A novel Ser *O*-glucuronidation in acidic proline-rich proteins identified by tandem mass spectrometry

Andreas P. Jonsson[a], William J. Griffiths[a], Per Bratt[b], Ingegerd Johansson[b], Nicklas Strömberg[b], Hans Jörnvall[a], Tomas Bergman[a,*]

[a] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden*
[b] *Department of Cariology, Umeå University, SE-901 87 Umeå, Sweden*

Received 15 May 2000

Edited by Pierre Jolles

Abstract Human acidic proline-rich salivary protein PRP-1 and its C-terminally truncated form PRP-3 were analyzed by electrospray tandem mass spectrometry. Post-translational modifications were detected and characterized. A pyroglutamic acid residue was demonstrated at the N-terminus, Ser-8 and Ser-22 were shown to be phosphorylated and an *O*-linked glucuronic acid conjugation was identified. The latter modification was located to Ser-17 and found to be present in approximately 40% of the polypeptides.

Key words: Tandem mass spectrometry; Electrospray; Acidic proline-rich protein; Post-translational modification; Phosphorylation; Glucuronidation

1. Introduction

Acidic proline-rich proteins (PRPs) are the predominant proteins in saliva. They are polymorphic with multiple functions in biofilm formation and innate immunity [1,2]. PRP-1 consists of 150 residues (Fig. 1) and PRP-3 is its C-terminally truncated 106 residue form [3–5]. The 30 residue N-terminal domain of acidic PRPs contains negatively charged amino acids and phosphorylations at Ser-8 and Ser-22 [3–5]. The N-terminal region binds calcium, mediates adsorption to hydroxyapatite and regulates calcium-phosphate homeostasis [1,2]. The C-terminal ProGln-sequence of PRP-1 mediates adhesion to commensal *Actinomyces* and *Streptococcus* species [6]. The Pro-rich middle domain promotes binding to and inactivation of ingested plant polyphenols (tannines) [1,2].

After secretion, the acidic PRPs are rapidly enriched on tooth surfaces and degraded as a consequence of bacterial proteolysis [7]. Recently, cleavage of acidic PRPs by a prolyl endoprotease activity of commensal *Streptococcus* and *Actinomyces* species was suggested to release an ArgGlyArgProGln pentapeptide that counteracts lactate production and desorbs bound bacteria [8].

Secreted proteins commonly contain *N*- and *O*-linked saccharides [9]. The human acidic PRPs are considered non-glycosylated proteins [5], although possible glycosylation has been noted [10]. Furthermore, acidic PRPs from rat saliva stimulated by β-adrenergic agonists are glycosylated, predominantly by glucuronic acid [11].

The aim of the present work was to investigate post-translational modifications in human acidic PRPs. To this end, we used nano-electrospray (nano-ES) tandem mass spectrometry with a hybrid quadrupole time-of-flight (QTOF) instrument.

2. Materials and methods

2.1. Isolation and tryptic digestion of PRP-1 and PRP-3

PRP-1 and PRP-3 were isolated from parotid saliva from three subjects (two normal and one with psoriasis and asthma on intermittent medication with Bricanyl, a β_2-adrenergic agonist, Draco). Parotid saliva (50 ml), collected by Lashley cups under mild acidic stimulation, was diluted (1:1, v/v) with 25 mM NaCl in 50 mM Tris–HCl, pH 8.0, and separated by DEAE–Sephacel chromatography (15×1.6 cm, Pharmacia) using a linear gradient of 25 to 1000 mM NaCl in 50 mM Tris–HCl, pH 8.0. The acidic PRP fraction was concentrated (Centriprep 10 concentrator, Amicon) and subjected to gel filtration (HiLoad 26/60 Superdex S-200 Prep grade, Pharmacia) in 20 mM Tris–HCl, pH 8.0, containing 500 mM NaCl. The PRP-1 and PRP-3 fractions collected were dialyzed against 50 mM Tris–HCl, pH 8.0 (Spectra/Pore membrane No. 4, Spectrum Medical Industries) and further purified on a Macroprep high Q column (15×1.6 cm, Bio-Rad) using a linear gradient of 25 to 1000 mM NaCl in 50 mM Tris–HCl, pH 8.0. The pure proteins were extensively dialyzed against water, lyophilized and stored at −20°C.

