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Research Article

Preventing Aggregation of Recombinant Interferon beta-1b in Solution by Additives: Approach to an Albumin-Free Formulation

Najmeh Mahjoubi[1], Mohammad Reza Fazeli[1]*, Rassoul Dinarvand[2], Mohammad Reza Khoshayand[1], Ahmad Fazeli[3], Mohammad Taghavian[1], Hossein Rastegar[4]

[1] *Department of Drug and Food Control, Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.*
[2] *Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.*
[3] *Research and Development Department, Zistdaru Danesh Company. Tehran, Iran.*
[4] *Food and Drug Research Center, Food and Drug Organization, MOH&ME, Tehran, Iran.*

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Abstract

Purpose: Aggregation suppressing additives have been used to stabilize proteins during manufacturing and storage. Interferonβ-1b is prone to aggregation because of being non-glycosylated. Aggregation behavior of albumin-free formulations of recombinant IFNβ-1b was explored using additives such as n-dodecyl-β-D-maltoside, Tween 20, arginine, glycine, trehalose and sucrose at different pH.

Methods: Fractional factorial design was applied to select major factors affecting aggregation in solutions. Box-Behnken technique was used to optimize the best concentration of additives and protein.

Results: Quadratic model was the best fitted model for particle size, OD350 and OD280/OD260. The optimal conditions of 0.2% n-Dodecyl-β-D-maltoside, 70 mM arginine, 189 mM trehalose and protein concentration of 0.50 mg/ml at pH 4 were achieved. A potency value of 91% ± 5% was obtained for the optimized formulation.

Conclusion: This study shows that the combination of n-Dodecyl-β-D-maltoside, arginine and trehalose would demonstrate a significant stabilizing and anti-aggregating effect on the liquid formulation of interferonβ-1b. It can not only reduce the manufacturing costs but will also ease patient compliance.

Introduction

Interferon beta (IFNβ) is a glycoprotein including 166 amino acid residues.[1] The recombinant IFNβ-1a has an identical sequence and is glycosylated similar to natural protein. In contrast, IFNβ-1b is produced in *Esherichia Coli* and is non-glycosylated with a molecular weight of 19 kDa. IFNβ -1b is prone to aggregation because of being non-glycosylated.[2]

Additives are widely used in therapeutic protein manufacturing processes and formulations to improve protein stability. Previous reports have shown that some small molecular weight additives such as sugars, polyols and amino acids can have positive effect on protein stability.[3-5] However, a universal formulation recipe does not work for all proteins.[6] When potential additives are identified, their concentration and use can be evaluated by screening assays for preventing aggregation of formulation.

Human serum albumin (HSA) is usually used for formulation of IFN as a strong stabilizer. However, it has several major drawbacks. Presence of a secondary protein such as HSA could promote aggregation of IFN and possibly immunogenicity reactions. Analysis of active protein becomes challenging, if the product contains HSA excipient.[7] In a previous report, aggregation behavior of the cytokine (such as IFNs and interleukins) was characterized to stabilize a HSA-free formulation of the cytokine by additives (glycine, Tween 20 and sucrose), changes in pH and ionic strength. The impact of these factors was investigated in the cytokine formulations.[7] We aimed to alter HSA in the formulation of IFNβ-1b by aggregation suppressing competent additives as well. Previous studies showed that some non-ionic surfactants (alkylsaccharides) such as n-Dodecyl-β-D-maltoside (DDM) reduce aggregation of IFNβ.[8,9] DDM has been used in the formulation of eye-drops (insulin).[9] Also some other additives such as sucrose, Tween 20, glycine, arginine and trehalose have been used in marketed products e.g. Leukine (GM-CSF), Actimmune (IFN-γ), IntronA (IFN-α), Avonex (IFN-β) and Herceptin (trastuzumab).[10]

Lyophilization of biopharmaceuticals most often lead to higher production cost as well as inconvenience of usage for patients.[11] Recently, the composition of liquid

***Corresponding author:** Mohammad Reza Fazeli, Fax: +98 21 66959096, Email: fazelimo@sina.tums.ac.ir

formulation of IFNβ was investigated comprising an additive from the group of amino acids, arginine and glycine at a pH between 4.0 and 7.2.[12] Conventional methods using trial-and-error experiments for development of new formulations are not only time and cost consuming, but also ignore interactions among different factors. In this work, statistical methods such as fractional factorial design and Response Surface Methodology (Box-Behnken design or BBD) were applied to determine the optimum levels of selected factors.

