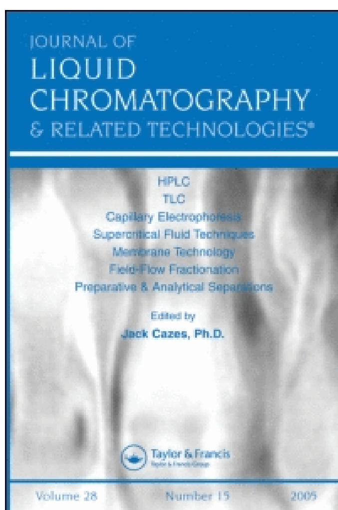
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



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

CAPILLARY ELECTROPHORESIS AS A MODERN TOOL FOR DETERMINING PROTEOLYTIC ACTIVITIES IN PURIFIED SPECIMENS AND IN REAL SAMPLES

Paolo Iadarola[a]; Laura Annovazzi[a]; Anna Lupi[a]; Maurizio Luisetti[b]; Begona Casado[c]; Gerd Doring[d]; Dieter Worlitzsch[d]; Giuseppe Cetta[a]; Simona Viglio[a]
[a] Dipartimento di Biochimica "A. Castellani", Università di Pavia, Pavia, Italy [b] Laboratorio di Biochimica e Genetica, Clinica di Malattie dell'Apparato Respiratorio, IRCCS Policlinico, Pavia, Italy [c] Department of Medicine, Georgetown University, Washington, DC, U.S.A. [d] Department of General and Environmental Hygiene, Hygiene Institute, University of Tuebingen, Tuebingen, Germany

Online publication date: 28 August 2002

To cite this Article Iadarola, Paolo , Annovazzi, Laura , Lupi, Anna , Luisetti, Maurizio , Casado, Begona , Doring, Gerd , Worlitzsch, Dieter , Cetta, Giuseppe and Viglio, Simona(2002) 'CAPILLARY ELECTROPHORESIS AS A MODERN TOOL FOR DETERMINING PROTEOLYTIC ACTIVITIES IN PURIFIED SPECIMENS AND IN REAL SAMPLES', Journal of Liquid Chromatography & Related Technologies, 25: 13, 1919 — 1945

To link to this Article: DOI: 10.1081/JLC-120013989

URL: http://dx.doi.org/10.1081/JLC-120013989

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.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
Vol. 25, Nos. 13–15, pp. 1919–1945, 2002

CAPILLARY ELECTROPHORESIS AS A MODERN TOOL FOR DETERMINING PROTEOLYTIC ACTIVITIES IN PURIFIED SPECIMENS AND IN REAL SAMPLES

Paolo Iadarola,[1,]* Laura Annovazzi,[1] Anna Lupi,[1] Maurizio Luisetti,[2] Begona Casado,[3] Gerd Doring,[4] Dieter Worlitzsch,[4] Giuseppe Cetta,[1] and Simona Viglio[1]

[1]Dipartimento di Biochimica "A. Castellani", Università di Pavia, Via Taramelli 3/B, 27100, Pavia, Italy
[2]Laboratorio di Biochimica e Genetica, Clinica di Malattie dell'Apparato Respiratorio, IRCCS Policlinico San Matteo, Pavia, Italy
[3]Department of Medicine, Georgetown University, Washington, DC 20007, USA
[4]Department of General and Environmental Hygiene, Hygiene Institute, University of Tuebingen, Germany

ABSTRACT

Owing to a series of interesting advantages, capillary electrophoresis (CE) is being widely used as an analytical tool for detecting enzymatic activities. On account of their resolving power, high speed, and small amount of sample required, several assays based on CE techniques have supported and/or replaced,

*Corresponding author. E-mail: piadarol@unipv.it

DOI: 10.1081/JLC-120013989
1082-6076 (Print); 1520-572X (Online)
Copyright © 2002 by Marcel Dekker, Inc.
www.dekker.com

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

during the past decade, traditional spectrophotometric or HPLC methods. In particular, a series of assays of practical utility have been designed for monitoring "in vitro" and "ex vivo", the hydrolytic activity of proteinases of different origin involved in the development of human diseases. The aim of the present article is to focus the interest of the reader on some recent applications of different CE modes (capillary zone electrophoresis—CZE; micellar electrokinetic chromatography—MEKC and capillary isoelectric focusing—cIEF) in the field of proteolytic enzymes to underline the wide applicability of this methodology. The article presents examples of particular interest for which the advantages and/or limitations of CE over established assays are discussed.

Key Words: Capillary electrophoresis; Proteolytic enzymes

Downloaded At: 20:43 23 January 2011

INTRODUCTION

From among the variety of rapid and sensitive procedures so far reported for measuring enzyme activity, capillary electrophoresis (CE) has been developed in recent years into powerful methods very useful for these determinations.[1–16] The requirement of small sample volumes, coupled to short analysis times and good quantification capabilities, make CE an excellent tool for "in vitro" assaying of a large number of enzymes. A wide series of applications of CE approaches to the study of minute enzymatic activities has been reported in a comprehensive review published by Bao et al. in 1997.[17] CE is, obviously, also well suited to the determination of enzyme activities in real samples: in fact, the very high resolution power of this technique often allows the decrease of substrate and the increase of product(s) of the reaction to be detected simultaneously, even in complex biological matrices.[18,19] This is particularly true for assays of proteinases, whose activity can be followed by monitoring the rate of hydrolysis of synthetic peptides used as specific substrates. Because these enzymes are often potential virulence factors, increased interest in a better understanding of their role in the development of human diseases has motivated the need of new techniques for determining their activity "ex vivo". A series of assays of practical utility have, thus, been developed for monitoring hydrolytic activity of proteinases of different origin in real samples. The data so far published show that capillary zone electrophoresis (CZE) and/or micellar electrokinetic chromatography (MEKC) can actually be considered, not only an attractive alternative to current analysis schemes but, in many instances an invaluable approach to the precise evaluation of enzyme activity.[20–24]

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

The aim of this review article is to focus the interest of the reader on some recent applications of CE in the field of proteinases, in order to highlight its wide applicability in this field, and to indicate advantages and/or limitations over other techniques. The report is divided into sections and subsections that will present examples of CZE, MEKC, and capillary isoelectric focusing (cIEF) applications from our laboratory and from the literature and determination of proteinases' activities in purified specimens, or in real samples.

DETERMINATION OF PROTEOLYTIC ACTIVITIES BY CZE

One of the most important features of capillary zone electrophoresis techniques is their ability to resolve mixtures of structurally related compounds, provided that the compounds have different electrophoretic mobilities. Indeed, because of its high resolution, CZE represents the simplest way of obtaining, at analytical level, the separation of analytes with slight differences of their charge-to-mass ratios and/or size or shape. This feature has been extensively utilized to separate intact substrates (generally synthetic peptides) of proteolytic enzymes from split products, thus allowing accurate assay of their activity to be performed. The series of applications presented in the following paragraphs prove the usefulness of this technique on purified specimens, as well as on real samples.

Downloaded At: 20:43 23 January 2011

Application on Purified Specimens

Although, the applications on purified enzymes reported in the literature are relatively complex, it should be underlined that, in most instances, the results achieved confirm the above statements. In fact, the newly developed CZE techniques, when compared with traditional chromatographic (HPLC) or spectrophotometric procedures, showed better separations and/or shorter analysis times. This was exactly what Krueger and co-workers[25] observed when they analyzed the specificity of cleavage of adrenocorticotrophic hormone (ACTH) peptide-bonds by commercial endoproteinase Arg-C, using both RP-HPLC and CZE. As evidenced by their results, the former technique exhibited lower efficiency than the latter; that was also found particularly appropriate for studying the kinetics of cleavage of the substrates used. Likewise, Lenz et al.[26] used CZE and RP-HPLC for monitoring the hydrolytic activity of trypsin and α-chymotrypsin toward high-molecular weight substrates in reversed micelles. They demonstrated that enzymatic transformations could occur in these non-polar multicomponent systems, using an intramolecularly quenched fluorogenic insulin derivative and comparing the uses of RP-HPLC and free CZE in the analysis

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

of microemulsions containing cationic or anionic amphiphiles. Their findings showed that CZE and RP-HPLC complemented each other in an ideal way to follow proteolysis in the above-mentioned systems. In another paper, Perron et al.[27] followed the activity of carboxypeptidase A on methotrexate-α-peptides with production of free methotrexate. This analyte was easily separated from the parent compound and the CE method was demonstrated to be faster and better reproducible than other separation techniques, such as HPLC.

