Combinations of charged amino acids and glycine as enhancers of the antibacterial properties of chicken lysozyme

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Abstract. It was found that glycine, glutamate, lysine, histidine, and arginine increase the rate of *Escherichia coli* cell lysis by chicken egg lysozyme. The highest cell lysis rates (lysis acceleration by 7.0-8.5 times) are provided by combinations of the following amino acids: 1) arginine, histidine, and lysine, 2) glutamate, histidine, and arginine, 3) glycine, lysine, and arginine (all charged amino acids are applied in concentrations of 5 mM, the concentration of glycine is 1.5 mM). These amino acids added into food products with lysozyme as a preservative can significantly increase antibacterial properties of the latter. This could help increase the shelf life of human food and also serve as a preventive measure against the spread of bacterial infections among farm animals.

1 Introduction

Food industry and agriculture are being rapidly developed all over the world. Growth of population of the Earth forces the mankind to increase livestock population and area of arable land. This extensive solution for the problem of food shortage is limited by natural resources. Concerning this, the problem of food conservation and preventing livestock deaths becomes especially relevant. To extend the shelf life of food and animal feed, people apply various food preservatives. It is widely known that synthetic preservatives cause harm to human health [1]. For instance, sodium nitrite found in ham, sausages, and bacon, can react with proteins and form cancerogenic nitrosamines [2-3]. Sulfur dioxide, which is applied as a preservative in beverage production, may cause asthma in susceptible individuals [4], while sulfite, added into some meat products, causes allergic diseases [5]. Such popular preservative as sodium benzoate can bind to human DNA, what also determines its cancerogenic properties [6]. Due to harm to health, which is induced by regular consumption of products containing cheap synthetic preservatives, application of safe and nutritious natural preservatives appears to be far more preferable. The bacteriolytic enzyme called lysozyme, found in tears, egg white and even phagocytic cells [7-10], is used as a natural preservative in the food industry. Lysozyme is added into food products, such as minced meat, poultry, cheese, smoked fish [11-13], including the way of applying a thin layer of enzyme to the surface of the cling film [14]. The antibacterial activity of lysozyme

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is an advantage compared to the properties of natamycin (which is also applied as a preservative in cheese and sausages), performing antifungal activities, but non-effective against bacteria [15]. Lysozyme is also applied in production of alcoholic beverages, such as wine and beer [16-17]. In agriculture, lysozyme serves as a preservative additive in animal feed, preventing infectious diseases among livestock [18]. Gram-negative enterobacteria, such as Salmonella, Shigella [19] and Yersinia [20], closely related to Escherichia, are especially dangerous to humans and animals. They get into food along with feces, for instance, being transferred on the surface of paws of flies. *Escherichia coli*, applied in the following research, is an indicator of water pollution [21]. Some *E. coli* strains can cause serious food poisoning [22], urinary tract infections, peritonitis [23], meningitis and sepsis [24]. Concerning this, search for remedies against *E. coli* appears to be an important practical problem. The main challenge is caused by bacteria developing mechanisms of resistance to antibacterial factors, including antibiotics and bacteriolytic enzymes, by means of mutational diversity [10, 25]. *E. coli* have also managed to develop resistance to lysozyme (for example, by excreting protein inhibitors for lysozyme) [26], thus it is necessary to find approaches for increasing effectiveness of its enzymatic activity. One of possible ways to achieve this is application of substances increasing bacteriolytic activity of lysozyme, i. e. effectors. Effectors increasing lysozyme activity are various chemical compounds, including surfactants (Tween-21) [27-28] and some peptide antibiotics, for example, bacitracin and polymyxin B [29]. Particularly significant effects of acceleration of cell lysis by lysozyme were discovered in the presence of glycine and charged amino acids [29-30]. It is well known that amino acids themselves are nutraceutical food additives [31-32]. For example, the use of lysine as a nutritional supplement in the diet of premature infants promotes their growth and organism development [33]. Enrichment of food with amino acids increases its nutritional value, and lysozyme-activating properties of amino acids make them promising food additives in food and animal feed containing lysozyme as a preservative. It is important to notice that co-presence of two or three amino acids in optimal conditions for lysozyme activities demonstrates higher increase in cell lysis rate than ones caused by a single amino acid presence [30]. In the following research, we investigate influence of triple combinations of amino acids on *Escherichia coli* cell lysis by chicken egg lysozyme in a biocompatible phosphate buffer solution under conditions close to physiological ones.

