SYNTHESIS, CLONING AND EXPRESSION IN *ESCHERICHIA COLI* OF A GENE CODING FOR MCoTI-II

Pham Thi Tran Chau, M.K.Reddy, Dao Thi Thuy, Hoang Thu Ha, Le Nguyen Dung, Laurent Chiche

1. Introduction

Proteinases are involved in different crucial living processes such as digestion, germination, growth, development, metamorphism, fertilisation, blood clotting, infection diseases, cancer etc. Therefore their protein inhibitors (PPIs) – an effective regulation factors have been used in medicine for treatment of diseases and in agriculture to improve plant resistance to pest insects.

The dormant seeds of *Cucurbitaceae* plant family contain small well characterized serine proteinase inhibitors [16,23,30]. Their molecule consist of about 30 amino acids, three disulfide bridges forming a compact structure, resistant to the action of various biological and physico – chemical agents.

Momordica cochinchinensis (MCo) belongs to *Cucurbitaceae* family. Their seeds are found to be the PPI – richest source among the tested *Cucurbitaceae* plants growing in Vietnam [17,21]. We have prepared a crude PPI preparation from MCo seeds named Momosertatin (Mo) which showed insecticidal and antibacterial activities [12,17,18,19,22]. A partly purified peptides from Mo (designated as MCoPIs) reduced inflammatory processes in both acute and chronic inflammatory models [14]. Moreover, none toxic effect of MCoPIs was found on tested animals [14].

Three trypsin inhibitors (TIs) named MCoTI-I,-II,-III from MCo seeds were isolated [15], purified, characterized and sequenced [5]. All of them are small polypeptides with molecular weight of about 3 kD and very stable. MCoTI-II is the most abudant inhibitor, consisting of 34 amino acid residues with 3 disulfide bridges, resistant to cleavage by thermolysin for more than 48 hours at 50^0C. Its conformational structure was also determined [1,10]. Although chemical synthesis of some squash TIs was found possible [11,27], but the overal procedure costly. Recombinant DNA technology was employed by several scientists groups to produce these inhibitors [2,3,26]. In this work, chemical synthesis, cloning and expression of the gene coding for MCoTI-II are presented.

2. Materials and methods

2.1. Materials

Qiagen kit, *E.coli strain* BL21(DE3), PCR reagents were purchased from Promega. Oligonucleotides were custom synthesized by Alpha DNA. pTYB12 vector, chitin beads were from New England Biolabs. Dithiothreitol, IPTG, DNA markers were from MBI Fermentas. PAGE reagents from Amersham. Other used chemicals were of highest purity grade.

2.2. Methods

Isolation of total DNA from MCo leaves was carried out as described in [4].

DNA techniques: plasmid isolation [24, 25, 29], plasmid transformation [6] and standard protocols were used as described by Sambrook et al [28]. PCR was preformed according to PCR protocol [8].

The inhibitory activity was determined by diffusion method as previously described [13]. Proteins was detected on polyacrylamide gel by Laemmli method [9].

Cells culture and fusion protein expression: use LB medium containing 100µg/ml ampicillin, incubated in shakers at 37°C until the OD_{600} reached 0.6. Induction by IPTG.

Expression and purification of recombinant MCoTI-II were followed the manufactures instruction of IMPACT-CN System, using chitin beads column chromatography [7].

3. Results

3.1. Design and construction of the gene coding for MCoTI-II

The nucleotide sequence coding for MCoTI-II was reversely translated from its amino acid sequence (fig 1, fig 2).

SGSDGGVCPKILKKCRRDSDCPGACICRGNGYCG

Fig 1: The amino acid sequence of MCoTI-II [5]

agcggcagcgatggcggcgtgtgcccgaaaattctgaaaaaatgccgccgcgatagcgattgcccgggcgcgt
gcatttgccgcggcaacggctattgcggc

Fig 2*:* The nucleotide sequence derived from MCoTI-II amino acid sequence

Based on the above nucleotide sequence four overlapping synthetic oligo nucleotides were designed. While desingning the oligo nucleotide primers Nde I restricsion site was introduced on the 5' end, stop codon and Xho I restriction site was introduced on the 5' end of the proposed synthetic trypsin inhibitor gene. The sequences of the forward and reversed primers are shown on fig 3 and of the synthetic gene is on fig 4.

