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Amino Acid Composition of Phosphopeptide produced in the Small Intestinal Tract of Rat fed on Casein

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(Figs. 1-a - 5-b, Tables 1-6)

Whether or not the nutritional value of protein or peptide is equal to that of the free amino acids mixture with an amino acid composition equivalent to that of protein or peptide is a significant problem in nutritional biochemistry. Although the presence of peptide in the digestive organ of mammalian has been reported, its nutritional significance however has not been elucidated yet.

Recently, MELLANDAR[1] and NAITO *et al.*[2] suggested that casein phosphopeptide contributes to a high utilization of calcium in milk. But what kinds of amino acid residues or functional groups contribute to the calcium-binding property of casein phosphopeptide is not yet evident.

In view of this, casein phosphopeptide samples were prepared from the intestinal contents of rats fed on casein and their amino acid compositions were determined.

MATERIALS AND METHODS

1. Preparation of β-casein

β-Casein was prepared by the urea method as modified by ASCHAFFENBERG[3] and about 20 mg of sample were obtained from 5 *l* of fresh skim milk. According to the method of GROVES *et al.*,[4] the purity of the samples was checked by disc electrophoresis performed with a 7% polyacrylamide gel containing 4 M urea. The sample was found to be almost homogeneous with β-casein, though α_S- and κ-casein could be detected slightly.

2. Brreeeding methods of rats

Eight rats of one weak old were fed by way of a space feeding for eight days on the diet as shown in Table 1. After the preparative breeding period, they were divided into two equal groups and the names of H and β were given under

Table 1. The diet of rats for preparative breeding periods.

Commercial first grade casein	(20%)	200 g
α-starch	(38%)	380 g
Vitamin Mineral Fibre	(5.5%)	55 g
soy bean oil	(6.5%)	65 g
sucrose	(30%)	300 g
sum		1000 g

Table 2. The diet for H-group.

Hammarsten casein	(20%)	18 g
α-starch	(38%)	34.2 g
Vitamin Mineral Fibre	(5.5%)	4.95 g
soy bean oil	(6.5%)	5.85 g
sucrose	(30%)	27 g
choline chloride		0.18 g
sum		90.18 g

Table 3. The diet for β-group.

β-casein	(20%)	18 g
α-starch	(38%)	34.2 g
Vitamin Mineral Fibre	(5.5%)	4.95 g
soy bean oil	(6.5%)	5.85 g
sucrose	(30%)	27 g
choline chloride		0.18 g
sum		90.18 g

Table 4. The growing records of each rat.

	H-1		H-2		H-3		H-4	
	body weight	weight of diet	body weight	weight of diet	body weight	weight of diet	body weight	weight of diet
1st day	120 g	15 g	172 g	20 g	188 g	20 g	188 g	20 g
2		17		16		20		20
3	138	0	168	0	196	4	194	4
4	128	5	164	10	186	10	188	8
5	128	7	162	10	182	10	186	8
6	124	6	162	10	178	9	183	7
7	121	9	162	10	173	10	180	8
8	122	7	164	9	173	9	178	9
9	119	10	163	10	173	7	180	6
10	119	10	160	9	169	9	176	5
just before killing	134	(0.7)*	173	(1.2)*	181	(0.1)*	189	(0.2)*

	β-1		β-2		β-3		β-4	
	body weight	weight of diet	body weight	weight of diet	body weight	weight of diet	body weight	weight of diet
1st day	172 g	20 g	180 g	20 g	184 g	20 g	142 g	20 g
2		20		20		20		20
3	186	4	194	4	192	8	162	4
4	176	8	182	12	186	12	152	10
5	173	8	182	14	184	14	156	9
6	172	8	181	9	185	12	158	9
7	172	11	176	12	184	13	158	12
8	172	12	175	13	188	12	162	12
9	173	10	176	10	187	10	164	9
10	171	10	173	10	183	9	164	10
just before killing	186	(0.2)*	192	(0.3)*	196	(0.3)*	182	(0.1)*

( )* the lost weight of diet

their respective diets. H- and β-group were space-fed for two days on the diets as shown in Table 2 and 3, respectively. The growing records of each rat are shown in Table 4.

