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Concise report on study of proteinase inhibitors and their utilization in practice (from 1986 – July 2007)

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Abstract. Proteinase inhibitors (PIs) are widely distributed in plants, microorganisms and mammalian species; they are known to play pivotal roles in regulating proteinase activities in these species. Accordingly, PIs are important molecular tools for many research disciplines. Studies of the correlation of PI structures and functions have lead to understanding of their inhibitory actions against physiological important proteases. There are potential therapeutic applications for such studies for diseases relating to excessive activities of proteases. Our studies on PIs in Viet nam have been begun since 1986, focusing on the following topics:

Quantitative analysis of proteinase inhibitory activity (PIA) in wild type and cultivated plants; tracing of PIs in the process of seed development and the distribution of PIs in different parts of seed; establishment of the procedure for isolation of the selected PIs; the procedure for using PIs preparations in medical and agricultural practice; characterization of the isolated PIs, study of their structure; investigation of their potential therapeutic and agricultural applications; clinical trial studies for the selected PIs preparations.

This paper is a brief summary of our 56 works published in the national and international journals as well as at the symposia and conferences .

Keywords: proteinase inhibitors, Momordica cochinchinensis, anti-inflamation, anti-bacterial activity, insecticide.

1. Introduction

Proteases are widely found in plants, and animals as well as in microorganisms. It is reported that 2% of all gene products are proteases (Barret et all 1998). They play a crucial role in the physiology and pathology of living organisms by controlling the synthesis, turnover and function of proteins.

Proteinase inhibitors are quite common in nature and also present in all life forms.The corresponding inhibitors of most proteases exist in nature. They are known to play a pivotal role in regulating proteolytic activity in living cells. Therefore, they are expected to be an effective tools for treatment of diseases caused by excessive activity of proteases, bloking proteolytic process involving an infecting, inflammation processes. Protease inhibitors (PIs) have also been used as insecticide. In Viet nam, our laboratory have

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been studying trypsin inhibitors (TIs) since 1986 as a continuance of our previous work [1]. The subjects of our research on PIs include:

Quantitative analysis of proteinase inhibitory activity (PIA) in wild type and cultivated plants [2-9]. Tracing of PIs in the process of seed development [10-17], and the distribution of PIs in different parts of seed [4, 18,19].

Establishment of the procedure for isolation of the PIs [20-26] and of the procedure for using PIs preparations in medical and agricultural practice [21,27-30].

Characterization of the isolated PIs, study of their structure [23,28,31-35] and their potential therapeutic and agricultural applications [36-42].

- Clinical trial studies for the selected PIs preparations [29,43,44].

2. Comprehensive Results (abridged)

2.1. *Quantitative analysis* trypsin inhibitor activity (TIA) of various wide and cultivated plants showed that *Momordica cochinchinensis* seeds (MCo) are the richest source of TIs [3].

It has been known that plant proteinase inhibitors, which play a potent defensive role against predators and pathogens, are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding. Several plant serine-proteinase inhibitors exhibit insecticidal as well as antibacterial activities . These findings along with our knowledge in the use of MCo seeds in traditional medicine prompted us to search for insecticidal and antimicrobial properties of a MCo TIs- rich preparation named "*Momorsetatin*" or "*Mos*".

2.2. The insecticidal activity of Mos.

The insecticidal activity experiments have been carried out in our laboratory and in green vegetable field and targeted the larvae of *Spodoprera litura* (Sl) and *Plutella xylostella* (Px), which severely destroy green vegetale field. The laboratory assays showed that after 2 days of feeding the 2nd instar larvae of Px by leaves treated with Mos, the insecticidal effect (calculated according Henderson Tillton) reached 70-80%. Moreover, Mos was able to inhibit the growth and the development of Sl and Px 2nd and 3rd instar larvae, thus reducing their destruction ability [23]. Besides, Mos also reduced the portion of larvae entering the pupa, and increased the rate of anomalous butterflies by half (16.42% while in control was 8.56%) [45-47].

Experiments on the field with mustard greens, cabbages, kohlrabi, cauliflowers showed that the insecticidal effect agains Px of Mos reached more than 60% [48].

