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Comparative Assessment of Peptide Concentration in Milk Protein Hydrolysates and Fractions

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ABSTRACT

A comparative study using the Kjeldahl method and two colorimetric methods for the determination of total protein/peptide concentrations in a range of intact and hydrolysed milk protein was carried out. The samples were goat whey, camel whey, buffalos milk retentate and hydrolysates of these made with different enzymes as well as hydrolysate fractions. Bicinchoninic acid (BCA) and o-phthaldialdehyde (OPA) were used as coloring agents to specifically react with proteins and peptides. The results obtained with the BCA method and bovine serum albumin as standard did not correlate with the results obtained by the Kjeldahl method ($r^2 = 0.17$). The OPA method showed better agreement with the Kjeldahl method ($r^2 = 0.69$) and with the use of a whey protein hydrolysate as standard good estimates of the peptide contents in the hydrolysed milk protein samples was obtained compared to the results obtained with the Kjeldahl method.

Key words: Milk protein hydrolysates, peptide concentration, Kjeldahl, colorimetric assays

INTRODUCTION

Milk proteins not only are an important nutrient and provide functional properties in food, they also represent an important source of bioactive peptides, that when liberated through enzymatic hydrolysis can have a positive impact on human health (Korhonen, 2009; Mils *et al*., 2011).

Since, the bioactivity exerted is dependent on the concentration of the active compound, it is important to relate the bioactivity of milk protein hydrolysates and peptides to the concentration of peptides in the sample. This is of particular importance in research work aiming to purify and identify bioactive peptides, which involves screening the bioactivity of a large number of fractions of hydrolysates and almost pure peptides in order to find the most active ones. Accordingly, quick and reliable determination of peptide content in samples of low protein content and small sample volume has become a major challenge.

The Kjeldahl method which is based on determination of the nitrogen content of the sample, is internationally recognized as the reference method for determination of protein concentration in dairy products (Dupont *et al*., 2013). Although this process is both time and sample consuming and when used for hydrolysates may suffer from interference from other N-containing compounds present in the sample, it has been used repeatedly for milk protein fractions (Contreras *et al*., 2010). Analysis of amino acids present in a fully digested sample is probably the

most reliable method for quantitation of the peptide content but it also requires dedicated instrumentation and is not suitable for screening purposes. Although, IR based quantitation of proteins and peptides based on the amide I band can be obtained with good reproducibility, such determination requires high sample amounts (Strug *et al*., 2012) and also needs special equipment and calibration.

Spectrophotometric methods based on the absorbance at 280 nm are simple and can be used with low protein concentrations or volumes especially with the nanodrop spectrophotometers (De Gobba *et al*., 2014a). However, a major fraction of milk protein derived peptides do not contain aromatic amino acid residues that absorb at 280 nm and will not be detected with this method. Furthermore, other compounds in milk and whey such as uric acid and nucleosides may absorb at this wavelength (De Gobba *et al*., 2014b) and lead to unreliable results.

Rapid methods based on formation of a complex between proteins/peptides and a color agent that can be determined spectrophotometrically, therefore, seem to be a more specific and thus a better choice for estimation of the peptide concentration in multiple samples. A great number of such methods have been developed for protein determination (Sapan *et al*., 1999). However, most of these require high sample volumes or concentrations of proteins are not suited for peptides or suffer from interference from other substances. Nevertheless, a number of these methods have been used for determination of peptide concentration in milk protein hydrolysates and fractions (Especio-Carpio *et al*., 2013; Gutiez *et al*., 2013; Hernandez-Ledesma *et al*., 2005; Quiros *et al*., 2007). Among these, the bicinchoninic acid (BCA) method is much used for its simplicity (Didelot *et al*., 2006). Even though this assay is sensitive and relatively easy to perform, it is still markedly influenced by protein to protein variation and by peptide properties such as peptide length and hydrophilicity (Kapoor *et al*., 2009). Furthermore, the BCA assay is affected by the presence of lactose (Milton and Mullen, 1992).

In order to determine the molar concentration of peptides, methods relying on agents reacting with free amino groups can be used. Spellman *et al.* (2003) evaluated the use of trinitrobenzene sulfonic acid (TNBS) and o-pthaldialdehyde (OPA), which react specifically with primary amino groups for measuring the DH of whey protein hydrolysates. They found that use of TNBS gave the most stable reaction but that it was more time consuming than the reaction with OPA. The OPA assay is based on the reaction of OPA and a thiol containing compound with amino groups released during proteolysis of a protein substrate. The reaction is specific for primary amines in amino acids, peptides and proteins and approaches completion within 1-2 min at 25°C (Nielsen *et al*., 2001) and requires no preliminary heating or separation of the hydrolysis products from the un degraded protein substrate prior to performing the assay. Both TNBS and OPA have been used to quantify peptides in milk protein hydrolysate fractions by use of a suitable standard such as a free amino acid, a dipeptide or a peptone hydrolysate (Ahn *et al*., 2009; Jiang *et al*., 2007; Quiros *et al*., 2007).

