Indian Journal of Biotechnology
Vol 10, October 2011, pp 468-479

Comparative elucidation of properties of sucrase-cellobiase co-aggregate produced in media containing sucrose by *Termitomyces clypeatus*

Samudra Prosad Banik[1], Swagata Pal, Shakuntala Ghorai[1], Rajib Majumder, Soumya Mukherjee, Sudeshna Chowdhury and Suman Khowala*

Drug Development Diagnostics and Biotechnology, Indian Institute of Chemical Biology, Kolkata 700 032, India

[1]Department of Microbiology, Maulana Azad College, Kolkata 700 013, India

Production profiles and characterization of the sucrase-cellobiase (S-C) co-aggregates from the filamentous fungus *Termitomyces clypeatus* were compared in media containing 1% and 5% sucrose to understand the effect of cellular regulation over secretion and aggregation of these two industrially important glycosidases. The enzymes were secreted constitutively but in a high sucrose medium (5%), cellobiase secretion was reduced to a basal level of 0.03 U/mL. In intracellular, cell-bound and extracellular milieus, S/C ratios gradually declined in a predictable trend indicating participation of more cellobiase subunits for secretion. Sucrase was secreted *via* vacuoles in the fungus, following the same route as that of cellobiase and thus co-aggregates of S-C were present in the vacuolar fraction. The extracellular co-aggregates showed similar molecular sizes (>550 kDa) on zymography; however, SDS-PAGE revealed substantial difference in their subunit assemblies. Sucrase from the 5% medium showed a 2.6 times lower K_m than 1% medium. These observations demonstrated the formation of a unique S-C co-assembly, optimally suited to its needs and accommodation of the constituent subunits, to be used for biotechnological applications.

Keywords: Constitutive secretion, industrial glycosidase, regulated secretion, sucrase-cellobiase co-aggregate, *Termitomyces clypeatus*

Introduction

Regulated secretion of glycosidases is a well known phenomenon in filamentous fungus[1,2], where both self aggregation and hetero-aggregation with other enzymes play important roles in controlling the secretory titer. In most cases, the activities and stabilities of the constituent enzymes are also profoundly affected by virtue of the association[2,3]. Most of the enzymes are secreted by the fungus constitutively, e.g., sucrase-cellobiase co-aggregate[3]; however, depending on the carbon source, the secretion of some of these enzymes, e.g., xylanase[4], cellulase[5], etc, was inducible. Cellular regulation plays a pertinent role in governing this differential secretion of the glycosidases in both the constitutive and inducible routes. Filamentous fungi are adapted to grow on complex natural sugars and as such the constitutive secretion of these glycosidases are obligatory in these organisms. However, in *Termitomyces clypeatus*, it has been observed that accumulation of carbon catabolite end products exerted a direct repression over secretion[6], which can be relieved by the presence of acids of Krebs cycle (under secreting conditions). The sucrase-cellobiase co-aggregate presented an interesting model in this respect.

It was shown in earlier studies that sucrase remained aggregated with cellobiase at all stages of secretion and until the last stages of purification of both the enzymes[2,7,8]. This association was found to be vital for the activity and stability of both the enzymes, especially that of cellobiase[2]. In cellobiose 1% (w/v) medium, the titer of both the enzymes secreted by the fungus increased significantly under secreting conditions as compared to non-secreting conditions with simultaneous increase in C/S ratios as observed with time characteristics of a regulated mode of secretion[1,2,8]. This trend was also evident in 1% (w/v) sucrose medium, which ensured that the rise was not due to a mere increase in the production of cellobiase in a favourable medium. The studies obviated the fact that cellular regulation of secretion is better decipherable under secreting conditions of the growth. In a separate study, production of sucrase

*Author for correspondence:
Te: 91-33-4995813; Fax: 91-33-4735197/4730284
E-mail: sumankhowala@iicb.res.in; sumankhowala@yahoo.com

Abbreviations: TCA, Trichloro acetic acid; 1S, Sucrase produced in 1% sucrose medium; 5S, Sucrase produced in 5% Sucrose medium; S/C, Sucrase/cellobiase activity ratio; kDa: Kilo Dalton (MW)

was boosted by about 10-times in a high sucrose medium, with a concomitant fall in cellobiase titer to a basal level; the rise in sucrase activity also led to the alteration of kinetic and physicochemical properties of purified extracellular sucrase[9]. However, corresponding changes in the attributes of cellobiase were not investigated in detail[10]. In conditions, where sucrase is liberated in high titers, the status of the enzyme in co-aggregated form cellobiase is one of the essential prerequisite to understand the phenomenon of co-aggregation in a wider perspective.

In view of the above fact and in order to have better insights into the complex cellular regulation in *T. clypeatus*, the present studies were taken up to ascertain the sorting of the co-aggregates in different cellular milieus (in intracellular, vacuolar, cell-bound and extracellular fractions) *en route* secretion both in an unbiased low sucrose medium and a biased high sucrose medium together with elucidation of the kinetic and physicochemical attributes of both the enzymes in an aggregated form.

Materials and Methods

Materials

Sephacryl S 200 (MW separation range 5-250 kDa for globular proteins), cellobiose, sucrose, *p*-nitrophenyl-β-D-glucopyranoside (pNPG), Bradford reagent, *p*-methyl sulfonyl fluoride (PMSF), pepstatin, iodoacetamide and 2,3,5 triphenyltetrazolium chloride monohydrate were purchased from Sigma (USA), and dialysis tubing (MW cut off 6 kDa) was purchased from Spectrapore (USA). Electrophoretic reagents except the prestained MW marker (SM 0441, Fermentas) were obtained from Bio-Rad (USA), filtration products were of Millipore (USA). All other chemicals and salts (AR grade) were procured locally.

Growth Conditions

T. clypeatus was grown in shake-flasks at 30°C in 25/500 mL synthetic medium, containing (%, w/v): sucrose , 1 (1S) or 5 (5S); $NH_4H_2PO_4$, 2.5; sodium succinate, 0.5; $CaCl_2 \cdot 2H_2O$, 0.037; KH_2PO_4, 0.087; $MgSO_4 \cdot 7H_2O$, 0.05; boric acid, 0.057; $FeSO_4 \cdot 7H_2O$, 0.025; $MnCl_2 \cdot 4H_2O$, 0.0036; $NaMoO_4 \cdot 4H_2O$, 0.0032; $ZnSO_4 \cdot 7H_2O$, 0.03; at *p*H 5.0, as described earlier[1]. To obtain the day-wise profile, 25 mL cultures from 1 and 5% media were harvested each day. For intracellular sucrase-cellobiase co-aggregates analysis, 500 mL cultures of each of 1 and 5% medium were terminated on the 3rd d of growth.

