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KINETICS STUDIES ON FLAVONE GLYCOSIDES: INHIBITORS OF α-CHYMOTRYPSIN

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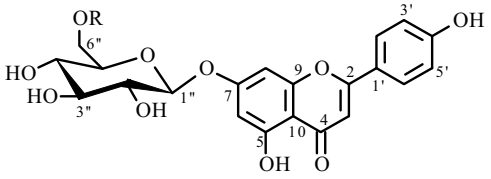
The mechanism of inhibition of α*-chymotrypsin enzyme by two flavone glycosides having an apigenin basic skeleton, namely, apigenin 7-*O-β-*D(-6″-*p-E*-coumaroyl)-glucoside (**1**) and apigenin 7-*O-β-*D-glucoside (**2**), isolated from* Clematis orientalis *L. (Ranunculaceae), was investigated. Lineweaver-Burk and Dixon plots and their secondary replots have shown that both of the compounds are competitive inhibitors of this enzyme.* K_i *values for* ***1*** *and* ***2*** *were found to be 71.75 ± 0.85 and 680.11 ± 0.11 μM, respectively.*

Keywords: α-chymotrypsin, *Clematis orientalis*, competitive inhibitors, flavone glycosides.

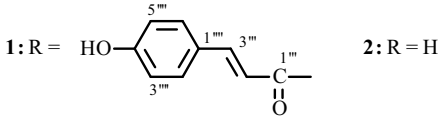
The genus *Clematis* Linn. (Ranunculaceae) consists of 280 species of climbing plants prized for their beautiful and showy flowers and distributed throughout the world. A number of species are acrid and poisonous [1], and various species are used as an antimalarial, and antirheumatic, as an antidote in snake bites, and in the treatment of dysentery and goiter [2].

The development of nontoxic protease inhibitors extracted from natural sources for *in vivo* application is quite important [3]. In the future, it is likely that numerous specific protease inhibitors will be tested clinically for the treatment of human disease such as emphysema, inflammation, dermatitis, and cancer. Since current serine protease (i.e., α-chymotrypsin) inhibitors are still far from perfect, interest in novel serine protease inhibitors is expected to continue. As part of our ongoing investigations on α-chymotrypsin inhibition by natural products, we now describe in detail the α-chymotrypsin inhibitory activity and kinetics of these potential compounds.

Apigenin 7-*O*-*β*-D(-6″-*p*-*E*-coumaroyl)-glucoside (**1**) [4], and apigenin 7-*O*-*β*-D-glucoside (**2**) [5], were isolated from the ethyl acetate soluble fraction of *Clematis orientalis* L. The structures of these reported glycosides from this genus were deduced through matching of their spectral (UV, IR, mass spectra, and NMR) data with the literature reports [4, 5].



1, 2



2: R = H

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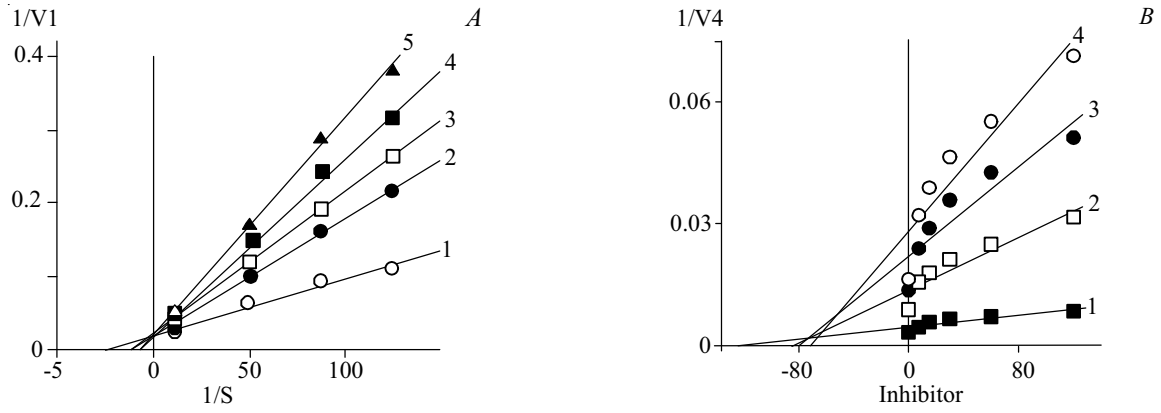


