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Recognition of riboflavin and the capsular polysaccharide of *Haemophilus influenzae* type b by antibodies generated to the haptenic epitope D-ribitol

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Abstract D-Ribitol, a five–carbon sugar alcohol, is an important metabolite in the pentose phosphate pathway; it is an integral part of riboflavin (vitamin B2) and cell wall polysaccharides in most Gram-positive and a few Gram-negative bacteria. Antibodies specific to D-ribitol were generated in New Zealand white rabbits by using reductively aminated D-ribose-BSA conjugate as the immunogen. MALDI-TOF and amino group analyses of ribitol-BSA conjugate following 120 h reaction showed ~27–30 mol of ribitol conjugated per mole BSA. The presence of sugar alcohol in the conjugates was also confirmed by an increase in molecular mass and a positive periodic acid–Schiff staining in SDS-PAGE. Caprylic acid precipitation of rabbit serum followed by hapten affinity chromatography on ribitol–KLH–Sepharose CL-6B resulted in pure ribitol–specific antibodies (~45–50 μg/mL). The affinity constant of ribitol antibodies was found to be 2.9×10^7 M^{-1} by non-competitive ELISA. Ribitol antibodies showed 100 % specificity towards ribitol, ~800 % cross–reactivity towards riboflavin, 10–15 % cross–reactivity with sorbitol, xylitol and mannitol, and 5–7 % cross–reactivity with L-arabinitol and *meso*-erythritol. The specificity of antibody to ribitol was further confirmed by its low cross-reactivity (0.4 %) with lumichrome. Antibodies to D-ribitol recognized the purified capsular polysaccharide of *Haemophilus influenzae* type b, which could be specifically inhibited by ribitol. In conclusion, antibodies specific to D-ribitol have been generated and characterized, which have potential applications in the detection of free riboflavin and ribitol in biological samples, as well as identification of cell-surface macromolecules containing ribitol.

Keywords *Haemophilus influenzae* type b · Immunogenicity · Polyribosyl ribitol phosphate · Reductive amination · Ribitol–specific antibodies · Riboflavin

Abbreviations

BSA	Bovine serum albumin
FAD	Flavin adenine dinucleotide
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FMN	Flavin mononucleotide
icELISA	Indirect competitive ELISA
KLH	Keyhole limpet hemocyanin
ncELISA	Non-competitive ELISA
OVA	Ovalbumin
PAS	Periodic acid–Schiff
PMF	Peptide mass fingerprint
PRP	Polyribosyl ribitol phosphate capsular polysaccharide
Rbt	Ribitol
RF	Riboflavin
RSA	Rabbit serum albumin
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing 0.05 % Tween-20
Tetvac	Tetanus toxoid
TNBS	Trinitrobenzenesulfonic acid

Introduction

D-Ribitol (1,2,3,4,5-pentanepentol; adonitol), a five-carbon sugar alcohol is an important metabolite formed from the

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intermediates such as D-ribulose-5-phosphate and D-ribose-5-phosphate in the pentose phosphate pathway. Ribitol (Rbt) is also formed in human fibroblasts and erythrocytes by the reduction of D-ribose, and is cleared from the body without metabolic conversion [1]. Ribitol concentration in blood (0.38–0.55 μM) is lower than in cerebrospinal fluid (3.01–4.45 μM) in normal conditions [2]. In Down's syndrome, an inborn error of metabolism, abnormal ribitol concentrations in body fluids have been reported [3]; it can be diagnosed by the assessment of urinary concentration of polyols [4]. Ribitol occurs naturally in very few plants and fungi in small amounts with the exception of sweet vernal (*Adonis vernalis*), where its concentration is 40,000 ppm [5, 6].

Ribitol is also a constituent component of an important water-soluble vitamin riboflavin (vitamin B2; 7,8-dimethyl-10-D-ribitylisoalloxazine; E-101). Riboflavin (RF) contains an isoalloxazine ring linked to the C1′ of D-ribitol (Fig. 1a). The principal forms of riboflavin in tissues and cells are flavin mononucleotide (FMN; riboflavin-5′-phosphate), and flavin adenine dinucleotide (FAD) [7]. Ribitol is a precursor for the synthesis of ribitol-teichoic acid of cell wall teichoic acids in Gram–positive bacteria, and also for the capsular polysaccharides of some Gram–negative bacteria [8, 9]. In the case of *Haemophilus influenzae* type b, ribitol is present as polyribosyl ribitol phosphate capsular polysaccharide (PRP), which is a linear copolymer of repeating units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, wherein n ranges from 6 to 10 (Fig. 1b), and the terminal ribitol group is free [10].

Given the importance of ribitol in body fluids for the diagnosis of inborn errors of metabolism [3, 4] and the potential utility of antibodies specific to ribitol for quantification of riboflavin in foods and biological samples, it appeared worth investigating whether antibodies to ribitol also recognize riboflavin, and characterize them in terms of cross-reactivity with other sugar alcohols, riboflavin, FMN and FAD, based on earlier studies from our laboratory on the immunogenicity of sugar alcohols such as mannitol, erythritol and xylitol [11, 12].

Anti-carbohydrate antibodies with specificity towards several monosaccharides, disaccharides and oligosaccharides have been generated using various conjugation methods including reductive amination with proteins [13–15]. In aqueous solution, D-ribose mainly exists in its pyranose form (α, 21 %; β, 59 %), but also in its furanose form in low concentrations (α, 6 %; β, 14 %); a small percentage of D-ribose also exists in the open-chain form (0.05 %) based on the circular dichroism study [16, 17]. Utilizing the reactive carbonyl form, D-ribose can be coupled to free amino groups of proteins at a slightly alkaline pH, by reductive amination. Reductively aminated D-ribose group has an acyclic structure and resembles D-ribitol as well as the corresponding Schiff base intermediate structurally with respect to carbon atoms 2 to 5 resulting in ribityl epitopes on the carrier protein (supplementary Fig. S1).

Haptens, normally defined as small molecules of <1,500 Da, can have one or more determinants (haptenic epitopes) as exemplified in the case of folic acid and chlorhexidine [18, 19]. However, there have been no reports on the generation of antibodies specific to ribitol and their recognition of riboflavin. Although number of assays have been developed for the determination of PRP content of *Haemophilus influenzae* type b conjugate vaccines, they mostly depend on physicochemical methods; biological tests have been carried out only to confirm the induction of immune response and to ensure vaccine safety [20]. The proposed antibodies to ribitol can find utility in the immunological detection of *Haemophilus influenzae* type b vaccine either alone or in combination with other vaccines to ensure the integrity and stability of the conjugate. In this investigation, ribityl conjugate of a carrier protein was prepared and used as the immunogen to produce polyclonal antibodies in rabbits. Antibody specific to ribitol was purified by hapten affinity chromatography on D-ribitol–KLH–Sepharose CL-6B matrix, followed by its characterization in terms of specificity and cross–reactivity with various sugar alcohols, ribitol, riboflavin and their analogs. Further, the specificity of antibodies to ribitol was evaluated for the detection of PRP of *Haemophilus influenzae* type b. The structures of some important compounds used in this study are given in Fig. 1.

