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Resorcinol as Protective Agent in Thermochemical Modification of Lysozyme

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Summary

Thermochemical modification of lysozyme enables it to retain its oligomers, showing a wide spectrum of increased antibacterial activities. It also results in significant loss of hydrolytic activity of the enzyme. The objective of this study is to analyse the protective effect of resorcinol on the hydrolytic activity of modified lysozyme using a high temperature method and the oligomerization degree in the obtained preparations. Resorcinol significantly affects the enzyme's hydrolytic activity. Samples containing resorcinol, without oxidizing agent, modified at 90 °C retained 75 % enzymatic activity of unmodified monomer, obtaining the final value of about 16 000 U/mg. The amount of lysozyme oligomers increased slightly in the samples produced under such conditions.

Key words: lysozyme, resorcinol, modification, oligomers, hydrolytic activity

Introduction

Lysozyme (*N*-acetylmuramide glycanhydrolase, EC 3.2.1.17) is a small hydrolytic enzyme, which functions by breaking specific bonds between polysaccharides that form the cellular wall of bacteria. Its catalytic properties allow a disintegration of the β-1,4-glycosidic bond between the *N*-acetylmuramic acid and *N*-acetylglucosamine in the structure of the polysaccharide-peptide complex. The antibacterial properties of lysozyme are highly interesting for its practical applications. The enzyme isolated from egg white is currently widely used in food industry as a biopreservative of food products and also in medicine and veterinary medicine in treating numerous diseases of bacterial and viral origin (*1*).

Naturally occurring lysozyme is mainly monomeric showing biocidal properties against Gram-positive bacteria. It was observed that under certain environmental conditions, the enzyme is able to form a dimer as well as higher oligomers, while at the same time obtaining new, valuable properties. The oligomeric form of lysozyme is characterized by increased antimicrobial activity, *e.g.* against Gram-negative bacteria and other strains that were so far resistant to the monomeric form. These properties provide for significantly broader and more practical application of the modified enzyme (*2–6*).

Lysozyme oligomers exhibit a new, but not completely defined action. Recent reports suggest that this unique antimicrobial activity of unfolded lysozyme is attributed to membrane binding and the subsequent perturbation of its functions (*7,8*). Probably, the interaction of lysozyme oligomers with the membrane of Gram-negative bacteria is caused by increased surface hydrophobicity of the modified enzyme.

There are some solutions to increase the oligomerization degree of enzymes, including *e.g.* aldehyde-dextran intermolecular cross-linking (*9,10*). Among the known modifications, lysozyme oligomerization was obtained as a result of applying the high temperature method using oxidants as an additional modifier besides temperature, which we described earlier (*3,4,11*). However, this modification resulted in a significant decrease of the enzyme

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hydrolytic activity in the obtained preparations, compared to the monomeric form not subjected to the process.

There is a paper showing how a proper immobilization may greatly improve the enzyme features (*12*). It may be used to improve enzyme performance by improving some enzyme limitations, *i.e.* enzyme purity, stability, specificity, selectivity, or activity. A recent study showed that some phenols, particularly alkylhydroxybenzenes like resorcinol, are good regulators of enzyme activity (*13,14*). It was proved that these compounds, due to their chemical structure and type of interactions with the protein molecule, alter the enzymatic activity of lysozyme, as well as the efficiency of hydrolysis of peptidoglycan, a substrate of the enzyme. This short communication presents first results and observations of extensive research about resorcinol as a protective substance used in thermal modification of lysozyme.

Materials and Methods

The experimental material was lysozyme monomer isolated from chicken egg white of hydrolytic activity of 21 252 U/mg produced by the Belovo company (Bastogne, Belgium). For modification, lysozyme was prepared as a 5 % (by mass per volume) water solution at pH=4.0±0.2. The modification was carried out for 30 min at 90 and 95 °C in the Syncore Analyst reactor (Büchi, Flawil, Switzerland) with the addition of: 0.03 % (by mass per volume) of resorcinol and 2.0 % of H_2O_2 (by volume) (both produced by Sigma-Aldrich, St. Louis, MO, USA) (sample no. 2), 0.03 % (by mass per volume) of resorcinol (sample no. 3), or without any additives (sample no. 1). Then the obtained preparations were freeze-dried (GT3 Leybold-Heraeus lyophilizer, Cologne, Germany) and subjected to analytical examination.

