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New Fluorogenic Substrates for Subtilisin[1)]

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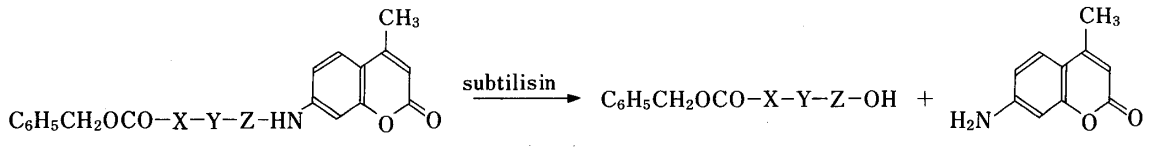
Two 7-amino-4-methylcoumarinamide derivatives of tripeptides were synthesized. They were shown to be potentially useful substrates for fluorometric microdetermination of subtilisins BPN′ and Carlsberg. The kinetic characteristics of the hydrolyses of these substrates by the enzymes were investigated.

Keywords——fluorometric enzyme assay; subtilisin; 7-amino-4-methylcoumarinamide derivative; fluorogenic substrate; peptidylamino-coumarin; kinetic study

Chromogenic and fluorogenic substrates are now widely used for the simple and sensitive assay of various proteases both in research laboratories and in clinical diagnostics.[2)] Peptidyl-*p*-nitroanilides (*p*NA) have been successfully applied to the convenient assay of serine proteases, *e.g.*, thrombin, plasmin, kallikrein, trypsin and blood-clotting factos.[3)]

Subtilisins are widely used as models for the study of enzyme–substrate interaction because the active sites and substrate specificities of subtilisins BPN′ and Carlsberg, have been well investigated. They have relatively broad substrate specificity[4,5)] and their three-dimensional structures have been extensively studied.[6)] The active site is considered to consist of several subsites,[7)] *i.e.* P_1,P_2, P_3, ... and P_1', P_2', P_3' ... as originally proposed for papain. On the basis of these studies, several tripeptidyl-*p*-nitroanilide substrates for subtilisins have been reported.[8)] Of the substrates reported so far, two compounds, Z–Asp–Pro–Leu–*p*NA and Z–Gly–Gly–Leu–*p*NA, attracted our attention, since the former is reported to be hydrolyzed much more efficiently by the Carlsberg enzyme than by the BPN′[8a)] while the latter seems to be similarly hydrolyzed by both enzymes, although full comparative data have not been presented. It might be possible, therefore, to design new substrates which are suitable for the detection of micro quantities of the enzyme and are cleaved preferentially by either of the subtilisins.

In the course of our studies of organic fluorescence reagents,[9)] we have become aware of the particular usefulness of amino–coumarin derivatives as fluorophores and a series of studies has been undertaken to develop fluorescence reagents employing 7-amino-4-methylcoumarin (AMC) as a key fluorophore. Thus, several AMC amides (MCA) of



I : X=Y=Gly, Z=Leu
II : X=Asp, Y=Pro, Z=Leu

Chart 1

appropriate amino acid derivatives have been synthesized and successfully employed for laboratory and clinical assay of corresponding proteolytic enzymes, *e.g.*, leucine aminopeptidase,[10] trypsin and papain,[11] cystine aminopeptidase[12] and γ-glutamyltranspeptidase.[13] As an extension of this work, we have newly synthesized two peptide–AMC amides, Z–Gly–Gly–Leu–MCA (I) and Z–Asp–Pro–Leu–MCA (II), and their use for the sensitive assay of subtilisins is described in the present paper (Chart 1).

Experimental

Materials——BPN′ and Carlsberg subtilisins [EC 3.4.31.14] were purchased from Nagase & Co., Osaka and Sigma Chemical Co., respectively, and used without further purification. The concentrations of active enzymes were determined by normality titration with *trans*-cinnamoylimidazole[14] to be 80.5% (BPN′) and 86.4% (Carlsberg). Protein concentrations was determined by measuring absorbance at 280 nm, based on the following values[15] ($E^{1\%}_{280\,nm}$): 11.7 for BPN′ with a molecular weight of 27500; 8.6 for Carlsberg with a molecular weight of 27300.