Forward primer:

5' GAATTCCATATGAGCGGCAGCGATGGCGGCGTGTGCCCGA 3'

Reverse primer:

5' CGGCTCGAGTTAGCCGCAATAGCCGTTGCCGCGGCAAAT 3'

Fig 3: The sequences of the forward and reversed primers for MCoTI-II gene

cttaaggtatactcgccgtcgcgtaccgccgcacacgggcttt
gaattccatatgagcggcagcgatggcggcgtgtgcccgaaa
M S G S D G G V C P K
taagacttttttacggcggcgctatcgctaacgggcccgcgc
attctgaaaaaatgccgccgcgatagcgattgcccgggcgcg
I L K K C R R D S D C P G A

acgtaaacggcgccgttgccgataacgccgattgagctcggc
tgcatttgccgcggcaacggctattgcggctaactcgagccg
C I C R G N G Y C G

Fig 4: The sequence of synthetic MCoTI-II gene

Amplification of MCoTI-II gene by PCR. The PCR conditions were established as :94^{0}C,3 min ; 61^{0}C 1min30;72^{0}C ,2 min for 40 cycles. The PCR product showed a single band corresponding to about 114bp as expected (fig 5)

Fig 5: Analysis of MCoTI-II gene on 5%polyacrylamide gel

Lane 1: DNA Marker 100 bp (100-1031) (#SMO241/2/3)

Lane 2 : PCR product of MCoTI-II gene .

2 **1**

MCoTI-II gene was also successfully prepared by using forward , reverse primers and the purified total MCo DNA as template. The conditions for this experiment were established as: 150ng of each primer; 200μM of dNTPs ;400ng of the purified total MCo DNA .

Cloning of the synthetic MCoTI-II .

The PCR amplified gene was double digested with NdeI and XhoI restriction enzymes (fig 6) and ligated with pTYB12 vector had been digested by the same restriction enzymes, then transformated into *E.coli* BL21 (DE3). The resulting recombinant *E.coli* was named PI-17 (fig 7).