Materials and methods

D-Ribose, ribitol, bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OVA), rabbit serum albumin (RSA), riboflavin, D-ribose-5-phosphate (Rib-5-*P*), flavin mononucleotide (riboflavin-5′-phosphate; FMN), flavin adenine dinucleotide (FAD), lumichrome (7,8-dimethylalloxazine), sodium cyanoborohydride ($NaCNBH_3$), Sepharose CL-6B, thionyl chloride ($SOCl_2$) and borane-pyridine complex solution were obtained from Sigma–Aldrich Chemical Co, St. Louis, MO. Periodic acid and trinitrobenzenesulfonic (TNBS) acid were products of Hi-Media Laboratories, Mumbai, India. Schiff reagent (1-fluoro-2,4-dinitrobenzene) and caprylic acid (octanoic acid) were procured from Sisco Research Laboratories, Mumbai, India. Flat–bottomed 96–well ELISA microtiter plates were from Greiner Bio-One, Frickenhausen, Germany. Goat anti-rabbit IgG-alkaline phosphatase (ALP) conjugate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP–NBT), *p*-nitrophenyl phosphate (pNPP), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were products of Bangalore Genei, Bangalore, India. Hiberix™ (GlaxoSmithKline Biologicals s.a., Rixensart, Belgium), Sii

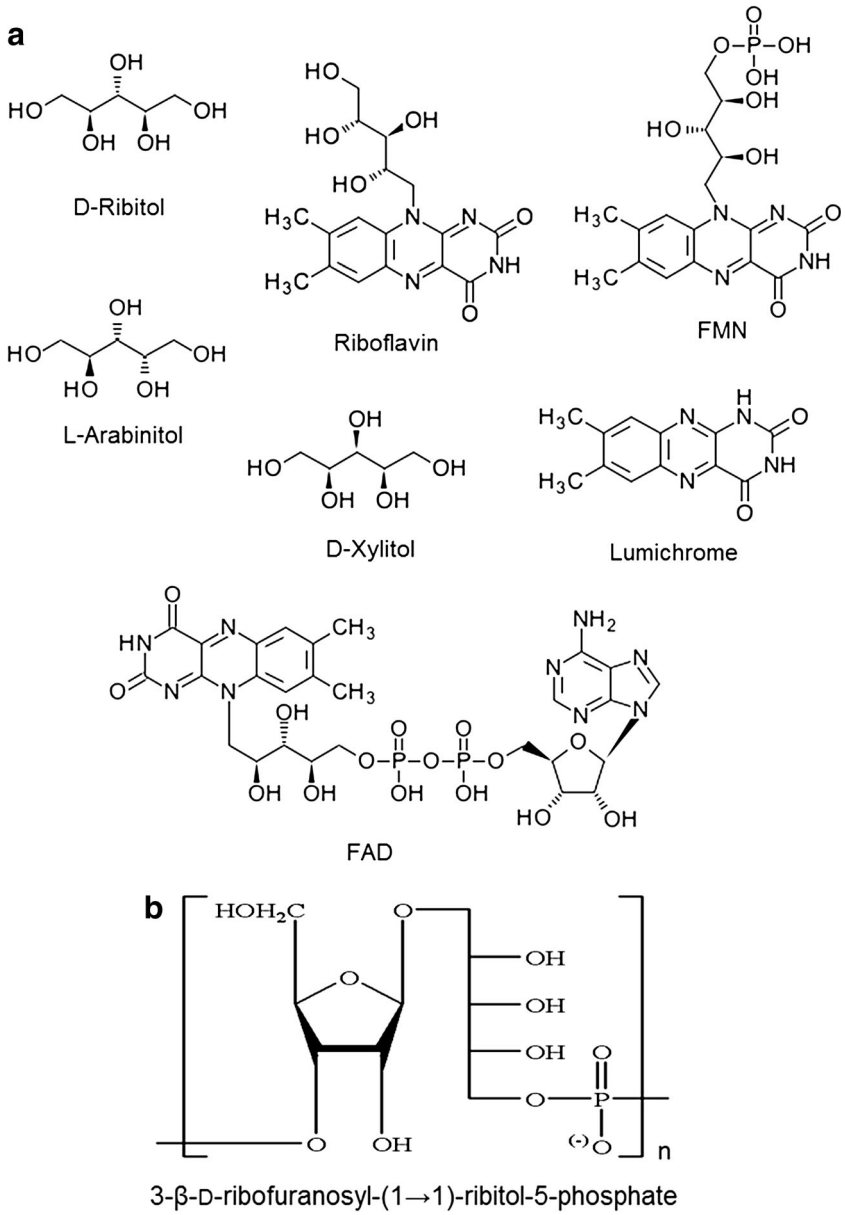

Fig. 1 Structures of selected compounds used in this study. **a** Structures of D-ribitol and its isomers, riboflavin and its coenzymes, and lumichrome. Chemical structures are taken from KEGG COMPOUND database (http://www.genome.jp/kegg/compound/). **b** Structure of PRP of *Haemophilus influenzae* type b; n=6–10

HibPro™, Pentavac SD® and Tetanus toxoid (all manufactured by Serum Institute of India Ltd., Hadapsar, Pune, India) were purchased from the local pharmacy. All other chemicals and reagents were of analytical grade.

Hiberix™ and Sii HibPro™ are lyophilized vaccines containing PRP from *Haemophilus influenzae* type b, covalently bound to tetanus toxoid. Pentavac SD® is a single-dose homogeneous preparation containing purified diphtheria and tetanus toxoids, inactivated whooping cough (pertussis) organism, highly purified, non-infectious particles of hepatitis B surface antigen and *Haemophilus influenzae* type b capsular polysaccharide chemically conjugated to tetanus toxoid. Each single dose of vaccine (0.5 mL) is formulated to contain 10 μg of purified PRP covalently bound to 20–40 μg tetanus toxoid.

Preparation of ribitol-protein conjugates by reductive amination

D-Ribose (10 mM) was conjugated to a carrier protein, BSA (100 μM), or OVA (100 μM) by reductive amination [12, 14, 15] in the presence of a mild reducing agent, $NaCNBH_3$ (100 mM) in 0.2 M borate buffer (pH 8.0) and incubated at 37 °C to obtain ribitol-BSA (Rbt-BSA) and ribitol-OVA (Rbt-OVA) conjugates. Aliquots were taken at various time periods and the reaction was stopped by adjusting the pH to 4 with dilute acetic acid followed by dialysis against phosphate–buffered saline (PBS) at 4 °C. As appropriate controls, BSA and OVA were incubated in the absence of D-ribose. Protein estimation of conjugates was carried out using BSA as a standard [21].

Similarly, D-ribose (10 mM) was conjugated to KLH (5 mg) using $NaCNBH_3$ (100 mM) to obtain ribitol-KLH (Rbt-KLH) conjugates, in which ~50 % of the amino groups of KLH have been modified. Since KLH is a very large protein with its exact molecular weight unknown [22], it is difficult to calculate the molar ratio of hapten to carrier. The large number of amino groups in KLH was exploited for the coupling of D-ribose to Sepharose CL-6B *via* KLH.

TNBS assay for free amino groups

The number of ε-amino groups remaining unreacted in reductively aminated BSA or OVA samples was determined using TNBS reagent [23]. To 1 mL of Rbt-BSA/Rbt-OVA conjugate (100 μg protein) solution, 1 mL of 4 % sodium bicarbonate (pH 8.5) and 1 mL of 0.01 % freshly prepared aqueous TNBS solution were added. The reaction was carried out at 42±2 °C for 2 h, followed by the addition of 1 mL of 10 % SDS and 0.5 mL of 1 N HCl. The absorbance was monitored at 335 nm in a spectrophotometer. The percent conjugation for a conjugate at a specified time was calculated from the decrease in absorbance, when compared with that of an identical concentration of standard BSA or OVA in the TNBS assay. Percent conjugation was converted to degree of substitution in terms of moles ribitol per mole of carrier protein, taking into account the molar extinction coefficient of single TNP–lysine complex for BSA as (0.995×10^4) and for OVA as (1.24×10^4) at 335 nm [24].

