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Evaluation of the Antibacterial Activity of Cystatin against Selected Strains of *Escherichia coli*

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The aim of this study was to analyze the antibacterial activity of hen egg white cystatin against selected *Escherichia coli* strains. We used a monomeric solution of hen egg white cystatin in bovine serum albumin (BSA) with added phosphate buffered saline (PBS), and three test strains: *Escherichia coli* ATCC 23811, *Escherichia coli* ATCC 8739 and *Escherichia coli* ATCC 25922. The effect of cystatin against the tested strains was determined on the basis of minimal inhibitory concentration (MIC) and survival curves of the microorganisms in a cystatin-containing environment during incubation at various temperatures. Our study confirmed the activity of cystatin against the analyzed *Escherichia coli* strains. taining environment, as compared to control samples incubated in a ovocystatin-deficient medium. Depending on the incubation temperature (20°C or 37°C) the reduction persisted up to 12 hours after incubation.

Key words: Hen egg white cystatin, biopreservatives, hen egg, *Escherichia coli.*

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In recent years, an increasing number of studies have dealt with the discovery and application of natural antimicrobial compounds. Hopes for possible application of biocompounds are mostly associated with the food and cosmetic industries and an array of medical disciplines. It is the food industry which shows particular demand for innovative solutions related to the microbiological safety of food. This is mostly associated with the processes of food preservation and growing expectations of consumers. Traditional methods of preserving food result in many unfavorable changes in food products. Exposure to high temperature causes loss of some nutrients, denaturation of proteins, and deterioration of organoleptic parameters. Freezing is also associated with decreased nutritional value of food, especially during long-term storage (HOLZAPFEL *et al.* 1995). In contrast, consumers expect natural, unprocessed, preservative-free food products with the longest possible shelf life. Consequently, manufacturers, technologists, and microbiologists of food focus on searching for novel solutions related to food safety.

Biopreservatives are chemical compounds originating from plant and animal tissues, and microorganisms, as well as their metabolites. The underlying mechanism of natural preservatives pertains to the inhibition of bacterial proliferation and some enzymatic reactions responsible for unfavorable changes in food (GWIAZDOWSKA & TROJANOWSKA 2005; MENDEZ *et al.* 2012; PRADEEP 2012; SINGH *et al.* 2010; GOULD 1996). The rationale behind using natural food preservatives is to obtain products characterized by high, unchanged nutritional value, simultaneously being free of any harmful chemicals and microbiologically safe. Furthermore, natural biocompounds are used in order to inhibit unfavorable changes in color, texture, and taste of food, as well as to improve other organoleptic parameters.

Hen eggs have raised the interest of researchers as a source of potential antimicrobial compounds. Due to wide availability of this raw material, possibility of extraction, and good solubility, egg proteins are well characterized (GOŁĄB & WARWAS 2005). In view of the wide spectrum of their biological effects, egg proteins are studied in the context of their potential therapeutic application. Egg proteins are characterized by antimicrobial and antineoplastic activity, and show an immunomodulatory effect (SAXENA 1997; GOŁĄB & WARWAS 2005; STEVENS 1996). Therefore, hen eggs are considered an important source of bioactive compounds with potential nutraceutic and health-related applications.

Cystatin is prevalent in human and animal cells, as well as in plants (fitocystatins). It is also present in hen eggs, both in white and yolk as ovocystatin. Egg white cystatin is an inhibitor of cysteine proteases. It was described by Fossum and Whitaker in 1968 for the first time. It represents the first and best understood inhibitor of cysteine proteases belonging to the papain family. Cystatin corresponds to only 0.05% of egg contents. It shows strong inhibitory activity against bacterial enzymes degrading the egg contents. The principal function of cystatin pertains to protecting eggs against bacterial and viral cysteine peptidases (WĘSIERSKA *et al.* 2005; ABDOU *et al.* 2007; BRYKNER *et al.* 2011; SALEH *et al.* 2003). Therefore, high hopes are associated with the potential utilization of antimicrobial properties of cystatin in the food industry.

