INVESTIGATION OF PROTEINASE PROTEIN INHIBITORS (PPIs) FROM SEEDS OF CERTAIN PLANTS BELONGING TO MORACEAE FAMILY AND OTHER ONES*

Pham Tran Chau, Nguyen Hong Anh, Le Trong Quang
Phan Thi Ha, Nguyen Tuyet Mai, Dao Trong Anh.

Biotechnology Center, College of Natural Sciences - VNU

I. INTRODUCTION

Proteinase protein inhibitors (PPIs) play an important role in living system and have several practical applications [7, 9, 11]. Therefore, the investigation of PPIs in plants has both scientific and realistic significance.

However, in Vietnam, the study on PPIs has started in our laboratory only since 1986. During that period, most of studies were focused on PPIs from *Cucurbitaceae* seeds.

As far as we know, there are more than 90 published papers about protein from *Artocarpus* seeds, but no one reported about PPIs.

This work presents some preliminary results of PPIs from seeds of certain Moraceae plants and from some plants of other families.

II. MATERIALS AND METHODS

1. Materials

The seeds of *Artocarpus melinoxyla* Gagn; *Artocarpus chaplasha* Roxb; *Artocarpus interger* Merr ; *Artocarpus lakoocha* Roxb; *Steblus asper* Lour; *Annona squamosa* L; *Diospyros kaki* Linn f; *Abelmoschus esculentus* L; *Syzygium jambos* L. ; *Delavaya toxocarpa; Citrus grandis* Osbeck; *Citrus sinensis* L; *Pyrus pyrifolia* Nakai; *Carica papaya* L; *Sechium edule Sw; Chrysophyllum cainito* L; *Persimmon kaki soft type ripe* were collected from mature fruits.

(+) Chemicals

α - chymotrypsin from Lab Leurquin (France); casein from BDH chemicals Ltd, Sephadex - G 75 from Pharmacia Fine chemical (Sweden); trypsin, albumin were supplied by Sigma Ltd (USA).

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Abbreviations: PA: proteolytic activity; TIA: trypsin inhibitor activity; ChIA; chymotrypsin inhibitor activity; DChIA (DTIA): TI or ChI peak.

- Chemicals for electrophoresis: acrylamide, bis - acrylamide, Tris - base...etc., from Sigma Ltd (USA). The other chemicals reached the PA grade.

(+) Methods

The finely ground seeds were extracted with distilled water (with an exception for *Diospyros kaki* Linn seeds, where pH of 4.5 Britton and Robinson buffer was used). The obtained suspensions were stirred for 30 minutes at room temperature and clarified by centrifugation (8000 rpm) for 10 minutes. The supernatant was collected for further analysis.

- Protein concentration was determined by the Lowry method [6], using bovine serum albumin as standard.

- Determination of proteolytic activity (PA) was carried out by Anson modified method [8]. PA was determined at 35.5^0C using pH 7.6 Srrensen buffer with 1% casein as substrate for 20 minutes. One unit of proteolytic activity was defined as the amount of enzyme after one minute of action at 35.5^0C generating TCA soluble products, giving colour reaction with Folin Ciocalteau reagent equivalent to one micromole of tyrosine.

Inhibitory activity was determined from the residual enzymatic activity after 10 min pre-incubation of the enzyme with the inhibitor solution. One unit of inhibitory activity was defined as that amount of inhibitor, which reduces the activity of two mg of enzyme by 50%.

- PA, ChIA, TIA were also determined by diffusion method [5,10].

Heat stability of TI and ChI was estimated as follows: the crude extract was treated at 100^0C for 15 min, cooling immediately in ice. The activity was measured in standard conditions. The remaining activity was calculated in comparing with the initial activity.

- Polyacrylamide gel electrophoresis for identification of protein bands was performed according to Laemmli method [4]. Protein pattern was analysed by Sharp JX. 330 scanner.

