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AGGLUTINATION OF BACTERIAL SPHEROPLASTS

I. EFFECT OF CONCANAVALIN A

HIROMI B. MARUYAMA*

Department of Vitamin and Nutritional Research, F. Hoffmann-La Roche and Co., Ltd, 4002 Basle (Switzerland)

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SUMMARY

Concanavalin A was found to agglutinate *Escherichia coli* spheroplasts prepared with EDTA and lysozyme and treated with proteases, in the range of population density from 10^8–10^9 per ml. Non-treated spheroplasts were not agglutinated by this agglutinin in the above range but, when the density was higher than 10^{10} per ml, they showed a spontaneous agglutination regardless of concanavalin A, which was diminished by protease treatment. Concanavalin A-specific agglutination was inhibited by α-methyl-D-glycoside and other sugars, while none of the tested sugars showed any significant inhibition of the spontaneous agglutination.

INTRODUCTION

During studies on the relation of cell membrane damage to ribosome degradation in *Escherichia coli*[1-5] (for review see refs. 4 and 5), we began to investigate protease-induced alteration on spheroplast membranes using a lectin, concanavalin A.

Concanavalin A is known to possess the properties of hemagglutination[6], precipitation with polysaccharides[7], and mitogenic induction in animal cells[8]. Recent progress around this lectin has been focused on the fact that this agglutinin can reveal the altered properties of the surface of transformed cells by differential agglutination[9,10] and that, after trypsin treatment, it can restore growth control in the malignant cells[11]. Therefore, if concanavalin A does act on the bacterial spheroplast in a similar manner, it should provide a suitable system for studying not only the effect of a specific surface alteration on cellular physiology, including ribosome degradation, but also the structural differences between mammalian and bacterial cell membranes about which, at present, little is known except for their gross chemical composition[12]. It is also a question whether this system could be regarded as a simpler model of the transformed and normal mammalian system with regard to its reaction with concanavalin A. In this paper we report that, under a given range of spheroplast concentration, concanavalin A causes the specific agglutination of bacterial spheroplasts treated with proteases.

* Present address: Department of Microbiology and Chemotherapy, Nippon Roche Research Center, Nippon Roche K.K., 200 Kajiwara, Kamakura, Kanagawa Pref., Japan.

MATERIALS AND METHODS

Escherichia coli K12:W3110 and *Proteus vulgaris* Bern X, grown in 200 ml of a nutrient medium[13] modified according to E. Semadeni by supplementation of 0.2% $MgSO_4$ and 10% sucrose (F. Hoffmann–La Roche and Co., Basle, unpublished) to a population of approx. 10^9 cells per ml, were harvested, washed twice with 0.03 M Tris, pH 7.4, and suspended to a final density of $2–5 \cdot 10^9$ cells per ml in the same buffer supplemented to have 20% sucrose (sucrose–Tris). The osmotically shocked cells were prepared by the method of Neu and Heppel[14], while lysozyme spheroplasts and EDTA–lysozyme spheroplasts were prepared with Lysozym (C. F. Boehringer and Söhne GmbH) according to Birdsell and Cota Robles[15], except that a prolonged incubation (1 h at 37 °C) was followed by extensive washing either with sucrose–Tris alone or sucrose–Tris supplemented with EDTA (a final concentration of 10^{-3} M). Penicillin spheroplasts were obtained from the culture grown with 50 (*P. vulgaris*) or 400 μg/ml (*E. coli*) Penicillin Novo (G, 1.6 I.U./μg) overnight and prepared in the same way. The final preparation of each type was suspended in various volumes of sucrose–Tris so as to give the desired cell density, ranging from 10^8 to $2 \cdot 10^{11}$ per ml. Protease treatment was carried out in 20 ml of sucrose–Tris, pH 7.4, at 37 °C for 30 min at the range of $10^9–10^{10}$ ml cell density with 50 μg/ml of either trypsin (20000 units/g), proteinase K (15 m Anson units/g) or pronase E (70000 PUK units/g), all purchased from Merck. The reaction was stopped by either 5 μg/ml of soy bean trypsin inhibitor (Merck) or by chilling, followed by washing twice with sucrose–Tris. The standard assay mixture for agglutination contained per ml: 30 μmoles Tris, pH 7.4, 2 mmoles sucrose, $10^8–10^{10}$ speroplasts and 500 μg of concanavalin A (Grade III, Sigma). Demetallized concanavalin A was prepared by the method of Kalb and Levitzki[16]. The agglutination was scored by microscopic observation (a Zeiss standard GFL) and expressed by the serological scale ranging from 0 to ++++ according to Benjamin and Burger[17]. In later experiments, quantitation of agglutination, based on differential migration towards natural gravity, was also applied and showed a good correspondence to the microscopic method, details of which will appear elsewhere (H. B. Maruyama, in preparation). The sugars used are products from Merck, except for the α-methyl-D-glycosides, which were obtained from Fluka AG, Buchs, Switzerland. Total viable number was measured by the "1% Agar method" of Tonomura and Rabinowitz[18].

