Generation of an antibody specific to erythritol, a non-immunogenic food additive

K. Sreenath¹, P. Prabhasankar², & Y. P. Venkatesh¹

¹Department of Biochemistry & Nutrition and ²Department of Flour Milling, Baking & Confectionery Technology, Central Food Technological Research Institute (CFTRI), Mysore — 570020, Karnataka State, India

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Abstract
Erythritol, a simple sugar alcohol, is widely used as a food and drug additive owing to its chemical inertness, sweetness and non-toxicity. Adverse reactions to erythritol are rare and only three cases of allergic reactions to foods containing erythritol have been reported. Being inert, erythritol cannot produce an immunological response. In order to explain the mechanism of immunogenicity of erythritol, a method to obtain erythritol epitopes on a carrier protein, which can serve as an immunogen to develop antibodies against erythritol, is described. D-Erythrose was conjugated to bovine serum albumin at pH 8 by reductive amination. The reduction product of the Schiff base of D-erythrose–bovine serum albumin conjugate creates erythritol groups. Rabbits immunized with erythritol–bovine serum albumin conjugate (29 hapten/molecule) showed good antibody response (detection of 1 ng antigen, erythritol–keyhole limpet haemocyanin conjugate possessing 50% modified amino groups, at 1:50000 dilution). Anti-erythritol immunoglobulin-G antibodies were purified from the immune serum using hapten-affinity chromatography on an erythritol-keyhole limpet haemocyanin-Sepharose CL-6B affinity matrix. The yield of erythritol-specific antibody was approximately 40 µg ml⁻¹ of rabbit antiserum. Enzyme-linked immunosorbant assay inhibition studies using sugars, sugar alcohols and L-lysine showed minimal cross-reactivity (approximately 4%) when compared with erythritol; only dithioerythritol showed a cross-reactivity of approximately 33%. D-Threitol and L-threitol (isomers of erythritol) had cross-reactivities of 15 and 11%, respectively. The inhibition studies confirmed the haptenic nature of erythritol and indicated that the erythritol group is a single epitope. The reaction scheme outlined here for the generation of erythritol epitopes appears to provide a basis for the immunogenicity of erythritol.

Keywords: Allergen, erythritol, erythritol-bovine serum albumin, erythritol-specific antibody, food additive, hapten, reductive amination, sugar alcohol, sweetener

Introduction
Owing to their chemical inertness, non-hygroscopicity, sweetness and non-toxicity, sugar alcohols (also known as polyhydric alcohols or polyols) are used as food additives and in pharmaceuticals. Due to their low caloric content, sugar alcohols are considered as low-calorie bulk sweeteners. Sugar alcohols commonly used as food additives are sorbitol (International Numbering System of Food Additives (INS) 420), mannitol (INS 421), xylitol (INS 967) and erythritol (INS 968). Polyglycitol syrup (INS 964), maltitol (INS 965) and lactitol (INS 966) are used to a lesser extent. Erythritol (1,2,3,4-butanetetrol; neso-erythritol) is 60–80% as sweet as sucrose; its caloric value for food labelling purposes is 0.2 cal g⁻¹ in the USA, and 0 cal g⁻¹ in Japan (De Cock 1999; De Cock and Bechert 2002). Erythritol is a self- affirmed generally recognized as safe substance (Berndt et al. 1996; Munro et al. 1998; European Commission 2003), and has been approved as a food additive in Japan since 1990, and recently in the USA, Canada, Europe and Australia. It has been part of the human diet for thousands of years as it is naturally present in fruits such as pears, melons and grapes, as well as mushrooms, and fermentation-derived foods such as wine, sake, beer, cheese and soy sauce at levels up to 0.13% (Shindou et al. 1988, 1989).
Mannitol, a hexitol, occurs naturally in many plant foods including mushrooms (Anon 2004). The use of mannitol in foods is broadly permitted by the food additive regulations of the US Food and Drug Administration (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. These appear to be non-immunoglobulin E (IgE)-mediated anaphylactic reactions caused by the direct action of mannitol at hyperosmolar concentrations (>100 mM) on mast cells or basophils. Recently, mannitol was identified as an allergen for a sensitized subject, and mannitol-specific IgE was demonstrated for the first time in the serum of the subject sensitized to mannitol (Hegde and Venkatesh 2004). Based on the inert nature of mannitol, a hypothesis has been proposed to explain the mechanism of sensitization and hypersensitivity to mannitol (Venkatesh and Hegde 2003). In this hypothesis, D-mannose acts as a prosensitizer, the Schiff base conjugate with proteins acts as a sensitizer, and D-mannitol acts as a non-sensitizing elicitor.

