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Chapter 2

Soybean-Derived Thyroglobulin As an Analyte Specific Reagent for *In Vitro* Diagnostic Tests and Devices

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Abstract

Presently, human thyroglobulin is extracted from cadaver or surgically resected thyroid tissue. Such reliance on human organs for recovery and purification of proteins required for diagnostic applications complicates the manufacturing process from acquisition, to purification and quality control. These limitations have defeated efforts to establish a universally recognized standard, preventing normalization of assays to detect circulating thyroglobulin or antibodies against this protein.

Unfortunately, producing intact, recombinant human thyroglobulin in protein expression systems is a daunting task that, until recently, has not been successful. We have achieved expression of full-length human thyroglobulin in stable lines of soy, and have been able to purify this protein from transgenic soybean seeds.

This accomplishment provides several manufacturing advantages, as well as some potential solutions for the problems encountered when using thyroid-extracted thyroglobulin.

The uniformity of recombinant protein is especially attractive when one considers the numerous post-translational modifications occurring in thyroid cells.

The ease of production and purification of soybean-derived thyroglobulin is cost effective, but also allows for manufacturing flexibility in that protein expression can be separated in time and geography from the final purification of this protein in soy powders. Taken together, soybean-derived thyroglobulin has characteristics consistent with those necessary and desired for an industry standard.

The availability of a renewable source of recombinant human thyroglobulin also has implications for solving some of the open-ended problems in diagnostic assays which

employ this protein. Immobilization or radiolabeling heterogeneous lots of thyroid-derived thyroglobulin are variables which should be minimized with more homogenous starting material. Furthermore, the ability to remove interfering anti-thyroglobulin antibodies from patients' sera or tissue homogenates could also be accomplished using devices constructed from a plentiful source of renewable thyroglobulin.

Our ability to express full length human thyroglobulin in transgenic soybean seeds provides, for the first time, a practical solution for the many limitations inherent in present day diagnostic assays that currently must rely on a protein isolated from primary thyroid tissue.

Introduction

Thyroglobulin is the most abundant protein in the thyroid gland and functions as a storage protein for thyroid hormones [1]. During its synthesis in thyrocytes, the 8.7 kb mRNA transcript is translated and routed to the endoplasmic reticulum where correctly folded monomers proceed to the golgi apparatus for glycosylation. Molecules which are not correctly folded are degraded in the endoplasmic reticulum. This immature thyroglobulin molecule then "matures" in the golgi to acquire variable amounts of carbohydrate residues (approximately 5%-10%) to a monomeric molecular weight of approximately 330 kDa. Following homodimerization, the 660 kDa protein is then exported into thyroid follicles where variable iodination of tyrosine residues occurs ranging from approximately 0.01% to 1.0% of the molecule [1]. In this manner "mature", dimeric thyroglobulin serves as a reservoir for the thyroid hormones, triiodothyronine (T3) and thyroxine (T4) [2].

The complexity of thyroglobulin's maturation as a thyroid storage protein results in a variety of forms being present in the thyroid gland. "Immature" thyroglobulin (i.e. no glycosylation and no iodination), along with a spectrum of maturing or "mature" forms of this protein containing varying amounts of carbohydrate and iodine residues are always present [1].

The presence of such a variety of thyroglobulin forms within the whole organ presents significant challenges when attempting to isolate a mature, dimeric protein for use in diagnostic assays.

Intact, Dimeric Human Thyroglobulin As an Analyte Specific Reagent: Thyroid Cancer and Thyroiditis

Thyroid cancer is the most common type of endocrine malignancy. Based on estimates from the National Cancer Institute, 62,980 new cases of thyroid cancer will occur in the United States in 2014 with an estimated 1,890 deaths (<http://seer.cancer.gov/statfacts/html/thyro.html>). From 2007-2011, the median age at diagnosis for cancer of the thyroid was 50 years of age with the median age for death being 73 years of age. Five year survival rates for thyroid cancer for 2004-2010 were 97.8%. While the prognosis for most thyroid cancer patients is very good, the rate of recurrence can be up to 30%, and recurrences can occur even decades after the initial diagnosis. Therefore it is recommended that patients

have routine follow-up examinations and monitoring for increased serum thyroglobulin levels throughout their remaining lifetime [3-7].

In summary, new thyroid cancers patients are identified daily, and most of these patients will live for decades following diagnosis. Since recurrence of thyroid cancers is common, routine monitoring of patients is essential. Immunoassays to quantify thyroglobulin levels are an important tool for diagnosis and the monitoring process for these patients [8].

As a group, thyroiditis is second only to diabetes as the most common endocrine disorder in the United States [9, 10]. Almost 100,000 new cases per year of clinically diagnosed thyroid disease occur, making the number of individuals with some form of thyroid disease an estimated 4-5 million people in the United States.

Similar percentages of thyroiditis are found in other developed countries with estimates of clinical disease ranging from 8-10 million in Europe. Hypothyroidism accounts for the majority of the cases of clinical thyroid disease in the United States and other developed countries, with autoimmune Hashimoto's thyroiditis accounting for about 80% of hypothyroiditis [11].

The prevalence of such types of spontaneous autoimmune thyroiditis range from 1% to 5% of the population in developed countries, with women having a 10 fold higher risk of developing clinical disease than men [12].

The incidence and severity of hypothyroidism in women also increases greatly during the latter years of life [13, 14].