2 Materials and methods

Materials applied are: chicken egg lysozyme ("Amresco", USA), NaCl, NaOH, Na_2HPO_4, KH_2PO_4, $MgSO_4$ ("Panreac", Spain), HCl ("Komponent-Reaktiv", Russia), L-arginine ("Merck", Germany), L-lysine, L-histidine ("Serva", Germany), glycine ("Roth", Germany), sodium L-glutamate ("MeiHua", China), glucose ("Rokett Frer", France), yeast extract ("Biospringer", France), bactoagar, tryptone, peptone ("BD Difco", USA).

Escherichia coli strain K-12 (B-3254) was received from All-Russian collection of industrial microorganisms. Bacteria were grown according to standard methods in LB nutritive medium for 16 hours [34]. 1 mL of night culture was added into 100 mL of sterile LB medium and cultivated during 3.5–4 hours until optical absorbance of the suspension equal to 1.2 optical unities is obtained. Then cells were separated from the nutritive medium by centrifugation during 30 minutes at the speed of 6000 rpm (5934 g) and the temperature of 4°C. Cell debris obtained by means of centrifugation was suspended in 12.5 mL of buffer solution containing 4 mM Na_2HPO_4, 5.4 mM KH_2PO_4, 5 mM NaCl, pH 7.0, and distributed into test tubes in 0.5 mL portions, which were subsequently frozen with liquid nitrogen. Suspension obtained had cell concentration equal to 10^9 CFU/mL. Cell

suspensions were defrosted right before the experiment. The pH value of amino acid solutions was adjusted to pH 7.0.

The rate of bacterial cell lysis was determined by the turbidimetric method by measuring the decrease in optical absorbance at a wavelength of 650 nm (A_{650}) over time ($-dA_{650}/dt$) [35, 36]. Decrease of optical absorbance of cell suspension ($v = -dA_{650}/dt$) is proportional to decrease of cell concentration (-dCFU/dt) [35,36]. The measurements were conducted at the wavelength of 650 nm and the temperature of 37 °C on a UV-1800 dual-beam spectrophotometer ("Shimadzu", Japan). The initial absorbance of bacterial cell suspension (A_{650}) was equal to 0.45, which corresponds to the cell concentration of $3 \cdot 10^8$ CFU/mL. After necessary volume of amino acid solutions were added to the cell suspension, background change in optical absorbance was recorded during 3—4 minutes. After this, the enzyme (lysozyme) solution was added up to the final concentration of 0.1 μg/mL of lysozyme in the cuvette, and the decrease in absorbance of the suspension was recorded for 3 minutes more. The rate of background decrease in absorbance did not exceed the value of experimental error of enzymatic cell lysis rate in all the experiments. Amino acids did not affect the absorbance value in absence of lysozyme. Each experimental measurement was repeated at least 4 times. Experimental errors were calculated using the Student distribution for a confidence interval of P = 0.95.

3 Results and Discussion

It is known from literature that the dependence of cell lysis rate on glycine concentration reaches a maximal value at the concentration of the amino acid of 1.5 mM, while charged amino acids cause a monotonic increase of cell lysis rate as the effector concentration increases to 5 mM and above [29, 30]. Figure 1 presents values of *Escherichia* cell lysis rates by chicken egg lysozyme in the simultaneous presence of three amino acids at pH 7.0.

