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Enzymatic production of 3’-sialyllactose in milk

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Highlights

- Enzymatic production of 3’SGL directly in milk using \textit{Trypanosoma rangeli} derived enzymes
- Kinetics of enzymatic conversion in milk is the same as that in aqueous systems
- Determination of activation energy ($E_a$) for enzymatic trans-sialylation reactions
- Robustness of 3’-SL at elevated temperatures (pasteurization) and freeze drying

Abstract

Human milk oligosaccharides (HMOs) are lactose-based glycan molecules present in human breast milk. HMOs are essentially not present in cow’s milk and hence not naturally available in infant formulas. HMOs possess several health and developmentally beneficial properties, and the sialylated HMOs are thought to play a particularly important role for infant brain development. Enzymatic transsialylation directly in cow’s milk, involving enzyme catalyzed transfer of sialic acid from a sialic acid donor to an acceptor, is a novel route for producing sialylated HMOs for e.g. infant formulas. The transsialidase (EC 2.4.1.) of \textit{Trypanosoma cruzi} is linked to trypanosomatid pathogenicity, but certain hydrolytic sialidases (neuraminidases), EC 3.2.1.18, from non-pathogenic organisms, can actually catalyze transsialylation. Here, we report enzymatic production of the HMO compound 3’-sialyllactose directly in cow’s milk using engineered transsialidases, Tr15 and Tr16, originating from the nonpathogenic \textit{Trypanosoma rangeli}. Both Tr15 and Tr16 readily catalyzed transsialylation in milk at 5 °C - 40 °C using $\kappa$ (kappa)-casein glycomacropeptide (cGMP) as sialyl donor substrate. Tr15 was the most efficient as this enzyme produced 1160 mg/L (1.8 mM) 3’-sialyllactose in whole milk during 10 minutes of reaction at 5 °C. The activation energy values, $E_a$, of the enzymatic
transsialylation reactions were similar in milk and in buffer solutions containing cGMP and lactose. The $E_a$ of the Tr15 catalyzed transsialylation reaction in milk was 16.5 kJ/mol, which was three times lower than the $E_a$ of Tr16 (66 kJ/mol) and of T. cruzi transsialidase (50 kJ/mol), corroborating that Tr15 was the fastest of the three enzymes and a promising candidate for potential industrial production of 3'-sialyllactose in milk. 3'sialyllactose was stable during pasteurization (30 min. at 62.5 °C) and freeze-drying.

Abbreviations

HMOs: Human Milk Oligosaccharides, 3’-SL: 3’-sialyllactose, 6’-SL: 6’-sialyllactose, SA: sialic acid, TcTS: Trypanosoma cruzi transsialidase, Tr15: engineered Trypanosoma rangeli sialidase 15 amino acids mutation, Tr16: engineered Trypanosoma rangeli sialidase 16 amino acids mutation, LC-MS: liquid chromatography coupled to mass spectroscopy.

Keywords: 3’-sialyllactose, transsialidase, Trypanosoma rangeli, organic whole milk, activation energy

1. Introduction

Human breast milk is characterized by the presence of human milk oligosaccharides (HMOs) in the range of 5-15 g/L [1]. HMOs are lactose-based oligosaccharides that beyond lactose generally contain one or more units of N-acetylglucosamine and galactose to form repeat backbone moieties of lacto-N-biose and N-acetyllactosamine. Further, the HMO backbone structures, including lactose, may be decorated with sialic acid (N-acetyl-neuraminic acid form) and/or fucose residues [1,2]. HMOs are presumed to play an important role in immunity and gut health of the breast fed baby as they function as bifidogenic prebiotics that appear to support the development of a healthy gut microflora and moreover prevent microbial infections by being decoy receptors that intercept epithelial attachment of enteric pathogens [1–3].

