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N-TERMINAL HALF MOLECULE OF L-TYPE BENCE JONES PROTEIN

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Abstract——A low molecular weight Bence Jones protein found in the urine of a patient with myeloma was isolated and its chemical and antigenic properties were compared with that of a usual 3.5 S L-type Bence Jones protein of the same patient. Sedimentation coefficient of the low-molecular-weight protein was 2.3 S and its molecular weight estimated by a thin layer gel-filtration in Sephadex G-75 or G-100 was 21,500 at neutral pH. On a gel-filtration in 1 M propionic acid or 0.5M acetic acid, the protein dissociated into two subunits with a molecular weight of 11,500. Antigenic analysis of 3.5 S and 2.3 S proteins showed that both the proteins shared common antigenic determinants and that 2.3 S protein lacked in some antigenic determinants derived from C-terminal half. This finding was also supported by comparison of peptide maps of both proteins. Furthermore, amino acid analysis clearly demonstrated that 2.3 S protein lacked in C-terminal half of λ-chain and had the same amino acid composition as that of N-terminal half of 3.5 S protein. On the basis of these results, it was concluded that the low-molecular-weight protein was the fragment protein derived from N-terminal half of 3.5 S protein and that the N-terminal half molecules associated to dimer by non-covalent bond at neutral pH but dissociated to two subunits in 1 M propionic acid or 0.5 M acetic acid. The origin of the fragment protein was discussed.

INTRODUCTION

Existence of low-molecular-weight protein together with Bence Jones protein in urine of patient with myeloma has been noted by some workers [1-7]. Recently, Baglioni et al. [5], Tan et al. [6] and Kozuru [7] reported that the low-molecular-weight

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proteins were identified as the N-terminal half molecule of the Bence Jones protein by chemical and immunological studies. On the other hand, Solomon et al. [3] and Williams et al. [4] presented the possibility that the low-molecular-weight protein was derived from "common" part of L-chain, i. e. C-terminal half on the basis of their antigenic analysis.

It is an interesting problem whether these fragment proteins are breakdown products or biosynthetic precursors of L-chain.

In our laboratory two cases were found in which low-molecular-weight proteins were present in the urine of patients who excreted L-type Bence Jones protein. Of these two cases one case was extensively studied to elucidate the structural relationship between the low-molecular-weight protein and Bence Jones protein.In this report, it was shown that the low-molecular-weight protein found in urine of a patient (Fu) was derived from N-terminal half of the Bence Jones protein existing together.

EXPERIMENTAL

Preparation of the Bence Jones protein and the low-molecular-weight protein Urine of a patient(Fu) with myeloma was treated with activated carbon, then filtered with hyflosupercel in order to remove the pigmented impurities. The filtrate was salted out in 3 M ammonium sulfate at pH 6. The precipitated protein was dialyzed against water and PBS*(pH 7.0). About 1 g of the protein was applied for gel-filtration to a Sephadex G-100 column (3.2×92cm) equilibrated beforehand with the same buffer and the column was developed upwards at a flow rate of 20 ml/hr and 5 C. Transmission tracing at 280 mμ was obtained with a LKB Uvicord ultraviolet absorptiometer and LKB recorder type 6520 H. The fraction B and C eluted (Fig.1) were collected, concentrated with collodion bag (Sartorius Membranfilter, No.13200), and dialyzed against 10 mM borate buffer, pH 8.5. The protein(0.1−0.2 g) was applied to DEAE-cellulose column(1.4×15−18 cm) equilibrated beforehand with the same buffer. Stepwise elution with the following buffers was carried out, successively: in the case of Fr.B, A) 30 mM borate buffer, pH 8.5; B) 60 mM borate, pH 8.4; C) 50 mM sodium chloride-40 mM borate, pH 8.4; D) 0.15 M sodium chloride-60 mM borate, pH 8.3 ; E) 0.5 M sodium chloride-40 mM borate, pH 8.2. The protein eluted with buffer C) was further purified by rechromatography on Sephadex G-100 column (3.5×93 cm) equilibrated with 20 mM borate, pH 8.0. The purified Fr. B was called Fr.2 (Fig.2). In the case of Fr.C, the following buffers were used for further purification: a) 10 mM borate, pH 8.5; b) 40 mM borate, pH 8.5; c) 60 mM borate, pH 8.3; d) 50 mM sodium chloride-60 mM borate, pH 8.3. The protein eluted with buffers b) and c) was called Fr. 3(Fig.3).