The objective of this study is to evaluate the effect of different factors on aggregation of HSA-free liquid formulation of IFNβ-1b, including the type of additives (surfactant, sugar and amino acid type), pH, concentration of additives and protein. IFNβ-1b formulation was then optimized by BBD and characterized.

Materials and Methods

Materials

The unformulated IFNβ-1b sample in sodium hydroxide buffer at a concentration of 0.92 mg/ml was obtained as a gift from Zistdaru Danesh Company. The native and monomer form of the protein sample was approved by SEC chromatography and UV spectroscopy tests as fully described in previous work.[13]

Trehalose and sucrose (Sigma, Sigma) were used as sugar additives. Tween 20 (Merck) and DDM (Sigma-Aldrich, US) were used as surfactants. L-arginine and glycine (Sigma, Sigma) were used as amino acids. Hydrochloric acid (Merck, Germany) was used for pH adjustment. All other chemicals were of pharmaceutical grade. Water for injection was used for all experiments.

Preparation of formulations

Based on previous literature, trehalose and sucrose as sugars, DDM and Tween 20 as surfactants, arginine and glycine as amino acids were used for the development of IFNβ-1b formulation in solution. In screening step, 16 formulations (samples) of IFNβ-1b solution at a concentration of 0.25 mg/ml were prepared by a combination of an additive from each type (200 mM arginine or glycine as amino acid; 0.2% Tween 20 or 0.2% DDM as surfactant; 200 mM trehalose or 200 mM sucrose as sugar) at room temperature. The pH of the formulations was adjusted at either 4.0 or 5.5, or 7.0 by 0.1 N HCl in order to perform thermal stress and then analysis.

In the optimization study, 30 formulations of IFNβ-1b were designed in three levels using concentrations of protein (0.25-0.50 mg/ml), DDM (0.06-0.20 %), trehalose (50-300 mM), and arginine (50-300 mM). Furthermore, the pH of formulations was adjusted at 4.0 by HCl 0.1N in order to perform thermal stress and then analysis.

Stability Studies

Stability of the optimized formulation was assessed after 14 and 30 days of sample storage at 2-8°C.

Stress Study

A standard procedure for inducing and increasing aggregation of native and non-aggragated protein is to heat solutions.[13] Hydrophobic interaction is entropy dependent. Therefore intramolecular interactions between proteins undergo aggregate formation due to elevate temperature of the system. Thermal-induced aggregation can be used for protein aggregation studies.[14,15]

One of the tools that can be used to develop a robust formulation is thermal stability studies. In order to find out the stable and unstable formulations, samples were incubated at 40°C (<Tm of IFNβ-1b previously reported in the literature[9]) for 30 minutes that might be reasonable.[16,17]

Total samples included 16 formulations in the screening step, 30 formulations in the optimization step and an optimized formulation (for OD350, OD280/OD260 and particle size determination) were investigated after incubation of them at 40°C for 30 minutes.

Experimental design study

Screening

Experimental design can identify and evaluate the most significant factors and their interactions in the experiments with reduction of the number of runs in the screening study by factorial design.

Preliminary selection was carried out based on previous knowledge from literature.

Experimental design studies might be a powerful tool for research on solution properties of osmolyte (for instance arginine, glycine, trehalose, sucrose) such as concentration and pH that turn this stabilizer to a potential destabilizer that they can help to develop proper biopharmaceutical formulations.[18]

A fractional factorial design was used for screening step with a resolution IV 2(4-1) that described by the $D = ABC$ generator and the major effects will be distinct of two-factor interactions. Resolution IV 2(4-1) represents that k = 4 factors (first in parentheses) have been shown and p = 1 of these factors (second in parentheses) was created from the interactions of a full factorial design.

It was found different factors (i.e., pH and additive types such as amino acids, sugars and surfactants) can be effective on aggregation. Factors including pH (A), sugar additives (B), amino acid additives (C) and surfactant additives (D) were applied in screening study to find significant factors in reduction of aggregation in rhIFNβ-1b formulations.

