Screening and Purification of a Chymotrypsin Inhibitor from Entrolobium Saman Seeds

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فحص وتنقيه مثبط الكا يموتربسين البروتيني من بذور انترولوبيوم سامان

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انترولوبيوم سامان كايموتربسين انهيبيتورقد تم فصله وتنقيته بالطرق التقليدية لتنقيه البروتينات مثل الترسيب بالحامضيه ،الحرارة، كبريتات الامونيوم، التبادل ألايوني الكروماتوجرافي (DEAE-Cellulose) وجل فلتريشن (Sephadex G75). النتائج النهائية وجدت بأنه متجانس من خلال استخدام ألفصل الكهربائي (SDS-PAGE و) Gel, filtration PAGE و Isoelectric focusing. الوزن الجزئي لمثبط الكايموتربسين كان 17978 دالتون بواسطة SDS-PAGE و Gel filtration . ألامينو اسيد لهذا المثبط وجدت بأنها سيرين. . انترولوبيوم سامان كايموتربسين انهيبيتور يفتقر إلى مجموعه الثايول (SH), ويظهر طيف امتصاصي في منطقه الاشعه فوق البنفسجية مع قيمه امتصاص منخفضة هي 2,99. الطيف القاعدي لهذا المثبط يظهر عدم وجود التربتوفان. (CD) Circular dichroic يشير إلى عدم وجود أي تركيب ثنائي للبروتين مع ثبات عالي في مختلف درجات الحرارة حتى 97 درجه مئوية كما اظهر هذا المثبط ثبات عالي عند المدى الهيدروجيني من 2,0 إلى 12,0. التخصص لهذا المثبط من انترولوبيوم سامان (Samanea saman) لاتظهر أي نشاط ضد السيستين، الكاربوكسيل وأيضا ميتالوبروتينيز. كايموتربسين يثبط بقوه بواسطة كايموتربسين انهيبيتور، كايموتربسين انهيبيتور يظهر معقد مترابط بثباته مع الكايموتربسين وهذا المعقد قد تم فصله ودراسته ونتائج هذه الدراسة دلت على وجود موقع واحد نشط على هذا المثبط (Single headed Inhibitor) لإنزيم الكايموتربسين . ثابت التفكك لمعقد الكايموتربسين والكايموتربسين انهيبيتور قد تم تعيينها وهي $9,05 \times 10^{-8}$ مولر . دراسة التحوير الكيميائي للمثبط كان نوع تايروسين. إلى جانب نشاط هذا المثبط تجاه الكايموتربسين ،فان هذا المثبط يظهر نشاطيه ضد بعض أنواع البكتيريا والفطريات الممرضه ولكن لم يتم كتابتها في هذا البحث.

ABSTRACT: A chymotrypsin inhibitor was isolated and purified from the seeds of *Enterolobium saman* (Leguminaceae family) by extraction with 100 mM phosphate buffer, heat treatment, ammonium sulphate precipitation, ion-exchange chromatography on DEAE-cellulose and filtration through Sephadex G-75. The final preparation appeared to be homogeneous by both chromatographic and electrophoretic analyses. ESCI had a molecular weight of about 17,890 and an isoelectric point of 5.8. ESCI inhibited bovine chymotrypsin at an inhibitor-enzyme molar ratio of 1:2. The inhibition mode of chymotrypsin inhibitor was competitive on bovine chymotrypsin. Investigation has been carried out on the complex formed between chymotrypsin and chymotrypsin inhibitor by physico-chemical methods. An apparent dissociation constant (Ki) of 9.05×10^{-8} M has been calculated for the complex. This enzyme-

inhibitor complex was isolated by gel filtration on Sephadex G-75 and a molecular weight of 43.000 was estimated for the complex. The inhibitor did not have any effect on other proteinases, such as papain, bromelin, elastase, α-amylase, trypsin and pepsin. The chemical modification of lysine residues indicated that –NH$_2$ groups are not essential for the activity of ESCI toward chymotrypsin. The inhibitor was an acidic protein and was stable over a wide pH range of 2-12 and temperature range of 10°C-97°C.

KEYWORDS: Samanea saman; Serine proteinase inhibitor; Chymotrypsin inhibitor; Single-headed inhibitor.

