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ANALYTICAL METHODS FOR ESTIMATING PROTEIN HYDROLYSATES QUALITY A REVIEW

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The article reviews information on analytical methods applied for estimating protein hydrolysates quality and focuses mainly on physico-chemical methods of determining degree of hydrolysis and distribution of molecular masses of the obtained hydrolysates as well as on immuno-chemical methods determining antigenicity and immunogenicity. A separate group of studies is made by clinical tests for determining hydrolysates allergenicity. The article also outlines available information on the hydrolysates currently used as formulas for infants with dietary allergies.

Keywords: allergenicity, degree of hydrolysis, exclusion chromatography, immunogenicity, protein hydrolysis

Protein hydrolysis is a chemical process during which peptide bonds within protein particles are cleaved to products with lower molecular weight, i.e. polypeptides, peptides and amino acids. The main hydrolysis types are: acidic, basic and enzymatic as well as acid-enzymatic. The first two proceed in a fast, spontaneous and difficult to control way. During chemical hydrolysis, L-form of amino acids are destroyed, while those of D-form are formed, occasionally, also toxic products, e.g. lysino-alanine, are formed.

Due to application of mild reaction conditions, enzymatic hydrolysis permits to precisely design a safe end product of the reaction. As a technological process, hydrolysis has been widely applied in various branches of food industry. The most popular protein sources for obtaining hydrolysed products are casein isolates, whey protein isolates and raw materials of plant origin, e.g. soybeans, peas, chickpeas (CLEMENTE, 2000).

Hydrolysis has been widely applied in many branches of food industry, among others in the production of formulas for patients suffering from all sorts of health problems: phenylketonuria, liver diseases or food allergies. On the market there are available hydrolysates, which make high-energy supplements used in diets aimed at body mass control, regulating intestinal peristalsis or geriatric supplements for the elderly.

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The article presents enzymatic hydrolysis in the aspect of its application for producing formulas with lowered allergenic potential for patients suffering from food allergies.

1. Methods of quality assessment of protein hydrolysates

Numerous studies have shown that products obtained by means of enzymatic hydrolysis reveal residual allergenic and antigenic activity. The immuno-chemical methods used for assessment of these activities are described below. Yet, in order to discuss the properties of protein hydrolysate, first it has to be defined, its degree of hydrolysis and distribution of molecular mass of its peptides have to be determined. For that purpose the physico-chemical methods described below are used.

1.1. Degree of hydrolysis

For a given substrate and enzyme, hydrolysis is described by five independent parameters: temperature, pH, substrate concentration, enzyme/substrate (E/S) ratio, and time of hydrolysis (ADLER-NISSEN, 1986). With so many parameters optimisation and control of the process are time consuming and expensive. ADLER-NISSEN (1986) theoretically described and experimentally confirmed that four of these parameters, i.e. temperature, substrate concentration, E/S ratio and time can be controlled simultaneously by determining the degree of hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved during the hydrolysis (ADLER-NISSEN, 1976)

$$DH = \frac{h}{h_{\text{tot}}} \times 100\%$$

where: h: number of peptide bonds cleaved, h_{tot}: total number of peptide bonds.

The remaining parameter, pH, is kept at a constant level throughout the process. Thus, in order to fully control hydrolysis it is enough to control DH.

From the above information it follows that there is a need for fast and convenient methods of DH determination. One group of methods is based on the measurement of free α-amino or, much more seldom, carboxyl groups in hydrolysate.

There is a number of simple methods of determining free amino groups based on their quantitative reactions. One of the oldest methods is the colourimetric reaction of these groups with ninhydrin in which a reduced ninhydrin particle joins ammonia and another ninhydrin particle, thus forming some dark blue product. Another commonly applied method is formic titration (KUEHLER & STINE, 1974; MAHMOUD et al., 1992). The classical form of this method is based on the reaction of formic aldehyde with amino acids and titration of protons released during the reaction with a base (SILVESTRE, 1997).

