POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

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Pol. J. Food Nutr. Sci.
2007, Vol. 57, No. 4(C), pp. 495-498

CHANGES IN COLOUR OF EXTRA-VIRGIN OLIVE OIL DURING STORAGE

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Key words: olive oil, colour, carotenoids, chlorophylls

The changes in colour parameters were studied for an extra-virgin olive oil stored for a period of one year in darkness and exposed to light. Colour parameters were found to correlate with the content of carotenoids and chlorophylls and may thus be used for oil quality control and fast quantitative analysis of the oil chromophores.

INTRODUCTION

Colour, flavour and texture are key factors in food acceptability. Additionally, colour may be used to evaluate composition and chemical changes in foodstuffs, being one of the indicators of product quality [Garcia & Yousfi, 2005; Maskan, 2003; Sinnecker *et al.*, 2002].

Extra-virgin olive oil is susceptible to oxidation, as all the vegetable oils. Oxidation is the predominant cause of oil quality deterioration during storage [Morello *et al.*, 2004]. These oxidative reactions result in a partial loss of minor constituents, primary cause of the health-promoting effects of oil consumption [Chen & Huang, 1998; Manna *et al.*, 1997; Psomiadou & Tsimidou, 2002a, b; Visoli & Galli, 1998]. The oxidation of olive oils leads to the degradation of natural pigments responsible for the oil colour, including carotenoids and pheophytins [Morello *et al.*, 2004]. Oxidation products absorbing in the visible spectral range may also contribute to colour changes. Therefore, the olive oil colour may become a critical tool in quality evaluation [Bilancia *et al.*, 2006; Ceballos *et al.*, 2003].

Colour is measured quantitatively using such equipment as spectrophotometers or tristimulus colorimeters. Usually the colour is quantified using the units proposed by the Commission International d'Eclairage (CIE). The colours are thus defined by their tristimulus values (X, Y, and Z), which may be used to calculate the respective derivative values, L*, a*, and b*colour space coordinates [Calvo, 2004]. The position of a colour in the CIELAB colour space is defined in a three-dimensional Cartesian coordinate system. The lightness value (L*) indicates how light or dark a colour is. The a* and b* values indicate the locations along the respective red-green and yellow-blue axes. The CIELAB space offers means for calculating two parameters directly related to the visually perceived colour attributes, namely, the hue angle h°, providing a quantitative measure of the hue; and chroma C, providing a quantitative measure of the colour saturation.

Our present objective was to evaluate the colour changes of an olive oil during storage. Additionally, we studied the relations between the pigment contents and the colour parameters of the same olive oil.

MATERIAL AND METHODS

Materials and sampling. We used extra-virgin olive oil from the *Coratina* cultivar, obtained in the crop season of 2001-2002 on a traditional plant using a stone mill and a hydraulic press. The oil was hermetically sealed in clear glass bottles, 150 mL each. The bottles were randomly divided into two sets. The first set was stored in darkness in a closed box, while the second one was stored under diffuse lighting, simulating the conditions of a supermarket shelf. The average temperatures were 15° and 25°C during winter and summer, respectively. The samples stored under light were irradiated at 1000 lux, ten hours per day. The samples were withdrawn from storage at fixed times: fresh (0 months) and after 1, 2, 4, 6, 8, 10, and 12 months of storage in the conditions described. Once withdrawn from storage and prior to the analysis, the samples were kept in darkness at -20°C. A single bottle was analysed for each of the storage times, and each analysis was performed with two replicates.

Analytical determinations. Chlorophyll concentration was calculated from the absorption spectrum of each oil sample dissolved in hexane, according to the AOCS method

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[AOCS, 1996], and expressed as mg of pheophytin "a" per kg of oil. The concentration of total carotenoids was evaluated by measuring the absorption at 449 nm of 0.25 g of oil dissolved in 5 mL of UV-quality hexane, using a calibration curve previously obtained with solutions of pure β-carotene. UV spectrophotometry was performed, using a Cary 5E spectrophotometer (Varian).

