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Preparation of prebiotics by lactose-malic acid and lactose-citric acid reaction

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1. SUMMARY

Prebiotics are indigestible food components that serve as nutrients for bifidobacteria and lactobacilli in the colon. Dietary fiber and oligosaccharides are typical prebiotics, therefore, prebiotics were prepared in our experiments by reacting lactose with malic acid and citric acid at the right concentration and for the right length of time at the optimal temperature. The ideal parameters of the reaction were determined, as well as the consumption of the starting materials and the increase in the concentration of the final product, and the total sugar content of the hydrolyzed prebiotic after hydrochloric acid hydrolysis. In vitro experiments have shown that the final product prepared by us is resistant to carbohydrate degrading enzymes (which is a basic requirement for a prebiotic) and thus can serve as a nutrient for the probiotic bacteria living in the colon.

2. Introduction

The international nomenclature of probiotics (probiotics, prebiotics and synbiotics) developed in the last two decades of the 20th century, while the formulations of the products have also become uniform in terms of their ingredients.

Probiotics are all intestinal bacteria that have a beneficial effect on the health of the host. Natural nutrients, which are typically the exclusive nutrients of probiotics, and hence promote their proliferation and preponderance, are called *Prebiotics*.

The term *synbiotics* means the sum total of probiotics and prebiotics. In synbiotic foods, the effects of the two aforementioned beneficial factors are added together, and their effects become synergistic. As a result, for example, dairy products that have been prepared using not only probiotics but also one or more prebiotics are synbiotic.

Prebiotics (formerly known as bifidus or bifidogenic factors) are oligosaccharides composed of 2-9 simple sugar units (monosaccharides). They are not metabolized in the stomach and small intestine, thus they reach the colon undigested as water-soluble dietary fiber. In addition to the dietary fiber function, their real value lies in the fact that they are the exclusive foods of probiotics. Since there is little digestible food residue in the colon, there is a relative lack of food for the microflora. However, the ingestion of prebiotics promotes the proliferation of human-friendly probiotics **[1]**.

In their natural state, prebiotics are found in many foods. Their rich sources include Jerusalem artichoke and chicory roots, but can also be found in onions, garlic, leeks, artichokes, oatmeal, wheat, bananas, milk and ripe cheeses. During the manufacture of formulations with a probiotic effect, pure products prepared by industrial technologies are typically used, which are marketed as liquid concentrates or powders with an active ingredient concentration of 40-95%. Natural industrial concentrates can be, for example, galacto-, fructo-, malto- or xylooligosaccharides, depending on the type of monosaccharide they are composed of. In 1995, more than 80,000 tonnes of prebiotics were produced worldwide, but production has increased to around 200,000 tonnes by now. The increase in world production indicates that interest in probiotic

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foods has been increasing steadily. About 40% of the amount produced is galacto-oligosaccharide (e.g. lactulose), the raw material of which is lactose **[2, 3, 4]**.

Thus prebiotics are indigestible polysaccharides and oligosaccharides. Reaching the colon, they inhibit the growth of *Salmonella* and *Escherichia coli*, while at the same time promoting the growth of bifido- and lactic acid bacteria. The name *„prebiotic"* comes from 1995 **[5]**. There are several conditions for the prebiotic effect of a nutrient **[6]**, the most important of which are:

- Resistance to gastric acid and the digestive effect of pepsin;
- The ability to serve as a nutrient to the beneficial microflora of the intestinal tract, the metabolic products of which contribute to the improvement of the health and well-being of the person consuming the probiotic.

Many food components met these criteria, such as inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactulose and polydextrose. Isomaltol oligosaccharides (IMO), xylooligosaccharides (XOS) and lactitol have been classified as potential prebiotics **[7]**.

Prebiotics are found in a wide variety of foods. For example, chicory root contains fructooligosaccharides of inulin origin, while wheat bran contains arabinoxylooligosaccharides (AOXS) and xylooligosaccharides (XOS). These substances are widely used in the manufacture of probiotic products **[8, 9, 10]**.

Mannitol, maltodextrin, raffinose, lactulose and sorbitol are also prebiotics with helath-protective effects **[11, 12, 13]**. Seeds rich in resistant starch are also considered prebiotics; they are not digested and are therefore not absorbed in the small intestine, but once in the colon, its microflora can utilize them during fermentation while short chain fatty acids (SCFA, such as propionic acid, butyric acid, valeric acid, caproic acid) are produced. These fatty acids inhibit the growth of putrefactive, toxin-producing bacteria in the colon by lowering the pH **[14]**.

Fermentable dietary fibers similar to the β-glucan of rye, as well as the gum-like polysaccharides of flaxseed and fenugreek can also be considered prebiotics and are also raw materials for the production of short chain fatty acids, and so they have a health-protective effect. Mannates present in large amounts in the cell wall of yeast cells can also be considered prebiotics **[15]**.

