Galactosyl-oligosaccharide formation during lactose hydrolysis: a review

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Enzymatic hydrolysis of lactose is accompanied by galactosyl transfer to other sugars, thereby producing oligosaccharides. These are hydrolyzed slowly, both in vitro and in vivo. They can be thought of as low molecular weight, non-viscous, water-soluble, dietary fibre. They are considered to be physiologically functional foods which promote the growth of bifidobacteria in the colon and a wide variety of health benefits has been claimed in connection with this effect. This article reviews the mechanism of oligosaccharide formation, and then discusses the amount and nature of the products as well as the factors which influence them. The appearance and disappearance of oligosaccharides is explored through consideration of the kinetics of transferase activity. The consequences of oligosaccharide formation for dairy processing, food analysis, nutrition and health are then briefly discussed.

INTRODUCTION

The enzymatic hydrolysis of lactose into its component monosaccharides—glucose and galactose—is of interest from both the nutritional and technological viewpoints. The resulting sugars are sweeter, more readily fermented and are absorbed directly from the intestine. This has led to the development of low-lactose milk, the production of sweeteners from hydrolyzed-lactose whey and the incorporation of both these products into other foods. The hydrolytic aspects of lactase (β-galactosidase) action have been studied extensively over the last several decades. The transferase reactivity by which the enzyme produces and subsequently hydrolyses a series of oligosaccharides containing galactose was reported in the early 1950s (Aronson, 1952; Pazur, 1953). Apart from theoretical aspects, early research was prompted by nutritional concerns about the presence of these compounds in low-lactose milk (Burvall et al., 1979, 1980). Other, later studies were based on the need to consider oligosaccharide formation when modeling lactose hydrolysis (Prenosil et al., 1987a). More recently, interest in the reaction has been raised by observation that oligosaccharides may have beneficial effects as ‘bifidus factors’—promoting the growth of desirable intestinal microflora. Also, the transferase reaction can be used to attach galactose to other chemicals and consequently has potential applications in the production of food ingredients, pharmaceuticals and other biologically active compounds.

This article will review the mechanism of oligosaccharide formation, the nature of the products, the kinetics of the process and the implications of this reaction within the context of lactose hydrolysis technology. No attempt will be made to catalog the amounts of oligosaccharides produced by many individual enzymes, since this information can be obtained from earlier reviews (Prenosil et al., 1987a; Zarate and Lopez-Levia, 1990). For the purpose of this review, oligosaccharides are taken to include all di-, tri- and larger galactosyl-saccharides present, other than lactose itself.

MECHANISM OF β-GALACTOSIDASE

β-Galactosidase (lactase, EC.3.2.1.23) has been isolated from a large variety of sources (Mahoney, 1996) and well characterized with regard to its kinetic behaviour in the hydrolytic mode. Much less is known about its mechanism of action. Most of what is known about the catalytic mechanism is based on studies with the Escherichia coli enzyme whose subunit structure, primary sequence and three dimensional structure have all been determined (Fowler and Zabin, 1978; Jacobson, 1993).

Early studies with the enzyme suggested a minimum of three steps were involved, the last of which allows for hydrolysis or transferase activity (Wallenfels and Malhotra, 1961), viz:

$$\text{enzyme + lactose} \rightarrow \text{enzyme − lactose} \quad (1)$$


enzyme $\rightarrow$ lactose $\rightarrow$ galactosyl $\rightarrow$ enzyme $+$ glucose

(2)

galactosyl $\rightarrow$ enzyme $+$ acceptor $\rightarrow$

galactosyl $-$ acceptor $+$ enzyme

(3)

Where the acceptor is water, free galactose is formed by hydrolysis. Where the acceptor is a sugar, the result is galactosyl-oligosaccharide formation.

Early research on the effect of pH on activity and inhibition of enzyme sulphydryl groups suggested that the active site of neutral pH enzymes contains a sulphydryl group acting as a general base and an imidazole group acting as a general acid which donates a proton to the glycosidic bond (Wallenfels and Malhotra, 1961; Mahoney and Whitaker, 1977).

