MOLECULAR ANALYSIS OF GLUCOSE ISOMERASE PRODUCING *STREPTOMYCES* SP.

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DECLARATION

Certified that the work incorporated in the thesis "**MOLECULAR ANALYSIS OF GLUCOSE ISOMERASE PRODUCING *STREPTOMYCES* SP.**" submitted by Mrs. Mohini S. Ghatge was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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ABSTRACT

D-glucose/xylose isomerase (GXI) (EC 5.3.1.5) reversibly catalyzes the isomerization of D-glucose to D-fructose and D-xylose to D-xylulose. Both the reactions are important industrially, the former in the production of high fructose corn sweetener and the latter in the fermentation of xylose-rich biomass wastes to ethanol.

GXI is widely distributed and is an inducible enzyme produced intracellularly in most of the microbes, capable of growing on xylose/xylan-containing raw materials. Few reports of extracellular as well as constitutive production of GXI are available. Glucose isomerases from *Bacilli* and *Streptomycetes* are quite stable at high temperature. They act at neutral pH and require metal ions such as Mg^{2+}, Co^{2+} and Mn^{2+} for their activity. GXI is usually a tetrameric enzyme and is reported to have a broad substrate specificity. In addition to xylose, it also isomerizes D-glucose, D-ribose, D-allose, L-arabinose and L-rhamnose.

GXI is one of the largest volume industrial enzymes used today and is commercially available as immobilized products such as Sweetzyme, Maxazyme, Takasweet, OptiSweet, Ketozyme, etc. where a diverse series of bacterial species

are being exploited.

Despite the industrial importance of GXI, relatively little information is available on purification, characterization, mechanism of action, and on structure-function relationship of the enzyme. Recently, X-ray-crystallography studies on GXI have led to the prediction of mechanistic details of the reaction.

As a part of our investigations on the structure-function relationship of GXI and isolation of its gene from a high GXI yielding *Streptomyces* sp. NCIM 2730, the present studies deal with the following aspects of the enzyme.

I. Structure-function relation of GXI

II. Immunoaffinity purification of GXI

III. Genome characterization of *Streptomyces* sp. NCIM 2730 and

IV. Construction of genomic library of *Streptomyces* in *E. coli*.

I. Structure-function relationship of GXI

Section A: Unfolding and refolding of GXI: The role of tertiary structure in biological activity

GXI from *Streptomyces* sp. NCIM 2730 is a tetramer of Mr 1,60,000.The unfolding and refolding of the tetrameric GXI from *Streptomyces* sp. NCIM 2730 has been investigated by correlating the biological activity with the protein

transitions as monitored by fluorometry, c.d. and by its retention volumes in molecular sieve chromatography. Treatment of the enzyme with SDS (0.1%) results in the dissociation of the tetramer (T) into an active dimer (D) with no gross change in the tertiary structure but change in the secondary structure. On removal of the denaturant, a part of the dimer reassociates to form a tetramer presenting, for the first time, an experimental evidence for the reversibility of the tetramer-dimer transition. Incubation of the enzyme with Gdn-HCl (2M) results in a complete loss in activity although the enzyme is in its dimeric form. The inactive dimer (D*) has conformational properties of a molten globule, namely, a native-like secondary structure and disordered tertiary structure. Regain of activity was observed on lowering of the concentration of the denaturant by dilution. Refolding of the Gdn-HCl treated enzyme results in the restoration of its tertiary structure and activity. The enzyme is completely inactivated by heating at 100°C for 5 min. The heated enzyme is a monomer and exhibits a distinct irreversible change in its structure. Thus, four distinct species have been identified and characterised during denaturation and renaturation of the GXI: (i) native tetramer (T) active and inactive dimers (D and D*) and (iii)

inactive monomer (M). The results suggest that the intact tertiary rather than the secondary structure is essential for GXI activity.

Section B: Evidence for specific interaction of guanidine hydrochloride with carboxy groups of GXI

GXI from *Streptomyces sp.* (NCIM 2730) was inhibited (50%) by a relatively low concentration of guanidine hydrochloride (Gdn.HCl) (0.6 M) without causing noticeable structural changes in the treated protein as deduced by its fluorescence spectra. A similar concentration of NaCl or KCl had little effect on the enzyme activity implying that the inhibition is not due to the salt effect. The Km (71.4 mM) of the enzyme increased to 285.7 mM in the presence of Gdn.HCl. Glucose and/or metal ions (Mg^{+2} and Co^{+2}) protect the enzyme against inactivation by Gdn.HCl suggesting that it is due to reversible inhibition of activity rather than due to denaturation and/or unfolding of the enzyme. The participation of carboxyl groups in the mechanism of action of the enzyme was demonstrated by its inactivation by Woodward's reagent K (WRK) and by consequent increase in the absorbance of the enzyme at 340 nm. Inaccessibility of the Gdn.HCl treated enzyme to WRK established, for the first time, the specific interaction of Gdn.HCl with the carboxylate residues of the enzyme.

II. Immunoaffinity purification of GXI

A limitation to more extensive investigations on the structure-function relationship of the enzyme is imposed by the scarce quantities of the purified enzyme available with classical purification techniques. A single step and easy-to-handle purification procedure was developed to purify GXI from cell extract of *Streptomyces* sp. NCIM 2730 using immunoaffinity chromatography. High titre polyclonal antibodies were raised in rabbit using electrophoretically homogeneous GXI as an antigen. The specificity of antibodies was confirmed by double immunodiffusion, rocket electrophoresis and western-blot ELISA, which revealed the presence of a single immunoreactive protein with an Mr of 40,000. The antibodies recognized 2-3 antigenic determinants/mol of enzyme and were found to partially neutralize the enzymatic activity in an immunotitration experiment. The affinity gel was prepared by coupling antibodies at pH 10.0 to divinyl sulfone-activated Sepharose CL-4B. The GXI purified by immunoaffinity chromatography yielded 75% recovery with a single enzymatically active protein band on gel electrophoresis and showed specific activity of 16 U/mg which is comparable with the specific activity of the enzyme purified by conventional techniques.

The crossreaction of the antibodies with glucose/xylose isomerase from other actinomycetes indicated that they share common epitopes.

III. Genome characterization of *Streptomyces sp.* NCIM 2730

The genome of *Streptomyces* sp. NCIM 2730 was characterized with respect to its DNA melting profile, (Tm), base composition, DNA heterogeneity, kinetic complexity, presence of repetitive DNA and its methylation status.

The melting profile of *Streptomyces* sp. NCIM 2730 DNA was smooth and monophasic with a Tm and G+C content of 98°C and 70%, respectively. The GC- rich nature of the genome was also revealed by its susceptibility to digestion with restriction endonucleases which recognize G and/ C containing sites. Digestion of the DNA with methylation specific enzymes showed the lack of A or C methylation in the GATC sequence and absence of C methylation in the CCGG sequence. Restriction analysis of the DNA revealed some intense bands on the gel suggestive of the occurrence of repeated DNA sequences in the genome. On renaturation analysis, these repeated DNA sequences were detected as 'rapidly reannealing sequences'. The latter accounted for approximately 25% of the total genome and had a copy number and kinetic complexity of 50 and 3×10^3 respectively. Bam HI restriction fragment distribution in the genome appeared to

be independent of the age of the culture. Compariative analysis of DNA restriction patterns of different *Streptomyces* species revealed that they were species-specific and hence could be used as a tool for taxonomic differentiation of different species. Hybridization of a rice repetitive DNA probe with *Streptomyces* DNA revealed the presence of a highly repeated DNA family in different *Streptomyces* species.

IV. Construction of genomic library of *Streptomyces* in *E. coli*

Isolation of GXI gene from *Streptomyces* sp. NCIM 2730 is important for the 'hyperexpression of the gene' and for the production of an altered protein with desirable properties by 'protein engineering'. The construction of *Streptomyces* genomic library is the first step in this direction. A standard approach of 'shot-gun' cloning was used for the construction of genomic library of *Streptomyces* in *E. coli* using a plasmid vector, pUC8. High molecular weight *Streptomyces* DNA was isolated using Kirby mixture (a slight modification of Hopwood's procedure). The conditions for partial digestion of the DNA using Bam HI were standardized and the partially digested DNA was size fractionated on a 10 to 40% sucrose density gradient to yield DNA fragments in the range of 2-10 kbp. The size

fractionated DNA was ligated with BamHI cut, dephosphorylated pUC8 using *E. coli* T_4 DNA ligase and the ligation mixture was transformed in *E. coli* JM 105 by the 'calcium chloride procedure'. The recombinants (white colonies) were analysed for the presence and origin of the inserts by (i) colony hybridization and (ii) by Southern hybridization of the digested plasmids with *Streptomyces* genomic DNA. Assuming the genome size of *Streptomyces* to be 10^7 bp and the average insert size of 4 kbp the total number of recombinant clones represent 45% of the genomic library . An eighteen-mer mixed oligonucleotide probe has been synthesized based on the sequence of a stretch of six amino acids, well conserved in the protein sequence of different GXIs from actinomycetes and related organisms to screen the library for the identification of the GXI gene.

A part of these studies has been published/communicated as:

1. Evidence for the essential histidine residue at the active site of glucose/xylose isomerase from *Streptomyces*

   Gaikwad, S.M., **More, M.W.**, Vartak, H.G. and Deshpande, V.V. (1988) Biochemical and Biophysical Research Communications **155:** 270-277.

2. Immunoaffinity purification of glucose/xylose isomerase from *Streptomyces*

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3. Genome characterization of *Streptomyces* sp. NCIM 2730: Evidence for the occurrence of repetitive DNA sequences

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CHAPTER I

INTRODUCTION

Xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) (XI) is an intracellular microbial enzyme that catalyzes *in vivo* the reversible isomerization of D-xylose to D-xylulose in order to utilize D-xylose from xylans as an energy source. In 1957, Marshall and Kooi (1) found that D-xylose isomerase also converts D-glucose to D-fructose. This reaction is of industrial interest for the production of high fructose corn syrup (HFCS) from starch (2) and hence the enzyme is often referred to as glucose isomerase (GI). The D-glucose/xylose isomerase (GXI) also attracts attention in the conversion of xylan-containing biomass to ethanol (3).

Production of ethanol

XI is a key to the economic production of ethanol from hemicellulose fraction of agricultural and hardwood biomass (4). D-xylose is easily obtained by dilute acid prehydrolysis of biomass or by enzymatic hydrolysis of xylan by xylanases, but it is difficult to ferment it to ethanol. In yeast, xylose to xylulose conversion is via xylitol - an oxidoreductive pathway (5). Thus, although many yeasts can grow on xylose, they can ferment it poorly, if at all.

Certain yeast species ferment xylose in a microaerophilic environment e.g. *Pachysolen tannophilus*, *Candida shehatae* and *Pichia stipitis* (6-9) but the low

ethanol tolerance of these species, slow rates of fermentation, along with catabolism of ethanol in the presence of oxygen by these species (10-12) limit the commercial application of this approach.

The conversion of xylose to the more fermentable xylulose, using the bacterial xylose isomerase, allows for totally anaerobic fermentation by ethanol-tolerant yeast strains, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Candida tropicalis* (3, 13-19).

Production of HFCS

Enzymatic isomerization of glucose to fructose in starch processing is carried out on an industrial scale world-wide, but predominantly in United Sates. The commercially obtained HFCS contains 42-55% fructose and is used as an alternative sweetner to sucrose/invert sugar in the food and beverage industry. HFCS which is an equilibrium mixture of glucose and fructose (1:1) is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose. The price of HFCS is typically 10-20% lower than that of sucrose, based on sweetening power. Though glucose isomerizing enzymes were developed first in Japan in the early 1960's, enzymatic glucose isomerization was first established on an industrial scale in 1967 by Clinton Corn Processing Co. in U.S. Around 1974, immobilized GI became commercially available. With the

increasing acceptance of HFCS, especially in the soft drink industry (as it does not pose the problem of crystallization like sucrose), the glucose isomerization process was rapidly adopted by practically all major starch processing companies in the Western World between 1975 to 1980. A substantial increase in HFCS consumption occurred around 1978 with the introduction of fructose enrichment by chromatographic technique. In 1988, the total amount of HFCS produced worldwide exceeded 7 x 10^6t, based on dry matter.

Immobilization of GXI

Currently glucose isomerase is one of the three highest value and tonnage commercial enzymes, amyloglucosidase and bacterial protease being the other two enzymes. The glucose to fructose conversion could be made economical only by reducing the cost of the enzyme by immobilization so that the enzyme could be utilized to convert much more glucose per unit of enzyme activity. The commercial immobilization processes are of two general types, viz. immobilization of GI associated with the microbes and immobilization of the cell-free enzyme (20). Some of the commercially available immobilized glucose isomerases are given in Table I.1.

Occurrence, production, purification and properties of the enzyme:

Glucose isomerase is an intracellular microbial enzyme

Table I.1 : Commercially available immobilized glucose isomerase

Trade Name	Company	Enzyme source	Method of immobilization	Enzyme form
Maxazyme	Gist-Brocades	*Actinoplanes missouriensis*	Whole cells entrapped in gelatin treated with glutaraldehyde	Hydrated spherical particles
Opti Sweet 22	Kali-Chemie	*Streptomyces rubiginosus*	Enzyme absorbed to inorganic support (silicate)	Hydrated
Taka Sweet	Miles Laboratories, Inc.	*Flavobacterium arborescence*	Flocculated whole cells treated with glutaraldehyde	Dry elliptical sphere particles
Sweetzyme	Novo Industry	*Bacillus coagulans*	Glutaraldehyde treated lysed cells	Dry cylindrical particles
Ketozyme	Universal Oil Products, Inc.	*Actinoplanes missouriensis*	Enzyme bound to inorganic support (alumina)	Hydrated

produced by most of the microbes which are capable of growing on xylose as a carbon source. Chen (2, 21) reported about 75 microorganisms as producers of the enzyme and among these, *Streptomyces* species have been the most extensively studied and used as a source of the enzyme. *Streptomyces glaucescenes* ETC-22794 (22) and *Chainia* (23) are the only known extracellular GI producers. Besides *Streptomyces*, *Actinoplanes missouriensis* belonging to actinomycetes is also a potent producer of GI. The wild type strain capable of producing GI constitutively, is used for commercial production of the enzyme by Gist Brocades N.V. (24).

GI from *Streptomyces* sp. is inducible and requires the presence of xylose in the fermentation medium. Glucose is the usual carbon source for constitutive producers. Where the xylose inducer is required, a supplementary carbon source is necessary e.g. *Streptomyces* spp. require sorbitol. Complex nitrogen sources are generally used for GI production e.g. peptone, tryptone, yeast extract, meat extract, corn steep liquor (CSL), soya fluour etc.; CSL being used commonly. Certain metal ions viz. Mg^{2+}, Co^{2+}, or Mn^{2+} are utilized very frequently by several organisms for the production of GI. GI is exclusively produced by submerged fermentation. Nearly all producers are mesophilic in nature

and are grown at around 30°C. Most strains are grown at pH around neutrality, without deliberate pH control. The main objective of any fermentation research on GI is to improve yields, replace xylose as an enzyme inducer by cheaper substances, such as xylan or xylan-containing raw materials (e.g. wheat bran, corn hulls or corn cobs) and elimination of the need for Co^{2+} ions in the fermentation media. Yield improvement is achieved by (i) the search for new efficient enzyme-producing organisms, (ii) mutation of existing microbes or (iii) by genetic engineering. The elimination of xylose in the fermentation helps to reduce the cost of enzyme production. This can be achieved by screening for constitutive mutants or for mutants capable of growing on cheaper xylose sources such as xylan-containing materials. Elimination of toxic Co^{2+} ions in the fermentation medium is needed because of health problems related to HFCS consumption and the fermentation waste disposal problems of the spent media.

GXI is extracted from microbial cells either by mechanical disruption (sonication, abrasive grinding, homogenization etc.) or by autolysis (using cationic detergents, lysozyme, toluene etc.). Enzyme purification has been reported from several organisms using classical purification techniques such as (i) heat treatment (to

denature heat labile proteases), (ii) precipitation with ammonium sulphate/acetone/Mg^{2+} or Mn^{2+} salts, (iii)ion exchange chromatography on DEAE-cellulose or DEAE-Sephadex and (iv) gel filtration using Sephadex. A few reports are also available on purification of GXI by affinity chromatography especially using D-xylitol (an inhibitor of GXI) as an affinity ligand (2).

The enzymatic and physicochemical properties of GXI from various microorgasnisms have been extensively studied. In addition to D-glucose and D-xylose, the enzyme also isomerizes D-ribose, D-allose, L-arabinose and L-rhamnose. Most of the glucose isomerases possess a fairly high optimum temperature, around 80°C with the optimum pH near neutrality. GXI in general requires divalent cataions such as Mg^{2+}, Co^{2+}, Mn^{2+} or a combination of these for the activity. The enzyme is generally multimeric (dimer or tetramer) especially from actinomycetes origin.

Xylose isomerases from Actinomycetaceae have distinct catalytic and physicochemical properties. To function catalytically, a stoichiometry of 2 mol of metal/mol of monomer is required in contrast with a stoichiometry of 1 mol of metal/mol of monomer for other isomerases. Their monomeric molecular masses of 43 kDa are lower than those

reported for GXI from other strains, having an average value of 50 kDa. The metal activating capacities further differ, Mg^{2+} being superior to Co^{2+} and Mn^{2+} with XI from Actinomycetaceae while the reverse is true for other XIs. In addition, XIs from Actinomycetaceae exhibit a superior thermostability and have higher pH optima than others (25).

Similarly, by comparing the primary structure of xylose isomerases from different microorganisms studied till 1990, Lee *et al* (26) showed the existence of two distinct classes of enzymes, viz. (i) thermolabile of the *Bacillus subtilis* and *Escherichia coli* type and (ii) thermostable of the *Streptomyces* type.

Lastick and Spencer (27) compared the coding regions of five sequenced XI genes (*B. subtilis, E. coli, S. violaceoniger, Ampullariella sp.* and *Actinoplanes missouriensis*) and their computer generated translation products. They found a good correspondence between *B. subtilis* and *E. coli* gene coding sequences despite the fact that they are taxanomically different. These genes and the enzymes which they code for were termed as 'Type-1'. XI-gene coding sequences of *S. violaceoniger, Ampullariella* and *A. missouriensis* showing significantly increased homology in primary structure were grouped as 'Type-2'. The *Type 1* enzymes, in general, have lower temperature optima and

stability, can function at lower pH levels and are more efficient at xylose isomerization than the *Type 2* enzyme (2) which makes them more suitable for fermentation processes using yeast. Type 1 enzymes were found to be the best for simultaneous isomerization and fermentation of xylose to ethanol (28). On the other hand, the *Type 2* enzymes are more efficient at glucose isomerization, and have other features that suit the production of HFCS.

Recently, a lot of work has been carried out on 'molecular enzymology' of glucose/xylose isomerase from different microbial sources. These include X-ray crystallography, active site studies and investigations on the requirement and binding of divalent metal ions to the enzyme.

Binding of metal ions to GXI

The activity of XI is generally dependent on divalent metal ions. The enzymes of *Streptomyces* sp. are best activated by Mg^{2+} than by Co^{2+} or Mn^{2+}. Sanchez and Smiley (29), Takasaki (30) and Kasumi *et al* (31) have recognized a stabilization effect of these cations on the tetrameric structure of the enzyme. In consonance with these results Callens *et al* (32) and Gaikwad *et al* (33) demonstrated that Co^{2+} is superior to Mg^{2+} as a protector against thermal

denaturation.

Direct metal ion binding studies were first reported by Danno (34) on XI of *Bacillus coagulans*. Studies on XI from *Lactobacillus brevis* by Schray and Mildvan (35) have shown the presence of 2.1 tight Mn^{2+}-binding sites and 4.4 weaker Mn^{2+}-binding sites/molecule of protein. Kasumi *et al* (31) have estimated 4 Co^{2+} ions to be present per tetramer of the GXI from *Streptomyces griseofuscus*. One of them was very tightly bound to the enzyme and had an essential role in maintaining the ordered conformation of the enzyme. Callens *et al* (36) have reported that the XI from *Streptomyces violaceoruber*, a tetramer, binds 2 moles of Co^{2+}/mole of monomer. Difference absorption spectrometry in the U.V. and visible regions indicated that the environment of the one Co^{2+} ion is markedly different from that of the other Co^{2+} ion. Marg and Clark (37) also observed two distinct metal-binding sites in GI from *B. coagulans*. One of the sites preferred Mn^{2+} (site 1) while the other showed preference for Co^{2+} (site 2). Enzyme activity towards fructose was highest when both sites were filled with Co^{2+} , whereas the activity towards xylose was highest when site 1 was occupied with Mn^{2+}. Recently, Sudfeldt *et al* (38) with the help of spectroscopic studies have shown the presence of two metal binding sites/subunit of the homotetrameric XI of *S.*

rubiginosus supporting its earlier X-ray crystallographic studies by Carrell *et al* (39).

X-ray crystallography and mechanism of enzyme action

X-ray crystal structure of D-xylose isomerases have been reported from five different actinomycetes and related organisms. In 1984, Carrell and co-workers (40) for the first time determined the structure of *Streptomyces rubiginosus* of 4-A° resolution. XI from *S. rubiginosus* is a tetramer (165,000) composed of four, identical polypeptide chains of approximately 370 amino acid residues. The folding of polypeptide chain has been established. It consists of two structural domains. The larger domain consists of eight β-strand ∝-helix (β∝) units arranged in a configuration similar to that found for triose-phosphate isomerase (another aldose-ketose transferase). The smaller domain forms a loop away from the larger domain but overlaps the larger domain of another subunit so that a tightly bound dimer is formed. The tetramer thus consists of two such dimers. Farber *et al* (41) determined the crystal structure of XI from *Streptomyces olivochromogenes* at 3.0 A° resolution. XI from *S. olivochromogenes* is also a tetramer and its three dimensional structure is very similar to that of the enzyme from *S. rubiginosus*. In 1988, Rey *et al* (42) and Glasfeld *et*

al (43) studied the structures of GI from *Actinoplanes missouriensis* and *Streptomyces violaceoniger*, respectively. In both the crystals, the asymetric unit contains the whole tetramer. Henrick *et al* (44) further studied the structure of XI from *Arthrobacter* B3728 containing inhibitors, xylitol and D-sorbitol at 2.5 A° and 2.3 A° resolution, respectively. The enzyme molecule is a tetramer and the asymmetric unit of the crystal contains a dimer similar to that for XI from *S. olivochromogenes*. The polypeptide folding is similar to that reported earlier for xylose isomerases. The enzyme is dependent on divalent cations for catalytic activity and two metal ions are required per monomer. The metal ion is complexed, at the high-affinity site, by carboxylate side chains of conserved glutamic and aspartic acid residues. The substrate binds in the active site in an extended open chain form. The active site lies in a deep pocket near the C-terminal ends of the β-strands of the barrel domain and includes residues from a second subunit. Thus, the tetramer is a dimer of 'active'dimers. Several internal salt bridges stabilize the tertiary and quaternary structure and one of these between Asp 23 and Arg 139, appear to play a key role in stabilizing the active dimer and is conserved in known sequences of this enzyme.

Carrell *et al* (39) further carried out the X-ray

analysis of XI from *S. rubiginosus* at 1.9 A° resolution and identified the active site, as well as the metal ion binding sites. The metal ions were shown to be important in maintaining the structure of the active site region. The study also revealed a very close contact between histidine and C^1 of a substrate, suggesting that this is the active site base that abstracts a proton from substrate; supporting cis-enediol intermediate formation during aldose-ketose transformation proposed by Rose *et al* (45). Similar base catalyzed enediol mechanism has been demonstrated for triose phosphate isomerase (46). However, in contrast to triose phosphate isomerase, xylose isomerase exhibits distinct biochemical and physical properties such as: (i) requirement for divalent cations for enzyme activity and stability; (ii) intramolecular hydrogen transfer without significant exchange with solvent; (iii) anomeric specificity for -configuration of substrate, etc. indicating that these two enzymes do not employ the same catalytic mechanism.

Based on the X-ray crystallographic studies Farber *et al* (47), for the first time, proposed a 1,2-hydride shift mechanism for aldose-ketose interconversion by GXI. Collyer and co-workers (48, 49) investigated the mechanism of xylose isomerization by determining the crystal structures of

Arthrobacter B3728 XI bound to various substrates, inhibitors and cations. Ring-opening (rate determining step) was found to be an obligatory first step of the reaction, where the active site histidine is involved. Of the two metal ion binding sites, one is permanently occupied under catalytic conditions and is co-ordinated by 4 carboxylate groups. Binding at second cation site is also necessary for catalysis and is believed to bind Co^{2+} more strongly than site 1. Metal ion-induced hydride shift from C_2 to C_1 is the second major step in aldose-ketose isomerization. After isomerization, ring closure (third step) is catalyzed as the reverse of ring-opening step.

Chemical modification of GXI

The data on X-ray crystallography has been substantially supported by chemical modification studies carried out by various research groups while determining the structure-function relationship of GXI. In 1988, three different groups of workers, Vangrysperre *et al* (50), Gaikwad *et al* (51) and Pawar *et al* (52) provided evidence for essential histidine residue at the active site of GXIs from Lactobacilli and Streptomycetes. Vangresperre *et al* (53) further located the single active-site histidine at position 54 in the primary structure of *Streptomyces violaceoruber* D-

xylose isomerase. In 1989, Vangryperre *et al* (54) for the first time provided an evidence for the modification of an active site carboxylate residue of GXI from different bacterial sources by reaction with Woodward's reagent K. The essential histidine and carboxylate group in the xylose isomerases from these sources were located by amino acid sequence analysis of peptides recognized by differential peptide mapping on ligand protected and unprotected derivatized enzyme. Both types of modified residues were found to be clustered in a region with consensus sequence: Phe-His-Asp-Xaa-Asp-Xaa-Xaa-Pro-Xaa-Gly, conserved in all XIs studied so far (25). A study of the pH-dependence of the kinetic parameters further suggested the participation of a histidine group in the substrate binding site and not in the isomerization process.

Cloning of GXI gene

The cloning of GXI gene from several microorganisms has been carried out with the aim of (1) over-production of the enzyme by gene-dosage effect by cloning it on a multicopy plasmid, (2) direct conversion of D-xylose (a major sugar in the hydrolysates of hemicelluloses) to ethanol by cloning of GXI gene in yeast cells or (3) for protein engineering of the enzyme to introduce properties desirable in the

industrial production of HFCS.

The enzyme is produced by almost all bacteria *in vivo* for the conversion of D-xylose to D-xylulose. D-xylulose is further phosphorylated to D-xylulose-5-phosphate and this phosphorylated intermediate is then metabolized by the pentose phosphate and Embden Meyerhoff pathways. Shamana and Sanderson (55) for the first time determined the genetics of D-xylose catabolism in *Salmonella typhimurium* LT2 and demonstrated the existence of three inducible activities for (i) D-xylose transport into the cell, (ii) its isomerization to D-xylulose (D-xylose isomerase) and (iii) for the phosphorylation of D-xylulose to D-xylulose-5-phosphate (D-xylulokinase), which are coded by genes *xylT, XylA* and *XylB*, respectively. These genes are closely linked to each other and to the *Xyl R* gene that codes for a positive regulatory factor (Fig. I.1). Further in 1984 (56) Ghangas and Wilson determined the gene order of *S. typhimurium* LT2 by cloning experiments and showed that it is consistent with that determined by genetic mapping.

There are several reports on cloning of GXI gene from *Escherichia coli*, Bacilli, Clostridia and Actinomycetes wherein GXI gene has been isolated by complementation of GXI negative mutants. 'Shotgun approach' was used as a cloning

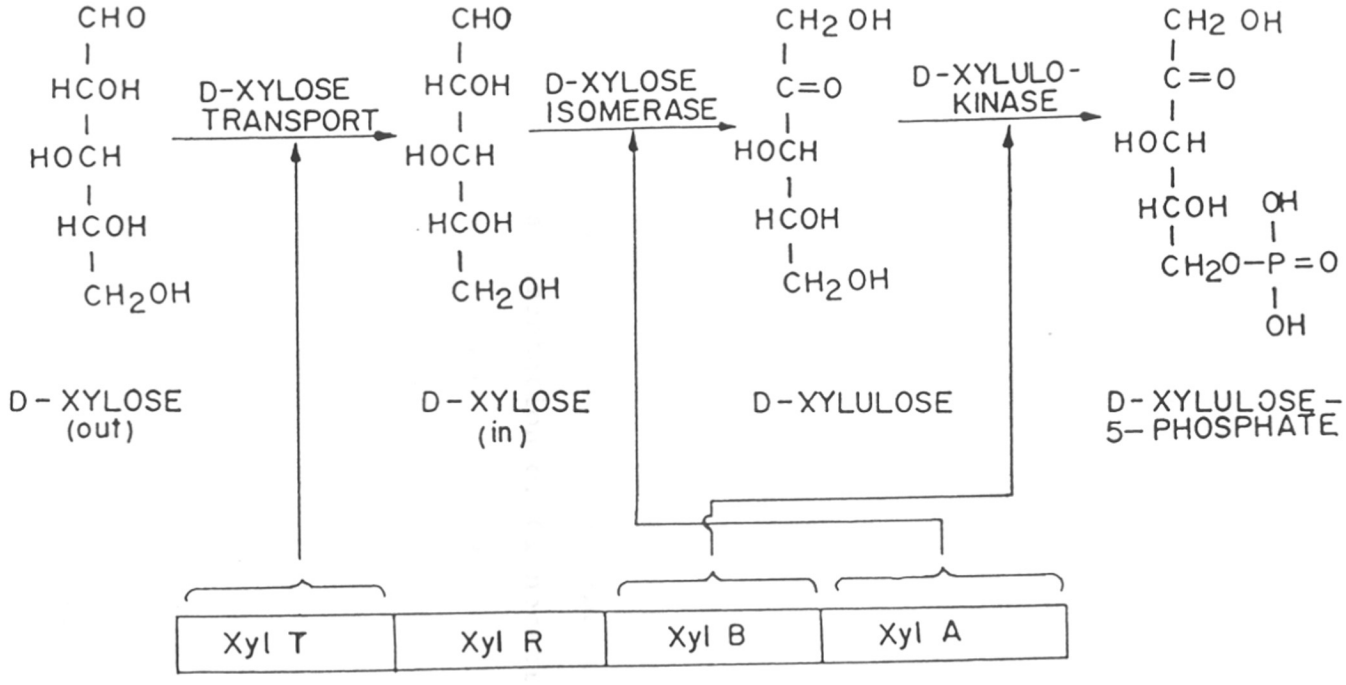

Fig. I.1 D-xylose catabolism pathway and organization of the relevant gene cluster in Salmonella typhimurium.

Xyl R: Regulatory gene coding for repressor.

strategy by most of the workers.

Cloning of *XylA* from *E. coli*

Earlier reports on cloning of GXI gene come from *E. coli*. The Clarke and Carbon bank of ColE1-*E. coli* DNA hybrid plasmids (57) was screened for complementation of D-xylose negative mutants of *E. coli*. Of the several plasmids obtained, the smallest, pRM10 was chosen for further studies and was shown to complement mutants of *E. coli* defective in either D-xylose isomerase or D-xylulokinase activity, or both (58). The behaviour of the plasmid was consistent with the existence of a D-xylose operon in *E. coli*. A recombinant plasmid, pUC 1002 was constructed by ligation of a Hind III restriction endonuclease fragment of *E. coli* chromosomal DNA to pMB9 (vector plasmid) (59). Strains carrying this plasmid were selected by complementation of GI negative mutant (JC 1553) and they showed coordinately elevated levels of D-XI and D-xylulokinase activities. The GXI activity was amplified > 5 fold relative to the activity of the donor strain. Ho *et al* (60) attempted separation of D-xylose isomerase gene of *E. coli* from D-xylulokinase by sub-cloning excercises. In 1984, Schellenberg *et al* (61) for the first time attempted sequencing of *E. coli* xylose isomerase gene. The second report on sequencing of xylose isomerase gene from

E. coli was of Lawlis *et al* (62). Briggs *et al* (63) cloned and isolated *E. coli* xylose isomerase gene by complementation of an isomerase deficient *E. coli* strain. Further, by subcloning, the gene was located in a 1.6 kbp Bgl II fragment. The fragment was sequenced and the gene was shown to be 1002 bp in size. The Bgl II fragment was also cloned into a yeast expression vector utilising the *CYCL* yeast promoter. However, no expression of GXI activity was detected in yeast cells.

Thus, the *E. coli* GXI gene was mainly isolated either using pBR 322 as a cloning vector (61,62) or by screening Clarke and Carbon's *E. coli* genomic library in ColE1 (58,60,63). Overproduction or hyperexpression of *E. coli* xylose isomerase has also been reported by several workers. Stevis and Ho (64) found that high levels of overproduction of GXI cannot be achieved simply by increasing the gene dosage. They fused *XylA* structural gene with strong promoters such as tac and lac resulting in a 20-fold overproduction of the enzyme. Similarly, Batt *et al* (65) achieved hyperexpression of *E. coli* xylose isomerase by cloning *XylA* under the control of tac promoter. XI accounted for approximately 28% of the total cell protein. Lastick *et al.* (66) obtained overproduction of *E. coli* XI by ligating

Xyl A and its ribosome binding site to a plasmid downstream from the strong p_L promoter.

Cloning of *Xyl A* in yeast cells

The yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are important in ethanol industry due to their high alcohol tolerance, rapid fermentation rate and high end product yield. However, they are not able to ferment D-xylose to ethanol. Therefore, cloning of xylose isomerase gene into these two yeasts shows promise for obtaining an organism that can directly ferment xylose to ethanol. Ueng *et al* (67) isolated the GI gene from Clarke and Carbon's *E. coli* genomic library in ColE1 and inserted into the Pst I site of pDB248, a shuttle plasmid between the bacterium *E. coli* and the fission yeast, *Schizosaccharomyces pombe*. The *E. coli* XI gene was maintained and expressed in *S. pombe*. The transformed *S. pombe* was found to be able to directly ferment D-xylose (10% w/v) to produce 3.0% (w/v) of ethanol (68). Further studies were undertaken to determine the factors which limit D-xylose utilization by the transformed yeast and it was shown that the low activity of xylose isomerase was the limiting step for D-xylose fermentation (69). An *in vitro* study showed that yeast proteases decreased the XI activity.