Ultracentrifugation Ultracentrifugal analysis was performed at 20 C and 59,780 rpm by a Spinco Model E analytical ultracentrifuge. Samples were dialyzed against

* Abbreviations. PBS, phosphate buffered saline. BJP, Bence Jones protein

0.2 M sodium chloride and analyzed at a concentration of 10.4 mg/ml.

Thin layer gel-filtration Thin layer gel-filtration using Sephadex G-75 and G-100 superfine was carried out according to the method described by Migita[8]. Molecular weight of the protein was estimated by the method of Andrews[9], using following proteins as standards of molecular weight: human albumin (M.W. 67,000); sperm whale myoglobin (17,800); ovalbumin (45,000; 2x cryst.); chymotrypsinogen A (25,000; beef pancreas; 6x cryst.). The human albumin was obtained from Nutritional Biochemicals Corp. and the other materials from Mann Research Lab. Inc. (New York).

Agar-electrophoresis Electrophoresis was performed by the method of Wieme[10] on a gel plate of Ionagar No. II (Oxoid Co.) in veronal buffer, pH8.6(μ=0.025) at gradient of 20 volt/cm for 25 min.

Amino acid analysis After performic acid-oxidation of proteins [11], hydrolysate of purified Fr.2 and 3 were prepared by heating materials *in vacuo* in doubly glass-distilled 6N-hydrochloric acid at 108 C for 24 hr in a sealed glass tube. Amino acid compositions were determined chromatographically by the procedure of Spackman et al.[12] in a Hitachi KLA-3B amino acid analyzer. The contents of Tyr and Trp were determined spectrophotometrically [13].

N-terminal amino acid N-terminal amino acids were determined by the dinitrofluorobenzene method of Sanger [14]. Two dimensional paper chromatography of dinitrophenyl amino acids was performed with the buffer system of Levy [15].

Peptide map Ten mg of performic acid-oxidized protein was digested with trypsin (bovine pancreas, Type III, 2x cryst., Sigma) with an enzyme: substrate ratio of 1:50, in 1 ml of 5% NH_4HCO_3 at 37 C for 6 hr. Two mg of the lyophilized digest was dissolved in water and applied to a Toyoroshi No. 51 filterpaper. Descending chromatography in the first dimension was performed with butanol-acetic acid-water(4:1:5). Electrophoretic separation was performed in the second dimension in pyridine-acetic acid-water(1:10:289) at pH 3.5 and a voltage gradient of 50 v/cm for 50 min. Peptides were detected with 0.2% ninhydrin in acetone solution.

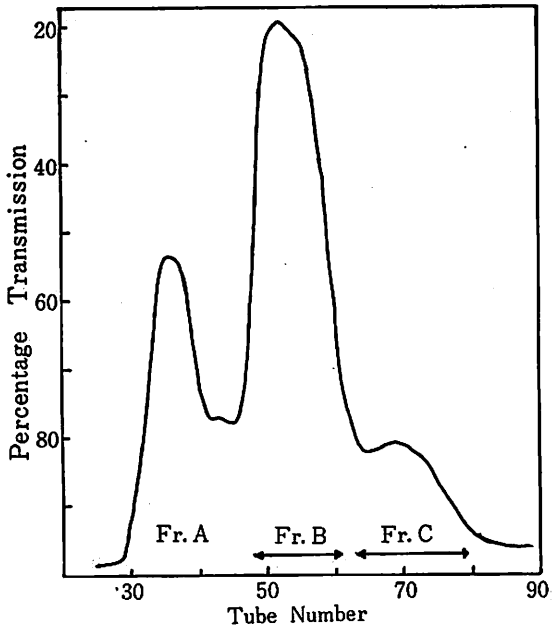
Antiserum Wister rats were immunized intradermally into footpads and intramuscularly into the thighs of the hind legs three times at 10 to 14 days intervals with each 0.5 mg of Fr.2 or 3 emulsified in complete Freund's adjuvant. Two weeks later these animals received booster injection of 0.5 mg of antigens in 0.15 M sodium chloride intravenously, then were bled by heart puncture 5 days after the booster injection.

RESULTS

(1) Preparation and purification of Fr.2 and 3

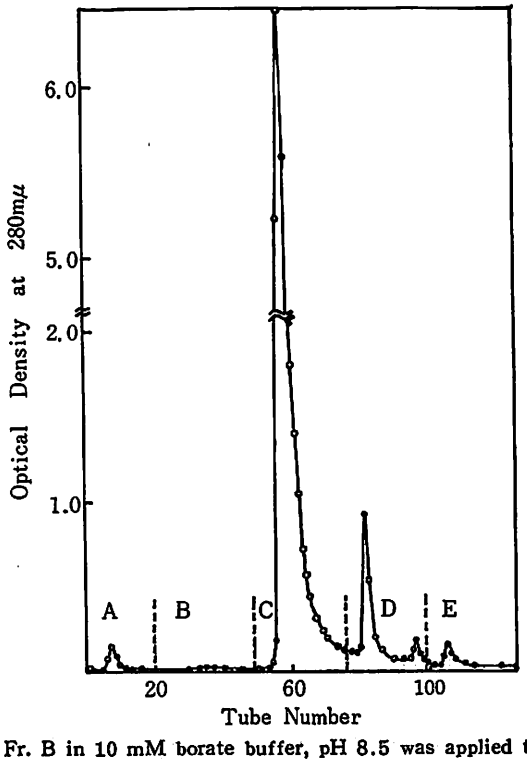
The proteins salted out from the urine in 3 M ammonium sulfate were separated into three peaks on Sephadex G-100 gel-filtration, as shown in Fig.1. The first peak (Fr.A) contained aggregated materials, IgG and albumin. The second and third peaks (Fr.B and C) were shown to contain the materials with molecular size of L-chain dimer and monomer, respectively. The recovery of the second and third peaks were 78

