Biochimica et Biophysica Acta 1780 (2008) 881–891

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Mass spectrometric and kinetic studies on slow progression of papain-catalyzed polymerization of L-glutamic acid diethyl ester

Asako Narai-Kanayama [a,*], Hiroyuki Koshino [b], Keiichi Aso [a]

[a] Faculty of Applied Life Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan
[b] Molecular Characterization Team, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

ARTICLE INFO

Article history:
Received 8 August 2007
Received in revised form 21 February 2008
Accepted 14 March 2008
Available online 20 March 2008

Keywords:
Papain
Protease-catalyzed peptide synthesis
Oligoglutamate
Glutamic acid
Induction period

ABSTRACT

Papain polymerizes L-glutamic acid diethyl ester (Glu-di-OEt) regioselectively, resulting in the formation of poly (γ-ethyl α-L-glutamic acid) with various degrees of polymerization of less than 13. Reaction temperatures below 20 °C were appropriate for the reaction in terms of suppression of non-enzymatic degradation of Glu-di-OEt and an increase in the peptide yield, while the reaction was preceded by a pronounced induction period. Mass spectrometric analyses of the reaction conducted at 0 °C revealed that the accumulation of the initial dimerization product, L-glutamyl-L-glutamic acid triethyl ester (Glu-Glu-tri-OEt), was limited during the induction period, and that a sequential polymer derived from a further elongation of the dimer was the tetramer, but not the trimer. Kinetic analyses of acyl transfer reactions with Glu-di-OEt and Glu-Glu-tri-OEt as acyl acceptors and Nα-benzoyl-L-arginine ethyl ester as an acyl donor affirmed that Glu-Glu-tri-OEt bound more strongly than Glu-di-OEt both to the S- and S′-subsites of papain. Therefore, what occurred during the initial stage of the polymerization was interpreted as follows: the rate of the papain-catalyzed dimerization of Glu-di-OEt was extremely slow, once Glu-Glu-tri-OEt was initially synthesized it exclusively bound to the active site of papain, and then papain utilized the dimer in polymerization effectively rather than the monomer.

1. Introduction

Protease-catalyzed acyl transfer reactions can be applied into the peptide synthesis, in which the acyl group of a substrate is transferred to water or to added nucleophile through the acyl-enzyme intermediate [1,2]. Using an amino component with a high affinity to the S1′ subsite in the catalyst as an acyl-group acceptor, deacylation of the acyl-enzyme intermediate by the acceptor would give peptide products in a good yield. In such a reaction, two different deacylation pathways, hydrolysis and aminolysis, occur competitively. For controlling the rate of protease-catalyzed peptide synthesis, yield, and degree of polymerization (DP) of peptide products, it is necessary to better understanding about the catalytic function of applicable proteases, the specificity of both their S- and S′- subsites, the reaction conditions responsible for promoting aminolysis, and so on. With regard to reaction conditions, a higher ionic strength is likely to increase the yield of peptide products [3–5]. A proper, alkaline condition of the reaction media is known to increase the reactivity of nucleophiles for aminolysis by making their amino group deprotonated [6]. Also, freezing reaction mixtures could significantly increase peptide yields [4,7–9]. In a frozen reaction mixture, hydrolysis of the acyl–enzyme intermediate and peptide products is likely to be suppressed, and concentration of reactants in the unfrozen liquid phase may accelerate aminolysis over hydrolysis.

It has been reported that papain (EC 3.4.22.2) and α-chymotrypsin (EC 3.4.21.1) polymerize L-glutamic acid diethyl ester (Glu-di-OEt) effectively [5,10]. Structural analyses had revealed that the polymerization gave the poly-α-peptide products exclusively [5,11]. From overall reaction yields based on the consumption of the substrate, it was found that papain polymerized Glu-di-OEt more efficiently than α-chymotrypsin [5,10]. This difference between the two proteases is probably attributed to the finding that the S′-subsite region of papain has a higher affinity to hydrophobic nucleophiles such as Glu-di-OEt and oligo-Glu-γ-OEt peptides than that of α-chymotrypsin [7,12,13]. In aqueous reaction mixtures, the synthesized Glu-γ-OEt polymers precipitated due to their hydrophobicity of ethyl ester groups in side chains. Uyama et al. (2002) reported that main DP values of the precipitated polymers were from 7 to 9 [5]. These polymers were insoluble not only in water but also in common organic solvents such as methanol, acetone, chloroform, tetrahydrofuran, and toluene, while soluble in *N, N*-dimethylformamide and dimethyl sulfoxide [5]. Insolubility of the peptide products might prevent themselves from secondary cleavage by proteases during the reaction. However, hydrolysis of esters in Glu-γ-OEt polymers provides us water-soluble L-glutamic acid oligomers, which are expected to have or enhance an

* Corresponding author. Tel.: +81 422 51 6121; fax: +81 422 51 9984.
E-mail address: a-narai@nvlu.ac.jp (A. Narai-Kanayama).

Abbreviations: BA, Nα-benzoyl-L-arginine; BA-Glu-Glu-tri-OEt, Nα-benzoyl-L-arginyl-Glu-Glu-tri-OEt; BA-Glu-di-OEt, Nα-benzoyl-L-arginyl-Glu-di-OEt; BAEE, Nα-benzoyl-L-arginine ethyl ester; DHBA, 2,5-dihydroxybenzoic acid; DQF-COSY, double quantum filtered correlation spectroscopy; DP, degree of polymerization; Glu-di-OEt, L-glutamic acid diethyl ester; Glu-γ-OEt, L-glutamic acid γ-ethyl ester; Glu-Glu-tri-OEt, L-glutamyl-L-glutamic acid triethyl ester; Glu-Glu, L-glutamyl-L-glutamic acid; HMBC, heteronuclear multiple bond correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy

0304-4165/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2008.03.009

umami taste [14,15] and possess a bitter taste masking effect against many kinds of bitter compounds [16]. They may also be available as biodegradable materials such as poly-γ-glutamic acid for purification of wastewater [17,18]. Therefore, the protease-catalyzed synthesis of oligo-Glu peptides is attractive in the fields of food chemistry and environmental cleanup.

Through investigating thermal influences on the papain-catalyzed polymerization of Glu-di-OEt, we found that the enzymatic consumption of Glu-di-OEt did not occur in the early period of the reaction at any temperatures. At higher temperatures, Glu-di-OEt was unstable and non-enzymatically degraded. On the other hand, the efficiency for peptide synthesis was good at lower temperatures, even though the induction period was extended. Thus in this study we attempted to characterize the induction period.

2. Materials and methods

2.1. Materials

L-Glutamic acid diethyl ester (Glu-di-OEt) hydrochloride was purchased from Tokyo Kasei Co. (Tokyo, Japan). *N*α-benzoyl-L-arginine ethyl ester (BAEE) hydrochloride was from Aldrich (Milwaukee, WI., U.S.A.). Papain from Papaya latex (EC 3.4.22.2), 2×crystallized suspension in acetate, pH 4.5, which was 17 U/mg protein determined at 25 °C and pH 6.5 with BAEE as a substrate, and ProteoMass™ peptide MALDI-MS calibration kit were purchased from Sigma Chemical Co. (St Louis, MO., U.S.A.). The molecular concentration of papain was determined from its absorbance at 278 nm using the value of E1%, 1 cm, 278 nm=25.0 and the molecular weight of 23700. Dipeptide of L-glutamyl-L-glutamic acid (Glu-Glu) was from Peptide Institute, Inc. (Osaka, Japan). Trifluoroacetic acid (TFA), thionyl chloride, ethanol, and 2,5-dihydroxybenzoic acid (DHBA) were purchased from Kanto Chemical Co. (Tokyo, Japan). For purpose of making dehydrated ethanol, molecular sieves 4A 1/16 from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) was added to ethanol and kept for over 24 h at room temperature.

2.2. Preparation of glutamyl glutamic acid triethyl ester (Glu-Glu-tri-OEt)

Glu-Glu-tri-OEt was synthesized as follows: desiccated powder of Glu-Glu of 0.5 g was dissolved in about 100 ml of dehydrated ethanol. After addition of 2.5 ml of thionyl chloride, it was hermetically sealed and incubated for 2 days at room temperature. These solvents were removed by evaporation. And then, viscous residue remaining like as syrup was rinsed with ethyl acetate repeatedly. After removal of the solvent by evaporation, Glu-Glu-tri-OEt was stored at −20 °C until before use. Complete esterification of Glu-Glu and its purity were checked by thin-layer chromatography (TLC) on silica gel to give a ninhydrin-positive single spot (a developing solvent, 1-butanol:acetic acid:water (4:1:2)). Molecular weight of Glu-Glu-tri-OEt hydrochloride was taken to be 396.8.

2.3. Enzymatic polymerization

Reaction conditions used were indicated in Table 1. Since Glu-di-OEt and Glu-Glu-tri-OEt were in the hydrochloride salt form, these substrates were dissolved in Na-phosphate buffer and pH of the substrate solutions was adjusted to 8.0 with an NaOH solution. Papain was activated by incubation with 11 mM DTT at 25 °C for 10 min before reaction. A substrate solution was preincubated at a given temperature for 1 min, and then the reaction was initiated by mixing with 1/10 volume of an activated-papain solution. During the reaction, aliquots (200 μl each) of the reaction mixture including precipitates were taken at appropriate intervals, added to 60 μl of 2 N HCl and then mixed well. These were kept on ice to prevent substrate and products from hydrolysis of ester groups. They were used as samples for matrix-assisted laser desorption and ionization time of flight-mass spectrometry (MALDI-TOFMS) and high-performance liquid chromatography (HPLC). To estimate the overall reaction yield, residual amino groups of Glu derivatives (Glu-di-OEt, Glu-mono-OEt and Glu) were measured using colorimetric potassium cyanide ninhydrin method [19].

