

Reductively Aminated D-Xylose–Albumin Conjugate as the Immunogen for Generation of IgG and IgE Antibodies Specific to D-Xylitol, a Haptenic Allergen

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Sugar alcohols are widely used as food additives and drug excipients. Xylitol, a five-carbon sugar alcohol, and a low-calorie alternative sweetener to sucrose (approx 40% fewer calories), has enjoyed an enviable record of safety, and allergic reactions to xylitol are very rare. A case of oral erosive eczema to xylitol has been reported recently [Hanakawa, Y., Hanakawa, Y., Tohyama, M., Yamasaki, K., Hashimoto, K. (2005) Xylitol as a causative agent of oral erosive eczema. *Brit. J. Dermatol.* 152, 821–822]. Xylitol does not contain any reactive groups; hence, it is nonimmunogenic. In order to explain the immunogenicity of xylitol, polyclonal antibodies to xylitol have been raised using the reductive aminated product of D-xylose conjugated to bovine serum albumin (BSA) as the immunogen. Rabbits immunized with xylitol–BSA conjugate (52 haptens/molecule) gave a good antibody response. Purification of anti-xylitol antibodies was carried out using hapten-affinity chromatography on xylitol–keyhole limpet hemocyanin–Sepharose CL-6B; the yield was $\sim 40 \mu\text{g/mL}$ of rabbit immune serum. Purified xylitol-specific antibodies appeared to be homogeneous by native PAGE with a pI of ~ 7.2 by isoelectric focusing. Although the purified antibodies are specific for the xylitol moiety of xylitol–protein conjugates, they reacted equally well with the Schiff base conjugate of xylosyl–protein conjugates (68% cross-reactivity) indicating that carbons 2 to 5 of xylitol act as an epitope. Xylitol antibodies showed excellent specificity towards xylitol and $<4.4\%$ cross-reactivity with D-xylose and various sugar alcohols except ribitol and galactitol, which showed $\sim 11\%$ and 8% cross-reactivity, respectively. D-Xylitol–BSA conjugate was used to raise IgE antibodies in BALB/c mice by repeated intradermal administration. Passive cutaneous anaphylaxis using the immune sera confirmed the haptenic nature of xylitol.

INTRODUCTION

Sugar alcohols (also known as polyhydric alcohols or polyols) are low-calorie bulk sweeteners. They are being used as food additives and drug excipients, as they are chemically inert, nontoxic, nonhygroscopic, and sweet in taste. Sugar alcohols commonly used as food additives are sorbitol (international numbering system: INS¹ 420), mannitol (INS 421), xylitol (INS 967), and erythritol (INS 968); maltitol (INS 965) and lactitol are used to a lesser extent. Xylitol, a pentitol, has been widely used in foods since its approval by the U.S. Food and Drug Administration (FDA) in 1960, because of its slow absorption, partial utilization, and reduced calories (2.4 calories/g) (1). Besides its uses as food additive and sweetener, xylitol has many medical uses such as prevention of dental caries (2), middle

ear infections (3), and experimental osteoporosis (4). Xylitol occurs naturally in many fruits and vegetables at concentrations as high as 935 mg (yellow plum) and as low as 14 mg (chestnut) per 100 g dry substance (5). Xylitol is a popular sweetener for the diabetic diet in some countries, as it is metabolized independently of insulin and is extremely safe. Xylitol is used in unlimited quantity for foods with special dietary purposes. Sugar-free chewing gums and candies made with xylitol as the principal sweetener have received official endorsements from six national dental associations.

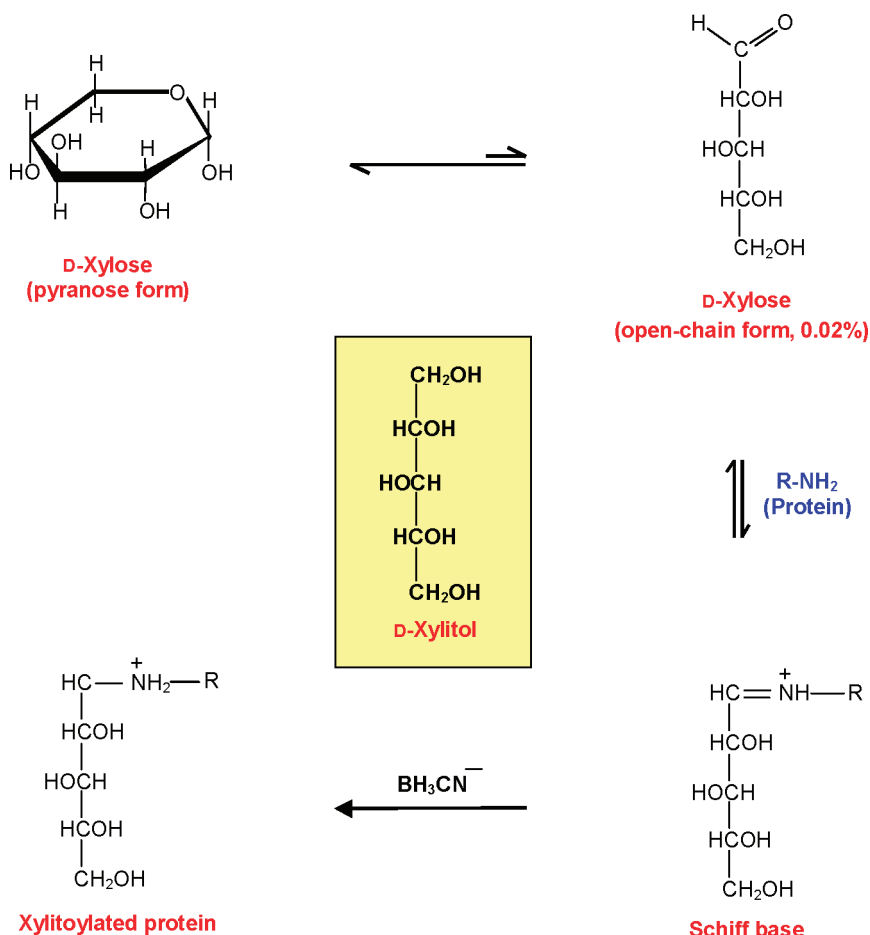
Mannitol, a hexitol, occurs naturally in many plant foods including mushrooms (6). The use of mannitol in foods is broadly permitted by the food additive regulations of FDA. In addition, intravenous infusion of 20% (w/v) mannitol (equivalent to 1100 mM) has been used as a therapeutic agent in many clinical situations due to its osmotically active property. Hypersensitivity reactions to 20% mannitol infusion have been reported in a small number of susceptible patients. Recently, mannitol was identified as an allergen for a sensitized subject (7, 8), and mannitol-specific IgE was demonstrated for the first time in the serum of the subject sensitized to mannitol (9). Allergic reactions caused by erythritol as a sweetener in processed foods have been reported in three cases (10, 11). On the basis of the inert nature of mannitol and erythritol, a hypothesis has been proposed to explain the mechanism of sensitization and hypersensitivity to these sugar alcohols (12). In this hypothesis, the sugar acts as a *prosensitizer*, its Schiff base adduct with proteins acts as a *sensitizer*, and the sugar alcohol acts as a *nonsensitizing elicitor*.

