited. Toyoda et al. reported that UTI has 10 chondroitin 0-sulfate chains and 5 chondroitin 4-sulfate chains (16). The 10 chondroitin 0-sulfate chains are located peripherally and the 5 chondroitin 4-sulfate chains are located sequentially. It is assumed that Ca^{2+} can bind all anions of chondroitin sulfate chains around physiological pH. Therefore, the binding site of UTI for Ca^{2+} is chondroitin sulfate chains. It is undeniable that the protein component of UTI can also bind Ca^{2+}. However, deglycosylated UTI or UTI with chondroitin sulfate monoclonal antibody bound Ca^{2+} less than UTI, the majority of Ca^{2+} binding sites are estimated to be on chondroitin sulfate chains. ITI bound Ca^{2+} nearly undetectable. Two heavy chains of ITI may mask or overlap the chondroitin sulfate chains, resulting in decreased affinity of ITI for Ca^{2+} compared to UTI.

Based on ionic strength, ionic binding between Ca^{2+} and SO_3^- is stronger than that between Ca^{2+} and COO^- or OH^-. It is known that carboxylic acids and hydroxide ions of carbohydrate can easily bind Ca^{2+} resulting in gel formation (17). Thus, the sulfuric acid-Ca^{2+} binding would form outline of multimeric UTI, and then carboxylic acid-Ca^{2+} or hydoxide ion-Ca^{2+} bindings may occur secondary.

The $^{45}Ca^{2+}$ binding study showed more than 70 cal-

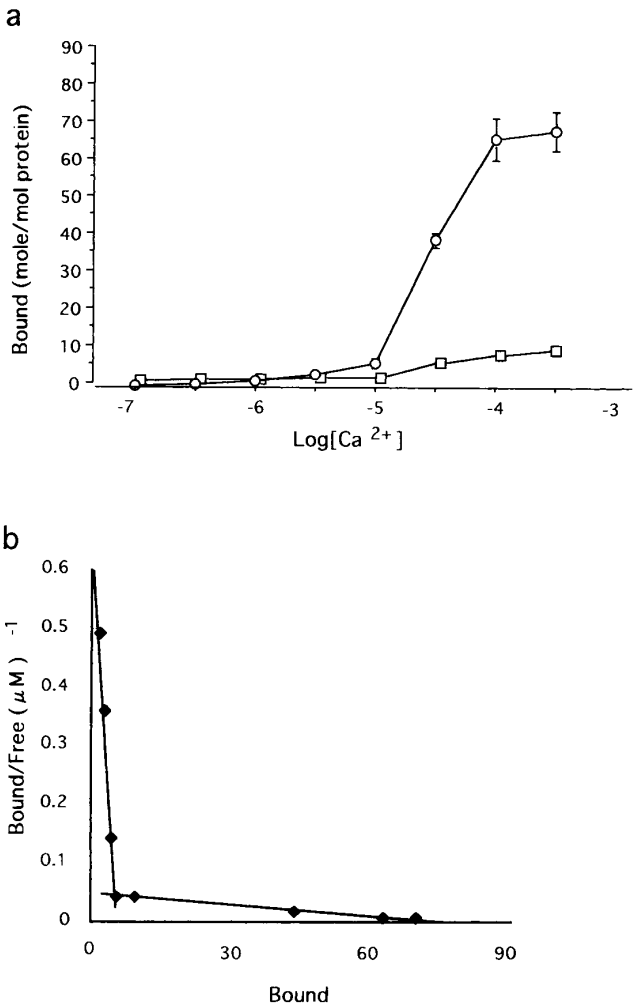

FIG. 2. (a) $^{45}Ca^{2+}$ binding to UTI. ○, UTI; □, UTI in the presence of excess amount of cold Ca^{2+}. (b) Scatchard plot for Ca^{2+} binding to the UTI. The regression curve for high affinity sites is $Y = -0.12 \times +0.6$ (Kd= 15 μM); that of low affinity sites is $Y = -5.1 \times 10^{-4} X + 0.045$ (Kd= 150 μM).

