Carbohydrate Composition in Bovine Milk Alkaline Phosphatase

Yasuo KUZUYA, Yoshihiro KANAMARU
and Tamotue TANAHASHI

Department of Poultry and Animal Sciences, Faculty of Agriculture Gifu University, Gifu-shi 501-11

(Received December 15, 1982)

Many studies have been carried out on the carbohydrate composition of alkaline phosphatase (EC 3.1.3.1) from various biological sources. It has been reported that most of the purified microbial and mammalian alkaline phosphatases contain fucose, mannose, galactose, glucosamine, galactosamine and sialic acid in varying amounts. However, most of these studies have been carried out only on bovine milk alkaline phosphatase and there is a complete paucity of data for the carbohydrate of bovine milk alkaline phosphatase.

In this report the determination of carbohydrate composition of bovine milk alkaline phosphatase and its comparison with those of calf intestinal alkaline phosphatase was conducted as a part of investigation on the role of the saccharide residue. The effect of hydrolysis by sialidase on bovine milk alkaline phosphatase activity is also discussed.

Materials and Methods

Bovine milk alkaline phosphatase was purified according to the procedures reported previously. The purification of commercial calf intestinal alkaline phosphatase (Boehringer Mannheim Co Ltd) was attained by polyacrylamide-gel electrophoresis. Calf intestinal alkaline phosphatase migrated on gel by disc-gel electrophoresis was cut into 1 mm thickness without staining and extracted with 0.5 ml of 20 mM Tris-HCl buffer, then dialyzed overnight against distilled water and lyophilized.

Estimation of molecular weight by filtration was performed as described previously.

Alkaline phosphatase activity was measured according to the method of Kitchen et al. modified by the use of a final substrate concentration of 1.58 mM p-nitro-
phenyl phosphate in 0.2 M carbonate/bicarbonate buffer, pH 10.2. Protein concentration was determined by the method of Lowry et al.\(^{21}\) as previously described\(^{19}\).

Quantitative determinations of hexose and hexosamine were carried out by gas-liquid chromatography of alditol acetates prepared from hydrolyzate of alkaline phosphatase as described by Niedermeier\(^{22}\) with a slight modification. Internal standard was arabinose for estimation of hexose and mannosamine for estimation of hexosamine, respectively.

The extraction of sialic acid was carried out by the method of Komoda et al.\(^{14}\) with a slight modification and quantitatively estimated according to the trifluoroacetylation method with N-methylbistrifluoroacetamide\(^{14}\). The sialic acid content was calculated by the method of Niedermeier\(^{22}\), using trans-stilbene as internal standard.

For the hydrolysis of alkaline phosphatase by sialidase\(^{14}\), a solution containing 0.5 unit sialidase/ml was added to an equal volume of the enzyme in 5 mM CaCl\(_2\)/24 mM acetate buffer, pH 5.5. The mixture was incubated at intervals of 10, 15, 20 and 25 hr at 37°C. Aliquots of the mixture taken at the time described were assayed for phosphatase activity\(^{23}\).

**Results and Discussion**

Alkaline phosphatase isolated from bovine skim milk was electrophoretically homogeneous and final preparation was almost 7,000 times as much as the activity in bovine milk.

By Sephadex G-200 gel filtration\(^{18}\), the molecular weight of this enzyme was estimated to be about 190,000. The purification of alkaline phosphatase using affinity chromatography was attempted by some workers\(^{15,24-26}\), but no report on the purification of bovine milk alkaline phosphatase with affinity chromatography has been presented except our report\(^{18}\). The commercial intestinal alkaline phosphatase was purified about 7.0 times by polyacrylamide gel electrophoresis compared to the starting material. But the activity of this purified enzyme was lower than that reported by Komoda et al.\(^{14}\).

