



Research Article

Glycopolymers from *Saccharomyces Cerevisiae* BIM Y-195 with Unusual Immunochemical Properties: Isolation, Structural Identification and Prediction of Their Role in Pathogenesis/Treatment of Autoimmune Thyroid Diseases

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Abstract

Molecular mimicry in the broad sense is structural, functional or immunologic similarity between unrelated macromolecules (Oldstone, 1998). In biomedical studies this phenomenon is discussed as the most proven mechanism of triggering/prevention of autoimmune diseases (Kivity et al., 2009). In this paper, we isolate and structurally identify the yeast biopolymers (BPs) that selectively bind human autoantibodies to thyroid peroxidase (TPO) and thyroglobulin (Tg) (anti-TPO and anti-Tg, respectively), *e.g.* immunologically mimic thyroid antigens. The BPs, *viz.*, BP_{anti-TPO} and BP_{anti-Tg}, were isolated from the soluble fraction of *S. cerevisiae* BIM Y-195 by affinity chromatography with anti-TPO or anti-Tg, respectively. Both affinity eluates, AE_{anti-TPO} and AE_{anti-Tg}, showed functional activity characteristic of BP_{anti-TPO} and BP_{anti-Tg}, *viz.* ability (*i*) to distinguish anti-TPO (anti-Tg) from other IgG and (*ii*) to compete with TPO (Tg) for binding of anti-TPO (anti-Tg) in ELISA tests. The semi-preparative size exclusion chromatography of AE_{anti-TPO} and AE_{anti-Tg} with detection by refractometer gave a 5000-7000 Da fractions containing pure BP_{anti-TPO} and BP_{anti-Tg}, respectively, according to ELISA data. Analysis by two-dimensional NMR spectroscopy including ¹H, ¹H COSY and ¹H, ¹³C HSQC experiments indicated that both substances are linear α -1, 6-glucans.

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It was shown for the first time an immunological similarity (molecular mimicry) of α -1, 6-glucans of *S. cerevisiae* BIM Y-195 and human thyroid proteins, TPO and Tg, just as it was shown early for α -1, 6-glucans of *Bifidobacterium bifidum* BIM B-733D and TPO/Tg (Kiseleva et al., 2013). On the whole, our data point to a possible role of wine yeast in the pathogenesis/treatment of autoimmune thyroid diseases (ATD).

Keywords: α -1, 6-glucan, autoantibodies, molecular mimicry.

Introduction

In the industrialized world, the most common causes of morbidity and mortality in 20th century became chronic diseases (Alberti, 2001; Heath, 2009). A special group of them are autoimmune diseases, affecting up to 5% of the population of the Western countries (Kivity et al., 2009). Autoimmune thyroid diseases (ATD) are the most common form of autoimmune diseases (Canaris et al., 2000; Cooper and Stroehla, 2003; Vanderpump et al., 1995). The clinical manifestations of ATD vary from thyroid hyperactivity (Graves' disease) to thyroid underactivity (chronic autoimmune thyroiditis or Hashimoto's thyroiditis, HT); term HT is used ordinary as a synonym of ATD. The two primary antigens in ATD are thyroglobulin (Tg) and thyroid peroxidase (TPO). The glycoprotein Tg (660 kDa) is the storage form of thyroid hormones within the thyroid follicle. TPO is an enzyme expressed at the apical border of the thyroid cells and catalyzes key reactions of thyroid hormones biosynthesis. The clinical diagnosis of ATD depends on physical and biochemical abnormalities as well as serological detection of autoantibodies to the antigens (anti-TPO and anti-Tg) (Davies and Amino, 1993).

It is generally accepted that ATD result from a complex interaction between genetic and environmental factors, most of which have not been identified. Eighty percent of the susceptibility to develop ATD are genetically determined and the immune-regulatory/thyroid-specific genes implicated in pathogenesis of ATD are identified (Ringold et al., 2002; Simmonds and Gough, 2004; Tomer, 2010). Environmental factors include excess dietary iodine; selenium deficiency; naturally occurring goitrogens found in the legumes and other plants; certain drugs

such as amiodarone, lithium and interferon- α ; synthetic chemicals such as pesticides, by-products of industry (polyaromatic hydrocarbons, polyhalogenated biphenols) and pollutants associated with smoking (Burek and Talor, 2009; Davies, 2008; Duntas, 2008). Interestingly, that the European literature shows the increasing incidence of HT during last decades, which supports the conclusion about role of environmental factors in triggering of the disease (Rizzo et al, 2010).

Different types of an infection have a special place among environmental factors implicated in the pathogenesis of ATD. Published reports involving serological data from human studies and suggesting association of ATD with seroreactivity against influenza virus, hepatitis C virus, enterobacteria, streptococci, staphylococci, *Yersinia enterocolitica*, *Helicobacter pylori* and protozoa *Toxoplasma gondii* have been reviewed (Tozzoli et al., 2008). Serologically detected infection agents also include *Mycoplasma sp.* (Sack, 1989) and herpes simplex virus (Thomas et al., 2008a). Direct evidences of retrovirus or herpes simplex virus DNA in the thyroid of ATD patients are rare and controversial (Al-Zarzur and Monem, 2011; Thomas et al., 2008b; Tomer and Villanueva, 2004). The homology between thyroid antigens and some proteins of *Yersinia enterocolitica*, *Borrelia burgdorferi* (Benvenga et al., 2006), *E. coli* (McLachlan and Rapoport, 1989) and hepatitis C virus (Martocchia and Falaschi, 2007) was established by an *in silico* analysis. Molecular mimicry of thyroid antigens by these microorganisms was proposed as a mechanism of ATD triggering. The fertile-field hypothesis explains how different microbial infections induce and exacerbate a single autoimmune disease (Von Herrath et al., 2003).

Multiple and mutually non-exclusive mechanisms, by which host infection by a pathogen can lead to autoimmunity were proposed: (i) molecular mimicry, (ii) epitope spreading owing to infective lysis of tissue, (iii) bystander activation, (iv) presentation of proteolytically formed cryptic self-epitopes (Ercolini and Miller, 2009; Getts and Miller, 2010; Münz et al., 2009). An adjuvant effect of pathogens (Getts and Miller, 2010; Rose, 2008) acting through pathogen-associated molecular patterns receptors (Den Dunnen et al., 2010; Jeong and Lee, 2011; Kawai and Akira, 2009; Zeuthen et al., 2008) or non-specific activation of immune system by bacterial superantigens (Münz et al., 2009) should also be taken into account.

The most plausible explanation of the role of microorganisms in the provocation of autoimmune diseases is molecular mimicry, *i.e.*, the emergence of autoreactive clones of T- and B-lymphocytes as a result of cross-immune response to homologous bacterial or viral antigen (Harkiolaki et al., 2009; Kohm et al., 2003; Roep, 2003; Wegner et al., 2009).

Under enhanced gut permeability for relatively high-molecular substances and even for whole cells of bacteria (Bruewer et al., 2003), known as "leaky gut" (Clayburgh et al., 2004), the list of microorganisms that could potentially influence the autoimmune process may be expended by intestinal symbionts.

In present study, the yeasts *S. cerevisiae* and *D. hansenii* are interesting for several reasons. Firstly, both yeasts are recognized antibiotic-resistant human commensals though their presence in the human gut has a transient character (Pecquet et al., 1991). People commonly consume live *S. cerevisiae* cells with sweet fruits and dairies including koumiss and acidophilus yeast milk (Wszolek et al., 2006), whereas live *D. hansenii* cells enter the body with cheese (Gori et al., 2012; Sibirny and Voronovsky, 2009) and dry-cured sausages and ham (Andrade et al., 2010; Cano-García et al., 2013). In addition, dried cells of *S. cerevisiae* are frequently used as nutritional supplement, so called

"nutritional yeast", that can be found in the most of the natural food stories. Products of processed *S. cerevisiae* cells (extracts, autolyzates) are widely used as food additives with flavoring properties, whereas yeast cell walls are used either as pharmaceuticals to activate the immune system (Jawhara et al., 2012) or as food additives with water-binding properties (Petravić-Tominac et al., 2011). Similarly to widely used *S. cerevisiae*, *D. hansenii* was found to have enormous biotechnological potential in agro-food sector (Breuer and Harms, 2006; Vetrisevi et al., 2010), which would lead to rapid expansion of their consumption as food additives worldwide.

Secondly, *S. cerevisiae* shares common bio-therapeutic properties with allied yeast *S. boulardii* (also known as *S. cerevisiae* var. *boulardii* (Mitterdorfer et al., 2002; Edwards-Ingram et al., 2007)) (Izadnia et al., 1998; Jahn et al., 1996; van der Aa Kühle et al., 2005), noted probiotic (Kelesidis and Pothoulakis, 2012). For present study, anti-inflammatory and immunomodulatory properties of *S. cerevisiae* are most interesting. The data obtained in animal models show that the administration of *S. cerevisiae* cells induces interleukin 10 (IL-10), which steers the immune system towards a regulatory T cell response (Generoso et al., 2010; Martins et al., 2009). It may have therapeutic benefits for patients suffering from Th1/Th17-mediated autoimmune diseases (in particular, HT) (Phenekos et al., 2004; Qin et al., 2012). In turn, *D. hansenii* has been found to stimulate immune response in fish (Reyes-Becerril et al., 2012). Moreover, same yeast preparations and substances providing immunomodulation are identified, *viz.*, β -1-3-glucan (Huang et al., 2009; Pelizon et al., 2005), chitin (Lee et al., 2008), and derivative of yeast cell wall known as zymoan (Kelly et al., 2010); all are shared by *S. cerevisiae* and *D. hansenii*.

Thirdly, specific antibodies to antigens of *S. cerevisiae* are used as serological markers to discriminate between Crohn's disease and ulcerative colitis (Desplat-Jégo et al., 2007). Patients with other intestinal and non-intestinal autoimmune disorders including celiac disease (Ashorn et al.,

2008), autoimmune hepatitis (Czaja et al., 2004), Behçet's disease (Fresko et al., 2005), ATD (Yazıcı et al., 2010; Mankai et al., 2013) and systemic lupus erythematosus (Dai et al., 2009) also have high seropositivity rates for anti-*S. cerevisiae* antibodies as compared with healthy donors. Elevated serum levels of anti-*S. cerevisiae* antibodies do not result primarily from a defect of the gut barrier (Harrer et al., 2004), which allow to assume implication of *S. cerevisiae* in common mechanisms underlying autoimmunity.

In this paper, we isolate and structurally identify the BPs of *S. cerevisiae* BIM Y-195 that selectively bind human anti-TPO and anti-Tg (BP_{anti-TPO} and BP_{anti-Tg}, respectively). The data on immunologic similarities between thyroid antigens and α -1, 6-glucans from *S. cerevisiae* point to a role of the wine yeast in the pathogenesis/treatment of ATD.

Materials and Methods

Methodological Steps of the Study are shown in Appendix 1.

