Published in final edited form as:
FEMS Microbiol Lett. 2013 April ; 341(2): 87–95. doi:10.1111/1574-6968.12097.

Identification of human plasma proteins associated to the cell wall of the pathogenic fungus *Paracoccidioides brasiliensis*

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Abstract

Paracoccidioides brasiliensis and *P. lutzii* are thermodimorphic species that cause paracoccidioidomycosis. The cell wall is the outermost fungal organelle to form an interface with the host. A number of host effector compounds, including immunologically active molecules, circulate in the plasma. In the present work we extracted cell wall-associated proteins from the yeast pathogenic phase of *P. brasiliensis*, isolate Pb3, grown in the presence of human plasma, and analyzed bound plasma proteins by liquid chromatography-tandem mass spectrometry. Transport, complement activation/regulation and coagulation pathway were the most abundant functional groups identified. Proteins related to iron/copper acquisition, immunoglobulins, and protease inhibitors were also detected. Several human plasma proteins described here have not been previously reported as interacting with fungal components, specifically, clusterin, hemopexin, transthyretin, ceruloplasmin, alpha-1-antitrypsin, apolipoprotein A-I, and apolipoprotein B-100. Additionally, we observed increased phagocytosis by J774.16 macrophages of Pb3 grown in plasma, suggesting that plasma proteins interacting with *P. brasiliensis* cell wall might be interfering in the fungal relationship with the host.

Keywords

Paracoccidioides brasiliensis; cell wall; human plasma proteins

Introduction

Paracoccidioides brasiliensis and *P. lutzii* are thermodimorphic species responsible for paracoccidioidomycosis (PCM), a prevalent systemic granulomatous mycosis in Latin America. The active disease occurs in 1 to 2% of infected individuals, whose number is estimated in 10 million throughout endemic areas (San-Blas, *et al.*, 2002). Once in the

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pulmonary alveolar epithelium, inhaled infectious particles can establish infection as long as they transform into the pathogenic yeast form.

The cell wall is the outermost fungal structure in contact with the host and its dynamic structure can rapidly change to adapt to the environment (Kapteyn, *et al.*, 2000). The yeast phase of *Paracoccidioides* cell wall is composed mainly of α-1,3-glucan and chitin, with a small proportion of β-1,3-glucan and galactomannan (Kanetsuna, *et al.*, 1972). Typical covalently linked structural proteins have not yet been described in *Paracoccidioides*; however, numerous non-covalently linked proteins have been shown in this compartment (Puccia, *et al.*, 2011).

Human plasma is composed by a large number of proteins, including both typical plasma proteins, such as albumin and lipoproteins, and tissue molecules that can be used in diagnosis and therapeutic monitoring (Anderson & Anderson, 2002). Although 1,175 proteins have been described in human plasma (reviewed in (Anderson, *et al.*, 2004)), 95% of protein abundance is represented by only ten (Putnam, 1984, Pieper, *et al.*, 2003): albumin (54%), immunoglobulin G (17%), alpha-1-antitrypsin (3.8%), alpha-2-macroglobulin (3.6%), immunoglobulin A (3.5%), transferrin (3.3%), haptoglobin (3%), apolipoprotein A-1 (3%), immunoglobulin M (2%) and alpha-1-acid-glycoprotein (1.3%).

Many plasma compounds, such as complement components and immunoglobulins, are immunologically active molecules and compose major defense lines of the host against invading microbes (Zipfel, *et al.*, 2007). Therefore, a better knowledge of the interactions between fungal cell wall and host plasma proteins may help us to understand infection development and host defense (Cottier & Pavelka, 2012).

The aim of the present work was to identify human plasma proteins that interact with *P. brasiliensis* yeast cell wall, since they might interfere in the host-pathogen relationship. For this we employed liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic analysis to identify proteins extracted with hot sodium dodecyl sulfate (SDS) from Pb3 cell wall, carefully isolated from yeasts cultivated in plasma-containing defined medium. We chose Pb3 as model because it represents *P. brasiliensis* cryptic species PS2, whose members are less virulent in B10.A mice (Carvalho, *et al.*, 2005). In this model, Pb3 evokes a predominant Th1-type protective immune response enriched in IgG2a, IgG2b, and IgG3 and high amounts of INF-γ (unpublished data).

1. Materials and methods

1.1. P. brasiliensis isolate and growth conditions

P. brasiliensis isolate Pb3 was maintained in the yeast phase at 36°C in solid modified YPD medium (0.5% yeast extract, 0.5% casein peptone, 1.5% glucose, pH 6.5). For cell wall isolation, yeast cells were cultivated in defined Ham's F12 medium (Invitrogen) added of 1.5% glucose (F12/Glc) and supplemented or not with 2% heat-inactivated (56°C, 30 min) human plasma, obtained from healthy donors of Hospital São Paulo (UNIFESP Ethics Committee, approval protocol number 0366/07). Although we started with 2% plasma, we observed protein precipitation, which was discarded by centrifugation (6,000x*g*, 30 min, 4°C). Cells were transferred from 7-day-old slants into F12/Glc (200 mL) and cultivated at 36°C for 4 days (pre-inoculum). Yeast cells from four pre-inoculums were transferred to 500 mL of fresh medium and cultivated for 2 days for cell wall purification. Yeast cells were analyzed for viability (>95%) with Trypan blue.