Published reports on the antibacterial activity of hen egg white cystatin against microorganisms responsible for food decay are sparse and inconclusive. The results of previous studies of some active compounds of hen eggs, e.g. lysozyme, point to the possibility of their application as natural food preservatives (MECITOFLU *et al.* 2006; PROCTOR & CUNNINGHAM 1988; YOU *et al.* 2010; CORBO *et al.* 2009). Therefore, the aim of this study was to analyze the effect of cystatin on the *in vitro* growth and proliferation of *Escherichia coli* strains. Moreover, we tried to verify if hen egg white cystatin could be used as an antibacterial compound in humans or food biopreservative.

Material and Methods

The study included biopreparations of cystatin isolated from hen egg white by a modified method based on affinity chromatography in the Department of Pharmaceutical Biochemistry of the Wroclaw Medical University. The mother liquor of egg white cystatin in monomeric form at a concentration of 1000 mg/ml was supplemented with 5% bovine serum albumin (BSA) and the addition of buffered saline solution (PSB). We used the stock monomeric solution of hen egg white cystatin in bovine serum albumin (BSA) with added phosphate buffered saline (PBS), at 1000 μg/ml concentration. The test strains used in the study were obtained from the collection of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wroclaw. The following three test strains were analyzed: *Escherichia coli* ATCC 23811, *E. coli* ATCC 8739, and *E. coli* ATCC 25922. The strains were proliferated in Mueller-Hinton broth incubated at 37°C for 18 hours, centrifuged three times (15 minutes, 3000 rotations per minute) and washed with physiological saline. Subsequently, bacterial cells were suspended in physiological saline and their counts were standardized to obtain concentrations required for analytical procedures. The minimal inhibitory concentration of hen egg white cystatin was determined by the agar dilution method. We prepared Petri dishes with Mueller-Hinton agar with cystatin at concentrations of 80 μg, 100 μg, 120 μg, 150 μg, and 300 μg per ml. Suspensions of bacterial strains of density corresponding to 0.5 McFarland turbidity standard, i.e. approximately 1×10^8 CFU/ml, were applied using the point-by-point method onto previously prepared Petri dishes containing various concentrations of ovocystatin. We applied as a spot 2 μl suspension of individual bacterial strains. The material was incubated for 24 hours under aerobic conditions at 37°C. Simultaneously, the control samples, i.e. Petri dishes with bacterial inoculum without the cystatin addition to agar medium, were incubated under the same conditions. The procedures were repeated five times for each test strain. After completing the incubation, we verified the growth of the analyzed test strains in agar media. The dishes without cystatin were considered as a positive control. Minimal inhibitory concentration (MIC) was defined as the minimal concentration of the studied compound causing inhibition of growth or reducing the count of the tested strain (CARSON *et al.* 1995).

In order to determine survival curves of the microorganisms, we standardized the previously prepared suspensions of test strains to approximately 10^4 CFU per ml. We added 1 ml of stock cystatin solution (concentration 1000 μg/ml) and 0.1 ml of previously prepared bacterial suspension to 8.9 ml of Mueller-Hinton liquid medium. The control samples were prepared according to a similar protocol, with 0.1 ml of bacterial cell suspension (concentration 10^4 CFU/ml) added to 9.9 ml of Mueller-Hinton liquid medium. Samples were incubated at 20°C and 37°C, with intermittent determination of bacterial counts at time 0, i.e. immediately after the inoculation, and following 2, 4, 8, 12, and 24 hours of incubation at each tem-

Table 1

Minimal inhibitory concentration (MIC) of cystatin monomer against *Escherichia coli* ATCC 23811

Sample no.	Biopreparate addition ($\mu g \times ml^{-1}$)				
	80	100	120	150	300
1	+	–	–	–	–
2	+	–	–	–	–
3	+	–	–	–	–
4	+	–	–	–	–
5	+	–	–	–	–
control	+	+	+	+	+

+ macroscopic growth

perature. The survivors were recovered in *Chromocult® Coliform Agar* selective medium (Merck), and their counts were determined using the most probable number (MPN) plate method (BURBIANKA *et al*. 1983). The procedures were repeated seven times for each test strain.