Today, the number of sick people and deaths due to malnutrition, smoking and alcoholism is significant. Diseases typical of our time include chronic obesity, gastrointestinal problems, diabetes, cardiovascular diseases, cancer and degenerative lesions, the number of which has increased significantly in recent times. To prevent these diseases or reduce their symptoms, food consumers are increasingly turning to health-protective foods that also contain prebiotics, from which they expect a significant improvement in their quality of life.

A significant proportion of consumers are looking for low-carb, high-fiber and high-protein foods, which has been accompanied by a growing interest in prebiotic foods. Good examples of this are food types containing blackcurrant leaf extract powder, lactoferrin and lutein, which are produced in large quantities all over the world. These products significantly increase the amount of bifidobacteria amd lactobacilli, and also significantly reduce the umber of Bacteroides and Clostridium species in the colon. In addition, they reduce the activity of β-glucuronidase enzymes and increase the activity of β-galactosidase enzymes in the small intestine, thus promoting, for example, the digestion of lactose in lactase-deficient individuals **[16]**.

Wheat germ supplementation resulted, after 20 days, in a significant lowering of the pH of the colon and the size of the clostridium population, and a significant increase in the number of lactobacilli and bifidobacteria, while also significantly improving the quality of life of the consumers of this type of product **[17]**. Gum arabic was found to reduce disease-related symptoms in patients with systolic blood pressure or diabetic renal failure **[18]**. It was also found that consumption of 25 g of gum arabic product per day for 8-12 weeks had a beneficial effect on the condition of diabetic patients and significantly reduced systolic blood pressure **[19]**.

Our group previously performed the structural and quantitative analysis of epoxypolysaccharides and oligosacchardies produced by lactic acid bacteria **[2, 3, 4]**. In the present paper, we report the results of our experiments in which prebiotics were prepared by the reaction of lactose with malic acid and citric acid. In the course of our work, the patent description found in sources **[20]** and **[21]** was considered as a starting point, in which it was sought to produce surfactants by forming ester bonds between carbohydrates and dicarboxylic acids, and the mechanism of these reactions was studied. The method was successfully tested by the authors in the case of sugar alcohols, sugars, oligosaccharides and polysaccharides as well **[20, 21]**.

3. Objective

Based on the literature and our own previous research, our objective was to produce prebiotics in which bonds were created between lactose and malic acid and between lactose and citric acid that resist the acidic medium and the attack of

carbohydrate-degrading enzymes in the human stomach and the anterior part of the intestinal tract, reach the colon, where then they serve as nutrients for the probiotic microorganisms. Our aim was to determine the optimal parameters of the reaction, such as temperature, time and the concentration of the reactants, to measure the loss of starting materials and the increase in the concentration of the final product, and to analyze the total sugar content of the hydrolyzed prebiotc after hydrochloric acid hydrolysis. It was demonstrated in in vitro experiments that the final product prepared by us is resistant to carbohydrate-degrading enzymes, which is a basic requirement for prebiotics.

4. Materials and methods

4.1. Materials used, analytical methods applied

Our experiments were performed using pharmacopoeia grade lactose, citric acid and malic acid. The purity of the malic acid used by us was 99.5%, containing less than 1% fumaric acid and less than 0.05% malonic acid. The certificate of analysis of malic acid (E number: E296) can be downloaded from the link http://bbbb.hu/spec/almasav.jpg. The material used by us meets the quality requirements of the US, European and Hungarian pharmacopoeias, as well as the specifications (no. 1-2-89/107) of the EU and Hungarian Food Codexes.

The citric acid used for our experiments was also of food and pharmacopoeia quality citric acid monohydrate (E330), the certificate of analysis of which and MSDS can be downloaded from the links http://www.bbbb.hu/spec/Citrom.jpg and http://www.bbbb.hu/spec/citrombizt.jpg, respectively. Its CAS no. is 5949-29-1, EU no. is 201-069-1. According to the certificate of analysis, its citric acid monohydrate content is close to 100%, water content is no more than 8.8%, and the oxalic acid content is less than 100 mg/kg. All of the parameters meet the requirements of the EU and Hungarian Food Codexes.

The lactose used in the experiments was 99.7% purity, food grade, finely powdered D(+)-lactose monohydrate isolated from bovine milk and spray dried. Its quality met the quality requirements of Ph.Eur 8.0.