More recent studies indicate that the mechanism is analogous to that of lysozyme, i.e. there is a group acting as a general acid which donates a proton to the glycosidic oxygen and another negatively charged group which stabilizes a positively charged carbonium galactosyl transition state intermediate, probably by forming a transient covalent bond (Sinnott, 1978). The general reaction mechanism can then be depicted as shown in Fig. 1.

Site-directed amino acid substitutions in the E. coli lac Z enzyme indicated that Tyr 503 functions as the general acid/general base and that Glu 461 functions as the carboxylate stabilizer of the carbonium ion galactosyl transition state intermediate (Cuppies et al., 1990).

The mechanism indicates that the enzyme will transfer galactose to nucleophilic acceptors containing a hydroxyl group. Transfer to water produces galactose; transfer to another sugar produces di-, tri- and higher galactosyl-saccharides, collectively termed oligosaccharides. These in turn become substrates for the enzyme and are slowly hydrolyzed. In this scheme galactosyl transfer is the general reaction and hydrolysis can be regarded as a special instance of galactosyl transfer to water. Under most conditions, hydrolysis predominates due to the high concentration of water, and oligosaccharide production is low. The yield of oligosaccharides can be increased by using higher substrate (acceptor) concentrations and/or by decreasing the water content (Monsan et al., 1989). The yield was also increased by chemical modification of the enzyme amino groups (Mozaffar et al., 1989) but the reason for this is not yet understood.

In the case of E. coli enzyme there is another mechanism of oligosaccharide production which leads directly to the formation of the disaccharide allolactose ($\beta$-D-galactose (1$\rightarrow$6) D-glucose). The major pathway for production of this compound is direct internal transfer of galactose from the 4 position to the 6 position of the glucose moiety without first releasing the glucose from the active site (Huber et al., 1976). Additional allolactose can be formed by transfer of galactose to free glucose (see Fig. 1) or by hydrolysis of trisaccharides containing the allolactose.

Quantitatively, allolactose is one of the major oligosaccharides produced by neutral pH $\beta$-galactosidases, so the direct internal transfer mechanism is an important one. Although it has been demonstrated only for the E. coli enzyme, it is likely that many other $\beta$-galactosidases with similarity to the lac Z enzyme use this mechanism.

**SEPARATION AND ANALYSIS**

The mechanism outlined above shows enzymatic transfer to a nucleophilic acceptor. Since all of the sugars present, as substrates or products, can act as acceptors, the result is a complex mixture of di-, tri-, tetra- and even higher saccharides. Separation of the products can be achieved by liquid, paper or gas chromatography. A preliminary separation of some size classes can be achieved by adsorption chromatography on charcoal and elution with ethanol (Pazar et al., 1958; Nakanishi et al., 1983; Toba et al., 1985).

Further resolution can be obtained with paper chromatography which has long been used for separation of oligosaccharides (Aronson, 1952; Pazar, 1953; Roberts and Pettinati, 1957; Mozaffar et al., 1984; Huh et al., 1990). It is especially good at separating oligosaccharides of similar size such as the disaccharides: lactose, allolactose and galactobiose (Asp et al., 1980; Greenberg and Mahoney, 1983). It is, however, lengthy and tedious, often requiring multiple runs. Furthermore, quantitation of results is often imprecise as compared to other methods.

Separation by gas liquid chromatography (GLC) requires prior methylation of the sugars (Li et al., 1983; Smart, 1990) and analysis/identification of peaks is complicated by the separation of anomers, unless they are first reduced (Huber et al., 1976). For structural

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**Fig. 1.** A proposed reaction mechanism for the action of $\beta$-galactosidase on lactose. E: enzyme; LAC, lactose; GAL, galactose; GAL$^+$: carbonium transition state; GLC: glucose; ROH acceptor sugar; GAL-OR: galactosyl sugar (oligosaccharide). Adapted from Cupples et al., J. Biol. Chem. (1990).
analysis of oligosaccharides, however, methylation followed by GLC/Mass Spectrometry is a preferred mode (Asp et al., 1980; Toba et al., 1985).

