

Immunological Analysis of the Alternate Rubber Crop *Taraxacum kok-saghyz* Indicates Multiple Proteins Cross-Reactive with *Hevea brasiliensis* Latex Allergens

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Abstract

The public health risk of Type I natural rubber latex allergy, caused by residual proteins in *Hevea brasiliensis* rubber latex (HNRL) products, has led to some medical examination and surgeon's gloves and other health-related products being made from synthetic polymers. However, they are generally not preferred by healthcare providers due to their physical limitations. Guayule latex (GNRL), from an alternate rubber crop *Parthenium argentatum*, has been proven to contain none of the protein antigens present in *H. brasiliensis* natural rubber latex and its products. Guayule latex has also exhibited excellent film properties. Another alternate rubber crop, *Taraxacum kok-saghyz*, is now under commercial development, and internet reports assume that, like guayule, its latex derived rubber is free of proteins that are cross-reactive with HNRL specific IgE antibodies. Thus, the assumption is that it will not trigger allergic reactions in Type I HNR latex allergic individuals. Using ELISA and immunoblot methods, we have tested the reactivity of HNRL protein specific murine monoclonal and polyclonal antibodies, rabbit polyclonal IgG antibodies and human IgE antibodies from clinically HNRL allergic individuals against *T. kok-saghyz* latex and its purified rubber particles. We demonstrate that *T. kok-saghyz* latex contains multiple HNRL cross-reactive proteins, which importantly react with HNRL latex specific human IgE antibodies from Type I latex allergic individuals. Exposure of HNRL allergic individuals to *T. kok-saghyz* latex may thus place them at risk for allergic reactions.

Keywords: Type I latex allergy; Dandelion; Guayule; *Taraxacum kok-saghyz*; Epitopes; Alternate rubber

Introduction

A quantity of at least 1.2 million MT/yr of irreplaceable natural rubber (defined as a strategic raw material) is currently required by the United States for its military, industrial, transportation, medical and consumer manufacturing sectors. There are over 40,000 different products made with natural rubber and over 400 medical devices [1].

Globally, almost all commercial natural rubber (>11 million MT/yr) is collected from a single source, *Hevea brasiliensis*, the Brazilian or para rubber tree. About 89% is converted to solid rubber of various grades. The remainder is concentrated into a stabilized form of latex with approximately 60% rubber content. However, the use of a single species of natural rubber plant to generate the global supply of this strategic commodity is not necessary, because many different plants [1] make natural rubber, as do some *Lactarius* sp. fungi [2-4]. Of the many plants capable of natural rubber production, two temperate species stand out as commercial candidates, *Parthenium argentatum* Gray (guayule) and *Taraxacum kok-saghyz* Rodin (Buckeye Gold, also known as Kazak dandelion, Russian dandelion and TKS). These alternate rubber species are under development for commercial use at a number of universities and companies on several continents. Both species make high quality rubber. *T. kok-saghyz* rubber is similar to *H. brasiliensis* rubber in composition and performance [5]. In contrast, guayule rubber is naturally softer and more elastic than the other two [6,7].

The highest value market for natural rubber latex is the medical arena, with medical examination and surgeon's gloves being the largest consumer product type made of latex [8]. Unfortunately, a processing change in the 1980's left high levels of soluble protein in the Hevea latex glove matrix which led to a large number of people becoming exposed

and sensitized (IgE antibody positive) with subsequent manifestations of Type I hypersensitivity referred to as natural rubber latex allergy [9-13]. HNRL proteins in products elicited high levels of IgE anti-HNR latex protein that when inhaled or adsorbed caused the release of vasoactive mediators from mast cells and basophils following HNRL allergen exposure. The clinical evidence that an individual had become "sensitized" was upper airway rhinitis and lower airway related asthma. Some individuals who were highly sensitized to HNRL proteins experienced life-threatening allergic symptoms involving anaphylaxis following an airborne or contact exposure to HNR latex allergens. This proved fatal in a number of high profile cases [13-17]. Most manufacturers now ensure that their latex gloves and other medical products are thoroughly leached and a lower number of new cases of Hevea latex allergy are now reported. The incidence of new cases in most developed countries has decreased dramatically in recent years as a result of effective avoidance tactics and lower allergenic content in rubber products [12,18]. Natural rubber latex, however, remains the best and most suitable protective material for high performance medical gloves during surgery [18].