4.2. Analytical methods applied

To monitor the reaction of lactose with malic acid and citric acid, the measurement of the lactose content was selected. The formation reaction of probiotics can be followed by monitoring the decrease in the amount of lactose. Lactose belongs to the group of reducing disaccharides, therefore, it exhibits the Fehling reaction. However, if the free glycosidic hydroxyl group of lactose forms a bond, then the sugar molecule will no longer participate in the reaction characteristic of reducing sugars. During the reaction, because of the aldehyde group of the sugar, Cu^{+} ions are produced from the Cu^{2+} ions. By determining the amount of Cu^{+} ions, the amount of sugar can be determined with analytical accuracy. During the analytical procedure, 2 g of the sample was placed in a 100 cm^3 volumetric flask, 50 cm^3 of water was added, and the mixture was shaken on a shaker for one hour. To remove substances that interfere with the determination of sugar, 20 cm^3 each of Carrez I and II solutions were added to the solution. The volumetric flask was then filled to mark with 80% ethanol, it was shaken and filtered through filter paper. 20 cm^3 of the filtrate was taken, from which the majority of the ethanol was evaporated, the evaporation residue was washed into a 20 cm^3 volumetric flask using distilled water at ca. 50 °C, it was allowed to cool and then filled to mark. This solution was later used to determine the reducing sugar content.

5 cm^3 of the solution thus prepared was placed in a 100 cm^3 Erlenmeyer flask, 5 cm^3 of Luff-Schoorl reagent was added and a few pieces of boiling stone, it was brought to a boil within 2 minutes by shaking over a free flame, boiled for 10 minutes, and then it was cooled down immediately. The precipitated copper(I) oxide was titrated iodometrically with 0.1 M sodium thiosulfate solution. The amount of lactose was calculated from the consumption of the titrant.

4.3. Design of the reactions between malic acid, citric acid and lactose

To the pharmacy grade lactose, 20% citric acid was added in the first step, then 20% malic acid in the second step. After reviewing the available literature and the patents, it was found that most of the reactions were carried out at 130-180 °C. Therefore, a reaction temperature of 170 °C was chosen for our experiments. The samples were mixed in a mortar to ensure adequate homogeneity of the samples. Following this, approximately 10 g each of the sample was placed in glass flasks and heat treated for 5, 10, 20, 30, 40, 50 and 60 minutes, and the lactose content of the samples was determined after cooling.

In the next experiment, the effect of the temperature on the reaction between citric acid and lactose and between malic acid and lactose was investigated. The samples containing 20% citric acid or 20% malic acid and 80% lactose were treated for 30 minutes at 130 °C in the first experiment, at 140 °C in the second experiment, at 150 °C in the third experiment, at 160 °C in the fourth experiment and at 170 °C in the fifth experiment. After cooling, the lactose content of the samples was determined in each case. The amount of residual lactose was given relative to 100 g of the reaction product (weight percent).

4.4. Results and their evaluation

4.4.1. Effect of the heat treatment time at 170 oC on the reaction between lactose and carboxylic acids

Table 1 shows the effect of heat treatment time at 170 °C on the reaction between lactose and citric acid.

By adding 20% citric acid to lactose and performing heat treatment at 170 °C for various periods of time it was found that upon heta treatment the starting white mixture turned yellow within 5 minutes, and within 10 minutes it turned brown and exhibited blisters. Following this, only the color of the mixture became deeper, its apparent volume remained virtually unchanged.

Table 2 shows the effect of heat treatment time at 170 °C between lactose and malic acid.

By adding 20% malic acid to lactose and performing heat treatment for various periods of time, similarly to the case with citric acid, it was found that the color of the samples hardly changed at all in the first 5 minutes, they turned slightly yellow within 10 minutes, yellowish brown within 20 minutes, then swelled continuously, and the color of the last sample changed to dark brown.

The residual amounts of lactose in the reactions with citric acid and malic acid are shown graphically in ***Figure 1***.

4.4.2. Effect of heat treatment at different temperatures for the same time on the reaction between lactose and carboxylic acids

Table 3 shows the effect of heat tretament at different temperatures for the same period of time (30 minutes) on the reaction between lactose and citric acid and between lactose and malic acid.

After heat treatment at 130 °C for 30 minutes, the samples practically retained their white color, at 140 °C samples containing either citric acid or malic acid turned yellow, at 150 °C the yellowing increased further for both carboxylic acids, at 160 °C both the citric acid and malic acid samples turned strongly brown, while upon heat treatment at 170 °C for 30 minutes, the citric acid sample formed a brown mass. The malic acid sample also turned brown, but the brown discoloration remained lighter compared to the other samples.

The percentages of residual lactose after reactions carried out at different temperatures for 30 minutes are shown in ***Figure 2***.

4.5. Conclusions

4.5.1. Time and temperature dependence

By determining the lactose content, it was possible to determine what percentage of the mixture that originally contained 80% lactose was converted to some kind of oligomer or polymer. If the lactose concentration decreased significantly during the heat treatment, the hydrohycarboxylic acids used presumably reacted with lactose to form molecules of different chain lengths.