Purified PRP-1 and PRP-3 were digested with modified trypsin (Promega) at an enzyme-to-substrate ratio of 1:10 (w/w) in 0.1 M ammonium bicarbonate for 4 h at 37°C. The reaction was quenched by addition of a few μl of 30% formic acid.

An aliquot of the tryptic digest was separated by HPLC using an ÄKTA explorer system (Pharmacia Biotech) equipped with a Hypersil Peptide C_{18} column (4.6×100 mm) (Pharmacia Biotech). A linear gradient of CH_3CN (0–67%, 60 min) in 0.1% aqueous trifluoroacetic acid (TFA) was employed at a flow rate of 1.2 ml/min (detection at 215 nm). The remainder of the digest was available for mass spectrometric analysis. Before mass spectrometry, the tryptic peptide solution

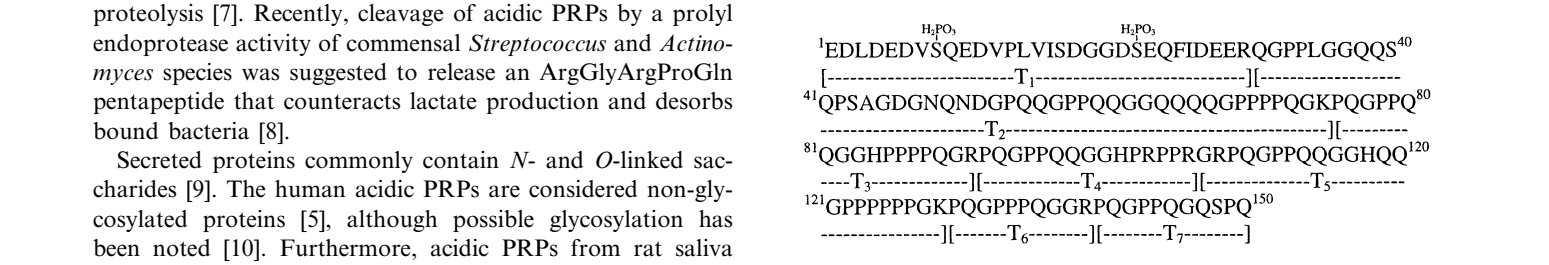

Fig. 1. Polypeptide structure of PRP-1 and PRP-3 [3–5]. PRP-3 is a C-terminally truncated form that corresponds to the first 106 residues. Tryptic peptides of PRP-1 are indicated (T_1–T_7) and Ser phosphorylation sites are shown (H_2PO_3). The glutamic acid residue at the N-terminus was cyclized to form a pyrrolidonecarboxylic acid (pyroglutamic acid).