Yeast Cells. *S. cerevisiae* BIM Y-195 and *D. hansenii* BIM Y-4 from the Scientific Collection of Model and Industrially-valuable Non-pathogenic Microorganisms (The Institute of Microbiology NAS of Belarus, Minsk, Republic of Belarus) were used. Both strains were grown on beer-wort agar slants at 26°C during 3 days. Cells were washed off with 0, 15 M NaCl, sedimented by centrifugation at 2000 × g for 20 min and weighed. Then cells (20-25 g) were washed (1 × 300 mL) with 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5 (buffer 1), separated by centrifugation as stated above and suspended in buffer 1 (50 mL). Portions of both cell suspensions (each 0.1 mL) were diluted 10-fold by buffer 1 and immediately used to adjust suspensions to a similar cell concentration.

Adjustment of *S. Cerevisiae* BIM Y-195 and *D. Hansenii* BIM Y-4 Cell Suspensions to a Similar Cell Concentration. Two series of dilutions were prepared and kept at 37°C for 1 h.

One of them contained *S. cerevisiae* BIM Y-195 cell suspension, another one contained *D. hansenii* BIM Y-4 cell suspension (each 1 – 10 μ L in 2.5 mL of 35% sulfuric acid). Absorption at 260 nm in a 1cm path length (A_{260}) was measured using 35% sulfuric acid as a control. The data were expressed as graphs in coordinates " A_{260} " (OY) – "cell suspension in probe, μ L" (OX). The slope was calculated for each graphs. The ratio of the slopes was used as dilution factor of the suspension that has higher cell concentration. The adjusted suspensions were used to obtain cell fractions.

Cell Fractions. The cells of both strains were used as pre-adjusted suspensions. They were manually homogenized (Wheaton, USA) and ultrasonicated on ice using UZDN-2T (UkrRosPribor, Ukraine) 6 times for 30 s at a frequency of 22 kHz. The cell wall and membrane fractions (CFs) referred through the text as CF-Sc and CF-Dh were sedimented by centrifugation at 40000 × g for 30 min, suspended in buffer 1 (50 mL) and immediately used for ELISA (see ELISA test 1 below). The supernatants (CFFs) referred through the text as CFF-Sc and CFF-Dh were used for ELISA (see ELISA tests 1 and 2 below). In addition, CFF-Sc was used for biospecific isolation of BPs.

Biospecific Isolation and Purification of the Bacterial Biopolymers. CFF-Sc was divided into two portions and loaded independently on anti-TPO and anti-Tg immobilized on CNBr-activated Sepharose 4B (Pharmacia, Sweden) (Anonymous, 1976). The BPs bound by immobilized autoantibodies were eluted with 0.2 M NH₄OH, pH 11.5. The affinity eluates (AEs) referred through the paper as AE_{anti-TPO} and AE_{anti-Tg} were freeze-dried, tested by ELISA (see ELISA tests 3 and 4 below) and used for BPs purification.

Dried AE_{anti-TPO} (5 mg) was dissolved in water (1 mL) and fractionated on the column (60 × 1.6 cm) of TSK-40 gel (Merck, Germany) equilibrated in water. Elution was monitored using a refractometer (Knauer, Germany). Dried AE_{anti-Tg} (5 mg) was fractionated in a similar way. The fractions corresponding to major peaks in

each size exclusion chromatography were referred to below as BP_{anti-TPO-2}, BP_{anti-TPO-3}, BP_{anti-Tg-2} and BP_{anti-Tg-3} and analyzed by NMR spectroscopy. Fractions BP_{anti-TPO-2} and BP_{anti-Tg-2} were tested by ELISA (see ELISA tests 5 and 6 below).

Human Serum Samples were obtained from the Minsk City Clinical Oncologic Dispensary (Republic of Belarus) and tested using the ELISA kits for anti-TPO and anti-Tg (Xema Co. Ltd, Russia). Serum samples were divided into three groups according to the data of ELISA. The first group was from healthy donors without clinical manifestations of ATD. Concentrations of anti-TPO and anti-Tg in these samples were less than the upper limits for healthy donors, *viz.*, 50 IU/ml and 100 IU/ml, respectively. The second and third groups included samples of blood serum of patients with clinically confirmed diagnosis of ATD. Concentrations of anti-TPO and anti-Tg in the samples of the second group were in the range 100 - 500 IU/ml and 200 - 500 IU/ml, respectively. The concentrations of antibodies to each thyroid antigen in the samples of the third group were higher than 500 IU/ml. Selected serum samples were used to prepare probes "healthy" and "ATD" and for ELISA test 1.

Serum Probes "Healthy" and "ATD" were prepared by combining 10 samples from the first group ("healthy") and combining 10 samples from the third group ("ATD"). Quantification of anti-TPO and anti-Tg in both probes was performed using the appropriate test kits for ELISA (Xema Co. Ltd, Russia). Probe "healthy" contained 25 IU/mL anti-TPO plus 52 IU/mL anti-Tg, probe "ATD" contained 1200 IU/mL anti-TPO plus 1500 IU/mL anti-Tg. Probe "healthy" was used for ELISA tests 2 and 3 and isolation of total human IgG. Probe "ATD" was used for ELISA tests 2 - 4 and isolation of anti-TPO and anti-Tg.

Total Human Immunoglobulins of Class G (IgG) were isolated from serum probe "healthy". The Igs were precipitated with ammonium sulfate (40% saturation of serum); the precipitate was dissolved in buffer 1 and ammonium sulfate was

removed by dialysis against the same buffer. The IgG were separated from crude Ig extract by affinity chromatography on Protein G Agarose (Pierce, USA) in accordance with product instruction manual. The anti-TPO and anti-Tg were removed by affinity chromatography using TPO or Tg, respectively (Tsyganova et al., 2006a) immobilized on CNBr-activated Sepharose 4B. IgG concentration was determined spectrophotometrically, where $D_{280\text{ nm}, 1\text{ cm}, 1\text{ mg/ml}} = 1.35$. Total IgG (without anti-TPO and anti-Tg) were used as a control probe in ELISA (see ELISA tests 5 and 6).

Autoantibodies to Thyroid Antigens were isolated from serum probe "ATD" by affinity chromatography with either TPO or Tg (Tsyganova et al., 2006a) immobilized on CNBr-activated Sepharose 4B (Pharmacia, Sweden) (Anonymous, 1976). Quantification of pure anti-TPO and anti-Tg was performed using the appropriate ELISA kits (Xema Co. Ltd, Russia). Additionally, concentrations of pure anti-TPO and anti-Tg were determined spectrophotometrically as stated above for pure total Igs. Pure anti-TPO and anti-Tg were used for (i) affinity gel synthesis and (ii) ELISA (see ELISA tests 5 and 6 below). Serum samples for antibodies isolation and purified anti-TPO (anti-Tg) were stored at -20°C and defrosted once immediately before use.

Thyroid Antigens. TPO and Tg were isolated from human thyroid tissue of patients with Graves' disease obtained from the Minsk City Clinical Oncologic Dispensary (Republic of Belarus). The soluble fraction of thyroid homogenate after sedimentation of nuclei, mitochondria and microsomes was used as source of TPO (Tsyganova et al., 2006b) and Tg. TPO was purified by affinity chromatography with mouse monoclonal antibodies (MAb) A1 and F8 (Tsyganova et al., 2006c) immobilized on CNBr-activated Sepharose 4B. Tg was isolated by ammonium sulfate (60% saturation) precipitation and gel-filtration of dissolved precipitate on P-200 (Bio-Rad, USA) (Simionescu et al., 1983). Purity of TPO and Tg was not less than 95% according to SDS-PAGE. TPO and Tg

were used for affinity gel synthesis and ELISA tests 1, 3-6.

ELISA. General ELISA protocol. Each item (TPO, Tg, CF-Sc, CF-Dh, CFF-Sc, CFF-Dh, AE_{anti-TPO}, AE_{anti-Tg}, BP_{anti-TPO-2}, BP_{anti-Tg-2}, human serum albumin (HSA) (Sigma, USA) and protein A from *Staphylococcus aureus* (Sigma, USA) was immobilized by passive adsorption on polystyrene microplates (Greiner bio-one, Germany) at 4°C overnight (0.1 mL per well). TPO and Tg were immobilized from solutions of 0.1 M NaHCO₃, pH 8.3, and 0.1 M citrate-phosphate buffer, pH 5.5, respectively, containing 0.5 mg/L of the appropriate antigen. Protein A and HSA were immobilized from 0.01 M sodium phosphate buffer, pH 7.5 that contained 1 mg/L of the protein. Cell fractions of both strains (each pre-diluted 50-fold) were immobilized from 0.01 M sodium phosphate buffer, pH 7.5, with A₂₆₀ values of 0.05 in a 1 cm pathlength. Both AEs (each 1 mg/L), BP_{anti-TPO-2} and BP_{anti-Tg-2} (each (0.4 - 2) mg/L) were immobilized from solutions of 0.05 M ammonium acetate, pH 8.5, supplemented with 1 mM CaCl₂ (buffer 2). The wells were washed (3 × 300 µL per well) with buffer 1 after immobilization and each stage of the analysis.

The immunochemical reactions were initiated by addition of solutions (0.1 mL per well) of primary reagents (selected serum samples, probe "healthy", probe "ATD" and "ATD" plus thyroid antigen, pure anti-TPO and anti-Tg, pure total IgG) in buffer 1 supplemented with 0.2% phenol and 3% casein (buffer 3) (first ELISA stage). Immobilized items and primary reagents added in wells at the first stage of ELISA tests are shown in Appendix 2.

The enzymatic conjugates (0.1 mL per well) in buffer 1 supplemented with 1% bovine serum albumin (Sigma, USA) were used as a developing reagents (second ELISA stage). All incubations were performed during 1 - 2 h at 37°C. The enzymatic peroxidation reaction was initiated by addition of 0.1 mL per well 3,

3',5,5'-tetramethylbenzidine liquid substrate system (Sigma, USA). The reaction was stopped by addition of 0.1 mL per well 4.8% H₂SO₄. The A₄₅₀ values were measured in each well using the automatic multi-channel spectrophotometer (Uniplan, Russia).

ELISA test 1 was performed to detect functional activity characteristic of BP_{anti-TPO} and BP_{anti-Tg} in cell fractions of both strains and thereupon select strain and cell fraction for BPs isolation. We used immobilized (i) CF-Sc, (ii) CF-Dh, (iii) CFF-Sc, (iv) CFF-Dh, (v) TPO and (vi) Tg (96 wells for each item). Human serum samples from the second and the third groups (n=48) were used as a source of anti-TPO and anti-Tg. They were diluted 100-fold by buffer 3 and added into appropriate wells (in duplicates) containing each of the immobilized items i - vi. The bound Igs were detected by means of MAb against human IgG conjugated with horseradish peroxidase (MAb-anti IgG - HRP) (Xema Co. Ltd, Russia) and the data set of A₄₅₀ values (n=48, each in duplicates) was obtained for each immobilized item. The correlations between the two data sets (one data set related to every one of cell fractions (i - iv) and another one related to TPO) were calculated by means of Excel. Similar correlations were calculated using the data set related to Tg instead of the data set related to TPO.