Abbreviations: ESCI, Enterolobium saman chymotrypsin inhibitor; CIA, Chymotrypsin inhibitory activity; ATEE, N-Acetyl-L-tyrosine ethyl ester; NB, Nutrient broth; SDS-PAGE, Sodium dodecyl sulphste-polyacrylamide gel electrophoresis; CD, Circular dichroism; TPCK, L-1-chloro-3-(4-tosylamide)-4-phenyl-2-butanone HC; APNE, N-acetyl-D,L phenylalanyl-β-naphthyl ester; TNBS, Trinitrobenzene sulfonic acid; CHD, 1, 2 cyclohexane dion, PDA, potato dextrose agar; MIC, Minimal Inhibitory Concentration; CIU, Chymotrypsin Inhibitory Unit; PVDF, (polyvinylidenedifluoride membrane); CAPS, [3-(cyclohexylamino) propane-1-sulphonic acid; PI, proteinase inhibitor; BSA, bovine serum albumin; TCA, trichloroacetic acid

1. Introduction

The protease inhibitor (PI) protein, the natural antagonists of protease, is a small protein which is quite common in nature and present in all life forms (Fritz, 2000). Plant protein inhibitors of proteases have been known for many years and their function is presently the subject of much interest. Serine proteinase inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore, the number of known and partially characterized inhibitors of serine proteinases is enormous (Haq *et al*., 2004). Serine proteinase inhibitors have been reported from a variety of plant sources and are the most studied class of proteinase inhibitors (Mello *et al*., 2002; Haq and Khan, 2003). Although the biological role of protein proteinase inhibitors is not still sufficiently clear, it has been suggested that they may perform three main functions-serving as storage proteins, being regulators of activity of endogenous proteinases and acting as agents protecting plants against insects and pathogenic microflora. These inhibitors are probably important physiologically and even in molecular evolution. Furthermore, they serve as excellent models for research on protein-protein interaction. The PIs are important tools to achieve a better understanding of fundamental principles and can be used to design new substances for the control of diseases and pathologic processes. Plant protein inhibitors of proteases have been known for many years and their function is presently the subject of much interest. *Enterolobium saman* (Rain tree) is a member of the Leguminaceae family. The seeds and the pulp are edible and nutritious for livestock and make an excellent feed supplement (George and Craig, 2005). The present paper describes the extraction, purification and biochemical characterization of chymotrypsin inhibitor from *Enterolobium saman* seeds.

2. Material and Method

Enterolobium saman (*Samanea saman*) seeds were procured from Bangalore (India), the local area. All biochemical's and enzymes used in this investigation were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals used were of analytical grade.

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2.1 Extraction of chymotrypsin inhibitor

The whole seeds were ground to fine powder and defatted with chilled acetone at 4°C. The defatted flour was extracted with 100 mM phosphate buffer (pH 7.6) for 3 h. at 4°C (1:10 W: V ratio). The supernatant was separated by centrifugation (30 min at 10.000 rpm) and the clear supernatant was used as a crude extract for estimation of chymotrypsin inhibitory activity and protein. Protein was estimated by the method of Lowry *et al*. (1951).

2.2 Assay of chymotrypsin and chymotrypsin inhibitory activity

The inhibition spectrum of ESCI was established by assay proteolytic or esterolytic activity of the enzyme on appropriate substrates. In principle, a fixed amount of the enzyme was incubated with various amounts of the inhibitor and the residual enzyme activity was assayed. The activity of chymotrypsin or its inhibition was routinely assayed by the method of Kakade *et al*., (1969) and the method suggested by Schwert and Takenaka (1955) using casein and ATEE as substrate, respectively.

