Stable β-Sheet Formation and Enhanced Hydrolytic Catalysis of a Sequential Alternating Amphiphilic Polypeptide Containing Catalytic Triads in a Serine Protease

Yasumasa Fukushima

Research & Development Center, Unitika Ltd., 23 Kozakura, Uji, Kyoto 611

(Received September 4, 1995)

The alternating amphiphilic copolypeptide poly(Asp–Leu–His–Leu–Ser–Leu) was prepared by the synthesis and polymerization of the respective hexapeptide in order to obtain a stable β-sheet polypeptide with hydrolytic catalytic activity, like that of a serine protease. The conformation and conformational transitions in aqueous solution and in water–organic solvent mixtures were determined by circular dichroism measurements. The polypeptide reveals a very strong tendency to adopt a β-sheet structure, which is accomplished at below pH 10.0, and even by adding sodium chloride or various alcohols. However, a partial α-helical conformation is observed in an aqueous solution at pH 12.0; also, an α-helix to β-sheet conformational transition occurs upon adding 1 M NaCl. The polypeptide is a more effective catalyst than the peptide hexamer, the amino acids mixture, or imidazole for the hydrolysis of *p*-nitrophenyl derivatives, and catalyzed the hydrolytic reaction of the substrates more effectively with increasing their hydrophobicity. It is likely that the enhanced hydrolytic catalyst of the polypeptide may be responsible for increasing the nucleophilicity by the electrostatic interactions or condensation effect of the substrates by hydrophobic interactions. The polypeptide would make good models for constructing novel proteins with catalytic properties involving β-sheet structures.

The β-sheet conformation is as common in natural proteins and peptides as is the α-helical conformation. However, little is known about which amino acid sequences adopt the β-sheet conformation, or about how the β-sheet conformation behaves in solution. Unlike the α-helical conformation, the β-sheet conformation is formed by nonlocal interactions, which are involved in intermolecular interactions of different chains as well as intramolecular interactions within a contiguous protein sequence. Many model studies concerning β-sheet conformational formation have been reported regarding synthetic polypeptides. In particular, poly(Ala–Glu), poly(Val–Lys), poly(Leu–Lys), poly(Phe–Lys), poly(Tyr–Lys), and poly(Tyr–Glu), sequential amphiphilic copolypeptides with alternating hydrophilic and hydrophobic amino acid residues can form a water-soluble stable β-sheet conformation with a hydrophobic interior and a hydrophilic exterior in aqueous solution.[1—5] It is presumed that the main driving forces of the formation of the bilayer β-sheet conformation is hydrophobic interactions between the side chains of the polypeptides as well as the conventional β-sheet hydrogen bondings between their main chains. We synthesized alternating amphiphilic copolypeptides with complementary ionic interactions, poly(Glu–Val–Lys–Val) and poly(Glu–Leu–Lys–Leu), and recently described that they formed much stabler β-sheet conformations.[6] The excellent stability of the β-sheet conformations would be caused by electrostatic interactions between opposite charged amino acid residues in addition to hydrophobic interactions.

On the other hand, the mammalian serine proteases have a catalytic triad of amino acid residues so positioned on the protein as to give hydrolase activity. X-Ray diffraction studies of α-chymotrypsin have indicated that aspartic acid residue (Asp^{102}) forms its β-carboxyl hydrogen bonding with the imidazole of the histidine residue (His^{57}). The imidazole ring possesses hydrogen bonding to the hydroxyl group of the serine residue (Ser^{195}); also, the oxygen of the hydroxyl group attacks the electropositive carbonyl carbon of the substrate. These three amino acid residues form catalytic triads, which play a key role in enzyme-catalyzed hydrolysis.[7—10]

Therefore, because an aspartic acid, a histidine and a serine are hydrophilic amino acid residues, alternating amphiphilic polypeptides which can adopt a stable β-sheet conformation can be obtained by combining these amino acid with hydrophobic amino acid residues, such as leucine. If these amono acids residues are positioned at places separated by every one residue (i, $i+2$, $i+4$ spacing) in the sequence of alternating amphiphilic polypeptides, the β-sheet conformation of the polypeptides can be stabilized by side-chain interactions among hydrophilic residues, which appear to be formed in catalytic triads in serine proteases.

In this study, we designed and synthesized a sequential alternating amphiphilic polypeptide bearing the serine protease catalytic site residues, an aspartic acid, a histidine, and a serine as hydrophilic amino acid residues, poly(Asp–Leu–His–Leu–Ser–Leu), in order to construct a stable β-sheet forming polypeptide with catalytic property, as shown Fig. 1. The positions of the hydrophilic residues were expected to form side-chain interactions between an aspar-

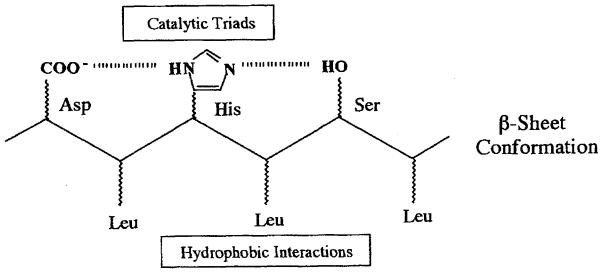


Fig. 1. Schematic model of catalytic triad formation of poly-(Asp–Leu–His–Leu–Ser–Leu).

tic acid and a histidine as well as between a histidine and a serine. We selected a leucine residue as a hydrophobic amino acid, because a leucine has high hydrophobicity among naturally occurring amino acids. Consequently, it is likely that the designed amphiphilic polypeptide adopts a stable β-sheet conformation by electrostatic and hydrophobic interactions in addition to conventional β-sheet hydrogen bonding. In addition, the polypeptide seems to have catalytic properties of the three hydrophilic residues, and the catalytic properties might be influenced by the conformation of the polypeptide. The synthesis and conformational studies of the polypeptide are described here. The conformational studies were carried out by circular dichroism in aqueous solution as well as water–organic solvent mixtures. In addition, in this report, we compared the ability of both poly-(Asp–Leu–His–Leu–Ser–Leu) and imidazole to catalyze the hydrolysis of AcONp as a function of the pH, or the addition of organic solvents, such methanol, ethanol, 2-propanol, or HFIP.[11] The dependence of the hydrolytic rate constants on the substrates was investigated as well.

Experimental

Materials and Methods. Boc–Asp(OBzl)–OH, Boc–His-(Tos)–OH, and Boc–Ser(Bzl)–OH were purchased from Peptide Institute, Inc. Z–Gly–ONp, Z–Ala–ONp, Z–Val–ONp, and Z–Phe–ONp were purchased from Bachem. All other reagents and solvents were of high purity and commercially available, and were used without further purification. Dialysis membranes (VT351), having a molecular mass cutoff of 3500, were from Nacalai. NMR spectra were obtained at 300 MHz with a Varian VXR300.

