Streptococcal Proteinase-catalyzed Hydrolysis of Some Ester and Amide Substrates*

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SUMMARY

Streptococcal proteinase was shown to belong to a group of enzymes such as trypsin, chymotrypsin, and subtilisin that exhibit a high ratio of esterase to peptidase activity. In this respect, streptococcal proteinase is different from other sulfhydryl enzymes, such as papain and ficin, and also different from pepsin. These enzymes hydrolyze a specific ester and amide substrate at comparable rates.

A spectrophotometric method is described for following the streptococcal proteinase-catalyzed hydrolysis of substrates of the general type benzyloxycarbonyl-X-Y, where X is lysine, norleucine, and glutamic acid, and Y is phenyl, p-nitrophenyl, aniline, and p-nitroaniline. This method was used to demonstrate that streptococcal proteinase is an efficient esterase in the cleavage of phenyl and p-nitrophenyl esters, and that in the cleavage of these esters the enzyme is favored by the presence of a cationic group in X. Phenyl esters were found to be more versatile substrates than p-nitrophenyl esters for the proteinase, since autolysis of phenyl ester substrates was not observed even at higher pH values (pH 9.0). The effect of pH on the proteinase-catalyzed hydrolyses of benzyloxycarbonyllysyl-p-nitrophenyl esters was examined. The data revealed that the ratio of log $k_{cat}/K_m$ for the enzyme depends on an ionizable group with a pK of 4.8.

When the leaving group, Y, in A-X-Y is changed from phenyl to p-nitrophenyl, $k_{cat}/K_m$ values increase 5-fold. However, when Y is changed from aniline to p-nitroaniline, the $k_{cat}/K_m$ value unexpectedly decreases 36-fold. Wang's theory of pretransition state protonation was used to interpret these data, and it is proposed that streptococcal proteinase hydrolyzes specific ester and amide substrates by different mechanisms. Tosynleucylphenylalanine and tosyllyysylphenyl ester were found to be resistant to cleavage by streptococcal proteinase. These two derivatives are competitive inhibitors of streptococcal proteinase, with $K_I$ values near the kinetically determined value of $K_{m(app)}$ for the corresponding benzyloxycarbonyl derivatives.

Proteolytic enzymes generally fall into two major categories. Those in one category hydrolyze the ester substrates at a rate at least $1 \times 10^4$ times faster than the corresponding amide, and those in the other group hydrolyze amide and ester substrates at comparable rates. Most of the “serine” enzymes, such as trypsin, chymotrypsin, and subtilisin, have been shown to belong to the first group (1–6), whereas the sulfhydryl enzymes such as pepsin (7, 8) belong to the second group. All of the “serine” enzymes mentioned have been shown also to have at least 1 histidine residue at the active site (6, 9, 10), whereas, for the sulfhydryl enzymes, papain and ficin, no compelling kinetic data are available to indicate that a histidine residue is involved in the catalysis of these enzymes. Although a histidine residue has been shown to be about 4 A away from the active site third group in papain both by x-ray crystallography (11) and chemical modification studies (12), no convincing evidence has yet been presented to implicate this particular histidine residue in the catalysis of papain. Kinetic studies (7, 13, 14) with papain suggest that acylation is dependent on a basic group with pK$_a$ of 4.3 and an acidic group with pK$_a$ of 8.4, and that deacylation is dependent on a single group with pK$_a$ of 3.9. The basic group seen both in acylation and deacylation has been suggested to be a carboxylate ion, while the acidic group is an un-ionized sulfhydryl group. Previously, the heat of ionization of these groups had been shown to be consistent with their being carboxyl and sulfhydryl groups (13, 14). In this respect, it is noteworthy that pepsin hydrolyzes ester and amide substrates at a comparable rate (15). The existence in pepsin of at least one carboxyl group which is essential for its catalytic function is well documented (16–18). It thus appears that, for the proteolytic enzymes that utilize imidazole simultaneously as a general base and a general acid for their catalytic function, a high ratio of esterase to peptidase activity is observed. For the proteolytic enzymes that show comparable rates of hydrolysis of a specific ester and amide substrate, a carboxyl group has been shown to be the catalytic entity.

A number of recent investigations on streptococcal proteinase, including both chemical modification (19–21) and kinetic studies (22), have led to the conclusion that both the —SH group of cysteine and the basic group of histidine are involved in the catalytic function of streptococcal proteinase. Thus, streptococcal proteinase is a “histidine” enzyme like trypsin, chymotrypsin, and subtilisin, and at the same time it is an “—SH” enzyme like papain and ficin.
We report here the syntheses of a number of specific ester and amide or anilide substrates for streptococcal proteinase. These substrates were used to demonstrate that streptococcal proteinase is an efficient esterase, and this activity has been compared with its peptidase activity.

EXPERIMENTAL PROCEDURE

Materials

Streptococcal proteinase, formed by the action of trypsin on the zymogen, was prepared according to the method of Elliott (23) and purified as described by Liu and Elliott (24). It was stored in the unreduced form and was activated by reduction prior to assay. Carboxypeptidase A treated with diisopropyl fluorophosphate was obtained from Worthington. Carboxypeptidase A, crystallized, was obtained from Worthington. N-Ethylmorpholine was distilled before use. Glass-distilled water was used throughout. Acetonitrile, ethanol, and ethyl acetate were of the highest purity available and were not distilled before use. Dithioerythritol was obtained from Calbiochem.

Kinetic Measurements

p-Nitrophenyl Ester of N\textsuperscript{\textalpha}-Z-lysine—Reactions were followed spectrophotometrically at 340 nm with the Cary model 14 CM recording spectrophotometer. Reactions were generally performed as described by Bender and Brubacher (25) with certain modifications. Enzyme (50 µl) was added to 3.0 ml of buffer solution in a 1.00-cm cell, and then substrate (50 µl) was added (see Tables II, III, and V for S\textsubscript{0} and E\textsubscript{0}). The solution was mixed for 2 to 3 sec with a stirring rod. Recording was started 5 sec after addition of substrate. The S\textsubscript{0} values used for calculation of kinetic constants were corrected for hydrolysis during the initial 5 sec before the actual recording of the reaction.

All reactions were followed to completion. The kinetic constants K\textsubscript{m} and V\textsubscript{m} were determined according to Equation 1 from Lineweaver-Burk plots of the data from each complete reaction (Fig. 1).

\[
\frac{1}{V} = \frac{1}{V_{\text{m}}} + \frac{K_m}{V_{\text{m}}} \frac{1}{S} \tag{1}
\]

Values for k\textsubscript{cat} were determined from the zero order part of the spectrophotometric trace where S\textsubscript{0} ≫ K\textsubscript{m}, or from double reciprocal plots using steady state rate data. The absence of significant product inhibition was demonstrated.

p-Nitrophenyl Esters of N\textsuperscript{\textalpha}-Z-N\textsuperscript{\textbeta}-Boc-lysine, N\textsuperscript{\textbeta}-norleucine, N\textsuperscript{\textbeta}-glutamic acid, N\textsuperscript{\textalpha}-Z-lysine, and N\textsuperscript{\textbeta}-lysine in 20% Acetonitrile—The procedure for following the streptococcal proteinase-catalyzed hydrolyses of these esters was the same as that for Z-Lys-ONp described above except that the hydrolyses were performed in buffer, pH 5.50, which contained 20% acetonitrile (see Table III for details).

