POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2003, Vol. 12/53, SI 2, pp. 32-39

EXAMINATION OF IMMUNOREACTIVE AND IMMUNOMODULATIVE PROPERTIES OF FOOD COMPONENTS WITH THE APPLICATION OF IMMUNOMETRIC METHODS

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Key words: immunometric methods, immunomodulative properties, ELISA, ELISPOT, immunoblotting, food antigens, food allergies

The impact of food antigens on the immune system of the organism is undisputable. The alimentary tract (GALT) is one of the most significant elements in the immune defence barrier of the organism. This is caused by a considerable role of the immune system related with the gastro-alimentary tract in the functioning of the whole immune system of the organism also in food allergy.

Due to many synergising health, genetic and environmental factors, the problem of food allergy has been arising an increasing interest. In the last two decades in Europe the number of allergic persons has doubled. The problem is becoming one of the most significant epidemiological and economic issues.

The above-mentioned threats cause that precise and sensitive methods appear to be more important in monitoring food components, which can induce the allergenic response.

In this work, the possibilities of application of immunometric methods for the examination of immunoreactive properties of foods, and their changes as a result of the processing employed as well as immunomodulative properties of selected food components (probiotics) were presented on the basis of the results obtained in an own research.

INTRODUCTION

The thesis about the effect of food on the organism's condition has seemed unquestionable and has been confirmed since the days of Hypocrites. Nutrients are necessary for keeping proper life functioning as they provide growth material and energy. There is no doubt that the demand for food is high.

There can be mentioned tree elements when discussing the effect of foods on the immune system. The first comprises the antigens and the possibilities of their modifications in technological and biotechnological processes. The second is connected with the immune system (cells, receptors, mediators and antibodies), next one, regarding the character of this effect with, the application of suitable (immunometric) methods.

The food taken and the digestive tract are major factors affecting the immunological system of the organism. This is due to the fact that, among others, throughout their life an average human consumes approximately 10 tons of food containing 10^2 to 10^{12} of cells of not always beneficial micro-organisms per 1 mL of chyme. Of great importance is wide antigenic diversification of food components and a huge area of contact between the human organism and food antigens: the alimentary tract mucosa area is about 200 m^2. For these reasons, the alimentary tract is one of the greatest protective barriers of the organism, which because of its large area of contact with strange antigens produces approximately 80% of all antibodies produced by the organism [Kirjavainen *et al.*, 2001].

INFLUENCE OF FOOD COMPONENTS ON THE IMMUNE SYSTEM

The problems of immunomodulative activity of food, and especially allergic responses caused by food components, are closely related with public health because the alimentary tract seems to be one of the greatest protective barriers of the organism. The food taken and the digestive tract are major factors affecting the immunological system of the organism.

Nutritional status and particular food components may significantly affect functioning of the immune system either indirectly – by modifying DNA synthesis substrates, energy metabolism, or hormones, or directly – by activating the cells of the system, changing their interactions, and producing some cytokines.

Among the constituents which have a significant influence on the functioning of the immune system the most often mentioned are: proteins [Stanga & Allison, 2000; Mayer *et a*l., 2001], some lipids [Erickson, 1998], natural bioactive compounds (beta-glucan, prebiotics, synbiotics), vitamins (A, B_6, C, D_3, E, pholic acid) [Mukhopadhyay; 2000, Benzie, 1999; Hughes, 1999], macro- and microelements (zinc, iron, selen, copper) [Failla & Hopkins, 1998; Rogala *et al*., 1999], and some probiotic bacteria [Kirjavainen *et al*., 2001].

The human organism has a highly efficient protective system against antigen threats of the environment, consisting of barriers of mechanical-physical kind, of the primary and secondary lymphatic system and specialised immune systems in this GALT- Gut associated lymphoid tissue.

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The defence mechanisms of the immune system are extremely complex. Local responses in the lymphatic system related to GALT may be transferred by the lymphatic and blood system to the peripheral elements where they finally determine the overall immunity of the organism [Roitt *et al.*, 1998]. There is a high number of expressions regarding immunity. Two kinds of immune responses are very important: humoral and cellular-mediated immunity in which numerous specialised cells of the immune system take part (mast cells, eosinophiles, B and T lymphocytes, macrophages, endothelium cells). Also mediators produced and released by these cells, which act as activators or information carriers, are of considerable importance [Kirjavainen *et al*., 2001]. For example, probiotic bacteria have been proven to affect the balance between Th1 and Th2 cells, which subsequently results in the changes and enhancement within the particular elements of the immune system [Kirjavainen *et al.*, 2001]. It is very essential in the case of food allergy.

