Advanced Journal of Microbiology Research Vol. 2010
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

A specific inhibitory protein to a restriction enzyme from *Saccharomyces cerevisiae*

Mukaram Shikara*

Biotechnology Division, Department of Applied Sciences, University of Technology, Baghdad, Iraq.
E-mail: mukaramshikara2010@yahoo.com.

Accepted 22 May, 2010

A specific protein inhibitor for the restriction enzyme (SacC1) has been purified from *Saccharomyces cerevisiae* approximately 21,000 fold and its inhibitory properties have been characterized. The isoelectric points (pI) of SacCI and its inhibitor are 9.0 and 5.22, respectively. The molecular weight of SacC1, the inhibitor and SacC1-inhibitor complex were estimated by gel filtration on a Sephadex G-100 column to be 64,000, 32,000 and 85,000, respectively. The inhibitor protein inhibits SacC1 catalytic activities efficiently, but has no effect on other restriction enzymes tested. Inhibition does not occur unless SacC1 enzyme is exposed to the inhibitor protein prior to the reaction of the enzyme with DNA. The inhibitory activity is independent of temperature. The inhibition increased linearly with the addition of inhibitor to various amounts of SacC1, up to 85% inhibition. The slope of inhibition was constant irrespective of the initial amount of SacC1 and Ki value of 3.45 x 10^{-12} was obtained. The inhibitor interacts strongly with SacC1 and this interaction could increase the stability of the complex, possibly manifesting itself as SacC1 decreases in the dissociation rate due to the electrostatic attraction between the two groups or the stability may increase by potentially stronger electrostatic interaction. The conformational specificity between SacC1 and its inhibitor seems to be essential for their interaction. The extremely strong affinity of the inhibitor to SacC1 is remarkable and stronger than the affinity of several restriction enzymes.

Key words: *Saccharomyces cerevisiae*, inhibitor, protein, restriction enzyme, yeast, purification, Ki.

INTRODUCTION

The yeast, *Saccharyomyces cerevisiae*, has become an important organism in molecular, biochemical and genetic analysis. The organism has specific requirements for growth under a variety of conditions to produce specific or non-specific inhibitors against their restriction enzymes. The characterization of the interaction between restriction enzyme and its inhibitor is of interest for at least three reasons. Firstly, the restriction enzyme has an important genetic roles and understanding inhibition could result in new strategies for the genetic engineering. Secondly, the activity of both restriction enzyme and its inhibitor will form a high affinity complex. Finally, the mechanism of interaction between the enzyme and its inhibitor is of great interest (Richard et al., 2003).

The wide range of restriction enzymes enables the researcher to manipulate DNA in such a simple yet specific ways. Natural inhibitors are better equipped than chemical artificial inhibitors in order to play an important role in genetic engineering. Several natural inhibitors were discovered during the past years in bacteria and seem to protect the bacterium from adventitious proteolysis, probably, during secretion (Braciak et al., 1988; Muralidhara et al., 2006; Richter and Conti, 2004).

The researcher purified a restriction endonuclease (SacC1) from *S. cerevisiae* (Shikara, 2010) and in due course an inhibitor protein was discovered, inhibiting the purified enzyme. Many natural DNases and RNases inhibitors were purified, but very few (to the author's knowledge) have been purified and the goal of this study is to purify the inhibitor and investigate its mechanism

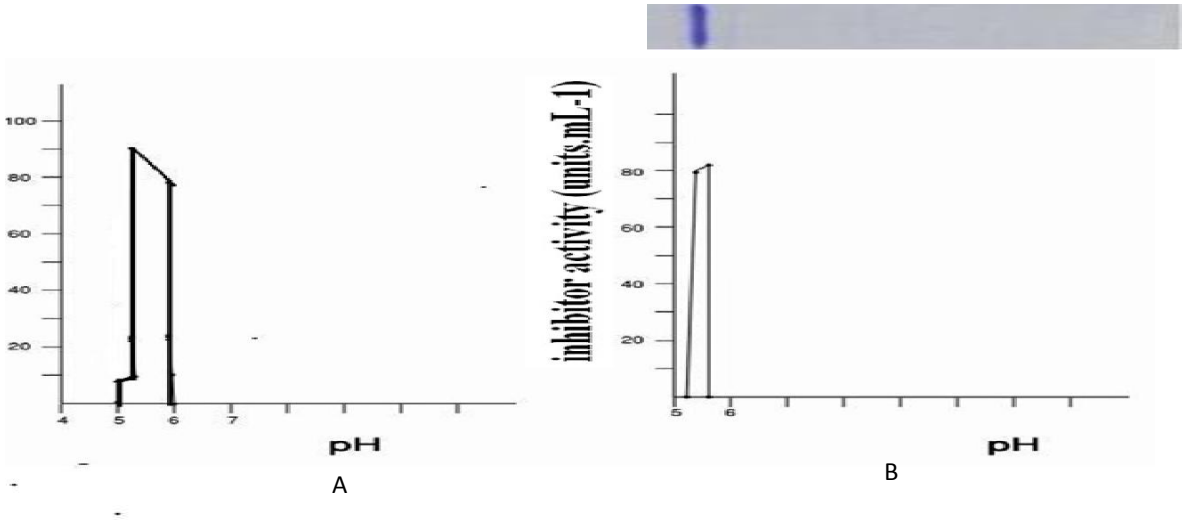

Figure 1. Isoelectricfocusing chromatography of the inhibitor A) Fraction IV was charged on an isoelectricfocusing column at a narrower pH range (4.0 to 6.0). The inhibitor was recovered with pH values of 5.15 to 5.40 and pooled (Fraction V). B) Fraction V was electrofocused to achieve at a narrower pH range (pH 5 to 6). The inhibitor was found to focus at pH 5.2 to 5.3 with the peak value being 5.22. The fractions of maximum specific activity were pooled, concentrated dialyzed against 0.02M Tris-HCl containing 0.1% glycerine, pH 7.5 with three changes for 5 h and 10 ml of dialysate (Fraction VI).

and properties.

