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Characterization of Catalytic Phenotype of β-Galactosidase From LacI Mutant, E. Coli CSH-36, as a Tool For The Management of Lactose Intolerance

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Abstract: Objectives: Lactose intolerance is a common public health problem with 5-100% prevalance among different populations of the world. Use of β-galactosidase to reduce the lactose content in food or consumption of special products of milk or exogeneous lactase enzyme are among the approaches taken to manage lactose intolerance. Recombinant β-galactosidase as well as β-galactosidase derived from different sources have variances in their bioefficiency. Thermodynamic properties of β-galactosidase determines the industrial efficiency.

In this β-galactosidase from E. coli CSH-36, a constitutive expressor of enzyme was investigated for enzyme biofunctionality.

Methods: The enzyme was purified by cell lysis, ammonium sulphate precipitation and anion exchange chromatography. Purity was assesed by SDS-PAGE and Western Blotting and the native molecular weight was determined by rate zonal sedimentation.

Results & Conclusions

E. coli β-galactosidase is characterized as a 410 kD protein consisting of 3 x 120 kD trimeric subunits complexed to a 50 kD peptide with a specific activity of 170 U/mg protein and 17% yield with the applied procedure. The Km app is 1 x 10-3 M for the substrate ONPG being slightly lower than the data in literature

On further investigation of thermodynamics of the reaction for the substrate lactose, E. coli CSH-36 may serve as a new source for in vivo and in vitro hydrolysis of lactose.

Key Words: lactose intolerance β-Galactosidase, E. coli, purification, kinetics.

Introduction

Lactose intolerance due to intestinal lactase (β-galactosidase) deficiency is a common public health problem with more than 60% prevalance in most parts of the world (1). The disaccharide lactose is presents as a natural component of milk and dairy products. In the gastrointestinal tract, lactose is hydrolyzed by the enzyme β-galactosidase into glucose and galactose. The lactase activity decreases in most people at an age of 4 to 6 years. Lactose intolerance clinically presents with the symtoms of bloating, flatulence, abdominal pain and diarrhea. The approaches to alleviate lactose maldigestion are numerous (2) Use of microbial β-galactosidases to degrade lactose content in milk and dairy products is among the approaches taken for management of maldigestion (3). Protein engineering and genetic manipulations are being used as a common approach to obtain recombinant enzymes and strains with higher efficiency of enzyme synthesis. However, there is significant misfolding in genetically engineered proteins (4) as well as variances of post-transcriptional steps (5) which disrupts biofunctionality. In E. coli, β-galactosidase is coded by the lac operon. i gene codes for lac I being the repressor protein of lac operon. i. mutant CSH36 is a constitutive expressor of β-galactosidase. In other words it is known to synthesize large amounts of β-galactosidase even in the absence of inducer, which leads to increased yield of enzyme synthesis on induction. Specifically, CSH36 lacks the functional tetramer of lac repressor to bind to lac operator DNA sqeuence even though it retains the oligomerization and IPTG binding capacity (6). This study is designed to find out if there are any structural changes reflected by kinetic observations in β-galactosidase CSH36 to evaluate the catalytic integrity of the enzyme derived from mutant source, under the given conditions. The results will serve for evaluation of CSH36 β-galactosidase as an alternative source for lactose hydrolysis in milk.

Materials and Methods

Bacterial Strain

E. coli K-12 strain CSH (E7074) Δ [F'Lac I proA+B+) (lac pro) sup E thi] (Cold Spring Harbor Laboratories) (7) which is constitutive for the lac enzymes was used, as a model, to study the structural impacts of lac i-mutant phenotype on the catalytic fucntionality of β-galactosidase.

