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Effects of Heat, Ultrasound and Microwave Pretreatments on the Antigenicity of Whey Protein Concentrate (β-lactoglobulin)

[*]Hamdy, A.M.; M.A. Mohran; A.I. Hassan and M.A. Fahmy

Dairy Science Department, Faculty of Agriculture, Assiut University, Assiut, Egypt
[*]Corresponding author: ahmed.hussien2@agr.au.edu.eg

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Abstract

The effects of heat (HT), ultrasound (US) and microwave (MW) pretreatments on the hydrolysis of whey protein concentrate (WPC) by Alcalase, Papain and Trypsin were analysed. Pretreatments were carried out for 10 min at 70ºC for both heat treatment and microwave, for ultrasound at 800 W and samples were jacketed with ice during sonication while control not being subjected to any pretreatments. The effects of heat, ultrasound and microwave pretreatments on the degree of hydrolysis and antigencity of β-lactoglobulin (β- LG) were evaluated. Pretreatments increased the degree of hydrolysis by all enzymes. Pretreatments of whey protein concentrate by heat, microwave and ultrasound before enzymatic hydrolysis decreased the antigencity for β-LG. Obtained results observed that microwave pretreatment was the most effective method in order to reduce whey protein antigencity (β- LG).

Keywords: *Antigencity; β-lactoglobulin; heat; ultrasound; microwave; ELISA*

Introduction

Cow milk and its products are rich in nutrient substance but contain approximately 30 potentially allergenic proteins Miciński *et al*., (2013) even at low concentrations Wal (2004). Cow's milk protein allergy (CMPA) is one of the most common food allergies in children under two years of age and can cause serious health problems Kleber *et al*., (2006). The incidence of CMPA ranges from 0.3% to 7.5% according to population based studies in different countries El-Agamy (2007).

The majority constituents of bovine whey proteins are β-lactoglobulin (55-60%) and α-lactalbumin (15-20%), but they also contain other minor proteins such as bovine serum albumin, immunoglobulins, lactoferrin, phospholipoproteins and bioactive factors and enzymes Smithers *et al*., (1996). It is reported that 66% of milk protein allergies were caused by β-LG, 57% by casein, and significantly less by α-LA and bovine serum albumin 18% Peñas *et al*., (2006). β-LG has been considered to be the principal milk allergen for a long time. It is the major whey protein in the milk of many mammals. However, it is normally absent from human breast milk Sawayer & kontopidis (2000). Moreover, β-LG can reach small intestine in its native form duo to its resistance to pepsin hydrolysis Wal (2001). Therefore, milk allergy often occurs in newborns and infants.

Several researchers attempted to reduce the antigenicity and allergenicity of milk proteins by applying different processing treatments including heat treatment, glycation reaction, irradiation, high pressure, enzymatic hydrolysis, lactic acid fermentation, microwave irradiation,

etc. Bu *et al*., (2010), Grar *et al*., (2009), Hu *et al*., (2011), Kasera *et al*., (2012) Shi *et al*., (2014) and Yao *et al*., (2015).

Heat denaturation changes the conformation of proteins and thus reduces the antigenicity of the protein Mondoulet *et al*., (2005). However, heat treatment may lead to the loss of nutritional quality of the product. Enzymatic treatment can reduce the allergenicity by hydrolyzing milk proteins, but development of bitterness and off-flavor in hydrolyzed milk makes it unacceptable for children El-Agamy (2007). A nonthermal technology is needed to reduce the allergenicity of milk proteins without damaging its nutritional quality.

Microwave irradiation (MWI) is another treatment that is known to affect protein structure. Proteolysis of dairy whey proteins with different enzymes in combination with MW treatment has also shown the potential to more efficiently produce hypoallergenic dairy hydrolysates. Izquierdo *et al*., (2008).

Microwave irradiation during the enzymatic hydrolysis could be an alternative to a conventional heating to reduce the antigenicity of milk proteins, since several studies on enhanced enzymatic proteolysis in solution under MWI have been reported. Izquierdo *et al*., (2005) and Pramanik *et al*., (2002).