Fig. 1. Sephadex G-100 gel-filtration of the urinary proteins of the patient Fu.



1 g of the urinary protein salted out in 3 M ammonium sulfate was applied for gel-filtration to a column (3.2 x 92 cm) equilibrated with PBS (pH 7.0). The column was eluted with the same buffer at 5 C and a flow rate of 20 ml/hr, and 6.6 ml fractions were collected. Ordinate is the reading of transmission at 280mμ. The arrows indicate the fractions pooled for following studies.

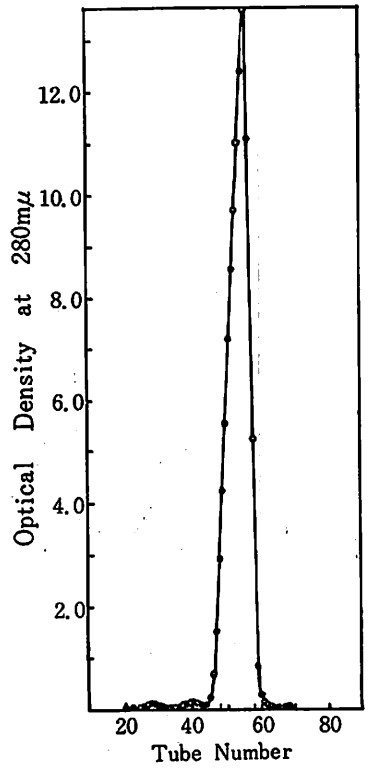
Fig. 2(a). Chromatography of Fr. B on a DEAE-cellulose column.



99 mg of Fr. B in 10 mM borate buffer, pH 8.5 was applied to a column (1.4 x 15cm) equilibrated with the same buffer and stepwise elutions were carried out, using the following buffers as eluants: (A) 30 mM borate, pH 8.5; (B) 60 mM borate, pH 8.4; (C) 50 mM NaCl-60 mM borate, pH 8.4; (D) 0.15 M NaCl-60 mM borate, pH 8.3; (E) 0.5 M NaCl-40 mM borate, pH 8.2. One tube was 3 ml.

% and 6.3 %, respectively. Fr.B and C were collected and concentrated with collodion bag with recovery of 97 % and 76 %, respectively. The elution patterns of Fr.B and C from DEAE-cellulose column were shown in Fig.2(a) and 3. The main component of Fr.B was eluted with 0.05 M NaCl-60 mM borate, pH 8.4 with a recovery of 82%, which was called Fr.2. Rechromatographic profile of the main peak was shown in Fig.2 (b), indicating the homogeneous molecular size. In the case of Fr.C, the main

Fig. 2(b). Rechromatography of Fr. B on a Sephadex G-100 column.



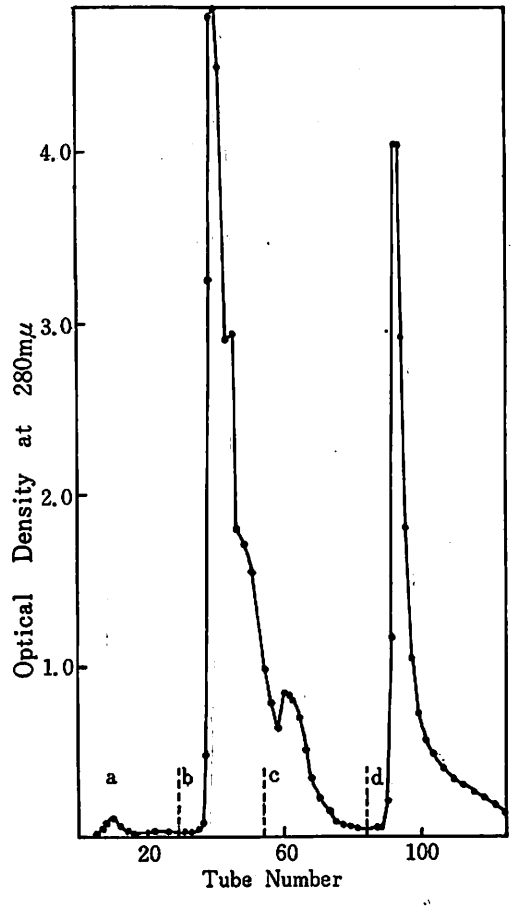
381 mg of the main component of Fr. B eluted with the buffer (C) (see Fig. 2(a)) was applied to a column (3.5 x 93 cm) equilibrated with 20 mM borate, pH 8.0. The column was eluted with the same buffer and 5.5 ml fractions were collected.