*Corresponding author. Fax: (46)-8-33 74 62.
E-mail: tomas.bergman@mbb.ki.se

PII: S0014-5793(00)01645-8

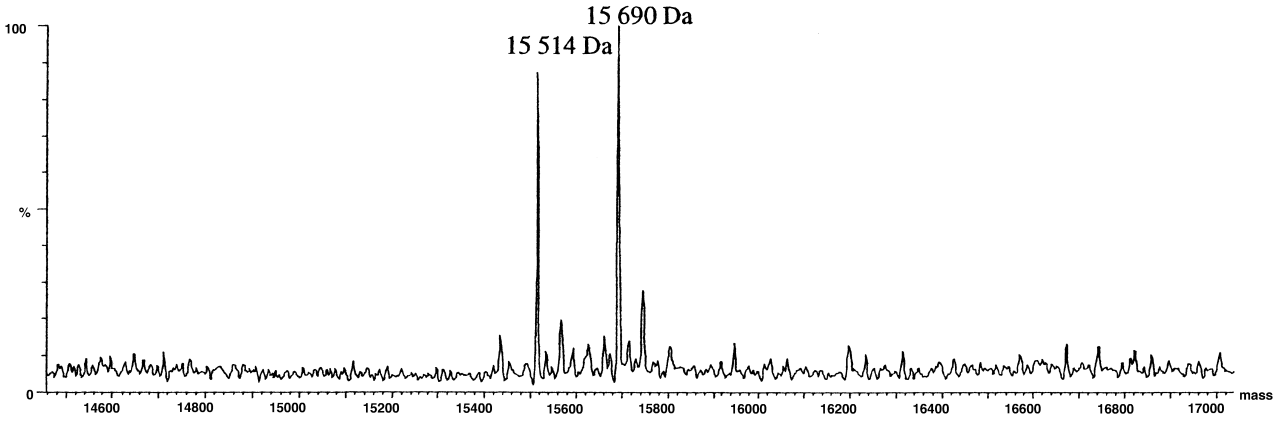

Fig. 2. Maximum entropy derived mass spectrum showing the two forms of PRP-1 that differ by 176 Da.

was diluted with 50% aqueous methanol to give an approximate concentration of 1 pmol/μl.

2.2. Mass spectrometry

Nano-ES mass spectra were recorded using a QTOF tandem mass spectrometer [12] (Micromass). The instrument was equipped with an orthogonal sampling ES-interface (Z-spray, Micromass). Gold coated nano-ES needles (Protana) were used and manually opened on the stage of a light microscope to give a spraying orifice of about 5 μm. This resulted in a flow of approximately 20–50 nl/min when a capillary voltage of about 1.3 kV was applied. Desolvation was facilitated by a nitrogen counter-current drying gas. The cone voltage was set at 40 V. For the acquisition of collision-induced dissociation (CID) spectra, the collision energy was optimized in the range 60–120 eV. Argon was used as the collision gas.

When necessary, contaminants and salts were removed from the samples by perfusion chromatography using micro-columns (200 nl bed volume) packed with POROS R3 (PE-Biosystems). Normally, 5 μl of sample was applied to the resin followed by washing with 30 μl 0.1% TFA and elution directly into the nano-spray needle with 60% CH_3CN containing 1% acetic acid.

3. Results

The deconvoluted ES mass spectrum of PRP-1 shows two major peaks at 15 514 Da and 15 690 Da, a mass separation of 176 Da (Fig. 2). Similarly, the mass spectrum of PRP-3 revealed two peaks, at 11 161 Da and 11 337 Da, also a mass separation of 176 Da. The 176 Da mass difference is diagnostic for a hexuronic acid conjugating group which means glucuronic acid or an isomer to glucuronic acid (e.g. galacturonic acid). The lower mass peaks correspond to PRP-1 and PRP-3 with an N-terminal pyroglutamic acid and phosphorylations of Ser-8 and Ser-22 [3–5].

Tryptic digestion of PRP-1 generated eight peptides of which six corresponded to residues 31–150 (T_2–T_7) (Figs. 1 and 3). The other two peptides both corresponded to residues 1–30 i.e. T_1 and T_1+176 Da. Tryptic digestion of PRP-3 generated five peptides, three of which covered residues 31–106. Similarly, the two peptides corresponding to residues 1–30 had a mass difference of 176 Da. The five tryptic peptides observed in the spectrum of PRP-3 were identical to their counterparts found in the spectrum of PRP-1, compatible with the fact that PRP-3 is a C-terminally truncated form of PRP-1.

