Enzymatic and Chemical Synthesis of New Anticoagulant Peptides

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In this study we report the enzymatic synthesis of N-α-*[Carbobenzyloxy]-Tyr-Gln-Gln (Z-YQQ), a new anticoagulant tripeptide. It was obtained using phytoproteases from the stems and petioles of* Asclepias curassavica *L. as catalyst in an aqueous–organic biphasic system formed by 50% (v/v) ethyl acetate and 0.1 M Tris–HCl buffer pH 8. The resulting peptide was compared with the analogous peptide Tyr-Gln-Gln (YQQ) produced by solid-phase chemical synthesis. The in vitro anticoagulant activity of the aforementioned peptides was determined using Wiener Lab Test (Wiener, Argentina). The toxicological activity of the peptides was also determined. The enzymatically synthesized Z-YQQ peptide acted on the extrinsic pathway of the coagulation cascade, delaying the conversion time of prothrombin to thrombin and fibrinogen to fibrin by 136 and 50%, respectively, with respect to the controls. The chemically synthesized YQQ peptide acted specifically on the intrinsic pathway of the coagulation cascade, affecting factors VIII, IX, XI, and XII from such cascade, and increasing the coagulation time by 105% with respect to the control. The results suggest that two new anticoagulant peptides (Z-YQQ and YQQ) can be useful for safe pharmaceutical applications. Nevertheless, some aspects related to peptide production should be optimized.*
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Introduction

Bioactive peptides are placed at the biotechnological forefront because they can replace chemical drugs. Peptides are considered to have an important competitive advantage over

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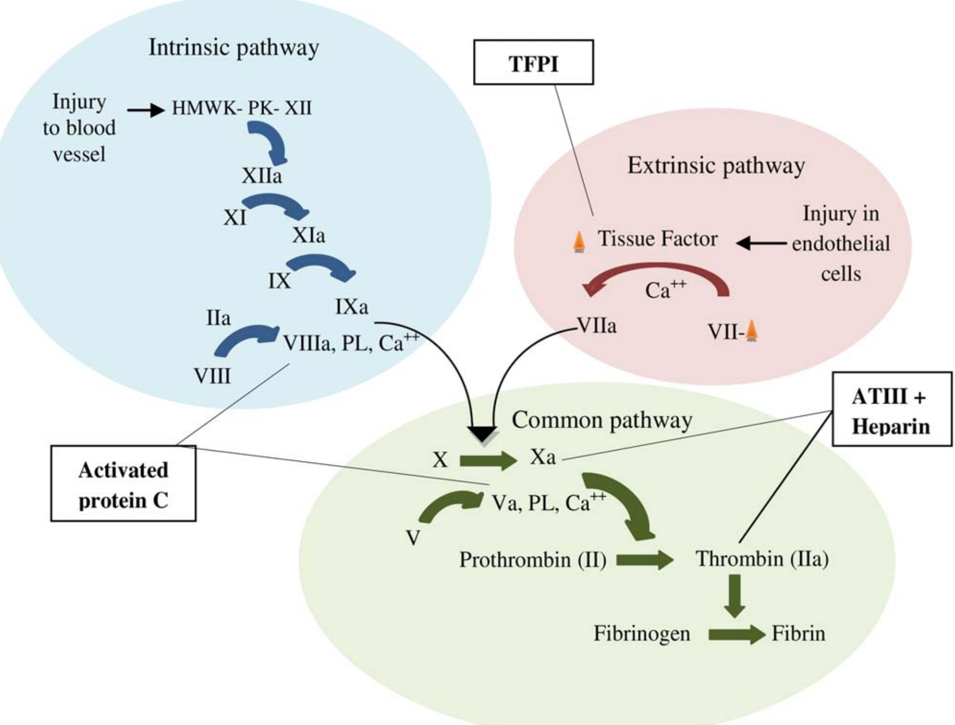


Figure 1. Schematic representation of the coagulation cascade. In bold are the anticoagulant factors.

HMWK: high-molecular-weight kininogen, PK: prekallikrein, ATIII: antithrombin, TFPI: inhibitor of the tissue factor pathway, PL: phospholipid.

traditional medications due to their high specificity in target tissues, none or little toxicity, low accumulation inside the organism, and easy degradation in the environment.[1]

Anticoagulant agents are widely used in medicine for the treatment of hemostatic impairments such as coronary thromboembolisms, myocardial infarction, and pulmonary embolism. All together, these arterial and venous thrombotic diseases are now the leading cause of death worldwide.[2]

Blood coagulation is the nature's defense mechanism that rapidly allows for the balance between flow and stasis depending on the need. Yet, this defense mechanism can be disrupted by several damaging internal triggers that induce pathologic clot thrombus, so that they can be life-threatening if not treated promptly.[3]

Figure 1 shows a scheme of the coagulation cascade which is nature's accurate mechanism which converts soluble into insoluble factors, by highly controlled aggregates called clots. The inactive forms (zymogens) of several enzymes that freely float in the bloodstream are activated in a sequential manner upon an initiating signal, such as injury, resulting in the formation of polymerized insoluble fibrin from soluble monomers.[4]

Two major classes of drugs have been used in the past century for treating thrombotic disorders: heparin and warfarin. Unfractioned heparin (UFH), its recent derivatives including low-molecular-weight heparins (LMWHs), and heparin-like pentasaccharide (Fondaparinux) are the most widely used anticoagulants worldwide with an annual market rate of at least US$10 billion.[5]