Growth Conditions

It is well-known that bacterial growth attained in minimal medium causes a cell physiology and protein composition markedly different from those in cells grown in the presence of amino acids (8). In this study, 2YT broth (16 g/lt tryptone, 10 g/lt yeast extract, 5 g/lt NaCl) was selected as enriched media for growth. Cultures were started from overnights grown at the same temperature, namely 37°C in LB medium (tryptone 10 g/lt, yeast extract 5 g/lt). 400 ml CSH 36 culture in 2YT Broth was grown to obtain a cell density of OD_{600}=1.2. With prior experience, this corresponds to the mid-exponential phase to optimize balanced growth to standardize the metabolic differences between batch cultures. For harvesting, cells were centrifuged for 10 minutes at 10.000 G and the pellet was stored frozen at -70°C. A streak was applied on LB-X gal agar (Tryptone 10 g/lt, yeast extract 5 g/lt, NaCl 5 g/lt, X-gal 40 μg 7ml) and LB agar for assesment of purity. All chemicals were from Sigma Chemical Company (St. Loius, MO, USA) unless otherwise indicated.

Purification of β-galactosidase

Cell lysis

All protein work was done at +4°C. Frozen E. coli CSH 36 was resuspended in 0.05 M NTM buffer (0.01 M Tris. HCl pH 7.6, 0.05 M NaCl, 0.01 M Mg Acetate, 0.01 Mβ-mercaptoethanol, 0.001 M Na2EDTA). Cells were sonicated for three minutes in 30 socends successions and a qualitative spot β-galactosidase assay was performed to see efficiency of sonication. On decision of sufficient lysis, the suspension was centrifuged for 15 minutes at 12.000 G and 0.8 ml of crude extract was saved at -20°C for quantitative enzyme assays, protein assays and electrophoresis.

Qualitative Spot assay for β-galactosidase activity

A spot test was developed through modification from the by Warren et al (9). Briefly, 200 μl of sonicated cell suspension was placed in an eppendorf tube and spun for 30 seconds at full speed in a microfuge. 20 μl of the crude extract was mixed with 100 μl of ONPG (o-nitrophenyl-B-D-galactoside) and the assay was allowed to proceed for 60 seconds. 50 μl of 1M Na_2 CO_3 was added and the intensity of orange color was scaled between 1-5.

Ammonium Sulphate Precipitation

The method was similar to that described by Every and Ashworth (10). Ammonium sulphate was added to the crude extract to 60% saturation, stirred for 30 minutes and centrifuged 10 minutes at 12.000 G. On observation of most of the β-galactosidase activity in the pellet, the pellet was resuspended in one-tenth volume of cold 0.05 M NTM. Solution was dialyzed against NTM buffer using cellulose tubing permeable to particles of 10.000 Daltons.

Anion Exchange Chromatography

The material obtained from the salt precipitation step is loaded on DEAE-Cellulose column (DE 52 anion exchange cellulose resin, Whatman) collected as 1.5 ml fractions and eluted with gradients of salt concentrations (upto 0.5 M NaCl). The graph in Figure 1 illustrates the elution profile of β-galactosidase, determined by qualitative β-galactosidase assay and proteins estimated by protein dye-binding assay (Bio-Rad).

Polyacrylamide Gel Electrophoresis

For the estimation of purification 7.5% SDS-PAGE was ran at 60 V for 30 minutes followed by 150 V for 50 minutes as described by Laemmli (11). 2.5 mg of proteins were loaded to each lane. Staining was done with Commassia Blue as described elsewhere (12). Myosin (205 kD), β-galactosidase (120 kD), phosphorylase B (97.4 kD), bovine albumin (66 kD), ovalbumin (45 kD) and carbonic anhydrase (29 kD) were used as molecular weight markers.

Western Blot was done as previously described (13) with 1/3000 dilution of the antibody (Boehringer-Mannheim Corp. USA) with 5 seconds exposure.

Rate Zonal Sedimentation

For the estimation of native size of the purified β-galactosidase a gradient of 5% sucrose in 0.05 M NTM buffer was prepared in 2.2 ml ultracentrifuge tubes and spun in the Beckman TL 100 in TLS55 swinging bucket rotor for 3 hours at 55.000 rpm at 4°C with brake. E. coli alkaline phosphatase (MW 80.000), catalase (MW 250.00), and jack bean urease (MW 480.000) were used as marker proteins. 100 μl fractions were collected and assayed for the markers and β-galactosidase. Alkaline phosphatase (14), catalase (15), and urease (16) assays were done spectrophotometricaly as previously described and the number of fractions were plotted against molecular weight on a semi-log scale.