atactcgccgtcgcg . .....taacgccgattg
tgagcggca attgcggctaactcga

Fig 6: Synthetic inhibitor gene digested by Nde I and Xho I

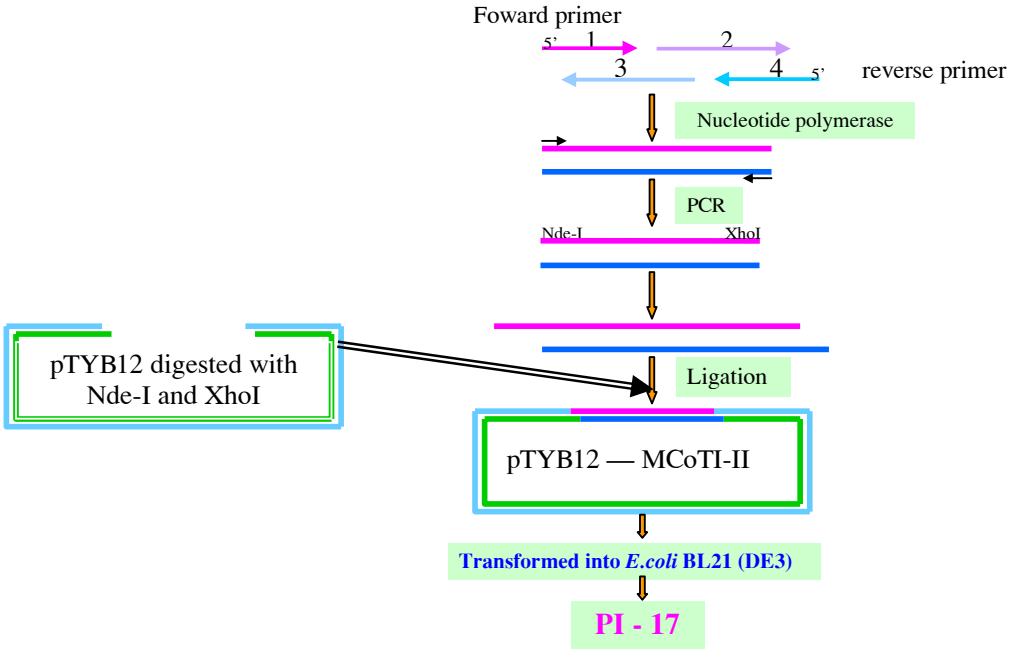
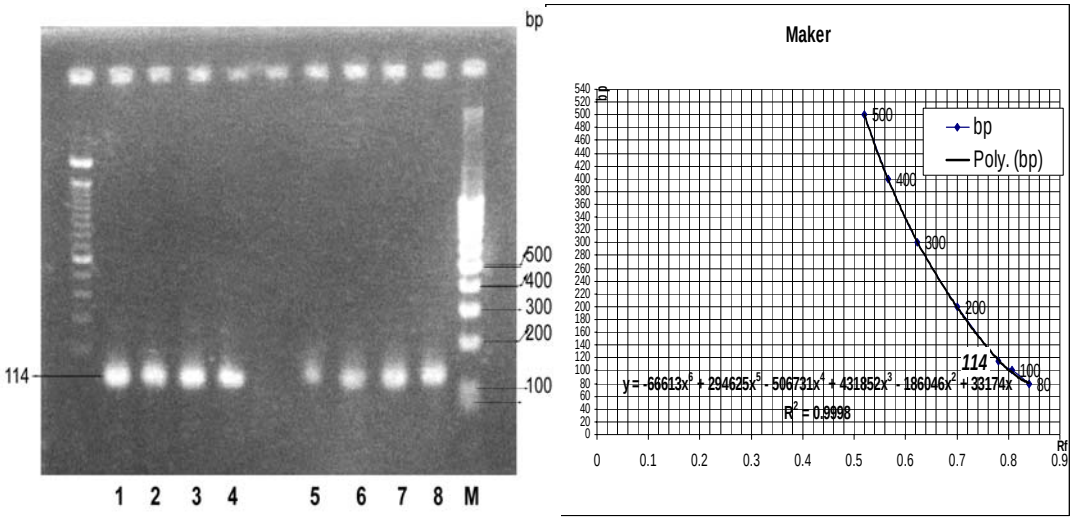


Fig 7: Scheme summarizing the steps in obtaining a construct system for expression of MCoTI-II

To identify the insert gene, we isolated plasmid from PI- 17, checking TI gene by PCR technique and sequencing . PCR product of recombinant plasmid DNA was shown on Fig 8, it corresponded to the expected size (#114bp) and showing correct sequence (Fig 9).



(a) (b)

Fig 8: Electrophoresis of the PCR product of MCoTI-II gene on 2% agarose gel (a) and estimation of its size by using SHARP JX-330 scanner (b)

