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Bioskin as an affinity matrix for the separation of glycoproteins☆

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Abstract

Bioskin is a natural product produced by a mixed culture of *Acetobacter xylinum*, *Saccharomyces cerevisiae* and *S. pombe* cultured on media containing sucrose. It is of fibrillar nature able to retain some proteins, such as cytochrome *c*, by adsorption, and mainly composed of glucosamine and *N*-acetyl-D-glucosamine. This makes it possible that, at an adequate pH value, proteins charged as polyanionic molecules, such as catalase, can be retained by ionic adsorption using the positively charged amino groups of the matrix. In addition, bioskin can also be used as an affinity matrix to retain glycoproteins able to perform specific affinity reactions with the amino sugars of the matrix, such as invertase, fetuin or ovalbumin. Its possible use as a chromatographic support is discussed.

Keywords: Bioskin; Adsorption; Affinity adsorbents; Glycoproteins; Proteins

1. Introduction

Many techniques have been successfully applied to separate proteins and peptides, the majority of which have been recently reviewed by Banks [1], such as RP-HPLC, hydrophobic interaction chromatography (HIC), size-exclusion chromatography, ion-exchange chromatography, and affinity chromatography. For affinity separations, the stationary phase is specifically modified to interact with the target analyte [2], and an affinity ligand is specific and covalently bonded to an inert support material. Bioskin is a natural product obtained from a mixed culture of *Acetobacter xylinum*, *Saccharomyces cerevisiae* and *S. pombe* cultured on media containing sucrose (patent DIMED/MS 8306340 Brazil, registered by Xavier Filho and Paulo) [3]. It is absolutely insoluble in water as well as in organic solvents, such as ethanol, methanol, acetone, acetonitrile, diethyl ether and hexane, extremely stable at alkaline pH values, 9.0 and over, slightly soluble in 6 *M* HCl at 25°C, and soluble in 6 *M* HCl at 60°C. It is described as a skin regenerating system and used in medicine to accelerate healing and as an antiseptic by adhering it to open injuries [4]. In addition, as many other polysaccharide matrices [5], it could be used for enzyme immobilization. For instance, high amounts of catalase can be immobilized by ionic adsorption on the bioskin surface at the adequate pH value [6] in such a manner that immobilisates can be used to visualize the arrangement of the four

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subunits of the tetrameric enzyme by scanning electron microscopy [7]. Since bioskin seems to be of polysaccharide nature, the possibility to use it for glycoprotein separation by affinity interactions is investigated.

2. Materials and methods

2.1. Biomaterial

Bioskin was kindly provided by Bioskin Produtos Micro-Biotecnologicos (Recife, Brazil) in sterile conditions. Bioskin was boiled in distilled water for 1 h and dried in air before use.

A sample of 100 mg of dried, boiled bioskin was treated with 20 ml 6 *M* HCl for 12 h at 60°C. After this, the remaining solid material was removed, the hydrolysate dried in air flow and the residue dispersed in 1.0 ml cold 80% (v/v) aqueous ethanol and stored at −13°C for 14 h. The precipitate was discarded and the supernatant heated at 60°C for 20 min. After this, 1.0 ml cold 80% ethanol was added and the material was then heated again to dryness. The residue was reconstituted with 1.0 ml cold 80% ethanol and centrifuged at 3000 *g* for 15 min. The supernatant was loaded onto the chromatographic column.

HPLC was performed with a Varian 5060 liquid chromatograph (Walnut Creek, CA, USA) equipped with a SpectraSystem UV2000 detector (SpectraPhysics, Freemont, CA, USA) and a Vista CDS 401 computer (Varian). The chromatographic conditions were as follows: column, MicroPak NH_2 10P/N (30 cm×3 mm, I.D.) from Varian; sample loading, 10 μl; mobile phase, acetonitrile–water (80:20 v/v) isocratically; flow-rate, 1.0 ml min^{-1}; temperature, 20°C; detector, UV 195 nm; attenuation, 64 [8].

Quantitation was performed by injecting different amounts of standards after filtration through an alumina column, according to Molina and Vicente [9], previously estimated according to Dubois et al. [10].

