PURIFICATION AND PRELIMINARY CHARACTERISATION OF β-GLUCOSIDASE

FROM ALCALIGENES FAECALIS (ATCC 21400)

by

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To my Mother

S.J. Day

Abstract

A β-glucosidase was isolated from A. faecalis and purified 880
fold by a combination of classical and medium pressure chromatographic
techniques to a specific activity of 31.6 units/mg. The protein was homo-
geneous by the criteria of SDS-PAGE and gel chromatography.

The sub-unit molecular weight was determined to be 51,000 by SDS-
PAGE. The apparent oligomeric molecular weight was determined to be
75,000 by Superose gel chromatography and 98,000 by Waters I-250 gel
chromatography, suggesting that the enzyme is a dimer.

The enzyme was shown to be a retaining β-glucosidase with exo-
glucanase activity only.

The kinetic parameters of a number of substrates and inhibitors
were determined allowing deductions to be made about the nature of the
active site and catalytic mechanism. The Km's determined for cellobiose
and PNPG were low for a bacterial β-glucosidase, 0.70 mM and 0.083 mM
respectively. In the cellodextrin series, cellobiose to cellopentaose,
the enzyme was most efficient (as defined by Vm/Km) with cellotriose as
substrate.

In common with other cellulolytic β-glucosidases, the glycone site
showed a high specificity for glucose (although it would tolerate some
modifications) and poor specificity at the aglycone site. Catalytic acti-
vity was (unusually) observed with p-nitrophenyl-β-D-mannopyranoside as
substrate.

Activation energies were determined by means of Arrhenius plots.

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CHAPTER I

Introduction

I.1   General

β-Glucosidase (β-D-glucoside glucohydrolase, 3.2.1.21) is an enzyme which catalyses the cleavage of β-glucosidic bonds, Fig. 1.
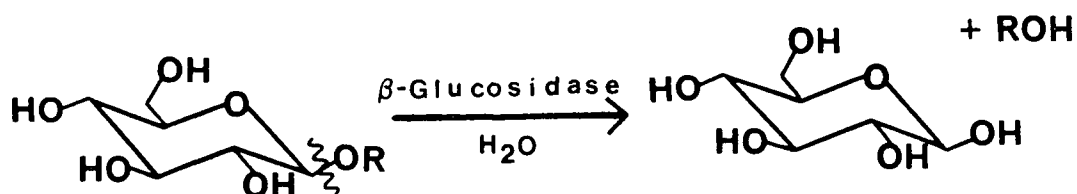
Figure 1:   Cleavage of Glucoside Bond.

The natural substrates for the enzyme have an aglycone (R) which may be either aryl or glycosyl.[1] β-Glucosidases are ubiquitous in nature and have been isolated from fungal, bacterial, yeast, plant and animal sources, including man.[1,2]

They are often found associated with, or as part of, the cellulase complex. This is an enzyme complex which catalyses the hydrolysis of cellulose, a β-1,4-linked polymer of glucose, to glucose units. It is known to be composed of at least three or four different classes of enzyme,[1] viz. endo-β-1,4-glucanase,exo-β-1,4-glucanase, cellobiohydrolase

(which is also an exo-glucanase) and β-glucosidase. Endo-β-1,4-glucanase catalyses the hydrolysis of the glucosidic bonds of cellulose at random internal points increasing the number of chain ends. Exo-β-1,4-glucanase and cellobiohydrolase catalyse the cleavage of oligosaccharide and cellobiose units, respectively, from the non-reducing ends of the polymer chains. β-Glucosidase catalyses the hydrolysis of cellobiose into glucose. Cellobiose inhibits both exo- and endo-gluconases and its hydrolysis by β-glucosidase, in addition to providing the last step in glucose production, is important to the efficiency of the cellulase complex.

Cellulose is a major component of industrial, municipal and agricultural waste. Recycling by degradation to glucose (and subsequent fermentation to ethanol) has been of increasing economic and environmental interest over the past one or two decades.

Endeavours to clone a cellulase complex from the bacterium Cellulomonas fimi into Escherichia coli have been embarked upon with a view to achieving expression of the complex at higher levels, thus producing a very efficient means of degrading cellulose. However, there have been problems with the cloning of the β-glucosidase component, and the co-cloning of a β-glucosidase from Agrobacter faecalis has been, and is still being, attempted.[3] Screening procedures used in this attempt would be facilitated by the use of pure protein and it was felt that a preliminary characterization of the purified enzyme, both physically and mechanistically would be useful. A discussion of some of the glycosidase mechanism literature follows.

I.2 Mechanism of Glycosidase Catalysis

In all of the glycosidases so far studied, bond cleavage has been shown (by ^{18}O labelling) to occur between the anomeric carbon and the glycosidic oxygen.[4]

Glycosidases may be divided into two categories, viz. those proceeding with retention of configuration at the anomeric centre and those proceeding with inversion, Fig. 2.
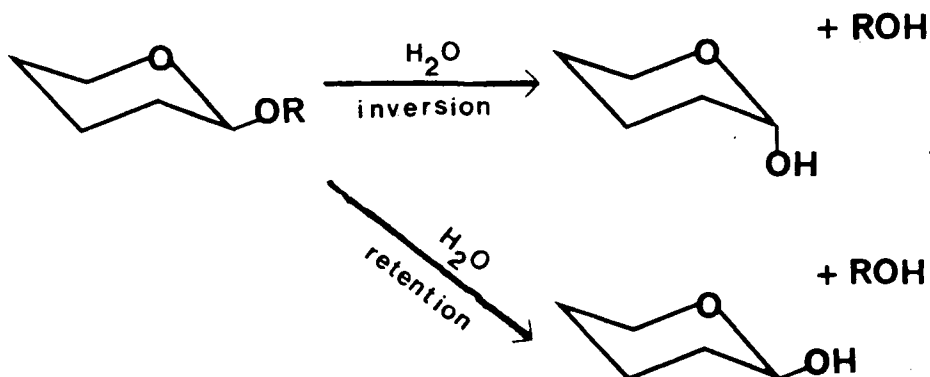
Figure 2: Retaining and Inverting Glucosidases.

Most mechanistic studies have been carried out on 'retaining' glycosidases and they are assumed to have common mechanistic features. This discussion will be limited to this class of enzymes.

Some of the processes that have been suggested[5] to be mechanistically important to catalysis by glycosidases are: a) intra-complex

general acid catalysis, b) intra-complex nucleophilic catalysis and c) electrostatic stabilisation of an oxocarbonium ion intermediate. These are illustrated below, Fig. 3.
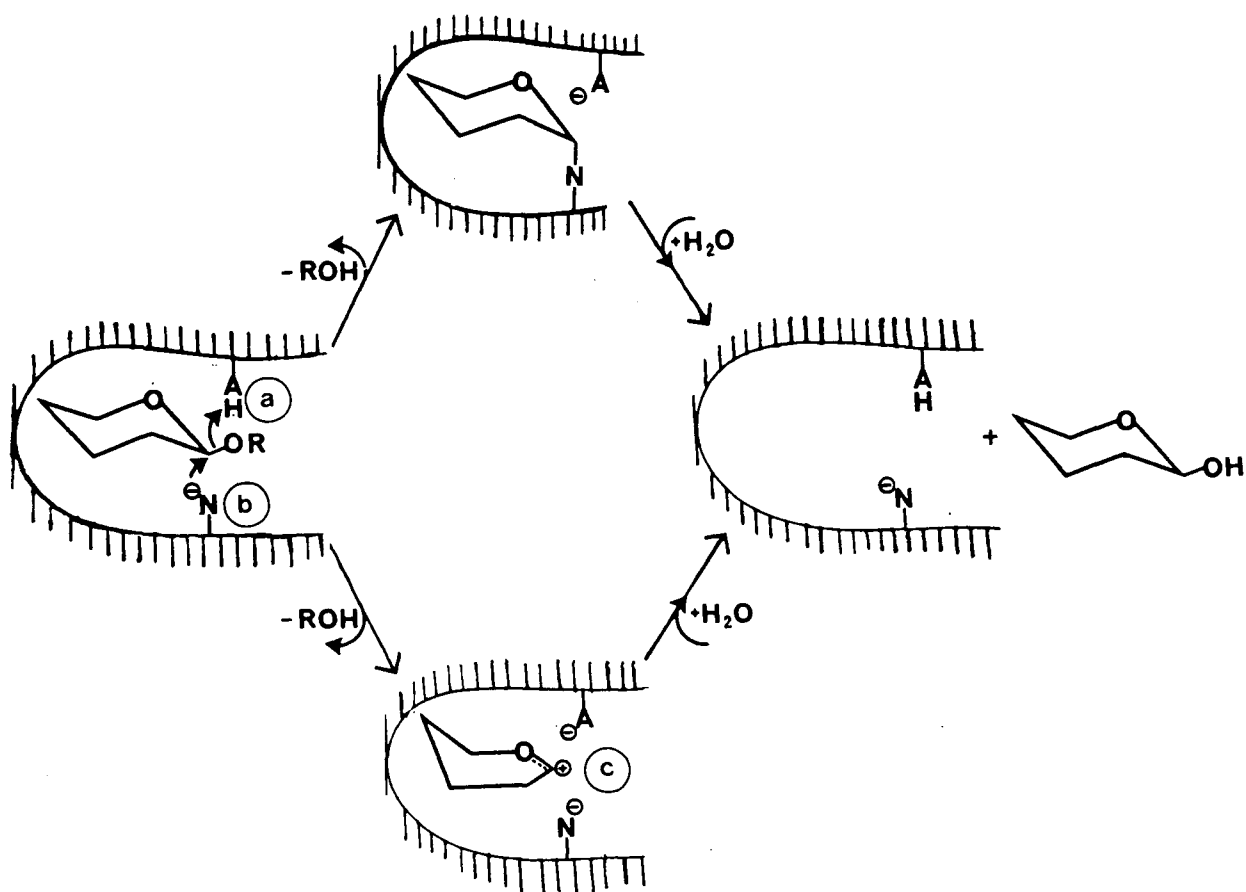
Figure 3: Proposed Glycosidase Mechanism.

Other factors that may be important are: d) conformational distortion of the substrate, i.e. some of the binding energy is used to distort or to

'pull' the ground state substrate towards a transition state conformation, and e) a microscopic medium effect in which any of the above might function more efficiently in the enzyme substrate complex than in an aqueous environment. The latter hypothesis, whilst being implied from the X-ray crystallographic data on the (e.g. hydrophobic) amino acid residues found at the active sites of a number of enzymes, is difficult if not impossible to prove or disprove unequivocally and will not be discussed further.

The only glycosidase that has been well characterised by means of <u>X-ray crystallography</u> to date is hen eggwhite lysozyme[6,7] and much of the evidence for the above postulated mechanisms has come from the data on this enzyme. The natural substrate for this enzyme is a β-1,4 linked co-polymer of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM), Fig. 4. Studies involving the building of substrate models into the active site of the enzyme (as determined crystallographically) suggest that it consists of six subsites (designated A-F) binding alternate NAG, NAM moieties. Cleavage takes place between the D and E subsites, with the D subsite binding a half-chair conformation of its sugar better than the preferred ground state conformation, a full-chair.[5,6] The amino acid residues thought to be important in catalysis are glutamic acid 35 and aspartate 52[5,6] since they are located in the active site on opposite sides of the glycosidic bond that is cleaved. This evidence implies that the substrate is bound at the active site and distorted toward the
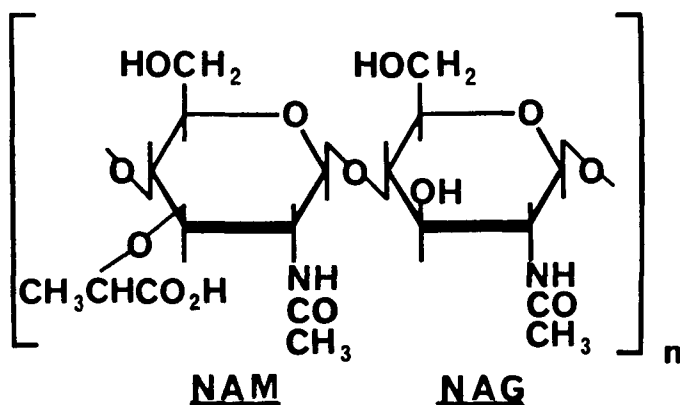
Figure 4: Substrate for Lysozyme.

conformation of an oxocarbonium ion like transition state. However it is

not clear whether the enzyme actually distorts the substrate ground state

or just 'pulls' it toward the transition state by binding the transition

state better than the undistorted substrate,[8] Fig. 5. The glycosidic bond

is then cleaved with general acid catalytic assistance from glutamic acid

35, accompanied either by direct nucleophilic participation, or less

directly, by oxocarbonium ion stabilisation by aspartate 52 as shown in

Fig. 3.

Crystallographic studies should, however, be interpreted with

caution. It had been suggested[6] from these studies that steric inter-

actions between the C_6OH and a group in the D subsite provide the main
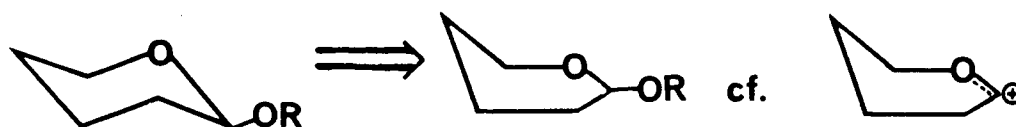
Figure 5: Oxocarbonium Ion Transition State.

source of distortion towards the half-chair sugar conformation in

lysozyme. However work by Capon[9] has shown that lysozyme is completely

inactive with substrate analogues having xylo, 6-chloro, 6-fluoro and

6-deoxy substitutions, suggesting a more subtle effect than simple steric

interactions.

The involvement of some kind of glycosyl-enzyme intermediate in

the glycosidase catalytic mechanism has been demonstrated in a number of

ways in different glycosidases.

Nucleophilic competition studies with β-galactosidase from E. coli

involving the use of ethanol and methanol as competing nucleophiles

(against water) have been carried out.[10] The product ratio measured

(alkyl-galactoside/galactose) was found to be independent of the aglycone

leaving group for a series of aryl galactosides. This implies a common

intermediate (E•G) in the solvolysis of these substrates, Fig. 6.

$$E + GOR \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot GOR \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E \cdot G \nearrow^{HOR'}_{k_{+3}} E + GOR'$$
$$\searrow^{H_2O}_{k_{+4}} E + GOH$$
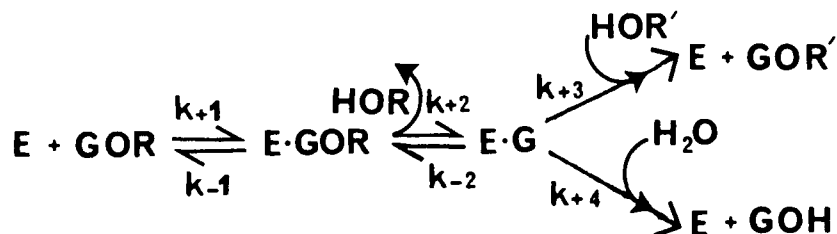
Figure 6: Minimal Kinetic Mechanism for Galactoside Hydrolysis.

Nucleophilic competition studies have also been used in the same system to show that the rate limiting step is degalactosylation (k+3,k+4) for 'fast' substrates[11] by measuring the increased rate of solvolysis observed in the presence of competing nucleophiles.

'Cryosolvents' (eg. 50% aqueous DMSO) at low temperature[12,13] and 'stopped flow' apparatus at $20°C$[14] have been used to investigate the hydrolysis of p-nitrophenyl-β-D-glucopyranoside catalysed by β-glucosidase from sweet almonds under conditions in which deglucosylation was expected to be rate limiting. An initial rapid release of p-nitrophenol (the first product) was observed, followed by a decrease in rate to the steady state rate of p-nitrophenol production. The initial 'burst' is assumed to be due to rate limiting cleavage of the glucosidic bond (k+2) equivalent to one enzyme turnover. The subsequent steady state rate is due to rate limiting deglucosylation (k+4). The activation energies, for both bond

cleavage and deglucosylation, calculated from low temperature pre-steady state kinetics, were consistent with those calculated from steady state kinetics at higher temperatures.[13]

Inhibitors have been utilised extensively to probe the catalytic mechanism of glycosidases.[15] There are two main classes of inhibitor of importance in mechanistic studies, viz. covalent (irreversible) and non-covalent competitive (reversible) inhibitors.

Within the former (covalent) class are two major types of inhibitor: the active site directed and the suicide inhibitors. While both resemble the natural substrate, the former are intrinsically reactive, whilst the latter remain unreactive until activated by the action of catalytic groups at the active site. Both classes, by virtue of their structural resemblance to the substrate, become covalently attached at the active site. Suicide inhibitors however, due to their requirement for catalytic activation, become attached at the active site almost exclusively. In order that a catalytically important glycosidase residue be labelled by a covalent inhibitor the reactive part of the molecule should be directly attached to the anomeric carbon.[16]

Epoxide derivatives have been used extensively.[15] Conduritol B cis- (Fig. 7) and trans-epoxides inhibit (β) and (α)-glucosidases respectively; incorporation of one mole of inhibitor per mole of enzyme active sites leading to complete inactivation. The inhibitor is released from the enzyme by the action of hydroxylamine and the stereochemistry of the released products is consistent with the proposed mechanism, Fig. 7.[15]

α-Glucosidases are inhibited 50-200 times more slowly (by the trans epoxide) than are β-glucosidases (by the cis epoxide) and this may be because the former do not go through a trans-diaxial ring opening in the proposed mechanism.[15]
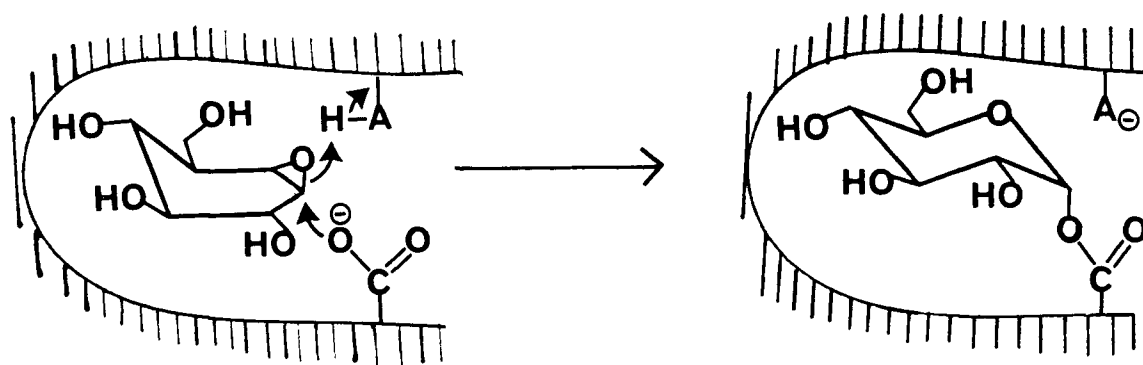
Figure 7:   Reaction of Conduritol B cis-Epoxide with β-Glucosidase.

Glucosidases that have been labelled with radioactive conduritol B epoxides have been subjected to proteolytic hydrolysis and the peptides containing radioactivity sequenced.[15] In all cases so far studied it is an aspartate residue that is labelled.[15] A shift in the epoxide function (eg. Fig. 8) diminishes, or even destroys inhibitory activity.[15] This suggests a fairly rigid orientation of catalytic groups at the active site.
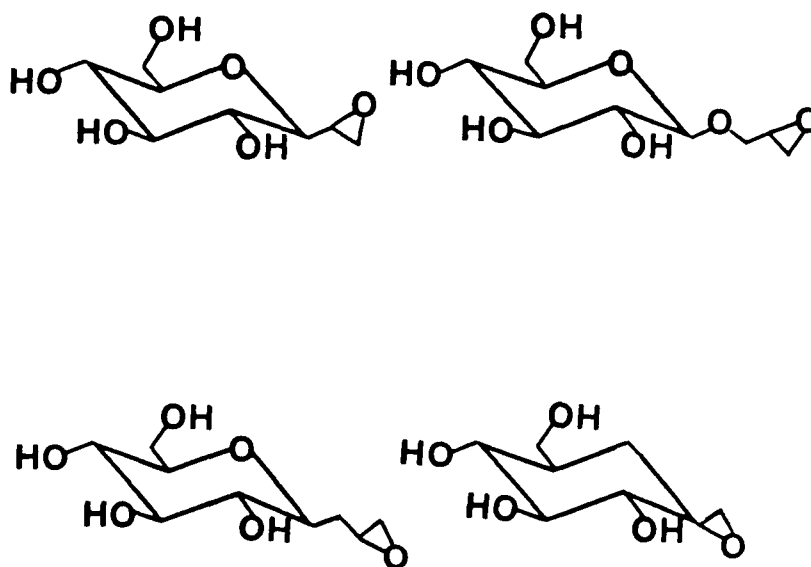
Figure 8: Epoxide Inhibitors of Glucosidases.

Isothiocyanate (-N=C=S), N-bromoacetyl (-NHCOCH$_2$Br) and triazene (-CH$_2$NHN=N-∅-NO$_2$) glycoside derivatives have also been used as covalent inhibitors although the results are less well defined.[15]

The use of competitive inhibitors to probe the mechanism of glycosidases is well illustrated by the work carried out on β-glucosidase from Aspergillus wentii by Legler et al.[17] The Ki* values determined for a

_____

* Ki is a dissociation constant for an inhibitor which is determined kinetically by quantifying its inhibition of the normal reaction. It should also be noted that Km is an apparent dissociation constant for the substrate and kcat is the maximal reaction rate at saturating substrate concentration. These terms and their derivation are discussed more fully in Appendix 1.

Table I[17]:   Inhibition Constants of some Cationic and Neutral

Inhibitors with β-Glucosidase.

Inhibitor	K_i (mM)	$-\Delta\Delta G°$ (kJ/mole)
β-glucose	2.8	-
β-2-amino-2-deoxyglucose	18	0
β-glucosylamine	0.0016	18.6
N-bromoacetyl-β-glucosylamine	0.25	6.6
D-glucono-δ-lactone	0.0096	14.6
5-amino-5-deoxy-D-gluconolactam	0.036	11.2
β-glucosylbenzene	96	-
N-β-glucosylpyridinium ion	0.30	14
N-β-glucosylpiperidine	0.091	17
N-β-glucosylimidazole	5.9	7

series of cationic and neutral inhibitors are shown (Table I).

D-Glucono-δ-lactone has an additional binding energy, compared to

glucose, of 14.6kJ mole^{-1}. This increase in binding energy was attributed

to this compound acting as a 'transition-state' analogue. Its preferred

ground state conformation, a half-chair, is analogous to that expected for

an oxocarbonium ion like transition state. Another explanation of this

tight binding is a reversible reaction with a catalytic group in the

active site of the enzyme.[18] Both of these possibilities are illustrated

below, Fig. 9.
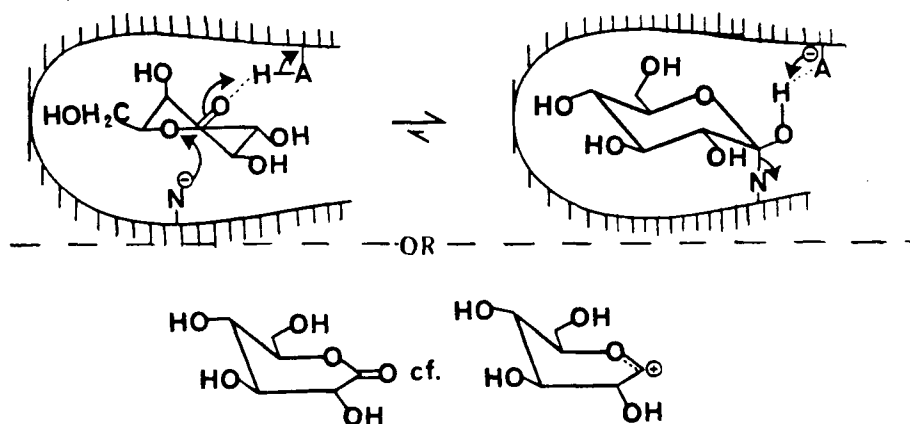
Figure 9: Inhibition of β-Glucosidase by D-Glucono-δ-lactone.

The tight binding of the intrinsically less reactive 5-amino-5-

deoxy-D-gluconolactam favours the former explanation. However

β-glucosidase from sweet almonds, whilst binding the lactone tightly,

binds the lactam only slightly better than β-D-glucose, and in this case

the latter explanation is favoured.[18]

Inhibitors bearing a positive charge on the exocyclic atom

attached to the anomeric carbon were found to bind tighter than analogous

neutral inhibitors. This is illustrated by the Ki ratios shown below,

Fig. 10. Support for the hypothesis that this increase in binding energy
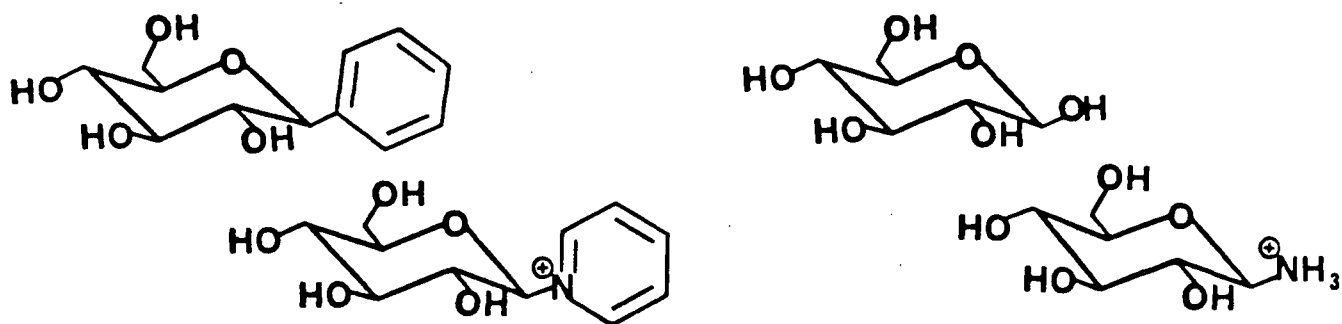
β-D-glucosyl benzene: β-D-glucosyl

pyridinium ion

β-D-glucose: β-D-glucosylammonium ion

$$K_i^0/K_i^+ = 320$$

$$K_i^0/K_i^+ = 4000$$

Figure 10: Ki Ratios of Some Isosteric Cationic and Neutral

Inhibitors.

is due to the influence of a single point negative charge (rather than the

contribution of a number of charges) is found in the increase in binding

energy ($\Delta\Delta G°$, Table I) observed for the β-D-glucosylimidazolium ion. The

increase observed is that anticipated for a half charge on the nitrogen

attached to the anomeric centre with no contribution from the charge on

N-3. Furthermore, the 2-amino-2-deoxyglucosyl cation binds no more

tightly than glucose, implying that the charge is located in the direct

vicinity of the anomeric carbon. From these, and other data, the
following transition state was suggested, Fig. 11.
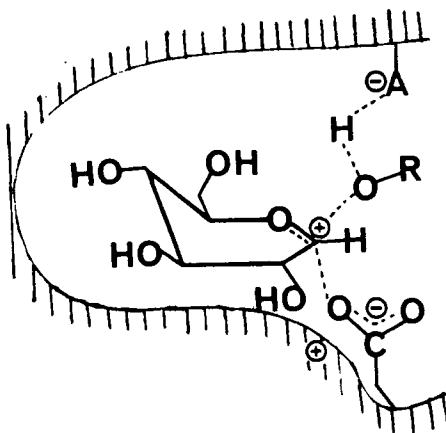
Figure 11: Proposed β-Glucosidase Transition State.

Summarising the implications of the evidence discussed so far, it
seems that for retaining glycosidases an enzyme intermediate is involved,
most likely having some degree of oxocarbonium ion character. Carboxylate
bearing groups are probably intimately involved with catalytic activity
and the catalytic groups are fairly rigidly placed with respect to the
bond to be cleaved. In short then, this evidence is consistent with the
mechanism proposed in Fig. 3.

Two unusual classes of substrate hydrated by some glycosidases are glycals[19,20] and heptenitols[21], Fig. 12.
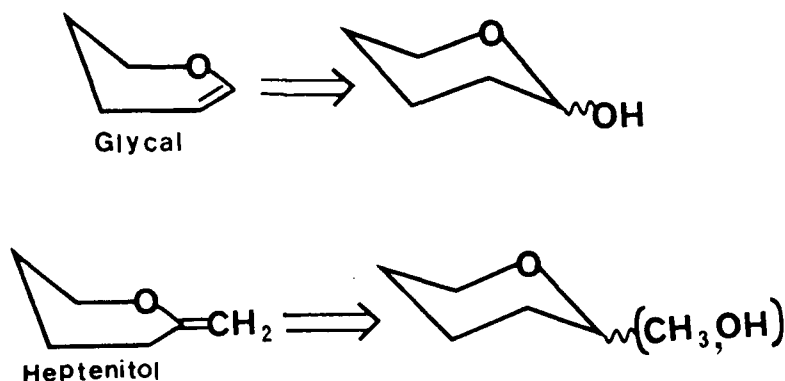
Figure 12: Unusual Glycosidase Substrates.

Glycals, by virtue of their sp^2 hybridization at the anomeric center, and therefore half-chair conformation, were originally thought to be 'transition state analogues'[15]. However the tight binding and time dependent (on the order of minutes) inhibition sometimes observed have been attributed to enzymatically catalysed hydration in which both enzyme glycosylation and deglycosylation are sometimes slow[19,20]. Denaturation and subsequent proteolytic digestion of β-glucosidase from A. wentii which had been incubated with radio labelled D-glucal yielded a radio-labelled peptide[20]. Amino acid analysis showed that the glucosyl moiety was attached to the same aspartate residue as had been labelled by conduritol

B epoxide. When the reaction was performed in D_2O, the stereochemistry of the products could be determined and a mechanism proposed, Fig. 13.[15]
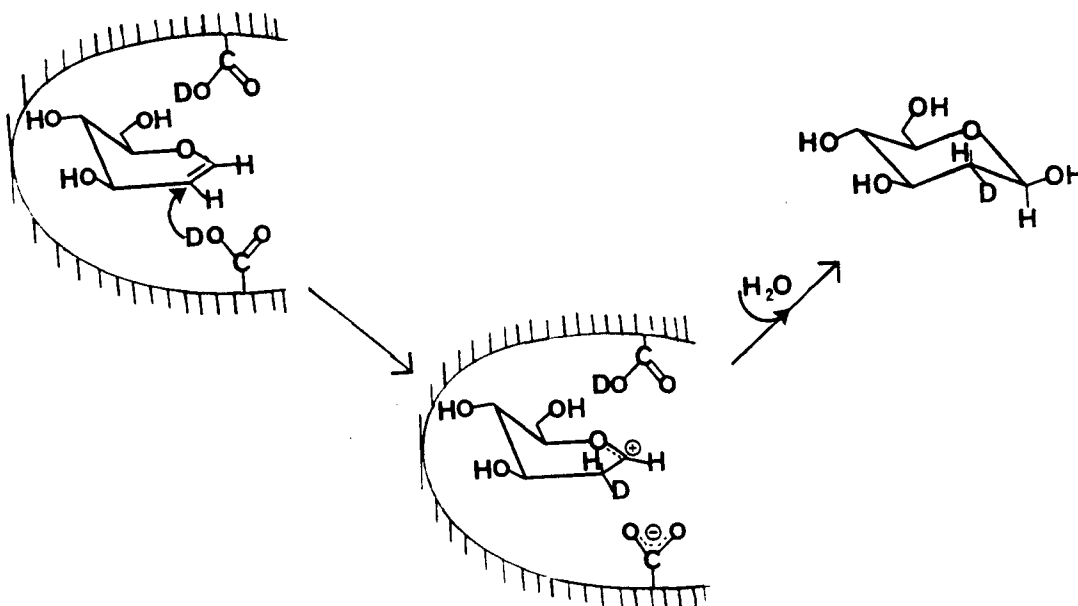
Figure 13: Hydration of Glucal by β-Glucosidase.

The less reactive 5-thio-D-glucal is not hydrated and binds only weakly.[22] Glycals are therefore not transition state analogues.

2,6-Anhydro-1-deoxy-D-gluco heptenitol is hydrated to 1-deoxy-D-gluco heptulose, Fig. 12, by both α- and β-glucosidases with 'retention'.[15,21] The reaction is proposed to go through the analogous catalytic pathway (to the normal reaction).[15,21]

Deoxy analogues of glucosyl substrates have been used to probe the mechanism of β-glucosidases.[23,24,25] Such substitutions, in addition

to altering Km, often lead to a decrease in kcat. The most marked

decrease in kcat. (up to 10^6 times) occurs when a 2-deoxy substitution

is made.[23] These effects have been variously attributed to: poor

substrate deformation on binding (2-deoxy),[23] lack of 'induced

fit'*[23,24], or incorrect alignment of catalytic groups due to increased

rotational freedom of the glucosyl moiety.[24,25].

