Partial Amino Acid Sequence of Cucumber Green Mottle Mosaic Virus Coat Protein†

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Chymotryptic peptides of CGMMV* protein were separated by chromatography on a AG50W-X2 column. Tryptic peptides obtained before and chymotryptic peptides which contain basic amino acids were aligned to yield a partial amino acid sequence (residue 41 to 129 in the corresponding TMV protein) of the protein. Amino acid sequences of coat proteins of TMV group were compared and CGMMV protein showed some variations even in such sections as 87 to 94 or 113 to 122, which is identical in the other coat proteins. Distributions of hydrophobicity in CGMMV, TMV, and HR proteins were found to be very similar each other in spite of fairly large difference in amino acid sequences. This suggests that the specific distribution of hydrophobicity in the primary structure would be significant for specific conformation of the protein which constructs the rod-shaped virus particle.

In the preceding paper,¹⁾ we reported that CGMMV belongs to TMV group and is a strain least related to TMV among viruses so far known to be in TMV group. As Inoue *et al.*²⁾ reported before, this virus is just similar in its shape (rod, $180 \times 3,000$ Å) and other physico-chemical properties to TMV but different in its pathological properties. The amino acid composition of the protein is also most different from TMV.¹⁾ These lead us to conclude that this virus protein is very good material for studying the relationship between the structure and the function of the protein. With the amino acid sequence of this protein, we will learn more essential structure to its function in virus particle.

In this paper, we report further study on amino acid sequence of the protein and discuss in relationship with its function.

MATERIALS AND METHODS

CGMMV protein. The coat protein was separated by the cold acetic acid method³⁾ from the virus¹⁾ and was lyophilized.

Chymotrypsin and trypsin. These enzymes were purchased from Worthington Biochemical Corporation. Chymotrypsin was used without further purification. Trypsin was treated with TPCK prior to use.

Nagarse. This enzyme was purchased from Nagase Sangyo Co.

Other reagent. Phenylisothiocyanate and trifluoroacetic acid were purchased from the Institute for Protein Research of Osaka University. Pyridine and benzene were of analytical grade. Hydrazine was purchased from Ishizu Chemical Industry Corp., and was redistilled prior to use. All other chemicals used were of reagent grade.

† Amino Acid Sequence of Cucumber Green Mottle Mosaic Virus Coat Protein. Part II.

* Abbreviations used: CGMMV, cucumber green mottle mosaic virus; TMV, tobacco mosaic virus (Vulgare); HR, Holmes ribgrass strain; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone.

Chymotryptic digestion of the protein. The suspension of the protein (200 mg) in 10 ml of water was adjusted to and maintained at pH 8.0 with 1 N NaOH at 38°C in the vessel of a Radiometer (Copenhagen, TTTIC) pH-stat. A 2 mg aliquot of chymotrypsin which was dissolved in water was added at zero time and another same aliquot after 1 hr of digestion. The digestion was stopped after 5.5 hr. The insoluble matter in the digest at pH 8 was separated by centrifugation and the supernatant was lyophilized. The peptides were then chromatographed on an AG 50-X2 column (0.9×150 cm) equilibrated with the 0.2 M pyridine-acetic acid buffer (pH 3.1). The peptides were eluted with a double linear gradient of increasing pH and ionic strength. The gradients were: (gradient I) 600 ml of 0.2 M pyridine-acetic acid (pH 3.1) followed by 600 ml of 0.2 M pyridine-acetic acid (pH 3.1) and 600 ml of 2 M pyridine-acetic acid (pH 5.0); (gradient II) 250 ml of 2 M pyridine-acetic acid (pH 5.0) and 250 ml of 2 M pyridine-acetic acid (pH 6.0). The column was stripped with 2 M NH₄OH. The column was maintained at 38°C and the flow rate was 40 ml per hour. The effluent was collected in 5.0 ml fractions. The separation was monitored by ninhydrin color at 570 m μ after alkaline hydrolysis and peptide-containing fractions were pooled accordingly. Sakaguchi reaction for arginine was also performed without the prior hydrolysis with alkali.

Digestion of peptides with nagarse or trypsin. A given amount of peptides was dissolved in 2–3 ml of water and was adjusted to pH 8.0 with 0.5 N NaOH or 1 N NH₄OH. A protease, approximately 2% of the peptide substrate by weight, was added and the mixture was stirred at 38°C for 2.5 to 3 hr. The pH of the mixture was maintained at pH 8.0 throughout digestion by an occasional addition of alkaline solution. Sub-digests were fractionated on a Dowex 1X2 column (1×50 cm) under almost the same conditions as those used for separation of tryptic peptides of the protein,¹¹ except that the column was operated at room temperature. Paper chromatography was also used to separate some of the sub-digests (pyridine-butanol-acetic acid-water, 10:15:3:12, v/v), and high voltage paper electrophoresis at pH 6.5 (pyridine-acetic acid-water, 10:0.4:90, v/v, 3,000 volts, 1 hr).