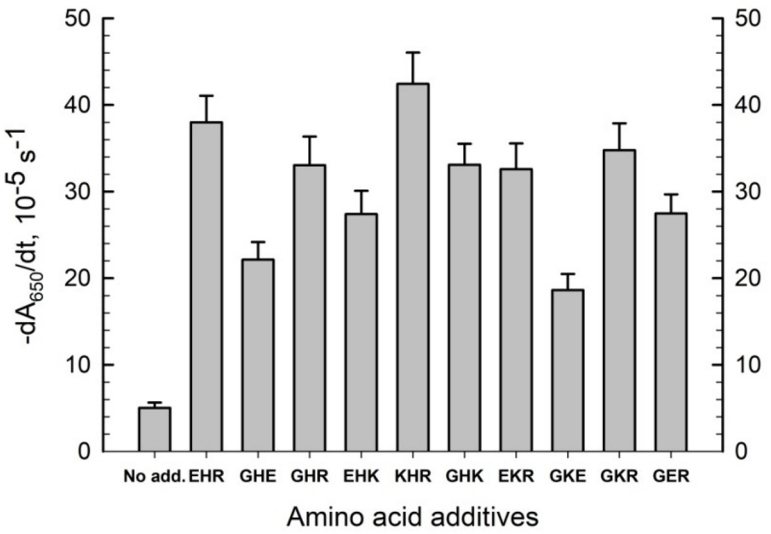

Fig. 1. Values of *E. coli* cell lysis rate by means of chicken egg lysozyme in the presence of mixtures of three amino acids and in the absence of additives (No add.). Amino acids are indicated by one-letter abbreviations (G - glycine, E - glutamate, K - lysine, H - histidine, R - arginine). The buffer solution contains 4 mM Na_2HPO_4, 5.4 mM KH_2PO_4, 5 mM NaCl, pH 7.0. The concentration of Gly is 1.5 mM, concentrations of other amino acids are 5 mM.

We can see that the cell lysis rate in the presence of all the triple combinations of amino acids is several times higher than the one in the absence of additives. Nevertheless, we can distinguish three triple combinations of amino acids, which induce the most significant effects of cell lysis acceleration. In the presence of lysine, histidine and arginine in concentrations of 5 mM of each amino acid the increase of cell lysis rate compared with the cell lysis rate without amino acids reaches 8.5 times. High effectivity is demonstrated by the addition of glutamate, histidine and arginine (increase of cell lysis rate by 7.5 times), and also glycine, lysine and arginine ((increase of cell lysis rate by 7.0 times). We should notice that all three combinations discussed include arginine. Its influence can be explained by interaction with both the enzyme and the cells [29]. I tis very important to mention that activation of lysozyme by charged amino acids is observed not only with lysis of *E. coli* cells. For instance, the rate of lysis of Gram-positive *Priestia megaterium* bacteria cells by lysozyme increases in the presence of glutamate and arginine [28]. *P. megaterium* bacteria inhabit soils, and they can easily get into farm animals feed with soil particles. Amino acids glutamate and arginine, which improve lysis of *P. megaterium,* are present in the mixture also containing histidine (this mixture increases *E. coli* cell lysis rate by 7.5 times). Arginine is also included in mixtures which accelerate *E. coli* cell lysis by 8 and 6.5 times respectively. Addition of the combinations of amino acids considered above into lysozyme-containing animal feed and food products can possibly increase the effectivity of antimicrobial lysozyme activity against Gram-negative Enterobacteria.

4 Conclusion

Amino acid combinations discussed in this research can be applied for the development of additives for animal feed, which can prevent bacterial epizootics and decrease risks of contamination of agricultural products. Application of charged amino acids as part of products for processing eggs before their admission to food production can be prospective in case to prevent spread of salmonellosis. Egg white and other egg-products enriched with combinations of amino acids, used in the baking and confectionery industry, can expand the regulations of technological processes. These compositions can also be added into food industry products in which chicken lysozyme is present as preservative in case to elongate their terms of shelf life.

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References

1. S.C. Mirza, U.K. Asema, S.S. Kasim, Journal of Medicinal Chemistry and Drug Discovery. **2(2)**, 610-616 (2017)
2. M.I. Anon, Food Irradiation – A technique for preserving and improving the safety of Food (WHO, Geneva, 1991)