Tu and Chu[28] used CE to assay the dipeptidase activity of Van X enzyme, one of the proteins involved in vancomicin resistance. They showed that this CE-based procedure was less time consuming than the conventional ninhydrin-based method.

This electrophoretic approach was also successfully applied by Hoffmann et al.[29,30] to develop a new peptidase assay for determining the specificity of cleavage of dipeptidylpeptidase IV (DP IV), an exopeptidase localized on the surface of leucocytes. Given the short analysis times of CE, the authors were able to perform a systematic investigation on a large series ($n = 14$) of oligopeptides analogous to the N-terminal structure of different cytokines, and searched for antibodies capable of inhibiting the hydrolysis of oligopeptides by dipeptidyl peptidase IV. More recently,[31] the same authors showed, that both soluble DP IV from blood plasma and the enzyme expressed on the surface of cells, were able to catalyze the hydrolysis of intact procalcitonin (PCT 1-116), forming an Ala-Pro dipeptide and a truncated form of procalcitonin (PCT 3-116) that is an important marker of diseases involving severe infections.[32,33] Owing to the excellent resolution offered by CZE, the peak corresponding to the Ala-Pro dipeptide, was well separated from all other signals present in the electrophoretic profile, and its linear, time-dependent increase could be exactly followed, thus allowing the kinetics of PCT 1-116 degradation to be determined. Similarly, the capillary electrophoretic separation of the peak of ubiquitin-valine (77 aminoacids) from that of ubiquitin (76 aminoacids) obtained in less than 10 min, led Franklin et al.[34] to conclude that this assay of ubiquitin carboxyl-terminal hydrolase was advantageous over other procedures. In fact, although these two molecules are structurally similar (their migration times differing by only 0.12 min), the truncated form of ubiquitin could be unambiguously identified, thus allowing the cleavage reaction to be monitored easily.

The CZE method developed by Vessillier et al.[35] to measure the activity of Las A protease from *Pseudomonas aeruginosa*, using pentaglycine as the model substrate, was also shown to be much faster and more sensitive than traditional ion-pair HPLC or spectrophotometric techniques. These authors then extended their CZE investigation to study a possible preference of this enzyme for glycine-rich substrates.[36] The 65 pentapeptides of human tropoelastin containing at least three glycines, were thus submitted to the hydrolysis test and the interesting qualitative and quantitative results achieved evidenced the successful use of this approach. Very recently, Walker et al.[37] have used CZE to investigate the low

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

oral bioavailability of luteinizing hormone-releasing hormone (LHRH) and its analogues, compounds currently being used in the treatment of several diseases, including prostate and breast cancers. Walker and his team were able to determine the relative contribution of pancreatic endopeptidases (chymotrypsin, trypsin, and elastase) present in the lumen of the small intestine to the degradation of LHRH and its analogues, by incubating these compounds with equal amounts of the individual proteinase.

Chen[38] proposed capillary electrophoresis with laser induced fluorescence detection (CE-LIF) for the characterization of proteases. Using a cyanine (Cy3) dye-labeled angiotensin I as a stable substrate, he studied the cleavage mechanism of various proteases (trypsin, carboxypeptidase P and Y, and proteinase K) by separating with CZE and detecting with LIF the fluorescent-labeled peptide fragments produced. This method could be of general interest and provide a tool for studying the mechanism of protease-catalyzed hydrolysis of peptides. Another method involving CE-LIF for assaying proteinases, was that proposed by Craig et al.[39] They coupled CZE to solid phase substrate extraction and, using chymotrypsin as the model protease, and a peptide biotinylated at its N-terminus as the substrate, they were able to monitor analytes with a LIF detector, thus increasing the sensitivity of the technique to quantities of protease as small as 10 pg/mL (4.6×10^{-13} M). According to these results, they suggested that the method could be extended to assay virtually any protease if the peptide substrate is biotinylated at one end and a group with a fluorescent dye can be attached on the other. CZE was also employed by Zhang et al.[40] and by Chang et al.[41] to determine both the activity and the inhibitory activity of angiotensin converting enzyme (ACE), a dipeptidylcarboxypeptidase that plays an important role in the regulation of blood pressure and fluid balance.[42] Using hippuryl-hystidyl-leucine as the substrate of the enzyme, the authors demonstrated that the CE separation of this analyte from products of reaction could be obtained with higher efficiency and shorter operation times than with previous techniques. Data derived from above mentioned applications let the author conclude that CZE can be compared favourably with established chromatographic assays.[43–45]

Application on Real Samples

The high complexity of biological matrices can often make identification or assay of proteinases problematic. In these cases, the most important feature that a separation technique should possess in order to be able to resolve substrate and/or products from all other components present in the mixture is a very high efficiency. The series of papers listed below demonstrate that CZE responds to this requirement and is well suited for assaying proteinase activity even in complex biological media.

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016
©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

One of the first papers dealing with monitoring proteolytic activites by CZE in a real sample was that by Banke et al.[46] As the first step of their procedure, they used free solution capillary electrophoresis to separate some minor side products from a complex fermentation broth of *Aspergillus oryzae*, collecting and identifying their enzymatic activities by reaction with substrates, such as synthetic peptides or casein. The second step consisted in the detection of the fragments produced from the cleavage reaction. In this way, as little as 3 ng of enzyme were identified as an alternative protease of the subtilisin family. Using tripeptide Gly-Gly-Phe as substrate, Mulholland et al.[47] were able to assay the activity of a tripeptidase from a crude extract of *Lactococcus lactis*. Given the reliability of this CE assay, it could also be demonstrated that the presence of contaminating enzymes in crude cell-free extracts might cause secondary reactions that were not evident from the ninhydrin assay data.

Another interesting paper by Shihabi and Kute,[48] presented a rapid and simple CZE method for analyzing cathepsin D, a proteolytic lysosomal enzyme, whose tissue levels can be used as markers of tumor malignancy in general, and of breast carcinoma in particular.[49,50] The amount of this enzyme was, thus, determined incubating breast tissue homogenate with hemoglobin as substrate; the cleaved hemoglobin gave origin to a specific peptide that was perfectly separated in less than 5 min. Shihabi also showed that CZE was well suited to the analysis of angiotensin-converting enzyme (ACE) in human serum, using hyppuryl-hystidyl-leucine as substrate.[51] The reaction was rapid and hippuric acid, one of the products of cleavage reaction, was separated from other components in about 4 min. For a comparison of this method with that developed by other authors on purified enzyme, the reader should refer to the previous paragraph in Refs. [40] and [41].

A paper containing a CZE method for determining the activity of aminopeptidase X, another important enzyme of the renin-angiotensin system, was published by Sim and Lim.[21] They investigated the activity of this enzyme in six tissues (lung, liver, kidney, plasma, endothelium, and smooth muscle) of normotensive and spontaneously hypertensive rats, obtaining very good electrophoretic profiles in which the peak of angiotensin I was perfectly separated from those of angiotensin peptides. Their determination contributed to verifying the differential distribution of aminopeptidase X between the tissues analyzed. Sol et al.[23] used CZE to separate the degradation products generated from incubating neuropeptide FF (NPFF, 8 aminoacids) and SQA-neuropeptide FF (SQA-NPFF, 11 aminoacids) with brain-slice suspensions. Identification and quantification of fragments determined that the degradation kinetics of these peptides was a function of the specific enzymatic content of brain regions. The assay of leucine aminopeptidase, a clinically significant enzyme, was performed by Miller et al.[52] on complex biological samples (human serum, urine and *E. coli* supernatant samples), using a technique called electrophoretically mediated

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

microanalysis (EMMA), in which the reaction is performed directly in the electrophoretic column. Very low concentrations (6×10^{-13} M) of enzyme were detected using this method.

Information concerning analytical CZE approaches for assaying proteinases activity is summarized in Table 1.