Thyroglobulin has been used as a reagent in kits for diagnosing thyroid diseases and cancers, making these assays some of the longest and most frequently used in the industry. Studies have suggested expanding the current guidelines toward more frequent diagnostic testing including expanded fine-needle aspirate tests [15], and for the use of thyroglobulin assays for prognosis [7]. To summarize, the following types of FDA approved assays contain thyroglobulin as a key component: 1) detection of thyroglobulin to diagnose recurrent thyroid cancer in patients who have recently had thyroid ablation; 2) detection of thyroglobulin in fine needle lymph node aspirates to diagnose metastatic thyroid cancers; 3) pre-screening sera for the detection of autoantibodies against thyroglobulin prior to performing diagnostic assays for thyroglobulin. The presence of such antibodies can make quantifying thyroglobulin using current diagnostic assays "indeterminant"; 4) yearly thyroglobulin screening for thyroid cancer survivors previously negative for recurrent disease; 5) quantification of thyroglobulin levels in thyroid cancer survivors as a possible prognostic indicator of future recurrent disease; and 6) detection of autoantibodies against thyroglobulin as a diagnostic for pre-clinical or active autoimmune thyroiditis.

Isolation of Mature, Dimeric Thyroglobulin from Human Tissue: Commercial Sources

Commercially available human thyroglobulin is presently isolated from human cadaver or surgically resected thyroid glands [16]. One such purification scheme was proposed in the mid 1990's, and resulted in an attempt to provide a reference standard for the industry, designated CRM 457 [17, 18].

The characterization of CRM 457 was consistent with standards for that era, but was limited to partial amino acid composition, mass determinations using the Lowry method, and absorbances to define this “world standard”. Unfortunately, present day requirements for analyte specific reagents are becoming more stringent [19].

As discussed below, CRM 457 has not solved the problems inherent in the batch-to-batch inconsistencies observed when isolating mature, dimeric thyroglobulin from vats of human thyroid glands over the last two decades.

In our ongoing investigations into the expression and purification of human thyroglobulin, we have made two observations. The first is the fact that different commercial lots of this protein vary in their contents. Figure 1 shows SDS-PAGE analyses of human thyroglobulin that was purchased from three different commercial sources.

Observations made from this representative example are consistent with what we have found over the years. Sometimes there is little protein material at the appropriate molecular weights for monomers (~330 kDa) in denaturing/ reducing gels or dimers (~660 kDa) in native gels. This has necessitated our screening of each lot of purchased material to ascertain what is actually in each vial.

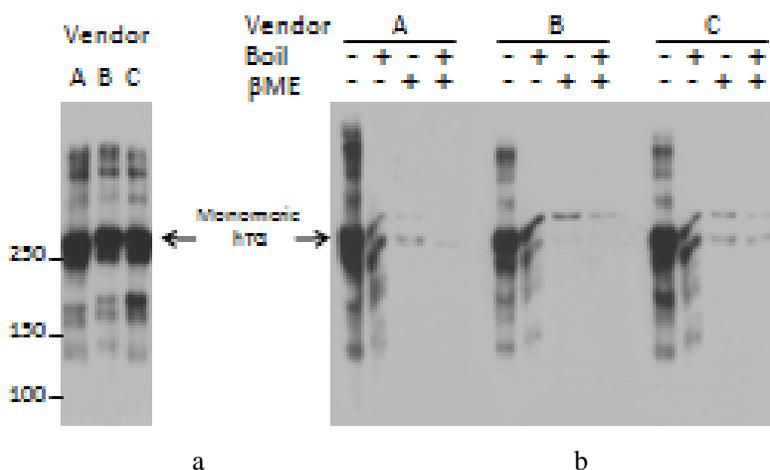


Figure 1. SDS PAGE and western analysis of commercially-prepared thyroglobulin. Panel A: Equivalent amounts (400 ng/lane) of purified human thyroglobulin purchased from Alpha Diagnostics International (Vendor A), AbD Serotec (Vendor B) and Sigma (Vendor C) were separated in 5% SDS-PAGE gels and transferred to membrane prior to western analysis employing a rabbit anti-thyroglobulin polyclonal antibody (primary antibody) and HRP-conjugated goat anti-rabbit secondary antibody for detection. The purified thyroglobulin samples were suspended in running buffer devoid of reducing agent and were not boiled prior to gel electrophoresis. The sizes of molecular weight standards (in kDa) are shown and the location of monomeric thyroglobulin protein is indicated by the arrow. Panel B: Equivalent amounts (400ng/lane) of purified human thyroglobulin protein was suspended in running buffer either devoid (-) or containing (+) reducing agent. Duplicate samples were either not boiled (-) or boiled (+) for 5 min. prior to loading into 5% SDS-PAGE gels. The location of monomeric protein is indicated with an arrow. Incubation with reducing agent or boiling significantly diminishes the signal.

Sometimes there is a smear of proteins that vary significantly in molecular weight, with some being much larger than monomers (~330 kDa) in denaturing gels, and some being much smaller in size. Presumably this is due to aggregation or to degradation of the intact molecule.

Taken together, these results demonstrate the variability in commercially available thyroglobulin preparations that claim to be purified. A second observation relates to the stability of isolated thyroglobulin preparations.

Figure 1 also shows SDS-PAGE analyses of human thyroglobulin that has been subjected to various manipulations. In general, the addition of a reducing agent and/or boiling thyroglobulin in SDS destroys the protein.