component was eluted with 40-60 mM borate, pH 8.5-8.3 from DEAE-cellulose column with a recovery of 63%, which was denominated Fr.3.The fraction eluted with 50 mM NaCl-60 mM borate, pH 8.3 with a recovery of 26% was shown to be contamination of Fr.2 by the agar-electrophoresis and thin layer gel-filtration on Sephadex G-100. Fr. 2 and 3 were used in the following studies.

(2) Physicochemical properties of Fr.2 and 3

Agar-electrophoresis Electrophoretic patterns of Fr.2 and 3 at pH 8.6 were shown in Fig.4, indicating that both the fractions were electrophoretically homogeneous and

Fig. 3. Chromatography of Fr. C on a DEAE-cellulose.



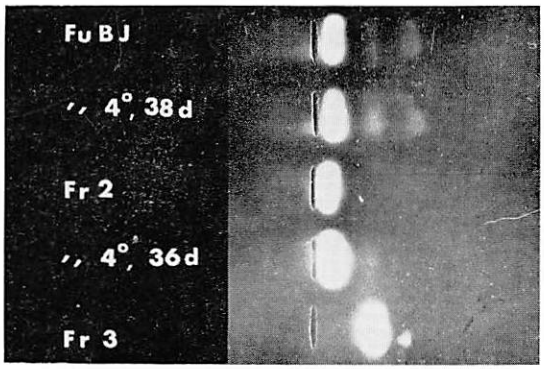
195 mg of Fr. C in 10 mM borate, pH 8.5 was applied to a column (1.4 x 18cm), equilibrated with the same buffer and stepwise elutions were performed, using the following buffers as eluants; a) 10 mM borate, pH 8.5; b) 40 mM borate, pH 8.5; c) 60 mM borate, pH 8.3 d) 50 mM NaCl-60 mM borate, pH 8.3. 3 ml fractions were collected.

differed in the electrophoretic mobility.

Sedimentation coefficient Ultracentrifugal patterns of Fr.2 and 3 were shown in Fig.5. Both the fractions showed symmetrical peaks and sedimentation coefficients, $S_{20,W}$, of Fr.2 and 3 were 3.6 and 2.3, respectively.

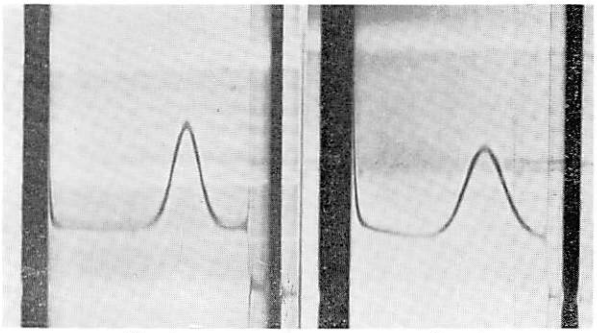
Molecular weight The molecular weight of Fr.2 and 3 were estimated by thin layer gel-filtration. An example of chromatogram in PBS (pH 7.0) and 1 M propionic acid was shown in Fig.6(a,b). The molecular weight values estimated in various conditions were listed in Table 1. Fr.2 showed the value of 45,000 in both PBS (pH 7.0) and 1 M propionic acid. However, when Fr.2 was reduced with 0.3 M 2-mercaptoethanol and alkylated with 0.36 M iodoacetamide, it showed the value of 45,000 in PBS but 25,000 in 1 M propionic acid. These results showed that Fr.2 was a dimer of λ-chain linked by disulfide bond.

Fig. 4. Agar-gel electrophoresis of the starting urinary proteins, purified Fr. 2 and 3.



Electrophoresis was carried out on a 1% Ionagar No. II gel plate in veronal buffer pH 8.6 (μ=0.025) for 25 min at a gradient of 20 volt/cm. The anode is to the left. FuBJ means the crude urinary protein of patient Fu. The second sample is the crude protein incubated in PBS (pH 7.0) for 38 days at 4 C. The fourth sample is Fr. 2 incubated in PBS (pH 7.0) for 36 days at 4 C.