Table 1
Reaction conditions

	Glu-di-OEt (mM)	Glu-Glu-tri-OEt (mM)	Papain (μM)	BAEE (mM)	Temperature (°C)
Enzymatic polymerization	100	–	20	–	−10–40
	100	10, 20, 40	10	–	0
Acyl transfer reaction	5–40	–	0.5	100	0
	–	4–40	0.5	100	0

Reactions were performed in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM dithiothreitol (DTT).

Table 2
Mass/charge of each peptide product in the papain-catalyzed polymerization of Glu-di-OEt

DP	Peptide product of which all ethyl ester groups are intact		Peptide product in which one of ester groups is deesterified	
	[M +Na^+]	[M +H^+]	[M +Na^+]	[M +H^+]
1	226.1	204.1	198.1	176.1
2	383.3	361.3	355.3	333.3
3	540.4	518.4	512.4	490.4
4	697.5	675.5	669.5	647.5
5	854.7	832.7	826.7	804.7
6	1011.8	989.8	983.8	961.8
7	1168.9	1146.9	1140.9	1118.9
8	1326.0	1304.0	1298.0	1276.0
9	1483.2	1461.2	1455.2	1433.2
10	1640.3	1618.3	1612.3	1590.3
11	1797.4	1775.4	1769.4	1747.4
12	1954.6	1932.6	1926.6	1904.6
13	2111.7	2089.7	2083.7	2061.7

(mass/charge).

2.4. Enzymatic acyl transfer reaction

Papain-catalyzed acyl transfer reactions were performed using 100 mM of BAEE as an acyl donor in 100 mM Na-phosphate buffer, pH 8.0, containing 0.5 μM papain and 1 mM DTT at 0 °C (Table 1). The pH of substrate solutions containing BAEE and either Glu-di-OEt or Glu-Glu-tri-OEt in Na-phosphate buffer was adjusted to 8.0 with an NaOH solution. After preincubation of substrate solutions for 1 min at 0 °C, the reaction was initiated by addition of an activated-papain solution prepared as described above. Aliquots (each 50 μl) of the reaction mixture were withdrawn at intervals of several minutes, added to 1 ml of 0.04 N HCl and kept on ice. The rates of hydrolysis and aminolysis were determined by time-course of the production of the acid (*N*α-benzoyl-L-arginine) and the peptide (*N*α-benzoyl-L-argininyl-Glu-di-OEt or *N*α-benzoyl-L-argininyl-Glu-Glu-tri-OEt), respectively, using HPLC. The composition analysis of the reaction mixture proved that secondary hydrolysis of the synthetic peptide did not occur under the conditions used.

2.5. Analysis of the reaction products by MALDI-TOFMS

MALDI mass spectral data were acquired using a KOMPACT/SEQ V1.2.2 (Shimadzu Co., Kyoto, Japan) operating in reflectron mode and positive ion mode. Accelerating voltage of 20 kV was employed. DHBA used as a matrix was dissolved in a mixture containing 0.1% TFA, 70% acetonitrile, and 30% water. Before spotting on a target plate, the sample solution including precipitates was premixed with the matrix solution for a final concentration of Glu residue to be ~50 nmol/μl. For a typical experiment, an aliquot (1 μl) of the prepared sample solution was spotted on a target plate and allowed to dry at room temperature prior to analysis. To analyze the precipitate in the sample solution, the precipitated products were rinsed twice with cold 0.01 M HCl aqueous solution after removal of supernatant by centrifugation (10,000 ×*g*, 4 °C, 30 min), and then suspended in the same volume of 0.01 M HCl as the original sample solution. This suspension was spotted on a target plate as described above. Bradykinin fragment 1–7 ($M+H^+$: 757.4), angiotensin II (1046.5) and P14R (1533.9) were used for mass calibration. DP values of synthesized peptides were assigned according to Table 2.

2.6. HPLC analysis

HPLC analyses were carried out using a Hitachi HPLC system (L-7100 pump, L-7400 UV detector, L-7200 autosampler with a cooling unit) equipped with a Cadenza CD-C18 (4.6×50 mm) column (Imtakt, Kyoto, Japan). The mobile phase was water/methanol mixture containing 0.1% TFA. To analyze the time-course consumption of substrates during the papain-catalyzed polymerization of Glu-di-OEt or Glu-Glu-tri-OEt, each substrate was eluted with 20% or 40% methanol/0.1% TFA, at a flow rate of 0.5 ml/min, and detected at 220 nm. Substrate and products in the papain-catalyzed acyl transfer reaction, BAEE, *N*α-benzoyl-L-arginine (BA), *N*α-benzoyl-L-arginyl-Glu-di-OEt (BA-Glu-di-OEt) and *N*α-benzoyl-L-arginyl-Glu-Glu-tri-OEt (BA-Glu-Glu-tri-OEt), were eluted with a linear gradient of methanol/0.1% TFA (40% to 55%) for 20 min, at a flow rate of 0.5 ml/min, and detected at 254 nm. Since they contain the same chromophore, their absorption coefficients were assumed to be equal [9]. To determine their concentrations, we have used the ratios of the peak areas calculated by Chromatocorder 21 from System Instruments Co. (Tokyo, Japan).

2.7. NMR analysis

All NMR spectral data were recorded at 25 °C on an ECA-600 NMR spectrometer (JEOL Ltd. Akishima, Tokyo), operating at 600.17 MHz and 150.91 MHz for ^1H and ^{13}C, respectively. Chemical shift values were referenced to a residual signal of deuterated dimethyl sulfoxide-d_6, 2.49 ppm and 39.5 ppm for ^1H and ^{13}C, respectively. Dissolved sample amount was 12.4 mg in 0.7 ml. ^1H-NMR data for precipitated polymers: 8.56 (br. d, J=6.5 Hz, NH of Glu—2nd), 8.33 (d, J=7.1 Hz, NH of Glu—2nd –from C-terminal), 8.23 (br. d,

J=7.6 Hz, NH of Glu—3rd), 8.18 (d, J=9.0 Hz, ca 0.5 H integration, NH of Glu of C-terminal in COOH form), 8.16 (d, J=7.5 Hz, ca 0.5H integration, NH of Glu of C-terminal in ester form), 8.10 (d. J=7.6 Hz, NH of Glu—4th), 8.04 (m, NH of Glu—3rd –from C-terminal), 8.08–8.02 (m, NH, ca 3.5H integration), 4.32 (m, α-CH of Glu—2nd), 4.27 (m, ca 0.5H integration , α-CH of C-terminal in ester form), 4.26 (m, α-CH of Glu—3rd), 4.25 (m, α-CH of Glu—4th), 4.25 (m, α-CH of other Glu), 4.22 (m, α-CH of Glu—2nd –from C-terminal), 4.17 (m, ca 0.5H integration , α-CH of C-terminal in COOH form), 4.03 (m, CH_2 of ethyl ester groups), 3.74 (t, α-CH of Glu—1st : N-terminal), 2.45–2.25 (m, γ-CH_2 of Glu), 2.05–1.70 (m, β-CH_2 of Glu), 1.15 (m, CH_3 of ethyl ester groups) ppm.

3. Results and discussion

3.1. Thermal influence on stability of Glu-di-OEt and on the papain-catalyzed polymerization of Glu-di-OEt

We conducted the polymerizing reaction with 100 mM of Glu-di-OEt and 10 μM of papain at pH 8.0 at different temperatures in the range of −10–40 °C. Every time-course substrate consumption curve exhibited a sigmoidal nature in varying degrees (Fig. 1A). Under a freezing condition at −10 °C or −20 °C, the reaction did not proceed even after 24 h (data not shown). The period during which Glu-di-OEt did not decrease in the early stage of the reaction was pronounced "induction period". At higher temperatures, the induction period was significantly shortened (Fig. 1A). However, Glu-di-OEt was decreased non-enzymatically with rise in temperature (Fig. 1B). Without papain, the $\ln(C/C_0)$ versus incubation time plot of Glu-di-OEt at each temperature gave a straight line (data not shown), where C is the concentration of Glu-di-OEt in mol/l at a given incubation time, and C_0 is initial concentration in mol/l. This indicated that non-enzymatic degradation of the substrate followed first- or pseudo-first-order kinetics [20]:

$$C = C_0 \exp(-kt)$$

where t is the incubation time in hour and k in hour^{-1} is the rate constant for thermal degradation of Glu-di-OEt. The k and half-life ($t_{1/2} = -\ln 0.5 \cdot k^{-1}$) values obtained for Glu-di-OEt were summarized in Table 3. It was suggested that the inefficient use of substrates by enzyme could not be avoided at higher temperatures due to non-enzymatic degradation. The papain-catalyzed polymerization of Glu-di-OEt at lower temperatures was favorable to improve its efficiency, since thermal degradation of substrates hardly occurred. Indeed, the yields of peptide products after 24-h reaction based on the consumed substrate were ~50%, ~80%, and almost 100%, at 40 °C, 30 °C, and below 20 °C, respectively. Characterization of the induction period would give us useful information for synthesis of a large amount of oligo-Glu peptides in a short reaction time at a low temperature even with a slight amount of enzyme.

