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A RE-EXAMINATION OF THE ISOLECTIN COMPONENTS OF THE FUCOSE-BINDING PROTEINS OF *Lotus tetragonolobus*

SHUN-ICHIRO OGATA, YOSHIRO KAMACHI, YUKO ARITA, MASAHIRO SATO, AND TAKASHI MURAMATSU
Department of Biochemistry, Kagoshima University School of Medicine, 1208-1 Usukicho, Kagoshima 890 (Japan)

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ABSTRACT

Fractionation of the fucose-binding proteins (FBP) of *Lotus tetragonolobus* (isolated by affinity chromatography on L-fucose–Sepharose) on porcine gastric mucin–Sepharose gave binding (FBP-M) and non-binding components (FBP-F). FBP-M gave three distinct spots (mol. wt. 26,000 for all three, and pI 5.40, 4.97, and 4.80) on two-dimensional gel electrophoresis, and their properties corresponded to those of FBP hitherto described. FBP-F migrated as a single spot (mol. wt. 25,000, pI 5.32). In contrast to FBP-M, FBP-F could not agglutinate type-H human erythrocytes. FBP-F reacted only with mouse extra-embryonic visceral endoderm of an egg cylinder-stage embryo and adult esophagus. Rabbit antiserum against FBP-F did not cross react with FBP-M, and antiserum against FBP-M did not cross react with FBP-F. Therefore, FBP-F appears to be substantially different from FBP-M both in structure and activity.

INTRODUCTION

Seeds of *Lotus tetragonolobus* contain isolectins which react with certain fucosyl residues[1–7]. These fucose-binding proteins (FBP) are useful in analysing the changes in cell surface which accompany differentiation[8–10]. For example, receptors for the lectin were expressed in stem cells of teratocarcinoma and disappeared from their differentiated derivatives[9]. Several affinity absorbents have been described for the preparation of the isolectins, and the isolectins obtained thus have been considered to be composed of identical components. We now describe a hitherto unnoticed component of FBP and point out that FBP preparations obtained by using different affinity absorbents are not necessarily identical.

EXPERIMENTAL

Preparation of fucose-binding proteins (FBP) from Lotus tetragonolobus. — Two kinds of affinity absorbents were used, and obtained by coupling L-fucose to Sepharose 4B[3] and porcine gastric mucin (Sigma; purified by phenol treatment) to BrCN-activated Sepharose 4B (1 mg of mucin/mL of gel).

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Seeds from *Lotus tetragonolobus* (F. W. Schumacher Co.) were milled, and relatively large pieces of the shell were removed. All subsequent procedures were carried out at ~4°. The flour (30 g) was extracted with Dulbecco's phosphate-buffered saline (PBS, 300 mL) for 3 h. The extract was filtered through 4 layers of gauze and centrifuged at 105,000*g* for 30 min. The supernatant solution was applied to a column (2 × 6.3 cm) of fucose–Sepharose equilibrated with PBS. The column was then washed with PBS (600 mL) and eluted with 0.05M L-fucose in PBS (100 mL). The eluate (designated FBP-F+M) was concentrated to 30 mL, dialysed extensively against PBS, and applied to a column (2 × 10 cm) of mucin–Sepharose equilibrated with PBS. The column was washed with PBS (1 L) and eluted with 0.05M L-fucose in PBS (150 mL). The void volume and the first 100 mL of the eluate were applied to the second mucin–Sepharose column containing 7.5 mL of the resin to ensure removal of isolectins that are able to bind to mucin–Sepharose. The void volumes and the first 20 mL of the eluate contained FBP-F (29.4 mg). The eluate from the first mucin–Sepharose column contained FBP-M (67.9 mg). An isolectin apparently identical to FBP-M could be prepared by affinity chromatography on the mucin–Sepharose without affinity chromatography on fucose–Sepharose (designated FBP-M'). FBP-M and FBP-F were stored at −20° after extensive dialysis against PBS and concentration to 4 and 1 mg/mL, respectively.

Preparation of rabbit antisera against FBP-F and FBP-M. — New Zealand White rabbits were immunised with either FBP-F (600 μg of protein per rabbit) or FBP-M (750 μg of protein per rabbit) in complete Freund's adjuvant by injection into the back. The rabbits were given 2 booster injections at intervals of 2 weeks with the same amount of antigen in complete Freund's adjuvant; 5 days after the last immunisation, the animals were bled and the antisera were stored at −20°.

