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OXIDOREDUCTASES AND LECTINS RECOGNIZING AND BINDING GLYCOCONJUGATES IN BIOPREPARATIONS ACCORDING TO CHEMILUMINESCENCE IN A REAL TIME

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ABSTRACT

Oxidoreductases and lectins recognizing and binding glycoconjugates are value biologically active compounds and substances. So screening of oxidoreductases of probiotic and plant origin in any biopreparations of preventive and medical importance is actual one. The procedures for such type screening were developed. Systems of weakly acidic lactobacilli oxidoreductases (pI 5-5.6) and strongly acidic plant oxidoreductases (pI 3.2-3.7) were identified and registered. Oxidoreductases showed sensitivity to glycoconjugates and could coincide with the location of lectins. Highly sensitive methods of identification, purification, separation and strong immobilization on membranes of oxidoreductase systems using isoelectrofocusing in gel, electroblotting, chemiluminescence of substrate oxidation products in the mode of registration of stepwise kinetics of luminescence were investigated and proposed. Methods of identification and registration of both oxidoreductases and lectins (as coupled processes) are promising for further standardization of biological products, assessment of the presence of glycoconjugates in them, and the use of drugs as biosensors. In an isolated, immobilized form, oxidoreductase systems cofunctioning to lectins and glycoconjugates are promising for use as tools in microanalysis and biosensors of any exposed glycoconjugates.

KEYWORDS: oxidoreductases; lectins; glycoconjugates; probiotics; phytopreparations; membranes; isoelectric focusing; electroblotting; chemiluminescence.

1. INTRODUCTION

Lectins belong to carbohydrate and glycoconjugate (GC)-recognizing proteins. Lectins include GC-recognizing protein/peptide-containing/associated biologically active substances; they are able to preserve and modify GC-recognizing activity in complexes at the level of different systemic forms. Among the lectins, phytolectins are the most studied, while the lectins of microorganisms are largely less studied.[1]

Lectins of human probiotic bacteria mimic the properties of probiotics, are metabolome-biotics, and belong to bacteriocin-similar Class IV agents[2], exhibit antimicrobial and anti-pathogenic properties against *Candida* and staphylococci[3-5], co-function with the body's defense systems[6, 7], and can be used for strain typing of microorganisms.[7]

Previously, we identified strongly acidic, slightly acidic and alkaline/cationic lectins in human probiotic bacterial cultures, and established their physico-chemical, biochemical and biological properties.[9] However, slightly acidic lectins (pI 5-5.6) of bacteria remain the least studied.

In biological sources, not only lectin systems (LS) are common, but also oxidoreductase systems (OS). The forms of both systems have varying activities, effective and manifested in the [ng/ml] scale, which is of interest for bionanotechnology. In addition, lectins and oxidoreductases, as affine binding carriers and target crosslinkers, individually or in complexes with effectors, exhibit multifunctionality: antimicrobial, antiviral, immunomodulatory, stimulating cytokine production, anti-allergenic, antitumor and anti-inflammatory properties are recorded.

Oxidative stress is an important highly sensitive factor influencing the vital activity of microbiocenoses of the human body, and lactobacilli producing peroxide increase the body's ability to resist pathogens. In addition, lactobacilli belonging to facultative anaerobes, unlike bifidobacteria (obligate anaerobes), are characterized by oxidoreductases involved in peroxide metabolism. In particular, primitive bifunctional (with undivided functions) oxidoreductases such as catalase peroxidase with pI in the 5-5.5 region were identified in lactobacilli. As a result of the functioning of oxidoreductases, discoloring pigments of activities are observed. This confirms the evidence that many lectins are able to exhibit the properties of enzymes, including oxidoreductases.[10-12]

GC, in turn, exhibit antioxidant properties (including, according to our data, GC are selective against a number of forms of LS). They are able to mask/preserve carbohydrate binding sites in target molecules, modify/regulate/modulate the resulting activity of their own targets.

The method of recording chemiluminescence of glycosylated oxidoreductases and lectins on the solid phase in live image mode (recording images on a computer display monitor in real time) is biosensory, comparable in sensitivity to isotopic methods and significantly exceeding the capabilities of common methods for protein determination. Previously, the method was optimized for the registration of chemiluminescence of the horseradish peroxidase on the blot.[13, 14]

The aim is to study OS of *Acylact* and phytopreparations of medical importance, the cofunctionation of human lactobacilli LS (metabolic ingredients of the multi–strain probiotic *Acylact*) and OS, identification and separation of OS and LS, which are part of phytopreparations promising for medicine using a set of standardized synthetic polymer polyvalent GC with a known structure (www.lectinity.com), as well as the application of the biosensor chemiluminescent method using the real time registration (live image mode).