Characterization of the kinetic behaviour of the enzyme

The appearant Km value was calculated by the double reciprocal plot method of Lineweaver and Burk.

Results

Figure 1 demonstrates the elution profile of ion-exchange chromatography. Only the protein peak at fractions 89-105 displayed β-galactosidase activity, determined by qualitative β-galactosidase assay. Commassia blue satined SDS-PAGE in Figure 2 gives more evidence of the discarded proteins on purification. The prominent 120 kD band in lanes 7-10, matches in mass with the pure β-galactosidase loaded on lane 11 as the β-galactosidase band on the marker lane. It is also observed that most of the protein present in the crude extract was eluted on batch additions of 0.1 and 0.2 M NaCl as observed on lane 6 loaded with fraction 38 on

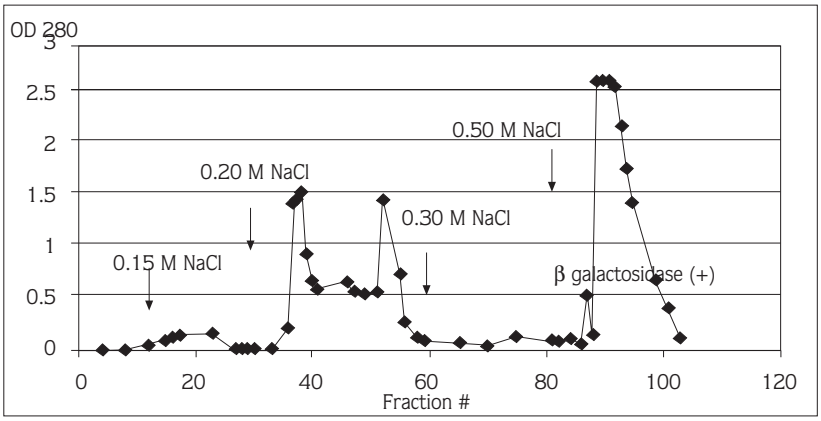

Figure 1. Ion exchange chromatography protein and b-galactosidase activity elution profile.

	Volume	Protein mg/ml	Enzyme U/ml	Specific Activity	Purfication Factor	% Yield
Crude Extract	30	2.5	83.3	33.3	1	100
Supernatant	25	1.62	35.7	21.5	1.3	36
Anion Exchange Chronatography	1	0.19	32.3	170	5.1	17

Table 1. Crude extract (10 μl). Lane 2. Supernatant (15 μl) Lane 3. Supernatant (10 μl) Lane 4. Pellet at 20% ammonium sulphate precipitation. Lane 5 Pellet at 30% ammonium sulphate precipitation. Lane 6. Fraction 38. Lane 7. Fraction 92. Lane 8. Fraction 99. Lane 10. Fraction 101. Lane 11. b-galactidose. Lane 12. Protein markers.

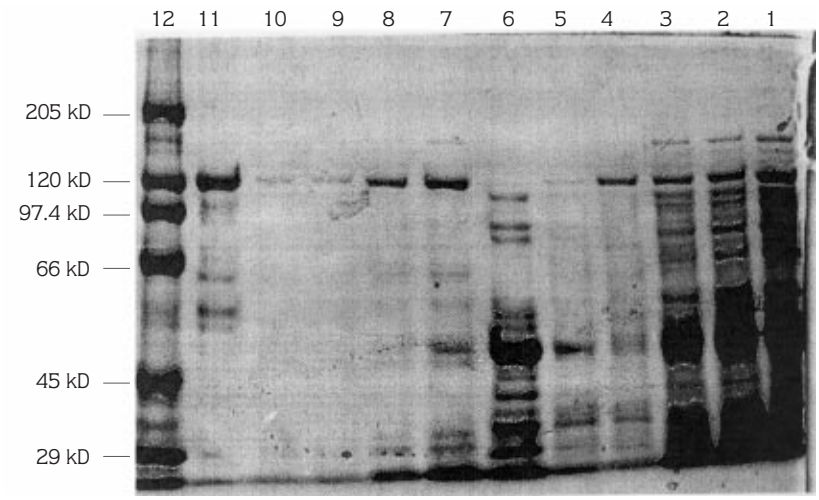

Figure 2. Commassia stained SDS-PAGE of fractions from protein purification steps.

