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Binding Ability of Bovine Milk Proteins to Mutagenic Heterocyclic Amine of 3-amino-1, 4-dimethyl-5*H*-pyrido[4, 3-*b*]indole

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Abstract The binding ability of bovine milk proteins with mutagenic heterocyclic amine was investigated. Binding was determined with 2 *mg* of protein and 20 *μg* of 3-amino-1, 4-dimethyl-5*H*-pyrido[4, 3-*b*]indole (Trp-P-1) in 0.4 *ml* of pH 7.4, 50 *mM* phosphate buffer, at 37℃, in a shaker for 10 *min*. The unbound Trp-P-1 in protein-free ultrafiltrate was prepared using Ultrafree UFC3LGC00 and was analyzed by HPLC method. The binding of whole casein, *αs*-casein, *β*-casein and *κ*-casein were 54.03, 40.02, 56.24 and 33.75%, respectively. *β*-Lactoglobulin A, *β*-lactoglobulin B, lactoperoxidase, lactoferrin-a and lactoferrin-b were 23.61, 17.87, 15.20, 22.53 and 6.92% respectively. However, *α*-lactalbumin could not bind Trp-P-1 in this experiment. The binding of these milk proteins which were prepared by ourselves were compared with purchased proteins. They showed similar tendency to our proteins.

INTRODUCTION

Recently milk minor proteins, such as *α*-lactalbumin, *β*-lactoglobulin A, *β*-lactoglobulin B, lactoperoxidase, lactoferrin, were easily isolated from bovine milk acid whey by using chromatographic procedures (YOSHIDA, 1990; YOSHIDA and YE-XIUYUN, 1991). *αs*-Casein, *β*-casein and *κ*-casein were also prepared by Diethylaminoethyl (DEAE) chromatography in the presence of urea and *β*-mercaptoethanol (THOMPSON, 1966). Some reports have been described the antitumor activities, such as yogurt, lactic acid bacteria and its cells (BONDA and RAO, 1990; HOSONO *et al*, 1987; HOSONO *et al*, 1988; REDDY *et al*, 1983; SHAHANI *et al*, 1983; ZHANG *et al*, 1990). However, little is known about the antimutagenity of milk proteins. 3-Amino-1, 4-dimethyl-5*H*-pyrido-[4, 3-*b*]indole (Trp-P-1) was isolated in pure state from heated D, L-tryptophan and was contained in cigarette smoke tar (SATO *et al*, 1977) and charred broiled and fried beef (NAGAO *et al*, 1977). Trp-P-1 has a carcinogenicity to rats and mice who were given a pellet diet containing .01 to .08 % of Trp-P-1 (SUGIMURA *et al*, 1989). This paper describes the binding of milk proteins against mutagenic heterocyclic amine of Trp-P-1.

MATERIALS AND METHODS

Milk proteins:

Whole casein was precipitated as acid casein. *αs*-Casein, *β*-casein and *κ*-casein were

isolated from whole casein by Thompson's method (1966). α-Lactalbumin, β-lactoglobulin A and β-lactoglobulin B were prepared from bovine milk acid whey by Aschaffenburg and Drewry's method (1957) and DEAE anion exchange chromatography at pH 6.8 and pH 8.5 (Yoshida, 1990). Lactoperoxidase, lactoferrin-a and lactoferrin-b were prepared from bovine milk acid whey by carboxymethyl (CM) cation exchange chromatography at pH 7.7 (Yoshida and Ye-Xiuyun, 1991). These isolated proteins were desalted by dialysis and freezedried. α-Casein so-called αs-casein (C-6780, Lot 78F9555), β-casein (C-6905, Lot 67F9645), κ-casein (C-0406, Lot 29F9540), α-lactalbumin (L-6010, Lot 89F8115), β-lactoglobulin A (L-7880, Lot 88F8095), β-lactoglobulin B (L-8005, Lot 108F8155), lactoperoxidase (L-2005, Lot 40H3778), and lactoferrin (L-9507, Lot 59F3915) were purchsed as standard proteins from Sigma Chemical Company (St. Louis, MO, USA).

Mutagen:

Trp-P-1 was kindly provided by the National Cancer Center Research Institute (Tokyo, JPN).

Determination of binding of the milk protein to mutagen:

Two milligram of lyophilized protein were suspended in .38 *ml* of pH 7.4, 50 *mM* phosphate buffer. This suspension was stored overnight in a refrigator at 4℃. Twenty microgram of a methanolic solution of Trp-P-1 (20*μg*/20*μl*) was added to the protein solution. This reaction mixture was incubated at 37℃ in a shaker for 10 min. After incubation, the reaction mixture was separated into protein free filtrate and retentate by centrifugation (2,000×*g*, 8 *min*) using Ultrafree UFC3LGC00 (Japan Millipore LTD. Tokyo, JPN) which was equipped with an ultrafilter of a molecular weight retention of 10,000. Twenty microliter of this ultrafiltrate was applied to a high pressure liquid chromatography apparatus for estimation of the amount of free mutagen. Trp-P-1 standard solution (20*μg*/.40*ml*) was prepared by addition of 20*μg* of methanolic Trp-P-1 solution (20*μg*/20*μl*) to .38 *ml* of pH 7.4, 50 *mM* phosphate buffer. Subsequently the mixture was filtrated with Ultrafree UFC3LGC00. The data in the table are means for duplicate experiments.

