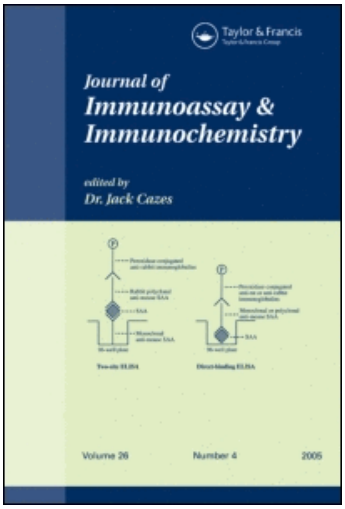

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 11 October 2004

To cite this Article Pourpak, Z. , Mostafaie, A. , Hasan, Z. , Kardar, G. A. and Mahmoudi, M.(2005) 'A Laboratory Method for Purification of Major Cow's Milk Allergens', Journal of Immunoassay and Immunochemistry, 25: 4, 385 — 397

To link to this Article: DOI: 10.1081/IAS-200033845

URL: http://dx.doi.org/10.1081/IAS-200033845

JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 25, No. 4, pp. 385–397, 2004

A Laboratory Method for Purification of Major Cow's Milk Allergens

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ABSTRACT

Food allergy is responsible for the most frequent allergic reactions in children under 1 year of age. Diagnostic tests such as skin test or specific IgE assay usually need highly purified preparations of allergenic proteins. The aim of the present study was to purify three main cow's milk proteins: casein, α-lactalbumin, and β-lactoglobulin as allergenic extracts for first time in Iran.

Key Words: Cow's milk; Allergen; Casein; α-Lactalbumin; β-Lactoglobulin.

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DOI: 10.1081/IAS-200033845 1532-1819 (Print); 1532-4230 (Online)

INTRODUCTION

Food allergy is a group of IgE-mediated and non-IgE-mediated disorders in which symptoms result from immunologic responses to specific food antigens.[1] Adverse reactions to foods are believed to be a relatively common occurrence.[2] Food allergy is the most important allergy in early childhood,[3] affecting 6–8% of young children.[4] Approximately 2% of the adult population experience food-induced allergic disorders.[5] Although many foods have been reported to cause allergic reactions in children,[6] a small number of allergens account for the majority of complaints, including cow's milk, hen's egg, wheat, and soy.[7] Other foods that cause allergic reactions frequently are nuts, fish, pork, beef, tomato, carrot, celery, mushroom, corn, yeast, and food colors and preservatives.[8]

Cow's milk is one of the most common food allergens in children, perhaps because it is usually the first foreign protein encountered by infants.[6] Prevalence of cow's milk allergy (CMA) varies from 0.3% to 7.5% in the general population.[9,10] Symptoms of CMA commonly appear during the first year of life and, in 75–90% of patients, during the first month of life,[6] coinciding with its introduction into the infant's diet.[11] Recent studies have shown that, although CMA in infants may disappear spontaneously with time, CM tolerance may remain in 28% of patients by 2 years of age, 33–56% by 4 years, and 78% by 6 years.[12–14]

The clinical features of patients allergic to cow's milk are mostly expressed as immediate symptoms,[15] which occur in 40–50% of children within the first hours after challenges.[16]

Most of the major food allergens have been found to be glycoproteins in the molecular weight range from 10 to 40 kDa.[17] Cow's milk contains more than 25 distinct proteins that may act as antigens in humans.[10] Milk allergens are proteins, and these are traditionally divided into two groups. Casein constitutes 80% of the total bovine milk proteins.[18] It corresponds to an association of four different proteins αs1-, αs2-, β- and κ-caseins in approximate proportions of 40%, 10%, 40%, and 10%, respectively.[19] The whey fraction of cow's milk consists of β-lactoglobulin, α-lactalbumin, bovine immunoglobulins, bovine serum albumin, and small amounts of various other proteins.[6] Bovine β-lactoglobulin exists as a dimmer and many species appear to possess genetic variants. In ruminants, the relative molecular weight of the subunit is $\sim$18 kDa, corresponding to a polypeptide of $\sim$162 residues.[20]

The purified α-lactalbumin gives a single band in electrophoresis. The molecular weight of bovine α-lactalbumin is about 14 kDa.[21] The most important allergens are β-lactoglobulin and casein, but clinical reactions have occurred to all of the major milk allergens; Lebanthol reviewed five studies and found sensitivity to β-lactoglobulin in 82%, to casein in 43%, to α-lactalbumin in 27%, and to bovine serum albumin in 18%.[10]

