Xanthine Oxidase Polymorphism in Bovine Milk

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Abstract

The biological activity of xanthine oxidase (XO) was determined polarographically in situ in milk from 92 Guernseys and 39 Holsteins. Individual assays of the milk from each breed formed three nonoverlapping distributions. Means and standard errors for Guernsey milk based on 21 animals with low XO activity, 50 animals with medium XO activity, and 21 animals with high XO activity were 30 ± 0.9, 52 ± 0.9, and 80 ± 1.5 μliters O₂ per ml milk per hr. Corresponding XO activities for Holstein milk from 8 low XO activity, 20 medium XO activity, and 11 high XO activity animals were 28 ± 2.4, 48 ± 1.9, and 75 ± 2.1 μliters O₂ per ml milk per hour. A nonsignificant interaction between the sires and the activity of their daughters was found. However, results with the daughters of five sires representing 48 of the 81 animals suggested that the two alleles present are not random; they depend upon the genotype of the parents. Genetic polymorphism of the gene responsible for this enzyme with two alleles showing no dominance and additive gene action is proposed with the following nomenclature: high activity allele XOA; low activity allele XO.B. Gene frequencies are: Guernsey, XOA = 0.50; XO.B. = 0.50, se ± 0.037; Holstein, XOA = 0.54; XO.B. = 0.46, se ± 0.058.

Introduction

Xanthine oxidase (XO) is a naturally occurring oxidizing enzyme in milk which controls biologically the last stages of purine catabolism by removal of hypoxanthine and xanthine in the form of uric acid from the general pool of purines (Fig. 1). The presence of this enzyme in excess, or its absence, inhibition, or stimulation, reflects on the chemistry of normal or abnormal cellular activity (4). Quantitative changes in this enzyme have been observed in neoplasia and nonmalignant hyperplasia. Xanthase oxidase activity was absent (normal: 0.81 μmoles uric acid formed/min/100 mg tissue) during careinogenesis in both liver and breast tumors (7, 10). Likewise, enzyme activity decreased fourfold in various mouse tumors (16). On the other hand, during rapid liver regeneration cellular XO approximately doubled (11). Haddow el al. (14) demonstrated that XO inhibited growth of spontaneous mammary carcinoma in mice even when XO was from a different species. They observed an increase in activity of approximately 2-5-fold for both tumor and liver tissue in the treated animals (14). Besides being present in relatively large amounts in bovine milk, XO is also present in milk of other ruminants such as sheep and goats but is devoid in human, sow, and mare milk (20). The fact that there are no reports on the occurrence of mammary tumors in cows, sheep, and goats may be correlated with XO.

Studies on the biochemical mechanism for the release of iron from hepatic ferritin stores have revealed participation of XO and were confirmed with intact guinea pigs, rabbits, and dogs (19). Administering XO substrates increased plasma iron in these animals. Individuals with xanthinuria are devoid of XO, excrete xanthine at the same rate as normal individuals excrete uric acids, and form xanthine stones. Xanthine oxidase is involved in the metabolic pathway for which several different defects have been responsible for development of gout in humans (25).

Xanthine oxidase has been implicated in the development of undesirable oxidized flavor in market milk and other dairy products (1, 2, 3). The limited data of Gudnason and Shipe (13) and preliminary data collected in this laboratory (26) indicated that the activity of this enzyme was distributed into three closely grouped populations rather than being randomly distributed over the entire range of activities. Additional investigations were undertaken to determine whether this distribution...
**Experimental Procedure**

Milk from 191 individual Guernsey and Holstein cows from the University of Delaware herds and two nearby herds\(^3\) were assayed. Of the 191 cows screened, 131 were suitable for genetic study. The remainder were eliminated either because they were suspects for mastitis, which increased XO activity (21, 28), or because they were daughters of sires for which few female offspring were available. Activity of XO was assayed polarographically by a modification of the method of Ball (5) using a Biological Oxygen Monitor, Model 534 equipped with the Clark type electrode (9). This method precisely analyzes oxygen tension in solution and measures the partial pressure of dissolved oxygen directly. This was accomplished in a closed system.