SDS-PAGE and MALDI-TOF MS analyses of ribitol-BSA conjugates

The conjugates were analyzed by SDS-PAGE [25] followed by Coomassie blue staining, to observe if there is an increase in the apparent molecular mass of ribitol-conjugates in comparison to control BSA.

Periodic acid–Schiff (PAS) staining following SDS-PAGE [26] was also carried out to confirm the glycated nature of Rbt–BSA conjugates. The gel was immersed in 12.5 % trichloroacetic acid solution for 30 min, transferred to 1 % periodic acid solution and kept in the dark at 25 °C for 50 min. Then the gel was washed thoroughly with distilled water to remove the periodic acid, followed by the addition of Schiff reagent and incubation at 4 °C for 1 h. After the color development, the reaction was arrested by the addition of 7 % glacial acetic acid.

The molecular mass of the Rbt-BSA conjugate (obtained after 120 h of reductive amination) was determined by MALDI–TOF MS [27] in a Kompact analytical SEQ MALDI–TOF mass spectrometer (Kratos, Manchester, UK).

Preparation of riboflavin-protein conjugates

Riboflavin was conjugated to proteins by coupling of the hydroxyl groups of the ribityl moiety with the amino groups of proteins as described by Holladay *et al*. [28]. Briefly, riboflavin (200 mg) was dissolved in pyridine (320 μL) with ice cooling and treated with thionyl chloride, heated at 65 °C for 16 h, and all the components were solubilized by the addition of 1 mL dimethylformamide. Seventy microliters of this reaction mixture was added to 10 mg protein (BSA, OVA or KLH) dissolved in 0.5 mL PBS; the pH was adjusted to 8.0 using 1 M NaOH and the solution was allowed to react at 25 °C for 3 h followed by centrifugation to remove particulates. Riboflavin-BSA (RF-BSA), riboflavin-OVA (RF-OVA) and riboflavin-KLH (RF-KLH) were separated from the corresponding starting materials using a desalting column (Sephadex G-25) equilibrated with PBS. The number of riboflavin molecules conjugated to each protein was determined spectrophotometrically from the difference in absorption of riboflavin-protein conjugate and unmodified protein at 445 nm using an extinction coefficient at this wavelength of $\varepsilon=4409$ [28].

Immunization and collection of antiserum

Three New Zealand white male rabbits (*Oryctolagus cuniculus*), 7–12 months-old kept in the animal house facility of CFTRI, Mysore was used for the immunization according to standard protocols [29], after obtaining approval from the institutional animal ethics committee (IAEC).

Initially, the immunogen, Rbt-BSA conjugate (~30 mol D-ribitol/mol BSA; 0.5 mL of 2 mg/mL conjugate) micro-emulsified with 0.5 mL of FCA was administered subcutaneously at 8–10 sites on the back of the animal. After 4 weeks, booster doses containing 0.5 mg of the immunogen micro-emulsified with FIA was administered intramuscularly at 15–day intervals. The animal was bled by marginal ear vein puncture 1 week before the first injection to obtain pre-immune serum and 1 week after each booster dose to obtain immune serum (antiserum). The pooled serum was stored in aliquots below −20 °C.

Dot-immunoblot and non-competitive ELISA (ncELISA) to measure antiserum titer

The antiserum titer was analyzed by dot-immunoblot [30] by spotting 1 μg of BSA, OVA and KLH along with their respective ribitol conjugates on nitrocellulose membrane. The blots were washed thrice extensively with Tris-buffered saline (TBS) containing 0.05 % Tween-20 (TBS-T) between each step. Blocking was carried out in TBS-T with 1 % gelatin (blocking buffer) for 2 h. The blots were incubated in Rbt-BSA antiserum diluted in blocking buffer (1:2,000, 1:10,000 and 1:20,000) for 2 h, followed by incubation with 1:5,000 dilution of goat anti-rabbit IgG-ALP conjugate for 1 h, and color development using NBT-BCIP solution.

The antiserum titer was determined by measuring the binding of serial dilutions of the antiserum to coated Rbt-KLH and

Rbt-OVA by ncELISA [31]. Microtiter wells were coated with D-ribitol conjugate at 1000, 100, 50, and 10 ng per well (100 μL volume) in 0.1 M carbonate-bicarbonate buffer, pH 9.6 by incubating at 4 °C overnight. The wells were washed thrice between steps using PBS containing 0.05 % Tween-20 (PBS-T). Blocking was done using 1 % gelatin in PBS-T (blocking buffer) at 37 °C for 30 min. Antiserum diluted in blocking buffer (1:10 to 1:100,000) was added (100 μL/well) and incubated at 37 °C for 2 h. Goat anti-rabbit IgG-ALP conjugate (1:5000 dilution in blocking buffer; 100 μL/well) was added and incubated at 37 °C for 1 h. Color development was done using pNPP (1 mg/mL; 100 μL/well) in 1 % diethanolamine buffer, pH 9.8, at 37 °C for 30 min. The reaction was stopped by the addition of 3 M NaOH (50 μL/well) and the absorbance was read at 405 nm in a microtiter plate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA).

A positive value in ELISA is defined as the absorbance value obtained with immune serum sample two-fold greater than that obtained in the case of pre-immune serum. Antibody titer is defined as the reciprocal of the highest dilution that gave a positive value in ELISA.

Preparation of D-Rbt–KLH–Sepharose CL-6B affinity matrix

Rbt-KLH conjugate in which ~50 % of amino groups carried the hapten was used for immobilization onto Sepharose CL-6B [32]. Sepharose CL-6B gel was first washed thoroughly with water to remove the preservative, and then suspended in water to obtain 0.2 g moist gel/mL (10 mL). The washed gel was then oxidized by adding solid sodium periodate ($NaIO_4$) to a final concentration of 25 mM. The mixture was swirled at 25 °C for 30 min; unreacted $NaIO_4$ was inactivated by the addition of an equimolar amount of ethylene glycol. After 15 min, the gel was thoroughly washed with water followed by 0.2 M phosphate buffer, pH 7.0. The moist gel (0.2 g/mL suspension) in phosphate buffer was mixed with D-Rbt-KLH conjugate (2 mg) followed by direct addition of borane-pyridine complex to a final concentration of 25 mM. The reaction mixture was mildly agitated at 25 °C for 12–15 h. Next, the gel beads were washed extensively with water to remove unreacted borane-pyridine and the conjugate. The gel was finally packed into a glass column (2 mL bed volume), and equilibrated with PBS and stored at 4 °C in the same buffer with 0.01 % sodium merthiolate as a preservative.

Caprylic acid precipitation of rabbit anti-Rbt-BSA serum and hapten-affinity chromatography for purification of ribitol-specific antibodies

Caprylic acid precipitation is generally carried out for the complete removal of albumin from any serum [29, 33]. Rbt-BSA antiserum was adjusted to pH 4.6 with 0.06 M sodium acetate buffer, pH 4. Caprylic acid was added slowly drop by drop (82 μL per mL of antiserum) for 30 min with constant stirring; next, the solution was filtered and centrifuged at 6,800×*g* at 4 °C for 1 h. Supernatant was collected and dialyzed against cold PBS and stored at 4 °C.

