Encapsulation of enzyme lysing group A streptococci to improve its stability

L.Yu. Filatova[1], A.V. Levashov[1], N.F. Dmitrieva[2], A.S. Eshchina[2], N.L. Klyachko[1]
[1]Department of chemical enzymology, M.V. Lomonosov Moscow State University, Moscow, Russia
[2]Medical Prophylaxis faculty, I.M. Sechenov Moscow Medical Academy, Moscow, Russia
Contact email: lubfil@rambler.ru

Introduction

Nowadays, even in the high-developed countries the increase of deceases caused by streptococci, are noted. The fact is connected with an appearance of antibiotic resistant bacterial strains. Bacteriophages, viruses of bacteria, and their lytic enzymes can be considered as potential alternative to the antibioticotherapy. But phage therapy has some disadvantages (a narrow range of bacterial host cells; occurrence of bacterial cells-phage resistant mutants; presence of bacterial debris in phage preparations; the immune responce of human organism). So, bacteriolytic enzymes are more suitable for medical purposes.

In 1971, the enzyme produced by bacteriophage C1 possessing lytic activity towards Streptococci A cells, was isolated by V. Fischetti. In the literature, it is referred to Lysin, or N-acetylmuramoyl-L-alanine amidase, or PlyC Lysin (D. Nelson, et al. 2006). The enzyme (PlyC Lysin) is able to cleave covalent bonds in peptidoglycan part of bacterial cell wall resulting in the effective streptococci cells lysis (D. Nelson, et al. 2006; N.L. Klyachko et. al. 2006). However, the enzyme has low temperature stability (figure 1). The aim of the present study was to improve the PlyC Lysin stability. Several approaches were developed: the use of micelle-forming matrices, cross-linking agents, and their combination.

Lipids or surfactant aggregates of different structure are well-known and widely used carriers for proteins (enzymes) and other bioactive substances. There is a long history of using micelles (normal and reverse) as well as liquid crystalline structures (lamellar, hexagonal, cubic, etc.) for enzyme regulation, protein modification applying the fundamental research to chemical syntesis, analysis, medical needs etc. Surfactants, both low molecular mass and polymeric, lipids, polysaccharides and polyelectrolytes, can all be used as a matrix material for enzyme entrapment, and the size and properties of the matrix can easily be varied by changing the nature and concentration of components. The sizes of particles of these compounds lie in nano-diapason and nanosized matrix influences the enzyme activity and stability (N.L. Klyachko et. al. 2006).

Materials and methods.

Recombinant PlyC Lysin (Lot 081) was prepared by Dr. Surekha Valhyam and provided by New Horizons Diagnostic Co. (USA) as a part of IPP Project. Preparation was stored in 20 mM phosphate buffer, pH 6.3 at + 4°C; protein content was 14 mg/ml. *Streptococcus pyogenes* cells of D-28/11 N62/59 bacterial strain (the Prague collection, cells M 29) were used. Cells were pretreated with rifampicin and lyophilized. All other reagents used were purchased from «Sigma».

Measurement of PlyC activity was based on turbidimetric determination of cell lysis. Rifampicin-pretreated cells were resuspended in water, giving a stable suspension with constant optical density (OD) during the measuring time. Cell lysis was followed spectrophotometrically measuring the OD

decrease at 600 nm (OD_{600}) for 30 min at 37ºC. An enzyme activity was expressed as a decrease of OD_{600} per minute which was the initial reaction rate of the cell lysis.

The influence on enzyme stability of a number of additives (salts, polyelectrolytes, carbohydrates, surfactants) was studied. Molar ratio reagent/PlyC was varied from 1/1 to 50/1. The mixture of PlyC and additive (in 20 mM phosphate buffer, pH 6.3) was incubated at 37ºC for different periods of time and after that the residual activity was measured.

PlyC was modified using of bifunctional cross-linking reagents: BS 3 (sodium bis-sulfosuccinimidine suberate), glutaraldehide, DMA (dimethyladipimidate), DMS (dimethylsuberimidate) in the wide range of pH values (6-9) and different molar ratios reagent/enzyme. Stability of cross-linked enzyme was studied in 20 mM phosphate buffer solution, and commercial mouthwashes: Tea Tree Oil Mouthwash, USA (F2); Mouthwash Vesna^{+}, Russia (F7); Oral rinse, Thera Breath, USA (F15); Mouthwash, Biotene with calcium, USA (F16). These mouthwashes contain some surface-active reagents, oils, inorganic salts, sugars, enzymes.