Results and Discussion

The literature on the antimicrobial activity of ovocystatin is sparse and inconclusive, mostly due to the variability of research methods. This variability, along with the paucity of previous studies dealing with this issue, and heterogeneity of analyzed material and results, hinder a comparison of our findings with data published by other authors.

Our experiments determined the minimal inhibitory concentration (MIC) of the cystatin solution which equaled 100 μg per ml for all studied *Escherichia coli* strains. We did not observe any strain-specific differences in the inhibitory effect of cystatin monomer against *E. coli.* Moreover, as expected, our study revealed that the solution of hen egg white cystatin efficiently inhibited growth and proliferation of the incubated bacterial test strains. This was reflected by survival curves of studied bacteria exposed to the biopreparation at 20°C and 37°C (Table 1, Figs 1-6).

WĘSIERSKA *et al*. (2005) analyzed the antimicrobial activity of ovocystatin against selected bacterial strains using the disc diffusion method and determining the inhibitory concentration (IC) of the biopreparation. The spectrum of studied microorganisms included such bacteria as *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus.* The inhibition zones of bacterial growth were observed at concentrations of cystatin amounting to 80 μg/disc (for *E. coli* ATCC 25922) and *S. aureus* ATCC 25923 strains) or 120 μg/disc (for *P. aeruginosa* ATCC2785). The use of different experimental methods and low number of studies of hen egg white cystatin as an antibacterial

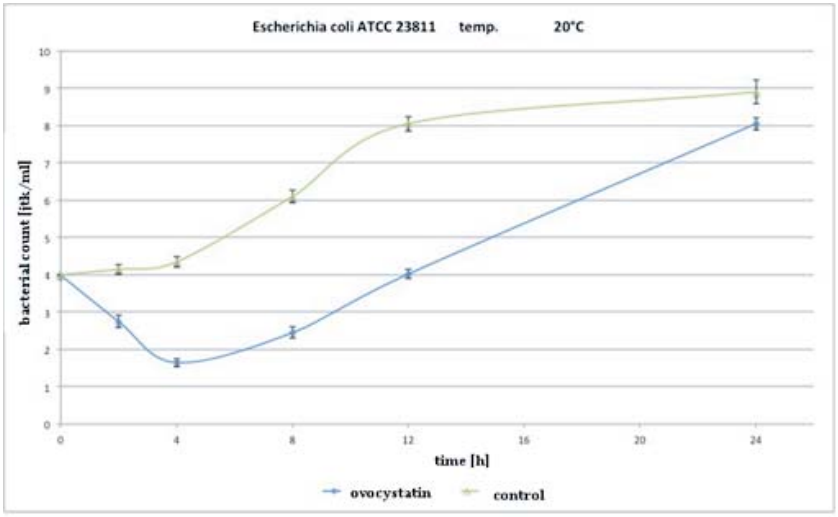

Fig. 1. Survival curve of *Escherichia coli ATCC 23811* strain in Mueller-Hinton medium with the addition of 100 μg of ovocystatin monomer and incubated at 20°C.