Fig. 1. Inhibition of chymotrypsin by **1**: (*A*) Lineweaver-Burk plot of the reciprocal of initial velocities versus the reciprocal of four fixed substrate concentrations in the absence (*1*) and presence of 50 μM (*2*), 25 μM (*3*), 12 μM (*4*), and 6 μM (*5*) of **1**; (*B*) Dixon plot at fixed substrate concentrations: 0.06 mM (*1*), 0.045 mM (*2*), 0.033 mM (*3*) and 0.01 mM (*4*).

Chymotrypsin catalyzes the hydrolysis of peptide bonds of proteins in the small intestine. It is selective for peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met) on the carboxyl side of this bond. Chymotrypsin also catalyzes the hydrolysis of ester bonds. X-ray studies have revealed a charge relay system of Asp-102, His-57, and Ser-195. This grouping has been found in a whole group of enzymes called serine proteases. Neutron diffraction studies on trypsin show that His-57 acts as a base in the catalytic process. The hydrolysis of peptide bonds occurs by general base-catalyzed nucleophilic attack on the carbonyl carbon of the substrate by the hydroxyl oxygen of Ser-195. At the same time, the hydroxyl proton of serine is transferred to the imidazole of His-57, the chemical base in the hydrolysis reaction. The hydroxyl group of Ser-195 attacks the carbonyl carbon atom of the peptide bond to give a tetrahedral intermediate. His-57 donates a proton to the nitrogen atom of the peptide bond, leading to cleavage and acylation of the enzyme. Deacylation then occurs with water taking the place of the amine group of the substrate [6]. Apigenin 7-*O*-*β*-D(-6″-*p*-*E*-coumaroyl)-glucoside (**1**) and apigenin 7-*O*-*β*-D-glucoside (**2**) inhibited chymotrypsin enzyme in a concentration-dependent manner with K_i values of 71.75 ± 0.85 and 680.11 ± 0.11 μM, respectively. Chymostatin (standard) K_i = 8.24 ± 0.11 μM. K_i values were calculated in three ways: first, the slopes of each line in the Lineweaver-Burk plot were plotted against different concentrations of **1** and **2**; secondly the $1/V_{maxapp}$ was calculated by plotting different fixed concentration of substrate versus ΔV in the presence of different fixed concentrations **1** and **2** in the respective assays of chymotrypsin; then K_i was calculated by plotting different concentrations of **1** and **2** versus $1/V_{maxapp}$. K_i is the intercept on the x-axis; in the third method, K_i was directly measured from the Dixon plot as an intercept on the x-axis. Determination of the inhibition type is critical for the identification of the mechanism of inhibition and the sites of inhibitor binding. Lineweaver-Burk plots, Dixon plots, and their replots indicated a purely competitive type of inhibition for both **1** and **2**, against chymotrypsin enzyme, as in both of these there was an increase in K_m without affecting the V_{max}. In other words, we can say that **1**, **2**, and *N*-succinylphenylalanine-*p*-nitroanilide bind at the same site of chymotrypsin.

The graphical analysis of steady-state inhibition data for **1** and **2** against chymotrypsin is presented in Fig. 1. The structure–activity relationship reveals that the high inhibitory potential of **1** as compared to that of **2** can be attributed to the presence of a *p*-*E*-coumaroyl group at C-6 of the glucose moiety in **1**, which was absent in **2**. Clearly, there are many questions regarding the mode of action of these ligands that need to be answered, and the answer to these questions will play an important role in the development of future generations of these inhibitors. For that reason, we are synthesizing several derivatives of **1** and **2** to be evaluated against chymotrypsin *in vitro* through co-crystallization to establish the in-depth mechanism of action of these flavone glycosides.

EXPERIMENTAL

General Techniques. For column chromatography (CC), silica gel (70–230 mesh) was used, and for flash chromatography (FC), silica gel (230–400 mesh) was used. TLC was performed on precoated silica gel G-25-UV_{254} plates.

