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Research Article

Role of Molecular Interactions and Oligomerization in Chaperone Activity of Recombinant Acr from *Mycobacterium tuberculosis*

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Abstract

Background: The chaperone activity of *Mycobacterium tuberculosis* Acr is an important function that helps to prevent misfolding of protein substrates inside the host, especially in conditions of hypoxia.

Objectives: The aim of this study was to establish the correlation of structure and function of recombinant Acr proteins both before and after gel filtration chromatography. The aim was also to find the oligomeric conformation of these samples and use this information to explain differences in activity

Material and Methods: *M. tuberculosis acr* gene was cloned with an N-terminal His-tag in pET28a and expressed with IPTG induction in BL2 (DE3) competent *Escherichia coli*. The activity of a recombinant Acr without gel filtration was checked by preventing thermal aggregation of citrate synthase at 45°C and the chaperone activity against insulin B chain aggregation at 60°C and 37°C. On further purification using gel filtration chromatography, the protein was again tested for chaperone activity using insulin as substrate at 37°C with two types of samples without and with gel filtration designated A and B respectively. The effects of pre–heat treatment at 60 °C on chaperone activity of both A and B samples were studied by performing the chaperone assay at 37°C.

Results: The level of expression was 40 to 50 mg /l. The protein was expressed in a soluble form at 37°C and subsequently purified by a 3 step gradient of imidazole using Ni-NTA resin. Gel filtration chromatography showed recombinant Acr to be a mixture of 9 to 15-mers, whereas Native-PAGE analysis showed a large proportion of 5 and 7 mers in the non gel-filtered sample, while non gel –filtered samples showed more proportions of higher size oligomers. The chaperone activity of non gel-filtered (A) samples was less than gel-filtered (B) samples at 37°C with 24 µM required of A for complete inhibition as compared to 6 µM of B. The chaperone activity of non gel–filtered samples at 60°C showed complete inhibition of activity at a concentration of 44 µM. Molecular interaction studies showed influence of size of oligomers on molecular coverage of insulin B chain. Pre-heat treatment improved the activity only after the gel filtration.

Conclusions: The larger proportion of monomers in the non gel-filtered sample could explain the difference in activity as compared to the gel-filtered samples in terms of molecular interaction with insulin. Increased oligomer size favorably affected secondary structure, a finding not reported so far, and warranting further investigation. A molecular level interaction of inhibition was predicted using Avogadro number of molecules and oligomer size. The difference in activity after pre–heat treatment seemed to indicate an important role for oligomerization.

Keywords: Acr; Insulin; Chaperone; Oligomer; pre-heat Treatment; *Mycobacterium tuberculosis*

1. Background

Tuberculosis (TB) is the oldest disease known to mankind with the earliest cases reported through skeletal remains found 4,000 years ago. It is caused by *Mycobacterium tuberculosis* (1). Unlike other known microbes that are cleared from the body by the host immune response, *M. tuberculosis* persists in a caseous granuloma for many years, leading to the phenomenon of latent TB (2). The protein dominant in the latent phase, is alpha-crystallin (Acr), the hspX or hsp16.3 protein. Acr belongs to the family of small heat shock proteins in the size range of 12-43 kDa which are known to function as oligomers that prevent misfolding of other proteins (3).

Earlier reports on cloning and expression of *M. tb.* Acr, showed that, Acr molecules can be used for preventing aggregation of the substrates (4-8). All

Acr preparations entailed the use of a gel filtration step to maximize the activity of the protein. However, there is no documentation of the oligomeric status of the recombinant protein being used in the assays. It is assumed that Acr is functionally active as a trimer of trimers; or a dodecamer; which was initially reported in 2005 and recently confirmed in 2016 (4, 7, 8). However, none of these studies mentions the actual oligomeric state of the protein during the assay, the ratio of different size of monomers and about the molecular level interactions except for few reports (9). There are reports on the mechanism by which the protein binds to the substrate *in vitro* (4, 10-12). It has been agreed that there is a dynamic association and dissociation of oligomers which helps in chaperone activity**.** Some interesting features of *M.tb* Acr include its ability to improve activity with pre-heat treatment due to increased exposure of hydrophobic surfaces to the substrate molecules that help them bind their substrates effectively (9). It has also been reported that pre-heating at 60°C enhances the activity due to a change in the secondary structure of β sheets and random coils (10, 13). However, few studies have been carried out so far on the interaction at the molecular level in terms of molecules binding to the substrate. Most of the assays in previous studies have used substrates like catalase, citrate synthase, alcohol dehydrogenase and insulin (4, 8-10). In addition, all the assays reported with insulin B chain were carried out at 25°C or 37°C except for a few studies performed at higher temperatures (10, 14).