Freeze thawing reconstituted protein is also deleterious and the shelf life of the protein maintained at 4°C is limited (data not shown). Taken together, these results demonstrate the labile nature of this storage protein once it has been isolated from the thyroid microenvironment.

Why are commercial preparations so variable? This results from several factors, including the apparent innate instability of this purified protein. It is logical to assume that the numerous molecular species of thyroglobulin present in thyroid tissue also contributes to these observed lot-to-lot variations. Protein purification schemes must distinguish between “immature” and “mature” forms of thyroglobulin, as well as incorrectly folded proteins targeted for degradation. In addition, mature thyroglobulin molecules also differ in their extent of glycosylation (5%-10%) and iodination (0.01% to 1.0%), making even the purified, homodimeric protein somewhat variable in composition.

Disadvantages of Isolating Thyroglobulin from Human Thyroid Tissue

With the advent of recombinant protein technologies, many proteins used in diagnostic tests are manufactured using various protein expression systems. However thyroglobulin must be purified from human cadaver or surgically resected thyroid tissue [16-18]. The necessity on isolating native thyroglobulin from human thyroid tissues has its limitations. First, the logistics of obtaining “fresh” human thyroid tissue and the precautions necessary for protection against transmissible diseases add to the cost of production. Second, the complexity of thyroid tissue dictates that thyroglobulin purification schemes are involved [17, 18] and result in the isolation of a myriad of thyroglobulin isoforms. As noted before, mature thyroglobulin molecules also differ in their extent of glycosylation (5%-10%) and iodination (0.01% to 1.0%) [1, 16], making even the purified, homodimeric protein somewhat variable in composition.

Due to this inherent limitation each lot of thyroglobulin isolated from human tissue can differ in its overall composition. Third, thyroglobulin is commercially available from numerous suppliers and there is evidence to suggest that each supplier’s thyroglobulin may not be interchangeable in a particular in vitro diagnostic test. For example, a relatively recent recall was issued for one of the AccessTM assays due to a change in the thyroglobulin that was used in these kits (<http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfres/res.cfm?id=92965>). Fourth, since thyroglobulin is isolated from human thyroid glands, each lot must be screened for infectious agents (e.g. HIV, hepatitis virus, etc.), and Material Safety Data Sheets (MSDS) routinely list this analyte specific reagents as a biohazard.

The necessity to screen lots for contaminating biological agents also adds to the cost of production, and increases possible product liability.

Limitations with Current Diagnostic and Prognostic Assays Which Use Human Thyroglobulin

In the mid 1970's radioimmunoassays (RIA) to quantify thyroglobulin in patient's sera were developed [20]. Soon after their development, limitations were recognized and included: 1) the presence of anti-thyroglobulin antibodies in some patients' sera which interfered with RIA detection of thyroglobulin [21]; 2) effects of the diluent used for thyroglobulin standard curves on RIA results [22]; 3) different results for thyroglobulin quantification depending on the kit that was used [23]; 4) the heterogeneity in I-125 labeling a large molecule that has already been iodinated to varying degrees in the thyroid gland [24]; 5) significant differences in thyroglobulin standards used in kits, demonstrating the need for a "*world standard for thyroglobulin*" [25]; and 6) the limited shelf life and the biohazardous nature of radiolabelled thyroglobulin.

Early attempts to address these problems met with limited success. In the mid 1980's, attempts to make thyroglobulin assays more sensitive, and to eliminate the need for radioactivity, resulted in the development of enzyme-linked immunosorbent assays (ELISA), also called immunometric assays (IMA). These assays typically included some form of immobilized anti-thyroglobulin antibodies for capturing and/or detecting thyroglobulin present in biological fluids [26, 27]. Unfortunately, these ELISA (IMA) assays were also affected by the presence of anti-thyroglobulin autoantibodies [26]. In the mid 1990's, an attempt was made to develop a "*world standard for thyroglobulin*" by isolating this protein from a vat of human thyroid tissue as a single lot, designated CRM 457 [17, 18]. Aliquots of this material have been used in an attempt to standardize assays between suppliers, but this attempt has not been successful [28, 29]. Decades later, many of these problems still exist. Initially, ELISAs (IMAs) replaced RIAs, but the use of RIA has lingered. The use of RIA seems counterintuitive, especially since these assays depend upon the consistent labeling of a protein with iodine-125 that is heterogeneously iodinated [24]. The rationale for such an approach is that RIAs are purported to have less interference from the presence of anti-thyroglobulin autoantibodies than do the ELISA based assays [30-32]. Studies by other investigators demonstrate that RIA may not be as sensitive as some ELISAs (IMAs) [33], and that RIA still suffer from significant interference by the presence of anti-thyroglobulin autoantibodies [34-37]. What seems more clear is the fact that both methods, RIA [34, 37] or ELISA (or IMA) [38, 39], continue to be discordant when attempting to quantify thyroglobulin in the presence of anti-thyroglobulin autoantibodies [40-44].

Despite the availability of CRM 457 [17, 18], results from assays made by different suppliers continue to provide different values for thyroglobulin and/or anti-thyroglobulin antibody levels [29, 38, 45].