Ultrasound technology that involves the use of sound frequency above the human hearing threshold (>16 kHz) has received great interest among food processors particularly application at high power (low frequency; 16–100 kHz) Kwiatkowska *et al*., (2011). The effects of ultrasound on functional properties of proteins largely depend on the nature of the protein Soria and Villamiel, (2010). Arzeni *et al*. (2012) observed that the effect of ultrasonic application was different in whey protein concentrate, egg protein and soy protein. Chandrapala *et al*. (2012). Application of ultrasound at 20 kHz showed greater effect on a-lactalbumin than on b-lactoglobulin but overall suggested changes to their structures.

The aim of this study was to investigate the effects of heat, ultrasound and microwave pretreatments on whey protein concentrate hydrolysate in order to reduce β-LG antigencity.

Materials and Methods

Commercial whey protein concentrate (WPC-8000) was obtained from Hilmar Ingredients (Hilmar 8000, Hilmar, CA, USA) with approximately 80.4% protein. Alcalase 2.4L (2.4AUg−1) and Papain (8×105 Ug−1) were obtained from Nanning Pangbo Biological Engineering Co., Ltd (Guangxi, China). Trypsin, 2,4,6-trinitrobenzenesulfonic acid (TNBS) and caffeine were purchased from Sigma-Aldrich China (Beijing, China) . Boric acid, sodium choloride, sodium dodecyl sulfate (SDS) were bought from Beijing Biodee Biotechnology Co. Ltd (Beijing, China). All chemicals and reagents used were of analytical grade.

Antigens and antibodies

The antigen proteins used for sensitization studies and enzyme linked immunosorbent assay (ELISA) were α -LA (L5385; purity >90%) and β -LG (L3908; purity >90%) purchased from Sigma Chemical Com-

pany (St. Louis, MO, USA). The first antibody from rabbits which contained polyclonal antibodies corresponding to α- LA and β-LG was prepared by animal science laboratory according to Liu *et al*., (2012). The second antibody (horseradish peroxidase conjugated goat anti-rabbit IgG) was also purchased from Sigma Chemical Company (St. Louis, MO, USA).

Preparation of thermal, ultrasound and microwave pretreatments

Whey protein concentrate (WPC80) was mixed with distilled water to a final concentration of 5% (w/v) on a protein basis. The solutions were stirred for 30 min and then allowed to equilibrate in a water bath at 40^{o}C for 30 min to allow for complete hydration. The samples were immersed in a water bath set at 70ºC for 10 min with stirring for heat treatment pretreatment. For ultrasound pretreatment samples were prepared following the procedure of Uluko *et al*., (2015). The samples were sonicated for 10 min while jacketed with ice using a cell disruptor (Ningbo Scientz, JY92-IIN, Zhejiang, China). For microwave pretreatment the solutions were subjected to microwave energy at 70ºC for 10 min using (Sineo, MDS-6, Shanghai, China).

Hydrolysis with different enzymes

The pH of WPC80 solutions were adjusted to optimum enzyme conditions (Table1) using 1mol/L NaOH and 1mol/L HCL. The solutions then immersed in a water bath at optimum temperature for each enzyme (Table 1). The enzyme/substrate (E/S) ratio of 1:100 (w/w). Samples were taken at after 30 min of hydrolysis. The pH of the mixture was maintained constant during hydrolysis using 1 mol/L HCl and 1 mol/L NaOH. The hydrolysis reaction was terminated by placing the mixtures in a water bath at 90^{o}C for 10 min with constant agitation. The hydrolysates were centrifuged (TGL-16C, Anting Science Instrument Co., Ltd, Shanghai, China) at 4000g for 15 min. The supernatants were lyophilized (FD-1 PF, Beijing Detianyou Technology Development Co., Ltd, Beijing, China) and stored at −18^{o}C before further analysis.

Table 1. WPC80 hydrolysis conditions.

