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Validated Sandwich Enzyme-Linked Immunosorbent Assay for Casein and Its Application to Retail and Milk-Allergic Complaint Foods

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ABSTRACT

Cows' milk is a commonly allergenic food. Cross-contamination of milk proteins into nondairy, kosher-pareve foods prepared on shared processing equipment can cause severe, life-threatening reactions in milk-allergic individuals. A sandwich-type enzyme-linked immunosorbent assay (ELISA; 96-well plate format) was developed for the detection of undeclared casein in foods. Rabbit anti-casein antibodies were used as the capture reagent. Food samples and standards were ground, extracted in 0.01 M phosphate-buffered saline, clarified by centrifugation, and added to the wells. Goat anti-casein antibodies were employed as the detector antibody, and the amount of antibody bound was determined with a commercial rabbit anti-goat immunoglobulin conjugated to alkaline phosphatase, with subsequent substrate reaction. Antibodies developed were specific to casein, with no cross-reaction observed with 30 foods and food ingredients. Non–milk-containing products such as fruit juices, fruit juice bars, sorbets, and dark and pareve-labeled chocolate were purchased from June 2002 through June 2003. In addition, samples allegedly causing eight milk-allergic consumer complaints were analyzed. The ELISA had a detection limit of less than 0.5 ppm of casein. The casein content in the analyzed foods ranged from less than 0.5 ppm to more than 40,000 ppm casein; undeclared casein residues were found in all of the samples implicated in allergic reactions. The levels of milk contamination in some of the other surveyed products could also be hazardous for milk-allergic consumers. This ELISA method provides a useful quality control tool for the food industry and could also be used as a validation of kosher-pareve status.

Cows' milk is one of the eight foods or food groups that are believed to cause at least 90% of the allergic reactions to foods *(7)*, the others being peanuts, tree nuts, fish, eggs, wheat, soybeans, and crustacean shellfish. At least 160 other foods *(13)* have been shown to cause allergic reactions, although most of these are rarely allergenic. The majority of studies estimate the prevalence of milk allergy among infants at about 2% *(14, 17, 19, 32)*. Although milk allergy is usually an allergy of childhood, occasionally it will persist into adulthood *(27, 28, 35)*. Individuals with severe cases of milk allergy are among those most likely to have persistent milk allergy *(33)*. Accordingly, allergic reactions to milk in adults and older children can be quite severe on occasion *(30, 31, 34)*. The naturally occurring protein causative agents in milk that cause allergic reactions include both casein and certain whey proteins *(2)*. These proteins retain their allergenicity following heating or other processing *(10)*. In addition, the fermentation processes associated with production of such dairy products as cheese and yogurt have virtually no effect on the allergenicity of milk proteins.

No treatment exists for food allergy, so strict avoidance is the key to preventing reactions for food-allergic individuals. Trace amounts of milk can pose a serious risk to milk-allergic individuals, and there have been several documented severe allergic reactions to contaminant milk proteins in nondairy foods, including products marked ''pareve'' *(8, 23)*. Fatalities have occurred with consumption of milk by allergic individuals *(3, 25, 29, 31)*. Milk-allergic consumers are faced with the dilemma of selecting from a variety of processed foods prepared or manufactured in facilities that might also be used to make milk-containing foods on shared equipment. For example, many dairy processors and chocolate manufacturers use the same processing equipment for milk-containing and non–milk-containing products. Milk-allergic consumers often use kosher designations and choose products marked ''pareve'' (free of milk, meat, or poultry products) as part of an avoidance diet. Although koshering is a well-defined religious procedure, it does not meet the critical standards necessary for the allergic consumer. The inadvertent presence of milk proteins, including casein, can result from practices in the food industry such as the use of contaminated raw materials, inadequate cleaning of shared equipment, allergen-containing ''rework'' (leftovers from food production), or other sources of cross-contact. Although the food industry has made large strides in allergen control over the past 10 years *(5, 18)*, U.S. Food and Drug Administration (FDA) and Department of Agriculture recalls still occur for undeclared milk. In 1999, of 68 FDA recalls because of allergens, 11 (16%) were the result of undeclared milk *(38)*.

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The enzyme-linked immunosorbent assay (ELISA) technique is a sensitive, specific, and convenient method to detect allergenic residues and has been used successfully to detect undeclared residues of peanut *(11, 21, 39),* egg *(12, 40),* hazelnut *(1, 16, 22),* almond *(15),* and whey *(6).* The objective of this study was to produce a rigorous, robust sandwich ELISA capable of detecting undeclared, potentially hazardous casein residues in non–milk-containing foods. A second objective was to do a survey of retail non–milk-containing foods and to investigate milk-allergic consumer complaints with the developed ELISA. The ELISA could also be used to assess tolerance or threshold limits in incidences of cross-contact and inadvertent exposure.