Allergic reactions to erythritol are also rare, similar to the case of mannitol. In recent years, three cases of allergic reactions to the ingestion of erythritol in processed foods (canned milk tea, beverages, and snack foods) have been reported based on case history and positive skin prick tests for immediate or type I (IgE-mediated) hypersensitivity (Hino et al. 2000; Yunginger et al. 2001). The reported allergic cases have occurred in Japan and the USA, where erythritol is widely used as a food additive. The estimated prevalence of allergy to erythritol is <1 per million (Yunginger et al. 2001).

Anti-carbohydrate antibodies with specificity towards various monosaccharides, disaccharides and oligosaccharides have been studied extensively (Schwartz and Gray 1977; Gray 1978; Roy et al. 1984; Pazur 1998, 2003; Pazur et al. 2001). 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 non-reducing monosaccharide (cyclic) determinant (Schwartz and Gray 1977; Gray 1978; Roy et al. 1984); 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. With this background, it appeared very interesting to demonstrate the formation of immunoglobulin G (IgG) antibodies to erythritol in laboratory animals. The present paper describes the preparation of antigen-bearing erythritol epitopes by the process of reductive amination of D-erythrose with bovine serum albumin (BSA), which was used for immunizing rabbits; antibodies were purified from the immune serum by hapten-affinity chromatography, and characterized for specificity by hapten-inhibition.

Materials and methods

D-Erythrose, Sepharose CL-6B, bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), sodium cyanoborohydride, periodic acid, and borane-pyridine complex were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Trinitrobenzenesulfonic acid (TNBS) was a product of HiMedia Laboratories (Mumbai, India). Schiff reagent (1-fluoro-2,4-dinitrobenzene) was procured from Sisco Research Laboratories (Mumbai, India). Maxisorp enzyme-linked immunosorbant assay (ELISA) microtitre plates (flat-bottom) were a product of Nunc A/S (Roskilde, Denmark). Goat anti-rabbit IgG–alkaline phosphatase conjugate, Freund’s complete adjuvant (FCA), and Freund’s incomplete adjuvant (FIA) were products of Bangalore Genei (Bangalore, India). All other chemicals were of analytical grade.

Preparation of hapten–protein conjugates containing erythritol epitopes

D-Erythrose was conjugated to a carrier protein, BSA, by reductive amination (also termed reductive alkylation) in the presence of a mild reducing agent, sodium cyanoborohydride, as described for other sugars, namely maltose, lactose and cellobiose (Gray 1978; Roy et al. 1984) to obtain stable erythritol epitopes on the conjugated carrier protein. BSA (68 mg), D-erythrose (100 mg), and sodium cyanoborohydride (100 mg) were dissolved in 5.0 ml of 0.2 M borate buffer (pH 8.0) and incubated at 37°C. Aliquots of the reaction mixture were withdrawn at various time periods; the reaction was stopped by adjusting the pH to approximately 4 using dilute acetic acid. The aliquots were dialysed against phosphate-buffered saline (PBS) at 4°C, and analysed by TNBS assay to determine the degree of substitution. As a control,
BSA was incubated as above, excluding D-erythrose (control BSA). Protein estimation of conjugates was carried out as per the procedure of Bradford (1976) using BSA as a standard.

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. The percent conjugation was converted to the degree of substitution in terms of moles of erythritol per mole of carrier, assuming that BSA has 61 TNBS-reactive amino groups (Sashidhar et al. 1994).

