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SSGE and DEE, new peptides isolated from a soy protein hydrolysate that inhibit platelet aggregation

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Abstract

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, reverse-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 480 and 460 μM, respectively, for SSGE and DEE.

Keywords: Peptide; Platelet aggregation

1. Introduction

It is generally accepted that platelet adhesion and aggregation plays an important role in the pathogenesis in thrombosis, particularly arteriothrombosis (Gorden, 1981; Knapp, Reilly, Alessandrini, & Fitzgerald, 1986). Peptides have been shown to inhibit platelet aggregation and thrombosis, as reviewed by Rutherfurd and Gill (2000) and Fiat et al. (1993). C-terminal sequence of human fibrinogen γ-chain (HHLGGAKQAGDV) and tetrapeptide sequences [RGD(S or F)], residues 572–575 or 95–98 of the fibrinogen α-chain (Andriex et al., 1989), a undecapeptide (MAIPPKKNQDK), glycomacropeptide (GMP) (Jollès et al., 1986) and a pentapeptide (KNQDK) (Jollès & Caen, 1991), corresponding, respectively, to residues 106–116 and 112–116 of cow κ-casein, and KRDS, a tetrapeptide with a sequence corresponding to residues 39–42 of human lactotransferrin (Mazoyer et al., 1990) inhibited platelet aggregation and fibrinogen binding to ADP-activated platelets. Peptide fractions from soybean paste inhibited ADP-induced platelet aggregation (Shon et al., 1996). Whole κ-casein inhibited thrombin-induced platelet aggregation and GMP inhibited both thrombin and ADP-induced platelet aggregation (Drouet et al., 1990). In vivo (in an experimental model of arteriolar thrombosis), intravenous injection of RGDS and KRDS (Mazoyer et al., 1990), and GMP (Drouet et al., 1990) inhibited thrombogenesis.

In the course of development of biologically active soy protein hydrolysates, 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 successively separated by gel filtration chromatography, reverse-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 have purified and identified two new peptides, DEE and SSGE, and determined their inhibitory effects on platelet aggregation in rat in vitro.

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2. Materials and methods

2.1. Materials

Soy protein hydrolysate manufactured by successive enzymatic hydrolysis of soy protein isolate by an endopeptidase, Promod 278 (Gist-brocades, Netherlands) and an exopeptidase, Promod 279 (Gist-brocades, Netherlands) was obtained from Nong Shim Co. Ltd. (Korea). HPLC grade water obtained with a Milli-*Q* water purification system (Millipore Corp., Bedford, MA) was used throughout the study. Acetonitrile was of HPLC grade (Honeywell/Burdick & Jackson, Muskegon, MI). All material for peptide sequencing and peptide synthesis was purchased from Perkin–Elmer (Applied Biosystems, Foster, CA). ADP was from Chrono-Log (Havertown, PA). RGDS (Arg–Gly–Asp–Ser) and all the other chemicals were from Sigma (St. Louis, MO).

2.2. Purification of peptides

2.2.1. Gel filtration chromatography

The soy protein hydrolysate dissolved in 0.1 N acetic acid at 30 g/100 ml (20 ml) was applied on a XK (Pharmacia Biotech) column (5.0×90 cm) filled with Sephadex G-25. The elution was realized at a flow rate of 10 ml/min at room temperature with 0.1 N acetic acid as the mobile phase. Eluted compounds were detected at 254 nm, 20 ml fractions being collected. Ten μl of some fractions was tested for inhibition of platelet aggregation. Active fractions were collected, freeze-dried, and stored at −20 °C until use.

2.2.2. Semi-preparative reverse-phase HPLC

The above was resolubilized in water (1 mg/ml) and filtered on an Acrodisc 0.45 μm PTFE filter (Gelman Lab., Ann Arbor, MI). Five hundred μl of the above was applied on a semi-preparative reverse-phase column (10×250 mm; C_{18}; Vydac 218TP510; The Separations Group, Hesteria, CA), using the HPLC apparatus with a UV monitor (UV-975) (Jasco, Tokyo, Japan). Isocratic elution at 5% acetonitrile in water containing 0.1% trifluroacetic acid was carried out at the flow rate of 1.5 ml/min. Fractions were freeze-dried, solubilized in water (100 μl) and 10 μl thereof was assayed for inhibition of platelet aggregation. An active fraction was collected, freeze-dried, and stored at −20 °C until use.