In addition, notably the sialylated HMOs are thought to be critical for brain development and cognition [4,5]. The two simplest sialylated HMOs are 3’-sialyllactose (3’-SL) and 6’-sialyllactose (6’-SL). The HMOs have a sialic acid moiety (N-acetyl-neuraminic acid) linked to the galactosyl subunit of lactose at position 3 or 6, respectively [1]. The concentration of sialylated HMOs in breast milk varies during the lactation period and also depends on the length of the pregnancy. The concentration of 3’-SL in breast milk usually range from 90-350 µg/mL during the first 35 lactation days [6,7], and then increases to 90-840 µg/mL during 0.5 to 8 months of breastfeeding [6,8]. However, breast milk of mothers who have given birth to preterm infants contain 13-23% more sialic acid than the milk of mothers who give birth at term [1,4,9,10].
Infant formula is used as a substitute when the infant cannot be breastfed and as a pediatric supplement to breastfeeding at the end of the breastfeeding period. The milk at the base of infant formulas is cow’s milk that in contrast to human breast milk contains only trace levels of HMOs. Interestingly, in cow’s milk, the concentration of sialylated oligosaccharides is much higher than that of fucosylated ones, with 3’-SL being the dominant type, but the concentration in cow’s milk is only 35-55 µg/mL [1,11]. The importance of HMOs for infant health and the significance of sialylated HMOs for cognition and brain development provide a strong incentive for producing biosynthetic HMOs for use as additives or ingredients in infant formulas.

A significant body of research has been performed to find ways to produce 3’-SL via biocatalytic synthesis. Some strategies use in vivo processes like those developed by Inbiose N.V. [12] and Jennewein Biotechnologie GMBH [13], respectively. Both companies have patented the use of engineered E. coli strains to synthesize 3’-SL via an intracellular biosynthesis route, but it is uncertain if this route is used commercially (for the sake of completeness it should be added that Glycom-DSM currently appear to mainly produce 2’-fucosyl lactose and lacto-N-neotetraose at large scale by E. coli fermentation [14]).

Another way is to produce 3’-SL through in vitro enzymatic reactions. Mcjarrow et al. [15] developed an enzymatic process already about 20 years ago using the sialidase from Arthrobacter ureafaciens. The patented reaction is accomplished in an aqueous system with κ(kappa)-casein glycomacropeptide (cGMP), a by-product from cheese manufacture, as sialic acid (Neu5Ac) donor [15].

Other classical enzyme-based methods for producing 3’-SL employ the native, true transsialidase from the human protozoan pathogen Trypanosoma cruzi (TcTS) [16–20]. The virulence of T. cruzi is tied to TcTS as the enzyme catalyzes transfer of sialic acid from host cell sialyl-glycoconjugates to the surface of T. cruzi cells to enable T. cruzi to evade the immune system in their mammalian hosts. Although the TcTS enzyme is not pathogenic on its own without T. cruzi cells, e.g. when produced recombinantly in a host organism such as the yeast Pichia pastoris, the original virulence property associated with the TcTS is considered an obstacle for the marketing of a food-grade HMO for infants resulting from a TcTS catalyzed reaction [21].

The idea behind the present study was to produce 3’-SL enzymatically directly in cow’s milk by transsialidase enzymes developed from the sialidase (TrSA) originating from the non-pathogenic protozoan Trypanosoma rangeli, using the lactose available in the milk as acceptor molecule, and cGMP as Neu5Ac donor in the reaction. TrSA was originally found to have no transsialidase activity, and to have only 70% sequence identity to TcTS, but an overall structure similar to TcTS [22]. However, TrSA mutants having only 5-6 amino acid mutations in the active site (M96V, A98P, S120Y, G249Y, Q284P (and I37L)) were reported to exhibit low transsialidase activity [21,23]. In further work, Nyffenegger et al. [24] carefully mutated
additional amino acids to give a total of 15 and 16 amino mutations in the TrSA to obtain the so-called Tr15 and Tr16 enzymes. These enzymes exhibit profoundly improved overall transsialidase activity by having reduced hydrolytic activity [24].

In the present study, we demonstrate that 3’-SL production directly in cow’s milk using Tr15 and Tr16 is indeed possible and moreover show that it is possible to achieve 3’-SL concentrations that are comparable to those found in breast milk. Different reactions were conducted to compare the enzymatic reactions in milk with those in aqueous buffer systems having lactose and cGMP added. In addition, different reaction temperatures were assessed to examine: (1) if the enzymatic transsialylation could take place even at low temperature, 5 °C, i.e. temperature at which milk is normally processed and stored and (2) to determine the activation energy, $E_a$, of the Tr15, Tr16 and TcTS transsialidase reactions. Lastly, 3’-SL stability studies were performed to examine the robustness of 3’-SL after pasteurization and freeze-drying.