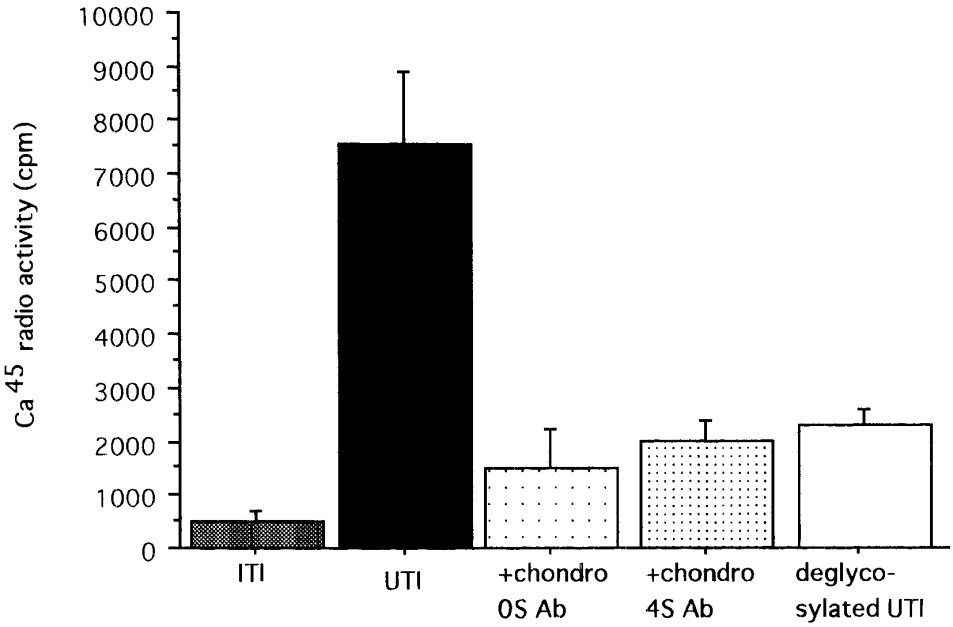

FIG. 3. $^{45}Ca^{2+}$ binding to UTI-related molecule. Black bar, 1 μM UTI; rough hatched bar, 1 μM UTI with excess amount of chondroitin 0-sulfate (Ch0S); fine hatched bar, 1 μM UTI with excess amount of chondroitin 4-sulfate (Ch4S) monoclonal antibodies; white bar, 1 μM UTI treated with chondroitinase ACII; gray bar, ITI. One μM UTI treated with chondroitinase ACII, UTI with Ch0S and Ch4S antibodies, and ITI bound $^{45}Ca^{2+}$ much less effectively than UTI.

cium ions bind UTI, and the gel filtration showed that UTI forms a large multimer in the presence of Ca^{2+}. Since the amount of Ca^{2+} bound to the UTI complex far exceeds the number of potential binding sites on a single monomer of UTI, the role of the UTI in this system must be provide the fundamental spatial rela-

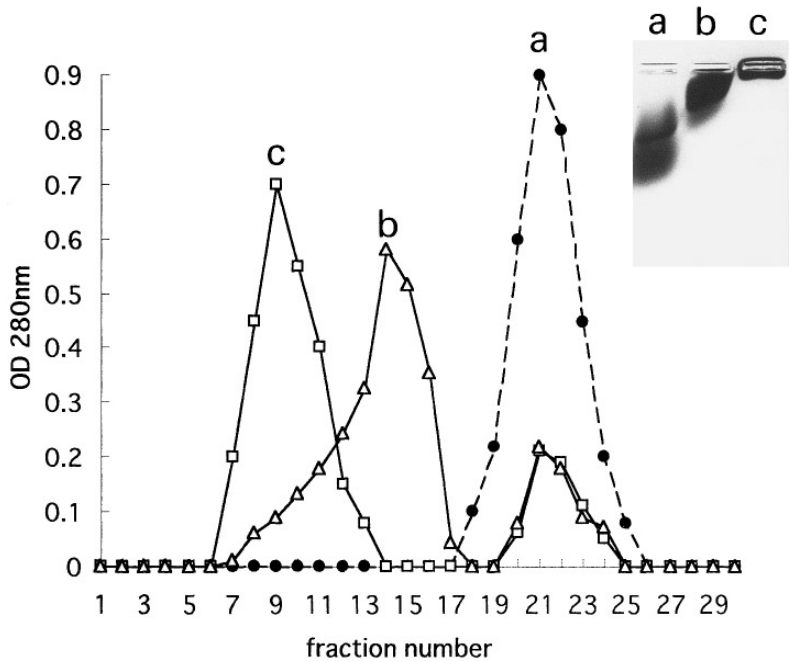

FIG. 4. The gel filtration pattern of UTI (a), UTI treated with $CaCl_2$ in Tris buffered saline pH7.0 (c) and UTI treated with $CaCl_2$ in 1.25 M NaCl (b). The electrophoresis patterns are shown in the upper right. Each letter in the graph corresponds to the agarose electrophoresis results.

tionship necessary to making Ca^{2+} chelation. The Ca^{2+} titration indicated that two sulfate groups are involved in the binding of each Ca^{2+} ion. The specific binding sites for Ca^{2+} are thought to be adjacent to the chondroitin sulfate chains of UTI. In other words, the Ca^{2+} is bound to the axial sulfate esters on the residues of different UTI chains rather than on their esidues of the same UTI chain. The binding capacity of UTI to other cations was less than that to Ca^{2+}. These findings suggest that GalNAc(SO_3^-) binds Ca^{2+} selectively in the presence of other cations. Presumably, Ca^{2+} is the selective cation for UTI.

