Research Article

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Prevention of protein aggregation by extracellular fungal sucrase of *Termitomyces clypeatus*
[Protein agregasyonunun ekstraselüler *Termitomyces clypeatus* fungal sükraz enzimi ile önlenmesi]

Abstract

Objective: Extracellular sucrase from *Termitomyces clypeatus* is known to impart stability and enhance activity of cellobiase, another secreted glycosidase of the fungus through co-aggregation with cellobiase. To explore whether sucrase can bind with some proteins like Insulin, BSA, alcohol dehydrogenase (ADH), carbonic anhydrase and whey proteins and prevent their dithiothreitol (DTT) induced/thermal aggregation and/or loss of activity measuring by spectrophotometry, gel filtration assay and activity assays.

Methods: MALDI-TOF and dynamic light scattering were used to assess the monomeric and aggregated molecular size of sucrase. Thermostatted spectrophotometric assays, gel filtration assays were used to study protein aggregation. Fluorescence of bound ANS was used to monitor temperature induced structural changes in sucrase together with determination of melting temperature.

Results: The mass of the monomeric unit of sucrase as 6649 Da. Enzyme inhibited DTT induced aggregation of insulin and suppressed the thermal aggregation of carbonic anhydrase, ADH and whey proteins, respectively by 83%, 68% and 70% at 70°C. Sucrase also protected about 84% activity of ADH.

Conclusion: An extracellular fungal sucrase with a low monomeric size can efficiently prevent protein aggregation. The studies can impart knowledge about potential therapeutic applications of this industrially important enzyme in protein misfolding disorders.

Keywords: Sucrase; Termitomyces clypeatus; Protein aggregation prevention; Thermal and non-thermal protein aggregation; Resolubilisation of proteins.

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Özet

Arka plan: Termitomyces clypeatus kaynaklı extraselüler sükrazın fungal kaynaklı glikozidaz ile agregatlaşarak selülaz aktivitesini ve stabilitesini artırdığı bilinmektedir.
Amaç: Sükrazın protein benzeri olan insülin, BSA, alkol dehidrogenaz, karbonik anhidraz, süt proteinini bağlayıp bağlamadığı ve DTT ile indüklenmiş/termal

agregasyonunun önlenip önlenmediğinin, buna bağlı olarak aktivitesinin aktivitesinin kaybolup kaybolmadığının spektrofotometrik, jel filtrasyon ve aktivite ölçümü ile incelenmesidir.

Metod: Sükrazın monomerik ve agregatlaşmış molekül büyüklüğünün değerlendirilmesi için MALDI-TOF ve Dinamik ışık saçılımı yöntemleri kullanılmıştır. Termostatlı spektrofotometre yöntemi ve jel filtrasyon yöntemi protein agregasyonunu ölçmek için kullanılmıştır. Bağlı ANS'nin floresans sinyali ile sıcaklıkla indüklenmiş yapısal değişikliklerle birlikte erime noktasının izlenmesinde kullanılmıştır.

Bulgular: MALDI-TOF analizine göre monomerik sükraz birimi 6649 Da olarak belirlenmiştir. DTT ile inhibe edilmiş enzimin insülin ile agragasyonu indüklenmiş ve karbonik andihraz, alkol dehidrogenaz ve süt proteininin termal agregasyonu 70°C de sırasıyla %83, %68 ve %70 olacak şekilde baskılanmıştır. Sükraz alkol dehidrogenazın aktivitesini %84 oranında korumuş ve agregatlaşmış insülinin tekrar çözünürlüğünü kolaylaştırmıştır. Sükrazın stabilize özelliği ile katalitik etkisi birbirinden bağımsızdır ve sükraz doğal yapısında olmasına rağmen 70°C de aktivitesini ANS bağlama ve erime noktası (Tm:68.3°C) çalışmaları verilerine göre kaybetmiştir.

Sonuç: Bu çalışmaya göre elde edilen veriler, düşük monomerik yapılı fungal sükrazın protein agregasyonunu önlediği ilke defa bu çalışmada belirtilmiştir. Bu çalışma ile proteinlerin hatalı katlanmasında ortaya çıkacak rahatsızlıklarda kulanılabilme potansiyeli olan yeni bir terapötik yöntem sunulmuştur.

Anahtar Kelimeler: Sükraz; Termitomyces clypeatus; Protein agregasyonunun önlenmesi; Termal ve termal olmayan protein agregasyonu; Protein tekrar çözünürlüğü.

Introduction

Sucrase/invertase (EC 3.2.1.26), belongs to the first proteins that were identified as biocatalysts and used to establish many principles of the enzyme kinetics [1]. The enzyme, widely present in the environment, gained importance for production of syrup and ethanol from sucrose and molasses by fermentation in industry. The enzyme, purified and characterized from several sources including fungi, also received attention as a model of protein secretion [2] and protein oligomerization in yeast and eukaryotes [3].

The sucrase was earlier purified and characterized from the filamentous fungus *Termitomyces clypeatus* [4] and its role in regulatory secretion of enzymes in the fungus was deciphered [5]. In this fungus, sucrase co-aggregated with cellobiase, another glycosidase, in extra- and intracellular milieu and affected its catalytic efficiency, stability and conformation [4]. Activity of native cellobiase purified from extracellular and intracellular domains decreased after separation from sucrase and was regained partially on in vitro addition of purified sucrase. This observation was not known for any other sucrase. The findings prompted us to test whether sucrase of *T. clypeatus* possessed aggregation-preventing activity on other proteins and enzymes of non-fungal origin. The sucrase was re-purified, from high sucrose medium, as a novel protein of 13.5 kDa, with lowest size in this category [6]. The goal of the present study was to examine if sucrase could prevent thermal and nonthermal aggregation of model substrate proteins like insulin, carbonic anhydrase, ADH and whey protein and hence to investigate the prospect of a low molecular protein having chaperone like activity.