Gel filtration by Sephadex-G75 column

The gel filtration column with a size of 1.7 x 92cm was equilibrated by pH 6.5 Srrensen buffer for *Artocarpus melinoxyla* Gagn and *Artocarpus chaplasha* Roxb pH 7.6 for *Abelmoschus esculentus* L. and *Delavaya toxocarpa*. Proteins were eluted by the equilibrated buffer. The fraction volume was 3ml. The running speed was 20ml/hour.

III. RESULTS

1- Screening proteinase inhibitory activity (PIA) of seed crude extracts

Seventeen seed samples of *Moraceae, Rutaceae, Annonaceae, Rosaceae, Ebenaceae, Caricaceae, Malvaceae, Sapindaceae, Myrtaceae, Sapotaceae, families* were subjected to PIA investigation. Nine of them showed inhibitory activity (Table 1) but in the eight others: pomelo, orange, pear, papaya, chayote, star apple, persimmon, kaki soft type ripe neither TI nor ChI was discovered.

Table 1, 4. .

No.	Seed samples (Latin name)	Family	% of dry matter	Protein			TIA			ChIA		
				mg p/g of air dry matter	mg p/g of dry matter	% of dry matter	mIU/g of air dry matter	mIU/g of dry matter	specific activity	mIU/g of air dry matter	mIU/g of dry matter	specific activity
1	Artocarpus melinoxyla Gagn	Moraceae	77.55	126.34	162.91	16.291	1896.16	2445.08	15.01	4401.60	5675.82	34.84
2	Artocarpus chaplasha Roxb	Moraceae	82.16	110.68	134.71	13.471	967.68	1177.80	8.74	3178.56	3868.74	28.72
3	Artocarpus lakoocha Roxb	Moraceae	80.62	47.36	59.08	5.908	197.19	244.59	4.14	-	-	-
4	Artocarpus integer (Thunb) Merr	Moraceae	78.34	102.61	130.98	13.098	-	-	-	1157.54	1477.58	11.28
5	Abelmoschus esculentus L.	Malvaceae	75.636	64.4	85.145	8.5145	1800	2380	29.95	2040	2700	31.68
6	Syzygium jambos L	Myrtaceae	45	97.218	216,04	21.604	1719	3820	17,68	1916	4257,78	19,71
7	Delavaya toxocarpa	Sapindaceae	86.95	66.75	76.768	7.6768	872	1003	13.06	-	-	-
8	Diospyros kaki L	Ebenaceae	63.77	20.16	31.61	3.161	851.2	1334.80	42.22	-	-	-
9	Annona squamosa L	Annonaceae	79.2	88.65	111.93	11.193	155	196	1.75	-	-	-

Table 1: Some biochemical properties of seeds belonging to several plant families

Samples		Protein			TIA				ChIA			
		volume (ml)	mg	%	mIU	%	the specific activity	the purified level	mIU	%	the specific activity	the purified level
Artocarpus melinoxyla Gagn	The crude extract	3,5	121,31	100					11900	100	98,1	1
	the active peak	84	64,03	52,78					9430	79,23	147,27	1,5
Artocarpus chaplasha Roxb	The crude extract	3	499,99	100					10830	100	21,66	1
	the active peak	93	252,99	56,22					8430	78	33,32	1,54
Abelmoscus esculentus L	The crude extract	3	204	100	3456	100	16,94	1	6120	100	30	1
	the active peak	63	72,85	35,71	3150	91,15	88,22	5,2	3590	58,6	100,57	3,35
Delavaya toxocarpa	The crude extract	3	35,4	100	1110	100	31,36	1				
	the active peak	51	9,384	26,51	1035	93,24	110,3	3,52				

Table 4: The Sephadex G75 column chromatography of seed extract from 4 samples of *Artocarpus melinoxyla* Gagn, *Artocarpus chaplasha* Roxb, *Delavaya toxocarpa*, *Abelmoscus esculentus* L

As shown in table 1, *Artocarpus melinoxyla* Gagn, *Artocarpus chaplasha* Roxb, *Abelmoschus exculentus* L and *Delavaya toxocarpa* contain both TIA and ChIA. However, in the first two samples, ChIA was 2-3 times higher than TIA, while in *Abelmoschus esculentus* L. and *Delavaya toxocarpa*, there was no clear distinction between TIA and ChIA.