Cloning of *Xyl A* from *Bacillus* into *E. coli*

Xylose operon was located in *Bacillus subtilis* by Wilhelm and Hollenberg (70) during their studies on 'cloning of *B. subtilis* XI gene in *E. coli*'. Also the *Xyl A* from *Bacillus* was found to be expressed in *E. coli* only after IS5 insertion. The XI gene from *Bacillus* was sequenced and compared with the derived amino acid sequence of the *E. coli* enzyme. 50% homology extending over the entire polypeptide (71) was observed. Shin and Kho (72) cloned and expressed *B. licheniformis* GI gene in *E. coli* and 20 fold increase in the activity was obtained.

B. subtilis is a safe microorganism which is traditionally used for the production of industrial enzymes. Hence, the cloning and expression of *E. coli Xyl A* in *B. subtilis* was attempted (73). *Xyl A* was subcloned into a *Bacillus-E. coli* bifunctional plasmid and it was found that the intact *E. coli* gene was not expressed in *B. subtilis*. An attempt was then made to express the *E. coli* gene in *B. subtilis* by fusion of the *E. coli* XI structural gene down stream to the promoter of the penicillinase gene isolated from *B. licheniformis*. Such fused genes were able to express the GI gene in both *B. subtilis* and *E. coli*.

Cloning of *Xyl A* from *Clostridium* in *E. coli*

The gene that encodes thermostable GI in *Clostridium thermosulfurogenes* was cloned and expressed both in *E. coli* and *B. subtilis* (74). Expression of the thermostable GI gene in both species was constitutive, whereas the enzyme is inducible in *Clostridium*. *B. subtilis* and *E. coli* produced higher levels of thermostable GI (1.54 and 0.46 U mg^{-1} of protein, respectively) than those produced by *Cl. thermosulfurogenes* (0.29 U mg^{-1} of protein). Lee *et al* (75) determined the complete nucleotide sequence of *Cl. thermosulfurogenes Xyl A* which encodes a polypeptide of 439 amino acids. Dekker *et al* (76) cloned, sequenced and expressed GXI gene from another thermophilic *Clostridium, Cl. thermohydrosulfuricum* in *E. coli*. Comparison of deduced amino acid sequences from both the Clostridia showed that the amino acids involved in substrate binding and isomerization are well conserved.

Cloning of GXI gene from other sources

Liu *et al.* (77) cloned and expressed *Xanthomonas* DNA in *E. coli* that encodes D-xylose catabolizing enzymes i.e. xylose operon and also expressed them in *Zymomonas mobilis* (78), an ethanol producing bacterium.

Other reports on cloning and sequencing of GXI gene include those from *Thermus thermophilus* - producing

exceptionally thermostable GXI (79) and from *Lactobacillus brevis* (80) showing homology with XIs from other bacteria, especially within the primary catalytic domains of the enzyme.

Cloning, sequencing and expression of GXI gene from Actinomycetes and related organisms will be discussed in Chapter V.

Site directed mutagenesis and protein engineering

The presence of essential histidine residue in the active site of GXI was further confirmed by its site directed mutagenesis. On the basis of sequence homology among xylose isomerases isolated from *Escherichia coli*, *Bacillus subtilis*, *Ampullariella* 3876 and *Streptomyces violaceusniger*, Batt *et al* (81) selectively replaced the two conserved histidine residues, His-101 and His-271 of *E. coli* XI (which appeared to be essential components in the active site of the enzyme) by site directed mutagenesis and showed them to be essential for activity. It was proposed that His-101 is the catalytic base mediating the reaction, and replacement of His-271 may render the enzyme thermolabile, since this residue appears to be a ligand for one of the metal ions in the active site of the enzyme. To identify the histidine residue at the active site and to elucidate its function during enzymatic

glucose/xylose isomerization, histidine residues at four different positions in the *Clostridium thermosulfurogenes* enzyme were individually modified by site-directed mutagenesis. Substitution of His 101 by phenylalanine completely abolished the enzyme activity whereas substitution of other histidine residues had no effect on enzyme activity (75). Meng *et al* (82) switched the substrate preference of thermophilic XI from *Clostridium thermosulfurogenes* from D-xylose (natural substrate) to D-glucose (industrial substrate) by redesigning the substrate binding pocket using site directed mutagenesis. Thus the alteration in substrate specificity would help in efficient catalysis in industrial processes.

High thermo-tolerance is desirable for production of HFCS because, at equilibrium, as the temperature of the enzyme reaction is increased, the ketose/aldose ratio increases proportionately (83). In addition, reactors running at high temperatures have less risk of microbial contamination, allowing for less frequent and less costly enzyme replacement. Such enhanced thermostability of GI from *A. missouriensis* by protein engineering has been reported recently (84, 85). Quax *et al* engineered recombinant GI from *A. missouriensis* by site directed mutagenesis to enhance its

thermostability both in the soluble and immobilized forms. Substitution of arginine for lysine at position 235, which lies at the dimer/dimer interface of the GI tetramer, produced the largest stabilization under model industrial conditions.

Recently in 1992, Lambier *et al* (86-88) have carried out protein engineering of substrate and metal ion binding sites of the GXI from *A. missouriensis* by site directed mutagenesis (after cloning and overexpressing it in *E. coli*) Together with the structural data derived from X-ray crystallography of the wild-type as well as mutant enzyme, they have demonstrated that the two metal ions play an essential part in binding of enzyme to substrates, in stabilizing their open forms, and in catalyzing hydride transfer between the C1 and C2 positions.

Folding of GXI

The primary structure of a protein refers to the specific amino acid sequence along the covalent polypeptide chain; the secondary defines regular arrangements of the polypeptide backbone in terms of α-helices and/β-structures; the tertiary structure refers to the three dimensional chain fold and the quaternary structure describes the arrangement of subunits. The cotranslational or the post translational

acquisition of the functional state of the nascent polypeptide chain based solely on the amino acid sequence and the solvent environment of the polypeptide is termed as 'protein folding'. For multimeric proteins, subunit association, has to be considered in addition to the protein 'folding' as the proper coordination of monomers is required for yielding a native functional protein molecule.

With the advent of technological improvements in biology, protein folding *in vivo* as well as *in vitro* has received increased attention in the past decade. The mechanism of *in vitro* protein folding and its implications in *de novo* protein folding are being investigated. The denaturation and renaturation of the protein (by the denaturants:Gdn.HCl, Urea, SDS, heat, acidic or alkaline pH etc.) accompanied with determination of the structural changes with the help of fluorescence and circular dichroism helps, to some extent, in understanding of the protein folding pathway. There exists a difference between folding of large, multidomain protein occurring cotranslationally (*in vivo*) and refolding *in vitro*, starting from the complete unfolded polypeptide. Earlier, it was believed that the native, functional conformation of a protein is determined by its amino acid sequence and the polypeptide chain requires

neither extrinsic factors nor the input of energy to attain this conformation (89). A number of proteins such as chaperonins, polypeptide chain binding proteins, foldases, unfoldases etc. involved in the maturation of folding of nascent protein *in vivo* have been discovered (90). Recent work connected with the protein folding pathway have also been focused on folding intermediates (91). Location of one such intermediate, a 'molten globule state' (an intermediate with high content of native-like secondary structure) of a protein is a clue for understanding the folding of globular proteins.

GXI being a multimeric protein, it would be interesting to study its unfolding and refolding. The effects of conformational changes and subunit dissociation on the biological activity of the GXI during denaturation have been studied (31, 92-94). However, the detailed characterization of the folding intermediates remains to be investigated.

Background and scope of the present work

Streptomyces sp. NCIM 2730 is isolated in National Chemical Laboratory from a soil sample from the laboratory campus. The organism produces high yields (10,000 UL^{-1}) of intracellular GXI on Callen's medium (95) with xylose (1%) as an inducer. Replacement of xylose by xylan containing

materials (wheat bran or bagasse hydrolysates, 5%) yields 30-40% of the maximum activity. The strain has no requirement for Co^{2+} ions for enzyme production. Immobilization of the enzyme on an inexpensive anion exchange resin, Indion 48-R considerably increases the thermostability of the enzyme. Concentrated glucose syrup (25%) can be efficiently converted to fructose by the immobilized enzyme suggesting its potential for use in HFCS production (96).

The enzyme is inducible and requires metal ions (Mg^{2+} and Co^{2+}) for its activity. Optimum temperature and pH are 75-80°C and 7.5-8.0 respectively. It acts only on glucose and xylose, its affinity being higher for xylose than for glucose.

Besides the commercial importance of the GXI from *Streptomyces* sp. NCIM 2730, the enzyme also serves as an interesting model for studying the structure-function relationship, which is important for understanding the catalytic mechanism of the enzyme. The GXI from *Streptomyces* sp. NCIM 2730 is a tetramer (Mr 160,000) and is made up of four identical subunits (Mr 40,000) (94). Previous work from our laboratory has shown the presence of an essential histidine residue at the active site (51) and requirement of a single active site for isomerization of both glucose and

xylose (97). Studies on differential roles of the metal ions showed that Mg^{2+} is superior to Co^{2+} as an activator while Co^{2+} acts as a stabilizer by holding the ordered conformation, and maintaining the quarternary structure of the enzyme (33).

In the present work, various molecular aspects of high glucose/xylose isomerase yielding *Streptomyces* sp. NCIM 2730 have been studied with special reference to the structure-function relationship of GXI and its possible cloning in *E. coli*. The thesis has been organized into five chapters as follows:

Chapter I: Introduction

Chapter II: Structure-function relationship of GXI

(a) The biological activity of GXI in relation with the structural changes in the protein consequent to its denaturation and renaturation have been studied.

(b) The reaction of guanidine hydrochloride with carboxyl groups of the protein has been demonstrated.

Chapter III: Immuno-affinity purification of GXI

In order to facilitate extensive investigations on GXI, a single-step purification procedure based on the immunoaffinity chromatography has been developed by raising a polyclonal antibody against electrophoretically homogeneous GXI.

Chapter IV: Genome characterization of *Streptomyces* sp. NCIM 2730

The *Streptomyces* genome has been characterized with respect to its kinetic complexity using optical reassociation and evidence for the occurrence of repetitive DNA has been presented.

Chapter V: Construction of genomic library of *Streptomyces* in *E. coli*

A partial genomic library of *Streptomyces* has been constructed in *E. coli* multicopy plasmid vector pUC8. Screening of the library for the identification of a putative gene using a synthetic oligoprobe and the specific antibodies is under way.

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CHAPTER II

STRUCTURE-FUNCTION RELATIONSHIP OF GLUCOSE/XYLOSE ISOMERASE

Section A

Unfolding and refolding of GXI: The role of tertiary structure in biological activity

SUMMARY

The unfolding and refolding of the tetrameric D-glucose/xylose isomerase (GXI) from *Streptomyces* sp. NCIM 2730 has been investigated by correlating the biological activity with the protein transitions as monitored by fluorometry, c.d. and by its retention volumes in molecular sieve chromatography. Treatment of the enzyme with SDS (0.1%) results in the dissociation of the tetramer (T) into an active dimer (D) with no gross change in the tertiary structure but change in the secondary structure. On removal of the denaturant, a part of the dimer reassociates to form a tetramer presenting, for the first time, an experimental evidence for the reversibility of the tetramer-dimer transition. Incubation of the enzyme with Gdn-HCl (2M) results in a complete loss in activity although the enzyme is in its dimeric form. The inactive dimer (D*) has conformational properties of a molten globule, namely, a native-like secondary structure and disordered tertiary structure. Regain of activity was observed on lowering of the concentration of the denaturant by dilution. Refolding

of the Gdn-HCl treated enzyme results in the restoration of its tertiary structure and activity. The enzyme is completely inactivated by heating at 100°C for 5 min. The heated enzyme is a monomer and exhibits a distinct irreversible change in its structure. Thus, four distinct species have been identified and characterised during denaturation and renaturation of the GXI: (i) native tetramer (T) active and inactive dimers (D and D*) and (iii) inactive monomer (M). The results suggest that the intact tertiary rather than the secondary structure is essential for GXI activity.

INTRODUCTION

Glucose/xylose isomerases have been studied extensively in view of their potential application in the conversion of glucose to fructose for the production of high fructose corn syrup (1). Moreover, isomerization of xylose to xylulose is a key step in the economic production of ethanol from hemicellulosic fraction of agricultural biomass (2). Besides its commercial importance the enzyme also serves as an interesting model for studying structure-function relationship. The extensive structural characterization of GXI carried out by X-ray crystallography (3,4) has made it an attractive subject for protein eingineering (5,6).

Protein folding, a process of aquisition of the functional state of the nascent polypeptide chain based on the amino acid sequence and the solvent environment, has received increased attention recently. The structural characterization of intermediate conformational states is one of the important aspects of protein folding studies (7). For a multimeric protein such as GXI, besides polypeptide folding, the subunit association and proper co-ordination of monomers is important for yielding a functional protein.

Few reports are available on subunit dissociation consequent to denaturation, accompanied with change in biological activity of the enzyme. Dissociation of the

tetramer/dimer to its constituent subunits (monomer) on denaturation results in loss of enzyme activity (8-10). The GXI (tetramer) from *Streptomyces griseofuscus*, S-41 dissociates into monomer by treatment with 1% SDS only when accompanied by heat or acidic pH (11). The stability of the enzyme to the SDS treatment may be due to the inaccessibility of the hydrophobic region of the native enzyme to SDS ions due to the rigid structure of the enzyme. Treatment of the enzyme with acid/heat may be responsible for loosening of the molecular structure of the enzyme, exposing hydrophobic regions for which SDS has more affinity and may result in inactivation of the enzyme accompanied by subunit dissociation. Callens *et al* (12) reported that native enzyme (tetramer, Mr 1,70,000) of *Streptomyces violaceoruber* on treatment with 0.1% SDS dissociates into a dimer (Mr 85,000) which is also an active species of the enzyme while monomer is an inactive species and cannot reassociate to either dimer/tetramer. Similar results were obtained by Rangarajan *et al* (13) in their studies on stability of *Arthrobacter* XI to denaturants and heat.

The aim of the present investigation has been to delineate the structure-function relationship of the *Streptomyces* GXI by correlating the changes in the biological

activity with the transitions in the protein structure consequent to denaturation and attempted renaturation. Our results suggest that the molecular basis of inactivation of GXI is a reflection of the disruption in the tertiary structure whereas the activity is independent of the changes in the secondary structure. The evidence for the occurrence of a unique conformational state resembling a molten globule has been presented, for the first time, in the folding pathway of GXI.

MATERIALS AND METHODS

Materials

Malt extract, yeast extract, peptone, casamino acids and Difco agar were obtained from Difco Laboratories (U.S.A.). D-Glucose and D-xylose were of analytical grade from E. Merck (Germany).

Acrylamide, bis-acrylamide, ammonium per sulphate, TEMED, guanidine hydrochloride, SDS and molecular weight markers were from Sigma Chemical Company (U.S.A.).

2:3:5 triphenyl tetrazolium chloride was from BDH Laboratory Chemicals (England). Coomassie Brilliant Blue G-250 was from Fluka (Switzerland). Sepharose CL-6B was obtained from Pharmacia (Sweden).

All other ingredients of media, buffers as well as different reagents were of analytical grade and were obtained from Qualigens or Sisco Research Laboratories, India.

All the buffers and reagents were prepared in double distilled water.

Microorganism

Streptomyces sp. NCIM 2730 was isolated from a soil sample from NCL campus by National Collection of Industrial Microorganisms (NCIM), Pune, India. The culture was maintained on MGYP (0.3% malt extract, 1.0% glucose, 0.3% yeast extract, 0.5% peptone and 2% agar) slants by periodic

subculturing.

Enzyme production

GXI from *Streptomyces* sp. NCIM 2730 was produced as described by Gaikwad *et al* (14). Vegetative inoculum was developed for 72 h on rotary shaker (200 rpm) at 28°C by transferring the culture grown on MGYP for one week to 100 ml medium reported by Callens *et al* (15) in 500 ml Erlenmeyer flasks. Composition of the medium is as follows:
Sorbitol, 2%; casamino acids, 1%; yeast extract, 0.5%; $MgSO_4.7H_2O$, 0.2%; K_2HPO_4, 0.52%; KH_2PO_4, 0.27%; NH_4NO_3, 0.3% and D-xylose, 1.0%; pH 7.5.

The inoculum (10%) was transferred to the fermentation medium having the same composition as that of inoculum medium. The culture was harvested after 96 h by centrifugation. Cells were washed twice with distilled water and preserved at -20°C for further use.

Enzyme purification

Enzyme purification was carried out as described by Gaikwad *et al* (14) and involved the following steps:

(i) Enzyme extraction:

Cells from 100 ml culture broth were suspended in 25 ml of 50 mM sodium phosphate buffer, pH 7.5 and subjected to sonication (Versonic) for cell disruption for 6 min (2 min at

a time, with 10 min time interval) at 4°C. The suspension was centrifuged in Sorvall (Model RC-5) at 12,000 rpm at 4°C for 15 min to remove the cell debris and the clear supernatant was used as the crude enzyme preparation.

(ii) Heat treatment

The crude enzyme preparation was subjected to heat treatment at 65°C for 15 min to denature the heat labile proteases. It was then cooled down to 4°C, centrifuged and the supernatant was used for further purification.

(iii) Ammonium sulfate precipitation

Ammonium sulfate required for 0.9 saturation (60 mg/100 ml) was added slowly with gentle stirring to avoid frothing. The solution was allowed to stand overnight at 20°C and the precipitate was collected by centrifugation at 15,000 rpm for 30 min. The precipitate was then dissolved and dialysed against 1 mM sodium phosphate buffer, pH 7.5 containing 1 mM Mg^{2+} and 0.5 mM Co^{2+} at 4°C for 24 h with three changes of buffer at every 8 h. The dialysate was concentrated by lyophilization.

(iv) Preparative PAGE

Preparative PAGE was carried out in 7.5% cylindrical gel (25 x 15 cm), pH 8.8 (16). The electrode buffer contained 6.0 mM Tris and 40 mM glycine, pH 8.3 whereas the gel contained 375 mM Tris-HCl, pH 8.8. 5 ml of enzyme

solution from the above step, containing 15-20 mg of protein was loaded on the gel and the electrophoresis was carried out (current 20 mA and voltage 200-250 V) at 4°C for 48 h. Electrode buffer in both anodic and cathodic compartments was replaced by fresh buffer after 16 h. The electrophoresis was continued till the tracking dye (bromophenol blue) moved upto 1 cm from the lower end of the gel. The cylindrical gel block was removed, a vertical thin strip of the gel was cut and used for *in situ* quick enzyme staining (17) to locate the protein band corresponding to GXI activity. A portion of the main cylindrical gel-block corresponding to the located enzyme band was sliced, extracted with 50 mM sodium phosphate buffer, pH 7.5 containing 1 mM Mg^{2+} and 0.5 mM Co^{2+} and filtered. The filtrate was dialysed against 5 mM sodium phosphate buffer, pH 7.5 containing 1 mM Mg^{2+} and 0.5 mM Co^{2+} for 24 h with three changes of dialysis buffer at 4°C. The dialysate was concentrated by lyophilization. This enzyme preparation was used as the purified enzyme for further studies.

Analytical PAGE

Analytical PAGE was carried out in 7.5% cylindircal gels (0.5 x 8.0 cm) at pH 8.8 according to Davis (16). Electrophoresis was run at 4°C with 3-5 mA current per gel

tube for a period of 3-4 h. Protein bands were stained with Coomassie Brilliant Blue G-250 (18).

***In situ* staining of GXI**

In situ staining of the gel for GXI activity was carried out according to Yamanaka (17) in a reaction mixture containing 5 ml of 0.1 M sodium phosphate buffer, pH 7.5; 1 ml of 2 M D-xylose; 0.5 ml of 0.1 M $MgSO_4.7H_2O$; 0.5 ml 0.02 M $CoCl_2.6H_2O$; 2.5 ml distilled H_2O at 70°C for 5 min for xylose isomerase activity. It was then rinsed with distilled water and immersed into a 0.1% solution of 2:3:5 triphenyl tetrazolium chloride in 1 N NaOH at room temperature, in dark for 1 min. The position of GXI was visualized by a dark pink band of formazan on the colourless background of the gel. The darkening of the background was prevented by dipping the gel into 1 N HCl followed by washing with distilled water.

Enzyme assay

GI was estimated by the colorimetric method of Takasaki (19). D-fructose produced in the reaction was determined by the method of Dische and Borenfreund (20), as modified by Marshall and Kooi (21).

Reaction mixture (2 ml) contained $MgSO_4.7H_2O$ (5 mM), $CoCl_2.6H_2O$ (1 mM), D-glucose (0.1 M), sodium phosphate buffer (0.05 M), pH 7.5 and suitably diluted enzyme solution. After incubation at 70°C for 30 min the reaction was terminated by

adding 2 ml of 0.5 M perchloric acid. To an aliquot of 0.05 ml from this mixture, 0.95 ml H_2O, 0.2 ml of 1.5% cysteine hydrochloride, 6 ml of 70% sulfuric acid and 0.2 ml of 0.12% alcoholic carbazole solution were added sequentially. After heating at 60°C for 10 min, optical density of the violet colour formed due to D-fructose was measured at 560 nm.

One unit of the enzyme activity was defined as the amount of enzyme which produces 1 µmole of D-fructose min^{-1} under assay conditions.

Protein estimation

Protein estimations were carried out by the following methods:

1. Bradford's method (22): Acrylamide impurities in the enzyme preparation do not interfere in the dye binding method of protein estimation, so this method was preferred to the method of Lowry *et al.* Bovine serum albumin (BSA) was used as a reference protein. The absorbance of the protein - Coomassie Brilliant Blue G-250 coloured complex was measured at 595 nm.

2. Spectrophotometric method (23): After removing the acrylamide impurities in the sample, final protein concentration was determined by spectrophotometry. A correction for nucleic acid and ultraviolet absorbing

impurities was made by the following equation:

$\frac{4}{7}$ [2.3 (OD_{280} - OD_{340}) - (OD_{260} - OD_{340})] = mg of protein ml^{-1}

Enzyme assay in the presence of denaturants

Loss of enzyme activity in the presence of denaturants (Gdn.HCl or SDS) was assayed with the same concentration of denaturant present in the assay mixture as in the original incubation mixture. The enzyme (0.40 U) was incubated with different concentrations of the Gdn.HCl for 15 min or with 0.1% SDS for 30 min at room temperature.

Renaturation of GXI

Renaturation of GXI after denaturation was estimated either by dilution of the denaturant in the assay mixture or by dialysis of the treated enzyme against 5 mM sodium phosphate buffer, pH 7.5 containing 1 mM Mg^{2+} and 0.5 mM Co^{2+} at 4°C for 24 h. Three changes of buffer were given at an interval of 8 h. The dialysed sample was then subjected to estimation of GI activity.

Fluorescence spectra

Fluorescence emission spectra of the enzyme (100 $\mu g.ml^{-1}$) in the presence and absence of the denaturants were recorded at excitation wavelengths of 274 and 295 nm using Aminco SPF-500 spectrofluorometer. The absorbance of the enzyme solution at 280 nm was kept below 0.1 for fluorometric

studies to obtain the emission spectra within the range of the instrument.

Circular dichroism measurements

All c.d. studies in the far ultraviolet range (195-250 nm) were carried out on Jasco J-500A spectropolarimeter equipped with Xenon lamp and attached with a data processing unit (DP-500 N). The instrument was set in standard operating conditions, after a 30 min warm up. The intensity and other parameters were optimised for best possible resolutions (S/N ratio) such as: the time constant (8 sec), sensitivity (1 m°/cm), concentration of protein (60 $\mu g.ml^{-1}$). All measurements were performed under constant nitrogen flush. The baseline for each spectra was corrected with blank. To improve the S/N ratio, each spectrum was obtained by substracting an average of 16 baseline scans from an average of 16 protein solution scans (16 repeats) using the data processor.

The protein concentrations of the sample used under different conditions were maintained constant for a particular set of experiments. Quartz cuvette of 1 mm path length was used for all measurements.

The data from CD measurements were expressed as Molar ellipticity [θ] for all wavelengths and were calculated from the equation:

$$[\theta] = \frac{100 \times \theta}{l \times c} \text{ deg.cm}^2\text{.dmol}^{-1}$$

where, $\theta = h \times s$
h = height observed (in cms)
s = sensitivity of CD scale (in m°/cm)
l = cell length (cm)
c = protein concentration (mol/lit)

Gel filtration

The changes in the subunit status of the enzyme were deciphered by gel filtration of the enzyme on a Sepharose CL-6B column (1.8 x 100 cm^2) at 4°C unless otherwise specified. The column was calibriated using proteins of known molecular mass as standards: β-amylase (200 kDa), bovine serum albumin (68 kDa) and ovalbumin (45 kDa). Unfolding of the enzyme (2 mg) was monitored by gel permeation in the presence of the respective denaturants while for refolding of the enzyme, the column was equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.5.

RESULTS AND DISCUSSION

Organism

Streptomyces sp. NCIM 2730 is a gram positive and highly aerobic sporoactinomycetes. It forms an extensive branching substrate as well as aerial mycelium. On solid medium (MGYP), initially, the colonies are of bacterial type. After 48 h of incubation white substrate mycelium starts appearing which fully sporulates generally after 7 days of incubation at 28°C. The colour of spores is grey (Fig.II.1).

Enzyme production

Streptomyces sp. NCIM 2730 grows in pellet form under submerged fermentation conditions after 96 h of incubation on a rotary shaker and the pH of the medium becomes alkaline. The organism produces 3000 UL^{-1} of the enzyme activity, under the assay conditions.

Enzyme purification

The enzyme purified by preparative PAGE was electrophoretically homogeneous as examined by analytical PAGE. The final recovery of the enzyme was 30% and the specific activity was 16 U/mg. The specific activity increases from 2.8 (of crude extract) to 16.0 with 5.6 fold purification.

The purified enzyme was used for further studies.

Fig. II.1 Streptomyces sp. NCIM 2730 grown on MGYP slant at 28°C for 7 days

Properties of native enzyme

GXI from *Streptomyces* sp. NCIM 2730 is a tetramer (Mr 1,60,000) of four identical subunits (Mr 40,000) (24). Intrinsic fluorescence of the enzyme was monitored at excitation wavelengths of 274 and 295 nm. The fluorescence of proteins is due primarily to emission from tyrosine and tryptophan residues. At 274 nm both tyrosine and tryptophan contribute to the emission spectrum (25) whereas at 295 nm, the resulting fluorescence emission is attributed to the tryptophan component of the protein alone (26). Excitation of the native enzyme at 274 and 295 nm showed emission maxima at 340 and 345 nm respectively (Fig.II.2). The far UV c.d. spectrum of the native enzyme showed double minima at 220 and 211 nm (Fig. II.3a), characteristic of a protein with significant α-helical content (27).

Changes in quaternary structure of the protein were monitored by its retention volumes in molecular seive chromatography on Sepharose CL-6B while those in the tertiary and secondary structure of the enzyme were followed with the help of fluorometry and circular dichroism respectively.

Treatment of the enzyme with SDS

The native GXI, retains almost all activity on treatment with 0.1% SDS (mild denaturing conditions). However, the gel filtration of the treated enzyme on

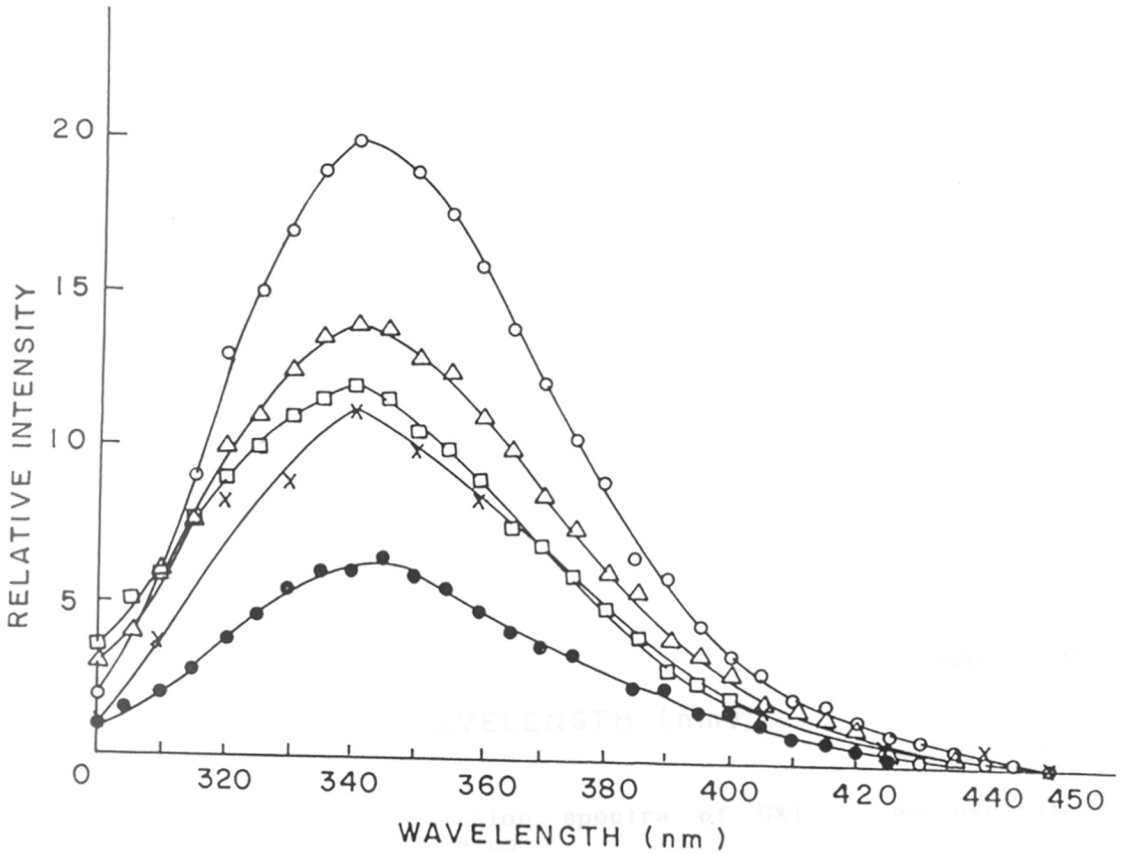


Fig.II.2(a) **Fluorescence-emmission spectra of GXI at an excitation wavelength of 274 nm**

GXI native (O); SDS (0.1%) treated (□); Gdn.HCl (2M) treated (●) and renatured (X); heat treated (△).

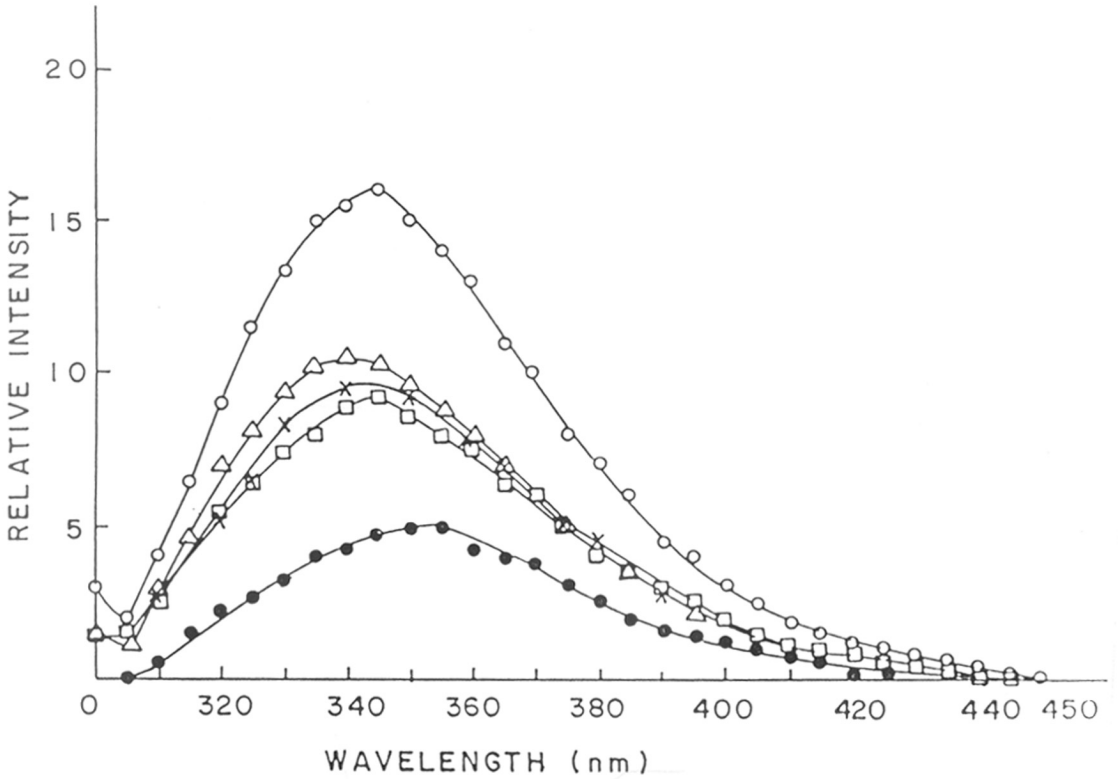


Fig.II.2(b) **Fluorescence-emission spectra of GXI at an excitation wavelength of 295 nm**

GXI native (O); SDS (0.1%) treated (□); Gdn.HCl (2M) treated (●) and renatured (X); heat treated (△).