Furthermore, lot variations in the quantity or quality of thyroglobulin isolated from human thyroid glands has led to recalls of some assay kits. In one case, a simple change in the thyroglobulin standard used in the kit was responsible, which led to the Beckman Access thyroglobulin Ab II assay being temporarily unavailable [46]. Furthermore, the lack of a clear reference standard for thyroglobulin has frustrated such fundamental efforts as to define the range of serum thyroglobulin levels in normal individuals [4].

Stated simply, there is no universal standard for thyroglobulin that has been successful for standardizing tests across the industry. Differences in the quantification of thyroglobulin between assays from different manufacturers are so great [34, 39, 46] that physicians must know which immunoassay system their particular clinical laboratory is using, and be careful not to compare results from one assay to another.

Recent studies have suggested the elimination of immunoassays altogether and propose the use of mass spectrometry for the quantification of serum thyroglobulin [47]. In retrospect, this is not surprising due to the nature of the thyroglobulin protein, the difficulty with its purification from whole thyroid tissue, and the lack of consistency from lot to lot.

In addition, RIA or ELISA assays use different monoclonal antibodies for detection of human thyroglobulin. Some of these monoclonal antibodies vary in their abilities to recognize “immature” versus “mature” (e.g. iodinated) forms of thyroglobulin [48]. Furthermore partial denaturation of thyroglobulin can alter binding and affinity of these antibodies [49].

The lot to lot variation in thyroglobulin standards can, therefore, affect the sensitivity and accuracy of thyroglobulin determinations.

Recombinant Human Thyroglobulin: Expression of a 660 kDa Protein in Transgenic Soybean Seeds

Many proteins and antibodies used in in vitro diagnostic tests are produced and purified from recombinant protein expression systems [50].

Such technologies provide a source of proteins that can be accurately characterized and quantified, allowing regulatory standards set for analyte specific reagents to be met [19].

Unfortunately, expression of full length human thyroglobulin has not been successful using standard recombinant protein expression systems.

The reasons for such failures likely result from the large size of this protein, the glycosylation needed for correct folding and dimerization, and its role as a storage protein. The lack of any source of recombinant, full length thyroglobulin necessitates the continued practice of isolating this protein from human thyroid tissues. Using a novel platform technology, we have successfully expressed full length human thyroglobulin in transgenic soybean seeds [51], and suggest that this renewable source of thyroglobulin will provide significant advantages for use in in vitro diagnostic tests.

There are several reasons to suggest that soy-derived thyroglobulin might be more homogenous than commercially available thyroid-isolated protein. As noted previously, thyroglobulin derived from thyroid glands contains a mixture of “immature” and “mature” species that vary in their glycosylation and iodination [1].

Using electrospray ionization to produce peptides, we have been able to perform mass spectrometry analyses on soy-derived and thyroid-derived thyroglobulin samples. Based on the number of spectra matched and on the MASCOT scores [52], both soy-derived and thyroid-derived proteins were identified as human thyroglobulin (data not shown). In fact, the MASCOT scores for soy-derived thyroglobulin were higher (i.e. more peptide matches to the database) than were the scores for the commercial thyroid-purified protein used for comparison. These initial studies demonstrate identity and suggest less heterogeneity for soy-derived thyroglobulin.

Since soybean seeds do not contain enzymes necessary for iodination of tyrosine, all soy-derived thyroglobulin will be uniform in its lack of this post-translational modification. The variability of iodination in thyroid-derived thyroglobulin likely contributes to the difficulty in consistently radiolabelling this protein with Iodine-125 for use in RIA [24]. Therefore it seems likely that the lack of iodination in soy-derived thyroglobulin would provide a substrate that would increase the uniformity of iodination reactions.

There is also reason to suggest that seed-derived thyroglobulin will be more uniformly glycosylated than its thyroid-derived counterpart. Purified thyroid-derived protein likely contains a spectrum of molecules from immature forms containing few sugar residues to mature forms containing as much as 10% sugar.

The uncertainty in this statement results from the lack of any routine characterization of commercially available protein for carbohydrate content, and the lack of any attempts to determine lot-to-lot heterogeneity. In contrast, soybean seeds function in nature to store mature proteins needed for future germination [53]. We anticipated that this property would also contribute to the homogenous nature of soy-derived thyroglobulin. Our preliminary results support this notion since we routinely observe tighter bands on Western analyses when comparing soy-derived versus thyroid-derived thyroglobulin (Figure 3). Furthermore, initial analyses to identify sites of glycosylation for soy-derived and thyroid-derived thyroglobulin have been performed.