However, UFH and LMWHs exhibit considerable off-target effects, such as the significantly enhanced risk of bleeding, heparin-induced thrombocytopenia, response variability between patients and lack of oral bioavailability.[6] Besides, warfarin therapy causes food-drug interactions and unpredictable pharmacokinetics which requires constant monitoring.[7]

In recent years, a number of peptides with well-established anticoagulant activity have been isolated from microbial, animal and plant sources.[2] Hydrolyzed protein with anticoagulant activity from amaranth,[8] peanut,[9] pacific salmon,[10] and *Mucuna pruriens*[11] were obtained using commercial enzymes. In our laboratory, peptide fractions isolated from soluble proteins of goat cheese have also shown in vitro anticoagulant activity.[12]

Peptides are currently produced mainly by solid-phase chemical synthesis. However, its production by fermentation with recombinant microorganisms and by enzymatic hydrolysis and synthesis has been studied as alternative technologies.

Enzymatic synthesis of peptides in organic media offers several advantages, such as high stereospecificity, mild reaction conditions, minimum side-chain protection, and absence of racemization. Small peptides, such as kyotorphin and cholecystokinin were obtained by enzymatic synthesis and they are available as pharmaceutical products.[13] However, no enzymatic synthesis of an anticoagulant peptide has been reported yet.

The aim of this work is to study the enzymatic synthesis of a new anticoagulant tripeptide (Z-YQQ) potentially useful for safe pharmaceutical applications, using phytoproteases from *Asclepias curassavica* L. as catalyst in aqueous–organic biphasic systems. The results are compared with an analogous peptide obtained by chemical synthesis, namely YQQ. A toxicological evaluation of both peptides derivatives is also carried out.

Materials and Methods

Preparation of crude and prepurified enzymatic extract

Proteases of *Asclepias curassavica* L. (*asclepain*) were extracted from the latex obtained by superficial incisions of the stems and petioles of the plant.[14]

Determination of proteolytic activity

Proteolytic assays were performed using *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) (Sigma-Aldrich, USA) as substrate. The reaction mixture was prepared by mixing 0.5 mL of prepurified *asclepain* with 0.5 mL of 10 mM BAPNA containing 20 mM cysteine in 0.1M Tris–HCl buffer pH 8. The initial rate of the reaction was measured within the linearity range at 37°C and 200 rpm. One international unit (IU) of proteolytic activity of *asclepain* was defined as the amount of enzyme which hydrolyzes 1 μmol of BAPNA per min under previously defined operating conditions. At the same time, a control under similar conditions but without BAPNA was carried out.

Stability assays in aqueous–organic biphasic systems

A statistical design by clustering 70 organic solvents according to their physicochemical properties (descriptors) was extracted from the literature[15] and used for performing *asclepain* stability assays.

Seven immiscible organic solvents which were representative of each cluster were selected and the residual proteolytic activity in each biphasic system was determined until reaching the half life-time of the enzyme.

Enzymatic synthesis of peptide

Substrates for the peptide synthesis reaction were chosen based on the preferences expressed by *asclepain* for the *N*-α-[carbobenzyloxy]-aminoacids-*p*-nitrophenyl esters.[14] *N*-α-[carbobenzyloxy]-tyrosine-*p*-nitrophenyl ester (Z-Y-pNO) and H-Gln-OH were selected as acyl donor and nucleophile, respectively.

The concentration of Z-Y-pNO was established on the basis of its maximal solubility in the organic phase of the chosen biphasic system and the partition coefficient between the phases.

The reaction of synthesis was carried out in a 50% (v/v) biphasic system, consisting of an aqueous phase (0.1M Tris–HCl buffer pH 8) containing 0.075 mg mL^{-1} of enzyme (*asclepain*), 20 mM 2-mercaptoethanol and 82.85 mM H-Gln-OH, and an organic phase (ethyl acetate) containing 82.85 mM of Z-Y-pNO. The reaction was conducted at 40°C in a GFL Shaking Incubator Orbital Motion (Model 3031, Germany) at 200 rpm.

Aliquots (1 mL) were taken from the aqueous and organic phase at different times, during 24 h, and mixed with 0.2 mL of 0.1% (v/v) trifluoroacetic acid (TFA) for quenching the reaction.

Simultaneously, three types of control were performed: one with only the enzyme in the reaction medium (in order to determine peptides which could have been released from the enzyme extract), one with each individual substrate, and one with all reagents but without the enzyme to account for the potential chemical synthesis.

The peptides were analyzed by RP-HPLC and elucidated by mass spectrometry (MS).

Chemical synthesis of peptide

For the chemical synthesis, 0.2 g of 2-chloro-trityl resin (loading: 1.6 mmol g^{-1}, particle size: 100–200 mesh) was used as a solid support for peptide synthesis with terminal carboxyl groups. The resin was placed in reactors (plastic syringes with a polypropylene filter) and Nα-Fmoc (9-Fluorenyl methoxycarbonyl) strategy was used in order to obtain YQQ peptide by chemical synthesis.

Two equivalents of the first amino acid (*N*-α-Fmoc-*N*-δ-trityl-L-glutamine) and five equivalents of diisopropyl ethylamine (DIEA) were added to a syringe containing the resin. The coupling was performed at 160 strokes per min at room temperature in a reciprocal shaker (IKA®-Werke KS 501 Digital) during 2 h. Then, methanol (0.5 mL g^{-1} resin) was added to the resin and removed from the reactor by vacuum filtration after 5 min (Vacuubrand ME 1C, Vacuum pump, Vac-Man® Laboratory Vacuum Manifold). The resin was washed twice with dichloromethane (DCM), twice with *N*,*N*-dimethylformamide (DMF), and twice again with DCM (for 1 min in all cases) for drying the resin. Organic solvents (DCM, DMF) were dried with 0.5 nm molecular sieve beads (Merck).

