Different glycan structures in prostate-specific antigen from prostate cancer sera in relation to seminal plasma PSA

Gloria Tabarés3,4, Catherine M. Radcliffe5, Silvia Barrabés3, Manel Ramírez4, R. Núria Aleixandre4, Wolfgang Hoesel6, Raymond A. Dwek5, Pauline M. Rudd1,5, Rosal Peracaula2,3, and Rafael de Llorens3

3Unitat de Bioquímica i Biologia Molecular, Departament de Biologia, Universitat de Girona, Campus de Montilivi s/n. 17071, Girona, Catalonia, Spain; 4Laboratori ICS Girona, Hospital Universitari Dr Josep Trueta, Avinguda de França s/n. 17007, Girona, Catalonia, Spain; 5Glycobiology Institute, Department of Biochemistry, Oxford University, Oxford OX1 3QU, UK; and 6Roche Diagnostics GmbH, Nonnenwanderstrasse 2, 82372 Penzberg, Germany

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Prostate-specific antigen (PSA), the tumor marker currently used for prostate cancer (PCa), is not specific enough to distinguish between PCa and benign prostate hyperplasia (BPH). Glycan processing is normally perturbed in tumors, therefore we investigated whether changes in glycosylation of PSA could be useful diagnostic indicators. Previously we determined that the glycosylation of PSA secreted by the tumor prostate cell line LNCApD differs significantly from that of PSA from seminal plasma (normal control). We therefore undertook a detailed glycan analysis of PSA derived from sera from PCa patients, and, importantly, established that the glycosylation of the PSA serum PSA was significantly different from the PSA from the LNCAp cell line. In comparison with seminal plasma PSA, the fucose content of PSA from the PCa patient sera was significantly lower and there was a decrease in α2,3-linked sialic acid. Differences in the glycosylation of PSA derived from PCa patients’ sera, seminal plasma, and LNCAp cells were further established by lectin detection, glycosylation immunosorbent assay, and two-dimensional electrophoresis. We also investigated whether the impact of glycosylation changes initiated by the tumor was reflected in the serum glycome. By comparing the glycans released from the total glycoproteins in PCa patient sera with those of normal serum we found an increase in the proportion of sialyl-Lewis x structures. Further analysis of the glycosylation of PSA from PCa and BPH sera will be required in order to determine the utility of these glycan differences to discriminate specifically between benign and malignant prostate states.

Key words: lectins/N-glycosylation/prostate cancer/prostate-specific antigen/two-dimensional electrophoresis

Introduction

Prostate diseases

Prostate cancer (PCa) is the most common male cancer in Western countries and the second leading cause of cancer death. The prevalence of PCa is proportional to age (Moul et al., 2003). After initially localized growth, the tumor penetrates the capsule along perineurals and lymphatic channels to reach the periprostatic tissues. The first metastases appear in the local obdurate lymph nodes. Then, circulating tumor cells lodge in the skeleton, forming osteosclerotic metastases (Ward et al., 2001).

Prostate-specific antigen (PSA) is currently considered the best tumor marker available in clinical medicine for the purpose of diagnosing early prostate carcinoma (Diamandis, 1998). However, it is not fully specific for PCa, as other prostatic pathologies, like benign prostate hyperplasia (BPH), can show serum PSA elevations. Thus different approaches have recently been developed to improve PSA specificity, such as the PSA index—defined as the free PSA (fPSA) expressed as a percentage of total PSA (tPSA)—PSA density, PSA velocity, complex PSA to tPSA ratio, or different PSA forms (Mikolajczyk et al., 2000). However, when PSA levels are between 4 and 10 ng/mL, these methods still do not allow clear differentiation between PCa and other benign prostate diseases (Brawer, 1999). Another emerging issue is that of the rising incidence of PCa in the low PSA levels. Recent studies have reported a mean PCa incidence of 20.5% when the PSA levels were between 2.5 and 4 ng/mL (Djavan et al., 2004).

PSA

PSA is a 28,400 Da glycoprotein comprising 237 amino acid residues, with five interchain disulphide bonds and ~8% carbohydrate in the form of an N-linked oligosaccharide side chain at Asn45. It exhibits serine protease activity similar to chymotrypsin and belongs to the kallikrein family. PSA is the human kallikrein 3 (hK3) (Ward et al., 2001) produced primarily by the prostatic epithelial cells and is secreted into the seminal plasma where it is present at relatively large concentrations (0.5–3 mg/mL) (Diamandis, 1998). Significant barriers are present between the prostatic lumen and the capillary blood. Several of these protective layers may be disrupted during disease processes, such as PCa, prostatic intraepithelial neoplasia, BPH, and prostatitis (Brawer, 1999).

The predominant molecular PSA form present in blood is the 80–90-kDa complex of PSA with alpha-1-antichymotrypsin (ACT), and minor fractions of PSA exist as complexes with other protease inhibitors, predominantly α2-macroglobulin and inter-α-trypsin inhibitor. fPSA represents a small but
variable proportion of the tPSA concentration, containing different proPSA forms.

Glycosylation of PSA from seminal plasma has been shown to differ from the glycosylation of PSA secreted by the prostate metastatic tumor cell line LNCaP (established from a metastasis of lymph node (Horoszewicz et al., 1983)). PSA from seminal plasma was reported to possess sialylated biantennary complex oligosaccharide structures (Bélanger et al., 1995; Okada et al., 2001; Peracaula et al., 2003b; Ohyama et al., 2004). However, PSA from LNCaP cells has been shown to contain a mixture of neutral biantennary and triantennary oligosaccharides (Prakash and Robbins, 2000), but recently other authors reported the presence of sialic acid on PSA from LNCaP cell culture media detected by lectin assays (Ohyama et al., 2004). However, we have previously also shown by glycan sequencing that LNCaP PSA oligosaccharides were not sialylated and presented a higher fucose (Fuc) content in contrast to PSA from seminal plasma (Peracaula et al., 2003b).

The glycan differences reported between PSA from normal (seminal plasma) and tumor origins (LNCaP cells) prompted us to investigate the glycan structures of serum PSA from PCa patients to determine whether the carbohydrate structures were altered during malignant transformation. So far few reports have been published on the glycan structures of PSA from PCa patients’ sera (Barak et al., 1989; Huber et al., 1995; Sumi et al., 1999; Basu et al., 2003; Ohyama et al., 2004). This is very likely due to the low PSA content in human sera, which makes these studies difficult.