The diagnosis of food hypersensitivity involves acquisition of a careful history, physical examination, in vivo or in vitro diagnostic tests, institution of elimination diets, and confirmation by blind oral food challenge.[22] Skin prick tests (SPTs) are commonly used to screen patients with suspected IgE-mediated food hypersensitivity. Radioallergosorbent tests (RASTs) detect the presence of food-specific IgE antibodies in patient sera and are often used by practitioners as a screen for IgE-mediated food hypersensitivity.[6] These tests need to use allergenic extracts. Also, purification of CM proteins is necessary for preparation of hydrolyzed formulas, which are used as CM substitutes for feeding babies with CMA.[23] In some studies, the production of recombinant β-lactoglobulin and its immunological properties have been described.[24] Although, in research centers in some countries, pure allergens were produced by recombinant technology,[17] in Iran, even purification of CM allergens (such as in this study) has not yet been accomplished. The purpose of this study is to start obtaining and purifying common allergenic extracts in the laboratory because of the importance and high prevalence of CMA, especially in children; major cow's milk allergens were purified.

EXPERIMENTAL

In this study, a favorite milk sample was selected and three important milk allergens were purified. Then, these extracts were evaluated with SDS–PAGE and rabbit immunization for determination of allergenic potential.

Milk Preparation

All samples for extraction were prepared from Holestein cow's milk (obtained from the Faculty of Veterinary, Tehran University). Fresh milk was selected, without any physical or chemical modification. The first step was the separation of residual fat from the milk, which was collected by centrifugation at $1000 \times g$ for 30 min at 4°C.

Allergens Purification

In order to separate casein by isoelectric precipitation, fresh skim milk was incubated at 37°C for an hour and the pH was decreased to 4.6 with 1 M Hcl. After centrifugation at $5000 \times g$ for 15 min, a casein pellet was separated from whey. The whey was stored frozen until required. Pellets were washed two times with distilled water, pH 4.6, and lyophilized.

For α-lactalbumin purification, the whey from cow's milk was added to an equal volume of saturated ammonium sulfate in 0.15 M phosphate buffered saline (PBS), pH 7. Then, it was centrifuged at $3000 \times g$ for 30 min at 4°C, dry ammonium sulfate was added to the supernatant solution up to 90% concentration, and then it was centrifuged. The pellet was then dissolved to a concentration of 10 mg/mL in PBS and loaded onto XK 26/100 columns of Sephadex G-50 (Pharmacia Biotech, Uppsala, Sweden) which had been previously equilibrated with the same buffer. The second peak of the chromatogram was collected as a fraction and then the previous steps were repeated.[21]

In order to purify β-lactoglobulin, first polyethylene glycol (PEG 6000) was added to whey to a 16% concentration (w/v), it was incubated 1 hr at 8°C with stirring, and then was centrifuged at $5000 \times g$ for 30 min at 8°C. After centrifugation, the pellet was separated and the PEG concentration in the supernatant was adjusted to 26%. A 10 mg/mL of protein in 0.03 M sodium phosphate buffer, pH 6.8, was loaded onto an XK 26/20 column of DEAE Sepharose CL-6B (Pharmacia Biotech, Uppsala, Sweden) that was previously equilibrated with the same buffer. The components in the column were eluted with a salt gradient of 0.0–0.5 M NaCl, at a flow rate of 30 mL/hr. Fractions which contained crude β-lactoglobulin were pooled.[25]

Product Evaluation

For evaluation of extraction purity, discontinuous SDS–PAGE was performed; 3% (w/v) acrylamide stacking gel and 12% (w/v) resolving gel were used. A 10 μg per well of sample extracts, standard samples (Sigma Co., USA), and a low molecular weight (LMW) marker including 14.4, 20.1, 30, 43, 67, 94 kDa (Sigma, USA), were loaded onto the gels. After electrophoresis, proteins were stained with Coomassie R-350 (Phast Gel Blue R, Uppsala, Sweden) and purity evaluation was performed by photo EP software densitometry (Photo EP verson 5.50, Amir Kabir Bio. Medical Eng. Group, Hoshmand Fanavar Co, Tehran, Iran).