Separation of Extracellular, Intracellular and Cell-bound Fractions

Subsequently, the mycelia were separated from the culture filtrate by filtering through a mesh with 30 μ pore size, followed by extensive washing with distilled water. Washed mycelia were collected on a pre-weighted filter paper and pressed to soak the water. Then they were macerated in 0.1 *M* acetate buffer *p*H 5 containing 1 m*M* PMSF and 15 μg/mL pepstatin A to obtain the crude intracellular and cell-bound extracts[1]. The culture filtrate was centrifuged at 8,000 rpm for 10 min and passed subsequently through a 0.22 μ filter disc.

Enzyme and Protein Assay

Cellobiase activity was assayed in terms of β-glucosidase activity[7]. The reaction mixture (1 mL) contained 4 m*M* *p*NPG in 0.1 *M* sodium acetate buffer, *p*H 5.0 and an appropriate amount of the enzyme. Incubation was carried out at 50°C for 30 min. Reaction was terminated by the addition of 0.5 mL Na_2CO_3 (1 *M*). Intensity of the yellow colour developed by liberation of *p*NP was measured at 400 nm. A unit (U) of enzyme activity was expressed by the enzyme that produced 1 μmole of *p*NP per min under the assay conditions. Sucrase activity[9] was assayed by estimating the liberation of glucose from sucrose (Sigma, USA) in 1 mL by the GOD-POD method at 505 nm and 50°C. The assay mixture containing 4 m*M* sucrose in 0.1 *M* sodium acetate buffer, *p*H 5.0 and an appropriate amount of the enzyme in a total volume of 40 μL was incubated at 45°C for 5 min. Subsequently, liberated glucose was converted by glucose oxidase (GOD) to D-glucono-1,4 lactone and hydrogen peroxide (POD), which imparted colouration in the added dye; one unit (U) of glucose oxidase is defined as the amount that converts 1 μmol of D-glucose into the product in 1 min at 25°C and *p*H 7.0. The above assay forms 1 μmol of o-dianisidine (dye in above scheme) (ε_{436} = 8.3 $mmol^{-1} \times 1 \times cm^{-1}$) per μmol of glucose oxidized. Alkaline phosphatase[11] activity was estimated by using 0.1 m*M* *p*-nitrophenyl phosphate in Tris buffer, *p*H 8.0, as a substrate. To 1 mL of the buffered *p*-nitrophenyl phosphate solution, the required amount of enzyme solution was added and readings were taken at 420 nm wavelength after keeping the solution at 27°C for 1 h. Activity of the enzyme was calculated on the basis of the amount of enzyme required to liberate 1 μmole of *p*-nitrophenol in 1 h, using a molar absorption coefficient for *p*-nitrophenol of 1.32×10^4.

Protease activity (non specific) was measured by the method of Anson[12]. In brief, requisite amount of enzyme aliquot was incubated with 0.65% casein in a 0.1 *M* potassium phosphate buffer (*p*H 7.5) in a total reaction volume of 1 mL for 30 min at 37°C. Subsequently, the reaction was stopped by addition of 0.5 mL 10% TCA. The white precipitate was centrifuged and the supernatant subsequently filtered through 0.45 µ*M* filter. Liberated tyrosine equivalents were calculated by measuring the OD_{660} of the supernatants in terms of a standard curve. Units of protease were calculated according to the following formula:

$$\text{Protease (U/mL)} = \frac{\mu\text{Moles of tyrosine equivalents released} \times \text{assay volume (mL)}}{\text{Volume of enzyme (mL)} \times \text{incubation time (min)} \times \text{volume (mL) used in colorimetric measurement}}$$

Protein was estimated by Bradford reagent according to manufacturer's technical bulletin.

Isolation of Vacuoles by Subcellular Fractionation

Vacuoles from mycelial samples were isolated according to a lab-standardized protocol[11]. In brief, fungal mycelia were macerated by hand or by bead-beater (BioSpec Products, Bartlesville, OK, USA). Filtered and centrifuged at 8,000 rpm for 5 min and the supernatant was collected as the mycelial extract (S8,000). This was again centrifuged for 30 min at 16,000 rpm at 8°C. The supernatant (S16,000) was run on a ficoll-sucrose gradient at 20,000 rpm for 4 h in a fixed angle T 865 rotor in a Sorvall Ultra 80 refrigerated centrifuge. The layers were examined for alkaline phosphatase activity (vacuolar marker enzyme). The layer with highest specific activity indicated presence of vacuoles.

Dialysis of Enzyme Aggregates

Sample replicates (4 for each 1S and 5S) were dialyzed in 6 kDa dialysis bags against 0.01 *M* acetate buffer in a volume ratio of 1:100 in separate beakers. Contents of individual bags along with their corresponding filtrates were assayed for sucrase, cellobiase and protein at defined time intervals of 2, 4, 8 and 16 h.

Partial Purification of Sucrase-Cellobiase Co-aggregate

Partially purified cellobiase from each of the extracellular mediums was obtained in the following manner. The culture filtrate was concentrated 5 times by ultrafiltration through a PM5 membrane under liquid nitrogen employing an AMICON stirred cell (Millipore, USA). The retentate was then loaded on a Sephacryl S 200 (MW separation range 5-250 kDa for globular proteins) gel permeation column in batches of 0.5 mL and subsequently eluted with 0.1 *M* acetate buffer, *p*H 5. Protein fractions (1.6 mL), eluted at the rate of 8.0 mL/h, were monitored for protein (A_{280}), sucrase and cellobiase activities; the peak pool (fractions 10-12) corresponding to the co-aggregate and containing both the enzyme activities was lyophilized, dialyzed against 0.01 *M* acetate buffer (*p*H 5) and used for further analyses as sephacryl stage.

Elucidation of Kinetic Constants

Kinetic parameters were determined using Lineweaver-Burk plot. 50 ng (for cellobiase) and 20 ng (for sucrase) coaggregate from the sephacryl stage were used for elucidation of kinetic parameters. Enzyme aliquots were incubated with 20 to 400 µ*M* pNPG for cellobiase and with 0.4 to 10 m*M* sucrose for sucrase activity measurements. Reciprocal of the corresponding enzyme activities were plotted against reciprocal of substrate concentrations to get a double-reciprocal linear plot.

Determination of *p*H and Temperature Optima

*p*H optima were determined by performing activity assays at 50°C in the *p*H range 3.0-9.0 (citrate/phosphate/tris buffer, 0.1 *M*) using 100 µg enzymes in the assay mixture. The temperature optimum of the enzymes was determined by assaying the enzymes (50 µg) in *p*H 5.0 at temperature ranges of 30-60°C.

