Membrane-Associated, Boron-Interacting Proteins Isolated by Boronate Affinity Chromatography

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Boron deficiency symptoms point to a role for boron in plant membranes, but the molecular partners interacting with boron have not yet been identified. The objective of the present study was to isolate and identify membrane-associated proteins with an ability to interact with boron. Boron-interacting proteins were isolated from root microsomal preparations of arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*) using phenylboronate affinity chromatography, subsequently separated by two-dimensional gel electrophoresis and identified using MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) peptide mass fingerprinting. Twenty-six boron-binding membrane-associated proteins were identified in *A. thaliana*, and nine in *Z. mays* roots. Additional uniden-tified proteins were also present. Common to both species were the β-subunit of mitochondrial ATP synthase, several β-glucosidases, a luminal-binding protein and fructose bisphosphate aldolase. In *A. thaliana*, binding of these proteins to boron was significantly reduced after 4 d of boron deprivation. The relatively high number of diverse proteins identified as boron interacting, many of which are usually enriched in membrane microdomains, supports the hypothesis that boron plays a role in plant membranes by cross-linking glycoproteins, and may be involved in their recruitment to membrane microdomains.

Keywords: Affinity chromatography • *Arabidopsis thaliana* • Boron • Membrane • Proteomics • *Zea mays*.

Abbreviations: ASB14, amidosulfobetaine-14,3-[*N,N*-dimethyl (3-myristoylaminopropyl) ammonio] propanesulfonate; B, boron; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate; 2-DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; GPI, glycosylphosphatidylinositol; BiP2, luminal-binding protein 2; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MBP, myrosinase-binding protein; MS, mass spectrometry; PBA, phenylboronic acid; POD, peroxidase; PBP, Pyk10-binding protein; RGII, rhamnogalacturonan II; TX100, Triton X-100.

Introduction

Currently the only established physiological role for boron (B) in plants involves cross-linking of the pectin rhamnoga-lacturonan II (RGII) in the cell wall (Ishii and Matsunaga 1996, Kobayashi et al. 1996, O'Neill et al. 1996, Ishii et al. 1999, Kobayashi et al. 1999, O'Neill et al. 2001, O'Neill et al. 2004). Nevertheless, the discovery that B is also essential for yeast (Bennett et al. 1999), bacteria (Chen et al. 2002) and several animals (Fort et al. 1998, Eckhert and Rowe 1999, Rowe and Eckhert 1999, Lanoue et al. 2000) which lack pectin strongly implies that additional roles for B in biology are also possible. In plants, many observations exist in which the effects of B perturbation cannot be explained solely by B cross-linking of RGII and which might suggest a role for B in membrane function and/or structure. For example, B deficiency has been reported to affect the swelling of liposomes (Parr and Loughman 1983), the fluidity of microsomes (Ferrol et al. 1993), the disruption of membrane transport processes (Goldbach 1985, Blaser-Grill et al. 1989, Goldbach et al. 1990, Barr et al. 1993, Ferrol et al. 1993), the activity of membrane-localized proteins (Goldbach 1997), the accumulation of secretory vesicles (Matoh et al. 1992), the stability of peribacteriod membranes (Bolanos et al.

1994) and the composition and permeability of the cell membrane (Blevins and Lukaszewski 1998, Mühling et al. 1998, Brown et al. 2002, Goldbach and Wimmer 2007). In animals, lack of B has been associated with membrane blebbing, cytoplasmic extrusion and death in Zebrafish zygotes (Rowe and Eckhert 1999), with growth retardation in trout (Rowe et al. 1998, Eckhert and Rowe 1999), and with impaired embryogenic development in rodents (Lanoue et al. 2000). In *Xenopus laevis*, B is required for normal oocyte maturation, embryonic growth and morphogenesis (Fort et al. 1998, Fort et al. 1999, Fort et al. 2002). It is particularly intriguing that B deficiency mainly affected processes with a high requirement for membrane synthesis and/or turnover. Taken together, these results support the emerging hypothesis that B could have a direct structural function in membranes, consistent with many reported effects in B deficiency studies as well as the physical and chemical characteristics of B.

An understanding of B chemistry has been paramount to most major discoveries in B biology including RGII cross-linking, quorum sensing in bacteria (Chen et al. 2002), as well as B transport in plants (Hu et al. 1997). These functions are all based on the ability of B to form reversible diester bonds with *cis*-diol-containing molecules (Loomis and Durst 1992, Shelp 1993, Woods 1996, Power and Woods 1997). Cellular membranes harbor a range of hydroxyl- and amine-containing molecules, such as glycoproteins, glycosylphosphatidylinositol (GPI)-anchored proteins or glycolipids. B could play a specific role in membranes by forming cross-links with such components, thereby dictating the membrane's physical state (Brown et al. 2002), which in turn can influence protein conformation, interaction and function (Mongrand et al. 2004).

Isolation of intact B complexes has proven to be challenging, probably due to the relatively weak interaction of B with most known biological molecules (Loomis and Durst 1992, Springsteen and Wang 2002). As far as we know, other than dimeric RGII–boron complexes from cell walls (Kobayashi et al. 1996, O'Neill et al. 1996) and complexes of polyalcohols with B in phloem sap (Hu et al. 1997), no other functional B complexes have been identified in plants. Membrane-associated complexes containing B so far remain elusive, and their discovery would be paramount to understanding a putative role for B in membrane function.

Agarose-immobilized phenylboronic acid (PBA) has been successfully used for the separation of glycosylated and non-glycosylated hemoglobins from human blood cells (Middle et al. 1983) and detergent-solubilized membrane proteins (Williams et al. 1982). In the latter work, some polypeptides were found to bind specifically to the PBA column only to be eluted using sorbitol-containing buffer, indicating that binding occurred through sugar residues containing suitable diol groups.

In this study we show that boronate affinity chromatography can be used to isolate membrane-associated proteins from arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*) roots that are capable of binding to B. Proteomic analysis revealed that several of the isolated B-binding proteins are typical components of membrane microdomains, and that their capacity to bind B seems to be influenced by B supply. It is expected that some of these proteins may be significant molecular partners of B in vivo and may be useful in additional studies which seek to characterize a role for B in membrane structure and function.

