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Polyphenol compounds belonging to flavonoids inhibit activity of coagulation factor X

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ABSTRACT

Blood coagulation consists of series of zymogens which can be converted by limited proteolysis to active enzymes leading to the generation of thrombin and conversion of fibrinogen into fibrin by this enzyme. The activated factor X (FXa) forms prothrombinase complex on phosphatidylserine containing surface which is responsible for conversion of prothrombin to thrombin. One molecule of FXa generates more than 1000 thrombin molecules. Therefore FXa is a novel target for modern anticoagulant therapy.

The aim of our present study is to examine the effects of the well-known plant polyphenolic compounds on factor Xa amidolytic activity and characterization of these interactions using bioinformatic ligand docking method.

We observed that only four polyphenols belonging to flavonoids group: procyanidin B2, cyanidin, quercetin and silybin, had inhibitory effect on FXa activity. Bioinformatic analyses revealed that procyanidin B2, cyanidin, quercetin and silybin bound in the S1–S4 pockets located in vicinity of the FXa active site and blocked access of substrates to Ser195.

The results presented here showed that flavonoids might be potential structural bases for design of new nature-based, safe, orally bioavailable direct FXa inhibitors.

1. Introduction

The modern cell based model of coagulation process demonstrates thrombin generation in three overlapping phases. The prothrombinase complex formed on phosphatidylserine containing surface plays the all major roles. The active factor X (FXa) together with its activated cofactor – factor V converts prothrombin to thrombin [1–4]. It has been demonstrated that one active molecule of FXa generates more than 1000 thrombin molecules [5]. The increased production and action of thrombin *in vivo* is observed in plasma of patients with high risk for venous or arterial thromboembolic disease [6–8]. Therefore FXa is a novel target for modern anticoagulant therapies.

Coagulation factor X, denominated also Stuart-Prower factor, is a member of S1 family of serine proteases called chymotrypsine-like with typical for this family active center (His57, Asp102 and Ser195) [9,10]. The first spatial structure of human activated factor X was determined in 1993 by Padmanabha et al. [11]. The structure of FXa protease domain is composed of two β-barrels similarly to chymotrypsin and the heavy chain of thrombin.

The active site of FXa are structured for specific catalytic cleavage of peptide bond located in specific sequences of physiological substrates. FXa cleaves a peptide bond located on the carboxyl side of arginine residue in FFNPRTF and YIDGRIV motif of prothrombin [12]. According to Schechter and Berger nomenclature [13] the substrate amino acid residues are P*n*, . . ., P2, P1, P1′, P2′, . . ., P*n*′, where P1–P1′ denote the hydrolyzed bond. The corresponding enzyme binding sites S*n*, . . ., S2, S1, S1′, S2′, . . ., S*n*′ are set in FXia for these residues respectively [14]. FXa strongly prefers arginine in P1 residue which binds to S1 pocket with Asp189 at its bottom and is surrounded by Tyr228 and Gly219 [15]. The S2 site is a shallow surface depression lined by Tyr99, which prefers small amino acids (like glycine) in the P2 position. The S3 site is flat and exposed to the solvent. The S4 site is also called aromatic box, as the key residues have hydrophobic character – Trp215, Tyr99 and Phe174 [12].

The researches accomplished in recent years have provided evidences that polyphenol compounds have been able to inhibit the activity of many enzymes, including serine proteases [16]. In our previous *in vitro* studies [17] we have shown that polyphenolic compounds from black chokeberry and grape seed extracts have antithrombin properties and block activity by competitive mechanism [18].

The aim of our present study is to examine the effects of the well-known plant polyphenolic compounds on factor Xa amidolytic activity and characterization of these interactions by bioinformatic ligand docking method.

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2. Materials and methods

2.1. Reagents

Chromogenic substrate S-2222® was purchased from Chromogenix (Italy). Bovine activated factor X (F9302), dimethyl sulfoxide (DMSO), polyphenol compounds: 4-hydroxyphenylacetic acid gallic acid, ferulic acid, caffeic acid, chlorogenic acid, coumaric acid, resveratrol, cyanin, cyanidin, (+)-catechin, (−)-epicatechin, procyanidin B2, naringenin, naringin, hesperetin, hesperidin, quercetin, rutin, genistein and silybin, were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were reagent grade or highest quality available products.

2.2. Factor Xa samples preparation

Factor Xa (0.75 U/ml in 50 mM TBS, pH 7.4) was preincubated with polyphenolic compounds (4-hydroxyphenylacetic acid, gallic acid, ferulic acid, caffeic acid, chlorogenic acid, coumaric acid, resveratrol, cyanin, cyanidin, (+)-catechin, (−)-epicatechin, procyanidin B2, naringenin, naringin, hesperetin, hesperidin, quercetin, rutin, genistein and silybin) at the concentration range of 0.125–1000 μM by 10 min at 37 °C. All tested compounds were initially dissolved in 50% DMSO to the preliminary concentration of 10 mM. Other solutions of compounds were also performed in 50% DMSO (prepared in 50 mM TBS, pH 7.4). The final concentration of DMSO in all samples was 5%. In control samples the same volume of solvent (50% DMSO prepared in 50 mM TBS, pH 7.4) was added as in the case of compound volume and was warmed 10 min at 37 °C.