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Guayule (*Parthenium argentatum*) latex (GNRL) is much lower in protein than HNRL, and it has no protein epitopes which cross-react with anti-HNRL protein specific antibodies [8]. A three year study of occupationally exposed workers found no evidence of sensitization [19]. HNRL allergic people can use dipped medical and consumer products made from GNRL, without exhibiting any allergy symptoms. This guayule-specific property has led to a widely disseminated assumption that other alternate rubber-producing species also will not contain proteins with epitopes which cross-react with anti-HNR latex protein antibodies. In this paper, we scientifically address the question of whether or not latex from *T. kok-saghyz* is free of allergenic proteins implicated in Type I HNRL allergy. This supposition is claimed in numerous internet listings, promoting use of *T. kok-saghyz* latex and rubber as a safer alternative to HNR latex, like GNRL, rather than as a supplement or addition to the *H. brasiliensis* rubber and latex supply. However, no scientific data support these assumptions.

Method

Latex was harvested from the roots of *T. kok-saghyz* and either mixed with sample buffer, or collected in sufficient quantities to prepare 3x washed rubber particles (WRP). For WRP, *T. kok-saghyz* latex was collected from its roots into a collection buffer of 100 mM Tris-HCl, pH 7.5, 5 mM Mg₂SO₄ buffer. Washed rubber particles (WRP) were prepared from *T. kok-saghyz* latex as described previously [20]. *H. brasiliensis* latex (from clone RIMM600) was obtained from a plantation in Malaysia and WRP also were prepared [20]. Latex and WRP of each species were mixed with 1x NuPAGE SDS sample buffer, with 50 mM dithiothreitol (DTT), vortexed, denatured for 5 min at 100°C. The samples were vortexed, then spun for 3 min to remove rubber, and these two steps were repeated twice more. Proteins were quantified using the modified Lowry method. The denatured proteins were loaded onto NuPAGE 4-12% Bis-Tris gels and run in a buffer of 50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3 for 35 min at 200V. Latex and WRP proteins latex and WRPs proteins were loaded onto the gels in the amount of 0.5 µg and 14 µg for silver stain and Coomassie Blue, respectively [20]. Similar gels were transferred onto PVDF membranes and reacted with various antibodies (Table 1).

Each gel was blotted to Immobilon-PVDF transfer membrane in a transfer buffer of 25 mM bicine, 25 mM Bis-Tris, 1 mM EDTA, 0.05 mM chlorobutanol (pH 7.2), 10% methanol for 60 min at 30V. The blots were then blocked for 30 min in 5% non-fat milk in 1xPBS/0.05% Tween-20. The blots were incubated with purified animal antibodies (mouse monoclonal antibodies, or rabbit IgG ASTM D6499, IRM#

914), or two distinct human serum pools: IgE anti-*H. brasiliensis* latex serum pool (n=53 children only with spina bifida, 1-16 yrs old, lot F8137); and serum from a mixed surgical pediatric and adult healthcare worker population containing IgE anti-*H. brasiliensis* latex (n=101, lot F8135). The antibodies used for the immunoblots blots were diluted at 1:20,000 (anti-AL); 1:500 (Hev b1, b3, b5, b6.02); 1:333 (F8137 and F8135); 1:15,000 (anti-rabbit IgG) and 1:15,000 (anti-Human IgE). All samples were incubated for 1 hr with shaking in 1xPBS/0.05% Tween-20, subsequently washed for three times for 10 min each in 1xPBS/0.05% Tween-20. The blots were then incubated with an anti-Human IgE peroxidase conjugate (Sigma-Aldrich) (1:15,000) and Bio-Rad Precision protein Streptactin-HRP conjugate (1:15,000) for 1 hr with shaking in 1xPBS/0.05% Tween-20 and then washed. The blots were then detected by Luminata Forte western HRP substrate (Millipore) and visualized by chemiluminescence imaging with Bio-Rad chemiDoc XRS.

For ELISA analysis, proteins were extracted from the *T. kok-saghyz* latex following the procedure outlined in the ASTM D1076 Standard. A 500 µL sample of the *T. kok-saghyz* latex was mixed with 450 µL extraction buffer (50 mM Na₂PO₄ pH 7.4) and 50 µL 20% SDS with agitation for 2 h at room temperature. The samples were then centrifuged for 15 minutes at 21,000 x g and the clarified aqueous phase removed. Total protein content was determined according to the ASTM D5712 procedure both with and without background subtraction.