Mouse embryos, tissues, and tumors. — Embryos were obtained by natural mating of random-bred Jcl:ICR mice. The day of vaginal plug was designated as day 0 of pregnancy. Preimplantation embryos were collected by flushing the oviduct–uterus with HEPES-buffered Whitten's medium[11]. Zonae pellucidae were removed using 0.5% pronase E (Kaken Kagaku), and the embryos were left for 3 h in Whitten's medium for recovery. Some egg cylinder embryos (day 6.5) were fixed with ethanol within the decidual tissue. The fixed embryos were processed according to standard histological methods, embedded in paraffin, and serially sectioned at 3-μm thickness. Other embryos were isolated from decidua and separated mechanically[12] into three fractions, namely, embryonic ectoderm, visceral endoderm, and parietal endoderm with Reichert's membrane. The organs were dissected from 2-month-old 129/Sv mice. Embryonal carcinoma cells, F9[13] and N4-1[14], were grown as solid tumors subcutaneously in 129/Sv mice. The organs and tumors were treated in the same way as the fixed embryos.

Staining of embryos with fluorescein isothiocyanate (FITC)–FBP-F. — Living preimplantation embryos and isolated fractions from the egg cylinder embryos were exposed to FITC-conjugated FBP-F (50 μg/mL) for 1 h at 4° under liquid paraffin. After washing with PBS, each embryo was transferred into a microdrop of PBS

under liquid paraffin on the glass slide and observed without coverslips. The section of an egg cylinder embryo was dewaxed and incubated with FITC–FBP-F for 1 h at room temperature. After washing in PBS, the sections were mounted in glycerol. Microscopic observation was performed with a fluorescence microscope equipped with an epi-illuminator (Olympus, Model BH-2). In control experiments, FITC–FBP-F was first incubated with an equal volume of 0.2M L-fucose in PBS and then applied to the specimen.

Staining of tissue section by the peroxidase–antiperoxidase (PAP) technique. — The sections of organs and tumors were dewaxed and exposed tp FBP-F (2.46 μg/mL) overnight. After washing with PBS, rabbit antiserum (1:160 dilution) against the lectins, swine anti-rabbit immunoglobulin (DAKO Immunoglobulin Ltd., Denmark, 1:10 dilution in PBS containing 1% of BSA) and PAP complex (DAKO, 1:10 dilution in PBS containing 1% of BSA) were serially mounted with thorough washing in PBS between each antiserum[15]. Binding sites of the lectins were detected[16] by reaction with 3-amino-9-ethylcarbazole.

Other biochemical methods. — Protein was determined[17], and SDS–polyacrylamide gel electrophoresis[18] and two-dimensional gel electrophoresis[19] under reduced conditions were performed, according to the literature methods.

FITC (Sigma) was coupled to FBP-F as follows. A solution of FBP-F (5 mg) in PBS (200 μL) was mixed with FITC (200 μL, 0.5 mg/mL in 0.1M $NaHCO_3$ containing M NaCl). The mixture was kept for 3 h at room temperature in the dark. FBP-F conjugated with FITC was separated from free FITC by column chromatography on Sephadex G-25. Fractions eluted in the void volume were collected.

Other immunological methods. — Ouchterlony double-diffusion analysis was performed in 1% agar gel containing 2% of Triton X-100, 0.15M NaCl, 50 μg/mL of phenylmethylsulfonyl fluoride, and 0.05% of sodium azide in 0.01M Tris-HCl buffer (pH 7.6).

Hemagglutination assays were performed in U-bottom hemagglutination titer plates, using a 1% suspension of human type-H erythrocytes in PBS (2 mL).

RESULTS

Comparison of the molecular properties of FBP-F and FBP-M. — FBP, isolated from *Lotus* seeds by affinity chromatography on fucose–Sepharose or on porcine gastric mucin–Sepharose, were analysed by SDS–polyacrylamide gel electrophoresis (Fig. 1). The lectin purified on fucose–Sepharose contained two species having mol. wts. of 25,000 (Fig. 1, arrow) and 26,000. The lectin purified on mucin–Sepharose showed only one band of mol. wt. 26,000. When fractionated on mucin–Sepharose, the lectin purified on fucose–Sepharose gave non-absorbed (FBP-F, mol. wt. 25,000) and absorbed material (FBP-M, mol. wt. 26,000); upon re-chromatography, FBP-F bound to fucose–Sepharose.