Results

Validation of the PBA affinity chromatography

The utility of immobilized PBA for the separation of glycosylated molecules has been previously demonstrated (Middle et al. 1983). We adapted the method in order to isolate proteins capable of binding B from plant membranes and other cellular compartments. Initially polycarbonate chromatography columns with approximately 1 ml of PBA resin were used, but results were hampered by poor reproducibility between replicates and a void volume that sometimes diluted the protein concentrations in collected fractions to below the level of detection. We therefore developed a modified spin cup method that allowed the use of very small sample and fraction volumes, resulting in very good reproducibility. The system was tested and calibrated using single and mixed standards of commercial bovine serum albumin (BSA) and peroxidase (POD). BSA is a non-glycosylated protein while POD is glycosylated. Both were used previously in a similar experimental set-up to investigate the characteristics of boronate binding (Bassil et al. 2004). The distribution of proteins in collected fractions of a mixed standard is shown in **Fig. 1**, and indicates that BSA was not significantly retarded by the PBA resin (**Fig. 1**, black arrow), while POD was significantly bound (**Fig. 1**, white arrow). Throughout this report, we define 'specific B-binding proteins' to be those proteins that are retained in the PBA resin by forming reversible ester-like linkages between *cis*-diol groups and the boronate ligand, such as POD. This should be distinguished from 'non-specific binding', such as for BSA, which probably involves hydrogen bonding, ionic and/or hydrophobic interactions (Middle et al. 1983). Specifically B-binding proteins are eluted from the PBA column by including sorbitol in the elution buffer, because the sorbitol competes for boronate-binding sites and therefore displaces bound proteins (Bassil et al. 2004).

Divalent cations such as Mg^{2+} greatly affect boronate–diol interactions (Middle et al. 1983, Bassil et al. 2004). We therefore examined the effect of Mg^{2+} on protein retention in the column. Decreasing the Mg^{2+} concentration in the equilibration and elution buffers reduced both specific and
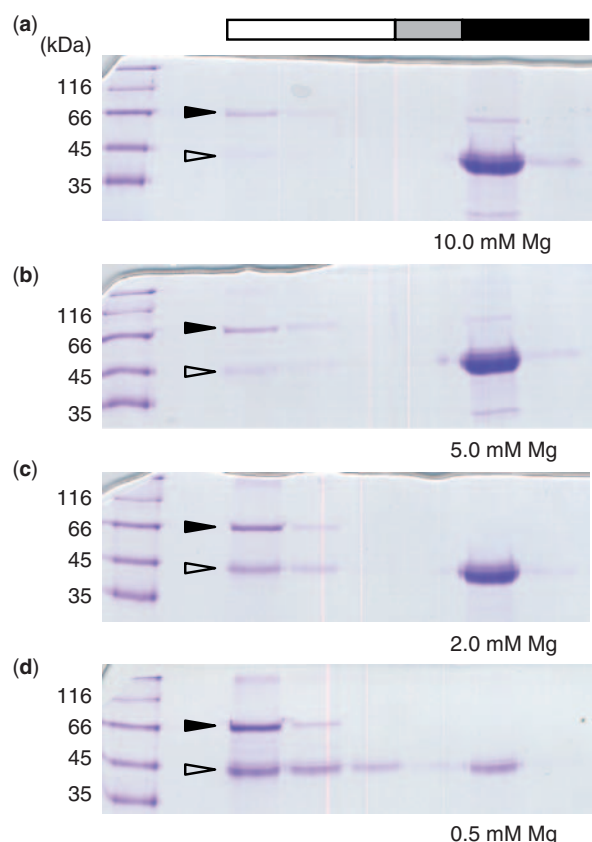
Fig. 1 Binding behavior of two protein standards [bovine serum albumin (BSA) and peroxidase (POD)] on spin cup columns with phenylboronic acid agarose resin at 10 (a), 5 (b), 2 (c) and 0.5 (d) mM Mg^{2+} in the equilibration and elution buffer. Lanes of the gels show the molecular weight markers and successive pooled fractions resulting from washes with taurine buffer (white bar), 1 N NaCl (gray bar) and sorbitol-containing taurine buffer (black bar); arrows indicate bands for BSA (black arrow) and POD (white arrow).

non-specific binding of proteins (**Fig. 1**). At concentrations of 5 or 10 mM Mg^{2+}, binding of POD was almost complete, but significant amounts of BSA were also bound (non-specifically) (**Fig. 1a**). On the other hand, including ≤2 mM Mg^{2+} in the loading buffer clearly resulted in incomplete binding of POD (**Fig. 1c, d**). We therefore chose 3 mM Mg^{2+} as optimal for our conditions and used this concentration from here on unless otherwise stated.

Identification of B-binding proteins from root microsomal preparations

Using boronate affinity chromatography and microsomal preparations from *A. thaliana* roots, we were able to separate two distinct protein fractions, one considered to be B binding and the other non-B binding. These fractions were subsequently resolved using two-dimensional gel electrophoresis (2-DE) and are shown in **Fig. 2**. The spot

pattern indicated in **Fig. 2** was consistently obtained in several independent experiments, including the separation of microsomal extracts. A total of 20 spots were resolved from samples corresponding to the B-binding fraction and subsequently analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) peptide mass fingerprinting. Of these, 16 spots were identified using the Mascot search function and contained proteins related to transport, metabolism and stress responses (**Table 1**). Silver staining of the same gels revealed additional spots which probably corresponded to proteins with an abundance that was too low for subsequent analysis.

In order to determine whether similar proteins can also be isolated from other species, we analyzed and compared microsomal preparations from *Z. mays* roots using a similar protocol. 2-DE of non-B-binding (**Fig. 3a**) and B-binding (**Fig. 3b**) fractions resulted in a lower percentage of identified proteins, which was probably due to the fewer annotated proteins in the available *Z. mays* databases.

The proteins identified using our most stringent criteria that were common to both *A. thaliana* and *Z. mays* B-binding fractions were mitochondrial ATP synthase subunit β (ATPB in *Z. mays* and At5g08670 in *A. thaliana*), β-glucosidases (Glu1 in *Z. mays* and Pyk10, Psr3.1 and Bglu22 in *A. thaliana*), luminal-binding protein (BIP2 in *Z. mays* and At5g28540 in *A. thaliana*) and fructose bisphosphate aldolase (ALF in *Z. mays* and At3g52930 in *A. thaliana*) (**Tables 1, 2**).

