Sonolytic hydrolysis of peptides in aqueous solution upon addition of catechol

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The sonolytic hydrolysis of peptides with addition of phenolic reagents to aqueous solutions is described. Sonolysis of an aqueous solution of peptides to which catechol (o-dihydroxybenzene) had been added resulted in hydrolytic products reflecting the amino acid sequence without any side reactions, while sonolysis without any additives resulted in oxidation analytes and degradation products caused by side reactions. Although the use of additives such as resorcinol (m-dihydroxybenzene), hydroquinone (p-dihydroxybenzene) and phenol was also effective in producing sequence related products, several degradation products were produced by side reactions. A characteristic of the sonolysis of peptides is that the N-terminal side of proline, Xxx-Pro, is more susceptible than other amino acid residues to the process. This characteristic of sonolysis is superior to that of acid hydrolysis in which cleavage at the C-terminal side of proline, Pro-Xxx is difficult, and where dehydration products result due to side reactions.

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1. Introduction

Ultrasound has been used for the degradation of organic pollutants in aqueous solutions [1,2]. It is believed that the sonolytic degradation occurs via oxidation with hydroxyl radicals [3–6]. In sonolysis in aqueous solutions, the hydroxyl radicals are produced via rapid collapse of bubbles, a process known as transient cavitation [7,8]. Although various chemical effects in sonochemistry of aqueous solutions can be explained by the high reactivity of hydroxyl and hydrogen radicals [8], the detailed mechanism(s) is not yet clear even for simple reactions such as hydrolysis. It has been reported that the rate of hydrolysis of certain organic compounds is considerably enhanced with ultrasound [9,10]. The enhancement of hydrolysis may be due to a cooperative effect of the reactions with radical species, high temperature and high pressure with transient cavitation.

Hydrolysis is a common chemical reaction with water in aqueous solutions. Hydrolytic reactions in biological systems are essential for maintaining metabolic pathways such as energy release from adenosine triphosphate, enzymatic digestion of food materials to the degradation of proteins by proteases [11]. In aqueous solution chemistry hydrolysis of the C–N bond of peptide backbone occurs in the presence of dilute hydrochloric acid [12,13], while in water the hydrolysis hardly occurs [14]. Acid hydrolysis of peptides results in faithful sequence related products, such that the hydrolysis is often used in sequence analysis of peptides and proteins [15,16] and specific cleavages of the peptide backbone [17]. It has been reported that the peptide bonds at the C-terminal side of aspartic acid (Asp-Xxx), the N-terminal side of serine and threonine (Xxx-Ser/Thr), and both sides of glycine (Xxx-Gly and Gly-Xxx) are relatively sensitive to acid hydrolysis [15–17]. These degradation characteristics of peptides in acid hydrolysis are informative in the analysis of primary structure and identification of peptides and proteins. However, the acid hydrolysis of peptides often leads to the formation of dehydration products as a result of side reactions. Furthermore, the peptide bond at the N-terminal side of proline (Pro-Xxx) is insensitive to acid hydrolysis [16].

Here we report sonolytic hydrolysis of peptides in aqueous solution and the degradation characteristics. Ultrasound is applied to the aqueous solutions of peptides upon addition of phenolic chemicals such as catechol and its analogues. Mass spectrometry was used to monitor the sonochemical reactions and resulting products.

2. Experimental

2.1. Sonolysis

The sonication was performed with a Branson model 450 sonifier (Danbury, CT, USA) in air. The frequency and maximum power
were 20 kHz and 400 W, respectively. The irradiation horn (3.2 mm diameter and 50 mm in length) was immersed into the sample solution 10 mm below the surface. The average power delivered to the aqueous solutions was 50 W. Analytes were prepared as an aqueous solution at 10 pmol/µL. The ultrasound was applied to 1000 µL of the aqueous solutions with or without addition of catechol at 0 °C for 0 to 60 min. 100 µmol of additives were added to 1000 µL of the sample solutions.

2.2. Mass spectrometry

Mass spectra were acquired on an AXIMA-CFR matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) instrument (Shimadzu Corp., Kyoto, Japan). A nitrogen laser was used to irradiate the sample for ionization. The spectra were obtained in positive-ion reflection mode. The acceleration potential was set to 20 kV using a gridless-type electrode. Analyte solution (5 µL) was mixed with 5µL of saturated solution of matrix in water/acetonitrile (1:1 v/v) with 0.1% trifluoroacetic acid (TFA). A volume of 1 µL of sample solution was deposited onto a MALDI sample plate, and the solvents were removed by allowing evaporation in air at room temperature. Spectra were the sum of 100 profiles automatically acquired rastering the sample spot. Each profile was the result of twelve consecutive single laser pulses.

