



Reduced IgE and IgG antigenic response to milk proteins hydrolysates obtained with the use of non-commercial serine protease from *Yarrowia lipolytica*

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ARTICLE INFO

Keywords:

Yarrowia lipolytica serine protease
Milk protein hydrolysis
Immunoreactivity reduction

ABSTRACT

The aim of the study was to investigate the use of serine protease from *Yarrowia lipolytica* yeast for reduction of milk proteins allergenicity. Whey protein concentrate (WPC-80), α s-casein and their hydrolysates were analyzed for the capacity to bind IgE and IgG antibodies present in sera from patients with cow milk protein allergy using a competitive ELISA. The hydrolysis of α s-casein and whey protein concentrate contributed to a significant reduction of their immunoreactive epitopes. In case of IgE antibodies, the lowest binding capacity was detected in the 24 h hydrolysates of both proteins in which the inhibition of the reaction was ≤ 20 and $\leq 68\%$ for α s-casein and whey protein concentrate respectively. One hour hydrolysis of WPC-80 reduced the protein antigenicity, while the longer time (5 h) might lead to the exposure of new IgE – reactive epitopes.

1. Introduction

In recent years an increasing occurrence of various types of allergic diseases is observed. Among them, allergies and adverse reactions to food ingredients are becoming more frequent (Devdas, Mckie, Fox, & Ratageri, 2018; Prescott & Allen, 2011). Typical food allergens include egg white proteins, legumes and cereals (wheat and soy), peanuts, fish and seafood. The earliest detected form of food allergy is a reaction to cow's milk protein (cow's milk protein allergy, CMPA), found in 1–17.5% of infants and children during early childhood (up to 5 years of age), 1–13.5% of teenagers (5–16 years), as well as in about 1–4% of adults (Luyt et al., 2014).

The prevalence of CMPA is strictly dependent on the population studied, diet and the adopted criteria (Bręborowicz & Sobkowiak, 2015). It is more frequent (by 3%–5%) in children fed with artificial food, more often in those with an allergy- positive family history.

Among small children, allergy to cow's milk protein is an important nutritional problem, because in the first months of life milk is the exclusive and later the basic component of the diet. The protein components of milk provide exogenous amino acids, easily digestible and

essential for the growth of all organs, involved in metabolism and ensuring the proper functioning of the dynamically developing child's body.

Clinical symptoms of allergy to milk proteins are very diverse and may affect many organs and systems, in extreme cases even leading to anaphylactic shock (Małaczyńska, 2000). The most common are skin lesions such as: eczema, rash, angioedema or atopic dermatitis, changes in the respiratory system and gastrointestinal changes and others. In chronic forms of CMPA, intestinal villi are destroyed what consequently leads to the development of so-called malabsorption syndrome with lactase deficiency. The complication of milk protein allergy may also include anemia, impaired nutrition and growth, as well as deficiency in bone calcification (Kaczmarek & Bartuzi, 2016).

Cow's milk contains about 30 proteins with potentially allergenic properties (Tsabouri, Douros, & Priftis, 2014). The main protein components of milk include: casein, found in four fractions: α s1-, α s2-, β - and κ - and whey proteins: β -lactoglobulin, α -lactalbumin, blood serum albumin, and immunoglobulins. The highest potential for allergy within casein proteins is characterized by the α s1-casein fraction, for which there is no equivalent in human milk proteins. So far, at least 7

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<https://doi.org/10.1016/j.foodchem.2019.125350>

Received 25 April 2019; Received in revised form 7 August 2019; Accepted 9 August 2019

Available online 10 August 2019

0308-8146/ © 2019 Published by Elsevier Ltd.

antigenic determinants have been identified within this fraction (Cerecedo et al., 2008; Cong, Yi, Qing, & Li, 2013). In the case of β -casein, four immunoreactive epitopes were identified in the N-terminal region, (Cerecedo et al., 2008). In human's milk there is also no β -lactoglobulin, which dominates among whey milk proteins. This protein is characterized by high resistance to enzymatic hydrolysis, and in the not fully developed digestive tract of infants, it may lead to the non-hydrolyzed BLG transfer to bloodstream and the induction of a particularly acute immune response (Lovegrove, Osman, Morgan, & Hampton, 1993). In the case of patients with chronic CMPA, seven epitopes for IgE antibodies and six epitopes binding IgG antibodies were identified in the structure of this protein (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). Studies carried out on a large group of infants showed that these two proteins: α s1-casein and β -lactoglobulin are the most common cause of allergic reactions to cow's milk proteins (Schulmeister et al., 2009; Wal, 2004).

The reduction of the allergenic properties of milk proteins can be carried out using a number of methods such as heat treatment, non-enzymatic glycosylation (glycation), pressurization, fermentation or enzymatic hydrolysis (Bu, Luo, Chen, Liu, & Zhu, 2013). The latter is the method most commonly used in the production of hypoallergenic nutrients.

Proteolytic enzymes cause the cleavage of selected peptide bonds, which in the case of conformational epitopes causes a significant reduction or complete elimination of the binding capacity of specific antibodies. The reduction of protein immunoreactivity by enzymatic hydrolysis depends primarily on the specificity of the enzyme used (Svenning, Brynhildsvold, Molland, Langsrud, & Vegarud, 2000), but also on the degree of hydrolysis and additional technological processes such as filtration or thermal treatment (Bu et al., 2013). However, based on the results of a randomized trial, it was shown that only deep enzymatic hydrolysis significantly reduced the incidence of CMPA (Businco, Bruno, & Giampietro, 1999; Pescuma et al., 1999).

The enzymes used to hydrolyze milk proteins are mainly commercial preparations of digestive enzymes (trypsin, chymotrypsin, pepsin), either of vegetable (papain) or microbiological origin (preparations Neutrase, Alcalase, Corolase). Their use, individually or in combination, and in combination with additional processes, such as heat treatment or pressurization, allows a significant reduction in the allergenicity of many proteins (Bu et al., 2013). Testing the allergenicity of milk protein hydrolysates is one of the critical factors analyzed in the production of milk replacer mixtures for children and infants diagnosed with CMPA (critical for the development of new milk derivative replacements for children with CMPA). Therefore, researchers are still looking for new enzymes from easily available sources that would be capable of efficient hydrolysis of milk proteins and a more effective reduction of their allergenicity. This is particularly important for whey proteins, which are of very high nutritional value but also exhibit a high allergenic potential, while low susceptibility to enzymatic degradation.