MATERIALS AND METHODS

Sources of media and analytical chemicals

All chemicals used were of analytical grade. Media and chemicals used in this study were purchased from Sigma. Lambda DNA 32300 KD, size 48502bp, concentration 250 mg/ml and standard restriction enzymes (EcoRI and SacI) were obtained from Sigma Chemical Company. Molecular weight markers were obtained from Boehringer, Mannheim, Germany.

Isolation of the inhibitor

Isolation of yeast

S. cerevisiae strain R-Z128 was used throughout the study. Yeast was grown in YPD medium (1% yeast extract, 2% Bacto-peptone and 2% dextrose) at 30°C with constant shaking for 3 d and then the cells were lysed by sonication in ultrasonic bath (Sonicator Branson 5210) for 20 x 10 s and broken cells were removed by centrifugation (2 min, 15,600 g). The supernatant was centrifuged at 3000 g for 30 min at 4°C and the supernatant has been used as the "crude extract", source of the enzyme and inhibitor.

Purification of the inhibitor

Solid ammonium sulphate was added to the “crude extract” to form 0 to 30%, 30 to 50% and 50 to 80% saturation fractions, respectively. After centrifugation at 4000 g for 15 min, the pellet (of each fraction) was suspended in 40 mM potassium phosphate (pH 7.5) containing 5 mM 2-mercaptoethanol and 10% glycerol (buffer A) and then dialyzed with two changes against 4 L of the above buffer for 24 h and measured for endonuclease activity.

The 50 to 80% saturation fraction was found to have a high inhibitor activity, so, it has been purified further by layering onto a 1.5 x 40cm phosphocellulose column that was previously equilibrated with 4 L of the same buffer. The inhibitor activity was eluted from the column with a linear gradient of 0-0.6N NaCl in buffer A. The peaked fractions were pooled together and dialyzed for 5 h against 4 L of 40 mM Tris-HCl, pH 8.0 containing 5 mM 2-mercaptoethanl and 10% glycerol (buffer B). The pooled fractions (Fraction II) were loaded onto a Sephadex G-100 column (1.5 x 18cm) that was previously equilibrated with buffer B. The enzyme was eluted with 120 ml linear gradient of 0-1N NaCl in buffer B and one major peak was observed.

The active fractions from Sephadex G-100 were pooled and dialyzed against 4 L of buffer B for 5 h with one change. The pooled fractions (Fraction III) were concentrated by filtration through collodion bag (Sartorious, Germany) almost to dryness (Fraction IV). This fraction was charged on to an isoelectricfocusing column at a narrower pH range from pH 4.0 to 6.0. The inhibitor was recovered with pH values of 5.15 to 5.40, pooled and then concentrated (Fraction V) and was electrofocused without the addition of an ampholyte to achieve at a narrower pH range (pH 5 to 6). The inhibitor was found to focus at pH 5.2-5.3 with the peak value being 5.22. The fractions of maximum specific activity were pooled, concentrated with collodion bag and dialyzed against 0.02M Tris-HCl containing 0.1% glycerine, pH 7.5 with three changes for 5 h and 10 ml of dialysate (Fraction VI) (Figure 1).

All operations were carried out at 4°C. Endonuclease activity, inhibitor activity, protein and carbohydrate concentrations were determined for all fractions.

Determination of endonuclease and inhibitor activities

Endonuclease activity determined by measuring the amount of acid-soluble nucleotide liberated from DNA according to Brown and Smith method (1980) by incubation of incubation of 0.3 pmol lambda DNA with 3.0 pmol of the purified endonuclease enzyme in

Table 1. Purification steps of the inhibitor.

Step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (unit/mg)	Purification	Recovery %
Fraction I	30	1656.93	162.44	10.2	1	100
Fraction II	65	1326.5	3.31625	400	39.21	80
Fraction III	80	1124	0.7025	1600	156.86	68
Fraction IV	20	900.5	0.225125	4,000	392.156	54
Fraction V	22	634	0.048	13,000	1274.50	38
Fraction VI	12	345.44	0.0164	21,000	2058.8	21

a final volume of 20 µl containing 40 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 0.1 mg/ml bovine serum albumin for 1h at 37°C. The reaction mixture was stopped by cooling at 0ºC and with the addition of 20mM EDTA. The cleavage products were analyzed using 0.7 to 1.2% TBE agarose, and 5% neutral polyacrylamide gels. DNA was visualized by staining with ethidium bromide.

One unit of the enzyme was defined as the amount of the enzyme that can digest 1 µg of Lamba DNA for 1 h at 37°C.