Each extract was dissolved to a concentration of 0.5 mg/mL in sterile 0.15 M PBS, pH 7, accompanied by adjuvant; it was then injected into young rabbits, subcutaneously. Four injections were performed: the first injection in the first week included 0.3 mL antigen in addition to 0.3 mL Freund's complete adjuvant; booster injections were administered during the 3rd, 5th, and 7th week including 0.3 mL antigen in addition to Freund's incomplete adjuvant. Then, sera were isolated from immunized rabbits' blood samples. Double immunodiffusion was performed on serum, in addition to this study, with extracts and standard, both 2 mg/mL.[26]

RESULTS

Allergen Purification

Casein isolated by the acidic procedure has a high purity, with 4–5 bands in SDS–PAGE. Result of densitometric analysis showed that this product has a higher purity (91–94%) than similar products, for example, a sample from Sigma Co. was only 80–85% pure (Fig. 1).

α-Lactalbumin was isolated in two steps of gel chromatography. SDS–PAGE on collected fractions from the first chromatographic separation demonstrated two curves, including a first curve that often was large, as β-lactoglobulin (Fig. 2). The second curve fractions were gathered and the previous steps were repeated. The purity of α-lactalbumin that was isolated in this study is very high (Fig. 3).

For purification of β-lactoglobulin, anion-exchange chromatography was used as the preferred procedure; results showed high purity that is comparable with other products, such as Sigma Co. (Fig. 4).

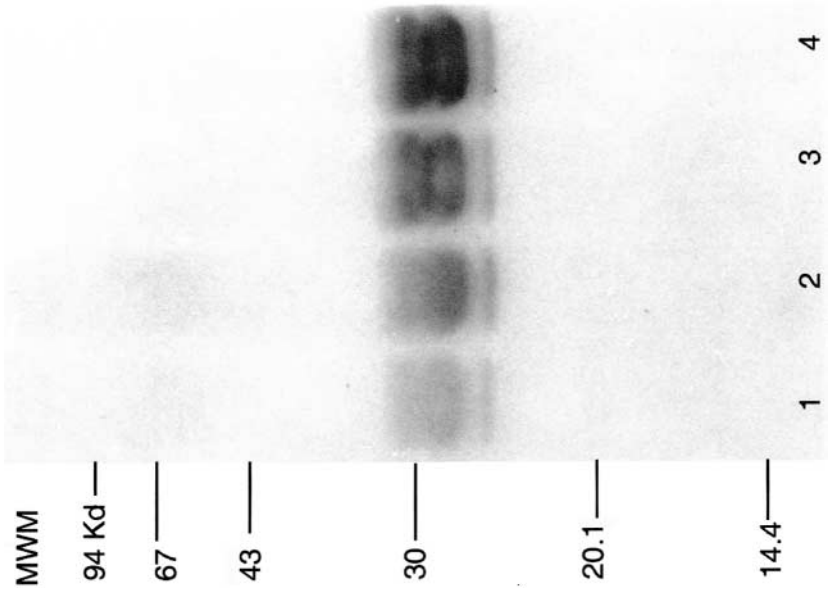

Figure 1. SDS–PAGE, comparison of casein extracts from fresh cow's skim milk. Casein standard (lanes 1 and 2), this study extract (lanes 3 and 4).