ELISA test 2 was performed to validate selection of CFF-Sc for BPs isolation. Immobilized (i) CFF-Sc, (ii) protein A and (iii) HSA (20 wells for each item) were used. Two series of dilutions were prepared. One of them contained probe "ATD", another one contained probe "healthy" (each (0.06 - 1) %). The dilutions were added into appropriate wells (in duplicates) containing each of the immobilized items i - iii. The bound Igs were detected by means of MAb-anti IgG - HRP (Xema Co. Ltd, Russia) (wells i and ii) and conjugate of protein L from *Peptostreptococcus magnus* with HRP (protein L - HRP) (Sigma, USA) (wells iii).

ELISA test 3 was performed to confirm that $AE_{\text{anti-TPO}}$ and $AE_{\text{anti-Tg}}$ contain substances, interacting selectively with anti-TPO and anti-Tg, respectively. Immobilized (i) $AE_{\text{anti-TPO}}$, (ii) $AE_{\text{anti-Tg}}$, (iii) TPO and (iv) Tg (16 wells for each item) were used. Two series of dilutions were prepared. One of them contained probe "ATD", another one contained probe "healthy" (each (0.04 – 1) %). The dilutions were added into appropriate wells (in duplicates) containing each of the immobilized items i – iv. The bound Igs were detected by means of MAb-anti IgG – HRP (Xema Co. Ltd, Russia).

ELISA test 4 (competitive) was performed to confirm that $AE_{\text{anti-TPO}}$ and $AE_{\text{anti-Tg}}$ contain substances, competing with TPO and Tg, respectively, for binding of the appropriate antibodies. We used the same immobilized items i – iv as in test 3 (8 wells for each item). Probe "ATD" was diluted 1000-fold and 0.05 mL was added into all wells. Next, 0.05 mL of buffer 3 was added into two wells containing each of the immobilized items i – iv (to give B_0 experimental points). Three TPO dilutions (40, 120 and 360 mg/L; each 0.05 mL) were added into strips i and iii (in duplicates) to give B_1 , B_2 and B_3 experimental points, respectively. Three Tg solutions (33, 100 and 300 mg/L; each 0.05 mL) were added into strips ii and iv (in duplicates) to give B_1 , B_2 and B_3 experimental points, respectively. The bound Igs were detected by means of MAb-anti IgG – HRP (Xema Co. Ltd, Russia). The data for each immobilized item were calculated as $(B_n/B_0) \times 100, \%$.

ELISA test 5 was performed to confirm functional activity of $BP_{\text{anti-TPO-2}}$. We used immobilized (i) $BP_{\text{anti-TPO-2}}$, (ii) TPO and (iii) protein A from *Staphylococcus aureus* (Sigma, USA) (16 wells for each item). Two series of dilutions were prepared. One of them contained pure anti-TPO, second one contained pure total IgG without anti-TPO and anti-Tg (each (1.3 – 35) mg/L). The dilutions were added into appropriate wells (in duplicates) containing immobilized items i – iii. The bound Igs were detected by means of MAb-anti IgG – HRP (Xema Co. Ltd, Russia) (wells i and ii)

and protein L – HRP (Sigma, USA) (wells iii).

ELISA test 6 was performed to confirm functional activity of $BP_{\text{anti-Tg-2}}$. We used immobilized (i) $BP_{\text{anti-Tg-2}}$, (ii) Tg and (iii) protein A from *Staphylococcus aureus* (Sigma, USA) (16 wells for each item). Test was performed similarly to test 5; pure anti-Tg (1.2 – 32) mg/L were used instead of pure anti-TPO.

NMR Spectroscopy. NMR spectra were recorded on a Bruker Avance II 600 spectrometer at 30°C using a 5 mm broadband inverse probe head for solutions in 99.95% D_2O after Deuterium exchange by freeze-drying sample solutions in 99.9% D_2O . Sodium 3-(trimethylsilyl)propanoate-2,2,3,3- d_4 (δ_H 0.0) and acetone (δ_C 31.45) were used as internal calibration standards for 1H and ^{13}C NMR chemical shifts, respectively. Two-dimensional NMR spectra were obtained using standard Bruker software, and Topspin 2.1 program was used to acquire and process the NMR data.

Statistics. Each ELISA test was done as three independent experiments; each well in duplicates. Results were evaluated by standard Student's t test.

Results

ELISA Testing of S. Cerevisiae BIM Y-195 and D. Hansenii BIM Y-4 Cell Fractions to Detect Functional Activity Characteristic of $BP_{\text{anti-TPO}}$ and $BP_{\text{anti-Tg}}$

S. cerevisiae BIM Y-195 and *D. hansenii* BIM Y-4 cell suspensions were adjusted to similar cell concentrations before ultrasound treatment for adequate comparison of two strains in subsequent ELISA tests.

Since we shown that yeast cells kept in 35% sulfuric acid for 1 h give clear solution with UV adsorption maximum at 260 nm (Figure 1, A), total nucleic acid concentration quantified by UV absorbance (Gallagher, 2011) was assumed as a measure of cells concentration. We used ratio of the slopes shown in Figure 1, B to

calculate the dilution factor of the suspension with higher cell concentration for adjustment of two suspensions.

Pre-adjusted cells of *S. cerevisiae* BIM Y-195 and *D. hansenii* BIM Y-4 were disrupted by ultrasound treatment and fractionated by high speed centrifugation. Cell fractions of two yeast strains (CF-Sc, CFF-Sc, CF-Dh and CFF-Dh) were immobilized on microplates and tested by ELISA to detect functional activity characteristic of BP_{anti-TPO}, namely the ability to interact selectively with anti-TPO (ELISA, test 1). In this test we used immobilized TPO as a positive control and human serum samples (n=48) from the second and third groups as source of anti-TPO. The bound Igs were detected by means of MAb-anti IgG – HRP since anti-TPO are generally of IgG class (Xie et al.,

2008). The data sets of values A_{450} (n=48) obtained for each immobilized item were used to calculate the correlation coefficients (ELISA, test 1). The calculated data are shown in Table 1.

The same cell fractions were tested by ELISA in a similar way to detect functional activity characteristic of BP_{anti-Tg}, namely the ability to interact selectively with anti-Tg (ELISA, test 1). We used immobilized Tg as a positive control and the same set of human serum samples (n=48) as source of anti-Tg. The bound Igs were detected by means of MAb-anti IgG – HRP since anti-Tg are generally of IgG class (Caturegli et al., 1994; Zhang et al., 2010). Similarly to the previous case, the correlation coefficients were calculated (ELISA, test 1) and shown in Table 1.

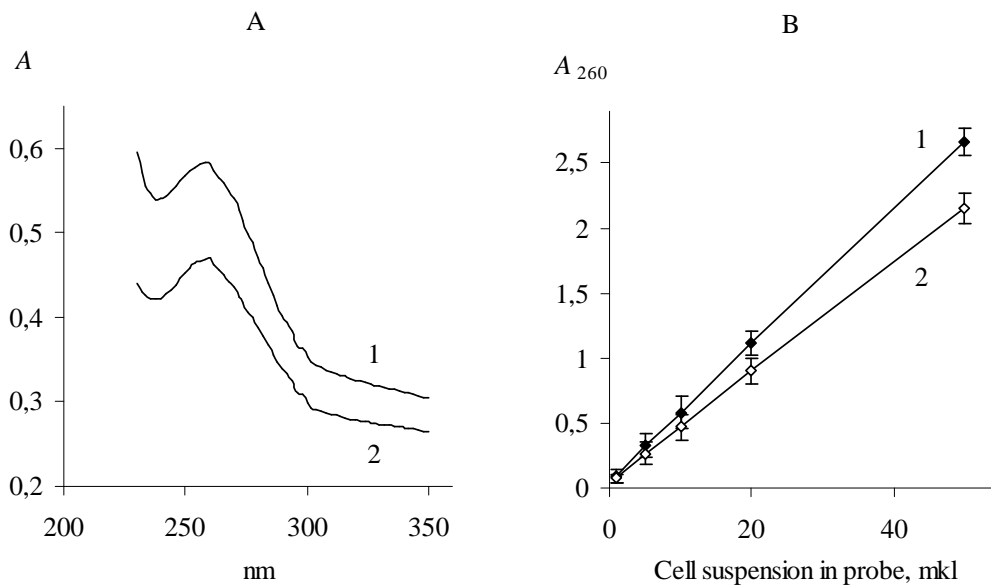


Figure 1. UV Adsorption Spectrum (A) and Adsorption at 260 nm (B) of *S. Cerevisiae* BIM Y-195 (1) and *D. Hansenii* BIM Y-4 (2) Cells Dissolved in 35% Sulfuric Acid. Representative Data from Three Independent Experiments are Shown. Values are Means \pm Range for Duplicate Tubes.

Table 1. ELISA Data for Functional Activity Characteristic of BP_{anti-TPO} and BP_{anti-Tg} in yeast Cell Fractions (ELISA, test 1). The Data are Expressed as Correlation Coefficients. Representative Data from Three Independent Experiments are Shown. Abbreviations: BP_{anti-TPO} and BP_{anti-Tg} – The Yeast Biopolymers that Selectively Bind Human Autoantibodies to Thyroid Peroxidase and Thyroglobulin.

Yeast	<i>S. cerevisiae</i> BIM Y-195		<i>D.hansenii</i> BIM Y-4	
Cell fraction	CF	CFF	CF	CFF
Correlation with TPO	0.56	0.60	0.25	0.28
Correlation with Tg	0.61	0.73	0.34	0.18

To interpret ELISA data (Table 1), we taken into account that the values of A_{450} in the wells with immobilized TPO and Tg are proportional to the concentration of anti-TPO and the anti-Tg, respectively, in serum samples used in ELISA. Consequently, the high correlation coefficients ($r \geq 0.55$) related to CF-Sc and CFF-Sc show that these fractions bind anti-TPO in a concentration-dependent fashion, like immobilized TPO, and thus contain BP_{anti-TPO}. Similarly, CF-Sc and CFF-Sc contain BP_{anti-Tg}. On the contrary, CF-Dh and CFF-Dh have considerably lower correlation coefficients and contain neither BP_{anti-TPO} nor BP_{anti-Tg}.

We select CFF-Sc as most preferably fraction for BP_{anti-TPO} and BP_{anti-Tg} isolation.

ELISA Testing of CFF-Sc to Validate Selection of the Fraction for BPs Isolation

To prove more convincingly that CFF-Sc contains BP_{anti-TPO} and BP_{anti-Tg}, CFF-Sc, protein A from *Staphylococcus aureus* and HSA were immobilized on microplates for ELISA and incubated with solutions of probes "ATD" or "healthy" (ELISA, test 2). We used protein A as a first negative control since it binds total Igs independently on their specificity (Yang et al., 2003) and HSA as a second negative control since it is irrelevant to human Ig on the whole and anti-TPO/anti-Tg specifically.

Total IgG in human serum samples (in our case, in probes "healthy" and "ATD") was assumed to be 7 – 16 g/L (Shakib and Stanworth, 1980). Concentrations of anti-TPO and anti-Tg in human serum of ATD

patients are in range from 0.05 to 1.4 g/L (Beever et al., 1989) and do not exceed 10% of the concentration of total IgG. Probe "ATD" containing 1200 IU/mL anti-TPO plus 1500 IU/mL anti-Tg was used as single source of both autoantibodies, since just a few of HT patients have serum anti-TPO without anti-Tg (Lindberg et al., 2001). Probe "healthy" containing 25 IU/mL anti-TPO plus 52 IU/mL anti-Tg was used as a negative control, though it has slightly low concentrations of anti-TPO and anti-Tg, so called "natural autoantibodies", which commonly present in serum of healthy donors (Lleo et al., 2010). Both probes were used as (0.06 – 1) % dilutions since this concentration range was found to be optimal to analyze substances interacting selectively with anti-TPO and anti-Tg (in our case, BP_{anti-TPO} and BP_{anti-Tg}) and to exclude bystander effect of total Igs (Kiseleva et al., 2013). The data are shown in Figure 2.