An attractive group of enzymes that can be used for such purposes are non-commercial proteases obtained from alternative sources. This group of enzymes includes extracellular proteases with high proteolytic activity isolated from yeast *Yarrowia lipolytica*. The species is able to synthesize proteolytic enzymes active in acid, neutral and alkaline pH [Ogrydziak, 1993]. The pH of the growth environment is one of the key factors influencing the kind of the produced and secreted protease, although other factors, like temperature and the composition of growth media are also important [Ogrydziak, 1993].

The yeast *Yarrowia lipolytica* are able to synthesize two extracellular proteases: serine (AEP), produced in alkaline pH and aspartyl (AXP), which is expressed in acid pH [Glover, McEwen, Thomas, & Young, 1997].

Serine protease is encoded by the XPR2 gene which encodes a 454-amino-acid (aa) prepro enzyme precursor containing a 15-aa signal sequence and a stretch of nine X-Ala or X-Pro dipeptides, followed by a 124-aa pro region that includes a glycosylation site (Asn123) and a

Lys156-Arg157 processing site, and finally the mature form itself.

AEP was first purified by Ogrydziak (1988). The molecular weight of this proteinase was 30 kDa as estimated by gel filtration chromatography. The pH optimum is 9.0 for caseinolytic activity and 8.0 for esterolytic activity, and the temperature optimum is 40 °C. AEP has maximum stability at a pH ranging from 5 to 9. Calcium ions help to stabilize AEP. The protease is completely inactivated by diisopropyl-fluorophosphate (DFP), but it is not inhibited by synthetic trypsin inhibitor, such as tosyl-L-lysine chloromethylketone (TLCK). Purified AEP is also partially inhibited by EDTA. The sulfhydryl protease inhibitor PCMB and several trypsin and microbial serine protease inhibitors have no effect on its activity. Therefore, it seems that AEP is different from other microbial serine proteases. It possesses also esterase activity against several arginine-, lysine-, or tyrosine-containing synthetic substrates (Ogrydziak, 1988). The enzyme is able to degrade many different proteins usually resistant to proteolysis and also release from their sequence biologically active peptides. The aim of our study was the application of *Yarrowia lipolytica* serine protease for reduction of the binding capacity of IgE and IgG antibodies of main milk allergens: whey proteins and α s-casein.

2. Materials and methods

2.1. The enzyme applied in the study

Preparation of serine protease was obtained from growth media of *Yarrowia lipolytica* yeast. The strain was isolated by us from a Polish blue-veined cheese "Rokpol" and is deposited in the Fungal Biodiversity Center, Institute of the Royal Netherlands Academy, Acc. no. CBS 140024. The growth medium contained (g/L): yeast extract (1.7), casein (4), post-refining fatty acids (10), KH_2PO_4 (0.5) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25) NH_4Cl (1). The yeast growth was carried out in a 9 L stirred-tank reactor (AK210) at a working volume of 2.0 L at 28 °C. The aeration rate was fixed at 1.00 vvm. The stirrer speed was adjusted to 500 rpm. The biomass was then centrifuged at $5000 \times g$ for 15 min at 4 °C and the obtained supernatant was subjected to microfiltration, ultrafiltration and diafiltration, followed by dialysis with the use of 18 kDa cut of membrane. The obtained concentrate was spray dried with the addition of 3% methylcellulose as a carrier.

2.2. Substrates

Alpha-s-casein was obtained according to the method of Thompson and Kiddy (1964) by a urea-calcium chloride procedure with a final precipitation in ethanol- ammonium acetate solution.

Whey protein concentrate (WPC-80) was provided by Davisco Foods International, Inc.

2.3. Sera

All sera patients were kindly provided by med. Dr Ewa Willak-Janc from the Institute and Clinic of Pediatric Allergology and Cardiology, Medical University in Wrocław.

2.4. Hydrolysis of α s-casein and WPC-80

Enzymatic hydrolysis was conducted using serine protease isolated from *Yarrowia lipolytica* at the enzyme to substrate ratio in reaction mixture E:S 1:10 [g/g]. The reaction was carried at 37 °C for 1, 5, 24 h in 0.1 M Tris-HCl buffer at pH 8.0. The hydrolysis reaction was terminated by thermal inactivation, 10 min. at 100 °C (for biological activity determinations) or by the addition of 10% trichloroacetic acid (TCA) (1:1 v/v).

2.5. The degree of hydrolysis [% DH]

The course of the hydrolysis was monitored by the determination of soluble peptide concentration in 5% TCA in relation to total protein. The concentration of the trichloroacetic acid-soluble product in the supernatant was measured spectrophotometrically at λ 280 nm (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003).

2.6. The free amino group's concentration

The concentration of free amino groups ($\mu\text{molGly/g}$) was determined using trinitrobenzene sulfonic acid (TNBS, Sigma) according to the method described by Kuchroo, Ramilly, and Fox (1983).

2.7. Reversed-phase high performance liquid chromatography (RP-HPLC)

Peptide profiles of hydrolysates were determined by RP-HPLC with an Agilent 1100 Series system. The hydrolysates were diluted in the even volume of phase A (0.1% TFA in H_2O) before loading on the chromatographic HPLC column (Zorbax Eclipse XDB-C18 Agilent column (50 $\times$ 4.6 mm). Separation was performed at a flow rate of 1 ml/min at 30 °C. Peptide fractions, varying in hydrophobicity, were eluted from the column in linear gradient of phase B (0.1% TFA in acetonitrile). Absorbance measurement was made at λ = 230 nm (DAD, G1315B).

2.8. Determination of protein content

Protein content was determined by colorimetric method of Lowry, Rosebrough, Farr, and Randall (1951), using BSA (Sigma, P0834) as a standard.

2.9. Analysis of α s-casein hydrolysates immunoreactivity.

The binding capacity of IgE and IgG by native α s-casein and α s-casein hydrolysates was determined using a competitive ELISA.

A pool of sera from 12 children (3–5 years old) with persistent cow's milk allergy, as defined by a positive result in a specific serum IgE were used in the study. Sera were selected from 37 analyzed samples and were characterized by the highest absorbance levels ($\text{OD}_{450\text{nm}} \geq 0.5$) in the reaction with α s-casein. The research material was also a pool of 5 sera, with high IgG reactivity ($\text{OD}_{450\text{nm}} \geq 0.5$) with α s-casein, which was taken from children not exhibiting any clinical symptoms of CMA.

