Arch Pharm Res Vol 32, No 6, 945-953, 2009
DOI 10.1007/s12272-009-1618-y

Archives of Pharmacal Research
www.springer.com/12272

Inhibitory Effects of Oligopeptides from Hen Egg White on Both Human Platelet Aggregation and Blood Coagulation

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(Received February 25, 2009/Revised May 21, 2009/Accepted June 3, 2009)

Egg white proteins have many biological functions and substantial nutritional benefits when used as a food source; however, they also contain allergens such as ovalbumin, ovomucoid, and ovotransferrin. We prepared oligopeptides without allergens from hen egg whites *via* the use of several proteases, and assessed their effects on platelet aggregation and blood coagulation, known to both of which are known to be major risk factors in thrombogenesis. Egg white oligopeptides (EWOP) inhibited collagen-induced human platelet aggregation in a dose-dependent manner. Additionally, we attempted to determine whether cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), aggregation-inhibiting intracellular molecules, regulate EWOP-inhibited platelet aggregation. EWOP caused an increase in cAMP levels, but did not affect cGMP levels, which suggests that the anti-platelet activity of EWOP operates in a cAMP-dependent manner, rather than *via* a cGMP-dependent process, in collagen-induced platelet aggregation. In addition, EWOP induced a significantly prolonged prothrombin time (PT) as compared with the controls. These data show that EWOP inhibits the conversion of fibrinogen to fibrin in a plasmatic atmosphere on an extrinsic pathway. Accordingly, these findings suggest that EWOP may be an excellent candidate as a crucial inhibitor of platelet activation, and its anti-platelet effects appear to involve the inhibition of both platelet aggregation and blood coagulation within the cardiovascular system.

Key words: Egg white oligopeptides, Platelet aggregation, Blood coagulation, Cyclic adenosine monophosphate (cAMP), Prothrombin time (PT)

INTRODUCTION

Platelet aggregation is absolutely essential to the formation of hemostatic plugs in cases of injury to normal blood vessels. However, the interaction between platelets and collagen can also cause circulatory disorders, including thrombosis, atherosclerosis, and myocardial infarction (Smith and Willis, 1971; Yamagami et al., 1999). Hence, the inhibition of the platelet-collagen interaction may be a promising target for the development of novel anti-thrombotic drugs (Schwartz et al., 1990). Current anti-platelet agents include aspirin, which inhibits the cyclooxygenase-mediated formation of proaggregatory prostaglandin endoperoxides and thromboxane A_2 (TXA_2) (Yamagami et al., 1999); TXA_2 synthetase inhibitors and TXA_2 receptor antagonists (Yamagami et al., 1999; Golino et al., 1988; Chang et al., 1997); ticlopidine and clopidogrel, which selectively inhibit the ADP-induced platelet aggregation and ADP-mediated amplification of other platelet agonists (Herbert et al., 1993; Yao et al., 1992; Berger, 1999); and the recently developed glycoprotein IIb/IIIa antagonists, which inhibit the final common pathway of platelet aggregation (Cannon et al., 1998; Kereiakes et al., 1998; Marin et al., 2000). Among these anti-platelet agents, aspirin has been widely utilized to prevent unstable angina, myocardial infarction, transient ischemic attack,

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stroke, and atherosclerosis (Hennekens et al., 1989; King, 2000) either alone or in combination with other platelet inhibitors. Vasodilators, including molsidomine, nitroprusside, and nitroglycerine, inhibit platelet aggregation by elevating the concentration of cyclic guanosine monophosphate (cGMP) (Llorens et al., 2002; Paolucci et al., 2003; Mullershausen et al., 2003). Anti-platelet drugs, such as theophylline and verapamil, inhibit platelet aggregation *via* an induced increase in the concentration of cyclic adenosine monophosphate (cAMP) in agonist-induced platelet aggregation (Clapp et al., 2002; Tertyshnikova and Fein, 1998; Naka et al., 1996; Oh-ishi et al., 1996). Therefore, cGMP- or cAMP-elevating agents are important regulators in the prevention of cardiovascular disease.

Eggs are one of the world's most popular foods not only due to their availability, but also because of their excellent nutritional qualities. Furthermore, egg white proteins have long been recognized as a healthy component of a balanced diet. However, the proteins in egg whites include several allergens, most notably ovalbumin, ovomucoid, and ovotransferrin (Langeland, 1982; Hoffman, 1983; Gutierrez et al., 1997). Interestingly, in our study, we determined that the oligopeptides obtained from hen egg white (EWOP) did not harbor allergenic proteins (Fig. 1-3). We reported previously that hen egg yolk proteins without allergens such as lipovitellenin and livetin inhibited collagen-induced platelet aggregation, which may be involved in the induction of elevated levels of both cGMP and cAMP (Cho et al., 2003). Therefore, the principal objective of this study was to determine whether EWOP inhibits platelet aggregation *via* the regulation of cyclic nucleotides (i.e. cAMP, cGMP) on collagen-induced platelets.

