

Development of an Enzyme-Linked Immunosorbent Assay Method To Detect Mustard Protein in Mustard Seed Oil

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ABSTRACT

An enzyme-linked immunosorbent assay for the detection of mustard protein was developed. The assay is based on a polyclonal antiserum directed against a mixture of mustard proteins raised in rabbits. The assay has a detection limit of 1.5 ppm (milligrams per kilogram) and is suitable for the detection of traces of mustard protein in mustard seed–derived flavoring ingredients. Limited cross-reactivity testing showed that no other plant proteins reacted significantly. From the animal proteins tested, only milk showed some cross-reactivity. With this sensitive assay, it was shown that refined mustard seed oil produced by steam distillation does not contain detectable amounts of mustard protein. Mustard seed oil is used as a flavoring in very low quantities, typically between 40 and 200 mg/kg. Thus, 100 g of a food product flavored with 200 mg of mustard seed oil per kg containing <1.5 mg of protein per kg would represent an amount of mustard seed protein of <30 ng. Taking into account the published literature on allergic reactions to the unintended ingestion of mustard, this conservatively low calculated level indicates that it is unlikely that food products containing mustard seed oil as a flavoring ingredient will elicit an allergic reaction in mustard-allergic individuals.

Allergy to mustard seed has gained considerable attention during the past decade, particularly by several investigators in Southern Europe. One of the first reports of mustard-induced allergy dates from 1980, when Panconesi et al. (14) described a severe anaphylactic reaction in a patient who had consumed pizza with mustard. Morisset et al. (13) showed that mustard allergy accounts for 1.1% of food allergies in children and that 23.3% of this group had clinical symptoms. Mustard-allergic patients may experience mild, oral, allergy-like symptoms, but more severe symptoms, such as systemic reactions, are reported to occur more frequently (5). Mustard allergy in other parts of the world has not been observed or reported as frequently as in Europe.

The allergenic component in mustard is the proteaceous fraction, particularly the seed storage proteins, similar to other allergenic seeds, nuts, and legumes (4). The major allergen in mustard is a 2S albumin (12). This protein has been characterized biochemically, and immunoglobulin E (IgE) epitopes have been described. There are no indications that any components other than protein of the mustard seed are allergenic.

In 2003, the European Parliament and the Council of the European Union issued new legislation related to the labeling of food ingredients from allergenic sources (2). One of the allergens listed is mustard. Mustard is used as a food ingredient in various forms. Whole seeds, ground seeds, and fractions thereof may be used to improve the

taste of soups, sauces, and other prepared foods. Highly refined mustard oil is also used as an ingredient to add mustard flavoring to a food. This flavor ingredient is composed mainly of allyl isothiocyanate, which gives the characteristic mustard taste. Mustard oil is obtained by grinding mustard seed; during this step, the allyl isothiocyanate is released from its precursor by the liberated endogenous enzyme myrosinase. Subsequently, the seed oil is removed by steam distillation at reduced pressure. The resulting distillate contains both water and volatile mustard oil, which is separated from the water phase by centrifugation and subsequently filtered and dried. During the steam distillation step, the allyl isothiocyanate evaporates, leaving behind nonvolatile material. Because of the nature of this isolation process, the mustard oil should not contain any appreciable proteins. The aim of this study was to develop a sensitive method for analyzing the residual protein content of highly refined mustard seed oil.

MATERIALS AND METHODS

Mustard seed and other reagents. A sample of mustard seed (*Brassica juncea*) that is commercially used to produce mustard seed oil by International Flavors and Fragrances (IFF Nederland BV, Hilversum, The Netherlands) was used to prepare a mustard extract. Mustard seed oil was obtained from IFF Nederland BV (Tilburg, The Netherlands). The ground mustard seeds were extracted at various pH values to obtain an extract that had a high extraction yield and a representative band pattern on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The extraction pH values were as follows: pH 4.0 (50 mM NaAc); pH 5.0 (50 mM NaAc); pH 6.0 (50 mM MES [morpholineethanesulfonic acid]); pH 7.0 (50 mM MOPS [morpho-