In this work we present different methods to concentrate and purify PSA from prostate cancer patients’ sera and characterize its carbohydrate structures by glycan sequencing of the released glycans using high performance liquid chromatography (HPLC) coupled with exoglycosidase digestions. This provides an analysis of both charged and neutral glycans including monosaccharide sequence and linkage information, which gives an accurate quantitative analysis of the different glycan populations. The glycosylation differences reported were further compared with the PSA glycans from seminal plasma and from LNCaP cells by using several approaches like lectin analysis, glycosylation immunosorbent assay (GISA), and 2D electrophoresis. Glycan sequencing was also performed on the total serum glycoproteins from a PCa patient and compared with those from control pooled serum.

Results

Purification and glycan sequencing of PSA from a PCa serum

The tPSA present in 35-mL prostate cancer patient serum (PCa A) was purified employing five chromatographic steps: two thiolphilic absorption chromatographies, one affinity chromatography to cibacron-blue, one with affinity to protein A, and one with affinity to heparin.

The first two chromatographic steps removed the main serum protein, albumin. The immunoglobulins present in human serum were removed by protein A chromatography. The other two chromatographic steps yielded partially pure tPSA (Figure 1). The different chromatographic steps were evaluated by silver staining sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and by western blotting with antibodies against tPSA. The results of the purification are shown in Figure 2.

Free PSA was released from the complex PSA–ACT by ethanalamine 2M treatment as it was described (Peter et al., 2000) and processed together with the free PSA already present in the serum sample to have more quantity of PSA and therefore to need less quantity of serum to proceed with the glycosylation analysis (Peter et al., 1999). A specific immunosorption with antibodies against free PSA allowed us to obtain pure free PSA (Figure 2c), which was then characterized by glycan sequencing.

All the purification steps use mild conditions that do not affect the integrity of PSA molecule and its glycosylation, as it has been checked by enzyme-linked immunosorbent assay (ELISA) and western blotting using PSA antibodies and lectins during the purification procedure. The last-step purification process has also been done for the PSA from seminal plasma and the results obtained for this sample have been the same as described before (Peracaula et al., 2003b).

Oligosaccharides from the purified PSA from PCa A serum and from seminal PSA were released by in-gel digestion with N-glycosidase F from Flavobacterium meningosepticum (PNGase F), fluorescently labelled with 2AB and subjected to normal phase HPLC and a series of exoglycosidase digestions (Figure 3).

The HPLC profile of the undigested seminal PSA together with the digestion profiles were compared with the profiles produced previously by us (Peracaula et al., 2003b). Despite the low quantities of ~300 femtomoles of glycans per profile, it was possible to assign glycan structures to the peaks. The undigested seminal plasma PSA shown in Figure 3A a, lower profile, shows the presence of biantennary (A2), galactosylated (G), mono- and disialylated glycans (S), some with N-acetylgalactosamine (GalNAc or GN on the figure), with and without core Fuc in very similar proportions to those seen previously (Peracaula et al., 2003b).

Some of the distinctive features between PSA from PCa patient serum, in relation to seminal plasma, could be attributed to differences in Fuc and GalNAc content and in the linkage of sialic acid. In PCa A glycans (Figure 3A a, c, and d, upper profiles), the fucosylated structures are greatly reduced, making up only 25–30% of the fucosylated glycans from the seminal PSA. Twenty to thirty percent of the glycan structures of PSA from PCa A serum were fucosylated versus 80–90% of the PSA glycans from seminal plasma. In PCa A glycans GalNAc structures were not detectable in the data presented in Figure 3A c and e, upper profiles, and in the Arthrobacter ureafaciens neuraminidase (AB+) bovine testes β-galactosidase (BTG) digestion (Figure 3A d, upper profile) the small peak eluting with a GU value corresponding to Fa2G1N1 was at the limits of detection and could not therefore be conclusively assigned. In seminal plasma PSA the GalNAc structures were clearly present (Figure 3A c, d, and e, lower profiles). PSA glycans from PCa A serum were mostly sialylated, as has been described for PSA from seminal plasma. There were no significant differences when comparing the mono- and disialylated structures between PCa A serum and seminal plasma PSA, the ratio of mono- and disialylated glycan structures being ~50:50 in both PSA.
samples. The digestions with *Streptococcus pneumoniae* neuraminidase (Nan1) (Figure 3A b), which removes only α2-3 linked sialic acid residues, and ABS (Figure 3A c), which cleaves both α2-3 and α2-6 linked sialic acid, clearly demonstrate the presence of both linkages in both samples. With PCa A there is ~15% α2-3 linked sialic acid and with the seminal plasma there is ~25% (Table I). Digestion with Jack bean β-N-acetyl-hexosaminidase (JBH), which removes both GlcNAc and GalNAc residues, trimmed both samples to the trimannosyl core (data not shown).

Comparison of the profiles of the serum glycans from PCa A and the control pooled serum (Figure 3B a and b) indicates that there is a difference in the percentage areas of the outlined peaks, 9% for the PSA serum and 6% for the control. Digestion with ABS, BTG, and *Xanthomonas manihotis* α1-2 fucosidase (XMF; Figure 3B c and d) confirmed the presence of the Lewis x antigen with an outer arm α1-3 Fuc and β1-4 galactose (Gal) linked to an N-acetyl glucosamine. The linkage of the Fuc was confirmed with α1-3,4 fucosidase and that of the Gal was confirmed with β1-4 galactosidase (data not shown). The serum from PCa A shows an increase to 6% for the Lewis x antigen from 3% in the control. There is also an increase in the oligomannose structures, particularly an increase to 2% of mannose9-N-acetyl glucosamine2 (M9) in the PCa A serum from <0.5% in the control.

**Methods for glycan characterization of PSA from PCa serum without previous purification**

The differences described above for PSA glycans from seminal plasma and a PCa patient serum could be useful for distinguishing between normal and tumor PSA. However, the amount of pure PSA needed for glycan sequencing requires large serum volumes and several purification steps. We therefore developed different methods to characterize and study PSA glycans from PCa patients’ sera avoiding these purification steps.
Glycan characterization of PSA was carried out using the lectins *Sambucus nigra* lectin (SNA) to detect α2,6-linked sialic acid, *Mackia amurensis* lectin (MAA) to detect α2,3-linked sialic acid, *Aleuria aurantia* lectin (AAL) for α-Fuc, and *Erithrina cristagalli* lectin (ECL) to detect the presence of the oligosaccharide Galβ1–4GlcNAc, without sialic acid bound to Gal.

