

# Assessment of IgE binding to native and hydrolyzed soy protein in serum obtained from dogs with experimentally induced soy protein hypersensitivity

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**Objective**—To assess binding of IgE to native, whole hydrolyzed, and separated hydrolyzed fractions of soy protein in serum obtained from dogs with experimentally induced soy protein hypersensitivity.

**Animals**—8 naïve Beagles (6 experimentally sensitized to native soy protein and 2 control dogs).

**Procedures**—6 dogs were sensitized against soy protein by administration of allergens during a 90-day period. After the sensitization protocol was completed, serum concentrations of soy-specific IgE were measured and intradermal skin tests were performed in all 6 dogs to confirm that the dogs were sensitized against soy protein. Serum samples from each sensitized and control dog underwent western blot analysis to assess the molecular mass band pattern of the different allergenic soy fractions and evaluate reactivities to native and hydrolyzed soy protein.

**Results**—In sera from sensitized dogs, a characteristic band pattern with 2 major bands (approx 75 and 50 kd) and 2 minor bands (approx 31 and 20 kd) was detected, whereas only a diffuse band pattern associated with whole hydrolyzed soy protein was detected in the most reactive dog. Reactivity was evident only for the higher molecular mass peptide fraction. In control dogs, no IgE reaction to native or hydrolyzed soy protein was detected.

**Conclusions and Clinical Relevance**—Data suggest that the binding of soy-specific IgE to the hydrolyzed soy protein used in the study was significantly reduced, compared with binding of soy-specific IgE to the native soy protein, in dogs with experimentally induced soy hypersensitivity. (*Am J Vet Res* 2006;67:1895–1900)

**F**ood hypersensitivity reactions (food allergies) refer to immunologically mediated adverse reactions to food that are unrelated to any physiologic effect of the food or food additives.<sup>1</sup> This implies that some components of the food, primarily glycoproteins, are recognized by cells of the immune system and an immune response develops.<sup>2,3</sup> In humans, food hypersensitivity

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## ABBREVIATIONS

ODU	Optical density unit
TBST	Tris-buffered saline solution with 0.07% Tween-20

is most commonly an IgE-mediated process.<sup>4,5</sup> In dogs, the pathogenesis of food hypersensitivity reactions has not been fully elucidated. Experimental canine models of IgE-mediated food hypersensitivity have been reported<sup>6,7</sup> as well as naturally occurring IgE-mediated food hypersensitivity in dogs.<sup>1,6</sup> A recent study<sup>8</sup> in dogs with food hypersensitivity revealed a low correlation between antigen-specific IgE test results and oral food provocation test results, which might be attributable to the presence of a non-IgE-mediated mechanism, such as type IV hypersensitivity, in the pathogenesis of food hypersensitivity in dogs.

Presently, the only method by which food allergies are diagnosed in dogs consists of feeding an elimination diet in which the offending allergen has been eliminated. Commercially available elimination diets that are based on hydrolyzed proteins have been developed. In those diets, the protein source is hydrolyzed into small peptides; these small peptides are thought to be less able to elicit an immune response. Hydrolyzed proteins have been proven to be effective hypoallergenic diets in children and could be a good option in the management of dogs with food hypersensitivity.<sup>9</sup>

Soy protein has been used in hydrolyzed hypoallergenic diets because it is highly digestible and has high nutritional value. Results of recent studies<sup>10,a</sup> in dogs have indicated that hydrolyzed soy protein diets are useful in the diagnosis and management of food adverse reactions in that species. However, to date, a major limitation in the use of these diets is a lack of data to determine whether dogs that are hypersensitive to the parent protein (eg, soy protein) could tolerate the hydrolyzed product.<sup>11</sup>