The serum concentrations of UTI and ITI are approximately 0.75 μM and 2.5 μM, respectively (18, 19). ITI is converted to UTI by enzymes such as elastase (8). UTI may be associated with pathological conditions such as inflammation and thrombus. If several micromolar concentratons of UTI exist in such regions, Ca^{2+} could cause UTI to form a complex, resulting in gel formation. Such a gel would be able to absorb high concentrations of Ca^{2+}. Since the concentratons of free Ca^{2+} is 1-2 mM in blood, UTI at micromolar concentrations should be able to absorb a large amount of Ca^{2+} in the pathological conditions.

In this study, we discovered that UTI regulates cytosolic free calcium in HL60 cells and HUVEC cells. This action can be explained that UTI has a binding capacity for Ca^{2+} on cell membranes and that it also forms a large polymer via calcium sulfate binding, resulting in a significant Ca^{2+} chelating effect. Thus, UTI could be a potent and physiological Ca^{2+} chelator on cell membranes. This result is in accord with several recent reports indicating that UTI has various biological functions besides protease inhibitor.

ACKNOWLEDGMENT

We thank Mr. Fujie M for technical advice and carbohydrate analysis.

REFERENCES

1. Gebhard, W., Hochstrasser, K., Fritz, H., Enghild, J. J., Pizzo, S. V., and Salvesen, G. (1990) *Biol. Chem. Hoppe-Seyler* **371,** 13–22.
2. Astrup, T. U., and Nissen, U. (1964) *Nature* **203,** 255–257.
3. Sumi, H., Takada, Y., and Takada, A. (1977) *Thromb. Res.* **11,** 747–754.
4. Enghild, J. J., Thøgersen, I. B., Pizzo, S. V., and Salvesen, G. (1989) *J. Biol. Chem.* **264,** 15975–15981.
5. Schreitmüller, T., Hochstrasser, K., Reisinger, P. W. M., Wachter, E., and Gebhard, W. (1987) *Biol. Chem. Hoppe-Seyer* **368,** 963–970.
6. Enghild, J. J., Salvesen, G., Thøgersen, I. B., Valnickova, Z., Pizzo, S. V., and Hefta, S. A. (1993) *J. Biol. Chem.* **268,** 8711–8716.
7. Ødum, L. (1990) *Biol. Chem. Hoppe-Seyler* **371,** 1153–1158.
8. Pratt, C. W., Swaim, M. W., and Pizzo, S. V. (1989) *J. Leuko. Biol.* **45,** 1–9.
9. Jessen, T. E., Ødum, L., and Jhonsen, A. H. (1994) *Biol. Chem. Hoppe-Seyler* **375,** 521–526.
10. Steinbuch, M. (1976) *Method in Enzymology* **45,** 760–772.
11. Perry, J. K., Scott, G. K., and Tse, C. A. (1994) *Biochim. Biophys. Acta* **1221,** 145–152.
12. Kanayama, N., Maradny, E. E. L., Halim, A., She, L., Maehara, K., Kajiwara, Y., and Terao, T. (1995) *Am. J. Obstet. Gynecol.* **173,** 192–199.
13. Maehara, K., Kanayama, N., Halim, A., Maradny, E. E. L., Oda, T., Fujita, M., and Terao, T. (1995) *Biophys. Biochem. Res. Comm.* **206,** 927–934.
14. Proksch, G. J., and Routh, J. I. (1972) *J. Lab. Clin. Med.* **79,** 491–499.
15. Dupont, Y. (1980) *Eur. J. Biolchem.* **109,** 231–238.
16. Toyoda, H., Kobayashi, S., Sakamoto, S., Toida, T., and Imanari, T. (1993) *Bio. Pham. Bull.* **16,** 945–947.
17. Angyal, S. J., and Davis, K. P. (1971) *Chem. Commun.* 500–515.
18. Ødum, L., Hansen-Nord, G., and Byrjalsen, I. (1987) *Clin. Chim. Acta* **162,** 189–198.
19. Nishino, N., Aoki, K., Tokura, Y., Sakaguchi, S., Fujie, M., Sugawara, Y., Takada, Y., and Takada, A. (1989) *Haemostasis* **19,** 112–119.