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Mechanism of papain-catalyzed synthesis of oligo-tyrosine peptides

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ABSTRACT

Di-, tri-, and tetra-tyrosine peptides with angiotensin I-converting enzyme inhibitory activity were synthesized by papain-catalyzed polymerization of L-tyrosine ethyl ester in aqueous media at 30 °C. Varying the reaction pH from 6.0 to 7.5 and the initial concentration of the ester substrate from 25 to 100 mM, the highest yield of oligo-tyrosine peptides (79% on a substrate basis) was produced at pH 6.5 and 75 mM, respectively. In the reaction initiated with 100 mM of the substrate, approx. 50% yield of insoluble, highly polymerized peptides accumulated. At less than 15 mM, the reaction proceeded poorly; however, from 30 mM to 120 mM a dose-dependent increase in the consumption rate of the substrate was observed with a sigmoidal curve. Meanwhile, each of the tri- and tetra-tyrosine peptides, even at approx. 5 mM, was consumed effectively by papain but was not elongated to insoluble polymers. For deacylation of the acyl-papain intermediate through which a new peptide bond is made, L-tyrosine ethyl ester, even at 5 mM, showed higher nucleophilic activity than di- and tri-tyrosine. These results indicate that the mechanism through which papain polymerizes L-tyrosine ethyl ester is as follows: the first interaction between papain and the ester substrate is a rate-limiting step; oligo-tyrosine peptides produced early in the reaction period are preferentially used as acyl donors, while the initial ester substrate strongly contributes as a nucleophile to the elongation of the peptide product; and the balance between hydrolytic fragmentation and further elongation of oligo-tyrosine peptides is dependent on the surrounding concentration of the ester substrate.

1. Introduction

L-Tyrosine (Tyr) is classified as a non-essential amino acid because it is formed *in vivo* by hydroxylation of dietary phenylalanine (Phe), except for in the case of some newborns. Thus, Tyr is an essential amino acid in patients with phenylketonuria, a genetic disorder in which Phe cannot be normally metabolized. Since the addition of Tyr can sustain synthesis of catecholamines, the potential usefulness of supplementary Tyr for the treatment of phenylketonuria, Parkinson's disease, and acute stress has been investigated [1,2]. However, the use of free Tyr is limited due to its low solubility and questionable efficacy. In contrast to free Tyr, dipeptides containing Tyr are generally water-soluble, therefore the development of facile operations for the synthesis of Tyr-containing peptides is desired [2–4]. Furthermore, a number of Tyr-containing di- or tri-peptides from several food protein sources exhibit angiotensin I-converting enzyme (ACE, EC. 3.4.15.1) inhibitory activity, which contributes to the regulation of blood pressure [5–8]. Among them, Tyr-Tyr is isolated from royal jelly treated with protease [8]. According to some research [9,10], Tyr-Tyr is effectively transported through PEPT1, a proton-coupled peptide transporter expressed on the intestinal epithelium. Thus, it is worth evaluating the bioactivity and safety of Tyr-Tyr for the development of novel therapeutic and/or nutraceutical products.

Pure dipeptides synthesized by chemical methods are commercially available; however, they are generally too expensive to be readily used. In the chemical synthesis of Tyr-containing peptides, not only the N- and C-terminal groups in amino acids but also other functional groups in the side chains are commonly protected [11–14]. Subsequently, coupling reaction and acid-catalyzed removal of the protecting groups lead to unwanted side reactions and the formation of byproducts. To overcome the problems of chemical peptide synthesis, protease-catalyzed reactions have been attracting considerable attention [14–18]. In protease-catalyzed peptide synthesis, the high specificity and high reactivity under mild conditions, which are characteristics of

Abbreviations: ACE, angiotensin I-converting enzyme; BA, Nα-benzoyl-L-arginine; BAEE, Nα-benzoyl-L-arginine ethyl ester; BA-Tyr, Nα-benzoyl-L-arginyl-L-tyrosine; DP, degree of polymerization; RP-HPLC, reverse-phase high-performance liquid chromatography; Tyr-OEt, L-tyrosine ethyl ester; Tyr-OH, free L-tyrosine.