Effect of B supply on B-binding membrane proteins in root microsomal preparations

In another experiment, *A. thaliana* plants were grown with either sufficient (30 μM) B or deficient (<1 μM) B supply for periods of between 1 and 5 d before harvest. Microsomal preparations from roots containing the same amount of total protein were solubilized with Triton X-100 (TX100) and subjected to PBA affinity chromatography. Different fractions were resolved by 2-DE and stained with hot Coomassie (**Fig. 4**). The protein patterns of the non-B-binding fractions were similar across treatments in terms of both abundance and distribution (**Fig. 4a–d**). However, overall abundance of B-binding proteins was clearly reduced after 4 and 5 d of B deficiency (**Fig. 4e–h**). While the typical spot pattern remained after 1 d of B withdrawal (**Fig. 4f**), only a few spots were weakly detectable after 4 and 5 d of B deficiency, respectively (**Fig. 4h**, arrows), corresponding to mitochondrial H^+-ATP synthase β chain (At5g08670; spots 1 and 2 in **Table 1** and **Fig. 2**), β-glucosidases (pyk10 and psr3.1; spots 4 and 5 in **Table 1** and **Fig. 2**) and endomembrane-associated protein (At4g20260; spot 17 in **Table 1** and **Fig. 2**). Re-staining of the gel shown in Fig. 4g with silver did not reveal additional spots typically most prominent in this fraction, indicating that the B-binding capacity of these proteins was at least significantly reduced (gel not shown).
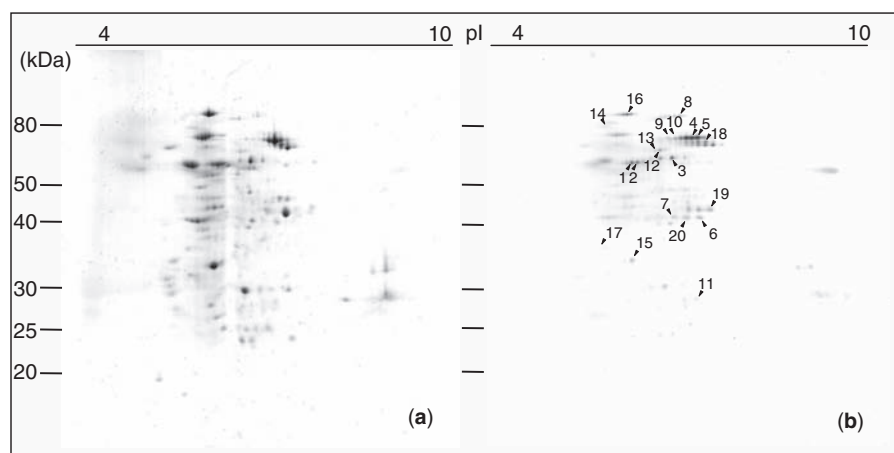
Fig. 2 Two-dimensional gels of non-B-binding (a) and B-binding (b) fractions of *Arabidopsis thaliana* root microsomal preparations after solubilization with Triton X-100 for 1 h. Arrows and numbers in (b) correspond to numbers of identified spots in **Table 1**.

Influence of different detergents on solubilization of B-binding proteins

The choice of detergents used during membrane solubilization can greatly influence the composition of proteins obtained (Herbert 1999, Santoni et al. 2000). Solubilization of integral membrane proteins for 2-DE presents added difficulties due to variabilities in the lipid content of samples and the inherently low solubility of hydrophobic membrane proteins (Pasquali et al. 1997, Morel et al. 2006). We therefore compared the ability of several detergents to solubilize B-binding proteins, including TX100, ASB14 {amidosulfobetaine-14, 3-[N,N-dimethyl(3-myristoylaminopropyl)ammonio propane-sulfonate} and CHAPS {3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate hydrate}, as well as their combinations (TX100 + ASB14, CHAPS + ASB14). From the 2-DE of B-binding fractions separated using boronate affinity chromatography, we found that depending on the detergent used to isolate microsomal proteins, distinctively different protein patterns were obtained (**Fig. 5**). Compared with TX100 alone (**Fig. 5c**), the combination of CHAPS and ASB14 (**Fig. 5b**), and especially of TX100 and ASB14 (**Fig. 5a**), solubilized larger amounts of protein. TX100 and ASB14 seemed to solubilize proteins of larger molecular weight (60–100 kDa; circle in **Fig. 5a**), while CHAPS and ASB14 solubilized proportionally more smaller molecular weight proteins (20–40 kDa; circle in **Fig. 5b**). It should be kept in mind, however, that the gel shown in **Fig. 5a** (TX100 + ASB14) was stained with Coomassie (for mass spectrometry analysis), whereas those in **Fig. 5b and c** were silver stained and therefore cannot be compared directly. Ten of the additional spots seen in **Fig. 5a** were identified using the Mascot search function (**Table 3**).

Discussion

B-binding proteins can be isolated using phenylboronate affinity chromatography

Binding of B via ester-like bonds to *cis*-diols of molecular partners is likely to be a prerequisite for any B function (Brown et al. 2002, Bassil et al. 2004, Bolanos et al. 2004). So far, the hypothesis that B might form cross-links between membrane-associated *cis*-diol-containing molecules, and thus contribute to membrane stability or function, has been based on a few indirect measurements and theoretical considerations regarding the presence of potential B binding partners.

The use of PBA affinity chromatography allows glycosylated proteins and other molecules capable of forming diester linkages with borate to be isolated (Williams et al. 1982, Middle et al. 1983, Bassil et al. 2004). The specificity of the borate–glycosyl interaction was demonstrated by POD retardation in the column, and BSA's lack of retardation (**Fig. 1**). The presence of cationic charges has been shown to affect the stability of borate–diol bonds (Kobayashi et al. 1999, Otsuka et al. 2003, Bassil et al. 2004, O'Neill et al. 2004). Similar to earlier studies using PBA resin (Middle et al. 1983, Bassil et al. 2004), we also found that high Mg^{2+} concentrations increased both specific and non-specific binding of proteins in the column, increasing the risk of false-positive identification of B-binding protein candidates. We therefore used 3 mM Mg^{2+} as a suitable compromise between strong specific and minimal non-specific binding (**Fig. 1**).