Protein bound Trp-P-1 (%) =(Total Trp-P-1−Free Trp-P-1)×100

Total Trp-P-1: Trp-P-1 content in reaction mixture (1*μg*/20*μl*)

Free Trp-P-1: Trp-P-1 content in ultrafiltrate (X *μg*/20*μl*)

High pressure liquid chromatography (HPLC):

HPLC was performed with Hitachi L-6000 pump and Hitachi L-4000 UV detector. The column was Inertsil ODS-2 (4.6×150 *mm*) (GL Sciences, Tokyo, JPN) and the mobile phase was acetonitorile-H_2O-triethylamine 50:50:.05 at a flow rate of 1*ml*/*min*. Absorbance at 254 nm was recorded with an integrator (Labchart 12, SIC System Instruments, Tokyo, JPN)

RESULTS AND DISCUSSION

The results are shown in Table 1. Caseins, whole casein, αs-casein, β-casein and κ-casein showed higher binding abilities against Trp-P-1 compared with other milk proteins such as β-lactoglobulin A and β-lactglobulin B. α-Lactalbumin could not bind Trp-P-1 in this experiment. Lactoferrin and lactoperoxidase which were prepared by ourselves showed higher binding abilities against Trp-P-1 compared with those standard proteins from Sigma.

These results meant the decrease of Trp-P-1 contents in the protein free ultrafiltrates. The remained Trp-P-1 were contained in the solutions with proteins. However, the relationship between those proteins and Trp-P-1 is not clear either Trp-P-1 is absorbed to a protein molecule or Trp-P-1 is combined with some chemical reaction.

Table 1. Binding ability of bovine milk minor proteins against Trp-P-1

Whole casein	54.03 (%)
αs-Casein	40.02
β-Casein	56.24
κ-Casein	33.75
α-Lactalbumin	0
β-Lactoglobulin A	23.61
β-Lactoglobulin B	17.87
Lactoperoxidase	15.20
Lactoferrin-a	22.53
Lactoferrin-b	6.92
α-Casein·sigma	59.46
β-Casein·sigma	40.43
κ-Casein·sigma	23.75
α-Lactalbumin·sigma	0
β-Lactoglobulin A·sigma	15.78
β-Lactoglobulin B·sigma	3.65
Lactoperoxidase·sigma	6.41
Lactoferrin·sigma	0

It has been proposed that the thiol group of hemoglobin and glutathione are related to the binding of heterocyclic amines (Umemoto, Grivas, *et al*, 1988; Umemoto, Monden, *et al*, 1988). However, αs-casein and β-casein, which could bind very high percentages of Trp-P-1, contained no -S-S- bond in their molecules. The binding between these proteins and Trp-P-1 is supposed the reaction of -COOH residue of protein and $-NH_2$ residue of amine. Further experiments are needed.

This investigation is only restricted *in vitro* and these milk proteins can not act as antitumor materials *in vivo*. However, it is possible that these proteins or partially digested proteins are able to transport the mutagenic heterocyclic amine from the bigining of digestive canal to large intestine. This investigation may suggest the existence of a new functional property of these milk proteins and blood serum proteins.

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牛乳蛋白質の発癌性ヘテロサイクリックアミンに対する結合性

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牛乳中の各種蛋白質，即ち whole casein, αs-casein, β-casein, κ-casein, β-lactoglobulin A, β-lactoglobulin B, α-lactalbumin, lactoferrin, lactoperoxidase を分離精製し，これらの蛋白質が発癌性ヘテロサイクリックアミンである 3-amino-1, 4-dimethyl-5*H*-pyrido[4, 3-*b*]indole (Trp-P-1) と結合するか否かについて実験を行った。さらに我々自身で分離調製した蛋白質と比較検討する為に Sigma 社製の α-casein, β-casein, κ-casein, β-lactoglobulin A, β-lactoglobulin B, α-lactalbumin, lactoferrin, lactoperoxidase についても同様の実験を行った。

凍結乾燥した蛋白質 2 *mg*, Trp-P-1 20 μg を0.4 *ml* の pH 7.4, 50 *mM* 燐酸バッファーに溶解し，37℃で10分間振盪し反応させる。つづいて，日本ミリポアリミテッド製の UFC3LGC00 を用いて反応液中の非結合型 Trp-P-1 を限外濾過により分離し，この Trp-P-1 を HPLC 法により測定する。HPLC は日立 L-6000 ポンプ・L-4000 UV 検出機に GL-Science 製 Inertsil ODS-2 (4.6×150 *mm*) カラムを使用し，acetonitrile-H_2O-triethylamine 50:50:0.05 で展開した。254 *nm* に於ける吸収を SIC System Instruments 製 Labchart 12 で記録する。

Whole casein:54.04%, αs-casein:40.02%, β-casein:56.24%, κ-casein:33.75%で反応液中の Trp-P-1

を比較的に高い割合で結合した。Sigma 社製の α-Casein:59.46%, β-casein:40.43%, κ-casein:23.75%, で類似の傾向を示した。β-Lactoglobulin A:23.61%, β-lactoglobulin B:17.87%, β-lactoglobulin A·Sigma:15.78%, β-lactoglobulin B·Sigma:3.65% であった。Lactoperoxidase:15.20%, lactoperoxidase·Sigma:6.41%, lactoferrin-a:22.53%, lactoferrin-b:6.92% であったが, α-lactalbumin, α-lactalbumin·Sigma, lactoferrin·Sigma は Trp-P-1 と結合しなかった。