The inhibitor was incubated briefly with the endonuclease prior to the addition of the substrate (DNA) and one unit of the inhibitor was obtained by subtracting the remaining endonuclease activity measured by the addition of the inhibitor from the original endonuclease activity of the enzyme. One unit of the inhibitor is defined as the amount that inhibits one unit of the enzyme.

Estimation of protein and carbohydrates

Protein contents were determined by the methods of Lowry et al. (1951) using Bovine serum albumin `as a standard. Carbohydrates contents were determined by the method of Dubois et al. (1956) using glucose as a standard.

Determination of Ki

The inhibitor constant (Ki) was determined by the method Lineweaver and Burk (1934) as modified by Oda et al. (2002) and Serap et al. (2006). The Inhibition was measured with lower concentration of the enzyme and inhibitor, where the dissociation of the enzyme-inhibitor complex became evident. Ki was calculated from the curves near the origin by the equation: $Ki= [I_t]/[V_0/V_{-1}]$, where $[I_t]$ is the concentration of the inhibitor. V_0 and V_{-1} are the velocities of the enzyme reaction in the absence and presence of the inhibitor, respectively.

Examination of purity and estimation of the molecular weight

The molecular mass of the enzyme was estimated by gel filtration on a Sephadex G-100 column according to the method of Andrews (1964) and by SDS-gel electrophoresis with 12% polyacrylamide gels as described by Laemmli (1970) as modified by Maizel (1971).

SacC1 and inhibitor samples were loaded (separately) onto a Sephadex G-100 column (1 x 50 cm) which was equilibrated with a buffer B. Separation was carried out at a flow rate of 0.2 ml/min. The molecular mass of the endonuclease (and the inhibitor) was estimated by comparing its elution volume with those of calibration standards, such as blue dextran blue (>100 kDa), bovine serum albumin (66.2 kDa), Egg albumin (45 KDa), chymotrypsinogen A (25 kDa), lysozyme (14.4 kDa) and cytochrome C (12.4 kDa). Elution profiles were monitored by measuring absorbance at 280 nm. For the interpolation of unknown molecular mass, a linear dependence of the logarithm of the molecular mass on the elution time was assumed.

RESULTS

Purification of the inhibitor

A summary of the purification steps (as in methods) is presented in Table 1.

Properties of the inhibitor

Homogeneity

The final preparation of the inhibitor (Fraction VIII) seemed homogenous on SDS-PAGE carried out in 12% acrylamide gels (Figure 1).

Ultraviolet absorption

The inhibitor showed a typical ultraviolet absorption curve of protein with maximum 270 nm and the A260:A280 ration was 1.40

SacC1-inhibitor complex formation

As long as the inhibitor was not obtained in high purified form, the existence of the specific complex between SacC1 and its inhibitor could not be ascertained because the association of the molecules could be mediated by other proteins.

When nearly equal volumes or amounts (in units) of the purified SacC1 and the inhibitor protein were mixed in buffer B and immediately applied to Sephadex G-100 column, SacC1-inhibitor complex was eluted at a position earlier than either of that of SacC1 or the inhibitor, whereas no SacC1 activity or an inhibitor activity were observed at their original position (Figure 2). The complex had little endonuclease activity by itself.

Molecular mass estimation

Molecular weights of SacC1, the inhibitor and the SacC1-

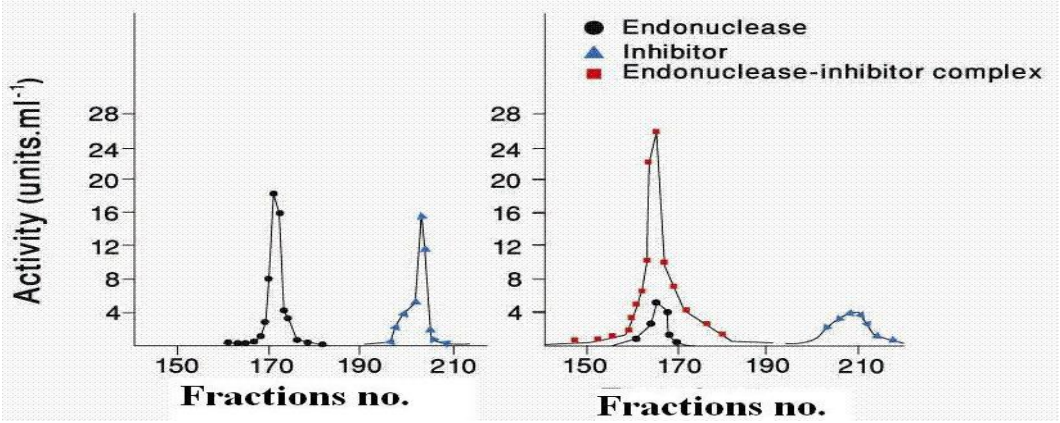

Figure 2. Sephadex G-100 column chromatography of SacC1, the inhibitor and SacC1-inhibitor complex. A) Each SacC1 and the inhibitor were chromatographed separately on the column; B) A mixture of SacC1 and the inhibitor was chromatographed on the column.

Table 2. Effect of the pH on the inhibitor.

pH	Activity (units)
4	20
5	40
6	45
7	76
8	100
9	70
10	12

inhibitor complex were estimated from their elution from Sephadex G-100 column to be 64,000, 32,000 and 85,000, respectively using standards (as in methods). By using SDS-PAGE, the molecular weight of the enzyme, the inhibitor and enzyme-inhibitor complex were 68,000, 35,000 and 87,000, respectively.