The experiment was conducted as described above. In the pre-incubation of sera with α s-casein protein solutions or its hydrolysates after 1 h, 5 h, 24 h of hydrolysis, the following starting dilutions of serum pool were used: 1:10 in the case of determining the IgE reactivity and 1:3000 for IgG in the group of children with CMA, and 1:2000 in the group of healthy children.

2.10. Analysis of WPC hydrolysates immunoreactivity

The ability to bind IgE and IgG antibodies by native WPC-80 and its hydrolysates was determined using a competitive ELISA test. A pool of sera from 20 children (3–5 years old) with persistent cow's milk allergy, as defined by a positive result in specific serum IgE were used in the study. Sera were selected from 37 analyzed samples and were characterized by the highest absorbance levels ($\text{OD}_{450\text{nm}} \geq 0.5$) in the reaction with WPC. The research material was also a pool of 5 sera, with high IgG reactivity ($\text{OD}_{450\text{nm}} \geq 0.5$) with WPC, derived from children not exhibiting any clinical symptoms of CMA.

Maxi-Sorp 96-well plates (Nunc) were coated with 100 μl of 1 $\mu\text{g/ml}$ WPC solution in 0.1 M carbonate buffer (pH 9.5) per well. The plates were incubated for 2 h at 37 °C and then overnight at 4 °C. In the next step the plates were washed with PBST buffer (PBS pH = 7.4 with 0.05% Tween 20) and blocked with a 2% solution of Tween 20 (300 μl

per well) for 2 h at 37 °C. Before use, sera were preincubated (initial dilution 1:10 for determination of IgE reactivity, 1:3000 for IgG – present in the serum pool of the CMA children; 1: 2000 for IgG – in the serum pool of healthy children) with solutions of WPC proteins or WPC hydrolysates after 1 h, 5 h, 24 h of hydrolysis (starting protein concentrations were: 2000, 1000; 500; 100; 50; 10; 5; 2.5; 1.25; 0.63; 0.31; 0 $\mu\text{g/ml}$).

A pool of sera were mixed at a ratio of 1:1 with hydrolysates solutions, which initial concentrations were: 2000, 1000; 500; 100; 50; 10; 5; 2.5; 1.25; 0.63; 0.31 $\mu\text{g/ml}$, and incubated for 2 h at room temperature on rotary platform (70 rpm) in 48-well plates. After incubation the mixture was loaded in triplicates on plates previously coated with WPC solution, blocked and incubated for approximately 18 h at 4 °C on a rotary platform (70 rpm). As the secondary antibodies goat anti-human IgE conjugated to HRPO (Sigma-Aldrich A9667, dilution 1:3000), or rabbit anti-human IgG conjugated to HRPO (Sigma-Aldrich A8792, dilution 1:60 000) were used in amount of 100 μl of conjugate per well. Afterwards the mixture was incubated for 1.5 h at room temperature with gentle rotation. The reaction was developed for 30 min using the TMB Super Sensitive substrate (Sigma-Aldrich, T4444). Readings were performed at 450 nm using a Quantum reader (Biotek).

The percentage of antigen binding inhibition was calculated using the following equation:

inhibition rate (%) = $[(\text{Abs}_0 - \text{Abs}_x) / \text{Abs}_0] \times 100$, where Abs_0 was the mean absorbance value of no-antigen triplicates (serum was incubated with PBST at a ratio of 1:1); Abs_x was the mean absorbance value of triplicates obtained with different antigen concentrations.

2.11. Determination of the binding capacity of goat anti-whey IgG/immune sera by WPC-80 hydrolysates

The binding capacity of goat anti-whey IgG by WPC-80 hydrolysates was determined using the competitive ELISA test. The analyzed immune serum obtained as a result of 5-time, intradermal immunization of goats with whey proteins. The obtained and purified antigenic preparations, emulsified with Freund's complete adjuvant (Calbiochem) were administered every 14 days. Twelve days after the last injection, blood was collected from the cervical vein and the obtained serum was stored at -20 °C until use (Stefaniak, Chrzanowska, & Nikołańczuk, 2000).

The experiment was conducted as described in previous section. The goat immune serum, diluted 1:15 000, was preincubated with WPC or WPC hydrolysates obtained after 1 h, 5 h, 24 h of hydrolysis. Secondary antibodies were used in the test were rabbit anti-goat IgG conjugated to HRPO (Sigma-Aldrich A5420, dilution 1:20 000).

2.12. Statistical analysis

All assays were conducted in triplicate. The results were analyzed using a Statistica 7.0 program Analysis of Variance (ANOVA), followed by a Duncan multiple range test to determine the significant difference between samples at $p \leq 0.05$.

3. Results

The protein substrates analyzed in the paper, i.e. α s-casein and the whey protein concentrate (WPC-80), i.e. a mixture of mainly β -lactoglobulin and α -lactalbumin, were susceptible to the yeast serine protease, which was introduced into the reaction in the E:S proportion of 1:10 [g/g]. Progressive proteolysis was shown by the degree of hydrolysis of the tested proteins and by the increase in the concentration of free amino groups (Fig. 1A).

As a result of the 5-hour proteolysis, the degradation level of α s-casein reached a degree of hydrolysis of 27.2% (Fig. 1B), whereas the WPC-80 fragmentation was less efficient where the DH was 17.7%.