Subsequently, 5–10 mg of dry resin coupled with the first amino acid was weighed in duplicate; 1 mL of 20% (v/v) piperidine in DMF was added, and stirred at 200 rpm for 20 min. Then, 30 μL of that solution was taken and brought to a final volume of 3 mL with DMF in a quartz cell. Absorbance was measured at 300 nm, using DMF as control.

Resin loading was calculated using the following equation[16]:

$$\text{Loading} = (\text{Fd}\times\text{A})/(\varepsilon\times\text{l}\times\text{m}) \qquad \text{(equation 1)}$$

Fd: Dilution factor (101)

A: Absorbance at 300 nm

ε: extinction coefficient dibenzofulvene (7.8 $mmol^{-1}$ cm^{-1})

l: optical path length (1 cm)

m: resin weight (mg)

The following amino acids were coupled one after the other at twice the concentration of the first amino acid, which had been previously deprotected with 20% piperidine and 1% X-100 Triton in DMF (twice for 7 min) and washed in DMF (three times for 1 min), in isopropyl alcohol (IPA) (once for 1 min), in 1% bromophenol blue (twice for 1 min), in DMF (twice for 1 min), and in DCM (twice for 1 min) at 200 rpm in an orbital shaker. O-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), Oxyma Pure® and DIEA were used as activator, as racemization suppressor (to preserve the chirality of the C-terminal of the coupled amino acid) and as base (to neutralize free protons), respectively. Alternatively, O-(benzotriazol-1-yl)-*N*,*N*,*N*’,*N*’-tetramethyluronium tetrafluoroborate (TBTU) or *N*,*N*′-diisopropylcarbodiimide (DIC) was also used as activator for double and triple couplings respectively, if needed.

In brief, the second amino acid and the following were assembled by coupling and deprotection cycles until the desired peptide sequence was obtained. The Nα group of the coupled amino acids was deprotected following the aforementioned protocol.

The completion of the coupling reaction was verified with the Kaiser test.[17]

Finally, deprotection of the amino acid side chains and cleavage of the peptide from the resin was performed using a TFA/H_2O/triisopropylsilane (95: 2.5: 2.5) (v/v) solution, during 90 min at 200 rpm and at room temperature.

The peptide was precipitated with cooled ethyl ether at −70°C, purified in a C_{18} cartridge (Merck), lyophilized and analyzed by RP-HPLC and MALDI-TOF to confirm its purity and molecular mass.

Analytical control of peptide synthesis

RP-HPLC. The separation of components (substrate, product, and by-products) of each phase in the enzymatic synthesis reaction was performed on a Gilson HPLC delivery system (Model 712) with UV detector equipped with a C_{18} column of 4.60 mm x 250 mm (Phenomenex). The injection volume was 20 μL, the flow rate of the mobile phase (formed by 50% (v/v) acetonitrile in 0.1 M buffer Tris-HCl pH 8) was 0.8 mL min^{-1} and the eluate was monitored at 254 nm and 25°C.

The purified peptide analyses obtained by enzymatic and chemical synthesis (Z-YQQ and YQQ) were performed on a Jasco HPLC delivery system (AS-2055 Plus Autosampler, PU-2089 Plus Quaternary Gradient Pump) equipped with a XBridge™ BEH C_{18} column (100 × 4.6 mm^2, 3.5 μm) (Waters). The injection volume was 20 μL, the flow rate of the mobile phase (A: Mili Q water containing 2.5% TFA; and 30–100% gradient of B: acetonitrile with 2.5% TFA, during 20 min) was 1mL/min, and the eluate was monitored with Photo Diode Array (PDA) Detector.

MS. Electrospray ionization-mass spectrometry (ESI-MS) was performed in a LCMS-2020 equipment (Shimadzu) loading 2 μg of each peptide under the following conditions: 4.5 kV, 350°C, positive ion mode, 20 min. The resulting data were analyzed using the LabSolutions software (version 5.42, Shimadzu).

MALDI-TOF. Samples were prepared in a micro scout plate by mixing 1 μL of peptide solution (1 $\mu g\ \mu L^{-1}$ in water) and 1 μL of the CHCA matrix (10 $\mu g\ \mu L^{-1}$ α-cyano-4-hydroxicinnamic acid in 50% (v/v) acetonitrile and 0.1% (v/v) formic acid) and allowing the mixture to air-dry. Samples were then measured in a MALDI-TOF Microflex (Bruker Daltonics) by reflexion detection under positive ion mode, calibrated beforehand with an external standard (700–1800 Da). Spectra were recorded using flexControl software (version 3.0, Bruker Daltonics GmbH).

Determination of anticoagulant activity

Anticoagulant activity of the synthesized peptides (Z-YQQ and YQQ) was assayed using Wiener Lab Test (Wiener, Argentina). Each peptide, 0.7 ppm in 0.1 M Tris-HCl buffer pH 8 (standard solution) was added to a pool of healthy people plasma and to Wiener Lab Test reagents for quantifying the following parameters: thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT). Controls under similar conditions and without peptides were also carried out.

Clotting tests

Thrombin Time Test (TTT). It evaluates the conversion time of fibrinogen to fibrin. This assay was carried out with 150 μL of plasma and 50 μL of the standard peptide solution (0.7 ppm) mixed and incubated for 2 min at 37°C. The clotting time (s) was determined after clotting induction by adding 200 μL of the TTT reagent (2.3 NIH/mL). NIH (National Institute of Health) is the commercial unit for thrombin and it is equivalent to 1.1 to 1.3 IU of thrombin.