RESULTS AND DISCUSSION

When rod cells, osmotically shocked cells, lysozyme spheroplasts, penicillin spheroplasts, and EDTA–lysozyme spheroplasts, at a population density of less than 10^9 per ml, were incubated overnight at 37 °C in the standard mixture, no agglutination was detected under microscopy. However, when EDTA–lysozyme spheroplasts were incubated after brief treatment with trypsin or proteinase, agglutination was clearly observed in the presence of concanavalin A. Typical aggregates thus formed contained many large clumps of spheroplasts, while less than 5% of the spheroplasts remained single. Phase contrast microscopy showed that the spheroplasts in the aggregates were evenly dense and their interlinkage was apparently not restricted to a limited number of binding points on the surface. A similar agglutination, though to a lesser degree, was obtained with protease-treated penicillin spheroplasts and lysozyme

spheroplasts, but not with others. Non-treated EDTA–lysozyme spheroplasts, however, showed a spontaneous agglutination when it was incubated at a population density of more than 10^{10} per ml, suggesting the existence of a sort of "agglutinin" on the spheroplast surface. Morphologically, these agglutinates were hardly distinguishable from those of protease-treated EDTA–lysozyme spheroplasts, except for the relatively infrequent occurrence of large aggregates of more than 20 EDTA–lysozyme spheroplasts in the spontaneous agglutination. Fig. 1 shows the concentration dependence of the agglutination in both non-treated and protease-treated EDTA–lyzozyme spheroplasts. It was found that, regardless of the presence or absence of concanavalin A, the same degree of agglutination occurs in non-treated EDTA–lysozyme spheroplasts, while the protease-treated EDTA–lysozyme spheroplasts usually showed no spontaneous agglutination when concanavalin A was omitted. This indicates that the presumable intrinsic "agglutinin" on the spheroplast was almost completely destroyed by treatment with protease (Fig. 1). It was also noted that older preparations of EDTA–lysozyme spheroplasts, which had been in cold storage for more than 3 days, increased the tendency toward spontaneous agglutination. Concanavalin A lost its agglutinability for protease-treated EDTA–lysozyme spheroplasts when demetallized by the method of Kalb and Levitzki[16].

Under the standard condition, the agglutination starts to occur within 30 min

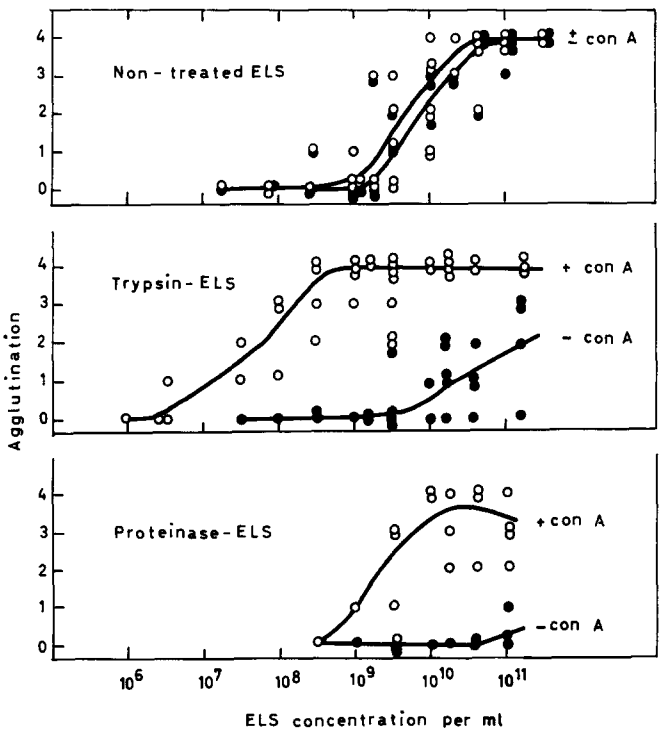

Fig. 1. Population density dependence of agglutinate formation of non-treated (top), trypsin-treated (middle) and proteinase-treated (bottom) EDTA–lysozyme spheroplasts (ELS). Incubation was done at 37 °C for 4 h in the standard mixture with (○) or without (●) concanavalin A (con A) at a final concentration of 0.5 mg/ml.

and is completed in 3 h, together with a slight decrease of turbidity and viability. However, in neither case was there any significant difference between these two parameters in the presence or absence of concanavalin A. The minimal concentration of concanavalin A and trypsin to give the full grade of agglutination was 50 μg (with 50 μg/ml trypsin-treated EDTA–lysozyme spheroplasts) and 10 μg (under 0.5 mg/ml concanavalin A) per ml, respectively. Longer incubation with, or a greater amount of trypsin and concanavalin A had no significant effect on the degree of agglutination; but considerable lysis was observed at a concentration of more than 2 mg/ml of concanavalin A and 100 μg/ml of trypsin. Incubation at 24 °C considerably retarded agglutination. The data presented here concern only *E. coli*; similar but less intensified agglutination was seen with *P. vulgaris* penicillin spheroplasts.