In remained samples, TIA was only discovered in seeds of *Artocarpus lakoocha* Roxb, *Diospyros kaki* Linn f, *Delavaya toxocarpa, Annona squamosa* L. while KIA existed in *Artocarpus melinoxyla* Gagn. Among the *Moraceae* seeds owing PPI, the highest TIA level (5675 mIU/g of dry matter) was found in *Artocarpus. Melinoxyla* Gagn. In opposition, *Steblus asper* Lour sample showed proteolytic activity (1.787U/g of dry matter) but was lacking in PA, PPIs. Thus in comparison with the *Momordica* seeds, TIA of *Artocarpus* was 10 times lower [12, 13] but it was higher than pumpkin, squash, water melon... TIA.

Basing on this experiment, we selected 4 samples of *Artocarpus melinoxyla* Gagn; *Artocarpus chaplasha* Roxb, *Delavaya toxocarpa* and *Abelmochus esculentus* L. for further analysis.

At first, SDS - PAGE was used for detecting the protein components.

Number	Samples / Rm	Artocarpus melinoxyla Gagn	Artocarpus chaplasha	Abelmoschus esculentus L	Delavaya toxocarpa
1	0,14	+ -	+ -		
2	0,18	+ -	+ -		
3	0,2				+
4	0,21	+ -	+ -		
5	0,23	+ -	+ -		
6	0,24				+
7	0,26	+ -	+ -		+
8	0,28				+
9	0,29	+ -	+ -		
10	0,32	+ -	+ -		
11	0,36			+++	
12	0,39	+			+++
13	0,44	++	++	+	+
14	0,45			+	
15	0,50			+	
16	0,54				+
17	0,58	++	++	+ -	
18	0,62	+++	+++		
19	0,70				+++
20	0,83				+
21	0,85			+ -	
22	0,90				+

Table 2: The ralative mobility (Rm) of protein bands from 4 samples.

2. Electrophoresis pattern of seed extract of Artocarpus melinoxyla Gagn; Artocarpus chaplasha Roxb; Abelmochus esculentus L; Delavaya toxocarpa L

For convenience, the Rm of protein bands was calculated and listed in table 2.

The data in photo 1 showed that the protein pattern of seed extract of *Artocarpus melinoxyla* Gagn and *Artocarpus chaplasha* Roxb was rather similar, including 10 protein bands in *Artocarpus melinoxyla* Gagn and 11 ones in *Artocarpus chaplasha* Roxb. Among these bands, the protein ones with Rm=0.39; 0.44; 0,58; and 0.62 were major bands.

It was interesting to note that there was only one difference in protein band with Rm=0.39 between *Artocarpus melinoxyla* Gagn and *Artocarpus chaplasha* Roxb. May be the differentiation between *Artocarpus melinoxyla* Gagn and *Artocarpus chaplasha* Roxb was defined by this protein.

The rate ratio of main protein bands (Rm = 0.39; 0.44; 0.58 and 0.62) was calculated and presented in table 3. In *Artocarpus melinoxyla* Gagn, the protein band with Rm = 0.39 accounts for approximately 6.16 of the total protein content, 6.16 % while for the Rm = 0.44 protein band it was 3.69 % lower than that of the corresponding one in *Artocarpus chaplasha* Roxb. Thus, a question arises: does the protein band with Rm = 0.39 originate from the one with Rm = 0.44? and the corresponding gen(s) identifying the difference between these two *Artocarfus species*?