The amino acid composition of the polypeptide was determined using a Hitachi 835 amino acid analyzer. The sample was hydrolyzed in an evacuated sealed tube with 6 M HCl (1 M = 1 mol dm^{-3}) at 110 °C for 24 h. The hydrolyzed polypeptide was derivatized by using ninhydrin.

Molecular-Weight Determination. The viscosity and size exclusion chromatography methods were used in determining the molecular weight of the polypeptide.

If we assume the viscosity–molecular weight relationships, studied by Doty, which were obtained in the case of poly(benzyl L-glutamate) in dichloroacetic acid, to also be valid in our case, we can estimate the order of the molecular weight of our polypeptide by measuring the intrinsic viscosity in dichloroacetic acid with an ubbelohde capillary viscometer at 25 °C.[12] The intrinsic viscosity $[\eta]$ was determined by graphically extrapolating plots of $(\eta_r - 1)/c$ vs. c, constructed from measurements at four concentrations. The symbol η_r stands for the ratio viscosities of the solution and solvent.

Size-exclusion chromatography of the polypeptide was carried out by using a combination of 0.74 cm by 30 cm Ultrahydrogel 250 and 0.74 cm by 30 cm Ultrahydrogel 500 columns (Waters). The eluent was monitored by measuring the absorbance at 214 nm. The polypeptides or protein standards were applied to the column in 0.15 M NaCl, 0.2 M phosphate buffer, pH 7.0, and eluted with the same buffer at a flow rate of 1.0 mL min^{-1}. The polypeptide was applied to the column at a concentration of approximately 850 μM. The apparent molecular weight was determined by interpolation from the standard curve.

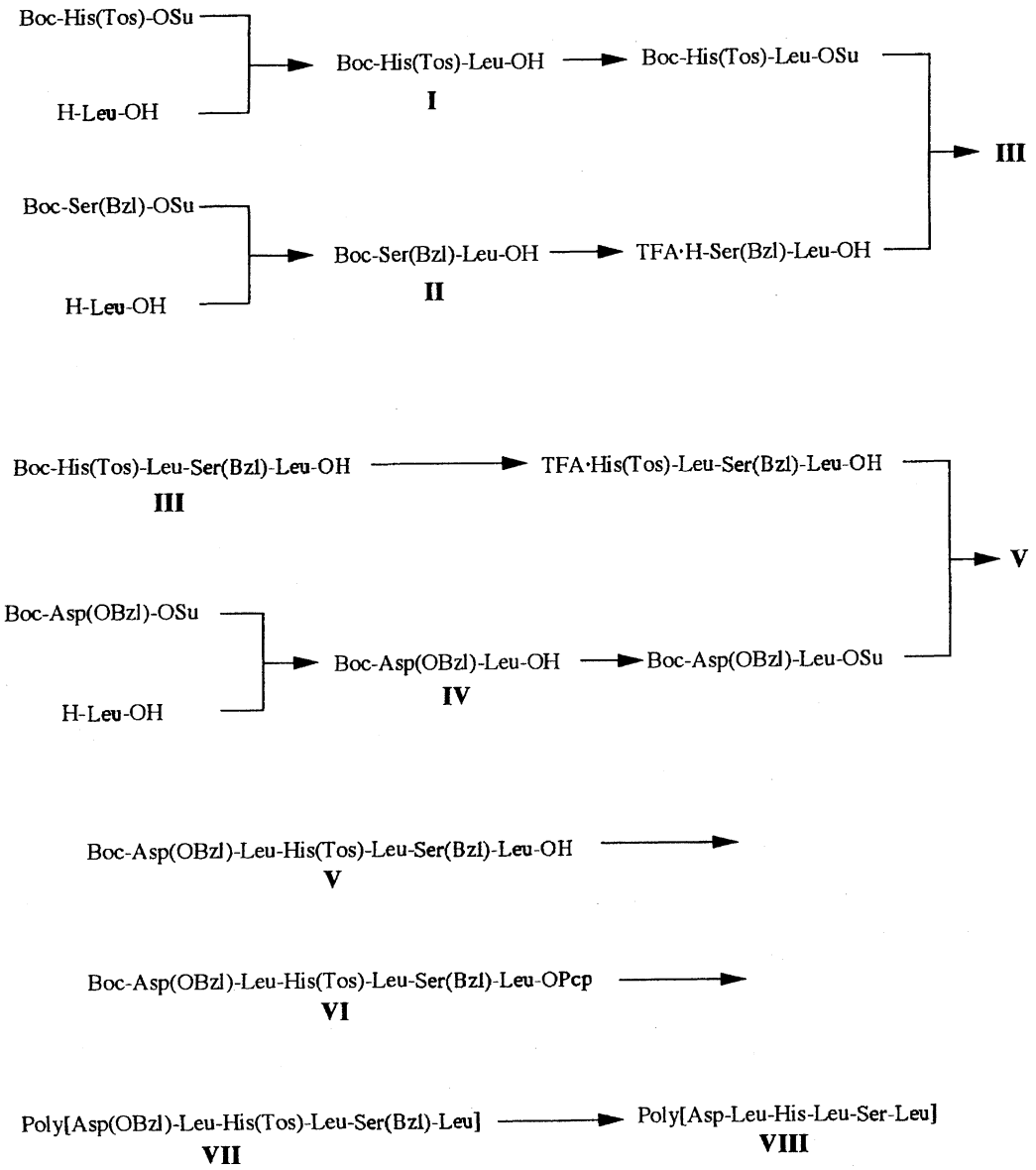
CD Measurements. CD spectra were obtained under a constant flow of nitrogen on a Jovin Ivon CD6 spectropolarimeter equipped with an interface and a personal computer using a quartz cuvette of 1 mm path length. The instruments were calibrated with an aqueous solution of ammonium *d*-camphorsulfate.[13] Polypeptide stock solution was prepared at a concentration of approximately 3 mg mL^{-1} in distilled deionized water. The precise polypeptide concentration of the stock solutions was determined by an ninhydrin analysis of a hydrolyzed polypeptide sample. CD samples were prepared by diluting the stock solutions either in water, various pH solutions, different concentrations of 5 M NaCl, or organic solvents such as methanol, ethanol, 2-propanol, TFE, and HFIP. The pH was measured with a Horiba pH meter (F-16) before the CD measurements. The observed ellipticity was expressed as the mean residue ellipticity $[\theta]$, which was normalized to units of degrees centimeter squared per decimole. The content of a β-sheet conformation was calculated on the basis of the reported values of molar ellipticity of 100% β-sheet peptides, [θ] (217 nm) = −20000 deg cm^2 dmol^{-1}.[14]

Kinetic Measurements. Five mM catalyst solutions, imidazole, and poly(Asp–Leu–His–Leu–Ser–Leu) were prepared in water with 1 M 2-amino-2-hydroxymethyl-1,3-propanediol. The pH was adjusted with concentrated hydrochloric acid. The substrates, (AcONp, Z–Gly–ONp, Z–Ala–ONp, Z–Val–ONp, and Z–Phe–ONp) were dissolved in ethanol at a concentration of 10 mM, respectively. After 100 μL of a catalyst solution (with or without a catalyst) was mixed with 890 μL of solvent (water or water/organic solvent mixture), 10μL of the substrate solution was added. The rates were studied in a Hitachi U-3210 spectrophotometer thermostated at 25 °C, by measuring the absorption of the 4-nitrophenoxide ion at 400 nm as a function of time. The rate data obeyed a pseudo-first-order rate profile. The second-order rate constants (k_{cat}) were obtained using the formula $k_{cat} = k_{measd} - k_{blank}$, in which k_{measd} is the second-order rate constant, measured in the presence of a catalyst, and k_{blank} is the second-order rate constant measured in its absence.