2.3. Reagents

The MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma Aldrich (Steinheim, Germany). Acetonitrile (HPLC Grade) and TFA were purchased from Wako Pure Chemical (Osaka, Japan). Water used was purified using a MilliQ water purification system from Millipore (Billerica, MA, USA). Catechol, resorcinol, hydroquinone and phenol were purchased from Tokyo Kasei (Tokyo, Japan). Adrenocorticotropic hormone fragment (ACTH18-39: RPVKVYPGADESAEAEFFL, Mr 2465.7) and angiotensin I (DRVYIHPFHL, Mr 1296.5) were purchased from Peptide Institute (Minoh, Osaka, Japan). All the reagents were used without further purification.

3. Results and discussion

3.1. Sonolysis on aqueous solutions of peptides and oxidation

The mass spectra obtained by treating aqueous solutions of ACTH18-39 without any additives with ultrasound for 0 to 45 min are shown in Fig. 1. The peak abundance of protonated [M+H]+ decreased with increasing irradiation time. Evidence for the occurrence of oxidation reactions with hydroxyl radicals is given by the fact that the number of peaks corresponding to protonated oxidation products [M+nO+H]+ (n > 1) increased with increasing irradiation time, as shown in inset A of Fig. 1. Fig. 2 shows the mass spectrum obtained upon irradiating with ultrasound for 40 min. The spectrum shows oxidation products [M+nO+H]+ (n > 6) as can be seen in inset B, peaks of sequence related products at m/z 499, 599, 1029 and 1473 and side reaction products at m/z 823, 943, 1071, 1200, 1444, 1601 and 2046 (inset A), as described in the next subsection. The oxidation of the analyte may be due to the oxidation of amino acid side chains by hydroxyl radicals. Reactivity of amino acid side chains with hydroxyl radicals has been reported by Chance’s group [18,19]. They have described that hydroxyl-attack at aromatic residues such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) results in oxidation products at +16 Da and +32 Da mass shifts [18]. The molecular mass shifts corresponding to the oxidation products M+nO (n > 6) in inset B of Fig. 2 suggest that Trp6, Phe18 and Phe22 of the analyte are modified via hydroxylation with hydroxyl radicals originating from H2O in the solution. The formation of oxidation products M+nO (n > 1) during sonolysis of aqueous solutions lacking additives was confirmed with another peptide, namely angiotensin I. The mass spectrum showed peaks corresponding to oxidation products M+nO (n > 4) (data not shown). Since angiotensin I contains aromatic residues such as Tyr4 and Phe8, the oxidation may occur in these residues with hydroxyl radicals.

3.2. Sonolysis of aqueous solutions of peptides upon catechol addition

Fig. 3 shows the change in mass spectra of an aqueous solution of ACTH18-39 to which catechol was added upon treatment with ultrasound for 0, 20, 40 and 60 min. The peak abundance of protonated analyte [M+H]+ decreased with increasing irradiation time and peaks reflecting the amino acid sequence of analyte were observed over the m/z range from m/z 499.5 of Arg1-Lys4 to m/z 2317.9 of Arg1-Glu21. The other peaks corresponding to Arg1, Arg1-Pro2 and Arg1-Val3 were not visible as they coincided with matrix peaks. The irradiation time dependence of the peak abundances of protonated analyte [M+H]+ and several informative ions at m/z 761.6, 1473.8 and 1978.8 is shown in Fig. 4. These informative peaks increased with increasing irradiation time, while the sonolysis on the aqueous solutions without any additives in Fig. 1 did not show any increase in the yields of those informative products as shown in Fig. 5. The ordinates in Figs. 4 and 5 represent the ratio of the abundance of each peak to the abundance of a reference peak at m/z 379 originating from the matrix. The maximum yields of the products in Fig. 4 were attained with a treatment time of 40 min.