M: DNA Marker 100bp (80-1031bp)(#SMO241/2/3)
1→8: PCR products of MCoTI-II gene

1	GCTCGCGGAT	TTATTTCGAG	TTCAGACCTG	TTCAGACCTG	40
41	TTATTATGGG	TATTACTTTA	TCTGATGATT	CTGATCATCA	80
81	GTTTTTGCTT	GGATCCCAGG	TTGTTGTACA	GAATGCTGGT	120
121	**CATATGA**GCG	GCAGCGATGG	CGGCGTGTGC	CGAAAATTCT	160
161	GAAAAAATGC	CGCCGCGATA	GCGATTGCCC	GGGCGCGTCA	200
201	TTTGCCGCGG	CAACGGCTAT	TGCGGCTAA**C**	**TCGAG**CCCGG	240
241	GTGACTGCAG	GAAGGGGATC	CGGCTGCTAA	CAAAGCCCGA	280
281	AAGGAAGCTG	AGTTGGCTGC	TGCCACCGCT	GAGCAATAAC	320
321	TAGCATACCC	CTTGGGGCCT	CTAAACGGGT	CTTGAGGGGT	360
361	TTTTTGCTGA	AAGGAGGAAC	TATATCCGGA	TATCCCGCAA	400
401	GAGCCCGGCA	GTACCGGCAT	AACCAAGCCT	ATGCCTACAG	440
441	CATCCAGGGT TTG	GACGGTGCCG	AGGATGACGA	TGAAGCGCCA	480

Fig 9. Sequence of recombinant plasmid DNA containing TI gene fragment

Expression of recombinant MCoTI-II (ReMCoTI-II)

The expression conditions such as ampicillin and IPTG concentrations, temperature and time of induction were tested . The growth and induction conditions of PI-17 giving maximal yield of the recombinant fusion protein established as follows : cells were grown in shaker flasks at 37^0C in LB medium with ampicillin (100µg/ml), induction at the mid-log phase with 0.5mM IPTG at 15^0C for 16h.

As mentioned in the methods, cloning and expression procedures were followed

the IMPACT-CN system and pTYB12 vector was used ,so it was expected to produce 55kD fusion of the cleavable intein tag to N-terminus of a synthetic inhibitor .As known, Mr of MCoTI-II is about 3.4kD, hence Mr of the obtained recombinant fusion protein should be about 58 kD (the sum of 55 + 3.4kD of MCoTI-II). As seen on Fig. 10 the 58 kD protein band was found in induced sample as the major band, acounted for 80% of total protein (lane 1,3,5 and 6).

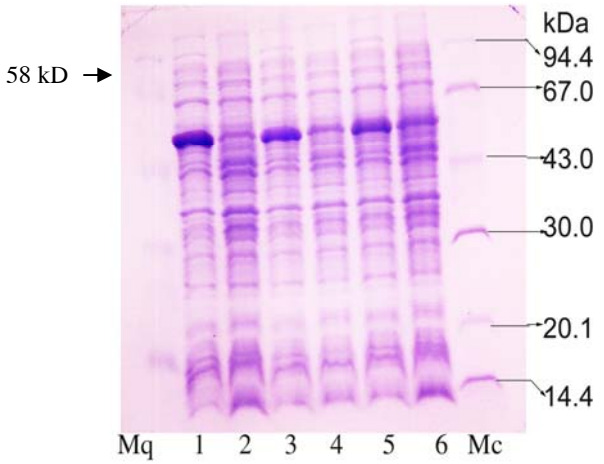


Fig 10. SDS – PAGE pattern of PI-17 proteins

Mc. Standard molecular markers
2, 4. Uninduced
1, 3, 5, 6. Induced (containing 58kD fusion protein band)

Cells were broken by sonication in 10mM Tris-HCl buffer pH8 containing 1mM EDTA (TE buffer). After centrifugation, the clarified supernatant was loaded onto chitin column .

On–column cleavage induction by 50mM 1,4 – dithiothreitol in TE buffer containing 500mM NaCl , at 4^0C for 40 hours (Fig 11).

Purification scheme of recombinant MCoTI-II following IMPACT™ - CN system using chitin beads column chromatography comprsed the following steps

1) Chitin beads column was equilibrated with 10mM Tris–HCl buffer pH=8.0 containing 1 mM EDTA, 500mM NaCl (CB buffer).

2) Loading cells clarified extract on the column.

3) Washing column by CB buffer until no trace of proteins were detected

4) On-column cleavage: CB buffer containing 50 mM DTT. After quick flush, stop the flow in the column, left at 4^0C for 40 hrs.

5) Elution of the target protein by CB buffer.