The proportions of the two forms of N-terminal tryptic peptide in the digest of PRP-1 were determined by reverse phase HPLC (Fig. 4). After collection and mass spectrometric analysis, the early eluting component was found to represent the higher mass peptide (triply charged ion at monoisotopic *m/z* 1233.1 (T_1+176 Da), corresponding to a mass of 3696 Da) while the later eluting component was 176 Da lighter (triply charged ion at monoisotopic *m/z* 1174.4 (T_1), corresponding to a mass of 3520 Da). The heavy (+176 Da) and the light form of the PRP-1 N-terminal fragment were present in the

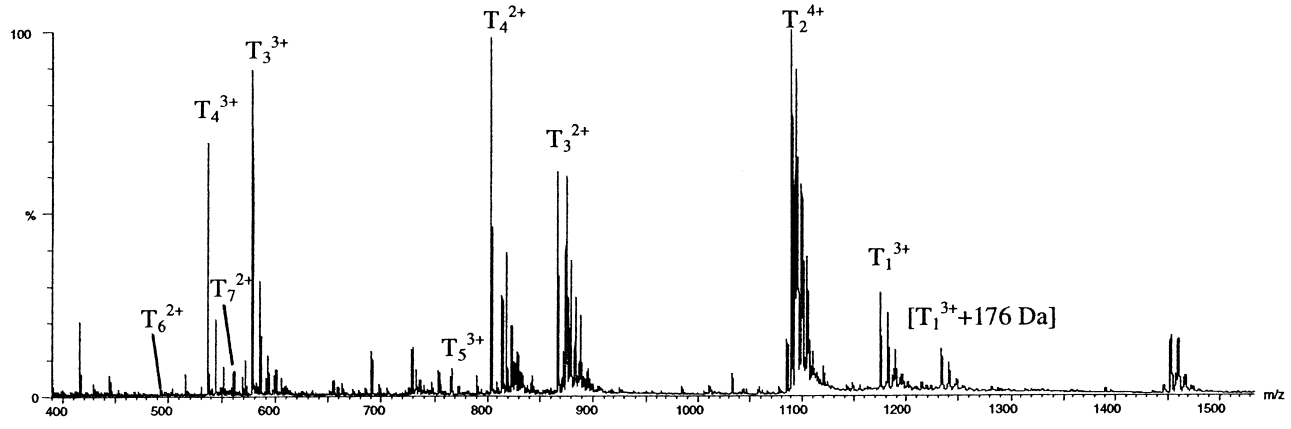

Fig. 3. Nano-ES mass spectrum of the complete tryptic digest of PRP-1. The proteolytic peptides are indicated T_1–T_7 and are also labeled with their respective charge.

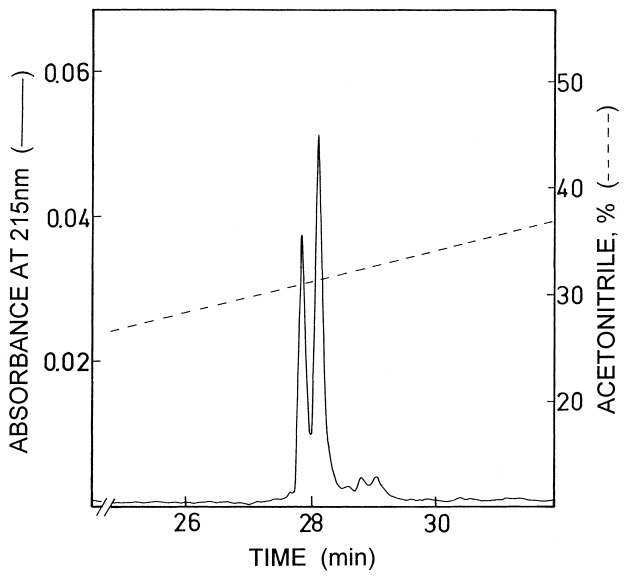

Fig. 4. Reverse phase HPLC of the two N-terminal tryptic peptides of PRP-1 (section of chromatogram). The early eluting component corresponds to the heavier N-terminal tryptic fragment (3696 Da) whereas the later eluting component corresponds to the lighter form (3520 Da). The proportions of the peak areas are 42 and 58%, respectively.

proportions 42 and 58%, respectively (Fig. 4). The proportion shifted in favor of the lighter form (3520 Da) under storage in acidic solution (pH 2–3), indicating loss of 176 Da.