1.2. Cell wall purification

Yeast cells cultivated in the presence (Pb3pl) or absence (Pb3) of heat-inactivated human plasma were harvested by centrifugation, washed three times with phosphate saline buffer (PBS) and mechanically disrupted with glass beads (425-600 μm, Sigma Aldrich) in B. Braun (6 times for 10 min, alternating with 10 min in ice) in the presence of PBS with protease inhibitors (100 mM ethylenediamine tetraacetic acid, EDTA, 10 mM 1,10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride, PMSF, 1 μM pepstatin A and 15 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, E-64). Cell wall was isolated from cytoplasmic contents and membranous structures by three sequential centrifugations (8,000×*g* for 45 min at 25°C) in 85% sucrose (Kanetsuna, *et al.*, 1969). Non-specifically bound components were eliminated by five sequential washes with each of the ice-cold solutions: deionized water, 5% NaCl, 2% NaCl, 1% NaCl, and 1 mM PMSF (Pitarch, *et al.*, 2002); final cell wall preparation was lyophilized.

1.3. SDS-extraction of cell surface-associated proteins

Isolated cell wall (100 mg) was extracted twice by boiling with SDS for 5 min in extraction buffer (100 mM EDTA, 50 mM Tris-HCl pH 7.8, 2% SDS). The SDS extracts were centrifuged, filtrated through a sterile 0.22-micron filter, and precipitated in ice-cold acetone (1 h at -20°C). After a 30-min centrifugation (16,000×*g* at 4°C), the protein pellet was removed, washed in acetone, and dried at room temperature.

1.4. Proteomic analysis

Protein digestion was carried out using the ammonium bicarbonate/methanol method (Russell, *et al.*, 2001). Tryptic peptides were desalted in POROS R2 microcolumns (Jurado, *et al.*, 2007) and dried in an Eppendorf vacuum centrifuge concentrator. Peptides were then dissolved in 0.1% formic acid (FA), loaded onto a reversed-phase trap column (1 cm×75 μm, Luna C18, 5 μm, Phenomenex), and separated in a capillary column (20 cm×75 μm, Luna C18, 5 μm, Phenomenex) coupled to a nanoHPLC (1D Plus, Eksigent). Peptides were eluted in a linear gradient from 8.75% to 35% acetonitrile in 0.1% FA over 200 min and directly analyzed in an electrospray-linear ion trap-mass spectrometer (LTQ XL/ETD, Thermo Fisher Scientific) equipped with a TriVersa NanoMate nanospray source (Advion). The nanospray was set at 1.45 kV and 0.25 psi N_2 pressure using a chip A (Advion). MS spectra were collected in centroid mode at the 400-1700 *m/z* range and the ten most intense ions were subjected twice to collision-induced dissociation with 35% normalized collision energy, before being dynamically excluded for 60s.

MS/MS spectra from peptides with 800 to 3,500 Da, more than 10 counts, and at least 15 fragments were converted into DTA files using Bioworks v.3.3.1 (Thermo Fisher) and searched against human (IPI v), porcine trypsin (GenBank) and Paracoccidioides (http://www.broadinstitute.org/annotation/genome/dimorph_collab.1/MultiHome) sequences, in both correct and reverse orientations, using TurboSequest (Bioworks 3.3.1, Thermo Fisher Scientific). The database search parameters included: i) trypsin cleavage in both peptide termini with one missed cleavage site allowed; ii) carbamidomethylation of cysteine residues as a fixed modification; iii) oxidation of methionine residues as a variable modification; and iv) 2.0 Da and 1.0 Da for peptide and fragment mass tolerance, respectively. TurboSequest outputs were filtered with DCn ≥ 0.05, peptide probability ≤ 0.05, and Xcorr ≥ 1.5, 2.0, and 2.5 for singly-, doubly-, and triply charged peptides, respectively. After filtering, the files were exported into XML formats and the peptide sequences were assembled into proteins using an in-house written script (Nakayasu, *et al.*, 2012). The protein hits were refiltered with the sum of peptide Xcorr ≥ 3.5. The false-discovery rate (FDR) was estimated as described previously (Rodrigues, *et al.*, 2008). Only proteins detected by at least two peptides exclusively in the Pb3pl cell wall were considered.

Functions and processes in which identified proteins are involved were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov) (Dennis, *et al.*, 2003), and Blast2GO (http://www.blast2go.org/) (Conesa, *et al.*, 2005) for Gene Ontology (GO). The exponentially modified protein abundance index (emPAI) (Ishihama, *et al.*, 2005) was used for protein abundance comparison considering the protein molecular masses.

1.5. In vitro phagocytosis assay

Phagocytosis assays were carried out with macrophage cell lineage J774.16 cultured in DMEM/10% inactivated FBS. $2x10^5$ cells were activated with 50 U/ml IFN-γ (PeproTech, Rock Hill, NJ) at 37°C overnight and incubated with *P. brasiliensis* yeasts at a ratio of 5:1 macrophages:fungi for 6 h at 37°C. Yeasts were cultivated in plasma-containing F12 medium. When grown in F12 alone, they were incubated with plasma (37°C, 1 h) before the assay. Fresh and heat-inactivated plasma (56°C, 1 h) were used. Three washes with 0.15 M α-methyl-mannopyranoside were performed to remove non-internalized yeasts bound via mannose receptor. Cells were fixed with methanol, stained with Giemsa (1:2 for 30 min) and phagocytosed yeasts were counted under light microscopy. Phagocytic index (PI) was defined as infected macrophages/counted macrophages and pairwise comparison between groups was done by the Student *t*-test.

2. Results and discussion

In order to identify human plasma proteins that interact with *P. brasiliensis* yeast surface, carefully isolated cell wall preparations were exhaustedly washed with salt to remove non-specifically bound proteins. Non-covalently interacting plasma proteins were extracted with hot SDS, and tryptic peptides were analyzed by LC-MS/MS (for raw data, see Supplemental Files). We identified 52 plasma proteins with two or more peptides present only in Pb3pl cell wall, annotated them into functional categories, and quantified them by relative emPAI (mass%) (Table 1). We chose the emPAI method for protein quantification since it provides an absolute abundance value that enabled us to compare our data with the literature. Proteins categorized as transport, complement activation/regulation and coagulation pathways were the most abundant. Proteins related to lipid metabolism, immune response, acute-phase response, and homeostasis were identified at lower relative amounts.