Determination of Thermal and *p*H Stability

For temperature stability analyses, the samples were incubated at temperatures ranging between 25 and 55°C for sucrase and between 35 and 60°C for cellobiase for 1 h, then brought to room temperature and immediately assayed for respective enzyme activities. *p*H stability was determined by putting the partially purified enzyme samples buffered with respective *p*H solutions at room temperature for 1 h before assaying for cellobiase and sucrase activities. 50 and 100 µg aliquots were used for sucrase studies, whereas 50 and 300 µg aliquot were used in case of cellobiase for samples from 1 and 5% media, respectively.

SDS-PAGE

SDS-PAGE [10% gel containing 0.1% (w/v) SDS] was carried out under reducing condition using a

Mini-protein system (Bio–Rad Laboratories), run at 20 mA[13]. A prestained MW marker was run in a parallel lane. Staining was done by silver stain kit, ProteoSilver™.

Zymography of Enzyme Aggregates

Sucrase activity was detected *in situ* in 5% native PAGE at 20 mA as earlier[14]. Duplicate lanes were run for Coomassie-staining and activity staining. Immediately after electrophoresis, gels were cut into two halves. One half was washed twice with distilled water and incubated with 10% sucrose in 0.1 *M* sodium acetate buffer, *p*H 5.0 at 45°C for 30 min. Then it was rinsed thoroughly with distilled water, treated with 0.1 *M* iodoacetamide for 5 min at room temperature, and then rinsed well again with distilled water. The gels were incubated in a freshly prepared solution of 0.2% (w/v) 2,3,5-triphenyltetrazolium chloride monohydrate in 1 *N* NaOH and then heated in a boiling water bath for 2-3 min. Immediately following the appearance of the red formazan bands, the gel was again rinsed with distilled water, fixed in 7.5% (v/v) acetic acid for 30 min and stored at 4°C. The other half was stained with Coomassie to detect the corresponding bands in parallel lanes.

Results and Discussion

Distribution of Sucrase, Cellobiase and Protein in Extracellular, Cell-bound and Intracellular Fractions

It was observed that increase in sucrase titer in a high sucrose medium was accompanied by a corresponding drop in cellobiase activity, which ultimately ceases to a minimal basal level. In order to ascertain whether the fall in cellobiase activity was indeed attributable to a decreased production or due to inhibition of secretion, fungal cultures were grown in 1S and 5S media under secreting conditions (by incorporation of an acid of Krebs' cycle, sodium succinate at 0.5% in the growth medium), and separated into three fractions, for comparative assessment of sucrase, cellobiase and protein profiles. The growth of the cultures in both mediums was continued till 6th d after which mycelial lysis was seen to occur.

The data revealed that, in 1S medium, secretion of both sucrase and cellobiase increased till the last day of the studies (6th d of growth) with overall rise in specific activities by 3.58 and 45.5 times for sucrase and cellobiase, respectively (Tables 1A & B). Total production of the enzymes as ascertained from the three cellular fractions increased by 7.8 and 45.5 times for sucrase and cellobiase, respectively; however, from the 4th d onwards, secretion of both the enzymes into the extracellular medium was enhanced by 1.5 and 2.2 times, respectively till the 6th d. Total sucrase (70%) and cellobiase (78.5%) synthesized on 5th day was released into the culture filtrate, while 22 and 15% of sucrase and cellobiase, respectively were being retained intracellularly. Residual enzyme titers were localized in the cell-bound fraction indicating that they were on the verge of secretion. In all the fractions, the S/C ratios were generally found to be declining gradually except a few, especially intracellular fraction of 3rd d mycelia and cell-bound fraction of 5th d mycelia, both harvested from 5% sucrose medium (Fig. 1). This has been a characteristic trend observed in earlier studies carried out in 1% cellulose medium elsewhere[1]. Sucrase governed the extracellular release of cellobiase and remained associated with it in all stages of secretion. However, the gradual decrease in S/C ratio indicated docking and conglomeration of more and more cellobiase active units, destined for secretion, to the sucrase-cellobiase aggregate.

In 5S medium, sucrase production reached a spike height almost instantaneously on the 2nd d of growth with a corresponding steep rise in the specific activity of the enzyme by 4.2 times in the extracellular medium (Table 1B). However, unlike the earlier medium, extracellular titer of the enzyme deteriorated drastically after 2 d. Sucrase synthesis continued till the 3rd d as evident from its intracellular distribution, but corresponding enzyme activity was not observed in the extracellular growth medium. It was confirmed through later studies that this sudden decline in sucrase activity was due to conspicuous rise in protease titer in the medium on the 3rd d of growth by 3.8 times (Fig. 2). However, the effect of development of protease activity was not reflected in the extracellular total protein titer, which kept on increasing till the 6th d with occasional halts (Table 2). This could be explained either by the appearance of huge amount of protease itself, which had a compensatory effect by contributing to the total protein titer or by its narrow substrate specificity for sucrase and some other closely-related extracellular enzymes. Production of protein in 1% sucrose medium followed a consistent trend and increased till 3rd to 4th d with the consequent secretion increasing till 5th d of growth.

The activity of cellobiase in 5% medium was observed as an initial flush on the 2nd d, which

Table 1—Day-wise distribution profile of sucrase and cellobiase in 1 and 5% sucrose medium

A. Distribution profile of sucrase

1% Sucrose medium

Days of growth	Total units	Culture filtrate			Intracellular			Cell bound		
		TA	SA	PD	TA	SA	PD	TA	SA	PD
1	1.802	1.023	1.43	56.77	0.527	5.49	21.78	0.252	1.051	10.41
2	4.42	1.64	3.34	37.1	1.862	10.55	49	0.918	0.927	24.15
3	9.408	2.96	3.08	31.46	5.16	12.8	59.52	0.848	3.44	9.01
4	7.662	3.48	4.06	45.41	3.32	11.08	43.33	0.862	4.72	11.25
5	9.595	6.804	2.95	70.91	2.13	13.36	22.13	0.661	1.31	6.88
6	14.06	9.7	5.13	68.99	2.44	14.57	17.35	1.92	5.48	13.65

5% Sucrose medium

Days of growth	Total units	Culture filtrate			Intracellular			Cell bound		
		TA	SA	PD	TA	SA	PD	TA	SA	PD
1	27.33	25.35	37.14	92.74	1.60	21.18	5.85	0.383	1.22	1.4
2	111.72	105.9	156.88	94.79	4.35	17.03	3.89	1.465	1.77	1.31
3	8.21	1.15	1.30	14.01	6.52	44.4	79.4	0.54	6.35	6.5
4	2.93	0.62	0.493	21.16	2.04	26.87	69.62	0.3	3.52	10.24
5	3.12	0.58	0.366	18.59	1.6	12.3	51.28	0.94	4.09	30.13
6	2.47	0.32	0.173	12.89	1.48	19.8	59.82	0.675	1.5	27.28