2.2.3. Cation exchange HPLC

The above from one injection of the reverse-phase HPLC was resolubilized in 100 μl of 5 mM potassium phosphate buffer (pH 3.0). The above was applied to a strong cation exchange sulphonic acid column (Vydac 400VHP575, 5 μm, 7.5×50 mm, The Separations Group, Hesperia, CA), using the same HPLC apparatus as in the reverse-phase HPLC. The flow rate was 1 ml/min. Elution was: 0–5 min, 5 mM potassium phosphate buffer (pH 3.0); 5–10 min, linear gradient from 0 to 0.5 M NaCl with the buffer. The fractions were freeze-dried, solubilized in water (100 μl) and 10 μl thereof was assayed for inhibition of platelet aggregation. Two active fractions were obtained, and each of them was desalted by the reverse-phase HPLC using a linear gradient elution (0–20 min, 0.1% trifluroacetic acid in water–20% acetonitrile in water containing 0.1% trifluroacetic acid), freeze-dried, and stored at −20 °C until use.

2.3. Identification of peptides by LC–MS and peptide sequencing

Molecular weights of the isolated peptides were determined by LC–electrospray ionization mass spectrometry (*MassLynx 2.1*, *Micromass Platform II*, Manchester, UK). Peptide sequencing was carried out with a Procise™ Protein Sequencing System (Perkin–Elmer, Applied Biosystems, Foster, CA).

2.4. Sequence comparisons

Our sequence data were compared with a protein database, NREF, using the Internet (Web page: www-nbrf.georgetown.edu/pir).

2.5. Synthetic peptide preparation

The identified peptides were prepared using a 433A peptide synthesizer (Perkin–Elmer, Applied Biosystems, Foster, CA). After final deprotection and purification by reverse-phase HPLC using a linear gradient elution (0–20 min, 0.1% trifluroacetic acid in water–20% acetonitrile in water containing 0.1% trifluroacetic acid), the free peptides appeared to be homogeneous on LC–electrospray ionization mass spectrometry and were in excellent agreement with their expected molecular weights (data not shown).

2.6. Platelet aggregation

Rat blood was drawn into a syringe in 1/6 volume of ACD-C (130 mM citric acid, 124 mM trisodium citrate, 110 mM glucose) and platelet-rich plasma (PRP) was obtained by centrifugation at 120*g*, 15 min at room temperature. Platelets were washed from plasmatic contaminants (Lee, Nurden, Thomaidis, & Caen, 1982), and resuspended in a buffer (11.9 mM $NaHCO_3$, 0.33 mM NaH_2PO_4, 16.3 mM NaCl, 2.8 mM KCl, 1.1 mM $MgCl_2$, 11.1 mM glucose, pH 7.4) to adjust the platelet counts to 5×10^8/ml with a hemacytometer (Superior, Germany).

Platelet aggregation was performed in a Chrono-Log aggregometer (Havertown, PA). A 470 μl volume of

PRP was placed in the cuvette of the aggregometer, and this was incubated at 37 °C for 5 min. To this, 10 μl of $CaCl_2$ solution (final concentration of 1.0 mM) and 10 μl of test solution were added successively with 2 min incubation after each addition. Platelet aggregation was induced by the addition of 10 μl of ADP solution (final concentration of 10 μM). Results were given in terms of change in light transmission 5 min after the addition of the inducer and expressed as percent inhibition of maximal intensity of control, and are expressed as the mean of two measurements.

3. Results and discussion

The soy protein hydrolysate had IC_{50} (median inhibitory concentration) of 2 mg/ml. The elution profile obtained on gel filtration of soy protein hydrolysate on Sephadex G-25 is given in Fig. 1, in which inhibitions of platelet aggregation of some fractions are also shown. 0.1 N acetic acid, the eluting solvent, showed a low inhibition of about 12% and the data were presented as is without adjustment of the solvent effects. All the fractions tested showed some inhibition, which suggests that most peptides in general have some inhibitory activity towards platelet aggregation. The inhibitory fractions (fractions 127–130), indicated by ↔ in Fig. 1, were combined and submitted to semipreparative reverse-phase HPLC.