Peptide Synthesis. The synthesis of poly(Asp–Leu–His–Leu–Ser–Leu) is summarized in Scheme 1.

Boc–His(Tos)–Leu (I) : To L-Leu (1.55 g, 11.8 mmol) and $NaHCO_3$ (2.00 g, 23.8 mmol) dissolved in 100 ml of water and 50 mL of dry THF was added Boc–His(Tos)–OSu (6.00 g, 11.9 mmol) in 50 mL of dry THF.[15] After 2 h of stirring at room temperature, the solution was concentrated, acidified with 1.0 M HCl, and then extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to give an oily product. The residual oil was triturated with hexane to obtain 4.16 g (67.2%) of **I** as a white solid. mp 146—148 °C. $[\alpha]^{25}_{565\ nm} - 13.6°$ (c 1.0, DMF). Elemental Analysis. Found: C, 55.02; H, 6.67; N, 10.75%. Calcd for $C_{24}H_{34}N_4O_7S$: C, 55.16; H, 6.56; N, 10.72%.

Boc–Ser(Bzl)–Leu (II) : To L-Leu (2.62 g, 20.0 mmol) and $NaHCO_3$ (3.36 g, 40.0 mmol) dissolved in 100 ml of water and 50 mL of dry THF was added Boc–His(Tos)–OSu (7.84 g, 20.0 mmol) in 50 mL of dry THF. After 2 h of stirring at room temperature, the



Scheme 1. Synthetic route of poly(Asp–Leu–His–Leu–Ser–Leu).

reaction mixture was treated as described above for the preparation of **I** to give 6.69 g (82.0%) of **II** as an oily product. $[\alpha]^{25}_{565\ nm} -8.2°$ (*c* 1.0, DMF).

Boc–His(Tos)–Leu–Ser(Bzl)–Leu (III) : Compound **I** (4.00 g, 7.66 mmol) was treated with *N*-hydroxysuccinimide (1.77 g, 15.3 mmol) and DCC (3.17 g, 15.3 mmol) in THF (120 ml) at 0 °C while being stirred for 20 h to give 4.11 g (86.7%) of Boc–His-(Tos)–Leu–OSu as a white solid.[16]

Compound **II** (2.66 g, 6.50 mmol) was treated with TFA (10 mL) at room temperature. After stirring for 1 h, TFA was removed in vacuo, and the residual oily product was triturated with ether and dried in vacuo. To the precipitate and $NaHCO_3$ (2.28 g, 26.0 mmol) dissolved in 100 ml of a mixture of water–THF (1 : 1) was added Boc–His(Tos)–Leu–OSu in 120 ml of THF. After 20 h of stirring at room temperature, the reaction mixture was treated as described above for preparing **I**, to give 4.86 g (92.1%) of **III**. Mp 47—49 °C. $[\alpha]^{25}_{565\ nm} -12.9°$ (*c* 1.0, DMF). Elemental Analysis. Found: C, 58.71; H, 6.88; N, 10.26%. Calcd for $C_{40}H_{56}N_6O_{10}S$: C, 59.02; H, 6.93; N, 10.32%.

Boc–Asp(OBzl)–Leu (IV) : To L-Leu (2.53 g, 19.3 mmol) and $NaHCO_3$ (3.25 g, 38.6 mmol) dissolved in 100 ml of water and 50 mL of dry THF was added Boc–Asp(OBzl)–OSu (8.00 g, 19.3 mmol) in 100 mL of dry THF. After 2 h of stirring at room temperature, the reaction mixture was treated as described above for the preparation of **I** to give 7.42 g (90.8%) of **IV** as an oily product. $[\alpha]^{25}_{565\ nm} -12.3°$ (*c* 1.0, DMF).

Boc–Asp(OBzl)–Leu–His(Tos)–Leu–Ser(Bzl)–Leu (V) : To a solution of compound **IV** (4.94 g, 11.5 mmol) and *N*-hydroxy-succinimide (1.45 g, 12.6 mmol) in dry THF (150 ml) was added DCC (2.60 g, 12.6 mmol) at 0 °C, after 20 h stirring, to give 5.45 g (89.8%) of Boc–Asp(OBzl)–Leu–OSu as a white solid.

Compound **III** (8.40 g, 10.3 mmol) was treated with TFA (20 mL) at room temperature for 1 h; TFA was removed in vacuo and the residual oily product was triturated with ether and dried in vacuo. To the precipitate and $NaHCO_3$ (3.61 g, 41.2 mmol) dissolved in 200 ml of a mixture of water–THF (1 : 1) was added Boc–Asp(OBzl)–Leu–OSu (5.37 g, 10.3 mmol) in 100 ml of THF. After 20 h of stirring at room temperature, the reaction mixture was

treated as described above for the preparation of **I** to obtain 5.04 g (43.6%) of **V**. Mp 81—83 °C. $[\alpha]^{25}_{565\ nm}$ −23.7° (*c* 1.0, DMF). Elemental Analysis. Found: C, 60.21; H, 6.88; N, 9.98%. Calcd for $C_{57}H_{78}N_8O_{14}S$: C, 60.46; H, 6.94; N, 9.90%.

Boc–Asp(OBzl)–Leu–His(Tos)–Leu–Ser(Bzl)–Leu–OPcp (VI) : To a solution of compound **V** (4.90 g, 4.38 mmol) and pentachlorophenol (1.28 g, 4.82 mmol) in 100 ml of THF was added DCC (0.99 g, 4.82 mmol) at 0 °C.[17] After 20 h of stirring, an oily product was triturated with ether to obtain 2.73 g (45.1%) of **VI**. Mp 117—118 °C. $[\alpha]^{25}_{565\ nm}$ −21.1° (*c* 1.0, DMF). Elemental Analysis. Found: C, 54.54; H, 5.56; N, 8.01%. Calcd for $C_{63}H_{77}Cl_5N_8O_{14}S$: C, 54.81; H, 5.62; N, 8.12%.

