

PROPERTY OF IMMUNOGLOBULIN BINDING SUBSTANCE (IBS)
FROM THE GRANARY WEEVIL

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*Fujisawa Pharmaceutical Co., Ltd.***Key words:** Fab fragment — IgG — weevil

(Received for Publication, April 13, 1985)

INTRODUCTION

We have previously reported on immunoglobulin binding substance (IBS) which binds to human Ig-Fab fragment¹⁾. IBS has been successfully separated on affinity chromatography using human IgG-immunosorbent. IBS has shown a single component on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), and its molecular weight was estimated to be 12,000-14,000. The major feature of IBS is its extraordinary affinity for all immunoglobulins through non-immunological binding to Fab. Physicochemical characterization of IBS is of importance for the elucidation of its biological functions. However, there is little information about it.

In this paper, we describe the chromatographic fractionation of partially purified IBS and the properties of its three fractions, two of which are considered to be the polymers of the smallest molecular weight fraction.

MATERIALS AND METHODS

Purification of IBS: Methods have been described elsewhere¹⁾. In brief, 15 g of *Sitophilus granarius* (SG) was ground with a hand mill and was sufficiently defatted in cold acetone. The dried SG powder was suspended in Coca solution²⁾ and homogenized by a glass rod homogenizer and then a sonicator (Ohtake Works, Tokyo) at 20 kHz with 50W for 5 min. After overnight incubation at 4°C, the homogenate was centrifuged at 15,000 ×g for 30 min. The supernatant was filtrated through filter paper (No. 6, Tokyo Roshi Kaisha Ltd., Tokyo) to eliminate the remaining fat. IBS was isolated from this crude SG extract by affinity chromatography using human IgG-Sepharose 4B (Pharmacia fine Chemicals AB, Uppsala, Sweden)³⁾. IBS was eluted with 6.0M urea, pH 9.4 (crude IBS). Fifty mg of eluted IBS was further purified on Sephadex G-100 column (3 × 30 cm, Pharmacia) equilibrated with 6.0M urea, pH 9.4. Collected fractions were dialyzed against distilled water (DM) and lyophilized.

Molecular weight determination: High pressure liquid chromatography (HPLC) using TSK-G3000SW column (7.5 × 600 mm, Toyo Soda Co., Ltd.) was performed to estimate its molecular weight. Lyophilized fractions were dissolved in 2.0 mM phosphate buffer (PB), pH 7.0, and applied to HPLC at 1.0 ml/min. SDS-PAGE⁴⁾ was also performed without or with 2-mercaptoethanol (SDS-PAGE/2ME).

Analysis of amino acid: Purified IBS was hydrolyzed in 6.0N HCl at 100°C for 20 hours, and then applied to Amino Acid Autoanalyzer (#835, Hitachi Ltd.).

Iodination of IBS: IBS Fr-III was radiolabeled with ¹²⁵I by the chloramine-T method⁵⁾, and the labeled material was separated from free iodine by gel filtration on Sephadex G-25 equilibrated with DW. Specific activity was 30-80 μCi/μg. 1.8 × 10⁶ cpm/50 μl of ¹²⁵I IBS Fr-III was mixed with 100 μl of DW and 0.1M phosphate buffered saline pH 7.4 (PBS), and 5.0 × 10⁶ cpm/50 μl of ¹²⁵I IBS Fr-III was mixed with 100 μl of 8% SDS with 20% 2ME (SDS-2ME), and applied to Sephadex G-100 column (1 × 25 cm) equilibrated with PBS. PBS was used as an elution buffer. The radioactivity of each fraction

Abbreviations: DW distilled water; HPLC high pressure liquid chromatography; IBS immunoglobulin binding substance; 2ME 2-mercaptoethanol; PAGE polyacrylamide gel electrophoresis; PB phosphate buffer; PBS phosphate buffered saline; SDS sodium dodecyl sulfate; SG *Sitophilus granarius*

collected at 15 drops per tube was measured by a gamma counter (ARC-221, Aloka Co., Ltd.).

Precipitability of IBS: Ten μ l of ^{125} I IBS Fr-III was mixed with 3.0 ml of the solution at various pH or salt densities. After three hours of incubation, the mixtures were centrifuged at $4,500 \times g$ for 30 min. Supernatants were discarded, and the radioactivity of the pellets was measured.