The data of Figure 2 suggest that CFF-Sc differentiate between probes "healthy" and "ATD" unlike protein A which does not distinguish between the same probes. HSA does not show interaction with both probes at all. We receive evidence that the data of Table 1 do not result from correlation between concentrations of anti-TPO (anti-Tg) and anti-*S. cerevisiae* antibodies (Desplat-Jégo et al., 2007) that were detected with higher frequency in serum samples of ATD patients as compared to healthy donors (Yazıcı et al., 2010; Mankai et al., 2013).

Thereupon, we validate selection of CFF-Sc for BPs isolation.

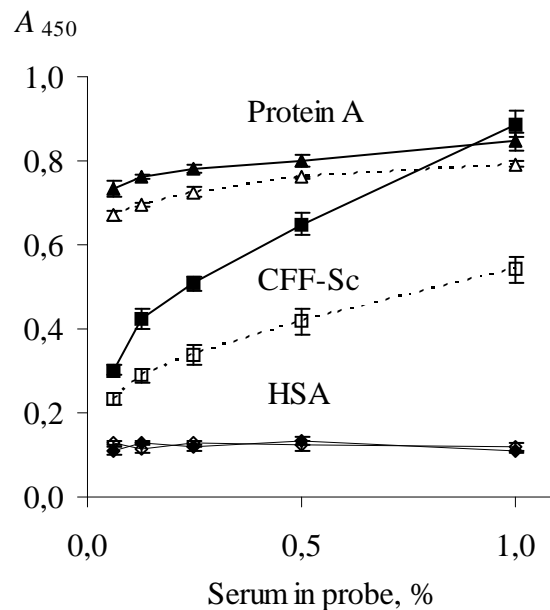


Figure 2. Binding of Human IgG with Immobilized CFF-Sc, Protein A and HSA (ELISA, Test 2). IgG Contained in Serum Probes “ATD” (Black Symbols, Solid Lines) and “Healthy” (Empty Symbols, Dashed Lines) were Used. CFF-Sc Bind Significantly More ($p < 0.05$) IgG from Probe “ATD” as Compared with Control Probe “Healthy”. Representative Data from Three Independent Experiments are Shown. Values are Means \pm Range for Duplicate Wells. Abbreviations: CFF-Sc – Cell Free Fraction of *S. Cerevisiae* BIM Y-195, HSA – Human Serum Albumin, ATD – Autoimmune Thyroid Disease.

ELISA Testing of $AE_{anti-TPO}$ and $AE_{anti-Tg}$ to Confirm Isolation of $BP_{anti-TPO}$ and $BP_{anti-Tg}$

$BP_{anti-TPO}$ and $BP_{anti-Tg}$ were isolated from CFF-Sc by affinity chromatography and referred to below as affinity eluates (AEs), $AE_{anti-TPO}$ and $AE_{anti-Tg}$, respectively. 1 mg of each AE was dissolved in water to prove that we actually isolate $BP_{anti-TPO}$ and $BP_{anti-Tg}$, viz., substances that (i) distinguish anti-TPO (anti-Tg) from other IgG and (ii) compete with TPO (Tg) for binding of anti-TPO (anti-Tg) in ELISA tests.

Direct ELISA test (ELISA, test 3) was performed to confirm that $AE_{anti-TPO}$ and $AE_{anti-Tg}$ contain substances that distinguish anti-TPO and anti-Tg, respectively, from other IgG. In this test we used immobilized $AE_{anti-TPO}$, $AE_{anti-Tg}$, TPO and Tg; thyroid antigens were used as a positive controls for $AE_{anti-TPO}$ and $AE_{anti-Tg}$, respectively. All immobilized items were incubated with dilutions of probes “ATD” or “healthy”. The data are shown in Figure 3.

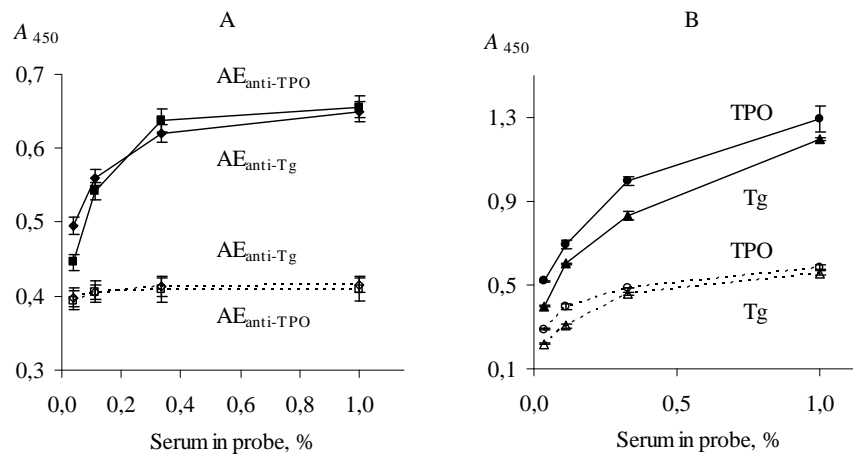


Figure 3. Binding of Human IgG with Immobilized $AE_{anti-TPO}$ and $AE_{anti-Tg}$ (A), TPO and Tg (B) (ELISA, Test 3). IgG Contained in Serum Probes “ATD” (Black Symbols, Solid Lines) and “Healthy” (Empty Symbols, Dashed Lines) were Used. All Immobilized Items Differentiate between Two Serum Probes ($p < 0.05$). Representative Data from Three Independent Experiments are Shown. Values are Means \pm Range for Duplicate Wells. Abbreviations: ATD - Autoimmune Thyroid Disease; TPO - Thyroid Peroxidase; Tg - Thyroglobulin; $AE_{anti-TPO}$ and $AE_{anti-Tg}$ - Affinity Eluates Obtained from the Soluble Fraction of *S. Cerevisiae* BIM Y-195 Cells by Affinity Chromatography with Either Human Autoantibodies to TPO or Autoantibodies to Tg, Respectively.

The data of Figure 3, A suggest that $AE_{anti-TPO}$ and $AE_{anti-Tg}$ bind IgG in a concentration-dependent and saturable fashion, like the human thyroid antigens do (Figure 3, B). $AE_{anti-TPO}$ and $AE_{anti-Tg}$ bind considerably less IgG from probe “healthy” in comparison with IgG from probe “ATD” containing more anti-TPO and anti-Tg (Figure 3, A), in a similar way to immobilized thyroid antigens (Figure 3, B). In whole, the data of Figure 3 prove that $AE_{anti-TPO}$ actually binds anti-TPO and $AE_{anti-Tg}$ actually binds anti-Tg, despite the presence of 10-fold excess of other IgG.

Competitive ELISA test (ELISA, test 4) was performed to confirm that $AE_{anti-TPO}$ and $AE_{anti-Tg}$ contain substances competing with TPO and Tg, respectively, for binding to the appropriate antibodies.

The $AE_{anti-TPO}$ and TPO as a positive control were immobilized on microplates for ELISA and incubated with probe “ATD” either in the presence of TPO solution or without TPO in liquid phase. Similarly, immobilized $AE_{anti-Tg}$ and Tg as a positive control were incubated with probe “ATD” either in the presence of Tg solution or without Tg in liquid phase. In this test, probe “ATD” was used as a source of anti-TPO and anti-Tg; TPO and Tg in liquid phase were used as a competitors, *e.g.* substances competing with immobilized items for anti-TPO and anti-Tg binding, respectively. For each immobilized item the data were calculated as $(B_n/B_0) \times 100\%$, where B_n and B_0 are IgG bound with the immobilized item in the presence and absence of thyroid antigen in liquid phase, respectively. The data are shown in Figure 4.

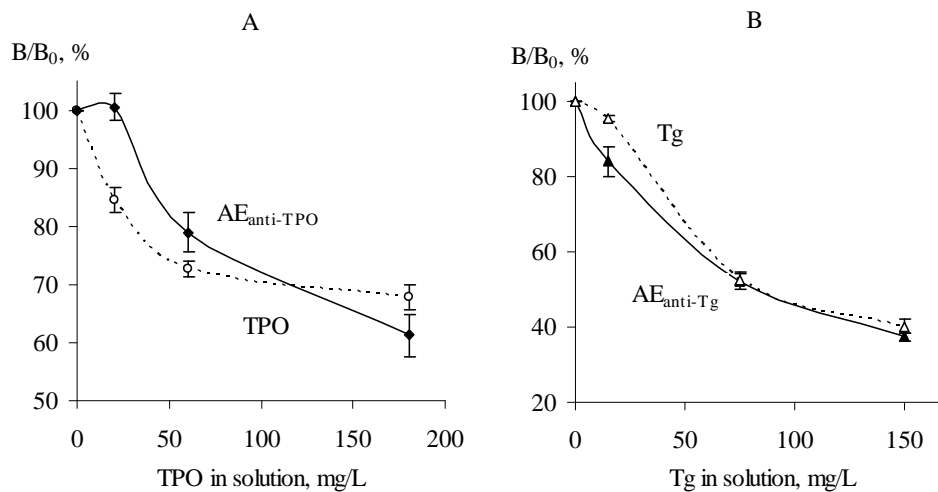


Figure 4. The Competition of Immobilized $AE_{anti-TPO}$ (A) and $AE_{anti-Tg}$ (B) with TPO (A) and Tg (B) Solutions, Respectively, for Binding of IgG Contained in Human Serum Probe "ATD". Immobilized TPO (A) and Tg (B) were Used as Positive Controls (ELISA, Test 4). $AE_{anti-TPO}$ and $AE_{anti-Tg}$ - Black Symbols, Solid Lines; TPO and Tg - Empty Symbols, Dashed Lines. Mean Data from Three Repeated Experiments are Shown. The Error Bars Represent the Range of Data. Abbreviations: ATD - Autoimmune Thyroid Disease; TPO - Thyroid Peroxidase; Tg - Thyroglobulin; $AE_{anti-TPO}$ and $AE_{anti-Tg}$ - Affinity Eluates Obtained from the Soluble Fraction of *S. cerevisiae* BIM Y-195 Cells by Affinity Chromatography with Either Human Autoantibodies to TPO or Autoantibodies to Tg, Respectively.

The data of Figure 4, A and B demonstrate the reduction of IgG binding with immobilized $AE_{anti-TPO}$ and $AE_{anti-Tg}$, respectively, in the presence of the appropriate thyroid antigens in liquid phase. The reduction is dependent on TPO concentration (Figure 4, A) and Tg concentration (Figure 4, B). Moreover, the immobilized $AE_{anti-TPO}$ and $AE_{anti-Tg}$ show similar effects as immobilized TPO and Tg (Figure 4, A and B).

