Biochimica et Biophysica Acta 905 (1987) 371–375
Elsevier

BAA 73819

Quantitative determination of the lectin binding capacity of small intestinal brush-border membrane. An enzyme linked lectin sorbent assay (ELLSA)

Henno G.C.J.M. Hendriks [a], Jos F.J.G. Koninkx [a], Marjan Draaijer [a], Jaap E. van Dijk [a], Jan A.M. Raaijmakers [b] and Johan M.V.M. Mouwen [a]

[a] *Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Utrecht, Utrecht, and* [b] *Department of Pulmonary Diseases, University Hospital, Utrecht (The Netherlands)*

(Received 23 July 1987)

Key words: Lectin binding; Brush-border membrane; Agglutinin; Soybean; Enzyme linked lectin sorbent assay (ELLSA); (Bovine intestine)

A test to determine quantitatively the lectin binding sites in brush-border membranes has been developed. Highy purified bovine small intestinal brush-border membranes were prepared, and subsequently coated directly to the bottom of a microtiter plate. Soybean agglutinin conjugated with peroxidase was coupled to its binding sites in the brush-border membranes and the peroxidase activity was determined in a spectrophotometer. The number of soybean agglutinin binding sites in the brush-border membranes has been established by means of iterized computer fit analysis of the data, indicating values for maximal binding of $7 \cdot 10^{-7}$ M soybean agglutinin per mg of brush-border membrane protein and a dissociation constant of $1.5 \cdot 10^{-5}$ M.

Introduction

Many food elements are known to contain specific carbohydrate binding proteins called lectins [1]. Lectins have been reported to cause severe damage to the digestive tract [2–4] growth problems [5–7] and even death [8].

The phenomenon of bloodgroup specificity of some lectins [9,10] suggests the possibility of a variability in the occurrence of lectin binding sites in other tissues, too. A quantitative test of these binding site has not been reported. The haemagglutination test commonly used to predict the undesirable effects of food lectins gives great discrepancies in agglutinating effects on erythrocytes of different species [11,12]. Moreover, since the surface of erythrocytes may not be representative for that of the small intestinal epithelium, the test probably lacks any predictive value with respect to the effect of lectin-containing food on growth.

This paper presents a test to determine quantitatively the occurrence of lectin binding sites in bovine small intestinal brush-border membranes.

Materials and Methods

Intestinal samples. Samples of small intestine (20 cm) of normal cows were taken 3 m. distal to the Treitz-ligament. They were collected in the slaughterhouse within half an hour after death. Subsequently the intestinal samples were cut open longitudinally at the mesenterial attachment, cleaned, sealed in plastic bags, frozen on solid CO_2 and stored at $-70\,^{\circ}C$ until use (within two months).

Preparation of the microvillus membrane frac-

Correspondence: H.G.C.J.M. Hendriks, Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 1, 3584 CL Utrecht, The Netherlands.

tion. Brush-border fragments were prepared according to the technique of Pinto et al. [13], with the following modifications: The intestinal specimens were partially thawed and the mucosa (and part of the submucosa) were stripped off from the underlying tissue with a razor blade. A 5% (v/v) homogenate was made using a Virtis blender (The Virtis Company, Gardiner, NY 12525, U.S.A.) at full speed during 5 min. All preparations were performed at 4°C. Brush-border fragments were used to prepare the microvillus membrane fraction according to the method of Schmitz et al. [14].

Preparation of samples for electron microscopy. For ultrastructural examination a 200 000 × *g* pellet of brush-border membrane fragments containing 1 mg of protein (absorbance 280 nm —260 nm) was prefixed with 1.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) and postfixed in 2% (v/v) OsO_4 in the same buffer. After dehydration the pellet was embedded in Durcupan plastic (A.C.M. Fluka), and routinely processed.

Enzyme activity assays. Alkaline phosphatase (EC 3.1.3.1) was used as brush-border membrane marker, and assayed according to Garen and Levinthal [15].

Enzyme linked lectin sorbent assay (ELLSA). Greiner (Alphen a.d. Rijn, The Netherlands), Titertek (Flow Laboratories, Herts, U.K.), Dynatech (Nutacon, Zug, Switzerland) and Omnilab (Breda, The Netherlands) flat bottom disposable polystyrene microtiterplates (96 wells) were tested for their suitability in the enzyme linked lectin sorbent assay.

