Studies of Bitter Peptides from Casein Hydrolyzate. I. Synthesis of Bitter Peptide BPIa Corresponding to Arg-Gly-Pro-Pro-Phe-Ile-Val from Casein Hydrolyzate by Alkaline Proteinase of *Bacillus subtilis*[1]

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A bitter heptapeptide BPIa was synthesized and compared with the natural peptide, isolated by Minamiura *et al.* from casein hydrolyzate, by means of thin layer chromatography, paper electrophoresis, and carboxymethylcellulose column chromatography. All results for the two peptides matched very closely each other. The synthetic BPIa has an extremely bitter taste with its threshold value 0.05 mM. It is one of the most bitter compounds such as phenylthiourea and quinine.

In recent years, many people have investigated chemical properties of various bitter peptides. Some bitter peptides have been isolated from natural foodstuffs such as cheese, natto, sake, and cocoa.[2-5] In addition, some workers have reported that hydrolysis of proteins with proteolytic enzymes is usually accompanied by formation of bitter taste.[6-9] Minamiura *et al.*[8,9] isolated bitter peptides from casein hydrolyzate by alkaline proteinase of *Bacillus subtilis*, and determined their amino acid sequences as shown in Fig. 1.

BPIa Arg-Gly-Pro-Pro-Phe-Ile-Val

BPIb Leu-Val-Pro-Arg-Tyr-Phe-Gly-----

BPIc Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His

BPII cyclo-(Trp-Leu-Trp-Leu)

Fig. 1. Amino acid sequence of bitter peptides from casein hydrolyzate by Minamiura *et al.*[8,9]

Although they reported that these peptides possessed a strong bitter taste, they did not report any other characteristics of these peptides. In connection with studies of the relationship between chemical structure and bitter taste, Shiba and Nunami[10] synthesized some cyclic oligopeptides, including Minamiura's BPII (cyclo-tryptophylleucyltryptophylleucyl). The results indicated that the structure of Minamiura's BPII was actually cyclo-tryptophylleucyl. They also reported that the threshold value of the synthetic cyclo-tryptophylleucyl was 0.063 mM (1 M=1 mol dm^{-3}). The value of the peptide anhydride is the same as that of phenylthiourea and quinine. They are one of the most bitter compounds known.

As for the linear decapeptide BPIc, one of the authors synthesized and compared it with natural BPIc. Details will be described in another investigation.[11] The present paper deals with synthesis of BPIa, measurement of its threshold value of bitter taste, and comparison between natural and synthetic BPIa.

The synthetic route for BPIa is shown in Fig. 2. *N*-(*t*-Butoxycarbonyl)isoleucine was coupled with valine benzyl ester by means of the mixed anhydride method to form *N*-(*t*-butoxycarbonyl)isoleucylvaline benzyl ester (**1**). Removal of the *t*-butoxycarbonyl group from **1** with hydrogen chloride in dioxane afforded the corresponding dipeptide ester hydrochloride (**2**). Compound **2** was coupled with *N*-(*t*-butoxycarbonyl)phenylalanine by the mixed anhydride method to yield acyltripeptide ester (**3**). Removal of the *t*-butoxycarbonyl group from **3** with hydrogen chloride in dioxane afforded the corresponding tripeptide ester hydrochloride (**4**). *N*-(*t*-Butoxycarbonyl)proline and proline benzyl ester were condensed to yield *N*-(*t*-butoxycarbonyl)prolylproline benzyl ester (**5**) by the dicyclohexylcarbodiimide method. Hydrogenation of **5** easily gave the corresponding acid (**6**). Compounds **6** and **4** were condensed by the same method as was used in the preparation of **1**, and the resulting acylpentapeptide ester (**7**) was converted to the corresponding pentapeptide ester hydrochloride (**8**) by action of hydrogen chloride in dioxane. Saponification of N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylglycine benzyl ester (**9**), derived from N^{α}-benzyloxycarbonyl-N^{G}-nitroarginine and glycine benzyl ester by the mixed anhydride method, gave the corresponding acid (**10**). Compounds **10** and **8** thus obtained were condensed by the dicyclohexylcarbodiimide method to yield the fully protected BPIa (**11**). Catalytic hydrogenation of **11** in methanol–acetic acid (1:1, v/v) gave a heptapeptide BPIa. The synthetic BPIa and its intermediates were subjected to measurement for melting point and specific rotation. Their purity was confirmed by thin layer chromatography on two and/or three solvent systems and by elemental analyses. The homogeneity of the final product was also confirmed by amino acid analysis, paper electrophoresis, and carboxymethylcellulose column chromatography.

