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Kinetic Specificities of BPN′ and Carlsberg Subtilisins

Mapping the Aromatic Binding Site

Yuji KARASAKI and Motonori OHNO

Laboratory of Enzyme Chemistry, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka, Fukuoka 812

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The kinetic specificities of BPN′ and Carlsberg subtilisins [EC 3.4.21.14] were examined with various nucleus-substituted derivatives of N^{α}-acetylated aromatic amino acid methyl esters for mapping their hydrophobic binding sites in comparison with that of α-chymotrypsin. The Carlsberg enzyme was generally much more reactive than the BPN′ enzyme due to the larger k_{cat} value. The fact that the two subtilisins hydrolyzed Ac-Tyr(PABz)-OMe, which is a derivative of tyrosine bearing a planar *trans-p*-phenylazobenzoyl group at the OH-function, with the smallest K_m value showed that these enzymes possess a more extended aromatic binding site than has so far been demonstrated. Ac-Phe(4-NO_2)-OMe was remarkable in being hydrolyzed with a particularly large k_{cat} value ($5,500 \pm 700\ s^{-1}$ at pH 7.8 for Carlsberg subtilisin). Ac-Phe(4-NO_2)-OMe and Ac-Tyr-OMe were distinguished by Carlsberg subtilisin in terms of k_{cat} but not by BPN′ subtilisin, suggesting that the specificity site of the former is more sensitive to a small change in size of substituent than that of the latter. Ac-Trp(NCps)-OMe and Ac-Trp(NCps)-OH were bound to the enzyme's active site but in a competitive manner. A difference in the standard free energies of binding between the two enzymes may indicate that the hydrophobic cleft of Carlsberg subtilisin is somewhat deeper and/or narrower than that of BPN′ subtilisin.

BPN′ and Carlsberg subtilisins [EC 3.4.21.14], proteolytic enzymes from *Bacillus subtilis* strains,[1] exhibit higher activity toward esters of aromatic amino acids than those of aliphatic ones (*1*, *2*). However, the low substrate specificity is in contrast to the high degree of specificity shown by α-chymotrypsin (*2*). X-ray crystallographic investigations of BPN′ subtilisin have shown that the specific substrate binding site is composed of a crevice and the carbonyl oxygen of Ser-125 which can form a hydrogen bond with a substrate (*3*). The crevice is formed by three short segments of the enzyme's backbone and is lined principally by hydrophobic groups. One side of the crevice is

Abbreviations: Ac, acetyl; OMe, methoxy; Trp(CHO), N^{in}-formyltryptophan; Trp(NCps), 2-(2-nitro-4-carboxyphenylsulphenyl)-tryptophan; Trp(OH), 2-hydroxytryptophan; Phe(4-NO_2), 4-nitrophenylalanine; Phe(4-NHAc), 4-acetamidophenylalanine; Dopa, 3,4-dihydroxyphenylalanine; Tyr(3-NO_2), 3-nitrotyrosine; *m*-Tyr, *meta*-tyrosine; *o*-Tyr, *ortho*-tyrosine; Tyr(Ac, 3-NO_2), *O*-acetyl-3-nitrotyrosine; Tyr(PABz), *O-trans-p*-phenylazobenzoyltyrosine; Cys(Bzl), *S*-benzylcysteine; DMSO, dimethyl sulfoxide; MeOH, methanol.

[1] A bacterium producing subtilisin BPN′ is classified at present as *Bacillus amyloliquefaciens* and distinguished from *Bacillus subtilis* (*10*).

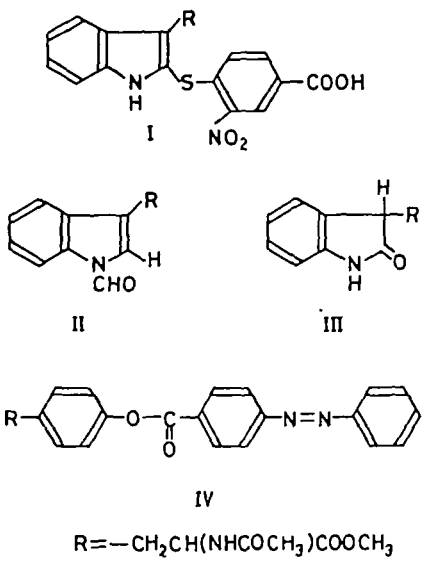

Diagram 1. Structures of Ac-Trp(NCps)-OMe (I), Ac-Trp(CHO)-OMe (II), Ac-Trp(OH)-OMe (III), and Ac-Tyr(PABz)-OMe (IV).