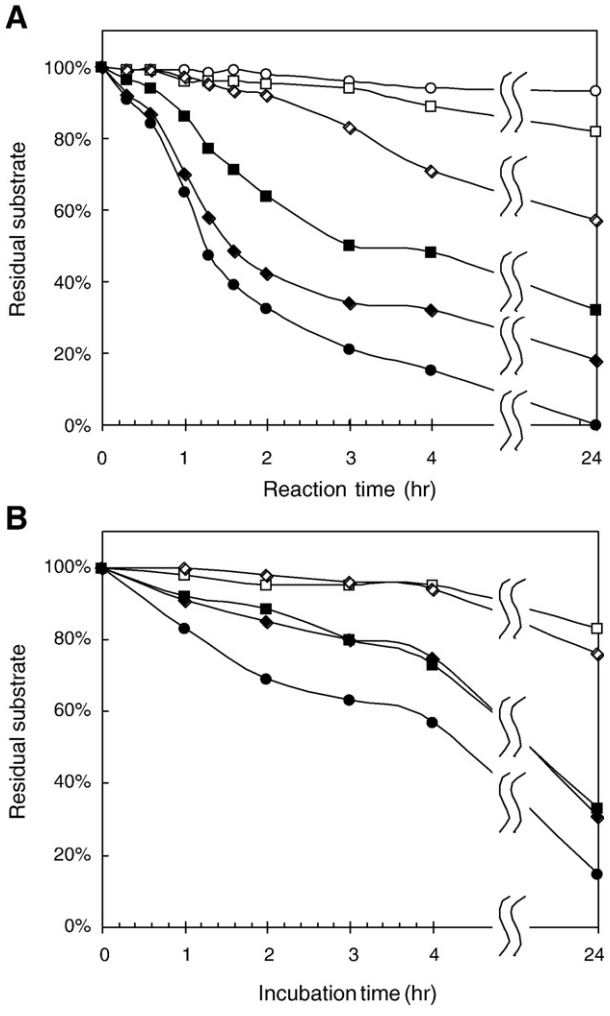


Fig. 1. Thermal effect on the papain-catalyzed polymerization of Glu-di-OEt. (A) Time-course consumption of substrate in the papain-catalyzed polymerization of Glu-di-OEt (100 mM). Reactions with 20 μM papain were conducted in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM DTT, at −10 °C (○), 0 °C (□), 10 °C (◇), 20 °C (■), 30 °C (◆), and 40 °C (●). (B) Non-enzymatic degradation of Glu-di-OEt (100 mM) in 100 mM Na-phosphate buffer, pH 8.0, during the incubation at 0 °C (□), 10 °C (◇), 20 °C (■), and 40 °C (●).

Table 3
Rate constant (k) for the thermal degradation of Glu-di-OEt and its half-life ($t_{1/2}$) without papain in 100 mM Na-phosphate buffer, pH 8.0, at different temperatures

°C	k (h^{-1})	$t_{1/2}$ (h)
0	1.06×10^{-2}	65.4
10	1.15×10^{-2}	60.3
20	6.26×10^{-2}	11.1
40	1.71×10^{-1}	4.0

3.2. Time-course polymerization of Glu-di-OEt catalyzed by papain

To investigate what sort of oligo-Glu-γ-OEt peptide papain synthesized time-dependently, we analyzed components in the reaction mixture using a MALDI-TOFMS. Reactions were conducted at 0 °C, where ester groups in substrate and products were stable and the induction period was so long that we could observe the process of polymerization of Glu-di-OEt. During the induction period, signals expressing peptide products could not be detected in mass spectra, except for a very little signal of [Glu-Glu-tri-OEt+Na^+] (Fig. 2A). As the reaction proceeded, peaks indicating polymers with DP more than 4, mainly as [M+Na^+] forms, gradually appeared (Fig. 2B and C). Therefore, it was hypothesized that the rate of dimerization of Glu-di-OEt by papain was very slow and a sufficient accumulation of the dipeptide induced the production of tetrapeptide. After 24-h reaction, the mass spectrum displayed signal peaks of polymers with DP from 3 to 6 (Fig. 2D). They were detected mainly as the form in which one of their carboxyl groups was free and the others were ethyl esters (Fig. 2D). This suggested that papain also hydrolyzed C-terminal esters or peptide bonds of the polymers once synthesized. It should be noted that the signal corresponding to the DP 3 product was solely [Glu-Glu-Glu-tri-OEt+Na^+] form. The tripeptide with one free carboxyl group might be occurred by the papain-catalyzed cleavage between 3rd and 4th Glu residues within some oligo-Glu-γ-OEt peptides. Analyzing the precipitate in the reaction mixture showed that insoluble products contained polymers with DP from 7 to 13 (Fig. 2E). Such insoluble polymers with high DP were probably synthesized through the papain-catalyzed transpeptidation between soluble oligomers.

3.3. Effect of Glu-Glu-tri-OEt on the induction period during the papain-catalyzed polymerization of Glu-di-OEt

In order to investigate whether papain could act on and polymerize the dipeptide, we added the enzyme into the substrate solution containing chemically synthesized Glu-Glu-tri-OEt, instead of Glu-di-

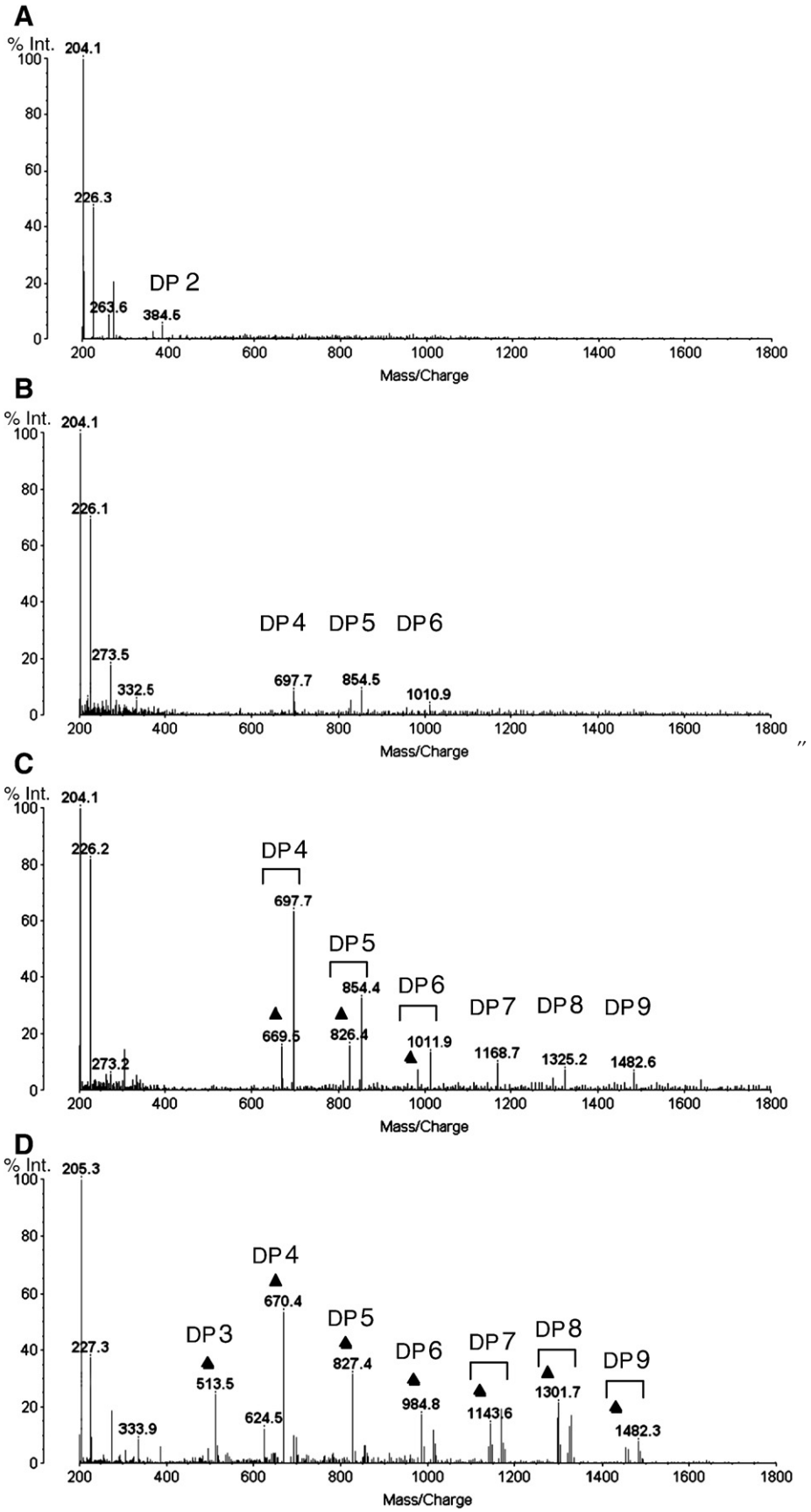


Fig. 2. Mass spectra of products in the papain-catalyzed polymerization of Glu-di-OEt, of which initial concentration was 100 mM. Reactions with 20 μM papain were conducted in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM DTT, at 0 °C, for 1 h (A), 4 h (B), 6 h (C), and 24 h (D). A mass spectrum of precipitated products after 24-h reaction is represented (E). Closed triangles indicate polymers in which one of ester groups is hydrolyzed.

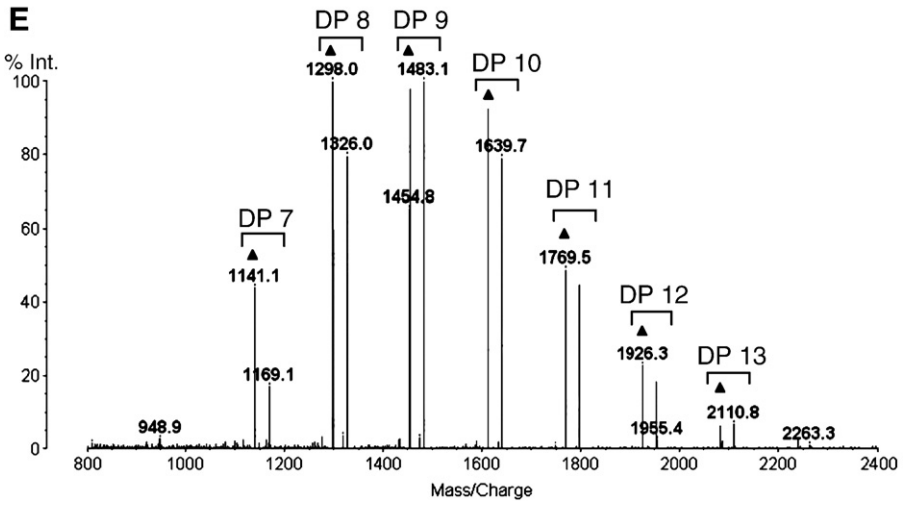


Fig. 2 (*continued*).

OEt. Contrary to the reaction with Glu-di-OEt (open squares in Fig. 3A), the consumption of Glu-Glu-tri-OEt by papain was significantly fast without apparent induction period (open circles in Fig. 3B).

