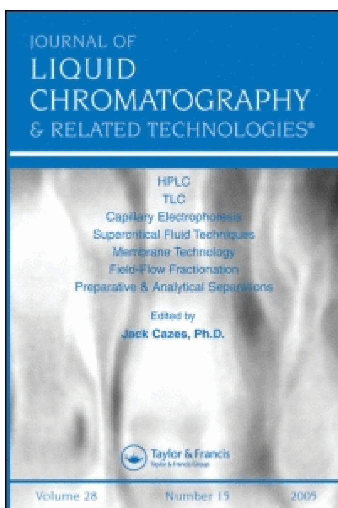
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

RAPID DETECTION OF A CASOMORPHIN AND NEW CASOMORPHIN-LIKE PEPTIDE FROM A PEPTIC CASEIN HYDROLYSATE BY SPECTRAL COMPARISON AND SECOND ORDER DERIVATIVE SPECTROSCOPY DURING HPLC ANALYSIS

Cécile Macaud[a]; Qiuyu Zhao[a]; Guy Ricart[b]; Jean Marie Piot[a]
[a] Université de La Rochelle, La Rochelle, France [b] Université Lille 1, Villeneuve d'Ascq, France

Online publication date: 22 February 1999

To cite this Article Macaud, Cécile , Zhao, Qiuyu , Ricart, Guy and Piot, Jean Marie(1999) 'RAPID DETECTION OF A CASOMORPHIN AND NEW CASOMORPHIN-LIKE PEPTIDE FROM A PEPTIC CASEIN HYDROLYSATE BY SPECTRAL COMPARISON AND SECOND ORDER DERIVATIVE SPECTROSCOPY DURING HPLC ANALYSIS', Journal of Liquid Chromatography & Related Technologies, 22: 3, 401 — 418
To link to this Article: DOI: 10.1081/JLC-100101668
URL: http://dx.doi.org/10.1081/JLC-100101668

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 22(3), 401–418 (1999)

RAPID DETECTION OF A CASOMORPHIN AND NEW CASOMORPHIN-LIKE PEPTIDE FROM A PEPTIC CASEIN HYDROLYSATE BY SPECTRAL COMPARISON AND SECOND ORDER DERIVATIVE SPECTROSCOPY DURING HPLC ANALYSIS

Cécile Macaud,[1] Qiuyu Zhao,[1]* Guy Ricart,[2] Jean Marie Piot[1]

[1] Laboratoire de Génie Protéique et Cellulaire
Pôle Sciences et Technologies
Université de La Rochelle
Avenue Marillac
17042 La Rochelle, France

[2] Laboratoire de Spectrométrie de Masse
Université Lille 1
59655 Villeneuve d'Ascq, France

Downloaded At: 10:35 24 January 2011

ABSTRACT

Detection of a casomorphin and a new casomorphin-like peptide from a casein enzymatic hydrolysate was achieved using second-order derivative spectra and UV-spectra comparison. In this method, synthetic casomorphins were used as standards. The ß 1-3 casomorphin and • 90-94 casomorphin-like, were detected and rapidly purified with RP-HPLC coupled with photodiode array detector. This technique simplifies the identification and the purification of bioactive peptides containing aromatic amino acids from complex hydrolysates. It could be applied to follow up the evolution of these peptides during casein hydrolysis.

Copyright © 1999 by Marcel Dekker, Inc. www.dekker.com

Downloaded At: 10:35 24 January 2011

INTRODUCTION

Since 1979, several authors have described bioactive peptides originating from casein hydrolysates. These peptides which are inactive within the sequence of the parent protein can be released during gastrointestinal digestion.[1,2] Once they are liberated in the body, bioactive peptides may act as regulatory compounds with hormone-like activity. Their possibly regulatory effects concern immunomodulatory,[3] antihypertensive,[4] antithrombotic,[5] nutrient uptake,[2] antibacterial,[6] and opioid properties.[7,8,9,20]

Most of the known bioactive peptides derived from caseins are opioid peptides called casomorphins. They can be released by casein enzymatic treatment both in vitro and in vivo.[1,10,11,12,13] It is known that casomorphins can directly or indirectly act on targets like hormones do. The physiological occurrence and the possible role of these peptides remain an open question. It would be interesting to follow up the evolution of their release during casein processing with a view to understand their significance and their dealing on the body. In this view, a rapid and simple detection method of casomorphins is necessary to be developed.

Until now, opioid peptides are detected via opioid receptor binding studies,[8] isolated organ preparations or immunoreactive materials.[1,12,15] These methods, though they are sensitive, are rather complex to be applied.

Structural characteristics of the casomorphins become very useful for their detection during HPLC analysis. Indeed, endogenous and exogenous opioid peptides present a common feature in their structure:[16,17] Tyr-X-AAA (AAA=aromatic amino acid) or Tyr-X1-X2-AAA which is essential for an opiate activity. Particularly, α-casomorphins have the fragment Tyr-Gly-Leu-Tyr while Tyr-Pro-Phe is involved in β-casomorphins.

