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Original article

A variant peptide of buffalo colostrum β-lactoglobulin inhibits angiotensin I-converting enzyme activity

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ABSTRACT

β-lactoglobulin is a rich source of bioactive peptides. The LC-MS separated tryptic peptides of buffalo colostrum β-lactoglobulin (BLG-col) were computed based on MS–MS fragmentation for *de novo* sequencing. Among the selected peptides (P1–P8), a variant was detected with methionine at position 74 instead of glutamate. The sequences of two peptides were identical to hypocholesterolemic peptides whereas the remaining peptides were in accordance with buffalo milk β-lactoglobulin. Comparative sequence analysis of BLG-col to milk β-lactoglobulin was carried out using CLUSTALW2 and a molecular model for BLG-col was constructed (PMDB ID-PM0076812). The synthesized variant pentapeptide (IIAMK, *m*/*z*-576 Da) was found to inhibit angiotensin I-converting enzyme (ACE) with an IC_{50} of 498 ± 2 μM, which was rationalized through docking simulations using Molgrow virtual docker.

1. Introduction

Milk, a mammalian specific biological fluid, gains considerable attention as its proteins and peptides exert a diverse range of nutritional and functional activities. Colostrum, the early lactation, has a nutritional profile and immunological composition that substantially differs from that of mature milk. It possesses major milk proteins like β-lactoglobulin, α-lactalbumin, glycomacropeptides, albumin proteose peptone along with immunoglobulins [1] and array of growth factors [2–4]. Recent studies suggest that colostral fractions, or individual peptides present in colostrum are useful for the treatment of a wide variety of gastrointestinal disorders including inflammatory bowel disease, nonsteroidal anti-inflammatory drug (NSAID) induced gut injury and chemotherapy-induced mucoscitis [5]. To date, several bioactive peptides have been identified in protein hydrolysates and fermented foods of milk but not of colostrum [6,7]. The multifunctional property associated with milk bioactive peptides includes antihypertensive, antioxidant, hypocholesterolaemic, opioid, antimicrobial, immunomodulatory and cytomodulatory activities [4].

Of the different proteins present in milk, β-lactoglobulin is a major soluble globular protein containing 162 amino acids secreted in the mammary gland during late pregnancy and lactation mainly in ruminants and other mammalian species except primates [8]. The functional advantage of β-lactoglobulin is implicated in the transport of vitamin D [9] and upon enzymatic digestion, the peptides of β-lactoglobulin are known to play pivotal role in human health and modulate several regulatory processes [10]. The peptides effectively exhibit antihypertensive [11], antimicrobial [12], antioxidant [13,14] and also immunostimulatory effects [15]. The peptides of β-lactoglobulin can suppress the cholesterol absorption as evidenced in Caco-2 cells, a model system used to study lipid metabolism. Furthermore, hypocholesterolemic activity exhibited by a tetrapeptide was even greater than the medicine β-sitosterol as studied in rats [16]. The high nutritional and functional value of β-lactoglobulin has therefore made it a preferred food ingredient in formulation of modern food supplement, BioZate [4].

Angiotensin I-converting enzyme (ACE) has a critical role in cardiovascular function by cleaving the C-terminal His-Leu dipeptide from angiotensin I to produce a vasoconstrictor angiotensin II. In the global scenario, inhibition of ACE is considered as one of the potential tasks in treating high blood pressure, heart failure, diabetic nephropathy, and type 2 *diabetes mellitus*. Consequently, synthetic ACE inhibitors such as lisinopril, captopril, enalapril and alecepril are effectively used in the treatment of hypertension despite their side effects. Hence, bioactive antihypertensive peptides of food origin are increasingly gaining importance rather than synthetic drugs in hypertension therapy. Although casein derived peptides from milk have been reported to have ACE

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inhibitory activity [17], the peptides from whey proteins are less studied and characterized except for β-lactoglobulin. Many peptides of β-lactoglobulin derived after treatment with trypsin, chymotrypsin, pepsin, thermolysin and proteinase K have been identified to possess moderate to high ACE inhibitory activity [13].

Realizing the biomedical importance of β-lactoglobulin, we have purified β-lactoglobulin (BLG-col) from buffalo (*Bubalus bubalis*) colostrum for the first time [18]. Buffalo, a major milking ruminant in the Asian countries, predominant in India contributes to 53% of the total bubaline population of the world. β-lactoglobulin, a major whey protein has been extensively studied from bovine milk but there is limited information from buffalo milk and no reports from buffalo colostrum. In the present study, we have employed LC-MS/MS with Collision Induced Dissociation (CID) and Electron Transfer Dissociation (ETD) based fragmentation on a 3D radio frequency (rf) ion trap [19–21] to unravel the sequence similarity and/or variation of BLG-col to buffalo and bovine milk β-lactoglobulin. Further, *in vitro* and *in silico* analysis validated the ACE inhibitory activity of a variant peptide detected in BLG-col for its possible use as a potent drug candidate in hypertension therapy.

2. Results and discussion

The "early" milk, which is known as 'colostrum' produced by mammals during early lactation, is exceptionally complex in its composition. The health promoting properties of the most abundant bovine milk β-lactoglobulin has been well documented [13] whereas no such detailed information is available from colostrum. Hence, we have purified β-lactoglobulin (BLG-col) to homogeneity from buffalo colostrum [18] and used it in the present study to delineate sequence similarity and/or variation between β-lactoglobulin derived from colostrum and milk employing tandem mass spectrometry and to determine its functional property.