Lymphocytes B affected by cytokines, can proliferate, differentiate and transform into cells producing the specific proteins–immunoglobulins with capacity to neutralize antigens [Roitt *et al.*, 1998].

IMMUNOMODULATING AND ANTIGENIC (ALLERGENIC) CHARACTERISTICS OF NUTRITIONAL COMPONENTS

The organism reaction to foods can be diversified. It can be tolerated (due to clone deletion mechanism, anergy mechanism and suppression of CD 8+ cells) or not tolerated (food intolerance) [Brandtzaeg, 2001; Rothenberg, 2001; Mayer *et al*., 2001].

All diseases caused by food intake (including the food intolerance and allergy) in this context are of high importance. It is confirmed by some statistical and epidemiological data, *e.g.*: (1) the prevalence of allergic rhinitis has increased substantially over the past 15 years [Linneberg, 2000]; (2) each year more than 50 millions of Americans suffer from allergic diseases [American Academy of Allergy, Asthma and Immunology – – The Allergy Report, 1996-2001]; (3) allergies are the 6th leading cause of chronic disease in the United States, costing the health care system $18 billion annually [American Academy of Allergy, Asthma and Immunology – The Allergy Report, 1996-2001]; (4) estimates of allergy prevalence in the United States are 9-16 percent [The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee Information, 1998]; (5) food allergy occurs in 8-9 percent of children 6 years of age or under, and in 1 to 2 percent of adults [Sampson, 1998]; (6) peanut or tree nut allergies affect approximately 3 millions of Americans and cause the most severe food-induced allergic reactions; (7) approximately 100 Americans, usually children, die annually from food-induced anaphylaxis [Information of the American Academy of Allergy, Asthma and Immunology (AAAAI) Board of Directors, 1998]; (8) approximately 16.7 million office visits to health care providers each year are attributed to allergic rhinitis [CDC. Vital and Health Statistics information, 1999].

Nowadays, the problem of food allergy becomes the most essential one also in Europe and Poland. About 1-2% of the adult population and 5-7% of babies/children have problems with the IgE-mediated form of food allergy. These specific reactions are directed to several proteins present in peanut, milk, soy, tree nuts, fish or egg white.

When considering the issues of food impact on the immune system, it should be remembered that deficiencies in particular food components, which are quite common these days due to a general tendency to consume highly processed food, use of all sorts of formulas, slimming diets and impaired absorption, adversely affect the immune system of the organism and cause disorders in the functioning of the humoral system.

There are lot of threats coming from the possibility of hidden allergens occurring in food or environment. Apart from the presence of hidden allergens there is also the problem of cross-reactions, cross-contact contamination during product processing. Great care and deep knowledge are necessary in the field of cross-reactions of some food (apples, carrots, celery, cherries, peach, potatoes, koper dill, kiwi, hazelnuts) and inhalent allergens mainly of pollen source (birch, grass, hazel, mugwort) [Pastorello *et al.*, 1997; 2001a; b]. This problem is equally important mainly with respect to allergens causing anaphylactic reactions [Wűthrich, 2000]. Therefore, estimation of food safety, including new generation and the so-called "functional foods", is crucial. Potential sources of threat are the previously mentioned products obtained using genetically modified organisms [Penninks *et al.*, 2001] as well as those made with the use of microorganisms (especially mould). Also some food additives have potential or already confirmed capability of inducing allergic or pseudoallergic responses [Jędrychowski & Wróblewska, 2002].

According to available data, one third of the European population suffers from allergies. In the last two decades, the number of allergic persons has doubled [Bogucki, 2000]. Allergic reactions to food are very dangerous. In the case of anaphylactic reactions, they make a life threat, at any age [Wűthrich, 2000]. The problem is becoming one of the most significant epidemiological and economic issues (the costs of recognising and treating allergies in the EU are *ca*. 30 bln ECU) and needs international-scale solutions [Bogucki, 2000].