As is known, several serine-proteinase inhibitors promote the insecticidal activity of the insect control proteins from *B. thuringinensis* (Bt) against their target insects. In fact, the insecticidal effect against Px of a mixed preparation of Mos with Bt (each ingredient 50% of its using dose, named MM) was higher than that of Mos or Bt preparation alone. Moreover MM had no negative effect on the leaves of green vegetables (The National Research project coded KHCN-02-08B conducted by the author)

2.3. The antimicrobial activity of Momosertatin preparation [38,43,49]

Mos preparation was partly purified by gel filtration chromatography on Sephadex G-75

column.The TI fractions were pooled (named MCoTIs).By using PAGE copolymerized with substrate, three TIs bands were detected on the gel. The Rm values of these TIs bands corresponded to those of protein bands detected by using PAGE Laemmli method.

In connection with the antimicrobial activity of McoTIs, 145 microbial samples including those isolated from soil, from pus of patient's burn wound have been tested. The results obtained have indicated that the antimicrobial activity of MCoTIs is unspecific: it inhibited the growth of both negative (G^-) and positive (G^+) bacteria, *Candida, Aspergillus* and *Streptomyces*. A study the sensitivity of the microorganisms to MCoTIs indicated that at low concentration, MCoTIs only inhibited bacteria but not fungi nor streptomyces [50]. Further study was carried out on bacteria isolated from infected wound.

Inhibition of MCoTIs on bacteria isolated from pus of patient's infected burn wound (Pibwp).

Among bacteria isolated from Pibwp, the apparition frequency of *Staphylococcus aureus* (Sa) and *Pseudomonas aeruginosa* (Pseu) were around 39% and 30% respectively [37]. These bacteria, particularly Pseu strongly inhibited by MCoTIs. Therefore, extracellular proteinases of Pseu (Pseu-PA) were isolated and subjected for study. The total proteolytic activity (PA) of cell-free broth strongly decreased in the presence of MCoTIs [38,43]. The partly purified two PA peaks (P1 and P2) obtained by fractionation of Pseu-PA on Sephadex G-75 column and followed by FPLC (with Q column) were also strongly inhibited by MCoTIs. MCoTIs- treated P1 and P2 before loading on polyacrylamide gel, and after running electrophoresis, soaking the gel in buffer containing MCoTIs, PA bands of P1 and P2 were ceased [38].

2.4. The anti-inflammatory action of proteinase inhibitors (PIs) preparations on experimental models.

As is known several PIs have been effectively used to diminish inflammatory process such as acute pancreatitis. Therefore, besides MCoTIs other PI named AT-04 have been investigated. AT-04* isolated from bovine lung inhibiting serine proteinases including trypsin, chymotrypsin, plasmin were subjected to study of their effect on experimental acute and chronic inflammatory models as well as experimental pancreatitis [36,40,44]. Both tested PIs preparations reduced inflammatory processes in both acute and chronic inflammatory models. In the experimental acute pancreatitis, a beneficial effect was

*AT-04 is a preparations produced by us [21,22,31], a product of the National Biotechnology Research project coded 52D-03-10 and KC-08-04) observed when using AT-04 in combination with Somatostatin (Vu Ha, Ph.D thesis, 2001) None toxical effect of AT-04 and MCoTIs was found on tested animal.

Thirty eight patients with infected burn wound were effectively treated by AT-04: inflammation was reduced, the process of wound healing was improved, the duration of treatment was shortened and no side effect was observed (Do L. Tuan, MSc. Thesis, 1999)

All above results have encouraged us to deeper study on PIs from MCo seeds.

2.5. Characterization of TIs from MCo seeds

Three TIs (MCoTI-I, MCoTI-II and MCoTI-III have been purified, characterized and sequenced [23,28,32]. All of them contain three disulfide bridges. The MCoTI-III (the

minor TI) is the linear polypeptide backbone composing of 30 amino acid residues. MCoTI-II and -III consisting of 34 amino acid residues with N to C termini ligation (head to tail ligation) [32] named a "cyclotides". They have been recorgnized to be the first known macrocyclic inhibitors of Cucurbitaceae TI family [32]. Further study indicated that MCoTI-II formed a new family of Cyclic Knottin [33,34]. The MCoTI-I was being synthesized by chemical method [51]. The gene coding for MCoTI-II was also synthesized, cloned and expressed in E.coli [52]. A recombinant Ti-plasmid carring MCoTI-II was also been constructed [53].