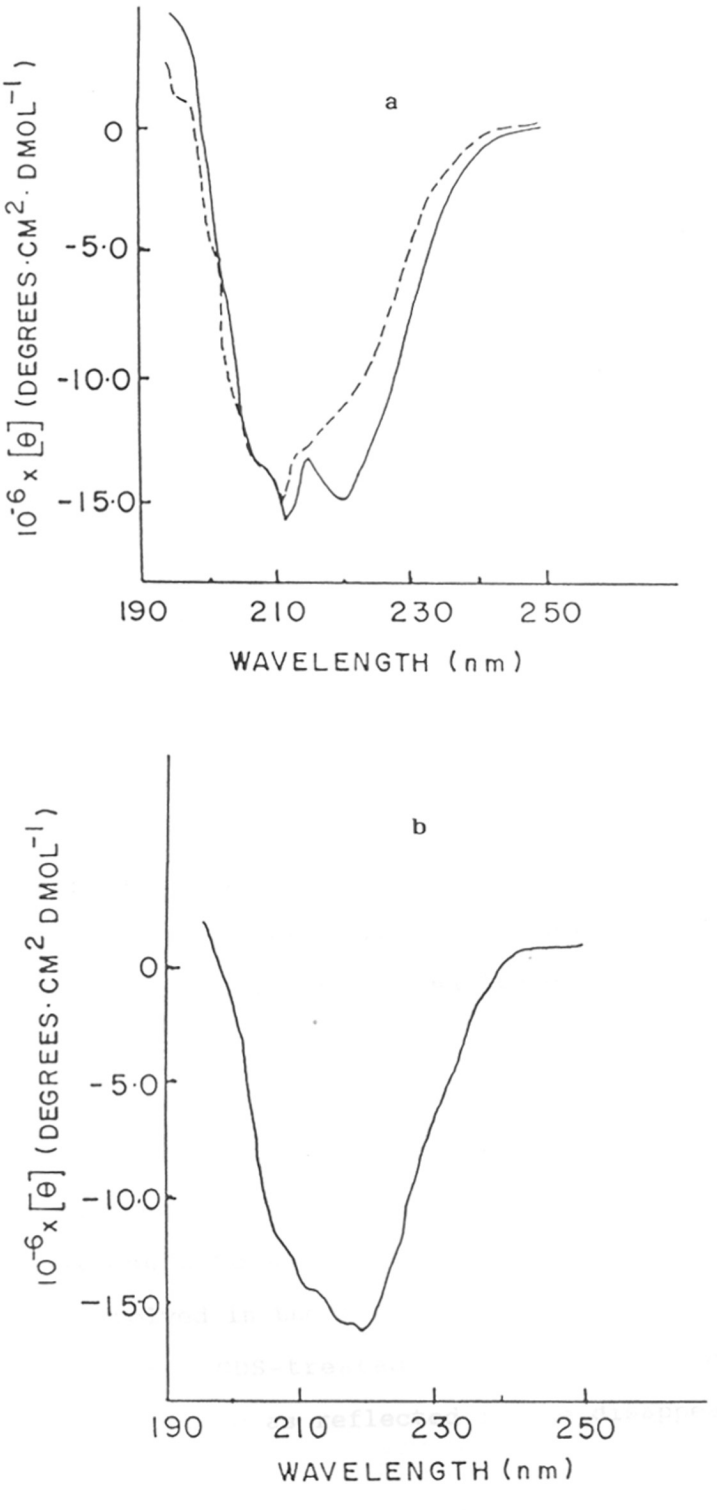


Fig.II.3 **Far UV c.d. spectra of GXI**

(a) Native (——); Heated (- - -)
(b) SDS (0.1%) treated

Sepharose CL-6B at 25°C revealed the presence of a predominant dimer (D) (Mr 80,000) along with small amounts of inactive aggregate (Mr 2,00,000) and inactive monomer (Mr 40,000) (Fig. II.4a).

X-ray crystallographic studies on GXI (tetramer) from actinomycetes (3, 28, 29) have shown that each subunit of the enzyme has two domians viz. large domain consisting of 8 stranded α/β barrels and a smaller domain (C-terminal domain) forming a loop away from the larger domain, but overlapping the larger domain of another subunit so that a tight bound dimer is formed. The active site lies in a deep pocket near the C-terminal ends of the β-strands of the barrel domain and includes residues from a second subunit. Thus, the tetramer is a dimer of 'active' dimers. The results on dissociation of the native enzyme (tetramer) into an active dimer on denaturation (12, 13, 24) confirm the X-ray crystallographic findings. Fluorescence spectra of the dimer are similar to that of a tetramer (Fig. II.2), except for a small decrease in the total fluorescence intensity, due to the exposure to the aqueous solvent of the tryptophan residues involved in the intersubunit faces. The far UV c.d. spectrum of the SDS-treated enzyme shows a change in the secondary structure as reflected in the disappearance of the

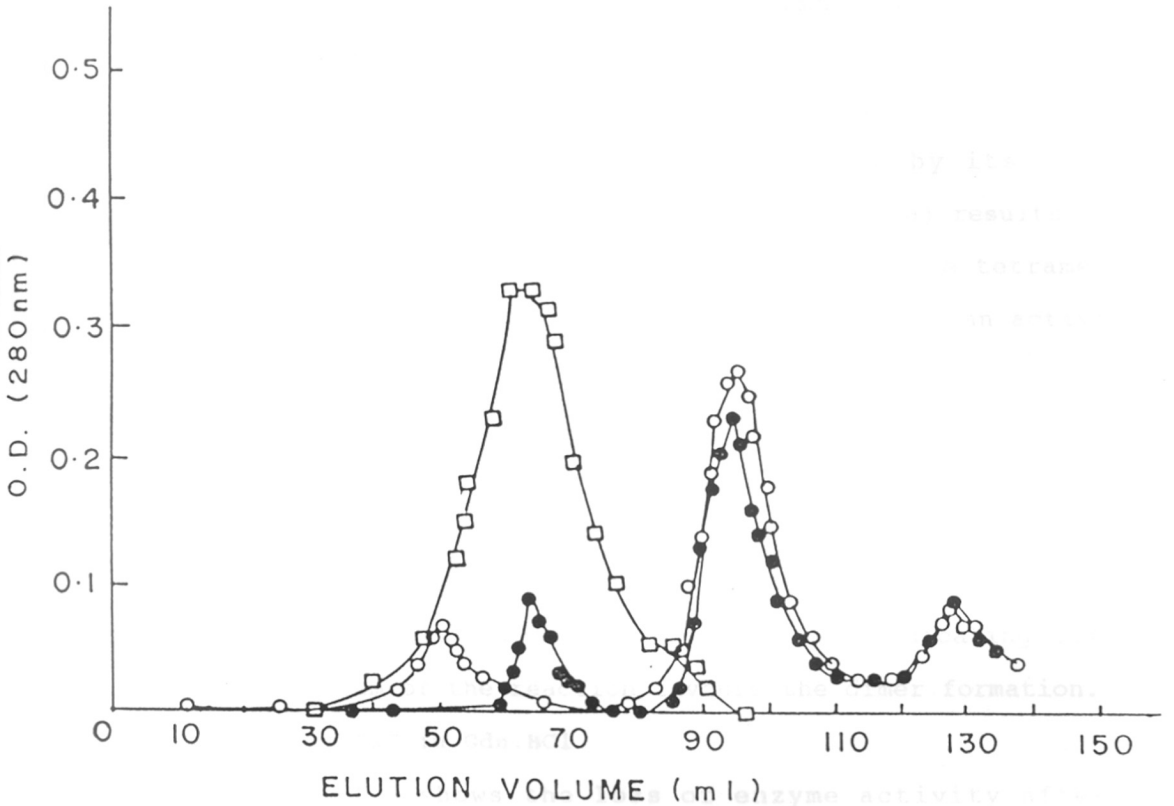


Fig.II.4(a) **Unfolding and refolding of GXI as monitored by gel filtration**

GXI native (□); in the presence (O) and absence (●) of SDS (0.1%).

minimum at 211 nm although the minimum at 220 nm is unaffected (Fig. II.3b).

Reversibility of the tetramer-dimer conversion

Refolding of the SDS-treated enzyme by its gel filtration under renaturing condition (Fig. II.4a) results in the partial reversion of the dimer into an active tetramer although a majority of the enzyme still exists as an active dimer.

Our results on refolding of the SDS-treated enzyme represent the first experimental evidence for the reversibility of the tetramer-dimer conversion. The tetramer is easily and almost fully converted into a dimer while dimer does not completely refold into the tetramer indicating that the equilibrium of the reaction favours the dimer formation.

Unfolding of GXI in Gdn.HCl

Fig. II.5 shows the loss of enzyme activity after incubation for 15 min with Gdn.HCl as a function of the concentration of the denaturant. The integrity of the active site was very easily disrupted by low concentrations of the denaturing agent (e.g. 0.3 M Gdn.HCl causes 55% loss in activity). Complete loss in activity occurs at 1.8 M Gdn.HCl. Therefore, the changes in protein structure of GXI were monitored after treating the enzyme with 2.0 M Gdn.HCl. The quaternary structure of the enzyme was assessed by gel

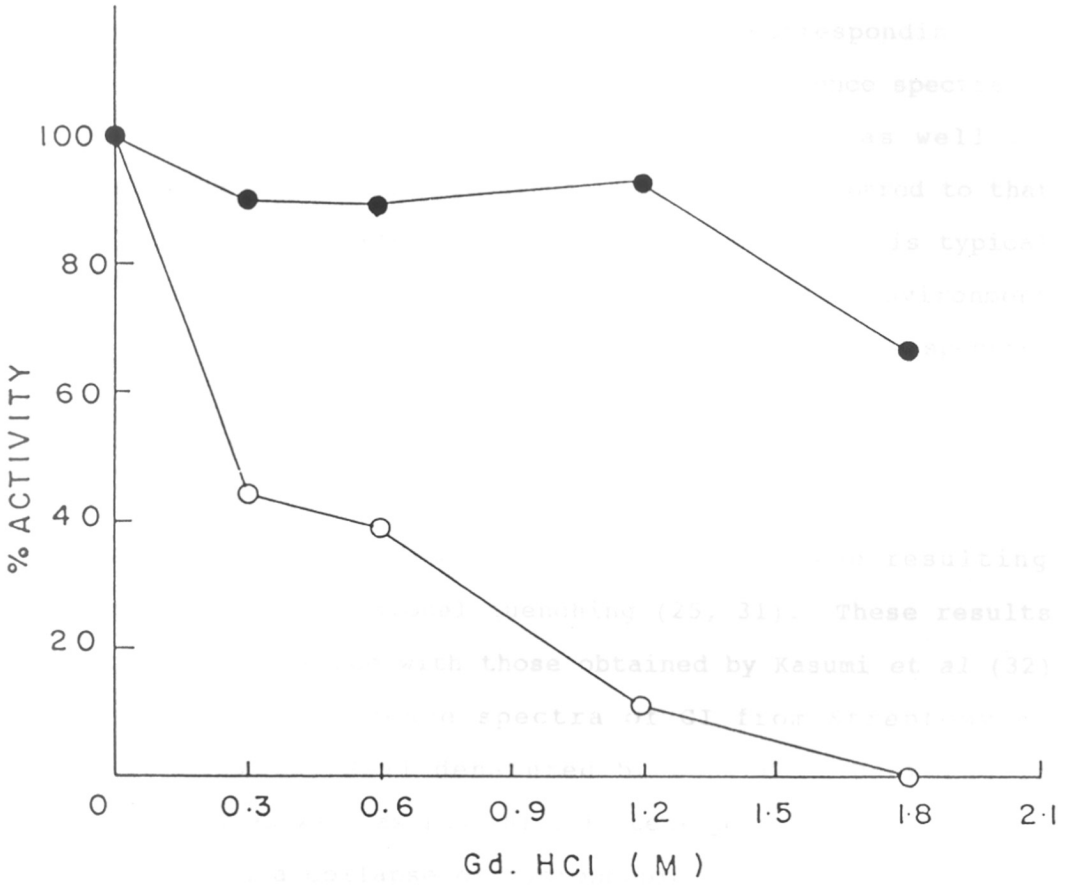


Fig.II.5 **Denaturation of GXI by Gdn.HCl and its renaturation by dilution (100 fold)**

Denaturation (O) and renaturation (●)

filtration on Sepharose CL-6B. In the presence of 2 M Gdn.HCl, the enzyme shows a major peak at the position of a dimer (Mr 80,000) (D*) and a small peak corresponding to a monomer (Mr 40,000) (Fig II.4b). The fluorescence spectra of the denatured enzyme exhibits a red shift as well as quenching of total fluorescence intensity as compared to that of the native enzyme (Fig. II.2). This behaviour is typical of tryptophan residues exposed to a more polar environment (30). Under denaturing conditions the fluorescence spectrum of most proteins shifts to lower energy (i.e. a red shift) due to the exposure of burried tyrosine and tryptophan residues to the more hydrophilic environment of the aqueous solvent and to a lower intensity due to the resulting increase in collisional quenching (25, 31). These results are in consistence with those obtained by Kasumi *et al* (32) for the fluorescence spectra of GI from *Streptomyces griseofuscus*, S-41 denatured by Gdn.HCl. Decrease in intensity as well as red shift in peak position were observed suggesting a collapse of hydrophobic region of the enzyme. Change in secondary structure of the native enzyme on treatment with Gdn.HCl (2 M) was monitored by far UV-c.d. spectra. Gdn.HCl treated enzyme shows a double minima at 211 and 220 nm similar to the native enzyme but has a decreased

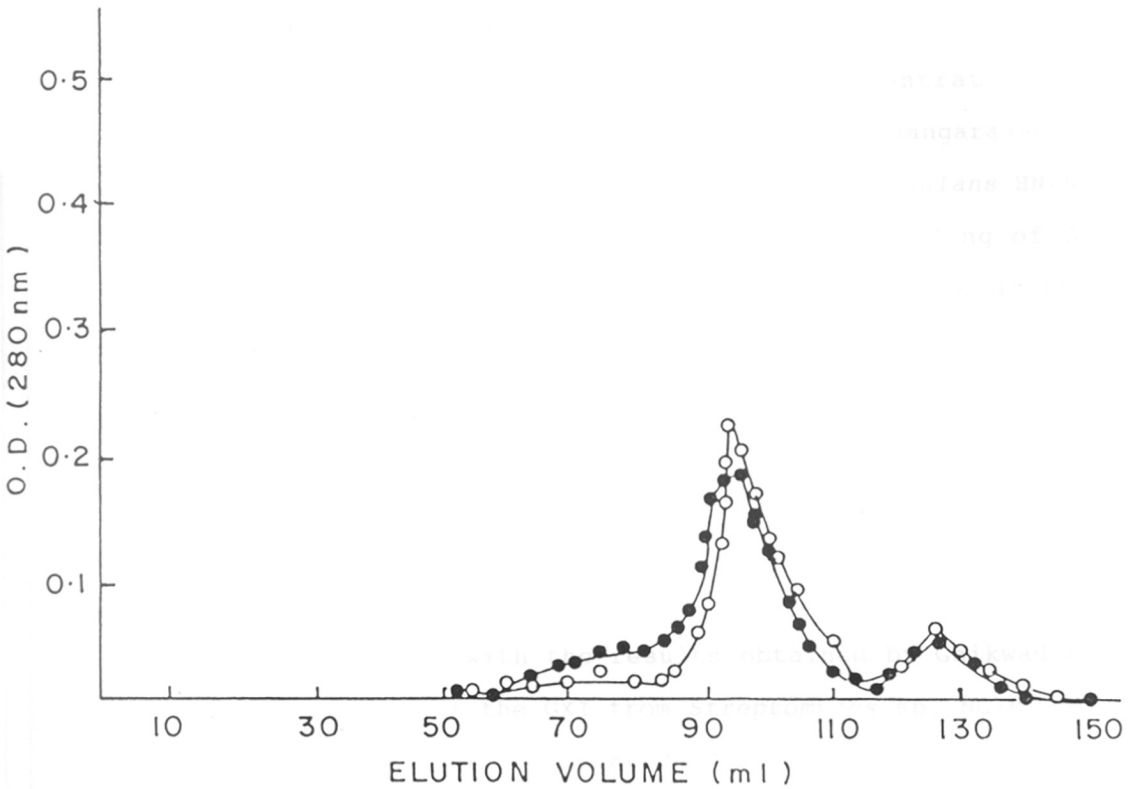


Fig.II.4(b) **Unfolding and refolding of GXI as monitored by gel filtration**

GXI in the presence (O) and absence (●) of Gdn.HCl (2M)

total ellipticity (Fig. II.3c).

Refolding of the Gdn.HCl treated enzyme

On dilution of the denaturant, 90% of the activity could be restored provided that initial concentration of Gdn.HCl was $\leq$ 1.2 M (Fig. II.5). Danno (8) and Rangarajan *et al* (13) have observed that GXI from *Bacillus coagulans* HN-68 and from *Arthrobacter* respectively showed refolding of GI activity by dilution only at lower concentrations of the denaturant (< 2M) whereas at higher initial concentration of the denaturant irreversible unfolding of the enzyme occurs. Gel permeation chromatography of the Gdn.HCl (2M) treated enzyme under renaturing conditions revealed the presence of predominantly a dimer (Mr 80,000) (Fig. II.4b) which is active.

In compliance with the results obtained by Gaikwad *et al* (24) treatment of the GXI from *Streptomyces* sp. NCIM 2730 with Gdn.HCl (2M) for 15 min at 25°C and subsequent dialysis did not result in renaturation of the enzyme. Little gain (28% of the initial activity) in enzyme activity was obtained upon dialysis of the denatured enzyme which further showed the presence of an inactive dimer on SDS-PAGE. This could be because of irreversible unfolding of the enzyme by Gdn.HCl when exposed for longer incubation period. Fluorescence spectrum of the enzyme refolded by gel filtration is similar

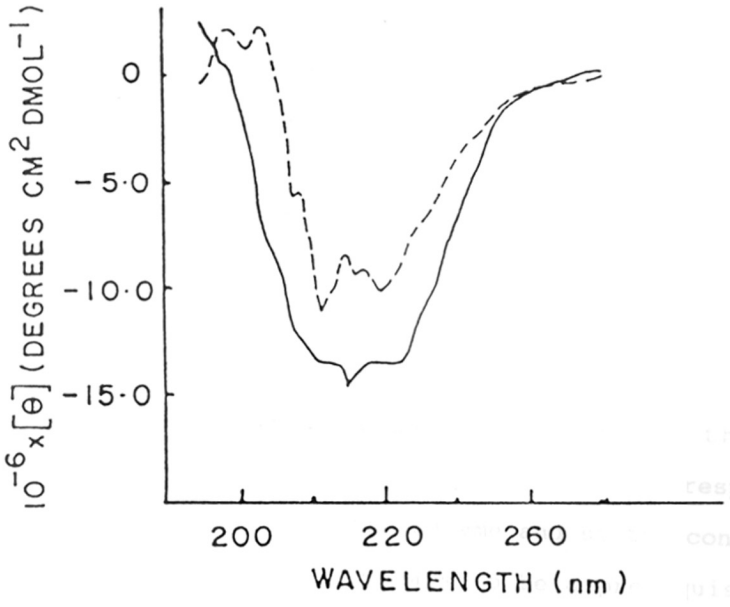


Fig. II.3(c) **Far UV c.d. spectra of GXI**

Gdn.HCl (2M) treated (– – –) and renatured (——).

to that of the active dimer (Fig. II.2). However, the c.d. spectrum of the enzyme exhibits an altogether different pattern, consisting of a single minimum at 215 nm revealing a B-type structure for the enzyme (Fig. II.3c).

Importance of the tertiary structure for biological activity

Comparison of the conformations of the native GXI and active dimer (D) obtained either by mild denaturation (0.1% SDS) or by reversible inactivation (2 M Gdn.HCl), reveals that the tertiary structure of the dimer is intact and it retains full activity inspite of perturbance in the secondary structure.

In case of the inactive dimer (D*) there is a full retention of the secondary structure but distoration in the tertiary structure. Thus, the change in the tertiary structure rather than secondary structure is responsible for the loss in activity of the enzyme and so the conservation of the tertiary structure appears to be a prerequisite for the GXI activity.

Occurrence of a molten globule intermediate

Earlier, only the native and the unfolded states of the protein were known to be the stable conformations in protein folding. However, recent studies have shown the existence of stable intermediates such as a molten globule for a few

proteins [e.g. α-lactalbumin (33), cytochrome C (34), carbonic anhydrase (35) and β-lactamase (36)] in the presence of intermediate concentrations of denaturants or under acidic or alkaline conditions. A particularly significant finding from the present investigations is the evidence, for the first time, for the occurrence of a molten globule type intermediate during the unfolding of GXI. The inactive dimer (D*) encountered during the denaturation of GXI by Gdn.HCl (2 M) possesses an intact secondary structure but distorted tertiary structure resembling a molten globule state. Although the occurrence of a molten globule state has been reported for a few proteins, its exact role in protein folding is still controversial. In order to verify the generality of the occurrence of this conformational state and to determine its role in protein folding it is important to locate such intermediates in the folding pathway.

Unfolding of GXI by heat

Heating of the GXI at 100°C for 5 min results in a complete loss in activity. When the heat denatured enzyme was loaded on a gel filtration column, it showed a major peak corresponding to a monomer (Mr 40,000) (Fig. II.4c). Danno (8) has shown that under drastic denaturing conditions, the loss of activity of GXI from *Bacillus coagulans* HN-68 was associated with subunit dissociation (i.e. the dissociation

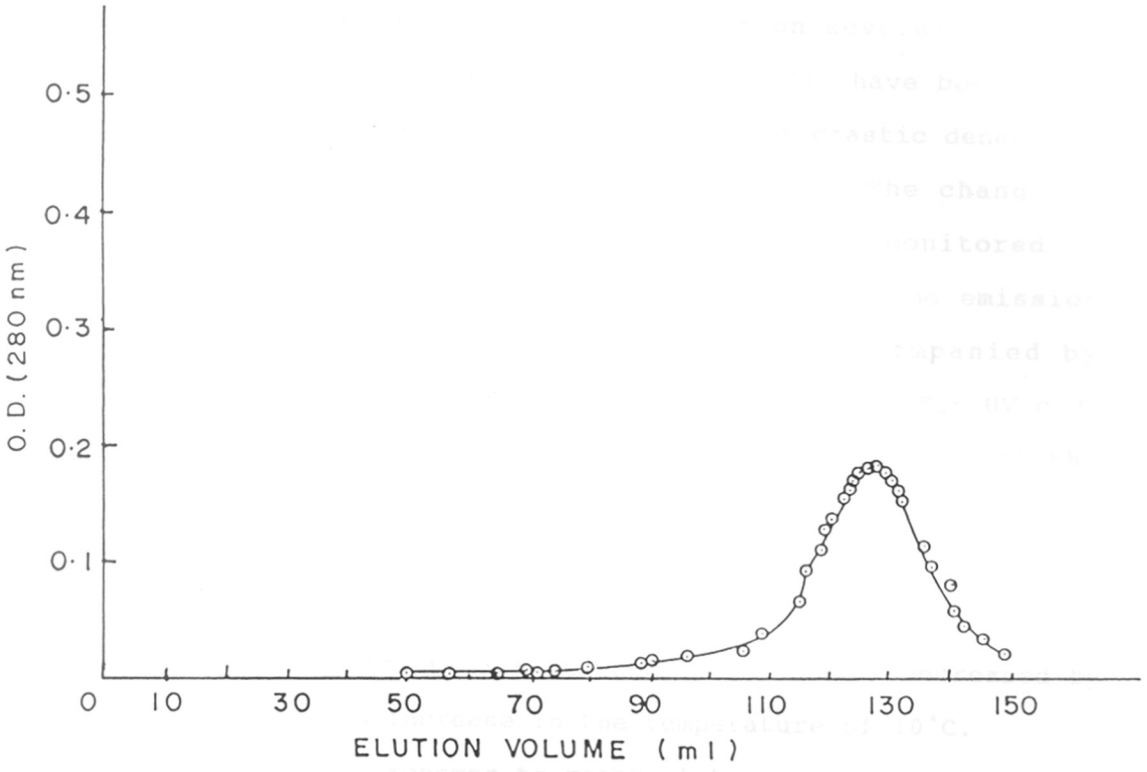


Fig.II.4(c) **Unfolding and refolding of GXI as monitored by gel filtration**

GXI heated at 100°C for 5 min.

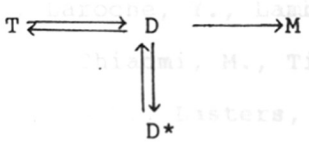
of the tetramer into monomer). Later on several XIs from different *Streptomyces* species (9, 11, 12) have been shown to dissociate into monomeric species under drastic denaturing conditions with loss of enzyme activity. The change in tertiary structure of the heated enzyme as monitored by fluorometry showed a distinct blue shift in the emission spectrum for an excitation at 295 nm, accompanied by quenching of total fluorescence (Fig. II.2b). Far UV c.d. spectrum of the heat denatured GXI showed absence of the minimum at 220 nm indicating an appreciable decrease in the α-helical content of the protein (Fig. II.3a). Hui et al (37) in their studies on thermostability of GI observed that the degree of α-helicity (38% at room temperature) decreased by about 2% with an increase in the temperature of 10°C.

Inability of the monomer to reassociate

Heating of the enzyme causes the dissociation of the tetramer into monomers accompanied by considerable structural changes and irreversible loss in activity. Inability of the monomers to reassociate may be due to their extensive unfolding and/or due to loss of information needed for refolding owing to the modification of certain amino acids (e.g. oxidation of tyrosine) as a consequence of heating.

In conclusion, the unfolding and refolding of the

tetrameric GXI from *Streptomyces* sp. NCIM 2730 has been studied using three different levels of denaturing conditions: (i) mild denaturation by 0.1% SDS, (ii) reversible inactivation by 2 M Gdn.HCl and (iii) irreversible inactivation by heat treatment. Our studies represent the first detailed report on the mapping of unfolding transitions of GXI and characterization of its non-native conformations. Comparison of the various changes in the structure and activity brought about by unfolding and refolding show that the loss or regain in activity parallel the structural changes detected by fluorescence but do not correlate with changes in the c.d. spectrum. Based on experimental results, the molecular mechanism of inactivation of GXI can be presented by the following model:



where, T is a tetramer, D is the active dimer, D* is an inactive molten globule and M is an inactive monomer.

The finding that the maintenance of tertiary structure is important for activity will be a useful guideline while engineering the protein for improving its properties to suit its commercial application.

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Section B

Evidence for specific interaction of guanidine hydrochloride with carboxy groups of glucose/xylose isomerase

SUMMARY

Glucose/xylose isomerase (GXI) from *Streptomyces sp.* (NCIM 2730) was inhibited (50%) by a relatively low concentration of guanidine hydrochloride (Gdn.HCl) (0.6 M) without causing noticeable structural changes in the treated protein as deduced by its fluorescence spectra. A similar concentration of NaCl or KCl had little effect on the enzyme activity implying that the inhibition is not due to the salt effect. The Km (71.4 mM) of the enzyme increased to 285.7 mM in the presence of Gdn.HCl. Glucose and/or metal ions (Mg^{+2} and Co^{+2}) protect the enzyme against inactivation by Gdn.HCl suggesting that it is due to reversible inhibition of activity rather than due to denaturation and/or unfolding of the enzyme. The participation of carboxyl groups in the mechanism of action of the enzyme was demonstrated by its inactivation by Woodward's reagent K (WRK) and by consequent increase in the absorbance of the enzyme at 340 nm. Inaccessibility of the Gdn.HCl treated enzyme to WRK established, for the first time, the specific interaction of Gdn.HCl with the carboxylate residues of the enzyme.

INTRODUCTION

As a part of our studies on 'unfolding and refolding of GXI from *Streptomyces sp.* NCIM 2730' we have investigated the effect of conformational changes and subunit dissociation during denaturation, on the biological activity of the enzyme. It was observed that the enzyme was rapidly inactivated by guanidine hydrochloride especially at lower concentrations of the denaturant (<1 M). The observation was similar to that observed by Callens *et al* (1) and Rangarajan *et al* (2) and suggests a specific interaction between the enzyme and the denaturant. Recently, Woodward *et al* (3) have postulated that the arginine-like guanidium moiety of Gdn.HCl may interact specifically with the carboxylate groups of enzymes. Vangrysperre *et al* (4) in 1989 for the first time showed rapid inactivation of D-xylose isomerases from *Streptomyces violaceoruber, Streptomyces sp., Lactobacillus xylosus, Lactobacillus brevis* and *Bacillus coagulans* by Woodward's reagent K (WRK), a reagent causing rapid and specific modification of aspartate or glutamate groups in proteins (5,6). Modification of a single carboxylic group per active site was demonstrated. Vangrysperre *et al* (7) further located the essential carboxylate group in D-xylose isomerases by amino acid sequence analysis of peptides recognized by differential peptide mapping on ligand

protected and unprotected derivatized enzyme.

In the present chapter, we provide experimental evidence, for the specific interaction of Gdn.HCl with the carboxyl groups of the enzyme and demonstrate the utility of Gdn.HCl in probing the functional carboxylate groups in the proteins.

MATERIALS AND METHODS

Chemicals

Gdn.HCl and WRK (N-ethyl-5-phenyl isoxazolium-3'-sulfonate) were from Sigma Chemical Company (USA). All other chemicals used were of analytical grade.

Enzyme production, purification and assay

The enzyme was produced, purified and assayed as described in Section A of this chapter.

Enzyme assay in the presence of denaturants

Loss of enzyme activity in the presence of denaturants (Gdn.HCl or urea) was estimated by incubating GXI at 70°C in the assay mixture containing different concentrations of the denaturant for 30 min. After 30 min the reaction was terminated and the fructose formed was estimated by cysteine-carbazol method.

Protection of the enzyme by substrate and/or metal ions against Gdn.HCl inhibition was studied by incubating 0.4 U of GXI with different concentrations of Gdn.HCl alone or with glucose (100 mM) and/or metal ions (Mg^{2+}, 5 mM; Co^{2+}, 1 mM) for 15 min at room temperature and then assaying with the same concentration of Gdn.HCl present in the assay mixture as in the original incubation mixture.

Kinetics of inhibition of Gdn.HCl treated GXI

The activity of GXI in the absence and presence of

Gdn.HCl (0.6 M) was carried out at different concentrations (25 to 100 mM) of glucose. The Km values of the enzyme were calculated from the graphs of 1/V vs 1/S.

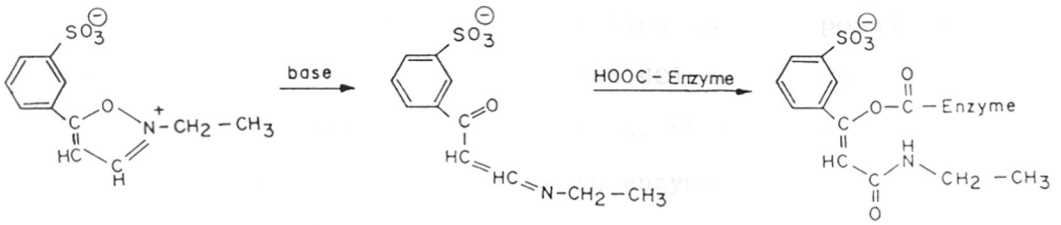
Fluorescence spectra

Fluorescence spectra of the enzyme in the presence and absence of Gdn.HCl were recorded at excitation wavelengths of 274 and 295 nm using Aminco SPF-500 spectrofluorometer.

Reaction of GXI with Gdn.HCl and WRK

Freshly prepared stock solution of 0.5 M WRK in 1 mM HCl was used. The modification reactions were carried out in 50 mM sodium phosphate buffer, pH 7.5. 50 ul enzyme solution (4 U/ml) was incubated with WRK (25 mM) in a reaction volume of 1 ml. In a parallel experiment the enzyme was preincubated with 0.6 M Gdn.HCl for 15 min and WRK was then added. The GXI activity and increase in the absorbance at 340 nm were measured.

Reaction mechanism of WRK



Enol ester shows an absorbance peak at 340 nm (8).

RESULTS AND DISCUSSION

Structure-activity relation of Gdn.HCl treated GXI

GXI was inhibited 50% by 0.6 M Gdn.HCl. The rate of inhibition of enzyme activity by Gdn.HCl was almost twice that by urea at lower (< 1.5 M) concentrations of the denaturants (Fig. II.6). The fluorescence of GXI was largely unaffected by 0.6 M Gdn.HCl treatment whereas treatment with 2 M Gdn.HCl resulted in complete loss of GXI activity accompanied by change in fluorescence (Fig. II.7). The molecular weight of the 0.6 M Gdn.HCl treated enzyme corresponds to that of a dimer (Mr 80,000) as determined by gel filtration in Sepharose CL-6B. We have shown earlier (9) that the dimer of the GXI is active while monomer is an inactive species. Thus the structural changes or alterations in the subunit status do not account for the observed loss in activity of the enzyme on treatment with Gdn.HCl at lower concentrations of the reagent. There was no inhibition of GXI by NaCl or KCl (0.5 M each), ruling out the possibility that the inhibition of enzyme by Gdn.HCl is due to the salt effect and strengthening the view-point that it is due to the specific interaction of Gdn.HCl with enzyme.