Synthesis of the Substrates——7-(N^{α}-Benzyloxycarbonyl-glycylglycyl-L-leucyl)amino-4-methylcoumarin (I): Dicyclohexylcarbodiimide (412 mg, 2 mmol) was added to a solution of *N*-benzyloxycarbonylglycylglycine (532 mg, 2 mmol), 7-(L-leucyl)amino-4-methylcoumarine hydrochloride[10] (662 mg, 2 mmol) and triethylamine (202 mg, 2 mmol) in *N*,*N*-dimethylformamide (DMF) (10 ml) at room temperature under stirring. The mixture was stirred for 12 h at room temperature. The solution was filtered and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate (100 ml) and the solution was washed successively with 1 N HCl, water, saturated $NaHCO_3$, and water, then dried (Na_2SO_4). The solvent was evaporated off, and recrystallization of the residue from MeOH–ether–pet. ether gave colorless fine needles, mp 115—118 °C, 902 mg (84%). $[\alpha]_D^{20}$ −33.8 (c=1.6 in AcOH). *Anal.* Calcd for $C_{28}H_{32}N_4O_7$: C, 62.67; H, 6.01; N, 10.44. Found: C, 62.45; H, 6.01; N, 10.46.

7-(N^{α}-Benzyloxycarbonyl-β-benzyl-L-aspartyl-L-prolyl-L-leucyl)amino-4-methylcoumarin (IIa): Dicyclohexylcarbodiimide (618 mg, 3 mmol) was added to a solution of N^{α}-benzyloxycarbonyl-β-benzyl-L-aspartyl-L-proline (1.4 g, 3 mmol), 7-(L-leucyl)amino-4-methylcoumarin hydrochloride (993 mg, 3 mmol) and triethylamine (303 mg, 3 mmol) in acetonitrile (20 ml) at 0 °C under stirring. The mixture was stirred for 12 h at room temperature, and the filtered solution was evaporated *in vacuo*. The residue was dissolved in ethyl acetate (20 ml) and the solution was washed successively with 1 N HCl, saturated $NaHCO_3$, and water, then dried (Na_2SO_4). The solvent was evaporated off, and recrystallization of the residue from ethyl acetate–per. ether gave colorless prisms, mp 154—156 °C, 1.65 g (76%). *Anal.* Calcd for $C_{40}H_{44}N_4O_9$: C, 66.28; H, 6.12; N, 7.73. Found: C, 66.35; H, 5.97; N, 7.43.

7-(N^{α}-Benzyloxycarbonyl-L-α-aspartyl-L-prolyl-L-leucyl)amino-4-methylcoumarin (II): A 2 N NaOH solution (1 ml) was added to a stirred solution of IIa (725 mg, 1 mmol) in MeOH (10 ml) at room temperature and the mixture was stirred for 2 h. The MeOH was evaporated off *in vacuo* and the residue was partitioned between water (10 ml) and chloroform (5 ml). The aqueous layer was neutralized with 6 N HCl under cooling, then extracted with ethyl acetate (10 ml). The ethyl acetate solution was washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (chloroform : MeOH = 10 : 1 as the eluent), followed by recrystallization from ethyl acetate–*n*-hexane to give a crystalline mass, mp 101—110 °C, 457 mg (72%). $[\alpha]_D^{20}$ −71.0 (c=3.1 in AcOH). *Anal.* Calcd for $C_{33}H_{38}N_4O_9$: C, 62.45; H, 6.04; N, 8.83. Found: C, 62.19; H, 6.28; N, 8.56.

Assays——Enzyme assays were conducted at 37 °C using 50 mM Tris–HCl buffer, pH 8.2, containing 7.5% DMF, in a total volume of 2.5 ml. The substrate was first dissolved in DMF and the solution was diluted to give a final concentration of 0.2 mM, using the buffer described above. The reaction was started by the addition of 10 μl of enzyme solution and the release of 7-amino-4-methylcoumarin (AMC) was monitored as a fluorescence increment at 460 nm (excited at 380 nm) using a Shimadzu RF-500 spectrofluorometer equipped with a thermostated cuvette holder, when the AMC substrates were employed. In the case of peptidyl-*p*-nitroanilides as substrates, the release of *p*-nitroaniline was measured as absorption increase at 410 nm (ε 8800), with a Hitachi 200-10 spectrophotometer. For the experiments in Table I, the conditions were as follows: enzyme concentrations were 0.24 μM (BPN′) and 0.33 μM (Carlsberg), and the substrate concentrations varied between 50 μM and 0.3 mM.