The ASTM D6499-12 ELISA Inhibition Assay was used to determine the amount of cross-reactive protein. The standard and test samples were serially diluted in a 96 well plate, after which an equal volume of diluted rabbit anti-HNRL polyclonal antibody was added. After a 2 h incubation, the sample from each well was transferred to the corresponding well in a HNRL-protein-coated plate which had been blocked with non-fat dry milk. After another 2h incubation, the plates were then washed with buffer and a 100 µl solution of Goat anti-Rabbit IgG-HRP was added. After a final incubation, plates were washed and the substrate O-phenylenediamine was added to each well. Color was allowed to develop until the reaction was stopped by the addition of H₂SO₄. The plates were then read at 490 nm and protein values were determined by interpolation from a reference calibration curve.

Results and Discussion

Protein concentration of *T. kok-saghyz* fractions

The total protein content of the *T. kok-saghyz* latex and WRP fractions (Table 2) were determined on the clarified aqueous fraction.

Protein	Animal	Anitibody	ASTM or lot #	# Samples
Total Hevea latex protein	Rabbit	Polyclonal IgG	D6499	multiple
ditto	Mouse	Polyclonal IgG	none	few
Hev b1	Mouse	Monoclonal (58/14.6 kD)	D7427	n/a
Hev b3	Mouse	Monoclonal (24-27 kD)	D7427	n/a
Hev b5	Mouse	Monoclonal (16 kDa)	D7427	n/a
Hev b6.02	Mouse	Monoclonal (4.7 kDa)	D7427	n/a
Glove	Adults,HCW, spina bifida	Polyclonal IgG	F8135	101
Glove	Children with spina bifida	Polyclonal IgG	F8137	53

Table 1: Summary of the different immunochemical antibodies used to test against *T. kok-saghyz* latex and WRP proteins.

Sample	5712 with bs (µg/mL)	5712 w/o bs (µg/mL)
Latex	293	1,891
WRP	785	1,398

Table 2: ASTM D5712 protein concentrations of *T. kok-saghyz* fractions with and without background subtraction (bs).

The more refined WRP clarified aqueous fraction was colorless while the crude latex fraction was brown in color. This pigmented material may be the reason for the higher background values seen in the crude latex fraction (Table 2). This pigmented crude *T. kok-saghyz*-latex material was assayed using the method in the ASTM D6499 ELISA which was developed to specifically detect HNRL proteins. The protein concentration of the extract varied between 32 and 55 µg/mL. This variation is likely due to the large dilution factors used as well as possible interference from those materials observed to interfere with the D5712 protein quantification test. Further purification of this material may be necessary to obtain more consistent results.

T. kok-saghyz latex and WRP contained similar amounts of protein to *H. brasiliensis*, and many proteins of different molecular weights are apparent in both their latex and rubber particles (Figure 1). In both gels, the staining was halted when any single lane verged on becoming

overstained, and so not all proteins present were visualized. The proteins in the *T. kok-saghyz* WRP lanes are smeared, which suggests that some hydrolysis has occurred in this particular protein preparation. The HNRL antigenic protein ELISA (ASTM D6499) was used to evaluate protein preparations similar to those visualized in Figure 1.

The rabbit anti-HNRL total protein IgG polyclonal antibodies (ASTM D6499, IRM# 914) reacted with *H. brasiliensis* and *T. kok-saghyz* proteins blotted onto nitrocellulose membranes from the gels (Figure 2).

Multiple proteins from *H. brasiliensis* latex were recognized by the polyclonal antibodies, indicating that these were antigenic in rabbits. As has been seen previously [18,21], a number of immunogenic proteins (Hev b1 and Hev b3, for example) are attached to the *H. brasiliensis* rubber particles and are not solely detected in the soluble fraction of the