Lysozyme hydrolytic activity was determined with the use of spectrophotometric method, the principle of which is based on the phenomenon of bacterial cell wall lysis by the enzyme (*15,16*). The lytic activity of lysozyme was determined by monitoring the decrease in turbidity of a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich) cells at 450 nm. The activity was presented as the rate of absorbance decrease per minute ($\Delta A_{450\,nm}$/min).

The lysozyme polymeric forms in the preparations after modification were determined by electrophoretic analysis in polyacrylamide gel using the SE-600 apparatus (Hoefer Scientific Instruments, Holliston, MA, USA). Polyacrylamide gel electrophoresis was carried out according to Laemmli (*17*) and Lesnierowski (*18*) using 6 % stacking gels at a current of 60 mA and 12.5 % separating gels at 90 mA. The gels were stained with 0.025 % Coomassie Brilliant Blue R solution. As molecular mass standards, the following were used (in kDa): lysozyme 14.3 (Sigma-Aldrich), Lydium-KLP 28 (Nika Health Products, Smigiel, Poland) and hen albumin 45 (Sigma-Aldrich).

The amounts of polymers in individual samples were calculated with the application of the TotalLab Quant software (Nonlinear Dynamics Ltd., Durham, NC, USA).

The obtained results of the determination were subjected to statistical analysis using STATISTICA software (*19*).

Results and Discussion

The presented study showed that the modifications carried out gave a new quality to the lysozyme. The electrophoretic analysis revealed that each preparation obtained after modification contained new forms of lysozyme (Fig. 1). Apart from the monomer, we found certain amounts of dimer, trimer and also tetramer of the enzyme. Based on densitometric analysis, the exact amount of the individual oligomeric forms of lysozyme was determined (Tables 1 and 2).

We observed that the amount of obtained oligomers is dependent on the temperature of the enzyme modification process. Without the use of chemical additives,

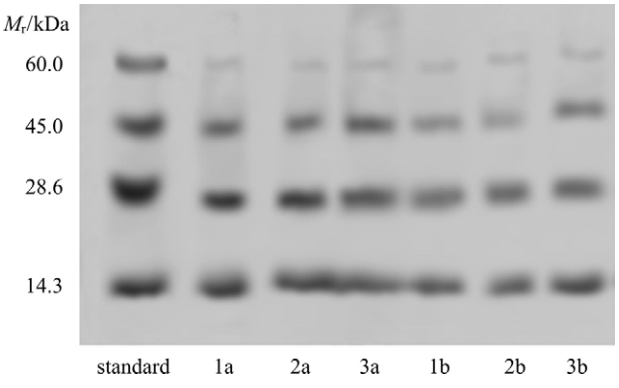

Fig. 1. Electrophoretic image of lysozyme samples modified thermally at 90 °C (1–3a) and 95 °C (1–3b) using: 1=no additives, 2=resorcinol and oxidant (H_2O_2), 3=resorcinol

Table 1. Average content of lysozyme oligomeric forms and hydrolytic activity of preparations obtained at the temperature of 90 °C

Sample	$\frac{m(\text{resorcinol})}{V(\text{water})}$	$\frac{m(H_2O_2)}{V(\text{water})}$	$\frac{m(\text{dimer})}{V(\text{water})}$	$\frac{m(\text{trimer})}{V(\text{water})}$	$\frac{m(\text{tetramer})}{V(\text{water})}$	$\frac{m(\text{oligomer})}{V(\text{water})}$	Hydrolytic activity	Hydrolytic activity decrease*
	%	%	%	%	%	%	U/mg	%
1a	0.00	0.0	27.5[a]	27.8[c]	8.2[a]	63.6[a]	7740[a]	63.6[c]
2a	0.03	2.0	31.4[b]	24.3[b]	15.9[c]	71.6[c]	10290[b]	51.6[b]
3a	0.03	0.0	31.0[b]	19.7[a]	15.4[b]	66.1[b]	15910[c]	25.1[a]