The two N-terminal tryptic peptides of PRP-1, triply charged at *m/z* 1174.4 and 1233.1, were further analyzed by CID (Fig. 5). The product–ion spectrum of the lighter precursor, triply charged at *m/z* 1174.4, showed serine residues 8 and 22 to be phosphorylated by the y-ion series following y_9^+ and y_{23}^{2+} (Fig. 5A) [13,14]. The y_9^+ and succeeding y^+-ions were 80 Th heavier than those which would be obtained in the absence of phosphorylation at Ser-22. From y_{23}^{2+} and above, the y^{2+}-ions were 80 Th heavier (i.e. 160 Da) than in an equivalent non-modified peptide, showing Ser-8 to also be phosphorylated. A series of b_3^+–b_7^+ ions was observed, indicating that the N-terminal residue was pyroglutamic acid. All higher b-ions were consistent with this finding. The b-ion series was compatible with Ser-8 being phosphorylated but did not extend to residue 22. To confirm that the serine residues were indeed phosphorylated as opposed to being sulfated, the doubly deprotonated peptide was analyzed in the negative ion mode. The CID spectrum (Fig. 5A, inset) showed a peak at *m/z* 78.9 characteristic of the PO_3-group. The CID spectrum of the heavier N-terminal peptide, triply charged at *m/z* 1233.1, showed b- and y-type ions that were identical to those of the lighter form in the low *m/z* range (Fig. 5B). The additional modification by glucuronic acid was found to be located to Ser-17 by a shift of 53 Da in the y-ion series following y_{14}^{2+}.