2. Objectives

We aimed to study the chaperone activity of Acr using citrate synthase as substrate at 45°C and insulin B chain as substrate at 60°C. At 60°C, a crucial energy barrier is overcome, allowing more hydrophobic surfaces to be exposed improving the activity of Acr (13). Here we report the findings on expression, purification, and activity of recombinant *M. tb* Acr on the substrate insulin at both 37°C and 60°C. Very importantly we also investigated the difference in chaperone activity of Acr with insulin at 37°C with 2 different types of samples non-gel-filtered and gel filtered. Pre-heat treatment studies at 37°C and 60°C followed by insulin assay at 37°C was carried out to investigate the role of oligomers in activity.

3. Materials and Methods

3.1. Expression and Purification of Acr

M. tb Acr was expressed as a full-length protein with N-terminal His-tag in pET28a. Two clones, #3 and #6 were tested for expression in BL21DE3 cells by inducing at 37°C with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The protein was checked for soluble expression by lysing a small volume of induced cell pellet and analysing the supernatant and pellet on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). 1mM (IPTG) induced cell pellet was lysed in 20mM Tris pH 7.0, 500 mM NaCl, 5% glycerol. The lysate was centrifuged at 20,000 g for 25 mins and the supernatant loaded on to Ni-NTA column (Thermo Scientific, USA), washed with 10 and 100 mM imidazole in the same buffer before eluting with a 3-step gradient of 300 mM, 400 mM and 500 mM imidazole in 20 mM Tris pH 7.0, 300 mM NaCl, 5% glycerol. The His-tag elutes were dialyzed against 20 mM Tris pH 7.0, 100 mM NaCl, 5% glycerol and checked for chaperone activity against insulin B chain. These samples were designated as '**A**' samples.

3.2. Gel Filtration of Acr

1.5 ml of the dialyzed His- tag elutes , equivalent to 3mg as was measured by the Bradford's dye binding method was loaded twice on Sephacryl S-200 HiPrep XK 16/70 column at a flow rate of 0.5 ml/min and the buffer passed for a total volume of 1.5 column volumes using the Akta Purifier System. A 15% denaturing SDS-PAGE was run to analyze the peaks obtained in the gel filtration runs. The gel-filtered elutes were designated as '**B**' samples.

3.3. Native-PAGE Analysis

A Native-PAGE analysis was carried out for both non-gel-filtered and gel-filtered samples, designated as (A) and (B), respectively using 8-16% gradient Tris Glycine gel and Bovine Serum Albumin (BSA) as a standard marker. The oligomer size was estimated using a plot of Log molecular weight versus distance migrated and using the antilog to estimate the exact oligomeric size.

3.4. Insulin Aggregation Assay

The chaperone activities of non-gel-filtered and gel-filtered Acr samples were checked using the 1,4-dithiothreitol (DTT) induced aggregation assay using 1.0 mg/ml insulin in buffer (20 mM Tris pH 7.0, 100 mM NaCl and 5% glycerol) at 60°C at 360 nm over 20 mins. Insulin R injection (BIOCON, India) was concentrated from a stock of 0.14 mg/ml to 1.0 mg/ml using 3kDa concentrators from Amicon (Millipore). Insulin in buffer without DTT and insulin with 25 µM DTT were used as controls. All the components except DTT were added to all these samples, the absorbance values were auto zeroed, baseline adjustment was done from 350 nm to 370 nm and 25 mM DTT added to start aggregation. Acr was used at 4 different concentrations of 12, 22, 39 and 44 µM in 3 different reaction volumes of 250, 300 and 400 µl. Likewise, chaperone activity was carried out by preventing DTT induced aggregation of insulin B chain concentration at 118 µM at the wavelength of 360 nm over 30 mins at 37°C. Aggregation was started by adding 25 mM DTT and

the absorbance recorded to look for inhibition of aggregation on addition of Acr. Two types of samples were used for the enzyme assays: (A) Non gel-filtered and (B) gel-filtered.

The activity of A samples was checked at concentrations of 6 and 24 µM and B samples at 1, 3 and 4 µM using the assay buffer 20 mM Tris pH 7.0, 100 mM NaCl, 5% glycerol.