On the whole, the data shown in Figure 4, A prove competition of immobilized $AE_{anti-TPO}$

vs. dissolved TPO for binding of the same IgG, viz., anti-TPO. Similarly, the data shown in Figure 4, B prove competition of immobilized $AE_{anti-Tg}$ vs. dissolved Tg for binding of anti-Tg.

Purification of $BP_{anti-TPO}$ and $BP_{anti-Tg}$

$AE_{anti-TPO}$ and $AE_{anti-Tg}$ (each 5 mg) were fractionated by size exclusion chromatography on TSK-40 gel with refractometric detection. The elution profiles are shown in Figure 5.

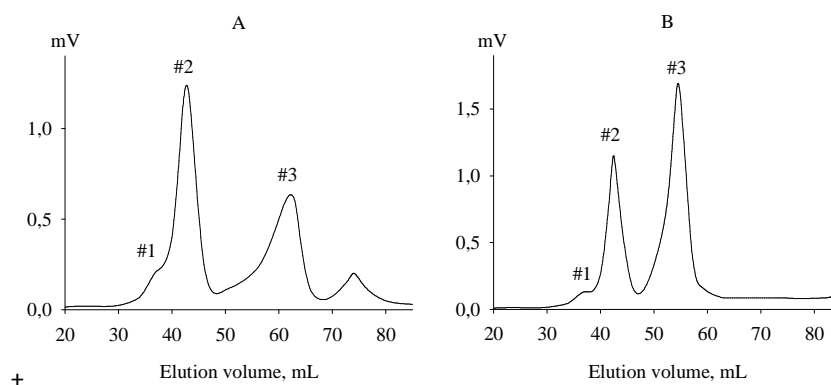


Figure 5. Size Exclusion Chromatography of Crude BP_{anti-TPO} (A) and BP_{anti-Tg} (B) on TSK 40 Gel (60 x 1.6 cm). The Elution Profiles were Detected by Differential Refractometer. Fractions #2 and #3 of Each BP were Used for NMR Spectroscopy. Abbreviations: BP_{anti-TPO} and BP_{anti-Tg} – The Yeast Biopolymers that Selectively Bind Human Autoantibodies to Thyroid Peroxidase and Thyroglobulin.

The elution profile of AE_{anti-TPO} contained 4 peaks (Figure 5, A). The first and last peaks corresponded to bad volume and low molecular weight contaminations, respectively; they were not collected. Other peaks were collected as fractions BP_{anti-TPO}-2 and BP_{anti-TPO}-3 corresponding to molecular weights 5000 – 7000 Da and 1000 – 1500 Da, respectively.

The elution profile of AE_{anti-Tg} had a similar form (Figure 5, B), though the retention times of peaks #3 in Figures 5, A and 5, B were inconsistent; the second and third peaks were collected as fractions BP_{anti-Tg}-2 and BP_{anti-Tg}-3.

All collected fractions contained BPs in quantities that were sufficient for NMR spectroscopy (2.75 mg, 1.5 mg, 2.15 mg and 2.3 mg for BP_{anti-TPO}-2, BP_{anti-TPO}-3, BP_{anti-Tg}-2 and BP_{anti-Tg}-3, respectively).

NMR Spectroscopy of BP_{anti-TPO} and BP_{anti-Tg}

Structure of BPs contained in fractions BP_{anti-TPO}-2, BP_{anti-TPO}-3, BP_{anti-Tg}-2 and BP_{anti-Tg}-3 was analyzed by NMR spectroscopy. ¹H NMR spectra of BP_{anti-TPO}-3 and BP_{anti-Tg}-3 were not interpretable. Since ¹H NMR and ¹³C NMR spectra of BP_{anti-TPO}-2 and BP_{anti-Tg}-2 were identical, only the data for BP_{anti-TPO}-2 are shown in Figure 6 and Table 2 as an example. ¹H NMR spectrum of BP_{anti-Tg}-2 is shown in Appendix 3. Analysis by two-dimensional NMR spectroscopy, including ¹H, ¹H COSY and ¹H, ¹³C HSQC experiments, indicated that the BPs contained in fractions BP_{anti-TPO}-2 and BP_{anti-Tg}-2 are linear α-1, 6-glucans.

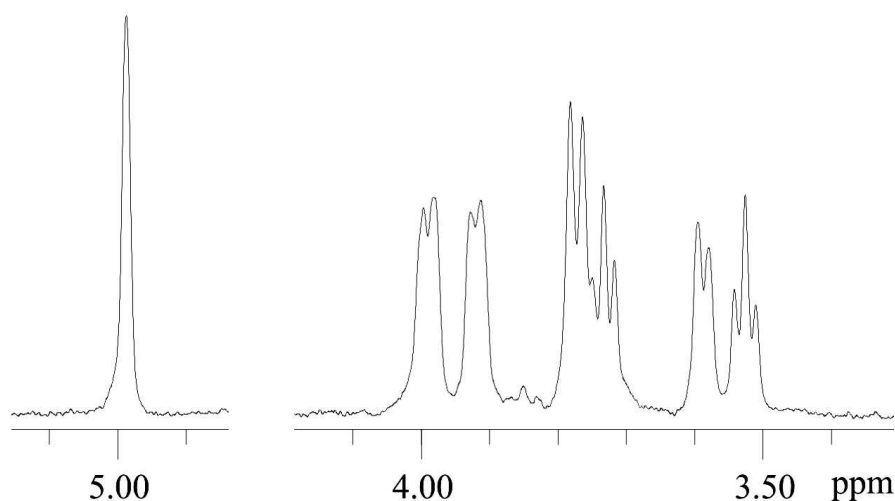


Figure 6. ¹H NMR Spectrum of BP_{anti-TPO}-2. Abbreviation: BP_{anti-TPO}-2 – Fraction #2 of the Yeast Biopolymers that Selectively Bind Human Autoantibodies to Thyroid Peroxidase and were Isolated by Affinity Chromatography with the Antibodies.

Table 2. ¹H NMR Data of the BP_{anti-TPO}-2 (δ, ppm). Abbreviation: BP_{anti-TPO}-2 – Fraction #2 of the Yeast Biopolymers that Selectively Bind Human Autoantibodies to Thyroid Peroxidase.

H-1	H-2	H-3	H-4	H-5	H-6a, 6b
4.99	3.59	3.73	3.53	3.92	3.77, 3.99

Confirmation of BP_{anti-TPO} and BP_{anti-Tg} Functional Activity

To confirm the suggestion that BP_{anti-TPO-2} actually contain BP_{anti-TPO}, ELISA test was performed. BP_{anti-TPO-2}, TPO as a positive control and protein A from *Staphylococcus aureus* as a negative control were immobilized on microplates and incubated with either pure anti-TPO or pure total human Igs (without anti-TPO and anti-Tg) (ELISA, test 5). The data are shown in

Figure 7. The data confirm the presence of BP_{anti-TPO} in the fraction.

To confirm the suggestion that BP_{anti-Tg-2} actually contains BP_{anti-Tg}, ELISA test was performed in a similar way; Tg and pure anti-Tg were used instead of TPO and pure anti-TPO, respectively (ELISA, test 6). The data are shown in Figure 8. The data confirm the presence of BP_{anti-Tg} in the fraction.

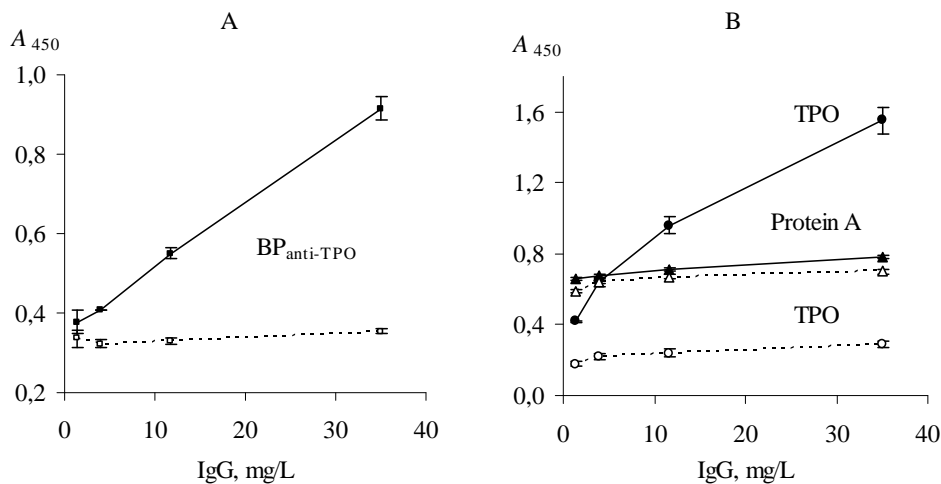


Figure 7. Interaction of Pure Anti-TPO (Black Symbols, Solid Lines) and Pure Total IgG (Empty Symbols, Dashed Lines) with Immobilized BP_{anti-TPO-2} (A), TPO and Protein A (B) (ELISA, Test 5). BP_{anti-TPO-2} and TPO Bind Significantly More (p<0.05) Anti-TPO Compared to Total IgG (Control). Representative Data from Three Independent Experiments are Shown. Values are Means ± Range for Duplicate Wells. Abbreviations: TPO - Thyroid Peroxidase; Anti-TPO - Human Autoantibodies to TPO; BP_{anti-TPO-2} - Fraction #2 of the

Yeast Biopolymers that Selectively Bind Anti-TPO and were Isolated by Affinity Chromatography with the Antibodies.

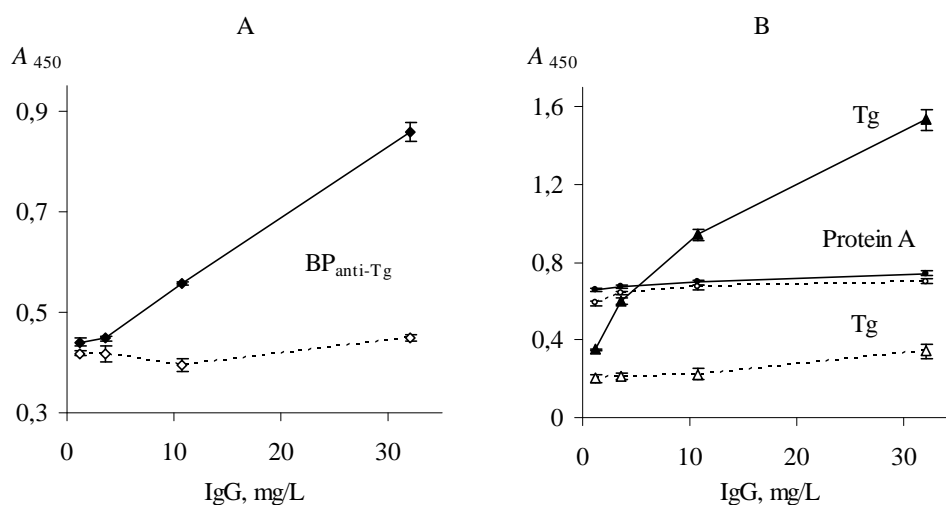


Figure 8. Interaction of Pure Anti-Tg (Black Symbols, Solid Lines) and Pure Total IgG (Empty Symbols, Dashed Lines) with Immobilized BP_{anti-Tg-2} (A), Tg and Protein A (B) (ELISA, Test 6). BP_{anti-Tg-2} and Tg Bind Significantly More ($p < 0.05$) Anti-Tg Compared to Total IgG (Control). Representative Data from Three Independent Experiments are Shown. Values are Means \pm Range for Duplicate Wells. Abbreviations: Tg - Thyroglobulin; Anti-Tg - Human Autoantibodies to Tg; BP_{anti-Tg-2} - Fraction #2 of the Yeast Biopolymers that Selectively Bind Human Autoantibodies to Tg and were Isolated by Affinity Chromatography with the Antibodies.