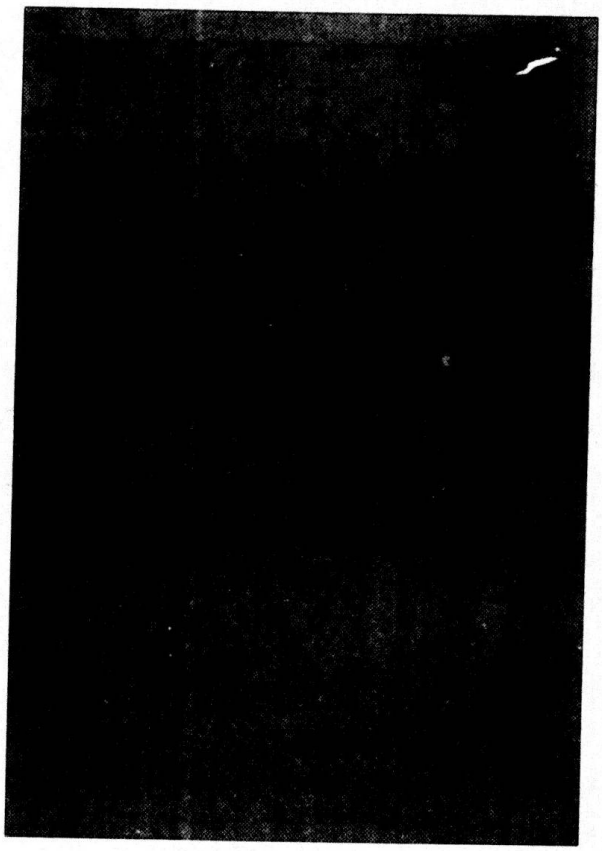


Photo 1. protein pattern of seed extract from Artocarpus melinoxyla Gagn (1, 3) and Artocarpus chaplasha Roxb (2, 4)
1, 2: each lane contain 10 μl of sample
3, 4: each lane contain 15 μl of sample

Rm / Samples	The major protein bands (Rm)			
	0,39	0,44	0,58	0,62
***Artocarpus chaplasha* Roxb (column2)**		22,29	23,65	36,47
***Artocarpus melinoxyla* Gagn (column 1)**	6,16	18,6	24,65	29,39

Table 3: The percentage ratio of major protein bands in comparison with the total of detected bands by Sharp JX-330 scanner.

The SDS - PAGE results also showed the difference in electrophoresis pattern among *Artocarpus melinoxyla* Gagn; *Artocarpus chaplasha* Roxb and *Delavaya toxocarpa*. The simplest protein pattern with 5 protein bands in which the major one with Rm = 0.36 was found in *Abelmochus esculentus* L. Although, ten protein bands were detected in protein pattern of *Delavaya toxocarpa*, almost these proteins were different from the corresponding ones of the other families in Rm value.

To preliminary separate PPIs from crude extract, Sephadex G75 column was used for chromatography.

3. Chromatogram of seed extract of samples through Sephadex - G75 column

As shown in fig. 1 and fig. 2, the chromatogram patterns of *Artocarpus melinoxyla* Gagn and *Artocarpus chaplasha* Roxb [5, 10] were similar in shape, consisting of 2 peaks (D_1, D_2), in which D_1 was an active peak. In *Artocarpus melinoxyla* Gagn, D_1 reached 52.78 % of the total protein loaded on the column while it was 55.75 % in *Artocarpus chaplasha* Roxb. Thus, at the back of separation, the purified level of ChI in *Artocarpus melinoxyla* Gagn was equivalent to that of *Artocarpus chaplasha* Roxb. After chromatography, since TIA both of *Artocarpus melinoxyla* Gagn, *Artocarpus chaplasha* Roxb was very low, it was not possible to detect by Anson method, TIA was only discovered by diffusion method.

It should be noted that TIA fraction shared the same peak with ChIA one. This phenomenon may be the cause of the similarity in Rm between TI and ChI in these samples? or there existed the double headed inhibitors in *Artocarpus:* one domain inhibits trypsin, the other is completed with chymotrypsin, following the type of BBI form in soybean seed [2, 3]?

Under the same condition, there were some differences in chromatogram pattern of *Delavaya toxocarpa* and *Abelmochus esculentus* L. PPIs fractions were eluted slowlier, thus it documented that molecular weight of PPI in these samples was lower than that of *Artocarpus.*

Fig. 1 showed two main protein peaks (D_1, D_2) in chromatogram pattern of seed extract of *Abelmochus esculentus* L.. TIA, ChIA were shown in D_1 As the protein content of D_1 reached 35.71 % of total studied protein, the specific activity of TIA and ChIA increased respectively 3.15 and 3.35 times.