The mass spectrum obtained from 40 min of sonolysis is shown in Fig. 6. Each peak observed corresponds to the protonated molecule [M+H]+ of a peptide fragment resulting from the sonolytic hydrolysis (Scheme 1). The sonolytic characteristics of the peptide upon sonolysis can be found by comparing the abundance of a peak with those of both side peaks in Fig. 6. The peaks at m/z 761.6 and 1978.8 corresponding to the N-terminal side peptides originating from the C–N bond cleavage at Tyr6-Pro7 and Phe18-Pro19, respectively, are higher in abundance than both side peaks. These peptide fragments originate from cleavage at the N-terminal side of the proline peptide bond (Xxx-Pro). To confirm this characteris-
tic, an aqueous solution of another proline-containing peptide, namely angiotensin I (DRVYIHPFHL) was treated with ultrasound for 40 min upon addition of catechol. The mass spectrum shows relatively abundant peaks at \( m/z \) 802.5 and 513.4 corresponding to the peptide fragments DRVYIH and PFHL, respectively, as well as the intense protonated analyte \([M+H]^+\) at \( m/z \) 1296.9 (data not shown). This suggests that the Xxx-Pro bond is more susceptible.

Fig. 2. Mass spectrum of sonolytic products of ACTH18-39 (Mr 2465.7) after 40 min of ultrasound treatment. Insets (A) and (B) represent the sonolytic products and the oxidation products \([M+nO+H]^+\) \((n > 6)\), respectively. Asterisk at \( m/z \) 379 indicates a peak originating from the matrix.

Fig. 3. The change in the mass spectra of the aqueous solutions of ACTH18-39 with added catechol with increasing time of ultrasound treatment (0–60 min). Asterisk at \( m/z \) 379 indicates a peak originating from the matrix.

Fig. 4. The change in the peak abundances of protonated analyte \([M+H]^+\) and degradation products as a function of the ultrasound treatment time, in the aqueous solutions of ACTH18-39 with added catechol.

Fig. 5. The change in the peak abundances of protonated analyte \([M+H]^+\) and degradation products as a function of the ultrasound treatment time, in the aqueous solutions of ACTH18-39 without any additives.

Fig. 6. Mass spectrum of sonolytic products of ACTH18-39 (Mr 2465.7) with catechol addition after 40 min of ultrasound treatment. All the peaks noted with \( m/z \) values correspond to protonated molecules \([M+H]^+\) of peptide fragments resulting from the sonolytic hydrolysis at peptide bonds.
3.3. Sonolysis of aqueous solutions of peptides with other phenolic additives

It is interesting that sonolysis of the aqueous solutions of peptides with catechol addition gave sequence related products as a result of hydrolysis of the peptide backbone without any side reactions. Instead of the catechol, other phenolic analogues such as phenol, resorcinol and hydroquinone were used as additives in the sonolysis of aqueous solutions of ACTH18-39. The mass spectra obtained upon the addition of phenol, resorcinol and hydroquinone showed unexpected peaks corresponding to the loss of 30 Da from sequence related products as well as other unexpected products as indicated by asterisks in Fig. 7, together with sequence related products as in Fig. 6. The peaks corresponding to ~30 Da were observed even in the aqueous solutions without any additives, as shown in Fig. 2. Although the sonochemical effect on the formation of unexpected products is unclear at present, it is clear that catechol as an additive to aqueous solutions of peptides is very useful for the specific and effective sonolytic hydrolysis at peptide bonds without any side reactions. Sonochemical effects in aqueous solutions are likely to be caused by hydroxyl and hydrogen radicals produced via transient cavitation [8]. However, the detailed mechanism is not clear.

4. Conclusions

The application of ultrasound to aqueous solutions of peptides with added catechol (o-dihydroxybenzene) resulted in hydrolytic products reflecting the amino acid sequence without any side reactions, while sonolysis of solutions without additives resulted in the formation of oxidation products M+nO (n > 1) and degradation products due to side reactions. Although the use of resorcinol (m-dihydroxybenzene), hydroquinone (p-dihydroxybenzene) and phenol as additives to the aqueous solutions of peptides was also effective for the formation of hydrolytic products reflecting sequence information, several interfering products occurred via side reactions. It was found that the Xxx-Pro bond was more susceptible to sonolysis than other amino acid residues. The sonolytic hydrolysis of peptides is superior to acid hydrolysis in which cleavage at Pro-Xxx is difficult to achieve and which in turn leads to dehydration products due to side reactions. Further study of the detailed mechanism(s) of sonolytic hydrolysis of peptides is in progress.

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