Applications in Food Science

Capillary electrophoresis can also be considered a useful analytical tool for investigating the proteolytic activity of different proteinases in food science. In particular, the technique has been applied to characterize a widely distributed class of enzymes with biotechnological application in the food industry. Krause et al.[53] in fact, used CE to investigate the hydrolytic process of whole casein and isolated casein compounds by aspartic proteases in rennin and pepsin to obtain information on the kinetics and specificity of these enzymes. Macedo et al.[54] showed that CZE could be used to study the action of aspartic proteinases on the C-terminal domain of β-casein, thus helping elucidation of their effect on cheese quality. The use of CZE to characterize proteolytic activity of chymosin and plasmin on casein was also described in a series of interesting papers by Kristiansen et al.[55] Recio et al.[56,57] and Molina et al.[58] The same technique was also applied by Otte et al.[59] to analyze the activity of bovine caseins and to separate their hydrolysis products in cheese. More recently, Larsen et al.[60] analyzed the proteolytic activity of thirty strains of *Penicillium roqueforti* used in the production of blue cheeses. For this study, they applied "in parallel" the agar diffusion test, the azocasein test, and capillary zone electrophoresis. CE proved to be the most valuable method for revealing qualitative differences between strains of *P. roqueforti* in the breakdown of casein. Given the importance of rennet in ripening of cheese, Irigoyen et al.[61] also developed a new technique to study the effect of the type of rennet (calf and lamb) upon the hydrolysis of the caseins. They found that CZE was more advantageous than existing methods, such as HPLC or PAGE. Once again, the separation efficiency provided by CZE allowed obtaining good electrophoretic profiles and following the degradation process of different caseins. Very recently, Morales et al.[62] used CE to characterize the proteolytic activity of wild *L. lactis* strains, including their specificities towards different caseins and their levels of production of hydrophilic and hydrophobic peptides. Fernandez-Garcia et al.[63] used CZE of raw milk Manchego cheese to analyze biogenic amines produced by the addition of proteinases and starter culture levels to the cheese milk.

The need for a fast and cheap technique to quantify the degree of proteolysis in meat products, as well as to evaluate the proteolytic activity of microbial origin, induced Martin et al.[64] to design a CZE method for these purposes. Their analyses

Downloaded At: 20:43 23 January 2011

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Table 1. Application of CZE to the Assay of Proteolytic Activities on Both Purified Specimens and Real Samples

Enzyme	Substrate(s)	Detection	Application on Purified Specimens
Endoproteinase Arg-C	Adrenocorticothropic hormone (ACTH)	UV at 200 nm	Kinetic analysis of proteolytic enzyme action on a peptide substrate.[25]
Trypsin and α-chymotrypsin	Native insulin	UV at 200 nm	Study of the activity of serine proteases toward substrates of high molecular weight after incorporation in reversed micelles.[26]
Carboxypeptidase A	Methotrexate-α-peptides	UV at 204 nm	Development of a novel method for monitoring carboxypeptidase A activity.[27]
Van X	Ala-Ala; Ala-Phe; AlaTyr and Ala-Trp.	UV at 200 and 280 nm	Development of a novel and convenient method for monitoring Van X activity.[28]
Dipeptidyl peptidase IV	Interleukin-2 (1-12)	UV at 200 nm	Studies of hydrolysis of oligopeptides and of enzymatic inhibition.[29,30]
	Procalcitonin (1-116)	UV at 200 nm	Detection of important markers of diseases involving severe infections.[31]

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016
©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Ubiquitin carboxy-terminal hydrolase (UCH)	Ubiquitin-valine	UV at 200 nm	Purification and identification of new members of UCH family.[34]
Protease Las A from *P.aeruginosa*	Pentaglycine	UV at 185 nm	Monitoring enzymatic hydrolysis of various peptides to investigate enzyme specificity.[35,36]
	Glycine-rich substrates obtained from human tropoelastin	UV at 185 nm	
Pancreatic serine endopeptidases	Luteinizing hormone-releasing hormones (LHRH)	UV at 215 nm	Monitoring of the activity of pancreatic endopeptidases toward LHRH.[37]
Endopeptidases and carboxypeptidases	Cyanine (Cy3)-labeled angiotensin	LIF-590 nm	Characterization of proteases by CE-LIF using a fluorescently labeled peptide.[38]
Chymotrypsin or other proteases	Peptide biotinylated at its N-terminus	LIF-535 nm	General method for detection of small amounts of proteases.[39]
Angiotensin converting enzyme (ACE)	Hyppuryl-Hystidyl-Leucine	UV at 228 nm	Determination of both the activity and the inhibitory activity of ACE.[40,41]
Alkaline protease	SucAla2ProPheNA	UV at 200 nm	Characterization of an enzymatic activity in crude fermentation broth from *Aspergillus oryzae*.[46]
Tripeptidase	Gly-Gly-Pro	UV at 200 nm	Measurement of protease activity in crude extracts.[47]
Cathepsin D	Hemoglobin	UV at 214 nm	Prediction of tumor malignancy.[48]
Angiotensin converting enzyme	Hyppuryl-hystidyl-leucine	UV at 214 nm	Determination of protease activity in human serum.[51]

(continued)

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Table 1. Continued

Enzyme	Substrate(s)	Detection	Application on Purified Specimens
Aminopeptidase X	Angiotensin I	UV at 185 nm	Study of the differential tissue distribution of aminopeptidase X.[21]
Enzymatic activities	Neuropeptide FF and SQA-neuropeptide FF	UV at 210 nm	Analysis of the specific enzymatic content of brain regions.[23]
Leucine aminopeptidase	L-leucine β-naphthylamide	LIF-450 nm	Electrophoretically mediated microanalysis of leucine aminopeptidase in complex matrices.[52]

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

contributed to evaluating the extent of changes by microorganisms in soluble nitrogen compounds. In addition, a capillary gel electrophoretic technique, developed "in parallel" was able to detect changes in sarcoplasmic and myofibrillar proteins due to endogenous and microbial enzymes.

From the series of examples reported above, it is clear that CZE can be considered a reasonable technique for obtaining fast, cheap, and reliable determinations of proteinase activities in biological matrices of various origins and of various complexity.

A schematic list of applications described in this paragraph, is reported in Table 2.

DETERMINATION OF PROTEOLYTIC ACTIVITIES BY MEKC

As pointed out above, since the separation mechanism of CZE is critically dependent on differences in charges between analytes, resolution of like-charged compounds is generally very poor. Thus, it can be often problematic to apply this technique to complex biological matrices containing a high number of structurally similar solutes. However, with the advent of micellar electrokinetic chromatography (MEKC), in which solutes partition between the micelles and the surrounding aqueous medium and separation is mainly based on the differential migration of these two phases, another dimension of selectivity has been introduced in CE.

This approach has been used in several laboratories to monitor the activity of different proteinases and the remainder of this section will address selected examples in this field, showing how its application on purified enzymes or on real samples has provided significant advantages over established techniques in terms of speed, resolution, and/or sensitivity.

Application on Purified Specimens

In recent years, a number of CE assays for detecting the activity of different proteolytic enzymes have been designed in our laboratory as a result of our growing interest in the research on proteinases responsible for the development of severe disorders in humans.

The first report we address here, is that of describing the determination of prolidase activity, a dipeptidase that specifically cleaves imidodipeptides containing Pro or Hyp at their C-terminus and whose role is essential in the final stages of protein catabolism. Since the traditional assay of prolidase activity (mainly based on the colorimetric reaction of proline following hydrolysis of X-Pro substrate[65]),

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Downloaded At: 20:43 23 January 2011

Table 2. Application of CZE to the Detection of Proteolytic Activities in Food Science

Enzyme	Substrate	Detection	Application
Aspartic proteases	Casein	UV at 214 nm	Study of substrate and binding specificity of aspartic proteinases with milk clotting properties.[53]
Aspartic proteases	Casein	UV at 214 nm	Study of the action of asparticproteinases on the C-terminal domain of β-casein.[54]
Chymosin and plasmin	Casein	UV at 214 nm	Determination of the action of chymosin and plasmin oncasein.[55–58]
Proteinases	Casein	UV at 214 nm	Study of the degradation of various caseins fractions.[59]
Proteinases	Casein	UV at 214 nm	Characterization of the proteolytic activity of starter culture of *P. roqueforti* for production of blue veined cheese.[60]
Rennet enzymes	Casein	UV at 214 nm	Study of the effect of different types of rennet upon the hydrolysis of caseins.[61]
Proteinases	Casein	UV at 214 nm	Chracterization of the proteolytic activity of wild *L. lactis* strains.[62]
Bacillus subtilis neutral proteinase and *Micrococcus* sp. Cysteine proteinase	Cheese	UV at 214 nm	Effect of added proteinases in the formation of biogenic amines in raw ewes' milk Manchego cheese.[63]
Proteinases	Meat slices	UV at 214 nm	Evaluation of microbial proteolysis in meat products.[64]