The above-mentioned threats cause that precise and sensitive methods appear to be more important in monitoring food components, which can induce the allergenic response, and legislative procedures are necessary to accept appropriate requirements concerning proper food labelling.

GENERAL CHARACTERISTICS OF IMMUNOMETRIC METHODS USED

Very useful tools in studying relations between immunological system and food and environmental antigens are immunometric methods, *i.e.* methods which make use of specific interactions between antigen and antibody produced by the organism.

History, theoretical considerations, development, terminology of immunoassays are broadly and sufficiently described in the publications of Rittenburg as well Morris and Clifford [Morris & Clifford, 1985; Rittenburg, 1990].

There are many methodological solutions available in this field [Rittenburg, 1990; Stepaniak, 1998; 2001; 2002]. New immunoassay methods have arisen from the desire to detect

and quantify complex biological molecules under conditions for which chemical and physical analytical techniques were either unsuitable or not available.

So far a great number of immunometric methods have been designed. Almost every technique that has been developed is based on the selection of an amplification method which will improve the sensitivity of detection of the antibody-antigen interaction. Double ImmunoDiffusion, Enzyme ImmunoAssay, Enzyme-Linked ImmunoSorbent Assay, Enzyme Multiplied Immuno-Assay Technique, ImmunoDiffusion, RadioImmuno-Assay, Single Radial ImmunoDiffusion belong to the most often mentioned [Rittenburg, 1990].

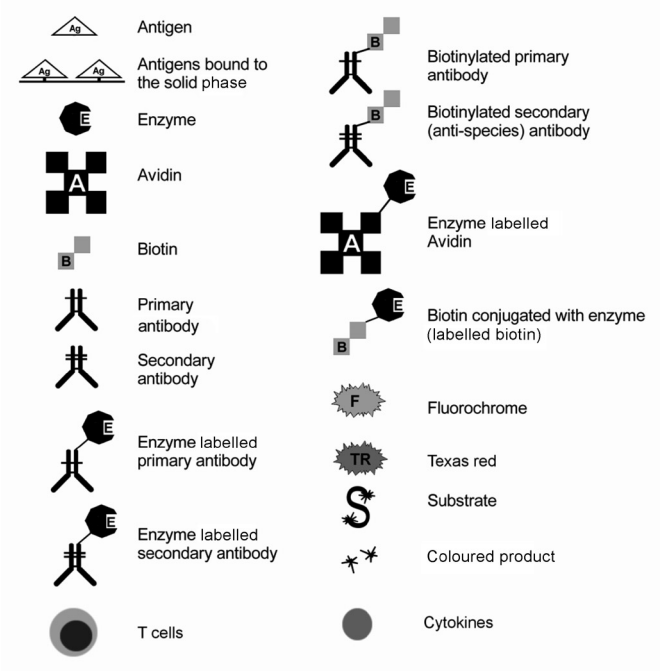
At present, immunometric methods comprise well developed techniques in which, depending on the circumstances, there can be applied: non-labelled antigens, polyclonal--monoclonal or, specific recombinant antibody; or the above--mentioned substances labelled with different compounds. Radioisotopes (^{131}I, ^{125}I, ^{3}H, ^{14}C), enzymes, fluorescent markers, luminescent molecules (chemiluminescent, bioluminescent), spin radicals, red blood cells, phages, colloid gold or silver belong to the most often applied in the immunometric methods labels [Rittenburg, 1990].

The present work is focused on the immunometric methods applied in the own research. The most frequently applied methods are: Direct Competitive ELISA, Indirect Competitive ELISA, Antibody Class Capture ELISA, Double Antibody Sandwich ELISA along with their modifications (Figure 1).

Immunometric methods are simple to carry out. Enzyme Linked Immunosorbent Assay (ELISA) can be divided into two kinds of assays: noncompetitive or competitive. The latter method may be direct or indirect depending of the kind of antibodies used in the assay. The use of competitive ELISA, reference labelled antibody and/or antigen are incubated with the sample. During incubation, they compete for limited quantity of the antigen or antibody bound to the solid phase of the microplate. The main steps in this assay are: coating the solid phase with antigen; incubation with the test sample and reference antibody; incubation with antiglobulin conjugate; incubation with an enzyme substrate; absorbance reading [Rittenburg, 1990]. High absorbance values indicate low antigen concentration in the test sample.