Allergic reactions to xylitol are also rare, similar to the case of mannitol and erythritol. However, one case of oral erosive

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¹Abbreviations used: INS, international numbering system for food additives; FDA, food and drug administration; IgG, immunoglobulin G; IgE, immunoglobulin E; PCA, passive cutaneous anaphylaxis; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; TNBS, trinitrobenzenesulfonic acid; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; NaIO₄, sodium periodate; *M_r*, relative molecular mass; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; pI, isoelectric point; Xyt-BSA, D-xylitol–BSA conjugate; Xyt-KLH, D-xylitol–KLH conjugate; GRAS, generally recognized as safe; HSA, human serum albumin; OVA, ovalbumin.

Scheme 1. Reaction of D-xylose with carrier protein by reductive amination to obtain xylitoyl epitopes. The structure of D-xylitol is shown in the box (carbons C1 to C5 should be viewed from top to bottom. The percentage of open-chain form of D-xylose is based on the circular dichroism study (22).



eczema to foods containing xylitol has been reported recently (13). Although the skin patch test was positive, the presence of xylitol-specific IgE has not been demonstrated in that study. Therefore, it is likely that the mechanism of formation of IgE antibodies to xylitol (Scheme 1) could follow the general scheme described for mannitol and other sugar alcohols (12).

Anticarbohydrate antibodies with specificity towards various monosaccharides, disaccharides, and oligosaccharides have been studied extensively (14–19). These are generally specific to cyclic pyranose or furanose structures of sugars. Only certain disaccharides have been conjugated to proteins by reductive amination with the aim of obtaining antibodies specific for a terminal nonreducing monosaccharide (cyclic) determinant (14–16); however, the acyclic monosaccharide arm formed by the reducing sugar unit was also involved in the antigenic determinant. Unlike saccharides, sugar alcohols possess *acyclic* straight chain structure; they are inert and do not have any reactive aldehyde or ketone groups. Hence, they cannot form conjugates with proteins to mount an immune response. This necessitates an indirect method to obtain sugar alcohol groups on a carrier protein for immunogenicity. Such a strategy was applied for the generation of polyclonal antibodies to erythritol and D-mannitol (20, 21). With this background, it appeared interesting to demonstrate the formation of IgG antibodies to xylitol in rabbits using a xylitol–protein conjugate as the immunogen and characterize the affinity-purified antibodies in terms of specificity and cross-reactivity. The allergenic nature of xylitol was investigated by repeated intradermal administration of xylitol–BSA conjugate in BALB/c mice followed by

homologous passive cutaneous anaphylaxis (PCA) assay for the demonstration of xylitol-specific IgE.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. D-Xylose, Sepharose CL-6B (6% beaded agarose, wet bead diameter: 40–165 μ m), bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), sodium cyanoborohydride, periodic acid, and borane–pyridine complex solution (C₅H₅N.BH₃; BH₃ concentration ~8M; excess pyridine ~20%) were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO. Trinitrobenzenesulfonic (TNBS) acid was a product of HiMedia Laboratories, Mumbai, India. Schiff reagent (1-fluoro-2,4-dinitrobenzene) was procured from Sisco Research Laboratories, Mumbai, India. Maxisorp ELISA microtiter plates (flat-bottom) were a product of Nunc A/S, Roskilde, Denmark. Goat antirabbit IgG–alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT), Freund's complete adjuvant (FCA), and Freund's incomplete adjuvant (FIA) were products of Bangalore Genei, Bangalore, India. Ampholines (pH 5–8) were procured from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

Animals. New Zealand white male rabbits (7 months old) and Swiss albino mice (6–8 weeks old) were obtained from the animal house facility of our institute. BALB/c mice aged 6–8 weeks were procured from Central Animal Facility (CAF), Indian Institute of Science, Bangalore, India. Each experimental group consisted of 6 mice.

Preparation of Hapten-Protein Conjugates Containing Xylitol Epitopes. D-Xylose was conjugated to a carrier protein, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH), by reductive amination (also termed as reductive alkylation) in the presence of a mild reducing agent, sodium cyanoborohydride, as described earlier for *meso*-erythritol and D-mannitol conjugates (20, 21) to obtain stable xylitol epitopes on the conjugated carrier protein.

In a separate experiment, D-xylose (100 mg) was incubated with BSA (68 mg) in the absence of sodium cyanoborohydride to obtain a Schiff base conjugate of D-xylosyl-BSA. The degree of substitution was determined by the decrease in amino groups as quantified by trinitrobenzenesulfonic acid (TNBS) assay. Protein estimation of conjugates was carried out as per the procedure of Bradford (23) using BSA as a standard.

Trinitrobenzenesulfonic Acid Assay for Amino Groups. The number of ϵ -amino groups remaining in conjugated BSA samples was determined using TNBS reagent (24). To 1 mL of D-xylitol-BSA conjugate/D-xylosyl-BSA Schiff base conjugate (100 μ g protein) solution, 1 mL of 4% NaHCO_3 (pH 8.5) and 1 mL of 0.01% freshly prepared aqueous TNBS solutions were added. The reaction was carried out at $42 \pm 2^\circ\text{C}$ for 2 h, followed by the addition of 1 mL of 10% SDS and 0.5 mL of 1 N HCl. The absorbance of the solution was monitored at 335 nm in a spectrophotometer, and a standard curve was obtained with unconjugated BSA (50–200 μ g range) after appropriate blank corrections. 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 in the TNBS assay. Percent conjugation was converted to degree of substitution in terms of moles of xylosyl Schiff base or xylitol per mole of carrier, assuming that BSA has 61 TNBS-reactive amino groups (24, 25).