Results and Discussion

As stated above, PlyC Lysin possesses a very low temperature stability. Figure 1 shows the dependence of logarithm of residual enzyme activity at 37ºC on time for two PlyC concentrations. Both dependences have a break, so dissociative mechanism of enzyme inactivation takes place at this temperature. Authors (D. Nelson, et al. 2006) found, that PlyC is composed of two separate gene products, PlyCA and PlyCB. Based on biochemical and biophysical studies, the catalytically active PlyC holoenzyme (116 kDa) consists of 8 PlyCB (8 kDa) subunits and 1 PlyCA (50 kDa). The aim of this work was to prevent PlyC dissociation and/or other inactivation. To achieve this purpose non-covalent linkage with carbohydrates (lactose, chitosane, sulfochitosane, heparin), polyelectrolites (polyacrylic acid), alcohols (sorbitol, polyvinyl alcohol), proteins (casein hydrolisate, BSA), nonionic surfactants (Brij 35, Brij 30, Tween 20, Pluronic P85, Pluronic F127), salts (sodium chloride) and glycerol were used. From all listed above reagents only Brij 30, Pluronic F127, polyacrylic acid (PAA) and glycerol increased the enzyme stability at its optimal pH value 6.3 (20 mM phosphate buffer was used). It was established, that enzyme stability depended on a reagent/enzyme molar ratio (in case of polyacrylic acid, the best enzyme stability was found at 10/1, for Pluronic F127 at 25/1). Not only these separate components, but their mixtures were used to provide experiments on enzyme stabilization as well.

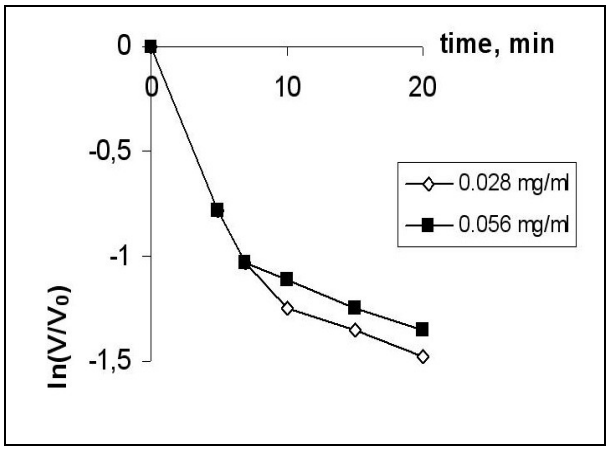


Figure 1: PlyC inactivation. Experimental conditions: the enzyme in 20 mM phosphate buffer solution (pH 6.3) was incubated at 37ºC, *Streptococcus pyogenes* cells were resuspended in 20 mM phosphate buffer, pH 6.3, OD_{600} = 0.3.

It was found, that PlyC Lysine stability could be increased 3 times in the system PAA/enzyme 10/1 (figure 2). An addition of 25% glycerol led to 10 times increase of PlyC Lysin stability, 0.1% Brij 30

– 14 times, if compared with this enzyme in buffer. The best result was achieved under using of Pluronic F127 (Pluronic F127/PlyC 25/1); PlyC Lysin stability increased 123 times (figure 2, table 1). Combination of Pluronic F127 with other additives did not give good results oppositely from PAA. The results obtained can be explained by the fact that polymeric molecules of PAA and Pluronic F127 covered enzyme molecule, thus preventing it from dissociation and following conformational changes.

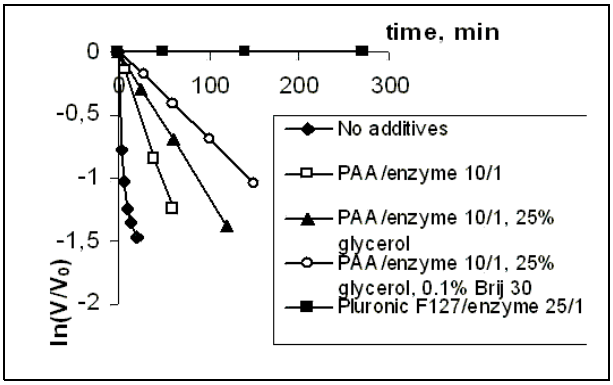


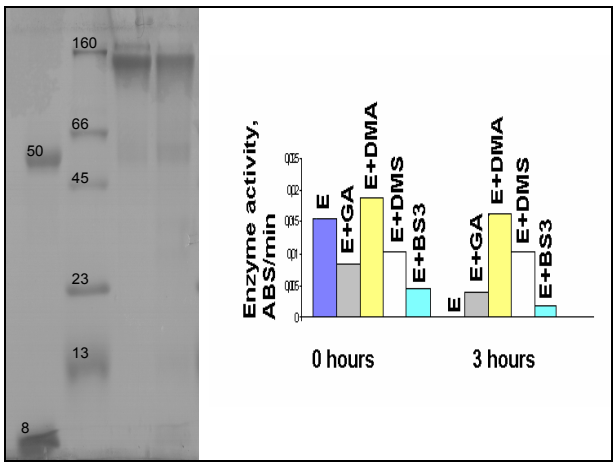
Figure 2: Inactivation of PlyC in buffer and encapsulated into micellar-polyelectrolyte compositions. Experimental conditions: the mixture of enzyme and additives in 20 mM phosphate buffer solution (pH 6.3) was incubated at 37ºC, *Streptococcus pyogenes* cells were resuspended in 20 mM phosphate buffer, pH 6.3, OD_{600} = 0.3.

