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# Anticoagulant activities of goby muscle protein hydrolysates

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ABSTRACT

The anticoagulant activities of protein hydrolysates prepared from goby muscle by treatment with various bacterial alkaline proteases were investigated. All proteases exhibited varying degrees of hydrolysis (DH) and all goby protein hydrolysates (GPHs) caused a significant prolongation of both the thrombin time (TT) and the activated partial thromboplastin time (APTT). The hydrolysate generated by the crude protease from *Bacillus licheniformis* NH1 displayed the highest anticoagulant activity, and the higher TT (about 32 s) at a concentration of 5 mg/mL was obtained with hydrolysate having a DH of 8.86%. This hydrolysate was then fractionated by size exclusion chromatography on a Sephadex G-25 column into five major fractions (F1–F5). Fraction F2, which exhibited the highest anticoagulant activity, was then fractionated by reversed-phase high-performance liquid chromatography. The molecular masses and amino acid sequences of four peptides in peptide sub-fraction F2–6, which exhibited the highest anticoagulant activity, were determined using ESI-MS and ESI-MS/MS, respectively. The structures of these peptides were identified as Leu-Cys-Arg, His-Cys-Phe, Cys-Leu-Cys-Arg and Leu-Cys-Arg-Arg.



## 1. Introduction

Coagulation is the second step of haemostasis. It helps stop the bleeding by the consolidation of the platelet aggregate obtained at the end of primary haemostasis (Allford & Machin, 2004). The coagulation system is triggered in response to endothelium rupture. It involves a cascade of enzymatic reactions involving clotting factors, many of which are serine proteases subjected to activation and inhibition (Davie & Ratnoff, 1964; Macfarlane, 1964). Human blood coagulation consists of an intrinsic and an extrinsic pathways. The two pathways converge at the formation of factor Xa (FXa) by factor IXa (FIXa) in an intrinsic pathway and FVIIa in an extrinsic pathway. The final step is the conversion of soluble fibrinogen into fibrin filaments by the action of thrombin. An imbalance in this balance leads to either bleeding or the formation of a thrombus (Allford & Machin, 2004). Accumulation of fibrin in blood vessels can interfere with blood flow and lead to a multitude of serious cardiovascular diseases. These are the leading causes of mortality. Indeed, studies conducted by the World Health Organisation in 2003, showed that heart attacks accounted for 30% of the total mortality rate in the world. To treat blood clots and prevent the damage they cause, doctors use anticoagulants, which are commonly called blood thinners, to decrease the clotting power of the blood and prevent growth of a clot. The most common anticoagulants used today are unfractionated heparin, low molecular weight heparin, and warfarin. Therapy with heparin prevents the extension and subsequent growth of a developing thrombus (Chiu, Hirsh, Yung, Regoeczi, & Gent, 1977). However, it does not demonstrate complete efficacy in all patients (Agnelli, Pascucci, Cosmi, & Nenci, 1990).

Therefore, research and development to find new antithrombotic agents (or drugs, or molecules) with reduced side risks is necessary for the prevention of thromboembolic events.

Several studies in the past few decades have established that bioactive peptides, beyond their nutritional value, exhibit biological activities, such as opioid (Bitri, 2004), antioxidant (Bougatef et al., 2010), antihypertensive (Balti, Nedjar-Arroume, Guillochon, & Nasri, 2010), etc., and may therefore serve therapeutic roles in the body (Erdman, Cheung, & Schröder, 2008). These peptides have a size of 2–20 amino acids (Meisel & FitzGerald, 2003). Based on the sequence of amino acids, these bioactive peptides exhibit diverse activities on the digestive system, the body's defences (e.g., antimicrobial or immunomodulatory effect), cardiovascular system (including antithrombotic and antihypertensive effects), and/or nervous system (such as a sedative and analgesic effect of opioid-like peptides) by inhibition of the key factors of these systems (enzyme, coenzyme, etc.).

Bioactive peptides, which are inactive within the sequence of the parent proteins, can be released by enzymatic hydrolysis,

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either during gastrointestinal digestion or during food processing (e.g., cheese ripening and milk fermentation) or in vitro by treatment with proteolytic enzymes. All food materials, especially those rich in proteins, can be used in the production of bioactive peptides. They are found in milk, egg, and fish of various kinds as well as in many plants.

Many of the known bioactive peptides are multifunctional and can exert several activities (Meisel & FitzGerald, 2003). Because of their health-enhancing potential and safety profiles they may be used as components in functional foods or nutraceuticals.