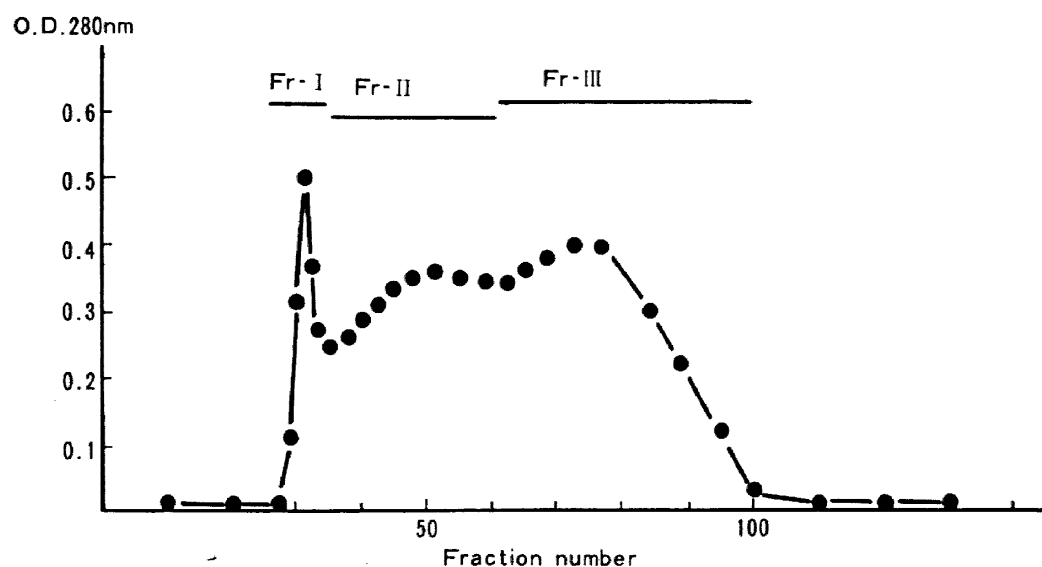


Fig. 1. Chromatography of IBS on a Sephadex G-100 equilibrated with 6.0M urea, pH 9.4. The volume of each tube was 2.0 ml. Each fraction named Fr-I, II and III, was dialyzed against DW and lyophilized.