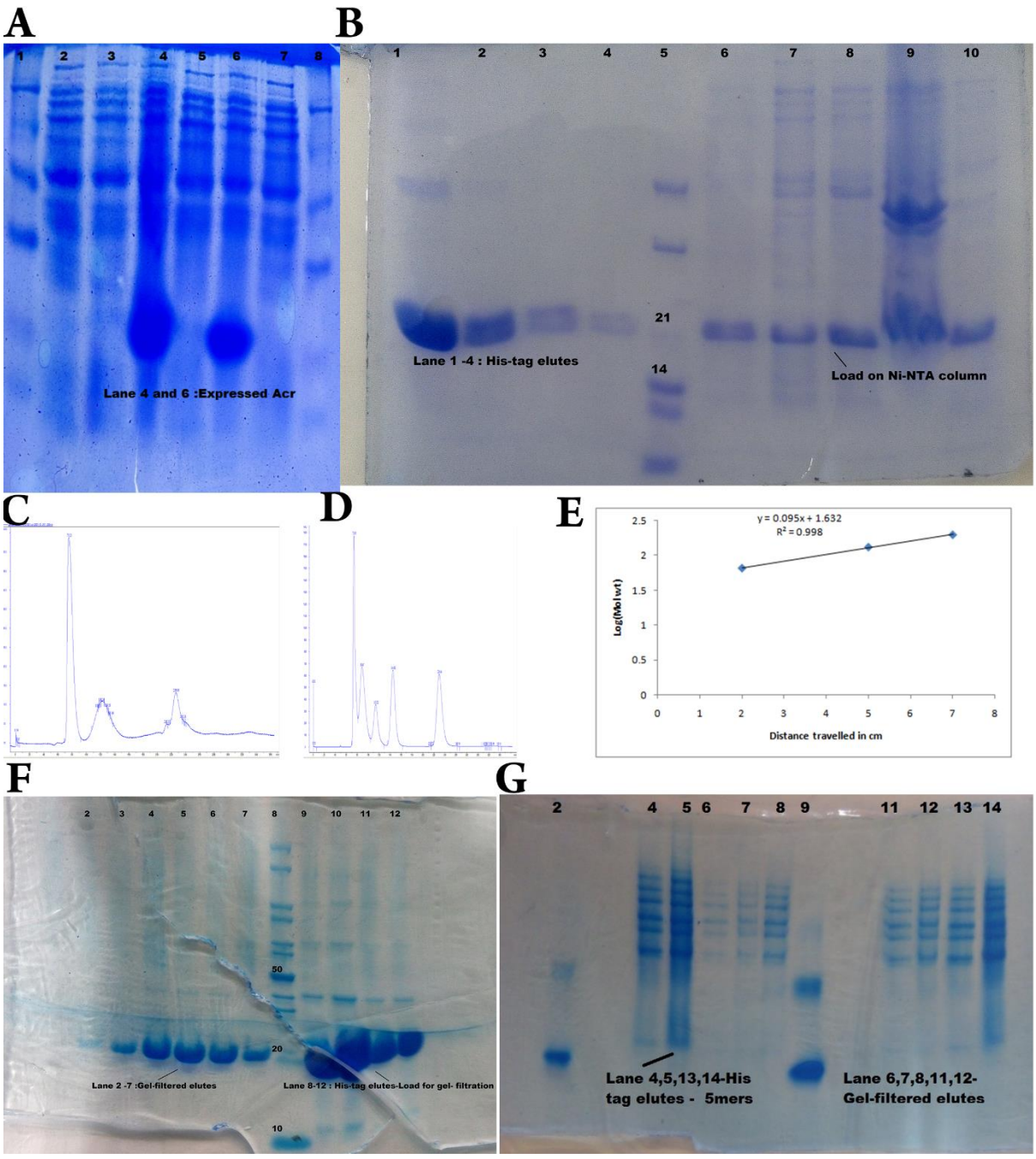

Figure 1. A) SDS-PAGE of Acr-pET28a expression: Lane 1, Marker 97, 66, 45, 30, 21, 14 kDa: Lane 2: uninduced vector; Lane 3: induced vector; Lane 4: induced clone #3; Lane 5: uninduced clone #3; Lane 6: induced clone #6; Lane 7: uninduced clone #6; Lane 8: Marker 97, 66, 45, 30, 21, 14 kDa. **B)** Lane 1: His-tag eluted fractions E1-E4; Lane 2: E5-E8; Lane 3,4: E9-E12; Lane 5: Marker (3, 6, 14, 21, 30, 43 kDa); Lane 6: Wash; Lane 7: Flow through; Lane 8: Sonicate supernatant; Lane 9: Sonicate pellet; Lane 10: Induced Acr. **C)** Gel Filtration Run 2 Chromatogram: X Axis: UV 280 nm; Y Axis: Elution time (mins). **D)** Bio-Rad Standards Chromatogram: X Axis: UV 280 nm; Y Axis: Elution time (mins) A - Aggregates + Thyroglobulin 670 kDa, 36.5 ml (73 mins); B - Globulin 158 kDa, 44 ml (88 mins); C - Ovalbumin 44 kDa, 55 ml (110 mins); D - Myoglobin 17 kDa, 77 ml (154 mins); E - vitamin B_{12} 1.5 kDa, 115 ml (230 mins). **E)** Plot of Log Mol wt versus distance migrated. The distance travelled by the three BSA monomers was plotted against the log molecular weight. **F)** 15% SDS-PAGE of gel filtration run: Lane 1: Gel filtered eluted fractions B2; Lane 2: B1; Lane 3: C1; Lane 4: C2; Lane 5: C3; Lane 6: C4; Lane 8: Marker (10, 20, 30, 40, 50, 60, 70, 85, 100, 150, 200 kDa); Lane 9, 10: Load of gel filtration run 10 and 30 µl; Lane 11,12: His-tag eluted sample (concentrated) 10 and 30 µl. **G)** Native-PAGE analysis of His-tag elutes and gel-filtered elutes. Lane 1: Empty; Lane 2: BSA; Lane 4, 5: His-tag elutes 10 and 20 µl; Lane 6, 7, 8: Gel-filtered elutes 20, 20 and 40 µl; Lane 9: BSA; Lane 11, 12: Gel-filtered elutes 20 and 40 µl Lane 13, 14: His-tag elutes 7 and 15 µl.

3.5. Thermal Aggregation Assay

It was carried out using A sample alone at a concentration of 5 µM and 2.5 µM citrate synthase substrate (Sigma) in a reaction volume of 300 µl. The assay buffer used was 20 mM Tris pH 7.0, 100 mM NaCl, 5% glycerol. The protein was added to citrate synthase and the assay was carried out at 45 °C by measuring absorbance at 360 nm over 30 mins.

3.6. Heat Treatment Assays

The recombinant Acr, after purification by gel filtration, was assayed at 37°C at a lower concentration of 18 µM to observe inhibition of insulin (1mg/ml). After pre-heat treatment at 60°C for 15 mins, the assay was repeated to check for chaperone activity. Another batch of His-tag purified protein was assayed at 37°C at 11 µM to check inhibition of insulin aggregation (1 mg/ml). After pre-heat treatment of samples at 60°C for 15 mins, the assay was repeated to check for chaperone activity.