**Immunological methods**

Before lectin characterization, sera were treated either by immunoprecipitation or by thiophilic absorption to increase the concentration of PSA in relation to the other serum proteins present.

Three immunosorbed PSA samples (from seminal plasma, from a PCa patient serum [PCa A], and secreted by the PCa cell line LNCaP) were compared by western blotting using SNA, AAL, and ECL lectins (Figure 4). The presence of α2,6 sialic acid, detected by SNA, was observed in PSA from both seminal plasma and PCa A. The lack of sialic acid detection in PSA from LNCaP medium corroborates...
the previous results on the glycan structures of PSA from this tumor cell line, which had shown only neutral structures (Peracaula et al., 2003b).

MAA lectin, which recognizes α2,3 sialic acid, did not yield a positive result with PSA from any sample, probably because of the fact that MAA shows only a strong binding

Table I. PSA glycans from PCa A and from seminal plasma

<table>
<thead>
<tr>
<th>Peak</th>
<th>Assignment</th>
<th>PCa A Digestions</th>
<th>Seminal plasma Digestions</th>
<th>Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GU</td>
<td>Undigested</td>
<td>Nan1</td>
</tr>
<tr>
<td>1</td>
<td>A2G2</td>
<td>7.19</td>
<td>84</td>
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</tr>
<tr>
<td>2</td>
<td>FcA2G1GN1</td>
<td>7.37</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>FcA2G2</td>
<td>7.56</td>
<td>6 16</td>
<td>7.58</td>
</tr>
<tr>
<td>4</td>
<td>A2G1GN1S1</td>
<td>7.83</td>
<td>6 3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>A2G2S1</td>
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<td>38 47</td>
<td>8.05</td>
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<td>FcA2G2S1</td>
<td>8.45</td>
<td>10 8</td>
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<tr>
<td>7</td>
<td>FcA2G1GN1S2 and A2G2S2</td>
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<td>48 35</td>
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</tr>
<tr>
<td>8</td>
<td>FcA2G2S2</td>
<td>9.3</td>
<td>4 5</td>
<td>9.32</td>
</tr>
</tbody>
</table>

See Figure 3 legend for key.

Fig. 4. Indirect immunosorption and western blotting results for PSA from seminal plasma, LNCaP cell culture medium, and a PCa A. Detection was done by the lectins SNA-Bi, AAL-Bi (biotinylated), ECL-Fl (Fl labelled), and by the rabbit polyclonal antibody anti-total PSA. 1, LNCaP media; 2, seminal plasma; and 3, PCa A.
PSA glycans from prostate cancer sera

when oligosaccharides contain more than a single recognition sequence (Knibbs et al., 1991).

The structure Galβ1–4GlcNAc, detected by ECL, was mainly present in PSA from LNCaP medium. This lectin gave a slight positive signal for PSA from PCa A, but it was negative for PSA from seminal plasma, also consistent with the glycan analysis. The reason why the ECL lectin detection was higher for PSA from a PCa patient serum than for PSA from seminal plasma could be due to two effects or a combination of them: (1) PSA from serum could carry less sialic acid than the one from seminal plasma and (2) PSA glycans from seminal plasma are known from the sequencing results to contain 25% GalNAc, whereas serum PSA carries only Gals, which are known to increase the sensitivity of the ECL detection (Rhodes and Milton, 1998).

The Fuc content, detected with AAL, was higher in PSA from LNCaP than in PSA from the other samples, which was in agreement with the previous sequencing study (Peracaula et al., 2003b), where it was reported that almost all the structures of LNCaP PSA had core Fuc and 10–15% of the structures had in addition outer-arm Fucs. However, no differences between PSA from a PCa A and seminal plasma could be detected using AAL although differences in core Fuc percentages were detected by glycan sequencing.

Sandwich ELISA, using fluorescein (Fl)-labelled ECL lectin (ECL–Fl) for detection revealed similar results to western blotting (Figure 5a). A strong positive reaction with ECL was observed for PSA from LNCaP medium and a low signal was seen with PSA from PCa A. A negative reaction was observed for PSA from seminal plasma.

The results using biotinylated SNA (SNA-Bi) in an ELISA capture assay were also consistent with those of the western blotting (Figure 5b). There were no significant differences observed between PSA from PCa A and seminal plasma with this lectin. When using AAL for capturing (Figure 5b), the sensitivity of the assay was not high enough to allow clear conclusions, although it seems that the Fuc content in PSA from LNCaP cell media is higher than for seminal plasma and PCa serum PSA, in agreement with western blot and sequencing results. 400 ng of PSA were necessary for ECL–Fl glycan detection, whereas only 25 ng of PSA were needed for SNA-Bi glycan capture.

GISA

To complement the results of the lectin assays described above, PSA samples were also analyzed with a sialyltransferase assay, which is able to transfer fluorescently labelled sialic acids onto nonsialylated terminal positions of N-glycans. The amount of sialic acid transferred is mostly dependent on the amount of nonsialylated N-glycan chains present in a glycoprotein. It can be further influenced by the ratio of Gal versus GalNAc present because the former monosaccharide is better sialylated by the sialyltransferase (Harduin-Lepers et al., 2001).

More samples were also treated by thiophilic interaction chromatography or by indirect immunosorption to increase the PSA content.

Figure 6 shows that there was fluorescent sialic acid transferred to the PSA sample from LNCaP medium, which is expected because the sequencing results had shown that it is not sialylated (Peracaula et al., 2003b). In contrast, the amount of fluorescent sialic acid transferred to PSA from seminal plasma in the GISa was very low and not greater than with the control patient serum, whereas PSA from PCa A was sialylated to some extent. These results corroborate the ECL results described above.

Thiophilic absorption chromatography, which was used to enrich the PSA content of the serum sample, revealed two PSA peaks: both reacted with anti-tPSA but only one with anti-fPSA antibodies. Before the GISa assay both were treated with ethanolamine to release fPSA from the PSA–ACT complex. When comparing these two PSA forms by GISa, the sialylation potential for fPSA was greater than for the PSA released from the complex.