The use of a direct immunometric method usually comprises the following stages [Rittenburg, 1990]: combining antibodies with sample containing antigen and enzyme-conjugated antigen; in this operation, the antigen studied competes with the added conjugated antigen for binding with antibody; possible multiple washing with buffer solution (in heterogeneous methods); adding substrate suitable for the marker-enzyme used for reaction mixture (the amount of colour product formed is proportional to the antigen amount in the sample); absorbance reading.

The major steps of an Indirect Double Antibody Sandwich ELISA include (Figure 1): coating solid phase with specific primary (from species first) antibodies; incubation with serial dilutions of reference antigen or sample antigen; incubation with constant amount of specific secondary antibodies (from species second); incubation with enzyme-labelled anti-secondary antibodies (conjugate); incubation with the enzyme substrate; absorbance reading.



Legend to Figures 1, 2 and 3.

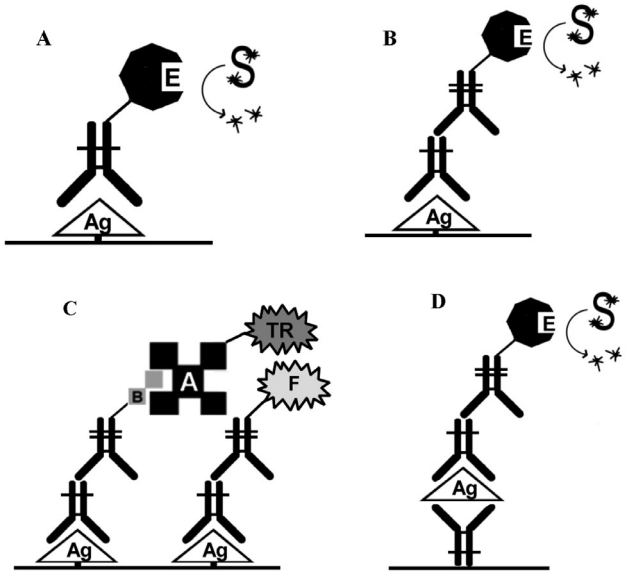


FIGURE 1. The examples of the heterogenic immune methods in antigen determination. The principle of antigen determination by direct method (A); two-stage indirect method (B); double labelling with fluorometric markers (C); and Sandwich ELISA method (D).

Between each step of the assay, careful washing is done using PBS-T buffer.

Among the immunological methods which use amplifying markers with high mutual affinity, *i.e*. avidin-biotin ones, the most often applied methods are: Labelled Avidin-Biotin (LAB), Bridged Avidin-Biotin (BRAB), Avidin-Biotin Complex (ABC) - the most sensitive of all avidin-biotin methods (Figure 2) [Pierce ImmunoTechnology Catalog & Handbook, 1994].

Enzyme Linked Immuno Spot Assay (ELISPOT) method (Figure 3) has been designed to investigate antibodies produced by Limphocytes B. Nowadays the method, in its im-

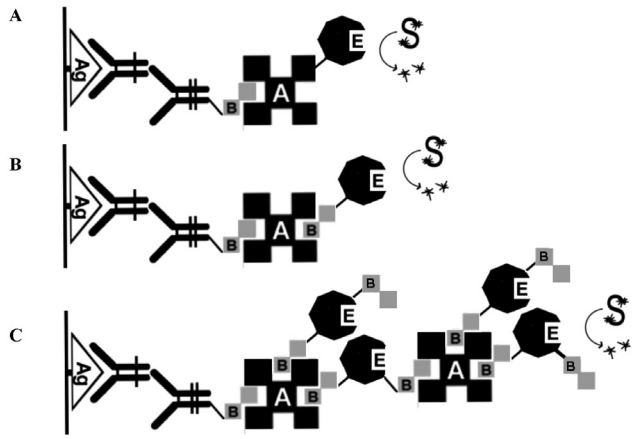


FIGURE 2. The examples of the immune methods with avidin-biotin signal amplication. (A) Labeled avidin-biotin assay (LAB assay); (B) Bridged avidin-biotin assay (BRAB assay); (C) Avidin-biotin complex method (ABC assay). Legend as in Figure 1.

proved and sophisticated form, can be commonly applied for the determination of metabolites produced by cells in minute quantities (*e.g.* cytokines) [Gudmundsson *et al.*, 1999; Hansson *et al.*, 1999; Herr *et al.*, 1996].