Colour measurements. Colour measurements were made on a Cary 5E spectrophotometer (Varian) in a glass cell with 0.5 cm optical path length. Transmittance measurements were made over the 380–780 nm range, at 120 nm/min scan speed. Colour parameters were calculated from the transmittance spectrum of each sample using the standard illuminant D65 and 10° observation angle. Final values for each sample were obtained by averaging over the three replicates analysed.

L*, a* and b* colour parameters were calculated in the CIELAB system, with L* indicating lightness, a* indicating hue on the green (-) to red (+) axis, b* indicating hue on the blue (-) to yellow (+) axis. Next, colour difference (ΔE) was calculated as $\Delta E = [(L_0\text{-}L)^2+(a_0^*\text{-}a^*)^2+(b_0^*\text{-}b^*)^2]^{1/2}$, where L_0, a_0^*, and b_0^* are the colour parameters of the fresh oil samples.

RESULTS AND DISCUSSION

Visible absorption spectra of extra-virgin olive oil

Figure 1 shows absorption spectra of an extra-virgin olive oil in n-hexane in the visible range in function of the oil storage period and conditions.

Usually, the absorption in the 450-520 nm range is attributed to carotenoid pigments. The carotenoid band overlaps with that of the chlorophylls at 380-450 nm, while the characteristic band at 650-700 nm belongs exclusively to chlorophylls and pheophytins. No significant changes in the visible absorption spectra were observed after storage in darkness over an entire year. Light, however, was rapidly degrading the olive oil pigments. Even after the first month of storage the intensity of the chlorophyll band decreased to less than half of its initial value; with this band eventually disappearing completely. In contrast, carotenoids were significantly more stable, although their absorption was partially reduced during storage. The absorption changes at specific wavelengths characteristic to carotenoids and chlorophylls were used to calculate the concentrations of these chromophores during storage.

Colour parameters

The colour of oils depends on the wavelengths of transmitted visible light, and is chiefly influenced by two groups of minor oil constituents, carotenoids and chlorophylls. The degradation of these constituents during storage obviously affects the oil colour. To evaluate such changes quantitatively, we have calculated the CIELAB colour parameters from the recorded transmission spectra of the olive oil samples. Table 1 shows the calculated colour parameters at different storage times and storage conditions.

Note that the colour is quite stable for the oil stored in darkness, as one would expect from visual inspection of the respective absorption spectra. On the other hand, the colour parameters of the samples stored under visible irradiation change continuously and almost monotonously during the storage period. The value of a* increases very slightly, whereas that of b* decreases in a much more pronounced way, which corresponds to a shift from green to red, and from yellow to blue, respectively. The value of lightness L* increases during storage, as the oil becomes more transparent as an effect of degradation of the oil chromophores. Finally, the colour difference ΔE was calculated between fresh extra-virgin olive oil and stored samples. The results are shown in Figure 2, in function of storage time and conditions. ΔE is a measure of distance between two colours in the colour space; it provides no indication of the direction in which the colours differ, evaluating only the amount of change.

The ΔE values are very low for the oil stored in darkness, indicating good colour stability in these storage conditions. On the other hand, the samples exposed to light exhibit pronounced changes in their colour, which are larger in the initial period of storage and tend to achieve a constant value after 10