MATERIALS AND METHODS

Antibody production. Polyclonal antibodies were produced at Covance Research Products Inc. (Denver, Pa.). New Zealand white rabbits, a goat, and a sheep were immunized with bovine casein (>90% purity, acid-precipitated, data not shown) obtained from Sigma Chemical Company (St. Louis, Mo.) with the use of a hyperimmunization protocol described by Cordle et al. *(4).* The immunogen consisted of casein in 0.01 M phosphate-buffered saline (PBS; 0.002 M NaH_2PO_4, 0.008 M Na_2HPO_4, 0.85% NaCl, 0.02% NaN_3, pH 7.4) emulsified with complete Freund's adjuvant. Polyclonal antibodies were partially purified from sera by precipitation with ammonium sulfate by the method described by Harlow and Lane *(9).* After partial purification, the antibodies were redissolved and dialyzed extensively (12,000 to 14,000 molecular weight cut-off, Spectrum, Gardena, Calif.) against PBS. To monitor antibody production, titers were routinely determined on collected sera with a noncompetitive ELISA (as described in the ''ELISA'' section). Titers were defined as the log reciprocal of the midlinear portion of the dilution curves from plates coated with 1.0 μg casein per ml. Antibody lots with titers of 10,000 or more were pooled according to species and stored at −20°C. Protein concentrations were obtained by the Lowry et al. *(24)* method.

Food samples and consumer complaint samples. Retail food samples for analysis and for cross-reactivity studies were purchased locally (Lincoln, Neb.) and at various locations in North America and Europe from June 2002 through June 2003. A total of 30 foods and food ingredients were examined for cross-reactivity in the ELISA. For retail samples, two lots of non–milk-containing products were obtained if possible; each lot was extracted in duplicate, and each extract was analyzed in duplicate. Food samples implicated in eight milk-allergic consumer complaints and reported to the Food Allergy Research and Resource Program at the University of Nebraska were obtained directly from the affected consumers. One of the implicated consumer complaint products was confirmed to contain milk with a skin prick test in the milk-allergic child who reported a reaction to it.

Reference food standards. Individual reference dark chocolate standards were made in an industrial setting (Barry Callebaut, St. Hyacinthe, Quebec, Canada). Dark chocolate standards containing 0, 1, 5, 10, 20, 50, and 100 ppm casein were prepared. The dark chocolate was prepared with chocolate liquor, butter oil, cocoa butter, soy lecithin, sugar, and vanilla (Barry-Callebaut), and all ingredients were previously tested to ensure the absence of casein. Tempered chocolate standards were thoroughly mixed (Hobart Corp., Troy, Ohio) before molding. Laboratory standards for the sorbet and juice were prepared by adding soluble casein in specific amounts to an extracted lemon sorbet control and serially diluting in the lemon sorbet control extract. All sorbet, fruit juice bar, and juice samples were adjusted to pH 7.0 ± 0.2 before extraction.

Extractions. Chocolate samples and reference chocolate standards were extracted 1:10 in 0.01 M PBS containing 0.1% Tween 20 at 60°C for 2 h with shaking (Cole-Palmer Instrument Co., Chicago, Ill.). Other foods and beverages were adjusted to pH 7.0 and extracted 1:1 in 0.01 M PBS at room temperature with shaking because of lower levels of contamination and simplicity of the matrix compared with chocolate samples. All extracts were centrifuged at 3,200 × *g* for 30 min at 10°C (International Equipment Company, Needham Heights, Mass.). Protein concentrations for food and reference standards were determined by the Lowry et al. *(24)* method. The supernatant solutions were stored at −20°C in polypropylene tubes until analyzed.