We also correlated the relative emPAI of cell wall-associated plasma proteins with their relative mass percentages in plasma (Pieper, *et al.*, 2003), as shown in Table 1 and Fig. 1. Note that proteins of the coagulation pathway (antithrombin-III), transport (hemopexin and transthyretin), and complement activation/regulation (alpha-2-macroglobulin) were abundantly enriched in the fungal cell wall. Of them, only the latter is among the most abundant in plasma, representing 3.6% of total plasma proteins mass (Pieper, *et al.*, 2003) versus 6.9% of cell wall-bound proteins (Fig. 1A).

Albumin, which is the most abundant plasma protein (54%), was responsible for only 13.9% of cell wall-associated protein mass (Table 1; Fig. 1B). Alpha-1-acid and alpha-2-HS glycoproteins, haptoglobin, transferrin, apolipoprotein A-1, alpha-1-antitrypsin, and immunoglobulins were also relatively more abundant in plasma (Fig. 1B). Together, these observations suggest that plasma proteins have not randomly bound to the cell wall and that our analysis generally identified specifically bound proteins.

The presence of albumin interacting with cell wall components is speculative, and unspecific binding cannot be disregarded in this particular case. However, it has already been shown that *Candida albicans* Ala1/Ala5 adhesin is able to bind to BSA-coated beads, probably because of free threonine, serine, or alanine patches (Gaur, *et al.*, 2002). Although an Ala1/

Ala5 adhesin ortholog has not been found in *Paracoccidioides* genome, there could be other(s) albumin-binding protein(s) not yet described. In *Paracoccidioides*, many proteins colocalize to the surface and bind to extracellular matrix-associated proteins (reviewed in (Puccia, *et al.*, 2011)), but none has apparently been tested to bind to BSA.

Many immunoglobulin chains were found on the cell wall; however, they were twice more abundant in plasma than among cell wall-associated plasma proteins (Fig. 1B). That is not surprising, considering that only a small amount of the total immunoglobulin repertoire would be able to recognize fungal surface antigens, leading to opsonization and activation of both the classical complement pathway and phagocytosis (Ehrnthaller, *et al.*, 2011).

Complement activation/regulation components, such as C3, C4b-binding protein alpha chain (C4BP), factors B and H were responsible for 38.6% of the cell wall-bound plasma protein mass. That corroborates with previously reported immunofluorescence data showing that C3, C3a, C3d, C3g, C4, C5b-9, and factors H and B are present on the *P. brasiliensis* yeast cell surface (Munk & Da Silva, 1992). The results in Fig. 2 showed that Pb3 cultivated in plasma-containing medium was 31% more internalized by J774.16 macrophages than Pb3 grown in the absence of plasma, while incubation in pure plasma caused a 78% increase in phagocytosis, corroborating previous data about the effect of serum in phagocytosis of a distinct isolate [32]. The effect was probably related to complement binding, considering that controls with inactivated plasma (both to grow and to assay the yeasts) were similar to a negative control with medium alone.

In *C. albicans*, C3b binds directly to the yeast surface or via mannan-specific antibodies (Zhang & Kozel, 1998), opsonizing and mediating recognition by host immune effector cells for phagocytosis (van Lookeren Campagne, *et al.*, 2007). To avoid an excessive response and subsequent self-damage to host tissues, the complement system is tightly regulated by soluble and membrane bound proteins, such as factor-I, factor-H, C4BP, vitronectin and clusterin (Carroll, 2004), presently identified. Complement regulators would help the pathogen to evade the immune system by down regulating complement activation. C4BP is a major plasma inhibitor of the classical and mannose-binding lectin-mediated complement pathways and its alpha-chain is responsible for binding to *C. albicans* cell wall (Meri, *et al.*, 2004). Some microorganism surface ligands of complement factors have already been elucidated, such as Pra1 and Gpm1 in *C. albicans* (Zipfel, *et al.*, 2007). In this fungus, interaction with vitronectin increased binding to and phagocytosis by macrophages (Limper & Standing, 1994).

The complement cascade is intimately connected to the blood coagulation system and their activation occurs simultaneously (Markiewski, *et al.*, 2007), thus explaining why we identified members of the coagulation cascade on *P. brasiliensis* cell wall preparations. In *C. albicans,* plasminogen bound to surface CaGpm1p was accessible for activation and was converted to active plasmin, which is a key enzyme of intravascular fibrinolysis and acts in the degradation of the host extracellular matrix (Poltermann, *et al.*, 2007). *P. brasiliensis* Pb3 has two CaGpm1p orthologs: fructose-2,6-biphosphatase (PABG_05093) and conserved hypothetical protein PABG_05096, whose localization and affinity for plasminogen remain unknown. Fibrinogen chains were detected in high abundance (3.1% emPAI mass%) among cell wall-associated plasma proteins. Als3p adhesin in *C. albicans* binds to fibrinogen (Nobbs, *et al.*, 2010), and although an ortholog in *P. brasiliensis* has not been found, other protein(s) might have similar functions.

Transport proteins such as hemopexin (discussed below) and transthyretin were more represented in the cell wall than in plasma (Table 1 and Fig. 1A). Transthyretin, involved in thyroxine and retinol transport, had altered expression in plasma during experimental

invasive pulmonary aspergillosis (Gonzales, *et al.*, 2010). It presents adhesive properties and binds to many compounds including plant flavonoids (Green, *et al.*, 2005). Possibly, transthyretin may bind to *P. brasiliensis* cell wall components via disulfide bridges (Ruiz-Herrera, *et al.*, 2006), considering it can form disulfide bonds with a thiol-Sepharose 4B column (Fex, *et al.*, 1977).