Periodic Acid Schiff (PAS) Staining for Glycoconjugates. This was performed to confirm the glycyated nature (26) of xylitol-BSA conjugate following the reductive amination. Following SDS-PAGE (27), the gel was immersed in 12.5% trichloroacetic acid solution for 30 min, transferred to 1% periodic acid solution, and kept in the dark 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 adding 7% glacial acetic acid.

Immunization and Collection of Antiserum. Three New Zealand white male rabbits (*Oryctolagus cuniculus*), 7 months old, housed in the animal house facility of this institute, were used for the immunization according to standard protocols (28, 29), after obtaining approval from the institutional animal ethics committee (IAEC).

Initially, the immunogen, D-xylitol-BSA conjugate (52 mol D-xylitol/mol BSA; 0.5 mL, 1 mg protein) microemulsified with 0.5 mL of $3\times$ concentrated Freund's complete adjuvant, was administered subcutaneously at 8–10 sites on the back of the animal. After 4 weeks, booster doses containing 0.5 mg of the antigen microemulsified with Freund's incomplete adjuvant were administered intramuscularly at 15 day intervals. The animal was bled by marginal ear vein puncture 1 week before the first injection (to obtain preimmune serum) and 1 week after each booster dose (to obtain immune serum). The pooled serum was stored in aliquots below -20°C .

Titration of Antiserum. To check for the formation of hapten-specific antibodies, the antiserum was analyzed initially by dot-immunoblot assay (30) and later by noncompetitive ELISA using the coated antigen format (31). The titer of the antiserum was determined by measuring the binding of serial dilutions of the antiserum to coated D-xylitol-KLH (conjugate in which 50% of the amino groups of KLH have been modified

with xylitol). Flat-bottom polystyrene microtiter wells were coated with D-xylitol-KLH conjugate at 1000, 100, 50, and 10 ng per well (100 μ L 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 0.5% gelatin in PBS-T at 37°C for 30 min. Antiserum diluted in blocking buffer (1:10 to 1:400 000) was added (100 μ L/well) and incubated at 37°C for 2 h. Goat antirabbit IgG-alkaline phosphatase secondary antibody (1:5000 dilution in blocking buffer; 100 μ L/well) was added and incubated at 37°C for 1 h. Color development was done using *p*-nitrophenyl phosphate (1 mg/mL; 100 μ L/well) in 1% diethanolamine buffer, pH 9.8, at 37°C for 30 min. The reaction was stopped by adding 3 M NaOH (40 μ L/well), and the absorbance was read at 405 nm in an ELISA microplate reader.

A positive value in ELISA is defined as the absorbance value obtained with immune serum samples (as measured by absorbance value at 405 nm) 2-fold greater than that obtained in the case of preimmune serum. Antibody titer is defined as the reciprocal of the highest dilution that gave a positive value in ELISA.

Purification of Antixylitol Antibodies. (a) *Preparation of D-Xylitol-Keyhole Limpet Hemocyanin (D-xylitol-KLH)-Sephacrose CL-6B Affinity Matrix.* D-Xylitol-KLH conjugate with 50% modification of amino groups was used for immobilization onto Sepharose CL-6B (32). Sepharose CL-6B gel was first washed thoroughly with water to remove preservative, and then suspended in water to obtain 0.2 g moist gel/mL (total 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-xylitol-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, first at 25°C for 3 h, and then at 4°C for 10 h. Next, gel beads were washed extensively with water to remove unreacted borane-pyridine and the ligand. The gel was finally packed into a glass column (2 mL bed volume), and equilibrated with 20 mM phosphate buffer, pH 7.4, containing 140 mM NaCl, and stored at 4°C in the same buffer with 0.01% merthiolate as a preservative.

(b) *Hapten Affinity Chromatography for Purification of Antixylitol Antibodies.* Antixylitol-BSA antiserum (6 mL) obtained from the immunized rabbit was diluted 1:1 in PBS (pH 7.4), and passed through the D-xylitol-KLH-Sephacrose CL-6B column (0.8 cm id $\times$ 4 cm; 2 mL bed volume) that had been pre-equilibrated with PBS at 4°C . The flowthrough was recycled twice through the column. After washing with 50 mL PBS, the bound antibodies were eluted with 0.1 M glycine-HCl buffer, pH 2.9; the eluate was neutralized immediately using 0.5 M Tris base. The eluate was aliquoted in 200 μ L portions and stored below -20°C . The concentration of specific antibody was calculated on the basis of the reference value of $A^{0.1\%, 1\text{ cm}}$ at 280 nm of 1.4 for rabbit IgG (33). The affinity-purified antixylitol antibodies were subjected to native-PAGE (basic) using Tris-glycine buffer system, and isoelectric focusing (29).

Titration of Hapten Affinity Purified Antibodies and Determination of Cross-Reactivity. The affinity-purified antibodies were tested for their ability to bind to Schiff base conjugate of D-xylosyl-BSA and its reductive aminated product, namely, D-xylitol-BSA conjugate. Therefore, the Schiff base conjugate of D-xylosyl-BSA, or its reduced product (D-xylitol-BSA conjugate) having approximately similar modification of

amino groups was used as the coating antigen; in the case of D-xylosyl-BSA, 20 amino groups had been substituted as compared to 19 amino groups in D-xylitol-BSA. The linear regression analysis was performed using *SigmaStat 3.1* software (Systat Software Asia Pacific Ltd., Bangalore, India).

The affinity-purified antixylitol antibodies were used for noncompetitive ELISA experiments to obtain the binding curves using D-xylitol-KLH (50 ng/well) conjugate as the coating antigen. Specificity and cross-reactivity of the affinity-purified antibodies were studied by inhibition-ELISA experiments. Here, purified antibodies were preincubated with various concentrations (0.01–1000 mM) of test compounds (competitors like D-xylitol, D-mannitol, sorbitol, D-ribitol, L-arabinitol, glycerol, ethylene glycol, D-xylose) and L-lysine at 37 °C for 1 h before adding to coated (D-xylitol-KLH, 50 ng/well) microtiter wells. Other steps were as described in the Titration of Antiserum section. The percent cross-reactivity for various inhibitors was calculated using the formula (IC_{50} for xylitol $\div$ IC_{50} for test compound) $\times$ 100 where IC_{50} is the concentration required for 50% inhibition in ELISA.