MATERIALS AND METHODS

Materials

Collagen was purchased from Chrono-Log Corp. (Chrono-Log, Havertown, PA 19083, U.S.A.). Prothrombin time (PT) and activated partial thromboplastin time (APTT) reagents were obtained from BioMérieux sa. (69280 Marcy l'Etoile-France). Other reagents were obtained from the Sigma Chemical Co. (St. Louis, U.S.A.).

Preparation of oligopeptides from hen egg white

We used fresh mature hen eggs obtained from a local market. The hen eggs were broken and the egg whites were collected. A total of 100 g of egg whites were denatured by 10 min of heating at 70°C, then digested with papain, trypsin, and peptidase at 60°C for 7 h. The reaction was stopped by 10 min of heating at 90°C, and then spray-dried (EYELA, SD-1000, Japan). The samples were dissolved with distilled water prior to use.

Separation and identification of EWOP

The EWOP and an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (5% mercaptoethanol, 2% SDS, 10% glycerol, 0.0625 M Tris, 0.01% bromphenol blue, pH 6.8) were boiled for 5 min in order to denature the proteins completely. The proteins were separated *via* SDS-PAGE (15%, 1.0 mm gel) in accordance with the method of Laemmli (1970). The separated proteins were stained with Ez Stain Silver (ATTO, Tokyo, Japan) and dried. Molecular weight standards were employed as protein size markers (Bio-Rad Laboratories). The resultant peptides from the hen egg whites were analyzed *via* GFC-HPLC (SHIMADZU Corp., Japan) by using a YMC-Pack Diol-60 (YMC Co., Ltd., Kyoto, Japan) (500 × 8 mm i.d., 5 μm) column equilibrated with 0.1 M KH_2PO_4, pH 7.0, containing 0.2 M NaCl/acetonitrile (70/30, v/v) at 0.7 mL/min. Absorbance was measured at 215 nm.

Comparative allergen scanning of EWOP with egg white protein

The resultant peptides from hen egg whites were separated *via* GPC-HPLC (RID-10A, SHIMADZU Corp., Japan) using a Cadenza CD-C18 (Imtakt Corp., Japan) (75 × 4.6 mm). The column was equilibrated with acetonitrile/water/trifluoroacetic acid=80/20/0.1, v/v) at 1.0 mL/min. Absorbance was measured at 220 nm. The egg white protein was utilized as a positive control.

Amino acid determination of EWOP

Oligopeptides were hydrolyzed in the presence of 6 M HCl at 110°C for 24 h and neutralized. The hydrolyzed proteins were converted to PITC (phenylisothiocyanate) derivatives and then mixed with PITC-amino acid standards. The PITC-amino acid mixtures were subsequently analyzed by HPLC (RID-10A, SHIMADZU Corp., Japan) on a ULTRON VX-ODS (Shinwa Chemical Industries, LTD., Japan) (250 mm × 4.6 mm) column at 0.8 mL/min. The column was maintained at 40°C, and the absorbance was monitored at 254 nm.

Preparation of human washed platelets

Blood from healthy human volunteers was collected

and anti-coagulated with 10% ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). Platelet rich plasma (PRP) was obtained *via* 10 min of centrifugation at 340 × g. The PRP was recentrifuged for 10 min at 1,300 × g to obtain the platelet pellets, and the platelets were washed twice in a washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 1 mM EDTA, pH 6.5) (Rittenhouse-Simmons & Deykin, 1976). The washed human platelets were suspended in a suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 0.49 mM $MgCl_2$, 5.5 mM glucose, pH 6.9) to a final concentration of 5×10^8 platelets/mL. All the procedures described above were conducted at 25°C to avoid platelet aggregation during cooling.

Measurement of human platelet aggregation

The washed human platelets (10^8 platelets/mL) were preincubated in a siliconized cuvette with gentle stirring for 3 min at 37°C in the presence of 2 mM $CaCl_2$ with or without EWOP, then stimulated with 10 μg of collagen/mL for 5 min with gentle stirring. Ten μg/mL of collagen was used as the threshold concentration to ensure maximal aggregation (Cho et al., 2004). Platelet aggregation was monitored with an aggregometer (Chrono-Log, Co., U.S.A.) in terms of the increase (%) in light transmission. The suspension buffer (pH6.9) was employed as a reference (100% transmission).