Using PBA affinity chromatography and 2-DE, we were able to isolate and resolve several membrane-associated proteins putatively capable of binding with B, including

Table 1 B-binding proteins identified by MALDI-TOF/MS in *Arabidopsis thaliana* root microsomal preparations

No.	Protein name	Gene name	Mascot score	Sequence coverage	MW$_{th}$[a] (kDa)	MW$_{OBS}$[b] (kDa)	pI[c]
Transport							
1	ATP synthase subunit beta (mitochondrial)	At5g08670	111	47%	59.6	50–60	6.18
2	ATP synthase subunit beta (mitochondrial)	At5g08670	109	54%	59.8	50–60	6.06
3	ATP synthase subunit alpha (mitochondrial)	AtMg01190	78	31%	55.0	50–60	6.23
Metabolism							
4	Thioglucosidase 3D precursor	pyk10	60	22%	59.7	60–70	6.45
5	β-Glucosidase	psr3.1	107	36%	60.0	60–70	6.95
6	Glyceraldahyde-3-phosphate dehydrogenase C subunit	At3g04120	80	44%	37.0	ca. 40	6.62
7	Fructose bisphosphate aldolase-like protein	At3g52930	105	48%	38.5	ca. 40	6.05
11	Carbonic anhydrase homolog	At1g70410	93	41%	28.4	30–40	6.66
Stress							
16	Jasmonate-inducible protein isolog (putative lectin protein, myrosinase-binding protein-like; similar to MBP1)	At3g16460	135	40%	72.4	80–90	5.31
Identified, but not always present							
8	Cobalamin-independent methionine synthase	At5g17920	56	21%	84.3	80–90	6.09
9	NADP-dependent malic enzyme-like protein	At5g11670	107	35%	64.4	60–70	6.01
10	NADP dependent malic enzyme-like protein	At5g11670	72	31%	64.4	60–70	6.01
12	Probable mitochondrial-processing peptidase subunit beta	At3g02090	112	45%	59.1	ca. 60	6.30
13	Probable mitochondrial-processing peptidase subunit beta	At3g02090	137	51%	59.1	ca. 60	6.30
14	Luminal-binding protein BiP2	At5g28540	88	30%	73.5	ca. 80	5.11
15	Jasmonate-inducible protein isolog (myrosinase-binding protein-like, putative lectin; PBP1)	At3g16420	109	68%	32.1	30–40	5.46
Putative, but highly regularly present							
17	Endomembrane-associated protein	At4g20260	58	58%	24.6	30–40	4.99
18	Putative β-glucosidase	Bglu22	39	25%	60.0	60–70	7.22
19	Putative myrosinase-associated protein	At1g54000	35	41%	43.1	30–40	7.12
20	Unknown (possibly peroxidase 39)	At4g11290	39	41%	35.6	40–50	6.53

The spot number corresponds to the numbers indicated in **Fig. 2b**.
[a]Theoretical molecular mass determined from the database.
[b]Approximate molecular mass observed in the gel.
[c]Theoretical isoelectric point determined from the database.

30 from *A. thaliana* and 15 from *Z. mays* (**Tables 1, 2, 3** and **Figs. 2, 3**). Similar protein patterns were consistently obtained from several independent experiments, including individual microsomal preparations. In both species, additional proteins were resolved in the B-binding fraction, but these could not yet be identified unequivocally due to their low abundance. Such a high number of proteins capable of B binding supports the hypothesis that many membrane-bound molecules share the ability to interact with B (Brown et al. 2002, Bolanos et al. 2004, Goldbach and Wimmer 2007). Whether such B–glycoprotein interactions have any physiological significance remains to be determined.

B-binding membrane proteins are related to transport, metabolism and stress response

From the B-binding fraction of TX100-extracted microsomal preparations, several proteins related to transport, metabolism and stress responses were identified (**Tables 1, 2**).

Among the most strongly B-binding proteins identified in both *A. thaliana* and *Z. mays* roots were several ATPase subunits, such as mitochondrial ATP synthase subunits α and β, and vacuolar ATPase subunit B and catalytic subunit (**Tables 1, 2, 3**). Subunits of ATP synthase are soluble (Alexandersson et al. 2004), and have been reported in many membrane
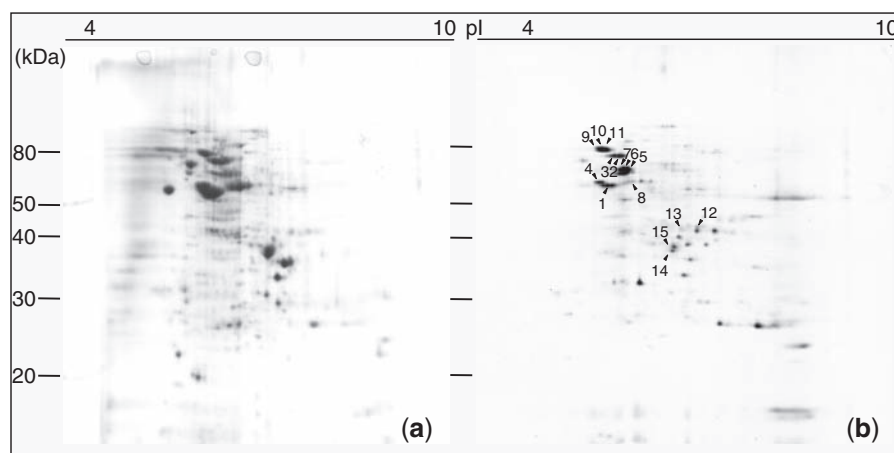
Fig. 3 Two-dimensional gels of non-B-binding (a) and B-binding (b) fractions of *Zea mays* root microsomal preparations after solubilization with Triton X-100 for 1 h. Arrows and numbers in (b) correspond to numbers of identified spots in **Table 2**.

