Volume 312, number 1, 100–104 FEBS 11711 November 1992

Bovine α_2-antiplasmin

N-Terminal and reactive site sequence

Søren Christensen and Lars Sottrup-Jensen

Department of Molecular Biology, University of Aarhus, Building 130, 8000 Århus C, Denmark

Received 8 September 1992

Bovine α_2-antiplasmin (α_2AP) has been purified and partially characterized. The amino acid composition is very similar to that of human α_2AP, and the N-terminal (23 residues determined) and reactive site loop sequences (42 residues determined) are highly homologous to those of the human protein. Compared with human α_2AP, bovine α_2AP has an 18-residue N-terminal extension, homologous with part of the pre-sequence of human α_2AP. A re-investigation of the N-terminal sequence of freshly prepared human α_2AP reveals a new form extended by 12 residues.

Plasma protein; Proteinase inhibitor; Sequence analysis; α_2-Antiplasmin

1. INTRODUCTION

The plasma protein, α_2-antiplasmin (α_2AP), is a member of the serpin class of inhibitors [1–3]. Human α_2AP is a 70 kDa single-chain protein containing 14% carbohydrate [4] and two disulfide bridges. Mature α_2AP consists of 452 residues [5], and pre-α_2AP appears to contain a 39 [5] or 37-residue [6] signal sequence. The reactive site peptide bond (P_1–P_1') cleaved during complex formation with plasmin is Arg^{364}–Met [5]. A secondary site interacting with plasmin(ogen) is located in the C-terminal part of α_2AP [7,8].

Bovine α_2AP has not previously been characterized, and commercial polyclonal anti(human α_2AP) antibodies do not react with bovine α_2AP. As part of a study of the components of the fibrinolytic system in bovine milk we have isolated bovine α_2AP from plasma. As a first step in the characterization of bovine α_2AP we report here its partial N-terminal sequence and 42 residues of internal sequence, covering its reactive site.

2. MATERIALS AND METHODS

2.1. *Materials*

Porcine pancreatic elastase type IV and DCI were obtained from Sigma. Bovine chymotrypsin A was from Boehringer-Mannheim. *Staphylococcus aureus* V8 protease was from Worthington. Tosyl-phenylalanine chloromethylketone-treated trypsin was from Cooper Biomedicals. Subtilisin was from Novo-Nordic. Urokinase from Abbott (20,000 CTA U/mg) was used for activation of plasminogen. Bovine blood was obtained from a local slaughter house and anticoagulated with trisodium citrate. After separation of plasma by centrifugation it was stored at −20°C. The substrate *H*-D-Pro-L-Phe-L-Arg-*p*-nitroanilide·2 HCl (S-2302) was from Kabi.

2.2. *Preparation of lysine–Sepharose and K1-3–Sepharose*

Lysine–Sepharose was prepared according to [9]. Bovine plasminogen was purified from plasma as described [10]. 3 g of plasminogen dissolved in 800 ml 0.1 M NH_4HCO_3 was digested with 1.5% w/w elastase and the fragments separated essentially according to [11]. K1-3 (858 A_{280} units) was coupled to 11 g of CNBr-activated Sepharose according to the instructions from Pharmacia.