Discussion

CFF of S. cerevisiae BIM Y-195 as Source of BP_{anti-TPO} and BP_{anti-Tg} and Affinity Chromatography as Biospecific Method for Isolation of Both BPs are Suitable

We used selected strains of *S. cerevisiae* and *D. hansenii* since these yeasts are consumed generally with traditional foods (Andrade et al., 2010; Cano-García et al., 2013; Gori et al., 2012; Sibirny and Voronovsky, 2009; Wszolek et al., 2006). Moreover, whole cells, compounds and substances of *S. cerevisiae* are used as nutritional supplements, food additives and pharmaceuticals with immunomodulatory properties, respectively (Petračić-Tominac et al., 2011; Jawhara et al., 2012). Similarly to widely used *S. cerevisiae*, *D. hansenii* was found to have enormous biotechnological potential in agro-food sector (Breuer and Harms,

2006; Vetrisevi et al., 2010). In addition, both yeasts are implicated in immunomodulation (Generoso et al., 2010; Martins et al., 2009; Reyes-Becerril et al., 2012). Moreover, *S. cerevisiae* strains share bio-therapeutic properties with well known probiotic *S. cerevisiae* var. *boulardii* (Izadnia et al., 1998; Jahn et al., 1996; Van der Aa Kühle et al., 2005).

In present study, we show that CFF and CF of *S. cerevisiae* BIM Y-195 contain BP_{anti-TPO} and BP_{anti-Tg} as opposed to cell fractions of *D. hansenii* BIM Y-4 (Table 1).

Enzymatic digestion with either thermal treatment (Núñez et al., 2006) or chemical extraction (Huang and Li, 2012; Pitarch et al., 2008) as well as chemical extraction itself (Klis et al., 2007; Huang, 2008; Zhang et al., 2011) are used traditionally for isolation of yeast cell wall polysaccharides and proteins. The procedures potentially

are capable of alter/disturb conformation and immunochemical properties of yeast biopolymers. As opposed to CF, CFF-Sc does not require additional treatment. In addition, CFF-Sc demonstrates higher correlation coefficients with TPO/Tg in ELISA tests as compared with CF-Sc (Table 1) and therefore it was selected as most preferable source of BPs. The additional testing of CFF-Sc allows proving more convincingly that the cell fraction actually contains BPs (Figure 1).

We used biospecific affinity chromatography on immobilized anti-TPO and anti-Tg to isolate BP_{anti-TPO} and BP_{anti-Tg}, respectively, from CFF of *S. cerevisiae* BIM Y-195, just as it was done early for isolation of BPs from CFF of *B. bifidum* BIM B-733D (Kiseleva et al., 2013). The combined data of Figure 2 and 3 prove unambiguously that AE_{anti-TPO} contains BP_{anti-TPO} and AE_{anti-Tg} contains BP_{anti-Tg}. Thus, biospecific affinity chromatography allows us to isolate crude BP_{anti-TPO} and BP_{anti-Tg}.

The BP_{anti-TPO} and BP_{anti-Tg} are Linear α -1, 6-glucans

To pure isolated BP_{anti-TPO} and BP_{anti-Tg}, we fractionated AE_{anti-TPO} and AE_{anti-Tg} by size exclusion chromatography (Figures 4 and 5). The data of ¹H NMR spectroscopy (Figure 6, Table 2, Appendix 3) prove that fractions #2 of both AEs contain identical substance, viz., linear α -1, 6-glucan with 5-7 kDa molecular weight.

The data of ELISA (Figure 7) prove that fraction #2 of AE_{anti-TPO} demonstrates functional activity characteristic of BP_{anti-TPO}, viz., ability to interact selectively with anti-TPO. Similarly, fraction #2 of AE_{anti-Tg} demonstrates ability to interact selectively with anti-Tg (Figure 8). Thus, BP_{anti-TPO} and BP_{anti-Tg} have identical structure and are linear α -1, 6-glucans.

Interestingly, that size exclusion chromatography profiles (Figures 4 and 5) show sharp and symmetrical peaks related to α -1, 6-glucan, whereas other studies generally demonstrate broad and asymmetrical elution peaks of bacterial/yeast dextrans (Moussa et al,

2012; Sarwat et al, 2008), at that average molecular weight of bacterial/yeast dextrans are significantly higher than in our study. Moreover, small peaks in bad volume (Figures 4 and 5) suggest that both AEs contained almost no substances with molecular weights over 7 kDa. Therefore, we assume that either *S. cerevisiae* BIM Y-195 does not produce high-molecular weight linear α -1, 6-glucan or we do not isolate such α -1, 6-glucan by biospecific chromatography since it does not have activity characteristic of BP_{anti-TPO} and BP_{anti-Tg}.

We unexpectedly show that the structure of both BPs is identical, which is in accord with (i) the presence of common B cell and T cell epitopes shared between TPO and Tg (Hoshioka et al, 1994; Kohno et al, 1988; McLachlan and Rapoport, 1989) and (ii) the existence of the circulating autoantibodies with dual specificity for Tg and TPO that were detected in the serum of rabbits immunized with Tg and commonly present in serum of peoples diagnosed with HT (Estienne et al., 1999; Ruf et al, 1994; Thrasyvoulides and Lymberi, 2004). Unfortunately, common B cell epitopes shared between TPO and Tg were not identified up to date, probably, due their conformational or/and cryptic nature or because of their production through the mechanism of epitope spreading. The mechanism was proven in the study (Thrasyvoulides and Lymberi, 2004), in which rabbits immunized with selected Tg peptide produced bispecific antibodies interacting with sites of Tg not coinciding with the peptide sequence of the immunogen.

One more unexpected finding is that BP_{anti-TPO} and BP_{anti-Tg} are carbohydrates. Taking into account that TPO and Tg are glycoproteins, one could assume that α -1, 6-glucan competes for anti-TPO (anti-Tg) binding with carbohydrate chains of TPO (Tg). The known data suggest that the carbohydrate chains of TPO do not contribute to TPO recognition by anti-TPO (Foti and Rapoport, 1990; Giraud et al., 1992; Kiso et al., 1992; Moura et al., 1991). On the contrary, improved glycosylation of Tg weakens interaction of the thyroid

antigen with anti-Tg due to distorting conformation of the polypeptide chain or masking its antigenic regions (Fenouillet, et al., 1986). Thus, our data suggest immunological similarity of α -1, 6-glucan and epitopes of TPO (Tg) polypeptide chain. The conclusion is in accordance with known studies (Alekseev et al., 2010; Goto-Tamura et al., 1976; Miyazaki et al., 1982; Ochi et al., 1982; Perrotti et al., 1986) where detection of cross-reactivity of antibodies against antigen 1 with unrelated antigen 2 was interpreted as immunologic similarity between these antigens.

Third unpredictable result is identity between BPs isolated from CFF-Sc in present study and BPs isolated recently from CFF of *B. bifidum* BIM B-733D (Kiseleva et al., 2013).

Carbohydrates as Peptidomimetics

Molecular mimicry, in the broad sense, is structural, functional or immunologic similarities between unrelated macromolecules (Oldstone, 1998). The carbohydrate nature of both BPs is in accord with the known cases of the immunological similarity between peptide and carbohydrate (Agadjanyan et al., 1997; Guilherme et al., 2006; Heimbürg-Molinario et al., 2009; Luo et al., 1998; Luo et al., 2000). Generally, the cases of molecular mimicry between protein and non-protein are rare and as a rule do not relate to autoimmunity. In our perusal of the literature on bacterial/viral triggering of autoimmunity via molecular mimicry mechanism, we found only one carbohydrate, *viz.*, streptococcal group A epitope, N-acetyl glucosamine, that structurally mimic cardiac protein myosin in human rheumatic carditis. On the contrary, molecular mimicry between bacterial/viral proteins and human protein antigens implicated in autoimmune disease pathogenesis is well known and most proven mechanism of autoimmune disease triggering (Atkinson et al., 1994; Cunningham, 2000; Dale and Beachey, 1985; Gautam et al., 1998; Katona-Durekovic, 2007; Root-Bernstein et al., 2009).

Recently, we show for the first time immunological similarity between α -1, 6-glucan isolated from CFF of probiotic bacterium *B. bifidum* BIM B-733D and thyroid proteins, TPO and Tg, implicated in ATD pathogenesis (Kiseleva et al., 2013). In the present study, we extended the list of commensals producing substances with the same properties. We take into account that α -1, 6-glucans are quite different from the glucans with other glycoside linkages because they have many possible conformations due to flexibility of 1-6 linkage (Zhang et al., 2007) that give an possible explanation for unusual property of the glycopolymer, *e.g.* ability to cross-react with anti-TPO and anti-Tg, thus mimicking natural thyroid proteins, TPO and Tg.

Microorganisms in Triggering/Abrogation of Autoimmune Diseases

In the experimental models, autoimmune disease occurs only in susceptible strains, under particular environmental conditions, and with the usage of adjuvant molecules (Pordeus et al., 2008). In humans, any autoimmune disease can and does occur only in genetically predisposed persons. They have a population of self-reactive T cells that are potentially intolerant but under special circumstances able to react with the host's antigens. One of the most common "special circumstances" is infectious agents that can induce or accelerate pre-existing autoimmune responses (Harkioliaki et al., 2009; Kohm et al., 2003; Roep, 2003; Wegner et al., 2009).

Interestingly, well known infection agents may protect from autoimmunity or even abrogate an ongoing autoimmune process (Gaisford et al., 2009; Kivity et al., 2009; Sfriso et al., 2010). Moreover, the same microorganism may induce one autoimmune disease and protect from another one. For example, Epstein-Barr virus can induce systemic lupus erythematosus and protect from type 1 diabetes mellitus (Kivity et al., 2009). Same possible mechanisms of abrogation an ongoing autoimmune process and

protection from autoimmunity are proposed to date (Sfriso et al., 2010). For example, infection at another location might keep autoaggressive cells from reaching the site of autoimmune destruction. In addition, several pathogens can shift the T cells balance toward a more immunosuppressive state, where Tregs might be induced. It may have therapeutic benefits for patients suffering from Th1/Th17-mediated autoimmune diseases (in particular, HT) (Phenekos et al., 2004; Qin et al., 2012). The last-named mechanism is triggered by ligands of pathogen-associated molecular patterns receptors expressed on/into antigen-presenting immune cells (Den Dunnen et al., 2010; Jeong and Lee, 2011; Kawai and Akira, 2009; Zeuthen et al., 2008). Since the ligands are shared between pathogens and commensals/probiotics (Didierlaurent et al., 2006; Rumbo et al., 2006), beneficial microorganisms can trigger/prevent autoimmunity just as certain pathogens do.