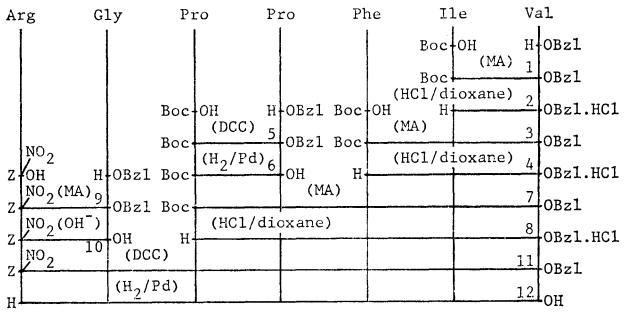

Fig. 2. Synthesis of BPIa.

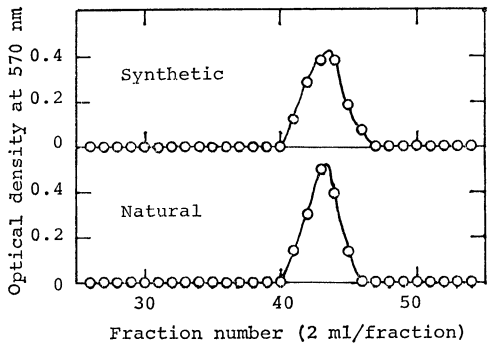

Fig. 3. Carboxymethylcellulose column chromatography of synthetic and natural BPIa.
Solvent: 0.2 M pyridinium acetate, pH 5.0.

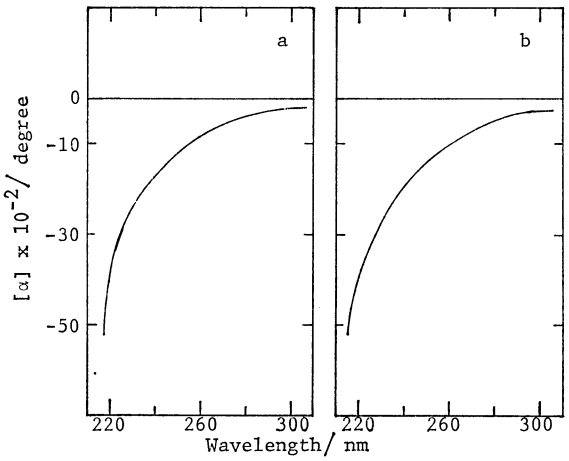

Fig. 4. ORD curves of synthetic BPIa.
Solvent: a; water, b; ethanol.

The synthetic BPIa was then compared with natural BPIa[12] isolated by Minamiura *et al.* Both compounds gave a single spot and identical R_f value on thin layer chromatography. The mobility of synthetic BPIa was indistinguishable from that of natural BPIa on paper electrophoresis. Both compounds also gave the same pattern on carboxymethylcellulose column chromatography as shown in Fig. 3. The ORD curves of synthetic BPIa measured in both aqueous and ethanol solutions are presented in Fig. 4. In both media BPIa exhibited similar curves.

The bitterness of synthesized BPIa was organoleptically determined *via* panel evaluation by four people; the threshold value of BPIa was found 0.05 mM. It is one of the most bitter compounds that are known now.