2.3. *Purification of bovine and human α_2AP*

As in [12] and [13], plasminogen-depleted plasma was fractionated with $(NH_4)_2SO_4$. The proteins precipitating between 0.8 and 2.7 M were dissolved in 40 mM sodium phosphate, 2 mM EDTA, pH 7.4, (buffer A) and dialyzed against 100 vols. of deionized water to precipitate fibrinogen. After centrifugation, the supernatant from 300 ml of plasma was made up to 40 mM in sodium phosphate, and 2 mM in EDTA, pH 7.4, and treated with 40 ml K1-3–Sepharose equilibrated with buffer A. The K1-3–Sepharose was washed on a Büchner funnel and poured into a column. Loosely bound proteins were eluted with buffer A containing 0.5 M NaCl, and α_2AP was eluted by including 20 mM ε-ACA in the buffer. The presence of α_2AP in the column fractions was monitored by observing an approx. 70 kDa band in reducing SDS-PAGE. 10-ml fractions containing α_2AP were applied to a Superose 12 column (2.5 × 40 cm) equilibrated and eluted with buffer A containing 0.5 M NaCl and 20 mM ε-ACA. Contaminating immunoglobulins were removed by passing the α_2AP preparation through a 4 ml protein A–Sepharose column equilibrated and eluted with the same buffer. Human α_2AP was purified by the same method as for bovine α_2AP except that a plasminogen–Sepharose column (60 mg plasminogen/ml Sepharose) was used instead of K1-3–Sepharose.

Correspondence address: L. Sottrup-Jensen, Department of Molecular Biology, University of Århus, C.F. Møllers Alle building 130, 8000 Århus C, Denmark. Fax: (45) 8619 6500.

Abbreviations: α_2AP, α_2-antiplasmin; serpin, serine protease inhibitor; DCI, 3,4-dichloroisocoumarin; CTA, committee of thrombolytic agents; ε-ACA, ε-aminocaproic acid; K, kringle region of plasminogen; HPLC, high performance liquid chromatograph(y); TFA, trifluoroacetic acid.

Published by Elsevier Science Publishers B.V.

2.4. *Assay of α_2AP*

Plasminogen (4.4 mg/ml in 0.1 M sodium phosphate, pH 7.3) was activated by incubation with urokinase (0.8 CTA U/μg plasminogen) for 2 h at room temperature. Fractions from Superose 12 were dialysed against buffer A and analyzed for α_2AP activity by incubating 50 μl samples with 50 μl plasmin (0.08 mg/ml in 0.1 M sodium phosphate, pH 7.3) for 30 min at 25°C. After the addition of 250 μl 0.2 mM S-2302 the change in absorbance at 405 nm was followed for 20 min.

2.5. *Preparation of cleaved α_2AP*

Reactive site cleaved α_2AP was prepared by incubation with plasmin and subtilisin (E:S = 1:1 and 1:2,000 w/v, respectively) for 1 min at 25°C. The enzyme was then inactivated by the addition of DCI. The C-terminal peptide was separated by SDS-PAGE [14,15].

2.6. *Amino acid analysis*

Hydrolyses were done at 110°C in 6 M HCl/1% thioglycolic acid for 3, 16, 24 and 72 h. To determine the half-cysteine content, samples were oxidized with 10 μl 1 M HCOOOH prior to hydrolysis. Free amino acids were separated by HPLC cation exchange using a pH gradient ranging from 3.10 to 10.10, as described [16].

2.7. *SDS-PAGE*

Protein purity and size estimation were performed in gels with linear gradients of acrylamide (10–20%, 2.5% bis-acrylamide) [14].

2.8. *N-Terminal sequence analysis*

Proteins and fragments were transferred from the SDS-PAGE gels to ProBlott membranes using electroblotting [17]. Edman degradations were done in an AB 477A sequenator equipped with a 120A on-line HPLC. The Donblot reaction and conversion cycles were used. Peptides were degraded on polybrene-coated glass-filters using the Normal-1 cycles. Initial yields of N-terminal amino acids were 20–200 pmol.