Kinetics of inhibition of Gdn.HCl treated GXI

The kinetics of inhibition of GXI by 0.6 M Gdn.HCl in the presence of different concentrations of glucose indicated

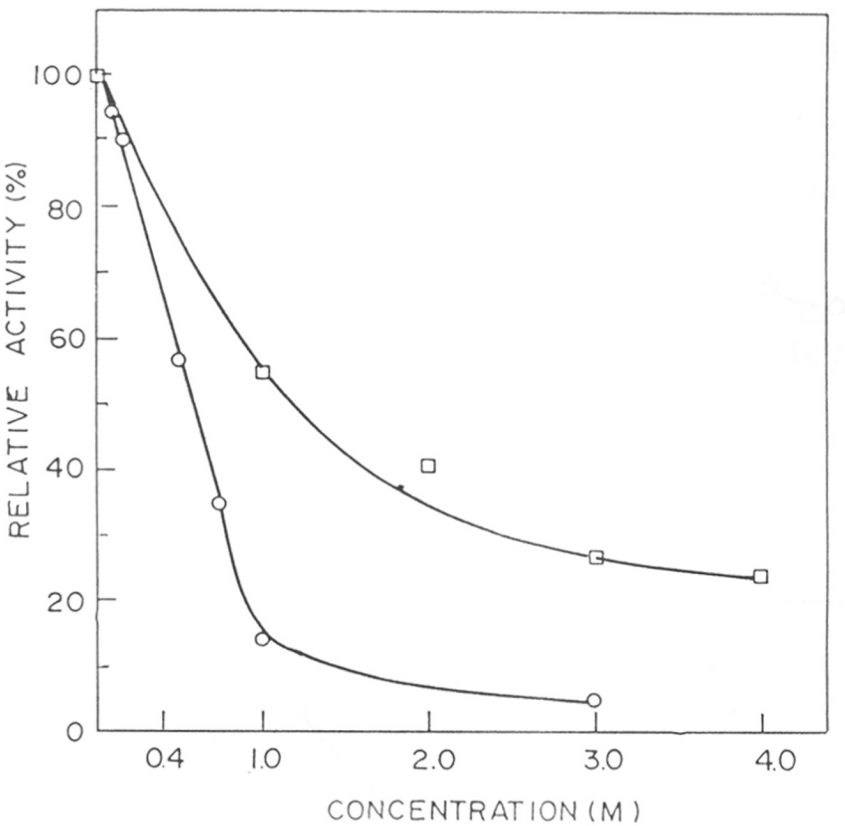
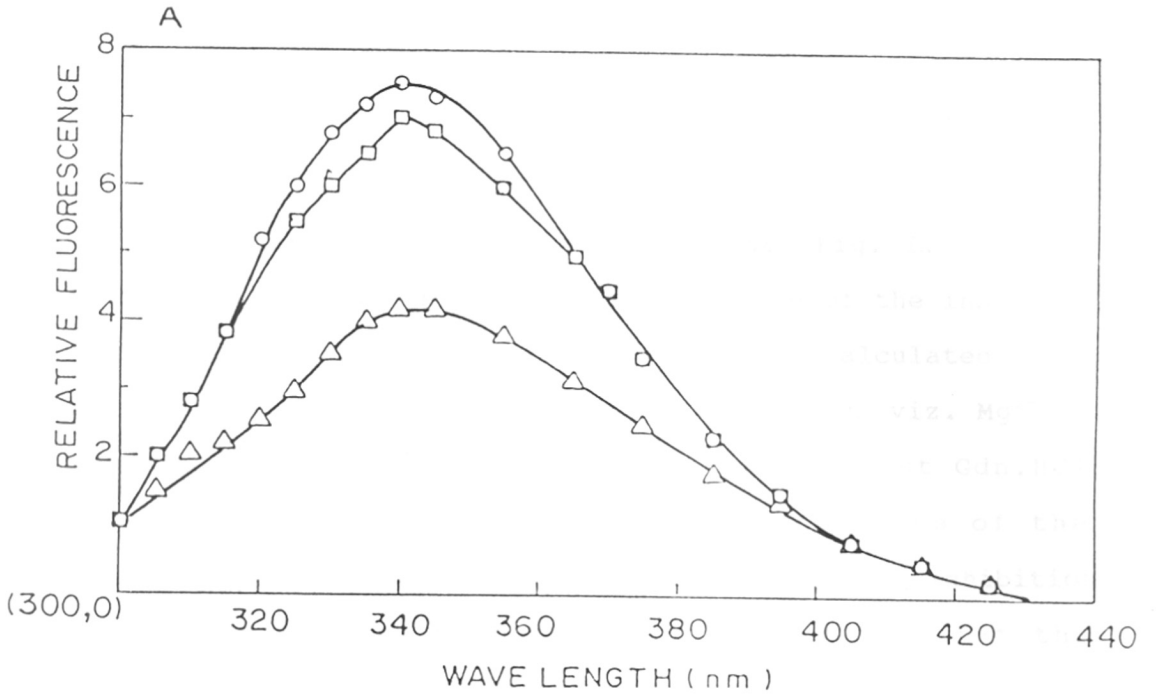


Fig.II.6 Effect of Gdn.HCl and urea on GXI

Gdn.HCl (O); urea (□).



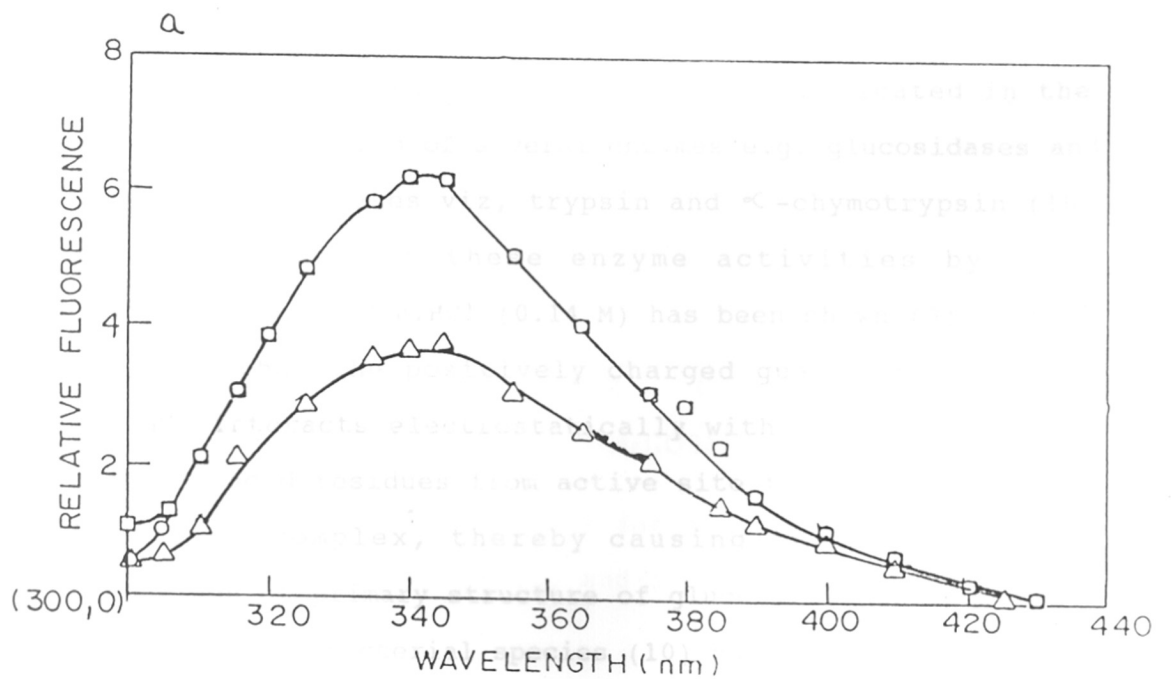


Fig.II(7) Fluorescence spectra of GXI

Fluorescence pattern at excitation wavelength of 274 nm (A) and 295 nm (a) of native enzyme (O) and enzyme treated with Gdn.HCl, 0.6 M (□) and 2 M (△).

that the inhibition was purely competitive (Fig. II.8). The Km of the enzyme in the absence and presence of the inhibitor were 71.4 and 285.7 mM respectively. Ki was calculated to be 87.5 mM. Both glucose (100 mM) and metal ions viz. Mg^{2+} (5 mM) and Co^{+2} (1 mM) protect the enzyme against Gdn.HCl inhibition especially at lower concentrations of the inhibitor (< 1.2 M) (Fig. II.9) revealing that the inhibition was reversible and supporting the view point that the reaction of Gdn.HCl with the enzyme occurs at the active site of the enzyme.

Evidence for the reaction of Gdn.HCl with carboxy groups in GXI

Carboxylic acid residues have been implicated in the catalytic mechanisms of several enzymes e.g. glucosidases and the serine proteases viz, trypsin and α-chymotrypsin (10) and inhibition of these enzyme activities by low concentration of Gdn.HCl (0.14 M) has been shown (3). It is proposed that the positively charged guanidium group of Gdn.HCl interacts electrostatically with aspartic acid and glutamic acid residues from active site to form the enzyme-inhibitor complex, thereby causing inhibition. The comparison of primary structure of glucose/xylose isomerases from different bacterial species (10) has shown that aspartic

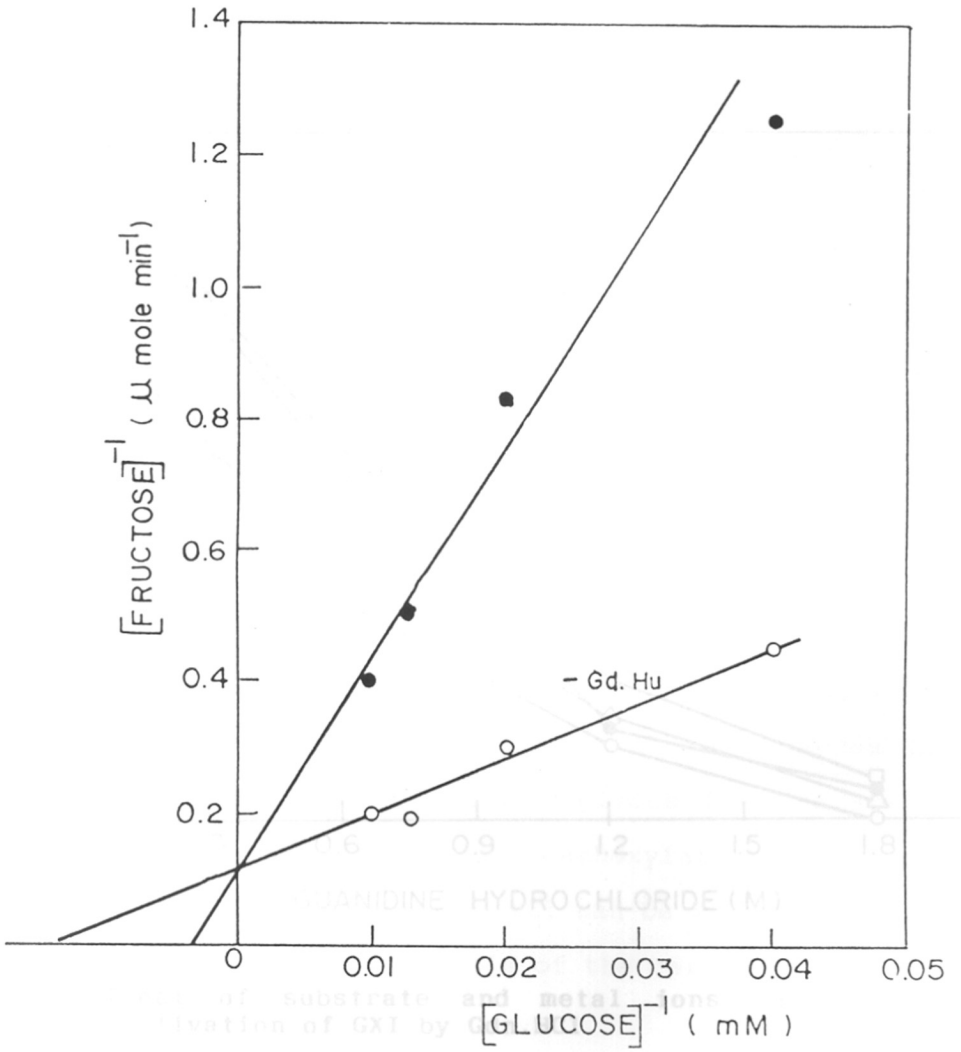
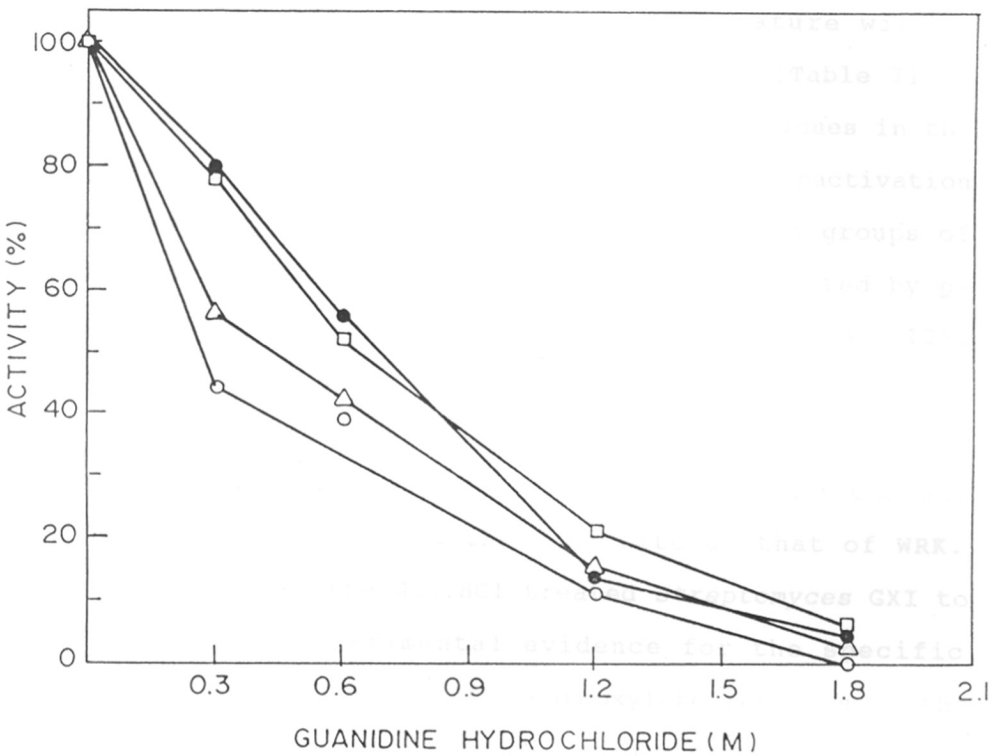


Fig. II.8 Lineweaver-Burk plot for glucose isomerase

In the absence (O) and presence (●) of Gdn.HCl.



II.9 **Effect of substrate and metal ions on the rate of inactivation of GXI by Gdn.HCl**

Gdn.HCl alone (O); Gdn.HCl + substrate (□); Gdn.HCl + metal ions (△) and Gdn.HCl + substrate + metal ions (●).

acid and glutamic acid are among the conserved amino acid residues and are also involved in catalysis.

GXI from *Streptomyces sp.* (NCIM 2730) was inactivated by treatment with WRK (25 mM) at room temperature with a simultaneous increase in absorbance at 340 nm (Table II.1) demonstrating the involvement of carboxylate residues in the active site. The possibility that the observed inactivation may be due to the reaction of WRK with cysteinyl groups of the enzyme is ruled out as the GXI was not inhibited by p-chloromercuribenzoate (5 mM) or iodoacetamide (1 mM) (12). Incubation of the enzyme with 0.6 M Gdn.HCl for 15 min at room temperature prior to the addition of WRK prevents increase in the absorbance at 340 nm showing that Gdn.HCl reacts with the enzyme at the same site as that of WRK. Inaccessibility of the Gdn.HCl treated *Streptomyces* GXI to WRK provides an experimental evidence for the specific interaction of Gdn.HCl with the carboxylate residues of the protein. This property of Gdn.HCl can be fruitfully utilized for probing the functional role of the caroboxylate residues in a protein.

Table II.1: Reaction of GXI with WRK

Sample	O.D. at 340 nm
Enzyme alone	0.014
Enzyme + WRK	0.222
Enzyme + Gdn.HCl (0.5 M)	0.085
Enzyme + Gdn.HCl (0.5 M) + WRK	0.085

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CHAPTER III

IMMUNOAFFINITY PURIFICATION OF GLUCOSE/XYLOSE ISOMERASE

SUMMARY

A procedure was developed to purify glucose/xylose isomerase (GXI) from cell extract of *Streptomyces* sp. NCIM 2730 using immunoaffinity chromatography. High-titer polyclonal antibodies were raised in rabbit using electrophoretically homogeneous GXI as an antigen. The specificity of antibodies was confirmed by double immunodiffusion, rocket electrophoresis and western-blot ELISA, which revealed the presence of a single immunoreactive protein with an Mr of 40,000. The antibodies recognized 2-3 antigenic determinants/mol of enzyme and were found to partially neutralize the enzymatic activity in an immunotitration experiment. The affinity gel was prepared by coupling antibodies at pH 10.0 to divinyl sulfone-activated Sepharose CL-4B. The GXI purified by immunoaffinity chromatography yielded 75% recovery with a single enzymatically active protein band on gel electrophoresis and showed specific activity of 16 U/mg. The crossreaction of the antibodies with glucose/xylose isomerase from other actinomycetes indicated that they share common epitopes.

INTRODUCTION

A limitation to more extensive investigations on the structure-function relationship of the enzyme is imposed by the scarce quantities of the purified enzyme available with classical purification techniques. Although numerous glucose isomerases have been produced from different microorganisms very few of them have been purified to homogeneity. The procedures reported in the literature for purification of GXI generally include: (i) Extraction of GXI from microbial cells by mechanical disruption (by sonication, abrasive grinding , homogenization or treatment with French Press) or by autolysis (by treatment with cationic detergents, lysozyme or toluene); (ii) Heat treatment of the cell extract to denature heat labile proteases; (iii) Precipitation with ammonium sulphate/acetone/Mg^{2+} or Mn^{2+} salts; (iv) Ion-exchange chromatography on DEAE-Sephadex/DEAE-cellulose or (v) Gel filtration on Sephadex (1).

Purification procedures involving such conventional chromatographic techniques are often tedious, time consuming and result in low yields. However, purification by affinity chromatography, based on the unique property of biological macromolecules, i.e. their ability to bind selectively and reversibly to other molecules, is an effective alternative to the above problem. A few reports are available on

purification of GI using affinity chromatography (2-6). Xylitol, an inhibitor of GXI, linked to CNBr activated Sepharose-4B by dicyclohexylcarbodiimide was used as the ligand for affinity chromatography on which GI was retained from a buffer solution. The enzyme could be eluted by NaCl, with a maximum purification of 1.5 fold (2). Ichiro *et al* (3) immobilized tannin for general affinity chromatography by coupling free tannin/CNBr-activated tannin with epichlorohydrin activated diaminoalkane ($C_2 \backsim C_{11}$) linked cellulose. Using this column 71% recovery of GI was obtained. In the purification of GI from *Streptomyces* sp. by affinity chromatography, GI present in 20,000 g supernatant from *Streptomyces* homogenate was purified by adsorbing GI activity on an affinity adsorbent, xylitol-Sepharose 4B. The purity of GI increased by >4 fold with a yield of 83% (4). A Japanese patent (5) has described the use of Biogel P-300 treated with 1,4-diaminobutane coupled to xylose as an affinity matrix to purify GI from *Thermus thermophilus* cell extract. Ulezlo *et al* (6) have reported purification of intracellular GI from *Actinomyces olivocenereus* 154 by an affinity chromatography. Mannitol, glucitol, xylitol and glucose were immobilized either on silochrome-based or epoxy-activated sorbents. Of these mannitol immobilized to

silochrome based sorbent gave optimum yield of purified GI when eluted with 0.4 M glucose. Purity of GI obtained was 60 fold with an yield of 90.2%.

In the present chapter, we describe a single step, rapid and easy-to-handle purification procedure for GXI from *Streptomyces* sp. NCIM 2730 based on immunoaffinity chromatography. The results have been compared with the conventional method of purification of the enzyme reported earlier (7).

MATERIALS AND METHODS

Enzyme production and purification

Production of GXI from *Streptomyces* sp. NCIM 2730 and its purification to homogeneity was carried out as described by Gaikwad *et al.* (7).

Glucose/xylose isomerases from *Streptomyces lividans* TK 24 and *Chainia* were also produced by the same procedure.

Enzyme assay

GI was estimated by the colorimetric method of Takasaki (8). D-Fructose/D-xylulose produced in the reaction was determined by the method of Dische and Borenfreund (9), as modified by Marshall and Kooi (10). The assay for D-xylose isomerase was carried out using 5 mM D-xylose as a substrate at pH 7.5 at 35°C for 20 min and the optical density was measured at 540 nm. One unit of GI/XI activity was defined as the amount of enzyme which produces 1 µmole of D-fructose/D-xylulose min^{-1} under the assay conditions.

Protein estimation

The protein content of the enzyme preparation was measured according to Bradford (11).

Antibody preparation

Antibodies against the enzyme purified to electrophoretic homogeneity were raised in New Zealand white rabbit. 1.0 mg of the enzyme was emulsified with equal

volume of complete Freund's adjuvant (Difco) and the emulsion was injected subcutaneously at multiple sites into the animal. Booster injections were given at fortnightly intervals for 10-12 weeks. The rabbit was bled intermittently from the ear vein in order to check the antibody titre using Oüchterlony double diffusion technique (12). When the titre reached to 32, the maximum amount of blood was collected from the rabbit by a cardiac puncture. Blood was allowed to clot, the serum was separated by centrifugation and stored in aliquots at -70°C.

Antibody from the antiserum was partially purified by ammonium sulphate precipitation to 0.5 saturation. The precipitate was dissolved and dialysed against phosphate buffer saline (PBS) (0.1 M Na_2HPO_4, 0.4 M NaH_2PO_4 and 0.14 M NaCl, pH 7.2). For the quantitation of antibodies, absorbance of the suitably diluted sample was measured at 200 and 310 nm. Antibody concentration was calculated using the following formula:

$$\frac{O.D._{280\ nm} - O.D._{310\ nm} \times \text{dilution factor}}{1.5}$$

Oüchterlony double diffusion

Double diffusion was carried out in 1% agarose gel in PBS, pH 7.2 at 4°C for 24 to 48 h. 30 - 300 µg of the antigens and 0.3 - 0.75 mg of antibody were used. Precipitin

lines formed were visualized after drying the gels and staining with 0.25% Coomassie Brilliant Blue R-250 in ethanol:acetic acid:water (4:1:5).

Rocket electrophoresis

0.45 mg of antibody was incorporated into 1% agarose gel. 0.3 mg of crude extract was applied in a well, cut at one end and electrophoresis was carried out at 8 mA, 5-10 VCm^{-1}. Precipitin arc formed was visualised as above.

Western blotting

Crude as well as purified enzyme preparations were subjected to SDS-PAGE on 10% gels according to Laemmli (13). After the run, proteins from the gel were electrophoretically transferred to nitrocellulose membrane (14) over a period of 2 h at 150 V in a buffer containing 20 mM Tris, 50 mM glycine and 20% methanol at pH 7.8 at 4°C. Protein bands on nitrocellulose membrane were visualised by staining with 1:10 diluted Ponceau S concentrate (Sigma).

Enzyme linked immunosorbent assay (ELISA)

The blot was treated overnight with 10% goat serum in PBS at 4°C and washed thoroughly with PBS containing 0.1% Tween-20. It was then treated with anti-GXI antibodies diluted 250-fold with 1% BSA in PBS for 2 h at room temperature, followed by incubation for 2 h with goat

antirabbit IgG horse radish peroxidase conjugate (Sigma) diluted 1000-fold in 10% goat serum. The bound peroxidase was detected with 0.5% 3,3-diaminobenzidine tetrahydrochloride in 0.5 M sodium citrate buffer, pH 5.0, containing 0.03% H_2O_2.

Determination of antigenic valency

Varying amounts of GXI ranging from 0 to 75 µg were added to a series of tubes containing 10 µl of antibody (22.4 mg/ml) to a final volume of 500 µl in PBS. The tubes were incubated at 37°C for 1 h, followed by an overnight incubation at 4°C. On centrifugation, precipitate was washed twice with cold PBS and dissolved in 0.1 N sodium hydroxide. Optical density of the samples was recorded at 280 nm. Antigenic valency was determined according to Heidelberger and Kendall (15). In an immunoprecipitation curve (a plot of varying concentrations of antigen Vs. O.D. of precipitate at 280 nm), at low antigen concentration, there is a relative antibody excess. Under such conditions, every antigenic determinant is likely to be covered by a separate antibody molecule and if we calculate the amount of antibody in the precipitate at this point, we can determine the ratio of antibody to antigen and so the relative number of molecules of each in the precipitate:

$$\text{i.e. Antigenic valency} = \frac{\text{Weight of antigen}}{\text{Molecular weight of antigen}} : \frac{\text{Weight of antibody}}{\text{Molecular weight of antibody}}$$

In a hyperimmune serum the major portion of antibody will be of the IgG class with a molecular weight of 150,000. The molecular weight of GI (the antigen in this case) is 160,000. The weight of antigen and antibody in the precipitate were calculated based on the extinction coefficients of 0.667 and 0.695 mg/ml respectively.

Immunotitration

GXI (20 µl, 10 U/ml) was added to antiserum (30-200 µg) in 0.05 M sodium phosphate buffer, pH 7.0, in a total volume of 500 µl. After incubation at 37°C for 1 h, each reaction mixture was assayed for GI as well as XI activity.

Immunoaffinity column chromatography

Ten grams of Sepharose CL-4B (Pharmacia, Sweden) in 10 ml of 1 M sodium carbonate, pH 11.0 was activated with 2 ml of divinyl sulphone (DVS) (Aldrich Chemical Co., USA) for 80 min at room temperature. The activated resin was washed with 0.3 M sodium carbonate, pH 10.0, to remove unreacted DVS. Finally, the activated resin was suspended in 3-4 ml of 0.3 M sodium carbonate, pH 10.0 and 2 ml of antibody (25 mg/ml) dialyzed against 0.3 M sodium carbonate, pH 10.0 was added to the resin. Coupling of antibodies to the resin was allowed

for 6 h at room temperature. The excess of antibody was removed by washing successively with 0.3 M sodium carbonate, pH 10, 0.3 M sodium chloride, 0.3 M glycine, pH 2.0 and PBS.

The coupled resin equilibrated with 0.05M sodium phosphate buffer, pH 7.0 containing Mg^{2+} (1 mM) and CO^{2+} (0.5 mM) was packed in a column (10 x 2 cm). The crude enzyme preparation was allowed to adsorb on it for 1 h at 4°C. The column was washed with 0.05 M sodium phosphate buffer, pH 7.0. The bound GXI was eluted with 0.3 M glycine, pH 2.0, with a flow rate of 20 mlh^{-1}. Fractions (2 ml each) were collected and immediately neutralized with solid Tris to pH 7.2 - 7.4. The fractions were assayed for GI activity, dialysed against 0.005 M sodium phosphate buffer, pH 7.0 containing Mg^{2+} (1 mM) and Co^{2} (0.5 mM), concentrated and checked for homogeneity on gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis in non-denaturing gels (7.5%) was carried out using Tris-glycine buffer, pH 8.3 (16). The *in situ* staining for GI activity in the gel was performed according to Yamanaka (17).

RESULTS AND DISCUSSION

Specificity of the antibody

It is critical to characterize the antiserum with respect to the antibody titer, specificity, affinity, etc. prior to its use for purification of the enzyme.

The specificity of antibody towards GXI was determined using conventional immunological techniques. A single precipitin arc was obtained by Oüchterlony double diffusion (Fig. III.1A) as well as by Rocket electrophoresis (Fig. III.1B), against both crude and purified GXI preparations. The sensitivity of western-blot ELISA technique is much higher than the gel precipitin test, hence it was used to further confirm the specificity of the antibody. A single band at 40 kd, corresponding to the Mr of the subunit of GXI, was immunoblotted from the crude and pure enzyme preparations (Fig. III.1c).

Antigenic valency of GXI

In an immune response, different groups on an antigen are recognised differentially by different lymphocytes which in turn proliferates to yield a clone responding to a single epitope/antigenic determinant on the antigen. Thus, in a hyperimmune serum the antibodies produced against the antigen are polyclonal. Antigenic valency is the number of antigenic determinants on the antigen that are recognised by the

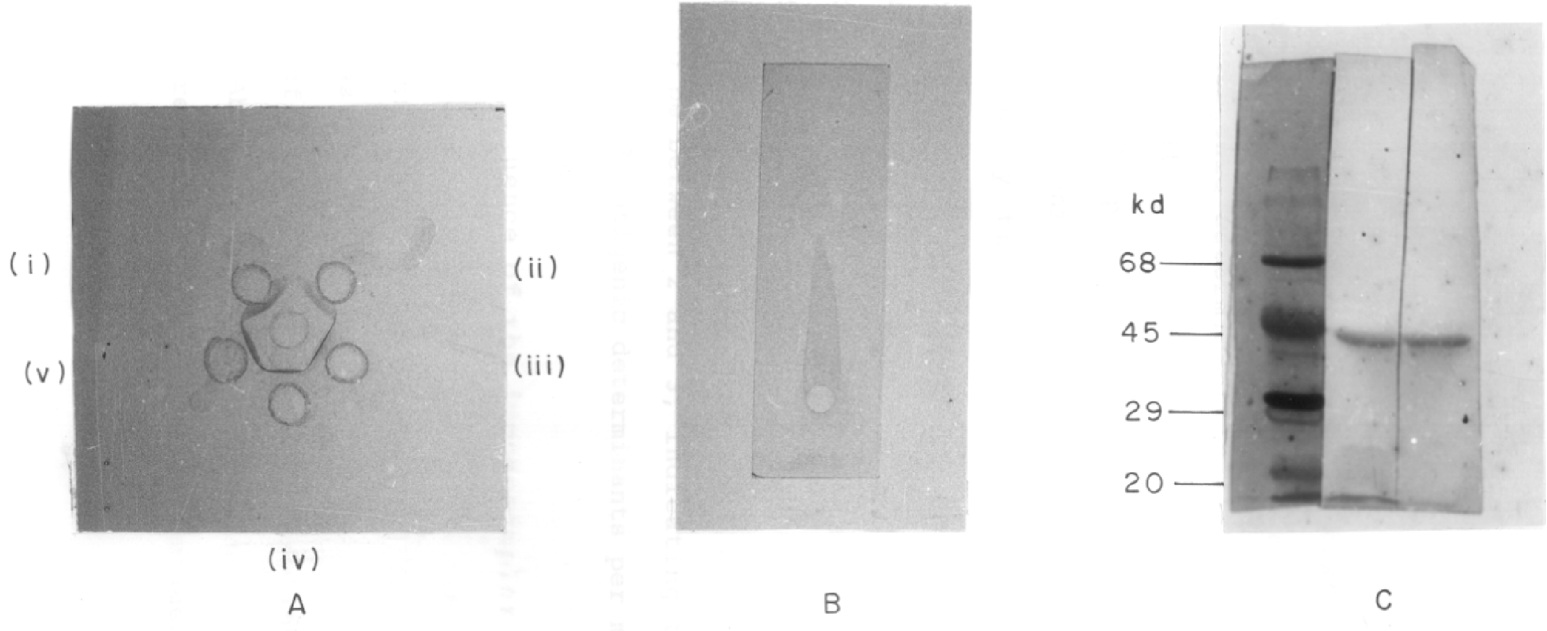

Fig.III.1 Specificity of the antibody against GXI

A. Oüchterlony double diffusion: The central well contained the antiserum. The peripheral wells contained (i) sonicated cell extract, (ii) heat treated, (iii) ammonium sulphate precipitated and dialysed, (iv) and (v) purified GXI, 10 µg and 20 µg.

B. Rocket electrophoresis of crude GXI preparation

C. Western-blot ELISA: Lane (i) molecular weight markers; Lane (ii) and (iii) immunoblots of crude and pure GXI, respectively.

antiserum.

In a quantitative precipitin test, at a low antigen concentration, there is a relative antibody excess wherein every antigenic determinant is likely to be covered by a separate antibody molecule. If we calculate the amount of antibody in the precipitate, we are likely to determine the number of antigenic determinants on the GXI molecule that are recognised by the antiserum. Fig. III.2 shows a precipitation curve of GXI with anti-GXI antiserum. The calculations for the antigenic determinants were made in the antibody excess region (viz, at the antigen concentrations of 5, 15 and 20 µg) before the equivalence point was reached. The average value of molar ratios of antibody to antigen was found to be between 2 and 3, indicating that the antiserum recognised 2-3 antigenic determinants per mol of enzyme.

Immunotitration

The influence of the immunocomplex formation on the enzyme activity helps to monitor the resulting modification of the enzyme. Neutralization of GI activity with the antibodies resulted in nearly 30% loss in activity, whereas in case of XI the loss in activity continued upto 50% (Fig. III.3). Absence of complete inactivation of the enzyme after antibody reaction suggests that the antigen-binding site does

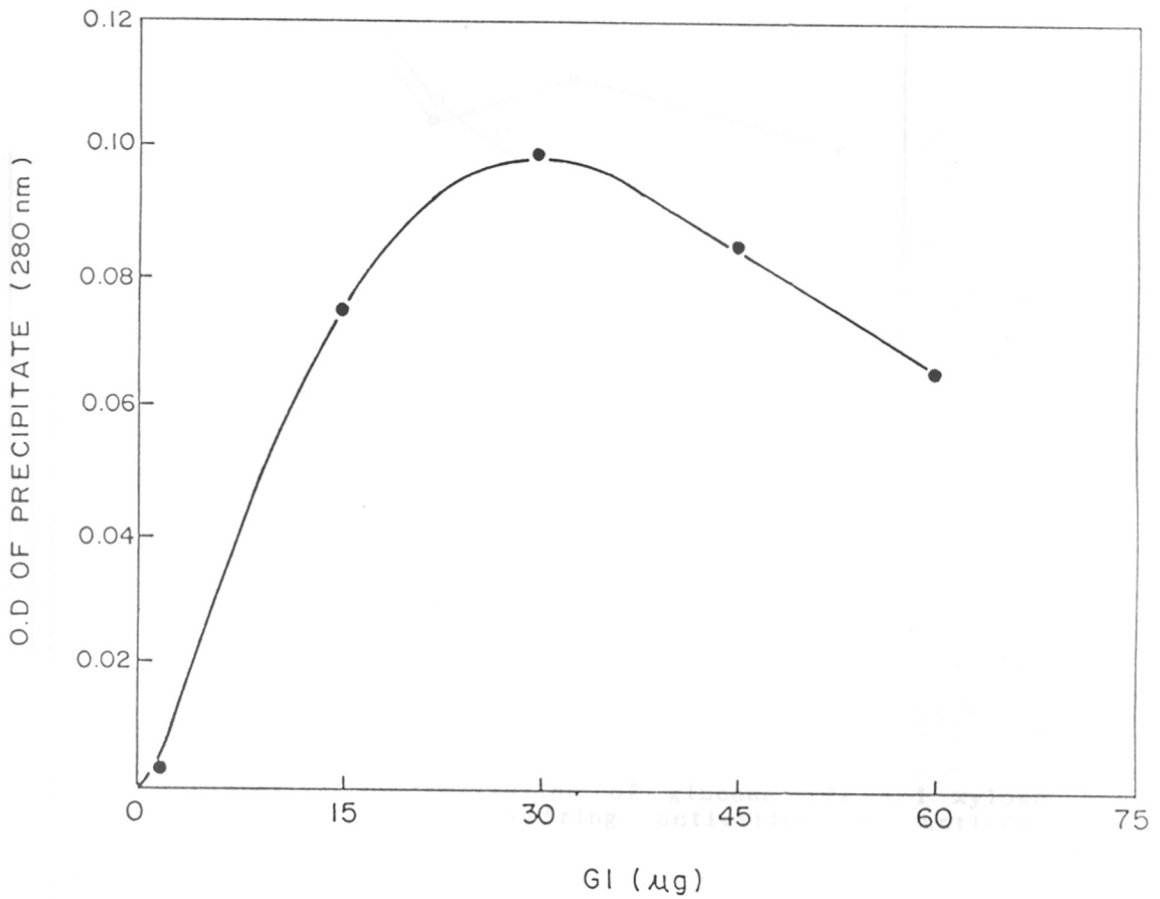

Fig.III.2 Precipitation curve of GXI with anti-GXI antiserum

Varying amounts of GXI (0 - 75 µg) were added to 10 µl (22.4 mg/ml) of antiserum. The precipitate was quantitated as described in Materials and Methods.

