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Determination of molecular-mass distribution of food-grade protein hydrolyzates by size-exclusion chromatography and chemiluminescent nitrogen detection

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Abstract

Size-exclusion chromatography (SEC) and chemiluminescent nitrogen detection (CLND) were used to estimate the molecular-mass distribution of food-grade protein hydrolyzates. Simultaneous CLND and UV (214 nm) detection is demonstrated for analytical SEC of an experimental casein hydrolyzate. In order to validate the estimated average M_r values derived from the SEC column data, a preparative SEC separation of an extensive casein hydrolyzate was pursued. Fractions were collected on a time basis and analyzed by time-of-flight (TOF) mass spectrometry. A plot of TOF M_r vs. SEC M_r indicated that the peptides below M_r of 1200 were eluted as estimated by the calibrated preparative SEC column. This paper demonstrates the power of using a dual CLND and UV detection system for analytical SEC analysis of protein hydrolyzates with a calibrated column.

Keywords: Detection, LC; Chemiluminescence, nitrogen detection; Proteins; Casein hydrolyzates; Peptides; Amino acids

1. Introduction

The advantage of using analytical size-exclusion chromatography (SEC) coupled to a dual detection system, i.e. UV (214 nm) and chemiluminescent nitrogen detection (CLND) to estimate molecular-mass distribution of an experimental casein hydrolyzate is demonstrated.

High-performance liquid chromatography (HPLC)–CLND was first reported in 1992 and the CLND reaction mechanism was also described [1]. Bizanek et al. [2] and Fujinari et al. [3] recently described a novel technique using CLND for determining the peptide content of crude synthetic peptides by reversed-phase (RP)-HPLC. Peptide mapping by RP-HPLC and the advantages of using CLND for peptides isolated from casein hydrolyzate have been reported [4].

SEC is often used for estimating average molecular masses and the M_r distribution of protein hydrolyzates. Food-grade protein hydrolyzates have been routinely characterized and studied by SEC [5–12]. The use of SEC mobile phases containing 10% alcohol, to reduce hydrophobic interaction with the silanized silica particles, was suggested by Dolan [13]. Methanol mobile phases are highly compatible with CLND and have been extensively used with a

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trifluoroacetic acid (TFA) modifier in related studies [14].

We report a useful nitrogen detection system for SEC analyses of an extensive casein hydrolyzate and an experimental casein hydrolyzate containing amino acids and peptides with a M_r of less than 1200 on a silica-based column.

2. Experimental

2.1. Apparatus

All HPLC mobile phases were filtered through a Millipore (Bedford, MA, USA) HV filter with a pore size of 0.45 μm. Analytical scale SEC of protein hydrolyszates was performed on a Waters Model 625 pump with an analytical pumphead, a Model 490 UV detector purchased from Waters Associates (Milford, MA, USA) and a nitrogen-specific detector, Model 7000 HPLC–CLND, from Antek Instruments (Houston, TX, USA). Samples were injected using a Model 9125-080 sample valve with a 50-μl loop from Rheodyne (Cotati, CA, USA) onto the analytical SEC column from TosoHaas (Philadelphia, PA, USA). The column effluent was passed through a gas chromatography (GC) capillary splitter from SGE (Austin, TX, USA) to achieve a post-column split for dual detection. The chromatographic data were collected using the Waters GPC Module and Millennium Sample Information software on a NEC Power Mate 486 computer.

Preparative scale SEC (prep-SEC) of extensive protein hydrolyzate was performed on a Model 660E pump, controller and a Model 480 UV detector with a prep flow cell purchased from Waters Associates. Samples were injected using a 5-ml loop from Rheodyne onto a Prep-SEC column from TosoHaas. SEC fractions were analyzed by plasma desorption ionization time-of-flight (TOF) MS on an ABI Bio-Ion 20 mass spectrometer (Foster City, CA, USA). MS analysis was performed by Multiple Peptide Systems (San Diego, CA, USA).

2.2. Reagents and standards

Extensive casein hydrolyzate and the experimental casein hydrolyzate were provided by undisclosed sources for this study. Carbonic anhydrase, α-lactalbumin, β-lactoglobulin, Met-Lys-bradykinin, insulin B, neurotensin (fragment 1–7), β-casomorphin (fragment 1–3), bombesin, α-lactorphin, tryptophan, glycyl-tyrosine and glycyl-glycine were purchased from Sigma (St. Louis, MO, USA); TFA was purchased from Pierce (Rockford, IL, USA), while methanol and 2-propanol were from Mallinckrodt (St. Louis, MO, USA). RO/deionized water was obtained from a Model Milli-QUV Plus system from Millipore.

