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Note

# A simultaneous two-colour detection method of human IgG- and IgE-reactive proteins from lactic acid bacteria

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ABSTRACT

Whole cell extracts of two *Lactobacillus* strains were tested with primary antibodies from two pooled sera from allergic patients. Fluorescently labelled anti-human IgG and anti-human IgE secondary antibodies applied in Western blotting, together with an appropriate image acquisition protocol facilitated imagining bacterial proteins that reacted with human IgG and IgE.



Understanding the interaction between bacteria and their hosts is vital for targeted and safe application of selected bacterial strains in foods. The proteins of bacteria (both beneficial and pathogenic) introduced into the digestive tract react with their host's immune system in the same way as food proteins. Secretion of IgG, IgA, or IgM antibodies is a natural response of the human host immune system to protein antigens; however, production of IgE antibodies may indicate that the immune system is oversensitive to some food proteins. A humoral response in sera of patients infected with *Clostridium perfringens* has been studied on the level of IgG, IgA, and IgM by Wright et al. (2008) and they identified several dozens of IgG-reactive *C. perfringens* proteins. However, a humoral IgE-dependent response to bacterial protein antigens has been described only for the *Staphylococcus aureus* superantigen (Liu et al. 2014). Yet, recent reports indicate a possible undesired IgE-dependent reaction to lactic acid bacteria (*Lactobacillus casei*) by the human immune system (Wróblewska et al. 2016). The basic issue in the immunoreactivity of bacterial proteins is differentiation between the protein fractions launching the physiological reaction (IgG-dependent) and the pathological reaction (IgE-dependent). The present study aimed at developing a method to detect simultaneously bacterial proteins reacting with the IgG and IgE proteins in sera of allergic patients.

The Western blot method, which is commonly used in specific protein detection, was used to separate bacterial proteins. Human sera were used as sources of primary antibodies to be tested for reactivity with the bacterial proteins. Differentiation of the primary antibodies bound to the immobilized proteins was by secondary antibodies specific for primary antibody classes, i.e. anti-human IgE and anti-human IgG. Obtaining a credible differentiation between the immobilized fractions of bacterial proteins reacting with primary antibody classes required simultaneous detection of proteins reacting with total IgG and IgE on the same membrane.

Proteins (whole cell extract; WC) obtained according to Klaassens et al. (2007) from two strains of lactic acid bacteria, *Lactobacillus bulgaricus* subsp. *lactis* 151 (L151) and *L. casei* LcY (LcY), were examined. The strains were routinely grown in MRS broth (BTL, Łódź, Poland) at the temperature of 37 °C for 18 h and fresh cultures after the second passage were used for the protein extraction. Both strains originated from commercially available fermented milk products and have been characterized (Markiewicz et al. 2006, 2010; Orłowski and Bielecka 2006). These strains were chosen because of their common use in the food industry and a high probability of their contact with allergic patients. For comparisons WC protein extracts were separated in 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue-R250 (B7920, Sigma, Poznań, Poland) (Wróblewska et al. 2011) (Fig. 1).

To detect the reactive bacterial proteins, the proteins (20 μg) were separated in the 12.5% polyacrylamide gel in the presence of the Tris-glycine buffer (192 mmol/L glycine, 25 mmol/l Tris and 0.1% SDS, pH 8.3) according to the methodology by Laemmli (1970) using 4 μl of Odyssey® MW marker 10–250 kDa (928–40,000, Li-COR Biotechnology, Bad Homburg, Germany) as a marker of molecular mass. The transfer of proteins onto a 15 × 15 cm nitrocellulose membrane with its pore diameter of 0.45 μm (N8267, Sigma) was performed using a method of wet

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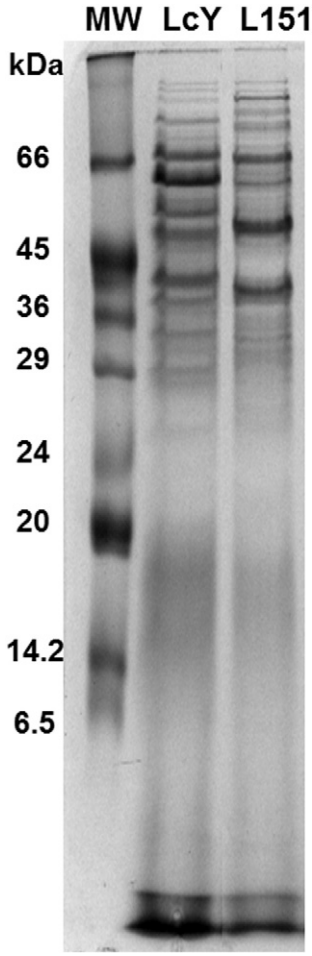


**Fig. 1.** Whole cell extracts of *L. casei* LcY (LcY) and *L. delbrueckii* subsp. *bulgaricus* 151 (L151) separated in a 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. MW - molecular weight marker (Sigma).