Measurement of cGMP and cAMP

The washed human platelets (10^8 platelets/mL) were preincubated for 3 min at 37°C in the presence of 2 mM $CaCl_2$ with or without EWOP, and were then stimulated with 10 μg/mL of collagen. The platelet reactions were halted *via* the addition of 80% ice-cold ethanol. The concentrations of cAMP and cGMP were determined using the respective [^{125}I]cAMP and [^{3}H]cGMP radioimmunoassay kits. The results were expressed as fmol/10^8 platelets.

Measurement of PT and APTT

Citrated-platelet poor plasma (PPP) was prepared by centrifuging the blood remaining after the removal of PRP at 1,300 × g for 10 min. The PPP (0.1 mL) was preincubated in a two-channel coagulator (KG Behnk Elektronik GMBH & Co., Germany) cup (BioMérieux, 95-662) with gentle stirring for 1 min at 37°C. PT was determined as the time interval between the addition of PT reagent (0.1 mL) to the PPP (0.1 mL) and the formation of a fibrin clot. After preincubation for the measurement of activated partial thromboplastin time (APTT), 0.1 mL of APTT reagent was added to the PPP and was incubated for 3 min at 37°C. After incubation, 0.1 mL of 25 mM $CaCl_2$ was added rapidly to the PPP solution containing APTT reagent. APTT was determined as the time required for the formation of a fibrin clot.

Protein analysis method

The protein contents were measured *via* the method of Lowry et al. (1951).

Statistical analysis

The experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations. Data were assessed *via* analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. A p value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the EWOP

As shown in Fig. 1, Hen egg white proteins are

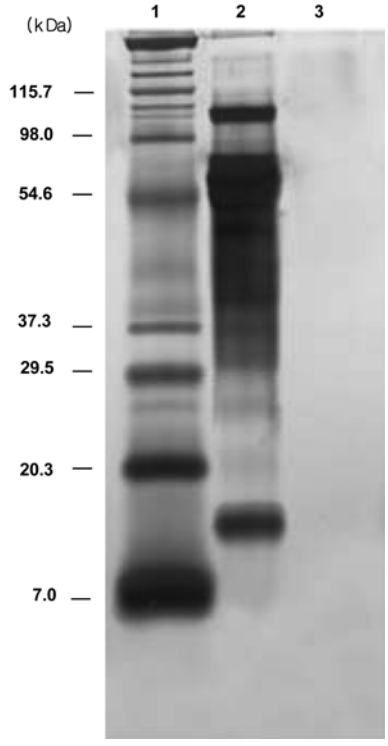

Fig. 1. SDS-PAGE of EWOP. The proteins of EWOP were separated *via* SDS-PAGE (15%, 1.0 mm gel) according to the method of Laemmli (Laemmli, UK, 1970). Separated proteins were stained with Ez Stain Silver (ATTO, Tokyo, Japan) and dried. Lane 1, standard marker; Lane 2, Egg white protein; Lane 3, EWOP.