Prothrombin Time Test (PTT). It evaluates the conversion time of prothrombin to thrombin. Plasma and PTT reagent were pre-incubated separately during 3 min at 37°C. Then, 25 μL of the standard peptide solution was added to 75 μL of plasma at 37°C. This mixture was then quickly added to the tube containing 200 μL of PTT reagent and the clotting time was recorded.

Activated Partial Thromboplastin Time Test (APTTT). It evaluates the action on VIII, IX, XI and XII factors from the coagulation Cascade. For this assay, 75 μL of plasma and 25 μL of the standard peptide solution were mixed and incubated for 1 min at 37°C. Then, 100 μL of APTTT reagent were added and the mixture was incubated for 3 min at 37°C. The clotting time was determined after adding 100 μL of 25 mM $CaCl_2$.

All assays were performed in quintuple and compared with a well-known anticoagulant compound (Heparin, 5000 IU mL^{-1} equivalent to 50 mg mL^{-1}, Veinfar Laboratory, Argentina) as positive control under equal conditions than those of the sample. Statistical analyses were made in each case. Differences were considered significant for values of $P < 0.05$.

Peptide stability in human plasma

The stability of Z-YQQ and YQQ was determined by incubating 0.7 ppm of each peptide in a pool of human plasma from healthy (nonanticoagulated) individuals at 37°C, and the anticoagulant activity was analyzed using the methodology previously described by Wiener Lab Test. The retained anticoagulant activity (APTT, TP and TT, s) of each peptide was plotted as a function of incubation time (min).

Quantitative determination of fibrinogen in plasma by immune turbidimetry method

Fibrinogen is a glycoprotein present in plasma and platelet alpha-granules. It is the clotting factor found in highest concentration in plasma. In the presence of trauma or vascular injury, thrombin cleaves fibrinogen, producing fibrin monomers which spontaneously polymerize and are stabilized, leading to insoluble fibrin mesh.

The fibrinogen immune turbidimetry method is based on the reaction between the fibrinogen in plasma and the polyclonal antibodies antihuman fibrinogen (goat) in phosphate buffer, forming insoluble immune complexes. The turbidity caused by these immune complexes is proportional to fibrinogen concentration in the sample and may be spectrophotometrically measured at λ: 340 nm.

A slightly modified version of Fibrinogen Turbiditest AA Line (Wiener Lab., Rosario, Argentina) was used to evaluate the inhibitory action of chemically and enzymatically synthesized peptides (Z-YQQ and YQQ) on the enzyme thrombin.

Reagent A: phosphate buffer, pH 7.4; Reagent B: polyclonal antibodies anti-human fibrinogen (goat) in phosphate buffer, pH 7.4; and the kit of Wiener Lab.'s Fibrinogen Calibrator Turbitest AA (for the fibrinogen calibration plot) were used in these assays.

Three controls were prepared and incubated together with the samples, as described below:

A Blank control (BC) consisting of 25 μL of 5.69 mg mL^{-1} fibrinogen (Wiener Lab., Argentina) in 165 μL of assay buffer (50 mM Tris-HCl pH 7.2 with 0.12 mM NaCl), based on the methodology reported by Sabbione et al., 2015.[18]

A Negative control (C -) consisting of 25 μL of 5.69 mg mL^{-1} fibrinogen (Wiener Lab., Rosario, Argentina) in 131 μL of assay buffer (50 mM Tris-HCl pH 7.2 with 0.12 mM NaCl) and 34 μL of 2.7 NIH/mL thrombin (Wiener Lab., Rosario, Argentina).

A Positive control (C +) consisting of 25 μL of 5.69 mg mL^{-1} fibrinogen (Wiener Lab., Rosario, Argentina) in 131 μL of 0.7 ppm heparin (5000 IU mL^{-1} or 50 mg mL^{-1}, Veinfar Laboratory, Argentina) and 34 μL of 2.7 NIH/mL thrombin (Wiener Lab., Rosario, Argentina).

The Samples (S) were prepared in the same manner as the positive control, but using 0.7 ppm of each synthetized peptide instead of heparin.

After incubation of controls and samples at 37°C for 10 min, 10 μL of each control/sample was mixed with 1.5 mL of Solution A, homogenized and measured at λ: 340 nm (OD_1), setting the instrument to zero with distilled water. Then, 300 μL of Solution B was added at that mixture, and it was incubated for 15 min at room temperature. The absorbance at λ: 340 nm was measured (OD_2), and the difference ($\Delta A = OD_2 – OD_1$) was calculated. Fibrinogen concentration of Samples and Controls was obtained from a calibration plot of ΔA absorbance differences vs. Fibrinogen concentration (Fibrinogen Calibrator Turbitest AA, Wiener Lab., Rosario, Argentina).

