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Subunit structure of bovine ESF (extracellular-matrix stabilizing factor(s))

A chondroitin sulfate proteoglycan with homology to human Iαi (inter-α-trypsin inhibitors)

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Two chondroitin sulfate-containing complexes have been isolated from fetal bovine serum and shown to contain the serine protease inhibitor bikunin. A complex of 126 kDa contains bikunin linked by a chondroitin sulfate chain to a protein with homology to the HC2 component of the human inter-α-trypsin inhibitor. This complex represents the extracellular matrix stabilizing factor recently described as a bikunin-containing fraction necessary for expansion of the cumulus matrix [(1992) J. Biol. Chem. 267, 12380–12386]. A second complex of 236 kDa contains, in addition to bikunin and HC2, a bovine homolog of HC3 of the human pre-α-trypsin inhibitor. Thus, bovine bikunin is a chondroitin sulfate proteoglycan that achieves multifunctionality by linkage to proteins homologous to human serine antiproteinase complexes.

Bikunin; Chondroitin sulfate proteoglycan; Carbohydrate crosslinking; Serine protease inhibitor

1. INTRODUCTION

Inter-α-trypsin inhibitor (IαI) is a serine protease inhibitor first isolated from human plasma [1] where it is present at about 450 mg/l in healthy individuals [2] and rises markedly in several disease states [3]. It contains three subunits, the smallest of which (30 kDa) carries the protease inhibitory activity. This subunit consists of two pancreatic trypsin inhibitor-type (Kunitz) domains and has been named HI-30 [4] or bikunin [5]. In human serum, bikunin is associated with two distinct heavier chains of 65–70 kDa [4] termed HC1 and HC2, that share 40% identity in their amino acid sequence [5]. A chondroitin sulfate chain links the three components into a 240 kDa complex; mild proteolysis of the Glu^{18}-Val^{19} bond of bikunin releases an HC1-HC2 dimer and extensive chondroitinase ABC treatment releases all three proteins [4]. The sequence around Ser^{10} of bikunin (EEGSG) is a partial consensus sequence (DEXSG) for glycosaminoglycan addition and is thought to be the site for chondroitin sulfate attachment [4]. Therefore, bikunin is a proteoglycan attached by chondroitin sulfate to HC1 and HC2 through linkages of unknown chemical structure.

Human plasma bikunin also occurs as a component of a second protease inhibitor known as pre-α-inhibitor (PαI). In studies with PαI, bikunin has been shown to carry a chondroitin-4-sulfate chain on Ser^{10} that is involved in a linkage with an 80 kDa protein named HC3 [6]. The linkage is an unusual covalent ester bond between the α-carboxyl group of the C-terminal Asp of HC3 and a C-6 of an internal *N*-acetylgalactosamine residue of the chondroitin sulfate chain [6]. The occurrence of similar linkages with HC1 and HC2 has not to our knowledge been investigated.

It has recently been found that cumulus cells of the mouse ovarian follicle secrete a hyaluronan-rich matrix following ovulation, that can be observed in vitro following stimulation with FSH and fetal bovine serum [7]. In the absence of serum, hyaluronan synthesis remains intact, but its retention in the matrix is impaired. A member of the IαI family with homology to the N-terminus of bikunin, when purified from bovine serum and added to minimal essential medium, was able to replace the serum requirement for matrix stabilization [7]. However, other trypsin inhibitors were unable to mimic the effect of IαI, and the anti-protease property of bikunin is not likely involved. Possibly this bovine extracellular matrix stabilizing factor (ESF) contains a bikunin proteoglycan that suits it for a role in the structural assembly or cross-linking of cumulus matrix. Alternatively, an unrecognized function of other components of the bikunin complex(es) could be responsible for these observations.