2.3. Determination of amidolytic activity of factor Xa

The activity of FXa was determined by measuring the hydrolysis of chromogenic substrate S-2222® (Bz-Ile-Glu(g-OR)-Gly-Arg-pNA·HCl) [19]. Absorbance measurements were performed using a 96-well microplate reader. Firstly, chromogenic substrate was added to the each microplate reaction well, 150 μl of 1.5 mM solution. Secondly, to initiate the chromogenic reaction, 150 μl of factor Xa samples were simultaneously inserted to every reaction well (final concentration of chromogenic substrate – 750 μM). Finally, absorbance measurement was started at 415 nm. The absorbance values were monitored every 12 s for 10 min. The velocity of the reaction (ΔmOD/min) for each absorbance curve was determined and presented as % of control sample rate. IC_{50} values for every polyphenolic compounds were estimated from inhibition curves. The results were presented as inhibition percent of control value according to polyphenolic compound concentration.

2.4. Ligand docking

The ligand docking for the selected polyphenolic compounds was calculated *in silico* with Autodock Vina 1.0, an algorithm released by Scripps Research Institute [20] (http://vina.scripps.edu/). Protein coordinates were found on RSCB Protein Data Bank (http://www.rcsb.org). All three-dimensional chemical structures were downloaded from PubChem (http://pubchem.ncbi.nlm.nih.gov/) and converted to pdb files using Avogadro 1.1.0, an open-source molecular builder and visualization tool (http://avogadro.openmolecules.net/) [21]. Molecular modeling was performed using human factor Xa PDB structure 2J4I [22] as a receptor. Hydrogen atoms and Gasteiger charges were assigned to the rigid protein and flexible ligand molecules. Vina was run with settings: center_x 10.283; center_y 3.028; center_z 23.295; size_x 44; size_y 44; -size_z 52. The binding places were computed and the binding affinity of the ligand to the receptor was counted in kcal/mol. The analysis and visualization of the three-dimensional structure of the protein with the bound ligand was performed with Python Molecular Viewer of Auto Dock Tools v 1.5.6rc1 (http://autodock.scripps.edu) [23] and Swiss-PdbViewer (http://spdbv.vital-it.ch/) [24].

2.5. Comparison of human and bovine FXa protease domain primary and tertiary structure

Protease sequence of human FXa was found on UniProt (http://www.uniprot.org/). NCBI protein BLAST – (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/) [25] was performed against bovine database. The alignment was edited to mark catalytic triad and pockets to underline an evolutionary conservation in primary structure. SwissModel [26,27] was used to simulate spatial structure of bovine FXa with 2J4I [22] as template in automated mode [24]. Three structures – human 2J4I, bovine modeled in SwissModel and bovine 1KIG [28] were used to compare the conservation of FXa tertiary structure.

3. Results and discussion

Fondaparinux (synthetic pentasaccharide activating ATIII to bind to FXa) introduced into research and clinical use have shown that anticoagulant treatment based only on factor Xa inhibition can give comparable therapeutic results as is the case of inhibition of thrombin. This discovery has contributed to the search for novel substances which could directly inhibit factor Xa activity [29]. In 1998 Bayer company commenced a program called “Factor Xa” which the main aim was to find orally active factor Xa inhibitors with satisfactory antithrombotic activity [30]. After a few years, this program resulted in the discovery of an oral, direct Factor Xa inhibitor: 5-chloro-N-([(5S)-2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl]methyl)thiophene-2-carboxamide, called rivaroxaban [31]. Following clinical trials RECORD 1–4 [32–35] and EINSTEIN [36] have shown that inhibition of factor Xa is more effective in preventing venous thromboembolic events compared with the use of low-molecular-weight heparin (LMWH).

Polyphenolic compounds are a broad group of organic secondary plant metabolites that have one or more aromatic rings in the molecule and from one to ten phenolic hydroxyl groups. Mozzicafreddo et al. [37] reported as a first group that flavonoids inhibited amidolytic activity of thrombin. A study of Jedinak et al. [38] presented that silybin and quercetin are able to inhibit thrombin ability to hydrolyze *N*-benzoyl-phenylalanyl-valyl-arginine-paranitroanilide (IC_{50} for silybin was 20.9 μM, and for quercetin was 30.0 μM respectively at 600 μM substrate concentration). They also showed the inhibitory effect of these compounds on trypsin and urokinase amidolytic activity (for trypsin: silybin IC_{50} was 3.7 μM and quercetin IC_{50} was 15.4 μM, while for urokinase: silybin IC_{50} was 21.0 μM and quercetin IC_{50} was 12.1 μM). In our previous study [17,18] we demonstrated inhibition of thrombin amidolytic and proteolytic activity by polyphenols of the flavonoid class. Factor Xa inhibition have been until now reported only for per-sulfated flavonoids [39] and for flavonoids with an oligopolysulfated moiety [40]. The inhibition of factor Xa was indirect and was mediated by ATIII.

In the present study we tested, for the first time, direct effect of polyphenolic compounds on factor Xa activity. Twenty polyphenolic compounds, which occur as the most abundant in nature and belong to the main group of polyphenols, were tested at the highest used concentration 1000 μM.

From the bulk of all polyphenolic compounds examined in presented study only four of them, belonging to flavonoid class (procyanidin B2, cyanidin, quercetin and silybin) have inhibitory