planar, while the other is less regular (*4*). On the other hand, the specific substrate binding site of α-chymotrypsin forms a deep pocket and both sides of this pocket are planar (*5–8*). Such differences presumably account for the less specific affinity of subtilisin for aromatic side chains than that of α-chymotrypsin.

The three-dimensional structure of Carlsberg subtilisin has not yet been determined. However, BPN′ and Carlsberg subtilisins show a 70% sequence homology with conservative substitutions of the hydrophobic residues in interior chain segments and are assumed to have the same amino acid residues in the active sites except that Val-165 of BPN′ subtilisin is replaced by Ile in Carlsberg subtilisin (*9*). This marked homology may be connected with the fact that the two enzymes are indistinguishable from the standpoint of substrate specificity (*1*). In this work, we examined kinetic specificities of BPN′ and Carlsberg subtilisins with a wide variety of nucleus-substituted derivatives of N^{α}-acetylated aromatic amino acid methyl esters for comparison with that of α-chymotrypsin and to map the aromatic binding sites of these three enzymes in regard to their differences and similarities. The structures of several complex substrates are shown in Diagram 1. The steady state kinetic studies were carried out at pH 7.8 and 6.5 in the presence of 5% dimethyl sulfoxide (DMSO).

MATERIALS AND METHODS

The substrates used here were prepared as reported previously (*11*) except for Ac-Tyr(PABz)-OMe.[1]

N^{α}-Acetyl-O-trans-p-phenylazobenzoyltyrosine Methyl Ester (Ac-Tyr(PABz)-OMe)—To a solution of Ac-Tyr-OMe (1.18 g, 5 mmol) in dimethylformamide (5 ml) containing triethylamine (0.70 ml, 5 mmol), a solution of *p*-phenylazobenzoyl chloride (*12*) (1.22 g, 5 mmol) in dioxane (15 ml) was added dropwise and the solution was stirred at room temperature for 2 h. The solution containing crystals was concentrated *in vacuo* and crystallization was completed by adding water. Crystals were collected and washed with sodium bicarbonate solution and water. The crude product was recrystallized from methanol (MeOH)-petroleum ether. Yield 1.63 g (73%), mp 267–268° (decomp.), $[\alpha]_D = +4.38°$ (MeOH). *Anal.* Calcd. for $C_{25}H_{23}O_5N_3 \cdot 1.5H_2O$: C, 63.55; H, 5.55; N, 8.89. Found: C, 63.03; H, 5.25; N, 9.04.

Enzymes—Salt-free, lyophilized preparations of subtilisins BPN′ (Lot 113C-3510) and Carlsberg (Lot 33C-3270) were purchased from Sigma and used without further purification. The effective normalities of the two enzymes were determined by titration with *trans*-cinnamoyl imidazole (*13*) to be 70.4% (BPN′) and 87.5% (Carlsberg), respectively, of the protein concentrations estimated using optical factors of 11.7 and 8.6 (280 nm) and molecular weights of 27,500 and 27,300, respectively (*14*).

Kinetics—The initial rates of hydrolysis of substrates by subtilisins were determined at pH 7.8 and 6.5 at 25°C (with a water-jacketed cell) under a stream of nitrogen using a Radiometer RTS-5 titrator. The buffer solution consisted of 95 volumes of 0.05 M phosphate buffer containing 0.2 M KCl and 5 volumes of DMSO. The substrate concentration was generally based on the weighed amounts of crystalline substrates. Concentrations of uncrystallizable Ac-*m*-Tyr-OMe and Ac-Dopa-OMe were determined spectrophotometrically using molar extinction coefficients obtained from crystalline compounds containing the

[1] All amino acids described in this paper are of the L-configuration.