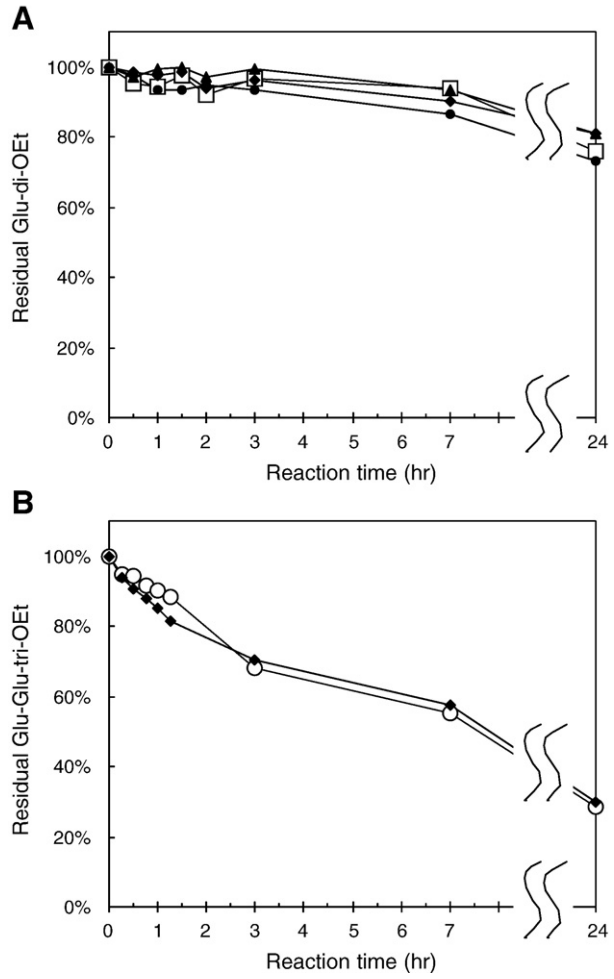


Fig. 3. Time-course consumption of Glu-di-OEt (A) and Glu-Glu-tri-OEt (B) during the reaction with 10 μM papain at 0 °C in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM DTT. (A) 100 mM Glu-di-OEt was mixed with Glu-Glu-tri-OEt of 0 mM (□), 10 mM (●), 20 mM (▲), and 40 mM (◆). (B) 40 mM Glu-Glu-tri-OEt was mixed with Glu-di-OEt of 0 mM (○) and 100 mM (◆).

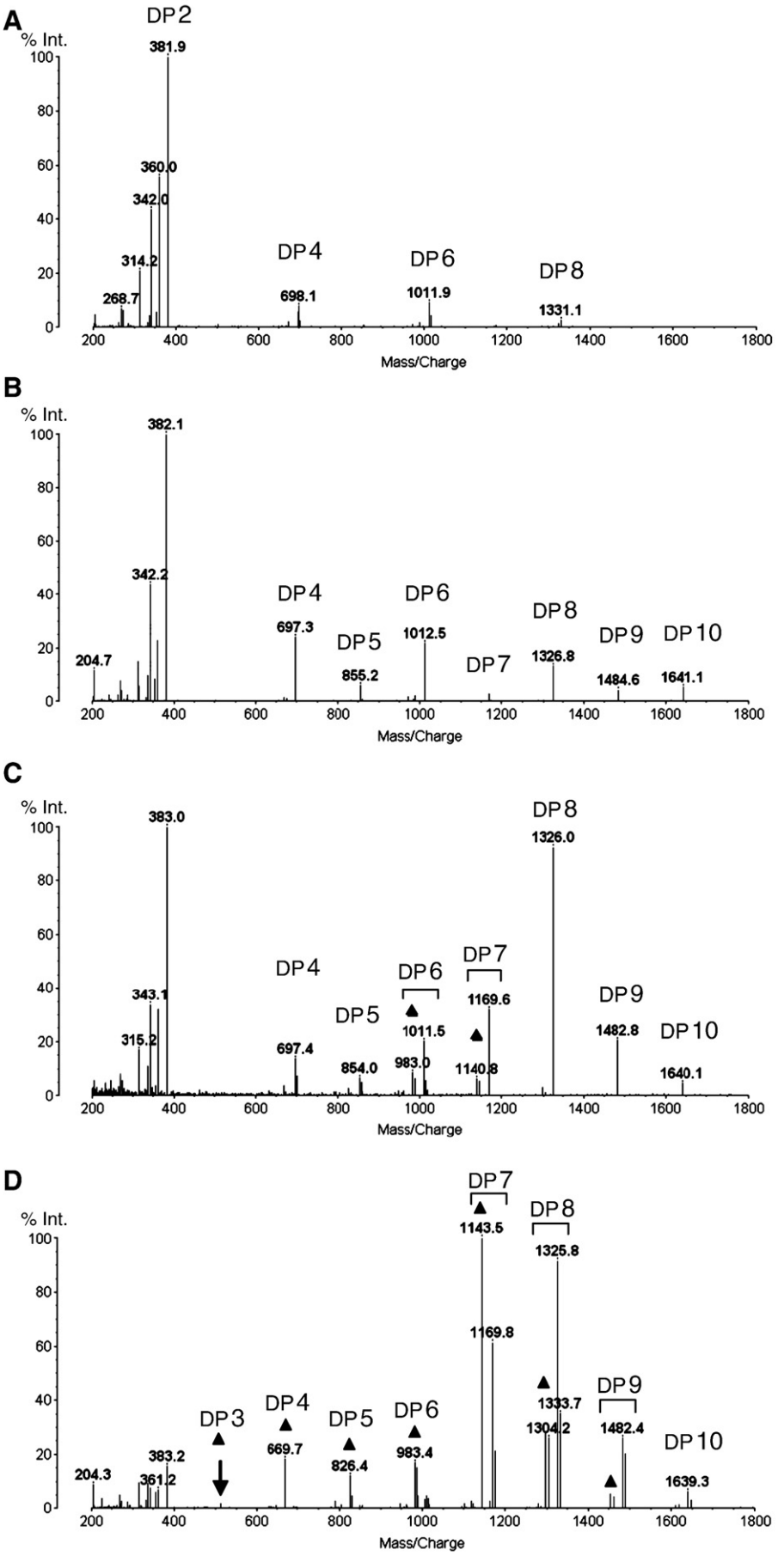
Insoluble polymers with high DP as precipitate synthesized from Glu-Glu-tri-OEt were characterized by 1D and several 2D NMR studies in deuterated dimethyl sulfoxide (DMSO-d_6) solution. 600 MHz ^{1}H NMR spectrum was identical to those reported in references ([5,11]) for oligo (γ-ethyl-L-glutamate). Sequential assignments of back bone signals from N-terminal to fourth residue were possible by analyses of 2D NOESY and DQF-COSY spectra. Two sets of signals were observed for C-terminal residues with ethyl ester and free carboxyl groups and sequential assignments from C-terminal to third residue were also possible. Based on HMBC spectral data, the presence of normal α-peptide structure and also γ-ethyl esters was confirmed.

MALDI-TOFMS analyses showed the rapid production of oligo-Glu-γ-OEt peptides, DPs of which counted on the basis of Glu residue were 4, 6 and 8 (Fig. 4A and B). Subsequently peaks of oligo-Glu-γ-OEt peptides with odd-numbered DP appeared in the mass spectra (Fig. 4C), then peaks of polymers in complete ester forms with DP from 4 to 7 shifted partially to those with one free carboxyl group in each polymer (Fig. 4D). The signal of DP 3 product was very weak (an arrow in Fig. 4D). Although a signal corresponding to Glu-di-OEt was only slightly detected by MALDI-TOFMS (Fig. 4A–D), time-dependent increase in Glu-di-OEt was not detected by HPLC analysis (data not shown), suggesting that papain rarely cleaved the peptide bond in Glu-Glu-tri-OEt.

Subsequently we examined whether coexistence of both Glu-Glu-tri-OEt and Glu-di-OEt molecules allowed papain to synthesize the tripeptide through an acyl transfer reaction with these two substrates. Unexpectedly, the time-course consumption of the one substrate was not notably affected by mixing with the other (Fig. 3A and B). Results from analyses using MALDI-TOFMS showed the distinct peak of DP 4 product, but not of DP3, prior to other products (Fig. 5A and B). It was difficult to think that the DP 3 product had been produced once and depleted by papain effectively and quickly, since neither enhancement nor suppression of consumption of the two substrates was observed. Therefore, it was assumed that papain could hardly synthesize the peptide product with DP 3 from Glu-Glu-tri-OEt and Glu-di-OEt. These results suggested that Glu-Glu-tri-OEt was dimerized quickly by papain due to its higher reactivity for both acylation and aminolysis, while the reverse was true for Glu-di-OEt.

3.4. Comparison of specificity for papain between Glu-di-OEt and Glu-Glu-tri-OEt

It was assumed that different specificities for papain between Glu-di-OEt and Glu-Glu-tri-OEt were involved in the induction period.



