The identification of a non-dialyzable proteinase inhibitor with the dialyzable from eggplant exocarps

MASAO KANAMORI, FUMIO IBUKI, MISAO TASHIRO[1] and MASAAKI YAMADA[2]

Summary Non-dialyzable proteinase inhibtor was purified from eggplant exocarp. The salting out, heat-treatment, DEAE-Sephadex and gel chromatographies were used, and the ultrafiltration was used instead of dialysis except first purification step. The molecular weight, inhibition behaviour for proteinases and amino acid composition were the same as those of purified inhibitor by the other methods.

These results show that the non-dialyzable and dialyzable inhibitors are identical and only one kind of inhibitor exists in eggplant exocarp as the main inhibitor.

However, the reason why the inhibitor of molecular weight of 6000 in crude stage remains in dialyzing tube, is still unclear.

Introduction

In previous papers[1,2], we have reported the occurrence and some characteristics of proteinase inhibitors from the eggplant exocarp. These inhibitors could be distinguished into dialyzable and non-dialyzable. They were at first regarded as distinct inhibitors and their purification studies were therefore carried out individually[3]. Recently, the dialyzable inhibitor was purified completely[4,5]. During the dialysis of the partially purified non-dialyzable inhibitor, a large amount of inhibitory activity appeared in outer solution through the membrane of Visking tube. This phenomenon suggests that the both inhibitors are identical. Therefore, we tried the purification of inhibitors without separating it into dialyzable and non-dialyzable with the use of the acetylated Visking cellulsoe tube and the only one purified inhibitor was obtained[6]. The properties of this inhibitor were in good agreement with those of the purified dialyzable inhibitor, indicating that the both inhibitors are the same.

However, the identification of non-dialyzable inhibitor with dialyzable is still not achieved. In this paper, the purification of the non-dialyzable inhibitor is examined by the methods without the use of dialysis except first step of purification, and the properties of purified inhibitor are compared with those of the inhibitors purified by the other methods.

Laboratory of Nutritional and Food Chemistry, Department of Agricultural Chemistry, Kyoto Prefectural University, Kyoto, Japan. [1]Present address, Laboratory of Nutrition, Department of Food Science, Kyoto Prefectural University, Kyoto, Japan. [2]Research Laboratories, The Green Cross Corp., Osaka, Japan. Received on July 29, 1977.

Materials and Methods

All materials and methods are almost the same as those described previously[4]. As the substrates, α-N-benzoyl-D, L-arginine-p-nitroanilide for trypsin, and α-N-benzoyl-L-tyrosine-p-nitroanilide for chymotrypsin were used.

Results and Discussion

1. Purification procedure of non-dialyzable proteinase inhibitor from eggplant exocarp.

The eggplant exocarp (11.8 kg) was ground with 0.1 M sodium acetate buffer, pH 5.5 (11.8 l). The extract was heated at 80°C for 10 min. After the filtration, ammonium sulfate was added to the filtrate to give 80 % saturation. Resultant precipitate was dissolved in water and the solution was dialyzed against water with Visking cellulose tube. During the dialysis, 40 to 50 % of total trypsin inhibitory activity passed through the cellulose tube. The dialysis for at least one week with the daily exchange of water was required in order to remove the dialyzable inhibitor completely. After the dialysis, the inner solution was concentrated and lyophilized.

Lyophilized sample (24.6 g) dissolved in 0.02 M phosphate buffer, pH 7.0 was applied on a column of DEAE-cellulose equilibrated with phosphate buffer above mentioned to remove the colored material which was strongly adsorbed on a column. The inhibitor fraction was eluted with the same buffer as non-colored solution. The fractions containing inhibitory activity were collected, desalted with a Diafilter (membrane G-01T, fractionation range of mol. wt. 1000) and lyophilized (226 mg).

This sample was dissolved in 0.1M acetate buffer, pH 5.4 and the solution was chromatographed on a column of DEAE-Sephadex A-25 equilibrated with 0.1 M acetate buffer, pH 5.4. The elution was performed with the same buffer. As shown in Fig. 1, a high protein peak with a slight proteinase inhibitory activity appeared first of all and then a strong inhibitory activity peak was revealed. Furthermore, the column was treated with the linear gradient of NaCl (0 to 1.0 M) and another inhibitory activity peak was gained but the activity was very low. The main inhibitor fractions were collected, desalted with a Diafilter and lyophilized (41.0 mg).