3. Isolation of casein phosphopeptide

On the 10th day, that is the last day of the breeding period, each individual rat was given its diet with a time lag of three minutes and then killed under ether anaethesia at exactly one hour after the meal. The intestinal contents of each rats were washed out with cold water. The washings were frozen in dry ice-acetone and stored at −20°C. To the frozen samples, after thawing to such an extent that small amounts of ice crystall remained, a few drops of 50% trichloroacetic acid (TCA) solution were added first and then larger quantities of an ice-cold TCA solution in order to bring the final concentration up to about 10%. The mixture was homogenized and centrifuged (10,000×g, 20 min, 10°C). A 5 m*l* portion of the supernatant was filtered with a Sephadex G–25 column previously washed with 0.05 N acetic acid and a N/P ratio of the fraction eluted at the void volume was measured. Each of the remaining supernatants of H- and β-group was eluted by successive gel filtrations with Sephadex G–25 and G–50 columns previously washed with 0.05 N acetic acid. The fractions eluted at the void volume were chromatographed on Dowex 50×2 columns equilibrated previously with 0.2 N pyridine-acetate buffer, pH 3.0,[5] and finally desalted by the Sephadex G–25 filtration. In the above gel filtrations and chromatography, the effluents were fractionated to 5 m*l* and 10 m*l* portion, respectively.

4. Determination of peptide and organic phosphorus

The amount of peptide was determined by the ninhydrin colorimetry,[6] and that of organic phosphorous by the modified ALLEN's method.[7]

5. Amino acid analysis

Amino acid analysis of the casein phosphopeptide sample was done by the method of SPACKMAN *et al.*[8] using an automatic amino acid analyzer (Hitachi Model 034). The sample of 1.1 mg was hydrolyzed with 3.3 m*l* of 6 N HCl in a sealed tube for 24 hrs at 110°C. After removing humin and HCl by filtration and evaporation, respectively, the hydrolyzate was kept *in vacuo* overnight and dissolved in 1.5 m*l* of 0.2 N citrate buffer (pH 2.2). A 0.5 m*l* portion of the hydrolyzate solution was used for the analysis of amino acid composition.

RESULTS

1. Phosphopeptide obtained from the H-group

The mean value of the N/P ratios of the supernatants in the TCA treatment was found to be about 100. The remaining supernatants from individnal rats

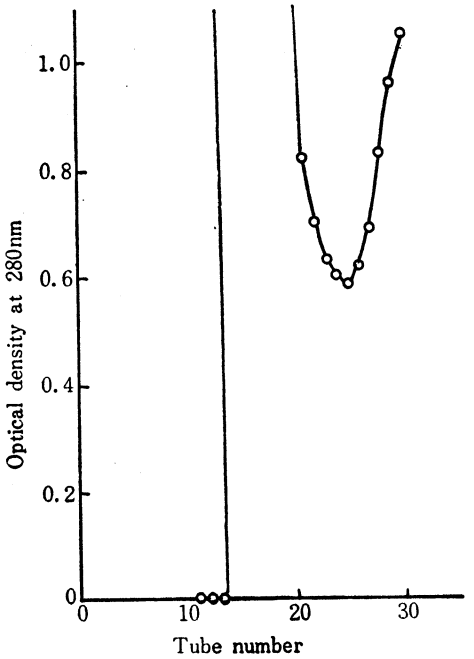

Fig. 1-a Gel filtration pattern of H-group on a Sephadex G-25 column.
Column : 2.5×40 cm
Eluent : 0.25 N acetic acid