Detection was carried out at 254 nm by ceric sulfate and aniline phthalate reagents. Purity was checked on TLC with different solvent systems using methanol, acetone, and $CHCl_3$ giving a single spot. Optical rotations were measured on a Jasco-DIP-360 digital polarimeter. UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrophotometers, respectively. ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra were run on Bruker spectrometers operating at 500, 400, and 300 MHz. The chemical shifts are given in δ in ppm, and the coupling constants in Hz. EI-MS and FAB-MS spectra were recorded on a JMS-HX-110 spectrometer, with a data system.

Plant Material. The plant *Clematis orientalis* L. was collected from Parachinar, Kurram Agency, Pakistan, in August 2005, and identified by Dr. Jahandar Shah (plant taxonomist), Department of Botany, Islamia College of Peshawar, Peshawar University. A voucher specimen (No. 5251) has been kept in the Herbarium of that Department.

Extraction and Purification. The shade-dried ground whole plant (27 kg) was exhaustively extracted with methanol at room temperature. The extract was evaporated to yield the residue (1.2 kg), which was dissolved in water and partitioned with hexane, chloroform, ethyl acetate, and *n*-butanol. The ethyl acetate extract (287 g) was subjected to CC over column silica gel using hexane with a gradient of $CHCl_3$ up to 100%, and then the polarity was increased with methanol in a similar fashion. Eighteen fractions (Fr. 1–18) were collected. Fraction 9 was loaded on flash silica gel and eluted with MeOH–$CHCl_3$ (8:92) to get two subfractions ($Fr_{sb.}$ 9.1–9.2). Fraction 9.1 was again flash chromatographed eluting with MeOH–$CHCl_3$ (8:92) to isolate purified **1** (35.4 mg). Fraction 12 was then submitted to repeated flash chromatograpy and eluted with MeOH–$CHCl_3$ (10:90), then divided it into three subfractions ($Fr_{sb.}$ 12.1–12.3). Fraction 12.2 was then passed through Sephadex LH-20 and eluted with H_2O–MeOH (1:1), and the resulting impure **2** was finally purified by repeated FC, eluting with MeOH–$CHCl_3$ (10:90), which yielded pure **2** (29.7 mg). The purity of these samples was also evident from their clear NMR spectra.

Apigenin 7-*O*-*β*-D(-6″-*p*-*E*-Coumaroyl)-glucoside (1). ^{1}H NMR (500 MHz, C_5D_5N, δ, ppm, J/Hz): 13.0 (1H, s, 5-OH), 7.91 (1H, d, J = 15.9, H-3‴), 7.87 (2H, d, J = 8.6, H-2′, H-6′), 7.46 (2H, d, J = 8.5, H-2⁗, H-6⁗), 7.16 (2H, d, J = 8.6, H-3′, H-5′), 7.06 (2H, d, J = 8.5, H-3⁗, H-5⁗), 7.04 (1H, d, J = 2.1, H-8), 6.90 (1H, d, J = 2.1, H-6), 6.84 (1H, s, H-3), 6.65 (1H, d, J = 15.9, H-2‴), 5.81 (1H, d, J = 7.2, H-1″). ^{13}C NMR (125 MHz, C_5D_5N, δ, ppm): 182.8 (C-4), 167.5 (C-1‴), 164.9 (C-2), 163.9 (C-7), 162.8 (C-9), 162.7 (C-4′), 161.4 (C-4⁗), 157.8 (C-5), 145.6 (C-3‴), 130.6 (C-2⁗, C-6⁗), 128.9 (C-2′, 6′), 125.9 (C-1⁗), 122.0 (C-1′), 116.9 (C-3′, 5′), 116.7 (C-3⁗, C-5⁗), 114.8 (C-2‴), 106.7 (C-10), 101.7 (C-1″), 100.6 (C-6), 95.4 (C-8), 78.3 (C-3″), 75.7 (C-5″), 74.6 (C-2″), 71.4 (C-4″), 64.5 (C-6″).