2. Materials and Methods

2.1. Materials

Engvang organic whole milk was purchased in Lidl (Lidl Denmark K/S, Kolding). The content of lactose in the milk was determined to be 112 mM by liquid chromatography coupled to mass spectroscopy (LC-MS) analysis (method described below). κ-casein glycomacropeptide (cGMP), in the form of the commercial product Lacprodan cGMP-20, was a gift from Arla Foods Ingredients Group (Viby, Denmark). cGMP was gently purified prior to use by ultrafiltration using a 10 kDa regenerated cellulose membrane (RC70PP; AlfaLaval, Nakskov, Denmark). The cGMP solution was concentrated to 37.1 g/L corresponding to a sialic acid content of 10 mM determined after 48 h desialylation using the sialidase from Arthrobacter ureafaciens (Sigma–Aldrich, Steinheim, Germany). 10 mM sialic acid concentration is the sum of the α2,3- and α2,6-bound sialic acid that are distributed 50:50 in cGMP [25]. The 3’-SL standard was purchased from Carbosynth Ltd. (Compton, United Kingdom). All other chemicals were from Sigma–Aldrich (Steinheim, Germany).

2.2. Enzymes

The TrSA sialidase from T. rangeli was mutatated with 15 and 16 amino acid substitutions to give the enzymes called Tr15 and Tr16, respectively, that each were cloned and expressed in P. pastoris as described previously [24]. A 5 L scale production of recombinant sialidases (Tr15 and Tr16) in P. pastoris were performed according to Zeuner et al. [26]. The total time for the fermentation process was 112 h. Tr15 and
Tr16 enriched fermentation broths were recovered by centrifugation at 5300 × g at 5 °C for 1 h and the supernatants were subjected to sterile filtration and concentrated by ultrafiltration, using a cross-flow bioreactor system with a 30 kDa cutoff polyethersulfone membrane (Millipore, Sartorius, Denmark), as described previously [26]. Protein concentrations were 16.2 mg/mL and 20.5 mg/mL for Tr15 and Tr16, respectively, as determined by the Bradford method (Thermo Fisher Scientific, Hvidovre, DK) using Bovine Serum Albumin as standard. The enzyme aliquots were stored at −80 °C until use.

The transsialidase from Trypanosoma cruzi, TcTS, was used as benchmark. TcTS was cloned and expressed in P. pastoris following the same fermentation process as described above for Tr15 and Tr16. Protein concentration of TcTS was also assessed with the Bradford method and found to be 5.8 mg/mL.

2.3. Transsialidase reaction and dose response experiments

Transsialylation reactions by Tr15 and Tr16 in the whole milk and in the aqueous solution, respectively, were performed in reaction mixtures with each enzyme added to a final concentration of 15 µg/mL in either milk or aqueous solution containing lactose (final concentration of 16 mM lactose in both systems) and cGMP (final concentration of 31.1 g/L corresponding to 4.2 mM of 2,3-bound Neu5Ac) to obtain an acceptor donor ratio of 3.8. In order to run the reactions in the milk and aqueous system at the same conditions, the pH of the organic whole milk reaction mixture was measured and found to be pH 6.7 which was slightly lower than the pH of the aqueous solution reaction mixture. Hence, the pH of the latter was adjusted with one drop of 0.5 mM HCl. Single reactions were run in 350 µL reaction volume (in Eppendorf tubes) with temperature control using a thermomixer, with individual tubes for each reaction time from 0 to 120 min, at 25 °C and shaking at 1400 rpm. The reactions were then halted by thermal inactivation at 90 °C for 10 minutes. The dose response experiments were run in the same way as described above, but using additional enzyme concentrations of 1.5 µg/mL and 150 µg/mL. Formation of 3’-SL and sialic acid was determined by LC-MS as described below.

