

IMMUNOFLUORESCENCE DETECTION OF MILK PROTEIN IN MEAT PRODUCTS

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ABSTRACT

Nowadays there are various vegetable protein additives intended for the manufacture of meat products in the food industry. These ingredients include both, plant-origin as well as animal-origin proteins. The most common vegetable additives include various types of flour, starch, fiber and plant protein. Among animal proteins, the most commonly used are plasma, collagen or milk protein. Milk protein is added to meat products due to its functional properties, such as emulsifying fats, improving the holding capacity of meat, improving juiciness, gel-forming capacity and affecting the taste of the product. Usage of these proteins, however, is currently limited by the effective legislation, not only in order to prevent consumer deception, but also because of their potential impact on consumers' health of. Thus, this issue has received considerable attention not only in the Czech Republic, but also globally. The main risk is the impossibility of selecting a suitable foodstuff for individuals with potential allergic reactions. The only option for allergic consumers to protect themselves is to strictly exclude the given allergen from their diet. Although the number of studies dealing with the reduction or loss of allergenicity is increasing, yet these practices are not common. Most of the population suffering from food allergies is thus still dependent on strict exclusion of foodstuffs causing adverse allergic reactions from their diet. Detection of allergens in foodstuffs is unfortunately quite difficult due to the fact that they occur in trace amounts and are often masked by different parts of the foodstuff. This research dealt with the detection of milk protein in meat products purchased in the market network of the Czech Republic, whereas declaration given by the manufacturer on the packaging for the small meat products purchased from the market was used to verify the detection of milk protein by the immunofluorescence method. 20 products were examined, these were selected with regard to the presence of milk protein that was declared by the manufacturer on the packaging. Method validation was performed by comparing the positive results from the investigated method with information on the packaging of the meat product. Milk protein was detected in 84.62 per cent of samples where the manufacturer declared the presence of milk or cheese on the package and additionally in 85.71 per cent of samples where the manufacturer declared the presence of milk protein. The results show that the immunofluorescence method is suitable for the detection of milk protein in meat products.

Keywords: immunohistochemistry; allergens; milk protein; fluorescence methods; meat products

INTRODUCTION

Protein formulations are frequently used in production of meat products. From among plant-origin proteins, meat products can thus contain e.g. wheat or soy protein. Of the animal-origin proteins, they often contain plasma, collagen or milk protein (caseinate, whey, powdered skim milk, etc.) (López et al., 2006). These proteins are added due to their functional properties such as emulsification of fats or improvement of holding capacity of meat. Milk proteins are also involved in improving juiciness, gel-forming capacity and affect the delicate flavor profile of the meat product. All properties are perfectly compatible with the meat systems. On the other hand, the best known milk protein – casein – which constitutes about 80 per cent of milk protein, is relatively expensive. Conversely, proteins in whey, representing about 20 per cent of milk protein, are more economical and provide good performance in meat systems. Whey protein is primarily beta-lactoglobulin, a globular protein that can be modified (its

structure can be changed) so that it changes the functional behavior of proteins used in food industry (López et al., 2006).

On the other hand, milk protein is classified among food ingredients, which are listed in Regulation 2011/1169/EC as regards indication of ingredients present in foodstuffs. Food allergy is an abnormal immune response to foodstuffs (Bruijnzeel-Koomen et al., 1995). In this case, one's immune system responds inappropriately to the stimulus provoked by the allergen, which can be a protein or carbohydrate, for example (Ferguson, 1992). In addition, food allergens contained in foodstuffs naturally are resistant to high temperatures, low pH in one's stomach, and enzymatic digestion in the digestive tract (Hefle et al., 2007). However, it has been reported that there is no correlation between in vitro digestibility and protein allergy (Fu et al., 2002). Allergies to specific foodstuffs may in some cases exhibit also after ingestion of food of similar origin, which is known as cross-reaction.

This occurs when IgE antibodies originally produced against one allergen are produced also upon contact with a similar protein from another source (**Aalberse et al., 2001**). Food allergies have become a major health problem worldwide. Adverse health effects due to allergic reactions to food products or food ingredients occur in about 1 per cent of population and in about 4 per cent of children (including food intolerance). Food allergy is therefore more common in children than in adults. In Central Europe, typical allergies include allergies to egg, milk, temperate-zone fruits, tree nuts, poppy seed, and root vegetables; in the Asian continent critical is surprisingly not rice, but rather highly allergenic soybean with its wide range of products – at least 50 per cent of the Asian food production is soy-based, the vast majority of other foodstuffs is at least contaminated with traces of soybean (**Fuchs, 2008**). In the United States, cow's milk (2.5%), eggs (1.3%), and peanuts (0.8%) are responsible for allergic reactions in children. In contrast, in the adult population the prevailing allergies include shellfish (2%), peanuts (0.6%), nuts (0.5%), and fish (0.4%) (**Sampson, 2004; Sicherer and Sampson, 2000**). Cow's milk, eggs, soy, wheat, peanuts, tree nuts, fish, crustaceans, and molluscs cause about 90 per cent of food allergies and are also the primary foodstuffs causing anaphylaxis (**Sicherer and Sampson, 2000**). Most food allergic reactions are induced immediately after exposure to the allergenic foodstuff. Even intake of a tiny amount of foodstuffs containing allergens may cause allergic reactions in sensitive individuals. It then includes a wide range of allergic symptoms, such as digestive disorders, respiratory problems, disorders of the circulatory system, and skin irritation. In some individuals it can even lead to anaphylactic shock (**Schubert-Ullrich et al., 2009**).