electrotransfer in a buffer of Tris-glycine (pH 8.3) with methanol [192 mmol/l glycine, 25 mmol/l Tris and 20% (v/v) methanol] and applying 25 mA for 20 h according to Towbin et al. (1979) and Gospodarska et al. (2015). To verify the effectiveness of the electrotransfer, the polyacrylamide gels were stained with the Coomassie Brilliant Blue R-250 solution. Membranes were washed in a phosphate buffered saline (PBS, pH 7.4, 5 min, room temperature), then blocked in the Odyssey® Blocking Buffer (pH 7.2–7.6, 2 h, room temperature; 927-40003, Li-COR Biotechnology) and incubated overnight (4 °C) in a solution of human sera. In this study the Odyssey® Blocking Buffer was used because it contains no mammalian proteins for the lowest cross-reactivity with mammalian antibodies. This is of special importance when working with human sera from patients suffering from cow milk protein allergy, which excluded the use of skim milk in blocking buffer. Two human sera pools were used for the examinations: sera A pool was obtained by combining the volumes of seven sera from allergic patients (aged 3–13, total IgE level of 985–3556 kU/l) and sera B pool was obtained from ten sera of allergic patients (nine children aged 1–16 and one person aged 37, total IgE level of 1165–5000 kU/l). All the patients were under the care of the Allergica and Atarax medical centres for allergic patients in Olsztyn. As a control, a pool of sera from six healthy participants (aged 1–6; sera H) with negative serum parameters for allergy diagnostic tests (total IgE level of 2–10.7 kU/l and specific-IgE not exceeded 0.7 kU/l), with no allergic manifestations and with defined negative family histories of atopy was used. Sera A, B and H were diluted twice in blocking buffer containing 0.1% Tween 20. After incubation, the membranes were rinsed four times for 10 min each in the PBS-T buffer (PBS, pH 7.4 with 20% Tween 20).

A simultaneous detection of bacterial proteins reacting with total IgG and IgE was performed by incubating (90 min., room temperature) the membranes in a solution containing two types of secondary antibodies labelled with different fluorescent markers, i.e. a 700 nm dye (red channel) and an 800 nm channel dye (green channel). Commercially available goat anti-human IgG antibodies already conjugated with IRDye® 800CW (926-32232, Li-COR Biotechnology) and mouse monoclonal anti-human IgE antibodies (I6510, Sigma) labelled using the IRDye® 680RD Protein Labelling Kit (928-38072, Li-COR Biotechnology) were used. The anti-human IgG and anti-human IgE secondary antibodies were diluted 1:15,000 and 1:500, respectively, with Odyssey® Blocking Buffer (pH 7.2–7.6) containing 0.1% Tween 20 and 0.01% SDS. Signal detection was carried out with the Odyssey Infrared Imaging System (Li-COR Biotechnology) using auto scan settings and Li-COR Image Studio (version 2.0) software. Scanning membranes that included marker proteins entailed detection in the red channel only for a marker whose signal strength was higher than that of the IRDye® 680RD-labelled anti-human IgE (Figs. 2 and 3; red + green panels with a marker). Therefore, scanning in the red channel was performed without marker proteins. Scanning in both channels was performed on membranes two times (with and without markers). The presence of noise in the membrane visible in the red channel also caused silencing of signal from WC protein fractions (Fig. 2; panels RED and RED and GREEN, section B). All analyses were conducted in triplicate. The repeatability of the method depended on the pool of the sera used and deemed acceptable (no differences in protein patterns acquired at both 700 and 800 nm) for at least six consecutive runs. The limiting factor for the repeatability were IgE-specific reactions, which we found to be unacceptable for the pool of sera B after the eighth run and for the pool of sera A after the tenth run. The IgG-specific reactions were reproducible even at the 15th analysis. This could be explained by differences in the total concentrations of those two antibody classes (80% of the total antibodies in human serum are IgGs, whereas IgEs constitute less than 0.001%).

The analyses detected bacterial proteins reacting with human IgG and IgE antibodies. The *Lactobacillus* strains were characterized by different profiles of cellular proteins (Fig. 1) and profiles of IgG-reactive proteins that were different for each sera pool (Figs. 2 and 3; green panels). All pooled sera produced different profiles of IgG-reactive proteins for the two bacterial strains, which may indicate a high number of active antigenic epitopes and the presence of differentiated immunoglobulin paratopes. When comparing the WC profile with the IgG, one can conclude that immune system recognizes only some bacterial antigens as potentially immunoactive, which might result from the process of releasing of bacterial proteins during gastrointestinal passage and availability of antigens to the host's immune system. Analyses performed for sera A and B showed more fractions reacting with human IgG than with IgE, whereas the analysis with control serum H produced no signal for IgE-reactive bacterial proteins (Fig. 3B). The latter reacts with proteins associated with potential pro-inflammatory allergenic activity. Analyses conducted for pooled sera A showed that only one fraction of the LcY strain proteins with molecular mass of ca. 36 kDa reacted both with IgG and IgE (Fig. 2; red + green panel; white arrow). However, the results of the analysis carried out for pooled sera B showed the presence of IgG- and IgE-reactive proteins in the whole cell extracts of both strains (Fig. 3A; red + green panel; white and black arrows). These reactive WC fractions had molecular masses of ca. 48 kDa and 125 kDa in the protein profile of the *L. bulgaricus* subsp. *lactis* 151 strain and the fractions of ca. 52 kDa and 63 kDa in the protein profile of *L. casei* LcY. These results indicate that bacterial proteins may possess different antigenic determinants with the same affinity to the antibodies. So far, most of data on IgE or IgG reactivity of bacterial proteins concern surface layer (S-layer) proteins A and G which bind to Fc region of human antibodies (Björck and Kronvall 1984; Peng et al. 1994). These phenomena