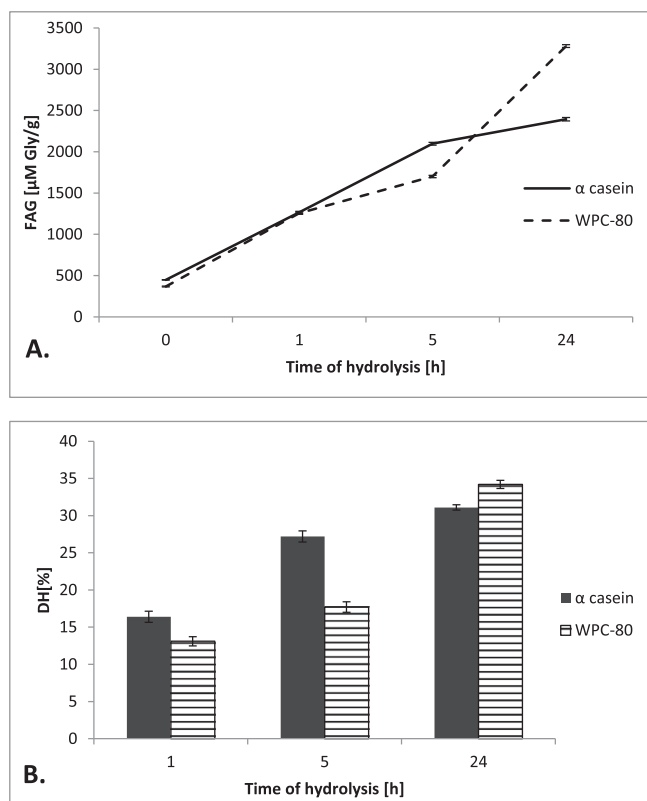


Fig. 1. A: Free amino groups concentration FAG ($\mu\text{M Gly/g}$) and B: Degree of hydrolysis DH (%) in hydrolysates obtained using serine protease isolated from yeast *Yarrowia lipolytica*.

These results were in correlation with the level of free amino groups (FAG, Fig. 1A), whose values were 2097 $\mu\text{M Gly/g}$ and 1700 $\mu\text{M Gly/g}$, respectively. Extending the reaction time to 24 h resulted in further progress in the hydrolysis of both analyzed preparations. DH after this time was 31.1% and 34.2% respectively, while depending on the protein substrate FAG level was 2394 $\mu\text{M Gly/g}$ – in the α -casein hydrolysate and 3280 $\mu\text{M Gly/g}$ in WPC-80 hydrolysate (Fig. 1AB).

The qualitative analysis of hydrolysates of the tested proteins was performed by RP-HPLC by the determination of protein-peptide profiles (Figs. 2 and 3). We observed numerous signals with varying hydrophobicity, obtained from degradation products of both proteins and absent in control samples. At the same time, incubation of substrate proteins with yeast protease resulted in the disappearance of signals from native proteins. Along with the extension of substrate hydrolysis duration, there was an increase in peptides with hydrophilic properties, released from the column at lower acetonitrile concentrations, which is particularly evident in the samples obtained after 24 h of hydrolysis (Figs. 2 and 3).

3.1. Evaluation of immunoreactivity of α -casein hydrolysates obtained with the use of *Yarrowia lipolytica* serine protease

Evaluation of the immunoreactivity of the obtained hydrolysates was performed against the sera of children diagnosed with CMPA and showing allergy to cow's milk proteins in the skin test. Analysis of the reactivity of IgE antibodies present in the patients' serum pool (Fig. 4A) showed that a 2-hour pre-incubation with native α -casein did not lead to binding of all IgE antibodies specific for this protein, and the reaction inhibition was $\leq 60\%$.

The enzymatic degradation of α -casein with the use of yeast protease reduced the ability of IgE antibodies to bind to the peptides released during hydrolysis of this protein (Fig. 4A). The most beneficial

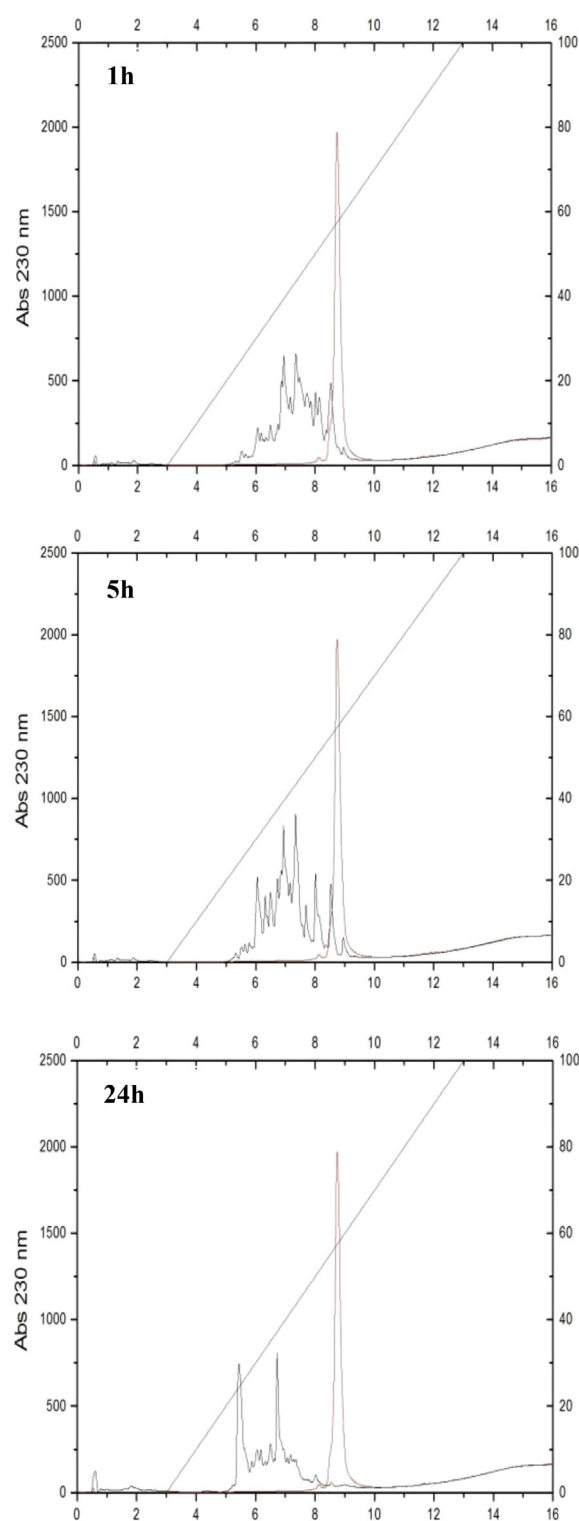


Fig. 2. RP-HPLC profiles of peptide fractions (in black) obtained after 1, 5, 24 h of α -casein hydrolysis with *Y. lipolytica* serine protease at the enzyme to substrate ratio E:S 1:10 [g/g]. Native-undigested 1% protein solution (in red) is presented as the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effects were observed in α -casein hydrolysates obtained after 1 h and 24 h of reaction. One hour of hydrolysis was already sufficient to reduce the antigenicity of α -casein; the inhibition of reaction with α -casein was $\leq 30\%$. Extension of the hydrolysis time to 5 h, despite the higher DH of the hydrolysate, did not further reduce the immunoreactivity of