Phenyl Esters of N\textsuperscript{\textalpha}-Z-lysine, N\textsuperscript{\textbeta}-norleucine, and N\textsuperscript{\textbeta}-glutamic acid—Reactions were followed spectrophotometrically, as described for p-nitrophenyl esters of Z-lysine, at 270 nm. Measurements were made in either the presence or absence of 20% acetonitrile (see Tables III and IV for E\textsubscript{0} and S\textsubscript{0}). All reactions were followed to completion, and thus it was possible to determine the Δε values at the pH of each reaction. (Δε for phenol at pH 5.5 is 1.49 × 10\textsuperscript{4}; at pH 7.6 it is 1.478 × 10\textsuperscript{4}).

pH-Stat Titration of Hydrolyses of p-Nitrophenyl Esters—The rates of hydrolysis of N\textsuperscript{\textalpha}-Z-Lys-ONp and N\textsuperscript{\textalpha}-Z-Nle-ONp were determined at pH 5.6 and 25.0° in a pH-stat (Radiometer TTT11e, SBR-2e, Abu-1e, 0.25-m1 piston). Titration was carried out in 5.4 ml of a solution containing 0.2 M KCl, 1.89 to 2.62 × 10\textsuperscript{-4} M substrate, and either 2.74 × 10\textsuperscript{-4} M streptococcal proteinase or 4.49 × 10\textsuperscript{-4} M trypsin. The rates of hydrolysis were essentially linear with respect to time for at least the first 20% of the reaction. The pH-stat titration was also performed in a solution containing 20% acetonitrile.

N\textsuperscript{\textalpha}-Z-lysine Anilide—The ultraviolet spectrum of Z-lysine anilide exhibits a maximum at 257 nm at pH 5.50, and at 255 nm at pH 7.0. The cleavage product anilide absorbs maximally at 280 nm at pH 5.50, and at 282 nm at pH 7.6. For the kinetic studies, the wavelength chosen (290 nm) was that at which the difference spectrum (cleavage products versus substrate) showed maximal change in absorbance; Δε\textsubscript{290} is 77.28 at pH 5.5 and 97.47 at pH 7.6 for the cleavage of Z-Lys-AN. Determinations of kinetic constants were carried out by continuous measurement of free aniline while the reaction proceeded in a Beckman DU spectrophotometer cuvette at 25.0° (see Fig. 2A and Table V for E\textsubscript{0} and S\textsubscript{0}).

Hydrolysis of Synthetic Peptides by Streptococcal Proteinase—All reactions were performed in 0.2 M N-ethylmorpholine acetate buffer containing 0.2 M NaCl at pH 7.6 and 37°. Assays to determine K\textsubscript{m}, V\textsubscript{m}, and k\textsubscript{cat} values were performed at substrate concentration of 0.40 to 3.70 × 10\textsuperscript{-4} M and enzyme concentration of 8.75 × 10\textsuperscript{-7} M (see Table I). Substrate (50 µl) was added to 0.90 ml of buffer solution in a 1.0-ml volumetric
Fig. 2. Lineweaver-Burk plots showing the effects of pH and substrate concentration on the velocity of hydrolysis of Z-Lys-OP and Z-Lys-AN by streptococcal proteinase at 25°C. Reaction conditions are those described in Table V.

For quantitative estimation of the amount of phenylalanine released from these substrates, a 1.0-ml portion of the sample was injected into the 60-cm column of the amino acid analyzer (27) equilibrated with pH 4.25 buffer. A time interval of 12 min was allowed between successive sample injections. For this purpose, a sample injector (Chromatronic model SV-8031) equipped with a 1.0-ml sample coil was found to be useful. For this purpose, a sample injector (Chromatronic model SV-8031) equipped with a 1.0-ml sample coil was found to be useful. The extent of hydrolysis of peptides was determined by quantitative amino acid analysis. All substrates used gave analytical values within 0 ± 0.5% of theory and yielded 95 ± 5% of the carboxyl-terminal residue upon hydrolysis with one of the proteolytic enzymes: trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B, or streptococcal proteinase. Z-lysine peptide or ester, when treated with streptococcal proteinase or trypsin, released Z-lysine which was quantitatively determined on the 6-cm column of the amino acid analyzer using pH 5.28 buffer. The amino acid analyzer constant was 53.2 on an instrument for which the phenylalanine constant was 89.9.

Syntheses of Substrates

p-Nitrophenyl N°-Z-N°-Boc-lysinate—This compound was prepared as described by Schwyzer and Rittel (28) and by Bodansky and du Vigneaud (29).

C_{14}H_{13}N_{4}O_{6} (501.54)
Calculated: C 59.9, H 6.23, N 8.38
Found: C 60.3, H 6.29, N 8.00

p-Nitrophenyl N°-Z-lysinate Hydrochloride—This compound was prepared from p-nitrophenyl N°-Z-N°-Boc-lysinate by treatment with excess anhydrous HCl in dry ethyl acetate at 25°C for 60 min. The product was recrystallized from acetonitrile; m.p. 150-152°C.

C_{16}H_{14}O_{4}N_{2}Cl (386.88)
Calculated: C 54.9, H 5.52, N 9.60
Found: C 55.4, H 5.49, N 9.65

p-Nitrophenyl Z-norleucinate—p-Nitrophenol (1.39 g, 10 mmole) and N°-Z-norleucine (2.65 g, 10 mmole) were coupled in the usual manner at 4°C for 20 hours in the presence of dicyclohexylcarbodimide (2.48 g, 12 mmole), with CH_{2}Cl_{2} (50 ml) as solvent. Glacial acetic acid (100 μl) was added at the end of the reaction. Upon removal of dicyclohexylurea, the filtrate was concentrated to dryness in a vacuum, the residue was dis-
the crystalline product was collected; yield, 1.35 g (35%); m.p. 71.5-72.5°C. Chromatography (Solvent A) gave a single spot of RF 0.76 (iodine).

C_{12}H_{25}N_{2}O_{8} (386.40)
Calculated: C 62.2, H 5.89, N 7.25
Found: C 62.9, H 6.01, N 7.31

p-Nitrophenyl N-Z-γ-t-butylglutamate—In our initial experiment, we used the compound given to us by Dr. P. G. Katsoyannis, to whom we express our gratitude. Subsequently, this compound was prepared essentially as described by Klieger and Gibian (30); m.p. 47-49°C.

C_{13}H_{16}O_{5}N
Calculated: C 60.6, H 5.82, N 6.21
Found: C 60.6, H 5.82, N 6.21

Phenyl N°-Z-N°-Boc-lysinate—Dicarboxylicarbodiimide (2.48 g, 12 mmoles) was added to a solution cooled at 0°C of N°-Z-N°-Boc-lysine (3.8 g, 10 mmoles) and freshly distilled phenol (0.95 g, 10 mmoles) in CH_{2}Cl_{2} (40 ml). The mixture was kept in the dark at 4°C for 20 hours, when 100 µl of glacial acetic acid were added. The dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in three 100-ml portions of ethyl acetate, and the organic layers were washed successively in countercurrent fashion with three portions of saturated KHC\_O\_3 and two 50-ml portions of saturated NaCl. The combined organic layers were dried over MgSO\(_4\) and evaporated to give a white powder; yield, 0.45 g (31.4%). Chromatography (Solvent A) gave a single spot of RF 0.78 (iodine).

C_{13}H_{16}O_{5}N\_2 (456.8)
Calculated: C 65.7, H 5.07, N 3.07
Found: C 65.4, H 5.12, N 3.12

Phenyl N°-Z-lysinate Hydrochloride—This compound was prepared from phenyl N°-Z-N°-Boc-lysinate (1.0 g) by treatment with excess anhydrous HCl (0.9 ml) in dry ethyl acetate (2 ml) at 25°C for 60 min. The product was collected by filtration after precipitation with ether (20 ml). The white powder obtained was dissolved in 30 ml of H\_2O, and the solution was extracted once with ether (20 ml). The aqueous layer was filtered and the clear filtrate was lyophilized; yield, 0.78 g (90%); m.p. 105.5-106.5°C. Chromatography (Solvent B) gave a single spot of RF 0.43 (iodine, ninhydrin). A sample for analysis was recrystallized from acetonitrile.