Recently, HPLC coupled with photodiode array detector extended many advances of ancillary spectroscopy in liquid chromatography because it led us to obtain real time spectra during a separation process. On one hand, the obtained spectra allowed us to identify an expected molecule with spectral comparison technology[18] and, on the other hand, it permitted us to investigate aromatic amino acids in the peptides by second order derivative spectroscopy.[19,21] As an example, these two techniques were effectively applied for the identification of hemorphins in a peptic hemoglobin hydrolysate.[18]

In this work, the detection of casomorphins in a complex casein hydrolysate is investigated. It indicates that the on-line detection becomes possible. This could be applied for the kinetic study of casomorphin release from casein digestion.

EXPERIMENTAL

All common chemicals and solvents were of analytical grade from commercial sources. Bovine casein and porcine Pepsin (E.C.3.4.23.1) were purchased from Sigma Chemicals and synthetic casomorphins from Bachem and Altergen.

Hydrolysate and Standard Casomorphins Preparation

Bovine casein (200mg) dissolved in 20mL HCl-KCl Buffer (50mM,pH2) was digested by addition of porcine pepsin (4mg), therefore, the enzyme/substrate ratio reached 1/50. Temperature reaction was maintained at 40°C and initial pH was 2.

The enzymatic hydrolysis was stopped by addition of an equal volume of 15% trichloroacetic acid (TCA) after 18 h incubation. The supernatant was then centrifuged, filtered, and analysed by RP-HPLC.

Downloaded At: 10:35 24 January 2011

Standard β-casomorphins (β1-3, β1-4, β1-5, β1-6, β1-7) and α-casomorphins (α90-94, α90-95, α90-96) were prepared at a concentration of 1µg/µL in MilliQ water.

HPLC System

The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisps 717 automatic sampling device, and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millenium software was used to plot, acquire, and analyse chromatographic data.

Mobile Phase for SynchroPak C4 Column (250X10 mm i.d.) and DeltaPak C18 Column (150X3,9 mm i.d.)

The mobile phase consisted of MilliQ water containing 0.1% trifluoro acid (TFA) as eluant A and acetonitrile containing 0.1% TFA as eluant B. The flow rate was 2.5mL/min for the semi-preparative column C4 (250X10 mm i.d.) and 1.2mL/min for the analytical column C_{18} (150X3,9 mm i.d.). For investigation of pH effect on the spectral comparison results (see casomorphins spectral library section), the samples (casomorphins dissolved in basic condition) were eluted from the analytical column with 10 mM acetate ammonium, pH 6.5 as eluant A and acetonitrile as eluant B. The gradient applied was 0-40% B in

40min, then 40-0% B in 5min. On-line instantaneous UV-absorbance spectral scans were performed between 200 and 300 nm at a rate of one spectrum/second. The resolution was 1.2 nm. Chromatographic results were analysed using Millenium software.

Casomorphins Spectral Library

Identification of peptides from the casein peptic hydrolysate was carried out by UV spectral comparison as previously described for the hemorphins.[18] Casomorphins standard solutions (10μL) were mixed with 15% TCA (10μL) and then injected in the C_{18} column (150X3,9mm i.d.). The spectra of synthetic casomorphins were stored in the Millenium library. The mathematical analysis of the differences between any two spectra was quantitatively expressed using the following criteria :

> Match Angle (MA): a measure of the difference in spectral shapes between an unknown spectrum and a library spectrum; the match angle can range from 0 to 90°. Lower values (about or under 1.000) indicate that spectra are similar. Larger values indicate greater degrees of spectral difference.
>
> Match Threshold (MT): sensitivity of the measurement. It can range from 0 to 180°. Larger values indicate a lower measurement sensitivity.

In general, if the Match Angle is greater than the Match Threshold, it indicates that the two spectra are different; if the MA is less than the MT, it indicates that the two spectra are the same or similar. Therefore, identical spectra allow us a quasi-identification of an expected peptide.

In order to investigate the effect of pH on the results of spectral comparison, 10μL of each casomorphin solution were mixed with the same volume of 1M NaOH. Then, 10μg casomorphin in 20μL were injected in the same column and under the conditions previously described.

Amino-Acid Analysis

Amino acids were analysed using a Waters PicoTag Work Station. Peptide hydrolysis was achieved with constant-boiling 6M HCl containing 1% phenol, for 24h at 110°C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids was performed on a Waters RP-Picotag column (150X3,9 mm i.d.) according to Bidlingmeyer et al.[13] The detection wavelength was 254 nm and the flow rate 1mL/min.

Downloaded At: 10:35 24 January 2011

Mass spectrometry analysis

MALDI mass spectra were measured on a reflectron-typeVision 2000 time-of-flight mass spectrometer (Finnigan MAT, Bremen, Germany). Sample was mounted on an x,y moveable stage allowing irradiation of selected sample areas. A nitrogen laser with an emission wavelength of 337nm and 3ns pulse duration was used. Spectra were recorded in the positive- ion mode and accelerated to an energy of 5 keV before entering the flight tube. Ions were post-accelerated for detection to an energy of 5 keV. Sample was prepared by mixing directly on the target 1 µL of the peptide and 1 µL of 2.5.dihydroxybenzoic acid (DHB) as matrix solution. The sample was allowed to dry for 3-4min at room temperature.