The <u>variation of kcat.</u> (reflecting the rate determining step) and

<u>kcat./Km</u> (reflecting the first irreversible step, see appendix I) <u>with pH</u>

is often measured. For glycosidases the plot is often 'bell' shaped, eg.

Fig. 14[29], and this is taken to imply that one protonated and one

deprotonated residue are necessary for catalysis. Attempts are often
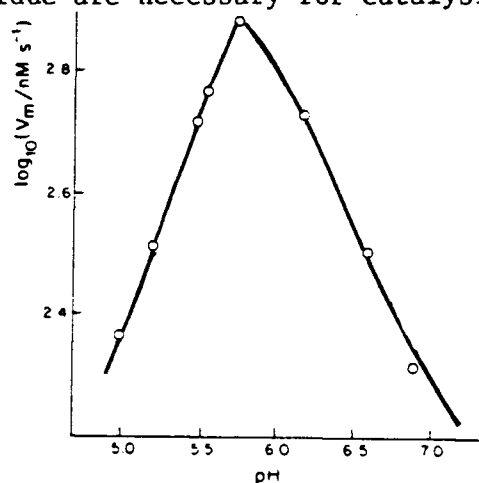
Figure 14[29]: Plot of Log_{10}(kcat/Km) vs. pH for β-Glucosidase.

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*   The hypothesis of 'induced fit' of a substrate was proposed by
Koshland[26] to explain enzyme specificity. The binding of the correct
substrate is proposed to induce a conformational change in the enzyme
which brings amino acid residues at the active site into the correct
alignment for catalytic activity. Evidence for this hypothesis was
first found for α-amylase.[27] This hypothesis has been disputed[28]

made to extract the pKa's of these residues[eg.29] from the points of

inflection of the plots. However Knowles, in an authoritative review of

the subject[30], has pointed out the folly of any simplistic interpretation

of pH profiles without corroborative data from other sources. Some work

which manages to avoid most of these pitfalls is that of Legler et al.[31]

All of the above mentioned work is consistent with the mechanism

proposed, Fig. 3.[31] However little has been said about the nature of the

enzyme intermediate and its conversion to products. Clearly, simple S_N1

heterolysis producing an isolated oxocarbonium ion is unlikely since the

predicted lifetime of such an intermediate is of the order of 10^{-11} –

10^{-15} seconds.[32] Kinetic studies have been carried out on β–galactosidase

from E. coli[18,33-37] with both galactosyl pyridinium salts (which cannot

undergo general acid catalysed hydrolysis) and aryl galactosides as

substrates. These studies involved correlation of kcat. with pKa of the

aglycone leaving group and determination of both α–deuterium and ^{18}O

kinetic isotope effects (reflecting bond order and bond scission in the

transition state). These studies gave rise to the following proposed

mechanism, Fig. 15. The existence of the ion-pair equilibrium represented

by K_4 has been well reviewed.[40]
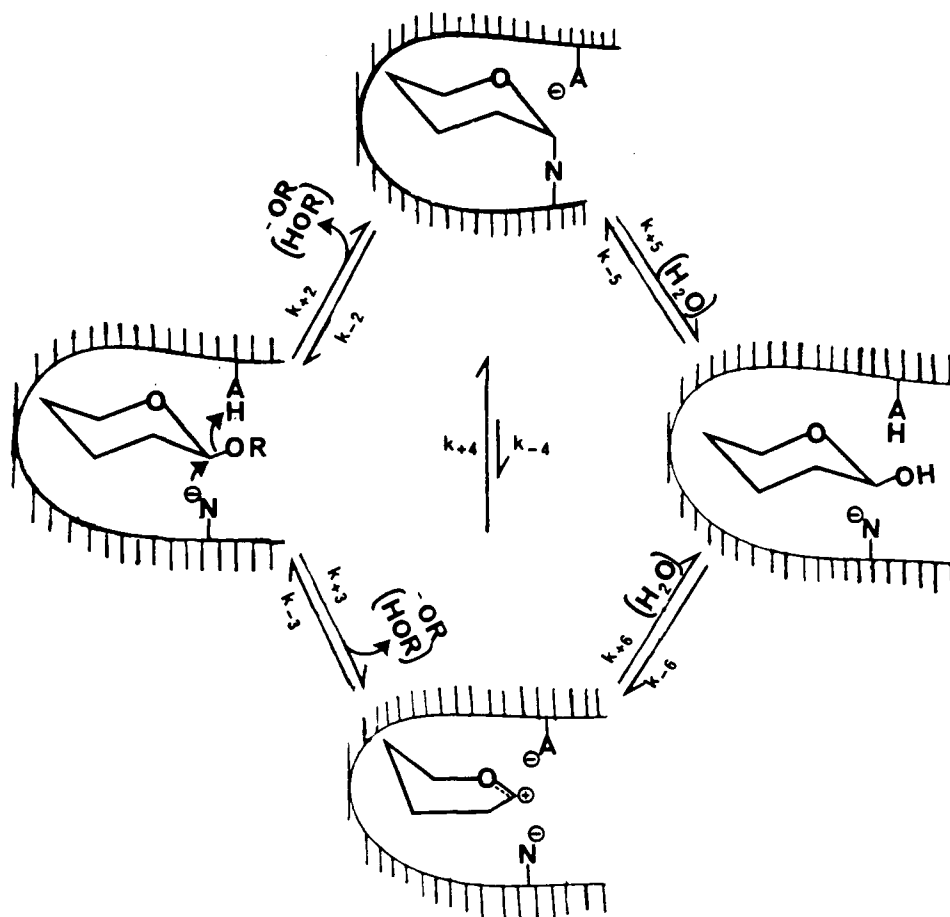
Figure 15: Proposed Catalytic Mechanism of β-Galactosidase from
E. coli.

The results of the ^{18}O KIE studies[39] and the pKa/kcat. studies[18] suggest different rate limiting steps. It is possible that this apparent contradiction may be resolved in the light of recent work[38] on the

specificity of the aglycone binding site. This suggests that in some

cases release of aglycone may be rate limiting.[40]

It is gratifying to find that some naturally occurring glycosidase

inhibitors isolated from bacterial sources,[15] Fig. 16, have structural

characteristics that might be expected in light of the proposed

glycosidase mechanisms.
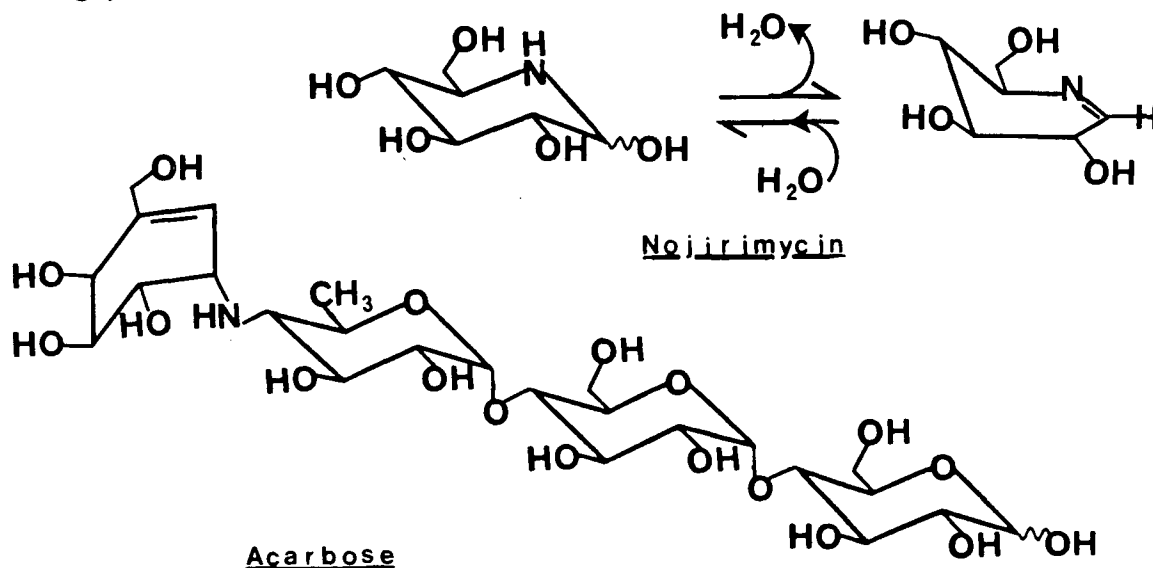
Figure 16: Nojirimycin and Acarbose.

Acarbose binds to α-glucosidases up to 10^5 times more tightly than

do the natural substrates.[15] The tight binding observed has been

attributed to both the conformation of the hydroxymethylcyclitol ring and

to interactions between the enzyme catalytic groups and the nitrogen

atom.[61] The tight binding of nojirimycin could be due to interactions

between the enzyme catalytic groups and the basic, endocyclic nitrogen

and/or by dehydration to a transition state analogue as shown, Fig.

16.[15]

Finally, it is of interest to note that the conformational changes suggested to occur within the enzyme substrate complex during catalysis are not consistent with the 'anti-periplanar lone pair' hypothesis.[42] Recent studies by Sinnott and Hosie[43] involving the use of β-deuterium kinetic isotope effects, which are geometry dependent, to probe conformational changes during catalysis by α-glucosidase from yeast, have cast further doubt upon this hypothesis.

I.3   Enzyme Isolation

Before an enzyme may fruitfully be studied mechanistically it is
necessary to purify it to homogeneity to ensure that the effects observed
are due to the enzyme under investigation, and not to contaminating
proteins.

Enzymes are biological molecules and consequently problems are
encountered with their 'denaturation' or breakdown if the conditions
encountered deviate too far from the physiological during their isolation,
and the methods employed reflect this constraint.

Enzymes are usually found either 'dissolved' in the cytoplasmic
fluid within the cell or bound to a membrane. The problems associated
with the latter are beyond the scope of this discussion.

Once the cytoplasmic fluid has been liberated from the cells by
the use of such techniques as homogenization, hydrolytic enzymes (eg.
lysozyme), freeze/thaw, ultrasonic disruption, osmotic shock, grinding
with glass beads or pressure differential (eg. French pressure cell), the
cell debris is removed by centrifugation. All procedures subsequent to
this are carried out in a buffer at a pH at which the enzyme is known to
be stable and at between 0-4°C to minimise proteolytic activity. Protease
inhibitors, such as diisopropyl phosphofluoridate, are often added in
order to inactivate proteolytic enzymes. The component of interest is
then isolated by a combination of techiques based on differences in

solubility, stability, charge, size/shape, affinity for a ligand or some
other physical property.

Nucleic acids are often removed from the cellfree extract by
precipitation with streptomycin sulphate. Following this, differential
precipitation is almost invariably carried out, usually with ammonium
sulphate as precipitant.

A plot of Log. (solubility) vs. ionic strength for haemoglobin,
Fig. 17,[44] shows an initial increase in solubility due to charge dispersal
by the salt ions. The subsequent decrease in solubility has been less
well quantified theoretically, but is thought to be due to a decrease in
available water as water becomes increasingly associated with the
hydration spheres of the salt ions.[45]
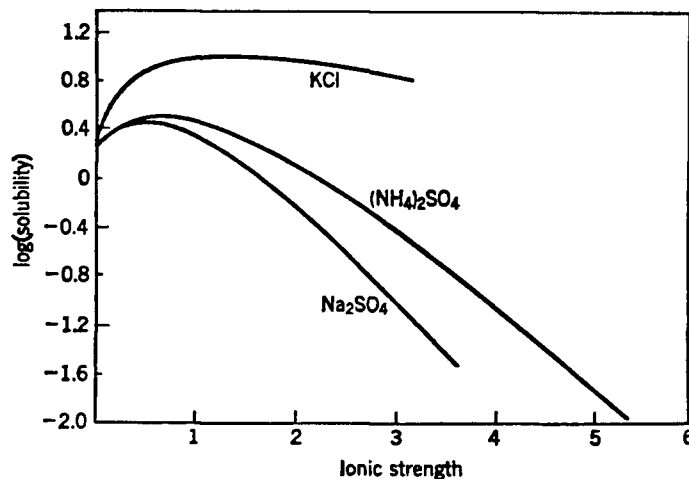
Figure 17[44]: Plot of Log (solubility) vs. Ionic Strength for
Haemoglobin.

Typically, ammonium sulphate would be added until the enzyme of interest was at the point of incipient precipitation; the contaminating proteins (and polysaccharide) precipitated would then be removed by centrifugation. More ammonium sulphate would be added until the protein of interest was just precipitated and the supernatant, containing contaminating protein, discarded. Ammonium sulphate is the salt of choice because of its relative inexpense, high solubility and lack of adverse effects on most proteins.

Proteins have also been fractionally precipitated by addition of polyethylene glycol and ethanol, and by adjustment of pH towards the isoelectric point (the pH at which a protein carries no net charge) of the protein of interest.[47] The latter method is of less use as many enzymes are denatured at their isoelectric points.[44]

The protein pellet obtained from the above procedure would be dissolved in a minimum volume of an appropriate buffer (possibly effecting a concentration at this stage) and the ionic strength lowered by either dialysis or gel filtration. If the enzyme were unusually stable to extremes of either heat or pH, an extra purification step, involving precipitation of contaminating proteins by heat or pH treatment, might be inserted at this point.[44,46]

Gel filtration may be used to effect a further purification, in addition to removing salt ions.[47,48] Gel filtration, or gel exclusion chromatography, is a chromatographic technique in which separation on the basis of size and shape takes place. The packing material is a solid support matrix, based on either a cross-linked polysaccharide (eg. agarose

or cellulose) or cross-linked polyacrylamide, which contains pores of such a size as to exclude larger molecules, while freely admitting smaller ones. Thus, on passing through a column of this material, larger molecules will have less solvent available to them than smaller molecules. A fractionation based on size (and shape) will take place with the larger molecules being eluted first.

The fractions from the above step containing enzyme activity might then be applied to an ion-exchange column. Ion-exchange chromatography effects a separation on the basis of charge.[47,49] Charged groups are covalently attached to a solid support matrix (of a similar type to that used for gel filtration) and the counter ions are freely exchangeable with those in solution. The functional groups commonly used are shown below, Table II.[49] The decision to bind a protein to a cation exchanger (pH below its isoelectric point) or an anion exchanger (pH above its isoelectric point) depends largely on its pH stability range.

Once the impure mixture has been bound to the top of the ion exchange column, unbound proteins are eluted with one or two bed volumes of start buffer. The column is normally developed with a gradient of increasing ionic strength. As the salt ions compete for binding sites on the ion exchange matrix, proteins are sequentially desorbed according to the strength of their affinity for the ion exchange resin.

Table II[49]: Functional Groups used in Ion-Exchange Materials.

Anion Exchangers	Functional Group
Aminoethyl (AE-)	$-OCH_2CH_2NH_3^+$
Diethylaminoethyl (DEAE-)	$-OCH_2CH_2^+NH(CH_2CH_3)_2$
Quaternary aminoethyl (QAE)	$-OCH_2CH_2^+N(C_2H_5)_2CH_2CH(OH)CH_3$
Cations Exchangers	
Carboxymethyl (M-)	$-OCH_2COO^-$
Phospho	$-PO_4H_2^-$
Sulphopropyl (SP-)	$-CH_2CH_2CH_2SO_3^-$

The above hypothetical purification scheme is representative of some of the more traditional techniques commonly used in protein isolation. Typical purification and yields obtained at each step may be seen in Chapter II.

Other techniques used successfully in protein purification are: affinity techniques (discussed later), hydroxyapatite chromatography (based on Ca^{2+} and non-specific interactions)[50], hydrophobic chromatography[51] and chromatofocussing[52]. The latter technique involves the setting up of a pH gradient across an ion exchange column. Proteins applied to the column migrate, and are focussed, according to their

isoelectric points. They are eluted at the pH of these points. Although in principle a very powerful technique, in practice it is found that many enzymes are denatured at their isoelectric points[44] lessening practical utility.

The affinity techniques commonly used are affinity adsorption and affinity elution chromatography. In the former, an enzyme substrate or inhibitor is covalently attached to a solid support (eg. agarose). The crude protein sample (usually after an initial ammonium sulphate purification step) is applied to the column, the enzyme of interest binds to the immobilised ligand and contaminating proteins are washed off in one bed volume of buffer. The enzyme is then desorbed and eluted with a pulse of either ligand solution or a (protein) 'deforming' buffer. The application of this technique is illustrated for the case of staphylococcal nuclease,[54] Fig. 18.

Although this technique has the potential for very large purifications in a single step, this must be weighed against the fact that a packing material, months in the preparation, may be of no use whatsoever.

Affinity elution chromatography is a technique in which an impure protein sample is adsorbed non-specifically to an ion exchange column and the enzyme(s) of interest is eluted with a pulse of enzyme substrate or
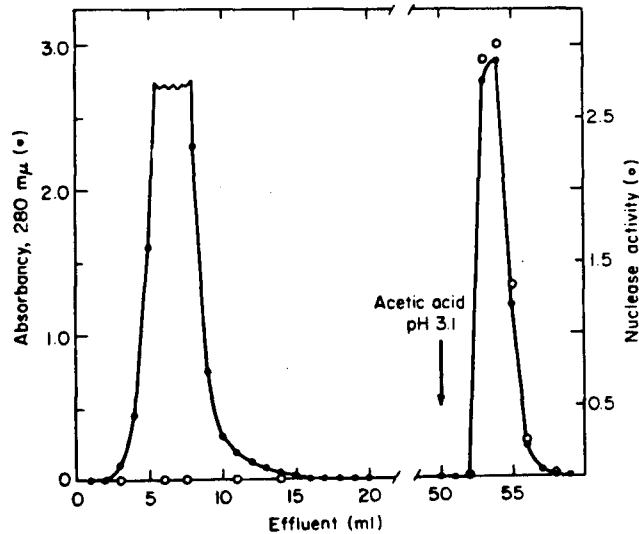
Figure 18: Purification of Stapylococcal Nuclease by Affinity
Adsorption Chromatography.

inhibitor solution. The advantages of this technique are the ease with

which it may be attempted (cf. affinity adsorption chromatography) and

the large capacity of the material. In a comprehensive study on the

purification of glycolytic enzymes[55] by affinity elution it was pointed

out that, in order to avoid ion exchange effects, the desorbing ligand

should have either no charge or the same charge as the ion exchange

material. Due to the non-specific nature of the adsorption this technique

is unsuitable for use with samples in which the enzyme of interest

comprises only a small portion of the total protein[55]. The basis of the technique is either a protein conformational change induced on binding of the desorbing ligand and/or the neutralisation of charge on the protein by charge on the desorbing ligand.[55] At least three charges (on the desorbing ligand) had to be introduced per 100,000 Daltons enzyme molecular weight for elution of glycolytic enzymes.[55]

For all of the above chromatographic techniques diffusion is decreased, and resolution increased, if the packing material is made up of small, monodisperse, spherical beads. The use of small bead size requires high pressure to obtain reasonable flow rates and at these (HPLC) pressures the shear forces generated may denature some enzymes. Some of these problems have been reduced by the recent introduction (by Pharmacia) of a new packing material (MonoBeads[TM]) for use at medium pressures with a, so called, Fast Protein Liquid Chromatography (FPLC[TM]) system. This material has been utilised in gel filtration chromatography, ion exchange chromatography and chromatofocussing. The increase in resolution that may be obtained by the use of this small, monodisperse packing material is considerable. A protein sample that eluted as one peak on a column (25 cm x 2.5 cm) of Whatman DE-52 anion exchange resin during a 25 hour run was resolved into fourteen peaks on a MonoBead anion exchange column (5.0 cm x 0.6 cm) in a 20 minute run[53]. The decrease in run time alone, obtained by the use of this material, allows for a much more detailed method development.

Once an enzyme has been purified by a combination of the above
techniques it is typically stored either as a solid suspension
crystallised with ammonium sulphate, a lyophilised powder, in solution
under toluene vapour or frozen, depending upon its stability to the
available conditions.

Demonstration of protein homogeneity (purity) is traditionally
achieved by observation of any, or a combination, of the following: a
single band on denaturing or non-denaturing electrophoresis gels, a single
band on an isoelectric focussing gel or the observation of a single,
symmetrical peak on any chromatographic procedure. Another criterion of
purity, for a previously isolated enzyme, is specific activity, i.e.
amount of enzyme activity per unit weight of protein. Weight of protein
is determined by a colorimetric assay (see II.3.1).

Electrophoresis is a method of separation based on charge to mass
ratio. The support is usually a porous, cross-linked polyacrylamide gel
whose pores are of such a size that the proteins experience a 'sieving'
effect when migrating under the influence of an applied potential
difference. The proteins are applied to one end of the gel and migrate
under the influence of an applied potential difference. Separation is
thus effected on the basis of both size and charge. If used as a crite-
rion of homogeneity the procedure should be carried out at a number of
pH's to vary the charge to mass ratio of the proteins. Proteins denatured
with the anionic detergent sodium dodecyl sulphate (SDS) and

mercaptoethanol are dissociated into their component subunits (if composed of such) and adsorb a constant weight ratio of SDS (1.4 g per g of protein).[56] Electrophoresis of these pretreated proteins in the presence of SDS thereby effects a separation on the basis of molecular weight alone, and in addition to being a criterion of purity, allows the subunit composition of the protein(s) to be determined.

Isoelectric focussing is a method of separation based on iso-electric point. A pH gradient is set up across a gel matrix, the protein sample loaded onto the gel and a potential difference applied. The proteins migrate, and are focussed, to a pH at which they have no net charge, the isoelectric point. Both isoelectric focussing and non-denaturing electrophoresis may be used preparatively, albeit with some difficulty.

I.4   Previous Studies on β-Glucosidase from A. faecalis

The first report of work carried out on a β-glucosidase from A.
faecalis was that of its purification and characterisation by Han and
Srinivasan.[57]   The β-glucosidase activity was found to be induced by
either lactose or cellobiose in the growth media.   However, due to the
number of mistakes (arithmetical, experimental and methodological) found
in this work, it is of only limited value.

Subsequent work on the immobilisation of partially purified
enzyme[58] and whole cells[59] onto solid supports has been published.

Work published as a Ph.D. thesis by E.O. Smith under the
supervision of Srinivasan[60] suggested that a number of carbohydrates are
actively transported across the cell wall membrane of A. faecalis.   Gel
chromatography of cell free extracts yielded two active fractions with
different β-glucosidase to β-galactosidase activity ratios.   The
β-glucosidase peak was purified to apparent homogeneity by preparative
disc-gel electrophoresis.   The specific activity of the pure protein was
55 units/mg; one unit of enzyme is that amount necessary to hydrolyse one
μmole of p-nitrophenyl-β-D-glucopyranoside in one minute at pH 6.5 in 0.1M
sodium phosphate buffer at 40°C.

A comparison of these results with those found herein will be made
in Chapter III.

CHAPTER II

Experimental

Abbreviations Used

PMR	proton magnetic resonance
TLC	thin layer chromatography
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
FPLC	fast protein liquid chromatography
IR	infra-red spectroscopy
UV	ultra-violet
TMS	tetramethylsilane
DSS	2,2-dimethyl-2-silapentane-5-sulphonic acid, sodium salt hydrate
PNP	p-nitrophenol
PMSF	phenylmethylsulphonyl fluoride
EDTA	ethylaminediamenetetraacetic acid
MOPS	3-(N-morpholino)-propanesulphonic acid
SDS	sodium dodecyl sulphate
PNPG	p-nitrophenyl-β-D-glucopyranoside
PNPGal	p-nitrophenyl-β-D-galactopyranoside
PNPMan	p-nitrophenyl-β-D-mannopyranoside
PY-G	β-D-glucopyranosyl pyridinium bromide
2,4-DNPG or DNPG	2,4-dinitrophenyl-β-D-glucopyranoside
2,4-TDNPG or TDNPG	2,4-dinitrophenyl-β-D-1-thioglucopyranoside
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
DNAse	deoxyribonuclease
2,4-DNP	dinitrophenol
2,4-TDNP	thiodinitrophenol
ppm	parts per million

All extinction coefficients are in units of: $1 \ mol^{-1} \ cm^{-1}$

II.1 <u>Synthesis</u>

II.1.1 <u>General Methods</u>

Infra-red spectra were recorded on a Nicolet 5D-X Fourier trans-
form instrument, samples being prepared as a nujol mull held between
sodium chloride plates.

FT proton magnetic resonance (PMR) spectra were recorded either on
a Bruker WP 80, a Bruker WH 400 or an instrument consisting of a 270 MHz
Oxford superconducting magnet, a Nicolet 1180 computer, a Nicolet 293b
pulse programmer and a Bruker WH 60 console. Deuterated solvents were
used with either TMS or DSS standard.

$C^{13}\{^1H\}$ magnetic resonance (CMR) spectra were recorded on a Bruker
WH 400 at 100.6 MHz with TMS as standard.

Melting points were determined on a Kofler micro heating stage and
are uncorrected.

Evaporations at reduced pressure were carried out in a Büchi
rotary evaporator at reduced pressure under water pump vacuum at the
optimum temperature for the removal of solvent without decomposition of
the material being prepared.

Purification by flash chromatography was carried out according to
the method of Clark et al[74] in the solvent specified.

Thin layer chromatography (TLC) was carried out on aluminum backed
Kieselgel 60 F_{254} plates supplied by Merck Chemical Company and visualised
by ultra-violet absorbance (UV) and/or charring by spraying with 10% H_2SO_4

in methanol and heating at 100°C for 5 minutes (H_2SO_4). Solvent A consists of ethyl acetate:ethanol:water 7:2:1 and is most useful for the separation of free sugars. Solvent B consists of pentane:ethyl acetate:-ethanol 20:9:1 and is most useful for the separation of acetylated sugars. Solvent C consists of chloroform:acetonitrile:ethyl acetate 3:1:1 and is an aprotic solvent suitable for acetylated sugars. Solvent D consists of methyl ethyl ketone:petroleum ether 30-60 1:3.

Solvents used were purified as described below: Acetone was dried by distillation from 4Å molecular sieves and stored over 4Å molecular sieves under dry nitrogen. Methanol was dried by distillation from magnesium methoxide and stored over 3Å molecular sieves under dry nitrogen. Pyridine was dried over potassium hydroxide, distilled from barium oxide and stored over potassium hydroxide under dry nitrogen. Dimethyl formamide was dried by distillation at reduced pressure from calcium hydride and stored over 4Å molecular sieves under dry nitrogen. Acetonitrile was dried by distillation from 4Å molecular sieves and stored over 4Å molecular sieves under dry nitrogen.

P-Nitrophenol was purified by repeated recrystallisation from 0.01M hydrochloric acid. MP = 115°C.

Nitrogen was dried by passage through a column of calcium chloride.

All chemicals used were general purpose grade reagents unless otherwise specified.

All products were dried for at least 24 hours in a vacuum dessicator over phosphorus pentoxide.

II.1.2 p-Nitrophenyl-β-D-cellobioside, IV

The title compound was prepared from cellobiose by the following

method.

1,2,3,6,2',3',4',6'-Octa-O-acetyl-α-D-cellobiose, I, was prepared

by the method of Wolfrom and Thompson[62] with the following modifications.

Cellobiose (10 g, 0.029 Mol.) was added to a stirred, cooled (0°C) mixture

of acetic anhydride (70 cm³, 0.74 Mol.) and pyridine (100 cm³). After 72

hours the reaction had not gone to completion (TLC, solvent A) and

undissolved solid remained in the mixture. A further 25 cm³ of pyridine

was added to effect dissolution. After 100 hours the reaction was worked

up as described[62].

Yield = 17.7 g, 90%. Melting point = 198-200°C. Lit. Melting point[63] =

202-202.5°C. Rf (Solvent A) = 0.75 (H_2SO_4).

2,3,6,2',3',4',6'-hepta-O-acetyl-α-D-cellobiosyl bromide

(acetobromocellobiose), II, was prepared according to the method of Stanek

and Kocourek[64] by reaction of I with hydrogen bromide in chloroform and

acetic acid.

Yield = 64%. Melting point = 186-188°C. Lit. Melting point[64] = 188°C.

Rf(solvent C) = 0.60 (H_2SO_4).

p-Nitrophenyl 2,3,6,2',3',4',6'-hepta-O-acetyl-β-D-cellobioside,

III, was prepared by the method of Capon[87] with the following

modifications. II(1.0 g, 0.0015 Mol.) and p-nitrophenol (0.41 g, 0.0029

Mol.) were dissolved in dry acetone (12 cm^3). Potassium carbonate (0.86 g, 0.0062 Mol.) was added and the mixture refluxed for 17 hours at which time TLC (solvent B) showed a major spot visualised by both UV and H_2SO_4. The reaction was worked up as described[87]. Yield = 0.30 g, 28%. Melting point = 238-240°C. Lit. Melting point[65] = 235°C. Rf (solvent C) = 0.54 (UV, H_2SO_4).

p-Nitrophenyl-β-D-cellobioside, IV, was prepared by catalytic deacetylation of III using sodium methoxide in methanol according to Zemplen[66]. The gum obtained was triturated with diethyl ether to induce crystallisation and recrystallised from water.

Yield = 94%. Melting point = 255-256.5°C. Lit. Melting point[65] = 255-256°C. Rf (solvent A) = 0.43 (UV, H_2SO_4). Elemental analysis calcd. for $C_{18}H_{25}NO_{13} \cdot 2H_2O$: C, 43.30%; H, 5.85%; N, 2.81%; O, 48.04%. Found: C, 43.09%; H, 5.95%; N, 2.78%; O, 48.10%. PMR (80 MHz, D_2O): 7.28, 8.30 (2 x dd; J = 9.0, 2.0 Hz; 2H each; aromatic H's), 5.35 (d; J = 6.2 Hz; 1H; H-1), 4.59 (d; J = 8.4 Hz; 1H; H-7), 3.3-4.2 (m; 12H; H-2-H-6 and H-2'-H-6').

II.1.3 p-Nitrophenyl-β-D-glucopyranoside, VIII

The title compound was prepared from glucose by the following method.

1,2,3,4,6-Penta-0-acetyl-α-D-glucopyranose, V, was prepared according to the method of Wolfrom and Thompson[62] by reaction of glucose

with acetic anhydride in the presence of perchloric acid.

Yield = 55%. Melting point = 111.5-112.5°C. Lit. Melting point[62] = 112-113°C.

2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide

(acetobromoglucose), VI, was prepared from glucose in a 'one pot' reaction according to the method of Lemieux[67]. Glucose was added to an acetylating mixture consisting of acetic anhydride and perchloric acid and subsequent addition of bromine and red phosphorus (to produce phosphorus tribromide in situ) converted the pentaacetate, V, to the bromide, VI.

An alternate procedure used[68] was conversion of the crystalline pentaacetate, V, to the bromide by reaction with hydrogen bromide in acetic acid and chloroform (c.f. preparation of II).

The material was stored at -20°C.

For both preparations: Yield > 80%. Melting point = 88°C. Lit. Melting point[67] = 88-89°C. Rf (solvent C) = 0.55 (H_2SO_4).

p-Nitrophenyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside, VII, was

prepared by the method of Capon[87] with the following modifications.

The bromide, VI, (15 g, 0.062 Mol.) and p-nitrophenol (10 g, 0.072 Mol.) were dissolved in dry acetone (150 cm^3) and potassium carbonate (12 g, 0.086 Mol,) added. The stirred mixture was refluxed for 4 hours. TLC (solvent D) showed 2 major spots, Rf = 0.26 (UV, H_2SO_4) and 0.20 (H_2SO_4). The reaction was worked up as described[87] and recrystallization of the product from methanol yielded the UV active material exclusively.

Yield = 4.0 g, 21%. Melting point = 174.5-175°C. Lit. Melting point[68] = 174-175°C. Rf (solvent D) = 0.26 (UV, H_2SO_4).

Cooling of the mother liquor induced crystallisation of the impurity which was identified by PMR (270 MHz) as the product of an elimination reaction, 2-acetoxy-3,4,6-tri-0-acetyl-D-glucal. PMR (270 MHz, $CDCl_3$): 2.13 (m; 12H; CH_3CO-), 4.28 (dd; J = 10, 3.3 Hz; 1H; H-5), 4.46 (m; 2H; H-6), 5.26 (t; J = 4.0 Hz; 1H; H-4), 5.61 (d; J = 4.0 Hz; 1H; H-3), 6.72 (s; 1H; H-1).

Carrying out the reaction at room temperature did not increase the yield of the desired product, VII.

p-Nitrophenyl-β-D-glucopyranoside, VIII, was prepared by catalytic deacetylation of VII using sodium methoxide in methanol according to the method of Zemplen[66].

Yield = 70%. Melting point = 164.5-165.5°C. Lit. Melting point[68] = 164°C. Rf (solvent A) = 0.63 (UV, H_2SO_4). PMR (80 MHz) identical to that of authentic sample.

II.1.4 Isopropyl-β-D-1-thioglucopyranoside, XII

The title compound was prepared from the bromide, VI, by the following method.