At least 16 potential soy antigenic peptides (molecular weights ranging from 20 to 78 kd) have been identified in humans, but their relative clinical importance is not known.<sup>12-14</sup> In humans, hydrolyzed soy peptides have to be > 20 kd to trigger an adverse allergic reaction. However, at present, similar data for dogs are not available, and the size of hydrolyzed soy peptides that can potentially trigger a hypersensitivity reaction in dogs has been extrapolated from human studies. In vitro studies allow the identification of the allergenic soy protein fractions that bind canine soy-specific IgE;

immunochemical methods have the advantages of high sensitivity and of being able to specifically target the proteins of interest (ie, the identified allergens).<sup>2,15,16</sup> The purpose of the study reported here was to assess binding of IgE to native, whole hydrolyzed, and separated hydrolyzed fractions of soy protein in serum obtained from dogs with experimentally induced soy hypersensitivity. By use of western blot techniques, native and hydrolyzed soy proteins were analyzed with soy-specific IgE from Beagles sensitized against soy protein. Moreover, the presence of antigenic peptides was also evaluated in several hydrolyzed soy protein fractions that were separated on the basis of their molecular weight. To evaluate the ability of the native and whole hydrolyzed soy proteins and hydrolyzed protein fractions to cross-link soy-specific IgE *in vivo*, all the protein sources were injected ID in sensitized Beagles and skin reactions were assessed.

### Materials and Methods

**Dogs**—Eight naive Beagles (1 male and 7 females) from 3 litters were enrolled in the study at birth and were maintained at the kennel of the Facultat de Veterinaria, Universitat Autònoma de Barcelona. All experimental procedures were approved by the Animal Research Ethical Committee of the University. Six dogs were experimentally sensitized against native soy protein (designated as S1 through S6), and 2 dogs were used as control dogs (designated as C1 and C2). There were no adverse effects following the induction of soy protein hypersensitivity, and no medication was needed to counter hypersensitivity reactions. The health conditions of dogs were monitored by a veterinarian throughout all the experimental procedures.

Dogs were fed commercial dry food without soy protein<sup>b</sup> twice a day, whereas water was available *ad libitum*. Guaranteed analysis for the food revealed that it contained 32% protein, 20% fat, 9% moisture, 7.5% minerals, and 2.5% crude fiber.

**Sensitization procedure**—Dogs were sensitized in accordance with a protocol described elsewhere.<sup>7</sup> Briefly, beginning when the 6 dogs in the sensitized group were 1 day old (designated day 1), an injection of 2 µg of native soy protein<sup>c</sup> in 0.2 mL of saline (0.9% NaCl) solution plus 0.2 mL of alum adjuvant<sup>d</sup> was administered SC in each axilla of each dog at intervals during a 90-day period; the 2 control dogs each received an injection of 0.2 mL of saline solution plus 0.2 mL of adjuvant in each axilla, according to the same schedule.

All dogs were inoculated with an attenuated vaccine against distemper-hepatitis-parvovirus<sup>e</sup> on day 21 and an attenuated vaccine against distemper-hepatitis-leptospirosis-parvovirus<sup>f</sup> on days 49 and 77. One and 7 days after each of the 3 inoculations (ie, on days 22, 29, 50, 57, 78, and 85), injections of the allergen extract were administered to the 6 dogs in the sensitized group in the same manner as that used on day 1; similarly, control dogs received injections of saline solution with adjuvant. After the sensitization protocol was completed on day 85, dogs in the sensitized group received a booster injection of 20 µg of native soy protein in alum adjuvant in each axilla bimonthly (beginning 30 days after completion of the sensitization protocol [day 115]) throughout the remainder of the study, as described.

**Determination of serum concentrations of soy-specific IgE and IgG**—Sera were obtained from dogs in the sensitized and control groups at intervals during the sensitization period (ie, on days 29, 57, and 85). Serum concentrations of soy-specific IgE were determined by use of a semiquantitative

enzyme-labeled immunodot assay, as described.<sup>7</sup> At the time that the immunoblotting assays were performed (ie, when the dogs were 2 years old), serum soy-specific IgE and soy-specific IgG concentrations in each dog were determined by use of an enzyme-labeled immunodot assay (assessed as ODUs by means of an optical densitometer<sup>g</sup>).