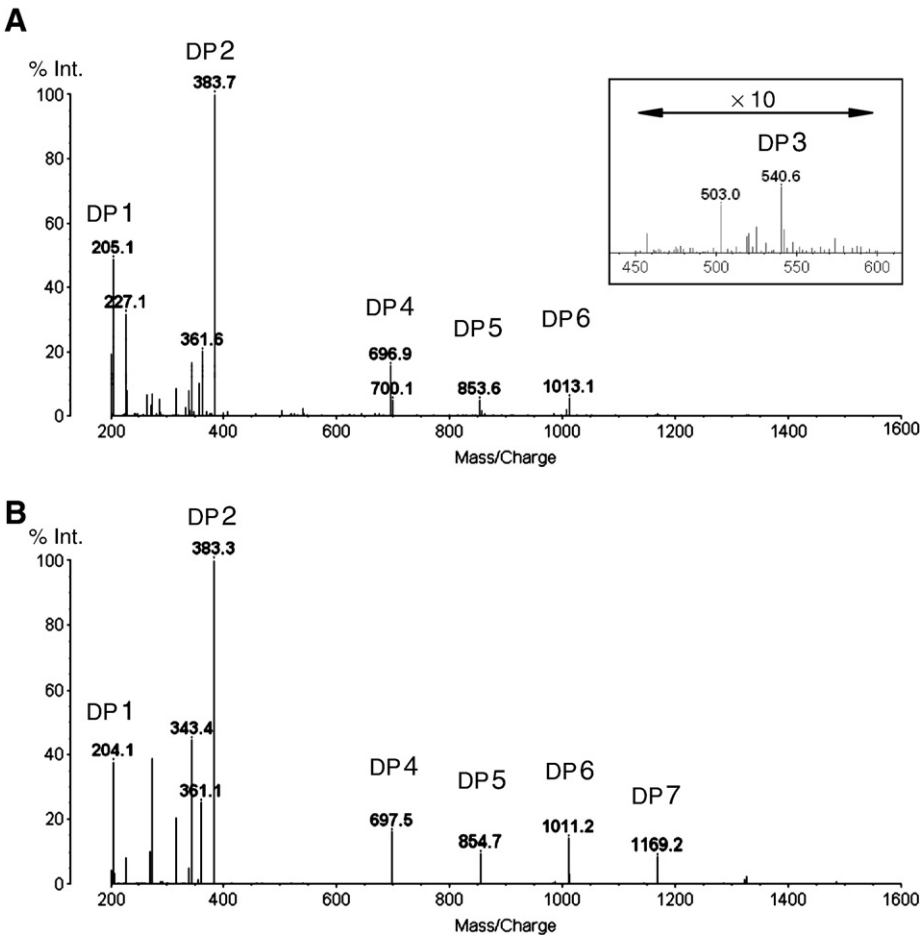
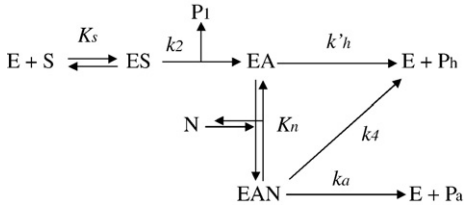


Fig. 5. Mass spectra of products in the reaction mixture containing both 100 mM Glu-di-OEt and 40 mM Glu-Glu-tri-OEt with 10 μM papain. Reactions were conducted in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM DTT, at 0 °C, for 15 min (A) and 45 min (B).

Thus in order to compare the aminolytic activities between Glu-di-OEt and Glu-Glu-tri-OEt, we performed the papain-catalyzed acyl transfer reaction with BAEE which has often been used as an acyl-group donor [8,21–25]. Due to charged arginine residue, the peptide products having hydrophobic residues can be analyzed by HPLC. In both cases of Glu-di-OEt and Glu-Glu-tri-OEt, with rise of these nucleophile concentrations the initial rates of aminolysis (*Va*) increased, while those of hydrolysis (*Vh*) decreased (Fig. 6A and B). The dependence of *Va* on nucleophile concentrations showing saturation curves in Fig. 6A and B can be interpreted by formation of the acyl–papain–nucleophile complex. Considering this point, we could follow the following scheme [12,26]:



Scheme 1.

where E represents the enzyme, S denotes the acyl-group donor, N is the added nucleophile, P1 and Ph are the products of hydrolysis, Pa is the peptide product, ES is the enzyme complex with S, EA is the acyl–enzyme intermediate, and EAN is the acyl–enzyme–nucleophile complex. The kinetic constants are indicated for the appropriate reactions in the scheme. We made the following assumptions for this model: the ES complex is in equilibrium with E and S (its equilibrium constant is *Ks*); the EAN is in equilibrium with EA and N (*Kn*); the concentration of EA do not change within time in the steady-state with an excess of S over E; and [S]=[S]0, [N]=[N]0, and d[Pa]/d[Ph]=*Va*/*Vh*. Fig. 6A and B indicated that Glu-di-OEt and Glu-Glu-tri-OEt induced aminolysis of the acyl–papain intermediate, competing against hydrolysis. Comparing these two nucleophiles, a larger amount of Glu-di-OEt than Glu-Glu-tri-OEt was required to enhance aminolysis and suppress hydrolysis. The pKa values of amino groups in both two nucleophiles were found to be about 7.4, determined by neutralization titration. Their similar pKa values indicated that proportions of their reactive molecules with deprotonated amino groups were comparable. Therefore, it was possible that more efficient aminolysis by Glu-Glu-tri-OEt was attributed to its higher affinity for papain.

Fig. 4. Mass spectra of products in the papain-catalyzed polymerization of Glu-Glu-tri-OEt, of which initial concentration was 40 mM. Reactions with 10 μM papain were conducted in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM DTT, at 0 °C, for 15 min (A), 30 min (B), 4.5 h (C), and 24 h (D). Closed triangles indicate polymers in which one of ester groups is hydrolyzed. An arrow shows the signal corresponding to the DP 3 product.

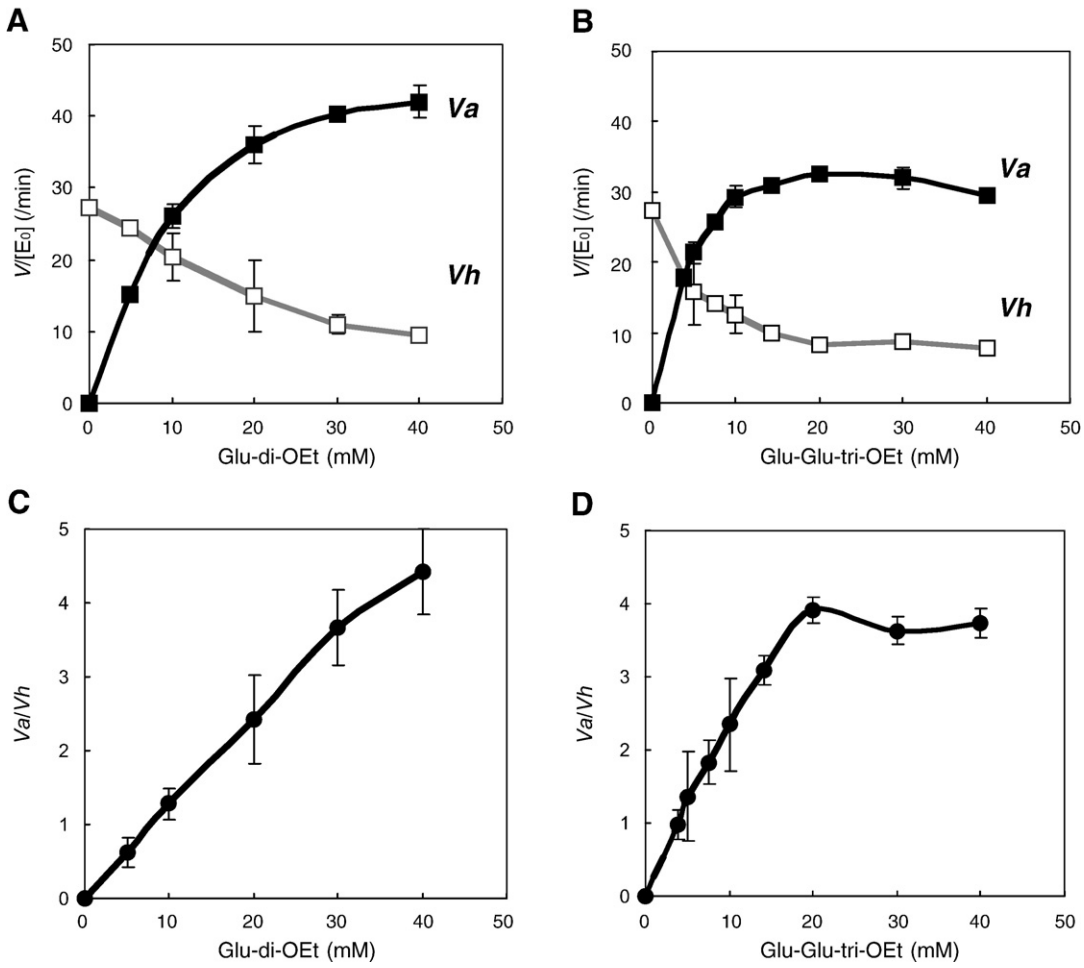


Fig. 6. Comparison of the aminolytic activities between Glu-di-OEt (A, C) and Glu-Glu-tri-OEt (B, D). *Vh* and *Va* values (A, B) and *Va*/*Vh* ratio values (C, D) were plotted against the nucleophile concentrations. 100 mM BAEE and various concentrations of nucleophiles were reacted with 0.5 μM papain in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM DTT, at 0 °C. Results are expressed as means ± S.D. of triplicate experiments.

According to Scheme 1, the ratio of *Va*/*Vh* is expressed as Eq. (1):

$$\frac{Va}{Vh} = \frac{k_a[\mathrm{N}]}{Kn \cdot k'_h + k_4[\mathrm{N}]} \tag{1}$$

With *k4* (or *k4*/*Kn*) being negligible, the *Va*/*Vh* ratio is dependent linearly on the concentration of nucleophiles with the coefficient meaning *ka*/(*Kn*·*k*′*h*). This might be true of the case of Glu-di-OEt (Fig. 6C). However, at concentrations of Glu-Glu-tri-OEt over 20 mM, the *Va*/*Vh* ratio against the nucleophile concentration did not show the overall saturated curve fit for Eq. (1) (Fig. 6D), and the sum of *Va* plus *Vh* gradually decreased (Fig. 6B). These results implied that the model proposed in Scheme 1 was defective. Since the dipeptide might interact with either enzyme or EAN complex, we should consider Scheme 2: where EN is the enzyme complex with N (its equilibrium constant is *Ki*) [26], and NEAN denotes the complex of the acyl–enzyme–nucleophile complex with another nucleophile molecule (*K*′*n*) [26]. This model gives us the following equation:

$$\frac{Va}{Vh} = \frac{Kn' \cdot k_a[\mathrm{N}] + k'_a[\mathrm{N}]^2}{Kn \cdot Kn' \cdot k'_h + Kn' \cdot k_4[\mathrm{N}] + k_5[\mathrm{N}]^2} \tag{2}$$

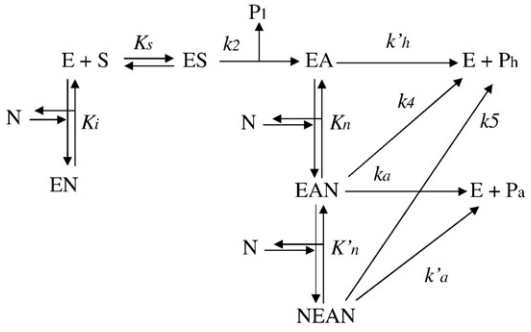
Here, Scheme 2 is an expansion of Scheme 1. Thus Eq. (2) coincides with Eq. (1) in case of the reaction without formation of NEAN.