2-(2,3,4,6-Tetra-0-acetyl-β-D-glucopyranosyl)-2-thiopseudourea hydrobromide, IX, was prepared by reaction of the bromide, VI, with thiourea in dry acetone according to the method of Cerny et al[69].

Yield = 78%. Melting point = 195°C. Lit. Melting point[70] = 205°C. PMR (270 MHz, D_2O): 2.10 (m; 12H; CH_3CO-), 4.12-4.60 (m; 3H; H-5, H-6), 5.26

(t; J = 8.5 Hz; 1H; H-=2), 5.38 (d, J = 8.5 Hz; 1H; H-1), 5.82 (t; J = 8.5 Hz; 2H; H-3, H-4).

Repeated attempts to prepare 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside, X, by addition of IX to a saturated solution of potassium carbonate in water according to the method of Cerny et al[69] failed. Addition of carbon tetrachloride to extract the product as it was formed according to the method of Cerny and Pacak[71] gave X in good yield. Yield = 83%. Melting point = 115.5-116.5°C. Lit. Melting point[70] = 115°C. Rf (solvent B) = 0.40 (H_2SO_4). PMR (270 MHz, $CDCl_3$): 2.05 (m; 12H; CH_3CO-), 2.34 (d; J = 10 Hz; 1H; SH), 3.75 (m; 1H; H-5), 4.13 (dd; J = 12, 2.8 Hz; 1H; H-6), 4.26 (dd; J = 12, 4.0 Hz; 1H; H-6), 4.56 (t; J = 10Hz; 1H; H-1), 5.00, 5.11, 5.22 (3 x t; J = 8.5 Hz; 2H each; H-2, H-3, H-4). On D_2O shake resonance at 2.34 ppm disappeared and that at 4.56 ppm collapsed to a doublet.

2,3,4,6-Tetra-O-acetyl isopropyl-β-D-1-thioglucopyranoside, XI, was prepared by the reaction of X with isopropyl iodide and potassium carbonate in dry acetone according to the method of Cerny and Pacak[72]. The discrepancy in the melting points can be ascribed to different crystal forms as the PMR spectrum is in accord with the structure. Yield = 82%. Melting point = 108.5-109°C. Lit. Melting point[73] = 91°C. Rf (diethyl ether) = 0.62 (H_2SO_4). Proton magnetic resonance (80 MHz, $CDCl_3$): 1.35 (d; J = 7.0 Hz; 6H; isopropyl CH_3), 2.06 (m; 12H; CH_3CO-), 3.21 (septuplet; J = 7 Hz); 1H; isopropyl CH), 3.75 (m; 1H; H-5), 4.23 (m; 2H; H-6), 4.63 (d; J = 10 Hz; 1H; H-1), 4.88-5.50 (m; 3H; H-2, H-3, H-4).

Isopropyl-β-D-1-thioglucopyranoside, XII, was prepared by catalytic deacetylation of XI using sodium methoxide in methanol according to the method of Zemplen[66]. The material was purified by flash chromatography[74] in methanol:chloroform 3:7. The hygroscopic gum obtained crystallised on standing at -20°C for 3 months.

Yield = 91%. Elemental analysis calcd. for (gum) $C_9H_{18}O_5S$: C, 45.37%; H, 7.61%; O, 33.56%. Found: C, 44.79%; H, 7.62%; O, 33.72%. PMR (400 MHz, D_2O): 1.35 (dd; J = 8.0, 2.0 Hz; 6H; isopropyl CH_3), 3.27-3.62 (m; 6H; SH, H-2, H-3, H-4, H-5, isopropyl CH), 3.74 (dd; J = 14, 5.6 Hz; 1H; H-6), 3.94 (dd, J = 14, 2.4 Hz; 1H; H-6): 4.65 (d; J = 9.2 Hz; 1H; H-1).

II.1.5 β-D-Glucopyranosyl Pyridinium Bromide, XIV

The title compound was prepared from the bromide, VI, by the following method.

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl pyridinium bromide, XIII, was prepared from the bromide, VI, by reaction with dry pyridine in the presence of m-cresol according to the method of Sinnott and Withers[34].

Yield = 25%. Melting point = 168-169°C. Lit. Melting point[75] = 170°C. PMR (80 MHz, D_2O) identical to that reported[75].

β-D-Glucopyranosyl pyridinium bromide, XIV, was prepared by deacetylation of XIII with methanolic hydrogen bromide according to the method of Lemieux and Morgan[75].

Yield = 80%. Melting point = 176°C. Lit. Melting point[75] = 176-177°C.

PMR (80 MHz, D_2O) identical to that reported[75].

II.1.6. β-D-Glucopyranosyl Azide, XVI

The title compound was prepared from the bromide, VI, by the following method.

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl azide, XV, was prepared from the bromide, VI, by reaction with sodium azide in dry acetonitrile according to the method of Szarek et al[76].

Yield = 72%. Melting point = 127-128°C. Lit. Melting point[76] = 127.5-129°C. Rf (solvent B) = 0.30 (UV, H_2SO_4). PMR (80 MHz, $CDCl_3$) identical to that reported[76]. IR identical to that reported[76].

β-D-Glucopyranosyl azide, XVI, was prepared by catalytic deacetylation of XV using sodium methoxide in methanol according to the method of Zemplen[66].

Yield = 81%. Melting point = 94°C. Lit. Melting point[76] = 89°C. ^{13}C magnetic resonance (acetone-d_6) identical to that reported[76]. IR identical to that reported. Elemental analysis calcd. for $C_6H_{11}O_5N_3$: C, 35.12%; H, 5.40%; N, 20.48%. Found: C, 35.11%; H, 5.36%, N, 20.33%.

II.1.7 β-D-Glycopyranosyl Fluoride, XVIII

The title compound was prepared from the bromide, VI, by the following method.

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl fluoride, XVII, was prepared from the bromide, VI, by reaction with silver fluoride (AgF) in

dry acetonitrile according to the method of Helferich[77].

Yield = 65%. Melting point = 86°C. Lit. Melting point = 98°C (after

repeated recrystallisation).

β-D-Glucopyranosyl fluoride, XVIII, was prepared by catalytic

deacetylation of XVII using sodium methoxide in methanol according to the

method of Zemplen[66] with the following modifications. The reaction was

only allowed to proceed for 5 minutes before working up. The material

had to be purified by flash chromatography[74] in solvent A. An alternate

procedure involved carrying out the deacetylation at 0°C. In this case

crystallisation could be induced without further purification. However,

this latter material inactivated the enzyme, presumably because it

contained trace amounts of silver.

Yield = 27%. Melting point = 90°C. Lit. Melting point[78] = 99-102°C

(after extensive recrystallisation). Rf (solvent A) = 0.40 (H_2SO_4).

Elemental analysis calcd. for $C_6H_{11}O_5F$: C, 39.56%; H, 6.08%. Found: C,

39.48%; H, 6.04%. PMR (270 MHz, D_2O) 3.35-3.60 (m;4H; H-2, H-3, H-4,

H-5), 3.75 (dd; J = 14, 4.4 Hz; 1H; H-6), 3.90 (dd, J = 14, 1.2 Hz; 1H;

H-6), 5.22 (dd, J = 53, 7.6 Hz; 1H; H-1).

II.1.8 β-D-Glucopyranosylamine, XIX

β-D-Glucopyranosylamine, XIX, was prepared from glucose by

reaction with methanolic ammonia according to the method of Cusack et

al[79].

Yield = 91%. Melting point = 126-129°C. Lit. Melting point[80] =

126-128°C.

II.1.9 2,4-Dinitrophenyl-1-thio-β-D-glucopyranoside, XXI

The title compound was prepared from the acetylated thio sugar, X, as follows.

2,3,4,6-Tetra-O-acetyl-1-(2,4-dinitrophenyl)-1-thio-β-D-glucopyranoside, XX, was prepared as follows. X (4g, 0.010 mol.), 2,4-dinitro-1-fluorobenzene (1.92 g, 0.010 mol.) and 1,4-diazabicyclo-[2,2,2]octane (0.45 g, 0.0040 mol.) were dissolved in dry dimethyl formamide (30 cm^3). The solution rapidly went red and after 100 minutes TLC (solvent B) indicated that the reaction had gone to completion. The reaction mixture was evaporated to a gum under reduced pressure, dissolved in chloroform (250 cm^3) and the solution washed with successive 100 cm^3 portions of saturated sodium bicarbonate solution and water. This solution was then dried over anhydrous magnesium sulphate, decolourised with charcoal and evaporated under reduced pressure to yield a faintly yellow gum. Crystallisation and recrystallisation were carried out by precipitation from chloroform with low boiling petroleum ether. Yield = 5.6 g; 59%. Melting point = 197°C. Lit. Melting point[81] = 200-201°C. Rf (solvent B) = 0.45 (UV, H$_2$SO$_4$). PMR (270 MHz, CDCl$_3$) 2.06 (m; 12H; CH$_3$CO), 3.96 (m; 1H; H-5), 4.22 (dd; J = 12, 2.8 Hz; 1H; H-6), 4.28 (dd; J = 12, 4.4 Hz; 1H; H-6), 5.00 (d; J = 9.6 Hz; 1H; H-1), 5.17, 5.21, 5.34 (3xt; J = 8.4 Hz each; 1H each; H-2, H-3, H-4), 7.98 (d; J = 8.8 Hz; 1H; H-6' of phenyl), 8.42 (dd; J = 8.8, 2.0 Hz; 1H; H-5' of

phenyl), 9.02 (d; J = 2.0 Hz; 1H; H-3' of phenyl).

2,4-Dinitrophenyl-β-D-1-thioglucopyranoside, XXI, was prepared by catalytic deacetylation of XX using sodium methoxide in methanol according to the method of Zemplen[66]. The final product was found to have one acetone of crystallisation associated with it. A peak in the proton magnetic resonance spectrum at 2.10 ppm, integrating to 3 protons, disappeared if the sample was evaporated to a gum and redissolved. The Elemental analysis is consistent with one acetone of crystallisation. Yield = 74%. Melting point (from methanol/acetone) = 111°C. Lit. Melting point (from methanol/diethyl ether)[81] = 184-185°C. Elemental analysis calcd. for $C_{12}H_{14}O_9N_2S:CH_3COCH_3$: C, 42.85%; H, 4.79%; N, 6.66%; S, 7.64%. Found: C. 42.59%; H, 4.72%; N, 6.72%; S, 7.79%. PMR (400 MHz, DMSO-d_6) 3.20-3.55 (m; 5H; H-2, H-3, H-4, H-5, H-6), 3.75 (dd; J = 12, 4.4 Hz; 1H; H-6), 4.55, 5.08, 5.23, 5.67 (4xs; 1H each; 2-OH, 3-OH, 4-OH, 6-OH), 5.00 (d; J = 7.6 Hz; 1H; H-1), 8.05 (d; J = 8.0 Hz; 1H; H-6' of phenyl), 8.40 (dd; J = 8.0, 1.6 Hz; 1H; H-5' of phenyl), 8.85 (d; J = 1.6 Hz; 1H; H-3' of phenyl). On D_2O shake resonances at 4.55, 5.08, 5.23 and 5.67 ppm disappeared.

II.2 Growth of A. faecalis, ATCC 21400, and Preparation of Cell Free Extract

II.2.1 General Methods

pH was measured on a Radiometer PHM-82 digital pH meter equipped with a Sigma Trizma glass/calomel combination electrode and standardised with Radiometer standard buffers.

All measurements of optical density were carried out on a Pye-Unicam PU-8800 ultra-violet/visible recording spectrophotometer in 1 cm path length glass or quartz cells.

Centrifugation was carried out on a Sorvall RC-5B refrigerated centrifuge equipped with either a SS-34 (350 cm^3 capacity) or a GS-3 (2.5 liter capacity) rotor at 4°C.

All media were sterilised at 121°C either in an autoclave (American Sterilizer Co. Model AS-DIT616GE) or directly in the 20 litre fermenter, which is steam jacketed. Small volumes of media were auto-claved at 121°C for 20 minutes and larger volumes (greater than 10 litres) for 45 minutes. The component solutions were sterilised separately as shown below (II.2.2).

The French pressure cell used was an American Scientific Bragg 6600.

pH 6.8 buffer refers to 100 mM sodium phosphate buffer. This was prepared by mixing appropriate amounts of di-sodium hydrogen ortho-phosphate (A.R.) and sodium di-hydrogen orthophosphate, making up to

volume in double deionised water and then further adjusting the pH with 1M sodium hydroxide or 1M hydrochloric acid as necessary.

The turbidity of the cell cultures was quantified by measuring the absorbance at 660 nm.

β-Glucosidase activity was quantified by adding between 10 and 100 μL (as necessary) of the sample to be assayed to an 1 cm pathlength cuvette containing 3.00 cm^3 of a solution of p-nitrophenyl-β-D-gluco-pyranoside (PNPG) (greater than 5.2 mM, ie. 12 Km) in pH 6.8 buffer. All weighings were carried out on either micro or semi-micro balances and all accurate volumes were measured with either grade A analytical glassware or Hamilton Microlitre syringes. The cuvette was equilibrated to 37.0°C (±0.1°C) in the spectrophotometer, by means of a Julabo VI circulating thermostat bath, prior to addition of the sample to be assayed. Optical density readings at a wavelength of 400 nm were taken every 100 s for up to 1000 s. Total units of β-glucosidase activity in the original sample were calculated from the following equation:

$$U = \frac{xy(ODc)(0.6\times10^6)}{7.28z\times10^3\times10^3} = \frac{0.0834xy(ODc)}{z}$$

where: U is the number of units β-glucosidase activity; 1 unit being defined as that amount of β-glucosidase which hydrolyses 1 μmole of p-nitrophenyl-β-D-glucopyranoside (PNPG) in 1 minute at pH 6.8 and 37°C; ODc is the average change in absorbance observed at 400 nM in 100 s;

x is the total sample volume;

y is the volume in the cuvette;

z is the sample volume added to the cuvette

and 7.28×10^3 is the extinction coefficient of p-nitrophenol at pH 6.8

and 37°C.

Media nutrients were obtained from Difco Laboratories. Lactose

was either BDH bacteriological grade or general purpose reagent grade.

Deoxyribonuclease (DNAse) was deoxyribonuclease I from bovine pancreas

containing 10,000 Dornase units/mg, obtained from Calbiochem-Behring,

#260912. p-Nitrophenyl-β-D-glucopyranoside (PNPG) was either prepared as

described (II.1.3) or obtained from the Sigma Chemical Company.

Phenylmethylsulphonyl fluoride (PMSF) and the stock bacterial cultures

were supplied by W. Wakarchuk, Microbiology department, UBC. All other

materials were general purpose reagent grade unless otherwise stated.

II.2.2 Growth of A. faecalis

A. faecalis stock culture was supplied as either a stab culture in

soft agar containing Difco nutrient (stored at 4°C) or as a 50% culture

suspension in glycerol (stored at -20°C). These preparations were used to

innoculate 100 cm^3 cultures which were themselves used either for tests on

a small scale or as innoculum for 10-20 litre cultures. Two media were

used:

1) Minimal Salts Medium[57]

NaCl	0.30%
$(NH_4)_2SO_4$	0.10%
KH_2PO_4	0.05%
K_2HPO_4	0.05%
$MgSO_4$	0.01%
$CaCl_2$	0.01%
Difco Yeast extract	0.50%
Lactose	3.00%

Autoclaved separately

2) Luria Broth[82]

Difco Tryptone	1.00%
Difco Yeast extract	0.50%
NaCl	0.05%
Lactose	3.00%

Autoclaved separately

pH adjusted to 7.0 with 1.0M sodium hydroxide.

N.B. a) 0.1% cellobiose could be used in place of lactose to induce

β-glucosidase production[82].

b) If yeast extract was omitted from the minimal salts medium

(preparation as given[57]), the cell yield was about 10% of that

obtained when it was included.

c) Both successful β-glucosidase isolations were carried out from

material cultured in Luria broth although this medium gave

slightly less total β-glucosidase activity (as measured in the

crude culture) and a slightly lower cell yield.

100 cm^3 Cultures were grown in 500 cm^3 Erhlenmeyer flasks on a thermostatted platform shaker with a 7/8" throw (built by the mechanical shop, Chemistry Department, U.B.C.) at 30°C and 200 rpm. 10-20 litre cultures were grown in impeller driven, steam-jacketed 20 litre fermenters (built by the mechanical shop, Chemistry Department, U.B.C.) at 30°C, 500 rpm and aerated at 500 cm^3 per litre. Polypropylene glycol (2-5 cm^3) was added to the 10-20 litre cultures in order to minimize foaming.

β-Glucosidase activity could be monitored in whole cells in the culture medium. The variation of cell density and β-glucosidase activity with time, for a 13 litre culture grown in minimal salts medium, innoculated with 150 cm^3 of late log phase culture, is shown below, Table III, Fig. 19. The three phases of bacterial growth: lag phase, log phase and stationary phase, are also marked, Fig. 19. It should be noted that the cell yield (and concomitant β-glucosidase activity) is somewhat lower in the 100 cm^3 cultures (OD 660~4) than in the 10-20 litre cultures (OD 660~6-8). This is due to the forced aeration in the 10-20 litre cultures.

Clearly it is important that the cultures be harvested at the late log growth phase for maximum β-glucosidase yield. The decrease in β-glucosidase activity observed (about 20% over 3 hours) after the stationary phase had been reached suggests that the organism actively metabolises the enzyme. Cell free extracts, in the absence of protease inhibitors, show only a 25% decrease in activity over 60 hours.

Table III: Variation of Cell Density and β-Glucosidase Activity With Time.

Time (hours)	OD 660	Total Activity in 10 L (β-glucosidase units)	Specific Activity (units/OD 660)
0.0	0.00	0	0
3.5	0.34	0	0
5.5	1.06	82	77
11.5	4.83	1414	292
12.5	5.32	1643	310
14.0	5.88	1847	311
16.5	5.92	1480	251
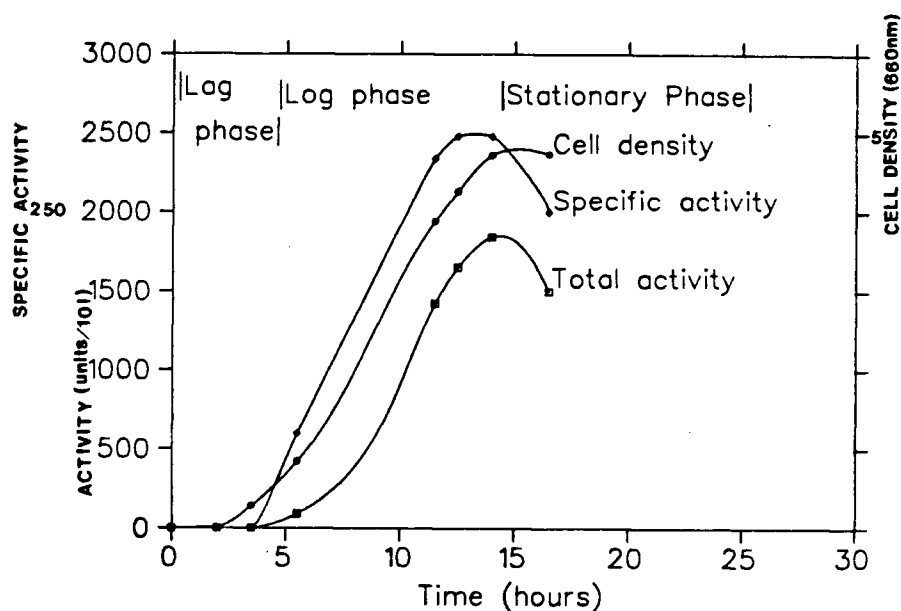
Figure 19. Variation of Cell Density, Specific Activity and

β-Glucosidase Activity with Time.

When centrifugation was carried out 4-5 hours after the stationary phase had been reached, 50-70% of the β-glucosidase activity was found to be associated with the supernatant. This suggests that cell autolysis had started to occur.

During the growth of one 10 litre culture aeration failed between the early and mid-log phases. The noxious smell, together with the reasonably high cell yield and β-glucosidase activity found in the mature culture, suggest that the organism is a facultative anaerobe.

Once a 10-20 litre culture had reached late log/early stationary growth phase it was transferred to the cold room (4°C) and the cells harvested immediately by centrifugation in 2.5 litre batches on GS-3 rotor at 5000 rpm for 20 minutes. The cell pellet was stored in the freezer at -20°C until it was required.

II.2.3   β-Glucosidase Induction Studies

It is known[83] that isopropyl-β-D-thiogalactose induces the synthesis of β-galactosidase in E. coli. This compound is not hydrolysed by β-galactosidase and therefore only a small amount has to be added to the culture in order to induce its production. It was thought that isopropyl-β-D-1-thioglucose, XII, might induce synthesis of β-glucosidase in A. faecalis and this was investigated.

A minimal salts medium, containing 0.5% sodium acetate as a carbon source in place of lactose, was prepared and the pH adjusted to 7 with glacial acetic acid. 100 cm³ of this medium was innoculated with

A. faecalis, incubated at 30°C on a platform shaker and the culture
allowed to reach early stationary phase. The cell yield was about 20%
that of a control culture grown in Luria broth and no β-glucosidase
activity could be detected. Five units of activity were found in the
control culture. The experiment was repeated and when the acetate culture
had reached early log phase (at the first sign of visible turbidity) 0.05%
isopropyl-β-D-1-thioglucose was added. No β-glucosidase activity was
detected at this or at any subsequent growth stage of this culture.
β-Glucosidase synthesis is probably not, therefore, induced in A. faecalis
by the thioglucose derivative, XII.

II.2.4 Preparation of Cell Free Extract From A. faecalis; Cell Breakage

Four methods of cell lysis were investigated; lysozyme and
freeze/thaw treatment, French pressure cell, sonication, and ballistic
disintegration with glass beads.

1) Lysozyme and Freeze/Thaw Treatment[84]

This method of cell breakage relies upon the hydrolysis of the
cell wall polysaccharide by hen eggwhite lysozyme and disruption of the
cell wall by repeated cycles of freezing and thawing.

Two 100 cm^3 cultures grown in minimal salts medium and allowed to
reach late log growth phase contained a total of 10 units of β-glucosidase
activity. After centrifugation for 10 minutes at 9000 rpm in a GS-3
rotor, no β-glucosidase activity was detected in the supernatant. The
cell pellet (3.6 g wet weight) was split into two equal portions. One

portion was resuspended in 20 cm^3 of pH 6.8 buffer. To this was added

20 mg of hen eggwhite lysozyme, 10 μL of a 250 mM EDTA solution, and 0.1

mg DNAse. This mixture was incubated at 30°C for 30 minutes, rapidly

frozen (in a dry ice/acetone bath) and thawed at 30°C. The process was

repeated twice more. After centrifugation for 15 minutes at 15,000 rpm in

a SS-34 rotor, the supernatant and pellet (resuspended in 20 cm^3 of pH 6.8

buffer) were both assayed. The total activity recovered (supernatant and

pellet) was 67% of the original activity. The activity associated with

the supernatant was 23% of the recovered activity. Cell breakage was

poor.

If this procedure was carried out on cells in which autolysis had

begun, cell breakage was greater than 90%.

2) French Pressure Cell[84]

In this method of cell disruption, the bacterial suspension is

placed in a steel cylinder fitted with a piston and a small nylon relief

valve which is connected to an outlet tube. The assembly is put in a

hydraulic press and a pressure of ~1200 lb/in^2 applied. The relief valve

is adjusted to give a slow steady flow. Breakage is effected by the shear

forces generated as the cells flow out of the small orifice and by

explosive decompression as the cells experience a rapid, 1200 lb/in^2

pressure drop.

The other half of the cell pellet prepared above (in 1) was

resuspended in 3 cm^3 of pH 6.8 buffer to give a total volume of 5.2 cm^3

and 0.1 mg DNAse added. The mixture was passed through a precooled (0°C)

French pressure cell at 1260 lb/in^2 and collected in a cooled (0°C) container. The process was repeated three times. After centrifugation for 15 minutes at 15000 rpm in a SS-34 rotor the supernatant and pellet (resuspended in 10 cm^3 pH 6.8 buffer) were both assayed. The total activity recovered (supernatant and pellet) was 91% of the original activity. The activity associated with the supernatant was 70% of the recovered activity. Cell breakage was therefore good.

3) Sonication[84]

In this method of cell disruption the rapid vibration of an ultrasonic probe induces the formation of gas bubbles moving at high velocity in the vicinity of the tip ('cavitation'). The high shear forces generated by these rapidly moving bubbles disrupts the cell wall.

The cell pellet (about 160 g wet weight) from a 10 litre late log phase culture grown on Luria broth was thawed at 30°C and resuspended to a total volume of 245 cm^3 in pH 6.8 buffer to which 0.5 mg DNAse had been added. The mixture was stirred in an ice bath and sonicated with the aid of a Bronwill Biosonik IV sonicator equipped with a 3/4" probe and set at high power. Sonication was carried out in 6 one minute bursts with one minute of cooling between each burst. The mixture was then assayed and the total activity recovered was 98% of the original. The mixture was centrifuged for 20 minutes at 9000 rpm in a GS-3 rotor. The supernatant was assayed and found to contain 10% of the recovered activity. Cell breakage was therefore poor.

4) <u>Ballistic Disintegration with Glass Beads</u>[84]

This method of cell disruption relies on the violent agitation of a cell suspension with small glass beads. The shear forces generated by the rapidly moving beads disrupt the cell walls.

A cell pellet (prepared as for 3, above) was thawed at 30°C and resuspended to a total volume of 400 cm^3 in pH 6.8 buffer to which 0.5 mg DNAse had been added. 100 g of 0.45-0.50 mm glass beads were added to the mixture. It was then blended in a precooled (0°C) three litre Waring commercial blender at high speed in two one minute bursts with one minute of cooling between the bursts. After centrifugation for 20 minutes at 9000 rpm in a GS-3 rotor the supernatant was assayed and found to contain 6% of the total activity. It was not possible to resuspend the pellet completely and thus ascertain the percentage recovery of activity. The cell breakage was therefore poor.

These results are summarized below, Table IV.

Table IV: Comparison of Cell Breakage Techniques.

Method	Percentage Activity Recovered	Percentage Cell Breakage (based on recovered activity)
Lysozyme and Freeze/Thaw	67%	23%
French Pressure Cell	91%	80%
Sonication	98%	10%
Ballistic Disintegration	-	approx. 6%

N.B.   a) Precooling was carried out in the latter three techniques to
           minimise heating effects associated with the shear forces
           generated.

       b) The DNA liberated from disrupted cells caused the mixture to be
           both viscous and mucilaginous. It was difficult to handle and
           an attempt was made to pass the mixture through a 21 gauge
           needle in order to break up the DNA by shear force. This was
           not practical because of the volumes involved and DNAse had to
           be added in order to hydrolyse the DNA polymer and thus reduce
           the viscosity.

The French pressure cell is obviously the method of choice. For
cultures from which β-glucosidase isolation was attempted, the following
methodology was followed. The cell pellet was thawed at 30°C and resus-
pended to a final volume of from 200-400 cm^3 in pH 6.8 buffer. 0.5 mg
DNAse and 0.1 mg of the protease inhibitor[85] phenylmethylsulphonyl
fluoride (PMSF) were added per 100 cm^3 of cell suspension. The mixture
was then sonicated twice, as described above, for 2.5 minutes. If the
sonication step was omitted the nylon valve in the pressure cell was found
to be worn away after processing only 30-50 cm^3 of the cell suspension.
The mixture was then twice passed through a 30 cm^3, self filling, pre-
cooled (0°C) French pressure cell at 1250 lb/in^2. This procedure and all
subsequent procedures were carried out at 0-4°C. The cell debris was
removed by centrifugation at 9000 rpm for 40 minutes on a GS-3 rotor. The
supernatant was assayed, the pellet resuspended in buffer and again
centrifuged down. This 'washing' of the pellet was repeated until the

amount of β-glucosidase activity liberated into the supernatant was less than 2% of that obtained from the first extraction. The supernatants were combined and this cell free extract was used immediately for subsequent isolation of β-glucosidase.

II.3 Purification and Isolation of β-Glucosidase from A. faecalis and Related Details

II.3.1 General Methods

Ammonium sulphate used was Canadian Scientific Products ultra pure special enzyme grade. 3-(N-Morpholino)-propanesulphonic acid (MOPS), triethanolamine, myo-inositol, streptomycin sulphate and cellobiose were obtained from the Sigma Chemical company. Electrophoresis reagents were obtained from BioRad with the exception of sodium dodecyl sulphate which was obtained from the Sigma Chemical Company. All other chemicals not previously described were general purpose reagent grade.

Buffers refer to 100 mM sodium phosphate buffers prepared as described (II.2.1) unless otherwise specified. Buffers other than sodium phosphate buffers were prepared by back titration of the buffering agent with either sodium hydroxide or hydrochloric acid as appropriate.

All water used was double deionised (except FPLC grade water).

Ultracentrifugation was carried out on a Beckman L-350 ultracentrifuge equipped with a 45-TI rotor and centrifugation was carried out as previously described (II.2.1).

DE-52 ion exchange resin and Sephacryl S-200 gel chromatography resin were packed and prepared, according to the manufacturers instructions, in LKB 2137 chromatography columns. Column dimensions are described in the main body of the text. All columns (excluding FPLC columns) were run in the cold room at 4°C. A constant flow rate was

maintained with a LKB 2132 Microperpex peristaltic pump. Eluent was monitored at 280 nm with a LKB 2138 Uvicord-S single beam UV monitor linked to a LKB 2210 single channel flat bed recorder. Fractions were collected with the aid of a LKB 2112 fraction collector.

DE-52 is a strong anion exchanger and the species immobilised is diethylaminoethyl. The DE-52 column was cleaned after each use by flushing with two bed volumes of the appropriate buffer containing 10% sodium chloride followed by at least three bed volumes of buffer containing no sodium chloride. After two or three runs a black band built up at the top of the column (possibly contaminant from the water purification treatment) and resolution decreased. When this happened the black portion of the packing material was discarded and the column repacked.

Both the DE-52 and S-200 columns were stored in solution containing 0.02% sodium azide (to prevent microbial growth) when not in use for periods longer than one week.

The fast protein liquid chromatography (FPLC) system is supplied by Pharmacia Fine Chemicals as a complete medium pressure chromatography unit optimized for use with MonoBead high resolution packing materials. The columns are supplied prepacked and include a cation exchange column, Mono S, any anion exchange column, Mono Q, a chromatofocussing column, Mono P and a gel chromatography column, Superose 12. Because of the short run times involved in the use of this system (of the order of 30-180 minutes) runs were carried out at room temperature. Water used was

distilled, double deionised, redistilled and filtered through Millipore

0.22 μm Fluoropore filters. If simply double deionised and filtered water

was used an intractable brown band built up at the top of the column. All

samples were filtered through 0.22 μm filters before application to the

column. Column regeneration and run procedures used were as recommended

by the manufacturer.

Fractions collected were assayed for β-glucosidase activity as

follows. 10μl aliquots of 5mM PNPG in 100 mM pH 6.8 sodium phosphate

buffer were spotted onto white waxed paper (Benchcote) and 10 μL of each

fraction added. If β-glucosidase activity was present a yellow colour

developed after between 30 seconds and 20 minutes. This assay was found

to be sensitive down to a least 0.025 units/cm^3. Following this the

enzyme activity in those fractions containing β-glucosidase was quantified

as described (II.2.1) with 5-10 μL samples. Units of activity expressed

on chromatograms are increase in absorbance measured at 400 nm in 50

seconds in a standard assay cuvette (II.2.1).

The units of specific activity are units of β-glucosidase activity

(previously defined II.2.1) per mg of protein.

Protein was assayed with the aid of BioRad protein assay kit

(based on measured absorbance upon binding of the dye Coomasie blue)

following the manufacturers instructions, using bovine serum albumin

(BSA) to construct a calibration curve. All assays were carried out in

duplicate with appropriate dilutions of the sample being assayed.

Dialysis was carried out in the cold room at 4°C in either 10 or

25 mm Spectrapor dialysis tubing with a molecular weight cut off of
12,000-14,000 Daltons. Prior to use the tubing was twice boiled in double
deionised water.

Sodium dodecyl sulphate denaturing polyacrylamide gel electro-
phoresis (SDS-PAGE) was carried out in a discontinuous Laemmli system as
described by Hames[86] using a BioRad Protean dual vertical slab gel
electrophoresis cell and a BioRad 500/200 power supply. The slab gels
prepared were 15% acrylamide, 1.5 mm thick and prepared according to
Hames[86]. The samples were diluted to allow loading of between 5 and 50 μg
of protein and pre-treated as described[86]. The gels were developed
through the stacking gel at 25 mA constant current and through the
resolving gel at 50 mA constant current. In order to determine which band
corresponded to β-glucosidase some samples were not boiled during
pre-treatment in order to avoid complete denaturation and the following
procedure applied. After the gel was run, and prior to Coomasie blue
staining, a piece of Whatman No.1 filter paper which had been soaked in 5
mM PNPG (in 100 mM pH 6.8 sodium phosphate buffer) was placed against the
gel and incubated at 37°C. After 30 minutes yellow bands were visible
which corresponded to protein bands on the subsequently stained gel.
There was no difference in band pattern between otherwise identical boiled
and unboiled samples.