C_{16}H_{20}O_{5}N\_2Cl (393.18)
Calculated: C 61.1, H 4.36, N 7.13
Found: C 61.4, H 4.22, N 7.09

Phenyl N°-Z-norleucinate—Dicarboxylicarbodiimide (2.48 g, 12 mmoles) was added to an ice-cold solution of N°-Z-norleucine (2.65 g, 10 mmoles) and freshly distilled phenol (0.95 g, 10 mmoles) in CH\_2Cl\_2 (40 ml). The mixture was kept at 4°C for 20 hours, when 100 µl of glacial acetic acid were added. The dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness, and the residue was dissolved in ethyl acetate (100 ml). The solution, extracted twice with 50 ml each of ice-cold saturated NaHCO\_3 and water, 50 ml, was dried over MgSO\(_4\) and evaporated to dryness in a vacuum. The phenyl N°-Z-norleucinate was crystallized from ethanol at -20°C; yield, 2.48 g (65.2%); m.p. 63°C. Chromatography (Solvent A) gave a single spot (iodine) of RF 0.30.

C_{13}H_{16}O_{5}N\_2 (341.40)
Calculated: C 70.4, H 6.79, N 4.10
Found: C 70.9, H 6.86, N 4.08

N°-ToS-N°-Boc-lysine Phenyl Ester—Dicarboxylicarbodiimide (0.62 g, 3 mmoles) in CH\_2Cl\_2 (10 ml) was added to a solution cooled at 0°C of N°-ToS-N°-Boc-lysine (1.0 g, 2.5 mmoles) in CH\_2Cl\_2 (50 ml). The mixture was kept in the dark at 4°C for 20 hours, when 100 µl of glacial acetic acid were added. The dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness. The residue was extracted three times with 100-ml portions of ethyl acetate, and the organic layers were washed successively in countercurrent fashion with three portions of saturated KHC\_O\_3 and two 50-ml portions of saturated NaCl. The combined organic layers were dried over MgSO\(_4\) and evaporated to give a white powder; yield, 0.45 g (31.4%). Chromatography (Solvent A) gave a single spot of RF 0.78 (iodine).

C_{13}H_{16}O_{5}N\_2S (476.6)
Calculated: N 5.88
Found: N 5.78

Phenyl N°-ToS-N°-Boc-lysinate Hydrochloride—This compound was prepared from phenyl N°-ToS-N°-Boc-lysine (0.3 g) by treatment with excess anhydrous HCl (0.9 ml) in dry ethyl acetate (1.0 ml) at 25°C for 60 min. The product was collected by filtration after precipitation with ether (50 ml). The solid was dissolved in 20 ml of H\_2O, which was extracted once with 20 ml of ether. The aqueous phase was filtered, and the clear filtrate was lyophilized to give a white powder; yield, 0.18 g (69%). Chromatography (Solvent B) gave a single spot of RF 0.41 (iodine, ninhydrin).

C_{13}H_{16}O_{5}N\_2S\_Cl (412.98)
Calculated: C 55.3, H 6.10, N 6.78
Found: C 55.1, H 6.21, N 6.73

Phenyl N°-Z-glutamate—This compound was prepared from phenyl N°-Z-glutamic acid anhydride (7.83 g, 30 mmoles), phenol (3.38 g, 36 mmoles), and 6.27 ml (36 mmoles) of dicyclohexylamine in absolute ether (20 ml), essentially according to the method of Klieger and Gibian (30); yield, 5.9 g (61%). Chromatography (Solvent C) gave a single spot (iodine) of RF 0.57.

C_{15}H_{18}O_{6}N\_2 (357.7)
Calculated: C 63.8, H 5.55, N 3.91
Found: C 63.8, H 5.54, N 3.85

N°-Z-N°-Boc-lysine Anilide—Dicarboxylicarbodiimide (2.46 g, 10 mmoles) was added to a solution of phenyl N°-Z-norleucine (3.8 g, 10 mmoles), and freshly distilled phenol (0.95 g, 10 mmoles) in CH\_2Cl\_2 (50 ml). The mixture was kept in the dark at 4°C for 20 hours, when 100 µl of glacial acetic acid were added. The dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness, and the residue was dissolved in ethyl acetate (100 ml). The solution, extracted twice with 50 ml each of ice-cold saturated NaHCO\_3 and water, 50 ml, was dried over MgSO\(_4\) and evaporated to dryness in a vacuum. The phenyl N°-Z-norleucinate was crystallized from ethanol at -20°C; yield, 2.48 g (65.2%); m.p. 63°C. Chromatography (Solvent A) gave a single spot (iodine) of RF 0.30.
filtration, and the filtrate was evaporated to dryness. The residue (88\%); m.p. 94°. Chromatography (Solvent B) gave a single spot of Rp 0.68 (iodine, ninhydrin).

A) gave a single spot of Rp 0.59 (iodine).

Aqueous phase was filtered, and the filtrate was evaporated to dryness. The residue was extracted with three 100-ml portions of ethyl acetate, and the organic phases were washed with three 50-ml portions of 20\% citric acid, two 50-ml portions of H2O, three 50-ml portions of 5\% KHCO3, and two 50-ml portions of H2O. The combined ethyl acetate layers were dried over MgSO4 and concentrated in a vacuum. The compound was crystallized from ethyl acetate; yield, 3.66 g (80\%). Chromatography (Solvent A) gave a single spot of Rf 0.71 (iodine).

\[ C_{10}H_{18}O_8N_2 (455.6) \]
Calculated: C 65.9, H 7.34, N 9.24  
Found: C 65.6, H 7.47, N 9.18

\( N^\circ-Z\)-lysine Amidate Hydrochloride—This compound was prepared from \( N^\circ-Z\)-N'-Boc-lysine anilide (0.454 g, 1 mmole), by treatment with an excess of dry HCl (0.97 \text{ n}) in ethyl acetate (3.0 ml) for 2 hours at room temperature. After precipitation with ether (50 ml), the product was collected by filtration. The solid was dissolved in 20 ml of H2O, which was extracted once with 20 ml of ether. The aqueous phase was filtered, and the clear filtrate was lyophilized to give a white powder; yield, 424 mg (88\%); m.p. 94°. Chromatography (Solvent B) gave a single spot of Rf 0.68 (iodine, ninhydrin).

\[ C_{13}H_{21}O_3N_2 (391.9) \]
Calculated: C 61.3, H 4.63, N 10.7  
Found: C 61.4, H 4.78, N 10.2

Ethyl \( N^\circ-Z\)-N'-Boc-lysylphenylalaninate—\( N^\circ-Z\)-N'-Boc-lysine (1.9 g, 5 mmoles) and phenylalanine ethyl ester, derived from 1.15 g (5 mmoles) of the hydrochloride, were coupled in the usual manner in the presence of diethylcarbodiimide (1.24 g, 6 mmoles), with CH2Cl2 (20 ml) as solvent. The mixture was stirred at room temperature for 20 hours, when 100 \% of glacial acetic acid were added. The dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness. The residue was extracted with three 2-ml portions of ethyl acetate, and the organic phases were washed with three 50-ml portions of 20\% citric acid, and two 50-ml portions of HzO. The combined ethyl acetate layers were dried over MgSO4 and concentrated in a vacuum. The compound was crystallized from aqueous ethanol (85\%, v/v); yield, 3.2 g (72\%); m.p. 113-114°. Chromatography (Solvent A) gave a single spot of Rf 0.72.