RESULTS

UV-Spectral Comparison Between Casomorphins

HPLC coupled with a photodiode array detector allowed us to obtain the instantaneous acquisition of UV-spectra at any time during a chromatographic analysis. This could be used for the identification of an expected molecule.[18]

It is known that the UV-spectra of a peptide is greatly dependent on its amino acid contents in such a manner that each molecule has a characteristic absorbance spectrum. It has been demonstrated that differences between two spectra could be calculated by mathematical analysis:[14] MA and MT values express these differences. The results of spectral comparison for casomorphins are shown in Table 1. These results display that the best matching values are obtained when identical casomorphins are compared between them.

Effect of pH

Table 2 presents spectral comparison results between casomorphins spectra obtained on different conditions: on one hand, casomorphin samples in basic conditions (NaOH) and eluted with neutral eluent (ammonium acetate), on the other hand, casomorphins dissolved in acid conditions (TCA) and eluted with acid eluent (TFA).

It indicates that for a same molecule, comparison between these spectra shows a great value of MA. Therefore, it is of great importance, when spectral comparisons are performed, to carry out experiments under the same pH conditions.

Downloaded At: 10:35 24 January 2011

Downloaded At: 10:35 24 January 2011

Table 1

Results of Spectral Comparison Between Casomorphins Eluted from Chromatography on Delta Pak C18 Column and Casomorphins from Spectral Library*

	β-1-3		**β 1-4**		**β 1-5**		**β 1-6**		**β 1-7**		**α 90-95**		**α 90-96**	
	MA	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**
β 1-3	**0.001**	**1.042**	2.878	1.049	3.435	1.035	3.619	1.185	3.596	1.105	6.768	1.718	7.469	1.041
β 1-4	2.854	1.047	**0.003**	**1.052**	0.567	1.041	1.091	1.186	1.644	1.106	6.789	1.718	6.238	1.045
β 1-5	3.494	1.034	0.590	1.042	**0.008**	**1.022**	0.806	1.183	1.280	1.100	6.797	1.718	5.836	1.032
β 1-6	3.752	1.193	1.145	1.195	0.831	1.192	**0.023**	**1.248**	0.951	1.205	7.167	1.734	5.292	1.192
β 1-7	3.645	1.114	1.684	1.118	1.294	1.112	0.870	1.214	**0.020**	**1.142**	7.517	1.724	4.516	1.117
α 90-95	7.156	1.753	7.054	1.748	7.091	1.851	7.432	1.787	7.782	1.758	**0.007**	**1.991**	0.427	1.753
α 90-96	7.416	1.042	6.397	1.049	5.942	1.035	5.216	1.185	4.486	1.102	0.411	1.713	**0.000**	**1.039**

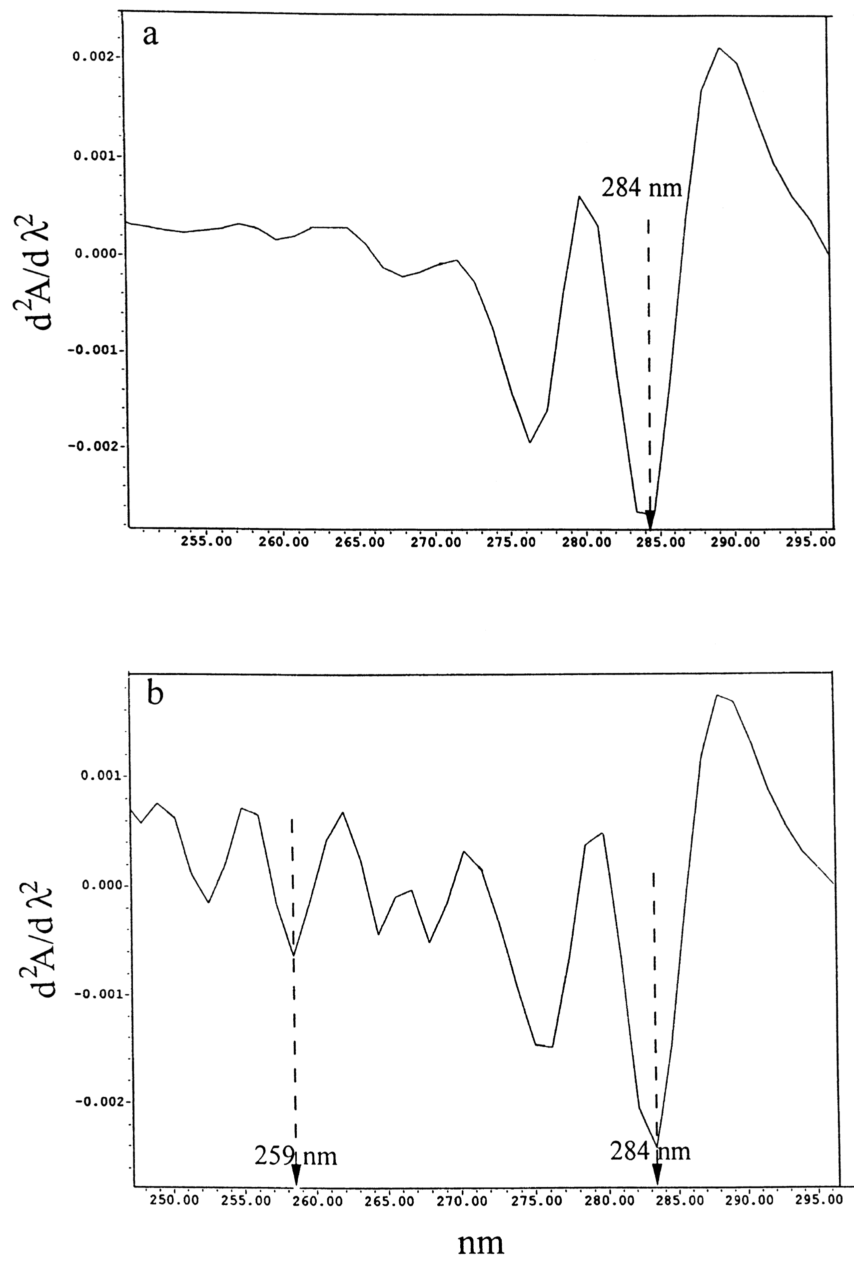
* MA: Match Angle; MT: Match Threshold.