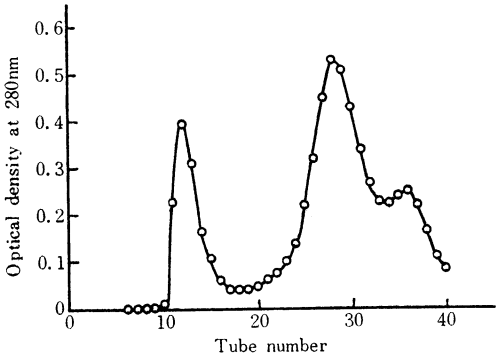

Fig. 2-a Gel filtration pattern of H-group on a Sephadex G-50 column.
Column : 2.5×40 cm
Eluent : 0.05 N acetic acid

were combined and concentrated. The concentrate was filtered with a Sephadex G–25 column. The elution curve is shown in Fig. 1–a. After concentration, the effluent (Tube Nos. 14–25) was filtered again with a Sephadex G–50 column. The elution curve is shown in Fig. 2–a. Among the three peaks, the fraction of the second peak was concentrated and filtered with a Sephadex G–25 column, because the second one contained phosphopeptide. The result obtained is shown in Fig. 3–a. The N/P ratio of the fraction eluted at the void volume was 17.6; the value is one-fifth of that of the original supernatant. To the fraction, after concentrated to 10 m*l* volume, 2.5 m*l* of 1 N pyridine-acetate buffer was added so as get 0.2 N pyridine in the final concentration. After the pH was adjusted to 2.5 with 6 N HCl, the sample was inserted on the upper bed of a column of Dowex 50×2.. The elution curve

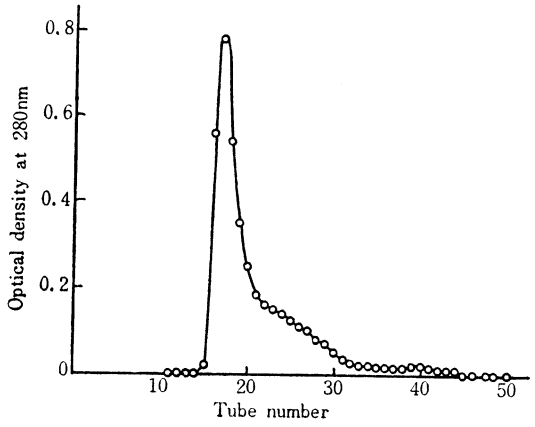

Fig. 3–a Gel filtration pattern of H-group on a Sephadex G–25 column.
Column : 2.5×40 cm
Eluent : 0.05 N acetic acid

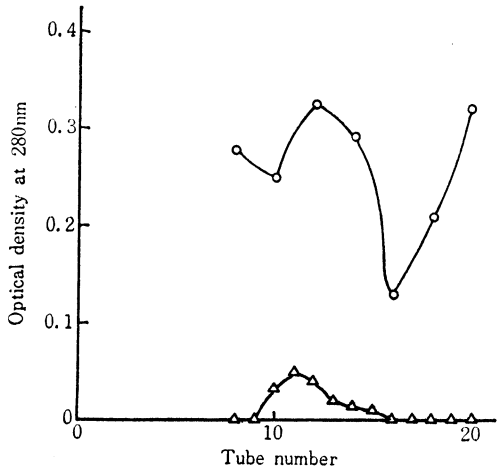

Fig. 4–a Chromatography of H-group with a Dowex 50×2 column.
Column : 1.8×50 cm
Eluent : 0.2 N pyridine-acetate buffer (pH 3.0)
○—○ peptide (O.D. at 570 nm)
△—△ organic phosphorus (O.D. at 660 nm)