2.9. *Trypsin digestion*

Bovine-α_2AP (280 μg) was dissolved in 200 μl 0.25 M Tris-HCl, 1 mM EDTA, 6 M guanidine-HCl, pH 8.5, and reduced by the addition of 10 μl 2-mercaptoethanol diluted 1:10 in water and incubated at room temperature for 2 h. Then iodoacetamide was added to 0.3 M. After reaction for 2 h at room temperature the alkylated α_2AP was desalted on a Nucleosil C-4 500-7 column (4 × 125 mm). The protein was eluted with a gradient of 0.1% TFA and acetonitrile and dried in a speed-vac centrifuge. The digestion was done in 0.2 M NH_4HCO_3 using 4% trypsin. After incubation for 5 h at 37°C the digest was lyophilized, dissolved in 0.1% TFA, and separated on a C-18 Nucleosil 100-5 column (4 × 250 mm). The elution was performed at 50°C with a linear gradient of acetonitrile from 4.5% to 76.5% in 0.1% TFA over 60 min at a flow rate of 1 ml/min.

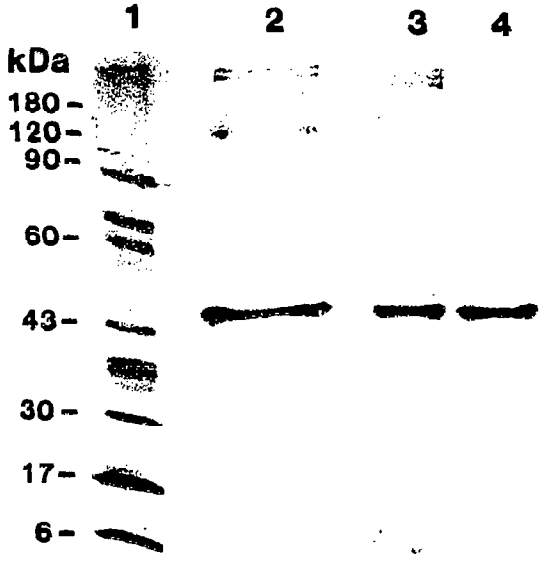

Fig. 1. SDS-PAGE of bovine α_2AP during purification. Lane 1, size markers, reduced. Lane 2, pool eluted from K1-3–Sepharose. Lane 3, after protein A–Sepharose chromatography. Lane 4, after Superose 12 gel chromatography. Lanes 2–4 are unreduced.

3. RESULTS AND DISCUSSION

3.1. *Purification of bovine α_2AP*

The purification of bovine α_2AP was based on affinity chromatography on K1-3–Sepharose as in [12,13]. However, when working with bovine plasma, contaminating fibrinogen and immunoglobulin were present in large amounts. To prevent saturation of the K1-3–Sepharose with fibrinogen, the major part of it was precipitated by dialysis before absorption. The α_2AP pool from K1-3–Sepharose was still contaminated with fibrinogen and immunoglobulin, but no tetranectin and histidine-rich glycoprotein could be detected (Fig. 1). However, in some preparations minor contaminants were present. A 28 kDa protein was identified as a fragment of bovine apolipoprotein(a) by N-terminal sequence analysis (DDPQSSXDRVK) and a 60 kDa protein with the N-terminal sequence (FQRGQVLSALPRTSR) might represent bovine plasma carboxypepti-

Table I

Amino acid composition of bovine and human α_2-antiplasmin

Amino acid	Bovine α_2AP (mol %)	Human α_2AP[d] (mol %)
Asx	10.1	7.7
Thr[a]	4.2	4.9
Ser[a]	7.3	7.7
Glx	13.2	13.7
Pro	8.5	8.2
Gly	5.3	5.8
Ala	7.3	6.4
Cys	1.0	0.9
Val[b]	4.6	6.2
Met	2.5	2.2
Ile[b]	2.3	2.0
Leu	15.0	14.8
Tyr	1.4	0.9
Phe	5.5	6.2
His	2.4	2.7
Lys	5.0	4.2
Arg	4.3	4.2
Trp	n.d.	1.3
GalcN	–	traces[e]
GlcN[c]	3.1	1.3[e]