2.4. Activation energy determination

The enzymatic transsialylation reactions in the whole milk and in the aqueous solutions by Tr15, Tr16, and TcTS were performed in reactions mixtures as described above (150 µg/mL of enzyme, 16 mM lactose, 31.1 g/L of cGMP), but at various temperatures of 5, 10, 25, 30 and 40 °C. Single reactions were run in reaction volumes of 350 µL in Eppendorf tubes with shaking at 1400 rpm in a thermomixer, using individual tubes for each reaction time: 0, 1, 2, 3, 4, 5 and 7 minutes. The reactions were then halted by thermal inactivation at 90 °C for 10 minutes.
Transsialylation in organic whole milk and in aqueous solution by TcTS was performed at the same reaction conditions as described above. A single reaction was run in a thermomixer for 3 hours with shaking of 1400 rpm and at various temperatures of 5, 10, 25, 30 and 40 °C.

The reactions at the two lower temperatures; 5 and 10 °C were run in a thermomixer placed in a cold room, that allowed the thermomixer to maintain the temperature at 5 °C and 10 °C, respectively. Initial rates of 3'-sialyllactose formation (3'-SL levels determined by LC-MS as described below) were determined after two minutes reaction for each temperature for the Tr15 and Tr16 reactions, while for TcTS initial rates were determined during 3 hours reaction because of the lower activity of this enzyme.

The enzyme activation energy, $E_a$, of each reaction was determined from the Arrhenius equation (equation 1) which was reorganized for linearization (equation 2):

$$k = Ae^{-\frac{E_a}{RT}}$$  
$$\ln(k) = -\frac{E_a}{RT} + \ln A$$

where $k$ is the initial 3'-sialyllactose formation rate (mM/s), $E_a$ is the activation energy (J/mol), $R$ is the gas constant (8.3145 J/(mol·K)), $T$ is the temperature (in Kelvin, K) and $A$ is a constant (“Arrhenius constant”).

### 2.5. 3'-SL stability during pasteurization and freeze-drying processes

Stability of 3'-SL was assessed at three different pasteurization temperatures: At 62.5 °C for 30 minutes, also known as Holder pasteurization [27], and after 1 minute at 80 °C and 90 °C, respectively. During production of milk powder the latter heat treatments are usually used instead of the classic pasteurization treatment [28]. For the heat treatments, 1 mM 3'-SL was diluted 1:1 in water and in organic whole milk, respectively to a final volume of 300 µL. Each prepared sample was subjected to heat at the selected temperatures for the specific time and then 3'-SL concentration was determined by LC-MS analysis as described below. The remaining sample was then freeze dried overnight, solubilized in water to a volume equal to the one measured before the freeze drying step and the 3'-SL concentration was determined again by LC-MS analysis.

Stability of 3'-SL after heat treatments and freeze-drying was also assessed. Transsialylation reactions of organic whole milk was run for 5 minutes at 5 °C as explained above and the reaction was halted by treatment at 90°C for 10 minutes, the reaction volume was measured and the sample was freeze-dried overnight. The powder (reaction mixture) was rehydrated adding water to a volume equal to the one measured before the freeze drying step and 3'-SL concentration was determined again by LC-MS analysis.
2.6. LC-MS analysis

Lactose content in the whole milk and the Tr15, Tr16 and TcTS transsialylation products were analyzed and quantified using LC-MS. Prior to analysis the samples were diluted 100 times in 70 % v/v acetonitrile and centrifuged for 5 minutes to precipitate insoluble proteins (for quantitative lactose analysis in the original whole milk, the milk was diluted 1000 times in 70% v/v acetonitrile). Aliquots of 5 μL were injected onto a TSK gel Amide 80 HILIC column (150 mm × 2 mm; 2 μm, TOSOH, Greisheim, Germany). Chromatography was performed on a Dionex UltiMate 3000 UPLC (Thermo Fischer Scientific, Sunnyvale, CA, USA) operated at 0.2 mL min\(^{-1}\) and 45°C with a two-eluent system consisting of eluent A, 50 mM ammonium acetate pH 4.5, and eluent B, acetonitrile. The elution was performed as follows: 0-2 min, isocratic 20% A, 80% B; 2–25 min, linear gradient to 50% A, 50% B; hereafter going directly to 25–28 min isocratic gradient with 50% A, 50% B followed directly by 28–40 min isocratic equilibration with 20% A, 80% B. The HPLC was connected to an ESI-iontrap (model Amazon SL from Bruker Daltonics, Bremen, Germany) and the electrospray was operated in full scan mode with target mass settings of 400 m/z and a scan range from 70 to 2200 m/z. Automatic MS2 events was executed for the two highest prevalent precursor ions. 100% amplitude for fragmentation reaction was selected in order to obtain sufficient fragmentation. The spray settings were: capillary voltage of 4.5 kV, end plate offset 0.5 kV, nebulizer pressure at 3.0 bar, dry gas flow at 12.0 L min\(^{-1}\), and dry gas temperature at 280 °C.