Our recent findings (Kiseleva et al., 2013) and the data of present study allow assuming that molecular mimicry *per se* can work as protective mechanism, which we will discuss in detail in section 4.6.

Dextrans from Other Microorganisms and Their Immunological Potency (Immunogenicity)

According to the comprehensive enzyme information system BRENDA (www.brenda-enzymes.info), the list of known dextran-producing microorganisms includes bacteria of genera *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Weissella*. Recently it was shown that *S. cerevisiae* NRRL Y-1534 produce secretory dextran with average molecular weight 67 kDa (Moussa and Khalil, 2012). The data on numerous applications of the dextrans and derivative substances in food industry and clinical practice suggest that the negative effects of dextrans on human health were not found to date (Liu et al., 2009; Neiser et al., 2011; Qader et al., 2005).

The linear bacterial dextrans are rare. Three known dextrans are the most similar

to linear α -1, 6-glucan obtained in present study. One of them is the water soluble dextran produced by *Leuconostoc mesenteroides* NRRL B-512, which have a molecular weight of 8×10^7 Da and contain 95% linear α -(1 \rightarrow 6) linkages and 5% α -(1 \rightarrow 3) linkages (Van Cleve et al., 1956); the latter are branch points for side chains including no more than two glucose units. Another one is dextran from *Weissella confusa*, strains E392 (Maina et al., 2008) and LBAE C39-2 (Amari et al., 2013) that contains only 2.7% - 2.8% α -(1 \rightarrow 3)-linked branches. The very similar to dextran shown in our study is an unique dextran from an indigenous *Ln. mesenteroides* CMG713 that contains 100% α -(1 \rightarrow 6)-linked glucopyranosyl units in the main chain and does not have any branching (Sarwat et al., 2008).

It has been shown that dextran-specific antibodies (anti-dextrans) commonly detected in the serum samples of healthy donors are produced as result of immune response to naturally occurring dextrans from caries-inducing *Streptococcus* species and intestinal bacteria (Neiser et al., 2011; Paul et al., 2009). Antigenic determinants of α -1, 6-glucans responsible for its specific interaction with human anti-dextrans involve six and perhaps seven glucosyl residues (Cisar et al., 1975). Thus, α -1, 6-glucan from *S. cerevisiae* BIM Y-195 is sufficiently large to act as an antigen, *e.g.* bind pre-existing anti-dextrans and cross-react with pre-existing anti-TPO/anti-Tg.

We assume that 5 - 7 kDa α -1, 6-glucan from *S. cerevisiae* BIM Y-195 has low immunogenicity, since it was shown that low molecular weight dextrans are appreciably less immunogenic than high molecular weight dextrans (Neiser et al., 2011). However, we can not exclude the presence of immunologically relevant BPs with the same structure but higher molecular weight in the cells of the yeast, which allow us to include antibodies to α -1, 6-glucan from *S. cerevisiae* BIM Y-195 in the list of the possible players in ATD pathogenesis.

α -1, 6-Glucan and Antibodies to α -1, 6-Glucan as Putative Players in ATD Pathogenesis

We discuss the possible role of α -1, 6-glucan and antibodies to α -1, 6-glucan in the molecular mechanisms of the immune

response typical of ATD by allowing either negative or positive impact of both substances on human health (triggering/acceleration and prevention/abrogation of ATD, respectively) (Figure 9).

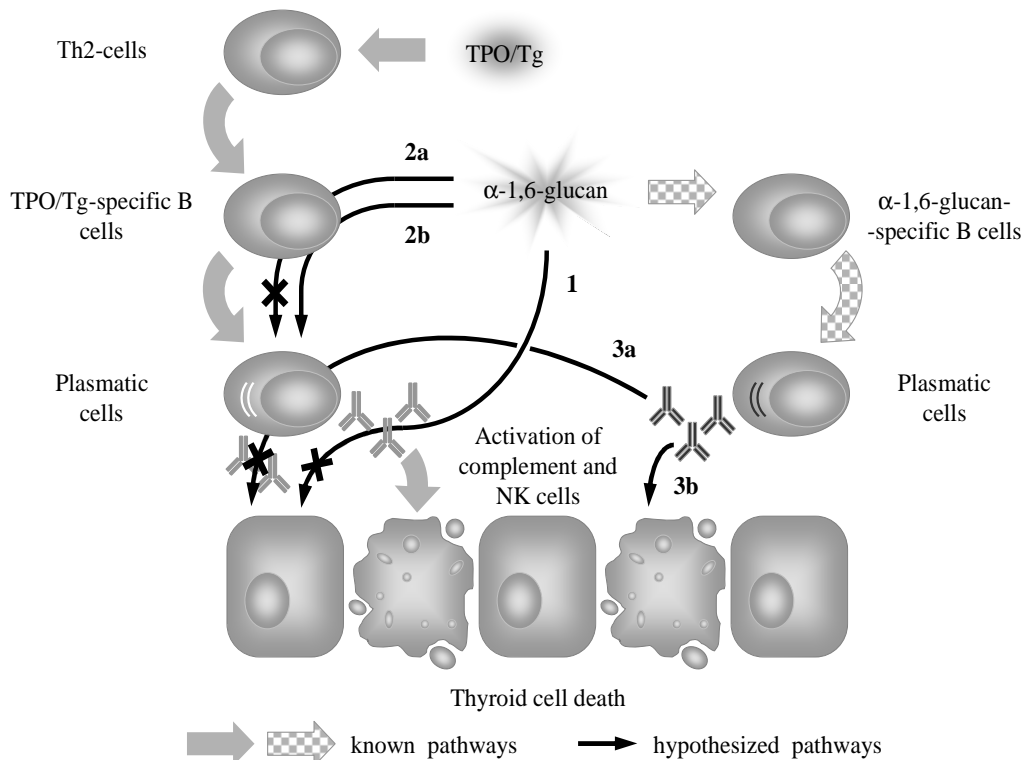


Figure 9. Presumptive Roles of α -1, 6-Glucan from *S. cerevisiae* BIM Y-195 and Natural Antibodies to α -1, 6-Glucan in ATD Pathogenesis: A Hypothetical Model. In “Normal” ATD Pathogenesis Anti-TPO and Anti-Tg Interact with TPO and Tg, Respectively, Presented by MHC II/Abnormally Expressed on Basolateral Membrane of Thyroid Cells, which Leads to Thyroid Cell Destruction through Complement Activation and Antibody-Dependent Cell Mediated Cytotoxicity (Green Arrows). Dextran-Specific Antibodies (Anti-Dextrans) are Produced as Result of Immune Response to Naturally Occurring Dextrans from Commensals, Including Yeasts (Chequered Arrows). The α -1, 6-Glucan (1) Bind Anti-TPO/Anti-Tg and Thereupon Prevents Their Interaction with TPO/Tg; (2) Interacts with TPO/Tg-Specific B Cells Receptors and Thereupon Either (2a) Blocks the B Cells as an Antagonist or (2b) Activates the B Cells as an Agonist. Natural Antibodies to α -1, 6-Glucan Either (3a) Reduce Anti-TPO/Anti-Tg Synthesis via Feedback Mechanism or (3b) Act Similarly to Anti-TPO/Anti-Tg. Thus, α -1, 6-Glucan and Natural Antibodies to α -1, 6-Glucan Either Induce/Accelerate ATD (1, 2a, 3a; Black Arrows) or Prevent/Abrogate ATD through a Mechanism of Molecular Mimicry (2b, 3b; 1, 2a, 3a; Crossed out Black Arrows). Abbreviations: Tg - Thyroglobulin; TPO - Thyroid Peroxidase; NK Cells - Natural Killer Cells; Th2 - T Helper Cells of Class 2.

Firstly, we assume that α -1, 6-glucan is able to bind pre-existing serum anti-TPO (anti-Tg) (Figure 9, 1). If it is true, α -1, 6-glucan prevents interaction of anti-TPO (anti-Tg) with either TPO (Tg) presented by MHC II (Salmaso et al., 2002; Weetman, 1997; Weetman, 2003) or TPO abnormally expressed on basolateral membrane of thyroid cells (Zimmer et al., 1997). Thus, α -1, 6-glucan can reduce thyroid cell destruction that in “normal” ATD pathogenesis occur through complement activation (Chiovato et al., 1993; Potluková and Limanová, 2007) or antibody-dependent cell mediated cytotoxicity (Rebuffat et al., 2008).

Secondly, α -1, 6-glucan, T cell independent antigen of class 2 (Mond et al., 1995), can interact with the receptors of B cells specific to TPO and Tg, since these receptors are identical to secreted forms (anti-TPO and anti-Tg) with the exception of the presence of an integral membrane domain (Kindt et al., 2007). To discuss this point in details, we take into account that polyspecificity is commonly recognized intrinsic property of B cell receptor recognition (Wucherpfennig et al., 2007) though the data on the role of the so called “altered ligand” (*e.g.*, partial homolog of the natural ligand, in our case TPO and Tg) in B cell receptor recognition are limited (Kouskoff et al., 1998; Liu et al., 2010; Wucherpfennig et al., 2007). Additionally, we take into account that the strength of signaling via the B cell receptor controls B cell fate in immunity and autoimmunity (Noelle et al., 2005), and in the immune response induced by T cell independent antigen of class 2 large differences in affinity (in our case, the differences in affinity of thyroid antigens and α -1, 6-glucan to receptors of thyroid antigens-specific B cells) produce only small differences in the intrinsic ability of B cells to respond to antigen (Shih et al., 2002). It is possibly that α -1, 6-glucan induce the same effect as natural thyroid antigens (negative impact on health) (Figure 9, 2b). Alternatively, we assume that it block thyroid antigens-specific B cells proliferation and synthesis of antibodies to thyroid antigens (positive impact on health) (Figure 9, 2a). Additionally, we do

not exclude that cross-reaction of α -1, 6-glucan with receptors of B cells specific to thyroid antigens is irrelevant to ATD.

At last, we take into account the possible role of antibodies to α -1, 6-glucan. Since α -1, 6-glucan mimic thyroid antigens, antibodies to α -1, 6-glucan can mimic anti-TPO and anti-Tg and either (*i*) induce the same effects as anti-TPO and anti-Tg including complement activation or antibody-dependent cell mediated cytotoxicity (negative impact on health) (Figure 9, 3b), or (*ii*) induce feedback inhibition of TPO-specific and Tg-specific B cells (positive impact on health) (Figure 9, 3a). It is known that some clones of anti-Tg are able to inhibit T-cell proliferative response and T cell cytotoxicity against thyroid cells and thus ameliorate ATD either via sequestration of antigenic epitopes of Tg or via blockade of peptide-MHC complexes (Dai et al., 2005; Salamero et al., 1987). The antibodies to α -1, 6-glucan may prevent or ameliorate ATD in a similar way. Additionally, we do not exclude that antibodies to α -1, 6-glucan do not mimic anti-TPO and anti-Tg and the effect of antibodies to α -1, 6-glucan is irrelevant to ATD.