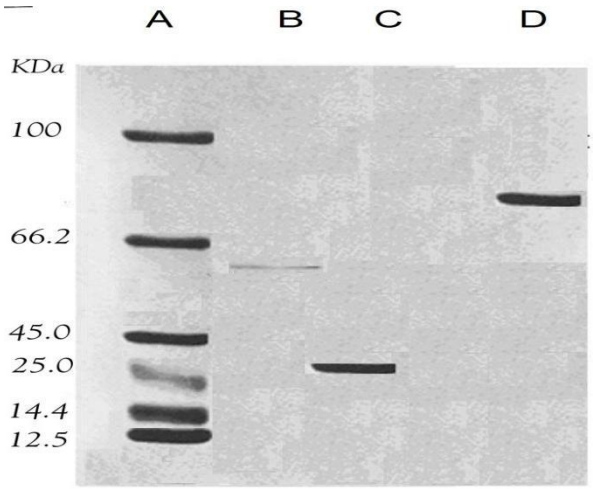

Figure 3. Determination of the molecular weight of SacC1 (B) its inhibitor (C) and SacC1-inhibitor complex(D), while (A) is the protein ladder (10-100 kDa) that was used as the protein molecular weight marker.

pH optimum and effect of different cations

The inhibitor has a maximum activity in buffer B, pH 8.0 (Table. 2). Mg^{+2} or Mn^{+2} reduced the inhibition rate, while all other cations have no effect on the inhibitor.

Isoelectric point

The isoelectric points (pI) of SacC1 and its inhibitor are 9.5 and 5.22, respectively at 4°C.

Properties of the inhibition reaction

Optimal conditions for inhibition

The primary requirement of the inhibition is a brief incubation of the SacC1 with the purified inhibitor protein prior to the addition of substrate DNA inhibition, is nearly maximal after 5 to 10 min. This level of maximum inhibition, as well as the rate at which it achieves, increased with the increasing amount of the inhibitor protein (Figure 3 and 4).

By contrast, if the inhibitor protein is added to the DNA before SacC1, or if the inhibitor protein added after the reaction has been initiated, no inhibitor is observed (Figure 5). It is clear that the inhibitor protein does not interact with the DNA, but rather with SacC1 before it initiates DNA degradation.

The mode of inhibition

The inhibitor inhibits SacC1 noncompetitively with Km of

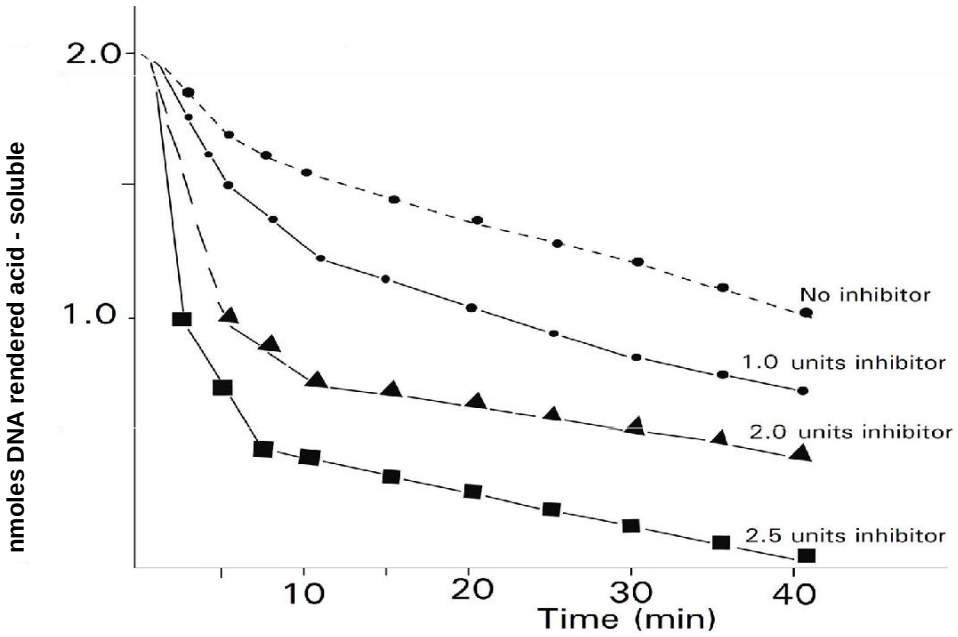

Figure 4. The rate and the limit of inhibition as a function of time of the incubation of SacC1 with the inhibitor protein, SacC1 (2 units) was incubated at 37°C with various amounts of the inhibitor protein in a standard mixture as described under methods.

