Different Acyl Specificity between Mold and Kidney Acylases: Correlation of Hydrolytic Rate to Molecular Size and Electronegative Property of Acyl Group in Acyl-L-phenylalanine Hydrolysis

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Synopsis. A series of acyl-L-phenylalanines were hydrolyzed by mold and hog kidney acylases. The formyl was found to be the most susceptible for mold acylase, and the butyryl for kidney acylase, indicating the different preferences of these acylases in molecular size and electronegative property of acyl group of substrates.

A variety of optically active amino acids have been prepared by resolving acyl-DL-amino acids with enzymes. Hog kidney acylase I and mold acylase are the most frequently utilized enzymes in this procedure, and their substrate specificity has been studied focusing mainly on the side chain of acetyl- or chloroacetylamino acids.[1] Recently, mold acylase has become increasingly useful because of its availability in large scale and easy immobilization even in an industrial scale. To obtain a high efficacy of the enzyme in such a system, it seems to be important to find more effective acyl groups other than the acetyl and chloroacetyl, since more susceptible acyl group may preserve or even elicit the hydrolytic efficiency when amino acids having unfavorable side chain for the enzyme should be resolved. However, no systematic studies have been reported for the substrate specificity related to acyl groups. Thus, in this study we measured the acylase-catalyzed hydrolytic rate of a series of synthetic acyl-L-phenylalanine in order to analyze the efficiency of acyl groups on the basis of their molecular size and electronegative property.

Acyl groups to derivatize L-phenylalanine were the formyl (For), acetyl (Ac), propionyl (Prp), butyryl (But), pentanoyl (Pen), hexanoyl (Hex), and chloroacetyl (ClAc). Urethane type acyl groups such as ethoxycarbonyl (Eoc), *t*-butoxycarbonyl (Boc), and benzyloxycarbonyl (Z)[2] were also examined. These derivatives were prepared by the usual methods as described in the literature. For hydrolysis of each acyl-L-phenylalanine, the optimum pH was determined (pH 6.0—7.5 for mold acylase: see Table 1). Although the optimum pH might be above pH 8.5 for kidney acylase, no definite values could be determined because of precipitation of cobalt hydroxide at this pH range. Under the conditions without cobalt ion, acylamino acids have been reported to show a rather broad pH maximum between pH 6.7 and 8.0.[3]

The hydrolytic rates were measured at the optimum pH of each substrate for mold acylase and at pH 7.0 for kidney acylase as reported by others.[3] The relative rates of hydrolysis were shown in Table 1. For mold acylase, the formyl was found to be very susceptible, showing a 3.6-fold larger hydrolytic rate than that of the acetyl. Moreover, the formyl was much more easily hydrolyzed than the chloroacetyl. The order of susceptibility of acyl groups towards the acylase was For > ClAc > Ac > Prp > But > Pen > Hex. A very little hydrolysis was observed for Eoc-derivative, and no hydrolysis for Boc- and Z-derivatives. When the rates were compared among a series of acyl groups, H-$(CH_2)_n$-CO-, it was found that a progressive decrease in the number n of methylene units causes an increase in the rate, and thus the formyl with n=0 became the most labile acyl group to the hydrolysis. This result indicates that mold acylase prefers smaller acyl group in molecular size.

For kidney acylase, the order of susceptibility was But > ClAc > Prp > Pen > Ac > Hex > For (Table 1), indicating that the rate was maximized with n=3 in a series of H-$(CH_2)_n$-CO-. Interestingly, the formyl was quite insusceptible toward kidney acylase in contrast to its high susceptibility toward mold acylase. Similar result was also observed by Fones and Lee.[3] These results show that mold and kidney acylases have different substrate-preferences in molecular size of acyl group.

Shimohigashi et al.[4] reported that electronegative property of acyl group can be estimated by the chemical shift of amide proton of acyl-L-phenylalanine in ^{1}H NMR. The order of the extent of electronegativity was found to be ClAc > For > Ac > Prp ≈ But ≈ Pen ≈ Hex. Although the molecular size of the chloroacetyl is almost equal to that of the propionyl, the susceptibility of these groups were apparently different,

Table 1. Relative Hydrolytic Rate of *N*-Acyl-L-phenylalanines by Acylases[a]

Substrate	Mold acylase[b]		Kidney acylase[b]
Acyl-L-Phe-OH	Optimum pH	Relative hydrolytic rate	Relative hydrolytic rate
For-L-Phe-OH	6.0	3.6	0.19
Ac-L-Phe-OH	7.5	1.0	1.0
ClAc-L-Phe-OH	6.2	1.5	3.3
Prp-L-Phe-OH	7.5	0.46	1.7
But-L-Phe-OH	7.5	0.31	3.7
Pen-L-Phe-OH	7.5	0.24	1.3
Hex-L-Phe-OH	7.5	0.03	0.23
Eoc-L-Phe-OH	7.0	0.004	0.09

a)Substrate conc., 10 mM; Used pH, optimum pH for mold acylase, pH 7.0 for acylase I. b) Enzyme concentrations varied depending on the susceptibility of each acyl derivative.

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namely, ClAc > Prp toward both acylases. This means that the electronic state of amide bond in the substrate might be an important rate-determining factor in the hydrolysis and a higher acidity of amide proton seems to be favorable for rapid hydrolysis. However, for kidney acylase, the butyryl was hydrolyzed with almost the same rate as the chloroacetyl. This result clearly indicates that kidney acylase has a much more restricted substrate specificity of molecular size of acyl group than that of its electronegative property. For mold acylase, both factors of molecular size and electronegativity appear equally important, since the formyl, which is fairly electronegative, was hydrolyzed more easily than the chloroacetyl.