ELISA. In polyclonal antibody production, titers were monitored with an indirect ELISA, generally by the method described by Hefle et al. *(12),* and defined as discussed in the section ''Antibody production.'' The sandwich-type ELISA for casein was modified from the methods described by Hefle et al. *(11, 12)* for peanuts and eggs and was applied as follows for chocolate samples: 100 μl of coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, 0.02% NaN_3, pH 9.6) containing 2 μg of rabbit anti-casein antibody was applied to each well of a 96-well microtiter plate (MaxiSorp, Nagle Nunc International, Roskilde, Denmark). For nonchocolate samples, 100 μl of coating buffer containing 0.175 μg rabbit anti-casein antibody was used. For all samples, the remainder of the protocol was the same: plates were incubated overnight at 4°C and then washed with 0.05% Tween 20 in 0.01 M PBS, pH 7.4 (PBS-T), with an automatic plate washer (Dynatech Laboratories Inc., Chantilly, Va.). All plates were washed with PBS-T after each incubation step described later in this section, except after substrate addition. After coating, remaining binding sites were blocked with 350 μl per well of 0.1% gelatin (300 bloom, porcine, Sigma) in PBS, and plates were incubated for 1 h at 37°C. Samples were diluted in the appropriate negative food extract and loaded in triplicate onto each plate. Following washing, 15 μg goat anti-casein antibody (100 μl per well) diluted in PBS containing 0.1% bovine serum albumin (PBS-B; RIA grade, Sigma) was added, and plates were incubated for 1 h at 37°C. The amount of casein bound was detected by subsequent incubations for 1 h (for chocolate samples) or 30 min (for nonchocolate samples) with 100 μl of rabbit anti-goat immunoglobulin conjugated to alkaline phosphatase (Pierce Chemical, Rockford, Ill.) and diluted 1:5,000 (vol/vol) in PBS-B, with the subsequent addition of *p*-nitrophenylphosphate substrate (Sigma Fast, Sigma). Color was allowed to develop for 30 min and was then stopped by the addition of 100 μl of 1 N NaOH. Absorbances were read at 410 nm on an MR600 plate reader (Dynatech). A series of standards were included on every plate, and standard curves were developed with the Prism (Windows-based) computer software program (GraphPad Software Inc., San Diego, Calif.).

RESULTS AND DISCUSSION

Antibodies and ELISA. Immunized rabbits and goats were responsive to the casein immunogen with high titers (5×10^5 or greater) occurring 4 weeks into the protocol and continuing for the duration of the antibody production schedule. Sheep did not produce high-titer antibodies against the immunogen. Analysis of preimmunization sera samples indicated that animals did not possess existing antibodies to casein (data not shown). Preliminary assay trials

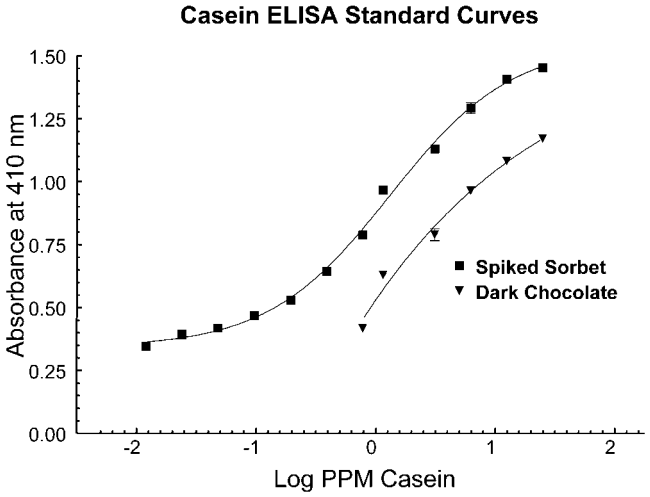

FIGURE 1. *ELISA standard curves for casein-in-dark chocolate standards and spiked sorbet samples. The values shown represent the average of 36 trials. For each reference point, the standard deviation was <0.01.*

indicated that an ELISA response optimal for the intended application was achieved with rabbit anti-casein antibodies at a concentration of 0.175 μg/ml as the capture antibody and goat anti-casein antibodies at 15 μg/ml as the detector antibody. The resulting ELISA had a detection limit of 0.5 ppm casein. Figure 1 shows the standard curves obtained with the reference casein-in-dark chocolate and sorbet standards. Dilutions were performed when necessary. The linear portion of the curves was used to estimate the level of undeclared casein in the commercial samples. The standard deviation of absorbances for each of the reference points was less than 0.01.

Cross-reactivity. The antibodies produced were specific for casein; no cross-reactivity was observed when 30 foods and food ingredients were analyzed in the ELISA (Table 1). The foods and food ingredients were tested after being extracted at 1:10 (wt/vol). This concentrated level assured that the true effect of potential cross-reactivity of a food or food ingredient in the ELISA was ascertained.