Furthermore, conducting the acyl transfer reactions at varied concentrations of BAEE with nucleophiles, we investigated inhibitory effects of Glu-di-OEt and Glu-Glu-tri-OEt on the acylation of papain with BAEE. Referring to the sum of *Vh* and *Va* as *Vs*, the $[E_0]/Vs \sim 1/[S]$ plot without inhibition of acylation by added nucleophiles would give us linear curves expressed as the following equation:

$$\frac{[E_0]}{Vs} = \frac{Ks}{k_2} \cdot \frac{1}{[S]} + \frac{1}{k_2} + \frac{1 + \frac{[\mathrm{N}]}{Kn} + \frac{[\mathrm{N}]^2}{Kn \cdot Kn'}}{k'_h + \frac{\left(k_a + k_4\right)}{Kn}[\mathrm{N}] + \frac{\left(k'_a + k_5\right)}{Kn \cdot Kn'}[\mathrm{N}]^2} \tag{3}$$

When competitive interaction of a nucleophile against an acyl-group donor occurred within the S-subsite region of papain, Eq. (3) would be modified as follows.

$$\frac{[E_0]}{Vs} = \frac{Ks}{k_2}\left(1 + \frac{[\mathrm{N}]}{Ki}\right) \cdot \frac{1}{[S]} + \frac{1}{k_2} + \frac{1 + \frac{[\mathrm{N}]}{Kn} + \frac{[\mathrm{N}]^2}{Kn \cdot Kn'}}{k'_h + \frac{\left(k_a + k_4\right)[\mathrm{N}]}{Kn} + \frac{\left(k'_a + k_5\right)[\mathrm{N}]^2}{Kn \cdot Kn'}} \tag{4}$$



Scheme 2.

Eq. (4) points out that coefficient constants for $[E_0]/Vs \sim 1/[S]$ linear plots are dependent on the nucleophile concentration. When Glu-di-OEt was added to the reaction mixture as a nucleophile, the coefficient constants of $[E_0]/Vs \sim 1/[S]$ linear curves were similar to that without any nucleophile (Fig. 7A). On the other hand, in the reaction with Glu-Glu-tri-OEt, the coefficient constants of $[E_0]/Vs \sim 1/[S]$ plots remarkably increased dependently on concentration of the dipeptide (Fig. 7B). The *Ki* values for Glu-di-OEt and Glu-Glu-tri-OEt were calculated to be approx. 99.5 mM and 8.8 mM, respectively, suggesting that Glu-Glu-tri-OEt had an extremely higher affinity to the S-subsite region of papain and behaved as a more effective acyl-group donor than Glu-di-OEt. This suggestion is supported by knowledge that papain is an endoprotease whose primary determinant of specificity is the nature of P2 residues such as Phe with a hydrophobic, large residue favored or accepted in the S2 subsite [27].

Scheme 2 also induced the following equation:

$$\frac{[E_0]}{Va} = \frac{Ks}{k_2}\left(1+\frac{[N]}{Ki}\right)\cdot\frac{1}{[S]} + \frac{\frac{Ks}{k_2}\left(1+\frac{[N]}{Ki}\right)\cdot\frac{1}{[S]}\left(k'_h + \frac{k_4[N]}{Kn} + \frac{k_5[N]^2}{Kn\cdot Kn'}\right) + 1 + \frac{[N]}{Kn} + \frac{[N]^2}{Kn\cdot Kn'}}{\frac{k_a[N]}{Kn} + \frac{k'_a[N]^2}{Kn\cdot Kn'}} \quad (5)$$

We estimated some kinetic constants, assigning a certain value to each parameter in Eqs. (2) and (5) so that calculated results approached to our experimental data on both $[E_0]/Va \sim [N]$ and $Va/Vh \sim [N]$ plots. Two assumptions were applied to the estimation: (1) *Ks* at 0 °C was experimentally determined to be 52.5 mM, (2) k_4 (or k_4/Kn) and NEAN formation for Glu-di-OEt were negligible, since the plot of *Va*/*Vh* ratio against Glu-di-OEt concentration showed a linear curve (Fig. 6C). Consequently, the *Kn* for Glu-Glu-tri-OEt was much smaller than that for Glu-di-OEt (Table 4), suggesting that Glu-Glu-tri-OEt more strongly interacted with the S′-region of papain than Glu-di-OEt. Considering high affinities of Glu-Glu-tri-OEt both to the S- and S′-subsites of papain, it can be thought that the dipeptide prevents the active site of papain from accommodating Glu-di-OEt. What happened during induction period and the reason why the tripeptide was not produced in the papain-catalyzed polymerization of Glu-di-OEt could be explained as follows: the induction period continued until a certain concentration of Glu-Glu-tri-OEt was synthesized by a very slow dimerization of Glu-di-OEt, and then Glu-Glu-tri-OEt was preferentially utilized by papain for synthesis of tetrapeptide. Furthermore, we are now exploring other reasons why papain could not synthesize the tripeptide through an acyl transfer reaction between Glu-di-OEt and Glu-Glu-tri-OEt. In some reports it has been suggested that the nature of the amino acids binding to one subsite can give some influences on specificities of the other subsites [28,29]. Indeed, the specificity of S1′ subsite of papain which takes a substantial role in the acyl trasfer reactions was slightly different from that observed in hydrolyses of a series of intramolecularly quenched fluorogenic substrates [12,13,28]. It is possible that binding of the one subsite region of papain to Glu-Glu-tri-OEt induces much higher affinity of the other subsite for the dipeptide.

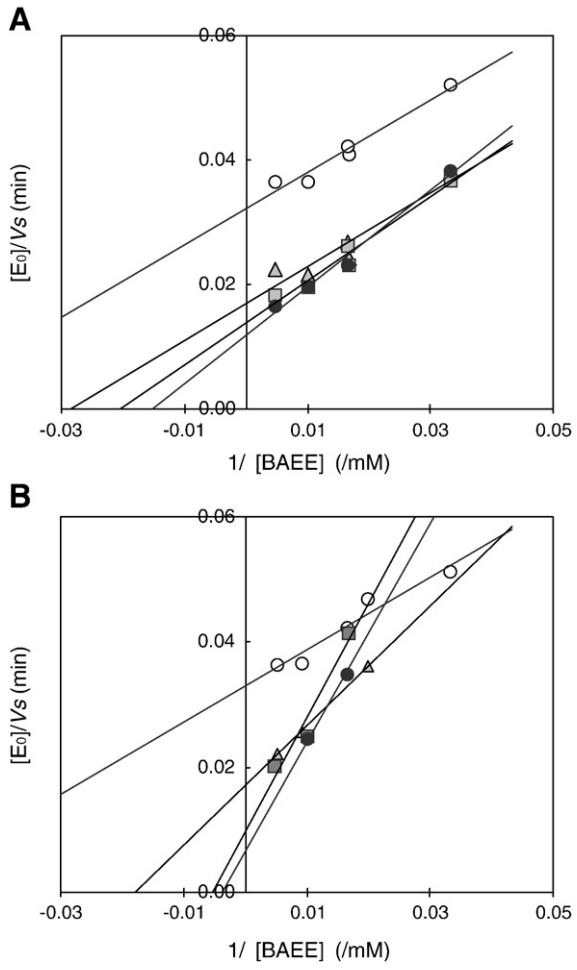


Fig. 7. Effects of Glu-di-OEt (A) and Glu-Glu-tri-OEt (B) on the acylation rate (*Vs*) of papain with BAEE. Reactions with 0.5 μM papain were conducted in 100 mM Na-phosphate buffer, pH 8.0, containing 1 mM DTT, at 0 °C. (A) Initial concentrations of Glu-di-OEt were 0 mM (○), 10 mM (▲), 20 mM (■), and 30 mM (●). (B) Initial concentrations of Glu-Glu-tri-OEt were 0 mM (○), 7.5 mM (▲), 14 mM (■), and 20 mM (●).

Table 4
Reactivity of Glu-di-OEt and Glu-Glu-tri-OEt in the papain-catalyzed acyl transfer reaction with BAEE (100 mM Na-phosphate buffer, pH 8.0, at 0 °C)

Kinetic constants	
Ks (mM)	52.3±0.3
k'_h (min^{-1})	45.3±0.1
k_2 (min^{-1})	99.2±4.6
Glu-di-OEt	
k_4/Kn (min^{-1})	≅0*
k_a/Kn (min^{-1})	5.4*
Kn (mM)	1112±374
Glu-Glu-tri-OEt	
k_4/Kn (min^{-1})	0.29±0.38
$k_5/(Kn\cdot K'n)$ (min^{-1})	0.49±0.09
k_a/Kn (min^{-1})	7.13±1.17
$k'_a/(Kn\cdot K'n)$ (min^{-1})	1.85±0.41
Kn (mM)	3.29±0.47
K′n (mM)	167±60

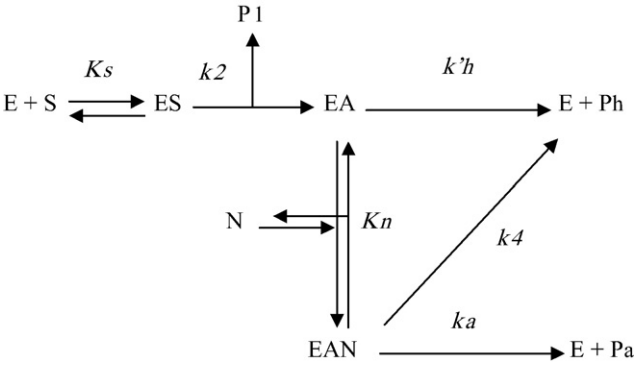
Data are given as the means±S.D. of 40 sets of values assigned to parameters in Eqs. (2) and (5) so that the results which were calculated with Microsoft Excel approached to experimental data on both $[E_0]/Va \sim [N]$ and $Va/Vh \sim [N]$ plots.
* Constants judged from data in Fig. 6C.