Materials and methods

Materials

BSA, insulin, carbonic anhydrase (CA), dithiothreitol (DTT), α-lactalbumin (from bovine milk), β-lactoglobulin (from bovine milk), ADH (EC1.1.1.1), NAD, NADH, sodium pyruvate and Mg-acetate were purchased from Sigma. All reagents used for making buffer solutions were of analytical grade. Whey protein isolate was procured from Ergogenic Nutrition, LLC.

Mycelial growth, production and purification of extra-cellular sucrase

Termitomyces clypeatus (Accession no. MTCC 5091, Institute of Microbial Technology, Chandigarh, India) was grown in synthetic medium containing 1% w/v sucrose, 0.5% w/v Na-succinate and other nutrients [6]. Cell free culture filtrate (as extracellular enzyme) was collected by filtration after 3–4 days of growth under secreting condition. The enzyme was purified by the following steps: ultra filtration, gel filtration chromatography, ion exchange chromatography and HPLC as reported [6].

Mass spectroscopy

In order to determine the molecular weight of the purified enzyme, 0.5 μL of a 250 fmol/μL sample of sucrase

obtained from HPGPLC [6] was mixed with 3.0 μL super DHB matrix solution and was spotted in five replicates of a 192-well MALDI sample plate. The data was analysed by a 4700 Proteomics Analyzer. Corresponding to each spot, 400 shots for MS acquisition were taken, and 10 precursors for MS/MS were selected and acquired. Data acquisition for the entire plate took 6.4 min with 76,800 laser shots taken per plate. MS/MS acquisition of the 10 precursors with 2000 shots each took 5.33 h per plate with 3.84 million laser shots taken. The "super-DHB" matrix consisted of a 9 : 1 (w/w) mixture of 2, 5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, respectively. Stock solutions of 2,5-DHB (10 mg/mL) was prepared in aqueous ACN (50%, v/v) containing 0.1% v/v TFA and 2-hydroxy-5-methoxybenzoic acid (10 mg/mL) was prepared with high purity absolute ethanol.

Enzyme activity, protein assays and optimum temperature determination

Sucrase was assayed as β-D fructofuranosidase activity using sucrose as substrate. The methods and units of enzyme activities were used as previously described [6]. To assay sucrase activities in presence of DTT, purified enzyme samples from extra-cellular fractions were incubated with 1 M DTT for 30 min and sucrase activity was checked at different intervals. ADH was assayed in 100 mM phosphate buffer, pH 7.0, containing 0.2 mM NAD^+ and 1 mM ethanol in final volume of 0.5 mL, and rate of reduction of NAD^+ was monitored at 340 nm [7]. Protein was determined by Bradford's reagent [8] using BSA as standard.

Assay of protein aggregation

Insulin dissolved in a minimum volume of 0.02 M NaOH was diluted to the required concentration (0.3 mg/mL) in 100 mM phosphate buffer (pH 7.0). The reduction of insulin was initiated by adding 20 μL of 1 M DTT to 1 mL of the sample in the spectrophotometric cuvette, and the extent of aggregation of the insulin B chain was measured as a function of time at 25°C by monitoring the apparent absorbance (scattering) at 360 nm in a UV-pharmaspec 1700 UV visible spectrophotometer from Shimadzu [9]. Thermal-induced aggregation of carbonic anhydrase (CA) was measured in the same spectrophotometer fitted with thermostatic cell holder assembly with electronic temperature control. CA solution and buffer with or without sucrase were mixed in the cuvette at room temperature and then placed in the thermostatic cell holder, and the apparent absorbance at 360 nm was monitored as a function of time [9]. Similar experiments were performed with sucrase which was preincubated at 100°C for 15 min and then cooled to room temperature in order to determine the role of temperature on aggregation-preventing properties of purified sucrase. Thermal aggregation of BSA (0.5 mg/mL in 10 mM phosphate buffer, pH 6.6) and whey protein isolate (0.5 mg/mL in 10 mM phosphate buffer, pH 6.6) were measured with and without sucrase at 400 nm at temperature 70°C [10]. Thermal aggregation of ADH (0.5 μM in 50 mM phosphate buffer, pH 7.0 with 100 mMNaCl) was monitored in absence and in presence of different molar ratios of sucrase at 50°C. Enzyme activity of ADH was determined by taking aliquots at different times from the assay mixture incubated at mentioned temperature.

The kinetics of DTT induced aggregation of 0.35 mg/mL insulin was followed at 360 nm. 1.5 mg sucrase was added to the reaction mixture at 30 min, and kinetics was continued for 10 min. For calculating the molar ratio of protein : sucrase, the molecular masses of sucrase, insulin, carbonic anhydrase and ADH were, respectively taken as 55, 5.7, 29, 150 kDa.

Gel filtration assay

The gel filtration assay of the samples was performed using a prepacked column (BIOSEP SEC S-2000 Phenomenex) attached to a protein liquid chromatography system of Shimadzu. Protein samples (α-lactalbumin and β-lactoglobulin), with or without sucrase, were heated at 70°C and immediately put on ice to bring the temperature to 25°C. The solution was filtered through a 0.45 μm filter and was loaded onto the column equilibrated with 50 mM phosphate buffer, pH 7.0. Samples were monitored by their absorbance at 280 nm.