The sampling technique was as follows: the first 35 to 40 ml of milk were collected at an afternoon milking directly into a 57 g plastic bottle and immediately placed in a water bath at 38 C. It is important that the freshly collected milk be kept at body temperature because upon cooling, the apparent XO activity is elevated (5, 13, 24, 27). The samples were returned to the laboratory and assayed within 100 min of sampling. All metal contact with the fresh milk was avoided since metal ions affect activity of this enzyme (6). Because the milk from each quarter of an animal differed slightly in XO activity (due to dilution effects, etc.), milk was obtained only from the right front quarter of each cow.

The polarographic assay of enzyme activity was: 3 ml of milk plus 0.2 ml of 0.1M phosphate buffer (pH = 7.2) and a magnetic stirring bar were placed in the sample chamber of the bath assembly. Three minutes were allowed for temperature equilibrium (38 C) and complete saturation with oxygen by bubbling air through the sample. The probe was carefully inserted into the sample chamber, and special care was taken to eliminate all air bubbles. A constant base line was then established on the strip chart recorder (no oxygen was being consumed by the sample). This permitted elimination of milk samples from those animals where spontaneous autooxidation was occurring. In most cases, this was due to high bacterial contamination and general symptoms of the various stages of mastitis (21, 28). Then, 50 \(\mu\)liters of \(3.13 \times 10^{-3} \) m hypoxanthine (in 0.05 m NaOH) were injected into the sample chamber.

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\(^{3}\) The contributions of University of Delaware project 604 A.S. (Dairy Cattle Management—Dr. G. F. W. Haenlein, supervisor; I. Huff, herdsman); Chr. Zeitler, H. Zeitler, Zeitler Farms; and R. Trivits, Winterthur Farms are gratefully acknowledged.

\(^{4}\) Obtained from the Yellow Springs Instrument Co., Yellow Springs, Ohio.
TABLE 1. Reproducibility of the polarographic method by quintuplicate determinations on milk from six cows.a

<table>
<thead>
<tr>
<th>Cow</th>
<th>Date Assayed</th>
<th>Activityb,c</th>
<th>Range</th>
<th>± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>H280</td>
<td>3/15/70</td>
<td>34.9</td>
<td>34.7–35.0</td>
<td>0.06</td>
</tr>
<tr>
<td>H197</td>
<td>3/15/70</td>
<td>34.7</td>
<td>34.7</td>
<td>0.00</td>
</tr>
<tr>
<td>H286</td>
<td>3/15/70</td>
<td>62.4</td>
<td>62.0–62.7</td>
<td>0.02</td>
</tr>
<tr>
<td>H273</td>
<td>3/15/70</td>
<td>45.9</td>
<td>45.7–46.7</td>
<td>0.19</td>
</tr>
<tr>
<td>H258</td>
<td>3/15/70</td>
<td>77.2</td>
<td>76.3–78.3</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>81.3</td>
<td>81.0–82.7</td>
<td>0.51</td>
</tr>
</tbody>
</table>

a 0.016% of the variance was due to the polarographic assay method and only one determination was necessary to classify accurately each animal (23).
b Each value is the average of five determinations.
c Activity in μliters oxygen per milliliter milk per hour.

with a microsyringe and 2 to 10 min were allowed for the completion of reaction. The pH of the final mixture (i.e., milk plus phosphate buffer plus alkaline substrate) was 6.7 to 6.8, varying slightly between individual animals. This is within the physiological pH range of normal milk. The rate of the reaction was monitored by following the oxygen consumption which was recorded directly on the strip chart recorder.

The reproducibility of the polarographic method to assay enzyme activity was examined by a series of determinations of five successive aliquots from a single milk sample from each of six cows. From the range of activity, the standard error, and one-way analysis of variance (23) (Table 1), the reproducibility of the assay method was excellent.