Extracellular proteases can play important roles in pathogenic fungal nutrition, tissue invasion, and host immune system evasion (Naglik, *et al.*, 2003). Recently, Maza and coworkers (Maza, *et al.*, 2012) showed that *P. brasiliensis* extracellular proteases degrade proinflammatory cytokines. Therefore, host protease inhibitors would be an obvious defense mechanism by neutralizing fungal proteases involved in infection. On the cell wall of *P. brasiliensis* grown in plasma-containing medium we identified plasma proteins with serine protease inhibitor activity, such as alpha-1-antitrypsin, inter-alpha-trypsin inhibitor, alpha-2-macroglobulin, and angiotensinogen (Table 1). *P. brasiliensis* extracellular thiol-dependent subtilysin-like protease (Carmona, *et al.*, 1995) and a secreted 66-kDa serine protease (Parente, *et al.*, 2010) could possibly be neutralized by the human plasma protease inhibitors during infection. These fungal serine protease activities cleave extracellular matrix-associated proteins *in vitro* and could play a role in tissue damage and dissemination.

Both iron and copper are key regulators of host-pathogen interactions (Doherty, 2007, Kim, *et al.*, 2008). We presently identified hemopexin and ceruloplasmin bound to *P. brasiliensis* cell wall. Hemopexin tightly binds to heme groups and scavenges the free heme in order to protect the body from oxidative damage. Ceruloplasmin is responsible for carrying about 70% of the total copper in human plasma and exhibits a copper-dependent oxidase activity, which possibly oxidizes Fe^{2+} into Fe^{3+}, thus participating in iron transport. Microorganism receptors for host Fe-binding proteins and ligands have been described (Nevitt, 2011). The presence of plasma iron and copper carriers in *P. brasiliensis* cell wall may be due to an attempt to accumulate these nutrients during growth. Iron availability is important for fungal growth (Arango & Restrepo, 1988), and the presence of siderophores has been demonstrated (Castaneda, *et al.*, 1988). *In silico* analysis showed that *P. brasiliensis* also has a high-affinity copper transport protein (Ctr3p) ortholog (Silva, *et al.*, 2011). The importance of copper homeostasis in *Cryptococcus neoformans* virulence was demonstrated, since it was linked to capsule production and inhibition of phagocytosis (Chun & Madhani, 2010).

In conclusion, by using a careful protocol employing sucrose centrifugation and successive washes with different NaCl concentrations, we isolated cell wall from Pb3 yeasts cultivated in the presence of human plasma. The non-covalently associated plasma proteins were extracted with boiling SDS and a proteomic analysis by LC/MS-MS was applied. Complement pathway components were identified, and their role in the phagocytosis was suggested. Several human plasma proteins described here have not been previously reported as interacting with fungal components, specifically, clusterin, hemopexin, transthyretin, ceruloplasmin, alpha-1-antitrypsin, apolipoprotein A-I, and apolipoprotein B-100. This report represents an initial step to understanding the *P. brasiliensis* cell wall interaction with host components and the possible role of plasma proteins in the host-parasite relationship and infection, especially in a low virulence isolate.

Data availability: Proteomic data will be available online upon the acceptance of the present manuscript

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been funded by FAPESP, CNPq, and NIH (grants # 5G12RR008124-16A1, 5G12RR008124-16A1S1, and 8G12MD007592). We thank the Biomolecule Analysis Core Facility at UTEP, supported by the Research Centers in Minority Institutions (RCMI) program, grant # 8G12MD007592, to the Border Biomedical Research Center (BBRC), from the National Institutes on Minority Health and Health Disparities (NIMHD), a component of the NIH, for the access to the LC-MS instrument.