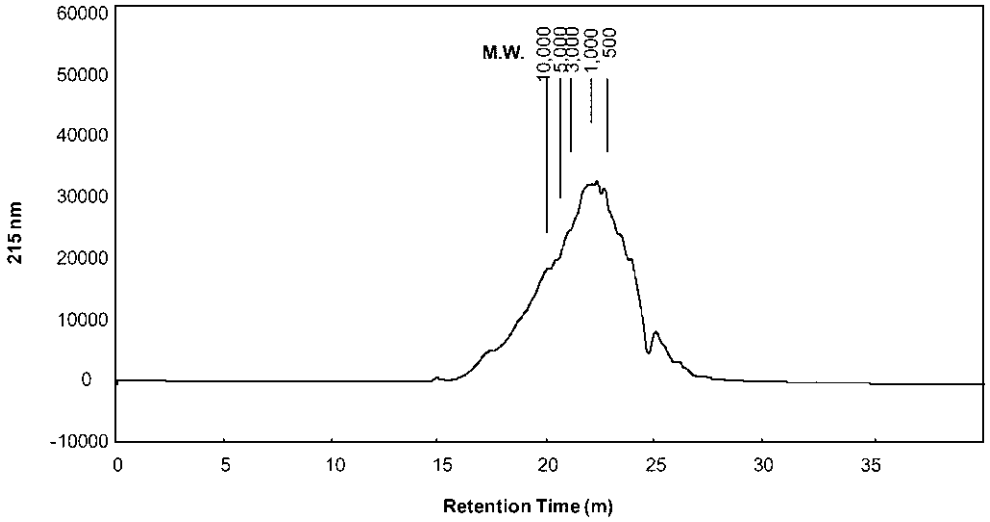

Fig. 2. Analysis of molecular weight distributions of EWOP. The resultant peptides from EWOP were analyzed *via* GFC-HPLC (SHIMADZU Corp., Japan) by using a YMC-Pack Diol-60 (YMC Co., Ltd., Kyoto, Japan) (500 × 8 mm) column equilibrated with 0.1 M KH_2PO_4, pH 7.0 containing 0.2 M NaCl/acetonitrile (70/30, v/v) at 0.7 mL/min. Absorbance was measured at 215 nm.

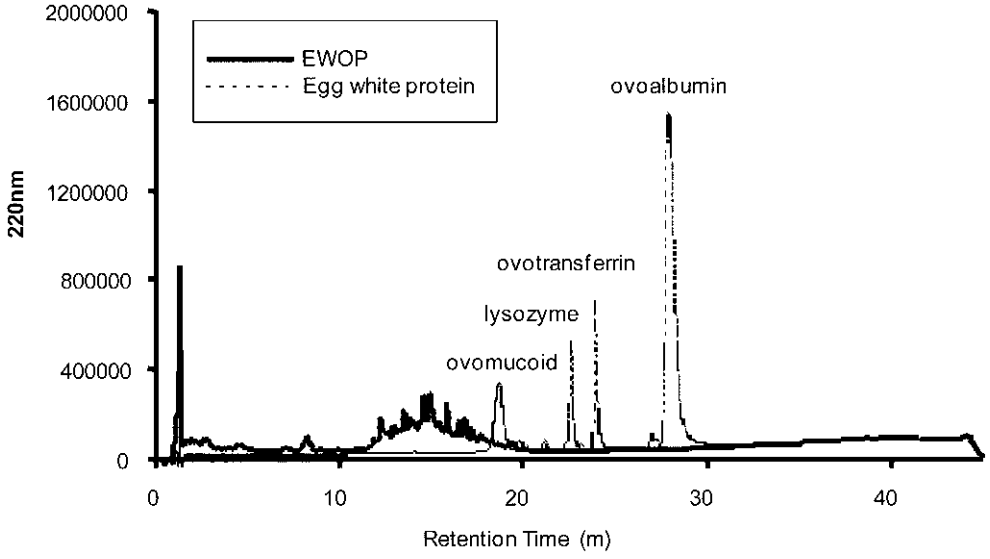

Fig. 3. The comparative allergens scanning between EWOP and egg white protein. The resultant peptides from hen egg white were separated by GPC-HPLC (RID-10A, SHIMADZU Corp., Japan) using a Cadenza CD-C18 (Imtakt Corp., Japan) (75 × 4.6 mm). The column equilibrated with acetonitrile/water/trifluoroacetic acid = 80/20/0.1, v/v) at 1.0 mL/min. Absorbance was measured at 220 nm. Egg white protein was used as a positive control.

digested completely by enzymes, as compared with egg white protein, and EWOP consists primarily of oligopeptides (protein conc., 100%), the molecular distributions of which are provided in Fig. 2. Furthermore, the digested egg white protein did not contain allergens such as ovomucoid, lysozyme, ovotransferrin, and ovoalbumin (Fig. 3). EWOP also contains all 8 essential amino acids in a favorable ratio for health (Table I).

Effects of EWOP on human platelet aggregation

When the washed human platelets (10^8 platelets/mL) were stimulated by collagen (10 μg/mL) in the presence of 2 mM $CaCl_2$, the platelet aggregation was 75% (Fig. 4). However, the platelet aggregation induced by collagen (10 μg/mL) in the presence of various concentrations (1, 3, 5, and 10 mg/mL) of EWOP was inhibited (Fig. 4) in a dose-dependent manner.