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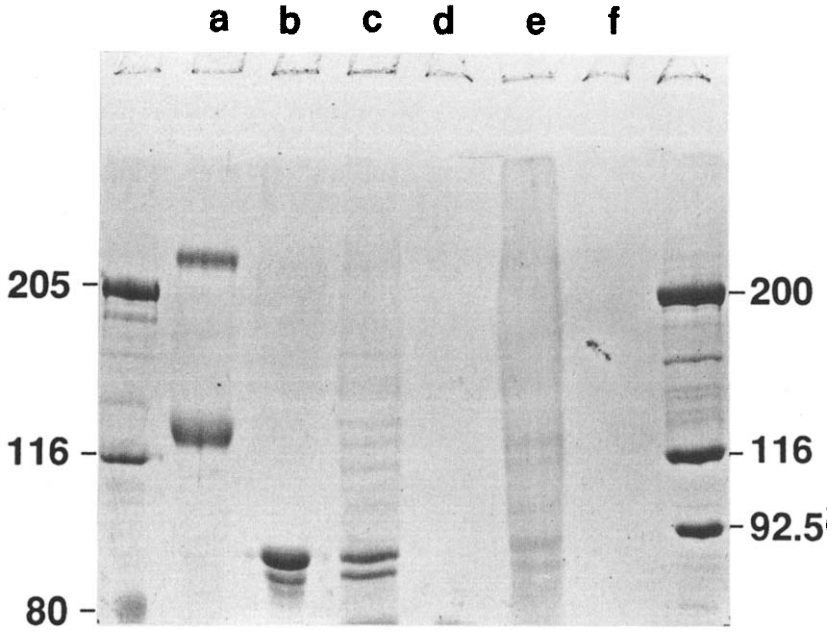

Fig. 1. Coomassie blue-stained 7% SDS-PAGE of chondroitin sulphate–bikunin complexes obtained from fetal bovine serum. Lanes (a) and (b) represent a mixture of the two complexes before and after deglycosylation with trifluoromethane sulphonic acid, respectively. Lane (c) is after a 4 h treatment at 37°C with chondroitinase ABC, and lane (e) is after similar treatment with testicular hyaluronidase. Lanes (d) and (f) are chondroitinase ABC and hyaluronidase alone, respectively, at the same concentrations used in lanes (c) and (e). Left- and right-most lanes are prestained and unstained molecular mass markers, respectively. The complete gel is shown in the photograph; lighter material was allowed to run off the gel to achieve resolution of the higher molecular mass components.

Here we report that bikunin in fetal calf serum occurs in two complexes, each linked by chondroitin sulfate. One is analogous to human IαI but containing homologs of HC2 and HC3 instead of HC1 and HC2. The other, ESF, is a dimer of bikunin and HC2 with no evidence of HC1 present. Interestingly, the first 20 residues at the N-terminus of bovine HC2 differ from the human protein in eight positions, resulting in a tandom concensus sequence (SGESGE) for glycosaminoglycan chain addition that nevertheless appears not to be glycosylated.

2. MATERIALS AND METHODS

2.1. *Proteoglycan purification*

Thirty ml of fetal bovine serum (Sigma, St. Louis, MO) was diluted to 200 ml with 50 mM Tris-HCl, 0.20 M NaCl, pH 8.0, and applied to a 2.0 ml DEAE-Sephacel column. After washing with 10 volumes of Tris buffer, proteoglycans were eluted with 5 volumes of 4 M guanidine-HCl, 50 mM sodium acetate, pH 5.8, and fractionated on Sephacryl S-300 (3 × 30 cm) in Tris buffer. Fractions between K_{av} of 0 and 0.30 were collected, desalted, lyophylized, and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli [8] on vertical polyacrylamide slab gels (7 × 8 × 0.075 cm). Gel concentrations are indicated in the legends of the appropriate figures. Molecular mass protein standards (Bio-Rad, Mississauga, Ont.) were electrophoresed along with samples.

2.2. *Deglycosylation and enzyme digestion*

Samples were digested with 1 mIU of chondroitinase ABC (E.C. 4.2.2.4; Sigma) in 10 μl of 50 mM Tris, 50 mM sodium acetate, pH 7.5, or with 1 μg of testicular hyaluronidase (E.C. 3.2.1.35; Sigma) in 10 μl of 50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4, before electrophoresis. As preliminary experiments indicated some protease activity in both enzyme preparations, a panel of protease inhibitors was added to each digestion mixture (10 mM disodium EDTA, 5 mM benzamidine-HCl, 3 mM *o*-phenanthroline, 5 mM tryptamine-HCl, and 1 mM phenylmethylsulfonylfluoride, all from Sigma). Proteoglycans were also deglycosylated using the method of Sojar et al. [9]. Briefly, 20 μl of trifluoromethane sulfonate (TFMS, Sigma) was added to the lyophilized samples under nitrogen atmosphere at 0°C. After 1.5 h, the reaction was neutralized by adding drop wise to the reaction mixture 60 μl of 60% (v/v) aqueous pyridine chilled in dry ice/ethanol. The neutralized reaction mixture was eluted from Sephadex G-50 in 25 mM ammonium carbonate and the void volume fractions were pooled and lyophilized for electrophoresis.