2.3. Standard preparation and analytical method

An analytical SEC (TSK G2000 SWXL, 300×7.8 mm I.D., 5 μm particle size, silica based) column (60°C) was calibrated using seven standards. The stock standard solution was prepared by dissolving carbonic anhydrase (1.04 mg), α-lactalbumin (1.00 mg), insulin B chain (1.00 mg), bombesin (1.20 mg), neurotensin (fragment 1–7, 0.50 mg), β-casomorphin (fragment 1–3, 0.50 mg) and tryptophan (0.08 mg) in 1 ml of water. In some cases, one drop of acetic acid was added to solvate the peptide. Extensive casein hydrolyzate and experimental casein hydrolyzate were each analyzed with a 5-μl partially filled injection made into a 50-μl sample loop of the SEC system. The mobile phase consisted of 2-propanol–methanol–water (15:15:70, v/v) containing 0.1% TFA; the flow-rate was 400 μl/min. The column effluent was passed through a capillary splitter from SGE at 185 μl/min to the CLND system and at 215 μl/min to the UV detector. CLND conditions: pyrolysis temperature, 1050°C; photomultiplier tube (PMT), 700 V; range×25 and detector output, 1 V; UV conditions: 214 nm, range, 0.2 a.u.f.s.

A Prep-SEC (TSK G2000 SW, 600×21.5 mm I.D., 13 μm particle size, silica based) column at 25°C was similarly calibrated with the following aqueous standards: carbonic anhydrase, β-lactoglobulin, α-lactalbumin, insulin B, Met-Lys-bradykinin, α-lactorphin and glycyl-tyrosine. Extensive casein hydrolysate (1 g in the mobile phase) was injected using a 5-ml sample loop and was chromatographed using a mobile phase consisting of water–2-propanol–methanol (60:20:20, v/v) with 0.1% TFA and a flow-rate of 4 ml/min. The separation was monitored with UV (215 nm) detection and fractions were collected at 1 min intervals starting from 22 min and

stopping at 44 min, which approximated the V_0 (void volume) and V_t (total volume) of the column.

The RP-HPLC peptide mapping conditions are as follows: A Synchropak RPP-100 (150×4.6 mm I.D., 5 μm particle size) column (37°C) was used with mobile phases of (A) 0.1% TFA in water and (B) acetonitrile–methanol (80:20, v/v) containing 0.1% TFA. A gradient elution of 0 to 50% B in 45 min, with a 5-min hold at 50% B and a flow-rate of 1 ml/min, followed by column regeneration was used. Prep-SEC fractions were resuspended in 100 μl of mobile phase (A) and 5 μl were analyzed. The separated peptide components were then detected by UV (215 nm, 2.0 a.u.f.s.).

3. Results and discussion

Casein hydrolyzates, for example, are widely used in cosmetic and food industries, with some being used in hypoallergenic formula for infants. Peptides of M_r<1200 are perceived as hypoallergenic with respect to the native proteins. SEC (analytical scale) was used to estimate the average molecular-mass (M_r<1200) distribution of extensively hydrolyzed casein. The SEC mode of separation was achieved for these peptides by careful selection of the mobile phase and the column.

Lemieux et al. [15] reported that it was not appropriate to characterize complex peptide mixtures such as casein hydrolyzates on the basis of the peptide's individual molecular mass by SEC using the TSK G2000 SW silica-based column. Their mobile phase consisted of 0.12 *M* phosphate buffer at pH 5.0 and 10% methanol with 0.1% TFA (22°C). Some of the amino acid groups of the peptides at pH 5.0 may not have been fully protonated and interaction with the silanol groups of the chromatographic silica support was evident. In this case, one would not expect an SEC mode of separation to occur. In fact, peptides were eluted beyond V_t for the SEC column under their conditions.

3.1. Analytical SEC

Several refractive index (RI) detectors were evaluated to compliment the CLND system and the UV detector in the preliminary phase of this study. The problem we experienced with RI detectors was that very slight differences in either the salt or water content of the hydrolyzates resulted in a large artifact peak that was either negative or positive at, or near, V_t. When RI detection was used to analyze extensively hydrolyzed materials, a large portion of the sample that eluted near V_t was masked by this interfering peak, contributing to significant errors in the molecular-mass distribution. In this case, the problem was easily circumvented by looking at the "nitrogen mass" using CLND in place of the RI detection.