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

is characterized by low specificity, we developed a CE technique to overcome these problems.[20] Given the small amount of sample required and the short migration time of substrates, this technique appeared ideally suited for "in vitro" characterization of prolidase activity. Because of the encouraging results, this method was extended to the determination of prolidase activity also in samples of cultured fibroblasts and haemolisates.[20] In both cases, the reproducibility of results was good and a comparison of data established that CE was more accurate and quicker than other methods. In the light of these results, our group devoted its efforts to designing a CE method for also determining the activity of elastases (human neutrophil elastase; cathepsin G, elastase from *P. aeruginosa*) responsible in humans for the development of destructive lung diseases, such as chronic obstructive pulmonary diseases (COPD).[66–69]

Systematic investigations carried out with CZE and MEKC to monitor "in vitro" proteolytic activity of commercial elastases from different sources, using synthetic peptides as enzyme substrates, revealed that CZE was unable to separate the intact substrate from the products of the proteolytic reaction. By contrast, MEKC appeared a very convenient tool for resolving these structurally related compounds, their fast and reliable separation being, in fact, obtained in less than 18 min. MEKC was, thus, proposed as a promising alternative to current spectrophotometric and HPLC-based techniques for monitoring activities of proteinases.[69] The feasibility and reproducibility of this method were so good that it was extended to the determination of activity of elastases with different bond cleavage specificity, simultaneously present in the assay mixture.[70] Given the high resolution offered by MEKC, all reaction products were distinguished clearly and their quantification was exploited to determine, with high precision, the amount of different elastases.

As a further extension of this study, MEKC was also used, (i) to investigate the interferences that these proteinases could exert on each other and, (ii) to analyze the active site specificity of *P. aeruginosa* elastase. In both cases, the answer of CE techniques was excellent.[71,72] In fact, if MEKC permitted verifying the existence of possible reciprocal inhibiting and/or potentiation effects of these proteinases when present as a mixture representative of "in vitro" lung secretions,[71] it also enabled the catalytic site geometry *of P. aeruginosa* elastase to be reexamined.[72] In this respect, a complete and detailed investigation, with a wide series of synthetic peptides differing from each other by only one or more aminoacids, was performed and the kinetic parameters K_m and k_{cat} of their hydrolysis were determined. The results were comparable to those available in the literature and, in addition, they also allowed the substrate specificity of this protease to be defined better.

Other interesting applications of MEKC to the study of proteinases on purified specimens are those reported in a series of papers by a research group coordinated by Michael Zeece.[73–75] These researchers were interested in the development of rapid and sensitive methods for determining the activity of

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016
©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

cathepsin D, an aspartyl protease of lysosomal origin. Using hemoglobin[73] or Oregon-Green labelled hemoglobin[74] as substrate of this proteolytic enzyme, they developed a procedure to determine Cathepsin D activity based on separation and quantification of reaction products by MEKC. Using the fluorescently labeled substrate, the detection limit was very low (approximately 10^{-9} M) and the method proved valid even when applied to real samples (bovine skeletal muscle cell cultures). For a comparison of this method with that developed by other authors, using CZE, the reader should refer to Ref. [48].

In another study[75] Zeece's group examined the action of μ- and *m*-calpains, calcium dependent thiol proteases that have been suggested to play a role in several physiological processes including muscle protein turnover.[76–78] Incubating both forms of calpain for various times with α_2- and β-casein fluorescently labelled with Oregon Green, they demonstrated that, although these enzymes had nearly identical proteolytic activities, there was an evident difference in the hydrolysis of the two substrates. The use of LIF detection allowed the activity of low ng quantities of μ- or *m*-calpain to be measured.

As a further contribution to the exploitation of the full potential of MEKC, a paper recently published by Freed et al.[79] should be mentioned in conclusion of this paragraph. It describes an "indirect" way of monitoring, in a fluid, the load of proteinases that cleave substance *P*, a neuroactive undecapeptide, with a wide spectrum of physiological actions in the central and peripheral nervous system.[80,81] Substance *P* is, in fact, cleaved at different sites by a variety of peptidases to yield several metabolites with different pharmacological actions. Separation and identification of all fragments obtained from the parent compound is a way of gaining information on the metabolism (i.e., on the peptidases involved in cleavage reactions) of this neurotransmitter. Under the experimental conditions used, the metabolites were separated from naphthalene-2,3-dicarboxy-aldheyde-derivatized-substance *P*. This MEKC method, in conjunction with LIF detection, has been used by the same authors to investigate the metabolism of substance *P* "in vivo" (see next paragraph).

Application on Real Samples

Waltham et al.[22] used the MEKC procedure to determine the activity of γ-glutamyl hydrolase (GGH), a lysosomal peptidase that catalyzes the cleavage of γ-glutamyl groups from the C-terminal end of methotrexate (MTX) polyglutamates. When the substrate was incubated with crude extract of a human T-cell leukemia cell line, it was cleaved into MTX and polyglutamylated forms. Distinct separation of these forms was achieved within 10 min and enabled simultaneous quantification of both reactant and products of the proteolytic reaction. The MEKC assay was, thus, successfully used to measure GGH levels in tumor

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

samples from patients and was shown to be more efficient and sensitive, respectively, than the previously described HPLC[82,83] and the colorimetric[84] techniques. Several MEKC methods originally developed for monitoring purified proteinases (see the appropriate paragraph) have also been successfully extended to the determination of these enzymes in real samples. For example, the procedure described in[74] for determining Cathepsin D activity was also found to be valuable when cultured bovine skeletal muscle cells were used as source of the proteolytic enzyme. Likewise, the previously described MEKC-LIF method[79] was also successfully applied to the investigation of substance *P* metabolism "in vivo," both in rat striatum during microdyalisis sampling and in bovine brain microvessel endothelial cell culture systems.[85,86] Furthermore, the methods previously mentioned for assaying prolidase[20] and serine elastases of different origin,[69–71] have given excellent results when used with real samples. In the case of prolidase, this enzyme's activity was easily detected in cellular extracts from cultured skin fibroblasts of both patients affected by prolidase deficiency and controls.[20] As far as the serine elastases are concerned, sputum from patients ($n = 40$) affected by obstructive pulmonary diseases have been used as a source of these proteolytic enzymes (unpublished results). Briefly, to check for the presence of HNE and Cat G, sputum sol phases were incubated with specific substrates of these enzymes (MeOSucAla$_2$ProValNA and SucAla$_2$ProPheNA, respectively) and electropherograms were recorded at different times of incubation.

As shown in Fig. 1, which is representative of the profiles typically obtained from the above indicated specimens, the high reproducibility and the good resolving power of the electrophoretic technique allowed an easy identification of the peaks of intact substrates (peaks 1 and 2 in traces **b**, **c** and **d**) and split products (peaks 3, 4 and 5 in traces **c** and **d**), although, endogenous components of sputum increased the profiles' complexity in comparison with those achieved using purified enzymes (see Ref. 70). Quantification of peaks, performed as indicated in[69] and[70] for purified enzymes, allowed calculation of the amount of free proteinases in expectorates from the patients investigated. MEKC was also used in our laboratory for detection of tripeptidylpeptidase-1 (TPP-I), a lysosomal enzyme that cleaves tripeptides from the N-terminus of proteins, and whose absence or deficiency in children, causes the onset of progressive neurodegenerative disorders known as late-infantile neuronal ceroid lipofuscinosis (LINCL). Systematic investigations have been carried out to optimize the experimental conditions for determining TPP-I activity in rat and bovine brains, in human specimens (brains) obtained from patients affected by LINCL, and in blood samples.[24] Compared to other methods,[87,88] MEKC was easier, faster, and simpler to use, thus, offering an interesting alternative to current methodologies for the diagnosis of LINCL.

The schematic list of applications described in this paragraph is reported in Table 3.

