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Review Article

# Residual Allergenicity Assessment of Cow's Milk Hydrolysates

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## Abstract

Hypoallergenic extensively hydrolyzed cow's milk formulas can be used as an alternative diet for infants who have mild to moderate cow's milk allergy in case breast-feeding is not possible. These products have been heat-treated and/or enzymatically hydrolyzed to breakdown the milk proteins into peptides and amino acids and disrupt antibody-binding structures. It is known that extensively hydrolyzed products can occasionally elicit an allergic reaction in cow's milk allergic infants.Therefore, understanding the factors that influence allergenic potential and how they are measured and reported are crucial to ensure efficacy and thus safety of cow's milk hydrolyses. In this overview, the required clinical study on hypoallergenicity, *in vivo* allergenicity models and several *in vitro* analytical and immunological methods are described and their suitability for the assessment of residual antigenicity/allergenicity of hydrolysates, and thus immune safety, is discussed.

**Keywords:** Cow's milk; Cow's milk allergy; Casein; Efficacy; Hydrolysate; safety; whey

## Introduction

Cow's Milk (CM) contains approximately 30 potentially allergenic proteins among which casein and whey proteins seem to be most important regarding allergy to CM [1]. The casein allergens comprise four different proteins, alpha-S1, alpha-S2, and beta and kappa casein and have a molecular weight between 19-25.2 kilodalton (kDa). The most important whey allergens include beta-lactoglobulin (BLG; 18.3 kDa) which is the most abundant CM whey protein (≈65%). BLG is present in CM but also in milk of many other mammals, however not in human milk although minor traces might be detected dependent on the diet of the lactating mother. Minor allergen fractions present in CM are alpha-lactalbumin (ALA; 14.2 kDa), bovine serum albumin (BSA; 66.3 kDa) and bovine immunoglobins (Igs; 150 kDa) [2].

CM hydrolyzed formulas are categorized according to the degree of hydrolysis into partially hydrolyzed formulas (pHF) or extensively hydrolyzed formulas (eHF). The term 'hypoallergenic formulas' was first used by Kleinman et al. to refer to formulas that are tolerated by 90% of subjects with proven Cow's Milk Allergy (CMA) with a 95% confidence interval, and are therefore used for the treatment of CMA [3]. This definition was subsequently adopted by the World Allergy Organization (WAO) [4]. In 2000, the American Academy of Pediatrics (AAP) defined an eHF as a formula containing only peptides that have a molecular weight of <3 kDa. These guideline states that formulas with 'most of the nitrogen in the form of free amino acids and peptides <1.5 kDa have been subjected to extensive clinical testing and meet the standard for hypoallergenicity [5].

Detection of allergens in food products can be very difficult, as they are mostly present in trace amounts or are masked by the food matrix [6]. Physico-chemical and immunological methods have been developed to confirm the safety of hydrolysates and to evaluate their residual antigenicity [7]. Generally, the allergenicity of hydrolysates is partially dependent on their molecular weight but, in addition, the control of the peptide length of peptides is important as well. There are several chemical analytical techniques to determine the degree of hydrolysis [8] and the molecular weight distribution (e.g. gel permeation chromatography (GPC), High-Performance Liquid Chromatography (HPLC) or Mass Spectrometry (MS) method such as MALDI-TOF [2,9,10] that can be used to analyze the proportion and the size of the hydrolyzedpeptides. However, none of the above mentioned techniques are sufficient to define the residual allergenicity in a biologically relevant manner [2]. Most of the immunological assays described for the detection of milk proteins such as immuno-diffusion, immuno-rocket, electrophoresis, immunoblotting and Enzyme-Linked Immunosorbent Assay (ELISA) can be suitable for testing hydrolysate formulas [9,10]. ELISA methods, based on allergen specific IgG or IgE detection, are the most conventional and sensitive *in vitro* techniques to estimate the residual antigenicity of aparticular hydrolyzed formula. *In vitro* cell-based allergenicity assays are newly developed and gaining in interest because among others these assays reflect the actual allergic response and are therefore labelled as a functional assay. This paper will discuss the suitability of allergen residue detection methods and their use specifically for CM hydrolysates safety assessment.