2.6. Quantification of LC-MS data

Quantification of the precursor ion was performed using Bruker Compass QuantAnalysis software (Bruker Daltonik GmbH), defining a method to quantify 3'-SL and SA. All ions were observed as [M – H]\(^{-}\). SA quantification was performed by defining an Extracted Ion Chromatogram (EIC) on All MS of masses m/z 307.91, m/z 289.87, m/z 219.73, m/z 169.7 and m/z 118.81 with a width of ± 0.5, retention time 13.5 min with a window of 1 min. 3'-SL quantification was performed by defining an EIC on All MS of masses m/z 632.09 and m/z 289.86 with a width of ± 0.5, retention time 18.4 min and a window of 1 min. In all the cases peak detection was done using algorithm version 2.1, S/N threshold 1, area threshold 0.1, intensity threshold 0.1, skim ratio 0.1 and smoothing width 1. Calibration curve was performed using 8 levels of concentrations varying from 1 to 100 μM and fitting the data with a linear equation.

2.7. Statistical analysis

One-way ANOVA was used to determine statistical significance of the activation energy and stability of 3'-SL before and after pasteurization and freeze drying using Tukey’s test. The ANOVA calculations were done with the aid of RStudio (RStudio Inc., Boston, MA, USA). Statistical significance was established at p ≤ 0.05.
3. Results and Discussion

3.1. Transsialylation in milk

Transsialylation in the organic whole milk with cGMP by Tr15 at 25 °C led to levels of 3′-SL during 2 hours of reaction that were comparable to those produced in the parallel transsialylation reaction in the pH adjusted aqueous solution containing lactose and cGMP (Figure 1 a); initial rates based on rates during the first 20 minutes at the enzyme dosage level of 15 µg/ml were thus 0.11 – 0.16 mM/min in the two media: milk and aqueous solution, respectively. Hence, although initial rates tended to be higher in the aqueous solution, the complexity of whole milk as a reaction medium did not appear to significantly affect the yields achieved in the extended Tr15 reaction. In contrast, 3′-SL formation from Tr16 catalyzed transsialylation initially progressed at similar rates in whole milk and in the aqueous reaction medium, but were slower and led to lower 3′-SL production than the Tr15 catalyzed reaction (Figure 1 b). Then, after 1 hour of reaction, the Tr16 catalyzed transsialylation in milk appeared to slow down, while the 3′-SL production in the aqueous solution continued at what appeared to a constant rate (0.07 mM/min), leading to higher 3'SL levels in the aqueous solution than in the whole milk after 2 hours of reaction (Figure 1 b).

Interestingly, using a concentration of Tr15 of 15 µg protein/mL the concentration of 3′-SL measured after 10 minutes of reaction was 1.7 mM (Figure 1 a) corresponding to 1160 mg/L, which is higher than the 3′-SL concentration in breast milk, which is typically 90-840 mg/L [6,8]. On the contrary by performing the reaction with 15 µg/mL of Tr16 only 0.1 mM of 3′-SL was formed after 10 minutes (Figure 1 b) corresponding to 63 µg/mL. The only difference between Tr15 and Tr16 is the single, structural mutation I37L, which is present in Tr16, but absent in Tr15 [24]. The finding that 3′-SL product formation was faster with Tr15 than with Tr16 agrees with previous data [22,24]. The data on the transsialylation of Tr15 and Tr16 in the aqueous system obtained in the present work were directly comparable to previously reported results for these enzymes [24]. Hence, after 1 hour of reaction the amount of 3′-SL obtained by Nyfferegger et al. [24] was 0.7 mM and 1.1 mM for 1.5 µg/mL of Tr15 and 15 µg/mL of Tr16, respectively. The amount of 3′-SL obtained in the present work were 1.1 mM and 1.2 mM for 1.5 µg/mL of Tr15 and 15 µg/mL of Tr16, respectively (Supplementary Figure S1), corresponding to 27.5% and 31.6% sialylation yield for Tr15 and Tr16. The yields were thus better, but in very good agreement with previously reported data.