The sensitivity of the method is sufficiently high to detect 100 of protein molecules produced in one second. The content of metabolites secreted by proliferating cells is determined by the use of specific antibodies. The application of optical microscopy and appropriate software for microtitreplate well image analysis enables rapid and automatic readings. Due to the application of microplates, the analyses can be readily run in replicates, which contributes to better statistical processing of the data obtained.

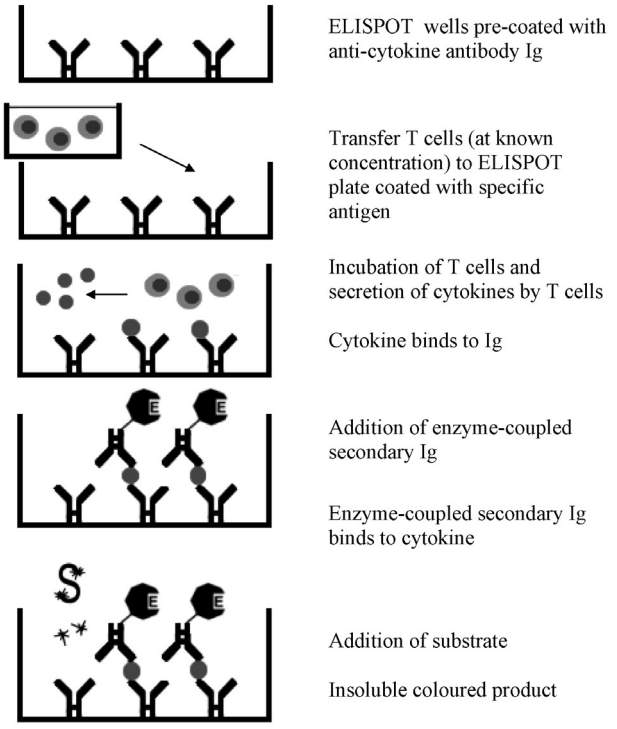


FIGURE 3. Diagram of the Elispot assay. Legend as in Figure 1.

One more immune method applied in food analysis and worth mentioning is immunoblotting (Figure 4). It comprises the following stages: electrophoresis, separated protein transfer onto the nitrocellulose membrane, and determination of the immunoreactive proteins with the use of antibodies and conjugates using one of the immunometric methods (Figures 1 and 2).

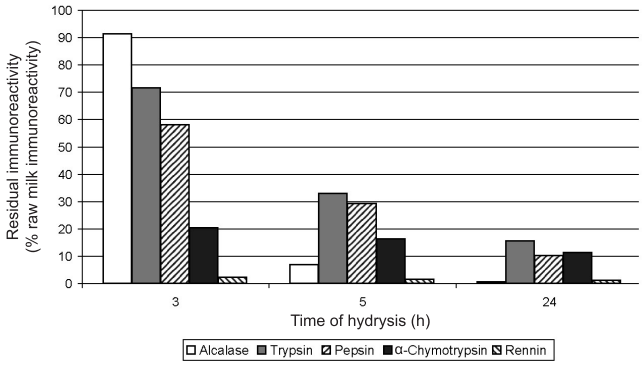


FIGURE 4. The effect of enzymatic hydrolysis of cow milk proteins on immunoreactive properties of α-lactalbumin.

As it can be seen from the above list, immuno-enzymatic methods make a large group of analytical methods with high sensitivity, specificity and multitude of applications.