The IR spectrum of bioskin was measured with an IR spectrophotometer Matson (Madison, IL, USA), Model Satellite, equipped with an ATR accessory.

2.2. Chemicals

All chemicals used for the preparation of the buffers, sodium acetate, sodium citrate, sodium phosphate, sodium borate, hydrochloric acid and sodium chloride, were of analytical reagent grade (Merck, Darmstadt, Germany) and were used as received. Water was of Milli-Q grade (Millipore, Bedford, MA, USA). Catalase, cytochrome *c*, fetuin, invertase, and ovalbumin were obtained from Sigma (St. Louis, MO, USA), as well as isoelectric point (p*I*) standards, amyloglucosidase, tyroglobulin, arginase, alcohol dehydrogenase, carbonic anhydrase, and myoglobin. Benzene, ethanol and acetonitrile were from Merck. D-Glucose, D-fructose, D-mannose, D-galactose, L-fucose, L-rhamnose, D-xylose, D-glucuronic acid, D-glucosamine, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine, were from Sigma.

2.3. Enzyme retention and removal

Fine sheets of bioskin, 20 cm in diameter and 0.1 mm thickness, were incubated for 60 min in 20 ml of a solution containing 4.0 mg ml^{-1} cytochrome *c* or catalase in distilled water (pH 5.8), or 2.5 mg ml^{-1} yeast invertase in 10 m*M* acetate buffer, pH 4.3, at room temperature and newly dried in air. When indicated, fetuin was immobilized in the same conditions on bioskin from a solution containing 2.5 mg protein ml^{-1} before immobilizing invertase.

Protein removal was attempted by immersion of the bioskin piece containing the immobilized enzyme in 20 ml of 10 m*M* citrate–phosphate buffer, pH 2.5; 10 m*M* acetate buffer, pH 4.3; distilled water, pH 5.8; distilled water–methanol (80:20 v/v); 10 m*M* phosphate buffer, pH 7.0; 10 m*M* borate buffer, pH 9.2, and borate buffer containing 2% (w/v) NaCl or, alternatively, using a series of 50 m*M* sugars and amino sugars at pH 7.0, for 30 min at room temperature. When indicated, 10 m*M* acetate buffer, pH 4.3, or 10 m*M* phosphate buffer, pH 7.0, contained increasing concentrations of NaCl. Elution of protein was monitored at 280 nm, or at 409 nm, where the haem group of cytochrome *c* and catalase absorbs.

2.4. Enzyme assays

Invertase activity was measured in reaction mixtures containing 60 μmol sucrose (or variable concentrations of substrate, when indicated), 30 μmol sodium acetate, pH 4.3, and 25 μg of soluble or immobilized protein in a final volume of 3.0 ml. Reaction was carried out for 15 min at 30°C with shaking at 250 oscillations min^{-1} to avoid the formation of a diffusion-resistant Nernst layer, and stopped by adding 1 ml 1 *M* NaOH. Reducing sugars were measured by reaction with dinitrosalicylic reagent [11] and quantified using the corresponding calibration curve made with fructose as standard.

Catalase activity was routinely assayed according to Herbert [12] in reaction mixtures containing 135 μmol H_2O_2, 0.1 mg soluble or immobilized catalase, and 40 μmol phosphate buffer, pH 7.5, in a final volume of 5 ml. Reaction was carried out at 40°C for 1 min and stopped by adding 2 ml 1 *M* H_2SO_4. Remaining H_2O_2 was determined by titrating liberated iodine from KI with sodium thiosulfate.

2.5. Capillary electrophoresis

A Hewlett-Packard (Waldbronn, Germany) capillary electrophoresis system was used. Fused-silica capillaries (50 μm I.D.) were obtained from Hewlett-Packard. A capillary just purchased was conditioned with 0.5 *M* NaOH for 5 min at 60°C and doubly distilled water for 5 min at 60°C and equilibrated by washing with 25 m*M* sodium borate buffer, pH 9.1, for 10 min at 25°C and, finally, with the same buffer for 10 min at 25°C under an applied voltage of 17 kV. Regeneration of the capillary surface between runs was performed by rinsing it with 0.05 *M* NaOH for 3 min, doubly distilled, deionized water for 5 min and 25 m*M* sodium borate buffer, pH 9.1, for 3 min.