same chromophores: Ac-DL-*m*-Tyr-OMe (ε = 2,040 $M^{-1}\cdot cm^{-1}$ at 275 nm in MeOH); Dopa-OMe·HCl (ε = 3,400 $M^{-1}\cdot cm^{-1}$ at 283 nm in MeOH) (*11*). Ac-Trp(CHO)-OMe is unstable above pH 7.8 due to deformylation (*15*), so the rate measurement was done at pH 7.2 instead of pH 7.8. The titrant used was 0.0130 M NaOH. Reactions were initiated by adding the enzyme in a volume of 20 μl to 3.0 ml of substrate solution. In every case, the initial portion of the alkali uptake *vs.* time plot was linear and rates were determined directly from the slopes. The values of $K_{m(app)}$ and k_{cat} were calculated from the rates at 8 different substrate concentrations according to Wilkinson's procedure (*16*) using a FACOM 230-75 computer. The dissociation constants (K_I) of inhibitors were determined by a modification of the above procedure, the inhibitors being introduced into the substrate solution at appropriate concentrations. Ac-Trp-OMe was employed as a substrate in these inhibition experiments.

RESULTS AND DISCUSSION

The kinetic studies were performed in the presence of 5% DMSO to keep substrates of a hydrophobic nature in solution. It is known that DMSO affects the catalytic properites of subtilisin BPN' in that the k_{cat} value decreases with increase of DMSO concentrations, while the K_m value remains almost constant (*17*).

Figure 1 shows Lineweaver-Burk plots for hydrolysis of Ac-Tyr-OMe and its derivatives by BPN' and Carlsberg subtilisins at pH 7.8. The linear relationship between e_0/v and $1/s_0$ indicates that the initial rates of hydrolysis of each substrate satisfy Michaelis-Menten kinetics. The kinetic parameters obtained at pH 7.8 and 6.5 for all substrates are summarized in Tables I and II, respectively. The parameters for α-chymotrypsin previously obtained (*11*) are listed together for comparison.

Carlsberg subtilisin showed considerably larger specificity constants (k_{cat}/K_m) toward the substrates examined than BPN' subtilisin, due to the particularly larger k_{cat} values. Based on the currently accepted Eq. 1 for chymotrypsin or subtilisin-catalyzed hydrolysis of an ester substrate, K_m and k_{cat} can be expressed by Eqs. 2 and 3, respectively,

$$E+S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E+P_2 \qquad (1)$$
$$\downarrow P_1$$

$$K_m = K_s k_3/(k_2+k_3) \qquad (2)$$

$$k_{cat} = k_2 k_3/(k_2+k_3) \qquad (3)$$

where ES represents an enzyme-substrate complex, K_s its dissociation contant, EA an acyl enzyme, P_1 an alcohol, and P_2 an acid. In the reaction of subtilisin and *trans*-cinnamoyl imidazole, the acylation is much faster than the deacylation in the case of chymotrypsin (*18*). The rate-controlling step for the α-chymotryptic hydrolysis of specific ester substrates is the deacylation process