Since KLH is a large polymeric protein and can undergo reversible dissociation into subunits, its exact molecular weight is not known. Hence, it is difficult to calculate the molar ratio of hapten to carrier. Therefore, KLH was subjected to reductive amination as described above for BSA under identical conditions. After 18 h of reaction, it was found that approximately 50% of its free amino groups had been modified compared with native KLH. This conjugate is designated as 50% modified KLH, and was used as the coating antigen.

### Trinitrobenzenesulfonyl acid assay for amino groups

The number of ε-amino groups remaining in conjugated BSA samples was determined using TNBS reagent (Sashidhar et al. 1994). To 1 ml of erythritol–BSA conjugate (100 μg protein) solution, 1 ml of 4% NaHCO₃ (pH 8.5) and 1 ml of 0.01% freshly prepared aqueous TNBS solutions were added. The reaction was carried out at 42 ± 2°C for 2 h, followed by the addition of 1 ml of 10% sodium dodecyl sulphate (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 μg range) after appropriate blank corrections.

### Periodic acid-Schiff (PAS) staining for glycoconjugates

PAS staining for glycoconjugates was performed to confirm the glycated nature (Zacharius et al. 1969) of erythritol–BSA conjugate following the reductive amination. Following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), 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.

### Preparation of erythritol-keyhole limpet haemocyanin (KLH)-Sepharose CL-6B affinity matrix

An erythritol–KLH conjugate with 50% modification of amino groups was used for immobilization onto Sepharose CL-6B (Stults et al. 1989).
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⁻¹ (a total of 10 ml). The washed gel was then oxidized by adding solid sodium periodate (NaIO₄) to a final concentration of 25 mM. The mixture was swirled at 25°C for 30 min; unreacted NaIO₄ 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⁻¹ suspension) in phosphate buffer was mixed with erythritol–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.

Hapten-affinity chromatography for the purification of anti-erythritol antibody

Hapten-affinity chromatography for the purification of anti-erythritol antibody was carried out at 4°C. Anti-erythritol-BSA antiserum (6 ml) obtained from the rabbit was diluted 1:1 in PBS (pH 7.4) and passed through the erythritol–KLH-Sepharose CL-6B column (0.8 cm i.d. x 4 cm; 2 ml bed volume), pre-equilibrated with PBS. The flow-through was recycled thrice through the column. After washing with 50 ml PBS, the bound antibody was eluted in 1 ml fractions of 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 μl portions and stored below −20°C. The concentration of specific antibody was calculated based on the reference value of A₄₉₀ of 1.4 for rabbit IgG (Johnstone and Thorpe 1996).

Titration of hapten affinity-purified antibody and determination of cross-reactivity

The affinity-purified anti-erythritol antibody was used for ELISA experiments to obtain the binding curves using the erythritol–KLH (50 ng per well) conjugate for coating, and to study the specificity and cross-reactivity by ELISA inhibition experiments. Here, purified antibody was pre-incubated with various concentrations (0.01–1000 mM) of test compounds (competitors such as meno-erythritol, D-threitol, L-threitol, dithioerythritol (DTE), DL-dithiodiethanol (DTT), D-xylitol, D-ribitol, D-sorbitol, D-ribitol, glycerol, ethylene glycol, D-erythrose, D-glucose, D-ribose, L-arabinose), and L-lysine at 37°C for 1 h before adding to coated microtitre wells. Other steps were as described under 'Titration of antisera'. The percent cross-reactivity for various inhibitors was calculated using the formula:

$$\text{IC}_{50} \text{ for erythritol/IC}_{50} \text{ for test compound} \times 100,$$

where IC₅₀ is the concentration required for 50% inhibition in ELISA.

Calculation of the affinity constant of the purified anti-erythritol antibody

The affinity constant of the purified anti-erythritol antibody was calculated using a computer program described by Raghava and Agrewala (1994). This is a simple and reliable method based upon law of mass action as for the calculating affinity constant of a monoclonal antibody directed against the haptenic 2,4,6-trinitrophenyl group using the data obtained from non-competitive ELISA (Raghava and Agrewala 1994).