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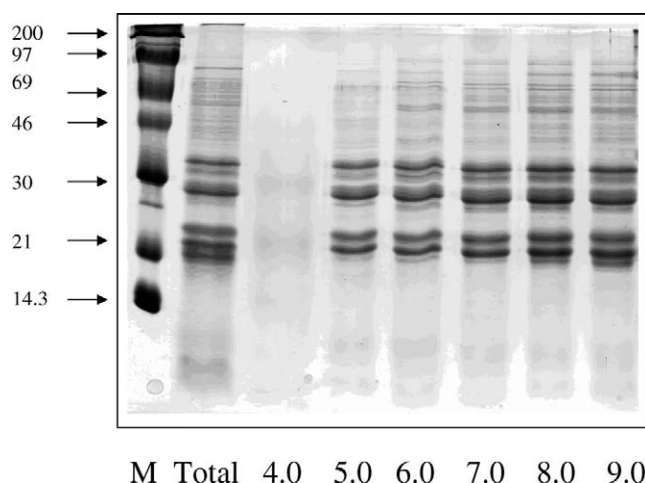


FIGURE 1. Extraction of mustard protein depends on pH. Mustard protein was extracted at different pH values. M, marker proteins indicated at the left margin (expressed in kilodaltons); total, a total extract with SDS under reducing conditions; 4.0, extraction at pH 4.0; 5.0, extraction at pH 5.0; 6.0, extraction at pH 6.0; 7.0, extraction at pH 7.0; 8.0, extraction at pH 8.0; 9.0, extraction at pH 9.0. The buffers used are described in "Materials and Methods."

linepropanesulfonic acid]); pH 8.0 (50 mM Tris); and pH 9.0 (50 mM Tris). The protein concentration of the mustard extracts was determined by Bradford analysis with bovine serum albumin as a standard. All reagents were from Sigma (St. Louis, Mo.). Extracts for testing cross-reactivity were prepared by mixing 1 part of food with 20 ml of extraction buffer (pH 8.0). Vegetables and meats were purchased from a local supermarket as fresh food. Egg yolk was a dried egg yolk powder used as an ingredient for the food industry, and whole egg was a lyophilized powder obtained from two eggs (both egg white and egg yolk, mixed gently) from a local supermarket. Nuts and legumes were obtained from a food ingredient supplier. Extracts were prepared by mixing 1 g of ground food with 20 ml of extraction buffer.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were performed essentially according to Towbin et al. (15). SDS-PAGE gels of 12 and 15% were used and were stained with Coomassie brilliant blue R250. Immunoblots were developed with a commercially available polyclonal antibody directed against rabbit IgG conjugated to peroxidase, similar to that described for hazelnut protein detection (10). The staining was performed with standard chemiluminescent technology.

Immunization and characterization of the antisera. Two rabbits were immunized with mustard extract (pH 8.0) by a classical immunization protocol at Genosphere Biotechnologies (Paris, France). Rabbits were immunized with mustard seed protein mixed with Freund complete adjuvant and subsequently boosted with mustard protein mixed with incomplete Freund adjuvant (three boosts, each after a 20-day period). Prebleeds and test bleeds were collected and stored at -20°C until use. For titer testing, enzyme-linked immunosorbent assay (ELISA) plates were coated with mustard extract (10 μg of mustard protein per ml in phosphate-buffered saline [PBS]), and dilutions of antiserum were incubated for 2 h. IgG bound to mustard protein was detected with a commercially available polyclonal antibody directed against rabbit IgG conjugated to peroxidase by the tetramethylbenzidine staining method according to the manufacturer's instruc-

TABLE 1. IgG responses in anti-mustard antisera

Serum dilution factor	Rabbit 1		Rabbit 2	
	Prebleed	Test bleed	Prebleed	Test bleed
1.000	0.153	1.950	0.134	2.040
10.000	0.051	1.370	0.042	1.370
100.000	0.039	0.750	0.028	0.770

tions (Sigma). Specificity was tested by immunoblotting methodology.