Two-dimensional electrophoresis followed by western blot

The presence of different protein isoforms, including some glycoforms, can also be revealed by two-dimensional electrophoresis. This method allows the detection of different isoforms separated by their isoelectric point (pI) and by their molecular weight. A decrease in pI may reflect an
increase in the sialic acid content of a glycoprotein, for example, because of the presence of more sialylated sugar structures (Packer et al., 1998).

First, PSA isoforms from purified seminal plasma were characterized with regard to the sialic acid linkage. To determine how the presence of sialic acid affects the pI, treatments with ABS which removes \( \alpha_2-6 \) and \( \alpha_2-3 \) linked sialic acid and Nan1 which removes only \( \alpha_2-3 \) sialic acid were performed. A full N-glycosylation release was also carried out using a combined ABS and PNGase F digestion, to compare the glycosylated and unglycosylated behaviour of PSA upon two-dimensional electrophoresis.

When treating seminal plasma PSA with Nan1 (Figure 7), the most acidic spots (pI 6.4 and 6.6) moved to more basic spots (pI 6.9 and 7.2). Thus, spots at pI 6.4 and 6.6 contain \( \alpha_2,3 \) sialic acid. ABS digested the PSA isoforms to two main spots at pI 7.2 and 7.4. These two PSA forms would not contain sialic acid. The completion of the ABS digestion was confirmed by a negative SNA lectin detection (data not shown). After N-glycan digestion by a combined ABS + PNGase F treatment the spots at pI 7.2 and 7.4 still remained indicating that the differences between the forms at pI 7.2 and 7.4 were not due to the N-glycosylation.

In summary, seminal plasma PSA contains two PSA isoforms at pI 7.2 and 7.4 that are not sialylated and three sialylated forms at lower pI, 6.4, 6.6, and 6.9 (Figure 7). The most intense band was at pI 6.9 (42%), followed by the spot at pI 7.2 (19%) when measuring the relative intensity of the different spots by using the Quantity-One software (BioRad, Hercules, CA).

Pretreated samples of PSA from LNCaP medium, seminal plasma, and PCa patients’ sera were compared by two-dimensional electrophoresis (Figure 8). PSA from LNCaP medium presented four PSA isoforms at pI 7.2, 7.4, 7.7, and 7.9. To check whether they corresponded to proPSA forms, they were probed with a monoclonal antibody against the \(-5\)proPSA and \(-7\)proPSA forms. The presence of these proPSA forms was observed in the more basic spots at pI 7.4, 7.7, and 7.9, and with higher intensity in the two last forms (data not shown).

Several PCa patients’ sera were analyzed. After pre-treating the serum samples with a thiophilic adsorption chromatography, the PSA detected by two-dimensional electrophoresis contained two main spots at pI 6.9 and 7.2 with intensities that range from 23 to 37% for the spot at pI 6.9 and 25 to 36% for the spot at pI 7.2, whereas in seminal plasma PSA the most intense spot at pI 6.9 was twice the intensity of that at pI 7.2. The 7.2 and 7.4 isoforms could be neutral forms. The 7.2 isoform in PSA from PCa patient’s sera had a higher spot intensity than that in seminal plasma PSA. The presence of spots at lower pI than 7.2 indicates that PSA from the different PCa patients’ sera contains sialic acid, as has been described previously for PCa A. Some basic PSA spots were detected in one of the PCa patients’ sera, which could correspond to proPSA isoforms.

A very recent study concluded that fPSA serum subforms separated by two-dimensional electrophoresis can be used to improve both sensitivity and specificity in PCa diagnostics compared to tPSA and fPSA to tPSA ratio (Jung et al., 2004).

Discussion

Only a few reports on the glycosylation of PSA from serum have been described so far and some are contradictory (Barak et al., 1989; Marrink et al., 1992; Pelt and van...
Dieijen-Visser, 1992; Huber et al., 1995; Sumi et al., 1999; Ornstein et al., 2000; Isono et al., 2002; Basu et al., 2003; Ohyama et al., 2004). Therefore studies on the glycosylation of PSA were undertaken to clarify conflicting results and, above all, to find whether glycan features could be useful for improving the diagnostic value of PSA. Examples have been reported where the glycosylation of other glycoproteins has been used for diagnostic purposes, like alpha-fetoprotein (AFP) for hepatocarcinoma (Poon et al., 2002) or human pancreatic ribonuclease, which has different oligosaccharide chains when produced by pancreatic tumor cells (Peracaula et al., 2003a). Because PSA produced by LNCaP tumor cells carries altered glycan structures compared to normal PSA (Peracaula et al., 2003b), it seems possible that these structures could also be present on the serum PSA of PCa patients.

Previous studies on serum PSA glycosylation

A previous study compared the PSA glycosylation between serum from PCa and BPH patients by affinity to the lectin concanavalin A (Con A) and reported significantly different PSA-Con A binding ratios for these two diseases (Barak et al., 1989). Other authors, following the same methodology, did not detect differences in the glycosylation pattern of PSA from benign and malignant prostate disease, and concluded that the PSA–Con A binding ratio could not be used to distinguish between benign and malignant prostate lesions (Marrink et al., 1992; Pelt and van Dieijen-Visser, 1992). Other recent work described that a low carbohydrate content (below 3 μg/mL) in the precipitate for the interaction between serum PSA–Con A indicated strong suspicion for PCa (Basu et al., 2003).

A serial lectin affinity chromatographic study compared the glycan binding of PSA from PCa and BPH tissues (Sumi et al., 1999). More multiantennary complex type glycans on PSA from PCa tissues were detected than in PSA from the BPH ones. A recent work analyzed the carbohydrate moiety of serum PSA from patients with PCa and BPH using lectin affinity chromatography and surface plasmon resonance analysis (Ohyama et al., 2004). It showed the presence of α2,3 sialic acid mainly on PSA from PCa patients’ sera and also suggested that differential binding of free serum PSA to MAA lectin between PCa and BPH patients could be a potential approach for diagnosing PCa.