3.7. Molecular Level Calculations

The molecular level calculations were done using the http://molbiol.edu.ru/eng/scripts/01_04.html

4. Results

4.1. Expression and Purification of Acr

The recombinant clone #3 was used for further studies because of its higher level of expression, which varied from 40-50 mg/l as determined by the Bradford's dye binding assay (**Fig. 1a**). The purification using the 3-step elution with imidazole gradient showed greater than 95% purity in the 500 mM imidazole fraction (**Fig. 1b**).

4.2. Gel Filtration of Acr

In the 1st run, a major peak was observed at 37.5 ml (75 mins) while the peak ended at 44 ml (88 mins), the position corresponding to the globulin peak of 158 kDa (data not shown). In the 2nd run, a peak was observed at the same time while it again ended at 44 ml. A few smaller peaks were observed later (**Fig. 1c**).

Table 1: Oligomer ratio calculation: Oligomer sizes have been calculated using BSA monomers, dimers and trimers as a reference (66, 132 and 198 kDa) and the log of molecular weight plotted versus the distance travelled in centimeter. From this calculation, the molecular weight has been extrapolated for the different bands observed in A (band 1 to 8) and B samples (band 1 to 5).

BSA (Mol. wt.)	Log Mol wt.	Distance (cm)			
66	1.82	2			
132	2.12	5		Monomer	
198	2.29	7		18	
			Size (kDa)	Oligomer size	% Fraction of total
A band 1		3	82	5	14
A band 2		5	129.93	7	9
A band 3		6	158.49	9	20
A band 4		6.5	177.82	10	15
A band 5		7	198.15	11	12
A band 6		7.5	221.3	12	8
A band 7		8	246.6	14	11
A band 8		8.5	275.4	15	
				Ratio of 11 to 15 mer	**43**
B band 1		6.5	177.82	9	15
B band 2		7	198.15	10	15
B band 3		7.5	221.3	11	15
B band 4		8	246.6	12	15
B band 5		8.5	275.4	16	40
				Ratio of 11 to 15 mer	**75**

Table 2. Molecular level interaction of Acr with insulin B chain at 60°C: Acr at 4 different concentrations was assayed with insulin at 60°C and the activity checked by measuring aggregation over 20 mins. The % inhibition measured was checked as a function of final OD divided by the final OD (minus insulin).The number of Acr molecules have beencalculated by entering the amount (µg) in the website http://molbiol.edu.ru/eng/scripts/01_04.html.