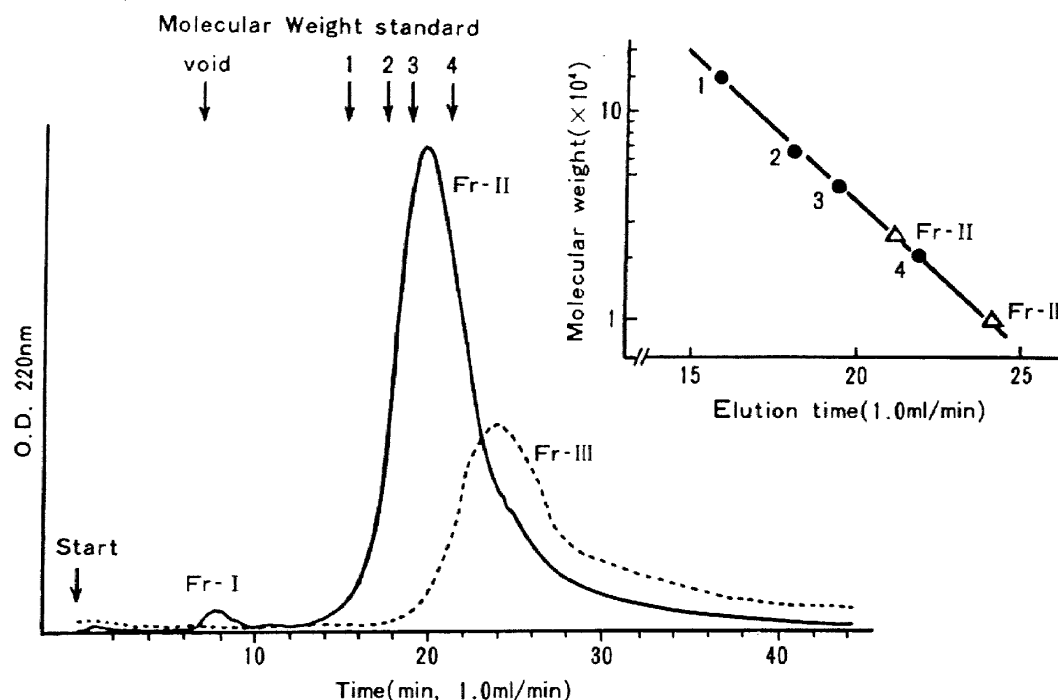


Fig. 2. High pressure liquid chromatography of IBS Fr-I, II and III on TSK-G3000SW column. Lyophilized fraction was dissolved with 20 mM PB, pH 7.0 and applied to HPLC at 1.0 ml/min. Molecular weight standards are glucose oxidase (150,000), bovine serum albumin (67,000), ovalbumin (45,000) and trypsin inhibitor (21,000). The molecular weights of Fr-I, II and III were calculated to be more than 150,000, 27,000 and 10,000 respectively.

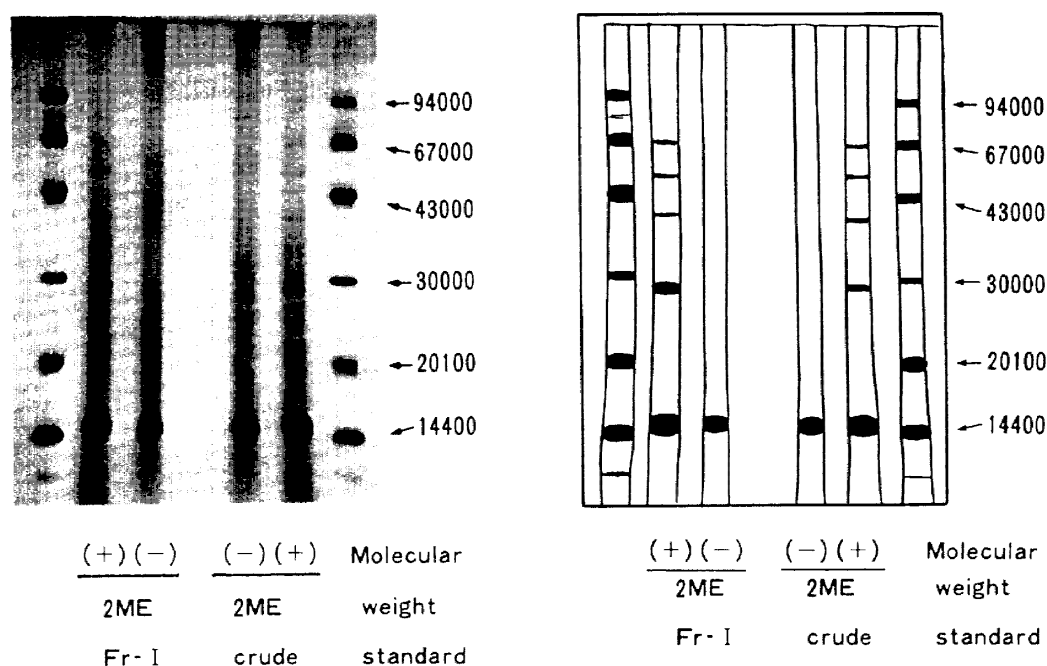


Fig. 3. SDS-PAGE of crude IBS and IBS Fr-I. Lyophilized crude IBS and IBS Fr-I were dissolved with 0.125M Tris-HCl buffer, pH 6.8, and applied to SDS-PAGE with or without 2ME. Molecular weight standards are phosphorylase B (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and alpha-lactalbumin (14,400). Crude IBS and IBS Fr-I showed a single band at same position, and the molecular weight was calculated to be 14,000-15,000.