Dumas *et al.*[13] determined the whole primary structure of bovine β-casein which contains a partial sequence of -Arg^{202}–Gly–Pro–Phe–Pro–Ile–Ile–Val^{209} in the C-terminal portion. Although Minamiura's BPIa cannot be found in the structure of Dumas' formula, it is similar to the C-terminal of that formula. Hashimoto *et al.*[14] synthesized a heptapeptide, H–Gly–Pro–Phe–Pro–Ile–Ile–Val–OH, corresponding to the C-terminal portion (203—209) and compared it with the natural peptide isolated from tryptic hydrolyzate of casein. He reported that the two peptides matched closely each other and supported Dumas' formula. In the same laboratory, Aoyagi and Izumiya[15] suggested, from a result that H–Arg–Gly–Pro–Phe–Pro–Ile–OH (202—207) of Dumas' formula possessed a bitter taste, that Minamiura's BPIa is to be changed so as to correspond to the C-terminal of that formula. However, we submit that Minamiura's findings are indentical with our own as shown herein. Therefore, we suggest that the sequence should be converted at this stage of investigation for the correct C-terminal sequence of β-casein.

Experimental

All the melting points are uncorrected. The thin layer chromatography was carried out on Merck silica gel G. The developing solvents used were 1) 1-butanol–acetic acid–pyridine–water (4:1:1:2, v/v), 2) 1-butanol–acetic acid–water (4:2:1, v/v), and 3) chloroform–methanol (5:1, v/v). Spots of materials possessing free amino group on a thin layer plate were detected by spraying ninhydrin, and those with blocked amino group, by spraying 25% hydrogen bromide in acetic acid and then ninhydrin. Optical rotations were measured on a JASCO automatic polarimeter, DIP-SL type. Amino acid analysis was performed with a Hitachi amino acid analyzer, KLA-5 type. Prior to analysis, compounds were dried over phosphorus pentaoxide at 66 °C and 2 mmHg (1 mmHg=133.332 Pa) for 2 h.

Synthesis of BPIa. N-*(t-Butoxycarbonyl)isoleucylvaline Benzyl Ester (**1**)*: Boc–Ile–OH·DCHA (9.08 g, 22 mmol) was dissolved in ethyl acetate (150 ml), and 1 M sulfuric acid (50 ml) was added to the mixture with stirring. The organic layer was washed with water and dried over anhydrous sodium sulfate. The solution was concentrated to dryness *in vacuo*, and the oily residue was dissolved in tetrahydrofuran (40 ml) and *N*-methylmorpholine (2.2 ml, 20 mmol). Ethyl chloroformate (2.0 ml, 20 mmol) was added to the mixture at −5 °C. After 10 min, a solution of H–Val–OBzl·TsOH (7.59 g, 20 mmol) and *N*-methylmorpholine (2.2 ml, 20 mmol) in chloroform (40 ml) was added to the mixture. The reaction mixture was stored in an ice bath for 1 h, and then at room temperature overnight. The mixture was evaporated *in vacuo*, and the oily residue was dissolved in ethyl acetate. The solution was washed successively with water, 4% citric acid, 4% sodium hydrogencarbonate, and water, and then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo*. The oily residue was crystallized with petroleum ether; yield 5.67 g (67%); mp 93—94 °C (lit,[14] 89—91 °C); $[\alpha]_D^{20}$ −50.0° (*c* 1, methanol) (Lit,[14] −48° (*c* 1.9, ethanol)); R_f^1 0.98, R_f^2 0.98, and R_f^3 0.93.

Found: C, 65.56; H, 8.61; N, 6.61%. Calcd for $C_{23}H_{36}O_5N_2$: C, 65.69; H, 8.63; N, 6.66%.

*Isoleucylvaline Benzyl Ester Hydrochloride (**2**)*: To a solution of **1** (4.21 g, 20 mmol) in dioxane (10 ml) 4 M hydrogen chloride in dioxane (40 ml) was added. The solution was allowed to stand at room temperature. After 1 h, the solution was evaporated *in vacuo*. The residual oil was solidified with ether: yield 3.36 g (95%); mp 154—155 °C; $[\alpha]_D^{20}$ −11.0° (*c* 1, methanol); R_f^1 0.80, R_f^2 0.90, and R_f^3 0.47.