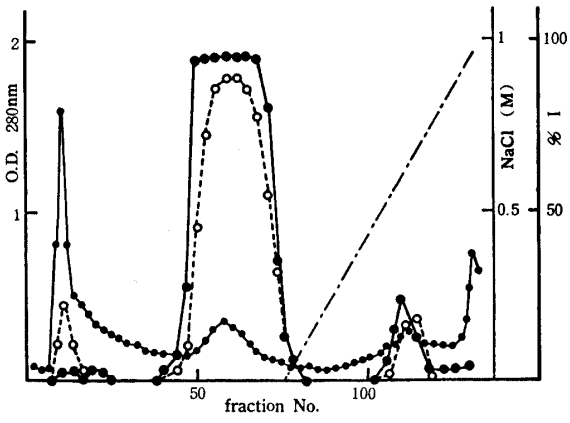

Fig. 1. DEAE-Sephadex A-25 chromatography. column size 2.5×41cm; flow rate 30ml/hr; one fraction 6.5ml; —●— absorbance at 280 nm; —●— trypsin inhibitory activity; ···○··· chymotrypsin inhibitory activity; —·—·— NaCl concentration.

2. Gel chromatography on Sephadex G-50.

The lyophilized inhibitor sample obtained by the methods above mentioned was dissolved in 0.1 M acetic acid and solution was applied to a column of Sephadex G-50 equilibrated

with acetic acid solution, followed by elution with the same solution. The protein peak was shown as a single peak and it was completely coincided with the inhibitory activity as shown in Fig. 2. The molecular weight of the inhibitor was calculated to be about 6000 from the elution position. The fractions of this peak were pooled and lyophilized. This sample was regarded as a final non-dialyzable inhibitor preparation (30.5 mg).

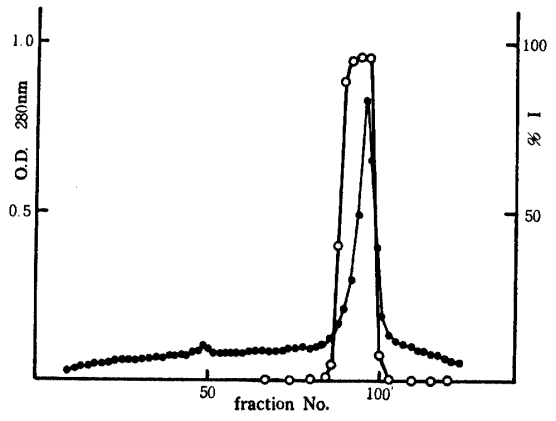

Fig. 2. Gel filtratlion of the inhibitor obtained by DEAE-Sephadex A-25 chromatography on Sephadex G-50. column size 2.5×96cm; flow rate 20ml/hr; one fraction 4ml; —●— absorbance at 280 nm; —○— trypsin inhibitory activity.

3. Inhibitory activity of the non-dialyzable inhibitor.

Fig. 3 shows the inhibitory activity of the purified non-dialyzable inhibitor against trypsin. Inhibitor A designates the purified dialyzable inhibitor[4], and inhibitor C means the inhibitor prepared with the use of acetylated cellulose tube for dialysis[6]. Inhibitor B is the one under consideration in this paper. One μg of the inhibitor completely inactivated 3.6 to 3.7 μg of trypsin in all cases. If it is assumed that 1 mol trypsin is inactivated by 1 mol inhibitor, Fig. 3 indicated that the molecular weight of the non-dialyzable inhibitor was calculated to be 6300 to 6400 on the basis of the molecular weight of 23300 for trypsin[7].

This inhibitor also inhibited chymotrypsin but the inhibition did not show stoichiometric reaction and the behaviour of inhibition for chymotrypsin was almost the same as those of inhibitor A and C.