Bioactive peptides with anticoagulant activity have been reported for certain food proteins. Peptide sequences which inhibit platelets aggregation and the binding of the human fibrinogen γ-chain to platelet surface fibrinogen receptors have been identified in caseinomacropeptide, which is split from κ-casein in milk coagulation with rennin (Fiat et al., 1993). Shimizu et al. (2009) upon papain treatment of pork meat identified a bioactive peptide with mean molecular weight 2500 which showed antithrombotic activity in vivo after oral administration to mice at 210 mg/kg body weight. Moreover, Deng et al. (2010) isolated a novel anticoagulant named AcaNAP 10, with potent inhibitive activity against both FXIa and FVIIa tissue factor from blood-feeding nematode *Ancylostoma caninum*. Jung and Kim (2009) isolated an anticoagulant oligopeptide from blue mussel (*Mytilus edulis*) with an approximate molecular mass of 2.5 kDa, which could prolong both the thrombin time and the activated partial thromboplastin time and specifically interact with blood coagulation factors FIX, FX and FII. More recently, Yang, Wang, and Xu (2007) reported that egg white protein hydrolysate possesses antithrombotic activity.

The goby (*Zosterisessor ophiocephalus*), is common in the Mediterranean Sea, Black Sea, and Sea of Azov. It reaches a maximum length of 25 cm and it is carnivorous; juveniles feed on small crustaceans, polychaetes and molluscs. It is relatively important in the fish-catches of Tunisia, and is utilised for human consumption. In Tunisia, goby catches were about 130 tonnes in 2004 (FAO, 2004).

Anticoagulant peptides derived from marine organisms by enzymatic treatment have rarely been isolated. In this study, we investigated the anticoagulant activity of goby muscle protein hydrolysates obtained by treatment with various proteases. The amino acids sequences of four peptides in the most active sub-fraction F2–6 were determined.

## 2. Materials and methods

### 2.1. Reagents

Thrombin and fibrinogen fraction I from bovine plasma were purchased from Sigma Chemical Co. (St. Louis, MO). CK-PREST was from Diagnostica Stago S.A.S. (Asnières sur Seine, France). Acetonitrile was of HPLC grade. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Water was obtained from a Culligan system; the resistivity was approximately 18 MΩ. All other chemicals and reagents used were of analytical grade.

### 2.2. Materials

Goby (*Z. ophiocephalus*) was purchased from the fish market of Sfax city, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample:ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. Muscle was separated, rinsed three times with distilled water to remove salts and other contaminants, and then stored in sealed plastic bags at −20 °C until they were used for protein hydrolysates production, less than 1 week later.

### 2.3. Proteolytic enzymes

Alcalase® 2.4 L serine-protease from *Bacillus licheniformis* was supplied by Novozymes® (Bagsvaerd, Denmark). Crude enzyme preparations from *B. licheniformis* NH1 (El Hadj-Ali et al., 2007), *Bacillus mojavensis* A21 (Haddar, Agrebi, Bougatef, Sellami-Kamoun, & Nasri, 2009), *Bacillus pumilus* A1 (Fakhfakh-Zouari, Haddar, Hmidet, Frikha, & Nasri, 2010) and *Bacillus subtilis* A26 (Agrebi et al., 2009) were prepared in our laboratory.

The protease activity in the crude enzyme preparations was determined by the method of Kembhavi, Kulkarni, and Pant (1993), using casein as a substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

### 2.4. Preparation of goby protein hydrolysates (GPHs) using various proteases

Goby muscle (500 g), in 500 mL distilled water, was first minced using a grinder (Moulinex Charlotte HV3, France) then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The cooked muscle sample was then homogenised in a Moulinex® blender for about 2 min. The samples were adjusted to optimal pH and temperature for each enzyme: Alcalase (pH 8.0; 50 °C), crude enzymes from *B. licheniformis* NH1 and *B. mojavensis* A21 (pH 10.0; 50 °C), crude enzyme from *B. pumilus* A1 (pH 8.5; 50 °C) and crude enzyme from *B. subtilis* A26 (pH 8.0; 45 °C). Then, the substrate proteins were digested with enzymes at a 3:1 (U/mg) enzyme:protein ratio for 5 h 30 min. Enzymes were used at the same activity levels to compare hydrolytic efficiencies. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of 4 N NaOH. After the required digestion time, the reaction was stopped by heating the solutions for 20 min at 80 °C to inactivate enzymes. Protein hydrolysates were then centrifuged at 5000*g* for 20 min to separate soluble and insoluble fractions. Finally, the soluble fractions, referred to as protein hydrolysates, were freeze-dried at −50 °C and 121 mbar (Modulyod-230, ThermoFisher Scientific, Waltham, MA) and then stored at −20 °C for further use.

### 2.5. Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds in the substrate studied ($h_{tot}$), was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen, 1986) according to the following equation.

$$\mathrm{DH}\ (\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times \mathrm{Nb}}{\mathrm{MP}} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where $B$ is the amount of NaOH consumed (mL) to keep the pH constant during the proteolysis of the substrate. Nb is the normality of the base, MP is the mass (g) of the protein ($N \times 6.25$), and α represents the average degree of dissociation of the α-$NH_2$ groups in the protein substrate expressed as:

$$\alpha = \frac{10^{\mathrm{pH-pK}}}{1 + 10^{\mathrm{pH-pK}}}$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds ($h_{tot}$) in a the protein substrate was assumed to be 8.6 meq/g (Adler-Nissen, 1986).