By chromatofocusing, another group detected the presence of sialic acid in PSA samples from PCa and BPH. The PSA forms with more basic pI were from PCa patients and more acid pI isoforms were from BPH patients’ sera (Huber et al., 1995). Other works were in agreement with these results when detecting, by two-dimensional electrophoresis, that PSA isoforms of a BPH patient tissue were very similar to seminal plasma PSA, and PSA isoforms from LNCaP cell lysate were more basic and similar to serum PSA from PCa patients (Isono et al., 2002). Moreover by two-dimensional electrophoresis with prostate tissue samples, other authors observed that the PSA from PCa tissue presented more acid pI than PSA from LNCaP cell lysates (Ornstein et al., 2000).

To clarify whether PSA glycosylation could be different in PCa serum, we have first characterized the glycans of PSA from a high PSA content PCa serum by glycan sequencing. The results obtained with PSA purified from PCa patients’ sera were compared with the glycan structures of PSA from seminal plasma and from PSA secreted by LNCaP cells to medium.

After describing glycan differences between the normal and serum tumor PSA, we then investigated whether these differences could be detected in serum using several technologies, which have allowed the study of the glycan structures of several PCa sera. In addition, we have also analyzed the N-glycans of total serum glycoproteins from a PCa patient and have shown that glycan differences were present in comparison to normal situation.

Fuc and GalNAc content

Glycan analysis uses a high resolution HPLC technique which when combined with exoglycosidase digestions enables the segregation and quantification of structures...
including those which overlap in the undigested glycan pool. The glycans from PSA from PCa, for example, showed a decrease in core fucosylation when compared with the control seminal plasma PSA and also showed the presence of terminal N-acetyl galactosamine in the control which was absent in the PCa (Figure 3A c). The more fucosylated structures detected on PSA from seminal plasma, could not be demonstrated by AAL on the immunological assays due to the low lectin sensitivity (Figures 4 and 5b).

ECL strongly recognizes the Galβ1–4GlcNAc epitope and it is more abundant in PSA from a PCa patient serum than in PSA from seminal plasma, which presented more GalNAc in its terminal oligosaccharide structures. ECL detection by western blotting or ELISA, therefore, allowed PSA of PCa A serum to be distinguished from that of seminal plasma (Figure 5a).

Sialic acid content

Glycan sequencing results showed that most of the glycan structures of PSA from seminal plasma and from a PCa patient serum were sialylated. However, digestion with Nan1, specific for α2,3 sialic acid, showed a higher proportion of α2,3 sialic acid in PSA from seminal plasma than in a PCa patient serum (Figure 3A b). These differences could not be detected by the lectin MAA, which recognizes α2,3 sialic acid, probably because of the fact that MAA shows preference for binding oligosaccharides that contain more than a single sequence Neu5Acα2,3Galβ1,4GlcNAc (Knibbs et al., 1991), and in this case, PSA glycan structures with α2,3 linked sialic acid contained only one α2,3 sialic acid per molecule. α2,6 linked sialic acid was identified in both samples by SNA lectin, whereas there was no sialic acid detected in LNCaP secreted PSA (Figure 5b).

Increased sialylation of tumor cell surfaces is well known and is due to either increased activity of the sia lysyltransferases, like the α(2,6)-sialyltransferase, or due to the increased branching of N-linked carbohydrates leading to further termini which can be sialylated (Orntoft and Vestergaard, 1999). However, in this study slight differences were detected between sialic acid content of PSA from a PCa patient serum and PSA from seminal plasma when explored by the GISA assay (Figure 6), where the sialic acid content is slightly higher for normal PSA compared with PCa sera. The sialylation potential measured by GISA was higher in PSA from a PCa patient serum, than in PSA from seminal plasma, as was expected from the previous lectin and sequencing results. GISA assay required only 25–50 ng of PSA, so it could be used to characterize serum PSA glycosylation in sera containing low amounts of PSA.

The two-dimensional electrophoresis results are in agreement with the above data. The spots found by sialidase treatment to contain α2,3 sialic acid (pI 6.4 and 6.6) were not found in all PCa patients’ sera. In both samples (PCa sera and seminal plasma) the α2,6 sialic acid is the most abundant PSA isoform (Figure 8). The proportion of the 6.9 spot versus 7.2 spot was higher for seminal plasma PSA (ratio of 2.2) than for PCa patients’ sera (ratios between 0.9 and 1.5).

PSA from LNCaP did not contain acidic spots in agreement with the published sequencing results (Peracaula et al., 2003b). However, other authors have detected sialic acid in the PSA secreted by LNCaP cells (Ohyama et al., 2004). These observations could be explained by differences between cells within the same cancer cell line, as some authors have already described for the PCa cells from the PC-3 cell line (Liu, 2000), where the heterogeneity in the protein expression may be an inherent population property of these cells. In addition, LNCaP cell line used in different laboratories could have deviated from the parent cell line after many passages (Ohyama et al., 2004).

Importantly, the lack of similarity between glycosylation of PSA from the tumor cell line LNCaP and PSA found in sera from PCa patients indicates that cell lines may not adequately represent the physiological conditions.

ProPSA and nicked PSA detected isoforms

Basic PSA isoforms were detected in LNCaP secreted PSA and could be assigned to proPSA by using specific antibodies. These isoforms had pI of 7.4, 7.7, and 7.9 (Figure 8). These findings are in agreement with the description of other authors about the proPSA secreted by LNCaP cells (Herrala et al., 1998; Wu et al., 1998; Väisänen et al., 1999). Some of these isoforms were also found in some of the PCa patients’ sera analyzed. These basic PSA isoforms were mainly assigned to proPSA (~5 and ~7) isoforms, which correspond to propeptides with basic amino acids. Different authors have related the presence of proPSA forms in sera with a greater probability of the presence of PCa (Mikolajczyk et al., 2000, 2004; Peter et al., 2000).

In our two-dimensional electrophoresis assays, some cleaved or nicked PSA forms were detected at low molecular weight in seminal plasma and also in most of the PCa sera (Figure 8). These cleaved PSA forms are inactive and may represent zymogen forms that are present in seminal plasma (Charrier et al., 1999, 2001).

PSA glycosylation from a PCa patient has been shown to be more similar to seminal plasma PSA glycosylation than to glycans from PSA secreted by LNCaP cells. PSA in serum could come from the tumor and also from the normal tissue cells. So, this similarity between glycans from normal- (seminal plasma) and tumor- (PCa patients’ sera) derived PSA could be explained because of the tissue disruption process described during cancer (Brawer, 1999), which could result in some PSA from the normal tissue around the tumor being shed into the blood.

