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

Agata Sokołowska[1], Agnieszka Kubiak[1], Tadeusz Trziszka[2], Antoni Polanowski[1]

[1]Faculty of Biotechnology, University of Wrocław; [2]Department of Animal Products Technology and Quality Management, Wrocław University of Environmental and Life Sciences

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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

Author's address for correspondence: Agata Sokołowska, Faculty of Biotechnology, University of Wrocław, ul. Tamka 2, 50-157 Wrocław, Poland; tel.: (48 71) 375 28 19; fax: (48 71) 375 26 08; e-mail: agata.sokolowska@biochem.uni.wroc.pl

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

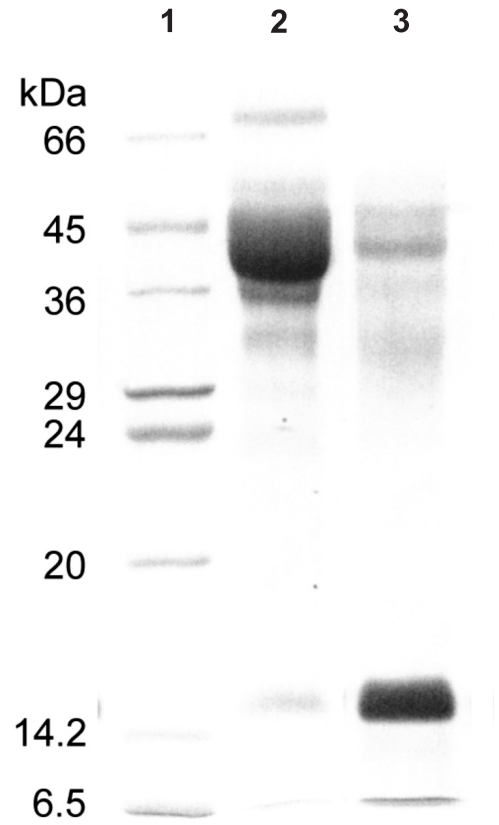

FIGURE 1. SDS-PAGE analysis of the antimicrobial preparation obtained from hen's egg white. Line 1 – protein standard, lane 2 – starting material, lane 3 – cystatin-lysozyme-trypsin inhibitor preparation (10 μg of protein / line was applied).

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.

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REFERENCES

1. Barrett A.J.,Cathepsin B and other thiol proteinases. 1977, *in*: Proteinases in Mammalian Cells and Tissues (ed. A.J. Barrett). Amsterdam: Elsevier/North-Holland Biomedicall Press, Inc., pp. 181-208.
2. Elliason D.J., Tatini S.R., Enhanced inactivation of śalmonella typhimurium and verotoxigenic Escherichia coli by nisin at 658C. Food Microbiol., 1999, 16, 257-267.
3. Erlanger B.F., Kokowsky N., Cohen W.,The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys., 1961, 95, 271-178.
4. Kopeć W., Skiba T., Korzeniowska M., Bobak Ł., Trziszka T., Activity of protease inhibitors and lysozyme of hen's egg white depending on feed modification and egg storage. Pol. J. Food Nutr. Sci., 2005, 14/55, SI 1, 79-83.
5. Laber B., Krieglstein K., Henschen A., Kos J., Turk V., Huber R., Bode W., The cysteine protease inhibitor chicken cystatin is a phosphoprotein. FEBS Lett., 1989, 248, 162-168.
6. Laemmli U.K., Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London), 1970, 227, 680-685.
7. Li-CHan E.C.Y., Powrie W.D., Nakai S., The chemistry of eggs and egg products. 1995, *in*: Egg Science and Technology (eds. W.J. Stableman, O.J. Cotterill). Food Products Press N.Y., London, pp. 105-175.
8. Maeda H., Role of Microbial proteases in pathogenesis. Microbiol. Immunol., 1996, 40, 685-699.
9. Mecitoglu C., Yemenicioglu A., Arslanoglu A., Elmaci Z.S., Korel F., Çetin A.E., Incorporation of partially purified hen egg white lysozyme into zein films for antimicrobial food packaging. Food Res. Int., 2006, 39, 12-21.
10. Pellegrini A., Thomas U., von Fellenberg R., Wild P., Bactericidal activity of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character. J. Appl. Bacteriol., 1992, 72, 180-187.
11. Pellegrini A., Thomas U., Bramaz N., Klauser S., Hunziker P., von Fellenberg R., Identification and isolation of a bactericidal domain in chicken egg white lysozyme. J. Appl. Microbiol., 1997, 82, 372-378.
12. Proctor V.A., Cunningham F.E., The chemistry of lyzosyme and its use as a food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr., 1988, 26, 359-395.
13. Shah N., Effects of milk-derived bioactives: an overview. Br. J. Nutr., 2000, 84, suppl. 1, S3-S10.
14. Smith P.K., Krohn R.I., Hermanson G.T., Mallia A.K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J., Klenk D.C., Measurement of protein using bicinchoninic acid. Anal. Biochem., 1985, 150, 76-85.
15. Turk V., Bode W., The cystatins: protein inhibitors of cysteine proteinases. FEBS Lett., 1991, 285, 213-219.
16. Turk B., Turk D., Salvesen G.S., Regulating cysteine protease activity: essential role of proteinase inhibitors as guardians and

regulators. Curr. Pharm. Des. 2002, 8, 1623-1637.
17. Węsierska E., Saleh Y., Trziszka T., Kopeć W., Siewiński M., Antimicrobial activity of chicken egg white cystatin. World J. Biotechn. Microbiol., 2005, 22, 59-64.
18. Whitaker J.R., Granum P.E., An absolute method for protein determination based on difference in absorbance at 235 and 280. Anal. Biochem., 1980.109, 156-159.
19. Wiesner B. Lysozyme (Muramidase). 1984, *in*: Methods in Enzymatic Analysis, vol. 4 (ed. H.U Bergmeyer), Verlag Chemie, Weiheim, pp. 189-195.

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

Agata Sokołowska[1], Agnieszka Kubiak[1], Tadeusz Trziszka[2], Antoni Polanowski[1]

[1]Wydział Biotechnologii Uniwersytetu Wrocławskiego; [2]Katedra Technologii Produktów Zwierzęcych i Zarządzania Jakością, Uniwersytet PrzyrodniczyWrocław

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.