3. M.M. Theron, J. F. Lues, Food Reviews International. **23**, 141-158 (2007)
4. B.J Freedman, Br. J. Dis. Chest. **74(2)**, 128-34 (1980)
5. T. D'Amore, A.D. Taranto, G. Berardi, V. Vita, G. Marchesani, A.E. Chiaravalle, M. Iammarino, Compr. Rev. Food Sci. Food Saf. **19(5)**, 2701-2720 (2020)
6. G. Zhang, Y. Ma, Food Chem. **141(1**), 41-7 (2013)
7. L. Callewaert, C.W. Michiels, J. Biosci. **35(1)**, 127-160 (2010)
8. E.M. Prager, P. Jollès, EXS. **75**, 9-31 (1996)
9. S. Browning, D. Housley, R. Richards, Acta Oto-Laryngologica. **117(6)**, 851-855 (1997)
10. S.A. Ragland, A.K. Criss, PLoS Pathog. **13(9)**, e1006512 (2017)
11. N. Khorshidian, E. Khanniri, M.R Koushki., S. Sohrabvandi, M. Yousefi, Front Nutr. **9**, 833618 (2022)
12. S. Mangalassary, I. Han, J. Rieck, J. Acton, X. Jiang, B. Sheldon, P. Dawson, J. Food Prot. **70(11)**, 2503-2511 (2007)
13. S. Min, T.R. Rumsey, J.M. Krochta, J. Food Eng. **84(1)**, 39-47 (2008)
14. P. Kaewprachu, K. Osako, S. Benjakul., S. Rawdkuen, Food Packag Shelf Life. **3**, 88-96 (2015)
15. H.M. Atta, Sh.M. S.M.S. Zayed, AJS. **8(2)**, 469-475 (2012)
16. K. Liburdi, I. Benucci, M. Esti, Compr. Rev. Food Sci. Food Saf. **13(5)**, 1062-1073 (2014)
17. T. Silvetti, M. Brasca., R. Lodi, L. Vanoni, F. Chiolerio, M. Groot, A. Bravi, J. Inst. Brew. **116 (1)**, 33-40 (2010)
18. C.M, Nyachoti, E. Kiarie, S.K. Bhandari, G. Zhang, D.O. Krause, J. Anim. Sci. **90(1)**, 252-260 (2012)
19. C. Parsot, FEMS Microbiol. Lett. **252(1)**, 8-11 (2005)
20. J. Jang, H.G. Hur, M.J. Sadowsky, M.N. Byappanahalli, T. Yan, S. Ishii, J. Appl. Microbiol., **123(3)**, 570-581 (2017)
21. D.M. Silva, L. Domingues, Ecotoxicol. Environ. Saf. **113**, 400-411 (2015)
22. J.P. Nataro, J.B. Kaper, Clin. Microbiol. Rev. **11(1)**, 142-201 (1998)
23. D.J. Evans, D.G. Evans, Medical Microbiology. Escherichia Coli in Diarrheal Disease (University of Texas Medical Branch, Galveston, 1996)
24. D. Liu, Encyclopedia of Microbiology (Academic Press, Oxford, 2019)
25. A.V. Grishin, A.S. Karyagina, D.V. Vasina, I.V. Vasina, V.A. Gushchin, V.G. Lunin, Crit. Rev. Microbiol. **46**, 703-726 (2020)
26. L. Vanderkelen, E. Ons, J.M. Van Herreweghe, L. Callewaert, B.M. Goddeeris, C.W. Michiels, PLoS One. **7(9)**, e45954 (2012)
27. W.-J. Lu, S.A. Smirnov, P.A. Levashov, BBRS. **575**, 73-77 (2021)
28. S.V. Iuzhakov, A.Y. Yakimov, N.V. Rastriga, D.A. Gasanova, D.A. Klimov, S.A. Smirnov, P.A. Levashov, Biotech. **39(4)**, 81-87 (2023)
29. P.A. Levashov, D.A. Matolygina, E.D. Ovchinnikova, D.L. Atroshenko, S.S. Savin, N.G. Belogurova, S.A. Smirnov, V.I. Tishkov, A.V. Levashov, Acta Naturae. **9(2)**, 82-87 (2017)
30. N.V. Rastriga, D.A. Klimov, D.A. Gasanova, P.A. Levashov, Process Biochemistry. **125**, 190-197 (2023)

31. M. Alagawany, S.S Elnesr, M.R Farag, R. Tiwari, M.I. Yatoo, K. Karthik, I. Michalak, K. Dhama, Vet. Q. **41(1)**, 1-29 (2020)
32. N. Tonouchi, H. Ito, Adv. Biochem. Eng. Biotechnol. **159**, 3-14 (2017)
33. N.W. Flodin, J. Am. Coll. Nutr. **16(1)**, 7-21 (1997)
34. T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982)
35. P.A. Levashov, S.A. Sedov, S.V. Shipovskov, N.G. Belogurova, A.V. Levashov, Anal. Chem. **82(5)**, 2161-2163 (2010)
36. D.A. Matolygina, N.S. Dushutina, E.D. Ovchinnikova, N.L. Eremeev, N.G. Belogurova, D.L. Atroshenko, S.A., Smirnov, S.S. Savin, V.I. Tishkov, A.V. Levashov, P.A. Levashov, Moscow Univ. Chem. Bull. **73(2)**, 47-52 (2018)