B. Distribution profile of cellobiase

1% Sucrose medium

Days of growth	Totalunits	Culture filtrate			Intracellular			Cell bound		
		TA	SA	PD	TA	SA	PD	TA	SA	PD
1	0.35	0.08	0.16	22.66	0.19	1.93	52.7	0.09	0.36	24.36
2	2.16	0.94	1.39	43.68	0.83	4.72	38.51	0.39	0.39	17.8
3	7.49	4.21	4.38	56.16	2.64	6.04	35.23	0.64	2.61	8.56
4	14.34	5.72	6.67	39.88	7.12	23.76	50.27	1.5	8.23	10.46
5	14.18	11.12	4.82	78.47	2.14	13.45	15.09	3.05	6.06	21.52
6	15.94	13.74	7.28	86.21	1.3	7.71	8.15	0.896	2.56	5.62

5% Sucrose medium

Days of growth	Totalunits	Culture filtrate			Intracellular			Cell bound		
		TA	SA	PD	TA	SA	PD	TA	SA	PD
1	0.23	0.12	0.17	51.1	0.07	0.96	3056	0.04	0.14	18.26
2	0.74	0.56	0.82	74.32	0.09	0.33	11.48	0.1	0.12	13.50
3	0.14	0.03	0.03	22.22	0.08	0.53	57.58	0.03	0.59	20.00
4	0.15	0.04	0.04	27.40	0.08	1.0	52.05	0.03	0.44	20.54
5	0.14	0.03	0.02	20.70	0.06	0.46	42.86	0.05	0.21	35.71
6	0.13	0.03	0.02	22.30	0.04	0.5	30.77	0.06	0.14	48.46

TA: Total activity, SA: Specific activity, PD: Percentage distribution

declined drastically by 18.6 times on the 3rd d and was barely noticeable henceforth (Table 1B). This was attributable either to the increased production of protease in the high sugar medium or to quenching of cellobiase production itself. It seems that the second reason was more probable since the titer of the enzyme in the intracellular fraction was also maintained at a basal level throughout the entire stage of growth, indicating that its synthesis was indeed repressed unlike that of sucrase, which was traced in the intracellular fraction in significant proportions till the 3rd and 4th d of growth. However, the S/C trends remained the same as observed in 1% medium, obviating the role of sucrase in secretion of whatever little cellobiase was produced (Fig. 1b).

The results clearly corroborated the extracellular secretion pattern of sucrase[9] and cellobiase[15] and indicated that cellobiase remained co-aggregated with

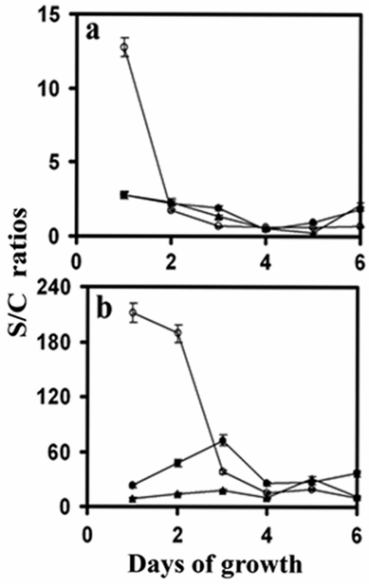

Fig. 1(a & b)—S/C ratios in 1% (a) and 5% (b) sucrose media in extracellular (○), cell-bound (▲) and intracellular (●) fractions.

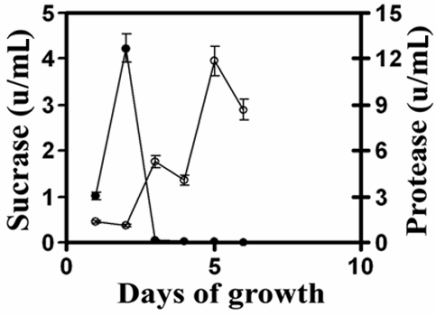

Fig. 2—Sucrase (●) and protease (○) production in 5% sucrose medium.

sucrase throughout the stages of its synthesis and secretion, even when its expression was reduced to a basal level. This is suggestive of the fact that the same cellular regulation over secretion of the co-aggregate was also operative in a biased high sugar medium (5S) where production of one of its constituent enzymes (cellobiase) was preferentially subdued.

Vacuolar Localization of Sucrase-Cellobiase Co-aggregate Prior to Secretion

Previous studies in this laboratory had successfully demonstrated that secretion of cellobiase-sucrase co-aggregate takes place through vacuoles and is regulated by cellular signaling prevailing in the fungus[11,15,16]. In an attempt to gain a proper perspective of the role of co-aggregation in secretion, vacuolar fractions were isolated and the enzyme titers were deciphered together with their corresponding specific activities.

Vacuoles were found to be concentrated in the third layer of the gradient as ascertained by the specific activity of the marker enzyme, alkaline phosphatase (Table 3). In the same layer, both sucrase and cellobiase activities were also found to be localized maximally, except for the non-secreting conditions where sucrase was barely detectable. As compared with the organellar fraction (S 10,000), specific activity of cellobiase in the vacuolar fraction increased for cellobiose by 1.32 times under secreting conditions, but it remained unchanged under non secreting conditions. However, it suffered 2- and 5-fold declines in 1 and 5% sucrose media, respectively. This may be attributed to the check imposed by the subtle cellular regulation over secretion of cellobiase in a medium (sucrose) where it is not required. The role of sucrase in shaping the aggregate *en route* secretion was more apprehensible in the vacuoles; S/C ratios increased by considerable proportions in the vacuolar layers in comparison with the organellar layers for all cases of secreting conditions. The rise was even better for the cellobiose medium, suggestive a more important role of sucrase in mediating the secretion of cellobiase rather than that of itself. In the preceding studies, it was observed that the S/C ratios had decreased for extracellular, intracellular and cell-associated fractions. This apparent contradiction can be explained in the lights of the indispensable role of sucrase in mediating the secretion of cellobiase as well as the co-aggregate on a broader sense. There is seemingly a constant deployment of sucrase in governing the enzyme assembly at the terminal stage of secretion (vacuolar exocytosis). Therefore, sucrase has to be maintained in a proportionately high concentration inside the vacuoles to accommodate for the constant turnover of the aggregate under secreting conditions. As a matter of fact, the observed titer of sucrase was less than the actual titer, since some enzyme activity was lost in the course of running the gradient layers through gel filtration columns for eliminating the free sugars. The significant role of sucrase in mediating the secretion of these glycosidases has also been observed in case of the sucrase-amyloglucosidase co-aggregate[16]. It appeared that sucrase served the role of guiding these aggregates into the extracellular milieu for concomitant utilization of a vast array of complex sugars on which these organisms thrive. Abiding by the cellular logic, this necessity of sucrase would not be a compulsion under non-secreting conditions of the enzyme. In the present study, this was indeed to be