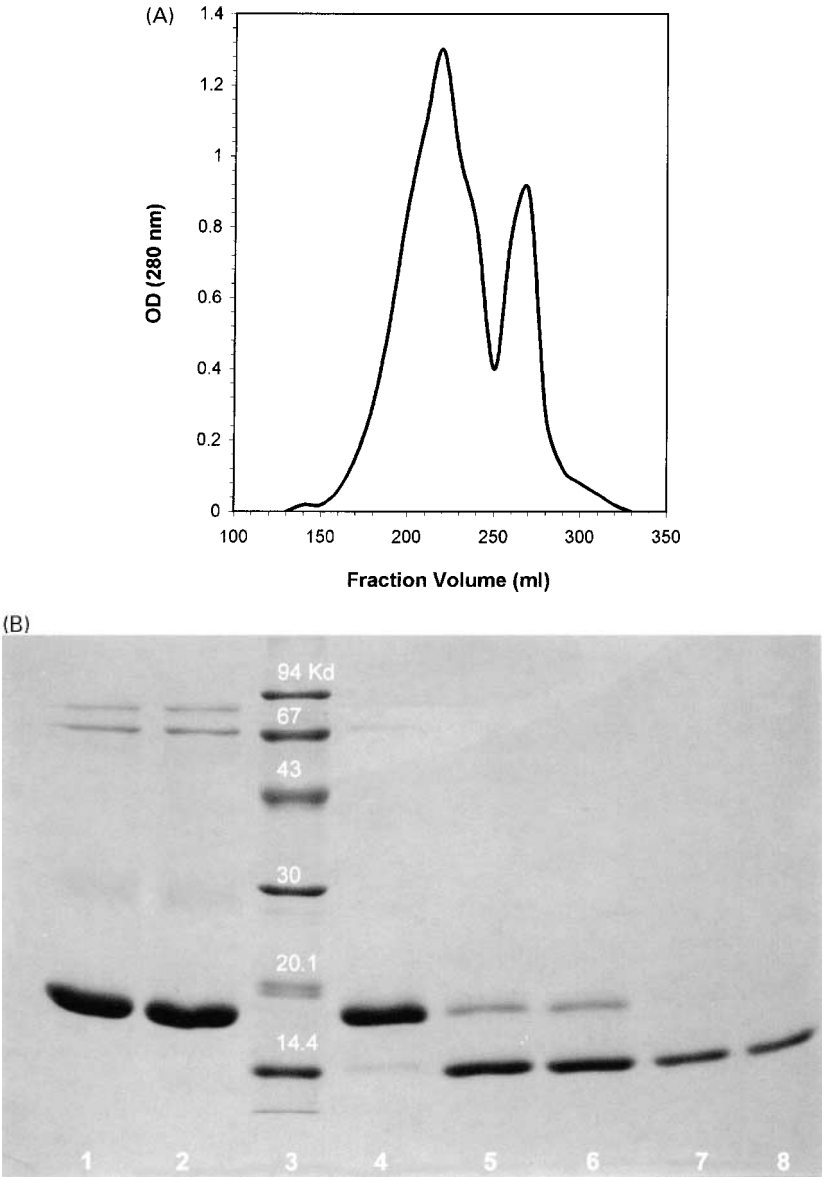

Figure 2. First step of α-lactalbumin purification. (A) Fractions of gel chromatography; (B) SDS–PAGE on fractions; primary shoulder (lane 1), ascending of first curve (lane 2), first peak (lane 4), ascending of second curve (lane 5), second peak (lane 6), descending of second curve (lane 7), α-lactalbumin standard (lane 8), and LMW (lane 3).