Table 2 B-binding proteins identified by MALDI-TOF/MS in *Zea mays* root microsomal preparations

No.	Protein name	Gene name	Mascot score	Sequence coverage	MW$_{th}$[a] (kDa)	MW$_{obs}$[b] (kDa)
Transport						
1	Mitochondrial ATP synthase subunit beta	ATPB	85	25%	59.2	50–60
2	Vacuolar H⁺-ATPase catalytic subunit	PcVHA-A1	92	18%	69.0	70–80
3	Vacuolar H⁺-ATPase catalytic subunit	PcVHA-A1	89	18%	69.0	70–80
4	Vacuolar ATPase B subunit	Q7FV25_ORYSA	43	6%	54.1	50–60
Metabolism						
5	β-Glucosidase	Glu1	134	32%	64.5	60–70
6	β-Glucosidase	Glu1	148	31%	64.5	60–70
7	β-Glucosidase	Glu1 or Glu2	118	23%	64.5	60–70
8	Probable UDPglucose-6-dehydrogenase	UGDH_SOYBN	63	17%	53.5	50–60
Stress						
9	Luminal-binding protein 2 (cBiPe2)	BIP2_MAIZE	182	30%	73.2	70–90
10	Luminal-binding protein 2 (cBiPe2)	BIP2_MAIZE	163	25%	73.2	70–90
11	Luminal-binding protein 3 (cBiPe3)	BIP3_MAIZE	82	19%	73.3	70–90
Putative						
12	Fructose bisphosphate aldolase, cytosolic	ALF_MAIZE	59	17%	39.0	ca. 40
13	Fructose bisphosphate aldolase, cytosolic	ALF_MAIZE	55	17%	39.0	40–50
14	Retrotransposon protein, putative, unclassified	Os11g22130	56	20%	31.3	30–40
15	Retrotransposon protein, putative, unclassified	Os11g22130	51	18%	31.3	30–40

The spot number corresponds to the numbers indicated in **Fig. 3b**.
[a]Theoretical molecular mass determined from the database.
[b]Approximate molecular mass observed in the gel.

preparations (Rouquie et al. 1998, Prime et al. 2000, Santoni et al. 2000, Froehlich et al. 2003). The strong B-binding capacity of ATPases is especially interesting, since an effect of the B supply on membrane H⁺ transport was an early observation under B deficiency (Blaser-Grill et al. 1989, Ferrol et al. 1993). Obermeyer et al. (1996) demonstrated that plasma membrane ATPase of lily pollen grains was activated by B only if it was supplied at very high concentrations (2–4 mM), which
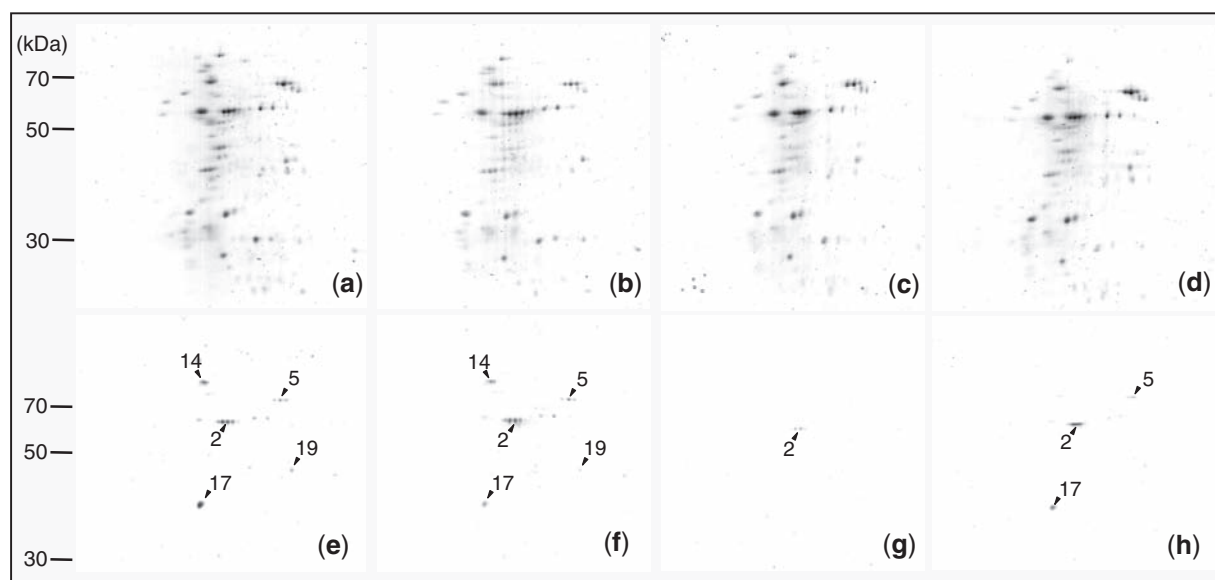
Fig. 4 Two-dimensional gels of non-B-binding (a–d) and B-binding (e–f) fractions of *Arabidopsis thaliana* root microsomal preparations after solubilization with Triton X-100 for 1 h. Initial sample volumes were chosen in such a way that they contained the same amount of total protein. Plants were subjected to either B-sufficient (30 µM B) (a, e), or to 1 d (b, f), 4 d (c, g) and 5 d (d, h) of B-deficient conditions (<1 µM B in the nutrient solution). Arrows and numbers in e–h are included for better orientation and correspond to those shown in **Fig. 2b**. All gels were stained with hot Coomassie.
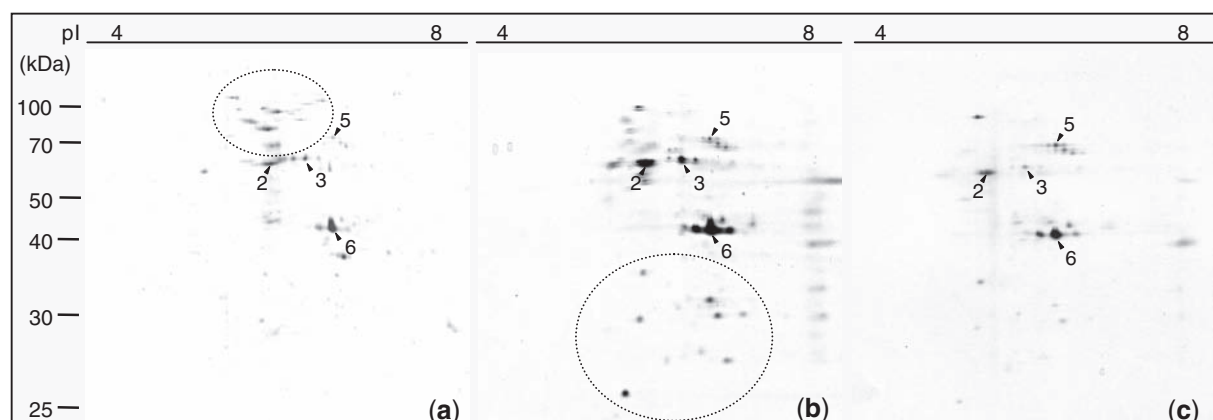