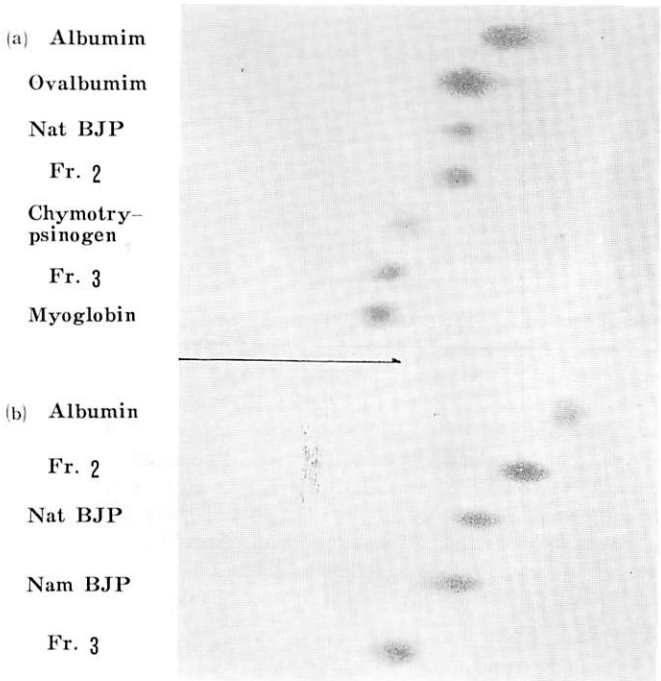
Fig. 5. Sedimentation patterns of Fr. 2 and 3.



Fr. 2 Fr. 3

Urtracentrifugations were performed in 0.2 M NaCl at a concentration of 10.4 mg/ml and photographs were taken 72 min and 120 min, respectively, after full speed at 59,780 rpm.

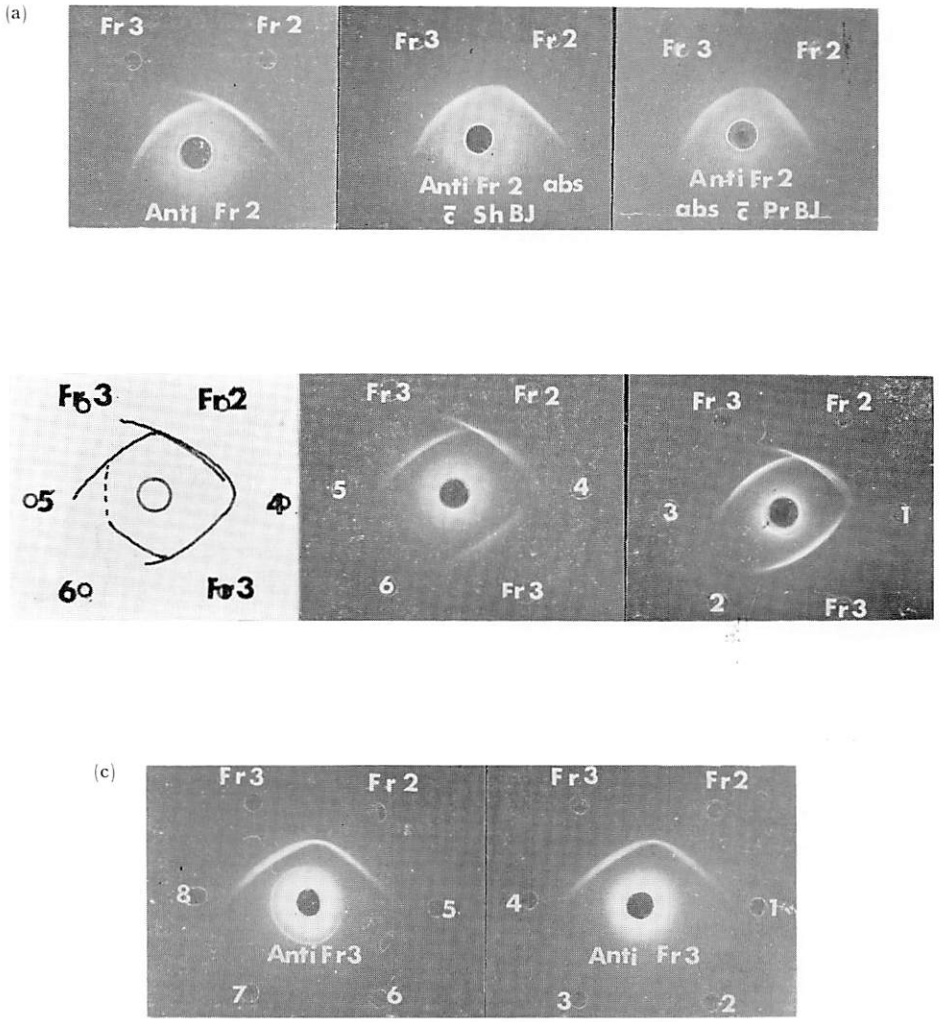
Fig. 6. Thin layer chromatography of Fr. 2 and 3 on Sephadex G-75 (superfine)



(a) Gel-filtration was performed in PBS (pH 7.0) for 3.5 hr and stained by Amidoblack 10 B. Standard substances used for molecular weight estimation were: albumin (67,000) ; chymotrypsinogen A (25,000) ; myoglobin (17,800); ovalbumin (45,000) ; Nat BJP (K-type dissociable dimer)

(b) Gel-filtration was performed in 1 M propionic acid for 3.5 hr. Nam BJP is K-type BJP of stable monomer type and has molecular weight of 24,800 determined by sedimentation equilibrium.