Besides, we also cloned and expressed in E. coli of the gene coding for other TI from pumpkin (Cucurbitaceae maxima) seeds named CMTI-V (TI specifically inhibited factor XIIa) [54-56]. In the presence of the recombinant CMTI-V, activated partial thromboplastin time (APTT) increased.

2.6. Trypsin inhibitors from medicinal plants .

Up to-day, we have assayed the trypsin inhibitory activity (TIA), chymotrypsin inhibitory activity (ChIA) and proteolytic activity (PA) of 200 samples from 129 medicinal plants coming from 55 plant families. Among the investigated ones, 70 plants have been used for treatment of skin diseases. Of the crude extracts, 41.8 % were found to possess PA, and 58.2% exhibited inhibitory activity against (TIA), 29.5 % against chymotrypsin (ChIA). However, there were only 4% of the leaf extracts found to possess both TIA and ChIA higher than 100mIU/gam of leaves. It is also been noticed that in the same extract, ChIA was higher than TIA (Pham thi Tran Chau Hoang Thu Ha et al., unpublisfed data).

Kalanchoe pinata leaves have been used for treatment of burn and pustule. From our premilinary study, their leaf extract exhibited high TIA and ChIA. The liophylized leaf juice possess TIA and ChIA of about 180 – 474 mIU/g and 635- 3060 mIU/g respectively. After fractionation on Sephadex G-25 column, two peaks were obtained. The first, major peak (D1) inhibited both trypsin, chymotrypsin and the growth of *Pseudomonas aeruginosa* isolated from patient's infected burn wound pus. Further study indicated that D1 also reduced the proteolytic activity of the *P. aeruginosa*. Moreover, the inhibitory specific activity (mIU/mg protein) against *P. aeruginosa* was about 2 – 8 fold higher than that against trypsin and chymotrypsin. Thus, it was suggested that PIs in the leaf juice may play an important role for the antibacterial activity of the Kalanchoe leaves [9].

At present, we focus our work on screening PIs potented therapeutic uses from medicinal plants and further study the selected subjects.

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Tóm lược kết quả nghiên cứu và ứng dụng các chất ức chế Proteinase (từ 1986–7/2007)

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Các chất ức chế proteinase (PI) khá phổ biến trong tự nhiên, chúng có vai trò quan trọng trong điều hòa hoạt động của proteinase của cơ thể sống. Do đó các PI có thể là công cụ quan trọng trong nhiều lĩnh vực nghiên cứu khác nhau. Nghiên cứu liên quan giữa cấu trúc và chức năng của các PI có thể làm sang tỏ tác dụng ức chế của chúng đối với các protease tham gia vào các quá trình sinh lý quan trọng. Các nghiên cứu này có ý nghĩa đối với việc nghiên cứu sử dụng các PI để điều trị các bệnh có liên quan với sự tăng hoạt độ của potease quá mức bình thường. Chúng tôi bắt đầu nghiên cứu các PI từ năm1986, chủ yếu tập trung vào một số vấn đề sau: định lượng các PI của các mẫu cây mọc dại và cây trồng; theo dõi sự biến đổi các PI trong quá trình phát triển của hạt và sự phân bố của các PI ở các phần khác nhau của hạt; xây dựng quy trình tách các PI, quy trình ứng dụng các chế phẩm PI trong y - dược và nông nghiệp; nghiên cứu thử nghiệm các chế phẩm PI đã tuyển chọn.

Bài này tóm tắt các kết quả nghiên cứu đã công bố trong 56 công trình đăng trong các Tạp chí khoa học trong và ngoài nước, và các báo cáo ở các Hội nghị quốc gia và quốc tế. Hiện nay, chúng tôi đang tiếp tục nghiên cứu các PI của các cây thuốc Việt Nam để tuyển chọn các PI có tiềm năng ứng dụng thực tế và tìm hiểu thêm về liên quan của chúng với tác dụng dược lý của cây.