Synthetic Identification of Bitter Heptapeptide in Tryptic Hydrolysate of Casein

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A heptapeptide, H–Gly–Pro–Phe–Pro–Ile–Ile–Val–OH (**1**), was synthesized and found to be indistinguishable from the natural peptide (**2**), isolated from a tryptic hydrolysate of Hammersten casein by means of chromatography, amino acid analyses, and mass spectrometric measurements. Both peptides (**1** and **2**) have a strong bitter taste.

Bitter peptides have been found widely in proteolytic hydrolysates of proteins. Two bitter peptides were isolated from the proteolytic hydrolysates of casein and their structures determined to be H–Gly–Pro–Phe–Pro–Val–Ile–OH (**3**)[1] and H–Arg–Gly–Pro–Pro–Phe–Ile–Val–OH (**4**).[2] Ribadeau Dumas *et al.* determined the whole primary structure of bovine β-casein[3] which contains the partial sequence of Arg(202)–Gly(203)–Pro–Phe–Pro–Ile(207)–Ile–Val(209)–OH in the *C*-terminal portion. No amino acid sequence of **3** or **4** can be found in the structure of the casein, but **3** and **4** are very similar to the *C*-terminal portion of the casein. Reports have been given on the synthesis of a natural hexapeptide (H–Arg–Gly–Pro–Phe–Pro–Ile–OH) as a sex factor in yeast, its sequence being identical with the 202—207 portion of casein.[4,5] Natural hexapeptide also has a strong bitter taste. Thus, we assumed that a heptapeptide, H–Gly–Pro–Phe–Pro–Ile–Ile–Val–OH (**1**), corresponding to the *C*-terminal portion (203—209) of casein and isolated from tryptic hydrolysate of casein, might show bitterness.

This paper deals with the synthesis of **1** by the conventional method and a comparison of the synthetic peptide with the natural peptide (**2**), isolated from tryptic hydrolysate of casein, by means of chromatography, amino acid analyses, mass spectrometric (MS) measurements and biological assays.

The synthetic route of **1** is shown in Fig. 1.[6] MA and EDC methods were used for the coupling reaction, Boc groups of the intermediates being removed by the action of HCl in EtOAc. Hydrogenation of **11** afforded **1** as dihydrate. The homogeneity of **1**·$2H_2O$ was confirmed by thin-layer chromatography (TLC) and paper chromatography (PPC). For tryptic digestion of casein and the subsequent isolation of the natural bitter peptide (**2**), the methods reported by Ribadeau Dumas *et al.*[3] and Matoba *et al.*[1] were used with slight modifications.

A comparison of **2** with **1** was carried out by TLC, PPC, and amino acid analysis. Compounds **1** and **2** are essentially the same, although **2** contains minor constituents as observed in TLC and amino acid analysis, and both have a strong bitter taste in almost the same threshold value. Field desorption mass spectra (FDMS) of **1** and **2** were measured at two different anode heating currents at 20 and 23 mA, respectively. Patterns of FDMS at 20 mA are shown in Fig. 2. Some peaks assigned to **1** and **2** at 20 mA are as follows: *m/e* 742 ([(M+H)$^+$] of the heptapeptide), 724 (loss of H_2O from [(M+H)$^+$]), and 697 (loss of CO_2 from [M$^+$]). A peak

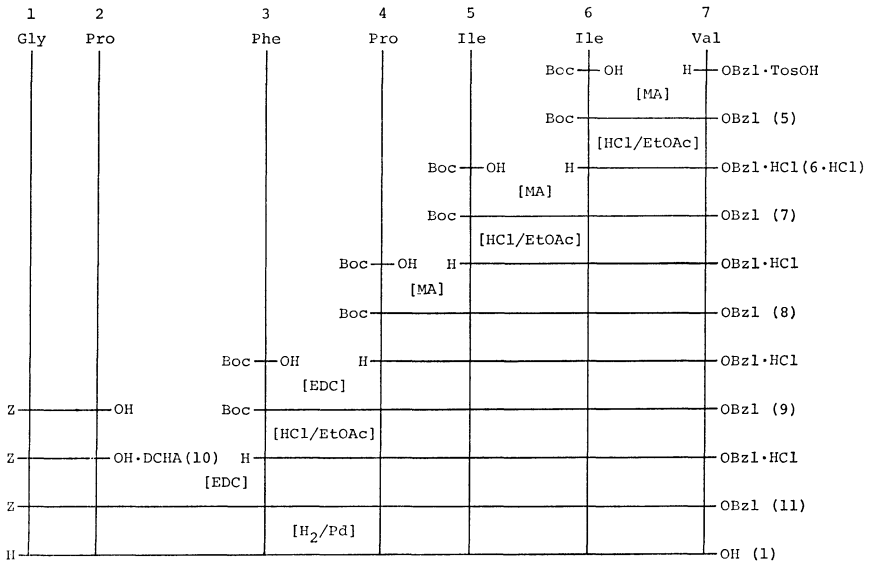

Fig. 1. Synthesis of a heptapeptide, **1**.