Conditions of electrophoretic analysis, as described by Legaz and Pedrosa [13], were as follows: uncoated fused-silica capillaries of 64 cm effective capillary length×50 μm I.D.; electrolyte, 25 m*M* sodium borate, pH 9.1; temperature, 25°C; applied voltage, 17 kV; on line diode array detector at 200 nm with 16 nm band width; injection, hydrodynamic by application of 50 mbar for 4–9 s at the end of the capillary. Benzene, at a concentration of 4% (v/v) in the same buffer was used as neutral marker. p*I* standards were amyloglucosidase (p*I* 3.6); tyroglobulin (p*I* 4.5); arginase (p*I* 5.1); alcohol dehydrogenase (p*I* 5.4); carbonic anhydrase (p*I* 5.9); and myoglobin (p*I* 7.0), all from Sigma.

3. Results

3.1. Chemical analysis of the bioskin

Analysis by HPLC of the acidic hydrolysate of bioskin produced only two peaks of retention time values of 3.0 and 3.65 min, which coincided with those of the standards *N*-acetyl-D-glucosamine and glucosamine, respectively (Fig. 1A and B). In effect, only the peak of 3.0 min increased after loading the sample of bioskin hydrolysate with *N*-acetyl-D-

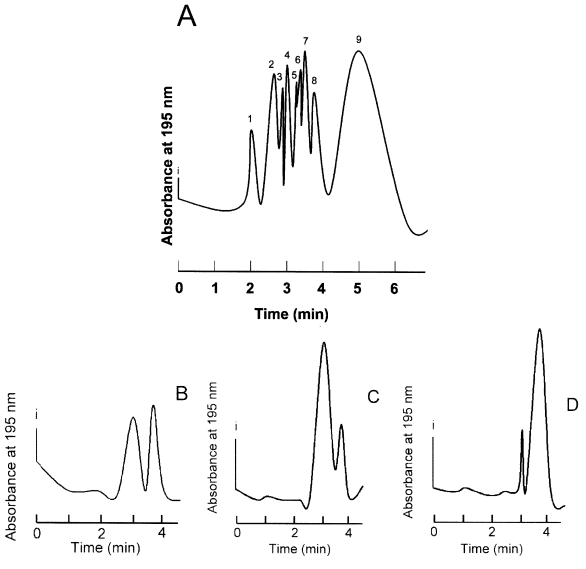

Fig. 1. HPLC analysis of chemical constituents of bioskin. HPLC separation of standards is shown in (A), where 1=D-glucuronic acids (t_R 2.03 min), 2=L-fucose (t_R 2.70 min); 3=D-galactose (t_R 2.86 min); 4=*N*-acetyl-D-glucosamine (t_R 3.04 min); 5=*N*-acetyl-D-galactosamine (t_R=3.24 min); 6=D-fructose (t_R 3.236 min); 7=D-mannose (t_R 3.49); 8=D-glucosamine (t_R 3.65 min), and 9=D-glucose (t_R 4.93 min). (B) HPLC profile of the acidic hydrolysate of bioskin. (C) Chromatographic profile of the acidic hydrolysate of bioskin loaded with 1.0 mg ml^{-1} glucosamine. (D) Chromatographic profile of the acidic hydrolysate of bioskin loaded with 1.0 mg ml^{-1} *N*-acetyl-D-glucosamine.

glucosamine (Fig. 1C), whereas the second peak, that of 3.65 min, selectively increased after loading the hydrolysate with glucosamine (Fig. 1D). These results were supported by the IR analysis of bioskin. Both the vibration of –OH and a signal of the secondary amide, this last diminished by the solid state of the sample, were revealed by a broad peak of transmittance at 3339 cm^{-1}, whereas the amine group of glucosamine was revealed by a peak at 1632 cm^{-1}. The peak at 2891 cm^{-1} was produced by the $-CH_3$ group of *N*-acetyl-D-glucosamine whereas the peaks at 1427, 1336 and 1204 cm^{-1} were vibration signals of –C–O functions (Fig. 2).