The gels were stained for protein with Coomasie blue dye as
described[86].

Other materials and methods have been described previously.

II.3.2 Purification and Isolation of β-Glucosidase from A. faecalis

The method used for the purification and isolation of β-glucosidase from A. faecalis is based on that of Han and Srinivasan[57]. The method was modified over eight attempts, two of which were successful. The successful procedure is summarised below, Fig. 20. A discussion of unsuccessful techniques tried, the rationale behind the modifications and the slight differences between the two successful procedures follows a detailed description of the method used for the purification of the β-glucosidase on which most of the characterisation was carried out.

The cell pellet from a late log phase culture grown in Luria broth (II.2.2) was thawed at 30°C and resuspended to a total volume of 330 cm^3 in pH 6.8 buffer and found to contain 1250 units of β-glucosidase activity. Cell free extract was prepared as described (II.2.4) and after washing the pellet twice 950 units were found to be associated with the combined supernatants and 28 units with the cell pellet. The cell free extract (450 cm^3) was cooled to 0°C and all subsequent operations were carried out at 0-4°C with the exception of the FPLC steps (room temperature).

To the stirred cooled solution streptomycin sulphate (6.75 g, 1.5% w/v) was slowly added and the stirring continued for 15 minutes after

Cell pellet

preparation of cell free extract

cell debris (discard)   cell free extract

1.5% streptomycin sulphate

precipitate (discard)   supernatant

35-55% $(NH_4)_2SO_4$ cut

precipitate

- redissolve

- dialyse

- DE-52 column

- S-200 column

'Mono-Q' column:

-1) phosphate

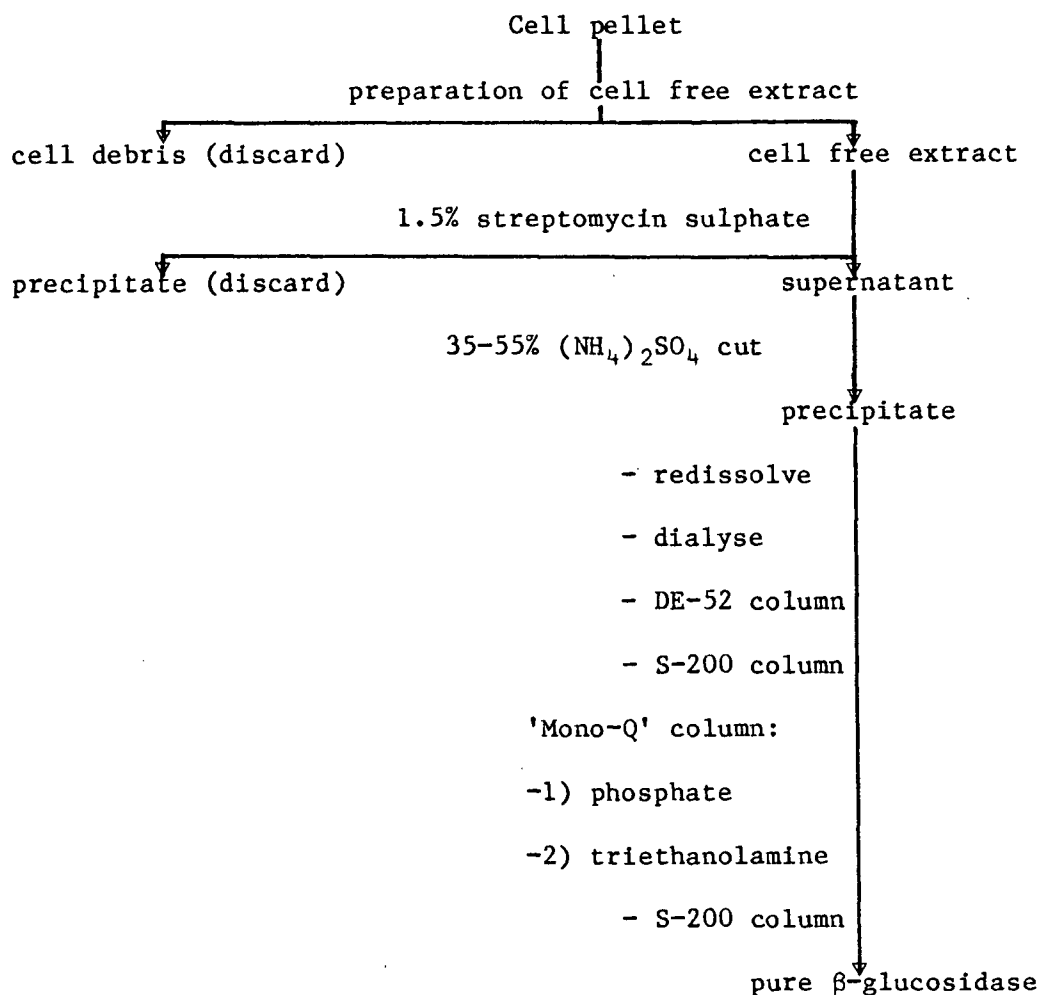-2) triethanolamine

- S-200 column

pure β-glucosidase

Figure 20:   Flow Chart for the Isolation and Purification of

β-Glucosidase from A. Faecalis.

addition was complete. The mixture was centrifuged for 60 minutes at

35,000 rpm in a TI-45 rotor and the precipitated DNA and RNA discarded.

The supernatant, containing 903 units of activity, was diluted to

610 cm^3 with double deionized water.

Ammonium sulphate was ground to a fine powder with a pestle and mortar and 127.5 g slowly added to the stirred cooled solution to give a 35% saturated solution[44]. Stirring was continued for 30 minutes after addition was complete and the mixture then centrifuged for 25 minutes at 9000 rpm in a GS-3 rotor. The precipitated protein pellet was redissolved in 100 cm pH 6.8 buffer and found to contain 54 units of activity. It was discarded. To the cooled stirred supernatant was slowly added a further 82 g ammonium sulphate to give a 55% saturated solution[44]. Stirring was continued for 30 minutes after addition was complete and the mixture again centrifuged for 25 minutes at 9000 rpm in a GS-3 rotor. The supernatant, containing 13 units in 610 cm^3, was discarded. The precipitated protein pellet was redissolved in a minimum volume of 122 cm^3 of 20 mM pH 7.4 buffer containing 10 $\mu g/cm^3$ PMSF and found to contain 670 units of activity. This solution was dialysed against 8 litres of the same buffer for 13 hours at which time 683 units were found in the 160 cm^3 solution.

The solution was centrifuged for 20 minutes at 9000 rpm in a GS-3 rotor to remove residual undissolved contaminants and loaded onto a DE-52 ion-exchange column (55 cm x 2 cm^2, pre-equilibrated with 20 mM pH 7.4 buffer) at 55 cm^3/hour. The sample was washed in (at 55 cm^3/hour) successively, with 100 cm^3 20 mM pH 7.4 buffer and 400 cm^3 of the same buffer containing 1.0% sodium chloride. The combined eluent from these washings contained 78 units of activity and was discarded. The column was washed with a further 500 cm^3 of 20 mM pH 7.4 buffer containing 1.0%

sodium chloride. The washings contained less than 1 unit of β-glucosidase

activity and most of the unbound and partially bound protein appeared to

have been washed off the column (as monitored by absorbance at 280 nm).

The column was developed with 20 mM pH 7.4 buffer containing 2.0% sodium

chloride at a flow rate of 10 cm^3/hour. 3.3 cm^3 fractions were collected

and the eluent was monitored at 280 nm at 2 OD full scale with a 3 mm

pathlength flow through cell. The fractions containing most of the enzyme

activity were combined (fractions 33-40 inclusive, Fig. 21), the tubes

being rinsed with 20 mM pH 7.4 buffer.

The fractions combined, containing 485 units of activity in 23.7

cm^3 were loaded (at 25 cm^3/hour) onto a Sephacryl S-200 gel chromatography

column (57 cm x 5.3 cm^2, pre-equilibrated with 20 mM pH 7.4 buffer). The

column was developed with 20 mM pH 7.4 buffer at 12 cm^3/hour. 4.0 cm^3

fractions were collected and the effluent was monitored at 280 nm at 0.5

OD full scale with a 3 mm pathlength flow through cell. The fractions

containing most of the enzyme activity were combined (fractions 33-43

inclusive, Fig. 22), the tubes being rinsed with 20 mM pH 7.4 buffer.

The fractions combined, containing 467 units of activity in 35 cm^3

were filtered through a Millipore Milex-GS 0.22 μm single use filter. The

solution was split into four equal portions and each was run separately
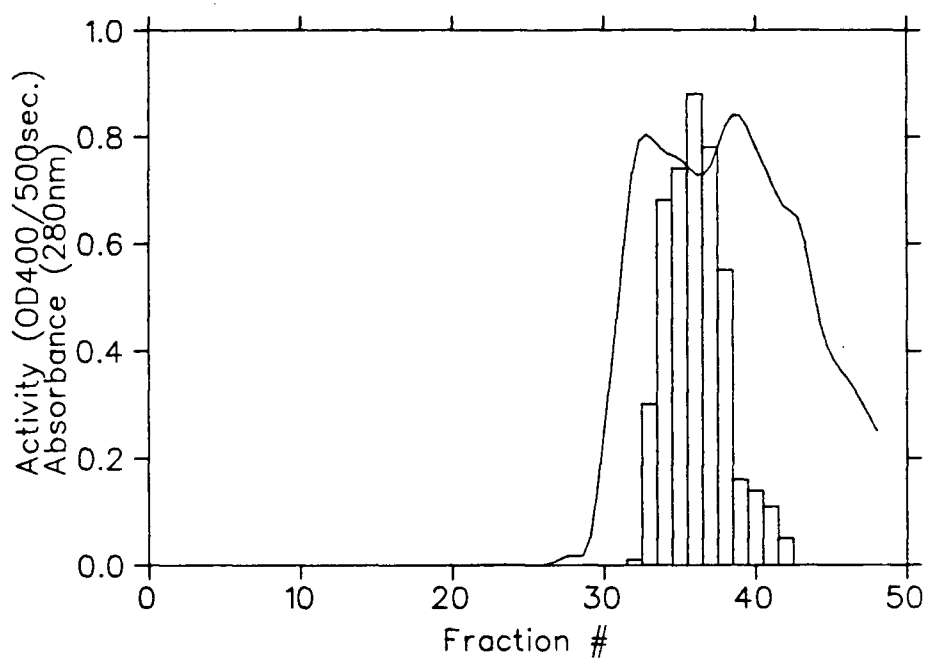
Figure 21: Protein/Activity Profile for DE-52 Chromatography

Step.
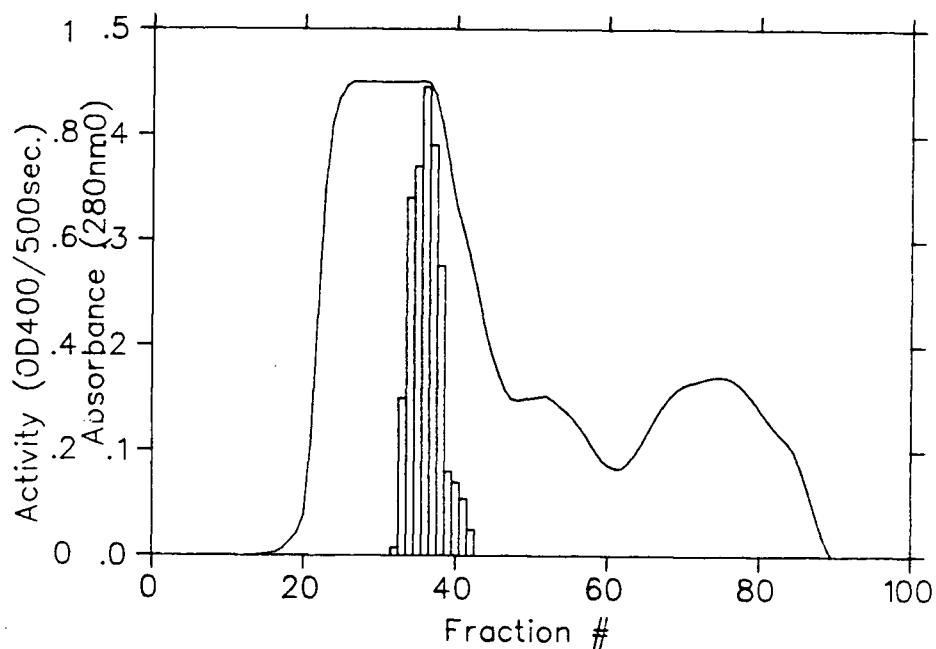
Figure 22: Protein/Activity Profile for First S-200

Chromatography Step.
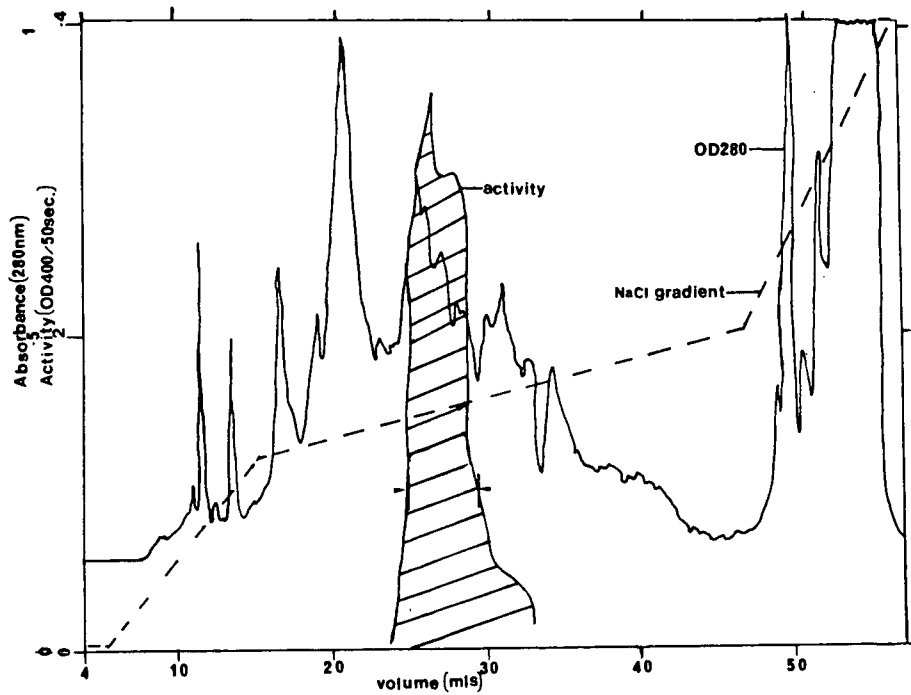
Figure 23:   Protein/Activity Profile for Mono Q (Phosphate)

Chromatography Step.
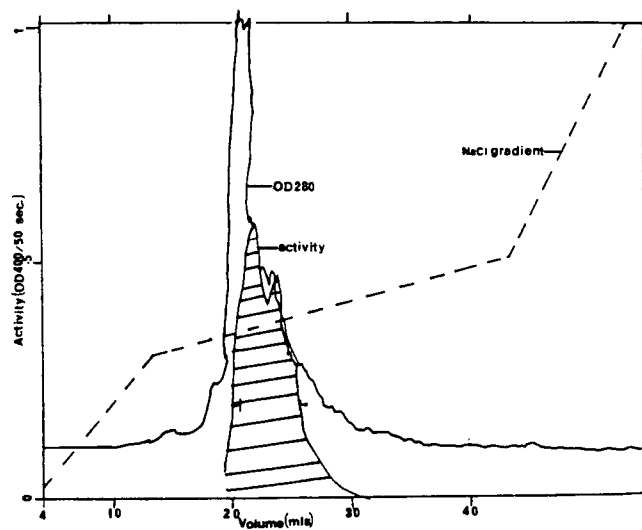
Figure 24:   Protein/Activity Profile for Mono Q (Triethanolamine)

Chromatography Step.
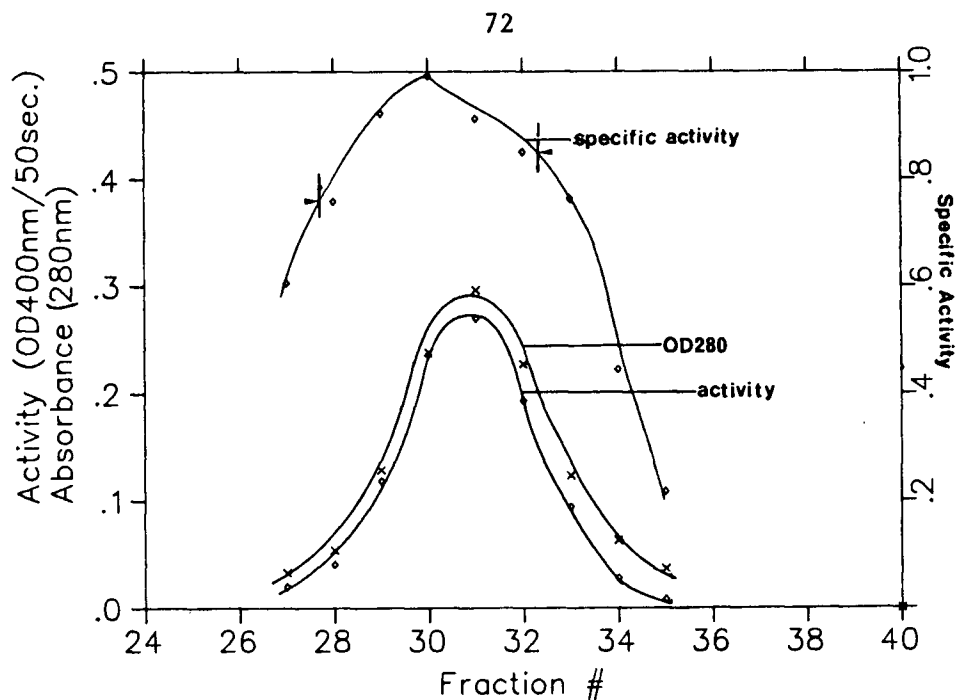
Figure 25: Protein/Activity Profile for Second S-200

Chromatography Step.

on the FPLC Mono Q anion exchange column (to avoid overloading the

column). The samples were loaded onto the column (5.0 cm x 0.36 cm^2,

pre-equilibrated with 20 mM pH 7.4 buffer) from a 10 cm^3 Superloop at 2

cm^3/minute. The column was developed at 2 cm^3/minute with a 0-5% sodium

chloride gradient in 20 mM pH 7.4 buffer and the eluent monitored at 280

nm at 1.0 OD full scale with a 10 mm pathlength flowthrough cell. Protein

profile (ie. absorbance at 280 nm), activity profile and the fractions

retained are shown below (Fig. 23) for one run. The fractions containing

most of the β-glucosidase were combined and the tubes rinsed with 20 mM pH

7.4 buffer.

The fractions combined, containing 249 units of activity in 29 cm^3 were loaded back onto the Mono Q column from a 50 cm^3 Superloop at 2 cm^3/minute and washed in with 30 cm^3 20 mM pH 7.4 triethanolamine buffer to reequilibrate the column. The column was developed with a 0-5% sodium chloride gradient in the triethanolamine buffer (Fig. 24). The fractions containing most of the β-glucosidase were combined and the tubes rinsed with 20 mM pH 7.4 buffer (sodium phosphate).

The fractions combined, containing 177 units of activity in 7.9 cm^3 were loaded and run on a Sephacryl S-200 gel chromatography column as described above. The protein was eluted as a single symmetrical 'peak'. The specific activity profile (enzyme activity/absorbance at 280 nm) together with the activity and protein (ie. absorbance at 280 nm) profiles are shown, Fig. 25. The fractions of highest specific activity were combined and this material, containing 98 units of actvity in 15.4 cm^3 was used for most of the characterisation carried out on β-glucosidase from A. faecalis.

A purification table for the whole procedure is shown below, Table V.

Table V: Purification of β-Glucosidase from A. faecalis.

	Purification step	Protein (mg/cm^3)	Volume (cm^3)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
1.	Streptomycin sulphate ppt	409	610	25000	903	0.036	--	(100)
2.	35-55% $(NH_4)_2SO_4$	109	122	13300	670	0.050	1.4	74
3.	DE 52	7	23.7	166	485	2.92	58	54
4.	S-200	1.6	35	56	467	8.34	2.9	52
5.	Mono-0 Phosphate	0.42	29	12.1	249	20.6	2.5	28
6.	Mono Q Triethanolamine	—	7.9	--	177	--	--	20
7.	S-200	0.20	15.4	3.1	98	31.6	1.5	11

N.B. a) Protein was assayed using a BioRad protein assay kit with bovine
serum albumin as standard.

b) The high loss of activity at the Mono Q (phosphate) step was
probably due to the large surface area of glass to which the
enzyme was exposed. It is not unusual for proteins to be
denatured by exposure to glass surfaces.[88]

c) The apparent high loss of activity at the final S-200 step was
because only fractions having the highest specific activity were
taken.

POSSIBLE HIGH MOL. WT. IMPURITY

MOL. WT. MARKERS

PHOSPHORYLASE b

AFTER STEP **1 2 3 4**   **7 7**
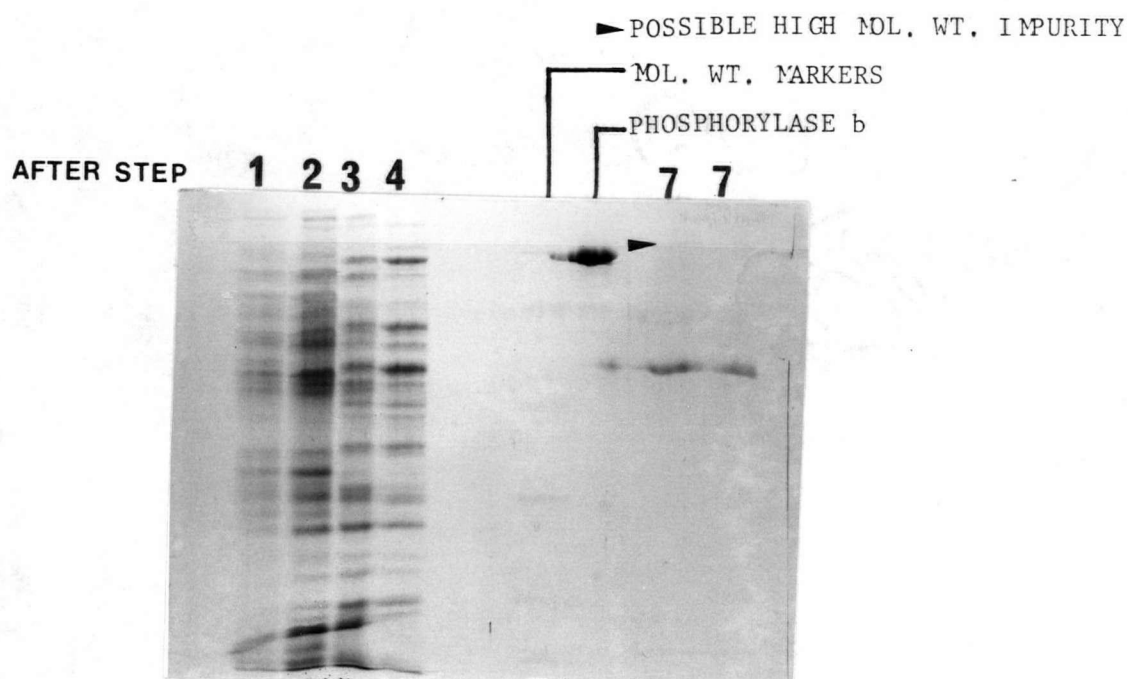
Figure 26: SDS-PAGE Gel of Pure and Partially Pure
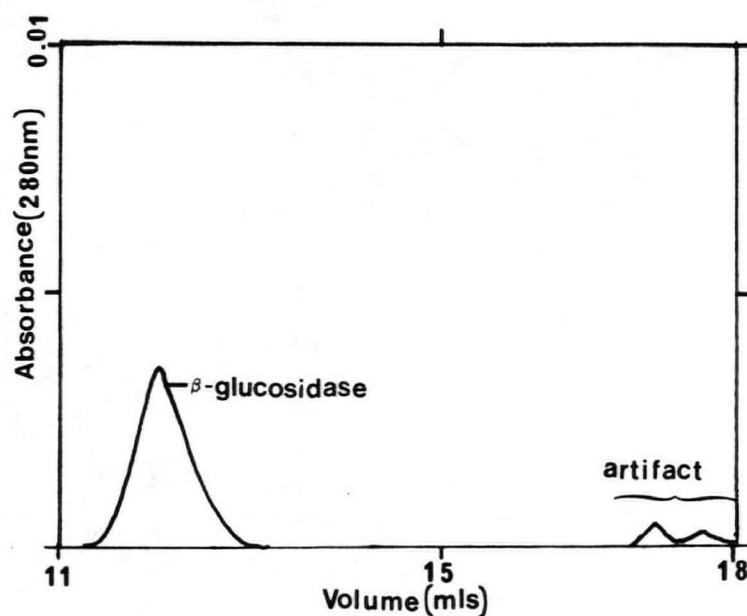
β-Glucosidase.

Figure 27: Analytical Gel Chromatography of Pure β-Glucosidase on

Superose 12 Column.

The protein was shown to be essentially homogeneous by SDS-polyacrylamide gel electrophoresis, Fig. 26, and by analytical gel chromatography on a MonoBead Superose 12 column, Fig. 27. The final preparation (6 and 9 µg protein loading) ran as one major band on a 15% acrylamide SDS gel with a small high molecular weight impurity band (marked on Fig. 26). The major protein band was shown to have a high β-glucosidase activity as described (II.3.1). Some of the cruder preparations were also run, Fig. 26. The conditions under which the gel was run are described elsewhere (II.3.1) as is the determination of sub-unit molecular weight on the same gel (II.4). The final preparation ran as a single symmetrical peak on the Superose 12 column with two artifacts of low apparent molecular weight, Fig. 27. The β-glucosidase also had an apparent molecular weight lower than expected and this is discussed elsewhere (II.4.2). The conditions are described in II.4.2.

Some other points that should be noted about this procedure are as follows.

1: The activity 'shoulder' observed on both of the Mono Q FPLC chromatograms may be due to: a) unrelated contaminating protein with β-glucosidic activity, b) sub-units of the (possibly) oligomeric β-glucosidase, c) partially hydrolysed β-glucosidase still retaining some activity or d) an artifact of the column. a) is unlikely as one of the other steps would most probably have resolved different proteins. It is not easy to distinguish between the latter three

possibilities.

2: The 'breakthrough' of 78 units on loading the sample during the DE-52 step is due to the intrinsic high ionic strength of the protein sample and could have been reduced by further dilution prior to this step.

3: A previous purification, identical except for the reversal of the two Mono Q steps and the omission of the final S-200 step, yielded β-glucosidase which ran as a single band on SDS-PAGE and had a specific activity of 45.7 units/mg. (cf. 31.6 units/mg). The reason for the higher specific activity and easier purification is not obvious (see III).

II.3.3 __Method Development__

The ammonium sulphate 'cut' found to give the maximum activity was 35-55% and not 40-60% as reported[57]. This may be due to the use of different tables of ammonium sulphate saturation than used in the reported preparation[57]. Published tables of ammonium sulphate saturation often give different values (dependent on the conditions under which they were measured).

Dialysis followed by ion exchange chromatography was chosen as the first step after ammonium sulphate precipitation, rather than gel filtration, because although the latter technique lowers the ionic strength concomitantly with effecting a purification, the resolution is dependent on small sample volume. The resolution in ion exchange chromatography is

essentially independent of sample volume and will, in addition to purification, effect a concentration of dilute samples.

The ion exchange column was developed isocratically after it was fortuitously observed during an early isolation attempt, when washing a sample in at 2% sodium chloride (as described[57]), that the β-glucosidase activity was eluted with resolution of the protein into 'peaks' (as monitored at 280 nm). This suggested that the β-glucosidase was partially bound to the column at this ionic strength and 'true chromatography' (partitioning between a mobile and stationary phase) was taking place.

Conditions for ion exchange chromatography on the FPLC Mono Q column were determined as follows. The buffers shown were prepared, Table VI. With the exception of 20 mM pH 7.4 sodium phosphate these are the buffers recommended by the manufacturer for use with the Mono Q column. A partially pure β-glucosidase preparation (specific activity = 10.1 units/mg) containing 2.3 units/cm^3 in 20 mM pH 7.4 sodium phosphate was divided into 0.5 cm^3 aliquots which were dialysed against 1 litre of each of the buffers (except pH 7.4 phosphate buffer) overnight. These aliquots were then run with a 0-5% linear sodium chloride gradient on the Mono Q column pre-equilibrated with buffers of the appropriate pH.

Table VI: Buffers Used in Determination of Conditions for Mono Q Chromatography.

Buffering agent	pH	Ionic strength (mM)
3-(N-Morpholino)-propanesulphonic acid	6.60	50
"	6.90	"
"	7.20	"
Triethanolamine	7.40	"
"	7.60	"
Sodium phosphate	7.40	20
"	6.90	"

Run conditions were as recommended by the manufacturer. The samples run at pH 6.6 and 7.6 were completely inactivated indicating that the enzyme is denatured at these pH's. The samples run at intermediate pH'S had essentially identical chromatographic profiles, the greatest difference being between the samples run at pH 7.4 in triethanolamine and sodium phosphate buffers. It was therefore decided to carry out the separation in the latter two buffers.

II.3.4 Enzyme Storage

A pure β-glucosidase sample with a specific activity of 45.7 units/mg containing 18.3 units/cm^3 in pH 7.4 20 mM buffer was split into two equal portions. One portion was made up to 1% w/v with myo-inositol,

frozen in liquid nitrogen, lyophilised and stored in the freezer at -20°C. 1% w/v myo-inositol was suggested to give complete protection of activity against lyophilisation[57]. The other portion was stored in solution under toluene vapour at 4°C in the refrigerator. After two months the lyophilised sample was made up to 1.0 cm^3 in 20 mM pH 7.4 buffer and assayed. It was found to contain 62% of the original activity. The sample stored under toluene vapour was found to contain 83% of the original activity. Myo-inositol showed no measurable inhibition of β-glucosidase activity at 2% w/v. Storage under toluene vapour appears to be the best method of medium term storage.

II.3.5 Unsuccessful Purification Techniques Attempted

1) Mono S Cation Exchange Chromatography

A partially pure β-glucosidase preparation (2.0 cm^3) was dialysed against 1 litre of 10 mM pH 6.7 (the lowest pH at which the enzyme is stable) buffer and applied to a Mono S cation exchange column (5.0 cm x 0.36 cm^2, pre-equilibrated with 20 mM pH 6.7 buffer). The enzyme was eluted in one bed volume of start buffer. This method was therefore of no utility in the purification of β-glucosidase.

2)   Chromatofocussing

A pure β-glucosidase sample (as described above, II.3.2) was dia-
lysed against the start buffer recommended by the manufacturer for chroma-
tofocussing on a FPLC Mono P column from pH 7-4. The column was developed
according to the manufacturers instructions. A single symmetrical protein
peak (monitored at 280 nm) was eluted at pH ~ 5 No β-glucosidase activity
was eluted at any pH (assays carried out at pH 7).

3)   Affinity Adsorption Chromatography

Approximately 1 cm^3 of Pierce Selectin 13 affinity support was
packed in a Pasteur pipette plugged with glass wool. This material con-
sists of cellobiose immobilised, with no spacer arm, onto a solid support
matrix of agarose at a concentration of 35-40 μmoles/cm^3 gel. This column
was equilibrated with 20 mM pH 7.4 buffer and a pure β-glucosidase sample
(as described above, II.3.2) containing 9 units of activity was applied.
All of the activity was eluted in one bed volume of start buffer. This
method was therefore of no utility in the purification of β-glucosidase.

4)   Affinity Elution Chromatography

It was hoped that addition of β-glucosidase substrates to the
developing buffer of an ion exchange column would specifically elute the

β-glucosidase by deformation and/or neutralisation of charge at the active site.