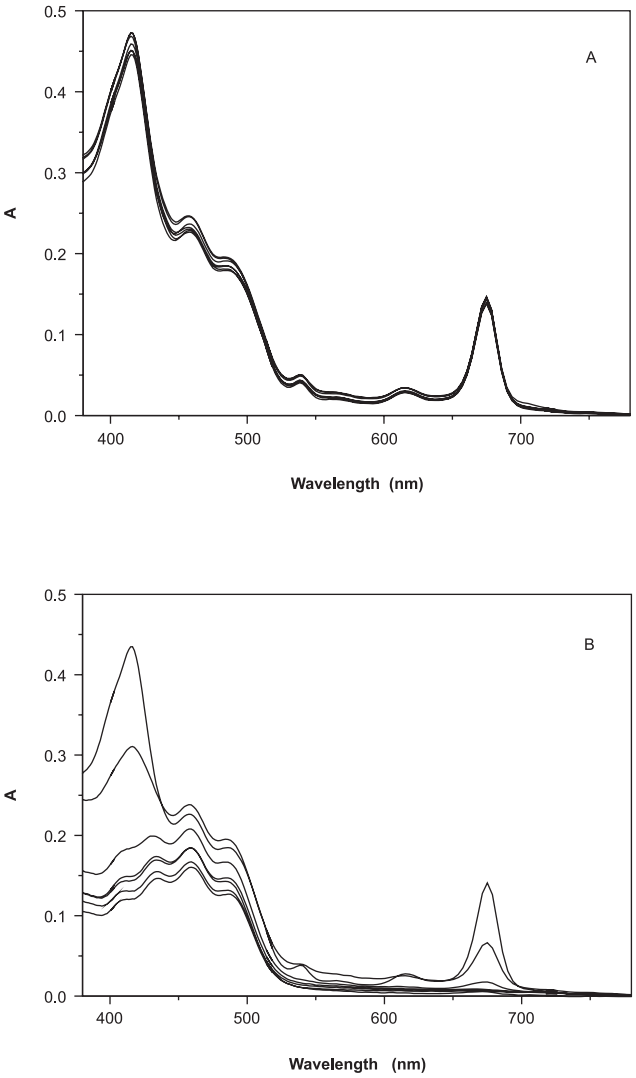

FIGURE 1. Evolution of the visible absorption spectra of extra-virgin olive oil during storage: A. oil stored protected from light, B. oil stored exposed to light.

TABLE 1. Evolution of the colour parameters of extra-virgin olive oil during storage.

Storage time (months) and conditions	L*	a*	b*
Stored in darkness			
0	94.0	-3.7	55.1
1	94.1	-3.5	56.1
2	94.5	-3.8	55.8
4	93.7	-3.8	56.4
6	94.2	-3.9	56.4
10	93.3	-3.6	55.5
12	94.4	-3.9	54.6
Stored under light			
0	94.0	-3.7	55.1
1	95.5	-3.0	52.1
2	97.3	-2.7	48.6
4	97.3	-3.0	44.7
6	98.0	-2.7	42.3
10	98.4	-2.7	39.3
12	98.2	-2.7	39.5

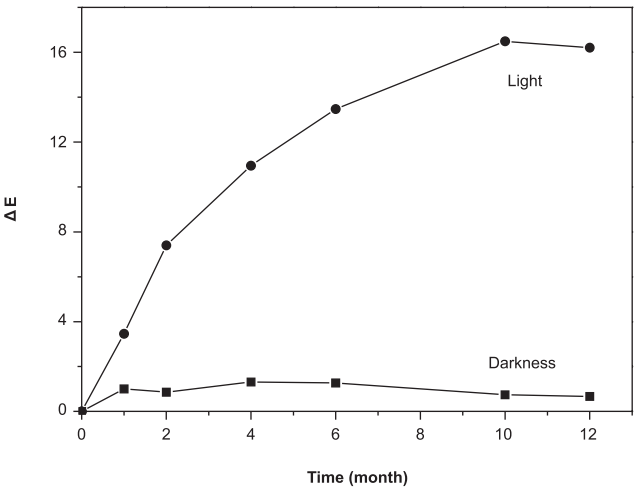

FIGURE 2. Evolution of the colour difference (ΔE) between fresh extra-virgin olive oil and stored samples with the storage time.

months. The visual threshold allowing an average observer to note the colour difference is at least 3 CIELAB units [Ceballos *et al.*, 2003]. According to this, the changes in colour of an olive oil stored under diffuse light become noticeable already after the first month of storage. Such rapid initial colour changes during storage are mainly caused by fast photodegradation of the minor oil constituents of the chlorophyll group. Ceballos *et al.* [2003] studied recently changes in chlorophyll and carotenoid indexes and CIELAB colour parameters of virgin olive oil from various varieties of oil fruits, subjected to an accelerated oxidation process. They found that the changes in olive oil colour depended on olives varieties and could become noticeable ($\Delta E > 3$) after about 1 month of storage at ambient temperature for most of the samples studied.