Inhibition ELISA for mustard protein. Microtiter plates (high-binding Costar 3590, 96-well plates, Costar, Bridgeport, N.J.) were coated with 10 μg of mustard protein per ml in PBS, pH 7.4. ELISA plates were blocked overnight at 4°C with PBS containing 1% bovine serum albumin. In separate test tubes, mixtures of diluted antiserum (1/10,000 final concentration) and known amounts of mustard protein were preincubated for 1 h. Preincubated mixtures were transferred to the ELISA wells coated with mustard protein and incubated for 2 h at 37°C . Bound IgG was detected with goat anti-rabbit IgG conjugated to peroxidase as described earlier for hazelnut protein (10) by the tetramethylbenzidine staining method according to the manufacturer's instructions (Sigma).

RESULTS

Extraction of mustard protein. The solubility of plant proteins depends on the pH of the extraction solution. Figure 1 shows the pH dependence of mustard protein extraction from ground mustard seed. With increasing pH, the solubility improved, similar to the observations for other seed and legume proteins (11). At pH 8.0 and 9.0, the SDS-PAGE band pattern and intensity were comparable with that of ground mustard seed extracted with SDS under reducing conditions, indicating that extraction at moderately basic pH values results in a representative mustard protein extract. The extraction method with pH 8.0 was chosen for all further experiments.

Characterization of the anti-mustard antisera. The two rabbits responded well to the immunization. Table 1 shows the IgG response of the test bleeds of the anti-mustard antisera at various dilutions. A serum dilution of 10,000 times showed an excellent response, and a dilution of 100,000 times still showed a good response, indicating that the sera were suitable for constructing a sensitive ELISA for mustard protein. Figure 2 shows the IgG reactivity of the antisera toward the different protein bands in mustard. Protein bands at approximately 30 and between 30 and 40 kDa, as well as an intense band at approximately 20 kDa, were recognized clearly, while several other bands reacted moderately. The band at 20 kDa probably represents the most intense member of the triplet, migrating at the height of the 21-kDa marker protein in Figure 1.

ELISA for mustard protein. With the anti-mustard antisera, an inhibition ELISA for the detection of mustard protein was constructed. IgG antibodies present in the anti-mustard antisera were allowed to bind to the mustard protein of the unknown sample in the liquid phase (preincu-

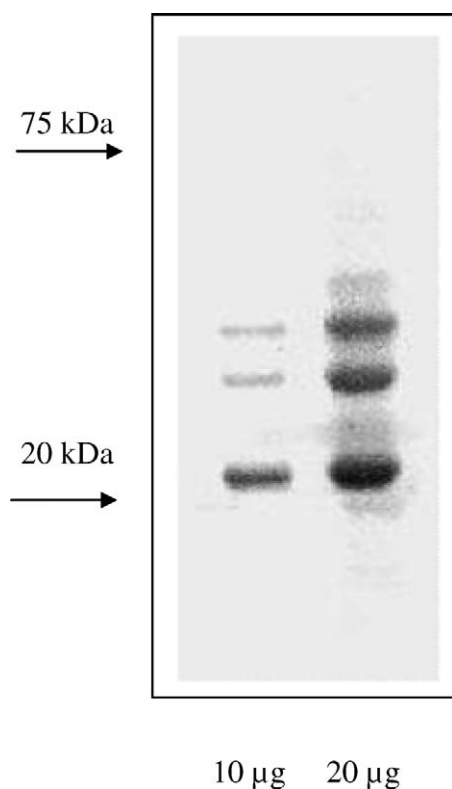


FIGURE 2. Characterization of the antisera by IgG immunoblotting. The antisera against mustard protein were analyzed by SDS-PAGE and IgG immunoblotting. First lane: 10 µg of mustard protein loaded on the gel; second lane: 20 µg of mustard protein loaded on the gel. Molecular weights are indicated in the left margin.