Table 1
Standards used to calibrate the analytical SEC chromatography silica-based column (TSK G2000 SWXL)

Peaks	Compounds	M_r	Retention time (min)
1	Carbonic anhydrase	29 000	13.47
		28 259[a]	
2	α-Lactalbumin	14 400	14.47
		18 288[a]	
3	Insulin B chain	3 550	18.15
		3 687[a]	
4	Bombesin	1 620	20.03
		1 627[a]	
5	Neurotensin (fragment 1–7)	777	22.63
		525[a]	
6	β-Casomorphin (fragment 1–3)	425	23.28
		395[a]	
7	Glycyl-glycine	132	24.63
	(Tryptophan)	220[a]	

[a] Calculated M_r from the retention time using the linear regression equation: Log M_r = −6.977−0.189×retention time (min), r=0.989; r^2=0.979.

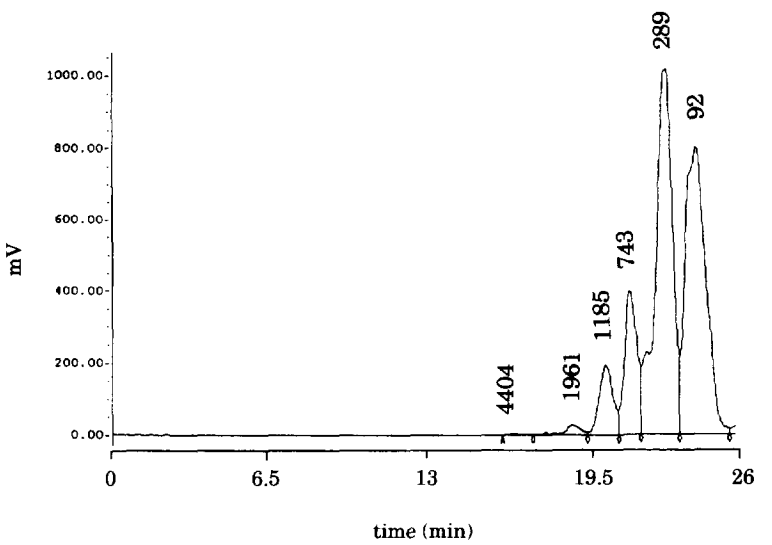

Fig. 1. SEC–CLND using a TSK G2000 SWXL silica-based analytical column showing the M_w distribution of an extensive casein hydrolyzate.

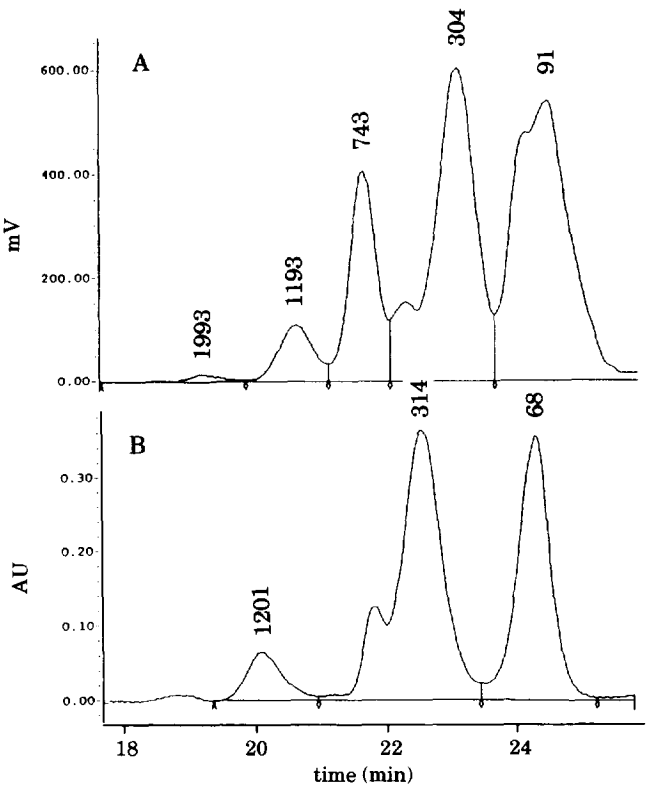

Fig. 2. SEC–CLND using a TSK G2000 SWXL silica-based analytical column with simultaneous detection, showing the M_w distribution of an experimental casein hydrolyzate. (A) CLND profile of total peptide and free amino acid content; (B) UV (214 nm) profile of components, mainly with strongly absorbing chromophores.