Found: C, 60.30; H, 8.18; N, 7.72%. Calcd for $C_{18}H_{29}O_3N_2Cl$: C, 60.57; H, 8.19; N, 7.85%.

N-*(t-Butoxycarbonyl)phenylalanylisoleucylvaline Benzyl Ester (**3**)*: This was prepared from Boc–Phe–OH·DCHA (4.91 g, 11 mmol) and **2** (3.57 g, 10 mmol) in the same way as described above. The product was solidified from ether–petroleum

ether: yield 5.16 g (91%); mp 148 °C; $[\alpha]_D^{20}$ −48.5° (*c* 1, methanol); R_f^1 0.99, R_f^2 0.99, and R_f^3 0.85.

Found: C, 67.93; H, 8.10; N, 7.48%. Calcd for $C_{32}H_{45}O_6N_3$: C, 67.70; H, 7.99; N, 7.40%.

*Phenylalanylisoleucylvaline Benzyl Ester Hydrochloride (**4**)*: This was prepared from **3** (5.68 g, 10 mmol) by the same procedure as described for the preparation of **2**: yield 4.96 g (98%); mp 231 °C; $[\alpha]_D^{20}$ −37.0° (*c* 1, methanol); R_f^1 0.92, R_f^2 0.94, and R_f^3 0.78.

Found: C, 64.28; H, 7.70; N, 8.17%. Calcd for $C_{27}H_{38}O_4N_3Cl$: C, 64.33; H, 7.60; N, 8.34%.

N-*(t-Butoxycarbonyl)prolylproline Benzyl Ester (**5**)*: To a suspension of Boc–Pro–OH (4.30 g, 20 mmol), H–Pro–OBzl·HCl (5.32 g, 22 mmol) and *N*-methylmorpholine (2.42 ml, 22 mmol) in acetonitrile (60 ml), dicyclohexylcarbodiimide (4.50 g, 22 mmol) was added at 0 °C with stirring. The reaction mixture was stirred at 0 °C for 3 h and then at room temperature overnight. *N,N'*-Dicyclohexylurea was filtered off and the filtrate was evaporated *in vacuo*. The oily residue was dissolved in ethyl acetate and the solution was washed successively with water, 4% citric acid, 4% sodium hydrogencarbonate, and water. The solution was dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residual oil weighed 8.0 g (100%). Deber *et al.*[16] also obtained this compound as an oily form: R_f^1 0.96 and R_f^3 0.79.

N-*(t-Butoxycarbonyl)prolylproline (**6**)*: Compound **5** (8.0 g, 20 mmol, oily form) was dissolved in methanol (50 ml) and hydrogenated in the presence of palladium black at room temperature for 48 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was crystallized from ether–petroleum ether. It was recrystallized from hot ethyl acetate: yield 4.60 g (74%); mp 177—180 °C (lit,[17] 187—187.5 °C); $[\alpha]_D^{20}$ −121.5° (*c* 1, methanol); R_f^1 0.70, R_f^2 0.93, and R_f^3 0.48.

Found: C, 57.41; H, 7.67; N, 8.84%. Calcd for $C_{15}H_{24}O_5N_2$: C, 57.67; H, 7.74; N, 8.79%.

N-*(t-Butoxycarbonyl)prolylprolylphenylalanylisoleucylvaline Benzyl Ester (**7**)*: Compounds **6** (1.56 g, 5 mmol) and **4** (2.52 g, 5 mmol) were coupled by the same method as described for the preparation of **1**: yield 3.49 g (92%); mp 109—110 °C; $[\alpha]_D^{20}$ −101.0° (*c* 1, methanol); R_f^1 0.96, R_f^2 0.99, and R_f^3 0.67.