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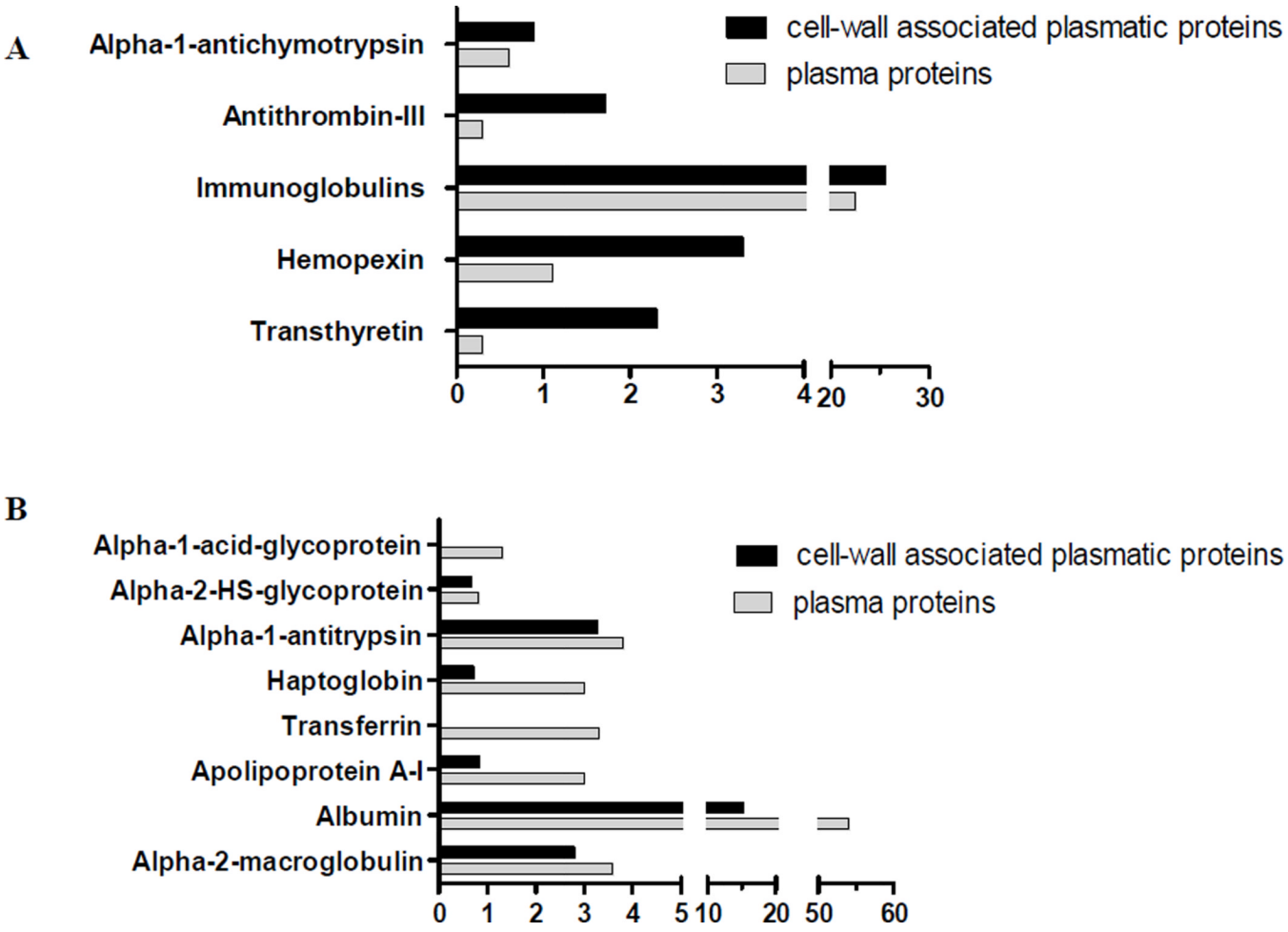

Fig. 1.

Relative abundance (relative emPAI mass%) of plasma proteins presently identified in *P. brasiliensis* (Pb3) isolated cell wall. Their percentage relative to total plasma proteins (Pieper, *et al.*, 2003) is shown in parallel. The figures show proteins relatively more abundant in the cell wall (A) or in plasma (B).

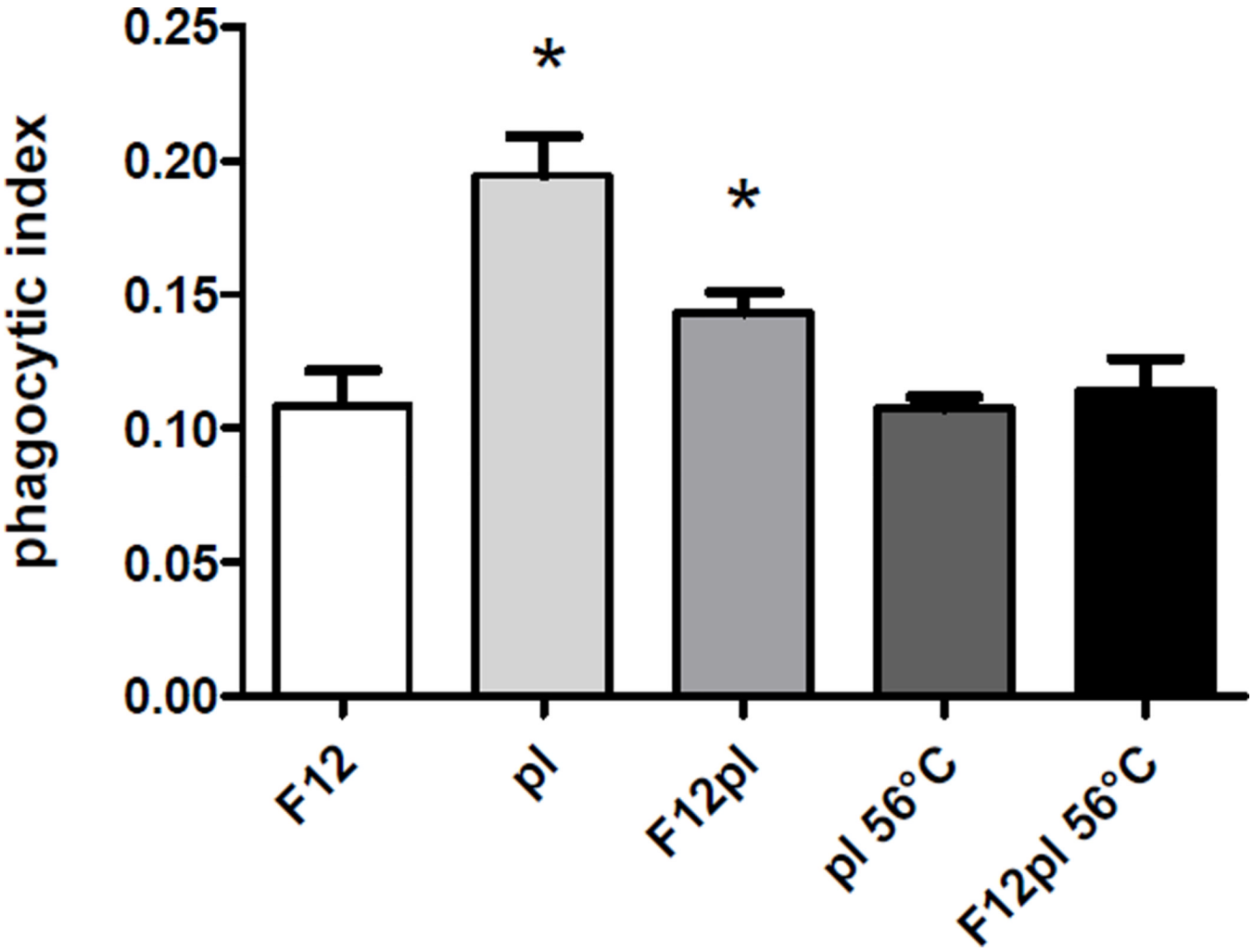

Fig. 2.