As shown in fig. 1, there were 3 peaks (D_1, D_2 and D_3) in chromatography pattern of *Delavaya toxocarpa.* Since D_2 had the lowest protein content (about 26.5 %) and was a TI peak, thus the specific activity of TI increased 3 times more than that of initial extract.

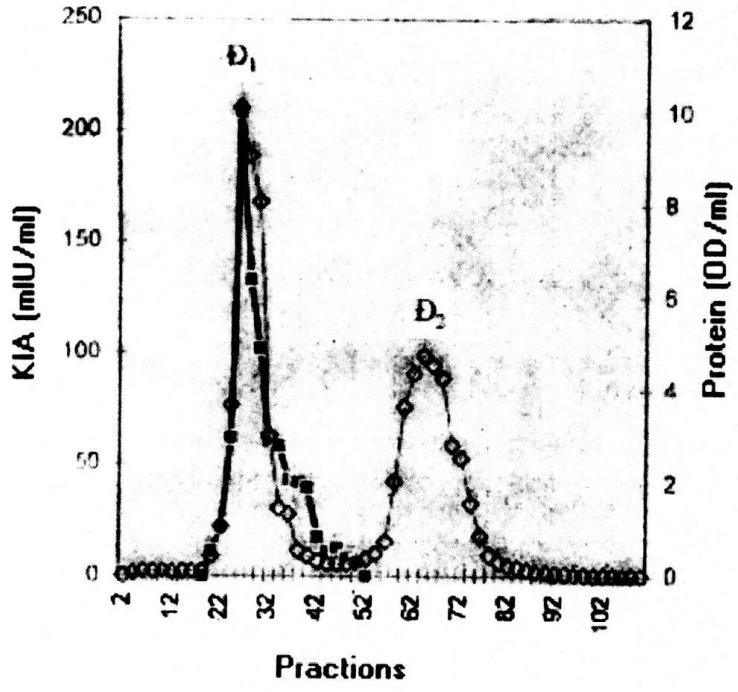
4. Effect of heat treatment on ChIA (or TIA) of studied samples

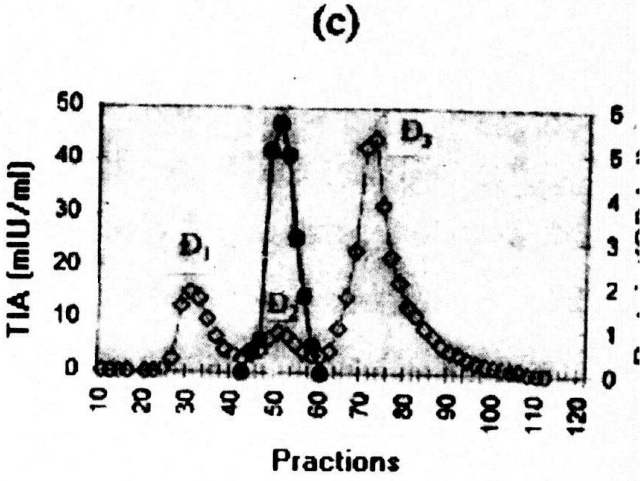
It is known that high PPI content in food might affect unfavorably digestion in animals and humans due to the decrease of enzyme activity by inhibitors.