Poly[Asp(OBzl)–Leu–His(Tos)–Leu–Ser(Bzl)–Leu] (VII) : Compound **VI** (2.50 g, 1.81 mmol) was treated with TFA (10 mL) at room temperature while being stirred for 1 h. After the oily product was triturated with ether to give a while precipitate, to the solution of the product in DMSO (2.0 mL) was added triethylamine (0.42 mL, 3.02 mmol). After stirring at room temperature for 10 d, the reaction mixture was triturated with ether. The precipitate was washed with water, methanol and ether to obtain 0.556 g (30.3%) of **VII**. Elemental Analysis. Found: C, 61.12; H, 6.88; N, 10.75%. Calcd for $(C_{52}H_{68}N_8O_{11}S)_n$: C, 61.58; H, 6.76; N, 11.05%.

Poly(Asp–Leu–His–Leu–Ser–Leu) (VIII) : Compound **VII** (0.200 g, 0.197 mmol) was treated with methanesulfonic acid (2 mL) and anisole (0.2 mL) at room temperature for 1 h; and the mixture was then triturated with ether to give a white precipitate.[18] The solid was first washed with methanol and ether, and then dried. After the powder was dissolved in water (20 mL), the solution was filtered through a 0.45-μm Millipore filter. The solution was dialyzed against 1 L of deionized distilled water for one week (the water was changed once a day) and lyophilized to obtain 0.057 g (42.4%) of **VIII**.

^{1}H NMR (δ, ppm from TMS in DMSO-d_6) δ =0.70—0.92 (18H, 6×δCH$_3$ in Leu), 1.30—1.67 (9H, 3×βCH$_2$ and 3×γCH in Leu), 2.62—3.20 (4H, βCH$_2$ in Asp and His), 3.50—3.64 (2H, βCH$_2$ in Ser), 4.11—4.36 (4H, αCH in Ser and Leu), 4.42—4.64 (2H, αCH in Asp and His), 7.13—7.23 (1H, C4H of imidazolyl in His), 7.70—8.25 (6H, all NH), 8.90—8.98 (1H, C2H of imidazolyl in His). 13.87—14.12 (1H, NH of imidazolyl in His). All NMR signals were broadened. The NMR spectra show a disappearance of the signals assigned to OBzl, Tos, and Bzl groups after the cleavage reaction of these protecting groups with methanesulfonic acid in the presence of anisole. The amino acid ratios found were $Asp_{1.07}His_{1.06}Ser_{1.01}Leu_{2.86}$.

Asp–Leu–His–Leu–Ser–Leu (IX) : After compound **V** (0.300 g, 0.268 mmol) was treated with methanesulfonic acid (3 mL) and anisole (0.3 mL) at room temperature for 1 h, the mixture was triturated with ether and dried to obtain 0.146 g (78.3%) of **IX** as a while precipitate. Mp 69—71 °C. $[\alpha]^{25}_{565\ nm}$ −19.3° (*c* 1.0, DMF). Elemental Analysis. Found: C, 53.11; H, 7.84; N, 15.89%. Calcd for $C_{31}H_{52}N_8O_{10}$: C, 53.45; H, 7.47; N, 16.09%.

Determination of the Optical Purity of Compound V. A solution of Compound **V** (0.224 g, 0.200 mmol) was hydrolyzed in an evacuated sealed tube with 6 M HCl (2 ml) at 110 °C for 24 h.[17] In a control experiment, L-Asp (0.023 g, 0.200 mmol), L-His (0.027 g, 0.200 mmol), L-Ser (0.017 g, 0.200 mmol), and L-Leu (0.068 g, 0.600 mmol) were treated under the same conditions. $[\alpha]^{25}_{565\ nm}$ for the sample and the control was 14.2° and 15.4°, respectively. The calculated optical purity was 92.2%.

Results and Discussion

Synthesis and Relative Size of the Polypeptide. The routes for synthesizing the hexapeptide monomer and the polymerization are given in Scheme 1. All of the α-amino groups of Asp, His, and Ser were protected with the *t*-butyloxycarbonyl (Boc) group. Both the β-carboxyl group of Asp and the hydroxyl group of Ser were protected with the Bzl group. The imidazolyl group of His was protected with the Tos group. The Boc group was selectively removed by anhydrous trifluoroacetic acid, while the Bzl and Tos groups were eliminated after completion of polymerization of the polypeptide by a treatment with methanesulfonic acid in the presence of anisole.[18]

Compound **I** dipeptide was synthesized by coupling Boc–His(Tos)–OSu with Leu in the presence of $NaHCO_3$. DCC has been used to synthesize *N*-hydroxysuccinimide active esters, which are colorless crystalline derivatives with good stability. Compounds **II** and **IV** dipeptides were synthesized by coupling leucine with active esters of Boc–Ser(Bzl)–OH and Boc–Asp(OBzl)–OH, respectively, in a similar manner to that described above. The N-terminal Boc group of compound **II** was removed by a treatment with TFA, and the product coupled with *N*-hydroxysuccinimide active ester of compound **I** to give compound **III**. In an analogy of the synthesis of compound **III**, compound **V** was synthesized by coupling the deblocked compound **III** with *N*-hydroxysuccinimide active ester of compound **IV**.

The polymerization of a sequential polypeptide having a defined repeating unit of amino acids is usually achieved by a self-condensation of monomer peptide active esters. For polymerizing the hexapeptide monomer, the free acid was activated by conversion to its pentachlorophenyl ester, since it has been shown that the active ester could be most active among the *p*-nitrophenyl-, pentachlorophenyl-, and *N*-hydroxysuccinimide esters. The high reactivity of the active ester could shorten the time for polymerization, and thereby reduce any possible side reactions. Compound **VI** was synthesized by coupling compound **V** with pentachlorophenol in the presence of DCC.

The polymerization was carried out in DMSO in the presence of triethylamine after removing the Boc group of compound **VI** by TFA. The polymerized product remains in the solvent long enough to allow the formation of a fairly high molecular-weight polypeptide. In addition, a sufficiently purified triethylamine needs to be used in order to avoid an early termination of the polymerization reaction. All signals of the NMR spectra of compound **VII** broadened due to polymerization.

The protecting groups, Bzl and Tos, of compound **VII** were removed by methanesulfonic acid in the presence of anisole. The NMR spectra of compound **VIII** show a disappearance of the signals assigned to the all protecting groups after the cleavage reaction.

Poly(Asp–Leu–His–Leu–Ser–Leu) showed an intrinsic viscosity of 0.060 dL g^{-1} in dichloroacetic acid, which is a random-coil forming solvent for polypeptides. The in-

trinsic viscosity value corresponds to a molecular weight of 6800, if we apply Eq. 1 proposed by Doty et al.,[12]

$$[\eta] = 2.78 \times 10^{-5}\ M^{0.87}. \quad (1)$$

Figure 2 illustrates the size-exclusion chromatography profile of the polypeptide. The size-exclusion chromatogram showed that the polypeptide was eluted into one fraction at the elution time corresponding to a molecular weight of approximately 6000 with 2.57 of the molecular-weight distribution calculated on the basis of M_w/M_n; no additional fraction was observed in the exclusion limit and low molecular-weight regions. The molecular weights of 6800 and 6000 correspond to the degree of polymerization of 10.4 and 8.9, respectively, on the hexamer Asp–Leu–His–Leu–Ser–Leu unit. Both of the molecular weights estimated by the two methods are in fair agreement.