The usefulness of the formyl group for resolution was demonstrated by resolving For-DL-Orn(Z)-OH. After complete hydrolysis by mold acylase, H-L-Orn(Z)-OH and For-D-Orn(Z)-OH were separated by extraction in a high yield, and the formyl of For-D-Orn(Z)-OH was selectively removed under the mild condition of treatment with *p*-toluenesulfonic acid without cleaving the Z group. The resolved L- or D-Orn(Z)-OH can be used as an important starting block for constructing bioactive peptides and their analogs containing these optically active nonproteinous amino acid. Owing to a high resolution yield and easy preparation of formyl amino acids, the formyl should be one of the acyl groups which are the candidates for an efficient resolution process using the most common mold acylase.

Experimental

Amino acids were determined with a Hitachi amino acid analyzer KLA-5. Mold acylase and acylase I were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively, and used without further purification.

Synthesis of Compounds. Acylamino acid derivatives of L-Phe were prepared according to the literatures: For,[6] Ac,[7] ClAc,[7] Prp,[8] But,[8] Pen,[9] Hex,[8] Boc,[10] and Z-L-Phe-OH.[11] Eoc-L-Phe-OH was prepared essentially with the same method described for Z-L-Phe-OH[11] using ethoxycarbonyl chloride; yield of DCHA salt, 89%; mp 146—147 °C, $[\alpha]_D^{20}$ +36.5° (*c* 2, EtOH).

Determination of Optimum pH. To a solution of acyl-L-amino acid (0.02 mmol) and 0.1 M NaOH (0.2 ml) (1 M=1 mol dm^{-3}) in a 2-ml flask were added 1/7 M sodium barbital buffer (1 ml) with a specified pH, 1/40 M $CoCl_2$ (0.1 ml), and an aqueous enzyme solution (0.2 ml). The amount of enzymes was varied depending on the susceptibility of the acyl group toward the particular enzyme. The solution was made up to 2.0 ml with water. The reaction mixture was incubated at 38 °C, an aliquot being withdrawn after 1 h. Appearance of L-amino acid was determined with an amino acid analyzer: column, 0.6×10 cm; buffer, standard 0.2 M sodium citrate at pH 4.25. The amount of L-amino acid produced was calculated on the basis of the color value determined for a corresponding authentic amino acid with the analyzer.

Determination of Relative Hydrolytic Rate of Acylamino Acids by Acylases. A mixture of an acyl-L-amino acid (0.02 mmol), 0.1 M NaOH (0.2 ml), 1.7 M sodium barbital buffer (1 ml) with optimum pH of each substrate for mold acylase or with pH 7.0 for acylase I, 1/40 M $CoCl_2$ (0.1 ml) and an aqueous enzyme solution (0.2 ml) was made up to 2.0 ml with water. The rate of hydrolysis at 38 °C was followed by the analyzer under the conditions described above. The hydrolysis of substrates followed zero-order kinetics within experimental error. The relative hydrolytic rate to that of Ac-L-Phe-OH was calculated (Table 1).

Resolution of For-DL-Orn(Z)-OH by Mold Acylase. H-L-Orn(Z)-OH: For-DL-Orn(Z)-OH[12] (0.74 g, 2.5 mmol) was dissolved in aqueous triethylamine, the pH being adjusted to 7.4 (optimum pH). To the solution were added acylase (16 mg), 1/40 M $CoCl_2$ (12.5 ml), and water. After the solution (250 ml) had been left to stand at 38 °C for 7 days, the precipitate formed was collected and washed with water. Combined filtrates were evaporated to a small volume. Aqueous formic acid was added to adjust the pH to 5. Separated oily product was extracted with EtOAc. The extract was used for the preparation of For-D-Orn(Z)-OH. The aqueous layer was loaded on a column (1.7×10 cm) of Dowex 50×8 (H^+ form) to elute with 2 M aqueous ammonia (60 ml). The eluate was evaporated to dryness and the residue was recrystallized from water-EtOH; yield; 0.32 g (97%); mp 244—246 °C (decomp); $[\alpha]_D^{20}$ +19.2° (*c* 2, 5 M HCl). Reported values, mp 246—248 °C (decomp); $[\alpha]_D^{20}$ +18.9° (5 M HCl).[5]

For-D-Orn(Z)-OH: The extract obtained above was dried over Na_2SO_4 and evaporated. The residual solid was recrystallized from EtOAc-ether; yield, 0.33 g (88%); mp 66—70 °C (decomp); $[\alpha]_D^{20}$ −15.6° (*c* 2, EtOH).

H-D-Orn(Z)-OH: To a solution of For-D-Orn(Z)-OH (0.34 mmol) in a mixture of dioxane (9 ml) and water (1 ml) was added TosOH·H_2O (0.37 mmol). After being left at 50 °C for 9 h, aqueous triethylamine was added to adjust the pH to 7. The solution was evaporated to dryness, and the residual solid was recrystallized from water-EtOH; yield, 88%; mp 246—248 °C; $[\alpha]_D^{20}$ −17.7° (*c* 2, 5 M HCl). Reported value of the L-isomer, $[\alpha]_D^{20}$ +18.9° (5 M HCl).[5]

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2) Abbreviations used: But, butyryl; ClAc, chloroacetyl; Eoc, ethoxycarbonyl; For, formyl; Hex, hexanoyl; Pen, pentanoyl; Prp, propionyl; TosOH, *p*-toluenesulfonic acid.

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