2.3. *Sequence analysis and peptide mapping*

The electrophoretogram of the TFMS-deglycosylated proteoglycans was electroblotted (TE22 Transphor; Hoefer Instruments, San Francisco, CA) to a polyvinylidine difluoride membrane (Bio-Rad), stained [10] and sequenced using a Porton 2090 gas-phase microsequencer (Porton Instruments; Tarzana, CA) with on-line analysis of phenylthiohydantoin derivatives. For the trypsin and cyanogen bromide digestion the samples were labelled with 0.2–1.0 mCi [^{125}I]NaI (ICN; Irvine, CA) in 100 μl of 0.5 M phosphate buffer (pH 7.5). Each iodination reaction was initiated by the addition of one Iodo-Bead (Pierce, Rockford, IL). The tubes were capped and left at room temperature. After 15 min the reaction was terminated by removing the bead and free ^{125}I was removed by desalting on Sephadex G-50/H_2O. The iodinated sample was deglycosylated and electrophoresed as described above. The corresponding core protein bands on the gels were cut, crushed, electroeluted [11] and desalted. Cyanogen bromide digestions were carried out in 20 μl of 70% formic acid containing 20 μg cyanogen bromide (J.T. Baker, Phillipsburg, NJ) overnight in the dark [12]. The digests were twice diluted to 0.5 ml and lyophilized for electrophoresis on 15% SDS-PAGE. Trypsin digestion was carried out with 0.25 μg of TPCK-treated trypsin (Sigma) in a total volume of 50

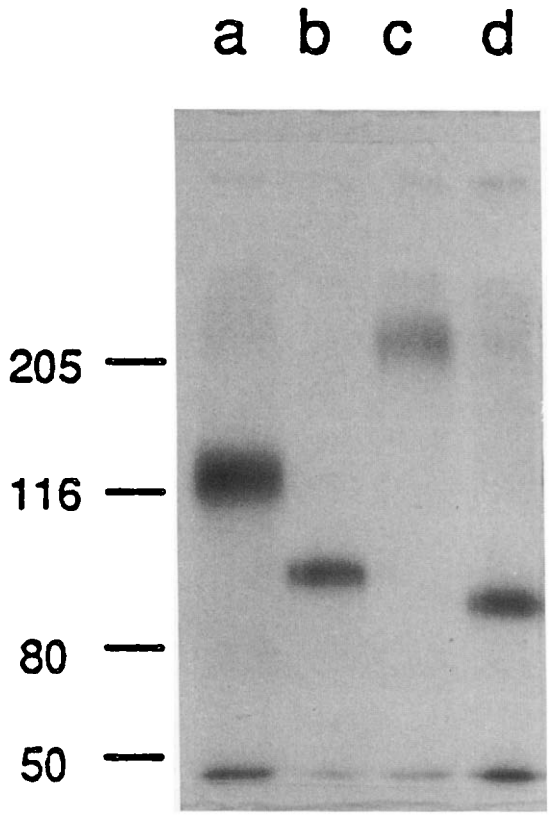

Fig. 2. SDS-PAGE (7%) autoradiogram of purified p126 and p236 after radioiodination. Lanes (a,b): p126. Lanes (c,d): p236. Lanes (a,c): before deglycosylation with trifluoromethane sulfonic acid. Lanes (b,d): after deglycosylation.

μl for 2 h at 37°C. The digests were lyophilized before electrophoresis on 20% SDS-PAGE. The dried electrophoretogram was exposed to Kodak X-OMAT X-ray film at − 70°C overnight.