## Clinical Study on Hypoallergenicity of CM Hydrolysates

eHFs are recommended as the first line substitute for CM-based formulas and widely used in infants with CMA who cannot be breastfed [11]. It is known that eHFs can occasionally elicit an allergic reaction in CMA infants. This occurs especially in those where CMA is part of the broader syndrome of multiple food allergies. In these circumstances amino acid based formulas are recommended [7,12]. In order to guarantee the hypoallergenicity of eHFs, different

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positioning and guideline documents have been published by authoritative national and international scientific platforms. One of the safety requirements of eHFs is that they must be tolerated by at least 90% of children with proven CMA with a 95% confidence interval [5]. Many commercial available hydrolysates for CMA management, either whey-based [13-19], casein-based [19,20] or a mixture of whey/casein [19,21] are found suitable for consumption by CMA infants and meet the requirements set out in European and international guidelines.

## In Vivo Animal Models

Animal models have played a valuable role in increasing our understanding of the immunology and pathology involved in allergic responses to food proteins. To assess the residual allergenicity of an eHF in a preclinical setting, animal models are available but not (fully) validated and embedded in guidelines to this date. Guinea pigs sensitized by the oral route have been used as a common model for the assessment of residual allergenicity of hypoallergenic formulas due to their innate responsiveness [22,23]. A main disadvantage of the guinea pig model is the generation of IgG1a subclass anaphylactic antibody responses instead of IgE antibody responses, which are the main pathophysiological antibody responses in allergic humans. This makes the suitability of the guinea pig model questionable with regard to the potential extrapolation to the human situation. Additionally, this guinea pig model has a higher discomfort in terms of the allergic symptoms and is for this reason less accepted for ethical reasons. More recently, a predominantly IgE-mediated mouse model of orally induced CMA was introduced [24] and interlaboratory evaluation was conducted as a first step to validate the model [25,26]. In addition, more recently rat models have been used for the assessment of the allergenicity of infant formulas as well [27,28].

## In Vitro Methods

Differences in CM hydrolysates arise as a result of the different protein sourcesused (whey, casein or mixture), the type of enzyme(s) (trypsin from porcine pancreas or bacterial/fungal proteases) and/or the process used for the manufacturing of the specific hydrolyzed formulas. The different formulas end up with a mix of many different peptides, ranging in peptide size and Amino Acid (AA) composition. This makes a hydrolysate a very complex matrix for *in vitro* analysis and comparisons, especially for methods using antibodies since specific peptides may inhibit e.g. a sandwich-type ELISA by binding to the solid phase antibody but not allowing the binding of the secondary antibody conjugate. Therefore, a method suitable for one hydrolysate does not necessarily mean that it is suitable for all different kinds of hydrolysates. Each method has to be validated specifically for the intended hydrolysate [28]. As a result of these complexities, one selected method of assessment is not suitable and enough to compare the potential allergenicity of the different commercial formula available.

Frequently used methods for allergen detection in a food matrix are Polymerase Chain Reaction (PCR) and Mass Spectrometry (MS) [10]. PCR is targeting a segment of the gene coding for the allergenic protein of interest and amplifying only this specific DNA fragment to make them detectable [29,30]. MS directly detects allergens by breaking them down into peptides, or short strings of amino acids that link together to form larger proteins [10]. This means that both PCR and MS are excellent methods to detect an intact allergen in a matrix in which this allergen should be absent, but not for hydrolysates which consist of only peptides. Therefore it becomes difficult to pinpoint a DNA fragment or peptide of interest which can cause an allergic reaction. A method still under development using MS is fingerprinting of the peptides in a hydrolysate which could be useful in the future to assess not only peptide size but also AA composition.