This design had three levels for pH as −1, 0 and +1and two levels for types of additives. All of the experiments were performed in triplicate and the averages were considered as the responses. The resultant responses were analyzed by Design- Expert® software (version 7.0.0; Stat-Ease, Inc., Minneapolis, Minnesota, USA).

Optimization Study

After selecting the most effective factors influencing OD350, OD280/OD260 and particle size of rhIFNβ-1b formulations, Design-Expert Software (version 7, Stat-Ease Inc., Minneapolis, USA) was used to generate the optimum level of these factors by BBD. Four factors namely: amount of DDM (A), amount of surfactant (B) and amount of sugar (C) in addition to three amounts of protein (D) as an important factor at pH 4 were selected in three levels. 30 formulations (samples) were prepared and analyzed following incubation, as shown in Table 1. All of the experiments were carried out in triplicate and the averages are presented in Table 2.

Table 1. Box-Behnken design in various runs.

Formulation No.	Arginine (mM)	DDM (%)	Trehalose (mM)	Protein (mg/ml)
1	50	0.06	175	0.38
2	300	0.2	175	0.38
3	50	0.06	175	0.38
4	300	0.2	175	0.38
5	175	0.13	50	0.25
6	175	0.13	300	0.25
7	175	0.13	50	0.5
8	175	0.13	300	0.5
9	175	0.13	175	0.38
10	175	0.13	175	0.38
11	50	0.13	175	0.25
12	300	0.13	175	0.25
13	50	0.13	175	0.5
14	300	0.13	175	0.5
15	175	0.06	50	0.38
16	175	0.2	300	0.38
17	175	0.06	50	0.38
18	175	0.2	300	0.38
19	175	0.13	175	0.38
20	175	0.13	175	0.38
21	50	0.13	50	0.38
22	300	0.13	50	0.38
23	50	0.13	300	0.38
24	300	0.13	300	0.38
25	175	0.06	175	0.25
26	175	0.2	175	0.25
27	175	0.06	175	0.5
28	175	0.2	175	0.5
29	175	0.13	175	0.38
30	175	0.13	175	0.38

A second-order polynomial function relationship between the responses (dependent factors) and the independent factors (*Xi*) was shown as follows second-order polynomial equation:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + \beta_{14}X_1X_4 \quad (1)$$

where Y is the predicted response, β_0 is the intercept term, β_1, β_2, β_3, and β_4 are linear coefficients, β_{11}, β_{22}, β_{33}, and β_{44} are the squared effects, β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and, β_{34} are cross product coefficients (interaction coefficients), and X_1, X_2, X_3, and X_4 are the independent factors. Using this equation, it may be to appropriately evaluate the linear, quadratic, and interactive effects of the independent factors on the response. Multiple correlation coefficients (R^2), F-value and adequate-precision were used as the quality indicators to assess the fitness of quadratic model. The optimized formulation which selected in optimization step was further prepared for characterization process.

Table 2. Responses of optimization design.

Formulation No.	OD350 (n=3)	OD280/OD260 (n=3)	Particle size (n=3)
1	0.1	1.4	128
2	1.15	1	>1000
3	0.03	1.4	115
4	0.99	1	>1000
5	0.64	1	>1000
6	0.44	1	>1000
7	1.16	1.2	>1000
8	0.99	1	>1000
9	0.89	1	>1000
10	0.85	1	>1000
11	0.33	1.5	120
12	0.58	1	>1000
13	0.06	1.45	53
14	1.1	1	>1000
15	0.92	1	>1000
16	0.91	1	>1000
17	1	1	>1000
18	0.74	1	>1000
19	0.71	1	>1000
20	0.7	1	>1000
21	0.06	1.35	24
22	0.12	1.4	31
23	0.02	1.5	78
24	0.1	1.3	33
25	0.02	1.4	9
26	0.02	1.4	26
27	0.4	1	460
28	0.06	1.4	312
29	0.05	1.4	226
30	0.09	1.4	227