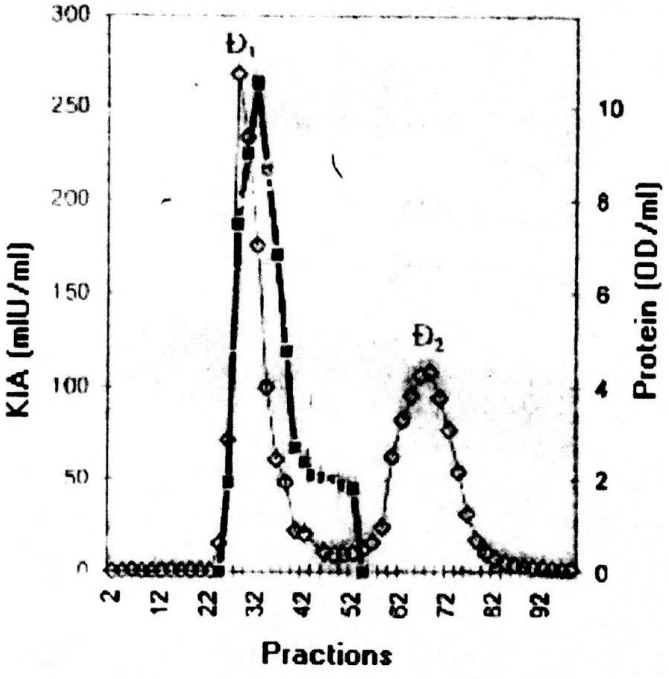
- *As Artocarpus melinoxyla* Gagn and *Abelmochus esculentus* L. has been used as a good nutrient, we studied the impact of heat treatment at 100^0C for 15 minutes on TI, ChI in these samples.

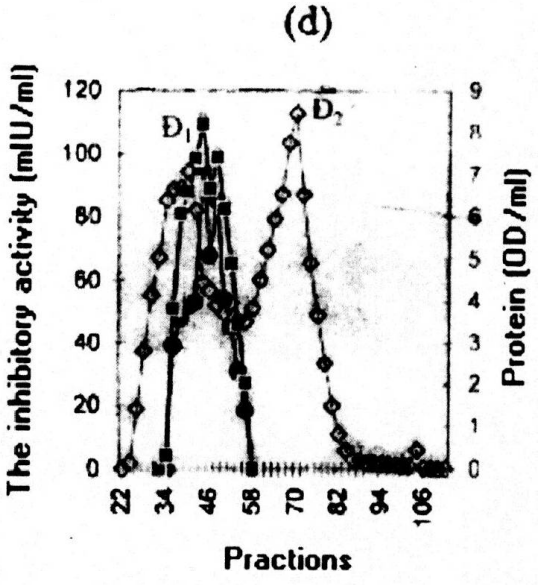
Fig. 1: Chromatogpaphy pattern of seed extract of studied samples by Sephadex G 75 column

a) Artocarpus mekinoxyla Gagn
b) Ratocarpus chaplasha Roxb
c) Delavaya toxocarpa
d Abelmochus esculentus









TIA KIA Protein

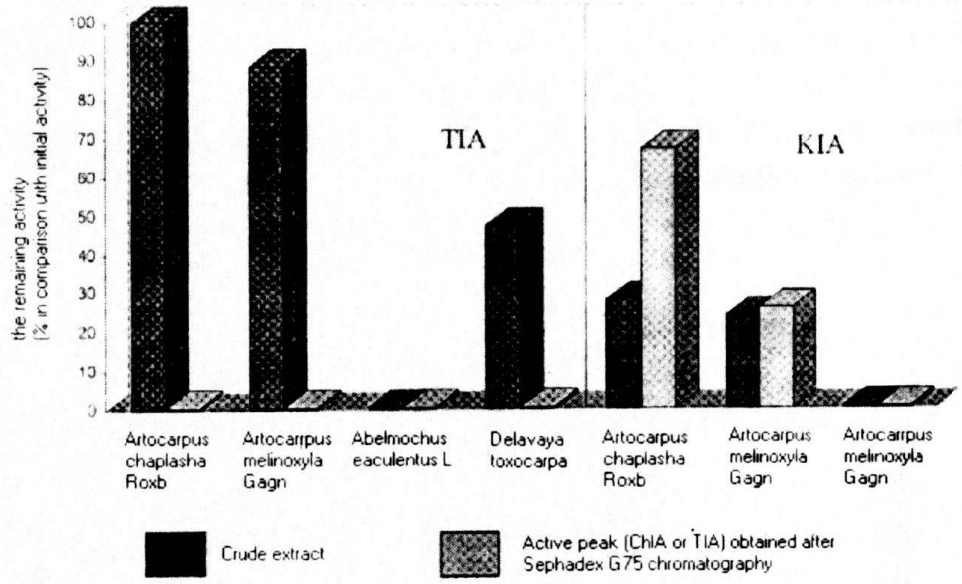


Fig.2: The effect of heat treatment on ChIA (or TIA) of studied samples.