Phagocytic index for Pb3 yeast cells after 6 h of incubation with J774.16 macrophages. The assay was carried out with yeasts grown in F12 (control), F12-containing either inactivated (F12pl 56°C) or fresh human plasma (F12pl), and also with yeasts grown in F12, but previously incubated for 1 h at 37°C in heat-inactivated (pl 56°C) or fresh (pl) human plasma. Values are averages of three measurements with standard deviations. *Significant differences ($P < 0.05$) comparing with F12 control.

Table 1

Plasma proteins detected by LC-MS/MS in *P. brasiliensis* (Pb3)-derived cell wall. Distribution into functional groups was performed according to Gene Ontology classification. Protein relative abundance in the sample (relative emPAI mass%) and mass percentage in plasma (Pieper, *et al.*, 2003) are shown.

Protein Code	Cellular Process	emPAI mass% cell wall	Plasma mass% (as in Pieper, *et al.*, 2003)
	Complement activation / regulation	**38.6**	
IPI00783987	Complement C3	10	
IPI00887739	Similar to complement C3	7.4	
IPI00739237	Complement C3	9.3	
IPI00478003	Alpha-2-macroglobulin	6.9	3.6
IPI00887154	Complement component 4B	1.4	
IPI00291262	Clusterin	1.3	
IPI00921523	Complement factor B	1.1	
IPI00021727	C4b-binding protein alpha chain	0.8	
IPI00029739	Complement factor H	0.4	
	Transport	**19.3**	
IPI00384697	Serum albumin	7.0	54
IPI00022434	Serum albumin	6.0	
IPI00022488	Hemopexin	2.6	1.1
IPI00878282	Serum albumin	1	
IPI00940791	Transthyretin	0.7	0.3
IPI00017601	Ceruloplasmin	2.1	
	Coagulation pathway	**14.7**	
IPI00790784	Alpha-1-antitrypsin	2.3	3.8
IPI00032179	Antithrombin-III	1.4	0.3
IPI00298971	Vitronectin	1.4	
IPI00877703	Fibrinogen gama chain	1.1	
IPI00298497	Fibrinogen beta chain	1.1	
IPI00019568	Prothrombin	1.1	
IPI00022418	Fibronectin splice variant E	1	

Protein Code	Cellular Process	emPAI mass% cell wall	Plasma mass% (as in Pieper, *et al.*, 2003)
IPI00339226	Fibronectin	3.4	
IPI00022371	Histidine-rich glycoprotein	0.7	
IPI00029717	Fibrinogen alpha chain	1	
IPI00019580	Plasminogen	0.4	
	Immunoglobulins (immune response)	**9.7**	**22.5**
IPI00852577	Ig lambda-1 chain C regions	0.7	
IPI00154742	Ig lambda-2 chain C regions	0.6	
IPI00386879	Immunoglobulin heavy constant alpha 1	2.3	
IPI00827560	HRV Fab N28-VL	0.5	
IPI00896380	Ig mu chain C region	1.8	
IPI00739205	Ig heavy chain V-I region HG3	0.5	
IPI00384407	Myosin-reactive Ig heavy chain variable region	0.4	
IPI00384409	Myosin-reactive Ig heavy chain variable region	0.4	
IPI00784950	Immunoglobulin heavy constant alpha 2	1.1	
IPI00785067	Immunoglobulin heavy constant alpha 2	1.1	
IPI00470652	Single-chain Fv	0.5	
	Lipid metabolism	**9.3**	
IPI00021841	Apolipoprotein A-I	1.4	3
IPI00847635	Alpha-1-antichymotrypsin	0.6	0.6
IPI00022229	Apolipoprotein B-100	6.8	
IPI00218732	Serum paraoxonase/arylesterase 1	0.5	
	Others/Unknown	**4.6**	
IPI00796830	UNKNOWN	0.6	
IPI00646384	UNKNOWN	0.5	
IPI00940494	Uncharacterized protein	0.5	
IPI00022895	Alpha-1B-glycoprotein	1.3	
IPI00879931	Serpin peptidase inhibitor	0.9	
IPI00292530	Inter-alpha-trypsin inhibitor heavy chain H1	0.7	

Protein Code	Cellular Process	emPAI mass% cell wall	Plasma mass% (as in Pieper, *et al.*, 2003)
IPI00935352	Uncharacterized protein	0.2	
	Acute-phase response	**2**	
IPI00218192	Inter-alpha-trypsin inhibitor heavy chain H4	1.5	
IPI00022431	Alpha-2-HS-glycoprotein	0.5	0.8
	Homeostasis	**1.7**	
IPI00032220	Angiotensinogen	1.3	
IPI00477597	Haptoglobin-related protein	0.4	3