The compositions of hexose, hexosamine and sialic acid of bovine milk and calf intestinal alkaline phosphatases were determined by gas-liquid chromatography. When the analytical condition for the measurement of the hexoses was applied in bovine milk and calf intestinal alkaline phosphatases, the four peaks of the fucose, arabinose, mannose and galactose, and an additional unknown peak at about 13 min of the retention time was observed. The carbohydrate compositions of bovine milk and calf intestinal alkaline phosphatases (assuming the molecular weight of bovine milk to be 190,000 and of calf intestinal enzyme to be 140,000\(^{27}\)) are shown in Table 1. Bovine milk and calf intestinal alkaline phosphatases contained total carbohydrates of 2.12 and 2.89% respectively, consisting of fucose, mannose, galactose, glucosamine, galactosamine and sialic acid. Scarcely any sialic acid was detected in the enzyme from calf intestine.
Carbohydrate in Bovine Milk Alkaline Phosphatase

Table 1. Quantitative estimation of carbohydrates in bovine milk and calf intestinal alkaline phosphatases

<table>
<thead>
<tr>
<th>Alkaline phosphatase</th>
<th>Fuc (%)</th>
<th>Man (mol)</th>
<th>Gal (mol)</th>
<th>Glu(NH₂) (mol)</th>
<th>Gal(NH₂) (mol)</th>
<th>NAcNeu (Sialic acid) (mol)</th>
<th>Total (mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine milk</td>
<td>0.08</td>
<td>0.65</td>
<td>0.34</td>
<td>0.26</td>
<td>0.32</td>
<td>0.52</td>
<td>2.12</td>
</tr>
<tr>
<td>(mol)</td>
<td>0.29</td>
<td>(6.86)</td>
<td>(3.60)</td>
<td>(2.19)</td>
<td>(2.71)</td>
<td>(2.84)</td>
<td>(18.51)</td>
</tr>
<tr>
<td>Calf intestine</td>
<td>0.78</td>
<td>0.61</td>
<td>0.11</td>
<td>0.05</td>
<td>1.33</td>
<td>0.01</td>
<td>2.89</td>
</tr>
<tr>
<td>(mol)</td>
<td>(6.68)</td>
<td>(4.76)</td>
<td>(0.84)</td>
<td>(0.40)</td>
<td>(8.40)</td>
<td>(0.04)</td>
<td>(21.12)</td>
</tr>
</tbody>
</table>

The results were expressed in grams of carbohydrate/100 g of protein and represented the average of triplicate analyses. Molecular weights of bovine milk and calf intestinal alkaline phosphatases were taken as 190,000 and 140,000, respectively. The values in parentheses were expressed in moles of carbohydrate/mol of protein.

Although the carbohydrate contents of alkaline phosphatase isolated from various biological sources including calf intestine were investigated, no detailed studies have been made on the composition of carbohydrate in bovine milk alkaline phosphatase. The sialic acid content in bovine milk alkaline phosphatase has been analysed only with the colorimetric method but not by gas-liquid chromatography. CRAVEN and GEHRKE showed that sialic acid content in κ-casein obtained by gas-liquid chromatography was about 10% higher than that of WARREN's colorimetric method. KOMODA et al. extended the method for the determination of sialic acid in human liver and placental alkaline phosphatases by combination of sialidase treatment and gas-liquid chromatography, and demonstrated that this method gave a higher value as compared with result of acid treatment. Their method was applied in this paper for the measurement of the carbohydrate contents in alkaline phosphatases, and found that the bovine milk alkaline phosphatase had more mannose, galactose, glucosamine and sialic acid and less fucose and galactosamine than the calf intestinal alkaline phosphatase and it was confirmed that bovine milk and calf intestinal alkaline phosphatases were identified as the sialoglycoprotein. The results on the calf intestinal alkaline phosphatase did not coincide with those obtained with same enzyme preparations. It is considered that this discrepancy may have resulted from the differences of experimental procedures and the purity of enzyme used.

In order to know the role of sialic acid in bovine milk alkaline phosphatase on the phosphatase activity, the enzyme was hydrolysed by sialidase. The loss of the enzyme activity was only within 5.7% of the controls in all cases. This indicates that the sialic acid is not required for the enzyme activity of bovine milk alkaline phosphatase. Effect of sialidase on bovine milk alkaline phosphatase activity has never been examined, but human and calf intestinal alkaline phosphatases have been confirmed to be not affected by sialidase treatment at 37°C for 24 hr.

References
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