The percentage of inhibition (fibrinogen which was not converted into fibrin by the inhibitory action of the anticoagulant peptides studied) was calculated as follows:

$$\% \text{ Inhibition} = \{[(S - C-)]/(BC)\} * 100 \qquad \text{(equation 2)}$$

Toxicity assays

Potential toxicity of peptides was assayed on the 293FT human cell line.[19]

Cells were grown in bottles using Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), incubated at 37°C in a 5% CO_2 enriched air atmosphere until near confluence (80%). Cells were trypsinized, recovered in DMEM medium, counted and prepared in a suspension of 5 × 10^4 cells/mL. Then, 100 μL/well were added to a 96-well microplate and grown overnight under the same conditions. Medium was removed and cells were washed twice with phosphate-buffered saline (PBS). Then, 100 μL of 0.7, 2.1, 7, and 21 ppm peptide solution in DMEM were prepared and added to the cell-containing wells. A number of wells were saved for survival controls (PBS in DMEM) and toxicity controls (20% DMSO in DMEM). The microplate was incubated under growth conditions for 2 h and 20 μL/well of MTS reagent (CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit, Promega) were added, and then incubated by additional 4 h under growth conditions. Absorbance at λ: 490 nm was measured every hour and up to 4 h after reagent addition. Cell survival was calculated as percentage using survival controls as a 100% reference.

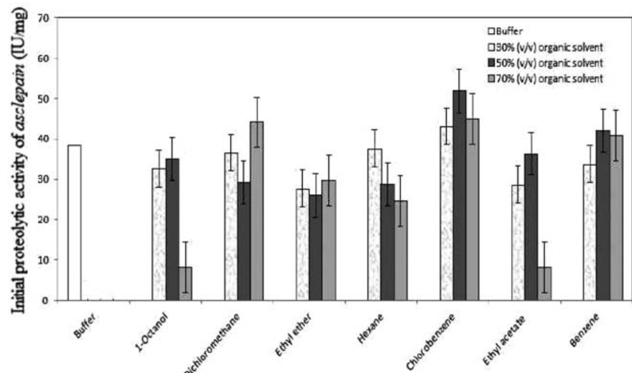


Figure 2. Initial proteolytic activity (IU mg^{-1}) of *asclepain* in biphasic systems formed by 30, 50, and 70% (v/v) of different immiscible organic solvents in 0.1 M Tris-HCl buffer pH 8.

Table 1. Half-Life Time Values ($t_{1/2}$, h) of *Asclepain* in Biphasic Systems Formed by 30, 50, and 70% (v/v) of Different Immiscible Organic Solvents in 0.1 M Tris-HCl Buffer pH 8

Immiscible organic solvents	Half-life Time Values (h)		
	30% (v/v)	50% (v/v)	70% (v/v)
Buffer	5.55	5.55	5.55
Benzene	6.18	4.52	6.06
1-Octanol	12.86	8.19	7.61
Dichloromethane	6.48	6.07	2.33
Ethyl ether	6.02	6.01	6.13
Hexane	6.07	12.18	5.99
Chlorobenzene	6.18	4.01	6.67
Ethyl acetate	6.17	11.07	2.22

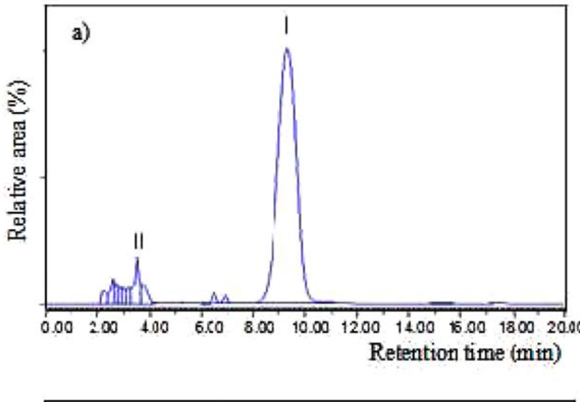
Results and Discussion

Asclepain stability in aqueous-organic biphasic systems

Initial proteolytic activity of *asclepain* in biphasic systems formed by 30, 50, and 70% (v/v) of different immiscible organic solvents in 0.1M Tris-HCl buffer pH 8 is shown in Figure 2. *Asclepain* initial activities in 50 and 70% (v/v) benzene, 70% (v/v) DCM and 30, 50 and 70% (v/v) chlorobenzene were 8 to 37% higher than in 0.1M Tris–HCl buffer pH 8. The values observed in the biphasic system ranged between 22 and 137% of the initial activity in buffer.

Table 1 shows the half-life time values ($t_{1/2}$, *h*) of *asclepain* in biphasic systems formed by 30, 50, and 70% (v/v) of different immiscible organic solvents in 0.1 M Tris-HCl buffer pH 8. *Asclepain* exhibited high stability in several biphasic media, such as 30% (v/v) 1-octanol, 50% (v/v) hexane, or 50% (v/v) ethyl acetate in 0.1M Tris–HCl buffer pH 8, which was evidenced by the high half-life values obtained. Nevertheless, when those organic solvent concentrations were increased to 70% (v/v), half-life values decreased. This effect may result from the fact that such immiscible organic solvents had partitioned to some extent into the aqueous phase and modified the surrounding microenvironment of the enzyme, altering the protein–water interactions and changing the enzyme tridimensional structure. This phenomenon is called molecular toxicity and it has been widely reported in the literature.[20] Furthermore, a major interfacial surface may have caused instability in those 70% (v/v) biphasic systems, which other authors have called toxicity of the phase.[21]

The high stability of *asclepain* in this system is consistent with the fact that proteases in water-organic solvent biphasic