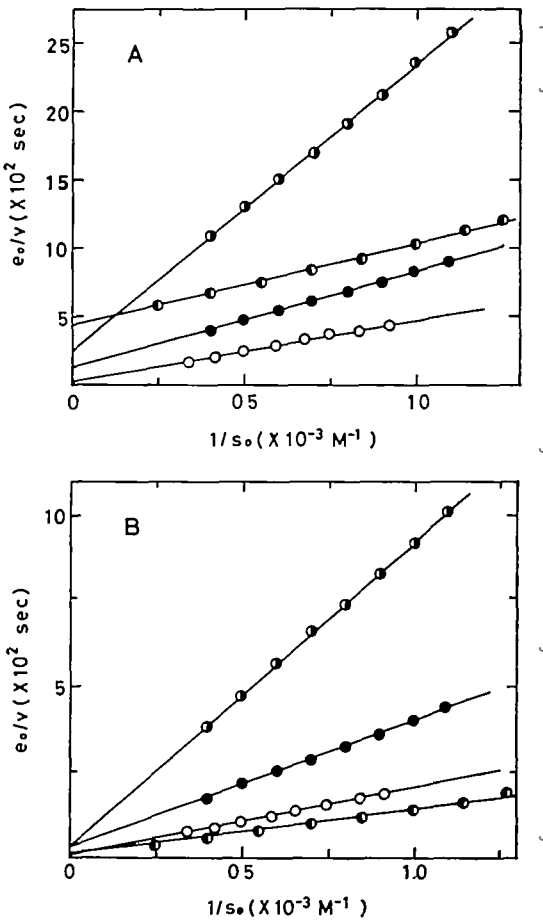

Fig. 1. Double-reciprocal plots for hydrolysis of Ac-Trp-OMe (○), Ac-Dopa-OMe (●), Ac-Tyr(Ac)-OMe (◐), and Ac-Tyr(3-NO_2)-OMe (◑) by BPN' (A) and Carlsberg (B) subtilisins at pH 7.8 in 0.05 M phosphate buffer containing 0.2 M KCl and 5% DMSO and 25°C.

TABLE I. Kinetic parameters for hydrolysis of phenylalanine and tryptophan derivatives by BPN′ and Carlsberg subtilisins and α-chymotrypsin at pH 7.8 and 25°C in the presence of 5% DMSO.[a]

Substrates	BPN′			Carlsberg			α-Chymotrypsin		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
Ac-Trp-OMe	37.9±0.9	4.0±0.1	9,500±400	444±12	7.2±0.3	62,000±3,100	45.3	0.60	76,000
Ac-Trp (NCps)-OMe		nh			nh		33.2	0.30	111,000
Ac-Trp (CHO)-OMe[b]	46.3±1.2	10.6±0.3	4,400±200	265±6	9.3±0.2	28,000±1,700	740	22	34,000
Ac-Trp(OH)-OMe	1.3±0.1	3.2±0.3	410±50	3.7±0.1	1.4±0.1	2,600±100	27.8	1.00	27,800
Ac-Phe-OMe	76.6±5.9	9.3±0.8	8,200±900	353±9	7.9±0.3	45,000±2,000	90	2.0	45,000
Ac-Phe(4-NO_2)-OMe	421±61	16.7±2.7	25,000±5,500	5,520±690	32.6±4.3	169,000±31,000	10.5	0.87	12,100
Ac-Phe(4-NHAc)-OMe	2.9±0.1	1.1±0.1	2,600±300	221±6	13.4±0.4	16,000±700		nh	
Ac-Tyr-OMe	418±13	18.6±0.6	23,000±1,000	862±36	16.4±0.8	53,000±3,400	200	2.0	100,000
Ac-Tyr(Ac)-OMe	22.7±0.3	1.31±0.04	17,000±600	721±72	9.5±1.2	76,000±9,900		nh	
Ac-Tyr(3-NO_2)-OMe	38.0±0.6	7.9±0.2	4,800±100	349±30	31.2±2.8	11,000±1,500	280	7.1	45,000
Ac-Tyr(Ac, 3-NO_2)-OMe	6.9±0.1	0.63±0.03	11,000±500	163±4	6.1±0.2	27,000±900		nh	
Ac-*m*-Tyr-OMe	59.1±2.4	5.03±0.29	12,000±800	187±3	3.0±0.1	62,000±2,300	660	13	51,000
Ac-*o*-Tyr-OMe	1.61±0.07	0.53±0.09	3,000±500	5.3±0.5	3.0±0.4	1,800±300	160	19	8,400
Ac-Dopa-OMe	80.2±0.6	5.6±0.1	14,000±200	370±17	14.0±0.7	26,000±1,800	170	1.9	89,000
Ac-Tyr(PABz)-OMe[c]	3.8±0.1	0.65±0.02	5,900±200	27.1±2.6	1.3±0.2	21,000±3,800		nh	

[a] The designation nh in the Table indicates no hydrolysis occurred. [b] Measured at pH 7.2. [c] Measured in the presence of 15% DMSO.