TABLE 2. Correlation coefficients between the concentrations of chlorophylls and carotenoids and the colour parameters.

Component	a*	b*	L*	ΔE
Chlorophylls (mg/kg)	-0.960	0.936	-0.975	-0.929
Carotenoids (mg/kg)	-0.547*	0.810	-0.639	-0.785

*insignificant correlation at $p<0.05$

The relation between colour parameters and chromophore concentrations

Regression analysis was performed in order to establish a correlation between colour parameters and concentrations of individual pigments in extra-virgin oil samples. We analysed the correlation between each of the colour parameters, and chlorophyll and carotenoid concentrations. The regression coefficients for all of the tested pairs of parameters are shown in Table 2.

Higher regression coefficients were obtained for the correlations between the colour parameters and the chlorophyll concentration. The best correlations were found for the L* and a* values. Considerably lower regression coefficients were obtained for the carotenoid concentration, due to their larger stability and significantly lower degradation rates. The best correlation for carotenoids was obtained with the b* value, no significant correlation was found in turn with the a* value.

CONCLUSIONS

We demonstrated that objective instrumental measurements of olive oil colour allow to quantitatively analyse chlorophylls and carotenoids in these complex foodstuffs. This information immediately translates into oil quality and may be used for fast quality control. Thus, colorimetry is a potentially much cheaper and faster alternative to quantitative analytic determinations involving chromatographic separations in oil quality control and quantitative analysis of certain minor components.

ACKNOWLEDGEMENTS

The grant No. 2P06T 112 26 (2004-2006) from the Polish State Committee for Scientific Research is gratefully acknowledged.

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ZMIANY BARWY OLIWY Z OLIWEK "EXTRA VIRGIN" W CZASIE PRZECHOWYWANIA

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W pracy przedstawiono wyniki badań zmian barwy oliwy z oliwek „extra virgin" przechowywanej przez okres 12 miesięcy w różnych warunkach: w szklanych bezbarwnych butelkach w ciemności oraz eksponowanych na działanie światła. Wykazano korelację pomiędzy zawartością barwników z grupy chlorofili i karotenoidów i parametrami barwy. Na podstawie uzyskanych wyników można stwierdzić, że pomiar barwy może stanowić prostą i szybką, metodę oceny zmian jakości oliwy z oliwek w czasie przechowywania.

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Pol. J. Food Nutr. Sci.
2007, Vol. 57, No. 4(C), pp. 499-502

RAPID ONE STEP SEPARATION OF PROTEINACEOUS AGENTS OF ANTIMICROBIAL ACTIVITY FROM HEN'S EGG WHITE

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Key words: cystatin, lysozyme, proteinase inhibitors, antimicrobial agents

A single-step method of separation of the mixture of cystatin, lysozyme and trypsin inhibitors from hen's egg white by alcohol extraction is demonstrated. The preparation was purified from the homogenate of egg white diluted threefold with 0.25% w/v NaCl and adjusted to pH 4.0 with 1 mol/L acidic acid, by adding to it ethanol to a final concentration of 30% v/v. The precipitated proteins were removed by centrifugation and the supernatant was subjected to evaporation at 30°C to remove ethanol. The remaining solution was adjusted to pH 7.2 and lyophilized. The obtained preparation contained about 40-80% of initial activity of both lysozyme and cystatin regardless of egg white formulation and 40-60% of trypsin inhibitor activity.

INTRODUCTION

The growing problem of resistance of microorganisms to conventional antibiotics has stimulated the search for new agents of antimicrobial activity. Many pathogens are known to produce an array of virulence factors of which extracellular proteinases are believed to play a crucial role in their pathogenicity [Maeda, 1996]. Therefore, bifunctional peptides with both antimicrobial and proteinase inhibitory activities could be considered as ideal candidates for future use in medicine and food industry.

Hen's egg white is a natural and rich source of proteins of proved and potential nutritional, technological and biomedical interest. Egg white is a mixture of about 40 different proteins of which cystatin, lysozyme and serine proteinase inhibitors are of particular significance [Li-Chan *et al*., 1995].