At present, the most popular methods are those based on the reaction of amino group with *o*-phtaldialdehyde (OPA) (GWIAZDA et al., 1994; PIHLANTO-LEPPÄLÄ et al., 1997) or with 2,4,6-trinitrobenzenosulfonic acid (TNBS) (GARCIA-RODENAS & CUQ,

1994; NEVES & LOURENCO, 1995; SÜLE et al., 1998). In the former, α- and ε-amino groups react with *o*-phtaldialdehyde and 2-mercaptoethanol in the presence of sodium dodecyl sulphate (SDS), forming 1-alkylthio-2-alkylisoindol, which is determined spectrophotometrically (CHURCH et al., 1983). Several modifications of this method exist. FRISTER and co-workers (1986) applied ethanothiol instead of 2-mercaptoethanol, while ALVAREZ-COQUE and co-workers (1989) used N-acetyl-L-cysteine, which forms more stable isoindoles with OPA. TNBS method is based on spectrophotometric measurement of chromophor, formed in the reaction of TNBS with primary amine in basic pH in the presence of SDS (ADLER-NISSEN, 1979). The methods with OPA and TNBS are presently in standard use due to their high sensitivity and reproducibility. According to PANASIUK and co-workers (1998) both methods give comparable results for determining α-amino nitrogen in pea protein hydrolysates and isolates, while results obtained with ninhydrin method are almost half as low.

Apart from the methods based on the reaction of free amino acid groups of hydrolysate, there are also other simple ways of determining DH, for example pH-stat and osmometer techniques. The pH-stat method (ADLER-NISSEN, 1986; ROZAN et al., 1997) is based on the fact that proteolysis results in releasing groups $-COO^-$ and $-NH_3^+$. If hydrolysis pH is higher than pK of α-amino groups, protons are released, and pH decreases. In order to maintain constant pH value, the reaction mixture is titrated during hydrolysis with a strong base. The amount of the base used is proportional to the amount of hydrolysed peptide bonds (ADLER-NISSEN, 1984). DH values determined with pH-stat method are usually slightly higher than those obtained with TNBS or OPA methods. Slightly higher DH values when using pH-stat method compared to TNBS were obtained by PONNAMPALAM and co-workers (1987) during hydrolysis of oat flour with Alcalase and Neutrase and by KARAMAĆ and co-workers (1998) during hydrolysis of pea protein isolate with trypsin. Both GWIAZDA and co-workers (1994), who hydrolysed rapeseed protein with Alcalase and Neutrase and POULIOT and co-workers (1995), who hydrolysed milk proteins with trypsin and chymotrypsin, obtained higher values using pH-stat technique than with colourimetric method with OPA.

The pH-stat method can be applied for DH control in neutral and alcaline pH or pH below 3 (ADLER-NISSEN, 1984). In the pH range 5–6, the phenomena of joining and releasing protons neutralise each other.

In acid environment, the osmometer technique (ADLER-NISSEN, 1984) can be used for DH control. According to POMMER (1995), this method is easier to apply in the industrial conditions than the pH-stat one due to its speed and simplicity of measurements. The osmometer method is based on the relation between solution peptide concentration and lowering its freezing point. The osmolality read from osmometer is next converted to DH according to ADLER-NISSEN's formula (1984).

Apart from DH other parameters are used, which are also referred to as degree of hydrolysis in contrast to the description of protein enzymatic hydrolysis course as seen by ADLER-NISSEN (1986). MAHMOUD and co-workers (1992) and LAHL and BRAUN (1994) define degree of hydrolysis as the ratio of amino nitrogen to total substrate nitrogen. It is then calculated from the following formula:

$$DH = \frac{AN}{TN} \times 100\%$$

where: AN: amount of α-amino nitrogen determined with the formic method, TN: amount of total nitrogen in substrate determined according to Kjeldahl method.

On the other hand, KIM and co-workers (1990) and BOMBARA and co-workers (1992) express DH as percentage of nitrogen compounds (determined according to Kjeldahl method) soluble in trichloroacetic acid (TCA) and describe it with the following formula:

$$DH = \frac{10\%\ TCA - soluble\ N}{total\ N} \times 100\%\ .$$

Another parameter characterising degree of hydrolysis course is the amount of α-amino nitrogen of hydrolysate soluble in TCA, determined with one of the colourimetric methods mentioned before and expressed in moles of leucine (BULMAGA et al., 1989; JIVOTOVSKAYA et al., 1996) or glycine equivalents (GALLAGHER et al., 1994).