2. MATERIALS AND METHODS

Fractions of components (27-220 kD, pI 4-8) of *Acylact* cultures, its ingredient strains (*Lactobacillus helveticus* 100ash, NK1 and *L. casei* K3III24) and strains of *Bifidobacterium adolescentis* MS-42, *B. bifidum* No.1, separated by isoelectrofocusing (600-700 volts, night, 10^{o}C, in pH gradient 4-8) in polyacrylamide (PAA) plate gel with 7-8 M urea and 5% (by weight) sucrose followed by electroblotting onto the membrane sandwich “hydrophilic Durapore (Millipore)—Immobillon P (Millipore)” were used. The proteins were stained with a fluorescent dye *SYPRO Ruby protein blot stain* (Bio-Rad Lab.). Reprobings of the blots was carried out after treatment of the blots with methanol hydrochloride (coincides with the procedure of preliminary degreasing of the blot when staining proteins with a fluorescent dye, as well as in the case of further use of affinity reagents), lectins were detected by GC based on PAA labeled with biotin (b) (Sugar$_n$-PAA-b; www.lectinity.com). The bound GC were revealed by streptavidin-peroxidase conjugate, and peroxidase was registered by chemiluminescent substrate *BioWest* (Pierce, USA). Lectin bleaching activity (LBA) was recorded in live image mode using the *BioChemi System* (UVP, Calif., USA).

Examples of phytopreparations were from the chair of phytotherapy of RUDN (Moscow University of Friendship of People, prof. Elena V. Korsun). Concanavalin A (ConA, Sigma), high molecular weight (more than 27 kD) concentrates (in phosphate-salt buffer pH 7.4 [PSB]) of fractions PC2- I, II of the roots of the *Baikal skullcap* with *chaga* (as Phytocomposition No. 2, PC2) and CHV-II of the extract of chaga (phytopreparation *Chagovite*, CHV) – both phytopreparations obtained as described in.[15]

The protein-containing fractions of *Acylact* and phytopreparations were separated by high-voltage horizontal isoelectrofocusing (IEF) (5 h, 10°C; the distance between the electrodes is 22-23 cm, the marker MR - methyl red with pI 3.75 passed 56-60% of the path between the electrodes) in a pH gradient of 2-6 (mixtures of equal volumes *Servalyt* 2.5-4 and 4-6 [Serva, Germany]). The linearity of the pH gradient was controlled in portions of 400 µl gel extracts in saline solution (grinding and extraction at 45^{o}C, 5-10 min) from sequentially cut gel fragments 0.5–1.0 cm along the track between the electrodes) in a polyacrylamide gel (PAG) block/plate in the presence of urea and sucrose under isoform separation conditions protein hormone - native highly glycosylated or recombinant human erythropoietin (rhEPO).[16] Samples (3-7 µl without heat treatment, in 1% Tween 80) were applied to cellulose applicators in the area of pI 4 (pH 4 in the pH gradient 2-6). After IEF, the gel was electroblotted through the *Durapore* hydrophilic membrane (Millipore) onto the *Immobillon P* hydrophobic membrane (Millipore). Profiles of absorbing components in tracks were recorded on the *Durapore* membrane in transmitted light (*Durapore* membrane as a tracking replica of visible tracks to control initially invisible tracks on the *immobillon P* membrane; also to assess the removal of background impurities on the *immobillon P*). The commercial drug rhEPO (a system of forms with pI in the pH range 4-4.3) served as an additional control of the distribution of the pH gradient in the gel plate. Immobilized OS was initiated with *BioWest* chemiluminescent substrate (UVP, USA) with increased sensitivity to oxidoreductases (14 times more sensitive to horseradish peroxidase compared to ECL plus; stable at room temperature during the day in working dilute solutions with pH > 8.5).

LS on *immobilone P* were treated with water-soluble pseudo-polysaccharide biotinylated (b) mucin-type GC [homo-pseudo-polysaccharides in the form of linear PAA with side branches in the form of a carbohydrate residue of the same type; in PSB; 5 mcg/ml] (www.lectinity.com): 1) Man_n-PAA-b (2 hours, 27^{o}C) with additional coloring by (Man-6-phosphate)$_n$-PAA-b (night, 5^{o}C), 2) additional coloring of the picture obtained in paragraph-1

by Gal_n-β-PAA-b (2 hours, 27°C), 3) In the case of LS, additional staining with a standard commercial GC-b of other structure was used to enhance the severity and discreteness of the stripes and spots identified in the previous paragraphs. After incubation with *Streptavidin-Peroxidase* conjugate (R&D; 1 h, 37-41°C) *BioWest* was added and kinetics was recorded in the form of sequential peroxidase chemiluminescent patterns (in the interval of a non-linear series of exposures of 40 sec – 20 min with regular visual monitoring) in the *BioChemi System* (UVP, USA) in live image mode. Digital paintings were edited. Inverting the patterns improved the discreteness of the bands, especially in cases of OS. Blocking and washing of the blots was carried out in the presence of a commercial recrystallized preparation of bovine serum albumin (BSA) in the PSB.