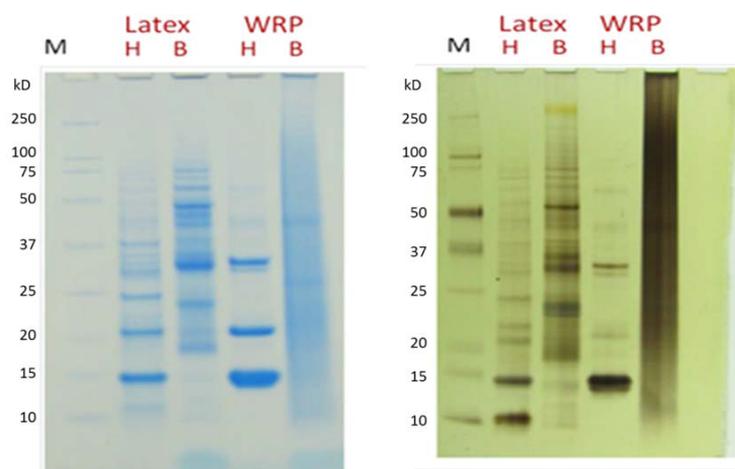


Figure 1: SDS-PAGE gels of latex and washed rubber particles (WRP) from *Hevea brasiliensis* (H) and *Taraxacum kok-saghyz* (B). The gel to the left is stained with Coomassie Blue, the gel to the right with silver stain. Lanes marked M is molecular weight markers with their molecular weights to the left of the lane.

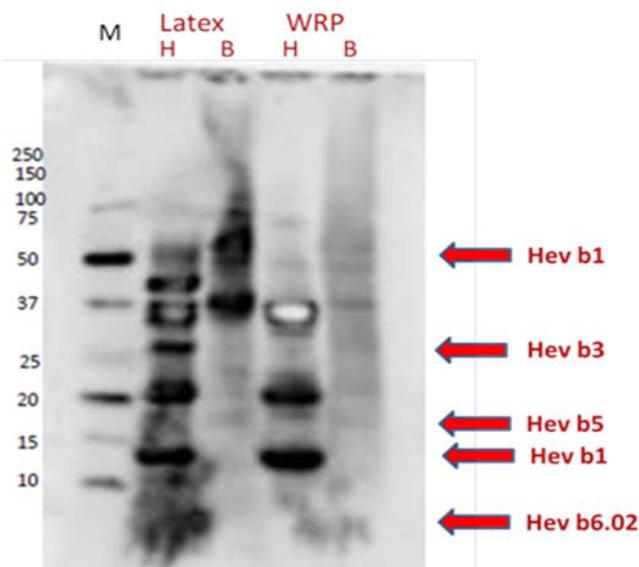


Figure 2: Immunoblot of proteins from latex and washed rubber particles (WRP) from *Hevea brasiliensis* (H) and *Taraxacum Kok-saghyz* (B). Lane M contains molecular weight markers. The red arrows indicate the positions of the most allergenic of the Hevea latex antigens.

cytosol (the C-serum). Similar results were obtained with mouse anti-HNRL latex protein polyclonal antibodies (immunoblot not shown). Of the many *H. brasiliensis* immunogenic latex proteins, 15 have been identified as being clinically significant in human Type I latex allergy [22]. Monoclonal antibodies were raised against recombinant versions of these allergenic proteins and four of them are included in the FIT kit (Icosagen). ASTM D7427 describes a standard ELISA method to use four of these antibodies to assess cross-reactivity. These antibodies were used to identify cross-reactive *T. kok-saghyz* latex and WRP proteins in immunoblots in direct comparison with *H. brasiliensis* latex and WRP proteins (Figure 3). Hev b1 is the 14.6 kD “rubber elongation factor” and is clearly seen in both the *H. brasiliensis* latex and WRP. This antibody also recognizes structurally-related proteins, including one at 58kD.

The Hev b1 specific antibody clearly cross-reacts with at least seven proteins in *T. kok-saghyz* latex, although no proteins were visualized for the WRP preparation (Figure 3). Hev b1 is the *H. brasiliensis* 14.6 kD REF (“rubber elongation factor”) protein, which often forms higher molecular weight aggregates, and shares sequence similarity with Hev b3, and a soluble CPT (cis-prenyl transferase, 37 kD). CPT also quite strongly associates with the rubber particle surface through hydrophobic interactions, but can be washed away without losing particle-bound rubber transferase activity. Multimers of CPT are also sometimes seen on SDS-PAGE gels. Hev b3 is the 24-27 kD SRPP “small rubber particle protein” and cross-reactive proteins to its monoclonal antibodies are detected in the *H. brasiliensis* latex and WRP, and in the *T. kok-saghyz* latex. This antibody cross-reacts with larger and smaller molecular weight proteins in both latex and WRP in *H. brasiliensis*, including with the 37 kD CPT. We hypothesize that the sequence similarity of these proteins also leads to epitopic similarity in the translated proteins. However, in *T. kok-saghyz*, multiple latex proteins contain epitopes which are recognized by the Hev b3 specific monoclonal antibody. It seems likely that some of these are multimers or aggregates of SRPP and or CPT. Hev b5 is the 16 kD acidic protein and although the immunoblot was not sufficiently sensitive to visualize