Mole ratio of Acr/insulin	% inhibition	% of insulin covered	Conc. of Acr (mg/ml)	Molecules of Acr	Molecules of 3 kDa insulin
0.144	53	14	0.2	1.8×10^{15}	1.26×10^{16}
0.26	56	47	0.36	4×10^{15}	0.85×10^{16}
0.35	100	70	0.8	1.05×10^{16}	1.5×10^{16}
0.46	95	94	0.7	5.9×10^{15}	0.63×10^{16}

Table 3. Molecular level interaction of **A** sample with insulin B chain. The assumption is that Acr is 18 kDa and the insulin B chain is 3 kDa. The number of molecules of insulin has been calculated based on an assay volume of 0.25 ml and the number of insulin molecules assumed to be 1.08×10^{16}.

% insulin B chain covered	% inhibition	Molecules of Acr covered	Acr concentration (µM)
0	0	0	0
1.68	28	1.81×10^{15}	6
3.37	90	3.64×10^{15}	24

Table 4. Molecular level interaction of **B** sample with insulin B chain. The assumption is that Acr was 18 kDa and the insulin B chain was 3 kDa. The number of molecules of insulin has been calculated based on an assay volume of 0.25 ml and the number of insulin molecules assumed to be 1.08×10^{16}.

% insulin B chain covered	% inhibition	Molecules of Acr	Acr concentration(μM)
0.42	48	4.54×10^{14}	3
1	55	6×10^{14}	4
1.68	97	1.81×10^{15}	6

Table 5. Molecular level interaction of A sample with citrate synthase. Assumption is molecular weights of Acr are 18 kDa and the substrate citrate synthase 85 kDa respectively. The number of molecules of citrate synthase molecules has been calculated based on an assay volume of 0.30 ml and the number of citrate synthase molecules is assumed to be 4.52×10^{14}.

% citrate synthase covered	%inhibition	Molecules of Acr	Acr concentration (μM)
200	95	9.53×10^{14}	5
0	0	0	0