\[ C_{12}H_{18}O_2N_2 (440.5) \]
Calculated: C 68.7, H 7.39, N 6.21  
Found: C 68.1, H 7.39, N 6.21

\( N^\circ-Z\)-norleucine Phenylalaninate—\( N^\circ-Z\)-norleucine, prepared from 4.46 g (10 mmoles) of the dicyclohexylamine salt of \( N^\circ-Z\)-norleucine, and ethyl phenylalaninate, prepared from 2.3 g (10 mmoles) of the hydrochloride, were coupled in the usual manner in the presence of diethylcarbodiimide (2.48 g, 12 mmoles) with CH2Cl2 (40 ml) as solvent. The mixture was kept at 0° for 30 min and at 25° for 20 hours, when 100 \% of glacial acetic acid were added and the dicyclohexylurea was removed by filtration. The filtrate was concentrated to dryness, and the residue was extracted with three 100-ml portions of ethyl acetate. The organic phases were washed with three 50-ml portions of 0.1 \text{n} HCl, two 50-ml portions of H2O, three 50-ml portions of 5\% NaHCO3, and two 50-ml portions of H2O. The combined organic layers were dried over MgSO4 and evaporated to near dryness in a vacuum. The compound was crystallized from aqueous ethanol (85\%, v/v); yield, 3.2 g (72\%); m.p. 113-114°. Chromatography (Solvent A) gave a single spot of Rf 0.72.

\[ C_{12}H_{18}O_2N_2 (440.5) \]
Calculated: C 68.7, H 7.31, N 6.35  
Found: C 68.1, H 7.39, N 6.21

\( N^\circ-Z\)-norleucynphenylalanine—To a solution of ethyl \( N^\circ-Z\)-norleucynphenylalaninate (1.1 g, 2.5 mmoles) in 10 ml of ethanol was added 1 \text{n} NaOH (2.7 ml). After 2 hours at 0°, ethanol was removed in a vacuum at 20°. The residue, dissolved in 90 ml of H2O, was extracted once with 30 ml of ether. The aqueous layer was acidified with 3.0 ml of 3 \text{n} HCl, and the precipitate that appeared was extracted into 100 ml of ether. The organic layer was dried over MgSO4 and evaporated to a small volume, to which petroleum ether was added. After 4 hours at 0°, crystals appeared and were collected. The compound was recrystallized from ether-petroleum ether; yield, 0.72 g (72\%); m.p. 140-141°. Chromatography (Solvent C) gave a single spot (iodine) of Rf 0.84.
Hydrolysis of synthetic peptide substrates by streptococcal proteinase

Experimental conditions were: pH 7.5; 0.2 m N-ethylmorpholine acetate buffer containing 0.2 m NaCl; 37; 1 X 10^-4 m EDTA, 5 X 10^-4 M dithioerythritol; enzyme concentration, 8.75 X 10^-6 M; S/E = 0 to 30 ml X 10^-4 M. Values for K_m and V_max were calculated with the CDC 6600 computer according to the method of Hanson, Ling, and Havier (26) as modified by K. Thompson. Na-Z-N'-Boc-lysine p-Nitroanilide-Dicyclohexylcarbodiimide layer was acidified with 2.0 ml of 3 normal HCl, and the organic phases were washed with three 50-ml portions of 5% NaHCO_3, one 50-ml portion of H_2O, three 50-ml portions of 0.1 normal HCl, and two 50-ml portions of H_2O. The combined ethyl acetate layers were dried over MgSO_4 and concentrated to dryness, the residue was extracted with ethyl acetate (3.0 ml) for 2 hours at room temperature. The oily product was dried in a vacuum over P_2O_5; yield, 2.8 g (65%). Chromatography (Solvent A) gave a single spot of RF 0.76 (iodine).

C_8H_8O_2N_4 (402.48)
Calculated: C 68.8, H 7.30, N 6.51
Found: C 68.8, H 7.30, N 6.51

N-Z-glutamylphenylalanine—This compound was prepared by saponification of N-Z-Glu-Phe-OEt, which was synthesized as described by Fruton and Bergmann (31). The product was re-crystallized from ethanol-water; m.p. 161-162°.

C_9H_9O_4N_2 (266.25)
Calculated: C 68.6, H 7.02, N 6.96
Found: C 68.6, H 7.02, N 6.96

Ethyl N-Tos-norleucinephenylalaninate—N-Tos-norleucine, 1.43 g (5 mmoles), and phenylalanine ethyl ester, 0.97 g (5 mmoles), were coupled in the usual manner in the presence of dicyclohexylcarbodiimide, 1.24 g (6 mmoles), with CH_2Cl_2 (20 ml) as solvent. The mixture was kept at 0-4° for 30 min and at 25° for 20 hours, when 100 ml of glacial acetic acid were added and the dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness, the residue was extracted with 100-ml portions of ethyl acetate, and the organic phases were washed with three 50-ml portions of 5% KHC_2O_4, one 50-ml portion of H_2O, three 50-ml portions of ice-cold 0.1 normal HCl, and two 50-ml portions of H_2O. The combined ethyl acetate layers were dried over MgSO_4 and concentrated in a vacuum. The yellowish residue was further titrated with 20% ethanol to remove residual amounts of p-nitroaniline. The oily product was dried in a vacuum over P_2O_5; yield, 2.8 g (65%). Chromatography (Solvent A) gave a single spot of RF 0.76 (iodine).

C_7H_7O_2N_4 (428.4)
Calculated: C 61.7, H 5.6, N 6.5
Found: C 62.1, H 5.82, N 6.63

RESULTS AND DISCUSSION

Hydrolyses of Dipptides—Examination of the kinetic data in Table 1 indicates that for the series of peptide substrates listed, where Y is phenylalanine and A is benzoyloxy carbonyl, the relative specificity as expressed by k_cat/K_m of streptococcal proteinase for the X group is NE-Boc-lysine > norleucine > glutamic acid > lysine. However, these variations are small, especially for the series where X is norleucine, glutamic acid, and lysine, considering the drastic change in the ionic environment of X that accompanies the change from a neutral group to a cationic or anionic group. Evidently, with this series of substrates, where the length of the side chain of the amino acid is similar, the enzyme recognizes only poorly the difference between a polar anionic group and a hydrophobic group. The data indicate that variation in the relative specificity of these substrates is due to K_m and not k_cat.

The most susceptible peptide, Z-Nle-Phe,
is the one that binds strongest, since it has the smallest value of $K_m$ and the least susceptible peptide, Z-Lys-Phe, is the one that binds least, with the highest $K_m$ value. It was of considerable interest to find a substantial increase in susceptibility when the $\epsilon$-amino group of lysine in $X$ was substituted with a $\beta$-butyloxycarbonyl group, which is a bulky group, as in N$^\alpha$-Z-N$^\beta$-Boe-Lys-Phe. The data in Table I indicate that the increase in susceptibility is due to an increase in $k_{cat}$ and not to a decrease in $K_m$. The compound has about the same $K_m$ value as Z-Nle-Phe, but it has a $k_{cat}$ value 3.5 times as large, which suggests that, for the two substrates with hydrophobic neutral side chains, the observed difference in the relative rate of hydrolysis is due very likely to the enhanced rate of decylation of the bulky hydrophobic residue. An acyl-enzyme intermediate of the type

\[
\begin{array}{c}
\text{O} \\
\text{N$^\alpha$-Z-N$^\beta$-Boe-Lys-C-S-enzyme}
\end{array}
\]

might undergo hydrolysis faster than

\[
\begin{array}{c}
\text{O} \\
\text{Z-Lys-C-S-enzyme}
\end{array}
\]