The objective of this work is to evaluate the BCA method as used routinely with BSA as standard and the OPA analysis applying standards of varying chain length (L-leucine, whey protein hydrolysate and BSA) for analysis of protein/peptide content in various milk and whey protein hydrolysates (from various species) and fractions there of in comparison to the Kjeldahl method.

MATERIALS AND METHODS

Materials: Three milk protein samples were used as substrates, i.e. goat whey, camel whey and buffalo milk protein. Goat cheese whey obtained from the Food Science Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt, was freeze-dried to obtain a goat whey concentrate.

Camel milk (12% total solids, 3.3% total protein, 3.3% fat) obtained from the local market, Bilbeis City, Sharkia government, Egypt, was heated to 37°C and immediately skimmed by centrifugation (5000×g, 15 min). The caseins (CNs) were precipitated by addition of 1 M HCl to pH 4.6 and centrifugation (5860×g, 60 min at 4°C). The whey was collected and dialyzed against 50 mM phosphate buffer, pH 7.8, lyophilized and stored at -20°C until use.

Skimmed buffalo milk retentate (17.5% protein) was prepared according to El-Zeini *et al*. (2007) and lyophilized and kept at -20°C until use.

The enzymes papain (from *Carica papaya),* Alcalase® 2.4 L (from *Bacillus licheniformis*), pepsin (from porcine gastric mucosa) and trypsin (from bovine pancreas) were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Production of milk protein hydrolysates and fractions: Enzymatic hydrolysis was performed using various enzymes (Alcalase, papain, pepsin or trypsin) at their optimal conditions (Table 1). The protein substrate was dissolved in the relevant buffer (Table 1, 2) at 100 g L^{-1} and the enzyme was added at an enzyme-to-substrate ratio of 1:200 (w/w). After 4 h of reaction, samples were heated in a boiling water bath for 10 min to inactivate the enzyme. The hydrolysate was centrifuged at 4000×g for 15 min and the supernatant was lyophilized and stored at -20°C.

The total hydrolysates of camel whey protein and goat whey protein were dissolved in deionized water and loaded onto a Sephadex G-25 gel filtration column (1.6×150 cm). Separation was obtained with deionized water at a flow rate of 20 mL h^{-1}. The eluate was monitored at 280 nm and fractions were collected (F1 referring to the fraction with the highest molecular weight), dialyzed and lyophilized.

Protein determination: A total of 14 samples were used for the analyses (Table 2). Each sample was dissolved in water at 5 mg mL^{-1} and stirred for 15 min before analysis.

The total nitrogen of the different samples (Table 2) was determined using the micro Kjeldahl method according to AOAC (1995) and total protein content was calculated by multiplying the total nitrogen by 6.38.

Table 1: Conditions used for hydrolysis of different milk protein samples (goat whey, camel whey and skimmed buffalo milk retentate)

Enzymes	Buffer	pH	Temperature (°C)
Alcalase	Distilled water	7.8	55
Papain	0.1 M Na_2HPO_4-NaH_2PO_4	6.0	37
Pepsin	0.1 M Glycine-HCl	2.0	37
Trypsin	0.1 M Na_2HPO_4-NaH_2PO_4	8.0	37

Table 2: Samples used for this study based on goat whey, camel whey and skimmed buffalo milk retentate, respectively

Sample No.	Sample contents	Information
1	Goat whey	
2	Goat whey hydrolysate	Made with Alcalase
3	Goat whey hydrolysate F1	F1-F4 are fractions obtained by size-exclusion
4	Goat whey hydrolysate F2	chromatography
5	Goat whey hydrolysate F3	
6	Goat whey hydrolysate F4	
7	Camel whey	
8	Camel whey hydrolysate	Made with papain
9	Camel whey hydrolysate F1	F1-F2 are fractions obtained by size-exclusion
10	Camel whey hydrolysate F2	chromatography
11	Skimmed buffalo milk retentate	
12	Skimmed buffalo milk retentate hydrolysate	Made with papain
13	Skimmed buffalo milk retentate hydrolysate	Made with pepsin
14	Skimmed buffalo milk retentate hydrolysate	Made with trypsin

A commercial kit (Pierce, Rockford, IL, USA) was used in microtiter plates (611F96, Sterilin Ltd., Newport, UK) according to the supplier's instructions and the absorbance was read at 562 nm using a Multiskan EX reader (LabsystemsOY, Helsinki, Finland). Each sample was analyzed in triplicate and converted into protein content by means of a standard curve based on bovine serum albumin as prescribed in the instructions.