Table 2—Distribution profile of total protein in media containing 1 and 5% sucrose

1% Sucrose medium

Days of growth	Total amount (mg)	Culture filtrate		Intracellular		Cell bound	
		TA	PD	TA	PD	TA	PD
1	0.85	0.5	58.8	0.1	11.76	0.25	29.41
2	1.86	0.68	36.56	0.18	9.6	1	53.76
3	1.65	0.96	58.18	0.44	26.67	0.25	15.15
4	1.34	0.86	64.18	0.3	22.39	0.18	13.43
5	2.96	2.30	77.7	0.16	5.4	0.5	16.9
6	2.41	1.89	78.42	0.17	7.06	0.35	14.52

5% Sucrose medium

Days of growth	Total amount (mg)	Culture filtrate		Intracellular		Cell bound	
		TA	PD	TA	PD	TA	PD
1	1.06	0.7	66.03	0.07	6.6	0.29	27.36
2	1.78	0.68	38.2	0.27	15.17	0.83	46.63
3	1.2	1	83.33	0.15	12.5	0.05	4.17
4	1.15	1	86.96	0.08	6.95	0.07	6.1
5	1.87	1.5	80.21	0.13	6.95	0.24	12.83
6	2.01	1.5	74.63	0.08	3.98	0.43	21.39

TA: Total activity, SA: Specific activity, PD: Percent distribution

Table 3—Vacuolar localization of sucrase and cellobiase in 1 and 5% sucrose medium

Sample	Gradient	Total protein (mg)	Total sucrase (units)	PD	Sp. activity (u/mg)	Total cellobiase (units)	PD	Sp. activity (u/mg)	S/C*
1% C NSc*	S 16,000	0.132	0.130	100	1.0	0.128	100	0.96	1
	1 L	0.010				0.001	0.7	0.1	
	2 L	0.040				0.027	21	0.675	
	3 L	0.028				0.07	59	2.5	
	4 L	0.016				0.022	17	1.375	
	Total	0.094				0.120			
1% C Sc	S 16,000	1.28	2.11	100	1.64	2.1	100	1.64	1
	1 L	0.06				0.01	0.4	0.108	
	2 L	0.47				0.396	19	0.586	
	3 L	0.48	1.32	63	2.75	1.5	71	2.17	0.6
	4 L	0.26				0.31	14.7	0.81	
	Total	1.27				2.2			
1% Su Sc	S 16,000	0.4	0.775	100	1.93	0.23	100	0.575	3.3
	1 L	0.05				0.007	3	0.034	
	2 L	0.09				0.034	14.7	0.086	
	3 L	0.12	0.5	64	4.17	0.134	58	0.255	1.38
	4 L	0.13				0.033	14.34	0.057	
	Total	0.39				0.208			
5% Su Sc	S 10,000	1.12	1.76	100	1.57	0.072	100	0.064	24.4
	1 L	0.01				0.007	9.72	0.22	
	2 L	0.26				0.015	20.8	0.02	
	3 L	0.72	1.18	67.04	1.64	0.028	38.9	0.013	42.5
	4 L	0.14				0.023	31.9	0.058	
	Total	1.13				0.073			

*C NSc: Cellobiose non-secreting medium, C Sc: Cellobiose secreting medium, Su Sc: Sucrose secreting medium, S/C: Sucrse/cellobiase activity ratio

the case as concentration of sucrase fell drastically in the vacuolar layer below detectable limit in the absence of sodium succinate (non-secreting conditions).

Dialysis of Co-aggregates

Aggregation of enzymes is known to be both reversible and irreversible in nature. Loose reversible aggregates are mediated by weak non-covalent forces[17] and thus can be disintegrated by sheer dilutions. In order to further understand the formation of the sucrase-cellobiase co-aggregate and the pivotal role of sucrase in precisely regulating the docking of cellobiase units therein, intracellular extracts from 1S and 5S media were dialyzed in a 6 kDa cut off membrane against the same buffer of lower ionic strength, to get rid of the ions (especially the divalent cations) and other intracellular small molecule stabilizers, which would be having a significant role in mediating the interactions leading to the formation and/or stabilization of the aggregates.

The results presented in Table 4 reveal significant functional differences between the constituent enzymes of the two aggregates, which were also reflected ostensibly in their changing S/C ratios. Sucrase activities were found to stay constant throughout the entire period of dialysis in both the cases. However, the activity of cellobiase decreased over time, resulting in a substantial rise of the S/C ratio. The effect was more intense for 5S medium where it increased by about 3.5 times after 16 h of dialysis as compared with 1S medium where the increase was a maximal of 1.7 times reached within 2 h. The extracts also underwent huge decreases in protein contents (~48 and 72% for 1S and 5S, respectively). However, none of the enzyme activities were detected outside the dialysis bag. This proved that the decline in activity of cellobiase was attributable to the changing conformations of the aggregates entailed as a consequence of filtering out of essential aggregation effectors and mediators and not due to actual loss of the cellobiase enzyme by way of dialysis. The data also indicate that the 1S sucrase was better adapted structurally than the 5S enzyme with respect to the dedicated assembly interfaces for docking and binding of cellobiase.

Table 4—Dialysis of extracellular aggregates from 1 and 5% sucrose media

Sample	Time	Volume	Protein	Sucrase	Cellobiase	S/C
1%	0	0.5	0.37	1.12	0.25	4.48
	2	0.5	0.32	1.22	0.158	7.72
	4	0.5	0.24	1.06	0.166	6.39
	8	0.7	0.24	1.07	0.173	6.18
	16	0.75	0.20	1.12	0.170	6.58
5%	0	1.0	0.58	0.8	0.1	8
	2	1.5	0.36	1.05	0.052	20.19
	4	1.8	0.19	0.82	0.04	20.5
	8	2.1	0.12	0.97	0.035	27.71
	16	2.48	0.17	1.1	0.04	27.5

Native PAGE and SDS-PAGE of Co-aggregates

Further characterization of the 1S and 5S aggregates were undertaken in a view of elucidation of the structural and functional attributes of the subunit assemblies. SDS-PAGE migration patterns of the two preparations showed different profiles of the disintegrated subunits (Fig. 3). The gels were subsequently silver stained in order to detect even the faintest of bands.

