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UNIVERSITY OF ALBERTA

I. CE(IV) ANALYSIS OF CARBOHYDRATES II. TRANSGALACTOSYLATION PRODUCTS OF β - GALACTOSIDASE FROM MAXILACT

by

Lee Shane Griffith

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Spring 1990

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **I. CE(IV) ANALYSIS OF CARBOHYDRATES II. TRANSGALACTOSYLATION PRODUCTS OF β - GALACTOSIDASE FROM MAXILACT** submitted by **Lee Shane Griffith** in partial fulfilment for the requirements for the degree of **Doctor of Philosophy in Food Chemistry.**

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ABSTRACT

Acidic solutions of Cerium (0.5 M nitric acid), when added in a 1 to 3 ratio to aqueous solutions of carbohydrates, oxidize different carbohydrates at dramatically different rates. Generally the rate of oxidation was such that monosaccharides were faster than oligosaccharides and reducing sugars much faster than non-reducing sugars. Using these differences cerium oxidimetry was investigated as a tool in the aid of determination of lactose hydrolysis. It was determined to be a very facile and easy method for the determination of lactose hydrolysis and original percentage lactose in fluid milk. The possibility of utilizing this simple technique to analyze carbohydrates in other foods led to the development of the use of cerium oxidimetry to determine dextrose equivalent in starch hydrolysates. The Ce(IV) test was also adapted to the rapid determination of percentage sucrose in honey samples.

The transgalactosylation activity of β-galactosidases has been of much interest in the last few years. It is hoped that this enzyme activity may be used to produce biologically interesting carbohydrates. The enzyme is also used to hydrolyze the lactose in milk and there could be nutritional concerns from the presence of oligosaccharides produced by transgalactosylation. In order to accomplish either goal the production and identity of these products must be determined.

The β-galactosidase from Maxilact L2000 was purified using Sephadex G200, away from other carbohydrase activities in the commercial preparation. The purified enzyme was determined to be a glycoprotein, possibly consisting of two isozymes of differing

thermal stability. The purified enzyme requires a divalent cation and sodium ion for maximal activity.

Temperature programmed gas chromatography was utilized to study the transgalactosylation products when the enzyme was exposed only to galactose as a substrate. Four disaccharides were determined to be produced by both β-galactosidase and Maxilact with little difference between the two enzyme preparations. Galactobiose was isolated from the reaction mixture and identified by NMR. Maximal production of total disaccharides was less than 0.4 % yield. Production of commercial quantities of disaccharides using the transgalactosylation activity of this β-galactosidase is not a viable alternative to chemical synthesis.

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I would like to acknowledge my wife, Marei, and my children, Kirsten and Meike, who were always there whether the experiment worked or not. Without their love and kindness I would not have found it possible to complete this thesis. It was vitally important to me that my success or failure in research did not in any way affect their love for me. They were always there and always seemed to understand the problems that any graduate student faces.

I must also acknowledge the expert technical assistance of my wife Marei for running and interpreting the NMR spectra and for her many helpful scientific suggestions. Joe Slupsky was also of invaluable assistance in the electrophoresis experiments that were performed. I must thank my supervisor, Dr. Peter Sporns, for allowing me to pursue this research in the manner I best saw fit and yet being there at all stages for consultation and advice.

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Introduction to lactose

Lactose is the major carbohydrate found in mammalian milk (Morrissey, 1985). While there are a few mammals; notably sea lions, some seals and opossums, that have no lactose in the milk these are rare exceptions (Nickerson, 1983). The percentage (generally reported as weight per volume, w/v) of lactose in the milk varies from species to species and can range from as low as 2 % in mink to 7 % in humans and monkeys (Nickerson, 1983).

The source of lactose of most interest to the food scientist is the milk from domesticated cattle. The lactose percentage in cows' milk averages 4.9 % anhydrous lactose but can vary, depending upon the breed of cow, from 4.4 to 5.2 % (Nickerson, 1983). The percentage of lactose in the milk is under osmotic regulation and this sugar, along with the charged milk salts (principally sodium, and potassium cations as well as chloride anions) contribute to the osmotic pressure of milk (Morrissey, 1985). This value must, because of the physiology of the mammary gland, be very close to, if not identical to, the osmotic pressure of the animals blood (Morrissey, 1985). Under some conditions of disease, for example mastitis (a microbial infection of the udder), the secretory epithelium is disrupted and the osmotic balance can no longer be so finely controlled (Morrissey, 1985) resulting in lactose and milk salt values quite different from the non diseased animal.

Lactose is a disaccharide constructed from the two

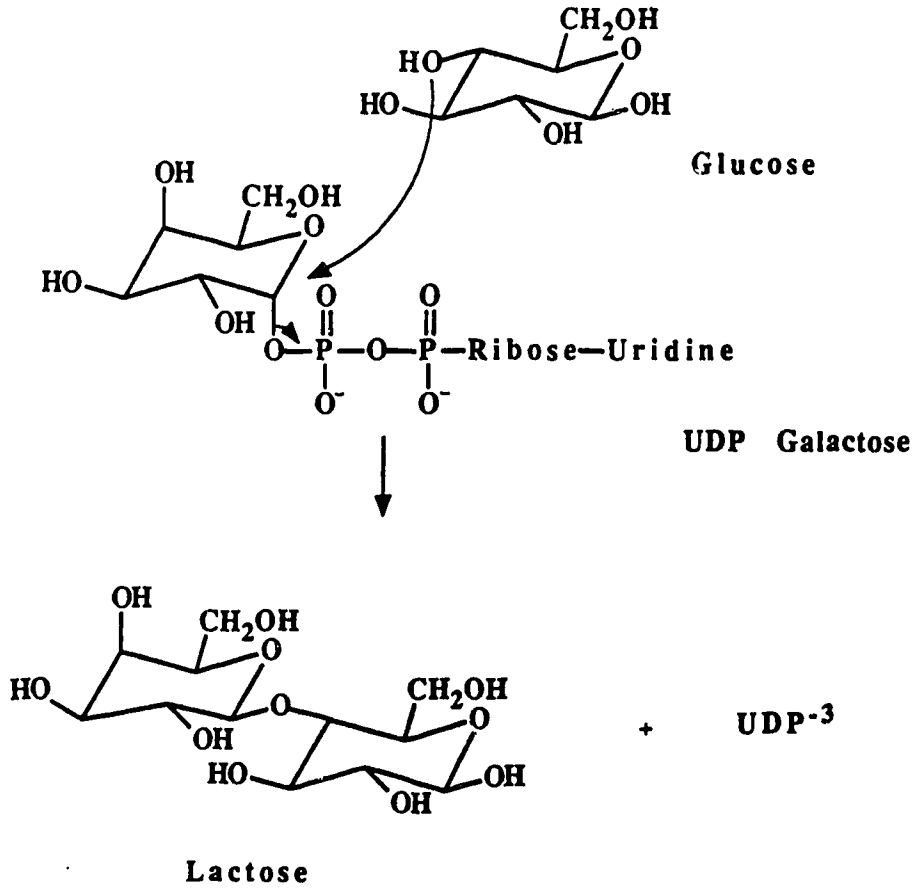
monosaccharides galactose and glucose, and is properly referred to in chemical nomenclature as β-D-galactopyranosyl-(1->4)-D-glucopyranose (JCBN, 1982). In solid, crystalline form it occurs most often as the α anomer of the glucose unit and is then referred to as α-lactose. If the glucose unit is in the β anomeric configuration then the molecule is referred to as β-lactose. These trivial names are acceptable for common usage (JCBN, 1982).

Lactose is biosynthesized in the mammary gland by the enzyme lactose synthetase (UDP-galactose-D-glucose 1-galactosyl transferase, E.C. 2.4.1.22). Lactose synthetase is the trivial name given to a combination of two enzymes; α-lactalbumin and UDP-galactosyl transferase (E.C. 2.4.1.22). UDP-galactosyl transferase will slowly transfer galactose from UDP-galactose to an acceptor hydroxyl group (which may be part of a glucose molecule) but in the presence of α-lactalbumin (the regulatory protein) the rate of this transfer is greatly enhanced (Walsh, 1979). An adaptation of the proposed mechanism is given in Figure I-1.

Lactose is relatively insoluble in water solutions when compared to other common disaccharides such as sucrose. The maximal solubility of lactose in aqueous solution at 25 °C is 21.6 grams per 100 mL of water. In contrast the maximal solubility of sucrose under these conditions is 62 grams per 100 mL water (Belitz and Grosch, 1988). This lack of solubility can lead to problems in the processing of dairy foods, which tend to be kept at refrigeration temperature or lower, with lactose crystallization adversely affecting the sensory characteristics of the product. This is especially prevalent in commodities such as ice cream mix that may be

Figure I-1

Lactose synthetase mechanism[1]



1. Mechanism adapted from Walsh, 1979

sweetened with sucrose. The combination of the two sugars further lowers the solubility of lactose (Keeney and Kroger, 1983).

The potential processing problems posed by lactose were the impetus to researchers studying lactose hydrolysis (Nickerson, 1983, Keeney and Kroger, 1983, Mahoney, 1985). Then, in the late 1950's and early 1960's, with the advent of enzyme technology and the discovery of hypolactasia (lactose intolerance) among large segments of the world population (Holzel et al, 1959, Bayless and Rosenweig, 1966) attention focused on hydrolyzing lactose to its constituent monosaccharides to overcome the nutritional problem of hypolactasia. A third reason for the interest in lactose hydrolysis was the increase in sweetness of the product gained by hydrolyzing lactose to glucose and galactose. This opened up the possibility of utilizing hydrolyzed cheese whey as a sweetener (Zadow, 1984). In comparison to sucrose, given a relative sweetness value of 1, lactose is at most 60 % as sweet with a relative sweetness value varying from 0.2 to 0.6 dependent upon the concentrations of the sugars being compared. In contrast the hydrolysis products; glucose and galactose, have relative sweetness values of 0.5 to 0.8 and 0.3 to 0.5 respectively (Belitz and Grosch, 1988). This would increase the sweetness of a hydrolyzed product approximately 2 times and would allow for the replacement of at least some of the sucrose used to sweeten some dairy products such as novelty ice creams (Zadow, 1984).

Introduction to hypolactasia

Contrary to the widely held belief in Europe and North America, it is not lactose intolerance but lactose tolerance which is the genetic abnormality. Except for a scant few races of humans, all originally concentrated in western Europe, there are no other mammals that have functional intestinal β-galactosidase (the carbohydrase responsible for hydrolyzing lactose to the absorbable monosaccharides, this enzyme is often referred to by its trivial name; lactase) as adults (Kretchmer, 1982). In European or North American culture there tends to be a pervading feeling that the members of those cultures are normal and that differences from that norm are abnormal. When faced with the statistic that over 70 % of the worlds population is lactose intolerant in late infancy and that this continues on into adulthood this idea must be rethought (Dahlqvist and Asp, 1975, Kretchmer, 1982, Welsh, 1982, Johnson, 1982, Simoons, 1982, Paige, 1982, Bolin and Davis, 1970).

In the early literature on lactose intolerance there was a great debate as to whether the deficiency of the enzyme was acquired because of weaning or a normal genetically controlled consequence of aging (Bolin and Davis, 1970, Hourigan, 1984, Lifshitz, 1982). There is still some controversy but careful experimentation has not shown enzyme adaptation in hypolactastic humans or rats (Hourigan, 1984, Lebenthal et al, 1973). A careful study of a tribe of hypolactastic North American aboriginal peoples tends to indicate that the trait (post weaning lactase deficiency) is an autosomally inherited

dominant trait (Johnson, 1982, Johnson et al, 1977, Johnson et al, 1978).

The physiological and nutritional importance of this phenomena is that it robs growing children of the available nutrients in milk (Paige and Bayless, 1982). This has led to a flurry of research into fermented dairy foods and into the use of β-galactosidase enzymes isolated from a variety of sources to hydrolyze the lactose in milk (Mahoney, 1985).

Introduction to β-galactosidase

β-galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.32) is an hydrolase acting upon lactose to yield the monosaccharides glucose and galactose. In the early literature on the enzyme, first isolated from *E. coli* , the mechanism was believed to involve participation of a cysteine group (Wallenfels and Weil, 1972) but more recent evidence (Loeffler et al, 1979, Legler and Herchen, 1981, Fowler and Smith, 1983, Herchen and Legler, 1984) has led researchers to believe that there is participation of an acidic amino acid in the catalysis. The candidate amino acid proposed in *E. coli* lacZ β-galactosidase is a tyrosine, the exact numbering of which is in dispute (Herchen and Legler, 1984, Loeffler et al, 1979). It is believed by these researchers that this is the explanation for the requirement of the *E. coli* β-galactosidase for a divalent cation. The divalent cation, usually magnesium, is in close proximity to the tyrosine and results in labilization of the phenolic hydroxy group. This tyrosine then acts as the hydrogen donor in the reaction. The pH effects upon *E. coli* β-galactosidase activity, whereby the enzyme is

only marginally active (if at all) under conditions of acidic pH is explained by the competition of the hydrogen cations for the divalent ion binding site and protonation of the ionizable phenol (Loontiens et al 1970, Tenu et al, 1971, Loeffler et al, 1979). It was this pH effect study that disproved the hypothesis of an active site cysteine (Loontiens et al, 1970). By use of *o*-mercuriphenyl chloride these researchers demonstrated only small effects upon the enzyme activity. This was proof that enzyme activity could not be requisite upon free cysteine at the active site. They were also unable to detect bound *o*-mercuriphenyl at the active site.

The situation in the yeast β-galactosidases is not so clear, mainly because of lack of research into the mechanism of the yeast enzymes. The enzyme from *Kluyveromyces fragilis* has been isolated as pure enzyme and partially characterized (Mahoney and Whitaker 1977, 1978). It is believed that this enzyme has an active site cysteine, in accordance with the earlier model of lactase action. This possibility was shown by titration of the enzyme with *p*-chloro mercuric benzoic acid (PCMB) which is a potent binding agent of free cysteines (Boyer 1954, Benesch and Benesch, 1962). Mahoney and Whitaker (1977) have proposed that the *K. fragilis* β-galactosidase enzyme consists of 10 subunits, each with a separate catalytic site, but as of this date this result has not been duplicated in the literature.

The β-galactosidase from *Kluyveromyces marxianus* (formerly *Kluyveromyces lactis* and earlier *Saccharomyces lactis*) has been purified and partially characterized. Dickson et al (1979) published a purification scheme for β–galactosidase from *K. lactis* Y1140. They

isolated the enzyme from freshly harvested cells using cell homogenization followed by centrifugation. The centrifuged pellet was suspended in buffer and chromatographed on DEAE Sephadex A25 and further purified on hydroxyapatite. They reported a final specific activity of 139 units/mg protein (1 unit = 10^{-6} moles *o*-nitrophenyl β-D-galactopyranoside [ONPG] hydrolyzed per minute) with an overall yield of 27 %. To further characterize the enzyme they determined the Michealis - Menton constant for ONPG and reported a value of 1.66 mM. These researchers also studied the cations necessary to achieve maximal enzyme activity and found that a monovalent and divalent cation were necessary. There was no definite requirement for specific ions but sodium and manganese had the greatest positive effect upon activity.

The enzyme has also been isolated from a different strain of *K. lactis* (Biermann and Glantz, 1968), the strain they called K12. The enzyme was isolated using ammonium sulfate precipitation and column chromatography on 2 columns; Sephadex G100 and DEAE Sephadex A50. These researchers report very little characterization of the enzyme other than showing a requirement for magnesium for optimal activity and reporting a pH maximum and Michealis - Menton constant (K_m). They reported a pH optimum of 7.2 in phosphate buffer and a K_m for ONPG of 1.18 mM.

A recent paper (Hussein et al, 1989) reports the isolation of β-galactosidase from an unspecified strain of *K. marxianus* . These researchers utilized acetone precipitation of homogenized cells followed by a second fractionation using ammonium sulfate. The precipitated proteins were suspended in buffer and

chromatographed on hydroxyapatite. The specific activity of their purified enzyme was 0.09 units/mg protein with an overall yield of protein of 9 %. They reported a pH optimum for this enzyme of 7.0 and conducted a determination of the enzyme extinction coefficient which they reported as 1.23 cm^2 mg^{-1} at 280 nanometers (nm). The K_m for ONPG reported by these researchers is 1.25 mM.

The active site configuration of the β-galactosidase from *K. marxianus* has not been determined. Neither has the amino acid composition or a theory of the active site amino acids. There is indirect evidence (Dickson et al, 1979), not corroborated in the literature to this date, for a catalytic cysteine.

The purification of the enzyme from bacterial and fungal sources has been widely published and a number of organisms have been studied (McFeters et al, 1967, Steers et al, 1971, Ohtakara et al, 1981, Mozaffer et al, 1984). The purification techniques employed have ranged from column chromatography coupled with isoelectric focussing (Ohtakara et al, 1981) to affinity column chromatography utilizing *p*-aminothiophenyl-β-D-galactopyranoside (PAPTG) covalently linked to agarose (Steers et al, 1971). Some researchers (McFeters et al, 1967) have utilized only precipitation techniques because of the lability of the enzyme.

The use of affinity chromatography for the yeast β-galactosidase from *K. fragilis* resulted in pseudo-affinity chromatography because of a low K_m of the enzyme for PAPTG (Mahoney and Whitaker, 1977). These researchers believe that most of the enzyme binding to the column was due to the linking arm used. More classical techniques (DEAE Sephadex and gel

chromatography) have been utilized for isolation of the enzyme from this source with success (Uwajimi et al, 1972).

Modern techniques, such as immunochemical chromatography and hydrophobic interaction chromatography have been attempted as tools to aid in the isolation of β–galactosidase from *K. marxianus* . These attempts have met with little, if any success (Peter Sporns, private communication). One probable reason for these failures is glycosylation of the enzyme.

The β–galactosidase enzyme from *E. coli* is known to be a tetramer of 520,000 daltons molecular weight (Loontiens et al, 1970, Walsh, 1979). The molecular weight of the β-galactosidase from *Bacillus circulans* is considerably less, at 240,000 daltons. It is believed that this protein is monomeric (Mozaffer et al, 1984). The enzyme from *Streptococcus lactis* 7962 is of relatively high molecular weight, because it is excluded from the column on Sephadex G200 column chromatography (McFeters et al, 1967) however no exact molecular weight was determined for the enzyme. The researchers who conducted this study believe that this enzyme is constructed from disporportionate molecular weight subunits, though no conclusive evidence was reported to substantiate this result (McFeters et al, 1967).

Dickson et al (1979) purified the β-galactosidase from *K. marxianus* by a combination of methods and using SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) determined that the subunit molecular weight of the enzyme was 135,000 $\pm$ 10 % (daltons) and that it was probably dimeric in structure. This gave a reported molecular weight of

270,000 daltons. Breunig et al (1984) report an identical molecular weight for the β-galactosidase enzyme from *K. marxianus*.

The amino acid sequence of the lacZ *E. coli* β-galactosidase is known (Fowler and Zabin, 1978, Hood et al, 1978). The exact molecular weight of the monomer has therefore been calculated and established as 116,353 (Fowler and Zabin, 1978), which gives a molecular weight for the tetrameric protein of 465,412. This weight was determined from a calculation based upon the molecular weight of the individual amino acids in the peptide backbone of the protein. The N terminal amino acid sequence of the β-galactosidase from *K. marxianus* has been determined from an analysis of the gene for this protein. No attempt was made to determine the N terminal amino acid by biochemical means on the protein itself. The sequence determined for the protein shows a high degree of homology with the *E. coli* lacZ β-galactosidase sequence in amino acids 66 to 116 (83 to 130 in the *E. coli* lacz β-galactosidase) where the homology is almost 65 % (Breunig et al, 1984). These researchers suggest that there might be divergent evolution of the yeast and bacterial enzymes from a common ancestor.

Introduction to the use of β-galactosidase in the food industry

The β-galactosidase enzyme from yeasts and fungal sources is being used to hydrolyze lactose in milk to accelerate yogurt production (O'Leary and Woychik, 1976), decrease the time of cheddar cheese ripening (Weaver and Kroger, 1978) and hydrolyze milk for lactose intolerant individuals (Kosikowski and Wierzbicki,

1973, Guy and Bingham, 1978, Morisi et al, 1973, Mahoney, 1985, Gist Brocades, 1988).

A number of different sources of the enzyme have been used industrially and a few bacterial sources, such as *Streptococcus thermophilus*, have GRAS (generally regarded as safe) status sought by industry (Table I-1) (Mahoney, 1985). The pH optima of the yeast enzymes is in the range 6.0 to 7.5 and for this reason they are generally used to hydrolyze lactose in fluid milk, either for use as fluid lactose hydrolyzed milk or as a source for later fermentation such as in cheese or yogurt manufacture (O'Leary and Woychik, 1976, Weaver and Kroger, 1978, Gist Brocades, 1988). The fungal enzymes, because their pH optima are considerably lower, in the range 3.0 to 5.5, are used to hydrolyze the lactose in acid whey to reduce the problems of lactose crystallization during concentration of this product (Nijpels et al, 1980).

A number of different firms and academic researchers have attempted to immobilize the enzyme from yeast sources with varying results. Enzyme immobilization is being actively researched for economic reasons; the batch method of enzyme utilization causes loss of the often expensive, and potentially reusable, enzyme unless expensive methodology such as ultrafiltration of the end product is used.

Valio, in Finland, have been successful in immobilizing the β-galactosidase enzyme from the fungus *Aspergillus niger* by adsorbing it onto phenol formaldehyde resin. This process is being used on a pilot plant scale to hydrolyze the lactose in whey ultrafiltrate (Gekas and Lopez-Leiva, 1985). The Corning Glass

Table I-1

Commercial sources of β-galactosidase

Source	Organism type
Kluyveromyces fragilis	Yeast
Kluyveromyces marxianus	Yeast
Candida pseudotropicalis	Yeast
Aspergillus niger	Fungus
Aspergillus oryzae	Fungus

Company in New Jersy, U.S.A. has a semi-industrial scale plant using *A. niger* β-galactosidase covalently linked to silanized glass through an imine bond to the protein. This system is used to hydrolyze the lactose in deproteinized acid whey to yield a high quality food source for bakers' yeast (Richmond et al, 1981, Gekas and Lopez-Leiva, 1985). The only industrial scale immobilized β-galactosidase reactor being used at the time of this writing is in Milano, Italy at the Snamprogetti dairy facility (Gekas and Lopez-Leiva, 1985). In this process the β-galactosidase from *K. marxianus* is entrapped in cellulose triacetate fibers (Morisi et al, 1973). The authors of this paper report no changes in the pH maxima for the enzyme after this treatment but do report a dramatically higher apparent Michealis - Menton (K_m) for lactose. They attribute this to diffusion limitations imposed upon the system by the fiber entrapment process. They report an enzyme half life of 40 days at refrigeration temperatures and the possibility to use the same reactor for up to 80 days under these conditions. The authors do report, as a note, that the enzyme was dramatically unstable under the conditions of immobilization at temperatures ranging from 25 to 30 ^{o}C. As of 1985 it was believed that the Gist Brocades Co. was developing an immobilized enzyme reactor utilizing *K. marxianus* β-galactosidase but actual results are unknown because of company secrecy regarding the project (Gekas and Lopez-Leiva, 1985).

Introduction to transgalactosylation

The mechanism of hydrolysis of the glycosidic linkage in lactose by the β-galactosidase enzyme is such that the galactosyl unit

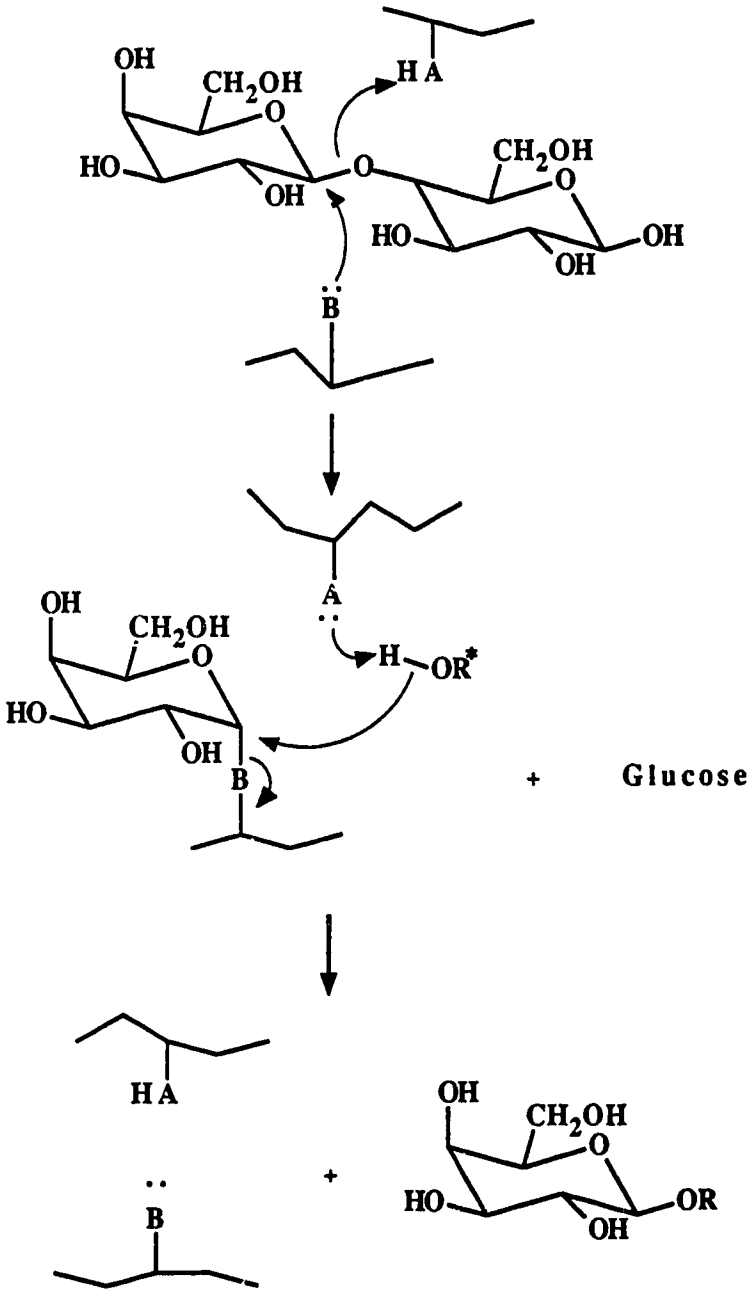
can be transferred to any nearby hydroxyl acceptor (Figure I-2). This hydroxyl acceptor can be water (the acceptor for hydrolysis leading to the expected end products, galactose and glucose) or it could be carbohydrate. If the acceptor were a carbohydrate the products of the reaction would be the monosaccharide glucose and a galactose containing oligosaccharide. The latter product is what is referred to as a transgalactosylation product (transfer of a galactose to a carbohydrate to create a sugar of one higher linkage than the acceptor sugar was originally).

This above mechanism explains formation of the lac operon promoter allolactose (β-D-galactopyranosyl (1->6) D-glucopyranose) (Huber et al, 1976). It is believed that in the *E. coli* β-galactosidase active site the transfer of the galactosyl unit to the glucose occurs immediately after hydrolysis of the β (1->4) bond without diffusion of the glucose unit into or out of the active site (Huber et al, 1976). These researchers reported that the transferase/hydrolase ratio of activity for the enzyme is affected by the pH of the buffer and postulate that conformational changes due to the protonation/deprotonation of susceptible amino acids may be the cause.

There have been a number of studies conducted on the transgalactosylation reaction of β-galactosidase from bacterial, yeast and fungal sources (Mozaffer et al, 1984, Huber et al, 1976, Greenberg and Mahoney, 1983, Jeon and Mantha, 1985, Ajisaka et al, 1987, Ajisaka and Fujimoto, 1989, Ajisaka et al, 1988, Toba and Adachi, 1978, Pazur, 1954, Pazur et al, 1961, Nilsson, 1988, Galensa, 1984, Kosikowski and Wierzbicki, 1973). Some interest has been

Figure I-2

β-galactosidase mechanism[1]



* R = Carbohydrate, the reaction is transgalactosylation
= H, (the molecule is water), the reaction is hydrolysis

1. Mechanism adapted from Walsh, 1979.

expressed in the nutritional aspects of these compounds (Toba and Adachi, 1978, Jeon and Mantha, 1985, Richmond and Gray, 1981) but there has not been a complete study along these lines. Most of the interest in the transgalactosylation reaction of the enzyme is in its exploitation to synthesize pharmacologically interesting compounds (Ajisaka and Fujimoto, 1989, Ajisaka et al, 1987, Nilsson, 1988, Pazur et al, 1961). There is also interest, mostly concentrated upon the *E. coli* enzyme, on the actual mechanism of the transgalactosylation side reaction of these hydrolase enzymes (Huber et al, 1976). Research has also been conducted on the production of oligosaccharides produced by β-galactosidase during lactose hydrolysis in dairy products (Kosikowski and Wierzbicki, 1973, Toba and Adachi, 1978, Galensa, 1984, Jeon and Mantha, 1985, Mozaffar et al, 1984) but a comprehensive study on the production of individual disaccharides and trisaccharides with purified enzymes has not yet been reported.

The possible nutritional concerns of these unstudied disaccharides and trisaccharides makes a study of their production necessary. It is possible that these newly formed disaccharides may not be metabolized in the upper intestine and pass on as free sugars into the lower intestine, where the metabolism of these carbohydrates by the gastrointestinal bacteria may cause exactly the same symptoms as a lactose dose in hypolactastic individuals (Phillips, 1982, Burvall et al, 1980). This would cause the same problem that the hydrolysis of lactose was supposed to solve.