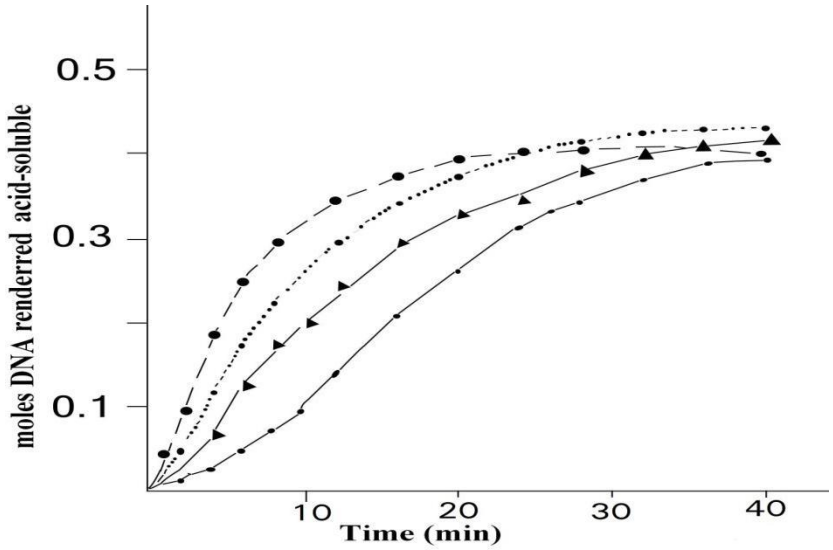

Figure 5. The rate and the limit of inhibition as a function of time of the incubation of SacC1 with the inhibitor protein.

The inhibitor protein SacC1 (2 units) was incubated at 37°C with various amounts of the inhibitor protein were added after the reaction has been initiated in a standard mixture as described under Methods;●- 2.5 units inhibitor; ●……2.0 units inhibitor; ◄-1.0 units inhibitor ◄…… no inhibitor.

the enzyme for DNA as a substrate is 2.5 x 10^{-8} M according to Lineweaver-Burk plot (Figure 6). When the increasing amounts of the inhibitor were added to a constant amount of SacC1, the inhibition increased linearly with the addition of the inhibitor up to 85% inhibition. The slope of inhibition was constant irrespective of the initial amount of SacC1. Further inhibition required disproportionably larger amounts of the inhibitor and the residual SacC1 activity cannot be entirely demolished (Figure 7).

When increasing amounts of SacC1 were added to a

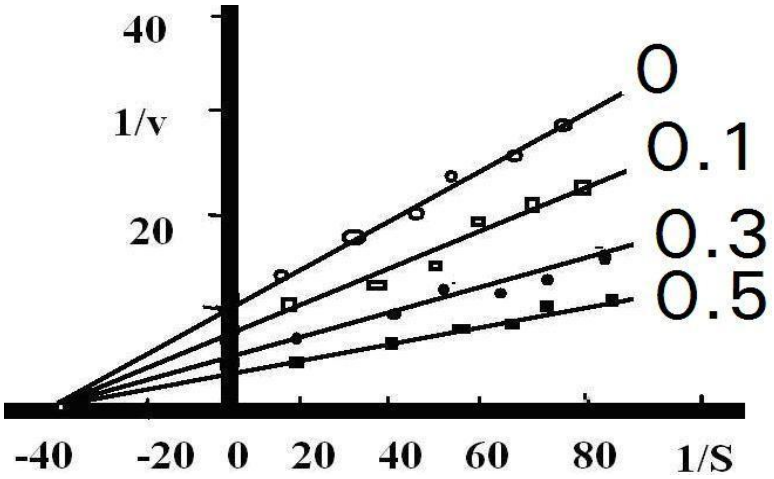

Figure 6. Lineweaver–Burk plot of SacC1 and the substrate with various amounts of the inhibitor. Each reaction mixture (1 ml) contains 0.4milliunits/ml of endonuclease, 0.05 milliunits.mL-^{1} of inhibitor and 0.01 mg/ml of substrate in buffer B. Reaction carried out at 30°C for 10 min. The reaction was stopped at zero and 10 min time by 0.2 ml uranyl reagent, cooled for 10 min and centrifuged at 10,000 g for 5 min. A260 was measured with zero time supernatant as a control. The reaction velocity (V) was expressed directly by A260. Substrate concentration was expressed as millimolar nucleotides. Inhibitor concentrations are in milliunits/ml.

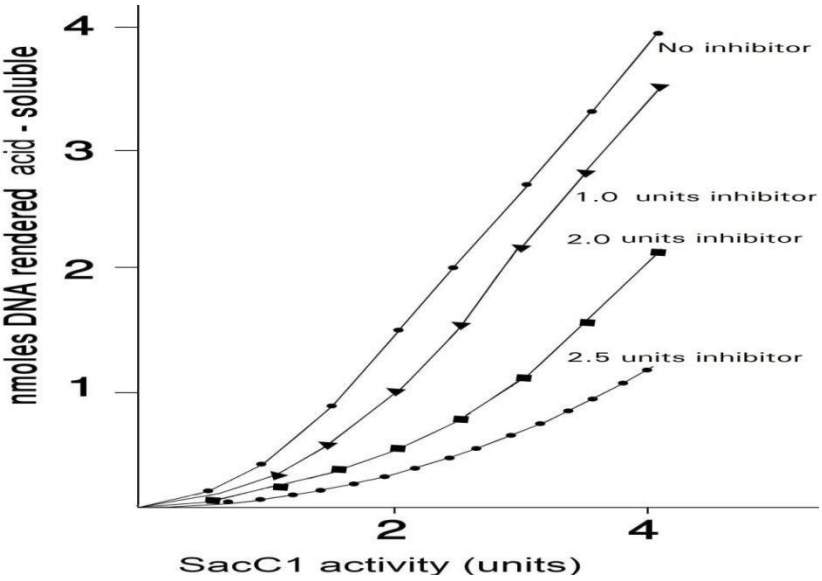

Figure 7. The effect of the inhibitor on various amounts of SacC1. SacC1 and the inhibitor protein were mixed in the amounts indicated and incubated at 37°C for 10min as described under “methods”.

fixed amount of the inhibitor protein, no or very little SacC1 activity was observed until the amount of SacC1 exceeded the amount of the inhibitor. After that, SacC1 activity increased linearly with the same slope as observed in the absence of the inhibitor (Figure 8).