On two-dimensional gel electrophoresis, FBP, isolated by using fucose–Sepharose, gave four protein components (*a–d*, Fig. 2A). FBP-F gave only one

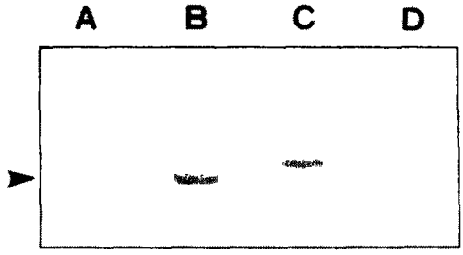

Fig. 1. SDS-polyacrylamide gel electrophoresis of FBP. Concentration of acrylamide in the running gel was 12% and staining was with Coomassie Brilliant Blue. Standard substances migrated as follows (cm): bovine serum albumin (67 k), 2.65; ovalbumin (45 k), 4.25; chymotrypsinogen A (25 k), 7.35; Bromophenol Blue, 10.5. FBP-F and FBP-M migrated 7.35 and 7.15 cm, respectively. Although only a part of the gel was shown, no other bands were detected on the gel. A, FBP-F+M (4.0 μg); B, FBP-F (2.5 μg); C, FBP-M (2.5 μg); D, FBP-M′ (2.0 μg).

component corresponding to *a* (Fig. 2B), and FBP-M gave three components corresponding to *b–d* (Fig. 2C). The pI values were estimated to be *a*, 5.32; *b*, 5.40; *c*, 4.97; *d*, 4.80.

Rabbit antiserum against FBP-F formed a single precipitation line with FBP-F, but not with FBP-M upon Ouchterlony double-diffusion analysis (Fig. 3A). Conversely, antiserum against FBP-M reacted with FBP-M, but not with FBP-F (Fig. 3B). These results suggested that FBP-F was substantially different from the components of FBP-M.

Reactivities of the isolectins to human type-H erythrocytes and to adult and embryonic tissues of the mouse. — FBP-M agglutinated human type-H erythrocytes

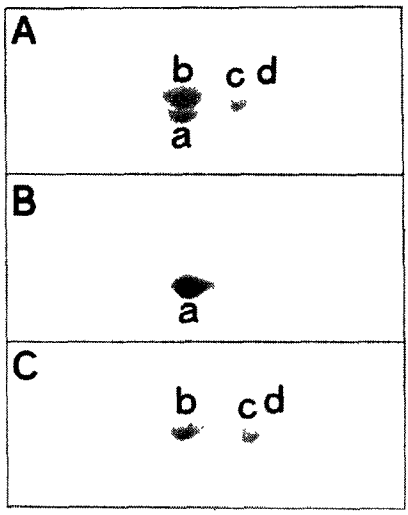

Fig. 2. Two-dimensional gel electrophoresis of FBP. For the second dimension, 12% polyacrylamide gel was used. Proteins were stained with Coomassie Brilliant Blue. A, FBP-F+M (10 μg); B, FBP-F (10 μg); C, FBP-M (8 μg).

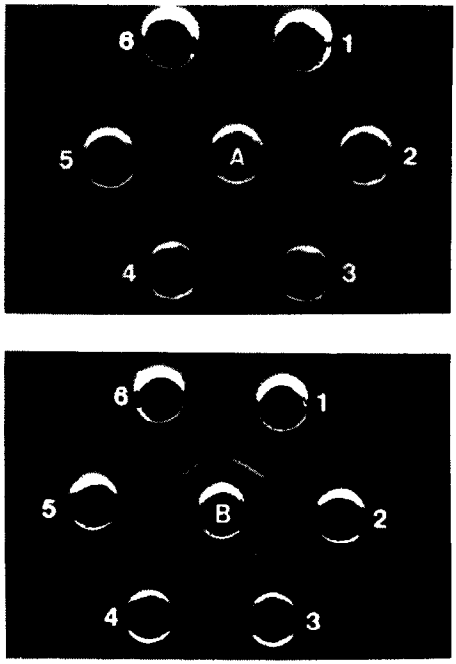

Fig. 3. Immunological independence of FBP-F and FBP-M by Ouchterlony double-diffusion analysis (see Experimental). Antisera were used without dilution. The peripheral wells contained 10 μg of FBP-F+M (1 and 4), FBP-F (2 and 5), or FBP-M (3 and 6). The centre well contained anti-FBP-F (A) or anti-FBP-M (B).

at a minimum concentration of 16.9 μg/mL, but FBP-F did not agglutinate the erythrocytes even at 760 μg/mL. Staining with the lectins labelled with FITC yielded the same result. The reactivity of FBP with human type-H erythrocytes has been well established[5]. Thus, FBP-F may be regarded as a new isolectin of FBP.