Thermal unfolding by ANS fluorescence

ANS at a concentration of 0.6 mg/mL was added to sucrase (0.1 mg/mL in 0.1 M phosphate buffer, pH 7) in dark and the resulting solution was filtered by using a syringe top filter of pore size 0.45 μm. ANS fluorescence was acquired in a Hitachi F7000 Fluorescence spectrophotometer fitted with a thermostatted cell holder. Five nanometer excitation and 5 nm emission slit widths were used; the fluorophore was excited with a wave length of 370 nm and emission was scanned from 420 nm to 550 nm.

Determination of melting temperature by absorption analysis

T_m value of sucrase was determined in T_m analysis software (module DLL, version 1.21) supported by UV-visible spectrophotometer (Shimadzu UV 2450). Protein samples in triplicate were loaded in the micro multi cell quartz cuvette. Prior to loading, degassing of the samples was done for 10 min and samplefilled cells were tightly stoppered with special siliconcaps. Absorbance was measured from 0 to 100°C at 280 nm and T_m value was calculated by the differential method.

Study of aggregation status by dynamic light scattering

Dynamic light scattering measurements were performed with different concentrations of the purified enzyme aggregates ranging between 6 and 60 μg/mL in 0.1 M phosphate buffer (pH 7). The concentration range was selected according to the response and sensitivity of the instrument and the selected range was used in further concentration dependent characterizations. Data were acquired in a nano zeta-sizer (Malvern Instruments) DLS instrument at a fixed 90° scattering angle using a Nd-doped solid state laser of 632.5 nm with 100 s of integration time; the intensity correlation function was obtained byacquiring data between 5 and 1000 ms in 200 channels.

Results and discussion

Molecular size of the sucrase

Size of the sucrase was calculated as 6649 Da through Mass-spectroscopy (Figure 1A). However, this 6.65 kDa protomer was functionally inactive and associated with higher oligomeric forms. The minimum active conformation of the enzyme was a stable dimer of 13.5 kDa as detected through gel filtration of the aggregated enzyme. Fungal glycosidases are prone to spontaneous concentration dependent aggregation once they are released into the culture medium [11]. Therefore, it is rather difficult to control their state of aggregation during an in vitro assay. Aggregation status of purified sucrase was investigated by dynamic light scattering (Figure 1B). Fitting with our expectation, a heterogenous size distribution of three different aggregated forms of the enzyme was obtained; about 16% of the enzyme had a hydrodynamic radius of

7.6 nm, whereas majority of the enzyme (80%) occurred as a higher molecular weight aggregate with radius of 134 nm. A meagre 4.7% of the population also had a huge radius of 5021 nm which can be attributed to the aggregation prone nature of the enzyme.

Prevention of temperature-independent and temperature-dependent aggregation of proteins by sucrase

To investigate the aggregation-preventing function of sucrase, the aggregation of insulin (0.3 mg/mL) induced by DTT in 100 mM phosphate buffer (pH 7.0), was monitored at 25°C in the absence and presence of purified sucrase. Insulin lost its native conformation upon cleavage of its disulfide bond by DTT (1 M) at 25°C accompanied by aggregation (Figure 2A, curve 1). Insulin aggregation was reduced by 70%, at insulin to sucrase weight ratio (I : S) of 1 : 1 (Figure 2A, curve 2) and at I : S ratio of 1 : 2, prevention of insulin aggregation was about 86% (Figure 2A, curve 3) in 30 min. Absorbance of insulin (without DTT) was 0.22 at 360 nm (set as zero in base line) (Figure 2A, curve 4). However, parallely, residual activity of sucrase decreased to 26% in presence of DTT in 50 min (Figure 2B). Aggregation of insulin was shown to be suppressed similarly in presence of tubulin, α-casein, spectrin, HSP proteins and α-crystallin etc [12]. Complete suppression of aggregation of insulin was detected at insulin to tubulin and insulin to α-casein ratios of 1 : 10 and 1 : 0.35, respectively [9, 10]. In presence of insulin to spectrin ratio of 1 : 0.5 suppression of insulin aggregation observed was about 45% and 26% with the dimeric and tetrameric forms of the spectrin, respectively [13]. The present observations suggested an analogous role of sucrase in regulating protein aggregation at stoichiometric ratios.