UV spectroscopy

Ultra violet absorbance of the formulations (concentration of 0.25 mg/ml protein) at three wavelengths including 350 nm (OD350 or optical density at 350 nm), 280 nm and 260 nm were measured using a Carry UV/VIS spectrophotometer in a 8-well quartz cuvette with a 1-cm path length (Varian, Australia). Then ratio of absorbance in 280 nm and 260 nm was calculated (OD280/OD260).The aggregate form of proteins can be observed as an increase in OD350 and decrease in OD280/OD260 as presented in the literature.[19,20] A decrease in OD280/OD260 is a sign of monomer protein aggregation in IFNβ-1b solutions.[15,19]

Dynamic Light Scattering (DLS)

Effective diameter of the particle (average size of particle or particle size) in rhIFNβ-1b formulations was measured by dynamic light scattering (DLS). Samples were diluted with water for injection to a concentration of 0.10 mg/ml protein and then analyzed using a 90 plus Brookhoven apparatus equipped with a red laser (λ=657nm), a detector at 90° by using 90 plus Software.

Osmolality Test

Osmolality of 50 µlit optimized formulation that diluted by water for injection to 0.25mg/ml rhIFNβ-1b was determined by using Osmomat 030. Reading was taken after measurement of calibration standard (NaCl /H_2O 300 mosm/Kg).The sample was repeated three times.

SDS-PAGE

The reduced SDS–PAGE was used to detect aggregates and/or impurities of optimized formulation. Two gel layers with different polyacrylamide concentrations were prepared that include of 4% Stacking gel and 14% resolving gel. Optimized formulation at concentration of 0.50 mg/ml IFNβ-1b was used for preparation of two samples (diluted and non-diluted formulations in Figure 1). Then heated at 95°C for 5 minutes and analyzed on SDS-PAGE gel. Unstained broad range molecular weight markers were included for molecular weight determination. Coomassie Brilliant Blue was used to visualize protein bands. The gel was scanned with a BIO-RAD Densitometer and Quantity one Software version 4.6.7.[15] The sample was repeated three times.

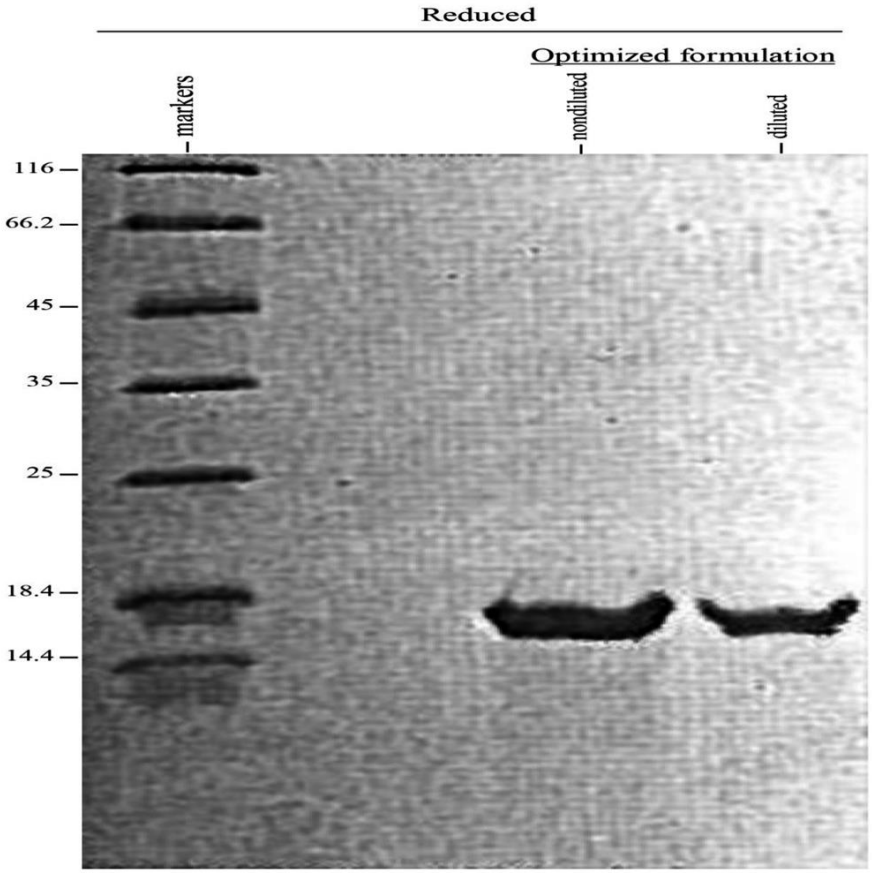

Figure 1. Reduced SDS-PAGE analysis of the optimized formulation that compared to markers.

