ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1513–1519

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Primary structure of bovine adenosine deaminase

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Received for review 8 August 1995; revised manuscript received 22 April 1996

Abstract

Derivatized bovine adenosine deaminase is used in enzyme replacement therapy and as an adjunct to gene therapy against severe combined immunodeficiency syndrome. Although a gene sequence is known for human adenosine deaminase, the structure of the bovine enzyme has not been characterized. Structure studies using mass spectrometry are reported here that evaluate sequence, processing, post-translational modifications and the extent of homology between the human protein and its therapeutic surrogate.

Keywords: Adenosine deaminase; Immumodeficiency; Mass spectrometry; Protein sequence; SCID

1. Introduction

Adenosine deaminase (ADA) deficiency, inherited as an autosomal recessive trait, is a major contributing cause of severe combined immunodeficiency syndrome, SCID. ADA (EC 3.5.4.4) catalyses the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. It is present in all mammalian cells and recently has been shown to be involved in T cell activation [1]. Both enzyme replacement therapy [2] and gene therapy [3] have been used to treat victims of this rare disease. Derivatized bovine ADA [4] is administered (with orphan drug status) to replace deficient human ADA. It is derivatized with polyethylene glycol to prolong its circulatory lifetime and provide slow release [5].

Gene sequences have been reported for human ADA [6,7], mouse ADA [8] and *E. coli* ADA [9]. Most of the human enzyme has also been sequenced directly, and the amino terminal is reported to be blocked [7]. The sequences of the two mammalian enzymes are 83% superimposable. The bacterial enzyme has about 33% identity, retaining however, the residues identified as critical in binding and catalysis by an X-ray crystallographic study [10] of mouse ADA complexed with a transition state analog.

The primary structure of bovine ADA has not been reported, although it is expected to resemble the other mammalian enzymes, and expecially human ADA with which it is functionally interchangeable. In the present study, the sequence of bovine ADA has been characterized in order to evaluate procesing, post-translational modifications and the extent of homology. Five proteolytic

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PII S0731-7085(96)01845-6

Table 1
Mass map of CNBr digestion products

Peptide fragment	MW_{found} by ES-MS	MW_{calc} from bovine sequence[a,b]	MW_{calc} from human sequence[a,b]
1–51	5649.1	5650.5	5525.5[c]
52–68	2029.3	2029.3	2030.4
69–88	2263.6	2262.6	2219.7
89–154	7391.8	7393.3	7192.2
155–256	11343.1	11342.5	11321.8
257–309	6261.3	6261.9	6142.8
310–314	–	573.6	602.8
315–352	4490.6, 4391.5	4490.1, 4391.1	4420.1
353–end	–	370.4	941.0

[a] Reduced and S-alkylated.
[b] Calculated for homoserine loctone termini.
[c] Calculated excluding initiator methionine.

mass maps [11] have been prepared and compared with those calculated for the human enzyme. When non-coincidental masses indicated different compositions, that region of bovine ADA was sequenced. In all, 76% of the protein sequence was experimentally determined from tandem mass spectra and specific cleavage sites. Based on sequencing and mapping, 93% identity is reported between the human and bovine enzymes. Acetylation of the amino terminus was the only modification found. This structural information provides the foundation for quality control and metabolism studies of the orphan drug.

2. Experimental

2.1. Materials

Bovine ADA was purchases from Boehringer–Mannheim Biochemicals (Indianapolis, IN) as catalog #102113, lot #12082820-41. Specifications from the supplier identified four protein contaminants, each at levels below 0.01%. Bovine ADA comes as a suspension in $(NH_4)_2SO_4$ solution. To remove the $(NH_4)_2SO_4$, an aliquot of suspension is centrifuged and the supernatant discarded. Endoproteinase Asp-N, and *S. aureus* V8 protease were pruchased from Boehringer–Mannheim Biochemicals. Iodoacetamide, clostripain, trypsin, chymotrypsin, CNBr, EDTA, DTT, guanidine hydrochloride, and ammonium bicarbonate were pruchased from Sigma Chemicals (St. Louis, MO). 3-Bromo-3-methyl-2-(*o*-nitrophenylsulfenyl)indolenine (BNPS-skatole) was purchased from Pierce Chemical Co. (Rockford, IL).