The reduced activity (of Tr16) caused by the I37L mutation has previously been tentatively suggested to be due to the mutation causing a conformational change of the nucleophile, which in turn bring about a less optimal fit of the substrate and the enzyme [24]. Apparently, this single amino acid difference between Tr15 and Tr16 also resulted in a larger difference in the reaction performance of Tr16 in milk versus in the aqueous reaction system (Figure 1b). The relatively lower transsialylation rate of Tr16 in milk compared to
Tr15 may be speculated to be a result of inhibition by milk components due to structural changes near the active site induced by I37L. Alternatively, it cannot be completely excluded that Tr16 may catalyze transfer of sialic acid to other acceptors than lactose in milk (relative to Tr15). A possible receptor could be the terminal galactose on cGMP, but this event would escape our analysis, and it is not immediately obvious why Tr16 would have a higher acceptor promiscuity than Tr15. In the present context, any such higher acceptor promiscuity by Tr16 would not change the overall conclusion, which remains that in milk Tr15 produced higher yields of 3′-SL than Tr16.

3.2. Effect of enzyme dose on transsialylation in milk

Dose response experiments of the transsialylation reactions catalyzed by Tr15 and Tr16 in whole milk verified that the concentration of 3′-SL was a function of the concentration of enzyme in the reaction mixture (Figure 1 c and d). The decrease of 3′-SL observed for the higher dosage of Tr15 (150 µg/mL) after 20 minutes of reaction is due to the hydrolytic activity of the enzyme beginning to catalyze degradation of
3'-SL. Hence, during the whole course of reaction two reactions take place: 1) transsialylation of lactose and 2) hydrolysis of the product 3'-SL or the SA donor cGMP.

**Figure 1.** Transsialylation by Tr15 and Tr16 in organic whole milk versus in an aqueous reaction system.

**a)** Comparison of the 3'-SL (circles) and SA (squares) concentrations during transsialylation of organic whole milk (close red symbols) and aqueous solution (open red symbols) by 15 µg/mL Tr15. **b)** Comparison of the 3'-SL (triangles) and SA (squares) concentrations during transsialylation of organic whole milk (close red symbols) and aqueous solution (open red symbols) by 15 µg/mL Tr16. **c)** Dose response of organic whole milk transsialylation by Tr15: 1.5 µg/mL, green circles; 15 µg/mL, red circles and 150 µg/mL, blue circles. **d)** Dose response of transsialylation in organic whole milk by Tr16: 1.5 µg/mL, green triangles; 15 µg/mL, red triangles and 150 µg/mL, blue triangles. Data are averages of true triplicate repeats and data are given ± standard deviations.
Clearly, the higher the enzyme concentration, the higher is the 3’-SL concentration and hence the more prominent is the hydrolytic reaction. Since Tr16 appeared to be a slower enzyme compared to Tr15, it was not possible to picture the same decrease of 3’-SL levels for Tr16 as for Tr15 reactions. However, the exact same pattern was obtained for the transsialylation in aqueous solution for both enzymes (Supplementary Figure S1).

3.3. Reaction temperature and enzymatic reaction activation energy

Transsialylation of organic whole milk was also tested at 5 °C (Figure 2). This temperature was chosen since 5 °C is the temperature at which milk is handled in almost all processes in order to reduce bacterial growth and keep unwanted enzymatic activities in check [29]. Both Tr15 and Tr16 showed transsialidase activity at 5°C and 150 µg/mL Tr15 formed 1.3 mM of 3’-SL after 5 minutes of reaction, which corresponds to 802 µg/mL. With longer reaction time the Tr15 reaction yielded to a total of 0.28 mg of 3’-SL in the final sample (Figure 2a). The Tr16 reaction was slow at 5°C; only 0.02 mM of 3’-SL were produced after 5 min reaction (Figure 2b).