Results

Preparation and characterization of erythritol–protein conjugates by reductive amination

D-Erythrose was coupled to BSA by reductive amination (Figure 1), as described under the Materials and methods. The time course of reductive amination reaction for D-erythrose is shown in Figure 2 (panel a). The reaction is faster initially, and slowly reaches a plateau after 60 h. At 20 h of reaction, approximately 35 moles of erythritol have been conjugated per mole of BSA. The conjugate was analysed by SDS-PAGE (Figure 2, panel b), wherein an increase in molecular mass was apparent for the 180 h reacted conjugate when compared with control BSA. The Rₖ values of the molecular weight markers, control and erythritol–BSA conjugates were calculated (data not shown). The increase in the relative molecular mass (Mₗ) of erythritol–BSA conjugate when compared with control BSA was found to be approximately 5.8 kDa; this increase translates to approximately 56 moles of erythritol coupled per mole of BSA, based on the molecular mass of 104 Da for an erythritol group. This value for erythritol conjugation is in good agreement with the value of 54 erythritol groups obtained by TNBS assay (Figure 2, panel a). Although identical amounts of BSA and erythritol–BSA conjugate were loaded on the gel, it is seen that the
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Figure 1. Reaction of D-erythrose with carrier protein by reductive amination to obtain erythritoyl epitopes. The structure of meso-erythritol is shown in the box (carbons C1 to C4 should be viewed from top to bottom). D-Erythrose exists in the furanose form (Maynard 1938; Hockett and Maynard 1939).

conjugate showed significantly less intensity than BSA (Figure 2, panel b).

Since BSA is devoid of glycans, we wanted to detect the glycation of erythritol-BSA conjugate by PAS staining. The PAS staining (Figure 2, panel c) clearly confirms the formation of erythritoyl groups on the carrier protein, BSA.

Immunochimical analyses of rabbit erythritol-BSA antiserum

The immunoreactivity of antiserum with erythritol-KLH can be detected easily at a 1:2 × 10^6 dilution, in the dot-immunoblot analysis (Figure 3, panel (a), strip a). The antiserum was positive for erythritol-BSA conjugate (column 1), erythritol-KLH conjugate (column 2), and BSA (column 4), but was negative for KLH (column 3). The highest dilution of antiserum for perceptible detection of dots was found to be a 1:5 × 10^4 dilution (strip b). Although the amount and the volume of the samples were kept constant, there appeared to be some spreading in the case of BSA.

The sensitivity of anti-erythritol-BSA antiserum was determined by checkerboard analysis of the immune serum where the concentrations of both antigen and antiserum are varied (Figure 3, panel (b)). The antiserum titration curves showed that the sensitivity of antibodies is seen even at a 1:5 × 10^4 dilution with 1000 ng per well of coating antigen, erythritol-KLH. An optimal titre of 1:100 antiserum dilution was evident at a coating antigen (erythritol-KLH) concentration of 100 ng per well.

Hapten affinity purification of anti-erythritol antibody

The erythritol-specific antibody was purified from the antiserum by hapten affinity chromatography on erythritol-KLH-Sepharose CL-6B. The antibody was found to elute in the first three to four fractions (data not shown). Erythritol-specific antibody yield was found to be in the range of 36–45 µg ml⁻¹ of rabbit antiserum, based on six chromatographic analyses.

The binding curve obtained with purified antibody showed the sensitivity of antibody even at 0.2 ng for a coating antigen (erythritol-KLH) concentration of 100 ng per well (Figure 4). However, the sensitivity of the antibody appeared to
be ideal at 0.4 ng, since there is a perceptible difference in the absorbance at all concentrations of the coating antigen. Therefore, an optimal concentration of 4 ng of affinity-purified antibody and 100 ng per well of erythritol–KLH coating antigen were selected from this checkerboard analysis for the inhibition studies with sugars, sugar alcohols, and other small molecules.