Several empty spot covering agents were tested since preliminary experiments indicated a high affinity of soybean agglutinin-peroxidase (E-Y Labs Inc. San Mateo, U.S.A.) to the uncoated bottom of the microtiterplate.

To establish the most suitable one, the following empty spot covering agents have been tested: 1, 5 and 10% (w/v) bovine serum albumin (BSA, fraction V) (Sigma, U.S.A.); 1% (w/v) fibrinogen (Sigma); 1% (w/v) β-alanine (Merck, F.R.G.); 0.5% (w/v) polylysine (Sigma); 0.5% (w/v) gelatin (Merck) and 1, 5 and 10% (w/v) ovalbumin (Sigma). Dilutions of empty spot covering agents and soybean agglutinin-peroxidase were made in 0.01 M phosphate-buffered saline (pH 7.45).

Optimal coating time as well as optimal coating temperature were established for the membrane fraction.

Actual experimental conditions. Overnight evaporation at 37°C of 2.5 μg of brush-border membrane protein in 100 μl of distilled water was performed.

Empty spots on the bottom of the plates were covered by adding 250 μl of 1% (w/v) bovine serum albumin to the wells. Subsequently the plates were sealed with clear tape and incubated at 37°C for 1 h. After incubation the plates were washed two times for 30 s with tap water and flick dried.

A saturation curve was made by incubation of the plates with 100 μl of soybean agglutinin-peroxidase/well with concentrations ranging from 0.15 nM to 40 nM soybean agglutinin-peroxidase. After that, the plates were washed two times for 30 s with 0.05% (v/v) Tween-20 (Serva, U.S.A.), flick dried, and after incubation of 60 min with 200 μl of 0.4 mM 2,2′-azino-bis-(3-ethylbenzthiazolinesulfonic acid) (ABTS) (Merck, F.R.G.) in 0.05 M citrate buffer (pH 4.0) and 1.5 mM hydrogen peroxide, peroxidase activity was measured in a multiscan spectrophotometer (Titertek Multiskan, Flow Laboratories) at 405 nm.

The molar absorption of soybean agglutinin-peroxidase was calculated from the absorbance at 405 nm displayed by serial dilutions of soybean agglutinin-peroxidase after incubaton for 60 min with 200 μl of ABTS substrate.

The data of saturation experiments were analysed using an iterative curve fit program [16] based on the Ligand program and executed on a Zenith Z-100 microcomputer. By means of this program the specific binding was calculated in saturation experiments from the untransformed total binding data and from the specific binding data, using corrected free ligand concentrations. The data were fitted in a one binding site and a two binding sites model. The best fit was chosen using least-squares values that were statistically compaired in an *F*-test.

Results

Morphological and biochemical characterization of the purified brush-border membrane fraction

Electron microscopical preparation of the

brush-border membrane pellets showed a pure fraction of mostly vesiculated membranes. The alkaline phosphatase specific activity of this fraction showed an 11.3-fold increase over the homogenate.

Enzyme linked lectin sorbent assay (ELLSA)

Of the several microtiterplates tested the Greiner-plate was found to produce the best results. So all experiments were performed with this plate.

The binding of soybean agglutinin-peroxidase to bovine serum albumin is the lowest of all empty spot covering agents tested and independent of the concentration used. Polylysine, on theoretical grounds the best empty spot covering agent since it contains no sugar-residues, did not attach to the bottom of the plate at all.

To make the test suitable for quantitative determination of the lectin binding capacity of unknown samples it is necessary to know the exact amount of brush-border membranes involved in the test. In Fig. 1 it is demonstrated that coating of brush-border membrane-protein present in 100 μl of distilled water is best achieved by overnight evaporation. The optimal temperature for the brush-border membrane-coating was 37 ° C.