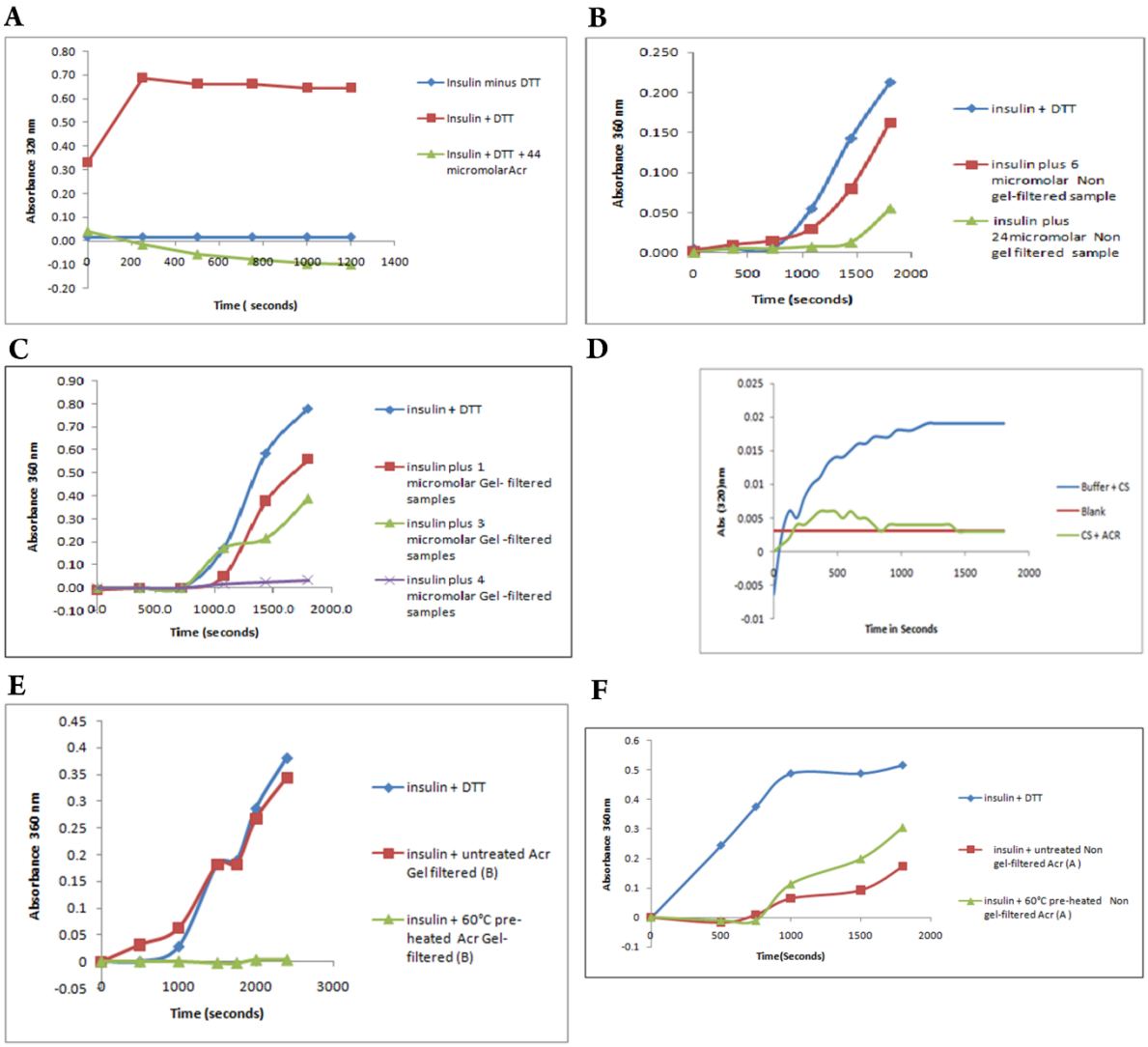

Figure 2. A) Chaperone assay of insulin aggregation (60°C) with non-gel-filtered samples: The assay was carried out using 125 μM insulin in an assay volume of 400 μl and of non-gel-filtered (A) and at Acr concentration of 44 μM along with controls of insulin without DTT and insulin with DTT. **B)** Chaperone assay of insulin aggregation (37°C) with non-gel-filtered samples: The assay of prevention of insulin B chain aggregation using A samples: insulin plus DTT; insulin with plus 6 μM of non-gel-filtered (A) sample; insulin with 24 μM of non-gel-filtered (A) sample. **C)** Chaperone assay of insulin aggregation (37°C) with gel-filtered samples: The assay of prevention of insulin B chain aggregation using B samples. Assay of prevention of insulin B chain aggregation using gel-filtered (B) samples. Insulin with DTT; 2: insulin plus 1μM gel-filtered (B) samples; 3: insulin with 3μM gel-filtered (B) samples; 4: insulin with 4μM gel-filtered (B) samples. **D)** Assay of thermal aggregation of citrate synthase using non gel-filtered (A) sample. Maroon dash indicates blank; light blue dash indicates buffer with citrate synthase light green dash indicates citrate synthase with Acr. **E)** Chaperone assay of insulin aggregation (37°C) after pre-heat treatment of gel-filtered samples: Inhibition of insulin aggregation using Acr- gel-filtered (B) sample (18 μM) and insulin 0.5 mg/ml was carried out at 37°C. Acr heated at 60°C was used to inhibit aggregation of insulin using DTT. **F)** Chaperone assay of insulin aggregation (37°C) after pre-heat treatment of non-gel-filtered samples: Inhibition of insulin aggregation using Acr-A sample (11μM) without gel filtration and insulin 1 mg/ml at 37°C. Acr heated at 60°C was used to inhibit aggregation of insulin using DTT.