Characterization of anti-erythritol antibody

The affinity constant of the anti-erythritol antibody was calculated using non-competitive ELISA by the method of Raghava and Agrewala (1994), and it was found to be $4.86 \times 10^6$.

Inhibition ELISA was performed to check the cross-reactivity of the purified anti-erythritol antibody (Figure 5). The purified antibody was
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2.0
1.6
1.2
0.8
0.4
0.0

No antigen
100 ng/well
50 ng/well
100 ng/well
1000 ng/well

Amount of antibody (ng)

Absorbance at 405 nm

Figure 4. Binding curves obtained with hapten-affinity-purified erythritol-specific antibody as a function of antibody concentration and varying coating antigen concentrations. Other details are as given in Figure 3, panel (b).

D-erythrose
D-xylitol
Dithioerythritol
Meso-erythritol

Figure 5. Specificity of the purified antibody as determined by inhibition enzyme-linked immunosorbent assay (ELISA). Coating antigen: erythritol-keyhole limpet haemocyanin (erythritol-KLH) conjugate, 100 ng per well. Purified anti-erythritol antibody (4 ng in 100 μl volume) was pre-incubated with various concentrations of meso-erythritol, D-erythrose, dithioerythritol or D-xylitol at 37°C for 1 h, and then transferred to ELISA wells. Other details are as given in Figure 3, panel (b).

tested for cross-reactivity with various sugars (D-erythrose, D-xylitol, L-arabinose, D-glucose, D-mannose, D-fructose) and sugar alcohols (glycerol, D-threitol, L-threitol, DTE, DTT, D-xylitol, L-arabinitol, D-ribitol, D-mannitol, sorbitol, D-galactitol), ethylene glycol, and L-lysine. The percent cross-reactivity for these sugars and sugar alcohols is listed in Table I. Among the sugars and sugar alcohols tested, DTE showed the highest (approximately 33%) cross-reactivity, followed by the isomers of erythritol, D-threitol (15.1%) and L-threitol (11.1%), and their dithiobenzoate DTT (13.8%). Among the other sugars and sugar alcohols, only xylitol exhibited approximately 8% cross-reactivity, whereas for all the others it was less than 4% (Table I).

Discussion

The reaction mechanism outlined in Figure 1 was utilized for the generation of erythritol groups by reductive amination of BSA using D-erythrose. Nearly 54 amino groups out of 61 amino groups of BSA can be reductively alkylated with D-erythrose. The erythritolation of BSA was confirmed by an increase in the relative molecular mass, quantification of remaining amino groups by TNBS assay, as well as by positive PAS staining for its glycation status. By silver staining, the erythritol-BSA conjugate showed significantly less intensity than BSA. This appears to be due to the effect of glycation on the propensity of protein staining. Similar observations in the decreased intensity of both Coomassie and silver staining have been reported in the case of other glycated proteins as well as proteins modified by Maillard reaction (Hodny et al. 1992; Syrovy 1992).

In order to remove antibodies to BSA from erythritol-BSA antiserum, an affinity matrix was prepared by conjugating D-erythrose to KLH, a carrier protein other than the one used for immunization, and then covalently coupling this conjugate (erythritol-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, which are absolutely essential for the coupling of D-erythrose to Sepharose CL-6B via KLH.

Based on the checkerboard analysis of purified antibody, the optimal conditions for inhibition ELISA were found to be 100 ng per well coating antigen erythritol–KLH, and 4 ng affinity-purified erythritol antibody in a volume of 100 μl. It is clear from the inhibition ELISA experiments that the antibody showed least cross-reactivity with most monosaccharides and sugar alcohols. meso-Erythritol showed maximum (100%) cross-reactivity, and among other sugars/sugar alcohols tested, dithioerythritol showed approximately 33% cross-reactivity, as expected from its structure. Dithioerythritol is remarkably similar to erythritol in that the thiomethyl groups replace the secondary alcohol groups at positions 1 and 4 of erythritol. Other sugar alcohols that showed somewhat significant cross-reactivity (11–15%) are D-threitol, L-threitol and DTT.
Table I. Specificity of hapten affinity-purified erythritol-specific antibody.