A partially pure β-glucosidase sample containing 186 units in 75 cm^3 of 20 mM pH 7.4 buffer and with a specific activity of 16 units/mg was loaded onto a DE-52 column (55 cm x 2 cm^2, pre-equilibrated with 20 mM pH 7.4 buffer) as described (II.3.2). The sample was washed in with 1% sodium chloride in start buffer. After all unbound protein had been eluted the column was developed with wash buffer which had been made up to 20 mM with cellobiose. No protein (monitored at 280 nm) or β-glucosidase activity was eluted in 500 cm^3. The column was then developed with 20 mM pH 7.4 buffer containing 0.68% sodium chloride and 0.32% β-D-gluco-pyranosyl pyridinium bromide (total salt concentration = 1%). No protein or β-glucosidase activity was eluted in 500 cm^3. Comparison of the UV spectra of the buffer before and after passage through the column, Fig. 28, indicated that no appreciable hydrolysis of β-D-glucopyranosyl pyridinium bromide had taken place. Development of the column with buffer containing 0.32% β-D-glucopyranosyl pyridinium bromide and 1.18% sodium chloride eluted a small amount of β-glucosidase activity in a large volume (~ 200 cm^3). This had previously been observed during development with buffer containing 1.5% sodium chloride. The β-glucosidase was finally eluted with buffer containing 1.68% sodium chloride and 0.32% β-D-glucopyranosyl pyridinium bromide. Therefore addition of β-glucosidase substrates to the developing buffer was no improvement on the use of buffers containing sodium chloride alone.
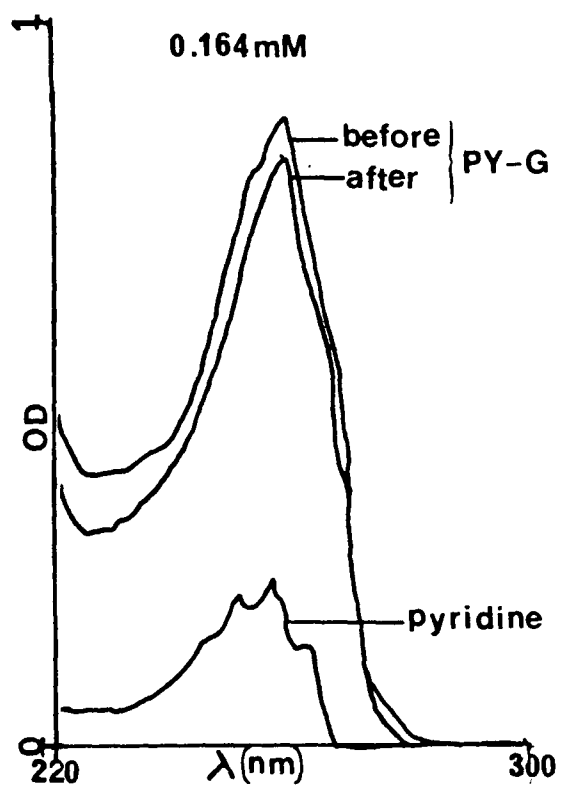
0.164mM

OD

before
after } PY-G

pyridine

λ(nm)
220   300

Figure 28:   UV Spectra of Affinity Elution Buffer Before and After

Passage Through A DE-52 Column Containing β-Glucosidase.

II.4   Characterisation of β-Glucosidase from A. Faecalis

II.4.1   General Methods

All kinetic measurements were made in 100 mM pH 6.8 sodium phosphate buffer at 37°C with the exception of glucosyl fluoride which was made in triethanolamine buffer.

The β-glucosidase used was that prepared as described in detail (II.3.2).

Water used was double deionised. All substrates and inhibitors not synthesised or obtained as already described were obtained from the Sigma Chemical Company with the following exceptions. 2,4-Dinitrophenyl-β-D-glucopyranoside was prepared by Dr. Paul Bird (UBC). Cellotriose, cellotetraose and cellopentaose were prepared by Neil Gilkes (UBC).

All volumes were measured with grade A analytical volumetric glassware and/or Hamilton Microlitre syringes and weights were measured on micro or semi-micro balances to at least four decimal places.

All samples were pre-equilibrated to 37°C ± 0.1°C for at least 10 minutes in quartz or glass cuvettes in a Pye-Unicam PU-8800 ultra-violet/visible recording spectrophotometer attached to a Julabo VI circulating thermostat bath unless otherwise stated.

Reaction was initiated by addition of between 20 and 50 μL of an appropriate dilution of β-glucosidase stock solution unless otherwise stated.

Initial reaction rates were, with the exception of the fluoride,

determined by measuring the rate of change of absorbance of the reaction

mixture at an appropriate wavelength in the PU-8800 spectrophotometer.

Extinction coefficients were determined at the appropriate

wavelengths, in duplicate at 37°C in 100 mM pH 6.8 sodium phosphate buffer

for β-D-glucopyranosyl pyridinium bromide/pyridine (distilled from

potassium hydroxide under nitrogen) ($\Delta\epsilon$ = -2925), p-nitrophenol

(recrystallised from 0.01 M hydrochloric acid) ($\Delta\epsilon$ = 7280) and

2,4-dinitrophenol (recrystallised from 0.01 M hydrochloric acid) ($\Delta\epsilon$ =

11,300). The extinction coefficient for 2,4-thiodinitrophenol was

determined (at 400 nm) by allowing a reaction mixture containing 2.956 mM

2,4-dinitrothiophenyl-β-D-glucopyranoside to react for 24 hours in the

presence of β-glucosidase ($\Delta\epsilon$ = 1698). The variation of extinction

coefficient with temperature (at 400 nm) for p-nitrophenol was also

determined, Fig. 29.
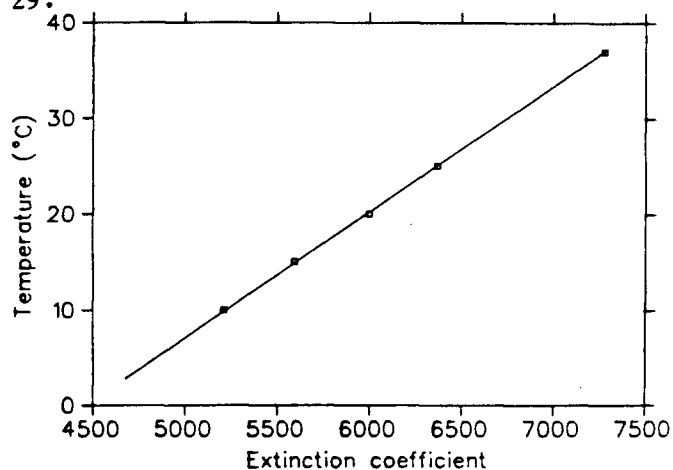
Figure 29: Variation of PNP Extinction Coefficient With

Temperature at pH 6.8.

All other materials and methods not previously described are described in the main body of the text.

II.4.2 Molecular Weight Determination

Sub-unit molecular weight was determined by running a Sigma MW-SDS-200 kit on the SDS-PAGE gel previously described (II.3.1) in a track adjacent to the β-glucosidase. The proteins, sub-unit molecular weights and RF's in the 15% sodium dodecyl sulphate gel are shown below, Table VII.

Table VII: Molecular Weight Markers for SDS-PAGE.

Protein	Sub-unit Molecular weight (Daltons)	Rf
Phosphorylase b	97,000	0.16
Bovine serum albumin	67,000	0.27
Ovalbumin	43,000	0.45
Carbonic anhydrase	30,000	0.68
Trypsin inhibitor	20,100	0.86
α-Lactalbumin	14,400	0.99
β-Glucosidase from A. faecalis	51,500 (determined)	0.40

A plot of log (molecular weight) against RF yielded a straight line from which the sub-unit molecular weight of the unknown protein could be determined, Fig. 30.

An attempt was made to determine the oligomeric molecular weight by using a MonoBead Superose 12 gel chromatography column in conjunction with the FPLC system. Samples (50 µL) were run at 0.25 cm^3/minute in 100 mM pH 6.8 sodium phosphate buffer. Blue dextran (a high molecular weight polysaccharide) and acetone were run to determine the exclusion volume and void volume of the column respectively. The following proteins from a Sigma non-denatured protein molecular weight marker kit were also run, Table VIII.

Table VIII: Molecular Weight Markers for Superose 12 Gel Chromatography.

Protein	Molecular weight (Daltons)	Rf
Bovine serum albumin (dimer)	132,000	1.37
Bovine serum albumin (monomer)	66,000	1.52
Phosphoglucomutase	61,600	1.61
Ovalbumin nhydrase	45,000	1.64
α-Lactalbumin	14,200	1.82
β-Glucosidase from A. faecalis	75,000 (determined)	1.50
Phosphorylase b	194,000	1.44

A logarithmic plot of molecular weight against RF yielded a straight line from which the molecular weight of the unknown protein was determined, Fig. 31. The molecular weight determined for the β-glucosidase was lower
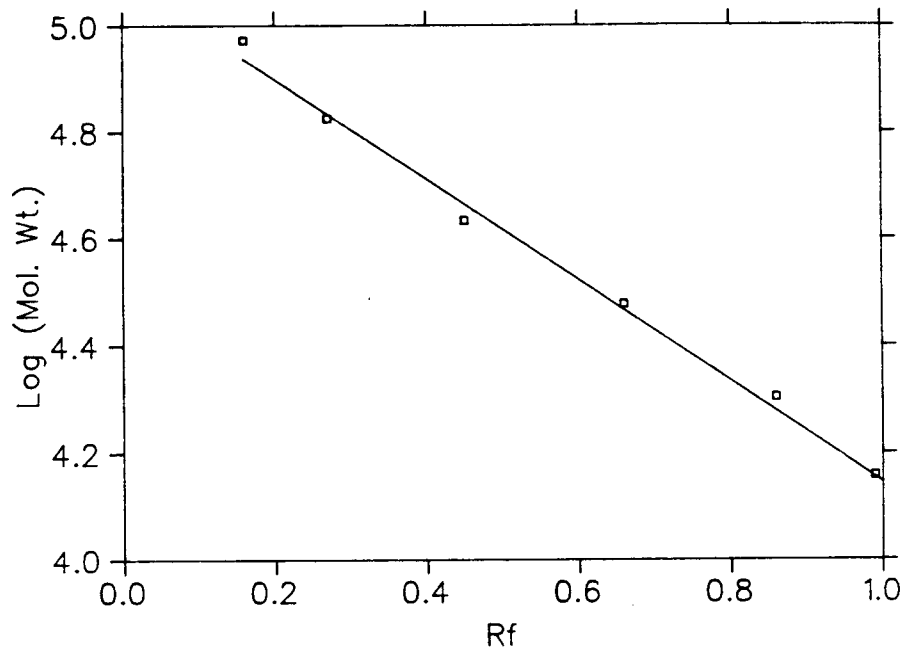
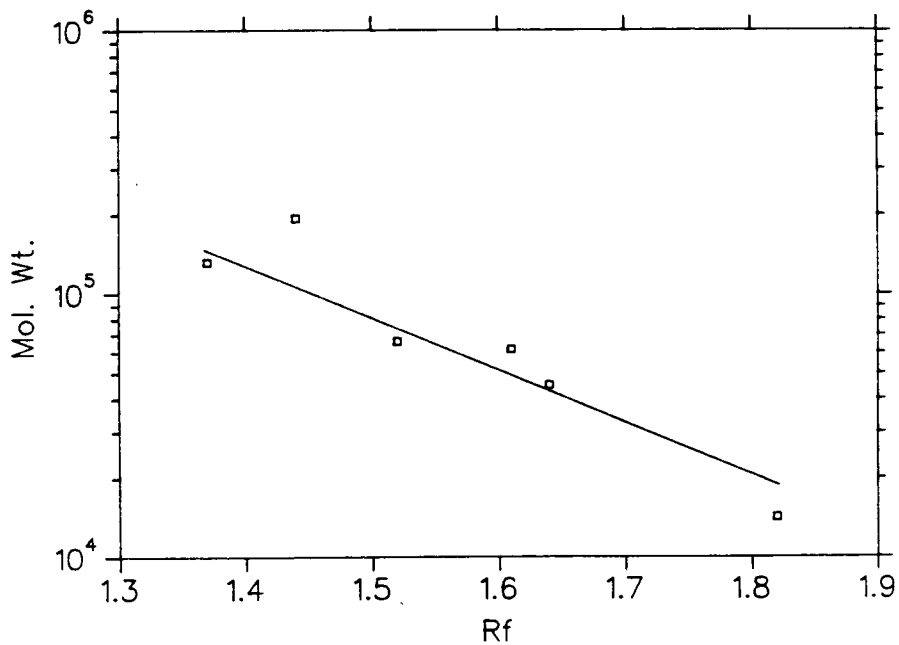Figure 30: Plot of Log (Molecular Weight) Against Rf For

SDS-PAGE.

Figure 31: Logarithmic Plot of Molecular Weight Against Rf For

Superose 12 Gel Chromatography.

than expected[57] at 75,000 Daltons. The apparent molecular weight of

phosphorylase b was 100,000 Daltons and not the known dimeric weight of

194,000 Daltons. Both phosphorylase and β-glucosidase bind oligo-

saccharides and it is probable that both are retarded on the agarose based

support of the Superose 12 column, leading to artificially low apparent

molecular weights. All runs showed two artifactual low molecular weight

peaks at the same Rf's. The reason for this is not clear.

II.4.3 <u>Kinetics</u>

    1:Substrates

    To measure the initial rate of reaction it is necessary to measure

the rate of production of one or more initial products. Three approaches

to this measurement were used in this study:

1) The rate of change of absorbance was monitored at an appropriate wave-

length where the substrate and one of the initial products had sufficient-

ly different extinction coefficients.

2) If the extinction coefficients were not sufficiently different at an

accessible wavelength a coupled assay was used. One of the initial

products (glucose) was a substrate for an enzyme catalysed reaction(s)

which produced a measurable change in absorbance.

3) The rate of production of the initial product of the hydrolysis of

β-D-glucopyranosyl fluoride (fluoride ion) was monitored by an ion-

specific electrode.

    All initial rates were measured at less than 5% reaction (linear

portion), with the exception of the fluoride which was measured as
described in the text.

Wavelengths or methods by which reactions were monitored are shown
below, Table IX.

Table IX: β-Glucosidase Substrates.

Substrate (all β-linked)	Cuvette type and pathlength (mm)	Wavelength (nm)	Reference
p-nitrophenyl-β-D-glucopyranoside	10	400	10
p-nitrophenyl-β-D-galactopyranoside	10	400	18
p-nitrophenyl-β-D-mannopyranoside	10(m)	400	18
β-D-glucopyranosyl azide	10(m)	245+G	87
β-D-glucopyranosyl pyridinium bromide	2	265	17
2,4-dinitrophenyl-β-D-glucopyranoside	10	400	--
2,4-dinitrophenyl-β-D-1- thioglucopyranoside	10(m)	400	--
β-D-glucopyranosyl fluoride	--	electrode	--
cellobiose	10(sm)	G	--
cellotriose	10(m)	G	--
cellotetraose	10(m)	G	--
cellopentaose	10(m)	G	--
lactose	10(sm)	G	--
sucrose	10(sm)	G	--
salicin	10(sm)	G	--
gentobiose	10(sm)	G	--

N.B. a) m indicates that micro cuvettes were used (absorbance could be measured on a volume as low as 0.22 cm^3) and sm designates that semi-micro cuvettes were used (nominal 1.0 cm^3). All other cuvettes used held a nominal 3 cm^3.

b) G indicates that a coupled glucose assay (described in text) was used to measure the rate of release of glucose.

c) Electrode indicates that an ion specific electrode was used to measure the rate of hydrolysis of this substrate.

d) 0.25-0.50% Bovine serum albumin (Sigma A 7906 or A 4378) was added to all reaction mixtures (except the fluoride, XVII). If bovine serum albumin was not added, a time dependent loss of enzyme activity was observed on dilution into the cuvette (particularly when dilute enzyme stock solution was used). This loss of activity is due to denaturation of enzyme on the glass surface and has been observed before[88] with other β-glucosidases at high dilution. A comparison of kinetic parameters in the presence and absence of bovine serum albumin was made with PNPG and no difference was found.

e) All β-glucosidase dilutions were made into 100 mM pH 6.8 sodium phosphate buffer containing 1% bovine serum albumin.

f) Before measuring kinetic parameters the β-glucosidase stock solution was standardised by measuring its activity against a reference of 1.34 mM PNPG (in duplicate) under the conditions used for determination of the kinetic parameters of PNPG. The exceptions are PNPGal and PY-G which had their kinetic parameters

determined using stock enzyme solutions that had been used for a full determination of the kinetic parameters of PNPG.

The coupled glucose assay used was based on the following reaction:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-PD}} \text{6-PG} + \text{NADPH}$$
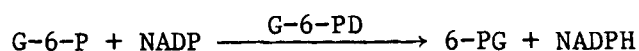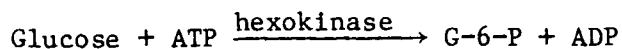
where: ATP is adenosine triphosphate, G-6-P is glucose-6-phosphate, NADP is the cofactor β-nicotinamide adenine dinucleotide phosphate, G-6-PD is glucose-6-phosphate dehydrogenase, 6-PG is 6-phosphogluconic acid, and NADPH is the reduced form of NADP.

The extinction coefficient difference NADPH-NADP at 340 nm is 6220 and the release of glucose can be quantified by the increase in absorbance at this wavelength.

The assay mixture used was: G-6-PD Sigma G-5760, 45 µL containing 90 units of activity; Hexokinase Sigma H-5875, 37 µL containing 150 units of actvity; 0.3 cm^3 12 mM ATP Sigma A-2383, in water; 0.3 cm^3 7 mM NADP Sigma N-0505, in water; 0.3 cm^3 20 mM MgCl$_2$ (AR), in water; and 0.6 cm^3 100 mM pH 6.8 sodium phosphate buffer, diluted no more than 4.3 times in the reaction mix. These proportions are based on those found in the Sigma 16-UV glucose assay.

In order for a coupled assay to effectively reflect the rate of
glucose release the auxiliary enzymes (G-6-PD and hexokinase) must be
present in sufficient excess. To determine this excess the concentration
of assay mix in a standard reaction mixture containing 1.34 mM cellobiose
was increased until further increases in concentration did not increase
the apparent initial reaction rate; i.e. until the initial rate observed
was dependent on the rate of hydrolysis of cellobiose by β-glucosidase and
independent of the concentration of the auxiliary enzymes. On addition of
β-glucosidase to the reaction mixture there was an immediate linear
increase in absorbance and no 'lag phase' could be detected. To further
confirm that the glucose assay was working as expected it was used with a
reaction mixture containing 0.137 mM PNPG. After measuring the relative
contributions of NADPH and p-nitrophenol to absorbance at 340 and 400 nm
the following initial reaction rates were measured:

based on glucose assay,   1.752 nmoles/minute

based on p-nitrophenol release, 1.691 nmoles/minute.

It should also be noted that pre-incubation of reaction mixtures
containing the glucose assay removes any residual glucose present before
hydrolysis is initiated by addition of β-glucosidase.

This assay has been developed independently[29].

The kinetic parameters of β-D-glucopyranosyl fluoride were
determined as follows. An old Orion Ionalyzer fluoride ion specific
electrode was regenerated[89]. The electrode and an Orion single junction
reference electrode were attached to a Radiometer PHM-82 digital pH meter

set to measure milliVolts. The pH meter was attached to a LKB 2210 single

channel flatbed recorder in series with a variable milliVolt supply (to

'zero' the recorder). Solutions were preequilibrated to 37°C in a

polythene beaker in a jacketted glass beaker attached to a Julabo VI

circulating thermostat bath. It was confirmed that the β-glucosidase had

the same activity (against PNPG) in 100 mM pH 6.8 triethanolamine buffer

as in sodium phosphate buffer and all measurements were made in

triethanolamine buffer containing no BSA. Phosphate ions decrease the

sensitivity of the electrode and it was felt that high protein

concentrations might also decrease its sensitivity. The system was

calibrated with 0-360 μM sodium fluoride at 37°C by standard additions,

both before and after the kinetic runs, eg. Fig. 32. The kinetic

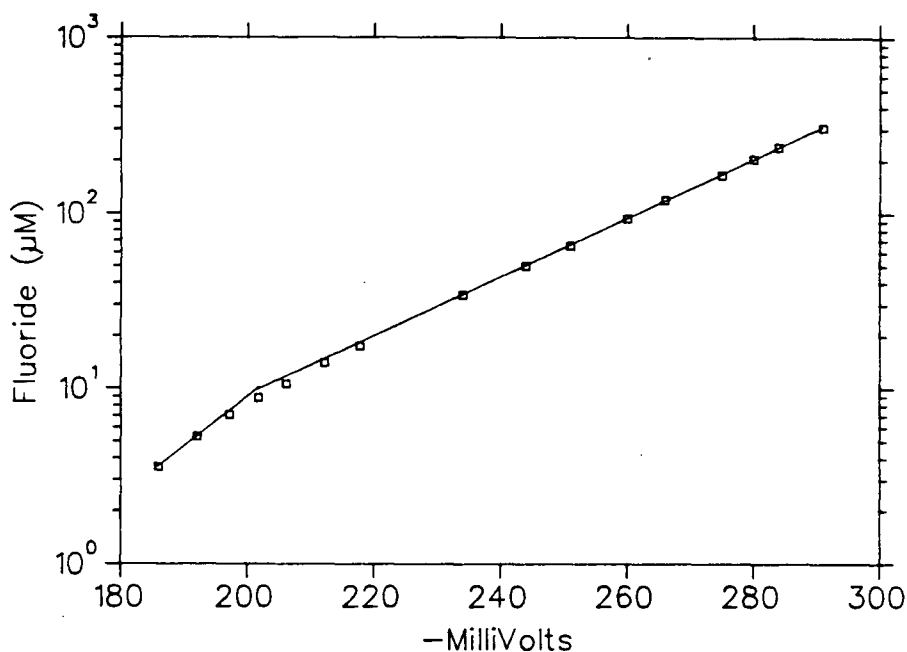parameters were determined by subtraction of the uncatalysed rate of

Figure 32: Calibration Curve For Fluoride Electrode.

hydrolysis of β-D-glucopyranosyl fluoride from the β-glucosidase catalysed
rate for each sample. The substrate concentration at which the rate was
measured was calculated by reference to the calibration curve. There was
a shift of ~18 mV between the calibration curves measured before and after
the kinetic run. This corresponds to a doubling of concentration and the
results obtained may therefore be out by a factor of two.

Attempts to determine the kinetic parameters of p-nitrophenyl-β-D-
cellobioside as a substrate of β-glucosidase by monitoring the release of
p-nitrophenol (as measured by increase in absorbance at 400 nM) yielded
unusual results. The initial reaction rates were not linear but increased
rapidly as the reaction proceeded and the rates observed decreased as
substrate concentration was increased. In an attempt to explain these
observations the inhibition constant, Ki, of this compound against PNPG as
substrate was determined (see 'Inhibitors' below).

In addition the products of the hydrolysis of p-nitrophenyl-β-D-
cellobioside by β-glucosidase were analysed by paper chromatography as
follows. It was confirmed that β-glucosidase was as active (against PNPG)
in a volatile 50 mM pH 6.8 triethylamine buffer as in a 100 mM sodium
phosphate buffer. Two 3 cm^3 aliquots of 1.43 mM p-nitrophenyl-β-D-
cellobioside in triethylamine buffer containing 0.3% BSA were hydrolysed
(by β-glucosidase) to 10% and 20% completion respectively (measured by
monitoring the release of p-nitrophenol as determined by absorbance at 400
nm). The reactions were stopped by heating at 100°C for 1 minute. A
blank containing no β-glucosidase was also heated at 100°C for 1 minute

and no p-nitrophenol was released (as determined by absorbance at 400 nm). The samples were repeatedly frozen (in a dry ice/acetone bath) and lyophilised to remove the volatile buffer. The samples were then redissolved in 40 µL water and 2.5 µL of each sample applied to an 1 m length of Whatman #1 filter paper together with 2.5 µg samples of glucose and cellobiose in order to carry out descending paper chromatography. The chromatogram was developed with ethyl acetate:acetic acid:formic acid:water 18:3:1:4 for 15 hours. The chromatogram was visualised by dipping it successively into solutions of 0.1% silver nitrate in acetone, 2% ethanolic potassium hydroxide and 5% aqueous sodium thiosulphate. This method of visualisation is specific for reducing sugars and did not visualise unreacted glycosides. Only glucose was found in the samples (and no cellobiose, RF relative to glucose = 0.43).

Kinetic parameters for PNPG with a β-glucosidase preparation that had been stored under toluene vapour for three months were also determined and compared with the parameters determined using the fresh preparation. Km was identical within experimental errors.

With PNPG and PNPGal, substrate inhibition was observed and quantified at high substrate concentrations. Where substrate inhibition was observed with other substrates it was not quantified.

2:Inhibitors

Inhibitors of β-glucosidase were quantified against PNPG as substrate at five substrate concentrations and four inhibitor concentrations (plus one determination with no inhibitor). Kinetic parameters were

accurately determined for the following inhibitors: β-D-glucosylamine, α-D-glucose, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-β-D-cellobioside and D-glucono-δ-lactone. The latter inhibitor was rapidly hydrolysed to D-gluconic acid under the reaction conditions used. In order to minimise this hydrolysis concentrated stock solutions were made up in water immediately prior to use and kept in an ice bath and 20-40 μL were added to the reaction mixture immediately prior to initiation of hydrolysis by addition of β-glucosidase. All other inhibitors were pre-equilibrated at 37°C in the reaction mixture prior to addition of the β-glucosidase. The approximate inhibition constants of cellobiose and p-nitrophenyl-β-D-mannopyranoside (PNPMan) were determined at four inhibitor concentrations and a single substrate concentration in order to demonstrate that hydrolysis of these compounds was taking place at the same site or a site interacting with that at which PNPG is hydrolysed.

II.4.4 Miscellaneous Characterisation

1:Effect of β-Glucosidase Concentration

The linearity of initial reaction rate with β-glucosidase concentration was checked. Initial reaction rate for a reaction mixture containing 1.34 mM PNPG run under standard conditions was plotted against β-glucosidase concentration over the range of concentrations used. The relationship was linear.

2:Temperature Dependence

The temperature dependence of the hydrolysis of PNPG was measured

between 5 and 45°C at five substrate concentrations and the activation

energy determined.

3:Metal Ion Sensitivity

The divalent metal ion dependency of the β-glucosidase was

investigated as follows. 1 cm^3 of the stock β-glucosidase solution was

dialysed against 1 litre of 20 mM pH 7.4 sodium phosphate buffer

containing 10 mM EDTA for 40 hours with two changes of buffer. Kinetic

parameters were determined for PNPG at four substrate concentrations. One

determination was carried out in the presence of 10 mM EDTA and the other

in the presence of 5 mM magnesium chloride; both determinations were

carried out in the absence of bovine serum albumin. The kinetic

parameters were identical within experimental error.

The sensitivity of the β-glucosidase to various metal ions was

determined in 50 mM pH 6.8 triethanolamine buffer in order to avoid

precipitation of insoluble metal phosphates. The activity of the

β-glucosidase was measured at 0.998 mM (∼14 Km) and 0.0832 mM (∼1 Km)

PNPG at 1.6 mM metal ion concentration. The following analytical grade

metal salts were used: ferrous chloride, calcium chloride, manganese(II)

chloride, mercuric chloride, cupric chloride and zinc sulphate. EDTA

treated β-glucosidase was used. When magnesium ion was added to the

β-glucosidase prior to the determination different results were obtained

(see III). All determinations were carried out in the absence of bovine

serum albumin.

4:The Anomeric Configuration of the Initial Product

The anomeric configuration of the initial product of hydrolysis was determined according to the method of Barnett[90] with β-D-gluco-pyranosyl fluoride as substrate as follows. Solutions of α-D-glucose (91 mg/100 cm^3), β-D-glucose (85mg/cm^3) in 5mM pH6.8 sodium phosphate buffer and β-D-glucopyranosyl fluoride in 20 mM pH 6.8 sodium maleate buffer were prepared. Immediately after preparation they were placed in a polarimeter microcell (1 cm^3 volume, 10 cm pathlength) and the optical rotation monitored (at the wavelength of the sodium D line) for 15-20 minutes in a Perkin-Elmer 141 polarimeter. 40 μL of 1M sodium carbonate was then added to the cell (and well mixed with the aid of a Pasteur pipette) to catalyse the mutarotation of any free sugar present, and the optical rotation again monitored. After confirming that β-glucosidase retains activity in sodium maleate buffer the process was repeated on a blank containing 0.2 cm^3 of stock enzyme solution and 0.8 cm^3 sodium maleate buffer. No change in optical rotation was detected on addition of the sodium carbonate. The process was then repeated on a mixture consisting of 0.2 cm^3 of stock enzyme solution and 0.8 cm^3 of 14 mM β-D-glucopyranosyl fluoride in 20 mM pH 6.8 sodium maleate buffer. The anomeric configuration of the first product of hydrolysis was inferred from the direction of the change in optical rotation on addition of the sodium carbonate (see III).

II.5 Treatment of Data

All values of Km and Vm, together with the errors associated with the scatter of the data, were calculated by fitting data to the normal, non-linear form of the Michaelis-Menten equation* by the procedure of Wilkinson[91]. This procedure was carried out with the aid of a program written for an Apple II computer in collaboration with I. Street (UBC) and listed in Appendix 2.

The reported values of Vm are for a standard amount of β-glucosidase; that amount necessary to hydrolyse 10 nmoles/minute of PNPG at saturating PNPG concentration. Reference rates were measured at 1.34 mM PNPG. The actual rate of saturating PNPG hydrolysis (Vm) was calculated assuming Km (PNPG) = 0.083 and is reported as 'enzyme concentration'.

The data were plotted according to Lineweaver and Burke* as shown in Appendix 3. Data points showing substrate inhibition were not considered in the determination of Km and Vm.

Approximate values of inhibition constants (Ki) were determined by measuring initial reaction rates at one substrate concentration (~ 1 mM PNPG) and 4 or 5 inhibitor concentrations and plotting the results according to Dixon*. For those inhibitors which were not also substrates

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*   These methods and terms and/or their derivation are explained in
    Appendix 1.

(for which Km and Vm were determined) the inhibition constants (Ki) were

accurately determined by measuring initial reaction rates at five

substrate (PNPG) concentrations, in the absence of inhibitor and at four

inhibitor concentrations (giving 25 data points in all). The data were

plotted according to Lineweaver and Burke in order to determine the

inhibition pattern (eg. competitive, non-competitive etc.)*.

The inhibition constant (Ki) for competitive inhibitors was deter-

mined by plotting the apparent Km (determined by the procedure of Wilkinson[91])

against inhibitor concentration*. The best straight line was found by

linear regression analysis and the correlation coefficient, R, is reported.

The inhibitor α-D-glucose showed partially non-competitive

kinetics; replots of both slope and apparent Vm were hyperbolic.

Secondary replots[92] yielded values for the kinetic constants. The best

straight lines for the secondary replots were found by linear regression

analysis.

The inhibitor p-nitrophenyl-α-D-glucopyranoside showed non-linear

kinetics from which it was not easily possible to determine kinetic

constants.

Substrate inhibition by the substrates PNPG and PNPGal was

quantified by plotting reciprocal initial reaction rates against substrate

concentration*. The values of Ki were determined by 'inspection' of the

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* These methods and terms and/or their derivation are explained in
  Appendix 1.

obviously inhibited data points by linear regression analysis. Since non-weighted linear regression is not valid in this case the analysis merely constitutes a convenient straight line.

All graphical plots related to inhibition (with the exception of the approximate Ki determinations) are shown in Appendix 3.

Arrhenius plots were analysed by linear regression (neglecting the obviously non-linear point at the highest temperature) and the correlation coefficient, R, is reported.

CHAPTER III

Results and Discussion

III.1 Purification (and Comparison with Previous Work)

Three schemes for the purification of β-glucosidase from

A. faecalis have been previously published[57,58,60], all by Srinivasan

and co-workers. The scheme published with Bumm[58] is a slight modification

of that published with Han[57]. Although these schemes claim to have

produced pure material, both the low specific activity[57], and also the

protein/activity profile of the final chromatographic step[58], suggest that

this is not so.

E.O. Smith, in a thesis submitted under the supervision of

Srinivasan, purified the β-glucosidase from A. faecalis to a specific

activity fifty times that reported by Han and Srinivasan[57], and stated

that the material prepared previously[57,58] was impure. The pure

material was prepared (in minute quantities) by E.O. Smith[60], utilizing

the unwieldy and inconvenient technique of disc-gel electrophoresis.

The specific activity of the material prepared during the course

of this work (31.6 or 45.7 units/mg of protein at pH 6.8 and 37°C) is in

reasonable agreement with that reported[60] (55 units/mg of protein at pH

6.5 and 40°C) considering the differences in pH, temperature and protein

assay method used to determine specific activities.

The difference in specific activities between the material

obtained from the two successful purification attempts (31.6 and 45.7

units/mg of protein) is not easily explained. However, considering that

both preparations ran as greater than 99% of a single band on SDS-PAGE it

is probable that the preparation of lower specific activity consisted

mainly of one protein, a portion of which was denatured (inactive) while

still retaining the physical properties of the active material.

III.2 Stability (and Comparison With Previous Work)

The range of pH at which the β-glucosidase is stable is suggested by the method development carried out on the FPLC Mono Q anion exchange column. Chromatographic runs carried out in pH 6.6 and pH 7.6 buffers yielded inactive material implying that the enzyme is only stable between these pH values. This result is in reasonable agreement with that of Han and Srinivasan[57] who reported that the enzyme is stable in sodium acetate and sodium phosphate buffers between pH 6.5 and pH 7.8.