Protein Code	Cellular Process		emPAI	Mr (Da)	emPAI*Mr	emPAI mass%	emPAI mol%	Plasma mass% (as in [21])
	Immunoglobulins (immune response)					**9.72**	**25.48**	**22.50**
IPI00852577	Ig lambda-1 chain C regions	P0CG04	0.70125428	11.35	7.96	0.67	3.92	
IPI00154742	Ig lambda-2 chain C regions	P0CG05	0.649648074	11.29	7.34	0.62	3.63	
IPI00386879	immunoglobulin heavy constant alpha 1	Q96K68	0.505836354	53.09	26.85	2.27	2.83	
IPI00827560	HRV Fab N28-VL	A2IPI3	0.467799268	12.28	5.75	0.49	2.61	
IPI00896380	Ig mu chain C region	P01871	0.435035831	49.31	21.45	1.81	2.43	
IPI00739205	Ig heavy chain V-I region HG3	P01743	0.42510267	12.95	5.50	0.47	2.37	
IPI00384407	Myosin-reactive immunoglobulin heavy chain variable region	Q9UL92	0.333521432	13.58	4.53	0.38	1.86	
IPI00384409	Myosin-reactive immunoglobulin heavy chain variable region	Q9UL94 Q9UL92	0.333521432	13.21	4.40	0.37	1.86	
IPI00784950	immunoglobulin heavy constant alpha 2	Q6MZV6 Q9UL92	0.251875026	51.64	13.01	1.10	1.41	
IPI00785067	immunoglobulin heavy constant alpha 2	Q6P089	0.245197085	52.00	12.75	1.08	1.37	
IPI00470652	Single-chain Fv	Q65ZC8	0.211527659	26.13	5.53	0.47	1.18	
	Transport	**Q9UL92**				**19.34**	**22.02**	
IPI00384697	Serum albumin	P02768	1.187761624	69.37	82.39	6.96	6.64	54
IPI00022434	Serum albumin	Q56G89	1.020949938	69.08	70.53	5.96	5.70	
IPI00022488	Hemopexin	P02790	0.59985872	51.68	31.00	2.62	3.35	1.1
IPI00878282	Serum albumin	Q9UL92	0.519911083	22.86	11.88	1.00	2.90	

Protein Code	Cellular Process		emPAI	Mr (Da)	emPAI*Mr	emPAI mass%	emPAI mol%	Plasma mass% (as in [21])
IPI00940791	Transthyretin	E7EW61	0.412537545	20.29	8.37	0.71	2.30	0.3
IPI00017601	Ceruloplasmin	P00450	0.201284899	122.21	24.60	2.08	1.12	
	Complement activation / regulation	**Q9UL92**				**38.63**	**18.42**	
IPI00783987	Complement C3	P01024	0.632172129	187.15	118.31	10.00	3.53	
IPI00887739	Similar to complement C3	Q9UL92	0.605016318	144.81	87.61	7.40	3.38	
IPI00739237	Complement C3	Q9UL92	0.584893192	187.15	109.46	9.25	3.27	
IPI00478003	Alpha-2-macroglobulin	P01023	0.502551161	163.29	82.06	6.94	2.81	3.6
IPI00887154	Complement component 4B	Q6U2L1	0.342078063	47.45	16.23	1.37	1.91	
IPI00291262	Clusterin	P10909	0.291549665	52.50	15.30	1.29	1.63	
IPI00921523	Complement factor B	P00751 Q9UL92	0.154781985	85.53	13.24	1.12	0.86	
IPI00021727	C4b-binding protein alpha chain	P04003 Q9UL92	0.149756995	67.03	10.04	0.85	0.84	
IPI00029739	Complement factor H	P08603 Q9UL92	0.034700871	139.10	4.83	0.41	0.19	
	Coagulation pathway	**Q9UL92**				**14.74**	**13.87**	
IPI00790784	Alpha-1-antitrypsin	P01009 Q9UL92	0.584893192	46.74	27.34	2.31	3.27	3.8
IPI00032179	Antithrombin-III	P01008	0.304321387	52.60	16.01	1.35	1.70	0.3
IPI00298971	Vitronectin	P04004	0.299081397	54.31	16.24	1.37	1.67	
IPI00877703	Fibrinogen gama chain	C9JC84	0.24782547	52.34	12.97	1.10	1.38	
IPI00298497	Fibrinogen beta chain	P02675	0.232846739	55.93	13.02	1.10	1.30	
IPI00019568	Prothrombin	P00734	0.184484581	70.04	12.92	1.09	1.03	
IPI00022418	Fibronectin splice variant E	A6YID6	0.163561851	70.22	11.48	0.97	0.91	
IPI00339226	Fibronectin	P02751	0.151650644	262.63	39.83	3.37	0.85	
IPI00022371	Histidine-rich glycoprotein	P04196	0.142068906	59.58	8.46	0.72	0.79	
IPI00029717	Fibrinogen alpha chain	P02671	0.119902793	94.97	11.39	0.96	0.67	
IPI00019580	Plasminogen	P00747	0.051908639	90.57	4.70	0.40	0.29	
	Lipid metabolism					**9.28**	**5.49**	
IPI00021841	Apolipoprotein A-I	P02647	0.528306733	30.78	16.26	1.37	2.95	3
IPI00847635	Alpha-1-antichymotrypsin	P01011	0.158323286	47.65	7.54	0.64	0.88	0.6