The 5S aggregate (lane 2) revealed lesser number of subunits than the 1S counterpart. The 110, 45 and 32 kDa subunits of the 1S aggregate (lane 3) were not detected in the 5S assembly. The 98 kDa band was more intense in 1S as compared to the 5S aggregate where the manifestation of the 74 kDa subunit seemed to be more pronounced. The profile collectively indicated that the composition of the 1S aggregate was significantly different from that of the 5S counterpart and this difference to a large extent was ascribable to the altered ratios of sucrase and cellobiase in the 1S and 5S enzyme assemblies. However, the same aggregates under native conditions were detected by sucrase zymography. Results demonstrated the presence of only a single

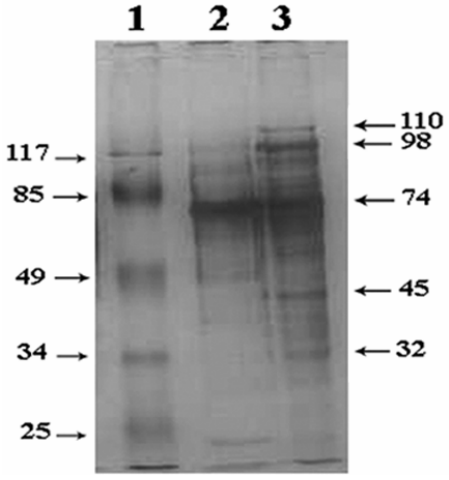

Fig. 3—SDS-PAGE of sucrase-cellobiase co-aggregates (sephacryl): MW marker (1 µg) (lane 1), and co-aggregate (5 µg) from 5% (lane 2) and 1% (lane 3) sucrose media.

band in each case at exactly the same positions (Fig 4). Size comparison with MW markers of native PAGE showed a mol wt of the aggregates, in both cases, in excess of 550 kDa. Therefore, the native aggregates were approximately of the same size irrespective of their differential subunit composition and enzyme activities. Sucrase seemed to play a significant role in modulating the interactions leading to the development of the subunit assemblies. In the 5S aggregate, absence of sufficient subunits supposedly having cellobiase activities was compensated for by increased participation of protein subunits pertaining to sucrase and or/other related enzyme activities.

Comparative Elucidation of Catalytic Efficiencies of Co-aggregates

After gaining some fundamental insight into the molecular nature of the two aggregates, it was essential to decipher the kinetic and physicochemical parameters of the two enzymes, especially sucrase to investigate whether there was any intrinsic difference between the enzymes active in 1S and 5S assemblies. The 5S sucrase was found to be catalytically superior to its 1S counterpart, especially because of its significantly better substrate affinity (2.6 times) (Table 5). This was a kind of rather obvious observation since either there were more sucrase units showing mutual cooperation in substrate binding in the 5S aggregate rather than the 1S aggregate or presence of a greater number of cellobiase subunits in the latter might have rendered the sucrase active sites inaccessible. The corresponding cellobiase enzymes had similar K_m values; however, maximal velocity of the 1S cellobiase was found to be 3-fold higher, which might be attributed to the steep increase in its specific activity (2.4) as compared to the 5S assembly (0.06).

Most of the known theories pertaining to evolution of enzyme structures in terms of functionality support the notion that, for enzymes conforming to Michaelis-Menten kinetics, evolutionary pressure acts in the direction of increased reaction fluxes. Such studies have led to a common hypothesis that a pressure for enhanced rate performances should tend to increase k_{cat}. However, there have been differences in opinion with regards to the likely effect of evolution on K_m. Fersht[18] proposed that K_m should tend to become as large as possible in comparison to the physiological substrate concentration. Cornish-Bowden[19], on the basis of certain assumptions relating to thermodynamic arguments, concluded that K_m values should exhibit no strong tendency to differ by more than a factor of 10 from the physiological substrate concentrations. One of the major contributing factors in controlling rates of enzymatic reactions is diffusion[20]; cellular micro-environment governs these interactions and regulates the docking of substrate molecules into the respective enzymes' active sites. Based on these factors, Albery and Knowles[20] had deduced a theoretical construct for defining enzyme efficiency, proposing that enzymes are said to reach catalytic perfection when they show a k_{cat}/K_m ratio of the order of 10^9 M^{-1} s^{-1} and K_m values somewhat above the *in vivo* concentration of substrate. All these factors have to be critically assessed before any long term conclusion can be drawn regarding the catalytic behaviours of sucrase and cellobiase present in the multisubunit 1S and 5S assemblies.

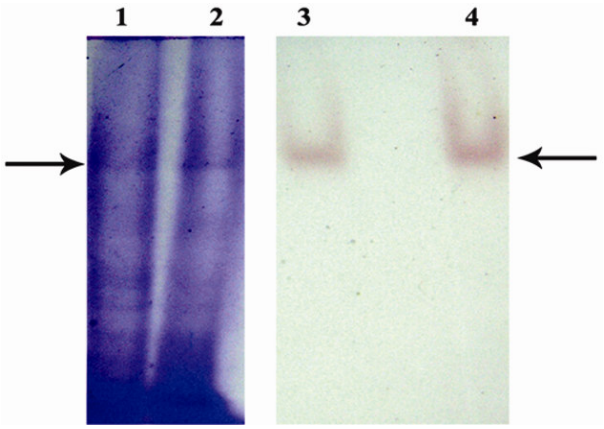

Fig. 4—Native PAGE zymography of sucrase-cellobiase coaggregate with respect to sucrase activity: Lanes 1 & 2, Coomassie staining of 1 and 5% coaggregate; and lanes 3 & 4, Sucrase activity staining of 1% & 5% coaggregate, respectively.

Table 5—Comparative kinetic efficiencies of sucrase and cellobiase in the extracellular sucrase-cellobiase coaggregate from 1 and 5% sucrose medium

Kinetic parameters	1%		5%	
	Sucrase	Cellobiase	Sucrase	Cellobiase
K_m (m*M*)	3.48	0.21	1.32 m*M*	0.17
V_{max} (u/mL)	0.148	0.095	0.094 u/mL	0.034
V_{max}/K_m	0.042	0.45	0.071	0.2