Some work has been performed on identifying the di- and trisaccharides produced in the transgalactosylation reaction during

the enzymatic hydrolysis of lactose (Pazur, 1954, Toba and Adachi, 1978, Burvall et al, 1979,1980, Asp et al, 1980, Greenberg and Mahoney, 1983) but most of the work has utilized monosaccharides or *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to produce the transgalactosylation products (Pazur et al, 1958, Pazur, 1961, Ajisaka et al, 1988, Ajisaka and Fujimoto, 1989, Ajisaka et al, 1987, Nilsson,1988, Asp et al,1980, Burvall et al, 1979,1980). Only a few of these studies have not been on the *E. coli* β-galactosidase enzyme. Toba and Adachi (1977) studied the β-galactosidase from *Saccharomyces fragilis* (now *Kluyveromyces fragilis*) and *Aspergillus oryzae* while Pazur (1961) studied a mixed β-galactosidase from *Saccharomyces, Torpulis* and *Candida* yeasts. Ajisaka et al (1988) have studied the enzyme from *Aspergillus oryzae* (a food grade enzyme) as well as the *E. coli* enzyme. Greenberg and Mahoney (1983) have studied the β-galactosidase from *Streptococcus thermophilus* and noted the production of galactobiose (β-D-galactopyranosyl (1->6) D-galactopyranose) and allolactose (β-D-galactopyranosyl (1->6) D-glucopyranose) as the two major oligosaccharides produced by the transgalactosylation reaction. Burvall et al (1979,1980) and Asp et al (1980) have studied the enzyme from *Kluyveromyces marxianus* and specifically from Maxilact preparations. They reported observing a number of oligosaccharides by paper chromatography and tentatively identified allolactose and galactobiose as the major disaccharides, other than lactose, formed by the transgalactosylation reaction of this enzyme. They conclude that β(1->6) galactosidic linkages are the only linkages formed in the transgalactosylation reaction by the β-galactosidase in

Maxilact (Asp et al, 1980). In their work there was no attempt made to purify the enzyme away from other potentially interfering carbohydrase activities in the preparation. Olano et al (1983) have reported a number of different enzymatic activities in Maxilact preparations ranging from proteases to other carbohydrases, including α-galactosidase and β-glucosidase. The assumption made in the work by Asp et al (1980) was that all of the glycosyl linkages were of the β form. The possibility of α-galactosidase action calls this into doubt. It is possible that one or more of the oligosaccharide products that they reported may be incorrectly identified because of the anomeric configuration of the glycosidic bond. These researchers determined the sugar structures by gas chromatography (GC) methylation analysis. In their work they reported retention times of the methylated monosaccharides (after acid hydrolysis of the permethylated oligosaccharides) on only one column. It is reported in the literature that proper GC methylation analysis requires comparison of retention times on at least two different columns of differing polarity (Geyer et al, 1982). If this is impossible the work should be conducted by co-injection of known methylated standards and unknowns (Toba and Adachi, 1978). The best method yet known for the methylation analysis of carbohydrates is GC-mass spectrometry using a GC-mass spectrometer (GC-MS) (Geyer et al, 1983). The reasons for these elaborate precautions is because of the possibility of misidentifying one of the closely eluting peaks relative to the standard which has been co-injected or used as a reference peak.

Other researchers, studying the enzyme from other sources, have noted that there is formation of β(1->6) linkages (Greenberg and Mahoney, 1983); β(1->6), β(1->2) and β(1->3) linkages (Toba and Adachi, 1978); and β(1->6) and β(1->3) linkages (Nilsson, 1988). Much earlier in the literature Pazur et al (1961) tentatively identified β(1->6) and β(1->3) linkages in transgalactosylation reactions catalyzed by β-galactosidase. Ajisaka et al (1988) in their work with *E. coli* and *A. oryzae* β-galactosidase enzymes have identified β(1->6), β(1->4), β(1->3), and β(1->2) glycosidic linkages by C-13 NMR (nuclear magnetic resonance) spectroscopy.

These results call into doubt the findings of Asp et al (1980) and Burvall et al (1979,1980) that the β-galactosidase from *K. marxianus* would catalyze only β(1->6) glycosidic linkages during transgalactosylation activity. This makes the need for further study of the transgalactosylation reaction of *K. marxianus* β-galactosidase necessary.

Introduction to gas chromatography (GC) of disaccharides

Most disaccharides, in fact most carbohydrates, are not volatile enough for use in GC. In order to analyze carbohydrates by GC the carbohydrate must be derivatized before injection onto the GC column. A number of different ethers have been used for this purpose, notably methyl ethers (Toba and Adachi, 1978, Geyer et al, 1983) and trimethylsilyl (TMS) ethers (Gianetto et al, 1986). As well there are useful ester derivatives; trifluoroacetic acid esters (Laegrid et al, 1986), and acetates (Whiton et al, 1985). There are a number of

other, less utilized derivativizing reagents including, trifluoroacetic acid to make the anhydrides of the carbohydrate and this acid, isopropylidene to make the isopropylidene acetals and butane borates to make the butane borate carbohydrate derivative (Lee et al, 1984).

The classic work on trimethylsilylation of carbohydrates was published in 1963 by Sweeley et al. In the reaction these researchers used hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) dissolved in pyridine (as the carrier solvent and as a general base to mop up acid produced in the reaction) and the sugar added. They noted silylation both with and without heating of the sample and found that for more hindered carbohydrates heating of the reaction mixture was necessary to achieve complete silylation of all the available hydroxyl groups. This mixture is now marketed world wide by the Pierce Chemical Company under the trade name Tri-Sil Z. In literature from the Pierce Chemical Company (1989) that accompanies the reagent, it was stated that silylation of most carbohydrates was complete at room temperature within ten minutes but that in some cases heating of the reaction mixture might be necessary. It has been shown, however, that carbohydrates as simple in structure as maltose require much longer reaction times or elevated temperatures to ensure complete silylation of the carbohydrate (Bhatti and Clamp, 1968).

The TMS reaction has been adopted as one of the most versatile methods of carbohydrate derivatization for GC (Pierce Chemical, 1968). The reaction is however adversely affected by water, which uses up the TMCS catalyst and renders silylation of the carbohydrate

incomplete. For sugars which are aqueous syrups and can not be dried without destruction of the sugar the method of choice for derivatization has therefore become N-trimethylsilyl-imidazole silylation catalyzed by excess TMCS (van Ling et al, 1967, van Ling, 1969). In most work determining carbohydrates by GC the method of Sweeley et al (1963) remains the most utilized (Pierce Chemical, 1989) even when compared to methylation or acetylation (Laker, 1979).

The largest problem with the GC analysis and identification of carbohydrates is that the detection methods used in most modern GC [flame ionization detection (FID), mass spectrometry (MS) and even infared (IR)] are so sensitive that each of the 5 major possible solution forms of a sugar are identified. Each carbohydrate can exist in at least 5 forms; an acyclic (straight chain) form and as the α or β anomer of each of the pyranose (6 membered) or furanose (5 membered) ring forms. This causes a great difficulty in interpretation of the collected data. The multiplicity of peaks makes integration of a mixed carbohydrate sample almost impossible because of peak overlap and makes identification of an unknown carbohydrate a formidable task (Angyal, 1984).

These isomeric forms have led to the necessity of including an extra step in the work up of samples for GC to reduce the anomeric carbon of the sugar to a sugar alditol. The resultant sugar alditols can be acetylated, methylated or trimethylsilylated and give only one peak in both packed and capillary GC (Ochiai, 1980, Holzer et al, 1980, Crowell and Burnett, 1967, Borchard and Piper, 1970). Other methods that have been used to reduce the complexity of the

spectrum obtained are; derivatization of the reducing sugar to an oxime before trimethylsilylation (Mason and Slover, 1971) and lithium perchlorate equilibration of the isomeric carbohydrate mixtures before TMS derivatization (Cowie and Hedges, 1984). Both of these methods give only a single peak for each derivatized sugar.

A number of studies have been performed in which TMS ethers of carbohydrates are used in GC analysis of different foods. Mason and Slover (1971) have used preparation of the oxime and TMS derivatization to determine the carbohydrates in wheat and wheat products. TMS derivatization has also been used to determine the identity and amount of sugars in corn starch hydrolysates, specifically low dextrose equivalent (DE) corn syrups. Foods as varied as chocolate (Demaimay and Lebouteiller, 1978) and chewing gum (Daniels et al, 1982) have been studied for carbohydrate content by using the TMS derivatives of the carbohydrates in a GC procedure.

Introduction to cerate oxidimetry

Cerium (Ce) is the first element in the lanthanide series of elements. It is a highly electropositive element, like all the lanthanides, and its chemistry is determined by the size of the Ce(III) [Ce^{3+}] ion. The lanthanides are often referred to as the rare earth elements but in fact they are more abundant than mercury (Hg), an element that is not commonly considered rare. Of all the lanthanide series Ce(IV) is the only tetrapositive species stable enough to exist in aqueous or solid form. The reasoning for this is simple. In this form the element attains the same configuration as the noble gas Xenon (Xe). Ce(IV) has a tendency to hydrate in

solution and can exist in a number of different complexes, the primary one being $[Ce(H_2O)_n]^{4+}$. The most common salt of tetrapositive Ce is the double salt, ceric ammonium nitrate or more specifically, hexanitratoammoniumcerate $[(NH_4)_2Ce(NO_3)_6]$. Other salts, such as the pyridinium salt, are also known to exist but they are much less stable than hexanitratoammoniumcerate (Cotton and Wilkinson, 1967).

The high electropositive charge of the Ce(IV) and the Ce(III) ions lends to a high redox potential for the Ce(IV)/Ce(III) couple. The redox potential of the couple is however dependant upon the nature and strength of the acid that the couple exists in. It is believed that this acid dependence occurs because of the multitude of anionic and cationic species that can exist in the different acid solutions (Rao and Rao, 1972). For example, the oxidation reduction potential of the Ce(IV)/Ce(III) couple in 1 M nitric acid is 1.61 V, while in 1 M perchloric acid the potential for the couple is 1.70 V. This is higher than the redox potential for water (1.23 V) (CRC Handbook, 1984) and therefore aqueous solutions of Ce(IV) salts have been called metastable (Cotton and Wilkinson, 1967) because of the possibility of the oxidation of water with the reduction of Ce(IV) to Ce(III).

A number of studies have been published on the Ce(IV) oxidation of carbohydrates and simple alcohols (Rao and Rao, 1972, Shukla and Beg, 1979, Shriner et al, 1980, Sharma, 1956, Virtanen et al, 1987). Sharma (1956) studied the complete oxidation of fructose and glucose with excess ceric ammonium nitrate and determined that there was a specific need for hydrogen and oxygen in the reaction. Sharma determined that the oxidation of carbohydrates proceeded

stepwise with each carbon-carbon bond breaking requiring 2 equivalents of Ce(IV). He also demonstrated the production of formic acid as the end product of the reaction from the complete oxidation of glucose.

Rao and Rao (1971) examined the kinetics and acid effects upon the Ce(IV) oxidation of methanol and ethanol. They observed pseudo-first order kinetics with a rate constant inversely affected by the concentration of free hydrogen ion. They reported that the rate of oxidation was inversely proportional to increased concentration of nitrate ion. They concluded that there were a number of different Ce(IV)-acid-nitrate complexes and that these different complexes could each react with an individual rate in the oxidation of alcohols. When they had completed these initial studies they then experimented with the use of Ce(IV) to determine methanol and ethanol quantitatively in solution. With both alcohols they report pseudo-first order kinetics. Pseudo-first order kinetics are kinetic plots that appear first order only when the concentration of one of the reactants is held in considerable excess over all the others. With the concentration of all other reactants held constant, and that the rate constant changes as the concentration of this reactant (the one held in excess) is varied. Using this technique the authors were successful in determining both ethanol and methanol in acidified aqueous solutions.

Shukla and Beg (1979) adapted the Ce(IV) oxidation for the determination of sugars in aqueous solution. They studied only one sugar at a time in solution and calculated the concentration of sugar from a titration of remaining Ce(IV) after they believed the oxidation

to be complete. In this work the oxidant [Ce(IV)] was added in excess to the carbohydrate solution and the mixture left for an indeterminate time at room temperature, usually less than one hour. When this time was complete, the excess, or remaining Ce(IV), was titrated. In this study the sugar being analyzed was known in advance because the structure of the sugar (the number of oxidizable carbons) must be known to correctly calculate the quantity of sugar because of the necessary back titration of unreacted Ce(IV). This renders the work of academic interest but of little practical application.

In the experimental protocol of Virtanen et al (1987) both the classical and the stopped flow times of the Ce(IV) oxidation of carbohydrates were studied. They determined the presence of two, separate and interchangeable, Ce(IV)-carbohydrate complexes that reacted at different rates. It was also shown, from a knowledge of the relative amounts of the different sugar isomers (straight chain, and the α and β anomers of the pyranose and furanose rings) in aqueous solution, that the open chain (straight chain) form of the carbohydrate probably reacted faster than the ring forms. These researchers observed second order rate kinetics for the reaction that could be made pseudo-first order by increasing the carbohydrate concentration high enough that complex formation was saturated. Most important, for the work on Ce(IV) oxidation presented in this thesis, they noted different rates of reaction for different carbohydrates. They noted faster oxidation of galactose when it was compared to glucose and faster oxidation of ribose in comparison to xylose. This complemented the observation by Shriner et al (1980)

that the red Ce(IV)-carbohydrate complex color was lost at different rates with different carbohydrates. This different reaction time for different carbohydrates offered the possibility of utilizing Ce(IV) oxidimetry of carbohydrates as an analytical tool for determinations of carbohydrates in foods.

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Determination of Lactose and Lactose Hydrolysis in Milk using Ce(IV)[1]

INTRODUCTION

Lactose is the major carbohydrate constituent of milk (Nickerson, 1983) and as such the analysis of the content of this carbohydrate is important both to food processors and nutritional researchers as well as the more chemically oriented dairy and food researchers (Nickerson, 1983). There are a variety of different tests for lactose in milk encompassing polarimetric techniques (Association of Official Analytical Chemists, [A.O.A.C.] 16.055, 1984), gravimetric (A.O.A.C. 16.057, 1984), enzymatic (A.O.A.C. 16.059, 1984) or infrared analysis (A.O.A.C. 16.090, 1984). In the dairy industry there are also a number of other techniques utilized to determine lactose in milk. None of these other techniques had been given first action status by the A.O.A.C. as of 1984. These techniques include chromatographic techniques; HPLC (high performance liquid chromatography), TLC (thin layer chromatography) and GC (gas chromatography) (Betshart and Prenosil, 1984; Robards and Whitelaw, 1986), as well as other analysis methods such as cryoscopy (Baer et al, 1980; Nijpels et al, 1980; Frank and Christen, 1984).

All of these methods, both those recognized by the A.O.A.C. and those not recognized, have advantages and disadvantages. The polarimetric technique is very accurate but it requires expensive

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instrumentation and considerable time for each analysis (Biggs and Szijarto, 1963). The gravimetric technique is inexpensive but is much less accurate and requires lengthy analysis times. (A.O.A.C. 16.057, 1984). The enzymatic method is relatively inexpensive but does require a longer amount of time for each analysis and uses perishable reagents (Boehringer Mannheim, 1986). The infrared method is rapid, accurate and can handle many samples yet it requires periodic calibration of the equipment with a polarimetrically determined lactose sample and it requires that the samples not vary greatly in protein or fat. The chromatographic methods are not widely used because of the expense of the equipment involved. There is also a requirement in some determinations for pre or post column derivatization of the sample and the reagents required can be expensive.

Only the chromatographic or enzymatic methods can be used both for monitoring lactose and lactose hydrolysis in milk and dairy products. There was a gap in the current analysis methods that could be filled by an inexpensive, simple test that could measure both parameters. With this in mind it was decided to investigate the advantages of the cerium(IV) [Ce(IV)] oxidation of carbohydrates (Shriner et al, 1980) for the determination of lactose and lactose hydrolysis in milk. It was noted that this test showed considerable differences in the oxidation times of monosaccharides and disaccharides and that this oxidation could be followed colorimetrically.

MATERIALS AND METHODS

All water was prepared using a Millipore Milli-Q system (Millipore Co.). β - galactosidase (Maxilact L2000 from *Kluyveromyces marxianus* (formerly *lactis*)) was a gift from Gist Brocades Co., North Carolina. All chemicals were reagent grade or better.

Zinc acetate, phosphotungstic acid (Znapta) precipitation solution

Znapta was prepared by adding 12.5 g. of zinc acetate [$Zn(C_2H_3O_2)_2 \cdot 2H_2O$] and 7.5 g. of phosphotungstic acid [$H_3PO_4 \cdot 12WO_3 \cdot 24H_2O$] to 8 mL of glacial acetic acid and diluting to 600 mL with water (Biggs and Szijarto, 1963).

Determination of factor affecting haze in Znapta solutions

Znapta was prepared as above. The final pH of the solution was 4.6. A problem with haze forming during the milk clarification procedure was traced to this reagent, which when examined closely appeared hazy to the eye. The haze removed the possibility of utilizing a spectrophotometric test. It was noted that solution that sat for 7 days at room temperature became clear and there was no haze formed later in the test.

To discover the cause of this haze clearing, a 600 mL batch of Znapta was divided into 4 lots. The first lot was placed at 4 ^{o}C in the dark in a laboratory refrigerator. The second lot was placed at room temperature in the dark and the third lot left at room temperature in

direct sunlight. All three of these solutions were left for one week and then visually inspected for haziness. A fourth solution was brought to a boil and left boiling for thirty minutes. Water was then added to this solution to make up the lost volume. This water loss did not exceed 10 to 15 milliliters.

All four solutions were also tested for pH before and after the treatment as pH of the solution is an integral part of the precipitation process (Garrison and Haigh, 1942; Biggs and Szijarto, 1963).

Titration of Ce(IV) solutions

First 0.025M ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2$] containing 0.001M ferroin [1,10 phenanthroline Fe(II) complex] indicator was standardized using a known amount of ammonium hexanitratocerium(IV) [$(NH_4)_2Ce(NO_3)_6$] (the salt is hereafter referred to as Ce(IV)] in 20 mL of 1M sulfuric acid (ammonium hexanitratocerium(IV) was a suitable primary standard if used quickly after preparation of the sample, Harris and Kratochvil, 1981). The solution developed a sky blue endpoint with no trace of green color when the Ce(IV) was completely reduced to Ce(III). A blank of 20 mL of 1M sulfuric acid was titrated in a similar manner and the blank value subtracted from all Ce(IV) titrations.

The standardized ferrous solution was then used to titrate 0.5 mL of ammonium hexanitratocerium(IV) added to 20 mL of 1M sulfuric acid. All titrations were carried out at least in triplicate.

Determination of interferences to the Ce(IV) test arising during milk testing

An immediate precipitate formed in the clarified, filtered milk samples treated with the Znapta solutions, upon addition of Ce(IV) oxidant to these solutions. A series of experiments with phosphorous (as potassium phosphate, K_3PO_4), zinc acetate, and tungsten (as phosphotungstic acid) were undertaken to determine the cause of this precipitate. In all of these experiments a solution of the possible interfering substances were added to a 0.39 M Ce(IV) solution in 0.5M nitric acid (HNO_3) and observed visually for formation of a precipitate.

Upon solving this immediate precipitate problem it became apparent that there was a second, late forming, precipitate occurring in the reactions. Ce(III) was the major cerium species present at this stage in the reactions. A series of compounds were investigated as causes of the late precipitate. Phosphotungstic acid, acetic acid, zinc acetate, and phosphorous were tested at the molarities found in the clarified solution. As well, calcium (as calcium chloride ($CaCl_2$)), sodium chloride (NaCl), magnesium (as magnesium chloride [$MgCl_2$]), and citric acid ($C_6H_8O_7$) were all tested at the levels that could be expected in the clarified, filtered milk samples, basing the results upon the reported values in mg/100mL of these constituents in milk (Johnson, 1983).

These solutions were added to a 0.39M solution of Ce(III) chloride ($CeCl_3$) in 0.5M nitric acid and the resultant solution observed visually for precipitation.

Determination of Ce(IV) use as an analytical tool for lactose/lactose hydrolysis

Before the Ce(IV) test was attempted on milk samples, simple carbohydrate solutions were examined. These solutions were galactose, glucose or lactose dissolved in water at a concentration of 1.5 % (w/v). A solution of 0.39M Ce(IV) in 0.5M nitric acid was added to the sugar solution in a 1 to 3 ratio and the time for absorbance at 445 nanometers to fall to 0.4 was measured.

Determination of optimal absorbance to measure as endpoint of the Ce(IV) oxidations

Solutions of 100 % lactose (29mM) and glucose:galactose:lactose (to represent hydrolysis levels of 50, 60, 70, 80, 90, and 100 %) were prepared. The solutions representing lactose hydrolysis were based upon an original lactose concentration of 29mM and therefore at 100 % hydrolysis represented a solution that contained 29mM glucose and 29mM galactose. The lactose solution and glucose:galactose solution representing 100 % hydrolysis were mixed in the appropriate ratios to give the required hydrolysis solutions.

A comparison of time to absorbance (from greater than 2.0 to 0.3) at 445 nanometers was then conducted and plotted. From these graphical representations of the experimental data the optical density (OD) giving the greatest difference between 10 % increments of lactose hydrolysis was determined.

Ce(IV) oxidations

Oxidations were carried out using a 1 to 3 ratio of 0.39M ammonium hexanitratocerium(IV) in 0.5M nitric acid to sugar solution. The Ce(IV) solution was at least 6 hours old and was shaken before use to suspend the small amount of fine precipitate that formed after this time. The original concentration of the Ce(IV) solution, before the occurrence of this precipitate was 0.4M. The mixed solution was placed in either a Baush and Lomb Spectronic 20 (zeroed with the original sugar solution) or a Pye-Unicam SP1800 spectrophotometer (sugar sample used as the reference) and the time in seconds for the absorbance at 445 nanometers to fall to 0.4 recorded. The Pye-Unicam spectrophotometer sample compartment was temperature controlled and maintained at 25.1 $\pm$ 0.1 ^{o}C using a LoTemptrol 154 bath (Precision Scientific Co.) containing ethylene glycol:water (50:50, v:v).

Determination of the optimal wavelength to measure the difference in Ce(IV)-carbohydrate complex and Ce(III)

A series of solutions of Ce(IV) in 0.5M nitric acid were created. In one solution excess carbohydrate in Znapta solution (glucose was the carbohydrate chosen) was added to the Ce(IV) solution and immediately a complete uv (ultraviolet) visible spectrum from 200 to 800 nanometers was taken using an Hewlett Packard 8451A Diode Array spectrophotometer. This procedure was repeated on this sample after reduction of the Ce(IV) was complete. This sample therefore represented a scan of a Ce(III) - oxidized carbohydrate

sample in 0.5M nitric acid. The range from 400 to 550 nanometers was seen to be of the most interest.

A complete uv-visible spectrum of Ce(IV) in 0.5M nitric acid, added to Znapta precipitation solution, was also performed.

Determination of optimal acid level and type of acid used in the Ce(IV) test

The utility of the ammonium hexanitratocerium(IV) test, using the acids, nitric acid (HNO_3) and sulfuric acid (H_2SO_4), at different molar concentrations of acid, was tested by oxidizing aqueous solutions of lactose, glucose and galactose . For solutions representing zero percent hydrolysis the concentration of lactose was 29 mM. For solutions representing 100 % hydrolysis the concentration of each monosaccharide was 29 mM. Solutions representing intermediate hydrolysis levels were prepared from the above two solutions. These solutions were made to represent different levels of lactose hydrolysis that could be tested with the procedure and were created using the experimental protocol given above.

The time for the absorbance to fall to 0.4 at 445 nanometers was recorded. If there was formation of a precipitate before the absorbance fell to 0.4, this was also recorded.

Determination of optimal Ce(IV) concentration to be used in the Ce(IV) test

Using the optimal acid level determined in earlier experiments, another set of experiments were undertaken to determine the optimal molarity of the Ce(IV) oxidant in the test solution.

A series of different molarity Ce(IV) solutions were created in 0.5M nitric acid and tested on 58 mM solutions of equimolar glucose:galactose with lactose to represent different lactose hydrolysis levels, equivalent to the solutions used before.

Ferroin as a visual indicator

Ferroin was added to 0.39M Ce(IV) in 0.5M nitric acid at a concentration of 0.001M. This caused the bright orange Ce(IV) solution to become brown-green in appearance.

This ferroin/Ce(IV) solution was then tested on a 58mM carbohydrate solution (equimolar glucose:galactose) to determine if the ferroin endpoint (green to blue in these circumstances) could be used as a visual indicator of completeness of the Ce(IV) oxidation of carbohydrate.

Temperature effects upon Ce(IV) rate

The effect of the reaction temperature upon the rate of the Ce(IV) reaction was studied in a temperature controlled spectrophotometer with the temperature varied from 10.9 ^{o}C to 41.1 ^{o}C. Two solutions, representing a precipitated 4.75 % lactose solution and a precipitated completely hydrolyzed 4.75 % lactose solution (original lactose concentration before hydrolysis) were studied. Oxidations were performed in triplicate with only temperature being varied between experiments.

Standard solutions

Aqueous solutions of lactose or lactose and equimolar amounts of glucose and galactose to represent hydrolyzed lactose solutions, were prepared and Znapta solution, barium chloride and cerium chloride were added as if the standard solutions represented actual milk samples. From these data standard curves for lactose percentage and percentage lactose hydrolysis were constructed.

Ce(IV) oxidations of milk

Milk (24 mL) was added to 38 mL of Znapta precipitating solution, 2 mL of 100 mg/mL cerium chloride solution, and approximately 10 g of barium chloride ($BaCl_2$). This mixture was diluted to 100 mL with water, shaken and filtered through Whatman #3 filter paper (Whatman Ltd.). The first 10 mL of filtrate were routinely discarded and the remaining solution tested as given above for Ce(IV) oxidation. Original lactose amounts in milk were determined by a comparison of the time for Ce(IV) oxidation to a predetermined standard curve. Percentage lactose hydrolysis in milk was calculated by a comparison of the Ce(IV) oxidation times with a standard curve for lactose hydrolysis.

Milk lactose hydrolysis analysis

The milk sample was tempered to 37 ^{o}C in a circulating water bath (Haake) and β-galactosidase added (either 2 units [2 μM o-nitrophenyl-β-D-galactopyranoside(ONPG) hydrolyzed/min] per mL milk for most samples or 0.8 units/mL milk for low enzyme hydrolyses). The milk was sampled at various intervals using the

Ce(IV) oxidation method and after similar precipitation, the enzyme method (glucose determination, Boehringer Mannheim, 1986).

RESULTS AND DISCUSSION

Utility of the Ce(IV) test for analysis of mono and disaccharides

A 0.39M Ce(IV) solution in 0.5M nitric acid was added to carbohydrate solutions (1.5% w/v) in a 1:3 ratio and the time for the absorbance at 445 nanometers to fall to 0.4 recorded (Table II-1). As can be seen there was a large difference in times between the monosaccharides glucose and galactose, which are the hydrolysis products of lactose, and the disaccharide lactose. This suggested that the test could be adapted to determine different levels of the monosaccharides in the presence of the disaccharide, therefore analysis of the level of lactose hydrolysis in the sample.

Optimal wavelength for discrimination between Ce(IV) and Ce(III) complexes

The Ce(IV)-carbohydrate reaction is believed to be a two electron radical mechanism whereby the Ce(IV) is reduced to Ce(III) in a 2:1 ratio as the carbohydrate is oxidized to a carbohydrate of one less carbon chain length and formic acid. There is a necessary involvement of the open chain form of the carbohydrate in this mechanism (Figure II-1, Virtanen et al, 1987). Virtanen et al have also proposed that two different complexes of Ce(IV)-carbohydrate may be involved in the oxidation. They propose an open chain

Table II-1.

Time for 1.5 % w/v aqueous carbohydrate solutions to reach an absorbance of 0.4 after Ce(IV) addition[1]

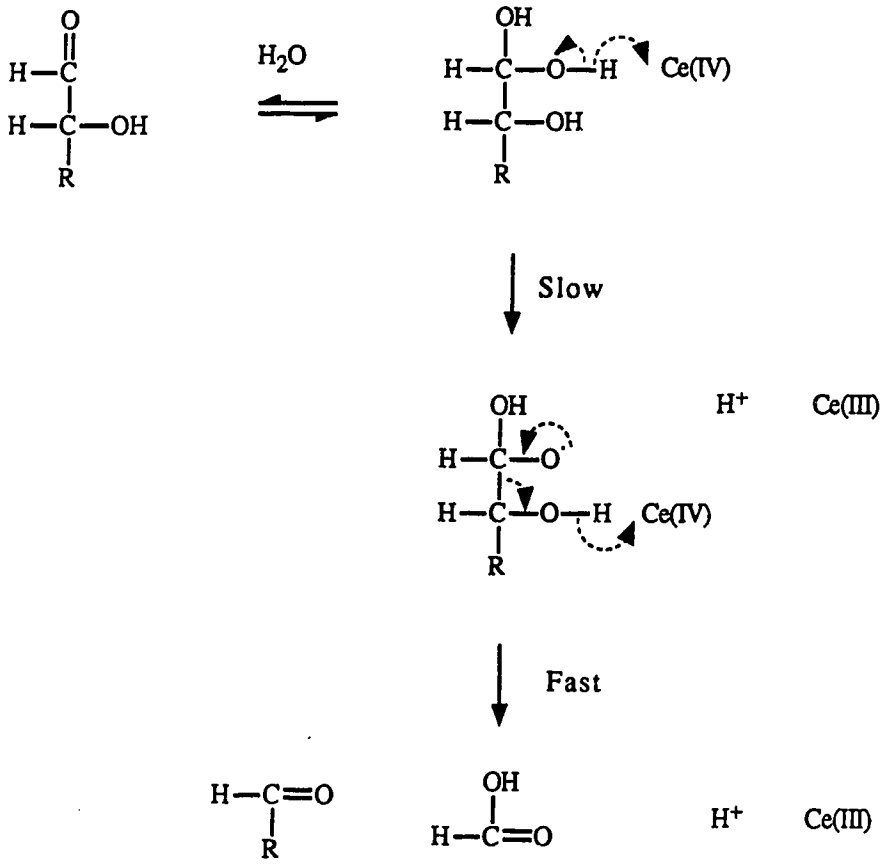
Carbohydrate	Time(sec)
Galactose	22.6 ± 0.5[2]
Glucose	52.7 ± 0.9[2]
Lactose monohydrate	293.1 ± 8.3[2]

1. 1 mL of 0.39M Ce(IV) in 0.5M nitric acid was added to 3 mL of the carbohydrate solution

2. standard deviation for triplicate analyses

Figure II-1

Proposed mechanism for the Ce(IV) oxidation of carbohydrates



carbohydrate Ce(IV) complex which is oxidized rapidly and a ring form Ce(IV) complex which is oxidized much slower. There can be interconversion of these two complexes (Figure II-2). This is in agreement with the results obtained for 1-O-methyl-β-D-glucopyranoside (Chapter 3), in which the carbohydrate, which is constrained to the pyranose ring form, does reduce Ce(IV) to Ce(III), albeit very slowly.

The complexes, when originally formed (as Ce(IV)-carbohydrate) were a deep and intense red color with a maximal absorbance over a large range from 300 to 520 nanometers. Over this range the absorbance recorded for a solution of 1.5 % (w/v) carbohydrate and 0.39M Ce(IV) (ratio of 3:1) was well over 2 (Figure II-3). The original deep red color probably resulted from the Ce(IV)-carbohydrate complexation. The loss of color resulted from the solution fading through the orange Ce(IV) color to the pale yellow Ce(III) color as the oxidation progressed. In water the Ce(III) color was so faint as to be hardly noticeable, while in Znapta precipitating solution it had a noticeable yellow tinge. The maximal absorbance for Ce(IV) in acid was 445 nanometers (Figure II-4). The maximal absorbance of a Ce(III) solution was 348 nanometers and it did not have as strong an absorbance, at equal molarities, as the Ce(IV) solution (Figure II-5).

This suggested that the absorbance that best distinguished between Ce(IV) and Ce(III) was in the 400 to 500 nanometers range. A scanning uv-visible spectrum of Ce(III) and glucose, Ce(IV) and glu[illegible]mediately after addition of the cerium solution to the car[illegible]) and of Ce(IV) and Znapta precipitating solution was [illegible]erformed in this absorbance range (Figure II-6).