APPLICATION OF IMMUNOMETRIC METHODS FOR THE EXAMINATION OF IMMUNOREACTIVE PROPERTIES OF FOODS

Due to its sensitivity and specificity (resulting from interactions between antigen and antibody), ELISA method has been proven very useful in food analyses [Allen, 1990]. This method has been used to determine the presence of: (1) various kinds of meat, food additives, among others addition of plant proteins to meat products [Kang'ethe,1990; Patterson & Jones, 1985]; (2) peptides, proteins and their relevant fractions (*e.g.* gluten, gliadins, casein, whey proteins) [Heppell, 1985; Stepaniak *et al.*, 1998; Szymkiewicz & Jędrychowski, 1998a]; (3) enzymes [Wróblewska & Jędrychowski, 2001a; 2001b]; (4) vitamins (A, D_3, B_{12}, B_6, pantothenic acid) hormones, glycoalcalloids, pesticides and herbicides in water and vegetables, antibiotics [Paraf & Peltre, 1992]; (5) micro-organisms and their metabolism products – enterotoxins, mycotoxins, including aflatoxins (ochratoxin A) [Morgan & Lee, 1990; Lee & Morgan, 1990; Wróblewska *et al.*, 1998]; (6) allergenic compounds [Szymkiewicz & Jędrychowski, 1998a; Wróblewska & Jędrychowski, 2001a, 2001b; Jędrychowski & Wróblewska, 2002].

Recently, they have been also used in biotechnology for, among others, determining metabolism, cell apoptosis, proliferation and necrosis [Apoptosis and Cell Proliferation- Boehringer Mannheim-catalogue, 1998].

In our own studies aimed at determining the possibilities of changing antigenic, immunoreactive properties and eliminating or considerable lowering of allergenicity of food products by means of the application of various physical,

chemical, enzymatic, technological and biotechnological processes, significant changes were found in the immunoreactive properties of these products [Szymkiewicz & Jędrychowski, 1998a; Jędrychowski & Wróblewska, 2002]; and their immunomodulative effect [Nagy *et al.*, 2002].

The observed changes indicate that the estimation of antigenic properties of food products is a vital part of the evaluation of food effect on the organism because of a substantial impact of food antigens on the immune system.

The application of proteolytic enzymes (Alcalase, pepsin and trypsin) allowed a significant reduction of the immunoreactive properties of pea (Figure 4) and milk (Figures 5 and 6) protein fractions. The most effective enzyme appeared to be trypsin whose application resulted in the highest lowering of immunoreactive properties of albumin, legumin and vicilin (by 99.97 and 98%, respectively) [Szymkiewicz & Jędrychowski, 2002].

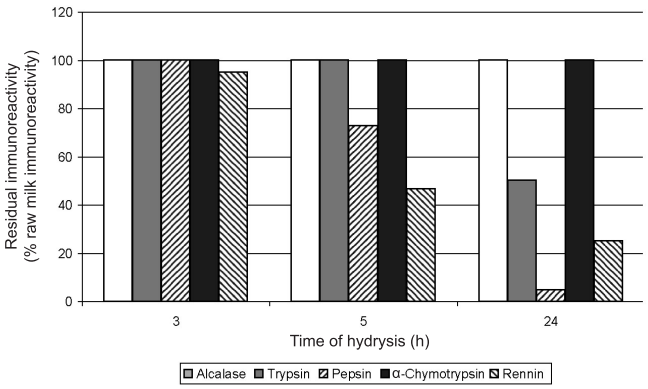
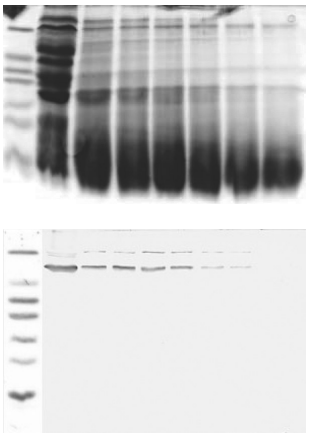


FIGURE 5. The effect of enzymatic hydrolysis of cow milk proteins on immunoreactive properties of β-lactoglobulin.

In the studies concerning modification of the immunoreactive properties of milk proteins it was found that the modification degree of the immunoreactive properties of the mentioned proteins through enzymatic hydrolysis was very high (especially with the application of trypsin and alkalase) but not sufficient to change whey protein immunoreactive properties to the degree required for their utilization as a hypoantigenic product (Figures 4 and 5).

In our studies with the use of ELISA (with avidin-biotin bridges) and ELISPOT methods, we observed a stimulating effect of selected probiotic bacteria (*Bifidobacterium longum*, *Bifidobacterium animalis*, *Lbc*. *Casei*, *Lbc. salivarius*) on the immune system of rats and mice (higher level of specific and total IgG). A similar tendency was found for IgA content (Figures 7, 8, 9 and 10) [Nagy *et al.*, 2002].