Downloaded At: 10:35 24 January 2011

Table 2

pH Effect on Spectral Comparison Results Between Acid and Neutral Chromatographic Condition

	Acid Medium (TFA)													
	β-1-3		**β 1-4**		**β 1-5**		**β 1-6**		**β 1-7**		**α 90-95**		**α 90-96**	
Neutral Eluent (Ammonium Acetate)	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**	**MA**	**MT**
β 1-3	**4.710**	**1.122**	4.559	1.150	3.463	1.094	5.990	1.430	5.333	1.185				
β 1-4	6.722	1.084	**4.803**	**1.107**	3.613	1.054	3.907	1.424	3.791	1.178				
β 1-5	8.008	1.094	5.764	1.116	**4.674**	**1.066**	4.103	1.425	3.347	1.178				
β 1-6			7.644	1.109	6.502	1.057	**3.713**	**1.424**	2.946	1.178				
β 1-7			9.102	1.117	7.874	1.066	5.558	1.425	**4.635**	**1.179**				
α 90-95											**1.623**	**3.140**	1.608	1.066
α 90-96											2.639	3.600	**2.629**	**1.067**



Downloaded At: 10:35 24 January 2011

Second-Order Derivative Spectroscopy

By using HPLC coupled with the photodiode array detector system, the derivation of peptides spectra gives rise to their corresponding second order derivative which can lead to identifying aromatic amino acids.[19] Since the presence of Tyr and Phe is characteristic of the casomorphin structure, second order derivative spectroscopy technique could complete the results of spectral comparison.

Figure 1 indicates the second order derivative spectra of α90-95 and α1-4 casomorphins. Analysis of the second-order derivative spectra between 240 and 300nm gave the same spectral characteristics as the free corresponding aromatic amino acids.[19] As an example, the presence of Tyr in the α90-95 casomorphin is indicated by the minimum at 284nm (Fig.1a). The minima at 284 and 259 nm demonstrated the presence of Tyr and Phe respectively, in β1-4 casomorphin (Fig.1b).

Downloaded At: 10:35 24 January 2011

Detection of a Casomorphin and a New Casomorphin-Like Peptide in the Casein Peptic Hydrolysate

RP-HPLC analysis of TCA supernatant

TCA supernatant (150µL) from the total casein hydrolysate was analysed by RP-HPLC on the C4 column (250X10mm i.d.). Since the aim of this study was to detect casomorphins which are known to contain aromatic amino acids, the chromatographic profile was analysed at 280nm and presented in Figure 2. It was observed that under these chromatographic conditions, a good resolution was obtained. Therefore, in order to detect casomorphins in this hydrolysate, the comparison of each peak spectrum with each casomorphin standard spectrum was carried out.

Detection of α90-94 casomorphin-like peptide and β1-3 casomorphin in the hydrolysate by UV- spectral comparison

A comparison between the retention time of each standard casomorphin and each eluted peptide was achieved in order to make a first selection among these peptides. At this stage, with these criteria, two peaks were selected : peak 1 and 2 (Fig.2, Table 3).

Figure 1 (left). Second order derivative spectra of casomorphins: α90-95 (a) and β1-4 (b). The minima at 284 nm indicated the presence of Tyr in both peptides and minima at 259 nm indicated the presence of Phe in β1-4.

Downloaded At: 10:35 24 January 2011

Table 3

Results of Spectral Comparison Between Casomorphins α 90-95, α 90-96, α90-94, β 1-3 and Peaks 1, 1a, 2, and 2b

Library Spectra	α 90-95 RT=34.012 min		α 90-96 RT=31.013 min		α 90-94 RT=26.193 min		β 1-3 RT=35.195 min	
	MA	MT	MA	MT	MA	MT	MA	MT
Sample Spectra								
-peak 1	1.125	1.779	0.976	1.030				
-peak 1a (RT=26.175 min)	1.072	1.779	0.644	1.090	**0.345**	**1.079**		
-peak 2							2.317	1.203
-peak 2b (RT=35.052 min)							**1.162**	**1.219**