Other methods. Amino acid analysis of peptides was carried out with a Hitachi Amino Acid Analyzer, Model KLA 3B. Sequence determination of peptides

by subtractive Edman degradation was performed according to the method of Konigsberg and Hill.⁴⁾ Hydrolysis of peptides with 0.03 N HCl was performed at 107°C for 16 to 20 hr in an evacuated, sealed tube.

RESULTS

Isolation of chymotryptic peptides

The separation of the soluble chymotryptic peptides is shown in Fig. 1. Each fraction was pooled and examined for purity by paper chromatography. Impure fractions were purified by paper chromatography or preparative high voltage paper electrophoresis. Peptides which contain basic amino acids and gave overlaps between tryptic peptides are listed in Table I.

Amino acid sequence of a chymotryptic peptide (C-13) containing a histidine residue

Asn-Ala-Thr-Lys-Arg-Asx-Asx-Asx-Ala-Ser-Thr-Ala-Ala-His-Asx-Asx-Ile-Leu-Pro-Leu-Leu

Since the tryptic peptide containing a histidine residue was difficult to get pure, peptide C-13 was used to determine the amino acid sequence around the histidine residue. Tryptic subdigestion of C-13 gave four peptides which were fractionated by a Dowex 1×2 column. The first peptide (C-13T-1) contained lysine. The second peptide (C-13T-2) contained lysine and arginine. Since the amino acid composition of these peptides were identical with those of the carboxyl-terminal portion of peptide T-10, peptide C-13 was found to be connected with peptide T-10.

Since third and fourth peptides contained histidine and the amino acid compositions of these peptides were identical except arginine, these peptides were combined and further submitted to nagarse-digestion. The digest yielded four peptides which were fractionated by a Dowex 1X2 column. The sequence of each peptide obtained was partially determined, mainly by Edman degradation. Four rounds of Edman degradation of C-13T-

FIG. 1. Elution Profile of the Chymotryptic Peptide from CGMMV Protein on a 1.0×150 cm Column of AG 50-X2 at 38° .

The column was developed at 50 ml/hr with a double linear gradient of pyridine acetate as described in the text. Fractions of 5.0 ml were collected and monitored by ninhydrin analysis after alkaline hydrolysis (solid line) and by Sakaguchi reaction (dotted line).

TABLE I. AMINO ACID COMPOSITIONS OF CHYMOTRYPTIC PEPTIDES^{a)} FROM CGMMV-PROTEIN

Peak	8	9	10	11	13	14	16	17		18	19	20	
Amino acid								a	b				
Lys	1.00			0.28	1.00	0.79	0.74	0.84	0.82				
His					0.84								
Arg	0.96	2.00	1.77	1.00	0.97		1.00	1.17	2.00	1.45	0.92	1.00	1.22
Asp	2.21	4.87	3.80	0.53	4.77		2.21	2.26	1.78	1.96	1.00		1.02
Thr	2.64	1.56	1.14	1.68	1.56		0.87	0.45	0.70				
Ser	4.75	3.60	3.80	2.83	1.52	1.05	1.10	1.17	2.12	1.17	0.94	1.29	2.50
Glu	1.71	3.73	2.70	0.75	0.85		0.92	1.00	1.36	0.70	1.00	0.71	1.16
Pro		2.43	1.83	0.93	1.08				0.86	1.00	0.98		
Gly	0.68	2.13	1.52	2.49	1.56		1.15	1.14	1.96				0.46
Ala	3.57	3.02	3.30	1.55	3.39	1.07	1.21	0.95	1.56	0.84	1.15		2.10
Val	0.94	1.08	1.16										
Ile	0.54	1.16	1.00	0.88	0.86		0.54	0.95	1.34				1.60
Leu	0.61	2.39	2.00	2.14	2.40		1.64	1.86	3.12				1.76
Tyr													
Phe				0.93		1.00	0.54		1.28	2.00	2.94		
Yield %	84	21	c)	4	56	7	17	c)	2	16	8	20	8
Purification ^{b)}	P	P	P	P	—	P, E	—	P, E	P	P	P	P	P
Overlap		T9, T1 T10	T9, T1 T10		T10, C13		TP-N, ^{d)} T6, T4	TP-N, ^{d)} T6, T4	TP-N, ^{d)} T6, T4	T4, T8	T4, T8	T6, T4	(T8, T9)

^{a)} In this table, only those peptides which contain basic amino acids and have possibilities to bridge tryptic peptides are summarized.

^{b)} Abbreviations used are: P, paper chromatography; E, high voltage electrophoresis.