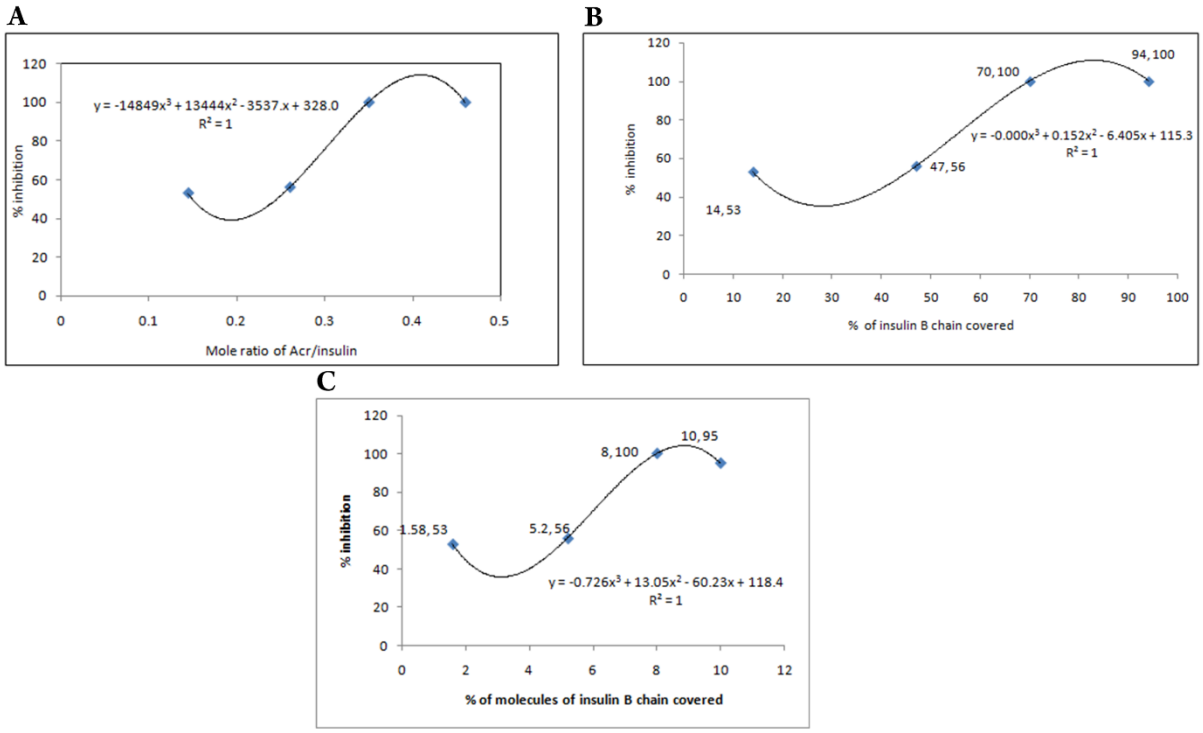

Figure 3. A) Table 2 data has been used to replot the mole ratio of gel-filtered Acr to insulin B chain in terms of percentage inhibition obtained. **B)** Table 2 data has been used to replot the number of Acr molecules that binds to insulin in terms of percentage of insulin B chain covered, assuming Acr to be an 18 kDa monomer and dividing the Avogadro number of molecules of Acr by the number of molecules of insulin B chain versus percentage inhibition. **C)** Table 2 data has been used to replot the number of molecules of Acr that binds to insulin B chain assuming Acr to be a nonamer 162 kDa and multiplying the number of molecules as shown in Figure 3b by 8.9 versus % inhibition.

The Sephacryl S-200 column was calibrated with BioRad standards (Catalogue no.151-1901), aggregates with thyroglobulin 670 kDa, globulin 158 kDa, ovalbumin 44 kDa, myoglobin 17 kDa and vitamin B12 1.5 kDa (**Fig. 1d**). A 15% denaturing SDS-PAGE was run to analyze the peak obtained throughthe gel filtration runs (**Fig. 1e**). Acr appeared as an oligomer from the preliminary data, with a size between 158 kDa and 670 kDa. No protein was observed in the later peaks.

4.3. Native-PAGE Analysis

The Native-PAGE analysis showed a predominance of 9 to 15 mers with a higher amount of 5 and 7 mers in the case of the A samples (**Fig. 1f**). The oligomeric calculation was performed by comparing the distance migrated by the oligomers with the BSA standards (**Fig. 1g**, **Table 1**)

4.4. Insulin Assays

Acr showed dose-dependent activity against insulin B chain at 60°C with 100% inhibition obtained at a mole ratio of 0.35 (**Fig. 2a**). A clear trend of increase in inhibition was observed with increase in molar concentration of Acr (**Table 2**). The amount of Acr molecules used to inhibit insulin was calculated in terms of number of molecules and it was found that this number ranged from 0.6 x 10^{16} to 1.5 x 10^{16} while the insulin B chain molecules ranged from 1.8 x 10^{15} to 1.05 x 10^{16}, wherein, more than 50% of the insulin B chain was covered, and the complete inhibition of aggregation was achieved (**Fig. 2a**). At 37°C, the chaperone activity of the A samples showed nearly 100% inhibition at a concentration of 24 µM while, the B samples showed 95% inhibition at a concentration of 6 µM (**Fig. 2b**, **2c**).