Effect of temperature on aggregation-preventing activity of sucrase

The thermal denaturation and subsequent aggregation of CA was monitored in presence and absence of sucrase. The denaturation of carbonic anhydrase (CA) was observed at 60°C followed by aggregation in absence of sucrase (Figure 3A, curve 1) showed denaturation of CA followed by aggregation in absence of sucrase. At CA : S weight ratio of 1 : 1, aggregation decreased approximately by 63% (Figure 3A, curve 2), however, at CA : S weight ratio of 1 : 2, suppression of thermal aggregation was about 83%
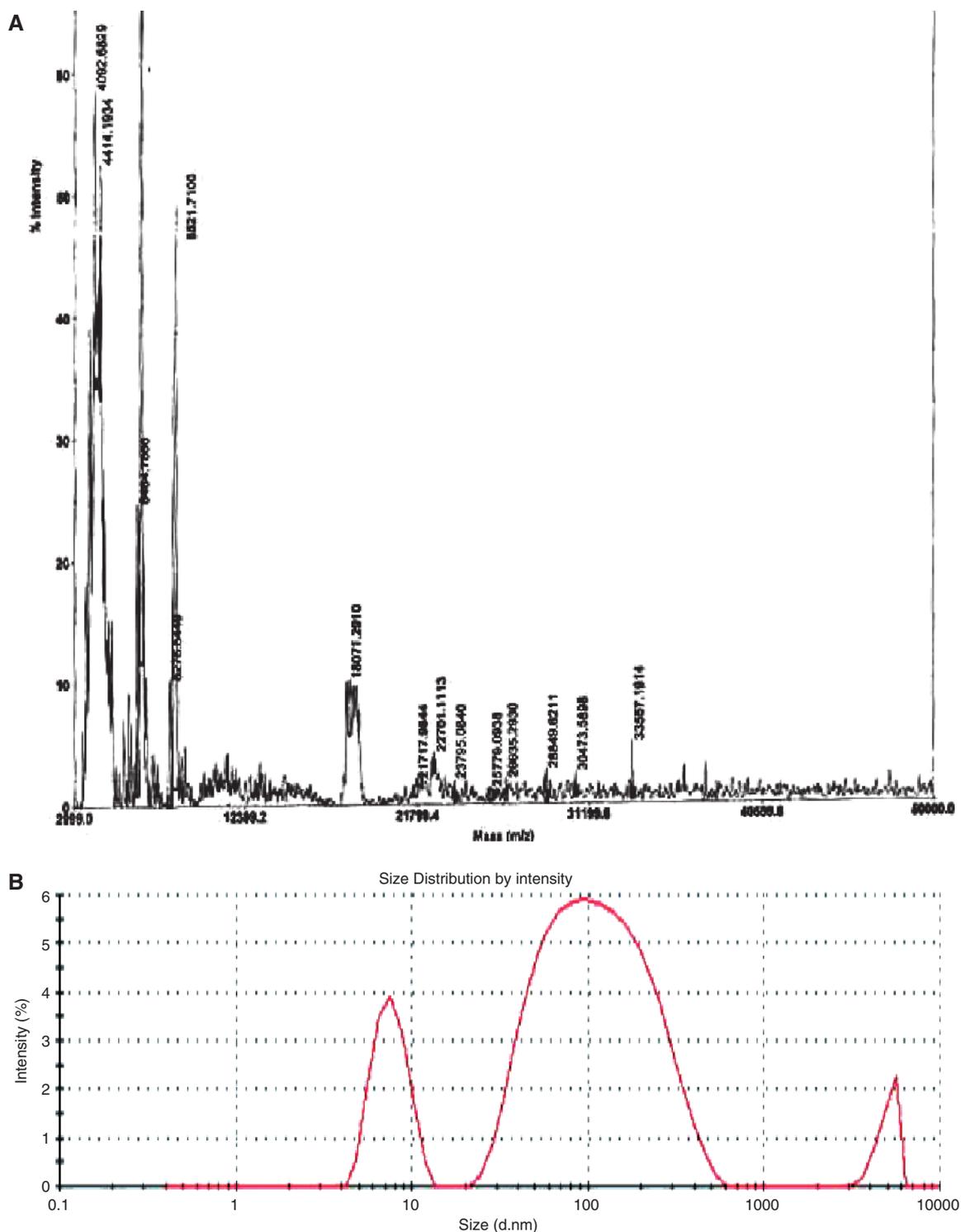
Figure 1: (A) Mass spectrometric analysis of HPGPLC pool sucrase: 10 μg sucrase obtained from HPGPLC was subjected to Applied Biosystem 4700 Proteomics Analyzer for MS analysis. (B) Dynamic light scattering to ascertain aggregation status and size of 5 μg HPGPLC sucrose.

(Figure 3A, curve 3) at 30 min. No aggregation of carbonic anhydrase was recorded at room temperature (Figure 3A, curve 5). In order to test whether native conformation of sucrase was required for its aggregation-preventing activity, the enzyme was denatured by preincubation at 100°C for 5–10 min, cooled to room temperature, and then used to prevent the thermal aggregation of carbonic anhydrase (0.1 mg/mL). Almost 90% prevention of aggregation
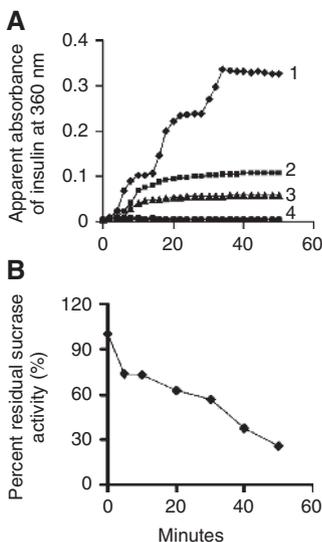
Figure 2: Aggregation of insulin in the absence and presence of sucrase.
(A) Aggregation of insulin (0.3 mg) B-chains was initiated using DTT in 100 mM phosphatebuffer (pH 7.0) at 25°C in a volume of 1 mL with: curve 1, only insulin; curve 2, plus 0.3 mg of purified sucrase; curve 3, plus 0.6 mg of purified sucrase. Curve 4 represents absorbance of only insulin (without DTT) which was adjusted as baseline. (B) Effect of DTT on sucrase activity.

Figure 3: Effect of temperature on protein-aggregation preventing property of sucrase on carbonic anhydrase and on enzyme activity of sucrase.
(A) Aggregation assay of carbonic anhydrase (0.1 mg/mL in 10 mM phosphate buffer, pH 7.0), at 60°C: curve 1, only carbonic anhydrase; curve 2, plus 0.1 mg/mL purified sucrase; curve 3, plus 0.2 mg/mL purified sucrase; curve 4, plus 0.2 mg/mL purified sucrase preincubated in 100°C for 15 min and then cooled to room temperature. Curve 5 represents absorbance of carbonic anhydrase at room temperature (27°C). Aggregation was monitored by measuring absorbance at 360 nm. (B) Temperature optima of purified sucrase. Activity of extra-cellular sucrase (-♦-) was assayed at different temperatures (30–70°C).

was observed (Figure 3A, curve 4) at weight ratio of 1:2, of CA:S in 30 min. The enzyme activity of the sucrase decreased sharply at temperature range 50–60°C and wasfully lost at 70°C (Figure 3B).