Protein Code	Cellular Process		emPAI	Mr (Da)	emPAI*Mr	emPAI mass%	emPAI mol%	Plasma mass% (as in [21])
IPI00022229	Apolipoprotein B-100	P04114	0.156051281	515.61	80.46	6.80	0.87	
IPI00218732	Serum paraoxonase/arylesterase 1	P27169	0.140624924	39.73	5.59	0.47	0.79	
	Acute-phase response					**1.97**	**1.62**	
IPI00218192	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	0.171190257	103.36	17.69	1.50	0.96	
IPI00022431	Alpha-2-HS-glycoprotein	F5H0Q5	0.118872212	46.60	5.54	0.47	0.66	0.8
	Homeostasis					**1.71**	**2.31**	
IPI00032220	Angiotensinogen	P01019	0.291549665	53.15	15.50	1.31	1.63	
IPI00477597	Haptoglobin-related protein	P00739	0.122018454	39.03	4.76	0.40	0.68	3
	Others/Unknown					**4.61**	**10.81**	
IPI00796830	UNKNOWN		0.519911083	12.993	6.76	0.57	2.90	
IPI00646384	UNKNOWN		0.42510267	13.16	5.59	0.47	2.37	
IPI00940494	Uncharacterized protein	F5GXM8	0.389495494	14.08	5.48	0.46	2.18	
IPI00022895	Alpha-1B-glycoprotein	P04217	0.291549665	54.25	15.82	1.34	1.63	
IPI00879931	Serpin peptidase inhibitor	E9PGN7	0.182298865	59.49	10.85	0.92	1.02	
IPI00292530	Inter-alpha-trypsin inhibitor heavy chain H1	P19827	0.079775162	101.39	8.09	0.68	0.45	
IPI00935352	Uncharacterized protein	F8W967	0.047615753	41.90	2.00	0.17	0.27	
	Not identified				**1183.21**			
	transferrin							3.3
	alpha-1-acid glycoprotein							1.3

Protein Code	Cellular Process	emPAI mass%	Plasma mass% (as in [21])	cw/pl	pl/cw
	Immunoglobulins (immune response)	9.72	22.5	0.432	2.314815
IPI00852577	Ig lambda-1 chain C regions	0.672563076			
IPI00154742	Ig lambda-2 chain C regions	0.620103392			
IPI00386879	immunoglobulin heavy constant alpha 1	2.269575171			
IPI00827560	HRV Fab N28-VL	0.485626254			
IPI00896380	Ig mu chain C region	1.81289135			
IPI00739205	Ig heavy chain V-I region HG3	0.465122774			

Protein Code	Cellular Process	emPAI mass%	Plasma mass% (as in [21])	cw/pl	pl/cw
IPI00384407	Myosin-reactive immunoglobulin heavy chain variable region	0.382790971			
IPI00384409	Myosin-reactive immunoglobulin heavy chain variable region	0.372220528			
IPI00784950	immunoglobulin heavy constant alpha 2	1.099261708			
IPI00785067	immunoglobulin heavy constant alpha 2	1.077556648			
IPI00470652	Single-chain Fv	0.467083877			
	Transport	19.34			
IPI00384697	Serum albumin	6.963384401			
IPI00022434	Serum albumin	5.961013305			
IPI00878282	Serum albumin	1.004441092			
	3 serum albumin	13.9288388	54	0.257941	3.876849
IPI00022488	Hemopexin	2.619847634	1.1	2.38168	0.419872
IPI00940791	Transthyretin	0.707569825	0.3	2.358566	0.423986
IPI00017601	Ceruloplasmin	2.078922683			
	Complement activation / regulation	38.63			
IPI00783987	Complement C3	9.999049156			
IPI00887739	Similar to complement C3	7.404535366			
IPI00739237	Complement C3	9.251239525			
IPI00478003	Alpha-2-macroglobulin	6.935546654	3.6	1.926541	0.519065
IPI00887154	Complement component 4B	1.371943478			
IPI00291262	Clusterin	1.293506619			
IPI00921523	Complement factor B	1.118902604			
IPI00021727	C4b-binding protein alpha chain	0.848425949			
IPI00029739	Complement factor H	0.407937086			
	Coagulation pathway	14.74			
IPI00790784	Alpha-1-antitrypsin	2.310338244	3.8	0.607984	1.644781
IPI00032179	Antithrombin-III	1.352922438	0.3	4.509741	0.221742
IPI00298971	Vitronectin	1.372699212			
IPI00877703	Fibrinogen gama chain	1.096228858			
IPI00298497	Fibrinogen beta chain	1.100620553			
	Fibrinogen alpha chain	0.962426618			
		3.159276029			
IPI00019568	Prothrombin	1.092007895			

Protein Code	Cellular Process	emPAI mass%	Plasma mass% (as in [21])	cw/pl	pl/cw
IPI00022418	Fibronectin splice variant E	0.97063572			
IPI00339226	Fibronectin	3.366033953			
IPI00022371	Histidine-rich glycoprotein	0.715357485			
IPI00029717	Fibrinogen alpha chain	0.962426618			
IPI00019580	Plasminogen	0.397335516			
	Lipid metabolism	9.28			
IPI00021841	Apolipoprotein A-I	1.37424672	3	0.458082	2.183014
IPI00847635	Alpha-1-antichymotrypsin	0.6376098	0.6	1.062683	0.941014
IPI00022229	Apolipoprotein B-100	6.800214728			
IPI00218732	Serum paraoxonase/arylesterase 1	0.47220433			
	Acute-phase response	1.97			
IPI00218192	Inter-alpha-trypsin inhibitor heavy chain H4	1.495399077			
IPI00022431	Alpha-2-HS-glycoprotein	0.46818096	0.8	0.585226	1.708741
	Homeostasis	1.71			
IPI00032220	Angiotensinogen	1.309744753			
IPI00477597	Haptoglobin-related protein	0.402496621	3	0.134166	7.453479
	Others/Unknown	4.61			
IPI00796830	UNKNOWN	0.570921874			
IPI00646384	UNKNOWN	0.472631708			
IPI00940494	Uncharacterized protein	0.46342725			
IPI00022895	Alpha-1B-glycoprotein	1.336849378			
IPI00879931	Serpin peptidase inhibitor	0.916601794			
IPI00292530	Inter-alpha-trypsin inhibitor heavy chain H1	0.68359158			
IPI00935352	Uncharacterized protein	0.168609529			
	Not identified				
	transferrin		3.3	0	3.3
	alpha-1-acid glycoprotein		1.3	0	1.3