In order to avoid misleading consumers and also to protect allergic consumers, analytical methods applicable to all types of foodstuffs have been developed. Among the available immunochemical methods, the Enzyme-Linked Immunosorbent Assay (ELISA) is the most frequently used method in laboratories to detect hidden allergens in foodstuffs. ELISA methods are still being improved and used in combination with other methods, as reported for example in the study by **Ben Rejeb et al., (2005)**. Polymerase chain reaction (PCR) is a method used for detection and quantification of DNA. This method is used for detection or quantification of allergens in processed foodstuffs where the DNA is generally more robust than proteins and therefore it is less likely to suffer damage or destruction during the processing of foodstuffs (**Walker et al., 2008**). There are also other immunochemical tests, for example Enzyme-Allergosorbent Test (EAST), followed by Radio-Allergosorbent Test (RAST) and Dot Immunoblotting which operate on a similar principle as ELISA.

MATERIAL AND METHODOLOGY

20 cooked meat products that, in harmony with their list of ingredients, should contain milk protein in various forms, e.g. milk protein or milk in general or that were marked: "May contain traces of milk protein", primarily sausages and pates purchased in the market network in the Czech Republic, were examined. The selected detection

method was immunofluorescence microscopy as a method more sensitive and selective than light microscopy. The samples were taken in a manner to be representative of the entire product. The samples were then processed in the accredited laboratory for investigation of foodstuffs at the Department of Vegetable Foodstuffs Hygiene and Technology, FVHE, VFU Brno. Using cryostat HM 550 (Germany, Microm) the sample was sliced into sections 10 µm thick. These sections were transferred to Thermo Superfrost slides (Germany, Thermo scientific). 9 sections were cut of each meat product. Each sample was constituted by three frozen blocks from which the microscopic sections were cut with 50 µm trimming. The selected detection method was immunofluorescence microscopy as a method more specific and selective than histochemical methods. The actual immunofluorescence procedure was launched by inserting the sections into cold acetone. After rinsing the preparations in PBS (phosphate buffer saline) for 2 x 5 min., sections were placed in humidified chamber in which blocking of nonspecific bond using Goat diluent normal serum (GB, VectorLaboratories) took place for 30 minutes. Afterwards, biotinylated primary antibody of Rabbit Anti-Beta-casein Polyclonal Antibody (USA, Bioss Antibodies) was applied to the sections, the humidified chamber was left in a refrigerator overnight. The next day, the sections were rinsed in PBS (2 x 5 min.). Thereafter, the sections were placed in the humidified chamber again and the secondary antibody (GB, VectorLaboratories) was applied to the sections for 30 min. at room temperature. Subsequently rinsing in PBS (2 x 5 min.) and application of fluorochrome followed. The fluorochrome used was Texas Red (GB, VectorLaboratories). Afterwards, the sections were mounted and examined using the fluorescence microscope of Leica DM 3000 (Germany, Leica) and further processed by Leica IM 50 software (Germany, Leica). Thus, 9 sections from each meat product was examined at a magnification of 40x and 100x.

RESULTS AND DISCUSSION

Milk protein was detected in 17 out of the total of 20 meat products samples where the manufacturer declared the presence of milk proteins or milk on the packaging. Cow's milk, wheat, eggs, soy, peanuts, tree nuts, fish, crustaceans, and molluscs cause about 90 per cent of food allergies and are also the primary foodstuffs causing anaphylaxis (**Sicherer and Sampson, 2000**). In order to protect consumers, European Commission adopted Regulation 2011/1169/EC amending Directive 2000/13/EC and Directive 2003/89/EC as regards indication of the ingredients present in foodstuffs. Annex IIIa of this guideline contains a list of food ingredients and products made from them, which are classified as potential allergens that could lead to potential intolerance, among these ingredients, is also milk (including lactose). Directive 2003/89/EC requires that each of the twelve described potentially allergenic ingredients is declared although they form less than 25 per cent of the food. The aim of the research was to verify the appropriate method for determination of milk proteins in meat products. Immunofluorescence method was selected as the examination method.

Table 1 Detection of milk protein in small meat products.

Ingredients	Milk, cheese		Milk protein	
	Declared by the manufacturer	Detected milk protein	Declared by the manufacturer	Detected milk protein
Number of samples	13	11	7	6
Percentage	100	84.62	100	85.71

Table 2 Meat products used for immunofluorescence detection.