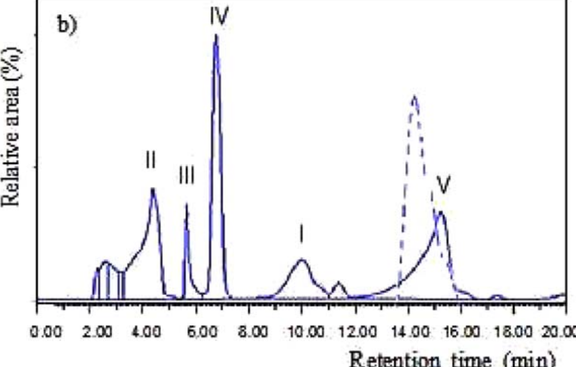


Figure 3. Component separation of the phases in the enzymatic synthesis reaction by RP-HPLC of: (a) aqueous phase and (b) organic phase of the sample after 5 min of reaction between N-α-[Carbobenzyloxy]–Tyr-p-nitrophenyl ester (Z-Y-pNO) and H-Gln-OH, using *asclepain* as catalyst at 40°C and 200 rpm. I: main product (t_R 9.8 min), II: enzyme (t_R: 2–4 min), III: Z-Y-pNO (t_R: 5.7 min), IV: *p*-nitrophenol (t_R: 6.8 min), V: byproduct (t_R: 15.2 min). Dotted line: reagents control.

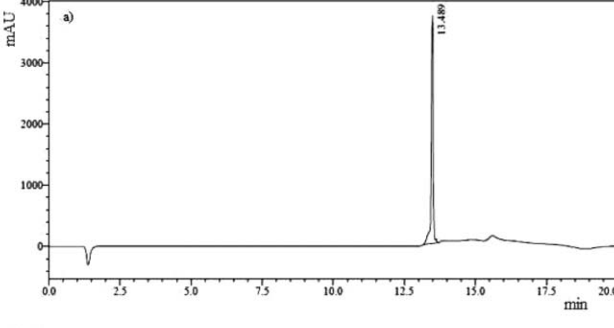
media are soluble in the aqueous phase, so that the catalyst is less exposed to the organic solvent.[15,22,23]

Ethyl acetate in 0.1 M Tris-HCl buffer pH 8 at 50% (v/v) was selected as the reaction medium for peptide synthesis based on the high stability of *asclepain* (Table 1), and the appropriate solubility and partition coefficient of the amino acid derivatives in that medium. The solubilities of Z-Y-pNO in ethyl acetate and H-Gln-OH in buffer were 82.85 mM and 122.85 mM, respectively. These solubility values were significantly higher than those found in 30% (v/v) 1-octanol and in 50% (v/v) hexane, so that 50% (v/v) ethyl acetate was selected as the best medium for performing the synthesis reaction. The partition coefficient of Z-Y-pNO in the biphasic medium of 0.1 M Tris-HCl buffer pH 8 and 50% (v/v) ethyl acetate was 1.28.

Enzymatic synthesis of peptide

Figure 3 shows the separation of reactants and products after 5 min of reaction from a representative sample of the peptide enzymatic synthesis, using Z-Y-pNO and H-Gln-OH as acyl donor and nucleophile, respectively.

According to Figure 3a, at retention time (t_R) of 9.4 min in the aqueous phase a peak of the main product (I) was observed, which was hydrolyzed after 3 h of reaction. Besides, a byproduct at t_R of 15.2 min (V) was observed in the organic phase, which remained invariable throughout the



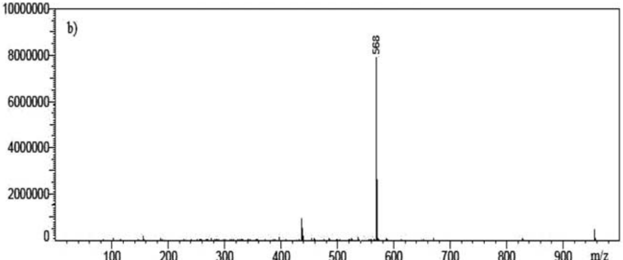


Figure 4. Representative analysis of Z-YQQ peptide obtained by enzymatic synthesis.

Peak I (t_R 9.4 min, aqueous phase) of the reaction between *N*-α-[Carbobenzyloxy]-Tyr-*p*-nitrophenyl ester (Z-Y-pNO) and H-Gln-OH as acyl donor and nucleophile respectively, using *asclepain* as a catalyst at 40°C and 200 rpm. Ion mass *m*/*z* 568 corresponded to Z-YQQ peptide. (a) RP-HPLC chromatogram, (b) MALDI-TOF MS spectra.

reaction time (Figure 3b). It was demonstrated that V was formed from the reagents in the absence of enzyme, by comparison with the reagent control (dotted lines in Figure 3b). However, I in the aqueous phase could be separated easily from the other reactants and from byproducts by stopping the agitation at the end of the reaction.

In the coupling reactions under kinetic control, an activated acyl donor reacts with the enzyme and forms an acyl-enzyme intermediate, which provides the N-terminal segment of the peptide product.[24] The acyl-enzyme intermediate undergoes aminolysis with the N-terminal of the nucleophile, which becomes the C-terminal segment of the peptide product. If these coupling reactions were carried out in an aqueous environment, nucleophile and water would compete by either cleaving the acyl-enzyme intermediate and forming a peptide bond or hydrolyzing the acyl-donor substrate. However, the hydrolyzed acyl-donor substrate (Z-Y) in this reaction of synthesis was undetectable and the tripeptide (Z-YQQ) was obtained with acceptable yields.

As shown in Figure 4, the mass spectrum of the main product of the reaction of synthesis (I, t_R 9.4 min), revealed an ion mass of *m*/*z*: 568, corresponding to the peptide Z-YQQ.

Chemical synthesis of peptide

YQQ peptide was chemically synthesized using Nα-Fmoc strategy, as it was previously described. After the first amino acid was loaded, a substitution of 0.6 mmol/g resin was obtained. The purity was higher than 95% as confirmed by RP-HPLC (Figure 5a). Peptide molecular mass was 438 Da, corresponding to the YQQ portion of the Z-YQQ peptide as confirmed by MALDI-TOF mass spectrometry (Figure 5b).