Hydrolysis conditions			
Enzyme	Source	pH	Temperature (^{o}C)
Alcalase	*Bacillus licheniformis*	8	60
Papain	Carica papaya	6.5	50
Trypsin	Porcine pancreas	7.5	45

Estimation of antigenicity of whey protein concentrate (β- LG) hydrolysates by inhibition enzyme-linked immunosorbent assay (ELISA)

Inhibition ELISA was used to estimate residual antigenicity of WPC hydrolysates. Microtiter plates with 96 wells (flat-bottomed Costar, Corning Inc., Corning, NY, USA) were coated with 100 µL/well of antigen diluted in 50 mmol/L carbonate buffer (pH 9.6) with the concentration determined earlier in the indirect ELISA method 2 µg mL^{-1} of β-LG and incubated overnight at 4^{o}C. In test tubes, 1 mg mL^{-1} solutions of various WPC

hydrolysates were incubated overnight at 4°C with an equivalent volume of rabbit anti-β-LG antiserum diluted in 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 1% BSA and 0.1% Tween 20 (PBS–BSA–Tween 20) (1:600 of anti-β-LG antiserum). The plates were washed 4 times the next day with 10 mM PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T). This washing procedure was repeated after each step of ELISA. After washing the plates, all wells were filled with 100 µL per well of PBS–BSA–Tween 20, for the purpose of blocking residual free binding sites in wells, and incubated for 37°C for 1 h. The plates were washed and then 100 µL per well of reactive mixtures of hydrolysates and polyclonal rabbit antibodies (IgG) were added and incubated for 1 h at 37°C. Meanwhile, the addition of 100 µL of anti-β- LG serum was taken for the noncompetitive model. After washing the plates, the wells were filled with 100 µL per well of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG in PBS–BSA–Tween 20. After incubation for 1 h at 37°C, the plates were washed again and 100µL per well of 3, 3', 5, 5'-tetramethylenbenzidine substrate solutions was added and then incubated at 37°C for 20 min. Finally, 50 µL per well of 2 mol/L H_2SO_4 were added to stop the reaction. Absorptions were read spectrophotometrically at dual wavelengths of 450 nm and 630 nm on a Multiskan MK3 ELISA plate reader (Thermo Labsystems, Franklin, MA, USA). All analyses were performed in duplicate.

In ELISA, the residual antigenicity of whey protein hydrolysates was evaluated by measuring the ability of the hydrolysates to inhibit binding of specific IgG antibodies of anti-β- LG serum to ELISA plates coated with β-LG.

The percentage of inhibition (%) anti-β-LG IgG binding to β-LG coated on ELISA plates by various WPC hydrolysates was calculated as follows:

Inhibition % = $(B_0 - B) / B \times 100$

Where B is the absorbance measured in the presence of WPC hydrolysate and B_0 is the absorbance measured in the absence of WPC hydrolysate. Low percentage of inhibition for α-LA and β-LG reflects low residual antigenicity of WPC hydrolysate for α-LA and β-LG.

Degree of hydrolysis

The degree of hydrolysis (DH) of the obtained hydrolysates was evaluated by determination free amino groups with trinitrobenzenesulfonic acid (TNBS) method using leucine as a standard Adler-Nissen, (1979). The hydrolysate samples and standard solutions were prepared in 10 mg/mL sodium dodecyl sulfonate (SDS). 1 mL sodium phosphate buffer (0.2125 mol/L, pH 8.2) and 1 mL TNBS solution in water (0.1% v/v) were added to the test tube which contained 125µL hydrolysate samples, followed by mixing and incubation at 50°C for 60 min in a covered water bath. The test tubes were covered with aluminum foil to avoid light. After incubation, 2.0mL of 0.1mol/L HC1was added to terminate the reaction. The test tubes were left at room temperature for 30 min before the absorbance values were measured at 340nm using a ultraviolet and visible spectrophotometerUV-

2600 (UNIC, Shanghai, China). The blank was treated in the same manner. DH was calculated as follows:

$DH = (h / h_{tot}) \times 100$

L-Leucine (0.0 - 5.0 mmol/L) was used to generate a standard curve. DH is expressed as disrupted peptide bonds (h)/total peptide bonds per weight unit (h_{tot}). The h_{tot} for whey protein concentrate is 8.8 meqv per g protein. Adler-Nissen, (1986, 17 pp.).