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Downloaded At: 20:43 23 January 2011

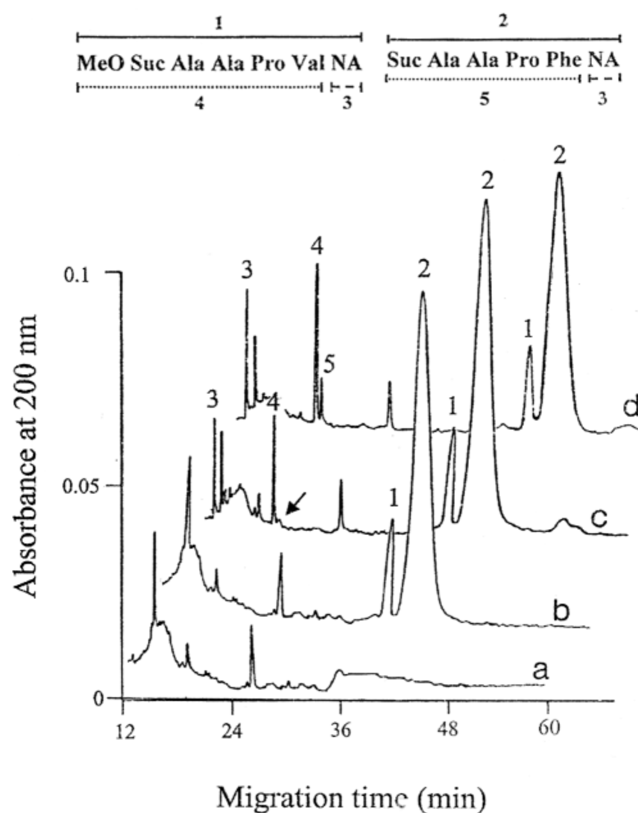


Figure 1. Trace **a**: Micellar electrokinetic chromatographic pattern obtained from intact sputum sol phase of a patient chosen as representative of all specimens examined (blank I). Trace **b**: electrophoretic pattern of the above sputum after 40 min incubation with substrates of HNE: $MeOSucAla_2ProValNA$ (peak 1) and of Cat G: $SucAla_2ProPheNA$ (peak 2) in the presence of their specific inhibitors (2 mM $PheGlyPheCH_2Cl$ and 2 mM $MeOSucAla_2ProValCH_2Cl$) (blank II). Traces **c** and **d**: electrophoretic patterns obtained incubating the above sample for 5 and 20 min respectively with the substrates indicated. Peaks 3, 4 and 5 represent the products of proteolytic reactions: NA, $MeOSucAla_2ProVal$ and $SucAla_2ProPhe$ respectively (for their identification see Ref. 69 and 70). Runs were carried out using 50 mM sodium tetraborate pH 9.3 containing 65 mM SDS and 15% methanol (v/v), applying a voltage of 25 kV and using a fused-silica capillary of 50 μm I.D. and 57 cm total length (50 cm to detector). The elution profile was monitored at 200 nm. Temperature: 20 ± 0.1°C. Positive to negative polarity (unpublished data).

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Table 3. Application of MEKC to the Assay of Proteolytic Enzymes on Both Purified Specimens and Real Samples

Enzyme	Substrate(s)	Detection	Application on Purified Specimens
Prolidase	Gly-Pro and Phe-Pro	UV at 200 nm	Determination of prolidase activity.[20]
Elastases from neutrophils and *Psedomonas aeruginosa* elastase	Specific synthetic peptides	UV at 200 nm	Determination of proteinases present singularly[69] or as a mixture[70] in the assay.
			Studies of reciprocal interferences of proteinases in mixtures representative of "in vitro" lung secretions.[71]
			Analysis of the active site specificity of *P. aeruginosa* elastase.[72]
Cathepsin D	Hemoglobin	UV at 214 nm	Monitoring of cathepsin D activity using hemoglobin as substrate.[73]
	Oregon Green labeled-hemoglobin	LIF-520 nm	Determination of cathepsin D activity using fluorescently labeled hemoglobin as substrate.[74]
μ- and m-calpains	Oregon Green labeled-α2 and β casein	LIF-520 nm	Development of a new very sensitive technique for detection of calpain activity.[75]

(continued)

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Table 3. Continued

Variety of peptidases	2,3-dicarboxaldheyde-derivatized substance P	UV at 246 nm	Development of an "indirect" way of monitoring the load of proteinases that cleave substance P.[79]
γ-glutamyl hydrolase	Methotrexate polyglutamates	UV at 300 nm	Evaluation of a new technique for monitoring GGH in patient tumor samples.[22]
Variety of peptidases	2,3-dicarboxaldheyde-derivatized substance P	LIF-488 nm	Investigation of the substance P metabolism "in vivo" both in rat striatum and at the blood-barrier.[85,86]
Cathepsin D	Oregon Green-labeled hemoglobin	LIF-520 nm	Development of an enzymatic assay to determine cathepsin D activity in biological samples.[74]
Prolidase	Gly-Pro and Phe-Pro	UV at 200 nm	Determination of prolidase activity in cultured skin fibroblasts.[20]
Elastases from neutrophils and *Pseudomonas aeruginosa* elastase	Specific synthetic peptides	UV at 200 nm	Determination of elastase activities in sputum samples (unpublished results).
Tripeptidylpeptidase I (TPP-I)	SucAlaAlaPheNA	UV at 200 nm	Determination of TPP-I activity in human and animal specimens.[24]

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016
©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

DETERMINATION OF PROTEOLYTIC ACTIVITIES BY MEANS OF ELECTROPHORETIC MODES OTHER THAN CZE OR MEKC

Application of cIEF

Capillary isoelectric focusing is a separation technique, in which charged species start migrating under the influence of an electric field in a pH gradient formed by carrier ampholytes. Migration stops when a zone is reached where the net charge is zero; at this isoelectric point (pI), the sample component is focused in a tight zone. In cIEF, the high resolving power of conventional gel IEF is, thus, combined with the advantages of capillary electrophoresis. Although, generally applied for the separation of proteins, the technique is also effective for certain types of peptides that focus well. The example reported below, shows how this technique can be used for an enzyme assay.

From among the variety of papers so far published concerning determination and/or assay of proteolytic activities, to our knowledge, that from Shimura et al.[89] is the only one in which cIEF was applied to obtain separation of fragments. In this report a synthetic nonapeptide, a substrate for trypsin, was labeled with an iodoacetyl derivative of Lissamone rhodamine B and incubated with a solution of trypsin. After having stopped the cleavage reaction by the addition of soybean trypsin inhibitor, the capillary was filled with the reaction mixture and the products separated by IEF with LIF detection. The method allowed the determination of trypsin amounts as low as 50–250 pg.

Downloaded At: 20:43 23 January 2011

CONCLUSIONS

The large number of examples reported in this article, shows that simple, yet extremely powerful CE separation systems can be designed for determining activity of proteolytic enzymes in both purified samples and in complex biological matrices. Although, CE will not replace traditional methods in this area, it can provide a useful complementary technique as a result of its versatility and minimal sample requirement. Furthermore, as underlined by the numerous applications mentioned in this article, very often these methods do not only represent a mere exercise of finding new analytical tools for separation science, but can also be of practical use in the diagnosis of specific diseases in which proteinases are responsible for the onset of severe disorders. The possibility of using the microchip technology for determining proteolytic activities recently described by Zugel et al.[90] makes these CE techniques even more attractive, and most likely will expand their role in this field.

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

ACKNOWLEDGMENTS

The authors would like to acknowledge Elaine Marchi (American Parkinson Disease Association, Staten Island, New York, USA) for helpful discussion during the preparation of this article. This project was funded in part with a grant from MURST-COFIN 2000.

