Changes in Zinc in Milk during Enzymatic Digestion

Hisao Matsui, Osamu Wada and Tetsu Ono

Department of Hygiene, Gunma University School of Medicine, Maebashi

INTRODUCTION

Zinc is known to be an essential element for human nutrition and good health¹⁾. Milk is its sole source in infants, and growing children as well as mature adults consume varying amounts of milk, which provide them with much of the needed zinc.

Investigators, using gel filtration chromatography^{2,3)}, have demonstrated that zinc in cow's milk can be associated with fractions of high molecular weight, whereas zinc in human milk is bound to fractions of low molecular weight. A low molecular weight zinc-binding ligand has also been reported in human milk, which, however, is found only in very small amounts in cow's milk. This ligand may improve zinc absorption by normal neonates as well as by children with acrodermatitis enteropathica, a zinc metabolism disorder which is genetic. Hurley et al.⁴⁾ have suggested that casein is the high molecular weight compound in cow's milk to which zinc mainly binds, and that such bound zinc is less available for absorption.

However, no attempt has been made to determine the form in which the zinc in milk can exist in the alimentary tract or the changes which occur on passage of the digesta along the tract. The chemical form of zinc during digestion affects its availability. In the present paper, the authors report the *in vitro* effects of digestive enzymes and pH on the forms of zinc in milk.

MATERIALS AND METHODS

Samples of human and cow's milk

Human milk was obtained from nursing mothers within three weeks after parturition. The milk was collected by hand expression into acid-washed glass or plastic containers and was stored at -20°C until used. Unprocessed cow's milk was donated by a local dairy (Gunma Prefecture).

Reagents

Pepsin (2,870 units/mg protein) and trypsin (12,000 BAEE units/mg protein) were obtained from Sigma Chemical Co., Ltd.. All chemicals used were of reagent grade. Buffers and reagents were prepared in distilled water.

Enzymatic digestions

The milk was centrifuged at $1,500 \times g$ for 15 min and the fat-free supernatant was withdrawn. Aliquots of 1.0 ml of fat-free milk were mixed with equal volumes of enzymes prepared as follows: pepsin (4.0 mg/ml) in 0.05 M hydrochloric acid solution containing 34 mM NaCl (pH 1.2); trypsin (2.0 mg/ml) in 0.1 M NaCl/sodium bicarbonate solution (pH 8.3). To study the effects of pH, aliquots (1.0 ml) of fat-free milk were mixed with equal volumes of the above solutions without enzymes. All of the mixtures were incubated at 37°C for 60 min. In addition, another aliquot (2.0 ml) of the milk was incubated with a one-half volume of pepsin solution, and then the mixture was digested with trypsin. After digestion, the reaction mixture was centrifuged at $12,000 \times g$ for 20 min and the supernatant obtained was filtrated on a dextran (Sephadex) gel.

Gel filtration chromatography

Separations were carried out on a 1.5×30 cm column of Sephadex G-75 (Pharmacia Ltd., Uppsala), which had been pre-equilibrated with 10 mM phosphate buffer (pH 7.4) containing 20 mM NaCl. The samples were eluted with the same buffer at a flow rate of about 6 ml/hr and 2.8 ml-fractions were collected. The concentration of zinc in each fraction was determined by atomic absorption spectros-

copy. Absorbance at 280 nm was measured for each fraction. The Sephadex G-75 column was calibrated with substances of known molecular weight — blue dextran, cytochrome C, bacitracin, leucine, $\text{Zn}(\text{NO}_3)_2$.

Metal analysis

Zinc concentration was measured on a flameless atomic absorption spectrophotometer (Shimazu AA-650) equipped with a graphite furnace atomizer (Shimazu GFA-2). Five μl samples were used in each

Fig. 1 Elution profiles of enzyme-treated cow's milk on a column of Sephadex G-75.

Concentrations of zinc (●—●), and A_{280} (△—△), and the position of $\text{Zn}(\text{NO}_3)_2$ eluted (↓).

analysis. Standard solutions were made by appropriate dilutions of the zinc with 1,000 mg/l as the reference standard (Wako, Japan).

RESULTS

Acid-peptic digestion

Gel filtration on Sephadex G-75 of both defatted human and cow's milk showed two peaks (Fig. 1a,

Fig. 2 Elution profiles of enzyme-treated human milk on a column of Sephadex G-75.

Concentrations of zinc (●—●), and A_{280} (△—△), and the position of $Zn(NO_3)_2$ eluted (↓).

Fig. 3 The effects of pH on the elution profiles of pepsin-digested cow's milk.

Milk was adjusted to pH 8.3 before gel filtration. Fig. 1b shows pepsin-digested cow's milk with no pH adjustment. Concentrations of zinc (●—●), and A_{280} (△—△), and the position of $Zn(NO_3)_2$ eluted (↓).

Sequential treatments of milk with pepsin and trypsin

Sequential digestion by pepsin and trypsin gave zinc elution profiles at a volume slightly less than that of cytochrome C (Fig. 1d, 2d). The position of the peak was similar to pepsin-digested milk with a pH adjustment. Milk treated with trypsin alone also gave a zinc elution profile similar to that of sequential digestion (Fig. 1c, 2c). As a control, a milk and $Zn(NO_3)_2$ solution (5 mg/ml) was adjusted to a pH of 8.3, and there were no significant changes before and after treatment. There was no difference between human and cow's milk in terms of the amount of zinc at any time during the experiments.