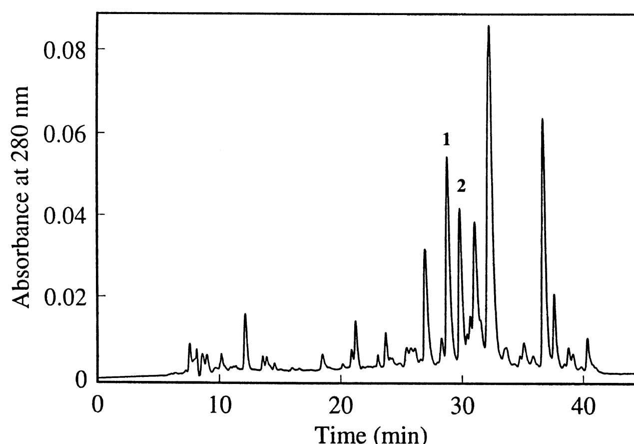


Figure 2. Chromatographic profile of the total peptic casein hydrolysate at 280 nm obtained from a semipreparative reverse phase column (Synchro Pack C4, 250 x 10 mm i.d.) under the conditions described in the experimental section.

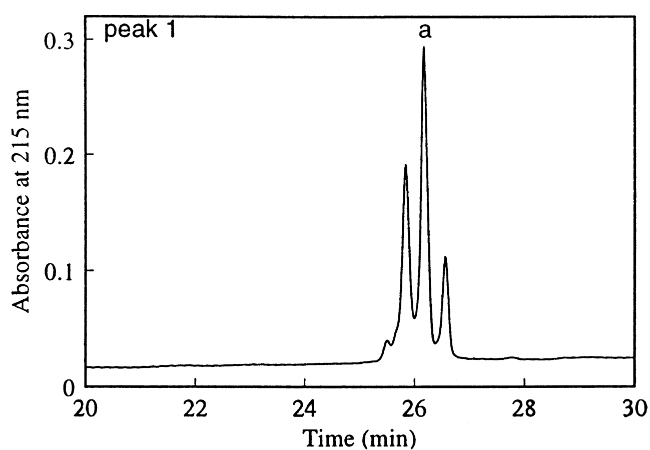
Downloaded At: 10:35 24 January 2011

As seen in Table 3, peak 1 and 2 are quite similar with α90-96 and β1-3 casomorphins respectively. As for peak 2, it could be β1-3 casomorphin, even if MA is higher than MT. In fact, it is the only peak exhibiting a MA value close to MT. The fact that in this case (peak 2) MA is higher than MT could be due to the presence of impurities. So in a second time, fractions 1 and 2 were re-injected on the column C_{18} (150X3,9mm i.d). The profiles are shown in Figure 3. From this figure, peak 1 gives rise to three peaks and peak 2 to two peaks. Each of these peaks has been compared with α90-95, α90-96 and β1-3 casomorphins. Two peaks (1a and 2b) were selected according to their retention time and matching values (Table 3).

For peak 2b, it could be the β1-3 casomorphin because of quasi identical retention time and matching results. However, for peak 1a, even if matching results indicated that this peptide was similar to α90-95 or α90-96 casomorphins, its retention time was very different. Therefore, it was interesting to identify this peptide with other techniques.

Second order derivative spectra analysis

Figure 4 shows the second order derivative spectrum of each selected peptide. For the peak 1a minimum at 284nm indicates the presence of Tyr as in α90-95 or α90-96 casomorphin. Minima at 284nm and 259nm demonstrate the presence of Tyr and Phe in the peak 2b as in β1-3 casomorphin.