TcTS was used as benchmark enzyme to compare the transsialidase activity of Tr15 and Tr16 (Supplementary Table S1). Longer reaction times had to be performed for the TcTS catalyzed transsialylation reactions in whole milk due to the inherent lower activity of this enzyme compared to Tr15 and Tr16. After 3 hours reaction at 5 °C only 0.04 mM of 3’-SL were produced by TcTS. Hence the 3’-SL formation with TcTS was 33 times lower than the 3’-SL concentration obtained in 5 minutes with Tr15 (Supplementary Table S1).

As expected, the yields of 3’-SL during transsialylation of whole milk by Tr15, Tr16 and TcTS were found to be a function of the reaction temperature, i.e. the higher the temperature the faster the reaction (Figure 2 and Supplementary Table S1). The same conclusion was obtained for parallel enzymatic transsialylation reactions in aqueous solution (Supplementary Figure S2 and Table S1).
Figure 2. Organic whole milk transsialylation by Tr15 and Tr16 at different reaction temperatures.

a) Tr15 catalysis (150 µg/mL): Concentration of 3'-SL at different temperatures of transsialylation in organic whole milk: 5°C, blue circles; 10°C, red circles; 25°C, yellow circles; 30°C, violet circles; 40°C, green circles.

b) Tr16 catalysis (150 µg/mL): Concentration of 3'-SL at different temperatures of transsialylation in organic whole milk: 5°C, blue triangles; 10°C, red triangles; 25°C, yellow triangles; 30°C, violet triangles; 40°C, green triangles. Data are averages of true triplicate repeats and data are given ± standard deviations.

Activation energies for each of the enzyme reactions in milk and aqueous solution were determined from Arrhenius plots (Figure 3). As anticipated, for all three enzymes Tr15, Tr16 and TcTS the slopes of the regression lines in the Arrhenius plot (logarithm of the initial rate vs the inverse of the temperature) for the transsialylation in aqueous solution and milk was the same. Hence, there was no statistically significant difference between the transsialylation activation energy, $E_a$, for the reaction in milk versus the one in aqueous solution for any of the three enzymes (Table 1).

Tr15 showed the lowest $E_a$ among the three enzymes, i.e. $E_a$ was 22.5 and 16.5 kJ/mol for transsialylation in aqueous solution and in milk, respectively, explaining the faster reaction of this enzyme compared to the two others. The additional mutation performed in Tr16 compared to Tr15 was already shown to be responsible of a lower transsialidase activity [24] and the present study clearly confirmed it, and expanded the explanation by showing a higher $E_a$ for Tr16 transsialylation then for Tr15 transsialylation.
Figure 3. Arrhenius plot for the transsialylation reaction of organic whole milk and aqueous solution by Tr15, Tr16 and TcTS. Transsialylation by Tr15, red circles; transsialylation by Tr16, blue triangles and transsialylation by TcTS, green squares. All open symbols refers to transsialylation in aqueous solution while all closed symbols refers to transsialylation in organic whole milk.

Table 1. Activation energy, $E_a$, of the transsialylation reactions in aqueous lactose solution and organic whole milk catalyzed by Tr15, Tr16 and TcTS. The $E_a$ values are given as averages of true triplicate determinations. Different roman superscript letters indicate significant differences ($p < 0.05$) between the $E_a$ values obtained.

|          | Lactose solution | Milk   |
|----------|------------------|--------|
| Tr15     | $22.5 \pm 1.5^c$| $16.5 \pm 3.1^c$ |
| Tr16     | $58.4 \pm 1.6^{ab}$| $66.0 \pm 3.3^a$ |
| TcTS     | $54.1 \pm 1.2^b$ | $49.8 \pm 4.1^b$ |

3.4. Stability of 3′-SL during pasteurization and freeze-drying processes

The stability of 3′-SL was assessed at three different common pasteurization temperatures. Breast milk, which is donated to breast milk banks, is normally pasteurized using the Holder method, i.e. at 62.5 °C for
30 minutes, a treatment which has been shown previously to not affect the concentration of HMO in the milk [27]. Cow’s milk used for the production of powdered milk is instead pasteurized at higher temperature for shorter times; typically at 80-90 °C for 1 minute [28]. After pasteurization, milk intended for infant formula will undergo a drying process. In this study, we used lyophilization as drying method.