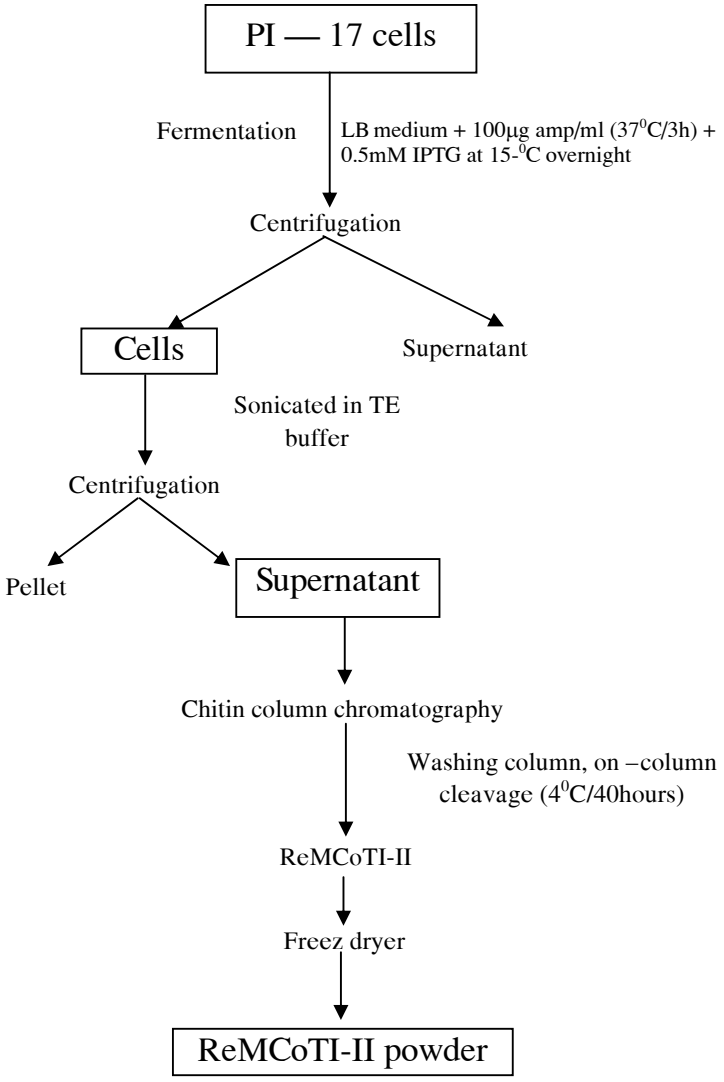


Fig 11: Purification scheme of recombinant MCoTI-II using chitin beads column chromatography

The target protein was released from the chitin column when the chitin-bound intein tag undergoes self-cleavage in the presence of DTT. *Fig 12* showed chitin gel before (N1) and after (N2) releasing MCoTI-II.

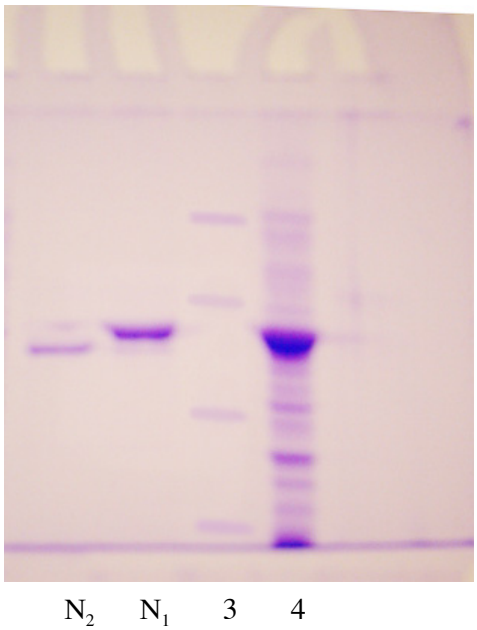


Fig 12. Changing in SDS-PAGE protein pattern of chitin gel before and after releasing ReMCoTI-II.