## Degree of Hydrolysis, Molecular Weight Distribution and Presence of Protein

The Degree of Hydrolysis (DH%) is defined as the percentage of peptide bonds cleaved during protein hydrolysis, or in simple terms, a measure of the breakdown of the protein by the enzyme(s). It is reported in terms of percentages/proportions of peptides with differing molecular weights. Various methods exist for determining DH%; the most used are the pH-stat, trinitrobenzenesulfonic acid (TNBS), o-phthaldialdehyde (OPA), trichloroacetic acid soluble nitrogen (SN-TCA), and formol titration (AN/TN%). The DH% value varies for the same hydrolyzed protein depending on the type of method adopted. Yet there is no consensus on the methodology to determine the DH%, so it is recommended to work with a standardized protocol to reach the most reliable measurement of DH% and allow a correct comparison [8].

Molecular weight distribution of hydrolysates can be determined by several different methods such as GPC [19,20,31] HPLC [14,22,33] and MS [34]. The peptides are usually grouped into different kDa ranges with the use of standard proteins of known molecular weight. It should be noted that different GPC columns will show different results for the same hydrolysate, therefore for comparison purposes, hydrolysates should only be characterized using the exact same column. This is also the case for molecular weight distribution assessment using different analytical methods.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein staining with e.g. Coomassie Blue (sensitivity 30-100 ng protein) or silver staining (sensitivity 5-10 ng protein) can also be used to detect the presence of residual proteins and/or peptides in a hydrolysate [14,19,33,35,36].

## Antigenicity Assessment

Antigenicity refers to the capacity of protein epitopes to bind to specific antibodies, in the used methods for allergen detection mostly of the IgG (antigenic integrity) and IgE (allergenic integrity) class. This binding capacity is used in both immunoblotting and ELISA.

For Immunoblotting polyclonal antiserum [37], monoclonal antibodies specific to CM proteins [38] but mostly sera from CMA patients [19,35,36] are used to assess residual antigenicity. It should be noted that a full characterization of the antibodies present in the CMA sera is needed to guarantee antibodies against the studied CM proteins are represented.

There are many commercially available ELISA kits for CM proteins available, however the majority is developed to detect intact CM in food stuff and none are specific for detection of allergenic peptides in CM hydrolysates. This is a point quite often overlooked. Most ELISAs are not validated for the specific hydrolysate of interest. For example, formulas were tested by ELISA for BLG and casein proteins using Ridascreen Fast test kits (R-Biopharm AG, Germany) [33]. However, when R-Biopharm validated the ELISA for Nutricia hydrolysates it was demonstrated that spike recoveries were very poor and that the ELISA seem to be not suitable for testing these hydrolysates. ALA was tested with the bovine alpha-lactalbumin ELISA quantitation set (Bethyl Laboratories, USA) which is used for detection of

ALA in milk and has not been tested for hydrolysates by the supplier. In another study, several different commercially available hydrolysates were compared on antigenicity. Residual bovine allergen content was quantified by high-sensitivity ELISA BLG and casein (EurocloneSpA) and ALA (Cloud-Clone Corp) [32].The BLG and casein ELISA from EurocloneSpA was not validated for hydrolysates by the supplier or the independent lab (Neotron Spa Italia) and a pre-validation of these ELISAs by MérieuxNutriSciences showed that these are not suitable to test Nutricia hydrolysates due to poor spike recoveries. The ALA ELISA from Cloud-Clone is developed for the detection of ALA in serum, plasma, breast milk, tissue homogenates, cell lysates, and cell culture supernates and other biological fluids, but not food matrixes. These ELISAs were used to assess antigenicity of a newly developed hydrolysate [14]. The BLG ELISA from ELISA Systems [38,39] was fully validated for Nutriciahy drolysates by Mérieux-NutriSciences, however, when validating the casein Residue ELISA from the same supplier for testing cross-contamination, this ELISA was found not suitable for the same hydrolysates. The above studies again stress the importance to validate each ELISA for each intended hydrolysate, especially when used for either comparing different hydrolysates or for release of the hydrolysate products in the factory.