Conformational Studies. The CD spectra of the polypeptide in aqueous solution at different pH values are shown in Fig. 3. The CD spectrum at pH 7.0 is characterized by a minimum around 217 nm (n–π* transition) and a maximum around 199 nm (π–π* transition), which indicates

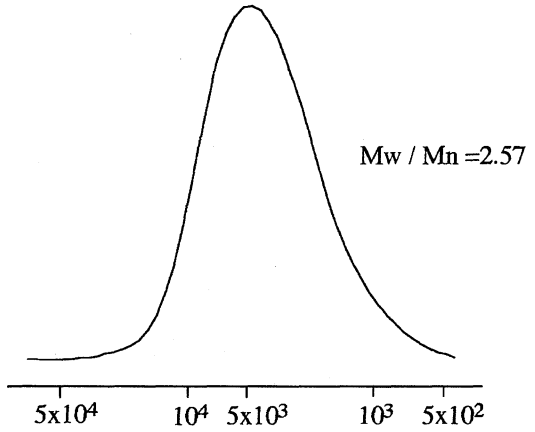


Fig. 2. Size exclusion chromatography profile of poly-(Asp–Leu–His–Leu–Ser–Leu).

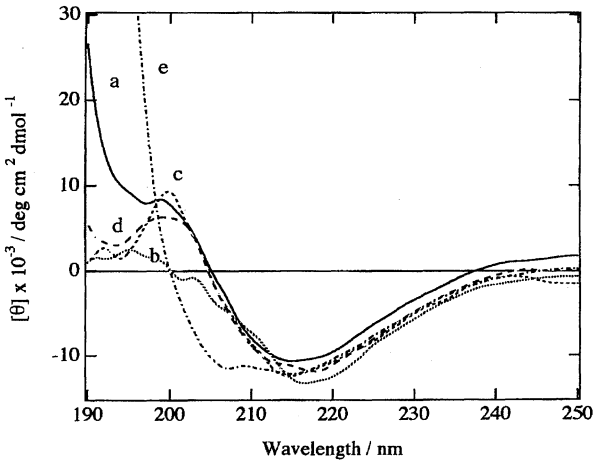


Fig. 3. CD spectra of poly(Asp–Leu–His–Leu–Ser–Leu) at a concentration of 524 μM in aqueous solution at different pH. pH: (a) 2.0, (b) 4.0, (c) 7.0, (d) 10.0, (e) 12.0.

that the polypeptide obviously exists in the β-sheet structure. Based on the residue ellipticity of −12000 at 217 nm, the β-sheet content at pH 7.0, was calculated using the β-sheet structure of poly-L-lysine as a reference, which was approximately 60%. The CD spectra at pH 4.0 and 2.0 are indicative of a predominant formation of a β-sheet structure with a content of 65 and 55%, respectively. The β-sheet content increases slightly from pH 7.0 to 4.0, while the content decreases from pH 4.0 to 2.0. In addition, the CD spectrum at pH 10.0 displays the typical feature in a β-sheet structure, and the β-sheet contents are almost constant in the pH 7.0 to 10.0 region. On the other hand, the polypeptide adopts a partially α-helical conformation containing a β-sheet structure in aqueous solution at pH 12.0. The α-helical and β-sheet structural contents at pH 12.0 cannot be estimated for the mixture of two secondary structures. Consequently, the polypeptide forms a β-sheet structure from pH 2.0 to 10.0, whereas the formation of the partly α-helical conformation is observed at pH 12.0. It is likely that a helix-sheet conformational transition takes place at around pH 11.0 and the polypeptide exhibits one pH-induced conformational transition.

Since poly(Asp–Leu–His–Leu–Ser–Leu) is presumed to exist as a β-sheet structure with a hydrophilic exterior and a hydrophobic interior, the β-sheet structure can be stabilized by a hydrophobic interaction in the hydrophobic interior comprising leucine residues and conventional hydrogen bonds. In particular, the hydrophobic interactions would play a key role in β-sheet formation for an alternating amphiphilic polypeptide. In addition, poly(Asp–Leu–His–Leu–Ser–Leu) has electrostatic interactions in a hydrophilic exterior for stabilizing the β-sheet structure.

The dependence of the secondary structure for the polypeptide on the pH may be explained by a consideration of the electrostatic interactions, as follows. The polypeptide contains one positively charged histidine and one negatively charged aspartic acid per repeating unit. Since a histidine has a pK_a of 6.0 and an aspartic acid has a pK_a of 3.9, a histidine is positively charged at pH 2.0 and 4.0, but is neutral above pH 7.0, while an aspartic acid is neutral at pH 2.0, but is negatively charged above pH 4.0. Therefore, because a histidine and an aspartic acid are oppositely charged in aqueous solution at pH 4.0, the β-sheet structure is most stabilized by complementary ionic pair bonding between a positively charged histidine and a negatively charged aspartic acid as well as a hydrogen bond between a charged histidine and a serine. On the other hand, in aqueous solution at pH 2.0, although the ionic pair bonding cannot form, hydrogen bonds can form between a charged histidine and an uncharged aspartic acid as well as between a charged histidine and a serine, since only a histidine residue is charged at pH 2.0. The polypeptide adopts a β-sheet structure at pH 2.0; however, the β-sheet structure may be slightly more destabilized than at pH 4.0, because the hydrogen bond forms a weaker attractive interaction than does the complementary ionic-pair bond. Because only the aspartic acid is charged above pH 7.0, the ionic-pair bond cannot form from neutral to alka-

line conditions. At pH 7.0 and 10.0, the β-sheet structure is formed by electrostatic attractive interactions, such as hydrogen bonds between an uncharged histidine and a charged aspartic acid as well as between an uncharged histidine and a serine; however, the β-sheet structural content decreases slightly more than at pH 4.0. In contrast, the formation of a partly α-helical conformation in aqueous solution at pH 12.0 cannot be understood.

A typical β-sheet CD profile was obtained in aqueous solution at pH 7.0 at a polypeptide concentration from 26.3 to 5260 μM (data not shown). Even at the lowest measurable polypeptide concentration (26.3 μM), the CD spectrum can be recorded to show β-sheet formation. Under these conditions, the ellipticities at 217 nm were held almost constant at approximately 12000 deg cm^2 dmol^{-1}, which corresponds to 60% of the β-sheet content (data not shown). These results suggest that the polypeptide forms a stable β-sheet structure in a very dilute solution.

The independence of the CD signal indicates that the β-sheet forming unit is monomeric, or that the lowest measurable polypeptide concentration has not attained the concentration required to dissociate an aggregated β-sheet structure.