**Isolation of peptide fractions from hydrolyzed soy protein**—To obtain peptide fractions from hydrolyzed soy protein (separated on the basis of their molecular mass), an ultrafiltration method was used.<sup>17</sup> Briefly, a suspension of 25 g of the hydrolyzed soy protein/L of water was centrifuged (10,000 × *g* for 10 minutes at 15°C) to eliminate insoluble material. The soluble fraction was filtered through a 50-kd pore membrane<sup>h</sup>; peptides with a molecular mass > 50 kd remained in the washing solution that was used, whereas peptides of lower molecular mass (< 50 kd) passed through the membrane. The filtered substances subsequently underwent ultrafiltration by use of 3 membranes as follows: a 30-kd pore membrane,<sup>i</sup> a 10-kd pore membrane,<sup>j</sup> and, finally, a 3-kd pore membrane.<sup>k</sup> All the separation operations were performed at 15°C and at 2 bars of pressure. On completion of the entire process, 5 protein fractions were obtained; the molecular weights of these fractions were < 3 kd, 3 to 10 kd, > 10 to 30 kd, > 30 to 50 kd, and > 50 kd. Each separated fraction was lyophilized.<sup>l</sup>

**IgE immunoblot analysis**—Serum samples obtained from the eight 2-year-old Beagles included in the study were analyzed via immunoblotting to assess IgE binding to the different soy protein sources. Sera were pooled according to source (sensitized or control dogs) for analysis. Furthermore, serum samples from the 6 sensitized dogs were each analyzed separately to assess individual variations.

Protein samples (native, whole hydrolyzed,<sup>m</sup> and hydrolyzed protein fractions) were analyzed via SDS-PAGE according to the method of Laemmli.<sup>18</sup> Each soy protein fraction (12.5 µg) was diluted in 1% SDS, boiled for 10 minutes, and then placed in ice for 5 minutes. Samples were analyzed in a 12% polyacrylamide gel under reducing conditions and run for 90 minutes at 100 mV. Commercial standard proteins (molecular mass range, 7 to 205 kd) were used as molecular mass markers.<sup>n</sup> After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes<sup>o</sup> and detected with Ponceau S stain.<sup>p</sup> Membranes were placed in a blocking solution consisting of 5% skimmed milk in TBST and incubated for 2 hours at room temperature (approx 23°C). After blocking, membranes were incubated for 16 hours at 4°C with 4 mL of diluted sera (1:4 [vol/vol] dilution in 5% skimmed milk in TBST) obtained from the study Beagles. Membranes were then washed and incubated with a secondary peroxidase-conjugated antibody anti-canine IgE<sup>q</sup> (1:10,000 [vol/vol] in 5% skimmed milk in TBST) for 1 hour at room temperature. After washing, peroxidase on the membrane was developed by use of a chemiluminescent process.<sup>f</sup>

**Intradermal challenge**—To evaluate the ability of each soy protein source to induce a skin reaction, an intradermal challenge test was performed in all the dogs included in the study. Each dog received ID injections of each of 3 dilutions (1, 10, and 100 µg/mL) of native, whole hydrolyzed, and hydrolyzed protein fractions in the ventral area of the abdomen, and wheals induced at the injection sites were measured 20 minutes later. Histamine<sup>s</sup> (25 µg/mL) was used as a positive control treatment, and the diluent (saline solution) was used as a negative control treatment. To improve delineation of the wheal perimeter, a 2% solution of Evans blue dye was injected IV (0.2 mL/kg) 30 minutes before the intradermal challenge. Wheal areas were assessed by applying a piece of acetate tape over the reaction site, onto which the outline

of the wheal was traced with indelible ink; wheal areas were subsequently calculated via computer-assisted image analysis.<sup>1</sup>

## Results

**Serum-specific IgE and IgG concentrations**—During the 90-day sensitization period, inoculation with native soy protein induced high concentrations of soy-specific IgE in treated Beagles. Control dogs had no detectable serum concentrations of soy-specific IgE throughout the study period.<sup>7</sup> At the time that the immunoblot assays were performed (ie, when the dogs were 2 years of age), serum concentrations of soy-specific IgE and soy-specific IgG in each dog were determined by use of an enzyme-labeled immunodot assay (assessed as ODUs). Both control dogs had no detectable serum concentrations of soy-specific IgE and soy-specific IgG. The IgE and IgG values for the sensitized dogs were as follows: S1, 49 and 57 ODUs, respectively; S2, 14 and 17 ODUs, respectively; S3, 11 and 15 ODUs, respectively; S4, 0 and 4 ODUs, respectively; S5, 28 and 40 ODUs, respectively; and S6, 29 and 32 ODUs, respectively.