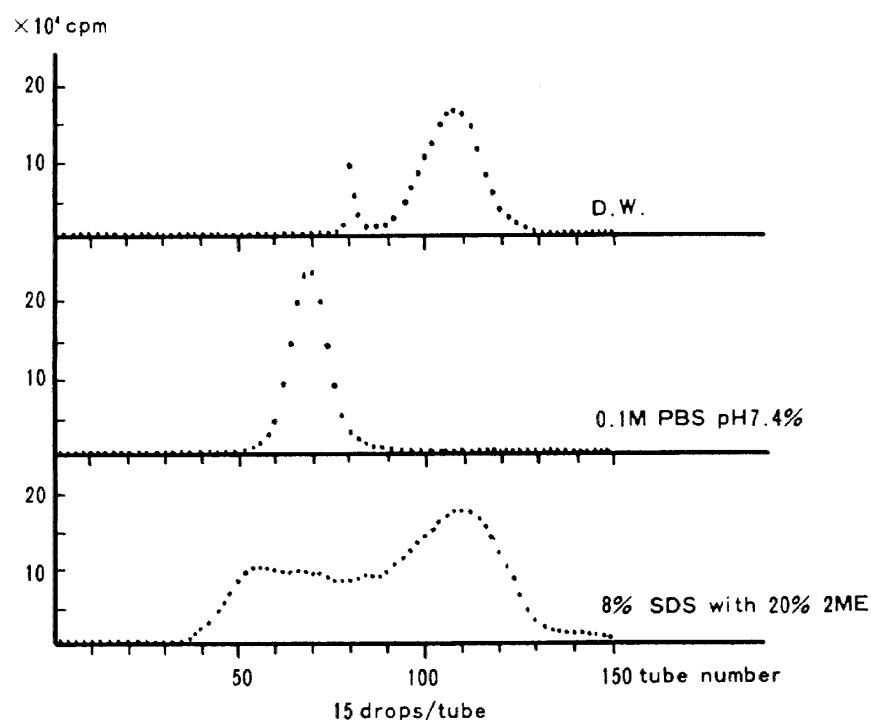


Fig. 4. The elution patterns of ^{125}I IBS Fr-III on a Sephadex G-100 column equilibrated with PBS. Fifty μl of ^{125}I IBS Fr-III was mixed with 100 μl of DW, PBS or SDS-2ME, and applied to the column. The radioactivity of aliquotes with 15 drops were measured by gamma-counter. The recoveries of applied radioactivity in the eluates were 90, 97 and 97%, respectively. As a result, the molecular size of IBS Fr-III was increased when it was mixed with PBS.

RESULTS

Gel filtration of Sephadex G-100: From 900 mg of crude SG extract, 240 mg of crude IBS was isolated by affinity chromatography. Fig. 1 shows a pattern of gel filtration of IBS on Sephadex G-100 column equilibrated with 6.0M urea, pH 9.4. Three fractions, Fr-I, II and III were separated.

HPLC: Fig. 2 shows the result of high pressure liquid chromatography. Although broader than the peaks of reference, the molecular weights corresponding to the peaks of Fr-I, II and III were calculated to be more than 150,000, 27,000 and 10,000, respectively.

SDS-PAGE: Crude IBS and IBS Fr-I showed a major band at the same position in SDS-PAGE without 2ME, and their molecular weight was estimated to be 14,000 (Fig. 3). However, in both cases, 4 additional minor bands were found in the regions corresponding to larger molecular size in the presence of 2ME (SDS/2ME).

Amino acid composition: The amino acid composition of Fr-II and Fr-III is shown in Table I. The yield of Fr-I was too little for amino acid analysis. IBS Fr-II and III showed a similar amino acid composition, and molecular weight of Fr-III was calculated to be 10,254 by amino acid analysis assuming one histidine residue per molecule.

Gel filtration of ^{125}I labeled IBS Fr-III: IBS Fr-III dissolved with DW shows two peaks on Sephadex G-100. The major peak in DW was totally or partially shifted to left when ^{125}I IBS Fr-III was mixed with PBS or SDS-2ME before gel filtration (Fig. 4). The change of the molecular size of IBS in SDS-2ME was also confirmed by the result of SDS-PAGE (Fig. 3).

Precipitability of ^{125}I IBS Fr-III: Table II shows the precipitability of IBS in various solutions. PH of DW and PB were adjusted at 7.4. IBS Fr-III precipitated at pH 5.0 and 6.0 in Tris system. IBS Fr-III dissolved in DW did not precipitate at 4°C for about one year, while it easily precipitated when dissolved with PBS.

