Biochemical characterization of proteases and anticoagulants from fig latex (*Ficus carica*) trees.

A Thesis

Submitted to Faculty of Science Ain Shams University in Partial Fulfillment of Master Degree in Biochemistry

Presented By

Mohamed Belal Hamed Soliman

B.Sc., Biochemistry, 2005

Under Supervision of

Prof. Dr. Ibraheem H. Borai

Professor of Biochemistry

Faculty of Science

Ain Shams University.

Prof. Dr. Afaf S. Fahmy

Professor of Biochemistry

Molecular Biology Department

Genetic Engineering and Biotechnology Division

National Research Centre.

Dr. Eman I. Kandil

Asst. Professor of Biochemistry

Faculty of Science

Ain Shams University.

Faculty of Science

Ain Shams University

2011

دراسة الخواص الكيميائية الحيوية لإنزيمات البروتييز ومضادات التجلط فى العصارة اللبنية لشجرة التين البرشومى.

رسالة مقدمة إلى

قسم الكيمياء الحيوية- كلية العلوم- جامعة عين شمس

كجزء متمم للحصول على درجة الماجستير فى الكيمياء الحيوية

من

محمد بلال حامد سليمان

بكالوريوس الكيمياء الحيوية (٢٠٠٥)

تحت إشراف

أ.د/إبراهيم حسن برعى
أستاذ الكيمياء الحيوية
كلية العلوم
جامعة عين شمس

أ.د/عفاف سعد الدين فهمى
أستاذ الكيمياء الحيوية
قسم البيولوجيا الجزيئية
شعبة الهندسة الوراثية والبيوتكنولوجيا
المركز القومى للبحوث

أ.م.د/إيمان إبراهيم قنديل
أستاذ مساعد الكيمياء الحيوية
كلية العلوم
جامعة عين شمس

كلية العلوم
جامعة عين شمس
٢٠١١

Abstract

The quantitative determination of alkaline proteases from the latex of *Ficus carica* was carried out revealing that *F. carica* crude extract could be a good starting material for protease production. Purification was carried out for the three proteases using columns of DEAE-cellulose, CM-cellulose and Sephacryl S-200. Biochemical characterization for FPIII with respect of molecular weight (48 kDa), pH optimum (8.5), temperature optimum (60ºC), activation energy (7 kcal/mol), substrate specificity, Michaelis constant, heat stability, effect of different metal cations and different compounds was carried out. The inhibition studies revealed that FPI and FPII belonged to cysteine proteases, while FPIII belonged to serine protease. The anticoagulant effect and fibrinogenolytic activity of *F. carica* crude extract and alkaline protease FPIII was studied.

Key Words: *Ficus carica*, Proteases, Purification, Characterization, FPI, FPII, FPIII, Anticoagulant and Fibrinogenolytic.

Contents

Introduction

The presence of latex in plants is one of the characteristic features belonged to the families *Euphorbiaceae*, *Asclepiadaceae*, *Moraceae* and *Apocyanaceae* **(Rajesh *et al*., 2005)**. The genus *Ficus* belongs to order *Urticales* and family *Moraceae* (the family of flowering plants) **(Robbins, 1930)**. *Moraceae* is composed of trees and shrubs which characteristically have a milky juice. At least five of the genera contain high proteolytic activity in their milky juice; these are *Maclura*, *Morus*, *Brosimum*, *Broussonetia* and *Ficus* **(Williams *et al*., 1968).**

Ficus genus consists of over 800 species and is one of about 40 genera (**Joseph and Justin Raj, 2011**) from pantropica and subtropical origins. The latin name, *carica*, is named for the location Caria in Asia Minor which is supposedly the home of the fig (**Nadel *et al*., 1992**). *Ficus* consists of numerous varieties with significant genetic diversity. There are diverse forms of *Ficus* such as the common edible fig (*F. carica*), the sycamore fig (*F. sycomorus*), the banyan tree (*F. indica* and *F. belonghalensis*), various strangling figs, which include *F. bonplandiana* and *F.*

padifolia, the rubber tree (*F. elastica*) and the creeping fig (*F. repens*) belong to this genus (**Salhi-Hannachi *et al*., 2006**).

Proteases (EC 3.4) are also referred to as peptidases or proteolytic enzymes. They have been isolated from vegetables (latex, fruits and seeds) and animals (bee venoms, snake venoms, scorpion and spider venoms) **(Costa *et al.*, 2010)**.