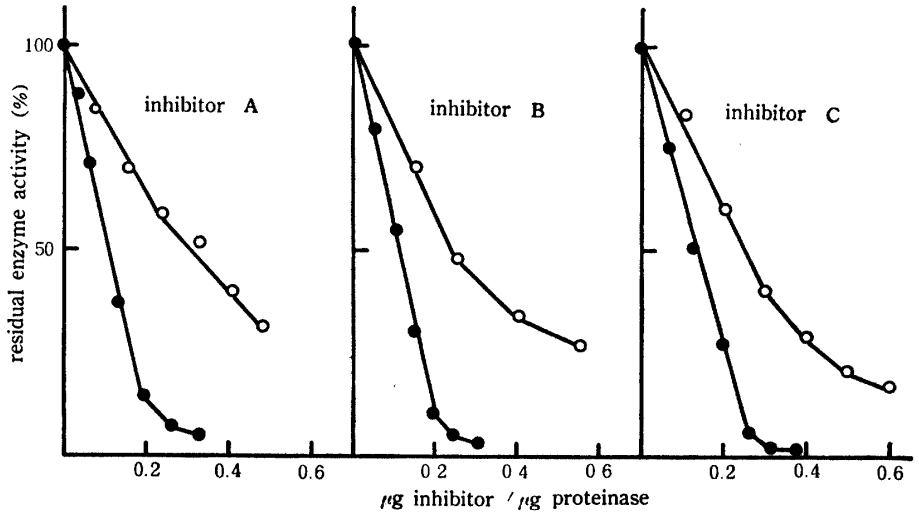

Fig 3. The inhibition of proteinases by purified inhibitors. —●—, trypsin; —○—, chymotrypsin. See in the text about inhibitor A B and C.

4. Amino acid composition.

The results of amino acid analyses of non-dialyzable are given in Table 1. The time for hydrolysis was 24 hr. They were computed based on the molecular weight of 6300. The inhibitor was composed of 58 amino acid residues, from which the molecular weight was calculated to be 6203. The amino acid composition of non-dialyzable inhibitor is in good agreement with that of inhibitor C.

In conclusion, the data of the molecular weight, inhibitory behaviour for trypsin or chymotrypsin and the amino acid composition mean that non-dialyzable inhibitor is identical with the dialyzable one or the inhibitor prepared with the acetylated cellulose tube for dialysis. Also these results suggest that only one kind of main inhibitor having the molecular weigh of about 6000 exists in eggplant exocarp.

However, the reason why the inhibitor of molecular weight of about 6000 in crude stage remains in cellulose tube during dialysis for long time, is still obscure.

Table 1. Amino acid composition of the eggplant trypsin inhibitor

amino acid	inhibitor*		
	A	C	B
Lysine	4	4	4.2 (4)**
Histidine	0	0	0
Arginine	3	3	3.0 (3)
Aspartic acid	7	7	7
Threonine	2	2	2.2 (2)
Serine	4	4	3.5 (4)
Glutamic acid	4	5	4.6 (5)
Proline	5	5	5.0 (5)
Glycine	7	7	6.7 (7)
Alanine	3	3	2.8 (3)
Cystine/2	8	8	8.3 (8)
Valine	0	0	0
Methionine	0	0	0
Isoleucine	4	4	3.6 (4)
Leucine	2	2	1.9 (2)
Tyrosine	2	2	1.8 (2)
Phenylalanine	2	2	2.2 (2)
Tryptophan	0	0	0
Total	57	58	(58)

* Inhibitor A, B and C are the same as those in Fig. 3.

** The nearest integers are expressed in parentheses.

References

1) M. Kanamori, F. Ibuki, M. Tashiro, M. Yamada and M. Miyoshi, J. Nutr. Sci. Vitaminol., **21** (1975) 421.

2) M. Kanamori, F. Ibuki, M. Yamada, M. Tashiro, and M. Miyoshi, J. Nutr. Sci. Vitaminol., **21** (1975) 429.

3) M. Kanamori, F. Ibuki, M. Tashiro, M. Yamada and M. Miyoshi, Agric. Biol. Chem., **40** (1976) 839.

4) M. Kanamori, F. Ibuki, M. Tashiro, M. Yamada and M. Miyoshi, Biochim. Biophys. Acta, **439** (1976) 398.

5) F. Ibuki, M. Tashiro, M. Yamada, M. Miyoshi and M. Kanamori, Sci. Rep. Kyoto Pref. Univ., Agr., **28** (1976) 172.

6) F. Ibuki, M. Yamada M. Tashiro and M. Kanamori, J. Nutr. Sci. Vitaminol., **23** (1977) 133.

7) K. A. Walsh, D. L. Kauffman, K. S. V. Sampath Kumar and H. Neurath, Proc. Natl. Acad. Sci. U. S., **51** (1964) 301.

要旨：ナスビ外皮より非透析性のプロテアーゼインヒビターを精製した。塩析，熱処理，DEAE- セファデックスおよびゲル漏過を用いたが，最初の段階を除いては，透析は行わず，脱塩はすべてダイヤフィルターを用いた。このようにして精製したインヒビターは，その分子量，トリプシンやキモトリプシンに対する挙動，またアミノ酸組成の点からみて，透析性およびアセチル化セルロースを透析に用いて精製したインヒビターと全く同じものであった。そしてナスビ外皮には主要なインヒビターとしては一種類しか存在しないことが明らかとなった。しかし分子量6000のインヒビターが，未精製のとき，何故，透析膜中に残存するかは依然として不明である。