**IgE immunoblotting**—Native, whole hydrolyzed, and hydrolyzed fractions of soy samples were prepared and analyzed by use of a western blot technique. The IgE antibodies in the pooled sera from the 6 sensitized dogs and the pooled sera from the 2 control dogs were analyzed for their reactivity against the 3 soy protein sources.

**IgE binding to native and whole hydrolyzed soy protein**—Immunoblot assays revealed that sera from the 6 sensitized dogs reacted with native soy protein

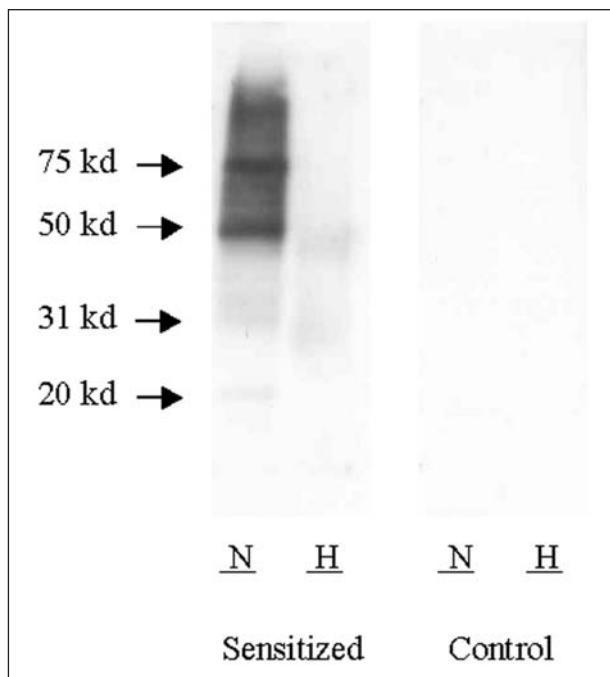


Figure 1—Identification of IgE binding to native (N) and whole hydrolyzed (H) soy protein in pooled serum from 6 soy-sensitized dogs and pooled serum from 2 control dogs. Equal amounts (12.5  $\mu$ g) of native and whole hydrolyzed soy protein were separated via 12% SDS-PAGE and analyzed via western blotting with pooled sera (1:4 [vol/vol]) as the soy-specific IgE source. Molecular mass markers are indicated on the left.

samples, whereas no IgE antibody binding to native soy protein samples was detected in sera from the 2 control dogs (Figure 1). In sensitized dogs, 2 major bands of approximately 75 and 50 kd and 2 minor bands of approximately 31 and 20 kd were identified in the native soy samples. However, when the whole hydrolyzed soy protein samples were analyzed, only 2 diffuse bands of approximately 50 and 30 kd were identified.

To assess differences in band patterns among the 6 sensitized dogs, serum from each dog was analyzed separately. The binding of IgE in each serum sample to the native and whole hydrolyzed soy proteins was analyzed (Figure 2). The native soy protein fraction with a molecular mass of about 75 kd was identified as a major IgE-binding protein fraction in all sensitized dogs. In the 6 serum samples analyzed, variable reactivity to the 50-, 37-, 31-, 25-, and 20-kd native soy protein fractions was detected.

In contrast to the evident reactivity bands identified with native soy protein, low-intensity diffuse band patterns were detected with whole hydrolyzed soy protein. Among the sera from the 6 sensitized dogs, only serum from dog S1 (the most reactive dog) revealed 2 diffuse bands with molecular masses of approximately 50 and 30 kd (Figure 3). However, the 75-kd major band previously associated with the native soy protein fraction was not visible. No binding reactivity was identified in the other analyzed sera.