The amount of inhibition is independent of temperatures and independent of the pH during the pre-assey incubation.

Determination of Ki

The inhibitor constant Ki was determined by measuring the inhibition with lower concentrations of SacC1 and the

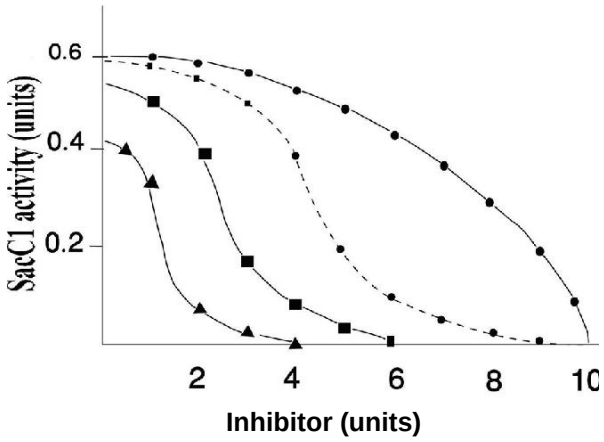

Figure 8. The effect of SacC1 on various amounts of the inhibitor. SacC1 and the inhibitor protein were mixed in the amounts indicated and incubated at 37°C for 10min as described under "Methods".SacC1 concentration 4 units. ● no inhibitor; ●…… 1.0 units inhibitor; ■- 2.0 units inhibitor and ◄…… 2.5 units inhibitor.

inhibitor, where the dissociation of SacC1–inhibitor complex becomes evident (Figure 9). From the curves near the origin, Ki was calculated with the equation $Ki=[It]/[v_o/v_{-1}]$, where [It] is the concentration of the total inhibitor and v_o and v_{-1} are the velocities of SacC1 reaction in the absence and presence of the inhibitor, respectively. As shown in Figure 9, a Ki value of 3.45×10^{-12} was obtained as the mean Table 3.

Specificity

The inhibitor nearly inhibited SacC1, but has no effect on EcoRI or SacI. This indicates the specificity of this inhibitor.

DISCUSSION

A protein inhibitor, which specifically inhibits SacC1 (Shikara, 2010*)* was purified by ammonium sulphate precipitation, dialysis and gel filtration using phosphor-cellulose, Sephadex G-100 and isoelectricfocusing columns. The affinity chromatography method was very effective and allowed the isolation of the inhibitor in homogenous state and the removal of all interacting proteins

The purity of the enzyme was judged by the appearance of one band in SDS-gel electrophoresis. SacC1, its inhibitor and SacC1-inhibitor complex have a molecular weight of 64,000, 32,000 and 85,000 by using gel filtration and 68,000, 35,000 and 80,000 by using SDS-PAGE, respectively. This deviation is expected since SDS-PAGE has its limitations and most proteins will give estimates within a few percentage of their actual weight (Sallantin et al., 1990).

Sac I (pI=6.2) and EcoRI (pI = 5.0)(Sallantin et al., 1990; Kim et al., 1990) have no interaction with the inhibitor of SacC1 (pI=5.22) since they have the same electric charge, while the inhibitor interacts with SacC1 (pI=9.0) and this interaction could increase the stability of the complex, possibly manifesting itself as a decrease in the dissociation rate due to the electrostatic attraction between the two groups (Sallantin et al., 1990; Zhuravleva et al., 1987) or the stability may increase by potentially stronger electrostatic interaction (Spector et al., 2000; Strickler et al., 2006).

This may facilitate the electrostatic force between SacC1 and the inhibitor, which lead to a complex formation since they charged oppositely at pH 8.0

The inhibition increased linearly with the addition of the inhibitor up to 85% inhibition. Further, inhibition required disproportionably larger amounts of the inhibitor and the residual SacC1 activity cannot be entirely demolished.

It is unclear why total inhibition (100%) is not achieved. It is unlikely that the uninhibited activity results from the presence of small amounts of SacC1, which is resistant to inhibition since the purified inhibitor protein inhibits various preparations of SacC1.

Alternatively, the uninhibited activity may be a consequence of an equilibrium established by a reversible interaction between the inhibitor and the enzyme in the form of E between the free and complexed (inhibited) forms of the enzyme. Other possibilities cannot be ruled out.

The stoichiometric measurements conclude that the SacC1 and the inhibitor have an extremely high affinity and constitute a mutual depletion system. The enzyme and the inhibitor combined stoichiometrically with each other form enzyme-inhibitor complex, which consists of one molecule of SacC1 and one molecule of the inhibitor until the concentration of one of them becomes near zero. This indicates that the inhibitor exerts a direct titra-tion effect upon any given quantity of SacC1.