3. RESULTS

Matrix stabilizing factor from bovine serum has been found to be homologous to bikunin, which exists in human serum in complexes containing acidic glycosaminoglycan. Partial purification of acidic components from fetal bovine serum by anion exchange chromatography provided two major protein bands of 126 kDa and 236 kDa on 7% SDS-PAGE under reducing conditions (Fig. 1), designated p126 and p236. Deglycosylation of the mixture with TFMS removes both these bands and gives rise to two major components of 82 and 86 kDa. After electroelution and radioiodination, both glycoproteins re-chromatographed in their original positions. On deglycosylation, the 86 kDa peptide was found to arise from p126, whereas p236 gave rise to the 82 kDa peptide (Fig. 2). In addition, a 30 kDa component was liberated from both glycoproteins by TFMS treatment that was observed on a 12.5% SDS-PAGE (not shown). Amino acid sequencing of the 30 kDa and 86 kDa components derived from p126 showed N-terminal homology with human bikunin and IαI-HC2, respectively (Table I). Sequencing of intact p126 gave both N-terminal sequences in approximately equimolar amounts (Table II), consistent with a carbohydrate linkage of two separate peptides. The 82 kDa peptide from p236 shows 65% identity with human IαI-HC3 in the N-terminal region (Table I). Although deglycosylation of iodinated p236 gave rise only to 82 and 30 kDa components, sequencing of intact p236 also revealed the presence of the N-terminal of the 86 kDa component of p126, and demonstrated that p236 consists of one chain each of the homologs of human IαI components HC2, HC3 and bikunin (Table II).

Treatment of the combined preparation of p126 and p236 with chondroitinase ABC released components of 82 and 86 kDa (Fig. 1) and 30 kDa (not shown). Therefore, the linkages in both complexes are chondroitin/dermatan sulfate in nature. Similar results were obtained with testicular hyaluronidase which randomly cleaves the internal β-*N*-acetylhexosamine-[1,4]-glycosidic bonds in hyaluronan and chondroitin sulfate.

Human HC2 and HC3, and also HC1 for which no bovine homolog was detected, are closely related proteins with considerable sequence identity that are nevertheless products of distinct genes on separate chromosomes [13]. The 86 and 82 kDa peptides described here as homologs of HC2 and HC3, respectively, also appear to be closely related as demonstrated by CNBr and tryptic fragmentation (Fig. 3). However, several unique bands in the CNBr-derived gel indicate that the proteins are distinct, and the smaller component is not a truncated version of the larger.

Table I

N-terminal amino acid sequences obtained from purified 30, 82 and 86 kDa proteins of fetal bovine serum bikunin-containing components

Residue	H_2N- 01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20
86 kDa	S	I	S	G	E	S	G	E	R	T	E	D	V	D	Q	V	T	V	Y	K
HC2	S	L	P	G	E	S	E	E	M	M	E	E	V	D	Q	V	T	L	Y	S
82 kDa	S	L	P	G	R	A	V	D	G	I	E	V	Y	S	T	K	V	N	X	K
HC3	S	L	P	E	G	V	A	N	G	I	E	V	Y	S	T	K	I	N	S	K
30 kDa	A	V	L	T	Q	E	E	E	G	X	G	X	G	Q	P					
Bikunin	A	V	L	P	Q	E	E	E	G	S	G	G	G	Q	L	V	T	E	V	T
ESF	X	V	L	G	Q	E	E	E	G	X	X	D	G	Q						

The sequences of the homologous components of human IαI and PαI [4] and ESF [7] are shown for comparison. A part of the sequence of the 30 kDa component (bold) was deduced by subtracting the known sequences of the 86 and 82 kDa proteins from the sequences of the 236 kDa complex. Identical residues are boxed.