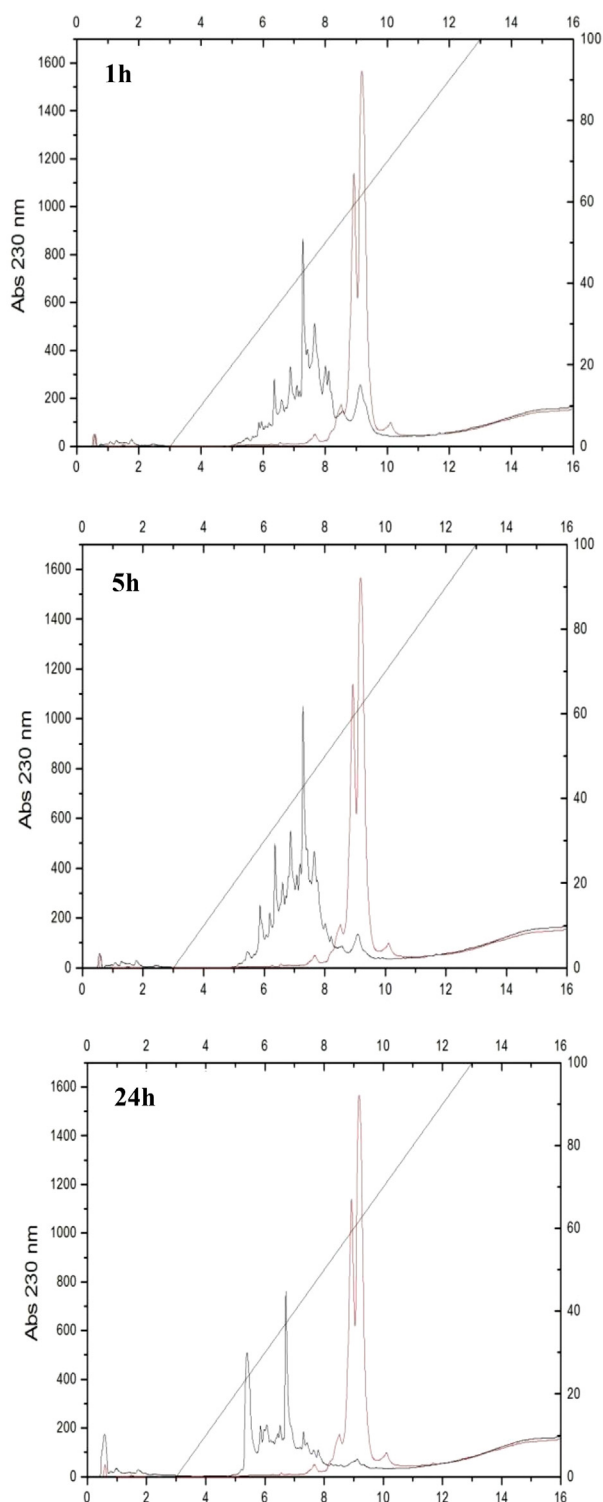


Fig. 3. RP-HPLC profiles of peptide fractions (in black) obtained after 1, 5, 24 h of WPC-80 hydrolysis with *Y. lipolytica* serine protease at the enzyme to substrate ratio E:S 1:10 [g/g]. Native-undigested 1% protein solution (in red) was used as the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydrolysates. The determined inhibition of the reaction was comparable to native protein, the IgG reactivity at high concentrations of 5 h hydrolysate was between 40 and 88.84%. Only after prolonging the protein fragmentation process to 24 h of enzymatic hydrolysis, the inhibition of interactions with α -casein decreased to the level of

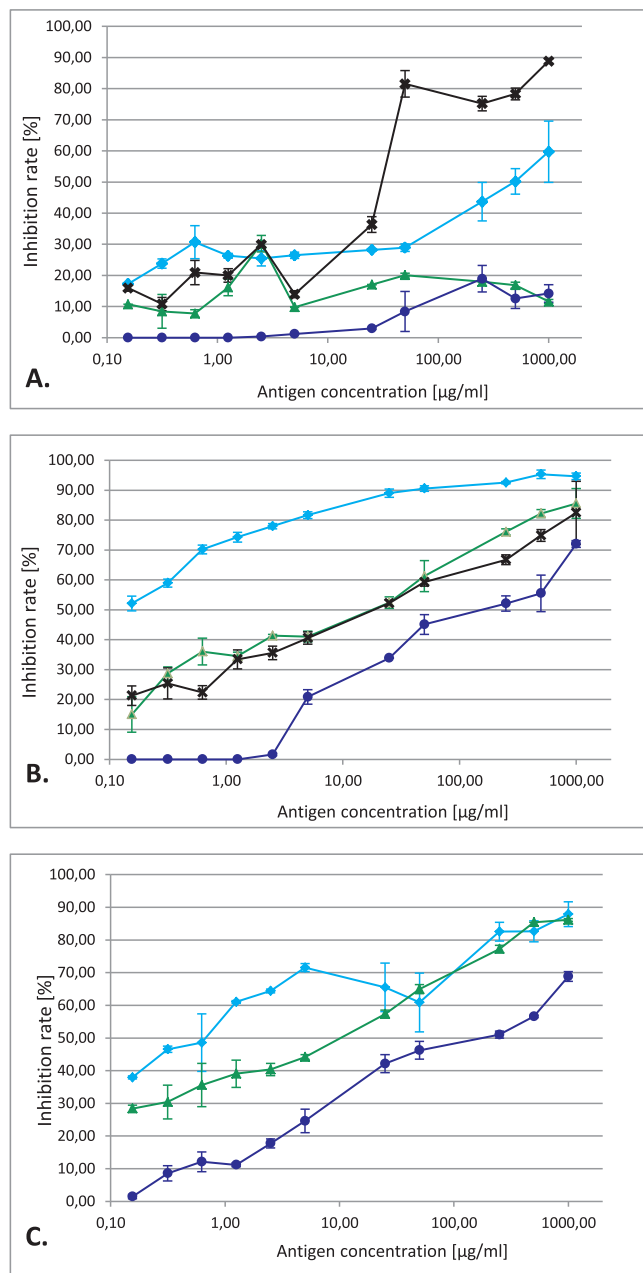


Fig. 4. Influence of pre-incubation of native protein/ α -casein hydrolysates with pool of children sera: with allergy to the reactivity of IgE class antibodies to α -casein [A], with allergy on the reactivity of IgG class antibodies to α -casein [B]; without allergic symptoms on reactivity of IgG antibodies to α -casein [C].; Legend: ▲ – hydrolysate after 1 h; × – hydrolysate after 5 h; ● – hydrolysate after 24 h; ◆ – native α casein.