Similar to other molecular chaperones, sucrase efficiently prevented thermal aggregation of carbonic anhydrase by about 83% at approximate CA:S molar ratio of 1:0.9 in 30 min (Figure 3A), whereas α-casein prevented up to 90% thermal aggregation of carbonic anhydrase at CA:α-casein molar ratio of 1:5 [10]. Proteins like α-crystallin, mammalian sHSPs etc. are reported to show pronounced activity at higher temperatures due to their structural alterations, leading to the exposure of hydrophobic sites that bind more efficiently with the target proteins [14–18]. Similarly heat denatured sucrase (denatured at 100°C for 5–15 min) could suppress temperature-induced aggregation of carbonic anhydrase almost completely (90%) at CA:S weight ratio of 1:2 in 30 min (Figure 3A). Mammalian sHSPs and extracellular clusterin were heterogeneous aggregates of high molar mass [19] and at higher temperature, protein aggregates of mammalian sHSPs dissociated to yield chaperone-active species with exposure of hidden hydrophobic surfaces and demonstrated higher capacity to bind with target proteins [15, 19, 20]. This theory seemed to be true for the extracellular sucrase, as the enzyme was co-aggregated with cellobiase
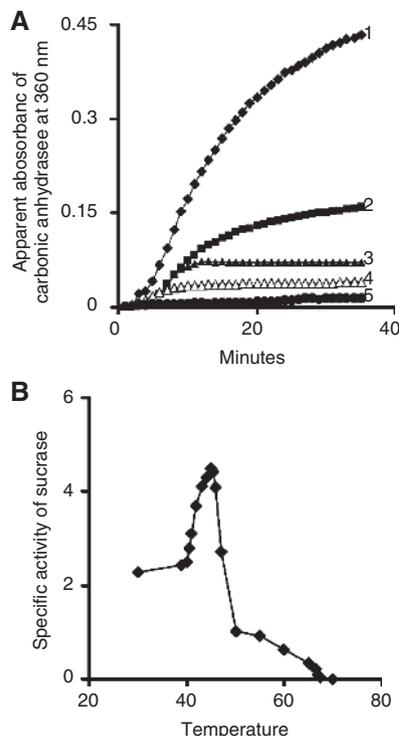
in extra and intracellular fractions and also demonstrated self-aggregating properties [5, 6]. At higher temperature oligomeric sucrase may dissociate into smaller protomeric forms and the exposed hydrophobic surfaces can bind more efficiently with target proteins, like carbonic anhydrase. This proposition was supported by the fact that about 57% of amino acid content of the sucrase was hydrophobic in nature, constituted mainly by tryptophan (25%) and cysteine (22%) [6].

Protection of the activity and aggregation of ADH

Role of sucrase in preventing the heat-induced aggregation as well as activity loss of ADH was investigated. At
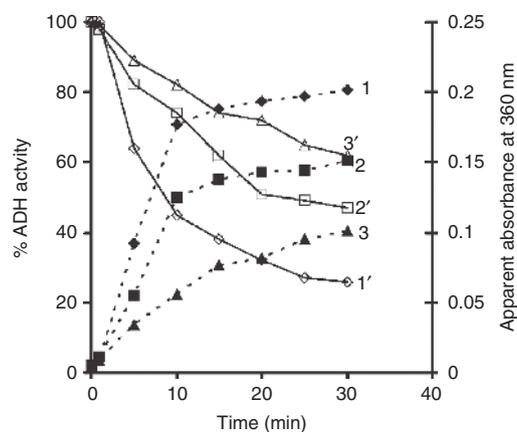
Figure 4: Effect of sucrose on thermal aggregation and inactivation of ADH.
Aggregation profiles were shown in dotted lines, and changes in enzyme activity were shown in solid lines. Curve 1 and 1′ ADH (5 μM) alone; curve 2 and 2′, ADH (5 μM) with 10 μM of sucrose; curve 3 and 3′, ADH (5 μM) with 20 μM of sucrose.

50°C ADH solutions got turbid due to the formation of large aggregates (Figure 4, curve 1) accompanied with the loss of enzyme activity. After 30 min, about 75% enzyme activity was lost (Figure 4, curve 1′). However in presence of different molar ratios of sucrose, aggregation (Figure 3, curves 2 and 3) as well as ADH activity was prevented (Figure 4, curves 2 and 3). In presence of 1 : 2 molar ratio of ADH : sucrose, approximately 30% protection of aggregation of ADH was found in 10 min (Figure 4, curve 2) and about 74% residual ADH activity was retained (Figure 4, curve 2′). Similarly when the enzyme was co-incubated with sucrose at molar ratio of 1 : 4, ADH retained 82% activity (Figure 4, curve 3′) and prevention of aggregation was about 68% (Figure 4, curve 3). All these experiments were performed in presence or in absence of ATP, ADP or GTP. In control experiments the activity of native enzyme did not change due to the presence of sucrose (data not given).

Prevention of thermal aggregation of BSA and whey proteins

0.5 mg/mL BSA solution in phosphate buffer (pH 6.6), showed increased scattering from 0.02 to 0.2 at 400 nm within 10 min (Figure 5A, curve 1) indicating the formation of BSA aggregates. However, in presence of sucrose (0.5 mg/mL) no increase of protein scattering was observed until 30 min (Figure 5A, curve 2). Turbidity of whey protein solution (0.5 mg/mL) at 70°C increased linearly with increase in time till 20 min in phosphate buffer

at pH 6.6 (Figure 5B, curve 1), which was prevented up to 70% by addition of sucrose (0.5 mg/mL) (Figure 5B, curve 2) within 10 min.