Immunometric methods have also been used at our Institute to: (1) determine mycotoxin (ochratoxin A) in foods, fodders, blood, urea, and tissues of pigs after slaughter; it was found that 68.4% of samples were contaminated with ochratoxin A (0.74 μg/kg was found in cereals and approx. 2.32 μg/kg in fodders), 3.5% of imported fodder samples were very highly contaminated (above standard level) as well as the content of the toxin in blood, urine, kidney tissue and muscles reached 26.21 μg/kg, 0.63-4.92 μg/L, 6.82-648 μg/kg and 13.26-102.92 μg/kg, respectively [Wróblewska *et al.*, 1998]; (2) examine the selected enzymes in food products (for instance: coagulating enzymes applied in the dairy industry, rapeseed lipases and



1. Preparations of protein samples (extract of proteins from pea - 1:4, pH 8, 2 h), hydrolysis with enzymes (pepsin 37°C, pH 2, 2 h, 30 mAU/g protein).
2. Gel electrophoresis (SDS-PAGE separation of pea proteins with pepsin - A, 50 V, ~70 mA, ~5 h).
3. Protein transfer from gel to membrane (25 nA, 24 h).
4. Blocking of nonspecific binding sites (3% solution of defated milk, 2 h).
5. Immunoblotting with anti-vicilin rabbit antibody - B (serum 1:5000, 2 h, conjugate of antibody with peroxidase 1:5000, direct immunoassay).
6. Detection of transferred proteins.

FIGURE 6. Procedure of immunoblotting method applied for determination of pea proteins.

lipoxygenases of sunflower, soybean, rape and pumpkin seeds as well wheat and barley grains. As to coagulation enzymes, the high antigenic similarities were found between enzymes with only one exception made for Fromase, and therefore it is considered to be impossible to differentiate the coagulation enzyme applied in the cheese production process [Wróblewska & Jędrychowski, 2001a, 2001b]; (3) determine the content of Ara h1 and Ara h2 allergens in peanuts, chocolate products and material used in the confectionery industry (their content varied from1000 ppm to 7000 ppm). In such studies, it was also found that Ara h1 and some oat protein were characterised by 2% and almond by 55% cross immunoreactivity.

Recently, the issues of antigenic (allergenic) properties of food and their immunomodulative effect on the human organism have been given a priority standing in Europe and world-wide due to their social and economic significance. Their applicable character is revealed in the practical dietetic recommendations in some diseases and convalescence.

CONCLUSIONS

Observing recent advances in applying immunometrical methods in medical science and their increasing role in food

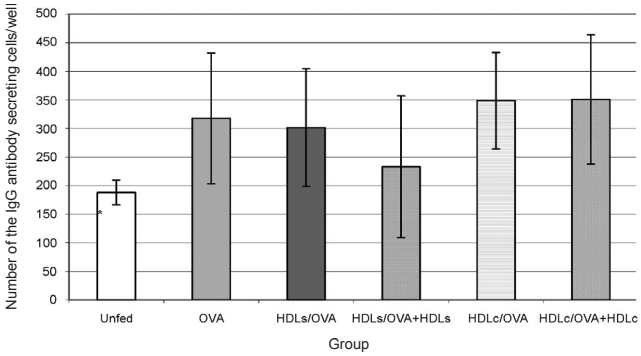


FIGURE 7. The effect of feeding probiotic bacteria on the IgG antibody secreting cell of murine spleenocytes response against the co-fed a marker antigen ovalbumin (OVA) enumerated by ELISPOT [Nagy *et al.*, 2002]. The data represent 5 mice per treatment group with duplicate measurments ± sd; significantly different from OVA group: *p < 0.05; ** p < 0.01.

industry and quality control, we have to take into account equally dynamic application of immunometric methods in food analysis.