Figure II-2

Complex formation in the Ce(IV) carbohydrate reaction

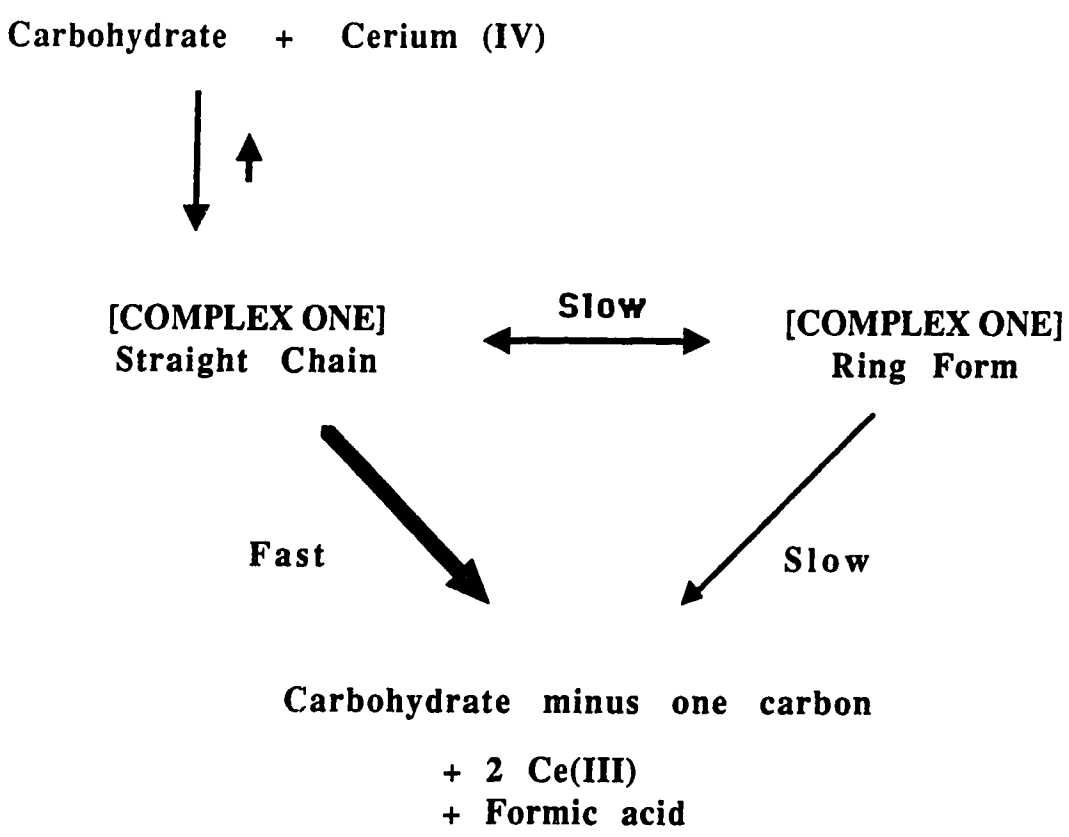
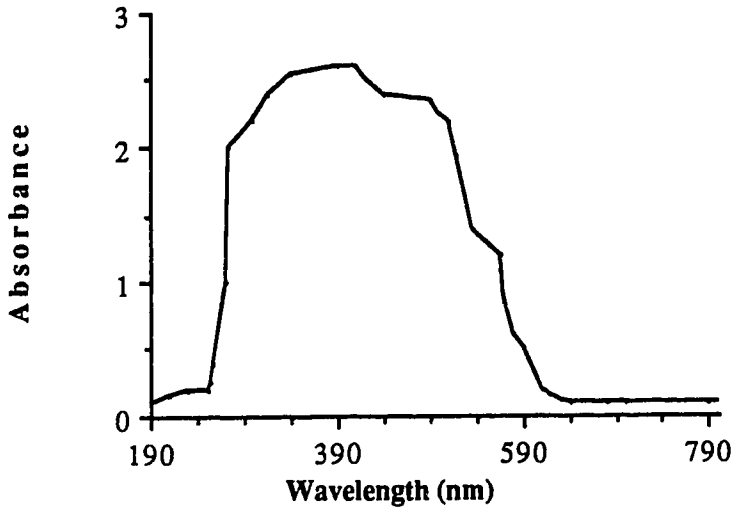


Figure II-3

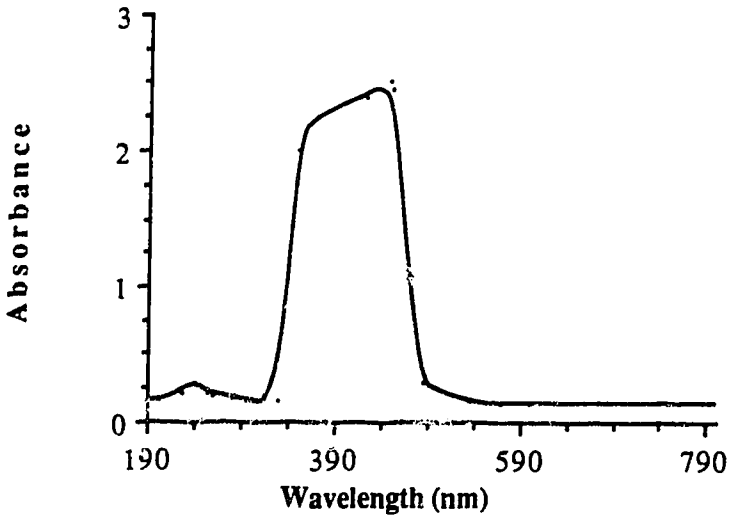
UV-visible spectrum of Ce(IV)[1] glucose complexes



1. 0.39 M Ce(IV) in 0.5M nitric acid added to a 29 mM aqueous glucose solution.

Figure II-4

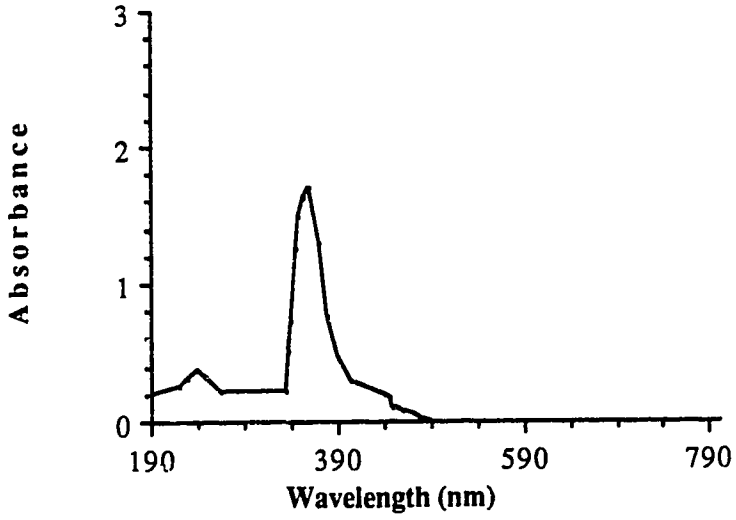
0.39M Ce(IV)[1] in Znapta



1. 0.39M Ce(IV) in 0.5M nitric acid added to Znapta precipitating solution at a ratio of 1:3

Figure II-5

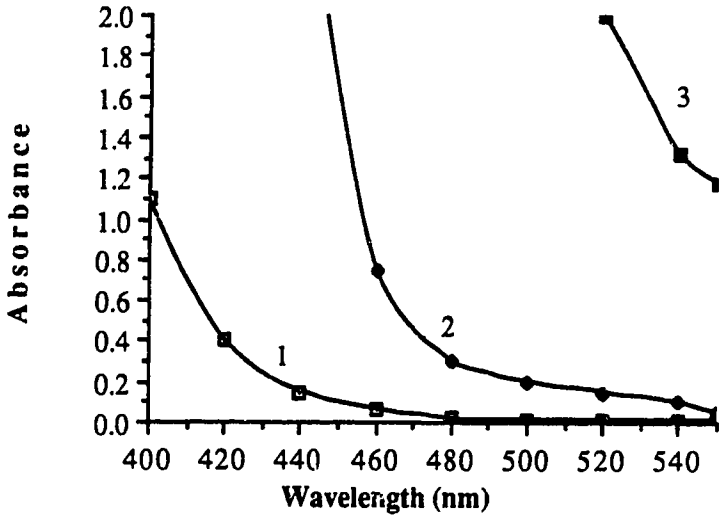
0.39M Ce(III)[1] in Znapta



1. 0.39M Ce(III) in 0.5M nitric acid added to Znapta precipitating solution at a ratio of 1:3

Figure II-6

Absorbance curves for the different complexes of cerium and carbohydrate



1. 1 mL of 0.39M Ce(IV) with 3 mL of 29mM glucose in Znapta after complete reduction of Ce(IV) to Ce(III). Therefore an absorbance curve of Ce(III) carbohydrate complexes.
2. 1 mL of 0.39M Ce(IV) with 3 mL of Znapta. Therefore an absorbance curve only of Ce(IV) in precipitating solution.
3. 1 mL of 0.39M Ce(IV) added to 3 mL of 29mM glucose in Znapta, 2 seconds after addition of oxidant. Therefore an absorbance curve of Ce(IV) carbohydrate complexes.

The results indicated that the optimal wavelength for studying the Ce(IV) to Ce(III) interconversion was 445 nanometers. For this reason all further experiments were conducted at this wavelength.

Optimal acid/acid concentration

The cerate reaction was kept at an acidic pH to ensure that there was no precipitation of ceric hydroxide [$Ce(OH_3)$] (Harris and Kratochvil, 1981). Precipitation of a flocular precipitate such as ceric hydroxide would ruin a spectrophotometric test. For this reason different acid concentrations and types of acids were tested against solutions representing 100, 90, 80, 70, 60 and 50 % hydrolysis of lactose as well as an unhydrolyzed lactose solution.

Both nitric and sulfuric acid at various molarities were studied by preparing a 0.39M solution of Ce(IV) in the appropriate molarity acid. The results of these tests are represented in Table II-2.

At all levels of sulfuric acid tested there was a precipitation of ceric hydroxide which rendered the spectrophotometric test useless. At the levels of nitric acid studied, 0.5M nitric acid was chosen because at this level the difference in oxidation times between 70 and 80 % hydrolysis of lactose, which is the determining factor for the dairy industry to label a product lactose hydrolyzed (Mahoney, 1985), was the greatest and allows for a more accurate result.

Ce(IV) concentration optimal for the test

The Ce(IV) level needed to give optimal results was then studied as well. A series of Ce(IV) solutions of 0.195, 0.39, and 0.78M Ce(IV)

Table II-2

Optimal acid and acid concentration for the Ce(IV) test

Acid[1]	% hydrolysis [2]						
	100	90	80	70	60	50	0
H_2SO_4							
1.5	ppte	ppte	ppte	ppte	ppte	ppte	ppte
1.00	ppte	ppte	ppte	ppte	ppte	ppte	ppte
0.75	ppte	ppte	ppte	ppte	ppte	ppte	ppte
HNO_3[4]							
1.00	120	135	168	179	195	195	720
0.75	120	130	150	180	210	250	468
0.50	156	168	185	240	300	408	>720
0.25	b.a.[3]	b.a.	b.a.	b.a.	b.a.	b.a.	b.a.

1. normality of acid solution
2. time in seconds to an absorbance of 0.4 at 445 nanometers
3. b.a. = background absorbance greater than 0.38
4. Lactose concentration was 4.0 % w/v

were created and studied by the determined experimental protocol on different sugar solutions (Table II-3).

The concentration of Ce(IV) that was optimal for testing lactose hydrolysis was 0.39M. At lower molarities (0.195M) there was not a large enough difference in times of reduction of Ce(IV) to Ce(III) by the carbohydrate solutions representing different levels of hydrolysis to allow for experimental error. At higher molarities of Ce(IV) the background absorbance (from the greater amount of the Ce(III) in the Znapta solution) was increased to approximately 0.36 to 0.38 OD from 0.2 to 0.24 (in the case of the Ce(IV) concentration being 0.39M). This made distinguishing the 0.4 OD endpoint very difficult and lead to large experimental error.

Optical density (OD) at 445 nanometers optimal for the test

Using 0.39M Ce(IV) in 0.5M nitric acid, a time study of the reaction was undertaken studying 100, 90, 80, 70, 60, and 50 % hydrolysis levels of a 4.75 % lactose solution as well as the lactose solution itself. Times versus absorbance at 445 nanometers were plotted and from these plots the most desirable optical density (OD) was determined (Figure II-7). The optimal OD was taken to be that at which there was the largest difference in oxidation time between solutions representing hydrolyzed lactose differing by 10 percent (i.e. maximal difference in oxidation times between 80 and 70 % hydrolysis solutions was greatest at 0.4 OD_{445}).

The absorbance level that best met both of these criteria was 0.4 OD. At higher absorbance levels there was less difference between

Table II-3

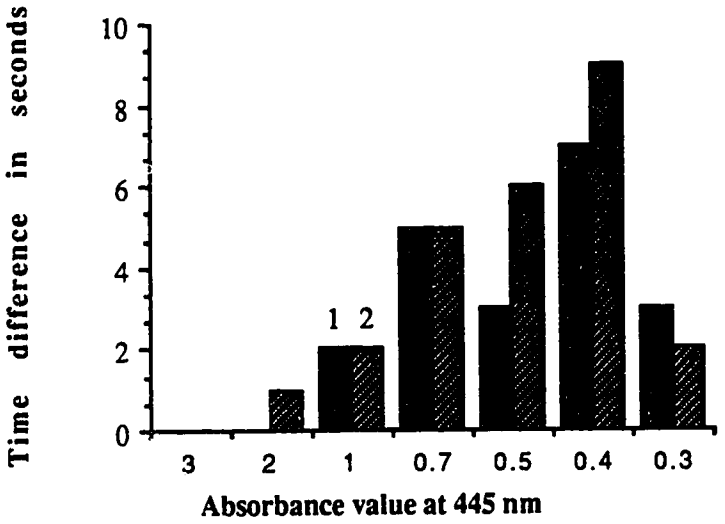
Optimal Ce(IV) molarity for Ce(IV) test

Ce(IV)[1]	hydrolysis[2]		
	100%[3]	90%[3]	70%[3]
0.195	96	100	108
0.39	156	168	240
0.78	225	227	230

1. molarity of Ce(IV) in 0.5M nitric acid
2. time in seconds for absorbance at 445 nanometers to fall to 0.4
3. based upon an original anhydrous lactose concentration of 4.75% w/v

Figure II-7

Determination of optimal OD_{445} to measure as endpoint of the Ce(IV) oxidation of carbohydrates[1]



1. The difference in time of oxidation between a sample representing a 90 % hydrolyzed lactose solution (lactose concentration = 4.75 % w/v) and a sample representing an 80 % hydrolyzed lactose solution.
2. The difference in time of oxidation between a sample representing an 80 % hydrolyzed lactose solution (lactose concentration = 4.75 % w/v) and a sample representing a 70 % hydrolyzed lactose solution.

hydrolysis solutions while at lower levels the reaction was slowing and the absorbance change for different samples began to coalesce. This did not allow for differentiation between samples.

Haziness in Znapta solutions

It was noted that freshly prepared solutions of Znapta were hazy and that this haziness persisted after the use of the solution to precipitate fats and protein from the milk. If the Znapta solution used to precipitate the milk were aged at room temperature for one week then no such result occurred. It was decided to study the haze to determine if it was due to light exposure or to temperature. A solution of fresh Znapta, was split into four lots as stated in the materials and methods section. The samples that were not boiled (4 ^{o}C in the dark, room temperature in the dark, and room temperature in sunlight) were observed for haziness after one week and the fourth sample (boiled for 30 minutes), immediately upon cooling. All samples were also tested for pH (Table II-4).

It was obvious from these results that temperature and not light exposure was responsible for the loss of haze. It was also obvious that the quickest means to ensure the loss of haze in the Znapta solutions was to boil them before use. During boiling the pH was not affected and in the method used, boiling in a narrow necked erlenmeyer flask, less than 15 mL of water was lost to evaporation. The results of these experiments do not, however, suggest what the cause of the original haze could be. No experiments, which would have involved determination of the different tungsten complexes possible, were undertaken to determine this.

Table II-4

Factor causing loss of haze in Znapta solution

Treatment	pH[1]	Haze remaining[2]
4 °C/dark/1 week	4.6	yes
25 °C/dark/1 week	4.6	no
25 °C/light/1 week	4.6	no
boiled/30 min.	4.6	no

1. determined after prescribed treatment

2. determined visually after the prescribed treatment

Ferroin as a visual indicator

The utility of the Ce(IV) test had been established from preliminary tests on milk samples but it was thought that a visual test, requiring no instrumentation, would be even less expensive than the spectrophotometric method. For this reason the redox indicator ferroin was tested for its potential to determine an endpoint.

The ferroin endpoint, under these conditions, was red to sky blue with no trace of green. Ferroin was regularly used as the indicator in titrations of cerate containing solutions (Rao and Rao, 1972; Shukla and Beg, 1979). The stoichiometry of Ce(IV) to oxidizable carbons when a 3:1 ratio of 29mM sugar solution to 0.39M Ce(IV) was studied was at least 10 to 1. This would ensure complete reduction of Ce(IV) to Ce(III) and should have resulted, as it did in titrations of cerium solutions, in a sharp transition from a red [Fe(II)] form of the indicator to a blue [Fe(III)] form of the indicator.

When this was attempted, however, the transition from a jade green Ce(IV) solution to a bluish tinged solution was slow and drawn out, making determination of an exact endpoint impossible. Adding the indicator to the sugar solution and then adding the Ce(IV) oxidant did not improve the results.

As a consequence it was decided not to attempt a direct visualization of the reaction and spectrophotometric studies were continued.

Determinations of interferences in milk

If only Znapta solution were used to clarify milk, even after pre-aging, Ce(IV) precipitated with the resulting solution. Carrez solution (Boehringer Mannheim, 1986) was then tested for its utility but was found to give filtrates which were cloudy and could not be used in the spectrophotometer.

A test of the possible interfering agents causing precipitation in the Znapta filtrates was undertaken, with the thought that it may be tungsten that caused the precipitation as tungsten was known to form highly insoluble lanthanide complexes (Cotton and Wilkinson, 1967). In case it was a different causative agent, or a combination of effects, all of the components of the Znapta precipitating solution were tested for causing Ce(IV) precipitation (Table II-5).

Tungsten was determined to be the problem. To remove the interference, excess tungsten in the filtrate, barium chloride was added to the Znapta and milk solution before filtration. Barium tungstate is insoluble in water.

This effectively stopped the early precipitate but then a late forming precipitate began to interfere. At this late point in the reaction the dominant species of cerium present was Ce(III). The precipitate was observed to be white in color and highly flocular in nature. From a study of literature on the lanthanides (Cotton and Wilkinson, 1967) it was discovered that organic acids such as citric acid form water insoluble complexes with the reduced forms of lanthanide ions and that these complexes are generally white, flocular precipitates in aqueous solutions. To ensure that this was the sole cause of the precipitation a number of possible causative agents

Table II-5

Possible interferences in precipitated milk solutions causing Ce(IV) precipitation

Compound	Concentration[1]	Precipitation[2]
Potassium phosphate	7.2	no
Acetic acid	66	no
Zinc acetate	95	no
Phosphotungstic acid	7.2	yes

1. mM concentration in water

2. observed visually immediately after addition of 1 mL of 0.39M Ce(IV) in 0.5M nitric acid to 3 mL of the solution being tested

(minor milk constituents) were tested, at the concentrations found in milk (Johnson, 1983), upon 0.39M solutions of Ce(III) (Table II-6).

The precipitate could be mimicked by adding citric acid to the Ce(III). As a preventative measure ceric chloride ($CeCl_3$) was routinely added to the milk precipitating solution at a concentration of 2 mg for every 24 mL of milk tested. The addition of Ce(III) to the solution affected the carbohydrate oxidation rate, because Ce(III) is a product of the reaction, so it was added at the same concentration to all of the standard solutions tested as well as the milk samples.

Standard curves for lactose/lactose hydrolysis

The standard curves were determined by the protocol in materials and methods.

The standard curve for lactose was linear over the region of 4.35 to 5.94 % lactose with a linear regression line calculated to be:

$$y = 1.72 + 1350x$$

where y was % lactose and x was 1/time in seconds. The correlation coefficient calculated for this line was 1.00 (Figure II-8).

Virtanen et al (1987) and Rao and Rao (1972) have shown that the reaction between organic alcohols and excess Ce(IV) is pseudo-first order. Therefore if a plot was made of concentration versus rate a straight line would be obtained. However, unlike these researchers, the experiments in this work require total, or near total, reduction of Ce(IV) to Ce(III) as these are the species observed spectrophotometrically. If the carbohydrates are considered as moles of oxidizable carbons rather than moles of complete carbohydrate (not unreasonable considering the reaction being studied) then there

Table II-6

Possible compounds, arising from milk, interfering with Ce(III)

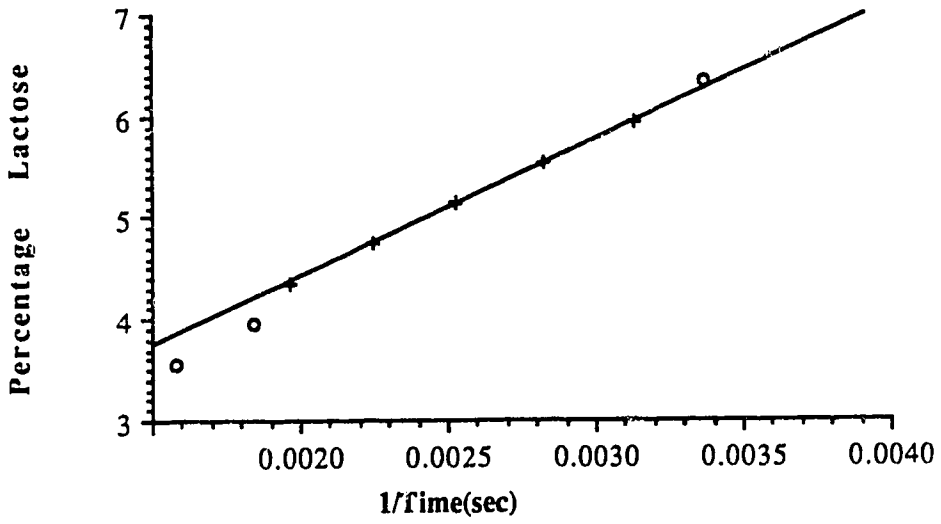
Compound	Concentration[1]	Precipitation[2]
Calcium chloride	132	no
Magnesium chloride	11	no
Citric acid	157	yes
Sodium chloride	60	no

1. mg/100 mL in water

2. observed visually immediately after addition of 1 mL of 0.39M Ce(III) in 0.5M nitric acid to 3 mL of the compound being studied.

Figure II-8

Standard curve for lactose determined by Ce(IV) oxidation



o. points outside the linear region used for lactose determination in milk

+. points within the linear region used for lactose determination in milk

is an excess of oxidizable carbons and the Ce(IV) will be stoichiometrically converted to Ce(III).

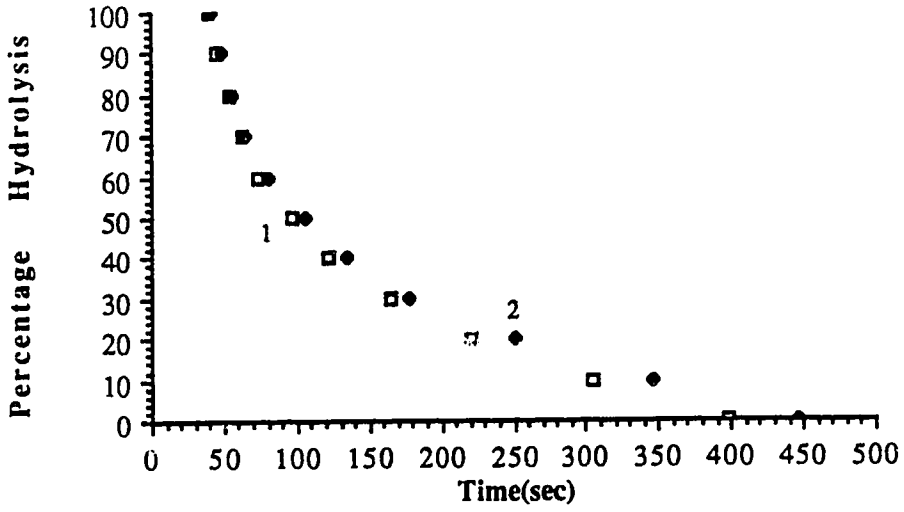
The standard curve is non-linear over a large concentration range. Concentration ratios of Ce(IV) and disaccharide were adjusted to approximate a linear region in the concentration range of interest (4.35 to 5.94 %, Figure II-8). This linear range was the best range for studying lactose in milk and that for this reason the ratios decided upon were kept for later experimentation.

As the Ce(IV) solution is prepared in 0.5 M nitric acid, the question arose whether the carbohydrate measured was indeed lactose or the hydrolysis products thereof. Therefore lactose was dissolved in 0.5 M nitric acid and left for 15 minutes at room temperature. This solution was then tested for glucose by the Boehringer Mannheim glucose oxidase enzymatic assay. There was no detectable level of glucose and it was therefore assumed that only lactose oxidation was occurring in the time frame of the experiment.

The standard curve for lactose hydrolysis (Figure II-9) was left non-linear as attempts to plot the curve linearly, such as log versus log, or double reciprocal plots, were unsuccessful. It could be expected that the curve would be non-linear as the reaction being observed is three-fold; the oxidation of lactose, the oxidation of galactose and the oxidation of glucose. All three of these

Figure II-9

Standard curve for lactose hydrolysis by Ce(IV) oxidation



1. standard curve simulating a milk of original 5.0% lactose concentration(w/v)

2. standard curve simulating a milk of original 4.75% lactose concentration(w/v)

carbohydrates have different rates of oxidation and would therefore contribute unequally to the reduction of Ce(IV) to Ce(III). At different points on the curve the relative contributions of each carbohydrate would be different and the rate observed would therefore be different and not linearly proportional to percentage hydrolysis. The other deciding factor in non-linearity was that this research did not concentrate upon initial rates but overall rates, and these were affected by the many individual rate constants in the mechanism [Complex one to complex two interconversion and the different rates of this oxidation as well as the differing rates of the complex formation (Virtanen et al, 1987)].

It was still possible to utilize this standard curve to determine % lactose hydrolysis in milk. A second standard curve, at 0.25 % lower lactose, 4.75 % instead of 5.0 %, was also determined to compare the effect of original lactose concentration upon the measured times. It can be seen that the differences in times, especially at the higher levels of hydrolysis (which are the most important industrially) were very small. This lead to the conclusion that percentage hydrolysis, could, if speed were a necessity, be determined on a milk sample with acceptable accuracy without the need for a determination of original percentage lactose in milk.

Cerium(IV) stability

The stability of the Ce(IV) oxidant was a concern since Shriner et al (1980) had stated that the reagent was unstable if left for a time and not used immediately. In fact a certain degree of instability was noted; in approximately 6 hours the 0.4M Ce(IV) (determined by

titration immediately after dissolution) had formed a small amount of orange precipitate. Upon shaking (to suspend the precipitate) and titration, the molarity of the solution was found to have fallen to 0.39M Ce(IV). If the oxidant solution were then filtered to remove the precipitate, upon standing more precipitate formed and the molarity of the solution dropped even further. If the precipitate were left in the solution, the solution was stable for up to 6 months, stored at room temperature in a brown bottle, with a titration value of 0.39M.

From these experiments it was determined that the Ce(IV) solution, if the precipitate (the composition of which is unknown) was left in the solution and resuspended before use, was stable under the conditions studied.

Effect of temperature

As could be expected for a reaction that involved an initial formation of a complex, and therefore interaction of the two compounds, temperature had an effect upon the reaction (Table II-7). For both lactose and galactose/glucose solutions a quicker reaction time (therefore a faster rate of reaction) was observed as the temperature was increased.

In the sample compartment of a Spectronic 21 (Bausch and Lomb) there is no temperature control and there can be warming of the sample from the bulb and electronics of the machine. This could cause difficulties in replication of samples. This temperature problem was noted, with the samples becoming progressively quicker in the rate of Ce(IV) reduction as time went on during the day. It was

Table II-7

Times for oxidations of lactose samples representing a 4.75 % lactose in milk at different temperatures

Temperature[1]	Time (sec)[2]	
	Lactose	100 % hydrolyzed lactose
10.9	2716 ± 24[3]	218.1 ± 5.3[3]
16.8	1256 ± 3	105.5 ± 1.0
20.9	743.8 ± 9.6	65.2 ± 1.0
28.1	336.0 ± 7.1	30.1 ± 0.4
34.6	165.4 ± 5.5	15.8 ± 0.5
41.1	87.0 ± 1.0	8.66 ± 0.01

1. temperature of the spectrophotometer sample compartment in °C
2. time for the absorbance at 445 nanometers to fall to 0.4 after addition of 1 mL of 0.39M Ce(IV) in 0.5M nitric acid to 3 mL of the carbohydrate
3. standard deviation in triplicate samples

discovered that pre-equilibrating the spectrophotometer for one hour before determination of samples (by turning on the machine one hour before any work was done), gave good replication between sample sets but not between runs made on one day and then the next. This was not a desirable effect, as it added the necessity of repeating the standard curve daily, so a temperature controlled spectrophotometer (Pye Unicam SP1800) was utilized for all further work.

The data from Table II-7 were taken and represented in an Arrhenius plot (Figure II-10), using the inverse of total oxidation time to represent the rate of reaction. There is an excellent linear correlation for both samples (R = 0.999 in both cases). The equations of the lines are:

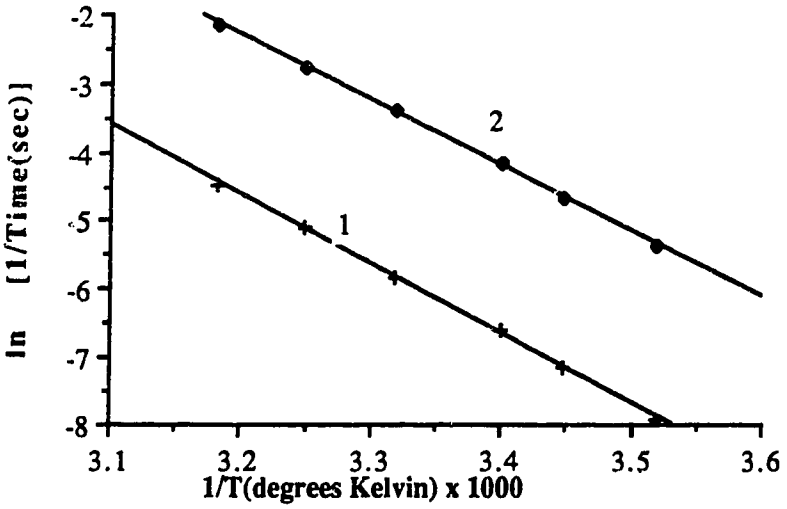
for the galactose/glucose mixture: $y = 28.3 - 9.6x$

for the lactose sample: $y = 28.0 - 10.2x$

These were essentially parallel lines. From the Arrhenius equation; $k = Ae^{-E/RT}$, the natural logarithm of which is; $\ln k = \ln A - E/RT$, the slope is the value -E/R where R is the gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$) and E is the activation energy (if ln rate is plotted against temperature). Within the limits of experimental error it could be assumed that both the monosaccharides and the disaccharides have relatively the same energy of activation. These values would be 1150 $J \cdot mol^{-1}$ for the monosaccharides and 1230 $J \cdot mol^{-1}$ for the disaccharide lactose. This would be equivalent to the energy required to form a complex between Ce(IV) and carbohydrate that could react to reduce Ce(IV) to Ce(III) and consequently oxidize the carbohydrate.