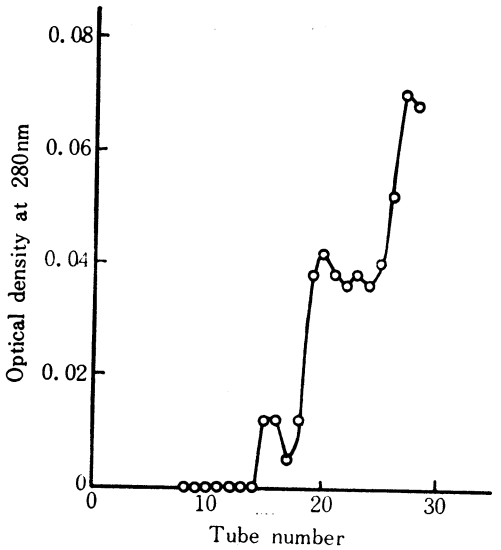

Fig. 5–a Desaltation of H-group with a Sephadex G–25 colum.
Column : 2.5×40 cm
Eluent : water

Table 5. The amino acid compositions of isolated phosphopeptides from the intestinal tract.

Amino Acid	H-group	β-group
Lys	−	−
His	−	−
NH_3	26	7.5
Arg	−	−
Asp	4	1.75
Thr	5.5	4.75
Ser	10	5.5
Glu	9.5	5.25
Pro	+	+
Gly	2.5	1.75
Ala	2.5	1.25
Cys	−	−
Val	2.5	1.75
Met	+	±
Ileu	3.0	1.0
Leu	1.0	1.0
Tyr	−	−
Phe	+	+
Trp	N.D.*	N.D.*

(Leu=1.0)
*N.D.=not determined

shown in Fig. 4-a was obtained. The fraction (Tube Nos. 10–15) containing organic phosphorus was desalted with a Sephadex G-25 column (Fig. 5-a). The fraction eluted at the void volume was lyophilized and about 6.3 mg of phosphopeptide was obtained as a purified sample. This sample of 1.1 mg was used for amino acid analysis and found to have the composition as shown in Table 5.

2. Phosphopeptide obtained from the β-group

The N/P ratios of the supernatants by the TCA treatment from individual rats of the β-group (β-1, β-2, β-3 and β-4) were 58.4, 54.0, 56.7 and 94.3,

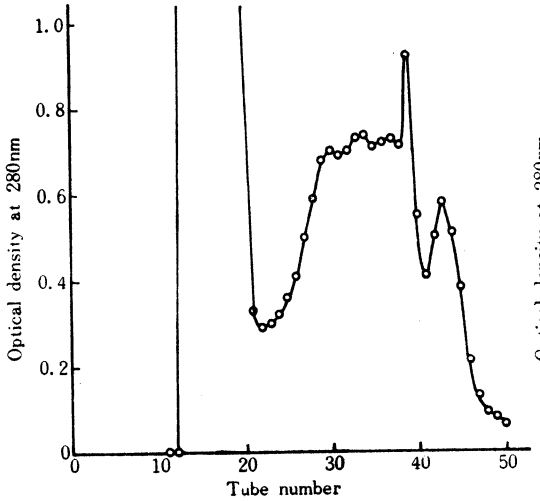

Fig. 1-b Gel filtration pattern of β-group on a Sephadex G-25 column.
Column : 2.5×40 cm
Eluent : 0.05 N acetic acid

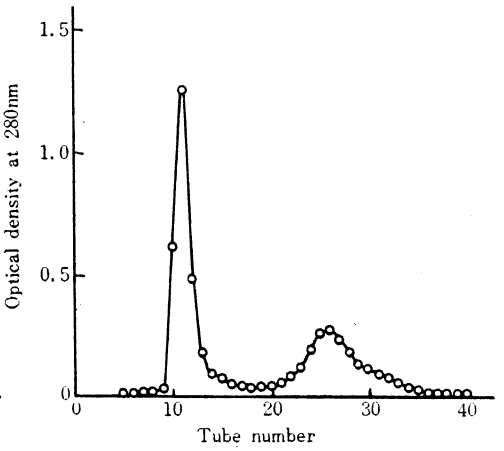

Fig. 2-b Gel filtration pattern of β-group on a Sephadex G-50 column.
Column : 2.5×40 cm
Eluent : 0.05 N acetic acid