Calculation of Affinity Constant of the Purified Antixylitol Antibodies. The affinity constants of the purified antixylitol antibodies were calculated using a computer program described by Raghava and Agrewala (34). This is a simple and reliable method based upon the law of mass action for calculating the affinity constant of a monoclonal antibody directed against the haptenic 2,4,6-trinitrophenyl group using the data obtained from noncompetitive ELISA.

D-Xylitol-KLH conjugate (10 μ g/mL) was prepared in 0.1 M carbonate-bicarbonate buffer. A series of 4 doubling dilutions were made so that the final concentration was 1/8 of the starting one. In a 96-well microtiter plate, rows A, B, C, and D were coated with 100 μ L D-xylitol-KLH containing 1000, 500, 250, and 125 ng, respectively. KLH (1000 ng in 100 μ L) was added in row E that worked as control. After overnight incubation at 4 °C, the plate was washed thrice with PBS-T. The concentration of the affinity-purified antibodies used was 4 ng in 100 μ L. All the other steps were followed as described in the Titration of Hapten-Purified Antibodies section.

The menu-driven computer program (Ab_affi program) for calculating the affinity constant of hapten-specific antibody from noncompetitive ELISA data written in GW-BASIC is available from the internet (<http://www.imtech.res.in/bic/achsw.html#serv3>). Absorbance data obtained from the microtiter plate can be fed to the computer either directly by interfacing or by using the keyboard. Before starting the calculation, a “dilution template” should be defined and the information about the serial dilutions of antibody added to the wells, and antigen coated on the plate must also be supplied.

Induction of Hypersensitivity in BALB/c Mice. BALB/c mice ($n = 6$ in each group) were immunized with 60 μ L of saline (negative control), 1 mg/mL ovalbumin (positive control), D-xylitol (1 mg/mL), D-xylitol-BSA conjugates (2 mg/mL), and 2 mg/mL BSA (carrier protein control) by intradermal injection. The use of an adjuvant was avoided in order to assess the inherent allergenicity of the xylitol-BSA conjugate. On days 5, 12, 19, 26, and 33, booster doses were given with 30 μ L of each of the antigens mentioned above (35). Blood was collected from the retro-orbital vein on days 8 and 22, and finally on day 36, and processed to obtain serum (1.8 mL per collection per group). Serum samples are pooled on a treatment group basis; equal volumes of serum from 3 collections contribute to the pool. Samples were stored at -70 °C until analysis.

Measurement of Serum Antixylitol IgE Antibody by PCA. The antixylitol IgE titer of BALB/c mouse immune serum was measured by the homologous passive cutaneous anaphylaxis (PCA) assay (36) performed in Swiss albino mice. For this

experiment, 100 μ L of 1:1 diluted pooled serum (with saline) obtained from each of the treatment groups (BALB/c mice that were administered separately with different antigens, viz., saline, ovalbumin, D-xylitol-BSA conjugates, BSA, D-xylitol) were injected intradermally on the shaved back of male Swiss albino mice ($n = 5$ for each group). After 24 h, 30 μ L of D-xylitol-BSA conjugates having different hapten densities or other antigens (saline, ovalbumin, BSA, D-xylitol) with 0.25% Evans blue were injected intravenously into the respective group ($n = 5$) of animals. The animals were sacrificed 30 min after the i.v. injection, and infiltration of the dye into the skin around the injection sites was observed. A serum sample (representative of a particular antigen) is identified as an IgE responder if the challenge resulted in a blue lesion in the skin with a mean diameter of 3 mm or greater (37).

RESULTS

Preparation and Characterization of Xylitol-Protein Conjugates by Reductive Amination. D-Xylose was coupled to BSA by reductive amination (Scheme 1) as described in the Experimental Procedures section. The time course of reductive amination reaction for D-xylose is shown in Figure 1 (panel a). The reaction is faster initially (up to 25 h), and slowly reaches a plateau thereafter. At 20 h of reaction, approximately 32 mol of xylitol have been conjugated per mole of BSA.

The xylitol-BSA conjugate was analyzed by SDS-PAGE (Figure 1, panel b), wherein an increase in molecular mass was apparent for the conjugate as compared to control BSA. The R_f values of the mol wt markers, control (BSA), and D-xylitol-BSA conjugate were calculated (data not shown). The increase in the relative molecular mass (M_r) of xylitol-BSA conjugate when compared to control BSA was ~ 8 kDa, which translates to 52 mol of xylitol coupled per mole BSA. Since BSA is devoid of glycans, the glycation of D-xylitol-BSA conjugates was detected by PAS staining. The PAS staining (Figure 1, panel c) clearly confirms the addition of glycans by way of xylitoyl groups on the carrier protein, BSA.

D-Xylose was incubated with BSA (without sodium cyanoborohydride) to form Schiff base conjugates. The time course of the reaction is shown in Figure 1, panel d. It is observed that the reaction of D-xylose with BSA is faster initially (up to 3 h) with the availability of almost all the accessible amino groups. As the reaction time progresses, the modification of the amino groups of BSA decreases due to the unstable nature of the Schiff base conjugate and the reversibility of the reaction. TNBS assay showed modification of 20 amino groups at the end of 3 h incubation, and as the time progresses, the rate of conjugation decreases. In the case of the reductive amination reaction (shown for comparison in Figure 1, panel d), the conjugation of D-xylose with BSA gradually increases with time. Approximately 19 and 32 amino groups have been modified in the reductive amination reaction at 3 and 6 h, respectively.

Immunochemical Analyses of Rabbit D-Xylitol-BSA Antiserum. The immunoreactivity of antiserum with D-xylitol-KLH can be detected easily at 1 in 2×10^4 dilution, in the dot-immunoblot analysis, which is shown in Figure 2 (panel A, strip a). The antiserum was positive for D-xylitol-BSA conjugate (Xyt-BSA), D-xylitol-KLH conjugate (Xyt-KLH), and BSA, but was negative for KLH. The highest dilution of antiserum for perceptible detection of dots was found to be 1 in 5×10^4 (strip b). The sensitivity of antixylitol-BSA antiserum was determined by checkerboard analysis of the immune serum where the concentrations of both the antigen and antiserum are varied (Figure 2, panel B). The antiserum titration curves show that the sensitivity of antibodies is seen at 1 in 1×10^4 dilution using 1000 ng per well of coating antigen, D-xylitol-KLH. An optimal titer of 1:100 antiserum dilution was evident at a coating antigen (D-xylitol-KLH) concentration of 100 ng/well.