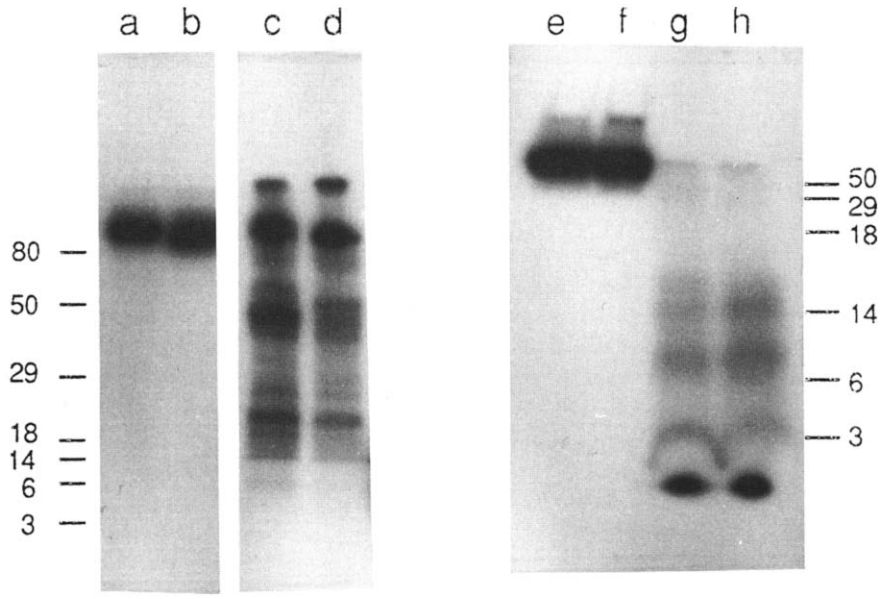

Fig. 3. Proteolysis patterns of the 82 and 86 kDa proteins associated with bikunin. Samples were run on 15% (a–d) or 20% (e–f) gels. Lanes (a,c,e,g): 86 kDa protein. Lanes (b,d,f,h): 82 kDa protein. Lanes (a,b,e,f) are untreated samples, lanes (c,d) are after cyanogen bromide cleavage, and lanes (g,h) are after trypsinization.

4. DISCUSSION

Chen et al. [7] have described a factor from fetal bovine serum that serves as a stabilizing factor for the extracellular matrix (ESF) of the expanding cumulus in stimulated preovulatory follicles, without which hyaluronan is not retained and normal ovulatory expansion does not occur. ESF was found to contain a protein homologous to bikunin, the inhibitory component of the human serine protease inhibitors IαI and PαI. The present study shows that bikunin occurs in at least two complexes – p126 and p236 - in fetal bovine serum,

Table II

Determination of subunit composition of p236 and p126 by sequencing of the intact complexes

	Cycle #1		Cycle #2		Cycle #3	
Complex	Amino acid	pmol	Amino acid	pmol	Amino acid	pmol
p236	Serine	93	Isoleucine	35	Serine	35
	Alanine	40	Leucine	32	Proline	32
			Valine	36	Leucine	32
p126	Serine	18	Isoleucine	12	Serine	12
	Alanine	11	Valine	15	Leucine	13

The first three cycles of Edman degradation are shown with the yield of amino acid given in pmol. Two sequences were obtained from the 126 kDa complex and three from the 236 kDa complex. Sequencing was carried out for at least 10 additional cycles (not shown) and the deduced sequences were identified from those of the individual components in Table I. Amino acid yields are consistent with 1:1(:1) peptide chain stoichiometry.

linked in each by a chondroitin sulfate chain to one or two larger proteins homologous to the 'heavy chain' peptides HC2 and HC3 of IαI and PαI, respectively. The smaller component, here designated p126, corresponds to ESF. HC1, HC2 and HC3 are closely related proteins [13] that are associated with bikunin in human serum. Although the nature of the bikunin-HC1-HC2 chondroitin sulfate linkages in IαI is not known, they may be similar to the bikunin-HC3 linkage of PαI, namely conventional attachment of the glycosaminoglycan chain to Ser^{10} of bikunin and a novel ester linkage between the glycosaminoglycan and the C-terminal of HC3 [6]. Based on the homologies described here, a model is proposed for the subunit arrangements of bikunin-containing complexes in both species (Fig. 4). Our sequence data indicate that Ser^{10} of bovine bikunin is also glycosylated, and chondroitin sulfate is shown as a rigid polyelectrolyte chain attached to this position and to the C-termini of the heavy chains.

The model emphasizes the absence of HC1 in the bovine material and the presence of a common heavy chain (HC2) in the two forms of the bovine bikunin proteoglycan. Thus, whereas the human structures IαI and PαI are distinct, the possibility exists that the bovine p126 arises from p236 by loss of HC3 or, alternatively, that p126 represents incomplete synthesis of p236. The partial sequence similarity of the three human heavy chains [13] and the similar patterns of proteolysis of bovine HC2 and HC3 (Fig. 3) suggest that these species differences in subunit arrangement may not produce divergent functions. However, the functional significance of the heavy chains is not known, nor indeed is the basis of the matrix stabilizing