$\leq 14.2\%$. Analysis of the immunoreactivity of 1 h and 5 h hydrolysates at $\leq 5 \mu\text{g/ml}$ did not show any clear differences in the IgE binding capacity. At low concentrations of hydrolysate obtained after 24 h of degradation, there was no inhibition of the reaction with α -casein, and the resulting peptides did not react with IgE antibodies.

Analysis of the reactivity of IgG antibodies present in the pool of sera of children showing symptoms of cow's milk allergy (Fig. 4B) showed high reactivity of this of antibodies to α -casein.

Hydrolysates of the main casein fraction of cow's milk obtained with the use of yeast serine protease showed a lower degree of inhibition of the reaction with α -casein than the control containing native α -casein (Fig. 4B). The lowest binding capacity regarding IgG antibodies was shown for a hydrolysate obtained after 24 h, for which the inhibition of

the reaction did not exceed 72%. At the low concentration of peptides of this hydrolysate, i.e. $\leq 2.5 \mu\text{g/ml}$, no inhibition of the reaction with $\alpha\text{s-casein}$ was observed. There were no clear differences in IgG binding capacity between 1- and 5-hour hydrolysates, while for the lowest concentration of hydrolysates the inhibition of the reaction with native $\alpha\text{s-casein}$ was still observed, which amounted to 15.05% and 21.31% respectively (Fig. 4B).

Analysis of the reactivity of IgG antibodies present in a pool of sera of healthy children (showing no allergy to milk protein in the skin test) (Fig. 4C) showed also high reactivity of these antibodies to $\alpha\text{s-casein}$.

For $\alpha\text{s-casein}$ hydrolysates obtained with the use of a yeast enzyme, it was found that the deeper degradation of proteins obtained with the extended hydrolysis time resulted in the reduction in IgG binding capacity of the resulting peptides (Fig. 4C). The lowest binding capacity of IgG antibodies specific for the native protein was shown by 24-hour $\alpha\text{s-casein}$ hydrolysate, and the observed inhibition of the reaction with $\alpha\text{s-casein}$, compared to the control, was at least 19% lower.

3.2. Evaluation of immunoreactivity of WPC-80 hydrolysates obtained as a result of hydrolysis with serine protease obtained from *Yarrowia lipolytica*

The immunoreactivity of IgE antibodies present in the pool of sera of children classified as having cow's milk allergy showed that 2-hour pre-incubation of antibodies with unmodified protein (native WPC-80) ($\geq 10 \mu\text{g/ml}$) resulted in the binding of all IgE antibodies specific for the tested protein (Fig. 5A).

The ability of the enzymatic hydrolysis of whey proteins to reduce their immunoreactivity was varied and also depended on the time of the hydrolysis reaction. In the case of the highest concentration of preparations used ($1000 \mu\text{g/ml}$), the difference in binding capacity of IgE antibodies compared to controls was demonstrated only for hydrolysates obtained as a result of 1- and 24-hour reactions, for which the reactivity inhibition was 87.38% and 67.72%, respectively. In comparison, 5-hour hydrolysate showed higher reactivity in the concentration range of 5– $1000 \mu\text{g/ml}$. The lowest binding capacity of IgE antibodies was demonstrated by the 24-hour WPC hydrolysate, characterized by the highest degree of degradation (DH = 34.2%), where the reaction inhibition with WPC, depending on the concentration used, ranged from 2.91% to 67.72% (Fig. 5A).

In the case of WPC-80 hydrolysates, the lowest binding capacity of IgG antibodies also showed a 24-hour hydrolysate, characterized by the highest degree of degradation (DH = 34.2%), and the reaction inhibition, depending on the hydrolysate concentration, ranged from 0.87% to 72.43% (Fig. 5B). In the case of preparations with the highest concentration tested ($1000 \mu\text{g/ml}$), the high binding capacity of this class of antibodies was demonstrated both by the native protein and the hydrolysates obtained from it. The analysis did not show significant differences in the binding capacity of IgG antibodies between the control and 1 and 5-hour hydrolysates (Fig. 5B).

Further analysis of the reactivity of IgG antibodies present in the pool of sera of healthy children revealed high reactivity of this class of antibodies to the whey protein concentrate (Fig. 5C).

In the hydrolysates obtained by introducing yeast *Yarrowia lipolytica* protease, a decrease in IgG binding capacity was observed. The lowest binding capacity of this class of antibodies showed a 24-hour WPC-80 hydrolysate, where at a concentration of $< 5 \mu\text{g/ml}$ no inhibition of the reaction with the native protein was observed (Fig. 5C).

The material for analysis of immunoreactivity of hydrolysates produced with the participation of yeast serine protease, was also the antiserum obtained as a result of immunization of goats with whey proteins. Analysis of the reactivity of IgG class antibodies present in the goat immune serum revealed that a two-hour pre-incubation of antibodies with a whey protein concentrate (at a protein concentration of $1000 \mu\text{g/ml}$) contributes to nearly 100% inhibition of their reactivity with WPC-80 (Fig. 6).