Recent advances in HPLC have made this the method of choice for rapid, quantitative analysis of the main oligosaccharide classes (Betschart and Prenosil, 1984; Jeon and Mantha, 1985). A typical elution pattern is shown in Fig. 2. HPLC columns are very effective at separation into size classes i.e. mono-, di-, tri- etc., but much less effective at separating within a size class. Consequently, allolactose and galactobiose are not readily resolved from lactose or from each other. In this respect, paper chromatography is more effective.

Each method has its advantages and the choice depends upon the requirements of speed, sample throughput and resolution.

AMOUNT AND NATURE OF OLIGOSACCHARIDES

The amount and nature of the oligosaccharides formed depends upon several factors including the enzyme source, the concentration and nature of the substrate, the degree of conversion of the substrate and the reaction conditions. With the E. coli enzyme, the transferase activity was also affected by pH, magnesium concentration and the anomeric configuration of lactose (Huber et al., 1976)

![Fig. 2. Separation of the products of b-galactosidase action on lactose by HPLC. A: tetrasaccharides; B: trisaccharides; C: disaccharides; D: glucose; E: galactose. Reprinted from Yang and Tang, Annals. N.Y. Acad. Sci. (1988).](image)

Amount

The amounts of oligosaccharides formed from different sources has been listed by Prenosil et al. (1987a) and by Zarate and Lopez-Leiva (1990). Comparisons between different enzyme sources is difficult because the reaction conditions are invariably different. Nevertheless, such comparisons indicate that the total amount of oligosaccharides depends on the source and can vary from 1–45% of the total sugar present (Zarate and Lopez-Leiva, 1990). Because the oligosaccharides are constantly being synthesized and degraded, the amounts are often expressed as peak levels or maximal amounts. These peak levels generally occur when a large part of the lactose has already been hydrolyzed (typically within the range 40–95% hydrolysis) since conversion to products is necessary to build-up the concentration of donor/acceptor molecules. In general, the highest levels are associated with the neutral pH enzymes from bacteria and yeast rather than acid pH enzymes from moulds. Levels of around 40% have been reported for the enzymes from Kluveromyces fragilis (Roberts and Pettinati, 1957); Bacillus circulans (Mozzafar et al., 1984); Streptococcus thermophilus (Smart, 1990); and Saccharopolyspora rectivagula (Nakao et al., 1994).

For a given enzyme, the peak level of oligosaccharides increases with increased starting lactose levels (Roberts and Pettinati, 1957; Huber et al., 1976; Burvall et al., 1979; Prenosil et al., 1987b; Smart, 1990). This effect is illustrated in Fig. 3 for two different enzyme sources. Consequently, highest levels of oligosaccharide production have been found using the highest starting lactose levels (15–50%). At lower lactose levels, such as those found in milk and whey transferase activity is reduced but peak oligosaccharide levels can still can reach as much as 25% of total sugars (Greenberg and

![Fig. 3. Maximum oligosaccharide production as a function of the initial lactose concentration L0. —- A. niger lactase; ○— A. oryzae lactase. Reprinted from Prenosil, Stuker and Bourne. Biotech. Bioeng. (1987). Reprinted by permission of John Wiley and Sons, Inc.](image)
Mahoney, 1983). Much lower values for oligosaccharide production, of around 5%, have been reported where only trisaccharides and higher oligosaccharides were measured and disaccharides other than lactose were ignored (Burvall et al., 1979).

The amount of oligosaccharides formed can also be influenced by the starting material. Oligosaccharide levels in milk were lower than in buffered lactose (5%) when using lactases from the yeasts Candida pseudotropicalis and Kluyveromyces lactis (Jeon and Mantha, 1985; Kwok and Jeon, 1986). However, this has not been confirmed in controlled comparisons for other sources of the enzyme.