bation). Preincubated samples were transferred to ELISA plates coated with mustard protein, and the unbound IgG that was directed toward mustard protein could bind to the ELISA plates. Figure 3 shows the calibration curve. At increasing concentrations of mustard seed protein, the signal decreased, as anticipated for the inhibition ELISA, as the presence of high concentrations of mustard proteins will deplete the anti-mustard antiserum. The sensitive domains of the calibration curve were in the nanograms per milliliter and micrograms per milliliter ranges. The lowest concentration that resulted in a significant inhibition was 38 ng/ml. Taking into account the necessary dilution step (20-fold during extraction and twofold for the ELISA), the detection limit for measuring the protein in mustard seed oil is 1.5 ppm.

Cross-reactivity of common food constituents. A set of 16 common food constituents of different origin (both plant and animal sources; see "Materials and Methods") was tested on the potential cross-reactivity in the mustard ELISA (Table 2). The cutoff of cross-reactivity testing was 0.015%. This cutoff level was determined as follows. The sensitivity of the ELISA is 38 ng/ml (see previous paragraph), equaling a mustard extract of 1/65,000. A food extract tested at a dilution of 1/10 with a similar reactivity of the sensitivity of the ELISA (65,000-fold diluted mustard extract) is then 6,500-fold less reactive, equal to 0.015% reactivity (1/6,500).

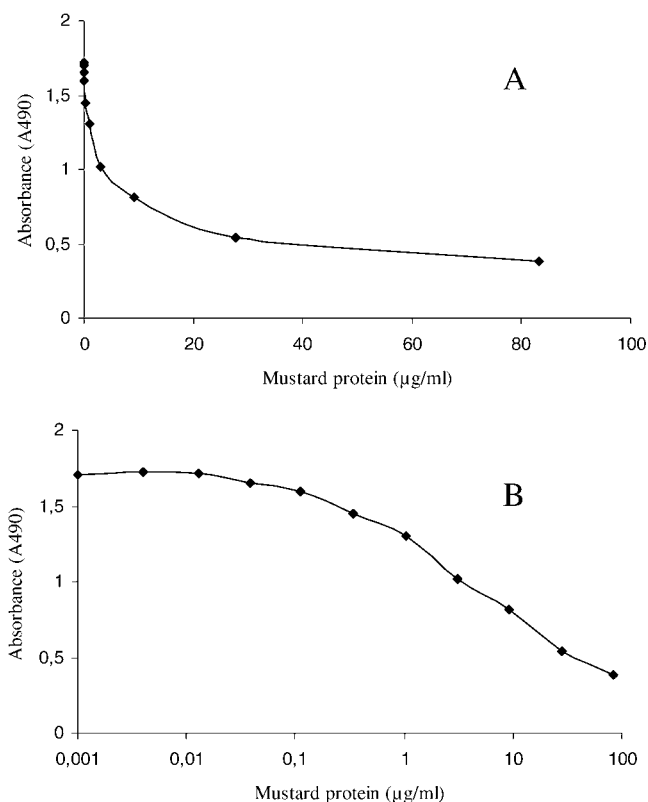


FIGURE 3. Calibration curves of the mustard seed protein inhibition ELISA. Panel A: linear x axis; Panel B: logarithmic x axis. Mustard protein was extracted at pH = 8.0 as described. The protein concentrations were measured, and dilutions of known concentrations were prepared as calibrators. Concentrations are expressed as micrograms of mustard protein per milliliter of buffer.

TABLE 2. Cross-reactivity of common food constituents

Species	Reactivity compared to mustard ^a
Soy	0.016%
Peanut	<
Walnut	<
Hazelnut	<
Brazil nut	<
Sesame seed	<
Corn	<
Milk	0.28%
Leek	<
Cauliflower	<
Cabbage	<
Pork	<
Beef	<
Poultry (chicken)	<
Egg yolk	0.20% ^b
Whole egg	<

^a Reactivity is expressed as a percent. A reactivity of 1% means that an extract reacts 100 times less than a mustard extract.