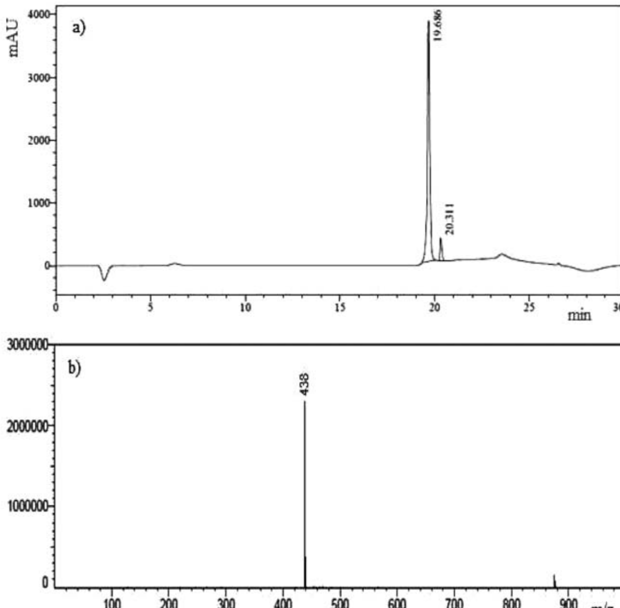


Figure 5. Representative analysis of YQQ peptide obtained by chemical synthesis, using Nα-Fmoc group removal optimized protocol.

Ion mass *m*/*z* 438 corresponded to YQQ peptide. (a) RP-HPLC chromatogram, (b) MALDI-TOF MS spectra.

Anticoagulant activity

The anticoagulant activity of Z-YQQ and YQQ peptides was determined by Wiener Lab Test, as previously described. APTTT, PTT, and TTT are usually used for clinical detection of the abnormality of blood plasma and for the primary screening of anticoagulant chemicals.[25] APTTT and PTT mostly reflect the inhibition effect of the tested samples on the intrinsic and extrinsic pathways, respectively, whereas TTT is considered an indicator of the content and coagulation activity of fibrinogen in plasma in the common pathway of the coagulation cascade.[26]

According to TTT, it was found that Z-YQQ peptide caused a 10 s delay in the conversion time from fibrinogen to fibrin. That is to say, the coagulation time in the sample (with peptide) was 50% higher than in the control (Table 2).

On the other hand, PTT showed that Z-YQQ peptide caused a 19 s delay in the conversion time from prothrombin to thrombin. In other words, the coagulation time was 136% higher in the sample with peptide than in the control (Table 2).

Finally, the action of Z-YQQ on some factors (VIII, IX, XI, and XII) from the coagulation cascade was evaluated using APTTT. Although Z-YQQ peptide caused a 9 s delay with respect to the control, there is no significant difference between the APTTT values obtained and the reference values. Thus, Z-YQQ did not act upon the intrinsic pathway. According to the results reported, Z-YQQ tripeptide acted on the extrinsic pathway of the coagulation cascade (Figure 1).

In contrast, the chemically synthesized YQQ peptide acted specifically on some factors (VIII, IX, XI, and XII) from the coagulation cascade, increasing the coagulation time by 105% with respect to the control (Table 2). YQQ tripeptide acted on the intrinsic pathway of the coagulation cascade (Figure 1).

According to the literature, the most widely used laboratory assay for monitoring unfractionated heparin therapy is APTTT. A fixed therapeutic range for the APTT of 1.5 to 2.5 times the control value has become widely accepted.[27]

Table 2. Clotting Tests Performed for Assessing the Anticoagulant Activity of Tripeptides Z-YQQ and YQQ, Obtained by Enzymatic and Chemical Synthesis Respectively

	APTT (s) Control: 36 ± 3.391	PT (s) Control: 14 ± 1.789	TT (s) Control: 20 ± 1.414
Reference values	30 − 43	10 − 14	17 − 21
Z-YQQ	45 ± 1.789	33 ± 3.050	30 ± 2.714
YQQ	74 ± 1.673	16 ± 2.000	16 ± 0.632
Heparin	56 ± 0.707	33 ± 2.280	31 ± 0.983

APTT: activated partial thromboplastin time; PT: prothrombin time; TT: thrombin time. Negative controls were carried out under same conditions as the samples, but with no peptides. Positive control was carried out under equal conditions than those of the samples, but with heparine.

According to our results, 0.7 ppm heparin showed similar TT and PT values than Z-YQQ, while APTT was 24% higher than in the last one. In addition, 0.7 ppm heparin showed 76% of the APTT value with respect to that of YQQ (Table 2).

Peptide stability in human plasma

Figures 6a,b shows the retained anticoagulant activity (TT, TP, and APTT, s) of Z-YQQ and YQQ peptides as a function of incubation time (min) in human healthy plasma. According to that Figure, Z-YQQ and YQQ peptides retained between 82 and 93% of thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) after 15 min of incubation in human healthy plasma. These results show the high stability of the chemically and enzymatically synthesized peptides in the time range studied.