Due to different ways of defining DH, it is difficult to compare DH values obtained by different authors. The reported DH values vary in the range of several percent. On hydrolysing oat flour with Alcalase and Neutrase, PONNAMPALAM and co-workers (1987) reported DH expressed as percentage ratio of free amino groups, measured with the pH-stat method, to the total number of peptide bonds after 180 min of hydrolysis conducted in stable conditions was 8%, while DH expressed as percentage of nitrogen soluble in 20% TCA reached 24%. When the degree of hydrolysis is expressed as amino to total nitrogen ratio, DH can reach the values of several percent, e.g. after 100 min of hydrolysis of casein with pancreatin, DH reported by MAHMOUD and co-workers (1992) was 70%.

It should, however, be considered that the ability to reach high DH values depends also on the selected enzyme-substrate system. For instance, oat proteins are more susceptible to Neutrase or Alcalase action with DH values being about 3% higher for the former enzyme (PONNAMPALAM et al., 1987). Degree of hydrolysis reached for casein-protease from *Bacillus subtilis* was over 5-fold higher than that of casein-bromelain system (GALLAGHER et al., 1994).

1.2. Distribution of molecular mass

Degree of hydrolysis provides information about hydrolysis extent but it does not solve the problem concerning the amount and size of peptides obtained through hydrolysis. Hydrolysates produced using different substrates and enzymes under different conditions of hydrolysis course may have a similar DH but different composition of peptides of the end product. Due to close correlation between the particle size and their molecular mass, chromatographic (size-exclusion chromatography) and electrophoretic methods can be used for that purpose.

Equipment suppliers offer a whole range of columns for size-exclusion chromatography working in FPLC and HPLC systems, which differ in kind of filling and range of molecular mass of separated products. The examples of columns used for size-exclusion chromatography of protein filtrates are Superose 12, on which CHOBERT and co-workers (1988) separated trypsin hydrolysates of whey proteins and casein; TSK 2000SW column, on which TURGEON and GAUTHIER (1990) separated trypsin and chymotrypsin hydrolysates of whey proteins; BioRad P-10 column used by ADLER-NISSEN (1993) to separate soybean proteins hydrolysed with Alcalase, or TSK G2000SW column which SLATTERY and FITZGERALD (1998) used to separate sodium caseinate treated with protease from *Bacillus* sp.

As a result of size-exclusion chromatography of hydrolysates obtained from denatured proteins, products with a wide spectrum of molecular mass are obtained. Hydrolysates contain large polypeptide particles as well as small peptides. The distribution of molecular mass depends on the degree of hydrolysis. At low values of DH, hydrolysates are dominated by proteins with low molecular mass and polypeptides which are next, at higher DH values, digested to smaller ones. The relation between DH and distribution of molecular mass of hydrolysates when using size-exclusion chromatography was determined for various enzyme-protein systems, e.g. for oat proteins hydrolysed with Alcalase and Neutrase (PONNAMPALAM et al., 1987), casein and proteins digested with trypsin (CHOBERT et al., 1988), whey proteins treated with Alcalase, protease 660L of bacterial origin or animal protease PEM 2500S (GONZALEZ-TELLO et al., 1994), sodium caseinate hydrolysed with proteinase from *Bacillus* sp. (SLATTERY & FITZGERALD, 1998).

There was no effect of DH found on distribution of molecular mass except in the case of hydrolysed native proteins. For them, size-exclusion chromatograms show peaks corresponding only to non-digested proteins and end products, but there are no peaks of intermediate fractions. PARRADO and co-workers (1993), who separated sunflower proteins hydrolysates with different DH on the column Superose 12 HR, observed only qualitative differences. Similarly, only qualitative differences were reported by DANILENKO and co-workers (1993) on chromatograms of proteins 11S of broad bean and their trypsin hydrolysates; in that case size-exclusion chromatography was made on the column TSK-64000SWG.