In case of the reaction with Glu-di-OEt, the signal of DP 5 product was rapidly detected before the mass spectrometric signals indicating peptide products with DP≥7 appeared (Figs. 2B, 5A, and B). It was assumed that papain could use Glu-di-OEt for peptide elongation after the DP 4 product accumulated. However, reasons why peptides with odd-numbered DPs, except for the DP3 product, occurred in the papain-catalyzed polymerization of Glu-Glu-tri-OEt (Fig. 4B and C) are still unclear. We need further studies to explain kinetic characteristics of the polymerization of monomer and short oligomers.

In conclusion, during the induction period in the papain-catalyzed polymerization of Glu-di-OEt, the substrate was dimerized slowly owing to its low reactivity with papain for both acylation and aminolysis. On the other hand, Glu-Glu-tri-OEt corresponding to the initially synthesized dimer could effectively interact with both S- and S′-subsites of papain, leading to production of the tetramer. Our study

clarified what is behind the induction period for the first time by kinetic data and mass spectrometric analyses.

Appendix



$$\frac{[E][S]}{[ES]} = Ks \tag{1}$$

$$\frac{d[EA]}{dt} = 0 \tag{2}$$

$$\frac{[EA][N]}{[EAN]} = Kn \tag{3}$$

$$Vh = k'_h[EA] + k_4[EAN]$$
$$Va = k_a[EAN]$$
$$Vs = k_2[ES] = Vh + Va$$

$$k_2[ES] = \left(k'_h + \frac{(k_a + k_4)}{Kn}[N]\right)[EA] \tag{4}$$

$$\frac{Va}{Vh} = \frac{k_a[EAN]}{k'_h[EA] + k_4[EAN]} = \frac{k_a[N]}{K'_h \cdot Kn + k_4[N]} \tag{5}$$

$$k_4 = 0 \rightarrow \frac{Va}{Vh} = \frac{k_a}{k'_h \cdot Kn}[N]$$

$$k_4 \neq 0 \rightarrow \frac{Va}{Vh} = \frac{\frac{k_a}{k_4}[N]}{\frac{k'_h \cdot Kn}{k_4} + [N]}$$

$$[E_0] = [E] + [ES] + [EA] + [EAN]$$

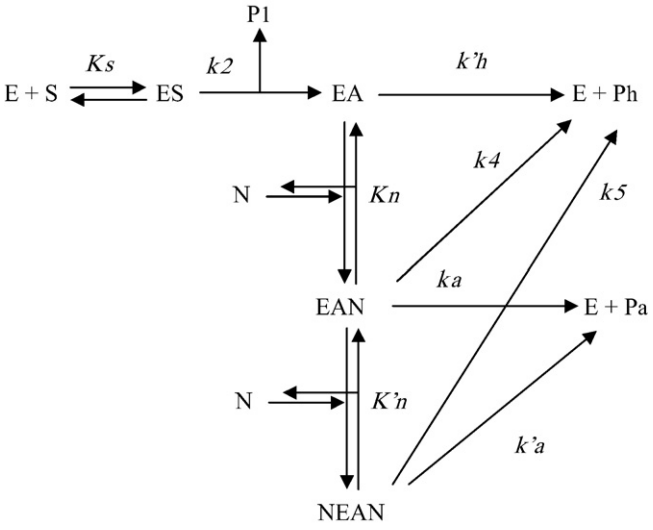
$$[E_0] = \left(\frac{Ks}{[S]} + 1\right)[ES] + \left(1 + \frac{[N]}{Kn}\right)[EA]$$

$$[E_0] = \left\{\frac{1}{k_2}\left(\frac{Ks}{[S]} + 1\right)\left(k'_h + \frac{(k_a + k_4)[N]}{Kn}\right) + \left(1 + \frac{[N]}{Kn}\right)\right\}[EA] \tag{6}$$

Eq. (6)/Eq. (4)—

$$\frac{[E_0]}{Vs} = \frac{\frac{1}{k_2}\left(\frac{Ks}{[S]} + 1\right)\left(k'_h + \frac{(k_a+k_4)}{Kn}[N]\right) + \left(1 + \frac{[N]}{Kn}\right)}{\left(k'_h + \frac{(k_a+k_4)}{Kn}[N]\right)}$$

$$\therefore \frac{[E_0]}{Vs} = \frac{Ks}{k_2} \cdot \frac{1}{[S]} + \frac{1}{k_2} + \frac{\left(1 + \frac{[N]}{Kn}\right)}{\left(k'h\frac{(k_a+k_4)}{Kn}[N]\right)} \tag{7}$$



$$\frac{[EAN][N]}{[NEAN]} = Kn' \tag{8}$$

$$Vh = k'_h[EA] + k_4[EAN] + k_5[NEAN] = \left(k'_h + \frac{k_4}{Kn}[N] + \frac{k_5}{Kn \cdot Kn'}[N]^2\right)[EA] \tag{9}$$

$$Va = k_a[EAN] + k'_a[NEAN] = \left(\frac{k_a}{Kn}[N] + \frac{k'_a}{Kn \cdot Kn'}[N]^2\right)[EA] \tag{10}$$

$$\frac{Va}{Vh} = \frac{\frac{k_a}{Kn}[N] + \frac{k'_a}{Kn \cdot Kn'}[N]^2}{k'_h + \frac{k_4}{Kn}[N] + \frac{k_5}{Kn \cdot Kn'}[N]^2} \tag{11}$$

$$Vs = k_2[ES] = Vh + Va = k'_h[EA] + (k_a + k_4)[EAN] + (k'_a + k_5)[NEAN]$$

$$Vs = \left(k'_h + \frac{k_a + k_4}{Kn}[N] + \frac{k'_a + k_5}{Kn \cdot Kn'}[N]^2\right)[EA] \tag{12}$$

$$[E_0] = [E] + [ES] + [EA] + [EAN] + [NEAN]$$

$$[E_0] = \left(\frac{Ks}{[S]} + 1\right)[ES] + \left(1 + \frac{[N]}{Kn} + \frac{[N]^2}{Kn \cdot Kn'}\right)[EA]$$

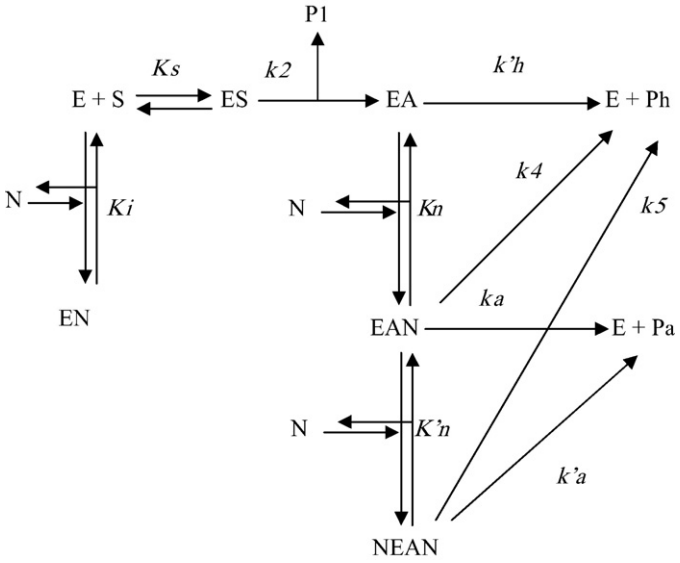
$$[E_0] = \left\{\frac{1}{k_2}\left(\frac{Ks}{[S]} + 1\right)\left(k'h + \frac{(k_a + k_4)[N]}{Kn} + \frac{(k'_a + k_5)[N]^2}{Kn \cdot Kn'}\right) + \left(1 + \frac{[N]}{Kn} + \frac{[N]^2}{Kn \cdot Kn'}\right)\right\}[EA]$$

$$\frac{[E_0]}{Vs} = \frac{\frac{1}{k_2}\left(\frac{Ks}{[S]} + 1\right)\left(k'_h + \frac{(k_a+k_4)[N]}{Kn} + \frac{(k'_a+k_5)[N]^2}{Kn \cdot Kn'}\right) + \left(1 + \frac{[N]}{Kn} + \frac{[N]^2}{Kn \cdot Kn'}\right)}{k'_h + \frac{(k_a+k_4)}{Kn}[N] + \frac{(k'_a+k_5)}{Kn \cdot Kn'}[N]^2} \tag{13}$$

$$\therefore \frac{[E_0]}{Vs} = \frac{Ks}{k_2} \cdot \frac{1}{[S]} + \frac{1}{k_2} + \frac{1 + \frac{[N]}{Kn} + \frac{[N]^2}{Kn \cdot Kn'}}{k'_h + \frac{(k_a+k_4)}{Kn}[N] + \frac{(k'_a+k_5)}{Kn \cdot Kn'}[N]^2} \tag{14}$$

$$\frac{[E][N]}{[EN]} = Ki \tag{15}$$

$$[EN] = \frac{[N]}{Ki}[E] = \frac{[N]}{Ki} \cdot \frac{Ks}{[S]}[ES]$$



$$Vh = k'_h[\mathrm{EA}] + k_4[\mathrm{EAN}] + k_5[\mathrm{NEAN}] = \left(k'_h + \frac{k_4}{Kn}[\mathrm{N}] + \frac{k_5}{Kn \cdot Kn'}[\mathrm{N}]^2\right)[\mathrm{EA}]$$