**IgE binding to hydrolyzed soy protein fractions**—Reactivity against the 5 hydrolyzed soy protein fractions was also examined. In the same experiment, we compared the reactivity of native and whole hydrolyzed soy protein and 5 hydrolyzed soy protein fractions (ie, fractions < 3, 3 to 10, > 10 to 30, > 30 to 50, and > 50 kd) to IgE from the most reactive sensitized dog (S1). Two major bands of approximately 37

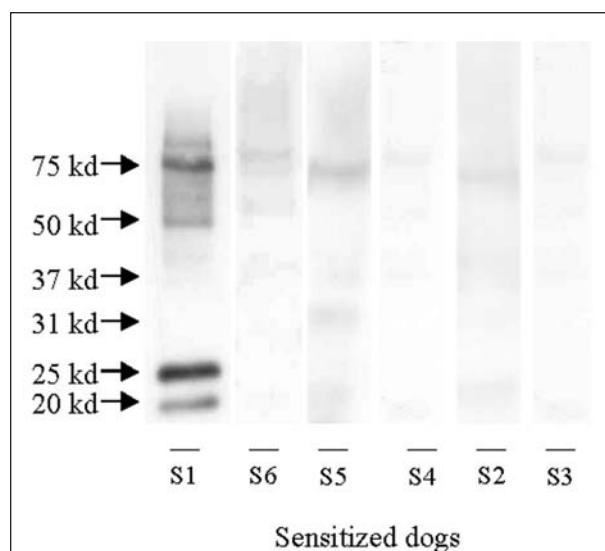


Figure 2—Identification of IgE binding to native soy protein in individual serum samples from 6 soy protein-sensitized dogs (S1 to S6). Equal amounts (12.5  $\mu$ g) of native soy protein were separated via 12% SDS-PAGE and analyzed via western blotting with serum (1:4 [vol/vol]; soy-specific IgE source) of each sensitized dog. Molecular mass markers are indicated on the left.

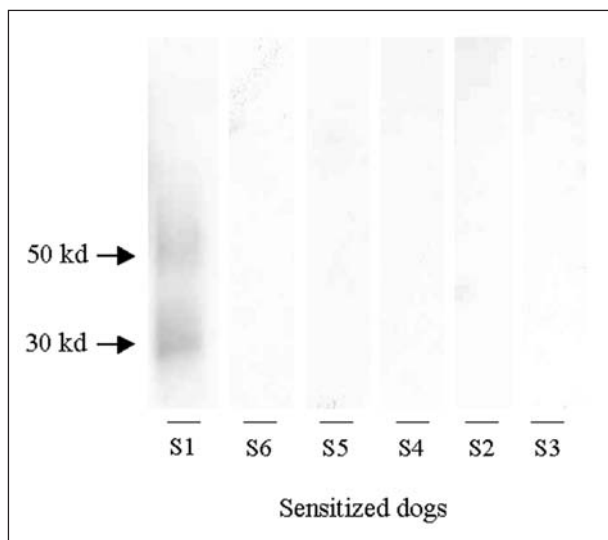


Figure 3—Identification of IgE specific for whole hydrolyzed soy protein in individual serum samples from 6 soy-sensitized dogs. Equal amounts (12.5  $\mu$ g) of whole hydrolyzed soy protein were separated via 12% SDS-PAGE and analyzed via western blotting with serum (1:4 [vol/vol]; soy-specific IgE source) of each sensitized dog. Molecular mass markers are indicated on the left.