Limited retail survey. Different brands of non–milk-containing chocolate, sorbets, frozen fruit drinks, fruit juice bars, and fruit juices were analyzed. It was impossible to obtain two lots of some products. Samples registered values ranging from less than 0.5 ppm to more than 12,000 ppm casein (Table 2). No non–milk-containing chocolate samples could be obtained or found that did not contain casein residues, except for the dark chocolate used to make the casein-in-chocolate standards (which is manufactured on a dedicated milk-free line). The coefficient of variation, more appropriate than the standard deviation for reporting immunoassays *(20),* for all samples analyzed in this study fell under the expected ranges for immunoassay methods *(20).* The detection limit of the casein ELISA was very low; thus, at high levels of casein contamination, small errors in pipetting, for example, could have a large effect on quantitation, causing a large coefficient of variation. In addition, it is observed from the retail chocolate samples for which two different lots could be obtained that the level of undeclared casein can be vastly different from one product lot to the next (Table 2). All of the fruit juice and sorbet samples analyzed in this study registered below the limit of detection of the ELISA. However, undeclared casein residues were detected in some lots of fruit juice bars or frozen fruit drinks, albeit at much lower levels than in the chocolate samples. Chocolate manufacturing poses special challenges to allergen control; for example, introduction of water into the manufacturing system for sanitation purposes is not usually an option because of microbiological and other concerns. Fruit juice products are usually manufactured with equipment that can be cleaned in place with aqueous solutions. Additionally, allergen contamination in chocolate products can be very nonhomogeneous because of the nature of the product and the process.

TABLE 1. *Cross-reactivity of foods and food ingredients in the casein ELISA*

Food[a]	Protein concentration (mg/ml)	Cross-reaction
Basil	9.7	None
Chocolate (dark)	2.87	None
Cocoa (baking)	9.9	None
Corn starch	0.06	None
Corn syrup (light)	0.07	None
Coconut cream	0.5	None
Eggs	32.2	None
Fennel seed	2.6	None
Garlic powder	2.7	None
Honey	0.9	None
Lemon juice	0.2	None
Malt extract A	5.7	None
Malt extract B	2.2	None
Malt extract C	5.4	None
Mango (canned)	0.3	None
Molasses (dark)	4.2	None
Mustard (ground)	72.5	None
Oats (rolled)	1.4	None
Onion powder	2.9	None
Oregano	64.8	None
Pepper (lemon)	0.7	None
Raspberries (frozen)	0.8	None
Rice	0.7	None
Salt	BLD[b]	None
Soybean oil	BLD	None
Strawberries (frozen)	0.5	None
Sugar (brown)	0.48	None
Sugar (white)	BLD	None
Thyme	3.8	None
Vegetable oil	BLD	None
Vinegar (white)	BLD	None
Yeast	0.07	None

[a] All food and food ingredients were extracted 1:10 (wt/vol) as described in the ''Materials and Methods'' section.

[b] BLD, below the detection limit of the protein determination assay *(24).*

Milk-allergic consumer complaint samples. Consumer complaint samples obtained from milk-allergic in-

TABLE 2. *Limited retail survey of non–milk-containing and kosher food products for undeclared casein residues by ELISA*[a]

Sample	Brand	Flavor	Kosher designation	Casein, lot 1 (ppm)	CV, lot 1	Casein, lot 2 (ppm)	CV, lot 2
Chocolate							
Bittersweet	A		None	1,857	14.5	4,265	12.2
	B		None	4,281	3.9	21	6.7
	C		None	7.8	16.7		
Dark	A		Certified kosher–dairy equipment	4,009	26.7	4,098	26.1
	B	1	Certified kosher	2,455	14.5	6,269	23.8
	B	2	None	3.6	19.4	27.5	43.6
	C		Certified kosher	238	10.9	390	42.6
	D		None	5,225	1.3	6,885	5.9
	E		None	10,814	20.3	6894	2.4
	F		None	203	7.9		
	G		None	571	15.8		
	H		None	2,561	19.5		
	I		None	312	25.3		
	J		None	96	8.9		
	K		None	71	19.7		
	L		None	57	17.5		
	M		None	34	23.5		
	N		None	4,232	19.9		
	O		None	126	3.7		
	P		None	5,108	10.0		
	Q		None	97	7.7		
	R		None	188	6.9		
	S		None	142	12.9		
	T		None	254	9.5		
	U		Certified kosher–dairy equipment	4,378	13.3		
Dark 70% cocoa	V		None	21	6.7	2.0	5.0
Semisweet	A		None	6,173	3.0	5,446	14.6
	B		None	3,491	15.9		
	C		None	186	11.8		
Sorbet	A	1	None	BLD		BLD	
	A	2	None	BLD		BLD	
	A	3	None	BLD		BLD	
	A	4	None	BLD		BLD	
	A	5	None	BLD		BLD	
	B	1	None	BLD		BLD	
	B	2	None	BLD		BLD	
	C		None	BLD		BLD	
	D		None	BLD		BLD	
Fruit juice bars	A	1	None	2.8	21.8	4.8	10.4
	A	2	None	6.3	7.9	4.0	12.5
	A	3	None	4.5	24.4	2.3	21.7
	B	1	None	BLD		BLD	
	B	2	None	BLD		BLD	
	B	3	None	BLD		BLD	
	C	1	None	BLD		BLD	
	C	2	None	BLD		BLD	
	C	3	None	BLD		BLD	
	D	1	None	BLD		BLD	
	D	2	None	BLD		BLD	
	E		None	BLD		BLD	
	F		None	BLD		BLD	
	G		None	BLD		BLD	
	H		None	BLD		BLD	
Frozen fruit drink	A		None	BLD		BLD	
	B		None	1.0	30.0		
	C		None	1.2	8.3		