Figure II-10

Arrhenius plot of oxidation of lactose and completely hydrolyzed lactose



1. Arrhenius plot for a lactose sample of 4.75% concentration(w/v)

2. Arrhenius plot for a completely hydrolyzed lactose sample representing an original lactose concentration of 4.75% (w/v)

Milk lactose determinations

A number of different milk samples were tested using the infared method (AOAC, 1984, method 16.090, using a Milko-Scan model 305, A.N. Foss, Denmark) as well as the above method to determine lactose percentages (Table II-8). There was generally good agreement between the two methods, although the Ce(IV) oxidation values were slightly lower than the values from the infared method. The slightly low values might have resulted because of the modified Znapta precipitation used for the Ce(IV) oxidation method. An assumption required with the Ce(IV) method was that the protein and fat precipitate contained the same amount of lactose as the clear aqueous solution on a volume basis. Of course, standard solutions of lactose did not contain this precipitate. Therefore, if the precipitate occluded or absorbed proportionately more lactose on a volume basis, lactose in milk samples would seem lower than the actual lactose levels when the Ce(IV) test was employed. It should be noted that unlike other methods (Biggs and Szijarto, 1963; Garrison and Haigh, 1942) no correction factors were used for the variation in protein and fat found in these samples and the effect that these variables might have upon the amount of lactose trapped in the precipitate.

Lactose was also added to some of the milk samples to further evaluate the accuracy of the Ce(IV) oxidation method (Table II-8). In all cases but one, recovery of the lactose was greater than 90 %.

1. 24 mL of milk used in the Ce(IV) test. For recoveries lactose was added to 100 mL of milk

2. percentage recovery

3. standard deviations of duplicate samples for the infared method and triplicate samples for the Ce(IV) oxidation method

4. this sample required twofold dilution with water before the infared method was used. The Ce(IV) oxidation was carried out upon undiluted sample.

Table II-8

Lactose amounts in milk calculated using the infared and the Ce(IV) oxidation methods

Milk sample[1]	Lactose percentage		
	Infared	Ce(IV)	Recovery[2]
1. full fat milk	4.99 ± 0.01[3]	4.87 ± 0.05[3]	
2. full fat milk	4.98 ± 0.01	4.91 ± 0.03	
3. 2% milk	5.05 ± 0.01	4.94 ± 0.01	
4. 2% milk	5.03 ± 0.01	4.90 ± 0.02	
5. half & half[4]	4.92 ± 0.04	4.74 ± 0.01	
6. skim milk	5.22 ± 0.01	5.03 ± 0.05	
6 + 0.6 g lactose	5.78 ± 0.01		93
6 + 0.6 g lactose		5.57 ± 0.03	90
6 + 1.2 g lactose	6.36 ± 0.02		95
6 + 1.2 g lactose		6.33 ± 0.03	108
7. 2% milk	5.12 ± 0.01	5.07 ± 0.02	
7 + 0.6 g lactose	5.70 ± 0.02		97
7+ 0.6 g lactose		5.61 ± 0.01	90
7+ 1.2 g lactose	6.28 ± 0.02		97
7 + 1.2 g lactose		6.19 ± 0.06	93
8. full fat milk	5.03 ± 0.02	5.04 ± 0.02	
8 + 0.6 g lactose	5.59 ± 0.02		93
8 + 0.6 g lactose		5.41 ± 0.02	62
8 + 1.2 g lactose	6.15 ± 0.02		93
8 + 1.2 g lactose		6.14 ± 0.04	92

Milk lactose hydrolysis determination

Several milk samples were hydrolyzed and the hydrolysis percentages at regular time intervals monitored using the Ce(IV) oxidation method and the enzyme method (Boehringer Mannheim, 1986). The results (Table II-9) were least comparable at low levels of hydrolysis but compared better at higher (75 to 80 %) hydrolysis levels. The reason for the differences between the two methods was because they measured different parameters to estimate hydrolysis rates. The enzyme method measured the production of glucose, which would tend to overestimate the degree of hydrolysis of the disaccharide lactose to the constituent monosaccharides glucose and galactose, especially early in the hydrolysis, when there was a large amount of formation of [illegible]to-oligosaccharides from transgalactosylation action of the [illegible]ne (Kwak and Jeon, 1986). These products can not be strictly [illegible]sidered hydrolysis products. The Ce(IV) oxidation method, if anything, probably underestimated the level of hydrolysis, especially under the early hydrolysis conditions where there was a high production of oligosaccharides, since trisaccharides oxidized even slower than lactose (Chapter 3). Later in the hydrolysis when oligosaccharide amounts were reduced by continued β-galactosidase action, both the enzyme method and the Ce(IV) oxidation method gave very similar results. The variation in Ce(IV) oxidation times for the different levels of lactose hydrolysis need not have been correlated to the artificially created standard curve but could have, if so desired, been correlated directly to results from the enzyme test (or other

Table II-9

Milk hydrolysis calculated using the Ce(IV) and enzyme methods

Milk sample[1]	Time (min)	Percentage hydrolysis	
		Ce(IV)[2]	Enzyme[3]
1, 2 units enz.	15	35	48
	30	58	69
	45	73	78
	60	80	80
2, 2 units enz.	15	42	47
	30	56	67
	45	73	77
	60	80	85
2, 0.8 units enz.	30	19	31
	60	28	43
	90	39	51
	120	47	61

1. same samples as found in Table 8 (by number)

2. average of triplicate analyses with standard deviations of values ≤ 1 %

3. average of duplicate analyses with standard deviations of values ≤ 2 %

appropriate method) so that the results matched. There were however, distinct advantages of the Ce(IV) method over the enzyme method. The Ce(IV) oxidation method was faster, more precise, did not deteriorate with time (the enzymes used for the enzyme method deteriorated and had to be standardized with a standard glucose solution) and was less expensive.

CONCLUSIONS

The Ce(IV) oxidation method was of facility for the determination of lactose and lactose hydrolysis in milk. It offered an alternative to other methods of measuring lactose and lactose hydrolysis in milk. The test was inexpensive, required a minimum of equipment (if temperature control was not deemed necessary it required only a very simple spectrophotometer and a stopwatch) and including the precipitation and filtration steps could be completed in under fifteen minutes. It should be of some utility in economically depressed areas where lactose intolerance is of high incidence and therefore the need to accurately determine lactose hydrolysis is paramount. The test was simple to perform and did not require elaborate and costly equipment.

Experimental attempts to use the test to determine the lactose in acid whey were unsuccessful because of problems with precipitation. The further uses of Ce(IV) oxidation to determine disaccharide amounts in differing foodstuffs is an experimental area that requires further study.

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Determination of Dextrose Equivalent in Starch Hydrolysates Using Cerium(IV)[2]

INTRODUCTION

Although time consuming, the Lane Eynon or similar copper complex oxidation-reduction reactions have been the traditional and official methods for measuring Dextrose Equivalent (DE) of Starch hydrolysates (De Whalley, 1964). Numerous other methods, notably, freezing point depression (cryoscopy) and High Performance Liquid Chromatography (HPLC) have been introduced as possible replacements for the traditional quality control methodology (Dlheye and Moreels, 1988). Although rapid, cryoscopy requires relatively expensive equipment and is affected by a variety of production parameters such as the raw material, production method, purification technique and salt content. HPLC gives quantitative information on oligosaccharides present, but also requires expensive equipment, greater expertise, time, and conversion of each oligosaccharide with a factor to establish the overall DE of the hydrolysate. This requires time consuming and elaborate determinations of known samples to determine response factors and retention times (Mariller et al, 1985). While working on a method for measuring lactose hydrolysis in milk, Griffith (Chapter 1) noted that hydrolysis to monosaccharides could be measured by determining the rapid reduction of Cerium(IV) to Cerium(III) using an excess of carbohydrate. The measurement of

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[2] A version of this chapter has been accepted for publication. Griffith, L.; Sporns, P. "Determination of Dextrose Equivalent in Starch Hydrolysates Using Ce(IV)" J. Agric. Food Chem.

dextrose equivalent is actually a measurement of monosaccharides in the presence of higher linked oligosaccharides formed during the hydrolysis of the starch molecule. The greater the degree of hydrolysis the greater the number of smaller oligosaccharides and free monosaccharide. The measurement of this free monosaccharide, as dextrose equivalent, gives the starch hydrolysate manufacturer an indication of the chemical composition of his product. Dextrose equivalent measurements actually measure the total number of reducing sugars in a product and have been defined as the percentage of reducing sugars in a product, expressed as the glucose (dextrose) in the sample calculated upon a basis of percentage total dry matter (Mariller et al 1985).

The Ce(IV) test was known to accurately measure monosaccharides in the presence of disaccharides (Chapter 1) and a project was therefore undertaken to adapt the test for measuring DE of starch hydrolysates. In the corn starch industry starch hydrolysates are identified as maltodextrins (DE less than 20), corn syrup solids (DE greater than 20, solids), or corn syrups (DE greater than 20, liquids). For products of DE greater than 10 the Lane-Eynon titration, involving the rapid titration of a boiling solution, is the traditional method of analysis. If the DE value is less than 10 then the method of Schoorl is usually the method chosen for the determination (Dlheye and Moreels, 1988).

MATERIALS AND METHODS

Materials

Carbohydrate standards (glucose and glucose oligosaccharides) were purchased from Sigma Chemical Co., St. Louis, MO. Starch hydrolysate materials were provided courtesy American Maize Products Co., Hammond, IN; A.E. Staley Manufacturing Co., Decatur, IL and Champlain Industries Ltd. Mississauga, Ontario. Water was prepared using a Millipore Milli-Q system and all other chemicals used were reagent grade or better.

Ce(IV) oxidations of starch hydrolysate products

Oxidations were carried out in a similar manner to the method reported by Griffith (1989) except that the time in seconds was recorded for the absorbance at 445 nm to return to 0.5 O.D. rather than 0.4 O.D.

The method involved addition of 0.4 M ammonium hexanitratocerium(IV) $[(NH_4)_2Ce(NO_3)_6]$ in 0.5 M nitric acid to carbohydrate solution in a 1 to 3 ratio. The Ce(IV) solution was aged at least 6 hours and shaken before use. The carbohydrate solution was prepared at a concentration of 2.00 $\pm$ 0.01 g in 50 mL of water, or equivalent. Before final dissolution of samples one drop of ammonium hydroxide (NH_4OH) was added to the solution to effect mutarotational equilibrium of the carbohydrates. These solutions were then made up to volume. The mixed solution was placed in a Pye-Unicam SP 1800 spectrophotometer (sugar solution as reference sample) and the time in seconds recorded for the absorbance at 445

nm to return to 0.5 O.D. The Pye-Unicam spectrophotometer sample compartment was temperature controlled and maintained at 25.1 ± 0.1 °C using a Lotemptrol 154 bath (Precision Scientific Co.) containing ethylene glycol:water (50:50, v:v).

Ce(IV) oxidations of carbohydrate standards

Solutions of glucose oligosaccharides, 4 % weight/volume (w/v), ranging from a dextrose polymerization (DP) number of 2 (maltose) to 7 (maltoheptaose) were prepared in Milli-Q water. These samples were then studied in the same manner as the starch hydrolysate samples.

Determination of moisture content of starch hydrolysate products

The percentage moisture content of the starch hydrolysates (for those not given by analytical determination from the starch manufacturer) were determined by weighing 5.0000 ± .0100 grams of starch hydrolysate into pre-dried, pre-weighed, capped aluminium pans. These pans were then placed in a forced air oven at 100 °C, at atmospheric pressure, for 4 hours. The pans were placed in a glass dessicator over Drierite ($CaSO_4$) to cool and then weighed. They were redried for 2 hours under the same conditions, cooled in a dessicator (same conditions) and reweighed. From these data the percentage moisture was determined (A.O.A.C. method 31.006, 1984).

Determination of optimal level of dissolution of starch hydrolysates

Time is an important factor in all analytical tests that may be conducted in an industry setting. For this reason it was decided that an optimal dissolution level of the sample had to be determined to give the best time differences in the Ce(IV) oxidation between differing DE levels yet with a quick total time of analysis.

The different dissolution levels were created by dissolving weighed portions of the corn starch hydrolysate in Milli Q water. The different levels studied were; 1 g in 50 mL water, 1 g in 25 mL water, and 1 g in 5 mL water. The 0.39M Ce(IV) solution was added to a pre tempered sample in the sample compartment of a Pye Unicam SP1800 spectrophotometer at a ratio of 1:3 Ce(IV) to sugar solution and the time recorded for the absorbance to fall to 0.5 at 445 nm.

RESULTS AND DISCUSSION

Ce(IV) oxidations of carbohydrate standards

In order to properly appraise the potential use of Ce(IV) oxidimetry for measurement of DE in starch hydrolysates, different glucose oligosaccharides were first examined using this test (Table III-1). At a 4 % (w/v) concentration, as the degree of glucose polymerization in the oligosaccharides increased, there was also an increase in the time required to reduce the absorbance (445 nm) of the colored complex (Griffith et al, 1989; Virtanen, et al, 1987) formed between Ce(IV) and carbohydrates. This loss of color was

Table III-1

Time for aqueous carbohydrate solutions to reach an absorbance of 0.5 O.D. at 445 nm after Ce(IV)[1] addition

Carbohydrate	Time (sec. + std. dev.)[2]
glucose	29.02 + 0.50
maltose	49.85 + 0.42
isomaltose	49.19 + 0.42
maltotriose	68.80 + 0.63
maltotetraose	78.84 + 0.02
maltopentaose	116.56 + 0.27
maltohexaose	176.42 + 1.35
maltoheptaose	208.81 + 1.01

1. 0.39 M Ce(IV) in 0.5M nitric acid. 3:1 sugar to Ce(IV).

2. All times are the means of duplicate analyses with the exception of times given for the first three carbohydrates which were the means of triplicate analyses.

caused by the reduction of Ce(IV) to Ce(III) with the concommitant oxidation of the carbohydrates. Although the times increased, even the largest oligosaccharide (maltoheptaose) required only about 3.5 minutes for completion of the reaction, and both disaccharides of glucose (maltose and isomaltose) required almost the same time. This experiment suggested that the Ce(IV) oxidation was dependent upon the concentration of reducing sugar. This is very similar to the traditional copper complex oxidation-reduction reactions, such as the Fehlings test or the Lane-Eynon titration.

Optimal dissolution level for starch hydrolysates

The Ce(IV) test for DE in starch hydrolysates must be both rapid and precise if it is to complement existing methods. To determine the optimal level of dissolution of the sample three different dilutions were prepared as stated in the materials and methods section.

The results indicated that a dissolution level of 1:25 gave the best compromise between rapidity of the test and discernment between different DE values (Table III-2). In all further work the dissolution level of the starch hydrolysate in water was 2 g in 50 mL. After dissolution the Ce(IV) test was performed by the stated experimental protocol. This technique was followed for both the standard curve determinations and the analysis of DE of unknown samples.

Table III-2

Dissolution level optimal for the Ce(IV) test

Dissolution Level	Time (sec.) to OD_{445} = 0.5[1]			
	10 DE	26 DE	34.5 DE	43.5 DE
1:5	139.7 ± 1.12	119.8 ± 1.28	n.d.[2]	n.d.
1:25	981.5 ± 25.4	115.6 ± 1.86	84.2 ± 1.98	67.6 ± 0.97
1:50	> 1 hour	n.d.	n.d.	n.d.

1. time in seconds ± standard deviation of triplicate analyses

2. not determined

Ce(IV) oxidations of starch hydrolysates used for the standard curve

The starch hydrolysate products supplied by the American Maize Products Company were used for preparation of the standard curve. These products were sent from the manufacturer with detailed analyses of DE by the Lane-Eynon method, percentage moisture and degree of polymerization by HPLC. The standard curve was constructed by comparing time for oxidation of the starch hydrolysate solution (time for the absorbance at 445 nm. to drop to 0.5) to the determined DE value.

The standard curve was plotted as DE versus 1/time, an indication of the rate of the reaction (Figure III-1). The line derived from this curve was a straight line and by linear regression analysis the equation of the line is:

$$y = 2161x + 9.58$$

where y is DE and x is 1/time in seconds. The correlation coefficient for the line was 0.997. This line was determined from triplicate analyses of duplicate samples. The results suggested an excellent agreement with a pseudo-first order rate mechanism, as seen with lactose (Griffith et al, 1989). The standard deviations in each point averaged approximately 1 % (Table III-3).

DE calculations of starch hydrolysates utilizing the Ce(IV) standard curve

The starch hydrolysate products from two other suppliers, AE Staley and Champlain Industries Ltd., were then tested to determine if the Ce(IV) oxidimetric test could be accomodated to analysing DE of

Figure III-1

Standard curve of starch hydrolysates for Ce(IV) oxidimetry

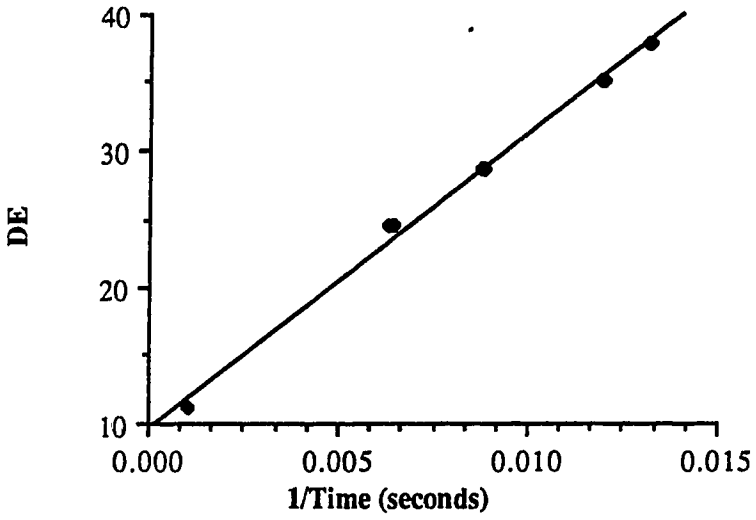

Table III-3

Standard curve for starch hydrolysate products using Ce(IV)

Product	DE[1]	% Moisture	Time[2]
Lo Dex 10	11.2	7.1	950.6 + 6.4
			945.5 ± 2.1
Fro Dex 25	24.6	5.7	155.1 ± 3.4
			158.2 ± 0.4
Fro Dex 24D	28.6	5.5	113.5 ± 1.1
			114.1 ± 2.3
Fro Dex 36P	35.1	5.4	83.3 ± 0.6
			83.8 ± 0.6
Fro Dex 42F	37.8	5.1	76.2 ± 0.6
			75.7 ± 0.8

1. Lane Eynon values.

2. means of triplicate analyses ± standard deviation.

starch hydrolysates, both maltodextrins and corn syrup solids. If the test were utilizable on these types of products it would be safe to assume that it would work for corn syrups as well. The DE values calculated by using the Ce(IV) standard curve compared very well with the DE value or range given by the companies (Table III-4).

None of the DE values reported has been corrected for the moisture in the sample. It was therefore possible to construct a standard curve for the analysis of DE in starch hydrolysates such that exact DE was reported without requirement for a moisture analysis. This would remove one more step from the analysis and decrease the time required to determine the DE of a product considerably. In the case of corn syrups the % solids in wet products would still be a required determination. If more exact data were required the determination of percentage moisture could be conducted and the results adjusted accordingly.

The different companies certainly used different techniques to treat their products and the Ce(IV) test appears to be unaffected by any difference there may have been in the processing. This test would however, as was shown for lactose and lactose hydrolysis measurements (Griffith et al, 1989) be affected by the temperature at which the test was conducted, the molarity of the Ce(IV), and the ratio of sugar to Ce(IV) oxidant.

Table III-4

Comparison of Expected DE Values From Commercial Starch Hydrolysates and DE Values Determined Using Ce(IV) Oxidimetry

Product	DE[1]	Ce(IV) Times[2]	Moisture[3]	Ce(IV) DE[4]
SD 10[5]	10	981.5 ± 25.4	5.2	11.8
SD 20[5]	21.5	208.2 ± 5.5	4.5	20.0
SD 24R[5]	26	115.7 ± 1.9	4.1	28.3
SD 35R[5]	34.5	84.2 ± 2.0	4.1	35.3
SD 42R[5]	43.5	67.6 ± 1.0	3.6	41.6
M100[6]	9.0-12.0	687.0 ± 13.7	5.9	12.7
M200[6]	20.0-23.0	170.7 ± 2.7	6.1	22.2
M250[6]	23.0-27.0	120.0 ± 0.5	4.6	27.6
M550[6]	13.0-17.0	375.5 ± 2.3	6.2	15.3

1. DE supplied by manufacturer (average or range for product)
2. seconds ± standard deviation
3. percentage by A.O.A.C. method
4. calculated by Ce(IV) oxidimetry using determined standard curve
5. supplied by A.E. Staley under the trade name, Star-Dri
6. supplied by Champlain Industries under the trade name, Maltrin

CONCLUSIONS

If special analytical needs should arise, the Ce(IV) oxidation method lends itself to facile modification to accommodate the needs of the starch producer. For products of low final DE values, a faster analysis could be achieved by either lowering the Ce(IV) concentration or increasing the hydrolyzed starch concentration. The simplicity of the test makes partial or total automation possible. Its dramatic color change (red to very pale yellow) makes it possible to also carry the test out visually, although a spectrophotometer as used in the above tests gives better accuracy. As already found earlier the oxidation times are affected by temperature (Griffith et al. ,1989) and thus temperature changes during the test or deviation from the temperature at which the standard curve was established lead to substantial error. If temperature control is impossible standard solutions and samples have to be run at the same time; a task most easily done during visual testing.

To conclude, the Ce(IV) oxidimetry test was fast, simple, inexpensive, did not require the use of boiling solutions and rapid titrations (such as the Lane Eynon), and could be modified as required by the processor. Therefore the Ce(IV) methodology is an alternative for present methods of determination of DE for starch hydrolysates, especially where required for process control.

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The Determination of Sucrose in Honey using Ce(IV) Oxidimetry

INTRODUCTION

The use of the color change during the reduction of Ce(IV) to Ce(III) with the concomitant oxidation of carbohydrate has been utilized in Chapter 3 of this thesis and by Griffith et al (1989) to determine the dextrose equivalent of corn starch hydrolysates and the percentage lactose and degree of lactose hydrolysis in milk. This technique allows the determination of quantities of di- or higher linked oligosaccharides in the presence of monosaccharides. It was therefore decided to adapt the test to determine sucrose in honey. Honey is essentially a solution of glucose, fructose and a minor quantity of sucrose plus very small amounts of other oligosaccharides.

The determination of sucrose in honey is of economic importance (Crane, 1979) as it is one of the major factors contributing to whether a honey is accepted or declared adulterated. The Codex Alimentarius, for example, allows a maximal sucrose content of 5 % before the honey is deemed to be adulterated. Another factor of great importance in the determination of a quality aspect of honey is the speed of determination (Crane, 1979). At present the major analytical tests used to determine sucrose are the Lane-Eynon titrimetric determination (A.OA.C. 31.128) and HPLC (high pressure liquid chromatography, A.O.A.C. 31.145). There is also a charcoal column absorption method that is

not as frequently used because of the lengthy analysis time of this test. The Lane-Eynon titrimetric test also has difficulties associated with it, the major being the requirement to quickly and accurately titrate a boiling solution. HPLC, while generally quick and accurate, requires the use of expensive equipment and reagents. The Lane Eynon test has one further drawback in comparison to the HPLC method. It does not measure true sucrose, rather it is a determination of total non-reducing sugars, and because a few of the minor oligosaccharides in honey are non-reducing sugars the value given by a Lane Eynon test must be properly referred to as apparent sucrose.

The difficulty, time, or expense of these tests makes the development of a quick, reliable and inexpensive test desirable. For that reason the feasibility of utilizing the Ce(IV) differential oxidation of carbohydrates to measure sucrose in honey was determined.

MATERIALS AND METHODS

All water was prepared using a Millipore Milli-Q water system (Millipore). Honey samples were generous gifts from the Alberta Honey Producers Co-Op (Edmonton) or the Alberta Government Food Lab (O.S. Longman Bldg., Edmonton). All chemicals used were of reagent grade or better.

Ce(IV) oxidations

All oxidations were carried out using a 1:3 ratio of 0.4M ammonium hexanitratocerium(IV) [$(NH_4)_2Ce(NO_3)_6$] in 0.5M nitric acid (HNO_3) to sugar solution. The Ce(IV) solution used was at least 6 hours old and shaken before each use to resuspend the fine precipitate. By ferrous ammonium sulfate titration it was determined that the actual concentration of the Ce(IV) solution was 0.39M (Chapter 1). The time (in seconds) from the addition of the Ce(IV) solution to sugar solutions until the absorbance at 445 nanometers which is the maximal absorbance difference for Ce(IV) to Ce(III) under these conditions (Chapter 3) fell to 0.5 optical density (OD) units was recorded. The measurements were made on a Pye-Unicam SP1800 spectrophotometer with the sugar solution as the reference sample and the temperature of the sample compartment maintained at 25.1 $\pm$ 0.1 ^{o}C by a LoTemptrol 154 bath (Precision Scientific Co.) containing a 50:50 mixture of ethylene glycol:water.

Optimal optical density for determinations of sucrose

Sucrose was dissolved in water containing equimolar ratios of glucose and fructose to give 5 solutions varying from 0 to 10 % weight/volume (w/v) sucrose (0, 2, 6, 8, and 10 %). These solutions were then oxidized at a 3:1 ratio of sugar solution to Ce(IV) and a time versus optical density (OD) curve determined for each concentration of sucrose. These data were then plotted and the optimal OD to give the largest difference in time between differing concentrations of sucrose was determined.

Optimal level of honey sample dissolution in the test

An artificial honey sample (glucose:fructose, 1:1, with no added sucrose) was sampled and dissolved in Milli-Q water at a number of different levels of dissolution (50:1, 100:1, and 200:1). These samples were then oxidized with Ce(IV) oxidant and the time for the absorbance to fall to 0.5 OD at 445 nanometers (nm) was recorded.

The same samples were then spiked with sucrose at increasing levels; 2 and 4 %. These samples were then oxidized and the time for the absorbance to fall to 0.5 OD determined.

Standard solutions

Artificial honeys containing glucose and fructose in equimolar ratios and increasing w/v % of sucrose were created. Glucose, fructose and sucrose were dissolved in the proper amounts in Milli-Q water to give a solution mimicking the dissolution level determined to be optimal for analyzing differences in sucrose percentage. The solutions were then oxidized at a 3:1 ratio of sugar solution to 0.39M Ce(IV) with monitoring of the absorbance at 445 nm and timing the reaction until the OD was 0.5. All oxidations were performed at least in triplicate.

Ce(IV) oxidations of honey

1.0000 g of honey $\pm$ 0.0010 g was carefully weighed into a 200 mL volumetric flask and diluted to volume with Milli-Q water. The solutions were left at room temperature for 30 minutes before sampling and all testing was done before 90 minutes had elapsed since dissolution. Honeys (12) were studied [the exact levels of

sucrose in the honeys were known from HPLC determination (A.OA.C. 31.145)] and the data determined from these oxidations were used in conjunction with the standard curve to determine percentage sucrose. All analyses reported are at least triplicate analyses of duplicate samples. Some honeys (those with sucrose values higher than 5 %) were spiked with solid sucrose, heated to ensure dissolution, and then studied by both HPLC and Ce(IV) oxidimetry for percentage sucrose. All honeys were boiled before testing, in loose covered jars, to ensure destruction of all enzyme activity without adversely affecting the moisture level.

HPLC determination of sucrose in honey

The determination of sucrose was performed using the A.O.A.C. standard method (31.145). The AOAC standard of glucose, fructose and sucrose was made up exactly as stated by the AOAC. The mobile phase was 83:17 acetonitrile : water, filtered through a 0.45 μM nylon 66 filter (Millipore) in a Millipore filter holder. The solution was then degassed by stirring under vacuum at room temperature. The acetonitrile was HPLC grade (Caledon Laboratories) with a determined UV cutoff of 190 nm. The water used was Milli-Q water.

The analysis was performed on a Phenomenex μ-Bondapak CHO-10 30 cm. column (Amino-propyl active phase, Phenomenex) protected by a Synchropak (Chromatographic Specialities) amino-propyl guard column . The solvent was delivered by a Beckman model 110A HPLC pump at a flow rate of 1.5 mL/min. and maximum pressure of 500 psi. The sample was injected onto the column by means of a Rheodyne 7125 injector with a 10 μL loop and detected

by a Waters optical refractometer R401 at 25 °C. Temperature in the refractometer was kept constant by a Polytemp Polyscience circulating bath filled with ethylene glycol.

Data was collected and integrated on a Varian model 4270 integrator.

Sugar acid effects upon the test

The two sugar acids; gluconic acid ($C_6H_{10}O_6$) and glucono-δ-lactone ($C_6H_{12}O_7$) were studied at the levels found in the average North American honey (Crane, 1979). These were 0.43 % w/v for gluconic acid and 0.14 % w/v for glucono-δ-lactone.

The two acids, 0.4286 g of gluconic acid and 0.1462 g of glucono-δ-lactone were dissolved in a 100 mL volumetric flask and made up to volume using water. A 1 mL sample was then taken and made up to volume in a 200 mL volumetric flask with water. This solution was then tested by the Ce(IV) test.

A second experiment was performed by adding 1 mL of the stock acid solution to the AOAC standard honey solution and diluting it to a ratio that replicated a honey diluted 200 fold. This solution was also tested by the experimental protocol above.

Determination of moisture in honey samples

The percentage moisture in each honey was determined by AOAC method 31.118 (AOAC 1984). The honey sample was placed on the sample window of an Abbe refractometer (Carl Zeiss, Germany) that was temperature controlled to 20.0 °C by a circulating water

bath (Haake F3, Karlsruhe, W. Germany) and the refractive index determined in triplicate for each sample.

The refractive indices were then used, by comparison with the published tables (AOAC, 1984), to establish the percentage moisture in each honey sample.

RESULTS AND DISCUSSION

Determination of optimal optical density for the test

In order to utilize the Ce(IV) test for determining sucrose in honey it was first necessary to determine the optimal optical density (OD) that gave maximal difference in times for oxidation between artificial honey solutions containing different levels of sucrose (Figure IV-1).