[a]Values extrapolated to 0 h.
[b]Values determined after 72 h of hydrolysis.
[c]Corrected for a loss of 25% during a 3 h hydrolysis period.
[d]Taken from the cDNA sequence [6].
[e]Data from [4].
n.d., not determined.

```
Bovine α2AP                    FSPVSQMEPLDLQLMDGPAQEKL
                               ::::: :::: :: :: ::
Human pre-α2AP    mallwqllvlswsclqgpcsvfspvsameplgrqltsgpNQEQVSPLTLLKLGN
Human α2AP(1)                          MEPLGXQLTSGPNQ
Human α2AP(2)                                  NQEQVSPLTLLKL
```

Fig. 2. Comparison of the N-terminal sequences of bovine and human α_2AP. Small letters are used to indicate the pre-sequence in human α_2AP [6]. Capital letters indicate the mature sequence. X, not identified. Human α_2AP(1) and human α_2AP(2), the two forms of α_2AP, are found in approx. equimolar amounts. Identical residues in bovine and human α_2AP are shown by a pair of dots; residues different in bovine α_2AP are shown in bold.

dase B [18] (results not shown). The contaminating immunoglobulin was removed by passage through a protein A–Sepharose column. Residual fibrinogen was removed by gel filtration on Superose 12 (Fig. 1). About 5 mg of bovine α_2AP was obtained from 1 l of plasminogen depleted plasma.

The amino acid composition of bovine α_2AP was determined and compared with the human α_2AP (Table I). The compositions of the two proteins agreed within ± 10% except for Asx, Val and Tyr which differed more than 30%. Bovine α_2AP contained *N*-acetyl-glucosamine, indicating the presence of N-linked carbohydrate as in human α_2AP. The SΔQ defined in [19] gives a value of about 12, indicating highly similar amino acid sequences. However, although bovine and human α_2AP have similar amino acid compositions no cross-reaction is found using polyclonal antibodies (Dako, Copenhagen) to the human protein (datasheet from Dako, and unpublished).

3.2. *N-Terminal sequence analysis of bovine α_2AP*

The sequence of the first 23 residues of bovine α_2AP and its alignment with the sequence of human α_2AP are shown in Fig. 2. Relative to human α_2AP, bovine α_2AP has an 18-residue extension which is clearly homologous to part of the presumed 39-residue pre-sequence of human α_2AP. Only one N-terminal sequence (NQEQ) was reported earlier for human α_2AP [4]. However, sequence analysis of a fresh preparation of human α_2AP revealed that, besides this sequence, another sequence in approx. equimolar yield was present (Fig. 2). That sequence included the last 12 residues of the presequence of human α_2AP. These findings are in line with the recent observation that human pre-α_2AP cDNA expressed in hamster kidney cells gives rise to active α_2AP containing that 12-residue extension [20].

In contrast to the Ala^{-13}–Met site the Pro^{-1}–Asn site of human pre-α_2AP fits poorly to the "−3 to −1 rule" [21], and it might be questioned whether the latter se-