^{c)} Not calculated.

^{d)} N-Terminal tryptic peptide of the protein.

TABLE II. CHARACTERIZATION OF PEPTIDE C-13

Sequence: Asn-Ala-Thr-Lys-Arg-Asx-Asx-Asx-Ala-Ser-Thr-Ala-Ala-His-Asx-Asx-Ile-Leu-Pro-Leu-Leu
Tryptic subdigest:
Peptide C-13T-1; ^{a)}
Sequence: (Asn-Ala-Thr-Lys)
Peptide C-13T-2; ^{a)}
Sequence: (Asn-Ala-Thr-Lys-Arg)
Peptide C-13T-3:
Composition: (Arg ₁ , Asp ₅ , Thr ₁ , Ser ₁ , Pro ₁ , Ala ₃ , Ile ₁ , Leu ₃ , His ₁)
Peptide C-13T-4: (Asp ₅ , Thr ₁ , Ser ₁ , Pro ₁ , Ala ₃ , Ile ₁ , Leu ₃ , His ₁)
Nagarse-subdigest of C-13T-3 and C-13T-4:
Peptide C-13T-(3,4)N-1:
Composition: (His ₁ , Asx ₂ , Pro ₁ , Ala ₁ , Ile ₁ , Leu ₃)
Hydrolysis with 0.03 N HCl:
Peptide C-13T-(3,4)N-1H-1:
Composition: (His, Ala)
Peptide C-13T-(3,4)N-1H-2:
Sequence: $\overrightarrow{\text{Ile}}\text{-}\overrightarrow{\text{Leu}}\text{-(Pro, Leu, Leu)}$
Peptide C-13T-(3,4)N-2:
Sequence: $\overrightarrow{\text{Ala}}\text{-}\overrightarrow{\text{His}}\text{-}\overrightarrow{\text{Asx}}\text{-}\overrightarrow{\text{Asx}}\text{-(Ile-Leu-Pro-Leu)}^b)$
Peptide C-13T-(3,4)N-3:
Composition: (Arg ₁ , Asp ₃ , Thr ₁ , Ser ₁ , Ala ₂)
Peptide C-13T-(3,4)N-4:
Sequence: $\overrightarrow{\text{Asx}}\text{-}\overrightarrow{\text{Asx}}\text{-}\overrightarrow{\text{Asx}}\text{-}\overrightarrow{\text{Ala}}\text{-}\overrightarrow{\text{Ser}}\text{-}\overrightarrow{\text{Thr}}\text{-}\overrightarrow{\text{Ala}}$

^{a)} The sequence was deduced from T-10. Asn was assumed from the behavior of the peptide on the Dowex 1×2 column.

^{b)} The sequence in the parentheses was deduced from C-13T-(3,4)N-1H-2 and the specificity of the enzyme.

(3,4)N-2 were accomplished before the remainder of the peptide was inexplicably lost. Then, C-13T-(3,4)N-1 was partially hydrolyzed with 0.03 N HCl to yield two smaller fragments which were fractionated by high voltage paper electrophoresis. The routine method of Edman degradation of C-13T-(3,4)N-1H-2 was not accomplished again before the inexplicable loss of the remaining peptide in the water layer. After the failure of the Edman method was shown to be caused by high solubility of the peptide in benzene, the subsequent Edman degradations were performed by analyzing amino acid compositions of the remaining peptide dissolved in the benzene used for elimination of excess PTH. The results are summarized in Table II.

Alignment of tryptic and chymotryptic peptides

The alignment of tryptic and chymotryptic peptides which have been purified is shown in Fig. 2. The alignment was also performed to maximize the homology with TMV protein. In each line, the tryptic peptides are positioned below and the chymotryptic peptides above the sequence. Thus, the amino acid sequence of the portion, residue No. 41 to 129 of the corresponding TMV protein was determined. The positions of T-2, T-3 and T-5 were not yet determined directly.

DISCUSSIONS

As shown in Fig. 2, sequences of CGMMV and TMV proteins⁵⁾ are similar in some respects. The locations of basic amino acids,

FIG. 2. Partial Sequence of CGMMV Protein.