In the case of lower concentrations of hydrolysates ($\geq 10 \mu\text{g/ml}$), to

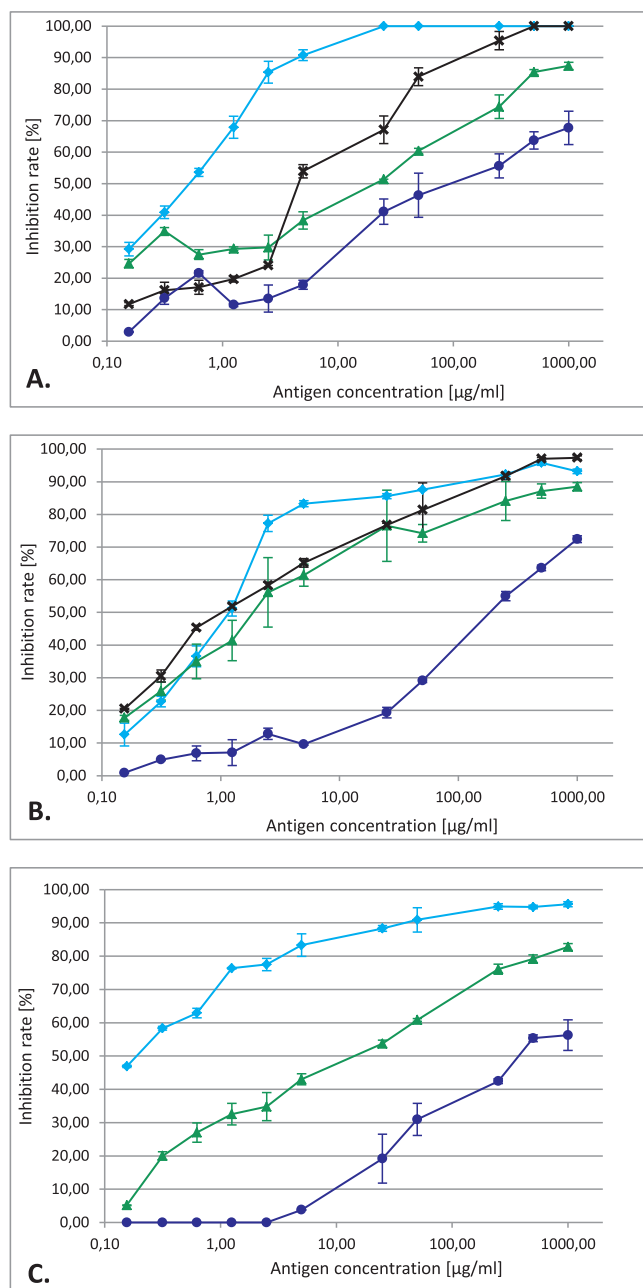


Fig. 5. The effect of preincubation of native protein and WPC hydrolysates with the pool of children sera: with allergy to the reactivity of IgE class antibodies against WPC [A], with allergy to reactivity of IgG class antibodies to WPC [B], without allergy symptoms on reactivity of IgG class antibodies to WPC [C]. Legend: ▲ – hydrolysate after 1 h; × – hydrolysate after 5 h; ● – hydrolysate after 24 h; ◆ – native WPC-80.

which the yeast enzyme was used, the relationship between hydrolysis depth and antigenicity decrease of whey proteins is clearly marked. The lowest binding capacity of antibodies was demonstrated by the hydrolysates obtained as a result of 5- and 24-hour degradation (Fig. 6).

4. Discussion

Due to attractive nutritional values and technological properties, milk proteins are commonly used component of food for special nutritional purposes. Their use, however, is significantly limited by the fact that they are also one of the main food allergens (Kneepkens & Meijer, 2009). For children and infants in a period of growth for which

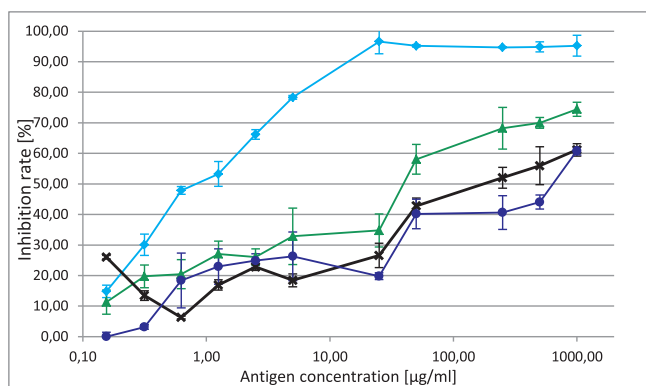


Fig. 6. Effect of preincubation of native protein/WPC hydrolysates with goat antiserum on reactivity of IgG class antibodies to WPC. Legend: ▲ – hydrolysate after 1 h; × – hydrolysate after 5 hours; ● – hydrolysate after 24 hours; ◆ – native WPC-80.

milk and dairy products are naturally an essential part of the diet, the complete exclusion of these products without appropriate substitutes is associated with a significant risk of malnutrition. For this reason, many studies are being carried out to develop an effective method for reduction, or preferably complete elimination of allergenicity in sensitizing proteins.

The immunoreactivity of proteins can to some extent be reduced by a number of technological processes, with proteolysis long seen as most promising for the improvement of their functional properties. Degree of hydrolysis (DH) is a basic parameter which directly gives the opportunity to assess the level of protein degradation. It also allows to estimate the length of released peptide chains and affects the physico-chemical, biological and nutritional properties of hydrolysates. Characteristics of hydrolysates are primarily influenced by the properties of the precursor protein, but also by the specificity of the protease used and the hydrolysis parameters (See, Hoo, & Babij, 2011).

In our study we obtained a significant degradation (DH) of α -casein and whey proteins by the non-commercial extracellular serine protease obtained from yeast *Yarrowia lipolytica*. Especially β -lactoglobulin, main component of WPC, usually more resistant to proteolysis, was hydrolyzed by yeast enzyme at a comparable level to plant-derived cysteine protease (Actinidin), what has been demonstrated by Vázquez-Lara, Tello-Solís, Gómez-Ruiz, García-Garibay, and Rodriguez-Serrano (2003). In contrast, a study by Ena, Van Beresteijn, Robben, and Schmidt (1995), showed a varying efficacy of peptidase from *Aspergillus* sp. (Corolase 7092 TM) against major whey protein fractions.

Proteolysis is a technique considered to be a potentially efficient method of eliminating epitopes that elicit an allergic response to milk proteins and increases their digestibility (Wróblewska & Kaliszewska, 2012). Milk substitutes based on highly hydrolyzed proteins are healthier for infants diagnosed with IgE-mediated allergy to milk protein, compared to those based on free amino acids (Koletzko et al., 2012).

IgG-dependent non-allergic food hypersensitivity is indicated by many researchers as a potential cause of many chronic conditions. The organism's response to food intake, during which IgG antibodies are involved, occurs, for example, in patients with celiac disease with a delayed IgG-dependent reaction to gluten. IgG antibodies may play a similar role in patients with hypersensitive bowel syndrome, atopic dermatitis and bronchial asthma (Shakib, Morrow Brown, Phelps, & Redhead, 1986).