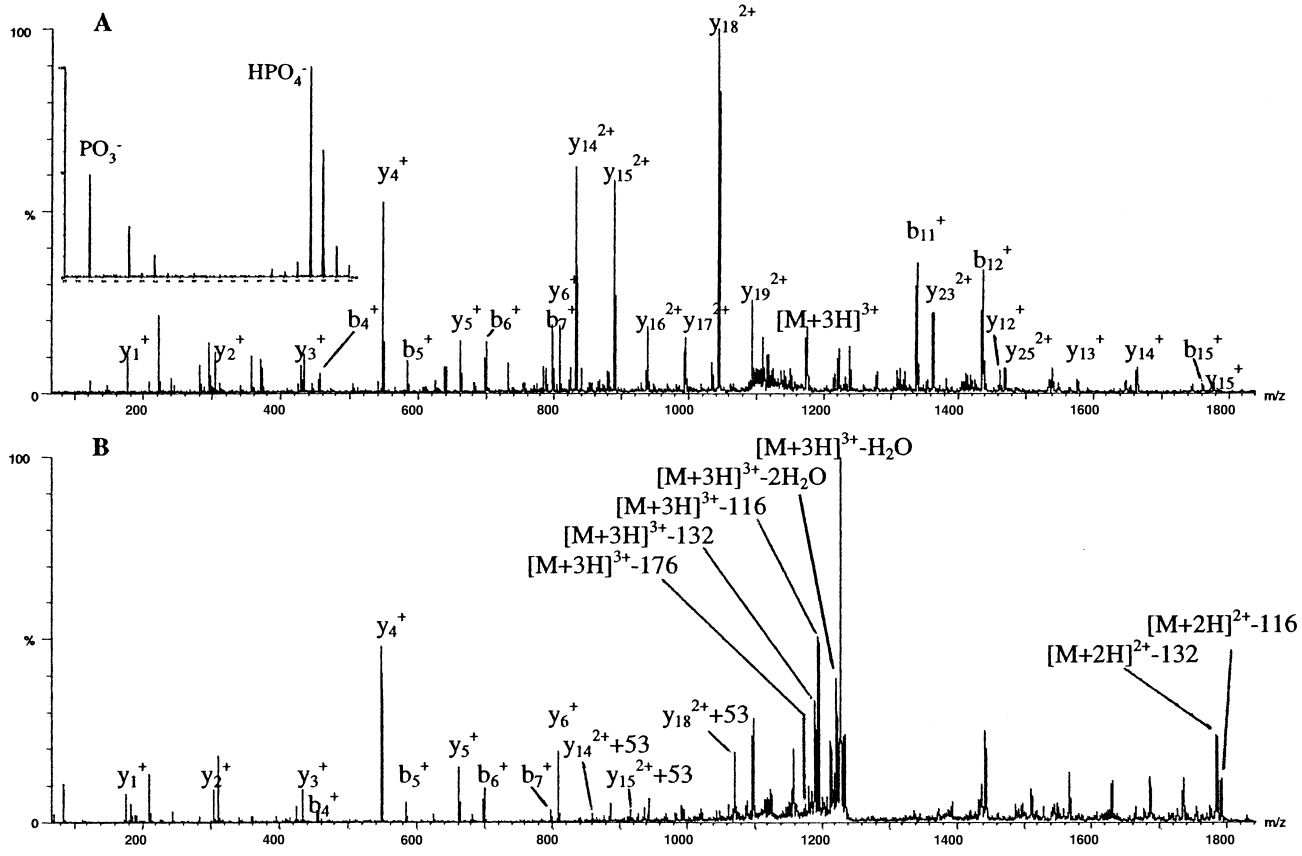

Fig. 5. CID spectra of the two N-terminal peptides generated via tryptic cleavage of PRP-1. A: The CID spectrum of the lighter N-terminal fragment (T_1), triply charged at monoisotopic *m/z* 1174.4. B: The CID spectrum of the heavier N-terminal fragment (T_1+176 Da), triply charged at monoisotopic *m/z* 1233.1. The inset in A shows the CID spectrum of the doubly deprotonated fragment T_1 analyzed in the negative ion mode.

The 53 Da shift corresponds to a cleavage after the 3rd and the 5th carbon atoms in glucuronic acid with concomitant loss of two water molecules ($^{3,5}X_1-2H_2O$) [15]. Further evidence for a glucuronic acid conjugation are the characteristic mass losses from the precursor ion that are indicated in Fig. 5B.

4. Discussion

We present mass spectrometric evidence for a novel post-translational modification of acidic PRPs by an *O*-linked hexuronic acid (i.e. glucuronic acid or an isomer) at Ser-17 in addition to an N-terminal pyroglutamic acid residue and phosphorylations at Ser-8 and Ser-22. The N-terminal pyroglutamic acid and the phosphorylations are known in acidic PRPs [5] and may contribute to their innate immunity properties [1,2]. However, to our knowledge this is the first structural evidence for a glycosylation of human acidic PRPs and for an *O*-glucuronidation of a serine residue.

The present findings indicate that about 40% of PRP-1 and PRP-3 carry a glucuronic acid residue *O*-linked to Ser-17. The mass difference of 176 Da of the two forms of PRP-1 and PRP-3 is indicative for glucuronic acid conjugation. Ions diagnostic for this modification and its location to Ser-17 were also present in the CID spectra. Furthermore, the loss of the 176 Da modification in acidic environment is consistent with the behavior of carboxylic acid sugars [9]. Glucuronic acid is commonly found in human proteoglycans [9]. It has also been reported in acidic PRPs from rats treated with a β-adrenergic agonist (isoproterenol) [11] but has not been studied in normal rats or localized to the position now found in human PRP. However, neither glucuronic nor hexuronic acid *O*-linked to Ser have so far been demonstrated in human glycoproteins [9].