Abbreviations used are: T, tryptic peptide; C, chymotryptic peptide; H, peptide released by partial hydrolysis with 0.03 N HCl. The sequence in parentheses is not yet determined.

lysine and arginine, are beautifully coincident and the corresponding tryptic peptides are composed of almost the same number of amino acid residues in both proteins. As we reported before,¹¹ CGMMV protein is composed of 160 amino acid residues, which are two more residues than that of TMV protein, and one of the additional two residues is located between residue 70 and 71 of TMV protein as serine. The addition of amino acid like serine is genetically interesting besides the deletion of amino acids in the protein of Holmes Ribgrass strain.⁶¹ In the structure of CGMMV

protein, Ser-Arg appears four times. This repetition is also genetically interesting suggesting that the proteins may have evolved from shorter peptide chains as discussed by Smith *et al.*⁷¹ on subtilisin Carlsberg. A comparison of the primary structures of coat proteins is shown in Fig. 3, in which the partial CGMMV protein sequence is added to the comparison originally proposed by Fraenkel-Conrat.⁸¹ It is seen that there are much fewer identical residues in the sequence of CGMMV protein than in the others. Portions, such as 87 to 94 and 113 to 122 as

		10										20												
V	Ac	Ser	Tyr	Ser	Ile	Thr	Thr	Pro	Ser	Gln	Phe	Val	Phe	Leu	Ser	Ser	Ala	Trp	Ala	Asp	Pro	Ile	Glu	Leu
D	Ac			Ser													Val	-						
U ₂	Pro	Thr	Asn	Ser								Tyr						Tyr	-		-	Val		
HR	Ac	Asn		Asn	Ser	Asn					Tyr	Gln	Tyr	Phe	Ala	Ala	Val			Glu	Thr	Pro	Met	
CV																								
CH				Ile		His	His	Leu	Val					Leu						Ala	Leu	Val		
V	Ile	Asn	Leu	Cys	Thr	Asn	Ala	Leu	Gly	Asn	Gln	Phe	Gln	Thr	Gln	Gln	Ala	Arg	Thr	Val	Val	Gln	Arg	
D	Leu	Val		Ser	Ser									-					Thr	-	Gln			
U ₂				-	-														Thr		Gln			
HR	Leu	Gln	Val	Ser				Ser	Gln	Ser	Tyr						Ala	Gly	Asp	Thr	Arg	Gln		
CV																			Asp	Ile	Leu	Arg	Glu	
CH	Val	Ser		Ile				Ser																
V	Gln	Phe	Ser	Gln	Val	Trp	Lys	Pro	Ser	Pro	Gln	Val	Thr	Val	Arg	Phe	Pro	Asp	Ser	Asp	Phe	Lys	Val	
D			Glu	-				Phe		Ser								Gly	Asp	Val	Tyr			
U ₂			Ala	Asp	Ala					Val	Met	-						Ala			Tyr			
HR			Ala	Asn	Leu	Leu	Ser	Thr	Ile	Val	Ala	Pro	Asn	Gln				Thr	Gly		Arg			
CV	Ser	Leu	Thr	Leu	Gly	Leu	Thr	Ser	Val	Ala	Ser	Leu	Asn	Ser				Ala	Asx	Glx	Phe			
CH																								
V	70	Tyr	Arg	Tyr	Asn	Ala	Val	Leu	Asp	Pro	Leu	Val	Thr	Ala	Leu	Leu	Gly	Ala	Phe	Asp	Thr	Arg	Asn	Arg
D													Ile					Thr					-	
U ₂													Ile					Asn	Ser					
HR													Tyr	Glu				Met	Lys	Ser				
CV		Trp	Ile	Ala	Ile	Ser	Asp						Ile	Ala	Ser	Asx	Ser	Leu	Ser					
CH		Ser		Ser																				
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FIG. 4. Hydrophobicity Distribution in Primary Structure of TMV (Vulgare), HR and CGMMV Protein. For CGMMV protein, partial sequence (42 to 129) was used.

well as cysteine residue, which have been believed to play some important role in functions of the subunit protein, were shown to permit some variation, while maintaining a functional conformation of the subunit protein. Residue 108-Leu to 112-Arg, about 22 residues from the carboxyl-terminus and 68-Lys of TMV protein are known to have immunological activities^{9,10)} and those corresponding parts in CGMMV protein are varied. Differences in immunological behavior of CGMMV from TMV could be partially due to these differences in amino acid sequence. In order to investigate the roles of variable regions in the sequence, distributions of hydrophobicity in the primary structure were compared in Fig. 4, using the value Δf_t , hydrophobicity of the side chain of each amino acid estimated by Tanford.¹¹⁾ It is apparent that patterns of hydrophobicity distribution in the primary structures of these proteins are similar to each other in spite of the great differences in their amino acid sequences. Generally, the regions

with high hydrophobicity and that with low hydrophobicity alternate except for the section, residue 110 to 123, that is composed of amino acids with comparatively low hydrophobicity. Therefore, the similarity in the hydrophobicity distribution appears to be important in the relationship between the sequence and the specific conformation of the protein. The specific distribution of hydrophobicity in the sequence would be necessary for maintaining a specific conformation of the subunit protein that is necessary for constructing the rod shaped virus particle.¹²⁾ This hypothesis may be proved valid when a crystallographic structure determination of TMV protein is done.

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