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Antithrombotic Soy Peptides

Kyung-Ae Lee and Seung-Ho Kim*

Korea Food Research Institute, Sungnam 463-746, Korea. E-mail: sehok@kfri.re.kr

INTRODUCTION

Thrombosis is a prime factor in the pathogenesis of a variety of cardiovascular disorders such as heart attacks, strokes and other vascular diseases. A thrombus is defined as a solid mass or plug formed during life within the heart or vessels from constituents of the blood. Thrombosis can be related to atherosclerosis in at least two possible ways. First, the "encrustation" or "thrombogenic" theory suggests that thrombosis is involved in the pathogenesis of the atherosclerotic lesion. Second, whatever the origin of atherosclerosis may be, thrombosis is clearly a most important complication of the established lesion and is responsible for a majority of the lethal and disabling features of the disease.

Therapeutic approaches intended to influence thrombosis have included the use of anticoagulents, antiplatelet agents, or thrombolytic agents. Warfarin, an anticoagulant, exert its effects by inhibiting the synthesis of coagulation factors II, VII, IX and X in the liver. Heparin, another anticoagulant, unlike warfarin, causes no deficiencies of coagulation factors, but instead interferes with the coagulation process at several points in the coagulation pathways.

Platelet aggregation is blocked by various agents. Asprin, an antiplatelet agent, interferes with the production of certain intermediate metabolites in platelets, and this intracelluar change may be associated with changes in platelet function. The effect of aspirin on platelets occurs as a consequence of acetylation of the platelet membrane. The duration of effects of aspirin lasts for the remainder of the exposed platelet, whose total life span is eight to ten days.

Peptides as Antiplatelet Agents

Fibrinogen interaction with platelets is essential for platelet aggregation and fibrinogen binds to a specific receptor on the platelet surface: the glycoprotein IIb-IIIa complex (GPIIb-IIIa) (Bennet, 1985; Peerschke, 1985). Unstimulated platelets do not bind fibrinogen. Two binding sites have been identified on the fibrinogen molecule: a decapeptide in the carboxy-terminal region of the γ-chain (LGGAKQAGDV; residues 402-411) and a tetrapeptide in the carboxy-terminal region of the α-chain (RGDS; residues 572-575). Both peptides inhibit aggregation and fibrinogen binding to ADP-activated platelets (Plow et al., 1987). RGDS becomes more chemically cross-linked to residues 109-171 of GPIIIa (D'Souza et al., 1988) while the γ-chain cross-linking site (positions 400-411) must reside within residues 294-314 of GPIIb (D' Souza et al., 1990).

RGDS inhibited platelet aggregation stimulated by ADP, collagen, and γ-thrombin without inhibiting platelet size change or secretion of serotonin and also inhibited fibrinogen binding to ADP-stimulated platelets as a partial competitive inhibitor (Gartner et al., 1985)

KRDS in human lactoferrin (residues 39-42) inhibited ADP-induced platelet aggregation and fibrinogen binding (Mazoyer et al., 1990). In contrast to RGDS, KRDS inhibited thrombon-induced serotonin release and did not inhibit the binding of monoclonal antibody PAC-1 to activated platelets. Both KRDS and RGDS inhibited 4β-phorbol-12-myristate-13-acetate (PMA)-induced aggregation and fibrinogen binding, while proteins were normally phosphorylated. Thus, KRDS is (a) an inhibitor of serotonin release by a mechanism independent of protein

phosphorylation and (b) an inhibitor of fibrinogen binding and, hence, aggregation by a mechanism that may not necessarily involve its direct binding to the glycoprotein IIb-IIIa-complex.

A undecapeptide (MAIPPKKNQDK), glycomacropeptide (GMP) (Jolls et al., 1986) and a pentapeptide (KNQDK) (Jolls & Caen, 1991), corresponding respectively to residues 106-116 and 112-116 of cow κ-casein inhibited platelet aggregation and fibrinogen binding to ADP-activated platelets. Whole κ-casein inhibited thrombin-induced platelet aggregation and GMP inhibited both thrombin and ADP-induced platelet aggregation (Drouet et al., 1990a). There were species differences in the ability of RGDS (Harfenist et al., 1988), RGDS and KRDS (Drouet et al., 1990b) to inhibit ADP-induced platelet aggregation.