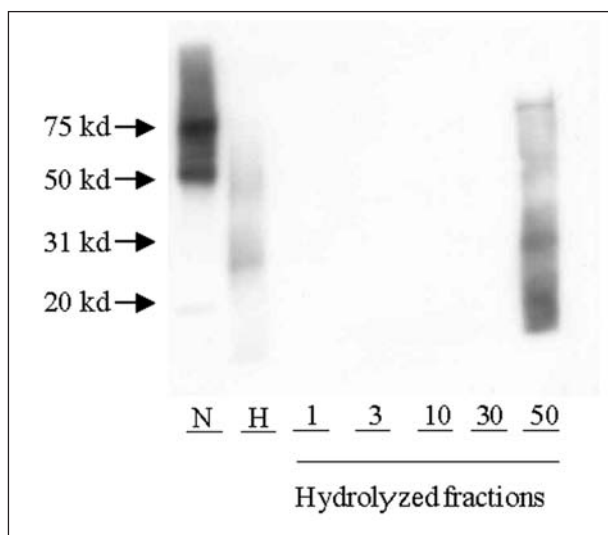


Figure 4—Identification of IgE specific for hydrolyzed soy protein fractions in the serum of the most reactive soy-sensitized dog (S1). Equal amounts (12.5  $\mu$ g each) of hydrolyzed soy protein fractions were separated via 12% SDS-PAGE and analyzed via western blotting with serum (1:4 [vol/vol]; soy-specific IgE source) of dog S1. Molecular mass markers are indicated on the left. Each numbered lane represents a hydrolyzed soy protein fraction of different molecular mass as follows: 1 = < 3 kd; 3 = > 3 to 10 kd; 10 = > 10 to 30 kd; 30 = > 30 to 50 kd; and 50 = > 50 kd. See Figure 1 for remainder of key.

and 20 kd were detected in the highest molecular mass fraction (> 50 kd; **Figure 4**). Other diffuse bands of 50 and 75 kd were also detected.

**Results of intradermal challenge**—Intradermal skin testing was performed in all dogs to compare responses to the native and whole hydrolyzed soy protein and the hydrolyzed soy protein fractions; each test substance was administered at 3 concentrations of soy protein (1, 10, and 100  $\mu$ g/mL). The response induced

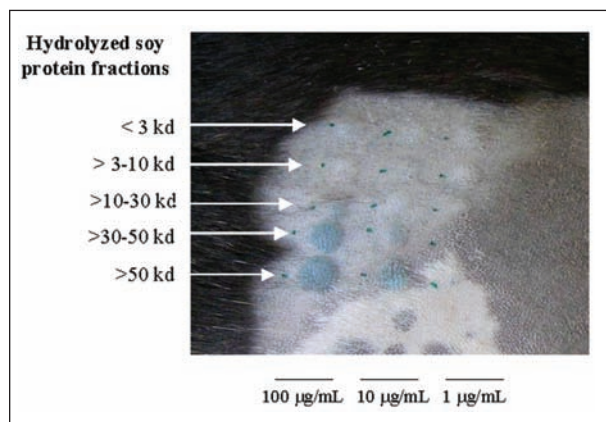


Figure 5—Photograph of a series of intradermal challenges with 5 hydrolyzed soy protein fractions in dog S1. Forty microliters of different dilutions (1, 10, and 100  $\mu$ g/mL) of each hydrolyzed soy protein fraction was injected ID in the skin of a ventral area of the abdomen, and wheal areas were assessed 20 minutes later; to improve delineation of the wheals, 2% solution of Evans blue dye (0.2 mL/kg) was injected IV 30 minutes before the skin test was begun.