*Hydrolysis of Tos-Lys-Phe and Tos-Nle-Phe.*—The work of Gerwin, Stein, and Moore (22) showed that the nature of the NH$_2$-blocking group has a decisive effect upon the rate of hydrolysis of the dipeptide substrates. They found that such a group is lacking altogether, or when it is small, hydrolysis is impaired. Further evidence of the exacting specificity of streptococcal proteinase with respect to $A$ is provided by our present observation that Tos-Nle-Phe and Tos-Lys-Phe were completely resistant to proteolytic cleavage. Moreover, Tos-Nle-Phe was found to be a strongly competitive inhibitor of Z-Nle-Phe cleavage. It has a $K_i$ value (4.2 mM) quite similar to the $K_m$ value for Z-Nle-Phe. The fact that a relatively small change in the nature of the $A$ group (tosyl in place of benzyloxycarbonyl) can produce such a large effect on the cleavage of the X-Y bond is interesting, especially when one considers the relatively small effect on the catalytic activity of the enzyme that results from a large change in the nature of the X group (neutral to cationic or anionic group). In this instance it is clear from the inhibition studies that the binding step is not the cause of the decreased rate of hydrolysis of tosyl peptide by streptococcal proteinase, since the $K_i$ values were quite similar to the $K_m$ values of the corresponding benzyloxycarbonyl peptides.

On the other hand, the acylation by the tosyl peptide proceeded too slowly to be detected. Two lines of evidence show that this step is the major cause of the lack of susceptibility of the tosyl peptide to the proteinase. First, if decylation had not occurred and acylation were appreciable, the acyl-enzyme intermediate would build up, and yet no acyl-enzyme intermediate could be detected. Second, a rate-determining decaying step should be accelerated by the addition of nucleophiles such as hydroxylamine, but no such acceleration was found. Therefore, the inertness of the tosyl peptide must be caused by the failure of the acylation step. In this case, it is tempting to speculate that the enzyme failed to induce the substrate to assume the proper conformation for its hydrolytic cleavage, rather than that the substrate failed to induce the enzyme to assume a productive structure to function as an enzyme. Since a tosyl derivative is more rigid in structure than a benzyloxycarbonyl derivative, it is less subject to conformational change when in touch with the enzyme.

*Esterase Activity of Streptococcal Proteinase: pH Activity Profile.*—The effect of pH on the streptococcal proteinase-catalyzed hydrolysis of Z-Lys-ONp was examined over a range between pH 4 and 6.0. The kinetic constants $k_{cat}$, $K_m$, and $k_{cat}/K_m$ for the hydrolysis of this substrate at various pH values are given in Table II. Examination of the data reveals that the $k_{cat}$ values are fairly constant above pH 5.0 and that the changes in the $K_m$ values for this substrate are not appreciable over the pH range studied.

The pH dependence of $k_{cat}/K_m$ for this substrate is shown in Fig. 3. Inspection reveals that the ratio $k_{cat}/K_m$ depends on an ionizable group with pK of 4.8. This pK value represents the ionization of the enzyme and thus should be identical for all of the substrates, provided that there is no pH-dependent interaction of the charged substrate with noncatalytic groups on the enzyme which would affect this value (32). Such an effect, if present at all, is small above pH 4.5 in this case, since both cationic and anionic substrates exhibited similar pH dependence of $k_{cat}/K_m$.

From the values of $K_m$ and $V_m$, for the streptococcal proteinase-catalyzed hydrolysis of Z-Phe-Tyr over the pH range 5 to 10, Gerwin et al. (22) have inferred a pK of 6.4 for a catalytically important group in the free enzyme. This group was suggested to be an unprotonated histidine residue at the active site of the enzyme. Direct chemical evidence for the involvement of a histidine residue in the active site of the enzyme was demonstrated by Liu (19).

The apparent discrepancy of 1.6 pK units (6.4 versus 4.8) between the results of Gerwin et al. (22) and the current studies on

\begin{table}
\centering
\caption{Kinetic constants of streptococcal proteinase-catalyzed hydrolyses of Z-lysine p-nitrophenyl ester}
\begin{tabular}{|c|c|c|c|}
\hline
pH & $K_m$ & $k_{cat}$ & $k_{cat}/K_m$ \\
& (mM) & (sec$^{-1}$) & (sec$^{-1}$ M$^{-1}$) \\
\hline
4.27 & 0.222 ± 0.028 & 29.7 ± 0.21 & 134 \\
4.40 & 0.494 ± 0.073 & 89.8 ± 0.86 & 182 \\
4.68 & 0.188 ± 0.043 & 55.0 ± 0.68 & 292 \\
4.96 & 0.250 ± 0.045 & 83.0 ± 0.95 & 359 \\
5.09 & 0.192 ± 0.036 & 169.7 ± 1.20 & 441 \\
5.29 & 0.247 ± 0.083 & 115.0 ± 2.2 & 465 \\
5.48 & 0.314 ± 0.031 & 148.0 ± 0.80 & 471 \\
5.69 & 0.248 ± 0.030 & 133.0 ± 0.92 & 531 \\
5.95 & 0.265 ± 0.078 & 132.0 ± 0.97 & 498 \\
6.13 & 0.233 ± 0.085 & 127.0 ± 1.29 & 502 \\
6.35 & 0.262 ± 0.075 & 142.0 ± 0.80 & 531 \\
6.55 & 0.277 ± 0.038 & 135.0 ± 1.35 & 487 \\
\hline
\end{tabular}
\end{table}


\footnote{T.-Y. Liu, unpublished observations.}
the pK of a catalytic entity in the streptococcal proteinase-catalyzed hydrolysis of peptide and ester substrates emphasizes the necessity of having independent chemical evidence for assigning a particular functional group at the active site of an enzyme. It should be noted that the conditions used by Gerwin et al. differ from ours in that they used a peptide substrate at 37°C, whereas we used an ester substrate at 25°C. On the basis of the kinetic data alone, one would hesitate to assign to an imidazole group a pK of 4.8. However, on the basis of the chemical evidence cited and the discussion given below, an imidazole group with pK of 4.8 may be present in the active site of streptococcal proteinase for the hydrolysis of Z-Lys-ONp. It is not possible to rule out the alternative that this group might actually be a carboxyl group. On the other hand, peptide and ester substrates of the type mentioned may not combine with the enzyme in the same manner, as is shown by the different order of susceptibility of various peptide substrates and ester substrates by streptococcal proteinase (see below).

In a recent paper, Hollands and Fruton (33) stress some of the anomalies connected with the action of pepsin on synthetic substrates. They point out that the pH optimum can vary widely from about pH 2 to around pH 4.5, depending upon the structure and the charge of the substrate. For two peptide substrates hydrolyzed by pepsin, Z-His-Phe-Phe-OEt and Ac-Phe-Tyr-OEt, a difference in the \( k_{cat}/K_m \) versus pH plots was observed. The possibility was suggested that, during the course of their hydrolysis by pepsin, these two substrates do not combine with the enzyme in the same manner.

### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>Initial rate ( V )</th>
<th>moles/sec/mole of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Lys-OP</td>
<td>5.5</td>
<td>2.63</td>
<td></td>
</tr>
<tr>
<td>Z-Nle-OP</td>
<td>5.5</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td>Z-Glu-OP</td>
<td>5.5</td>
<td>0.965</td>
<td></td>
</tr>
<tr>
<td>Z-Lys-ONp</td>
<td>5.5</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Z-Nle-ONp</td>
<td>7.6</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Z-Glu-ONp</td>
<td>5.5</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Z-Lys-ONp</td>
<td>7.6</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Z-Nle-ONp</td>
<td>5.5</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>Z-Glu-ONp</td>
<td>5.5</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td>Z-Lys-ONp</td>
<td>5.5</td>
<td>1.40</td>
<td></td>
</tr>
<tr>
<td>Z-Nle-ONp</td>
<td>5.5</td>
<td>0.186</td>
<td></td>
</tr>
<tr>
<td>Z-Glu-ONp</td>
<td>5.5</td>
<td>0.346</td>
<td></td>
</tr>
<tr>
<td>Z-Lys-ONp</td>
<td>5.5</td>
<td>0.226</td>
<td></td>
</tr>
</tbody>
</table>

* The syntheses of Tos-Nle-OP, Tos-Nle-Phe, and their use as inhibitors will be described in detail elsewhere (N. Nomura and T.-Y. Liu, unpublished results).

