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Cleavage specificity of cucumisin, a serine protease, with synthetic substrates

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Abstract

The substrate specificity of a plant serine protease, cucumisin (EC 3.4.21.25), was studied by the use of synthetic oligopeptides and peptidyl-*p*NA substrates. Since P1′-Ser, Ala, and Gly substrates were hydrolyzed rapidly, cucumisin appears to prefer a small side chain at the P1′ position of the oligopeptide substrate. The k_{cat}/K_m for the hydrolysis of P1-Leu, Ala, Phe, and Glu substrates demonstrated that they were preferentially cleaved over P1-Lys, diaminopropionic acid (Dap), Gly, Val, and Pro substrates. From the digestion of peptidyl-*p*NAs, the specificity of the protease was determined to be broad, but the preferential cleavage sites were hydrophobic amino acid residues at the P1 position.

Keywords: *Cucumis melo*; Cucurbitaceae; Melon fruit; Serine protease; Substrate specificity

1. Introduction

Several useful endopeptidases have been isolated from plant sources, including papain and bromelain, which are used extensively in the food industry. Most of the isolated plant endopeptidases have been classified as cysteine endopeptidases, whose activity is readily reduced by air oxidation or metal ions. Cysteine endopeptidases require reductants and chelating agents, whereas serine endopeptidases do not require these reagents. For this reason, serine proteases may also be of use in the food industry. Whereas the cysteine proteases have been studied extensively (Arnon, 1970; Liener and Friedenson, 1970; Murachi, 1970), relatively little is known about other types of plant proteases. Previously, we found cucumisin [EC 3.4.21.25] in melon fruit, *Cucumis melo L.* var. *Prince* (Kaneda and Tominaga, 1975; Uchikoba et al., 1995). This protease is strongly inhibited by DFP but is unaffected by reducing compounds such as cysteine and 2-mercaptoethanol. Therefore, the enzyme seems to be a serine protease. The amino acid sequence around the reactive serine and histidine of cucumisin has been identified (Kaneda et al., 1984; Yonezawa et al., 1995), and the primary structure has been predicted from its cDNA (Yamagata et al., 1994), and was found to be homologous to those of proprotein processing proteases such as Kex2 from yeast (Mizuno et al., 1988) and human furin (Van den Ouweland et al., 1990). Cucumisin is present in high concentrations in melon fruit, and appears to be very stable allowing a large scale preparation (Kaneda and Tominaga, 1975; Uchikoba et al., 1995).

Cucumisin seems to prefer carboxylic amino acid residues at P1 position (Kaneda and Tominaga, 1975), but has a broad specificity for P1 and P1′ as is common in other plant proteases. However, the amino acid sequences around the cleavage sites of naturally occurring substrates differed, and exact specificity was

Abbreviations: Dap, diaminopropyonyl; DFP, diisopropyl fluorophosphate; *p*NA, *p*-nitroanilide; Suc-, succinyl.

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not obtained by the use of naturally occurring substrates (Uchikoba et al., 1995).

In this paper, we report the cleavage specificity of P1′ and P1 positions for cucumisin against oligopeptide substrates and peptidyl-*p*NAs.

2. Results and discussion

In the previous paper, we reported the hydrolysis rates for several commercially available peptidyl-*p*NA substrates (Uchikoba et al., 1995). The k_{cat} increased with the increase of peptide chain length; tetrapeptidyl-*p*NA was cleaved about 25 times faster than tripeptidyl-*p*NA. It was noted that some subsites of cucumisin had substantial effects on its activity. Moreover, cucumisin preferred Pro at the P2 position as described above, and so, in the present study, the effect of the P1 position on the hydrolysis rate was determined by using nine substrates, I–IX (Ala-Ala-Pro-X-Y; X, Y = Ala, Ser, and Leu). Two oligopeptides were detected in each oligopeptide hydrolysate to apply with an ion-exchange column or a reverse phase HPLC column. It was suggested that only one cleavage site was found for each substrate by cucumisin. P1-Ala substrate, Ala-Ala-Pro-Ala-Ser (I) is the most favorable for cucumisin among them, as shown in Table 1. The substrates containing Leu at the P1 position (VII, VIII, and IX) were minimally hydrolyzed by cucumisin. The effect of the P1′ position on the hydrolysis rates were compared in the nine substrates (I–IX). Among them, the most favorable substrates for cucumisin were Ser-P1′, which have Ala (I) and Ser (IV) at the P1 position. However, Ser-P1′ substrate containing Leu at the P1 position (VII) was a poor substrate for cucumisin. Moreover, the effect of the P1′ position on the hydrolysis rate was determined. Eleven substrates (Ala-Ala-Pro-Ala-W; W = Gly, Ala, Val, Leu, Ile, Ser, Phe, Tyr, Pro, Asp, and Lys) were designed for the basis of the results of the S1-preference (P1-Ala) of cucumisin. As shown in Table 2, the P1′-Ser (I), Ala (II), and Gly (X) substrates were hydrolyzed rapidly. The P1′-Asp (XIV) substrate was hydrolyzed slowly, so it seems that cucumisin did not prefer a charged amino acid at the P1′ position.