Noteworthy that we take into account only those of the known mechanisms of autoimmune destruction of thyroid cells and thyroid autoimmunity in whole (Blanchin et al., 2003; Londei et al., 1985; MacKenzie et al., 1987; Stassi and De Maria, 2002) which are relevant to the possible targets of either α -1, 6-glucan or antibodies to α -1, 6-glucan, *viz.*, anti-TPO/anti-Tg/receptors of thyroid antigen-specific B cells and TPO/Tg, respectively.

It is importantly that immunologic similarity of thyroid antigens and α -1, 6-glucan from *S. cerevisiae* BIM Y-195 and other human symbionts (Kiseleva et al., 2013) is necessary but not sufficient prerequisite for triggering/acceleration or prevention/abrogation of autoimmune disease (in our case, ATD) by molecular mimicry mechanism, as it was accentuated in known review of Oldstone M. B. (Oldstone, 2005). The main requirements are hypothesized to be (*i*) genetic

predisposition to ATD (Muixi et al., 2008) and (ii) intestinal epithelium penetration by α -1, 6-glucan.

Conclusion

In this paper, we show that *Saccharomyces cerevisiae* BIM Y-195 (but not *Debaryomyces hansenii* BIM Y-4) contains BPs with unusual immunochemical property, notably ability to distinguish anti-TPO (anti-Tg) from other IgG and compete with TPO (Tg) for binding of anti-TPO (anti-Tg) in ELISA tests. The BPs, viz., BP_{anti-TPO} and BP_{anti-Tg}, were isolated, purified and identified as linear α -1, 6-glucans. The unusual immunochemical property of BP_{anti-TPO} and BP_{anti-Tg} suggests that they cross-react with anti-TPO and anti-Tg, respectively, which in turn points to immunological similarity between BP_{anti-TPO} and epitopes of TPO and between BP_{anti-Tg} and epitopes of Tg. Since both BPs have identical chemical structure, we assume that each BP immunologically mimic common epitopes shared between TPO and Tg and binds autoantibodies with dual specificity to TPO and Tg.

Thus, it was shown for the first time an immunological similarity (molecular mimicry) of α -1, 6-glucans of *S. cerevisiae* BIM Y-195 and human thyroid proteins, TPO and Tg. Our data point to a possible role of wine yeast in the molecular mechanisms of the immune response typical of ATD, which we discussed in this paper allowing for both negative and positive impact of yeast α -1, 6-glucans on human health (triggering/acceleration and prevention/abrogation of ATD, respectively).

The data obtained and known mechanisms of ATD pathogenesis are insufficient to draw conclusions in context of human health. It appears to be advisable to extend this study by using of animal models and clinical trials to investigate whether α -1, 6-glucan from *S. cerevisiae* is a possible candidate for antigen-specific immunosuppressive therapy against ATD or, alternatively, recommend limiting the consumption of traditional foods, food

additives and pharmaceuticals containing cells/components of the yeast in order to avoid triggering/acceleration of ATD.

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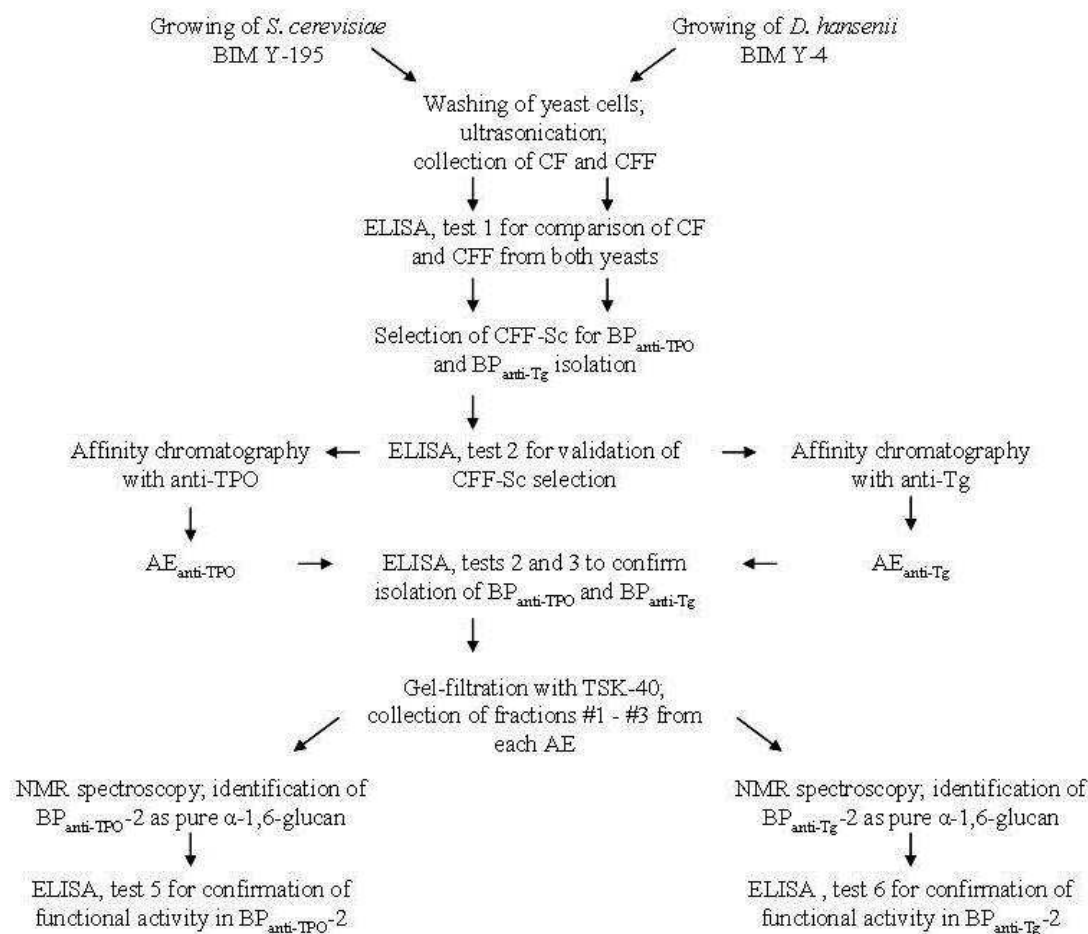
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Appendix 1. Methodological Steps of the Study

Appendix 2. Immobilized Items and Primary Reagents Added in Wells at the First Stage of ELISA Tests.

Table 3. Immobilized Items and Primary Reagents Added in Wells at the First Stage of ELISA test 1. Test 1 was Performed to Detect Functional Activity Characteristic of BP_{anti-TPO} and BP_{anti-Tg} in the Cell Fractions of *S. cerevisiae* BIM Y-195 and *D. hansenii* BIM Y-4. Each Table Cell Means Two Identical Wells.

		Immobilized item					
		<i>S. cerevisiae</i> BIM Y-195		<i>D. hansenii</i> BIM Y-4			
		CF	CFF	CF	CFF	TPO	Tg
Solution at the first stage of analysis	sample	Serum samples from second and third groups, diluted 100-fold by buffer 3					
	sample number	1	1	1	1	1	1
		2	2	2	2	2	2
		3	3	3	3	3	3
		47	47	47	47	47	47
		48	48	48	48	48	48

Table 4. Immobilized Items and Primary Reagents Added in Wells at the First Stage of ELISA Test 2. Test 2 was Performed to Validate Selection of CFF-Sc for BP_{anti-TPO} and BP_{anti-Tg} Isolation. Each Table Cell Means Two Identical Wells.

		Immobilized item					
		CFF-Sc		Protein A		HSA	
Solution at the first stage of analysis	serum probe	"ATD"	"healthy"	"ATD"	"healthy"	"ATD"	"healthy"
	concentration, %	0.063	0.063	0.063	0.063	0.063	0.063
		0.125	0.125	0.125	0.125	0.125	0.125
		0.250	0.250	0.250	0.250	0.250	0.250
		0.500	0.500	0.500	0.500	0.500	0.500
		1.0	1.0	1.0	1.0	1.0	1.0

Table 5. Immobilized Items and Primary Reagents Added in Wells at the First Stage of ELISA Test 3. Test 3 was Performed to Prove that AE_{anti-TPO} and AE_{anti-Tg} Selectively Bind Anti-TPO and Anti-Tg. Each Table Cell Means Two Identical Wells.

		Immobilized item							
		AE _{anti-TPO}		AE _{anti-Tg}		TPO		Tg	
Solution at the first stage of analysis	serum probe	"ATD"	"healthy"	"ATD"	"healthy"	"ATD"	"healthy"	"ATD"	"healthy"
	concentration, %	0.0370	0.0370	0.0370	0.0370	0.0370	0.0370	0.0370	0.0370
		0.1111	0.1111	0.1111	0.1111	0.1111	0.1111	0.1111	0.1111
		0.3333	0.3333	0.3333	0.3333	0.3333	0.3333	0.3333	0.3333
		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table 6. Immobilized Items and Primary Reagents Added in Wells at the First Stage of ELISA Test 4. Test 4 was Performed to Prove that AE_{anti-TPO} and AE_{anti-Tg} Compete with TPO and Tg, Respectively, for Binding of the Appropriate Autoantibodies. Each Table Cell Means Two Identical Wells.

		Immobilized item			
		AE _{anti-TPO}	AE _{anti-Tg}	TPO	Tg
Solution at the first stage of analysis	source of Ig competitor	probe "ATD"			
	concentration of competitor, mg/L	TPO	Tg	TPO	Tg
	B ₀	0	0	0	0
	B ₁	40	33	40	33
	B ₂	120	100	120	100
	B ₃	360	300	360	300

Table 7. Immobilized Items and Primary Reagents Added in Wells at the First Stage of ELISA Test 5. Test 5 was Performed to Confirm that the Fraction BP_{anti-TPO-2} Contains BP_{anti-TPO}. Each Table Cell Means Two Identical Wells.

		Immobilized item						
		BP _{anti-TPO-2}		TPO		Protein A		
Solution on the first stage of analysis	pure IgG	anti-Tg	tIgG	anti-Tg	tIgG	anti-Tg	tIgG	
	concentration, mg/L		1.2963	1.2963	1.2963	1.2963	1.2963	1.2963
			3.8889	3.8889	3.8889	3.8889	3.8889	3.8889
			11.6667	11.6667	11.6667	11.6667	11.6667	11.6667
			35.0	35.0	35.0	35.0	35.0	35.0

Table 8. Immobilized Items and Primary Reagents Added in Wells at the First Stage of ELISA Test 6. Test 6 was Performed to Confirm that the Fraction BP_{anti-Tg-2} Contains BP_{anti-Tg}. Each Table Cell Means Two Identical Wells.

		Immobilized item						
		BP _{anti-Tg-2}		Tg		Protein A		
Solution on the first stage of analysis	item	anti-Tg	tIgG	anti-Tg	tIgG	anti-Tg	tIgG	
	concentration, mg/L		1.19	1.19	1.19	1.19	1.19	1.19
			3.56	3.56	3.56	3.56	3.56	3.56
			10.67	10.67	10.67	10.67	10.67	10.67
			32.0	32.0	32.0	32.0	32.0	32.0

Appendix 3. ¹H NMR Spectrum of BP_{anti-Tg-2}