Electrophoretic techniques enable separation of protein mixtures according to molecule size. The principle here is the application of polyacrylamide matrix as a carrier and appropriate voltage. For separation of denatured proteins the method with SDS is used (so-called SDS-PAGE). This kind of analysis provides a possibility of practical determination of peptides with molecular weight above 5000 Da, but it cannot be used for hydrolysates with high DH. Here, electrophoresis with urea or two-dimensional electrophoresis, in which the obtained electrophoregram is stained with silver, are applicable.

2. Immunochemical methods

Non-clinical immunochemical tests are used for determining antigenicity and immunogenicity of hydrolysates.

2.1. Antigenicity

Antigenicity is defined as a specific reaction of antigen with antibody. Determining residual antigenicity is based on determining reacting possibility of antibody obtained in animals immunised with native protein, which was the material for obtaining hydrolysate. The methods used are ELISA and RIA. For analysis of hydrolysates, methods determining individual epitopes are used (competitive inhibition). The principle of this method is competition of hydrolysate proteins with native protein for the site of binding with antibody. The residual antigenicity is inversely proportional to the signal received during analysis.

The estimation of hydrolysis end products with the ELISA method can be applied based on the kinetics of epitopes destruction during hydrolysis. Results are given in antigen equivalents, since the sites of specific enzyme binding on native protein undergo both qualitative and quantitative changes revealed during hydrolysis.

One of the most popular and cheapest methods not requiring high-tech apparatus is immunodiffusion. Although it is less sensitive than ELISA (even 10 000-fold), it is still used for analysis of residual antigenicity. Yet, the method may be burdened with an error. The presence of non-protein substances, e.g. lipids, can have an effect on apparent precipitation. Forming of precipitation arch is a result of antigen's reaction with antibody; nevertheless, it depends on the analysed peptide epitope density, mutual relations of reagents concentrations, pH conditions and ion strength.

Also an animal model can be used for determining the residual hydrolysate antigenicity. The results are not as precise as those obtained with ELISA. The most commonly used animals are guinea pigs orally allergised with cow's milk, for example. The residual allergenicity is established by means of intracardiac administration of the hydrolysate examined followed by systemic anaphylaxis observation. Another possibility is active or passive anaphylaxis test instead of hydrolysate intracardiac administration. In in vivo tests, parenteral immunisation with adjuvant is necessary to induce the state of allergy to cow's milk prior to further antigenic testing.

2.2. Immunogenicity

Immunogenicity is provoking a response in the organism of a non-allergic host. Evaluation of immunogenicity is always connected to the use of animal model. Although parenteral immunisation with adjuvant was applied by some researchers, feeding guinea pigs is more common. They can be fed at different time intervals, their immunological response can be checked in vivo by means of intracardiac administration, test of active or passive anaphylaxis, or serum checked in vitro with ELISA. Four-week guinea pigs were given 100 ml milk and hydrolysates instead of

water for 5 weeks. Next, they were transferred to water for 1 week, and then were stimulated intracardially. All animals fed milk had anaphylaxis. The experiment showed that deeply hydrolysed protein was a weaker allergen than native protein (LEE LEARY, 1992).

2.3. Allergenicity

Allergenicity can be determined only in clinical tests. Correct recognition of allergy kind and introduction of appropriate treatment are conditioned by identifying the actual etiological agent. Basic methods used in vivo, apart from anamnesis, are skin tests of three kinds: intradermal, intracutaneous and epidermal. The principle is provoking a reaction between allergen and IgE antibody bound by specific receptors (mainly FceR1) with cell membrane of skin mastocytes. In 1 cm^3 of skin there are 5 000–12 000 mast cells, whose degranulation leads to releasing allergic reaction mediators and, consequently, to increased vessel permeability, swelling and itching (BIAŁEK et al., 2001a).

Intradermal tests are made on the forearm, by injecting 0.02–0.05 ml solution of the allergen studied until a blister is formed. This kind of test is rather dangerous for patients as it may lead to anaphylactic shock.