Since XO activity is elevated by cooling milk, the effect of holding time at 38 C on XO activity was studied. Figure 2 represents activity versus time for milk samples from three animals. This figure indicated that milk samples can be maintained at 38 C for approximately 5 hr after sampling without a significant change in XO activity. Significant increases in XO activity became evident subsequent to 5 hr incubation in all samples at the three activities, but it appeared that elevation in XO activity was greatest in the high activity XO milk sample. All milk samples subsequently assayed for XO activity were analyzed individually within 100 min after sampling.

The reaction rate for XO was determined by calculating the actual oxygen consumption:

a) Assuming a change from 85 to 50% of saturation in 3 min. This means that 85 – 50

Fig. 2. Time study of xanthine oxidase activity in fresh milk at 38 C.
XANTHINE OXIDASE

= 35 or 35/85 = 41% of oxygen in solution was consumed in 3 min.

b) Assuming that the content of dissolved oxygen in milk at 38°C was 7.5 mg/liter (12) or 7.27 µliters/ml; the density of milk was 1.0313 g/ml.

c) The total volume of the mixture was 3.20 ml (3 ml milk plus 0.2 ml of phosphate buffer). When a 3.20 ml of this mixture was saturated with air, it contained approximately (7.27 µliters/ml) (3.20 ml) = 23.26 µliters O₂. A change of 41% in saturation means (23.26) (41%) = 9.54 µliters O₂/3 ml milk was consumed in 3 min or (9.54) (60/3) = 190.80 µliters O₂/3 ml milk/hr or 190.80/3 = 63.60 µliters O₂/ml milk/hr.

Results

Three typical determinations are shown in Figure 3. This graph represents per cent oxygen saturation as a function of time, and the three determinations are high (214), medium (121), and low (68) enzyme activity expressed in µliters O₂/3 ml milk/hr. The data from the milk of the 131 cows are summarized in Table 2. Assays of milk of individual animals from each breed formed three nonoverlapping distributions. Means and standard errors for the Guernsey milk based on 21 cows with low XO activity, 50 cows with medium XO activity, and 21 cows with high XO activity were 30 ± 0.9, 52 ± 0.9, and 80 ± 1.5 µliters O₂/ml milk/hr. The corresponding activities for Holstein milk from 39 cows are also in Table 2. Figure 4 depicts more clearly the distribution of animals for the Guernsey breed (data from Table 2) for the three activity groups. Xanthine oxidase activity formed a distribution curve with three peaks at 59, 156, and 239 µliters O₂/3 ml milk/hr indicating three distinct populations.

Table 3 shows the distribution of daughters by sire according to XO activity. The criterion to classify XO activity in milk of animals assayed into three groups was as follows. Cows whose milk gave a XO activity range of 10 to 35 were classified low, those with a XO activity range of approximately 40 to 65 were classified medium, and those with a XO activity range of about 70 to 100 (µliters O₂/ml milk/hr) were classified high. From Table 3, one can determine the most probable genotype of the particular sire by the distribution of XO activity of his daughters. For example, Sires 2, 3, and 4 were most probably homozygous for high XO activity since all of their daughters assayed had either high or medium activity, and none

Fig. 3. Typical rates of oxygen uptake measuring in situ xanthine oxidase activity of the milk from high, medium, and low activity animals.
had low. Likewise, Sires 1 and 5 were most probably homozygous for low XO activity since all of their daughters had either low or medium XO activity, and none had high. The remainder of the sires (6 to 10) were heterozygous for XO activity since their daughters were distributed into all three groups.

Statistical analysis of the data of Table 3 showed a nonsignificant interaction between sires and XO activity in the milk of their daughters, as determined by the Fisher's Exact Test (22), a Chi Square test correcting for continuity. However, results with the daughters of Sires 1 to 5 (representing 48 of the 81 cows assayed) suggested that the two alleles involved segregated nonrandomly in these sire groups. In three daughter-dam comparisons only one of the three dams was homozygous for low (23 μliters O₂/ml milk/hr) XO activity. Two of her daughters were assayed and were likewise homozygous for low (22 and 20 μliters O₂/ml milk/hr) XO activity. Also, a pair of confirmed identical twins with different freshening dates both had medium XO activity of 58 and 59 μliters O₂/ml milk/hr.