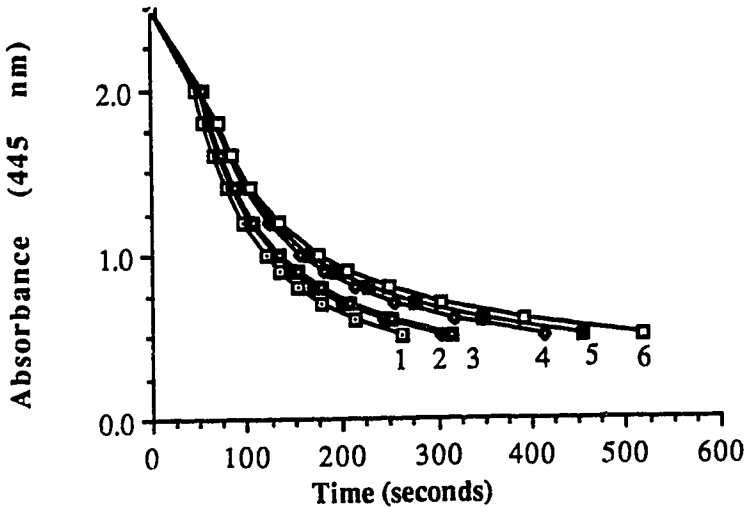
The OD giving the greatest difference in times of oxidations between artificial honeys containing 0, 2, 6, 8, and 10 % sucrose was shown to be 0.5 OD at 445 nm. The choice of 445 nm to determine the Ce(IV) to Ce(III) transition is explained in earlier chapters of this thesis (Chapters 2 and 3) as is the explanation for the color change of Ce(IV) to Ce(III).

Optimal level of honey dissolution

In order to increase the facile nature of the Ce(IV) test an ideal level of dissolution of honey was determined. It was decided that this level must give both a rapid time for the test at all levels of sucrose being studied and easily distinguish between these levels. In summary the test had to be both rapid and precise.

Figure IV-1

Optimal optical density (445 nm) for the Ce(IV) test for sucrose in honey



1. 0 % sucrose added to standard honey solution
2. 2 % sucrose added to standard honey solution
3. 4 % sucrose added to standard honey solution
4. 6 % sucrose added to standard honey solution
5. 8 % sucrose added to standard honey solution
6. 10 % sucrose added to standard honey solution

A series of dissolutions of artificial honeys were created as stated in the materials and methods. The levels studied were 50:1, 100:1 and 200:1. These were then tested using the Ce(IV) test (Table IV-1). From these data it was clear that the best level of dissolution of the honey to give the most favorable differences in time was 200:1. In all further experimentation with actual or artificial honeys the dissolution level used was 200:1.

Ce(IV) test on carbohydrates/effect of non-reducing sugar upon the Ce(IV) analysis

A number of different carbohydrates, including some that would not be expected in honey samples, were dissolved in water to give 1.5 % solutions (w/v) and tested using the Ce(IV) method of analysis (Table IV-2). In this investigation, because many of the sugars studied had been tested earlier (Chapter 2), the times of oxidation were measured to 0.4 OD at 445 nm.

Sucrose was placed in 0.5M nitric acid for fifteen minutes and the solution then tested for the presence of reducing sugars. The labile disaccharide sucrose is hydrolyzed under this acid condition and would therefore be hydrolyzed under the conditions of the Ce(IV) test, unlike the disaccharide lactose (Griffith et al, 1989). The hydrolysis of sucrose into monosaccharides is therefore the most likely explanation for why the disaccharide sucrose, and the trisaccharides raffinose and melezitose (both containing sucrose units), which are all non-reducing sugars, have oxidation times so much faster than the non-reducing sugars trehalose and 1-O-methyl-β-D-glucopyranoside which do not contain sucrose units. The acid

Table IV-1

Optimal dissolution level for the Ce(IV) test

Dissolution level[1]	Time to OD_{445} = 0.5[2]	
	0 % sucrose	2 % sucrose
50:1	21.37 ± 0.02[3]	21.35 ± 0.03
100:1	60.96 ± 1.13	66.96 ± 1.51
200:1	262.9 ± 10.09	310.79 ± 4.80

1. equivalent to 1 gram of honey dissolved in 50, 100, or 200 mL water
2. measured in seconds at 25.1 °C
3. average of triplicate determinations ± standard deviation

hydrolysis products; glucose and fructose, both considerably faster in oxidation times than disaccharides, were being oxidized in the case of carbohydrates containing the sucrose moiety. As can be seen from Table IV-2 fructose is the hexose most susceptible to Ce(IV) oxidation. Release of a fructose because of acid hydrolysis of the glycosidic linkage in sucrose would cause a faster loss of color [reduction of Ce(IV) to Ce(III)] and the reaction would appear to be much faster than expected.

It is of interest that the acid stable methyl glycoside; 1-*O*-methyl-β-D-glucopyranoside, though a monosaccharide, is oxidized slower than the non-reducing di and trisaccharides. This suggests that even in the case of trehalose there may be some hydrolysis of the glycosidic linkage taking place under the acidic conditions and that this accounts for the faster times of oxidation observed.

In the Ce(IV) test there was a general trend of monosaccharides oxidizing faster than disaccharides which in turn were oxidized faster than trisaccharides and so on. There was another trend that suggests that the reducing moiety on the sugar is essential for rapid reduction of Ce(IV) to Ce(III) and therefore rapid oxidation of the sugar. Virtanen et al (1987) have suggested that there are two oxidizable forms of the Ce(IV)-carbohydrate complex; Complex One and Complex Two. These researchers postulate that one of the complexes is with the open chain form of the carbohydrate and oxidizes rapidly while the other complex is with the ring form of the carbohydrate and is oxidized slowly. The results presented in Table IV-2 agree with this hypothesis since the methyl glycoside,

1. 1.5 % w/v carbohydrate in water
2. average of triplicate analyses except where noted below
3. non-reducing carbohydrates
4. average of duplicate determinations

Table IV-2

Oxidation times of some carbohydrates by the Ce(IV) oxidimetric test

Carbohydrate[1]	Time (sec) to OD445 = 0.4[2]
Monosaccharides	
Ribose	11
Arabinose	18
Glucose	53
Fructose	13
Galactose	23
Rhamnose	36
1-O-Me-β-D-glucopyranoside[3]	19,800[4]
Disaccharides	
Maltose	151
Lactose	293
Lactulose	119
Sucrose[3]	1883
Turanose	497
Trehalose[3]	13,500[4]
Melibiose	100
Gentiobiose	92[4]
Palitinose	39[4]
Trisaccharides	
Raffinose[3]	2560
Melizitose[3]	6362[4]

which is constrained to the ring form, does reduce Ce(IV) to Ce(III), albeit at a much reduced rate in comparison to other carbohydrates.

In food systems sugar analyses are generally reported as percentage values based upon weight/volume (w/v) or weight/weight (w/w) of a dry, moist or liquid sample. For this reason the sugars tested above were reported on an equal w/v basis instead of on a molar basis.

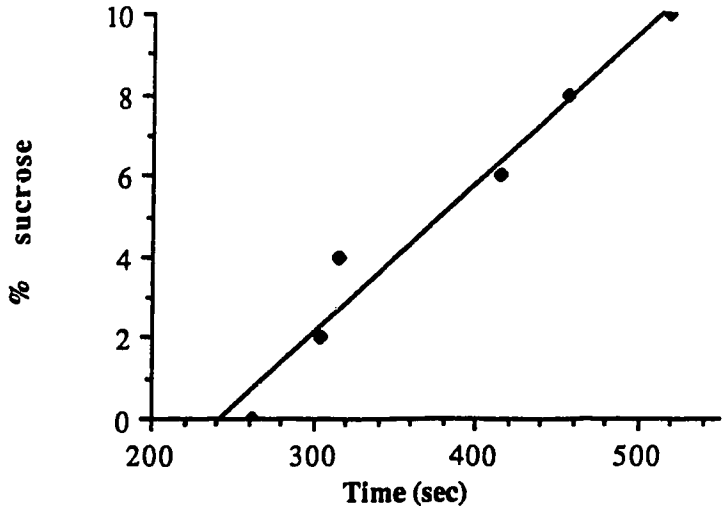
In honey analysis the sugars that are of the most interest are glucose, fructose, sucrose and maltose. Minor disaccharides in honey include; isomaltose, nigerose, turanose, gentiobiose, palitinose and kojibiose (Siddiqui and Furgala, 1967). All of these sugars are oxidized much faster than sucrose under the Ce(IV) experimental protocol. There has been a rigorous work, utilizing capillary gas chromatography and C^{13} nuclear magnetic resonance (NMR) to identify the minor oligosaccharides in honey (Low, 1986) but these constituents have been shown to be of very limited quantity in honey. Therefore only the sugars listed in Table IV-2 were studied with the cerium oxidimetric method.

Standard curve

Artificial honeys that mimicked the AOAC standard, except for the percentage sucrose, (38 % fructose and 30 % glucose) were created. Instead of a fixed sucrose percentage the sucrose concentration was varied from 0 to 10 % at a dilution level (honey:water) of 1 to 200. From these samples a standard curve was created of percentage sucrose in the sample versus time in seconds (Figure IV-2) using the experimental protocol. The curve is linear

Figure IV-2

Standard curve for sucrose by the Ce(IV) oxidimetric method



over the region studied and has a regression calculated equation of the line of:

$$y = -8.934 + 0.0368x$$

where y is the percentage sucrose in the sample and x is the time for the absorbance at 445 nm. to fall to 0.5 OD. The linear regression coefficient calculated for this line is 0.964.

Determination of sucrose in honey by the Ce(IV) method

The standard curve established above was used to discern the percentage sucrose in a number of different honeys (Table IV-3). These honeys were also studied for percentage sucrose by HPLC analysis (Table IV-3). It was obvious that in all but two cases the Ce(IV) method underestimated the percentage sucrose by varying degrees. Various possible factors (see below) were studied and their effects upon the times of oxidation, as detailed below, were found to be inconsequential. It was not discounted however that perhaps these factors all act in synergy. The possibility of synergistic action was not studied further as it was decided that the test would best be used as a preliminary screen instead of a final action on percentage sucrose and therefore it was not necessary to fully characterize these possibilities.

Sugar acid effects upon the Ce(IV) test

The first factors studied were the two sugar acids present in honey; gluconic acid and glucono-δ-lactone. The data accumulated by Crane (1979) shows that the acid composition of the average North

Table IV-3

Percentage sucrose in honey by HPLC and Ce(IV) determination

Honey	HPLC % sucrose	Ce(IV) % sucrose
1	0.5 ± 0.06[1]	-0.05 ± 0.01[1]
2	0.5 ± 0.04	0.09 ± 0.04
3	4.3 ± 0.01	4.53 ± 1.07
4	10.1 ± 0.2	11.09 ± 0.87
5	4.8 ± 0.18	2.83 ± 0.45
6	1.48 ± 0.03	1.02 ± 0.19
7	0.8 ± 0.01	-0.28 ± 0.06
8	2.3 ± 0.05	1.61 ± 0.35
9	0.8 ± 0.01	-0.13 ± 0.02
10	2.1 ± 0.05	1.87 ± 0.21
11	1.1 ± 0.02	0.42 ± 0.04
12	1.7 ± 0.03	1.09 ± 0.1

1. average of triplicate determinations ± standard deviation

American honey is 0.5748 % total acid, with gluconic acid being the major constituent acid at approximately 0.43 %.

The stock solution described in the materials and methods section was then used, as described, in the Ce(IV) test. There was no initial reaction of the Ce(IV) oxidant with the two sugar acids, as evidenced by the lack of formation of an initial red color in the solution and the lack of any absorbance change at 445 nm.(Shriner et al, 1980; Virtanen et al, 1987; Griffith et al, 1989) even when the solutions were left for as long as one hour at room temperature.

A second test was performed whereby the acid solution was added to the 0 % sucrose standard solution used for the determination of the standard curve, in a ratio mimicking an actual honey. This solution was then tested by the Ce(IV) experimental protocol. Upon analysis of the data no significant increase or decrease in the time of oxidation of the sample was noted. There was a slight increase in the time of oxidation (averaging 11 seconds) but the two values, with addition of sugar acids and without, were within the limits of experimental error of each other. The time for the oxidation for the sample with no sugar acids was 290 $\pm$ 10 seconds and that for the sample with added sugar acids was 305 $\pm$ 6 seconds.

By itself this is not a significant effect, but as stated before, it could be possible that a synergism between effects could have been responsible for the observation of decreased times of oxidation for actual honeys studied when compared to the standard curve.

Effect of moisture level upon the test

The next constituent of honey that was studied was moisture level. The moisture values for the honeys varied from 16.4 to 18.0 % moisture (Table IV-4). There was not any obvious correlation between the moisture level of the honey and the difference in sucrose values established. It was concluded therefore that the moisture level of the honey was not having a significant effect upon the results determined by the Ce(IV) test.

Fructose/glucose ratio in the honeys

Fructose is by far the quickest oxidized monosaccharide by Ce(IV) under the conditions employed. It has a much faster time of oxidation than glucose, the other major carbohydrate in honey (Crane, 1979). This large difference in oxidation behavior by equimolar solutions of these two monosaccharides could be part of the explanation for why the Ce(IV) oxidimetric determined values for percentage sucrose were so different than those determined by HPLC.

Variations in the fructose/glucose ratio between honey samples and between the samples and the AOAC standard solution used to ascertain the standard curve could have large effects upon the time of oxidation in the Ce(IV) method. The honeys studied did vary in the fructose/glucose ratio and this ratio was in some instances quite different from that of the AOAC standard used for the standard curve (Table IV-5). This could not be the entire answer to the problem either. Honeys 1 and 2, both considerably lower in fructose/glucose ratio than the standard solution were considerably

Table IV-4

Moisture levels in honey samples

Honey	% moisture[1]
1	17.9
2	17.2
3	16.4
4	16.7
5	16.5
6	17.6
7	18.0
8	17.5
9	17.7
10	17.6
11	17.9
12	17.9

1. Average of duplicate analyses

Table IV-5

Fructose/glucose ratio in honey samples

Honey	Fructose/glucose ratio[1]
AOAC standard	1.26
1	0.99
2	1.09
3	1.13
4	1.12
5	1.13
6	1.15
7	1.05
8	1.13
9	1.12
10	1.20
11	1.09
12	1.13

1. determined by HPLC analysis

faster in oxidation times than would be expected if they were to match the standard curve. This was the opposite effect expected from a lower fructose/glucose ratio (with less fructose it could be expected that the time of oxidation was longer). The conclusion to this experiment was two-fold; first, that it would be technically impossible to establish a separate standard curve for each level of fructose/glucose ratio in the honeys, defeating the purpose of developing a rapid method, and secondly that the fructose/glucose ratio could not be entirely responsible for the difference in results between the two methods.

CONCLUSIONS

The conclusion of this work was that the Ce(IV) oxidimetric technique could be used to determine sucrose in honey only as a rapid pre-screen test. If the desired result from the analysis is a level of sucrose, for example less than or greater than 5 %, the Codex Alimentarius establish a honey as adulterated or actual if greater than 5 % sucrose is present (Crane, 1979) then the Ce(IV) method could be a rapid means to establish that answer. Honeys with levels of sucrose that appeared to be very close to 5 % would have to be analysed again using a different method because of the errors in the Ce(IV) oxidimetric method for this determination.

The determination of a relatively minor disaccharide in the presence of fluctuating levels of two differently oxidized (when rate is used as the comparison) monosaccharides is not the optimal use of the Ce(IV) test. The nature of the method is such that it works best for determining a single carbohydrate in a non carbohydrate matrix,

such as lactose in milk, or for determining the breakdown of a disaccharide to its constituent monosaccharides (a situation where the molar ratios of the monosaccharides in question remain relatively constant, Griffith et al, 1989). Alternatively the Ce(IV) method performs well in determining a minor monosaccharide constituent in the presence of various oligosaccharides, as in the determination of the dextrose equivalent of corn starch hydrolysates (Chapter 3).

The striking differences in the oxidation times of the different carbohydrates, both monosaccharides and higher linked saccharides, opens up the possibility that the Ce(IV) test may, in the future, be adapted for determination of carbohydrates in different foodstuffs.

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Purification and Partial Characterization of β-Galactosidase from Maxilact

INTRODUCTION

β-galactosidase (E.C.3.2.1.32) is an important enzyme in the dairy processing industry. The most commonly used β–galactosidases in the dairy industry include the enzymes from yeasts, *Kluyveromyces fragilis* and *Kluyveromyces marxianus*, formerly *lactis*, (Mahoney, 1985). The enzyme from *K. fragilis* has been well studied in the literature (Mahoney and Whitaker, 1977 and 1978). While some characterization of the enzyme from *K. marxianus* has been carried out (Hussein et al., 1989; Dickson et al., 1979; Olano et al., 1983; Jacober-Pivarnik and Rand, 1984; Mahoney and Wilder, 1988), there has not been a study on the effect of mono and divalent cations, temperature, and inhibitor action on purified enzyme. In addition, the amino acid composition of this enzyme has not been reported. The economic significance of this enzyme to the food industry makes the determination of these factors, on purified enzyme, of importance to researchers and producers who study or use this enzyme. This work describes the purification of β – galactosidase from the commercial preparation Maxilact L2000 (Gist-Brocades, N. Carolina) and the partial characterization of the enzyme.

MATERIALS AND METHODS

Maxilact L2000 was a gift from the Gist-Brocades Company (North Carolina). Glutathione, Blue Dextran, inhibitors, and all enzyme substrates were purchased from Sigma Chemical Co. (St. Louis, MO.). Sephadex G200 was purchased from Pharmacia chemicals (Lund, Sweden). Electrophoresis grade reagents, Bio-Rad Mini-Protean II apparatus and molecular weight standards were obtained from Biorad (Richmond, CA). The Glycan Detection Kit was purchased from Boehringer Mannheim (Boehringer Mannheim, Mannheim, W. Germany). All other reagents were analytical grade or better. All water was Milli-Q (Millipore, Bedford, MA) purified.

Enzyme purification

Sephadex G200 gel (approximately 25 grams) was swelled and the chromatography column (5 cm by 90 cm) packed according to the manufacturers instructions. The column was pre-equilibrated before void volume determination and before enzyme purification experiments with 50 mM sodium phosphate buffer, 0.2 % sodium azide (w/v), pH 6.8 to 7.0 (dependent upon the experimental run.). The void volume (90 mL) of the column was determined with Blue Dextran. The sample of Maxilact L2000 (1 mL) was loaded onto the column from a three way valve by means of an attached 1 mL syringe. The column was run in a reverse flow mode at a flow rate of 5.0 mL/h, with a collection rate of one tube/h. The purity tests for contaminating carbohydrases were conducted by a variation of the Mahoney and Whitaker method (1978) using appropriate

carbohydrate substrates (o-nitrophenyl-β-D-galactopyranoside, ONPG, for determination of β-galactosidase activity; p-nitrophenyl-α-D-galactopyranoside, for determination of α-galactosidase activity; o-nitrophenyl-β-D-glucopyranoside, for determination of β-glucosidase activity; p-nitrophenyl-α-D-glucopyranoside, for determination of α-glucosidase activity). Contaminating protease was determined using thetrichloracetic acid (TCA) soluble nitrogen after casein digestion test (Annunziato and Mahoney, 1987).

Dialysis and lyopholization of the enzyme.

After purification on the Sephadex G200 column an aliquot of the enzyme, in 50 mM sodium phosphate, 0.2% sodium azide, adjusted to pH 7.0, was placed in a Spectrapor 3, dialysis membrane (Spectrum Medical Industries Ltd., Los Angeles, CA) that was sterilized and washed according to the suppliers instructions. The filled membrane was sealed with Spectrapor tubing clamps and placed in 150 volumes of water at 4 °C. The dialysis was continued for 48 h with 4 changes of solution. The solution contained in the dialysis bag was then tested for enzymatic activity. Lyophilization of the remaining enzyme was conducted in a FTS Systems Flexi Dry Apparatus (FTS Systems, Stone Ridge NY).

Buffer, cation, and inhibitor effects.

All experiments to determine the effects of buffer, inhibitors and cations on enzyme activity were conducted at 37 °C using a Pye Unicam SP1800 UV spectrophotometer. Enzyme activity was monitored at 420 nm using a modification of the procedure of

Mahoney and Whitaker (1978). The inhibitors studied; p-aminophenyl-thio-β-D-galactopyranoside (PAPTG), galactosamine, galactonolactone, galactose and glucose were dissolved in 0.05 M phosphate buffer, pH 7.0, at two concentrations, 5 and 12.5 mM, with 4 concentrations of o-nitrophenyl-β-D-galactopyranoside (ONPG) from 0.5 to 8 mM (1.25, 2.5, 5.0, and 8.0 mM) used in the determination. For each experiment a zero inhibitor concentration sample was also determined. The inhibition constants and type of inhibition were determined by a double reciprocal plot (Lineweaver and Burk, 1934).

Michaelis Menton constant determination.

The K_m for ONPG was determined according to the method described by Whitaker (1972) in the 37 ^oC UV spectrophotometer by continously monitoring the increase in absorbance at 420 nm. The Michaelis Menton constant was determined from a double reciprocal plot (Lineweaver and Burk, 1934).

SDS gel electrophoresis

The sodium dodecyl sulfate (SDS) gel electrophoresis was performed according to the procedure of Laemmli (1970) on 5 to 15 % acrylamide gradient gels or 12 % acrylamide gels for nongradient work. Except for the gels run in nonreducing medium, the buffer used to load protein samples was Laemmlis buffer (0.125 Tris HCL, pH 6.8; 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol). No 2-mercaptoethanol was used for the nonreducing gels. Molecular weights of the bands were determined by comparison to a standard

curve constructed from molecular weight standards. The distance of migration of both the standards and samples were measured on wet gels immediately after destaining using vernier calipers from the bottom of the stacking gel to the center of the stained band. Bands were stained with Coomassie Blure R250 (Sigma, St. Louis, MO) and destained in methanol/acetic acid/water (300/300/2400) until the background color was no longer visible on the gel.

Nondenaturing gel electrophoresis and activity determination

The slab gel electrophoresis of the protein under nondenaturing conditions was performed on a 3 mm slab gel with a 2 to 4 % acrylamide gradient. A maximum applied voltage of 50 V for 30 hours was used with complete changes of the running buffer every 12 hours and cooling supplied by having the gel apparatus in a 4 ^{o}C cold room. The running buffer and the buffer used to pour the gel were identical and the buffer was; 40 mM Tris.HCl, 20 mM potassium acetate, and 2 mM EDTA adjusted to pH 7.4 (Morrow and Haigh, 1983). A standard 160 by 160 mm gel was prepared with a 10 fingered comb and the samples loaded as mirror images (ie: samples 1 to 5 mirrored the order of samples 6 to 10). After electrophoresis was complete, the gel was cut in half and lanes 1 to 5 were subject to staining in Coomassie Blue R250, while the gel with lanes 6 to 10 was submerged, with gentle shaking, at room temperature for 15 min in 1.25 mM ONPG and 50 mM potassium phosphate adjusted to pH 6.8. The latter activity stained gel was immediately photographed after gentle washing in water as there

was rapid fading of the stain. After 2 hours at room temperature the yellow stain was no longer visible.

Glycoprotein functionality determination

The determination of glycan moieties on the protein was conducted with the Glycan Detection Kit. All reagents and samples were prepared using the manufacturer's instructions. The SDS PAGE was performed on 12 % acrylamide gels in a Bio-Rad Mini-Protean II apparatus (Bio-Rad, Richmond CA) at a constant voltage of 150 V for one hour. The blotting onto Immobilon (Millipore, Bedford, MA) was performed in a Bio-Rad Mini Trans-Blot apparatus following the instructions for trans blotting accompanying the apparatus. Staining of the membrane to detect carbohydrates was conducted by the protocol determined by the supplier with the exception that the volumes of reagents used were all doubled to ensure immersion of the membrane. The staining was completed within 3 min.

Extinction coefficient of β-galactosidase determination

The extinction coefficient of β-galactosidase was determined on an exactly weighed portion of the dialysed, lyopholized enzyme dissolved in sodium phosphate buffer, adjusted to pH 7.0. Three different concentrations of the enzyme were used. The complete UV scan of the enzyme was determined on a Hewlett Packard 8451A Diode Array Spectrophotometer (Hewlett Packard, Pao Alto, CA).

Protein concentration determination

The protein concentration was determined either by the Bio Rad assay method (standardized against Bovine Serum Albumin) for the Maxilact L2000 or by determination of the concentration of the purified enzyme in solution by use of the determined extinction coefficient.

Temperature stability determinations.

Effect of temperature on the enzyme stability in buffer was determined by measuring the activity remaining after the enzyme had been left for different times at temperatures varying between 22.5 to 60 ^{o}C. Activity of the enzyme was determined as above.

Amino Acid Analysis

The amino acid determination on purified, water dialyzed, lypholized enzyme was conducted by the Biochemistry Department of the University of Alberta on a Dionex D-502 amino acid analyzer (Dionex, Sunnyvale, CA), with complementary runs on an Applied Biosystems 13A Separation System, 42-Derivatizer (Foster City, CA). The chromatograms were analysed on an Applied Biosystems 920 Data Analyzer. Tryptophan was determined spectrophotometrically (Beaven and Holiday, 1952).

p-Chloromercuric benzoic acid (PCMB) titration

The PCMB titration was performed according to the method of Boyer (1954). PCMB (8 mg.) was dissolved in 1 equivalent of sodium hydroxide and then diluted to 25 mL. This solution was then

standardized by titration against glutathione and used in the procedure for spectrophotometric titration of cysteines outlined by Benesch and Benesch (1962). The concentration of titrant was 8.9 x 10^{-4} M. The PCMB titrant was added to a 2.4 x 10^{-6} M solution of β-galactosidase by means of a 1, 10 or 100 μL gas tight Hamilton syringe (Hamilton Syringe, Reno, NV) depending upon the experiment being performed. In experiments comparing the titration value to activity, 100 μL aliquots of the enzyme (undialyzed before the experiment and the concentration of protein known) were removed from the cuvette in which the titration was being performed after 15 min of incubation at 37 °C with the added PCMB and tested for activity by the above method. Experiments were performed with longer times of PCMB protein incubation (up to one hour) but the titer was unaffected.

pH effect on activity.

For the pH range 2 to 4.8, the buffer utilized was 50 mM sodium acetate. The buffer used in the rest of the pH range was 50 mM sodium phosphate. In all cases 1.2 x 10^{-10} moles of enzyme were added to a 2.0 mL solution of 1.25 mM ONPG dissolved in the appropriate buffer.

RESULTS AND DISCUSSION

Purification of β - galactosidase from Maxilact L2000

The purification of the enzyme is summarised in Table V-1. The purity of the enzyme was assessed by determination of

Table V-1: Purification of β-Galactosidase

Sample	Activity[1]	Protein[2]	Specific Activity[3]	Purification	Recovery[4]
Maxilact	1272	128	9.94	n.a.	n.a.
Purified Enzyme	21.6	0.5	43.2	4.34	21 %

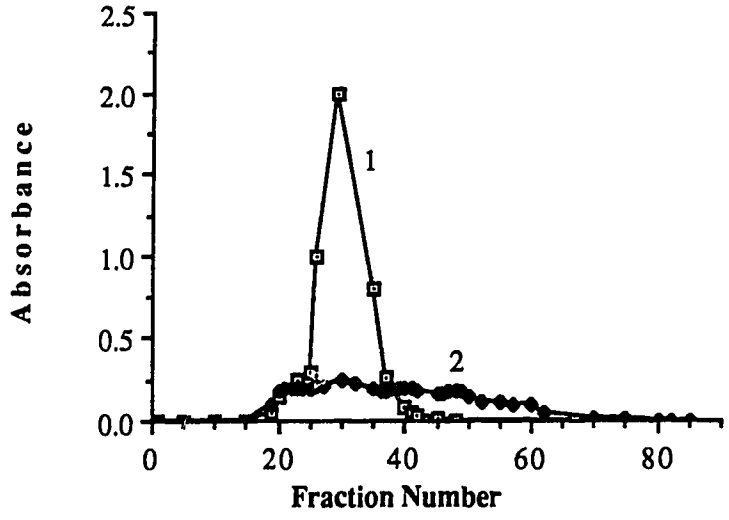
1. a unit is measured as micromoles ONPG hydrolyzed/minute. Activity is units/mL
2. Measured spectrophotometrically at 280 nm. using the calculated extinction coeffecient. Expressed as mg/mL
3. Expressed as units/mg protein
4. Based upon mg. of protein recovered.

contaminating enzyme activities, specifically other carbohydrases and protease (Table V-2). The elution profile of the Sephadex G200 column (Figure V-1) showed a leading shoulder eluting before the β-galactosidase with a small amount of enzyme activity. This protein eluted at the breakthrough volume of the column and was closely followed by the protein peak containing the desired enzyme activity. There was also a tailing shoulder with some activity, albeit minor, and a second peak of protein eluting within a few fractions of the major β-galactosidase containing fraction. After the tailing no further protein was detectable.

The peak was cut and only the centre fractions with the highest activity were pooled as purified enzyme. The pooled enzyme was tested for interfering carbohydrases and protease (Table V-2) and for protein concentration by absorbance at 280 nm. Purification was stopped at this stage because for the work envisaged the enzyme was pure enough to continue.

In initial experiments the protein was determined using the Bio-Rad protein assay test. Using the calculated extinction coefficient for determining the amount of protein, the purified enzyme had a specific activity of 43.2 units/mg. This is one of the highest reported specific activities for purification of this enzyme in the literature. The best purification earlier reported was 139 units/mg (Dickson et al., 1979). In some experiments (data not shown) the purification was at a level of 533 units/mg but the yield was substantially reduced to below 2 percent.

Figure V-1.
Sephadex G200 purification of β-galactosidase



1. Absorbance at 420 nm. (Enzyme activity)
2. Absorbance at 220 nm. (Maximal enzyme absorbance)

Table V-2:
Contaminating Enzyme Activities in Maxilact and purified β-Galactosidase

Enzyme Activity	Maxilact L2000	β-Galactosidase
α-Galactosidase	1.15[1]	zero[3]
α-Glucosidase	0.01[1]	zero[3]
β-Glucosidase	0.02[1]	zero[3]
Protease	2.0[2]	zero[2]

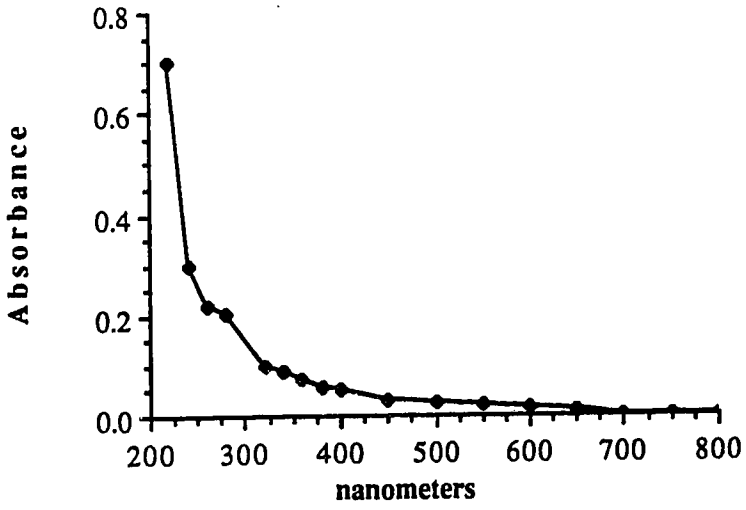
1. specific activity. micromoles ONPG hydrolyzed per min./mg. protein The test conditions were 25 °C, 5 minutes incubation.
2. units/ml enzyme solution. A unit is increase in Absorbance at 280 nm/hr/ml
3. the activity determinations were at 25 °C, 30 minutes incubation.

Determination of the extinction coefficient of β-galactosidase.