- The obtained results showed that when the seed extract was treated at 100^0C for 15 minutes, TIA and ChIA of *Abelmochus esculentus* L. were almost destroyed. In *Artocarpus melinoxyla* Gagn, *Artocarpus chaplasha* Roxb, ChIA in both crude extract and active peak ($D_{\text{ChIA}}, D_{\text{TIA}}$) were reduced by around 70 % but the remained TIA of seed extract in these samples was still high. This phenomenon might be due to the fact that sample concentration for determining TIA was 5 times higher than that one for ChI detection. Therefore, TIA, ChIA in boiled seeds were determined in a further step.

After boiling, TIA and ChIA reduced to 10 times in comparison with initial activity, corresponding to 0.1 - 0.2 IU. The minimum of trypsin content released from human pancreas per day is 782 mg (782 U) [1], therefore the remaining TI, ChI in *Artocarpus* seed after boiling has no considerable impact on trypsin and chymotrysin activities in human body.

IV. CONCLUSION

1- Among 17 investigated samples, TI and ChI are found in 9 samples, consisting of *Artocarpus melinoxyla* Gagn, . *Artocarpus chaplasha* Roxb; *Artocarpus integer* Merr; *Artocarpus lakoocha* Roxb; *Streblus asper* Lour; *Annona squamosa* L; *Dispyros kaki* Linn f; *Diospyros kaki.* In which 4 samples of *Artocarpus melinoxyla* Gagn, *Artocarpus chaplasha* Roxb Syzygium jambos L., Abelmoschus esculentus L., had both TI and ChI.

2- pH 8,3 SDS - PAGE 12,5 % has enabled the identification of 10 protein bands from seed extract of *Artocarpus melinoxyla* Gagn, . *Artocarpus chaplasha* Roxb. However, there was another protein band with Rm = 0.39 in *Artocarpus melinoxyla* Gagn, at the same time, the protein with Rm = 0.44 was decreased.

At least, 5 protein bands were found in *Abelmochus esculentus* L. and 9 protein ones were discovered in *Delavaya toxocarpa.* Almost these bands differed in terms of Rm value to the corresponding other samples.

3- Chromatogram pattern of *Artocarpus melinoxyla* Gagn; *Artocarpus chaplasha* Roxb

were rather similar, consisting of 2 peaks (D_1, D_2) in which D_1 had both TIA and ChIA. 4- TI and ChI of seed extract of *Artocarpus melinoxyla* Gagn; *Artocarpus chaplasha* were rather stable with heat treatment. After heat treatment at 100^0C for 15 minutes the remained activity was over 30%.

V. REFERENCES

[1] R.M. Berne , M. N. N. Levy. *Physiology* , Mosby - Year Book, Inc., USA. (1993)669-676.

[2] Y. Birk , A. Gerler. *Proc. Int. Res. Conf. Inhibitor., Int. ed. H. fritz. H. Tes chesche 142-148.* Berlin: De Grufter (1971)304.

[3] D.E Bowman. Differentiation of Soybean antitryptic factors, *Proc. Soc. Exp. Biol. Med.* **63**(1946) 547-550.

[4] V.K. Laemmli. Cleavage of structure protein during the assembly of the head of bacterophage T4, *Nature* **227**(1970) 680-685.

[5] Leluk Jacek, Pham Thi Tran Chau, Kieleczawa Jacek. Zastosowanie edestyny do vadania aktywno'sc' proteinaz i ich inhibitorow, *XXI Zjazd P.T.Bioch. Krako'w. Streszczenie* **140**(1985).

[6] Q.H. Lowry, N.J. Rose brough, A.L. Farr , R.J. Randall. Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* **193**(1951) 265-270.