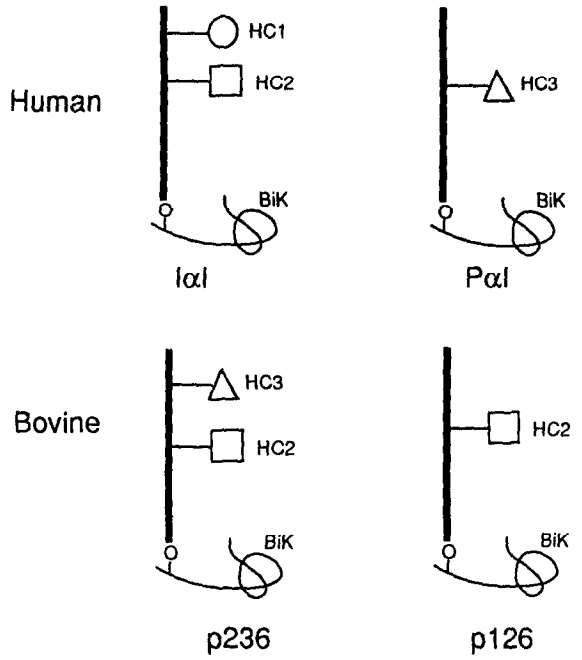

Fig. 4. Proposed model of the subunit arrangements of bovine p126 (also known as ESF) and p236. Protein components are named for their homology with components of the human IαI and PαI complexes following the terminology of Enghild et al. [4]. The chondroitin sulfate chain (vertical bold line) is in extended conformation. Linkages are to the C-termini of the HC proteins and to Ser^{10} of both human and bovine bikunin (BiK).

activity of p126. Several other protease inhibitors were unable to substitute for ESF in stabilizing cumulus matrix [7] and a role of the non-bikunin components is indicated. The unusual linkages of the HC components to the proteoglycan bikunin may hold the proteins in a spatial arrangement that facilitates a crosslinking function.

The failure to observe the 86 kDa HC2 protein by autoradiography after iodination and deglycosylation of p236 (Fig. 2) requires comment. Its presence in stoichiometric amounts was demonstrated by sequencing (Table II) and a second chain is necessary to account for the mass of p236. HC2 may be destabilized by iodination and more fully degraded by TFMS under the conditions for extensive deglycosylation employed in Fig. 2. Alternatively, partial degradation could lead to failure to resolve the two components.

In summary, bovine bikunin is a chondroitin sulfate proteoglycan that achieves multifunctionality by virtue of its association with a family of related heavy chain proteins. Matrix stabilizing function may reside in these heavy chains, or in the spatial arrangement dictated by their disposition along the glycosaminoglycan chain. In this regard, it will be interesting to determine if matrix-stabilizing activity is also associated with p236.

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REFERENCES

[1] Steinbuch, M. and Loeb, J. (1961) Nature 192, p. 1196.
[2] Ødum, L. (1991) Danish Med. Bull. 38, 68–77.
[3] Ødum, L. (1990) Biol. Chem. Hoppe-Seyler's 371, 1135–1138.
[4] Enghild, J.J., Thøgersen, I.B., Pizzo, S.V. and Salvesen, G. (1989) J. Biol. Chem. 264, 15975–15981.
[5] Gebhard, W., Schreitmüller, T., Hochstrasser, K. and Wachter, E. (1989) Eur. J. Biochem. 181, 571–576.
[6] Enghild, J.J., Salveses, G., Hefta, S.A., Thørgersen, I.B., Rutherfurd, S. and Pizzo, S.V. (1991) J. Biol. Chem. 266, 747–751.
[7] Chen, L., Mao, S.J.T. and Larsen, W.J. (1992) J. Biol. Chem. 267, 12380–12386.
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Sojar, H.T. and Bahl, O.M. (1987) Arch. Biochem. Biophys. 259, 52–57.
[10] Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.
[11] Hunkapiller, M.W., Lujan, E., Ostrander, F. and Hood, L.E. (1983) Methods Enzymol. 91, 227–236.
[12] Gross, E. (1967) Methods Enzymol. 11, 238–255.
[13] Diarra-Mehrpour, M., Bourguignon, J., Sesboüé, R., Matteï, M.-G., Passage, E., Salier, J.-P. and Martin, J.-P. (1989) Eur. J. Biochem. 179, 147–154.