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Purification and Partial Characterisation of Glycosylated Bovine Alpha Iactalbumin

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Abstract

Bovine alpha lactalbumin exists in four different forms. These are F, M, S_1 and S_2, so named because of relative position on non-denaturing polysaccharide gels. F, S_1 and S_2 are minor components that make up 15% of the total alpha lactalbumin and S_1 and S^2 have shown to be glycoforms of protein. The lectin affinity column chromatography (Concanvalin A) was used to seprate glycosylated protein from non glycosyalated contaminants from acid whey. Size exclusion chromatography was then used to separate the glycosylated alpha lactalbumin from the immunoglobulins and other contamnants. In order to analyse the number of oligosaccharide chains bound to protein, they were enzymatically cleaved from the protein using peptide-N4-(N-acetyl-β-glucosamnyl) asparagines aminase (PNGase F), then separated using high pH anion exchange chromatography with pulsed amperometic detection (HPEA/PAD) and fluorophore assisted carbohydrate electrophoresis (FACE). Electrospray mass spectroscopy was used to confirm the results of these techniques. The structure of the main sialic acid containing glycan and neural glycan wete postulated, which represent S_1 and S_2 respectively. It is interesting to note that these molecules differs only one sialic acid residue.

Key words : Glycosylated bovine alpha lactalbumin, PNGase F, HPEAC/PAD, ACE

1. Introduction

Recent development in the field of glycobiology suggest that the N-linkes glycan of alpha lactalbumin could have a variety of functions. Due to the current concern in improving human milk substitute, the determination of the role of the glycan on alpha lactalbumin is of interest. However, elucidation of the number and structure of the glycan linked to the protein must be thoroughly investigated before the possible effect of different glycan structures on protein function can be establish.

Bovine alpha lactalbumin exists in four different forms (1). These are F, M, S_1 and S_2 so named because of relative position on non-denaturing polyacrilamide gels. F, S_1 and S_2 are minor components that make up 15% of the total alpha lactalbumin and S_1 and S_2 have shown to be of protein. This study was to purify the glycoforms of alpha lactabumin and partially characterise main glycans of S_1 and S_2.

2. Materials and Methods

Purification of glycosylated alpha lactalbumin

Lectin affinity column chromatography

Two liters of fresh bovine milk was used to make one half liter of acid whey. This was then dialysed against loading buffer (20mM ammonium acetate, 1mM Ca^{2+}, 1mM Mn^{2+}, 1 mM Mg^{2+}, pH 6.5). All the unbound protein was removed by washing with loading buffer until the absorbance at 280nm was less than 0.1. Bound glycosylated protein were eluted from the Concanvalin A (Con A) column by an isocratic elution using 0.5M methyl α-D mannopyranoside in loading buffer. The protein content of the fractions from the Con A affinity chromatography were estimated using the absorbance at 280nm and a plot of fraction number verses absorbance at 280nm was drawn. Peaks were analysed by native-PAGE. Those containing glycosylated alphalactalbumin were pooled and lyophilized

size-excludion charomatography

The lyphoysed fractions were further purified using size-exclusion chromatography and fast protein liquid chromatography (FPLC) according to following procedure.

Protein of the lyophilized protein (25mg) were dissolved in a minimum of distilled deionised water and filtered through a 0.22μm filter before being applied to a Superdex 75 column (HR 10/30) using a Phamacia EPLC. A 500μl sample of protein solution (10mg/ml) was injected onto the column which had been equilibratd in phosphate buffer (20mM sodium phosphate, 150mM NaCl, pH 7.0) at room temperature and eluted using a flow rate of 0.4ml/min. Fractions of 1.2ml were collected and the elution monitored by measuring the absorbance of the eluent at 280nm. Multiple injections of 500μl were then carried out, using the same conditions as above.

Separation of oligosaccharide mixtures

Purified glycosylated alpha lactalbumins (S_1 and S_2)were enzymatically cleaved from the protein using peptide-N4-(N-acetyl-β-D-glucosaminyl) asparagines aminase (PNGase F), anlysed using following techniques.