2.3 Purification of chymotrypsin inhibitor

Enterolobium Saman chymotrypsin inhibitor was isolated in an apparently homogeneous form by the following procedure. Acetone defatted *Enterolobium Saman* flour was stirred with 100 mM phosphste buffer (pH 7.6) for 3 hr at 4°C. The extract was centrifuged at 10.000 rpm for 30 min. The pH of the supernatant was adjusted to 5.0 with 0.2 M acetic acid and the suspension was heated to 70°C with stirring. After 10 min at 70°C the supernatant was rapidly chilled in an ice-bath and centrifuged at 10.000 rpm for 10 min to remove the precipitate. The pH of the supernatant from the previous step was adjusted to 7.0 with diluted ammonium hydroxide. Solid ammonium sulphate was added to 80% saturation with constant stirring at 0°C. The suspension was centrifuged at 10.000 rpm for 15 min .The precipitate was dissolved in 10 mM Tris-HCl buffer (pH 8.0) and dialyzed. The ammonium sulphate fraction (80 mg protein) was applied to the DEAE-Cellulose column and eluted with a starting buffer. Thereafter, a gradient elution with increasing strength (0.2M NaCl) was performed. The fraction containing CIA was pooled, dialyzed and lyophilized. Protein (20 mg) from DEAE-preparation in 1.0 ml phosphate buffer (pH 7.0) was applied to a sephadex G-75 column and chromatographed using 100 mM phosphate buffer (pH 7.0) as the effluent. The active fractions were pooled, dialyzed and lyopholilized. The final preparation showed a single protein band in PAGE and this band was shown to possess CIA by a specific staining method.

2.4 PAGE analysis of inhibitors

Polyacrylamide gel electrophoresis was performed according to the method of Davis (1964). For visualization of the inhibitor in acrylamide gels, after electrophoresis the gels were first incubated with trypsin solution (100 µg /ml) and then with the substrate (N-acetyl-D,L phenylalanyl-β-naphthyl ester)-dye (Diazoblue-B mixture by the method of Filho and Moreira, (1978). The CIA appeared as a clear zone against a pink background.

2.5 Determination of molecular weight

For estimation of M.W. the sample and the marker proteins were incubated at 50°C for 1 hr in 0.1 ml phosphate buffer, with 10 µl 20% SDS and 10 µl β-mercaptoethanol by the method of Weber and Osborn (1969). The MW marker proteins used were BSA (66 KDa), Ovalbumin (43KDa), Carbonicanhydrase (29 KDa), SoybeanTI (20.1 KDa) and Lysozyme (14.3 KDa) for SDS-PAGE.

2.6 Isoelectric point

Isoelectro focusing was done by the method of Wringley (1969) in 7.5 % PAGE tube gels with 2% ampholyte gradient between pH 3 and pH 10. The upper and lower reservoirs were 0.2 % sulphuric acid and 0.4 %

ethanolamine, respectively. The run was performed in room temperature. After the run, the gels were stained for CIA and protein.

2.7 N-terminal sequencing

The purified (ESCI) was separated by SDS-PAGE in 12% gel and stained with Coomassie Brilliant Blue , then electro blotted on to a PVDF in 10 mM CAPS as described by Matsudaira, (1987) . The protein band was cut out and loaded on to the sequencer.

2.8 Stability of the inhibitor

The stability of inhibitors at different pH (2-12) values was assayed by incubating chymotrypsin inhibitor solution at different pH values at 5^oC overnight. Then the pH of the mixture was brought to 7.0 and its inhibitor activity towards chymotrypsin was assayed as described above. To study the thermostability, the inhibitor prepared was incubated at different temperatures $(10-121^0 C)$ by 10^oC interval in a water bath for 10 min.

2.9 Circular dichroism Spectra (CD-Spectra)

The CD spectra were recorded on a Jasco-810 CD spectropolarimeter with a cell length of 0.1 cm at 10^oC. The inhibitor (2 mg/ml) in distilled water was placed in a 2 mm Quartz cuvette and spectra were recorded from 200-300 nm at intervals of 1 nm and a well time of 2 seconds.

2.10 Chemical modification

2.10. 1 Modification of the amino groups

The free amino groups were modified according to the method of Haynes *et al*., (1967). 500 μg of ESCI was incubated in different test tubes with 0.1 % TNBS in 2 ml of 4 % $Na_2 CO_{3,}$ (pH 8.5) at 40^oC for different time intervals. The reaction was stopped by the addition of 1 ml of 10 % SDS followed by 0.5 ml of 1 N HCl. The extent of modification of each sample was determined by measuring the absorbance of the solution at 344 nm. The residual CIA was determined during the course of modification by removing aliquots from each reaction mixture at pre determined time intervals. The control inhibitory activity was estimated from an inhibitor solution incubated in the same manner without TNBS.