The lactose contents of the 24 samples were determined to obtain the following results. In the first experiment, 20 g of citric acid was mixed with 80 g of lactose, in the second experiemnt, 20 g of malic acid was mixed with 80 g of lactose, and then heat treatment was performed at 170 °C for 5, 10, 20, 30, 40, 50 and 60 minutes. In a subsequent experiment, the temperature dependence was investigated, and during this the samples listed above (with citric acid or malic acid) were treated for 30 minutes at 130, 140, 150, 160 and 170 °C. In this series of experiments we wanted to find out what the optimal temperature was at which the reaction between lactose and the added carboxylic acid produces the most oligomers and polymers.

For our experiments, a control sample mixture containing 20% erythritol and 80% lactose was prepared in order to determine the thermal decomposition of lactose during heat treatment at 170 °C for 30 minutes, as lactose does not react with erythritol. After the heat treatment, the lactose content of the mixture was found to be 79.1%.

After the heat treatment of lactose with citroc acid for 5 minutes, the amount of lactose decreased to 73.6%, and after 60 minutes it decreased to 7.1%. Based on this, it is believed that during the heat treatment with citric acid, approximately 93% of the lactose was converted to some type of oligomeric or polymeric compound.

In the second experiment, the lactose content of the sample with malic acid, treated for 5 minutes, was measured to be 70.6%, and the residual lactose after 60 minutes of treatment was measured to be 16.4%. After 60 minutes, 83-84% of the lactose was converted to a reaction product. Based on our experimental results, it is believed that oligomers and polymers can be prepared by the reaction of lactose with both malic acid and citric acid.

Regarding the temperature dependence of the reaction, the following results were obtained. By the heat treatment of the citric acid sample at 130 °C for 30 minutes, only about 1-2% of the lactose was converted to the desired reaction products. However, only 16.4% of the lactose could be recovered from the same sample after heat treatment at 170 °C, so

that more than 80% of the lactose was converted to oligomers or polymers. Repeating the experiment with malic acid at 130 °C, about 30% of the lactose was converted in 30 minutes. Over the same period of time, 70% of the lactose reacted with malic acid at 170 °C.

From the second experiment, it can be concluded that at higher temperatures, both citric acid and malic acid proved to be suitable partners for the formation of lactose oligomers and polymers. The temperature of 130 °C appears to be too low, so heat treatment at 160-170 °C for 30 minutes, or possibly at 150-160 °C for 1 hour is recommended for ideal oligomer and polymer yields. The control sample experiment with erythritol showed that under the applied temperature conditions and duration, the lactose did not decompose, since almost all of the lactose mixed with the sample was recovered.

4.5.2. Determination of the sugar content of the prepared prebiotic after hydrolysis with hydrochloric acid

From the obtained reaction products, the release of alctose from the chemical bonds was attempted by hydrolysis with hydrochloric acid. Our hypothesis was that the results of sugar determination after hydrolysis would show whether lactose was indeed incorporated in the non-redcing polymers or whether other unexpected chemical transformations took place during the reaction. Following hydrochloric acid hydrolysis, the total sugar content determination yielded favorable results. For the sample heat treated at 170 °C for 60 minutes in the presence of 20% citric acid, the residual lactose content was 7.1%, which increased to 46.3% as measured by total sugar content after hydrochloric acid hydrolysis. After heat treatment with 20% malic acid at 170 °C for 60 minutes, the residual lactose content expressed as total sugars increased from 16.4% to 51.2% after hydrochloric acid hydrolysis. After heat treatment at 170 °C for 30 minutes in the presence of 20% citric acid, the residual lactose content expressed as total sugars increased from 16.4% to 54.9%, while in the case of malic acid it increased from 25.5% to 53.8%.

It follows from our measurement results that during the heat treatment reactions most of the lactose did not decompose but was converted to oligomers and polymers that give the Fehling reaction only to a minimal extent. However, when the oligomers and polymers were conveted to mono- or disaccharides by hydrochloric acid hydrolysis, the resulting sugar-like substances (probably mostly glucose and galactose and, to a lesser extent, lactose) gave the Fehling reaction and cold be determined as total sugars.

4.5.3. Treatment of the prebiotic produced with amlyase

In another series of experiments, the products obtained in the heat treatment reactions were also hydrolyzed with amlyase, modeling the reactions that take place in the anterior intestinal tract. Following hydrolysis with amylase, the total sugar content remained virtually unchanged. It follows that a natural reaction did not take place between the amylase and the disaccharide lactose, and it was also unable to cleave the oligo- and polysaccharide derivatives produced in our experiments. Thus, the as-yet-unidnetified, presumably oligomeric and/or polymeric product prepared in our experiments possesses the properties that are the basic prerequisites of a probiotic effect. That is, it does not break down in the anterior part of the human intestinal tract, it most likely reaches the colon, where it can serve as a nutrient for the probiotics that live there.

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