by the whole hydrolyzed soy protein was approximately half that induced by the native soy protein. At soy protein concentrations of 1, 10, and 100  $\mu$ g/mL, the mean wheal areas induced by whole hydrolyzed soy protein were 2.5, 16.2, and 39.8  $\text{mm}^2$ , respectively, whereas the wheal areas induced by native soy protein were 5.9, 30.9, and 60.6  $\text{mm}^2$ , respectively, in the 3 most reactive soy-sensitive dogs (S1, S3, and S5). No inflammatory responses to any of the 3 concentrations of hydrolyzed soy protein fractions of < 3 kd and > 3 to 10 kd were induced in any of the sensitized dogs. Also, at soy protein concentrations of 1 and 10  $\mu$ g/mL, no inflammatory responses to the hydrolyzed soy protein fraction of > 10 to 30 kd were induced in any of the sensitized dogs; however, at the soy protein concentration of 100  $\mu$ g/mL, the mean wheal area induced by the > 10 to 30 kd fraction in the 3 most reactive dogs was 17.9  $\text{mm}^2$ . For the hydrolyzed soy protein fractions of > 30 to 50 kd and > 50 kd, the mean wheal areas induced in the 3 most reactive dogs were 18.0 and 45.5  $\text{mm}^2$ , respectively, at soy protein concentration of 10  $\mu$ g/mL and 27.1 and 49.8  $\text{mm}^2$ , respectively, at soy protein concentration of 100  $\mu$ g/mL. The most pronounced inflammatory skin response was observed after the > 50-kd fraction was administered (**Figure 5**). No skin reactions were observed in control dogs after ID injection of native or whole hydrolyzed soy protein or the hydrolyzed soy protein fractions.

## Discussion

Food hypersensitivity reactions are relatively common in dogs. Clinical signs include atopic dermatitis, nausea and vomiting, diarrhea, and, rarely, anaphylaxis. Homemade elimination diets have been reported<sup>10,19</sup> to be the only reliable tool for diagnosis of adverse food reactions in dogs despite the inconveniences associated with this practice. Increasing evidence suggests that commercial hydrolyzed-protein-based diets could be useful and more balanced substitutes for the diagnosis and management of food allergic reactions in humans and dogs.<sup>10,11,20</sup>

However, more data are needed to support that protein hydrolysis is an appropriate method to decrease the allergenic properties of proteins.<sup>11</sup> Therefore, the main goals of the present study were to determine the main allergenic fractions of native soy protein that affect dogs and investigate the presence of those fractions in hydrolyzed soy protein. Previous work<sup>7</sup> in our laboratory revealed that soy protein hypersensitivity could be induced in naive Beagles; in 3 of 6 experimentally sensitized dogs in that study, clinical signs of soy protein hypersensitivity (diarrhea and vomiting) were detected following an oral challenge with native soy protein but not with soy protein hydrolysate.

Via western blot analysis of the sera of sensitized dogs in the present study, 2 major bands of approximately 75 and 50 kd and 2 minor bands of approximately 31 and 20 kd were identified as IgE binding sites in native soy protein. No reactivity was associated with any peptide with a molecular mass < 20 kd. In soybean-sensitive humans, serum soy-specific IgE has been found to bind a 46-kd native soy fraction and, to a lesser extent, a 21-kd fraction.<sup>12</sup> In another study<sup>13</sup> in humans, 5 proteins with a molecular mass of 20 or 58 kd in the soy whey fraction and 26, 31, or 78 kd in the soy globulin fraction were considered major allergens in IgE-mediated reactions to soy protein. Results of our study in dogs, in which no soy-specific IgE reactivity was detected for peptides < 20 kd, are therefore very similar to findings in humans.

Comparison of serum reactivity among the 6 sensitized study dogs revealed that serum from dog S1 had the most consistent and intense band pattern. This dog also had the highest serum concentrations of soy-specific IgE and soy-specific IgG and the largest inflammatory response following intradermal challenge with native soy protein or the hydrolyzed soy protein fractions. In contrast, the sera from dogs S3 and S4 lacked reactivity, which correlated with the dogs' low serum concentrations of soy-specific IgE and soy-specific IgG. In dog S5, another band of approximately 31 kd was detected. This indicated that although band patterns may vary among sensitized dogs, no bands < 20 kd were identified. Those results agree with findings of another study<sup>21</sup> in dogs, in which serum IgE binding to whey or globulin fractions of soy protein (as determined via western blot analysis) was variable.

