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Fluorometric determination of free and total isocitrate in bovine milk

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ABSTRACT

Isocitrate is an intermediate metabolite in the citric acid cycle found both inside the mitochondria as well as outside in the cytosolic shunt. Oxidation of isocitrate is believed to deliver large fractions of energy [i.e., reducing equivalents (NADPH) in the bovine udder] used for fatty acid and cholesterol synthesis. This study describes a new analytical method for determination of free and total isocitrate in bovine milk where time-consuming pretreatment of the sample is not necessary. Methods for estimation of both total isocitrate and free isocitrate are described, the difference being the esterified or even lactonized isocitrate. On average, 20% (6–27%) of cow milk isocitrate was esterified and free isocitrate correlated significantly with total isocitrate (r = 0.98). The present fluorometric determination correlated well with the traditional spectrophotometric determination of isocitrate. Milk samples from Danish Holstein cows (984) contained significantly less isocitrate than milk from Danish Jersey cows (760; i.e., 0.134 vs. 0.211 mmol/L). Isocitrate in milk is correlated to milk protein, fat, and citrate, and it is speculated, based on biochemistry, former studies, and the present, that isocitrate may reflect the energy situation in the mammary gland. The use of isocitrate as a biomarker of the energy status in the dairy cow is warranted.
Key words: esterified isocitrate, milk, fluorometry, biomarker, energy status

INTRODUCTION

Isocitric acid is widespread in living material (e.g., bacteria, plant, and animal tissues). The formation of isocitrate is, in mammalian metabolism, closely connected to citric acid, which in turn is an important intermediate in energy metabolism. Isocitrate is oxidized by the enzyme isocitrate dehydrogenase (**ICDH**), where carbon dioxide is liberated and 2-oxoglutarate is the oxidized product. Three main groups of ICDH are known: (1) NAD^+-dependent (EC 1.1.1.41), abundant inside the mitochondria and an important regulatory region of the citrate cycle (TCA cycle); (2) $NADP^+$-dependent form, also inside the mitochondria (EC 1.1.1.42, 2), and (3) $NADP^+$-dependent form in the cytosol (EC 1.1.1.42, 1). However, both citrate and isocitrate are able to cross the mitochondrial membrane, alleviating intra- and intermitochondrial exchange of energy precursors.

Lipogenesis in the ruminant is very different from lipogenesis in nonruminant animals, both considering precursors for the FA and considering the origin of energy supply. In ruminants, about one half of the milk FA (molar percent) are derived from de novo synthesis (Bauman and Griinari, 2003). β-Hydroxybutyrate and acetic acid are important precursors in ruminant lipogenesis. Fatty acid synthesis is an energy-demanding series of reductive steps using reducing equivalents in the form of NADPH, namely 2 mol per 2C-incorporation. A significant part of the NADPH-production in ruminant mammary glands is believed to be oxidative decarboxylation of isocitrate (Bauman et al., 1970; Gumaa et al. 1973; Chaiyabutr et al., 1980). The latter process is mediated by extra mitochondrial ICDH (EC 1.1.1.42; 1), also believed to be the main energy source for synthesis of cholesterol (Moore and Christie, 1979). Cytosolic $NADP^+$-dependent ICDH activity is therefore abundant in ruminant mammary cells (Bauman et al. 1970; Gumaa et al. 1973).

Some attention has been given to isocitrate and ICDH activity in ruminant mammary gland and milk to describe the physiological status of the animal or the production conditions. Most of these observations support the important role of citrate-isocitrate-2-oxoglutarate in the milk energy turnover.

Measurements of isocitrate in milk have so far all been based on spectrophotometry, which is not an ideal method considering the opaque protein and fat matrix. Analyses are performed after a pretreatment of the sample (i.e., centrifugation to separate the fat), followed by precipitation of protein with perchloric acid ($HClO_4$) to obtain a clear fraction and subsequent neutralization (Faulkner, 1980; Faulkner and Clapperton, 1981; Chaiyabutr et al., 1981; Faulkner et al., 1986).

The present analytical method for determination of isocitrate is principally based on the spectrophotometric

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(UV) method (Beutler, 1989); however, the produced NADPH is subsequently used in a second reaction for reduction of the nonfluorescent precursor resazurin to produce the fluorescent substance resorufin, which is detected by fluorometry. The present fluorometric technique renders pre-treatment of the sample superfluous. It is well recognized from food analyses that a certain fraction of the isocitrate may be esterified or lactonized (internal esterification; Boehringer Mannheim, 1995). We have used this obtained knowledge to distinguish between free and total isocitrate by the fluorometric method.