3. RESULTS

3.1. OS and LS of *Acylact and its strains*[17-19]

3.1.1. In the slightly acidic region (pI 5.0-5.6), Man_n-PAA (pseudomannan)-binding lectins were detected (in *Acylact* as a conditional sum of lectins of ingredient strains). During the prolongation of the staining process, the lectin regions of lactobacilli (but not bifidobacteria) were strain-dependent exposed to LBA and completely disappeared. The effect of LBA was maximal (in terms of the length of the pI interval within the track) in the case of *Acylact*, included a combination of LBA of strains 100ash (dominant contributor of OS forms) and K3III24 (contributor of more acidic forms), was absent in the NK1 strain.

3.1.2. There was a gradient development (slow - in the "seconds" scale) of continuous LBA in the tracks of lactobacilli in the direction from pI 5.6 towards more acidic values (the most acidic in the case of *Acylact*). At the same time, a "point/spot" zone initiating the LBA cascade was identified, characterizing the beginning of the location of a series of oxidoreductases within the track (presumably, localization of the oxidoreductase as disaggregated as possible with maximum LBA). This zone of LBA spread over the entire area of the track until the end of the location of the series of oxidoreductases in a more acidic region and characterized the presumably maximally aggregated form of the oxidoreductase family with minimal LBA (other oxidoreductase activities in the detected supramolecular ensembles are likely). As a result, clearly limited areas of comparison of LBA of serial oxidoreductases of *Acylact* and its ingredient strains were formed in the tracks, proving the contribution of LBA of strains in the composition of LBA of *Acylact*.

LBA patterns were repeatedly reproduced during reprobing of blots (including repeated reprobing of the same blot), and an increase in the contour boundaries of strain-dependent families of slightly acidic oxidoreductases was observed.

3.2. OS and LS of phytopreparations[20-22]

IEF-PAG, followed by electroblotting, provided significant purification of OS and LS from colored impurities (not far from anode), and other light-absorbing impurities according to the analysis of the *Durapore* membrane (Millipore) in combination with an assessment of the results of reducing the background and increasing the discreteness of band separation on the *Immobillon P* membrane.

Unique (not completely coincident with each other) OS with pI 3.2-3.7 (F2-II, CHV-II) and LS were identified. Anionic OS showed high sensitivity (biosensory features) and were characterized by symmetrical discrete spots in the form of series, including in the form of a gradient with "attenuated" chemiluminescence in the direction from pI 3.7 to pI 3.2 (in the direction from less aggregated to more aggregated forms).

The (Phospho)mannan-binding systems ConA and F2-II in the region of pI 5-6 were characterized by diffuse (less discrete compared to OS) spots and were located in a similar way. In the case of ConA, additional bands were observed. Among the plant LS, the ConA system (LS from seeds) was characterized by the maximum number of bands compared to phyropreparations from the whole bodies.

Additional coloring of OS and LS with galactan GC practically did not change the chemiluminescence pattern that indicate similarity of specificity of ConA and LS of other phytopreparations.

4. CONCLUSION

New opportunities are opening up for the identification and diagnostics of serial bacterial and plant oxidoreductases and their participation in oxidative stress conditions involving and/or using GC, against the background of studies of lectins of probiotic bacteria, including using the registration of "delayed" (seconds) chemiluminescent kinetics of LBA.

The results are of interest for the development of medical bio(nano)technologies, strain diagnostics and strain typing of *Gram* positive bacteria (probiotic, conditionally pathogenic and pathogenic), as well as the design of anaerobic multi-pro/synbiotics.

The proposed experimental approaches demonstrate the effectiveness of the developed procedures for the detection of LS and OS in the crude plant fractions.

Simple technologies (IEF in PAG, electroblotting) are highly sensitive procedures for microanalysis, purification and separation of lectins, oxidoreductases, SL and CO from raw materials for the development of methodologies, design and evaluation of biological products for their further preventive and therapeutic use.

The results demonstrate the possibilities of designing synergistic spatially separated biocompatible (OS+LS)-containing biologics.

OS and LS using the proposed methodology can be studied as systemic territorial carriers of promising endogenous and exogenous GC, for ordering and clustering GC in space that will increase potential of GC as effectors.

Membrane active GC-sensitive SO and LS as immobilized systems can be used in the development of biosensor (biocatalytic, others) constructions, affinity-assembly microanalysis tools and for other aspects of bionanotechnology.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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