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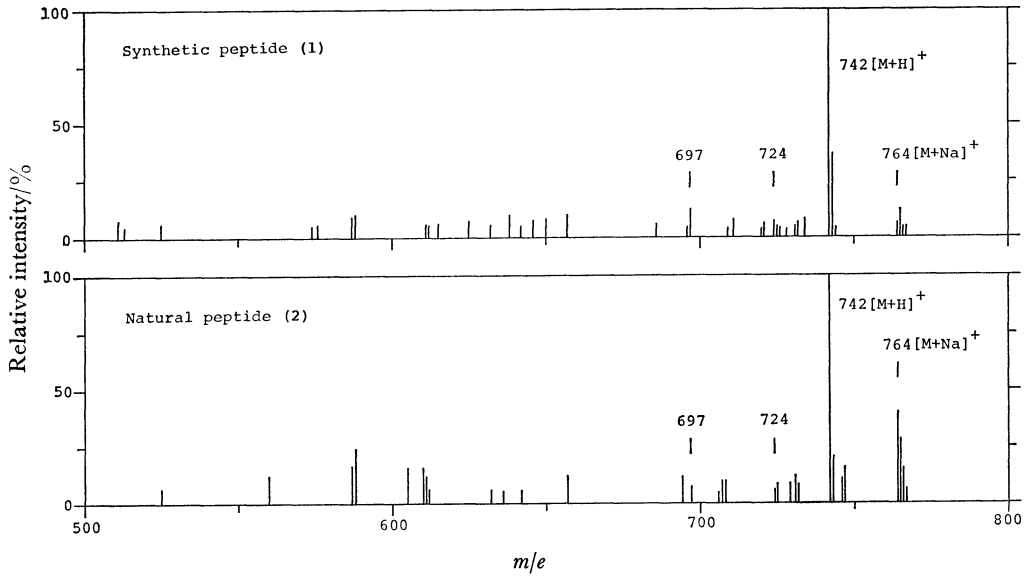

Fig. 2. FDMS of synthetic peptide (**1**) and natural peptide (**2**) measured at anode heating current of 20 mA.

at 23 mA is as follows: *m/e* 640 (loss of CO_2 from H–Pro–Phe–Pro–Ile–Ile–Val–OH which revealed *N*-terminal amino acid as Gly).

The results indicate that **2** is a new bitter peptide having the structure of H–Gly–Pro–Phe–Pro–Ile–Ile–Val–OH, the presence of several hydrophobic amino acids being important for showing bitterness as pointed out by Clegg *et al.*[7,8]

Experimental

TLC was carried out on Merck silica gel G with the systems, R_f^1, $CHCl_3$–MeOH (5 : 1); R_f^2, $CHCl_3$–MeOH (9 : 1); R_f^3, *n*-BuOH–AcOH–pyridine–H_2O (15 : 3 : 10 : 12), and on micro crystalline cellulose (AVICEL) (Funakoshi Yakuhin Co. Ltd., Tokyo) with the system R_f^4, *t*-pentyl alcohol–pyridine–H_2O (10: 4: 5). PPC was performed on Toyo Roshi No. 52 paper with the system R_f^5, the same solvent as used for R_f^4. Optical rotations were measured on a Union high sensitivity polarimeter, PM-71. Amino acid analyses were performed with a Hitachi amino acid analyzer, KLA-5. Crystalline trypsin (Sigma Chemical Co., U. S. A.) and Hammersten casein (E. Merck AG, Darmstadt, Germany) were used.

Boc–Ile–Val–OBzl (5). **5** was prepared from Boc–Ile–OH and H–Val–OBzl·TosOH by the usual MA method;[9] yield, 75%; R_f^1 0.95. Physical constants and elemental analyses of crystalline compounds are given in Table 1.

Boc–Ile–Ile–Val–OBzl (7). Compound **5** (5.0 mmol) was dissolved in 2 M HCl in EtOAc (25 ml). The solution was left to stand at room temperature for 1 h, and evaporated; yield of a solid H–Ile–Val–OBzl·HCl (**6**·HCl), 98%. **7** was then prepared from Boc–Ile–OH and **6**·HCl by the MA method; yield, 75%; R_f^1 0.75.

Boc–Pro–Ile–Ile–Val–OBzl (8). **8** was prepared from Boc–Pro–OH and H–Ile–Ile–Val–OBzl·HCl derived from **7** by the MA method; yield, 84%; R_f^2 0.75.

Boc–Phe–Pro–Ile–Ile–Val–OBzl (9). **9** was prepared from Boc–Phe–OH and H–Pro–Ile–Ile–Val–OBzl·HCl derived from **8** by the usual EDC method;[10] yield, 93%; R_f^2 0.67.

Z–Gly–Pro–OH·DCHA (10). **10** was prepared from Z–Gly–Pro–OH[11] and DCHA by the usual procedure;[12] yield, 66%.