** These three compounds were gifts from Dr. P. G. Katsoyannis.

Fig. 3. pH rate profiles for the streptococcal proteinase-catalyzed hydrolysis of Z-Lys-ONp at 25°C. Reaction conditions are described in Table II.

The results in Table III show that streptococcal proteinase catalyzes the hydrolyses of phenyl ester substrates of the type \( A-X-Y \) where \( Y \) is either phenyl or p-nitrophenyl, under reaction conditions which gave initial rates directly proportional to the concentration of both enzyme and substrate. All experiments were performed in the presence of 20% acetonitrile, since the neutral substrates were relatively insoluble.

The data in Table III indicate that streptococcal proteinase catalyzes the hydrolyses of phenyl ester substrates of the type \( A-X-Y \) where \( Y \) is phenyl, A is benzoyloxycarbonyl, and X is lysine, norleucine, or glutamic acid, at essentially identical rates at pH 5.5 and 7.6 (see Fig. 2b). These results are in agreement with those of the pH activity study with Z-Lys-ONp.

The results in Table III show that streptococcal proteinase exhibits a preference for X, the acyl component of the sensitive bond, in the decreasing order lysine > norleucine > glutamic acid. This sequence is not the same as for peptide bond cleavage, where the most susceptible peptide was the one in which X is norleucine, and the least susceptible one was where X is lysine. Since, for peptide bond cleavage, acylation is the rate-limiting step (7), it might be possible that a positive charge on X enhances the rate of deacylation, and a hydrophobic side chain favors the formation of acyl enzyme intermediates. However, the differences in the rates of hydrolysis of these substrates are relatively small compared with the drastic decrease in the rate of hydrolysis of the ester substrates when A is tosyl, as in Tos-Lys-OP and Tos-Nle-OP. These two compounds were found to be resistant to hydrolysis by streptococcal proteinase under the conditions specified in Table III. Tos-Lys-OP was found to be a competitive inhibitor of enzymic hydrolysis of Z-Lys-OP with a \( K_i \) value of 3.1 \( \times 10^{-4} \) M (see Fig. 4).

When X is glycine, as in the case of p-nitrophenyl Z-glycinate, the susceptibility to enzymic hydrolysis decreases dramatically. This is in agreement with the finding of Mycek, Elliott, and Fruton (34), who observed that glycine-containing dipeptides are resistant to streptococcal proteinase. Removal of A from X and substitution of e-NH of lysine with benzoyloxycarbonyl results in an ester substrate \( N^+Z-Lys-ONp \), which was found to be resistant to hydrolysis by streptococcal proteinase.
substrate

Effect of Dielectric Constant—Lowering of the dielectric constant has a profound effect on the enzymic cleavage of ester substrates by streptococcal proteinase. The effect of acetonitrile on the esterase activities is shown in Table IV. The rates of esterase activities are being determined.

Inhibition Studies—The spectrophotometric method for following streptococcal proteinase kinetics, with Z-Lys-OP as the substrate, provides a convenient procedure for the study of the inhibitor effect of test compounds. As will be seen from Fig. 4, Tos-Lys-ONp behaved as a competitive inhibitor of streptococcal proteinase in this assay, and a value of $K_i$ of 3.1 mM (pH 5.5, 25°C) was determined. Similar linear plots have been obtained with Tos-Lys-Phe ($K_i$ = 4.2 mM) as an inhibitor of the hydrolysis of substrate Z-Lys-Phe. When either Z-Lys-AN or Tos-Lys-AN was used as inhibitor for the hydrolysis of Z-Lys-OP, an inhibition other than the competitive type was observed. $K_i$ values for this system were not determined. These results would suggest that the ester and anilide substrates both use the same catalytic site on streptococcal proteinase for their hydrolysis but perhaps by different mechanisms.

**Effect of Dielectric Constant**—Lowering of the dielectric constant has a profound effect on the enzymic cleavage of ester substrates by streptococcal proteinase. The effect of acetonitrile on the esterase activities is shown in Table IV. The rates of substrate hydrolyses in this case were determined with a pH-stat as described under "Experimental Procedure." Enzymic cleavage of the substrates was performed in the presence and absence of acetonitrile. Comparison of the rates of hydrolysis of Z-Lys-ONp showed that the hydrolysis of this substrate was retarded almost 6-fold when 20% acetonitrile was present in the assay mixture. Under identical conditions, trypsin-catalyzed hydrolysis of this substrate was reduced only by about 2-fold. With Z-Nle-ONp, a neutral ester, the presence of up to 20% acetonitrile seems to have no effect. (It should be noted, however, that Z-Nle-ONp has a low solubility in water and, in the absence of acetonitrile, the substrate appeared as a fine suspension in the assay medium.) The effects of dielectric constant on the kinetic parameters $K_m$, $k_{cat}$, etc., are being determined.

![Graphical determination of $K_i$ for Tos-Lys-OP. The substrate was Z-Lys-OP. Enzyme concentration was 2.85 × 10^{-7} M.](image)

**TABLE IV**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Solvent</th>
<th>Substrate</th>
<th>Initial $V^a$</th>
<th>Activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcal proteinase</td>
<td>0.2 M KCl</td>
<td>Z-Lys-ONp</td>
<td>38</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20% acetonitrile in 0.2 M KCl</td>
<td>Z-Lys-ONp</td>
<td>0.37</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>0.2 M KCl</td>
<td>Z-Nle-ONp</td>
<td>2.91</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20% acetonitrile in 0.2 M KCl</td>
<td>Z-Nle-ONp</td>
<td>2.91</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.2 M KCl</td>
<td>Z-Lys-ONp</td>
<td>2.18</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20% acetonitrile in 0.2 M KCl</td>
<td>Z-Lys-ONp</td>
<td>1.31</td>
<td>60.1</td>
</tr>
</tbody>
</table>

$^a$Expressed as moles of substrate hydrolyzed per sec per liter per mole of enzyme.

$^b$The $V$ value of 0.2 M KCl as 100% was used to calculate the percentage of $V$ in 20% acetonitrile-0.2 M KCl.

**TABLE V**

**Comparison of esterase and peptidase activities of streptococcal proteinase**

Experimental conditions were: ionic strength, 0.200; 25°C; 0.2 M sodium acetate buffer for pH 7.6; 1 × 10^{-3} M EDTA.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>$K_m$</th>
<th>$k_{cat}</th>
<th>k_{cat}/K_m</th>
<th>Relative reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Lys-OP$^a$</td>
<td>5.5</td>
<td>0.826</td>
<td>80.8</td>
<td>97.6</td>
<td>1</td>
</tr>
<tr>
<td>Z-Lys-ONp$^b$</td>
<td>7.6</td>
<td>0.483</td>
<td>45.8</td>
<td>93.2</td>
<td>0.98</td>
</tr>
<tr>
<td>Z-Lys-ONp$^b$</td>
<td>5.5</td>
<td>0.314</td>
<td>148</td>
<td>472</td>
<td>4.83</td>
</tr>
<tr>
<td>Z-Lys-AN$^c$</td>
<td>5.5</td>
<td>7.19</td>
<td>1.62 × 10^{-1}</td>
<td>2.25 × 10^{-4}</td>
<td>2.31 × 10^{-4}</td>
</tr>
<tr>
<td>7.6</td>
<td>4.53</td>
<td>1.03 × 10^{-1}</td>
<td>2.20 × 10^{-4}</td>
<td>2.32 × 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>Z-Lys-NPA$^d$</td>
<td>5.5</td>
<td>4.28</td>
<td>2.67 × 10^{-1}</td>
<td>6.23 × 10^{-4}</td>
<td>6.37 × 10^{-4}</td>
</tr>
<tr>
<td>7.6</td>
<td>3.35</td>
<td>1.91 × 10^{-1}</td>
<td>6.88 × 10^{-4}</td>
<td>6.04 × 10^{-4}</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Enzyme concentration, 3.75 × 10^{-3} M, $S_i$ = 1.07 to 8.37 × 10^{-4} M.