*in relation to lysozyme monomer (21 252 U/mg)
Different letters in columns denote significant differences of the mean values at $p \leq 0.05$
V(water)=100 mL

Table 2. Average content of lysozyme oligomeric forms and hydrolytic activity of preparations obtained at the temperature of 95 °C

Sample	$\frac{m(\text{resorcinol})}{V(\text{water})}$	$\frac{m(H_2O_2)}{V(\text{water})}$	$\frac{m(\text{dimer})}{V(\text{water})}$	$\frac{m(\text{trimer})}{V(\text{water})}$	$\frac{m(\text{tetramer})}{V(\text{water})}$	$\frac{m(\text{oligomer})}{V(\text{water})}$	Hydrolytic activity	Hydrolytic activity decrease*
	%	%	%	%	%	%	U/mg	%
1b	0.00	0.0	29.7[a]	28.6[b]	9.1[a]	67.4[a]	4960[a]	76.7[c]
2b	0.03	2.0	32.9[c]	27.3[a]	10.4[b]	76.6[c]	9690[b]	54.4[b]
3b	0.03	0.0	31.6[b]	27.1[a]	10.6[b]	69.3[b]	14600[c]	31.3[a]

*in relation to lysozyme monomer (21 252 U/mg)
Different letters in columns denote significant differences of the mean values at $p \leq 0.05$
V(water)=100 mL

more than 63 % of oligomers were obtained at 90 °C and approx. 67.5 % at 95 °C. Through the use of additional chemicals, we increased the efficiency of resorcinol by 2–3 % and even up to 9 % in the case of simultaneous use of resorcinol and H_2O_2 (sample 2b, temperature 95 °C). This preparation contained the highest amount of dimer (33 %), which was the main oligomeric form obtained after the modification. According to previous studies, this form of lysozyme is the most important in shaping a new, much stronger antibacterial activity of modified lysozyme (*1*,*2*,*5*).

As a result of the modifications carried out, we also observed a significant decrease in the hydrolytic activity of the obtained samples. The mean values of these activities and percentage reduction compared to the unmodified monomer are presented in Tables 1 and 2. The observed changes were probably caused by radical modification factors such as high temperature and oxidizing substance, which work destructively on the enzyme's active centre.

Modification temperatures of 90 and 95 °C led to the decrease of the enzyme's hydrolytic activity by 64 and 77 %. However, the addition of resorcinol resulted in the stabilization process and then a marked decline in the destruction of hydrolytic activity was observed. Lysozyme modified with resorcinol at 90 and 95 °C showed 25–30 % higher activity compared to the enzyme modified without this additive. The protective properties of resorcinol were also very significant when it was used together with an oxidizing agent. Under these conditions, a greater number of oligomers was obtained and hydrolytic activity still remained high (Tables 1 and 2).

Comparing the results of the research so far, we found that the best conditions for the modification of lysozyme were when the process was carried out at 90 °C with the addition of resorcinol as a protective agent and an oxidizer as a substance supporting the oligomerization process. There is a need for further research on the optimization of the process, especially determining the optimal dose of resorcinol, modification parameters and other substances from the group of phenols, which may be even more effective. It is also necessary to assess the physicochemical properties of the produced oligomers, the antibacterial spectrum and the strength of their actions. Such studies are currently being conducted in our laboratory.

Conclusions

In the light of the findings concerning the protection of lysozyme during its thermal modification, we found that resorcinol, an organic compound from the group of phenols, exhibits good protective properties. It both protects the hydrolytic activity of lysozyme and helps in its oligomerization. Further studies, especially those concerning optimization of enzyme modification process and finding other substances that could protect lysozyme during thermal modification, need to be conducted. And finally, it is essential to evaluate the physicochemical properties of the produced preparation.

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