CONCLUSIONS

In the present study, the effects of acid-peptic digestion on zinc in milk was studied to consider the possibility that hydrochloric acid and pepsin secreted by the gastric mucosa react with milk present in the stomach. Pepsin digestion in an HCl solution showed that most zinc eluted at a volume similar to that observed for $Zn(NO_3)_2$. Milk treated with an enzymeless HCl solution peaked at the same place as in pepsin-digested samples. The results suggest that in the stomach, zinc is either free or bound to low molecular weight moieties. Parkash and Jenness⁵⁾ have reported that most zinc in cow's milk is associated with casein, and casein-bound zinc is completely released by acidification to a pH of 2.0.

Milk was then sequentially digested by pepsin and trypsin. Most zinc eluted at a volume slightly less than that of cytochrome C (12,000 molecular weight). Moreover, pepsin-digested milk, adjusted to pH 8.3 prior to gel filtration, had an elution profile similar to that of sequentially digested milk, suggesting that when pepsin-digested milk passes into the duodenum, zinc may bind to substance (s) with molecular weights of approximately 12,000.

Hurley et al.^{2,4,7)} and Evans et al.^{3,6)} have demonstrated that human milk contains much zinc-binding ligands which may facilitate zinc absorption. However, changes in zinc were not reported during milk digestion.

The present experiments show that the digestion of human and cow's milk results in identical changes in elution profile. Zinc-binding substance (s), therefore, may be formed in the gastrointestinal

2a). Most of the zinc (cow's milk, >90 %; human milk, >70 %) eluted at the first peak which was located at the void volume of the gel. The second peak eluted at the column's total volume, where zinc was present in greater quantities in human milk obtained early in lactation than in cow's milk. During separation of pepsin-digested milk, the first peak disappeared and most of the zinc eluted at a volume similar to that observed for $Zn(NO_3)_2$ (Fig. 1b, 2b). The zinc elution profiles obtained after pepsin digestion were similar for human and cow's milk. Changes in elution pattern were observed by adjusting the pH (8.3) of the peptic digest before gel filtration (Fig. 3). The zinc eluted at a volume slightly less than cytochrome C with a molecular weight of 12,000. However, an HCl solution with no enzymes gave a main zinc peak at the same place as those in pepsin-digested samples. Adjusting the pH to 8.3 prior to gel filtration demonstrated that most of the zinc eluted at the void volume.

tract, although digestion *in vivo* is perhaps not comparable to *in vitro* experiments using a simple enzyme system. Furthermore, it may be impossible to estimate zinc availability simply from its presence in raw milk.

It has been suggested that enzyme-resistant peptides are formed during the intestinal digestion of milk casein and that they can form complexes with various other substances⁹⁾. Mellander⁹⁾ demonstrated that the peptides form complexes with calcium 70 % or more of which is then absorbed from the intestinal tracts of infants. To what extent these peptides form complexes with zinc is an interesting problem for further research.

Zinc-binding substance (s) here probably bind other metals as well. The extent to which the fraction obtained participates in the intestinal absorption of zinc is presently being investigated.

SUMMARY

The amount of zinc in human and cow's milk was evaluated in *in vitro* experiments using gel chromatography on Sephadex G-75 to simulate the action of gastrointestinal enzymes. The HCl-peptic digestion of both types of milk demonstrated that most of the zinc eluted at the same position as $\text{Zn}(\text{NO}_3)_2$. The sequential digestion of both types of milk by pepsin and trypsin demonstrated that most of the zinc eluted at a position corresponding to a molecular weight slightly less than 12,000. The results suggest that zinc may be liberated in the stomach and that when the pepsin-digested milk passes into the duodenum, which has an alkaline pH and where trypsin is secreted, zinc may bind to substance (s) formed during intestinal digestion. There was no difference between the elution profiles of the two kinds of milk. This indicates that differences in zinc availability in human and cow's milk is not responsible for the molecular weight of zinc-binding substances.

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消化による乳汁中亜鉛の存在形態の変化

群馬大学医学部衛生学教室

松井寿夫・和田 攻・小野 哲

人乳および牛乳中の亜鉛が消化過程でどのような存在形態の変化を示すかを明らかにする目的で *in vitro* の実験で検討した。人乳および牛乳をペプシン、トリプシン、あるいはその両方で消化し、消化生成物を Sephadex G-75 でゲルろ過クロマトグラフィーを行った。ペプシン消化では、乳汁中亜鉛の大部分は $\text{Zn}(\text{NO}_3)_2$ と同じ位置に溶出した。また、乳汁を塩酸酸性にするのみでも同様に亜鉛の遊離がみられた。このことは、胃において乳汁中亜鉛が遊離することを示し

ている。乳汁をペプシン消化した後、トリプシンで消化した場合には、大部分の亜鉛はチトクローム C(分子量: 12,000)よりわずかに早い位置に溶出された。また、ペプシン消化した乳汁を pH8.3 に調整した場合にも、ペプシン・トリプシンの連続消化したときと同様な溶出パターンを示した。これらのことから、アルカリ性の十二指腸中では亜鉛は消化生成物と結合すると考えられる。また、消化後の亜鉛溶出パターンに人乳と牛乳とで差がみられなかったことは、人乳と牛乳における亜鉛吸収率の差が亜鉛結合物の分子量の大きさによっているのではないことを示唆している。

Key words: Human milk, Cow's milk, Pepsin digestion, Trypsin digestion, Zinc
人乳, 牛乳, ペプシン消化, トリプシン消化, 亜鉛

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