Rbt-BSA antiserum or the supernatant from caprylic acid–precipitated antiserum was diluted 1:2 in PBS, and loaded on Rbt-KLH-Sepharose CL-6B column (1.2 cm i.d. × 2.5 cm; 2 mL bed volume) that had been pre-equilibrated with PBS at 4 °C; the flow-through was recycled twice. After washing with 50 mL PBS, the bound antibodies were eluted with 0.2 M glycine-HCl buffer, pH 2.9; the eluate was neutralized immediately using 1 M Tris base. The purified antibody was concentrated using 100 kDa MWCO centricon (Amicon, Beverly, MA), aliquotted and stored below −20 °C. The affinity-purified antibodies were analyzed by SDS-PAGE under both reducing and non-reducing conditions. The concentration of ribitol-specific antibody was calculated based on the reference value of $A^{0.1\%,\ 1\,cm}$ at 280 nm of 1.4 for rabbit IgG [29].

In-gel trypsin digestion and Peptide Mass Fingerprint (PMF) analysis

This was carried out as described by Shevchenko *et al.* [34]. Coomassie-stained band of 66 kDa contaminant protein or RSA was cut into small cubes, destained and digested with TPCK-treated trypsin. PMF analysis was carried out with the extracted peptides to obtain peptide mass values using Ultraflex MALDI-TOF (Brucker Daltonics, Billerica, MA). Database search was carried out using Mascot search engine (http://www.matrixscience.com) against NCBInr sequence database.

Measurement of antibody titer and affinity constant of ribitol-specific antibodies

Antibody titer of affinity-purified ribitol antibodies was obtained by two-dimensional checkerboard titrations, in which different amounts of the purified antibody (100, 50, 25, 10, 5, 2 and 1 ng) were titrated against varying amounts of the coating antigen (Rbt-KLH conjugate) in ncELISA as described in an earlier section.

The affinity constant of the purified anti-ribitol antibodies from ncELISA was calculated using a computer program (Ab_affi program written in GW-BASIC) available from the internet (http://www.imtech.res.in/raghava/progs/abaffi/README.html) as described by Raghava and Agrewala [35], which is a simple and reliable method for calculating the affinity constant based upon law of mass action. Rbt-KLH conjugate was coated using a series of two–fold dilutions (1,000, 500, 250 and 125 ng/100 μL) so that the final concentration was one-eighth of the starting concentration. KLH (1,000 ng/100 μL) as control coating antigen lacking the hapten, and 10 ng/100 μL affinity-purified ribitol antibodies were used in ncELISA as described in an earlier section.

Specificity and cross-reactivity of ribitol-specific antibodies

Specificity and cross-reactivity of the affinity-purified antibodies were studied by indirect competitive ELISA (icELISA). Here, purified antibodies (50 ng/well) were pre-incubated with various concentrations (0.1 nM to 1 mM) of test compounds (competitive inhibitors like ethylene glycol, glycerol and various sugar alcohols, monosaccharides) at 37 °C for 2 h before adding to coated microtiter wells (Rbt-KLH, 100 ng/well). All other steps were followed as described in an earlier section. IC_{50} value of each compound was calculated using 4-parameter logistic nonlinear regression curve fit mode in the GraphPad Prism 5. IC_{50} value is defined as the concentration required for 50 % inhibition in ELISA. The percent cross-reactivity for various inhibitors was calculated using the formula (IC_{50} for ribitol ÷ IC_{50} for test compound) × 100. Other compounds tested for cross-reactivity include riboflavin, FMN, FAD, lumichrome, and L-lysine.

Similarly, specificity of the affinity-purified antibodies to riboflavin-protein conjugates was carried out by coating microtiter wells with 100 ng/well ribitol-protein conjugates (Rbt-BSA, Rbt-OVA or Rbt-KLH) and riboflavin-protein conjugates (RF-BSA, RF-OVA or RF-KLH) along with the respective unconjugated proteins (BSA, OVA and KLH) as negative controls. Ten ng/well purified ribitol-specific antibodies were used in ncELISA as described in an earlier section.

Detection of PRP of *Haemophilus influenzae* type b by ribitol-specific antibodies

Microtiter wells were coated with 100 ng/well of one of the following: KLH, OVA, Rbt-KLH and Rbt-OVA, while Hiberix™, HibPro™, Penatvac SD® and Tetvac were coated at 1,000 ng/well concentration. ncELISA was carried out, as described in an earlier section, using 10 ng/well of purified ribitol-specific antibodies for the detection of PRP of *Haemophilus influenzae* type b.

icELISA was carried out to determine the percent inhibition of ribitol-specific antibodies (10 ng/well) with PRP of *Haemophilus influenzae* type b by ribitol. Antibodies were pre-incubated with various concentrations of ribitol (1 nM, 1 μM and 1 mM) at 37 °C for 2 h before adding the mixture to coated microtiter wells containing one of the following: 100 ng/well of Rbt-KLH, 1,000 ng/well each of Hiberix™, HibPro™, Pentavac SD® and Tetvac.

Statistical analyses

Statistical treatment of ELISA data was carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 5.

Results

Characterization of ribitol-protein and riboflavin-protein conjugates

D-Ribose was conjugated to BSA as well as OVA by reductive amination to obtain ribitol epitopes on the respective proteins as outlined earlier in Fig. S1. The degree of substitution as determined by the decrease in the number of amino groups on the carrier protein (as quantified by TNBS assay) is shown in Fig. 2a. The time course of the reaction indicated that the reaction was faster initially up to 72 h, and then slowly reached a plateau after 120 h. After 120 h of reductive amination, ~30 and ~15 mol of ribitol has been conjugated per mole of BSA and OVA, respectively.

SDS–PAGE analysis (reducing) of Rbt–BSA conjugates clearly showed an increase in apparent molecular weight as compared to control BSA (Fig. 2c). Increase in relative molecular weight (M_r) of 120 h Rbt–BSA conjugate calculated from the R_f values was found to be ~4 kDa; this translates to ~30 mol of ribitol conjugated per mole BSA (data not shown). MALDI–TOF analysis of 120 h Rbt–BSA conjugate showed a molecular mass of 69,927 Da which translates to 26 mol of ribitol per mole BSA (Fig. 2b). This is in good agreement with the values obtained by TNBS assay and SDS–PAGE.

Since BSA is not a glycoprotein, the glycation of BSA as a result of reductive amination with D-ribose was confirmed by PAS staining. The PAS staining of Rbt–BSA conjugates as indicated by pink bands (Fig. 2d) confirmed the addition of ribitol groups on the carrier protein, BSA.

Riboflavin was conjugated to proteins as described under 'Materials and methods', and the number of riboflavin molecules conjugated was determined by an increase in absorbance at 445 nm relative to unconjugated proteins. All the three conjugates appeared as orange-yellow in color indicating the presence of covalently bound riboflavin. In the case of RF-BSA and RF-OVA conjugates, approximately 7±1 and 3± 1 mol of riboflavin were found to be conjugated per mole protein, respectively. However, the number of riboflavin conjugated to KLH could not be determined due to the uncertainty in the exact molecular mass of KLH [22].