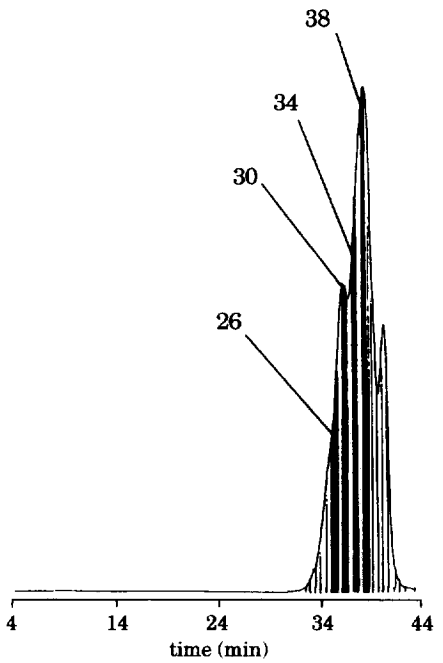

Fig. 3. SEC–CLND (UV) profile of extensive casein hydrolyzate using a TSK G2000 SW silica-based preparative column showing the M_r fractions (4 ml) collected at 1 min intervals. RP-HPLC peptide maps for fractions 26, 30, 34 and 38 are shown in Fig. 6.

An analytical SEC (TSK G2000 SWXL) silica-based column was used with an eluting solvent consisting of 2-propanol–methanol–water (15:15:70, v/v) containing 0.1% TFA. Table 1 shows the SEC analytical column calibration data based on molecular mass elution of the seven reference standards. The dipeptides and free amino acids in the hydrolysates eluted within V_t=24.63 min. At the beginning of the chromatographic process, V_o was 13.47 min. Fig. 1 is the CLND profile of an extensive casein hydrolyzate showing the molecular-mass distribution (SEC separation mode) with major peaks at M_r 1961, 1185, 743, 289 and 92. The average molecular mass was calculated (CLND values: number-average molecular mass M_n=178; weight-average molecular mass M_w=408; Z average molecular mass M_z=809).

A very powerful technique is demonstrated using SEC of an experimental casein hydrolyzate with an analytical column and a dual CLND and UV (214 nm) detection system (Fig. 2). CLND exhibits a complete nitrogen content profile of the experimental hydrolyzate showing average molecular mass (CLND values: M_n=163; M_w=346; M_z=965) with a SEC mode of separation. The UV profile, on the other hand, displayed only a part of the hydrolyzate profile, while clearly showing the presence of components with strong UV chromophores. The average molecular mass was also obtained (UV values: M_n=138; M_w=317; M_z=982). This dual detection technique can provide useful and practical solutions to other industrial applications that are currently problematic.

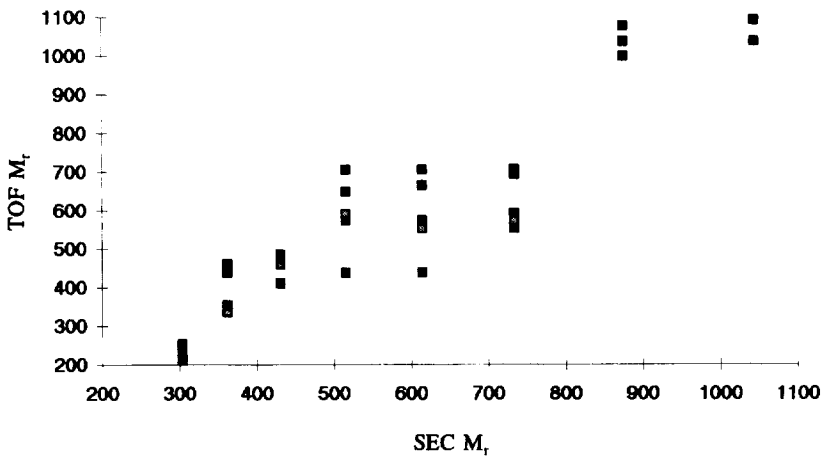

Fig. 4. Extensive casein hydrolyzate fractions from a prep-SEC (TSK G2000 SW) silica-based column were analyzed by TOF–MS. Data of up to the five most abundant TOF M_r ions from each fraction were compared to the corresponding SEC M_r values obtained from the calibrated preparative column.