<table>
<thead>
<tr>
<th>Test compound</th>
<th>IC$_{50}$ (mM)</th>
<th>Percentage of cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>meso</em>-Erythritol</td>
<td>7.2</td>
<td>100.0</td>
</tr>
<tr>
<td>Dithioerythritol</td>
<td>21.7</td>
<td>33.2</td>
</tr>
<tr>
<td>D-threitol*</td>
<td>47.7</td>
<td>15.1</td>
</tr>
<tr>
<td>DL-dithiothreitol*</td>
<td>52.0</td>
<td>13.8</td>
</tr>
<tr>
<td>L-threitol*</td>
<td>65.1</td>
<td>11.1</td>
</tr>
<tr>
<td>D-xylitol</td>
<td>92.3</td>
<td>7.8</td>
</tr>
<tr>
<td>D-erythrose</td>
<td>171.4</td>
<td>4.2</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>175.6</td>
<td>4.1</td>
</tr>
<tr>
<td>D-sorbitol</td>
<td>240.0</td>
<td>3.0</td>
</tr>
<tr>
<td>D-glucose</td>
<td>400.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Glycerol</td>
<td>450.0</td>
<td>1.6</td>
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<tr>
<td>Ethylene glycol</td>
<td>480.0</td>
<td>1.5</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>560.0</td>
<td>1.3</td>
</tr>
<tr>
<td>D-ribitol</td>
<td>600.0</td>
<td>1.2</td>
</tr>
<tr>
<td>D-galactitol</td>
<td>655.0</td>
<td>1.1</td>
</tr>
<tr>
<td>L-lysine</td>
<td>900.0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Tested up to 100 mM concentration due to solubility problems.

In the reductive amination reaction, the epsilon amino groups of L-lysyl residues of BSA react with the aldehyde group of the sugar D-erythrose. 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 I show that the cross-reactivity of L-lysine is only 0.8%, which clearly indicates that the erythritol antibody does not recognize the lysyl residue.

In summary, the results presented in this paper demonstrate that erythritol epitopes can be easily formed on a suitable carrier protein by the reductive amination reaction. The erythritol–BSA conjugate thus formed (with 25–30 erythritol groups) has been used as an immunogen in rabbits to generate anti-erythritol antibody, which has been purified from erythritol–BSA antiserum on a hapten-affinity column (erythritol–KLH-Sepharose CL-6B) for characterization. The purified antibody shows excellent specificity for erythritol; only its isomers and dithioderivatives were found to give significant cross-reactivity. It may be noted here that D-erythrose exhibits only 4.2% cross-reactivity since it exists predominantly as the furanose form (Maynard 1938; Hockett and Maynard 1939). Based on the low cross-reactivity of 2-carbon alcohol (ethylene glycol), 3-carbon alcohol (glycerol), and 5-carbon sugar alcohols (pentitols), it appears that erythritol molecule most likely represents a single epitope. The affinity constant of the purified anti-erythritol antibody was comparable with that of monoclonal antibodies developed for hapten (Raghava and Agrewala 1994), indicating that the purified antibody is a monospecific polyclonal antibody that has specificity for erythritol. An antibody specific to saccharides or saccharide derivatives should prove useful as an analytical reagent for immunoassays for the identification of corresponding analytes in biological samples, foods/processed foods (Pazur and Li 2004), and pharmaceuticals. Using an anti-erythritol antibody, a competitive immunoassay for the quantification of erythritol that occurs in low abundance in natural foods is currently being developed.