CD spectroscopy technique

Circular dichroism (CD) technique was used as an ideal method to probe the secondary structural changes in protein samples (samples containing optimized formulation and control protein). Circular dichroism (CD) was measured using an Aviv CD spectropolarimeter in the far-UV regions (190–260 nm). Optimized formulation compared with control protein at 0.25 mg/ml concentration. Each spectrum was defined as the average of 3 repeated scans, and the background spectrum of the buffer was subtracted. Changes of ellipticity at 222 nm wavelength were selected to specifically analysis of the opening up of helical regions in the protein.[21] To quantify the structural changes, each spectra of CD spectroscopy was deconvoluted by the method of Bohm et al. employing CDNN CD Spectra Deconvolution Software.

Antiviral activity (Invitro bioassay for potency determination)

Human lung carcinoma (A549) cell line and encephalomyocarditis virus were used to measure antiviral activity of optimized formulation. Cytopatic effects were determined by colorimetric assay using

ELISA reader (Bio-TEK Instrument, Inc). Antiviral activity of optimized formulation was evaluated by comparison of its anti-CPE (anti-cytopathic effect) with that of the NIBSC interferon beta Ser17 mutein standard in 3 replicates (code: 00/574).[21]

Results and Discussion

Experimental Design Study

Fractional Factorial Design

A suitable selection of surfactant, sugar and amino acid types at proper pH in rhIFN β-1b formulations were prepared using a fractional factorial screening design. To screen the effective factors, a normal plot of parameters was employed (data was not shown). OD280/OD260 increased in formulations containing arginine at acidic pH and also particle size decreased in combination with trehalose. A combination of arginine, DDM and trehalose decreased OD350 compared with combination containing glycine, sucrose and Tween 20. Three significant factors including arginine (X1), DDM (X2) and trehalose (X3) at pH 4 were selected from fractional factorial design.

Box-Behnken Design and Response Surface Methodology for Optimization

After screening process, the most significant factors were selected. Four factors including arginine (X_1), DDM (X_2) and trehalose (X_3) as well as protein concentration (X_4) at pH 4 were used to minimize particle size, OD350 and to maximize OD280/OD260, As summarized in Tables 1(a) and 1(b). Box-Behnken design was used to optimize IFNβ-1b of the independent factors.

As shown in Table 3, Quadratic model was the best fitted model for particle size, OD350 and OD280/OD260 that had favorable adeq-precision, R^2 and F-value. The predictive and experimental responses for optimized formulation are given in Table 4. Experimental design studies showed that the effect of additives efficiency in combination with protein solution might be dependent on concentrations and/or pH.[22]

Table 3. Characteristics of models fitted to responses.

Dependent factors (responses)	Best-fitted model	Model F-value	R^2	Adeq-precision
OD 350	Quadratic	2.96	0.80	8.48
Particle size	Quadratic	3.28	0.79	8.24
OD280/OD260	Quadratic	4.48	0.83	8.23

Table 4. Comparative values of predicted and experimental responses for optimized formulation.

Dependent variables (Responses)	Predicted responses	Experimental responses	Predicted error (%)
Particle size	49nm	50nm	2.0
OD350	0.02	0.02	0
OD280/OD260	1.5	1.65	6.6

Increasing concentration of some proteins generally affect protein aggregation. As shown in Figures 2(b) and 2(c), increasing the concentration of IFNβ-1b from 0.25 mg/ml to 0.50 mg/ml did not have a great effect on protein aggregation behavior. Previous studies have shown that by increasing human IFN-γ concentration from 1 μM to 4 μM, the time of reaching maximum aggregation was increased.[23]