4.5. Thermal Aggregation Assay

The Thermal aggregation assay showed complete inhibition of activity at mole ratios of 2:1 of Acr to citrate synthase (**Fig. 2d**).

4.6. Heat Treatment Assays

The gel-filtered sample showed complete inhibition of insulin B chain after pre–heat treatment at 60°C for 15 mins (**Fig. 2e**). His-tag eluted sample failed to exhibit improvement in chaperone activity after pre-heat treatment, suggesting the need for secondary structure analysis to explain this difference (**Fig. 2f**).

4.7 Molecular Level Calculations

The molecular level calculations showed a typical pattern. Even though the percentage of insulin B chain covered was less in the gel-filtered (B) samples, the percentage inhibition was more than the non-gel-filtered (A) samples (**Table 3**, **4**, **5**). The molecular level calculations showed better coverage of insulin B chain if we assume Acr to be a nonamer instead of monomer (**Fig. 3a**, **3b** and **3c**).

5. Discussion

High levels of purified Acr were obtained after His-tag and gel filtration. Earlier studies have shown Acr to be a nonameric 158 kDa in the form of trimer of trimers, 210 kDa and 193 kDa (4, 7, 8, 10). The activity of A samples was two times less than that of B samples at 37°C. The difference in activity between A samples and B samples could be explained by the difference in the ratio of oligomers in both the samples. The A samples showed 43% of 11 to 15 mers as compared to the B samples which had 75% of 11 to 15 mers. In addition, the presence of 5 and 7 mers in the A samples was recorded. This could explain the higher activity of the B samples. As the ratio of oligomers increases, the activity increases due to better molecular interaction with the substrate. This is due to the physical coverage of the substrate and prevention of aggregation. In the case of citrate synthase, as the molecular weight is larger, the mole ratio required is higher than insulin.

In the pre-heat treatment assays, it was conjectured that a more favourable secondary structure might aid in improving the chaperone activity of gel-filtered Acr. Our observations indicate that pre-heating the sample might reduce the percentage of β sheets and increase the randomness and disorderliness which corroborates with previous findings (8, 10, 12). However non-gel-filtered samples did not show any improvement suggesting an analysis of secondary structure that is required to interpret this data. These results also suggest that more number of molecules of Acr can bind to insulin B chain and increase in oligomer size of Acr enhances activity after pre-heat treatment.

In order to understand the molecular interactions preliminarily, a mole ratio of Acr to insulin versus percentage inhibition was plotted (Figure 3a). The assumption in the calculation of Acr activity is that the molecular weight is 18 kDa**.** However, the number of molecules would increase if it is calculated in terms of an oligomer. In this regard, figures plotted are presented in terms of monomer and 9 mers in terms of molecules of insulin covered and % inhibition (**Fig. 3b, 3c**). It has been reported from several previous studies about the occurrence of Acr as a nonamer or dodecamer (4, 7). Our observation supports the previous findings that monomers aggregate to form oligomers that are able to bind effectively to inhibit insulin B chain aggregation. It also suggests that a higher ratio of oligomers would help binding insulin better. This is probably the basic mechanism by which heat shock proteins can prevent unfolding of proteins. The status of oligomerization of recombinant Acr protein affects chaperone activity against insulin B chain aggregation.Recent *in silico* studies on Acr have again assumed it to be a dodecamer (15).The potential of Acr as a diagnostic and vaccine is largely unfulfilled even 20 years after its discovery (16, 17).

6. Conclusions

Soluble active Acr was purified by both Ni-NTA and the gel filtration chromatography to nearly 95% purity. The chaperone activity of A samples (non-gel-filtered) against insulin at 37°C was 4 times less than that of B samples (gel-filtered). The difference in activity could be explained by the difference in the ratio of oligomers in both the samples as revealed by the Native-PAGE analysis. The activity of non-gel-filtered samples (A) at 60°C showed 100% inhibition at a molar concentration of 44 µM which is higher than 24 µm achieved with non-gel-filtered samples A at 37˚C.This difference in activity needs to be investigated by secondary structure analysis. To predict molecular level interaction of Acr, polynomial graphs can be plotted from the preliminary activity data we obtained. Molecular level interaction may be predicted in future for the *in vivo* condition in the host with the help of these polynomial graphs. These could be useful tools to detect Acr protein inside the host and to verify our postulates. Secondary structure analysis could be carried out to explain the correlation between structure and function. Further verification can be carried out by *in vivo* isolation of *M. tb* Acr and studying its interactions with insulin, citrate synthase and other substrates used *in vitro* to further strengthen the hypothesis.

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