REFERENCES

1. Bao, J.; Regnier, F.E. Ultramicroenzyme Assay in a Capillary Electrophoretic System. J. Chromatogr. **1992**, *608* (1–2), 217–224.
2. Wu, D.; Regnier, F.E. Native Protein Separations and Enzyme Microassays by Capillary Zone and Gel Electrophoresis. Anal. Chem. **1993**, *65* (15), 2029–2035.
3. Roberts, M.W.; Preiss, J.; Okita, T.W. A Capillary Zone Electrophoresis Assay for the Nucleoside Transfer Enzyme Adenosine Diphosphate-Glucose Pyrophosphorylase. Anal. Biochem. **1995**, *225* (1), 121–126.
4. Pattanaargson, S.; Roboz, J. Determination of Hyaluronidase Activity in Venoms Using Capillary Electrophoresis. Toxicon **1996**, *34* (10), 1107–1117.
5. Suh, M.J.; Kim, Y.S.; Yoo. Y.S. Application of Capillary Electrophoresis to Determination of Enzyme Activity and Other Properties of Phosphatidylinositol-Specific Phospholipase C. J. Chromatogr. A **1997**, *781* (1–2), 263–270.
6. Taylor, M.R.; Westwood, S.A.; Perrett, D. Direct Monitoring of Enzyme Reactions Using Micellar Electrokinetic Capillary Chromatography. Optimisation of Drug Glucuronide and Sulfate Conjugate Hydrolysis. J. Chromatogr. A **1997**, *768* (1), 67–71.
7. Kanie, Y.; Kirsch, A.; Kanie, O.; Wong, C-H. Enzymatic Assay of Galactosyltransferase by Capillary Electrophoresis. Anal. Biochem. **1998**, *263* (2), 240–245.
8. Fujima, J.M.; Danielson, N.D. Determination of Creatine Kinase Activity and Phosphocreatine in Off-Line and On-Line Modes with Capillary Electrophoresis. Anal. Chim. Acta **1998**, *375* (3), 233–241.
9. Snow, D.M.; Shaper, J.H.; Shaper, N.L.; Hart, G.W. Determination of β-1,4-Galactosyltransferase Enzymatic Activity by Capillary Electrophoresis and Laser-Induced Fluorescence Detection. Anal. Biochem. **1999**, *271* (1), 36–42.
10. Saillard, S.; Gareil, P.; Jozefonvicz, J.; Daniel, R. Development of a Capillary Electrophoresis Assay Based on Free Sulfate Determination

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Downloaded At: 20:43 23 January 2011

for the Direct Monitoring of Sulfoesterase Activity. Anal. Biochem. **1999**, *275* (1), 11–21.

11. Choi, S.; Lee, Y.S.; Na, D.S.; Yoo, Y.S. Determination of Enzymatic Activity and Properties of Secretory Phospholipase A_2 by Capillary Electrophoresis. J. Chromatogr. A **1999**, *853* (1–2), 285–293.
12. Glatz, Z.; Bouchal, P.; Janiczek, O.; Mandl, M.; Cesková, P. Determination of Rhodanase Activity by Capillary Zone Electrophoresis. J. Chromatogr. A **1999**, *838* (1–2), 139–148.
13. Viglio, S.; Valentini, G.; Zanaboni, G.; Cetta, G.; De Gregorio, A.; Iadarola, P. Rapid Detection of Ornithine Transcarbamylase Activity by Micellar Electrokinetic Chromatography. Electrophoresis **1999**, *20* (1), 138–144.
14. Glatz, Z.; Marini, M.V.; Wimmerová, M.; Damborsky, J.; Nagata, Y. Determination of Haloalkane Dehalogenase Activity by Capillary Zone Electrophoresis. J. Chromatogr. A **2000**, *895* (1), 219–225.
15. Murakami, Y.; Morita, T.; Kanekiyo, T.; Tamiya, E. On-Chip Capillary Electrophoresis for Alkaline Phosphatase Testing. Biosens. Bioelectron. **2001**, *16* (9–12), 1009–1014.
16. Starkey, D.E.; Han, A.; Bao, J.J.; Ahn, C.H.; Wehmeyer, K.R.; Prenger, M.C.; Halsall, H.B.; Heineman, W.R. Fluorogenic Assay for β-Glucuronidase Using Microchip-Based Capillary Electrophoresis. J. Chromatogr. B. **2001**, *762* (1), 33–41.
17. Bao, J.J.; Fujima, J.M.; Danielson, N.D. Determination of Minute Enzymatic Activity by Means of Capillary Electrophoretic Techniques. J. Chromatogr. B **1997**, *699* (1–2), 481–497.
18. Adam, T.; Sevcik, J., Fairbanks, L.D.; Bartak, P. Determination of Purine Nucleoside Phosphorylase Activity in Human Erythrocytes by Capillary Electrophoresis. J. Chromatogr. B. **1997**, *698* (1–2), 308–311.
19. Wu, X.; Loganathan, D.; Linhardt, R.J. Sensitive Method for the Quantification of β-Glucuronidase Activity in Human Urine Using Capillary Electrophoresis with Fluorescence Detection. J. Chromatogr. B **1998**, *708* (1–2), 61–66.
20. Zanaboni, G.; Viglio, S.; Dyne, K.M.; Grimm, R.; Valli, M.; Cetta, G.; Iadarola, P. Direct Monitoring of Prolidase Activity in Cultured Skin Fibroblasts Using Capillary Electrophoresis. J. Chromatogr. B **1997**, *695* (1), 77–84.
21. Sim, M.K.; Lim, B.C. Determination of Aminopeptidase X Activity in Tissues of Normo- and Hypertensive Rats by Capillary Electrophoresis. J. Chromatogr. B **1997**, *697* (1–2), 259–262.
22. Waltham, M.C.; Lin, S.; Li, W-W.; Göker, E.; Gritsam, H.; Tong, W.P.; Bertino, J.R. Capillary Electrophoresis of Methotrexate Polyglutamates and Its Application in Evaluation of γ-Glutamyl Hydrolase Activity. J. Chromatogr. B **1997**, *689* (2), 387–392.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

23. Sol, J.-C.; Roussin, A.; Proto, S.; Mazarguil, H.; Zajac, J.-M. Enzymatic Degradation of Neuropeptide FF and SQA-Neuropeptide FF in the Mouse Brain. Peptides **1999**, *20* (10), 1219–1227.
24. Viglio, S.; Marchi, E.; Wisniewski, K.; Casado, B.; Cetta, G.; Iadarola, P. Diagnosis of Late-Infantile Neuronal Ceroid Lipofuscinosis: a New Sensitive Method to Assay Lysosomal Pepstatin-Insensitive Proteinase Activity in Human and Animal Specimens by Capillary Zone Electrophoresis. Electrophoresis **2001**, *22* (11), 2343–2350.
25. Krueger, R.J.; Hobbs, T.R.; Mihal, K.A.; Tehrani, J.; Zeece, M.G. Analysis of Endoproteinase Arg C Action on adrenocorticotropic Hormone by Capillary Electrophoresis and Reversed-Phase High-Performance Liquid Chromatography. J. Chromatogr. **1991**, *543* (2), 451–461.
26. Lenz, V.J.; Gattner, H.G.; Leithäuser, M.; Brandenburg, D.; Wollmer, A.; Höcker, H. Proteolyses of a Fluorogenic Insulin Derivative and Native Insulin in Reversed Micelles Monitored by Fluorescence Emission, Reversed-Phase High-Performance Liquid Chromatography, and Capillary Zone Electrophoresis. Anal. Biochem. **1994**, *221* (1), 85–93.
27. Perron, M.J.; Pagé, M. Measurement of the Enzymatic Specificity of Carboxypeptidase A by Capillary Zone Electrophoresis. J. Chromatogr. A **1994**, *662* (2), 383–388.
28. Tu, J.; Chu, J. Vancomicin Resistance in *Enterococcus faecium*: a Capillary Electrophoresis-Based Assay for Van X Enzyme. Anal. Biochem. **1998**, *264* (2), 293–296.
29. Hoffmann, T.; Faust, J.; Neubert, K.; Ansorge, S. Dipeptidyl Peptidase IV (CD 26) and Aminopeptidase N (CD 13) Catalyzed Hydrolysis of Cytokines and Peptides with N-Terminal Cytokine Sequences. FEBS Lett. **1993**, *336* (1), 61–64.
30. Hoffman, T.; Reinhold, D.; Kähne, T.; Faust, J.; Neubert, K.; Frank, R.; Ansorge, S. Inhibition of Dipeptidyl Peptidase IV (DP IV) by Anti-DP IV Antibodies and Non-Substrate X-X-Pro-Oligopeptides Ascertained by Capillary Electrophoresis. J. Chromatogr. A **1995**, *716* (1–2), 355–362.
31. Wrenger, S.; Kähne, T.; Bohuon, C.; Weglöhner, W.; Ansorge, S.; Reinhold, D. Amino-Terminal Truncation of Procalcitonin, a Marker for Systemic Bacterial Infections, by Dipeptidyl Peptidase IV (DP IV). FEBS Lett. **2000**, *466* (1), 155–159.
32. Assicot, M.; Gendrel, D.; Carsin, H.; Raymond, J.; Guilbaud, J.; Bohuon, C. High Serum Procalcitonin Concentrations in Patients with Sepsis and Infection. Lancet **1993**, *341* (8844), 515–518.
33. Gendrel, D.; Raymond, J.; Assicot, M.; Moulin, M.; Iniguez, J.L.; Lebon, P.; Bohuon, C. Measurement of Procalcitonin Levels in Children with Bacterial or Viral Meningitis. Clin. Infect. Dis. **1997**, *24* (6), 1240–1242.