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enzymes, can reduce the number of operational steps required for protection–deprotection and avoid side reactions such as racemization. The acyl group of a substrate specific to the S subsite region in a protease is transferred to an added nucleophile with a high affinity for the S' subsite in the catalyst through the acyl-enzyme intermediate [18–22]. The deacylation by amino components such as amino acid derivatives or peptides is called aminolysis, generating a new peptide bond. In an aqueous reaction, hydrolysis is unavoidable due to the presence of water. Temperature, pH and nucleophile concentration are important parameters that affect peptide yield by regulating the rate of aminolysis by reactive nucleophiles with deprotonated amino group.

With respect to the efficient synthesis of Tyr-Tyr, Narai-Kanayama et al. [23] previously proposed the two-step enzymatic reaction, in which papain (EC 3.4.22.2)-catalyzed synthesis of Tyr-polymers from L-tyrosine ethyl ester (Tyr-OEt) in a buffer solution [24] was followed by their hydrolytic cleavage by α-chymotrypsin (EC 3.4.21.1) in aqueous DMSO media [23]. Overall, these two reactions were carried out at 25 °C and pH 7.5. Starting with 100 mM of Tyr-OEt, the final reaction products contained di- and tri-Tyr peptides in good yield, reaching 65% on an initial ester substrate basis [23]. Each of these two oligo-Tyr peptides exhibited a mixture of competitive and noncompetitive inhibitions of ACE from rabbit lung, with IC_{50} values of 34 μM and 48 μM, respectively [21]. However, the two-step enzymatic reaction proceeds slowly, at least 4 days. Furthermore, centrifugation is necessary between the two reactions to collect the water-insoluble Tyr-polymers with degrees of polymerization (DPs) of between 5 and 10, and DMSO is used as a solvent to dissolve Tyr-polymers. The 2nd reaction step needs a substantial amount of α-chymotrypsin to bring its hydrolytic activity enough in a 50% DMSO/buffer solution. Ultimately, DMSO and some peptide fragments derived from autoprocessing of α-chymotrypsin must be removed from the oligo-Tyr peptides.

Therefore, the development of a high throughput method for the synthesis of oligo-Tyr peptides using only papain-catalyzed polymerization of Tyr-OEt without both DMSO and α-chymotrypsin has been anticipated. In the present study, we investigated the effects of reaction temperature, pH and initial Tyr-OEt concentration on the yields of oligo-Tyr peptides. Also, we kinetically analyzed the mechanism through which papain polymerized Tyr-OEt and elongated oligo-Tyr peptides to Tyr-polymer.

2. Materials and methods

2.1. Materials

L-Tyrosine ethyl ester (Tyr-OEt) hydrochloride was purchased from Tokyo Chemical Industry (Tokyo, Japan). Papain from papaya latex, a 2× crystallized suspension in sodium acetate, pH 4.5, and *N*α-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma–Aldrich (St Louis, USA). 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 23,700 [25]. Chemically synthesized Tyr-Tyr, >95% purity, was purchased from Thermo Fisher Scientific (Ulm, Germany). Trifluoroacetic acid (TFA) was purchased from Kanto Chemical (Tokyo, Japan). All other reagents used were of analytical grade.