On the other hand, the determination of interaction parameters *in vitro* indicates that the SacC1 possesses a higher affinity for the substrate than for the inhibitor. That means one molecule of SacC1 will bind to one molecule of the inhibitor. These results, as well as the kinetic data are consistent with the model of enzyme-inhibitor complex, which composed of catalytic and regulatory subunits. The values obtained from molecular mass estimation strengthened the hypothesis that the complex is formed from one molecule of SacC1 and one molecule of the inhibitor. It has been shown that the hydrophobic effect, hydrogen bonding and packing interactions between residues in the protein interior are dominant factors that define protein stability. These results suggest that surface charge−charge interactions are important for protein stability and that rational optimization of charge−charge interactions on the protein surface can be a viable strategy for enhancing protein stability. Charge-charge interactions on the surface of native proteins

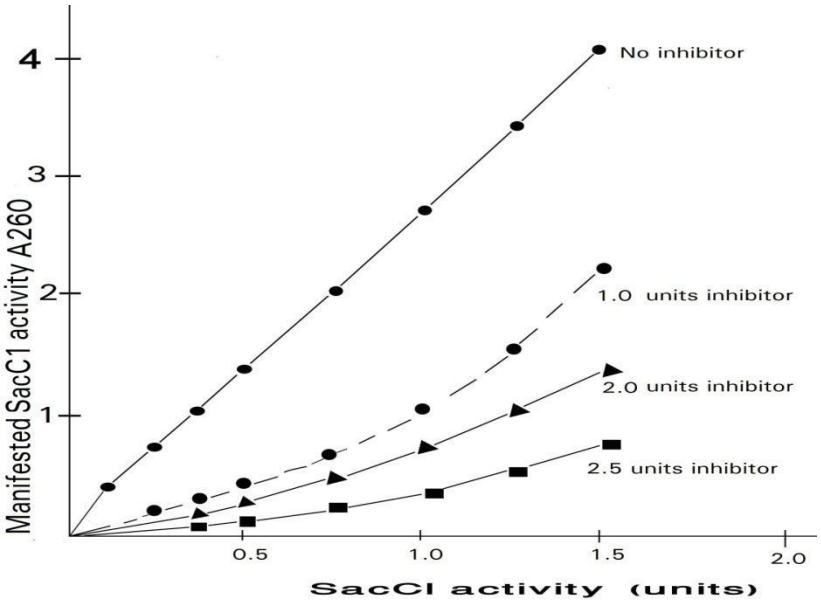

Figure 9. Residual SacC1 activity in the presence of different concentrations of the inhibitor. The inhibitor constant Ki was calculated as described under methods.

Table 3. Determination of Ki.

Total inhibitor		Ki	
Milliunits/ml	**M**	**V_0/V_{-1}**	**M**
0.1	3.6×10^{-12}	9.8	3.67×10^{-12}
0.3	4.2×10^{-12}	12.7	3.30×10^{-12}
0.5	4.9×10^{-12}	14.5	3.37×10^{-12}
0.7	5.7×10^{-12}	15.1	3.77×10^{-12}
1.0	6.6×10^{-12}	21.1	3.12×10^{-12}
Mean value			3.45×10^{-12}

are important for protein stability (Gribenko and Makhatadze, 2007).

The conformational specificity between SacC1 and its inhibitor seems to be essential for their interaction. The extremely strong affinity of the inhibitor to SacC1 (Ki= 3.45×10^{-12}) is remarkable and weaker than the affinity of several restriction enzymes, such as EcoRI (Ki=1.38×10^{-11}) (Malin et al., 1999), Serine Protease (Ki= 2.3×10^{-10}) (Milstone et al., 2000), λ Integrase (Ki= 3.8×10^{-10}) (Boldt et al., 2004) and Astacin Metalloproteinase (Ki= 1.9×10^{-11}) (Tsai et al., 2004), so, it is safe to say that the enzyme activity is regulated by the content of the inhibitor. The inhibitor does not affect other restriction enzymes.

The development of inhibition required 3 to 4 h in order to reach completion at the mM concentrations of the proteins. The mechanism of inhibition is consistent with a noncompetitive, single-step bimolecular reaction between enzyme and inhibitor. The rate constants for formation and breakdown of the enzyme-complex along with values for the apparent dissociation constant and change in Gibbs free energy of binding derived from these constants are decreased affinity for the inhibitor. The decrease in binding affinity resulted predominantly and definitely from an increase in the rate of dissociation, with an increase in association rate of ~2- and ~5-fold, respectively, whereas the rate of association of the enzyme-complex was about the same as that for the weight protein.

It seems that SacC1 and its inhibitor is a good system for the study of physiological role inside yeast cell and is of interest to compare the effects with similar studies.

ACKNOWLEDGMENTS

The author is grateful for the skillful technical assistance of Mr. Jalal Ahmad Odeh, Faculty of Pharmacy, University of Al-Zaytoonah, Amman, Jordan. The author express his profound gratitude to professor Julia Bejar Alvarado, Department of Cellular Biology, Genetics and

Physiology, University of Malaga and professor Manchikatla Venkat Rajam, Department of Genetics, University of New Delhi India for their valuable suggestions and criticism in kinetics studies.

REFERENCES

Andrews P (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J., 91: 222-233.

Boldt JL, Pinilla C, Segall AM (2004). Reversible Inhibitors of λ Integrase-mediated Recombination Efficiently Trap Holliday Junction Intermediates and Form the Basis of a Novel Assay for Junction Resolution. J. Biol. Chem., 279: 3472-3483.