Meat product	Declaration	Number of products	Number of detection
hamburger	milk protein content	2	2
frank	modicum of milk protein	4	3
hotdog	milk protein content	6	5
paté	Milk protein content	8	7

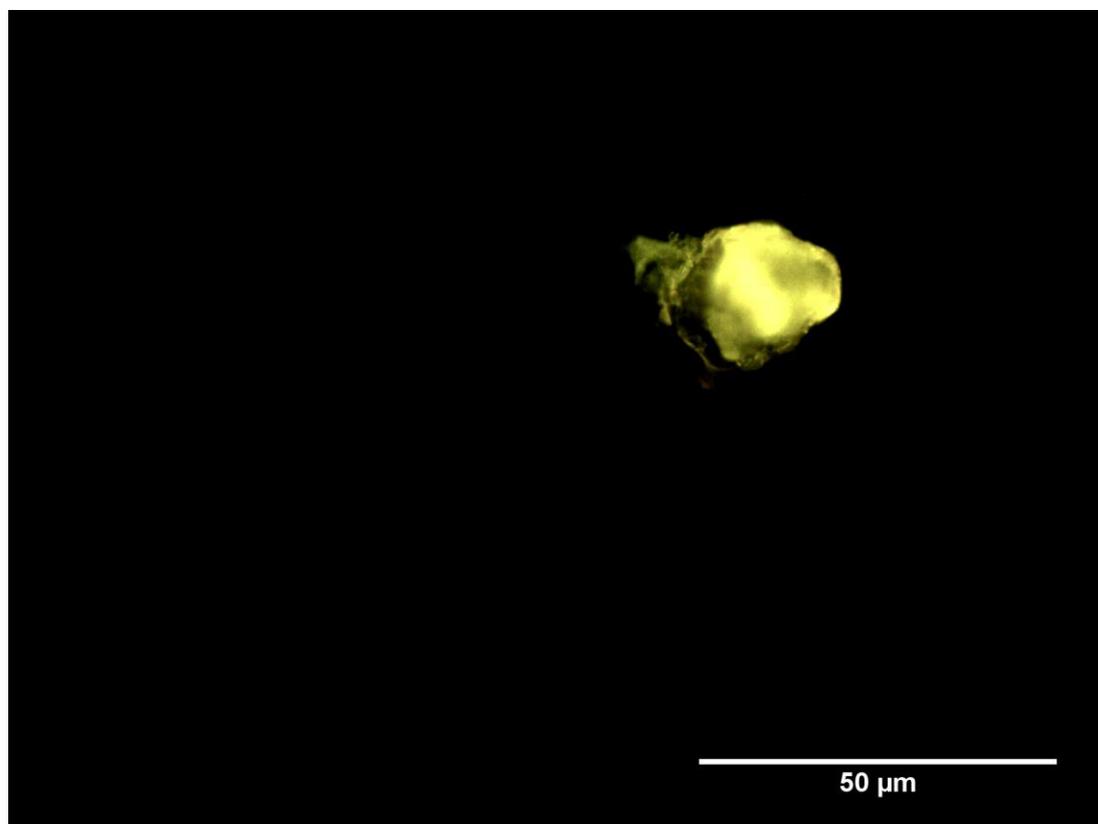


Figure 1 Milk protein green and yellow – Texas Red (magnification 400 x).

Detection was based on fluorescence which was achieved by immunohistochemical procedure and staining using fluorochromes. Immunohistochemical procedures are generally based on the reaction between the allergen and the corresponding labeled antibody (Petrašová et al., 2014; Bednářová et al., 2015). Binding of the labeled antibody was evaluated in a fluorescence microscope with a fluorescence filter I3. The examination was based on the formation of a fluorescent color, which indicates a positive reaction of the antigen with the antibody. To visualize milk protein by staining, Texas Red fluorochrome was applied. Fig. 1 shows a microphotograph of the milk protein, which differs in color from the black background that is formed by muscle, and other component of the meat product. Hereby it possible to differentiate between the milk protein and meat protein which is not fluorescent but black. We compared the results obtained in our examination with the information supplied by the manufacturer on the product packaging. The values obtained in the milk protein detection are given in Tab. 1. As apparent from this Table, the fluorescence immunohistochemical method appears suitable for determining milk protein in small meat products. Out of 13 samples where the manufacturer had declared the presence of milk or cheese, we detected milk protein in 11 products. Additionally, 7 products where the manufacturer directly declared the presence of milk protein were examined. In 6 of these products, milk protein was really detected. In one sample the presence of milk protein was not detected, which could be e.g. because of mere preventive warning on the package protecting the manufacturer for example in the production process where cross-contamination could occur, or because of deactivation of the binding sites of milk protein during the manufacturing process.

CONCLUSION

Cryosections were cut of each sample to be examined. Texas Red was used as the fluorochrome due to minimal background fluorescence. Immunofluorescence method for the detection of milk protein was verified by examination of 20 small meat products (Tab. 2) purchased from the market network. Our results obtained in this pilot study was compared with information on the packaging of the product when milk protein was declared on 7 packagings and general content of milk or cheese was stated on the packagings of 13 manufacturers. In total, milk protein was detected in 17 products. Out of that, in 11 products where milk protein was directly declared on the packaging and in 6 products where contained milk or cheese was declared in general. The results point to the possibility of using this method for the detection of milk protein in meat products. To use this method in practice, however, further validation of the method in more parameters, such as repeatability and reproducibility, is still necessary.

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