There are four kinds of intradermal tests depending on a technique:

- Scarification test – a drop of allergen is placed on the skin which is scarified with a needle or scalpel. These tests have low sensitivity and are not recommended.
- Prick tests – a drop of allergen is placed on the skin which is pricked with a needle within the drop range. The diameter of allergen blister is compared to histamine blister. The tests are sensitive and safe for patients and therefore are recommended in clinical diagnosing.
- Prick by Prick test – allergenic substance is used in its natural form. Fruit pricking is immediately followed by patient's skin pricking.
- Gronomeyer-Debelic test – solution of allergen is rubbed into the inner forearm side. It is controlled by rubbing the skin with a clean palm.

Epidermal tests are applied in contact allergies diagnosing. The substance checked is applied on the skin surface in powder, solid or liquid form (BIAŁEK et al., 2001a).

In the lab practice, many in vitro tests are used depending on cell hyperallergenicity evaluation. Among the known methods lymphocyte blastic transformation test, histamine release test, leukocyte and microphage migration inhibition test, rosette test and Cast-ELISA (Cellular Antigen Stimulation Test) test (BIAŁEK et al., 2001b) should be mentioned.

3. Milk proteins hydrolysis in view of research studies

Hydrolysed formulas were introduced on the market about 50 years ago by Nestle Research Centre (EXL et al., 2000) as a form of dietary prevention of cow's milk allergy

and other atopic diseases induced by food. They are marked H.A.; their residual antigenicity is higher than that of formulas obtained through extensive hydrolysis, therefore they are not suitable for patients with cow's milk allergy. Nevertheless, their antigenicity is significantly lowered compared to native milk proteins, which allows for increased oral tolerance. At the same time, such formulas have attractive organoleptic properties and their production cost is not high. Formulas H.A. have different antigenicity and nutritional value, depending on the material and hydrolysis method used for their production.

The problem of food allergy due to allergy to milk has brought about a need to develop hypoallergenic formulas. At present, there is a range of such products available on the market, although many studies pointed out their residual allergenic and antigenic activity. Therefore, numerous studies focus on the key problem, which is determining the molecular weight of peptides that may trigger allergic reaction and which have in vivo effect on binding IgE antibodies. The direct aim is to search for the possibilities of producing safe standardised diet based on milk proteins. One of the most effective processes at present is enzymatic hydrolysis. However, there are some studies which show increased immunoreactivity of α-la and β-lg, two major milk allergens, after hydrolysis (SHARMA et al., 2001; JĘDRYCHOWSKI et al., 2000).

It was observed that casein hydrolysates reveal non-antigenic properties of peptides at the analysis of protein molecules <1200 Da. According to VAN BERENSTEJN and co-workers (1994), the minimum molecular weight required for peptides obtained from whey for obtaining a response was between 3000 and 5000 Da. ENA and co-workers (1995) showed in their study that peptides with molecular weight below 3400 Da did not provoke allergic reactions. The residual antigenicity depended mostly on the enzyme action specificity and not on hydrolysis degree or molecular distribution of molecular weights of the compounds formed.

VAN HOEYVELD and co-workers (1998) reported that peptides with molecular weight above 2600 Da provoke positive skin response and inhibit IgE binding. Peptides below 1400 Da do not provoke positive skin response, although they are still potent to partly inhibit IgE binding with hydrolysate. The authors concluded that the minimum molecular weight of antigen which can react with IgE in vitro is within the range 970–1400 Da. They also suggested the use of proteins hydrolysed this way for production of formulas safe for allergic patients. Such observations are significant in the case of patients in whom IgE is a mediator. In the case of infants, allergies are caused by T cells reaction to peptides. Therefore, in such cases the most suitable is alternative diet or diet based on amino acids (VAN HOEYVELD et al., 1998).