Fig. 5 Two-dimensional gels of B-binding fractions of *Arabidopsis thaliana* root microsomal preparations after solubilization with Triton X-100 + ASB14 (a), CHAPS + ASB14 (b) and Triton X-100 (c). For better comparison with Fig. 2, spot numbers 2 and 3 (ATP synthase), 5 (β-glucosidase) and 6 (glyceraldehyde-3-phosphate dehydrogenase) are marked by arrows. Circles in (a) and (b) indicate areas of increased number of spots resolved after solubilization with a combination of detergents as compared with Triton X-100 alone. Gel a was stained with hot Coomassie for further mass sprectrometric analysis; gels b and c were silver stained.

are unlikely to exist in vivo. However, if B does not directly affect the ATPase activity upon binding, but rather is involved in establishing membrane stability and/or correct orientation of the ATPases, enzyme activities could be altered by much lower B concentrations, such as observed in tobacco protoplasts (Matoh et al. 1992).

β-Glucosidases were also consistently found within the B-binding fraction (**Tables 1, 2**). They belong to family 1 of glycoside hydrolases (http://www.cazy.org/fam/GH1.html),

which hydrolyze glycosidic bonds, and which are involved in plant defense, activation of phytohormones, signaling, remodeling of the cell wall and lignification (Minic 2008, Morant et al. 2008). Several spots from the B-binding fraction of *A. thaliana*, representing β-glucosidases (**Fig. 2**, spots 4, 5 and 18), belong to the group of β-thioglucoside glucohydrolases, commonly referred to as myrosinases, which are important components of the glucosinolate–myrosinase defense system (Rask et al. 2000). Myrosinases are commonly glycosylated in

Table 3 B-binding proteins additionally identified by MALDI-TOF/MS in *Arabidopsis thaliana* root microsomal preparations after solubilization with Triton X-100 plus ASB14

Protein name	Gene name	Mascot score	Sequence coverage	MW$_{th}$[a] (kDa)	MW$_{obs}$[b] (kDa)
V-type proton ATPase catalytic subunit A	P49087	107	19%	62	70–80
Vacuolar proton ATPase B subunit	Q7FV25_ORYSA	126	21%	54	50–60
H⁺-transporting two-sector ATPase	At4g38510	68	21%	54	50–60
ATP synthase subunit beta-3, mitochondrial	At5g08670	96	16%	60	50–60
Allene oxide synthase	Q6RW10_MAIZE	176	27%	53	50–60
Lipoxygenase	Q9LKL4_MAIZE	93	12%	98	100–120
Ferripyochelin-binding protein-like	At5g66510	70	41%	29	30–40
Protein kinase APK1A	At1g07570	70	25%	46	40–50
Methionine synthase (fragment)	Q8W529_MAIZE	70	9%	84	80–100
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	At5g37510	65	13%	82	90–100

Spots were analysed from a replicate gel to that shown in **Fig. 5a**. Proteins already identified in **Table 1** are not included.
[a]Theoretical molecular mass determined from the database.
[b]Approximate molecular mass observed in the gel.

dicot (but not in monocot) plants (Morant et al. 2008). In *A. thaliana*, thioglucosidases were reliably represented in the B-binding fraction as several spots in close proximity (**Fig. 2**), similar to the pattern in membrane-associated fractions from *A. thaliana* callus homogenate (Prime et al. 2000). Such a spot pattern is typical for proteins with different co- or post-translational modifications, and multiple myrosinase iso-forms (20 in *Brassica napus*: Xue et al. 1992, Rask et al. 2000) are commonly present in plants, indicating tight differential and tissue-specific regulation (Xue et al. 1995).

The presence of soluble enzymes related to metabolism (**Tables 1, 2**) was observed previously in membrane preparations (Chivasa et al. 2002, Alexandersson et al. 2004) and could be explained either by contamination with highly abundant proteins or by an often tight association of peripheral proteins with membranes (Pasquali et al. 1997, Alexandersson et al. 2004). Of the B-binding proteins identified here, ATPases, β-glucosidases, endomembrane-associated protein, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, myrosinase-binding protein and luminal-binding protein BiP2 have been previously reported in membrane-associated fractions (Santoni et al. 2000, Prime et al. 2000, Borner et al. 2005), but most of them have not been shown to be integral to the membrane (Santoni et al. 2000). In order to determine whether integral membrane proteins can also bind B, we used either TX100, TX100 plus ASB14 or CHAPS plus ASB14 (**Fig. 5**) for solubilization. ASB14 is an amidosulfobetaine surfactant well suited for the recovery of strongly hydrophobic proteins such as PIPs (aquaporins) (Santoni et al. 2000). In our experiments, using the combination of either TX100 and ASB14 or CHAPS and ASB14 increased the amount of detectable spots in the B-binding fraction,

suggesting that the additional spots seen in **Fig. 5a and b** might represent integral membrane proteins. Ten of these additional spots could be identified by mass spectrometry and peptide mass fingerprinting, including different V-type ATPase subunits (**Table 3**), which are known to represent integral membrane components (Santoni et al. 2000). We were surprised by the relatively low number of positive identifications of these spots. Since it is likely that B-binding proteins carry glycosyl side chains, we hypothesize that the difficulties in identifying these spots arose from post-translational modifications combined with an inherently low abundance of this protein fraction. Whether or not membrane-associated B-binding proteins are integral or peripheral components of membranes, or rather associated by some other mechanism, remains to be elucidated.