Proteases are hydrolytic enzymes that are catalyzing the hydrolysis of peptide bonds in proteins. According to the position of the peptide bond to be broken, they can be classified as endopeptidases or exopeptidases. The endopeptidases (EC 3.4.21-99) hydrolyze peptide bonds within polypeptide structures, while exopeptidases (EC 3.4.11-19) act at the N or C terminal of the polypeptide chain (**Costa *et al.*, 2010**).

According to the active site involved, they may be further classified as serine, cysteine, aspartic and metalloproteases. In serine and cysteine proteases, the nucleophilic agents are part of an amino acid of the catalytic site, but in aspartic acid and metalloproteases, the nucleophilic is an activated water molecule **(Priolo *et al*., 2000)**.

The physiological functions of proteases ranging from generalized protein digestion to more specific regulated processes such as the activation of zymogen, blood coagulation, complement activation, inflammation process and liberation of physiological peptides from precursor protein by specific proteases, since the cleavage occur between certain pairs of amino acids **(Pèrez and Sanchez, 2000)**. In addition, the proteolytic enzymes are ubiquitous in nature **(Neurath, 1984; Devaraj *et al.*, 2008)** and constitute nearly half of the commercially utilized enzymes, frequently used in food processing, tenderization of meat, brewing, cheese elaboration, bread manufacturing, leather and textile industry **(Devaraj *et al.*, 2008).**

Cardiovascular diseases such as high blood pressure, acute myocardial infraction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, stroke, etc. are the primary causes of death throughout the world **(Mine *et al*., 2005; Simkhada *et al*., 2010)**. In thrombosis, largely unknown conditions promote the apparently spontaneous formation of clots large enough to block circulation in arteries which supplied vital organs, such as the heart or brain. Anticoagulants are pivotal for the prevention and treatment of thrombotic disorders, and approximately

0.7% of the western population receives oral anticoagulant treatment **(Gustafsson *et al.*, 2004)**.

Anticoagulants have contributed for understanding of molecular mechanisms of blood coagulation and have provided potential new leads for the development of drugs to treat or to prevent unwanted clot formation. Some of these anticoagulants exhibit various enzymatic activities whereas others do not. They interfere in normal blood coagulation and prolonged the blood-clotting process by different mechanisms either by specifically inactivating zymogen, one of the blood coagulation factors, or by directly destruction of fibrinogen **(Gustafsson *et al.*, 2004**; **Devaraj *et al.*, 2008)**.

The treatment of thrombotic diseases with fibrinolytic therapy has been often employed mainly with use of plasminogen activators such as streptokinase, urokinase, and the genetically engineered tissue plasminogen activator (t-PA). Intravenous administration of urokinase and streptokinase has been widely used but these enzymes have low specificity to fibrin and are expensive. However, t-PA has been developed for this therapy because of its efficacy and stronger affinity to fibrin **(Sherry, 1987)**. The other thrombolytic agents used are the plasmin-like protein, e.g., nattokinase **(Sumi *et al.*, 1987)** and lumbrokinase **(Sumi *et al.*, 1987)**, which can directly degrade the fibrin of blood clots. On the basis of catalytic mechanisms, most of fibrinolytic enzymes are classified into serine proteases or metalloproteases (**Simkhada *et al.*, 2010**). Therefore, the search for new potential plant serine proteases still continues, in order to make these industrially and pharmaceutically applicable and cost-effective, as well as to understand their physiological role in plants.

Review of literature

The fig tree (*Ficus carica*) is cultivated for its fruit in temperate zones and has been investigated for its proteolytic enzyme ficin (**Porcelli, 1967; Hatano, 2006**), organic acids (**Shiraishi *et al.,* 1996**), sugars (**Kim *et al.,* 1992**), and natural rubber (**Kim *et al.,* 2003**). The dried fruit has been a very familiar food for human beings since 11,000 years (**Kislev *et al.,* 2006**) and California is especially famous for the production. The nutritional characteristic of dried figs is known for the abundance of calcium, potassium, and dietary fiber as compared with other fruits. Despite its high agricultural, economic, and medical values, little attention has been given to investigate the physiological and biochemical traits of the dry fruit and latex (**Kim *et al.,* 2003**).