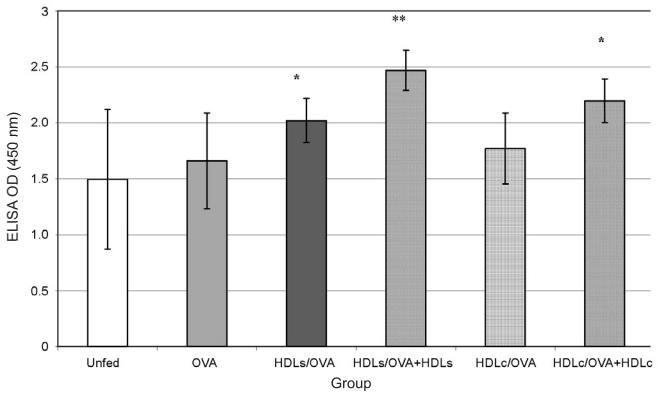


FIGURE 8. The effects of feeding probiotic bacteria on the serum IgG response against the co-fed marker antigen ovalbumin (OVA) measured by ELISA [Nagy *et al.*, 2002]. The data represent 5 mice per treatment group with duplicate measurments ± sd; significantly different from OVA group: *p < 0.05; ** p < 0.01.

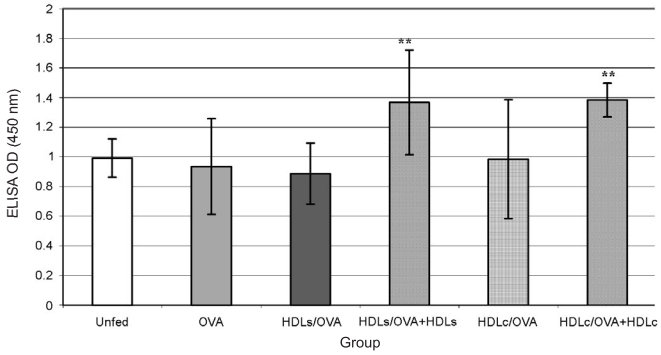


FIGURE 9. The effects of feeding probiotic bacteria on gut IgA response against the co-fed marker antigen ovalbumin (OVA) measured by ELISA [Nagy *et al.*, 2002]. The data represent 5 mice per treatment group with duplicate measurments ± sd; significantly different from OVA group: *p < 0.05; ** p < 0.01.

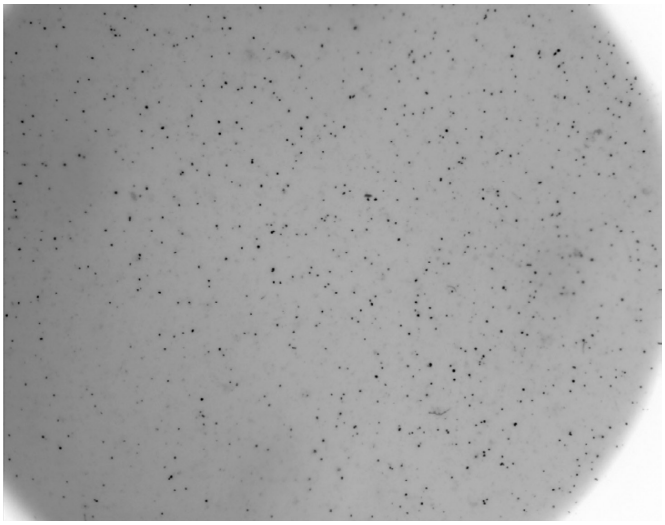


FIGURE 10. An example of IgG2 ELISPOT.

Studies on determining antigenic, allergenic and immunomodulative properties of food are worthwhile both in the cognitive and applicable aspect.

On the basis of our own experience as well as on that of other researchers using immunometric methods, we can unquestionably state the usefulness of these methods for investigating:

1. antigens alone (enzymes, other proteins, allergens, metabolites of micro-organisms including mycotoxins, metabolites-mediators produced by the immune system cells);
2. transformations of the immunoreactive properties of antigens through technological processes;
3. the quantity of residual immunoreactivity of protein- and indirect potential food protein allergenicity;
4. the effects caused by food components in the immune system of the organism.

In addition, it can be concluded that immunoblotting is a simple method but gives only qualitative results (the knowledge about the presence or absence of the examined antigen) and that ELISPOT method (using avidin-biotin amplification) appears to be useful in determination of metabolites secreted by cells (immunocells) in small quantities.

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