Synopsis of the thesis submitted for the award of Ph D degree (Biochemistry) of the University of Mysore, Mysore, India.

Title of the thesis: Antibodies to erythritol and xylitol: their characterization and applications

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The main uses of additives in foods are to ensure safety and wholesomeness, to improve keeping quality, to maintain nutritional value, to provide leavening or control acidity/alkalinity, to enhance flavor or impart desired color, and to maintain product consistency for preparation of processed foods. A food additive is a chemical (natural / synthetic) that is added to food to improve various parameters of the food. Sweeteners are a type of food additives added to food to replace sugar completely or in part. A variety of sweeteners exist to help consumers satisfy their desire for sweetness. Sugar alcohols (also known as polyols) are widely used as food additives and drug excipients due to their chemical inertness, non-hygroscopicity, sweetness and non-toxicity. They are considered as low-calorie bulk sweeteners due to their low calorific content. Since sugar alcohols do not increase blood glucose level, they are beneficial to diabetics.

Sugar alcohols have enjoyed an enviable record of safety. However, in recent times some adverse reactions due to sugar alcohols, though rare, have been reported in the medical literature. Hypersensitivity reactions to 10% or 20% (w/v) mannitol intravenous infusion have been reported in a small number of patients. An unusual case of mannitol hypersensitivity arising from ingestion of pomegranate and cultivated mushroom has been reported. Mannitol-specific IgE was demonstrated in the sensitized subject. There are reports of three well-described cases (two from US and one from Japan) of severe allergic or allergic-like adverse reactions after erythritol ingestion. All the three subjects experienced generalized urticaria. Puncture skin tests, and patch tests with different lots of erythritol were positive in all the cases. Basophil histamine release was high with erythritol in two subjects. The estimated prevalence of adverse reactions to erythritol-containing products is less than 1 per million.
One rare case of allergic reaction to xylitol has also been reported, recently. A 45-year-old Japanese man presented with oral erosions. An area of erythema with bullae was also found on his right thigh and on the right side of his chest. The subject also had an allergic reaction to the wrapping paper of the xylitol-containing chewing gum. Patch tests with various components of the chewing gum confirmed that xylitol was the causative agent of the allergic reaction. Sugar alcohols do not contain any reactive groups; hence, they are unreactive and non-immunogenic. A hypothesis for the mechanism of hypersensitivity to mannitol has been proposed which should be applicable to all sugar alcohols including erythritol and xylitol. Based on this hypothesis, attempts have been made to develop IgG and IgE antibodies to erythritol and xylitol in vivo, with the following objectives for the present study:

1. To develop monospecific polyclonal antibodies to erythritol in rabbits using the conjugate of the respective sugar on a carrier protein and to characterize the antibodies in terms of specificity.
2. To develop monospecific polyclonal antibodies to xylitol (a pentitol) in rabbits using the conjugate of the respective sugar on a suitable carrier protein and to characterize the antibodies in terms of homogeneity and specificity.
3. To develop an immunoassay for the detection and quantification of erythritol in certain foods utilizing the specific antibodies.
4. To develop a quantitative immunoassay for xylitol in some selected foods, using the specific antibodies.

The main focus of the present study is to demonstrate the immunogenicity and/or allergenicity of erythritol and xylitol in laboratory animals by utilizing hapten-carrier conjugates containing these sugar alcohol epitopes. The rabbit IgG antibodies obtained against erythritol and xylitol were utilized to develop immunoassays to detect and quantitate the respective sugar alcohols in some selected foods.
Chapter 1: General Introduction

This chapter starts with a general account on food additives, especially on sugar alcohols that are used as sweeteners in various foods and pharmaceuticals.

Some small molecules (<1000 Da) or haptens can generate immune response by conjugating themselves with macromolecular proteins. The structures of certain mono-/oligo- saccharides are immunogenic. Carbohydrates (mono-/oligo-saccharides) can be easily coupled to proteins by various chemical methods. Reductive amination is one such reaction where the Schiff base formed between the free amino groups of the protein and the aldehyde group of carbohydrate can be reduced by a mild reducing agent like sodium cyanoborohydride to form stable hapten-protein conjugates. Poly/m monoclonal antibodies have been raised against various mono-/oligo- saccharides using the respective hapten-carrier conjugates as immunogens.