comparison with figure 1. It is also important to notice a 50 kD band that shows up at fraction 92, however can hardy be observed on later fractions most probably due to decrease in concentration. This band is discussed later in the text as a 4th subunit of β-galactosidase with variation of post-translational modification under the experimental conditions. In order to check if the 120 kD band observed on SDS-PAGE corresponds to β-galactosidase molecule, 50 ng/μl protein concentration of fraction 94 was run on a 7.5% SDS-gel and Western transferred to be detected with anti-β-galactosidase antibody. The image reveals not only the 120 kD band but the 50 kD band as well to perfectly provide evidence of the suggested molecular structural modifications, as seen in Figure 3. Table 1 demonstrates the purification scheme. It is worthwhile to note that the extract was fairly pure from excess proteins as seen on SDS-PAGE. The actual protein concentration decreased from 2.5 mg/ml in the crude extract to 0.19 mg/ml in the pool after chromatography which counts for a decrease by a factor of 13. However, the specific activity was increased only by a factor of 5, indicating loss of enzyme activity during the procedure. It has been stated that β-galactosidase from E. coli has a flat pH optimum, which is somewhat dependent on the type of buffer used and on the added ions (17). In the presence of alkali ions (sodium ions inhibit the enzyme) which are necessary for the activity of β-galactosidase and magnesium ions which result in an increase in activity, the pH optimum of β-

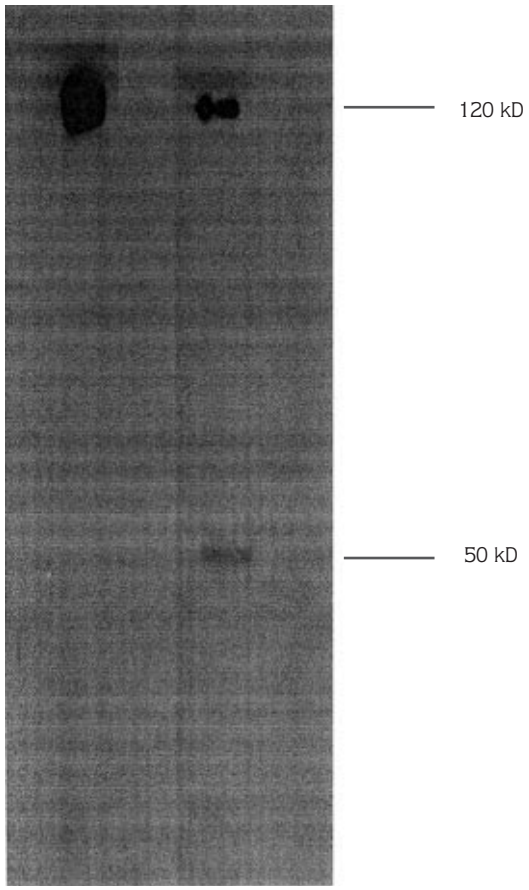

Figure 3. Western Blot of fraction 92 with anti-β-galactidose antibody.