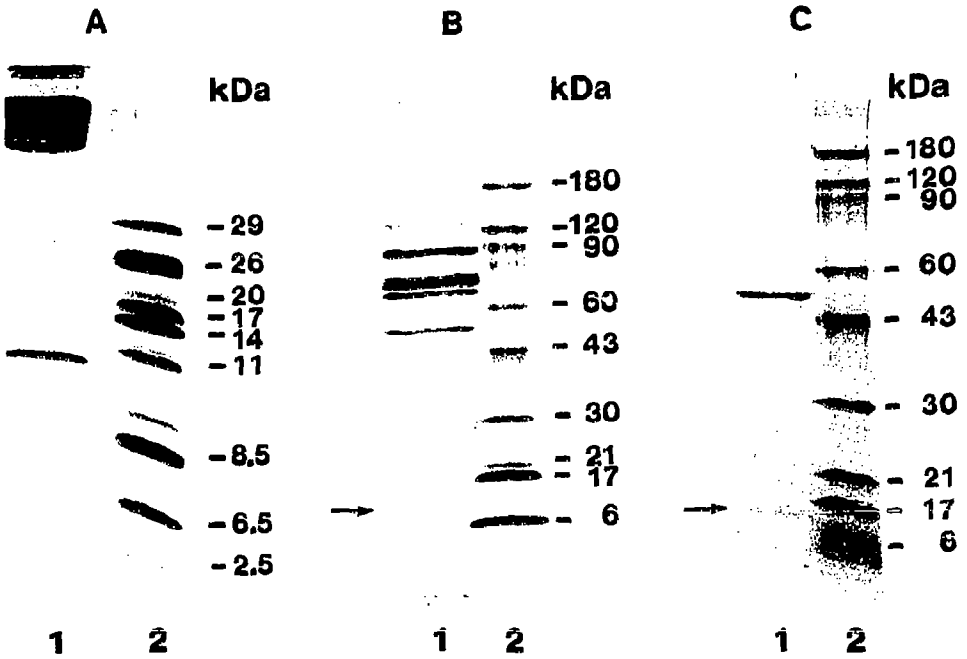

Fig. 3. SDS-PAGE of cleaved preparations of bovine α_2AP. (A) lane 1, size markers; lane 2, cleaved bovine-α_2AP preparation. (B) lane 1, plasmin-bovine α_2AP complexes, plasmin (different forms), cleaved α_2AP and cleavage peptide; lane 2, size markers. (C) Lane 1 subtilisin cleaved bovine α_2AP and peptide; lane 2, size markers. Arrows indicate the C-terminal peptides resulting from cleavage.

```
C-α2AP1                                          MS-RMSLSSFIVNRP
C-α2AP2                                            MSLSSFIVNRP
C-α2AP3                                            SLSSFIVNRP
T52                    LVVSSVQHQSALELSEAGVQAAAATSTA
T58SP1                 LVVSSVQHQSALE
T58SP2                              LSEAGVQAAAATSTAMS-R

Bovine α2AP            LVVSSVQHQSALELSEAGVQAAAATSTAMS-RMSLSSFIVNRP
                       :::: ::::: ::::: :: :::::: ::: :::::::: ::::
Human α2AP             LVVSGVQHQSTLELSEVGVEAAAATSIAMS-RMSLSSFSVNRP
P and P' sites                        i8  i0  s  i1'  s'  i0'
```

Fig. 4. Reactive site sequence of bovine α_2AP. C-α_2AP^1, C-α_2AP^2, C-α_2AP^3, C-terminal peptides from α_2AP cleaved with an elastase-like enzyme, plasmin and subtilisin, respectively. T52 and T58, see Fig. 5. T58SP1 and T58SP2, peptides from *S. aureus* V8 proteinase digestion of T58. Bovine and human α_2AP are aligned according to [3] which introduces a gap between P_1 and P_2 in α_2AP. Identical residues in bovine and human α_2AP are shown by a pair of dots; residues different in bovine α_2AP are shown in bold. The P and P' sites are numbered.

quence represents a site recognized by the processing enzyme. One possible explanation is that processing initially occurs at Ala^{-13}–Met, and that the Pro^{-1}–Asn peptide bond is unstable and slowly cleaved by solvent.

The change of the Ala^{-13}–Met and Pro^{-1}–Asn sites in human α_2AP to Gln–Met and Pro–Ala, respectively, in bovine α_2AP, is compatible with that protein having a single N-terminal extended by 18 residues when compared with human α_2AP.