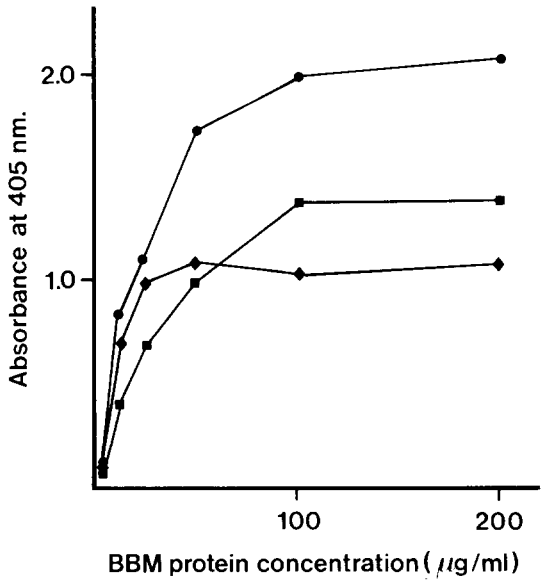

Fig. 1. Time dependence of the coating of bovine brush-border membranes (BBM) to the bottom of the microtiterplates. The coating of the brush-border membranes was performed at 37 ° C by incubation during 1 h (■), 16 h (◆) and evaporation (●). After covering the empty spots with 1% (w/v) bovine serum albumin, the brush-border membranes were incubated during 1 h at 37 ° C ith 100 μl of 5 μg of soybean agglutinin-peroxidase/ml and peroxidase activity was determined.

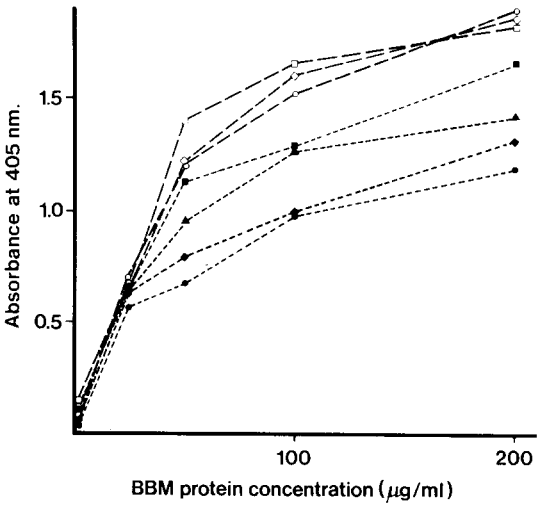

Fig. 2. Determination of the soybean agglutinin-peroxidase (SBA-PO) saturationpoint for bovine brush-border membranes. Brush-border membranes (100 μl aquadest) were coated to the bottom of the microtiterplates by evaporation. After covering the empty spots with 1% (w/v) bovine serum albumin, the brush-border membranes were incubated during 1 h at 37 ° C with 100 μl of 10 μg (●), 20 μg (◆), 30 μg (▲), 40 μg (■), 60 μg (□), 80 μg (○) and 100 μg (◇) of soybean agglutinin-peroxidase/ml, respectively. Subsequently peroxidase activity was determined.

For obvious reasons quantitative determinations can only be made when saturation curves of the soybean agglutinin-peroxidase binding capacity are made. This point has been reached when offering more soybean agglutinin-peroxidase does not cause more binding to brush-border membranes. When using up to 20 μg of brush-border membranes per well, 6 μg of soybean agglutinin-peroxidase per well appears to be sufficient to produce saturation (Fig. 2).

The reproducability of the method was determined by testing the same brush-border membrane sample in duplicate six times within two weeks. A typical experiment in this series is presented in Fig. 3. After computer analysis of the individual curves the mean B_{max} and K_d values $\pm$ S.E. of six experiments were $(683 \pm 51) \cdot 10^{-9}$ M per mg of brush-border membrane protein and

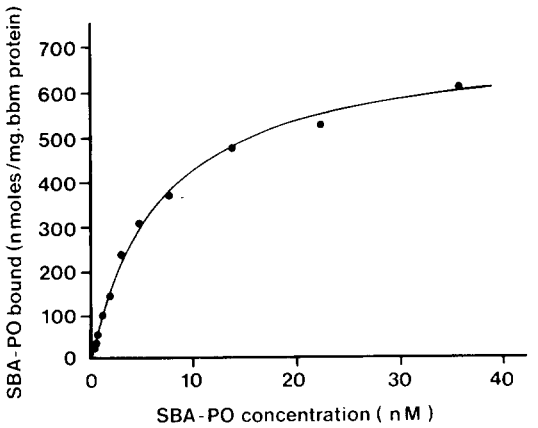

Fig. 3. Specific binding of soybean agglutinin-peroxidase (SBA-PO) to bovine brush-border membranes. Experimental conditions were optimal with respect to coating of the brush-border membranes and the use of bovine serum albumin as empty spot covering agent. Specific binding amounted to 70% of total binding.

$(1.52 \pm 0.26) \cdot 10^{-5}$ M, respectively. The data fitted best the one-binding site model.