Linear gradient elution (0–60 min, 0.1% trifluroacetic acid in water–60% acetonitrile in water containing 0.1% trifluroacetic acid) was initially used in the separation of the above by reverse-phase HPLC. Early eluting fractions, around 5% acetonitrile, showed most of the inhibitory activities and the resolution of peaks eluting around here was poor (data not shown). Therefore, isocratic elution at 5% acetonitrile was used for the separation.

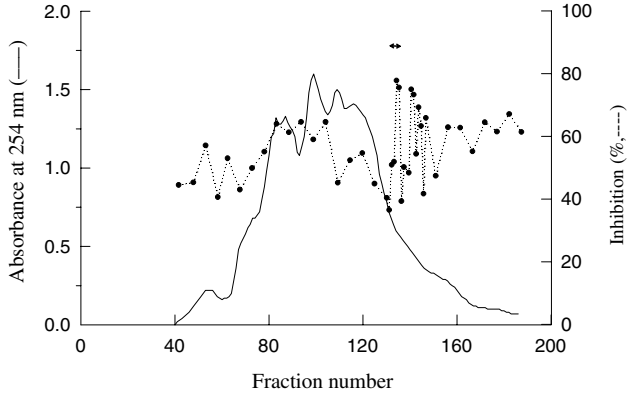

Fig. 1. Sephadex G-25 gel filtration chromatogram of a soy protein hydrolysate with inhibition of platelet aggregation.

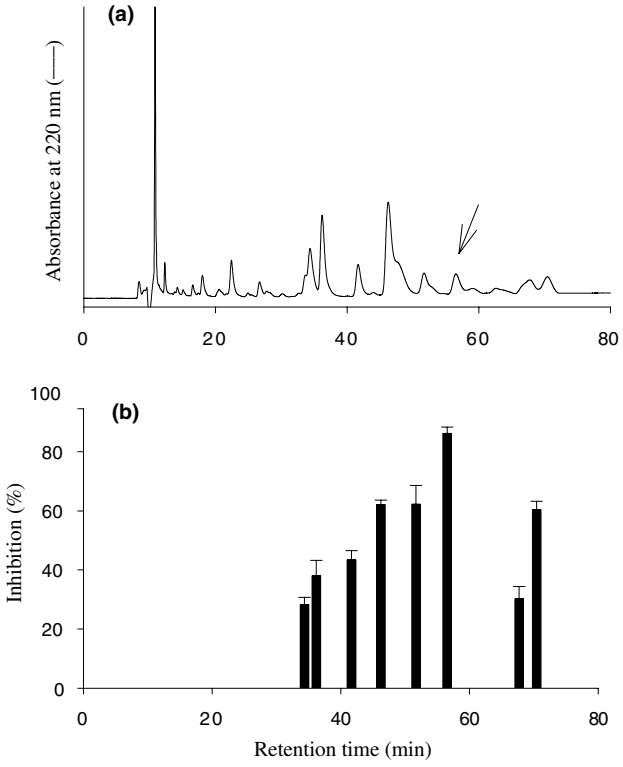

Fig. 2. Reverse-phase HPLC chromatogram (a) and inhibition of platelet aggregation (b). Results are expressed as mean ± SEM of two measurements in the bottom figure.

The chromatogram with inhibitory activities of fractions of concern is presented in Fig. 2. 5% acetonitrile in water containing 0.1% trifluroacetic acid, the eluting solvent, showed a low inhibition of about 18% and the data were presented as is without adjustment of the solvent effects. Again, all the fractions tested showed some inhibitory activities. The fraction with the most inhibitory activity, indicated by ↓ in Fig. 2, was further separated by cation exchange HPLC (Fig. 3), which showed two inhibitory fractions.

The two fractions from cation exchange HPLC were, respectively, desalted by reverse-phase HPLC, and subsequently each was subjected to LC–electrospray ionization mass spectrometry for molecular weight determination. Molecular weights of first and second fractions from cation exchange HPLC were 381.3 and 391.3 Da, respectively.

Their amino acid sequences were determined by Procise™ Protein Sequencing System to be SSGE (Ser–Ser–Gly–Glu) and DEE (Asp–Glu–Glu), respectively, and their calculated molecular weights were in agreement with their respective experimentally determined values.