(a) High-pH anion-exchange chromatography with detection by pulsed amerometry (HPAEC/PAD)

The chromatograph system (Dionex) used consists of a gradient pump, a detector (PAD-II) and an eluent degas module (EDM).

(b) Fluorophore Assissted Carbohydrate Electrophoresis (FACE)

The protocols used in this study were based on the procedures described previously (2). 4 µl of 8-amino-napthalene-1,3,6, trisulphonic acid (ANTS) solution (0.15M in 15% acetic acid) was added to 10-20µg of oigosaccharide followed by of cyanoborohydride solution (74mg/ml in dimethylsulphoxide) and the reaction incubated at 37°C overnight. After incubation the reaction mixture was dried in speed-vac then re-suspended in an appropriate volume of loading buffer (80% glycerol, 20% water). Typically 1-2µg of oligosaccharides were loaded per lane, 4µl of standard glucose ladder was used as a marker. Electrophoresis was performed at 5-8°C for about 1.5 h with running buffer (192mM Glycine, 25mM Tris, pH 8.5). This was achieved by immersing the electrophoresis chamber in ice. After running the gel, the bands were visualized under UV light, and the image recorded using an Alpha Innotech gel documentation system.

Electrospray ionization mass spectrometry (ES/MS)

Electrospray mass spectra was acquired on a triple quadruple electrospray mass spectrometer operating with an API ion source in the positive ion electrospray mode. Sample were diluted into acetonitrile/0.25 formic acid (1:1 by volume), to a concentration o f 20 pM/µl and infused at 5µl/min, using a syringe pump.

3. Results

Native-PAGE analysis showed that there was immunoglobulin contaminated with the glycosylated alpha lactalbumin. Contaminated immunoglobulins were successfully removed using size exclusion chromatography as described in method section. Results are shown in Figure 1 and 2.

HPAEC/PAD profiles of alpha lactalbumin oligosaccharides that were enzymatically released from bovine glycosylated alpha lactalbumin using PNGase F, showed that the oligosaccharides of alpha lactalbumin can be separated in to two groups: neutral and sialic acid containing species.

The sialic acid containing oligosaccharides are probably represented by the S_1 band of alpha lactalbumin due to their expected higher mobility on native PAGE. The FACE techniques was used to get a idea of main oligasaccharide structures. Results are shown in Figure 3. Electrospray mass spectroscopy of purified alpha lactalbumin was used to confirm the results. The correlation between the mass spectrometry results and FACE gel results are shown in Table 1.

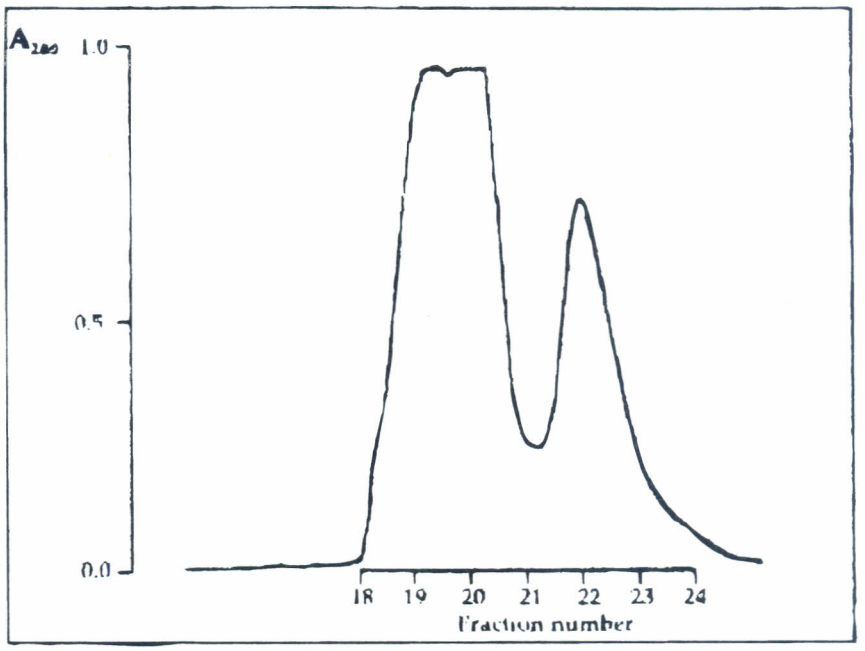

Figure 1 Chromatogram of the elution from Superdex-75 of glycosylated alphalactabumin.