Figure 4 shows the CD spectra of the polypeptide at pH 12.0 in the presence of 1 M NaCl. The polypeptide exhibits a β-sheet structure upon the addition of the salt, and the β-sheet content is almost equal to that at pH 7.0 in the absence of a salt. Accordingly, the polypeptide shows a salt-induced helix-sheet conformational transition at pH 12.0, and the β-sheet formation may be caused by an increase in the hydrophobic interaction between leucine residues by affecting the order of the water molecules with NaCl.

In the pH 2.0 to 10.0 region, as well, the polypeptide showed CD spectra typical of a β-sheet structure in the presence of 1 M NaCl (data not shown). Table 1 lists the extent of β-sheet formation (measured by the ellipticity of 217 nm) in aqueous solution from pH 2.0 to 12.0 in both the absence and presence of the salt. The β-sheet contents other than at pH 4.0 slightly increase upon the addition of the salt, whereas at

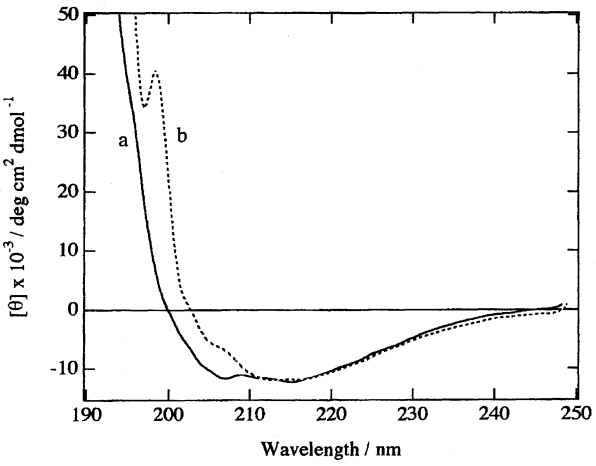


Fig. 4. CD spectra of poly(Asp–Leu–His–Leu–Ser–Leu). (a) in water at pH 12.0 and (b) of a solution in 1 M NaCl at pH 12.0.

Table 1. Ellipticity at 217 nm of Poly(Asp–Leu–His–Leu–Ser–Leu) in Water or in 1 M NaCl Solution at Various pH

pH	$-[\theta]_{217}$/deg cm^2 dmol^{-1}	
	No NaCl	1 M NaCl
2.0	10600	12600
4.0	13000	11700
7.0	12700	13800
10.0	12200	12500
12.0	Partly helix	12600

pH 4.0 the content in the presence of the salt is slightly lower than that in the absence of the salt. These results are very complicated for forming an explanation. Only in the case of pH 4.0 the complementary ionic bond between an aspartic acid and a histidine can form; at a pH other than 4.0 though the ionic bond cannot form, the hydrogen bonds can form. It is likely that a decrease of the content at pH 4.0 is responsible for the destabilization of a β-sheet structure by shielding the ionic bond with a salt. On the other hand, except for at pH 4.0, it appears that the contents increase due to an increase in hydrophobic interactions rather a decrease of the hydrogen bonds.

Figure 5 shows the CD spectra for the polypeptide at pH 7.0 in the presence of various alcohols. In spite of 80% methanol, ethanol, 2-propanol, or TFE solution, the CD spectra are characteristic of a β-sheet structure in all cases, and the contents are almost equal to that in pure water at pH 7.0. In addition, Fig. 6 shows the CD spectra at pH 7.0 in the presence of HFIP. The polypeptide adopts a β-sheet structure irrespective of the ratio of water and HFIP, and the contents are independent of the ratio. Because these organic solvents enhance the hydrogen bonding, and electrostatic interactions reduce hydrophobic interactions, these solvents destabilize a β-sheet structure for alternating amphiphilic polypeptides by collapsing the hydrophobic interactions.[19,20] Therefore, an

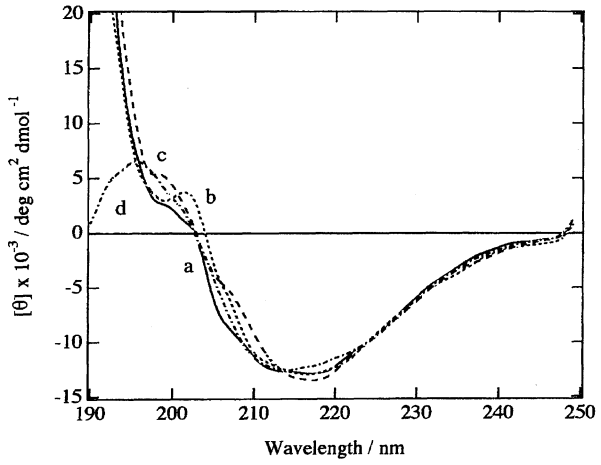


Fig. 5. CD spectra of poly(Asp–Leu–His–Leu–Ser–Leu) in water at pH 7.0 and alcohol mixtures (v/v). (a) water–methanol (20 : 80), (b) water–ethanol (20 : 80), (c) water–2-propanol (20 : 80), (d) water–TFE (20 : 80).

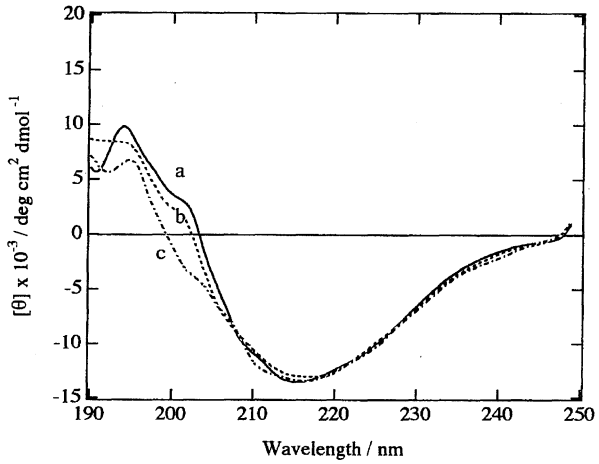


Fig. 6. CD spectra of poly(Asp–Leu–His–Leu–Ser–Leu) in water at pH 7.0 and HFIP mixtures. HFIP concentrations (v/v) of: (a) 40%, (b) 60%, (c) 80%.

alternating amphiphilic peptide is difficult to form a β-sheet structure in organic solvent–water mixtures or organic solvents, because the main driving force of β-sheet formation is the hydrophobic interaction. However, because the polypeptide exists as a β-sheet structure in organic solvent–water mixtures, the polypeptide is unusually stable against organic solvents.

