Brazilian Journal of Medical and Biological Research (2002) 35: 727-730
ISSN 0100-879X Short Communication

The use of protein structure/activity relationships in the rational design of stable particulate delivery systems

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Abstract

The recombinant heat shock protein (18 kDa-hsp) from *Mycobacterium leprae* was studied as a T-epitope model for vaccine development. We present a structural analysis of the stability of recombinant 18 kDa-hsp during different processing steps. Circular dichroism and ELISA were used to monitor protein structure after thermal stress, lyophilization and chemical modification. We observed that the 18 kDa-hsp is extremely resistant to a wide range of temperatures (60% of activity is retained at 80ºC for 20 min). N-Acylation increased its ordered structure by 4% and decreased its ß-T1 structure by 2%. ELISA demonstrated that the native conformation of the 18 kDa-hsp was preserved after hydrophobic modification by acylation. The recombinant 18 kDa-hsp resists to a wide range of temperatures and chemical modifications without loss of its main characteristic, which is to be a source of T epitopes. This resistance is probably directly related to its lack of organization at the level of tertiary and secondary structures.

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Research supported by CNPq, UNDP/World Bank/WHO Special Program for Research and Training in Tropical Disease (TDR), and FAPESP (Nos. 94/5854-5, 00/10970-7, and 00/14228-3 to M.H.B. Costa, and 96/1405-7 to P.S. de Araujo).

Received February 15, 2002
Accepted March 15, 2002

Key words
- Protein stability
- Hydrophobic modification
- Vaccine delivery system
- Drug delivery system
- Adjuvant

Modern vaccinology combines recombinant DNA technology and protein chemistry to obtain safe subunit vaccines. Most vaccines are poorly immunogenic because a large number of antigens are membrane proteins and consequently they are not present in their native conformation in the vaccine. Others that are not as potent because they contain only B epitopes cannot stimulate cell memory. Their potential usefulness as vaccines depends on adjuvants that increase cell-mediated and humoral responses (1). Some of these antigens will need to be conjugated with protein carriers to provide long-term protection in young children (2). It is known that the heat shock proteins are involved in immunity (3) and particularly in T cell stimulation (4).

The recombinant heat shock protein (18 kDa-hsp) from *Mycobacterium leprae* contains some sequences that are similar to those of other heat shock proteins (4). Furthermore, the 18 kDa-hsp binds to T cells, which may confer to the bound carried antigen a proper presentation to induce B/T cell immunity (5,6). We developed the large-scale production of 18 kDa-hsp at low cost in order to be able to introduce this T cell

stimulation into our vaccines (7,8). Our main goal was to co-encapsulate the 18 kDa-hsp together with poor antigens within safe and pluripotent supports, such as liposomes (9,10) or biodegradable microspheres (11). These supports can also be excellent vehicles with adjuvant and controlled release capacity. As pharmaceutical products they should be stable under storage conditions. The liposomes containing the 18 kDa-hsp are prepared in a stable condition by lyophilization (10). In addition to vehicle stability (10) it is important to study the chemical and thermal stability of the T-epitope source, i.e., the 18 kDa-hsp.

To elicit the preferential production of IgM, the protein must be associated with the outer layer of liposomes. To make this association with the liposome membrane possible, the 18 kDa-hsp was modified by acylation of the lysine residues to increase its hydrophobicity (9). The N-acyl-18 kDa-hsp circular dichroism spectra were obtained using a JASCO spectropolarimeter model J720. One 0.1-cm optical path circular cell was used to obtain all spectra.

The recombinant 18 kDa-hsp (1 mg/ml in PBS) was frozen, lyophilized, resuspended in water (final concentration of 1 mg/ml in PBS) and incubated for 30 min at different temperatures up to 80ºC. Protein stability was monitored by circular dichroism and ELISA.

For ELISA, the samples were added to plates and blocked with 0.1% BSA after 2 h at 37ºC. Subsequently, L5 (a monoclonal antibody; kindly provided by Dr. Carlos Alberto Moreira Filho, ICB, USP), anti-mouse peroxidase and TMB were added at 30-min intervals. After 15 min at room temperature, the reaction was stopped with H_2SO_4. Absorbance was read automatically at 450 nm (9).

When considering 18 kDa-hsp stability, its particular structural features should be taken into account. The primary structure of 18 kDa-hsp was derived from the nucleotide sequence (12). It is composed of 10 peptide regions delimited by nine proline residues intercalated by nine glycine residues. Both proline and glycine are known to destabilize the α-helix. It does not contain S-S bonds that would maintain a rigid tertiary structure.