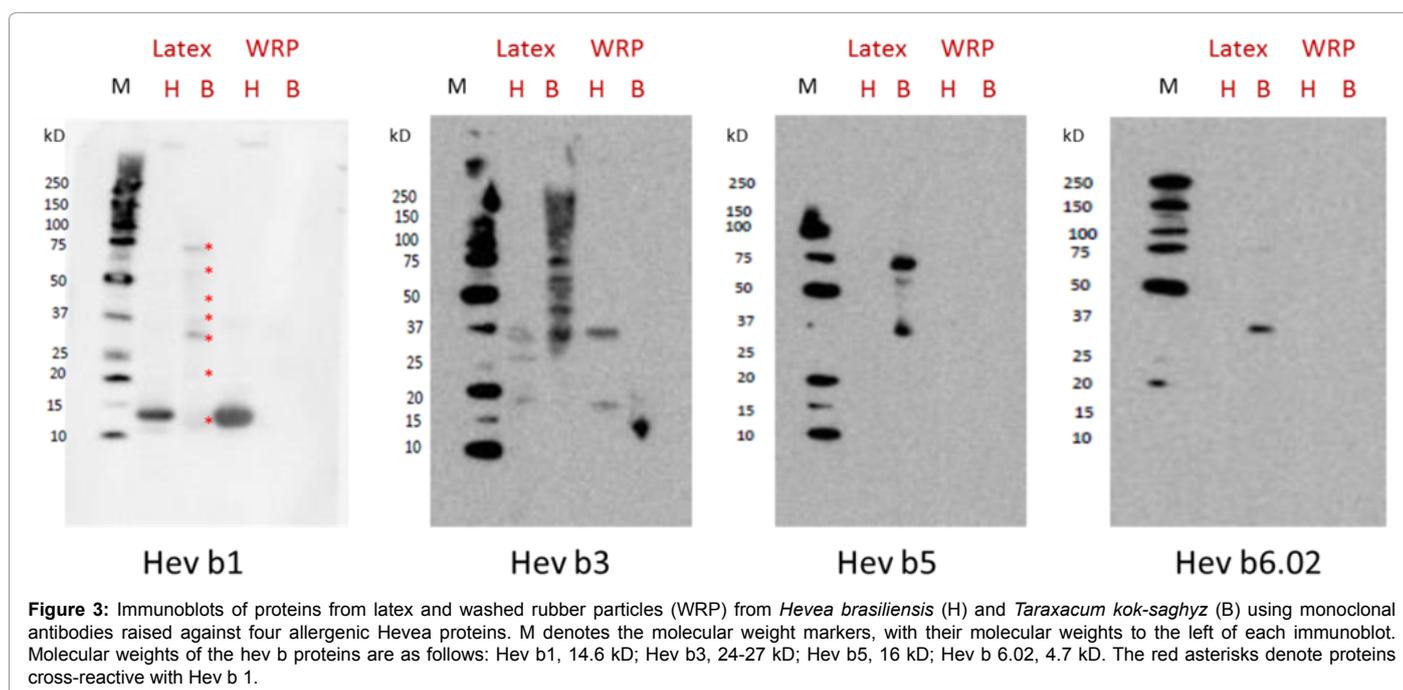
this in the *H. brasiliensis* latex lane, the monoclonal antibodies to this protein quite strongly cross-reacted with three proteins in *T. kok-saghyz* latex (specific identities not known at this time), suggesting that these proteins might pose a risk of triggering allergic reaction in latex-allergic patients. A similar issue arises with the anti Hev b6.02 immunoblot (Figure 3). Although Hev b6.02 is a very small protein, the 4.7 kDa antifungal hevein, cross-reactive epitopes of at least three other proteins in *T. kok-saghyz* latex were visualized, especially a protein of about 30 kD. The much fainter higher molecular weight bands suggest aggregates or multimers of this protein. However, hevein is a protein derived from prohevein, with is 21.9 kD. It seems possible that the cross-reactive *T. kok-saghyz* 30 kD latex protein could be related to this larger progenitor.