The heat stability of the enzyme is hinted at by the Arrhenius plots. The data measured at 40°C lay on the linear portion of the plot. The data measured at 45°C deviated from linearity with a lower rate, Fig. 34. This suggests that the enzyme is either being denatured or is less active (but still stable) at 45°C than at 40°C. Although Han and Srinivasan[57] noted the discontinuity in the Arrhenius plot at 45°C they claimed that the enzyme was not denatured until temperatures above 55°C were reached. However, a subsequent study published by Srinivasan and Bumm[58] reported that the β-glucosidase was rapidly inactivated above 45°C.

Enzyme which had been dialysed against EDTA to remove any enzyme-bound divalent metal ions (β-galactosidase from E. coli requires magnesium ion for complete activity[93]) was found to have identical kinetic parameters, with PNPG as substrate, in the presence and absence of magnesium ion. The K_m (PNPG) was identical, within experimental error, to that

measured with untreated enzyme. These results imply that either no divalent metal ion co-factor is necessary for catalysis, or that any divalent cation co-factor is too tightly bound to be removed by EDTA.

The treated enzyme was found to be inactivated by iron(II), manganese(II), mercury(II), copper(II) and zinc(II) ions, but not by calcium ions. When untreated enzyme was used, activity was observed in the presence of manganese(II) and zinc(II) ions (the measurement was not made in the presence of other ions). A tentative explanation for the increased stability of the untreated enzyme to inactivation by metal ions could involve a non-catalytic, structural role for a metal ion.

Han and Srinivasan[57] found that the enzyme was completely inactivated by mercury, copper and iron divalent metal ions only. This inactivation may have been non-specific (eg. by interaction with non-catalytic sulphydryl groups). In this case some protection might be given by the addition of BSA as this would lower the effective concentration of metal ions reaching the β-glucosidase; some metal ions would bind non-specifically to the BSA.

Further work needs to be carried out in order to clarify the sensitivity of this enzyme to divalent metal ions. In any event, protection from most of these metal ions was afforded by working in sodium phosphate buffer as most of the metal ion phosphates are almost completely insoluble in aqueous solution.

The β-glucosidase was found to be relatively stable to storage in solution (under toluene vapour to inhibit microbial growth); the Km

measured (PNPG) using freshly prepared enzyme was identical to that measured using enzyme which had been stored in solution for two months, and which retained 83% of its original activity. It should be noted that dilute β-glucosidase solutions were inactivated by exposure to surfaces unless BSA was added to increase the protein concentration.

Lyophilisation in the presence of myo-inositol gave β-glucosidase that retained 62% of its original activity on redissolution. Lyophilisation may therefore be of some use in long term storage of the enzyme. Han and Srinivasan[57] reported that myo-inositol completely protected the enzyme activity against lyophilisation. This difference may be due to the impure β-glucosidase used by Han and Srinivasan[57]; contaminating proteins may afford some protection, analogous to the protection against surfaces afforded by BSA.

Freezing as a method of storage was not investigated although the cell free extract and whole cells could be frozen with only a minimal loss of β-glucosidase activity.

III.3 Molecular Weight (and Comparison with Previous Work)

The sub-unit molecular weight of the β-glucosidase determined by SDS-PAGE is 51,500 Daltons. The molecular weight determined under native conditions using a Superose gel chromatography column is 75,000 Daltons. This latter value is probably low. The support material of the Superose column is based on the polysaccharide agarose. It is possible that the β-glucosidase is retarded on the column by specific interactions between the (oligosaccharide binding) active site and the polysaccharide backbone of the column packing material. Support for this hypothesis is the low apparent molecular weight of phosphorylase b as determined by Superose gel chromatography. Phosphorylase b binds oligosaccharides and is known to exist as a dimer (mol. wt. 194,000) and tetramer (mol. wt. 380,000) in solution; its apparent molecular weight is 100,000 Daltons.

Han and Srinivasan[57] reported a molecular weight for the β-glucosidase of 160,000 Daltons by gel filtration and 120,000 Daltons by sucrose density gradient centrifugation. However in both these cases only two standards were used for calibration, i.e. the calibration curve was the best straight line drawn between two points!

In order to clarify the above results the molecular weight should be determined by another technique or using a gel chromatography column with a non polysaccharide support (e.g., one based on polyacrylamide). This work is being undertaken.

It should be noted that if the enzyme is an oligomeric protein,

then the observation of β-glucosidase activity in the denaturing SDS-PAGE gel would suggest that the dissociated sub-units retain some enzymatic activity.

III.4   Anomeric Configuration of Initial Products

The anomeric configuration of the initial products was determined as described (II.4.4). The results are shown in Fig. 33. It may be seen that the change in rotation observed on addition of sodium carbonate suggests that the initial product has the β configuration. The β-glucosidase is therefore a retaining β-glucosidase.
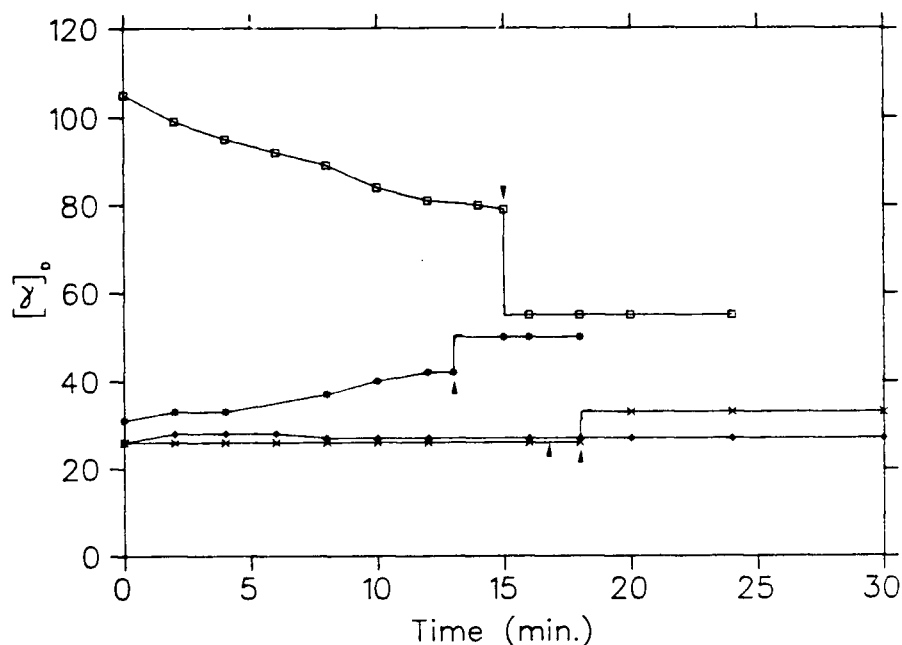
Figure 33.   Specific Optical Rotational Changes During Hydrolysis by β-glucosidase. (□), α-D-glucose; (●), β-D-glucose; (◆), β-D-glucopyranosyl fluoride alone; (×) β-D-glucopyranosyl fluoride and β-glucosidase from A. faecalis. The measured rotations were converted into specific rotations by using the initial weight of glucose or β-D-glucopyranosyl fluoride. The rotation of the sugars was measured at ~35°C and of the fluoride (with and without β-glucosidase) at ~17°C to minimise non-enzymic hydrolysis. The arrows mark the time at which sodium carbonate was added.

III.5 Kinetic and Thermodynamic Data

II.5.1 Results

The kinetic parameters of the substrates and inhibitors studied are shown in Table X and XI. Arrhenius plots are shown in Fig. 34.

Table X: Kinetic Parameters of β-Glucosidase with Several Substrates at 37°C and pH 6.8.

Substrates	Km (mM)	Vm[d] (nmoles/minute)	Enzyme[e] conc.	Substrate conc.(mM)	Vm/Km[f]	ΔG°[g] (kJ/mole)
PNPG[a,b]	0.083±0.006	(10.00±0.16)	40.56	0.98-0.016	120	24.23
PNPGal[b]	2.9±0.5	12.4±0.7	43.49	40-1.4	4.3	15.07
PNPMan	0.020±0.002	0.00743±0.00016	121.1	1.7-0.005	0.37	27.90
PY-G[c]	4.9 ± 1.2	0.17±0.03	43.49	2.5-0.25	0.035	13.72
cellobiose	0.702±0.061	9.60±0.38	3.307	2.7-0.13	13.6	18.73
cellotriose	0.387±0.014	8.11±0.10	10.19	4.1-0.06	21.1	20.27
cellotetraose	0.332±0.012	7.52±0.08	10.19	3.5-0.35	22.7	20.66
cellopentaose	0.312±0.008	6.73±0.05	10.19	2.3-0.043	21.6	20.82
lactose	73.2±6.0	7.48±0.48	30.69	32-1.1	0.102	6.74
sucrose	3.82±0.80	1.49±0.14	3.114	10-1.3	0.390	14.36
salicin	0.382±0.025	3.30±0.07	3.114	3.3-0.85	8.64	20.30
gentiobiose	3.85±0.68	0.71±0.06	29.36	10-0.74	0.18	14.34
glucosyl[a] azide	3.40±0.33	0.49±0.02	118.9	10-0.81	0.14	14.66
glucosyl[a] fluoride	3.69±0.19	13.0±0.4	219.0	5.1-0.16	3.52	14.45
2,4-DNPG	0.031±0.002	11.1±0.3	10.25	0.17-0.0097	360	26.77
2,4-TDNPG[a]	3.69±0.19	7.78±0.63	32.92	3.3-0.11	2.11	14.45

[a] Substrate inhibition was observed with these compounds and obviously inhibited points were neglected in the calculation of Km and Vm. These points are circled in the Lineweaver-Burke plots (Appendix 3).

[b] The Km determined for PNPG was 0.083 mM; however, subsequent determinations (during inhibition studies) yielded a Km of ~0.075 mM. This may be due to the use of older enzyme or due to experimental error.

[c] The large error in Km is due to the low substrate concentrations necessitated by the high background absorbance.

[d] Vm is given relative to that amount of enzyme necessary to hydrolyse 10 nmoles of PNPG in one minute.

[e] The enzyme concentration is given in terms of nmoles PNPG hydrolysed in one minute (at saturating PNPG, Vm).

[f] The units of Vm/Km are $min.^{-1} L^{-1} \times 10^{-6}$.

[g] ΔG° was calculated from the equation $\Delta G^{\circ} = -RT \ln Km$.

Table XI: Kinetic Parameters of β-Glucosidase with Several Inhibitors at 37°C and pH 6.8.

Inhibitor	Ki (mM)	R[d]	Type	Substrate conc. (mM)	Inhibitor conc. (mM)	ΔG°[a] (kJ/mole)
p-nitrophenyl-β-D-cellobioside	0.029	0.999	competitive	1.3-0.090	0-0.42	26.94
β-D-glucosylamine	0.40	0.999	competitive	1.3-0.090	0-3.6	20.18
glucono-δ-lactone	0.0017	0.999	competitive	1.1-0.075	0-0.013	34.25
α-D-glucopyranose	6.4 (β=0.44)	--	partially non-competitive	1.1-0.075	0-27	13.03
p-nitrophenyl-α-D-glucopyranoside	b	--	c	1.0-0.045	0-15	--

[a] ΔG° was determined from the equation $\Delta G^{\circ} = -RT \ln Ki$.

[b] No kinetic parameters were determined for this inhibitor.

[c] The type of inhibition is discussed in the text.
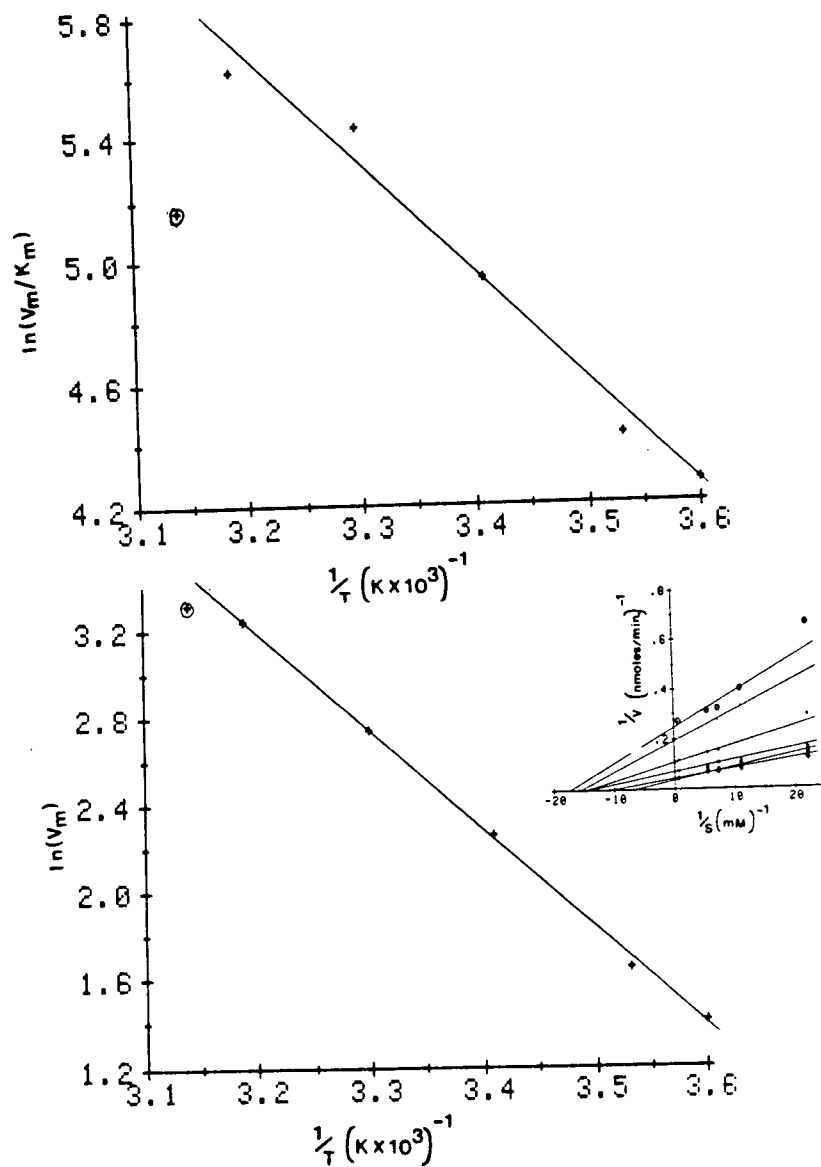
[d] R is the correlation coefficient of the replot.

Figure 34. Arrhenius plot of lnVm and ln(Vm/Km) against 1/T. Lineweaver-Burke plots of data shown in inset.

III.5.2 <u>Comparison with Previous Work</u>

The value of Km obtained for PNPG is somewhat lower than that obtained by Han and Srinivasan[57] of 0.125 mM. This may be because their kinetic studies were carried out on enzyme which had only been purified 50 to 80 fold. Han and Srinivasan[57] only investigated cellobiose as an inhibitor of PNPG hydrolysis. The value of Ki that they found from an approximate determination (a single inhibitor concentration and several substrate concentrations) of 1 mM is in reasonable agreement with the Km of 0.702 mM determined in this investigation. An approximate Ki determination was also carried out and yielded a comparable value of ~1 mM. The value of Ki determined by Han and Srinivasan[57] for glucose of 1 mM is in poor agreement with the value of 6.4 mM determined in this study. This is no doubt because an approximate determination assumes that any inhibition is competitive in nature.

The activation energy determined by Han and Srinivasan[57] of 39.7 kJ/mole is in reasonable agreement with that calculated from a plot of lnVm against reciprocal temperature (37.9 kJ/mole, coefficient of correlation = 0.999). It is possible that Han and Srinivasan[57] did not consider the variation of PNP extinction coefficient with temperature and this may be the cause of the slight differences in activation energy calculated.

Of 42 β-glucosidases isolated from microbial sources whose activity against PNPG has been measured[1,94] only 3 have smaller Km's

than that measured with β-glucosidase from A. faecalis, and of 39 whose

activity against cellobiose has been measured[94], only 4 have smaller Km's

than that measured with β-glucosidase from A. faecalis. The efficiency

of the β-glucosidase from A. faecalis, as determined by Km, is therefore

one of the highest measured for a bacterial β-glucosidase; bacterial

β-glucosidases tend to have higher Km's (weaker binding) than those

isolated from fungal sources[3]. This tight binding increases the poten-

tial utility of this enzyme for co-cloning into a cellulase complex.

The enzyme was found to be the most active (as defined by Vm/Km)

in the cellodextrin series (G2-G5) against cellotriose. It is not

unusual for β-glucosidases associated with cellulase complexes to have

higher activity against higher oligomers of glucose than cellobiose[1,95].

III.5.3 Substrates; Implications for the Active Site and Mechanism

A number of deductions may be made about the active site binding

specificity and mode of action from the kinetic parameters determined.

First of all it is necessary to confirm that the enzyme is a

'typical' β-glucosidase, i.e. a retaining β-glucosidase having an

exo-glucanase activity and a catalytic mechanism similar to that

postulated in Chapter I (Fig. 3).

Evidence that the enzyme is a retaining β-glucosidase has already

been discussed (III.4).

Evidence for exo-glucanase activity was obtained during the

attempt to measure the kinetic parameters of p-nitrophenyl-β-D-cello-

bioside as a substrate. The initial reaction rate (as measured by

release of PNP) was found to <u>increase</u> with time and to be <u>higher</u> at <u>low</u> substrate concentrations, Fig. 35. At first this observation was thought to arise from high substrate inhibition. However product analysis
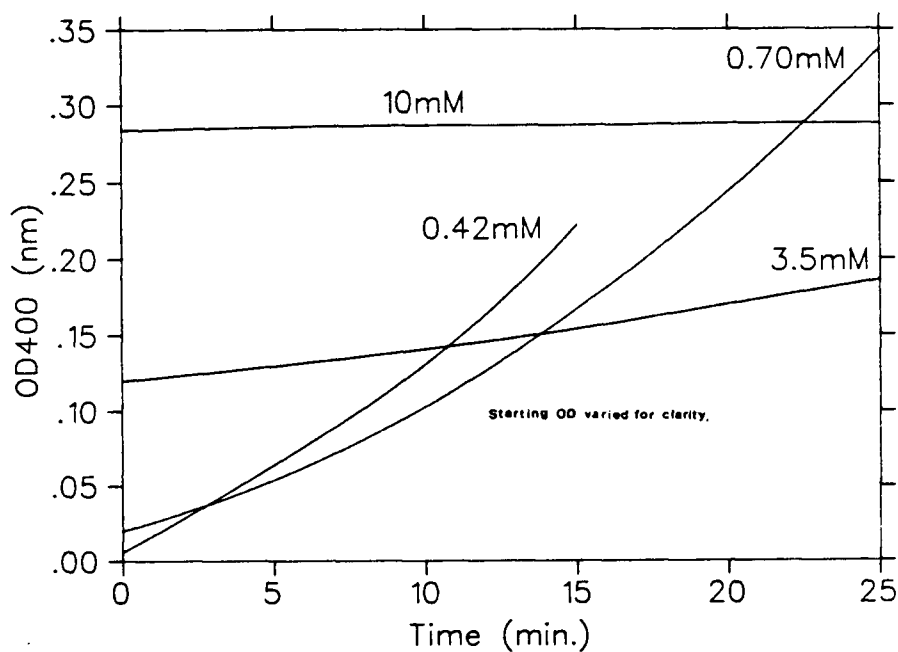

Figure 35. Initial Rate of Hydrolysis of p-Nitrophenyl-β-D-Cellobioside at a Number of Concentrations.

showed that only glucose (and not cellobiose) was produced and this suggested the following explanation for the observed kinetics. The enzyme cleaves single units from the non-reducing end of the substrate only, i.e. glucose is cleaved from the PNP-cellobioside to give PNPG and glucose. The concentration of PNPG is initially low (below Km PNPG). As the reaction proceeds more PNPG is produced and as its concentration

approaches and exceeds Km (PNPG) it is better able to compete with PNP-cellobioside as a substrate and hence the rate of PNP release increases. This enzyme therefore has exo-glucanase activity (exclusively). The kinetic parameters of PNP-cellobioside as an inhibitor of the hydrolysis of PNPG were measured and the results are discussed later.

The glycone binding site appears to be able to tolerate a range of sugar moieties, viz. glucosyl, galactosyl, mannosyl (and possibly fructosyl), however the specificity of the enzyme, as defined by Vm/Km, is at least 30 times higher for glucose than any other sugar.

The galactosyl moiety is bound somewhat more weakly than the glucosyl moiety; the difference in binding energy is 12 kJ/mole for the pair cellobiose/lactose and 9 kJ/mole for the pair PNPG/PNPGal. This is close to the binding energy expected from one hydrogen bond[101]. This suggests that the inversion of the 4-hydroxyl either removes one hydrogen bonding interaction or introduces a negative interaction of the same energy. The enzyme's ability to tolerate galactose may be related to the fact that the β-glucosidase activity is induced by addition of lactose to the bacterial growth medium.

The aglycone binding site has poor specificity and can tolerate a wide variety of 'aglycones' and linkages viz. aryl (PNP, DNP, TDNP, PY, salicyl); inorganic (azide, fluoride); β-D-(1,6) (gentiobiose); α-D-(1-1)-β(sucrose, i.e. glucosyl-α-(1-1)-β-fructoside). This poor specificity is not uncommon[95] in cellulolytic β-glucosidases; an enzyme which has evolved to degrade cellulose for use as a food source does not need the specificity of a metabolic enzyme.

The β-glucosidase was, however, found to be completely inactive to p-nitrophenyl-α-D-glucopyranoside as a substrate. Glucosidases are normally very specific towards the anomeric configuration of substrates and the activity observed against sucrose here, and by other β-glucosidases elsewhere[95], may be due to binding of fructose into the glucoside (glycone) site to give a fructosyl-β-(1-1)-α-glucoside configuration at the active site.

The increase in binding energy on increasing the chain length from one glucose to two (β-D-glucopyranosyl fluoride or azide to cellobiose) is 4.2 kJ/mole, and a further increase in chain length to cellotriose gives rise to a further increase in binding energy of 1.5 kJ/mole. Subsequent increases in chain length (cellotetraose and cellopentaose) do not significantly increase the strength of binding. This evidence suggests that there are three binding sites with some affinity for glucose and that the active site is arranged such that any additional moieties do not sterically hinder binding to the enzyme. The active site might be envisaged as a tunnel or angled cleft, three glucose sub-sites in length (with hydrolysis taking place between the first and second sub-sites) and from which any additional glucose moieties protrude out from the enzyme surface into solution, Fig. 36.
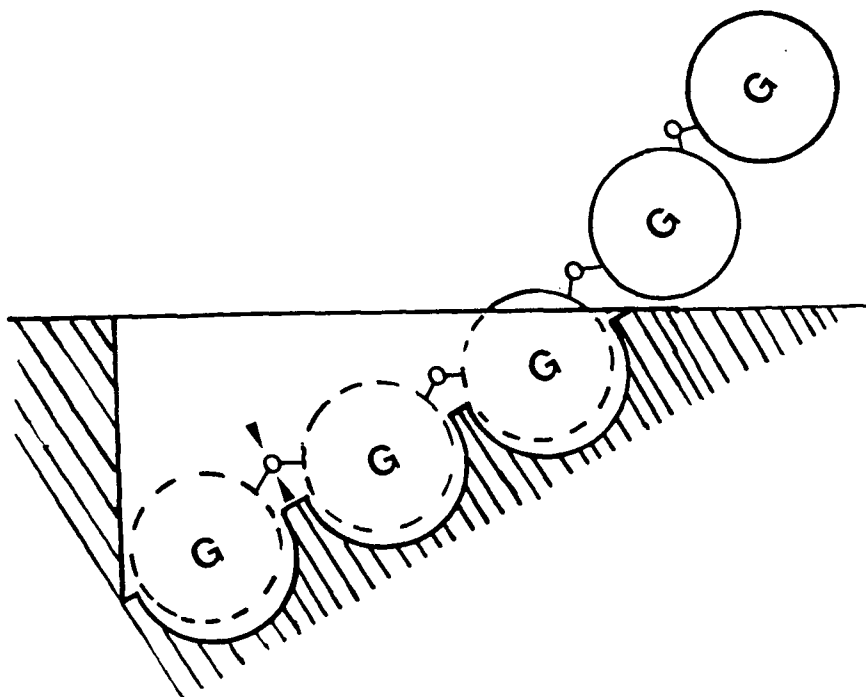
Figure 36. Schematic Representation of β-Glucosidase Binding

Site.

Gentiobiose binds no more tightly than a substrate having only one

glucose moiety. This suggests that the change from a β-1-4 linkage to a

β-1-6 linkage either completely destroys the enzyme's ability to bind the

second glucose moiety or, that a negative interaction of equivalent energy

to any positive interactions is introduced.

PY-G also binds no more tightly than substrates having a small

aglycone (fluoride or azide) and this again suggests either that the

'aglycone' site cannot bind the pyridinium ring or, that any positive

interactions are counteracted by equivalent negative interactions
(possibly between the positive charge on the substrate and a group in the
active site as discussed in III.5.4).

Similarly 2,4-TDNPG has a binding energy 12.3 kJ/mole less than
2,4-DNPG, and in fact, not signifcantly greater than that of the azide or
fluoride. The longer C-S bond length and larger atomic radius of the
sulphur must distort the structure sufficiently to either completely
destroy the binding interactions between the thiophenolic moiety and the
active site, or introduce negative interactions that completely neutralise
them.

If glucose is replaced by PNP the binding energy is increased by
5.5 kJ/mole in the case of cellobiose and 6.7 kJ/mole in the case of
cellotriose (from Ki of PNP-cellobioside). The observation that replace-
ment of a glucose moiety with a phenolic moiety increases the binding
energy hints that the binding interactions might be predominantly hydro-
phobic in nature, although one fortuitous additional hydrogen bond could
give rise to this increase in binding energy[101] (it is also possible that
the tighter binding is occurring fortuitously at another site). A similar
structure for the second and third binding sites might be postulated on
the basis of the similarity in binding energy difference between glucose
and PNP found at the two sites.

It may be seen from the range of substrates that the enzyme can
catalyse the heterolytic cleavage of C-O, C-N, C-S and C-F bonds. Bond
lengths in model compounds are[96,97]: $H_3C-(OCH_3)_2$, 1.42Å; H_3C-F, 1.385Å;

H_3C-N, 1.47Å; H_3C-SH, 1.82Å; $(pBrC_6H_4-)_2$-S, 1.75Å. This shows that the enzyme can tolerate appreciable changes in an important molecular dimension of the substrate (in common with other glycosidases[17,98]). This implies that the acid catalytic groups have some degree of freedom of movement (or that catalysis does not have to involve direct interaction with the exocyclic hetero atom to be cleaved; PY-G cannot undergo acid catalysed hydrolysis[34]).

The evidence discussed so far suggests a binding site as described (Fig. 36) in which the two 'aglycone' sites have some specificity for glucose, possibly, by predominantly hydrophobic interactions.

It is helpful if the discussion of the <u>mechanism</u> of glycoside hydrolysis (and the evidence for it) by β-glucosidase from A. Faecalis be carried out with reference to following hypothetical kinetic mechanism, Fig. 37, and hypothetical chemical mechanism, Fig. 38. Where K_2 represents a non-covalent conformational change (postulated in the mechanism of some glycosidases, e.g.[18]), K_3 represents bond cleavage (Fig. 38) and $k+4$ represents enzyme deglucosylation.
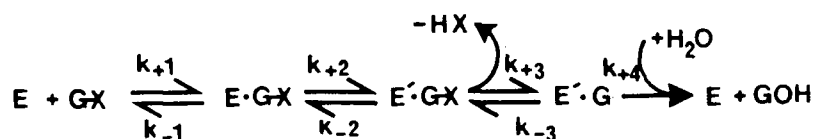
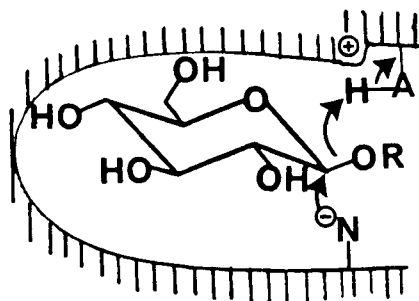Figure 37. Hypothetical β-Glucosidase Kinetic Mechanism.

Figure 38. Hypothetical β-Glucosidase Chemical Mechanism.

The Vm measured for all glucosyl substrates does not exceed 10-11 nmoles/min. (glucosyl fluoride falls into this range if the possible systematic error is taken into account; see II.4.2). If bond cleavage (k_{+3}) were rate limiting then some correlation between Vm and leaving group ability, as defined by pKa, would be expected[17,18,34-36]. PNPG, cellobiose, cellotriose, cellotetraose, β-D-glucopyranosyl fluoride, 2,4-DNPG and 2,4-TDNPG all have comparable values of Vm. As the leaving groups have considerably different pKa's the rate limiting step cannot be bond cleavage but must be some other step, e.g. (k+4) deglucosylation, (K_2) a slow protein conformational change, release of aglycone etc.

Further evidence that bond breaking is not rate limiting comes from the values obtained from the Arrhenius plots for the hydrolysis of PNPG, Fig. 34. A plot of ln Vm (representing rate limiting step; see

Appendix 1) against reciprocal temperature yielded an activation energy
of 37.9 kJ/mole (correlation coefficient = 0.999). A plot of ln(Vm/Km)
(representing first irreversible step; see Appendix 1) against reciprocal
temperature yielded an activation energy of 29.6 kJ/mole (correlation
coefficient = 0.990). As the activation energy determined for the first
irreversible step (presumably bond heterolysis) is different to that
determined for the rate limiting step, bond cleavage cannot be rate
limiting.

It is therefore postulated that for most substrates of this
enzyme, including the natural substrate, some other step than bond
cleavage is, at least partially, rate limiting.

The activation energy determined for bond cleavage in the
enzymatically catalysed hydrolysis of PNPG is considerably less than that
reported for the non-enzymic, acid catalysed reaction, 127 kJ/mole[99] and
this shows the high catalytic power of this enzyme.

The behaviour of the substrate PNPMan is unusual and deserves some
comment. The only difference between this substrate and PNPG is the axial
position of the 2-hydroxyl. β-Glucosidases are known to be sensitive to
changes at the 2 position of the glucose moiety[23,25], and a review of
the literature has revealed no other glucosidases which have activity
against mannosyl substrates. In the case of β-glucosidase from A.
faecalis PNPMan has an increased binding energy over PNPG of 3.7 kJ/mole
and a maximal rate 1/1350 that of PNPG. An approximate inhibition
constant was determined (Ki = 0.03) to confirm that hydrolysis was

taking place at the same site as PNPG hydrolysis. Application of Occam's razor to these observations might give rise to the following explanation. The axial 2-hydroxyl may interact with the catalytic group -BH (Fig. 38) thereby both increasing binding interactions and also decreasing catalytic efficiency (by interfering with acid catalysis).

The similarity in Vm observed for the substrates 2,4-DNPG and 2,4-TDNPG deserves some explanation. The acid catalysed rate of hydrolysis of phenyl-β-D-glucopyranoside is 300 times that of 1-thiophenyl-β-D-glucopyranoside[99]. The reason cited for this[99] is that the thio derivative is a weaker conjugate acid than the oxygen analogue and is consequently more resistant to protonation prior to bond heterolysis. In a study involving the enzymically catalysed hydrolysis of these compounds[23] the thio analogue was hydrolysed about 6000 times more slowly, and this is probably because both substrates have to undergo acid hydrolysis in the enzyme active site. Thiophenoxide ion is, however, a better leaving group (pKa = 6.61[100]) than phenoxide ion (pKa = 9.94[100]). If the 2,4-DNP ion, whose pKa = 4.09[100], is that much poorer a leaving group than the 2,4-TDNP ion, then neither substrate will require acid catalytic assistance for hydrolysis and the thio derivative should undergo bond cleavage at a greater rate than the oxygen derivative. However, since it has been shown that bond cleavage is not rate limiting for 2,4-DNPG, the observed rate will depend on the rate limiting step not involving bond cleavage.

Another, less likely, explanation is that while the enzymic rate of C-S bond cleavage might be considerably slower than C-O bond cleavage,

the enzymic rate limiting step is slower than both and thus no difference is observed.

III.5.4 Inhibitors; Implications for the Active Site and Mechanism

Inhibitors are extremely useful in probing both the mechanism and active site environment of β-glucosidases[15].

The tight binding of the inhibitor glucono-δ-lactone (19.5 kJ/mole more binding energy than the azide or fluoride), whose preferred half chair conformation makes it an analogue for the glucosyl oxocarbonium ion, suggests that the transition state is a stabilised oxocarbonium ion (possibly in equilibrium with a covalent glycosyl-enzyme species as discussed in I.2).