Immunochemical analyses of ribitol antiserum

Rabbit antiserum was analyzed for the specific antibody response by dot–immunoblot analysis (Fig. 3a). The immunoreactivity of antiserum was found to be positive for Rbt-BSA, Rbt-KLH, Rbt-OVA and BSA, but was negative for both OVA and KLH. The highest dilution of antiserum with which Rbt-KLH can easily be detected was found to be 1:20000. Pre-immune serum at 1:2000 dilution showed no reactivity towards all the conjugates, indicating the presence of specific antibodies to ribitol in the antiserum.

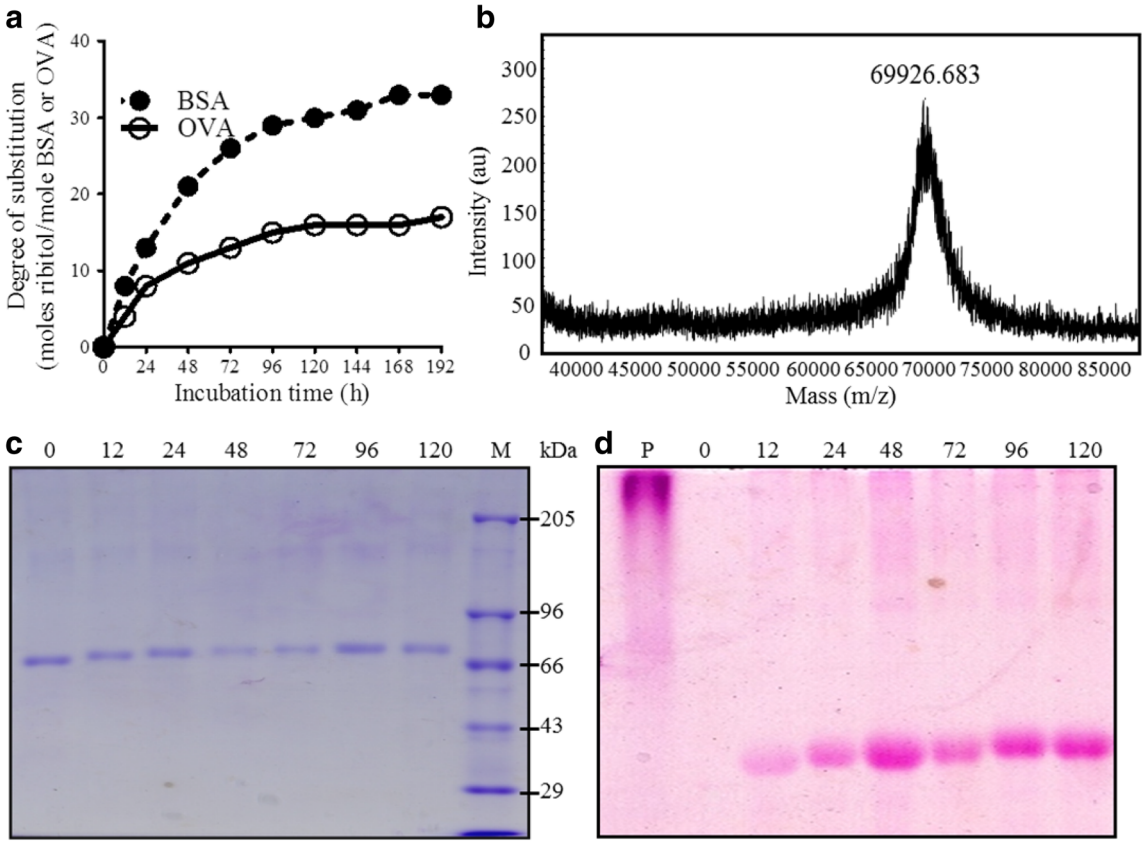

Fig. 2 Preparation and characterization of ribitol-protein conjugates. **a** Time course (TNBS assay) for the formation of Rbt-BSA or Rbt-OVA conjugates by reductive amination of BSA or OVA, respectively, with D-ribose. **b** MALDI-TOF-MS of Rbt-BSA obtained by reductive amination at 120 h. **c** Coomassie-stained SDS-PAGE (10 %, reducing) of Rbt-BSA conjugates; lane M: mol. wt. markers. The *numbers* at the top represent incubation times (in h) during the reductive amination reaction. **d** PAS-stained SDS-PAGE (10 %, reducing) of Rbt-BSA conjugates; lane P: positive control (yeast invertase). Other details are as given in panel **c**

The antiserum was also analyzed for its sensitivity in detecting both Rbt–KLH and Rbt–OVA conjugates using a checkerboard analysis by ncELISA (Fig. 3b), where the concentrations of both antigen and antiserum are varied. The antiserum titration using 100 ng/well of coating antigen showed the presence of ribitol–specific antibodies sensitive at 1:1000 and 1:10000 dilutions for Rbt–KLH and Rbt–OVA conjugate, respectively.

Affinity purification of ribitol–specific antibodies

Purification of antibodies specific to ribitol from rabbit Rbt-BSA antiserum was carried out by hapten affinity chromatography on Rbt-KLH-Sepharose-CL-6B; the elution profile showed a single peak with a yield of ~80–85 μg protein/mL of rabbit antiserum. The affinity–purified preparation showed an IgG band around 160 kDa and a 66 kDa protein band was observed as a contaminant (marked X) on SDS-PAGE under non-reducing conditions (Fig. 4a).

Alternatively, caprylic acid precipitation of rabbit Rbt-BSA antiserum followed by hapten affinity chromatography on Rbt-KLH-Sepharose-CL-6B resulted in homogeneous specific antibodies to ribitol with a yield of 45–50 μg/mL of antiserum. The affinity–purified antibody preparation showed a single band around 160 kDa on non-reducing SDS–PAGE and the absence of the 66 kDa contaminant (Fig. 4b).

Further, the 66 kDa contaminant protein was subjected to in–gel trypsin digestion followed by PMF analysis (supplementary Fig. S2a) which was found to be identical to the results obtained by using pure RSA as a positive control (supplementary Fig. S2b). Thus, PMF analysis confirmed that the contaminant 66 kDa protein is RSA (gi 372467098; PDB entry: 3V09_A).

Characterization of ribitol-specific antibodies

The affinity constant of ribitol–specific antibodies was calculated using ncELISA by the method of Raghava and Agrewala [35] and found to be at 2.9×10^{7} M^{-1}. Binding curve obtained with checkerboard analysis using purified ribitol-specific antibodies and Rbt–KLH conjugate indicated that the reactivity of antibodies (at 10 ng/100 μL) for coating