SSGE was located in six soybean proteins: for example, residues 597–600 of plasma membrane Ca^{2+}-ATPase. DEE had 151 matches in soybean proteins with a few of them redundant: for example, residues 157–159 and 186–188 of β-conglycinin α-subunit.

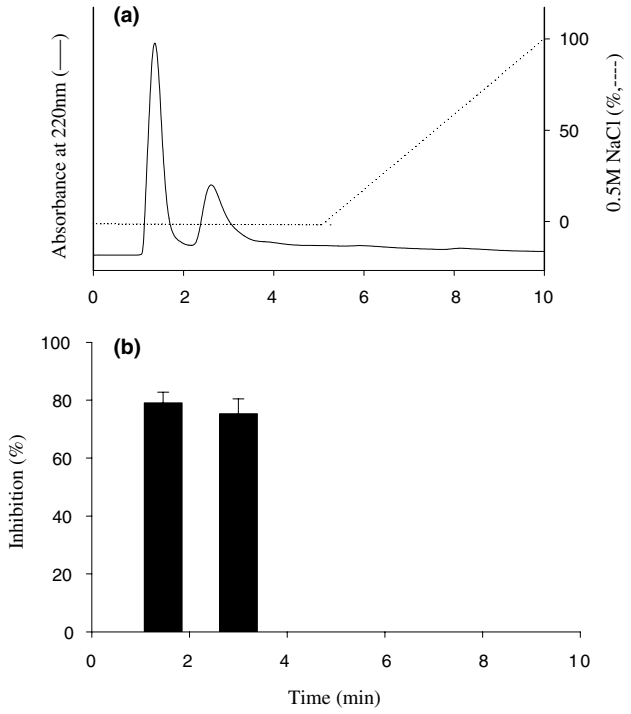

Fig. 3. Cation exchange HPLC chromatogram (a) and inhibition of platelet aggregation (b). Results are expressed as mean ± SEM of two measurements in the bottom figure.

SSGE and DEE peptides were both highly hydrophilic as indicated by their respective average hydropathy scores of – ↑ 1.38 and – ↑ 3.5, calculated according to Kyte and Doolittle (1982). High hydrophilicity may be a common feature of antiplatelet peptides, since Mazoyer et al. (1990) also found that their antiplatelet peptides, KRDS and RGDS, were highly hydrophilic.

Both SSGE and DEE were synthesized and studied for their effects on inhibition of washed rat platelet aggregation induced by ADP (10 μM). These peptides inhibited the aggregation in a concentration-dependent manner (Fig. 4) and the concentrations to obtain 50% inhibition (IC_{50}) of the aggregation intensity were approximately 480 and 460 μM, respectively, for SSGE and DEE. Similar inhibition was found in the presence of RGDS used as a control (IC_{50} 475 μM). This is in disagreement with the findings of Mazoyer et al. (1990), who reported that RGDS at a concentration of 500 μM was without any significant effect on ADP-induced rat platelet aggregation. This discrepancy could be due to differences in the test protocol: i.e., they used platelets suspended in their own plasma-induced by 1.2 μM ADP while we used platelets suspended in a buffer-induced by 10 μM ADP.

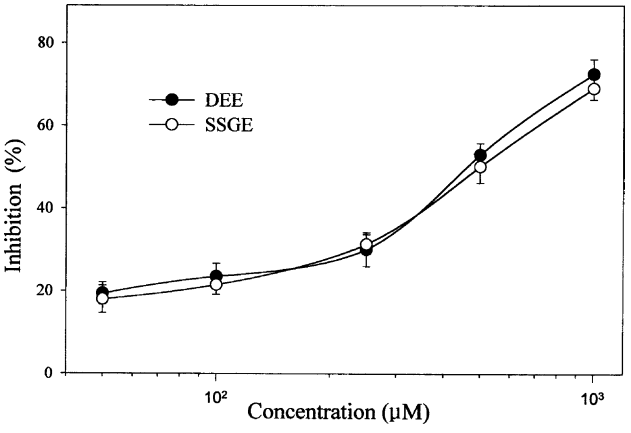

Fig. 4. Concentration-dependent inhibition of ADP-induced platelet aggregation. Results are expressed as mean ± SEM of two measurements.

Acknowledgements

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