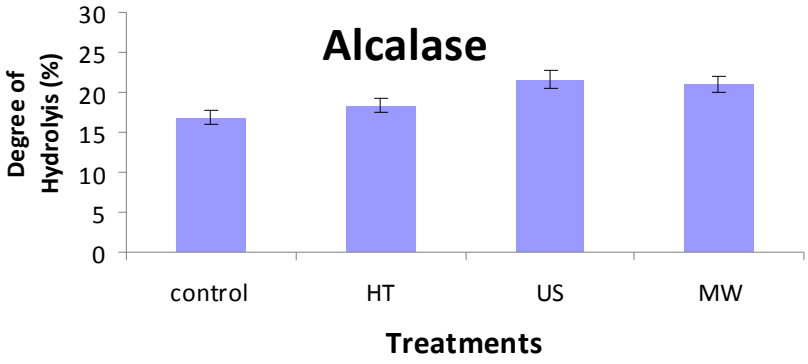
Statistical analysis

All treatments in the present study were performed in three replicates. All results were was performed with a one-way analysis of variance (ANOVA) using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). The least significant difference (LSD) method was used to separate significant treatment means.

Results and discussion

Degree of hydrolysis (DH)

Results of degree of hydrolysis obtained for pretreatment of WPC before hydrolysis with heat, ultrasound and microwave are presented in (Fig1). It has been found that the Pretreatments of WPC with heat, ultrasound and microwave before hydrolysis with enzymes had different effects on degree of hydrolysis. Kwiatkowska *et al*., (2011) observed that strength, frequencies and duration of exposure for ultrasound and microwave treatments cause many chemical and biological effects. Obtained results can show a statically significant $P<0.05$.Combination between heat pretreatment and enzymatic hydrolysis had the highest hydrolysis values for Papain followed by Alcalse and Trypin. Ultrasound pretreatment obtained the highest DH values with Alcalase. The degree of hydrolysis ranged from 16.87% to 21.61% while microwave pretreatment samples gave heights DH values with Trypsin. Changes in the degree of hydrolysis depend on the type of the enzyme employed and the length of pretreatment used.