The results of our experiment indicate that the hydrolysis of the main milk allergen fractions, i.e. α -casein and whey proteins using non-commercial serine proteases obtained from yeast *Y. lipolytica* led to a significant reduction in their ability to bind IgE and IgG antibodies specific for cows' milk proteins. The lowest immunoreactivity of hydrolysates was observed after 24-hour degradation, where deep protein

fragmentation resulted in the elimination of the largest number of reactive epitopes. The obtained results may, however, suggest the participation of other classes of specific antibodies, e.g. IgG and IgM, which could have strongly competed with the analyzed IgE class antibodies for the same epitopes (Hochwallner et al., 2011).

Analyzing BLG hydrolysates obtained in fermentation processes using the *Lactobacillus delbrueckii* subsp. *bulgaricus*, Pescuma et al. (2011) demonstrated that this method was effective in the degradation of major epitopes recognized by IgE antibodies, identified in the structure of this protein. The immunoreactivity of the obtained hydrolysate was 32% lower than for the substrate subjected only to thermal denaturation. Despite obtaining a relatively high DH of the hydrolysate (35%), however, the binding of IgE antibodies by the enzymatic degradation products was still observed.

In patients with milk protein allergy, the elevated level of specific IgE antibodies is often accompanied by a high concentration of IgG (Meulenbroek et al., 2013). IgG antibodies can play an important role in the formation of complexes allergen-IgG. Oldaeus, Björkstén, Jenmalm, and Kjellman (1999) analyzed the presence of BLG-specific IgE and IgG antibodies in the serum of 94 infants with food allergy in family history. These children were fed with modified milk, diversified due to the DH of its proteins. The studies conducted by these authors showed that the IgG response to BLG was strictly dependent on the degree of hydrolysis, i.e. very low in the group administered a highly hydrolyzed mixture and very high for native modified milk. A strong immune response was associated with the observed development of atopic dermatitis. The low antigenicity and allergenicity of the highly hydrolyzed blend of modified milk justified its prophylactic use in children with familial predisposition to allergies. According to the American Academy of Pediatrics, only a mixture of peptides below 3 kDa is considered to be highly hydrolyzed, because for peptide mixtures with this molecular weight in 90% of infants examined in clinical trials, no allergy symptoms were observed (Lifschitz & Szajewska, 2015).

In our study, the concentration of IgG antibodies specific for WPC present in the serum of children with allergies was high. In addition, equally high concentration of these immunoglobulins was found in the sera of children who had no history of allergic symptoms. This is confirmed by the work of Jenmalm and Björkstén (1998), who showed that exposure to milk proteins in the first three months of life results in the high levels of IgG antibodies, identified even in children aged 8 years. Similar conclusions were presented by Nentwich, Szepefalusi, Kunz, Spuerger, and Urbanek (2004), demonstrating the presence of IgG specific for milk proteins, not only in serum obtained from children and adults with allergy to milk proteins, but also in healthy people consuming dairy products. The presence of α -casein specific IgG and IgE antibodies was also confirmed in allergic to milk proteins as well as IgG antibodies in non-allergic individuals (Meulenbroek et al., 2013; Nentwich et al., 2004).

In our research increased degradation of milk proteins clearly correlated with the observed reduction of allergenicity of proteins subjected to hydrolysis. Pre-incubation of a pool of sera and antiserum with high-concentration hydrolysates revealed that proteolysis lasting less than 5 h may be insufficient to eliminate epitope immunoreactivity.

Nentwich et al. (2004) showed that for commercial casein hydrolysate found in available hypoallergenic nutrients, immunoreactivity of IgG antibodies assessed by ELISA was only 18% lower than for native casein. Among patients with milk protein allergy, the lowest level of IgG reactivity, among all casein fractions, was shown α -casein (Nentwich et al., 2004). Hydrolysis of whey protein concentrate for 24 h using yeast protease resulted in the degradation of epitopes reactive with IgG antibodies. Potentially, therefore, the administration of this type of hydrolysate may not elicit the immune response of the infant.

For α -casein hydrolysates obtained using a non-commercial enzyme from *Y. lipolytica*, one hour hydrolysis was found to be sufficient to significantly reduce the allergenicity of this protein. However,

extension of the hydrolysis time to 5 h most likely led to the appearance of new epitopes reactive to polyclonal anti- α -casein IgE antibodies. It is possible that longer hydrolysis led to the release of linear epitopes that were then degraded by further hydrolysis. This phenomenon was not observed in the analysis of IgG antibody reactivity.

As other researchers have shown, hydrolysis of milk proteins can lead to both reduction and increase in immunoreactivity, which is directly dependent on the specific activity of the enzyme used (Wróblewska & Kaliszewska, 2012). During hydrolysis, protein is fragmented into peptides of varying length, the immunogenicity of which is conditioned by their amino acid sequence and secondary structure. Sequential BLG hydrolysis using trypsin and chymotrypsin reduced its allergenicity, but at the same time some of the peptides released during this process showed immunoreactivity against IgE (Selo et al., 1999). The structure of the epitopes can be linear, overlapping or spatial (Wróblewska & Kaliszewska, 2012). The spatial structure is characteristic of most epitopes identified in many natural allergens. The deformation or destruction of the spatial structure of a protein allergen molecule under the influence of e.g. denaturation leads to the destruction of spatial epitopes. However, it can also lead to the formation of new epitopes or the appearance of previously hidden variants, which affects the change of immunogenicity. Linear epitopes show high resistance to denaturing agents, but may change as a result of the breakdown of the polypeptide chain, e.g. during hydrolysis (Wróblewska & Kaliszewska, 2012).

5. Conclusions

The obtained results indicate that the non-commercial serine protease isolated from yeast *Yarrowia lipolytica* expresses the activity which significantly reduces the antigenic IgE and IgG response to major allergens of bovine milk (α s-casein and whey proteins). These findings points that the enzyme may be use for production of infant milk formulas and obtained hydrolysates with such characteristics might be used for infants with a high risk of allergy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The research was undertaken in the project POIG.01.03.01-02-080/12. "The use of yeast *Y. lipolytica* and *D. hansenii*, their enzymes and toxins for application in industry and agriculture" co-financed by the European Union under the European Regional Development Fund, 2007–2013.

Publication was financially supported by Wrocław Centre of Biotechnology programme "The Leading National Research Centre (KNOW)" for 2014–2018 granted by Polish Ministry of Science and Higher Education.

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