$^b$Enzyme concentration, 1.25 × 10^{-7} M, $S_i$ = 2.14 × 10^{-5} to 1.33 × 10^{-4} M.

$^c$Enzyme concentration, 1.25 × 10^{-6} M, $S_i$ = 2.0 × 10^{-4} to 2 × 10^{-3} M.

$^d$Enzyme concentration, 8.9 × 10^{-6} M, $S_i$ = 2.0 × 10^{-4} to 2 × 10^{-3} M.

**Phenyl versus p-Nitrophenyl Ester**—Table V lists some kinetic constants for substrates of the type A-X-Y that differ in Y. The change of Y from phenyl to p-nitrophenyl produces relatively little change in $K_m$, but $k_{cat}/K_m$ increases 5-fold. Since $K_m$ is related to the enzyme-substrate complex dissociation constant, it can be concluded that the binding of the substrate A-X-Y to the enzyme is not affected appreciably by the presence or absence of the nitro group on Y. The fact that a p-nitrophenyl ester is hydrolyzed nearly 5 times as fast as the phenyl ester can be attributed to the powerful electron-withdrawing property of the nitro group, which enhances the rate of acylation of these substrates by the enzyme. For ester substrates of this type, the rate-limiting step is presumably the deacylation step (7).
FIG. 5. Mechanism of the streptococcal proteinase-catalyzed hydrolysis of peptides or anilide substrates. Adopted from Wang (35).

Anilide versus p-Nitroanilide—A more striking feature of the data summarized in Table V is a change in $k_{cat}/K_m$ that accompanies the change of $Y$ from anilide to p-nitroanilide. Here again, the lack of large variation in the $K_m$ values between these two substrates would suggest that both substrates have similar enzyme-substrate dissociation constants. However, the fact that $k_{cat}/K_m$ for anilide is more than 36 times as large as for p-nitroanilide is somewhat puzzling. For, if acylation is the rate-limiting step of anilide and the rate of acylation is enhanced by an electron-withdrawing group in $Y$, a nitroanilide substrate should be more susceptible than the anilide. This was indeed found to be the case when these two substrates were subjected to trypsin-catalyzed hydrolysis; the $k_{cat}/K_m$ value for p-nitroanilide was larger than for anilide.2 The reverse effect of a nitro group found for streptococcal proteinase is a unique feature of this enzyme. A theory that may explain this finding is that proposed by Wang (35), who postulated a protonation of the substrates in chymotrypsin-catalyzed hydrolysis. Thus, by measuring the rate constants, $K_m$ in Equation 2, for a series of substituted anilides in chymotrypsin-catalyzed hydrolysis, Inagami, York, and Patchornik (36) concluded that a proton transfer was involved in the acylation step.

$$E + S \xrightarrow{K_1} ES \xrightarrow{K_2} ES' + P_1 \xrightarrow{K_3} E + P_2 \quad (2)$$

Based on the observation that $K_2$ is relatively constant above pH 7, Parker and Wang (37) proposed a simple theory of chymotrypsin-catalyzed hydrolysis of peptide, amide, and anilide substrates. According to this theory, a pretransition state protonation of the substrate followed by the usual nucleophilic attack of the protonated substrate by the alkoxy group of serine is postulated.

Assuming that streptococcal proteinase catalyzes the hydrolysis of peptide, amide, and anilide substrate along the similar path shown for chymotrypsin in Fig. 5, let us define the first order rate constants $K_e$ and $K_a$ by the following equivalent reaction scheme

$$I \rightleftharpoons II \rightleftharpoons III \quad K_e \leq K_a$$

We obtained the following rate equation according to the reasoning given by Wang (35).

$$Rate = K_e [II] + K_a [III]$$

$$= \left[ K_e \frac{K_{IMH}^+}{K_{sub}^-} + K_a \frac{K_{cysH}^+}{K_{sub}^-} \right] [I] \quad (3)$$

where $K_{IMH}^+$, $K_{sub}^-$, and $K_{cysH}^+$ are the acid dissociation constants of cysteine and imidazole in streptococcal proteinase and the protonated substrate, respectively.

Parker and Wang (37) showed that the over-all acylation rate constant for chymotrypsin is given by

$$K_a = \frac{K_{ser}}{K_{sub}^-} \cdot K_a \quad (4)$$

where $K_{ser}$ and $K_{sub}^-$ represent the acid dissociation constants of serine-195 and the protonated substrate, respectively. $K_a$ is the first order rate constant characteristic of the attack on the protonated substrate by the adjacent alkoxy group. For the streptococcal proteinase catalyzed hydrolysis

$$K_a' = \frac{K_{ser}}{K_{sub}^-} \cdot K_a \quad (5)$$

At pH 5.5 to 7.6, the concentration of Cys$^-\text{H}$ in streptococcal proteinase is higher than that of Ser-O$^-\text{H}$ in chymotrypsin by a factor of $10^{-3}/10^{-11}$ or $\sim 10^4$, therefore

$$K_a' \approx K_a \times 10^4$$

This implies that for “sulfhydryl” enzymes such as streptococcal proteinase, the contribution of $K_{IMH}^+/K_{sub}^-$ to the over-all rate of acylation according to Equation 3 is significantly larger than the contribution of $K_{ser}/K_{sub}^-$ to the “serine” enzyme such as chymotrypsin or trypsin. Thus, for streptococcal proteinase-catalyzed hydrolysis of peptide, amide, or anilide, the pretransition state protonated path $I \rightleftharpoons III \rightarrow V$ will be relatively more important than the concerted and post-transition state protonation path $I \rightleftharpoons II \rightarrow V$. The finding that Z-Lys-NpA is hydrolyzed 36-fold slower than Z-Lys-AN by streptococcal proteinase would suggest that both substrates are hydrolyzed by the enzyme by the same mechanism: the pretransition state protonated path $I \rightleftharpoons III \rightarrow V$.

Since the nitrogen in the susceptible C$^-=N$ bond of Z-Lys-NpA is a much weaker base than the nitrogen in the susceptible C$^-=N$ bond of Z-Lys-AN, protonation of the p-nitroanilide substrate is much more difficult than the protonation of the anilide substrate, hence the decrease in the rate of hydrolysis on p-nitroanilide substrate by the enzyme.