^b A further dilution of 10-fold completely abolished the reactivity of the egg yolk extract. < means <0.015%.

TABLE 3. Levels of mustard protein in mustard seed oil

Sample code ^a	Concn of mustard protein ($\mu\text{g/g}$, ppm)	Recovery of spike (%)
03001	<1.5	102
03002	<1.5	107
44602-401	<1.5	125
03004	<1.5	142
03005	<1.5	113

^a Sample codes refer to different batch numbers.

Soy reacted slightly (0.016%), and milk reacted moderately (0.28%). Egg yolk reacted with 0.20%, but a further 10-fold dilution completely abolished this reactivity. It might be that the high fat content of egg yolk powder disturbs the assay, indicating that the 0.20% is not reflecting true cross-reactivity, but rather, a matrix effect. The hypothesis of an effect caused by the fatty matrix is supported by the absence of cross-reactivity of whole egg powder, which consists of the major part of egg yolk.

It is interesting to note that the other tested foods did not cross-react. Mustard seed oil is used as a flavoring mainly in soups and sauces. In such foods, vegetables and legumes (soy rather than peanut) are other plant proteins that are potentially cross-reactive with mustard. However, only soy showed a minor cross-reactivity. An explanation might be that mustard oil is obtained from the seeds in contrast to the vegetable. It is well known that the composition of seeds is quite different from other plant tissues, but also, nuts and seeds from other plants did not give rise to cross-reactivity. Meat may be another ingredient in soups and sauces. Pork, beef, and poultry did not cross-react in this mustard ELISA.

Analysis of mustard oil. By the above-described inhibition ELISA, five samples of mustard seed oil were analyzed for the potential presence of mustard protein. Samples were mixed in a 1/20 ratio with aqueous buffer, gently shaken for 15 min at 50°C, and subsequently stirred at room temperature for 1 h. The aqueous phase was collected, diluted twofold, and analyzed. Table 3 summarizes the results, showing that all five samples were below the detection limit (second column). To exclude the possibility that the sample matrix influences the detection method, known amounts of mustard protein were spiked to the extracted samples. All five samples were extracted and diluted twofold. Mustard protein was added to a final concentration of 3.1 $\mu\text{g/ml}$, a concentration leading to an approximately half-maximal inhibition (Fig. 3, the fourth from the last value of the calibration curve). The recovery of the spikes added to the samples was 118% (average), and the individual values are listed in Table 2. The recoveries were all above 100%, indicating that the matrix did not inhibit the analysis. It is not known why the recoveries of samples 3 and 4 were considerably higher than 100%. Recoveries for ELISA methodology are reported to vary, especially at low concentrations of analyte, because of analytical imprecision. Earlier reported recoveries for similar food allergen detection ELISA tests varied from 82.7 to 138.7% (3).

DISCUSSION

Allergy to mustard has gained considerable attention during the past decade, because of several cases of severe allergic reactions to the inadvertent ingestion of mustard. Therefore, mustard is subject to the new legislation related to the labeling of food ingredients from allergenic sources by the European Parliament and the Council of the European Union in 2003 (2). Although the minimal provoking dose of mustard to elicit a clinical reaction is not known from case reports, a recent study by Morisset et al. (13) showed that one patient reacted to a dose as low as 0.8 mg of mustard protein in a prospective placebo-controlled food challenge study. This dose is about 20 to 30 times higher than the minimal provoking dose for peanut (16). The allergenic fraction of mustard is the protein fraction. The major allergen is the 2S albumin seed storage protein, Sin a 1, in yellow mustard (*Sinapis alba*) and Bra j 1 in Indian or leaf mustard (*B. juncea*) (6, 12). There are no indications that components other than protein are related to the allergenic character of mustard.