Table I. Amino acid components of EWOP

Amino acid	Amino acid (weight %)	
	Egg white protein	EWOP
Aspartic acid	10.1	11.3
Glutamic acid	12.9	15.0
Serine	6.2	7.8
Glycine	3.5	4.0
Histidine	2.4	2.2
Arginine	5.6	5.8
Threonine	4.3	4.7
Alanine	6.0	6.2
Proline	3.5	3.6
Tyrosine	3.9	3.7
Valine	7.0	6.2
Methionine	3.8	3.5
Isoleucine	5.4	4.5
Leucine	8.6	7.6
Phenylalanine	5.7	4.6
Lysine	6.6	7.0
Tryptophan	1.5	0.3
Cysteine	3.0	2.0
Total	100	100

Effects on the level of both cAMP and cGMP

We attempted to determine whether the inhibitory effects of EWOP on collagen-induced platelet aggregation are mediated by cGMP or cAMP, both of which are known to inhibit agonist-induced Ca^{2+} increases (Geiger et al., 1992). The level of cAMP in unstimulated platelets was 117.6 ± 5.2 fmol/10^8 platelets (Fig. 5A), but collagen (10 μg/mL) reduced this level to 79.3 ± 0.2 fmol/10^8 platelets. However, EWOP (5 and 10 mg/mL) increased cAMP levels in collagen-induced platelet aggregation in a dose-dependent manner (Fig. 5A). On the other hand, the cGMP level in unstimulated platelets was 0.65 ± 0.02 fmol/10^8 platelets (Fig. 5B), but collagen (10 μg/mL) reduced this level to 0.28 ± 0.01 fmol/10^8 platelets. However, the addition of EWOP (5 and 10 mg/mL) did not alter the cGMP level as compared with that (0.28 ± 0.01 fmol/10^8 platelets) observed in collagen-induced platelet aggregation.

Effects of EWOP on blood coagulation

Platelet aggregation depends on the coagulation factors surrounding the platelets (Sinha et al., 1983). PT was defined as the time interval between the addition of thromboplastin to plasma and the formation of the fibrin clot. PT is dependent on the coagulation factors II, V, VII and X. As shown in Fig. 6, after pretreatment with 10 mg/mL of EWOP, the PT was 21.5 ± 0.9 sec, 9 sec longer than that (12.7 ± 0.2 sec) of the control. It has been suggested that EWOP might prolong PT in order to inhibit fibrin clot

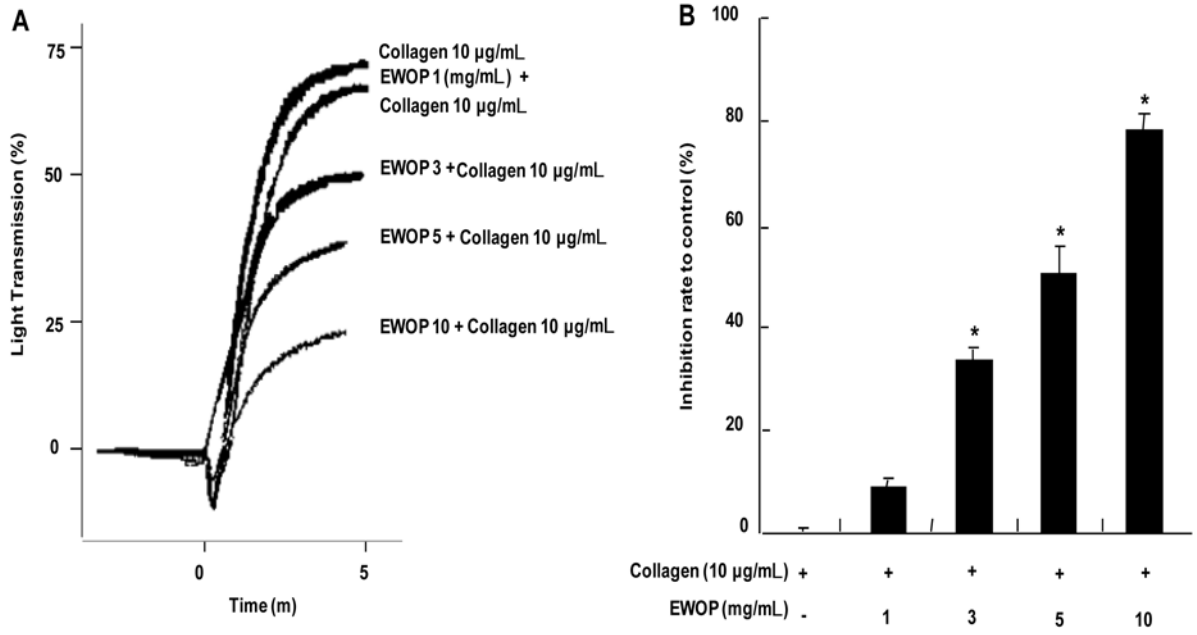

Fig. 4. Effects of EWOP pretreatment on collagen-induced platelet aggregation. Washed human platelets (10^8/mL) were preincubated with or without EWOP in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, and then stimulated with collagen for 5 min. (A) Effect of EWOP on collagen-induced platelet aggregation. (B) Inhibitory effects of EWOP on collagen-induced platelet aggregation. Platelet aggregation was recorded as an increase in light transmission. Inhibition by EWOP was recorded as the percentage of the collagen-induced aggregation rate. Data are expressed as the mean ± S.E.M. (n = 4). $^*p<0.05$ compared with that of only collagen-induced aggregation.