$$Va = k_a[\mathrm{EAN}] + k'_a[\mathrm{NEAN}] = \left(\frac{k_a}{Kn}[\mathrm{N}] + \frac{k'_a}{Kn \cdot Kn'}[\mathrm{N}]^2\right)[\mathrm{EA}]$$

$$[E_0] = [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{EN}] + [\mathrm{EA}] + [\mathrm{EAN}] + [\mathrm{NEAN}]$$

$$[E_0] = \left(\frac{Ks}{[\mathrm{S}]} + 1 + \frac{Ks[\mathrm{N}]}{Ki[\mathrm{S}]}\right)[\mathrm{ES}] + \left(1 + \frac{[\mathrm{N}]}{Kn} + \frac{[\mathrm{N}]^2}{Kn \cdot Kn'}\right)[\mathrm{EA}]$$

$$[E_0] = \left\{\frac{1}{k_2}\left(\frac{Ks}{[\mathrm{S}]} + 1 + \frac{Ks[\mathrm{N}]}{Ki[\mathrm{S}]}\right)\left(k'_h + \frac{(k_a + k_4)[\mathrm{N}]}{Kn} + \frac{(k'_a + k_5)[\mathrm{N}]^2}{Kn \cdot Kn'}\right) + \left(1 + \frac{[\mathrm{N}]}{Kn} + \frac{[\mathrm{N}]^2}{Kn \cdot Kn'}\right)\right\}[\mathrm{EA}] \quad (16)$$

$$\frac{[E_0]}{Vs} = \frac{1}{k_2}\left(\frac{Ks}{[\mathrm{S}]} + 1 + \frac{Ks[\mathrm{N}]}{Ki[\mathrm{S}]}\right) + \frac{1 + \frac{[\mathrm{N}]}{Kn} + \frac{[\mathrm{N}]^2}{Kn \cdot Kn'}}{k'_h + \frac{(k_a + k_4)[\mathrm{N}]}{Kn} + \frac{(k'_a + k_5)[\mathrm{N}]^2}{Kn \cdot Kn'}}$$

$$\therefore \frac{[E_0]}{Vs} = \frac{Ks}{k_2}\left(1 + \frac{[\mathrm{N}]}{Ki}\right) \cdot \frac{1}{[\mathrm{S}]} + \frac{1}{k_2} + \frac{1 + \frac{[\mathrm{N}]}{Kn} + \frac{[\mathrm{N}]^2}{Kn \cdot Kn'}}{k'_h + \frac{(k_a + k_4)}{Kn}[\mathrm{N}] + \frac{(k'_a + k_5)}{kn \cdot Kn'}[\mathrm{N}]^2} \quad (17)$$

$$\frac{[E_0]}{Va} = \frac{Ks}{k_2}\left(1 + \frac{[\mathrm{N}]}{Ki}\right) \cdot \frac{1}{[\mathrm{S}]} + \frac{\frac{Ks}{k_2}\left(1 + \frac{[\mathrm{N}]}{Ki}\right) \cdot \frac{1}{[\mathrm{S}]}\left(k'_h + \frac{k_4[N]}{Kn} + \frac{k_5[\mathrm{N}]^2}{Kn \cdot Kn'}\right) + 1 + \frac{[\mathrm{N}]}{Kn} + \frac{[\mathrm{N}]^2}{Kn \cdot Kn'}}{\frac{k_a[\mathrm{N}]}{Kn} + \frac{k'_a[\mathrm{N}]^2}{Kn \cdot Kn'}} \quad (18)$$

References

[1] V. Schellenberger, A. Görner, A. Könnecke, H.–D. Jakubke, Protease-catalyzed peptide synthesis: Prevention of side reactions in kinetically controlled reactions, Pept. Res. 4 (1991) 265–269.

[2] J. Bongers, E.P. Heimer, Recent applications of enzymatic peptide synthesis, Peptides 15 (1994) 183–193.

[3] V. Schellenberger, A. Aaviksaar, H.–D. Jakubke, Salt-enhanced aminolysis of acyl-α-chymotrypsins by dipeptides, Biocatalysis 4 (1991) 291–296.

[4] P. Lozano, J.L. Iborra, A.J.P. Rocha, Cryo-enzymatic synthesis of kyotorphin in saline environments, Biochem. Mol. Biol. Int. 33 (1994) 91–97.

[5] H. Uyama, T. Fukuoka, I. Komatsu, T. Watanabe, S. Kobayashi, Protease-catalyzed regioselective polymerization and copolymerization of glutamic acid diethyl ester, Biomacromolecules 3 (2002) 318–323.

[6] Y.V. Mitin, N.P. Zapevalova, E.Y. Gorbunova, Peptide synthesis catalyzed by papain at alkaline pH value, Int. J. Pept. Prot. Res. 23 (1984) 528–534.

[7] V. Tõugu, H. Meos, M. Haga, A. Aaviksaar, H.–D. Jakubke, Peptide synthesis by chymotrypsin in frozen solutions. Free amino acids as nucleophiles, FEBS Lett. 329 (1993) 40–42.

[8] M. Hänsler, G. Ullmann, H.–D. Jakubke, The application of papain, ficin, and clostripain in kinetically controlled peptide synthesis in frozen aqueous solutions, J. Pept. Sci. 1 (1995) 283–287.

[9] G. Ullmann, M. Haensler, M. Gruender, M. Wagner, H.–J. Hofmann, H.–D. Jakubke, Influence of freeze-concentration effect on proteinase-catalyzed peptide synthesis in frozen aqueous systems, Biochim. Biophys. Acta 1338 (1997) 253–258.

[10] K. Aso, T. Uemura, Y. Shiokawa, Protease-catalyzed synthesis of oligo-L-glutamic acid from L-glutamic acid diethyl ester, Agric. Biol. Chem. 52 (1988) 2443–2449.

[11] G. Li, A. Vaidya, K. Viswanathan, J.R. Cui, W.C. Xie, W. Gao, R.A. Gross, Rapid regioselective oligomerization of L-glutamic acid diethyl ester catalyzed by papain, Macromolecules 39 (2006) 7915–7921.

[12] M. Schuster, H.-D. Jakubke, V. Kasche, Nucleophile specificity in papain-catalyzed acyl transfer reactions, Biomed. Biochim. Acta 50 (1991) S122–S126.

[13] M. Schuster, V. Kasche, H.-D. Jakubke, Contribution to the S'-subsite specificity of papain, Biochim. Biophys. Acta 1121 (1992) 207–212.

[14] M. Noguchi, S. Arai, M. Yamashita, H. Kato, M. Fujimaki, Isolation and identification of acidic oligopeptides occurring in a flavor potentiating fraction from a fish protein hydrolysate, J. Agric. Food Chem. 23 (1975) 49–53.

[15] M. Kawai, A. Okiyama, Y. Ueda, Taste enhancements between various amino acids and IMP, Chem. Senses 27 (2002) 739–745.

[16] M. Noguchi, S. Yamashita, S. Arai, M. Fujimaki, On the bitter-masking activity of a glutamic acid-rich oligopeptide fraction, J. Food Sci. 40 (1975) 367–369.

[17] M. Taniguchi, K. Kato, A. Shimauchi, X. Ping, H. Nakayama, K. Fujita, T. Tanaka, Y. Tarui, E. Hirasawa, Proposals for wastewater treatment by applying flocculating activity of cross-linked poly-γ-glutamic acid, J. Biosci. Bioeng. 99 (2005) 245–251.

[18] S.S. Mark, T.C. Crusberg, C.M. Da Cunha, A.A. Di Iorio, A heavy metal biotrap for wastewater remediation using poly-γ-glutamic acid, Biotechnol. Prog. 22 (2006) 523–531.

[19] E.W. Yemm, E.C. Cocking, The determination of amino-acids with ninhydrin, Analyst 80 (1955) 209–213.

[20] S.R. Logan, Fundamentals of Chemical Kinetics, Addison Wesley Longman, Harlow, U.K., 1996

[21] B.C. Wolthers, Kinetics of inhibition of papain by TLCK and TPCK in the presence of BAEE as substrate, FEBS Lett. 2 (1969) 143–145.

[22] K.G.D. Allen, J.A. Stewart, P.E. Johnson, D.G. Wettlaufer, Identification of the functional ionic groups of papain by pH/rate profile analysis, Eur. J. Biochem. 87 (1978) 575–582.

[23] S.D. Lewis, F.A. Johnson, A.K. Ohno, J.A. Shafer, Dependence of the catalytic activity of papain on the ionization of two acidic groups, J. Biol. Chem. 253 (1978) 5080–5086.

[24] S. Tsukamoto, M. Ohno, Modification of papain with tetranitromethane, J. Biochem. 84 (1978) 1625–1632.

[25] Y. Yuthavong, W. Suttimool, Rate constants of individual steps in papain-catalysed reactions, Biochim. Biophys. Acta 523 (1978) 198–206.

[26] M.Y. Gololobov, V.M. Stepanov, T.L. Voyushina, P. Aslercreutz, The second nucleophile molecule binds to the acyl-enzyme-nucleophile complex in α-chymotrypsin catalysis. Kinetic evidence for the interaction, Eur. J. Biochem. 217 (1993) 955–963.

[27] I. Schechter, A. Berger, On the active site of proteases. 3. Mapping the active site of papain; specific peptide inhibitors of papain, Biochem. Biophys. Res. Commun. 32 (1968) 898–902.

[28] R. Ménard, E. Carmona, C. Plouffe, D. Brömme, Y. Konishi, J. Lefebvre, A.C. Storer, The specificity of the S1′ subsite of cysteine proteases, FEBS Lett. 328 (1993) 107–110.

[29] P. Abato, J.L. Conroy, C.T. Seto, Combinatorial library of serine and cysteine proteases inhibitors that interact with both the S and S ' binding sites, J. Med. Chem. 42 (1999) 4001–4009.