Allergic reactions to sugars and sugar alcohols are rare. However, hypersensitivity reactions to some carbohydrates viz., dextrose, galactose etc., and to some sugar alcohols viz., mannitol, erythritol, and xylitol have been reported in the medical literature. Sugar alcohols do not contain any reactive groups, hence they are not immunogenic. A hypothesis has been proposed for the mechanism of hypersensitivity of these sugar alcohols. Mono/polyclonal antibodies raised against various carbohydrates can be used as diagnostic markers for the detection of carbohydrate antigens in diseased tissues, as analytical reagents for determining glycan constituents in food items and as models for studying genetic aspects of the biosynthesis and assembly of various plant and animal glycoconjugates.

The subsequent chapters have a general format of Introduction followed by sections on Materials and methods, Results, Discussion and a brief Summary and conclusions. Literature cited has been listed in an alphabetical order at the end (after General summary and conclusions) as Bibliography.
Chapter 2: Generation and characterization of IgG antibodies specific to erythritol

Erythritol is a four-carbon sugar alcohol, which is a white, anhydrous, non-hygroscopic, crystalline substance that is 60-70% as sweet as sucrose. Erythritol occurs naturally in a wide variety of fruits, vegetables and fermented foods. D-Erythrose 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. The conjugation was followed by the decrease in lysine groups using trinitrobenzenesulfonic acid assay. Periodic acid-Schiff (PAS) staining was performed to confirm the glycated nature of erythritol-BSA conjugate following the reductive amination. New Zealand white male rabbits (7-month-old) were immunized with 1 mg of the immunogen, erythritol-BSA conjugate. The immune serum was analyzed for hapten-specific antibodies, initially by dot-immunoblot later by non-competitive ELISA using the coated antigen format.

Purification of anti-erythritol antibodies was performed on erythritol-KLH-Sepharose CL-6B affinity column. The yield of erythritol-specific antibodies was found to be in the range of 36-45 µg/mL of rabbit antiserum. The affinity constant of the anti-erythritol antibodies as calculated by non-competitive ELISA was found to be $4.86 \times 10^6$. The isoelectric point of the anti-erythritol antibodies was found to be approximately 7.2. Inhibition ELISA was performed using various sugars and sugar alcohols to check the cross-reactivity of the purified anti-erythritol antibodies. Among the sugars and sugar alcohols tested, dithioerythritol showed the highest (~33%) cross-reactivity, followed by the isomers of erythritol, D-threitol (15.1%) and L-threitol (11.1%), and their dithioderivative, dithiothreitol (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%. The monospecific polyclonal antibodies to erythritol can be used in immunoassays for the identification and quantification of erythritol in biological samples, foods/processed foods and pharmaceuticals.
Chapter 3: Generation of IgG and IgE antibodies specific to xylitol, a haptenic allergen

Xylitol is a sugar alcohol that has been used as a food additive and sweetening agent since 1960s. The US FDA has approved the use of a ‘does not promote tooth decay’ health claim in labeling for sugar-free foods that contain xylitol. It is widely present in various fruits and vegetables, and human body produces 5-15 g xylitol/day. D-Xylose was conjugated to a carrier protein, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH), by reductive amination as described for erythritol. Immunization of New Zealand white male rabbits followed by immunochemical analysis of immune serum revealed the presence of specific antibodies to xylitol. Purification of anti-xylitol antibodies was performed on xylitol-KLH-Sepharose CL-6B affinity column. The yield of xylitol-specific antibodies was ~40 µg/mL of rabbit antiserum with an isoelectric point of ~7.2 and an affinity constant of 3.86x10^6. Xylitol-specific antibodies showed excellent specificity towards xylitol and <4.4% cross-reactivity with d-xylose and various sugar alcohols except ribitol and galactitol which showed ~11% and 8% cross-reactivity, respectively. Although the purified antibodies are specific for the xylitoyl moiety of xylitol-protein conjugates, they reacted to an extent of two-thirds with the Schiff base conjugate of xylosyl-protein conjugates.

D-Xylitol-BSA conjugates were tested for their intrinsic ability to induce IgE response in BALB/c mice by repeated intradermal administration without the use of an adjuvant. Passive cutaneous anaphylaxis (PCA) assay performed in Swiss-albino mice using the immune sera from BALB/c mice showed that BALB/c mice immunized with high hapten density D-xylitol-BSA conjugate (52 haptens/mole) produced a positive reaction (5-9 mm range) when compared to 2-4 mm range seen in mice immunized with moderate hapten density D-xylitol-BSA conjugate (32 haptens/mole). Ovalbumin was used as a positive control (6-11 mm range), whereas, D-xylitol, BSA and saline were used as negative controls (0-3 mm range). These studies using xylitol-protein conjugates confirmed the haptenic nature of xylitol.
Chapter 4: Development of an immunoassay for the detection and quantitation of erythritol in foods