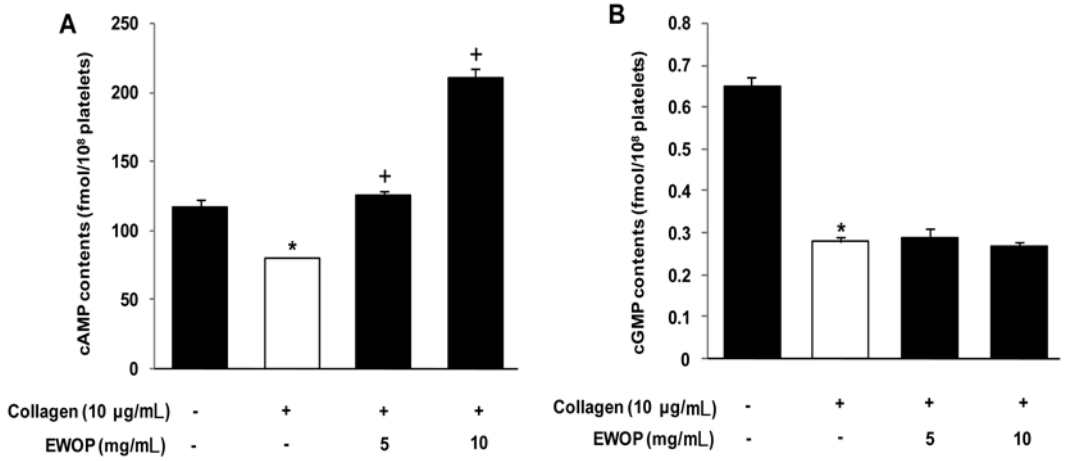

Fig. 5. Effects of EWOP on cAMP and cGMP production. Washed human platelets (10^8/mL) were preincubated for 3 min with or without EWOP in the presence of 2 mM $CaCl_2$, and then stimulated with collagen (10 μg/mL) for 5 min at 37°C. The reactions were terminated *via* the addition of 80% ice-cold ethanol. cAMP and cGMP contents were measured by using [^{3}H-cAMP] or [^{3}H-cGMP] radioimmunoassay kits. (A) Effects of EWOP on cAMP production in collagen-stimulated platelets. (B) Effects of EWOP on cGMP production in collagen-stimulated platelets. Data are expressed as means ± S.E.M. (n = 4). $^*p<0.05$ compared with that of intact platelets. $^+p<0.001$ compared with that of collagen only-induced platelets.

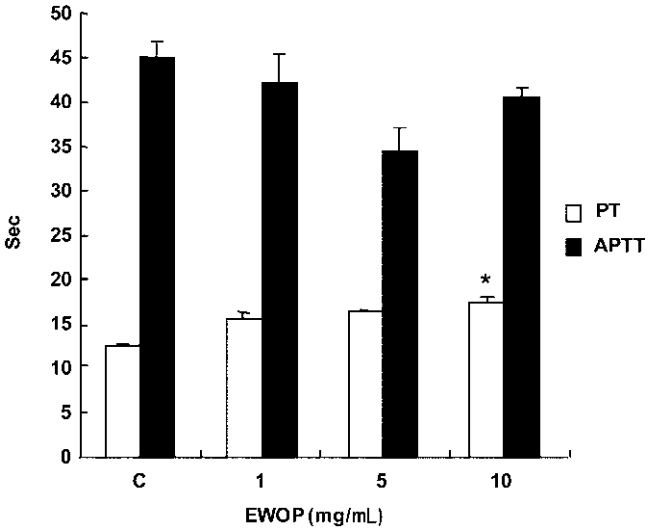

Fig. 6. Effects of EWOP on prothrombin time (PT) and activated partial thromboplastin time (APTT). PT and APTT were measured as described in "Materials and Methods". The data are expressed as the mean ± S.E.M. (n = 4). $^*p<0.05$ compared with that of control. PT, prothrombin time; APTT, activated partial thromboplastin time; C, control (plasma only).

formation in the presence of thromboplastin with the coagulation factors II, V, VII, and X. APTT is defined as the blood coagulation time in the intrinsic pathway, as differentiated from PT, which involves the extrinsic pathway. Both intrinsic and extrinsic thromboplastins convert prothrombin to thrombin to induce fibrin clot formation. APTT is dependent on the coagulation factors VIII and IX. As shown in Fig. 6, APTT, significantly, was not prolonged as compared with that of control (45.0 ± 2.0 sec). Thus, we demonstrate that EWOP might exert an anti-coagulative effect by delaying fibrin clot formation in the presence of coagulation factors in the extrinsic pathway.