The enzyme also suppressed the thermal aggregation of citrate synthase by 42% in 20 min at weight ratio of the enzyme to sucrose as 1 : 1 (data not shown). Therefore, sucrose of *T. clypeatus* was able to protect the activity of proteins similar to the other known chaperones like tubulin [7], α-crystallin [21], NAD synthase [22], BSA [18], the C-terminal extension of fibrinogen (α_EC) [23] etc.

Prevention of formation of soluble aggregates

To study the aggregation-preventing activity of sucrose on whey protein aggregation, α-lactalbumin and β-lactoglobulin, two major constituents of whey proteins, were heated in presence and in absence of sucrose. To check if purified sucrose can prevent formation of soluble aggregates in appreciable size, a gel filtration assay was carried out since α-lactalbumin forms soluble aggregates with β-lactoglobulin in early stages of heat treatment which is undetectable through light scattering. A mixture of α-lactalbumin (1 mg/mL) and β-lactoglobulin (1 mg/mL) at pH 7.0 was heated to 70°C for 5 min and rapidly cooled to room temperature. The sample on gel filtration showed the presence of aggregated proteins of high molecular masses (≥ 350 kDa) eluting at the void volume, some intermediate aggregates of size around 100 kDa, unreacted proteins corresponding to dimeric β-lactoglobulin (36.5 kDa, elution at 9.243 min) and monomeric α-lactalbumin (14.4 kDa, elution at 9.719 min) (Figure 6, curve 1). In the presence of purified sucrose (2 mg/mL) a considerable reduction in the higher molecular mass species (350–100 kDa) was observed, size of the dimeric peak of β-lactoglobulin was reduced and most of the proteins were eluted in a single peak centered around 16 kDa (elution at 9.693 min) (Figure 6, curve 2). With increasing concentration of sucrose (4 mg/mL), there was almost no trace of higher molecular mass species and most of the proteins were eluted at 9.682 min corresponding to molecular size 16.9 kDa (Figure 6, curve 3).

Solubilization of aggregated insulin

The effect of sucrose in resolubilization of aggregated protein was investigated using insulin. Aggregation reaction was initiated by adding DTT to the native insulin (0.35 mg/mL), and when almost 80% aggregation
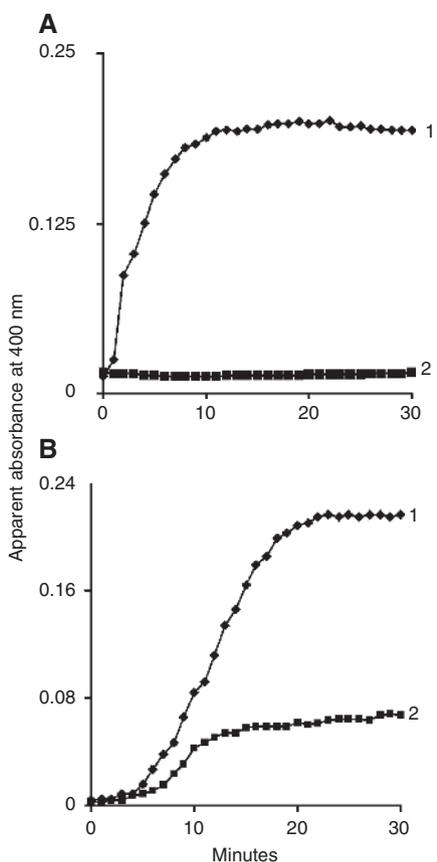
Figure 5: Prevention of thermal aggregation of whey proteins by purified sucrase.
(A) Aggregation assay of BSA. BSA (0.5 mg/mL) in 10 mM phosphate buffer, pH 6.6 at temperature 70°C in a volume of 1 mL with the following conditions: curve 1, only bovine serum albumin; curve *2*, plus 0.5 mg/mL purified sucrase. (B) Aggregation assay of whey protein isolate. Whey protein isolate (0.5 mg/mL) in 10 mM phosphate buffer, pH 6.6, temperature 70°C in a volume of 1 mL with the following conditions: curve 1, only whey protein isolate; curve 2, plus 0.5 mg/mL purified sucrase.

Figure 6: Effect of sucrase on the soluble aggregate mixture of α-lactalbumin and β-lactoglobulin. α-lactalbumin (1 mg/mL) and β-lactoglobulin (1 mg/mL) were heated in 50 mM phosphate buffer of pH 7.0.
Curve 1, mixture of α-lactalbumin and β-lactoglobulin heated at 70°C for 5 min; curve 2 mixture of proteins plus sucrase (2 mg/mL); curve 3 mixture of proteins plus sucrase (4 mg/mL). Flow rate in HPGPLC was 1.0 mL/min.

occurred at 30 min as reflected by increase in absorbance at 360 nm, excess amount of purified sucrase (1.5 mg) was added to the reaction mixture (Figure 7). It was found that sucrase not only prevented further aggregation of insulin, but it also solubilized the already-aggregated insulin up to 21% within 10 min.