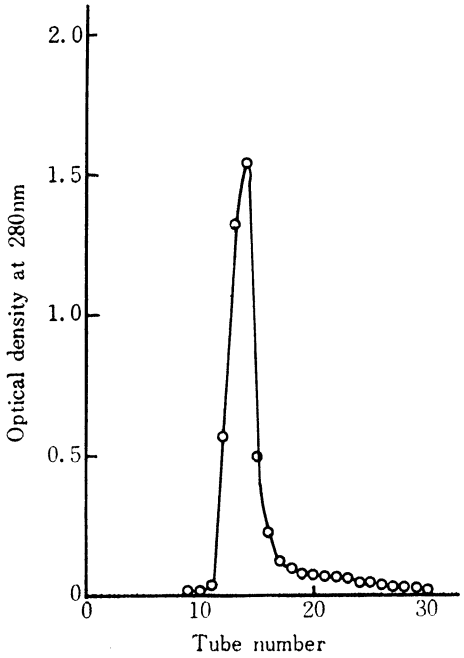

Fig. 3-b Gel filtration pattern of β-group on a Sephadex G-25 column.
Column : 2.5×40 cm
Eluent : 0.05 N acetic acid

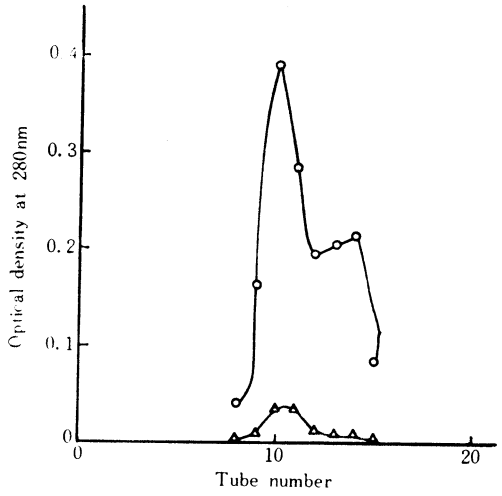

Fig. 4-b Chromatography of β-group with a Dowex 50×2 column.
Column : 1.8×50 cm
Eluent : 0.2 N pyridine-acetate buffer (pH 3.0)
○—○ peptide (O.D. at 570 nm)
△—△ organic phosphopus (O.D. at 660 nm)

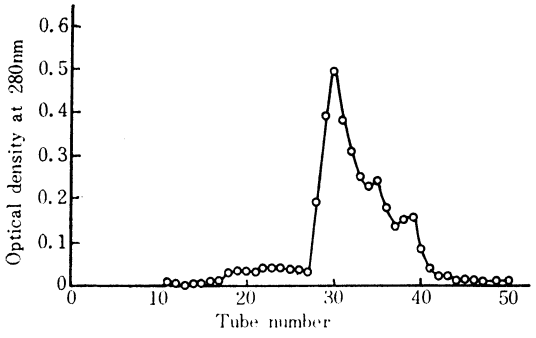

Fig. 5-b Desaltation of β-group with a Sephadex G-25 column.
Column : 2.5×40 cm
Eluent : water

of the purified phosphopeptide was 4.1 mg. The amino acid composition is shown in Table 5.

respectively. Among them, the three supernatants with a similar N/P ratio were combined and concentrated. The concentrate was filtered with Sephadex G-25 and G-50 columns successively and chromatographed with a Dowex 50×2 column in the same way as used on the above H-group. The elution curves are shown in Figs. 1-b, 2-b, 3-b, 4-b and 5-b. The yield

CONSIDERATIONS AND CONCLUSIONS

1) Two casein phosphopeptide preparations, i.e., H.C.P. and β.C.P. were

prepared from the contents of small intestinal tracts of rats by successive gel filtrations. In the case of the H. C. P. which was prepared from the 4 rats fed on Hammarsten casein, the N/P ratio of the phosphopeptide fraction obtained through the first gel filtration with a Sephadex G–25 column was found to be 10 times higher than that of the one reported by NAITO *et al.*[2] Although the ration used in this experiment contained both sucrose and starch as appetizers for the rats, the one used by NAITO *et al.*[2] contained starch only. The difference in ration seems to influence the digestive conditions of rats, but the cause for the above difference of N/P ration is still not clear.