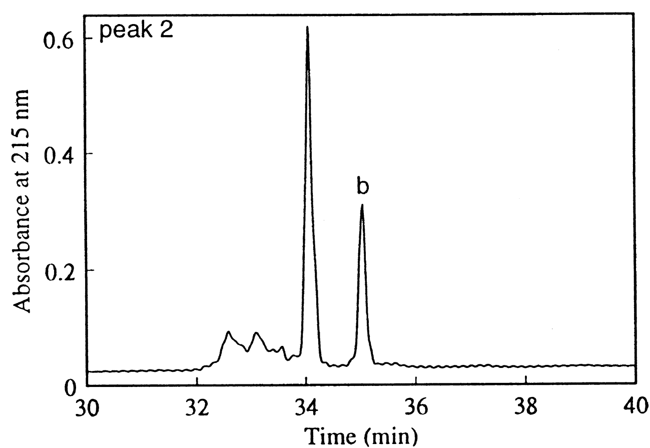
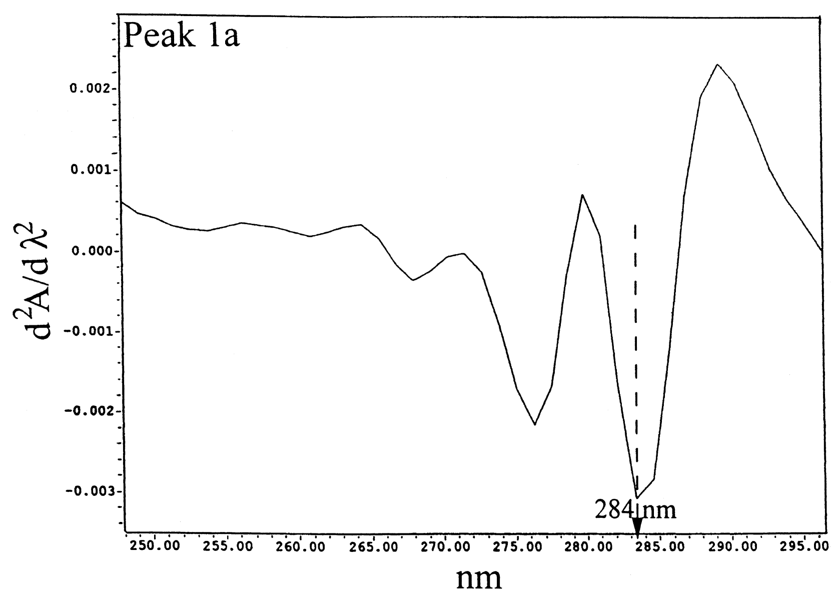
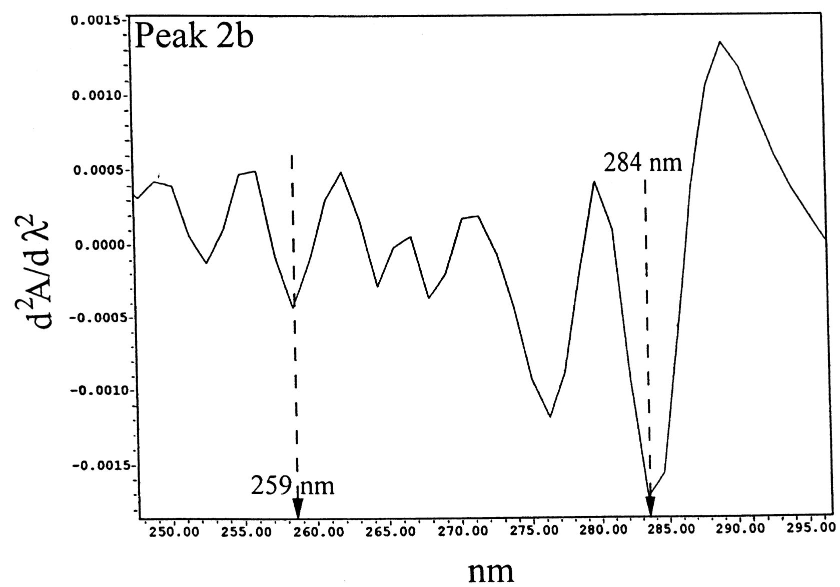


Figure 3. Chromatographic profiles from the analysis of peaks 1 and 2 (Figure 2) with an analytical reverse phase column (Delta pak C18, 150 x 3.9 i.d.) under the conditions described in the experimental section.

Figure 4 (right). Second order derivative spectra of peaks 1a and 2b. The minima at 284 nm indicated the presence of Tyr in both peptide and minima at 259 nm indicated the presence of Phe in peak 2b.

Downloaded At: 10:35 24 January 2011





Downloaded At: 10:35 24 January 2011

Downloaded At: 10:35 24 January 2011

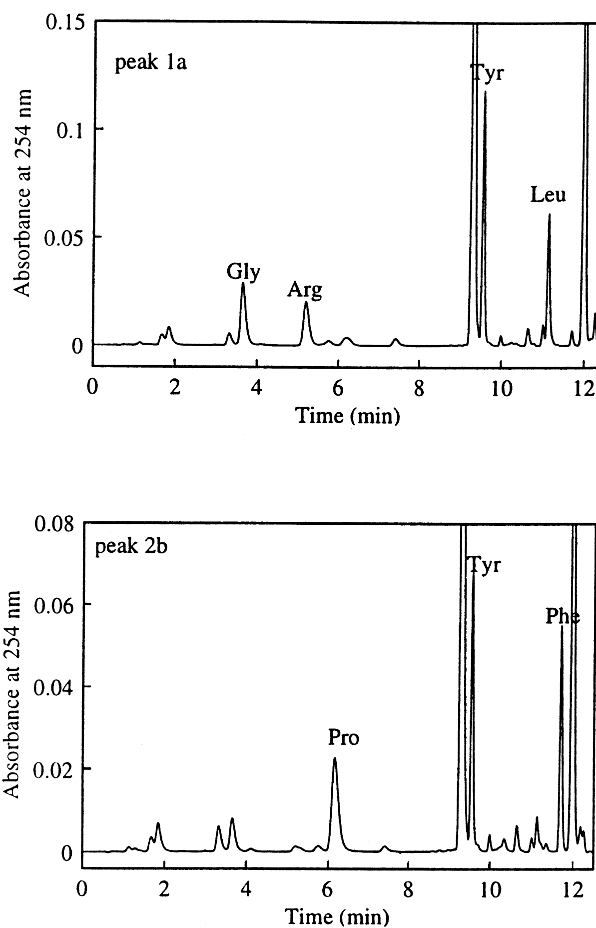


Figure 5. Elution profiles of PTC amino acids from peak 1a and 2b obtained by HPLC with a Waters Pico Tag column.