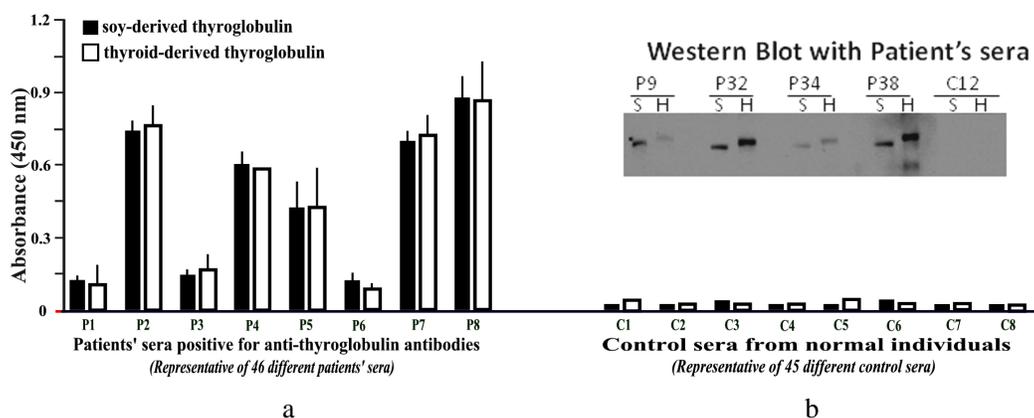


Figure 2. Reactivity of patient's sera to soy-thyroglobulin ELISA. Panel A: Equivalent amounts (1 microgram/well) of purified, soy-derived thyroglobulin or commercially available thyroid-derived thyroglobulin were coated onto ELISA plates. Plates were incubated with dilutions of patients' sera which had been previously determined to be anti-thyroglobulin autoantibody positive (P1, P2, P3, etc.), or similar dilutions of sera from individuals with no detectable thyroid disease or anti-thyroglobulin antibodies (C1, C2, C3, etc.). After incubation, any bound antibody was detected using a peroxidase-conjugated anti-human IgG antibody, followed by washing and addition of substrate. Results are presented as mean absorbance values (\pm standard error) for each representative patients' sera assayed on soy-derived (solid bars) or commercially available thyroid-derived (open bars) thyroglobulin. Panel B: For Western blot analyses, equivalent amounts (200 ng) of purified, soy-derived thyroglobulin (S) or human thyroid-derived thyroglobulin (H) were resolved by 5% SDS-PAGE and transferred onto Immobilon-P membrane. Separate strips from the same membrane were blocked and incubated with the indicated sera samples (P9, P32, P34, P38, and C12) for 2 hours. Unbound antibody was washed away, and an HRP-conjugated goat anti-human secondary antibody was used to detect bound antibody. After washing, a chemiluminescent substrate was added and binding visualized by exposing to X-ray film.

The majority of glycosylated residues in thyroid-derived thyroglobulin corresponded to those in the soy-derived counterpart, with a few differences (data not shown). The exact location of each glycosylated amino acid, and the composition of sugar residues at each glycosylation site will require additional investigation. Soybean-derived thyroglobulin will clearly differ in its post-translation modifications when compared to thyroid-derived protein since it will not contain iodine and will have soy-specific glycosylation [54].

To date, we have no evidence that these differences affect the immunogenicity of soy-derived thyroglobulin when used in *in vitro* diagnostic assays. Specifically, we have found that antibodies used in FDA approved ELISAs recognize soy-derived thyroglobulin with substantially equivalent reactivity as thyroid-derived protein. Not only does soy-derived thyroglobulin function in FDA approved kits to detect thyroglobulin [51], but 46 sera from different patients with anti-thyroglobulin autoantibodies recognized soy-derived thyroglobulin.

To demonstrate the ability of patients' sera to recognize thyroglobulin, equivalent amounts (1 microgram/well) of purified, soy-derived thyroglobulin or commercially available thyroid-derived thyroglobulin were coated onto ELISA plates.

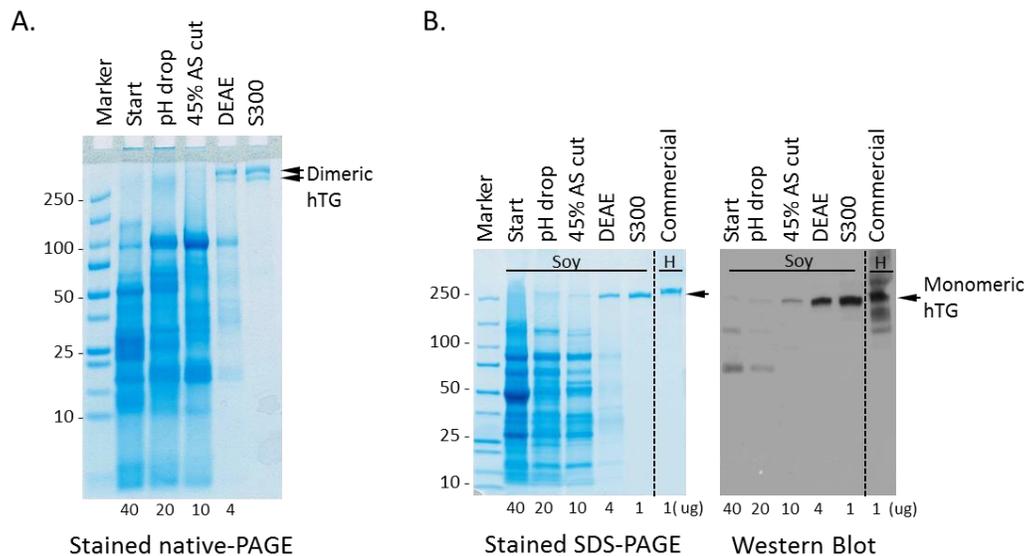


Figure 3. Purification of soybean-derived recombinant human thyroglobulin. Total protein was extracted from ground soybean seeds expressing human thyroglobulin. Purification involved a series of biochemical steps including pH drop, ammonium sulfate precipitation, DEAE ion exchange chromatography, and a Sephacryl 300 sizing column. Panel A: 3-20% native-PAGE. Amounts of protein loaded in each lane (indicated at the bottom of each lane) from different fractionation steps are indicated. Molecular weight standards (kDa) are shown and the arrows indicate location of dimeric protein. Panel B: Left, 4-15% SDS-PAGE. Lanes and amounts of protein loaded are identical to those in panel A. In addition, 1 μ g thyroid-purified protein (Calbiochem) was included for comparison. Protein samples were suspended in running buffer devoid of reducing agent and were not boiled prior to loading into the gel. Right, western blot of 4-15% gel. Arrow indicates monomeric form of protein. Note the heterogeneity of the commercial sample.