Temperature and *p*H Dependence of Co-aggregate

Inspection of the physico-chemical properties of sucrase did not reveal appreciable difference between the enzymes active in 1S and 5S assemblies (Fig. 5). The sucrases from 1S and 5S assemblies were found to be optimally active at *p*H 5.4 (Fig. 5b) and had temperature optima values of 53 (1S) and 51°C (5S),

respectively (Fig. 5a). In a separate study, temperature optima of the corresponding extracellular enzymes were reported to be 46°C for both the enzyme forms[10]. This shift in the temperature optima denoting the thermal energy required for optimal activation of the enzymes owed its origin to the differential aggregational behaviour of the extracellular and intracellular aggregates. The specific activities, however, were higher in all cases for the 5S sucrase as also observed previously. Both the sucrase enzymes were found to be moderately thermostable and retained full activities upto 45°C (Fig. 6a). The corresponding cellobiase activities were found to be more heat labile than sucrase; their activities declined steeply at temperatures above 40°C (Fig. 6b). The thermodynamic stability of the native protein conformation is only marginal, differing by a meager 5-20 kcal/mole of free energy than the unfolded, biologically inactive conformations under physiologic conditions, much weaker than covalent or ionic bonds (~150 kcal/mole)[21]. This may be accounted for the fact that the native structures of protein are held together by a culmination of various non-covalent forces involving hydrogen bonds, hydrophobic interactions, van der Waal's interactions and salt bridges in subtle synchronies (other than the disulphide and bityrosine bridges). Even a slight thermodynamic undulation can cause sufficient disturbance leading to destabilization of the entire native state. The situation can be worsened by presence of non-native aggregates whose propensity towards thermal unfolding is generally believed to be higher. However, the extracellular protein aggregates of filamentous fungi present a unique scenario in this respect; the activity and stability of these glycosidases are hugely dependent on mutual aggregation and as such the highly aggregated states resemble the native physiologic conformations for these enzymes. Therefore, thermal denaturation characteristics of either enzyme can only be interpreted with respect to thermal unfolding of the aggregates as a whole. In order to decipher the same for the individual enzymes and correlate it with the impact of their interdependence, one has to resort to molecular dynamic simulation studies which require prior knowledge of their crystal structures.

The two sucrase preparations were found to be fully functional in the *p*H range between 4 and 7; however, their activities declined drastically beyond *p*H 8 (Fig. 6c). Same trends were observed for cellobiase also; however, the effects of alkaline environment were more severe for the 5S cellobiase (Fig. 6d). The better retention of activity in the 1S cellobiase could be explained in terms of stoichiometric resistance offered by the cellobiase subunits against denaturation by altered charge distributions. Proteins are generally rendered ineffective by changes in *p*H due to altered

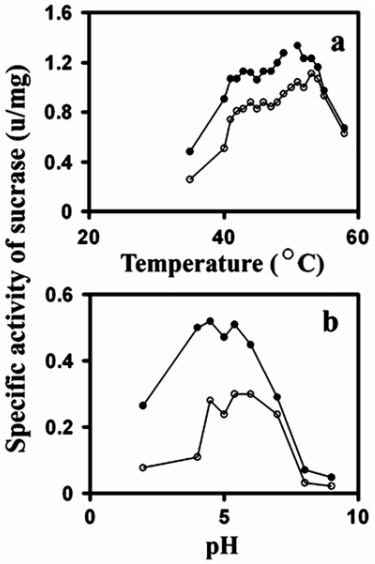

Fig. 5 (a & b)—Temperature (a) and *p*H (b) optima of sucrase in sucrase-cellobiase coaggregates (sephacryl preparations) from 1 (○) and 5% (●) sucrose media.

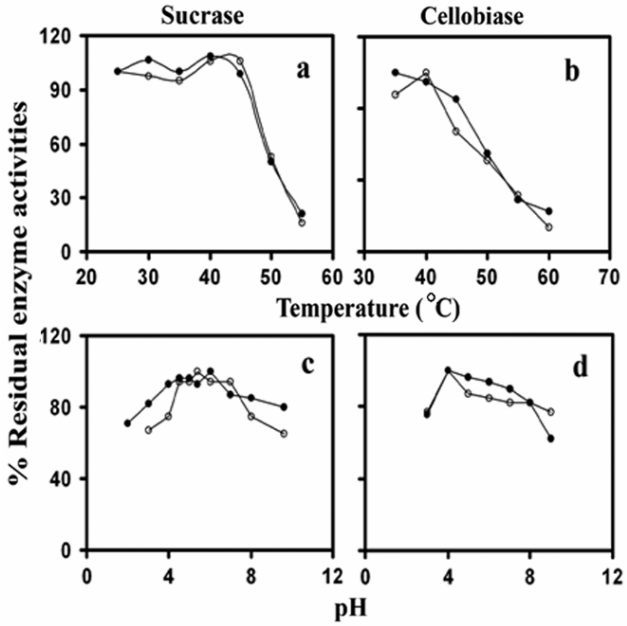

Fig. 6 (a-d)—Temperature and *p*H stabilities of sucrase and cellobiase in the sucrase-cellobiase coaggregate (sephacryl preprartions). Sucrase [temperature (a), *p*H (c)], Cellobiase [temperature (b), *p*H (d); 1% (○) and 5% (●).

electrostatic interactions governing their structures and subsequent stabilities. This can again be achieved by either of the two ways. First, classic electrostatic effects are the nonspecific repulsions that arise from charged groups on a protein when it is highly charged, for example, at *p*H far removed from the isoelectric point (pI) of the protein[22]. As the number of charged groups on a protein is increased by increasing the acidity or basicity of the solution, increased charge repulsion within the protein destabilizes the folded protein conformation because the charge density on the folded protein is greater than on the unfolded protein. Thus, *p*H-induced unfolding of proteins leads to a state of lower electrostatic free energy[22]. Secondly, specific charge interactions, such as salt bridges (or ion pairing), can also affect protein conformational stability. In contrast to the nonspecific electrostatic effect, where increasing charges destabilize the folded state, salt bridges stabilize it[23]. In addition to their effects on protein conformation, charges on protein molecules also give rise to electrostatic interactions between protein molecules. When proteins are highly charged, repulsive interactions between proteins stabilize the protein solution colloidally, making assembly processes, such as, aggregation, energetically unfavourable[24]. At *p*H values close to the pI, when proteins possess both positively and negatively charged groups, anisotropic charge distribution on the protein surfaces could give rise to dipoles. In such cases, protein-protein interactions could be highly attractive, making assembly processes, such as, aggregation energetically favourable[25].

The present observations collectively demonstrated the formation of a unique sucrase-cellobiase co-assembly which was optimally suited according to its needs and had a precise control in terms of localizing the constituent subunits. Although, in the culture medium, these aggregates were able to develop into large molar mass complexes by spontaneous protein-protein interactions, their sizes were restricted to a finite extent, at least in the intracellular domain, by some subtle cellular regulation. This in turn was achieved through provision of limited docking interfaces and/or through chaperone assisted differential folding. The central role of sucrase in governing the formation of these aggregates was bolstered by its strong chaperonic activity as studied elsewhere through *in vitro* assisted refolding experiments[10]. Further understanding of the mechanisms of aggregate formations might be possible after elucidation of the crystal structure of the aggregates and simulation studies of aggregate formation.