The specificity of cucumisin obtained by synthetic peptide substrates was in agreement with that obtained by the naturally occurring peptides and proteins. Yamagata et al. (1994) reported that the isolation of a putative native form of cucumisin has a molecular mass of 67 kDa. This 67-kDa enzyme shows limited autolysis between Thr (505) and Gly (506) to produce a 54-kDa enzyme (Yamagata et al., 1994). This cleavage site is in agreement with the specificity of cucumisin.

In order to determine the effect of the P1 substitutions, peptidyl-*p*NA substrates (Ala-Ala-Pro-Z-*p*NA; Z = Ala, Leu, Phe, Glu, Lys, Val, Gly, Pro, and diaminopropinonic acid (Dap)) were synthesized. As shown in Table 3, P1-Ala, Leu, Phe, and Glu substrates were hydrolyzed much faster than the P1-Lys and Dap substrates. The substrates that contained Val and Pro at the P1 position were minimally hydrolyzed by cucumisin. The kinetic parameters (k_{cat}/K_m) for the hydrolysis of nine Ala-Ala-Pro-X-*p*NA substrates by cucumisin are shown in Table 3. The most favorable substrate for the protease was Ala-Ala-Pro-Leu-*p*NA. It is shown that the k_{cat}/K_m of Ala-Ala-Pro-Ala-*p*NA for cucumisin was of almost the same order as that of porcine elastase (data not shown). The substrates Ala-Ala-Pro-Phe-*p*NA, Ala-Ala-Pro-Glu-*p*NA, Ala-Ala-Pro-Dap-*p*NA, and Ala-Ala-Pro-Lys-*p*NA were also hydrolyzed by the enzyme. However, the substrates Ala-Ala-Pro-Gly-*p*NA and Ala-Ala-Pro-Val-*p*NA showed low parameters under the same conditions. Ala-Ala-Pro-Lys-*p*NA was hydrolyzed by bovine trypsin but Ala-Ala-Pro-Dap-*p*NA was not (data not shown). Bovine α-chymotrypsin hydrolyzed Ala-Ala-Pro-Phe-*p*NA and Ala-Ala-Pro-Leu-*p*NA, but not Ala-Ala-Pro-Ala-*p*NA (data not shown). Moreover, subtilisin BPN′ cleaved Ala-Ala-Pro-Leu-*p*NA and Ala-Ala-Pro-Phe-*p*NA faster than Ala-Ala-Pro-U-*p*NA (U =

Table 1
Hydrolysis rates of oligopeptide substrates with P1 or P1′ substitutions by cucumisin

No.	Substrates				Hydrolysis rate (nmol/min/nmol protein)	Relative activity (%)
	P4	P3	P2	P1 ↓ P1′		
I	Ala	Ala	Pro	Ala-Ser	2.22	100
II	–	–	–	Ala-Ala	1.62	73
III	–	–	–	Ala-Leu	0.044	2
IV	–	–	–	Ser-Ser	2.07	93
V	–	–	–	Ser-Ala	1.07	48
VI	–	–	–	Ser-Leu	0.088	4
VII	–	–	–	Leu-Ser	0.044	2
VIII	–	–	–	Leu-Ala	0.067	3
IX	–	–	–	Leu-Leu	0.156	7

Table 2
Hydrolysis rates of oligopeptide substrates with P1′ substitutions by cucumisin

No.	Substrates					Hydrolysis rate (nmol/min/nmol protein)	Relative activity (%)
	P4	P3	P2	P1 ↓	P1′		
I	Ala-	Ala-	Pro-	Ala-	Ser	2.22	100
II	–	–	–	–	Ala	1.62	73
X	–	–	–	–	Gly	0.67	30
XI	–	–	–	–	Tyr	0.36	16
XII	–	–	–	–	Phe	0.31	14
XIII	–	–	–	–	Lys	0.27	12
XIV	–	–	–	–	Asp	0.13	6
XV	–	–	–	–	Val	0.11	5
XVI	–	–	–	–	Ile	0.044	2
III	–	–	–	–	Leu	0.044	2
XVII	–	–	–	–	Pro	0	0

Lys, Ala, Dap) (data not shown). In the case of peptidyl-*p*NA substrates, the P1 specificity of cucumisin is different from that of trypsin and α-chymotrypsin, but is similar to that of subtilisin BPN′. The specificity of cucumisin on the insulin B-chain differs from that of trypsin and chymotrypsin (Kaneda and Tominaga, 1975; Uchikoba et al., 1995). In the amino acid sequence, cucumisin resembles the subtilisin family, however, subtilisin BPN′ did not cleave the C-terminal sides of cysteinic acid and glutamic acid with insulin B-chain as a substrate. The sites of cleavage by papain (Kaneda et al., 1995a), a cysteine protease from a plant source, are similar to those of cucumisin in this regard. The amino acid sequence around the catalytic amino acid residues of cucumisin is homologous with those of subtilisin, e.g. the four amino acids, Gly-Thr-Ser*-Met in sequence around the reactive serine of cucumisin (Kaneda et al., 1984). The sequence around the reactive histidine of cucumisin (Yonezawa et al., 1995) is also identical to that of subtilisin BPN′ (Yamagata et al., 1994), e.g. the four amino acids, His*-Gly-Thr-His. However, the molecular size, the chain length of D-H-S (Yamagata et al., 1994; Noda et al., 1994), and the enzymatic properties are distinct between cucumisin and subtilisins. The thermal stability of cucumisin (60°C) is higher than that of subtilisin BPN′ (40°C) at pH 11 (Kaneda et al., 1995b), and cucumisin has high stability at acidic pH levels, where subtilisin is unstable. Cucumisin preferentially cleaved a peptide bond on the carboxyl side of negatively charged amino acid residues (Uchikoba et al., 1995) which were not hydrolyzed by subtilisin BPN′ as described above. The above results seem to classify cucumisin into the subtilisin subfamily.