Clearly, significant cross-reactivity was detected between *T. kok-saghyz* proteins and anti-HNRL antibodies, in both the soluble protein fraction and the rubber particle bound fractions. However, since these results were obtained with rabbit polyclonal and mouse monoclonal antibodies, the potential impact on humans, specifically Type I latex allergic patients, is only inferred.

We directly examined this issue, by using human IgE antibodies from two groups of Type I HNRL allergic patients. One group consisted of a mixture of children sensitized during surgery and HNRL allergic healthcare workers while the other consisted strictly of spina bifida patients. The IgE from both groups cross-reacted with multiple proteins in *T. kok-saghyz* latex and WRP (Figure 4). Interestingly, the most predominant reactive protein in *H. brasiliensis* latex is around 37 kD in size, and so is likely to be CPT. This protein is not yet implicated in clinically symptomatic Type I latex allergy, but seems quite clearly to not be large enough to be the Hev b13, 43 kD esterase – the closest in size on the allergen list.

It should be noted, that even if we do not fully understand the basis for the observed cross-reactions, the fact that they exist at all, may pose a significant of allergic reaction in Type I latex-sensitized humans.

The presence of cross-reactive proteins does not mean that *T. kok-saghyz* latex and rubber cannot be used effectively in commercial



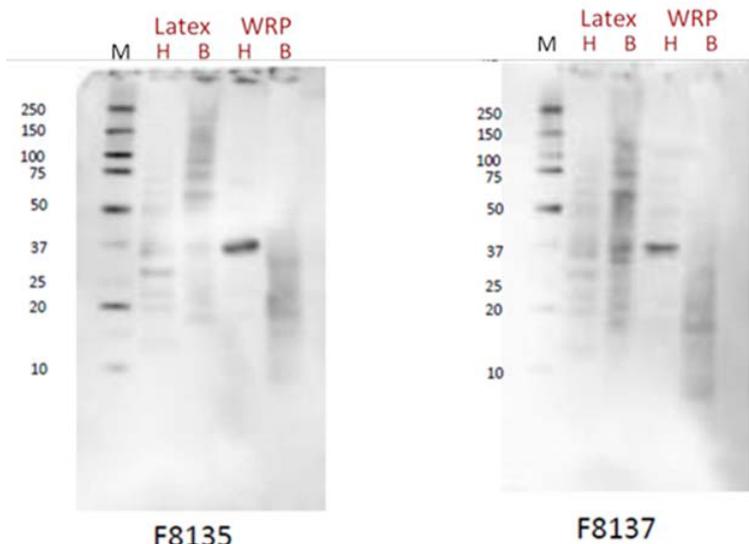


Figure 4: Immunoblot of proteins from latex and washed rubber particles (WRP) from *Hevea brasiliensis* (H) and *Taraxacum Kok-saghyz* (B), using IgE antibodies from two human serum pools. Lane M contains molecular weight markers. F8135 is pooled from 101 patients in a mixed surgical pediatric and adult healthcare worker population. F8137 is pooled from 53 children with spina bifida, 1-16 yrs old.

products. They should just be used in the same way that *H. brasiliensis* latex and rubber products are currently used. Moreover, most of the rubber in soil-grown *T. kok-saghyz* roots has been irreversibly coagulated before harvest, and is not in latex form [23]. It thus seems unlikely that latex production from such plants will present a viable commercial opportunity to serve as a substitute for Hevea latex, and that efforts should concentrate on solid rubber applications.

Conclusion

Rubber and latex from *T. kok-saghyz* contain multiple proteins which share epitopes with antigenic and allergenic proteins from *H. brasiliensis* latex. This does not mean that the latex and rubber cannot be used commercially. The application of good manufacturing practices currently in use, should serve to keep protein levels low and prevent products from sensitizing people. However, as is the case for products made with *H. brasiliensis*, latex and rubber, the proteins in *T. kok-saghyz* products pose a risk for inducing allergic reactions in previously Hevea latex sensitized patients. Thus, while *T. kok-saghyz* rubber can substitute for *H. brasiliensis* rubber, it is not a Hevea latex allergy-safe alternative, a position still uniquely held by guayule.

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