The concentration of 3’-SL (0.5 mM initial concentration) dissolved in water and milk, respectively, appeared not to be affected significantly by neither the pasteurization nor the freeze drying step (Table 2).

Freeze-drying of the reaction mixture resulting after 5 minutes transsialylation reaction in organic whole milk by Tr15 at 5 °C was performed to study the stability of 3’-SL in the reaction mixture. The concentration of 3’-SL was measured before (1.39 ± 0.06a mM) and after (1.40 ± 0.06a mM) freeze-drying and no statistical difference between the two concentrations was found. Therefore, the freeze-drying process is considered a safe and gentle drying process that does not affect the stability of 3’-SL.

Table 2. Stability of 3’-SL after pasteurization and freeze-drying processes. Concentration of 3’-SL before, after pasteurization and after freeze-drying are reported for two systems: 1 mM 3’-SL dissolved in water and 0.5 mM 3’-SL dissolved in organic whole milk. Different superscript letters indicate significant differences (p<0.05) between the 3’-SL concentration before, after pasteurization and after freeze-drying. Statistical analysis was performed for each distinct pasteurization temperature and system.

| Heat treatment | 3’-SL [mM] in water | 3’-SL [mM] in milk |
|----------------|---------------------|-------------------|
| Before         | 0.55 ± 0.08a        | 0.37 ± 0.07b      |
| After          | 0.54 ± 0.09a        | 0.47 ± 0.04a      |
| After Freeze Drying | 0.61 ± 0.05ab | 0.37 ± 0.01b      |
| Before         | 0.55 ± 0.08a        | 0.37 ± 0.07a      |
| After          | 0.62 ± 0.07a        | 0.40 ± 0.07a      |
| After Freeze Drying | 0.60 ± 0.06a | 0.45 ± 0.01a      |
| Before         | 0.55 ± 0.08a        | 0.37 ± 0.07a      |
| After          | 0.53 ± 0.11a        | 0.26 ± 0.03ab     |
| After Freeze Drying | 0.56 ± 0.06a | 0.20 ± 0.01b      |
4. Conclusions

Transsialylation reaction in organic whole milk by Tr15 and Tr16, using cGMP as the right Neu5Ac-type sialic acid donor, was verified and it was shown that for the Tr15 enzyme the 3’-SL concentrations achieved in milk were comparable to those achieved in aqueous solution. Hence, the complex milk structure, including presence of casein micelles and lipid droplets, did not inhibit the enzyme. It was also established that the enzymes were able to work even at 5°C. For the specific case of Tr15, a reaction time of only 5 minutes with an enzyme concentration of 150 µg/mL was sufficient to produce a concentration of 3’-SL which was higher than the one found in human breast milk.

$E_a$ values of the two *T. rangeli* sialidases Tr15 and Tr16 were compared with the $E_a$ of the *T. cruzi* transsialidase TcTS. Among the three enzymes, Tr15 had the lowest $E_a$, which corroborated the faster transsialylation reaction of this enzyme compared to Tr16 and TcTS.

Finally, neither pasteurization nor freeze-drying affected the 3’-SL concentration in a remarkable manner.

The finding that it is possible to run the Tr15 catalyzed transsialylation reaction effectively at 5 °C, combined with the high stability of 3’-SL after pasteurization and drying are promising options for designing direct enzymatic transsialylation in milk for industrial production of 3’-SL enriched infant formulas.

Declaration of competing interests

Dr. Valentina Perna and Dr. Christian Dehlholm are employed by Mille International, a company that trade specialized foods including products for infants. The authors declare no conflict of interest.

Author agreement.

All authors agree to this submission.

CrediT author statement

VNP, CD, ASM: Conceptualization. VNP: Experiments and Analytical Work. VNP, ASM: Data Interpretation. CD, ASM: Supervision, Funding. VNP: Original Draft Preparation. VNP, ASM: Manuscript Writing, Editing.

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