In the *ficus* fruit, root and leaves are used in the native system of medicine in various disorders such as gastrointestinal (colic, indigestion, loss of appetite and diarrhea), respiratory (sore throats, coughs and bronchial problems), inflammatory, cardiovascular disorders, ulcerative diseases, and cancers (**Pèrez *et al.,* 1999; Canal *et al.,* 2000; Rubnov *et al.,* 2001; McGovern 2002; Gilani *et al.,* 2008**). *Ficus carica* has been reported to have numerous bioactive compounds such as psoralen, bergapten, umbelliferone (**Seong-Kuk *et al.*, 1995; Louis *et al.*, 2000**), arabinose, β-amyrins, β-carotines, glycosides, xanthotoxol (**Ross and Kasum, 2002; Vaya and Mahmood, 2006; Gilani *et al.*, 2008**). β-sitosterol, campesterol, stigmasterol, fucosterol, fatty acids (**Jeong and Lachance, 2001**), 6-(2-methoxy-Z-vinyl)-7-methyl-pyranocoumarin and 9,19-cycloarlane triterpenoid as an anticancer (**Weiping *et al.*, 1997; Lansky *et al.*, 2008**) and antiproliferative agent: 6-O-acyl-β-D-glucosyl-β-sitosterol (**Shai *et al.*, 2001**), calotropenyl acetate, and lupeol acetate (**Saeed and Sabir, 2002**). The researchers reported the hypoglycemic action of a fig leaf decoction in type-I diabetic patients and used a chloro- form extract, obtained also from a decoction of *F. carica* leaves, to decrease the cholesterol levels of rats with diabetes (**Ross and Kasum, 2002**). *F. carica* has been reported to include antioxidant, antiviral, antibacterial, hypocholesterolaemic, hypotriglyceridaemic, and anthelmintic effects (**Pèrez *et al.*, 1999; Canal *et al.*, 2000; Rubnov *et al.*, 2001; Wang *et al.*, 2004; Jeong *et al.*, 2005; Solomon *et al.*, 2006**). It has also been investigated for its proteolytic enzymes, amino acids, minerals, sugars, triter-penes, organic acids, and allergens (**McGovern, 2002; Vaya and Mahmood, 2006**). The methanol extract of the leaves of *F. carica* Linn. (*Moraceae*) was evaluated for hepatoprotective activity in rats with liver damage induced by carbon tetrachloride (**Krishna- Mohan *et al.*, 2007**).

Latex is the cytoplasm of the laticiferous cells; its organominerat composition has all the features of ordinary plant cytoplasm except that it contains 30-45% rubber and the nuclei and mitochondria are not expelled during tapping.

Among flowering plants, over 20,000 species (from over 40 families in multiple lineages) contain latex (**Farrell *et al.*, 1991; Lewinsohn, 1991; Hunter, 1994**). Latex is found in dicotyledonous and monocotyledonous (e.g., *Liliaceae*) plants. This finding, that nearly 10% of families and species produce latex, implies that latex is a highly convergent trait (that is, has evolved independently multiple times) and that latex is likely encountered by many herbivore species (**Agrawal and Konno, 2009**).

These species (angiosperms) exude latex upon tissue damage due to action of turger pressure and this latex has no known function in primary metabolism (in terms of plant resource acquisition and allocation) (**Farrell *et al.*, 1991; Lewinsohn, 1991; Hunter, 1994**).

Latex is sometimes colored yellow, orange, or red, such as that of *Cannabis* (*Cannabaceae*). Latex is well known for its sticky properties, which have been used to produce rubber (from *Hevea brasiliensis*, *Euphorbiaceae* and other species), chicle from *Manilkara spp.* (*Sapotaceae*) used in chewing gum, and lacquers from phenols in the latex of plants in the *Anacardiaceae*. Latex from various plant species contains bioactive compounds including alkaloids such as asmorphine in *Papaver spp*. (*Papaveraceae*); cardiac glycosides in *Asclepias spp*. (*Apocynaceae*); terpenes such as the sesquiterpene lactone, lactucin, from lettuce (*Lactuca spp. Asteraceae*); and digestive cysteine proteases in *Carica papaya* (*Caricaceae*) and *Ficus spp.* (*Moraceae*) (**Agrawal and Konno, 2009**).

As early at the 1600s, the term latex was used by English-speaking physicians, its function was analogized to lymphatic vessels of animals, and it was studied in several plant families (**Mahlberg, 1993**). Aboriginal cultures of Southeast Asia have used exudates of *Antiaris spp*. containing cardiac glycosides as a poison for arrow tips (**Evert, 2006**). Opium poppy (*Papaver somniferum*) owes both its fame and notoriety to opiates found in latex (**Evert, 2006**).