β-Glucosidases have been divided into two groups with regard to the binding of cationic and basic substrates and inhibitors[15]. The first group binds cationic glycosyl derivatives with much higher affinity than their neutral counterparts. The positive charge may be either permanent or can be formed by proton transfer from the enzyme active site. Enzymes from the second group have a high affinity only for basic glycosyl derivatives; permanently cationic ones bind similarly to their uncharged analogues. This behaviour in the second group has been postulated[15] to be due to the presence of a cationic group located near the putative catalytic carboxylate (marked -BH in Fig. 38). This cationic group is proposed to neutralise the influence of the carboxylate group on the

permanently cationic inhibitor. Proton donation to the basic glucosyl

derivatives could be from this cationic group or from a neutral acid.

For both groups the observed effects can be taken as evidence for an

active site that is strongly shielded from the aqueous environment since

electrostatic interactions are very weak in water. The β-glucosidase

from A. faecalis can be thought of as belonging to the second group. The

permanently cationic pyridinium derivative (PY-G) binds no more tightly

than substrates having no charge (azide and fluoride) whereas the basic

inhibitor β-D-glucosylamine has an additional binding energy of 5.7

kJ/mole.

The glucose used for the inhibition studies was supposed to be an

anomeric mixture; however, during the 'retention/inversion' study it

became apparent that it was predominantly in the α-configuration. The

kinetics observed were best explained as partially competitive, Fig. 39.

One possible explanation is that the β-glucose binds into the third

glucose site and while PNPG can still bind into the first two sites,

release of products is hindered by the inhibiting α-glucose, Fig. 40.

$$
\begin{array}{ccccc}
E + S & \underset{}{\overset{K_S}{\rightleftharpoons}} & E{\cdot}S & \xrightarrow{K_p} & E + P \\
+ & & + & & \\
I & & I & & \\
K_i \Updownarrow & & K_i \Updownarrow & & \\
E{\cdot}I + S & \underset{}{\overset{K_S}{\rightleftharpoons}} & E{\cdot}I{\cdot}S & \xrightarrow{\beta K_p} & E + P
\end{array}
$$
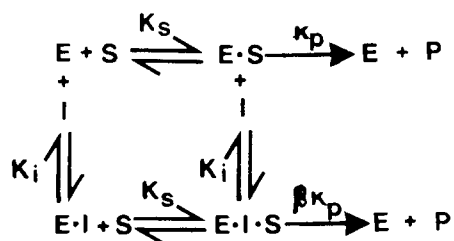
Figure 39. Kinetic Mechanism for Partially Non-Competitive
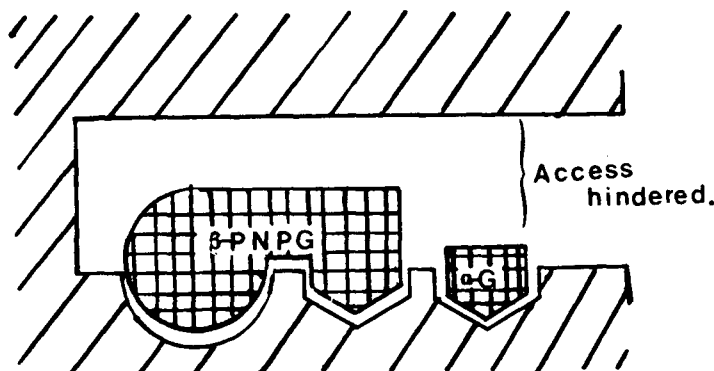
Inhibition.

Figure 40.   Schematic Model for Partially Non-Competitive

Inhibition.

The inhibition observed with p-nitrophenyl-α-D-glucopyranoside

was non-linear (see Appendix 3). A kinetic mechanism which fits these

data is one involving equivalent, non-interacting sites only one of which

can be (competitively) inhibited at one time.[92] A physical picture of

this behaviour is not obvious.

The slight, but real trend of decreasing Vm with increasing

glucose oligomer chain length is also not readily interpreted.

The Ki's observed for substrate inhibition by PNPGal and PNPG, of

160 mM and 60 mM respectively, are high enough to have no mechanistic

significance and are probably due to incorrect (unproductive) binding

of a second substrate molecule (ESS).

II.6 Conclusion

Summing up, the active site may have the following features. An angled, hydrophobic cleft with three glucose sub-sites from which longer oligomers can protrude into solution. The last two sub-sites are probably similar and the binding interactions in these latter two sites may be predominantly hydrophobic in nature. The transition state is probably a stabilised oxocarbonium ion (possibly in equilibrium with a covalent intermediate as discussed in I.2). The catalytic groups might be arranged as shown in Fig. 38 and the rate limiting step for most substrates is one which does not involve glycosidic bond cleavage.

It should be stressed that this is only a preliminary study and any or all of the above conclusions might be incorrect. Further investigation by such techniques as nucleophilic competition studies, kinetic isotope effects, site directed covalent inhibitors, substrate analogues etc. will be needed to further clarify the mechanism of this enzyme.

It is hoped that this work will be carried out when cloning yields a plentiful source of this β-glucosidase.

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APPENDIX 1

Basic Enzyme Kinetics

The Briggs-Haldane Study State Assumption

The following process is assumed:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$
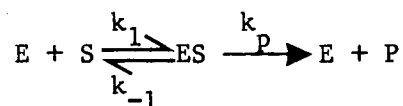
where E is free enzyme;

S is free substrate;

ES is enzyme-substrate complex

and P is product(s).

(The reverse reaction, E + P $\longrightarrow$ ES, may be neglected as the concentration of P is negligible at the start of the reaction)

At steady state:

$$\frac{d[ES]}{dt} = 0$$

$$\therefore k_1[E][S] = (k_{-1} + k_p)[ES]$$

Rearranging: $$[ES] = \frac{k_1[S]}{(k_{-1} + k_p)} [E] \quad \ldots\ldots \text{(I)}$$

Initial Velocity, v is given by:

$$v = k_p[ES] \quad \ldots\ldots(II)$$

The total enzyme concentration, $[E]_t$ is given by:

$$[E]_t = [E] + [ES] \quad \ldots\ldots(III)$$

Dividing II by III and substituting the right hand side of I for $[ES]$:

$$\frac{v}{k_p[E]_t} = \frac{\dfrac{k_1[S]}{(k_{-1} + k_p)}}{[E] + \dfrac{k_1[S]}{(k_{-1} + k_p)}[E]}$$

Cancelling $[E]$ and substituting Vm (maximal initial velocity) for $k_p[E]_t$:

$$\frac{v}{Vm} = \frac{\dfrac{k_1[S]}{(k_{-1} + k_p)}}{1 + \dfrac{k_1[S]}{k_{-1} + k_p}}$$

Grouping the rate constants as Km, the Michaelis constant:

$$v = \frac{Vm[S]}{Km + [S]} \quad \ldots\ldots(IV)$$

$$\text{where } Km = \frac{k_{-1} + k_p}{k_1}$$

IV is the Michaelis-Menten equation and Km is a pseudoequilibrium

constant.

If a <u>rapid preequilibrium</u> is assumed, $k_1 \approx k_{-1} \gg k_p$, then the same

equation may be derived, however, Km, is a true equilibrium (dissociation)

constant: $Km = \dfrac{k_{-1}}{k_1}$

Equation IV, as given, is non-linear, however inversion gives:

$$\frac{1}{v} = \frac{Km}{Vm} \frac{1}{[S]} + \frac{1}{Vm} \quad \ldots\ldots(V)$$

A plot of $\dfrac{1}{v}$ vs. $\dfrac{1}{[S]}$ gives a straight line. The $\dfrac{1}{v}$ intercept gives $\dfrac{1}{Vm}$ and

the $\dfrac{1}{[S]}$ intercept $\dfrac{-1}{Km}$. This is the Lineweaver-Burke plot (see Appendix 3

for examples).

Inhibition

<u>Competitive inhibition</u> arises when an inhibitor competes for the

same site as the substrate. Thus, the rate at saturating substrate (Vm)

is unaffected but the amount of substrate necessary to saturate the site

(Km) in the presence of inhibitor is greater.

Equation IV becomes:

$$v = \frac{Vm[S]}{[S] + Km \left(1 + \frac{[I]}{Ki}\right)}$$

where [I] is the inhibitor concentration and Ki is the dissociation

constant for the process: $E + I \underset{K_i}{\overset{\longrightarrow}{\rightleftharpoons}} EI$

It may be shown that:

$$Km_{app} = \frac{Km}{Ki} [I] + Km$$

where Km_{app} is the apparent Km.

A replot of Km_{app} against [I] will be linear and the [I] intercept will

give −Ki (see Appendix 3 for examples).

Another plot commonly used to determine Ki is that of Dixon. $\frac{1}{v}$ is plotted

against [I]. The point at which the lines for various values of [S]

intersect gives a value of −Ki on the [I] axis. If Vm is known only one

value of [I] need be used in order to calculate an approximate value of

Ki.

The treatment of kinetic data obtained when <u>partially</u>

non-competitive inhibition takes place is non-trivial and has already been referenced[91].

Substrate inhibition occurs when a second substrate molecule binds to the ES complex to produce an inactive ES.S complex. Equation IV becomes:

$$v = \frac{Vm[S]}{Km + [S] + \dfrac{[S]^2}{K_{si}}} \qquad \ldots\ldots(VI)$$

where K_{si} is the inhibition constant (dissociation constant) for the binding of the second substrate molecule. Inversion of VI gives:

$$\frac{1}{v} = \frac{Km}{Vm[S]} + \frac{1}{Vm} + \frac{[S]}{K_{si}}$$

at high substrate concentrations the first term becomes negligible and it may be shown that a plot of $\frac{1}{v}$ against [S] (at high [S]) will extrapolate to $-K_{si}$ on the [S] axis, (see Appendix 3 for examples). More detailed discussions of enzyme kinetics may be found in references 103 and 104.

The Significance of Vm and Vm/Km

Vm is clearly related to the slowest forward step on the enzyme catalysed pathway (k_p in the scheme given). The significance of Vm/Km is less clear intuitively, however it may be demonstrated to be related to the first irreversible step on the catalytic pathway[102]. This demonstration is non-trivial and will not be given here.

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APPENDIX 2

Program for Apple II Computer Used to Determine Km and Vm

```
10   HOME
15   GOSUB 2500
20   PRINT "THIS PROGRAM PROVIDES ESTIMATES"
30   PRINT "OF KM AND VMAX FROM A WEIGHTED"
40   PRINT "LINEAR REGRESSION ANALYSIS.
50   DIM S(10),V(10),S$(10),V$(10),IY(10),IX(10)
60   D$ = CHR$ (4) +  CHR$ (13)
70   KI = 0:FG = 0:FH = 0
80   PRINT : PRINT
90   PRINT "1)---KI DETERMINATION"
100  PRINT : PRINT "2)___KM DETERMINATION"
110  PRINT
120  GET Q$
130  IF  ASC (Q$) = 49 THEN KI = 1: GOTO 160
140  IF  ASC (Q$) = 50 THEN 160
150  GOTO 120
160  HOME :S0 = 0
170  PRINT "SAMPLE #"; TAB( 11)"SUBSTRATE"; TAB( 23)"RATE"; TAB( 30)"CALC
     ULATED"
180  PRINT  TAB( 13)"CONC."; TAB( 32)"RATE"
190  POKE 34,3
200  HOME
210  N = 0:FG = 0:FH = 0
220  IF KI = 1 THEN IT$ = "INHIBITOR CONC.:      ":L = 7:IL = 3:FL = 3: GOSUB
     1000:IN$ = B$
230  PRINT
240  GOSUB 900
245  IF N > 10 THEN 260
250  IF  VAL (B$) < > 0 THEN 240
255  IF N < 3 THEN 240
260  GOSUB 1370
280  IT$ = "ADD A POINT ? (Y/N) "
290  GOSUB 1540
300  IF Q$ = "Y" THEN  GOSUB 1440: GOSUB 900
305  IT$ = "CONVERT DATA ?(Y/N) ": GOSUB 1540
307  IF Q$ = "Y" THEN FG = 1: GOSUB 2000: GOSUB 1440
308  IT$ = "CALCULATE VM/KM?    ": GOSUB 1540
309  IF Q$ = "N" THEN S0 = 1: GOTO 840
310  A = 0:B = 0:C = 0:D = 0:E = 0
320  FOR Z = 1 TO N
330  X = V(Z) ↾ 2
340  Y = X / S(Z)
350  A = A + (V(Z) * X)
360  B = B + X ↾ 2
370  C = C + (Y * V(Z))
380  D = D + (X * Y)
390  E = E + Y ↾ 2
400  NEXT Z
410  DE = ((A * E) - (D * C))
420  KM = ((B * C) - (A * D)) / DE
430  VM = ((B * E) - (D * D)) / DE
440  A = 0:B = 0:C = 0:D = 0:E = 0:F = 0:G = 0:F1 = 0
450  FOR IC = 1 TO N
460  F = VM * S(IC) / (S(IC) + KM)
470  F1 =  - VM * S(IC) / ((S(IC) + KM) ↾ 2)
480  A = A + F ↾ 2
490  B = B + F1 ↾ 2
500  C = C + (F * F1)
```

```
500 C = C + (F * F1)
510 D = D + (V(IC) * F)
520 E = E + (V(IC) * F1)
530 G = G + V(IC) ^ 2
540  NEXT IC
550 DE = (A * B) - (C * C)
560 B1 = ((B * D) - (C * E)) / DE
570 B2 = ((A * E) - (C * D)) / DE
580 VR = B1 * VM
590 KR = KM + (B2 / B1)
600 S2 =  SQR ((G - B1 * D - B2 * E) / (N - 2))
610 EK = S2 / B1 *  SQR (A / DE)
620 EV = VM * S2 *  SQR (B / DE)
630 IL = 3:FL = 3:NO = KR: GOSUB 1250:KM$ = B$
640 IL = 2:FL = 3:NO = EK: GOSUB 1250:EK$ = B$
650 IL = 3:FL = 3:NO = VR: GOSUB 1250:VM$ = B$
660 IL = 2:FL = 3:NO = EV: GOSUB 1250:EV$ = B$
670  GOSUB 1500
680  FOR Z = 1 TO N
690 VC = VR * S(Z) / (KR + S(Z))
700  VTAB (Z + 3)
710  HTAB (32)
720 IL = 3:FL = 3:NO = VC: GOSUB 1250: PRINT ;B$
730  NEXT Z
735  GOSUB 1370
736  IF  VAL (B$) <  > 0 THEN 310
737 IT$ = "REFERENCE? (Y/N)       ": GOSUB 1540
738  IF Q$ = "Y" THEN FH = 1: GOSUB 2200
740 IT$ = "PRINT RESULTS (Y/N) ": GOSUB 1540
750  IF Q$ = "N" THEN 840
760 IT$ = "PRINTER SLOT #       ":IL = 1:FL = 0:L = 1: GOSUB 1000:SL =  VAL
    (B$)
770  PR# SL
775  HOME
780  PRINT ;"SAMPLE 3"; TAB( 11)"SUBSTRATE"; TAB( 22);"RATE"
790  PRINT  TAB( 13)"CONC"
800  GOSUB 1450
810  GOSUB 1500
820  PR# 0
840 IT$ = "GRAPH MENU? (Y/N)       ": GOSUB 1540
845  IF Q$ = "Y" THEN  GOTO 3000
850 IT$ = "ANOTHER SET OF DATA? ": GOSUB 1540
855  IF Q$ = "Y" THEN 200
860  END
880 IL = 1:FL = 0:L = 1:IT$ = "PRINTER SLOT #       ": GOSUB 1000:SL =  VAL
    (B$)
900 N = N + 1
910  VTAB (N + 3): PRINT  TAB( 3);N
920 IT$ = "ENTER RATE :       ":L = 7:FL = 3:IL = 3: GOSUB 1000
930  IF  VAL (B$) = 0 THEN  VTAB (N + 3): HTAB (3): PRINT ;" ":N = N - 1:
    RETURN
940  VTAB (N + 3): HTAB (22): PRINT ;B$
950 V(N) =  VAL (B$):V$(N) = B$
960 IT$ = "SUBSTRATE CONC.:       ":L = 7:IL = 3:FL = 3: GOSUB 1000
970 S(N) =  VAL (B$):S$(N) = B$
980  VTAB (N + 3): HTAB (11): PRINT ;B$
990  RETURN
1000 M$ = "_____"
```

```
1000 M$ = "_____"
1010 S$ = "_____"
1020 B$ =  MID$ (M$,1,L)
1030  POKE 35,24: VTAB (23): HTAB (1)
1035  PRINT S$
1037  VTAB (23): HTAB (1)
1040  PRINT ;IT$;
1050 I = 1
1060  VTAB (23): HTAB (20): PRINT S$
1065  HTAB (20): VTAB (23)
1070  PRINT  MID$ (B$, LEN (B$) - L + 1, LEN (B$))
1080  VTAB (23)
1090  HTAB (20 + L)
1100  GET X$
1110  IF X$ =  CHR$ (13) THEN 1220
1120  IF X$ <  >  CHR$ (8) THEN 1170
1130  IF I = 1 THEN 1060
1140 B$ =  MID$ (B$,1, LEN (B$) - 1)
1150 I = I - 1
1160  GOTO 1060
1170  IF I = L + 1 THEN 1060
1180  IF  ASC (X$) < 46 OR  ASC (X$) > 57 THEN 1060
1190 B$ = B$ + X$
1200 I = I + 1
1210  GOTO 1060
1220 B$ =  MID$ (B$, LEN (B$) - I + 2, LEN (B$))
1230 B$ =  MID$ (S$,1,L -  LEN (B$)) + B$
1235  RETURN
1240 NO =  VAL (B$)
1250 S =  SGN (NO)
1260 N2 = 10 �½ FL
1270 N1 =  ABS (NO) + .5 / N2
1280 IP =  INT (N1)
1290 FP =  INT (N2 * (N1 - IP)) + N2
1300 B$ =  STR$ (IP):L =  LEN (B$):B$ =  RIGHT$ (B$,L)
1310  IF IL = 0 AND IP = 0 THEN B$ = "":L = 0
1320  IF S < 0 THEN L = L + 1:B$ = "-" + B$: IF IP = 0 AND IL = 1 THEN B$
     = "-":L = 1
1330  IF IL > L THEN B$ = " " + B$:L = L + 1: GOTO 1330
1340  IF FL > 0 THEN B$ = B$ + "." +  RIGHT$ ( STR$ (FP),FL)
1350  POKE 35,22
1360  RETURN
1370 IT$ = "DELETE POINT #       ":L = 2: GOSUB 1000:P =  VAL (B$)
1380  IF P = 0 THEN  RETURN
1390 N = N - 1
1400  FOR Z = P TO N
1410 V(Z) = V(Z + 1):V$(Z) = V$(Z + 1)
1420 S(Z) = S(Z + 1):S$(Z) = S$(Z + 1)
1430  NEXT
1440  HOME
1450  FOR Z = 1 TO N
1460  VTAB (Z + 3): PRINT  TAB( 3);Z;
1470  PRINT  TAB( 11);S$(Z); TAB( 22);V$(Z)
1480  NEXT
1490  RETURN
1500  VTAB (N + 5): HTAB (2): PRINT "KM = ";KM$;" +/- ";EK$
```

```
1500   VTAB (N + 5): HTAB (2): PRINT "KM = ";KM$;" +/- ";EK$
1510   VTAB (N + 7): HTAB (2): PRINT "VM = ";VM$;" +/- ";EV$;
1515   IF FG = 1 THEN  PRINT " NMOLES/MIN."
1520   IF KI = 1 THEN  VTAB (N + 9): HTAB (2): PRINT "[INHIBITOR] = ";IN$
1522   IF KI = 1 AND FH = 1 THEN  VTAB (N + 11): HTAB (2): PRINT "REFERENC
       E VM=";VE;" NMOLES/MIN."
1525   IF KI = 0 AND FH = 1 THEN  VTAB (N + 9): HTAB (2): PRINT "REFERENCE
       VM=";VE;" NMOLES/MIN"
1530   RETURN
1540   POKE 35,24
1550   VTAB (23): HTAB (1)
1560   PRINT IT$;
1565   VTAB (23): HTAB (20): PRINT S$
1570   VTAB (23): HTAB (21): GET Q$
1580   IF Q$ = "Y" OR Q$ = "N" THEN  POKE 35,22: RETURN
1620   GOTO 1550
2000   REM   DATA TREATMENT SUBROUTINE
2001 IT$ = "NEW VALUES?(Y/N)     ": GOSUB 1540
2002   IF Q$ = "N" GOTO 2080
2005 L = 6
2010 IT$ = "E.COEFF.? " + "(" + EB$ + ")": GOSUB 1000
2020   IF  VAL (B$) = 0 THEN  GOTO 2030
2025 EB =  VAL (B$):EB$ =  STR$ (EB)
2030 IT$ = "CELL VOLUME? " + "(" + VL$ + ")": GOSUB 1000
2040   IF  VAL (B$) = 0 THEN  GOTO 2050
2045 VL =  VAL (B$):VL$ =  STR$ (VL)
2050 IT$ = "PATH LENGTH? " + "(" + LE$ + ")": GOSUB 1000
2060   IF  VAL (B$) = 0 THEN  GOTO 2070
2065 LE =  VAL (B$):LE$ =  STR$ (LE)
2070   REM   TREAT DATA
2080   FOR K = 1 TO N
2090 V(K) = (V(K) * VL * 1E6) / (EB * LE)
2095 V$(K) =  STR$ (V(K))
2100   NEXT
2110   RETURN
2200   REM   REFERENCE SUBROUTINE
2205 L = 6
2210 IT$ = "E.CO.REF.?" + "(" + ES$ + ")": GOSUB 1000
2220   IF  VAL (B$) = 0 THEN  GOTO 2230
2225 ES =  VAL (B$):ES$ =  STR$ (ES)
2230 IT$ = "REF. KM? " + "(" + KT$ + ")": GOSUB 1000
2240   IF  VAL (B$) = 0 THEN  GOTO 2250
2245 KT =  VAL (B$):KT$ =  STR$ (KT)
2250 IT$ = "CELL VOLUME? " + "(" + VOS$ + ")": GOSUB 1000
2260   IF  VAL (B$) = 0 THEN  GOTO 2270
2270 IT$ = "[STOCK]? " + "(" + ST$ + ")": GOSUB 1000
2280   IF  VAL (B$) = 0 THEN  GOTO 2290
2285 ST =  VAL (B$):ST$ =  STR$ (ST)
2290 IT$ = "STOCK VOL.? " + "(" + SO$ + ")": GOSUB 1000
2300   IF  VAL (B$) = 0 THEN  GOTO 2305
2302 SO =  VAL (B$):SO$ =  STR$ (SO)
2305 IT$ = "INIT. RATE? " + "(" + VA$ + ")": GOSUB 1000
2307   IF  VAL (B$) = 0 THEN  GOTO 2310
2308 VA =  VAL (B$):VA$ =  STR$ (VA)
2310 SV = (ST * SO) / VOS
2320 VT = (VA * (SV + KT)) / SV
2330 VE = VT * VOS * 1E6 / ES
2335   GOSUB 1520
2340   RETURN
2500   REM   SET VARIABLES TO AVOID DIVISION BY ZERO
2510 EB = 6220:EB$ = "6220":VL = 1.05:VL$ = "1.05":LE = 1:LE$ = "1":ES =
     7280:ES$ = "7280":KT = .066:KT$ = ".066":
2515 VOS = 3.05:VOS$ = "3.05":ST = 2.724:ST$ = "2.724":SO = 1.5:SO$ = "1.
     5":VA = .07:VA$ = ".07"
2520   RETURN
```

```
3000    REM  GRAPHPLOT SUBROUTINE
3010    POKE 34,0: POKE 35,24: HOME :XMAX =  - 1E8:YMAX =  - 1E8
3020    PRINT "GRAPHPLOT ROUTINE"
3030    PRINT : PRINT
3040    PRINT "1)---L/B PLOT"
3050    PRINT : PRINT "2)---HILL PLOT"
3060    PRINT
3070    GET Q$
3080    IF  ASC (Q$) = 49 THEN  GOTO 3200
3090    IF  ASC (Q$) = 50 THEN  GOTO 3400
3100    GOTO 3070
3200    REM  L/B PLOT
3210    PRINT "L/B PLOT"
3220    PRINT "--------"
3230    FOR K = 1 TO N
3240 IY(K) = 1 / V(K):IX(K) = 1 / S(K)
3250    IF YMAX < IY(K) THEN YMAX = IY(K)
3260    IF XMAX < IX(K) THEN XMAX = IX(K)
3270    NEXT
3280    PRINT "XMAX=";XMAX: PRINT
3290    PRINT "YMAX=";YMAX
3300    GOSUB 3600
3310 IT$ = "REPLOT SAME DATA?": GOSUB 1540
3320    IF Q$ = "Y" THEN  GOTO 840
3340    GOTO 160
3400    REM  HILL PLOT
3410    PRINT "HILL PLOT"
3420    PRINT "---------"
3421    IF SO = 0 THEN  GOTO 3430
3422 IT$ = "VM?": GOSUB 1000
3425    IF  VAL (B$) = 0 THEN  GOTO 3422
3427 VR =  VAL (B$)
3430    FOR K = 1 TO N
3440 IY(K) =  LOG (V(K) / (VR - V(K))):IX(K) =  LOG (S(K))
3450    IF YMAX < IY(K) THEN YMAX = IY(K)
3460    IF XMAX < IX(K) THEN XMAX = IX(K)
3470    NEXT
3475    VTAB (10): HTAB (1)
3480    PRINT "XMAX=";XMAX: PRINT
3490    PRINT "YMAX=";YMAX
3500    GOSUB 3600
3510 IT$ = "REPLOT SAME DATA?": GOSUB 1540
3520    IF Q$ = "Y" THEN  GOTO 840
3530    GOTO 160
3600    REM FILE ROUTINE
3610 S1 = 2 * N
3620 D$ =  CHR$ (4)
3625    PRINT
3630    INPUT "FILE NAME     ?";NA$
3640    PRINT D$;"OPEN";NA$
3650    PRINT D$;"WRITE";NA$
3660    PRINT S1
3670    FOR K = 1 TO N
3680    PRINT IX(K): PRINT IY(K)
3690    NEXT
3700    PRINT D$;"CLOSE";NA$
3710    RETURN
```