1,2: N1, N2 (chitin gel before and after on column cleavage, respectively)
3: Maker LMW electrophoresis 17-0446 (14,4-94)
4: PI-17 cells extract

Eluted fractions from chitin column were pooled, lyophilysed, desalted and freez-dried. The obtained protein showed a single band of Mr about 3 kD (Fig 13).

ReMCoTI showed inhibitory activity against trypsin as well as proteinase from *Spodoptera litura (Fig 14).*

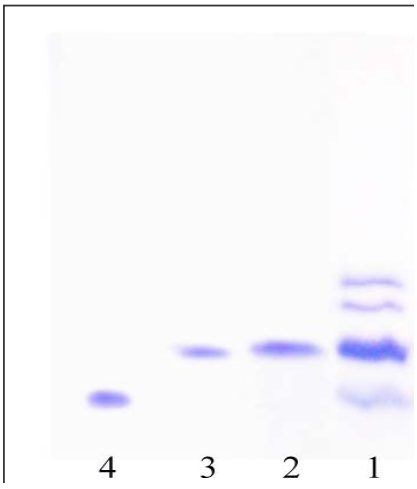


Fig 13. Checking the purity and size of ReMCoTI-II by SDS – PAGE
1. Maker
2, 3. Aprotinin
4. Re MCoTI-II

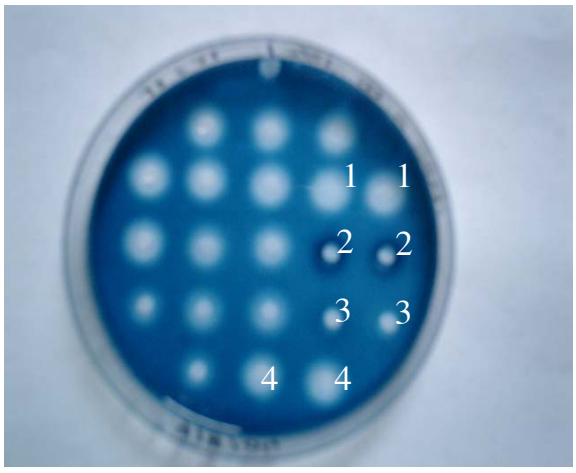


Fig 14. Checking the inhibiting activity of ReMCoTI-II by using diffusion method
1. PA from *S. litula*
2. PA of *S. litula* + ReMoTI-II
3. Trypsin
4. Trypsin + ReMCoTI-II

4. Conclusion

♦ The MCoTI-II gene was synthesized by four overlapping primers ,transformated into pTYB12 vector .

♦*E.coli BL21(DE3*) strain was used as the host for cloning and expression of the recombinant gene .

♦The recombinant MCoTI-II was firstly synthesized in a 58kD fusion protein, then released from it and purified following IMPACT-CN system manufactures instruction by using chitin beads column chromatography .

♦ The obtained purified recombinant MCoTI-II showing inhibitory activity against trypsin,and proteinases from *Plutela xylostella* .