Food manufacturers commonly use food allergen detection assays to verify sanitation procedures. Test kits for peanut, milk, nuts, gluten, egg, and other allergens are commercially available and have detection limits of between 1 and 10 ppm (9). Taking into account the various case reports on food allergy and considering the lowest eliciting doses established by placebo-controlled food challenges, Hefle and Koppelman (7) postulated that this range of 1 to 10 ppm was indeed a useful figure for establishing detection limits for food allergens. The detection method described in this article had a detection limit of 1.5 ppm, suitable for appropriate mustard protein detection. The anti-mustard antiserum recognizes all main protein bands that are observed in mustard seed. The major mustard allergen, 2S albumin, has a molecular weight of between 16 and 20 kDa. The band at approximately 20 kDa on the immunoblot (Fig. 2) may represent this protein, although supportive evidence, such as protein identification with N-terminal sequencing, is not available. That the anti-mustard antiserum recognizes all main protein bands indicates that other potentially allergenic mustard proteins are recognized as well. We tested several food ingredients that are commonly used in recipes in which mustard flavoring is used, such as soups and sauces. Only milk showed some cross-reactivity, but this was only minor (0.28%). Nevertheless, when this ELISA is used for analyzing milk, this cross-reactivity should be taken into account. We have not investigated possibilities to reduce cross-reactivity—for example, by affinity purification of the antibodies from the hyperimmune serum.

We demonstrated that steam-distilled mustard oil does not contain substantial amounts of mustard protein. In fact, all tested samples were below the detection limit of the assay (<1.5 ppm). Mustard seed oil is used as a flavoring with a powerful sensory impact, and a typical concentration of mustard oil will vary from 40 to 200 mg per kg of food product. Considering a serving of 200 g of a food containing the upper limit of mustard seed oil (200 ppm), the intake of mustard seed oil in such a meal would be 40 mg,

and the exposure to mustard protein would be <60 ng, given the finding of <1.5 ppm of mustard protein in the tested samples. This estimated exposure level is several orders of magnitude below the minimal eliciting dose reported for mustard of 0.8 mg (13) and is orders of magnitude below the minimal eliciting dose known for peanut protein of 100 µg (16).

It is not surprising that highly refined mustard oil does not contain a detectable protein residue because of the nature of the production process. Mustard seed is ground and suspended in water, and the volatile flavoring compounds, mainly allyl isothiocyanate, are recovered by steam distillation at reduced pressure. The seed oil is subsequently separated from the water phase by centrifugation. Proteins are not volatile; therefore, their presence in a product obtained by steam distillation under reduced pressure is highly unlikely. This production process differs from seafood processing, when allergens become aerosolized during processing (peeling, prawn “blowing” by water jets or compressed air, boiling, and steaming). In aerosols found in open-process facilities of seafood manufacturers, levels of up to 5 µg/m³ have been found (8). Because mustard seed oil is processed by well-controlled steam distillation, only the evaporated fraction passes over the distillation column, thus eliminating the potential contamination of the distilled product with protein. The European Food Safety Authority, which is the independent scientific advisory agency of the European Parliament and Council, follows this rationale. Related to the new food allergen legislation, the European Food Safety Authority has concluded that mustard seed oil obtained by a properly controlled steam distillation process is not very likely to cause a severe allergic reaction in susceptible individuals (1). Calculating the amounts of mustard seed oil used in food products, it is very unlikely that mustard seed oil would provoke any sort of allergic reaction at all, based on the available case reports of allergic reactions to mustard and the lowest dose causing an adverse reaction.

We conclude that the described method is useful for assessing the possible allergen content of mustard-derived products and that mustard seed oil as produced by means of steam distillation does not contain substantial amounts of mustard protein.

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This article is dedicated to the life and research of Dr. Sue Hefle, Food Allergy Research and Resources Program, University of Nebraska, who lost her battle with cancer. Dr. Hefle’s research led to the development and successful implementation of numerous assays for the detection of allergens in food, using similar technology as is described in this article. She inspired me and many others in the field of allergen detection to provide tools to safeguard the life of food-allergic consumers. Her

achievements are of great value to the food industry.—Stef Koppelman, September 2006.

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