TABLE II. Kinetic parameters for hydrolysis of phenylalanine and tryptophan derivatives by BPN′ and Carlsberg subtilisins and α-chymotrypsin at pH 6.5 and 25°C in the presence of 5% DMSO.[a]

Substrates	BPN′			Carlsberg			α-Chymotrypsin		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Ac-Trp-OMe	2.4±0.1	2.0±0.1	1,200±80	54±3	13 0±0.7	4,200±400	12.5	0.34	37,000
Ac-Trp(NCps)-OMe		nh			nh		9.9	0.11	90,000
Ac-Trp(CHO)-OMe	15.0±0.6	4.2±0.2	3,600±200	38.1±0 3	5.1±0.6	7,500±900	69	3.0	23,000
Ac-Phe-OMe	6.9±0.1	6.2±0.1	1,100±20	18.8±0.3	3.4±0.1	5,500±200	24.5	1.50	16,300
Ac-Phe(4-NO_2)-OMe	25.1±0.3	7.8±0.1	3,200±60	805±93	37.3±4.5	22,000±3,900	5.3	0 82	6,500
Ac-Phe(4-NHAc)-OMe		nd		18.3±0.4	10.5±0.3	1,700±50		nh	
Ac-Tyr-OMe	36.4±2.4	17.2±1.2	2,100±200	88±6	15.9±0.3	5,500±500	51	2.3	22,000
Ac-Tyr(Ac)-OMe	2.5±0.1	0.8±0.1	3,000±400	59±5	6.2±0.7	9,500±1,300		nh	
Ac-Tyr(3-NO_2)-OMe	31.4±1.6	5.1±0.4	6,200±600	22±1	2.2±0.2	10,000±1,000	120	6.4	19,000
Ac-Tyr(Ac, 3-NO_2)-OMe	0.90±0.0	0.39±0.01	2,300±60	66±7	22.7±2.6	2,900±500		nh	
Ac-*m*-Tyr-OMe	16.0±2.3	12 5±2.0	1,300±300	19.9±0.4	4.0±0.1	5,000±200	57	3.1	19,000
Ac-*o*-Tyr-OMe		nd			nd		16.2	3.35	4,800
Ac-Dopa-OMe	8.4±0.1	4.1±0.1	2,000±60	27±1	8.7±0.4	3,100±200	48	2.5	19,000

[a] The designation nh indicates no hydrolysis occurred. The designation nd means that the parameters could not be determined because of extremely slow hydrolysis.

(*19*). The same relationship should hold for ester hydrolysis by subtilisin as well. Thus the k_{cat} represents the deacylation rate constant (k_3).

The substrates possessing a group consisting of a chain of four atoms at the *para* position of the phenyl nucleus, such as Ac-Phe(4-NHAc)-OMe, Ac-Tyr(Ac)-OMe, and Ac-Tyr(Ac,3-NO_2)-OMe, were not hydrolyzed by α-chymotrypsin but hydrolyzed by BPN′ and Carlsberg subtilisins. This shows that the latter two enzymes possess a longer side chain binding site than α-chymotrypsin. The k_{cat} values for Ac-Phe(4-NHAc)-OMe are significantly smaller than those for Ac-Tyr(Ac)-OMe with the two subtilisins, although pronounced with the BPN′ enzyme. Structural difference in the substituent lies only in substitution of NH of the former by oxygen in the latter. It is probable that rotation around the bond between the ζ-carbon atom (numbered from the α-carbon) of the phenyl nucleus and the η-atom is favorable with Ac-Tyr(Ac)-OMe rather than Ac-Phe(4-NHAc)-OMe as regards orientation of the scissile ester bond to the catalytic residues of enzymes. However, the addition of a nitro group at the *meta* (or 3) position of Ac-Tyr(Ac)-OMe considerably reduces the k_{cat} value. When k_{cat} values for Ac-Tyr-OMe are taken as standards, all its derivatives with a nitro or hydroxyl group at the *meta* position, such as Ac-Tyr(3-NO_2)-OMe and Ac-Dopa-OMe, exhibited smaller k_{cat} values. Ac-*m*-Tyr-OMe also gave smaller k_{cat} values than Ac-Tyr-OMe with subtilisins. This is the reverse of the observation with α-chymotrypsin in which the k_{cat} values for substrates bearing a nitro or hydroxyl group at the *meta* position of the phenyl nucleus are either comparable with or larger than those for the substrates with no substituent at this position. These results suggest that the substituent at the *meta* position of the phenyl nucleus is stereochemically unfavorable to the catalytic process with BPN′ and Carlsberg subtilisins and these enzymes have a narrower binding cleft than α-chymotrypsin.