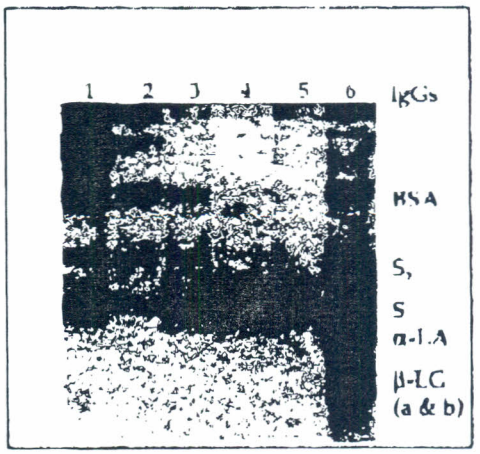

Figure 2 Fractions from size exclusion chromatography, L ane 1: Sample after Con A Chromatography, Lane 2: Fraction 21, Lane 3: Fraction 22, Lane 4: Fraction 23, Lane 5: Fraction 24, Lane 6: Marker

Table 1 The correlation between the mass spectrometry results and FACE gel results.

Molecular weight predicted by ES/MS	Glycan mass*	Calculated FACE gel values	Proposed glycan structures
16031.0	1854.0	7.7 ± 2 (band c)	GalNAc-GlcNAc-Man GalNAc-GlcNAc-Man Man-GlcNAc-GlcNAc- Fuc S_2
16322.0	2146.0	6.7±2 (band d)	Gal-GlcNAc-Man Sia-Gal-GlcNAc-Man Man-GlcNAc-GlcNAc- Fuc S_1

*Glycan mass = Observed glycoforms mass-observed non-glycosylated alpha lactalbumin mass (14176.0)

Man : Mannose, Gal : Galactose, GalNAc : N-acetyl galactosamine, GlcNAc : N-acetyl glucosamine, Sia : Sialic acids

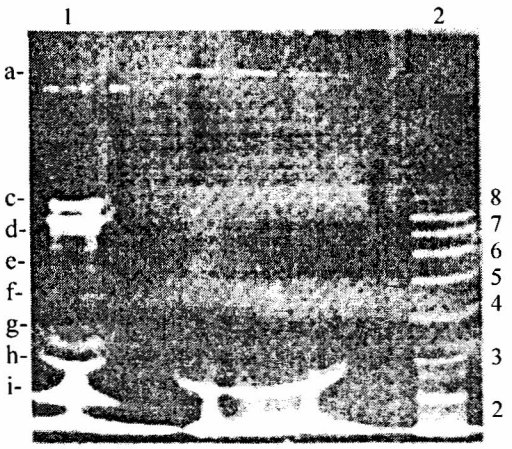

Figure 3 FACE pattern of oligosaccharide released from bovine alpha lactlbumin
Lane 1: Oligosaccharides released from bovine alpha lactabumin.
Lane 2: standard

4. Discussion

To my knowledge, this is the first study in which the attempt to made purification and characterization of glycosylated bovine alpha lactalbumin. HPEAC/PAD results thows that S_1 and S_2 band represent a families of glycoforms not just one glycoform. There was a very intense signal with a glycoprotein mass of 16322.0 Da

and a very intense band in the FACE gels of around degree of polymerization (Dp) =7 (3)(4). It can be concluded that the main bovine alpha lactalbum glycan containing sialic acid (representing S_1) is the structure shown in Table 1. There was another significant signal, which corresponds to the molecular weight of 16032. From the ES/MS data and the calculated FACE gel values this species should be the main neutral glycoform represented by S_2. The structures of the main sialic acid containing glycan and neutral glycan were postulated, which represent S_1 and S_2 respectively (Table 1). It is interesting to note that these molecules differ by only one sialic acid residue.

5. Acknowledgment

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