Therefore, its five lysine residues could be easily acylated, introducing at least five hydrophobic tails through its side chain. Furthermore this protein is not rich in aromatic residues, which would contribute to its rigidity by hydrophobic interactions. In fact, this protein is flexible and has no α-helix content as determined by circular dichroism (Table 1). N-Acylation decreased disorder (component 3) by 2.0% and enhanced ß-T1 (component 4) by 4.5%. In general, the organizational level was not significantly changed by N-acylation (Table 1).

The N-acylation of the 18 kDa-hsp did not alter its T-epitope conformation, as confirmed by its reactivity measured by ELISA (9).

The 18 kDa-hsp T-epitope conformation

Table 1. The 18 kDa-hsp and N-acyl-18 kDa-hsp secondary structures.

Spectral signal (expressed as component number)	Related structure	18 kDa-hsp (% of Θ)	N-acyl-18 kDa-hsp (% of Θ)
1	α-helix	0	0
2	ß-sheet or ß-T2	0	0
3	Disordered	39.6	37.6
4	ß-T1	33.7	37.2
5	S-S or aromatic	26.7	26.2

Θ in 10^{-3} degree cm^2 $decimol^{-1}$.

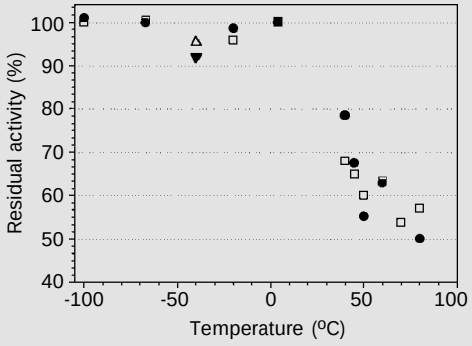

Figure 1. Residual activity as a function of temperature. The 18 kDa-hsp was frozen in the absence (squares) or the presence (circles) of trehalose and assayed by ELISA after thawing. Another assay was performed with the sample frozen in the absence (open triangle) or presence (filled triangle) of trehalose and then lyophilized. The sample was resuspended and incubated at the temperatures indicated.

- expressed as immunological integrity *in vitro* - is preserved during entrapment within vehicles such as PLGA microspheres, liposomes, and protein supramolecular aggregates (9,10). The residence time in the vehicles also does not alter its immunological integrity. The immune response induced by N-acyl-18 kDa-hsp was as high as those mediated by the formulation within liposomes (9). This means that the simple introduction of hydrophobic tails increased the activity of 18 kDa-hsp. These ELISA tests were conducted using polyclonal antibodies, so it is impossible to distinguish if the antibodies are conformational or not. But, in spite of the small structural changes introduced by acylation, the N-acyl-18 kDa-hsp probably adopted the aggregation state of a mixed micelle, which is as good as a liposomal formulation (9,10).

In countries with a warm climate, vaccine stability is mainly correlated with the cold chain, i.e., all transport steps from the production center to the patient at the clinical center during which the vaccine must be conserved refrigerated. Thermostability is an essential prerequisite for the successful development and dissemination of inexpensive, effective vaccine formulations. In many instances in the past, potential candidates have failed due to substantial losses at the production or downstream processing stages. In other cases, labile entities have been successfully produced at the commercial level, only to be inactivated by inadequacies in the handling procedures during transportation and distribution. Such occurrences have contributed to the failure of many vaccination campaigns (13,14).

The use of trehalose was also investigated as a protective factor for the 18 kDa-hsp to permit its pasteurization and to improve its stability under storage and transport conditions, but it did not provide additional protection to the epitope activity.

The first question addressed was whether trehalose would protect the 18 kDa-hsp in the dry state, thus improving storage conditions. The particular conditions chosen mimic the thermal stress that dry vaccine formulations could endure during transportation. Laboratory storage is usually performed in low temperature freezers.

The 18 kDa-hsp is an extremely resistant protein. It resisted a wide range of temperatures, remaining active from -100°C to 80°C. After 30 min at 80°C, 60% of its activity could be recovered (Figure 1). The use of trehalose for the preservation of this protein proved to be redundant, since the protein by itself is extremely resistant to thermal stresses.

In terms of 18 kDa-hsp vehiculation, formulations within supramolecular aggregates, PLGA microspheres, liposomes and the N-acyl-derivative can be used to transport poor immunogens. The recombinant 18 kDa-hsp resists a wide range of temperatures and chemical modifications without loss of its main characteristic, which is to be a source of T epitopes. In this study, we demonstrated by circular dichroism that this resistance is directly related to its lack of organization at the level of tertiary and secondary structures.

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