2.10.2 Modification of Arginine group

Arginine groups were modified by the method of Liu *et al*., (1968). 10 mg CHD was allowed to react with 5.0 mg ESCI in 5.0 ml 200 mM triethanolamine buffer, (pH 7.8) in the dark for 12 h. The residual CIA was determined in relation to an inhibitor control without CHD after dialysis in cold condition for 36 hours.

2.11 Isolation of the chymotrypsin-ESCI complex

The enzyme-inhibitor complex was isolated by gel filtration on sephadex G-75. A mixture of inhibitor (2mg) and chymotrypsin (1mg) in 2 ml, 100 mM phosphate buffer (pH 7.6) was allowed to stand at room temperature for 15 min. This solution was then chromatographed on a sephadex G-75 column equilibrated with 100 mM phosphate buffer (pH 7.0) and the column was then developed with the same buffer solution. The A_{280} nm of the fractions was measured. CIA and chymotrypsin activity in the fraction was determined. Estimation of the apparent dissociation constant (Ki) of the ESCI-Chymotrypsin complex was performed according to the method of Grob (1950). Fixed concentrations were calculated assuming of a M.W of 17890 for ESCI and 25000 for bovine chymotrypsin.

2.12 Kinetic measurement

The nature of inhibition of chymotrypsin by ESCI was studied by incubating chymotrypsin with varying concentrations of substrate (Casein) in the presence and absence of the inhibitor. The substrate (1-5 mg) was

incubated with 40 µg chymotrypsin for 10 min at 37 ^{o}C .After the incubation period, the reaction was stopped by adding 6 ml 5% TCA. A $_{280}$ nm of the solution was read against a blank incubation sample, but without enzyme.

The assay was repeated in the presence of 10 and 20 µg of the inhibitor in the reaction mixture.

2.13 Specificity of ESCI

The inhibitory activity of ESCI towards α-chymotrypsin was determined using casein (Kakade and Liener, 1970) or ATEE (Schwert and Takenaka, 1955) as substrate. The casein digestion method (Murachi, 1970) was used to determine the activity or inhibition of papain and bromelin. Elastolyatic activity of elastase was determined by the method of (Naughton and sanger, 1961) using elastin-Congo red as substrate. The activity of pepsin was assayed by the method of (Anson, 1938). The esterolytic activity of subtilisin-BPN' was assayed using ATEE as substrate in a manner similar to the α-chymotrypsin assay; α-Amylase (Pancreatic) activity or its inhibition was measured by the method of (Saunders and Lang, 1973).

3. Result and Discussion

ESCI was purified from mature seeds based on chymotrypsin inhibitory activity. A procedure has now been evolved for the purification of chymotrypsin inhibitor from Enterolobium *saman* seeds as summarized in Table 1. The CIA was eluted by a linear gradient of increasing ionic strength. The chymotrypsin inhibitor was desorbed from the DEAE-Cellulose column at an ionic strength of 0.063 M NaCl (Figure 1), and then was finally separated by Sephadex G-75 column (Figure 2).

Table 1. Summary of purification of *Enterolobium saman Chymotrypsin inhibitors* (ESCI) (from 100gm of defatted flour) *Yield and fold purification are calculated on the basis of CIU using Casein as substrate.

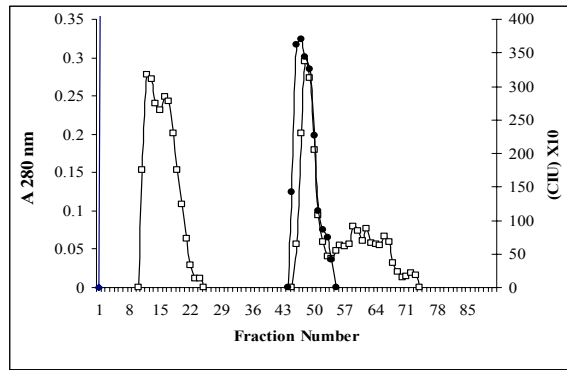| Purification steps	Total protein (mg)	Total activity units CIU X10^4	Specific activity CIU/mg	Fold purification* CIU	*Yield % CIU
Crude extract	50400	215.88	42.83	1.0	100
pH&heated treated extract	28820	197.31	68.47	1.59	91.4
80% (NH$_4$)$_2$SO$_4$	5320	138.26	259.90	6.06	64.0
DEAE-Cellulose	1220	103.32	846.80	19.77	47.86
Sephadex G-75	760	64.86	853.49	20.0	25.6