Composition	K_{in}, min^{-1}	$\tau_{1/2}$, min
Enzyme	0.097	7
PAA/enzyme 10/1	0.021	33
PAA/enzyme 10/1, 25% glycerol	0.01	69
PAA/enzyme 10/1, 25% glycerol, 0.1% Brij 30	0.0068	101
Pluronic F127/enzyme 25/1	0.0008	866

Table 1: Inactivation of native PlyC and that included into micellar-polyelectrolyte compositions

Since the enzyme was found to be a multimer, the use of cross-linking agents could be helpful to improve the enzyme stability. Several bifunctional cross-linking reagents such as BS 3 (sodium bis-sulfosuccinimidine suberate), glutaraldehyde, DMA (dimethyladipimidate), DMS (dimethylsuberimidate) were used at different pH values and agent/protein molar ratios. Modification process was controlled by SDS/PAGE. Under the enzyme optimal conditions (20 mM phosphate buffer, pH 6.3), the stability of modified enzyme was checked in comparison with the initial PlyC Lysin in buffer. Significant increase (several times) in the enzyme stability was found upon its modification with DMA and DMS (figure 3). The enzyme, modified by BS3 and glutaraldehyde possessed lower stability than that one cross-linked with DMA and DMS.

Cross-linked PlyC stability was studied in some medical mouthwashes. Modified with DMA and DMS PlyC stability was studied in these mouthwashes at 25ºC (list of mouthwashes see in Materials and methods). It was estimated, that in these mouthwashes cross-linked by DMA and DMS enzyme kept its activity during 10 days (figure 4). Mouthwashes contained different surfactants, polyelectrolytes, salts etc. So the combination of the enzyme modification and its accommodation into micelle-forming matrices shows promises in using PlyC Lysin as a component of commercially available oral hygiene liquids.



A B

Figure 3: A) An SDS/PAGE of t the purified PlyC demonstrates that PlyC is composed of PlyCA (50 kDa) and PlyCB (8 kDa) (track 1). Tracks 3 and 4 are PlyC treated with glutaraldehyde and BS3 (50x molar abundance of both reagents) respectively. B) Stability of native and modified PlyC in 20 mM phosphate buffer at initial time and after 3 h of incubation at 37ºC. E-native PlyC in buffer, E+GA-PlyC, cross-linked by glutaraldehyde (50x molar abundance of reagent), E+DMA-PlyC, cross-linked by dimethyladipimidate (50x molar abundance of reagent), E+DMS-PlyC, cross-linked by dimetylsuberimidate (50x molar abundance of reagent), E+BS3-PlyC, cross-linked by sodium bis-sulfosuccinimidine suberate (50x molar abundance of reagent)

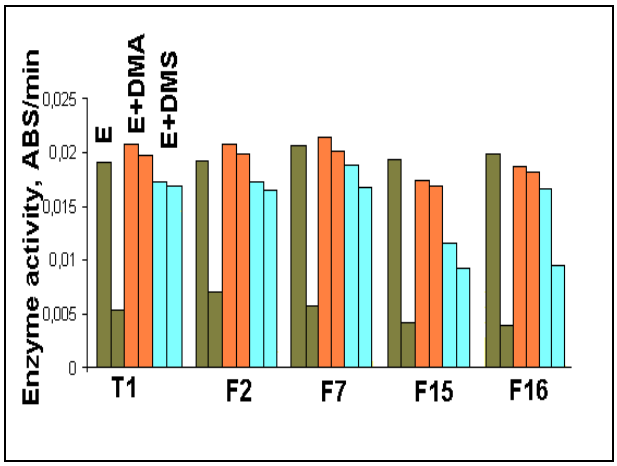


Figure 4: Cross-linked PlyC stability in mouthwashes compositions at 25ºC. Enzyme activity was measured at the initial time moment (the first columns) and 10 days later (the second columns). E-native PlyC, E+DMA-PlyC, cross-linked by dimethyladipimidate (50x molar abundance of reagent), E+DMS-PlyC, cross-linked by dimetylsuberimidate (50x molar abundance of reagent). Abbreviations of mouthwashes are given in Material and methods.

Conclusions

As a result, to increase PlyC stability its chemical modification by a number of cross-linking reagents, confurmed by SDS/PAGE, was used. It was established, that enzyme stability depended on its modification type. The essential increase of enzyme modified by DMA and DMS stability in commercial mouthwashes was confirmed. Entrapment of PlyC in micellar compositions allowed to increase its stability at 37º C. The enzyme stability was found to dependent on the concentration of an additive. The best result (100 times increase of PlyC stability) was achieved with Pluronic F127.

Acknowlegements

The work has been done in the frames of IPP Contract N 405911-A-K5.

References

1. D. Nelson et al. (2006) *PlyC: a multimeric baceriophage lysin*. Microbiology 103 10765 – 10770.
2. N.L. Klyachko et al. (2006) *Matrix design for bacteriolytic enzyme encapsulation.* J. Drug Del. Sci. Tech. 16(4) 293 – 299.