3.2. Ionic adsorption of catalase and cytochrome c

Sheets of bioskin with an average dry mass of 80 mg were used to immobilize 80 mg of catalase in 20 ml of distilled water, pH 5.8, at room temperature. About 70% of the protein was retained on the support. Desorption was attempted by using 10 m*M* acetate buffer, pH 4.3; the same buffer containing different amounts of NaCl; distilled water, pH 5.8; distilled water–methanol (80:20 v/v); 10 m*M* phosphate buffer, pH 7.0, for 30 min at room temperature. Only saline acetate buffer desorbed the protein with an efficiency of 100%, as is shown in Fig. 3. Simultaneous adsorption on bioskin of both cytochrome *c* and catalase, dissolved in 10 m*M* acetate buffer, pH 4.3, was easily achieved. Cytochrome *c* was desorbed using NaCl-containing acetate buffer,

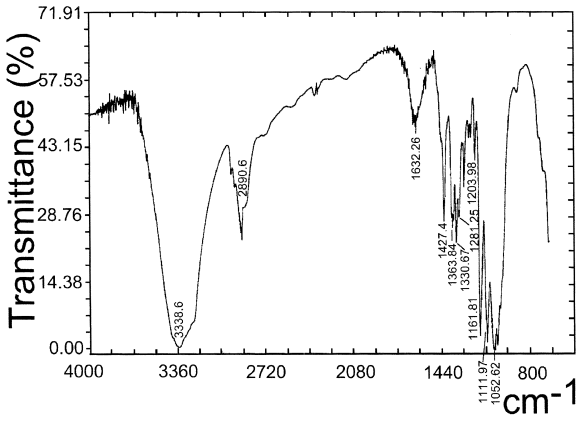

Fig. 2. Transmittance IR spectrum of bioskin.

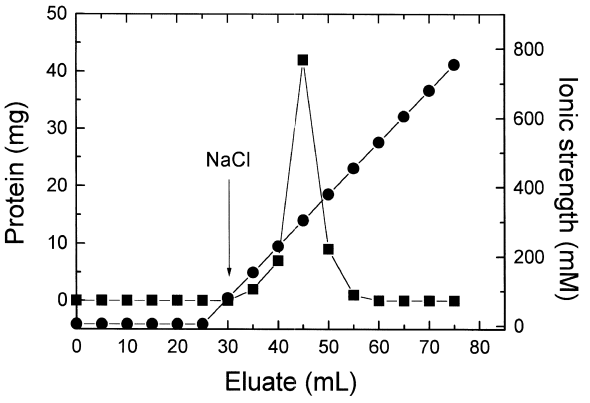

Fig. 3. Elution profile of catalase (■) previously retained on bioskin, using as eluent 10 m*M* acetate buffer, pH 4.3, containing, from the fraction indicated by the arrow, increasing concentrations of NaCl, which form a linear gradient of ionic strength (●).

pH 4.3, with an ionic strength value of 80 m*M* whereas catalase was eluted at 320 m*M*.

3.3. Invertase retention by affinity

Identical pieces of bioskin were used to immobilize invertase from 20 ml of a bulk solution containing 5 mg of pure enzyme in 10 m*M* acetate buffer, pH 4.3, at room temperature. About 86% of protein was retained in bioskin at a mean of 59 μg protein mg^{-1} dry matrix. Yeast invertase migrates in capillary electrophoresis as a polyanionic protein: its migration value was 13.23 min (the migration time value of the neutral marker was 8.28 min) at 17 kV. Its electrophoretic mobility was calculated to be 3.03×10^{-4} cm^2 s^{-1} V^{-1}. This value was interpolated in a straight line of migration time versus electrophoretic of standards and a p*I* value of 3.23 was then calculated for invertase. Thus, invertase behaved as a polyanion in the range of pH values from 4.3 to 9.2, but as a polycation at pH 2.5. No desorption was achieved by using 10 m*M* citrate–phosphate buffer, pH 2.5; 10 m*M* acetate buffer, pH 4.3; distilled water, pH 5.8; distilled water–methanol (80:20 v/v); 10 m*M* phosphate buffer, pH 7.0; 10 m*M* borate buffer, pH 9.2, and borate buffer containing 2% (w/v) NaCl for 30 min at room temperature. A series of 50 m*M* xylose, galactose, fructose, glucose and glucosamine at pH 7.0 removed minimal amount of protein from bioskin. Removal was en-