In the study of this report, only 2 diffuse bands of approximately 50 and 30 kd were identified in whole hydrolyzed soy protein, and these were only detected in the serum from the most reactive dog (S1). The diffuse appearance of both bands could be explained by the presence of many different molecular weight peptides in the soy protein hydrolysate. The major band of 75 kd identified in the native soy protein was not evident after the hydrolysis process. Those results suggest that in dogs sensitized against the native soy protein, whole hydrolyzed soy protein would be recognized to a much lesser degree than the native form, as evident in humans who have food allergic reactions to soy protein but do not respond to ingestion of infant formula containing soy protein hydrolysate.<sup>14</sup>

With regard to the various hydrolyzed soy protein fractions, western blot analysis revealed diffuse bands of 50 and 75 kd only in the highest molecular mass fraction (> 50 kd). In this same fraction, there were also 2 bands of 37 and 20 kd, which were probably a result of proteolysis of high-molecular-mass peptides (> 50 kd) during the assay.<sup>22</sup>

In the soy-sensitized Beagles, the inflammatory response to intradermal challenge with whole hydrolyzed soy protein was approximately half that detected after ID injection of native soy protein, in agreement with previously published data.<sup>7</sup> When hydrolyzed soy protein fractions were administered ID, an inflammatory response was induced by the > 10- to 30-kd fraction (100 µg/mL dose) and, in general, wheal areas directly correlated to the molecular mass of the hydrolyzed soy protein fractions (the maximal response was detected after administration of the fraction of highest molecular mass [> 50 kd]). Therefore, intradermal skin testing revealed reactivity to the hydrolyzed soy protein fractions that could not be detected by use of western blot analysis and that was not evident in the < 10-kd hydrolysate fraction. This lack of correlation between in vivo and in vitro test results was also apparent in a study<sup>23</sup> of allergic responses to soybean in asthmatic patients, from which it was concluded that western blot analysis is less sensitive to the shiny phenotype allergenic soybean varieties than other solid-phase assays, such as the skin prick test and specific IgE determinations. However, differences between results of intradermal skin testing and western blot analysis could be attributable to soy-specific IgG responses.<sup>8,24</sup>

In the present study, sera of soy protein-sensitized dogs were analyzed against whole hydrolyzed soy protein and hydrolyzed soy protein fractions with an immunoblotting technique and only low-intensity diffuse bands were detected. These results agree with results of our previous in vivo study<sup>7</sup> and also with findings that a soy hydrolysate diet was well tolerated by most dogs with food allergic reactions to soy protein.<sup>11</sup> Our results suggest that low-molecular-weight soy peptides (< 10 kd or at least < 30 kd) could further improve the treatment benefits of hydrolyzed soy protein-based diets. The protocol for experimental induction of soy protein sensitivity used in the present study appears to be a useful tool for investigations of the effect of hydrolyzed soy protein and assessment of soy-specific IgE reactivity in soy protein-sensitized dogs.

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- e. Canigen MHA2 Puppy, Virbac, Nice, France.
- f. Canigen MHA2PL Cuadruple, Virbac, Nice, France.
- g. FAG VIPDENS 111 Densitometer, Lausanne, Switzerland.
- h. Vivaflow 50 30000 MWCO, Vivascience-Sartorius, Goettingen, Germany.
- i. Vivaflow 50 kDa-psu, Vivascience-Sartorius, Goettingen, Germany.

- j. Vivaflow 50 10000 MWCO, Vivascience-Sartorius, Goettingen, Germany.
- k. Vivaflow 50 3000 MWCO, Vivascience-Sartorius, Goettingen, Germany.
- l. USIFROID SMH 15, Laboratoires Lyocentre, Aurillac Cedex, France.
- m. Nurish 1500, Solae, Iper, Belgium.
- n. Prestained SDS-PAGE standard broad range, Bio-Rad Laboratories, Hercules, Calif.
- o. Immun-blot PVDF, Bio-Rad Laboratories, Hercules, Calif.
- p. Ponceau S, Sigma Chemical Co, Barcelona, Spain.
- q. Anti-canine IgE, Bethyl, Montgomery, Tex.
- r. ECL System, Amersham Pharmacia Biotech, Uppsala, Sweden.
- s. Histamine diphosphate salt, Sigma Chemical Co, Barcelona, Spain.
- t. MIP4 Advanced Version, MICROM ESPAÑA SL, Barcelona, Spain.

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