Fig. 7. Ouchterlony double diffusion tests of Fr. 2 and 3.



(a) Double diffusion precipitation reaction of Fr. 2 and 3 against rat anti-Fr. 2 serum and the serum after absorption with L-type BJPs. Fr.2 ; 0.5 mg/ml Fr.3 : 0.25mg/ml. Anti-Fr. 2 serum well (left) contains 40 μl of the serum and 5 μl of 0.15 M NaCl. Center antiserum well, 40 μl of anti-Fr. 2+5 μl of Sh BJP (2 mg/ml) ; Right antiserum well, 40 μl of anti-Fr.2 + 5 μl of Pr BJP (3 mg/ml) (b) Double diffusion pattern of 6 different L-type BJPs against anti-Fr. 2 serum. Center wells contain anti-Fr. 2 serum. The other wells contain: 1, Ba BJP at 2 mg/ml; 2, Sh BJP 2 mg/ml; 3, Ha BJP 2 mg/ml; 4, Ko BJP 0.2 mg/ml; 5, Yo BJP 0.2 mg/ml; 6, Pr BJP 0.2 mg/ml ; Fr.2, 0.2 mg/ml; Fr. 3, 0.1 mg/ml. (c) Double diffusion patterns of Fr.2, Fr.3 and other 8 different L-type BJPs against anti-Fr. 3 serum. Center wells contain anti-Fr.3 serum and the other wells contain; 1, Yo; 2, Ko; 3, As; 4, Pr; 5, To; 6, Ba; 7, Sh; 8, Ha BJP, 0.2 mg/ml of each; Fr.2, 0.2 mg/ml; Fr. 3, 0.1 mg/ml.

Table 1. Molecular weight of Fr. 2 and 3 estimated by thin layer gel-filtration on Sephadex G-75 or 100 superfine.

Sample	Gel	Buffer	Molecular weight
Fr. 2	G-100	PBS	45,000±1,000
	G-75	PBS	45,000
	G-75	1 M propionic acid	45,000
Fr. 3	G-100	PBS	23,000
	G-75	PBS	21,000±2,000
	G-75	1 M propionic acid	11,500±1,500
	G-75	0.5 M acetic acid	9,000
	G-75	30 mM citrate, pH 3.5	18,500± 500
	G-75	30 mM borate, pH 9.3	26,000
	G-75	30 mM borate, pH 10.1	26,000
	G-75	30 mM borate, pH 11.0	22,000

Fr.3, however, showed the value of 21,500 in PBS (pH 7.0) and 11,500 in 1 M propionic acid or 0.5 M acetic acid. Therefore, it was suggested that Fr.3 was present as dimer form of a subunit which has a half molecular weight of λ-chain, in PBS(pH 7.0) and dissociates into the subunits with molecular weight of 11,500 in "dissociating" solvent, such as 1 M propionic acid or 0.5 M acetic acid. The dissociation of Fr.3 into its subunits was not occurred in the other buffers of pH 3.5 to 11 examined by thin layer gel-filtration.

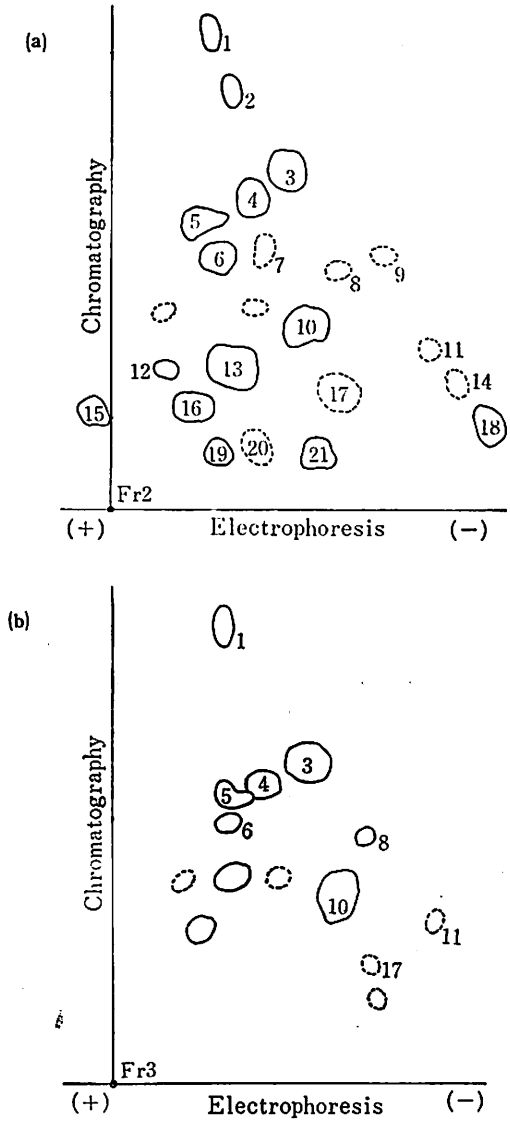
(3) Immunological analysis of Fr.2 and 3