3.3. *Reactive site sequence*

Unlike human α_2AP [13] the preparation of bovine α_2AP did not convert to forms of smaller size upon storage. However, one preparation was inactive, due to proteolysis in the reactive site loop as also seen with other serpins [22,23]. Sequence analysis of a 10 kDa peptide present in that preparation (Fig. 3) suggested that it resulted from cleavage of bovine α_2AP by an elastase-like enzyme, that bovine α_2AP had a similar reactive site (Arg^{364}–Met), and that the 10 kDa peptide originated from cleavage 3 residues upstream of this site (Fig. 4). Sequence analysis of the 10 kDa peptide generated by incubation of bovine α_2AP with bovine plasmin confirmed that the reactive site of bovine α_2AP was identical to that of human α_2AP. Sequence analysis of the peptide generated by incubation of bovine α_2AP with subtilisin revealed that cleavage had occurred at the P_1' Met residue.

To obtain sequence information covering the N-terminal part of the reactive site of bovine α_2AP, tryptic peptides were analyzed (Fig. 5). Since the sequence analysis had indicated high similarity of the reactive site sequences of bovine and human α_2AP the amino acid composition of all HPLC fractions were determined. In human α_2AP the corresponding tryptic peptide would be 37 residues long and contain 13% Ala residues. On the basis of a high content of Ala residues two 31-residue peptides, T52 and T58, were selected (Fig. 5). Except for the lower content of methionine in T52 their composition were identical. Sequence analysis of T52 (28 residues determined) revealed a strong similarity with residues 373–414 of the reactive site loop of human α_2AP (Fig. 4). By determining the sequences of peptides obtained from T58 by digestion with *S. aureus* V8 proteinase (T58SP1 and 2, separation not shown) the complete sequence of a 42 residue segment of bovine α_2AP containing its reactive site loop was established (Fig. 4).

Among serpins and other serine proteinase inhibitors the reactive site sequences of orthologous proteins show

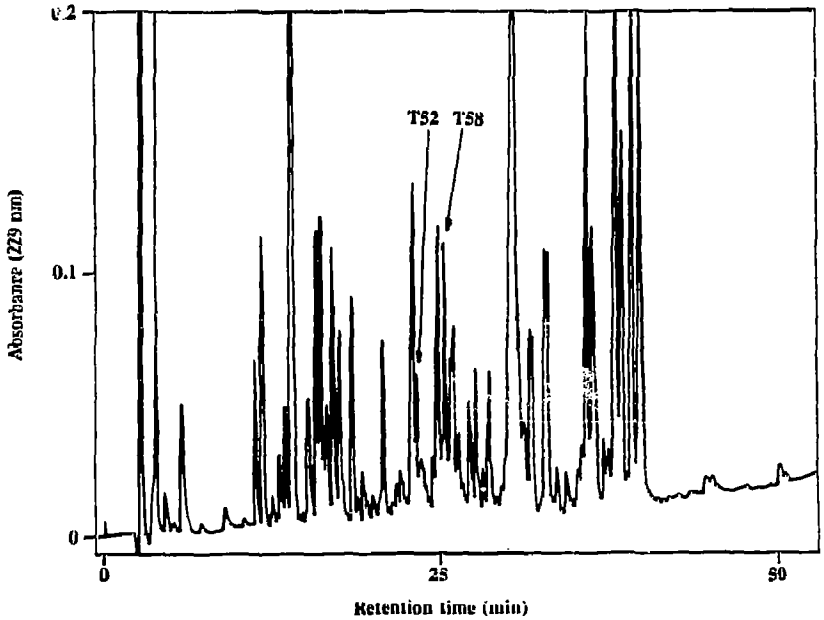

Fig. 5. HPLC chromatogram of tryptic peptides from bovine α_2AP. T52 and T58 were used for sequence analysis. The two Met residues in T52 were probably oxidised to the sulfoxide form.

the greatest variation [24,25]. However, it is evident from the present work that for α_2AP both the N-terminal sequences and the reactive site sequences of the bovine and human proteins are strongly conserved.