In vivo (in an experimental model of arteriolar thrombosis), intravenous injection of RGDS and KRDS to rat and guinea-pig synergistically (Drouet et al., 1990b), and GMP to rat (Drouet et al., 1990a) inhibited thrombogenesis. In other animal models of thrombosis, an antithrombotic effect of RGDS or related peptides was reported (Cook et al., 1988; Cadrey et al., 1989; Shebuski et al., 1989).

Soy peptides with antiplatelet or antithrombotic activities have been studied in our laboratory: 1) most peptide fractions from a soybean paste had *in vitro* antiplatelet activity, 2) most peptides from a soy protein hydrolysate had *in vitro* antiplatelet activity and two of them have been identified, 3) a soy protein hydrolysate orally administered had antithrombotic activity. These are described in some more detail in the following.

Peptide Fractions from a Soybean Paste (Doenjang)

This was published (Shon et al., 1996). *In vitro* inhibitory activity of ultrafiltered water extract of a soybean paste and its peptide fractions on ADP-induced aggregation of washed rat platelets was investigated. Ultrafiltered soybean paste water extract (mol. wt. cut off of 3000 Dalton) exhibited an inhibition 90% at a concentration of 96 μg/mL, using a turbidometric aggregometer. The soybean paste extract was fractionated into nineteen fractions by Dowex 50W X-2 ion exchange chromatography and the inhibitory activities of these fractions were assessed by a microplate method. All had inhibitory activities and IC_{50} (median inhibitory concentration) values were between 10 and 1000 μg/mL with most fractions having greater activity than arginine-glycine-aspartic acid-serine (RGDS), used in the study as a positive control (IC_{50}: 205 μg/mL). Fractions 16～18 exhibited higher inhibition activity than the soybean paste extract per se (IC_{50} 10～20 vs. 30 μg/mL). The fraction 16, which had the highest inhibitory activity (IC_{50}: 10 μg/mL) was purified successively by Sep-pak C_{18} cartridge and C_{18} reverse-phase HPLC to an apparent homogenecity but could not be sequenced, which suggests necessity of more purification. Analysis of amino acid composition of the partially purified fraction showed that histidine, arginine and alanine were the major residues present in the peptide part of the fraction.

SSGE and DEE from a Soy Protein Hydrolysate

This will be published (Lee et al., 2005). A soy protein hydrolysate was found to inhibit rat platelet aggregation induced by ADP, an aggregating agent. To find out its principal antiplatelet peptide(s), the soy protein hydrolysate was separated successively by gel filtration chromatography, revere-phase HPLC, and cation exchange HPLC. During the course of separation, we observed that most fractions had antiplatelet effects, which suggests that most peptides have some degree of antiplatelet effect. Following the inhibitory fractions, we purified and identified two new peptides, SSGE and DEE, by LC-electrospray ionization MS and peptide sequencing. Both peptides were highly hydrophilic. The concentrations to obtain 50% inhibition (IC_{50}) of the aggregation intensity were approximately 458 μM and 485 μM, respectively, for SSGE and DEE.

Production of an Antithrombotic Soy Protein Hydrolysate

This was submitted (Lee et al.). Since we found that most peptides had some degree of antiplatelet effect (Lee & Kim, 2005), we tried to develop antithrombotic protein hydrolysates without extensive separation steps. After screening food protein hydrolysates produced from soy protein isolate, gelatin and egg white using various proteases, we found that a soy protein isolate hydrolysed with pancreatin was superior in inhibiting rat platelet aggregation induced by ADP, an aggregating agent.

Hydrolysis of soy protein isolate with pancreatin was optimized to produce a hydrolysate with maximum *in vitro* inhibition of ADP-induced rat platelet aggregation. A hydrolysate with the maximum inhibition was ultrafiltered and both retentate and filtrate showed lower inhibition than before the fractionation. Antithrombotic activity of the orally administered hydrolysate was evaluated by *in vivo* mouse thrombotic model. The synergistic effects shown in the ultrafiltration experiment supports a hypothesis that peptides may act on different sites on the platelet. The hydrolysate had IC_{50} (median inhibitory concentration) of 0.3 mg/mL in the *in vitro* assay and produced a significant inhibition of thrombotic death at 100～500 mg/kg body weight. To our knowledge, this is the first report that showed peptides orally administered had *in vivo* antithrombotic effects. Aspirin, a representative antiplatelet drug used as a control, had about an order higher activity both in *in vitro* and *in vivo* assays.

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