Bosshard and Berger (*20*) reported that benzyloxycarbonyl (Z)-alanyl-alanyl-*S*-benzylcysteine (Cys(Bzl)) is strongly bound to BPN′ and Carlsberg subtilisins rather than α-chymotrypsin with orientation of the Cys(Bzl) residue to the specific hydrophobic site. This also proves that subtilisins possess a more extended aromatic binding site than α-chymotrypsin. In the present experiment, Ac-Tyr(PABz)-OMe, which is the substrate with the longest substituent at the *para* position of the phenyl nucleus, exhibited a moderate reactivity to subtilisins due to the small K_m value. This shows that the side chain binding site of subtilisins is capable of interacting effectively with a planar *trans-p*-phenylazobenzoyl (PABz) moiety far removed from the phenyl nucleus orienting to the central part of the binding cleft and is far more elongated than has been demonstrated so far. Orientation of the ester bond to the catalytic site of enzymes, however, is less favorable because of comparatively smaller k_{cat} values than the others. It has not been ascertained whether or not the PABz group interacts only with the residues in the cleft.

The specificity constants for Ac-Phe(4-NO_2)-OMe were largest in all substrates examined at pH 6.5 and 7.8 due to remarkably large k_{cat} values. The k_{cat} value ($5{,}500 \pm 700\ s^{-1}$) at pH 7.8 for Carlsberg subtilisin is particularly noteworthy. Such a high turnover number has never been reported for the substrates hitherto examined. This implies that a 4-nitrophenylmethyl side chain of this substrate interacts with the hydrophobic cleft of the enzymes the most favorably as regards the deacylation process. BPN′ subtilisin hydrolyzed Ac-Tyr-OMe and Ac-Phe(4-NO_2)-OMe with comparable k_{cat} values, whereas Carlsberg subtilisin hydrolyzed Ac-Tyr-OMe with 6 to 8 times smaller k_{cat} values than those for Ac-Phe(4-NO_2)-OMe. The ability of the Carlsberg enzyme to distinguish these substrates in terms of k_{cat} is ascribed to the fact that the aromatic binding site of this enzyme responds more sensitively to a small difference in size of substituent as large as a nitro or hydroxyl group at the *para* position of the phenyl nucleus than the BPN′ enzyme. The k_{cat} values of Carlsberg subtilisin for all substrates are inherently larger than those of BPN′ subtilisin. These phenomena suggest that the active site of the Carlsberg enzyme possesses more relevant rigidity and flexibility than the BPN′ enzyme and thus is capable of accommodating the substrate in such a way as to fit the reacting groups in a more proper position and form the more stabilized transient complex and then enhance the rate.

BPN′ subtilisin is characteristic, when compared with Carlsberg subtilisin, in hydrolyzing the

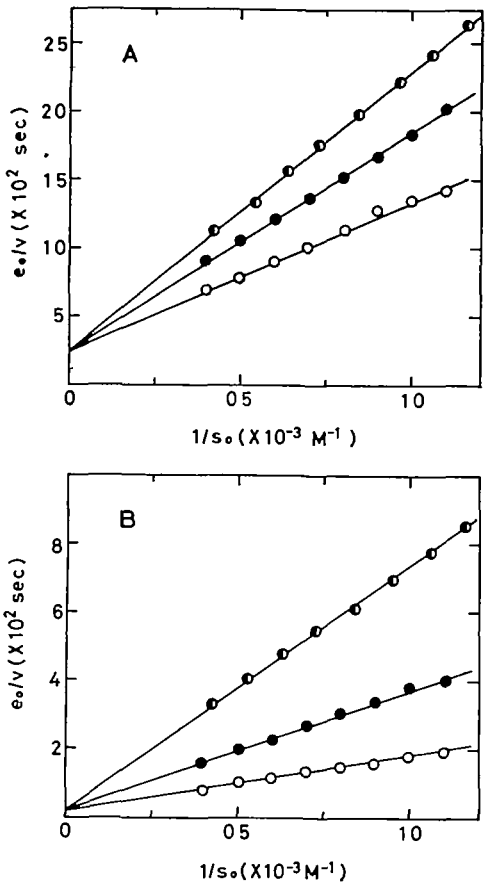

Fig. 2. Double-reciprocal plots for hydrolysis of Ac-Trp-OMe by BPN′ (A) and Carlsberg (B) subtilisins in the absence and presence of Ac-Trp(NCps)-OMe at pH 7.8 in 0.05 M phosphate buffer containing 0.2 M KCl and 5% DMSO at 25°C. Concentrations of Ac-Trp(NCps)-OMe: 0 mM (○), 0.5 mM (●), 2.0 mM (◐).