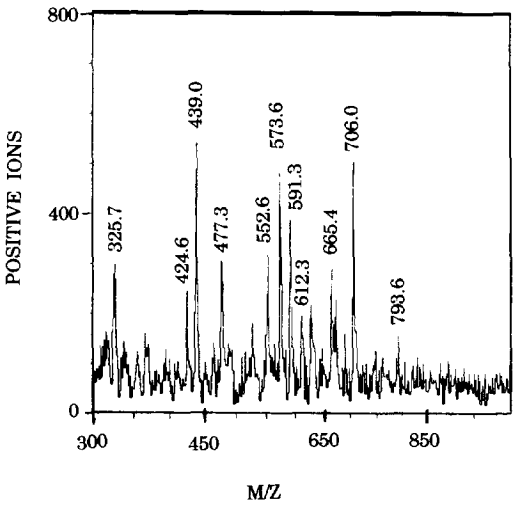

Fig. 5. TOF–MS profile of an extensive casein hydrolyzate fraction, fraction 34, collected from a prep-SEC (TSK G2000 SW) silica-based column. SEC M_r=614 for this fraction.

3.2. Prep-SEC

In the next phase of our study, a prep-SEC (TSK G2000 SW) silica-based column was used with a mobile phase consisting of water–2-propanol–methanol (60:20:20, v/v) with 0.1% TFA, to show that true SEC separation of complex samples is actually attainable. Due to the large dimensions of the Prep-SEC column and particle size (13 μm rather than 5 μm), the resolution of the extensive casein hydrolyzate (Fig. 3) by SEC was less than that obtained on the analytical column (Fig. 1). Area slices shown in Fig. 3 represent 4 ml fractions that were collected at 1 min intervals starting at 20 min and stopping at 44 min. The calibrated column provided the estimated SEC M_r for each collected fraction. Fractions of the extensive casein hydrolyzate were also analyzed by TOF MS in order to compare the distribution of the SEC M_r vs. TOF M_r (Fig. 4). In this plot, up to five of the most abundant TOF M_r (m/z) ions from each fraction were compared to the corresponding SEC M_r of the collected fractions. Results confirmed that peptides in the casein hydrolyzate eluted selectively according to size. A representative TOF–MS profile (fraction 34) of an extensive casein hydrolyzate is shown in Fig. 5. The extensive casein hydrolyzate fractions (Fig. 6) obtained by reversed-phase peptide mapping (UV, 215 nm) illustrate the complexity of the component mixtures.

4. Conclusion

SEC with a calibrated analytical column and CLND were used to estimate the molecular-mass distribution of both the experimental and extensive casein hydrolyzates. A dual SEC–CLND and UV

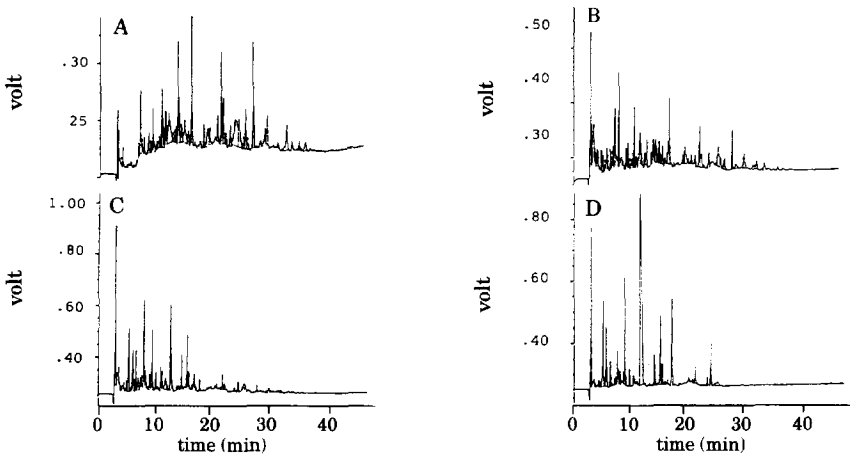

Fig. 6. Reversed-phase peptide mapping with UV (215 nm) detection showing complex peptide mixtures in the extensive casein hydrolyzate fractions obtained from a prep-SEC (TSK G2000 SW) silica-based column. Fractions collected are illustrated in Fig. 3: (A) 26; (B) 30; (C) 34 and (D) 38.

(214 nm) detection system provided valuable information: (1) CLND provided an accurate peptide content profile, (2) although UV detection did not provide a complete profile, UV detection clearly shows the groups of peptides containing primarily strong aromatic chromophores.

Prep-SEC of extensive casein hydrolyzate with a proper choice of mobile phase and a calibrated column was accomplished. Results of the TOF–MS ($m/z<1200$) analysis of the collected fractions indicated that components in the casein hydrolyzate eluted in the expected SEC mode of separation. These hydrolyzate fractions consisted of complex mixtures, as shown by a conventional RP-HPLC (UV detection) peptide mapping technique.

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