Table 1
Desorption of yeast invertase from bioskin using 50 m*M* sugars or amino sugars solution

Protein adsorbed (μg)	Desorbed with	Amount (μg)
192±12.18		
	Water	0
	Fructose	32.23±2.71
	Fucose	60.56±5.47
	Galactose	15.01±1.32
	Glucosamine	20.21±2.02
	Glucose	30.93±2.56
	Mannose	115.6±9.87
	Rhamnose	60.12±5.12
	Xylose	11.31±0.98
	N-Acetyl-D-glucosamine	127.33±11.68
	Mannose + *N*-acetyl-D-glucosamine	190.0±14.45

hanced by 50 m*M* fucose and rhamnose but it reached its maximum value by using mannose or *N*-acetyl-D-glucosamine, or a mixture of both compounds (Table 1). No immobilization of invertase was obtained by using a bioskin piece in which 0.18 mg of fetuin were previously immobilized (data not shown), although retained fetuin was removed from the support by rinsing it with 10 ml of 15 m*M* of

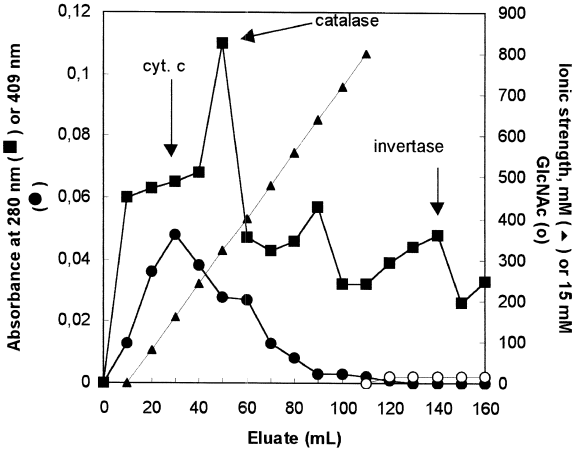

Fig. 4. Retention by bioskin of proteins from a mixture of cytochrome *c*, catalase and invertase. Desorption was carried out using 10 m*M* acetate buffer, pH 4.3, for the first 10 ml, followed by 10 m*M* phosphate buffer, pH 7.0, containing NaCl from 80 m*M* to 800 m*M*, from 20 ml to 110 ml of eluate, and, finally, the same buffer containing 15 m*M* *N*-acetyl-D-glucosamine, from 120 ml to 160 ml eluate. Arrows indicate the fraction developing the major value of the corresponding enzyme activity.

N-acetyl-D-glucosamine for 30 min at room temperature, with a recovery of about 80%. Similar results were obtained using ovalbumin instead of fetuin.

A mixture of cytochrome *c*, catalase and invertase (10 mg of each one in 20 ml distilled water) were adsorbed on bioskin and eluted with acetate buffer, acetate buffer containing increasing amounts of NaCl or 10 m*M* phosphate buffer, pH 7.0, containing 15 m*M* *N*-acetyl-D-glucosamine. Cytochrome *c* was eluted by acetate buffer containing 160 m*M* NaCl whereas catalase only was removed from bioskin by increasing the ionic strength of the solvent to 320–400 m*M*. However, invertase elution required the addition of *N*-acetyl-D-glucosamine to the solvent to be efficiently achieved (Fig. 4).