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Downloaded At: 20:43 23 January 2011

34. Franklin, K.; Layfield, R.; Landon, M.; Ramage, R.; Brown, A.; Love, S.; Muir, T.; Urquhart, K.; Bownes, M.; Mayer, R.J. Capillary Electrophoresis Assay for Ubiquitin Carboxyl-Terminal Hydrolases with Chemically Synthesized Ubiquitin-Valine as Substrate. Anal. Biochem. **1997**, *247* (2), 305–309.
35. Vessillier, S.; Bernillion, J.; Saulnier, J.; Wallach, J. Capillary Electrophoresis in the Assay of the Hydrolysis of Glycine-Containing Peptides by a Protease from *Pseudomonas aeruginosa.* J. Chromatogr. A **1997**, *776* (1), 133–137.
36. Vessillier, S.; Delolme, F.; Bernillion, J.; Saulnier, J.; Wallach, J. Hydrolysis of Glycine-Containing Elastin Pentapeptides by LasA, a Metalloelastase from *Pseudomonas aeruginosa.* Eur. J. Biochem. **2001**, *268* (4), 1049–1057.
37. Walker, G.F.; Ledger, R.; Tucker, I.G. Activity of Pancreatic Endopeptidases Towards Luteinizing Hormone-Releasing Hormones. Int. J. Pharm. **2001**, *216* (1–2), 77–82.
38. Chen, F-T.A. Characterization of Protease-Catalyzed Hydrolysis of Cyanine-Labeled Angiotensin Using Capillary Electrophoresis with Laser-Induced Fluorescence Detection. Anal. Biochem. **1995**, *225* (2), 341–345.
39. Craig, D.B.; Wong, J.C.Y.; Polakowski, R.; Dovichi, N.J. General Protease Assay Method Coupling Solid-Phase Substrate Extraction and Capillary Electrophoresis. Anal. Chem. **1998**, *70* (18), 3824–3827.
40. Zhang, R.; Xu, X.; Chen, T.; Li, L.; Rao, P. An Assay for Angiotensin-Converting Enzyme Using Capillary Zone Electrophoresis. Anal. Biochem. **2000**, *280* (2), 286–290.
41. Chang, B-W.; Chen, R.L.C.; Huang, I-J.; Chang, H-C. Assays for Angiotensin Converting Enzyme Inhibitory Activity. Anal. Biochem. **2001**, *291* (1), 84–88.
42. Ryan, J.W.; Ryan, U.S.; Schultz, D. R.; Whitaker, C.; Chung, A. Subcellular Localization of Pulmonary Angiotensin-Converting Enzyme (Kininase II). Biochem. J. **1975**, *146* (2), 497–499.
43. Lieberman, J. Elevation of Serum Angiotensin-Converting-Enzyme (ACE) Level in Sarcoidosis. Am. J. Med. **1975**, *59* (3), 365–372.
44. Lantz, I.; Glamsta, E.L.; Talback, L.; Nyberg, F. Hemorphins Derived from Hemoglobin have an Inhibitory Action on Angiotensin Converting Enzyme Activity. FEBS Lett. **1991**, *287* (1–2), 39–41.
45. Meng, Q.C.; Balcells, E.; Dell'Italia, L.; Durand, J.; Oparil, S. Sensitive Method for Quantitation of Angiotensin-Converting Enzyme (ACE) Activity in Tissue. Biochem. Pharmacol. **1995**, *50* (9), 1445–1450.
46. Banke, N.; Hansen, K.; Diers, I. Detection of Enzyme Activity in Fractions Collected from Free Solution Capillary Electrophoresis of Complex Samples. J. Chromatogr. **1991**, *559* (1–2), 325–335.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

47. Mulholland, F.; Movahedi, S.; Hague, G.R.; Kasumi, T. Monitoring Tripeptidase Activity Using Capillary Electrophoresis. Comparison with Ninhydrin Assay. J. Chromatogr. **1993**, *636* (1), 63–68.
48. Shihabi, Z.K.; Kute, T.E. Analysis of Cathepsin D from Breast Tissues by Capillary Electrophoresis. J. Chromatogr. B **1996**, *683* (1), 125–131.
49. Nazeer, T.; Malfetano, J.H.; Rosano, T.G.; Ross, J.S. Correlation of Tumor Cytosol Cathepsin D with Differentiation and Invasiveness of Endometrial Adenocarcinoma. Am. J. Clin. Pathol. **1992**, *97* (6), 764–769.
50. Duffy, M.J.; Reilly, D.; Brouillet, J.P.; McDermott, E.W.; Faul, C.; O'Higgins, N.; Fennelly, J.J.; Maudelonde, T.; Rochefort, H. Cathepsin D Concentration in Breast Cancer Cytosols: Correlation with Disease-Free Interval and Overall Survivors. Clin. Chem. **1992**, *38* (10), 2114–2116.
51. Shihabi, Z.K. Analysis of Angiotension-Converting Enzyme by Capillary Electrophoresis. J. Chromatogr. A **1999**, *853* (1–2), 185–188.
52. Miller, K.J.; Leesong, I.; Bao, J.; Regnier, F.E.; Lytle, F.E. Electrophoretically Mediated Microanalysis of Leucine Aminopeptidase in Complex Matrices Using Time-Resolved Laser-Induced Fluorescence Detection. Anal. Chem. **1993**, *65* (22), 3267–3270.
53. Krause, W.; Partzsch, M.; Hassan, Z.M.; Haufe, T. Substrate and Binding Specificity of Aspartic Proteases with Milk Clotting Properties. Nahrung **1998**, *42* (3–4), 162–165.
54. Macedo, Q.; Jovilet, P.; Meunier, J.C. Capillary Electrophoresis of Caseino-Peptides: Influence of Applied Voltage and Column Temperature. Biotech. Tech. **1999**, *13* (10), 647–651.
55. Kristiansen, K.R.; Otte, J.; Zakora, M.; Qvist, K.B. Capillary Electrophoresis Used to Monitor the Enzymatic Hydrolysis of Caseins and the Fractionation of Hydrolysis Products. Milchwissenschaft **1994**, *49* (12), 683–687.
56. Recio, I.; Amigo, L.; Ramos, M.; Lopez-Fandiño, R. Application of Capillary Electrophoresis to the Study of Proteolysis of Caseins. J. Dairy Res. **1997**, *64*, 221–230.
57. Recio, I.; Garcia-Risco, M.R.; Ramos, M.; Lopez-Fandiño, R. Characterization of Peptides Produced by the Action of Psychrotrophic Proteinases on Kappa-Casein. J. Dairy Res. **2000**, *67* (2), 625–630.
58. Molina, E.; De Frutos, M.; Ramos, M. Capillary Electrophoresis Characterization of the Casein Fraction of Cheeses Made from Cows', Ewes', and Goats' Milks. J. Dairy Res. **2000**, *67* (4), 209–216.
59. Otte, J.; Zakora, M.; Kristiansen, K.R.; Qvist, K.B. Analysis of Bovine Caseins and Primary Hydrolysis Products in Cheese by Capillary Zone Electrophoresis. Le Lait **1997**, *77*, 241–257.
60. Larsen, M.D.; Kristiansen, K.R.; Hansen, T.K. Characterization of the Proteolytic Activity of Starter Cultures of *Penicillium roqueforti* for

Downloaded At: 20:43 23 January 2011

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016
©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Production of Blue Veined Cheeses. Int. J. Food Microbiol. **1998**, *43* (3), 215–221.