These respective ELISA plates were incubated with dilutions of patients' sera which had been previously determined to be anti-thyroglobulin autoantibody positive or similar dilutions of sera from individuals with no detectable thyroid disease or anti-thyroglobulin antibodies. After incubation, any bound antibody was detected using a peroxidase-conjugated anti-human IgG antibody, followed by washing and addition of substrate. Figure 2A shows the results of one such assay which includes individual patients' sera positive for anti-thyroglobulin antibodies (P1, P2, P3, etc.) as well as individual sera with no detectable anti-thyroglobulin antibody (C1, C2, C3, etc.).

We concluded from these studies that: 1) soy-derived thyroglobulin and thyroid-derived thyroglobulin were immunologically indistinguishable.

If a patient's sera could detect thyroid-derived thyroglobulin, it could also detect soy-derived thyroglobulin (i.e. no false negatives); and 2) sera from normal individuals did not contain antibodies which could bind to soy-derived thyroglobulin.

If an individual's sera could not detect thyroid-derived thyroglobulin, it could also not detect soy-derived thyroglobulin (i.e. no false positives). Together, these results suggest the equivalence of soy-derived thyroglobulin when compared to thyroid-derived thyroglobulin using ELISA assays.

Western blot analyses were also performed to demonstrate the ability of patients' sera to recognize thyroglobulin (Figure 2B). For these studies, equivalent amounts (200 ng) of purified, soy-derived thyroglobulin (S) or human thyroid-derived thyroglobulin (H) were resolved by 5% non-reducing SDS-PAGE and transferred onto Immobilon-P membrane. Separate strips from the same membrane were blocked and incubated with the indicated sera samples (P9, P32, P34, P38, and C12) for 2 hours. Unbound antibody was washed away, and an HRP-conjugated goat anti-human secondary antibody was used to detect bound antibody. After washing, a chemiluminescent substrate was added and binding visualized by exposing to X-ray film. The results of one representative Western blot are shown in Figure 2B. We concluded from these studies that: 1) if a patient's sera could detect thyroid-derived thyroglobulin, it could also detect soy-derived thyroglobulin (i.e. P9, P32, P34, and P38); and 2) if an individual's sera could not detect thyroid-derived thyroglobulin, it could also not detect soy-derived thyroglobulin (i.e. C12). Results of this Western blot are consistent with those of the ELISA, and further suggest the equivalence of soy-derived thyroglobulin when compared to thyroid-derived thyroglobulin.

The substantial equivalence of soy-derived thyroglobulin in immunoassays is not surprising since it is the protein backbone, not carbohydrate or iodinated residues that are recognized by these antibodies [49, 55]. Finally, it is unlikely that soybean-specific glycosylation patterns will be recognized by sera from normal individuals. Assays of sera samples from 45 individuals without thyroid disease have detected no such false positives (Figure 2A).

It is logical to assume that such findings will be consistent throughout the human population since soy protein as a foodstuff or as a protein additive is common in the diets of most developed countries [56]. The consumption of soy products likely contributes to oral tolerance as evidenced by the fact that very few individuals have immunity against soy proteins [57].

The same glycosylation machinery which places carbohydrate residues on normal soy proteins will perform similar additions to soy-derived thyroglobulin.

There is little reason to imagine the presence of antibodies specific for carbohydrate residues on soy-derived thyroglobulin in the general population.

Many of the additional advantages for soy-derived thyroglobulin derive from the unique nature of this protein expression system and the subsequent protein purification. For example, the FDA requires each lot of an analyte isolated from human tissue to be screened for the absence of transmissible agents (e.g. HIV, Hepatitis B, etc.).

Since thyroglobulin is isolated from human thyroid glands, each lot must be screened for such infectious agents, and Material Safety Data Sheets (MSDS) must list this reagent as a biohazard. This adds to the cost of production, and increases possible product liability. Soy-derived thyroglobulin poses no such risk of spreading transmissible diseases, and will not require such screening.

Presently we have transgenic soybean lines that express approximately 4 grams of thyroglobulin per liter of raw soy powder. This translates into greater than 10 kilograms of production per greenhouse acre. Such production in BioSafety Level 2 greenhouses would result in a cost of less than 2 cents per milligram [58]. For comparison, retail prices for purchasing research-grade thyroglobulin purified from human thyroid glands range from more than \$100 to over \$1,000 per milligram. Furthermore, at expression levels greater than 10 kilograms per acre, it will be possible to contain the growth of transgenic soybeans in Biosafety Level 2 greenhouses without the need for production in open fields. Secure greenhouse growth would provide containment, and be consistent with good manufacturing practices for production of an FDA approved analyte. Such greenhouse production could be considered a “green” technology, as the growth of these plants consumes CO₂, and releases O₂.

The purification of human thyroglobulin from thyroid tissue is an involved process [17, 18], and results in the isolation of “immature” as well as species that vary in their glycosylation and iodination [1].

The thousands of contaminating proteins which must be removed from homogenized thyroid tissue complicate the purification process, and leads to variability in composition and purity between lots.