Formation of the soybean agglutinin-brush-border membrane complex could be prevented and the complex already formed could be dissociated by using concentrations of 10^2 till 10^8 nM of the soybean agglutinin specific sugar (*N*-acetyl-D-galactosamine, Sigma) (1 to 10^6 times the concentration of soybean agglutinin).

Discussion

Of the brush-border associated enzymes sucrase-isomaltase is normally used as marker enzyme for brush border membranes. This marker enzyme could not be used in cows since bovine intestinal epithelium lacks sucrase-isomaltase activity [17]. In this study alkaline phosphatase was used as a marker enzyme for brush-border membranes. An established 11.3-fold enrichment of the specific activity of alkaline phosphatase in brush-border membranes over the homogenate is in agreement with other investigations [18].

Both *Phaseolus vulgaris* haemagglutinin and soybean agglutinin bind specificially to *N*-acetyl-D-galactosamine. The dissociation constant found for *Phaseolus vulgaris* haemagglutinin in rat intestine by other investigators [19] is in agreement with our findings for soybean agglutinin binding in bovine intestine.

In this paper a highly reproducible test is presented with which the number of binding sites for soybean agglutinin can be determined. Slight modifications will make the test suitable to detect the number of binding sites for other lectins as well. In comparison with other methods our method permits to study glycoprotein-lectin complexes without previous purification of glycoproteins or isolated cells [20] and to screen the whole scala of glycoproteins present in the brush-border membranes in one test. For studies on the interaction between intestine and lectin a great advantage of this test is the direct homology between the test-material (brush-border membranes) and the object in study (intestinal epithelium). The small amount of brush-border membrane protein (approx. 25 μg) required to quantify the lectin binding sites enables the testing of biopsies. Modifications will make the test also suitable for the determination of lectins in food using brush-border membranes of any species.

Studies on the predictive value of this test towards the relationship between the degree of binding of lectins to brush-border membranes and the damaging effects on the intestinal epithelium are in progress.

Acknowledgements

The authors are grateful to Ton Ultee for electronmicroscopical preparations, to Henk Halsema for his help with the figures and to Marijke van Dijk for typing the manuscript.

References

1 Boyd, W.C. and Shapleigh, E. (1954) Science 119, 419
2 Lorenzsonn, V. and Olsen, W. (1982) Gastroenterology 82, 838–848
3 King, T.P., Pusztai, A. and Clarke, M.M. (1980) J. Comp. Pathol. 90, 585–595
4 Lorenz-Meyer, H., Roth, H., Elsässer, P. and Hahn, U. (1985) Eur. J. Clin. Invest. 15, 227–234
5 Liener, I.E. (1974) J. Agric. Food Chem. 22, 17–22
6 Evans, R.J., Pusztai, A., Watt, W.B. and Bauer, D.A. (1973) Biochim. Biophys. Acta 303, 175–184
7 Andrews, A.T. and Jayne-Williams, D.J. (1974) Br. J. Nutr. 32, 181–189
8 Pusztai, A. (1985) Nutr. Abstr. Rev. (Ser. B) 55, 363–369

9 Wurster, K., Peschke, P. and Kuhlmann, W.D. (1983) Virchows Arch. (Pathol. Anat.) 402, 1–9
10 Etzler, M.E. and Kabat, E.A. (1970) Biochemistry 9, 869–877
11 Jaffé, W.G., Brücker, O. and Palozzo, A. (1972) Z. Immun. Forsch. Bd. 142, 439–447
12 Grant, G., More, L.J., McKenzie, N.H., Stewart, J. and Pusztai, A. (1983) Br. J. Nutr. 50, 207–214
13 Pinto, M., Robine-Leon, S., Appay, M., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) Biol. Cell. 47, 323–330
14 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Carda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112
15 Garen, A. and Levinthal, C. (1960) Biochim. Biophys. Acta 38, 470–483
16 Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220–239
17 Roy, J.H.B. (1980) Studies in the Agricultural and Food Sciences, The Calf, 4th Edn., pp. 205–207, Butterworths, London
18 Crooker, B.A. and Clark, J.H. (1986) J. Diary Sci. 69, 58–70
19 Boldt, D.H. and Banwell, J.G. (1985) Biochim. Biophys. Acta 843, 230–237
20 Köttgen, E. (1979) Klin. Wochenschr. 55, 359–373