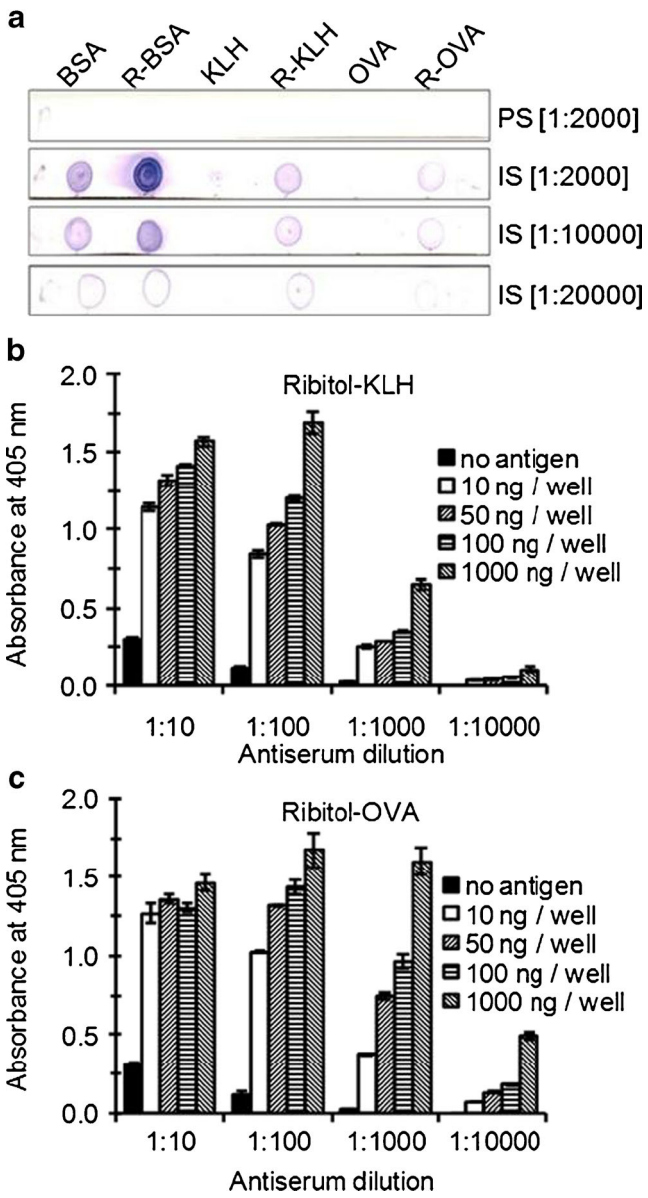

Fig. 3 Immunochemical analyses of rabbit Rbt–BSA antiserum. **a** Dot-blot on nitrocellulose membrane. Antigen amount: 1 μg; PS: pre-immune serum; IS: immune serum; secondary antibody: goat anti-rabbit IgG-ALP (1:5000 dilution). **b** and **c** Checkerboard analysis by ncELISA at different amounts of antigen (shown as *inset*) and antiserum dilutions. Coating antigen: Rbt-KLH (**b**) and Rbt-OVA (**c**). Secondary antibody: as in panel **a**. Absorbance values are means of triplicates

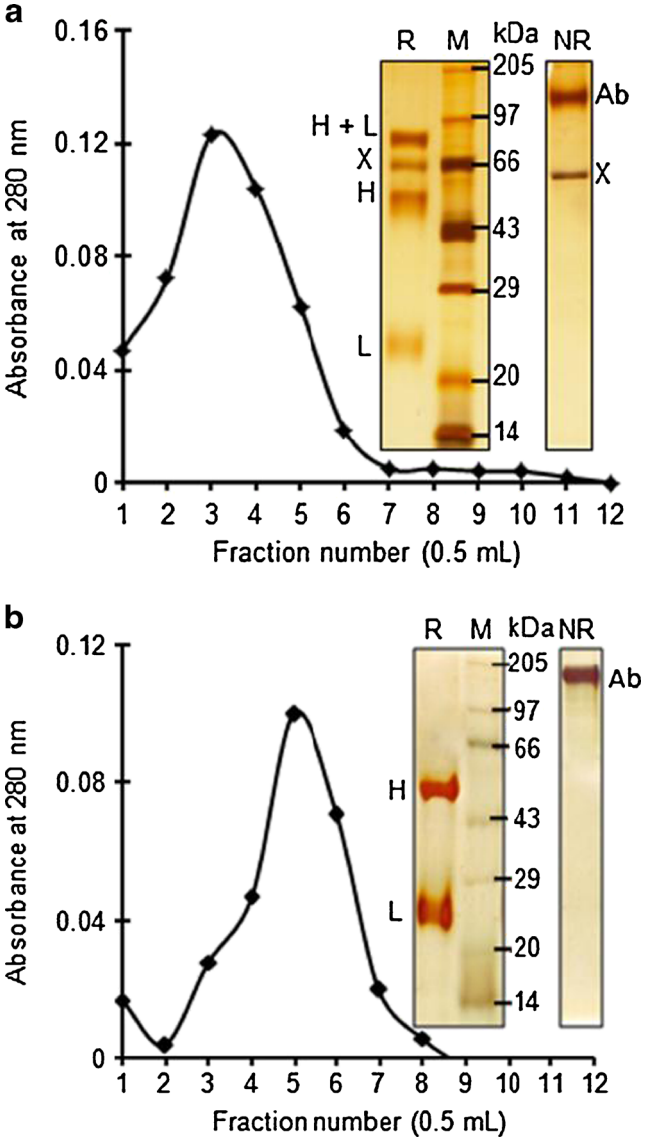

Fig. 4 Affinity purification of ribitol–specific antibodies. **a** Hapten affinity chromatography of rabbit Rbt-BSA antiserum on Rbt–KLH–Sepharose CL-6B. Sample volume: 4 mL antiserum; elution buffer: 0.2 M glycine–HCl buffer, pH 2.9; fraction volume: 0.5 mL. Inset: 10 % SDS-PAGE, silver staining of affinity-purified ribitol-specific antibodies with 66 kDa contaminant (band X). *Lane R*: reducing condition; *lane NR*: non-reducing condition; *lane M*: mol. wt. markers under reducing condition. Bands *marked L and H* represent light and heavy chains of the antibody, Ab. **b** Caprylic acid precipitation of rabbit Rbt-BSA antiserum followed by hapten-affinity chromatography on Rbt-KLH-Sepharose-CL-6B. Inset: 10 % SDS-PAGE, silver staining of affinity-purified ribitol-specific antibodies

antigen (Rbt–KLH) level of 50 ng/well and above is detectable. In order to obtain an optimal detection in icELISA for cross–reactivity studies, 50 ng of purified antibodies and 100 ng/well of coating antigen were considered for further analysis (Fig. 5a).

In order to demonstrate the specificity and cross–reactivity of purified ribitol antibodies, icELISA (Fig. 5b) was performed with various sugars (D-ribose, D-xylose, L-arabinose), sugar alcohols (D-ribitol, D-xylitol, *meso*-erythritol, D-mannitol, sorbitol, L-arabinitol, D-galactitol), glycerol, ethylene glycol, L-lysine, riboflavin and its derivatives (FMN, FAD and lumichrome).

The percent cross–reactivity of ribitol-specific antibody towards these compounds is summarized in Table 1. The specificity of ribitol antibodies towards ribitol is taken as 100 % for the calculation of percent cross–reactivity. Ribitol–specific antibodies showed 5–15 % cross-reactivity with arabinitol, *meso*-erythritol, sorbitol, xylitol and mannitol,