Drugs, such as β_2-adrenergic agonists, are metabolized by glucuronidation [16], and β-adrenergic agonists (isoproterenol) enhance rat salivary gland PRP [17] and parotid proteoglycan synthesis [18]. One of the three saliva donors suffered from psoriasis and asthma which are diseases associated with altered glycosylation patterns [19,20]. This subject was also medicated with a β_2-adrenergic agonist intermittently. Such individual differences or a post-translational modification that, similar to truncation of PRP-1, affects only a subset of molecules may explain the occurrence of *O*-glucuronidation in about 40% of the acidic PRPs. Functionally, *O*-glycosylation of the N-terminal domain of acidic PRPs may interfere with its Ca-binding and hydroxyapatite adsorption properties or introduce properties typical of glycoproteins such as resistance to proteolysis and ligand binding [9].

Acknowledgements: This work was supported by Grants from the Swedish Medical Research Council (projects 03X-3532, 03X-10832, 03X-12551, 03X-10906), the Swedish Cancer Society (project 1806), the Foundation for Strategic Research (Cell Factory), the European Commission (BIO4-CT97-2123), the Emil and Wera Cornell Foundation and the GLIBS (Glycoconjugates in Biological Systems) program.

References

[1] Lamkin, M.S. and Oppenheim, F.G. (1993) Crit. Rev. Oral Biol. Med. 4, 251–259.
[2] Hay, D.I., Moreno, E.C. (1989) in: Human Saliva: Clinical Chemistry and Microbiology (Tenovuo, J., Ed.), vol. I, pp. 131–150, CRC, Boca Raton, FL.
[3] Bennick, A. (1975) Biochem. J. 145, 557–567.
[4] Wong, R.S.C., Hofmann, T. and Bennick, A. (1979) J. Biol. Chem. 254, 4800–4808.
[5] Hay, D.I., Bennick, A., Schlesinger, D.H., Minaguchi, K., Madapallimattam, G. and Schluckebier, S.K. (1988) Biochem. J. 255, 15–21.
[6] Li, T., Johansson, I., Hay, D.I. and Strömberg, N. (1999) Infect. Immun. 67, 2053–2059.
[7] Madapallimattam, G. and Bennick, A. (1990) Biochem. J. 270, 297–304.
[8] Li, T., Bratt, P., Jonsson, A.P., Ryberg, M., Johansson, I., Griffiths, W.J., Bergman, T. and Strömberg, N. (2000) Infect. Immun., in press.
[9] Gabius, H-J., Gabius, S. (1997) Glycosciences. Status and perspectives, Chapman and Hall, Weinheim.
[10] Strömberg, N., Borén, T., Carlén, A. and Olsson, J. (1992) Infect. Immun. 60, 3278–3286.
[11] Bedi, G.S. and Bedi, S.K. (1995) Prep. Biochem. 25, 119–132.
[12] Morris, H.R., Paxton, T., Dell, A., Langhorne, J., Berg, M., Bordoli, R.S., Hoyes, J. and Bateman, R.H. (1996) Rapid Commun. Mass Spectrom. 10, 889–896.
[13] Roepstorff, P. and Fohlman, J. (1984) Biomed. Mass Spectrom. 11, 601.
[14] Biemann, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99.
[15] Gillece-Castro, B.L. and Burlingame, A.L. (1990) Methods Enzymol. 193, 689–712.
[16] Zhang, M., Fawcett, J.P., Kennedy, J.M. and Shaw, J.P. (2000) Br. J. Clin. Pharmacol. 49, 152–157.
[17] Johnson, D.A. (1984) Arch. Oral Biol. 29, 215–221.
[18] Blair, E.A., Castle, A.M. and Castle, J.D. (1991) Am. J. Physiol. 261, C897–C905.
[19] Dabelsteen, E., Broby-Johansen, U., Jeppe-Jensen, D. and Mandel, U. (1990) APMIS 98, 221–228.
[20] Axford, J.S. (1999) Biochim. Biophys. Acta 1455, 219–229.