61. Irigoyen, A.; Izco, J.M.; Ibañez, F.C.; Torre, P. Evaluation of the Effect of Rennet Type on Casein Proteolysis in an Ovine Milk Cheese by Means of Capillary Electrophoresis. J. Chromatogr. A **2000**, *881* (1–2), 59–67.
62. Morales, P.; Fernandez-Garcia, E.; Gaya, P.; Medina, M.; Nuñez, M. Hydrolysis of Caseins and Formation of Hydrophilic and Hydrophobic Peptides by Wild *Lactococcus lactis* Strains Isolated from Raw ewes' Milk Cheese. J. Appl. Microbiol. **2001**, *91* (5), 907–915.
63. Fernandez-Garcia, E.; Tomillo, J.; Nuñez, M. Effect of Added Proteinases and Level of Starter Culture on the Formation of Biogenic Amines in Raw Milk Manchego Cheese. Int. J. Food Microbiol. **1999**, *52* (3), 186–196.
64. Martin, A.; Cordoba, J.J.; Rodriguez, M.M.; Nuñez, F.; Asensio, M.A. Evaluation of Microbial Proteolysis in Meat Products by Capillary Electrophoresis. J. Appl. Microbiol. **2001**, *90* (2), 163–171.
65. Chinard, P. Photometric Determination of Proline and Ornithine. J. Biol. Chem. **1952**, *199* (1), 61–65.
66. Kolattukudy, P.E.; Lee, J.D.; Rogers, L.M.; Zimmerman, P.; Ceselsky, S.; Fox, B.; Stein, B.; Copelan, E.A. Evidence for Possible Involvement of an Elastolytic Serine Protease in Aspergillosis. Infect. Immun. **1993**, *61* (6), 2357–2368.
67. Jaffar-Bandjee, M.C.; Lazdunski, A.; Bally, M.; Carrere, J.; Chazalette, J.P.; Galabert, C.J. Production of Elastase, Exotoxin A, and Alkaline Protease in Sputa During Pulmonary Exacerbation of Cystic Fibrosis in Patients Infected by *Pseudomonas aeruginosa.* J. Clin. Microbiol. **1995**, *33* (4), 924–929.
68. Groeneveld, A.B.; Raijmakers, P.G.; Hack, C.E.; Thijs, L.G. Interleukin 8-Related Neutrophil Elastase and the Severity of the Adult Respiratory Distress Syndrome. Cytokine **1995**, *7* (7), 746–752.
69. Viglio, S.; Zanaboni, G.; Luisetti, M.; Cetta, G.; Guglielminetti, M.; Iadarola, P. Micellar Electrokinetic Chromatography: a Convenient Alternative to Colorimetric and High Performance Liquid Chromatographic Detection to Monitor Protease Activity. Electrophoresis **1998**, *19* (12), 2083–2089.
70. Viglio, S.; Luisetti, M.; Zanaboni, G.; Döring, G.; Worlitzsch, D.; Cetta, G.; Iadarola, P. Simultaneous Determination of *Pseudomonas aeruginosa* Elastase, Human Leukocyte Elastase and Cathepsin G Activities by Micellar Electrokinetic Chromatography. J. Chromatogr. A **1999**, *846* (1–2), 125–134.
71. Viglio, S.; Lupi, A.; Luisetti, M.; Zanaboni, G.; Cetta, G.; Iadarola, P. Activity of Different Proteases in a Complex Mixture and "in vitro" Study of Their Reciprocal Interferences by Micellar Electrokinetic Chromatography. J. Microcol. Sep. **2000**, *12* (5), 302–307.

Downloaded At: 20:43 23 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

72. Viglio, S.; Zanaboni, G.; Lupi, A.; Gianelli, L.; Luisetti, M.; Casali, L.; Cetta, G.; Iadarola, P. Micellar Electrokinetic Chromatography for Analyzing Active Site Specificity of *Pseudomonas aeruginosa* Elastase. Electrophoresis **1999**, *20* (7), 1578–1585.
73. Chu, Q.; O'Dwyer, M.; Zeece, M.G. Capillary Electrophoretic Analysis of Cathepsin D Action on Hemoglobin. J. Cap. Elec. **1997**, *4* (3), 117–122.
74. Chu, Q.; Jones, S.; Zeece, M. Capillary Electrophoretic Determination of Cathepsin D Activity Using Oregon Green-Labeled Hemoglobin. Electrophoresis **1999**, *20* (14), 2945–2951.
75. Gu, X.; Whipple-VanPatter, G.; O'Dwyer, M.; Zeece, M. Capillary Electrophoretic Analysis of μ- and *m*-Calpain Using Fluorescently Labeled Casein Substrates. Electrophoresis **2001**, *22* (11), 2336–2342.
76. Goll, D.E.; Thompson, V.F.; Taylor, R.G.; Christiansen, J.A. Role of the Calpain System in Muscle Growth. Biochimie **1992**, *74* (3), 225–237.
77. Koohmaraie, M. The Role of Ca(2+)-Dependant Proteases (Calpains) in Post Mortem Proteolysis and Meat Tenderness. Biochimie **1992**, *74* (4), 239–245.
78. Morgan, J.B.; Wheeler, T.L.; Koohmaraie, M.; Savell, J.W.; Crouse, J.D. Meat Tenderness and the Calpain Proteolytic System in Longissimus Muscle of Young Bulls and Steers. J. Anim. Sci. **1993**, *71* (6), 1471–1476.
79. Freed, A.L.; Lunte, S.M. Separation of Napthalene-2,3-Dicarboxaldehyde-Derivatized-Substance P and Its Metabolites by Micellar Electrokinetic Chromatography. Electrophoresis **2000**, *21* (10), 1992–1996.
80. Gerard, N.P. Characterization of Substance P Contractile Activity on Isolated Guinea Pig Lung Tissue. J. Pharmacol. Exp. Ther. **1987**, *243* (3), 901–906.
81. Otsuka, M.; Yoshioka, K. Neurotransmitter Functions of Mammalian Tachykinins. Physiol. Rev. **1993**, *73* (2), 229–308.
82. Samuels, L.L.; Goutas, L.J.; Priest, D.G.; Piper, J.R.; Sirotnak, F.M. Hydrolytic Cleavage of Methotrexate Gamma-Polyglutamates by Folylpolyglutamyl Hydrolase Derived from Various Tumors and Normal Tissues of the Mouse. Cancer Res. **1986**, *46* (5), 2230–2235.
83. Wang, Y.; Rotundo, R.F.; Nimec, Z.; Ryan, T.J.; Galivan, J. Two Novel HPLC Methods which Rapidly Detect the Substrates and Cleavage Products of Gamma-Glutamyl Hydrolase. Adv. Exp. Med. Biol. **1993**, *338* (1), 655–658.
84. Wagh, P.V.; Kalman, T.I. A Rapid Colorimetric Assay for Gamma-Glutamyl Hydrolase (Conjugase). Anal. Biochem. **1992**, *207* (1), 1–5.
85. Freed, A.L.; Cooper, J.D.; Davies, M.I.; Lunte, S.M. Investigation of the Metabolism of Substance P in Rat Striatum by Microdialysis Sampling and Capillary Electrophoresis with Laser-Induced Fluorescence Detection. J. Neurosci. Meth. **2001**, *109* (1), 23–29.

Downloaded At: 20:43 23 January 2011

Marcel Dekker, Inc. • 270 Madison Avenue • New York, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

86. Freed, A.L.; Audus, K.L.; Lunte, S.M. Investigation of the Metabolism of Substance P at the Blood-Brain Barrier Using Capillary Electrophoresis with Laser-Induced Fluorescence detection. Electrophoresis **2001**, *22* (17), 3778–3784.
87. Sohar, I.; Sleat, D.E.; Jadot, M.; Lobel, P. Biochemical Characterization of a Lysosomal Protease Deficient in Classical Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL) and Development of an Enzyme-Based Assay for Diagnosis and Exclusion of LINCL in Human Specimens and Animal Models. J. Neurochem. **1999**, *73* (2), 700–711.
88. Junaid, M.A.; Sklower Brooks, S.; Wisniewski, K.; Pullarkat, R.K. A Novel Assay for Lysosomal Pepstatin-Insensitive Proteinase and Its Application for the Diagnosis of Late-Infantile Neuronal Ceroid Lipofuscinosis. Clin. Chim. Acta **1999**, *281* (1–2), 169–176.
89. Shimura, K.; Matsumoto, H.; Kasai, K. Assay of Trypsin Activity by Capillary Isoelectric Focusing with Laser-Induced Fluorescence Detection. Electrophoresis **1998**, *19* (13), 2296–2300.
90. Zugel, S.A.; Burke, B.J.; Regnier, F.E.; Lytle, F.E. Electrophoretically Mediated Microanalysis of Leucine Aminopeptidase Using Two-Photon Excited Fluorescence Detection on a Microchip. Anal. Chem. **2000**, *72* (22), 5731–5735.

Received April 19, 2002
Accepted May 30, 2002
Manuscript 5832

Downloaded At: 20:43 23 January 2011