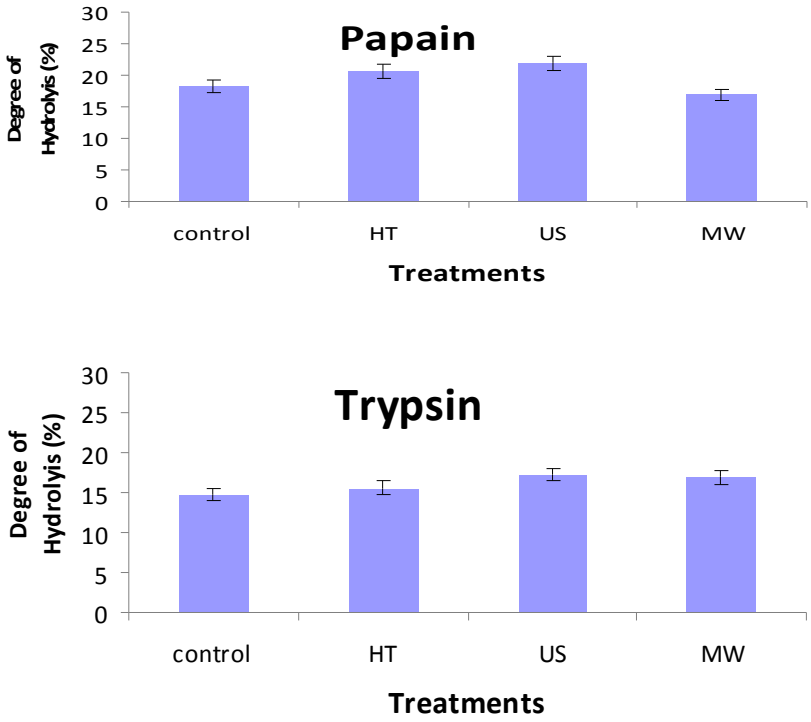


Fig 1. The degree of hydrolysis of WPC after hydrolysis using Alcalase, Papain and Trypsin after pretreatment with heat (HT), ultrasound (US) and microwave (MW). Control had no pretreatment.

These results are in agreement with the results of Dryakova *et al*. (2010). Usually, pretreatments will increase the rate of hydrolysis of a protein because of the protein unfolding associated with pretreatment. Therefore, the degree of hydrolysis has important correlation to the bioactivity of the hydrolysate. Uluko *et al*., (2015).

Effects of heat, ultrasound and microwave pretreatments on the antigenicity of β-lactoglobulin (β-LG)

Effects of heat treatment on β-LG antigencity

The change of β-LG antigenicity is shown in Fig (2). The antigenicity of β-LG was sharply increased by heat treatment at 70ºC / 10 min. The antigenicity of β-LG increased from 50.81% to 73.54% compare to unheated sample. Similar observations were made by Bu *et al*., (2009) who reported that the antigenicity increased initially from 50°C to 90°C.

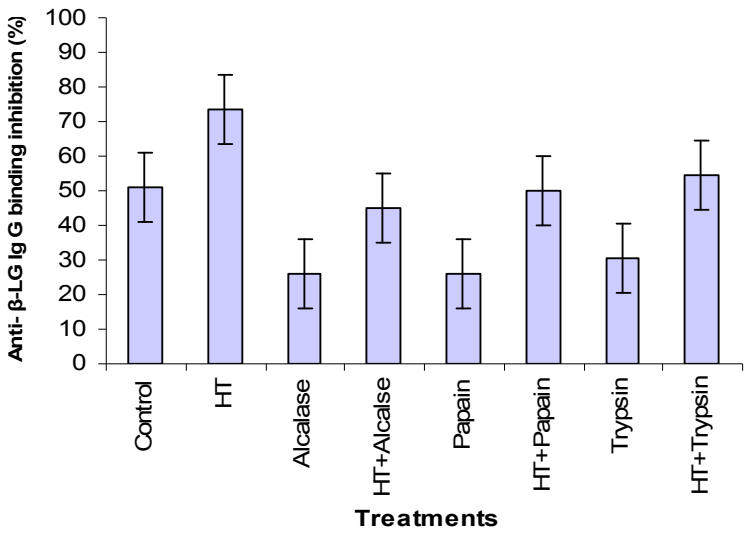


Fig 2. Effects of heat treatment (HT) on β-LG antigencity hydrolysed with different enzymes. Control had no pretreatment, the experiments were done in triplicate.

Taheri-Kafrani *et al*., (2009) suggested that when heating temperatures above 60°C it will cause the destabilization of the β-sheets, the unfolding of the β-barrel, the exposition of disulfide bonds and the free cysteine to the solvent. Kleber *et al*., (2007) also claimed during the heat denaturation of β-LG, firstly the unfolding of conformational structure, the allergenic epitopes which buried inside of the native β-LG molecule may be exposed and in turn increased antigenicity.

Sharma *et al*., (2001) reported that heat-denatured β-LG has been found to have at least one new epitope not found in the native state. All those structure changes may lead to increased antigenicity. The combination between heat treatment and prototypic enzymes caused a significant decreased for β-LG antigencity. The lowest value was found for Alcalase followed by Papain and Trypsin. Our results are in good agreements with results obtained by Bu *et al*., (2009) who mentioned that Enzymatic hydrolysis of whey protein offers a practical way to destroy allergenic epitopes and reduce the antigenicity.