Several B-binding proteins form larger aggregates by interacting with other proteins

Certain myrosinases interact with specific myrosinase-binding proteins (MBPs) in order to form large insoluble complexes (Eriksson et al. 2002). Pyk10, a root and hypocotyl-specific β-glucosidase in *A. thaliana* (Nitz et al. 2001, Morant et al. 2008), which was also present in the B-binding fraction (**Table 1**), is the major component of so-called endoplasmic reticulum (ER) bodies (Matsushima et al. 2003). In analogy to the myrosinase/MBP system, Pyk10 interacts with a binding protein (PBP1) (Nagano et al. 2008). Both PBP1 and a protein similar to MBP1 were represented in the B-binding fraction in *A. thaliana* (**Table 1**, spots 15 and 16), as was the luminal-binding protein 2 (BiP2) (**Table 1**, spot 14 and **Table 2**, spots 9–11), which has a role in facilitating the assembly of multimeric protein complexes (http://www.uniprot.org).

Since both β-glucosidases (Pyk10 and Psr3.1) as well as MBP1 and PBP1, but also BiP2 were present in the B-binding fraction, it is tempting to speculate whether B might be involved in the formation of larger aggregates or interaction with binding proteins through formation of B cross-links.

B supply affects the abundance of B-binding proteins in root microsomal preparations

Our results indicate that after 4 and 5 d of B deprivation, the B-binding capacity of proteins was significantly reduced (**Fig. 4e–h**). Although an effect on gene expression and/or translation cannot be ruled out, we favor the possibility that changes occurred in post-translational glycosylation, because the abundance of proteins also present in the non-B-binding fraction (ATPases, glycosyl hydrolases) was not significantly affected compared with control plants (**Fig. 4a–d**). Small changes in the glycosylation pattern of molecules can greatly affect their B-binding capacity, as was shown in the *A. thaliana mur1-1* mutant, where substitution of (methyl-)fucose residues by (methyl-)galactose resulted in greatly reduced formation of dimeric RGII and reduced growth (O'Neill et al. 2001, Iwai et al. 2002). Bolanos et al. (2001) also observed that in B-deficient pea nodules, two glycoproteins lacked the carbohydrate epitope required for incorporation into peribacteriod membranes of healthy symbiosomes, while the protein itself was not down-regulated. The reduction of B-binding capacity was not observed after 1 d of B deficiency (**Fig. 4**), indicating that, different from rapid effects upon B binding (see below), it might represent medium- or long-term changes in the glycosylation process.

B cross-links could be involved in stabilization of membrane microdomains

Serious consideration of a function for B in membranes emerged when the essentiality of B in animals was established (Brown et al. 2002, Goldbach and Wimmer 2007). Such a function would be likely to be based on the formation of B cross-links with membrane-localized diol-containing molecules and could be related to (i) physical stabilization of membranes and/or microdomains; (ii) incorporation of diol-containing molecules into membranes; or (iii) a direct effect on membrane-localized enzyme activity.

A regulatory role for B in enzymatic reactions has been postulated, but the evidence was either indirect (Hunt and Idso 1999) or observed under non-physiological B concentrations (e.g. 2–4 mM; Obermeyer et al. 1996), raising doubts about whether such conditions can occur in vivo. In our opinion, the multitude of proteins identified as B binding rather supports the hypothesis of a more general function for B, where B cross-links glycoproteins in cell membranes. A change in B supply could in turn alter membrane physical properties, an effect observed in vitro in artificial liposomes by Verstraeten et al. (2005). In contrast to the effect of

B deficiency on the B-binding capacity described above, such effects could happen rapidly and could explain the observed early effects of B deficiency on membrane-bound enzymes (Findeklee and Goldbach 1996, Goldbach et al. 2001).

Cross-linking of glycoproteins could also be involved in the formation and insertion of specific proteins in membrane microdomains. Such domains have been implicated in root hair and pollen tube growth (Kost et al. 1999), as well as in animal processes characterized by rapid membrane development (Simons and Ikonen 1997), all of which are especially prone to B deficiency (Blevins and Lukaszewski 1998, Fort et al. 1998, Rowe and Eckhert 1999). Since the ATP synthase α and β subunit, vacuolar ATPase B subunit, β-glucosidases and jacalin lectin family proteins have been previously described as components of membrane rafts (Bhat and Panstruga 2005, Borner et al. 2005, Morel et al. 2006), it is tempting to speculate that B might indeed have a function in cross-linking and thus 'fixing' these proteins in membrane microdomains, or in modulating their function indirectly by affecting membrane physical properties. A lack of B might then release proteins usually attached via B cross-links, leading to perturbations in a range of diverse physiological processes. Such a pleiotropic effect of B supply has been postulated earlier based on the many observed B-dependent processes (Bolanos et al. 2004).

Conclusions

We are aware of the fact that the mere observation of B binding to proteins does not per se demonstrate any physiological relevance. It is, however, one of the premises required for any function of B at membranes to occur (Brown et al. 2002, Koshiba et al. 2008). The in vitro formation of B complexes with different biomolecules (Ralston and Hunt 2001) has been used to suggest many possible functions for B in plant and animal metabolism (Hunt 2002, Hunt 2007). However, most B esters of sugar alcohols are relatively weak (Springsteen and Wang 2002), and their occurrence under physiological conditions may have been overestimated. Nevertheless, the stability of weak B esters can be substantially increased in non-aquous environments by hydrophobic interactions (Deuel and Neukom 1949). This could be relevant for B-binding proteins attached to membranes, where hydrophobic regions occur in close proximity to a high number of B-binding sites and the presence of stabilizing cationic charges. Taken together, all our results are in line with the hypothesis that B indeed has a function in the stabilization of membranes or membrane domains.

Here we have demonstrated a novel approach in combining PBA affinity chromatography and proteomic analysis for the isolation and identification of B-interacting membrane-associated proteins. We expect this method to be a useful tool in additional studies seeking to identify functions and molecular partners of B not only in plants, but also in animals.