2.1. Chemistry

The pure protein was subjected to in-solution trypsin digestion and the peptide mass fingerprint (PMF) of MS analysis revealed a match of 67% to β-lactoglobulin of buffalo and bovine milk [18]. The LC-ESI-MS/MS analysis was performed with CID and ETD capabilities to derive the sequence information of the tryptic peptides of BLG-col. The separation of peptides by LC, facilitated detection of 4, 2 and 1, single, double and triple charged ion pairs respectively (P1–P7, Fig. 1, Table 1) in analogy to the PMF of the BLG-col [18]. Accordingly, the identified protonated molecular ions were m/z-576^{1+} (P1), m/z-674^{1+} (P2), m/z-916^{1+} (P3), m/z-933^{1+} (P4), m/z-818.6^{2+} (P5), m/z-851.5^{2+} (P6) and m/z-772.2^{3+} (P7). Except for P1 (Fig. 2, Table 1), the masses of all other peptides match the expected masses of BLG-col tryptic peptides. Hence, molecular ion m/z-576^{1+} along with other peptides was analyzed to determine the sequence information. This suggests the presence of isoforms of β-lactoglobulin with possible sequence variations; as such information is not available either from buffalo milk or colostrum.

2.1.1. De novo sequencing of BLG-col peptides

The protonated molecular ion (m/z-576.5) detected after 8.3 min by LC-MS showed variation to the PMF of BLG-col [18] and tryptic peptides of bovine milk β-lactoglobulin. As shown in Fig. 2, the molecular ion of m/z-573.3 in bovine milk β-lactoglobulin was detected as 576.5 in BLG-col and hence, MS/MS analysis was carried out for this ion to unravel the sequence information. The CID based MS/MS fragmentation revealed almost complete *b*- and *y*-ion series corresponding to m/z-576.5 ($M + H^+$) with "AMK/Q" motif (b_2–b_5) and "AI/L" motif (y_2–y_4) and the resultant sequence was I/LAMK/Q (Mr calc. = 575.35, Fig. 3A). Submission of .mgf file to Mascot MS/MS ion search database did not show any match to β-lactoglobulin sequence although it has close match to hypocholesterolemic peptide "IIAEK" from bovine milk β-lactoglobulin. This was further supported by the identification of b_4 and y_2 ions with 429.4 and 278.2 for "IIAMK" instead of 427.25 and 276.15 for "IIAEK" respectively (Supplementary file: Table 1). Thus, we could identify single amino acid variation in P1 with methionine at position 74 instead of glutamate between the CID derived sequence data of the present study and the available sequence for bovine and buffalo milk β-lactoglobulin in the database. Although bovine β-lactoglobulin is known to express in multiple forms with one or two amino acid variations [23], none of the isoforms have been identified in buffalo β-lactoglobulin. But, Vohra et al. [22] have reported the presence of genetic variants of β-lactoglobulin gene and studied its association with milk composition traits in riverine buffalo. The single amino acid variation identified in the present study could be a genetic variant. Incidentally, the sequence tag refers to a potent candidate molecule that decreases the absorption of cholesterol in comparison to well-known drugs as evidenced by both *in vivo* and *in vitro* studies [16]. Hence, biological implication of the deduced sequence offers further investigation.

The CID based MS/MS analysis of other single charged ions (P2–P4: Fig. 3B–D; Supplementary files: Tables 2–4) did not reveal any variations and were in accordance with the β-lactoglobulin of buffalo milk [24] and cDNA sequence [25]. Further, the sequence deduced for P4 represented N-terminus of β-lactoglobulin and precisely matched to the N-terminal sequence data and PMF [18]

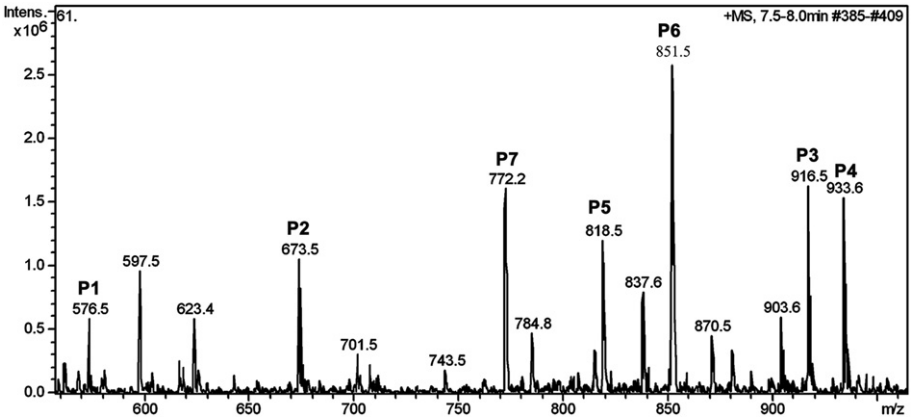

Fig. 1. LC-MS analysis of tryptic peptides derived from BLG-col. Insert letters P1–P7 denotes single $[M + H]^+$-576, $[M + H]^+$-673, $[M + H]^+$-916, $[M + H]^+$-933, double $[M + H]^+$-818 (m/z-1636), $[M + H]^+$-851 (m/z-1702) and triple $[M + H]^+$-772 (m/z-2313) charged ions.