A relationship between Fr.2 and 3 was also elucidated in antigenic properties. Fig.7 (a) showed a double diffusion precipitation patterns of Fr.2 and 3 tested with anti-Fr.2. Fr.2 formed spur over Fr.3, indicating that Fr.3 was antigenically deficient compared with Fr.2. However, when the anti-Fr.2 serum was absorbed with heterologous L-type Bence Jones proteins, the spur of Fr.2 over Fr.3 was no longer observed but the two proteins formed a fused line. These facts suggested that the antigenical deficiency of Fr.3 was due to the lack of the antigenic determinants of the "common" part of λ-chain. The anti-Fr.2 serum used, however, reacted with only two heterologous Bence Jones proteins out of six examined, forming feeble precipitation line and with the other proteins did not form precipitation line (Fig 7(b)). With anti-Fr.3 serum, Fr.2 and Fr.3 showed an identical line, indicating that the two proteins have the "common" antigenic determinants. The anti-Fr.3 serum did not produce precipitation line with 8 heterologous Bence Jones proteins examined.

(4) Peptide map of tryptic digest of Fr.2 and 3

Fig.8(a) and (b) showed the schematic diagrams of peptide maps of tryptic digest of performic acid-oxidized Fr.2 and 3. Some peptides (spots 1,3,4,5,6,8,10,11,17) were

Fig. 8. Peptide map analysis of tryptic digests of performic acid-oxidized Fr. 2 and 3.



Paperchromatography in the first dimension was performed with butanol-acetic acid-water (4:1:5). Electrophoresis was carried out in the second dimension in pyridine-acetic acid-water (1 : 10 : 289) at pH 3.5 and a voltage gradient of 50 V/cm for 50 min. (a), peptide map of Fr. 2. (b), peptide map of Fr. 3.

found in both maps. However, the map of Fr.3 lacked in some peptides which were observed in Fr.2. The peptide spots 13, 15 and 18 which were found in the map of Fr.2 but not in Fr.3, were also seen in all of four other L-type Bence Jones proteins examined. These peptides were considered to be "common" peptides of λ-chain including C-terminal peptide which was identified as spot 15 from criteria of the position[20].

(5) Amino acid analysis of Fr.2 and 3

The amino acid composition of Fr.2 and 3 were presented in Table 2. The total amino acid residues of subunit chains of Fr.2 and 3 are supposed to be 213 and 108,

Table 2. Amino acid composition of Fr. 2 and 3.

	Fr. 2	Fr. 3	Fr. 2−Fr. 3	Common part of BJP (Sh)
Lys	11.8	2.3	9.5	8
His	2.4	—	2.4	2
Arg	7.7	7.2	0.5	1
$CySO_3H$	5.0	1.7	3.3	3
Asp	12.1	6.6	5.5	5
Thr	19.6	8.2	11.4	11
Ser	35.6	18.7	16.9	16
Glu	19.3	8.3	11.0	12
Pro	17.2	8.7	8.5	9
Gly	17.0	13.3	3.7	3
Ala	16.6	7.1	9.5	11
Val	14.3	5.5	8.8	9
Ileu	5.6	4.4	1.2	1
Leu	15.1	9.0	6.1	6
Phe	4.4	2.7	1.7	2
Tyr	5.5	2.0	3.5	4
Trp	4.2	2.3	1.9	2
Total residues	213	108	105	105

respectively, from the molecular weight observed and the known sequence of the Bence Jones protein(Sh) [16]. If subunit chain of Fr.3 corresponds to "variable" part of λ-chain of Fr.2, the amino acid composition of C-terminal half of λ-chain is obtained by subtracting that of Fr.3 from that of Fr.2. As seen in the Table 2, Fr.2 minus Fr.3 was almost identical with the amino acid composition of the "common" part of λ-chain calculated from the amino acid sequence of L-type Bence Jones protein (Sh) decided by Titani et al.[16].