Conclusions

The observations collectively suggested a sucrase dependent novel co-aggregation phenomenon in a constitutive route of enzyme secretion precisely controlled by the regulatory mechanism of the fungus. In a 1% sucrose medium, the C/S (cellobiase/sucrase) ratio was much higher than that in a 5% sucrose medium, since little cellobiase would be required by the organism in the latter case. However, the aggregates purified from the two media gave activity bands at similar positions and, therefore, were of similar sizes. It seemed that a minimal size of the coaggregate was required for effective sequestering through the secretory pathway before final release into the extracellular medium by the vacuoles. Sucrase played a pertinent role in shaping the aggregate and determining its size along with other uncharacterized cellular chaperones. Therefore, the sucrase-cellobiase co-aggregate presented a lucrative model for studying protein-protein interaction and protein aggregation in a wider scientific niche. The studies conducted on the formation and secretion of these uniquely adapted enzyme assemblies are thought to provide important clues both for optimizing production of these commercially exploited enzymes as well in designing optimal production processes for therapeutics and pharmaceutical industries. Most of the commercially produced therapeutics suffer from undesired aggregation throughout the stages of production, including storage and shipping, which render them either functionally inactive or significantly decrease their shelf life. Unlike other cases, the sucrase-cellobiase coaggregate represents the optimally active native state of both the enzymes. However, the exact molecular mechanism governing their association *in vitro* and *in vivo* can only be understood through their crystal structures. This in turn will provide with cardinal breakthroughs in studies of protein aggregation associated with a host of neurodegenerative diseases.

Acknowledgement

The fellowship granted to SPB by University Grant Commission (UGC), New Delhi is duly acknowledged.

References

1 Mukherjee S & Khowala S, Regulation of cellobiase secretion in *Termitomyces clypeatus* by co-aggregation with sucrase, *Curr Microbiol,* 45 (2002) 70-73.

2 Mukherjee S, Basak S & Khowala S, Hetero-aggregation with sucrase affects the activity, stability and conformation of extra- and intra-cellular cellobiase in the filamentous fungus *Termitomyces clypeatus, Enzyme Microb Technol,* 29 (2001) 213-224.

3 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 Prog*, 18 (2002) 404-408.

4 Mukherjee M & Sengupta S, Regulation of *Termitomyces clypeatus* protoplast regeneration by Krebs cycle acids, *J Gen Microbiol*, 131 (1985) 1881-1885.

5 Mukherjee M, Khowala S, Ghosh A K & Sengupta S, Regulation of endo-β-1,4 glucanase secretion from *Termitomyces clypeatus*, *Folia Microbiol*, 40 (1995) 475-480.

6 Khowala S & Sengupta S, Secretion of β-glucosidase by *Termitomyces clypeatus*—Regulation by carbon catabolite product(s), *Enzyme Microb Technol*, 14 (1992) 144-149.

7 Ghorai S, Pal S, Chowdhury S, Banik S P & Khowala S, Enhanced activity and stability of cellobiase (β-glucosidase: EC 3.2.1.21) produced in presence of 2-deoxy *D*-glucose from the fungus *Termitomyces clypeatus*, *Carbohydr Res*, 345 (2010) 1015-1022.

8 Pal S, Banik S P, Ghorai S, Chowdhury S & Khowala S. Purification and characterization of a thermostable intracellular β-glucosidase with transglycosylation properties from filamentous fungus *Termitomyces clypeatus*, *Bioresour Technol,* 101 (2010) 2412-2420.

9 Chowdhury S, Ghorai S, Banik S P, Pal S, Basak S *et al*, Characterization of a novel low molecular weight sucrase from filamentous fungus *Termitomyces clypeatus, Process Biochem*, 44 (2009) 1075-1082.

10 Chowdhury S, *Co-aggregation of sucrase with cellobiase in the regulated secretary pathway of filamentous fungus Termitomyces clypeatus*. Ph D Thesis, Jadavpur University, Kolkata, 2009.

11 Mukherjee S & Khowala S, Secretion of cellobiase is mediated *via* vacuoles in *Termitomyces clypeatus*, *Biotechnol Prog*, 18 (2002) 1195-1200.

12 Anson, M L, The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin, *J Gen Physiol,* 22 (1938) 79-89.

13 Laemmli U K, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature (Lond)*, 227 (1970) 680-685.

14 Mukherjee S, Chowdhury S, Ghorai S, Pal S & Khowala S, Cellobiase from *Termitomyces clypeatus*: Activity and secretion in presence of glycosylation inhibitors, *Biotechnol Lett*, 28 (2006) 1773-1778.

15 Banik S P, Pal S, Chowdhury S & Ghorai S, Evidence of an alternative route of cellobiase secretion in the presence of brefeldin A in the filamentous fungus *Termitomyces clypeatus*, *J Microbiol Biotechnol*, 21 (2011) 412-420.

16 Sengupta T, Hazra P P, Mukhopadhyaya A & Sengupta S, *Termitomyces clypeatus* controls secretion of extracellular amyloglucosidase by regulating exocytosis of vacuolar enzyme, *FEMS Microbiol Lett*, 158 (1998) 101-105.

17 Moore J M, Patapoff T W & Cromwell M E M, Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody to vascular endothelial growth factor, *Biochemistry*, 38 (1999) 13960-13967.

18 Fersht A R, Catalysis, binding and enzyme-substrate complementarity, *Proc R Soc Lond (B) Biol Sci,* 187 (1974) 397-407.

19 Cornish-Bowden A, Estimation of the dissociation constants of enzyme-substrate complexes from steady-state measurements. Interpretation of *p*H-independence of K_m, *J Biochem*, 153 (1976) 455-461.

20 Albery W J & Knowles J R, Evolution of enzyme function and the development of catalytic efficiency, *Biochemistry*, 15 (1976) 5631-5640.

21 Pace C N, Shirley B A, McNutt M & Gajiwala K, Forces contributing to the conformational stability of proteins, *FASEB J*, 10 (1996) 75-83.

22 Dill K A, Dominant forces in protein folding, *Biochemistry*, 29 (1990) 7133-7155.

23 Takano K, Tsuchimori K, Yamagata Y & Yutani K, Contribution of salt bridges near the surface of a protein to the conformational stability, *Biochemistry*, 39 (2000) 12375-12381.

24 Chi E Y, Krishnan S, Kendrick B S, Chang B S, Carpenter J F *et al*, Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor, *Protein Sci*, 12 (2003) 903-913.

25 Striolo A, Bratko D, Wu J Z, Elvassore N, Blanch H W *et al*, Forces between aqueous nonuniformly charged colloids from molecular simulation, *J Chem Phys,* 116 (2002) 7733-7743.