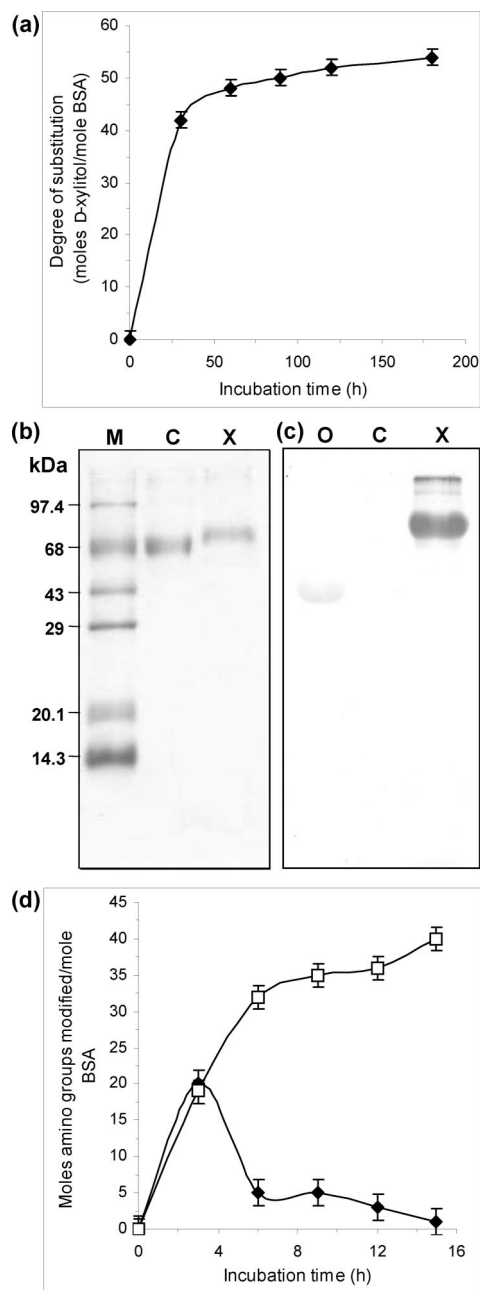


Figure 1. Preparation of D-xylitol-bovine serum albumin (BSA) conjugate by reductive amination at pH 8 and 37 °C. Panel a: Time course of reaction for the formation of D-xylitol-BSA. Panel b: SDS-PAGE (15%; reducing) analysis of D-xylitol-BSA sample from the 180 h reductive amination reaction. Staining: Coomassie blue. Lane M, molecular weight markers in kDa; lane C, control BSA (10 μ g); lane X, D-xylitol-BSA conjugate (10 μ g). Panel c: Periodic acid-Schiff (PAS) staining of D-xylitol-BSA conjugate analyzed by SDS-PAGE (15%; reducing). Lane O, ovalbumin, a glycoprotein (positive control); lane C, BSA, a nonglycoprotein (negative control); lane X, D-xylitol-BSA conjugate. Protein load: 15 μ g in all cases. Panel d: Time course of reaction for the formation of Schiff base conjugate of D-xylose with BSA (●) or D-xylitol-BSA conjugate by reductive amination (□).

Hapten Affinity Purification of Antixylitol Antibodies. The xylitol-specific antibodies were purified from the antiserum by hapten affinity chromatography on D-xylitol-KLH-Sepharose CL-6B. The elution of bound antibody, after loading the sample and washing, is presented in Figure 3. The antibodies were found to elute in the first 3–4 fractions. The yield of xylitol-specific antibodies was found to be in the range of 36–42 μ g per milliliter of rabbit antiserum, based on six chromatographic analyses.

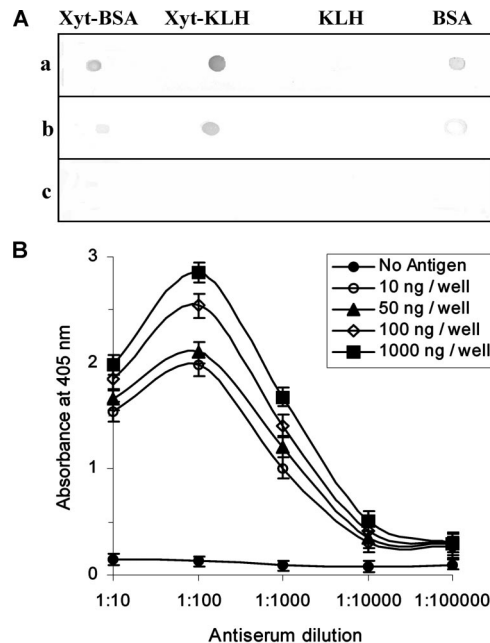


Figure 2. Immunochemical analyses of rabbit antiserum to xylitol-BSA conjugate. Panel A: Dot-immunoblot analysis on nitrocellulose strips. Antigens: 5 μ g each of D-xylitol-BSA (Xyt-BSA); D-xylitol-KLH (Xyt-KLH); KLH; BSA. Antixylitol-BSA antiserum dilutions: a, 1:20 000; b, 1:50 000; c, preimmune serum as control (1:2000 dilution). Secondary antibody: goat antirabbit IgG-alkaline phosphatase (1:4000 dilution). Panel B: Checkerboard analysis by ELISA at different coated antigen concentrations and antiserum dilutions. Coating antigen: D-Xylitol-KLH conjugate (concentrations shown as inset). Secondary antibody: goat antirabbit IgG-alkaline phosphatase (1:4000 dilution). The absorbance values are the means of triplicate analysis.