Although segregation at this locus is independent, these observations suggest the two alleles that control XO activity are not segregating randomly. They depend upon the genotype of the parents. Genetic polymorphism of the gene responsible for this enzyme with two alleles showing no dominance and additive action is proposed as an explanation for these observations. The following nomenclature is suggested: high activity allele XOₐ; low activity allele XO₀. The gene frequencies (as calculated by the binomial expansion, a² + 2ab + b²) were as follows: Guernsey, XOₐ = 0.50; XO₀ = 0.50, se ± 0.037 and that for Holstein, XOₐ = 0.54, XO₀ = 0.46, se ± 0.058.

Table 2. Distribution of xanthine oxidase activities of individual cows.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Activity Group</th>
<th>Number</th>
<th>Range</th>
<th>x</th>
<th>± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guernsey (92)</td>
<td>Low</td>
<td>21</td>
<td>18-35</td>
<td>30</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>50</td>
<td>39-64</td>
<td>52</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>21</td>
<td>69-97</td>
<td>80</td>
<td>1.5</td>
</tr>
<tr>
<td>Holstein (39)</td>
<td>Low</td>
<td>8</td>
<td>20-36</td>
<td>28</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>20</td>
<td>37-63</td>
<td>48</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>11</td>
<td>67-86</td>
<td>75</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*μLiters oxygen per milliliter milk per hour.
Table 3. Distribution of xanthine oxidase activities within Guernsey sires.

<table>
<thead>
<tr>
<th>Sire identification</th>
<th>Total no. of daughters for each sire</th>
<th>X.O. activity levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<td>0</td>
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<td>9</td>
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<td>1</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Chi square (P > 0.05) was calculated by the Fisher's Exact Test (22) which corrects for continuity.

<sup>b</sup> Low activity $\bar{x} = 30 \mu$liters O<sub>2</sub>/ml milk/hr.

<sup>c</sup> Medium activity $\bar{x} = 52 \mu$liters O<sub>2</sub>/ml milk/hr.

<sup>d</sup> High activity $\bar{x} = 80 \mu$liters O<sub>2</sub>/ml milk/hr.

Discussion

The data suggest that the enzyme XO in cow's milk is under direct genetic control, as has been demonstrated with many other enzymes. Two alleles showing no dominance with additive activity can explain the observations. For example, a cross between a homozygous cow for high XO activity and a sire homozygous for low XO activity would result in an offspring with medium XO activity (heterozygous). This medium activity can be explained on the basis that the animal possesses an equal mixture of two enzyme molecules, one with high activity and another with low which total to an apparent medium activity.

In nearly all studies concerning milk XO, the enzyme has been purified either from pooled fresh milk or from pooled pasteurized buttermilk. To the knowledge of the authors, the only exception is the work of Hart and Bray (15). They purified the enzyme from the milk of three individual cows and found at least two forms of XO, one active and another with negligible activity (which they assumed was devoid of molybdenum). It was assumed that the latter form was secreted by cows along with the active form. This is not in disagreement with the observations in this study, providing the three cows they assayed were heterozygous. Their observation supports the assumption that the heterozygous animal has a mixture of two XO molecules.

Results from such studies of XO have been varied and inconsistent in molecular weight and content of flavin adenine dinucleotide, molybdenum, and iron (8, 15, 18). Since present in situ findings, comparison of daughter-dams homozygous for either low or high XO activity and subsequent characterization of purified XO from the milk of such individual cows would conclusively prove or disprove the hypothesis. If the hypothesis is proven true, it would clarify existing discrepancies in the literature concerning XO. The present findings also have the following immediate practical application: in a genetic improvement program, one could select for animals that synthesize milk with either low or high XO activity.

References