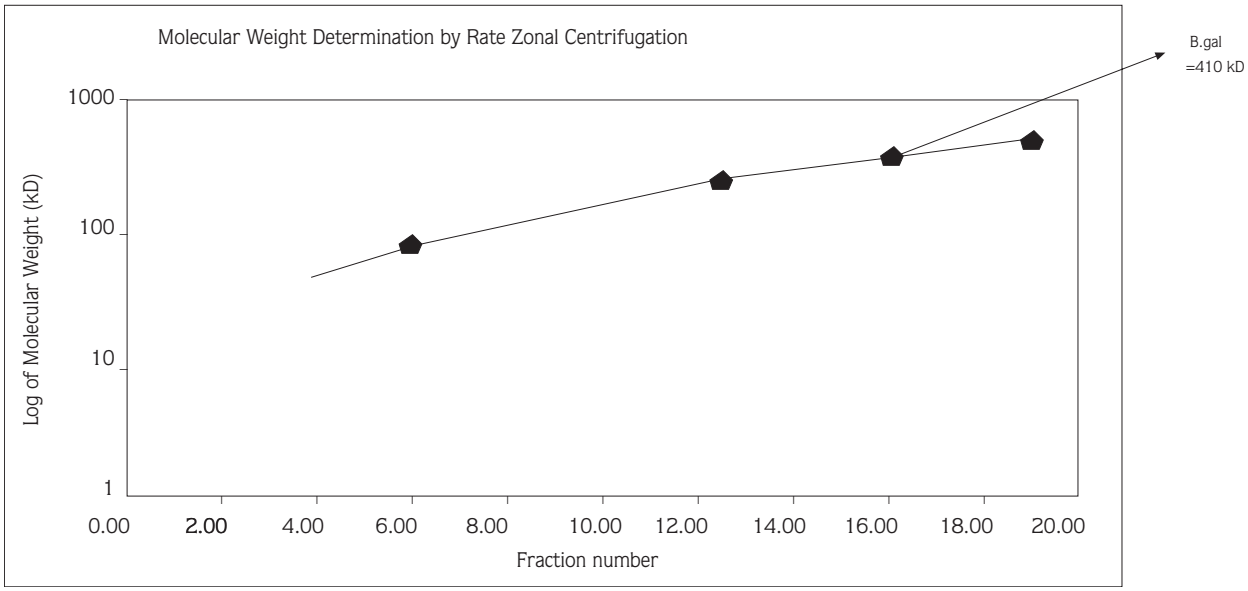

Figure 4. Specific activity of CSH36 β-galactosidase after each step of purification.

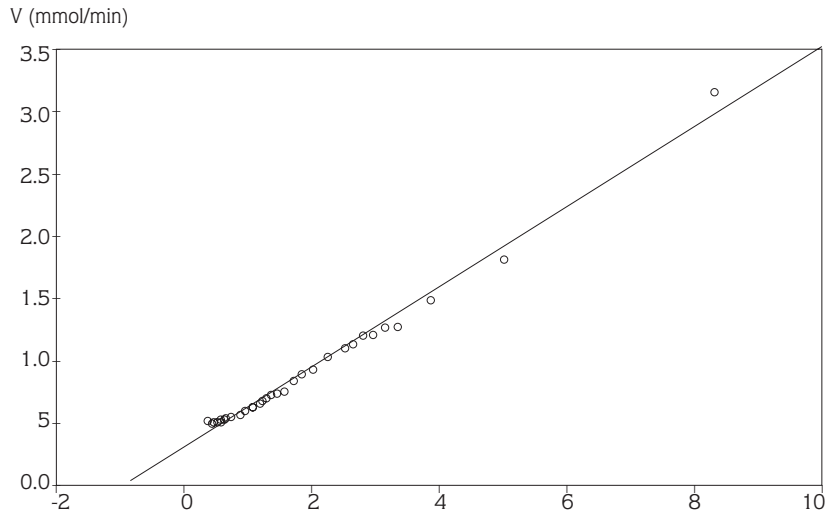

Figure 5. Molecular weight determination by rate zonalcentrifugation.

galactosidase in phosphate buffer is 6.5. The activity is said to be 85% in phosphate buffer and 90% in imidazole buffer at pH 7.5 whereas it is 85% in imidazole buffer, pH 8.0 β-galatosidases from other sources show different pH optimum properties. This study was conducted in 0.05 M NTM buffer pH 7.6. Especially after the elution from ion-exchange chtomatography Na+ concentration of the solution medium increased to as high as 0.5 N which is inhibitory on β-galactosidase. Figure 4 demonstrates the migration profile of β-galactosidase obtained on rate zonal centrifugation. β-galactosidase activity for the substrate ONPG is detected at 410 kD. The appearant Km of β-galatosidase purified is calculated as $1x10^{-3}$ from double reciprocal plot as seen in Figure 5.