Before determination of the extinction coefficient of the enzyme a complete UV spectrum was determined from 220 nm to 800 nm. on a 0.5 mg/ml concentration of the enzyme at pH 7.0 (Figure V-2).

From these data it was decided to determine the extinction coefficient at 220 and 280 nm. The extinction coefficient was determined for three different dilutions of the enzyme. This experimental protocol was repeated on a second dissolved portion of the lyopholized enzyme. The extinction coefficients determined from this experiment were: at 220 nm the ξ_{220} was 2.60 $cm^2 \cdot mg^{-1} \pm 0.04$ $cm^2 \cdot mg^{-1}$ protein, and at 280 nm the ξ_{280} was 0.728 $cm^2 \cdot mg^{-1} \pm$ 0.004 $cm^2 \cdot mg^{-1}$ protein. Hussein and coworkers (1989) have reported an extinction coefficient at 280 nm for purified *Kluyveromyces marxianus* β–galactosidase of 1.23 $cm^2 \cdot mg^{-1}$ protein. However, their experimental protocol did not involve determination of the extinction coefficient on lyopholized sample and in fact their protein concentration was based upon a Folin-Coicalteau standard curve of BSA. It is probable that the β-galactosidase enzyme does not have the same response to the Folin-Coicalteau reagent as does BSA. This has been shown to be the case with the Bradford method based Bio-Rad protein determination kits (Bio-Rad Protein Assay insert 1988). A difference in response to the reagent would mean an inaccurate determination of the protein concentration and could account for the discrepancy in the extinction coefficients between the paper of Hussein et al. (1989) and this work.

Figure V-2. UV scan of β-galactosidase



Km determination for ONPG

Earlier reports in the literature have given the Km for ONPG as 1.18 mM (Biermann and Glantz, 1968), 1.25 mM (Hussein et al., 1989.), and 1.66 mM (Dickson et al., 1979) for various purification levels of the β-galactosidase from *Kluyveromyces marxianus*. The Km reported here for the purified enzyme was 1.58 mM. This was determined by a double reciprocal plot (Figure V-3). This was in good agreement, especially with the last value, where the specific enzyme activity was higher. The higher value for specific activity probably represented an enzyme of greater purity and therefore would more closely agree with the results presented here.

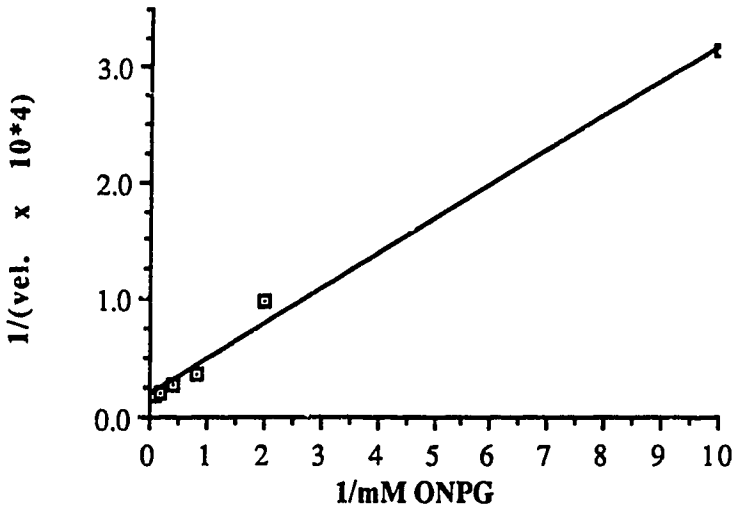
Inhibition constant determination.

All of the inhibition constants for the reported chemicals were determined by double reciprocal plots (galactonolactone, [Figure V-4], galactosamine [Figure V-5], galactose [Figure V-6], and PAPTG [Figure V-7]). All of the inhibitors studied, with the exception of glucose, which was not an inhibitor, acted as competitive inhibitors. The inhibition constants are given in Table V-3. The best inhibitor of β-galactosidase action was galactonolactone. Mahoney and Whitaker (1977) also reported D-galactonolactone as the best inhibitor of *K. fragilis* β-galactosidase. The K_i these researchers reported was 0.172 mM. The value of 0.19 mM reported here for *K. marxianus* β-galactosidase is in very good agreement. These researchers also reported that high concentrations of galactose acted as a competitive inhibitor of enzyme action, as was observed for β-galactosidase from

Table V-3: Inhibition Constants

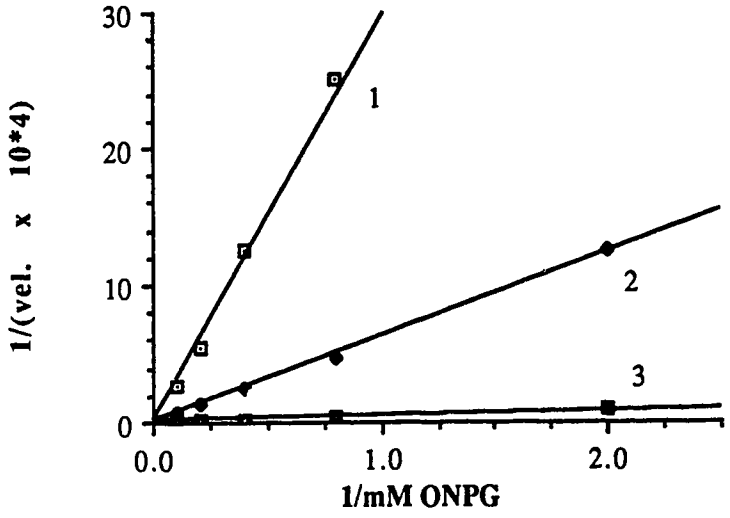
Compound.	K_i (mM)
Glucose	none
Galactose	31.06
Galactosamine	3.04
p-Amino thio-β-D-Galactopyranoside	15.17
Galactono-γ-1,4-lactone	0.19

Figure V-3. Lineweaver Burk plot for ONPG[1]



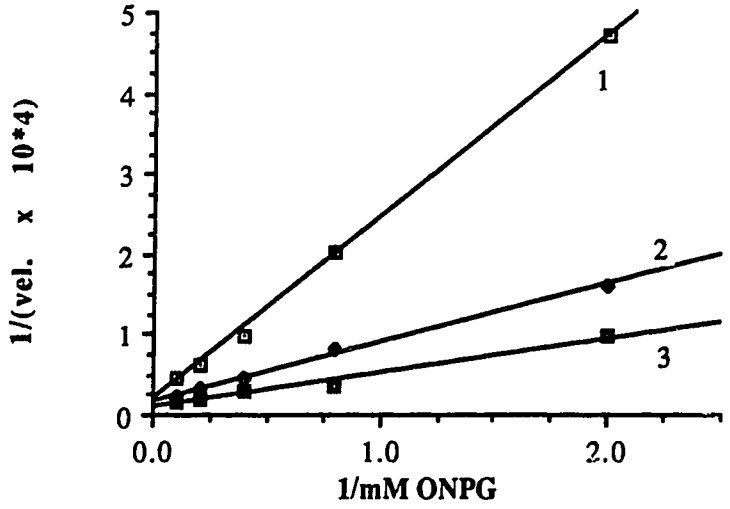
1. Determined at pH 7.0 in sodium phosphate buffer

Figure V-4. Lineweaver Burk plot of galactonolactone inhibition



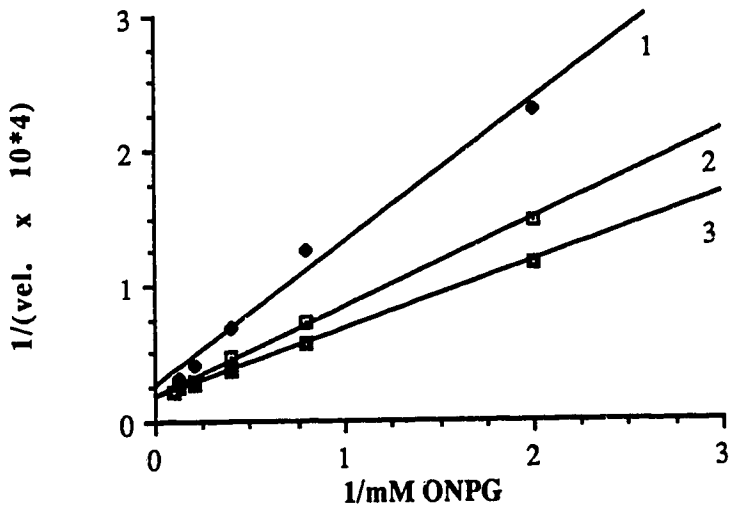
1. 12.5 mM galactonolactone added to solutions
2. 5.0 mM galactonolactone added to solutions
3. 0 mM galactonolactone added to solutions

Figure V-5. Lineweaver Burk plot of galactosamine inhibition



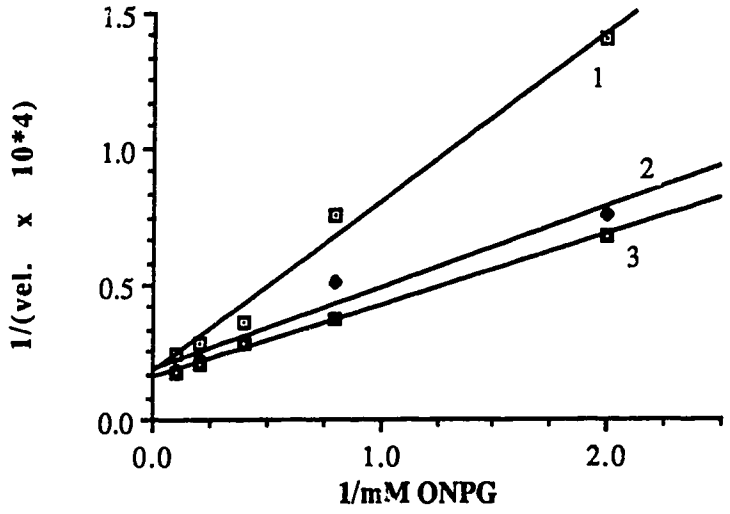
1. 12.5 mM galactosamine added to solutions
2. 5.0 mM galactosamine added to solutions
3. 0 mM galactosamine added to solutions

Figure V-6. Lineweaver Burk plot of galactose inhibition



1. 35 mM galactose added to solutions
2. 10 mM galactose added to solutions
3. 0 mM galactose added to solutions

Figure V-7. Lineweaver Burk plot of PAPTG inhibition



1. 12.5 mM PAPTG added to solutions
2. 5.0 mM PAPTG added to solutions
3. 0 mM PAPTG added to solutions

K. marxianus. Substrate analogues of lactose, such as the monosaccharide galactose and the thiogalactosides have been shown to act as inhibitors of *E. coli* β–galactosidase (Kuby and Lardy, 1953).

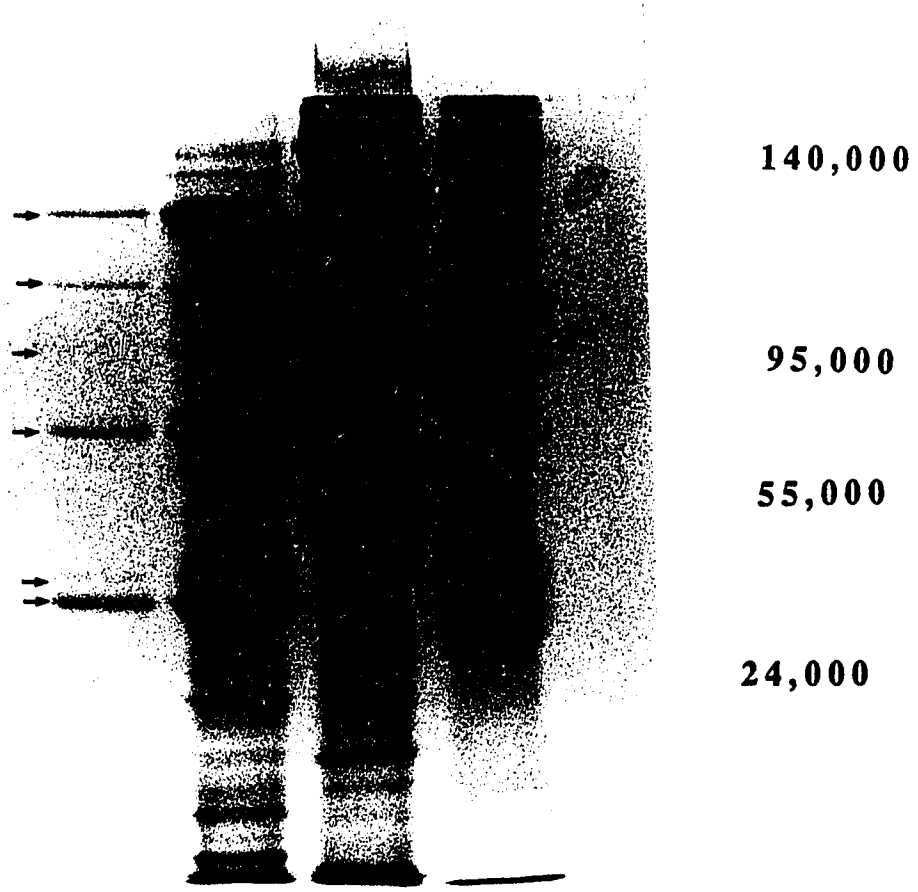
SDS and nondenaturing PAGE experiments

In all of the SDS PAGE experiments an anomalous 6 band pattern was observed. There were 5 major bands and a minor band which was not visible once the gels dried or if the protein loading was at a low level (Plate V-1). Resolution of the SDS pattern was not improved by utilizing gradient gel electrophoresis.

The TCA soluble nitrogen experiments performed on the purified enzyme seem to discount the possibility of protease contamination causing protein degradation and an increase in the number of observed bands due to proteolysis. Resolution of the bands was not improved whether the gel was run in reducing or non-reducing sample buffer. It would be expected that if there were disproportionate molecular weight fragments attached by sulfhydryl bonds or if the protein units were of the same molecular weight, that there would be the appearance of new bands of higher molecular weight, in the non-reduced media and the concomitant disappearance of the lower molecular weight bands corresponding to the subunits. This type of SDS pattern was also observed in the SDS PAGE of the β-galactosidase from *K. fragilis* (Mahoney and Whitaker, 1978), a closely related organism to *K. marxianus*. These researchers had no definite explanation for this phenomena.

The 5 major bands and minor band all correspond to the major staining bands in the Maxilact L2000 preparation. The molecular

Plate V-1. SDS PAGE of Maxilact and β-galactosidase



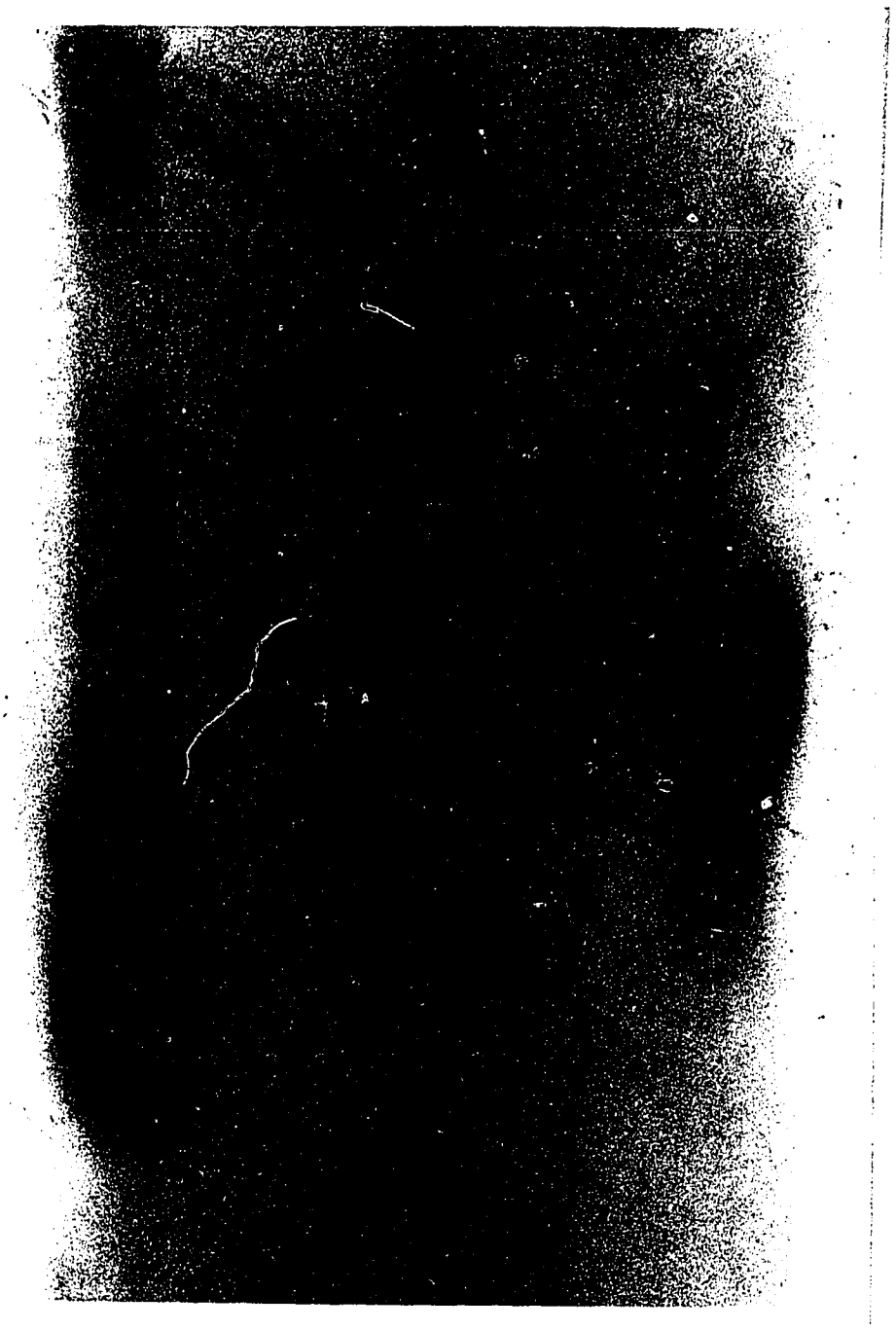
Lane 1. β-galactosidase
Lane 2. Maxilact L2000
Lane 3. Bio Rad High Molecular Weight Standards
Lane 4. Bio Rad Pre-Stained Standards

weights of the bands, from the highest molecular weight (the top of the gel) to the lowest was as follows: 125,000; 104,000; 99,000 (the minor band); 55,000; 36,500; and 34,500 daltons. The error in dupicate analyses of these bands was less than 10 %. It could be suggested from the SDS PAGE pattern that the protein consisted of 2 subunits of approximately 125,000 molecular weight and that the subunits readily dissociate into smaller fragments. This SDS pattern was different than that reported by Dickson et al. (1979). They report that the protein stained as one major band on 3 % SDS PAGE with a molecular weight of 135,000 $\pm$ 10 %. This difference could be due to the strain of *K. marxianus* used in the experiments.

Under non-denaturing conditions the Maxilact L2000 preparation ran as four distinct bands with the major band consisting of two discernable regions. The purified β-galactosidase ran as two bands, one major band and a barely discernable second minor band (Plate V-2). Both of these bands represented the major bands in the Maxilact preparation and both stained for activity when tested with ONPG. The third lane on the figure gives the result for the molecular weight standards used.

The 2 bands of major protein staining in the Maxilact preparation are the only areas staining for activity by the ONPG activity assay. These two bands of protein with activity in both samples suggests that there may be an isozyme of β-galactosidase present in the Maxilact L2000. This isozyme could be of a different molecular weight because of differences in the primary structure or it may migrate differently because of a variation in glycosylation.

Plate V-2. Non-denaturing PAGE of Maxilact and β-galactosidase



Lane 1. Bio Rad High Molecular Weight Standards
Lane 2. Maxilact L2000
Lane 3. β-galactosidase

This could perhaps be another explanation for the anomalous SDS PAGE patterns.

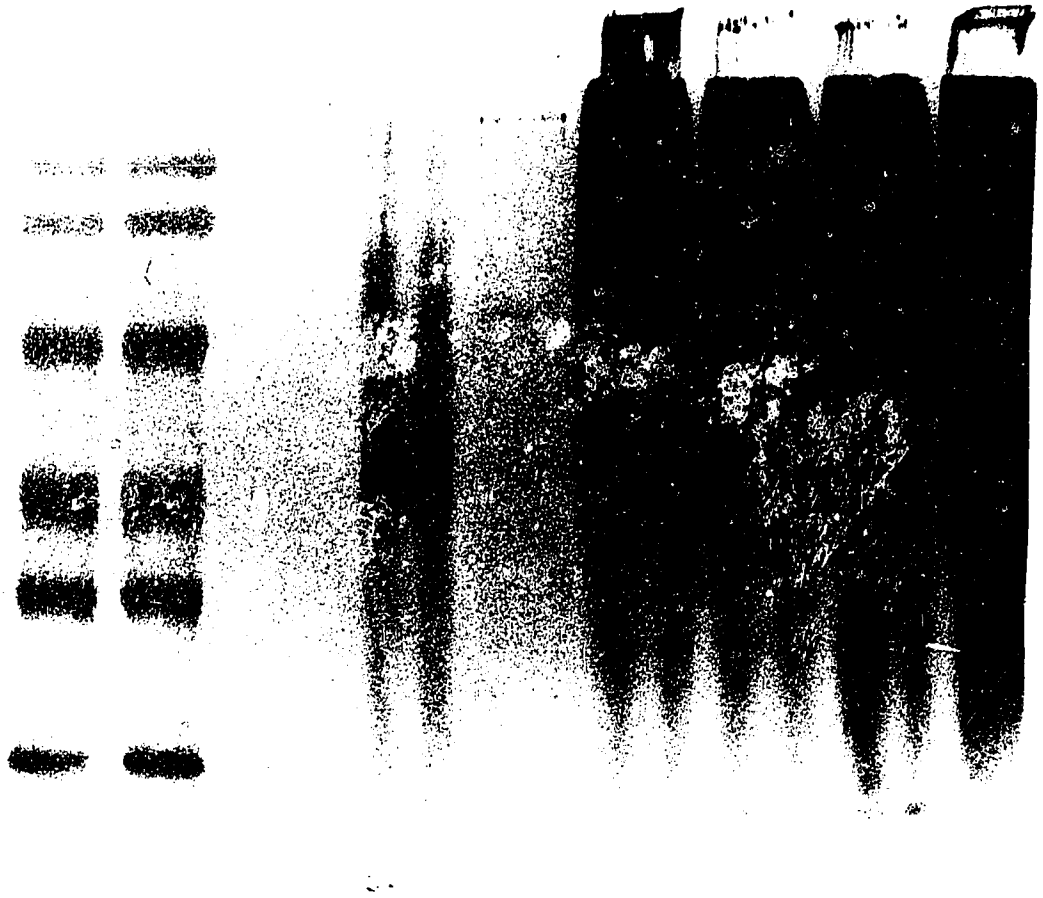
Glycoprotein determination

During the partial characterization of this enzyme it was determined that a knowledge of the glycosylation of the protein would be of interest to researchers in this field. The Boehringer Mannheim Glycan detection kit was therefore used for determination of the glycosylation of the enzyme.

The western blot of the SDS PAGE electrophoresed sample, when subjected to the experimental protocol, clearly showed the presence of carbohydrate on all but one of the 5 major protein staining bands (Plate V-3). There was a molecular weight shift upon attachment of the antibody and the exact band that did not contain carbohydrate could not be determined. It appeared that the band that did not stain for carbohydrate was one of the two lower molecular weight protein bands and it was possible that an explanation for the difference in mobility of these two bands, similar in molecular weight, was caused by glycosylation of one protein unit while the other remained non-glycosylated.

The only protein bands staining for carbohydrate in the Maxilact preparation were those corresponding to the β-galactosidase. This suggested that an affinity chromatography procedure, utilizing the proper lectin, could be of use in the purification of the enzyme from Maxilact L2000 or perhaps even from the organism itself.

Plate V-3. Glycan determination



Lanes 1 and 2.	Bio Rad Pre-Stained Standards
Lane 3.	Fibrinogen
Lane 4.	Maxilact L2000 (2.5 μg loading)
Lanes 5 to 8.	β-galactosidase (2.5, 5, 10, and 20 μg loadings)

pH effect on enzyme activity.

β-galactosidase from *E. coli* has been shown to have maximal activity at pH 7.2 (Kuby and Lardy, 1953) and to be stable in the pH range 6 to 8. The β-galactosidase from *K. marxianus* has been shown to have a pH optimum of pH 7.2 in 0.1 M phosphate buffer (Biermann and Glantz, 1968) and pH 7.0 in phosphate buffer (Hussein et al. 1989). The pH optimum for the purified enzyme from Maxilact L2000 in 50 mM phosphate buffer is pH 7.2 (Figure V-8). The enzyme appears to be more active in alkaline than acidic conditions although this may be only an indication of the relative activities in phosphate and acetate buffer.

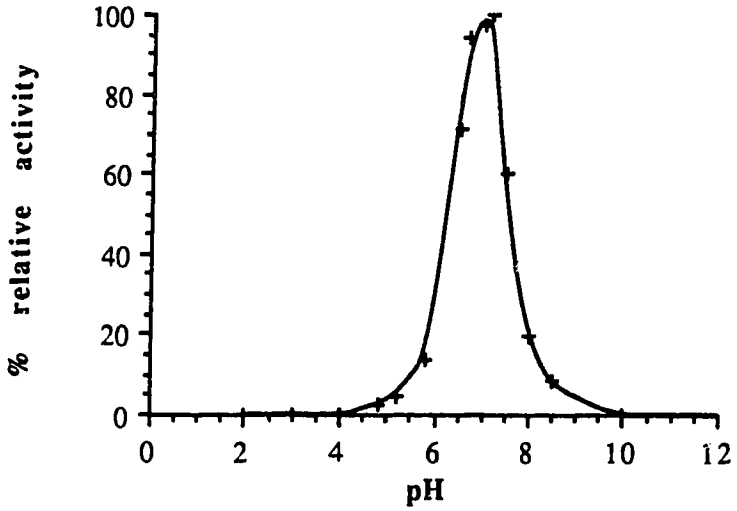
Dialysis and lyopholization of the enzyme

The dialysis of the enzyme against water was carried out according to the experimental protocol. After dialysis was complete the contents of the dialysis bag were carefully transferred to a screw top vial and stored at 4 °C. The dialysed enzyme was inactive against ONPG even after 30 min incubation at 37° C. It appears that the enzyme is either denatured or rendered inactive by loss of an essential co-factor by dialysis. Addition of magnesium or manganese up to an addition level of 0.01 M did not restore enzyme activity.

Temperature effects on the activity of the enzyme

The enzyme was reasonably stable up to a temperature of 47 °C. After this point, denaturation was quite rapid and at 60 °C there was less than 1 % discernable activity remaining after 2 min. incubation. Mahoney and Whitaker (1979) have reported that the

Figure V-8. pH versus activity of β-galactosidase

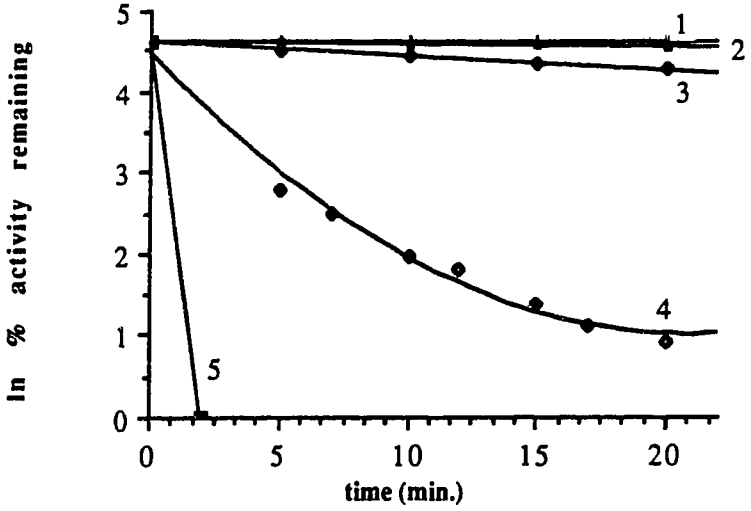


rate of inactivation of the β-galactosidase from *K. fragilis* is rapid after 40 °C.

It has been reported that the thermostability of the enzyme from *K. marxianus* is increased as much as 50 times in milk over phosphate buffered controls (Mahoney and Wilder, 1988). The results reported in this paper are for enzyme in phosphate buffer and would therefore differ from those reported in the above research. Also, the results presented here are for ONPG and would be different than those obtained if lactose were the substrate, as it was in the research conducted by Mahoney and Wilder (1988)..

The inactivation rate appeared to be first order at temperatures of 22.5 to 41 °C by graphical representation (Figure V-9). The line for 60 °C was not extended past zero, for values of remaining activity less than 1 %. At 49 °C there was a definite break in the line when time of incubation was plotted against the natural logarithm of the remaining activity. At 49 °C it appeared that there was a rapid denaturation of over 90 % of the enzyme activity. This denaturation appeared to be first order and the slower denaturation of the remaining activity also appeared first order in nature but of a different magnitude. Whether this represented isozymes of differing thermal stability or an inherent physical property of the enzyme could not be conclusively demonstrated by this experiment. It has been reported in the literature (Mahoney and Wilder, 1988) that there were isozymes of the β-galactosidase in the lactase preparation from *K. marxianus* and that they had differing thermal stabilities in milk. The authors did not report a differing thermal stability for the isozymes in buffer. This could be due to the method they used to

Figure V-9. Temperature dependent denaturation of purified β-galactosidase



1. 22.5 °C
2. 37 °C
3. 41. °C
4. 49 °C
5. 60 °C

determine activity or a difference in the preparation studied by those researchers from the one reported here. The non denaturing PAGE of the purified enzyme indicated isozyme activity so it was not unlikely that the explanation for this result was that there was an isozyme of the enzyme of differing thermal stability in the purified preparation.

Cation effects on activity of the enzyme

The effects of some monovalent and divalent cations on the activity of β-galactosidase from *K. marxianus* are summarised in Table V-4. The controls used were enzyme in 50 mM pottasium phosphate at pH 6.74 and enzyme in 50 mM Tris pH 6.74, dependent upon the buffer being studied.

In Tris buffer the effect of the monovalent ions sodium (Na^+) and potassium (K^+) were different. Activation by the sodium cation could not be determined within the limits of experimental error. At high levels of potassium (10^{-3} M or greater), this cation was inhibitory to enzyme action.

For the divalent ions studied it was found that calcium (Ca^{++}) was moderately inhibitory while mercury (Hg^{++}) completely inhibited the enzyme at 10^{-4} M. Magnesium (Mg^{++}) and manganese (Mn^{++}) were both activating to the enzyme activity at relatively low levels (10^{-5} M) with manganese having a much greater effect than magnesium.

In phosphate buffer a mild activation was observed by sodium at 5×10^{-4} M but at higher concentrations it began to inhibit enzyme

Table V-4. Cation Effects.