To obtain stable formulation in acidic condition, arginine concentrations were varied from 50 mM to 300 mM. As depicted in Figures 2(b), 2(a), and 2(c), the results of arginine combined with DDM and trehalose in formulations showed the lowest concentration of arginine at acidic pH significantly reduced particle size and OD350 and, considerably increased OD280/OD260. It seems that decrease in concentration of arginine strongly increases the stability of IFNβ-1b protein. Experimental data showed that IFNβ-1b formulation containing arginine in combination with other additives had a unique complicated behavior proving that arginine is concentration and protein-dependent.[24,25] Previous studies have shown that arginine could be an effective agent in inhibiting aggregation and has successfully been used in Enbrel liquid parenteral formulation at a low concentration of 5.3 mg/ml arginine HCl.[10] Arginine slows protein association. It suppresses aggregation by affecting attractive protein–protein interactions.[26,27] Two important mechanisms are proposed for the effect of arginine on stability of proteins. Tsumoto et al. suggested arginine affects suppressing aggregation due to interactions between the guanidine group of arginine and tryptophan side chains of protein surface. On the other hand, Shukla and Trout proposed the "gap effect" hypothesis.[28-31] Arginine may form a number of varying interactions with protein. Arginine increases surface tension and is larger than water (volume exclusion). Because of having guanidinium, it can interact with protein surface and has two other ionic charge locations since it is zwitterionic. Its amino group, also enables another location for donating hydrogen bonds and has a hydrophobic alkyl chain three carbons long. Studies show that guanidinium and carboxylate moieties interact and subsequently Arg-Arg clusters are formed. It seems that there is a correlation between clustering and aggregation suppression. However, none of the mechanisms has been accepted because most mechanisms cannot show all complex behavior of arginine.[25]

Based on the results of the optimization study, concentration of trehalose was found to be a critical factor in IFNβ-1b formulation. To obtain stable formulation in acidic conditions, trehalose concentrations were varied from 50 mM to 300 mM. Figures 2(f) and 2(e) has showed that by increasing the concentration of trehalose up to 190 mM, there was a great decline in particle size as well as increase in OD280/OD260. However, trehalose at a higher concentration in combination with arginine and DDM at pH 4 indicated significantly induced aggregation. At high concentrations many osmolytes generally destabilize or move towards destabilize proteins.[32] It is important to look into both aspects of trehalose to determine their precise role

in different concentrations. A survey of literature shows that trehalose at high concentrations destabilizes protein as a result of change in the property of trehalose.[33-35] It has also been reported that trehalose forms clusters that reduce the peptide backbone and increase side chain interaction and subsequently protein destabilization.[22,36] The stabilizing effect of trehalose depends not only on the concentration of its molecule but also on the pH of the protein solution.[37] Trehalose has been reported to destabilize some proteins at high pH and to have pH-dependent stabilizing effects.[22,38] This could help minimize aggregation using trehalose additive in optimized concentration at low pH in formulations by experimental design. In fact, protein hydrophobicity increases with decreasing pH due to the protonation of COO− groups.[39]