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References


Generation of an antibody specific to erythritol 869
Erratum

Generation of an antibody specific to erythritol, a non-immunogenic food additive

K. SREENATH¹, P. PRABHASANKAR², & Y. P. VENKATESH¹

¹Department of Biochemistry & Nutrition, and ²Department of Flour Milling, Baking & Confectionery Technology, Central Food Technological Research Institute (CFTRI), Mysore – 570020, Karnataka State, India


Taylor and Francis wish to apologise to the authors of this article, as Figure 3(b) was published incorrectly. It is presented in its correct form below:

Figure 3. Immunochemical analyses of rabbit antiserum to erythritol-bovine serum albumin (BSA) conjugate. (b) Checkerboard analyses at different coated antigen concentrations and antiserum dilutions. Coating antigen: erythritol-KLH conjugate; concentrations are shown in the inset. Secondary antibody: goat anti-rabbit IgG-alkaline phosphatase (1: 4000 dilution). The absorbance values are the mean of triplicate analysis.
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 antixylitol antibodies was carried out using haptens-affinity chromatography on xylitol-keyhole limpet hemocyanin-Sepharose CL-6B; the yield was ~40 μg/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 ~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 polyhydroxy 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: INS 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
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 μm), 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. Trinitrobenzensulfonic (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 Xylosyl 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 xylosyl 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 Amido 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 μg protein) solution, 1 mL of 4% NaHCO₃ (pH 8.5) and 1 mL of 0.01% freshly prepared aqueous TNBS solutions 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 of the solution was monitored at 355 nm in a spectrophotometer, and a standard curve was obtained with unconjugated BSA (50–200 μg 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 xylosyl 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 glycosylated nature (26) of xylosyl–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-xylose–BSA conjugate (52 mol D-xylitol/mol BSA, 0.5 mL, 1 mg protein) microemulsified with 0.5 mL of 3× 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 at −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-xylosyl–KLH (conjugate in which 50% of the amino groups of KLH have been modified with xylosyl). Flat-bottom polystyrene microtiter wells were coated with D-xylosyl–KLH conjugate at 1:1000, 1:100, 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 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 μL/well) and incubated at 37 °C for 2 h. Goat antirabbit IgG-alkaline phosphatase secondary antibody (1:5000 dilution in blocking buffer; 100 μL/well) was added and incubated at 37 °C for 1 h. Color development was done using p-nitrophenyl phosphate (1 mg/mL; 100 μL/well) in 1% diethanolamine buffer, pH 9.8, at 37 °C for 30 min. The reaction was stopped by adding 3 M NaOH (40 μL/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 Antixylose Antibodies. (a) Preparation of D-Xylosyl–Keyhole Limpet Hemocyanin (D-xylosyl–KLH)–Sepharose CL-6B Affinity Matrix. D-Xylosyl–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 (NaIO₄) to a final concentration of 25 mM. The mixture was stirred at 25 °C for 30 min; untreated NaIO₄ 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-xylosyl–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 Antixylose Antibodies. Antixylosyl–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–Sepharose CL-6B column (0.8 cm id × 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 μL portions and stored below −20 °C. The concentration of specific antibody was calculated on the basis of the reference value of A₄₀⁵ of 1.1 cm at 280 nm of 1.4 for rabbit IgG (33). The affinity-purified antixylosyl 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 reduced aminated product, namely, d-xylosyl–BSA conjugate. Therefore, the Schiff base conjugate of d-xylosyl–BSA, or its reduced product (d-xylosyl–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 the carrier protein, 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-xyitol-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 nM) of test compounds (competitors like D-xyitol, D-mannitol, sorbitol, D-ribose, L-arabinose, glycerol, ethylene glycol, D-xylose) and L-lysine at 37 °C for 1 h before adding to coated (D-xyitol-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 (IC50 for xylitol + IC50 for test compound) x 100 where IC50 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-Xylosyl-KLH conjugate (10 μg/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 μL of D-xylose-KLH containing 1000, 500, 250, and 125 ng, respectively. KLH (1000 ng in 100 μL) 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 μL. 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 μL of saline (negative control), 1 mg/mL ovalbumin (positive control), D-xylose (1 mg/mL), D-xylosyl-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 xylosyl-BSA conjugate. On days 5, 12, 19, 26, and 33, booster doses were given with 30 μL 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 were 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 μL 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-xylosyl-BSA conjugates, BSA, D-xylosyl) were injected intradermally on the shaved back of Swiss albino mice (n = 5 for each group). After 24 h, 30 μL of D-xylosyl-BSA conjugates having different hapten densities or other antigens (saline, ovalbumin, BSA, D-xylosyl) with 0.25% Evans blue were injected intravenously into the same 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 xylose 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 Ry values of the mol wt markers, control (BSA), and D-xylosyl-BSA conjugate were calculated (data not shown). The increase in the relative molecular mass (Mr) of xylitol–BSA conjugate when compared to control BSA was ~8 kDa, which translates to 52 amino groups per mole of BSA. The D-xylosyl-BSA conjugate was formed by Schiff base conjugation. 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-Xylosyl-BSA Antiserum. The immunoreactivity of antiserum with D-xylosyl-KLH can be detected easily at 1 in 2 × 10⁸ dilution, in the dot-immunoblot analysis, which is shown in Figure 2 (panel A, strip a). The antiserum was positive for D-xylosyl-BSA conjugate (Xyt-BSA), D-xylosyl-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 × 10⁸ (strip b). The sensitivity of antixylosyl-BSA antiserum was determined by checkerboard analysis of the immune serum where the concentrations of both the antigens and antiserum are varied (Figure 2, panel B). The antiserum titration curves show that the sensitivity of antibodies is seen at 1 in 1 × 10⁶ dilution using 1000 ng per well of coating antigen, D-xylosyl-KLH. An optimal titer of 1:100 antiserum dilution was evident as a coating antigen (D-xylosyl-KLH) concentration of 100 ng/well.
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 μg per milliliter of rabbit antiserum, based on six chromatographic analyses.