TABLE 2. *Continued.*

Sample	Brand	Flavor	Kosher designation	Casein, lot 1 (ppm)	CV, lot 1	Casein, lot 2 (ppm)	CV, lot 2
Fruit juice	A	1	None	BLD		BLD	
	A	2	None	BLD		BLD	
	A	3	None	BLD		BLD	
	A	4	None	BLD		BLD	
	A	5	None	BLD		BLD	
	B	1	None	BLD		BLD	
	B	2	None	BLD		BLD	
	B	3	None	BLD		BLD	
	B	4	None	BLD		BLD	
	C	1	Certified kosher	BLD		BLD	
	C	2	Certified kosher	BLD		BLD	
	D		None	BLD		BLD	
	E		None	BLD		BLD	

[a] BLD, below the detection limit of the protein determination assay *(24)*.

dividuals had undeclared residues ranging from 5,500 to 44,500 ppm casein (Table 3). One of the products, a kosher chocolate, was evaluated by skin prick testing, an in vivo allergy diagnostic method. The kosher chocolate sample elicited a positive skin prick test reaction in the milk-allergic child who reported a serious allergic reaction after ingestion of this chocolate, further confirming the presence of potentially hazardous levels of milk. The results of the limited retail survey of dark (non–milk-containing) and pareve-labeled chocolate in this study illustrate that it can be difficult for a milk-allergic individual to avoid undeclared milk residues in chocolate products. In addition, the limited survey of other non–milk-containing food products indicates that juices, sorbets, and fruit juice bars often register below the limit of detection of the casein ELISA, although occasional exceptions do exist. However, the roster of consumer complaint samples analyzed show that a wider variety of products could contain undeclared milk residues on occasion.

The ELISA successfully detected residues of casein at concentrations pertinent to allergenic concerns in foods. The minimum provoking dose of milk is as yet not fully defined, although in one clinical trial the minimum provoking dose of milk protein was 0.6 mg, whereas other studies show that levels higher than 15 to 180 mg of milk protein are capable of eliciting reactions in other groups of patients *(36)*. A concentration as low as 0.06% undeclared milk in sausage was documented as causing a fatal reaction *(25)*. The developed ELISA has a detection limit of 0.5 ppm, equivalent to 0.00005%. Some have argued that foods containing in excess of 10 ppm undeclared allergen pose a significant risk to food-allergic individuals *(26, 37)*, although more research on statistically based clinical threshold studies needs to be done. Despite existing uncertainty about the precise threshold dose for milk in milk-allergic individuals, this ELISA offers an appropriate detection limit suitable for the protection of milk-allergic consumers.

This ELISA would be useful to the food manufacturer in evaluating finished products and raw materials and in assessing the effectiveness of cleaning and scheduling strategies in the development of an allergen control program. This analytical technique could be useful in developing risk assessment strategies for dealing with allergens in food production and manufacturing environments. In addition, this technique would be useful to food regulatory agencies in investigating milk-allergic consumer complaints. It is important that immunochemical methods for allergenic food residues are validated with the use of reference standards (such as the casein-in-dark chocolate standards used in this study) made in actual industrial or pilot plant settings to ascertain the true extraction efficiencies, usefulness, and robustness of the method. Most methods reported in the lit-

TABLE 3. *Casein levels of products associated with milk-allergic consumer complaints as determined by ELISA*

Product	Kosher designation	Casein (ppm)
Honey whole wheat roll	None	5,500
Tofu chocolate cheesecake	None	16,100
Soy coffee drink	None	43,500
Organic dark chocolate	None	10,400
Dark chocolate	Certified kosher	12,700[a]
Baker confectionary icing	Made in kosher bakery	44,500
Apple-flavored energy bar	None	27,300
Dairy-free chocolate cupcake	None	13,700

[a] Positive skin prick test to product in milk-allergic child.

erature and available commercially have not been evaluated with the use of standards such as these, and this should be a prerequisite to implementing analytical techniques in any exercise involving detection of allergenic residues in food matrices.

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