The findings presented in this study suggest a general approach to develop potential biomarkers for PCa. The PSA glycosylation differences demonstrated by lectin analyses, GISA, 2D electrophoresis, and particularly quantitative glycan analyses could be used to determine the level of serum PSA that has altered glycosylation and is cancer related. These methods could be used for diagnostic purposes when combined with the described pretreatment of serum samples, which allow increasing PSA concentration for the subsequent analyses. In addition, analysis of the glycans of whole serum provides a rapid method for detecting glycosylation changes from ~10 μL of serum that may be optimized to obtain a biomarker for PCa.

Further studies (in progress) on PSA glycosylation in sera from patients affected with PCa and BPH will reveal
whether there are differences that could be used to improve the specificity of PSA as a PCa tumor marker.

Materials and methods

PSA samples

PSA purified from human seminal plasma was purchased from Lee Scientific (St. Louis, MO). LNCaP cells (ATCC CRL-1740) were a generous gift from the Department of Urology (Hospital Universitario de Getafe, Madrid, Spain) and from the Catalán Institute of Cardiovascular Sciences (CID-CSIC, Barcelona, Spain). These cells were cultured in Dulbecco’s modified eagle medium (DMEM) (Gibco, Paisley, Scotland) with 10% foetal bovine serum (FBS) (Gibco), and the conditioned medium was collected. Secreted PSA was purified as described previously (Peracaula et al., 2003b).

Serum from a PCa patient with high tPSA content (1.8 μg/mL) (PCa A), seminal plasma from a healthy donor (~2 mg/mL free PSA), and a control serum from a healthy patient (without PSA) were from the Protein Chemistry Department of Roche Diagnostics (Penzberg, Germany). The other sera from PCa patients were from the Hospital Universitari Dr. J. Trueta (Girona, Catalonia, Spain), and its Ethics Committee approved their use. Patients were diagnosed with PCa with bone metastasis by biopsy and digital rectal examination by the Urology and Pathology departments. They presented bone metastasis and received a combined androgen blockade. Their tPSA concentrations were 640 ng/mL for PCa B, 650 ng/mL for PCa C, and 890 ng/mL for PCa D. Control pooled serum was from healthy donors from the discarded blood bank material.

Antibodies and lectins

The different antibodies against PSA used in this work were a biotinylated mouse monoclonal antibody anti-free PSA M-30 (Fab fragments), a biotinylated mouse monoclonal antibody anti-tPSA M-36 (Fab fragments), a horseradish peroxidase (HRP)-conjugated mouse monoclonal antibody anti-tPSA M-66 (all from Roche Diagnostics), and a rabbit polyclonal antibody anti-tPSA (Dako, Glostrup, Denmark).

Mouse monoclonal antibodies anti-proPSA forms ~5 and ~7 were from the Protein Chemistry Department of Roche Diagnostics.

The different HRP-conjugated antibodies used were antidigoxigenin (anti-Dig), anti-Fl (Roche Diagnostics), and anti-rabbit immunoglobulins (Pierce, Rockford, IL). To detect Bi, a complex of HRP and streptavidin was used (Roche Diagnostics).

The lectins were used were AAL from A. aurantia specific for Fuco ± GlcNAc; ECL from E. crista-galli specific for α1-2,6Galβ1-4GlcNAc or Galβ1-4GalNAc; SNA from S. nigra specific for Siaα2,6-Galβ1-4Glc or Siaα2,6-Galβ1-4GlcNAc; MAA from M. amurenensis specific for Siaα2,3-Galβ1-4GlcNAc or Siaα2,3-Galβ1-4Glc (lectin specificities reported in Knibbs et al., 1991; Kobata and Yamashita, 1993; Rhodes and Milton, 1998). Dig-conjugated or Bi-conjugated lectins were from Roche Diagnostics and Fl-conjugated lectins were from Vector (Burlingame, CA).

Purification of PSA from a PCa serum

tPSA was purified from 35 mL serum of a PCa patient (PCa A) with high tPSA content (1.8 μg/mL). The purification protocol consists of five chromatographic steps followed by an indirect immunosorption specific for fPSA. After each chromatographic step, fractions were analyzed by SDS–PAGE with silver staining (Blum et al., 1987) and the fractions containing PSA were detected and quantified by sandwich ELISA as described in Peracaula et al. (2003b). Briefly, free or tPSA were captured with biotinylated M-30 or M-36 antibodies, respectively, and then detected with the HRP-conjugated anti-tPSA antibody M-66.

The first chromatographic step was by biotinylated gel chromatography. It was performed using a 3S, T-gel slurry (Fractogel EMD TA, Merck, Germany) following the protocol described before (Kawinski et al., 2002). PSA fractions were pooled and freeze-dried.

These fractions were applied to a Cibacron-Blue 3GA (Sigma, St. Louis, MO) affinity chromatography column, following the protocol described previously (Peracaula et al., 2003b).

A third chromatography was performed with a protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) affinity column, which was equilibrated with 3 column volumes (CV) of buffer A (100 mmol/L Tris–HCl, pH 8) before sample loading. Unbound sample was eluted with 5 CV of buffer A, and bound sample (antibodies) was eluted with 3 CV of buffer B (100 mmol/L glycine, pH 3).

The next purification step was performed using a HiTrap Heparin column HPLC system (Amersham Biosciences) because previous assays had shown that PSA binds to a Heparin column (Tabarés, 2004). The column was equilibrated with 10 CV of buffer C (50 mmol/L Tris–HCl, 20 mmol/L NaCl, pH 7) before sample injection, and then elution was performed using three gradient steps: 5 CV from 0 to 35% buffer D (50 mmol/L Tris–HCl, 1 M NaCl, pH 7), 15 CV from 35 to 70% of buffer D, and 5 CV from 70 to 100% of buffer D.

The last chromatography step was another thiolipic absorption column 3S, T-gel, which was performed as described above.

tPSA fractions were collected, dialyzed against distilled water and freeze-dried. They were then subjected to an ethanolamine treatment to release PSA from the PSA–ACT complex, as described previously (Peter et al., 2000). To isolate the tPSA for glycans characterization, this treatment was followed by a specific immunosorption with M-30 anti-fPSA antibodies (Peter et al., 1999).