Cation M	Tris [1]	KPO_4 [2]
Na^+ 5×10^{-6}	------	100
5×10^{-5}	98	102
5×10^{-4}	105	104
5×10^{-3}	100	62
10^{-2}	99	-----
K^+ 10^{-6}	100	---
10^{-5}	99	---
10^{-4}	99	---
5×10^{-4}	75	---
10^{-3}	56	---
2×10^{-2}	3	---
Ca^{++} 10^{-6}	100	100
5×10^{-5}	---	101
10^{-4}	100	108
10^{-3}	93	108
10^{-2}	62	---
10^{-1}	ppte.	---
Mg^{++} 10^{-7}	---	100
10^{-6}	100	104
10^{-5}	108	113
10^{-4}	117	145
10^{-3}	142	190
10^{-2}	132	182
5×10^{-2}	131	---
10^{-1}	30	---
Mn^{++} 10^{-7}	101	101
10^{-6}	125	111
10^{-5}	155	130
10^{-4}	170	141
10^{-3}	130	139
Hg^{++} 10^{-11}	---	100

10^{-10}	100	90
10^{-9}	99	81
10^{-7}	93	77
10^{-6}	79	31
10^{-5}	11	1
10^{-4}	0	0

1. Relative to Tris 50 mM, pH 6.74, no added cations
2. Relative to KPO_4 50 mM, pH 6.74, no added cations

activity. The divalent cation calcium was not inhibitory at the levels of addition that were possible to study. At levels higher than 10^{-3} M calcium caused precipitation in the phosphate buffer and the activity of the enzyme could not be spectrophotometrically determined. In phosphate buffer mercury was even more inhibitory than in Tris buffer, with almost all activity being inhibited at 10^{-5} M. As seen for Tris buffer, both magnesium and manganese cations activated the enzyme at lower levels of addition. In both buffers the maximal activation was at the same level. Analagous results have been obtained for the β-galactosidase from *K. fragilis* (Mahoney and Whitaker, 1978). In their work a much greater magnitude of activation was reported for magnesium and manganese cation than that reported here, yet maximal activation was seen at relatively the same levels.

The effect of the potassium cation, in 50 mM sodium phosphate buffer, pH 6.74, is summarised in Table V-5. At high levels of potassium addition, 250 mM and greater, substantial inhibition of the enzyme began to be observed. There was no attempt to stabilize the ionic strength of the buffer with another non-reactive ion. The ionic strength of the solution could therefore be responsible for the observed effect. Jacober-Pivarnik and Rand (1984) have reported that commercially obtained enzyme preparations of *K. marxianus* were stimulated by the addition of 36 mM KCl. These researchers saw stimulation of the β-galactosidase in Maxilact L2000 up to as high a level of potassium addition as 1.26 M. At levels of addition that were this high, the effects of buffer ionic strength could not be discounted. The assay utilized by these researchers was not the ONPG

Table V-5. K^+ Effect.

K^+ mM[1]	Relative activity[2]
500	39
250	74
100	90
10	99
1	92

1. mM KCl added to 50 mM $NaPO_4$ buffer pH 6.74

2. Relative to the activity in buffer with no added KCl

assay, but a milk assay and this may in itself have accounted for the differences in data. Mahoney and Whitaker (1977) have reported an effect similar to that presented in this paper, of enzyme inhibition at higher levels of potassium addition to sodium phosphate buffer, for the enzyme from *K. fragilis*.

To achieve maximal activity of the enzyme, both sodium and manganese must be present in the buffer. Reithel and Kim (1960) have shown that both magnesium and sodium must be present in the buffer for maximal activity of the *E. coli* enzyme. Kuby and Lardy (1953) have reported that activation of the *E. coli* β-galactosidase is achieved by the divalent cations manganese and magnesium.

Effect of buffers on activity

The relative activity of β-galactosidase in different buffers is reported in Table V-6. The Tris buffer was inhibitory relative to potassium phosphate buffer, while sodium phosphate buffer was activating. Mahoney and Whitaker (1977) reported that Tris buffer was activating under these circumstances for the enzyme from *K. fragilis*.

Effect of dithiothreitol (DTT) and ethylenediamine tetraacetic acid (EDTA) on activity

At all levels studied dithiothreitol (DTT) was slightly activating for ONPG hydrolysis. There was not, however, a marked increase in activity (Table V-7).

EDTA, a metal chelator, might be expected from the cation experiments reported earlier, to have an inhibitory effect upon the

Table V-6. Buffer Effect on Enzyme Activity

Buffer[1]	Relative Activity[2]
KPO_4	100
$NaPO_4$	175
Tris·HCl	35

1. 0.2 M pH 6.74 for all buffers
2. Relative to 0.2 M KPO_4 buffer, pH 6.74

Table V-7. DTT Effect

DTT M[1]	Relative Activity[2]
2×10^{-6}	108
2×10^{-5}	108
2×10^{-4}	108
2×10^{-3}	107

1. DTT added to 0.2 M $NaPO_4$ buffer, pH 6.74
2. Relative to buffer with no added DTT

Table V-8. Effect of EDTA

EDTA mM[1]	Relative Activity[2]
1.0	79
0.1	90
0.01	100

1. EDTA added to 0.2 M $NaPO_4$ buffer, pH 6.74
2. Relative to buffer with no added EDTA

enzyme activity. However, against a control containing no divalent catons, the effect of EDTA was only slightly inhibitory (Table V-8).

PCMB Titration

The β-galactosidase enzyme, purified from Maxilact L2000, was titrated with PCMB to determine both titratable cysteines and the effect this compound would have upon enzymatic activity.

There are very few titrable cysteines in this enzyme and the low titration values encountered lead to a possibility of error in the titration. However, repetitive experiments with different lots of the lyopholized enzyme and different PCMB titrants gave results of the same titer. After calculation of the titratable cysteines, the moles of cysteine that were titrated with PCMB were 2.04 $\pm$ 0.09 moles per mole of enzyme. The molecular weight of the enzyme used for these determinations was 270,000 (Dickson et al. 1979). Only 2 titratable cysteines was less than the value of 5 reported for *K. fragilis* β-galactosidase (Mahoney and Whitaker,1978).

An interesting result was obtained from the comparison of the PCMB titer against remaining activity. At the same level of PCMB addition that fully titrates the enzyme there was still 84 % enzyme activity remaining. This appeared to discount the direct involvement of cysteines in the active site of the enzyme. At higher levels of PCMB addition there was marked inhibition of the enzyme (Table V-9). It could be that a higher level of PCMB, and at the levels of mercury seen to inhibit the enzyme, the cysteines that are disrupted have to do with protein structure. It is possible that as structure was

Table V-9. PCMB Titration

µL PCMB[1]	Activity Remaining[2]
1	100
2	90
3	88
4	84
5	80
7	71
10	55
100	8

1. Added to the enzyme (concentration of 2.4×10^{-9} moles/mL in $NaPO_4$ buffer, pH 6.74)

2. Measured against a control with no added PCMB.

lost because of the interaction with ionic mercury or PCMB, the enzyme activity was affected.

Amino acid composition of β-galactosidase

The amino acid composition of the β-galactosidase is given in Table V-10. The amino acid values were calculated by the procedure of Moore and Stein (1963). The cysteine value reported was determined from the 24 h hydrolysis phenyl-isothiocyanate (PITC) determination and not a performic acid oxidation. For this reason it could be assumed that it was a lower value than would be determined by performic acid oxidation and subsequent determinaton of the cysteine. Tryptophan was determined spectrophotometrically by the method of Beaven and Holiday (1952).

The molecular weight calculated from these data for the β-galactosidase from *K. marxianus* would be 252,900 ± 8 %. This was in agreement with the molecular weight postulated by Dickson et al. (1979) of 270,000 ± 10 %.

CONCLUSIONS

The experimental work conducted on the enzyme purified from Maxilact L2000 has yeilded information not before known about the enzyme and its catalytic activity. Of a more academic than practical usage is the determination of the inhibition constants of the synthetic carbohydrates, galactonolactone, galactosamine and PAPTG. The low inhibition constant for PAPTG (15.17 mM) for this enzyme would probably mean that PAPTG affinity chromatography would not be of

Table V-10.
Amino Acid Composition of β-Galactosidase from *K. marxianus*. [1]

Amino Acid	Average[2]
Asx	284
Glx	253
Pro	132
Gly	167
Ala	117
Val	148
Leu	152
Tyr	107
Phe	105
His	72
Lys	140
Arg	63
Met	14
Ser[3]	185
Thr[3]	212
Cys	7
Ile	140
Trp[4]	7

1. number of residues per mole of protein.
2. Average of determinations at 24, 48, 75, and 96 hours. Reported as interger value
3. Determined by extrapolation to zero time.
4. Determined spectrophotometrically.

much use as a new method of enzyme isolation. However, due to the fact that the enzyme is a glycoprotein (and apparently the only one in the Maxilact L2000 preparation) there is a possibility that lectin affinity chromatography could be used, perhaps even upon cell free extracts directly from the organism, to isolate the enzyme. This may prove to be of use industrially. The need for further purification of the enzyme was shown from the large degree of protease contamination of the commercial preparation and the other carbohydrase activities in the Maxilact.

The PCMB titration data would seem to discount an active site model requiring a free cysteine and would seem to favor the more modern interpretation of β-galactosidase activity proposed by Herchen and Legler (1984) of an acid base mechanism (see introduction to this thesis). This opens up the possibility of very fruitful research into the mechanism of action, both hydrolase and transgalactosylase, of the yeast β-galactosidases. The gross amino acid structure of the enzyme is reported for the first time and is the first step that must be taken if further sequencing work to continue. The eventual sequencing of this enzyme, along with the data on buffer, cation, and inhibitor effects, could give enzyme engineers a handle on designing point mutations to make a more thermally stable or more hydrolase (or transgalactosylase) active enzyme.

The basic research conducted here not only gives the researcher a better feel for the enzyme he is working with it also has some practical applications to industry, suggesting new ways of isolating the enzyme, discovering the possibilty of two isozymes of differing thermal stability in the preparation, and determining the

ionic conditions most conducive to enzyme activity. It has also opened up many new avenues of research to pursue. These include a study of the active site residues of the enzyme and pursual of the possibility of purifying the enzyme with lectin affinity chromatography.

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Transgalactosylation products from the transferase enzyme activity of Maxilact β-galactosidase

INTRODUCTION

β-galactosidase (E.C. 3.2.1.32) is known to have transferase as well as hydrolase activity (Walsh, 1979, Huber et al., 1976). In *E. coli* this activity is responsible for the production of allolactose (β-D-galactopyranosyl (1->6) D-glucopyranose), the natural promoter of the lac operon (Walsh, 1979, Huber et al., 1976). This transferase activity is believed to occur because the free galactosyl unit in the enzyme active site (some researchers postulate that it may be bound covalently to the enzyme [Walsh, 1979]) can be transferred to any hydroxyl acceptor. The acceptor can be water with the production of glucose and galactose (during lactose hydrolysis) or another carbohydrate with the production of an oligosaccharide and free glucose. The latter activity is referred to as transferase or transgalactosylation activity (Walsh, 1979). The production of di, tri and higher linked oligosaccharides has been noted in the literature for the β-galactosidase from *K. marxianus* (Asp et al, 1980, Jeon and Mantha, 1985, Burvall et al, 1980) as well as from a number of other other sources (Huber et al, 1976, Toba and Adachi, 1978, Nilsson, 1988, Ajisaka et al, 1988, Greenberg and Mahoney, 1983).

There is a two-fold interest in the study of these transgalactosylation products. First, they may have nutritional significance in milk products that have been treated with the enzyme

(Toba and Adachi, 1978, Mahoney, 1985). Secondly, that transferase enzyme activity may be exploited to produce complex oligosaccharides of a medical or biological significance in a more cost effective manner than complex and lengthy protective group synthesis methods (Nilsson,1988, Ajisaka and Fujimoto, 1989).

An HPLC method has been used to monitor the transgalactosylation reaction (Jeon and Mantha, 1985) of β-galactosidase from *K. marxianus* but it suffered from the low sensitivity of the detection method used, refractive index (RI), and from incomplete separation of the carbohydrates. In this study no attempt was made to characterize the oligosaccharides that were produced other than to classify them as di or trisaccharides based upon their retention behavior. Gas chromatography (GC) has been effectively used to separate the minor oligosaccharides in honey using persilylation, temperature programming and flame ionization detection (FID) (Low, 1986). The sensitivity in this study was at least one order of magnitude better than RI detection and there was excellent separation of compounds differing by only the position of the glycosidic linkage. It was therefore decided to study the production of transgalactosylation disaccharides by *K. marxianus* β-galactosidase by persilylation GC. Preliminary results with lactose hydrolysis gave 7 discernable GC peaks in the area where disaccharides would be expected to elute. To simplify the analysis and identification of these disaccharides it was decided to study only the production of galactose disaccharides by the enzymes transferase activity. By using only the monosaccharide galactose as a carbohydrate in the reaction mixture, only galactose-galactose

disaccharides could be produced. It was believed that this would make identification of the disaccharides simpler.

It is well known that enzyme rates are affected by temperature (Walsh, 1979, Whitaker, 1972) and that product ratios (if there are byproducts of the reaction) can be affected under different temperature conditions. For this reason the enzymatic production of transferase products was studied at the enzyme optimal temperature for hydrolysis (37 °C, Gist-Brocades, 1988) as well as temperatures both above and below this value. Olano et al (1983) have also reported a number of other enzyme activities in a commercial *K. marxianus* β-galactosidase preparation (Maxilact) and these other activities were confirmed in Chapter 5 of this thesis. To ensure that only β-galactosidase transferase products were being studied the disaccharide production of the purified enzyme was compared to the production from Maxilact L2000. Both enzymes were studied at the same temperatures and levels of enzyme addition.

MATERIALS AND METHODS

Maxilact L2000 was a gift from Gist-Brocades (Charlotteville, NC). Purified β-galactosidase was isolated from Maxilact as stated in Chapter 5. All water used was Milli-Q (Millipore, Bedford, MA) quality. All reagents were reagent grade or better.

Enzymatic production of galactose disaccharides

A 20 percent (w/v) solution of galactose ($C_6H_{12}O_6$) (1.11 M) was prepared in pH 7.0 sodium phosphate ($NaPO_4$) buffer, containing 0.2 % (w/v) sodium azide (NaN_3). This solution was pretempered to 4, 37 or 50 °C dependent upon the experiment. Solutions were either pretempered in a refrigerator (4 °C) or in a Haake Thermomix 1420 temperature controlled waterbath (Haake, W. Germany) at the stated temperature. The total volume of each solution used for the experiments was 30 mL. To each solution 60 units (1 unit = 1 μmole ONPG hydrolyzed per minute at 37 °C) of either purified β-galactosidase (Chapter 5) or Maxilact L2000 were added. This level of enzyme addition approximates the level of enzyme used in batch hydrolyses of lactose in milk (Gekas and Lopez-Leiva, 1985). Immediately after addition of enzyme the solution was sampled to give a zero time indication.

The solutions were stirred gently throughout the time course of the experiment by magnetic stirrers (Ika Werke, Janke and Kunkel GMBH, Staufen. W. Germany). Each 1 mL sample taken from the solution was sealed and placed in a boiling water bath for 5 minutes. A test solution of buffer attained an internal temperature of 70 °C within 1 minute of immersion, a temperature high enough to cause complete thermal denaturation of the enzyme within this time (Chapter 4). After boiling each sample was labelled and frozen at -20 °C until needed for workup for gas chromatography (GC).

The sampling scheme utilized for each experiment was different. At 4 °C samples were taken at zero time, 1, 2, and 3 hours and a final sample taken at 24 hours. At 37 °C samples were taken

every fifteen minutes for the first hour (as well as a zero time sample), every thirty minutes from 1 hour until 3 hours of incubation, at the 4, 5, and 6 hour times of incubation and a final sample at 24 hours of incubation. The 50 °C experiment was sampled every fifteen minutes for 1 hour.

Sodium borohydride reduction of carbohydrate standards

Sucrose (10 mg, 0.029 mmoles) was dissolved in 5 mL of Milli-Q water. To this solution 50 ± 5 mg of sodium borohydride ($NaBH_4$) was added to give a 45 fold molar excess of borohydride to sucrose. Sucrose is a non-reducing sugar and needs no reduction to simplify the GC chromatogram. However the addition of $NaBH_4$ was conducted to ensure that sample and standard treatment were identical.

The above solution was left at room temperature for one hour with gentle stirring and then excess $NaBH_4$ was destroyed by the dropwise addition of 10 % acetic acid (Borchardt and Piper, 1970). To remove sodium ions, which would interfere later in the procedure, the solution was quickly passed over a Baker C267 H^+ column (15 mL, 86 mEq). From GC analysis it could be seen that this procedure did not cause hydrolysis of the acid labile glycosidic linkage in sucrose. Sucrose recovery varied from 97 to 102 %, as determined by both weighing of pre-dried, pre-weighed flasks after sucrose recovery and GC analysis.

After the column procedure the solution was evaporated to dryness (Buchi Rotovapor R, Buchi, Flawil, Switzerland) with a temperature reaching no higher than 40 °C during the evaporation.

The dry, white solid was then dissolved in methanol (10 to 15 mL) and again evaporated to dryness. This methanol treatment was repeated 4 to 6 times to leave a clear oil in the flask. This oil was dissolved in a 50:50 mixture of methanol - toluene and the solvent evaporated. A faint yellow white solid or oil (depending upon the experiment, in the case of sucrose it was a solid) remained coating the walls of the flask. This oil or solid was the sample that was silylated for GC analysis.

The galactose disaccharides are, with the exception of β-D-galactopyanosyl (1->6) D-galactopyranose (galactobiose), unavailable commercially. The available disaccharide, galactobiose, is available only in limited quantities and at very high cost. This makes it prohibitive to use as a GC standard. For this reason the disaccharide lactose was used as a standard to test the linearity of the flame ionization response (FID) to a reducing disaccharide and to compare the integrated values for equal concentrations of lactose and sucrose.

The lactose sample (10 mg, 0.029 moles, dissolved in 5 mL water) was treated in exactly the same manner as sucrose. The end product of this treatment is lactitol, the reduced form of lactose. This alditol will give only one peak in GC analysis (Borchardt and Piper, 1970, Geyer et al, 1983, Lee et al, 1984).

Silylation of carbohydrate standards

The samples of sucrose and lactitol were subjected to a silylating procedure that was a slight modification of the procedure accompanying Tri-Sil Z reagent (Pierce Chemical Co., 1989). The manufacturer of this reagent suggests silylating up to 10 mg of

carbohydrate with 1 mL of the reagent at room temperature for 5 to 10 minutes. These conditions did not give complete silylation of sucrose and the disaccharide alditols observed in this study. This effect has been noted before in the literature (Lee et al, 1984, Borchardt and Piper, 1970).

A series of conditions, varying time and temperature of silylation, were studied and it was determined that the optimal time/temperature ratio to ensure complete silylation of the disaccharides was 10 minutes at 90 °C. Longer times at this temperature did not have any effect upon the chromatograms.

Therefore the silylation procedure used in this study was as follows. The sugar alditols were stored in serum capped small round bottomed flasks at 4 °C until needed. To silylate these samples they were removed from the refridgerator and left at room temperature for 10 to 20 minutes. Tri Sil Z (1 mL) was added through the septum cap by means of a gas tight syringe (Hamilton, Reno, NV). The samples were gently rotated by hand to ensure that all of the sugar alditol was brought into the pyridine solvent. In some, but not all cases, the oil did dissolve at this stage but in most determinations the sugar did not dissolve in the pyridine before it was at least partially silylated. The samples were then placed in a peanut oil bath held at 90 °C by a stabilizing thermometer (Janke and Kunkel GMbH, Staufen, W. Germany) connected to an Ika Combimag hot plate/stirrer (Janke and Kunkel GMBH, Staufen, W. Germany) and left for 10 minutes. After 5 minutes of reaction the samples were gently rotated to ensure dissolution of all the sugar alditol and placed back in the bath. Upon removal from the oil bath the samples were

further sealed with parafilm and placed at 4 °C or used immediately for GC analysis. Under these storage conditions there was no appreciable breakdown of the samples for as long as one month storage. If the samples were not sealed with parafilm there was the possibility that the septa would not properly reseal and under the moist conditions of the fridge there would be breakdown of the persilylated sugars. This breakdown was noted in some samples that were not sealed in the above manner.

Standard curve for carbohydrate standards

The silylated samples of sucrose and lactitol (10 mg/mL concentration) were used, by serial dilution with Tri-Sil Z reagent, to construct a standard curve for each sugar from 0.1 to 3.33 mg/mL. At higher concentration levels the curve was not linear. Since lower concentrations were of more interest in this study it was decided to use only the lower linear portion of the curve. If a sample of higher concentration were encountered during the experiments it was diluted into the proper range for analysis.

These standard curves were then used in the quantification of disaccharides produced by the transgalactosylation reaction of β-galactosidase from Maxilact L2000 and enzyme purified from this source.

Preparation of enzyme treated samples for GC analysis

Reduction and silylation of 50 μL samples (1 mg galactose if the sample was only galactose) was conducted as above with the exception that 1 mL of a 1 mg/mL solution of sucrose was added to

the sample before $NaBH_4$ reduction of the sample. The sucrose was added as an internal standard for quantification of the disaccharides and because of the labile nature of the glycosidic bond of sucrose, to test for possible acid hydrolysis of the disaccharides somewhere in the procedure. This would be especially prevalent during the columning step.

Gas Chromatographic procedure

All GC runs were performed on a J and W Scientific 30 m. DB-5 glass capillary, surface coated column with a 0.25 μM film thickness (J and W Scientific, Folson, CA) except for test chromatograms on the same column in 60 m. format to ensure that there was separation of all possible disaccharides. The gas chromatograph utilized was a Varian 3700 (Varian, Georgetown, Ontario) in temperature program mode. Injections were split at a splitter ratio of 30:1 through a fritted glass splitter insert (Varian, Pao Alto, CA). Flow rate in the column (at 215 °C) was 0.13m/sec. The carrier gas was UHP Helium (Linde, Edmonton, AB) passed through an oxygen scrubber (Chromatographic Specialities, Toronto, ON). The injection port and detector were held at 300 °C. Split injection was necessary because of the low capacity of surface coated open tubular columns (SCOT) (Lee et al, 1984, Jennings, 1978). The flow rate was the optimal flow rate for the column as determined from a Jones-Van Deemter plot for the 30 m. column (Jennings, 1978, Jonsson, 1987). The splitter ratio used gave the best compromise between sensitivity and separation.

After prolonged testing the temperature program that was best for resolving the disaccharides and separating them well away from

monosaccharides was: 215 °C held for 10 minutes followed by a temperature ramp of 2 °C/min. until a final temperature of 295 °C was attained. Disaccharides were found to elute with temperatures between 260 and 290 °C with sucrose, the internal standard, eluting well before the galactose disaccharides.

To ensure that there was complete separation of all possible disaccharides the analysis was performed both isothermally and at a slower temperature ramp of 1 °C/min. Neither procedure improved the resolution of the peaks or increased the number of observed peaks. A third test was performed on a 60 m. DB-5 column. The optimal splitter and flow rates were determined for this column before comparisons were made to the data from the 30 m column. Both a 2 °C/min and a 1 °C/min temperature ramp were utilized upon samples that had been determined on the 30 m column. There was no increase in the number of observable peaks under these conditions.

In all cases studied there were five disaccharide peaks. Sucrose was the first eluting peak followed by 4 galactose disaccharides. All possible parameters to increase the resolution (length of the column, isothermal rather than temperature programmed elution, reduction of sample volume on the column) were attempted and no greater definition of the four galactose disaccharide peaks could be achieved. It was believed therefore that there were only four galactose disaccharides formed in both enzyme treatments.

Test of possible GC interferences

There was a possibility that one or more of the four observed disaccharides may have been a contaminant from some step of the workup and silylation of the samples. It was also possible that one of the variably occurring, non-integrating noise peaks in the 260 to 290 °C range of the chromatogram was actually a very minor disaccharide.

To test this possibility GC analyses were performed upon galactose with no enzyme treatment, enzyme, Tri-Sil Z heated for 10 minutes at 90 °C with no carbohydrate, and $NaBH_4$/ methanol/ toluene. The small non-integrating peaks in the chromatogram (variably present) were determined to be from the $NaBH_4$/ methanol/ toluene mixture so each was silylated alone, after evaporating to dryness for the liquids, and analysed by GC. The variably appearing peaks seemed to arise from the methanol but pre-distillation of the methanol before use did not remove them. The appearance of the peaks was dependent upon the volume of methanol used in the workup and in samples where smaller volumes of methanol were used the peaks did not appear.

Separation of transgalactosylation product disaccharides

Galactose (20.00 $\pm$ 0.01 g) was dissolved in 90 mL of pH 7.0 $NaPO_4$ buffer (0.05 M) and after dissolution was complete the solution was made up to 100 mL volume in a volumetric flask. This yielded a 20 % w/v solution of galactose. Purified enzyme (Chapter 4) was added to this solution (pre-tempered to 37 °C) at a level of 10

units/g of galactose. The solution was stirred gently for 3 hours and then placed in a boiling water bath until the temperature of the galactose solution was 85 °C (approximately 15 minutes) to destroy enzyme activity.

Two similar techniques of carbohydrate isolation were attempted. In the first method the 100 mL solution was added directly to a 70 by 2.6 cm column of activated carbon:Celite 545 1:1 (carbon was supplied by Atlas Chemical Co., Wilmington, DW; Celite 545 was a product of Johns Manville Chemical Division, supplied by Fisher, Edmonton, AB). The column was washed with water several times to remove monosaccharides (Siddiqui and Furgala, 1967). Washing was stopped when concentrated fractions were no longer positive to the Ce(IV) test (Chapter 3). The column was then washed with 20 % ethanol and 25 mL fractions collected. These samples were evaporated to dryness to ensure removal of ethanol, rehydrated with water to a volume no greater than 1 mL and tested for the presence of carbohydrates by the Ce(IV) test. Fractions that tested positve were rotovapped to dryness and taken up in fully deuterated water (D_2O) (Fisher, Edmonton, AB). High field nuclear magnetic resonance (NMR) spectra were collected, in ^1H mode, on these isolates. All NMRs' were performed on a Bruker 360 MHz FT-NMR (Fourier transform NMR) (Bruker, Switzerland). The majority of carbohydrate positive fractions were only galactose and those that did contain disaccharide were so heavily contaminated with galactose that no data could be obtained from the spectra.

The second isolation procedure attempted was a batch process of the carbon-celite method (Siddiqui and Furgala, 1967). Activated

carbon (25 g) and Celite 545 (25 g) were mixed as a water slurry. 10 mL of the enzyme treated solution was added and the solvent removed by vacuum filtration. Larger volumes caused unacceptable increases in time of filtration. The dried cake was resuspended in the minimum volume of water necessary to slurry it (50 mL). It was then stirred for 1 hour and the solvent again removed by vacuum filtration. This procedure was continued with water for five resuspensions past the point where concentrated fractions were no longer positive to Ce(IV).

The cake was then resuspended in 50 mL of 95 % ethanol and stirred for 1 hour before removal of the solvent by vacuum filtration. This was repeated, with each 50 mL wash being collected as a seperate fraction, for 10 washes. Each fraction was evaporated to dryness, dissolved in 1 mL of water and tested for the presence of carbohydrates by Ce(IV). Those fractions testing positive for carbohydrate (7) were then evaporated to dryness and dissolved in 1 ml of D_2O. NMRs' were performed on all of these samples.

The majority of samples from this isolation were again heavily contaminated with galactose (6) or were galactose alone (4) (because of the low production of disaccharides it was possible that these 4 samples also contained disaccharide but no peaks for disaccharide were noticeable in the galactose spectra). One sample, weighing 1.1 mg after drying over phosphorous pentoxide (P_2O_5), had only a very minor contamination of galactose. ^1H decoupling experiments were performed on this sample to identify the disaccharide. ^1H NMR was also performed on a standard of galactobiose purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

GC quantification of transgalactosylation disaccharides produced by purified β-galactosidase and Maxilact L2000 at 4 °C.

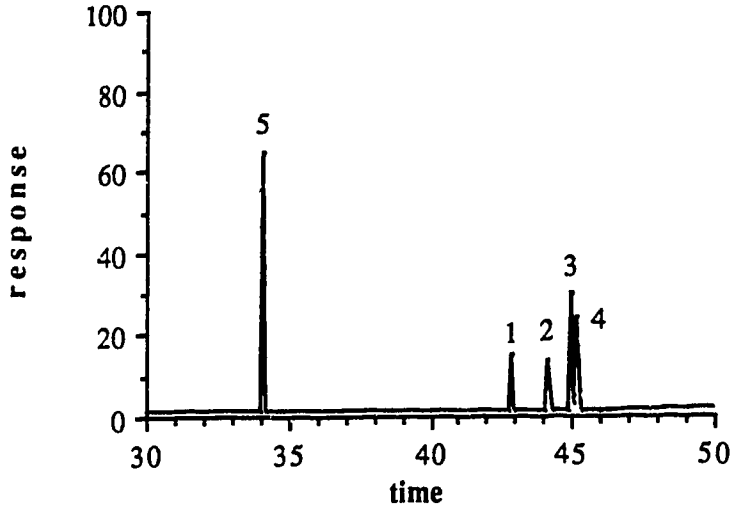
The experiments were prepared and sampled as stated in the materials and methods. At 4 °C it was decided to sample only at one hour intervals because of the decreased enzymatic rate at this temperature. Samples were taken for the first three hours and then at 24 hours. Industrially, milk is often lactose hydrolyzed in a batch process at 4 °C for 24 hours, thus the rationale for the final 24 hour sample (Mahoney, 1985, Kwak and Jeon, 1986). In this study both purified β-galactosidase (Chapter 4) and Maxilact L2000 were tested.

Each frozen sample was brought to room temperature and a 50 μL aliquot removed for production of the alditols and subsequent GC determination. At 4 °C for all samples there was production of 4 disaccharides at all times tested. For the sample that had been reacted with purified enzyme all 4 disaccharides were present in the first hour but two of the disaccharide peaks did not integrate.

The disaccharides were labelled 1, 2, 3, and 4 based upon their retention time relative to sucrose (Figure VI-1). The differences in retention times between these disaccharides and sucrose are as follows: disaccharide 1 elutes 8.80 ± 0.11 min. after sucrose; disaccharide 2 elutes 10.05 ± 0.06 min. after sucrose; disaccharide 3 elutes 10.90 ± 0.10 min. after sucrose and disaccharide 4 elutes 11.13 ± 0.05 min after sucrose. These peaks were so reproducible in their retention times that there was no overlap between peaks, even those as closely eluting as disaccharides 3 and 4. This allowed for

Figure VI-1

Gas Chromatograph trace of the disaccharide region



1. disaccharide 1
2. disaccharide 2 (β-D-galactopyranosyl (1->6) D-galactopyranose)
3. disaccharide 3
4. disaccharide 4
5. sucrose internal standard (1 mg/mL concentration)

identification of each peak even if all of the peaks had not integrated or were not present. As stated in the materials and methods section, the only commercially available galactose-galactose disaccharide is β-D-galactopyranosyl (1->6) D-galactopyranose (galactobiose). This compound (1.0 mg) was treated as stated for reduction and silylation and co-injected with sucrose upon the GC. The retention time difference between this known carbohydrate and sucrose was 10.05 ± 0.05 min. This corresponds in retention time to disaccharide 2. When this standard was co-injected with samples from the experimental run only disaccharide 2 increased in peak area. This evidence, though inconclusive because there were determinations only upon a column of one polarity (Geyer et al, 1983, Jennings, 1978, Lee et al, 1984), suggests that disaccharide peak 2 may be galactobiose.