Z–Gly–Pro–Phe–Pro–Ile–Ile–Val–OBzl (11). **11** was prepared from **10** and H–Phe–Pro–Ile–Ile–Val–OBzl·HCl derived from **9** by the EDC method; yield, 88%; R_f^2 0.52.

H–Gly–Pro–Phe–Pro–Ile–Ile–Val–OH (1). Compound **11** (0.31 g, 0.31 mmol) dissolved in a mixture of MeOH (8 ml), AcOH (4 ml), and H_2O (1 ml) was hydrogenated in the presence of Pd black. The filtrate from the catalyst was evaporated to leave a solid. Recrystallization from EtOH–EtOAc gave 0.22 g (91%); R_f^3 0.75, R_f^4 0.81, R_f^5 0.71; mass spectrum, *m/e* 741 (calcd, 741.4). Amino acid ratios in an acid hydrolysate: Pro (2.08), Gly (1.00), Val (1.03), Ile (2.09), Phe (0.99).

Isolation of Bitter Peptide from Tryptic Hydrolysate of Casein. A mixture of casein (10 g) and trypsin (150 mg) in H_2O (1000 ml) was incubated at pH 8.5 and 37 °C for 2 d. After the

Table 1. Analytical data of synthetic peptides

Compound	Mp/°C	$[\alpha]_D^{20}$ in EtOH	Formula	Found (%)			Calcd (%)		
				C	H	N	C	H	N
5	89—91	−48° (*c* 1.9)	$C_{23}H_{36}O_5N_2$	65.55	8.58	6.54	65.69	8.63	6.66
7	173—174	−77° (*c* 2.2)	$C_{29}H_{47}O_6N_3$	64.83	8.84	7.75	65.26	8.88	7.87
8	191—193	−82° (*c* 1.1)	$C_{34}H_{54}O_7N_4$	64.58	8.59	8.78	64.73	8.63	8.88
9	165—166	−81° (*c* 2.0)	$C_{43}H_{63}O_8N_5 \cdot 1/2H_2O$	65.94	8.11	8.79	65.62	8.20	8.90
10	125—126	−37° (*c* 2.6)	$C_{27}H_{41}O_5N_3$	66.15	8.45	8.45	66.50	8.48	8.62
11	133—134	−106° (*c* 1.0)	$C_{53}H_{71}O_{10}N_7 \cdot H_2O$	64.75	7.42	9.86	64.68	7.48	9.96
1	160—163	−116° (*c* 0.5)	$C_{38}H_{59}O_8N_7 \cdot 2H_2O$	58.58	7.99	12.30	58.67	8.16	12.60

solution had been adjusted to pH 7.0, it was extracted with 1-butanol (1000 ml × 3). The butanol phase was concentrated *in vacuo* and lyophilized. A solution of the residue in H_2O (100 ml) was adjusted to pH 10, and then acidified slowly to pH 5.4. The precipitates were removed by filtration, and the filtrate was concentrated and lyophilized. The residue obtained was purified by a series of column chromatography as follows: Sephadex G-25 (2 × 35 cm) with 0.01 M phosphate buffer (pH 7.5), Dowex 50X8 (1 × 45 cm) with 1 M pyridine–AcOH (pH 4.1), Dowex 50X8 (1 × 45 cm) with 0.4 M pyridine–AcOH (pH 3.5), Sephadex G-10 (1.6 × 12 cm) with H_2O, AVICEL (1.6 × 17 cm) with *t*-pentyl alcohol–pyridine–H_2O (10 : 4 : 5), Dowex 1X2 (1.6 × 17 cm) with 0.1 M AcOH and finally Sephadex G-10 (2.0 × 10 cm) with H_2O. In each case, the eluate fractions showing the same R_f values as that of **1** by TLC were collected, concentrated and lyophilized. The residue was dissolved in a small amount of EtOH. EtOAc was added to the solution until precipitates appeared. The precipitates were collected by filtration; yield, 8 mg; R_f^3 0.73, R_f^4 0.81, R_f^5 0.71; mass spectrum, *m/e* 741 (calcd, 741.4). Amino acid ratios in an acid hydrolysate: Pro (2.27), Gly (1.00), Val (1.35), Ile (2.15), Phe (1.27). Minor amounts of Asp, Thr, Ser, Glu, and Ala were also found in the hydrolysate.

FDMS Measurements. Measurements were carried out on a Nihondenshi mass spectrometer Model JMS-01SG-2 equipped with a combined EI/FI/FD source. The mass spectra of **1**·2H_2O and **2** in MeOH (5 mg/ml) were measured at anode heating currents at 20 and 23 mA, respectively.

Bitterness Evaluation. The degree of bitterness was determined according to the method reported by Matoba *et al.*[1] Compounds **1**·2H_2O and **2** have the same degree of bitterness at 0.17—0.34 mM (0.13—0.25 mg/ml).

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