[7] Nguyen Khoa Dieu Thu, Pham Thi Tran Chau. Preliminary researches on the effect of trypsin inhibitor product (HN) separating from Momodica seed to Rat's reproduction, *Journal of Biology-Soil science; Hanoi National University* (1989).

[8] J.S. Pietrowa, M.M. Wincjunajte. Opredelenie proteoliticheskoi aktivnosti ferment-nykh preparatov mikrobiologicheskovo proiskhozhdenia, *Priklad. Biochem. Mikro-bilo.* **2**(1966) 232.

[9] Pham Thi Tran Chau. *Trypsin inhibitors of white bush (Cucurbita pepo var, patisso-nia) fruit anh seeds.* (Doctor habilitatus thesis), Acta Universitatis Wratislaviensis (1985) 912.

[10] Pham Thi Tran Chau, L. Konopska, J. Leluk. Trypsin inhibitors in the aleurone graines of Cucurbita pepo var. patissonia (White bush) cotyledons, *Biochem. Phys-iol.*, Pflanzen, **181**(1986) 565-569.

[11] Pham Thi Tran Chau, Nguyen Tuyet Mai, Phan Thi Ha. Purification, characteriza-tion of antilysin from bovine lung - preliminary study regarding the determination of its practical uses, *Journal of Biology* **15(4)**(1993) 8-10 (in Vietnames).

[12] Pham Thi Tran Chau, V.T.Hien, P.T.Ha, N.H.M.Quyen, N.T.Mai. Preliminary data of trypsin inhibitors (Tls) from squash seeds of Vietnam, *10th FAOB sympo-sium on protein Research Taipei* 7-10/12/1993, (1993)111-21.

[13] Phan Tuan Nghia, Pham Thi Tran Chau (1987): Changes in trypsin inhibitory ac-tivity (TIA) and proteolytic activity of development seeds and fruits of Momordica charantia L, *Journal of Biology* **9(3)**, 12-17 (in Vietnames).

TẠP CHÍ KHOA HỌC ĐHQGHN, KHTN, t.XV, $n^0$3 - 1999

ĐIỀU TRA CÁC PROTEIN ỨC CHẾ PROTEINAZ (PPI) Ở HẠT MỘT SỐ CÂY THUỘC HỌ MORACEAE VÀ MỘT VÀI HỌ KHÁC.

Phạm Trân Châu, Nguyễn Hồng Anh, Lê Trọng Quang
Phan Thị Hà, Nguyễn Tuyết Mai, Đào Trọng Anh

Trung Tâm Công nghệ Sinh học
Đại học KH Tự nhiên - ĐHQG Hà Nội

Chúng tôi đã tiến hành điều tra sơ bộ PPI của 17 mẫu hạt thuộc các họ Dâu tằm, Bông, Sim, Bồ hòn, Thị và Na. Trong đó 4 mẫu hạt Mít mật, Mít dai, Roi, Đậu bắp có chứa cả chất ức chế tripxin và kimotripxin. Năm mẫu hạt khác: Chay, Mít tố nữ, Hồng đỏ, Na có chứa TI hoặc KI. Từ các kết quả trên cúng tôi đã chọn hạt Mít mật và Mít dai để nghiên cứu tiếp. Kết quả điện di dịch chiết hạt 2 mẫu Mít mật và Mít dai cho thấy phổ điện di protein 2 mẫu này khá giống nhau, trừ băng protein có Rm = 0.39 (Mít dai không có băng protein này). Phải chăng băng protein trên qui định sự sai khác giữa Mít mật và Mít dai. Sắc ký qua cột Sephadex G75 cũng cho thấy phổ sắc kí 2 mẫu này khá giống nhau gồm 2 đỉnh protein chính, trong đó đỉnh thứ nhất có cả KIA và TIA nhưng ở mức độ thấp hơn. TI và KI từ hạt mít khá bền với nhiệt, sau khi sử lý ở 100^0C trong 15 phút, hoạt độ kìm hãm chỉ còn lại khoảng 30 % so với hoạt độ ban đầu.