A peptide array is a collection of peptides displayed on a solid surfaceu sed to map antibody-binding epitopes or to find key residues for protein binding, and has become a versatile tool for high throughput screening assays in biomedical and pharmaceutical research. Peptide arrays that contained linear peptide fragments reported as IgE-binding epitopes for CMA have been developed as well. Various peptides with different solubility in aqueous solutions were dissolved in the buffer solutions containing SDS, and the IgE-binding patterns were detected by observing the binding of Alexa 647-labeled anti-human IgE using sera from CMA patients. This provides a potent capability for the development of a peptide array for mapping IgE-epitopes in CM proteins and will help better understand the IgE-epitopes associated with the clinical outcome of CMA [40]. It has been further developed to detect IgG4-epitopes as well [41], to predict safety and efficacy of e.g. oral immunotherapy with CM [42] and to evaluate antigenicity of CM hydrolysates [43]. After full validation and a link to clinical relevance this could be a promising technique to be used in the near future.

## AllergenicityAssessment

Allergenicity is the potential of a material to cause sensitization and as a consequence allergic reactions, mostly associated with allergen-specific IgE antibodies. The main advantage of assessing allergenicity over antigenicity is the possibility to investigate the capacity of a protein/peptide to cause an actual allergic reaction providing a more biologically relevant prediction of allergenicity.

An *in vitro* model using huFcεRIα-RBL-2H3 cells, sensitized with an oligoclonal pool of chimeric human (chu)IgE antibodies covering the allergenic epitopes of bovine β-lactoglobulin (BLG) was developed to assess the residual allergenicity of whey hydrolysates [44]. This *in vitro* model has been partly validated for the intra- and inter-laboratory variation and showed that this degranulation assay is robust and reproducible within and between different laboratories [38]. This validation was performed with several different hydrolysates (Friesland Campina and Nutricia) with known (pre)clinical allergic outcomes. It was demonstrated that when the minus+10% degranulation (variation of the assay) is used as cut-off value, all hydrolysates with no clinical allergy symptoms were below this cut-of, whereas all hydrolysates with known allergic reaction (pHFs) were above this cut-off. This assay is currently used as a part of the safety strategy by companies producing eHF for CMA infants [14,39]. However, in some studies [14,32] the RBL-assay was adapted and the way the assay was performed and calculated/interpreted was different than how the assay was developed and validated originally. In these studies a reference curve of intact BLG was produced and samples were calculated with a 4-PL-fit to have a result in 'allergenicity µg/ml'.The reference curve concentration is up to 1 µg/ml, whereas samples have a concentration up to 10,000 µg/ml and makes extrapolation unreliable. In a comparison of allergenicity of different hydrolysates [32] it is demonstrated that hydrolysates with higher BLG ELISA results show lower 'allergenicity' result than hydrolysates with low BLG ELISA results. This indicates that these assays seem to be not complementary and more validation should be performed and clinical relevance of the 'allergenicity µg/ml' should be assessed before the method is used to compare different hydrolysates for their allergenic capacity.

The utility of a mouse basophil activation test (BAT) in the evaluation of residual allergenicity in hypoallergenic formulas was assessed as well [45]. Whole blood samples derived from BLG or casein-immunized mice were incubated with intact CM formula, pHF or eHF. Basophilic activation was analyzed by flow cytometry using CD200R1 (IgE-dependent activation marker) and CD200R3 (IgG-dependent activation marker). Intact CM formula induced pronounced changes in marker expression on basophils and induced anaphylaxis, pHF also induced basophilic activation and systemic anaphylaxis but the magnitude of these effects was smaller than with intact CM formula, whereas eHF did not elicit any changes in marker expression and did not induce anaphylaxis. The mouse BAT might be a useful tool for the evaluation of allergenicity of hydrolysate formulas, however, it would be difficult to standardize since whole blood samples of immunized mice have to be used freshly for each experiment.

## Closing Remark

As there is no single or series of method(s) available that are validated for testing of the different hypoallergenic formulas, it is recommended that for any newly developed CM hydrolysate, manufacturers assemble a toolbox or decision tree of *in vitro* methods, validated specifically for the intended hydrolysate, to assess the presence of residual protein/peptides, antigenicity and allergenicity [39]. As an extra check on allergenicity an *in vivo* animal [39] can be considered, however the final proof of hypoallergenicity is in its efficacy in the target population and this has to be assessed and proven in a clinical study [18] as proposed by the WAO and AAP.

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