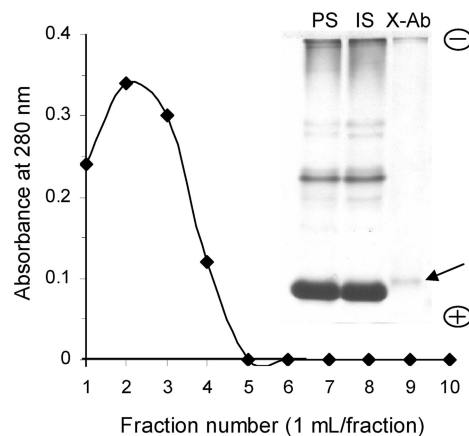


Figure 3. Elution profile of antixylitol antibodies from D-xylitol-KLH-Sepharose CL-6B affinity column (0.8 cm id $\times$ 4 cm; 2 mL bed volume). Sample load, 6 mL rabbit antixylitol-BSA antiserum; elution buffer, 0.1 M glycine-HCl, pH 2.9. Inset: Native PAGE (7.5%, basic) analysis of affinity-purified xylitol-specific antibodies. Staining: Coomassie blue. PS, preimmune serum (10 μ g); IS, immune serum (10 μ g); X-Ab, affinity-purified xylitol-specific antibodies (5 μ g); arrow indicates the position.

Affinity-purified antixylitol antibodies were analyzed by native-PAGE (basic), which showed a single band on the gel indicating the homogeneity of the antibodies (Figure 3, inset). Isoelectric focusing (IEF) analysis of affinity-purified antixylitol antibodies showed only a single band at approximately pI 7.2, suggesting a single specific clone and isotype (data not shown).

Characterization of Antixylitol Antibodies. The affinity constant of antixylitol antibodies was calculated using noncom-

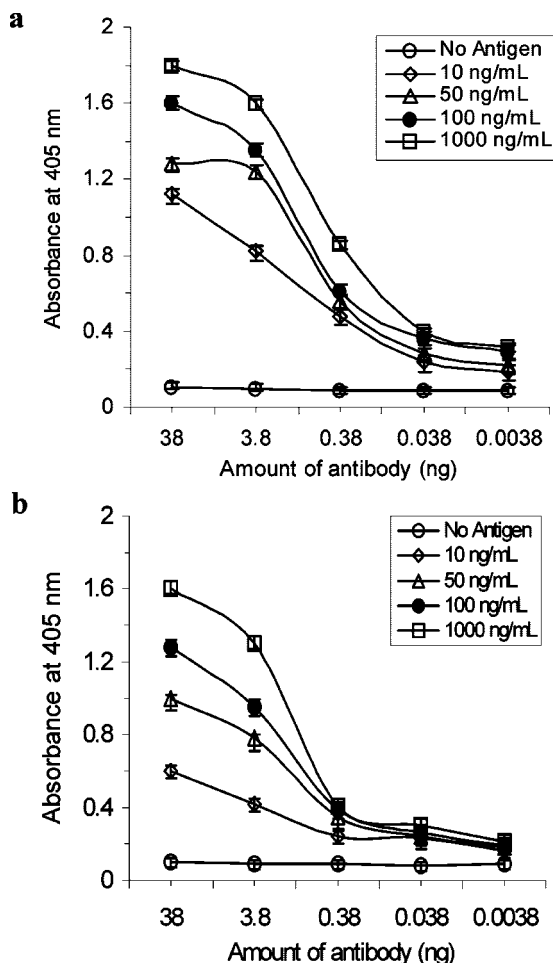


Figure 4. Comparison of binding curves for affinity-purified xylitol antibodies with xylitol-BSA/xylosyl-BSA conjugates. The absorbances at 405 nm for all the antigen concentrations at three different antibody concentrations (38, 3.8, and 0.38 ng) were analyzed between the two data sets by linear regression analysis. The R values were found to be 0.979 (at 38 ng), 0.991 (at 3.8 ng), and 0.699 (at 0.38 ng). Panel a: Binding curves obtained with xylitol-specific antibodies as a function of protein concentration and varying coated antigen, D-xylitol-BSA conjugate concentrations. Hapten density: 19 mol/mol BSA. Other details are as given in Figure 2, panel B. Panel b: Binding curves obtained with xylitol-specific antibodies as a function of protein concentration and varying coated antigen, D-xylosyl-BSA (Schiff base conjugate) concentrations. Hapten density: 20 mol/mol BSA.

petitive ELISA by the method of Raghava and Agrewala (34) and was found to be 3.86×10^9 .

In order to find out whether the xylitol-specific antibodies can recognize the Schiff base conjugate of xylosyl-BSA, the binding curves with both xylitol-BSA conjugate and xylosyl-BSA conjugate (Schiff base) were examined. The binding curves are shown in Figure 4. It is seen that the affinity-purified antibodies recognize the Schiff base conjugate of xylosyl-BSA (panel b) to a slightly lesser extent—approximately two-thirds the reactivity of xylitol-BSA conjugate (panel a).

Cross-Reactivity of Antixylitol Antibodies. The binding curves obtained with purified xylitol antibodies (Figure 5, panel a) shows that the sensitivity of antibodies can be observed even at ~ 200 pg (data not shown) for a coating antigen (D-xylitol-KLH) concentration of 100 ng/well. Optimal concentrations of ~ 4 ng of affinity-purified xylitol antibodies and 100 ng/well of D-xylitol-KLH coating antigen were selected from this checkerboard analysis for the inhibition ELISA studies with various sugars, sugar alcohols, and other small molecules.