Table 3
Kinetic parameters for hydrolysis of peptidyl-*p*NA substrates with P1 susbtitutions by cucumisin

Substrates	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/Ms)
Ala-Ala-Pro-Leu-*p*NA	1.28	2.70	2,110
– – – Ala –	0.82	1.07	1,300
– – – Phe –	1.25	1.58	1,260
– – – Glu –	3.70	3.31	894
– – – Dap –	1.56	0.69	442
– – – Lys –	2.33	0.28	120
– – – Gly –	2.50	0.081	32
– – – Val –	0.56	0.010	18
– – – Pro –	1.19	0.017	14

In a previous report, some peptidyl-*p*NA substrates were synthesized, and the hydrolysis rate for an endopeptidase from melon fruit and cucumisin was determined by Noda et al. (1994). Cucumisin preferred Ala, Phe, Gln, and Asn at the P1 position and this specificity for peptidyl-*p*NA substrates was in agreement with our present data. However, it was in conflict with the data obtained with the present peptide substrates. It must be noted that peptidyl-*p*NA substrates have a *p*NA group at the P1′ position, which is not a naturally occurring amino acid, and the substrate specificity obtained by peptidyl-*p*NA substrates does not always agree with peptides and protein substrates.

3. Experimental

3.1. Materials

Prince melon (*Cucumis melo* var. *Prince*) was obtained in Kagoshima Prefecture, Japan. DEAE-cellulose was purchased from Whatman. CM-Sepharose was a product of Pharmacia. Casein was obtained from MERCK. TLCK was a product of Sigma. TPCK was from Seikagaku Kogyo. Other materials were purchased from Wako Pure Chemical.

3.2. Enzyme purification

Cucumisin was purified by the method previously

reported by Uchikoba et al. (1995). The active fractions were collected and kept in −20°C until required.

3.3. Substrate synthesis

The oligopeptide substrates, Ala-Ala-Pro-X-Y (X, Y = Ala, Ser, and Leu); Ala-Ala-Pro-Ala-W (W = Gly, Val, Ile, Phe, Tyr, Pro, Asp, and Lys) and peptidyl-*p*NA substrates, Ala-Ala-Pro-X-*p*NA (X = Phe, Leu, Ala, Gly, Lys, Glu, Val, Pro, and Dap) were prepared by the solution method (Yonezawa et al., 1994; Delmar et al., 1979) and purified by HPLC on a reverse phase octadecyl silica column. Homogeneity of the synthetic substrates was demonstrated by HPLC, amino acid analysis and elemental analysis.

3.4. Hydrolysis of oligopeptides

To 0.2 ml of substrate solution (20 mM, in 0.1 M Tris–HCl buffer pH 8.0), 0.2 ml of cucumisin solution of each concentration (0.1 M Tris–HCl buffer pH 8.0) was added and incubated at 37°C. Then, 0.1 ml of the reaction mixture was taken out and added to 25 μl of formic acid. The reaction mixtures were applied to a Hitachi amino acid analyzer 034, attached to a cation-exchange column (1.0 × 500 mm, Hitachi) and equilibrated with 0.20 M citrate buffer, pH 3.3. The adsorbed oligopeptides were eluted from the column with 0.35 M Na–citrate buffer of pH 5.3. Some digested peptides were separated on a DYNAMAX-60A C18 column (4.6 × 250 mm, Rainin Instrument) with a linear gradient in acetonitrile containing 0.1% TFA, 0–40% for 30 min by using a Model 150A HPLC system (Applied Biosystems). The eluate was monitored by measuring the absorbance at 215 nm. The resulting products and hydrolysis rates were determined by comparison with the authentic peptides.

3.5. Hydrolysis of peptidyl-pNAs

Enzymatic hydrolysis was done in 0.2 M Tris–HCl buffer, pH 7.5 at 25°C. The enzyme solution (0.1 ml) was added to 0.8 ml of 0.5 mM substrate solution. The rate of enzymatic hydrolysis for peptidyl-*p*NA substrates was followed spectrophotometrically with a Hitachi U-1100 spectrophotometer. An extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm was used for the yield of the hydrolysis product, *p*NA (Erlanger et al., 1961). A unit of activity was defined as the activity giving 1 μmol of the product per min under the above conditions.

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