ANS binding assay to probe thermal denaturation of sucrase

ANS binding was used to probe the structural changes associated with thermal unfolding of sucrase. There was a sharp blue shift from 510 nm to 450 nm concomitant
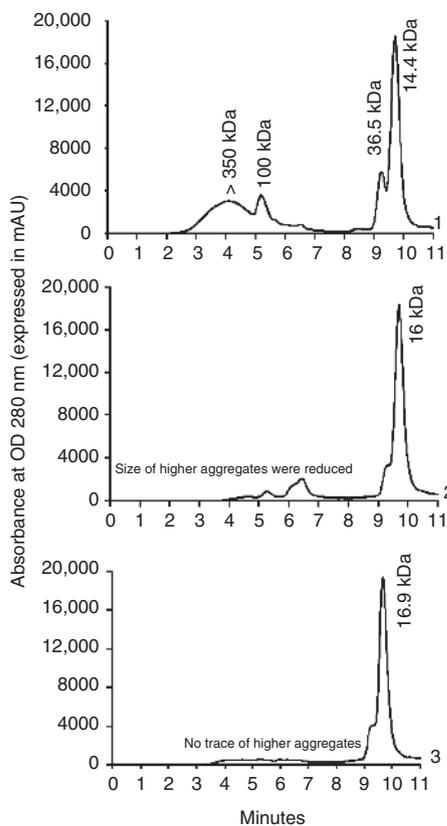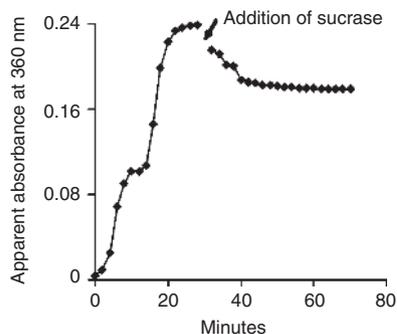

Figure 7: Effect of addition of purified sucrase on partially aggregated insulin.
Aggregation of insulin (0.35 mg) B-chains was initiated using DTT in 100 mM phosphate buffer (pH 7.0) at 25°C in a volume of 1 mL. The kinetics of DTT induced aggregation of 0.35 mg/mL insulin was followed at 360 nm (-♦-). 1.5 mg/mL purified sucrase was added at 30 min, and kinetics were continued for 10 min.
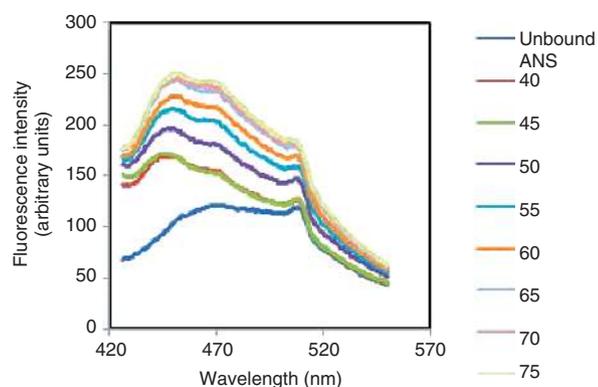
Figure 8: ANS binding profile accompanying thermal denaturation of sucrose.
ANS at a concentration of 0.6 mg/mL was added to sucrase (0.1 mg/mL) in dark in 0.1 M phosphate buffer (pH 7). The solution was excited at 370 nm and emission was recorded between 420 nm and 510 nm. The blue spectral line indicates fluorescence of free ANS in buffer and the subsequent blue shifted lines are representative of ANS fluorescence bound to sucrase scanned at intervals of 5°C at temperatures between 40°C and 75°C.

with binding of free ANS to hydrophobic sites of sucrase (Figure 8) Parallely, there was a marked increase in fluorescence intensity which continued with scans at progressively higher temperatures. This was testimonial to thermal unfolding of native sucrase resulting in availability of more and more hydrophobic sites for binding of ANS. Fluorescence intensity was saturated at about 70°C indicating complete loss of the enzyme's native structure. This data was also supported by the mean melting temperature of sucrase (68.3°C ± 4.25) as assessed by increase in absorbance at 280 nm. These data together with earlier observations on the loss of catalytic activity of sucrase indicated that the aggregation preventing activity of sucrase was independent of its catalysis and therefore was not lost on collapse of the native structure of the enzyme.

Apart from protein chaperones, prevention of non-native aggregation of proteins is known to be executed efficiently by a diverse group of small molecules which include mostly amino acids such as arginine, serine and proline, amines such as betaine and trimethyl amine N oxide, and disaccharides such as trehalose and sucrose [24, 25]. In order to be classified as a full-fledged chaperone, a molecule ought to have two properties, (a) unfolded state contraction to facilitate attainment of folded state and (b) denaturant like property to open up a misfolded state [24]. Although most of the small molecule stabilizers lack the second feature, they are efficiently applied in pharmaceutical and other industrial formulations for successful prevention of non-native aggregation

of proteins [26]. Most of these molecules are believed to interact with solvent exposed interfaces of partially unfolded proteins thus efficiently preventing their further unfolding. In order to sterically access a protein's folded contour, a smaller size of the chaperone like molecule is desirable. The smallest protein chaperones discovered and established till date are the small heat shock proteins (sHSPs) having molecular weights between 12 kDa and 43 kDa and all of them are characterized by formation of large oligomers [27]. In this respect, the present observations relating to the thermal stabilization imparted by the 6.65 kDa sucrase are promising clues for discovery of the lowest molecular weight non-synthetic chaperone which can be produced in industrial level. However, the establishment of sucrase as a novel low molecular weight chaperone satisfying the two aforesaid criteria requires further studies.

Conclusion

Sucrase is a well known glycosidase for its use in commerce and industry, and this study showed its properties on prevention of protein aggregation. The enzyme from other fungal sources is much bigger in size [28] without any proven in vitro protein stabilizing property. Earlier the C-terminal domain of mammalian membrane brush border sucrase-isomaltase complex was reported to exhibit similar property [29]. The present studies reported for first time that a fungal sucrase purified from high sucrose medium with the lowest molecular weight of its kind can successfully bind to and impart stability to other proteins of non-fungal origin. The observations also provided important insights for development of novel therapeutic interventions in a number of protein misfolding disorders. Further mechanistic insights about the interaction of native/unfolded sucrase with other proteins can only be obtained after crystal structure elucidation of the enzyme.