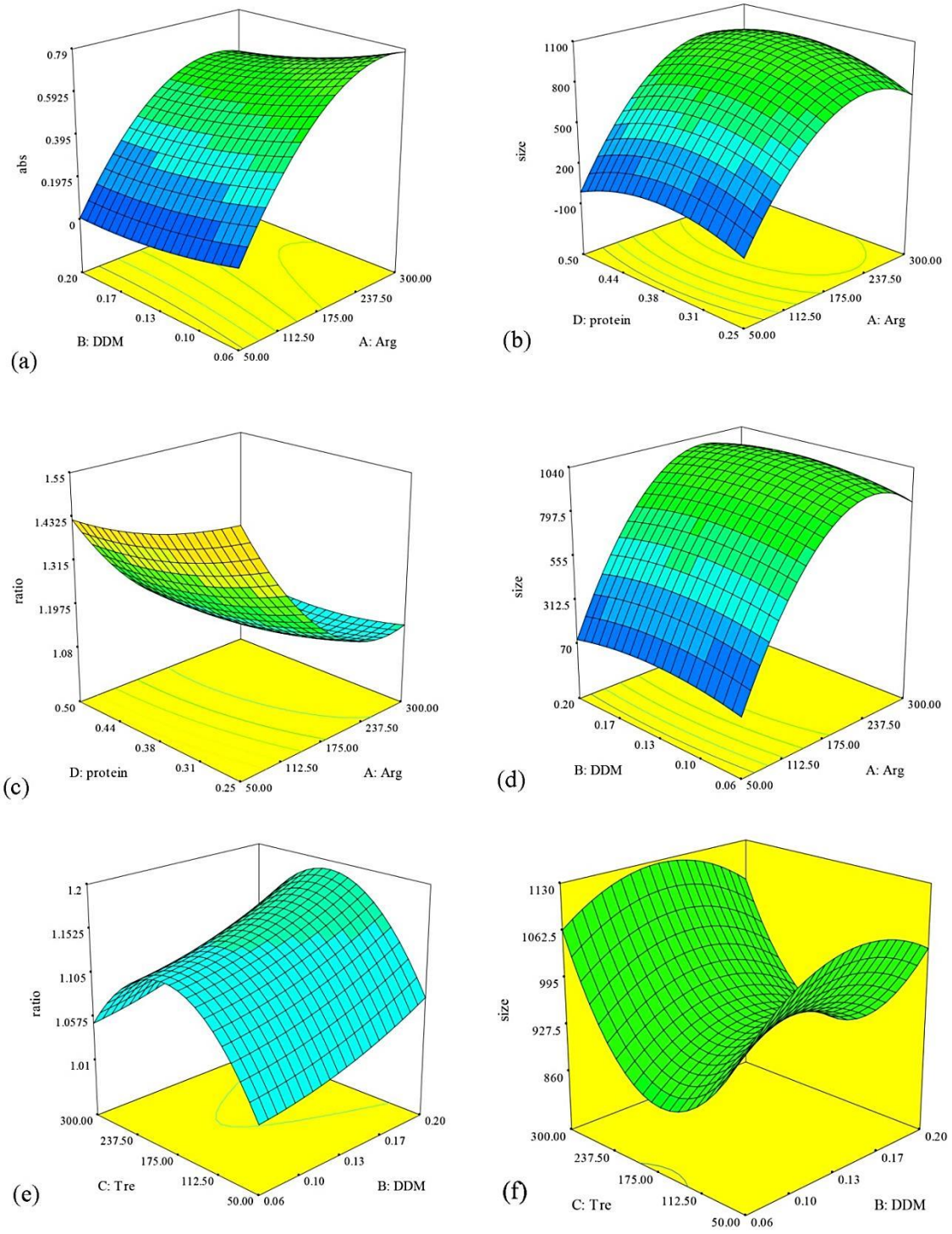

Figure 2. Response surface plot including the effects of interactions between (a) arginine and DDM on OD 350nm (abs), (b) protein and arginine, (d) DDM and arginine and (f) DDM and trehalose on size, (c) protein and arginine and (e) DDM and trehalose on OD280/OD260 (Ratio).

Because of various hydroxyl groups in trehalose, hydrogen bonding with water and other trehalose molecules can occur. The structural and dynamical properties of trehalose alter by the interaction between trehalose and water. Compared to other sugars, trehalose highly affects the water tetrahedral coordination and creates networks of extensive hydrogen bonding.[40-42] Lerbret et al. found that trehalose clusters size increases as a function of concentration due to the formation of cages that trap water molecules in solution.[24] The stability of IFNβ-1b was possibly increased by preferential exclusion of trehalose and creation of a rigid hydration shell around the protein. The CD spectrum depicting the conformational integrity of IFNβ-1b showed 16% decrease in the helical structure of protein in the absence of trehalose.[21]

As depicted in Figures 2(a) and 2(e) formulations containing DDM concentration of 0.06% to 0.20% in combination with arginine and trehalose showed reduction in OD350 and increase in OD280/OD260 indicating an improvement in stability of protein. Particle size decreased slightly by increasing the concentration of DDM in predetermined range indicated in Figure 2(d). Previous studies have shown dissolution of aggregate of IFNβ1-b improve at an increasing temperature due to add 0.1% DDM to protein.[9] Also the absorption of DDM on hydrophobic surfaces of IFNβ-1b makes the surface change to hydrophilic and non-ionic, and shows improvement in the stability of the protein.[9] Furthermore DDM has been demonstrated to prevent self-association of protein.[8,43]

Characterization of Optimized Formulation

The experimental results of UV spectroscopy, DLS, osmolality test, reduced SDS-PAGE and potency test of optimized formulation has been observed in Table 5. The characterization data showed 0.021, 1.65 nm and 50 nm for OD350, OD280/OD260 and particle size, respectively. As depicted in Figure 1, the reduced SDS-PAGE showed there is no aggregate or impurity for both samples of the optimized formulation (100% in purity) compared to the markers. Osmolality of the sample was 320 mosm/kg considered iso-osmotic.