Found: C, 66.46; H, 7.83; N, 8.97%. Calcd for $C_{42}H_{59}O_8N_5$: C, 66.22; H, 7.81; N, 9.19%.

*Prolylprolylphenylalanylisoleucylvaline Benzyl Ester Hydrochloride (**8**)*: Compound **7** (3.80 g, 5 mmol) was treated in the same way as described for the case of **2**: yield 3.40 g (97%); mp 191 °C; $[\alpha]_D^{20}$ −99.0° (*c* 1, methanol); R_f^1 0.81, R_f^2 0.50, and R_f^3 0.45.

Found: C, 63.82; H, 7.45; N, 10.25%. Calcd for $C_{37}H_{52}O_6N_5Cl$: C, 63.64; H, 7.51; N, 10.03%.

N^{α}-*Benzyloxycarbonyl-*N^{G}-*nitroarginylglycine Benzyl Ester (**9**)*: To a solution of Z–Arg(NO_2)–OH (7.06 g, 20 mmol) and *N*-methylmorpholine (2.2 ml, 20 mmol) in tetrahydrofuran (20 ml), ethyl chloroformate (2.0 ml, 20 mmol) was added at −5 °C. After 10 min, a solution of H–Gly–OBzl·TsOH (6.74 g, 20 mmol) and *N*-methylmorpholine (2.2 ml, 20 mmol) in *N,N*-dimethylformamide (20 ml) was added to the mixture. The reaction mixture was stored in an ice bath for 1 h, and then at room temperature overnight. The mixture was evaporated *in vacuo*. The precipitate which was formed upon addition of water to the residue was collected, washed with 0.5 M hydrochloric acid, 4% sodium hydrogencarbonate, and water, and dried. It was dissolved in *N,N*-dimethylformamide (10 ml) and solidified with ethyl acetate–ether: yield 8.70 g (87%); mp 150—151 °C (lit,[18] 145—147 °C); $[\alpha]_D^{20}$ −3.5° (*c* 1, *N,N*-dimethylformamide) (lit,[18] −14.7° (*c* 1, methanol)); R_f^1 0.95, R_f^2 0.99, and R_f^3 0.66.

Found: C, 55.31; H, 5.69; N, 16.79%. Calcd for $C_{23}H_{28}O_7N_6$: C, 55.19; H, 5.64; N, 16.79%.

N^{α}-*Benzyloxycarbonyl-*N^{G}-*nitroarginylglycine (**10**)*: To a suspension of **9** (5.00 g, 10 mmol) in methanol (20 ml), 1 M sodium hydroxide (12 ml, 12 mmol) was added. The mixture was allowed to stand at room temperature. After 3 h, the mixture was evaporated *in vacuo* and 2 M hydrochloric and (7 ml) was added to it. The precipitate thus obtained was collected and washed with water. It was recrystallized from hot ethanol: yield 3.72 g (91%); mp 110—112 °C (Lit,[19] 112 °C); $[\alpha]_D^{20}$ −10.0° (*c* 1, methanol) (lit,[20] −10° (methanol)); R_f^1 0.81, R_f^2 0.85, and R_f^3 0.07.

Found: C, 46.69; H, 5.58; N, 20.66%. Calcd for $C_{16}H_{22}O_7N_6$: C, 46.82; H, 5.40; N, 20.48%.

N^{α} - *Benzyloxycarbonyl* - N^{G} - *nitroarginylglycylprolylprolylphenylalanylisoleucylvaline Benzyl Ester (**11**)*: To a solution of **10** (0.82 g, 2 mmol) in *N,N*-dimethylformamide (10 ml), dicyclohexylcarbodiimide (0.45 g, 2 mmol) was added at 0 °C. After 20 min, a solution of **8** (1.39 g, 2 mmol) and *N*-methylmorpholine (0.22 ml, 2 mmol) in chloroform (10 ml) was added to it. The reaction mixture was stirred at 0 °C for 3 h and then at room temperature overnight. *N,N*-Dicyclohexylurea was removed by filtration, and the filtrate was diluted with ethyl acetate. The solution was washed successively with 0.5 M hydrochloric acid, 4% sodium hydrogencarbonate, and water, dried over anhydrous sodium sulfate, and evaporated *in vacuo*. The residue was solidified with ether. The product was dissolved in methanol (2 ml) and solidified with ethyl acetate–ether: yield 1.45 g (69%); mp 120 °C; $[\alpha]_D^{20}$ −44.0° (*c* 1, *N,N*-dimethylformamide); R_f^1 0.86 and R_f^3 0.64.