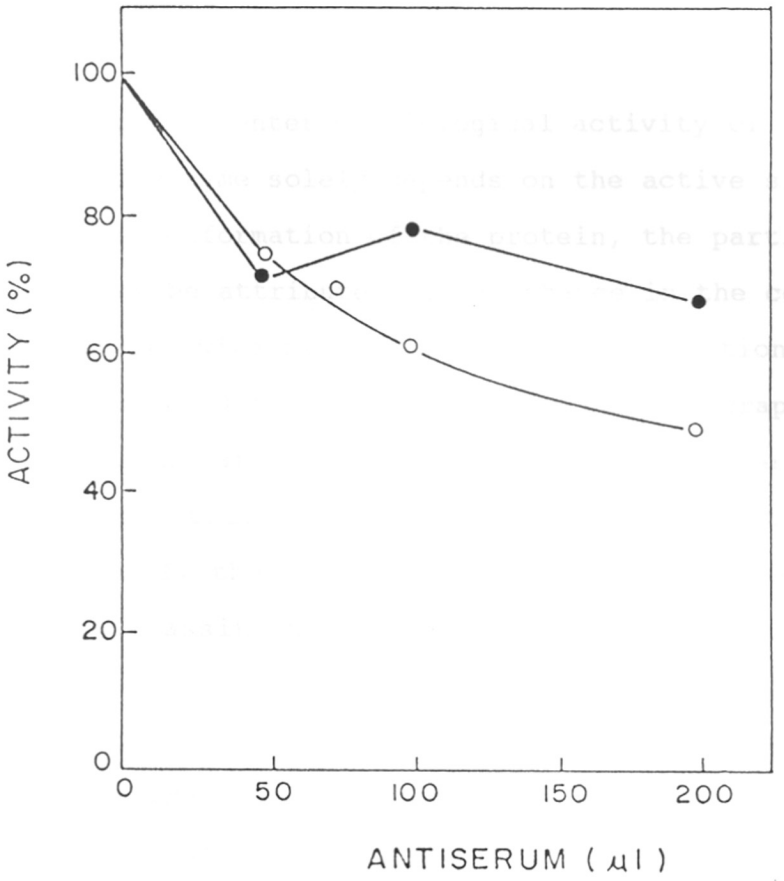

Fig.III.3 Neutralization of glucose (●) and xylose (○) isomerizing activities by anti-GXI antiserum.

not overlap the center of biological activity of GI. As the activity of enzyme solely depends on the active site and the appropriate conformation of the protein, the partial loss in activity may be attributed to the change in the conformation of the enzyme owing to the immunocomplex formation.

Purification of GXI by Immunoaffinity chromatography

Since the antibodies recognized a single antigenic protein, viz. GXI, from the crude enzyme extract, they were used to purify the enzyme by coupling them to an insoluble matrix and passing the crude extract over it. Fig. III.4 shows the elution profile of the bound enzyme. 75% of the enzyme activity was recovered in fractions 4 and 5. When these two fractions were pooled and subjected to gel electrophoresis, a single protein band that showed GI activity on *in situ* staining was observed (Fig. III.5). The specific activity of the purified enzyme was 16 U/mg, which is similar to the enzyme purified by gel electrophoresis (7). As evident from Table III.1, immunoaffinity chromatography yields 75% of the purified enzyme as compared to 41% by gel electrophoresis, which requires an additional step of DEAE-chromatography to eliminate the acrylamide impurities. Although there was some loss in enzyme activity after immunotitration, the activity was regained after the dissociation of antigen-antibody complex by glycine

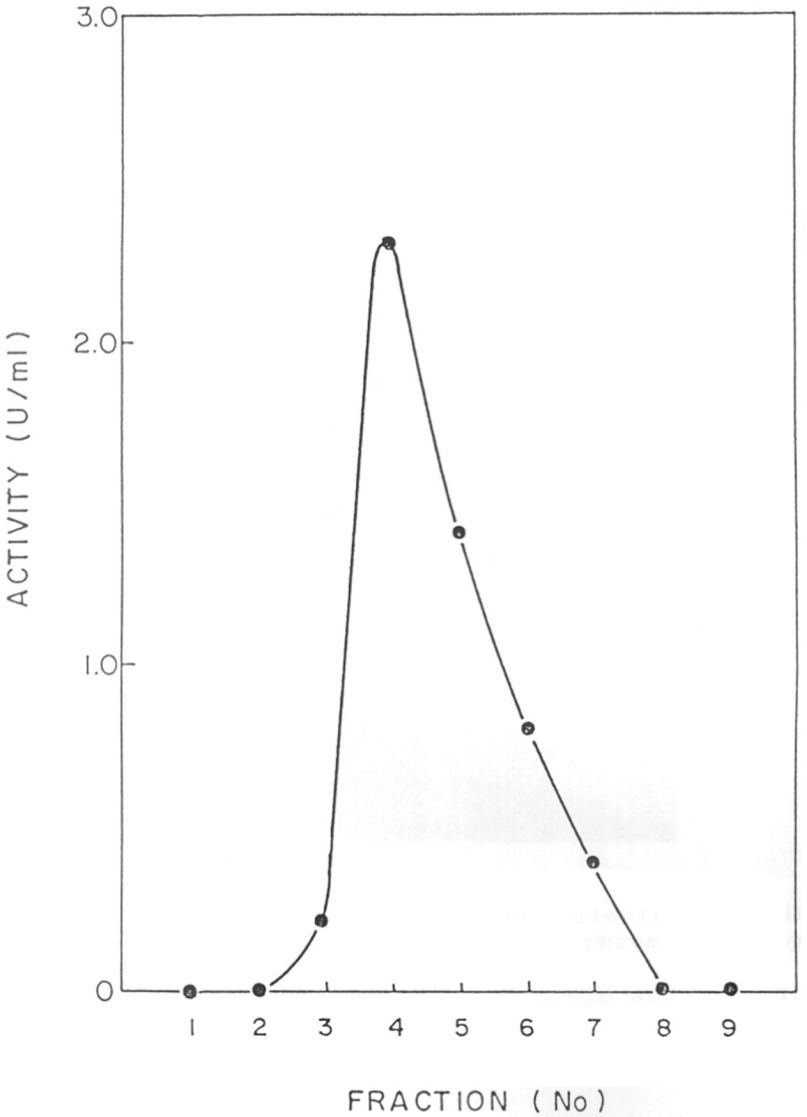

Fig.III.4 Elution profile of GXI bound to the immunoaffinity matrix

Antibodies raised against GXI were coupled to DVS activated Sepharose CL-4B (affinity matrix) and the crude enzyme preparation (10 U/0.9 ml) was allowed to adsorb on it for 1 h at 4°C. After washing the column (0.05 M sodium phosphate buffer, pH 7.0) the bound GXI was eluted with 0.3 M glycine, pH 2.0.

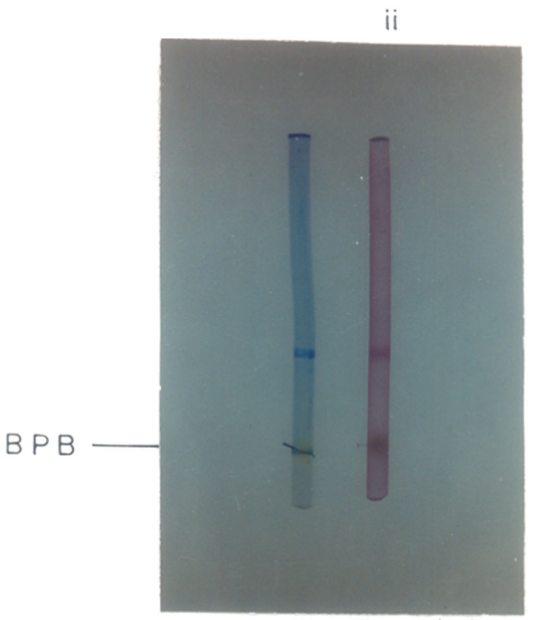

Fig.III.5 **Polyacrylamide gel electrophoresis of GXI purified by affinity chromatography**

Stained for (i) protein and (ii) activity

Table III.1 : Purification of *Streptomyces* GXI

Method	Sample Volume ml	Total protein concentration mg	Total enzyme activity U	Specific activity	Recovery %
Crude extract	0.89	0.89	9.8	11.00	100
Affinity eluate	4.0	0.48	7.4	15.5	75.5
Crude extract	30	66	697	10.6	100
Gel[a] electro-product	18	18	285	16.0	41

[a] Data from previous work (Ref. 7)

(reflecting the reversible nature of antigen-antibody reaction), resulting in a resonably good yield of purified enzyme.

The method proposed by us for the purification of GXI by immunoaffinity chromatography is rapid and virtually an instantaneous method for obtaining large quantities of purified enzyme. The column after re-equilibriation with 0.05 M sodium phosphate buffer, pH 7.0 was subsequently used for enzyme purification without any observable loss in efficiency.

Cross reactivity of the antibody with other GI/XI preparations

The antibodies raised against electrophoretically purified GXI of *Streptomyces* sp. NCIM 2730 reacted with (i) extracellular GI and XI of *Chainia* sp., (ii) intracellular GXI of *Chainia* sp., and intracellular GXI of *S. lividans* TK 24 (Fig. III.6), indicating the presence of common antigenic determinants among the GI/XI preparations of different actinomycetes tested. A sandwitch immuno-enzymic method has been proposed by Mosichev *et al* (18) for primary screening of actinomycetes strains for their GI synthesizing ability in a plate assay. The antibodies raised against GXI from *Streptomyces* sp. NCIM 2730 can be used for screening high glucose isomerase producers among actinomycetes.

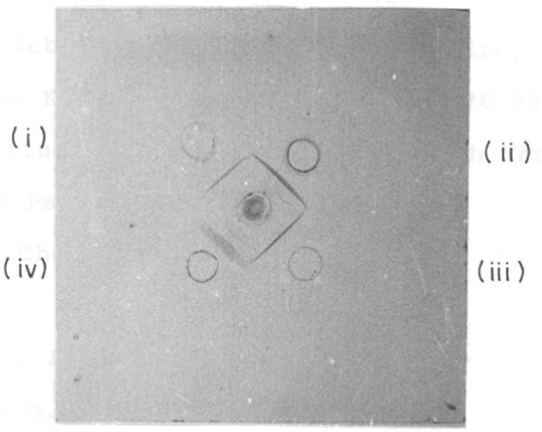

Fig.III.6 Crossreactivity of the antibody with GI/XI preparations from different actinomycetes

The central well contained the antiserum. The peripheral wells contained crude extracts of (i) Streptomyces sp. NCIM 2730, (ii) Chainia (extracellular), (iii) Chainia (intracellular) and (iv) S. lividans TK24.

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CHAPTER IV

GENOME CHARACTERIZATION OF *STREPTOMYCES* SP. NCIM 2730

SUMMARY

The genome of *Streptomyces* sp. NCIM 2730 was characterized with respect to its DNA melting profile, (Tm), base composition, DNA heterogeneity, kinetic complexity, presence of repetitive DNA and its methylation status.

The melting profile of *Streptomyces* sp. NCIM 2730 DNA was smooth and monophasic with a Tm and G+C content of 98°C and 70%, respectively. The GC- rich nature of the genome was also revealed by its susceptibility to digestion with restriction endonucleases which recognize G and/ C containing sites. Digestion of the DNA with methylation specific enzymes showed the lack of A or C methylation in the GATC sequence and absence of C methylation in the CCGG sequence. Restriction analysis of the DNA revealed some intense bands on the gel suggestive of the occurrence of repeated DNA sequences in the genome. On renaturation analysis, these repeated DNA sequences were detected as 'rapidly reannealing sequences'. The latter accounted for approximately 25% of the total genome and had a copy number and kinetic complexity of 50 and 3 x 10^3, respectively. Bam HI restriction fragment distribution in the genome appeared to be independent of the age of the culture. Compariative analysis of DNA restriction patterns of different *Streptomyces* species revealed that they

were species-specific and hence could be used as a tool for taxonomic differentiation of different species. Hybridization of a rice repetitive DNA probe with *Streptomyces* DNA revealed the presence of a highly repeated DNA family in different *Streptomyces* species.

INTRODUCTION

The occurrence of a significant proportion of repeated DNA in eukaryotes is well documented. The heterogeneity of complex eukaryotic DNA has been studied extensively by Britten and Kohne (1). On denaturation, the reassociation of eukaryotic DNA was expected to require a longer time as compared to that for prokaryotic DNA. In reality, however, a large fraction of eukaryotic DNA was found to reassociate many times faster than the bacterial DNA at the same concentration, indicating the presence of certain nucleotide sequences in many fold concentrations in eukaryotes. This DNA was termed as 'repeated DNA'. Later on, it was also termed as a selfish DNA or junk DNA as no direct function could be attributed to this DNA. Repeated DNA sequences are an unusual phenomenon in prokaryotes with the exception of Myxobacteria, Archaebacteria and *Thiobacillus* (2-4).

The *Streptomyces* are amongst the most complex prokaryotes which undergo a diverse series of irreversible morphological and secondary metabolic changes during their developmental cycle. Their genomes exhibit certain distinct characters such as large genome size, high G+C content and presence of repetitive DNA sequences (5). Benigni *et al* in 1975, for the first time reported the presence of repeated DNA sequences in *Streptomyces coelicolor* (6,7). The genome

complexity in *S. coelicolor* was further analysed by Antonov *et al* (8). It was deduced that 2% of the genome consisted of 'foldback' DNA and that about 5% was present as repetitive sequences in a few copies per haploid genome. Kuroedov *et al* (9), in their work with *Streptomyces chrysomallus*, also estimated about 5% of the genome to consist of diverged repeated DNA, occurring on an average of ten times per haploid genome. In later studies, repeated DNA sequences were detected as rapidly reannealing sequences in several *Streptomyces* species where these sequences made up approximately 4% to 11% of the total DNA (10). Interestingly enough, the repeated DNA sequences were not detected in *S. rimosus*.

The DNA content in *Streptomyces* has also been determined by various research groups based on the kinetics of reassociation. The experimentally calculated genome size estimates varied from 3.3 to 7.3 x 10^3 mDa (Table IV.1). The G+C content of *Streptomyces* DNA was found to be very high viz. 70 to 74% (6, 9-11).

This chapter deals with the genome characterization and presents evidence for the occurrence of the repetitive DNA sequences in the *Streptomyces* genome. The results of hybridization of *Streptomyces* DNA from different species with a rice repetitive DNA probe are also presented.

Table IV.1: Genome size of *Streptomyces* DNA

Species	Genome size (mDa x 10^3)	References
S. coelicolor A3(2)	7.09 ± 0.73	6
S. rimosus ATCC 10970	6.77 ± 0.26	6
S. coelicolor A3 (2)	5.2 - 7.3	8
Streptomyces strains (13 strains) and		11
Streptoverticillium strains (2 strains)	3.69 ± 5.15	
S. coelicolor Isp 5049	3.28	12
S. crysomallus A-590	5.54	9

MATERIALS AND METHODS

Chemicals, materials and enzymes

All chemicals used throughout the work were of analytical grade and were obtained from Qualigens, Sisco Research Laboratories, India or E. Merck, Germany.

Fine chemicals such as SDS (Sodium dodecyl sulphate) Sarkosyl, Bovine serum albumin (BSA), Ficoll, Polyvinylpyrrolidone and calf thymus DNA were obtained from Sigma Chemical Co. (USA). 4-Aminosalicylic acid (sodium salt, dihydrate) was obtained from Aldrich Chemical Co. (USA). Triisopropylnaphthalene sulfonic acid (sodium salt) was from Eastman Kodak Co. (USA).

Media components viz. tryptone, peptone, yeast extract, malt extract and Bacto agar were from Difco Laboratories (USA).

All restriction endonucleases, DNA molecular weight markers were from Amersham (UK), Boehringer-Mannheim (Germany) or New England Biolabs (NEB) (USA).

Enzymes such as lysozyme, RNase A (free of DNase activity) and Pronase were from Sigma Chemical Co. (USA). Rice repetitive DNA probe (1.5 kbp) was obtained from Dr. P.K. Ranjekar. α^{32}PdCTP was from Bhabha Atomic Research Centre, Bombay (India). Multiprime DNA labelling kit was from Boehringer Mannheim (Germany).

X-ray films used for autoradiography were from Konica (Japan). Commercially prepared developer (IPC163) was from Kodak (USA). Sodium thiosulphate (2%) was used as a fixer.

Preparation of reagents

I. Organic reagents

(i) Neutral phenol: 8-hydroxyquinolene (antioxidant) (0.1%) was added to the melted phenol and the phenol was equilibrated with equal volumes of TE buffer (Tris, 40 mM; EDTA, 1 mM, pH 8.0) several times till the pH of aqueous phase was around 7.5. Phenol solution was stored at 4°C under equilibration buffer while in use.

(ii) Chloroform-isoamyl alcohol (IAA): A mixture of chloroform and isoamyl alcohol (24:1 v/v) was used to eliminate proteins from preparations of nucleic acid.

(iii) Phenol-chloroform mix: Neutral phenol, chloroform and IAA were mixed in a ratio of 25:24:1.

II. Liquid media

(i) YEME medium

Yeast extract	0.3%
Peptone	0.5%
Malt extract	0.3%
Glucose	1.0%
Sucrose	34.0%

pH adjusted to 7.5 with NaOH

(ii). LB (Luria-Bertani) medium

Tryptone	1.0%
Yeast extract	0.5%
NaCl	1.0%

pH adjusted to 7.5 with NaOH.

III. Enzymes

(i) Pronase: A stock of pronase (10 mg/ml) in H_2O was prepared and the solution was self-digested before use for 2 h at 37°C for elimination of any contaminating DNAse and/RNase activities.

(ii) RNase A: A stock of RNase A (10 mg/ml)in Tris-HCl (10 mM) pH 7.5 and NaCl (15 mM) was prepared and heated at 100°C for 15 min for elimination of any contaminating DNase activity.

All other solutions and buffers were prepared according to Maniatis *et al.* (13).

Cultivation of organisms

Streptomyces was inoculated from a well sporulated MGYP slant into YEME medium containing $MgCl_2.6H_2O$ (5 mM) and glycine (0.5%) (both autoclaved separately) in a spring-baffled flask and incubated at 28°C at 200 rpm for 40 h.

Streptomyces cells tend to pellet in a submerged fermentation. When such pellets are subjected to lysozyme

treatment for break-opening the cells, exposure of the cells to lysozyme is insufficient leading to the inefficient cell breaking, which ultimately affects the final yields of DNA. So a non-pelletal growth of the organism was preferred. The presence of high percentage of sucrose (34%) in the medium and the use of spring-baffled flask for cultivation resulted in bacterial type of growth for *Streptomyces*.

The cells were harvested by centrifugation, washed once with STE buffer (sucrose, 0.3 M; Tris, 25 mM, pH 8; EDTA, 25 mM, pH 8.0) and finally suspended in STE for cell lysis.

Escherichia coli was grown in LB medium overnight at 37°C at 200 rpm.

Isolation of *Streptomyces* DNA

Various methods of cell lysis with slight modifications were attempted for the isolation of high molecular weight DNA from *Streptomyces* sp. NCIM 2730.

(i) Hopwood's method (14) using SDS-EDTA

Mycelium (1 g, wet weight) of *Streptomyces* was suspended in TE buffer (5 ml) containing lysozyme (10 mg) and incubated at 28°C for 1 h. After ensuring complete cell lysis, 1.2 ml EDTA (0.5 M) was added to it; followed by the addition of 0.13 ml pronase solution for deproteinization. After incubation for 15 min at 28°C, 0.7 ml of SDS (10%) was added to the tube and was further incubated at 28°C for 2 h.

Then equal volume of phenol-chloroform mixture was added, the tube was gently shaken and centrifuged at 15,000 rpm for 20 min at 4°C. The upper aqueous layer was gently removed by broad tip pasteur pipette and again treated with phenol-chloroform mixture. The aqueous layer containing nucleic acids was then dephenolised by treatment with chloroform-IAA. The aqueous layer was further treated with RNase A (final concentration 50 µg/ml) for 1 h at 37°C and deproteinized again. 0.1 volume of 3 M sodium acetate, pH 5.2 was added and the DNA was precipitated by adding 2 volumes of ethanol. DNA was spooled on a glass rod, washed with 70% ethanol, air dried and dissolved in 5.0 ml TE buffer.

The routine technique used for the isolation of *Streptomyces* DNA invariably yielded a DNA preparation containing small molecular weight DNA fragments besides high molecular weight (> 23 kbp) DNA as examined by agarose gel electrophoresis. In order to obtain a pure high molecular weight DNA preparation, various methods of DNA isolation and purification were attempted.

Electroelution

The DNA was electrophoresed in 1% agarose gel. The 23 kb band was cut and the agarose block was put in a dialysis bag filled with electroelution buffer (TAE) [Tris-acetate EDTA

buffer:Tris acetate, 0.04 M, EDTA, 0.001 M, pH 7.8]. The dialysis bag was then immersed in a shallow layer of electrophoresis tank buffer (TAE) and the electric current was allowed to pass through the bag (100 V for 2-3 h) to electroelute the DNA into the buffer inside the dialysis bag (13). The DNA from the solution contained in the dialysis tubing was precipitated with ethanol, washed with 70% ethanol, air dried and dissolved in TE buffer.

The electroelution of the DNA fragment yielded a pure high molecular weight DNA preparation. However the DNA yield was low (14%) and contained agarose impurities.

Sucrose density gradient centrifugation

The crude DNA was subjected to sucrose density gradient (10 to 40%) centrifugation (14). The sucrose gradient was prepared as follows: 6 ml of 10% sucrose in TNE buffer (10 mM Tris-HCl, pH 8.0; 1 mM sodium EDTA, 1 M NaCl) was carefully loaded on the top of 6 ml of 40% sucrose in TNE buffer. The tube was sealed and rotated slowly to the horizontal position and kept at room temperature for 4 h. The tube was then gently turned back to the vertical position, the DNA was loaded on the top and then centrifuged at 36,000 rpm for 16 h at 17°C in a Beckman SW41 rotor. After centrifugation, fractions of 500 ul each were collected from bottom of the tube and an aliquot from alternate fraction was analysed on

0.7% agarose gel. High molecular weight DNA fractions (around 23 kbp) were pooled, the DNA was precipitated with ethanol, washed with 70% ethanol, air dried and dissolved in TE buffer.

Fig. IV.1 shows the size fractionation of the crude DNA preparation by sucrose density gradient centrifugation. Though the procedure yielded good amount (50%) of pure high molecular weight DNA, it is tedious and time consuming.

(ii) Urea-sarkosyl method

To break open *Streptomyces* cells, the mycelium was suspended in urea-phenol extraction buffer [urea (8.0 M), NaCl (0.35 M), Tris-HCl (0.05 M), pH 7.5, EDTA (0.02 M); sarkosyl (2%) and Tris saturated phenol (5%) were added freshly to the buffer] (15). The suspension was incubated at 65°C for 15 min and then allowed to cool down to room temperature for extraction with equal volume of phenol-chloroform mixture. After centrifugation, the upper aqueous phase was removed and was dephenolized by extracting twice with chloroform-isoamyl alcohol mixture. The DNA was precipitated with ethanol, washed with 70% ethanol, air dried and suspended in TE.

The treatment of the cells with urea-sarkosyl lysis buffer was not effective in breaking the Streptomyces cells.

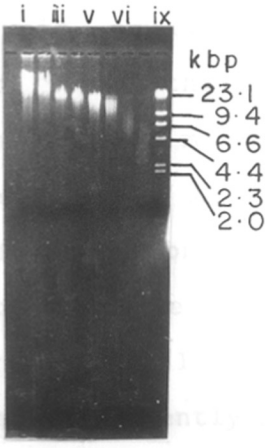

Fig. IV.1 Size fractionation of the crude DNA preparation by sucrose density gradient

DNA from alternative fraction of sucrose density gradient (Lane i to Lane viii).

λ Hind III digest (Lane ix)

Lysozyme treatment, before adding urea-sarkosyl, failed to yield single band DNA.

(iii) Hopwood's method (14) using Kirby mixture

1 g (wet weight) mycelium of *Streptomyces* from fresh culture/frozen stock was suspended in 5 ml lysozyme solution (2 mg/ml STE buffer) and incubated at 28°C for 1 h. After ensuring complete cell lysis, equal volume of '2x' Kirby mixture (sodium tri-isopropyl naphthalene sulphonate, 2 g; sodium 4-amino-salicylate, 12 g; 5 ml Tris-HCl, pH 8, 2 M; neutral phenol, 6 ml; made up to 100 ml with water) was added to the tube and agitated for 1 min on a vortex mixer. Equal volume of phenol-chloroform mixture was added to the tube, mixed on vortex and centrifuged at 15,000 rpm for 20 min at 4°C. The upper aqueous layer was gently removed by broad tip pasteur pippette and deproteinized twice. The upper aqueous phase was then transferred to a fresh tube, 0.1 volume of 3 M sodium acetate, pH 5.2 was added and the nucleic acids were precipitated by adding equal volume of isopropanol. The pecipitate was spooled on a glass rod, washed with 70% ethanol, air dried and dissolved in 5.0 ml TE buffer. The DNA preparation was treated with RNase A (final concentration 50 µg/ml) followed by treatment with pronase (final concentration 1 mg/ml) for 1 h at 37°C. Deproteinization and DNA precipitation were carried out as above.

Each DNA preparation was monitored for protein and polysaccharide contamination by carrying out wavelength scanning in the range of 220-320 nm on a Shimadzu double beam spectrophotometer, model UV 210-A. Only those DNA preparations which exhibited optical density ratios of A230/A260 $\leq$ 0.45 and A280/A260$\leq$0.55 and with an absorbance of $\leq$ 0.1 at 300 nm were used for further studies.

Isolation of *E. coli* DNA

E. coli DNA was isolated by Marmur's procedure (16).

Thermal denaturation (TD)

Thermal denaturation of unsheared *Streptomyces* DNA was performed in sodium phosphate buffer (SPB)(0.012 M), pH 6.8 in Beckmann DU-8B spectrophotometer (17), while that of *E. coli* DNA (control) was performed in both 0.12 M and 0.012 M SPB, pH 6.8. About 300 ul of DNA solution (A260 < 1.0 O.D.) was filled in thermal cuvettes. Cuvettes were stoppered with teflon stoppers to prevent evaporation of the DNA solution. The temperature of cuvettes was raised from 62°C to 100°C at a heat rate of 1°C/min with the help of a thermoprogrammer and the increase in absorbance at every °C rise was recorded manually. The total percent hyperchromicity (%H) of the DNA sample was calculated using the formula:

$$\% H = \frac{A260(98^\circ C) - A260\ (62^\circ C)}{A260\ (98^\circ C)} \times 100$$

This value of %H was normalised to 100%. The hyperchromicity at each temperature was calculated using the formula:

$$\% H(T^{\circ}C) = \frac{A260(T^{\circ}C) - A260\ (62^{\circ}C)}{A260(98^{\circ}C) - A260\ (62^{\circ}C)} \times 100$$

A graph of temperature Vs % H at that temperature was plotted.

Tm was calculated as the temperature at which there is a 50% absorbance rise or 50% hyperchromicity. Transition width (ΔT) of melting was calculated as the temperature difference between 16.5% and 83.5% hyperchromicity.

The G+C content of the DNA was calculated using the formula:

$$\% G+C = (Tm - 69.3) \times 2.44$$

High resolution thermal denaturation (HRTD)

DNA in SPB (0.012 M), pH 6.8 was denatured at a heat rate of 0.2°C/min and absorbance values were recorded at 0.2°C increment from 60°C to 99°C. Data smoothening and differentiation were performed by fitting ten contiguous data points at a time to a simple polynomial by the least squares method. A linear function was used as a fitting polynomial (18). The differential curves were obtained by plotting the temperature derivative of DNA absorption against temperature (dA/dT vs T).

HRTD analysis of *E. coli* and calf thymus DNA served as control.

Optical reassociation kinetics

The high molecular weight (>23 kbp) *Streptomyces* DNA dialysed against 1 X SSC was fragmented to an average length of 550 bp using a Ralsonic fitted with a half inch probe. 30 ml (20-30 µg/ml) of the DNA solution was sonicated at a maximum intensity for 3 min, using of 1 min pulse at a time and at an interval of 10 min between successive pulses of sonication. The temperature of the DNA solution was strictly maintained below 4°C by constant cooling in an ice bath to prevent its denaturation due to heating. The average size of the fragmented DNA was determined by agarose gel (1%) electrophoresis.

Ethylene glycol was added to the sonicated DNA preparation to a final concentration of 20%. Reassociation kinetics was carried out by spectrophotometric method using Beckmann DU-8B spectrophotometer. Thermal cuvettes were filled with 300 µl DNA solution (15-20 µg/ml) and were heated to 99°C. The increase in absorbance of DNA solution was monitored at 260 nm and % hyperchromicity was calculated as described earlier. The DNA samples were maintained at 99°C for 10 minutes to ensure complete strand seperation and then

quickly cooled to 65° (Tm - 25°C). The change in absorbance of the DNA solution was continuously monitored as a function of time. The Cot values at different time intervals were calculated using the formula:

$$\text{Cot} = \frac{A}{2} \times t \quad (h) \quad (1)$$

A - abosrbance at 260 nm.

Thus, a Cot value of one is equivalent to incubation of DNA solution at a concentration of 83 µg/ml (approximately 2 O.D.) for 1 h. The percent reassociation at different Cot values was calculated according to the formula:

$$\% R = \frac{\text{A260 of denatured DNA - A260 of DNA at a given time}}{\text{A260 of denatured DNA-A260 of native DNA at 65°C}} \times 100$$

Restriction digestion of DNA

For all restriction enzyme digestions, the DNA was incubated overnight with restriction enzyme at a ratio of 1 ug:10U. The enzyme to DNA and enzyme volume to reaction volume ratios were carefully adjusted to avoid non-specific (star) activity of restriction enzymes (19). Following incubation, the enzyme reaction was stopped by addition of 10 X reaction terminating buffer [glycerol (50%), EDTA (100 mM), bromophenol blue (0.25%)] to a final concentration of 1X.

Agarose gel electrophoresis

The restriction endonuclease digests of genomic DNA were

analysed on 0.7% neutral agarose horizontal slab gels in 1X TAE buffer at a constant current of 40 mA for 3-4 h.

After electrophoresis, the agarose gels were stained with ethidium bromide (0.5 µg/ml) in dark for half an hour. The gels were visualized on a long wavelength (302 nm) UV transilluminator (UV products, San Gabriels, California, USA) and photographed with a 35 nm SLR camera (Minolta x 700 with macrophotography and 200 m lens system) using a red filter and ORWO 125 ASA, black and white film.

Southern blotting

The transfer of DNA from agarose gel to Hybond-N (Amersham) membrane was carried out essentially according to Southern (20) and Maniatis *et al.* (13). After agarose gel electrophoresis, the gel was soaked in HCl (0.25 N) at room temperature for 15 min for depurination of the DNA. The DNA was then denatured by soaking the gel in several volumes of NaCl (1.5 M) and NaOH (0.5 M) for 1 h at room temperature with gentle shaking. Neurtalization of the gel was done by soaking the gel in several volumes of Tris.HCl (1M) , pH 8.0 and NaCl (1.5 M) for 1 h at room temperature with gently shaking. The mechanical/capillary transfer of DNA onto the membrane was carried out for 16-18 h in 20 x SSC, after which the membrane was rinsed in 2 x SSC to remove any adhering agarose. The membrane was then allowed to air dry, wrapped

in saran-wrap and placed DNA side down on a UV transilluminator for 10 min for crosslinking of DNA to the membrane. The membrane was stored at room temperature between sheets of whatman No. 3 paper till further use.

Labelling of DNA

The rice repetitive DNA (1.5 kbp PstI fragment) was labelled with [α^{32}P]dCTP (specific activity 3000 Ci/m mol) by random primer method of Fienberg and Vogelstein (21). This method is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labelled. The hybridized portion of the DNA acts as a primer for synthesis of a complementary strand of DNA in which [α^{32}P] dCTP is incorporated. The input DNA serves solely as template for the synthesis of labeled DNA, and is not degraded during the reaction, making it possible to label minimal amounts of DNA (10 ng) using this method.

25 ng (10 µl) of the probe was denatured by heating for 10 min at 95°C and by subsequent cooling on ice. To the denatured DNA, following reagents from Boehringer Mannheim multiprime DNA labelling kit were added in series: 3 µl of dATP, dGTP, dTTP mixture, 2 µl primer, 5 µl [α^{32}P]dCTP, 3000 Ci/mmol (50 µCi) and 1 µl Klenow enzyme (2 U). The reaction mixutre (20 µl) was incubated at 37°C for 30 min. Reaction

was stopped by adding 2 µl EDTA (0.2 M pH 8.0). To remove the unincorporated deoxyribonucleotide triphosphates, the DNA was precipitated with ethanol. The precipitate was dissolved in 100 µl T_{10} pH 8.0

Counting of radioactivity

The radioactivity of the purified labelled probes was counted in Rack-beta liquid scintillation counter. The specific activity of the samples ranged between 1-5 x 10^8 cpm/µg DNA.

Prehybridization, hybridization, and washing of blot

Prehyrbridization of the blot was carried out to block the sites on the Hybond-N membrane that bind single or double stranded DNA non-specifically, giving high background to the autoradiogram.

The Southern blot was prehybridized overnight at 42°C in heat-sealed plastic bag containing: 5x SSC, 0.5% SDS, 5 x Denhardt's solution (50 x Denhardt's solution:Ficoll polyvinyl pyrrolidone and BSA, 1% each) and 100 µg/ml denatured, sonicated calf thymus DNA in a total volume of 5 ml.

The purified labelled probe was denatured and added to the prehybridization solution in a bag for hybridization. The blot was incubated at 42°C with shaking for another 24 h.