Discussion

It is suggested that lactose intolerance does not become cilinically appearant unless the susceptible person attains a higher amount of dairy consumption. Thus basically diminishing the daily intake is the simplest approach in alleviating the symptoms. However being a well-established calcium source as well as other nutrients dairy products are irreplacable ingredients of natural healthy diet. The other approach taken is maneuvers to reduce the lactose content in food or consumption of special products of milk and exogeneous lactase enzyme (18). Among the available substitutes, yogurt prepared by fermenting milk with Lactobacillus Bulgaricus, sweet acidophilus milk, microbial lactase hydrolyzed milk, and commercial lactase tablets can be counted (19). In the study conducted to compare the relative efficiency of these products, it was demonstrated that best response in breath hydrogen levels was attained by yogurt, hydrolyzed lactose tablets added whole milk and sweet acidophilus milk in decreasing order. The main concern influencing the product choice of the consumer is product-acceptance. The same study indicates that reservations for plain yogurt were due to loss of palatability whereas hydrolyzed-milk was also considered to be sweeter than whole milk. It is well known that lactose milk can be hydrolyzed upto 90%. However 62% hydrolyzed milk is found to be clinically as effective as 100% hydrolyzed milk (20) estimated as breath hydrogen excretion. β-galactosidases from several bacterial sources have been experimented as candidates for lactose milk hydrolysis. Solomons and his collegues have reported a β-galactosidase from Aspergillus niger to be less effective than the enzyme from Klurveromyces lactis (Lactaid) for in vivo hydrolysis (21). E. coli β-galactosidase and the lac operon has been extensively studied by bacterial geneticists and the knowledge has served immensely in the development of procaryotic and eucaryotic protein expression systems. However this is the first study suggesting methodology to evaluate the utilization of lac mutants as a possible source to manage clinical lactose intolerance.

It is well known that in bacterial expression systems, not only introducing peptide sequences, but also culture conditions, temperature, growth medium composition and genetic factors have significant impact on protein integrity (22). This is a concern in recombinant protein functionality (23). Reasons for this is thought to be a consequence of the absence of stabilizing ligands or more likely presence of a different cellular environment which is incompatible with correct protein folding due to expression of different stress factors. It has previously

been stated that small amount of a single abnormal protein affects the cell's heat shock protein expression (23) which leads to an increased protein degradation rate. With this notion, when manipulating lac i to maximize yield of β-galactosidase synthesis special attention should be paid to conserve the native structure and kinetic efficiency of β-galactosidase as much as possible, for industrial purposes such as treatment of milk in clinical management of lactose intolerance. This study is a simple demonstration of the extent of conservation of structure under the stated conditions. The lac i-mutant CSH36 used in this study lacks the functional tetramer of lac repressor to bind specifically to lac operator DNA sequences even though it retains oligomerization and IPTG binding capacity (7). Purified β-galactosidase yielded a Km appearant of 1x10-3 M for ONPG as a substrate.

The stated Km values for β-galactosidase in E. coli are $0.95x10^{-4}$ M for ONPG (o-nitrophenyl-β-D-galactosidase), $3.23x10^{-3}$ M for phenyl-β-galactoside, $3.85x10^{-3}$ M for Lactose and $4.45x10^{-4}$ M for p-nitrophenyl-β-galactoside (25). The molecular weight for the enzyme has been determined as 540.000 at pH 7.5 (26) and 518.000 at pH 7.6. In this study the molecular weight of β-galactosidase was estimated as 410.000 kD. There is a 120.000 kD band and a 50.000 kD band retained on the purest fraction obtained after ion-exchange chromatography. This totals upto 410.000. This indicates possible proteolysis at the stated conditions on lac i-mutant. It also possible that some post-translational modifications involving possible glycosylation/acylation are missed in the experimental bacterial environment. The Km appearant was found to be $1x10^{-3}$ M for ONPG as a substrate, higher than what Weber has found. This is an indication of lower affinity for substrate as the structure is varied as seen in the results. It is esential to check the Km for lactose for the convenience for industrial utility as well as further tests to compare the efficiency of in vivo hydrolysis with present alternatives. The purity required for industrial standard is specific activity >=30 U/mg protein (17). In this study 170 U/mg protien was achieved with 17%. Thus, the suggested methodology using the lac i-strain. E. coli CSH36 is acceptable, however as an alternate to existing sources for delactosidation performance would be expected to be low with a low affinity for tested substrate.

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