The OPA method used was carried out according to Nielsen *et al.* (2001). Each sample was analyzed in duplicate and the absorbance was converted into protein or peptide content by means of standard curves based on either BSA, a whey protein hydrolysate (Peptigen® IF-3080, Arla food ingredients, Denmark), or L-leucine (L-2-Amino-4-methylpentanoic acid, Sigma Aldrich, Germany), respectively.

RESULTS AND DISCUSSION

Comparison of results obtained by the bicinchoninic acid (BCA) assay and the Kjeldahl method: The standard curve used for the BCA assay obtained with concentrations of BSA up to 2 mg mL^{-1} showed a good linearity with a correlation coefficient of 0.996. In Fig. 1 the protein contents as determined by the BCA method are plotted against the results obtained by the Kjeldahl procedure. The results obtained with the BCA assay using BSA as standard did not correlate with the protein determination by Kjeldahl, as seen by the low correlation coefficient

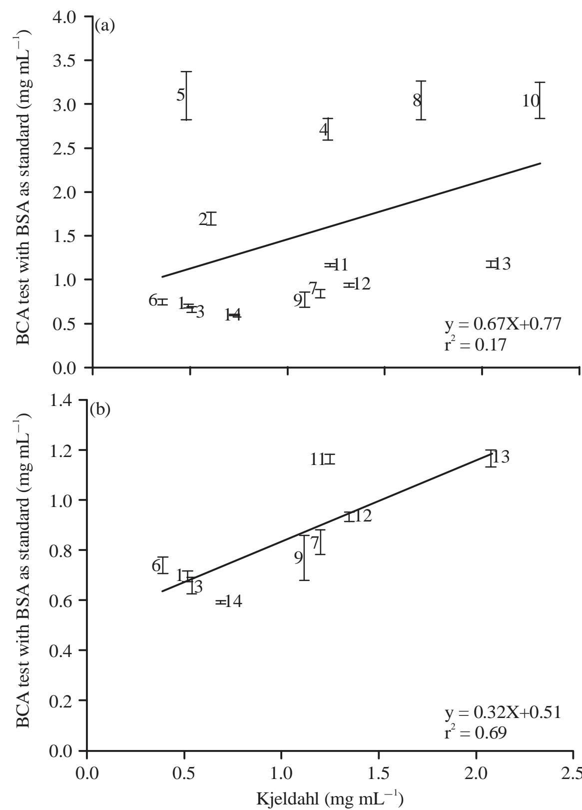

Fig. 1(a-b): Protein determination in milk protein hydrolysate samples from various animals using the bicinchoninic acid (BCA) method as function of results obtained with the Kjeldahl analysis. Samples are represented by numbers as given in Table 2

($r^2 = 0.17$, Fig. 1a). The discrepancy seemed to stem from some whey hydrolysates (samples 2 and 8) and some of the purified whey fractions (samples 4, 5 and 10). Maybe these samples contain a higher number of cysteine, tyrosine and tryptophan residues which led to higher reduction of copper and more intense purple color formation with BCA. Omitting these samples from the plot, there was a good correlation ($r = 0.69$, Fig. 1b). However, the actual values for peptide content were underestimated by the BCA method in comparison to the Kjeldahl method, which might be due to the presence of shorter peptides not detected by the BCA method or may be due to a slight over estimation by the Kjeldahl method from non-peptide N present in the samples.

Other authors have compared colorimetric methods to the Kjeldahl method for determination of the protein concentration. For instance, Keller and Neville (1986) evaluated four spectrophotometric methods for suitability in determining total protein in human milk samples, and compared the results with those obtained by determination of total nitrogen by a micro Kjeldahl method. Values for total protein by all four methods significantly correlated with micro Kjeldahl giving correlation coefficients of 0.96, 0.97, 0.89 and 0.99, respectively, for the biuret assay, Lowry Peterson assay, Bio-Rad Coomassie Blue assay, and Pierce BCA assay, respectively. Since the Pierce BCA assay showed least affected by different protein types and gave the greatest correlation with the micro Kjeldahl method, they recommended this method.

In contrast, to the best of our knowledge, no studies have previously compared methods with respect to the determination of the concentration of peptides of various lengths as resulting from hydrolysis of various milk proteins by commercial enzymes.

Comparison of results obtained by the o-phthaldialdehyde (OPA) assay and the Kjeldahl analysis: The standard curves obtained with the OPA method using the three different protein standards gave good correlations ($r^2>0.99$) for concentrations up to 2 mg mL^{-1} for BSA and the whey protein hydrolysate and up to 0.5 mg mL^{-1} for leucine, respectively. The protein/peptide content of the 14 milk protein hydrolysate samples as obtained by use of the three standard curves are shown in Fig. 2 as function of the protein content obtained with the Kjeldahl procedure.