Conflict of interest statement: The authors declare no conflict of interest.

References

1. Michaelis L, Menten ML. Die kinetik der invertin-wirkung. Biochem J 1913;49:333–96.
2. Kelly RB. Pathways of protein secretion in eukaryotes. Science 1985;230:25–32.
3. Kern G, Schulke N, Schmid FX, Jaenicke R. Stability, quaternary structure and folding of internal, external and core glycosylated invertase from yeast. Protein Sci 1992;1:120–31.
4. Mukherjee S, Basak S, Khowala S. Regulation (Alteration) of activity and conformation of sucrase by coaggregation with cellobiase in culture medium of Termitomyces clypeatus. Biotechnol Progr 2002;18:404–8.
5. Mukherjee S, Khowala S. Regulation of cellobiase secretion in Termitomyces clypeatus by co-Aggregation with sucrase. Curr Microbiol 2002;45:70–3.
6. Chowdhury S, Ghorai S, Pal S, Banik SP. Characterization of a novel low molecular weight sucrase from filamentous fungus Termitomyces clypeatus. Process Biochem 2009;44:1075–82.
7. Manna T, Sarkar T, Poddar A, Roychowdhury M, Das KP, Bhattacharyya B. Chaperone-like activity of tubulin: binding and reactivation of unfolded substrate enzymes. J Biol Chem 2001;276:39742–7.
8. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
9. Guha S, Manna TK, Das KP, Bhattacharyya B. Chaperone-like activity of tubulin. J Biol Chem 1998;273:30077–80.
10. Bhattacharyya J, Das KP. Molecular chaperone-like properties of an unfolded protein, as-casein. J Biol Chem 1999;274:15505–9.
11. Banik SP, Pal S, Ghorai S, Chowdhury S, Majumder R, Mukherjee S, et al. In situ reversible aggregation of extracellular cellobiase in the filamentous fungus Termitomyces clypeatus. Biotechnol Bioproc Eng 2012;17:925–36.
12. Vekshin NL. How a crystallin prevents the aggregation of insulin. Biochemistry (Moscow) 2008;73:562–7.
13. Bhattacharyya M, Ray S, Bhattacharya S, Chakrabarti A. Chaperone activity and prodan binding at the self-associating domain of erythroidspectrin. J Biol Chem 2004;279:55080–88.
14. Ganea E. Chaperone-like activity of alpha-crystallin and other small heat shock proteins. Curr Protein Pept Sci 2001;3:205–25.
15. Carver JA, Lindner RA, Lyon C, Canet D, Dobson CM, Redfield C. The interaction of the molecular chaperone a-crystallin with unfolding a-lactalbumin: a structural and kinetic spectroscopic study. J Mol Biol 2002;318:812–27.
16. Fu X, Chang Z. Temperature-dependent subunit exchange and chaperone-like activities of Hsp16.3, a small heat shock protein from Mycobacterium tuberculosis. Biochem Biophys Res Commun 2004;316:291–9.
17. Kumar MS, Kapoor M, Sinha S, Reddy GB. Insights into hydrophobicity and the chaperone-like function of alphaA- and alpha B-crystallins: an isothermal titration calorimetric study. J Biol Chem 2005;280:21726–30.
18. Marini I, Moschini R, Corso AD, Mura U. Chaperone-like features of bovine serum albumin: a comparison with a-crystallin. Cell Mol Life Sci 2005;62:3092–9.
19. Jones SE, Jomary C. Clusterin. Int J Biochem Cell Biol 2002;34:427–31.
20. Gu LX, Abulimiti A, Li W, Chang ZY. Monodisperse Hsp16.3 nonamer exhibits dynamic dissociation and reassociation, with the nonamer dissociation prerequisite for chaperone-like activity. J Mol Biol 2002;319:517–26.
21. Moschini R, Marini I, Malerba M, Cappiello M, Corso AD, Mura U. Chaperone-like activity of a-crystallin toward aldose reductase oxidatively stressed by copper ion. Arch Biochem Biophy 2006;453:13–7.
22. Zha RG, Zhang F, Hiesinger PR, Cao Y, Haueter CM, Bellen HJ. NAD synthase NMNAT acts as a chaperone to protect against neurodegeneration. Nature 2008;452:887–91.
23. Tang H, Fu Y, Zhan S, Luo Y. aEC, the C-terminal extension of fibrinogen, has chaperone-like activity. Biochemistry 2009;48:3967–76.
24. Sharma S, Sarkar S, Paul SS, Roy S, Chattopadhyay K. A small molecule chemical chaperone optimizes its unfolded state contraction and denaturant like properties. Sci Rep 2013;3:3525.
25. Bondos SE, Bicknell A. Detection and prevention of protein aggregation before, during, and after purification. Anal Biochem 2003;16:223–31.
26. Gomes CM. Protein misfolding in disease and small molecule therapies. Curr Top Med Chem 2012;12:2460–9.
27. Zhang K, Ezemaduka AN, Wang Z, Hu H, Shi X, Liu C, et al. A novel mechanism for small heat shock proteins to function as molecular chaperones. Sci Rep 2015;5:8811.
28. Uma C, Gomathi D, Muthulakshmi C, GopalaKrishnan VK. Production, purification and characterization of invertase by Aspergillus flavus using fruit peel waste as substrate. Adv Biol Res 2010;4:31–6.
29. Jacob R, Purschel. B, Naim HY. Sucrase is an intramolecular chaperone located at the C-terminal end of the sucrase-isomaltase enzyme complex. J Biol Chem 2002;277:32141–8.