Sample pretreatment by indirect immunosorption

The indirect immunosorption protocol is based on the one described previously (Peter et al, 1999, 2001). A suspension of 0.5 mL streptavidin magnetic beads (1 mg/mL) was washed three times in washing buffer (phosphate-buffered saline [PBS], 0.1% Tween-20) using magnetic separation. The beads were then incubated with 0.25 mL of biotinylated anti-Dig M-1.71.256 monoclonal antibody 15 μg/mL, dissolved in incubation buffer (PBS, 1% bovine serum albumin [BSA], 0.02% Tween-20, pH 7.2) for 30 min at room temperature with shaking (1000 rpm). The beads were washed and incubated with 0.25 mL digoxigenylated mouse anti-Dig, anti-Fl (Roche Diagnostics).
monoclonal antibody M-30, diluted 15 μg/mL in incubation buffer, for 1 h at room temperature with shaking. The beads were washed, 0.5 mL of PSA samples were diluted in incubation buffer and were incubated for 1 h at room temperature with shaking. The beads were washed as described before and treated with 0.1 mL of saturated Dig-Lysine solution, prepared as described in Peter et al. (1999) for 1 h at room temperature with shaking. The supernatant was removed after magnetic separation of the beads and analyzed by gel electrophoresis and western blotting or stored at -20°C until use.

When the indirect immunosorption was performed before ELISA with lectin detection, the M30-anti-tPSA and the M36-anti-tPSA antibodies used were double labelled, with Bi and Dig. The Dig labelling of the biotinylated M30 and M36 antibodies was performed following the protocol described in Peter et al. (1999).

Glycan sequencing of PCa serum PSA
The purified PSA sample from PCa serum (PCa A) and seminal plasma PSA were electrophoresed on SDS-PAGE on a 12% acrylamide gel. The oligosaccharides from a pure electrophoretic band were analyzed as described previously (Kuster et al., 1997; Radcliffe et al., 2002). Briefly, the N-glycans were released by in-gel digestion with PNGase F recombinant in Escherichia coli (PNGase F, Roche Diagnostics) fluorescently labelled with 2-aminobenzamide (2AB, Ludger Ltd, Oxford, UK) and subjected to normal-phase HPLC. Glycans were analyzed on the basis of their elution positions and measured in glucose units (GU) (Guile et al., 1996). Exoglycosidase digestions were carried out using A. ureafaciens sialidase (EC 3.2.1.18), with broad specificity for α-sialic acids; BTG (EC 3.2.1.23), specific for Gal linked β1-3/4; bovine kidney fucosidase (BKF) (EC 3.2.1.51), with broad specificity for α-Fuc; Nan1 (EC 3.2.1.18), with specificity for α2,3 sialic acids; and with JBH (EC 3.2.1.30), which releases GalNAc and GlcNAc. (All enzymes from Glyko Inc., Upper Heyford, Oxfordshire, UK.)

The glycans from 20 μL of serum from both patient PCa A and from control pooled serum were also released by in-gel digestion by PNGase F and sequenced by exoglycosidase digestions. XMF (EC.3.2.1.51) (New England Biolabs, Hitchin, UK) was also used with these glycan pools.

Lectin detection by ELISA
Biotinylated mouse monoclonal antibodies M-30 against tPSA or M-36 against tPSA were diluted at 4 μg/mL in ELISA buffer (PBS, 1% BSA, 0.05% Tween-20, pH 7.2) and bound to streptavidin-coated microplates for 30 min at room temperature. After washing the plates three times with saline (0.9% NaCl solution and 0.05% Tween-20), PSA samples were diluted to a final volume of 100 μL/well of ELISA buffer and were incubated for 1 h at room temperature. Plates were then washed three times with saline and 100 μL/well of the Dig or Fl-labelled lectins were added to a final dilution of 2 μg/mL in lectin buffer (Tris–HCl 0.1 M, NaCl 150 mmol/L, MgCl2 1 mmol/L, MnCl2 1 mmol/L, CaCl2 1 mmol/L, pH 7.6) and incubated for 2 h at room temperature. The lectins used were SNA, MAA, ECL, and AAL. Plates were washed three times with saline and the labelled lectins were detected with 100 μL/well of 20 μM/mL of the corresponding HRP-conjugated antibodies, anti-FI-HRP-Fab fragments, or anti-Dig-HRP-Fab fragments (Roche Diagnostics), diluted in ELISA buffer. Colorimetric detection was carried out using 100 μL/well of BluePeroxidase substrate soluble (Roche Diagnostics), and the absorbance was read at 450 nm with reference of 620 nm in an automated microplate reader (Bio-Tek, Winooski, VT) after stopping the reaction with 100 μL/well of 0.25 M H2SO4. Pure glycoproteins were used as positive controls for this assay (fetuin for SNA and MAA, asialofetuin for ECL and erythropoietin for AAL). BSA, an unglycosylated protein, and the control serum from a healthy patient (without PSA) were used as negative controls.

When immunosorbed PSA samples were used, the ELISA protocol began with the binding of the samples directly to the streptavidin-coated microplates, because they were bound to anti-PSA biotinylated antibodies. Then Dig or Fl-labelled lectins were added as described above.

ELISA with lectin capture
The ELISA for PSA glycan detection could be performed inversely, with biotinylated lectins for capturing the sample. Lectins were bound to the streptavidin-coated microplates at 2 μg/mL in lectin buffer for 1 h at room temperature. After washing with saline three times, PSA samples from indirect immunosorption were added and incubated for 1 h at room temperature. After washing three times with saline, HRP-conjugated anti-PSA antibodies were used for the detection, after 1 h incubation at room temperature. Colorimetric detection was carried out as before.