Amino acid composition analysis

Figure 5 presents the results of the PicoTag analysis. The amino acid composition was deduced from the comparison with a standard curve (not shown). Peak 1a consists of Gly(1), Arg(1), Tyr(2), and Leu(1). Peak 2b

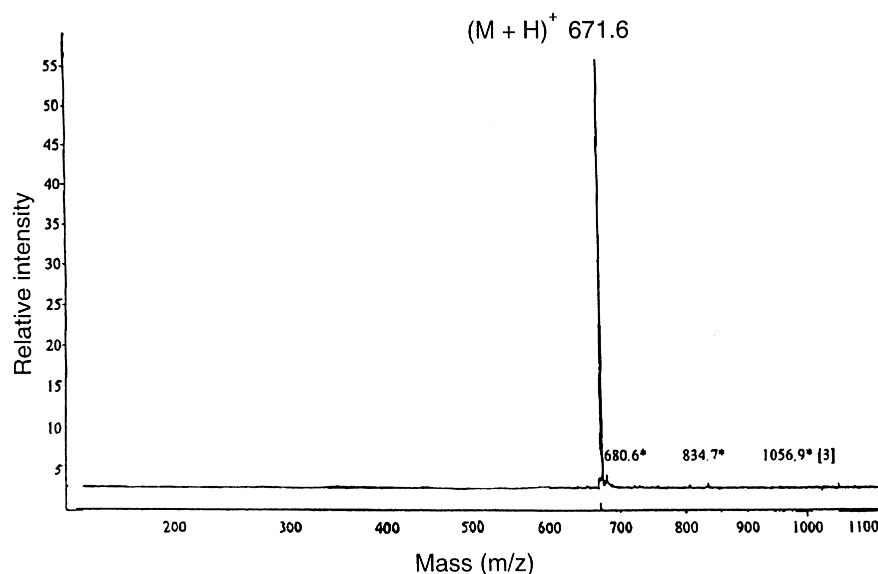


Figure 6. Mass spectrometry of peak 1a. The abundant ion at 671.6, designated as a molecular cation $(M + H)^+$, suggested the peak 1a has a molecular weight of 670.

Downloaded At: 10:35 24 January 2011

consists of Pro(1), Tyr(1), and Phe(1). Regarding the four chain structures of bovine casein, the only sequence compatible with peak 1a amino acid composition is Arg-Tyr-Leu-Gly-Tyr and it represents αs1 90-94 bovine casein fragment. Deduced sequence of peptide 2b is Tyr-Pro-Phe which represents the β1-3 bovine casein fragment.

The synthetic αs1 90-94 peptide was loaded on the C_{18} column. The spectral comparison with peak 1a was carried out and the results are shown in Table 3. From these results, peak 1a appears to correspond closely to •s1 90-94 sequence.

Analysis of peak 1a by mass spectrometry

Figure 6 shows the positive mass spectrum of peak 1a. Exact molecular weight deduced from the m/z value of $[M+H]^+$ by subtraction of one mass unit for the attached proton is 670. This molecular weight corroborates, absolutely, the sequence previously determined for peak 1a: Arg-Tyr-Leu-Gly-Tyr (αs1 90-94).

DISCUSSION

In this work, a rapid method of detection of casomorphin and casomorphin-like peptide from a peptic bovine casein hydrolysate was applied. Such technique was successfully carried out for the identification of hemorphins from a peptic hemoglobin hydrolysate.[18] Only one casomorphin (β1-3) has been detected in our peptic casein hydrolysate. Another peptide very close to the well-known α casomorphins (α90-95 and α90-96),[7] was also identified in our hydrolysate. This casomorphin-like peptide corresponded to the α casomorphin sequence α90-94. Although this α90-94 peptide has never been described in the literature, it could be named α-casomorphin since it contains the sequence Tyr-X-X-Tyr which is essential for the opiate activity. Nevertheless, it would be interesting to carry out the Guinea-Pig Ileum test (GPI) in order to evaluate its opioid activity.

It can be noticed that the UV-spectral matching results, second order derivative spectroscopy and the retention time always gave rise to complementary data that lead to a constant result. Moreover, biochemical analysis, such as amino acids composition or mass spectrometry, were used to corroborate these results and were also necessary to identify this molecule (α90-94).

As far as α-casomorphins are concerned, it was reported that α-casein fragments with an opioid activity were generated after one hour of peptic α-casein hydrolysis.[8] The peptides responsible for such activity were identified as α90-95 and α90-96 casein fragments.[7] These peptides were then synthesized and their opioid activity tested. It was similar to that of purified casomorphins.

However, only α90-94 casomorphin was identified in our peptic casein hydrolysate and it was obtained after 18 h of hydrolysis. The hypothesis of α90-95 and α90-96 casomorphins as precursors of α90-94 peptide could be logically suggested. Then the hydrolysis of α90-95 and α90-96 casomorphins by pepsin was carried out.

Preliminary results (not yet published) indicated that about 2.5% of α90-95 and 44% of α90-96 casomorphins were hydrolysed to produce α90-94 peptide after 24h of hydrolysis. Therefore, one may hypothesize that the generation of α90-94 could result either directly from α casein or from α90-95 and α90-96 hydrolyses.

In 1979, Zioudrou et al.[8] realized an α-casein hydrolysis during only one hour while our hydrolysis was stopped after 18 hours. Hence, we can wonder about the sufficient time for the appearance of α90-94 casomorphin. Maybe one

Downloaded At: 10:35 24 January 2011

hour is too short to produce this peptide. Moreover, we can not affirm that, before its absorption, opioid material described by Zioudrou will not be degraded anymore by the pepsin.