2..2 Electrospray mass spectrometry

Electrospray mass spectrometry was performed with a Vestec (Houston, TX) electrospray source retrofitted onto a Hewlett Packard (Palo Alto,

Table 2
Mass map of clostripain digestion products

Peptide fragment	MW_{found} by ES-MS	MW_{calc} from bovine sequence[a]
2–33	3722.1	3721.3
34–80	5249.7	5251.1
81–100	2245.3	2345.7
81–148	7659.4	7660.6
101–148	5333.0	5332.9
149–210	7018.8	7018.9
156–210	6098.5	6097.7
211–234	2511.1	2509.8
235–250	1890.0	1890.0
251–281	3771.4	3773.2
253–281	3502.0	3503.9
282–323	5054.2	5054.6
324–351[b]	3282.6	3282.8
324–354[c]	3499.0	3499.0
324–356[c]	3666.4	3667.2

[a] Reduced and S-alkylated.
[b] Arginine at position 351.
[c] Glycine at position 351.

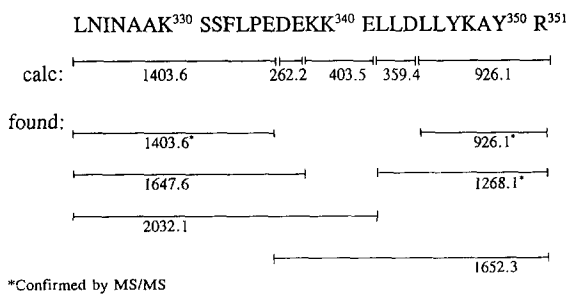

Fig. 1. Peptide map of an S. aureus V8 digest of clostripain fragment [324–351] from bovine ADA. Peptides denoted with a superscript asterisk were sequenced by tandem mass spectrometry.

CA) 5988A single quadrupole mass spectrometer, also equipped with a Phrasor (Duarte, CA) high energy dynode (HED) detector and a Teknivent (St. Louis, MO) Vector/Two™ data system. Samples were typically dissolved in 50:50 MeCN:6% HOAc in H_20 at concentrations of 10–100 μM. Injections of 5 μl of analyte were analyzed via a loop-injection apparatus. The analyzer was scanned (6 s) at unit resolution through the mass ranges 300–1700 for peptides and 700–2000 for proteins.

2.3. Tandem mass spectrometry

Collisionally induced dissociation spectra were measured using FAB ionization on a JEOL (JEOL Ltd., Tokyo, Japan) HX110/110 four-sector mass spectrometer with a Complement data system was used. Peptides were dissolved at 10 ng μl^{-1} in 0.1% TFA, and 2μl was added to a 50:50 glycerol:thioglycerol matrix. Precursor ions were attenuated 80% with helium collision gas. The collision cell was floated to 4 kV and the accelereating voltage in MS1 was 10 kV. A resolution of 1000 was used in both MS1 and MS2. The analyzers were scanned from 0 to 4500 in 90 s.

2.4. Capillary electrophoresis

The protein was characterized on an Advanced Technologies Inc. (Madison, WI) capillary electropohoresis system with the detector at 200 nm. The protein was desalted using an Econo-Pac 10DG column from Bio-Rad (Richmond, CA) equilibrated and eluted with 20 mM ammonium bicarbonate, frozen, and lyophilized. The protein dissolved in 18 mM sodium phosphate, pH 7.6, and was pressure-injected (19 s) into a 50 μm (ID) 8 × 45 cm capillary. The separation buffer was 350 mM sodium phosphate buffer (pH 7.6).

2.5. High pressure liquid chromatography

Separation of chemical and enzymatic digests was performed on a Shimadzu (Columbia, MD) HPLC system using a Brownlee Aquapore RP-300 column (250 × 4.6 mm) (Perkin Elmer, Foster City, CA), or on a Beckman (Fullerton, CA) system using a Poros RH II (2.11 × 33 mm) column (Perseptive Biosystems, Cambridge, MA) and a Shimadzu UV detector. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.08% TFA in acetonitrile. A typical gradient elution went from 5% B to 60% B in 30 min for the Aquapore column and in 9 min for the Poros column. Fractions were collected and lyophilized prior to mass analysis.

2.6. Reduction and alkylation

Cysteine residues in the protein were reduced with dithiothreitol and alkylated with iodacetamide [12]. The product was desalted on an Econo-Pac 10DG column form Bio-Rad equilibrated with 20 mM ammonium bicarborate.