Found: C, 60.46; H, 6.71; N, 14.62%. Calcd for $C_{53}H_{71}O_{12}N_{11}$: C, 60.38; H, 6.79; N, 14.62%.

*Arginylglycylprolylprolylphenylalanylisoleucylvaline Diacetate (**12**)*: A solution of **11** (1.05 g, 1 mmol) in methanol—acetic acid (1:1, 10 ml) was hydrogenated in the presence of palladium black at room temperature for 48 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was crystallized by the aid of acetone. It was recrystallized from methanol–ether: yield 0.76 g (97%); $[\alpha]_D^{20}$ −92.5° (*c* 1, water); R_f^1 0.59. The spot was detected by Sakaguchi reagent as well as ninhydrin. Amino acid analysis:[21] Arg 1.06; Gly 1.07; Pro 1.83; Phe 1.11; Ile 1.00; Val 1.05.

Found: C, 55.60; H, 7.71; N, 15.34%. Calcd for $C_{38}H_{60}O_8N_{10}2CH_3COOH$: C, 55.74; H, 7.57; N, 15.48%.

Comparison of Synthetic and Natural BPIa. Thin Layer Chromatography: Both compounds gave a single spot with the R_f^1 value 0.59.

Paper Electrophoresis: This was carried out under the following conditions: paper, Toyo Roshi No. 51 A chromatography paper; solvent, pyridine–acetic acid–water (10:0.4:90, v/v) (pH 6.4); voltage gradient, 15 V/cm; charge period, 2 h. The mobility of synthetic BPIa was 7.8 cm and indistinguishable from that of natural BPIa.

Carboxymethylcellulose Column Chromatography: A portion (*ca.* 1 mg) of synthetic or natural BPIa was dissolved in 0.3 ml of 0.2 M pyridinium acetate (pH 5.0). The solution was applied to a column (1.0 cm × 60 cm) of carboxymethylcellulose. Elution was carried out with the same solvent and 2 ml fractions were collected at a flow rate of 8 ml h^{-1}. The peptide content was determined by the method described by Yemm and Cocking.[22] The results shown in Fig. 3 indicate that the pattern of chromatogram of synthetic BPIa

is indistinguishable from that of natural one.

ORD Measurement: This was performed with a JASCO spectropolarimeter Model ORD/UV-5 over a range 215—300 nm. A cell of path length 0.1 cm was used and runs were made at ambient temperature. Patterns in both aqueous and ethanol solutions are shown in Fig. 4.

Sensory Test: The bitterness of the synthetic BPIa was organoleptically determined *via* panel evaluation by four people. A series of solutions of decreasing concentration was prepared in which each solution was half as strong as its proceeding one. Before testing the sample, the mouth was thoroughly rinsed with deionized water. The sample size was usually 2—3 ml. The sample solution was held in the mouth for *ca.* 10 s and then spit out, and the threshold value of the sample was determined. The threshold value of synthetic BPIa was 0.05 mM, the same as that of phenylthiourea and quinine.

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References

1) The abbreviations recommended by the IUPAC-IUB commission of Biochemical Nomenclature (*J. Biol. Chem.*, **247**, 977 (1972)) have been used. Amino acid symbols except glycine denote L-configuration. Additional abbreviations: DCHA, dicyclohexylamine; TsOH; *p*-toluenesulfonic acid.

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