Catalyst Properties. Figure 7 shows the dependences of the second-order rate constants of the hydrolysis of *p*-nitrophenyl acetate on the pH for Poly(Asp–Leu–His–Leu–Ser–Leu), Asp–Leu–His–Leu–Ser–Leu hexamer, a mixture of four amino acids, and imidazole. For all catalysts, the rate constants are higher with increasing pH because the nucleophilicity of the free base species of imidazole, and the imidazolyl group is higher than that of

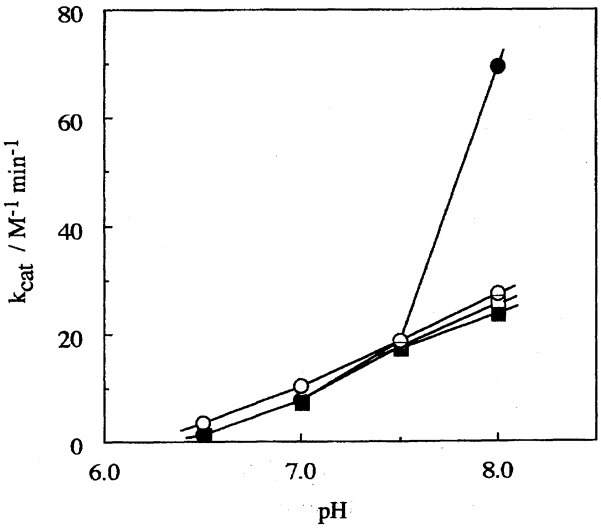


Fig. 7. pH dependence of *p*-nitrophenyl acetate hydrolysis catalyzed by poly(Asp–Leu–His–Leu–Ser–Leu) (●), Asp–Leu–His–Leu–Ser–Leu (■), imidazole (○), and Asp/His/Ser/Leu mixture (□). Molar ratio of the mixture is [Asp]/[His]/[Ser]/[Leu]=1/1/1/3 and its k_{cat} is calculated on His concentration.

their protonated species; also, the amounts of their free base species increased with the pH. From pH 6.5 to 7.5, the rate constants of all the catalysts are almost the same. At pH 8.0, there is a strong enhancement of the rate constant of the polypeptide over the other catalysts, and the rate constant for the polypeptide is higher than those for the other catalyst by a factor of about 2.5. This enhancement may be responsible for an increase in the nucleophilicity of a histidine residue by electrostatic interactions between a histidine and an aspartic acid as well as between a histidine and a serine. The interactions would lead to draw a proton from a histidine to an aspartic acid and a serine, thus making the nucleophilicity greatly increased. If an aspartic acid and the serine residues adjacent to a histidine residue interacts with a histidine to draw its proton, β-sheet structure formation of the polypeptide may play an important role in enhancing its catalytic properties based on the results of no formation of the β-sheet structure for the peptide hexamer and the amino acid mixture.

The polypeptide more strongly catalyzes *p*-nitrophenyl acetate hydrolysis than do the other catalysts in all cases of adding organic solvents, such as methanol, ethanol, 2-propanol, and HFIP, as shown in Table 2. This may indicate that a histidine residue interacts with an aspartic acid and serine residues, even in organic/aqueous solutions due to the stable β-sheet structure formation of the polypeptide in those media, and that the stable β-sheet structure may contribute to an enhancement of the hydrolytic properties of the polypeptide.

Table 3 summarizes the second-order rate constants for the hydrolysis of five kinds of substrates with four catalysts in an aqueous solution containing 25 volume% ethanol. Because these substrates precipitate in making the reaction solution when the ethanol content of the reaction solution is less than 25 volume%, the hydrolytic reactions are performed in solution containing ethanol of 25 volume% or above. The rate constants of the hydrolysis of Z–Phe–ONp for polypeptide and the peptide hexamer are higher than those for the other catalysts by a factor of approximately 7 and 2, respectively. The rate constants follow the order Z–Phe–ONp>Z–Val–ONp>Z–Ala–ONp>Z–Gly–ONp> AcONp for all of the catalysts. This order is identical with the hydrophobicity of these substrates, whereas the steric hindrance for the hydrolytic reaction varies inversely with the order. These results may indicate that condensation of the substrates on the polypeptide and the hexamer due to the hydrophobic interactions between the hydrophobic leucine residue of the catalysts and the substrates cancel the steric hindrance. Therefore, the hydrophobic interactions between the hydrophobic leucine residue of the catalysts and the substrates play an important role in the hydrolytic reactions.

Figure 8 shows the effects of organic solvents, such as methanol, ethanol, 2-propanol, and HFIP, on *p*-nitrophenyl acetate hydrolysis by Poly(Asp–Leu–His–Leu–Ser–Leu). The rate constants decrease along with increasing organic solvent content for all cases, and with decreasing dielectric constant of the organic solvents. Because the addition of

Table 2. Rate Constants for Hydrolysis of *p*-Nitrophenyl Acetate in Organic Solvent/Water (pH 8.0) Mixtures

Organic solvent volume %		$k_{cat}/M^{-1}\,min^{-1}$			
		poly(DLHLSL)[a]	DLHLSL[b]	D/H/S/L[c] mixture	Imidazole
Methanol	10%	48.54	23.37	24.98	27.21
	25%	34.21	14.90	14.72	15.62
	50%	14.25	7.03	7.33	7.61
Ethanol	10%	34.95	21.73	21.26	23.16
	25%	29.67	9.65	10.02	11.93
	50%	7.73	5.48	6.14	6.83
2-Propanol	10%	32.35	18.98	19.32	20.53
	25%	25.78	11.95	11.18	11.87
	50%	3.89	4.12	4.46	4.29
HFIP	10%	23.53	14.25	14.85	15.43
	25%	3.15	2.56	2.84	2.55
	50%	1.34	2.06	1.74	1.96

a) Poly(Asp–Leu–His–Leu–Ser–Leu). b) Asp–Leu–His–Leu–Ser–Leu. c) Mixture of Asp, His, Ser, and Leu. Molar ratio: [Asp]/[His]/[Ser]/[Leu]=1/1/1/3. k_{cat} was calculated on His concentration.

Table 3. Rate Constants (k_{cat} ($M^{-1}\,min^{-1}$)) for Hydrolysis of Five Kinds of Substrates in Water (pH 8.0) Containing 25 Volume% Ethanol at 25 °C

Catalyst	AcONp	Z–Gly–ONp	Z–Ala–ONp	Z–Val–ONp	Z–Phe–ONp
Poly(Asp–Leu–His–Leu–Ser–Leu)	29.67	36.88	79.21	104.36	127.24
Asp–Leu–His–Leu–Ser–Leu	9.65	12.45	23.39	29.08	34.83
Asp/His/Ser/Leu mixture[a]	10.02	10.57	14.87	17.26	18.17
Imidazole	11.93	12.24	15.56	16.88	17.79

a) Molar ratio: [Asp]/[His]/[Ser]/[Leu]=1/1/1/3. k_{cat} was calculated on His concentration.