Cystatins are reversible, tight-binding competitive inhibitors of the papain-like cysteine proteases, *e.g.* cathepsins B, H, L, [Turk *et al*., 2002] exhibiting also antimicrobial activity [Węsierska *et al*., 2005]. Chicken egg white cystatin is a single-chain, nonglycosylated, low-molecular weight protein comprising 116 amino acid residues, having two disulfide bridges [Turk & Bode, 1991]. It exists in two isoelectric forms, namely, unphosphorylated form 1 with p*I* of 6.5 and phosphorylated form 2 with p*I* of 5.6 [Laber *et al*., 1989].

Lysozyme (EC 3.2.1.17) is an antibacterial enzyme widely used in food and pharmaceutical industries [Proctor & Cunningham, 1988]. It is capable of hydrolysing the β-1,4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetyloglukosamine in the peptidoglycan layer of the cell wall of some Gram-positive bacteria, resulting in lysis of microbial cells [Shah, 2000]. It was found however, that the bactericidal spectrum of lysozyme was broader than the bacteriolytic and included also Gram-negative bacteria [Pellegrini *et al*., 1992]. A factor responsible for this feature was isolated from clostripain-digested lysozyme as a pentadecapeptide bearing antimicrobial properties but without muramidase activity [Pellegrini *et al*., 1997]. In spite of that, lysozyme alone is not considered effective against Gram-negative bacteria due to the presence of a lipopolisaccharide layer in the outer membrane, which acts as a permeability barrier for this enzyme to penetrate the cell interior [Elliason & Tatini, 1999]. Due to this, in recent years, the food industry applies a combination of factors to achieve effective food preservation [Węsierska *et al*., 2005, Mecitoglu *et al*., 2006]. Therefore, the aim of this work was to obtain from the chicken egg white of different formulation (fresh, frozen, lyophilized, vacuum dried) a preparation containing several bioactive compounds exhibiting antimicrobial properties, for use as food preservative.

MATERIALS AND METHODS

Fresh eggs were supplied by the Department of Animal Products Technology and Quality Management, Wrocław University of Environmental and Life Sciences. The whole eggs were broken and separated albumen from yolk. The albumen was homogenized for 5 sec at low speed with Warring Commercial Blendor and used immediately for separation of cystatin-lysozyme-trypsin-inhibitors preparation (CLTI).

Sterilized and unsterilized vacuum dried egg white was supplied by Ovopol, Nowa Sól Poland.

Protein content was determined either by the bicinchonic acid method [Smith *et al*., 1985] or spectrophotometrically

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according to Whitaker & Granum [1980]. SDS-PAGE was carried out under reducing conditions according to Laemmli [1970]. The low molecular weight range marker calibration kit was used as a reference. Staining was done with 0.1% w/v Coomassie Brilliant Blue G 250 (Sigma-Aldrich Co., St. Louis, MO, U.S.A.)

Papain and trypsin inhibitors activities were measured spectrophotometrically using chromogenic turn-over substrates *N*-α-benzoyl-DL-Arg-β-naphtylamide (BANA) for papain [Barrett, 1977] and *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BApNA) for trypsin [Erlanger *et al*., 1961].

Papain (0.4 μg) was incubated for 10 min at 37°C in 200 mmol/L phosphate buffer, pH 6.4, containing 2 mmol/L EDTA and 10 mmol/L Cys HCl to activate the enzymes. Then the activated proteinase was allowed to react with suitable amount of inhibitor for 10 min in a final volume of 1.0 mL. The residual activity of the enzyme was measured by addition of BANA (1.5×10^{-4} mol/L) and after 20 min incubation the reaction was stopped with 1 mL of 1% DMBA (7,12-dimethylbenzanthracene) in 50% v/v acetic acid and released naphtylamine was measured at 450 nm.