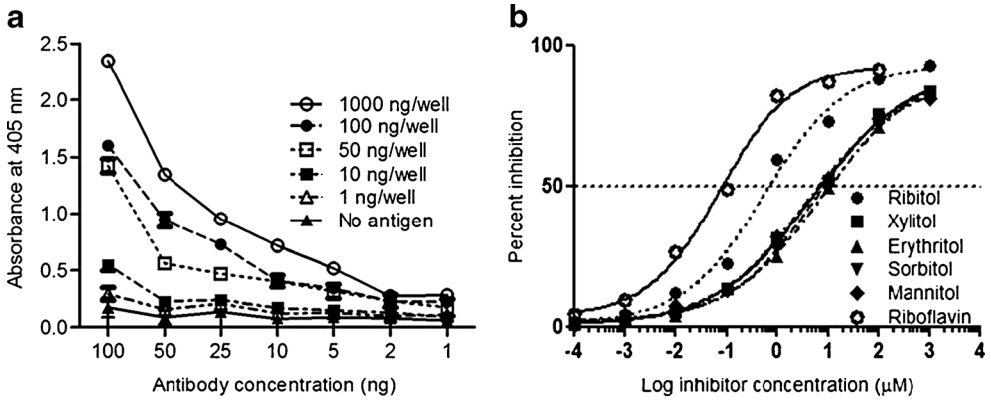

Fig. 5 Characterization of affinity–purified ribitol–specific antibodies. **a** Binding curves obtained by checkerboard analysis with ribitol-specific antibodies by ncELISA at different concentrations of coating antigen (shown as *inset*) and antiserum dilutions. Coating antigen: Rbt-KLH; secondary antibody: goat anti-rabbit IgG-ALP (1:5000 dilution). Absorbance values are means of triplicates. **b** Specificity of ribitol–specific antibodies as determined by icELISA. Coating antigen: Rbt-KLH, 100 ng/well. Purified antibodies specific to ribitol (50 ng in 100 μL volume) were pre-incubated with various concentrations of inhibitor (as shown in *inset*) at 37 °C for 1 h, then transferred to ELISA wells. Other details are as given in panel **a**

~2 % cross-reactivity with FMN and D-galactitol, and <1 % for all other compounds tested (D-ribose, D-xylose, Rib-5-*P*, FAD and lumichrome). Surprisingly, riboflavin containing ribityl group as an integral part of its structure showed ~800 % cross-reactivity.

Ribitol-specific antibodies were tested for their ability to bind RF-protein conjugates along with the respective Rbt-protein conjugates by ncELISA. The results are shown in Fig. 6. It is evident from the data that the affinity-purified antibodies specifically recognize all the three protein conjugates prepared by reductive amination, and does not show binding of the RF-protein conjugates as well as the unconjugated proteins.

Table 1 Specificity and cross-reactivity of affinity-purified ribitol-specific antibodies

Test compound	IC_{50} (μM)	Percent cross-reactivity
D-Ribitol	0.57	100.00
D-Mannitol	3.80	14.87
D-Xylitol	4.96	11.40
D-Sorbitol	5.65	10.02
meso-Erythritol	8.10	6.99
L-Arabinitol	11.50	4.92
D-Galactitol	29.60	1.91
D-Threitol	48.90	1.16
Glycerol	576.00	0.10
Ethylene glycol	891.00	0.06
D-Ribose	124.00	0.46
D-Xylose	172.00	0.33
D-Ribose-5-*P*	192.00	0.29
D-Arabinose	196.00	0.29
L-Lysine	8.98[a]	0.01
Riboflavin	0.07	774.05
FMN	26.30	2.15
FAD	71.20	0.79
Lumichrome	165.00	0.34

[a] This value is represented in mM

Detection of PRP of *Haemophilus influenzae* type b by ribitol-specific antibody

The binding of ribitol-specific antibodies to three commercial sources of *H. influenzae* type b vaccine containing PRP conjugated to tetanus toxoid was evaluated by ncELISA, and the results are shown in Fig. 7. It is seen that Hiberix™, Sii HibPro™ and Pentavac SD® are recognized by ribitol-specific antibodies compared to tetanus toxoid which served

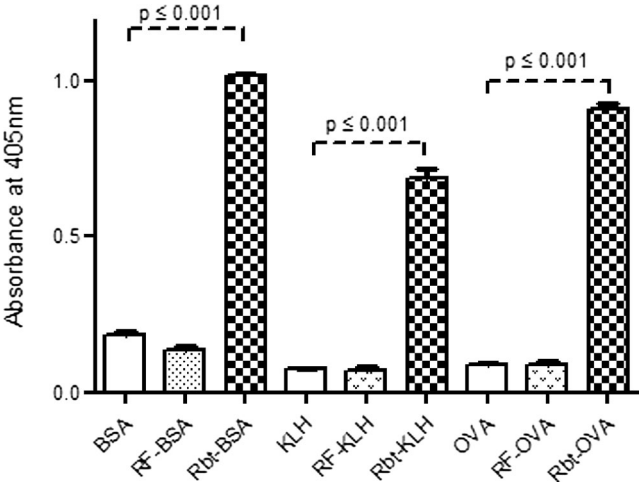

Fig. 6 Evaluation of binding of ribitol–specific antibodies to riboflavin-protein conjugates using ncELISA. Coating antigens:100 ng/well riboflavin-protein conjugate (RF-BSA, RF-OVA or RF-KLH) along with 100 ng/well ribitol-protein conjugate (Rbt-BSA, Rbt-OVA or Rbt-KLH) as positive controls and 100 ng/well unconjugated proteins (BSA, OVA or KLH) as negative controls. Primary antibody: 10 ng/well affinity-purified ribitol antibodies; secondary antibody: goat anti-rabbit IgG-ALP (1:5000 dilution). Absorbance values are means of triplicates

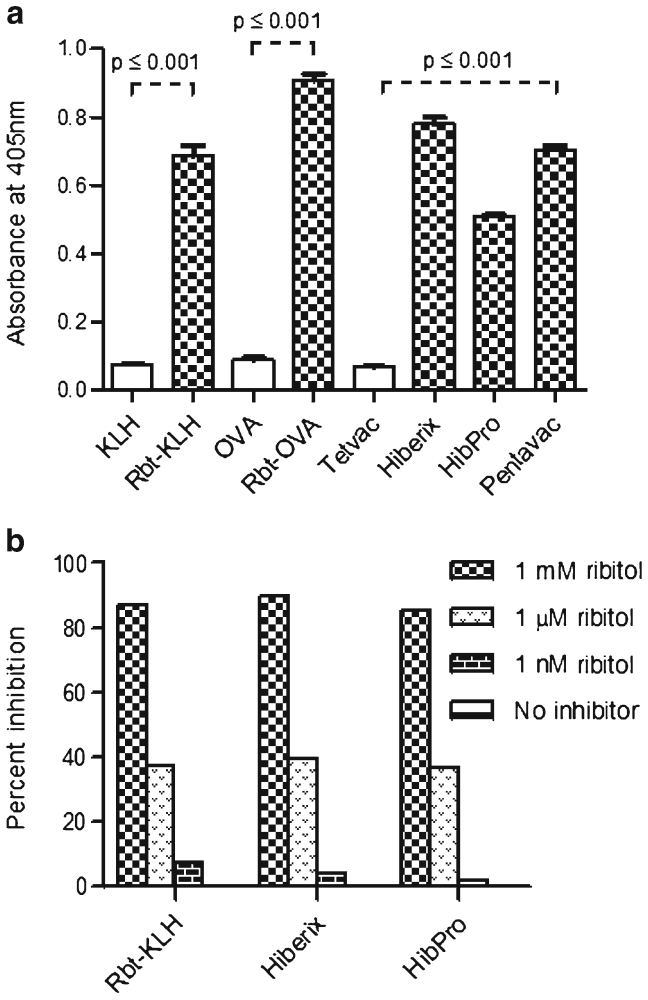

Fig. 7 Detection of PRP conjugate of *Haemophilus influenzae* type b by ribitol-specific antibodies. **a** ncELISA using three different commercial vaccine preparations of PRP conjugate. Coating antigens: Rbt-KLH, Rbt-OVA, KLH, OVA (each at 100 ng/well) and test samples – Hiberix™, Sii HibPro™, Pentavac SD® and Tetanus toxoid (Tetvac) (each at 1 μg/well). **b** icELISA for determination of the percent inhibition of ribitol-specific antibodies towards PRP conjugate. Coating antigen: 100 ng/well of Rbt-KLH and 1 μg/well of Hiberix™, Sii HibPro™. Purified ribitol-specific antibodies (10 ng in 100 μL) were pre-incubated with various concentrations (1 nM, 1 μM and 1 mM) of D-ribitol at 37 °C for 2 h, and then transferred to ELISA wells. The percent inhibition for the sample labeled 'no inhibitor' is zero. Other details are as given in legend to Fig. 6

as a negative control; the binding of these vaccine preparations was comparable to that of ribitol-conjugates of KLH and OVA, which served as positive controls (Fig. 7a). Further, the binding of ribitol-specific antibodies to Hiberix™ and Sii HibPro™ could be inhibited by D-ribitol in a dose-dependent manner (Fig. 7b).