The inhibitory effects of various sugars on agglutination were tested in two ways. In haptenic experiments, sugar incubated for 1 min with concanavalin A at 24 °C was added to the standard mixture of EDTA–lysozyme spheroplasts and the aggregate formation was scored after an additional 4 h incubation at 37 °C. On the other hand, the effect of sugar on the dispersion of aggregates was examined after 2 h of incubation at 37 °C on the agglutinated EDTA–lysozyme spheroplasts with sugar. Data given in Table I indicate that α-methyl-D-glucoside had a strong inhibition on both tests at a concentration of $2 \cdot 10^{-3}$ M, which is considerably low in comparison with that of mammalian cells[19]. Others, such as α-methyl-D-mannoside, D-mannose, D–galactose and *N*-acetyl-D-galactosamine, represent poorer inhibitors. sucrose, which is necessary in the standard mixture for the protection of spheroplast, is known to be a moderate inhibitor of α-glucan precipitation by concanavalin A[20]. The presence of 20% sucrose throughout our studies may be attributable to some different

TABLE I

EFFECT OF SUGARS ON AGGLUTINATION AND AGGREGATE DISPERSION

Sugars	*Agglutination**			*Dispersion***	
	ELS	*Trypsin-ELS*	*Proteinase-ELS*	*ELS*	*Trypsin-ELS*
None (no concanavalin A)	++++***	±	o	—§	—§
None (with concanavalin A)	++++	+++++	+++	++++	±+++
D-Glucose	−±++	+++	±+	+±++	−++
D-Mannose	±+±+	+±	+	−+++	+±+
G-Galactose	−+++	++	++	−+++	±+
L-Fucose	−+++	+++	++	++±	+−
N-Ac-D-glucosamine	+±++	+++	+	−++	−+
N-Ac-D-galactosamine	++++	++	++	−++	++
α-Me-D-glucoside	+++	+	o	+±+	±
α-Me-D-mannoside	−+−	++	+	−++	−

* The standard mixture containing 10^{10} EDTA–lysozyme spheroplasts (ELS) per ml was incubated for 4 h at 37 °C with 0.5 mg of concanavalin added by sugars (a final concn of 10 μmoles/ml).

** To the aggregates formed by overnight incubation with 0.5 mg/ml of concanavalin, sugar was added and incubated for an additional 2 h. The concentration of sugars added and ELS was the same as in *.

*** Agglutination was the average of two observations. Thus, the score of degree 1 *plus* zero gives ±.

§ Not carried out.

features in the agglutination between mammalian tumor and the present system: such as delayed completion of the reaction and inhibition by monosaccharides at a lower concentration in our system as well as the fact that α-methyl mannoside is a poorer inhibitor than α-methyl glucoside. It is noted that, in case of non-treated EDTA–lysozyme spheroplasts, no inhibitory effects of any of the tested sugars was observed, suggesting the structural difference around the presumable receptor sites for concanavalin A and for spontaneous agglutinin. Among several mono- and divalent cations tested, Mg^{2+} and Ca^{2+} were found to inhibit the agglutination considerably at the concentration of 10^{-3} M, regardless of whether EDTA–lysozyme spheroplasts was previously treated with protease or not.

This study indicates that the surface of EDTA–lysozyme spheroplasts contains a proteinous "agglutinin" system as well as the receptor site for the concanavalin A integrated on it in a topographically different manner. Although Brenner *et al.*[21] suggested that the spheroplasts from Gram-negative bacteria are not completely lacking all cell wall compounds, it has been confirmed that EDTA–lysozyme spheroplasts have a single layer of cytoplasmic membrane[15] over large areas of their surface. Therefore, one of the most likely interpretations would be: there is an intrinsic "agglutinin" somewhere on the EDTA–lysozyme spheroplasts surface. This can be digested or inactivated by a short treatment with protease which, in turn, also reveals the cryptic receptor sites for concanavalin A integrated in the membrane, or changes the conformation of a certain area of the membrane, thereby allowing agglutination in the presence of concanavalin A. The actual digestion by proteases—not merely their absorption on the surface—seems to be necessary for the agglutination, since the treatment at 0 °C or in the presence of trypsin inhibitor causes a lesser or negligible agglutination. The contribution of lipopolysaccharides in the outmost membrane layer on this agglutination, though not ruled out, seems to be very small in the light of the negligible, or lesser agglutination found in osmotically shocked spheroplasts, lysozyme spheroplasts, and penicillin spheroplasts. Recently, Tomasz and Zanati[22] reported the appearance of a trypsin-sensitive agglutinin on the spheroplast of competent *Pneumococci*. Whether the agglutinin resembles the present spontaneous one in its properties is still to be determined.

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