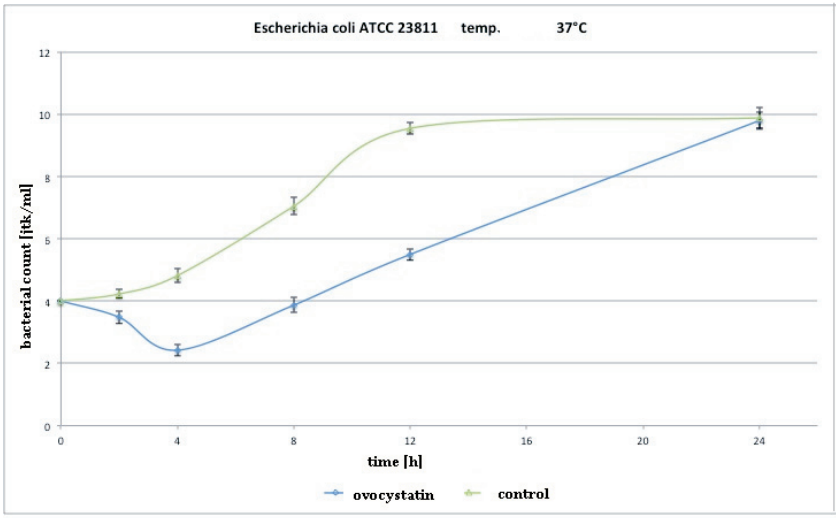

Fig. 2. Survival curve of *Escherichia coli ATCC 23811* strain in Mueller-Hinton medium with the addition of 100 μg of cystatin monomer and incubated at 37°C.

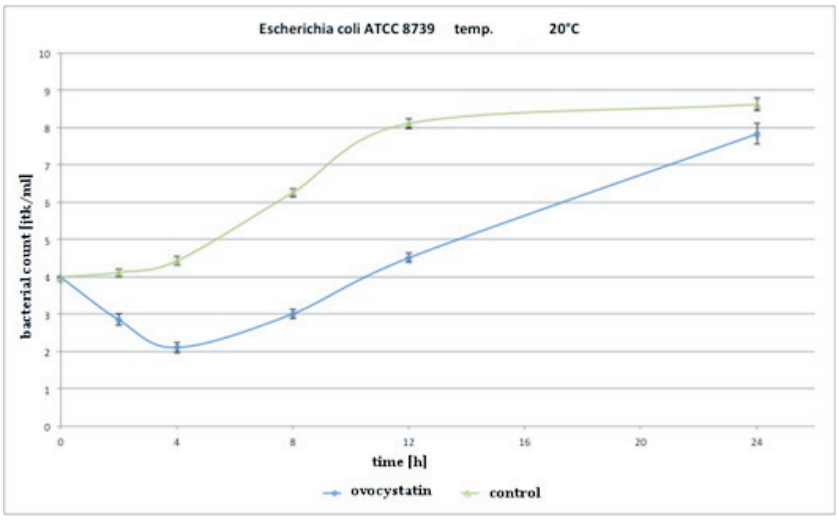

Fig. 3. Survival curve of *Escherichia coli ATCC 8739* strain in Mueller-Hinton medium with the addition of 100 μg of cystatin monomer and incubated at 20°C.

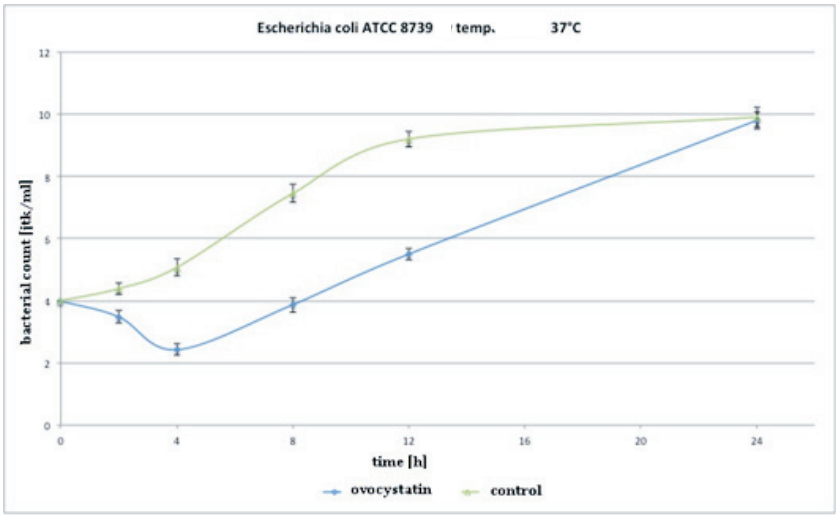

Fig. 4. Survival curve of *Escherichia coli ATCC 8739* strain in Mueller-Hinton medium with the addition of 100 μg of ovocystatin monomer and incubated at 37°C.