Results and Discussion

Methylcoumarinamide derivatives have been reported to be very sensitive substrates for some peptidases.[10–13,16] Similarly the newly synthesized peptide substrates (I,II) are highly fluorescent, as is their hydrolytic product, 7-amino-4-methylcoumarin (AMC), but their excitation and emission maxima are distinctly different. When excited at 380 nm and measured at 460 nm, the relative fluorescence intensity of AMC is at least 500-fold higher than

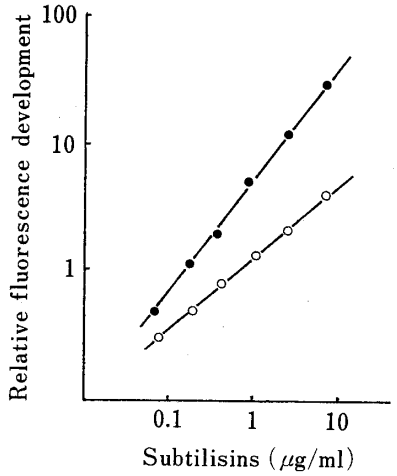


Fig. 1. Relationship between Subtilisin Concentration and Fluorescence Development

Experiments were carried out at 37 °C in 50 mM Tris-HCl (pH 8.2) containing 7.5% DMF. The substrate was 0.2 mM Z–Gly–Gly–Leu–MCA.
○, subtilisin Carlsberg; ●, subtilisin BPN′.

TABLE I. Kinetic Parameters of the Fluorogenic Substrates for Subtilisin

Substrate	Subtilisin BPN′			Subtilisin Carlsberg		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Z–Gly–Gly–Leu–MCA	0.55	0.52	9.5×10^2	0.74	0.65	8.8×10^2
Z–Gly–Gly–Leu–*p*NA	0.84	0.54	6.4×10^2	0.78	0.53	6.8×10^2
Z–Asp–Pro–Leu–MCA	1.7	0.31	1.8×10^2	0.79	2.38	30×10^2
Z–Asp–Pro–Leu–*p*NA	1.8	0.29	1.6×10^2	0.82	2.56	31×10^2

50 mM Tris–HCl pH 8.2 (7.5% DMF), 37 °C.

that of the substrates so that the fluorescence of the substrates does not interfere with these fluorometric assays.

As shown in Fig. 1, the rate of hydrolysis was proportional to enzyme concentration over at least a 50-fold range. The detection limits of subtilisins BPN′ and Carlsberg were nearly 0.1 μg/ml under conditions where Z–Gly–Gly–Leu–MCA was incubated for 10 min at pH 8.2, while with Z–Gly–Gly–Leu–*p*NA, 0.2 μM or 5.5 μg/ml of subtilisin BPN′ was needed.[8a] This comparison clearly illustrates the advantage of the fluorogenic substrate (MCA) over the chromogenic counterpart (*p*NA) in the case of subtilisin microdetermination.

Kinetic parameters in Table I show that the two subtilisins behave quite differently toward Z–Asp–Pro–Leu–MCA, as in the case of the corresponding *p*-nitroanilide, while they hydrolyze Z–Gly–Gly–Leu–MCA indistinguishably. These observations confirm the hypothesis that the specificity and catalytic susceptibility of the substrates for subtilisins could be predominantly determined by their peptidyl moieties (P_1—P_3).[8] Table I also shows that the kinetic parameters of the MCA substrates were quite similar to those of the *p*NA substrates. Pozsgay *et al.* reported a large difference (*ca.* 2000-fold) in the hydrolyses of Z–Asp–Pro–Leu–*p*NA by the BPN′ and Carlsberg enzymes,[8a] but we did not observe such a difference under similar conditions (20-fold). In any case, the present data demonstrate that substitution of an MCA moiety for *p*-nitroaniline at the P_1' site does not cause any significant change in the specificity or hydrolytic efficacy of the subtilisins towards these peptidyl substrates.

The two fluorogenic substrates described here appear to be selective to subtilisins, since no detectable hydrolysis was observed after a 20 min incubation with 1 μg/ml of chymotrypsin, whose substrate specificity is directed toward P_1 amino acid residues with aromatic or large hydrophobic side chains, as is the case with subtilisins. In addition, these substrates

showed no detectable fluorescence increase resulting from spontaneous hydrolysis even after incubation for 5 h at pH 8.2. Thus, Z–Asp–Pro–Leu–MCA and Z–Gly–Gly–Leu–MCA are potentially useful substrates for fluorometric microdetermination of subtilisins.

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