2.7. Chemical and proteolytic digestions

BNPS-skatole was used as a tryptophan-specific cleavage agent using conditions reported recently [12,13]. Cyanogen bromide reactions were carried out for 20 h at room temperature following published procedures [14]. The protein was cleaved with trypsin [15] or chymotrypsin [16], incubating at 37°C for 2 h. Proteolytic cleavage of 4 mg of reduced and alkylated ADA was carried out with 7 units of clostripain [17] incubated at 37°C for 30 min. *S. aureus* V8 protease and endoproteinase Asp-N were also used under standard conditions [16].

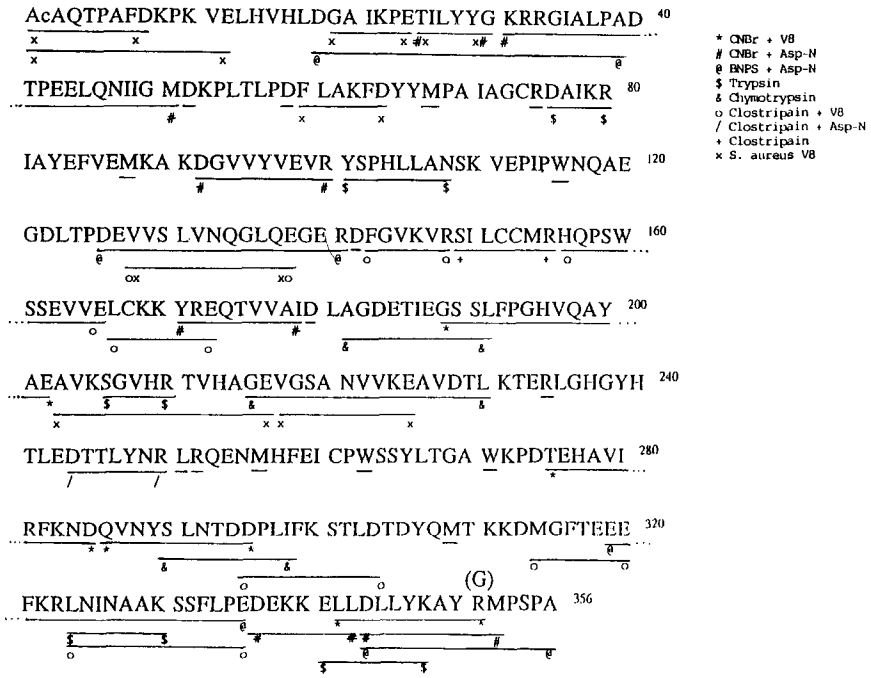

Fig. 2. Summary of peptides sequenced from bovine ADA by tandem mass spectrometry and characterized by specific cleavage reactions.

3. Results and discussion

The electrospray mass spectrum of bovine ADA purified from the commercial preparation, reduced and alkylated, contained a broad set of molecular ions with a mass of 40549 ± 16 Da ($n = 12$). This differs from the molecular weight of cysteine-alkylated human ADA, predicted from the gene sequence as 40933 Da. (The initiator methionine was excluded in this calculation.) Capillary electrophoresis without reduction and alkylation indicated that 97% of the sample comprised a set of three closely eluting unresolved species.

The first step in the strategy used was to weigh the entire protein and cleave it with reagents that cut the amide backbone specifically at infrequently occuring residues. Cyanogen bromide was used, which cuts on the carboxy terminal side of methionine: BNPS-skatole, which cleaves at the carboxy terminal side of tryptophan; and clostripain, specific for the carboxy terminal side of arginine and lysyl-lysine.

The mass map of peptide products produced by incubation with CNBr and analyzed by electrospray mass spectrometry is summarized in Table 1 and compared with the map calculated for the human enzyme. The theoretical masses of the product peptides are also provided in Table 1, based on the sequence finally determined for bovine ADA. Overall, the two maps are similar in both the number of petides found and the magnitudes of their molecular weights, suggesting that the positions of methionine residues (specifically cleaved by CNBr) are conserved in the two proteins. However the exact masses observed in most of the pairs of peptides indicate differences in the specific sequences of the two proteins. The order proposed for these peptides in bovine ADA was further supported by characterizaton of larger, incompletely cleaved peptides from the CNBr experiment. Two low molecular weight peptides were not characterized in this first map, which focussed on heavier products. The heterogeneity assigned to peptide [315–352] is based on sequencing experiments described below.