In this study, we have shown that a casomorphin-like peptide can be isolated from casein, a food protein, after treatment with the stomach proteinase, pepsin. Conditions of digestion of casein are quite similar to conditions to which food proteins may be processed in the stomach after a meal. Through the discovery of this new casomorphin-like peptide, many questions persists. More knowledge about digestion physiology and reactions leading to the generation of casomorphins is still necessary.

REFERENCES

1. H. Meisel, FEBS Lett., **196**, 223-227 (1986).

2. H. Meisel, H. Frister, J. Dairy Res., **56**, 343-345 (1989).

3. D. Migliore-Samour, P. Jollès, Experientia, **44**, 188-193 (1988).

4. H. Meisel, “Casokinins as inhibitors of Angiotensin-Converting-Enzyme,” in **New Perspectives in Infant Nutrition,** G. Sawatzki, B. Renner, eds., Stuttgart:Thieme, Stuttgart, New York, 1993, pp.153-159.

5. P. Jollès, J. P. Caen, Trends Food Sci. Technol., **2**, 42-43 (1991).

6. H. D. Zucht, M. Raida, K. Adermann, H. J. Mägert, W. G. Forssman, FEBS Lett., **372**, 185-188 (1995).

7. S. Loukas, D. Varoucha, D. Zioudrou, R. A. Streaty, W. A. Klee, Biochemistry, **22**, 4567-4573 (1983).

8. C. Zioudrou, R. A. Streaty, W.A., Klee, J. Biol. Chem., **254**, 2446-2449 (1979).

9. V. Brantl, H. Teschemacher, A. Henschen, F. Lottspeich, Hoppe Seylers Z. Physiol. Chem., **360**, 1211-1216 (1979).

10. H. Teschemacher, G. Koch, V. Brantl, in **Beta-Casomorphins and Related Peptides,** V. Brantl, H. Teschemacher, eds., VCH Verlag, New York, Basel, Cambridge, 1994, pp. 3-17.

11. A. M. Fiat, P. Jollès, Mol. Cell. Biochem., **87**, 5-30 (1989).

Downloaded At: 10:35 24 January 2011

12. M. Umbach, H. Teschemacher, K. Praetorius, R. Hirshhauser, H. Bostedt, Regul.Peptides, **12**, 223-230 (1985).

13. B. A. Bidlingmeyer, S. A. Cohen, T. L. Tarvin, J. Chromatogr., **336**, 93-104 (1984).

14. Q. Zhao, F. Sannier, J. M. Piot, Biochim. Biophys. Acta, **1295,** 73-80 (1996).

15. J. Svedberg, J. De Haas, G. Leimenstoff, F. Paul, H. Teschemacher, Peptides, **6**, 825-830 (1985).

16. H. Chiba, M. Yoshikawa, "Biologically Functional Peptides from Food Proteins: New Opioid Peptides from Milk Proteins," in **Protein Tailoring for Food and Medical Uses**, R. E. Feeney, J. R. Whitaken, eds. Marcel Dekker, Inc., New York, 1986, pp.123-153.

17. K. J. Chang, A. Killian, E. Hazum, P. Cuatrecasas, Science, **212**, 75-77 (1981).

18. Q. Zhao, F. Sannier, G. Ricart, J. M. Piot, J. Liq. Chromatogr., **18**, 93-103 (1995).

19. Q. Zhao, F. Sannier, I. Garreau, C. Lecoeur, J. M. Piot, J. Chromatogr. A, **723**, 35-41 (1996).

20. A. Henshen, F. Lottspeich, V. Brantl, H. Teschemacher, Hoppe Seylers Z. Physiol. Chem., **360**, 1217-1224 (1979).

21. E. Perrin, L. Miclo, A. Driou, G. Linden, J. Chromatogr. B, **664**:267-276 (1995).

Received April 27, 1998
Accepted May 19, 1998
Manuscript 4784

Downloaded At: 10:35 24 January 2011

Downloaded At: 10:35 24 January 2011

Request Permission or Order Reprints Instantly!

Interested in copying and sharing this article? In most cases, U.S. Copyright Law requires that you get permission from the article's rightsholder before using copyrighted content.

All information and materials found in this article, including but not limited to text, trademarks, patents, logos, graphics and images (the "Materials"), are the copyrighted works and other forms of intellectual property of Marcel Dekker, Inc., or its licensors. All rights not expressly granted are reserved.

Get permission to lawfully reproduce and distribute the Materials or order reprints quickly and painlessly. Simply click on the "Request Permission/Reprints Here" link below and follow the instructions. Visit the U.S. Copyright Office for information on Fair Use limitations of U.S. copyright law. Please refer to The Association of American Publishers' (AAP) website for guidelines on Fair Use in the Classroom.

The Materials are for your personal use only and cannot be reformatted, reposted, resold or distributed by electronic means or otherwise without permission from Marcel Dekker, Inc. Marcel Dekker, Inc. grants you the limited right to display the Materials only on your personal computer or personal wireless device, and to copy and download single copies of such Materials provided that any copyright, trademark or other notice appearing on such Materials is also retained by, displayed, copied or downloaded as part of the Materials and is not removed or obscured, and provided you do not edit, modify, alter or enhance the Materials. Please refer to our Website User Agreement for more details.

Order now!

Reprints of this article can also be ordered at

http://www.dekker.com/servlet/product/DOI/101081JLC100101668