Figure 1. Ion-exchange chromatography on DEAE-Cellulose Column of the ESCI. □□ Absorbance at 280 nm;●●Chymotrypsin inhibitory activity.
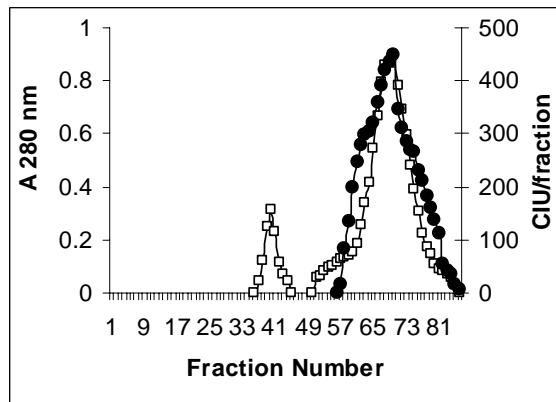
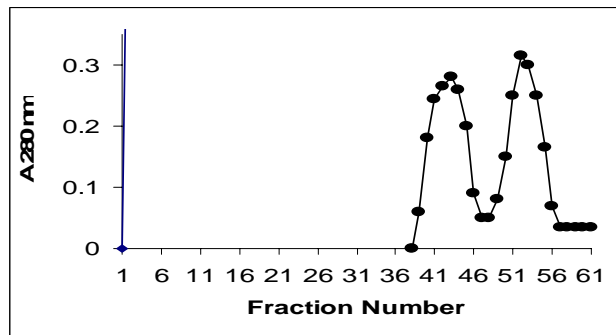Figure 2. Gel filtration on sephadex G-75 of ESCI. □□ Absorbance at 28n nm, ●●Chymotrypsin inhibitory activity

Figure 4. Gel filtration on Sephadex G-75 of a mixture of bovine chymoptrypsin and ESCI. Protein was monitored at 280 nm ● ●

Table 2. Effect of pH and Temperature on ESCI. * Inhibitory activity at 10°C was taken as 100%. †
Boiling water. ‡ Autoclaving at 1.04 Kg/cm^2 pressure

Temp.^{o}C	% CIU	pH	CIU/mg
10*	100	2.6	1673.3
25	100	3.2	1666.6
30	100	5.0	1670.0
40	99.3	6.0	1753.0
50	98.2	7.0	1703.5
60	97.9	7.6	1780.0
70	95.5	8.6	1640.0
80	95.31	9.6	1620.0
97†	92.62	10.6	1680.0
> 121‡	0.0	12.6	1576.6

A   B   C   D
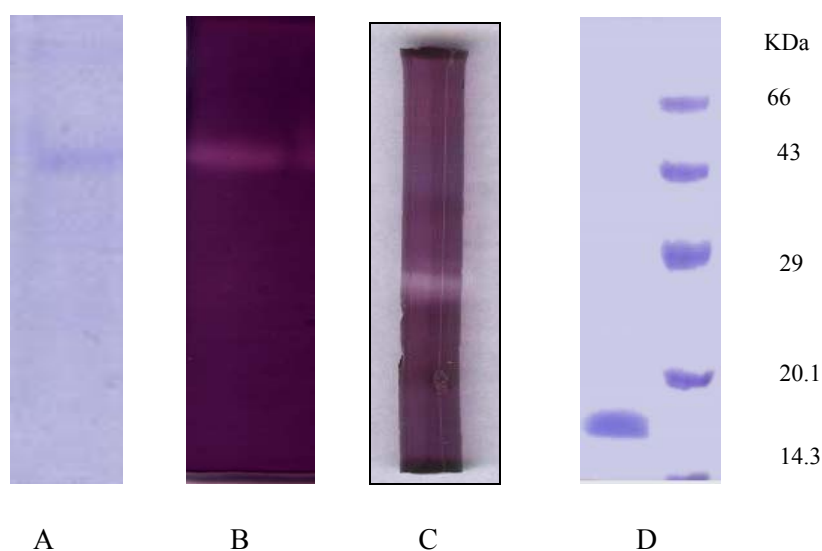
Figure 3. Electrophoretic analysis of ESCI. (a) PAGE of purified ESCI in 10% gel stained with coomassie brilliant blue (b) Visualization of the chymotrypsin inhibitory band on the gel. (c) Isoelectric point in a 7.5% tube gel. (d) Molecular weight determination of purified ESCI by SDS-PAGE in 12% gel