Materials and Methods:

Plant growth conditions

Arabidopsis thaliana L. seeds were sown on wet rock wool packed in Eppendorf tubes whose tips had been removed and were fitted into a styrofoam sheet floating on tap water. Plants were grown in a climate chamber at day/night regimes of 20°C/18°C, 10 h/14 h light/dark and 50%/70% relative humidity. One week after germination, seedlings were transferred to an aerated hydroponic system and supplied with nutrient solution containing: 4 mM N [as $Ca(NO_3)_2$], 1.5 mM K (as K_2SO_4, KCl and KH_2PO_4), 1.2 mM S (as $MgSO_4$ and K_2SO_4), 2 mM Ca [as $Ca(NO_3)_2$], 1 mM Mg (as $MgSO_4$), 1 mM P (as KH_2PO_4), 100 µM Fe (as FeNaEDTA), 30 µM B (as H_3BO_3), 5 µM Mn, 1 µM Cu, 1 µM Zn (all as sulfates) and 0.7 µM Mo [as $(NH_4)_6Mo_7O_{24}$] and adjusted to pH 5.5–6.0. Plants were supplied with one-quarter strength solution for the first week, with one-half strength solution for the second week, and with full-strength solution thereafter, and were harvested 5 weeks after germination. B deficiency treatments were applied during the last 1–5 d of growth by exchanging the nutrient solution with solution lacking B. Boron was removed by treating with B-specific exchange resin Amberlite IRA743 (Sigma-Aldrich, Munich, Germany) until the final concentration in the nutrient solution was <1 µM B. Maize (*Z. mays* L.) seeds were surface sterilized, germinated between filter paper placed upright in water and then supplied with one-quarter strength nutrient solution. *Zea mays* plants were harvested 1 week after germination.

Membrane isolation

Microsomal membrane fractions were extracted according to Sandelius and Morré (1990) with modifications. Plant roots were briefly washed in ice-cold water, cut and homogenized in a pre-cooled Waring blender in homogenization buffer (pH 7.5) containing 50 mM HEPES, 5 mM Na_2-EDTA, 0.6% polyvinyl polypyrrolidone (PVPP), 5 mM dithiothreitol (DTT), 5 mM ascorbate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 330 mM sucrose. The homogenate was filtered through miracloth and centrifuged at 8,000×*g* for 15 min at 4°C to remove cell debris. A microsomal fraction was obtained by ultracentrifugation of the homogenate at 90,000×*g* (4°C, 35 min), resuspended in Tris/EDTA buffer (10 mM Tris–HCl, 1 mM Na2-EDTA, pH 7.5) and stored at −80°C. The final protein concentration was determined using a Bradford assay (Bradford 1976).

Solubilization of membrane proteins

Before gel electrophoresis, membranes were solubilized by adding a final concentration of 1% (w/v) TX100 and incubated for 60 min on ice with repeated agitation. The influence of including different detergents or combinations of these was tested by replacing TX100 in individual experiments with CHAPS (2% w/v), ASB14 (1% w/v), TX100 (1% w/v) + ASB14 (1% w/v) or CHAPS (2% w/v) + ASB14 (1% w/v).

Affinity chromatography

Proteins with the ability to bind to B were purified using PBA-bound agarose (Sigma-Aldrich, Munich, Germany) based on the method of Middle et al. (1983), but with modifications. Instead of using a column format, we developed a spin cup format, where 500 µl of sample previously diluted (1:1) with equilibration buffer (50 mM taurine/NaOH, pH 8.7, containing 3–10 mM $MgCl_2$) was incubated with 200 µl of pre-washed immobilized ligand resin for 1 h on ice and with gentle shaking. After transfer of the resin into spin columns (Pierce Chemical, Rockford, IL, USA), the non-binding fraction was collected by low speed centrifugation (10 s, 500×*g*). The resin was then thoroughly rinsed with equilibration buffer (six washes of 150 µl each) and 1 N NaCl (three washes of 150 µl each). For final elution of the bound fraction, a total of six washes (150 µl each) with taurine buffer containing 50 mM sorbitol was used. Three successive fractions (150 µl each) were pooled before further analysis.

Protein determination and gel electrophoresis

Protein concentrations in pooled fractions were determined using the bicinchoninic acid assay (Pierce Chemical) with BSA as standard. For electrophoretic separation, all fractions were precipitated overnight with ice-cold acetone containing 7.7 mg ml^{-1} DTT in a freezer (−20°C). Subsequently, the samples were centrifuged at 18,400×*g* for 25 min at 4°C and the resulting pellets were resuspended in sample buffer (63 mM Tris, 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.05% bromophenol blue, pH 6.8) for 1D PAGE and in lysis buffer [7 M urea, 2 M thiourea, 0.5% TX100, 20 mM DTT, 5 mM Pefabloc protease inhibitor mix (Fluka, Deisenhofen, Germany), 20 mM Tris base, 0.2% pharmalytes pH 3–10] for 2-DE. For 1D SDS–PAGE, samples were boiled for 4 min and separated by standard protocols on 9% polyacrylamide gels in the buffer system of Laemmli (1970). 2D PAGE was carried out using 11 cm pH 3–10 IPG strips in conjunction with an IPGPhor Isoelectric Focusing System (Amersham Biosciences, Freiburg, Germany). IPG strips were rehydrated for 11 h and focused for a total of 33,700 V h. After isoelectric focusing, strips were equilibrated for 15 min in 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 1% DTT. This was followed by a second equilibration step with 4% iodacetamide instead of DTT. Separation in the second dimension was carried out using 12.5% polyacrylamide gels (35 mA per gel). A total of 40–100 µg of protein were ideally applied per gel, but in B-binding fractions the total protein content was sometimes less. Molecular mass standards were

from Fermentas (St Leon-Rot, Germany) (1D gels) and Invitrogen (Karlsruhe, Germany) (2D gels). Gels were hot Coomassie or silver stained and scanned (Umax PowerLook 1120). For protein identification, spots from Coomassie-stained 2D gels were excised, digested with trypsin and analyzed using MALDI-TOF peptide mass fingerprinting. Proteolytic digests were analyzed using α-cyano-4-hydroxy cinnamic acid (HCCA; Fluka) as matrix on an Ultraflex I MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). For external calibration, a standard peptide calibration mixture was used (Bruker Daltonics). Up to 500 shots were summarized. Data processing was performed with the Compass 1.2 and BioTools 3.1 software (Bruker Daltonics). Proteins were identified by Mascot searches (version 2.2.04, Matrix Science, London, UK) using locally installed MSDB or NCBInr databases. Searching parameters included carbamidomethylation of cysteine residues, partial methionine oxidation, one missed cleavage and a mass accuracy of 75 p.p.m. Proteins were considered as matches only if they had a significant Mascot score and their theoretical molecular weight corresponded to the apparent molecular weight after SDS–PAGE.

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