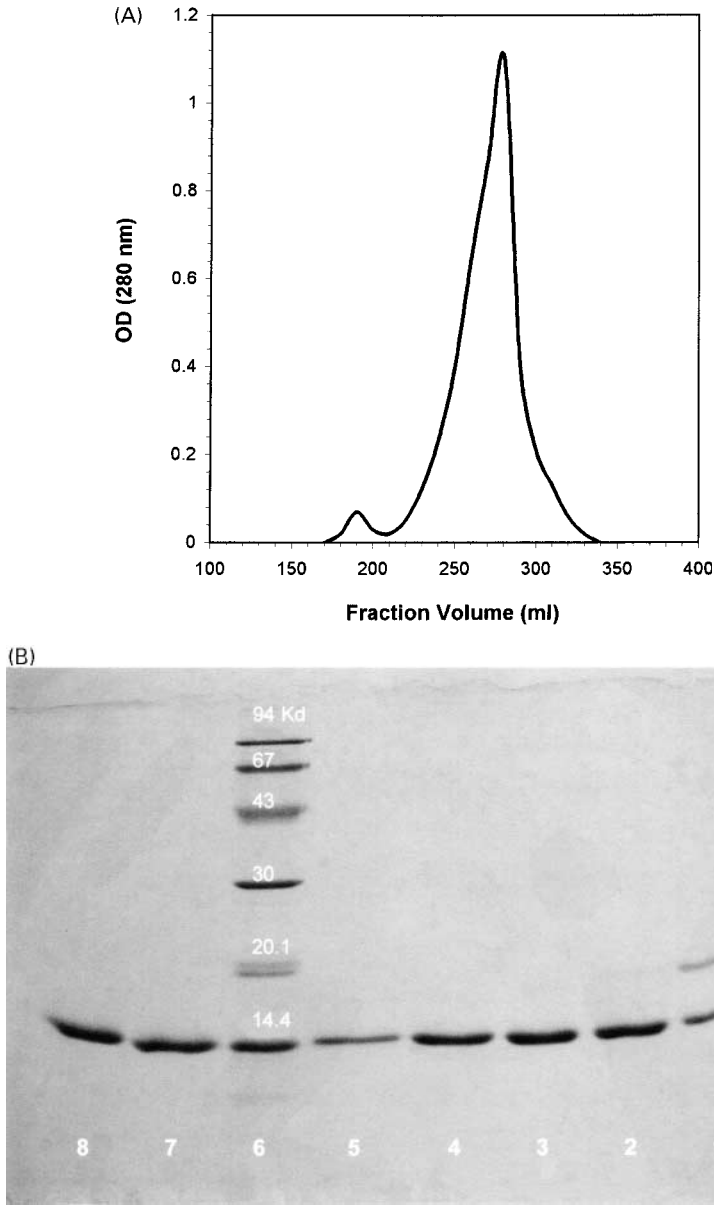

Figure 3. Second step of α-lactalbumin purification. (A) Fractions of gel chromatography; (B) SDS–PAGE on fractions, primary shoulder (lanes 1 and 2), ascending (lane 3), peak (lane 4), descending (lane 5), all of (curves or 3, 4, and 5) (lane 7), and α-lactalbumin standard (lane 8).

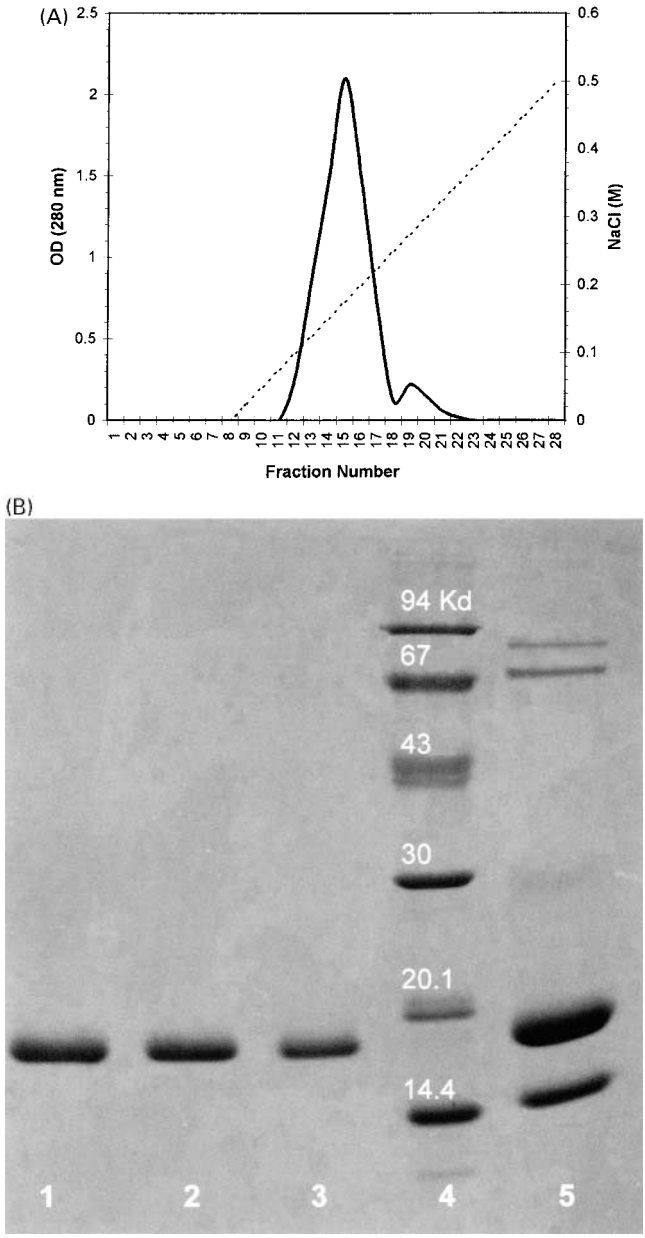

Figure 4. β-Lactoglobulin purification. (A) Fractions of anion-exchange chromatography, fraction size: 10 mL. (B) SDS–PAGE on fractions, peak of curve (lanes 1 and 2), β-lactoglobulin standard (lane 3), and whey for comparison (lane 5).

Immunization Results

Preservation of antigenic potential of extracts is important, so extract immunogenicity was evaluated by means of a double immunodiffusion method. Immunized rabbits produced specific antibodies that showed the same specificity as standard samples (Fig. 5).