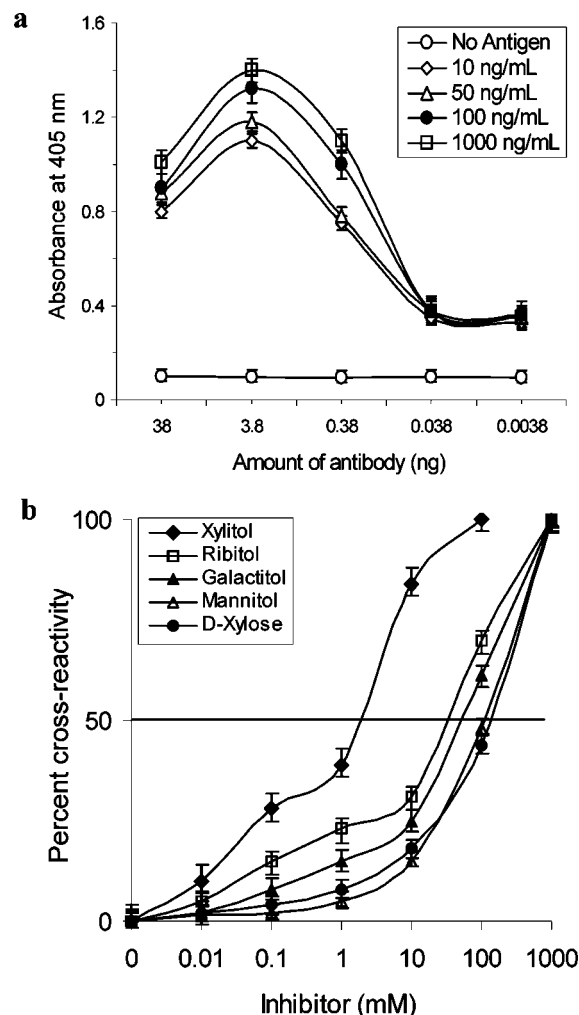


Figure 5. Immunochemical analyses of affinity-purified xylitol antibodies. Panel a: Binding curves of xylitol-specific antibodies as a function of protein concentration and varying coated antigen, D-xylitol-KLH (reductively aminated product) concentrations. Other details are as given in Figure 2, panel B. Panel b: Specificity of purified xylitol antibodies as determined by inhibition ELISA. Coating antigen: D-xylitol-KLH conjugate, 100 ng/well. Purified antixylitol antibodies (~ 4 ng in 100 μ L volume) were preincubated with various concentrations of D-xylitol, ribitol, galactitol, and so forth, at 37 $^{\circ}$ C for 1 h and then transferred to ELISA wells. Other details are as given in Figure 2, panel B.

Table 1. Specificity and Cross-Reactivity of Affinity-Purified Antixylitol Antibodies

test compound	IC ₅₀ (mM)	% cross-reactivity
D-xylitol	4.0	100.0
D-ribitol	35.0	11.4
D-galactitol	50.0	8.0
D-mannitol	90.0	4.4
D-xylose	102.5	3.9
L-arabinitol	108.0	3.8
meso-erythritol	108.1	3.8
D-sorbitol	135.1	3.7
D-threitol	350.0	3.0
glycerol	380.0	1.1
ethylene glycol	400.0	1.0
L-lysine	500.0	0.8

Inhibition ELISA was performed to check the cross-reactivity of the purified antixylitol antibodies (Figure 5, panel b). The panel of compounds tested (competitive inhibitors) included sugars like D-xylose and various sugar alcohols (*meso*-erythritol, D-xylitol, L-arabinitol, D-ribitol, D-mannitol, sorbitol, D-galactitol), ethylene glycol, glycerol, and L-lysine. The percent cross-

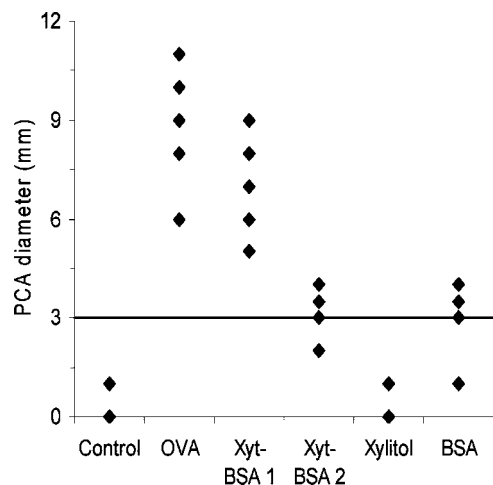


Figure 6. Passive cutaneous anaphylaxis (PCA) assay performed in Swiss albino mice ($n = 5$ per group) of different sera obtained on days 8, 22, and 36 from BALB/c mice ($n = 6$ per treatment group) that received repeated intradermal administration of antigens. The amount of different antigens used for immunization is $60 \mu\text{g}$. Xyt-BSA 1, D-xylitol-BSA conjugate with 52 haptens; Xyt-BSA 2, D-xylitol-BSA conjugate with 32 haptens; OVA, ovalbumin; BSA, bovine serum albumin; control, saline. Horizontal line denotes cutoff for positivity at 3 mm. The PCA response from each animal is represented as a data point. Note the overlaps in the data points of control, xylitol, Xyt-BSA 2, and BSA groups.

reactivity for D-xylose and sugar alcohols is summarized in Table 1. Among the sugar alcohols tested, only D-ribitol showed 11.4% and galactitol showed 8% cross-reactivity, whereas for D-xylose and other sugar alcohols, the cross-reactivity ranged from 3.0% to 4.4%. The cross-reactivity was found to be 2–4% in the case of some monosaccharides such as D-glucose, D-mannose, and D-ribose (data not shown). The cross-reactivity was $\sim 1\%$ in the case of ethylene glycol, glycerol, and lysine.

Measurement of Antixylitol-IgE Antibody by Passive Cutaneous Anaphylaxis (PCA). BALB/c mice were immunized with various antigens, saline (negative control), ovalbumin (positive control), D-xylitol-BSA conjugates (Xyt-BSA 1, 52 haptens/mole; Xyt-BSA 2, 32 haptens/mole), D-xylitol, and BSA to evaluate the inherent potential of these antigens to produce IgE response. BALB/c mice immunized with high hapten density D-xylitol-BSA conjugate (Xyt-BSA 1, $60 \mu\text{g}$) showed a positive reaction (5–9 mm range) in passive cutaneous anaphylaxis (PCA) when compared to 2–4 mm range seen in mice immunized with moderate hapten density D-xylitol-BSA conjugate (Xyt-BSA 2, $60 \mu\text{g}$). Ovalbumin showed a strong positive reaction (6–11 mm range), whereas D-xylitol, BSA, and saline showed a negative reaction (Figure 6).

DISCUSSION

The hypothesis proposed for the mechanism of sensitization as well as IgE-mediated type I (immediate) hypersensitivity to mannitol (12) appears to be applicable to xylitol also (Scheme 1). Nearly 52 out of 61 amino groups of BSA can be reductively alkylated with D-xylose. The xylitoylation of BSA was confirmed by an increase in the relative molecular mass as well as positive PAS staining.