REFERENCES

1. Annie Heitz, Jean-Francois Hernandez, Jean Gagnon, T.H. Thai, Pham Thi Tran Chau, N.T.Mai, L.N. Dung and Laurent Chiche. Solution Structure of the Macrocyclic Squash Trypsin Inhibitor MCoTI-II, the first member of a new family of cyclic Knottins. *Peptides:: The Wave of the Future*, Michal Lebl and Richard A. Houghten (Editors), American Peptide Society, 2001, pp. 387-388.
2. Bozenna Rempola, T-Wilusz, W. Markiewicz and M.Fkus, Synthesis, cloning and expression in E.coli of the gene coding for the Trypsin inhibitor from *Cucurbita pepo, Acta Biochimica Polonica* 42, $N_0$1(1995), pp. 109-114.
3. Chen, X-M., Qian Y-W., Chi C-W., Gan K-D. E., Zhang M-F., Chen C-Q., Chemical synthesis, molecular cloning and expression of the gene coding for the Trichosantes trypsin inhibitor. *J. Biochem*, V.112 (1991). pp. 45-51.
4. Deana Namuth, Kimmo Koivu, Jelena Arbatova, Viktor Kuvshinov and Eija Pehu, *Advanced plant Molecular Biology Laboratory Manual*, University of Helsinki, Finland, (1998), pp. 4-7.
5. Hernandez, J.F., Gagnon, J., Chiche, L., Nguyen, T.M, Andrieu, J.P., Heitz, A., Trinh H.T., Pham T.T. Chau., and Le Nguyen D., V. Squash trypsin inhibitors from *Momordica cochinchinensis* exhibit an atypical macrocyclic structure. Biochemistry, V.39(2000), pp. 5722-5730.
6. Hioraki Inoue, Hiroshi Nojima and Hiroto Okayama, Transformation of E. coli with plasmids, *Gene* V.96 (1990), pp. 23-28.
7. *IMPACT™-CN. Instruction Manual,* New England BioLabs Inc. Version 1.9, 7/2001 (50pp).
8. Innis M.A., Gelfand D.H., Sninsky J.J and White T.J., eds. *PCR Protocols, A guide to Methods and Appications*. Academic Press, Inc., Harcourt Brace Jovanovich, Publ. San Diego, New York, Berkeley, Boston 1990, 482p
9. Laemmli U.K.,Cleavage of structure proteins during the assembly of the head of bacteriophage T4, *Nature* V.227 (1970) pp 680-685
10. Laurent Chiche, A. Heitz, J. C. Gelly, J.Gracy, T.C. Pham, T. H, Phan, J. F. Hernandez and Le nguyen, D. Squash Inhibitors: From Structural Motifs to Macrocyclic Knottins, *Current Protein and Peptide Science,* V.5(2004), pp. 341-349.
11. Le nguyen, D., L.G., Barry, J.P.Tam, A. Heitz, L. Chiche, J. F. Hernandez, T.C. Pham, *In* "*Peptides*" (Benedelii, E., and Pedone, C., Eds), Endizioni Ziino, Napoli, Italy, 2002, pp182 -183.
12. Le nguyen, D., A. Heitz, L. Chiche, J. F. Hernandez, T. H. Phan, T.C. Pham. Antimicrobial and insecticidal effects of crude extract momosertatin from *Momordica cochinchinensis. in* "*Peptide Revolution: Genomics, Proteomics & Therapeutics*" Michael Chorev & Tomi K. Sawyer (Eds). American Peptide Society, (2003).