Effects of microwave pretreatment on antigencity of β-LG

The antigenicity of treated whey proteins expressed as a percentage is illustrated in Fig. (3). Our results showed that the microwave pretreatment had significant effect to decrease antigencity of β-LG. The antigencity was decreased from 50.81% to 35.16% after 10 min of treatment. This reduction in antigenic response of β-LG may be explained by different phenomena. There is evidence that microwave irradiation speeds up rates of folding and unfolding for β-LG. Bohr and Bohr (2000). That conformational change of this protein is highly dependent on pH. This could lead to the loss of tertiary protein structures that play an important role in maintaining conformational epitopes and, therefore, leads to a de-

creased antigenic potential. Monaci *et al*., (2006).

Microwave pretreatment decreased the antigenicity of whey protein hydrolysate using different enzymes. The lowest value obtained of anti- β-LG Ig G binding inhibition using microwave pretreatment and Alcalase was 15.56% while was 18.65% and 23.48% for Papain and Trypin respectively.

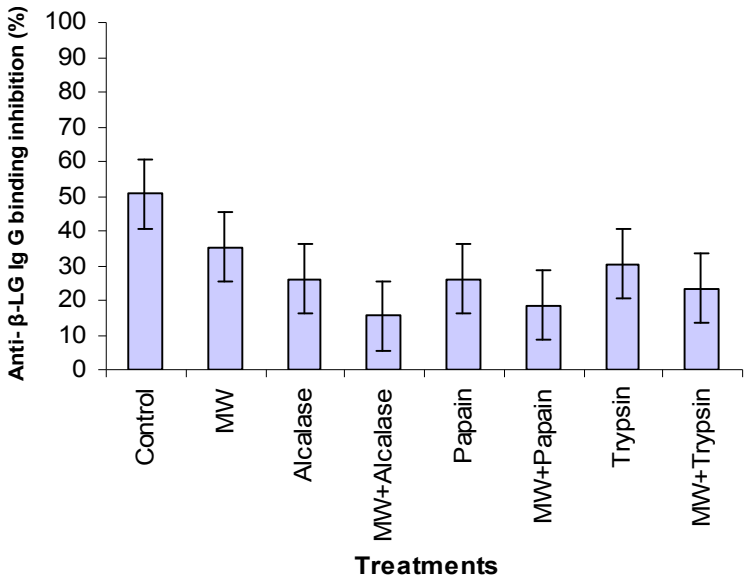


Fig 3. Effects of microwave (MW) on β-LG antigencity hydrolysed with different enzymes. Control had no pretreatment, the experiments were done in triplicate.

The combined enzymatic and MWI treatments, compatible with retention of enzyme activity and reversibility of structural modifications of proteins, may have a practical relevance by decreasing their immunoreactivity, Bonomi *et al*., (2003). The unfolded proteins formed under MWI could represent an ideal substrate for the action of proteases, making some epitopes more accessible to enzymatic hydrolysis.

Effects of ultrasound pretreatment on antigencity of β-LG

Effects of ultrasound pretreatment on the antigencity of β-LG presented in Fig (4). The effects of ultrasound on functional properties of proteins largely depend on the nature of protein Soria and Villamiel (2010). Obtained results showed slight decrease in β-LG antigencity after pretreatment with ultrasound, which decreased from 50.81% to 48.82%. The results are in good agreement with Jambark (2008) who mentioned that applications of ultrasound at 20KHz showed greater effect on α-LA than on β-LG. Combination effects between ultrasound and hydrolysis enzymes also mentioned. The lowest value obtained of anti- β-LG Ig G binding inhibition using ultasound pretreatment and Alcalase was

22.11% while was 25.11% and 29.67% for Papain and Trypin respectively. Li *et al*. (2005) stated that high intensity ultrasound treatment was effective in reducing the allerenicity of shrimp after 180 min of treatment at 30 Hz frequency and 800 W powers. Higher intensities and longer treatment time might yield better results with high intensity ultrasound treatment.