Table 2 lists the mass map produced by incubation with arginine-specific clostripain. Heterogeneity near the carboxy terminus led to multiple products, this time reflecting clostripain cleavage when arginine is present at position 351 and no cleavage in variants with glycine at position 351 (see below). Partial loss of N- and C-terminal residues is attributed to artifactual chemical hydrolysis. Products from cleavage with BNPS-skatole were consistent and allowed tryptophan -residues to be located in the sequence. The molecualr weight determined for the protein allowed all proteolytic products to be accounted for by the mass balance strategy [18].

Human	MAQTPAFDKPK	VELHVHLDGS	IKPETILYYG	RRRGIALPAN	40
Bovine	AcAQTPAFDKPK	VELHVHLDGA	IKPETILYYG	KRRGIALPAD	
Mouse	MAQTPAFNKPK	VELHVHLDGA	IKPETILYFG	KKRGIALPAD	
	TAEGLLNVIG	MDKPLTLPKF	LAKFDYYMPA	IAGCREAIKR	80
	TPEELQNIIG	MDKPLTLPDF	LAKFDYYMPA	IAGCRDAIKR	
	TVEELRNIIG	MDKPLSLPGF	LAKFDYYMPV	IAGCREAIKR	
	IAYEFVEMKA	KEGVVYVEVR	YSPHLLANSK	VEPIPWNQAE	120
	IAYEFVEMKA	KDGVVYVEVR	YSPHLLANSK	VEPIPWNQAE	
	IAYEFVEMKA	KEGVVYVEVR	YSPHLLANSK	VDPMPWNQTE	
	GDLTPDEVVA	LVGQGLQEGE	RDFGVKARSI	LCCMRHQPNW	160
	GDLTPDEVVS	LVNQGLQEGE	RDFGVKVRSI	LCCMRHQPSW	
	GDVTPDDVVD	LVNQGLQEGE	QAFGIKVRSI	LCCMRHQPSW	
	SPKVVELCKK	YQQQTVVAID	LAGDETIPGS	SLLPGHVQAY	200
	SSEVVELCKK	YREQTVVAID	LAGDETIEGS	SLFPGHVQAY	
	SLEVLELCKK	YNQKTVVAMD	LAGDETIEGS	SLFPGHVEAY	
	QEAVKSGIHR	TVHAGEVGSA	EVVKEAVDIL	KTERLGHGYH	240
	AEAVKSGVHR	TVHAGEVGSA	NVVKEAVDTL	KTERLGHGYH	
	EGAVKNGIHR	TVHAGEVGSP	EVVREAVDIL	KTERVGHGYH	
	TLEDQALYNR	LRQENMHFEI	CPWSSYLTGA	WKPDTEHAVI	280
	TLEDTTLYNR	LRQENMHFEI	CPWSSYLTGA	WKPDTEHAVI	
	TIEDEALYNR	LLKENMHFEV	CPWSSYLTGA	WDPKTTHAVV	
	RLKNDQANYS	LNTDDPLIFK	STLDTDYQMT	KRDMGFTEEE	320
	RFKNDQVNYS	LNTDDPLIFK	STLDTDYQMT	KKDMGFTEEE	
	RFKNDKANYS	LNTDDPLIFK	STLDTDYQMT	KKDMGFTEEE	
	FKRLNINAAK	SSFLPEDEKR	ELLDLLYKAY	GMPPSASAGQ (G)	NL 362
	FKRLNINAAK	SSFLPEDEKR	ELLDLLYKAY	RMPSPA	356
	FKRLNINAAK	SSFLPEEEKR	ELLERLYREY	Q	351

Fig. 3. Primary sequences of human [6,7], bovine, and mouse [8] ADA.

Further cleavages of these large peptides with *S. aureus* V8 protease and endoptroein- Asp-N provided additional mapping, and peptides of appropriate size for sequencing by tandem mass spectrometry [19]. An example is shown in Fig. 1 in which the peptide of mass 3282 produced by clostripain cleavage of bovine ADA (Table 2), is further mapped by subsequent cleavage by *S. aureus* V8 protease. Three of these V8 products were also sequenced by tandem mass spectrometry, as indicated in Fig.1. Sequencing was carried out on smaller peptides to explain all the mass differences observed between the bovine and human ADA peptide maps.