A recent study indicated that peak oligosaccharide levels could be increased by removal of glucose. Resting cells of Sterigmatomyces elviae produced 37% oligosaccharides from lactose. When the cells were allowed to grow on the glucose, however, the level of oligosaccharides increased to 64% (Onishi et al., 1995).

**Nature of the oligosaccharides**

Although several oligosaccharides have been observed as spots on paper or chromatographic peaks, their structural identification has been reported in only a few cases. A list of oligosaccharide structures identified is shown in Table 1.

The primary transferase product is allolactose (Jobe and Burgeoise, 1972; Huber et al., 1976). This will be formed along with galactobiose (β-D-galactose (1→6) galactose) at all substrate concentrations. Other disaccharides have been observed in much smaller amounts and at higher starting lactose levels. Disaccharides containing galactose linked β(1→3) and β(1→2) to glucose and β(1→3) to lactose have been identified using lactase from Aspergillus oryzae (Toba and Adachi, 1978). These disaccharides, together with lactose, serve as acceptors for the synthesis of tri- and higher saccharides. It would seem that galactose can be transferred to any of the hydroxyl groups on acceptor sugars, except for the C1 hydroxyl. Quantitatively, however, the 1→6 linkage is preferred for oligosaccharide synthesis. The same bond is the most susceptible for hydrolysis among β-galactose-glucose isomers when using the E. coli enzyme (Wallenfels and Malhotra, 1961).

Trisaccharides, especially galactosyl (1→6) lactose can be identified at most lactose levels, including those in milk and whey. Tetra- and higher saccharides have been reported only when using much higher starting lactose levels and only in very small amounts. It is likely that the larger oligosaccharides are formed at all lactose levels but that the amounts are too small for detection except at the higher starting levels. Using 30% lactose, as many as 20 di-, tri-, tetra- and penta saccharides have been isolated (Toba et al., 1985).

Quantitatively, the peaks amounts present appear to follow the order: di- > tri- > tetra > higher saccharides and the linkages synthesized are β(1→6) > β(1→3) and β(1→2) (Prenosil et al., 1987b; Toba and Adachi, 1978; Smart, 1990).

**Kinetics of transferase activity**

The actual amount of oligosaccharide present at any time depends upon the relative rates of synthesis and breakdown. This in turn depends upon both the amount of lactose and the extent of lactose hydrolysis which provides different levels of acceptors as hydrolysis proceeds. An example of the kinetics is shown in Fig. 4A, where the concentration of sugars is shown as a function of (a) time and (b) degree of conversion. In this system the oligosaccharides were almost entirely disaccharides and their peak level (25% total sugars) was not reached until almost all the lactose had been hydrolyzed. Disaccharides are formed directly from monosaccharides whose concentration rises as lactose is hydrolyzed. They are also formed from degradation of trisaccharides. Accordingly it is to be expected that they would peak late in the conversion process.

The concentrations of the species depicted in Fig. 4 is shown in Table 2. During the first hour, when over 90% of the lactose was converted, the ratio of free glucose to free galactose was close to 2:1. It only approached parity when the reaction had gone to completion and most

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**Table 1. Structures of some oligosaccharides formed during β-galactosidase action on lactose**

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-Gal</td>
<td>6' digalactosyl-glucose</td>
<td>6' galactosyl-lactose</td>
<td>6' galactotriose</td>
<td>3' galactosyl-lactose</td>
</tr>
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<td>3' galactosyl-lactose</td>
</tr>
</tbody>
</table>

Gal, galactose; Glc, glucose. Structures from Asp et al. (1980), Toba et al. (1985) and Onishi et al. (1995).
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At higher starting lactose levels, substantial amounts of trisaccharides are formed early on (at low % conversion) using the lactose as an acceptor, as illustrated in Fig. 5. The trisaccharides typically reach their peak and start to decline before the disaccharide peak, reflecting their degradation to di- and mono-saccharides (Betschart and Prenosil, 1984; Yang and Tang, 1988; Smart, 1990).

The pattern of tetrasaccharide formation and decline is similar to that of the trisaccharides but the peak is slightly delayed since synthesis is dependent on trisaccharide formation first.