Trypsin (2.0×10^{-7} mol/L) was allowed to complex with a suitable amount of inhibitor in 50 mmol/L Tris-HCl, 20 mmol/L $CaCl_2$, 0.005% Triton X-100 buffer, pH 8.3 at 22°C in a final volume of 1 mL. The residual enzyme activity was measured by addition of BApNA (2×10^{-4} mol/L) and after 20 min incubation the reaction was stopped with 50 μL of glacial acidic acid and the release 4-nitroaniline was measured at 412 nm.

The inhibitory activity was expressed in units (u). One unit of antipapain activity corresponded to that amount of inhibitor which quenched activity of papain capable of hydrolyzing 1.0 mmol of substrate per min under the above conditions. One unit of antitrypsin activity was defined as the amount of inhibitor which reduced by half the activity of 2 μg of trypsin.

Lysozyme activity was determined turbidimetrically by measuring the decrease in absorbance at 600 nm of a suspension of *Micrococcus lysodeikticus* lyophilized cells according to Weisner [1984] with small modification. The reaction was carried out at 25°C for 6 min and measurements were taken every 30 sec. One unit of enzyme activity was defined as the amount of lysozyme which yielded decrease in absorbance A_{600} by 0.01 per min.

RESULTS AND DISCUSSION

Nowadays strategies in reduction of pathogenic and spoilage bacteria tend to eliminate or at least to diminish the dosage of traditionally used preservatives such as *e.g.* salts of lactic or acetic acid in food storage. To attain this goal however, safer and more effectively acting bactericidal substances have to be used instead. Bifunctional peptides exhibiting both antimicrobial and antiproteinase activities in combination with lysozyme and other natural products derived from nature could be considered as ideal candidates for future use. However, largely due to their high cost they have a very restricted application for industrial purposes. The objective of the research was to develop a very simple and inexpensive method of separation from egg whites a bactericidal preparation consisting mainly of bioactive compounds known for their antimicrobial properties, namely cystain, lysozyme and serine proteinase inhibitors. The study was conducted using different formulations of egg white i.e fresh and vacuum dried with and without sterilization.

According to this new protocol the liquid albumen, upon homogenization, was diluted threefold with 0.25% w/v NaCl. In case of vacuum dried egg white the powder was first rehydrated with distilled water at ratio of 1:5 (w/v) followed by dilution with saline solution as mentioned above. The solution was adjusted to pH 4.0 with 1 mol/L acidic acid, and then an equal volume of 60% v/v ethanol was added, while stirring, to a final concentration of 30% v/v. The mixture was left at ambient temperature for 3.5 h. The precipitated proteins were removed by centrifugation at 1 500 ×*g* for 30 min (4°C) and the supernatant was subjected to evaporation under reduced pressure at 30°C to get free of ethanol. The remaining solution was adjusted to pH 7.2 and lyophilized.

As shown in Table 1 after adding ethanol to the material (pH 4.0) up to the final concentration of 30% v/v about 90% of egg white protein, irrespective of formulation, precipitated. The resulting supernatant contained about 40-80% of initial activity of both lysozyme and cystatin regardless of egg white formulation and 40-60% of trypsin inhibitor activity determined only in fresh albumen. The specific activity of preparations obtained from fresh albumen increased against papain trypsin and lysozyme about 8, 10 and 7-fold respectively, as compared to

TABLE 1. Cystatin, lysozyme and trypsin inhibitors activity in the preparations obtained from 5 hen's eggs of different albumin formulation*.

Activity	Formulation of albumen	Protein[3] (mg)	Activity (u)	Specific activity (u/mg)	Purification factor	Yield (%)
cystatin	Fresh	742.0	14 940	20.1	10.6	71.2
	Dried[1]	950.0	9 575	10.0	3.8	37.0
	Dried[2]	870.0	7 560	8.7	4.8	51.0
lysozyme	Fresh	742.0	102 600	138.0	7.8	54.4
	Dried[1]	950.0	320 000	337.0	9.0	89.0
	Dried[2]	870.0	228 000	262.0	7.9	84.0
trypsin inhibitors	Fresh	862.5	1 875 000	2 174.0	6.6	44.0
	Dried[1]	nd	nd	nd	nd	nd
	Dried[2]	nd	nd	nd	nd	nd