Galactobiose has been identified as a transgalactosylation product of Maxilact L2000 (Asp et al, 1980) and as a product of the transferase activities of *Saccharomyces fragilis* (Pazur et al, 1958), *Streptococcus thermophilus* (Greenberg and Mahoney, 1983), *Aspergillus niger* and *Saccharomyces fragilis* (Toba and Adachi, 1978) and *Eschericia coli.* (Nilsson, 1987, Huber et al, 1976). It is not unlikely then that it would be identified. It is of interest that although Asp and co-workers (1980) identify carbohydrates of only β(1->6) linkage most of the other reports cited above identify carbohydrates with β(1->2), β(1->3), β(1->4), and β(1->6) glycosidic linkages. In this work the GC traces have 4 peaks in the area of disaccharide elution. To ensure that these 4 peaks were disaccharides a number of experiments were performed as controls.

The first control was to check that there had been complete silylation of the product. A sample that had given four peaks in the disaccharide area was taken and 1 mL more Tri-Sil Z was added. The solution was reheated for the appropriate time and re-injected. There was no decrease in the number of peaks, only the expected decrease (due to dilution) in the integration area of each peak. This eliminated the possibility of incomplete silylation causing an increase in the number of peaks.

The second control was to test that there had been complete reduction of the sample to alditols before injection. If reduction were incomplete as many as 5 peaks could be expected for each disaccharide (Angyal, 1984) and this would cause an increase in the number of observed disaccharides if it were assumed that each peak were one separate and distinct carbohydrate. A second 50 μL aliquot of a sample having 4 suspected disaccharide peaks was taken and treated with 500 mg (13.2 mmoles, a 2400 fold molar excess if all the carbohydrate were galactose) of $NaBH_4$ to ensure complete reduction of the disaccharides. After workup and silylation this sample gave 4 peaks corresponding to those seen earlier. This discounted the possibility of incomplete reduction causing an increase in the number of peaks.

The third control, as stated in the materials and methods section, was to test the solvents and reagents. All of the possible compounds that could lead to interferences were silylated and tested by GC. Tri-Sil Z was also tested alone, after heating, to see if there were any breakdown products that eluted in the range of interest. Only methanol gave reproducible noise peaks and none of these

integrated unless large amounts of methanol were tested. When these peaks did integrate they did so at a retention time difference from sucrose of 5.73 ± 0.11 min and 6.25 ± 0.09 min. These peaks did not interfere with any of the peaks corresponding to disaccharides 1 to 4.

The temperature range of interest for disaccharides is 260 to 290 °C, corresponding to times of 33 to 47 minutes. Tri Sil Z, even at very low attenuations (high sensitivity) did not have any peaks eluting from the column at temperatures above 235 °C. None of the other reagents or the enzyme itself had peaks eluting above this temperature.

These results would seem to indicate that these 4 peaks, eluting at the above mentioned times in the chromatogram, were disaccharides. This contradicts the work of Asp et al (1980). As stated in the introduction to this thesis there is some doubt as to the identification of the linkage position in the oligosaccharides identified by Asp and co-workers (1980). These researchers used permethylation analysis by GC to identify the linkage position but did not perform the analysis on two columns of differing polarity, nor did they perform co-injections of known methylated standards with their unknowns as is the procedure ussually adopted in the identification of complex carbohydrates (Jennings, 1978, Geyer et al, 1983). There is also some doubt attached to their contention that the enzyme has only transgalactosylation activity for the β(1->6) glycosidic linkage from the work of Huber et al (1976). The hydrolase activity of the enzyme is to break a β(1->4) glycosidic linkage and it therefore binds the disaccharide in such a way that

this can be accomplished (Huber et al, 1976, Walsh, 1979). It has been postulated, from kinetic studies (Huber et al, 1976) that this bond breakage is reversible. It could therefore be expected that two glycosidic linkages would be produced, the β(1->4) and the β(1->6), if not more. These researchers postulated the formation of these two glycosidic linkages and proved the formation of β(1->6) linkages. It is possible that the binding of galactose to the glucose binding site of the enzyme is such that only the 6 hydroxyl is available for transfer but this has not been noticed in the transferase activity of β-galactosidases from other yeasts (Toba and Adachi, 1978).

Jeon and Mantha (1985) have performed an HPLC study of the transgalactosylation action of β-galactosidase from *Kluyveromyces marxianus*, the source organism for Maxilact L2000, during lactose hydrolysis. In this study 5 oligosaccharides other than lactose were noted and 4 are, by retention time analysis in comparison to standards, disaccharides. The fifth oligosaccharide has a retention time, under the conditions these researchers used, of a trisaccharide. If only β(1->6) linkages could be formed by the transgalactosylation activity then only two other disaccharide peaks (as well as lactose) would be expected. These would be galactobiose and allolactose (β-D-galactopyranosyl (1->6) D-glucopyranose). However, these researchers note 4 disaccharide peaks. Under the conditions employed in HPLC there is only peak for each carbohydrate, not 5 or more as seen in GC (Jeon and Mantha, 1985). This data also disagrees with the work of Asp et al (1980) and though it does not report as many disaccharides as would be expected from the work conducted in this study (8 would be expected if galactose-glucose disaccharides

formed as readily as galactose-galactose disaccharides), this discrepancy may be due to the much reduced sensitivity of an RI detector in comparison to a FID. The HPLC method also had much reduced resolution in comparison to the GC method used here.

It was concluded therefore, from this study and after a study of the literature, that the β-galactosidase from *K. marxianus* produced 4 β linked galactose disaccharides at 4 °C and that these disaccharides were still detectable in solution after 24 hours of enzyme incubation. In the case of Maxilact L2000 it can not be conclusively demonstrated that all 4 of these disaccharides are β linked because of the α-galactosidase impurity in the Maxilact preparation (Chapter 4). There is a possibility that even though the 4 peaks noted in Maxilact treated samples were identical in retention time between samples treated with purified enzyme that an α linked galactose disaccharide elutes at the same temperature (temperature is equivalent to time in temperature programmed GC). This was however unlikely due to the high efficiency of the modern SCOT GC column (Jennings, 1978, Jonsson, 1987). The DB-5 column (a SCOT column) had an efficiency of 2100, as determined by Jones - Van Deemter analysis (Jennings, 1978).

It was also interesting to note that the maximally produced disaccharide under these conditions was not galactobiose (disaccharide 2) but rather the unidentified disaccharide 3. This occurred for both the purified enzyme and Maxilact (Table VI-1). All four disaccharides are produced and begin to decline slightly in quantitation in relatively the same proportions for purified enzyme

Table VI-1

Quantity of disaccharides produced by β-galactosidase and Maxilact L2000 at 4 °C

Disaccharide	Time (hr)[1]			
	1	2	3	24
1 (Maxilact)	0.29	0.30	0.30	0.29
1.(Purified)	n.i.[2]	0.30	0.31	0.31
2.(Maxilact)	0.30	0.31	0.33	0.31
2.(Purified)	n.i.[2]	0.31	0.33	0.31.
3.(Maxilact)	0.35	0.37	0.37	0.35
3.(Purified)	0.33	0.36	0.38	0.37
4.(Maxilact)	0.33	0.34	0.34	0.33
4.(Purified)	0.31	0.33	0.35	0.34

1. mg/mL concentration, as determined by GC

2. n.i. = peak visible in chromatograms but did not integrate

and for Maxilact treated galactose solutions. Disaccharide 3 is always produced in the highest amount at all times studied.

For both enzyme sources the maximal production of disaccharides from a 20 % w/v galactose solution at 4 °C occurred after 3 hours (Figure VI-2). It was possible, because of the lack of later data points, that this maxima could have been shifted to a later time. The maximal production of oligosaccharides, based upon molar yield (1.11 mmoles/mL of disaccharide would then be 100 % yeild because 200 mg/mL galactose is 1.11 mmoles/mL) was 0.36 % for Maxilact L2000 and the same number - 0.36 % - for the purified enzyme. There was no difference in the production of total oligosaccharides by the purified enzyme or Maxilact L2000 at 4 °C. Each sample was injected at least in duplicate (most were triplicate analyses) and the quantity of each disaccharide determined by comparison with the standard curve for lactose. The equations of the lines for the standard curves were as follows:

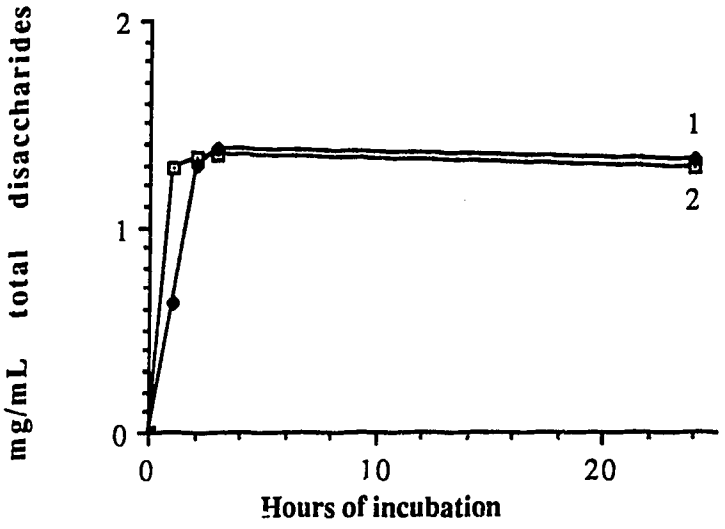
$$\text{sucrose:} \quad y = -0.14 + 4.67*10^{-4}\,x$$

$$\text{lactose:} \quad y = 0.25 + 3.54*10^{-4}\,x$$

where y is concentration of the disaccharide and x is integration area of the peak. The correlation coefficient for the sucrose standard curve is 0.99 and for the lactose standard curve is 0.98 (Figure VI-3). Reduced and silylated samples that had a higher discrepancy than 10 % between the expected sucrose recovery and that observed were discarded and the analysis repeated. The error in replicate GC analyses of standard samples was 10 %, therefore the rationale of using this value as a cutoff.

Figure VI-2

Production of total disaccharides at 4 °C by purified β-galactosidase and Maxilact L2000



1. Disaccharide production by purified β-galactosidase
2. Disaccharide production by Maxilact L2000

After 24 hours of incubation at 4 °C all 4 disaccharides remained in both experiments. The total oligosaccharide yield (molar basis) after 24 hours was 0.33 % for Maxilact L2000 and 0.34 % for the purified enzyme. This was a very slight difference but it was not significant at the levels of error (± 0.03 %) attached to these two values.

There were a number of conclusions that could be drawn from this series of experiments. First, that at 4 °C there is no difference in enzymatic production of transgalactosylation disaccharides between Maxilact L2000 and the β-galactosidase isolated from it. There appear to be no interferences from the other carbohydrase activities in the Maxilact preparation (in this case the only one of any interest is α-galactosidase) upon the transgalactosylation products detected. Secondly, that the transferase activity of β-galactosidase from Maxilact would not be of commercial interest in the production of biologically interesting galactose disaccharides because of the very low yield.

GC quantification of transgalactosylation disaccharides produced by purified β-galactosidase and Maxilact L2000 at 37 °C.

With the exception of the times of incubation at which samples were taken, all of the samples in this experiment, both Maxilact and purified enzyme, were treated in the same manner as for the experiments performed at 4 °C. The identification of disaccharides was exactlty the same, based upon differences in retention time

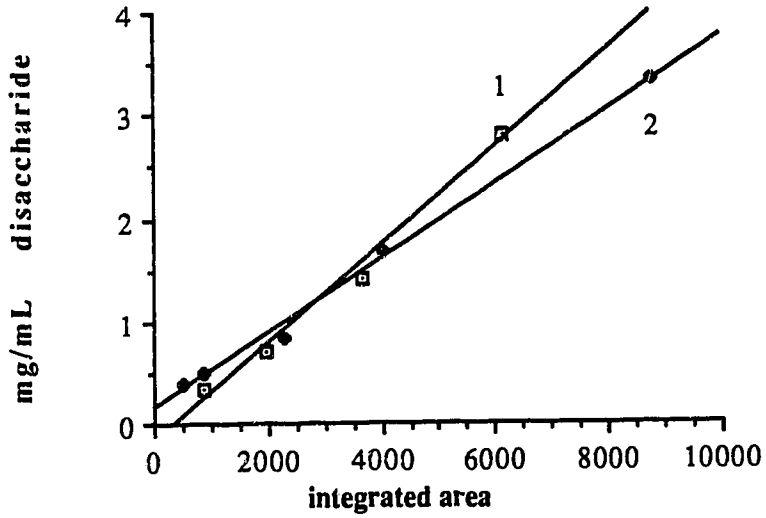
relative to sucrose. Quantification was conducted by comparison to the standard curve.

In this work the FID was being utilized at maximal sensitivity. At these levels of sensitivity (at least part per million, ppm) the possibility of ghost peaks and injection carry over can not be ignored. For this reason, all peaks not greater than 4 times noise were rejected as noise (Jennings, 1978, Sevcik, 1976).

FID response is linear in proportion to the number of carbon atoms in the molecule being detected (Sevcik, 1976) and therefore it is reasonable to use lactose (fully silylated it contains 39 carbon atoms/molecule) as a quantification standard for the galactose disaccharides (fully silylated the number of carbons/molecule is 39). The slight difference in the standard curves of persilylated sucrose and persilylated lactose (Figure VI-3) can be explained because the number of carbons per molecule of persilylated sucrose is only 36. The fact that the curves became non-linear (data not shown) at higher levels of concentration was also expected. Though the FID is theoretically linear over a concentration range of six orders of magnitude this is not a linear response to actual concentration but rather to effective carbon number (ECN) (Sevcik, 1976). The ECN has not been determined for either lactose or sucrose and this was not undertaken in this work. Linearity of FID response is also affected by high concentrations of persilylated molecules because of the deposition of silicon dioxide (SiO_2) onto the electrode surface during combustion of the compound. The deposited SiO_2 affects the electronic nature of the collector and can cause the detector response to be non-linear (Sevcik, 1976).

Figure VI-3

Standard curve for sucrose and lactose



1. Sucrose response
2. Lactose response

For the enzyme studies at 37 °C analagous results were obtained to the experimental results at 4 °C. Maximal production of galactose disaccharides occurred at 150 minutes of incubation for both the purified and the Maxilact preparations. From this time until the final sample was taken at 24 hours of incubation there was a gradual, albeit minor, decline in concentration of all 4 disaccharides. It may be possible that this decline was due to the transferase production of trisaccharides. The number of possible trisaccharides that could be formed was 16. If all of these were being formed the concentrations of each trisaccharide would be in such a small amount that they would be not detectable utilizing the method used for these experiments. This would mean that the equilibrium condition between all the possible products and activities is so complex that it is probably not achieved, especially with regard to the fact (Chapter 4) that at this temperature there is also enzyme denaturation.

The maximal production of total disaccharides by the Maxilact L2000 preparation (based upon molar yield) was 0.30 % $\pm$ 0.03 %. At the levels of error in these determinations this was only slightly different than that determined for the same preparation at 4 °C. The level of maximal production of oligosaccharides by purified enzyme was 0.31 % $\pm$0.03 %. As stated for the Maxilact preparation this was only a slightly different value from that reported for the same preparation at 4 °C. These two values; the amount of disaccharides produced by Maxilact and the amount of disaccharides produced by purified β-galactosidase, as was the case at 4 °C, are not greatly different. In both cases, purified enzyme and Maxilact L2000, the

time of incubation for maximal production of oligosaccharides was 150 minutes.

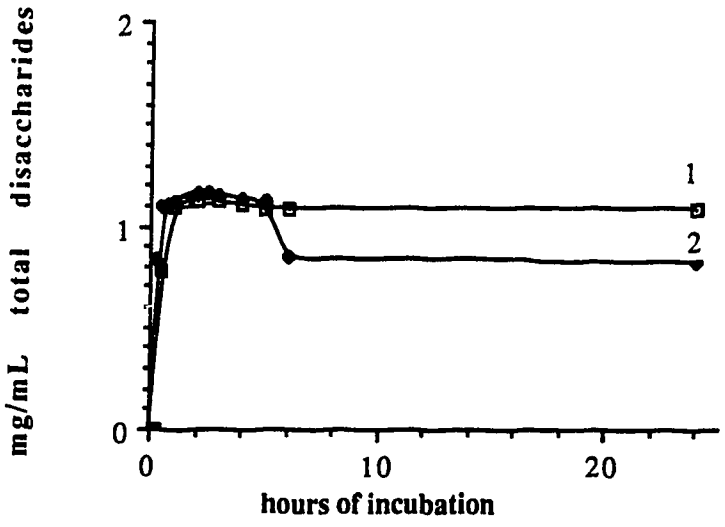
After 24 hours of incubation the levels of total disaccharides remaining (molar yeild) were 0.29 % ± 0.02 % for Maxilact L2000 and 0.22 % ± 0.02 % for the purified enzyme (Figure VI-4). The difference between these two enzyme treatments (Maxilact and purified enzyme), in regard to total disaccharide production, at 24 hours incubation, is minimal. The difference between these values and those for the same sample at 4 °C are however quite different. The total disaccharides remaining at 24 hours at 4 °C are greater than those remaining at 37 °C at the same time. An explanation for this phenomena may be that the rate of production of trisaccharides from the disaccharides is slower at 4 °C than at 37 °C and therefore more disaccharides could be expected to remain at the lower temperature.

As was the case for the runs at 4 °C disaccharide 3 was produced in the highest quantity (Table VI-2) and throughout the incubation the relative ratios of disaccharide 3 to the other disaccharides remained relatively constant. Only the 1, 2.5, 6 and 24 hr. concentrations are shown to simplify the table.

The conclusions that can be drawn from these experiments are the same as those from the 4 °C determinations, namely that there is no significant difference in total oligosaccharide production between purified β-galactosidase and Maxilact L2000 and that even at 37 °C the use of this enzyme to produce commercial quantities of galactose disaccharides would not be viable.

Figure VI-4

Production of total disaccharides at 37 °C by β-galactosidase and Maxilact L2000



1. Production of disaccharides by Maxilact L2000
2. Production of disaccharides by purified β-galactosidase

Table VI-2

Quantity of disaccharides produced by β-galactosidase and Maxilact L2000 at 37 °C

Disaccharide	Time (hr)[1]			
	1	2.5	6	24
1 (Maxilact)	0.28	0.28	0.27	0.27
1.(Purified)	0.28	0.29	0.28	0.27
2.(Maxilact)	0.27	0.28	0.27	0.27
2.(Purified)	0.28	0.29	0.28	0.28
3.(Maxilact)	0.29	0.31	0.29	0.29
3.(Purified)	0.30	0.31	0.30	0.28
4.(Maxilact)	0.26	0.27	0.26	0.26
4.(Purified)	0.26	0.27	n.i.[2]	n.i.[2]

1. mg/mL concentration, as determined by GC

2. peak visible in chromatogram but did not integrate

GC quantification of transgalactosylation disaccharides produced by purified β-galactosidase and Maxilact L2000 at 50 °C.

The collection of samples and their workup was identical to that of the samples at other temperatures studied except for the sampling times. Upon injection of the first sample, 15 minutes incubation, it was noted that there was no production of disaccharides. This was repeated for the 30 minute incubation with the same result. The 45 and 60 minute incubations were not worked up as the enzyme would have been destroyed within 10 minutes (Chapter 4).

The purpose of studying the disaccharide production at this temperature was to determine if there was any production before the thermal denaturation of the enzyme. It appears that there was not. If there had been production of disaccharides before the thermal denaturation of the enzyme it would be expected that they would be detected by the GC procedure, so this temperature was not studied further.

Isolation and identification of β - D - galactopyranosyl (1->6) D - galactopyranose by high field NMR

The procedure described in the materials and methods was utilized to isolate one disaccharide free of galactose and other disaccharides. The low yield of disaccharides in this procedure made isolation very difficult. Using the GC quantification method the maximally produced disaccharide at both 4 and 37 °C is disaccharide 3 and it is produced in a maximal yeild of 0.384 mg/mL. Therefore,

in the 100 mL solution there could be no more than approximately 40 mg of this disaccharide, and no more than 115 mg of all 4 disaccharides. In the same solution there would still be 19,885 mg of galactose. This is almost a 200 fold excess of monosaccharide to total disaccharides.

The monosaccharide contamination was attempted to be removed by charcoal adsorption chromatography (Siddiqui and Furgala, 1967). After the monosaccharide had been removed the collected disaccharides were evaporated to dryness and then peracetylated (Tate and Bishop, 1962) and a thin layer chromatographic (TLC) procedure employed to separate the acetylated sugars (Tate and Bishop, 1962). Four separate spots were developed by TLC on silica gel. Preparative plate TLC was performed on the mixture, the spots isolated and in the one case where there was enough material, identified by high field NMR. This isolated spot was peracetylated galactose.

Flash chromatography on silica gel was the second attempt. The acetylated compounds eluted very close together under all conditions attempted and because of this there was peak overlap in all fractions obtained. Flash chromatography was repeated on all partially purified fractions until only single spots were visuallized on TLC. This caused a drastic reduction in yeild and only one compound could be isolated pure. By high field NMR it was determined to be peracetylated galactose.

Silica gel chromatography of acetate derivatives of the carbohydrates was therefore abandoned and the two charcoal isolation methods described in the materials and methods utilized.

These procedures allowed for the separation of only one disaccharide. High field NMR of this compound gave the following NMR ^1H spectrum: (all numbers are ppm relative to tetramethylsilane (TMS), ppm of TMS is zero) 3.48, multiplet of 8; 3.70, doublet of doublets, $J_{1,2}$ = 3.2 Hz; 3.75, doublet, J = 3.5 Hz; 3.80, multiplet of 5; 3.82, multiplet of 5, 3.85, shouldered singlet; 3.90, doublet, J = 3.4 Hz; 4.00, multiplet of 5; 4.05, doublet of doublets, $J_{1,2}$ = 9.5 Hz; 4.25, multiplet of 8; 4.42, doublet of doublets, J_1 = 7.8 Hz, J_2 = 7.5 Hz; 4.55, doublet, J_1 = 7.5 Hz; and 5.25, doublet, J_1 = 3.2 Hz.

Decoupling experiments (^1H) identified the structure of this unknown disaccharide to be β-D-galactopyranosyl (1->6) D-galactopyranose. To further ensure identification of the disaccharide the commercially available compound, β-D-galactopyranosyl (1->6) D-galactopyranose, was purchased and a high field NMR of it determined. High field NMR of this compound gave the following NMR ^1H spectrum: (all numbers are ppm relative to tetramethylsilane (TMS), ppm of TMS is zero) 3.48, multiplet of 8; 3.70, doublet of doublets, $J_{1,2}$ = 3.2 Hz; 3.75, doublet, J = 3.5 Hz; 3.80, multiplet of 4; 3.82, multiplet of 4, 3.85, shouldered singlet; 3.90, doublet, J = 3.4 Hz; 4.00, triplet, J = 3.2; 4.05, doublet of doublets, $J_{1,2}$ = 9.5 Hz; 4.25, multiplet of 8; 4.42, doublet of doublets, J_1 = 7.8 Hz, J_2 = 7.5 Hz; 4.55, doublet, J_1 = 7.5 Hz; and 5.25, doublet, J_1 = 3.2 Hz.

These two spectra are virtually identical, except for the minor contaminating peaks in the isolated sample. These probably arise from contaminating galactose. This conclusively identifies the isolated disaccharide as galactobiose and in this respect agrees with

the report of Asp et al (1980) for the production of galactobiose by Maxilact.

Other researchers, notably Asp et al (1980) and Toba and Adachi (1981), have managed to isolate and identify oligosaccharides produced by yeast and microbial lactases. With the exception of galactobiose this study was unable to identify any of the three other disaccharides produced. ^{13}C NMR (Toba et al, 1981, Prakash et al, 1989) identification of unknown disaccharides present in minor quantities, requires hours of NMR time and considerable skill in the interpretation of results. GC-Fast Atom Bombardment (FAB) mass spectrometry (MS) can give linkage positions of oligosaccarides (Prakash et al, 1989) but this equipment was not readily available and as well this technique requires very involved computational determination of the spectra obtained.

The difficulty in isolating minor disaccharides out of a monosaccharide matrix is the largest impediment to research in this field. A number of different isolation methods were attempted to obtain pure disaccharides but in only one case was one of the minor disaccharides isolated in a large enough quantity and a pure enough form for identification.

CONCLUSIONS

The interfering carbohydrase activities in the Maxilact L2000 preparation do not interfere in the production, by transgalactosylation activity, of galactose disaccharides from galactose. At 4 and 37 ^{o}C there are slight differences in the

production levels of the disaccharides which may be even more pronounced if later times were studied in the 4 °C experiment.

In contrast to the work of Asp et al (1980) upon transgalactosylation by Maxilact L2000 this study has identified 4 disaccharides, not only 1. This tends to agree with the work of Jeon and Mantha (1985), who noted but did not identify, 4 disaccharides produced by Maxilact during lactose hydrolysis. There are 5 possible linkage positions on a pyranose ring form of a carbohydrate, the 1, 2, 3, 4, and 6 hydroxyls. It is likely that a non-reducing disaccharide (a 1-1 linkage), because of conformational restraints, would elute close to the sucrose peak on GC. No such peak was noted in the GC chromatograms. This assumption, along with the possible conformational restraints of the active site of the enzyme would seem to suggest that the β(1->1) linkage is not formed. The identity of the 4 disaccharides, one of which has been identified as β-D-galactopyranosyl (1->6) D-galactopyranose by NMR, would therefore be: β-D-galactopyranosyl (1->6) D-galactopyranose, β-D-galactopyranosyl (1->4) D-galactopyranose, β-D-galactopyranosyl (1->3) D-galactopyranose and β-D-galactopyranosyl (1->2) D-galactopyranose. Which one of these disaccharides was the one that was produced in maximal quantity to the rest is still unknown, it was only determined that it was not β-D-galactopyranosyl (1->6) D-galactopyranose. It is possible that the maximally produced disaccharide may be β-D-galactopyranosyl (1->4) D-galactopyranose.

The maximal concentration of total disaccharides produced in this reaction was 1.14 mg/mL, for a maximal molar yield of 0.36 %. This is not a great enough production to use the transgalactosylation

activity of the enzyme to synthesize commercial quantities of galactose disaccharides.

Finally, it can be concluded that the α-galactosidase activity in Maxilact is not great enough to interfere in the analysis of galactose disaccharides produced by β-galactosidase transgalactosylation activity. It can not be stated, from these experiments, whether the other interfering carbohydrase activities (Chapter 5) would not interfere during a study of lactose hydrolysis.

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Conclusions

Cerium(IV)

The use of Cerium(IV) as an analytical tool for food carbohydrates has been shown to be of wide possible utilization for foods as varied as milk, honey and corn starch hydrolysates. Lactose hydrolysis and lactose percentage in full fat, 2 % and skim milk can all be determined quickly and relatively easily in comparison to some of the standard methods used in the food industry today (i.e.: A.O.A.C. 16.055, 1984; polarimetry). The dextrose equivalent value (DE) of solid corn starch hydrolysate samples has been determined over the DE range 10 to 40 with excellent agreement between the Ce(IV) determined values and the classical Lane-Eynon determined values. The Ce(IV) test is much quicker and easier to perform than some of the classical analysis methods, most notably the Lane-Eynon titration (De Whalley, 1964). The final use of this test determined during this work was the prescreening of the sucrose level in honey. Honey is not as stable in general carbohydrate nature as milk and corn starch hydrolysates and therefore did not lend itself as well to analysis by the Ce(IV) test, for reasons stated in the thesis.

Transgalactosylation activity of the β-galactosidase from Maxilact L2000

It was originally thought that the interfering carbohydrase activities in the Maxilact, through their own transferase action, might produce more disaccharides than would be produced by β-

galactosidase alone. The enzyme was therefore purified away from these interferences and partially characterized. It appears that the β-galactosidase in Maxilact L2000 is a glycoprotein consisting of two isozymes having different thermal stability. This is the first report of glycosylation on the β-galactosidase from Maxilact and may open up new avenues of isolation of this enzyme. It is a confirmation of the work of Mahoney and Wilder (1988), who noted isozyme activity in Maxilact when studying milk hydrolysis. The transgalactosylation activity of β-galactosidase is not markedly different in the pure or impure form. The enzyme forms 4 disaccharides, one of which was identified by NMR. This disaccharide, β-D-galactopyranosyl (1->6) D-galactopyranose (galactobiose) has been reported as a transgalactosylation product of the lactase from Maxilact (Asp et al, 1980) and in this regard the work reported here agrees with those researchers. They however, report that galactobiose is the only galactose disaccharide formed while the results of this thesis are that there are 4 galactose disaccharides formed. The major disaccharide formed was not identified but it is not galactobiose.

The transgalactosylation activity of the β-galactosidase from Maxilact L2000, when acting upon galactose (not during lactose hydrolysis) is not sufficient to cause a large enough concentration of oligosaccharides to accumulate to cause nutritional concern. It is also not of a high enough activity, either at 4 or 37 °C, to be utilized as an alternative to synthetic methods of obtaining galactose disaccharides.

Future Research

The isolation and identification of the three unidentified galactose disaccharides should be carried out. If, as seems likely, they can not be isolated from this source, then there is the possibility of synthesizing the standard compounds needed for mixed GC injection. It is also possible to perhaps use another source of β-galactosidase to produce the disaccharides in large enough quantities to isolate them. These could then be identified by ^1H NMR and then used as co-injection standards on the GC to identify the remaining disaccharides.

The disaccharides formed during lactose hydrolysis would also be galactose-glucose disaccharides and these should be quantitated and studied in a manner similar to that presented in this work. It would be possible, by using a very low concentration of galactose, in the presence of saturating glucose, to cause the enzyme to form galactose-glucose disaccharides through its' transgalactosylation activity. These compounds could then be quantitated and identified using the methodology suggested above. Once these compounds had also been identified it would be relatively easy to study the transgalactosylation reaction, with the idea of quantifying and identifying all of the disaccharides produced by this reaction. It may be that during action on lactose the other carbohydrase activities in Maxilact do produce identifiable amounts of their own transgalactosylation products. The work on galactose disaccharides and galactose-glucose disaccharides outlined above would account for that possibility.

There is also the possibility for further research into the Cerium(IV) reaction and its use as an analytical test. There are a number of foods, notable among them ripening cheese, that undergo carbohydrate changes during aging or processing that are indicative of quality or nutritional balance of the product. These changes, at least in the case of cheese are constant and could concievably be measured with the Ce(IV) test. There is also a need in the food industry for a simple and rapid test to determine lactose and lactose hydrolysis in cheese whey. Perhaps the Ce(IV) test can be adapted to that end. Research into the mechanism and kinetics of the Ce(IV) carbohydrate reaction in classical and stopped flow time frames would also be of interest to the academic community and may have some applied end.

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