4. Discussion

The interfibrillar spaces of bioskin are sufficiently large to permit absorption of cytochrome *c* and catalase by capillarity from the external solution. Their retention probably involves attractive electrostatic interactions since bioskin is positively charged at pH 5.8–7.0 on the basis of the $-NH_3^+$ groups of both glucosamine and *N*-acetyl-D-glucosamine (Fig. 1). But interaction through hydrogen bonds is probably established, mainly for catalase. This can be deduced from assays of protein elution (Figs. 3 and 4), since this enzyme is not removed from bioskin after reaching a net positive charge, but only when a strong increase of the ionic strength breaks other classes of binding [6]. However, cytochrome *c* is eluted at values of ionic strength lower than that required for catalase desorption. This can easily be explained since cytochrome *c* has a p*I* value of 10.6 and, thus, it is charged as a polycation at pH 4.3 (Fig. 4). This implied that cytochrome *c* is adsorbed on bioskin whereas catalase is electrostatically bound to the support [6,7].

The p*I* value of commercial yeast invertase has been determined as 3.23 by capillary electrophoresis, a procedure even more accurate than the conventional electrofocusing on column [14]. This value is similar to those found for other plant invertases, such as that ionically bound to the cell wall of *Daucus carota* parenchymatous cells [15] or some acidic isozymes from rice grains [16]. Thus, invertase

behaves as a polyanion for a range of pH values from 4.3 to 9.1, used here for desorption protocols, and as a polycation for a pH=2.5. However, no desorption was achieved in the overall range 2.5–9.1. On this basis, invertase cannot be only electrostatically bound to bioskin, as previously described for catalase [6]. Since invertase is a glycoprotein, the alternative of a mechanism of affinity immobilization could be considered.

Exocellular invertase from baker yeast is formed by 12 *N*-acetyl-D-glucosamine-containing glycopeptides linked at 4-L-aspartyl positions. Nine of them are defined as *N*-glycosidic chains that can be removed by an endo-β-*N*-acetyl-D-glucosaminidase [17]. The composition of the different oligosaccharides on invertase molecule has been elucidated by sequential treatment with endo-β-*N*-acetyl-D-glucosaminidase and peptide-*N*4-*N*-acetyl-β-glucosaminyl asparagine amidase. The first enzyme releases about 17% of the carbohydrate as Glc3Man8GlcNAc2, while the second releases the remainder as Hex8GlcNAc2 and Man5GlcNAc2 in a 1:4 ratio. The pool Hex8GlcNAc2 contained Glc3Man5GlcNAc2 and Man8GlcNAc2 in a 6:4 ratio [18]. In yeast Golgi compartment, at least five, and potentially several additional mannosyl transferases, are involved in elongating to ''mannan'' the core Man8GlcNAc2 oligosaccharide trimmed from Glc3Man8GlcNAc2 in endoplasmic reticulum [19]. The high number of both mannosyl and *N*-acetyl-D-glucosamine residues explains the high amount of enzyme removed from bioskin when the immobilisates are washed with mannose or *N*-acetyl-D-glucosamine solutions. The enzyme molecule is displaced by competition with sugars identical to those specific residues with affinity for the ligand, the bioskin matrix (Table 1). Immobilization by affinity was confirmed by using a bioskin piece in which fetuin was previously attached. Since two asialo-triantennary glycopeptides are predominant in bovine fetuin, containing large amounts of both mannose and *N*-acetyl-D-glucosamine [20], it is proposed that the sites in bioskin for the binding of the ligand have previously been occupied by fetuin and then the binding of invertase is completely impeded. Similar results were obtained using ovalbumin, since its oligosaccharide moiety is largely composed by mannose and *N*-acetyl-D-glucosamine [21,22].

In this way, bioskin behaves as the oligosaccharide moiety of a lectin, able to bind glycoproteins containing mannose and *N*-acetyl-D-glucosamine and, thus, to separate these glycoproteins from others of different oligosaccharide composition. Sugar–sugar interactions are in the basis of the biological activity of plant lectins and they have been used not only to develop particular systems of affinity chromatography but also capillary affinity electrophoresis in order to investigate molecular interactions of glycoproteins in free solution [23]

As a conclusion, bioskin exhibits some of the ideal conditions to be a matrix for affinity separation of glycoproteins. It is suited for ligand immobilization, it is stable for long time periods, even in extreme chemical environments, and it does not disrupt either the matrix or the ligand [24]. However, their use absolutely required them to be manufactured as uniform spheres instead of uniform sheets, as has been used in this study.

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