After hybridization, the blot was washed for removal of

the unbound/non-hybridized probe using solutions of different stringencies. The membrane was washed twice with 2 X SSC, 0.1% SDS at room temperature for 10 min and once at 42°C with 1 x SSC, 0.1% SDS for 15 min.

Autoradiography

Moist filters were wrapped in Saran wrap and exposed to X-ray film for 40 h at -70°C using X-ray cassette (Sigma) with builtin intensifying screens.

RESULTS AND DISCUSSION

The use of Kirby mixture is known to protect the DNA against nucleolytic activity. The method yielded high molecular weight DNA (>23 kb) with a sharp single band as determined by agarose gel electrophoresis (Fig. IV.2). The yield of the DNA was 1 mg/g of mycelium. The UV-absorption spectrum of the DNA isolated by using Kirby mixture is shown in Fig. IV.3.

DNA melting analysis

In the DNA double helix, two polynucleotide chains are associated through hydrogen bonding and hydrophobic interactions. These bonds can be easily disrupted by heating, and the process is called as DNA 'denaturation' or 'melting'. Melting of the DNA (separation of two strands) is generally monitored as the change in ultraviolet absorbance caused by elevating the temperature of the DNA solution. The thermal melting profile of DNA is primarily characterized by following parameters:

(i) Tm/melting temperature

(ii) $\Delta T/6$ 2/3rd, and

(iii) Hyperchromicity

The melting of DNA is generally carried out in SPB (0.12 M), pH 6.8 (17). As the melting of *Streptomyces* DNA was not complete under these conditions, it was carried out in a low

Fig.IV.2 High molecular weight Streptomyces DNA isolated using Kirby mixture

λ Hind III digest (Lane 1)
Streptomyces DNA (Lane ii).

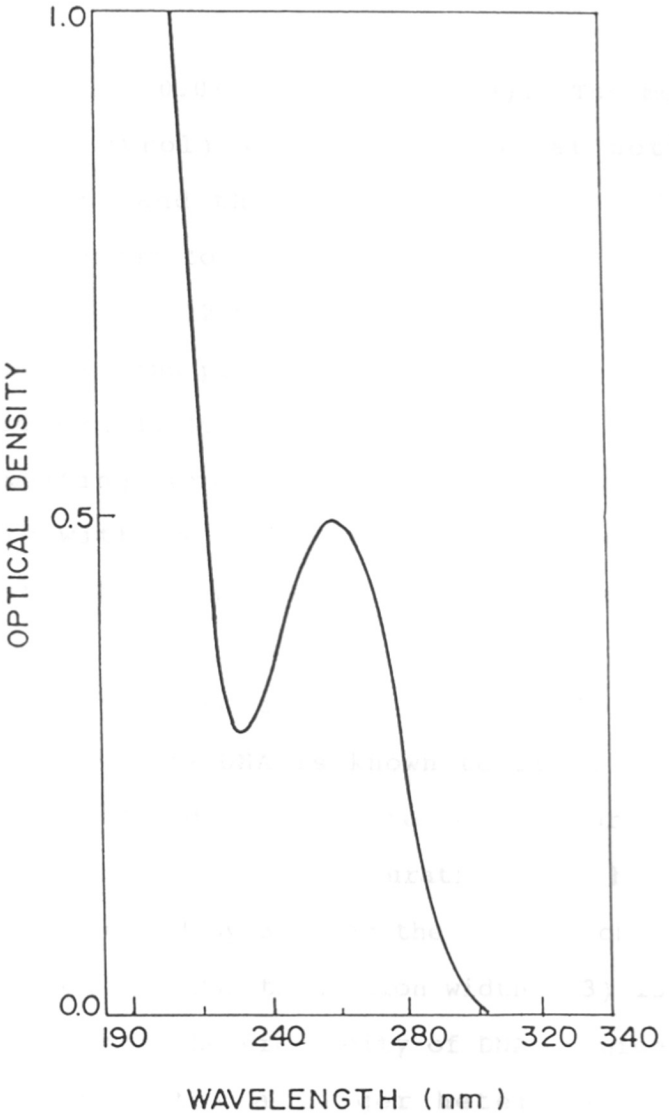

Fig.IV.3 UV absorption spectrum of Streptomyces DNA.

salt buffer (SPB, 0.012 M, pH 6.8) (23). The melting of *E. coli* DNA (control) was carried out at both the salt concentrations and the difference in Tm values (13°C) obtained was added to that of *Streptomyces* Tm value (85°C) obtained in SPB (0.012 M), pH 6.8.

The DNA melting profiles of *Streptomyces* and *E. coli* are depicted in Fig. IV.4.

The melting curve of *Streptomyces* DNA is smooth and monophasic with a Tm of 98°C. There exists a linear relationship between the melting temperature and base composition of DNA, higher the G+C content of DNA more will be its Tm. The G+C content of *Streptomyces* is 70%. Denaturation of the DNA is known to start from A+T rich region of the DNA and to propagate to the more difficult G+C rich region (22). During denaturation, the hyperchromicity of the DNA increased by 30% and the transition width (ΔT) is found to be 4.5°C. The transition width (23) is an estimate of base composition heterogeneity of DNA; a greater ΔT value generally indicates a larger heterogeneity in base composition ΔT is found to increase from phages to animals to higher plants.

The sharp melting profile of *Streptomyces* DNA and absence of a 'foot' or shallow absorbance rise before the

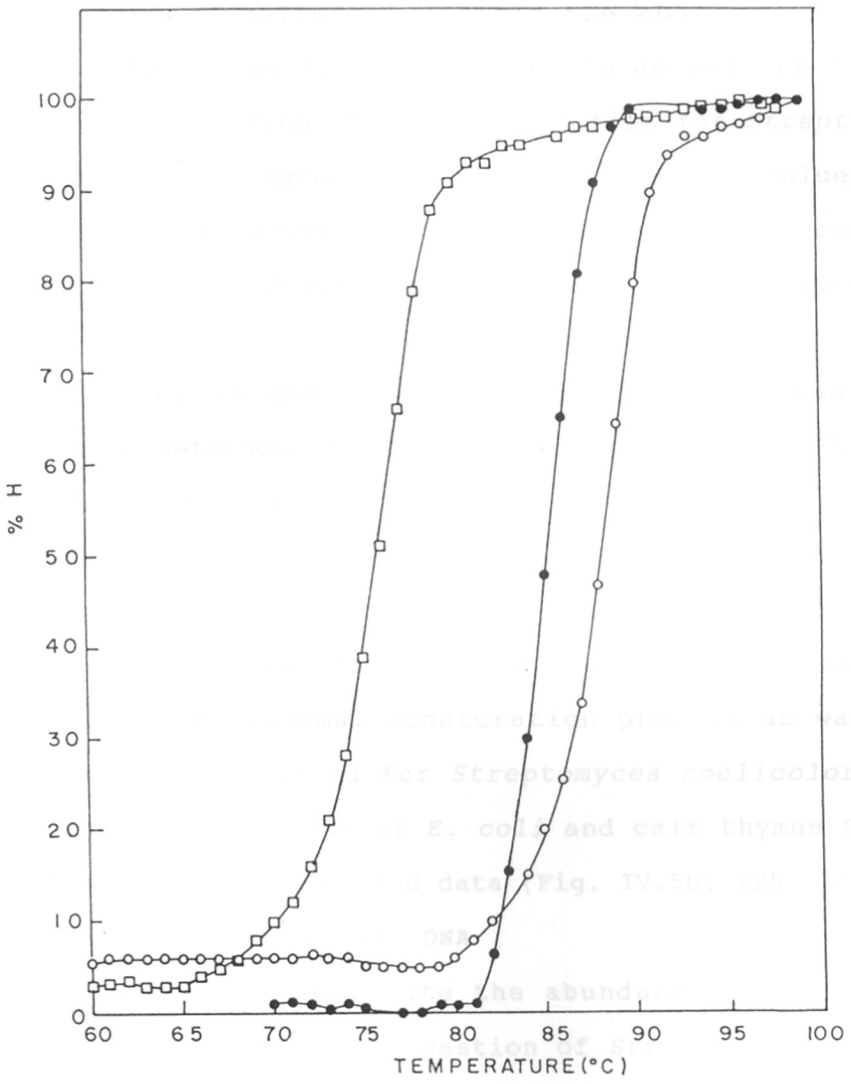

Fig.IV.4 Thermal melting profile of Streptomyces DNA

Thermal denaturation of Streptomyces DNA in 0.012 M SPB, pH 6.8 (●) and of control DNA (E. coli DNA) in 0.12 M (O) and 0.012 M (□) SPB, pH 6.8.

actual start of melting indicated the absence of single stranded DNA and/or RNA (24). The Tm as well as the G+C content obtained from the melting profile for *Streptomyces* sp. NCIM 2730 DNA agree well with the reported values (5). The melting parameters of *E. coli* DNA (used as a control) compare well with those of their reported literature values (25).

In order to get a better insight into the base composition heterogeneity of *Streptomyces* sp. NCIM 2730 DNA, the DNA was denatured at a heat rate of 0.2°C/min and the derivative analysis of the melting data was carried out (Fig.IV.5a). The curve is smooth and monophasic, which is typical of bacteria. No blocks of low G+C content could be detected in the thermal denaturation profile as was also observed by Usdin *et al* for *Streptomyces coelicolor* (10). The derivative profiles of *E. coli* and calf thymus DNA are comparable with the reported data (Fig. IV.5b) (25, 26).

Restriction analysis of the DNA

In order to investigate the abundance of particular bases in the genome, the digestion of *Streptomyces* DNA with restriction enzymes having specificity for AT or GC rich regions was carried out. The restriction enzymes which recognize sites low in G+C content (e.g. Hind III and HpaI) cut the DNA into a relatively few and predominantly large

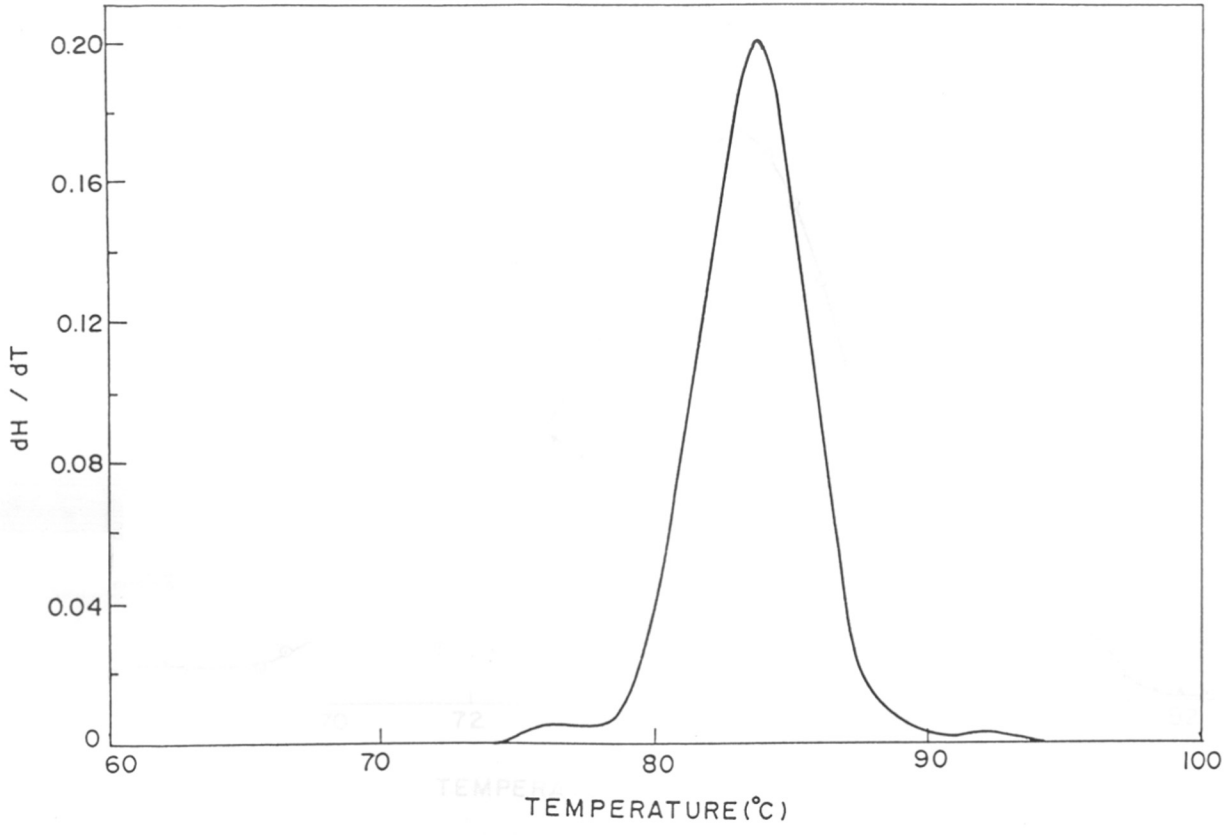

Fig. IV.5a Derivative melting profile of Streptomyces DNA.

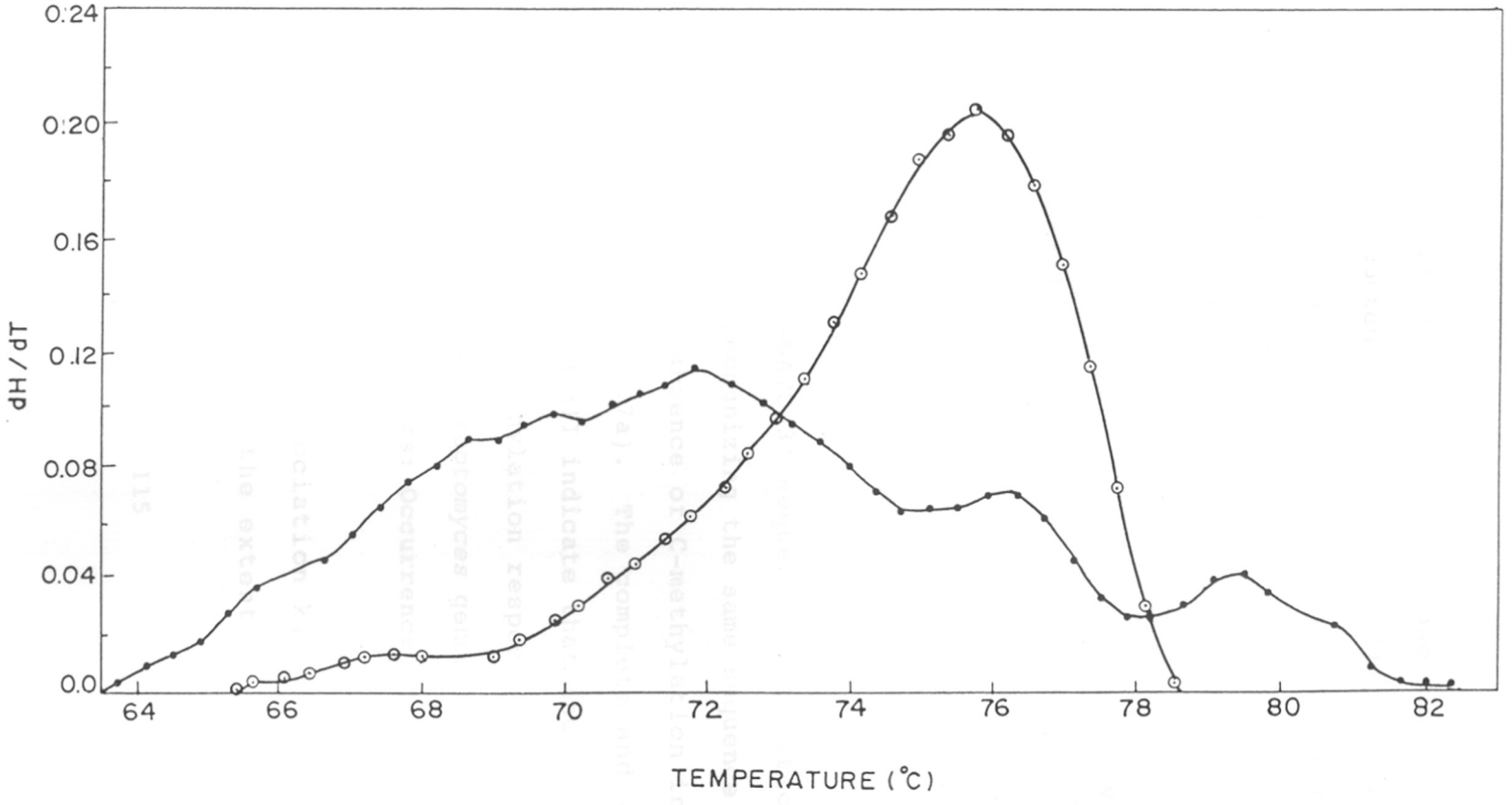

Fig.IV.5b Derivative melting profiles of E. coli (O) and Calf thymus (O) DNA (controls).

fragments (data not shown), whereas those which recognize sites high in G+C content (e.g. SmaI, Hae III, Pst I, Bam HI, Ava II and Hinc II) gave many small fragments (Fig. IV.6). Thus, the G+C rich nature of the genome is also revealed by its susceptibility to digestion by restriction enzyme which recognize G and /C containing sites.

Certain restriction endonucleases are DNA base-methylation specific and use of sets of such restriction endonucleases provides an information about the methylation status of the DNA. The *Streptomyces* DNA was digested with DpnI, MboI and Sau 3A I. The lack of digestion with Dpn I and extensive digestion with MboI shows the absence of A-methylation in the 5′-GATC-3′ sequence. Digestion of the genome with Sau 3A I (recognizing the same sequence of bases viz. GATC) denotes the absence of C-methylation in the 5′-GATC-3′ sequence (Fig. IV.7a). The complete and identical digestion with MspI and HpaII indicate that there is neither internal nor external C-methylation respectively in the 5′-CCGG-3′ sequence of the *Streptomyces* genome (Fig. IV.7b).

Optical reassociation kinetics: Occurrence of repetitive DNA sequences

The technique of reassociation kinetics provides a valuable information about the extent of repetitive and

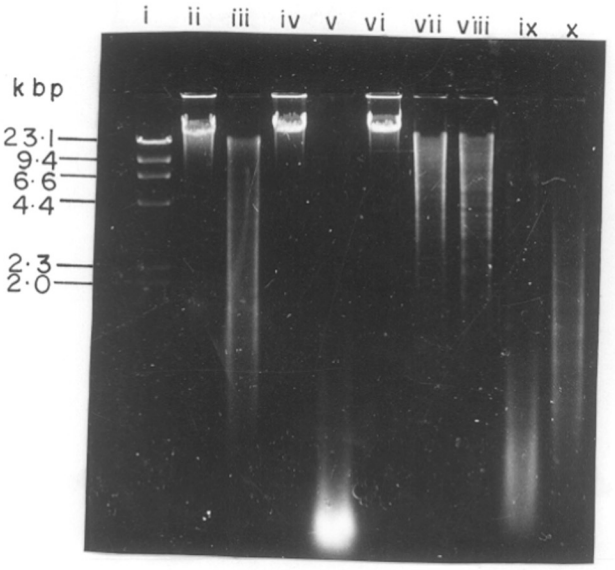

Fig.IV.6 Digestion of Streptomyces DNA by restriction endonucleases recognizing sites high in G+C content

λ Hind III digest (Lane i); Streptomyces DNA (Lane ii, iv and vi) digested with Sma I (Lane iii), Hae III (Lane v), Pst I (Lane vii), Bam HI (Lane viii), Ava II (Lane ix) and Hinc II (Lane x).

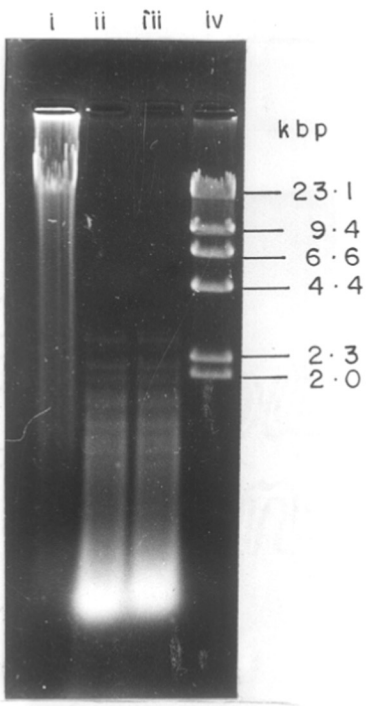

Fig.IV.7a **Digestion of Streptomyces DNA with 'base-methylation specific restriction endonucleases'**

Streptomyces DNA digested with Dpn I (Lane i), Sau 3A I (Lane ii), and Mbo I (Lane iii); λ Hind III digest (Lane iv).

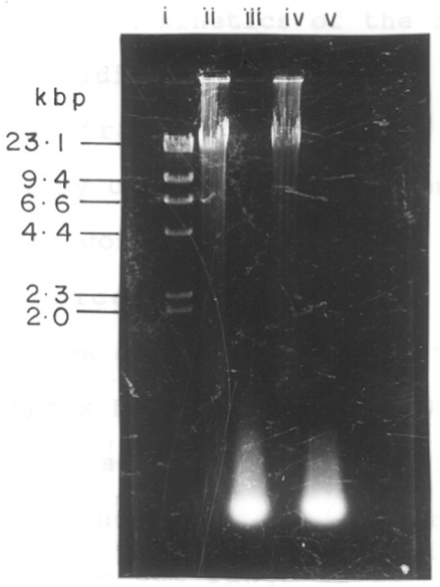

Fig.IV.7b Digestion of *Streptomyces* DNA with 'base-methylation specific restriction endonucleases'

λ Hind III digest (Lane i); *Streptomyces* DNA (Lane ii and iv) digested with Msp I (Lane iii) and Hpa II (Lane v).

single copy DNA in the genome and also gives an idea about the size of the genome. We have used an optical method for studying the reassociation kinetics of the *Streptomyces* DNA due to the ease and rapidity of the method.

The size of DNA fragments is one of the important parameters in the study of DNA reassociation kinetics (27). The fragment size was controlled to 550 bp by shearing the DNA by means of sonication. Ethylene glycol (final concentration, 20%) was added to the sonicated DNA to reduce the Tm of *Streptomyces* DNA at least by 10°C (28), and to ensure complete strand separation during DNA denaturation.

Figure IV.8 includes the reassociation curve of *Streptomyces* DNA in the Cot range of 10^{-3} to 10^{0} M.S.L^{-1}. The curve appears to be biphasic and a maximum of 65% reassociation has been achieved. We have assumed that the first component of the reassociation curve represents the fast reassociating DNA which accounts for about 25% of the total DNA. The second component is a slow reassociating DNA and presumably consists of single copy DNA sequences. The rate of reassociation of fast reassociating DNA is 50 times faster than that of slow reassociating DNA and hence it appears that the average copy number of this DNA is about 50 (Table IV.2). The Cot 1/2 value of the slow reassociating DNA is compared with that of *E. coli* DNA under our

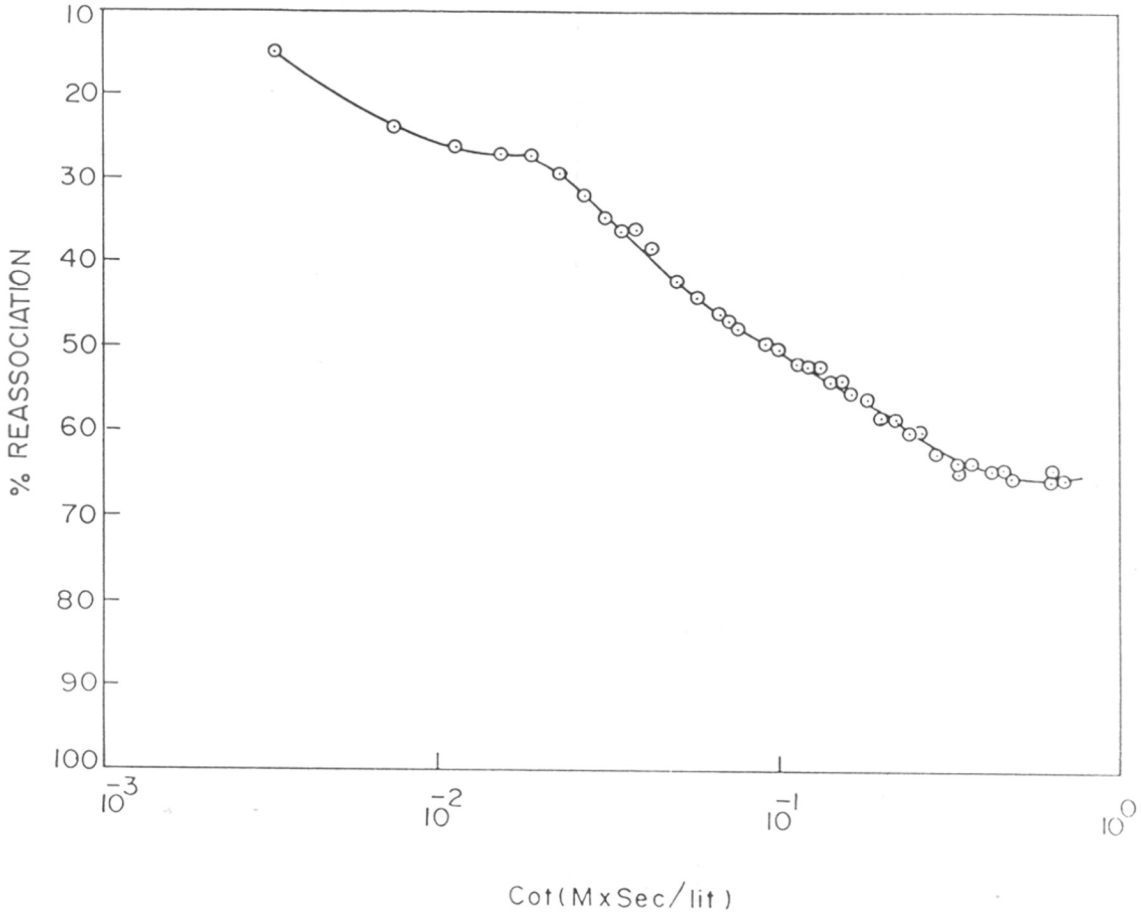

Fig.IV.8 Reassociation curve of <u>Streptomyces</u> DNA (500 bp) in 1 X SSC, 20% ethylene glycol.

The graph represents average of five different experiments.

Table IV.2 : Kinetic estimates of *Streptomyces* sp. NCIM 2730 genome

Component	Fraction of the genome[a]	$Cot_{1/2}$ observed mol x sec/L	$Cot_{1/2}$ pure[b]	K_{pure}	Frequency of repetition[c]	Kinetic complexity[d]
Fast reassociating < Cot 10^{-2}	0.25	0.002	0.0005	2×10^{3}	50	3×10^{3}
Slow reassociating Cot 2×10^{-2} to Cot 3×10^{-1}	0.35	0.1	0.035	2.9×10^{1}	1	0.15×10^{6}

[a] Values obtained from the Cot curve

[b] $Cot_{1/2}$ observed x fraction of the DNA

[c] $Cot_{1/2}$ of slow reassociating DNA/$Cot_{1/2}$ of fast

[d] Estimated using *E. coli* as a standard (29).

experimental conditions and the kinetic complexity of the *Streptomyces* DNA is estimated to be .15 x 10^6 bp. The calculated value for the genome size of *Streptomyces* sp. NCIM 2730 is lower than the values reported for *Streptomyces* genome. Variations in genome complexity may arise due to the gene amplification or deletion which is known to occur frequently in *Streptomyces* (5).

Our findings from the data on colony hybridization support the estimate of the content of repetitive DNA (25%) derived from reassociation kinetics of DNA. The colony hybridization of clones lifted randomly from the partial genomic library of *Streptomyces* in *E. coli* is shown in Fig.V.4 of Chapter V. The clones for repetitive sequences can be indentified based on their strong hybridization signals while remaining clones revealed faint hybridization mainly indicative of low/single copy sequences. Thus, the library presented approximately 25% repeat clones and 75% low/single copy clones.

Streptomycetes are known to undergo a diverse series of irreversible morphological and secondary metabolic changes during their life cycle. The repetitive DNA sequences may be involved in the control of these changes as well as in the organization and packaging of large *Streptomyces* genome.

Streptomycetes are subject to a high degree of genetic instability too. One manifestation of this phenomenon is the occurrence of tandemly reiterated DNA stretches within the chromosome (30). Reiteration is shown to be associated with interspecies protoplast fusion (31), mutations carrying defects in unstable genes (32-35) and plasmid loss (36) within *Streptomyces*. Thus, the repetitive DNA sequences are also implicated to have a role in the genetic instability and the recombinational events that are commonly encountered in *Streptomyces* (10).

Specificity of banding pattern

In order to verify whether the DNA of a particular strain is an intrinsic property of that strain or is subject to changes in the extrinsic factors such as time of harvesting of a culture, the restriction patterns of DNA isolated from a culture grown for different time intervals (24 to 120 h) were studied. Comparative analysis of Bam HI restriction endonuclease digestion patterns of *Streptomyces* sp. NCIM 2730 DNA was found to be independent of the age of the culture (Fig. IV.9) revealing that it can furnish a useful DNA fingerprint for a given strain.

Comparison of the (Bam HI) restriction analysis of DNA from different *Streptomyces* species [viz. (i) *Streptomyces sp.* NCIM 2730; (ii) *S. thermonitirificans* NCIM

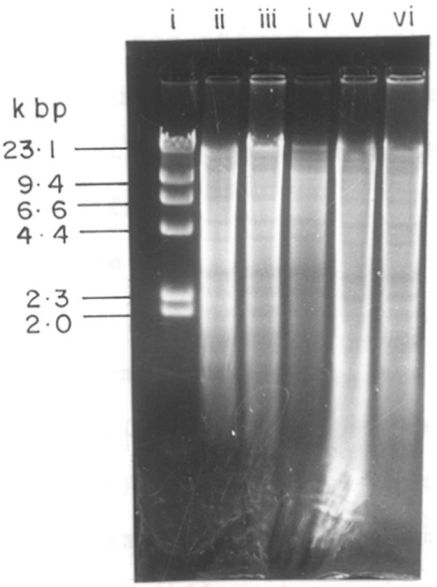

Fig.IV.9 Banding pattern of restriction fragments with respect to the age of culture

λ Hind III digest (Lane i); Bam HI restriction digestion of Streptomyces sp. NCIM 2730 DNA isolated from culture grown for 24 h (Lane ii), 48 h (Lane iii), 72 h (Lane iv), 96 h (Lane v) and 120 h (Lane vi).

2007; (iii) *S. lividans* TK64 and (iv) *Chainia*] revealed a distinct pattern for each species (Fig.IV.10). Defining a species with the help of 'numerical taxonomy' is an extremely difficult task, owing to the high degree of genetic instability, frequently encountered in *Streptomyces* genome. Therefore, such comparative analyses of restriction pattern of DNA from different *Streptomyces* species can be of great use in distinguishing different *Streptomyces* species.

Hybridization with a rice probe

A rice repetitive DNA probe (1.5 kbp) was used to hybridize with DNA from *Streptomyces* sp. NCIM 2730, *S. thermonitrificans* NCIM 2007, and *S. lividans* TK 64 digested with Bam H1 (Fig. IV.11a). The probe detects a large number of DNA fragments superimposed on a background smear (Fig. IV.11b), in all the three different species suggestive of the presence of a highly repeated DNA family in *Streptomyces* homologous to the rice probe. The observation that the rice repetitive DNA probe exhibits homology with *Streptomyces* DNA is an unusual finding and not reported hitherto. The only precedence for such type of observation is the detection of DNA fingerprints of cultivated rice by hybridization with a human minisatellite DNA probe (37) and may be of evolutionary significance.

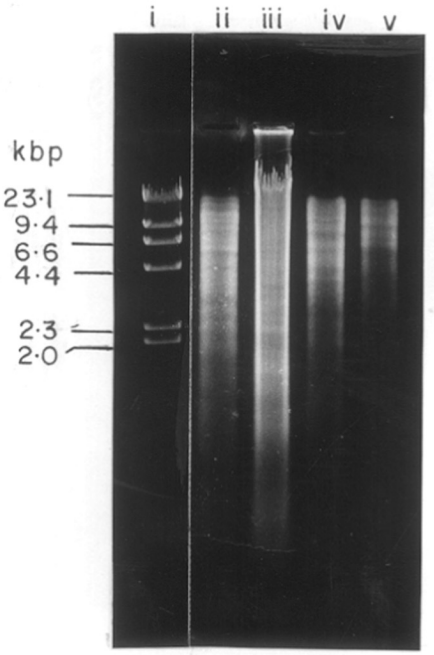

Fig.IV.10 Comparative analysis of DNA restriction patterns of different Streptomyces species

λ Hind III digest (Lane i). Bam HI restriction digestion of DNA isolated from Streptomyces sp. NCIM 2730 (Lane ii), S. thermonitrificans NCIM 2007 (Lane iii), S. lividans TK 64 (Lane iv) and Chainia (Lane v).