2.2. Papain-catalyzed polymerization of Tyr-OEt

Unless otherwise specified, papain-catalyzed polymerization of Tyr-OEt was performed using 0.2 M Na-phosphate buffer, pH 6.5, containing 1 mM DTT at 30 °C without pH control. It was confirmed that the reaction pH changed by only 0.2–0.4 during the reaction. Papain solution was prepared at 330 μM in 11 mM DTT and was then activated by incubation at 30 °C for 10 min before the reaction. Tyr-OEt was dissolved in 0.22 M Na-phosphate buffer and the pH of this substrate solution was adjusted to the predetermined value with a NaOH solution. After preincubation of the substrate solution at a given temperature for 1 min, the reaction was initiated by mixing with an activated enzyme solution at a 10:1 volume ratio, with a final papain concentration of 30 μM, and the reaction proceeded mildly enough to allow us to temporally analyze the reaction products. Aliquots of the reaction mixture were withdrawn at appropriate intervals, added into 1/5 volume of 5 M HCl, and then mixed well to make L-tyrosine (Tyr-OH), a hydrolysis product of Tyr-OEt, soluble and distinctive from insoluble Tyr-polymers. These sample solutions were kept on ice to prevent the ester group hydrolysis of Tyr-OEt and products. These were used for the quantitative analysis of the reaction products by RP-HPLC, which was carried out using a Hitachi HPLC system (L-7100 pump, L-7400 UV detector, and L-7200 autosampler) equipped with a SUS line filter (GL Science, Tokyo, Japan) and a Cadenza CD-C18 (4.6 mm × 50 mm) column (Imtakt, Kyoto, Japan). Tyr-OEt, Tyr-OH, and oligo-Tyr peptides with DP 2, 3 and 4 were eluted using a linear gradient (0–50% for 40 min) of methanol/0.1% TFA at a flow rate of 0.5 mL/min, and detected at 280 nm. The concentration of each component on the basis of Tyr residues was determined from peak areas as described previously [20], except for the analysis of chromatograms, which used the data processing software Chromato-PRO (Run Time Corporation, Kanagawa, Japan).

2.3. Preparation of oligo-Tyr peptides with DP 2, 3, and 4

The acidified reaction mixture (as described in Section 2.2) was centrifuged at 10,000 × *g* for 30 min at 25 °C, and the supernatant was mixed with 10 M NaOH at a 17:3 volume ratio. This alkaline de-esterification of residual Tyr-OEt for 30 min at 25 °C in the dark was stopped by mixing with 1/5 volume of 5 M HCl. After the removal of sediment by centrifugation, the solution was loaded onto a NOBIAS RP-OD1 cartridge (Hitachi High Technologies, Tokyo, Japan). The absorbed matters were eluted using 50% ethanol and then evaporated. From this concentrate, which mainly contained Tyr-Tyr (DP2), Tyr-Tyr-Tyr (DP3) and tetra Tyr peptide (DP4), each of the oligo-Tyr peptides was purified by RP-HPLC using a preparative column (InertSustain™ C18 (7.6 mm × 150 mm); GL Science) with a linear gradient (4.5–54% for 35 min) of acetonitrile/0.1% TFA at a flow rate of 0.5 mL/min. The retention time of DP 2 was confirmed using the commercially obtained Tyr-Tyr.

2.4. Kinetic analysis of papain-catalyzed acyl transfer reaction

Papain-catalyzed acyl transfer reactions were conducted using 1 μM papain and 100 mM of BAEE as an acyl donor in 0.2 M Na-phosphate buffer, pH 6.5, containing 1 mM DTT at 30 °C. BAEE of 110 mM and a specified concentration of nucleophile such as Tyr-OEt or oligo-Tyr peptides were dissolved in 0.22 M Na-phosphate buffer and the pH was adjusted to 6.5 with a NaOH solution. After preincubation of the substrate solution at 30 °C for 1 min, the acyl transfer reaction was initiated by mixing with an activated papain solution of 11 μM at a 10:1 volume ratio. Aliquots (each 100 μL) of the reaction mixture were withdrawn at intervals of 5 min within 25 min, added to 1.5 mL of 0.05 M HCl and kept on ice. RP-HPLC analysis of BAEE, *N*α-benzoyl-L-arginine (BA), Tyr-OEt, DP2 and synthetic peptides (BA-Tyr-OEt or BA-DP2) were performed using a Cadenza CD-C18 (4.6 mm × 50 mm) column (Imtakt) with linear elution (0–54–90–90% of methanol/0.1% TFA: 0–30–40–45 min) at a flow rate of 0.5 mL/min, and all eluted components were detected at 260 nm (Fig. 1A and B). DP3 and BA-DP3 were analyzed using an InertSustain™ C18 (4.6 mm × 75 mm) column (GL Science) under a different elution condition (0–0–54–90–90% of methanol/0.1% TFA: 0–5–30–40–45 min) (Fig. 1 C). Products other than BA and BA-X peptides (X: Tyr-OEt, DP2 or DP3) were not detected under the conditions used, as shown in Fig. 1. Velocities