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 pH 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-
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 conjugate) 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 a) to a slightly lesser extent—approximately two-thirds the reactivity of xylitol-BSA conjugate (panel b).

**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) of concentration ~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.

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

<table>
<thead>
<tr>
<th>test compound</th>
<th>IC50 (mM)</th>
<th>% cross-reactivity</th>
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<tbody>
<tr>
<td>D-xylitol</td>
<td>4.0</td>
<td>100.0</td>
</tr>
<tr>
<td>D-ribose</td>
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<tr>
<td>t-arabinol</td>
<td>108.0</td>
<td>3.8</td>
</tr>
<tr>
<td>mezE-erythritol</td>
<td>108.1</td>
<td>3.8</td>
</tr>
<tr>
<td>D-sorbitol</td>
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</tr>
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</tr>
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<td>glycerol</td>
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<td>1.1</td>
</tr>
<tr>
<td>ethylene glycol</td>
<td>500.0</td>
<td>0.8</td>
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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-xylene and various sugar alcohols, meso-erythritol, D-xylitol, L-arabinol, erythritol, D-mannitol, L-rhamnose, D-galactitol, ethylene glycol, collagen, and L-lysine. The percent cross-
Antibodies Specific to Xylitol

Figure 6. Passive cutaneous anaphylaxis (PCA) assay performed in Swiss Webster mice (n = 6 per treatment group) that received repeated intradermal administration of antigens. The amount of different antigens used for immunization is 60 μg. 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 1, 52 haptens/mole; Xyt-BSA 2, 32 haptens/mole), D-xylitol, 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 ~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 haptens density D-xylitol-BSA conjugate (Xyt-BSA 1, 60 μg) 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 haptens density D-xylitol-BSA conjugate (Xyt-BSA 2, 60 μg). 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 xylitol-oxydation 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 antigen, 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 x 10^6, 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-xyllosyl-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-xyllosyl-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 μl. 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 haptens 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 haptens density (32 haptens/mole), D-xylose, and BSA showed a negative reaction, indicating that they cannot induce IgE response under identical conditions. Thus, the xylitol moeity 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-xylene 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.

IgE 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 xylolyl–lyxyl linkages (aldimine) and xylolyl–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 xylitolyl 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 homologous PCA.

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LITERATURE CITED

Antibodies Specific to Xylitol