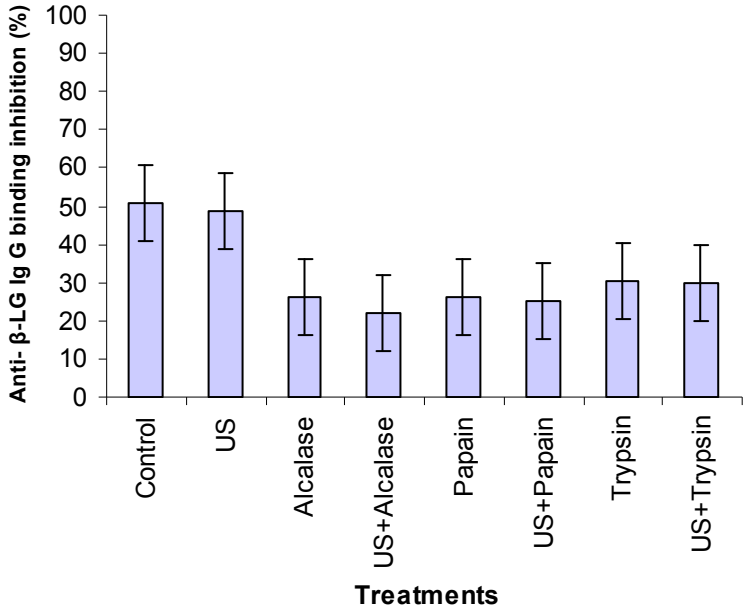


Fig 4. Effects of ultrasound (US) on β-LG antigencity hydrolysed with different enzymes. Control had no pretreatment, the experiments were done in triplicate.

Conclusions

Pretreatment of whey protein concentrate by heat, microwave or ultrasound before hydrolysis enhanced the enzymatic hydrolysis. The degree of hydrolysis increased for all treated samples but it was vary according to kind of enzyme. Papain showed the highest proteolysis followed by Alcalase and Trypsin. Pretreatment of whey protein concentrate by heat treatment, microwave and ultrasound before enzymatic hydrolysis decreased the antigencity of β-LG. obtained results observed that microwave pretreatment was the most effective method in order to decrease β-LG antigencity.

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تأثير المعاملات الأوليه بالحرارة والموجات فوق الصوتية والميكروويف على القدرة الانتيجنية لمركز بروتينات الشرش (β-lactoglobulin)

أحمد محمود حمدى ، محمد عطية مهران ، على اسماعيل حسن ومحمد عبد العزيز فهمى

قسم الألبان – كلية الزراعة – جامعة أسيوط

الملخص

تم دراسة تأثير المعاملات الأولية بالحرارة والموجات فوق الصوتية والميكروويف على التحلل لمركز بروتين شرش اللبن بواسطة انزيمات الـ Alcalase و Papain و Trypsin وأجريت المعاملة لمدة ١٠ دقائق على ٧٠ درجة مئوية لكل من المعاملة الحرارية والميكروويف، بينما أجريت المعاملة بالموجات فوق الصوتية عند ٨٠٠ وات وتمت تغطية العينات بالثلج أثناء ذلك. العينة الكنترول لم تخضع لأي من المعاملات السابقة. تم تقييم تأثير المعاملات السابقة على درجة التحلل الانزيمى لمركز بروتين شرش اللبن وكذلك قدرته الانتيجينية للبروتين المتحلل انزيمياً. ووجد أن المعاملات أدت الى زيادة درجة التحلل بواسطة جميع الإنزيمات التى استخدمت. كما ان معاملة مركز بروتين شرش اللبن بالحرارة والميكروويف والموجات فوق الصوتية قبل التحلل الإنزيمي أضعفت من القدرة الانتيجنية له. كما اظهرت النتائج أيضاً أن المعاملة الأولوية بالميكروويف هى الأكثر فاعلية للحد من القدرة الانتيجينية لبروتين β-lactoglobulin.