MATERIALS AND METHODS

Samples and Standard Analyses

Milk samples (n = 1,744) were from the resident herd at the Danish Cattle Research Centre (Tjele, Denmark). Analyzed samples were used to investigate the correlation among free isocitrate, selected milking data, other milk components, and udder health as indicated by milk SCC. The milking system is a robotic system, where representative milk samples are taken in a 10-mL tube, predosed with the preservative Bronopol (Myacide, Pharma BP, BSF Ltd., Nottingham, UK) to obtain a 100-mg/kg sample. The samples were stored at 4°C and brought to the laboratory every morning. Milk citrate, lactose, fat, and protein were determined by infrared spectroscopy (CombiFoss 4000, Foss Electric Ltd., Hillerød, Denmark). Determination of SCC was performed at a commercial laboratory (Eurofins, Holstebro, Denmark) using standard Fossomatic cell counter (EN ISO 13366–3; Foss Electric Ltd.).

Intrinsic milk enzymes were inactivated (denatured) by heating the milk samples in a water bath. Isocitrate in milk is found in a free state and in an esterified state. Samples analyzed for free isocitrate were heat-treated at 75°C for 10 min. Samples analyzed for total isocitrate were adjusted to pH 10 with 1 mol/L of NaOH and heat-treated at 96°C for 20 min, because heat and the alkaline environment breaks the chemical ester bond between lactate and other intrinsic components. Thus, total isocitrate is the sum of free isocitrate and esterified isocitrate.

Isocitrate Analysis

Two reagents were necessary for the detection of isocitrate by fluorometry. Reagent 1 contained the enzyme that mediates the conversion of isocitrate to 2-oxoglutarate (i.e., ICDH; EC 1.1.1.42), Tris-buffer, pH 7.2, $NADP^+$, and Mn-ions that activates the enzyme. Reagent 2 contained the necessary fluophore, resazurin, and the enzyme mediating the reduction of resazurin, diaphorase (EC 1.6.99.1), both dissolved in Tris-buffer with Triton X-100 (Merck, Darmstadt, Germany).

Eighty microliters of diluted sample (1:2 water) was pipetted into a microplate, then 60 μL of reagent 1 was added ($t = 0$) and the mixture was incubated 3 min (incubation 1) at room temperature. After this, 40 μL of reagent 2 was added and the plate was incubated for an additional 6 min (incubation 2) and read (excitation: 544 nm; emission: 590 nm) in a fluorometer (FluoStar Galaxy, BMG Labtechnology, Germany). Incubation conditions for incubation 1 were: sample fraction = 0.19; isocitrate maximum = 0.076 mmol/L; Mn^{++} = 0.77 mmol/L; Tris-buffer (pH 7.2) = 31 mmol/L; ICDH activity = 0.11 U/mL; β-$NADP^+$ = 0.123 mmol/L. Incubation 2 conditions were: diaphorase activity = 2.2 U/L; resazurin = 0.28 mmol/L; Triton X-100 = 0.0024%; Tris-buffer = 34 mmol/L.

Analytical Details

The ICDH oxidizes isocitrate to 2-oxo-glutarate, whereas $NADP^+$ is reduced equivalently to NADPH + H^+ at pH 7.2. The reaction is activated by Mn ions. The developed NADPH + H^+ equivalently reduced resazurin to resorufin, mediated by the enzyme diaphorase; the amount of resorufin is detected by fluorometry and compared with a standard curve. All dilutions and additions of reactants were performed in a laboratory robotic system (Biomek 2000; Beckman Coulter, Pasadena, CA).

Standards and Controls

Standards were prepared in isocitrate-free milk: 45 mL of milk were supplied with 5 mL of 1 mol/L Tris-buffer, pH 7.2, 25 mg of Mn-sulfate, 14 U of ICDH, and 10 mg of $NADP^+$ and incubated for 60 min at 40°C. Terminally, 1.0 mL of hydrogen peroxide (15%) was added, mixed immediately, and the mixture was heat-treated at 75°C for 10 min. Standards were prepared by addition of DL-isocitrate (Acros 205010010, MW 258.07; Acros Organics, Geel, Belgium) to the isocitrate-free milk; concentrations of standards were 0, 0.02, 0.04, 0.08, 0.10 0.20, 0.30, and 0.40 mmol/L of D-isocitrate. Control samples were produced likewise, using 0.069 and 0.227 mmol/L of D-isocitrate. Standards and controls were measured in duplicate on every microplate.

Verification of the Assay

Four hundred thirty milk samples were analyzed for both total isocitrate and free isocitrate according to

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