The chymotrypsin inhibitor isolated from *Enterolobium saman* by the procedures described above appeared to be homogeneous by the criteria of PAGE (Figure 3a), SDS-PAGE (Figure 3d), gel filtration on Sephadex G-75 (Figure 4) and isoelectric-focussing (Figure 3c). The final preparation showed one protein band in PAGE and this band was shown to possess CIA by a specific staining method (Figure 3b). The studies with pure preparation have shown that the CIA has a broad pH stability and thermo stability (Table 2).

The M_r of ESCI was determined as 17890 ± 100 SDS-PAGE and gel filtration on sephadex G-75. Many legume inhibitors have their molecular weight in the range of 15,000-20,000 Da. Single headed inhibitors have been isolated from barley bean (Prakash *et al*., 1996), *Crotalaria paulina* seeds ((Luiza *et al*., 1999), and *Caesalpinia echinata* (Pau-brasil) seeds (Cruz-Silva .*et al.,* 2004)????. The Kunitz-type inhibitor found in *E.saman* supports the notion that there is a relationship between the types of inhibitors found in leguminous seeds and the evolution of the leguminous plants, since only Kunitz-type inhibitors are present in the relatively primitive plants of Caesalpinoideae. This conclusion was reached by Norioka *et al*. (1988) who investigated the presence of both Kunitz and Bowman-Birk inhibitors in seeds of 34 legumes by gel filtration. The results were compared to the morphological classification of Leguminosae: the seeds of the more primitive Leguminosae (Caesalpinoideae and Mimosoideae) contain mainly Kunitz-type inhibitors, whereas those of a more advanced family (Papilinoideae) contain only Bowman-Birk-type inhibitors.
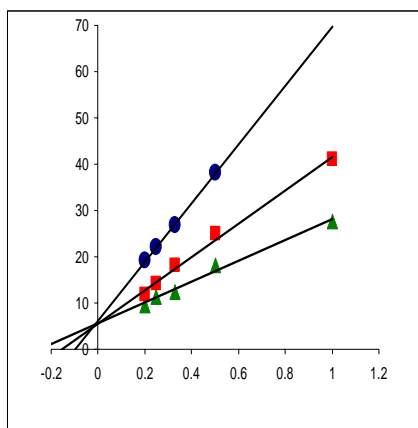

Figure 5. Lineweaver-Burk plot of the inhibition of caseinolytic activity of chymotrypsin by ESCI. ▲▲ without inhibitor, ■ ■ with 10 µg of ESCI, ●● with 20 µg of ESCI.
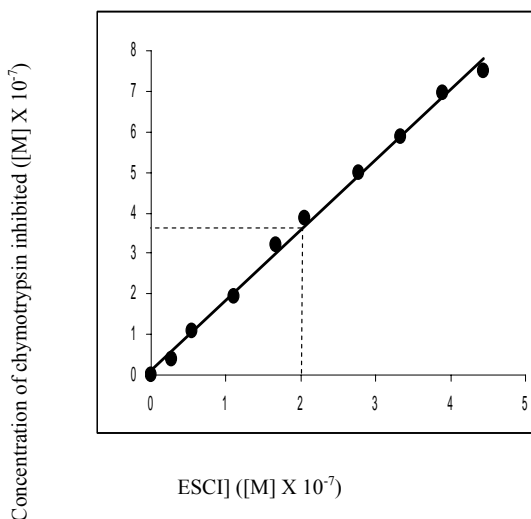

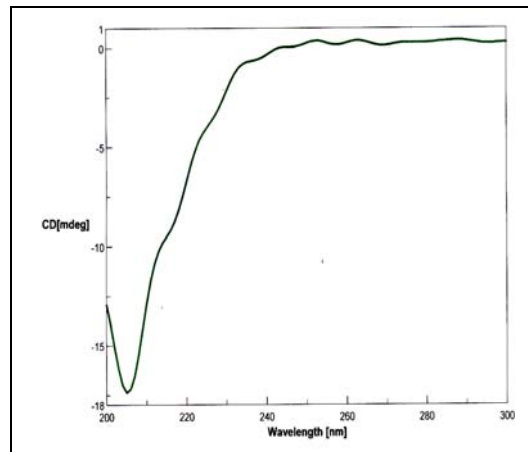Figure 6. Inhibition of bovine chymotrypsin by ESCI.

