Peptide synthesis with halophenylalanines by thermolysin

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Abstract. Thermolysin was able to catalyze enantioselective peptide synthesis with non-natural amino acids, halophenylalanines. However, the reactivity of thermolysin was considerably influenced by the kind and position of halogen substituents on these analogues. The manner of the recognition of the amino component by the enzyme was different from that of the carboxyl component in the synthesis of peptides with non-natural phenylalanine analogues. The phenomena observed are discussed, based on the kinetic parameters obtained.

Introduction

Natural peptides have many kinds of important biological activities, such as metabolic control, antibacterial activity, antiviral activity, and enzyme inhibition. If any amino acids (AAs; L form unless otherwise stated) of these peptides are exchanged for their analogues, namely, non-natural AAs that are not present in the natural world, the modified peptides may exhibit different functions from the natural one and have new functions, because a non-natural AA itself has different stereochemical and electric characteristics (Abeles and Alston 1990; Bovy et al. 1991). Thus, synthesis of peptides including non-natural AAs is very interesting. Furthermore, it is very attractive to discover how proteases recognize non-natural AAs as substrates, which may also provide important information on how modified peptides behave in vivo. However, there have been few studies on enzymatic synthesis of peptides with non-natural AAs.

In this study, synthesis of benzyloxycarbonyl (Z)-Phe-Phe-OMe analogues with halophenylalanines as non-natural AAs by thermolysin was chosen as a model reaction (Fig. 1). Thermolysin is thermostable and suited for the industrial production of peptides. Furthermore, its structure and substrate specificity are well known (Izquierdo and Stein 1990; Kester and Matthews 1977). We also systematically investigated how thermolysin recognizes non-natural AAs such as halophenylalanines and discuss the effects of the kind and position of substituents of phenylalanine analogues on reactivity and enantioselectivity of thermolysin in terms of the carboxyl and amino components.

Materials and methods

Materials. Thermolysin (from Bacillus thermoproteolyticus Rokko) was a product of Daiwa Kaso (Osaka, Japan). Chemicals were obtained from the following sources: Z-L-Phe from the Peptide Institute (Osaka, Japan); L-Phe-OMe·HCl, Z-L-Tyr, and L-Tyr-OMe·HCl from the Kokusan Chemical Works (Osaka, Japan).

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Fig. 1. Synthesis of peptides with non-natural amino acids by thermolysin
Celite adsorption of thermolysin. Thermolysin (18 mg) was suspended in 0.018 ml 2-(N-morpholino)ethanesulfonic acid (MES)/NaOH buffer (50 mM, pH 6.5) containing 5 mM CaCl₂ and the paste obtained was mixed with 45 mg Celite no. 535.

Synthesis of Z-AA-AA-OMe. AA-OMe was prepared from AA-OMe·HCl according to the method of Nakanishi et al. (1985). The carboxyl component, Z-AA (40 mM) and amino component, AA-OMe (40 mM) were dissolved in 10 ml ethyl acetate saturated with 50 mM MES/NaOH buffer (pH 6.5) containing 5 mM CaCl₂. The reaction was carried out by adding thermolysin (18 mg) adsorbed on Celite at 30°C with shaking (120 strokes/min). In the case of enantioselective synthesis of peptides with racemic AAs, a racemic Phe or a racemic non-natural Phe analogue (40 mM) was used as either the carboxyl or amino component, while the counter component was optically pure l-Phe (40 mM).

Measurement of kinetic parameters. The Phe(X)-OMe concentration was varied from 20 mM to 250 mM at a fixed Z-Phe concentration (40 mM), and Lineweaver-Burk plots were obtained from the initial reaction rates. The other conditions were the same as those mentioned above.

Analytical methods. Products were quantified by HPLC (Waters 600) with an octadecyl silane (ODS) column (10 X 300 mm; Yamamura Chemical Laboratories, Kyoto, Japan). The eluent was a mixture of acetonitrile/water (60/40, v/v) adjusted to pH 2.5 with phosphoric acid and monitored at 214 nm. The flow rate was 0.8 ml/min.

To determine the diastereomeric excess, the products were analyzed by HPLC using a Sumichiral OA-4600 column. The eluent was a mixture of hexane/1,2-dichloroethane/ethanol (100/201, v/v/v) and monitored at 254 nm. The flow rate was 1.0 ml/min. The diastereomeric excess (%) de was calculated from peak areas of the respective amino components.

Results and discussion

Thermolysin was found to catalyze successfully the synthesis of Z-Phe-Phe-OMe analogues having a halophenyl group (Fig. 1) in ethyl acetate saturated with 50 mM MES/NaOH buffer (pH 6.5). The time courses of the reaction with a non-natural carboxyl component [Z-Phe(X)] and natural amino component (Phe-OMe) are shown in Fig. 2. Although the reactivity was different from one another, all the non-natural analogues tested were good substrates as a carboxyl component. The conversion ratios were 75–90%. The reactivity toward Z-Tyr was low, but its conversion reached 85%.

To make the difference in reactivity of these substrates clear, relative activity was calculated from the initial reaction rates (Table 1). The initial rate for Z-Phe was expressed as 100. The effect of substitution was greatest in the ortho position and smallest in the para position. Among the fluorides, Z-Phe (pF) was the best substrate and had almost the same activity as natural phenylalanine; Z-Phe(mF) was the second, and the activity for Z-Phe (oF) was half that for Z-Phe (pF). As for chlorides, the ortho-substituted substrate [Z-Phe(oCl)] also exhibited almost half the activity of that for the para-substituted one [Z-Phe(pCl)]. Z-Phe(pI) having a larger iodine atom at the para position served as a substrate, even though less active than Z-Phe(pF) and Z-Phe(pCl). These results suggested that the site for carboxyl component in the active site of thermolysin might have a relatively large space toward the direction of the para position of the substrates. The reactivity of Z-Tyr having a hydroxyl group in the para position, which is smaller than chlorine, was lower than that of Z-Phe(pCl). From the viewpoint of the steric effect of the substituent group, Z-Tyr should react more actively than Z-Phe(pCl). This result showed that the hydroxyl group had other effects that lowered its reactivity.

The time courses of the reaction with the natural carboxyl component (Z-Phe) and the non-natural amino component [Phe(X)-OMe] are shown in Fig. 3. Different from the case of the carboxyl component, Phe(pCl)-OMe and Phe(pI)-OMe hardly served as substrates. Except for these non-reactive substrates, conversion reached 70–90% after 96 h of reaction. The relative activity of the respective amino components is