Conversely, the number of proteins present in soybean seeds is quite limited [59, 60] suggesting that purification schemes for soy-derived human thyroglobulin would be greatly simplified and rapid. Figure 3 shows the results of one such purification scheme. For these studies, transgenic soybean seeds expressing full length human thyroglobulin were ground to a fine powder in a coffee mill.

Total protein was extracted in buffer with sonication, followed by a pH drop to 5.8 and clarification by centrifugation. Ammonium sulfate precipitated proteins were collected and buffer exchanged to 50 mM Tris buffer (pH 7.5).

DEAE cellulose chromatography was performed, followed by sizing of the thyroglobulin containing fractions on Sephacryl 300 column. Figure 3 shows the results of one such analysis. Fractions containing purified, soy-derived thyroglobulin were pooled, concentrated, and quantified using the Bradford reagent and in-house ELISAs.

Protein from each purification step was separated by PAGE using 3-20% native (Figure 3A) and 4-15% SDS-PAGE gradient gels, and then visualized with NuSep dye. The arrows indicate purified soy-derived thyroglobulin. The sizes of protein molecular weight standards are shown as kDa. Companion Western blot analyses were also performed to demonstrate the

identity of purified thyroglobulin (Figure 3B). Note that this particular lot of commercial thyroid-derived thyroglobulin was quite heterogeneous.

In summary, we have used a novel platform technology to successfully express full length, recombinant human thyroglobulin in transgenic soybean seeds [51]. Advantages of this protein expression system include the relative homogeneity of the product, ease of purification, low cost of production, elimination of human pathogen transmission, as well as being a renewable, green technology.

This accomplishment has promise to enhance and/or standardize present day immunoassays that have human thyroglobulin as a component.

A Device Capable of Removing Anti-Thyroglobulin Antibodies from Patient Serum Samples

The most significant limitation with present day thyroglobulin immunoassays is the quantification of thyroglobulin levels in patients' sera that contain autoantibodies against thyroglobulin [28, 29].

This is true regardless of whether the assay format is an RIA [34, 37] or an ELISA (or IMA) [38, 39], and the problem of discordance between values obtained with varying immunoassays remains [40-44]. Estimates are that 20%-40% of thyroid cancer patients develop anti-thyroglobulin antibodies at some time during their disease or recovery. These autoantibodies can bind thyroglobulin in sera or to thyroglobulin in the assays themselves and interfere with the detection and capture monoclonal antibodies' ability to detect this molecule in all current FDA-approved thyroglobulin immunoassays. Presently there is no clear solution for this significant problem [40-44].

Antibody-antigen interactions are non-covalent ones, and subject to the laws of mass action [61]. Immunoaffinity chromatography schemes have long been used in the isolation of proteins for diagnostic and therapeutic use [62]. Therefore it should be possible to remove autoantibodies from sera, even if they are already bound to circulating thyroglobulin.

In recent studies, we have demonstrated the ability of immobilized, soy-derived thyroglobulin to remove anti-thyroglobulin autoantibodies from patients' sera.

For these studies, 100 micrograms of BSA or soy-derived thyroglobulin were immobilized onto resin contained within mini-affinity columns. Sera from patients containing anti-thyroglobulin antibodies could then be passed over each column to assess the ability to remove these autoantibodies.

Figure 4 shows representative ELISA results that detected the presence of anti-thyroglobulin antibodies. Passing the sera over a control, bovine serum albumin (BSA) conjugated column resulted in no reduction in the presence of these autoantibodies.

However when patients' sera were incubated on affinity columns conjugated with soy-derived thyroglobulin, it was clear that there was a significant reduction in the level of anti-thyroglobulin autoantibodies (Figure 4).

These studies suggested that a device can be constructed which would be capable of removing anti-thyroglobulin antibody interference from patients' sera so that human thyroglobulin can be measured more accurately.

To demonstrate this possibility, separate two microliter aliquots of sera from 3 different patients' (P32, P36, and P38) containing anti-thyroglobulin antibodies were spiked with 1.0 ng/ml of commercially available thyroid-derived thyroglobulin, and the mixtures incubated for 60 minutes to allow antibody-antigen complexes to form.

One aliquot from each patient was incubated overnight on affinity columns conjugated with BSA, while the second aliquot was incubated overnight on affinity columns conjugated with soy-derived thyroglobulin. Material that did not bind to the respective column was eluted and assayed by ELISA to detect the present of unbound thyroglobulin.

Figure 5 shows the results of one such experiment.

As expected, incubation on BSA-conjugated affinity columns did little to dissociate thyroglobulin which had complexed with the patients' autoantibodies. Clearly the presence of the anti-thyroglobulin autoantibodies present in patients' sera (P32, P36 and P38) interfered with the ability of the ELISA to detect soluble thyroglobulin.