Although the samples were different with respect to purity and degree of hydrolysis, there was a quite good correlation between the protein concentration as determined by the Kjeldahl method and the OPA method ($r = 0.69$) when all samples were taken into account. However, depending on the standard used for protein determination in the OPA assay the protein concentration was either over or under estimated. With BSA as standard less free amino groups were present for a certain mass of protein and therefore the absorbance of the OPA derivatives was low, resulting in a standard curve with shallow slope and therefore high protein concentration readings at low absorbance. Conversely, the use of the free amino acid L-leucine results in many free amino groups per gram and a high absorbance in relation to protein concentration. Therefore, by use of a protein hydrolysate with similar size peptides as those in the sample should give the best result. In the present study, the use of a commercial WPH indeed gave the best values, though slightly lower in comparison to the values obtained with the Kjeldahl analysis, which as mentioned before could be due to overestimation by the Kjeldahl method. The samples giving the lowest results in the OPA test in comparison to the Kjeldahl measurement are samples no. 4 and 11. Sample 4 contains a goat whey protein fraction (F2) which probably contains a large peptide and therefore will give a low response in the OPA assay in comparison to smaller peptides. Sample 11 contains buffalo milk protein retentate expected to contain mainly caseins, thus large proteins, which explains the low

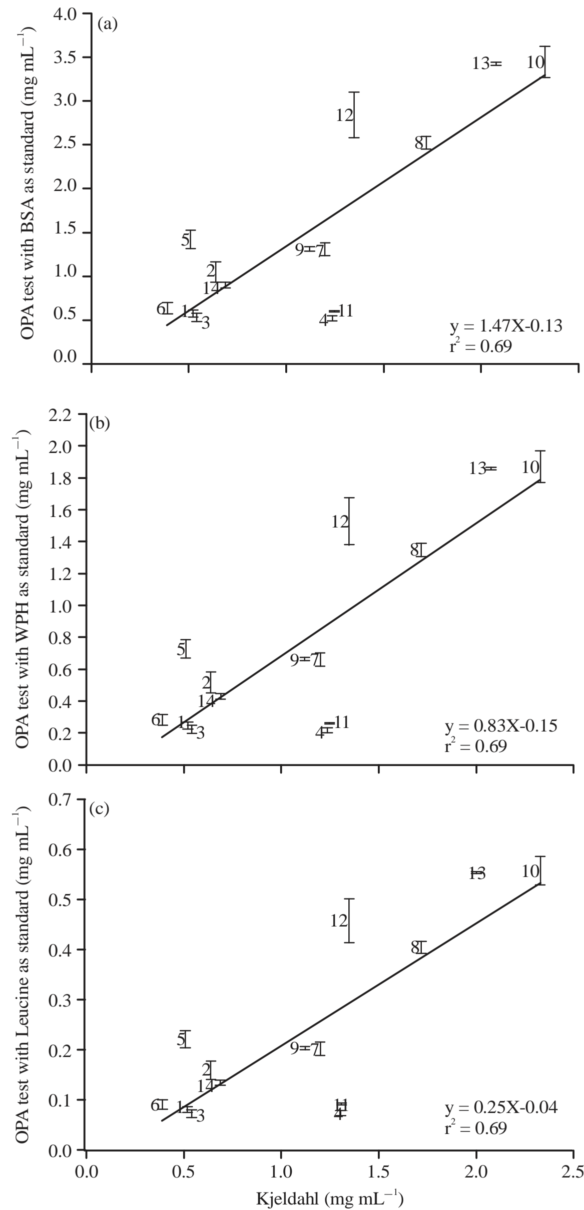

Fig. 2(a-c): Protein determination in milk protein hydrolysate samples from various animals using the ortho-phthaldialdehyde method with different standards as function of results obtained with the Kjeldahl analysis. Numbers represent the samples given in Table 2. Standards used were (a) Bovine serum albumin, (b) Whey protein hydrolysate and (c) Leucine

response in the OPA assay. Omitting these samples from the plot, the linear correlation between the values obtained by the two methods was 0.88 with a regression coefficient of 0.87.

The OPA analysis thus seems to provide good estimates of the protein contents in milk protein samples with varying degree of hydrolysis and composition when compared to the results obtained by the Kjeldahl analysis. The most accurate results for milk protein hydrolysate samples containing many peptides were obtained when the commercial whey protein hydrolysate was used as standard.

CONCLUSION

The BCA test seems to overestimate the protein/peptide contents in samples containing isolated peptides in comparison to the Kjeldahl analysis showing its dependence on the peptide properties. However, for samples containing mixtures of peptides reasonably similar values were obtained by the two methods and the BCA assay seems to be useful for an approximation.

With the OPA test, a standard curve made with a sample as similar as possible in composition and DH as the sample to be analyzed should be used. With a commercial whey protein hydrolysate as standard this method gives a good estimate of the peptide content in hydrolysates of goat and camel whey proteins and of total buffalo milk proteins.

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