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Figure 7. CD-spectra of the purified chymotrypsin inhibitor (ESCI) (2.0 mg/ml) in distilled water.

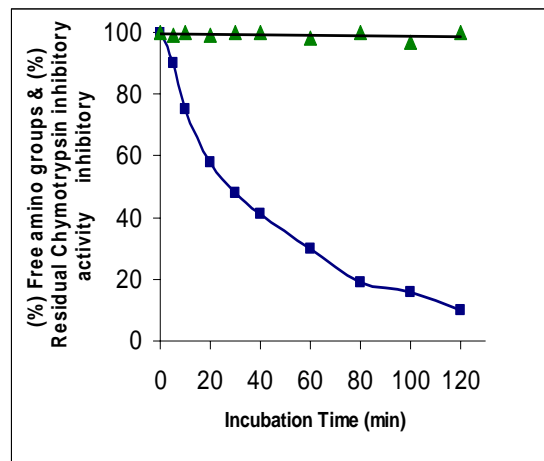
Figure 8. Time course of modification of free amino groups with TNBS % free amino groups ■■% residual CIA ▲ ▲ .

Inhibitor	N-terminal sequences
ESCI	S-N-L-L-L

Figure 9. N-terminal sequences of ES.

Chymotrypsin activity in the presence (10 and 20 μg) and absence of ESCI was measured at different substrate concentrations. The double-reciprocal plot of the kinetic data is shown in (Figure 5). It can be seen that ESCI inhibited trypsin by a competitive mechanism involving competition of the substrate and inhibitor for the site on the enzymes. Most naturally occurring proteinase inhibitions inhibit the respective enzymes competitively.

The dissociation constants (Ki) of the ESCI-chymotrypsin was calculated to be 9.05×10^{-8} M. The low K_i indicates a high affinity between the inhibitor and chymotrypsin.

The extreme stability of the ESCI in acidic pH and higher temperatures suggests that the ESCI has little or no ordered structure. The CD-spectra of the ESCI (Figure 7) in distilled water shows the complete absence of α-helical content. The changes in the conformation of the ESCI in different pH and temperatures could not be carried as the proteins exhibited random coil secondary structures in CD-spectra.

The modification of arginine residues by a group specific reagent does not have any significant effect on the trypsin inhibitory activity of ESCI. The modification of lysine residues showed the participation of fast acting NH_2 groups in the reactive site of ESCI (Figure 8). This suggests that lysine is perhaps at the active site of the ESCI. ESCI most probably belong to the class of tyrosine type inhibitor.
Specificity of the inhibition of different classes of proteinases by ESCI was tested against the enzymes from various sources (Table 3). ESCI inhibited only bovine chymotrypsin and showed no activity against other proteolytic enzymes tested. This establishes that the inhibitory activity of ESCI against bovine chymotrypsin with ATEE as substrate (Figure 6) is stoichiometric.

N-terminal groups of the *Enterolobium saman* inhibitors have been detected by precise protein sequencing system IISc, Bangalor (Figure 9). The N-terminal sequence up to 5-residues was S-N-L-L-L.

4. Conclusions

In this work we describe the purification and characterization of a new chymotrypsin inhibitor from *Enterolobium saman* (Samanea saman) which belongs to the class of tyrosine inhibitor. The inhibitor existed as a monomer with molecular weight of 17,890 Da. Judging from its molecular mass and the inhibitory activity against chymotrypsin, chymotrypsin can be considered as a Kunitz type inhibitor. This inhibitor highly inhibited bovine chymotrypsin, but did not show any activity against other proteolytic enzymes tested.

5. Acknowledgments

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