Detection by western blotting
Western blotting analysis of PSA was performed following the standard western blotting protocol explained previously (Peracaula et al., 2003b). For lectin detection, PSA samples obtained after indirect immunosorption were electrophoresed in a 12% SDS-PAGE and transferred to a polyvinylidine difluoride membrane (Millipore, Bedford, MA) at a constant voltage of 30 V, overnight at 4°C, in 192 mmol/L glycine/Tris 25 mmol/L/methanol 20%. Filters were blocked in 1% (w/v) BSA and 0.02% Tween-20 in Tris-buffered saline for 1 h at room temperature. The blots were incubated for 2 h at room temperature with Dig, Fl, or Bi-labelled lectins diluted in lectin buffer to a final concentration of 2 μg/mL. After washing three times with 0.1% Tween-20 in Tris-buffered saline, different HRP-conjugated antibodies were used for the detection. These were 20 μM/mL of the antibodies anti-FI-HRP-Fab fragments or anti-Dig-HRP-Fab fragments or 5 μM/mL of the streptavidin-HRP conjugate (Roche Diagnostics) diluted in 1% (w/v) BSA and 0.1% Tween-20 in Tris-buffered saline for 1 h incubation at room temperature. After washing five times with 0.1% Tween-20 in Tris-buffered saline, the detection was performed using a chemiluminiscence kit (Super Signal West Dura, Pierce).
GISA

The protocol used for the glycan characterization of PSA is a modification of the already described GISA for alphafetoprotein glycan studies (Poon et al., 2002). Biotinylated mouse monoclonal antibody M-30 against PSA was diluted at 4 μg/mL in incubation buffer (PBS, 1% BSA, 0.05% Tween-20) and 100 μL/well were bound to streptavidin-coated microplates for 30 min at 37°C. After washing the plates with washing buffer (PBS, 0.05% Tween-20), different pretreated PSA samples diluted in incubation buffer were added to the microplate (100 μL/well) and incubated for 1 h at 37°C. The plate was washed four times with washing buffer and once with incubation buffer (Tris–HCl 15 mmol/L, NaCl 45 mmol/L, Triton X-100 1 mL/L, BSA 1 g/L, pH 7.2). Then 45 μL of incubation buffer containing 2.5 mM/ml α2-6 (N) rat sialyltransferase recombinant in Spodoptera frugiperda (Calbiochem, La Jolla, CA) and 0.75 mg/L CMP-5-fluoresceinyl-neuraminic acid (which was a generous gift from Dr. Brossmer) were added to each well and incubated for 2 h at 37°C. After washing, FL was detected using 100 μL/well of a 1:2000 dilution of the secondary antibody anti-FL-HRP-Fab fragments in incubation buffer for 1 h at 37°C. The plate was washed and the colorimetric detection was carried out as before. Asialofetuin was used as a positive control for this assay. As negative controls, recombinant BSA and the control serum from a healthy patient (without PSA) were used.

Two-dimensional electrophoresis

Pretreated PSA samples were characterized by two-dimensional electrophoresis followed by specific western blotting detection of PSA and proPSA. The samples were diluted to 100 μL in rehydration buffer containing 8 M urea, 0.5% (v/v) Triton X-100, 13 mmol/L dithiothreitol (DTT) (USB, Cleveland, OH), and 1% (v/v) IPG buffer pH 6–11 (Amersham Biosciences). The Immobiline Dry Strips pH 6–11 (Amersham Biosciences) were also immersed in rehydration buffer overnight at 20°C. Isoelectrofocusing was carried out in a Multiphor II (Amersham Biosciences) with sample cup loading and a three steps running protocol, first from 0 to 300 V in 1 min, second from 300 to 3500 V in 90 min, and finally 3 h at 3500 V. After that, the strips were placed in an equilibration solution (containing 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, 21 mmol/L DTT, 50 mmol/L Tris–HCl, pH 8.8) for 15 min with gentle shaking. The strips were equilibrated with running buffer (192 mmol/L glycine, 25 mmol/L Tris–HCl, 0.1% SDS, and pH 8.3–8.5) with traces of bromophenol blue for 5 min and were placed on a 12% acrylamide electrophoresis gel prepared as described before (Peracaula et al., 2003b) in a mini-protein system (BioRad) and sealed in plate with 0.5% (w/v) agarose dissolved in running buffer. The second dimension (SDS–PAGE) was run in two steps, first 15 min at 15 mA/gel and then for 90 min at 30 mA/gel. The western blotting PSA detection was performed, as described previously (Peracaula et al., 2003b), using rabbit polyclonal antibodies anti-PSA (Dako) diluted 1:4000, followed by anti-rabbit IgG HRP-conjugated antibodies (Pierce) 1:40,000. To detect −5 and −7 pPSA forms, biotinylated monoclonal antibodies against pPSA (Roche Diagnostics) at 2.4 μg/mL were used. They were detected with streptavidin HRP-conjugated antibodies diluted at 5 μM/mL. In both cases detection was performed using chemiluminescence. The protein molecular weight markers were See-Blue Pre-stained standards (Invitrogen, Carlsbad, CA).

Glycosydasde digestions before two-dimensional electrophoresis

ABS (Roche Diagnostics) digestion: 5 μL of 0.5 M sodium acetate buffer, pH 5, 7 μL of 10% Triton X-100, and 50 μL of ABS were added to 15 μL of PSA sample (~300 ng of PSA), and the final volume was adjusted to 50 μL with double distilled water.

Nan1 (Glyco, Novato, CA) digestion: 5 μL of 0.2 M sodium phosphate buffer, pH 6, and 50 μL of Nan1 were added to 15 μL of PSA sample (~300 ng of PSA), and the final volume was adjusted to 50 μL with double distilled water.

For both digestions, samples were incubated for 16 h at 37°C and then frozen to stop the digestion. They were analyzed by two-dimensional electrophoresis followed by western blotting detection as described above.

PNGase F + ABS digestion: This protocol needed a denaturing treatment where 10 μL of sample (1 μg of PSA) was added to 90 μL of denaturing solution (0.1 M 2-mercaptoethanol, 0.1% SDS, 0.05% BSA) and boiled for 5 min at 90°C. After cooling the sample, 7.2 μL of 0.2 M sodium phosphate buffer, pH 7.2, 6 μL of 10% Triton X-100, 2 μL of PNGase F, and 50 μL of ABS were added to 50 μL of PSA sample (~500 ng of PSA) and the final volume was adjusted to 72 μL with double distilled water. The digestion was performed by incubation at 37°C for 20 h and the samples frozen to stop the digestion.

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Abbreviations

ABS, Arthrobacter ureafaciens neuraminidase; ACT, alpha-l-antichymotrypsin; BTG, bovine testes β-galactosidase; ELISA, enzyme-linked immunosorbent assay; IPSA, free PSA; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GISA, glycosylation immunosorbent assay; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; MAA, Mackia amurensis lectin; Nan1, Streptococcus pneumoniae neuraminidase; NP, normal phase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered...
References


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