The sequence proposed for bovine ADA is shown in Fig. 2, along with a summary of peptides that were sequenced by tandem mass spectrometry and residues assigned by specific cleavage reactions (see Section 2). 76% of the residues were ordered experimentally. The remaining sequence is proposed to be analogous to that of human ADA (see Fig. 3), based on the coindicence of overlapping mass maps produced by cleavages of the reduced and alkylated protein with CNBr, clostripain, BNPS-skatole, trypsin and *S. aureus* V8, and combinations of the first three with endoproteinase Asp-N and V8 protease. Cleavage with trypsin and chymotrypsin of protein that has not been reduced and alkylated characterized a disulfide bond between cys-152 and cys-153 in part of the sample. This is may be formed artifactually, and it may contribute to electrophoretic microheterogeneity.

The amino terminus was shown to be acetylated alanine in two peptides that were sequenced by collisional activation. No other post-translational modifications were found.

The carboxyl terminus was more difficult to characterize because of microheterogeneity (arginine/glycine) at position 351, six residues from the end. Two peptides were detected corresponding to [315–352] in the CNBr map (Table 1). This heterogeneity was also reflected in the peptide products of clostripain cleavage (Table 2) [20]. Collisional activation spectra [19] were obtained for the two peptides [344–352] (obtained by cleavage of the CNBr products with Asp-N protease) and these spectra are shown in Fig. 4.

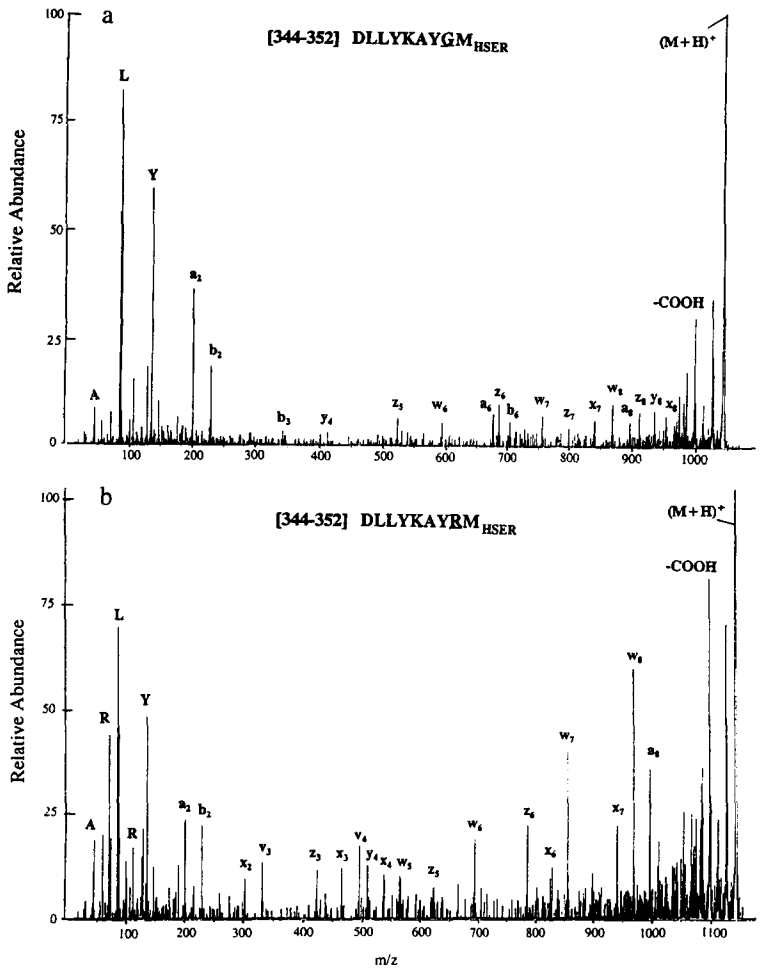

Fig. 4. Collisional activation mass spectra of peptides [344–352] from bovine ADA: a, the peptide with glycine at position 351; b, the peptide with arginine at position 351. Both peptides are terminated by homoserine derived from cleavage of methionine by CNBr.

Sequence ions are annotated following Biemann [19]. The molecular weights are different [20] as are the masses of all sequence ions that contain residue 8 [ADA residue 251], and glycine/arginine heterogeneity is assigned to that position. The dominance of carboxyl-terminus ions (x, y and z series) is consistant with the presence of a strong base, arginine, near that terminus. The proportions of the two variants were estimated from HPLC as 60:40, R:G. Glycine/argtinine heterogeneity can result from a single base mutation and has been reported in other proteins (see e.g. Ref. [21]).