13. Leluk J., and Pham T.T.C., Zastosowanie edestyny do badania aktywnosc proteinaz i ich inhibitorow (Polish) in *XXI Meeting of Polish Biochemical Society Abstracts*, pp.139 (1985), Krakow, Poland .
14. N.V.Nguyên, V.Hà, L.Năm, Đ.L.Tuấn, P.T.Trân Châu, P.T.Hà, Nghiên cứu khả năng ứng dụng chế phẩm protein ức chế proteinaz trong điều trị viêm, Trong quyển: *Những vấn đề nghiên cứu cơ bản trong khoa học sự sống Y-Dược học*, Nhà xuất bản Khoa học và Kỹ thuật, Hà nội 2004 (507 trang), tr. 287-290.
15. Nguyễn Tuyết Mai, Phạm Thị Trân Châu, So sánh các chất ức chế tripxin (TI) của hạt gấc chín sinh lý trước (Go) và sau khi xử lý nhiệt, *Tạp chí Khoa học, Đại học Quốc gia Hà nội*. Vol. XII, $N_0$3 (1996), tr. 33-41.
16. Pham Thi Tran Chau, Trypsin inhibitors of white bush *(Cucurbita pepo var. patisonina) fruits and seeds* (Doctor habilitatus thesis, 1985), *Acta Universitatis Wratistaviensis* N_0-912, Wroclaw University, Wroclaw, 1987, 110p.
17. Pham Thi Tran Chau, V. T . Hien, P.T. Ha, N. H. M. Quyen, N. T. Mai, Preliminary of trypsin inhibitors (TIs) from squash seeds of Viet nam 10th FAOB symposium on Protein Resarch. Taipei 7 – 10/12/1993, *Abstracts PIII* – 21.
18. Pham Thi Tran Chau, M. N. Toan, T. Q. Tan. Effect of Momosertatin on the growth and mortality of insect pests, Plutella xylostella (PX) and Spodoptera litura (SL).17th International Congress of Biochemistry and Molecular Biology. *Annual Meeting of the American Society of Biochenistry and Molecular Biology*, August 24-29, San Franciso, California, Program N^0 - 2130, 1997.
19. Pham Thi Tran Chau, M. T. Hang, V. T. Hao, N. H. Ha, N. L. Dung, Effect of Momosertatin on the growth of microorganisms. *8th FAOBMB Congress*, Kula Lumpur, Malaysia. *Abstract C 26*, November 22 – 27/1998.
20. Phạm Thị Trân Châu, T. Q. Tấn , P.T. Hà, H. T. Việt, M. N. Toàn. N. Đ. Toàn, T. H. Thái, P. T. Hạnh, Tác dụng trừ sâu hại rau của chế phẩm Momosertain tách từ hạt gấc (*Momordica cochinchinensis*), *Tạp chí khoa học,* Khoa học Tự nhiên, ĐHQGHN, T XVI, $N_0$1,(2000) tr.1 -11.
21. Phan Tuấn Nghĩa, Phạm Thị Trân Châu, Sự biến đổi hoạt độ kìm hãm tripxin (TIA) ở quả và hạt mướp đắng trong quá trình phát triển của chúng, *Tạp chí Sinh học*, Vol.9, $N_0$3 (1987), tr. 12-17.
22. Phan Thị Hà, Phạm Thị Trân Châu, Tác dụng của Momosertatin đến proteinaz ngoại bào của *Pseudomonas* phân lập từ mủ bỏng, *Tạp chí Sinh học*, Vol.22, $N_0$3 (2000), tr 31- 37.
23. Polanowski, A., Otlewski, J., Leluk, J., Wilimowska – Pelc, A., and Wilusz, T.,. A new family of serine proteinase inhibitors from squash seeds. *Biol. Zentralbl* Vol.107(1988) pp. 45-49.
24. *Promega Protocols and Applications Guide* (1989), pp. 52-56.
25. *Qiagen Mini Hand book* (1999), 34p
26. Rempola B., Trypsin inhibitor gene cloned and expressed in *Saccharomyces cerevisiae. Acta Biochim.Polon.* 40(1993), pp. 8-11.
27. Rolka, K., Kupryszewski G., Ragnarsson U., Otlewski J., Krokoszynska I., Wilusz T.E. Chemical synthesis of new trypsin, chymotrypsin and elastase inhibitors by amino acid substitutions in a trypsin inhibitor from squash seeds (CMTI-V) *Biol. Chem. Hoppe-Seyler* 372 (1991), pp. 63-58.
28. Sambrook J., Fritsch E.J. and Maniatis T. in: *Molecular cloning: Laboratory Manual, 2nd edition.* New York: Cold Spring Habor Laboratory Press 1989. Cold Spring Harbor.
29. Ulrike Schindler and Anthony R. Cashmore. *Methods in Plant Molecular Biology A Laboratory Course Mannual*, Cold Spring Harbor laboratory Press in USA, (1995), pp. 275-297.
30. Wieczorek M., Otlewski J., Cook J., Parks, K., Leluk J., Wilimowska-Pelc A., Polanowski A., Wilusz T., and Laskowski M., Jr. The squash family of serine proteinase inhibitors. Amino acid sequences and association equilibrium constants of inhibitors from squash, summer squash, zucchini and cucumber seeds. Biochem. Biophys. Res. Commun. 126(1985), pp. 646-652.