2) Another phosphopeptide preparation was the β. C. P., prepared from the 3 rats fed on β-casein. Borh the two preparations, H. C. P. and β. C. P., had a similar amino acid composition, and contained considerable amounts of glutamic acid and phosphoserine. Accordingly, they were acidic peptides. The calculated N/P ratios of H. C. P. and β. C. P. on the basis of their phosphoserine contents were 6.5 and 5.7, respectively. These values are similar to those of NAITO *et al.*[2] and of others[9)~13)] (Table 6). Considering their amino acid compositions,

Table 6. The amino acid composition of phosphopeptide from casein (by literature).

Amino Acid	MELLANDAR[1)]	ÖSTERBERG[9)]	SCHORMÜLLER[10)]		DUMAS[11)12)]			MANSON[13)]
			α-casein	α-casein	α_{S_1}-casein	α_{S_1}-casein	β-casein	β-casein
Lys		2	1	1	1	1		
His								
NH_3							2	2
Arg	2.8	4	1	1	1	3	1	1
Asp	1.7	1				1	1	1
Thr	9.5	5	5	4	4	2	4	5
Ser	10.3	10	7	5	7	4–5	7	7
Glu	1.0	1	1		1		1	1
Pro	1.2	1				1	1	1
Gly	1.0	2	1	1	1	1		
Ala								
Cys	2.0	2	2	1	2		2	2
Val		2	1	1	1	1	2	
Met	3.7	4	2	1	2	2		2
Ileu	1.0						3	3
Leu	(Leu=1.0)							
Tyr								
Phe								
Trp								
P	18.6	6	5	4	5	3	5	4
Ser/P	0.5	0.8	1	1	0.9	0.7	0.8	1.25
N/P	2.6	5.9	4.45					
Amino–N/P	1.8	6.0	4.4	4	4	5–6	5.8	7.75

some acidic peptides seem to be present in the samples as impurities. When tryptic digestion occured, lysine and arginine must have been present in the peptide preparations. As shown in Table 5, however, they could not be observed in the amino acid compositions of the two phosphopeptide preparations.

3) The localized presence of a phosphoric acid group on the casein phosphopeptide chain has been reported by DUMAS[11)12)] and MANSON[13)] and the formation of the Ca-complex or the Ca-binding property of the casein phosphopeptide by MELLANDAR[1)] and ÖSTERBERG.[9)] A Ca-binding protein containing much glutamic acid had been isolated from chick intestinal mucosa.[14)] On the basis of these findings, the casein phosphopeptide preparations in this study must have a significant physiological activity.

This study was carried out under the guidance of Dr. H. NAITO previously assistant professor in our laboratory.

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カゼイン摂取マウス腸管内で生成するホスホペプチドのアミノ酸組成

今村経明・鈴木秀男・内藤　博

著者らは既に，牛乳カルシウムの利用性が高いことの理由のひとつに，小腸管内に生成するカゼインホスホペプチドが考えられることを指摘した．その機構を知る第一歩として本研究では，カゼイン摂取マウスの小腸管内容物からホスホペプチドを単離し，アミノ酸組成を調べた．

マウスには体重 130～200 g の雄ウイスター種を用い，β-カゼインもしくは Hammarsten カゼインを含む飼料を space-feeding し，その腸内容物からホスホペプチドを分離した．ホスホペプチドの単離並びに精製はゲル泳過法および Dowex 50 イオン交換クロマトグラフィーによった．またアミノ酸組成は，ホスホペプチドの加水分解物について日立アミノ酸自動分析器034型によって分析した．

単離したホスホペプチドは，グルタミン酸とホスホセリンが多く，これらが Ca 結合性残基となる可能性が考えられた．また N/P 比は，トリプシン消化ペプチドについての文献値に近似していた．