APPENDIX 3

Graphical Representation of Kinetic Data

1. Lineweaver-Burke Plots for Substrates.
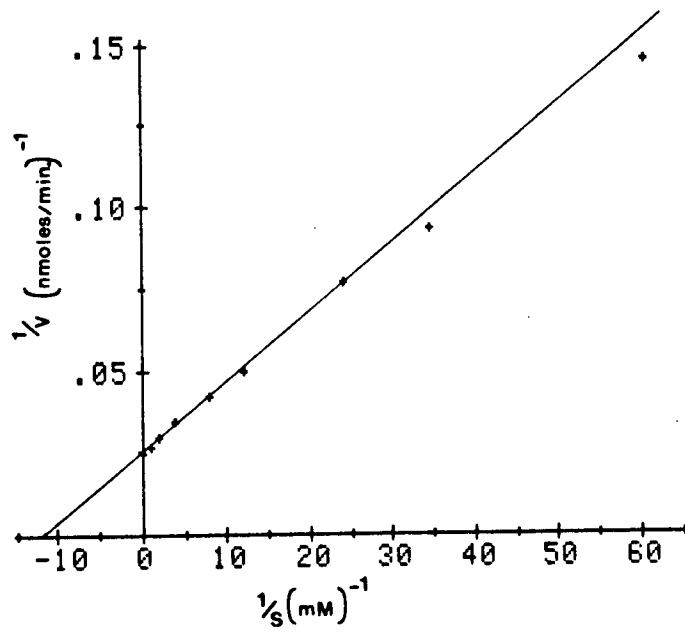
Figure 41: PNPG.
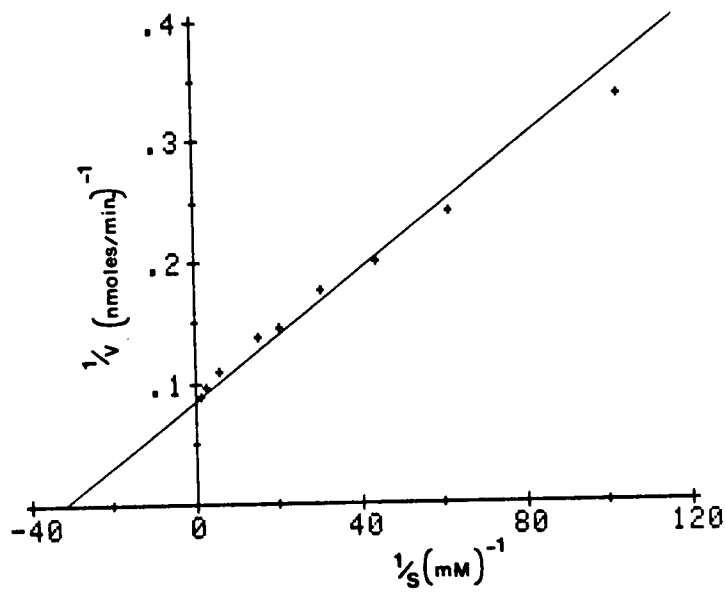
Figure 42: DNPG.
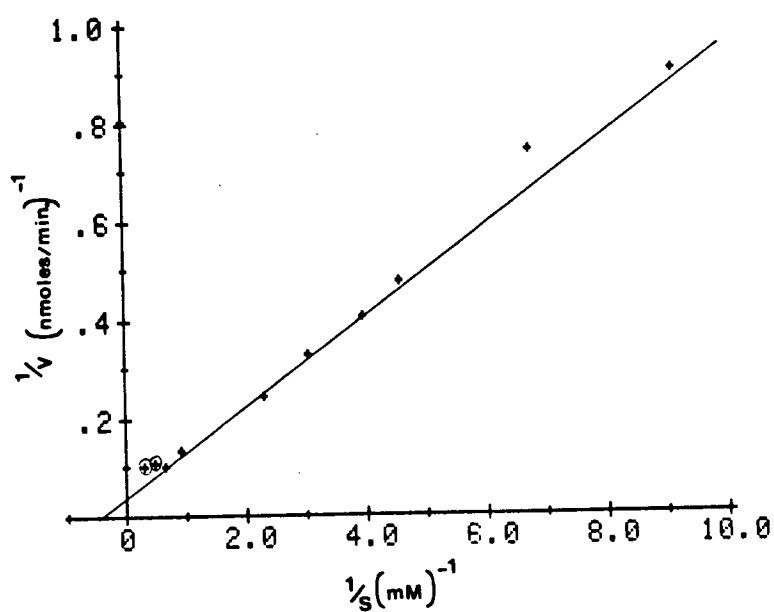
Figure 43: DNPTG.
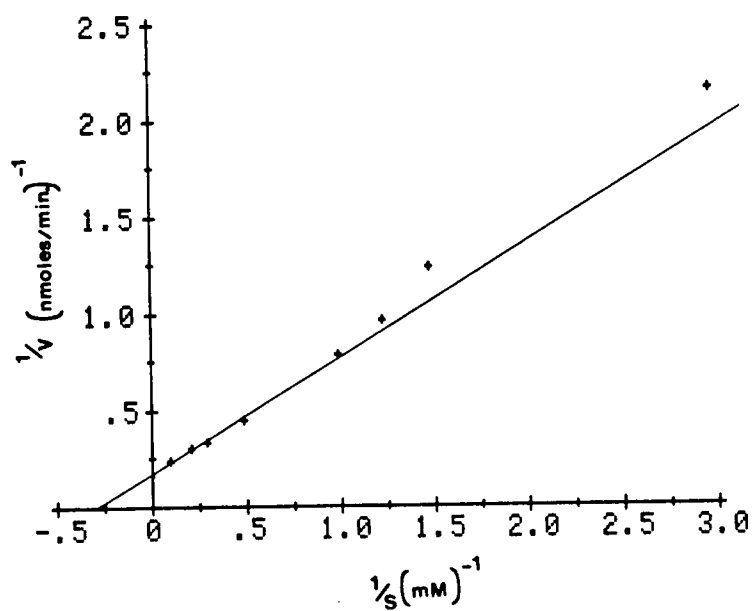
Figure 44: β-D-Glucopyranosyl azide.
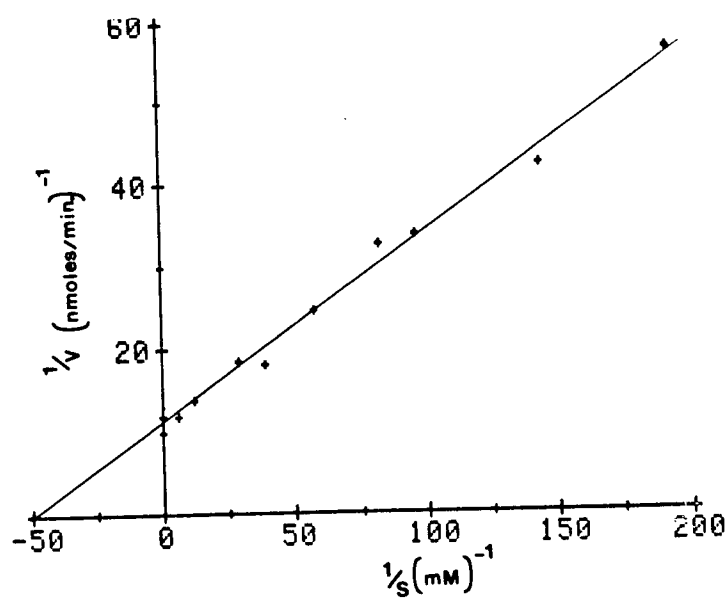
Figure 45: PNPMan.
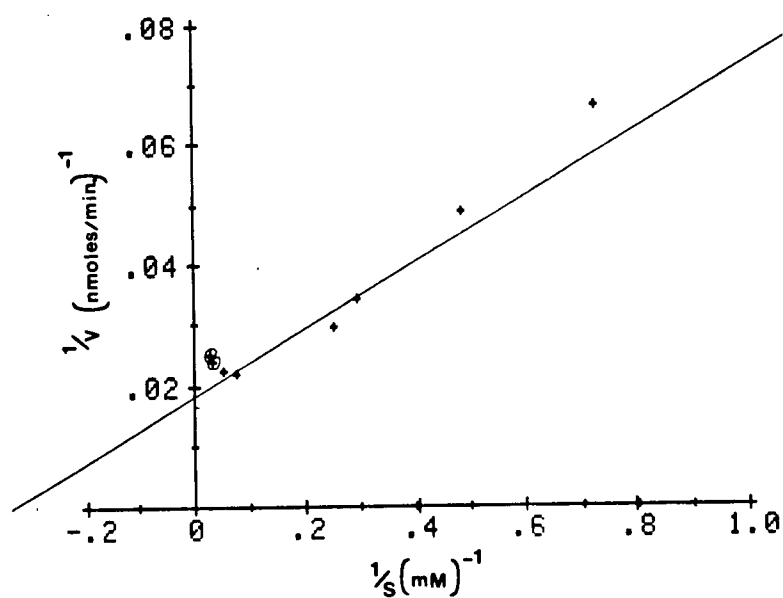
Figure 46: PNPGal.
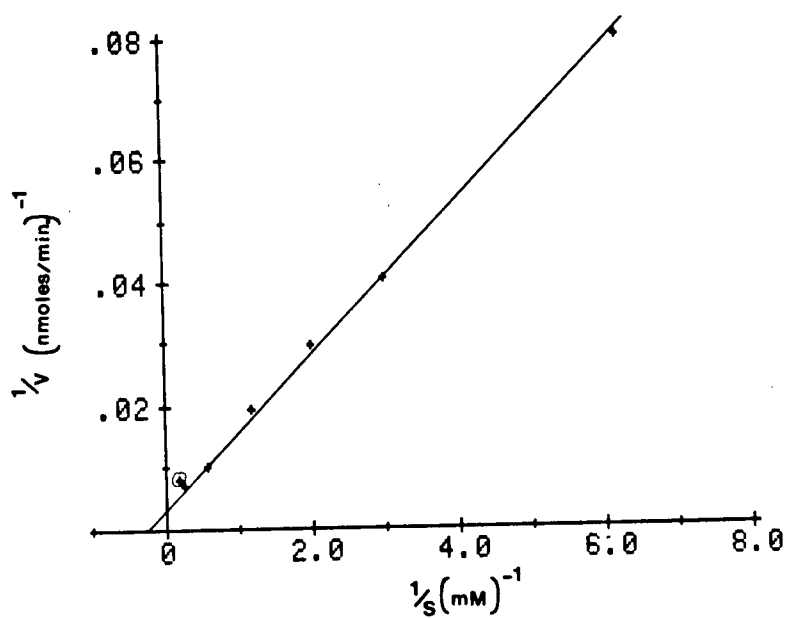
Figure 47: β-D-Glucopyranosyl fluoride.
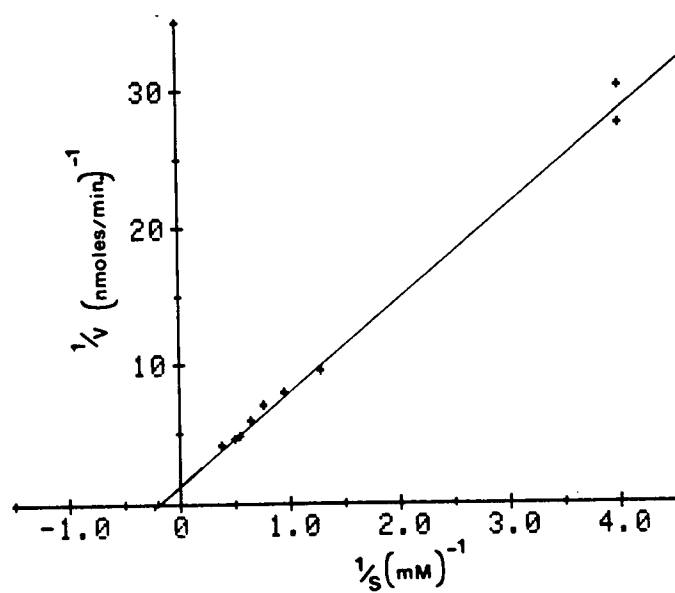
Figure 48: PY-G.
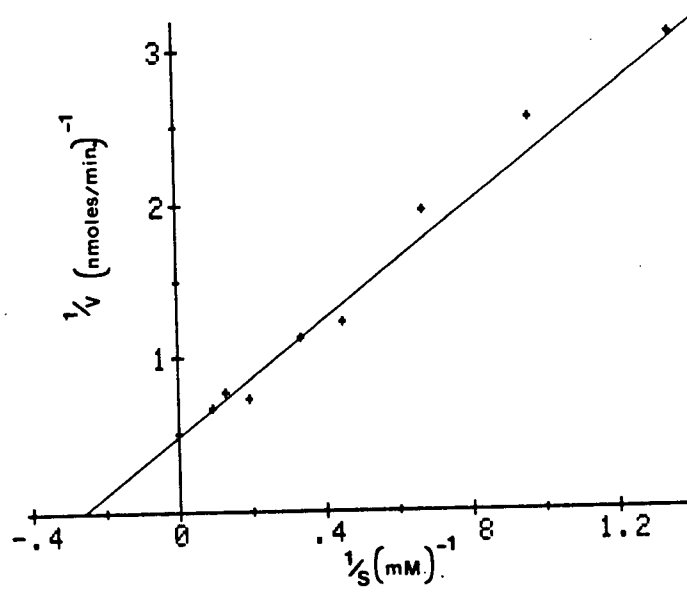
Figure 49: Gentiobiose.
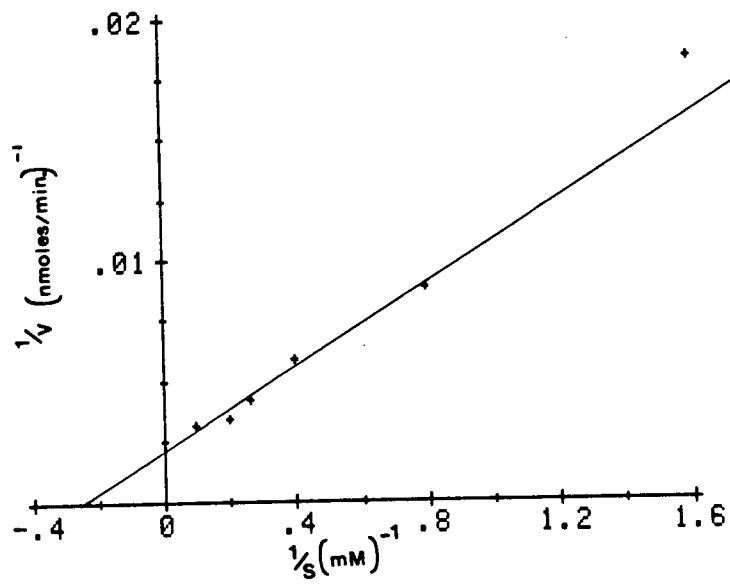
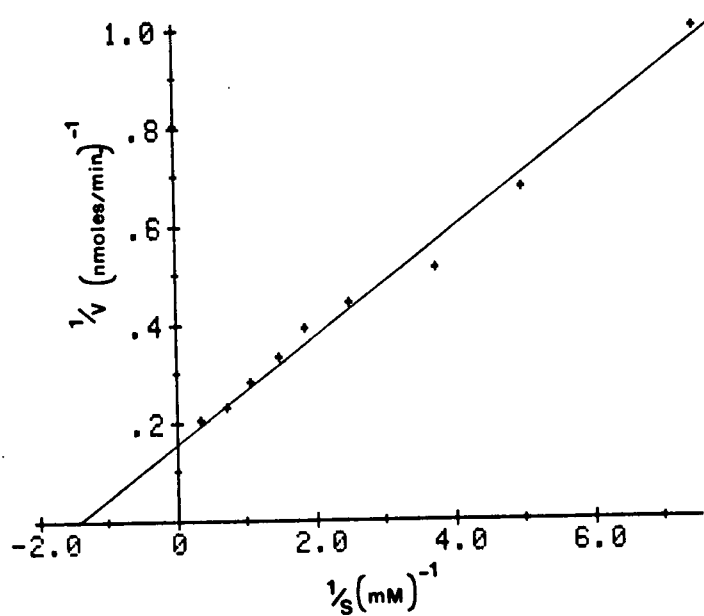Figure 50: Sucrose.
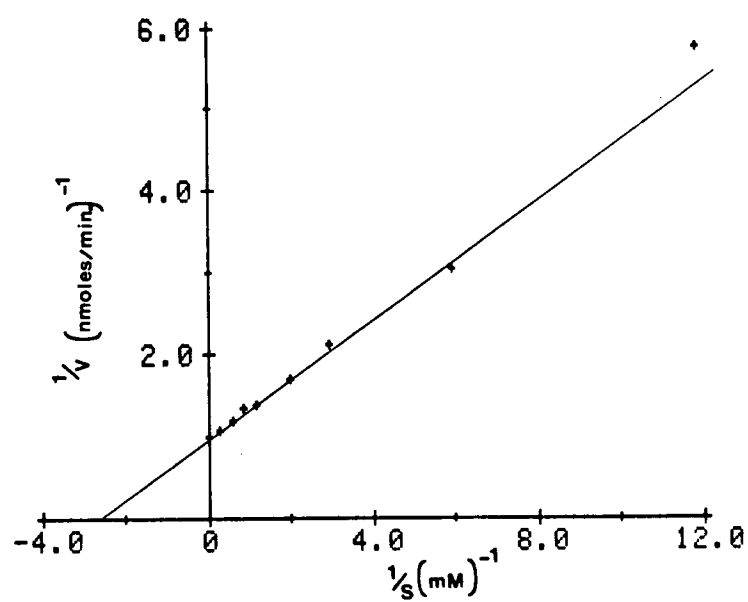
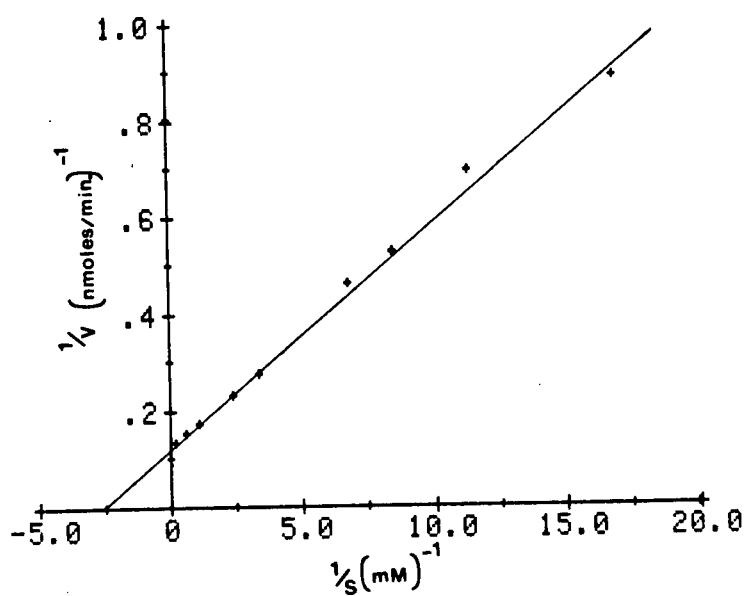Figure 51: Salicin.
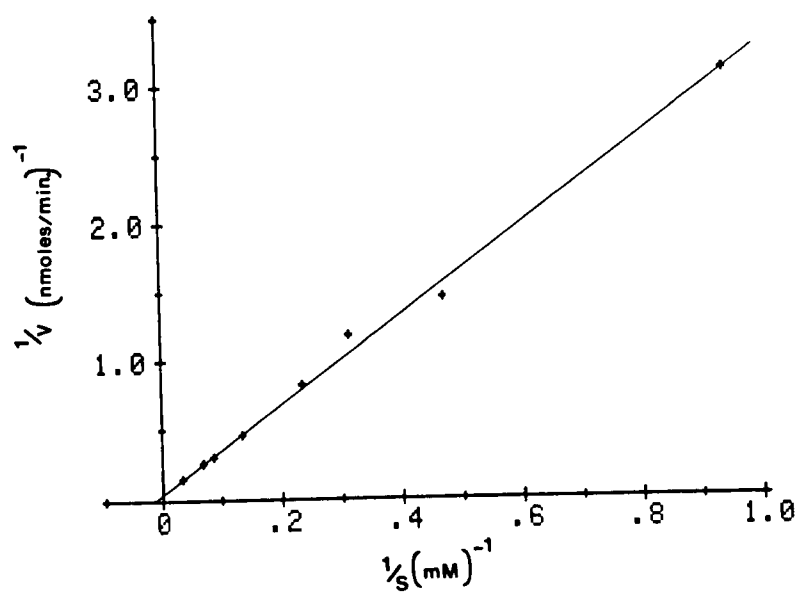
Figure 52: Lactose.
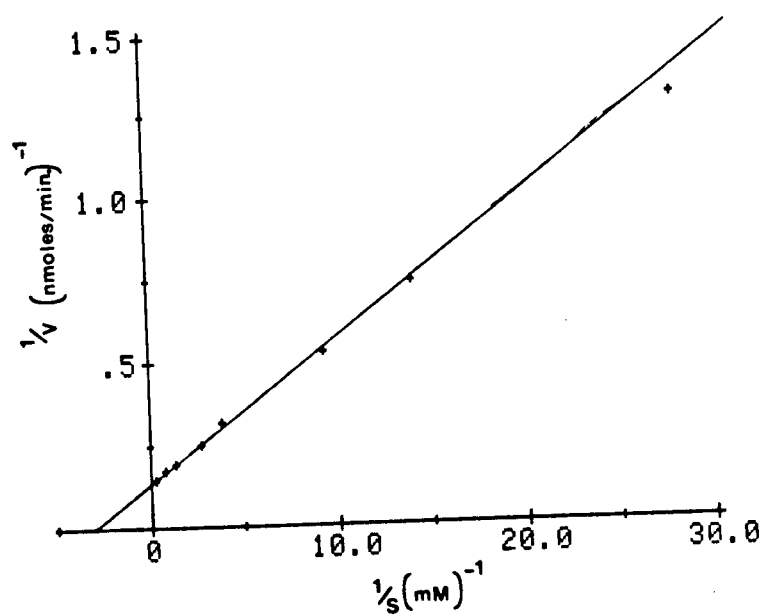
Figure 53: Cellobiose.
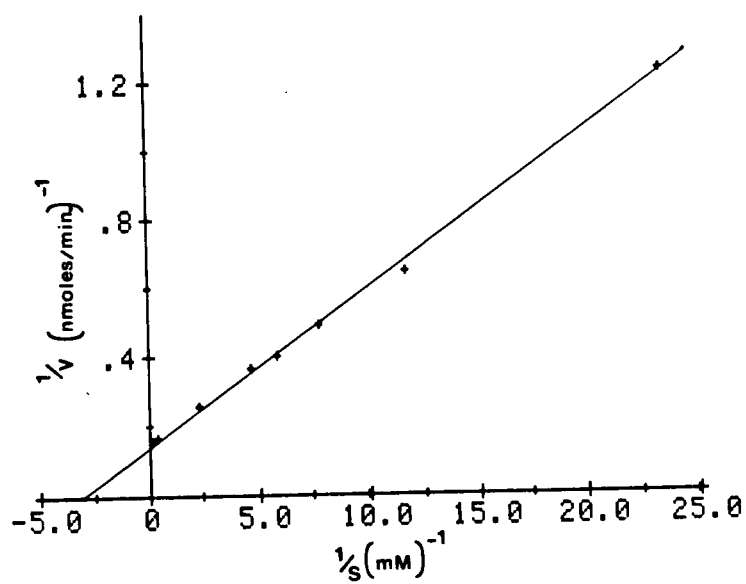

Figure 54: Cellotriose.

Figure 55:   Cellotetraose.

Figure 56: Cellopentaose.

2. Lineweaver-Burke Plots for Inhibitors.

Replots shown in insets. Inhibitor concentrations shown in titles.
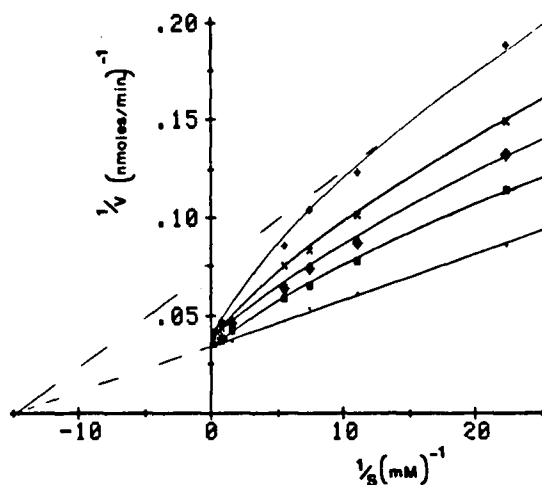
Figure 57: p-Nitrophenyl-α-D-glucopyranoside. (·), No inhibitor;

(+), 14.56 mM; (✗), 9.860 mM; (♦), 7.043 mM (■); 4.930 mM.
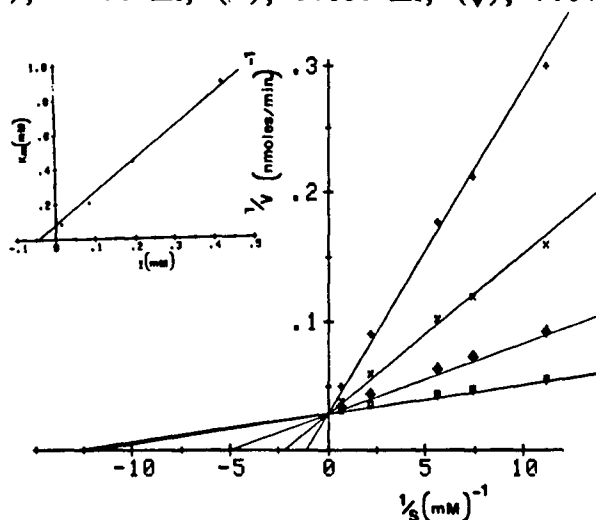
Figure 58: p-Nitrophenyl-β-D-cellobioside. (·), No inhibitor; (+),

0.4222 mM; (✗), 0.1971 mM; (♦), 0.08447 mM; (■), 0.01408 mM.
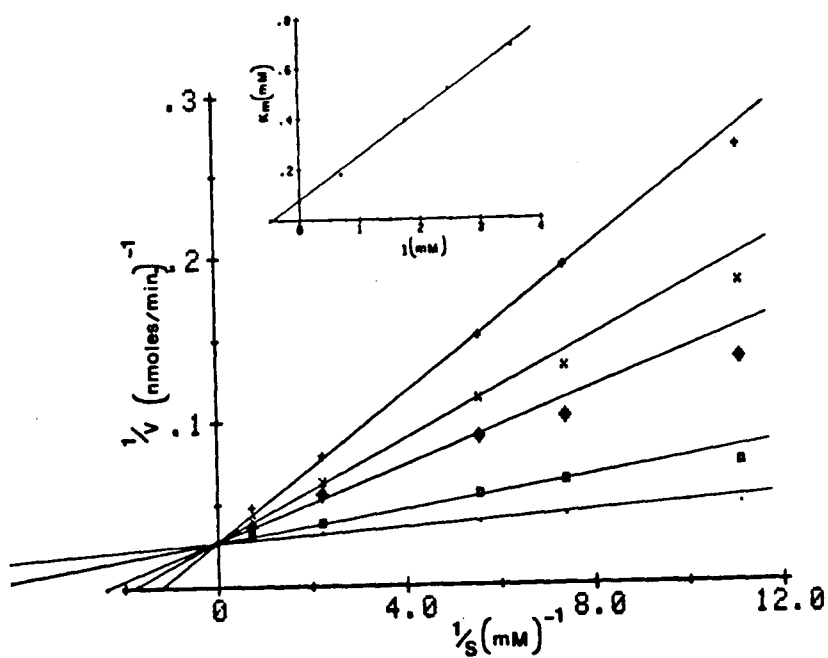
Figure 59:   β-D-Glucosylamine. (·), No inhibitor; (+), 3.560 mM;
(×), 2.494 mM; (♦), 1.780 mM; (■), 0.7125 mM.
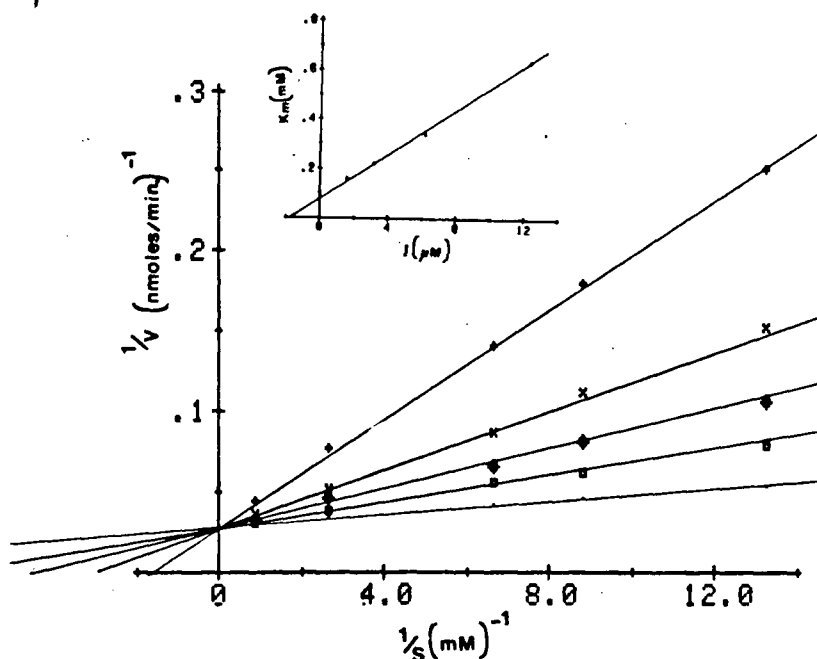
Figure 60:   D-Glucono-δ-lactone. (·), No inhibitor; (+), 12.29 μM;
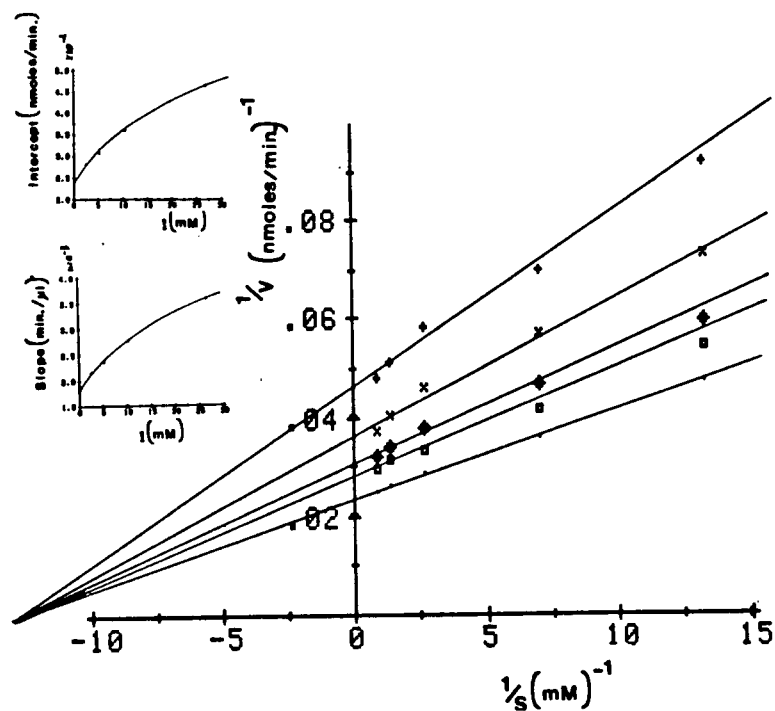(×), 6.147 μM; (♦), 3.192 μM; (■), 1.596 μM.

Figure 61: α-D-Glucopyranose. (·), No inhibitor; (+), 26.81 mM;
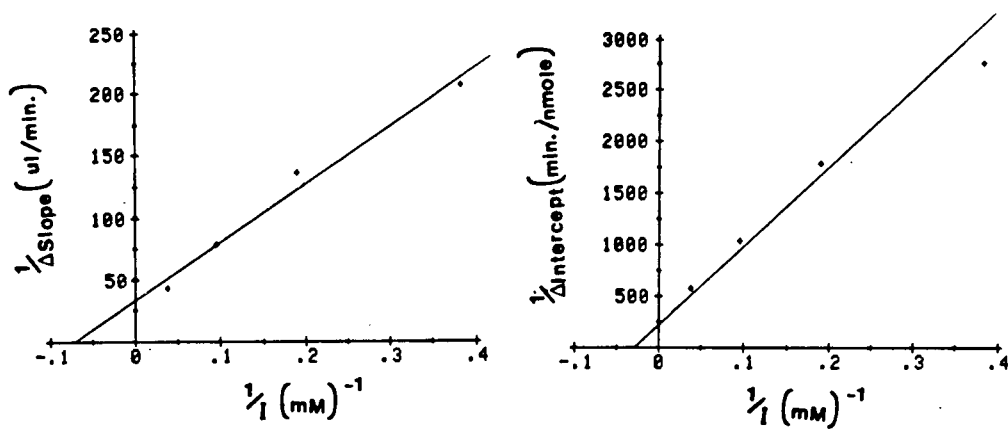
(×), 10.44 mM; (♦), 5.221 mM; (■), 2.611 mM.

Figure 62: α-D-Glucopyranose. Secondary replots.

3. Substrate Inhibition; Reciprocal Plots.

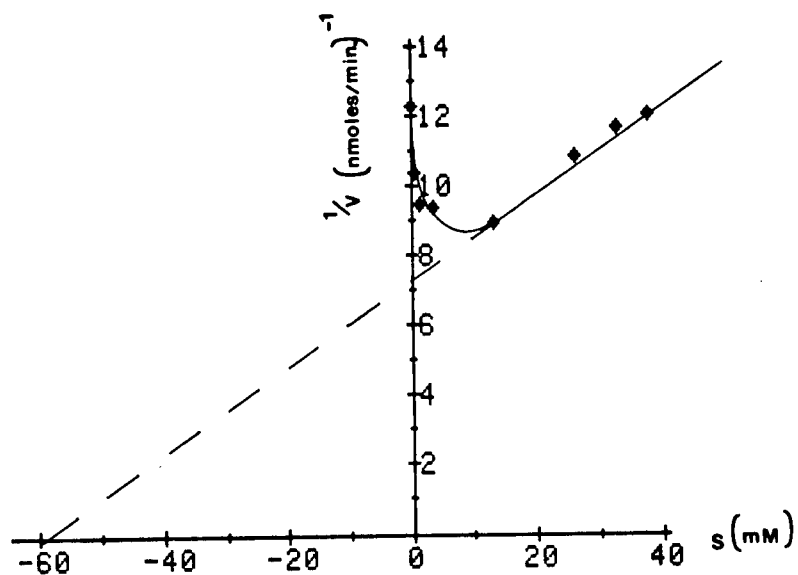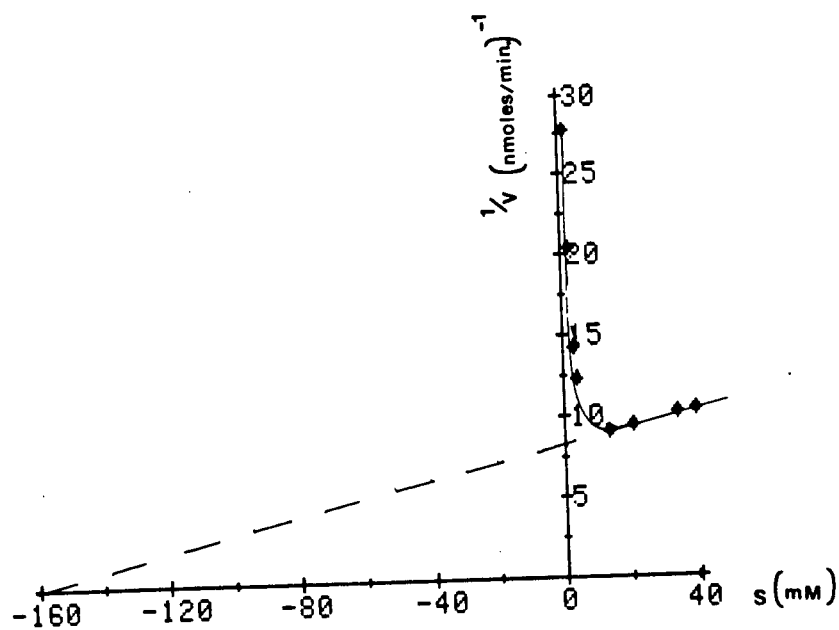Figure 63:   PNPG.

Figure 64:   PNPGal.

Addendum

Molecular Weight Determination on Waters I-250 HPLC Gel column

Molecular weight was determined on a Waters Silica based HPLC gel column (in order to avoid specific interactions).

Table XII: Molecular Weight Markers for I-250 column.

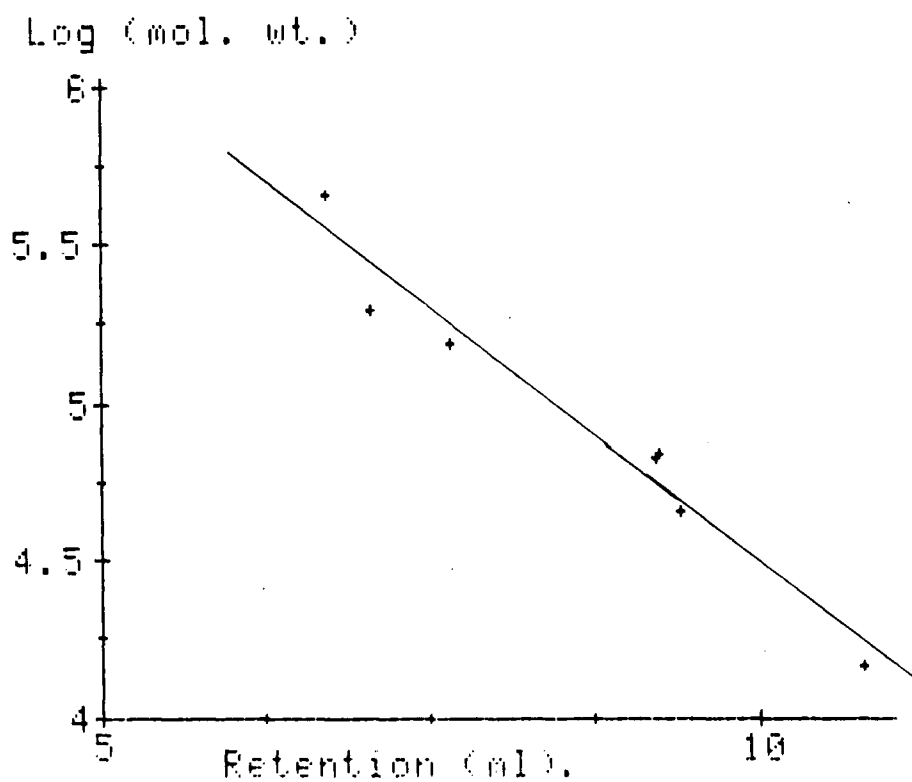| Protein	Molecular weight (Daltons)	Log (Molecular weight)	Retn. (ml)
Ferritin	450,000	5.653	6.73
Phosphorylase	198,000	5.288	7.07
Aldolase	158,000	5.199	7.66
Black Albumin	68,000	4.833	9.25
BSA	66,000	4.820	9.22
Ovalbumin	45,000	4.653	9.41
Lactalbumin	14,500	4.161	10.78
β-Glucosidase from A. faecalis	98,000 (Determined)		8.55

Figure 65: Plot of Log (Molecular Weight) Against Rf for
I-250 Gel Chromatography.

The enzyme is therefore a dimer of subunit molecular weight 50,000

Daltons.