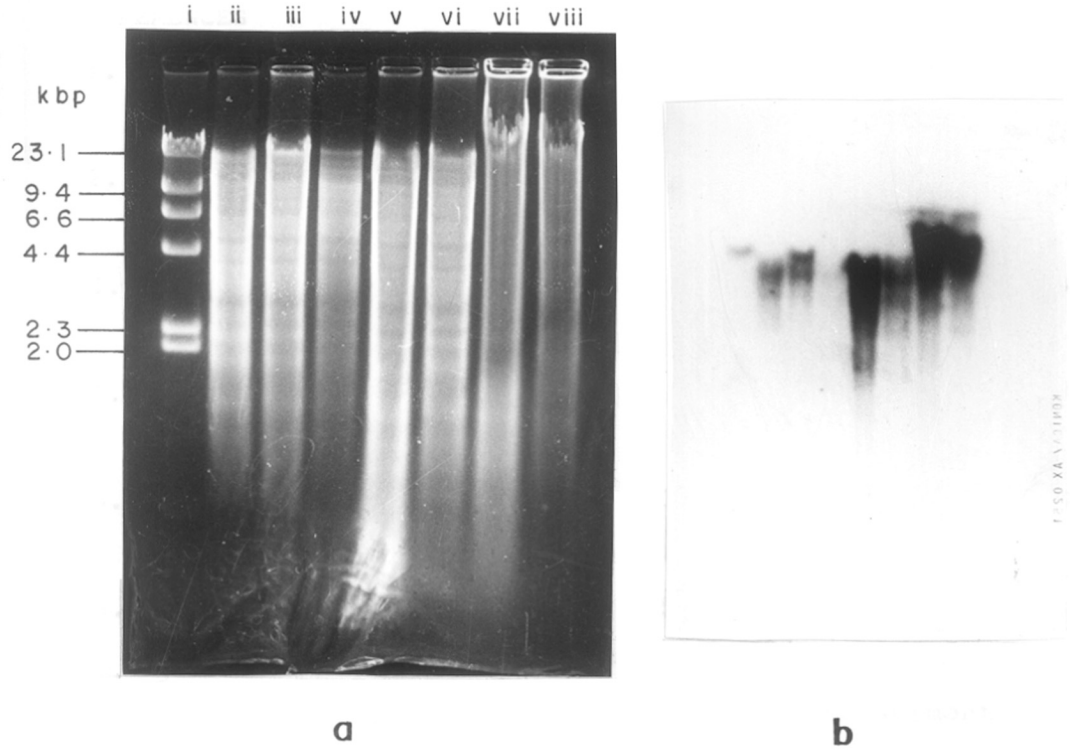

Fig.IV.11 **Hybridization of Streptomyces DNA digests with α-^{32}P labelled rice repetitive DNA probe**

a. λ Hind III digest (Lane i). Bam HI restriction digestion of DNA from Streptomyces sp. NCIM 2730, culture grown for 24 h (Lane ii), 48 h (Lane iii), 72 h (Lane iv), 96 h (Lane v) and 120 h (Lane vi); S. thermonitrificans NCIM 2007 (Lane vii) and S. lividans TK 64 (Lane viii).

b. Autoradiogram of Southern hybridization gel, Fig.IV.11a.

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CHAPTER V

CONSTRUCTION OF GENOMIC LIBRARY OF *STREPTOMYCES* IN *E. COLI*

SUMMARY

Isolation of GXI gene from *Streptomyces* sp. NCIM 2730 is important for the 'hyperexpression of the gene' and for the production of an altered protein with desirable properties by 'protein engineering'. The construction of *Streptomyces* genomic library is the first step in this direction. A standard approach of 'shot-gun' cloning was used for the construction of genomic library of *Streptomyces* in *E. coli* using a plasmid vector, pUC8. High molecular weight *Streptomyces* DNA was isolated using Kirby mixture (Hopwood's procedure). The conditions for partial digestion of the DNA using Bam HI were standardized and the partially digested DNA was size fractionated on a 10 to 40% sucrose density gradient to yield DNA fragments in the range of 2-10 kbp. The size fractionated DNA was ligated with BamHI cut, dephosphorylated pUC8 using *E. coli* T_4 DNA ligase and the ligation mixture was transformed in *E. coli* JM 105 by the 'calcium chloride procedure'. The recombinants (white colonies) were analysed for the presence and origin of the inserts by (i) colony hybridization and (ii) by Southern hybridization of the digested plasmids with *Streptomyces* genomic DNA. Assuming the genome size of *Streptomyces* to be 10^7 bp and the average insert size of 4 kbp the total number

of recombinant clones represent 45% of the genomic library. An eighteen-mer mixed oligonucleotide probe has been synthesized based on the sequence of a stretch of six amino acids, well conserved in the protein sequence of different GXIs from actinomycetes and related organisms to screen the library for the identification of the GXI gene.

INTRODUCTION

Production of high fructose corn syrup using bacterial thermostable xylose isomerases that have high activity for glucose, is a multi-billion dollar industry. The understanding of the molecular structure of the enzyme in relation to its catalytic properties helps in the improvement of the industrial applications of the enzyme. Cloning of the gene for xylose isomerase (*Xyl A*), is the first step for improving its yield and properties. Actinomycetes and related organisms are known to be super producers of XIs and cloning of GXI gene from a few of them is reported.

Kho (1) has reported cloning of GI gene from *Streptomyces phaeochromogenes* using pIJ 702 (a multicopy *Streptomyces* cloning vector which carries genes for thiostrepton resistance and melanin pigmentation as markers) by complementation of GI negative mutant of *Streptomyces lividans* 66. The GI activity of the cloned strain was found to be approximately 50-fold higher than that of the wild type. *Xyl* A gene from *Ampullariella* sp. strain 3876 was isolated by its ability to complement a xylose isomerase defective *E. coli* strain (2). The sequence analysis revealed that the gene has a high GC content (70%) and an exceptionally strong preference (97%) for G/C in the third position of the codons. The results also present evidence

for the occurrence of xyl-operon in *Ampullariella*. A gene for XI from *Streptomyces violaceoniger* has been cloned (3) and sequenced (4) by Tiraby and co-workers. The gene coding for xylose isomerase from *Actinoplanes missouriensis* has been isolated by complementation of a XI negative *E. coli* mutant using pBR 322 as a cloning vector and was shown to be expressed in *E. coli* under the control of its own promoter (5). The gene was further fused to the yeast GAL 1 promoter to investigate whether the XI of *A. missouriensis* could be expressed in *Saccharomyces cerevisiae*. Analysis of the total RNA from yeast transformants containing this construct showed presence of xylose isomerase specific mRNA. The nucleotide sequence of the gene (6) was similar to that for GXI from *Ampullariella* (2). The total GC content of the gene is 68% with 94% of the bases in the third wobble position of the codons being G/C. The gene shows 74-92% homology with other XIs from Actinomycetes (2,4) whereas the homology with *Bacillus* and *E. coli* XI genes is weak (7,8). The gene coding for XI from *Streptomyces griseofuscus* S-41 was cloned in *E. coli* MN522 using pUC 13 as a cloning vector (9). Nucleotide sequence analysis of the gene showed high GC content (71%) and 93% of the bases in the third wobble position of the codons were either G or C, which is common in XI genes from

Actinomycetes (2,6). As observed for *A. missouriensis*, the gene shows significant homology with the genes for XIs from different Actinomycetes. Recently, a gene for GXI from *Arthrobacter* strain N.R.R.L. B-3728, has been cloned, sequenced and expressed in *E. coli* under the control of its own promoter (10).

Streptomyces sp. NCIM 2730 isolated in our laboratory is one of the highest GXI producing *Streptomyces* species ($10,000UL^{-1}$ of culture broth) and possesses several properties useful from commercial point of view. Fermentation studies have shown that although maximum activity is obtained with xylose as an inducer, it is possible to replace it at least to some extent by cheaper materials like wheat bran or bagasse hydrolysates (11). Secondly, it has been shown that the Co^{2+} ions are not required for enzyme production (12), eliminating the health problems related to HFCS and also the problem of waste disposal of the spent media. Immobilization of the enzyme on an inexpensive anion exchange resin, Indion 48-R considerably increases the thermostability of the enzyme (11). High thermo-tolerance is desirable for HFCS production as, at equilibrium, the increase in reaction temperature, results in an increase in the ketose to aldose ratio proportionately (13). In addition, reactors running at high temperatures

have minimum of microbial contamination, allowing for less frequent and less costly enzyme replacement. Concentrated glucose syrup (25%) was efficiently converted to fructose by the immobilized enzyme and shows a considerable potential for use in HFCS production. Isolation of *Xyl* A from *Streptomyces* sp. NCIM 2730 DNA is important for the overproduction of the enzyme and offers new opportunities to manipulate the enzyme to make it better suited for its industrial application. The construction of *Streptomyces* genomic library is the first step in this direction.

The present chapter, describes the construction of the genomic library of *Streptomyces* sp. NCIM 2730 in *Escherichia coli* and discusses the steps to be followed towards the isolation of glucose/xylose isomerase gene.

MATERIALS AND METHODS

Materials and Methods which are in common with the work on 'Genome Characterization' are listed in Chapter IV. While the others are listed here.

Materials

Ampicillin (sodium salt), X-gal (5-bromo-4 chloro 3-indolyl beta D-galactoside), IPTG (isopropyl thio-D-galactoside) and $CaCl_2$ were from Sigma Chemical Co. (USA). Hybond-N was from Amersham (UK). T_4 DNA ligase was obtained from New England Biolabs (NEB) (USA). Whereas pUC8 was obtained from Pharmacia (Sweden).

E. coli host and vector system used for cloning

Streptomyces genomic library was constructed in *E. coli* JM 105 using pUC 8 as a vector. The details of the genotype of the host are as follows:

E. coli strain	Genotype
JM 105 (K-12 derivative)	*thi*, *rsp*L, *end* A, *sbc* B15, *hsp* R4, (*lac*-*pro*AB), [F', *tra* D36, *pro*AB, *lac* I^qZ M15]

pUC8

pUC plasmids developed by Messing and his collagues (14,16) permit the histochemical identification of recombinant clones. They carry a segment of DNA from the *lac* operon of *E. coli* that codes for amino terminal fragment of β-galactosidase. Embeded in this coding region is a

polycloning site that does not disrupt the reading frame of β-galactosidase. This fragment whose synthesis can be induced by IPTG is capable of intra-allelic (α) complementation with a defective form of β-galactosidase encoded by the host. Bacteria exposed to IPTG, synthesize both fragments of the enzyme and form blue colonies when plated on media containing X-gal (17). Insertion of foreign DNA into the polyclonal site of the plasmid inactivates the amino-terminal fragment of B-galactosidase and abolishes α-complementation. Bacteria carrying recombinant plasmid therefore give rise to white colonies. The size of the plasmid is 2.7 kbp and it carries *amp*^{r} as a selection marker. In Col E1 derived plasmids such as pBR322, the copy number is regulated by the hybrid formation of RNAI: RNAII and a small peptide of 66 amino acids named as Rom/Rop which stabilizes this hybrid. Rom/Rop gene is absent in pUC plasmids. In addition, they carry a mutation (G → A transition) one nucleotide upstream of the normal site of intiation of transcription of RNAI (18). As a result, these plasmids replicate to a much higher copy number (500-700).

Maintenance of cultures

The working stock of *E. coli* was stored on LB agar slant at 4°C for short term while for long term storage, 15%

glycerol suspension of the liquid culture was kept frozen at -70°C. The presence of F′ episome in JM 105 was checked intermittently by subculturing on minimal medium plates (M-9) supplemented with thiamine-HCl. F′ episome carries *lac* Z, M15 and *lac* I^q on it which are necessary for histochemical identification of recombinant clones.

Streptomyces sp. NCIM 2730 was maintained on MGYP slants at 4°C and subcultured every three months.

Preparation of reagents

Heat labile compounds such as antibiotics (Ampicillin, sodium salt) and IPTG were filter sterilized through autoclaved millipore (0.45 µm) membranes and added freshly to the cooled media (45°C).

10 µl of 100 mM IPTG and 40 µl of 2% X-gal (2 g/100 ml dimethyl formamide) were used per LB plate of 85 mm diameter.

Composition of 10X M-9 salt (pH 7.0)

Anhydrous Na_2HPO_4	70 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g
Distilled water	1 L

Composition of M-9 medium

10X M-9 salt	100 ml
0.01 M $CaCl_2$	10 ml

0.1 M $MgSO_4.7H_2O$ 10 ml

(Each solution was autoclaved separately at 15 psi for 20 min)

20% casamino acids 20 ml

(Sterilized by autoclaving at 10 psi for 10 min)

H_2O 1000 ml

0.2 ml of 10 mg/ml thiamine (Vit B1) (filter sterilized) was added to cooled (45°C) medium.

2.0% Difco agar was added to get solid medium.

Isolation of *Streptomyces* DNA

Streptomyces DNA was isolated using Kirby mixture as described earlier in Chapter IV.

Partial digestion of *Streptomyces* DNA

To generate 2-10 kbp DNA fragments, *Streptomyces* DNA was partially digested with Bam HI restriction endonuclease. Standardisation of conditions for partial digestion of DNA was carried out according to Maniatis *et al* (19). A solution containing 10 µg of genomic DNA with 10 µl high salt buffer (10X) was adjusted to a final volume of 100 µl. It was dispensed in eppendorf vials with 40 µl in the first vial and 20 µl in the remaining vials and kept on ice. 20 U of Bam HI were added to the first vial and the contents were mixed thoroughly but gently. The tube was microfuged and 20 µl of the sample was transferred to the next vial. Serial

transfers of 20 µl were carried out upto the 4th vial. Half of the contents of 4th vial were transferred to a new vial (5th vial). All the vials were briefly microfuged and incubated at 37°C for 1 h. The reaction was terminated by chilling to 0°C and by adding 10X reaction terminating buffer to a final concentration of 1X. The DNA was analysed by electrophoresis on 0.7% agarose gel, using λ Hind III digest as a standard molecular weight marker.

Large scale partial digestion of *Streptomyces* DNA (50 µg) was carried out with Bam HI (5.0 U μg^{-1} DNA) at 37°C for 1 h. The reaction was stopped with EDTA (10 mM).

Size fractionation of restricted DNA

The above restricted DNA was subjected to size fractionation by sucrose density gradient (10-40%) centrifugation (20) at 36,000 rpm for 10 h at 17°C in a Beckman SW 41 rotor. After centrifugation, fractions of 500 ul each were collected from the bottom of the tube and an aliquot from each fraction was analysed on 0.7% agarose gel. Fractions ranging from 2 to 10 kbp were pooled and precipitated with two volumes of ethanol. The pellet was air dried and dissolved in T_{10} buffer (10 mM Tris-HCl, pH 7.5).

Preparation of vector DNA

Restriction digestion of pUC8

pUC8 (1 µg) was linearized with Bam HI (10 U) in a

reaction volume of 20 µl containing 2 ml of 10 X high salt buffer, at 37°C overnight to ensure complete plasmid digestion. The enzyme digestion was stopped by the addition of EDTA to a final concentration of 10 mM, deproteinized and precipitated with equal volume of isopropanol. The pellet was dissolved in 90 ml of 10 mM Tris.Cl, pH 8.3.

Dephosphorylation

Linearized plasmid vector was dephosphorylated with calf intestinal alkaline phosphatase (CIP). 10 µl of 10 X CIP buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris.Cl, pH 8.3) was added to 90 µl of the above linearized plasmid preparation and incubated with 1 U of enzyme at 37°C for 1 h. The phosphatase was removed by the treatment with phenol/chloroform.

Vector-Insert ligation

Size fractionated (2-10 kbp) *Streptomyces* DNA (Insert) was ligated to Bam H1 treated dephosphorylated pUC 8 (vector) in a reaction volume of 20 µl such that the total DNA concentration was 20 ng/µl. A series of test ligations was conducted to determine the optimum ratio of vector:insert. In each case, the ligation mixture consisted of the vector-insert mixture together with 1/10th volume of 10X ligation buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 20 mM

dithiothreitol, 1 mM ATP, 50 µg/ml BSA) and T_4 DNA ligase (10 U) and was incubated overnight at 14°C.

Transformation of *E. coli* with plasmid DNA

Plasmid molecules can be introduced in a suitable *E. coli* host by transformation of the competent cells. Though a number of transformation protocols are available, the following protocol was used which is a slight modification of the procedure described by Maniatis *et al.* (19).

25 ml of Luria broth in a 250 ml flask was inoculated with 250 ul of an overnight bacterial culture. The flask was incubated at 37°C with vigorous shaking to a density of approximately 5 x 10^7 cells/ml (i.e. A_{550} of 0.5). The culture was chilled on ice for 10 min and centrifuged at 6,000 rpm for 10 min at 4°C in Sorvall SS34 screw capped tubes. The supernatent was discarded and the pellet was suspended in 12.5 ml of sterile, chilled 50 mM $CaCl_2$ in 10 mM Tris.HCl, pH 8.0. The cell suspension was kept on ice for about half an hour and then centrifuged at 6,000 rpm for 20 min at 4°C in the same tube. The supernatant was discarded and the cells were resuspended in 1/15th of the original volume, in an ice cold, sterile 50 mM $CaCl_2$ in 10 mM Tris-HCl, pH 8.0 solution. Aliquots of 0.2 ml were dispensed in prechilled tubes. Plasmid DNA (20 ng) in T_{10} buffer or the ligation mixture was added to each aliquot, mixed and the

tube was incubated on ice for 30 min. A heat shock was given at 42°C for 2 min and the tubes were chilled immediately on ice. 2 ml of Luria broth was added to each tube, followed by an incubation at 37°C for 75 min without shaking. Different aliquots were plated on LB plates containing ampicillin-Xgal-IPTG and incubated at 37°C for 16 h. The transformation efficiency was expressed as the number of transformants per ug of DNA.

Selection of recombinants and their storage

Colonies of *E. coli* harbouring chimeric plasmids (plasmids with insert) were selected as white colonies on Xgal-IPTG-ampicillin LB plate; while blue colonies were dismissed as non-recombinants. Individual white colonies were then inoculated with sterile toothpicks on plates containing LB agar with 100 µg/ml ampicillin. The plates were incubated overnight at 37°C and stored at 4°C as a master plate. For long term storage, recombinant clones were stored frozen at -70°C as 15% glycerol stocks.

Plasmid minipreparations

Plasmid DNA extractions were done by the alkaline lysis protocol described by Birnboim and Doly (21). 5 ml of LB containing ampicillin (50 µg/ml) was inoculated with a single bacterial colony and the tube was incubated at 37°C overnight

with vigorous shaking. 1.5 ml of the culture was poured into an eppendorf tube and centrifuged for 2 min. Supernatant was discarded and the cell pellet was suspended in 100 µl of GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). The tube was stored at room temperature for 5 min and then 200 µl of freshly prepared solution of 0.2 N NaOH, 1% SDS was added to it. Contents of the tube were mixed rapidly by inverting the tube two to three times and the tube was incubated on ice for 15 min. The suspension was neutralized by adding 150 µl of the ice cold solution of potassium acetate, pH 4.8 (which was prepared by mixing 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of H_2O) and further stored on ice for 10 min. The tube was centrifuged for 10 min in an Eppendorf centrifuge, the supernatant was transferred to a fresh tube, deproteinized by phenol/chloroform mix and precipitated with ethanol. The DNA was pelleted by spinning, the pellet was air-dried and dissolved in a suitable amount of TE. Before restriction digestion, the plasmid preparations were subjected to RNase A treatment.

Plasmid digestion

For all plasmid DNA digestions with restriction endonucleases, minimum 10 U of restriction enzyme per microgram of plasmid DNA were used in a rection volume of 20

µl and incubated overnight at 37°C to ensure excision of insert from the chimeric plasmids. The reaction was stopped by addition of EDTA to a final concentration of 10 mM.

Agarose gel electrophoresis

The restriction endonuclease digests of recombinant plasmids were analysed on 0.7% agarose gels.

Southern blotting

The transfer of DNA from agarose gel to Hybond-N membrane was carried out as described in Chapter IV.

Colony blotting

Hybond-N was placed carefully onto the agar surface. Both the membrane and agar were marked carefully so as to ensure correct orientation of the colonies. The membrane was removed after 1 min and placed on Whatman No. 3 soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 min with the colony side up. It was then kept on neutralizing solution pad (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M EDTA) for 3 min twice. The membrane was washed with 2XSSC for 1 min, allowed to dry at room temperature, wrapped in saran wrap and exposed to UV with colony side down for 7 min. The membrane was stored at room temperature between sheets of Whatman No. 3 paper till further use.

Labelling of DNA

The *Streptomyces* total genomic DNA was labelled with ($\alpha^{32}P$)^{dCTP} (specific activity 3000 Ci/mmol) by multiprime DNA labelling kit as described in Chapter IV.

Prehybridization, hybridization and washing of blots

The Southern as well as colony blots were prehybridized overnight at 65°C in a heat sealed plastic bag containing 5 X SSPE, 0.5% SDS, 5X Denhardt's solution (50 x Denhardt's solution: Ficoll, Polyvinyl pyrrolidone, BSA, 1% each) and 100 µg/ml denatured, sonicated calf thymus DNA in a total volume of 5 ml.

The purified labelled DNA probe was denatured and added to the prehybridization solution in a bag for hybridization. The blot was incubated at 65°C with shaking for another 24 h.

After hybridization, the blot was washed for removal of the unbound/non-hybridized probe using solutions of different stringencies. The blot was washed sequentially as follows: twice with 2 X SSPE, 0.1% SDS at 28°C for 10 min; once with 1 x SSPE, 0.1% SDS at 65°C for 15 min; once with 0.1 X SSPE, 0.1% SDS at 65°C for 10 min and finally with 0.1 X SSPE at 65°C for 10 min. It was allowed to air dry, wrapped in Saran wrap and subjected to autoradiography.

Synthesis of oligonucleotide probe

An eighteen-mer mixed oligonucleotide sequence was,

synthesized on an automatic synthesizer, Gene Assembler Plus, Pharmacia (Sweden) in collaboration with Dr. A.A. Natu, NCL, Pune. Synthetic oligonucleotides are single-stranded DNA fragments which are not isolated from natural sources but assembled from mononucleotide units by chemical synthesis according to a defined sequence. The sequence chosen for this purpose is a stretch of six amino acids well conserved in the protein sequence of GXIs from actinomycetes and related organisms (2,4,6,9,10). The amino acid sequence and corresponding DNA sequence are as follows:

Val-Thr-Phe-His-Asp-Asp

Codons: 5'....GTG-ACC-TTC-CAC-GAC-GAC....3'

C G

RESULTS AND DISCUSSION

Streptomyces sp. NCIM 2730 is one of the highest GXI yielding *Streptomyces* species. As a premonitory to cloning of GXI gene from this organism, the main objective of the present work has been to construct a genomic library of *Streptomyces* in a suitable host. A standard approach of shot-gun cloning was used for the construction of library.

Choice of host and vector

The selection of an appropriate host-vector system is important for successful cloning of a gene. Considering the high G+C content of *Streptomyces* genome and for efficient recognition of the promoters in the homologous system, it would have been ideal to choose *S. lividans* as a host. Since *S. lividans* produces GXI, it would be necessary to isolate a GXI negative mutant of *Streptomyces* for screening of the cloned GXI gene. In the present work, *E. coli* was used as a host for cloning in view of (i) the possibility of using a wide range of plasmid vectors (ii) availability of simple but highly efficient methods of transformation of *E. coli* cells, and (iii) the possibility of using the GXI negative *E. coli* mutants for screening of the gene by complementation. Such GI negative mutants are available with the *E. coli* Genetic Stock Center, Yale University, U.S.A. The problem of recognition of *Streptomyces* promoters by *E. coli* can be

overcome by selecting a vector containing strong promoters and by cloning the gene next to it so that the gene can be expressed under the control of a powerful promoter recognized by *E. coli*. Moreover, it has been shown that *Streptomyces* contains a few *E. coli* type A+T rich promoters capable of being recognized by *E. coli* RNA polymerase (22). Several *Streptomyces* genes [e.g. neomycin phosphotransferase gene from *S. fradie* (23), isopenicillin N synthetase gene from *S. lipmanii* (24), phosphinothricin N-acetyltransferase gene from *S. viridochromogenes* (25) and glutamine synthetase gene from *S. coelicolor* (26)] have been shown to be expressed in *E. coli* under the control of vector promoter.

pUC8 is a multicopy expression vector with multiple cloning sites and offers an efficient visual blue-white screening for differentiating between the non-recombinants and recombinants. Cloning of the DNA fragment in one of the cloning sites ensures its expression under the control of B-galactosidase promoter. In order to facilitate efficient expression of the cloned fragment pUC8 was chosen as a vector for cloning of the *Streptomyces* DNA.

Construction of genomic library

High molecular weight *Streptomyces* DNA ($\geq$ 23 kbp) was isolated using Kirby mixture (as described in Chapter IV) and

digested with Bam HI. To avoid the ligation of very low molecular weight DNA fragments with the vector, the DNA fragments in the range of 2 to 10 kbp were prepared. The standardization of conditions for partial digestion revealed that digestion for 1 h with 5.0 U of Bam HI μg^{-1} of DNA was sufficient to generate DNA fragments of the required range (Fig. V.1). For large scale preparation of partially digested *Streptomyces* DNA, 50 µg of the DNA was digested with 250 U of Bam HI at 37°C for 1 h. After terminating the enzyme reaction by the addition of EDTA, the DNA-digest was subjected to sucrose density gradient (10-40%) centrifugation for size fraction. The size of the DNA in each fraction was monitored by agarose gel electrophoresis (Fig. V.2). The fractions (6 to 11) showing 2 to 10 kbp DNA fragments were pooled, precipitated, dissolved in T_{10} buffer and used as the insert DNA. 15 µg of DNA was obtained in the range of 2-10 kbp.

The insert (size fractionated DNA) and the vector (Bam HI treated, dephosphorylated pUC8) (Fig. V.3) were ligated. *E. coli* JM 105 was transformed with the chimeric plasmid molecules and the cells were plated on LB plates containing Xgal, IPTG and ampicillin.

Transformation efficiencies of 10^5 to 10^6 and 10^3 to 10^4 μg^{-1} of vector DNA were obtained with pUC 8 (control

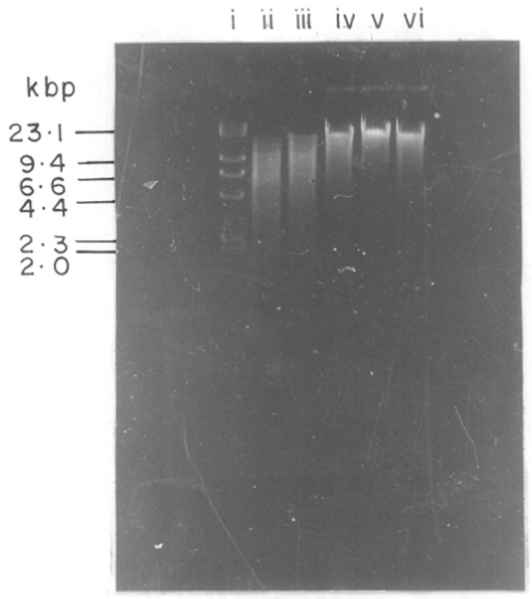

Fig.V.1 Partial digestion of Streptomyces DNA with Bam HI

λ Hind III digest (Lane i); Streptomyces DNA digested with decreasing units of Bam HI (Lane ii-vi).

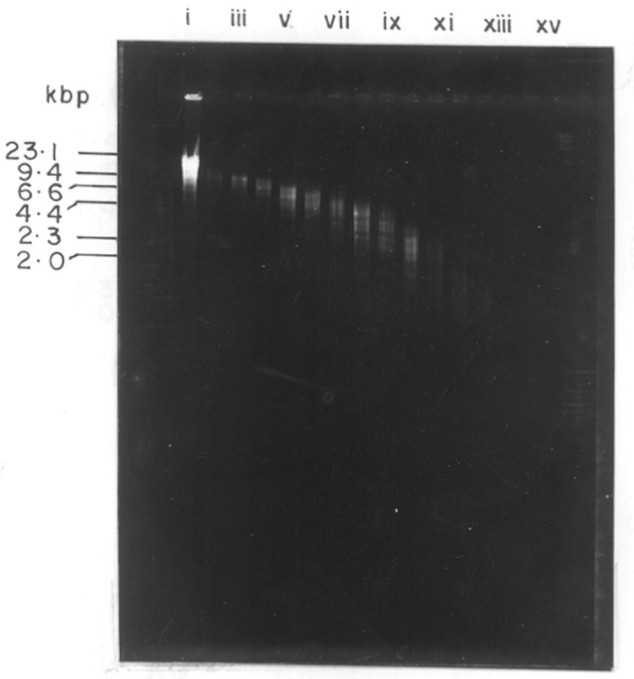

Fig.V.2 Size fractionation of partially digested Streptomyces DNA on sucrose density gradient

λ Hind III digest (Lane i); DNA from consecutive fractions of sucrose density gradient (Lane ii-xiv).

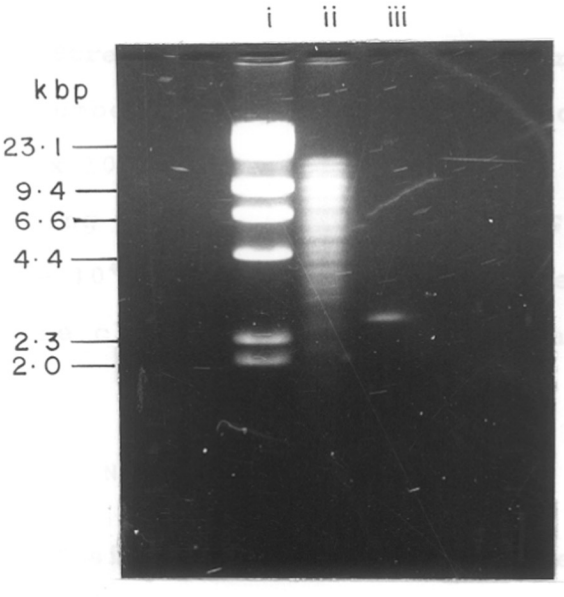

Fig.V.3 Insert and vector DNA before ligation

λ Hind III digest (Lane i); insert (size fractionated Streptomyces DNA) (Lane ii) and vector (Bam HI treated, dephosphorylated pUC8) (Lane iii).

experiment) and with the ligation mixture, respectively. The ratio of blue to white colonies (transformants:recombinants) was 2:1.

Representation of *Streptomyces* genome in the library

The total number of white colonies (recombinants) obtained was 1.5×10^3. For an average insert size of 4.0 kbp and considering the value reported for *Streptomyces* genome size to be 10^4 kbp (27), the percentage of library represented by the clones was calculated using following formula :

$$N = \frac{\ln (1-P)}{\ln (1-f)}$$

where, P is the desired probability, f is the fractional proportion of genome in a single recombinant, and N is the necessary number of recombinants.

Thus, the total number of clones represent 45% of the *Streptomyces* sp. NCIM 2730 genome in the library.

Analysis of the library

Colony hybridization

Hybridization of genomic DNA from *Streptomyces* sp. NCIM 2730 with the recombinants (white colonies), selected randomly, confirmed the presence of the inserts in the recombinants and that they originated from *Streptomyces* sp.

NCIM 2730 (Fig. V.4).

Southern hybridization

Plasmid DNA was isolated from ten different recombinants (as representatives of the library) and digested with Bam HI to excise the insert. The undigested plasmids showed retarded mobility due to the increase in size of the chimeric-plasmid compared to that of pUC8. The digested plasmids showed the presence of inserts in the molecular weight range of 2 to 6 kbp (Fig. V.5). On Southern blotting of plasmid digestion gel followed by hybridization with *Streptomyces* sp. NCIM 2730 DNA and autoradiography, only the insert bands gave positive signal, confirming the origin of inserts from *Streptomyces* (Fig. V.6).

Screening of the library for identification of GXI gene (*Xyl A*)

The nucleic acid hybridization method as well as methods based on expression of the cloned gene have been planned to be employed for identification of the GXI gene from the *Streptomyces* library.

Nucleic acid hybridization method

(i) Use of synthetic oligonucleotide probe:

One of the most widely used methods to identify clones within a library carrying specific fragments is by DNA-DNA hybridization with radio-active gene-specific DNA probes.

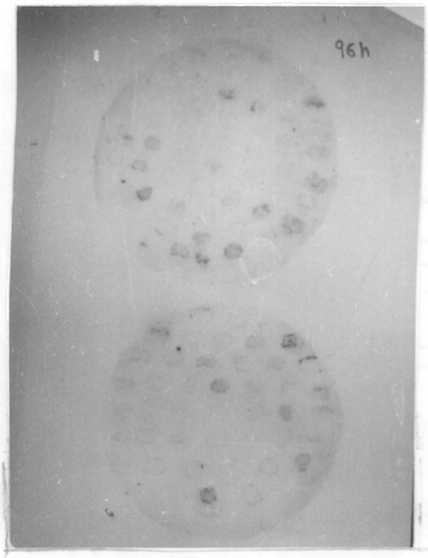

Fig.V.4 Colony hybridization of recombinants with Streptomyces DNA.

Recombinants picked up randomly from Streptomyces genomic library in E. coli JM 105 were challenged with α-^{32}P labelled Streptomyces DNA.

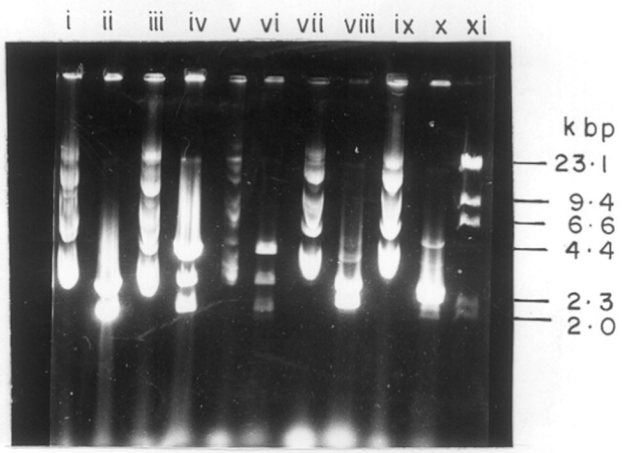

Fig.V.5 Isolation of plasmid from recombinants and its digestion with Bam HI

Plasmids, undigested (Lane i, iii, v, vii and ix) and digested with Bam HI (Lane ii, iv, vi, viii and x); λ Hind III digest (Lane xi).

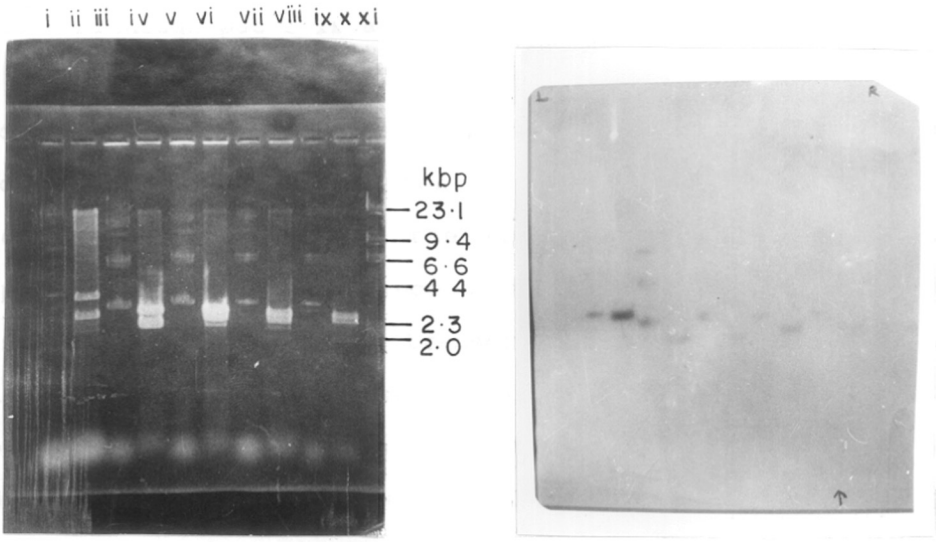

Fig.V.6 Hybridization of chimeric plasmids and their digests with α - ^{32}P labelled Streptomyces DNA

a. Plasmids, undigested (Lane i, iii, v, vii and ix) and digested with Bam HI (Lane ii, iv, vi, viii and x); λ Hind III digest (Lane xi)

b. Autoradiogram of Southern hybridization gel, Fig.V.6a.