A recent survey of serpin sequences [26] has pointed out the presence in functionally active inhibitors of a consensus sequence, Thr-Glu-Ala-Ala-Ala, corresponding to the P_{14}–P_{10} sites. This sequence is believed to be important for insertion of part of the reactive site loop into the extended A-sheet [3,26], thereby changing the loop from being a substrate to an inhibitor of proteinases. Of 20 proteins examined [3] only C1-inhibitor, corticosteroid binding globulin and human α_2AP have a Val-residue in P_{14}. With regard to P_{13}, heparin cofactor II and angiotensinogen have a Gln residue in this position; however, neither corticosteroid binding globulin or angiotensinogen are active as inhibitors. Bovine α_2AP is a functionally active inhibitor, even though it contains two changes in the 5-residue consensus sequence (a Gln residue in P_{13} and a Val residue in P_{14}).

Acknowledgements: We thank Tove Wiegers for excellent technical assistance. This work was supported by The Danish Ministry of Agriculture and The Danish Dairy Board.

REFERENCES

[1] Travis, J., Guzdek, A., Potempa, J. and Watorek, W. (1990) Biol. Chem. Hoppe-Seyler 371, suppl. 3–11.
[2] Travis, J. and Salvesen, G. (1983) Annu. Rev. Biochemistry 83, 655–709.
[3] Huber, R. and Carrell, R.W. (1989) Biochemistry 28, 8951–8966.
[4] Wiman, B. and Collen, D. (1977) Eur. J. Biochem. 78, 19–26.
[5] Holmes, W.E., Nelles, L., Lijnen, H.R. and Collen, D. (1987) Thrombos. Haemostas. 48, 311–314.
[6] Hirosawa, S., Nakamura, Y., Miura, O., Sumi, Y. and Aoki, N. (1988) Proc. Natl. Acad. Sci. USA 85, 6836–6840.
[7] Sasaki, T., Morita, T. and Iwanaga, S. (1986) J. Biochem. (Tokyo) 99, 1699–1705.
[8] Hortin, G.L., Trimpe, B.L. and Fok, K.F. (1989) Thrombos. Res. 54, 621–632.
[9] March, S.C., Parikh, I. and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149–152.
[10] Deutsch, D.G. and Mertz, E.T. (1970) Science 170, 1095–1096.
[11] Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T.E. and Magnusson, S. (1977) Progr. Chem. Fibrinol. Thrombol. 3, 191–209.
[12] Wiman, B. (1980) Biochem. J. 191, 229–232.
[13] Clemmensen, I., Thorsen, S., Müllertz, S. and Petersen, L.C. (1981) Eur. J. Biochem. 120, 105–112.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[16] Barkholt, V. and Jensen, A.L. (1989) Anal. Biochem. 177, 318–322.
[17] Matsudaira, P.J. (1987) J. Biol. Chem. 262, 10035–10038.
[18] Eaton, D.L., Malloy, B.E., Tsai, S.P., Henzel, W. and Drayna, D. (1991) J. Biol. Chem. 266, 21833–21838.
[19] Marchalonis, J.J. and Weltman, J.K. (1971) Comp. Biochem. Physiol. 38B, 609–625.
[20] Sumi, Y., Ichikawa, Y., Nakamura, Y., Miura, O. and Aoki, N. (1989) J. Biochem. (Tokyo) 106, 703–707.
[21] von Heijne, G. (1984) J. Mol. Biol. 173, 243–251.
[22] Mast, A.E., Enghild, J.J., Pizzo, S.V. and Salvesen, G. (1991) Biochemistry 30, 1723–1730.
[23] Mast, A.E., Enghild, J.J. and Salvesen, T. (1992) Biochemistry 31, 2720–2728.
[24] Hill, R.E. and Hastie, N.D. (1987) Nature 326, 96–99.
[25] Laskowski Jr., M., Kato, I., Kohr, W.J., Park, S.J., Tashiro, M. and Whatley, H.E. (1987) Cold Spring Harbor Symposia on Quantitative Biology 52, 545–553.
[26] Carrell, R.W., Ewans, D.L. and Stein, P.E. (1991) Nature 353, 576–578.