DISCUSSION

The hemostatic system is composed of platelet aggregation, coagulation, and fibrinolysis. Platelet activation results from exposure to collagen from vascular endothelium damaged by hypertension, diabetic mellitus, etc. Because collagen is the first major platelet aggregatory factor (the primary step in the hemostatic system) operating *via* damaged endothelial cells, and its inhibition is a promising target for the development of antithrombotic drug (Schwartz et al., 1990), we employed collagen as an agonist to assess the effects of EWOP on anti-platelet function. EWOP inhibited collagen-induced platelet aggregation (Fig. 4A, B). In particular, a concentration of 10 mg/mL (1%) of EWOP exerted a potent inhibitory effect on collagen-induced platelet aggregation (Fig. 4). Therefore, in another experiment, we have assessed the effects of a dietary supplement containing EWOP at a concentration of 1% of the total diet on rat platelet aggregation. As a consequence, when platelets from rats administered EWOP (1%) for 30 d were stimulated by collagen (10 μg/mL), the degree of aggregation (55%) was low as compared with that (69%) of the controls, which indicates that dietary EWOP (1%) inhibited collagen-induced rat platelet aggregation by up to 20%

(Data not shown). Therefore, it is apparent that EWOP exerts an antiplatelet effect both *in vitro* (Fig. 4) and *in vivo*.

Of two aggregation-inhibiting intracellular molecules, cAMP and cGMP, EWOP increased the level of cAMP in collagen-induced platelet aggregation (Fig. 5A). It has been demonstrated that an increase in the level of cAMP or cGMP is involved in most of the inhibitory pathways of Ca^{2+} mobilization required for platelet aggregation (Cavallini et al., 1996; Nakamura et al., 1995). An increase in cAMP in the platelets also lowers the intracellular Ca^{2+} concentration by stimulating both Ca^{2+} extrusion from the cells and Ca^{2+} uptake into the dense tubular system (Leoncini and Signorello, 1999; Johnson et al., 1985) *via* the activation of cAMP-dependent protein kinase (Heemskerk et al., 1994). Therefore, we suggest that EWOP may exert an inhibitory effect on platelet aggregation by increasing the level of cAMP to suppress Ca^{2+}-elevation in a bidirectional system (Berridge, 1975; Kaser-Glanzman et al., 1979; Nakamura et al., 1995). However, the mechanism of action by which EWOP increases cAMP has yet to be clearly elucidated. cAMP is generated *via* the activation of adenylate cyclase or the inhibition of phosphodiesterase (PDE) in a variety of cells (Stones and Marletta, 1996; Maurice and Haslam, 1990). Adenylate cyclase generates cAMP by using adenosine triphosphate as a substrate. There are at least four different isoforms--including cGMP-stimulated PDE (PDE2), cGMP-inhibited PDE (PDE3), cAMP-specific PDE (PDE4), and cGMP-specific PDE (PDE5) (Maurice and Haslam, 1990; Beavo, 1995). PDE2 hydrolyzes both cAMP and cGMP. PDE3 is thought to be responsible for the hydrolysis of cAMP rather than that of cGMP (Schwarz et al., 2001). PDE4 hydrolyzes cAMP to AMP, and PDE5 is involved in the hydrolysis of cGMP to GMP. When cAMP or cGMP is hydrolyzed to AMP or GMP by PDEs, their normal actions are abolished. As EWOP was shown to increase cAMP levels and does not alter cGMP levels in collagen-induced platelet aggregation (Fig. 5-A, B), it could be inferred that EWOP may be involved, at least, in the activation of adenylate cyclase and the inhibition of PDE4, thus increasing the level of cAMP in collagen-induced platelet aggregation. Because EWOP was not shown to be involved in the elevation of cGMP levels (Fig. 5B), the inhibitory effect of EWOP on collagen-induced platelet aggregation does not appear to be associated with PDE3 inhibition. The results of this study show that EWOP inhibits collagen-induced human platelet aggregation *via* the activation of a cAMP/PKA-dependent process. However, because the precise action mode of EWOP in cAMP elevation remains to be clearly elucidated, further studies into this issue will be necessary. Additionally, we noted that PT, which involves the extrinsic pathway, was prolonged to a profound degree by EWOP in a dose-dependent manner (Fig. 6). However, EWOP does not prolong APTT significantly. APTT is defined as the blood coagulation time in the intrinsic pathway. APTT is dependent on coagulation factor VIII and IX. As shown in Fig. 6, APTT, was not significantly prolonged as compared with that of the controls. These results show that EWOP may exert an anti-coagulative effect by delaying the formation of fibrin clots in the presence of coagulation factors in the extrinsic and common pathway. In the common pathway, thromboplastin accelerates the conversion of prothrombin to thrombin in the presence of the blood coagulation factors Xa and Va, and then thrombin initiates fibrin formation, and induces the intra-platelet synthesis of TXA_2 (Cavallini et al., 1996), a potent platelet aggregating factor, thereby stimulating platelet aggregation. It can be inferred that EWOP delays PT by inhibiting the conversion of prothrombin to thrombin and the formation of TXA_2, which is generated from the arachidonic acid released when the PIP_2 membrane is broken down by Ca^{2+}-dependent PLC activated with collagen, thrombin, ADP, and others (Maurice and Haslam, 1990; Doni et al., 1993). Accordingly, these results indicate that EWOP may modulate both platelet aggregation and blood coagulation within a common region of thrombus generation.