Fibrinogen in plasma by immune turbidimetry method

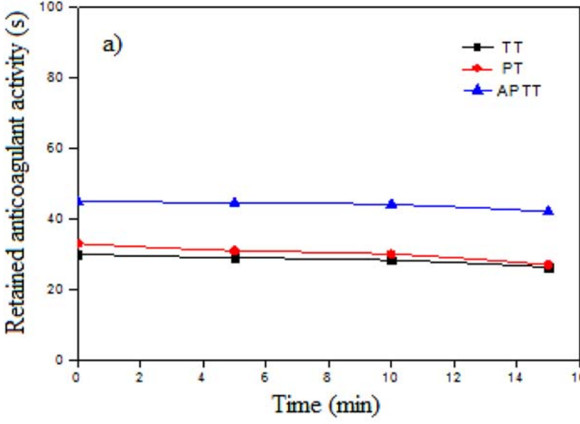
Table 3 shows the effect of the synthetized peptides (Z-YQQ and YQQ) on thrombin enzyme. According to our results, thrombin was inhibited by Z-YQQ (17%), delaying the depolymerization time from fibrinogen to fibrin and clot formation, in the same manner than that observed with heparin (18%) under the study conditions. Besides, YQQ only caused 4% of thrombin inhibition. This fact explains the action of YQQ on the intrinsic pathways of the coagulation cascade at some earlier point of factor X, as it was previously demonstrated by APTTT values (Table 2). Factor X produces the activated factor Xa which allows for the conversion of prothrombin (II) into thrombin (IIa) (Figure 1).

Toxicity activity

Figure 7 displays the percentage of cell viability after exposure of 293FT human cell line to different concentrations of Z-YQQ and YQQ peptides (0.7 to 21 ppm), for 4 h at 37°C in 5% CO_2 enriched air atmosphere.

Kruskall-Wallis method was used to test for significant differences in cell viability values after exposing the 293FT human cell line to different peptide concentrations and (C+) positive and (C−) negative controls.[28]

The synthesized peptides (Z-YQQ and YQQ) did not produce any significant decrease ($P \leq 0.05$) in cell survival under the conditions of the toxicity test. Under some treatment conditions, survival rates above the control (100%) were measured. However, as these differences are less than 10%, they are not considered to be a significant proliferative



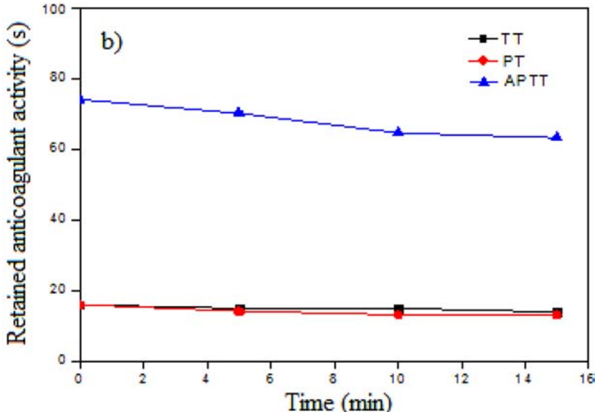


Figure 6. Retained anticoagulant activity (APTT, TP, and TT) of (a) Z-YQQ and (b) YQQ peptides in human healthy plasma as a function of incubation time. APTT Control: 36 s; TP Control: 14 s; TT Control: 20 s.

Table 3. Effect of the Synthetized Peptides (Z-YQQ and YQQ) on Thrombin Enzyme, Using Quantitative Determination of Fibrinogen in Plasma by Immune Turbidimetry Method

	Fibrinogen Concentration (mg mL^{-1})	Inhibition Percentage (%)
Blank control (BC)	0.83 ± 0.000	(a)
Negative control (C–)	0.50 ± 0.000	(b)
Positive control (C+)	0.65 ± 0.000	18
Z-YQQ	0.64 ± 0.122	17
YQQ	0.53 ± 0.000	4

(a) Total content of fibrinogen in the sample (without thrombin), (b) Content of the remaining fibrinogen in the sample after thrombin action, under study conditions.

effect.[29] Probably, this effect may be related to the hormesis phenomenon, which is a commonly term used by toxicologists to refer to a biphasic dose response to an environmental agent. It is characterized by low dose stimulation or beneficial effect and high dose inhibitory or toxic effect.[30] According to Kruskall-Wallis test, Z-YQQ and YQQ were not cytotoxic in in vitro assays at the concentrations analyzed.

Conclusion

Z-YQQ and YQQ, two new anticoagulant peptides potentially useful for safe pharmaceutical applications, were obtained by enzymatic and chemical synthesis, respectively.

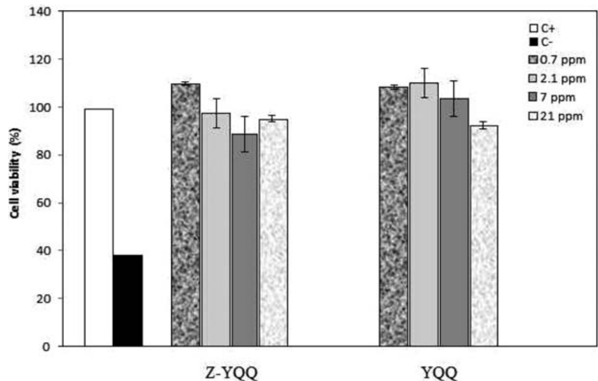


Figure 7. In vitro cytotoxicity activity of Z-YQQ and YQQ over 293FT cell line.

Nevertheless, some aspects of their production should be optimized. Other issues such as absorption, effectiveness, bioavailability, and the molecular mechanisms of activity should be further investigated before using these tripeptides as drugs. Finally, this study demonstrated that phytoproteases from *Asclepias curassavica* L., an indigenous plant from South America, can be used as a new biocatalyst for bioactive peptide synthesis.

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