Apigenin 7-*O*-*β*-D-Glucoside (2). ^{1}H NMR (500 MHz, C_5D_5N, δ, ppm, J/Hz): 13.6 (1H, s, 5-OH), 7.80 (2H, d, J = 8.7, H-2′, H-6′), 7.16 (2H, d, J = 8.7, H-3′, H-5′), 7.09 (1H, d, J = 2.1, H-8), 6.90 (1H, s, H-3), 6.85 (1H, d, J = 2.1, H-6), 5.80 (1H, d, J = 7.1, H-1″). ^{13}C NMR (125 MHz, C_5D_5N, δ, ppm): 182.8 (C-4), 164.9 (C-2), 164.0 (C-7), 162.8 (C-9), 162.5 (C-4′), 157.8 (C-5), 128.9 (C-2′, 6′), 122.0 (C-1′), 116.9 (C-3′, 5′), 106.5 (C-10), 101.8 (C-1″), 100.6 (C-6), 95.3 (C-8), 79.2 (C-3″), 78.4 (C-5″), 74.8 (C-2″), 71.4 (C-4″), 62.4 (C-6″).

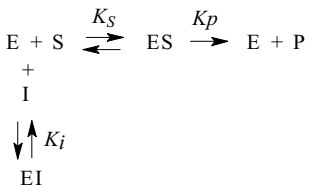
Chymotrypsin Assay. The chymotrypsin inhibitory activity of compounds **1** and **2** was performed by the method of Cannel et al. [7]. Chymotrypsin (9 units/mL of 50 mM Tris-HCl buffer pH 7.6; Sigma Chemical Co. USA) was preincubated with the compounds for 20 min at 25°C. Then 100 μL of substrate solution (*N*-succinylphenylalanine-*p*-nitroanilide 0.01–0.06 mM of 50 mM Tris-HCl buffer pH 7.6) was added to start the enzyme reaction. The absorbance of the released *p*-nitroaniline was continuously monitored at 410 nm until a significant color change was achieved. The final DMSO concentration in the reaction mixture was 7%.

Estimation of Kinetic Parameter. Two different methods were applied to monitor the effect of the inhibitor (test sample) on both K_m and V_{max} values. This was done firstly by plotting the reciprocal of the reaction rate against the reciprocal of the substrate concentration as a Lineweaver–Burk plot, and secondly by a Dixon plot in which the reciprocal of the reaction rates was plotted against the inhibitor concentrations [8]. The secondary replot of the Dixon plot was constructed as the slope of each line of the substrate concentration in the original Dixon plot against the reciprocals of the substrate concentrations. K_i values (the dissociation constant of dissociation of the enzyme–inhibitor complex into free enzyme and inhibitor) were determined by interpretation of the Dixon plot, the Lineweaver–Burk plot, and its secondary replots by using the initial velocities. The dependence of V_{max}/K_m and V_{max} on inhibitor [I] is given by

$$V_{max}/K_m = \left(\frac{(V_{max}/K_m)K_i}{K_i+[I]}\right) \rightarrow I = \frac{K_i}{K_i+[I]} \rightarrow K_i = K_i + [I]$$

These velocities were obtained over a range of substrate concentrations between 0.01 and 0.06 mM. The assay conditions for measurement of the residual activities of all inhibitors were identical to the mentioned spectrophotometric assay

procedure except that fixed concentrations of inhibiting compounds **1** and **2** were used in the assay medium. The types of inhibition were determined by the graphical views of Dixon plots, Lineweaver–Burk plots, and their secondary plots. The interaction of **1** and **2** with chymotrypsin can be described by the following:



$$K_i = \frac{[E][I]}{[EI]}, \qquad K_S = \frac{[E][S]}{[ES]}$$

where ES is the chymotrypsin-substrate complex and P is the product, K_i is the inhibition constant reflecting the interaction of **1** and **2** with the free enzyme and enzyme/ substrate complex, while EI is the enzyme/inhibitor complex.

Statistical Analysis. All assays were conducted in triplicate. Graphs were plotted using the GraFit program [9]. Values of the correlation coefficient, slope, intercept, and their standard errors were obtained by linear regression analysis using the same software. The correlation coefficient for all the lines of all graphs was > 0.99; each point in the constructed graphs represents the mean of three experiments.

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