*data refer to average values from different preparations; [1]pasteurised; [2]unpasteurised; [3]determined spectrophotometrically; nd - not determined

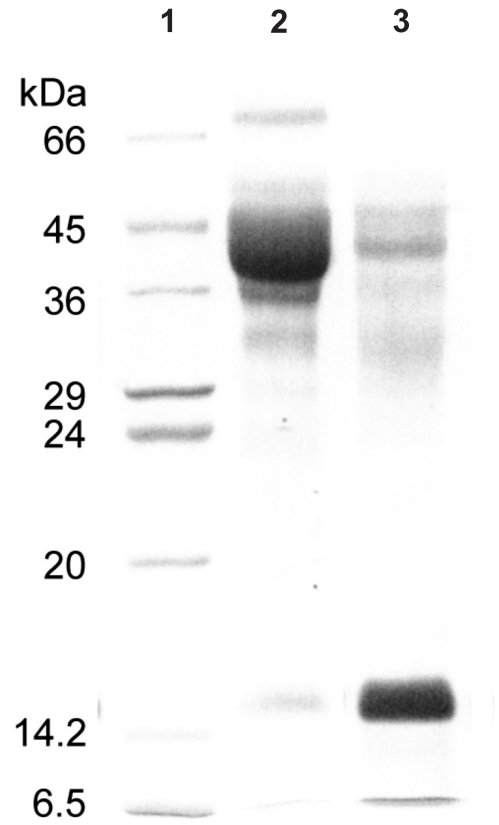

FIGURE 1. SDS-PAGE analysis of the antimicrobial preparation obtained from hen's egg white. Line 1 – protein standard, lane 2 – starting material, lane 3 – cystatin-lysozyme-trypsin inhibitor preparation (10 μg of protein / line was applied).

the starting material (Table 1). Electrophoresis of the preparation under denatured conditions (Figure 1) revealed the presence of one major and a few minor bands in the molecular weight range of approximately 14 kDa, 30 kDa and about 45 kDa. The most intense band of about 14 kDa certainly corresponds to cystatin (14 kDa) and lysozyme (14.4 kDa).

It is worth noting that the storage of eggs up to 4 weeks at 15°C causes decreasing the activity of serine proteinase inhibitor, lysozyme and cystatin by approximately 50, 10 and almost 100%, respectively [Kopeć *et al*., 2005]. Activity of the same compounds in the preparation stored at 0-4°C remained almost unchanged for several weeks.

CONCLUSIONS

1. Fractionation of egg white homogenate (pH 4.0) with 30% v/v ethanol yielded a preparation of bactericidal properties consisting of cystatin, lysozyme and serine protease inhibitors concentrated about 10 times as compare to the initial material.

2. Since the product could be obtained quite inexpensively by means of a very simple procedure in a reasonable yield, therefore it would be widely applicable as a food preservative.

3. The activity of all active components in the preparation was found to be more stable than in stored albumen, therefore, it seems to be a promising step in purification of bioactive compounds.

ACKNOWLEDGEMENTS

This work was financially supported by the Ministry of Science and High Education (State Committee for Scientific Research), project No R 1201401

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SZYBKA, JEDNOETAPOWA METODA WYDZIELANIA BIAŁEK O AKTYWNOŚCI ANTYBAKTERYJNEJ Z BIAŁKA JAJA KURZEGO

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W pracy opisano jednoetapową metodę otrzymywania preparatu zawierającego cystatynę, lizozym i inhibitory trypsyny z białka jaja kurzego. Do trzykrotnie rozcieńczonego 0,25% w/v roztworem NaCl białka o pH 4,0 dodawano równą objętość 60% v/v etanolu o temperaturze pokojowej. Powstały osad usuwano przez wirowanie a supernatant odparowywano pod próżnią w 30°C celem pozbycia się alkoholu. Pozostały roztwór doprowadzano do pH 7,2 i liofilizowano. Preparat otrzymany ze świeżych białek jaj zawierał 40-80% aktywności wyjściowej lizozymu i cystatyny niezależnie od formy białka i około 60% inhibitorów trypsyny.