Extended reaction times lead to very low residual oligosaccharide levels, but some will always be present, even after all the lactose has been hydrolyzed, due to synthesis from free galactose and glucose. This reverse reaction was demonstrated by Prenosil et al. (1987b) who started with 7.5% each of galactose and glucose and detected disaccharides at a maximum level of 3.8% of total sugar after 7h reaction time. The reverse reaction was much slower than lactose hydrolysis or oligosaccharide formation via the other routes.

**Immobilization**

There are relatively few reports of oligosaccharide production by immobilized lactase but these suggest that there are few, if any, significant differences when the initial lactose concentration is low (5%). At higher starting lactose levels (10–20%), however, the peak concentration of trisaccharides is reduced. Rugh (1982) observed a decrease of 23% in trisaccharides and of 33% in tetrasaccharides using immobilized lactase from a Bacillus spp. Similar results have been reported by Prenosil et al. (1987b) who used A. niger lactase.

The decreases can be attributed to diffusional resistance in the enzyme support particles which produce concentration gradients of possible acceptors (Rugh, 1982). Lactose concentration inside the particle is lower than in bulk solution but since local conversion is higher some oligosaccharides are hydrolyzed before they reach the bulk solution. As a consequence, oligosaccharide peak levels are reduced. With the A. niger enzyme the peak level was shifted toward a lower percent conversion (Prenosil et al., 1987b) but with the Bacillus enzyme the opposite trend was observed (Rugh, 1982).

**Table 2. Distribution of sugars during hydrolysis of lactose in milk by S. thermophilus β-galactosidase at 37°C**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Lactose (%)</th>
<th>Glucose (%)</th>
<th>Galactose (%)</th>
<th>Glucose/galactose ratio</th>
<th>oligosaccharides (%)</th>
<th>Galactose/glucose ratio in oligosaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17</td>
<td>55.7</td>
<td>14.6</td>
<td>8.7</td>
<td>1.67</td>
<td>13.1</td>
<td>1.78</td>
</tr>
<tr>
<td>0.42</td>
<td>35.3</td>
<td>25.0</td>
<td>11.4</td>
<td>2.19</td>
<td>22.7</td>
<td>2.85</td>
</tr>
<tr>
<td>1.0</td>
<td>6.16</td>
<td>40.2</td>
<td>21.3</td>
<td>1.89</td>
<td>24.8</td>
<td>3.81</td>
</tr>
<tr>
<td>2.0</td>
<td>1.74</td>
<td>46.4</td>
<td>37.1</td>
<td>1.25</td>
<td>13.8</td>
<td>4.41</td>
</tr>
<tr>
<td>4.50</td>
<td>1.17</td>
<td>46.7</td>
<td>47.5</td>
<td>0.98</td>
<td>3.61</td>
<td>—</td>
</tr>
</tbody>
</table>
Oligosaccharides in fermented milk products

Oligosaccharide production has been studied primarily in lactose solutions, milk and whey. However, oligosaccharides have also been found in fermented milk products such as yoghurt even though the percentage of lactose conversion is modest (~20%). Toba et al. (1982, 1983) detected allolactose and galactobiose in commercial yoghurt at low levels ranging from 0.03–0.09%. They concluded that these oligosaccharides were formed by lactases in the lactic acid starter bacteria. In a study with yoghurt prepared in the laboratory, Toba et al. (1983) found that oligosaccharide content increased with time of fermentation and during storage.

When low-lactose yoghurt was made by adding lactase from A. oryzae along with the starter culture, the level of oligosaccharides after storage was 4–19 times higher than that found in control yoghurt (Toba et al., 1986). This could be of interest to yoghurt producers who wish to include oligosaccharides in their product for health benefits (see below).

CONSEQUENCES

Oligosaccharide production has been considered as an interesting but ultimately unimportant sideline activity during lactose hydrolysis, since eventually they are mostly hydrolyzed. There are, however, some relevant and practical consequences.