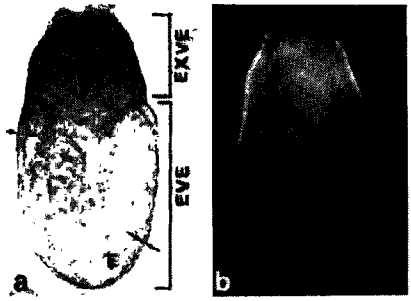

Fig. 4. Immunofluorescent staining of FITC-conjugated FBP-F on a living egg cylinder (day 6.5) deprived Reichert's membrane. After mechanical removal of Reichert's membrane, the visceral endoderm monolayer (arrows) covering the inner ectoderm layer was exposed. This specimen was incubated *in situ* with the FITC-labelled lectin and observed as described in the Experimental. FBP-F reacted strongly with extra-embryonic visceral endoderm (EXVE) cells, but not with embryonic visceral endoderm (EVE) cells. Phase-contrast optics (a) and u.v. illumination (b) (× 73).

Fig. 5 Staining of a section of esophagus by FBP-F, using anti FBP-F and the PAP technique (see Experimental): (a) stained specimen. (b) control specimen. where FBP-F was preincubated with 0 1M fucose (× 40)

The reactivity of FBP-F toward a number of adult and embryonic tissues of the mouse was examined (Table I). Only two sites, namely, extra-embryonic visceral endoderm of early post-implantation embryos (Fig. 4) and esophagus of adult mice (Fig. 5) reacted with FBP-F, so that its reactivity, compared to that of FBP-M, is much more restricted (Table I).

DISCUSSION

The fucose-binding isolectins (FBP) of *Lotus tetragonolobus* have been isolated by several different methods. Yariv *et al.*[1] precipitated the lectin with a dye containing three α-L-fucopyranose residues. Blumberg *et al.*[2] isolated the lectin by affinity chromatography on a column of *N*-(ε-aminocaproyl)-β-D-fucopyranosyl-amine coupled to agarose gel. FBP has also been purified by adsorption on to insoluble polyleucyl hog A + H blood-group substance[5]. In spite of differences in methodology, the same three isolectins, separable by means of DEAE-cellulose column chromatography or isoelectrofocusing, were obtained. Using sodium dodecyl sulfate(SDS)-polyacrylamide gels, the mol. wts. of the three isolectins were estimated[5] to be in the range 27,000–28,000. More recently, Allen and Johnson[3] used an affinity adsorbent (prepared by coupling[20] L-fucose to Sepharose 6B) to isolate FBP and reported the presence of isolectins revealed by polyacrylamide gel electrophoresis at acidic and alkaline pH. The authors asserted that the results were in accordance with those of Pereira and Kabat[5]. We have isolated FBP by using an affinity chromatography adsorbent devised by Allen and Johnson[3]. The lectins isolated were further fractionated by affinity chromatography on mucin–Sepharose into two groups, FBP-F and FBP-M. On two-dimensional gel electrophoresis, FBP-M was separated into three components, which were probably identical to those reported by Kalb[6], Blumberg *et al.*[2], and Pereira and Kabat[5], as judged on the basis of their isoelectric points, apparent mol. wts. on SDS-polyacrylamide gel electrophoresis, and hemagglutination activity. No isolectin described hitherto was similar to FBP-F, which was not bound to mucin–Sepharose

TABLE I

COMPARISON OF DISTRIBUTION OF BINDING SITES[a] FOR FBP-F AND FBP-M ON EMBRYONIC AND ADULT TISSUES OF THE MOUSE

		FBP-F	*FBP-M*[b]
Embryos			
2-Cell (1.6-day)		−	−
8-Cell (late stage, 2.6-day)		−	+
16-Cell (2.9-day)		−	++
Early blastocyst (3.7-day)			
Trophectoderm		−	++
Inner cell mass		−	±
Egg cylinder (6.5-day)			
Visceral endoderm	extra-embryonic	++	++
	embryonic	−	+
Ectoderm	extra-embryonic	−	−
	embryonic	−	++
Parietal endoderm		−	−
Reichert's membrane		−	−
Adult mice			
Esophagus		+	+
Stomach		−	++
Small intestine		−	++
Large intestine		−	−
Kidney		−	−
Liver		−	++
Lung		−	−
Heart		−	−
Skeletal muscle		−	−
Testis		−	−
Ovary		−	−
Teratocarcinomas			
F9		−	++
N4-1		−	++

[a]The intensity of fluorescence and reactive products resulting from PAP: −, negative; ±, faint stain; +, moderate stain; ++, intense stain. [b]Ref. 21.

and did not show hemagglutination activity against human type-H erythrocytes. Pereira and Kabat[5] showed that their component I had the lowest hemagglutinating activity (450 μg/mL) with type-H erythrocytes. Even at higher concentrations, FBP-F did not agglutinate type-H erythrocytes. FBP-F reacted with few tissues in the mouse and its specificity may be of value in detecting or isolating certain subpopulations of cells. It will be of interest to examine human malignant cells for receptors for FBP-F.

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