substrates possessing an acetamido or acetoxy substituent at the *para* position of the phenyl nucleus, such as Ac-Phe(4-NHAc)-OMe, Ac-Tyr(Ac)-OMe, and Ac-Tyr(Ac,3-NO_2)-OMe, with significantly smaller K_m values than those for the other substrates. However, this does not simply reflect the large affinity of the substrates to the aromatic site because the k_{cat} values representing k_3 are also small.

No large difference in k_{cat} values has been observed between Ac-Trp-OMe and Ac-Trp(CHO)-OMe with the two subtilisins except for that at pH 6.5 with the BPN′ enzyme. With α-chymotrypsin, Ac-Trp(CHO)-OMe was hydrolyzed with a much larger k_{cat} value than Ac-Trp-OMe. Ac-Trp(OH)-OMe, which is a substrate with a non-planar indolinone ring, exhibited less reactivity due to an extremely small k_{cat} value. Ac-o-Tyr-OMe was also less reactive for the same reason. The latter two substrates are identical with respect to modification at the δ-position and such a modification makes the scissile ester bond orient in the wrong way to the catalytic residues.

Ac-Trp(NCps)-OMe, which is an effective substrate for α-chymotrypsin, was not hydrolyzed by subtilisins. Reversely, as shown in Fig. 2, it inhibited the hydrolysis of Ac-Trp-OMe by subtilisins in a competitive manner. Ac-Trp(NCps)-OH is also bound to the enzymes and inhibits competitively (Fig. 2). The dissociation constants (K_I) and the standard free energies of binding are listed in Table III. The free energies of binding of Carlsberg subtilisin for the two inhibitors are approximately 1 kcal/mol larger than those of BPN′ subtilisin, indicating the higher binding affinity of the former. This can be accounted for by assuming that the hydrophobic cleft of Carlsberg subtilisin is somewhat deeper and/or nar-

TABLE III. Inhibition constants (K_I) and standard free energies of binding of Ac-Trp(NCps)-OMe and Ac-Trp(NCps)-OH for subtilisin BPN′ and Carlsberg at pH 7.8 and 25°C in the presence of 5% DMSO.

Inhibitors	Inhibitor conc. (mM)	BPN′		Carlsberg	
		K_I (mM)	$-\Delta G°$ (kcal/mol)	K_I (mM)	$-\Delta G°$ (kcal/mol)
Ac-Trp(NCps)-OMe	0.5	1.54±0.11	3.8	0.36±0.02	4.7
	2.0	1.71±0.08	3.8	0.65±0.10	4.4
Ac-Trp(NCps)-OH	0.5	2.28±0.24	3.6	0.53±0.15	4.5
	2.0	3.25±0.30	3.4	0.93±0.17	4.2

rower than that of BPN′ subtilisin and thus energetically more favorably interacts with an aromatic moiety of inhibitors. This is qualitatively consistent with the finding by Bosshard and Berger (*20*). They found that Novo subtilisin[3] associates strongly with Z-Ala-Ala-diphenylmethylcysteine but Carlsberg subtilisin hardly does and concluded that the aromatic binding site of the latter is narrower than that of the former. No significant difference in K_I values of free energies of binding between Ac-Trp(NCps)-OMe and Ac-Trp(NCps)-OH indicates that there is little effect of a negative charge due to α-carboxyl group of Ac-Trp(NCps)-OH in the interaction with the enzymes and the presence of a charged group in the counterpart of the enzymes is excluded.

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[3] Novo subtilisin is identical with BPN′ subtilisin in sequence (*14*) and crystal structure (*21*).