(6) The N-terminal amino acids of Fr.2 and 3

The N-terminal amino acids were determined on Fr.2 and 3 by the method of Sanger. Both fractions showed no free N-terminus, but trace amount of Phe was found: 0.026 moles of Phe/22,000 of Fr.2; 0.024/11,000 of Fr.3.

DISCUSSION

From the urine of a patient with myeloma, two kinds of protein, Fr.2 and 3 were isolated by Sephadex G-100 and DEAE-cellulose chromatography. These fractions

were homogeneous on agar-electrophoresis and ultracentrifugation. Sedimentation coefficients were 3.6 S for Fr.2 and 2.3 S for Fr.3 at a concentration of 10.4 mg/ml. Molecular weight of Fr.2 and 3 were 45,000 and 21,000, respectively when estimated on Sephadex G-75 or G-100 thin layer gel-filtration in neutral pH. On gel-filtration in 1 M propionic acid, Fr.3 was dissociated into two subunits with a molecular weight of 11,000, while Fr.2 showed the same molecular weight as in neutral pH. These results indicated that Fr.2 was a stable dimer of λ-chain and that Fr.3 was present as a dimer of the subunit with a molecular weight of 11,000 at pH 7.0.

Ouchterlony test with rat anti-Fr.2 serum showed that Fr.3 shares common antigenic determinants with Fr.2 but is antigenically deficient compared with Fr.2. This antigenic deficiency was due to the lack of antigenic determinants derived from "common" part of λ-chain i.e. C-terminal half, since anti-Fr.2 sera absorbed with heterologous L-type Bence Jones protein showed identical reaction with both Fr.2 and 3.

These findings were also supported by comparison of Fr.2 with Fr.3 by means of peptide maps. Furthermore, amino acid analysis clearly demonstrated that Fr.3 lacks in C-terminal half of λ-chain.

On the basis of these data, it was concluded that Fr.3 was the fragment protein derived from N-terminal half of Fr.2 and that at neutral pH, Fr.3 existed in dimer form of N-terminal half molecule but in monomer form in 1 M propionic acid or 0.5 M acetic acid.

The fact that Fr.2 and 3 had no free N-terminus was compatible with the finding by Hood et al.[17] that N-terminus of λ-chain is mostly pyrrolid-2-one-5-carboxylic acid.

As mentioned above, Fr.3 had the characteristic property that it was present in dimer form of subunits at neutral pH but monomer form in 1 M propionic acid. In the previous papers, the same properties of Bence Jones protein were described[5-7]. Tan and Epstein reported that the fragment derived from N-terminal half of K-type Bence Jones protein had the sedimentation coefficient of 1.9 S at pH 8.0 but 1.0 S in 1 M propionic acid. The fragment protein described by Kozuru was demonstrated to be N-terminal half of K-type Bence Jones protein and had a half molecular weight of L-chain monomer in 1 M acetic acid, though molecular size of the fragment at neutral pH was not described. Baglioni et al. observed the dissociation of the fragment protein i.e. dimer of N-terminal half, into its subunits at pH above 9.8. Fr.3, however, did not dissociate at alkaline pH.

Generally, Bence Jones protein exists in dimer form of L-chain except for stable κ-chain monomer. When two kinds of Bence Jones protein monomer obtained by reduction with 2-mercaptoethanol are mixed, preferential dimerization of homologous Bence Jones protein monomer is observed but dimerization between heterologous Bence Jones protein monomers is never observed[18]. In such preferential dimerization of homologous L-chain, the interaction by non-covalent bond between homologous L-chain in the moiety of variable part may play an important role, because it appears that N-terminal half molecule has a tendency to form dimer at neutral pH.

As to the origin of the fragment protein, we have no reliable information at present. It is an attractive hypothesis that the fragment protein is a biosynthetic unit and a precursor of the L-chain. The other possibility is that the fragment is a breakdown product[2]. Recently, Cioli and Baglioni[19] demonstrated that some fragment proteins could be considered to be products of catabolism of Bence Jones protein dimer during excretion through the kidney and that this breakdown did not occur in the serum or urine of the patient who excreted the fragment.

It is an interesting problem to study what is the mechanism of the specific cleavage of peptide bond at middle point of L-chain. When the crude Bence Jones protein (Fu) and Fr.2 were incubated in PBS(pH 7.0) at 4 C for 38 and 36 days, respectively, partial conversion of them into Fr.3 occurred and was detected by agar-electrophoresis(Fig.4). This observation suggested that Fr.3 occurred by some enzymatic cleavage of Fr.2. In our preliminary experiment, however, we could not obtain the fragment corresponding to Fr.3 by enzymatic digestion of Fr.2 with any of trypsin, chymotrypsin, pepsin, papain, pronase and Nagarse.

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