Optimum condition was obtained when the concentration of protein, DDM, arginine and trehalose was set at 0.50 mg/ml, 0.2%, 70 mM and 189 mM, respectively.

Circular dichroism analysis

The CD spectra obtained of optimized formulation showed about 17% increase in helical structure of optimized formulation compared to control protein (unformulated IFNβ-1b sample), as can be seen in Figure 3.

Table 5. Characteristic results of optimized formulation.

No.	OD350	OD280/OD260	Particle size (nm)	Osmolality (mosm/kg)	SDS-PAGE purity(%)	Potency (%)
Optimized formulation	0.021	1.65	50	320	100	91

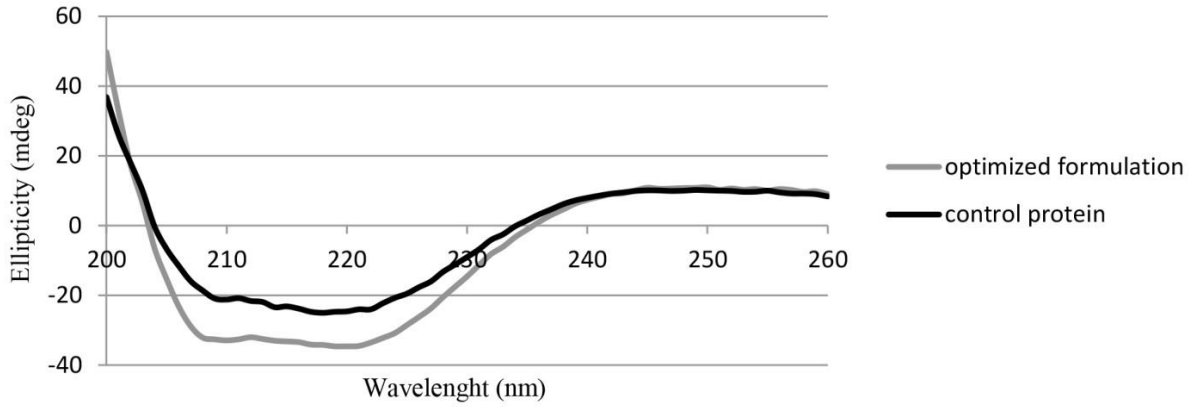

Figure 3. Far-UV CD spectra of optimized formulation and control protein without additives.

Stability studies

The biological activity of the sample kept at 2-8°C for 14 and 30 days showed a 12% and 19% decrease respectively when compared to fresh sample. Other quality control tests were also within the acceptable limits.

Conclusion

Aggregation behavior of IFNβ-1b in HSA-free formulation containing additives such as DDM, Tween 20, arginine, glycine, trehalose and sucrose was investigated at different pH. A significant reduction in aggregate formation was observed for the formulation containing DDM and trehalose in combination with arginine at pH 4.

Arginine at lower concentration in combination with other additives in formulation showed a complicated behavior and significantly protected the protein compared with most protein formulations using higher concentration of arginine. Therefore,arginine has concentration and protein dependent stabilizing effect.

Trehalose showed to have a great effect on aggregation due to concentration dependent behavior.

DDM was particularly effective to prevent aggregation of protein due to the absorption of DDM on hydrophobic surfaces of IFNβ-1b leading to hydrophilic and non-ionic surface change, and increases the stability of the IFNβ1-b. Increasing protein concentration from 0.25 mg/ml to 0.50 mg/ml did not affect protein aggregation pattern.

Particle size, OD350, OD280/OD260, osmolality and invitro bioassay of the optimized formulation did not significantly changed during 1 month of storage and were within the acceptable limits.

These results tend to suggest that HSA-free liquid formulation of interferon beta-1b could be prepared using anti-aggregation additives such as DDM, arginine and trehalose. This will not only reduce the manufacturing costs but will also ease patient compliance.

Acknowledgments

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Ethical Issues

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

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