DISCUSSION

The public perception of importance of allergic reactions to foods substantially exceeds the prevalence of such reactions identified in clinical studies.[5] Cow's milk generally represents the first foreign food proteins encountered by infants. It is one of the most common food allergens in young children and has been implicated in a variety of hypersensitivity reactions.[27] The overall incidence of CMA ranges from 0.3% to 7.5% in population-based studies.[9,10] Fatal and severe food-allergic reactions are well documented in children. If 5% of the child population have food allergy, the risk that a food-allergic child will die from a food-allergic reaction is 1 in 800,000 per year. Milk causes the greatest number of fatal reactions (four out of eight); this is in line with reports of both the frequency and the severity of reactions to milk.[28] After the diagnosis of food hypersensitivity is established, the only proven therapy is strict elimination of the offending allergen. SPTs are highly reproducible and are frequently utilized to screen

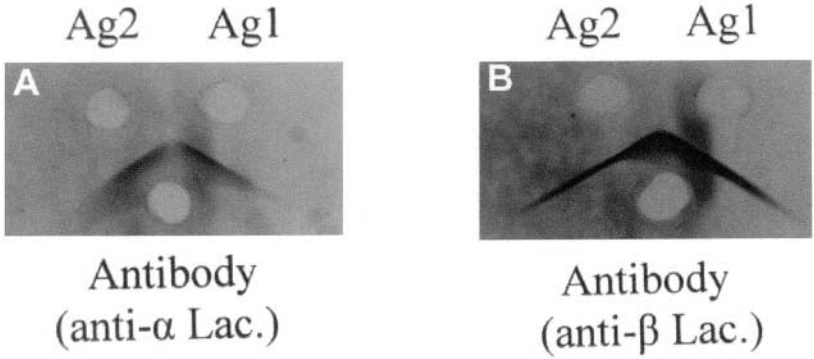

Figure 5. Double immunodiffusion. (A) Ag1: purified α-lactalbumin; (B) Ag1: purified β-lactoglobulin; Ag2: standard; antibody: serum of immunized rabbit by purified allergen.

patients with suspected IgE-mediated food allergies. RASTs and similar in vitro assays for identifying food-specific IgE antibodies are also frequently used to screen for IgE-mediated food allergies.[9]

This study is a starting point for obtaining and purifying common allergenic extracts in Iran. In this study, we purified major cow's milk allergens and we hope to use them in diagnosis and immunotherapy in the future. Results showed that allergenic extracts which were obtained in this study were extremely pure and, even in the case of casein, densitometric analysis demonstrated that this study's product has higher purity than that of similar trade products (Sigma Co), and evaluation of extracts' immunogenicity by means of a double immunodiffusion method showed the antigenic potential of extracts was preserved.

Techniques are required to obtain and control extracts to retain all potential allergens and exclude irrelevant materials under conditions that preserve all biological activities. The source material should be selected to guarantee the content of all potential allergens; but, non-relevant material (i.e., material that does not have the ability to sensitize patients) must be avoided. The preparation of an allergenic extract imposes a number of constraints upon both source material and the physiochemical conditions used during the extraction. This process must neither denature the proteins/allergens nor alter, significantly, the ratio between the components that are present. Identification, purification, and characterization of food allergens have generally been somewhat limited compared with other allergens. This has partly been due to the problems of establishing the pathogenesis of some reactions to foods. There are great geographical and cultural variations in the consumption of foods; also, allergic reactions have been reported for a great number of food sources. In cow's milk, the bovine whey alone has been shown to contain at least 36 antigenic components, of which four (α-lactalbumin, β-lactoglobulin, bovine serum albumin, and immunoglobulin) had the strongest IgE binding capacity. Also, the production of food extracts raises some special questions concerning food processing (source materials) and oral intake. The above-mentioned problems generate special demands for future standardization of food extracts and their eventual application as diagnostic tools. Having pure major allergens would facilitate standardization of allergenic extracts. Some studies consider the ease with which pure allergens could be produced by recombinant technology.[17,29] Currently, casein is precipitated from raw milk by acidification; it constitutes 80% of milk protein synthesized by the mammary secretory epithelium. Casein aggregates in micelles that also contain colloidal calcium phosphate, whey proteins, and milk serum proteins; they remain soluble after acid precipitation and constitute 20% of total milk proteins.[30]

The isolation of individual proteins would allow production of more consistent and reliable products by the food industry and, possibly, would also increase their use in the pharmaceutical industry.[31]

We hope to be able to purify other allergens and following purification, and standardization, using them in experimental animals. We will be able to use them for diagnosis and immunotherapy of allergic diseases. The significance of the present study is more notable when we consider the allergens' geographical distribution and morphologic differences among the allergens of each area. In addition, it is noteworthy that such a study has not yet been accomplished in Iran. On the other hand, because of the problems involved in preparing diagnostic kits in third world and underdeveloped countries, this study can lead to diagnostic kits.

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Received March 13, 2004
Accepted April 19, 2004
Manuscript 3131