The calculated molecular weights for cysteine-alkylated bovine ADA, based on the primary sequence determined in this study, are 40516 Da with glycine at positon 351, and 40615 Da with arginine at position 351. The average of these two values is 40565 Da, close to the mass determined by elctrospray (see above[6]) [22].

[6] The difficulty in resolving isozymes at high charge states on unit resolving quadrupole analyzers has been discussed in Ref. [22].

Acknowledgements

The authors would like to thank Dr. Philip Smith for discussion about the interpretation of tandem mass spectra, and Drs. Yetrib Hathout and Thomas Wheat for the capillary electrophoresis analysis. This research was supported by the National Institutes of Health (GM21248) and the National Science Foundation.

References

[1] J. Kameoka, T. Tanaka, Y. Nojima, S.F. Schlossman and C. Morimoto, Science, 261 (1993) 466–469.
[2] Y. Levy, M.S. Hershfield, C. Fernandez-Mejia, S.H. Polmar, D. Scudiery, M. Berger and R.U. Sorensen, J. Pediatr., 113 (1988) 312–317.
[3] E. Marshall, Science, 267 (1995) 1588.
[4] N. Pfrogner, Arch. Biochem. Biophys. 119 (1967) 141–146.
[5] M.S. Hershfield, R.H. Buckley, M.L. Greenberg, A.L. Melton, R. Schiff, C. Hatem, J. Kurtzberg, M.L. Markert, R.H. Kobayashi, A.L. Kobayashi and A. Abuchowski, N. Engl. J. Med., 316 (1987) 589–596.
[6] D.A. Wiginton, G.S. Adrian and J.J. Hutton, Nucleic Acids Res., 12 (1984) 2439–2446.
[7] P.E. Daddona, D.S. Shewach, W.N. Kelley, P. Argos, A.F. Markham and S.H. Orkin, J Biol. Chem., 259 (1984) 12101–12106.
[8] C.Y. Yeung, D.E. Ingolia, D.B. Rot, C. Shoemaker, M.R. Al-Ubaidi, J.Y. Yen, C. Ching, C. Bobonis, R.J. Kaufman and R.E. Kellems, J. Biol. Chem., 260 (1985) 10299–10307.
[9] Z. Chang, P. Nygaard, A.C. Chinault and R.E. Kellems, Biochemistry, 30 (1991) 2273–2280.
[10] D.K. Wilson, F.B. Rudolph and F.A. Quiocho, Science, 252 (1991) 1278–1284.
[11] C. Fenselau, in (I. Cornides, Gy. Horvath, K. Vekey Eds.), Advances in Mass Spectrometry, Vol. 13, John Wiley & Sons Ltd, Sussex, England, 1995, pp. 129–145.
[12] M. Vestling and C. Fenselau, Anal. Chem., 66 (1994) 471–477.
[13] M.M. Vestling, M.A. Kelly and C. Fenselau, Rapid Commun,. Mass Spectrom., 8 (1994) 786–790.
[14] D.R. Goodlett, F.B. Armstrong, R.J. Creech and R.B. van Breemen, Anal. Biochem., 186 (1990) 116–120.
[15] M.M. Vestling, C.M. Murphy and C. Fenselau, Anal. Chem., 62 (1990) 2391–2394.
[16] T.D. Lee and J.E. Shively, Methods Enzymol., 193 (1990) 361–374.
[17] W.M. Mitchell, Methods Enzymol., 47 (1977) 165–170.
[18] S. Hua, M.M. Vestling, C.M. Murphy, D.K. Bryant, J.J. Height and C.C. Fenselau, Int. J. Peptide Protein Res., 40 (1992) 546–550.
[19] K. Biemann, Biol. Envir. Mass Spec., 16 (1988) 99–111.
[20] C. Fenselau and M. Kelly, in P. Snyder (Ed.), Biological and Biotechnological Applications of ESI-MS, ACS Symposium Series, American Chemical Society, Washington, D.C. 1995, in press.
[21] K.A. Resing, R.S. Johnson and K.A. Walsh, Biochemistry, 32 (1993) 10036–10045.
[22] R. Feng, Y. Konishi and A.W. Bell, J. Am. Soc. Mass spectrom., 2 (1991) 387–401.