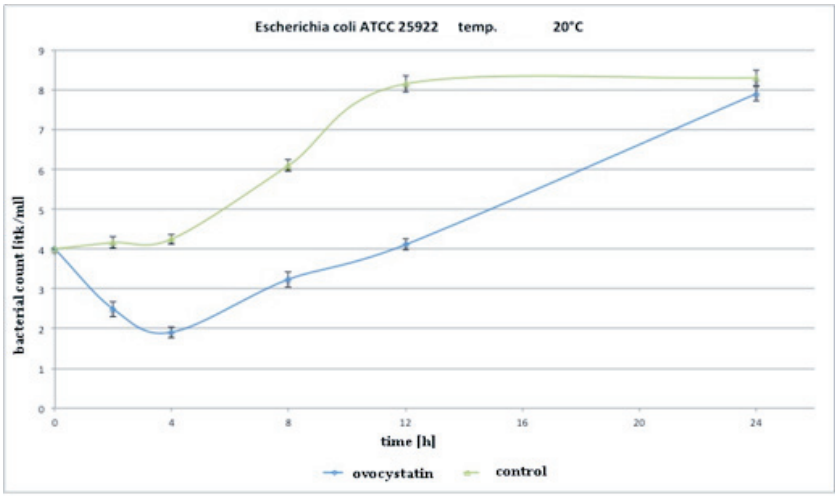

Fig. 5. Survival curve of *Escherichia coli ATCC 25922* strain in Mueller-Hinton medium with the addition of 100 μg of ovocystatin monomer and incubated at 20°C.

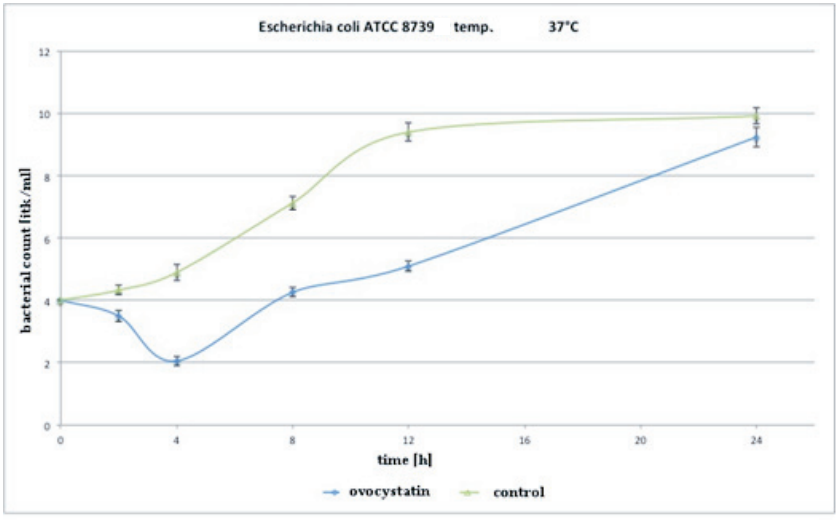

Fig. 6. Survival curve of *Escherichia coli ATCC 25922* strain in Mueller-Hinton medium with the addition of 100 μg of ovocystatin monomer and incubated at 37°C.

compound hinder direct comparison of our findings with those from previously published reports.

Our study confirmed the efficacy of ovocystatin against the analyzed strains of *Escherichia coli.* Our findings, confirming the antibacterial activity of ovocystatin, substantiate further research addressing the possible applications of this substance as a biopreservative in the food industry. Undoubtedly, the duration of the antibacterial effect of ovocystatin, which decreased during longer incubation times of the test strains, is a factor limiting the application of ovocystatin as a biopreservative of foods. However, according to the literature, the antimicrobial effect could be extended due to synergy with other compounds of natural origin. This hypothesis needs to be verified by further research.

Conclusions

The following conclusions can be formulated on the basis of our findings:

1. The monomer of ovocystatin efficiently inhibits the proliferation of the analyzed *E. coli* strains at concentration of 100 μg x ml^{-1} or higher, even under optimal nutritional and thermal conditions for these bacteria.

2. The antibacterial effect of ovocystatin monomer against the analyzed *E. coli* strains is determined by the time and temperature of incubation.

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