The advantage of the hybridization method is that it is independent of expression of the foreign DNA by the *E. coli* host. Although pUC 8 is an expression vector, the expression of the gene may not be under the control of B-galactosidase promoter if, by chance, the fragment is not in frame with the promoter or if the orientation of the fragment is not right. Under such circumstances, the expression of the gene will depend solely on the presence of an indegenous promoter and its recognition by *E. coli* transcriptional and translational machinery. In such a case the presence of a DNA fragment containing the desired gene can be effectively identified by using a labelled oligonucleotide probe (28). An eighteen-mer mixed oligonucleotide sequence has been synthesized coding for a sequence of a stretch of six amino acids that are conserved in the protein sequence of GXIs from actinomycetes and related organisms. The screening of *Streptomyces* genome library in *E. coli*, using the oligonucleotide probe is in progress.

Methods based on expression of the gene

(i) Complementation of GXI negative *E. coli* mutants:

In majority of the reports on cloning of GXI gene from actinomycetes and related organisms into *E. coli*, the GXI gene was identified by complementation of GXI negative *E.*

coli mutants (2,5,10). In the present work the host used for cloning (*E. coli* JM 105) is not deficient in GXI. Therefore, direct isolation of GXI gene by complementation of GXI activity is not possible. To overcome this problem, a pooled plasmid preparation from recombinants will be used to transform the GXI negative *E. coli* mutants. Alternately, the ligation mixture which yields a good ratio of recombinants to transformants and a high efficiency of transformation will be used directly to transform the *E. coli* GXI negative mutants allowing direct selection of the GXI gene by complementation of xylose isomerase activity.

(ii) Immunoscreening:

Immunochemical method of detection can be successfully employed for identification of the desired clone provided the gene is expressed and the specific antibody is available. Polyclonal antibodies have been raised against the electrophoretically homogeneous GXI preparation from *Streptomyces sp.* NCIM 2730. The specificity and the sensitivity of the antibodies for the antigen (GXI) have been confirmed by ouchterlony double diffusion, rocket electrophoresis and by western blot ELISA (Chapter III). These antibodies can be used for screening of a clone from the library expressing the GXI activity even at a low level.

Scope of the future work

Streptomyces sp. NCIM 2730 has several novel features such as ability to produce high GXI activity, production of enzyme on a Co^{2+} deficient medium and on a medium containing hemicellulose-rich agricultural wastes. The GXI can be immobilized on an inexpensive support Indion 48-R and can be reused efficiently. These properties show that the organism has a potential for use in HFCS production. The results reported in this thesis provide basis for cloning and hyper-expression of the GXI gene in *E. coli* which will facilitate its application in biotechnology. Transfer of the gene (*xyl A*) to yeast will obviate a major hurdle in fermentation of xylose to ethanol by these high yielding, ethanol tolerating microorganisms. Manipulation of the GXI gene by site directed mutagenesis will lead to an altered protein with properties desirable for its industrial application in HFCS production.

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EVIDENCE FOR THE ESSENTIAL HISTIDINE RESIDUE AT THE ACTIVE SITE OF GLUCOSE/XYLOSE ISOMERASE FROM STREPTOMYCES*

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Modification of glucose/xylose isomerase from Streptomyces sp. NCIM 2730 by diethylpyrocarbonate (DEPC) or its photo-oxidation in presence of rose bengal or methylene blue caused rapid loss in its activity. The inactivation of the enzyme was accompanied by an increase in the absorbance at 240 nm and was reversed by hydroxylamine. Glucose and xylose but not Mg^{++} and Co^{++} afforded significant protection to the enzyme from inactivation by DEPC. Inactivation followed pseudo-first-order kinetics and modification of a single histidine residue per mole of enzyme was indicated.

D-glucose/D-xylose isomerase (GI) (EC 5.3.1.5) catalyses the interconversion of D-glucose and D-xylose to D-fructose and D-xylulose respectively. The enzyme is currently used in the industrial production of high fructose corn syrup from starch and in the conversion of xylose to ethanol. Recently, efforts have been made to identify the catalytic residues of the enzyme (1). Dyson and Noltmann (2) proposed general acid catalysis of ring-opening by a protonated lysine residue and proton transfer by a deprotonated histidine. In contrast, O'Connell and Rose (3) suggested that a glutamate residue mediates the proton transfer in the isomerization process.

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In the present paper, we report on the modification of a histidine residue of the GI from Streptomyces sp. NCIM 2730 by a histidine acylating reagent, DEPC and by its photo-oxidation in the presence of rose bengal or methylene blue. Our results support the involvement of a histidine residue for the catalytic activity of GI.

MATERIALS AND METHODS

DEPC and hydroxylamine hydrochloride were obtained from Sigma Chemical Company, St. Louis MO, USA. Glucose and xylose were from E. Merck, Darmstadt, Germany. Other chemicals were of the highestpurity commercially available.

Microorganisms : Streptomyces sp. NCIM 2730 was isolated in National Chemical Laboratory, Pune, by National Collection of Industrial Microorganisms.

Enzyme assay : GI was estimated by the colorimetric method of Takasaki and Tanabe (4). D-fructose or D-xylose produced in the reaction was determined by the method of Dische and Borenfreund (5) as modified by Marshall and Kooi (6). The enzyme was estimated at pH 7.5 at 70°C for 30 min. The unit of GI is defined as the amount of enzyme that produces 1 µmole of D-fructose or D-xylulose per min under the assay condition.

Protein assay : The protein concentration was measured according to Brandford (7).

Enzyme production and purification : The organism was grown for 96 h on a medium (8) containing 1% xylose as an inducer. The cells were sonicated and extract was heated at 65°C for 15 min. The enzyme was purified by ammonium sulphate precipitation and by preparative gel electrophoresis followed by DEAE cellulose chromatography. The purified enzyme showed a single band on gel electrophoresis (Unpublished results).

Carbethoxylation : The enzyme (400 µg) in 20 mM potassium phosphate buffer pH 7.0 was incubated with indicated concentrations of DEPC diluted in absolute ethanol at 30°C The ethanol at this concentration was found to have no effect on the activity and stability of the enzyme during incubation time. The extent of carethoxylation was monitored by recording the increase in absorbance at 240 nm of the reaction mixture and a control containing an equal amount of the untreated enzyme which was incubated under identical conditions with ethanol but without DEPC. Ten microliter aliquots were withdrawn from the experimental and control at different time intervals and the residual activity was determined.

Decarbethoxylation : The enzyme was treated with 2 mM DEPC for 10 min. The reactivation of the modified

histidine residues was achieved by incubation with 400 mM hydroxylamine hydrochloride at pH 7.0 at 4°C for 16 h.

Photo-oxidation : Photo-oxidation was carried out (9) at room temperature by exposing the enzyme solution in 20 mM phosphate buffer pH 7.0 containing rose bengal or methylene blue to 200 W flood-light bulb held at 10 cm from the sample. After particular time intervals the samples were estimated for the activity. The controls were similarly treated in the dark.

RESULTS AND DISCUSSION

The pH dependence of kinetic parameters for isomerization of glucose was studied in the pH range of 5.0 to 8.5. The plot of log $\left\{\frac{Vmax}{Km}\right\}$ vs pH results in a bell-shaped curve (Fig.1) and the apparent pKa values obtained were 6.8 and 8.4. The former value suggests that imidazole moiety of histidine is involved in catalytic function of the enzyme.

Treatment of glucose isomerase with DEPC led to inactivation and the extent of which was dependent both upon time and reagent concentration. Plot of residual activity versus time at all concentrations of the reagent was linear indicating that the inactivation follows first-order kinetics (Fig.2). A double logarithmic plot of the observed pseudo-first-order

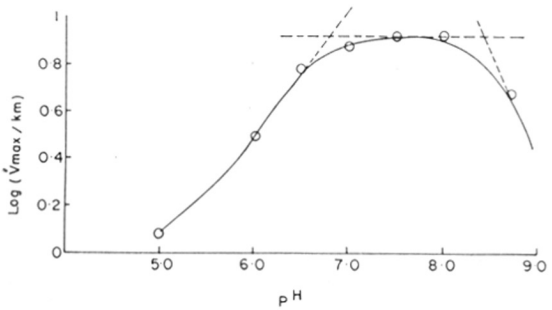

Figure 1. Plot of log $\left\{\frac{Vmax}{Km}\right\}$ vs pH. Km and Vmax at different pH were determined from Lineweaver-Burk plots.

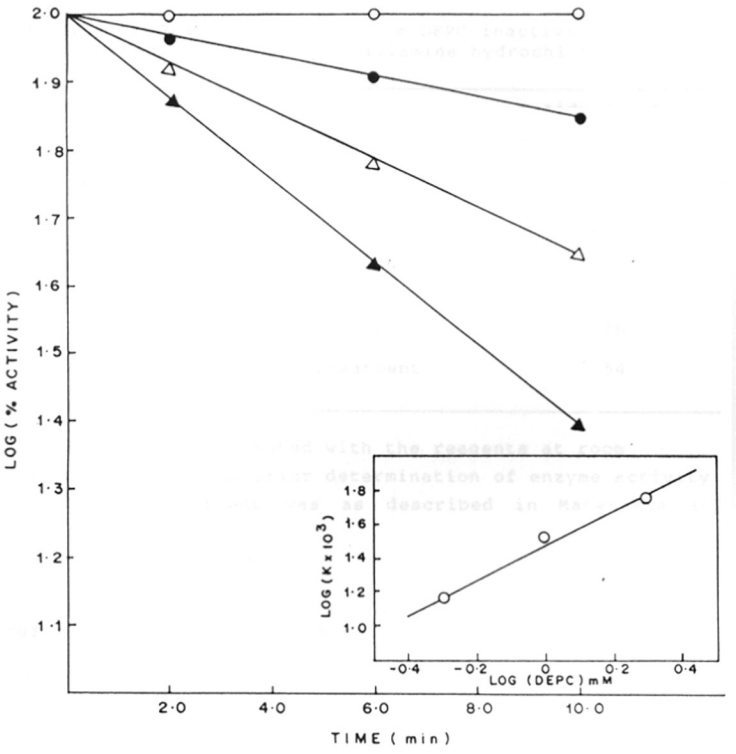

Figure 2. Kinetics of inactivation of GI by DEPC. The enzyme (400 ug) at pH 7.0 was incubated with DEPC 0 mM (o); 0.5 mM (●); 1 mM (△) and 2.0 mM (▲).
Inset - Apparent order of reaction with respect to reagent concentration. The observed pseudo-first-order rate constants calculated from Fig. 2 were plotted.

rate constants against reagent concentrations yielded a reaction order of 1.0 (Fig.2 inset) indicating that the modification of a single histidine residue results in the loss of enzyme activity (10).

The possibility of the inactivation of enzyme due to some non-specific interactions of DEPC with other amino

Table 1 : Protection of GI from DEPC inactivation and its reactivation by hydroxylamine hydrochloride

Additions	Residual activity (%)
None (control)	100
DEPC (2 mM)	26 ± 4
Glucose (100 mM) + DEPC	94
Xylose (5 mM) + DEPC	95
Mg^{++} (5 mM) + Co^{++} (1 mM) + DEPC	26
Hydroxylamine hydrochloride treatment	54

Enzyme (20 μg) was incubated with the reagents at room temperature for 10 min prior determination of enzyme activity. Hydroxylamine treatment was as described in Materials and Methods.

acid residues such as cysteinyl, arginyl or tyrosyl was also considered. GI was not inhibited by pCMB (5 mM) or iodoacetamide (1 mM) ruling out the possibility that the inactivation may be due to the modification of cysteine residues. The inactivation of enzyme due to modification of amino acids other than histidyl or tyrosyl residues cannot be reversed by hydroxylamine. Treatment of carbethoxylated GI with hydroxylamine resulted in 54% reactivation of the enzyme (Table 1) indicating that the inactivation of the enzyme is due to the modification of either histidyl or tyrosyl residues. The possibility of the modification of tyrosyl residues was ruled out by studying the absorbance of DEPC treated enzyme at 278 nm. The modification of the tyrosyl residues by DEPC leads to the formation of 2-O-carbethoxy-tyrosyl which causes a large decrease in the absorbance at 278 nm. No such decrease was observed in the DEPC inactivated enzyme indicating that tyrosyl residues of GI are not modified and the inhibition of enzyme activity

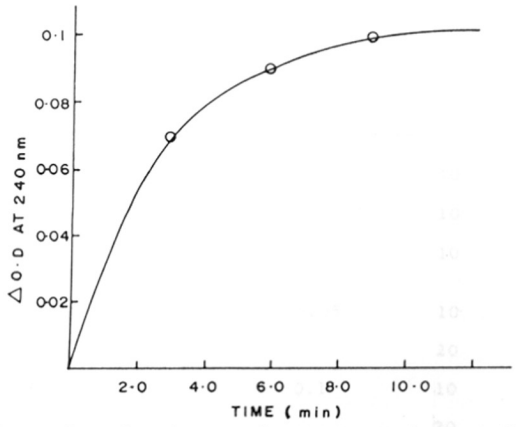

Figure 3. Absorbance of DEPC-treated GI at 240 nm.

was most likely due to specific modification of the histidyl residues.

The inactivation of GI by DEPC was accompanied by a significant increase in absorbance at 240 nm (Fig.3) characteristic for ethoxylcarboxylation of histidine residues which reinforced the evidence of histidine modification at the active site and eliminated the possibility of gross conformational changes.

GI was protected by its substrates glucose and xylose, against inactivation by DEPC (Table 1). However, Co^{++} and Mg^{++} fail to protect the enzyme from inactivation suggesting that the catalytic site is distinct from the cofactor binding site.

Photo-oxidation of the enzyme in the presence of methylene blue or rose bengal caused loss in activity which was completely prevented by shielding from irradiation (Table 2). The inactivation was directly proportional to the concentration of dye and was protected by glucose or xylose. With increase

Table 2 : Photo-oxidation of GI by photo-sensitizing dyes

Photo-oxidizing dye		Conc. (%)	Time (min)	Residual activity (%)
Methylene blue	(pH 7.0)	0.05	10	60
		0.1	10	58
	(pH 8.5)	0.1	10	40
Rose Bengal		0.05	10	76
			20	60
	(pH 7.0)	0.1	10	40
			20	25
		0.2	10	30

in pH the rate of inactivation by methylene blue was increased but that by rose bengal was decreased as observed by Tsai et al. (11).

CONCLUSIONS

pH dependence of kinetic parameters indicated likely involvement of imidazole group at the catalytic site of GI. However, results from such studies are not conclusive since the pKa value of an amino acid residue depends upon its microenvironment. In the present study, the confirmation of active site histidine residue was sought by involving histidine specific reactions. DEPC caused a strong inhibition of the enzyme activity. The inactivation is considered to be specificially due to the modification of the histidine and not of other amino acid residues with which DEPC is known to react because (a) the loss in enzyme activity was associated with an increase in absorbance of the enzyme at 240 nm indicating formation of carbethoxy-imidazole; (b) hydroxylamine treatment which decarboethyoxylates histidine and tyrosine residues only, reactivates the DEPC treated enzyme; (c) no increase in absorbance of the enzyme at 278 nm indicates that carbethoxytyrosine residues are not formed and (d) GI was hot inhibited by pCMB or iodoacetamide. Another confirmatory evidence for the involvement of histidine residue at the active site of GI comes from its inactivation by photooxidation in the presence of a dye. Protection of inactivation by substrates provides convincing evidence that a functional histidyl residue is ncessary for the catalytic activity of the enzyme and most likely is situated at or near the substrate binding.

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Immunoaffinity Purification of Glucose/Xylose Isomerase from *Streptomyces*

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ABSTRACT

A procedure was developed to purify glucose/xylose isomerase from cell extract of *Streptomyces sp.* NCIM 2730 using immunoaffinity chromatography. High-titer polyclonal antibodies were raised in rabbit using electrophoretically homogeneous glucose/xylose isomerase as an antigen. The specificity of antibodies was confirmed by double immunodiffusion, rocket electrophoresis, and Western-blot ELISA, which revealed the presence of a single immunoreactive protein with an M_r of 40,000. The antibodies recognized 2–3 antigenic determinants/mol of enzyme and were found to partially neutralize the enzymatic activity in an immunotitration experiment. The affinity gel was prepared by coupling antibodies at pH 10.0 to divinyl sulfone-activated Sepharose CL-4B. The glucose/xylose isomerase purified by immunoaffinity chromatography yielded 75% recovery with a single enzymatically active protein band on gel electrophoresis and showed specific activity of 16 U/mg. The crossreaction of the antibodies with glucose isomerase from other actinomycetes indicated that they share common epitopes.

Index Entries: Glucose/xylose isomerase; antibody specificity, immunoaffinity purification; *Streptomyces*.

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INTRODUCTION

D-Glucose/D-xylose isomerase (GI/XI) (E.C. 5.3.1.5) is generally an intracellular enzyme that catalyzes the reversible isomerization of D-glucose to D-fructose and D-xylose to D-xylulose. Both these reactions are important industrially, the former in the production of high fructose corn syrup and the latter in the production of ethanol from hemicelluloses. Despite its industrial importance, relatively little is known about the structural basis of its catalysis. Recently, based on the crystallographic data, the possibility of a 1,2-hydride shift mechanism in the isomerization of sugars by XI has been suggested (*1,2*). Involvement of a single histidine residue in the catalytic mechanism has been reported (*3,4*). Based on the kinetic analysis, we have reported that the enzyme has a single active site for the isomerization of both D-glucose and D-xylose (*5*). A limitation of more extensive investigations on the structure-function relationship of this system is imposed by the scarce quantities of the purified enzyme available with classical purification techniques.

In the present paper, we report a single step and easy-to-handle purification procedure for GI based on immunoaffinity chromatography. The enzyme purified by this method yielded 75% recovery with a specific activity (SA) of 16 U/mg, which is the same as that for the enzyme purified by conventional method of purification (*5*).

MATERIALS AND METHODS

Enzyme Production and Assay

The intracellular GI/XI from *Streptomyces* sp. NCIM 2730, *Chainia* and *Streptomyces lividans* TK24 were produced as described by Gaikwad et al. (*5*). GI/XI was estimated by colorimetric method of Takasaki and Tanabe (*6*). D-fructose/D-xylulose produced in the reaction was determined by the method of Dische and Borenfreund (*7*), as modified by Marshall and Kooi (*8*). The assay for GI was carried out at pH 7.5 at 70°C for 30 min, and that for XI at pH 7.5 at 35°C for 20 min. The unit of GI/XI activity is defined as the amount of enzyme that produces 1 μmol of D-fructose/D-xylulose under the assay conditions. The protein content of enzyme preparations was measured according to Bradford (*9*) and that of antibodies was measured by using the formula: IgG (mg/mL) = (λ 280– λ 310)/1.5 × dilution factor, where λ 280 and λ 310 represents the optical densities at respective wavelengths.

Antibody Preparation

Antibodies against electrophoretically pure GI/XI from *Streptomyces* sp. NCIM 2730 (*5*) were raised in a New Zealand white rabbit by injecting sc the enzyme (1 mg) emulsified with equal vol of complete Freund's adjuvant. Booster injections were given at fortnightly intervals for 10–12 wk. The rabbit was bled when the antibody titer reached 1:32.

Specificity of the Antibody

Oüchterlony double-diffusion was carried out in 1% agarose gel in PBS (phosphate buffered saline: 0.1*M* Na_2HPO_4, 0.4*M* NaH_2PO_4, 0.14*M* NaCl, pH 7.2) at 4°C for 24–48 h. Rocket electrophoresis was performed by incorporating the antibody into 1% agarose gel. Antigen was applied in a well, cut at one end and electrophoresis was carried out at 8 mA, 5–10 V/cm. Precipitin lines formed were visualized after drying the gels and staining with 0.25% Coomassie brilliant blue R-250. Western-blotting and the following immunodetection were performed in a modified version of the method of Towbin et al. (*10*). Proteins from SDS-PAGE (*11*) were electrophoretically transferred to nitrocellulose paper over a period of 2 h at 150 V in a buffer containing 20 m*M* Tris, 50 m*M* glycine, and 20% methanol at pH 7.8 at 4°C. The blot was treated overnight with 10% goat serum in PBS at 4°C and washed thoroughly with PBS containing 0.1% Tween-80. It was then treated with anti-GI antibodies diluted 250-fold with 1% BSA in PBS for 2 h at room temperature, followed by incubation with 1000-fold diluted goat antirabbit IgG horseradish peroxidase conjugate (Sigma Chemical Co., USA) in 10% goat serum, for 2 h. Bound peroxidase was detected with 0.5% 3,3-diaminobenzidine tetrahydrochloride in 0.5*M* sodium citrate buffer, pH 5, containing 0.03% H_2O_2.

Determination of Antigenic Valency

Varying amounts of GI ranging from 0 to 75 μg were added to a series of tubes containing 10 μL of antibody (22.4 mg/mL) to the final vol of 500 μL in PBS. The tubes were incubated at 37°C for 1 h, followed by an overnight incubation at 4°C. On centrifugation, the precipitate was washed twice with cold PBS and dissolved in 0.1N sodium hydroxide. The antigenic valency was determined according to Heidelberger and Kendall (*12*).

Calculation of Antigenic Valency

In an immunoprecipitation curve, at low antigen concentration, there is a relative antibody excess. Under such conditions, every antigenic determinant is likely to be covered by a separate antibody molecule and the

'antigenic valency' can be determined by calculating the molar ratio of antibody to antigen using following equation

$$\text{Antigenic Valency} = \frac{\text{Wt of antigen}}{\text{Mol wt of antigen}} \bullet \frac{\text{Wt of antibody}}{\text{Mol wt of antibody}}$$

The mol wt of antibody and antigen were assumed to be 1,50,000 and 1,60,000, respectively. The wt of antigen and antibody in the precipitate were calculated based on the extinction coefficients of 0.695 and 0.667 mg/mL, respectively.

Immunotitration

GI (20 μL, 10 U/mL) was added to antiserum (30–200 μL) in 0.05*M* sodium phosphate buffer, pH 7, in a total vol of 500 μL. After incubation at 37°C for 1 h, each reaction mixture was assayed for GI as well as XI activity.

Immunoaffinity Column Chromatography

Ten grams of Sepharose CL-4B (Pharmacia, Sweden) was activated with 2 mL of divinyl sulfone (DVS) (Aldrich Chemical Co., USA) for 80 min at room temperature. The activated resin was washed with 0.3*M* sodium carbonate, pH 10, to remove unreacted DVS. Two milliliters of antibody (15 mg/mL) dialyzed against 0.3*M* sodium carbonate, was added to the resin and was allowed to react for 6 h at room temperature. The excess of antibody was removed by washing successively with 0.3*M* sodium carbonate, pH 10, 0.3*M* sodium chloride, 0.3*M* glycine, and PBS.

The coupled resin equilibrated with 0.05*M* sodium phosphate buffer, pH 7, containing magnesium sulfate (1 m*M*) with cobalt chloride (0.5 m*M*) was packed in a column (10×2 cm). The crude enzyme preparation was allowed to adsorb on it for 1 h at 4°C. The column was washed with 0.05*M* sodium phosphate buffer, pH 7. The bound GI/XI was eluted with 0.3*M* glycine, pH 4, with a flow rate of 20 mL/h. Fractions (2 mL each) were collected and immediately neutralized with solid Tris to pH 7.2–7.4. The fractions were assayed for GI activity, dialyzed against 0.005*M* sodium phosphate buffer, pH 7.0, containing magnesium sulfate (1 m*M*) and cobalt chloride (0.5 m*M*), concentrated and checked for homogeneity on gel electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis in nondenaturing gels (7.5%) was carried out using Tris-glycine buffer, pH 8.3 (*13*). The *in situ* staining for GI activity in the gel was performed according to Yamanaka (*14*).

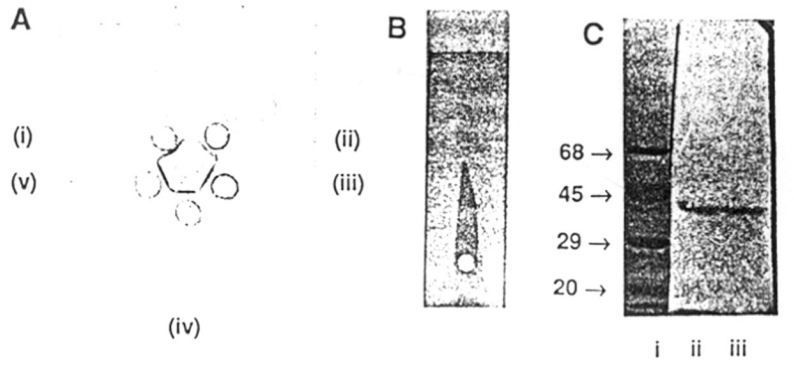

Fig. 1. Specificity of the antibody against GI/XI. A. Double immunodiffusion: The central well contained the antibodies. The peripheral wells contained (i) sonicated cell extract, (ii) heat treated, (iii) ammonium sulfate precipitated and dialysed, (iv) purified GI (10 μg), and (v) purified GI (20 μg). B. Rocket immunoelectrophoresis of crude GI/XI. C. Western-blot-ELISA of GI/XI. Lane i, mol wt markers, lanes ii and iii, immunoblots of crude and pure GI/XI.

RESULTS AND DISCUSSION

Specificity of the Antibody

The specificity of antibody toward GI/XI was determined using conventional immunological techniques. A single precipitin arc was obtained by Oüchterlony double-diffusion (Fig. 1A), as well as by Rocket electrophoresis (Fig. 1B), against both crude and purified GI/XI preparations. The sensitivity of Western-blot-ELISA technique is much higher than the gel-precipitin test, hence it was used to further confirm the specificity of the antibody. A single band at 40 K_d, corresponding to the M_r of the subunit of GI/XI, was immunoblotted from the crude and pure enzyme preparations (Fig. 1C).

Antigenic Valency of GI/XI

In a quantitative precipitin test, when the antigen concentration is low, there is a relative antibody excess wherein every antigen determinant is likely to be covered by a separate antibody molecule. Figure 2 shows the graph of antigen–antibody complex against the amount of GI/XI used. The calculations for the antigenic determinants were made in the antibody excess region (viz, at the antigen concentrations of 5, 15, and 20 μg) before the equivalence point was reached. The average value of molar ratios of antibody to antigen were found to be between 2 and 3, indicating that the antiserum recognizes 2–3 determinants/mol of enzyme.

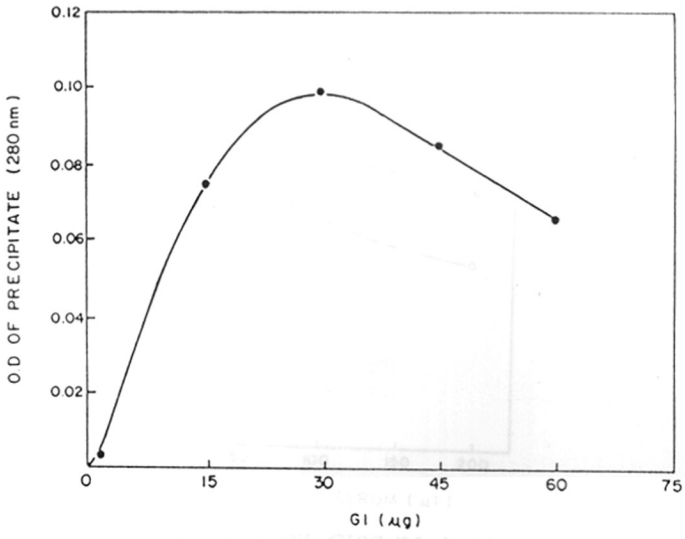

Fig. 2. Precipitation curve of GI/XI with anti GI/XI. Varying amounts of GI (0–75 µg) were added to 10 µL (22.4 mg/mL) of antibody. The precipitate was quantitated as described in Materials and Methods.

Immunotitration

The influence of the immunocomplex formation on the enzyme activity helps to monitor the resulting modification of the enzyme. Neutralization of GI activity with the antibodies resulted in nearly 30% loss in activity, whereas in case of XI the loss in activity continued to 50% (Fig. 3). Absence of complete inactivation of the enzyme after antibody reaction suggests that the antigen-binding site does not overlap the center of biological activity of GI. As the activity of the enzyme solely depends on the active site and the appropriate conformation of the protein, the partial loss in activity may be attributed to the change in conformation of the enzyme owing to the immunocomplex formation.

Purification of GI/XI by Immunoaffinity Chromatography

Since the antibodies recognized a single antigenic protein, viz, glucose/xylose isomerase, from the crude enzyme extract, they were used to purify the enzyme by coupling them to an insoluble matrix and passing the crude extract over it. Figure 4 shows the elution profile of bound enzyme. Seventy-five percent of the enzyme activity was recovered in fractions 4 and 5. When these two fractions were pooled and subjected to gel

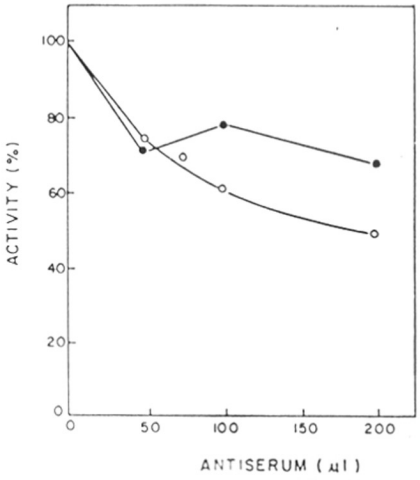

Fig. 3. Immunotitration of GI/XI. GI/XI (20 μL, 10 U/mL) was added to varying amounts (50–200 μL, 15 mg/mL) of antibody and the reaction mixture was estimated after 1 h at 37°C ● GI, ○ XI.

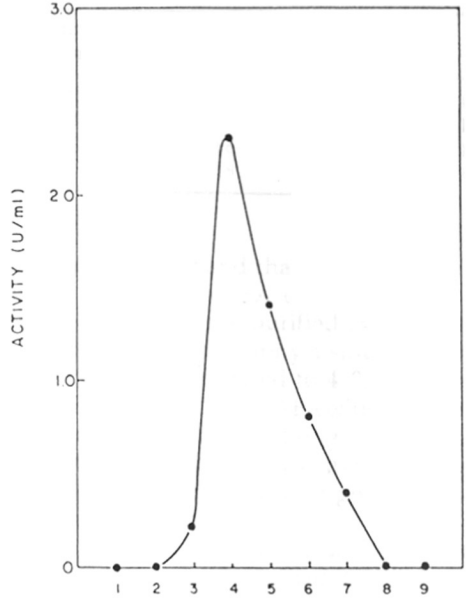

Fig. 4. Purification of GI/XI by immunoaffinity chromatography.

i ii

Fig. 5. Polyacrylamide gel electrophoresis of GI/XI purified by affinity chromatography stained for: i, protein and ii, activity.

Table 1
Purification of *Streptomyces* GI/XI

Method	Sample vol, mL	Total protein concn., mg	Total enzyme activity, U	SA	Recovery %
Crude extract	0.89	0.89	9.8	11.00	100
Affinity eluate	4.0	0.48	7.4	15.5	75.5
Crude extract	30	66	697	10.6	100
Gel[a] electroproduct	18	18	285	16.0	41

[a]Data from previous work (ref. 5).

electrophoresis, a single protein band that showed GI activity on *in situ* staining was observed (Fig. 5). The SA of the purified enzyme was 16 U/mg, which is similar to the enzyme purified by gel electrophoresis (5). As evident from Table 1, chromatography using immunoaffinity yields 75% of the purified enzyme as compared to 41% by gel electrophoresis, which requires an additional step of DEAE-cellulose chromatography to eliminate the acrylamide impurities. Although there was some loss in enzyme activity after immunotitration, the activity was regained after the dissociation of antigen-antibody complex by glycine, resulting in a reasonably good yield of purified enzyme.

Ours is the first report on the rapid, virtually instantaneous method for obtaining the large amount of purified GI/XI by immunoaffinity chromatography. The column after reequilibration with 0.05*M* sodium

Fig. 6. Crossreaction of the antibody with other GI/XI preparations. The central well contained the antibodies. The peripheral wells contained crude extracts of (i) *Streptomyces* sp. NCIM 2730, (ii) *Chainia* (extracellular GI and XI), (iii) *Chainia* (intracellular GI/XI), and (iv) *S. lividans* TK24.

phosphate buffer, pH 7.0, was subsequently used three times without any observable loss in efficiency.

Crossreactivity of the Antibody with Other GI/XI Preparations

The antibodies raised against electrophoretically purified GI/XI of *Streptomyces* sp. NCIM 2730 reacted with (i) extracellular GI and XI of *Chainia* sp., (ii) intracellular GI/XI *Chainia* sp., and (iii) intracellular GI/XI of *S. lividans* TK24 (Fig. 6), indicating the presence of common antigenic determinants among the GI/XI preparations of different actinomycetes tested. As such, the antibodies raised against GI/XI from *Streptomyces* sp. NCIM 2730 can be used for screening high glucose isomerase producers among actinomycetes.

CONCLUSION

Polyclonal antibodies raised in rabbit against electrophoretically homogeneous glucose/xylose isomerase were used for preparing immunoaffinity matrix for purification of the enzyme. The specificity of the antibodies was confirmed by various immunotechniques, and antibodies were found to recognize 2–3 antigenic determinants/mol of enzyme. Purification using immunoaffinity chromatography yielded 75% recovery of electrophoretically homogeneous GI/XI of SA 16 U/mg.

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