Discussion

Antibodies to saccharide haptens have been generated by coupling to carrier proteins using various chemistries [14]. In the present study, the direct coupling of a reducing sugar (D-ribose) to proteins by reductive amination was used to obtain ribitol epitopes on carrier proteins. After 120 h of reductive amination, ~30 and ~15 mol of ribitol have been conjugated per mole of BSA and OVA, respectively; the degree of substitution was also confirmed by an increase in apparent molecular weight by SDS–PAGE and MALDI–TOF analyses as well PAS staining for the glycated nature of conjugates. Rbt–BSA conjugate used as an immunogen in rabbits produced an excellent immune response with marked specificity and sensitivity to D-ribitol as demonstrated with dot immunoblot and checkerboard ncELISA.

In order to obtain antibodies specific to ribitol, an affinity matrix was prepared by conjugating Rbt-KLH conjugate to Sepharose CL-6B that had been subjected to periodate oxidation; KLH was used as the matrix protein as it is a non–homologous protein compared to BSA, and belongs to a different phylum. The hapten affinity purification of Rbt-BSA antiserum showed the presence of an antibody band at 160 kDa and a 66 kDa contaminant protein by SDS–PAGE (non-reducing) analysis; the contaminant protein was confirmed to be RSA by in-gel trypsin digestion and PMF analysis using authentic RSA for comparison. Alternatively, caprylic acid precipitation of Rbt–BSA antiserum followed by hapten–affinity chromatography yielded homogeneous specific antibodies to ribitol (45–50 μg/mL of rabbit antiserum) devoid of the 66 kDa contaminant. Since serum albumin has been clearly shown to bind riboflavin in several studies [36, 37], it can be postulated that one arm of ribitol antibody binds to the hapten on the affinity matrix and the other arm binds the exposed ribitol portion of RF–RSA complex during hapten affinity chromatography of rabbit antiserum not subjected to caprylic acid precipitation. Alternatively, RSA may have binding sites for ribitol though this has not been shown experimentally.

The affinity constant of ribitol-specific antibodies, 2.9×10^{7} M^{-1}, is comparable to those of xylitol-specific antibodies (3.86×10^{9} M^{-1}) and erythritol-specific antibodies (4.86×10^{8} M^{-1}) [38, 39], although one to two orders of magnitude lower compared to the antibodies specific to these sugar alcohols. Generally, a large variation in the affinities for different antibodies elicited to the same antigen exists and differences of a factor of 10^{3} to 10^{5} are frequently found between the lowest and the highest value of K_a for the same hapten as reported in the literature [40].

The cross-reactivity values carried out by icELISA using purified ribitol antibodies was calculated considering 100 % specificity for ribitol. Antibodies specific to D-xylitol, *meso*-erythritol, and D-mannitol showed cross-reactivity of 11.4, 1.2, and 1.3 %, respectively towards D-ribitol indicating greater similarities between the pentitols [38, 39, 41]. L-Lysine showed very low cross-reactivity (0.01 %); although the ε-amino group of L-lysyl residues was part of the Rbt-

BSA conjugate, the ribitol antibodies do not recognize the lysyl residue as an epitope, and hence it can be inferred that the ribityl group represents a single epitope.

Riboflavin which contains ribityl moiety as an integral part of its structure showed higher cross–reactivity (~800 %) than the hapten used for immunization; this may be due to the presence of hydrophobic residues in the proximity of the binding sites of ribitol-specific antibody interacting with the isoalloxazone ring [42, 43]. This situation may be analogous to the case of riboflavin–IgG^{GAR} complex where the isoalloxazine ring is trapped in a narrow slot in the combining site, and the ribityl side chain rests on the outer surface of the combining site [44]. The coenzymes FMN and FAD as well as lumichrome (a riboflavin analog lacking the ribityl moiety) showed <2 % cross–reactivity indicating that ribitol antibodies are specific to the ribityl portion of riboflavin. It may be noted that normal human IgGs contain one or more species that bind riboflavin and FAD with very high affinity [42].

Riboflavin has been conjugated to proteins through the hydroxyl groups of the ribityl moiety of riboflavin to BSA [28]; such protein conjugates are not recognized by our antibody which is specific for the ribityl moiety. Ribitol-specific antibodies may likely bind 3-carboxymethyl riboflavin-protein conjugate wherein the ribityl moiety is not derivatized [45].

Though protein–bound FMN and FAD are absent in rat hepatocyte plasma membranes, protein–bound riboflavin has been identified as a source of autofluorescence [46]. Antibodies to riboflavin have been prepared in rats using RF–BSA conjugate as the immunogen, and such antibodies have been utilized for investigating the anatomical distribution of conjugated riboflavin in monkey brain [47, 48]. Riboflavin bound to plasma membrane proteins of several higher plants have been studied, and the probable biological significance of such complexes appears to be related to photoreceptor function and antioxidant effects of riboflavin [49, 50]. Though majority of bound flavins (FMN and FAD) appear to be specifically associated as coenzymes with certain proteins, bound riboflavin has also been detected in a few protein complexes [51]; their biological significance are still unknown. It would be interesting to investigate whether ribitol–specific antibodies can detect such protein–bound riboflavin.

Antibodies to ribitol specifically bind to the PRP conjugate *in vitro* as tested by ELISA. This was confirmed using PRP conjugates obtained from different manufacturers including one commercial vaccine wherein PRP conjugate is present as a component of the combined vaccine. Binding of PRP conjugate to ribitol-specific antibodies was found to be inhibited by D-ribitol in a dose-dependent manner. This indicates that ribitol–specific antibodies could be potentially useful for the immunological detection of *Haemophilus influenzae* type b vaccines either alone or in combination with other vaccines, and cell-surface macromolecules containing ribitol.

In conclusion, Rbt-BSA conjugates carrying ribityl epitopes were prepared using reductive amination to obtain ribitol-specific antibodies in rabbits. Ribitol-specific antibodies have been purified from Rbt-BSA antiserum on a hapten-affinity column (Rbt–KLH–Sepharose CL-6B) and characterized in terms of specificity and cross–reactivity. Antibodies showed excellent specificity towards ribitol and also higher cross–reactivity towards riboflavin indicating that the ribityl group of riboflavin is identified as a unique haptenic epitope. Ribitol–specific antibodies appear to have potential in the development of ELISA and immunohistochemistry for the identification and quantification of D-ribitol, free and bound riboflavin in biological samples. Additionally, they can be exploited for the detection of cell-surface complex molecules containing ribitol such as PRP.

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Conflict of interest The authors have declared that they do not have any conflict of interest.

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