D-Xylitol-BSA conjugate as the immunogen produced good antibody response in rabbits, as evidenced by the sensitivity and specificity in dot immunoblots. Xylitol-specific antibodies were purified by affinity chromatography for characterization studies. In order to remove antibodies to BSA from D-xylitol-BSA antiserum, an affinity matrix was prepared by conjugating D-xylose to KLH, a carrier protein other than the one used for immunization, and then covalently coupling this

conjugate (D-xylitol-KLH) to Sepharose CL-6B. Another advantage of using KLH for the affinity matrix is its high molecular weight possessing a large number of amino groups (38), which are absolutely essential for the coupling of D-xylitol to Sepharose CL-6B, through KLH. The affinity matrix, xylitol-KLH-Sepharose CL-6B, should be of value in purifying xylitol-specific IgE from the serum of allergic subjects sensitized to xylitol. The affinity constant of antixylitol antibodies was 3.86×10^9 , which appears to be in the range of affinity constants reported for many monoclonal antibodies raised against haptens.

Interestingly, the affinity-purified antibodies also reacted with the Schiff base conjugate of D-xylosyl-BSA, indicating that antixylitol IgE can react with the Schiff base conjugates of D-xylose with serum proteins which may be formed in vivo by nonenzymic glycation of proteins by xylose. However, the reactivity of purified antibodies with the Schiff base conjugate of D-xylosyl-BSA was roughly two-thirds compared to xylitol-BSA conjugate (Figure 4, panel b).

On the basis of the checkerboard analysis of purified antibodies, the optimal conditions for inhibition ELISA were found to be 100 ng/well coating antigen D-xylitol-KLH, and 4 ng affinity-purified xylitol antibodies in a volume of $100 \mu\text{L}$. It is clear from the inhibition ELISA experiments that the antibodies showed the least cross-reactivity with most monosaccharides including D-xylose and sugar alcohols. D-Xylitol showed maximum (100%) cross-reactivity, and among other sugars/sugar alcohols tested, only ribitol and galactitol showed 11.4% and 8% cross-reactivity, respectively. In the reductive amination reaction, the epsilon amino groups of L-lysyl residues of BSA react with the aldehyde group of the sugar D-xylose. Therefore, L-lysine was tested in the cross-reactivity studies to check whether the antibody also recognizes a portion of lysyl residue as an epitope. The results from Table 1 show that the cross-reactivity of L-lysine is only 0.8%, which clearly indicates that the xylitol-specific antibody is not recognizing the lysyl residue. On the basis of the low cross-reactivity of 2-carbon alcohol (ethylene glycol), 3-carbon alcohol (glycerol), and 4-carbon alcohol (*meso*-erythritol), it appears that the portion of xylitol molecule representing carbon atoms 2 to 5 most likely represents a single epitope.

Immunization study using D-xylitol-BSA conjugate in BALB/c mice (without the use of an adjuvant) demonstrated that the conjugate with high hapten density (52 haptens/mole) has the potential to induce IgE response, which is evidenced by the positive reaction in the passive cutaneous anaphylaxis. However, D-xylitol-BSA conjugate with moderate hapten density (32 haptens/mole), D-xylitol, and BSA showed a negative reaction, indicating that they cannot induce IgE response under identical conditions. Thus, the xylitol moiety as a hapten appears to possess allergenicity.

Only a single case of oral erosive eczema to xylitol has been reported so far, based on case history and skin patch test for IgE-mediated hypersensitivity (13). However, the presence of xylitol-specific IgE in the serum of the xylitol-sensitized subject has not been demonstrated. Xylitol is currently being used as a low-calorie alternative sweetener, and is listed as a GRAS (generally recognized as safe) substance under food additives. Xylitol and its parent monosaccharide D-xylose are considered safe even at high concentrations as evidenced by several studies (1–3, 39–41). However, the incidence of allergic reactions could slightly increase in the future due to the increased consumption of a variety of processed foods containing D-xylose and/or covalently bound xylitol. Additionally, xylitol may undergo oxidation to xylose in vivo, which can then follow the reactions outlined in Scheme 1. On the basis of the results of our study, it should be possible to demonstrate by ELISA the presence of xylitol-specific IgE in serum using D-xylitol-protein

as the coating antigen. In fact, the presence of mannitol-specific IgE in the serum of the allergic subject sensitized to mannitol has been demonstrated earlier (9).

Xylitol conjugate of human serum albumin (HSA) or other endogenous proteins may be formed in vivo, which can give rise to an IgE immune response in humans who are susceptible to sensitization to xylitol. Alternatively, xylitol conjugates or corresponding Schiff bases formed under certain food processing conditions may also act as sensitizing agents. Although not tested, it is possible that the Schiff base conjugate of xylosyl-protein may act as an immunogen to produce antibodies which can recognize both the Schiff base as well as free xylitol, albeit not to a similar extent. In the rare case of oral erosive eczema caused by xylitol as a contact allergen described by Hanakawa et al. (13), wherein the presence of xylitol-specific IgE has not been shown, it is highly probable that the afore-mentioned possibilities are operating. On the basis of our earlier hypothesis enunciated for mannitol sensitization and hypersensitivity (12), it appears very likely that D-xylose acts as a noneliciting prosensitizer and free D-xylitol acts as a nonsensitizing elicitor. This means that, although xylitol cannot act as a sensitizing agent, it will elicit an allergic reaction in persons sensitized to xylosyl-protein (Schiff base) conjugates. The question of how Schiff base conjugates get reduced in vivo is yet to be investigated.

IgG antibodies specific to xylitol described here should prove useful in developing immunoassays for D-xylitol or D-xylitol epitopes on modified proteins in biological samples, natural and processed foods (42), and pharmaceuticals. It is interesting to note in this context that xylosyl-lysyl linkages (aldimine) and xylosyl-seryl linkages are present on core proteins of dermatan sulfate of connective tissues (43), and reducible lysine-carbohydrate condensation products have been identified in collagen as a result of age-related changes (44).

CONCLUSIONS

The results from this study demonstrate that xylitol epitopes can be easily formed on a suitable carrier protein by reductive amination. D-Xylitol-BSA conjugate thus formed (with 52 xylitol groups) has been used as an immunogen in rabbits to generate antixylitol antibodies, which have been purified from D-xylitol-BSA antiserum on a hapten-affinity column (D-xylitol-KLH-Sepharose CL-6B) for characterization. The purified antibodies show excellent specificity for xylitol; only ribitol was found to give significant cross-reactivity. Schiff base conjugates of xylosyl-protein are also recognized although to a slightly lesser extent. Immunization study in BALB/c mice has clearly shown that D-xylitol-BSA conjugate with high hapten density induces an IgE response as confirmed by homologous PCA.

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