(a) Peak oligosaccharide levels often occur when most of the lactose has been hydrolyzed (see Fig. 4). Reduction to low levels can only be achieved by prolonging the reaction time far beyond that required for sufficient lactose conversion (typically 70–90%), thereby increasing the cost of processing.

(b) Concentration of lactose substrates such as whey permeate will lead to higher oligosaccharide levels which can only be removed by extended processing. If not removed the lower solubility of oligosaccharides could cause crystallization problems during storage of concentrated syrups (Prenosil et al., 1984).

(c) In vitro experiments indicate that oligosaccharides are hydrolyzed very slowly by the β-galactosidase of the human intestinal mucosa. Several oligosaccharides were hydrolyzed at less than 10% of the rate for lactose, while allolactose was hydrolyzed at less than 5% of the rate for lactose (Burvall et al., 1980). As a result ingested oligosaccharides will largely escape digestion in the small intestine and will pass on to the colon where bacterial degradation could lead to the gastrointestinal discomfort associated with lactose intolerance, if the levels were high enough.

(d) Oligosaccharide formation complicates estimates of lactose hydrolysis. Routinely the presence of one mole of glucose (or galactose) is taken to indicate that one mole of lactose has been hydrolyzed. Reference to the data in Table 2 shows this is not correct. Incorporation of glucose and galactose (especially) into oligosaccharides leads to errors if the concentration of either one is used to follow the reaction. The value for glucose tends to underestimate the extent of lactose hydrolysis, especially at higher residual lactose levels (>35%). Use of galactose values leads to even greater errors except when the reaction is essentially complete and almost all the oligosaccharides have been degraded. On the whole, it is better to use the values for glucose if only one sugar is to be used. Estimation of total monosaccharides (glucose + galactose) also gives errors in lactose hydrolysis due to the preferential incorporation of galactose into the oligosaccharides. Nonetheless it
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is widely used as a practical measure of the reaction progress since the whole purpose of the reaction is to produce monosaccharides.

(e) Oligosaccharides also interfere with the estimation of lactose hydrolysis by the freezing point depression method. The difference in percentage hydrolysis estimated by this method and also estimated by HPLC ranged from 0.2% to 13.7% (Jeon and Saunders, 1986). In general, the differences were greater for milk and whey permeate than for lactose solution. For each substrate, the difference tended to increase as the reaction progressed, so final estimates by the freezing point method were often the least accurate.

(f) Kinetic modelling of enzymatic lactose hydrolysis is complicated by the formation of oligosaccharides, due to the complexity of the equations and the number of kinetic constants. Models can be simplified by considering only the production of trisaccharides and ignoring the internal transformation of lactose to allolactose, in which case agreement with experimental data can be obtained (Prenosil et al., 1987b; Yang and Tang, 1988).

Health benefits

While the preceding consequences indicate that oligosaccharides are largely a problem/nuisance it must be noted that they are also believed to benefit human health. Oligosaccharides are largely indigestible in the upper intestine so they can be thought of as low-molecular weight, non-viscous, water-soluble dietary fiber. As such they are considered physiologically functional foods, especially in Japan where they are very popular.

Oligosaccharides serve as growth factors for indigenous bifidobacteria in the colon. The increased population of bifidobacteria antagonistically suppresses the activity of putrefactive bacteria such as Clostridia and thereby reduces the formation of toxic fermentation products (Tomomatsu, 1994). A wide variety of health benefits including anticarcinogenic effects, reduction in serum cholesterol, improved liver function and improved intestinal health are associated with oligosaccharides/bifidobacteria (Hawkins, 1993; Tomomatsu, 1994). In Japan production of oligosaccharides from all sources (including fructo-oligosaccharides and soybean oligosaccharides) reached 18 million kg in 1990. They were included in a wide variety of foods including soft drinks, cookies, cereals, and candies (Tomomatsu, 1994). If the claims for health benefits are confirmed, the public demand for dairy foods containing galactosyl-oligosaccharides is likely to increase very significantly. In the long run, what was once considered a problem may turn out to be a very beneficial aspect of lactose hydrolysis.

REFERENCES