On the other hand, it has been shown that the final pathway (outside-in signal pathway) of platelet aggregation occurs when fibrinogen binds to activated glycoprotein IIb/IIIa ($\alpha IIb/\beta_3$) receptors on the surface of platelets and thereby cross-links platelets (Cutton et al., 2001). $\alpha IIb/\beta_3$ activation is known to be stimulated by endogenous platelet activators including TXA_2, serotonin, and ADP, which bind to their respective receptors to stimulate the binding of fibrinogen to the $\alpha IIb/\beta_3$ receptor (Cutton et al., 2001). Because EWOP was shown to inhibit collagen-induced platelet aggregation (Fig. 4), increase the level of the intracellular Ca^{2+} antagonist cAMP (Fig. 5A), and prolonged PT (Fig. 6), which indicates an inhibition of thrombin formation, one could speculate that EWOP might inhibit the product of TXA_2 and the release of serotonin and ADP. Serotonin and ADP are released out of dense bodies in platelets activated by collagen, thrombin, and ADP (Mustard and Packham, 1970). Therefore, as another action mode of EWOP, we suggest that EWOP might be associated with the inhibition of $\alpha IIb/\beta_3$. This is supported by the finding that EWOP contains amino acids (Table I) including arginine (Arg), glycine

(Gly), aspartic acid (Asp), and serine (Ser), which form αIIb/β_3-antagonistic peptides such as Arg-Gly-Asp or Arg-Gly-Asp-Ser (Cutton et al., 2001).

The physiological functions of hen egg white have been well characterized, because hen egg proteins perform crucial functions as growth promotion factors, which are necessary for the development of the chicken. However, our current knowledge of the biological functions of egg white peptides remains quite limited. Although the main allergens in egg white reported thus far are ovalbumin (MW, 45 kDa), ovomucoid (MW, 28 kDa), and ovotransferrin (MW, 76 kDa) (Langeland, 1982; Hoffman, 1983; Gutierrez et al.,1997), as shown in Fig. 2 and Fig. 3, oligopeptides from hen egg whites did not harbor allergens as compared with egg white proteins. Furthermore, EWOP as an egg white hydrolysate is highly nutritious and contains all 18 amino acids in a ratio favorable for health, because the well-balanced amino acid profile of egg whites remains as it is (Table I), and EWOP is readily absorbed as a physiologically active substance in the human body, owing to its small molecular size. In conclusion, the results of this study suggested that EWOP, as compared with hen egg whites, does not harbor allergenic proteins such as ovalbumin, ovomucoid, and ovotransferrin (Fig. 3), and it might be developed into a potent modulator that operates *via* the inhibition of both platelet aggregation and blood coagulation.

ACKNOWLEDGEMENTS

This study was supported (in part) by a grant from the Ministry of Commerce, Industry and Energy (MOCIE) and Korean Institute of Industrial Technology Evaluation & Planning (ITEP) through the Biohealth Products Research Center (BPRC) of Inje University, Korea.

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