The Subcommittee on Nutrition and Allergic Disease of the American Academy of Paediatrics defined the notion of hypoallergenic formula (HØST et al., 1999) as a product based on elementary protein modified in such a way that 90% of patients with diagnosed allergy to this protein could tolerate the formula without occurrence of allergy symptoms. Although hypoallergenic milk formulas based on this criterion are safe for most infants, allergic reactions may still occur in some allergic patients. IgE antibodies circulating in the organism and recognising protein fragments in

hypoallergenic formulas were found in allergic patients. This may account for occasional heavy reactions to hypoallergenic formulas and increasing doubts concerning their absolute safety. Therefore, use of hypoallergenic formulas in patients with advanced milk allergy should be introduced very cautiously, preferably under clinical supervision.

Further studies on the presence of the remaining immunogenic epitopes in so-called hypoallergenic protein hydrolysates are necessary. The epitopes may give a beginning to heavy anaphylactic reactions in infants with cow milk allergy.

ROSENDAL and BARKHOLT (2000) analysed in vitro 12 infant formulas based on various proteins with various hydrolysis degree: non-hydrolysed Nan 1 (cows milk proteins), partially hydrolysed Nan HA, Beba HA, Nutrilon Pepti, Nutrilon Pepti Plus (whey proteins), Aptamil Hypoantigen (whey proteins and casein), Aptamil HA (bovine collagen and soybean proteins), extensively hydrolysed Alfare, Pepti Junior, Profylac (whey proteins), Pregomin (bovine collagen and soybean proteins), Nutramigen, Pregestimil (casein). Using gel filtration, the authors separated molecules sized 7 to > 30 kDa for further analysis. SDS-PAGE electrophoresis revealed the presence of molecules with molecular weight above 20 kDa, while ELISA the presence of antigen β-lg. Antigen material was observed mainly in partly hydrolysed formulas.

In medical press time and again worrying articles report cases of heavy allergy induced by consumption of milk hydrolysates. NILSSON and co-workers (1999) describe the case of a 3-year-old girl who, being allergic to milk, was given such formulas as Profylac, Nutramigen and Neocate. Both in boiled and non-boiled form, all the formulas provoked a positive reaction in RAST test. During a 3-year observation, the girl's level of IgE antibodies increased towards casein an Nutramigen.

Allergies induced by formulas are also reported on the Internet (http://www.aarrc.com). There were described results of skin tests carried out at Allergy and Asthma Rochester Resource Center (USA), in which infant formulas were used (Similac – all milk proteins, Isomil – soybean proteins, Good Start and Alfare – whey proteins hydrolysates, Nutramigen – deeply hydrolysed casein) as allergen samples, and histamine (positive control) and physiological saline (negative control). Three out of the above-mentioned formulas caused very strong allergic reactions (from the alimentary and respiratory systems to the skin). The most suitable milk substitute for infants suffering from dietary allergies to milk and soybean was amino acid mixture Neocate. The basic problems related to this kind of formulas are their unpleasant taste, high price and limited availability.

In their publication on hypoallergenic formulas GIAMPIETRO and co-workers (2001) stressed that none of the so far prepared and available formulas for allergic infants is completely safe. When recommending such formulas, the American Academy of Paediatrics should at the same time be responsible for carrying out tests with double-blind samples in which tolerance is confirmed in at least 90% of infants with formerly diagnosed milk allergy. Such the point of view is also represented by the European organisations such as ESPGAN and ESPACI. In both centres trials were made on a group of 32 infants allergic to milk. Skin Prick test conducted with such formulas as

Nutrilon Pepti, Profylac (deeply hydrolysed whey protein) and Nan HA (whey proteins partly hydrolysed) caused positive reaction in 19%, 15% and 32% of infants, respectively. Oral administration of these formulas made it possible to determine tolerance level at 97%, 94% and 64%, respectively. The conclusion was to apply skin tests prior to introducing hydrolysed formulas to allergic infants' diet, as not all the available formulas meet hypoallergenic requirements.

The issue of searching for the best solution concerning optimum formula for allergic patients is still open. An optimized infant formula should contain sufficient allergens to induce oral tolerance and low allergen content to minimize sensitization (EXL, 2001). The above literature review indicates the need for continuation of this research area.

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