DISCUSSION

IBS yielded three peaks, named Fr-I, II and III, respectively on gel column chromatography, equilibrated with 6.0M urea, pH 9.4 and on HPLC in 20 mM PB. However, crude IBS showed a single band on SDS-PAGE and its molecular weight was calculated to be about 14,000. Gel filtration patterns of ^{125}I IBS Fr-III revealed that its molecular size was in-

Table 1 Amino acid composition of IBS
Fr-II and III hydrolyzed in 6.0N HCl
at 100°C for 20 hours

Amino acid	Mol ratio ($\times 100$)		No./mol
	Fr-II	Fr-III	Fr-III
Aspartic acid	3.63	3.64	9
Threonine	2.53	2.13	5
Serine	3.39	2.86	7
Glutamic acid	4.07	4.38	11
Proline	2.36	2.71	7
Glycine	3.08	3.05	8
Alanine	2.87	2.57	7
Cystine	0.00	0.00	0
Valine	2.28	2.01	5
Methionine	0.00	0.00	0
Isoleucine	1.20	0.87	2
Leucine	2.66	2.19	6
Tyrosine	0.77	0.82	2
Phenylalanine	1.22	1.07	3
Lysine	1.91	1.79	5
Histidine	0.61	0.39	1
Arginine	1.60	1.36	4

Table 2 Precipitability of ^{125}I IBS Fr-III
in the solution of various
salt density and pH

Salt density	%Precipitability*	pH**	%Precipitability
DW	1.6	2	5.4
0.005M PB	1.3	3	7.9
0.05 M PB	1.6	4	14.2
0.5 M PB	1.5	5	81.3
0.5 M NaCl	1.7	6	63.1
1.0 M NaCl	1.4	7	2.4
2.0 M NaCl	1.5	8	1.3
3.0 M NaCl	1.4	9	1.1
4.0 M NaCl	1.4	10	1.0
5.0 M NaCl	1.7	11	1.0
6.0 M NaCl	1.5		

* : %Precipitability = (Pellet cpm)/(total cpm) $\times 100$

** : pH of 0.1 M Tris solution was adjusted by 10.0 N HCl

creased when Fr-III was mixed with PBS or SDS-2ME. Furthermore, the amino acid composition of Fr-II was similar to that of Fr-III. IBS Fr-I, II and III were often precipitated in PBS when they were stored at 4°C for several months. These data imply that the three fractions of IBS are polymer homologues. That is, IBS Fr-II is a dimer of Fr-III, and Fr-I might be a polymer or aggregate of Fr-III.

Amino acid analysis has shown that IBS contains neither cysteine nor cystine. This suggests that IBS is not composed of polypeptide chains held together by disulfide bridges. The mechanism of polymerization of IBS is still unknown, but it is interesting that IBS was polymerized in the presence of 2ME (Fig. 3, Fig. 4).

It is well known that staphylococcal protein A binds to human IgG-Fc region via non-immunological mechanism⁵. Protein A does not contain amino acids containing sulfur atoms⁶ and the tyrosines have an important role in the reaction with the Fc region of human IgG⁷. Protein A is a cell wall component of *S. aureus* and is covalently linked to the peptidoglycan structure⁸. 500 mg of protein A was obtained from 300 g of wet bacteria⁶, while the same amount of IBS was obtained from 30 g of SG. The localization of IBS is still unknown, but IBS may be a major component of SG because the recovery of IBS from SG (1.6%) is higher than that of protein A (0.17%).

SUMMARY

Immunoglobulin binding substance (IBS) which binds to human Ig-Fab fragment via non-immunological mechanism was separated from the granary weevil (SG) extract on affinity chromatography using human IgG-immunosorbent. 240 mg of IBS was obtained from 15 g of SG or 900 mg of SG extract, and the recovery of IBS (1.6%) was higher than that of staphylococcal protein A (0.17%). IBS yields three peaks, named Fr-I, II and III on gel column chromatography, equilibrated with 6.0M urea, pH 9.4 and on high pressure liquid chromatography (HPLC). The molecular weights of Fr-I, II and III were calculated to be more than 150,000, 27,000 and 10,000, respectively. However, the three fractions show a single band at the same position on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), and the molecular weight was calculated to be 14,000. Furthermore, gel filtration patterns of ¹²⁵I-IBS Fr-III revealed that its molecular size was increased when Fr-III was dissolved with PBS. These data suggested that two of the three fractions of IBS should be considered to be polymers of the smallest molecular weight fraction.

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