Braciak TA, Northemann W, Hudson GO, Shiels BR, Gehring MR, Fey GH (1988). Sequence and acute phase regulation of rat alpha 1-inhibitor III messenger RNA. J. Biol. Chem., 263: 3999-4012.

Brown NL, Smith M (1980). A general method for defining restriction enzyme cleavage and recognition site. Methods Enzymol., 65: 391-404.

Dubois M, Gilles KA, Hamilton JK, Rebers, RA, Smith F (1956). Colorimetric method for determination of sugars and related substances, Anal. Chem., 28: 350.

Gribenko AV, Makhatadze GI (2007). Role of the Charge-Charge Interactions in Defining Stability and Halophilicity of the CspB Proteins. J. Mol. Bio., 366(3): 842-856.

Kim YC, Grable JC, Love R, Greene PJ, Rosenberg JM (1990). Refinement of EcoRI endonuclease crystal structure. A revised protein chain tracing. Science, 249(4974): 1307-1309.

Lammeli UK (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature, 227: 680-685.

Lineweaver H, Burk D (1934). The Determination of Enzyme Dissociation Constants. J. Am. Chem., Soc. 56: 658-666.

Lowry OH, Rosebrough NJ, Furr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 1209-1220.

Maizel JV (1971). Polyacrylamide gel electrophoresis of viral protein. In: Maramorosch, K, Koprowshi H (Eds.). Meth. Virol., 5: 179-246.

Malin G, Lakobashvili R, Lapidot A (1999). Effect of tetrahydropyrimidine Derivatives on Protein-Nucleic Acids Interaction: Type II restriction endonuclease as a model system. J. Biol. Chem., 274: 6920-6929.

Milstone AM, Harrison LM, Bungiro RD, KuzmičP, Cappello M (2000). A Broad Spectrum Kunitz Type Serine Protease Inhibitor Secreted by the Hookworm Ancylostoma ceylanicum J. Biol. Chem. 275: 29391-29399.

Muralidhara BK, Negi S, Chin CC, Braun W, Halpert JR (2006). Conformational Flexibility of Mammalian Cytochrome P450 2B4 in Binding Imidazole Inhibitors with Different Ring Chemistry and Side Chains: Solution thermodynamics and molecular modeling. J. Biol. Chem. 281: 8051-8061.

Oda M, Tamura A, Kanaori, K, Kojima S, Miura KI, Momma K, Tonomura B, Akasaka K (2002). Functional tolerance of *Streptomyces subtilism* inhibitor toward conformational and stability changes caused by single-point mutations in the hydrophobic core. J. Biochem. 132: 991-995.

Richard JR, Belfort M, Bestor T, Bhagwat AS, Bickle TA, Bitinaite J, Blumenthal RM, Degtyarev SKH, Dryden DTF, Dybvig K, Firman K, Gromova ES, Gumport RI, Halford SE, Hattman S, Heitman J, Hornby DP, Janulaitis AJ, Jeltsch A, Josephsen J, Kiss A, Klaenhammer, Kobayashi I, Kong H, Krüger DH, Lacks S, Marinus MG, Miyahara M, Morgan RD, Murray NE, Nagaraja V, Piekarowicz A, Pingoud A, Raleigh E, Rao DN, Reich N, Repin VE, Selker EU, Shaw PC, Stein DC, Stoddard BL, Szybalski W, Trautner TA, Van Etten, JL, Vitor, JMB, Wilson GG, Xu SY (2003). Survey and Summary: A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. Nucleic Acids Res. 31(7): 1805-1812.

Richter W, Conti M (2004). The Oligomerization State Determines Regulatory Properties and Inhibitor Sensitivity of Type 4 cAMP-specific Phosphodiesterases. J. Biol. Chem. 279: 30338-30348

Sallantin M, Huet JC, Demarteau C, Pernollet JC (1990). Reassessment of commercially available molecular weight standards for peptide SDS polyacrylamide gel electrophoresis using electroblotting and microsequencing. Electrophoresis, 11(1): 34-36

Serap Doğan S, Turan P, Doğan M (2006). Some kinetic properties of polyphenol oxidase from *Thymbra spicata* L. var. *spicata*". Process Biochem. 41(12): 2379-2385.

Shikara M (2010). Purification of a restriction enzyme from *Saccharomyces cerevisiae*. J. Yeast and Fungal Res. 1(7): 127-135.

Spector S, Wang M, Carp SA, Robblee J, Hendsch ZS, Fairman R, Tidor B, Raleigh DP (2000). Rational Modification of Protein Stability by the Mutation of Charged Surface Residues. Biochemistry, 39(5): 872-879.

Strickler SS, Gribenko AV, Gribenko AV, Keiffer TR, Tomlinson J, Reihle T, Loladze VV, Makhatadze GI (2006). Protein Stability and Surface Electrostatics: A Charged Relationship, Biochemistry, 45(9): 2761-2766.

Tsai PL, Chen CH, Huang CH, Chou CM, Chang GD (2004). Purification and Cloning of an Endogenous Protein Inhibitor of Carp Nephrosin, an Astacin Metalloproteinase J. Bio. Chem. 279: 11146-11155.

Zhuravleva LI, Oreshkin EN, Bezborodov AM (1987). Isolation and purification of restriction endonuclease Sac I from *Streptomyces achromogenes* ATCC 12767. Prikl. Biokhim. Mikrobiol. 23(2): 208-215.