Watermelon and commercial red wine were selected as the food sources for the detection and quantitation of erythritol. Watermelon was crushed without the addition of any buffer to obtain watermelon extract. The watermelon extract or red wine was subjected to stirred-cell ultrafiltration using 3K membrane (molecular weight cutoff = 3 kDa). Watermelon 3K-ultrafiltrate was analyzed by Ca\(^{2+}\)-moderated cation exchange chromatography on Dowex-50 W by elution with water. Polyol assay (using chromotropic acid reagent) was employed to detect sugar alcohols. A peak was observed exactly at the same position where meso-erythritol eluted on the same column in a separate run under identical conditions. The presence of erythritol was confirmed by subjecting the peak fractions (corresponding to the erythritol peak) from the Dowex-50 W column to gas-liquid chromatographic (GLC) analysis.

Monospecific polyclonal anti-erythritol antibodies were utilized to develop an indirect competitive immunoassay for erythritol. A calibration curve was prepared by creating competition between rabbit anti-erythritol antibodies (which are supposed to bind to the erythritoyl epitopes on erythritol-BSA conjugate used as coating antigen) and known concentrations of pure erythritol (0.1 to 100,000 ng). A linear range was obtained between 1-1000 ng of meso-erythritol concentrations. Both watermelon extract and wine were subjected to stirred-cell ultrafiltration using 3K-membrane. Both the 3K-ultrafiltrate and the whole extract of the two foods (containing erythritol) were used as the source of competing analyte in the immunoassay. Use of watermelon extract did not produce the expected results suggesting interference from macromolecular substances due to matrix effect. The amounts of erythritol from the immunoassay (3K-ultrafiltrates as competing analytes) were found to be 2.36 mg/100 g fresh wt. in watermelon and 206.7 mg/L of red wine which are in good agreement with the values reported in the literature by HPLC and GC methods (2.2-2.4 mg/100 g fresh weight for watermelon and 130-300 mg/L for red wine). Since apple contains only sorbitol as the main polyol, apple 3K-ultrafiltrate was used as a negative control in this immunoassay.
Chapter 5: Development of an immunoassay for the detection and quantitation of xylitol in foods

Onion and strawberry were selected as the food sources for the detection and quantitation of xylitol. The onion (or strawberry) extract was subjected to stirred-cell ultrafiltration using 3K membrane. Immunoaffinity column was prepared by coupling rabbit anti-xylitol antibodies (~1.5 mg) to 2 mL of Sepharose CL-6B. Onion extract (3K-ultrafiltrate) was passed through the column and eluted using 0.1 M glycine-HCl, pH 2.9. The eluate from the immunoaffinity column was analyzed for polyols by Ca\(^{2+}\)-moderated cation exchange chromatography on Dowex-50 W by elution with water. A peak was seen exactly at the same position where D-xylitol eluted from the same column in a separate run under identical conditions. Analysis of the eluate by GLC showed that the capacity of immunoaffinity column is only 68% of the theoretical value (2.85 \(\mu\)g xylitol). Although the immunoaffinity method is good for detection and quantification of small amounts of xylitol in low abundance foods, its capacity is low and is not cost-effective.

Immunoassays are useful in detecting and quantitating very small amounts of analytes. Specificity and sensitivity are the two main parameters that determine the efficiency of an immunoassay. Rabbit anti-xylitol antibodies were utilized to develop an indirect competitive immunoassay for D-xylitol. A calibration curve was prepared by creating competition between anti-xylitol antibodies (which are supposed to bind to the xylitol epitopes on xylitol-BSA conjugate used as coating antigen) and known concentrations of pure xylitol (0.1 to 100,000 ng). A linear range was obtained between 1-1000 ng of xylitol concentrations. Onion and strawberry 3K-ultrafiltrates and their whole extracts (containing xylitol) were used as sources of competing analyte in the immunoassay. Use of onion and strawberry extracts did not produce the expected results suggesting interference from macromolecular substances due to matrix effect. The amounts of xylitol from the immunoassay (3K-ultrafiltrates as competitors) were found to be 12.6 mg/100 g in onion and 44 mg/100 g strawberry which are in good agreement with the reported values by HPLC and GC methods (12.91 mg/100 g fresh weight for onion and 44.16 mg/100 g fresh weight for strawberry).
General summary and conclusions

The thesis ends with a comprehensive summary and conclusions describing the key observations and salient aspects that emanated from the present study described in Chapters 2-5.

List of publications


4. Sreenath K, and Venkatesh YP. Utility of antibodies specific for xylitol in the quantification of xylitol in foods by indirect competitive enzyme-linked immunosorbant assay. (to be submitted to *J. Agric. Food Chem*.)

List of patents submitted