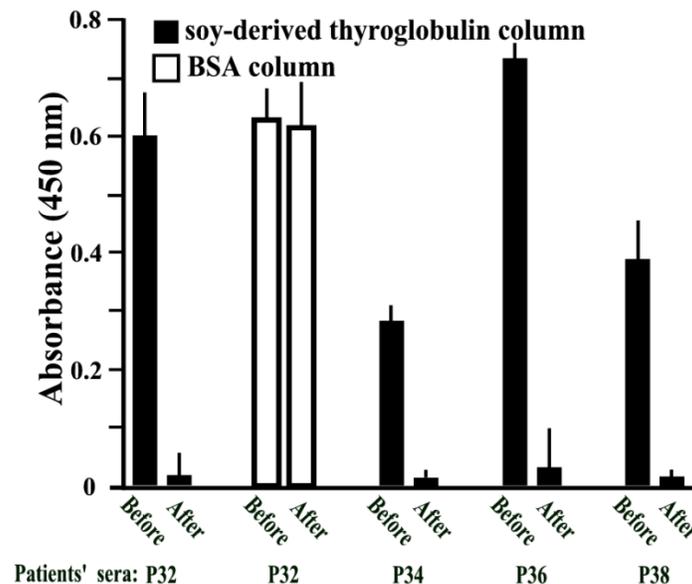


Figure 4. Ability of soy-derived thyroglobulin conjugated affinity columns to remove anti-thyroglobulin autoantibodies from patients' sera. Bovine serum albumin (BSA) or soy-derived thyroglobulin (100 micrograms) were immobilized onto resin contained within mini-affinity columns (MicroLink Protein Coupling Kit, Pierce Chem. Co.) using the instructions supplied by the manufacturer. Aliquots of representative patients' sera (P32, P34, P36, and P38) were assayed by ELISA for the presence of anti-thyroglobulin autoantibodies before or after a 60 minute incubation on the indicated mini-affinity column. Results are presented as mean absorbance values (\pm standard error) for each representative patients' sera after incubation on a BSA (open bars) or soy-derived thyroglobulin (closed bars) columns.

However when duplicate aliquots were incubated on soy-thyroglobulin-conjugated affinity columns, a significant amount of bound thyroglobulin was released from these samples, and could easily be detected by ELISA.

We concluded from these studies that an excess of immobilized soy-derived thyroglobulin on an affinity matrix could compete with the autoantibodies present in patients' sera, resulting in a release of the bound thyroglobulin in each sample.

These results provide compelling evidence that affinity devices can be constructed that remove anti-thyroglobulin antibody interference from patients' sera so that human thyroglobulin can be measured more accurately.

Conclusion

Using a novel platform technology, we have successfully expressed full length human thyroglobulin in transgenic soybean seeds. To our knowledge, this is the only source of recombinant human thyroglobulin, and is the only successful expression of this protein using any protein expression system. We propose that the unique advantages of transgenic soybean-derived proteins (e.g. homogeneity, ease of purification, low cost of production, elimination of human pathogen transmission, renewable "green technology", etc.) will allow, for the first time, a practical solution to solve two of the most significant problems plaguing immunoassays which utilize thyroglobulin.

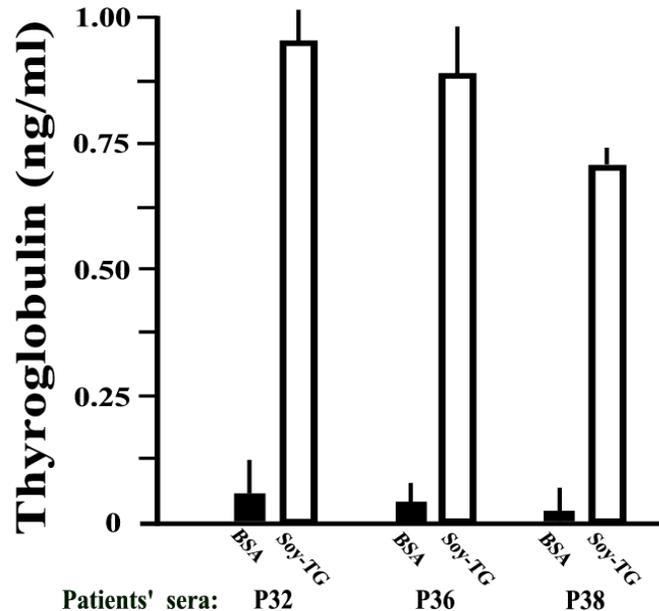


Figure 5. Reduction of anti-thyroglobulin autoantibody interference in patients' sera by incubation on soy-derived thyroglobulin conjugated affinity columns. Separate two microliter aliquots of sera from 3 different patients' (P32, P36, and P38) containing anti-thyroglobulin antibodies were spiked with 1.0 ng/ml of commercially available thyroid-derived thyroglobulin, and the mixtures incubated for 60 minutes to allow antibody-antigen complexes to form. One aliquot from each patient was incubated overnight on affinity columns conjugated with BSA (BSA), while the second aliquot was incubated overnight on affinity columns conjugated with soy-derived thyroglobulin (Soy-thyroglobulin). Material that did not bind to the respective column was eluted and assayed by ELISA (Kronus, Inc.) to detect the present of unbound thyroglobulin. Results are presented as mean thyroglobulin concentrations (ng/ml \pm standard error) for each representative patients' sera following incubation on BSA-conjugated (solid bars) or soy-thyroglobulin-conjugated (open bars) affinity columns.

Soybean-derived thyroglobulin provides a renewable source of this analyte with properties superior to thyroid-derived protein, providing a universal standard for the industry. Furthermore, the ability to construct affinity devices provides a practical solution for the elimination of anti-thyroglobulin autoantibodies that can interfere with immunoassays.

These accomplishments should significantly enhance present day thyroglobulin immunoassays designed to diagnose and monitor patients with thyroid cancers and autoimmune thyroiditis.

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