The finding that trypsin hydrolyzes Z-Lys-NpA faster than Z-Lys-AN suggests that the two substrates are hydrolyzed by different mechanisms. For the hydrolysis of Z-Lys-NpA catalyzed by trypsin, the concerted post-transition state path $I \rightleftharpoons II \rightarrow V$ dominates. In this mechanism, the effect of the electron-
Comparison of specific ester and amide substrates hydrolyzed by some proteolytic enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_{cat}$</th>
<th>$10^6$</th>
<th>$k_{cat}/K_m$</th>
<th>$k_{cat}/K_m$</th>
<th>Proposed catalytic entity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcal proteinase</td>
<td>Z-Lys-OEt&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.8</td>
<td>283</td>
<td>95.2</td>
<td>4.23 x 10^4</td>
<td>His&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Z-Lys-AN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.162</td>
<td></td>
<td>0.0225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papain</td>
<td>Bz-Arg-OEt&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.7</td>
<td>1.85</td>
<td>10.8</td>
<td>4.12</td>
<td>—COOH&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Bz-Arg-A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.48</td>
<td>1.15</td>
<td>2.62</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Bz-Arg-OEt&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5</td>
<td></td>
<td>0.1</td>
<td>2.18</td>
<td>—COOH&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Bz-Arg-A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2</td>
<td></td>
<td>0.046</td>
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<tr>
<td>Bromelin</td>
<td>Bz-Arg-OEt&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.50</td>
<td>140</td>
<td>2.94 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>1.02</td>
<td></td>
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<tr>
<td></td>
<td>Bz-Arg-A&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.0035</td>
<td></td>
<td>2.92 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td></td>
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</tr>
<tr>
<td>Pepsin</td>
<td>Z-His-Phe(NO&lt;sub&gt;2&lt;/sub&gt;)-Pla-OMe&lt;sup&gt;g&lt;/sup&gt;</td>
<td>77 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.66</td>
<td>19.2</td>
<td>3.0</td>
<td>—COOH&lt;sup&gt;h&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Z-His-Phe(NO&lt;sub&gt;2&lt;/sub&gt;)-Phe-OMe&lt;sup&gt;g&lt;/sup&gt;</td>
<td>29 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
<td>6.3</td>
<td></td>
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<tr>
<td>Trypsin</td>
<td>Bz-Arg-OEt&lt;sup&gt;i&lt;/sup&gt;</td>
<td>8.4</td>
<td>195</td>
<td>8.4 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>6.05 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>His&lt;sup&gt;i&lt;/sup&gt;, m</td>
</tr>
<tr>
<td></td>
<td>Bz-Arg-A&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.043</td>
<td></td>
<td>13.9</td>
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<td>Z-Lys OP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82</td>
<td>280</td>
<td>1.13 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>9.42 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>His&lt;sup&gt;m&lt;/sup&gt;, m</td>
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<tr>
<td></td>
<td>Z-Lys-AN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.293</td>
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<td>0.12</td>
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<td>Chymotrypsin</td>
<td>Ac-Phe-OMe&lt;sup&gt;e&lt;/sup&gt;</td>
<td>52.5</td>
<td>1.62 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.53 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>2.75 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>His&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Ac-Phe-A&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.046</td>
<td></td>
<td>1.62 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.53 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Subtilisin</td>
<td>Ac-Tyr-OEt&lt;sup&gt;p&lt;/sup&gt;</td>
<td>&gt;1 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td>4.53 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ac-Tyr-A&lt;sup&gt;p&lt;/sup&gt;</td>
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<tr>
<td>Carboxypeptidase A</td>
<td>Ci-Phe&lt;sup&gt;v&lt;/sup&gt;</td>
<td>9.45 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Ci-Phe&lt;sup&gt;v&lt;/sup&gt;</td>
<td></td>
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</tbody>
</table>

† pH 5.5, 25° (see text).
‡ Liu (19), Gerwin et al. (22).
§ pH 5.2, 25°, Whittaker and Bender (7).
¶ Smith and Parker (13), Stockell and Smith (14).
* pH 6.0, 25°, Hammond and Gutfrend (8).
†† pH 6.0, 25°, Inagami and Murachi (38). $K_a$ was used instead of $k_{cat}$.
†‡ pH 6.0, 25°, Inagami and Murachi (38). $K_a$ was used instead of $k_{cat}$.
†§ Pla, β-phenyl-L-lactyl; OMe, methyl ester; pH 4.0, 37°, Innere and Fruton (15).
†¶ Rajagopalan, Stein, and Moore (17).
†‖ Delpierre and Fruton (16).
†¥ pH 8.0, 25°, Inagami (1). 
†€ pH 7.6, 25°, Bernhard (2). 
†† Bender and Kezdy (5).

withdrawing Phe(NO<sub>2</sub>) group is to weaken the susceptible C—N bond and enhance the rate of acylation. For the hydrolysis of Z-Lys-AN catalyzed by trypsin, the pretransition state protonated path I → III → V is the major mechanism. Thus, the hydrolysis of the p-nitroanilide by trypsin proceeds by a mechanism similar to that for the esters.

**Esterase versus Peptidase Activity**—Streptococcal proteinase belongs to a group of enzymes that exhibit a relatively high ratio of esterase to peptidase activity. Table VI lists ratios of esterase to peptidase activity for some representative proteolytic enzymes. Comparison of the rates of hydrolysis of an ester substrate and the corresponding amide or anilide indicates that proteolytic enzymes generally fall into two major categories. In one, they hydrolyze the ester substrates at a rate at least 1000 times faster than the corresponding amide, and in the other they hydrolyze amide and ester substrates at comparable rates. Most of the serine enzymes such as trypsin, chymotrypsin, and subtilisin have been shown to belong to the first group, whereas the sulfhydryl enzymes such as papain and ficin belong to the second group. Streptococcal proteinase is unique in this respect, since it is a sulfhydryl enzyme and yet it belongs to the first group of enzymes. Comparison of the rate of hydrolysis of Bz-Arg-OEt<sup>c</sup> and Bz-Arg-A<sup>c</sup> catalyzed by papain and trypsin reveals that both enzymes hydrolyze the ester substrate Bz-Arg-OEt<sup>c</sup> at about the same rate ($k_{cat} = 15.7$ sec<sup>-1</sup> versus $8.4$ sec<sup>-1</sup> for papain and trypsin), but the rate of hydrolysis of the amide substrate Bz-Arg-A<sup>c</sup> differs considerably ($k_{cat} = 8.48$ sec<sup>-1</sup> versus 0.043 sec<sup>-1</sup> for papain and trypsin). The observed difference between these two types of enzyme toward ester and amide bond cleavage is due, therefore, to the lower efficiency of the trypsin type enzyme for the hydrolysis of amide bond, rather than to the higher efficiency of the papain type enzyme for the hydrolysis of ester substrate.

All of the serine enzymes mentioned have been shown also to have at least 1 histidine residue at the active site (5, 9, 10), whereas for the sulfhydryl enzymes papain and ficin, in addition to the —SH group, a carboxyl group has been shown kinetically by various authors (5, 7, 8, 13, 14) to participate in the mechanism. A number of recent investigations have led to the conclusion that both the —SH group of cysteine and the basic group of histidine are involved in the catalytic function of streptococcal proteinase. Thus, streptococcal proteinase is a "histidine"-
enzyme like trypsin, chymotrypsin, and subtilisin, and at the same time it is an —SH enzyme like papain and ficin.

It is particularly noteworthy that pepsin hydrolyzes ester and amide substrates at a comparable rate. The existence in pepsin of at least one carboxyl group which is essential for its catalytic action is well documented (16–18). It thus appears that, for the proteolytic enzymes that utilize imidazole simultaneously as a general base and the protonated form of the same imidazole group as a general acid for their catalytic function, a high ratio of esterase to peptidase activity is observed. For the proteolytic enzymes that show comparable rates of hydrolysis of a specific ester and amide substrate, a carboxyl group has been proposed to be the catalytic entity.

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38. INAGAMI, T., and MURACHI, T., Biochemistry, 2, 1439 (1963).
Streptococcal Proteinase-catalyzed Hydrolysis of Some Ester and Amide Substrates
Teh-Yung Liu, Noboru Nomura, Elsy K. Jonsson and Bruce G. Wallace

J. Biol. Chem. 1969, 244:5745-5756.

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