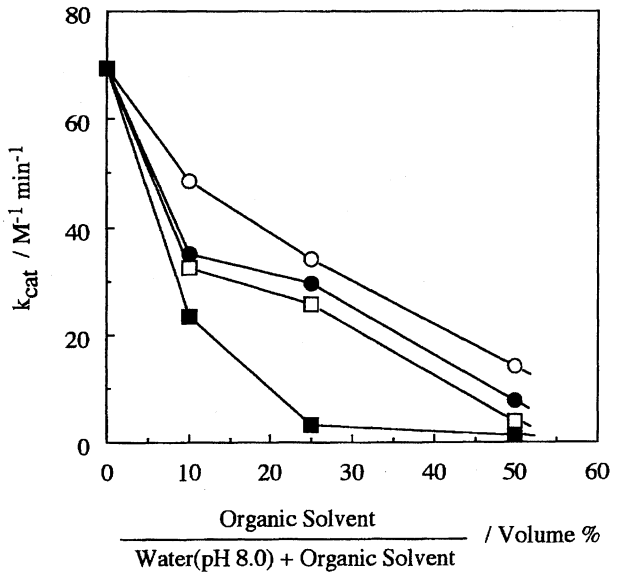


Fig. 8. Effects of organic solvents on p-nitrophenyl acetate hydrolysis by poly(Asp–Leu–His–Leu–Ser–Leu) at 25 °C. methanol (○); ethanol (●); 2-propanol (□); and HFIP (■).

organic solvents to water lowers the dielectric constant of a solvent, and, therefore, the electrostatic interactions become greater, the state of orderliness of the water molecules increases.[21] Consequently, a reduction of the catalytic activity may reflect an increase in the restriction of free movements of the catalysts and the substrate molecules by the ordering of the water molecules in organic solvents/water mixtures. Another explanation is that if in organic/aqueous mixtures pK_a of an imidazolyl group may increase compared to that in a pure aqueous solution, the amount of free base species of a imidazolyl group would become lower, and, therefore, the rate constants would become lower.[21]

Figure 9 shows the effect of the ethanol content on the hydrolytic rate constants for the five substrates. The rate constants decrease along with increasing ethanol content for all of the substrates. These results may be responsible for the decrease in the hydrophobic interaction along with increasing ethanol content, in addition to the two possible explanations described above.

Since the alternating amphiphilic polypeptide, poly-(Asp–Leu–His–Leu–Ser–Leu), forms a stable β-sheet structure in water or a water/alcohol mixture, the stability could be caused by electrostatic interactions between hydrophilic residues in addition to hydrophobic interactions between leucine residue. The polypeptide is a more effective catalyst than is imidazole for the hydrolysis of *p*-nitrophenyl acetate in water or a water/alcohol mixture, and catalyzed hydrolytic reactions of the substrates more effectively with increasing hydrophobicity. The enhanced hydrolytic catalyst of the polypeptide would be responsible for increasing

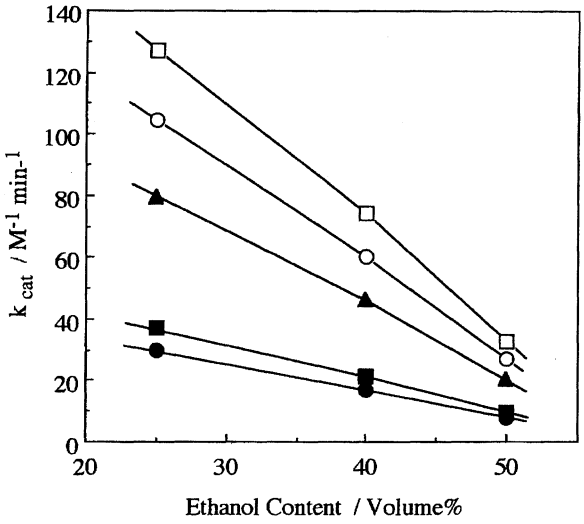


Fig. 9. Dependence of rate constants for hydrolysis of five kinds of substrates by poly(Asp–Leu–His–Leu–Ser–Leu) on ethanol content at 25 °C. AcONp (●); Z–Gly–ONp (■); Z–Ala–ONp (▲); Z–Val–ONp (○); and Z–Phe–ONp (□).

the nucleophilicity by either electrostatic interactions or the condensation effect of the substrates by hydrophobic interactions. The polypeptide can be a good model for constructing de novo designed proteins and peptides with catalytic properties involving β-sheet structures.

I thank Dr. M. Hirami of Unitika Ltd. for many helpful discussions and valuable comments concerning this work.

References

1) A. Brack and L. E. Orgel, *Nature*, **256**, 383 (1975).
2) W. B. Rippon, H. H. Chen, and A. G. Walton, *J. Mol. Biol.*, **75**, 369 (1973).
3) S. L. Kielland, L. A. Slotin, and R. E. Williams, *Can. J. Chem.*, **56**, 2657 (1978).
4) Y. Trudelle, *Polymer*, **16**, 9 (1975).
5) S. Saint-Pierre, R. T. Ingwall, M. S. Verlander, and M. Goodman, *Biopolymers*, **17**, 1837 (1978).
6) Y. Fukushima, *Polym. J.*, in press.
7) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature*, **221**, 337 (1969).
8) A. R. Fersht and Y. Requena, *J. Am. Chem. Soc.*, **93**, 7079 (1971).
9) D. M. Shotton and H. C. Watson, *Nature*, **225**, 811 (1970).
10) A. R. Fersht and J. Sperling, *J. Mol. Biol.*, **74**, 137 (1973).
11) Abbreviations: HFIP, hexafluoro-2-propanol; TFE, trifluoroethanol; OBzl, benzyl ester; Bzl, benzyl; Tos, tosyl; OSu, *N*-hydroxysuccinimde ester; Boc, *t*-butoxycarbonyl; OPcp, pentachlorophenyl ester; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; ONp, *p*-nitrophenyl ester; AcONp, *p*-nitrophenyl acetate; CD, circular dichroism; DCC, dicyclohexylcarbodiimide; THF, tetrahydrofuran; TMS, tetramethylsilane; M_n, number-average molecular weight; M_w, weight-average molecular weight.
12) P. Doty, J. H. Bradbury, and A. M. Holtzer, *J. Am. Chem. Soc.*, **78**, 947 (1956).
13) T. Takakura, T. Konno, and H. Meguro, *Anal. Sci.*, **1**, 215 (1985).
14) N. Greenfield and G. D. Fasman, *Biochemistry*, **8**, 4108 (1969).
15) T. Sugihara, Y. Imanishi, T. Higashimura, and Y. Shimonishi, *J. Org. Chem.*, **41**, 2584 (1976).
16) K. Nakajima and K. Okawa, *Bull. Chem. Soc. Jpn.*, **46**, 1811 (1973).
17) J. Kovacs, R. Giannotti, and A. Kapoor, *J. Am. Chem. Soc.*, **88**, 2282 (1966).
18) M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 539 (1978).
19) R. F. Epand and H. A. Sheraga, *Biopolymers*, **6**, 1383 (1968).
20) J. R. Parrish and E. R. Blout, *Biopolymers*, **11**, 1001 (1972).
21) H. J. Goren and T. Fletcher, *Biopolymers*, **17**, 1679 (1978).